



UNIVERSIDAD DE MURCIA

FACULTAD DE MEDICINA

Opiate Addiction: Neuronal Plasticity on Brain
Reward System and Emotional Memory–Related
Areas

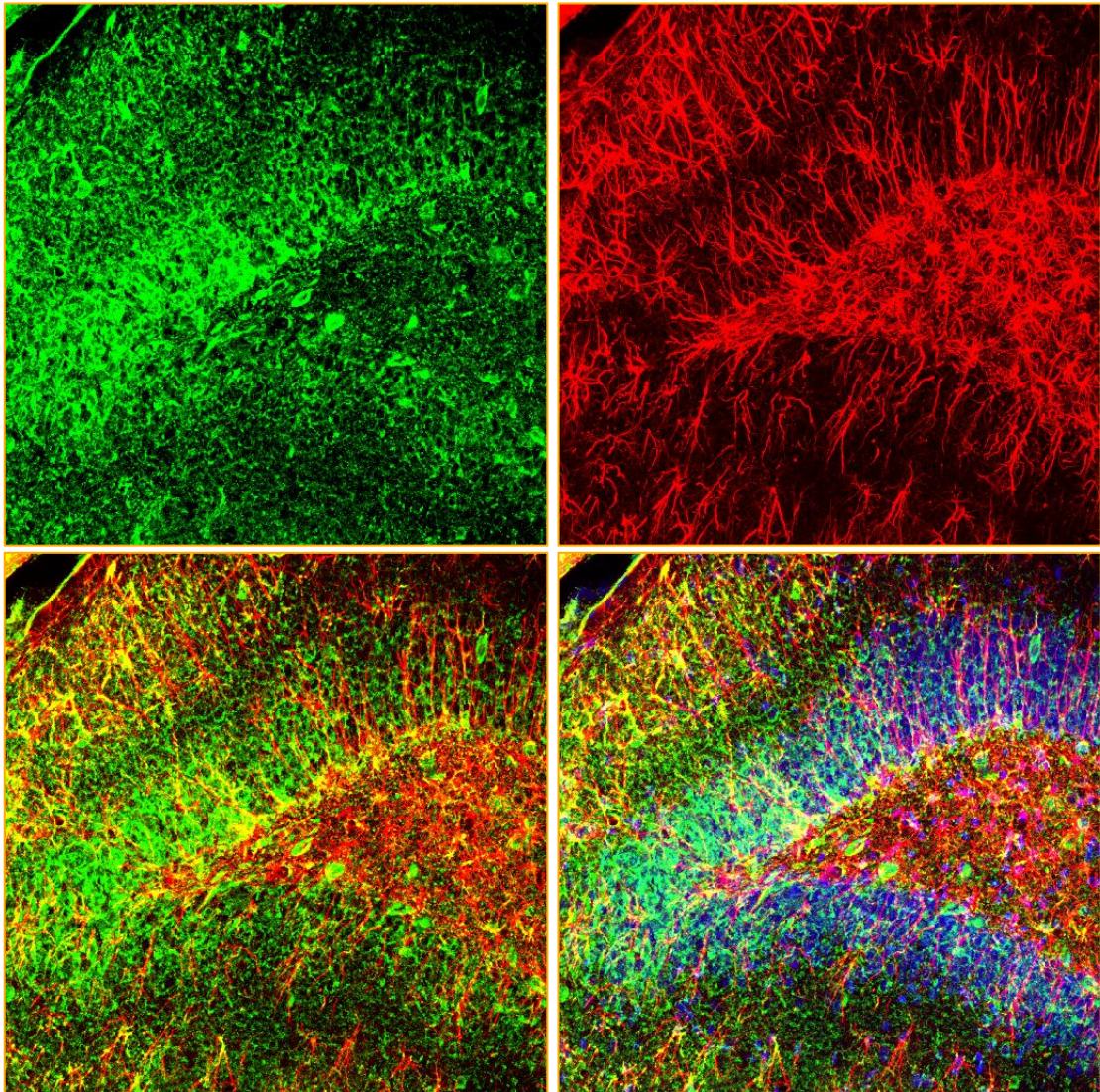
Adicción a Opiáceos: Plasticidad Neuronal en
los Circuitos Neuronales de Recompensa y en
Áreas de Memoria Emocional

D. Daniel García Pérez

2015

Adicción a opiáceos: plasticidad neuronal en los circuitos neuronales de recompensa y en áreas de memoria emocional

Opiate addiction: neuronal plasticity on brain reward system and emotional memory-related areas



Daniel García Pérez
Tesis Doctoral (con Mención Europea)

A mis padres, los mejores del mundo

El presente trabajo de investigación ha dado lugar a las siguientes publicaciones:

García-Pérez D, Laorden ML, Milanés MV, Núñez C. Glucocorticoids regulation of FosB/ Δ FosB expression induced by chronic opiate exposure in the brain stress system. PLoS One. 2012;7(11):e50264. doi: 10.1371/journal.pone.0050264.

García-Pérez D, Sáez-Belmonte F, Laorden ML, Núñez C, Milanés MV. Morphine administration modulates expression of Argonaute 2 and dopamine-related transcription factors involved in midbrain dopaminergic neurons function. Br J Pharmacol. 2013;168(8):1889-901. doi: 10.1111/bph.12083.

García-Pérez D, López-Bellido R, Hidalgo JM, Rodríguez RE, Laorden ML, Núñez C, Milanés MV. Morphine regulates Argonaute 2 and TH expression and activity but not miR-133b in midbrain dopaminergic neurons. Addict Biol. 2015;20(1):104-19. doi: 10.1111/adb.12083.

García-Pérez D, López-Bellido R, Rodríguez RE, Laorden ML, Núñez C, Milanés MV. Dysregulation of dopaminergic regulatory mechanisms in the mesolimbic pathway induced by morphine and morphine withdrawal. Brain Struct Funct. 2014;220(4):1901-19. doi:10.1007/s00429-014-0761-5.

García-Pérez D, Núñez C, Laorden ML, Milanés MV. Regulation of dopaminergic markers expression in response to acute and chronic morphine and to morphine withdrawal. Addict Biol. 2014. doi: 10.1111/adb.12209.

García-Pérez D, Luisa Laorden M, Núñez C, Victoria Milanés M. Glial activation and midkine and pleiotrophin transcription in the ventral tegmental area are modulated by morphine administration. J Neuroimmunol. 2014;274(1-2):244-8. doi: 10.1016/j.jneuroim.2014.07.017.

García-Pérez D, Laorden ML, Milanés MV. Acute morphine, chronic morphine and morphine withdrawal differently affects pleiotrophin, midkine and receptor protein tyrosine phosphatase β/ζ regulation in the ventral tegmental area. Mol Neurobiol (under review).

García-Pérez D, Laorden ML, Milanés MV. Regulation of pleiotrophin, midkine, receptor protein tyrosine phosphatase β/ζ and their intracellular signalling cascades in the nucleus accumbens during opiate administration. Int J Neuropsychopharmacol. 2015. doi: 10.1093/ijnp/pyv077.

García-Pérez D, Ferenczi S, Kovács KJ, Laorden ML, Milanés MV, Núñez C. Glucocorticoid homeostasis in the dentate gyrus is essential for opiate withdrawal-associated memories. Biol Psychiatry (under review).

García-Pérez D, Ferenczi S, Kovács KJ, Laorden ML, Milanés MV, Núñez C. Different contribution of glucocorticoids in the basolateral amygdala to the formation and expression of opiate withdrawal-associated memories. Addict Biol (under review).

El presente trabajo de investigación ha dado lugar a las siguientes comunicaciones a Congresos:

7th Forum of European Neuroscience (FENS) 2010

Amsterdam, The Netherlands, July, 3-7th, 2010

Title: Corticotropin-releasing factor-2 receptor (CRF2R) mediates morphine withdrawal-induced increased noradrenaline turnover in the hypothalamic paraventricular nucleus.

European Opioid Conference (EOC) 2011

Cracow, Poland, April, 13-14th, 2011

Title: Δ fosb expression in the brain stress system from adrenalectomized rats during morphine dependence.

Published in: Pharmacological Reports, 2011; 63: 255

XIV Congress of the Spanish Neuroscience Society (SENC) 2011

Salamanca, Spain, September, 28-30th, 2011

Title: Role of glucocorticoids in Δ FosB expression in CRF neurons during morphine dependence.

XXXIII Congress of the Spanish Pharmacology Society (SEF) 2011

Málaga, Spain, October, 3-5th, 2011

Title: Glucocorticoids regulation of Δ FosB expression in TH and dynorphin neurons during morphine dependence.

Published in: Basic & Clinical Pharmacology & Toxicology, 2011; 109: 43

8th Forum of European Neuroscience (FENS) 2012

Barcelona, Spain, July, 14-18th, 2012

Title: Tyrosine hydroxylase activity is modified in the dopaminergic reward circuit during morphine dependence and withdrawal.

8th Forum of European Neuroscience (FENS) 2012

Barcelona, Spain, July, 14-18th, 2012

Title: Chronic morphine administration modulates the expression of transcription factors involved in midbrain dopaminergic neurons function.

European Opioid Conference (EOC) 2013

Guildford, United Kingdom, April, 10-12th, 2013

Title: Morphine regulates Argonaute 2 and TH expression but not miR-133b in midbrain dopaminergic neurons (Oral presentation).

European Opioid Conference (EOC) 2013

Guildford, United Kingdom, April, 10-12th, 2013

Title: Orexins receptor type 2 (OX2R) antagonist, TCSOX229, attenuates somatic expression of naloxone-precipitated morphine withdrawal syndrome.

European Opioid Conference (EOC) 2013

Guildford, United Kingdom, April, 10-12th, 2013

Title: μ opioid receptor (mor) regulation by microrna let-7a in the hypothalamus during morphine dependence and withdrawal.

European Opioid Conference (EOC) 2013

Guildford, United Kingdom, April, 10-12th, 2013

Title: Argonaute 2 regulates TH expression during chronic morphine and naloxone-induced morphine withdrawal.

XXV Congreso Nacional Sociedad Española Farmacología (SEF 2013)

San Pedro del Pinatar-Murcia, Spain, September, 16-19th, 2013

Title: Morphine administration modulates expression of dopamine-related transcription factors involved in midbrain dopaminergic neurons function.

XXV Congreso Nacional Sociedad Española Farmacología (SEF 2013)

San Pedro del Pinatar-Murcia, Spain, September, 16-19th, 2013

Title: Assessing students' knowledge and understanding of pharmacology by a re-evaluation with different types of exams.

Reunión Científica Red Trastornos Adictivos 2013 (RTA 2013)

Oviedo, Spain, September, 25-28th, 2013

Title: Morphine regulates Argonaute 2 and TH expression and activity but not miR-133b, in midbrain dopaminergic neurons.

IX Congreso Nacional Estudiantes de Farmacia de Alicante (CEFA 2013)

San Juan de Alicante, Spain, October, 23-25th, 2013

Title: Midkine and pleiotrophin expression in the brain reward system is modulated by morphine administration (Oral presentation).

XVI Congress of the Spanish Neuroscience Society (SENC) 2015

Granada, Spain, September, 23-25th, 2015

Title: Glucocorticoids signaling is essential for opiate withdrawal-associated memories.

Daniel García Pérez ha sido beneficiario de las siguientes becas y ayudas:

- “Beca para estudios de máster universitario conducente al doctorado” del Programa Séneca 2009. Fundación Séneca - Agencia de Ciencia y Tecnología de la Región de Murcia. 2009/2010.
- Ayuda para la Formación de Profesorado Universitario (FPU) Ref. AP2009-2379. Ministerio de Educación, Cultura y Deporte. 2010-2014.

El presente trabajo de investigación ha sido financiado con las siguientes ayudas de Organismos Públicos:

- Proyecto SAF/FEDER 2007-62758, Ministerio de Ciencia e Innovación, MICINN. Implicación del CRF extrahipotalámico en la tolerancia/dependencia de morfina.
- Proyecto SAF/FEDER 2009-07178, Ministerio de Ciencia e Innovación, MICINN. Implicación del sistema cerebral del estrés en la dependencia de opiáceos. Mecanismos celulares y moleculares.
- Proyecto SAF/FEDER 2010-17907, Ministerio de Ciencia e Innovación, MICINN. Mecanismos neurobiológicos implicados en la preferencia de plaza a morfina y en la aversión a naloxona. Papel de los receptores de CRF1.
- Proyecto SAF/FEDER 2013-49076-P, Ministerio de Ciencia e Innovación, MICINN. Estrés y memoria: Implicación en los estados aversivos de la adicción y en las recaídas; mecanismos moleculares.
- Proyecto 15405/PI/10, Fundación Séneca - Agencia de Ciencia y Tecnología de la Región de Murcia. Relación entre estrés y adicción a opiáceos: papel de las orexinas y su interacción con los sistemas de CRF y NA.
- Red de Trastornos Adictivos (RETICS RD12/0028/0003), Instituto de Salud Carlos III.
- Instituto Murciano de Investigación en Biomedicina (IMIB) - Hospital Clínico Universitario Virgen de la Arrixaca (HCUVA) (GI/IMIB/C040/2011), Región de Murcia.

RESUMEN

Los opiáceos, como la morfina, son unos potentes analgésicos que se usan para tratar diversas formas de dolor agudo y crónico. Sin embargo son también muy adictivos, lo que limita su uso médico. Además, el uso recreativo de los opiáceos y opioides (morfina, heroína), ha aumentado notablemente en los últimos años. El uso crónico de opiáceos produce cambios neuroadaptativos a nivel cerebral que conllevan efectos indeseables, como la adicción, la cuál es un notable problema médico y sanitario. La creciente evidencia implica a varios mecanismos de regulación génica (incluyendo epigenéticos, moleculares, celulares y a nivel de circuitos neuronales) en los cambios que las drogas de abuso provocan en el cerebro, siendo el conocimiento de éstos una posible estrategia terapéutica para el tratamiento de la adicción.

Recientemente se ha sugerido que la patología adictiva es el resultado de procesos maladaptativos de plasticidad neuronal causados por el consumo de sustancias de abuso, y que conducen a cambios a largo plazo en la expresión génica en áreas concretas del sistema nervioso central (CNS), dando lugar a profundas anormalidades del comportamiento que persisten a lo largo de toda la vida del adicto. Debido a estas características de la adicción, se ha sugerido el importante papel que podrían desempeñar los mecanismos epigenéticos (metilación de DNA, modificación de histonas y RNAs no codificantes) en dicha patología. Así, actualmente ya se sabe que las sustancias adictivas pueden alterar mecanismos epigenéticos que modularían (silenciando o sobre-expresando) la expresión de genes implicados en la neuroplasticidad, perturbando los niveles intracelulares de proteínas y modificando los procesos de señalización dependientes de diferentes neurotransmisores, alterando en último término el procesamiento de la información en diferentes circuitos neuronales: circuitos de recompensa, circuitos del estrés, memoria y control de la función ejecutiva, entre otros. Además, en la última década se ha propuesto la importancia del estrés en la génesis y mantenimiento de los procesos adictivos a sustancias de abuso, así como en las recaídas en el consumo. Por otra parte, cada día es más evidente que las drogas de abuso producen alteraciones en el sistema inmune del CNS. Por ello, se ha propuesto que los astrocitos contribuirían a la plasticidad sináptica que se produce durante el desarrollo de la adicción mediante la síntesis y liberación de sustancias, como las citoquinas. De hecho, los opiáceos inducen cambios en la morfología de las células de la glia y expresión del marcador inmunohistoquímico proteína glial fibrilar ácida (GFAP) de una forma región-específica. Estos datos apuntan la posibilidad de que los astrocitos contribuyan a la plasticidad sináptica durante el desarrollo de la adicción.

En base a estos hallazgos, nos hemos planteado el estudio diferentes circuitos neuronales, neurotransmisores, factores de transcripción y microRNAs (miRs) que contribuirían a la plasticidad neuronal y memorias aversivas que llevarían al establecimiento del fenotipo adictivo. Esta Tesis aborda: 1) la caracterización de los cambios en factores de transcripción responsables del fenotipo dopaminérgico en la vía mesolímbica (Nurr1 y Pitx3) tras la administración aguda de morfina (intoxicación/binge), durante la dependencia y tras la inducción del síndrome de abstinencia. Además

se ha estudiado la posible relación entre dichos cambios y factores epigenéticos (miR-133b); 2) la contribución de los astrocitos a la plasticidad sináptica durante la adicción a opiáceos, mediante la síntesis y liberación de citoquinas (midkina –MK- y pleiotrofina –PTN-) en los circuitos de recompensa. 3) los aspectos subyacen en las modificaciones transcripcionales, post-transcripcionales y epigenéticas en el sistema cerebral del estrés y en los circuitos responsables de diferentes tipos de memorias, tanto a nivel neuroquímico, como celular y de comportamiento. Hemos centrado el presente estudio en varios neurocircuitos implicados en la adicción a opiáceos: i) el eje hipotálamo-hipófisis-adrenal (HPA) y el sistema extrahipotalámico del estrés, ii) el sistema dopaminérgico de recompensa, y iii) circuitos relacionados con las memorias aversivas.

- El uso crónico de las drogas de abuso altera el sistema cerebral del estrés. La exposición repetida a opiáceos conlleva la acumulación del factor de transcripción Δ FosB, particularmente en áreas cerebrales relacionadas con la recompensa y el estrés. La evidencia sugiere que las hormonas relacionadas con el estrés (como los glucocorticoides –GCs-) podrían producir adaptaciones en el sistema cerebral del estrés que podrían incluir alteraciones a nivel de la expresión génica y de factores de transcripción. El primer objetivo del presente estudio se diseñó después de considerar lo anterior. Examinamos el papel de los GCs en la expresión de Δ FosB en poblaciones neuronales específicas del sistema cerebral del estrés durante la dependencia de morfina. Para ello, la expresión de Δ FosB se cuantificó en ratas control (sham) y adrenalectomizadas (ADX) que desarrollaron dependencia de morfina.

- En los últimos años, diferentes estudios resaltan las modificaciones post-transcripcionales mediadas por los miRs en la adicción y otras patologías neurológicas y enfermedades neurodegenerativas. Algunos de ellos, como el miR-133b, han sido identificados como mediadores de la diferenciación, maduración y función de las neuronas dopaminérgicas del mesencéfalo. miR-133b actuaría mediante la regulación a la baja de factores de transcripción implicados en el desarrollo y el fenotipo de estas neuronas, como Nurr1 y Pitx3, que poseen una función crítica a nivel mesencefálico al regular la transcripción de diferentes genes implicados en el metabolismo de la dopamina (DA). Los cambios epigenéticos, como el silenciamiento génico mediado por miRs/Argonauta 2 (Ago2), también representan complejos mecanismos que regulan la plasticidad neuronal. Entre los miembros de la familia Ago, sólo Ago2 parece tener un papel importante en la ejecución del silenciamiento mediado por los diferentes miRs. Segundo objetivo: Dada la importante implicación de la transmisión dopaminérgica en los desórdenes adictivos, hemos centrado esta parte de nuestro estudio en identificar los marcadores dopaminérgicos que resultan alterados con la administración aguda y crónica de morfina, así como durante el síndrome de abstinencia inducido por naloxona, tanto en el área tegmental ventral (VTA) como el núcleo accumbens (NAc). Para ello, hemos determinado: i) la expresión del miR-133b y Ago2 en el VTA; ii) el contenido de tirosina hidroxilasa (TH) y su

actividad; iii) la actividad dopaminérgica (recambio de DA y activación de TH) en el sistema mesolímbico de ratas tratadas con morfina aguda, ratas dependientes de morfina y durante el síndrome de abstinencia inducido por naloxona. El tercer objetivo fue estudiar: i) posibles cambios en los niveles de mRNA y proteína de Nurr1 y Pitx3, así como la expresión de Ago2 y de TH en regiones específicas del sistema mesolímbico; ii) actividad dopaminérgica en el NAc; iii) co-localización cuantitativa de Nurr1 y Pitx3 en el VTA en las neuronas que expresan TH, iv) cambios en la plasticidad de subpoblaciones neuronales dopaminérgicas del VTA en respuesta a morfina aguda, crónica o durante el síndrome de abstinencia.

- Las neuronas dopaminérgicas localizadas en el mesencéfalo y que proyectan al estriado se caracterizan por la expresión de autoreceptores receptor de dopamina tipo 2 (DRD2) que inhiben la síntesis y liberación de DA. Además, transportador de dopamina (DAT; que media la recaptación de DA), TH (enzima limitante de la síntesis de catecolaminas) y transportador vesicular de monoaminas 2 (VMAT2), han sido identificados como importantes reguladores de la función dopaminérgica. Por otro lado, Nurr1 y Pitx3 son esenciales para la expresión de genes implicados en el metabolismo de la DA, como *Th*, *Dat*, *Vmat2* and *Drd2*. El cuarto objetivo fue: Los efectos del síndrome de abstinencia a morfina sobre la expresión de Nurr1 y Pitx3 nos llevó a investigar la expresión de otros genes y proteínas que están implicadas en la regulación de la función dopaminérgica, algunos de los cuáles son dianas de Nurr1 y Pitx3. Para ello, las ratas recibieron morfina de forma aguda o crónica. Otro grupo fue sometido a un síndrome de abstinencia a morfina. Se estudió: i) la expression de *Dat*-DAT, *Vmat2*-VMAT2, *Drd2*-DRD2 y DRD1 en VTA/NAc; ii) la co-localización de Nurr1 y/o Pitx3 con neuronas que expresan TH en el VTA, así como el porcentaje de neuronas dopaminérgicas que expresan Nurr1 y Pitx3; iii) la posible correlación entre la expresión de Nurr1/Pitx3 y los niveles de los diferentes marcadores dopaminérgicos.

- También se ha propuesto que los astrocitos, mediante la síntesis y liberación de factores de crecimiento/citoquinas, como MK y PTN, jugarían un papel fundamental en los cambios en la plasticidad sináptica del CNS durante el desarrollo de la adicción. Por ello, el quinto objetivo fue: Dado que PTN y MK tienen los mismos efectos que las neurotrofinas y que los astrocitos pueden liberar MK y PTN después de diversos estímulos, nuestro siguiente objetivo fue: i) estudiar posibles cambios en la expresión de PTN, MK, su receptor receptor proteína tirosina fosfatasa β/ζ (RPTP β/ζ), así como alteraciones en sus vías de señalización intracelular en respuesta a morfina aguda, crónica o durante el síndrome de abstinencia en el VTA y NAc; ii) la posible activación de los astrocitos durante los tratamientos descritos, que podría conllevar la liberación de diferentes factores; iii) identificar las subpoblaciones celulares que producen y secretan PTN y/o MK, así como aquellas que expresan RPTP β/ζ .

• En ratas dependientes de morfina, las memorias aversivas del síndrome de abstinencia pueden generar un estado motivacional que dé lugar a una búsqueda y consumo compulsivo de la droga. En la actualidad, los mecanismos que subyacen en la generación de las memorias aversivas del síndrome de abstinencia permanecen desconocidos. Es interesante recalcar que entre las regiones cerebrales que se ven afectadas por las drogas de abuso, se encuentran aquellas que son sustratos neuronales de la memoria, incluyendo el hipocampo, la amígdala y la corteza prefrontal. Esto coincide con los hallazgos de que algunas de las alteraciones más importantes observadas clínicamente en la adicción (por ejemplo el deseo compulsivo de consumir droga y las recaídas) reflejan anomalías en los circuitos tradicionales de la memoria, actuando las memorias a largo plazo de las experiencias de consumir la droga como conductores de la patología adictiva. Las hipótesis actuales de los mecanismos moleculares que intervienen en el aprendizaje y la memoria, sugieren que la rápida regulación génica y síntesis de nuevas proteínas conllevan modificaciones sinápticas persistentes que constituyen un mecanismo para la estabilización de memorias a largo plazo. *Egr1* y *Arc* son genes de expresión inmediata que juegan un papel crucial en la plasticidad y la memoria. Se ha propuesto que *Arc* tendría un papel fundamental en la consolidación de diferentes tipos de memoria, con algunos trabajos recientes implicando el mRNA y la proteína *Arc* en los procesos adaptativos que ocurren tras el estrés, así como en la plasticidad maladaptativa que ocurre en la adicción a drogas. Por otra parte, se ha demostrado que los GCs modulan la consolidación de la memoria. Los GCs ejercen sus acciones en diferentes regiones cerebrales, incluyendo el hipocampo, la amígdala y la corteza prefrontal, que tienen alta densidad de receptores de glucocorticoides (GRs) y son regiones importantes para la formación de la memoria. En roedores, el componente afectivo negativo de la dependencia de opiáceos se puede ver reflejado en diferentes tests comportamentales. Entre ellos, el test de aversión condicionada de plaza (CPA) es el más sensible para la medida del estado negativo emocional que produce el síndrome de abstinencia. Con estos antecedentes el sexto objetivo fue: En esta parte del estudio, hipotetizamos que los GCs en diferentes estructuras límbicas (como BLA y el DG en el hipocampo) podrían mediar el componente afectivo negativo del síndrome de abstinencia a morfina, y que por tanto jugarían un papel crucial en las memorias aversivas relacionadas con la abstinencia a opiáceos. Para comprobar nuestra hipótesis utilizamos el paradigma de CPA en animales ADX y que desarrollaron dependencia de morfina. A continuación estudiamos: i) posibles modificaciones epigenéticas (miR-124a y miR-212) y activación o inhibición de diferentes vías intracelulares (pCREB, *Egr1* y BDNF) en amígdala basolateral (BLA) y giro dentado (DG) durante la consolidación de memoria y la recuperación de memoria; ii) cambios en la expresión de *Arc* como marcador de plasticidad neuronal; iii) identificación de las diferentes subpoblaciones neuronales donde se expresaban las proteínas de interés; iv) correlaciones entre los miRs y la regulación de las diferentes proteínas.

Las conclusiones de nuestro trabajo se pueden resumir en:

1. El presente trabajo demuestra que los GCs están críticamente involucrados en la acumulación de FosB/ Δ FosB en el sistema cerebral del estrés durante la administración crónica de morfina, lo que resultaría en cambios duraderos en los patrones de expresión génica en las áreas relacionadas con el estrés.

2. Aunque no hallamos cambios significativos en los niveles de miR-133b en el VTA, proponemos el papel de Ago2 en combinación con otros miRs en la regulación de la estabilidad y/o traducción del mRNA de TH en respuesta a la administración crónica de morfina y al síndrome de abstinencia a opiáceos provocado por la administración de naloxona. Además, la dependencia de morfina y el síndrome de abstinencia están asociados con alteraciones en los factores de transcripción involucrados en el mantenimiento de las neuronas dopaminérgicas del sistema mesolímbico de recompensa. Por ello, nuestros resultados sugieren un papel importante de Nurr1 y Pitx3 en los cambios en la expresión génica durante la dependencia y abstinencia a opiáceos, y que la regulación epigenética de TH puede estar asociada, al menos en parte, a los mecanismos moleculares que contribuyen a los cambios en la función dopaminérgica inducida por los opiáceos.

3. La dependencia de morfina y el síndrome de abstinencia a la misma están asociados con alteraciones de la mayoría de los marcadores dopaminérgicos (DAT, VMAT2, DRD2) en la vía mesolímbica de recompensa, que se correlacionan con alteraciones en los factores de transcripción encargados del mantenimiento de las neuronas dopaminérgicas (Nurr1 y Pitx3). La correlación entre los marcadores dopaminérgicos y Nurr1/Pitx3 añaden evidencia a nuestros anteriores resultados, y pueden reflejar el papel de estos factores de transcripción durante la dependencia y abstinencia a morfina.

4. Debido a la activación glial en el VTA durante la administración aguda y crónica de morfina, junto con el incremento de los niveles de MK y PTN, proponemos la importancia de estas citoquinas al mediar, al menos en parte, las adaptaciones tróficas que se observan durante el fenómeno de adicción.

5. Nuestros experimentos demuestran que la expresión de Egr-1 y Arc está asociada con el recuerdo de la memoria del síndrome de abstinencia y sugieren que estos genes contribuirían a la plasticidad y a la reconsolidación que acompaña el proceso de recuerdo. Además, los GCs juegan un papel esencial durante la formación de la memoria aversiva a largo plazo del síndrome de abstinencia, ya que los animales ADX-dependientes muestran reducida aversión de plaza y niveles de Egr-1 y Arc disminuidos en el DG. Además nuestros datos demuestran que los GCs participan en los procesos transcripcionales que se requieren para la consolidación de memoria del síndrome de abstinencia e influyen mecanismos epigenéticos que siguen al recuerdo en el BLA.

ABSTRACT

Opiate drugs, such as morphine, are a class of powerful analgesics that are used for treating many forms of acute and chronic pain. However, they are also highly addictive, which limits their medical use. The non-medical use of opiates (heroin, morphine) has increased greatly in recent years. Chronic use of opiates causes brain neuroadaptation that lead to undesirable effects, namely opiate addiction that is a significant medical and public health problem. Increasing evidence implicates various mechanisms of gene regulation (including epigenetic, molecular, cellular and circuit level effects) in the changes that drugs of abuse induce in the brain, indicating a potential therapeutic strategy for addiction therapy.

It has been suggested that drug addiction is a result of maladaptive synaptic plasticity mechanisms that are initiated during drug taking. Long-term changes in gene expression in different brain areas would mediate the long-lasting addictive behaviors. Because of these altered behaviors are long-lasting, epigenetic mechanisms have been proposed as mediators of this phenomenon. Thus, it has been described that several drugs of abuse can modulate some epigenetic mechanisms that, in turn, would enhance or repress different genes related with neuroplasticity, intracellular protein levels and/or signaling cascades depending on neurotransmitters action. All these changes would lead to altered functioning of different neuronal circuits: brain reward system, brain stress system, learning and memory or decision-making. In the last decade, it has been suggested a prominent role for stress on drug seeking and taking and the development of drug addiction, as well as on drug reinstatement. On the other hand, there is increasing evidence that drugs of abuse produce alterations in CNS immunology. Thus, it has been proposed that astrocytes contribute to the synaptic plasticity during the development of drug addiction by the synthesis and release of substances, such as cytokines. Thus, opiates induce profound changes in glial cellular morphology and phenotypic receptor/immunohistological marker expression, glial fibrillary acidic protein (GFAP) in a region specific-manner. These data raise the possibility that astrocytes contribute to the synaptic plasticity during the development of drug addiction.

All these data prompted us to study different brain networks, neurotransmitters, transcription factors and microRNAs (miRs) that would contribute to neuronal plasticity and aversive memories during the development of the addicted phenotype. The current Thesis investigates: 1) changes in the transcription factors that characterize the dopaminergic phenotype (Nurr1 and Pitx3) in the mesolimbic circuit after acute and chronic morphine and during morphine withdrawal. Moreover, the possible relationship between such transcription factors and epigenetic mechanisms (miR-133b) was also studied; 2) the role of astrocytes in synaptic plasticity during morphine dependence by means of the synthesis and release of cytokines (midkine –MK- and pleiotrophin –PTN-) in the brain reward system; 3) an altered epigenetic, transcriptional and post-transcriptional regulation of limbic and memory circuits, and the relationship between behavioral and molecular changes. We have focused the present study on several neurocircuits involved in opiate addiction: i) the hypothalamic-pituitary-

adrenal (HPA) and extrahypothalamic stress systems; ii) the dopaminergic reward pathways; and iii) aversive memories-related neuronal circuits.

- Chronic use of drugs of abuse profoundly alters stress-responsive system. Repeated exposure to opiates leads to accumulation of the transcription factor Δ FosB, particularly in brain areas associated with reward and stress. Recent evidence suggests that stress-related hormones (e.g. glucocorticoids –GCs-) may induce adaptations in the brain stress system that are likely to involve alterations in gene expression and transcription factors. The first objective of the present study was designed after considering the above. We examined the role of GCs in regulation of Δ FosB expression in specific populations of the brain stress system during morphine dependence. For that, expression of Δ FosB was measured in control (sham-operated) and adrenalectomized (ADX) rats that were made dependent on morphine.

- In recent years, studies highlight post-transcriptional modifications mediated by miRs in addiction and other neurological disorders and neurodegenerative diseases. Some of them, such as miR-133b, have been proposed to regulate the differentiation, maturation and function of dopaminergic neurons by downregulating transcription factors involved in the development and physiological function of midbrain dopaminergic neurons, such as Nurr1 and Pitx3, which are critical for transcription of a set of genes involved in dopamine (DA) metabolism in the mesolimbic pathway. Epigenetic changes such as miRs/Argonaute 2 (Ago2)-induced gene silencing represent complex molecular signature that regulate cellular plasticity. Among the Ago family members, only Ago2 seems to have an important role in miRs generation and execution of miR-mediated gene silencing. Our second objective was: Given the important implications of DA neurotransmission in addiction disorders, we have focused this part of our study on identifying the DA markers that are altered in association with acute and chronic morphine exposure, as well as with morphine withdrawal in the ventral tegmental area (VTA) and nucleus accumbens [NAc(medial shell)]. For that, we have determined i) the expression of miR-133b and Ago2 in VTA; ii) tyrosine hydroxylase (TH) content and activity; and iii) dopaminergic activity (DA turnover and TH activation) in the mesolimbic system from rats acutely injected with morphine, in morphine-dependent rats and during naloxone-induced morphine withdrawal. Thus, the third objective was to study: i) Nurr1 and Pitx3 mRNAs and proteins changes as well as the expression of Ago2 and TH mRNA and protein levels in specific region of the mesolimbic system; ii) dopaminergic activity in the NAc; iii) quantitative co-localization of Nurr1 and Pitx3 in the VTA TH-positive neurons; and iv) the plasticity changes in VTA DA neurons subpopulations in response to morphine, morphine dependence and morphine withdrawal.

- DA neurons located in the midbrain and projecting to the striatal complex are primarily characterized by expression of dopamine receptor 2 (DRD2) autoreceptor subtype, which inhibits DA synthesis and release. In addition, dopamine transporter (DAT), which mediates the reuptake of

DA, TH (the rate-limiting enzyme for DA synthesis) and vesicular monoamine transporter 2 (VMAT2) have been identified as important regulators of DA function. On the other hand, Nurr1 and Pitx3 are crucial for expression of the set of genes involved in DA metabolism, such as *Th*, *Dat*, *Vmat2* and *Drd2*. Our fourth objective was: The long-lasting effects of opiate withdrawal on Nurr1 and Pitx3 expression prompted us to investigate expression of other genes and proteins that are involved in the regulation of DA function, some of which represent putative targets of Nurr1 and Pitx3. For that, rats were exposed to acute and chronic morphine administration as well as to morphine withdrawal and analyzed: i) the expression of *Dat*-DAT, *Vmat2*-VMAT2, *Drd2*-DRD2 and DRD1 in VTA/NAc(shell), dysfunction of which is causally linked to addiction; ii) the co-localization of Nurr1 and/or Pitx3 with TH-positive neurons in the VTA as well as the percentage of DA neurons expressing Nurr1 and Pitx3; and iii) the possible correlation between Nurr1/Pitx3 expression and DA markers levels in the VTA and/or NAc(shell).

- It has also been proposed that astrocytes, through the synthesis and release of growth factors/cytokines, such MK and PTN, would play a critical role in long-term synaptic plasticity in the CNS during drug addiction. Given these facts, the fifth objective was: Given the important implications of DA neurotransmission in addiction disorders and the complexity of opiate-induced neuroadaptive responses in the brain reward dopaminergic system, the present study was focused on: i) identifying whether the expression of PTN, MK, Receptor protein tyrosine phosphatase β/ζ (RPTP β/ζ) and their intracellular signaling pathways [thymoma viral proto-oncogene (Akt) and extracellular-signal regulated kinase (ERK)] are altered in association with acute and chronic morphine exposure as well as with morphine withdrawal in the VTA and NAc; ii) the possible activation of astrocytes, which could lead to the release of astrocyte-related soluble factors; iii) we also aimed to identify those cell subpopulations that produced and secreted PTN and/or MK and those that expressed RPTP β/ζ in response to morphine administration or morphine withdrawal.

- In opiate-dependent rats, aversive memories of drug withdrawal can generate a motivational state leading to compulsive drug seeking and taking. However, the mechanisms underlying generation of drug withdrawal memories remain unclear. Interestingly, among the brain regions affected by drugs of abuse are those that are key neural substrates for behavioral memory, including the hippocampus, amygdala and prefrontal cortex. This coincides with the increasing realization that some of the most important features of addiction seen clinically (eg, drug craving and relapse) reflect abnormalities in traditional memory circuits, with long-term memories of the drug experience serving as potent drivers of addiction pathology. Current hypothesis on the molecular mechanisms of learning and memory suggest that rapid regulation of gene programs and synthesis of new proteins leading to persistent synaptic modifications constitute a key mechanism for the stabilization of long-term memory. Early growth response 1 (Egr1) and activity-regulated cytoskeletal-associated protein (Arc) are Immediate-

early gene (IEG) known to play major roles in plasticity and memory. Arc has been proposed to have an important role in consolidation of many forms of memory, with recent works implicating *Arc* mRNA and protein in adaptation to stress as well maladaptive plasticity connected to drug addiction. GCs mediate and modulate memory consolidation. They exert their actions on brain regions, including the hippocampus, amygdala and prefrontal cortex, that are enriched in glucocorticoid receptor (GR) and are important for long-term memory formation. In rodent, the negative affective component of opiate dependence could be reflected by several behavioral alterations, among which conditioned place aversion (CPA) is the most sensitive measurement for the negative motivational state produced by opiate withdrawal, which is considered as a Pavlovian conditioned paradigm. For these reasons the sixth objective was: We hypothesized that GCs in limbic structures (eg., the basolateral amygdala – BLA- and hippocampal dentate gyrus –DG-) could specifically mediate the negative motivational component of opiate withdrawal and might play a crucial role in the aversive memories of opiate withdrawal. To test this hypothesis, we used the CPA paradigm in control (sham-operated) and ADX animals that were rendered dependent on morphine. Then, we studied: i) epigenetics modifications (miR-124a y miR-212) and intracellular pathways activated or inhibited (phosphorylated cAMP response element binding protein –pCREB-, Egr1 y brain derived neurotrophic factor –BDNF-) in the BLA and the DG during memory consolidation and memory retrieval; ii) changes in Arc expression as a marker of neuronal plasticity; iii) we also aimed to identify those cell subpopulations that produced and secreted the different proteins that we studied; iv) correlation analysis between different miRs and target proteins levels.

The conclusions from present work are:

1. Present work provides evidence that GCs are critically involved in FosB/ Δ FosB accumulation in the brain stress system after chronic morphine exposure, which might result in lasting changes of gene expression pattern in stress-related areas.

2. Although no significant changes of miR-133b levels are detected in the VTA, a role for Ago2 and specific miRs is hypothesized in regulating TH mRNA stability and/or translation in response to chronic morphine administration and naloxone-induced morphine withdrawal. Moreover, morphine dependence and withdrawal are associated with consistent alteration of transcription factors involved in the maintenance of dopaminergic neurons in the mesolimbic drug-reward pathway. Thus, our results suggest an important role for Nurr1 and Pitx3 in contributing to the changes in gene expression during opiate dependence and withdrawal and that epigenetic regulation of TH could be associated, at least in part, to the molecular mechanisms contributing to opiate-induced changes in midbrain dopaminergic function.

3. Morphine dependence and withdrawal are associated with consistent alteration of most of the DA markers (DAT, VMAT2, DRD2) in the mesolimbic drug-reward pathway which correlated with alteration of transcription factors involved in the maintenance of dopaminergic neurons (Nurr1 and Pitx3). The correlations between DA markers and Nurr1/Pitx3 add evidence onto previous results, and may reflect engagement of these transcription factors during morphine dependence and withdrawal.

4. Because of the glial activation in the VTA during acute and chronic morphine administration besides the enhancement in MK and PTN, we propose a role for these cytokines in mediating, at least in part, the trophic adaptations that are observed during drug addiction.

5. Our experiments demonstrate that Egr-1 and Arc expression in the DG are associated with morphine-withdrawal memory retrieval and suggest that these genes may contribute to plasticity and reconsolidation accompanying the retrieval process. Besides, GCs play an essential role during the formation of long-term withdrawal-aversive memories, given that ADX-dependent animals exhibit reduced place aversion and decreased Egr-1 and Arc levels in the DG. In addition, our data provide the first evidence that GCs mediate transcriptional events required for morphine-withdrawal memory consolidation and influence epigenetic mechanisms following retrieval in the BLA, supporting the idea that targeting GCs in this amygdalar area may provide important insights into the role of essential signaling cascades mediating aversive drug memories.

INDEX

| | |
|--|-----------|
| RESUMEN | 11 |
| ABSTRACT | 19 |
| ABBREVIATIONS | 33 |
| PRESENTACIÓN | 39 |
| INTRODUCTION | 43 |
| I. BACKGROUND | 47 |
| 1. DRUG ADDICTION | 49 |
| 1.1. DRUG-CENTERED THEORIES | 50 |
| 1.2. INDIVIDUAL DIFFERENCES IN THE ETIOLOGY OF ADDICTION | 51 |
| 2. NEUROCIRCUITRY OF ADDICTION | 53 |
| 2.1. MESOCORTICOLIMBIC CIRCUIT | 53 |
| 2.2. THE HYPOTHALAMIC-PITUITARY-ADRENAL (HPA) AXIS | 56 |
| 2.3. THE EXTRAHYPOTHALAMIC CRF CIRCUIT | 57 |
| 2.4. NORADRENERGIC PATHWAYS | 58 |
| 3. NEUROPLASTICITY UNDERLYING TRANSITION TO ADDICTION | 58 |
| 3.1. CELLULAR PLASTICITY AND TRANSCRIPTIONAL MECHANISMS | 60 |
| 3.1.1. CREB | 60 |
| 3.1.2. Δ fosB | 61 |
| 3.1.3. Nur transcription factors subfamily and Pitx3 | 63 |
| 3.1.4. Neurotrophins | 64 |
| 3.2. SYNAPTIC PLASTICITY | 66 |
| 3.3. STRUCTURAL PLASTICITY | 67 |

| | |
|--|----------------------|
| 4. STRESS AND ADDICTION | 68 |
| 4.1. STRESS AND DRUG TAKING | 69 |
| 4.2. STRESS AND WITHDRAWAL | 69 |
| 4.3. STRESS AND REINSTATEMENT OF DRUG SEEKING | 70 |
| 4.4. STRESS AND REWARD PATHWAYS | 70 |
| 4.5. STRESS AND THE NORADRENERGIC SYSTEM | 71 |
| 4.6. STRESS AND MOLECULAR MECHANISMS | 72 |
| 5. EPIGENETIC MECHANISMS | 72 |
| 5.1. OPIATES AND miRs | 74 |
| 6. MEMORY MODULATION..... | 74 |
| 6.1. MEMORY CONSOLIDATION | 75 |
| 6.2. MEMORY RETRIEVAL | 77 |
| 6.3. EGR1 | 78 |
| 6.4. ARC AND MEMORY | 79 |
| 6.5. DRUG ADDICTION AND MEMORY | 80 |
| 7. GLIA AND ADDICTION | 81 |
| 7.1. ASTROCYTES AND GLUTAMATE HOMEOSTASIS DURING ADDICTION | 82 |
| 7.2. PLEIOTROPHIN AND MIDKINE | 83 |
| <u>II. OBJETIVOS</u> | <u>85</u> |
| <u>III. AIMS</u> | <u>91</u> |
| <u>IV. ARTICLES</u> | <u>97</u> |
| 1. GLUCOCORTICOIDS REGULATION OF FOSB/ΔFOSB EXPRESSION INDUCED BY CHRONIC OPIATE EXPOSURE IN THE BRAIN STRESS SYSTEM..... | 99 |
| 2. MORPHINE ADMINISTRATION MODULATES EXPRESSION OF ARGONAUTE 2 AND DOPAMINE-RELATED TRANSCRIPTION FACTORS INVOLVED IN MIDBRAIN DOPAMINERGIC NEURONS FUNCTION..... | 100 |

| | |
|---|------------|
| 3. MORPHINE REGULATES ARGONAUTE 2 AND TH EXPRESSION AND ACTIVITY BUT NOT MIR-133B IN MIDBRAIN DOPAMINERGIC NEURONS | 101 |
| 4. DYSREGULATION OF DOPAMINERGIC REGULATORY MECHANISMS IN THE MESOLIMBIC PATHWAY INDUCED BY MORPHINE AND MORPHINE WITHDRAWAL..... | 102 |
| 5. REGULATION OF DOPAMINERGIC MARKERS EXPRESSION IN RESPONSE TO ACUTE AND CHRONIC MORPHINE AND TO MORPHINE WITHDRAWAL | 103 |
| 6. GLIAL ACTIVATION AND MIDKINE AND PLEIOTROPHIN TRANSCRIPTION IN THE VENTRAL TEGMENTAL AREA ARE MODULATED BY MORPHINE ADMINISTRATION..... | 104 |

V. CONCLUSIONES **105**

VI. CONCLUSIONS **109**

VII. APPENDIX **113**

| | |
|---|------------|
| 1. OBJETIVOS SUPLEMENTARIOS | 115 |
| 2. SUPPLEMENTAL AIMS..... | 117 |
| 3. ACUTE MORPHINE, CHRONIC MORPHINE AND MORPHINE WITHDRAWAL DIFFERENTLY AFFECTS PLEIOTROPHIN, MIDKINE AND RECEPTOR PROTEIN TYROSINE PHOSPHATASE β/ζ REGULATION IN THE VENTRAL TEGMENTAL AREA..... | 119 |
| 4. REGULATION OF PLEIOTROPHIN, MIDKINE, RECEPTOR PROTEIN TYROSINE PHOSPHATASE β/ζ AND THEIR INTRACELLULAR SIGNALING CASCADES IN THE NUCLEUS ACCUMBENS DURING OPIATE ADMINISTRATION | 165 |
| 5. GLUCOCORTICOID HOMEOSTASIS IN THE DENTATE GYRUS IS ESSENTIAL FOR OPIATE WITHDRAWAL-ASSOCIATED MEMORIES | 167 |
| 6. DIFFERENT CONTRIBUTION OF GLUCOCORTICOIDS IN THE BASOLATERAL AMYGDALA TO THE FORMATION AND EXPRESSION OF OPIATE WITHDRAWAL-ASSOCIATED MEMORIES..... | 227 |

VIII. REFERENCES

289

ABBREVIATIONS

| | |
|-------------------|--|
| ACTH: | Adrenocorticotrophin hormone |
| ADX: | Adrenalectomized |
| Ago: | Argonaute |
| Akt: | Thymoma viral proto-oncogene |
| AMPA: | α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |
| Arc: | Activity-regulated cytoskeletal-associated protein |
| ATP: | Adenosine triphosphate |
| BDNF: | Brain derived neurotrophic factor |
| BLA: | Basolateral amygdala |
| BNST: | Bed nucleus of the stria terminalis |
| CaMKII: | Calcium/calmodulin-dependent protein kinase type II |
| cAMP: | Cyclic adenosine monophosphate |
| CDK5: | Cyclin-dependent kinase 5 |
| CeA: | Central nucleus of the amygdala |
| CNS: | Central nervous system |
| CORT: | Corticosterone |
| CPA: | Conditioned place aversion |
| CREB: | cAMP response element binding protein |
| CRF: | Corticotropin-releasing factor |
| CRFR1: | CRF receptor 1 |
| DA: | Dopamine |
| DAT: | Dopamine transporter |
| DG: | Dentate gyrus |
| DNA: | Deoxyribonucleic acid |
| DOR: | Delta opioid receptor |
| DR: | Dopamine receptor |
| DRD1: | Dopamine receptor 1 |
| DRD2: | Dopamine receptor 2 |
| DSM: | Diagnostic and Statistical Manual of Mental Disorders |
| e.g.: | For example |
| Egr: | Early growth response |
| ERK: | Extracellular-signal regulated kinase |
| GABA: | Gamma-aminobutyric acid |
| GABAergic: | GABA releasing |
| GCs: | Glucocorticoids |

| | |
|--------------------|----------------------------------|
| GFAP: | Glial fibrillary acidic protein |
| GLT: | Glutamate transporter |
| GR: | Glucocorticoid receptor |
| HPA: | Hypothalamic-pituitary-adrenal |
| HRs: | High responders |
| i.c.v.: | Intra-cerebroventricular |
| IEG: | Immediate-early gene |
| IP3: | Inositol-1,4,5-trisphosphate |
| IRS2: | Insulin receptor substrate-2 |
| ISuE: | Intensified Sustained Escalated |
| kDa: | Kilodaltons |
| KO: | Knock out |
| KOR: | Kappa opioid receptor |
| LC: | Locus coeruleus |
| LoC: | Loss Control |
| LRs: | Low responders |
| LTD: | Long-term depression |
| LTP: | Long-term potentiation |
| MAPK: | Mitogen-activated protein kinase |
| mGluR: | Metabotropic glutamate receptor |
| miRs: | microRNAs |
| MK: | Midkine |
| MOR: | Mu opioid receptor |
| MR: | Mineralocorticoid receptor |
| MSN: | Medium spiny neuron |
| NA: | Noradrenaline |
| NAc: | Nucleus accumbens |
| NAc(Core): | Core of the NAc |
| NAc(Shell): | Shell of the NAc |
| NeuroD: | Neurogenic differentiation |
| NFκB: | Nuclear factor kappa B |
| NGF: | Nerve growth factor |
| NMDA: | N-Methyl-D-aspartate |
| nt: | Nucleotide |
| NT3: | Neurotrophin-3 |

| | |
|--------------------------------------|---|
| NT4/5: | Neurotrophin-4/5 |
| NTS: | Nucleus tractus solitarius |
| p75NTR: | p75 neurotrophin |
| PFC: | Prefrontal cortex |
| PI3K: | Phosphatidylinositol 3'-kinase |
| PLCγ: | Phospholipase C γ |
| POMC: | Proopiomelanocortin |
| PTN: | Pleiotrophin |
| PVN: | Paraventricular nucleus |
| ReS: | Recreational Sporadic |
| RNA: | Ribonucleic acid |
| RPTPβ/ζ: | Receptor protein tyrosine phosphatase β/ζ |
| SN: | Substantia nigra |
| SUDs: | Substance Use Disorders |
| TH: | Tyrosine hydroxylase |
| TLR4: | Toll-like receptor 4 |
| TOR: | Target of rapamycin |
| TORC2: | TOR complex 2 |
| TrkB: | Tropomyosin-related kinase B |
| UTR: | Untranslated region |
| VGluT: | Vesicular glutamate transporter |
| VMAT2: | Vesicular monoamine transporter 2 |
| VP: | Ventral pallidum |
| VTA: | Ventral tegmental area |

PRESENTACIÓN

El Grupo de Farmacología Celular y Molecular se ha ocupado, durante años, del estudio de diferentes factores transcripcionales y post-transcripcionales relacionados y/o responsables de la dependencia de opiáceos y del síndrome de abstinencia a los mismos. En la última década se ha propuesto la importancia del estrés en la génesis y mantenimiento de los procesos adictivos a sustancias de abuso, así como en las recaídas en el consumo. Nuestro Grupo se ha interesado por las bases neurobiológicas que relacionan estrés y adicción, y ha profundizado en el estudio de los diferentes factores implicados en dicha conexión: genes de expresión inmediata, proteínas quinasas, así como neurotransmisores y hormonas.

Recientemente se ha sugerido que la patología adictiva es el resultado de procesos maladaptativos de plasticidad neuronal causados por el consumo de sustancias de abuso, y que conducen a cambios a largo plazo en la expresión génica en áreas concretas del sistema nervioso central (CNS), dando lugar a profundas anormalidades del comportamiento que persisten a lo largo de toda la vida del adicto. Debido a estas características de la adicción, se ha sugerido el importante papel que podrían desempeñar los mecanismos epigenéticos (metilación de DNA, modificación de histonas y RNAs no codificantes) en dicha patología. Así, actualmente ya se sabe que las sustancias adictivas pueden alterar mecanismos epigenéticos que modularían (silenciando o sobre-expresando) la expresión de genes implicados en la neuroplasticidad, perturbando los niveles intracelulares de proteínas y modificando los procesos de señalización dependientes de diferentes neurotransmisores, alterando en último término el procesamiento de la información en diferentes circuitos neuronales: circuitos de recompensa, circuitos del estrés, memoria y control de la función ejecutiva, entre otros.

En base a estos hallazgos, nos hemos planteado el estudio de diferentes circuitos neuronales, neurotransmisores, factores de transcripción y microRNAs (miRs) que contribuirían a la plasticidad neuronal y memorias aversivas que llevarían al establecimiento del fenotipo adictivo. Esta Tesis aborda: 1) la caracterización de los cambios en factores de transcripción responsables del fenotipo dopaminérgico en la vía mesolímbica (Nurr1 y Pitx3) tras la administración aguda de morfina (intoxicación/binge), durante la dependencia y tras la inducción del síndrome de abstinencia. Además se ha estudiado la posible relación entre dichos cambios y factores epigenéticos (miR-133b). Para realizar estos estudios se estableció una colaboración con la Dra. Raquel E Rodríguez (INCYL, Salamanca); 2) los aspectos que probablemente subyacen en las modificaciones transcripcionales, post-transcripcionales y epigenéticas en el sistema cerebral del estrés y en los circuitos responsables de diferentes tipos de memorias, tanto a nivel neuroquímico, como celular y de comportamiento. Para ello se han utilizado modelos animales con déficit de glucocorticoides [(GCs), hormona del estrés] y el paradigma de la aversión condicionada de plaza (CPA) como modelo experimental de memorias aversivas. Para llevar a cabo este trabajo se estableció una colaboración con la Dra. Krisztina Kovács (Institute of Experimental Medicine, Budapest, Hungría); 3) la contribución de los astrocitos a la

plasticidad sináptica durante la adicción a opiáceos, mediante la síntesis y liberación de citoquinas (midkina –MK- y pleiotrofina –PTN-) en los circuitos de recompensa.

Este trabajo constituye la presente Tesis Doctoral cuyos resultados han sido parcialmente publicados en siete manuscritos y tres en revisión.

INTRODUCTION

Cellular and Molecular Pharmacology Group has focused on the research of different transcriptional and post-transcriptional factors influencing opiate dependence and withdrawal. In the last decade, it has been suggested a prominent role for stress on drug seeking and taking and the development of drug addiction, as well as on drug reinstatement. We have focused our interest on the neurobiological basis connecting stress and addiction and the relationship between different factors that mediate this disease: neurotransmitters, hormones, immediate early genes...

It has been suggested that drug addiction is a result of maladaptive synaptic plasticity mechanisms that are initiated during drug taking. Long-term changes in gene expression in different brain areas would mediate the long-lasting addictive behaviors. Because of these altered behaviors are long-lasting, epigenetic mechanisms have been proposed as mediators of this phenomenon. Thus, it has been described that several drugs of abuse can modulate some epigenetic mechanisms that, in turn, would enhance or repress different genes related with neuroplasticity, intracellular protein levels and/or signaling cascades depending on neurotransmitters action. All these changes would lead to altered functioning of different neuronal circuits: brain reward system, brain stress system, learning and memory or decision-making.

All these data prompted us to study different brain networks, neurotransmitters, transcription factors and miRs that would contribute to neuronal plasticity and aversive memories during the development of the addicted phenotype. The current Thesis investigates: 1) changes in the transcription factors that characterize the dopaminergic phenotype (Nurr1 and Pitx3) in the mesolimbic circuit after acute and chronic morphine and during morphine withdrawal. Moreover, the possible relationship between such transcription factors and epigenetic mechanisms (miR-133b) was also studied. For this purpose, we have collaborated with Dr. Raquel Rodriguez (INCYL, Salamanca); 2) an altered epigenetic, transcriptional and post-transcriptional regulation of limbic and memory circuits, and the relationship between behavioral and molecular changes. For that purpose we have used bilaterally adrenalectomized rats and the CPA paradigm, as expression of aversive memories related to morphine withdrawal. We collaborated with Dr. Krisztina Kovács (Institute of Experimental Medicine, Budapest, Hungary) to develop this experiments; 3) the role of astrocytes in synaptic plasticity during morphine dependence by means of the synthesis and release of cytokines (MK and PTN) in the brain reward system.

All these results that compose the current Thesis, have been published in seven research articles and three manuscripts that are under revision currently.

I. BACKGROUND

1. DRUG ADDICTION

Opiates are the most potent compounds known today to control pain, and are also among the most used drugs of abuse (1). Drug addiction is a chronic relapsing disorder characterized by compulsive drug seeking and taking (2,3). *Diagnostic and Statistical Manual of Mental Disorders* (DSM) provides a series of behavioral symptoms (items) that if present in an individual, allows making the diagnosis of specific pathological behaviors such as drug dependence. DSM-IV (4) describes possible behavioral alterations characterizing loss of control that can be grouped into three main categories: 1) difficulty on limiting drug use and seeking; 2) extremely strong motivation to procure the drug; 3) maintaining drug use despite awareness of negative consequences. The DSM-V (5) replaces the two separate DSM-IV categories of abuse and dependence with a single category: Substance Use Disorders (SUDs) along with different severities of SUD.

The compulsive use of drugs despite serious negative consequences defines addiction as a mental illness (6). Research supports the idea that addiction is a true psychiatric pathology and not an iatrogenic disease resulting from a vice. Thus, addiction should not be considered a condition for which the individual is largely responsible and for which he or she should be punished, but as a disease that needs treatment (7).

Recently, Piazza and Deroche-Gamonet (2) proposed a general theory of transition to addiction that is composed of three phases (Figure 1). The first step is a non-pathological phase of drug-taking in which there is Recreational Sporadic (ReS) drug use. The ReS phase starts when the individual learns to take drugs as one among many of his or her recreational activities. Drug use is then sporadic and occupies a small portion of the behavioral repertoire. This can be considered normal behavior and is present in a large proportion of the human population when both legal and illegal drugs are taken into account. The second step, Intensified Sustained Escalated (ISuE) drug use, starts with an increase in frequency, amount, and motivation to take drugs. Drug-taking intensifies and becomes sustained; additionally, drug-related problems appear but are not sufficient to promote spontaneous abstinence. The ISuE phase is the first moderate pathological state, one in which the individual takes too much drug but behavior is still organized and the individual is, generally, well integrated into society. The third and final step signals the beginning of the most serious pathological state. In this case, the individual largely has Loss Control (LoC) of drug intake and becomes fully addicted. In this phase, drug-taking becomes the individual's major goal-directed activity, invading most of the space normally occupied by other components of his or her normal behavioral repertoire. Then, social degradation is inevitable, and relapses to addiction -even after prolonged abstinence- is the rule. When the individual goes from liking drugs to pathologically mourning them when they are not available, the process of transition to addiction is complete. It is important to emphasize that in the

transition from ReS to ISuE, the individual progresses from taking drugs sporadically and moderately to taking them frequently and in large quantity. Thus, drug intake first changes principally quantitatively (from recreational to sustained). In contrast, in the transition from ISuE to LoC, the principal difference is not how much drug is taken but where the individual is able to confine drug-taking (qualitatively changes). In this theory, physical and/or emotional withdrawal is observed as an additional process that promotes excessive drug intake, thus contributing to the transition to addiction (2).

The establishment of an allostatic state has been proposed by Le Moal and Koob as the crucial phenomenon in the addiction process (8,9,10). The idea is that following extended drug use, reward systems adapt to the daily overexposure of the brain to drugs by shifting the homeostatic set point (allostasis) to adapt to this continuous overstimulation. Because of this shift, the drug state progressively becomes the normal state and the nondrug state is now perceived as a pathological, or at least, as an unpleasant state, so drugs progressively shift from being strongly wanted to also strongly needed (8,11,10).

1.1. DRUG-CENTERED THEORIES

Drug-centered theories of addiction include all views of addiction for which taking a drug repeatedly is the major cause of addiction. According to these theories, addiction is principally a consequence of drug intake and results from the psychopharmacological changes that follow chronic drug use. These theories focused on specific drug-induced changes, such as tolerance (12), sensitization (13,14), withdrawal and allostasis (10,15,16) or drug-induced cognitive changes in impulsivity (17), decision making (18,19) and conditioning (20). These views of addiction were reinforced by the very significant neurobiological alterations at molecular, cellular (21,22,23,24), synaptic (25,26), and network levels (27,28,29,30) that follow chronic drug intake.

Phases and Process of Transition to Addiction

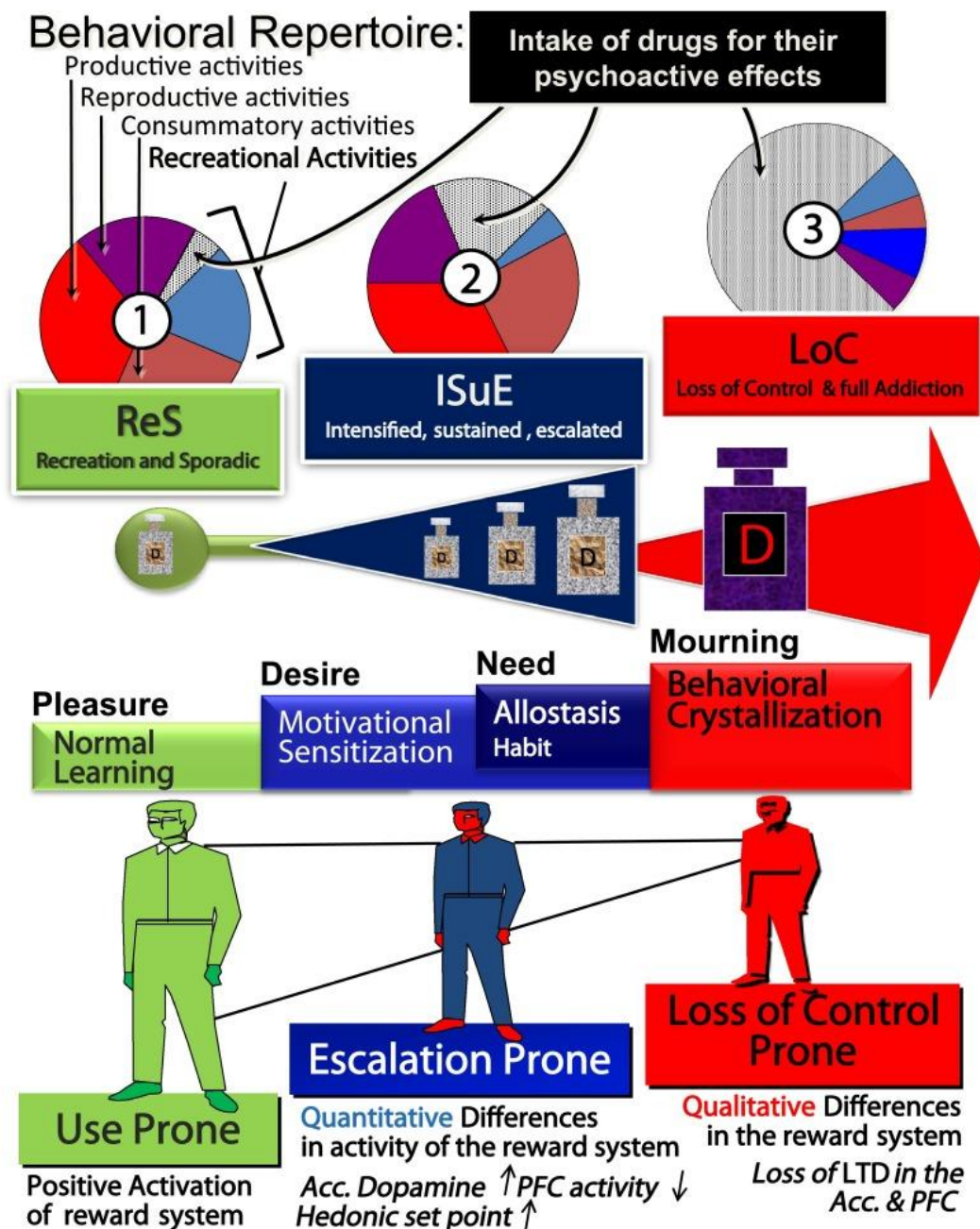


Figure 1: Recently, Piazza and Deroche-Gamonet proposed a general theory of transition to addiction that is composed of three consecutive and independent phases: consecutive, because entering one phase is a necessary condition to go to the next one; independent, because entering one phase is not a sufficient condition to progress to the next one.

Reproduced from ref (2): Piazza PV, Deroche-Gamonet V. A multistep general theory of transition to addiction. *Psychopharmacology*. 2013. 229:387-413.

1.2. INDIVIDUAL DIFFERENCES IN THE ETIOLOGY OF ADDICTION

It has been proposed that a “vulnerable” phenotype that predisposes to addiction exists (31). Individual differences in the responses to drugs of abuse have been widely demonstrated in humans (32,33) and in laboratory animals (31,34,35). It was showed that when low unitary doses of drugs are

made available, some rats (high responders, HRs) rapidly escalated their drug intake, reaching and maintaining a high level, whereas others (low responders, LR) do not (36) (Figure 2). Moreover, self-administration-prone subjects can be identified because of their behavioral reactivity to a stress challenge (36). Thus, animals with a higher locomotor response to novelty (HR) compared with low responder animals (LR), showed the highest sensitivity to drugs (36,31). These observations were confirmed when it was showed that HR rats had a higher preexisting dopaminergic activity (37,38,39) and a higher corticosterone (CORT) secretion in response to stress (40) than did LR rats.

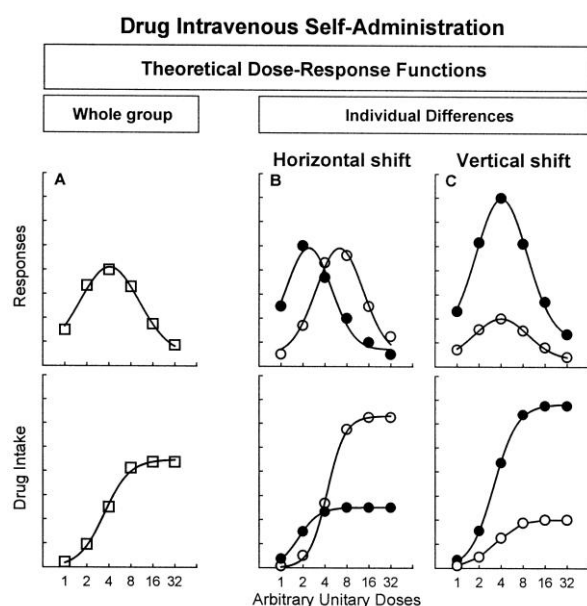


Figure 2: According to the model of horizontal shifts, vulnerable subjects are the most sensitive, i.e., the ones leftward shifted, and these individuals would develop self-administration at low unitary doses of the drug (Fig. 2B, top). However, at higher doses, “resistant” subjects, which are the less sensitive and rightward shifted, would provide an equal rate of self-administration (Fig. 2B, top) and even take in vertical shifts (Fig. 2C), vulnerable subjects, which are the upward shifted ones, present a higher rate of responding across doses (Fig. 2C, top) and consume the highest quantities of the drug (Fig. 2C, bottom). Consequently, these vulnerable individuals would have the highest chances to develop drug abuse also in environmental conditions in which both low and high drug doses are available.

Reproduced from ref (35): Piazza PV, Deroche-Gamonet V, Rouge-Pont F, Le Moal M. Vertical shifts in self-administration dose-response functions predict a drug-vulnerable phenotype predisposed to addiction. *J Neurosci.* 2000. 20:4226-32.

The role of individual vulnerabilities has been also established for the most severe SUDs, which is characterized by loss of control (41). After three months of self-administration, the three addiction-like behaviors according to the DMS-IV [1) high motivation for the drug, measured by a progressive ratio reinforcement schedule; 2) inability to refrain from drug seeking even if the drug was not available, measured by active responses during periods of signaled drug non-availability; 3) drug use despite negative consequences, measured by resistance to foot-shock-induced punishment at cocaine self-administration], appeared in a small percentage of rats, whereas the largest proportion of rats maintained good control over drug intake (41). Importantly, vulnerability to loss of control was completely unrelated to vulnerability to sustained drug use (41,42). Recent publications support the existence of these two independent vulnerable phenotypes by showing that vulnerability to loss of control is associated with a different type of biological modification than the vulnerability to develop sustained escalated drug use (43,44).

Thus, individual-centered theories of addiction support that addiction results from a pathological response to the drug that is generated in a few individuals by a vulnerable biological

phenotype. The main differences among theories in this family principally concern different degrees of etiological relevance given to genetic factors (45,46,47,48), environmental factors (31,49,50), developmental factors (51,52,53), and gene plus environment interactions (54,55,56).

2. NEUROCIRCUITRY OF ADDICTION

2.1. MESOCORTICOLIMBIC CIRCUIT

All drugs of abuse exert their primary rewarding effects on the mesolimbic dopamine (DA) reward pathway, which consists of DA neurons originating in the ventral tegmental area (VTA) and extending to the striatal complex including the nucleus accumbens (NAc), the prefrontal cortex (PFC), the hippocampus and the amygdala (57). Addictive substances increase, directly or indirectly, firing of dopaminergic neurons in the VTA of the midbrain and a subsequent increase of DA release in the NAc (58,59). The NAc not only receives dopaminergic projections from the VTA, but also glutamatergic projections from a number of neocortical, allocortical, and thalamic areas, and plays a central role in the neurobiological processes of reward, learning, impulsivity, and addiction (60). Opiates bind to the three classical opioid receptors, mu (MOR), delta (DOR), and kappa (KOR), and mediate DA release by reducing the inhibitory gamma-aminobutyric acid (GABA) release on VTA DA neurons (61,62,63). It has also been established that morphine increases DA level through the MOR in the NAc, which may mediate reinforcing effects of morphine (64). Relevant to these observations, endogenous opioid peptides, such as enkephalins or dynorphins, are upregulated in the NAc after exposure to morphine, and modulate DA release in the midbrain (65). Two major distinctions exist between DA release following motivational biological stimuli versus following exposure to an addictive drug. First, the release of DA by addictive drugs is of greater amplitude and duration than the one that can be achieved through physiological mechanisms. The second major difference is that tolerance develops to the release of DA by biological stimuli, whereas addictive drugs release DA every time the drug is taken. In chronic users, increased dosage is required due to tolerance, but with sufficient dose, a DA increase reliably occurs (66,67).

The association between increased DA transmission and learning behaviors to obtain reward has led to an understanding that DA release is a key event to facilitate learning. The release of DA has been proposed to imbue an event with salience, creating an internal sense that this event requires the development of a behavioral response (68). Thus, once a behavior designed to obtain a reward or avoid a negative consequence has been learned, the role of DA changes from promoting new learning to enabling the use of learned information to efficiently execute the adaptive behavioral response (69).

Thus, the increase in DA is backward-shifted and is activated by discriminative stimuli that predict drug availability more than by the drug itself (70). Consequently, conditioned stimuli associated with drug delivery become important discriminative stimuli of drug availability and, under some training conditions, secondary reinforcers. It is noteworthy that exactly the same phenomenon happens with natural reinforcers, showing that these neurobiological mechanisms activated by drugs are part of the normal learning sequence (71).

More than 90% of the cells in the NAc are GABA releasing (GABAergic) medium spiny neurons (MSNs) with a small portion of cholinergic aspiny cells and GABAergic interneurons (72). MSNs in the striatum are typically divided into two groups, based on DA receptors (DR) (Figure 3). To date, five different subtypes of DR have been cloned, and based on their structural and pharmacological properties, a general subdivision into two groups has been made: the D1-like receptors (DRD1)-like, which stimulate intracellular cyclic adenosine monophosphate (cAMP) levels, comprising D1 (73,74) and D5 (75,76), and the D2-like receptors (DRD2)-like, which inhibit intracellular cAMP levels, comprising D2 (77,78), D3 (79), and D4 (80) receptors. In contrast to dorsal striatum, the discrete separation of DRD1-MSNs in the direct pathway and DRD2-MSNs in the indirect pathway does not apply in NAc (72) (Figure 4). DRD2 are located postsynaptically in soma and dendrites to regulate firing rate of neurons and presynaptically in axons to regulate DA synthesis and release (81). However, it has been shown that DRD2 located postsynaptically in GABA projecting neurons of the striatum also regulate DA release (82).

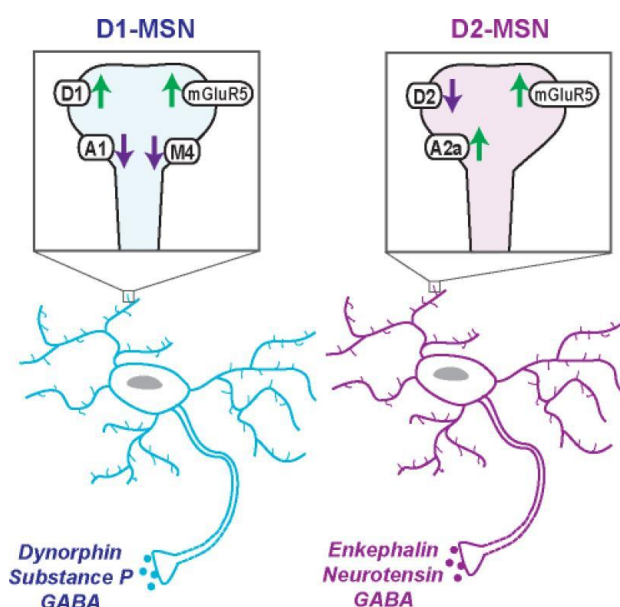


Figure 3: DRD1 and DRD2 MSNs in the NAc with co-localized receptors and neuropeptides. DRD1-MSNs also express M4 cholinergic receptors, A1 adenosine receptors, dynorphin, and substance P, whereas DRD2-MSNs co-express A2a adenosine receptors, enkephalin, and neurotensin.

Reproduced from ref (72): Smith RJ, Lobo MK, Spencer S, Kalivas PW. Cocaine-induced adaptations in D1 and D2 accumbens projection neurons (a dichotomy not necessarily synonymous with direct and indirect pathways). *Curr Opin Neurobiol.* 2013. 23:546-52.

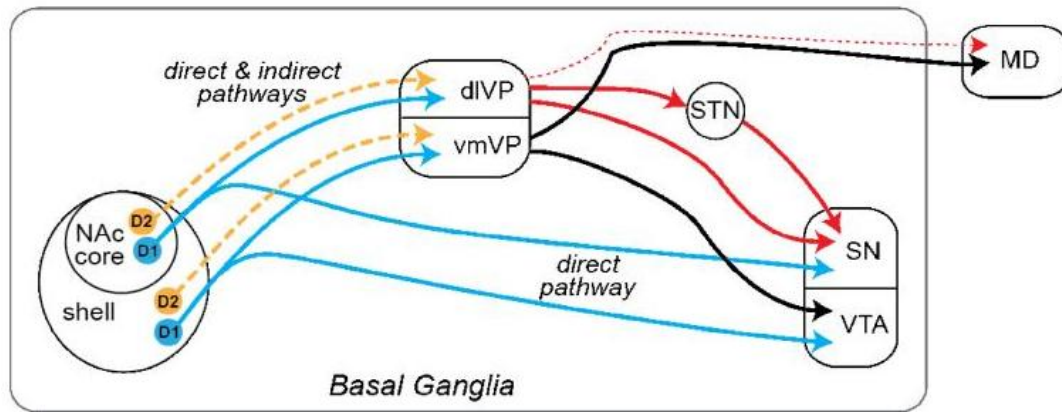


Figure 4: Output pathways of NAc DRD1-MSNs and DRD2-MSNs. Both, DRD1-MSNs and DRD2-MSNs in NAc project to ventral pallidum (VP), whereas DRD1-MSNs alone project to ventral mesencephalon output structures.

Reproduced from ref (72): Smith RJ, Lobo MK, Spencer S, Kalivas PW. Cocaine-induced adaptations in D1 and D2 accumbens projection neurons (a dichotomy not necessarily synonymous with direct and indirect pathways). *Curr Opin Neurobiol.* 2013. 23:546-52.

DA has different affinity for DRD1-like and DRD2-like receptors. DRD1 receptor, which is known as the low-affinity DA receptor, is thought to be preferentially activated by the transient, high concentrations of DA mediated by phasic bursts of dopaminergic neurons (83,84). In contrast, it is hypothesized that DRD2-like receptors, which are known to have a high affinity for DA, can detect the lower levels of tonic DA release (85). The effects of DR activation are complex and distinctions exist between activation of DRD1-like versus DRD2-like receptors (81). One signaling pathway of particular interest in neurons is the mitogen-activated protein kinases (MAPK), extracellular-signal regulated kinases (ERK), which are activated by DRD1 and DRD2 (81)(Figure 5,6).

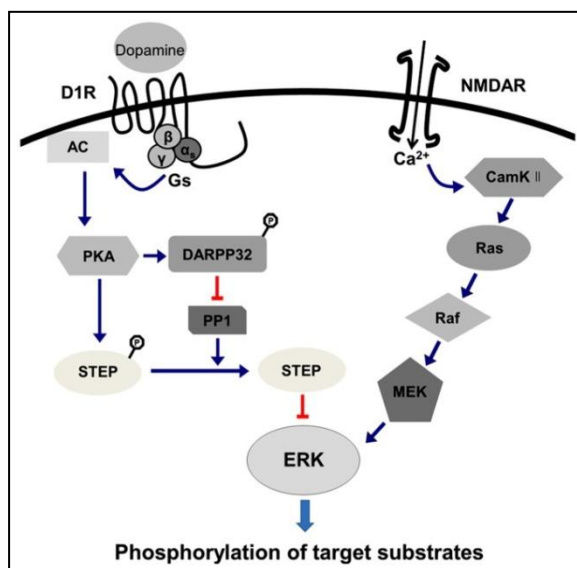


Figure 5: DRD1 receptor-mediated ERK activation signaling pathway.

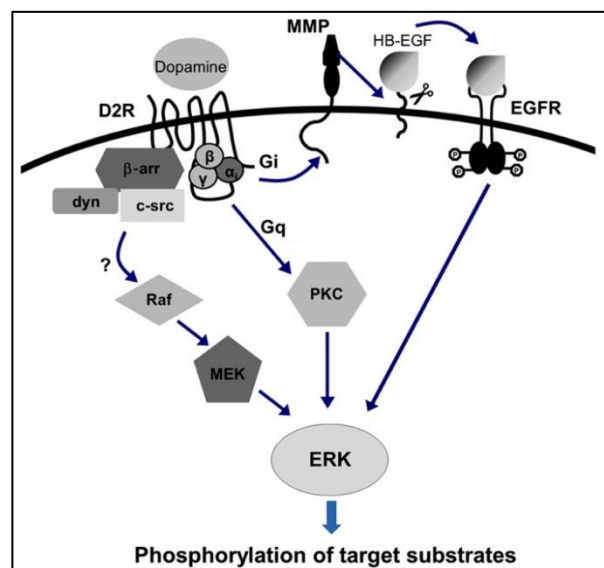


Figure 6: DRD2 receptor-mediated ERK activation signaling pathway.

Figure 5 and 6 reproduced from ref (81): Baik JH. Dopamine signaling in reward-related behaviors. 2013. 7:152.

Importantly, DRD1-MSN and DRD2-MSN cell populations are implicated in distinct aspects of NAc function. NAc DRD1-MSNs are critical for acquiring reward-based learning, and blockade of DRD2-MSNs is associated with loss of behavioral flexibility when the task calls for a switch in learning strategies (86). Opposing roles for the cell types in learning is also supported by the finding that selective stimulation of DRD1-MSNs in dorso-medial striatum induces persistent reinforcement, while stimulating DRD2-MSNs causes transient punishment (87). Concerning drug-associated behaviors, DRD1-MSNs in the NAc or dorso-medial striatum are involved in the development of place preference and sensitization, whereas DRD2-MSNs oppose these behaviors (88,89,90,91).

2.2. THE HYPOTHALAMIC-PITUITARY-ADRENAL (HPA) AXIS

In response to stressful stimuli, secretory neurons of the paraventricular nucleus (PVN) of the hypothalamus discharge corticotropin-releasing factor (CRF) that in turn increases both the secretion of adrenocorticotrophin hormone (ACTH) and the transcription of its precursor, the proopiomelanocortin (POMC) gene, in the anterior pituitary. ACTH stimulates the release of GCs (cortisol in humans and CORT in rodents) and the transcription of genes encoding several steroidogenic enzymes in the adrenal gland. GCs exert diverse effects on target tissues to mobilize energy for the body to deal with the stressor, and also exert a negative feedback through the inhibition of the synthesis and secretion of CRF and POMC (92,93,94,95) (Figure 7). GCs are highly lipophilic and, thus, rapidly enter the brain and bind directly to mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs) (96,97). MRs have a high affinity for the natural steroids CORT and aldosterone and are mostly saturated during basal CORT levels, whereas GRs become occupied by higher levels of CORT (98,99).

The HPA axis is activated following acute administration of many addictive substances, causing increased ACTH and CORT levels in plasma (100). Chronic administration of drugs of abuse in the same animal models results in either a sustained increase in HPA axis function, in the case of cocaine and amphetamine, or a reduced effect of the initial activating effects of the drug, in the case of morphine, nicotine, and alcohol (101,102,103,104). Human studies demonstrate similar perturbations following illicit drug use, with slight differences. As in animal models, acute administration of cocaine (105), alcohol (106) and nicotine (107) increases cortisol levels, whereas acute exposure to opiates decreases cortisol levels (108,109). Activation of the HPA axis is maintained in cocaine addicts (110), whereas following chronic opiate use, HPA responses are reduced over time (111), a more typical response to repeated exposure to a stressor (112,113).

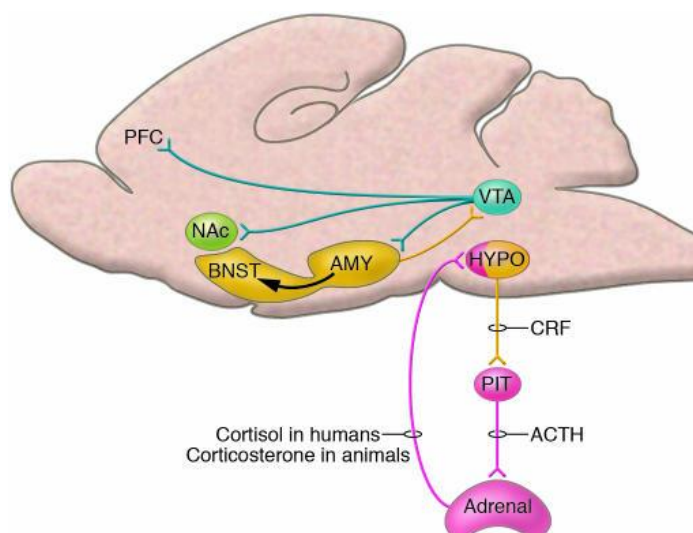


Figure 7: The mesolimbic DA reward pathway is depicted in blue. The hypothalamic CRF projections are directed to the pituitary gland (PIT) and stimulate the endocrine output of the HPA axis (red) including the release of ACTH, which acts on the adrenal gland to stimulate the secretion of GCs. Central CRF circuitry (yellow) consists of CRF-containing cell bodies located in the central nucleus of the amygdala, which projects to the BNST. CRF projections from the amygdala also innervate the VTA, thus completing the circuit.

Reproduced from ref (114): Cleck JN, Blendy JA. Making a bad thing worse: adverse effects of stress on drug addiction. 2008. 118:454-461.

2.3. THE EXTRAHYPOTHALAMIC CRF CIRCUIT

In addition to its crucial role in the activation of the HPA axis, CRF functions as a neurotransmitter/neuromodulator within the CNS, coordinating extra-hypothalamic aspects of the stress response (115,116). The placement of CRF and its receptors, CRF receptor 1 (CRFR1) and CRFR2, throughout the limbic system and neocortex suggests a critical role for this peptide in affective disorders, including addiction (117). Both CRF receptors are seven transmembrane domain G-protein coupled receptors that function by interacting with G_s -protein, thus increasing cAMP levels, although may also couple other G-proteins (118).

The extrahypothalamic CRF circuit, also called the extended amygdala, is an important structure for emotional and affective behavior, and has been delineated in mediating stress-induced relapse. Structures comprising the extended amygdala overlap with those of the reward pathway, including the central nucleus of the amygdala (CeA), the bed nucleus of the stria terminalis (BNST), and the shell of the NAc [NAc(shell)] (119,120) (Figure 7). Thus, the extended amygdala represents a common anatomical substrate where brain arousal-stress systems with hedonic processing systems are integrated in order to produce the negative emotional states that promote negative reinforcement mechanisms, associated with the development of addiction (60).

CRF from extrahypothalamic sources was demonstrated to play a key role in the behavioral responses to stressors (121). Cocaine, morphine and alcohol tend to increase CRF expression acutely (122,123,124,125,126); however, the direction in which CRF expression is altered chronically and following withdrawal from these drugs depends on both the brain region and the drug studied. For example, following chronic morphine administration increased CRF levels were observed in the

hypothalamus, while decreased levels have been reported in the BNST (124,125). The differences in CRF expression among the different drugs highlight the distinctive pharmacological and molecular mechanisms throughout the CNS that each drug uses in exerting its addictive properties (114).

2.4. NORADRENERGIC PATHWAYS

Literature points for a role of noradrenergic transmission within the reward system in drug addiction. α 1-adrenergic receptors control a neural pathway responsible for the release of DA in the NAc (127). Additionally, noradrenergic neurons modulate the DA cell firing pattern via excitatory postsynaptic α 1-adrenoceptors (128). Moreover, DA release in the PFC is regulated by local noradrenergic nerve terminals, and electrical stimulation of the locus coeruleus (LC) neurons increases both, extracellular DA and noradrenaline (NA) in the PFC (129).

On the other hand, NA also augments CRF release in different nuclei such as PVN, BNST and CeA (60), and this CRF would induce the release of NA by the brainstem noradrenergic areas (feed-forward system). Interestingly, CRF1R expression in the VTA, LC and nucleus tractus solitarius (NTS) catecholaminergic neurons has been observed (130,131). Thus, CRF neurons would activate the NA cell firing rate, and noradrenergic neurons would stimulate CRF release (132,133). Moreover, an augment of noradrenergic activity in the PFC was detected as a consequence of CRF intra-cerebroventricular (i.c.v.) administration (134). Noradrenergic afferents from the NTS to the extended amygdala and PVN mediate not only the development of the motivational effects of opiate withdrawal, but also the stress-induced reinstatement of drug seeking behavior (135,136).

Previous works from our laboratory found an increased enzymatic activity of tyrosine hydroxylase (TH; the rate limiting enzyme in catecholamine synthesis) and TH activation in the NTS during morphine withdrawal (137,138). In addition, α 1- and α 2-receptor antagonists blocked the increased NA turnover in the PVN, the enhancement in CORT levels and c-Fos expression in CRF cells from the PVN during naloxone-precipitated morphine withdrawal, suggesting that the HPA axis is activated during morphine withdrawal through the modulation of noradrenergic connections from the NTS to the PVN (139,140,141).

3. NEUROPLASTICITY UNDERLYING TRANSITION TO ADDICTION

Different types of molecular and cellular adaptations occur in specific brain regions to mediate addiction-associated behavioral abnormalities. These include alterations in gene expression achieved in part via epigenetic mechanisms, plasticity in the neurophysiological functioning of neurons and

synapses, and associated plasticity in neuronal and synaptic morphology mediated in part by altered neurotrophic factor signaling (142).

It has been reported a relationship between neuroplasticity, motivated learning, brain circuitry, and the stages of addiction (Figure 8) (143). Two different categories of neuroplasticity have been proposed: first, relatively transient changes in neuronal function that continue for hours up to weeks of drug abstinence. Transient neuroplasticity corresponds to the necessary changes that are antecedent to develop a new behavior, and is referred to as social use. Secondly, relatively stable changes lasting from weeks to being relatively permanent changes. Thus, repeated drug insults cause enduring changes in the synaptic physiology of brain circuits regulating cognitive and emotional responding to important environmental stimuli, which guides the execution of learned behavior. This is illustrated in Figure 8a as two phases of relapse. The first phase of relapse (regulated relapse) refers to a relatively declarative decision-making process whereby the addict consciously decides to relapse. In the second phase (compulsive relapse) the addict is not making a conscious choice. Figure 8b maps the current understanding of biological reward memory and learning processes onto the stages of addiction. Thus, the acquisition of memories and developing adaptive behavioral responses to important stimuli is referred to as acquisition and corresponds to social drug use. The counterpart to regulated relapse is the retrieval of declarative memories, that is, memories that are verbalized and are used in conscious decision-making. Finally, compulsive relapse can be considered equivalent to habit or procedural memories. The retrieval of procedural memories is not verbalized, and guides the unconscious execution of adaptive motor behaviors. Figure 8b also illustrates how key brain circuits and corresponding neurotransmitters map onto stages of addiction. Thus, learning to become addicted through social drug use critically involves dopaminergic cells in the VTA that release DA. As drug-seeking becomes well-learned, a reliance of the behavior on glutamatergic projections from PFC to the NAc emerges. Thus, regulated relapse strongly depends on the retrieval of drug-associated memories and the integration of these declarative memories through glutamatergic projections from the PFC to the NAc. While glutamate continues to play a dominant role in compulsive relapse in this model, the glutamatergic circuit shifts from more declarative, executive prefrontal circuitry to habit circuitry involving classic cortico-striato-thalamic motor pattern generators, and the procedural memories that drive the unconscious engagement of well-learned behaviors.

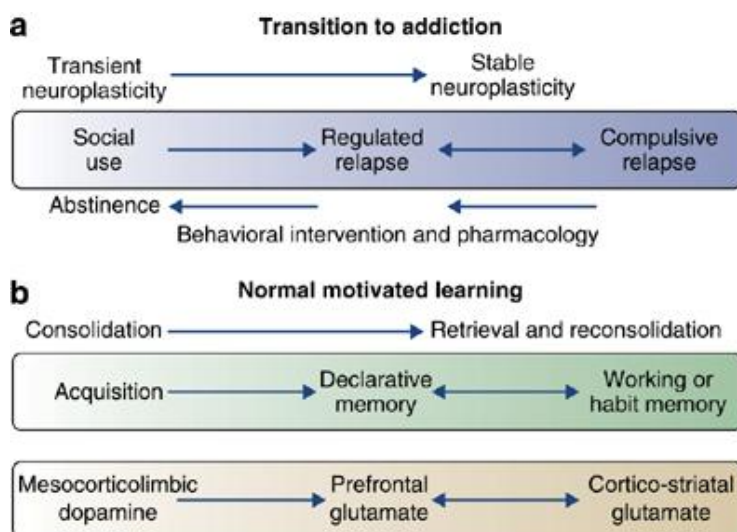


Figure 8: Relationship between neuroplasticity, brain circuitry, and the stages of addiction

Reproduced from ref (143): Kalivas PW, O'Brien C. Drug addiction as a pathology of staged neuroplasticity. *Neuropsychopharmacology*. 2008. 33:166-180.

3.1. CELLULAR PLASTICITY AND TRANSCRIPTIONAL MECHANISMS

Opiate-induced plasticity in different regions includes cellular plasticity (homeostatic changes in intracellular signaling cascades). Regulation of gene expression is considered a plausible mechanism of drug addiction, given the stability of behavioral abnormalities that define an addicted state (144). Numerous transcription factors, proteins that bind to regulatory regions of specific genes and thereby control levels of their expression, have been implicated in the addiction process (145). Among several others, a Fos family protein (Δ FosB), cAMP response element binding protein (CREB), Nur transcription factors subfamily and Pitx3 display very different regulation by drugs of abuse within the brain's reward circuitry, and in turn mediate, distinct aspects of the addiction phenotype.

3.1.1. CREB

CREB is a transcription factor implicated in diverse aspects of neural plasticity (146). Both psychostimulants and opiates increase CREB activity, acutely and chronically, in multiple brain regions, including the NAc and dorsal striatum (147,148,149) (Figure 9). In addition, CREB activation occurs in both the dynorphin and enkephalin subtypes of MSN (147). Drug activation of CREB in NAc has been shown to represent a classic negative feedback mechanism, whereby CREB serves to reduce the animal sensitivity to the rewarding effects of drugs (tolerance) and to mediate a negative emotional state during drug withdrawal (dependence)(150,151,152). These effects have been shown recently to drive increased drug self-administration and relapse, presumably through a process of negative reinforcement (153). However, some studies show that CREB is necessary for the rewarding effects of addictive drugs and biological reinforcement (154,155,156), raising the possibility that

whereas acute regulation of CREB is required for motivated behaviors, repeated upregulation of CREB induces tolerance to the reinforcing effects of rewarding stimuli(143). CREB activity has also been directly linked to the functional activity of NAc MSNs, since CREB overexpression increases the electrical excitability of these neurons (157).

Drugs of abuse activate CREB in several brain regions beyond the NAc. In the VTA, chronic administration of cocaine or opiates activates CREB within dopaminergic and non-dopaminergic neurons. This effect seems to promote or attenuate the rewarding responses of drugs of abuse, depending on the subregion of the VTA affected (145). Interestingly, a large body of literature has shown that CREB, acting in the hippocampus and the amygdala, is a key molecule in behavioral memory (158,159,160).

Numerous target genes for CREB have been identified (161,162,149). CREB mediates the induction of dynorphin expression in NAc neurons, which results in activation of KOR on VTA DA neurons and thereby suppresses dopaminergic transmission to the NAc and impairs reward (150). Certain glutamate receptor subunits are also implicated, such as the GluA1 α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) subunit and GluN2B N-Methyl-D-aspartate (NMDA) subunit, as well as K^+ and Na^+ ion channel subunits, which together would be expected to control NAc cell excitability (157,163). Brain derived neurotrophic factor (BDNF) is another target gene for CREB in the NAc (148). CREB induction has also been shown to contribute to cocaine induction of dendritic spines on NAc MSNs (164).

3.1.2. Δ fosB

The 35-37 kilodaltons (kDa) Δ FosB isoforms accumulate with chronic drug exposure due to their extraordinarily long half-lives (165,166,167,168). The stability of the Δ FosB isoforms provides a novel molecular mechanism by which drug-induced changes in gene expression can persist despite relatively long periods of drug withdrawal (Figure 9). Thus, Δ FosB is proposed to function as a sustained "molecular switch" that helps to initiate and then to maintain an addicted state (169,170).

Δ FosB-overexpressing mice selectively in the dynorphin-containing MSNs (DRD1-type) show augmented locomotor responses to cocaine after acute and chronic administration (171). They also show enhanced sensitivity to the rewarding effects of cocaine and of morphine in place conditioning assays (171,161,172), and work harder for cocaine (173). Additionally, Δ FosB overexpression in NAc exaggerates the development of opiate physical dependence and promotes opiate analgesic tolerance (172). In contrast, Δ FosB-overexpressing mice are normal in several other behavioral domains, including spatial learning as assessed in the Morris water maze (171). On the

other hand, targeting Δ FosB expression to the enkephalin-containing MSNs in NAc and dorsal striatum (DRD2-type) fail to show most of these behavioral phenotypes (172).

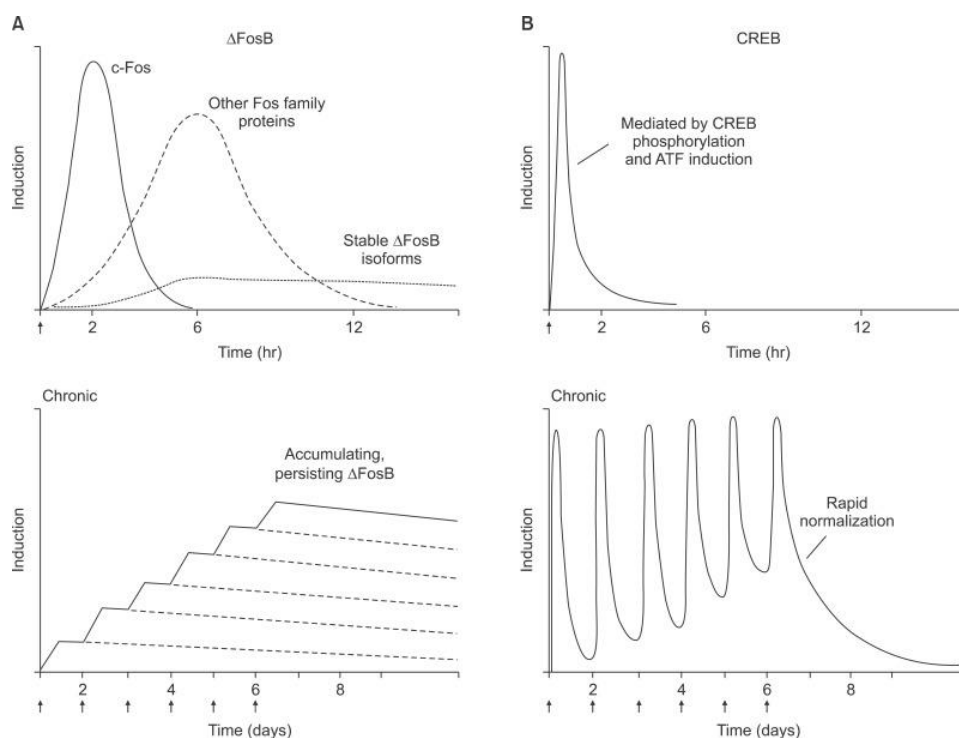


Figure 9: A) Fos family transcription factors, which include c-Fos, FosB, Fra1, and Fra2, heterodimerize with Jun family proteins (c-Jun, JunB, or JunD) to form active activator protein-1 (AP1) transcription factors. Fos family proteins are highly unstable and return to basal levels within hours of drug administration. However, after chronic drug administration, Δ FosB accumulates within the same brain regions whereas all Fos family members show tolerance. B) CREB forms homodimers that can bind to genes at cyclic AMP response elements (CREs), but activates transcription after it has been phosphorylated at Ser133. CREB activation is highly transient in response to acute drug administration and reverts to normal levels within a day or two after withdrawal.

Reproduced from ref (145): Nestler, EJ. Transcriptional mechanisms of drug addiction. Clin Psychopharmacol Neurosci. 2012. 10:136-143.

Δ FosB functions primarily as a transcriptional activator, while it serves as a repressor for a smaller subset of genes (161). Δ FosB appears to be induced by drugs of abuse (174). In dynorphin-producing cells in the NAc, Δ FosB represses dynorphin gene expression (172). Since dynorphin is thought to activate KOR on VTA dopaminergic neurons, which inhibits dopaminergic transmission and thereby downregulate reward mechanisms (175,176), Δ FosB repression of dynorphin expression could contribute to the enhancement of reward mechanisms. Another Δ FosB target is cFos: as Δ FosB accumulates represses the *c-Fos* gene, which helps to create the molecular switch: from induction of several short-lived Fos family proteins after acute drug exposure to the predominant accumulation of Δ FosB after chronic drug exposure (167).

3.1.3. Nur transcription factors subfamily and Pitx3

Nur transcription factors subfamily (Nur77 -NGFI-B, NR4A1-, Nurr1 -NR4A2- and Nor-1 -NR4A3-) are orphan members of the nuclear receptor superfamily(177,178,179,180), and share their classic structural organization (181)(Figure 10). Although Nur transcription factors have a well-recognized ligand binding domain structure, their transcriptional activity is not regulated by endogenous ligands (Benoit et al., 2004). Since the binding of ligands does not trigger the transcriptional activity of Nur factors, changes of their expression levels and post-transcriptional modifications appear keys to regulate their activity (182). Nur factors show some basal expression in specific nuclei of the rodent brain (183), but also behave as immediate early genes. Thus, their mRNA expression is induced independently of protein synthesis in several cell types by multiple kinds of stimuli (184,185,186,187).

Nur factors are associated to several functions (187), such as inflammatory (188,189) and oncogenic processes (190). However, some of the functions are exclusive for one factor, like the essential role of Nurr1 in the induction and maintenance of midbrain DA neurons. Unlike Nur77 and Nor-1, Nurr1 is expressed under basal physiological conditions in dopaminergic neurons of the substantia nigra (SN) and the VTA (191,192,193,194), and Nurr1 absence results in agenesis of dopaminergic neurons (195,196,197). Dopaminergic markers like TH, the dopamine transporter (DAT), the vesicular monoamine transporter 2 (VMAT2) are not expressed in the ventral mesencephalon of Nurr1 null mice at birth (198,199).

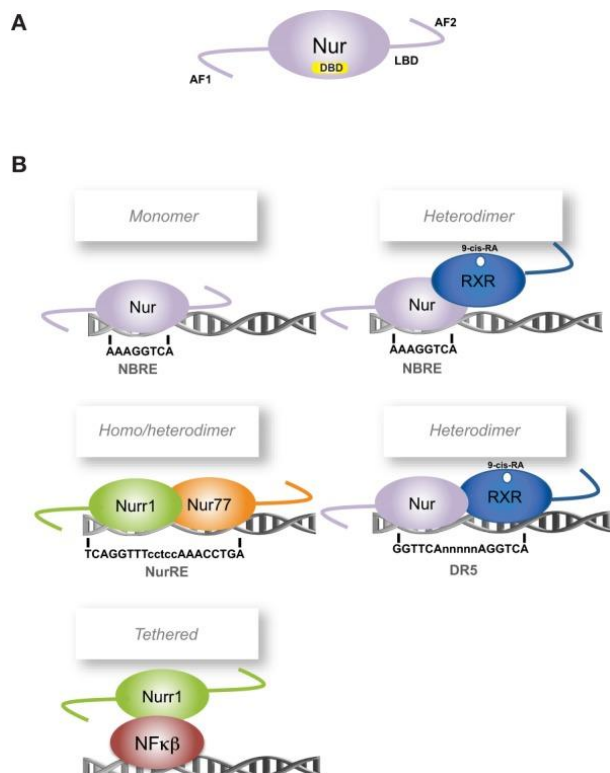


Figure 10: A) Nur transcription factors, structural organization: (1) a non-conserved N-terminal region containing the transcriptional activation function-1 (AF-1), (2) a conserved DNA binding domain (DBD) located in the middle of the proteins, and (3) a moderately conserved C-terminal domain, which encloses the ligand-binding domain (LBD) and the ligand-dependent transcriptional activation function 2 (AF-2). B) To exert their function as transcriptional regulators, Nur factors bind to nerve-growth-factor inducible gene B (NGFI-B)-responsive element (NBRE) as monomers, and to Nur-responsive element (NurRE) as homodimers or heterodimers between Nur factors.

Reproduced from ref (182): Campos-Melo D, Galleguillos D, Sánchez N, Gysling K, Andrés ME. Nur transcription factors in stress and addiction. *Front Mol Neurosci*. 2013. 6:44.

On the other hand, a progressive decline in the expression of the same genes in the SN and VTA is observed when Nurr1 is ablated during adulthood (200,201,202). For instance, aged Nurr1-deficient mice have analogous motor impairment to Parkinson's disease associated with decreased DA levels in the striatum (203). Moreover, the decreased number of TH positive cells in SN observed in aged Nurr1-deficient mice correlates with decreased DA release in the striatum (204). Nurr1-deficient mice also show some symptoms related to schizophrenia, associated with dysfunctions in DA neurotransmission (205,206). Importantly, it has been described that different set of genes are controlled by lower versus higher Nurr1 levels (207).

Evidence suggests that Nurr1 expression is regulated by DA signaling, mainly through DRD2. DRD2 knock out (KO) mice have increased Nurr1 expression in midbrain dopaminergic neurons (208) and the loss of DA in rat striatum generates a rapid increase of Nurr1 expression in DA neurons of the SN (194). Regarding drug administration, studies in cocaine abusers showed a reduction of Nurr1 expression in the SN (209). Similarly, rats chronically treated with cocaine show a down-regulation of the expression of Nurr1 mRNA and protein in the ventral midbrain (210). Additionally, chronic use of heroin in humans decreases Nurr1 mRNA in the VTA (211).

Another critical transcription factor for the development, survival and maintenance of dopaminergic neurons is the homeobox protein Pitx3 (212). At birth, Pitx3-deficient mice have a virtually complete loss of SN dopaminergic neurons, resulting in a 90% reduction in dorsal striatal DA (213,214,215,216). Although normal at birth, the VTA is progressively affected postnatally with a noticeable decline in DA cells (217,216), while no other brain regions are affected (213,214). Interestingly, within the CNS, Pitx3 is only expressed within the SN and VTA DA cells (218).

The gene encoding for Pitx3 activates the transcription of genes directly involved in the differentiation of dopaminergic neurons (219,220). A binding site for Pitx3 has been identified at the promoter region of *Th* gene (221), and a role for the transcription factor Pitx3 in TH regulation has been shown (220). Other genes identified as important regulators of DA function, such as *Dat* and *Vmat2*, are also targets of Pitx3. It has been indicated that robust expression of *Dat* and *Vmat2* genes can be detected when both Nurr1 and Pitx3 coexist (219,221). On the other hand, alterations in Pitx3 transcription and protein levels have been detected after acute and chronic cocaine and methamphetamine administration (Leo et al., 2007; Krasnova et al., 2011).

3.1.4. Neurotrophins

Neurotrophic signaling has been shown to modulate neural plasticity and behavior (222). Neurotrophin family members share significant homology (223), and include: nerve growth factor

(NGF) (224), BDNF (225,226), neurotrophin-3 (NT3) (227), and neurotrophin-4/5 (NT4/5) (228). Importantly, the pro- (immature) forms of the neurotrophins mediate signaling cascades distinct from the mature peptides (229). The p75 neurotrophin (p75NTR) receptor binds both the immature and mature forms of all four neurotrophins (230,231,232). BDNF also binds the tropomyosin-related kinase B (TrkB) receptor (233). BDNF receptor activation induces stimulation of different signaling pathways: the phosphatidylinositol 3'-kinase (PI3K), MAPK, phospholipase C γ (PLC γ) and nuclear factor kappa B (NF κ B), which influence a range of cellular functions including neuronal survival, growth, differentiation, and structure (234).

Studies using conditional deletions of BDNF or the TrkB receptor show that they are required for proliferation and maturation of dendritic spines in developing neurons as well as the maintenance and proliferation of spines on neurons throughout the adult brain (235,236,237,238). According to this, accumulating evidence suggests an essential role for this neurotrophic factor signaling in the neuronal remodeling that occurs after chronic drug administration. Stimulants produce a widespread, but transient, induction of BDNF protein in the NAc, PFC, VTA, CeA and the basolateral amygdala (BLA) (148,239,240). In contrast, after chronic morphine treatment, it has been shown that the number of BDNF immunoreactive cells in the VTA is decreased (241). Moreover, several neurotrophin signal cascades have been shown to be regulated within the mesolimbic DA system by opiates and stimulants. Stimulants dramatically increase ERK phosphorylation in numerous brain regions following acute or chronic drug administration (242,243,244,245,246,247). The effects of opiates on ERK signaling are less clear. Recently, it has been reported that ERK phosphorylation is decreased in the NAc (248), PFC (249), and VTA (234) after chronic morphine, an effect that is consistent with decreased neurite branching seen in these regions. In contrast, it has also been reported increased ERK activity in the VTA after chronic morphine (250,251,252).

It is well established the influence of BDNF on various phases of the addiction process. Local infusion of BDNF into the VTA or NAc augments locomotor and rewarding responses to cocaine, while global loss of BDNF exerts the opposite effect (253,254,255). Importantly, BDNF signaling may produce profoundly different effects on neuronal morphology and behavior depending on the brain region examined. For example, increased BDNF in the NAc enhances cocaine-induced behaviors (148,254), whereas in the PFC BDNF suppresses these same behaviors (256). The level of BDNF in the VTA, as well as NAc and amygdala, progressively increases during abstinence (239). This progressive increase has been hypothesized to underlie the progressive increase in drug-seeking that occurs during cocaine withdrawal (257,240). Interestingly, it has been described that stimulating BDNF receptors in the amygdala, NAc, or VTA promotes (254,255,148,258), whereas microinjection of BDNF into the PFC inhibits drug-seeking (256). Moreover, BDNF infusions in the hippocampus

are antidepressant-like, whereas infusions of BDNF in the VTA or NAc produce prodepressant-like effects (259,260,261).

3.2. SYNAPTIC PLASTICITY

Opiate-induced plasticity also includes synaptic plasticity, described as persistent changes in glutamatergic and GABAergic synaptic transmission (262,263). Focused on glutamate transmission, decreased levels of basal extrasynaptic glutamate are observed in rodents in the NAc(Core) following chronic exposure to drugs of abuse (264), resulting in a lack of tone on inhibitory presynaptic mGluR2/3 subtype of metabotropic glutamate receptors(mGluR) and a dramatic enhancement of excitatory transmission at PFC-NAc(Core) synapses (264).

Moreover, there is a general reduction in prefrontal cortical measures of cellular metabolism and blood flow in individuals addicted to a variety of different drugs (265). This hypofrontality has been characterized as a strong indicator of reduced ability to regulate drug-seeking (266). A reduction in frontal cortical neurons has also been reported in drug-free cocaine addicts (267). Interestingly, when exposed to a cue previously associated with drug use that precipitates the desire for drug, there is marked activation in the PFC (265,268,269), which has been positively correlated with the intensity of cue-induced desire for the drug. Moreover, consistent with addiction being characterized in part by reduced response to biological rewards, when cocaine addicts were presented with a sexual stimulus, prefrontal activation was significantly impaired compared to controls (270). Also, addicts show reduced levels of DRD2 receptors in the striatum (271). Since reduced DRD2 receptors indicates blunting of DA transmission, it is not surprising that addicts report reductions in high or pleasure in response to methylphenidate relative to control subjects. In contrast, whereas the methylphenidate induces strong cravings in the addicts, there is no craving in comparison subjects (143).

There is also increasing evidence for the importance of whole cell plasticity, also referred to as homeostatic plasticity (272), which involves changes in the intrinsic excitability of an entire nerve cell in a manner that it is not synapse-specific. Chronic opiates increase the intrinsic excitability of noradrenergic neurons of the LC (273). This increased excitability is mediated via CREB-dependent induction of certain isoforms of adenylyl cyclase (273,274,275,276). This hyperexcitability of LC neurons represents a classic mechanism of tolerance and dependence and drives some of the signs and symptoms of opiate withdrawal. Interestingly, CREB mediates a similar form of whole cell plasticity in NAc MSNs, which are also rendered hyperexcitable by chronic exposure to drugs of abuse (157). Another example of whole cell plasticity is the hyperexcitability of VTA dopaminergic neurons that occurs after chronic exposure to opiate drugs (277,278). This adaptation is not mediated by CREB, but achieved instead via regulation of neurotrophic signaling cascades (277).

3.3. STRUCTURAL PLASTICITY

Opiate-induced plasticity also includes a third type of plasticity: structural plasticity (long-lasting changes in neuronal morphology) (279). Changes in synaptic plasticity are associated with morphological changes at synapses. For example, long-term depression (LTD) and the generation of silent synapses are associated with the formation of thin or stubby dendritic spines, whereas long-term potentiation (LTP) is associated with larger, mushroom-shaped spines (280,281). Chronic exposure to stimulant drugs of abuse increases the dendritic spine density of MSNs in the NAc (282,283,279). However, particular circuits display different forms of plasticity (Table 1). Literature suggests very different spine changes occurring at different withdrawal time points and in the NAc(Shell) versus NAc(Core) subregions (264,284,285,286). Although the induction of dendritic spines after repeated treatment with psychostimulants occurs in both DRD1 and DRD2 MSNs, the long-term stability of new spines appears to be greater in DRD1 neurons, supporting the idea that intracellular signaling pathways downstream of DRD1 may mediate longer-term stabilization of spines than in DRD2 neurons (287,288). Δ FosB has been shown to be both necessary and sufficient for the induction of immature spines on DRD1 NAc neurons (289,290,282). Indeed, the persistence of increased dendritic spines in DRD1 MSNs highly correlates with the persistent induction of Δ FosB in DRD1 MSNs and sensitized behavioral response to chronic drug exposure (291,170). Interestingly, cocaine regulation of several genes known to control the reorganization of the actin cytoskeleton, including induction of cyclin-dependent kinase 5 (CDK5), calcium-/calmodulin-dependent protein kinase type II (CaMKII) and NFkB, and repression of the dimethyltransferase G9a, are mediated via Δ FosB (292,289,290,293). In contrast, opiates exert the opposite effect and reduce dendritic spine density of NAc MSNs (283). This phenomenon is surprising, given that CREB and Δ FosB are induced by both stimulants and opiates and are both implicated in stimulant-mediated induction of NAc dendritic spine density.

Other major form of morphological plasticity is the physical reduction in cell soma size of VTA DA neurons induced by chronic opiate administration.(277,294,295) (Table 1), which seems to mediate reward tolerance and is associated with reduced DA release in the NAc. It has been directly linked the opiate-induced VTA neuron shrinkage to reduced activity of downstream BDNF signaling cascades, specifically reduced activity of the insulin receptor substrate-2 (IRS2), thymoma viral proto-oncogene (Akt), and TOR (Target of rapamycin) complex 2 (TORC2) (277,295). The downregulation of BDNF signaling directly modulates the increased excitability that morphine induces in these neurons (277,278). In contrast, stimulants induce BDNF signaling to the NAc, an effect due to increased local synthesis of BDNF as well as increased release from several afferent regions (148).






| Decrease ← → Increase | | |
|---|--|---|
| Opiates | Neuron Function | Stimulants |
| ? | Amygdala spiny neuron  | ? |
| Dendrite complexity and spine density/morphometry | NAc medium spiny neuron  | Dendrite complexity and spine density/morphometry |
| Cell body size | VTA dopamine neuron  | Spine density/morphometry |
| Spine density | CA3 pyramidal neuron  | ? |
| Dendrite complexity and spine density/morphometry | PFC pyramidal neuron  | Dendrite complexity and spine density/morphometry |

Table 1: In several regions of the central nervous system opiates are observed to reduce neuronal size or extensions, while stimulants (e.g., cocaine and amphetamine) exert the opposite types of changes.

Reproduced from ref (234): Russo SJ, Mazei-Robison MS, Ables JL, Nestler EJ. Neurotrophic factors and structural plasticity in addiction. *Neuropharmacology*. 2009. 56 Suppl 1:73-82.

The regulation of the actin cytoskeleton, that can both stabilize or change spine morphology, is a candidate for a process that may underlie changes in spine density (296,297,298). Accordingly, there is an enduring increase in actin cycling after withdrawal from chronic psychostimulant administration (299). In addition to alterations in spine morphology, another consequence of increased actin cycling would be alterations in the trafficking of proteins into the postsynaptic membrane (300). A potentially critical change in postsynaptic receptor trafficking is an enduring increase in membrane insertion of glutamate receptors (301,302,303).

4. STRESS AND ADDICTION

Although many factors can contribute to initial and continued drug use, exposure to either psychological or physiological stress at any point in the addiction cycle seems to worsen this disease, augmenting all drug-seeking behaviors, including initial drug taking, drug craving, and relapse (304,305).

4.1. STRESS AND DRUG TAKING

Stress affects different stages of drug addiction. Exposure to physiological as well as physical stressors, including social isolation, tail pinch or footshock, can enhance initial amphetamine and cocaine self administration (306,307,308). Furthermore, repeated exposure to forced swim stress can augment the rewarding properties of cocaine (309). Thus, these studies implicate stress in modulating the initial rewarding effects of addictive drugs. Moreover, CORT release via the HPA axis is vital to the acquisition of drug administration. Inhibiting CORT release by adrenalectomy or pharmacologic treatment blocks cocaine self administration in rats (310,311). Furthermore, CORT release following drug administration in rats increases neuronal activity above the critical levels needed for self administration to occur (312). The ability of CORT to modulate drug reward can be mediated by GRs located on neurons throughout the mesolimbic DA reward pathway (313). Adrenalectomized (ADX) animals exhibit a blunted DA response in the NAc following either drug exposure (314) or stress (315). Similarly, GR antagonists decrease extracellular DA levels in the NAc (316). In addition, GR antagonists locally injected into the VTA decrease morphine-induced increases in locomotor activity (316), thus indicating that activation of GRs in the VTA can mediate DA-dependent behavioral outputs. Interestingly, in mice where the *GR* gene was deleted specifically in the CNS, a decrease in motivation to self-administer cocaine was observed (317). A role for CRF has also been proposed in stress-induced drug taking. For example, pharmacological blockade of CRFR1 inhibits cocaine-induced DA release as well as reductions in the rewarding properties of cocaine and locomotor activating effects (318,319).

4.2. STRESS AND WITHDRAWAL

Activation of the HPA axis and increased CORT levels occur following acute withdrawal from most drugs of abuse, both in humans and in animal models (100). During long-term withdrawal, the HPA axis displays an augmented response upon exposure to a stressor (320,321). These data suggest that the stress response can be sensitized by drug exposure and subsequent withdrawal. In addition, previous results from our laboratory showed that the physical signs of opiate withdrawal are modulated by GR signaling (322). Moreover, the somatic signs of opiate withdrawal were also modulated by MR signaling (323).

Also alterations in the CRF peptide and mRNA levels throughout the CNS were observed following acute withdrawal from several drugs of abuse, and these alterations vary depending on the brain region as well as the drug administered. Interestingly, increases in CRF mRNA in the PVN correlate with increases in anxiety behaviors during ethanol, cocaine, and morphine withdrawal, and blockade of the CRF system decreases the anxiety observed in this acute withdrawal phase

(324,325,326). These results suggest that the CRF1R subtype may be involved in the behavioral and somatic signs and in ACTH release (partially) during morphine withdrawal (327,326). Also, CRF2R signaling modulated physical signs of opiate withdrawal (328).

4.3. STRESS AND REINSTATEMENT OF DRUG SEEKING

Alterations in the responsiveness of the HPA axis to a stressor during long-term withdrawal might play a role in the ability of stressors to reinstate drug seeking long after the drug is removed (304,305). Exposure to an acute stressor can effectively reinstate drug seeking of various drugs (329,330,331,332). Stress facilitates relapse by activating central CRF brain circuits, since animals reinitiate lever pressing following an i.c.v. CRF injection (333).

The significance of the extended amygdala in the addiction cycle is evident primarily in relapse. Inactivation of the CRF projection from the CeA to the BNST blocks stress-induced cocaine reinstatement (334,335), and local injections a nonspecific CRF receptor antagonist into the BNST, but not the amygdala, attenuates footshock-induced reinstatement (335). Interestingly, selective CRFR1 antagonists attenuate footshock-induced reinstatement of cocaine or opiate seeking (329,336) but have no effect on drug-induced reinstatement (333,337). These data demonstrate that stress stimulation of the CRF-containing pathway, originating in the CeA and extending into the BNST, and subsequent activation of CRFR1 localized in the BNST, triggers drug seeking in previously addicted animals.

4.4. STRESS AND REWARD PATHWAYS

It is well established that the mesolimbic DA reward pathway participates in the response to stress. Acute exposure to a stressor, such as footshock (338) or tail pinch (315), produces an increase in DA release in the NAc. Moreover, exposure to either drugs of abuse or stress produces similar alterations in the electrophysiology of neurons in the mesolimbic reward pathway in animals. Enhanced excitatory synaptic transmission, as evidenced by an increase in glutamate receptor activation, occurs in VTA dopaminergic neurons following exposure to either stress or different drugs of abuse (339). In addition, both stress and drugs of abuse cause alterations in dendrites (340,341,342). Thus, rats subjected to chronic restraint stress exhibit decreases in dendritic branching in the medial PFC (342). As stated above, alterations in dendritic branching are also observed following exposure to addictive drugs, with an increase occurring following exposure to cocaine and amphetamine, whereas reductions in branching occur following exposure to morphine (340,341). Together, these findings indicate that stress and drugs of abuse act similarly to affect the neurochemistry, electrophysiology and morphology of neurons involved in reward pathways. Also, common mechanisms can be observed

at the molecular level. Chronic stress increases Δ FosB in the NAc as well as in the PFC and BLA (343). CREB is regulated by both acute and chronic drug treatment throughout brain reward areas (344,155) and various stressors, including shock, repeated immobilization or forced swim are associated with increased CREB phosphorylation in several regions of the brain (345), including the NAc (151).

Importantly, CRF has been detected in the VTA (346). In both, cocaine-naïve and cocaine-experienced rats, CRF is released into the VTA following an acute footshock, but only in cocaine-experienced animals glutamate and DA are released in the VTA in conjunction with CRF in response to a stressor (347). This release of glutamate and DA is dependent upon CRF, as local injections of CRF antagonists into the VTA attenuated the release of these two neurotransmitters (346,347). In addition, administration of CRFR2 antagonists, but not CRFR1 antagonists, locally into the VTA blocked the ability of footshock to reinstate cocaine seeking in a self-administration paradigm (347). Thus, these studies suggest a role for CRF in modulating DA cell activity, specifically following drug experience.

4.5. STRESS AND THE NORADRENERGIC SYSTEM

The noradrenergic system has been implicated in stress-induced reinstatement of drug seeking. Animal models have demonstrated that an α 2-adrenergic receptor agonist (lofexidine) reduced opiate withdrawal symptoms by decreasing noradrenergic outflow in the CNS (348) and attenuated footshock-induced reinstatement of drug seeking (349). Moreover, selectively lesioning noradrenergic projections to forebrain areas effectively blocks stress-induced reinstatement in rats (350). Furthermore, local injections of noradrenergic antagonists into the BNST and CeA inhibited the ability of footshock to reinstate drug seeking (351).

Previous results from our laboratory have proposed a role for CRF and GR signaling in noradrenergic-mediated opiate withdrawal. Thus, we showed that the TH activation and stimulation of noradrenergic pathways innervating the PVN are modulated by GR signaling (322). However, pretreatment with spironolactone (a MR antagonist) resulted in no significant modification of the increased NA turnover or TH activation that occurred during morphine withdrawal (323). Interestingly, increased GCs levels are responsible for induction of TH mRNA expression, phosphorylation, and enzyme activity in the NTS during morphine withdrawal (138). Also, CRF2R signaling modulated not only the physical signs of opiate withdrawal, but also the TH activation and stimulation of noradrenergic pathways innervating the PVN (328).

4.6. STRESS AND MOLECULAR MECHANISMS

The mechanisms by which stress affects the addiction process are not well understood. It is important to elucidate the molecular mechanisms underlying the interactions between stress and drug abuse, as an understanding of this may help in the development of novel and more effective therapeutic approaches to block the clinical manifestations of drug addiction. Some knowledge on this area has focused on stress-regulation of different transcription factors.

CREB-deficient mice do not exhibit stress-induced reinstatement of cocaine-conditioned place preference, but do exhibit reinstatement of drug seeking to a priming dose of cocaine (331). This deficit in stress- but not drug-induced reinstatement indicates a specific requirement for CREB in stress-induced behavioral responses to drugs of abuse. BDNF, a putative CREB target gene, was increased following withdrawal from chronic cocaine (239). The increase in BDNF in these brain areas positively correlated with the response of the rats to drug-associated cues and relapse to drug-seeking (239,148).

Several lines of evidence indicate that Nur transcription factors play a prominent role in adaptive responses to stress, regulating the transcription of target genes in the HPA axis: CRF and POMC (352,353) and different enzymes essential for the production of GCs in the adrenal gland (354,355,356). In addition, the expression of Nur transcription factors increases in the limbic nuclei associated to the extra-hypothalamic aspects of the stress response, suggesting that Nur factors could also underlie enduring changes induced by chronic stress (357,358). Interestingly, the increased Nurrl expression has been suggested as a possible compensatory mechanism that may counteract the observed reduction of DA levels induced by stress (359,360,361). Importantly, Nur factors have been proposed to integrate stress and drug addiction signaling (362).

5. EPIGENETIC MECHANISMS

A crucial role for epigenetic mechanisms in driving lasting changes in gene expression has been proposed, which has prompted research aimed at characterizing the influence of epigenetic regulatory events in mediating the lasting effects of drugs of abuse (363). There is robust evidence that repeated exposure to drugs of abuse induces changes within the brain in three major modes of epigenetic regulation: non-coding RNAs, histone modifications such as acetylation (364,365,366) and methylation (290,367) and DNA methylation (368,369).

The complete sequencing of the mammalian genome and its transcriptional products has revealed a large number of expressed RNAs that are not translated into proteins. Such non-coding RNAs have been shown to play crucial regulatory roles in cell function (370,371). Most studied are miRs, ~22 nucleotide (nt) non-coding RNAs that participate in gene regulation. They bind to 3' untranslated regions (UTRs) of their mRNA targets, inhibiting the transcripts translation and/or destabilizing them (372,373)(Figure 11). However, miR targeting can also occur in coding regions (374,375). Interestingly, deletion of Argonaute (Ago), a protein crucial for the processing of miRs, alters behavioral responses to cocaine, with distinct effects observed for DRD1- versus DRD2-type MSNs (376).

Since the discovery that miRs are important regulators of gene expression, these molecules have been linked to biological and pathological processes such as drug addiction (377,378). Multiple miRs are reported to be up- or down-regulated by drugs of abuse. For instance, cocaine increases levels of miR-181a and decreases miR-124 and miR-let-7d in rat striatum (379,380,376,381,382), and mimicking the direction of each of these changes enhances cocaine reward. miR-212 is induced in rat dorsal striatum after cocaine self-administration, and serves to inhibit cocaine intake (383). This action was attributed to the ability of miR-212 to indirectly lead to the activation of CREB, a transcription factor that antagonizes cocaine reward (384). Moreover, chronic ethanol suppresses the expression of big potassium channels via induction of miR-9 in striatum and hypothalamus (385). Several additional genes implicated in addiction models, such as Δ FosB, DAT and glutamate receptor subunits, have also been related to drug-triggered alterations in specific miRs (380,386). However, there is very little knowledge about the intracellular signaling pathways through which synaptic transmission and neural activity are translated into epigenetic modifications.

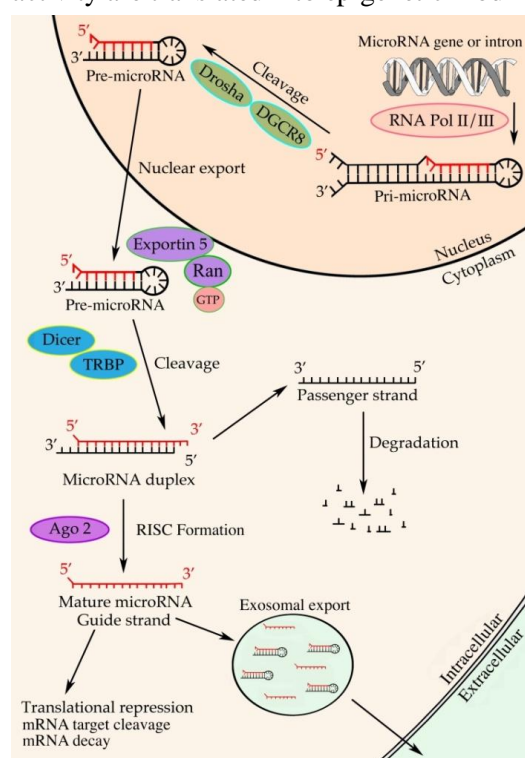


Figure 11: miR biogenesis involves transcription of pri-miR by RNA polymerase II/III, cleavage by the Drosha-DGCR8 complex to pre-miR, followed by export to the cytoplasm by Exportin-5. In the cytoplasm, pre-miR is cleaved by the Dicer-TRBP complex to a miR duplex, which is unwound to a guide strand that is bound to Ago2 and incorporated into the RISC, and a passenger strand, which is degraded. Ultimately, miR binding to target mRNAs results in mRNA target cleavage, translational repression, or mRNA decay.

Reproduced from ref (373): Møller HG, Rasmussen AP, Andersen HH, Johnsen KB, Henriksen M, Duroux M. A systematic review of microRNA in glioblastoma multiforme: micro-modulators in the mesenchymal mode of migration and invasion. *Mol Neurobiol.* 2013;47:131-44.

5.1. OPIATES AND miRs

As stated above, miRs have been linked to drug addiction. Thus, literature supports a role for several miRs in mediating different aspects of opiate addiction. miR-let-7 represses MOR expression and works as a mediator moving MOR mRNA to P-bodies, leading to translation repression (377). It has been identified that miR-23b represses MOR translation efficiency (387), whereas fentanyl regulates the expression of miR-190 and one of its targets, neurogenic differentiation 1 (NeuroD) (388,389).

miR-133b, regulates the differentiation, maturation, and function of dopaminergic neurons by downregulating the transcription of its target Pitx3 in the dopaminergic system (390). In zebrafish embryos, morphine decreases miR-133b level and increases the mRNA levels of Pitx3 and Dat. Moreover, naloxone effectively abolished the morphine-induced changes in the expression levels of miR-133b, Pitx3, Th, and Dat (391,392)(Figure 12). In addition, inhibition of ERK 1/2, even when the MOR is activated by morphine, produces the same effect as knockdown of the receptor, i.e., the expression of miR-133b increases, and hence, the expression of Pitx3, TH, and DAT decreases, which reduces the level of dopaminergic neuron differentiation (392).

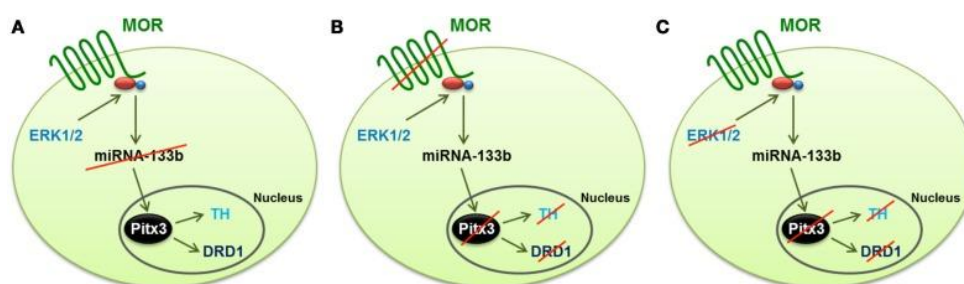


Figure 12: Schematic representation of the mechanism by which morphine regulates the differentiation of dopaminergic neurons through the control of the miR-133b.

Reproduced from ref (392): Rodríguez RE. Morphine and microRNA Activity: Is There a Relation with Addiction? Front Genet. 2012. 3:223.

6. MEMORY MODULATION

Addiction-related molecular and cellular adaptations involve most of the same brain regions that mediate classic forms of memory. This coincides with the increasing realization that some of the most important features of addiction seen clinically (eg, drug craving and relapse) reflect abnormalities in traditional memory circuits, with long-term memories of the drug experience serving as potent drivers of addiction pathology (143,269,20). Memory modulation systems influence different

neurobiological processes underlying the consolidation of new information, memory extinction, memory recall and working memory. Hormones of the adrenal medulla (adrenaline) and adrenal cortex (cortisol or CORT) are released during and immediately after the kind of emotionally arousing stimulation typically used in training tasks (393,394,395), and there is extensive evidence that these hormones enhance memory consolidation (396,397). Drugs and hormones affecting several other systems also enhance memory consolidation, e.g., CRF (398) or ACTH (399).

Adrenaline effects are most likely mediated by activation of β -adrenoceptors located on vagal afferents that project to the NTS in the brain stem (400). Early studies also reported evidence that adrenaline induces the release of NA in the brain (401). Thus, the NTS appears to be an interface between peripheral adrenergic activation and brain processes regulating memory consolidation. It has also been proposed that opioids and GABA impair memory by decreasing NA release in the brain (402,403,404).

Post-training injections of GCs produce dose- and time-dependent enhancement of memory (405,406,407). The memory-modulating effects of GCs appear to involve the selective activation of the low-affinity GRs (408,409,410). GCs are known to act through intracellular and intranuclear receptors and can affect gene transcription by direct binding of receptor homodimers to DNA (411,412) or via protein-protein interactions with other transcription factors such as Jun or Fos (413). However, GCs may also act more rapidly by interacting with membrane receptors and/or potentiating the efficacy of the NA signal cascade via an interaction with G-protein-mediated actions (414,415,416). Adrenergic activation is essential in enabling GC enhancement of memory consolidation (417), and training-induced emotional arousal is required in enabling GC effects on memory consolidation (405).

6.1. MEMORY CONSOLIDATION

In rodents, when an affective component is paired with a particular environment, it triggers the onset of association between affective consequences with the context, and results in memory formation/consolidation. It has long been known that noradrenergic activation of the BLA is crucially involved in strengthening the consolidation of long-term memory (418,419,397) (Figure 13). NA or a β -adrenoceptor agonist infused into the BLA immediately post-training enhances the retention of many different types of emotionally arousing training experiences, including inhibitory avoidance (420,421), contextual fear conditioning (422,423) or water-maze spatial training (403). In contrast, intra-BLA infusions of a β -adrenoceptor antagonist impair the consolidation of memory for these training experiences (403,424). However, it has been recently published that the BLA noradrenergic innervation also modulates long-term memory consolidation of object-in-context recognition training, a

low-arousing behavioral task designed to assess episodic-like memory in rats (425). Evidence that post-training intra-amygdala infusions of a synthetic cAMP analog enhance retention (426) is consistent with the hypothesis that activation of β -adrenoceptors modulates memory via a direct coupling to adenylate cyclase.

There is extensive evidence that the BLA is a critical locus of the synergistic actions of GCs and emotional arousal-induced noradrenergic activation in influencing memory consolidation (Figure 13). Systemic or intra-BLA infusions of GCs modulate memory consolidation (406,427). A β -adrenoceptor antagonist infused post-training into the BLA blocks the memory-enhancing effects of GCs (417). It has been suggested that activation of GRs in the BLA may facilitate memory consolidation by potentiating the NA-induced signaling cascade through an interaction with G-protein-mediated effects (428). *In vivo* microdialysis suggested that GCs also facilitate the training-induced release of NA in the amygdala (429).

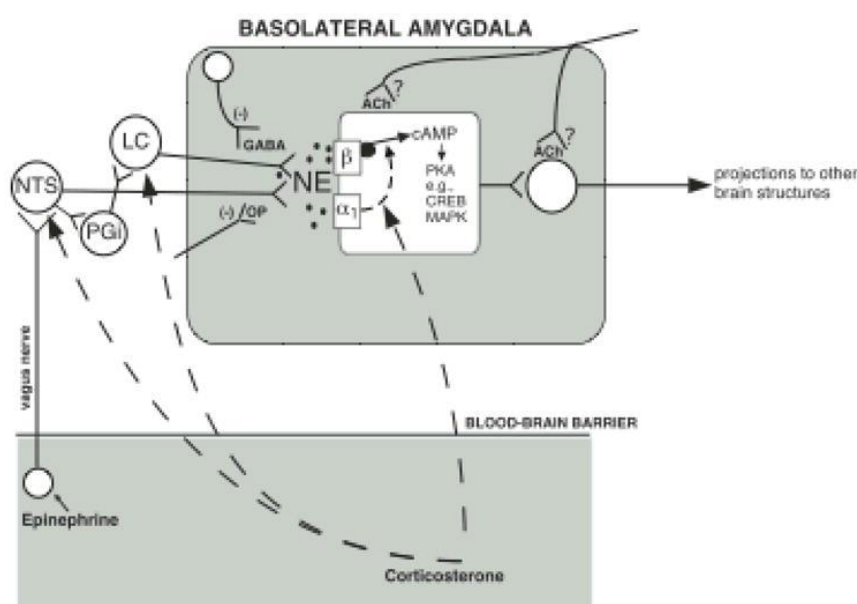


Figure 13: Role of the noradrenergic system of the BLA in memory consolidation. NA is released in the BLA and binds to both β -adrenoceptors and α_1 -adrenoceptors at postsynaptic sites. The β -adrenoceptor is coupled directly to adenylate cyclase to stimulate cAMP formation. The α_1 -adrenoceptor modulates the response induced by β -adrenoceptor stimulation.

Reproduced from ref (430):
Roosendaal B, McGaugh JL.
Memory modulation. *Behav Neurosci.* 2011. 125:797-824.

As was found with adrenaline, GC effects on memory consolidation also appear to involve brain stem nuclei, including the NTS, which send noradrenergic projections to the BLA. A GR antagonist infused into the NTS attenuates the memory-enhancing effects of systemically administered dexamethasone (431). Moreover, the finding that post-training infusions of the GR agonist RU 28362 into the NTS enhance inhibitory avoidance retention and that intra-BLA infusions of a β -adrenoceptor antagonist block the enhancement (431) provides additional evidence that the NTS influence on memory consolidation involves noradrenergic activation of the BLA (432).

It is recognized that spatial information about a given environment is acquired through the hippocampus and then moves to the cortex for long-term storage (433). The amygdala projects directly and indirectly to the hippocampus (434,435). Evidences of BLA-hippocampus interactions in memory consolidation have been proposed since unilateral post-training intra-hippocampal infusions of the specific GR agonist enhance rats retention of inhibitory avoidance training and the enhancement is blocked selectively by ipsilateral infusions of a β -adrenoceptor antagonist into the BLA (430). Lesions of the BLA, BNST or NAc also block the enhancement induced by GR activation in the hippocampus (436). Studies of BLA influences on hippocampal neuroplasticity provide additional important evidence of amygdala-hippocampal interactions. Electrical stimulation of the BLA enhances the induction of LTP in the dentate gyrus (DG) of the hippocampus (437,438). Also, selective lesions of the BLA or infusions of a β -adrenoceptor antagonist into the BLA block the induction of LTP in the DG (439,440). Importantly, the effects of BLA stimulation on DG LTP are influenced by NA and CORT (441,442).

6.2. MEMORY RETRIEVAL

Memory retrieval describes when animals are re-exposed to a previously paired environment, and thus, they can prefer or avoid the paired environment due to the association between the context and positive or aversive memories. Stress exposure or CORT administered systemically shortly before testing for memory of different training tasks produces temporary impairment of retention performance (443,444,445), indicating that GCs impair retention by influencing memory retrieval. Since the β -adrenoceptor antagonist propranolol administered systemically 30 minutes before inhibitory avoidance retention testing blocks the memory retrieval impairment induced by concurrent injections of CORT (446), and the stimulation of β_1 -adrenoceptors with systemic injections of the selective agonist xamoterol induces memory retrieval impairment comparable to that seen after CORT administration (447), GC effects on memory retrieval impairment involve activation of noradrenergic mechanisms. Peripheral administration of the opioid antagonist naloxone or DRD2 antagonists also blocks the impairing effect of concurrently administered CORT or dexamethasone on memory retrieval (444,448).

Many studies have reported evidence that the hippocampus is involved in retrieval of spatial and contextual information (449,450). Inactivation of the hippocampus with local infusions of the glutamatergic AMPA/kainate receptor antagonist or the GABAergic agonist muscimol impairs memory retrieval (451,452). Since administering a GR agonist into the hippocampus shortly before retention testing also impairs retrieval of spatial memory (453,447), the GC-induced memory retrieval impairment depends, in part, on GR activation in the hippocampus. Moreover, a β -adrenoceptor antagonist infused into the hippocampus prevents the retrieval-impairing effect of a GR agonist

administered concurrently (447). In contrast, infusions of the protein-synthesis inhibitor anisomycin do not block CORT effects on memory retrieval (454), suggesting that stress and CORT may influence memory retrieval through a protein synthesis-independent mechanism, a finding consistent with the rapid onset of stress and GC effects on memory retrieval.

Memory retrieval of emotionally arousing information also induces the activation of the BLA (455,456). Furthermore, intra-BLA infusions of NA or AMPA receptor antagonist affect retrieval of memory for inhibitory avoidance training (457,458). In contrast, intra-BLA infusions of a GR agonist do not appear to affect memory retrieval (453). However, the BLA interacts with the hippocampus in mediating GC effects on memory retrieval. Lesions of the BLA or infusions of a β -adrenoceptor antagonist into the BLA block the impairing effect of a GR agonist infused into the hippocampus on memory retrieval (447,453) indicating that, similarly to memory consolidation, BLA regulates memory retrieval via interactions with other brain regions.

6.3. EGR1

Among the studies of the functional role of inducible transcriptional regulators in synaptic plasticity and memory processes, many have focused the Early Growth Response (Egr) family (459,460). The Egr family comprises four members: Egr1 (also known as NGFI-A, Krox-24, Zif268, Tis8 or ZENK), Egr2 (Krox-20), Egr3 (Pilot) and Egr4 (NGFI-C) (461,462). In addition to their basal expression, the expression of Egr family members can be induced in different brain areas after a variety of neuronal stimulation paradigms, such as the LTP induction or maximal electroconvulsive shocks inducing seizure activity, or in behavioral circumstances after exposure to novel environments of specific learning experiences (461,463,464).

Egr1 is rapidly induced in dentate granule cells following the induction of LTP (465,466) and its expression has been widely used as a marker of neuronal plasticity in a learning context (463,464). Interestingly, Egr1 is involved in the maintenance of LTP (467), in the consolidation of different forms of long-term memory (460,468,467,469) and in the reconsolidation of memory after reactivation during retrieval (470,471). In addition, enhanced memory strength was reported in mice over-expressing Egr1 (472). In parallel, Egr1-deficient mice show inability to form long-term memories in a variety of behavioral tasks, while short-term memory, as well as early-LTP, is intact (467).

Although a wide range of neurotransmitter systems can induce expression of Egr family members (461), the cell-signaling pathways leading to the regulated expression of Egr members in synaptic plasticity and learning have not been precisely defined. In the hippocampus, activity-dependent expression of Egr1 is dependent upon NMDA receptor (465,473,466) and MAPK/ERK

activation (474), both of which are known to play a key role in synaptic plasticity and learning. Conversely, *Egr1* can be negatively regulated by the protein phosphatase calcineurin, a negative regulator of neuronal signaling and of memory formation (472). In addition, *Egr1* has been shown to be regulated by BDNF/TrkB and GCs can enhance the memory of stressful events driving *Egr1* expression (475,476).

6.4. ARC AND MEMORY

Changes in dendritic spine morphology are thought to be involved in synaptic plasticity (477), and cytoskeletal actin is the major structural component of the dendritic spine (478). Cytoskeletal actin role in synaptic plasticity is mediated by dynamic transformation between G-actin (monomeric globular actin) and F-actin (polymeric filamentous actin) through polymerization and depolymerization (actin rearrangement) (479,480,481). Literature supports a role for actin rearrangement in LTP maintenance and behavioral adaptations (479,482,483) and demonstrates that changes in the actin cytoskeleton in certain neuronal cell types alter behavioral responses to different drug of abuse (299,484). Importantly, actin rearrangements in the amygdala and the hippocampus are required for the acquisition and consolidation of the aversive memories of morphine withdrawal (485).

The immediate-early gene (IEG) activity-regulated cytoskeletal protein (*Arc*) is found at recently activated synapses and interacts with the cytoskeletal protein actin (486,487) (Figure 14). For instance, *Arc* controls LTP consolidation through regulation of local actin polymerization in the DG in vivo (488). Like *Egr1*, *Arc* is required for synaptic plasticity and for several forms of long-term memory (489). *Arc*, however, is a direct effector protein at the synapse. Upon cell activation, *Arc* mRNA traffics to dendrites and accumulates at sites of synaptic activity, where it is locally translated (490,486,487) and plays important roles in the structural modifications of the synapse (491,492). The expression of *Arc* is regulated as an IEG (493), but also as a late-response gene by a protein synthesis-dependent mechanism (494,495). Moreover, *Arc* has been identified as a direct target of the *Egr1* (496,495). Furthermore, *Arc* synthesis is required for both the induction and consolidation of LTP elicited by local BDNF infusion, thus identifying *Arc* as a key molecular effector of BDNF in synaptic plasticity (488).

Noradrenergic stimulation of the BLA, which enhances memory consolidation, also increases dorsal hippocampal levels of *Arc* (497). Additionally, inactivation of the BLA impairs memory consolidation and decreases *Arc* protein levels or actin rearrangements in the dorsal hippocampus (497,485)}. The finding that intra-BLA infusions of muscimol attenuate the increase in *Arc* mRNA induced by contextual fear conditioning provides further evidence that the BLA modulates memory consolidation via regulation of *Arc* expression in the hippocampus (498).

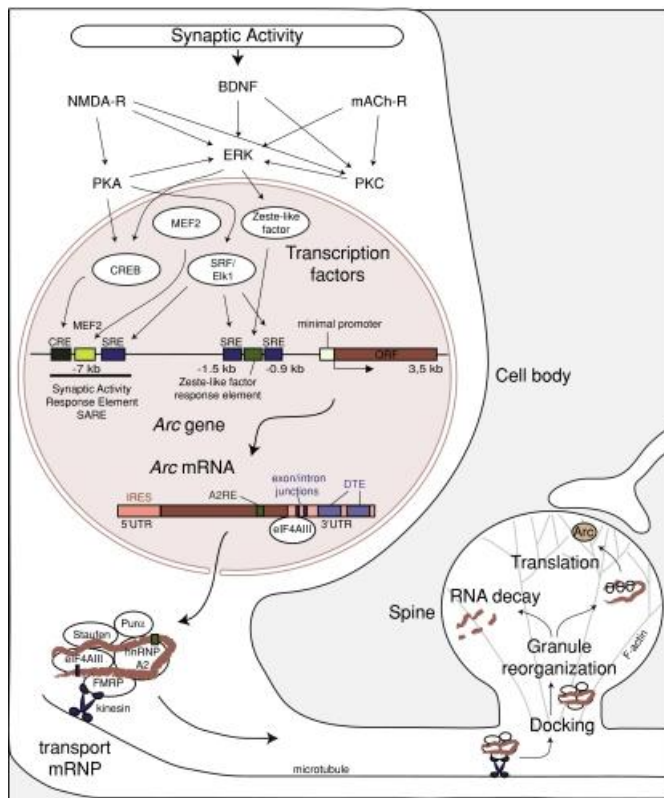


Figure 14: *Arc* expression is induced by signaling cascades that regulate transcription factors in response to synaptic activity. The diagram depicts the genomic organization of the synaptic activity-responsive element (*SARE*) and other regulatory elements upstream of the *Arc* open reading frame that serve as binding sites for these transcription factors. Following transcription, *cis*-regulatory elements of the *Arc* mRNA regulate its assembly into transport mRNPs. The dendritic localization and stability of the mRNA in dendrites is a result of active microtubule-based transport and local F-actin-dependent docking. Upon translation, *Arc* RNA is subject to rapid nonsense-mediated RNA decay.

Reproduced from ref (499): Bramham CR, Alme MN, Bittins M, Kuipers SD, Nair RR, Pai B, Panja D, Schubert M, Soule J, Tiron A, Wibrand K. The Arc of synaptic memory. *Exp Brain Res*. 2010. 200:125-40.

6.5. DRUG ADDICTION AND MEMORY

The aversive memories of drug withdrawal play a key role in the induction of a motivational state leading to drug seeking and taking (60). CPA test is one of the most sensitive measurement for the negative motivational state produced by opiate withdrawal in both chronically dependent (500) and acutely dependent animals (501). CPA is a pavlovian conditioning paradigm in which drug withdrawal is paired with a particular environment, which triggers the onset of association between the negative affective consequences of withdrawal with context (aversive memory formation), and then the animals are reexposed to the paired environment in drug-free state, leading to avoidance of the paired environment (aversive memory retrieval). Thus, CPA can be used to explore the neurobiological mechanisms underlying the formation and retrieval of aversive memories of drug withdrawal in drug-dependent animals. Place aversion is the reflection of the aversive memories formed by the negative affective consequences of morphine withdrawal with a particular environment (502). Interestingly, the amygdala and dorsal hippocampus are critically involved in mediating the negative affective component of morphine withdrawal in morphine-dependent rats (485).

7. GLIA AND ADDICTION

Astrocytes, a subset of glial cells, preserve homeostasis of the extracellular space by buffering potassium and glutamate (503). Astrocytes extend thousands of fine membranous processes, many of which make contact with synapses, to provide metabolic support and maintain the integrity of neuronal communication (503). Astrocyte metabolism operates via the generation of adenosine triphosphate (ATP) from glycolysis (504). Lactate serves as a key component of the neuronal metabolic cycle used to generate the ATP serving the energy demands of synaptic transmission (504). Following an action potential, astrocytes act to terminate signaling by clearing glutamate from the synaptic cleft via the Na⁺-dependent glial glutamate transporter (GLT) family, in particular GLT-1 (505). Once removed from the synapse, glutamate is converted to glutamine, which is then released by astrocytes into the extracellular space, where it can be taken up by neurons (506). Following uptake, the enzyme glutaminase converts glutamine back to glutamate so that it can then be loaded into vesicles by the vesicular glutamate transporter (VGluT), restarting the cycle of glutamate synaptic transmission (506). Unlike neurons, astrocytes are connected to each other via gap junction pore proteins, forming functional ensembles of interconnected cells (507). As a result, Ca²⁺ and other small molecules like inositol-1,4,5-trisphosphate (IP3) can flow between adjacent cells in the reticular astrocyte network. This allows for the propagation of transient increases in internal calcium concentration across connected cells, termed calcium waves. Calcium waves play a role in initiating organized gliotransmission in order to synchronize neuronal firing patterns (508). In fact, astrocytes participate in bidirectional communication with neurons and release chemical transmitters (gliotransmission), including taurine, ATP, D-serine, and glutamate (509). Thus, activation of astrocytes induces the synthesis and release of substances capable of modulating the surrounding cells, like neurotransmitters, neurotrophic factors, cytokines, chemokines and extracellular matrix factors (510,511,512).

Moreover, glial cells are considered the immune competent cells of the CNS as well as crucial components of synaptic plasticity (513,514). There is increasing evidence that drugs of abuse produce alterations in CNS immunology. For example, opiates induce profound changes in glial cellular morphology and phenotypic immunohistological marker expression (GFAP: glial fibrillary acidic protein, a cell surface marker of astrocyte reactivity) in specific brain areas (515). Importantly, it has been proposed that the actions of opiates through glial reactivity are involved in the development of opiate dependence (516,517,518) (Figure 15). In addition, morphine has been shown to promote the expression of pro-inflammatory cytokines and chemokines in the CNS (519,520), where these peptides are synthesized and released mostly by glial cells. Some of these molecules exhibit a profile similar to that of neuromodulators or behave like neurotrophic factors (521,522). Interestingly, morphine-mediated increase of cytokines/chemokines appears to be more pronounced in brain areas that

participate in the neuronal network involved in opiate abuse/reward, including the NAc (523). These data raise the possibility that astrocytes would contribute to the synaptic plasticity during the development of drug addiction.

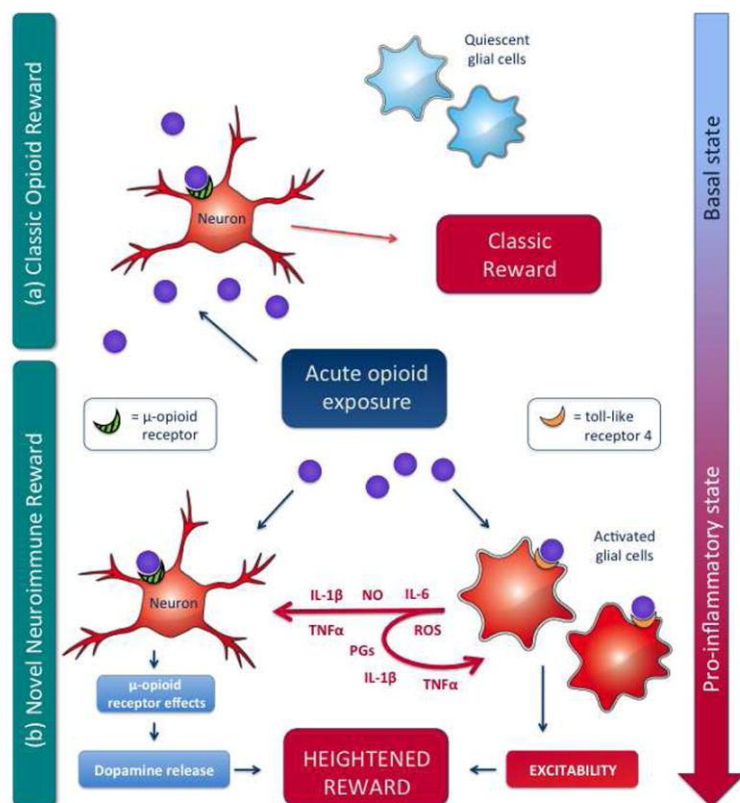


Figure 15: a) In the classic view of opioid reward, glia is not accounted for, beyond their homeostatic role. b) Recent research describes non-neuronal actions of opioids and opioid-induced proinflammatory glial activation. The neuronal opioid receptor-dependent and complementary opioid-Toll-like receptor 4 (TLR4) dependent mechanisms produce a heightened opioid reward signal and ensuing presentation of altered behavior.

Reproduced from ref (524): Hutchinson MR, Watkins LR. Why is neuroimmunopharmacology crucial for the future of addiction research? *Neuropharmacology*. 2014. 76 Pt B:218-27.

7.1. ASTROCYTES AND GLUTAMATE HOMEOSTASIS DURING ADDICTION

Glutamate homeostasis is defined as the balance between synaptic and nonsynaptic glutamate, with nonsynaptic glutamate acting to regulate neuronal communication (264). As such, astrocytes tightly regulate synaptic and nonsynaptic glutamate levels through the coordinated release and uptake of glutamate (525,503). Presynaptically, glial-glutamate release can activate Gi/Go-coupled group II mGluR (mGluR2/3) located on cortical glutamatergic neurons synapsing in the NAc (526). Stimulation of glutamate autoreceptors attenuates synaptic release through the activation of presynaptic K⁺ channels, the inhibition of presynaptic Ca²⁺ channels, and by directly inhibiting vesicular release (526). Glial-derived glutamate can also potentially activate mGluR5, a Gq coupled group I metabotropic glutamate receptor located postsynaptically on GABAergic MSNs in the NAc (527). Activation of these receptors has been linked to synaptic plasticity (528) and the induction of LTP in the NAcCore (529). In addition to mGluRs, glial glutamate release can also activate nonsynaptic ionotropic GluN2B containing NMDA receptors. The mainly synaptic GluN2A containing NMDA receptors have been linked to the induction of LTP, whereas extrasynaptic GluN2B

containing subtypes mediate the induction of LTD (530). Evidence suggests that these receptors play a role in mediating drug-related plasticity, as antagonizing GluN2B-containing NMDA receptors has been shown to inhibit opiate (531), nicotine (532), and alcohol reinstatement (533).

7.2. PLEIOTROPHIN AND MIDKINE

MK is a heparin-binding cytokine that promotes growth, survival, differentiation and migration of different target cells (534). MK has an important role in development and repair of the CNS (535). PTN is a secreted, highly conserved cytokine which shares over 50% identity in amino acid sequence with MK, the only other member of the PTN/MK developmentally-regulated gene family (536). The expression of both PTN and MK genes is constitutive and limited to only a few cell types in adults (537). Both genes are up-regulated at sites of injury and repair in inflammatory macrophages, glia, dermal fibroblasts, endothelial cells and other cells (538,539,540), suggesting that PTN and MK signaling may be critical in different steps of differentiation of different cells, both in development and in wound repair.

It has been suggested that MK and PTN could be involved in the addictive cycle to drugs of abuse (Table 2). Thus, PTN mRNA and/or MK mRNA levels are up-regulated after acute amphetamine administration (541) and after injection of delta-9-tetrahydrocannabinol (542) or morphine (543) in brain areas related to addiction, such as the NAc(shell), the PFC and the hippocampus, respectively. Likewise, increased mRNA and protein levels were found in the PFC of alcoholics and smokers (544). Given that these cytokines exert effects that are similar to those of neurotrophins, these findings support the hypothesis that these two cytokines are up-regulated in order to induce neurotrophic or neuroprotective effects during drug consumption (545). MK and PTN bind common receptors, including the protein tyrosine phosphatase β/ζ (RPTP β/ζ) receptor (546), which is abundantly expressed in the CNS. The interaction of MK or PTN with RPTP β/ζ establishes a “ligand-dependent inactivation” of RPTP β/ζ , presumably consequence of RPTP β/ζ dimerization (547). As a consequence, PTN or MK signaling through RPTP β/ζ leads to the activation of ERK and PI3K-Akt (548,549), important axes inducing morphological changes and modulating addictive behaviors.

| | Drug addiction | | Neurodegenerative disorders | |
|------------|--|---|--|---|
| | Neurotoxicity | Addictive behavior | Parkinson's Disease | Alzheimer's Disease |
| <i>PTN</i> | ↓ Amphetamine nigrostriatal toxicity ↓ Cocaine neurotoxicity | ↓ Amphetamine relapse ↓ Morphine reward | ↓ nigrostriatal degeneration ↑ functional nigrostriatal recovery | |
| <i>MK</i> | ↓ Amphetamine striatal toxicity | ↓ Cocaine relapse | ↓ neurochemical and behavioral dysfunctions in nigrostriatal pathways ↓ olfactory and cognitive dysfunctions of early stages | ↓ amyloid β -peptide plaques cytotoxicity ↓ amyloid β -peptide plaques deposition |

Table 2: Proposed roles and effects of PTN and MK in drug addiction, Parkinson's disease and Alzheimer's disease

Reproduced from ref (545): Herradón G, Pérez-García C. Targeting midkine and pleiotrophin signalling pathways in addiction and neurodegenerative disorders: recent progress and perspectives. *Br J Pharmacol.* 2014. 171:837-48.

II. OBJETIVOS

Los opiáceos, como la morfina, son unos potentes analgésicos que se usan para tratar diversas formas de dolor agudo y crónico. Sin embargo son también muy adictivos, lo que limita su uso médico. Además, el uso recreativo de los opiáceos y opioides (morfina, heroína), ha aumentado notablemente en los últimos años (550). El uso crónico de opiáceos produce cambios neuroadaptativos a nivel cerebral que conllevan efectos indeseables, como la adicción, la cuál es un notable problema médico y sanitario. La creciente evidencia implica a varios mecanismos de regulación génica (incluyendo epigenéticos, moleculares, celulares y a nivel de circuitos neuronales) en los cambios que las drogas de abuso provocan en el cerebro, siendo el conocimiento de éstos una posible estrategia terapéutica para el tratamiento de la adicción (550,384,551).

Por otra parte, cada día es más evidente que las drogas de abuso producen alteraciones en el sistema inmune del CNS. Por ello, se ha propuesto que los astrocitos contribuirían a la plasticidad sináptica que se produce durante el desarrollo de la adicción mediante la síntesis y liberación de sustancias, como las citoquinas (279,552,545). De hecho, los opiáceos inducen cambios en la morfología de las células de la glia y expresión del marcador inmunohistoquímico GFAP de una forma región-específica(515). Estos datos apuntan la posibilidad de que los astrocitos contribuyan a la plasticidad sináptica durante el desarrollo de la adicción.

Hemos centrado el presente estudio en varios neurocircuitos implicados en la adicción a opiáceos: i) el eje HPA y el sistema extrahipotalámico del estrés, ii) el sistema dopaminérgico de recompensa, y iii) circuitos relacionados con las memorias aversivas.

- El uso crónico de las drogas de abuso altera el sistema cerebral del estrés. La exposición repetida a opiáceos conlleva la acumulación del factor de transcripción Δ FosB, particularmente en áreas cerebrales relacionadas con la recompensa y el estrés. La evidencia sugiere que las hormonas relacionadas con el estrés (como los GCs) podrían producir adaptaciones en el sistema cerebral del estrés que incluirían alteraciones a nivel de la expresión génica y de factores de transcripción.

1. **El primer objetivo** del presente estudio se diseñó después de considerar lo anterior. Examinamos el papel de los GCs en la expresión de Δ FosB en poblaciones neuronales específicas del sistema cerebral del estrés durante la dependencia de morfina. Para ello, la expresión de Δ FosB se cuantificó en ratas control (sham) y ADX que desarrollaron dependencia de morfina.

- En los últimos años, diferentes estudios resaltan las modificaciones post-transcripcionales mediadas por los miRs en la adicción y otras patologías neurológicas y enfermedades neurodegenerativas (553). Algunos de ellos, como el miR-133b, han sido identificados como mediadores de la diferenciación, maduración y función de las neuronas dopaminérgicas del mesencéfalo. miR-133b actuaría mediante la regulación a la baja de factores de transcripción implicados en el desarrollo y el fenotipo de estas neuronas, como Nurr1 y Pitx3, que poseen una función crítica a nivel mesencefálico al regular la transcripción de diferentes genes implicados en el metabolismo de la DA. Los cambios epigenéticos, como el silenciamiento génico mediado por miRs/Ago2, también representan complejos mecanismos que regulan la plasticidad neuronal. Entre los miembros de la familia Ago, sólo Ago2 parece tener un papel importante en la ejecución del silenciamiento mediado por los diferentes miRs (554).

2. **Segundo objetivo:** Dada la importante implicación de la transmisión dopaminérgica en los desórdenes adictivos, hemos centrado esta parte de nuestro estudio en identificar los marcadores dopaminérgicos que resultan alterados con la administración aguda y crónica de morfina, así como durante el síndrome de abstinencia inducido por naloxona, tanto en el VTA como el NAc. Para ello, hemos determinado: i) la expresión del miR-133b y Ago2 en el VTA; ii) el contenido de TH y su actividad; iii) la actividad dopaminérgica (recambio de DA y activación de TH) en el sistema mesolímbico de ratas tratadas con morfina aguda, ratas dependientes de morfina y durante el síndrome de abstinencia inducido por naloxona.

3. El **tercer objetivo** fue estudiar: i) posibles cambios en los niveles de mRNA y proteína de Nurr1 y Pitx3, así como la expresión de Ago2 y de TH en regiones específicas del sistema mesolímbico; ii) actividad dopaminérgica en el NAc; iii) co-localización cuantitativa de Nurr1 y Pitx3 en el VTA en las neuronas que expresan TH, iv) cambios en la plasticidad de subpoblaciones neuronales dopaminérgicas del VTA en respuesta a morfina aguda, crónica o durante el síndrome de abstinencia.

- Las neuronas dopaminérgicas localizadas en el mesencéfalo y que proyectan al estriado se caracterizan por la expresión de autoreceptores DRD2 que inhiben la síntesis y liberación de DA (82). Además, DAT (que media la recaptación de DA), TH (enzima limitante de la síntesis de catecolaminas) y VMAT2, han sido identificados como importantes reguladores de la función dopaminérgica. Por otro lado, Nurr1 y Pitx3 son esenciales para la expresión de genes implicados en el metabolismo de la DA, como *Th*, *Dat*, *Vmat2* and *Drd2* (220,555,221).

4. **Cuarto objetivo:** Los efectos del síndrome de abstinencia a morfina sobre la expresión de Nurr1 y Pitx3 nos llevó a investigar la expresión de otros genes y proteínas que están implicadas en la regulación de la función dopaminérgica, algunos de los cuáles son dianas de Nurr1 y Pitx3. Para ello, las ratas recibieron morfina de forma aguda o crónica. Otro grupo fue sometido a un síndrome de abstinencia a morfina. Se estudió: i) la expresión de *Dat-DAT*, *Vmat2-VMAT2*, *Drd2-DRD2* y *DRD1* en VTA/NAc; ii) la co-localización de Nurr1 y/o Pitx3 con neuronas que expresan TH en el VTA, así como el porcentaje de neuronas dopaminérgicas que expresan Nurr1 y Pitx3; iii) la posible correlación entre la expresión de Nurr1/Pitx3 y los niveles de los diferentes marcadores dopaminérgicos.

- También se ha propuesto que los astrocitos, mediante la síntesis y liberación de factores de crecimiento/citoquinas, como MK y PTN, jugarían un papel fundamental en los cambios en la plasticidad sináptica del CNS durante el desarrollo de la adicción (524,545).

5. **Quinto objetivo:** Dado que PTN y MK tienen los mismos efectos que las neurotrofinas y que los astrocitos pueden liberar MK y PTN después de diversos estímulos, nuestro siguiente objetivo fue: i) estudiar posibles cambios en la expresión de PTN, MK, su receptor RPTP β/ζ , así como alteraciones en sus vías de señalización intracelular en respuesta a morfina aguda, crónica o durante el síndrome de abstinencia en el VTA y NAc; ii) la posible activación de los astrocitos durante los tratamientos descritos, que podría conllevar la liberación de diferentes factores; iii) identificar las subpoblaciones celulares que producen y secretan PTN y/o MK, así como aquellas que expresan RPTP β/ζ .

III. AIMS

Opiate drugs, such as morphine, are a class of powerful analgesics that are used for treating many forms of acute and chronic pain. However, they are also highly addictive, which limits their medical use. The non-medical use of opiates (heroin, morphine) has increased greatly in recent years (550). Chronic use of opiates causes brain neuroadaptation that lead to undesirable effects, namely opiate addiction that is a significant medical and public health problem. Increasing evidence implicates various mechanisms of gene regulation (including epigenetic, molecular, cellular and circuit level effects) in the changes that drugs of abuse induce in the brain, indicating a potential therapeutic strategy for addiction therapy (550,384,551).

On the other hand, there is increasing evidence that drugs of abuse produce alterations in CNS immunology. Thus, it has been proposed that astrocytes contribute to the synaptic plasticity during the development of drug addiction by the synthesis and release of substances, such as cytokines (279,552,545). Thus, opiates induce profound changes in glial cellular morphology and phenotypic receptor/immunohistological marker expression, GFAP in a region specific-manner (515). These data raise the possibility that astrocytes contribute to the synaptic plasticity during the development of drug addiction.

We have focused the present study on several neurocircuits involved in opiate addiction: i) the HPA and extrahypothalamic stress systems; ii) the dopaminergic reward pathways; and iii) aversive memories-related neuronal circuits.

- Chronic use of drugs of abuse profoundly alters stress-responsive system. Repeated exposure to opiates leads to accumulation of the transcription factor Δ FosB, particularly in brain areas associated with reward and stress. Recent evidence suggests that stress-related hormones (e.g. GCs) may induce adaptations in the brain stress system that are likely to involve alterations in gene expression and transcription factors.

1. **The first objective** of the present study was designed after considering the above. We examined the role of GCs in regulation of Δ FosB expression in specific populations of the brain stress system during morphine dependence. For that, expression of Δ FosB was measured in control (sham-operated) and ADX rats that were made dependent on morphine.

- In recent years, studies highlight post-transcriptional modifications mediated by miRs in addiction and other neurological disorders and neurodegenerative diseases (553). Some of them, such as miR-133b, have been proposed to regulate the differentiation, maturation and function of

dopaminergic neurons by downregulating transcription factors involved in the development and physiological function of midbrain dopaminergic neurons, such as Nurr1 and Pitx3, which are critical for transcription of a set of genes involved in DA metabolism in the mesolimbic pathway. Epigenetic changes such as miRs/Ago2-induced gene silencing represent complex molecular signature that regulate cellular plasticity. Among the Ago family members, only Ago2 seems to have an important role in miRs generation and execution of miR-mediated gene silencing (554).

2. **Second objective:** Given the important implications of DA neurotransmission in addiction disorders, we have focused this part of our study on identifying the DA markers that are altered in association with acute and chronic morphine exposure, as well as with morphine withdrawal in the VTA and NAc (medial shell). For that, we have determined i) the expression of miR-133b and Ago2 in VTA; ii) TH content and activity; and iii) dopaminergic activity (DA turnover and TH activation) in the mesolimbic system from rats acutely injected with morphine, in morphine-dependent rats and during naloxone-induced morphine withdrawal.

3. **The third objective** was to study: i) Nurr1 and Pitx3 mRNAs and proteins changes as well as the expression of Ago2 and TH mRNA and protein levels in specific region of the mesolimbic system; ii) dopaminergic activity in the NAc; iii) quantitative co-localization of Nurr1 and Pitx3 in the VTA TH-positive neurons; and iv) the plasticity changes in VTA DA neurons subpopulations in response to morphine, morphine dependence and morphine withdrawal.

- DA neurons located in the midbrain and projecting to the striatal complex are primarily characterized by expression of DRD2 autoreceptor subtype, which inhibits DA synthesis and release (82). In addition, DAT, which mediates the reuptake of DA, TH (the rate-limiting enzyme for DA synthesis) and VMAT2 have been identified as important regulators of DA function. On the other hand, Nurr1 and Pitx3 are crucial for expression of the set of genes involved in DA metabolism, such as *Th*, *Dat*, *Vmat2* and *Drd2* (220,555,221).

4. **Fourth Objective:** The long-lasting effects of opiate withdrawal on Nurr1 and Pitx3 expression prompted us to investigate expression of other genes and proteins that are involved in the regulation of DA function, some of which represent putative targets of Nurr1 and Pitx3. For that, rats were exposed to acute and chronic morphine administration as well as to morphine withdrawal and analyzed: i) the expression of *Dat*-DAT, *Vmat2*-VMAT2, *Drd2*-DRD2 and DRD1 in VTA/NAc(shell), dysfunction of which is causally linked to addiction; ii) the co-localization of Nurr1 and/or Pitx3 with TH-positive neurons in the VTA as well as the percentage of DA neurons expressing

Nurr1 and Pitx3; and iii) the possible correlation between Nurr1/Pitx3 expression and DA markers levels in the VTA and/or NAc(shell).

- It has also been proposed that astrocytes, through the synthesis and release of growth factors/cytokines, such MK and PTN, would play a critical role in long-term synaptic plasticity in the CNS during drug addiction.

5. **Fifth Objective:** Given the important implications of DA neurotransmission in addiction disorders and the complexity of opiate-induced neuroadaptive responses in the brain reward dopaminergic system, the present study was focused on: i) identifying whether the expression of PTN, MK, RPTP β/ζ and their intracellular signaling pathways (Akt and ERK) are altered in association with acute and chronic morphine exposure as well as with morphine withdrawal in the VTA and NAc; ii) the possible activation of astrocytes, which could lead to the release of astrocyte-related soluble factors; iii) we also aimed to identify those cell subpopulations that produced and secreted PTN and/or MK and those that expressed RPTP β/ζ in response to morphine administration or morphine withdrawal.

IV. ARTICLES

García-Pérez D, Laorden ML, Milanés MV, Núñez C. Glucocorticoids regulation of FosB/ Δ FosB expression induced by chronic opiate exposure in the brain stress system. PLoS One. 2012;7(11):e50264. doi: 10.1371/journal.pone.0050264.

Plos One

Abstract

Chronic use of drugs of abuse profoundly alters stress-responsive system. Repeated exposure to morphine leads to accumulation of the transcription factor Δ FosB, particularly in brain areas associated with reward and stress. The persistent effects of Δ FosB on target genes may play an important role in the plasticity induced by drugs of abuse. Recent evidence suggests that stress-related hormones (e.g., glucocorticoids, GC) may induce adaptations in the brain stress system that is likely to involve alteration in gene expression and transcription factors. This study examined the role of GC in regulation of FosB/ Δ FosB in both hypothalamic and extrahypothalamic brain stress systems during morphine dependence. For that, expression of FosB/ Δ FosB was measured in control (sham-operated) and adrenalectomized (ADX) rats that were made opiate dependent after ten days of morphine treatment. In sham-operated rats, FosB/ Δ FosB was induced after chronic morphine administration in all the brain stress areas investigated: nucleus accumbens(shell) (NAc), bed nucleus of the stria terminalis (BNST), central amygdala (CeA), hypothalamic paraventricular nucleus (PVN) and nucleus of the solitary tract noradrenergic cell group (NTS-A(2)). Adrenalectomy attenuated the increased production of FosB/ Δ FosB observed after chronic morphine exposure in NAc, CeA, and NTS. Furthermore, ADX decreased expression of FosB/ Δ FosB within CRH-positive neurons of the BNST, PVN and CeA. Similar results were obtained in NTS-A(2) TH-positive neurons and NAc pro-dynorphin-positive neurons. These data suggest that neuroadaptation (estimated as accumulation of FosB/ Δ FosB) to opiates in brain areas associated with stress is modulated by GC, supporting the evidence of a link between brain stress hormones and addiction.

<http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0050264>

García-Pérez D, Sáez-Belmonte F, Laorden ML, Núñez C, Milanés MV. Morphine administration modulates expression of Argonaute 2 and dopamine-related transcription factors involved in midbrain dopaminergic neurons function. *Br J Pharmacol*. 2013;168(8):1889-901. doi: 10.1111/bph.12083.

British Journal of Pharmacology

Abstract

BACKGROUND AND PURPOSE:

Alterations in transcription factors that regulate the development and maintenance of dopamine (DA) neurons (such as Nurr1 and Pitx3) play an important role in the pathogenesis of addiction diseases. We have examined the effects of acute and chronic morphine and morphine withdrawal on TH expression and activity as well as expression of Nurr1, Pitx3 and Ago2 in the ventral tegmental area (VTA) and nucleus accumbens (NAc) of the rat.

EXPERIMENTAL APPROACH:

Rats were injected acutely with morphine and decapitated 1 or 2 h later. Another set of rats were made dependent on morphine by implantation of two morphine pellets. Precipitated withdrawal was induced by injection of naloxone. Ago2, Pitx3, Nurr1, total TH (tTH), TH phosphorylated at Ser31 and at Ser40, and 3,4-Dihydroxyphenylacetic acid, and DA determination in the VTA and/or NAc were measured using immunoblotting, HPLC and immunofluorescence.

KEY RESULTS:

Acute morphine produced a marked increase in TH activity and DA turnover in the NAc, concomitantly with increased Nurr1 and Pitx3 expression in the VTA. In contrast, precipitated morphine withdrawal decreased TH activation, TH expression and did not increase DA turnover in the NAc. These effects paralleled decreases in Ago2 expression, which was accompanied by increased Nurr1 and Pitx3, TH activity and normalized TH protein levels in the VTA.

CONCLUSIONS AND IMPLICATIONS:

The combined decrease in Ago2 and increases in Nurr1 and Pitx3 might represent some of the mechanisms that served to protect against accumbal TH regulation observed in morphine withdrawn rats, which may be critical for DA bioavailability to influence behaviour.

<http://onlinelibrary.wiley.com/doi/10.1111/bph.12083/abstract;jsessionid=F1A1F51070CEE548FD60818818D67F6A.f01t04>

García-Pérez D, López-Bellido R, Hidalgo JM, Rodríguez RE, Laorden ML, Núñez C, Milanés MV. Morphine regulates Argonaute 2 and TH expression and activity but not miR-133b in midbrain dopaminergic neurons. *Addict Biol.* 2015;20(1):104-19. doi: 10.1111/adb.12083.

Addiction Biology

Abstract

Epigenetic changes such as microRNAs (miRs)/Ago2-induced gene silencing represent complex molecular signature that regulate cellular plasticity. Recent studies showed involvement of miRs and Ago2 in drug addiction. In this study, we show that changes in gene expression induced by morphine and morphine withdrawal occur with concomitant epigenetic modifications in the mesolimbic dopaminergic (DA) pathway [ventral tegmental area (VTA)/nucleus accumbens (NAc) shell], which is critically involved in drug-induced dependence. We found that acute or chronic morphine administration as well as morphine withdrawal did not modify miR-133b messenger RNA (mRNA) expression in the VTA, whereas Ago2 protein levels were decreased and increased in morphine-dependent rats and after morphine withdrawal, respectively. These changes were paralleled with enhanced and decreased NAc tyrosine hydroxylase (TH) protein (an early DA marker) in morphine-dependent rats and after withdrawal, respectively. We also observed changes in TH mRNA expression in the VTA that could be related to Ago2-induced translational repression of TH mRNA during morphine withdrawal. However, the VTA number of TH-positive neurons suffered no alterations after the different treatment. Acute morphine administration produced a marked increase in TH activity and DA turnover in the NAc (shell). In contrast, precipitated morphine withdrawal decreased TH activation and did not change DA turnover. These findings provide new information into the possible correlation between Ago2/miRs complex regulation and DA neurons plasticity during opiate addiction.

<http://onlinelibrary.wiley.com/doi/10.1111/adb.12083/abstract>

García-Pérez D, López-Bellido R, Rodríguez RE, Laorden ML, Núñez C, Milanés MV. Dysregulation of dopaminergic regulatory mechanisms in the mesolimbic pathway induced by morphine and morphine withdrawal. *Brain Struct Funct.* 2014;220(4):1901-19. doi:10.1007/s00429-014-0761-5.

Brain Structure and Function

Abstract

Dopamine (DA) is thought to represent a teaching signal and has been implicated in the induction of addictive behaviours. Previously, it has been proposed that the transcription factors Nurr1 and Pitx3, which are critical for transcription of a set of genes involved in DA metabolism in the mesolimbic pathway, are associated with addiction pathology. The aim of our study was to investigate abnormalities in the mesolimbic pathway associated with morphine dependence and withdrawal. Using quantitative real-time PCR, immunofluorescence, HPLC and Western blotting, here we studied the effects of single morphine administration, morphine dependence and morphine withdrawal on Nurr1 and Pitx3 expression as well as on the DA marker tyrosine hydroxylase (TH) and the turnover of DA in the ventral tegmental area (VTA) and/or nucleus accumbens. We showed that the three experimental conditions caused induction of Nurr1 and Pitx3 in the VTA, which correlated with changes in TH expression during chronic morphine administration. Present data also confirmed the colocalization of Nurr1 and Pitx3 with TH-positive neurons in the posterior VTA. Furthermore, during morphine dependence, Nurr1 was detected in the nucleus compartment of VTA TH-positive neurons, whereas Pitx3 was strongly detected in the nucleus of TH-positive neurons after single morphine administration and during morphine withdrawal. The number of TH neurons, number of Nurr1 or Pitx3-positive cells, and the number of TH neurons expressing Nurr1 or Pitx3 were not modified in the subpopulations of DA neurons. Present data provide novel insight into the potential correlation between Nurr1 and Pitx3 and DA neurons plasticity during opiate addiction in the mesolimbic pathway.

<http://link.springer.com/article/10.1007%2Fs00429-014-0761-5>

García-Pérez D, Núñez C, Laorden ML, Milanés MV. Regulation of dopaminergic markers expression in response to acute and chronic morphine and to morphine withdrawal. *Addict Biol.* 2014. doi: 10.1111/adb.12209.

Addiction Biology

Abstract

Dopamine (DA) is thought to represent a teaching signal and has been implicated in the induction of addictive behaviours. Dysfunction of DA homeostasis leading to high or low DA levels is causally linked to addiction. Previously, it has been proposed that the transcription factors Nurr1 and Pitx3, which are critical for transcription of a set of genes involved in DA metabolism in the mesolimbic pathway, are associated with addiction pathology. Using quantitative real-time polymerase chain reaction, immunofluorescence and Western blotting, we studied the effects of single morphine administration, morphine dependence and withdrawal on the DA markers DA transporters (DAT), vesicular monoamine transporters (VMAT2) and DA 2 receptor subtype (DRD2), DA 1 receptor subtype as well as tyrosine hydroxylase (TH) in the ventral tegmental area (VTA) and/or nucleus accumbens (NAc). In addition, Nurr1 and Pitx3 expression was also measured. Present data showed a high degree of colocalization of Nurr1 and Pitx3 with TH⁺ neurons in the VTA. We found that the increased Nurr1 and/or Pitx3 levels during morphine dependence and in morphine-withdrawn rats were associated to an increase of DAT, VMAT2 and DRD2. Altogether, present data indicate that morphine dependence and withdrawal induced consistent alterations of most of the DA markers, which was correlated with transcription factors involved in the maintenance of DA neurons in drug-reward pathways, suggesting that Nurr1 and Pitx3 regulation might be associated with controlling adaptation to chronic morphine and to morphine withdrawal-induced alterations of DA neurons activity in the mesolimbic pathway.

<http://onlinelibrary.wiley.com/doi/10.1111/adb.12209/abstract>

García-Pérez D, Luisa Laorden M, Núñez C, Victoria Milanés M. Glial activation and midkine and pleiotrophin transcription in the ventral tegmental area are modulated by morphine administration. *J Neuroimmunol.* 2014;274(1-2):244-8. doi: 10.1016/j.jneuroim.2014.07.017.

Journal of Neuroimmunology

Abstract

Opiates cause persistent restructuring in the mesolimbic reward system. Although a possible role for midkine and pleiotrophin cytokines in the field of synaptic plasticity has been proposed, it has not been assessed whether morphine administration regulates astrogliosis and midkine and pleiotrophin transcription. We observed that single morphine injection and chronic morphine increased glial fibrillary acidic protein expression in the ventral tegmental area (VTA). Interestingly, single morphine injection and chronic morphine increased VTA midkine and pleiotrophin mRNA expression. Given these results, we hypothesize a role for these cytokines in mediating, at least in part, acute neuroprotective effects and chronic neurotrophic adaptations that contribute to drug dependence.

[http://www.jni-journal.com/article/S0165-5728\(14\)00232-X/abstract](http://www.jni-journal.com/article/S0165-5728(14)00232-X/abstract)

V. CONCLUSIONES

1. El presente trabajo demuestra que los GCs están críticamente involucrados en la acumulación de FosB/ Δ FosB en el sistema cerebral del estrés durante la administración crónica de morfina, lo que resultaría en cambios duraderos en los patrones de expresión génica en las áreas relacionadas con el estrés.

2. Aunque no hallamos cambios significativos en los niveles de miR-133b en el VTA, proponemos el papel de Ago2 en combinación con otros miRs en la regulación de la estabilidad y/o traducción del mRNA de TH en respuesta a la administración crónica de morfina y al síndrome de abstinencia a opiáceos provocado por la administración de naloxona. Además, la dependencia de morfina y el síndrome de abstinencia están asociados con alteraciones en los factores de transcripción involucrados en el mantenimiento de las neuronas dopaminérgicas del sistema mesolímbico de recompensa. Por ello, nuestros resultados sugieren un papel importante de Nurr1 y Pitx3 en los cambios en la expresión génica durante la dependencia y abstinencia a opiáceos, y que la regulación epigenética de TH puede estar asociada, al menos en parte, a los mecanismos moleculares que contribuyen a los cambios en la función dopaminérgica inducida por los opiáceos.

3. La dependencia de morfina y el síndrome de abstinencia a la misma están asociados con alteraciones de la mayoría de los marcadores dopaminérgicos (DAT, VMAT2, DRD2) en la vía mesolímbica de recompensa, que se correlacionan con alteraciones en los factores de transcripción encargados del mantenimiento de las neuronas dopaminérgicas (Nurr1 y Pitx3). La correlación entre los marcadores dopaminérgicos y Nurr1/Pitx3 añaden evidencia a nuestros anteriores resultados, y pueden reflejar el papel de estos factores de transcripción durante la dependencia y abstinencia a morfina.

4. Debido a la activación glial en el VTA durante la administración aguda y crónica de morfina, junto con el incremento de los niveles de mRNA de MK y PTN, proponemos la importancia de estas citoquinas al mediar, al menos en parte, las adaptaciones tróficas que se observan durante el fenómeno de adicción.

VI. CONCLUSIONS

1. Present work provides evidence that GCs are critically involved in FosB/ Δ FosB accumulation in the brain stress system after chronic morphine exposure, which might result in lasting changes of gene expression pattern in stress-related areas.

2. Although no significant changes of miR-133b levels are detected in the VTA, a role for Ago2 and specific miRs is hypothesized in regulating TH mRNA stability and/or translation in response to chronic morphine administration and naloxone-induced morphine withdrawal. Moreover, morphine dependence and withdrawal are associated with consistent alteration of transcription factors involved in the maintenance of dopaminergic neurons in the mesolimbic drug-reward pathway. Thus, our results suggest an important role for Nurr1 and Pitx3 in contributing to the changes in gene expression during opiate dependence and withdrawal and that epigenetic regulation of TH could be associated, at least in part, to the molecular mechanisms contributing to opiate-induced changes in midbrain dopaminergic function.

3. Morphine dependence and withdrawal are associated with consistent alteration of most of the DA markers (DAT, VMAT2, DRD2) in the mesolimbic drug-reward pathway which correlated with alteration of transcription factors involved in the maintenance of dopaminergic neurons (Nurr1 and Pitx3). The correlations between DA markers and Nurr1/Pitx3 add evidence onto previous results, and may reflect engagement of these transcription factors during morphine dependence and withdrawal.

4. Because of the glial activation in the VTA during acute and chronic morphine administration besides the enhancement in MK and PTN mRNAs, we propose a role for these cytokines in mediating, at least in part, the trophic adaptations that are observed during drug addiction.

VII. APPENDIX

1. OBJETIVOS SUPLEMENTARIOS

- En ratas dependientes de morfina, las memorias aversivas del síndrome de abstinencia pueden generar un estado motivacional que dé lugar a una búsqueda y consumo compulsivo de la droga (9). En la actualidad, los mecanismos que subyacen en la generación de las memorias aversivas del síndrome de abstinencia permanecen desconocidos. Es interesante recalcar que entre las regiones cerebrales que se ven afectadas por las drogas de abuso, se encuentran aquellas que son sustratos neuronales de la memoria, incluyendo el hipocampo, la amígdala y la corteza prefrontal. Esto coincide con los hallazgos de que algunas de las alteraciones más importantes observadas clínicamente en la adicción (por ejemplo el deseo compulsivo de consumir droga y las recaídas) reflejan anormalidades en los circuitos tradicionales de la memoria, actuando las memorias a largo plazo de las experiencias de consumir la droga como conductores de la patología adictiva (20,142). Las hipótesis actuales de los mecanismos moleculares que intervienen en el aprendizaje y la memoria, sugieren que la rápida regulación génica y síntesis de nuevas proteínas conllevan modificaciones sinápticas persistentes que constituyen un mecanismo para la estabilización de memorias a largo plazo (556). *Egr1* y *Arc* son genes de expresión inmediata que juegan un papel crucial en la plasticidad y la memoria. Se ha propuesto que *Arc* tendría un papel fundamental en la consolidación de diferentes tipos de memoria, con algunos trabajos recientes implicando el mRNA y la proteína *Arc* en los procesos adaptativos que ocurren tras el estrés, así como en la plasticidad maladaptativa que ocurre en la adicción a drogas (499). Por otra parte, se ha demostrado que los GCs modulan la consolidación de la memoria (396). Los GCs ejercen sus acciones en diferentes regiones cerebrales, incluyendo el hipocampo, la amígdala y la corteza prefrontal, que tienen alta densidad de GRs y son regiones importantes para la formación de la memoria.

En roedores, el componente afectivo negativo de la dependencia de opiáceos se puede ver reflejado en diferentes tests de comportamiento. Entre ellos, el test de CPA es el más sensible para la medida del estado negativo emocional que produce el síndrome de abstinencia (557).

1. **Sexto objetivo:** En esta parte del estudio, hipotetizamos que los GCs en diferentes estructuras límbicas (como BLA y el DG en el hipocampo) podrían mediar el componente afectivo negativo del síndrome de abstinencia a morfina, y que por tanto jugarían un papel crucial en las memorias aversivas relacionadas con la abstinencia a opiáceos. Para comprobar nuestra hipótesis utilizamos el paradigma de CPA en animales ADX y que desarrollaron dependencia de morfina. A continuación estudiamos: i) posibles modificaciones epigenéticas (miR-124a y miR-212) y activación o inhibición de diferentes vías intracelulares (pCREB, *Egr1* y BDNF) en BLA y DG durante la consolidación de memoria y la recuperación de memoria; ii) cambios en la expresión de *Arc* como marcador de plasticidad neuronal; iii) identificación de las diferentes subpoblaciones neuronales donde

se expresaban las proteínas de interés; iv) correlaciones entre los miRs y la regulación de las diferentes proteínas.

2. SUPPLEMENTAL AIMS

- In opiate-dependent rats, aversive memories of drug withdrawal can generate a motivational state leading to compulsive drug seeking and taking (9). However, the mechanisms underlying generation of drug withdrawal memories remain unclear. Interestingly, among the brain regions affected by drugs of abuse are those that are key neural substrates for behavioral memory, including the hippocampus, amygdala and prefrontal cortex. This coincides with the increasing realization that some of the most important features of addiction seen clinically (eg, drug craving and relapse) reflect abnormalities in traditional memory circuits, with long-term memories of the drug experience serving as potent drivers of addiction pathology (20,142). Current hypothesis on the molecular mechanisms of learning and memory suggest that rapid regulation of gene programs and synthesis of new proteins leading to persistent synaptic modifications constitute a key mechanism for the stabilization of long-term memory (556). *Egr1* and *Arc* are IEG known to play major roles in plasticity and memory. *Arc* has been proposed to have an important role in consolidation of many forms of memory, with recent works implicating *Arc* mRNA and protein in adaptation to stress as well as maladaptive plasticity connected to drug addiction (499). GCs mediate and modulate memory consolidation (396). They exert their actions on brain regions, including the hippocampus, amygdala and prefrontal cortex, that are enriched in GR and are important for long-term memory formation.

In rodent, the negative affective component of opiate dependence could be reflected by several behavioral alterations, among which CPA is the most sensitive measurement for the negative motivational state produced by opiate withdrawal, which is considered as a Pavlovian conditioned paradigm (557).

1. **Sixth Objective:** We hypothesized that GCs in limbic structures (eg., the BLA and hippocampal DG) could specifically mediate the negative motivational component of opiate withdrawal and might play a crucial role in the aversive memories of opiate withdrawal. To test this hypothesis, we used the CPA paradigm in control (sham-operated) and ADX animals that were rendered dependent on morphine. Then, we studied: i) epigenetics modifications (miR-124a y miR-212) and intracellular pathways activated or inhibited (pCREB, *Egr1* y BDNF) in the BLA and the DG during memory consolidation and memory retrieval; ii) changes in *Arc* expression as a marker of neuronal plasticity; iii) we also aimed to identify those cell subpopulations that produced and secreted the different proteins that we studied; iv) correlation analysis between different miRs and target proteins levels.

Molecular Neurobiology

Acute morphine, chronic morphine and morphine withdrawal differently affects pleiotrophin, midkine and receptor protein tyrosine phosphatase β/ζ regulation in the ventral tegmental area

--Manuscript Draft--

| | |
|-----------------------|--|
| Manuscript Number: | |
| Article Type: | Original Research |
| Keywords: | Opiate dependence; reward pathway; pleiotrophin; midkine; glial fibrillary acidic protein. |
| Corresponding Author: | Daniel García Pérez SPAIN |
| First Author: | Daniel García Pérez |
| Order of Authors: | Daniel García Pérez M Luisa Laorden M Victoria Milanés |
| Abstract: | Pleiotrophin (PTN) and midkine (MK) are secreted growth factors and cytokines, proposed to be significant neuromodulators with multiple neuronal functions. PTN and MK are generally related with cell proliferation, growth, and differentiation by acting through different receptors. PTN or MK, signaling through receptor protein tyrosine phosphatase β/ζ (RPTP β/ζ), lead to the activation of extracellular signal-regulated kinases (ERKs) and thymoma viral proto-oncogene (Akt), which induce morphological changes and modulate addictive behaviors. Besides, there is increasing evidence that during the development of drug addiction, astrocytes contribute to the synaptic plasticity by synthesizing and releasing substances such as cytokines. In the present work we studied the effect of acute morphine, chronic morphine and morphine withdrawal on PTN, MK, and RPTP β/ζ expression and on their signaling pathways in the ventral tegmental area (VTA). Present results indicated that PTN, MK and RPTP β/ζ levels increased after acute morphine injection, returned to basal levels during chronic opioid treatment and were up-regulated again during morphine withdrawal. We also observed an activation of astrocytes after acute morphine injection and during opiate dependence and withdrawal. In addition, immunofluorescence analysis revealed that PTN, but not MK, was overexpressed in astrocytes and that dopaminergic neurons expressed RPTP β/ζ . Interestingly, p-ERK 1/2 levels during chronic morphine and morphine withdrawal correlated RPTP β/ζ expression. All these observations suggest that the neuroprotective and behavioral adaptations that occur during opiate addiction could be, at least partly, mediated by these cytokines. |

Acute morphine, chronic morphine and morphine withdrawal differently affects pleiotrophin, midkine and receptor protein tyrosine phosphatase β/ζ regulation in the ventral tegmental area

Daniel García-Pérez^{*}, M. Luisa Laorden and M. Victoria Milanés

Group of Cellular and Molecular Pharmacology, University of Murcia, Campus de Espinardo, 30100 Murcia, Spain. IMIB, Instituto Murciano de Investigación Biosanitaria, Murcia, Spain.

* Corresponding author at: Department of Pharmacology, Faculty of Medicine, Campus de Espinardo, 30100 Murcia, Spain. Phone number: +34868887183. E-mail address: daniel.garcia9@um.es (D. García-Pérez)

Short title: Morphine regulates pleiotrophin and midkine.

Key words: Opiate dependence; reward pathway; pleiotrophin; midkine; glial fibrillary acidic protein.

Abbreviations: Pleiotrophin (PTN); midkine (MK); receptor protein tyrosine phosphatase β/ζ (RPTP β/ζ); ventral tegmental area (VTA); glial fibrillary acidic protein (GFAP); GFAP-immunoreactivity (GFAP-IR); dopamine (DA).

Abstract

Pleiotrophin (PTN) and midkine (MK) are secreted growth factors and cytokines, proposed to be significant neuromodulators with multiple neuronal functions. PTN and MK are generally related with cell proliferation, growth, and differentiation by acting through different receptors. PTN or MK, signaling through receptor protein tyrosine phosphatase β/ζ (RPTP β/ζ), lead to the activation of extracellular signal-regulated kinases (ERKs) and thymoma viral proto-oncogene (Akt), which induce morphological changes and modulate addictive behaviors. Besides, there is increasing evidence that during the development of drug addiction, astrocytes contribute to the synaptic plasticity by synthesizing and releasing substances such as cytokines. In the present work we studied the effect of acute morphine, chronic morphine and morphine withdrawal on PTN, MK, and RPTP β/ζ expression and on their signaling pathways in the ventral tegmental area (VTA). Present results indicated that PTN, MK and RPTP β/ζ levels increased after acute morphine injection, returned to basal levels during chronic opioid treatment and were up-regulated again during morphine withdrawal. We also observed an activation of astrocytes after acute morphine injection and during opiate dependence and withdrawal. In addition, immunofluorescence analysis revealed that PTN, but not MK, was overexpressed in astrocytes and that dopaminergic neurons expressed RPTP β/ζ . Interestingly, p-ERK 1/2 levels during chronic morphine and morphine withdrawal correlated RPTP β/ζ expression. All these observations suggest that the neuroprotective and behavioral adaptations that occur during opiate addiction could be, at least partly, mediated by these cytokines.

Introduction

Astrocytes perform numerous functions: they provide structural support for nerve cells, modulate the environment around neurons, regulate the production of synapses, maintain the blood--brain barrier and release a range of neuronal growth factors [1, 2]. Since glia play a variety of roles in the central nervous system (CNS), a stimulus that affects their morphology and function has widespread consequences including changes in neurotransmission, metabolism of neurotransmitters, synaptic plasticity and propagation of action potentials [2]. Morphine administration is established as a stimulus that affects glial activity. For example, morphine administration induces region-specific upregulation of glial fibrillary acidic protein (GFAP), a protein constituent found specifically in astrocytes, in specific brain areas [3]. Interestingly, evidence has demonstrated that tolerance and dependence are associated with opioid-induced increases in glial cell activity, resulting in neuroadaptations that may directly contribute to the reinforcing effects of these agonists [4].

Activation of astrocytes induces the synthesis and release of substances capable of modulating the surrounding cells (including neurons) like neurotransmitters, neurotrophic factors, cytokines, chemokines and extracellular matrix factors [5-7]. Morphine not only increases glial activation, but also the consequent production of immune factors (chemokines and cytokines) [8]. In addition, some of these molecules exhibit a profile similar to that of neuromodulators or behave like neurotrophic factors [9, 10]. These data raise the possibility that astrocytes contribute to the synaptic plasticity during the development of drug addiction. Moreover, cytokines, in addition to dopamine (DA) and neurotrophins, have been proposed as key mediators of addiction-related neuronal plasticity [11].

Pleiotrophin (PTN) is a secreted cell signaling cytokine that acts as growth factor and as a neuromodulator with multiple neuronal functions [12]. PTN shares high homology with

another peptide, denominated Midkine (MK) [13]. PTN and MK have been involved in neurodegenerative disorders and in response to chronic drug consumption. PTN is upregulated in cortex and caudate-putamen after injection of a cannabinol [14], and in NAc after acute administration of amphetamine [15]. Likewise, increased mRNA and protein levels were found in the prefrontal cortex of alcoholics and smokers [16]. In addition, PTN is also highly upregulated in substantia nigra of patients with Parkinson disease [17] and treatment with L-Dopa increases PTN levels in striatum [18]. Given that these cytokines exert effects that are similar to those of neurotrophins, these findings support the hypothesis that these two cytokines are up-regulated in order to induce neurotrophic or neuroprotective effects during drug consumption [19]. Different membrane receptors could function as a multi-molecular complex coordinated to transduce the PTN-MK signal into the cell by different signaling pathways [12]. So, PTN and MK bind common receptors, including receptor protein tyrosine phosphatase β/ζ (RPTP β/ζ) [20], which is abundantly expressed in the CNS. The interaction of MK or PTN with RPTP β/ζ establishes a “ligand-dependent inactivation” of RPTP β/ζ , presumably consequence of RPTP β/ζ dimerization [21]. As a consequence, PTN or MK signaling through RPTP β/ζ leads to activation of ERK and phosphatidylinositol 3-kinase (PI3K)-Akt [22, 23], important axes inducing morphological changes and modulating addictive behaviors.

To date, despite the fact that morphine causes a full addiction syndrome and persistent restructuring in the mesolimbic reward system, the role of PTN and MK in the context of morphine administration has only been assessed in morphine-induced antinociceptive effects [24, 25]. The action of many addictive substances converges on the mesolimbic dopaminergic reward pathway, inducing increased firing of dopaminergic neurons in the ventral tegmental area (VTA) of the midbrain [26, 27]. Given the important implications of DA neurotransmission in addiction disorders and the complexity of opiate-induced neuroadaptive

responses in the brain reward dopaminergic system, the present study was focused on identifying whether the expression of PTN, MK, RPTP β/ζ and their intracellular signaling pathways (Akt and ERK) are altered in association with acute and chronic morphine exposure as well as with morphine withdrawal in the VTA. Then, we assessed the possible activation of astrocytes, which could lead to the release of astrocyte-related soluble factors. Finally, we also aimed to identify those cell subpopulations that produced and secreted PTN and/or MK and those that expressed RPTP β/ζ in response to morphine administration or morphine withdrawal.

Materials and Methods

Subjects

Male Wistar rats ($n = 65$, Harlan, Barcelona, Spain) initially weighting 220–240 g were housed (2-3/cage) on arrival in a room with controlled temperature ($22 \pm 2^\circ\text{C}$) and humidity ($50 \pm 10\%$), with free access to water and food (Harlan Teklad standard rodent chow; Harlan Interfauna Ibérica, Barcelona, Spain). Animals were adapted to a standard 12 h light-dark cycle (lights on: 08:00 h – 20:00 h) for 7 days before the beginning of the experiments. All surgical and experimental procedures were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC), and were approved by the local Committees for animal research (REGA ES300305440012).

Drug treatment and experimental procedure

Following habituation, rats were implanted subcutaneously (s.c.) with placebo pellets (lactose) for six days. Another set of rats were made dependent on morphine by implantation (s.c.) of two 75 mg morphine pellets under light ether anaesthesia. This procedure has been shown to produce consistent plasma morphine concentrations beginning a few h after the implantation of the pellets and a full withdrawal syndrome after acute injection of opiate antagonists [28]. On day 7, rats were injected intraperitoneally (i.p.) with either morphine HCl (20 mg/kg; in a volume of 1 ml/kg body weight), naloxone (1 mg/kg; 1 ml/kg body weight) or an equivalent volume of 0.9% saline and sacrificed 2 h later. There were five experimental groups: chronic placebo + acute saline, chronic placebo + acute morphine, chronic morphine + acute saline, chronic placebo + acute naloxone, and chronic morphine + acute naloxone. The weight gain of the rats was checked during chronic treatment to ensure that the morphine was liberated correctly from the pellets because it is known that chronic morphine treatment

induces a decrease in body weight gain due to lower caloric intake [29]. In addition, the animals were observed for opioid withdrawal behaviors for 30 min before and after naloxone injection.

Electrophoresis and Western blotting

Animals were killed by rapid decapitation. The brains were removed, placed (with its ventral surface facing up) on a plaque over crushed ice, and tissue samples of the VTA were dissected out as described in [30]. Brain regions were placed in individual wells, frozen immediately on dry ice and stored at -80°C until assaying. Samples were placed in homogenization buffer, homogenized and sonicated for 30 s prior to centrifugation at 10 000 g for 10 min at 4°C. Samples containing equal quantities of total proteins (20–40 mg, depending on the protein of interest) were separated by 6%, 10% or 12% SDS-PAGE (depending on the molecular weight of the protein of interest) and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). Membranes were blocked in TBS containing 0.15 % Tween-20 (TBS-T), 1% BSA for 90 minutes at room temperature (RT), and incubated overnight at 4°C with the primary antibody diluted in 1% BSA in TBS-T. The following primary antibodies were used: goat polyclonal anti-PTN (1:1000; AF-252-PB, R&D Systems, Minneapolis, MN, USA); rabbit polyclonal anti-MK (1:500; sc-20715, Santa Cruz Biotechnology, Santa Cruz, CA, USA); mouse monoclonal anti-RPTP β/ζ (1:750; 610180, BD Transduction Laboratories, Erembodegm, Belgium); mouse monoclonal anti-phospho-ERK 1/2 (p-ERK 1/2; 1:1000; sc-7383; Santa Cruz Biotechnology); mouse monoclonal anti-ERK 1/2 (1:1000; sc-135900; Santa Cruz Biotechnology); rabbit monoclonal anti-phospho-Akt (p-Akt; 1:2000; #4060, Cell Signaling Technology Inc., Danvers, MA, USA); rabbit polyclonal anti-Akt (1:1000; #9272, Cell Signaling Technology Inc.). Blots were washed and incubated for 90 min at RT in TBS-T with 1% BSA with appropriate horseradish peroxidase (HRP)

conjugated secondary antibodies: anti-rabbit (1:5000; sc-2004, Santa Cruz Biotechnology) anti-mouse (1:5000; sc-2005, Santa Cruz Biotechnology) or anti-goat (1:5000; sc-2350, Santa Cruz Biotechnology). After washing, immunoreactivity was detected with an enhanced chemiluminescent/chemifluorescent western blot detection system (ECL Plus, GE Healthcare, UK) and visualized by a Typhoon 9410 variable mode Imager (GE Healthcare). We used GAPDH or α -Tubulin as our loading control. Before reprobing, blots were stripped by incubation with stripping buffer (glycine 25 mM and SDS 1%, pH 2) for 1 h at 37°C. Blots were subsequently reblocked and probed with rabbit polyclonal anti-GAPDH (1:5000; #2118, Cell Signaling Technology Inc.) or rabbit polyclonal anti- α -Tubulin (1:2500; #2144, Cell Signaling Technology Inc.). For Akt or ERK experiments, the same membrane was processed in the following order: incubation with anti-p-Akt or anti-p-ERK 1/2 antibody, stripping, incubation with anti-Akt or anti-ERK antibody, stripping and incubation with anti-GAPDH antibody. The ratios of PTN/GAPDH, MK/GAPDH, RPTP $\beta/\zeta/\alpha$ -Tubulin, p-Akt/t-Akt, t-Akt/GAPDH, p-ERK 1/t-ERK 1, t-ERK 1/GAPDH, p-ERK 1/GAPDH, p-ERK 2/t-ERK 2, t-ERK 2/GAPDH, p-ERK 2/GAPDH were plotted and analyzed. Protein levels were corrected for individual levels.

Brain Perfusion and Sectioning

Another set of rats was deeply anaesthetised with an overdose of pentobarbital (100 mg/kg i.p.) and perfused transcardially with saline following by fixative containing paraformaldehyde (4% paraformaldehyde in 0.1 M borate buffer, pH 9.5). After removal of the perfused brains, they were post fixed in the same fixative for 3 h and stored at 4°C in PBS containing 30% sucrose until coronal sections (30- μ m thickness) were cut rostrocaudally on a freezing microtome (Leica, Nussloch, Germany). The atlas of Paxinos and Watson (2007)

[31] was used to identify the VTA. The sections were cryoprotected and stored at -20°C until use.

GFAP Immunohistochemistry

Sections of the VTA were used for immunohistochemistry to detect astrocytes. Brain sections were rinsed in PBS and an antigen retrieval procedure was applied by treating sections with citrate buffer (10 mM citric acid in 0.05% Tween 20, pH 6.0) at 60°C for 20 min. Endogenous peroxidase activity was inhibited with 0.3% H₂O₂ and non-specific binding sites were blocked in 3% normal goat serum (Sigma, USA)/0.3% Triton-X-100 in PBS for 1 h at RT. The sections were incubated for 72 h (4°C, constant shaking) with primary antibody: mouse monoclonal anti-GFAP (1:400; sc-33673, Santa Cruz Biotechnology). The sections were then incubated for 2 h at RT in biotinylated secondary antibody: anti-mouse (1:500; BA-2000, Vector Laboratories, Burlingame, CA, USA). Tissue was processed by the avidin-biotin immunoperoxidase method (1:250; Vectastain ABC Elite Kit, Vector Laboratories) and immunopositive cells were visualized by addition of the chromogen 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma, USA) and 0.005% H₂O₂. Sections were mounted on chrome-alum gelatin-coated slides and dehydrated in graded ethanol series and xylene before being coverslipped.

Quantification GFAP-positive cells

Neuroanatomical sites were identified using the Paxinos and Watson (1997) atlas. Photomicrographs were captured by means of Leica microscope (DM 4000B; Leica) connected to a video camera (DFC290, Leica). GFAP-positive cell nuclei were counted using a computer-assisted image analysis system (QWIN, Leica). Positive cells were counted at 20X magnification. A square field (325 µm) was superimposed upon captured image to use as

reference area. The number of astrocytes was counted bilaterally in four to five sections from each animal, and averaged to obtain a single value for each rat. The whole histological quantification was performed blindly. Total counts for different brain regions are expressed as mean \pm SEM.

GFAP Densitometric Analysis

The same conventional light microscopy described above was used for optical density (OD) study of the nuclei and processes. Photomicrographs were captured at 10X magnification (image size, 936 μ m x 702 μ m). To avoid observer bias, all sections were quantified by a blinded investigator. The intensity of the different brain areas was evaluated through densitometric analysis using ImageJ 1.43 software (NIH ImageJ, Bethesda, MD, USA). The region of interest was outlined in each image, and a mean optical density value was obtained. Background was determined from unstained areas in the tissue section. The corrected densitometric signal was calculated by subtracting the background from each densitometric determination. Four to five sections from each animal were evaluated. A mean value for different regions of each animal was then obtained. The area outlined in each image was also calculated to assure that there were no differences between the regions of interest analyzed in different groups.

Immunofluorescence

Triple immunofluorescence study was carried out to detect GFAP, PTN and MK. Sections were treated with citrate buffer (60°C for 20 min). Non-specific Fc binding sites were blocked with 2% normal horse serum/0.3% Triton-X-100 in PBS for 1 h at RT, and the sections were incubated for 72 h (4°C, constant shaking) with primary antibodies: mouse monoclonal anti-GFAP (1:400; sc-33673, Santa Cruz Biotechnology), goat polyclonal anti-

PTN (1:400; AF-252-PB, R&D Systems) and rabbit polyclonal anti-MK (1:250; sc-20715, Santa Cruz Biotechnology). Secondary antibodies were applied sequentially for 4 h: Alexa Fluor 488 anti-rabbit IgG (1:1000; A-21206, Invitrogen, Eugene, OR, USA), Alexa Fluor 594 anti-goat IgG (1:1000; A-11058, Invitrogen) and Alexa Fluor 405 anti-mouse IgG (1:1000; A-31553, Invitrogen). After washing, the sections were mounted in ProLong® Gold antifade reagent (Invitrogen).

Double-fluorescent labeling was processed as previously explained. Mesencephalic sections were incubated (72 h, 4°C) with mouse monoclonal anti-RPTP β/ζ (1:50; 610180, BD Transduction Laboratories) and goat polyclonal anti-tyrosine hydroxylase (TH; 1:4000; ab101853, Abcam, Cambridge, UK) to identify midbrain dopaminergic neurons. Appropriate secondary antibodies were used: Alexa Fluor 488 anti-goat IgG (1:1000; A-11055, Invitrogen) and Alexa Fluor 594 anti-mouse IgG (1:1000; A-21203, Invitrogen). Sections were incubated in DAPI (1:100 000) for 1 min, and mounted in ProLong® Gold antifade reagent (Invitrogen).

Confocal analysis

The brain sections were examined using a Leica DMIRE2 confocal microscope and Leica Confocal Software (Leica Microsystems). Images were captured from low magnification to high magnification (20X to 63X oil objective). Confocal images were obtained using 405-nm excitation for Alexa Fluor 405 or DAPI, 488-nm excitation for Alexa Fluor 488 and 543-nm excitation for Alexa Fluor 594. Emitted light was detected in the range of 450 nm for DAPI, 515-530 nm for Alexa Fluor 488 and 605 nm for Alexa Fluor 594. Every channel was captured separately to avoid spectral crosstalk. Series of optical sections were performed determining an upper and lower threshold using the Z/Y position for Spatial Image Series setting. The optical series covered 20 μ m of thickness through the tissue. Images were

deconvolved using Huygens Essential 3.6 by Scientifica Volume Imaging (SVI, Hilversum, The Netherlands).

Materials

Morphine HCl and morphine base were supplied from Alcaliber Laboratories (Madrid, Spain) in cooperation with the Área de Estupefacientes y Psicotropos, Agencia Española del Medicamento y de Productos Sanitarios (Madrid, Spain). Naloxone HCl was purchased from Sigma-Aldrich (Sigma Chemical Co, St Louis, MO, USA). Morphine HCl and naloxone HCl doses are expressed as the weight of the salt. Protease inhibitors were purchased from Boehringer Mannheim, (Mannheim, Germany); phosphatase inhibitor Cocktail Set was purchased from Calbiochem (Darmstadt, Germany). Morphine HCl and naloxone were prepared fresh each day by reconstitution in sterile saline (0.9% NaCl; ERN Laboratories, Barcelona, Spain).

Statistical Analysis

Data are presented as mean \pm standard error of the mean (SEM). Data were analyzed using one-way or two-way analysis of variance (ANOVA) followed by a post hoc Newman–Keuls test. Correlations between changes in protein expression were assessed using Pearson correlation. Differences with a $p < 0.05$ were considered significant. Statistical analyses were performed with GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA).

Results

PTN and MK were up-regulated in the VTA after morphine administration and during precipitated morphine withdrawal

PTN and MK have been found to be up-regulated in different brain areas after amphetamine, alcohol or delta-9-tetrahydrocannabinol administration (see Introduction); however, their possible alteration after morphine administration or during morphine withdrawal in the mesolimbic pathway has not been determined. This experiment addressed two questions regarding PTN and MK expression. The first was whether acute or chronic morphine treatment was associated with changes in PTN and/or MK in VTA. ANOVA showed significant effect after acute morphine for PTN ($F(2,20) = 8.656$; $p = 0.0023$) and MK ($F(2,19) = 6.949$; $p = 0.0062$) in the VTA. As shown in Fig. 1A,C, *post hoc* comparisons showed that acute morphine administration significantly elevated PTN ($p < 0.01$) and MK ($p < 0.05$) expression in the VTA. This elevation was not seen during chronic morphine administration ($p < 0.01$) compared with acute morphine injection. Two-way ANOVA for PTN expression showed a significant effect of chronic pretreatment ($F(1,21) = 12.65$; $p = 0.0019$), acute naloxone injection ($F(1,21) = 6.73$; $p = 0.0170$) and an interaction between pretreatment and acute treatment ($F(1,21) = 4.92$; $p = 0.0376$). *Post hoc* test revealed that PTN levels in the VTA were increased after naloxone precipitated morphine withdrawal ($p < 0.01$), as shown in Fig. 1B. Two-way ANOVA for MK showed a significant effect of acute naloxone injection ($F(1,23) = 5.885$; $p = 0.0235$) and an interaction between pretreatment and acute treatment ($F(1,23) = 4.516$; $p = 0.0445$). *Post hoc* test revealed that MK levels in the VTA were increased after naloxone precipitated morphine withdrawal ($p < 0.05$), as shown in Fig. 1D.

As our first goal was to establish whether acute morphine administration, chronic morphine or morphine withdrawal affect PTN and or MK VTN expression, it was important

to know if VTA express RPTP β / ζ , one of the main receptors for PTN/MK, as well as whether morphine, morphine dependence and/or morphine withdrawal alter its expression. So, we next used semiquantitative Western blot to examine whether morphine and or morphine withdrawal affected RPTP β / ζ expression protein levels. ANOVA showed significant effect after acute morphine ($F(2,18) = 14.6456$; $p = 0.0002$). As shown in Fig. 1E, *post hoc* comparisons showed that acute morphine administration significantly elevated RPTP β / ζ ($p < 0.001$) expression. However, there was a decrease in its expression during morphine dependence compared with the control group receiving placebo plus saline ($p < 0.05$) and with acute morphine-treated rats ($p < 0.001$). Two-way ANOVA for RPTP β / ζ expression showed an interaction between pretreatment and acute treatment ($F(1,23) = 4.75$; $p = 0.03976$). *Post hoc* test revealed that RPTP β / ζ levels in the VTA were decreased in morphine-dependent rats ($p < 0.05$), as compared with the control group (Fig. 1F). No changes were observed in morphine-withdrawn rats.

We next compared the expression of PTN and MK with the induction of RPTP β / ζ protein levels by Pearson correlation. There were not significant correlations in the different experimental groups between MK expression and RPTP β / ζ protein levels in the VTA (data not shown). In contrast, we observed that during morphine dependence and withdrawal, the expression of PTN was significantly positively correlated with RPTP β / ζ levels (Fig. 2).

It is important to notice that robust behavioral signs of the morphine withdrawal syndrome were seen in all of chronically morphine-treated animals after administration of naloxone, indicating the development of dependence.

Astrocytes were activated by morphine and morphine withdrawal in the VTA

Astrocytes undergo a process of proliferation, morphological changes, and enhancement of GFAP expression, termed the activation of astrocytes or astrogliosis [32]. In

this work we assessed the activation of astrocytes in the VTA by both, measuring the number of astrocytes (as the number of GFAP-positive cells) in the different groups and studying the levels of GFAP-immunoreactivity (GFAP-IR) by densitometric analysis. ANOVA showed significant effect of morphine administration for GFAP-positive cells ($F(2,12) = 5.909$; $p = 0.0202$) and GFAP-IR ($F(2,14) = 8.632$; $p = 0.0048$) in the VTA. *Post hoc* test revealed that acute morphine elevated the number of GFAP-positive cells and GFAP-IR ($p < 0.05$; $p < 0.01$, respectively) (Fig. 3G,I). In addition, chronic morphine administration also increased GFAP-IR ($p < 0.05$). Two-way ANOVA for GFAP revealed main effects for chronic pretreatment ($F(1,14) = 22.16$; $p = 0.0003$), and significant interaction between acute and chronic treatment ($F(1,14) = 10.530$; $p = 0.0059$). We found that precipitated morphine withdrawal significantly increased the number of GFAP-positive cells in the VTA compared with its control group ($p < 0.001$) and with chronic morphine-treated rats receiving saline ($p < 0.01$; Fig. 3H). Two-way ANOVA for GFAP-IR in the VTA revealed main effect for chronic pretreatment ($F(1,16) = 17.08$; $p = 0.0008$). *Post hoc* test showed that both chronic morphine and morphine withdrawal elevated ($p < 0.05$) GFAP-IR (Fig. 3J).

PTN but not MK was overexpressed in astrocytes during acute morphine administration and morphine withdrawal in the VTA

Activation of astrocytes produces the accumulation of intermediate-filament GFAP, consequently they can be recognized by their stellate morphology. Triple immunofluorescence study revealed that acute morphine (Fig. 4A-A''') or morphine withdrawal (Fig. 4B-B''') mediated the activation of astrocytes that expressed high levels of PTN protein, but not MK protein in the VTA. Cells that produced MK are supposed to be

neurons, due to their size and morphology and the fact that MK and RPTP β/ζ colocalized (data not shown).

RPTP β/ζ was expressed in midbrain dopaminergic neurons

RPTP β/ζ is a transmembrane tyrosine phosphatase, the extracellular domain of which carries chondroitin sulphate chains. The anti-RPTP β/ζ antibody used recognizes only the intracellular portion of the RPTP β/ζ to avoid non-specific labeling of other proteoglycans and specially of phosphacan, a secreted peptide which has the same molecular structure as the extracellular portion of the RPTP β/ζ .

In order to determine where this receptor is located, we immunolabeled RPTP β/ζ in combination with specific cell markers on the mesencephalon of morphine-treated rats. At low magnification, we observed that following morphine injection there was a region-specific increase of RPTP β/ζ protein in the VTA, but weak or absent staining was detected in the interpeduncular nucleus (IP) or red nucleus (RN) (Fig. 5A-A'''). At high magnification, RPTP β/ζ immunoreactivity was widely distributed throughout the VTA, and double-labeling with anti-TH (the rate-limiting enzyme of DA synthesis) showed that RPTP β/ζ is located on cell bodies and proximal processes of almost all dopaminergic neurons in the VTA (Fig. 5B-B'''). There were some RPTP β/ζ^+ /TH $^-$ cells, that might represent GABAergic interneurons in the VTA.

ERK pathway but not Akt was activated by morphine and by precipitated morphine withdrawal in the VTA

In each experiment, the specific signal of p-Akt or p-ERK proteins was normalized to the corresponding Akt or ERK signals, respectively, and then to the level of GAPDH measured in the same preparation. Previously, it has been described that PTN or MK

signaling through RPTP β/ζ leads to activation of ERK and Akt pathways [22, 23]. ANOVA for p-Akt showed significant effect after acute or chronic morphine administration ($F(2,18) = 5.265$; $p = 0.0175$) failed to detect significant changes in t-Akt expression in the VTA ($F(2,18) = 2.845$; $p = \text{ns}$) (Fig. 6A,C). *Post hoc* test revealed that p-Akt levels in the VTA were decreased in morphine-dependent rats ($p < 0.05$). Two-way ANOVA revealed that morphine withdrawal had a significant effect on p-Akt ($F(1,21) = 5.190$; $p = 0.0332$). *Post hoc* test revealed that p-Akt levels in the VTA were significantly ($p < 0.05$) elevated in morphine-withdrawn rats compared with the morphine dependent group receiving saline instead naloxone (Fig. 6B). Two-way ANOVA revealed that morphine pre-treatment ($F(1,21) = 1.83$; $p = 0.0539$), acute naloxone injection ($F(1,21) = 0.04$; $p = 0.8352$) or the interaction between pre-treatment and acute treatment ($F(1,21) = 0.13$; $p = 0.7172$) had no significant effects on t-Akt (Fig. 6D).

Because t-ERK showed changes after the different treatments, p-ERKs 1/2 were quantified by Western blot using both t-ERK and GAPDH as loading controls (Fig. 7). ANOVA showed significant effect after acute or chronic morphine for p-ERK 1/t-ERK 1 ($F(2,19) = 11.65$; $p = 0.0007$) and for p-ERK 2/t-ERK 2 ($F(2,19) = 15.26$; $p = 0.0002$). As shown in Fig. 7A,G, *post hoc* comparisons showed that both acute and chronic morphine administration significantly elevated p-ERK 1/ t-ERK 1 ($p = 0.001$; $p = 0.01$) and p-ERK 2/ t-ERK 2 ($p = 0.001$; $p = 0.001$) expression in the VTA. Two-way ANOVA for p-ERK 1/ t-ERK 1 and p-ERK 2/ t-ERK 2 expression showed significant effect for morphine pre-treatment (p-ERK 1: $F(1,24) = 21.15$; $p = 0.0001$; p-ERK 2: $F(1,24) = 41$; $p < 0.0001$) and acute naloxone injection (p-ERK 1: $F(1,24) = 4.62$; $p = 0.0419$; p-ERK 2: $F(1,24) = 8.85$; $p = 0.0066$). *Post hoc* comparisons showed that morphine withdrawal significantly ($p < 0.001$) elevated p-ERK 1 and p-ERK 2 levels compared with their respective controls and with morphine-dependent rats ($p < 0.05$) (Fig. 7B,H). In addition, p-ERK 2 levels were also

increased ($p < 0.01$) in morphine-dependent rats compared with its control (Fig. 7H). Similar results were seen by using the p-ERK 1/GAPDH and p-ERK 2/GAPDH. Thus, ANOVA showed significant effect after acute morphine for p-ERK 1/GAPDH ($F(2,19) = 17.87$; $p < 0.0001$) and after acute and chronic morphine for p-ERK 2/ GAPDH ($F(2,19) = 14.91$; $p = 0.0002$). As shown in Fig. 7E,K, *post hoc* comparisons showed that acute morphine administration injection significantly elevated p-ERK 1/GAPDH and p-ERK 2/GAPDH ($p < 0.001$) as well as and p-ERK 2/ GAPDH after chronic morphine treatment ($p < 0.01$) expression in the VTA (Fig. 7K). Two-way ANOVA showed significant effect of chronic pretreatment for p-ERK 1/GAPDH ($F(1,24) = 12.41$; $p = 0.0017$) and p-ERK 2/GAPDH ($F(1,24) = 21.31$; $p = 0.0001$) and acute naloxone injection (p-ERK 1/GAPDH: $F(1,24) = 6.14$; $p = 0.0207$; p-ERK 2/GAPDH: $F(1,24) = 6.81$; $p = 0.0154$). As shown in Fig. 7, there was an increase ($p < 0.01$) of p-ERK 1 and p-ERK 2 after naloxone administration to morphine-dependent rats. p-ERK 2 was also elevated during morphine dependence (Fig. 7L).

We next used semiquantitative Western blot to examine the effect of acute morphine administration, chronic morphine and morphine withdrawal on t-ERK 1 (ratio t-ERK 1/GAPDH) and t-ERK 2 (ratio t-ERK 2/GAPDH) protein levels (Fig. 7C-J). ANOVA showed significant effects after acute morphine for t-ERK 1 ($F(2,19) = 6.160$; $p = 0.0097$) and for t-ERK 2 ($F(2,19) = 4.459$; $p = 0.0277$). As shown in Fig. 7C,I, *post hoc* comparisons showed an increase of t-ERK 1 ($p < 0.01$) and t-ERK 2 ($p < 0.05$) after acute morphine administration. This effect was suppressed in chronic morphine-treated rats. Two-way ANOVA for VTA t-ERK1 and t-ERK2 levels failed to detect any significant effects of chronic morphine pretreatment or morphine withdrawal (Fig. 7D,J).

Relationship between PTN, MK and/or RPTPβ/ζ and p-ERK or t-ERK protein levels

We first compared the expression of PTN, MK and/or RPTP β/ζ with the induction of p-ERK or t-ERK protein levels in control rats that were injected an acute dose of morphine. There were significant negative correlations between PTN expression and t-ERK 1 and t-ERK 2 protein levels in the VTA (Fig. 8A,B). On the other hand, MK or RPTP β/ζ expression in acute morphine-injected rats was not correlated with changes on p-ERK or t-ERK levels.

Interestingly, PTN expression during morphine dependence was also negatively correlated with t-ERK 1 (Fig. 8G), and during morphine withdrawal we detected that the increased PTN expression was associated with a decrease in t-ERK 2 levels in the VTA (Fig. 8N).

When we studied MK expression, we found that this cytokine was negatively correlated with p-ERK 1 and p-ERK 2 levels only in morphine withdrawn rats (8O,P), while we observed not significant correlation for MK in other experimental groups.

During morphine dependence and withdrawal, expression of RPTP β/ζ tended to be positively correlated with p-ERK. There was a significant positive correlation between RPTP β/ζ and both, p-ERK 1 and p-ERK 2 protein levels during chronic morphine administration (Fig. 8K,L). As depicted in Fig. 8Q,R, a similar trend for positive correlation between RPTP β/ζ and p-ERK 1/ p-ERK 2 was found after naloxone-induced morphine withdrawal.

Discussion

The data presented in this study show that different paradigms of morphine treatment promote a diverse profile of activation of cytokines in the brain. While we observed that a single dose of morphine and morphine withdrawal increased the protein levels of PTN and MK in the VTA, chronic morphine administration had the opposite effect, since protein levels returned to basal levels or even decreased. Our results are supported by previous findings showing that acute morphine or withdrawal and not chronic morphine promote the expression of pro-inflammatory cytokines [33]. The present study showed that, PTN up-regulation following morphine administration or during morphine withdrawal is restricted to astrocytes. In agreement with this data, PTN mRNA levels were up-regulated in reactive astrocytes in a model of cryo-injured mouse brain [34]. Besides, in primary cultures of mesencephalon, GFAP-positive astrocytes express PTN mRNA and protein [35] and recently, it has been described that PTN is over-expressed only in astrocytes following an adenoviral vector injection [36]. In contrast, we observed that MK is produced and secreted by non-astrocytic cells following a morphine challenge. Hippocampal pyramidal neurons in adult mouse brain expressed MK, but kainic acid injection induced cell death of pyramidal neurons and enhanced expression of MK by astrocytes [37]. A possible explanation is that, depending on the nature of the insult or the damage/cell death it may produce, MK can be over-expressed by neurons or astrocytes. Moreover, there is evidence that nearly every cell of the CNS, including neurons, is capable of contributing and modifying the central immune signaling [38], for example, by secreting cytokines. For instance, morphine stimulates chemokine CCL2 production by neurons [39].

We also found that the expression of PTN- and MK- target receptor (RPTP β/ζ) was regulated in the same way that these cytokines by morphine administration. Mainly, PTN can

bind and signal via RPTP ζ [40, 41]. Thus, we detected that PTN enhanced expression after chronic morphine and morphine withdrawal would lead to adaptative responses, such as the increase in RPTP β/ζ levels, which might try to compensate the ligand-dependent inactivation. Although the role and the functions of RPTPs are barely characterized, it has been suggested that each of these enzymes has different and specific functions in neurons [42]. Regarding the RPTP β/ζ signal, we found a staining pattern in neurons. Our results are in line with previous studies where this protein was found to be located in neurons but not in astrocytes [42, 43]. Moreover, TH-positive neurons in primary cultures of mesencephalon have been described to express RPTP β/ζ [35]. Although it has been reported that adult control rats do not express RPTP β/ζ in the VTA [44], we detected (by WB and IF) that systemic morphine injection induced RPTP β/ζ in VTA-dopaminergic neurons in a region-specific manner. Thus, it suggests that RPTP β/ζ is tightly up-regulated in the VTA after determinate challenges. This expression pattern supports our hypothesis of an interaction between glial and neuronal function during morphine administration and withdrawal.

PTN and MK have been involved in the regulation of the survival and function of dopaminergic neurons [45, 46]. In addition, many reports attribute neuroprotective roles against drug-induced neurotoxicity to these two cytokines. For instance, amphetamine-induced loss of dopaminergic fibers in the striatum was enhanced in PTN knockout (PTN $^{-/-}$) mice and even caused dopaminergic cell loss in the substantia nigra (SN) of PTN $^{-/-}$ mice [47]. It is important to note that moderate glial activation is thought to be neuroprotective, while exacerbated activation can be deleterious for the brain [48-50]. Accordingly, amphetamine-induced astrogliosis in the nigrostriatal pathway was increased in PTN $^{-/-}$ mice and in MK $^{-/-}$ mice compared to wild type mice [47, 51, 52]. As exposed above, morphine withdrawal may promote cytokines and other inflammatory responses that have the potential of exacerbating neuronal damage [33]. The increase in pro-inflammatory cytokines may explain why

withdrawal causes caspase-3 dependent apoptosis [53]. So, the enhanced expression of PTN and MK that we observed during acute morphine injection and morphine withdrawal can be regarded as a physiological response to prevent the possible neurotoxic effects of morphine, an excessive astrogliosis or pro-inflammatory cytokines release. Moreover, it has been demonstrated that PTN increases the expression of nuclear receptor related-1 (Nurr1; a transcription factor which is critical for the generation, survival and maintenance of DA neurons) in neural stem cells to promote production of dopaminergic neurons [45]. Previously, we have observed an increase of Nurr1 after acute morphine administration and withdrawal [30], which parallels that of PTN. So, given present results, it is tempting to speculate that neuroprotective effects of PTN during acute morphine injection and morphine withdrawal may be, in part, mediated through the induction of Nurr1.

Astrocytes can display both, hypertrophy and proliferation upon treatment with drugs of abuse. Chronic morphine exposure, achieved by surgically implanted morphine pellets and previously verified to produce opioid tolerance and dependence [54], increased GFAP immunoreactivity in the VTA [3]. This effect was not observed when morphine tolerance and dependence was blocked by concomitant administration of the mu-opioid antagonist, naltrexone [3]. Accordingly, in the present study acute and chronic morphine caused a robust activation of astrocytes, as evidenced by an increase in the level of GFAP-IR in the VTA, while morphine withdrawal maintained the increase GFAP-IR levels. Regarding to proliferation, repeated methamphetamine administration induces proliferation of astrocytes in rodent striatum and SN [47, 51]. Moreover, morphine and opioid signaling have been shown to promote proliferation of astroglia in the postnatal brain [55]. We observed a rapid astrocyte proliferation in the VTA, the brain area where the rewarding properties of morphine are believed to be firstly mediated (via binding to μ opioid receptors located on inhibitory neurons) [56]. A question that arises is whether glial activation (GFAP-IR) observed during

chronic morphine is maintained throughout a longer time or, on the contrary, it slowly decreases. Data supporting this second hypothesis is the evidence that, after two months, methamphetamine induced a long-lasting astrocytic activation and behavioral sensitization, while morphine produced a reversible activation of astrocytes and a reversibility of behavioral sensitization [57].

It has been proposed that PTN/MK signaling may function through a multi-receptor complex, combining different receptors and most probably other adaptor proteins; then, PTN/MK action over previously mentioned receptors could in turn signal through different signal pathways [12]. It has been documented that the interaction of RPTP β / ζ with PTN or MK blocks the intrinsic tyrosine phosphatase activity of RPTP β / ζ [19], leading to an increase in the phosphorylation levels in RPTP β / ζ target proteins. Interestingly, these target proteins include the PI3K-Akt and Ras-ERK signaling, which have been demonstrated to play a crucial role in opiate addiction (for review see [58]).

PTN neuroprotective effects constitute a relevant role, suggesting that PTN signaling pathways are involved in neurodegenerative disorders, as well as in response to injuries and chronic drug consumption [12]. PTN protective effects against amphetamine-induced toxicity in PC12 cells (which express RPTP β / ζ and synthesize and release DA) were mediated by the ERK 1/2 signaling pathway [51]. The same neuroprotective action could be conferred to RPTP β / ζ during chronic morphine administration and morphine withdrawal given the positive correlation between RPTP β / ζ and p-ERK 1/2. Cytokines have also been implicated in withdrawal-related behavior. For example, it was showed that corticotrophin releasing factor (CRF) and cytokines work together to worsen ethanol withdrawal phenotypes [59]. Although the role of PTN and MK in conditioned place aversion (CPA) remains uncharacterized, future studies should address this issue, since robust increased levels of PTN and MK were also

observed during morphine withdrawal. Other studies have shown the important role of ERK signaling in memory formation [60] and the acquisition of CPA [61]. Interestingly, pERK 1/2 levels in the VTA during morphine withdrawal (acquisition of CPA) seem to be controlled through RPTP β / ζ signaling. In contrast, we assessed that during morphine withdrawal, MK is negatively related to p-ERK 1/2. In agreement, literature shows that although both cytokines could present overlapping or similar functions, they are also clearly involved in different roles [12].

On the other hand, a decrease of Akt signaling was detected in the VTA during chronic morphine. This down-regulation is considered critical to mediate the effects of chronic morphine on soma size and electrical excitability [62, 63]. PTN and MK are also known to be neurotrophic factors for DA neurons [35]. Initial up-regulation of PTN promoted regeneration of axons, but subsequent decline in the expression of PTN has been proposed to be responsible for failure to regenerate denervated nerves [64], highlighting the importance of sustained levels of these growth factors to accomplish their trophic function. Nevertheless, we did not find any correlation between PTN, MK or RPTP β / ζ and p-Akt levels, so it is reasonable to think that these cytokines do not participate in the decreased dendrite complexity and spine density and cell body size that chronic opiates produce in the mesolimbic system [58].

In summary, given that PTN, MK and RPTP β / ζ levels increase after acute morphine injection, return to basal levels during chronic opioid treatment and are up-regulated again during morphine withdrawal, we hypothesize that signaling through these cytokines plays a role in mediating, at least in part, neuroprotective and behavioral adaptations that are observed during opiate addiction.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Acknowledgments

This work was supported by grants from Ministerio de Ciencia e Innovación (SAF/FEDER 2009-07178; SAF/FEDER 2010-17907), Spain; Red de Trastornos Adictivos, Spain; Fundación Séneca (15405/PI/10) and Instituto Murciano de Investigación en Biomedicina (IMIB), Región de Murcia, Spain. Daniel García-Pérez was supported by a fellowship from Ministerio de Ciencia e Innovación (AP2009-2379).

Authors Contribution

DG-P designed and performed the research, analyzed the data and wrote the paper. MLL revised the manuscript. MVM conducted the astrocyte quantification and contributed to editing the paper.

References

- [1] Svendsen CN. Neurobiology: The amazing astrocyte. *Nature* 2002 May 2;417:29-32.
- [2] Ullian EM, Sapperstein SK, Christopherson KS, Barres BA. Control of Synapse Number by Glia. *Science* 2001 Jan 26;291:657-61.
- [3] Beitner-Johnson D, Guitart X, Nestler EJ. Glial fibrillary acidic protein and the mesolimbic dopamine system: regulation by chronic morphine and Lewis-Fischer strain differences in the rat ventral tegmental area. *J Neurochem* 1993;61:1766-73.
- [4] Watkins LR, Hutchinson MR, Johnston IN, Maier SF. Glia: novel counter-regulators of opioid analgesia. *Trends in Neurosciences* 2005 Jan 1;28:661-9.
- [5] Fellin T, Carmignoto G. Neurone-to-astrocyte signalling in the brain represents a distinct multifunctional unit. *The Journal of Physiology* 2004 Aug 15;559:3-15.
- [6] Bohn MC. Motoneurons crave glial cell line-derived neurotrophic factor. *Experimental Neurology* 2004 Dec;190:263-75.
- [7] Vesce S, Bezzi P, Volterra A. Synaptic Transmission with the Glia. *Physiology* 2001 Aug 1;16:178-84.
- [8] Hutchinson MR, Coats BD, Lewis SS, Zhang Y, Sprunger DB, Rezvani N, et al. Proinflammatory cytokines oppose opioid induced acute and chronic analgesia. *Brain, Behavior, and Immunity* 2008 Nov 2;22:1178-89.
- [9] Adler MW, Rogers TJ. Are chemokines the third major system in the brain? *Journal of Leukocyte Biology* 2005 Dec 1;78:1204-9.
- [10] Rostene W, Kitabgi P, Parsadaniantz SM. Chemokines: a new class of neuromodulator? *Nat Rev Neurosci* 2007 Nov;8:895-903.
- [11] Russo SJ, Dietz DM, Dumitriu D, Morrison JH, Malenka RC, Nestler EJ. The addicted synapse: mechanisms of synaptic and structural plasticity in nucleus accumbens. *Trends in Neurosci* 2010 Jun 11;33:267-76.

- [12] Gonzalez-Castillo C, Ortuño-Sahagun D, Guzman-Brambila C, Pallas M, Rojas-Mayorquin AE. Pleiotrophin as a central nervous system neuromodulator, evidences from the hippocampus. *Front Cell Neurosci* 2014 Jan 8;8:443.
- [13] Kadomatsu K, Muramatsu T. Midkine and pleiotrophin in neural development and cancer. *Cancer Letters* 2004 Oct 10;204:127-43.
- [14] Mailleux P, Preud'homme X, Albala N, Vanderwinden JM, Vanderhaeghen JJ. delta-9-Tetrahydrocannabinol regulates gene expression of the growth factor pleiotrophin in the forebrain. *Neurosci Lett* 1994;175:25-7.
- [15] Le Grevès P. Pleiotrophin gene transcription in the rat nucleus accumbens is stimulated by an acute dose of amphetamine. *Brain Research Bulletin* 2005 May 30;65:529-32.
- [16] Flatscher-Bader T, Wilce PA. Impact of Alcohol Abuse on Protein Expression of Midkine and Excitatory Amino Acid Transporter 1 in the Human Prefrontal Cortex. *Alcoholism: Clinical and Experimental Research* 2008 Oct 1;32:1849-58.
- [17] Marchionini DM, Lehrmann E, Chu Y, He B, Sortwell CE, Becker KG, et al. Role of heparin binding growth factors in nigrostriatal dopamine system development and Parkinson's disease. *Brain Res* 2007 May 25;1147:77-88.
- [18] Ferrario JE, Taravini IRE, Mourlevat S, Stefano A, Delfino MA, Raisman-Vozari R, et al. Differential gene expression induced by chronic levodopa treatment in the striatum of rats with lesions of the nigrostriatal system. *Journal of Neurochemistry* 2004 Sep 1;90:1348-58.
- [19] Herradón G, Pérez-García C. Targeting midkine and pleiotrophin signaling pathways in addiction and neurodegenerative disorders: Recent progress and perspectives. *Br J Pharmacol* 2013 Jul 1;171:837-48.
- [20] Muramatsu T. Midkine and Pleiotrophin: Two Related Proteins Involved in Development, Survival, Inflammation and Tumorigenesis. *Journal of Biochemistry* 2002 Sep 1;132:359-71.

- [21] Deuel TF, Zhang N, Yeh HJ, Silos-Santiago I, Wang ZY. Pleiotrophin: A Cytokine with Diverse Functions and a Novel Signaling Pathway. *Archives of Biochemistry and Biophysics* 2002 Jan 15;397:162-71.
- [22] Polykratis A, Katsoris P, Courty J, Papadimitriou E. Characterization of Heparin Affin Regulatory Peptide Signaling in Human Endothelial Cells. *J Biol Chem* 2005 Jun 10;280:22454-61.
- [23] Qi M, Ikematsu S, Maeda N, Ichihara-Tanaka K, Sakuma S, Noda M, et al. Haptotactic migration induced by midkine. Involvement of protein-tyrosine phosphatase zeta. Mitogen-activated protein kinase, and phosphatidylinositol 3-kinase. *J Biol Chem* 2001 May 11;276:15868-75.
- [24] Vicente-Rodríguez M, Pérez-García C, Gramage E, Herradón G. Genetic inactivation of pleiotrophin but not midkine potentiates clonidine-induced alpha-2 adrenergic-mediated analgesia. *Pharmacology Biochemistry and Behavior* 2013 Sep;110:185-91.
- [25] Gramage E, Martín YB, Herradón G. The heparin binding growth factors midkine and pleiotrophin regulate the antinociceptive effects of morphine through $\alpha(2)$ -adrenergic independent mechanisms. *Pharmacology Biochemistry and Behavior* 2012 May;101:387-93.
- [26] Di Chiara G, Imperato A. Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *PNAS* 1988 Jul 1;85:5274-8.
- [27] Ikemoto S. Dopamine reward circuitry: Two projection systems from the ventral midbrain to the nucleus accumbens-olfactory tubercle complex. *Brain Res Brain Res Rev* 2007;56:27-78.
- [28] Frenois F, Cador M, Caille S, Stinus L, Le Moine C. Neural correlates of the motivational and somatic components of naloxone-precipitated morphine withdrawal. *Eur J Neurosci* 2002;16:1377-89.
- [29] Houshyar H, Manalo S, Dallman MF. Time-Dependent Alterations in mRNA Expression of Brain Neuropeptides Regulating Energy Balance and Hypothalamo-

- Pituitary-Adrenal Activity after Withdrawal from Intermittent Morphine Treatment. *J Neurosci* 2004 Oct 20;24:9414-24.
- [30] García-Pérez D, Sáez-Belmonte F, Laorden ML, Núñez C, Milanés MV, Milanés MV. Morphine administration modulates expression of Argonaute 2 and dopamine-related transcription factors involved in midbrain dopaminergic neurons function. *Br J Pharmacol* 2013;168:1889-901.
- [31] Paxinos G, Watson C. The rat brain in stereotaxic coordinates. 6th edn. Amsterdam: Academic Press; 2007.
- [32] Raivich G, Bohatschek M, Kloss CUA, Werner A, Jones LL, Kreutzberg GW. Neuroglial activation repertoire in the injured brain: graded response, molecular mechanisms and cues to physiological function. *Brain Research Reviews* 1999 Jul;30:77-105.
- [33] Campbell LA, Avdoshina V, Rozzi S, Mocchetti I. CCL5 and cytokine expression in the rat brain: Differential modulation by chronic morphine and morphine withdrawal. *Brain, Behavior, and Immunity* 2013 Nov;34:130-40.
- [34] Iseki K, Hagino S, Mori T, Zhang Y, Yokoya S, Takaki H, et al. Increased syndecan expression by pleiotrophin and FGF receptor-expressing astrocytes in injured brain tissue. *Glia* 2002 Jul 1;39:1-9.
- [35] Mourlevat S, Debeir T, Ferrario JE, Delbe J, Caruelle D, Lejeune O, et al. Pleiotrophin mediates the neurotrophic effect of cyclic AMP on dopaminergic neurons: Analysis of suppression-subtracted cDNA libraries and confirmation in vitro. *Experimental Neurology* 2005 Jul;194:243-54.
- [36] Taravini I, Chertoff M, Cafferata E, Courty J, Murer M, Pitossi F, et al. Pleiotrophin over-expression provides trophic support to dopaminergic neurons in parkinsonian rats. *Molecular Neurodegeneration* 2011;6:40.
- [37] Kim Y, Ryu J, Lee H, Lim I, Park D, Lee M, et al. Midkine, heparin-binding growth factor, blocks kainic acid-induced seizure and neuronal cell death in mouse hippocampus. *BMC Neuroscience* 2010;11:42.

- [38] Hutchinson MR, Watkins LR. Why is Neuroimmunopharmacology crucial for the future of addiction research? *Neuropharmacology* 2013 Jan 1;76 Pt B:218-27.
- [39] Rock RB, Hu S, Sheng W, Peterson P. Morphine stimulates CCL2 production by human neurons. *Journal of Neuroinflammation* 2006;3:32.
- [40] Maeda N, Nishiwaki T, Shintani T, Hamanaka H, Noda M. 6B4 Proteoglycan/Phosphacan, an Extracellular Variant of Receptor-like Protein-tyrosine Phosphatase + \hat{A} /RPTP+, Binds Pleiotrophin/Heparin-binding Growth-associated Molecule (HB-GAM). *J Biol Chem* 1996 Aug 30;271:21446-52.
- [41] Maeda N, Ichihara-Tanaka K, Kimura T, Kadomatsu K, Muramatsu T, Noda M. A Receptor-like Protein-tyrosine Phosphatase PTPzeta/RPTPbeta Binds a Heparin-binding Growth Factor Midkine: Involvement of arginine 78 of midkine in the high affinity binding to PTPzeta. *J Biol Chem* 1999 Apr 30;274:12474-9.
- [42] Lorenzetto E, Moratti E, Vezzalini M, Harroch S, Sorio C, Buffelli M. Distribution of different isoforms of receptor protein tyrosine phosphatase g (Ptp γ -RPTP γ) in adult mouse brain: upregulation during neuroinflammation. *Brain Struct Funct* 2013;219:875-90.
- [43] Hayashi N, Oohira A, Miyata S. Synaptic localization of receptor-type protein tyrosine phosphatase zeta/beta in the cerebral and hippocampal neurons of adult rats. *Brain Res* 2005 Jul 19;1050:163-9.
- [44] Ferrario JE, Rojas-Mayorquín AE, Saldaña-Ortega M, Salum C, Gomes MZ, Hunot S, et al. Pleiotrophin receptor RPTP-z/b expression is up-regulated by L-DOPA in striatal medium spiny neurons of parkinsonian rats. *Journal of Neurochemistry* 2008 Oct 1;107:443-52.
- [45] Jung CG, Hida H, Nakahira K, Ikenaka K, Kim HJ, Nishino H. Pleiotrophin mRNA is highly expressed in neural stem (progenitor) cells of mouse ventral mesencephalon and the product promotes production of dopaminergic neurons from embryonic stem cell-derived nestin-positive cells. *FASEB J* 2004 Jun 4;18:1237-9.

- [46] Prediger R, Rojas-Mayorquin A, Aguiar A, Chevarin C, Mongeau R, Hamon M, et al. Mice with genetic deletion of the heparin-binding growth factor midkine exhibit early preclinical features of Parkinson's disease. *J Neural Transm* 2011;118:1215-25.
- [47] Gramage E, Rossi L, Granado N, Moratalla R, Herradón G. Genetic inactivation of Pleiotrophin triggers amphetamine-induced cell loss in the substantia nigra and enhances amphetamine neurotoxicity in the striatum. *Neuroscience* 2010 Sep 29;170:308-16.
- [48] Ridet JL, Malhotra SK, Privat A, Gage FH. Reactive astrocytes: cellular and molecular cues to biological function. *Trends Neurosci* 1997;20:570-7.
- [49] Pekny M, Nilsson M. Astrocyte activation and reactive gliosis. *Glia* 2005 Jun 1;50:427-34.
- [50] Ahlemeyer B, K+Älker S, Zhu Y, Hoffmann GF, Kriegstein J. Increase in glutamate-induced neurotoxicity by activated astrocytes involves stimulation of protein kinase C. *Journal of Neurochemistry* 2002 Aug 1;82:504-15.
- [51] Gramage E, Putelli A, Polanco MJ, González-Martín C, Ezquerra L, Alguacil LF, et al. The neurotrophic factor pleiotrophin modulates amphetamine-seeking behaviour and amphetamine-induced neurotoxic effects: evidence from pleiotrophin knockout mice. *Addiction Biology* 2010 Oct 1;15:403-12.
- [52] Gramage E, Martín YB, Ramanah P, Pérez-García C, Herradón G. Midkine regulates amphetamine-induced astrogliosis in striatum but has no effects on amphetamine-induced striatal dopaminergic denervation and addictive effects: functional differences between pleiotrophin and midkine. *Neuroscience* 2011 Sep 5;190:307-17.
- [53] Emeterio EPP-S, Tramullas M+, Hurl+® MaA. Modulation of apoptosis in the mouse brain after morphine treatments and morphine withdrawal. *J Neurosci Res* 2006 May 15;83:1352-61.
- [54] Rasmussen K, Beitner-Johnson DB, Krystal JH, Aghajanian GK, Nestler EJ. Opiate withdrawal and the rat locus coeruleus: behavioral, electrophysiological, and biochemical correlates. *J Neurosci* 1990;10:2308-17.

- [55] Sargeant TJ, Miller JH, Day DJ. Opioidergic regulation of astroglial/neuronal proliferation: where are we now? *Journal of Neurochemistry* 2008 Nov 1;107:883-97.
- [56] Johnson SW, North RA. Opioids excite dopamine neurons by hyperpolarization of local interneurons. *The Journal of Neuroscience* 1992 Feb 1;12:483-8.
- [57] Narita M, Miyatake M, Shibasaki M, Tsuda M, Koizumi S, Narita M, et al. Long-lasting change in brain dynamics induced by methamphetamine: enhancement of protein kinase C-dependent astrocytic response and behavioral sensitization. *Journal of Neurochemistry* 2005 Jun 1;93:1383-92.
- [58] Russo SJ, Mazei-Robison MS, Ables JL, Nestler EJ. Neurotrophic factors and structural plasticity in addiction. *Neuropharmacology* 2009;56, Supplement 1:73-82.
- [59] Knapp DJ, Whitman BA, Wills TA, Angel RA, Overstreet DH, Criswell HE, et al. Cytokine involvement in stress may depend on corticotrophin releasing factor to sensitize ethanol withdrawal anxiety. *Brain, Behavior, and Immunity* 2011 Jun 1;25, Supplement 1:S146-S154.
- [60] Shiflett MW, Balleine BW. Contributions of ERK signaling in the striatum to instrumental learning and performance. *Behav Brain Res* 2011 Mar 17;218:240-7.
- [61] Longoni R, Spina L, Vinci S, Acquas E. The MEK inhibitor SL327 blocks acquisition but not expression of lithium-induced conditioned place aversion: a behavioral and immunohistochemical study. *Psychopharmacology* 2011;216:63-73.
- [62] Russo SJ, Bolanos CA, Theobald DE, DeCarolus NA, Renthal W, Kumar A, et al. IRS2-Akt pathway in midbrain dopamine neurons regulates behavioral and cellular responses to opiates. *Nat Neurosci* 2007 Jan;10:93-9.
- [63] Mazei-Robison MS, Koo JW, Friedman AK, Lansink CS, Robinson AJ, Vinish M, et al. Role for mTOR Signaling and Neuronal Activity in Morphine-Induced Adaptations in Ventral Tegmental Area Dopamine Neurons. *Neuron* 2011;72:977-90.
- [64] Mi R, Chen W, Höke A. Pleiotrophin is a neurotrophic factor for spinal motor neurons. *PNAS* 2007 Mar 13;104:4664-9.

Figure Legends

Fig. 1. PTN, MK and RPTP β/ζ protein expression are altered by acute and chronic morphine administration and during morphine withdrawal in the VTA. Over a 7 day period, control (pla) and morphine (mor)-dependent rats received saline (sal), morphine (mor; 20 mg/kg i.p.), or naloxone (nx; 1 mg/kg s.c.) on day 7 and were sacrificed 2 h later. Semi-quantitative analysis and representative immunoblots of PTN (A, B), MK (C, D) and RPTP β/ζ (E, F) protein in VTA isolated from rats receiving the above treatments. Each bar corresponds to mean \pm SEM. Values are expressed as % of controls. * p < 0.05, ** p < 0.01 vs. pla+sal; ++ p < 0.01, +++ p < 0.001 vs. pla+mor; ## p < 0.01 vs. pla+nx; & p < 0.05, && p < 0.01 vs. mor+sal

Fig. 2. Correlation between PTN and RPTP β/ζ . The percent increase in PTN levels was positively correlated with RPTP β/ζ protein during morphine dependence and morphine withdrawal. No significant correlation was found between PTN expression and RPTP β/ζ levels after acute morphine injection. # p < 0.05: PTN levels vs. RPTP β/ζ levels.

Fig. 3. GFAP expression is enhanced by acute and chronic morphine administration and maintained during morphine withdrawal in the VTA, while astrocyte proliferation only occurs in acute morphine-injected and morphine-withdrawn rats. Over a 7 day period, control (pla) and morphine (mor)-dependent rats received saline (sal), morphine (mor; 20 mg/kg i.p.), or naloxone (nx; 1 mg/kg s.c.) on day 7 and were sacrificed 2 h later. The analyzed region within the VTA is schematically illustrated in A (modified from Paxinos and Watson, 2007). A rectangle indicates the size of the photomicrographs. (B-F): Representative photomicrographs showing immunohistochemical detection of GFAP⁺ nuclei and fibers in midbrain coronal sections (scale bar: 200 μ m). (B'-F'): high magnifications from each photomicrograph (scale bar: 100 μ m). (G, H): Quantitative analysis of astrocytes in the VTA. (I, J): Mean optical

density measurement of GFAP-immunoreactivity in the VTA from rats receiving the treatments mentioned above. (K, L): Reference area used in the densitometric analysis did not differ between groups. SN: Substantia nigra. Each bar corresponds to mean \pm SEM. * $p < 0.05$, ** $p < 0.01$ vs. pla+sal; # $p < 0.05$, ### $p < 0.001$ vs. pla+nx; && $p < 0.01$ vs. mor+sal

Fig. 4. PTN but not MK is overexpressed in astrocytes during acute morphine administration and morphine withdrawal in the VTA. (A-B): Stack of confocal images from the midbrain areas immuno-stained for GFAP (blue), PTN (red) and MK (green) in rats treated with acute morphine or morphine-dependent rats injected with naloxone. Scale bar: A-B, 50 μ m

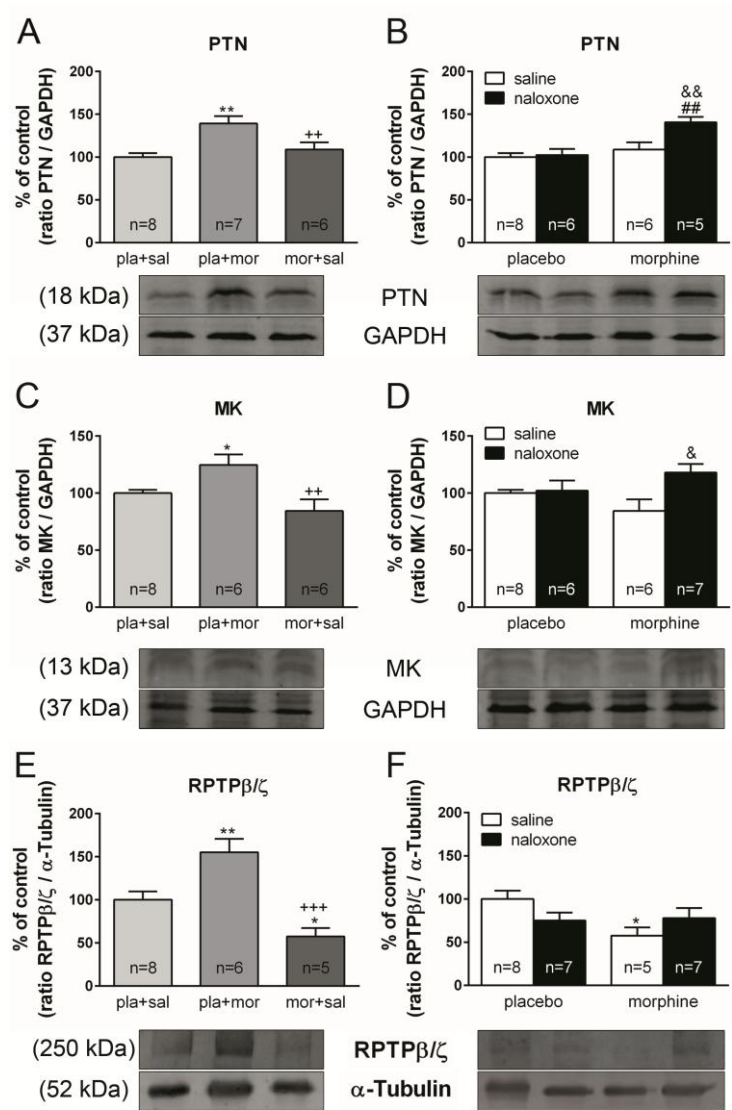
Fig. 5. RPTP β/ζ is expressed in midbrain dopaminergic neurons. (A): RPTP β/ζ (red) protein in the VTA is region-specific since strong staining in the VTA contrasts with weak or absent staining in the interpeduncular nucleus (IP) or red nucleus (RN). (B): Double-labeling with anti-TH (green) showed that RPTP β/ζ is located on cell bodies and proximal processes of almost all dopaminergic neurons in the VTA and some non-dopaminergic cells. DAPI (blue) was used as a counterstaining in both nuclei. Scale bar: A, 200 μ m; B, 50 μ m

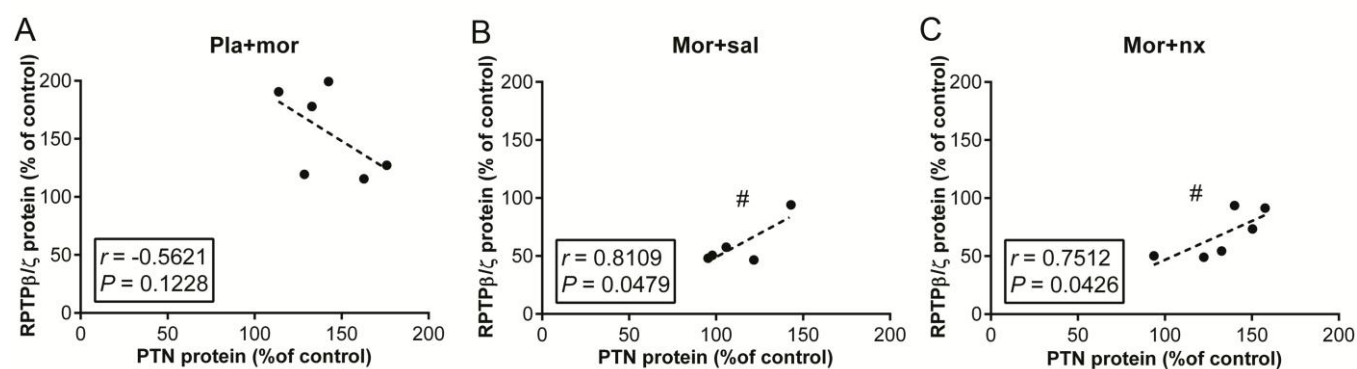
Fig. 6. p-Akt levels but not t-Akt levels are diminished during chronic morphine administration in the VTA. Over a 7 day period, control (pla) and morphine (mor)-dependent rats received saline (sal), morphine (mor; 20 mg/kg i.p.), or naloxone (nx; 1 mg/kg s.c.) on day 7 and were sacrificed 2 h later. Semi-quantitative analysis and representative immunoblots of p-Akt / t-Akt ratio (A, B) and t-Akt levels (C, D) in VTA isolated from rats receiving the above treatments. Each bar corresponds to mean \pm SEM. Values are expressed as % of controls. * $p < 0.05$ vs. pla+sal; + $p < 0.05$ vs. pla+mor; & $p < 0.05$ vs. mor+sal

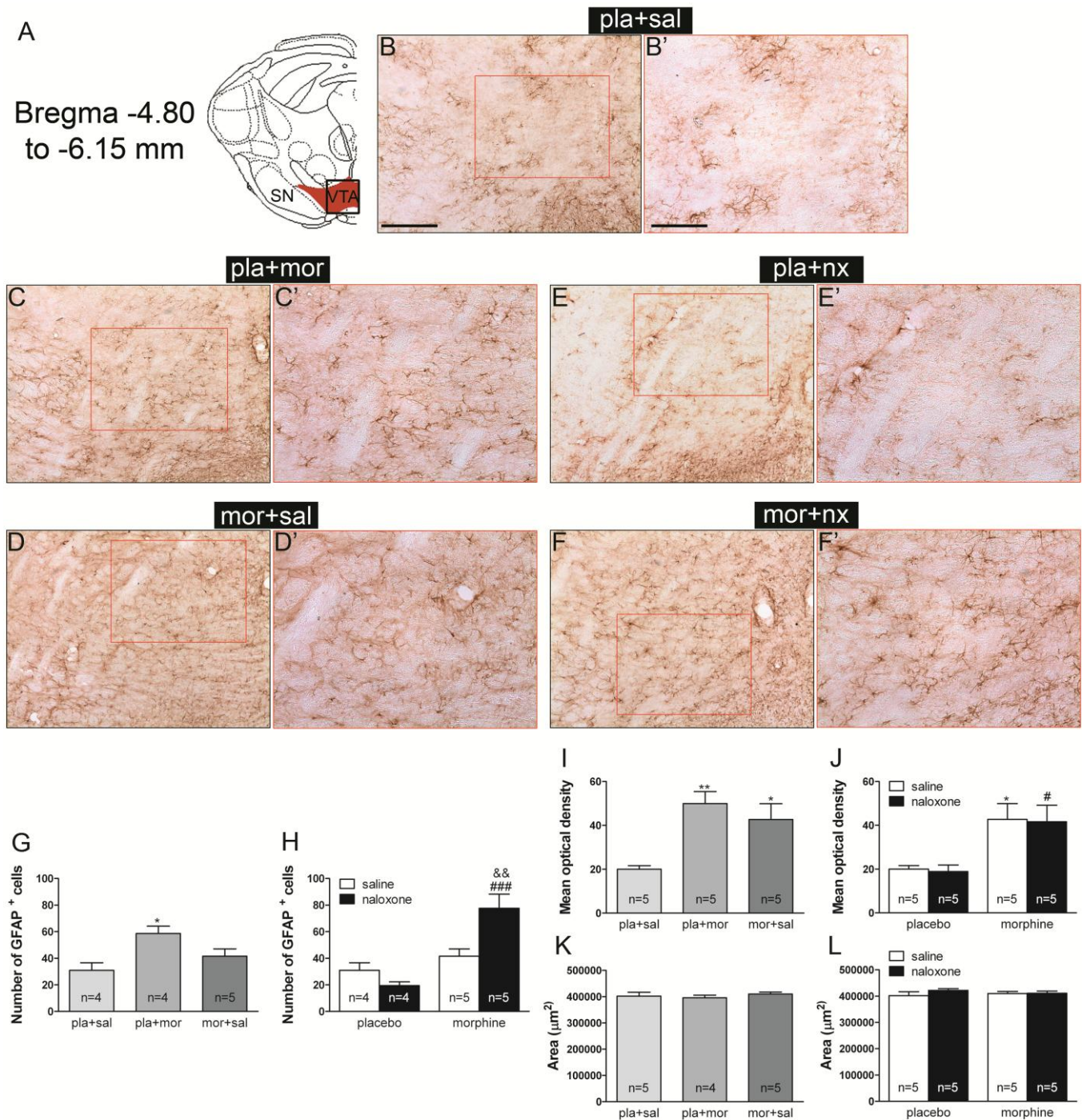
Fig. 7. p-ERK / t-ERK ratio, t-ERK levels and p-ERK absolute levels are altered by acute and chronic morphine administration and during morphine withdrawal in the VTA. Over a 7 day period, control (pla) and morphine (mor)-dependent rats received saline (sal), morphine (mor; 20 mg/kg i.p.), or naloxone (nx; 1 mg/kg s.c.) on day 7 and were sacrificed 2 h later. Semi-quantitative analysis and representative immunoblots of p-ERK 1 / t-ERK 1 ratio (A, B), t-ERK 1 levels (C, D), p-ERK 1 absolute levels (E, F), p-ERK 2 / t-ERK 2 ratio (G, H), t-ERK 2 levels (I, J), p-ERK 2 absolute levels (K, L) in VTA isolated from rats receiving the above treatments. Each bar corresponds to mean \pm SEM. Values are expressed as % of controls. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. pla+sal; $^+p < 0.05$, $^{++}p < 0.01$ vs. pla+mor; $^{##}p < 0.01$, $^{###}p < 0.001$ vs. pla+nx; $^{\&}p < 0.05$ vs. mor+sal

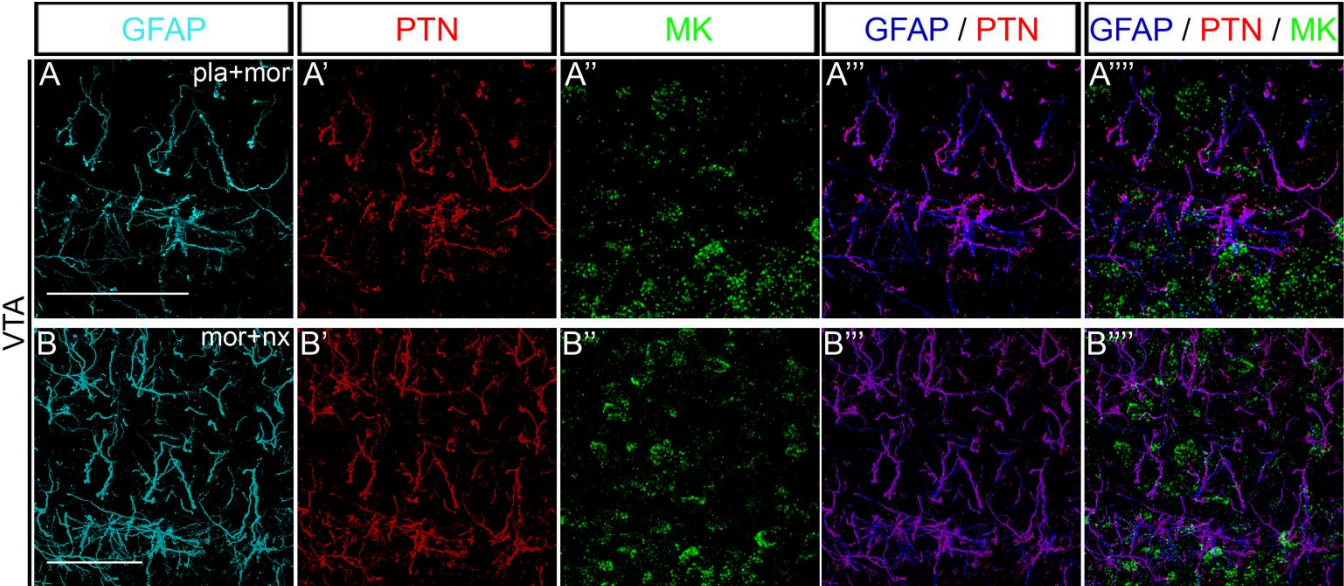
Fig 8. Correlation between PTN, MK and/or RPTP β/ζ and p-ERK or t-ERK protein levels in different experimental groups. PTN tended to be negative correlated with t-ERK 1 and t-ERK 2 expression, while MK was negatively correlated with p-ERK 1 and p-ERK 2 levels only in morphine withdrawn rats. On the other hand, during morphine dependence and withdrawal, expression of RPTP β/ζ tended to be positively correlated with p-ERK. $^{\#}p < 0.05$: PTN, MK and/or RPTP β/ζ vs p-ERK 1-2 / t-ERK 1-2; $^{\&}p < 0.05$: PTN, MK and/or RPTP β/ζ vs p-ERK 1-2 / GAPDH; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$: PTN, MK and/or RPTP β/ζ vs t-ERK 1-2 / GAPDH.

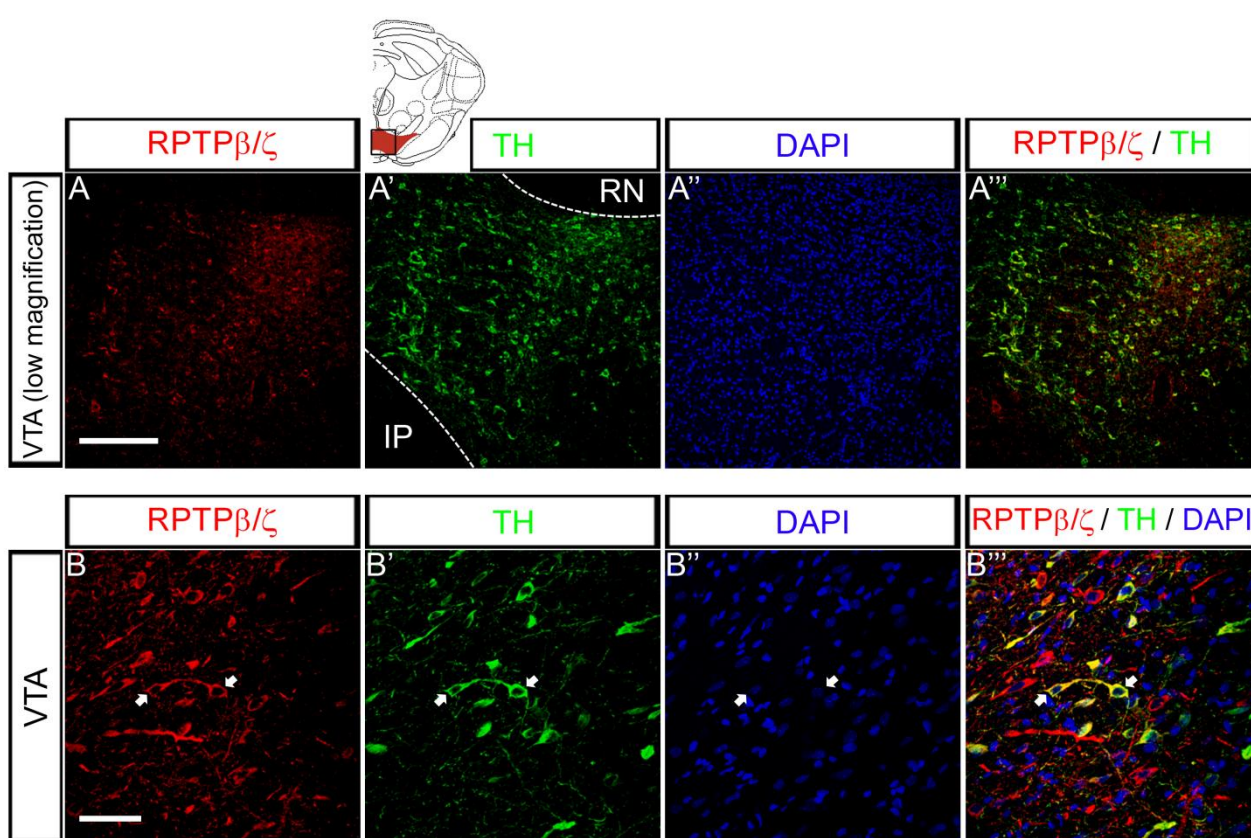
VTA

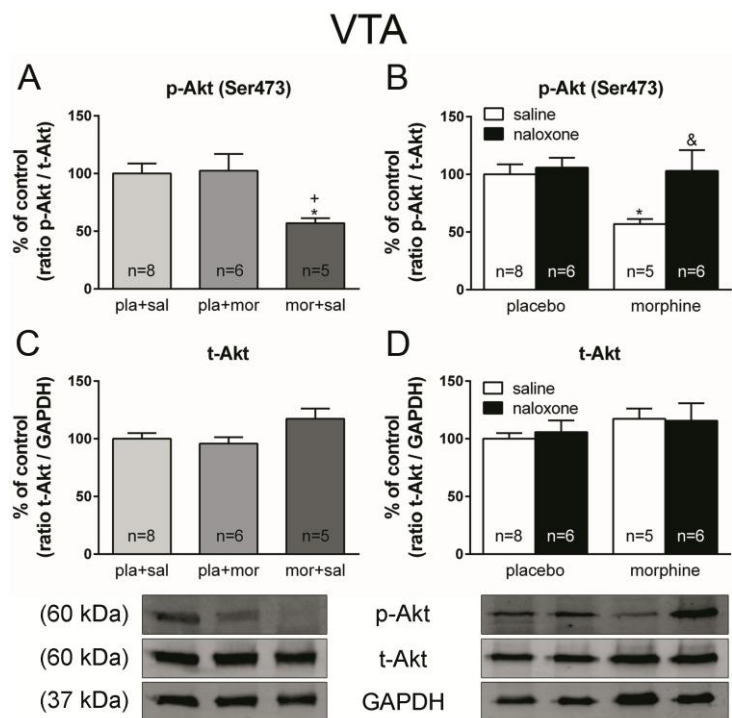




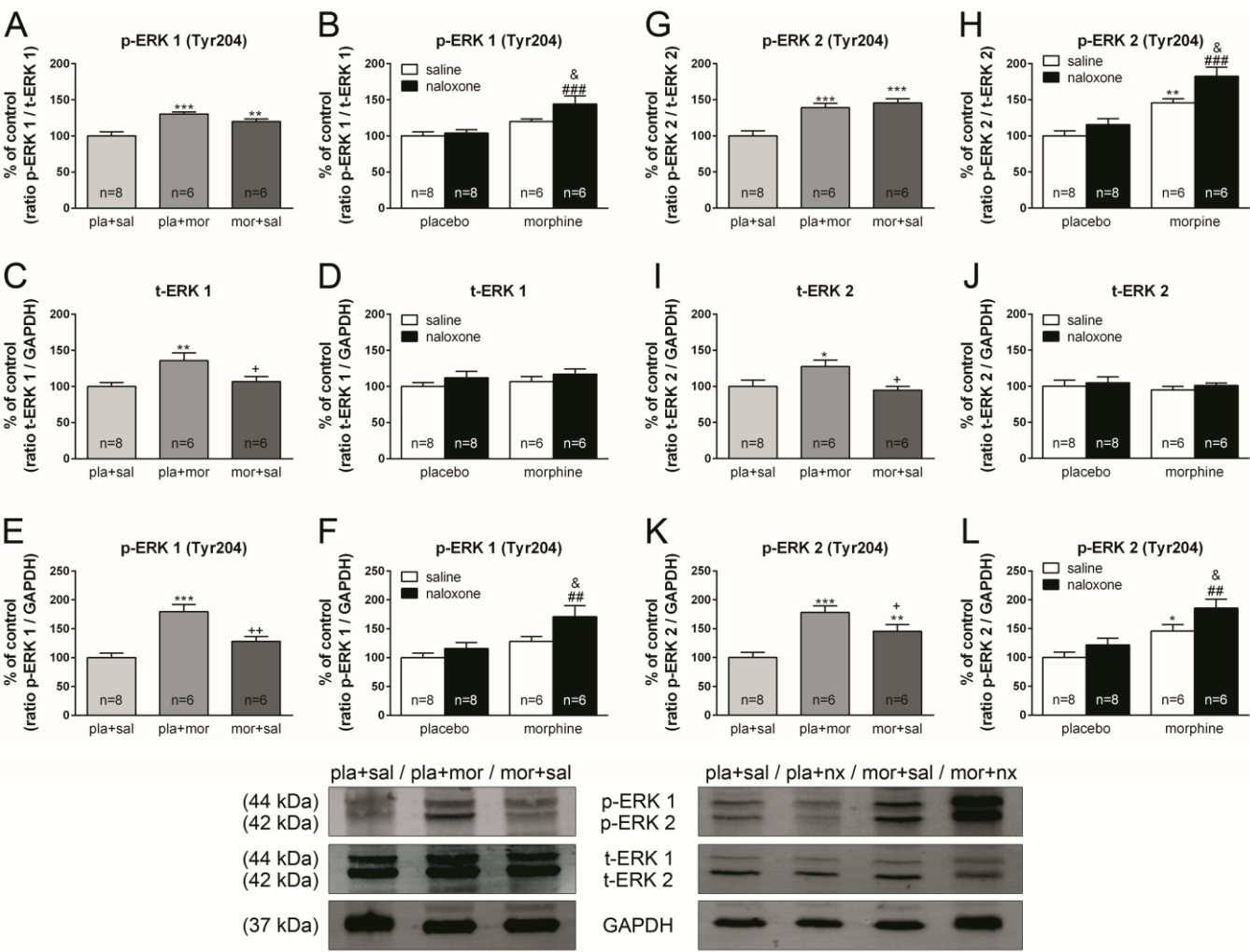


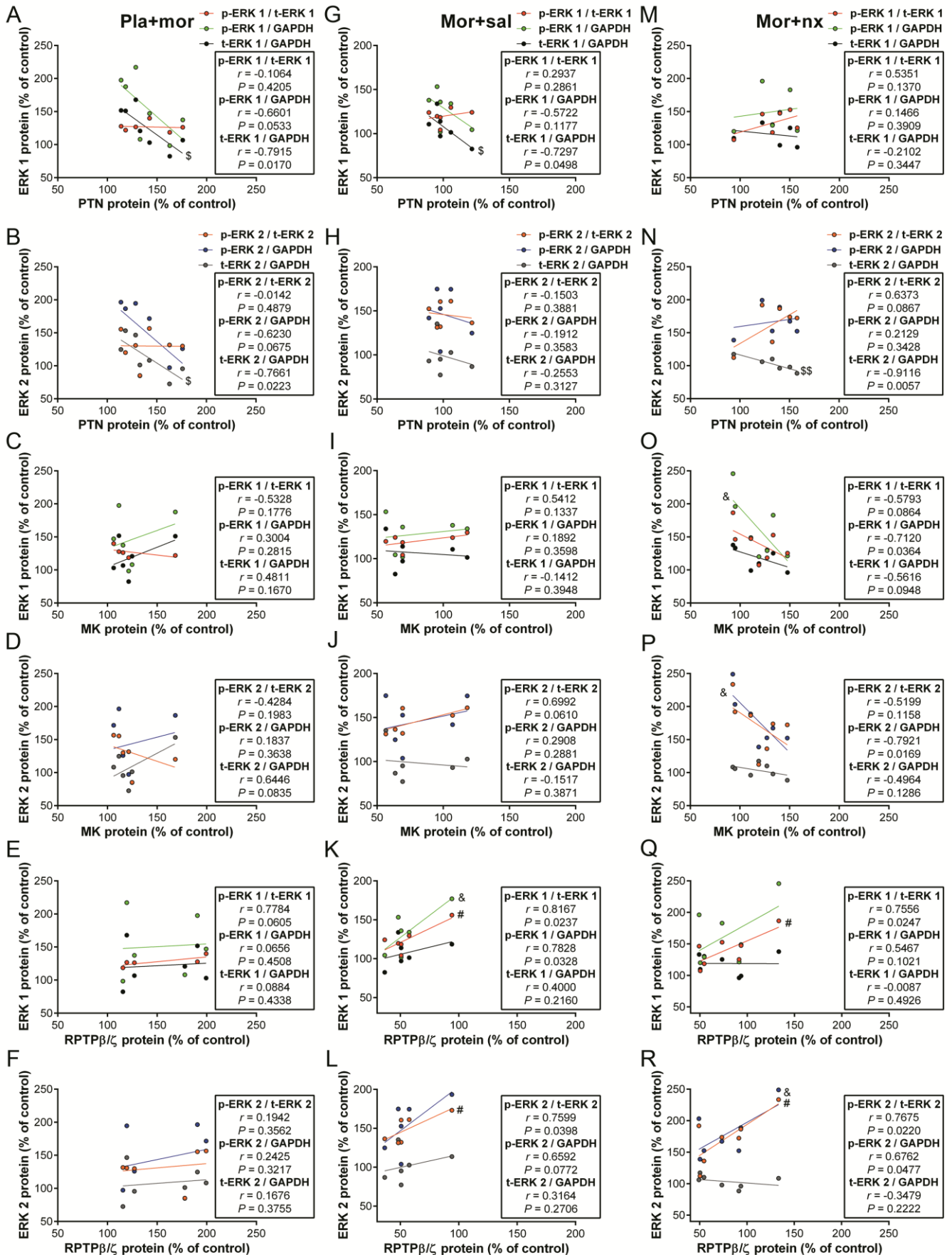






VTA





García-Pérez D, Laorden ML, Milanés MV. Regulation of pleiotrophin, midkine, receptor protein tyrosine phosphatase β/ζ and their intracellular signalling cascades in the nucleus accumbens during opiate administration. *Int J Neuropsychopharmacol*. 2015. doi: 10.1093/ijnp/pyv077.

International Journal of Neuropsychopharmacology

Abstract

BACKGROUND:

Most classes of addictive substances alter the function and structural plasticity of the brain reward circuitry. Midkine (MK) and pleiotrophin (PTN) are growth/differentiation cytokines which, similarly to neurotrophins, play an important role in repair, neurite outgrowth, and cell differentiation. PTN or MK signaling through receptor protein tyrosine phosphatase β/ζ (RPTP β/ζ), leads to the activation of extracellular signal-regulated kinases and thymoma viral proto-oncogene. This activation induces morphological changes and modulates addictive behaviors. Besides, there is increasing evidence that during the development of drug addiction, astrocytes contribute to the synaptic plasticity by synthesizing and releasing substances such as cytokines.

METHODS:

In the present work we studied the effect of acute morphine administration, chronic morphine administration, and morphine withdrawal on PTN, MK, and RPTP β/ζ expression and on their signaling pathways in the nucleus accumbens.

RESULTS:

Present results indicated that PTN, MK, and RPTP β/ζ levels increased after acute morphine injection, returned to basal levels during chronic opioid treatment, and were up-regulated again during morphine withdrawal. We also observed an activation of astrocytes after acute morphine injection and during opiate dependence and withdrawal. In addition, immunofluorescence analysis revealed that PTN, but not MK, was overexpressed in astrocytes and that dopaminoceptive neurons expressed RPTP β/ζ .

CONCLUSIONS:

All these observations suggest that the neurotrophic and behavioral adaptations that occur during opiate addiction could be, at least partly, mediated by cytokines.

<http://ijnp.oxfordjournals.org/content/early/2015/07/29/ijnp.pyv077.long>

Elsevier Editorial System(tm) for Biological Psychiatry
Manuscript Draft

Manuscript Number:

Title: Glucocorticoid homeostasis in the dentate gyrus is essential for opiate withdrawal-associated memories

Article Type: Archival Report

Keywords: Conditioned place aversion; adrenalectomy; memory consolidation and retrieval; addiction; transcription factors; miRNAs

Corresponding Author: Mr. Daniel García-Pérez,

Corresponding Author's Institution: University of Murcia

First Author: Daniel García-Pérez

Order of Authors: Daniel García-Pérez; Szilamer Ferenczi; Krisztina J Kóvacs; Maria L Laorden; Maria V Milanés; Cristina Núñez

Abstract: **BACKGROUND:** Drug-withdrawal associated aversive memories might trigger relapse to drug-seeking behavior. However, changes in structural and synaptic plasticity, as well as epigenetic mechanisms, which may be critical for long-term aversive memory, have yet to be elucidated. **METHODS:** We used conditioned place aversion (CPA) paradigm to uncover the role of glucocorticoids (GCs) on plasticity-related processes that occur within the dentate gyrus (DG) during opiate-withdrawal conditioning (memory formation-consolidation), and after reactivation by re-exposure to the conditioned environment (memory retrieval).

RESULTS: Rats subjected to conditioned morphine-withdrawal robustly expressed CPA, while adrenalectomy impaired naloxone-induced CPA. However, increased locomotor activity was observed in all the animals receiving morphine. Accordingly, activity-regulated cytoskeletal-associated protein (Arc) expression was induced in sham- and ADX-dependent animals during the conditioning phase in the DG. In contrast, memory retrieval elicited increased Arc and early growth response 1 (Egr-1) expression in sham+morphine rats, but not in ADX+morphine animals. Importantly, Arc seemed to regulate CPA score and was selectively expressed in the granular zone of the DG in dopaminoceptive, glutamatergic and GABAergic neurons. We further found that brain derived neurotrophic factor was regulated in the opposite way during the test phase. Our results also suggest a role for epigenetic regulation on the expression of GC receptors and Arc during memory retrieval.

CONCLUSIONS: Our data provide the first evidence that GC homeostasis is important for the expression of long-term morphine-withdrawal memories. Moreover, our results support the idea that targeting Arc and Egr-1 in the DG may provide important insights into the role of these signaling cascades in CPA reconsolidation.

Glucocorticoid homeostasis in the dentate gyrus is essential for opiate withdrawal-associated memories

Daniel García-Pérez, Szilamer Ferenczi, Krisztina J. Kovács, M. Luisa

Laorden, M. Victoria Milanés and Cristina Núñez

Department of Pharmacology (DG-P, CN, MLL, MVM), University of Murcia, Murcia, Spain; Murcia Institute of Biomedical Research (IMIB) (CN, MLL, MVM), Murcia, Spain; Laboratory of Molecular Neuroendocrinology (SF, KJK), Institute of Experimental Medicine, Academy of Sciences, Budapest, Hungary.

Address correspondence to Daniel García-Pérez, PhD Student; Department of Pharmacology, Faculty of Medicine, Campus de Espinardo, 30100 Murcia, Spain. Phone number: +34868887183; Email: daniel.garcia9@um.es

Key words: Conditioned place aversion; adrenalectomy; memory consolidation and retrieval; addiction; transcription factors; miRNAs

Short Title: Glucocorticoids and opiate withdrawal-associated memory

Number of words in the Abstract: 250

Number of words in the article body: 4.108

Number of Figures: 8

Number of Tables: 0

Supplemental information: 1

ABSTRACT

BACKGROUND: Drug-withdrawal associated aversive memories might trigger relapse to drug-seeking behavior. However, changes in structural and synaptic plasticity, as well as epigenetic mechanisms, which may be critical for long-term aversive memory, have yet to be elucidated.

METHODS: We used conditioned place aversion (CPA) paradigm to uncover the role of glucocorticoids (GCs) on plasticity-related processes that occur within the dentate gyrus (DG) during opiate-withdrawal conditioning (memory formation-consolidation), and after reactivation by re-exposure to the conditioned environment (memory retrieval).

RESULTS: Rats subjected to conditioned morphine-withdrawal robustly expressed CPA, while adrenalectomy impaired naloxone-induced CPA. However, increased locomotor activity was observed in all the animals receiving morphine. Accordingly, activity-regulated cytoskeletal-associated protein (Arc) expression was induced in sham- and ADX-dependent animals during the conditioning phase in the DG. In contrast, memory retrieval elicited increased Arc and early growth response 1 (Egr-1) expression in sham+morphine rats, but not in ADX+morphine animals. Importantly, Arc seemed to regulate CPA score and was selectively expressed in the granular zone of the DG in dopaminoceptive, glutamatergic and GABAergic neurons. We further found that brain derived neurotrophic factor was regulated in the opposite way during the test phase. Our results also suggest a role for epigenetic regulation on the expression of GC receptors and Arc during memory retrieval.

CONCLUSIONS: Our data provide the first evidence that GC homeostasis is important for the expression of long-term morphine-withdrawal memories. Moreover, our results support the idea that targeting Arc and Egr-1 in the DG may provide important insights into the role of these signaling cascades in CPA reconsolidation.

INTRODUCTION

Drug addiction is a chronic, relapsing disorder in part due to the strong associations formed between drugs and the stimuli associated with drug use through Pavlovian conditioning (1). These stimuli become strong drivers of continuous use and relapse after abstinence (2). One of the factors thought to be responsible for this vulnerability is conditioned withdrawal, which consists of somatic and affective withdrawal signs elicited in the presence of cues that were previously paired with drug withdrawal (3). Reactivation of these withdrawal memories has been suggested to trigger the relapse of drug seeking behavior in abstinent opiate addicts (4,5). Although much has been learned about the neural basis of reinforcement during drug administration, the neural circuitry that underlies conditioned-cued relapse is only now being clarified.

It has been proposed that the reinforcing properties of drugs of abuse are due, in part, to their ability to enhance memory consolidation. In addition, several molecular and cellular adaptations involved in addiction are also implicated in models of learning and memory. Therefore, recent efforts to develop effective treatments for addiction have focused on manipulations of learning and memory processes involved in encoding cue-drug or cue-drug withdrawal associations (4). Conditioned-place aversion (CPA) paradigm is a highly sensitive animal model for measurement of the negative affective component of drug withdrawal as well as to investigate the neural substrates underlying the aversive memory associated with drug withdrawal (6,7). In rodents, the negative affective component of opiate dependence could be reflected by CPA, in which opiate withdrawal is paired with a particular environment, which triggers the association between negative affective consequences of withdrawal with context (aversive memory formation). When animals are re-exposed to the paired environment in a drug-free state, they avoid the paired environment due to the association between the context and aversive memories of drug withdrawal (aversive memory retrieval). Among the limbic structures that are likely to mediate these component of drug addiction, the basolateral amygdala (BLA) and the hippocampus represent critical neural substrates (8,9). It is well established that the hippocampus is crucially important for memory encoding/consolidation but also for episodic memory retrieval (10). In

this context, much experimental evidence has demonstrated that the granular zone (GZ) of the hippocampal dentate gyrus (DG) has a critical role in learning and memory formation (11,12).

On the other hand, stress hormones such as glucocorticoids (GCs) and noradrenaline mediate and modulate memory consolidation (13,14). GCs exert this action directly on brain regions, including the hippocampus, amygdala and prefrontal cortex, which are enriched in glucocorticoid receptors (GRs) and are important in long-term memory formation (15).

Most of the existing knowledge focuses on the molecular and cellular adaptations that occur in the brain reward regions (VTA and NAc) in response to drugs exposure and withdrawal, with much less information available about other key limbic brain regions also crucial for drug addiction and the molecular mechanisms that are critical to transform opiate withdrawal into a long-term aversive memory.

CPA develops through associative learning and requires synaptic plasticity (16), which is implicated in adapted responses in brain, including memory formation and drug addiction and withdrawal (17). Therefore, we have used CPA to study the plasticity-related processes that occur within the DG during conditioning of the aversive properties of opiate withdrawal (memory formation-consolidation; MC), and after allowing their reactivation by re-exposure to the conditioned environment (memory retrieval; MR). For that, first we have assessed activity-regulated cytoskeletal (*arc*) mRNA expression and effector protein [a plasticity marker; (18)], as well as other transcription factors and receptors involved in memory formation: GR, phosphorylated cAMP response element binding protein (pCREB), brain derived neurotrophic factor (BDNF) and early growth response 1 (Egr-1), among others. Second, we hypothesized that GCs might play a crucial role in the aversive memories of drug withdrawal. To test this hypothesis we have used adrenalectomized (ADX) rats and examining the same neurobiological parameters and determining the role of GC in the induction of CPA.

METHODS AND MATERIALS

Details are provided in the Supplementary Information (SI).

Animals

All experimental procedures were performed in accordance with the European Community Council Directive (2010/63/EU), and local Committees for animal research (REGA ES300305440012). Male Wistar rats (Harlan, Barcelona, Spain) were used.

Adrenalectomy

Rats were bilaterally ADX as described previously (19) and implanted subcutaneously (s.c.) with slow-release corticosterone pellets at surgery that provide stable corticosterone levels corresponding to circadian nadir. Control rats were subjected to the same surgical procedure (sham) without adrenal extirpation.

Induction of Opiate Dependence

Morphine dependence was induced by s.c. implantation (lower back) of two slow-release, morphine-containing pellets (each morphine pellet contains 75 mg of morphine base) under light ether anaesthesia. Placebo-pelleted rats received lactose pellets also implanted subcutaneously under anaesthesia.

Conditioned Place Aversion Protocol

Pre-testing Phase

In the pre-testing phase (day 0), animals were placed in the central corridor and allowed to explore the apparatus freely for 20 min. For each rat, one room was randomly chosen to be paired with naloxone and the other to vehicle. Rats were ADX on day 1 and placebo or morphine pellets were implanted on day 4 according to the experimental protocol depicted on Figure 1A.

Conditioning Phase

Rats received injection of saline on days 7 and 9, prior to being confined to their preselected saline-paired compartment for 1 hour. On days 8 and 10, rats received 0.1 mg/kg s.c. of naloxone immediately prior to confinement in the naloxone-paired compartment for 1 hour. In addition, opiate withdrawal behaviors on sham or ADX animals receiving naloxone on days 8 and 10 were measured for 15 min after the conditioning phase. A cohort of animals, named conditioning memory rats (CM), were decapitated on day 10 (15 min after leaving the naloxone-paired compartment).

Testing Phase

The test was conducted on day 11, exactly as in the preconditioning phase (free access to each compartment for 20 min). The difference ($\Delta D = D - D_0$) between the time spent in the naloxone-paired compartment after conditioning (D) minus the time spent in the same compartment before conditioning (preconditioning test D_0) reflects the change of preference induced by opiate withdrawal. Conditioned place aversion animals (CPA groups) were killed 1 hour after starting the testing phase.

Tissue processing

Brains were sliced on a cryostat and kept at -20°C until each region of interest comes into the cutting plane. For DG study, three consecutive 500- μ m coronal slides were made corresponding to approximately -2.9 to -4.4 mm from bregma, according to the atlas of (20). Tissues of interest were dissected using a punching device with a 1-mm internal diameter. A second set of animals from each treatment group was used for immunofluorescence staining.

RNA extraction and quantitative real-time PCR (qPCR)

qPCR was performed as previously described (21).

List of the genes: gapdh, arc, egr-1, bdnf

List of the microRNA (miRNA) assays: U6 snRNA (cat.: 001973, 4427975), miR-212 (cat.: 002551, 4427975), miR-124a (cat.: 001182, 4427975)

The gene expression was analyzed by ABI Step One 2.1 program. The amplicon was tested by Melt Curve Analysis on ABI Step OnePlus Instrument. Experiments were normalized to gapdh (glyceraldehyde-3-phosphate dehydrogenase) and U6 snRNA expressions.

gapdh for ACAGCCGCATCTTCTTGTGC

gapdh rev GCCTCACCCCATTTGATGTT

arc for CCCCCAGCAGTGATTCATAC

arc rev CAGACATGGCCGAAAGACT

egr-1 for CACCTGACCACAGAGTCCTTTT

egr-1 rev ACCAGCGCCTTCTCGTTATT

bdnf for AAACGTCCACGGACAAGGCA

bdnf rev TTCTGGTCCTCATCCAGCAGC

U6 snRNA cat:001973

mmu-miR-212 cat: 002551

mmu-miR-124a cat: 001182

Western Blotting

Western Blotting protocol (22) and a list of the primary antibodies used in this study can be found at SI. We used GAPDH as our loading control.

Immunofluorescence study and confocal microscopical analysis

The tissue for immunofluorescence was processed as previously described (22) (see details in the SI). Equipment and settings are detailed in the SI. Final confocal images are illustrated as they appear throughout the stack of sections as a simple layer or as a transparency of all layers merged together.

Data Analysis

Data are presented as mean \pm SEM. Data were analyzed using one-way or two-way analysis of variance (ANOVA) followed by a *post hoc* Newman–Keuls test. Student’s *t*-test was used when there were two experimental groups. Correlations between different parameters were assessed using linear regression. Differences with a $p < 0.05$ were considered significant (GraphPad Prism 6: GraphPad Software Inc., San Diego, CA, USA).

RESULTS

GCs are released during and immediately after emotionally arousing stimulation used in training tasks, and there is extensive evidence that these hormones enhance memory consolidation (23,24). The animals used in this experiment did not exhibit natural preference for neither the dots nor stripes chambers before the conditioning sessions (Fig. 1B-D,F-I). During the test, morphine dependent rats spent less time in the naloxone-paired compartment than controls (Fig. 1E). The negative score of these animals correlated with reduced number of entries in the naloxone-paired compartment (Fig. 1M). Remarkably, adrenalectomy diminished naloxone-induced CPA in opiate-dependent animals (Fig. 1E). Additionally, in the test phase, the total entries to any chamber were higher in animals receiving opiates (Fig. 1J-L).

ADX animals gained significantly less weight after surgery than sham-operated rats (Fig. 2A). After pellets implantation, sham- and ADX-operated animals receiving morphine showed lower body weight gain than their respective controls (Fig. 2B,C). In addition, ADX reduced the body weight gain from days 4 to 7 in control rats compared with sham+placebo rats (Fig. 2B). In agreement with other studies (25), naloxone administration to sham- or ADX-operated rats dependent on morphine evoked an increase of the body weight loss (Fig. 2D), as well as the appearance of a number of behavioral symptoms characteristic of opiate withdrawal (Fig. 2E-G). It is noteworthy that, on day 10, the ADX+morphine group exhibited an enhancement of the withdrawal score when compared with both sham+morphine and ADX+morphine (day 8) groups (Fig. 2G).

Regarding the activity of the hypothalamic-pituitary-adrenal (HPA) axis after memory formation and retrieval processes, our results revealed that naloxone administration to morphine dependent rats induced an increase in GCs plasma levels, which was not observed in ADX animals after conditioning sessions on day 10 due to adrenalectomy (Fig. 2H). The process of memory retrieval after naloxone-induced CPA did not alter the blood levels of adrenocorticotroph hormone (ACTH) and corticosterone, although ADX rats displayed increased ACTH concentration because of the lack of GCs inhibition on HPA axis (Fig. 2I,J).

To determine which molecular pathways are coupled to the learning-dependent GCs activation required for long-term memory, we examined the effect adrenalectomy on numerous molecular changes that were previously established to underlie long-term synaptic plasticity and memory formation. Our results show that memory consolidation process did not modify neither miR-124 levels nor miR-212 expression in the DG from sham+morphine rats (Fig. 3A,B), but induced a significant increase in Arc mRNA and protein levels (Fig. 3G,J). No other changes were seen in the rest of the parameters studied in sham-operated morphine-dependent animals compared with placebo-pelleted rats. Nonetheless, in ADX animals we observed a diminution of miR-212 in DG from both control and morphine-dependent rats (Fig. 3B), which paralleled an increase of Arc mRNA and protein in morphine-dependent ADX animals (Fig. 3G,J). In addition, Egr-1 protein levels in ADX animals treated with morphine were significantly higher than those in ADX+placebo and sham+morphine groups.

After CPA memory retrieval, the levels of both miR-124a and miR-212 decreased in ADX animals (Fig. 4A,B). These data were accompanied by increasing GR protein levels in ADX animals (Fig. 4K). Both miRNAs were negatively correlated with the levels of GR in the sham+morphine group (Fig. 4F,G). Besides, we observed an enhancement of Arc mRNA and protein levels in sham morphine-dependent animals that did not appear in animals with lack of GCs (Fig. 4E,O), as well as a positive correlation between miR-124a and Arc protein levels (Fig. 4H). Then, we measured the expression of other factors in the DG after the test phase. We observed that both Egr-1 mRNA and protein levels tended to augment in sham+morphine-treated rats, while in ADX animals dependent on morphine there was a significant decrease of Egr-1 mRNA and protein expression (Fig. 4C,M). CREB phosphorylation also augmented after CPA in morphine-treated and in ADX rats (Fig. 4L). In addition, BDNF protein levels were increased in ADX morphine-dependent animals (Fig. 4N). As shown in Fig. 4J, the regulation of BDNF mRNA is opposite to Egr-1 and Arc mRNAs. Interestingly, when we evaluated the correlation between the score and the proteins studied, we found a negative correlation with Arc only in sham+morphine rats (Fig. 4P-U).

As seen in Fig. 5A,C, D1 and D2 receptors were localized in the GZ of DG, although D1 was also found in other regions of the DG. Distinct staining pattern for D1 and D2 was also demonstrated in rat cortex (Fig. S1A). GLS2 and GAD, markers of glutamatergic and GABA-ergic neurons, respectively, were colocalized in the DG, mainly in the GZ (Fig. 5D,E). Besides, D1-positive or GLS2-positive signals were colocalized with GFAP (astrocytic marker) only in the molecular zone, but not in the GZ of the DG (Fig. 5F,G).

According to what has been set out above, we confirmed the co-localization of GR with pCREB, Egr-1, and BDNF in the GZ of the DG after the CPA in sham- and ADX-operated morphine-dependent rats. Representative images are shown in Figure 6. Additionally, positive correlations were obtained between GR and pCREB protein levels in the sham-morphine group (Fig. 6I), as well as between GR and Egr-1 protein levels in ADX-operated rats dependent on morphine (Fig. 6L). In contrast, sham+placebo and ADX+placebo rats displayed negative correlation between GR and Egr-1 (Fig. S2C,D). In addition, GR and BDNF protein levels were negatively correlated in the ADX+morphine group (Fig. 6N).

As seen in Fig. 7 and supplementary Fig. S3A, the immunofluorescence study indicated that neurons exhibiting GR staining in the DG after naloxone-induced CPA displayed low Arc signal. Besides, there was a negative correlation between GR and Arc only in sham morphine-dependent rats (Fig. 7H). Arc was found in GABAergic, glutamatergic and dopaminergic neurons (Fig. 7A-C). Moreover, all neurons that expressed Arc were pCREB- or Egr-1-positive in the GZ of the DG after the test phase (Fig. 8A-D; Fig. S3B-C). While all groups exhibited positive correlation between Arc and pCREB (Fig. 8I-J; Fig. S4A-B), Egr-1 was positively correlated with Arc only in sham+placebo rats (Fig. 8K-L; Fig. S4C-D). The majority of Arc-positive neurons in sham+morphine rats did not express BDNF (Fig. 8E). On the other hand, the few Arc⁺ neurons in ADX+morphine animals were also BDNF⁺ (Fig. 8F). However, there was no correlation between Arc and BDNF expression neither in sham+morphine rats nor in ADX+morphine rats (Fig. 8M-N). In addition, there was no colocalization of Arc and pTrkB in the DG of morphine-dependent animals (Fig. 8G,H).

DISCUSSION

Relapse to drug-taking following prolonged abstinence constitutes an important problem for long-term drug-addiction treatment. Factors contributing to relapse are exposure to conditional drug cues and stress (2). Present report highlights the impact of opiate withdrawal-associated aversive stimuli on DG activity, which has a pivotal role in mediating memory consolidation and recall. Collectively, the present study determined the role of GCs (by using ADX animals) in the formation of morphine withdrawal-associated conditioned memory, as well as in the expression of morphine withdrawal-associated CPA. GCs have profound influence on different stages of opiate dependence, such as the relapse to opiate-seeking (26). However, opiate withdrawal, as such, did not reinstate drug-seeking behavior (27), thus highlights the essential role of withdrawal-related memories on drug reinstatement.

Drugs of abuse stimulate HPA axis during acute withdrawal (28,29). A GR antagonist alleviated the somatic signs of naloxone-induced opiate withdrawal, without altering plasma GCs concentration (30). Our current results indicate that brain GCs levels do not modulate the somatic expression of opiate withdrawal, thus highlighting the essential role for GR signaling but not for GCs levels on the expression of withdrawal-associated somatic signs. Increased withdrawal score observed in ADX+morphine rats on day 10 could underlie increased GR levels in stress-related areas, as described by our group (30). Naloxone injection to morphine-dependent rats decreased the levels of hippocampal GR mRNA (31,32,33). However, sham+morphine rats did not exhibit increased GR levels during conditioning, which could be explained by a lower naloxone dose used in our experiments.

On the other hand, enhanced GCs levels are known to impair memory retrieval (10). Importantly, sham or ADX animals implanted with morphine do not differ in plasma corticosterone levels from their controls during the test phase. Chronic opiate exposure did not alter GR mRNA in the DG (33). In contrast, we observed that ADX-animals exhibited enhanced GR levels during the test phase, which may be explained by the lack of endogenous GCs (34,35). Corticosterone secretion is required for the enhanced locomotor response to opiates (36,37). Our results show that withdrawal-induced behavioral sensitization occurred both, in sham and ADX-dependent animals, although we detected differences in

the room preference during the test phase. Thus, the reduced aversion in ADX+morphine rodents was not a result of nonspecific locomotor changes.

Abused drugs usurp the synaptic machinery of hippocampus circuits and may induce maladaptive plasticity (38). Hippocampal long-term potentiation (LTP) is a form of synaptic plasticity proposed as a cellular substrate for learning and memory (39). Previous studies have shown that chronic morphine can both reduce (40,41,42) or augment (43,44,45,46) LTP in rat hippocampal synapses. Our results demonstrate that morphine-dependent rats display associative learning through a LTP-dependent mechanism, with endogenous GCs playing a pivotal role.

Memory consolidation refers to the time-dependent stabilization process that leads to long-term storage of a newly acquired memory (15). Strengthening effects of GCs on memory consolidation are well known, but the underlying molecular mechanisms during CPA conditioning remain poorly understood. We uncovered that ADX-dependent group showed increased Egr-1 expression, while both sham and ADX-addicted animals displayed increased Arc levels during consolidation process. Corticosterone and GR are essential to control increased learning-dependent Arc (47,48,14), suggesting that GR-signaling should be active in ADX-rats. Arc and Egr-1, which are induced during memory consolidation (49,50,51), are required in late LTP and long-term memory (52,53). Thus, our results support that sham- and ADX morphine-dependent animals are able to encode the aversive experience through LTP induction. Moreover, we detected that BDNF or pCREB levels remained unaltered during consolidation, also described for other conditioning tasks (54), but in contrast with increased pCREB-133 levels (14,55,54) and BDNF pathway recruitment (14) during fear training. We conclude that fear and morphine-withdrawal training only partially engage the same signaling pathways.

Post-transcriptional processes play essential roles in neuronal plasticity and contribute to addiction (56). We observed temporal dissociated miRNA regulation during the CPA protocol. miR-212 was downregulated in ADX groups, independently of the experimental phase. By contrast, daily injection of ACTH upregulated the expression of miR-212 in rat adrenal glands (57). A possible explanation to

this discrepancy is that ADX animals in our experiments displayed sustained high ACTH plasma levels, which could dysregulate the acute ACTH-induced miR-212 expression.

While advances towards understanding memory consolidation have been made, less is known about the molecular processes underlying plasticity during memory retrieval (58), a very rapid process in which a previously acquired memory trace is reactivated (59). Retrieval-dependent activation occurs in the dorsal hippocampus (60,58,8) and induces protein synthesis-dependent memory reconsolidation (61,62). We tested memory recall one day after the last conditioning session, since hippocampal GRs regulate essential mechanisms for long-term fear memory formation, without affecting short-term retention (14).

Compelling evidence shows that gene expression programs are, in part, orchestrated by epigenetic mechanisms. GCs induce the expression of miR-124, which in turn downregulates GR (63,64). In concordance, we observed a negative correlation between miR-124a and GR levels in sham+morphine rats during the test phase. Moreover, the extirpation of adrenal glands seems to lead to reduced miR-124a levels and increased GR expression. The negative relationship observed between miR-212 and GR raises the possibility that reduced miR-212 could participate in GR upregulation in ADX rats. Several genes related with synaptic plasticity and drug addiction have been described among miR-124 and mir-212 targets, such as CREB or BDNF (65,66,67,68). However, our results do not point to a direct regulation of BDNF or pCREB by these miRNAs during the CPA expression. Importantly, miRNAs can attract a variety of activities to their targets, even stimulating translation (69). It is noteworthy that miR-124 and miR-212 seem to promote Arc protein levels in sham+morphine animals.

Our results show that CPA expression in sham+morphine rats is accompanied by increased CREB activation, indicating that pCREB accompanies memory retrieval and plays a role in memory re-consolidation following retrieval (70). On the other hand, ADX animals show increased pCREB levels, which may be due to increased GR expression instead of memory-related mechanisms, since ADX-dependent animals do not display enhanced pCREB levels compared to their controls.

While GR-dependent memory-related pCREB molecular changes were nongenomic, Egr-1 or Arc induction required transcription (14). Thus, our results clearly show that IEG induction in the hippocampus during testing reflects the genomic activation of neurons relevant to the contextual memory retrieval (60). Previous reports demonstrated an increase in Egr-1 expression levels at the similar and more prolonged time-points (51,71,72). GR in the hippocampus enhance fear memory consolidation via Egr-1 activation (73,74). However, post-conditioning Egr-1 levels did not increase in sham+morphine rats, suggesting that morphine-withdrawal memory consolidation does not require Egr-1, whereas reconsolidation recruits Egr-1, as proposed by Lee and colleagues (72). Moreover, our results point out that homeostatic endogenous GCs are critical for retrieval-induced Egr-1 expression.

Similar data were obtained for Arc induction during fear-memory retrieval (51), although other reports failed to detect these signaling event at late time-points (75). Arc KO animals results in an unusual phenotype: short-term learning is normal but lasting memories cannot be formed (76). Late-phase LTP is considered to be the underlying mechanism of long-term memory, and LTP consolidation in the DG requires sustained period of Arc synthesis translation since Arc protein underlying LTP consolidation is rapidly degraded (77,78). Given that Arc learning-dependent expression in the hippocampus is controlled by corticosterone and GR (47,48,14), we propose that ADX-dependent animals are able to induce Arc-mediated LTP, but in the absence of withdrawal-induced corticosterone are unable to maintain LTP and form long-term memory. A role for Arc in reconsolidation following memory retrieval has been proposed (79). Our data indicated that Arc plays a pivotal role in morphine-withdrawal memory retrieval and reconsolidation: rats with higher Arc expression significantly exhibited enhanced aversion. Arc identifies genomically activated neurons as a result of retrieval, so if associative memory retrieval is reduced and reconsolidation processes are not invoked, then Arc expression might be selectively diminished, as occurs in ADX+morphine animals.

Increased GCs levels displayed consequent reduction of GR expression (34,35). Given that we detected a selective negative correlation between GR and Arc in sham+morphine rats, it is tempting to speculate that GCs levels during memory retrieval enhance Arc expression, as described by

McReynolds (47). Moreover, correlation analysis in all the experimental groups supports that pCREB regulates Arc expression (80). We observed that Arc was found in glutamatergic neurons of the GZ, which extends previous evidence (81). Moreover, expression of Arc in GAD65/67-positive neurons suggests that CPA retrieval and subsequent reconsolidation represented a strong-learning stimulus (81). On the other hand, associative learning is dependent on D1 signaling (82), which indicates that Arc induction occurs in the same neurons that become activated through D1-dependent learning.

In concordance with previous data (72), we demonstrate that reconsolidation does not recruit BDNF. It could be surprising that, while BDNF enhances LTP in the DG (83,84,85) and triggers protein synthesis-dependent LTP (86,87), we observed reduced BDNF levels in sham+morphine group after the CPA test. However, literature describes that chronic morphine treatment represses BDNF (88,89,90). On the other hand, opiates decrease the number/complexity of dendritic spines on hippocampus neurons (91,92,93). Given the role of BDNF in dendrite development (94,93), reduced BDNF levels could account for this effect. Similarly, intermittent stress decreases dendritic branching (95), raising the possibility that the lack of increased GCs and enhanced BDNF in ADX-dependent rats could revert this consequence. In addition, chronic opiate exposure negatively impacts subgranular zone (SGZ) neurogenesis (96,97,98,99), in direct opposition with BDNF (100). Thus, cognitive defects are described in animals chronically exposed to opiates (101,102,103), suggesting that reduced neurotrophic factors may mediate the morphine-induced cognitive deficit (101). However, our data indicate that sham+morphine rats can perform associative learning and recall long-term memory. It has been suggested that BDNF effects on memory are downstream or independent of Arc (14). We did not observe relationship between BDNF and Arc levels in morphine-dependent animals and immunofluorescence study revealed no colocalization between Arc and pTrkB.

In summary these experiments represent the first demonstration that Egr-1 and Arc expression in the DG are associated with morphine-withdrawal memory retrieval and suggest that these genes may contribute to plasticity and reconsolidation accompanying the retrieval process. Besides, GCs play an

essential role during the formation of long-term withdrawal-aversive memories, given that ADX-dependent animals exhibit reduced place aversion and decreased Egr-1 and Arc levels.

ACKNOWLEDGMENTS

This work was supported by grants from Ministerio de Ciencia e Innovación (SAF/FEDER 2009-07178; SAF/FEDER 2010-17907; 2013-49076-P), Spain; Red de Trastornos Adictivos, Spain; Fundación Séneca (15405/PI/10) and Instituto Murciano de Investigación en Biomedicina (IMIB), Región de Murcia, Spain. In Hungary, the work was supported by the National Research, Development and Innovation Fund 109622 to KJK and 109744 to SF. Daniel García-Pérez was supported by a fellowship from Ministerio de Ciencia e Innovación (AP2009-2379).

FINANCIAL DISCLOSURES

Mr García-Pérez reported no biomedical financial interests or potential conflicts of interest. Dr. Ferenczi reported no biomedical financial interests or potential conflicts of interest. Dr. Kovács reported no biomedical financial interests or potential conflicts of interest. Dr. Laorden reported no biomedical financial interests or potential conflicts of interest. Dr. Milanés reported no biomedical financial interests or potential conflicts of interest. Dr. Núñez reported no biomedical financial interests or potential conflicts of interest.

REFERENCES

1. Robbins TW, Everitt BJ (2002): Limbic-Striatal Memory Systems and Drug Addiction. *Neurobiology of Learning and Memory* 78:625-636.
2. Shaham Y, Shalev U, Lu L, de Wit H, Stewart J (2003): The reinstatement model of drug relapse: history, methodology and major findings. *Psychopharmacology* 168:3-20.
3. O'Brien CP, Testa T, O'Brien TJ, Brady JP, Wells B (1977): Conditioned narcotic withdrawal in humans. *Science* 195:1000-1002.
4. Everitt BJ (2014): Neural and psychological mechanisms underlying compulsive drug seeking habits and drug memories -- indications for novel treatments of addiction. *Eur J Neurosci* 40:2163-2182.
5. Koob GF, Le Moal M (2005): Plasticity of reward neurocircuitry and the 'dark side' of drug addiction. *Nat Neurosci* 8:1442-1444.
6. Myers KM, Bechtholt-Gompf AJ, Coleman BR, Carlezon WA (2012): Extinction of conditioned opiate withdrawal in rats in a two-chambered place conditioning apparatus. *Nat Protocols* 7:517-526.
7. Stinus L, Caille S, Koob GF (2000): Opiate withdrawal-induced place aversion lasts for up to 16 weeks. *Psychopharmacology* 149:115-120.
8. Frenois F, Stinus L, Di Blasi F, Cador M, Le Moine C (2005): A Specific Limbic Circuit Underlies Opiate Withdrawal Memories. *The Journal of Neuroscience* 25:1366-1374.
9. Lucas M, Frenois F, Vouillac C, Stinus L, Cador M, Le Moine C (2008): Reactivity and plasticity in the amygdala nuclei during opiate withdrawal conditioning: differential expression of c-fos and arc immediate early genes. *Neuroscience* 154:1021-1033.
10. Roozendaal B, McGaugh JL (2011): Memory modulation. *Behav Neurosci* 125:797-824.
11. Aimone JB, Deng W, Gage FH (2011): Resolving new memories: a critical look at the dentate gyrus, adult neurogenesis, and pattern separation. *Neuron* 70:589-596.
12. Kee N, Teixeira CM, Wang AH, Frankland PW (2007): Preferential incorporation of adult-generated granule cells into spatial memory networks in the dentate gyrus. *Nat Neurosci* 10:355-362.
13. Roozendaal B (2000): 1999 Curt P. Richter award. Glucocorticoids and the regulation of memory consolidation. *Psychoneuroendocrinology* 25:213-238.
14. Chen DY, Bambah-Mukku D, Pollonini G, Alberini CM (2012): Glucocorticoid receptors recruit the CaMKII[alpha]-BDNF-CREB pathways to mediate memory consolidation. *Nat Neurosci* 15:1707-1714.

15. McGaugh JL (2000): Memory--a Century of Consolidation. *Science* 287:248-251.
16. Hou YY, Lu B, Li M, Liu Y, Chen J, Chi ZQ, Liu JG (2009): Involvement of Actin Rearrangements within the Amygdala and the Dorsal Hippocampus in Aversive Memories of Drug Withdrawal in Acute Morphine-Dependent Rats. *The Journal of Neuroscience* 29:12244-12254.
17. Hyman SE, Malenka RC, Nestler EJ (2006): Neural Mechanisms of Addiction: The Role of Reward-Related Learning and Memory. *Annu Rev Neurosci* 29:565-598.
18. Korb E, Finkbeiner S (2011): Arc in synaptic plasticity: from gene to behavior. *Trends in Neurosciences* 34:591-598.
19. García-Pérez D, Laorden ML, Milanés MV, Nuñez C (2012): Glucocorticoids Regulation of FosB/ Δ FosB Expression Induced by Chronic Opiate Exposure in the Brain Stress System. *PLoS ONE* 7:e50264-
20. Paxinos G, Watson C (2007): The rat brain in stereotaxic coordinates 6th
21. Polyák Á, Ferenczi S, Dénes A, Winkler Z, Kriszt R, Pintér-Kübler B, Kovács KJ (2014): The fractalkine/Cx3CR1 system is implicated in the development of metabolic visceral adipose tissue inflammation in obesity. *Brain, Behavior, and Immunity* 38:25-35.
22. García-Pérez D, López-Bellido R, Hidalgo JM, Rodríguez RE, Laorden ML, Núñez C, Milanés MV (2015): Morphine regulates Argonaute 2 and TH expression and activity but not miR-133b in midbrain dopaminergic neurons. *Addiction Biology* 20:104-119.
23. Roozendaal B, McEwen BS, Chattarji S (2009): Stress, memory and the amygdala. *Nat Rev Neurosci* 10:423-433.
24. McGaugh JL, Roozendaal B (2002): Role of adrenal stress hormones in forming lasting memories in the brain. *Current Opinion in Neurobiology* 12:205-210.
25. Núñez C, Földes A, Pérez-Flores D, García-Borrón JC, Laorden ML, Kovács KJ, Milanés MV (2009): Elevated glucocorticoid levels are responsible for induction of tyrosine hydroxylase (TH) mRNA expression, phosphorylation and enzyme activity in the nucleus of the solitary tract (NTS-A2) during morphine withdrawal. *Endocrinology* 150:3118-3127.
26. Shaham Y (1993): Immobilization stress-induced oral opioid self-administration and withdrawal in rats: role of conditioning factors and the effect of stress on "relapse" to opioid drugs. *Psychopharmacology (Berl)* 111:477-485.
27. Shaham Y, Rajabi H, Stewart J (1996): Relapse to heroin-seeking in rats under opioid maintenance: the effects of stress, heroin priming, and withdrawal. *J Neurosci* 16:1957-1963.
28. Koob G, Kreek MJ (2007): Stress, Dysregulation of Drug Reward Pathways, and the Transition to Drug Dependence. *Am J Psychiatry* 164:1149-1159.

29. Houshyar H, Cooper ZD, Woods JH (2001): Paradoxical Effects of Chronic Morphine Treatment on the Temperature and Pituitary-Adrenal Responses to Acute Restraint Stress: A Chronic Stress Paradigm. *Journal of Neuroendocrinology* 13:862-874.
30. Navarro-Zaragoza J, Hidalgo JM, Laorden ML, Milanés MV (2012): Glucocorticoid receptors participate in the opiate withdrawal-induced stimulation of rats NTS noradrenergic activity and in the somatic signs of morphine withdrawal. *Br J Pharmacol* 166:2136-2147.
31. Sapolsky RM, Krey LC, McEwen BS (1984): Stress down-regulates corticosterone receptors in a site-specific manner in the brain. *Endocrinology* 114:287-292.
32. Sapolsky RM, Krey LC, McEwen BS (1985): Down-regulation of neural corticosterone receptors by corticosterone and dexamethasone. *Brain Res* 335:161-165.
33. McNally GP, Akil H (2003): Selective down-regulation of hippocampal glucocorticoid receptors during opiate withdrawal. *Molecular Brain Research* 118:152-155.
34. Numakawa T (2009): Glucocorticoid receptor interaction with TrkB promotes BDNF-triggered PLC-gamma signaling for glutamate release via a glutamate transporter. *Proc Natl Acad Sci USA* 106:647-652.
35. Chiba S, Numakawa T, Ninomiya M, Richards MC, Wakabayashi C, Kunugi H (2012): Chronic restraint stress causes anxiety- and depression-like behaviors, downregulates glucocorticoid receptor expression, and attenuates glutamate release induced by brain-derived neurotrophic factor in the prefrontal cortex. *Progress in Neuro-Psychopharmacology and Biological Psychiatry* 39:112-119.
36. Deroche Vr, Piazza PV, Moal ML, Simon H (1994): Social isolation-induced enhancement of the psychomotor effects of morphine depends on corticosterone secretion. *Brain Res* 640:136-139.
37. Deroche V, Marinelli M, Maccari S, Le Moal M, Simon H, Piazza PV (1995): Stress-induced sensitization and glucocorticoids. I. Sensitization of dopamine-dependent locomotor effects of amphetamine and morphine depends on stress-induced corticosterone secretion. *The Journal of Neuroscience* 15:7181-7188.
38. Kauer JA, Malenka RC (2007): Synaptic plasticity and addiction. *Nat Rev Neurosci* 8:844-858.
39. Bliss TVP, Collingridge GL (1993): A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* 361:31-39.
40. Pu L, Bao GB, Xu NJ, Ma L, Pei G (2002): Hippocampal Long-Term Potentiation Is Reduced by Chronic Opiate Treatment and Can Be Restored by Re-Exposure to Opiates. *The Journal of Neuroscience* 22:1914-1921.
41. Salmanzadeh F, Fathollahi Y, Semnani S, Shafizadeh M (2003): Dependence on morphine impairs the induction of long-term potentiation in the CA1 region of rat hippocampal slices. *Brain Res* 965:108-113.

42. Bao G, Kang L, Li H, Li Y, Pu L, Xia P et al (2007): Morphine and Heroin Differentially Modulate In Vivo Hippocampal LTP in Opiate-Dependent Rat. *Neuropsychopharmacology* 32:1738-1749.
43. Mansouri FA, Motamedi F, Fathollahi Y, Atapour N, Semnani S (1997): Augmentation of LTP induced by Primed-Bursts tetanic stimulation in hippocampal CA1 area of morphine dependent rats. *Brain Res* 769:119-124.
44. Mansouri FA, Motamedi F, Fathollahi Y (1999): Chronic in vivo morphine administration facilitates primed-bursts-induced long-term potentiation of Schaffer collateral-CA1 synapses in hippocampal slices in vitro. *Brain Res* 815:419-423.
45. Ito Y, Tabata K, Makimura M, Fukuda H (2001): Acute and chronic intracerebroventricular morphine infusions affect long-term potentiation differently in the lateral perforant path. *Pharmacology Biochemistry and Behavior* 70:353-358.
46. Lu W, Dong HJ, Bi GH, Zhao YQ, Yang Z, Su RB, Li J (2010): Effect of agmatine on long-term potentiation in morphine-treated rats. *Pharmacology Biochemistry and Behavior* 96:125-129.
47. McReynolds JR (2010): Memory-enhancing corticosterone treatment increases amygdala norepinephrine and Arc protein expression in hippocampal synaptic fractions. *Neurobiol Learn Mem* 93:312-321.
48. Molteni R (2010): Depression-prone mice with reduced glucocorticoid receptor expression display an altered stress-dependent regulation of brain-derived neurotrophic factor and activity-regulated cytoskeleton-associated protein. *J Psychopharmacol* 24:595-603.
49. Huff NC, Frank M, Wright-Hardesty K, Sprunger D, Matus-Amat P, Higgins E, Rudy JW (2006): Amygdala Regulation of Immediate-Early Gene Expression in the Hippocampus Induced by Contextual Fear Conditioning. *The Journal of Neuroscience* 26:1616-1623.
50. McIntyre CK, Miyashita T, Setlow B, Marjon KD, Steward O, Guzowski JF, McGaugh JL (2005): Memory-influencing intra-basolateral amygdala drug infusions modulate expression of Arc protein in the hippocampus. *Proc Natl Acad Sci U S A* 102:10718-10723.
51. Antoine B, Serge L, Jocelyne C (2014): Comparative dynamics of MAPK/ERK signalling components and immediate early genes in the hippocampus and amygdala following contextual fear conditioning and retrieval. *Brain Struct Funct* 219:415-430.
52. Guzowski JF, Lyford GL, Stevenson GD, Houston FP, McGaugh JL, Worley PF, Barnes CA (2000): Inhibition of Activity-Dependent Arc Protein Expression in the Rat Hippocampus Impairs the Maintenance of Long-Term Potentiation and the Consolidation of Long-Term Memory. *The Journal of Neuroscience* 20:3993-4001.
53. Jones MW, Errington ML, French PJ, Fine A, Bliss TVP, Garel S, et al (2001): A requirement for the immediate early gene Zif268 in the expression of late LTP and long-term memories. *Nat Neurosci* 4:289-296.

54. Impey S (1998): Stimulation of cAMP response element (CRE)-mediated transcription during contextual learning. *Nat Neurosci* 1:595-601.
55. Taubenfeld SM, Wiig KA, Bear MF, Alberini CM (1999): A molecular correlate of memory and amnesia in the hippocampus. *Nat Neurosci* 2:309-310.
56. Schratt G (2009): Fine-tuning neural gene expression with microRNAs. *Current Opinion in Neurobiology* 19:213-219.
57. Hu Z, Shen WJ, Cortez Y, Tang X, Liu LF, Kraemer FB, Azhar S (2013): Hormonal Regulation of MicroRNA Expression in Steroid Producing Cells of the Ovary, Testis and Adrenal Gland. *PLoS ONE* 8:e78040-
58. Thomas SA (2015): Neuromodulatory signaling in hippocampus-dependent memory retrieval. *Hippocampus* 25:415-431.
59. Cammarota M, Bevilaqua LR, Barros DM, Vianna MR, Izquierdo LA, Medina JH, Izquierdo I (2005): Retrieval and the extinction of memory. *Cell Mol Neurobiol* 25:465-474.
60. Murchison CF, Schutsky K, Jin SH, Thomas SA (2011): Norepinephrine and b1-adrenergic signaling facilitate activation of hippocampal CA1 pyramidal neurons during contextual memory retrieval. *Neuroscience* 181C:109-116.
61. Nader K, Schafe GE, Le Doux JE (2000): Fear memories require protein synthesis in the amygdala for reconsolidation after retrieval. *Nature* 406:722-726.
62. Miller RR, Matzel LD (2000): Memory involves far more than 'consolidation'. *Nat Rev Neurosci* 1:214-216.
63. Kim J, Jeong D, Nam J, Aung TN, Gim JA, Park KU, Kim SW (2015): MicroRNA-124 regulates glucocorticoid sensitivity by targeting phosphodiesterase 4B in diffuse large B cell lymphoma. *Gene* 558:173-180.
64. Ledderose C, Möhnle P, Limbeck E, Schütz S, Weis F, Rink J et al (2012): Corticosteroid resistance in sepsis is influenced by microRNA-124--induced downregulation of glucocorticoid receptor- α . *Critical Care Medicine* 40:2745-2753.
65. Rajasethupathy P, Fiumara F, Sheridan R, Betel D, Puthanveetil SV, Russo JJ et al (2009): Characterization of small RNAs in *Aplysia* reveals a role for miR-124 in constraining synaptic plasticity through CREB. *Neuron* 63:803-817.
66. Chandrasekar V, Dreyer JL (2011): Regulation of MiR-124, Let-7d, and MiR-181a in the Accumbens Affects the Expression, Extinction, and Reinstatement of Cocaine-Induced Conditioned Place Preference. *Neuropsychopharmacology* 36:1149-1164.
67. Hollander JA, Im HI, Amelio AL, Kocerha J, Bali P, Lu Q et al (2010): Striatal microRNA controls cocaine intake through CREB signaling. *Nature* 466:197-202.

68. Im HI, Hollander JA, Bali P, Kenny PJ (2010): MeCP2 controls BDNF expression and cocaine intake through homeostatic interactions with microRNA-212. *Nat Neurosci* 13:1120-1127.
69. Vasudevan S, Tong Y, Steitz JA (2007): Switching from Repression to Activation: MicroRNAs Can Up-Regulate Translation. *Science* 318:1931-1934.
70. Hall J, Thomas KL, Everitt BJ (2001): Fear memory retrieval induces CREB phosphorylation and Fos expression within the amygdala. *Eur J Neurosci* 13:1453-1458.
71. Hall J, Thomas KL, Everitt BJ (2001): Cellular Imaging of zif268 Expression in the Hippocampus and Amygdala during Contextual and Cued Fear Memory Retrieval: Selective Activation of Hippocampal CA1 Neurons during the Recall of Contextual Memories. *The Journal of Neuroscience* 21:2186-2193.
72. Lee JL, Everitt BJ, Thomas KL (2004): Independent cellular processes for hippocampal memory consolidation and reconsolidation. *Science* 304:839-843.
73. Revest JM (2005): The MAPK pathway and Egr-1 mediate stress-related behavioral effects of glucocorticoids. *Nat Neurosci* 8:664-672.
74. Revest JM (2010): The enhancement of stress-related memory by glucocorticoids depends on synapsin-Ia/Ib. *Mol Psychiatry* 15:1140-1151.
75. Mamiya N, Fukushima H, Suzuki A, Matsuyama Z, Homma S, Frankland PW, Kida S (2009): Brain Region-Specific Gene Expression Activation Required for Reconsolidation and Extinction of Contextual Fear Memory. *The Journal of Neuroscience* 29:402-413.
76. Plath N, Ohana O, Dammermann B, Errington ML, Schmitz D, Gross C et al (2006): Arc/Arg3.1 Is Essential for the Consolidation of Synaptic Plasticity and Memories. *Neuron* 52:437-444.
77. Messaoudi E, Kanhema T, Soulé J, Tiron A, Dagey G, da Silva B, Bramham CR (2007): Sustained Arc/Arg3.1 synthesis controls long-term potentiation consolidation through regulation of local actin polymerization in the dentate gyrus in vivo. *J Neurosci* 27:10445-10455.
78. Bramham CR, Alme MN, Bittins M, Kuipers SD, Nair RR, Pai B et al (2010): The Arc of synaptic memory. *Exp Brain Res* 200:125-140.
79. Zhang WP, Guzowski JF, Thomas SA (2005): Mapping neuronal activation and the influence of adrenergic signaling during contextual memory retrieval. *Learn Mem* 12:239-247.
80. Kawashima T, Okuno H, Nonaka M, Adachi-Morishima A, Kyo N, Okamura M et al (2009): Synaptic activity-responsive element in the Arc/Arg3.1 promoter essential for synapse-to-nucleus signaling in activated neurons. *Proc Natl Acad Sci U S A* 106:316-321.

81. Vazdarjanova A (2006): Spatial exploration induces ARC, a plasticity-related immediate-early gene, only in calcium/calmodulin-dependent protein kinase II-positive principal excitatory and inhibitory neurons of the rat forebrain. *J Comp Neurol* 498:317-329.
82. Ortiz O, Delgado-García JM, Espadas I, Bahí A, Trullas R, Dreyer JL et al (2010): Associative Learning and CA3-CA1 Synaptic Plasticity Are Impaired in D1R Null, *Drd1a*^{-/-} Mice and in Hippocampal siRNA Silenced *Drd1a* Mice. *The Journal of Neuroscience* 30:12288-12300.
83. Korte M, Carroll P, Wolf E, Brem G, Thoenen H, Bonhoeffer T (1995): Hippocampal long-term potentiation is impaired in mice lacking brain-derived neurotrophic factor. *Proc Natl Acad Sci U S A* 92:8856-8860.
84. Korte M, Kang H, Bonhoeffer T, Schuman E (1998): A role for BDNF in the late-phase of hippocampal long-term potentiation. *Neuropharmacology* 37:553-559.
85. Ying SW, Futter M, Rosenblum K, Webber MJ, Hunt SP, Bliss TVP, Bramham CR (2002): Brain-Derived Neurotrophic Factor Induces Long-Term Potentiation in Intact Adult Hippocampus: Requirement for ERK Activation Coupled to CREB and Upregulation of Arc Synthesis. *The Journal of Neuroscience* 22:1532-1540.
86. Bramham CR, Messaoudi E (2005): BDNF function in adult synaptic plasticity: The synaptic consolidation hypothesis. *Progress in Neurobiology* 76:99-125.
87. Lynch G, Rex CS, Gall CM (2007): LTP consolidation: Substrates, explanatory power, and functional significance. *Neuropharmacology* 52:12-23.
88. Chu NN, Zuo YF, Meng L, Lee DY, Han JS, Cui CL (2007): Peripheral electrical stimulation reversed the cell size reduction and increased BDNF level in the ventral tegmental area in chronic morphine treated rats. *Brain Res* 1182:90-98.
89. Koo JW, Mazei-Robison MS, Chaudhury D, Juarez B, LaPlant Q, Ferguson D et al (2012): BDNF is a Negative Modulator of Morphine Action. *Science* 338:124-128.
90. Koo JW, Mazei-Robison MS, LaPlant Q, Egervari G, Braunscheidel KM, Adank DN et al (2015): Epigenetic basis of opiate suppression of *Bdnf* gene expression in the ventral tegmental area. *Nat Neurosci* 18:415-422.
91. Robinson TE, Gorny G, Savage VR, Kolb B (2002): Widespread but regionally specific effects of experimenter- versus self-administered morphine on dendritic spines in the nucleus accumbens, hippocampus, and neocortex of adult rats. *Synapse* 46:271-279.
92. Robinson TE, Kolb B (2004): Structural plasticity associated with exposure to drugs of abuse. *Neuropharmacology* 47, Supplement 1:33-46.
93. Russo SJ, Mazei-Robison MS, Ables JL, Nestler EJ (2009): Neurotrophic factors and structural plasticity in addiction. *Neuropharmacology* 56, Supplement 1:73-82.

94. Wang L, Chang X, She L, Xu D, Huang W, Poo Mm (2015): Autocrine Action of BDNF on Dendrite Development of Adult-Born Hippocampal Neurons. *The Journal of Neuroscience* 35:8384-8393.
95. McEwen BS (1999): Stress and hippocampal plasticity. *Annu Rev Neurosci* 22:105-122.
96. Arguello AA, Harburg GC, Schonborn JR, Mandyam CD, Yamaguchi M, Eisch AJ (2008): Time course of morphine's effects on adult hippocampal subgranular zone reveals preferential inhibition of cells in S phase of the cell cycle and a subpopulation of immature neurons. *Neuroscience* 157:70-79.
97. Eisch AJ, Barrot M, Schad CA, Self DW, Nestler EJ (2000): Opiates inhibit neurogenesis in the adult rat hippocampus. *Proc Natl Acad Sci U S A* 97:7579-7584.
98. Kahn L, Alonso G, Normand E, Manzoni OJ (2005): Repeated morphine treatment alters polysialylated neural cell adhesion molecule, glutamate decarboxylase-67 expression and cell proliferation in the adult rat hippocampus. *Eur J Neurosci* 21:493-500.
99. Mandyam CD, Norris RD, Eisch AJ (2004): Chronic morphine induces premature mitosis of proliferating cells in the adult mouse subgranular zone. *J Neurosci Res* 76:783-794.
100. Scharfman H, Goodman J, Macleod A, Phani S, Antonelli C, Croll S (2005): Increased neurogenesis and the ectopic granule cells after intrahippocampal BDNF infusion in adult rats. *Experimental Neurology* 192:348-356.
101. Spain JW, Newsom GC (1991): Chronic opioids impair acquisition of both radial maze and Y-maze choice escape. *Psychopharmacology (Berl)* 105:101-106.
102. Miladi Gorji H, Rashidy-Pour A, Fathollahi Y (2008): Effects of morphine dependence on the performance of rats in reference and working versions of the water maze. *Physiology & Behavior* 93:622-627.
103. Lu G, Zhou QX, Kang S, Li QL, Zhao LC, Chen JD et al (2010): Chronic Morphine Treatment Impaired Hippocampal Long-Term Potentiation and Spatial Memory via Accumulation of Extracellular Adenosine Acting on Adenosine A1 Receptors. *The Journal of Neuroscience* 30:5058-5070.

FIGURE LEGENDS

Figure 1. Effects of adrenalectomy on the naloxone-induced conditioned place aversion in morphine dependent rats. (A) Schematic overview of the animals' treatment and behavioral experiments time course. (B) Time spent in the dot chamber during pretest phase. (C) Time spent in the stripes chamber during pretest phase. (D) Time spent in the naloxone-paired chamber during pretest phase. (E) Difference between the time spent in the naloxone-paired compartment after conditioning sessions minus the time spent in the same compartment before conditioning sessions. Morphine treatment: $F_{(1,48)} = 10.05$, $P = 0.0027$; Interaction adrenalectomy X morphine treatment: $F_{(1,48)} = 10.44$, $P = 0.0022$. Newman-Keuls' post hoc: *** $P < 0.001$ vs sham+pla; ++ $P < 0.01$ vs sham+mor. (F) Number of entries to the dots chamber during pretest phase. (G) Number of entries to the stripes chamber during pretest phase. (H) Number of entries to the naloxone-paired chamber during pretest phase. (I) Percentage of total entries to the dots and stripes chambers during pretest phase. (J) Difference between the number of total entries after conditioning sessions minus the number of total entries before conditioning sessions. Morphine treatment: $F_{(1,48)} = 13.72$, $P = 0.0005$. Newman-Keuls' post hoc: * $P < 0.05$ vs sham+pla; # $P < 0.05$ vs ADX+pla. (K) Difference between the number of entries to the saline-paired chamber after conditioning sessions minus the number of entries to the same chamber before conditioning sessions. Morphine treatment: $F_{(1,48)} = 11.11$, $P = 0.0017$. Newman-Keuls' post hoc: ** $P < 0.01$ vs sham+pla. (L) Difference between the number of entries to the naloxone-paired chamber after conditioning sessions minus the number of entries to the same chamber before conditioning sessions. Morphine treatment: $F_{(1,48)} = 11.68$, $P = 0.0013$. Newman-Keuls' post hoc: ## $P < 0.01$ vs ADX+pla. (M) Correlation between the score and the number of entries to the naloxone-paired chamber. The number of entries to the opiate antagonist-paired chamber was positively correlated with the decrease of the score in the sham+mor group.

Figure 2. Effects of adrenalectomy on metabolic parameters and on HPA-axis activity in morphine dependent rats. (A) Body weight gain of rats 4 d after ADX or sham surgery. $t_{50} = 12.57$, $P < 0.0001$.

(B) Body weight gain of rats from the day of pellets implantation to the first day of conditioning sessions. Adrenalectomy: $F_{(1,48)} = 6.802$, $P = 0.0121$; Morphine treatment: $F_{(1,48)} = 62.98$, $P < 0.0001$; Interaction adrenalectomy X morphine treatment: $F_{(1,48)} = 11.52$, $P = 0.0014$. Newman-Keuls' post hoc: *** $P < 0.001$ vs sham+pla; ## $P < 0.01$ vs ADX+pla. (C) Body weight gain of rats during the days of conditioning sessions. **Day 8:** Morphine treatment: $F_{(1,48)} = 32.60$, $P < 0.0001$; Interaction adrenalectomy X morphine treatment: $F_{(1,48)} = 10.91$, $P = 0.0018$. Newman-Keuls' post hoc: ++ $P < 0.01$ vs sham+mor; ### $P < 0.001$ vs ADX+pla. **Day 9:** Morphine treatment: $F_{(1,48)} = 41.99$, $P < 0.0001$. Newman-Keuls' post hoc: ** $P < 0.01$ vs sham+pla; ### $P < 0.001$ vs ADX+pla. **Day 10** Morphine treatment: $F_{(1,48)} = 56.47$, $P < 0.0001$. Newman-Keuls' post hoc: *** $P < 0.001$ vs sham+pla; ### $P < 0.001$ vs ADX+pla. **Day 11:** Morphine treatment: $F_{(1,48)} = 60.27$, $P < 0.0001$. Newman-Keuls' post hoc: *** $P < 0.001$ vs sham+pla; ### $P < 0.001$ vs ADX+pla. **Sham+pla:** Day: $F_{(4,55)} = 22.21$, $P < 0.0001$. Newman-Keuls' post hoc: &&& $P < 0.001$ vs day 7; \$ $P < 0.05$, \$\$\$ $P < 0.001$ vs day 8; @ $P < 0.05$, @@@ $P < 0.001$ vs day 9. **Sham+mor:** ns. **ADX+pla:** Day: $F_{(4,55)} = 12.70$, $P < 0.0001$. Newman-Keuls' post hoc: & $P < 0.01$, &&& $P < 0.001$ vs day 7; \$\$ $P < 0.01$ vs day 8; @ $P < 0.05$ vs day 9. **ADX+mor:** ns. (D) Body weight loss during conditioning sessions: **Day 7:** Morphine treatment: $F_{(1,48)} = 4.572$, $P = 0.0376$; **Day 8:** Morphine treatment: $F_{(1,48)} = 77.30$, $P < 0.0001$. Newman-Keuls' post hoc: *** $P < 0.001$ vs sham+pla; ### $P < 0.001$ vs ADX+pla; **Day 9:** ns; **Day 10:** Adrenalectomy: $F_{(1,48)} = 5.406$, $P = 0.0243$; Morphine treatment: $F_{(1,48)} = 131.8$, $P < 0.0001$. Newman-Keuls' post hoc: *** $P < 0.001$ vs sham+pla; + $P < 0.05$ vs sham+mor; ### $P < 0.001$ vs ADX+pla. **Sham+pla:** ns; **Sham+mor:** Naloxone injection: $F_{(3,60)} = 43.30$, $P < 0.0001$. Newman-Keuls' post hoc: &&& $P < 0.001$ vs day 7 (saline); @@@ $P < 0.001$ vs day 9 (saline). **ADX+pla:** ns; **ADX+mor:** Naloxone injection: $F_{(3,44)} = 35.75$; $P < 0.0001$. Newman-Keuls' post hoc: &&& $P < 0.001$ vs day 7 (saline); @@@ $P < 0.001$ vs day 9 (saline). (E) Graded signs of opiate withdrawal in sham- and ADX-operated animals treated with morphine during 15 min after the conditioning sessions with naloxone. ++ $P < 0.01$ vs sham+mor (day 10); \$ $P < 0.05$ vs ADX+mor (day 8). (F) Appearance of signs of morphine withdrawal in sham- and ADX-operated animals treated with morphine during 15 min after the conditioning sessions with naloxone. ++ $P < 0.01$ vs sham+mor (day 10). (G) Global opiate withdrawal score in sham- and ADX-operated animals

treated with morphine during the conditioning sessions with naloxone. Day of conditioning session: $F_{(1,26)} = 5.387$, $P = 0.0284$. Interaction adrenalectomy X day of conditioning session: $F_{(1,26)} = 7.487$, $P = 0.0111$. Newman-Keuls' post hoc: $^{++}P < 0.01$ vs sham+mor (day 10); $^SP < 0.05$ vs ADX+mor (day 8). (H) Corticosterone plasma levels 15 min after leaving the naloxone-paired compartment on day 10. Adrenalectomy: $F_{(1,21)} = 224.3$, $P < 0.0001$. Morphine treatment: $F_{(1,21)} = 244.2$, $P < 0.0001$; Interaction adrenalectomy X morphine treatment: $F_{(1,21)} = 236.9$, $P < 0.0001$. Newman-Keuls' post hoc: $^{***}P < 0.001$ vs sham+pla; $^{+++}P < 0.001$ vs sham+mor. (I) ACTH plasma concentration 1 hour after starting the testing phase. Adrenalectomy: $F_{(1,32)} = 219.2$, $P < 0.0001$. Newman-Keuls' post hoc: $^{***}P < 0.001$ vs sham+pla; $^{+++}P < 0.001$ vs sham+mor. (J) Corticosterone plasma concentration 1 hour after starting the testing phase.

Figure 3. miR, mRNA and protein expression during memory consolidation process. (A) miR-124a: ns. (B) miR-212: Adrenalectomy: $F_{(1,14)} = 17.06$, $P = 0.0010$. Newman-Keuls' post hoc: $^*P < 0.05$ vs sham+pla; $^+P < 0.05$ vs sham+mor. (C) GR protein: ns. (D) pCREB protein: ns. (E) Egr-1 mRNA: ns. (F) BDNF mRNA: ns. (G) Arc mRNA: Morphine treatment: $F_{(1,15)} = 17.53$, $P = 0.0008$. Newman-Keuls' post hoc: $^*P < 0.05$ vs sham+pla; $^{\#}P < 0.05$ vs ADX+pla. (H) Egr-1 protein: Morphine treatment: $F_{(1,22)} = 5.257$, $P = 0.0318$; Interaction adrenalectomy X morphine treatment: $F_{(1,22)} = 5.155$, $P = 0.0333$. Newman-Keuls' post hoc: $^{++}P < 0.01$ vs sham+mor; $^{\#}P < 0.05$ vs ADX+pla. (I) BDNF protein: ns. (J) Arc protein: Morphine treatment: $F_{(1,19)} = 16.07$, $P = 0.0008$. Newman-Keuls' post hoc: $^*P < 0.05$ vs sham+pla; $^{\#}P < 0.05$ vs ADX+pla.

Figure 4. miR, mRNA and protein expression during memory retrieval process. (A) miR-124a: Adrenalectomy: $F_{(1,23)} = 6.621$, $P = 0.0170$. Newman-Keuls' post hoc: $^+P < 0.05$ vs sham+mor. (B) miR-212: Adrenalectomy: $F_{(1,25)} = 16.83$, $P = 0.0004$. Newman-Keuls' post hoc: $^*P < 0.05$ vs sham+pla; $^+P < 0.05$ vs sham+mor. (C) Egr-1 mRNA: Adrenalectomy: $F_{(1,23)} = 5.123$, $P = 0.0334$. Newman-Keuls' post hoc: $^+P < 0.05$ vs sham+mor. (D) BDNF mRNA: Interaction adrenalectomy X

morphine treatment: $F_{(1,24)} = 6.521$, $P = 0.0174$. (E) Arc mRNA: Adrenalectomy: $F_{(1,24)} = 6.878$, $P = 0.0149$; Interaction adrenalectomy X morphine treatment: $F_{(1,24)} = 4.740$, $P = 0.0395$. Newman-Keuls' post hoc: $**P < 0.01$ vs sham+pla; $^+P < 0.05$ vs sham+mor. (F-I) Correlation analysis between miR-124a and miR-212 and protein levels in sham+mor rats: significant negative correlations were observed between both miRs and GR protein levels; significant positive correlation was observed between miR-124a and Arc protein levels. (J) Correlation analysis between Egr-1, BDNF and Arc mRNAs in sham+mor animals: negative correlation between BDNF and Egr-1 or Arc mRNAs is observed. (K) GR protein: Adrenalectomy: $F_{(1,28)} = 7.502$, $P = 0.0106$. (L) pCREB protein: Interaction adrenalectomy X morphine treatment: $F_{(1,26)} = 4.373$, $P = 0.0464$. Newman-Keuls' post hoc: $*P < 0.05$ vs sham+pla. (M) Egr-1 protein: Adrenalectomy: $F_{(1,28)} = 11.94$, $P = 0.0018$. Newman-Keuls' post hoc: $^+P < 0.05$ vs sham+mor. (N) BDNF protein: Interaction adrenalectomy X morphine treatment: $F_{(1,29)} = 6.208$, $P = 0.0187$. Newman-Keuls' post hoc: $^+P < 0.05$ vs sham+mor. (O) Arc protein: Adrenalectomy: $F_{(1,28)} = 6.807$, $P = 0.0144$; Interaction adrenalectomy X morphine treatment: $F_{(1,28)} = 4.460$, $P = 0.0438$. Newman-Keuls' post hoc: $*P < 0.05$ vs sham+pla; $^{++}P < 0.01$ vs sham+mor. (P-U) Correlation analysis between aversion score and the different proteins analysed. Only significant correlation between Arc protein and aversion score in sham+mor rats was observed.

Figure 5. Characterization of dopaminoceptive, glutamatergic and GABAergic neurons in the DG from sham-operated animals treated with morphine after naloxone-induced CPA. (A) Representative confocal image showing DG coronal sections of rat immunostained for D1 in the polymorphic zone. (C-C'') D1 and D2 staining in the DG. While D1 expression was ubiquitous (molecular, granular, and polymorphic zone) (C), D2 signal was restricted to the granular zone (C'). (B) Schematic illustration showing the subdivisions of the DG (diagram modified from (20)). (D-E'') Representative confocal images showing DG rat coronal sections immunostained for GLS2 (green; D) and GAD (red; D'). Colocalization is shown by yellow neurons in the merged images (D''). High magnification images of GLS2 (E) and GAD (E') immunostaining, and merged (E'') in the DG. (F-F'') Representative

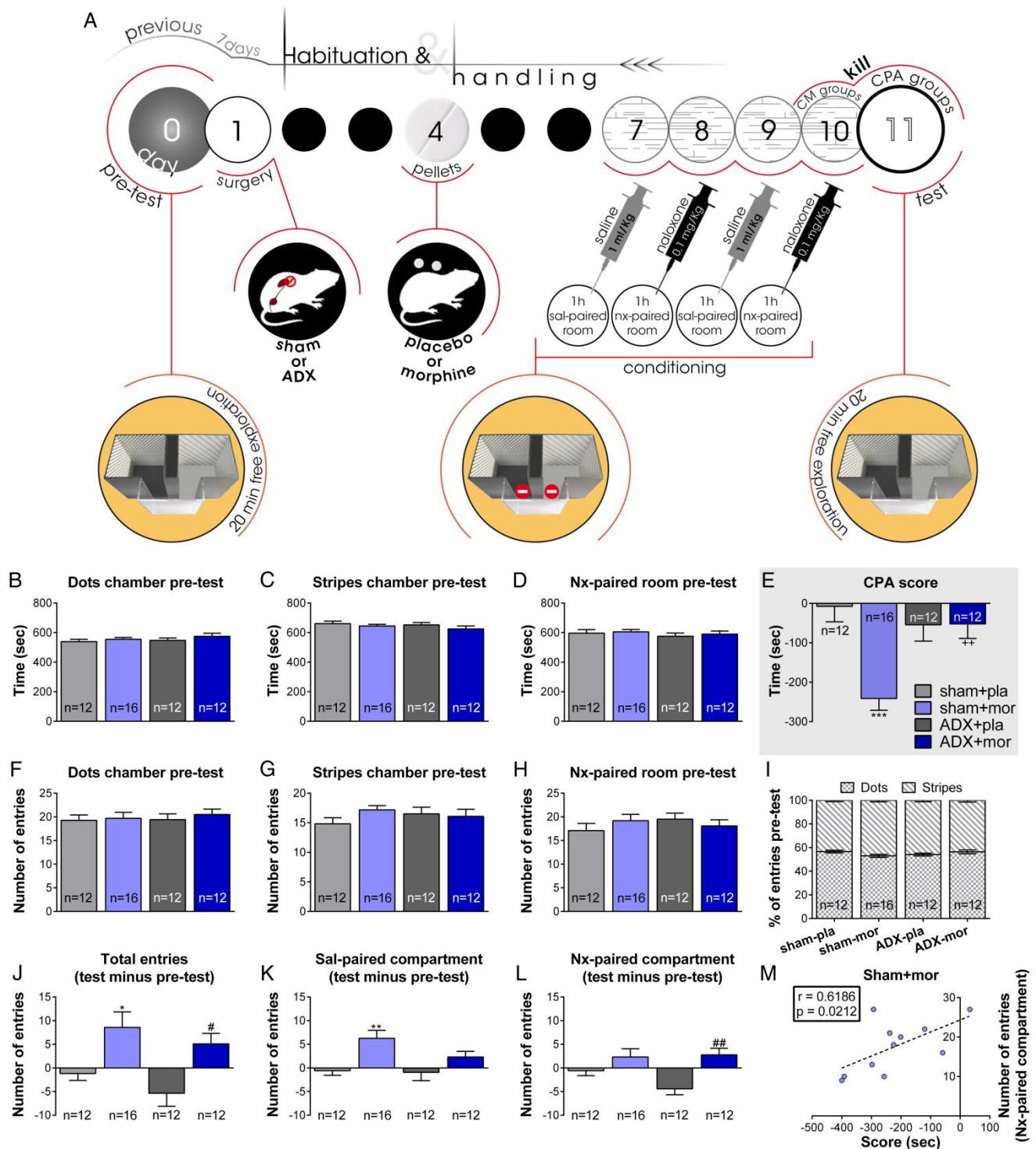
confocal images showing DG coronal sections of rat immunostained for D1 (green; F), GFAP (red; F'), merged (F'') and DAPI (nuclear stain, blue; F'''). (G-G''') Representative confocal images showing DG rat coronal sections immunostained for GLS2 (green; G), GFAP (red; G'), merged (G'') and DAPI (nuclear stain, blue; G'''). Scale bars, 100 μ m.

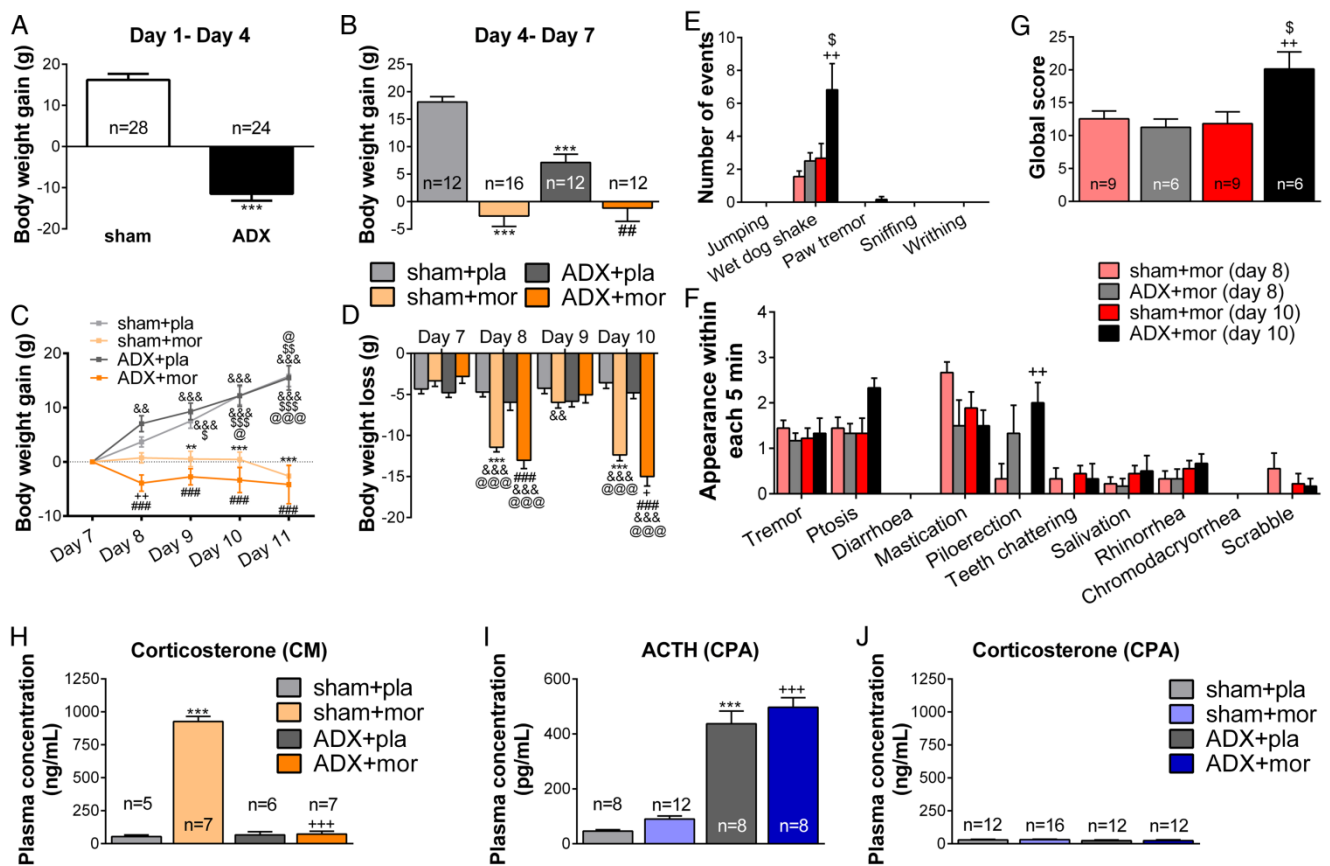
Figure 6. Effects of naloxone-induced CPA on GR colocalization with pCREB, Egr-1, BDNF or pTrkB in the DG of sham- and ADX-operated morphine-dependent rats. Representative confocal images of GR (red) and pCREB (green) (A–B'), GR (red) and Egr-1 (green) (C–D'), GR (red) and BDNF (green) (E–F') and GR (red) and pTrkB (green) (G–H') immunostaining in the DG. Merged images are shown in A'',B'' (GR/pCREB), C'',D'' (GR/Egr-1), E'',F'' (GR/BDNF), and G'',H'' (GR/pTrkB). (I–J) Correlation between GR and pCREB protein levels in the DG after naloxone-induced CPA in sham- (I) and ADX-operated (J) morphine-dependent animals. GR protein levels were positively correlated with pCREB protein levels in the sham+mor group. (K–L) Correlation between GR and Egr-1 protein levels in the DG after naloxone-induced CPA in sham- (K) and ADX-operated (L) morphine-dependent rats. GR protein levels were positively correlated with Egr-1 protein levels in the ADX+mor group. (M–N) Correlation between GR and BDNF protein levels in the DG after naloxone-induced CPA in sham- (M) and ADX-operated (N) morphine-dependent animals. GR protein levels were negatively correlated with BDNF protein levels in the ADX+mor group. Scale bars, 100 μ m.

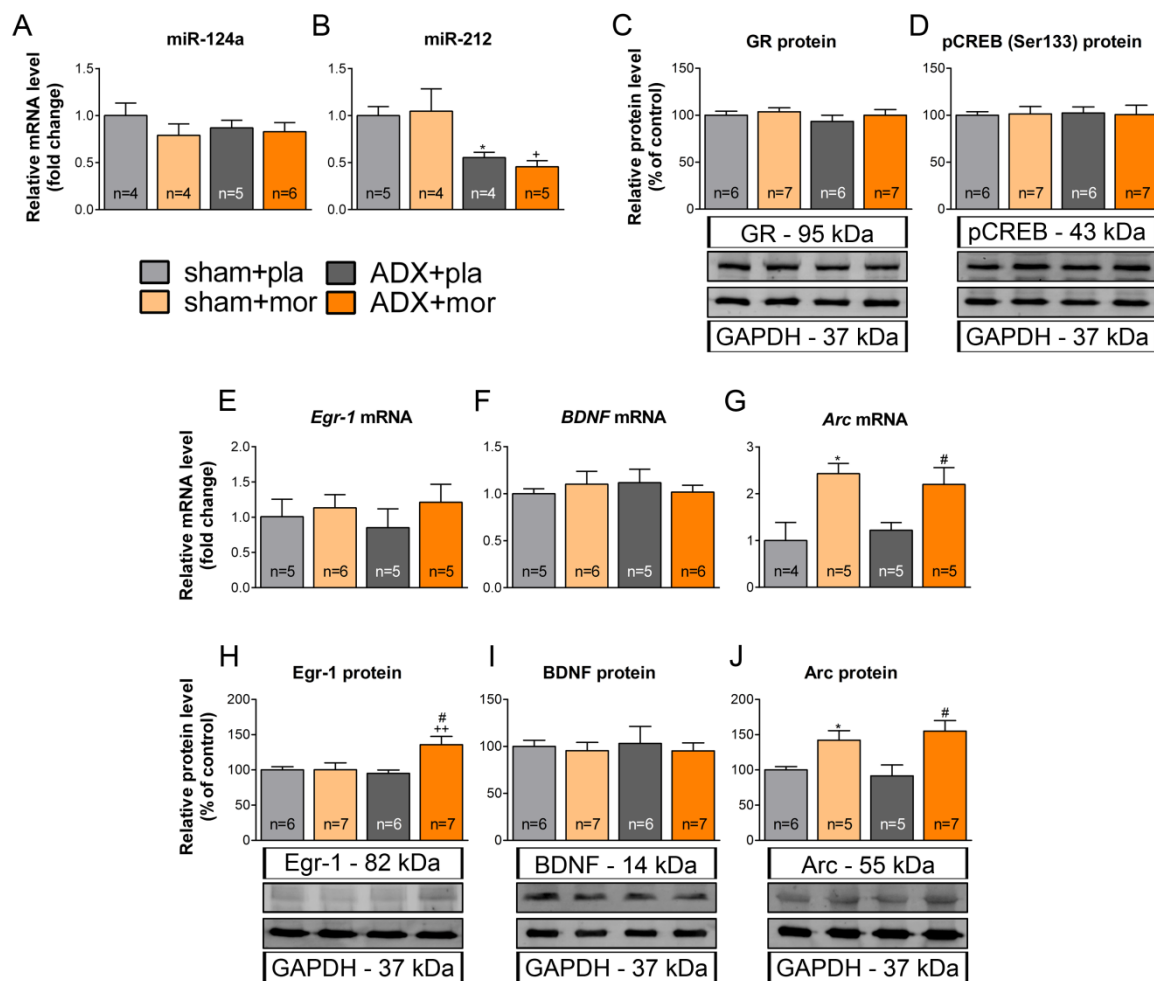
Figure 7. Arc colocalization with D1, GLS2, GAD or GR in the DG of morphine dependent rats during the expression of naloxone-induced CPA. Representative confocal images of Arc (red) and D1 (green) (A,A'), Arc (red) and GLS2 (green) (B,B') and Arc (red) and GAD (green) (C,C'); DAPI immunostaining (nuclear stain, blue; A''–C''). Merged images are shown in A''' (Arc/D1), B''' (Arc/GLS2), C''' (Arc/GAD); merged images with DAPI are also depicted (A''''–C'''). Representative confocal images of Arc (red) and GR (green) (D–D') in sham+mor animals and Arc

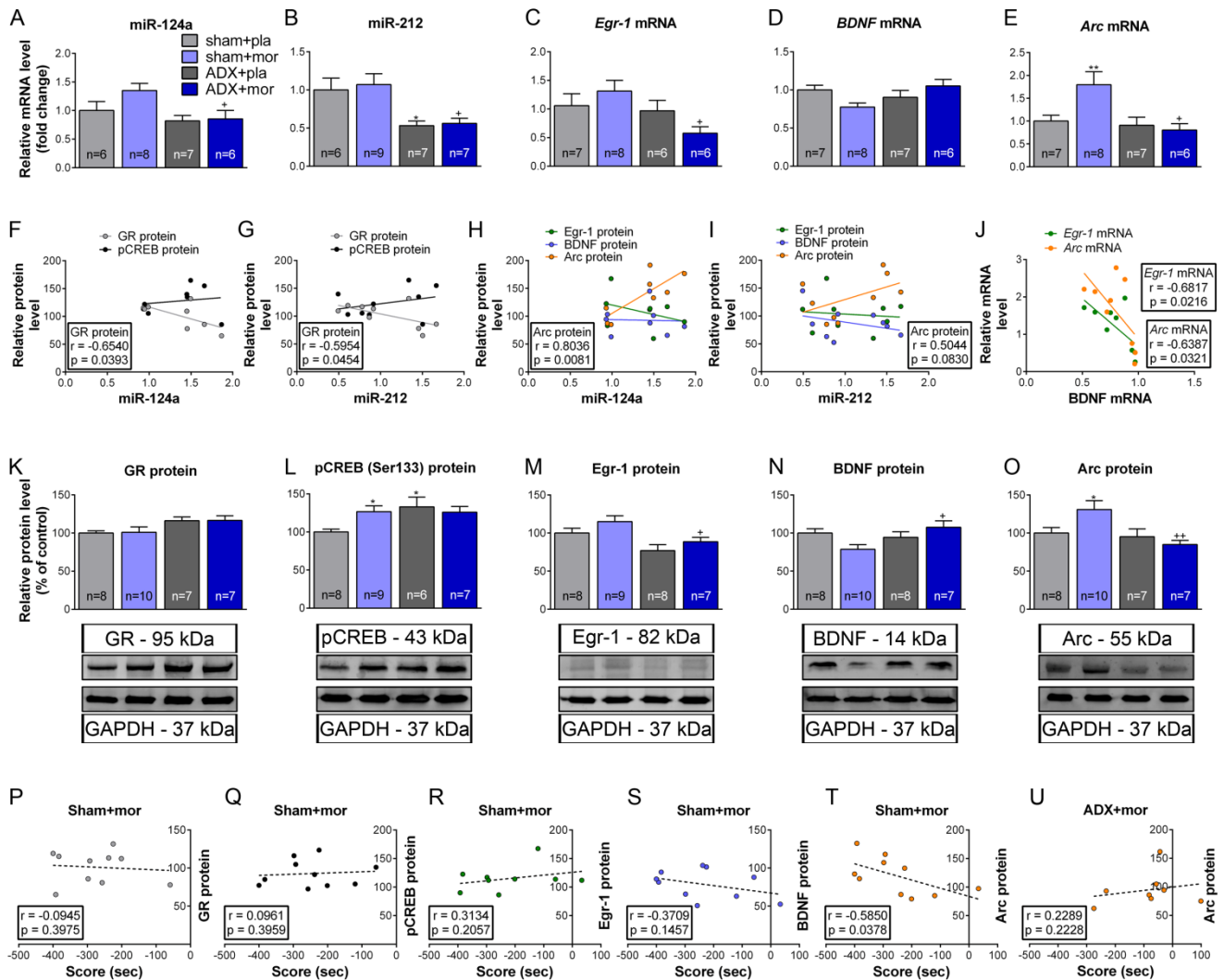
(red; F) and GR (green; F') in ADX+mor rodents. Immunostaining for DAPI (D'',F''), merged (D''',F''') and merged with DAPI (D''''-F'''). (E-E''') High magnification images from sham+mor group. (G-J) Correlation between Arc and GR protein levels in the DG after naloxone-induced CPA in sham-pla (G), sham-mor (H), ADX-pla (I), and ADX-mor (J) groups. Arc protein levels were negatively correlated with GR protein levels in the sham+mor group. Scale bars, 100 μ m (A-D,F); 25 μ m (E).

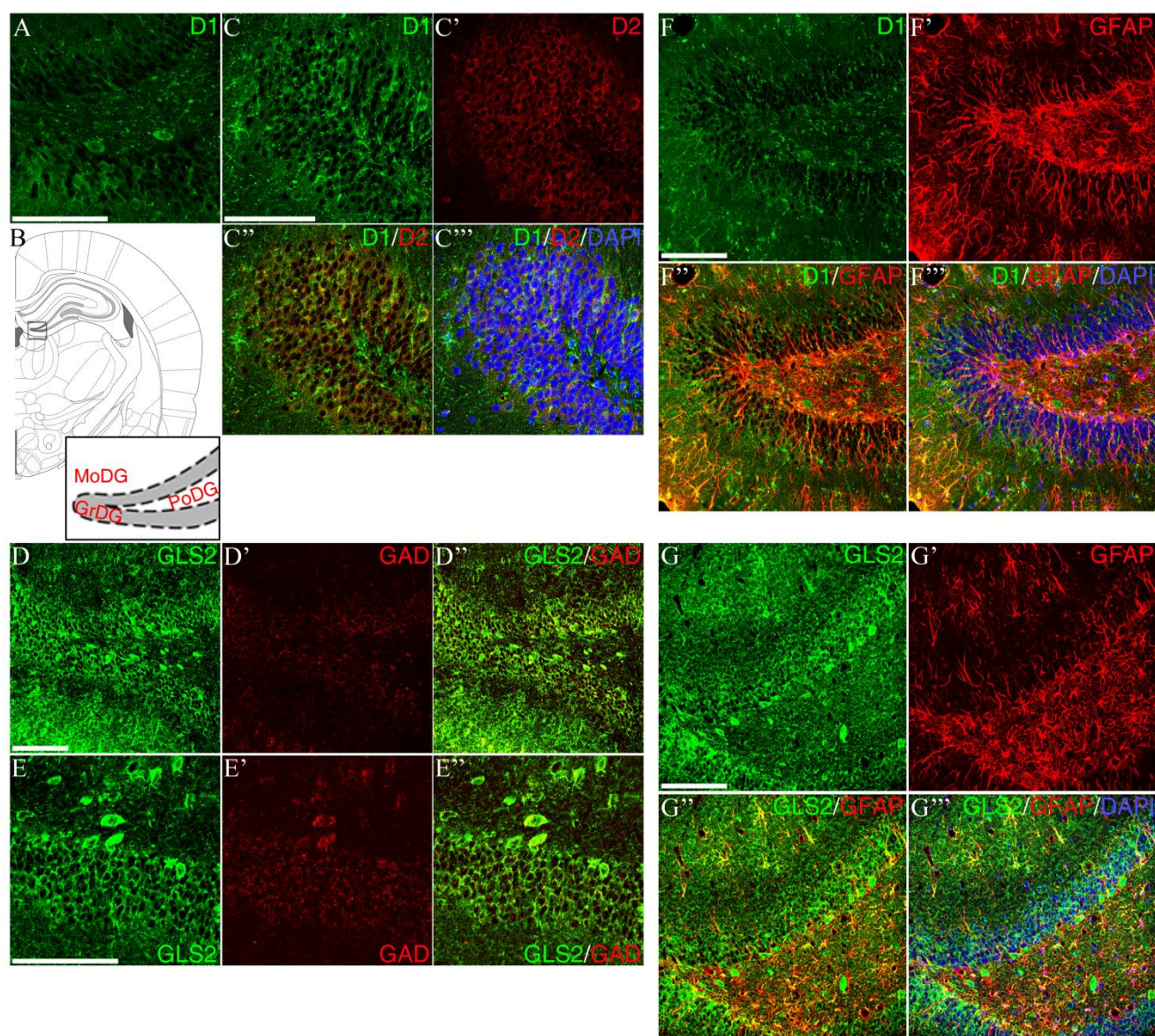
Figure 8. Effects of naloxone-induced CPA on Arc colocalization with pCREB, Egr-1, BDNF or pTrkB in the DG of sham- and ADX-operated morphine dependent rats. Representative confocal images of Arc (red) and pCREB (green) (A-B'), Arc (red) and Egr-1 (green) (C-D'), Arc (red) and BDNF (green) (E-F') and Arc (red) and pTrkB (green) (G-H') immunostaining in the DG. Merged images are shown in A'',B'' (Arc/pCREB), C'',D'' (Arc/Egr-1), E'',F'' (Arc/BDNF), and G'',H'' (Arc/pTrkB). (I-J) Correlation between Arc and pCREB protein levels in the DG after naloxone-induced CPA in sham- (I) and ADX-operated (J) morphine-dependent animals. Arc protein levels were positively correlated with pCREB protein levels in both sham- and ADX-operated morphine-treated group. (K-L) Correlation between Arc and Egr-1 protein levels in the DG after naloxone-induced CPA in sham- (K) and ADX-operated (L) morphine-dependent rats. (M-N) Correlation between Arc and BDNF protein levels in the DG after naloxone-induced CPA in sham- (M) and ADX-operated (N) morphine-dependent animals. . Scale bars, 100 μ m.

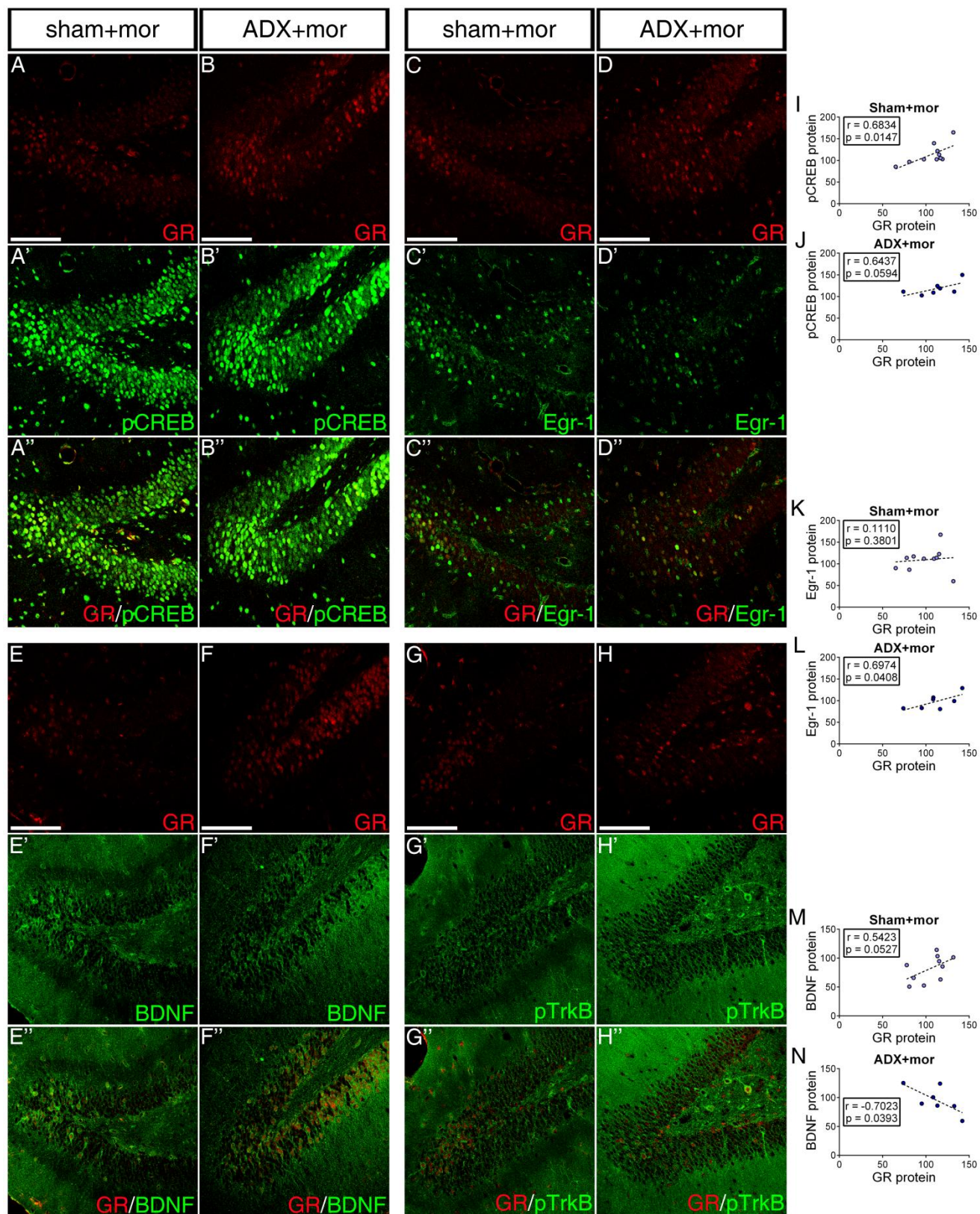


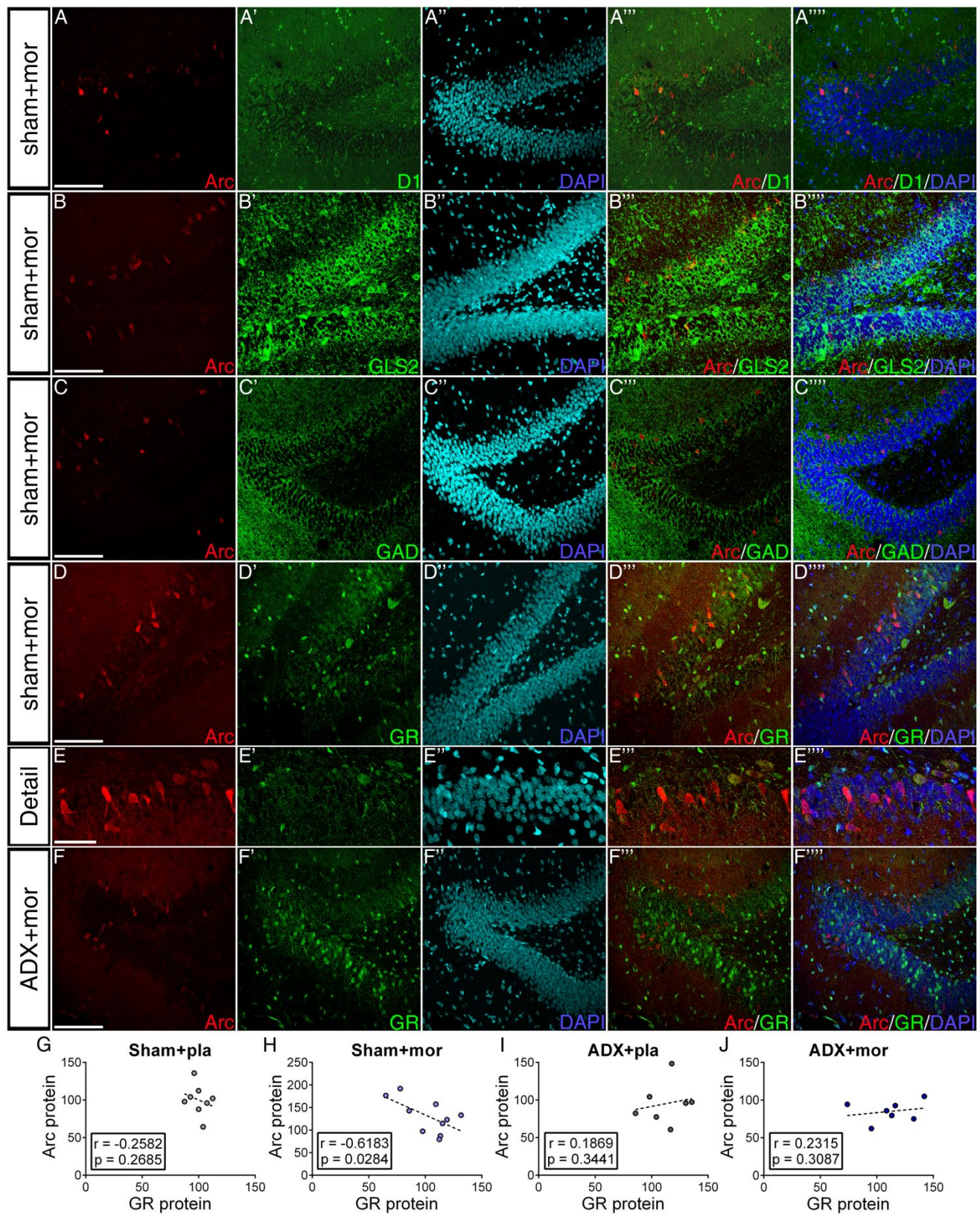


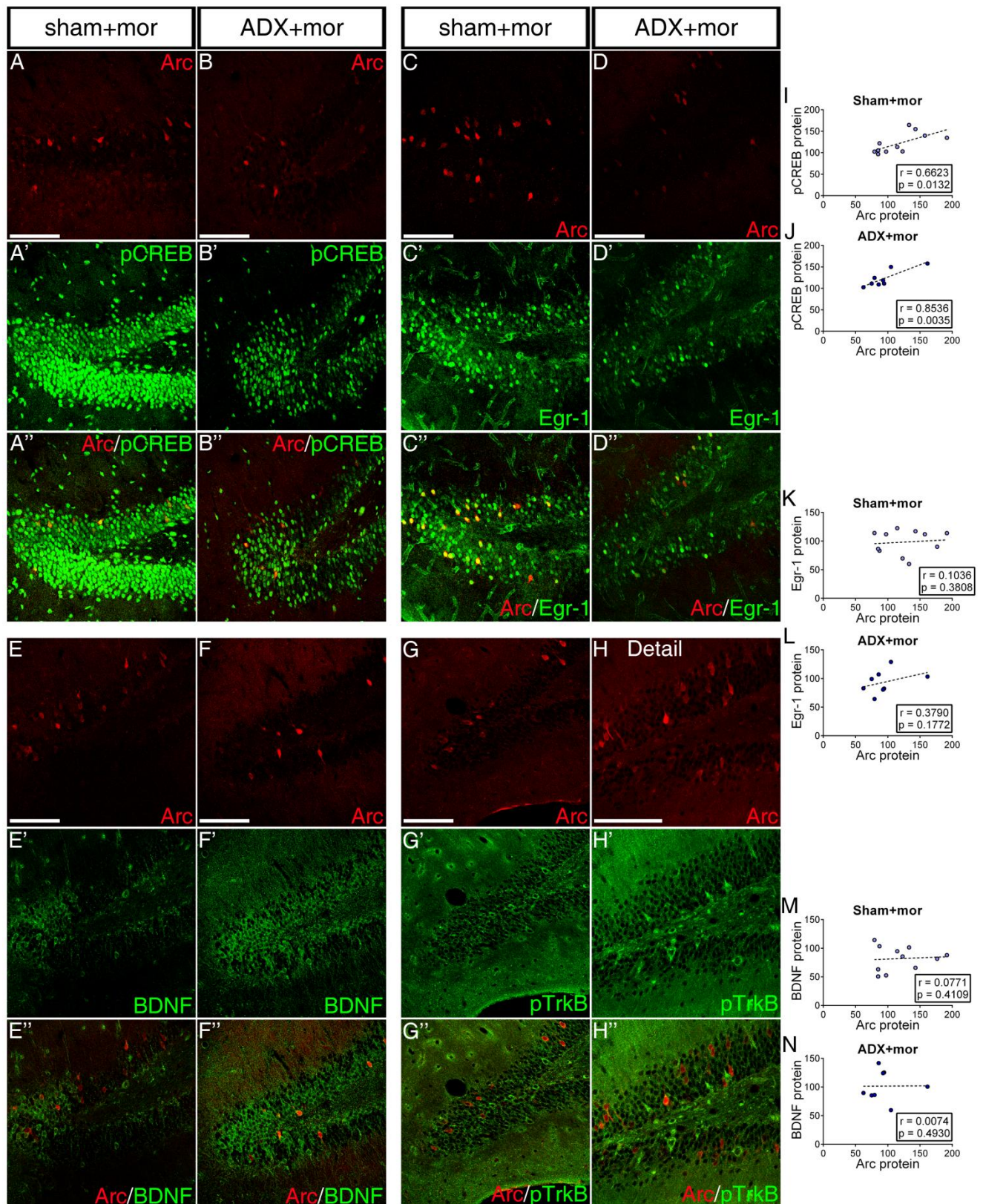












SUPPLEMENTAL INFORMATION

SUPPLEMENTAL METHODS AND MATERIALS

Subjects

All surgical and experimental procedures were performed in accordance with the European Community Council Directive (2010/63/UE), and were approved by the local Committees for animal research (REGA ES300305440012). Male Wistar rats ($n = 78$, Harlan, Barcelona, Spain) initially weighting 220–240 g were housed (2-3/cage) on arrival in a room with controlled temperature ($22 \pm 2^\circ\text{C}$) and humidity ($50 \pm 10\%$), with free access to water and food (Harlan Teklad standard rodent chow; Harlan Interfauna Ibérica, Barcelona, Spain). Animals were adapted to a standard 12 h light-dark cycle (lights on: 08:00 h – 20:00 h) for 7 days before the beginning of the experiments.

Adrenalectomy

Rats were bilaterally adrenalectomized (ADX) via a dorsal approach under 100 mg/kg ketamine chlorhydrate (Imalgene 1000, Merial, Lyon, France) and 8 mg/kg xylazin (Xilagesic® 2%, Lab. Calier S.A., Barcelona, Spain) intraperitoneally anaesthesia, and implanted subcutaneously (s.c.) with slow-release corticosterone pellets at surgery. The composition of steroid pellets (25 mg corticosterone plus 75 mg cholesterol) was chosen to provide stable corticosterone concentration corresponding to circadian nadir up to 20 days after implantation (1). ADX rats with corticosterone replacement (ADX plus corticosterone) do not mount a challenge-induced increase of plasma corticosterone. After surgery, ADX plus corticosterone rats had free choice to drink isotonic saline (0.9% NaCl) to replace depleted sodium secondary to the loss of aldosterone because of adrenalectomy. Control rats were subjected to the same surgical procedure (sham) without adrenal extirpation. Sham and ADX plus corticosterone rats were allowed to recover from surgery for 3 days before morphine or placebo pellet

implantation. Successful bilateral adrenalectomy was confirmed by plasma concentration of corticosterone and ACTH and by post-mortem examination of the ADX animals.

Induction of Opiate Dependence

Morphine base was supplied from Alcaliber Laboratories (Madrid, Spain) in cooperation with the Área de Estupefacientes y Psicotropos, Agencia Española del Medicamento y de Productos Sanitarios (Madrid, Spain). Morphine dependence was induced by subcutaneous implantation (lower back) under light ether anaesthesia of two slow-release, morphine-containing pellets (each morphine pellet contains 75 mg of morphine base). Full dependence on morphine has been previously operationally defined in this model by using a complete naloxone dose effect on various behavioral parameters (spontaneous locomotor activity, operant responding for food, intracranial self-stimulation threshold, conditioned place aversion) (2), and the rating of abstinence signs showed that opiate dependence was achieved 24 h after implantation of the morphine pellets and remained constant for 15 d (3). Placebo-pelleted rats received lactose pellets also implanted subcutaneously under anaesthesia. There were four experimental groups: sham + placebo pellets, sham + morphine pellets, ADX + placebo pellets and ADX + morphine pellets.

Drugs

Naloxone hydrochloride (N-7758, Sigma Chemical Co, St Louis, MO, USA) was dissolved in sterile saline (0.9% NaCl; ERN Laboratories, Barcelona, Spain) and injected subcutaneously. Naloxone was administered at a dose of 0.1 mg/kg, 1 ml/kg body weight. Naloxone HCl doses are expressed as the weight of the salt. Sterile 0.9% saline was also injected subcutaneously at a dose of 1 ml/kg. Note that a slightly higher naloxone dose (0.120 mg/kg) has previously been shown to be without effect on place aversion conditioning in placebo control rats (4).

Conditioned Place Aversion Paradigm

Briefly, the conditioned place aversion apparatus (Panlab, Barcelona, Spain) used to induce a reliable aversion consists in a box with two equally sized chambers (40 *L* x 34 *W* x 45 *H* cm) interconnected by a rectangular corridor (25 *L* x 13 *W* x 45 *H* cm). Distinctive visual and tactile cues distinguish the compartments: the motifs painted on the walls (either black dots or grey stripes), the floor coloring (black or grey) and the floor texture (smooth or rough). The sensory cues combination that produces a balanced choice are for walls and floor coloring and texture, respectively: (A) black dots, black smooth floor; (B) grey stripes, grey rough floor. Transparent walls are also used to minimize the time the animal spent in the corridor. The weight transducer technology (and PPCWIN software) allows for detection and analysis of animal position throughout the test and the number of entries in each compartment. The experimental protocol consists of three distinct phases: a preconditioning phase, a conditioning phase, and a testing phase. The weight gain of the rats was checked during the entire protocol to ensure that the morphine was liberated correctly from the pellets because it is known that chronic morphine treatment induces a decrease in body weight gain due to lower caloric intake (5,6). In addition, the animals were observed for body weight loss and opioid withdrawal behaviours for 15 min after the conditioning phase.

Pre-testing Phase

In the pre-testing phase (day 0), animals were placed in the central corridor and allowed to explore the apparatus freely for 20 min. Animals showing strong unconditioned aversion (less than 40% of the session time) or preference (more than 60% of the session time) for any compartment were discarded. For each rat, one room was randomly chosen to be paired with naloxone and the other chamber to vehicle. Importantly, after the compartment assignments were completed, there were no significant differences between time spent in the naloxone-paired and the vehicle-paired compartments during the

preconditioning phase. This is an important step in the experimental procedure that avoids any preference bias prior to conditioning. Rats were adrenalectomized on day 1 and placebo or morphine pellets were implanted on day 4 according to the experimental protocol depicted on Figure 1A.

Conditioning Phase

In the second phase (conditioning), rats received injection of saline on days 7 and 9, prior to being confined to their preselected saline-paired compartment for 1 hour. On days 8 and 10, rats received 0.1 mg/kg s.c. of naloxone immediately prior to confinement in the naloxone-paired compartment for 1 hour. In addition, opioid withdrawal behaviours on sham or ADX animals receiving naloxone on days 8 and 10 were measured for 15 min after the conditioning phase. A cohort of animals, conditioning memory rats (CM), was decapitated on day 10 (15 min after leaving the naloxone-paired compartment).

Testing Phase

The test was conducted on day 11, exactly as in the preconditioning phase (free access to each compartment for 20 min). The difference ($\Delta D = D - D_0$) between the time spent in the naloxone-paired compartment after conditioning (D) minus the time spent in the same compartment before conditioning (preconditioning test D_0) reflects the change of preference induced by opiate withdrawal. A negative score indicates a place aversion; a positive score indicates a place preference. Conditioned place aversion animals (CPA groups) were killed 1 hour after starting the testing phase.

Measurement of the withdrawal syndrome

Experiments were carried out in a quiet room. The observer was unaware of the drug combination used. Sham + mor and ADX + mor rats were individually placed into transparent plastic cages after

the conditioning phase and observed continuously for the occurrence of somatic signs of opiate withdrawal up to 15 min on days 8 and 10 (naloxone injection). Subsequently, previously identified behavioural characteristics of the rat opiate abstinence syndrome (7) were evaluated, including jumping, wet-dog shakes, paw tremor, sniffing, writhing, tremor, ptosis, diarrhoea, mastication, piloerection, teeth chattering, salivation, rhinorrhea, chromodacryorrhea and scrabble. The number of jumping, wet-dog shakes, paw tremor, sniffing and writhing was counted as the number of events occurring during the total test time period (graded signs). Tremor, ptosis, diarrhoea, mastication, piloerection, teeth chattering, salivation, rhinorrhea, chromodacryorrhea and scrabble were scored 1 for appearance or 0 for non-appearance within each 5 min time. To obtain a comprehensive index of the severity of somatic opiate withdrawal including all the signs examined, a global withdrawal score was calculated for each animal by giving each individual sign a relative weight as previously reported (8): jumping, x0.8; wet-dog shakes, x1; paw tremor, x0.35; sniffing, x0.5; writhing, x0.5; tremor, x1.5; ptosis, x1.5; diarrhoea, x1.5; mastication, x1.5; piloerection, x1.5; teeth chattering, x1.5; salivation, x1.5; rhinorrhea, x1.5; chromodacryorrhea, x1.5; and scrabble, x1.5. Body weight loss was determined as the difference between the weight determined immediately before naloxone injection and that determined 1 hour later, after the conditioning phase.

Preparation of tissue extract

Rats were decapitated on days 10 and 11 (between 10:00-12:00 h to avoid circadian variations in plasma levels of the hormones), the brains were rapidly removed, and stored immediately at -80°C until use for high performance liquid chromatography (HPLC), quantitative real-time PCR (qPCR) and Western blot analysis. Brains were sliced on a cryostat and kept at -20°C until each region of interest comes into the cutting plane (Fig. 3A). For DG study, three consecutive 500-µm coronal slides were made corresponding to approximately -2.9 to -4.4 mm from bregma, according to the atlas of (9). Tissues of interest were dissected using a punching device with a 1-mm internal diameter. The anatomical locations and boundaries of each region were determined using the rat brain Atlas of (9).

Bilateral punches of the DG were collected into Eppendorf tubes, according to the method of (10). A second set of animals from each treatment group was used for immunofluorescence staining.

RNA extraction and quantitative real-time PCR (qPCR)

One punch from the DG was placed in an Eppendorf tube containing 30 µl of Trizol® reagent (Invitrogen Corp., Carlsbad, CA, USA) and rapidly stored at -80°C. Frozen brain tissue samples were homogenized in Trizol® reagent (Invitrogen Corp., USA) and total RNA was isolated with QIAGEN miRNeasy Mini Kit (Qiagen, Valencia, CA, USA) according the manufacturer's instruction. To eliminate genomic DNA contamination DNase I treatment were used and 100 µl Rnase-free DNase I (1 unit DNase) (Thermo Scientific, USA) solution was added. Sample quality control and the quantitative analysis were carried out by NanoDrop (Thermo Scientific, USA). Amplification was not detected in the RT-minus controls. The cDNA synthesis was performed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Primers for the comparative Ct experiments were designed by Primer Express 3.0 Program. The primers (Microsynth, Balgach) were used in the Real-Time PCR reaction with Fast EvaGreen® qPCR Master Mix (Biotium, USA) on ABI StepOnePlus instrument was listed. The reverse transcription for microRNA assays were carried out by TaqMan MicroRNA Reverse Transcription Kit according the manufacturer's instruction. The microRNA expressions were detected using TaqMan MicroRNA assays.

List of the genes: gapdh, arc, egr-1, bdnf

List of the miRNA assays: U6 snRNA (cat.: 001973, 4427975), miR-212 (cat.: 002551, 4427975), miR-124a (cat.: 001182, 4427975)

The gene expression was analyzed by ABI Step One 2.1 program. The amplicon was tested by Melt Curve Analysis on ABI Step OnePlus Instrument. Experiments were normalized to gapdh (glyceraldehyde-3-phosphate dehydrogenase) and U6 snRNA expressions.

gapdh for ACAGCCGCATCTTCTTGTGC

gapdh rev GCCTCACCCCATTTGATGTT

arc for CCCCCAGCAGTGATTCATAC

arc rev CAGACATGGCCGGAAAGACT

egr-1 for CACCTGACCACAGAGTCCTTTT

egr-1 rev ACCAGCGCCTTCTCGTTATT

bdnf for AAACGTCCACGGACAAGGCA

bdnf rev TTCTGGTCCTCATCCAGCAGC

U6 snRNA cat:001973, 4427975

mmu-miR-212 cat: 002551, 4427975

mmu-miR-124a cat: 001182, 4427975

Western Blotting

Four bilateral punches from VTA were placed in homogenization buffer. The Eppendorf tubes contained 100 µl of homogenization buffer. The tubes were frozen immediately on dry ice and stored

at -80°C until assaying. Samples were sonicated, vortexed and sonicated again prior to centrifugation ($6000\times g$; 10 minutes at 4°C). Samples containing equal quantities of total proteins (20–40 mg, depending on the protein of interest) were separated by 10% or 12% SDS-PAGE (depending on the molecular weight of the protein of interest) and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). Membranes were blocked in TBS containing 0.15 % Tween-20 (TBS-T), 1% BSA for 90 minutes at room temperature (RT), and incubated overnight at 4°C with the primary antibody diluted in 1% BSA in TBS-T. The primary antibodies used are referenced in Table S1. Blots were then washed and incubated for 90 min at RT in TBS-T with 1% BSA with appropriate horseradish peroxidase (HRP) conjugated secondary antibodies: goat anti-rabbit (1:5000; sc-2004, Santa Cruz Biotechnology) or goat anti-mouse (1:5000; sc-2005, Santa Cruz Biotechnology). After washing, immunoreactivity was detected with an enhanced chemiluminescent/chemifluorescent western blot detection system (ECL Plus, GE Healthcare, UK) and visualized by a Typhoon 9410 variable mode Imager (GE Healthcare). We used GAPDH as our loading control. Before reprobing, blots were stripped by incubation with stripping buffer (glycine 25 mM and SDS 1%, pH 2) for 1 h at 37°C . Blots were subsequently reblocked and probed with GAPDH. The ratios of protein of interest/GAPDH were plotted and analyzed. Protein levels were corrected for individual levels.

Table S1. Primary antibodies used in this study

| Antibody | Host | Supplier | Catalog Number | WB dilution | IF dilution |
|-----------------|-------------|--------------------------|-----------------------|--------------------|--------------------|
| GR | Rabbit | Santa Cruz Biotechnology | sc-1004 | 1:500 | 1:600 |
| pCREB | Rabbit | Millipore | # 06-519 | 1:2,000 | 1:3,000 |
| Egr-1 | Rabbit | Santa Cruz Biotechnology | sc-189 | 1:500 | 1:400 |
| BDNF | Rabbit | Santa Cruz Biotechnology | sc-546 | 1:500 | 1:400 |
| Arc | Mouse | Santa Cruz Biotechnology | sc-17839 | 1:500 | 1:400 |

| | | | | | |
|-----------|--------|--------------------------------|----------|---------|---------|
| GAPDH | Rabbit | Cell Signaling Technology Inc. | #2118 | 1:5,000 | -- |
| GR | Mouse | Abcam | ab2768 | -- | 1:600 |
| pTrkB | Rabbit | Abcam | ab81288 | -- | 1:600 |
| DRD1 | Goat | Santa Cruz Biotechnology | sc-1434 | -- | 1:200 |
| DRD2 | Mouse | Santa Cruz Biotechnology | sc-5303 | -- | 1:200 |
| GLS2 | Rabbit | Abcam | ab113509 | -- | 1:1,000 |
| GAD-65/67 | Goat | Santa Cruz Biotechnology | sc-7513 | -- | 1:200 |
| GFAP | Mouse | Santa Cruz Biotechnology | sc-33673 | -- | 1:400 |

Immunofluorescence study

Another set of rats was deeply anaesthetized with an overdose of pentobarbital (100 mg/kg i.p.) and perfused transcardially with 250 ml 0.9% saline following by 500 ml cold fixative solution containing paraformaldehyde (4% paraformaldehyde in 0.1 M borate buffer, pH 9.5). After removal of the perfused brains, they were post fixed in the same fixative solution containing sucrose (30%) for 3 h and stored at 4°C in PBS containing 30% sucrose until coronal sections (30-mm thickness) were cut rostrocaudally on a freezing microtome (Leica, Nussloch, Germany). The atlas of Paxinos and Watson (2007) (19) was used to identify different brain regions. The sections were cryoprotected and stored at -20°C until use.

Brain sections were rinsed in PBS and an antigen retrieval procedure was applied by treating sections with citrate buffer (10 mM citric acid in 0.05% Tween 20, pH 6.0) at 60°C for 20 min. Non-specific Fc binding sites were blocked with 3% normal horse serum/0.3% Triton-X-100 in PBS for 1 h at RT, and the sections were incubated for 72 h (4°C, constant shaking) with primary antibodies described in Table S1. Alexa Fluor 488 donkey anti-rabbit IgG (1:1000; A-21206, Invitrogen, Eugene, OR, USA), Alexa Fluor 488 donkey anti-goat IgG (1:1000; A-11055, Invitrogen), Alexa Fluor 594 donkey anti-goat IgG (1:1000; A-11058, Invitrogen) and Alexa Fluor 594 donkey anti-mouse IgG (1:1000; A-

21203, Invitrogen) labelled secondary antibodies were applied for 4 h. After washing, sections were incubated in 4, 6-diamino-2-phenylindole (DAPI, 1:100,000) for 1 min and the sections were mounted in ProLong® Gold antifade reagent (Invitrogen). Specificity for D1 and d2 antibodies was tested in rat cortex, where both receptors are known to display only partial overlapping signal (Fig. S1A). Moreover, given that we required two different antibodies raised against GR, we tested if both of them displayed the same immunofluorescence signal (Fig. S1B-C).

Confocal analysis

The brain sections were examined using a Leica DMIRE2 confocal microscope and Leica Confocal Software (Leica Microsystems). Images from the DG were captured from low magnification to high magnification (40X to 63X oil objective). Confocal images were obtained using 405-nm excitation for DAPI, 488-nm excitation for Alexa Fluor 488 and 594-nm excitation for Alexa Fluor 594. Emitted light was detected in the range of 450-460 nm for DAPI, 515-530 nm for Alexa Fluor 488 and 610-630 nm for Alexa Fluor 594. Every channel was captured separately to avoid spectral crosstalk. Series of optical sections were performed determining an upper and lower threshold using the Z/Y position for Spatial Image Series setting. The optical series covered 20 µm of thickness through the tissue. The confocal microscope settings were established and maintained by Leica and local technicians for optimal resolution. Final confocal images can be illustrated as they appear throughout the stack of sections as a simple layer or as a transparency of all layers merged together.

Radioimmunoassay

Blood was collected on days 10 and 11 into ice-cooled tubes containing 5% EDTA and was then centrifuged (500 g; 4°C; 15 min). Plasma was separated, divided into two aliquots and stored at -80°C until assayed for corticosterone or ACTH. Plasma concentration of corticosterone and ACTH were quantified using specific corticosterone and ACTH antibodies for rats ($[^{125}\text{I}]$ -CORT and $[^{125}\text{I}]$ -ACTH

RIA; MP Biomedicals, Orangeburg, NY, USA). The sensitivity of the assay was 7.7 ng/mL for corticosterone and 5.7 pg/mL for ACTH.

Reagents

Protease inhibitors were purchased from Boehringer Mannheim, (Mannheim, Germany); phosphatase inhibitor Cocktail Set was purchased from Calbiochem (Darmstadt, Germany); HPLC reagents were purchased from Sigma Chemical Co.

Data Analysis

Data are presented as mean \pm standard error of the mean (SEM). Data were analyzed using one-way or two-way analysis of variance (ANOVA) followed by a *post hoc* Newman–Keuls test to determine specific group differences. Student's *t*-test was used when comparisons were restricted to two experimental groups. Correlations between different parameters were assessed using linear regression. Differences with a $p < 0.05$ were considered significant. Statistical analyses were performed with GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA).

SUPPLEMENTAL REFERENCES

1. Kovács KJ, Földes A, Sawchenko PE (2000): Glucocorticoid negative feedback selectively targets vasopressin transcription in parvocellular neurosecretory neurons. *J Neurosci* 20:3843-3852.
2. Schulteis G, Markou A, Gold LH, Stinus L, Koob GF (1994): Relative sensitivity to naloxone of multiple indices of opiate withdrawal: a quantitative dose-response analysis. *J Pharmacol Exp Ther* 271:1391-1398.
3. Gold LH, Stinus L, Inturrisi CE, Koob GF (1994): Prolonged tolerance, dependence and abstinence following subcutaneous morphine pellet implantation in the rat. *Eur J Pharmacol* 253:45-51.
4. Frenois F, Cador M, Caille S, Stinus L, Le Moine C (2002): Neural correlates of the motivational and somatic components of naloxone-precipitated morphine withdrawal. *Eur J Neurosci* 16:1377-1389.
5. Houshyar H, Manalo S, Dallman MF (2004): Time-Dependent Alterations in mRNA Expression of Brain Neuropeptides Regulating Energy Balance and Hypothalamo-Pituitary-Adrenal Activity after Withdrawal from Intermittent Morphine Treatment. *J Neurosci* 24:9414-9424.
6. Núñez C, Földes A, Pérez-Flores D, García-Borrón JC, Laorden ML, Kovács KJ, Milanés MV (2009): Elevated glucocorticoid levels are responsible for induction of tyrosine hydroxylase (TH) mRNA expression, phosphorylation and enzyme activity in the nucleus of the solitary tract (NTS-A2) during morphine withdrawal. *Endocrinology* 150:3118-3127.
7. Lu L, Liu D, Ceng X, Ma L (2000): Differential role of corticotropin-releasing factor receptor subtypes 1 and 2 in opiate withdrawal and in relapse to opiate dependence. *Eur J Neurosci* 12:4398-4404.
8. Maldonado R, Blendy JA, Tzavara E, Gass P, Roques BP, Haoune J, Schütz G (1996): Reduction of morphine abstinence in mice with a mutation in the gene encoding CREB. *Science* 273:657-659.
9. Paxinos G, Watson C (2007): The rat brain in stereotaxic coordinates 6th
10. Leng A, Feldon J, Ferger B (2004): Long-term social isolation and medial prefrontal cortex: dopaminergic and cholinergic neurotransmission. *Pharmacol Biochem Behav* 77:371-379.

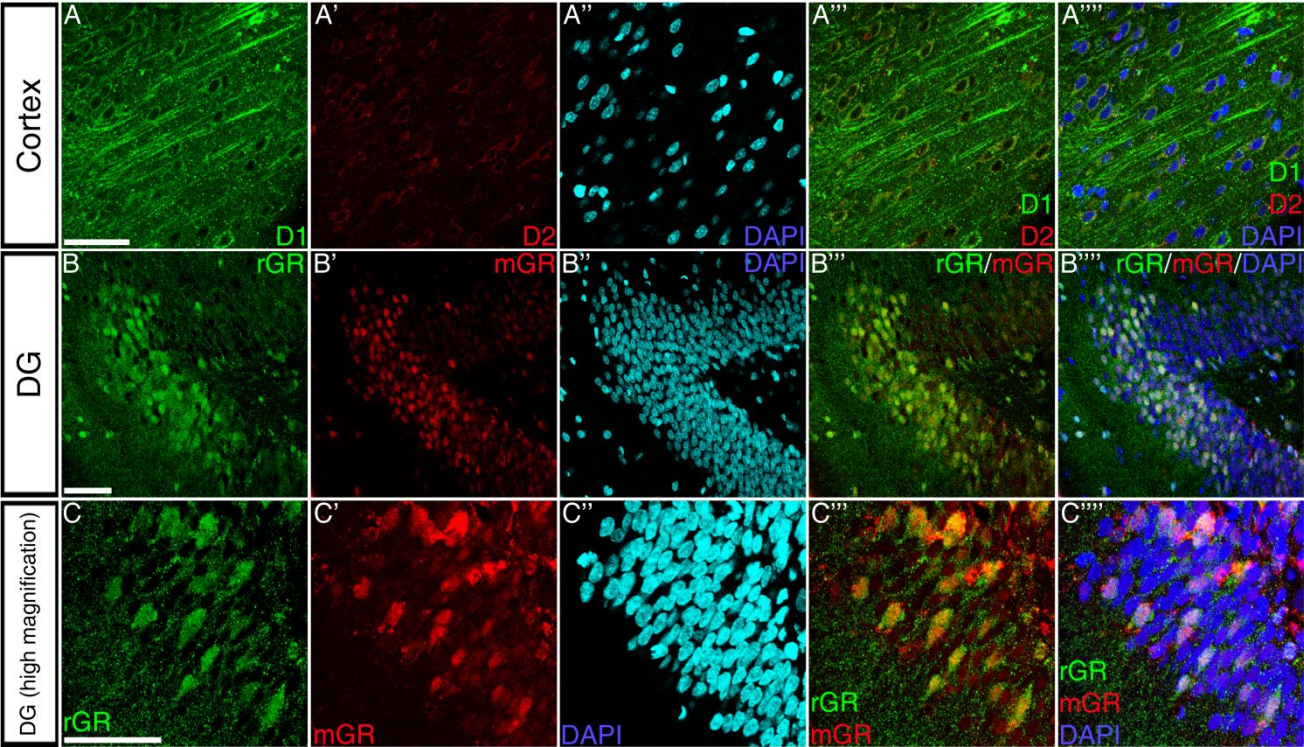
LEGENDS TO SUPPLEMENTAL FIGURES

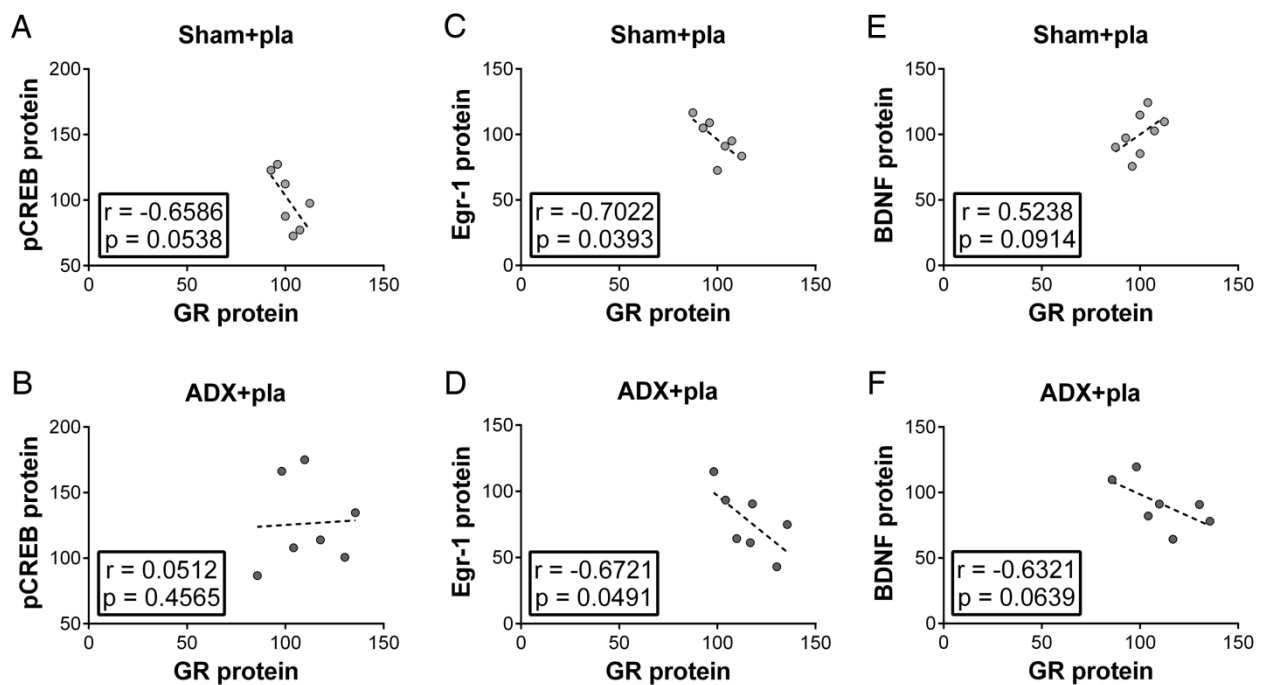
Supplemental Figure 1. (A-A''') Representative confocal images showing cortex coronal sections of rat immunostained for D1 (green; A) and D2 (red; A'), DAPI (nuclear stain, blue; A''), merged (A''') and merged with DAPI (A'''). (B-B''',C-C''') Low and high magnification confocal images showing GR overlapping signal between an antibody originated in rabbit (green; B,C) and an antibody originated in mouse (red; B',C'). DAPI (nuclear stain, blue; B''C''), merged (B'''C''') and merged with DAPI (B''''',C'''''). Scale bars, 50 μ m.

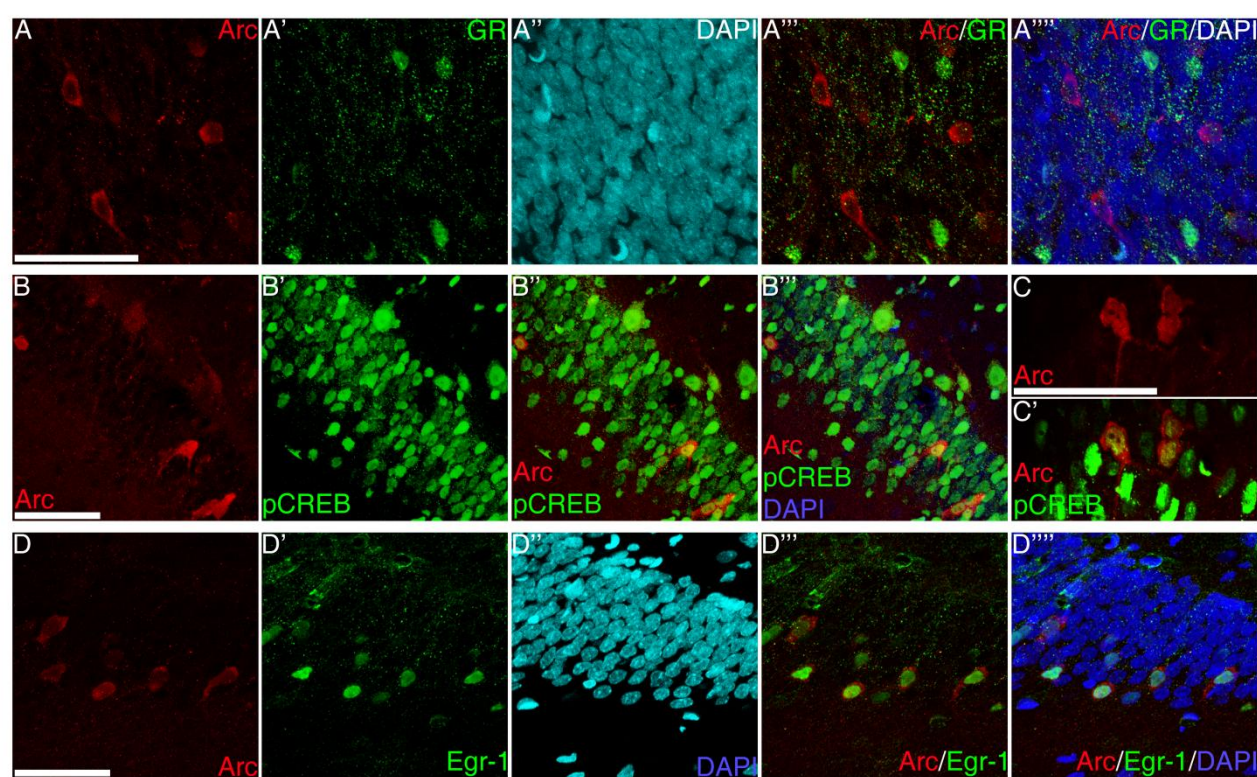
Supplemental Figure 2. Correlation of GR protein levels with pCREB (A,B), Egr-1 (C,D) or BDNF (E,F) protein levels in the DG of sham- and ADX-operated control animals. (A) Correlation between GR and pCREB protein levels in the sham+pla group. (B) Correlation between GR and pCREB protein levels in the ADX+pla group. (C) Correlation between GR and Egr-1 protein levels in the sham+pla group. GR protein levels were negatively correlated with Egr-1 protein levels. (D) Correlation between GR and Egr-1 protein levels in the ADX+pla group. GR protein levels were negatively correlated with Egr-1 protein levels. (E) Correlation between GR and BDNF protein levels in the sham+pla group. (F) Correlation between GR and BDNF protein levels in the ADX+pla group.

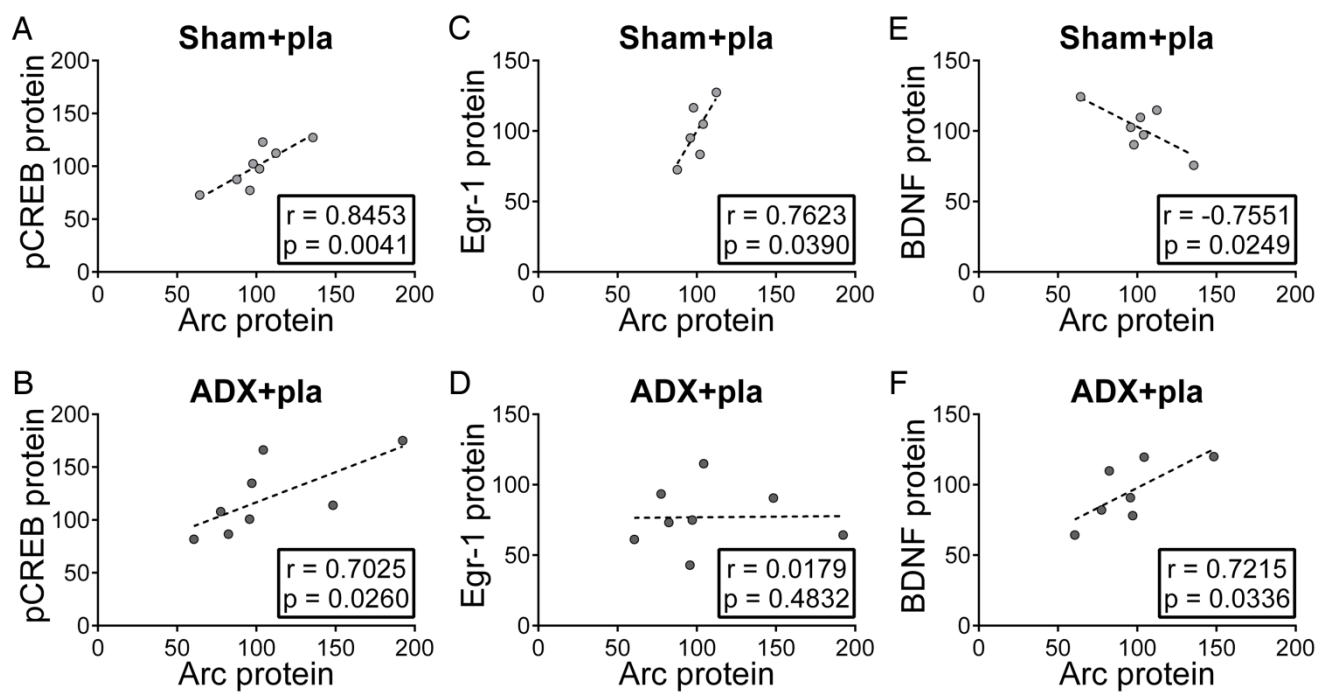
Supplemental Figure 3. (A-A''') Representative confocal images showing DG coronal sections of rat immunostained for Arc (red; A) and GR (green; A'), DAPI (nuclear stain, blue; A''), merged (A''') and merged with DAPI (A'''). (B-B''') Representative confocal images showing DG coronal sections of rat immunostained for Arc (red; B) and pCREB (green; B'), merged (B'') and merged with DAPI (B'''). (C-C') High magnification images of Arc and Arc/pCREB immunostaining. (D-D''') Representative confocal images showing DG coronal sections of rat immunostained for Arc (red; D) and Egr-1 (green; D'), DAPI (nuclear stain, blue; D''), merged (D''') and merged with DAPI (D'''''). Scale bars, 50 μ m.

Supplemental Figure 4. Correlation of Arc protein levels with pCREB (A,B), Egr-1 (C,D) or BDNF (E,F) protein levels in the DG of sham- and ADX-operated control animals. (A) Correlation between Arc and pCREB protein levels in the sham+pla group. Arc protein levels were positively correlated with pCREB protein levels. (B) Correlation between Arc and pCREB protein levels in the ADX+pla group. Arc protein levels were positively correlated with pCREB protein levels. (C) Correlation between Arc and Egr-1 protein levels in the sham+pla group. Arc protein levels were positively correlated with Egr-1 protein levels. (D) Correlation between Arc and Egr-1 protein levels in the ADX+pla group. (E) Correlation between Arc and BDNF protein levels in the sham+pla group. Arc protein levels were negatively correlated with pCREB protein levels. (F) Correlation between Arc and BDNF protein levels in the ADX+pla group. Arc protein levels were positively correlated with BDNF protein levels.









Addiction Biology



Different contribution of glucocorticoids in the basolateral amygdala to the formation and expression of opiate withdrawal-associated memories

| | |
|-------------------------------|---|
| Journal: | <i>Addiction Biology</i> |
| Manuscript ID | Draft |
| Wiley - Manuscript type: | Original Article |
| Date Submitted by the Author: | n/a |
| Complete List of Authors: | García-Pérez, Daniel; University of Murcia, Pharmacology Ferenczi, Szilamer Kovacs, Krisztina Laorden, M. Luisa; University of Murcia, Department of Pharmacology Milanés, M. Victoria; University of Murcia, School of Medicine, Department of Pharmacology Núñez, Cristina; University of Murcia, Department of Pharmacology |
| Keywords: | Adrenalectomy, conditioned place aversion , memory consolidation and retrieval |
| Abstract: | <p>Drug-withdrawal aversive memories generate a motivational state leading to compulsive drug taking, with plasticity changes involved in the formation of aversive memories. The basolateral amygdala (BLA) is involved in aversive motivational learning. We examined the influence of glucocorticoids (GCs) on memory consolidation and retrieval-reconsolidation for a morphine-withdrawal-paired environment through conditioned place aversion (CPA) paradigm, along with plasticity changes in the BLA, in sham-operated and adrenalectomized (ADX) animals. We demonstrated that sham+morphine animals robustly expressed CPA. In contrast, ADX-dependent animals lacked the affective-like signs of opiate withdrawal but displayed increased somatic signs of withdrawal. Following the conditioning phase, ADX-animals displayed reduced phosphorylated cAMP response element binding protein (pCREB). The consolidation process was accompanied by increased early growth response 1 (Egr-1) mRNA, activity-regulated cytoskeletal-associated protein (Arc) mRNA and protein both, in sham-dependent and ADX-dependent animals. Moreover, glucocorticoid receptor (GR) levels were positively correlated with pCREB, Egr-1 and Arc mRNA only in sham+morphine animals upon naloxone-induced corticosterone secretion. In contrast, memory retrieval elicited increased pCREB levels in sham+morphine and ADX animals, and enhanced Arc levels in sham+morphine, but not in ADX+morphine animals. Arc was mainly expressed in glutamatergic neurons. Corticosterone levels were unaltered following memory retrieval, and consequently GR no longer regulated pCREB or Arc. However, we uncovered that pCREB levels positively correlated Arc mRNA/protein expression following memory recall. In conclusion, context-withdrawal associations are accompanied by</p> |

| | |
|--|---|
| | structural and functional plasticity in the BLA, which are, in part, regulated by GR signaling. These findings might have important implications for drug-seeking behavior. |
| | |

SCHOLARONE™
Manuscripts

For Review Only

Different contribution of glucocorticoids in the basolateral amygdala to the formation and expression of opiate withdrawal-associated memories

Daniel García-Pérez¹, Szilamer Ferenczi³, Krisztina J. Kovács³, M. Luisa Laorden^{1,2}, M. Victoria Milanés^{1,2} and Cristina Núñez^{1,2}

¹Department of Pharmacology, University of Murcia, Murcia, Spain. ²Murcia Institute of Biomedical Research (IMIB), University of Murcia, Spain. ³Laboratory of Molecular Neuroendocrinology, Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest, Hungary.

Running head: Withdrawal memory, amygdala

Correspondence to:

Daniel García-Pérez

Department of Pharmacology, Faculty of Medicine, Campus de Espinardo, 30100 Murcia, Spain.

Phone number: +34868887183.

E-mail address: daniel.garcia9@um.es

Abstract

Drug-withdrawal aversive memories generate a motivational state leading to compulsive drug taking, with plasticity changes involved in the formation of aversive memories. The basolateral amygdala (BLA) is involved in aversive motivational learning. We examined the influence of glucocorticoids (GCs) on memory consolidation and retrieval-reconsolidation for a morphine-withdrawal-paired environment through conditioned place aversion (CPA) paradigm, along with plasticity changes in the BLA, in sham-operated and adrenalectomized (ADX) animals. We demonstrated that sham+morphine animals robustly expressed CPA. In contrast, ADX-dependent animals lacked the affective-like signs of opiate withdrawal but displayed increased somatic signs of withdrawal. Following the conditioning phase, ADX-animals displayed reduced phosphorylated cAMP response element binding protein (pCREB). The consolidation process was accompanied by increased *early growth response 1* (*Egr-1*) mRNA, activity-regulated cytoskeletal-associated protein (*Arc*) mRNA and protein both, in sham-dependent and ADX-dependent animals. Moreover, glucocorticoid receptor (GR) levels were positively correlated with pCREB, *Egr-1* and *Arc* mRNA only in sham+morphine animals upon naloxone-induced corticosterone secretion. In contrast, memory retrieval elicited increased pCREB levels in sham+morphine and ADX animals, and enhanced *Arc* levels in sham+morphine, but not in ADX+morphine animals. *Arc* was mainly expressed in glutamatergic neurons. Corticosterone levels were unaltered following memory retrieval, and consequently GR no longer regulated pCREB or *Arc*. However, we uncovered that pCREB levels positively correlated *Arc* mRNA/protein expression following memory recall. In conclusion, context-withdrawal associations are accompanied by structural and functional plasticity in the BLA, which are, in part, regulated by GR signaling. These findings might have important implications for drug-seeking behavior.

Key words: Adrenalectomy; conditioned place aversion; memory consolidation and retrieval; miRNAs; morphine dependence; transcription factors.

Introduction

Evidence supports that some of the most important features of addiction reflect abnormalities in traditional memory circuits, with long-term memories of the drug experience serving as potent drivers of addiction pathology (Robbins et al. 2008). One factor responsible for this vulnerability is reactivation of withdrawal memories, which consists in somatic and affective withdrawal signs elicited in the presence of cues that were previously paired with drug withdrawal (O'Brien et al. 1977). The conditioned-place aversion (CPA) paradigm is a highly sensitive animal model for measurement of the negative affective component of drug withdrawal as well as to investigate neural substrates underlying the aversive memory associated with drug withdrawal (Myers et al. 2012; Stinus et al. 2000). CPA triggers association between negative affective consequences of withdrawal with context (aversive memory formation or consolidation). When the animals are re-exposed to the paired environment in a drug-free state, they avoid the paired environment due to the association between the context and aversive memories of drug withdrawal (aversive memory retrieval). Besides, reconsolidation is the process by which memories are re-stabilized and maintained after retrieval, the disruption of which can result in the permanent loss of a previously established memory (Nader et al. 2000).

Re-exposure to a withdrawal-paired environment induces conditioned responses in a specific limbic circuit, which can be partially dissociated from the structures involved in acute withdrawal (Frenois et al. 2005b). The amygdala is a heterogeneous brain region critically involved in processing emotional response (LeDoux 2000), which includes the basolateral amygdala (BLA), a brain area implicated in addictive behavior. Importantly, BLA neuronal activity is correlated with the acquisition and recall of opiate-related associative memories (Frenois et al. 2005a). On the other hand, it is well known that glucocorticoids (GCs) effects on memory are mediated by the BLA. Infusions of a glucocorticoid receptor (GR) agonist into

the BLA enhance memory consolidation (Roosendaal and McGaugh 1997), whereas activation of GC pathway in the BLA is important in the impairing effects of stress on memory retrieval (de Quervain et al. 1998).

CPA develops through associative learning and requires synaptic plasticity (Hou et al. 2009). It has been demonstrated that the reconsolidation of memories during retrieval requires both transcription and translation in the amygdala in a manner analogous to that seen during the initial consolidation of learning to form long-term memories (Miller and Matzel 2000;Nader et al. 2000). However, the molecular processes underlying plasticity during opiate-withdrawal memory consolidation and retrieval have been little studied. Converging evidence supports a role for cAMP response element binding protein (CREB), a transcription factor that becomes activated when phosphorylated on Ser 133 residue, in both consolidation and reconsolidation of fear (Bourtchuladze et al. 1994;Dash et al. 1990;Hall et al. 2001b;Kida et al. 2002;Mamiya et al. 2009;Yin et al. 1994). It is therefore important to examine the specific role for CREB-regulated signaling in amygdala-dependent aversive-withdrawal memory processes. On the other hand, immediate early genes (IEGs) are inducible genes which can be divided into two functional classes: a) regulatory transcription factors, such as early growth response 1 (Egr-1), and b) “effector” proteins, such as activity-regulated cytoskeletal-associated protein (Arc) (Guzowski et al. 2001). Egr-1 and Arc are required in learning and long-term memory (Guzowski et al. 2000;Jones et al. 2001), and are necessary for the consolidation and reconsolidation of a variety of amygdala-dependent memory tasks (Hellemans et al. 2006;Maddox and Schafe 2011;Plath et al. 2006;Ploski et al. 2008). Importantly, pCREB, Egr-1 and Arc have been proposed to be targeted by activation of GRs, which leads to fear memory consolidation (Finsterwald and Alberini 2014). In addition, microRNAs (miRNAs) are involved in the development of synaptic connections and

plasticity, direct dendrite formation in neurons (Schratt et al. 2006), and have an important role in the development of addiction-related behaviors (Chandrasekar and Dreyer 2011).

To date, despite the fact that GCs modulate memory consolidation and retrieval (Roosendaal and McGaugh 2011), the role of GCs in the BLA in the context of morphine-induced CPA has not been assessed. Therefore, we hypothesized that GCs might modulate context-drug associations and could affect memory-associated plasticity in the BLA, a brain region known to mediate the negative motivational component of opiate withdrawal. The present study was undertaken to test this hypothesis by examining morphine-withdrawal aversive memories in sham-operated and adrenalectomized (ADX) rats, and changes in miR-124a, GR, pCREB, Egr-1 and Arc during consolidation and retrieval-reconsolidation memory.

Materials and methods

Details are provided in Supplementary Materials and Methods.

Animals

Experimental procedures were performed in accordance with the European Community Council Directive (2010/63/UE), and were approved by the local Committees for animal research (REGA ES300305440012). Male Wistar rats (n=78) were used.

Adrenalectomy

Rats were bilaterally ADX as described in (García-Pérez et al. 2012) and implanted subcutaneously (s.c.) with slow-release corticosterone pellets at surgery. Control rats were subjected to the same surgical procedure (sham) without adrenal extirpation.

Induction of Opiate Dependence

Morphine dependence was induced by s.c. implantation (lower back) under light ether anaesthesia of two, slow-release, morphine-containing pellets (each morphine pellet contains 75 mg of morphine base). Placebo-pelleted rats received lactose pellets also implanted subcutaneously under anaesthesia.

Conditioned Place Aversion Protocol

Pre-testing Phase

Animals were placed in the central corridor and allowed to explore the apparatus freely for 20 min. For each rat, one room was randomly chosen to be paired with naloxone and the

other chamber to vehicle. Rats were adrenalectomized on day 1 and placebo or morphine pellets were implanted on day 4 according to the experimental protocol depicted on Figure 1A.

Conditioning Phase

Rats received injection of saline on days 7 and 9, prior to being confined to their preselected saline-paired compartment for 1 hour. On days 8 and 10, rats received 0.1 mg/kg s.c. of naloxone immediately prior to confinement in the naloxone-paired compartment for 1 hour. Opiate withdrawal behaviors were measured for 15 min after the conditioning phase. A cohort of animals, named conditioning memory rats (CM), were decapitated on day 10 (15 min after leaving the naloxone-paired compartment).

Testing Phase

The test was conducted on day 11, exactly as in the preconditioning phase (free access to each compartment for 20 min). A negative score indicates a place aversion; a positive score indicates a place preference. Conditioned place aversion animals (CPA groups) were killed 1 hour after starting the testing phase.

Tissue processing

Brains were rapidly removed, and stored immediately at -80°C until use for quantitative real-time PCR (qPCR) and Western blot analysis. For BLA study, three consecutive 500-μm coronal slides were made corresponding to approximately -1.9 to -3.4 mm from bregma, according to the atlas of (Figure S1) (Paxinos and Watson 2007). Tissue samples of interest

were dissected using a punching device with a 1-mm internal diameter. A second set of animals from each treatment group was used for immunofluorescence staining.

RNA extraction and qPCR

qPCR was performed as previously described (Polyák et al. 2014).

List of the genes: *gapdh*, *arc*, *egr-1*

List of the miRNA assays: U6 snRNA, miR-124a

The gene expression was analyzed by ABI Step One 2.1 program. The amplicon was tested by Melt Curve Analysis on ABI Step OnePlus Instrument. Experiments were normalized to *gapdh* (glyceraldehyde-3-phosphate dehydrogenase) and U6 snRNA expressions.

gapdh for ACAGCCGCATCTTCTTGTGC

gapdh rev GCCTCACCCCATTTGATGTT

arc for CCCCCAGCAGTGATTCATAC

arc rev CAGACATGGCCGGAAAGACT

egr-1 for CACCTGACCACAGAGTCCTTTT

egr-1 rev ACCAGCGCCTTCTCGTTATT

U6 snRNA cat:001973, 4427975

mmu-miR-124a cat: 001182, 4427975

Western Blotting

Western Blotting protocol (García-Pérez et al. 2015) and a list of the primary antibodies used in this study can be found at Supplementary Materials and Methods. We used GAPDH as our loading control.

Immunofluorescence study and Confocal analysis

The tissue for immunofluorescence was processed as previously described (García-Pérez et al. 2015) (see details in the Supplementary Materials and Methods). Equipment and settings are detailed in the SI. Final confocal images can be illustrated as they appear throughout the stack of sections as a simple layer or as a transparency of all layers merged together.

Data Analysis

Data are presented as mean \pm SEM. Data were analyzed using one-way or two-way analysis of variance (ANOVA) followed by a *post hoc* Newman–Keuls test. Student's *t*-test was used when comparisons were restricted to two experimental groups. Correlations between different parameters were assessed using linear regression. Differences with a $p < 0.05$ were considered significant. Statistical analyses were performed with GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA).

Results

Effect of GCs on opiate withdrawal-associated somatic and affective behavior

The sequence of events during the CPA assay can be conceptualized as follows: (1) the morphine-dependent animal is administered naloxone and develops naloxone-precipitated morphine withdrawal [an unconditioned stimulus (US)], (2) experiences a subjective aversive dysphoric effect [an unconditioned response (UR)], (3) learns to associate this UR with a specific environmental context [a conditioned stimulus (CS)], and when subsequently presented with the CS, (4) the animal exhibits avoidance behavior [a conditioned response (CR)] to the earlier withdrawal-paired CS. Importantly, before the conditioning sessions, the different experimental groups used in this study did not exhibit different locomotor activity (Figure 1A), spent similar amount of time in the naloxone-paired room (Figure 1B) and did not display natural preference for neither the dots or stripes chambers (Figure 1C).

Opiate withdrawal is characterized by both somatic and motivational behavioral signs upon cessation of drug use, leading to the emergence of an aversive state that can be conditioned to a specific environment (Frenois et al. 2005b). As expected, our results showed that naloxone administration to sham- or ADX-operated rats dependent on morphine evoked an increase of the body weight loss ($P < 0.001$; Figure 1E) when compared to their control groups also receiving naloxone dose or compared to the same experimental groups after saline injection. As well, opiate antagonist administration to sham- or ADX-operated rats dependent on morphine elicited the appearance of a number of behavioral symptoms characteristic of opiate withdrawal (Figure 1F). It is noteworthy that, on day 10, the ADX+morphine group exhibited an enhancement of the withdrawal score when compared with both sham+morphine (day 10; $P < 0.01$) and ADX+morphine (day 8; $P < 0.05$) groups (Figure 1F).

Since CPA expression occurs on a naloxone-free test day, the behavior is dependent upon the retrieval of the association between the environment and the affective state produced by the drug (White and Carr 1985). Therefore, behavior in the place aversion method depends upon both the affective and the memory improving properties of the reinforcers under test (White and Carr 1985). During the test, morphine-dependent rats spent less time in the naloxone-paired compartment than controls ($P<0.001$; Figure 1G). However, adrenalectomy diminished the naloxone-induced CPA in opiate-dependent animals ($P<0.01$). Additionally, in the test phase the total entries were higher in animals receiving opiates ($P<0.05$; Figure 1H), which might indicate an increased locomotor activity of the morphine-dependent rats.

Gene expression and protein synthesis in the BLA during memory consolidation and retrieval-reconsolidation processes

We focused our study on the BLA, because among the limbic areas that are likely to mediate motivational features of opiate dependence, the BLA represents a critical neural substrate (Frenois et al. 2005a; Lucas et al. 2008). Furthermore, the BLA is part of an integrated circuit of neural structures implicated in the formation and storage of CS–US associations and in the processing of emotional events in relation to environmental stimuli that guide motivated behaviour (Everitt et al. 2003). Moreover, it is well known that GC effects on memory are mediated by the BLA (Schwabe et al. 2012).

We studied GR expression in the amygdala, given that the BLA is enriched in GRs, which are known to play critical roles in encoding, processing and retaining the information of emotional events (Finsterwald and Alberini 2014). Western blot analysis did not reflect any change on GR protein between the different groups after the last conditioning session (Figure

2D). In contrast, after memory recall, two-way ANOVA revealed a main effect of surgery ($P < 0.05$, Figure 2E) on GR expression, although no differences between groups were observed with the *post hoc* test. In addition, no correlation was observed between GR induction and the aversive score (Figure 2F).

miRNAs and post-transcriptional processes have an important role in the development of addiction-related behaviors (Chandrasekar and Dreyer 2011). Here we directly assessed the specific role of amygdalar miR-124a in memory consolidation and retrieval-reconsolidation. Our results show that during the memory consolidation process (day 10), miR-124a levels were not modified (Figure 2A). In contrast, during the test phase, miR-124a levels were significantly diminished in sham animals treated with morphine ($P < 0.05$; Figure 2B), whereas adrenalectomy reversed this effect. Furthermore, we observed that miR-124a was positively correlated with the aversion score in the sham+morphine group (Figure 2C). Thus, rats that expressed higher aversion in the CPA test and spent less time in the naloxone-paired room, displayed the lowest miR-124a levels.

We next have investigated whether activation of the transcription factor CREB is induced in the BLA during the formation and recall of Pavlovian cued morphine-withdrawal associations. We uncovered that the lack of adrenal glands resulted in decreased pCREB levels during memory consolidation ($P < 0.05$; Figure 3A). On the other hand, after memory retrieval, pCREB levels were increased both in sham+morphine animals ($P < 0.05$; Figure 3B) and ADX animals ($P < 0.05$). However, pCREB levels showed no direct relationship with the aversive index (Figure 3C). Furthermore, we performed an immunofluorescence study to investigate if CREB activation after memory retrieval occurred in GR⁺ cells. As depicted in Figure 3D-E, we found that pCREB⁺ neurons were also GR⁺ cells, although some cells that expressed GR did not exhibit CREB activation.

To extend our results, the present study aimed to assess the cellular reactivity and plasticity of amygdala nuclei during the opiate withdrawal conditioning process. *Egr-1* and *Arc* IEGs are required in long-term potentiation (LTP) and long-term memory (Guzowski et al. 2000; Jones et al. 2001). For this, we have quantified *Egr-1* and *Arc* expression using PCR and Western-blot analysis, following the last training session and after re-exposure to the withdrawal-paired environment. During the consolidation process, we detected that sham or ADX morphine-dependent rats exhibited increased *Egr-1* mRNA levels ($P<0.05$), although these changes were not observed at the protein level (Figure 4A,D). Following retrieval, ADX+placebo animals exhibited decreased *Egr-1* mRNA levels ($P<0.01$; Figure 4B). However, no differences in *Egr-1* protein were observed at the same time-point (Figure 4E). In addition, immunofluorescence analysis demonstrated that *Egr-1* immunoreactivity was widely distributed throughout the BLA, and double-labeling with anti-GR showed that *Egr-1* is expressed on a high percentage of GR⁺ neurons (Figure 4G-H).

BLA tissue from sham+morphine ($P<0.01$) and ADX+morphine ($P<0.05$) animals showed an increase in the expression of the plasticity-related *Arc* gene during conditioning (Figure 5A), and these changes were paralleled in *Arc* protein ($P<0.05$; Figure 5D). Interestingly, following memory retrieval, ADX groups showed a significant reduction in *Arc* mRNA expression ($P<0.05$ or 0.01 ; Figure 5B). *Arc* protein was also increased in sham-dependent rodents by re-exposure to the withdrawal-paired environment ($P<0.05$), although addicted animals that had been adrenalectomized displayed a significant reduction in protein levels ($P<0.05$; Figure 5E). Correlation analysis between the different IEGs mRNA or protein content and CPA index failed to detect any significant correlation. As shown in Figure 5G-H, according to our results, sham+morphine animals displayed a higher number of *Arc* positive neurons compared to ADX-dependent subjects. In addition, double immunofluorescence study revealed that *Arc* protein was expressed in a reduced number of GR⁺ cells.

Distinct GR-dependent non-genomic and genomic regulation following conditioning and testing

Importantly, mineralocorticoid receptors (MRs) have a tenfold higher affinity for GCs than GRs and are largely occupied by the ligand in basal conditions, whereas GRs occupation highly depends on increases in glucocorticoid levels following stress response (de Kloet et al. 2005). Thus, we studied the activity of the hypothalamic-pituitary-adrenal (HPA) axis throughout the CPA paradigm. Our results revealed that naloxone administration to morphine dependent rats before conditioning induced an increase in GCs plasma levels ($P < 0.001$) which was not observed in ADX animals due to adrenalectomy ($P < 0.001$; Fig. 6A). In contrast, following memory retrieval of aversive withdrawal memories did not alter the corticosterone blood levels (Figure 6B). On ligand binding, GRs undergo conformational changes that lead to their homodimerization and nuclear translocation, or their binding to other cytoplasmic proteins. In the nucleus, GRs can bind to specific sequences and directly activate transcription of target genes (Zalachoras et al. 2013). In addition, GRs can control rapid cellular responses by mechanisms that are independent of nuclear translocation and gene expression regulation, but instead occur through genomic-independent actions (Groeneweg et al. 2011). During the consolidation process, we detected that GR levels were positively correlated with pCREB, *Egr-1* and *Arc* mRNA only in sham+morphine animals (Figure 6C-E), indicating that genomic and non-genomic GR-dependent regulation during memory consolidation depends on increased GCs levels. In contrast, in other experimental groups with basal GC levels, correlation analysis revealed that GR might exert inhibitory genomic regulation (Figure 6F-H,O). Following memory retrieval, we observed a lack of GR regulation of the same targets, except for ADX+morphine animals, where GR seemed to negatively regulate pCREB levels (Figure 6I-N,P).

Given the absence of positive GR regulation of Arc, we next compared pCREB induction with that of Arc, the expression of which can be regulated by pCREB (Kawashima et al. 2009). We confirmed the co-localization of pCREB and Arc in the BLA after the CPA in sham- and ADX-operated morphine-dependent rats (Figure 7A-B). In addition, the present results showed that CREB activation correlated with increased Arc mRNA and protein in sham-dependent animals, while in ADX+morphine animals pCREB did only correlate Arc protein levels, but not Arc mRNA (Figure 7E-I). However, greater numbers of nuclei showed induction of pCREB than of Arc, suggesting that pCREB may regulate the expression of other IEGs. Indeed, the CRE consensus sequence is present in Egr-1 promoter (Changelian et al. 1989), raising the possibility that CREB activation may regulate Egr-1 during reconsolidation. Nonetheless, we observed no relationship between pCREB and Egr-1 levels after the reactivation test (data not shown). On the other hand, Egr1 can also regulate Arc (Li et al. 2005). Although we observed that Egr-1 and Arc were expressed on the same cells (Figure 7C-D), our results reject that Egr-1 controls Arc induction during memory retrieval-reconsolidation (Figure 7J).

Characterization of Arc-expressing neurons

The BLA mainly contains glutamate output neurons (GAD-) together with GABA interneurons (GAD+) (Pitkänen et al. 1997). We performed a phenotypic analysis to characterize the neuronal populations involved in Arc expression to better understand the cellular events underlying these processes. We uncovered that the majority of Arc-positive cells were glutamatergic neurons (Figure 8A). Nevertheless, Arc was also stained in few GABAergic neurons (Figure 8B).

Discussion

Somatic and affective components of the opiate withdrawal syndrome are dissociable at different levels of analysis (Frenois et al. 2002; Mucha 1987). Our findings of a clear-cut dissociation of affective-like and somatic signs of opiate withdrawal in sham and ADX rats provide robust evidence in support of the latter notion. It has been previously demonstrated that CRF1^{-/-} mice, which displayed basal and stress-related plasma corticosterone deficits (Smith et al. 1998; Timpl et al. 1998), showed increased somatic expression of opiate withdrawal (Papaleo et al. 2007). Moreover, intake of nonstressful amounts of corticosterone reduced the exacerbated somatic reactions of CRF1^{-/-} mice to opiate withdrawal (Papaleo et al. 2007). Thus, the use of adrenalectomy also allowed us to unravel a HPA axis-dependent modulatory (inhibitory) role for GCs upon the somatic expression of opiate withdrawal. These findings clearly indicate that failure to adequately activate stress-responsive HPA axis may lead to exaggerated somatic reactions to opiate withdrawal. In contrast, CRF1^{-/-} mice lack the negative affective states of opiate withdrawal (Contarino and Papaleo 2005)). Together, our results indicate that a fully functional HPA system is required for the negative affective properties of opiate withdrawal.

The lack of affective-like behaviors in ADX-dependent animals cannot be attributed to decreased levels of somatic opiate withdrawal. Alternatively, GCs activate regions enriched in GRs, which play critical roles in encoding, processing, and retaining the information of emotional events (Finsterwald and Alberini 2014). Thus, we next focused on the molecular processes accompanying withdrawal memory consolidation and retrieval-reconsolidation in the BLA for several reasons: GC effects on memory are mediated by the BLA (Schwabe et al. 2012), GRs are highly expressed in this brain area, and the BLA is a neural region strongly involved in emotional learning and affective conditioning (LeDoux 2000). In the hippocampus, several long-lasting molecular modifications required for fear memory

consolidation are dependent on GR activation. Specifically, GRs control the rapid non-genomic learning-dependent increase of pCREB (Chen et al. 2012) and the genomic expression of the IEGs Arc and Egr-1 (Chen et al. 2012;Revest 2005;Revest et al. 2010). However, GRs actions highly depend on increases in GCs levels (de Kloet et al. 2005;Lupien et al. 2007). In agreement, we uncovered that GR expression correlated with pCREB, Egr-1 and Arc induction during aversive-withdrawal memory consolidation only in sham+morphine animals. Since delayed genomic GR actions suppress competing information processing after learning and hence promote memory consolidation (Schwabe et al. 2012), GCs secretion and GR-signaling are likely to mediate the differences in the CPA index between sham- and ADX-dependent animals.

CREB plays a critical role in memory consolidation (Bourtchuladze et al. 1994;Dash et al. 1990;Yin et al. 1994). In the context of opiate-withdrawal CPA paradigm, it has been previously reported that despite a global inhibition of *c-fos* expression in the BLA after conditioning session, a subpopulation of output neurons was activated by conditioned withdrawal (Frenois et al. 2005b). In concordance, in the present study sham+morphine rodents did not display a net increase in pCREB levels during memory formation, but the fact that training-dependent GR activation seemed to be coupled to CREB phosphorylation suggests that pCREB signaling is engaged in a subset of BLA cells during memory consolidation. Furthermore, our data demonstrate that adrenalectomy mediates CREB hypoactivation within the BLA, which might underlie the impairment of withdrawal-memories consolidation in ADX+morphine animals.

In addition, synaptic plasticity within the BLA is critical in the process of a CS becoming associated with a US during conditioning (Blair et al. 2005). We tested this hypothesis by assessing Arc gene expression, which has been widely implicated in synaptic plasticity, LTP and memory formation (Korb and Finkbeiner 2011). Importantly, Arc has been

extensively described in the consolidation phases of a variety of memory tasks (Guzowski et al. 2000;Plath et al. 2006;Ploski et al. 2008). Our findings are consistent with previous data suggesting a strong increase in the number of output neurons expressing *Arc* mRNA (Lucas et al. 2008) and enhanced *Arc* protein expression (Hou et al. 2009) in the BLA following withdrawal pairing. Particularly, late-phase LTP is considered to be the underlying mechanism of long-term memory, and LTP consolidation requires sustained period of *Arc* synthesis translation since *Arc* protein underlying LTP consolidation is rapidly degraded (Bramham et al. 2010;Messaoudi et al. 2007). Actually, evidence points to a second wave of *Arc* induction hours after the degradation of the first wave of *Arc* (Ramírez-Amaya et al. 2005), which may indicate the reactivation of circuits during consolidation. Besides, *Arc* learning-dependent expression is controlled by corticosterone and GR (Chen et al. 2012;McReynolds 2010;Molteni et al. 2010). Accordingly, in sham+morphine animals, we observed that activated GR lead to genomic activation of *Arc* gene, which would lead to sustained *Arc* transcription required for consolidation. Because of the lack of endogenous GCs in ADX+morphine animals, GR did not mediate genomic activation. It is likely that enhanced *Arc* mRNA levels in ADX+morphine animals are induced by neural activity (Abraham et al. 1993;Worley et al. 1993). Together, our data suggest that ADX-dependent animals are able to induce *Arc*-mediated LTP, but in the absence of withdrawal-induced corticosterone are unable to maintain LTP and form long-term memory.

Memory retrieval additionally engages a protein synthesis-dependent re-consolidation of memories (Nader et al. 2000). However, the transcriptional mechanisms necessary for initial memory consolidation and stability after retrieval differ. For instance, stress is known to have an inhibitory effect on memory retrieval. Activation of GC pathway in the BLA is important in the impairing effects of stress on memory retrieval (de Quervain et al. 1998;Roosendaal et al. 2004). Moreover, the severity of memory impairments has been

correlated with the concentration of circulating plasma corticosterone at the time of testing (de Quervain et al. 1998; Diamond et al. 1999). In agreement, we showed that GRs acted as transcriptional regulators specifically during memory consolidation upon activation by GCs, but not after memory retrieval, when GCs levels remained at basal levels.

Emerging evidence supports a role for miRNAs in different steps of addiction. Chronic cocaine suppresses miR-124 levels throughout the brain, including the BLA (Chandrasekar and Dreyer 2009). Our mir-124a findings are particularly important for several reasons. First, a role for mir-124a in addicted behavior has been proposed. For instance, miR-124 is suppressed after cocaine CPP and during the reinstatement, and miR-124 over-expression attenuates cocaine CPP (Chandrasekar and Dreyer 2011). Second, our data indicated that miR-124a downregulation occurred selectively following a re-exposure to withdrawal context in sham-dependent animals, but was reversed by adrenalectomy. Third, miR-124a is likely to play a pivotal role in morphine-withdrawal memory retrieval and reconsolidation since lesser miR-124a expression significantly correlated with enhanced aversion.

GR no longer regulated pCREB during memory retrieval. However, CREB can be phosphorylated on Ser 133 by different kinases (De Cesare and Sassone-Corsi 2000; Shaywitz and Greenberg 1999), which are required in the amygdala for reconsolidation of fear or drug-paired cues (Duvarci et al. 2005; Sanchez et al. 2010; Tronson et al. 2006). Previous studies have demonstrated that CREB is required for reconsolidation of contextual and cued fear memories (Kida et al. 2002; Mamiya et al. 2009). Furthermore, it has been postulated that CREB activity in the BLA alone is required for reconsolidation of auditory memories, but is not required for retrieval or extinction of fear (Tronson et al. 2012). The present study extends these findings, and given that CREB activation promotes *c-fos* transcription (Mayr and Montminy 2001), is in agreement with the notion that the re-exposure to a withdrawal-paired

environment increased *c-fos* expression of most BLA output neurons (Frenois et al. 2005b). ADX-animals also showed increased pCREB levels. By all means, pCREB levels in ADX+morphine rats do not seem to be due to memory-related mechanisms, since ADX-dependent animals do not display enhanced pCREB levels compared to their controls.

Our findings contrast with studies showing that cue-induced opiate withdrawal memories undergo Egr-1 protein synthesis-dependent reconsolidation in the BLA (Hellemans et al. 2006). Given that Egr-1 expression following retrieval is described to be more prolonged than that of Arc (Besnard et al. 2014), our observations of unaltered Egr-1 levels during memory reactivation are not likely due to a time-course effect. It could be argued that Egr-1 within the BLA is only involved in the reconsolidation of cue-induced, but not context-induced, aversive withdrawal memories (Hellemans et al. 2006). However, Egr-1-activation in the BLA after re-exposure to CS is described in a great variety of training tasks, such as contextual and cued fear learning (Hall et al. 2001a; Hoffman et al. 2015; Lee et al. 2005), withdrawal-aversive (Hellemans et al. 2006) or appetitive (Lee et al. 2005; Lee et al. 2006; Thomas et al. 2003) drug-associated memories. A possible explanation is that Egr-1 activation evolves as a function of the number of pairings. After the last (second) withdrawal conditioning, we observed that opiate-dependent animals exhibited increased Egr-1 mRNA but unaltered Egr-1 protein levels, which raises the possibility that additional pairings resulted in increased strength of the association and Egr-1 protein levels. Actually, Egr-1 activation during opiate-withdrawal memory reconsolidation was detected after five conditioning sessions (Hellemans et al. 2006).

The increased plasticity within the BLA may contribute not only to the consolidation of CS–US associations, but also to facilitate BLA reactivation by conditioned environmental stimuli in order to initiate appropriate behavioral responses. While *Arc* induction is not necessary for memory retrieval, it likely identifies neurons genomically activated as a result

of retrieval (Zhang et al. 2005). When analyzing the expression of the plasticity-related *Arc* IEG after aversive withdrawal-memory retrieval, we observed that the group of animals that exhibited robust place aversion also displayed increased *Arc* levels, in agreement with (Lucas et al. 2008), suggesting that sustained *Arc*-induced BLA plasticity during the consolidation phase may well participate in the retrieval of learned associations in sham+morphine animals. This notion is supported by data showing that the same neurons in the BLA are activated both by learning and by memories retrieval in a fear conditioning paradigm (Reijmers et al. 2007). As expected, *Arc* mRNA and protein levels were significantly decreased within the BLA in the ADX+mor group. As proposed above, in the absence of GCs, GR do not activate sustained *Arc* induction during the consolidation phase, and subsequently exposure CS does not reactivate BLA neurons to facilitate memory retrieval.

The expression of CRE-driven genes after CREB phosphorylation is described to be essential for the stability of conditioned memories after retrieval (Kida et al. 2002), and miR-124 inhibition is crucial for long-term synaptic facilitation by regulating CREB (Chandrasekar and Dreyer 2011; Rajasethupathy et al. 2009). Together, our observations suggest that miR-124 downregulation may enhance the maladaptive drug-associated memories in sham+morphine animals, in line with the findings that miR-124 inhibition act as the switch necessary to convert short-term facilitation into long-term facilitation (Rajasethupathy et al. 2009). Moreover, our results suggest that sustained CREB phosphorylation following retrieval mediates *Arc* expression. This observation is of particular interest given the prominent role of *Arc* in fear memory reconsolidation (Maddox and Schafe 2011). Thus, dysregulation of CREB signaling in the amygdala may enhance reconsolidation, resulting in the maintenance of excessive aversive states.

In agreement with previous studies (Frenois et al. 2005b; Lucas et al. 2008), double-labeling showed that the majority of Arc-positive neurons during the re-exposure to withdrawal-paired stimuli were glutamatergic neurons, while very few Arc⁺/GAD⁺ neurons were detected. Plasticity in glutamate output neurons in the amygdala is of great interest since the apparent variety of functions of the amygdala are based on its diverse extended connections with cortical and subcortical structures (Noori et al. 2012). Thus, our results suggest that plasticity processes within BLA output neurons during the consolidation and reconsolidation of withdrawal aversive memories may participate in increasing the BLA reactivity to conditioned stimuli, which could in turn (by the control of downstream nuclei) reinforce and drive the motivational properties of withdrawal over drug consumption.

Defining the differential molecular mechanisms of consolidation and retrieval-reconsolidation allows for the possibility of separately targeting both of these processes to reduce persistent memories and maladaptive behavioral responses. Our data provide the first evidence that GCs mediate transcriptional events required for morphine-withdrawal memory consolidation and influence epigenetic mechanisms following retrieval in the BLA, supporting the idea that targeting GCs in this amygdalar area may provide important insights into the role of essential signaling cascades mediating aversive drug memories. Together, disrupting these processes might lead to effective treatments in drug addiction (Milton and Everitt 2010; Tronson and Taylor 2007) thereby rapidly and persistently reducing invasive memories and subsequent drug-seeking.

Acknowledgements

This work was supported by grants from Ministerio de Ciencia e Innovación (SAF/FEDER 2009-07178; SAF/FEDER 2010-17907; 2013-49076-P), Spain; Red de Trastornos Adictivos, Spain; Fundación Séneca (15405/PI/10) and Instituto Murciano de Investigación en Biomedicina (IMIB), Región de Murcia, Spain. In Hungary, the work was supported by the National Research, Development and Innovation Fund 109622 to KJK and 109744 to SzF. Daniel García-Pérez was supported by a fellowship from Ministerio de Ciencia e Innovación (AP2009-2379). Authors declare no conflicts of interest.

Figure legends

Figure 1. Experimental procedures and behavioral parameters. (A) Diagram showing the experimental protocol: after 7 habituation and handling days, on day 0 animals were placed in the central corridor and allowed to explore the apparatus freely for 20 min. For each rat, one room was randomly chosen to be paired with naloxone and the other chamber to vehicle. Rats were adrenalectomized on day 1 and placebo or morphine pellets were implanted on day 4. In the second phase (conditioning), rats received injection of saline on days 7 and 9, prior to being confined to their preselected saline-paired compartment for 1 hour. On days 8 and 10, rats received 0.1 mg/kg s.c. of naloxone immediately prior to confinement in the naloxone-paired compartment for 1 hour. The test was conducted on day 11, exactly as in the preconditioning phase (free access to each compartment for 20 min). (B) **Number of entries during pre-test phase:** Two-way ANOVA: surgery ($F(1, 48) = 0,1450$; $P = 0,7050$), treatment ($F(1, 48) = 0,7319$; $P = 0,3965$), interaction ($F(1, 48) = 0,2800$; $P = 0,5991$). (C) **Time in naloxone-paired room during pre-test phase:** Two-way ANOVA: surgery ($F(1, 48) = 0,7422$; $P = 0,3932$), treatment ($F(1, 48) = 0,3054$; $P = 0,5831$), interaction ($F(1, 48) = 0,01515$; $P = 0,9026$). (D) **% of time in dots or stripes rooms during pre-test phase:** Two-way ANOVA: surgery ($F(1, 48) = 0,8195$; $P = 0,3699$), treatment ($F(1, 48) = 1,906$; $P = 0,1738$), interaction ($F(1, 48) = 0,1204$; $P = 0,7301$). (E) **Body weight loss during conditioning sessions: Day 7 (saline):** Two-way ANOVA: surgery ($F(1, 48) = 0,004669$; $P = 0,9458$), treatment ($F(1, 48) = 4,572$; $P = 0,0376$), interaction ($F(1, 48) = 0,4938$; $P = 0,4856$). Post hoc test: ns. **Day 8 (naloxone):** Two-way ANOVA: surgery ($F(1, 48) = 3,246$; $P = 0,0779$), treatment ($F(1, 48) = 77,30$; $P < 0,0001$), interaction ($F(1, 48) = 0,05720$; $P = 0,8120$). Post hoc test: *** $P < 0.001$ versus sham+pla; ### $P < 0.001$ versus ADX+pla. **Day 9 (saline):** Two-way ANOVA: surgery ($F(1, 48) = 0,1954$; $P = 0,6604$), treatment ($F(1, 48) = 0,3916$; $P = 0,5344$), interaction ($F(1, 48) = 2,762$; $P = 0,1030$). **Day 10 (naloxone):** Two-

way ANOVA: surgery ($F(1, 48) = 5,406$; $P = 0,0243$), treatment ($F(1, 48) = 131,8$; $P < 0,0001$), interaction ($F(1, 48) = 0,7116$; $P = 0,4031$). Post hoc test: $***P < 0.001$ versus sham+pla; $^+P < 0.05$ versus sham+mor; $^{###}P < 0.001$ versus ADX+pla. **Sham+pla:** One-way ANOVA: acute injection ($F(3, 44) = 0,5766$; $P = 0,6335$). **Sham+mor:** One-way ANOVA: acute injection ($F(3, 60) = 43,30$; $P < 0,0001$). Post hoc test: $^{\&\&\&}P < 0.001$ versus day 7 (saline); $^{@@@}P < 0.001$ versus day 9 (saline). **ADX+pla:** One-way ANOVA: acute injection ($F(3, 44) = 0,7106$; $P = 0,5509$). **ADX+mor:** One-way ANOVA: acute injection ($F(3, 44) = 35,75$; $P < 0,0001$). Post hoc test: $^{\&\&\&}P < 0.001$ versus day 7 (saline); $^{@@@}P < 0.001$ versus day 9 (saline). **(F) Withdrawal score after conditioning sessions:** Two-way ANOVA: day ($F(1, 26) = 5,387$; $P = 0,0284$), surgery+treatment ($F(1, 26) = 3,964$; $P = 0,0571$), interaction ($F(1, 26) = 7,487$; $P = 0,0111$). Post hoc test: $^{++}P < 0.01$ versus sham+mor same day; $^{\$}P < 0.05$ versus ADX+mor day 8. **(G) Aversion score:** Two-way ANOVA: surgery ($F(1, 48) = 3,789$; $P = 0,0574$), treatment ($F(1, 48) = 10,05$; $P = 0,0027$), interaction ($F(1, 48) = 10,44$; $P = 0,0022$). Post hoc test: $***P < 0.001$ versus sham+pla; $^{++}P < 0.01$ versus sham+mor. **(H) Number of entries during test phase:** Two-way ANOVA: surgery ($F(1, 48) = 0,08893$; $P = 0,7668$), treatment ($F(1, 48) = 14,09$; $P = 0,0005$), interaction ($F(1, 48) = 0,0006481$; $P = 0,9798$). Post hoc test: $^*P < 0.05$ versus sham+pla; $^{\#}P < 0.05$ versus ADX+pla.

Figure 2. miR-124a and GR regulation during the conditioning and test phase. (A) miR-124a expression during the conditioning phase: Two-way ANOVA: surgery ($F(1, 18) = 1,819$; $P = 0,1942$), treatment ($F(1, 18) = 0,7172$; $P = 0,4082$), interaction ($F(1, 18) = 3,137$; $P = 0,0934$). **(B) miR-124a expression during the test phase:** Two-way ANOVA: surgery ($F(1, 25) = 0,3312$; $P = 0,5701$), treatment ($F(1, 25) = 4,068$; $P = 0,0546$), interaction ($F(1, 25) = 4,306$; $P = 0,0484$). Post hoc test: $^*P < 0.05$ versus sham+pla. **(C) Correlation analysis between aversion score and miR-124a. (D) GR expression during the conditioning**

phase: Two-way ANOVA: surgery ($F(1, 22) = 0,1916$; $P = 0,6659$), treatment ($F(1, 22) = 0,0006138$; $P = 0,9805$), interaction ($F(1, 22) = 0,01153$; $P = 0,9155$). **(E) GR expression during the test phase:** Two-way ANOVA: surgery ($F(1, 29) = 4,628$; $P = 0,0399$), treatment ($F(1, 29) = 1,411$; $P = 0,2446$), interaction ($F(1, 29) = 0,2657$; $P = 0,6102$). Post hoc test: ns. **(F) Correlation analysis between aversion score and GR.**

Figure 3. pCREB regulation during the conditioning and test phase and identification of pCREB-expressing cells during conditioned place aversion (CPA) paradigm. (A) pCREB expression during the conditioning phase: Two-way ANOVA: surgery ($F(1, 20) = 16,15$; $P = 0,0007$), treatment ($F(1, 20) = 0,01732$; $P = 0,8966$), interaction ($F(1, 20) = 0,3543$; $P = 0,5584$). Post hoc test: $^*P < 0.05$ versus sham+pla; $^+P < 0.05$ versus sham+mor. **(B) pCREB expression during the test phase:** Two-way ANOVA: surgery ($F(1, 28) = 2,725$; $P = 0,1100$), treatment ($F(1, 28) = 1,664$; $P = 0,2076$), interaction ($F(1, 28) = 5,629$; $P = 0,0248$). Post hoc test: $^*P < 0.05$ versus sham+pla. **(C) Correlation analysis between aversion score and pCREB. (D-E)** Representative confocal microphotographs showing coronal sections of rats immunostained in: red (GR), green (pCREB) and blue (DAPI, nuclear stain); scale bar, 50 μ m. Merged images are also depicted.

Figure 4. Egr-1 mRNA and protein regulation during the conditioning and test phase and identification of Egr-1-expressing cells during conditioned place aversion (CPA) paradigm. (A) Egr-1 mRNA expression during the conditioning phase: Two-way ANOVA: surgery ($F(1, 17) = 0,06232$; $P = 0,8059$), treatment ($F(1, 17) = 15,78$; $P = 0,0010$), interaction ($F(1, 17) = 0,1141$; $P = 0,7397$). Post hoc test: $^*P < 0.05$ versus sham+pla; $^{\#}P < 0.05$ versus ADX+pla. **(B) Egr-1 mRNA expression during the test phase:** Two-way ANOVA: surgery ($F(1, 25) = 8,343$; $P = 0,0079$), treatment ($F(1, 25) = 4,229$; $P =$

0,0503), interaction ($F(1, 25) = 5,999$; $P = 0,0217$). Post hoc test: $^{**}P < 0.01$ versus sham+pla; $^{##}P < 0.01$ versus ADX+pla. **(C) Correlation analysis between aversion score and Egr-1 mRNA. (D) Egr-1 protein expression during the conditioning phase:** Two-way ANOVA: surgery ($F(1, 20) = 0,01518$; $P = 0,9032$), treatment ($F(1, 20) = 0,9763$; $P = 0,3349$), interaction ($F(1, 20) = 0,5218$; $P = 0,4784$). **(E) Egr-1 protein expression during the test phase:** Two-way ANOVA: surgery ($F(1, 29) = 0,2768$; $P = 0,6028$), treatment ($F(1, 29) = 0,01433$; $P = 0,9055$), interaction ($F(1, 29) = 0,7424$; $P = 0,3959$). **(F) Correlation analysis between aversion score and Egr-1 protein. (G-H)** Representative confocal microphotographs showing coronal sections of rats immunostained in: red (GR), green (Egr-1) and blue (DAPI, nuclear stain); scale bar, 50 μ m. Merged images are also depicted.

Figure 5. Arc mRNA and protein regulation during the conditioning and test phase and identification of Arc -expressing cells during conditioned place aversion (CPA) paradigm. (A) Arc mRNA expression during the conditioning phase: Two-way ANOVA: surgery ($F(1, 17) = 0,4034$; $P = 0,5338$), treatment ($F(1, 17) = 20,01$; $P = 0,0003$), interaction ($F(1, 17) = 0,6690$; $P = 0,4247$). Post hoc test: $^{**}P < 0.01$ versus sham+pla; $^{#}P < 0.05$ versus ADX+pla. **(B) Arc mRNA expression during the test phase:** Two-way ANOVA: surgery ($F(1, 26) = 16,96$; $P = 0,0003$), treatment ($F(1, 26) = 1,044$; $P = 0,3162$), interaction ($F(1, 26) = 0,1477$; $P = 0,7039$). Post hoc test: $^{*}P < 0.05$ versus sham+pla; $^{++}P < 0.01$ versus sham+mor. **(C) Correlation analysis between aversion score and Arc mRNA. (D) Arc protein expression during the conditioning phase:** Two-way ANOVA: surgery ($F(1, 19) = 2,625$; $P = 0,1216$), treatment ($F(1, 19) = 14,14$; $P = 0,0013$), interaction ($F(1, 19) = 0,03367$; $P = 0,8563$). Post hoc test: $^{*}P < 0.05$ versus sham+pla; $^{#}P < 0.05$ versus ADX+pla. **(E) Arc protein expression during the test phase:** Two-way ANOVA: surgery ($F(1, 29) = 1,591$; $P = 0,2172$), treatment ($F(1, 29) = 0,7737$; $P = 0,3863$), interaction ($F(1, 29) = 7,545$; $P =$

0,0102). Post hoc test: $^*P < 0.05$ versus sham+pla; $^+P < 0.05$ versus sham+mor. **(F)**

Correlation analysis between aversion score and Arc protein. (G-H) Representative confocal microphotographs showing coronal sections of rats immunostained in: red (**Arc**), green (GR) and blue (DAPI, nuclear stain); scale bar, 50 μ m. Merged images are also depicted.

Figure 6. Corticosterone secretion and correlation analysis between GR and pCREB, Egr-1 mRNA and Arc mRNA during the conditioning and test phase. (A) Corticosterone levels during the conditioning phase: Two-way ANOVA: surgery ($F(1, 21) = 224,3$; $P < 0,0001$), treatment ($F(1, 21) = 244,2$; $P < 0,0001$), interaction ($F(1, 21) = 236,9$; $P < 0,0001$). Post hoc test: $^{***}P < 0.001$ versus sham+pla; $^{+++}P < 0.001$ versus sham+mor. **(B) Corticosterone levels during the test phase:** Two-way ANOVA: surgery ($F(1, 48) = 1,732$; $P = 0,1944$), treatment ($F(1, 48) = 0,02623$; $P = 0,8720$), interaction ($F(1, 48) = 0,01722$; $P = 0,8962$). **(C-H) Correlation analysis during the conditioning phase.** GR was positively correlated with pCREB, Egr-1 mRNA and Arc mRNA in sham+morphine animals, whereas a trend for negative correlation was observed in ADX+morphine group. **(I-N) Correlation analysis during the test phase.** No relationship was observed, except a negative correlation between GR and pCREB in ADX+morphine rodents. **(O-P) Table summarizing the results obtained in the different experimental groups during the conditioning phase (O) and test phase (P).**

Figure 7. Identification of Arc in pCREB- and Egr-1-expressing cells and correlation analysis between pCREB or Egr-1 and Arc mRNA/protein during the test phase. (A-B)

Representative confocal microphotographs showing coronal sections of rats immunostained in: red (Arc), green (pCREB) and blue (DAPI, nuclear stain); scale bar, 50 μ m. Merged images are also depicted. Confocal images demonstrated that all Arc neurons in sham or ADX morphine-dependent animals were pCREB positive. **(C-D)** Representative confocal microphotographs showing coronal sections of rats immunostained in: red (Arc), green (Egr-1) and blue (DAPI, nuclear stain); scale bar, 50 μ m. Merged images are also depicted. Immunofluorescence experiments showed that Arc-expressing neurons in sham or ADX morphine-dependent animals were Egr-1⁺. **(E-I) Correlation analysis between pCREB and Arc mRNA/protein levels.** Positive correlation between pCREB and Arc mRNA was observed only in sham+morphine animals, whereas Arc protein was positively correlated in both morphine-dependent groups. **(J) Correlation analysis between Egr-1 and Arc mRNA/protein levels.** No relationship was detected.

Figure 8. Identification of different cellular subtypes that express Arc during memory retrieval. Representative confocal microphotographs showing coronal sections of rats immunostained in: red (Arc), green [GLS2 **(A)** or GAD **(B)**] and blue (DAPI, nuclear stain); scale bar, 50 μ m. Merged images are also depicted. **(A)** The majority of Arc-positive cells were glutamatergic neurons. **(B)** Arc was stained only in few GABAergic neurons. White arrows: Arc⁺ and GLS2⁺ or GAD⁺ neurons. Orange arrows: Arc⁺ and GLS2⁻ or GAD⁻ neurons. Blue arrows: Arc⁻ and GAD⁺ neurons.

References

- Abraham WC, Mason SE, Demmer J, Williams JM, Richardson CL, Tate WP, Lawlor PA, Dragunow M (1993) Correlations between immediate early gene induction and the persistence of long-term potentiation. *Neuroscience* 56:717-727
- Besnard A, Laroche S, Caboche J (2014) Comparative dynamics of MAPK/ERK signalling components and immediate early genes in the hippocampus and amygdala following contextual fear conditioning and retrieval. *Brain Struct Funct* 219:415-430
- Blair HT, Sotres-Bayon F, Moita MAP, LeDoux JE (2005) The lateral amygdala processes the value of conditioned and unconditioned aversive stimuli. *Neuroscience* 133:561-569
- Bourtchuladze R, Frenguelli B, Blendy J, Cioffi D, Schutz G, Silva AJ (1994) Deficient long-term memory in mice with a targeted mutation of the cAMP-responsive element-binding protein. *Cell* 79:59-68
- Bramham CR, Alme MN, Bittins M, Kuipers SD, Nair RR, Pai B, Panja D, Schubert M, Soule J, Tiron A, Wibrand K (2010) The Arc of synaptic memory. *Experimental Brain Research Experimentelle Hirnforschung Experimentation Cerebrale* 200:125-140
- Chandrasekar V, Dreyer JL (2009) microRNAs miR-124, let-7d and miR-181a regulate Cocaine-induced Plasticity. *Molecular and Cellular Neuroscience* 42:350-362
- Chandrasekar V, Dreyer JL (2011) Regulation of MiR-124, Let-7d, and MiR-181a in the Accumbens Affects the Expression, Extinction, and Reinstatement of Cocaine-Induced Conditioned Place Preference. *Neuropsychopharmacology* 36:1149-1164
- Changelian PS, Feng P, King TC, Milbrandt J (1989) Structure of the NGFI-A gene and detection of upstream sequences responsible for its transcriptional induction by nerve growth factor. *Proc Natl Acad Sci U S A* 86:377-381
- Chen DY, Bambah-Mukku D, Pollonini G, Alberini CM (2012) Glucocorticoid receptors recruit the CaMKII[alpha]-BDNF-CREB pathways to mediate memory consolidation. *Nat Neurosci* 15:1707-1714
- Contarino A, Papaleo F (2005) The corticotropin-releasing factor receptor-1 pathway mediates the negative affective states of opioid withdrawal. *PNAS* 102:18649-18654
- Dash PK, Hochner B, Kandel ER (1990) Injection of the cAMP-responsive element into the nucleus of Aplysia sensory neurons blocks long-term facilitation. *Nature* 345:718-721
- De Cesare D, Sassone-Corsi P (2000) Transcriptional regulation by cyclic AMP-responsive factors. *Prog Nucl Acid Res Mol Biol* 64:343-369
- de Kloet ER, Joels M, Holsboer F (2005) Stress and the brain: from adaptation to disease. *Nat Rev Neurosci* 6:463-475
- de Quervain DJF, Roozendaal B, McGaugh JL (1998) Stress and glucocorticoids impair retrieval of long-term spatial memory. *Nature* 394:787-790

- Diamond DM, Park CR, Heman KL, Rose GM (1999) Exposing rats to a predator impairs spatial working memory in the radial arm water maze. *Hippocampus* 9:542-552
- Duvarci S, Nader K, LeDoux JE (2005) Activation of extracellular signal-regulated kinase-mitogen-activated protein kinase cascade in the amygdala is required for memory reconsolidation of auditory fear conditioning. *Eur J Neurosci* 21:283-289
- Everitt BJ, Cardinal RN, Parkinson JA, Robbins TW (2003) Appetitive Behavior: Impact of Amygdala-Dependent Mechanisms of Emotional Learning. *Ann NY Acad Sci* 985:233-250
- Finsterwald C, Alberini CM (2014) Stress and glucocorticoid receptor-dependent mechanisms in long-term memory: from adaptive responses to psychopathologies. *Neurobiology of Learning and Memory* 0:17-29
- Frenois F, Cador M, Caille S, Stinus L, Le Moine C (2002) Neural correlates of the motivational and somatic components of naloxone-precipitated morphine withdrawal. *Eur J Neurosci* 16:1377-1389
- Frenois F, Le Moine C, Cador M (2005a) The motivational component of withdrawal in opiate addiction: role of associative learning and aversive memory in opiate addiction from a behavioral, anatomical and functional perspective. *Rev Neurosci* 16:255-276
- Frenois F, Stinus L, Di Blasi F, Cador M, Le Moine C (2005b) A Specific Limbic Circuit Underlies Opiate Withdrawal Memories. *The Journal of Neuroscience* 25:1366-1374
- García-Pérez D, Laorden ML, Milanes MV, Nuñez C (2012) Glucocorticoids Regulation of FosB/DFosB Expression Induced by Chronic Opiate Exposure in the Brain Stress System. *PLoS ONE* 7:e50264
- García-Pérez D, López-Bellido R, Hidalgo JM, Rodríguez RE, Laorden ML, Núñez C, Milanes MV (2015) Morphine regulates Argonaute 2 and TH expression and activity but not miR-133b in midbrain dopaminergic neurons. *Addiction Biology* 20:104-119
- Groeneweg FL, Karst H, de Kloet ER, Joels M (2011) Rapid non-genomic effects of corticosteroids and their role in the central stress response. *J Endocrinol* 209:153-167
- Guzowski JF, Lyford GL, Stevenson GD, Houston FP, McGaugh JL, Worley PF, Barnes CA (2000) Inhibition of Activity-Dependent Arc Protein Expression in the Rat Hippocampus Impairs the Maintenance of Long-Term Potentiation and the Consolidation of Long-Term Memory. *The Journal of Neuroscience* 20:3993-4001
- Guzowski JF, Setlow B, Wagner EK, McGaugh JL (2001) Experience-Dependent Gene Expression in the Rat Hippocampus after Spatial Learning: A Comparison of the Immediate-Early Genes Arc, c-fos, and zif268. *The Journal of Neuroscience* 21:5089-5098
- Hall J, Thomas KL, Everitt BJ (2001a) Cellular Imaging of zif268 Expression in the Hippocampus and Amygdala during Contextual and Cued Fear Memory Retrieval: Selective Activation of Hippocampal CA1 Neurons during the Recall of Contextual Memories. *The Journal of Neuroscience* 21:2186-2193

- Hall J, Thomas KL, Everitt BJ (2001b) Fear memory retrieval induces CREB phosphorylation and Fos expression within the amygdala. *Eur J Neurosci* 13:1453-1458
- Hellemans KGC, Everitt BJ, Lee JLC (2006) Disrupting Reconsolidation of Conditioned Withdrawal Memories in the Basolateral Amygdala Reduces Suppression of Heroin Seeking in Rats. *The Journal of Neuroscience* 26:12694-12699
- Hoffman AN, Parga A, Paode PR, Watterson LR, Nikulina EM, Hammer J, Conrad CD (2015) Chronic stress enhanced fear memories are associated with increased amygdala zif268 mRNA expression and are resistant to reconsolidation. *Neurobiology of Learning and Memory* 120:61-68
- Hou YY, Lu B, Li M, Liu Y, Chen J, Chi ZQ, Liu JG (2009) Involvement of Actin Rearrangements within the Amygdala and the Dorsal Hippocampus in Aversive Memories of Drug Withdrawal in Acute Morphine-Dependent Rats. *The Journal of Neuroscience* 29:12244-12254
- Jones MW, Errington ML, French PJ, Fine A, Bliss TVP, Garel S, Charnay P, Bozon B, Laroche S, Davis S (2001) A requirement for the immediate early gene Zif268 in the expression of late LTP and long-term memories. *Nat Neurosci* 4:289-296
- Kawashima T, Okuno H, Nonaka M, Adachi-Morishima A, Kyo N, Okamura M, Takemoto-Kimura S, Worley PF, Bito H (2009) Synaptic activity-responsive element in the Arc/Arg3.1 promoter essential for synapse-to-nucleus signaling in activated neurons. *Proc Natl Acad Sci U S A* 106:316-321
- Kida S, Josselyn SA, de Ortiz SP, Kogan JH, Chevere I, Masushige S, Silva AJ (2002) CREB required for the stability of new and reactivated fear memories. *Nat Neurosci* 5:348-355
- Korb E, Finkbeiner S (2011) Arc in synaptic plasticity: from gene to behavior. *Trends in Neurosciences* 34:591-598
- LeDoux JE (2000) Emotion Circuits in the Brain. *Annu Rev Neurosci* 23:155-184
- Lee JLC, Di Ciano P, Thomas KL, Everitt BJ (2005) Disrupting Reconsolidation of Drug Memories Reduces Cocaine-Seeking Behavior. *Neuron* 47:795-801
- Lee JLC, Milton AL, Everitt BJ (2006) Cue-Induced Cocaine Seeking and Relapse Are Reduced by Disruption of Drug Memory Reconsolidation. *The Journal of Neuroscience* 26:5881-5887
- Li L, Carter J, Gao X, Whitehead J, Tourtellotte WG (2005) The Neuroplasticity-Associated Arc Gene Is a Direct Transcriptional Target of Early Growth Response (Egr) Transcription Factors. *Mol Cell Biol* 25:10286-10300
- Lucas M, Frenois F, Vouillac C, Stinus L, Cador M, Le Moine C (2008) Reactivity and plasticity in the amygdala nuclei during opiate withdrawal conditioning: differential expression of c-fos and arc immediate early genes. *Neuroscience* 154:1021-1033

- Lupien SJ, Maheu F, Tu M, Fiocco A, Schramek TE (2007) The effects of stress and stress hormones on human cognition: Implications for the field of brain and cognition. *Brain and Cognition* 65:209-237
- Maddox SA, Schafe GE (2011) The activity-regulated cytoskeletal-associated protein (Arc/Arg3.1) is required for reconsolidation of a Pavlovian fear memory. *J Neurosci* 31:7073-7082
- Mamiya N, Fukushima H, Suzuki A, Matsuyama Z, Homma S, Frankland PW, Kida S (2009) Brain Region-Specific Gene Expression Activation Required for Reconsolidation and Extinction of Contextual Fear Memory. *The Journal of Neuroscience* 29:402-413
- Mayr B, Montminy M (2001) Transcriptional regulation by the phosphorylation-dependent factor CREB. *Nat Rev Mol Cell Biol* 2:599-609
- McReynolds JR (2010) Memory-enhancing corticosterone treatment increases amygdala norepinephrine and Arc protein expression in hippocampal synaptic fractions. *Neurobiol Learn Mem* 93:312-321
- Messaoudi E, Kanhema T, Soulé J, Tiron A, Dągryte G, da Silva B, Bramham CR (2007) Sustained Arc/Arg3.1 synthesis controls long-term potentiation consolidation through regulation of local actin polymerization in the dentate gyrus in vivo. *J Neurosci* 27:10445-10455
- Miller RR, Matzel LD (2000) Memory involves far more than 'consolidation'. *Nat Rev Neurosci* 1:214-216
- Milton AL, Everitt BJ (2010) The psychological and neurochemical mechanisms of drug memory reconsolidation: implications for the treatment of addiction. *Eur J Neurosci* 31:2308-2319
- Molteni R, Calabrese F, Chourbaji S, Brandwein C, Racagni G, Gass P, Riva MA (2010) Depression-prone mice with reduced glucocorticoid receptor expression display an altered stress-dependent regulation of brain-derived neurotrophic factor and activity-regulated cytoskeleton-associated protein. *J Psychopharmacol* 24:595-603
- Mucha RF (1987) Is the motivational effect of opiate withdrawal reflected by common somatic indices of precipitated withdrawal? A place conditioning study in the rat. *Brain Res* 418:214-220
- Myers KM, Bechtholt-Gompf AJ, Coleman BR, Carlezon WA (2012) Extinction of conditioned opiate withdrawal in rats in a two-chambered place conditioning apparatus. *Nat Protocols* 7:517-526
- Nader K, Schafe GE, Le Doux JE (2000) Fear memories require protein synthesis in the amygdala for reconsolidation after retrieval. *Nature* 406:722-726
- Noori HR, Spanagel R, Hansson AC (2012) Neurocircuitry for modeling drug effects. *Addiction Biology* 17:827-864

- O'Brien CP, Testa T, O'Brien TJ, Brady JP, Wells B (1977) Conditioned narcotic withdrawal in humans. *Science* 195:1000-1002
- Papaleo F, Kitchener P, Contarino A (2007) Disruption of the CRF/CRF(1) receptor stress system exacerbates the somatic signs of opiate withdrawal. *Neuron* 53:577-589
- Paxinos G, Watson C (2007) The rat brain in stereotaxic coordinates. Academic Press, Amsterdam
- Pitkänen A, Savander V, LeDoux JE (1997) Organization of intra-amygdaloid circuitries in the rat: an emerging framework for understanding functions of the amygdala. *Trends in Neurosciences* 20:517-523
- Plath N, Ohana O, Dammermann B, Errington ML, Schmitz D, Gross C, Mao X, Engelsberg A, Mahlke C, Welzl H, Kobalz U, Stawrakakis A, Fernandez E, Waltereit R, Bick-Sander A, Therstappen E, Cooke S, Blanquet V, Wurst W, Salmen B, B+Äsl M, Lipp HP, Grant S, Bliss TVP, Wolfer DP, Kuhl D (2006) Arc/Arg3.1 Is Essential for the Consolidation of Synaptic Plasticity and Memories. *Neuron* 52:437-444
- Ploski JE, Pierre VJ, Smucny J, Park K, Monsey MS, Overeem KA, Schafe GE (2008) The Activity-Regulated Cytoskeletal-Associated Protein (Arc/Arg3.1) Is Required for Memory Consolidation of Pavlovian Fear Conditioning in the Lateral Amygdala. *The Journal of Neuroscience* 28:12383-12395
- Polyák Á, Ferenczi S, Dénes A, Winkler Z, Kriszt R, Pintér-Kübler B, Kovács KJ (2014) The fractalkine/Cx3CR1 system is implicated in the development of metabolic visceral adipose tissue inflammation in obesity. *Brain, Behavior, and Immunity* 38:25-35
- Rajasethupathy P, Fiumara F, Sheridan R, Betel D, Puthanveetil SV, Russo JJ, Sander C, Tuschl T, Kandel E (2009) Characterization of small RNAs in *Aplysia* reveals a role for miR-124 in constraining synaptic plasticity through CREB. *Neuron* 63:803-817
- Ramírez-Amaya V, Vazdarjanova A, Mikhael D, Rosi S, Worley PF, Barnes CA (2005) Spatial Exploration-Induced Arc mRNA and Protein Expression: Evidence for Selective, Network-Specific Reactivation. *The Journal of Neuroscience* 25:1761-1768
- Reijmers LG, Perkins BL, Matsuo N, Mayford M (2007) Localization of a Stable Neural Correlate of Associative Memory. *Science* 317:1230-1233
- Revest JM (2005) The MAPK pathway and Egr-1 mediate stress-related behavioral effects of glucocorticoids. *Nat Neurosci* 8:664-672
- Revest JM, Kaouane N, Mondin M, Le Roux A, Rougé-Pont F, Vallée M, Barik J, Tronche F, Desmedt A, Piazza PV (2010) The enhancement of stress-related memory by glucocorticoids depends on synapsin-Ia/Ib. *Mol Psychiatry* 15:1140-1151
- Robbins TW, Ersche KD, Everitt BJ (2008) Drug Addiction and the Memory Systems of the Brain. *Ann NY Acad Sci* 1141:1-21

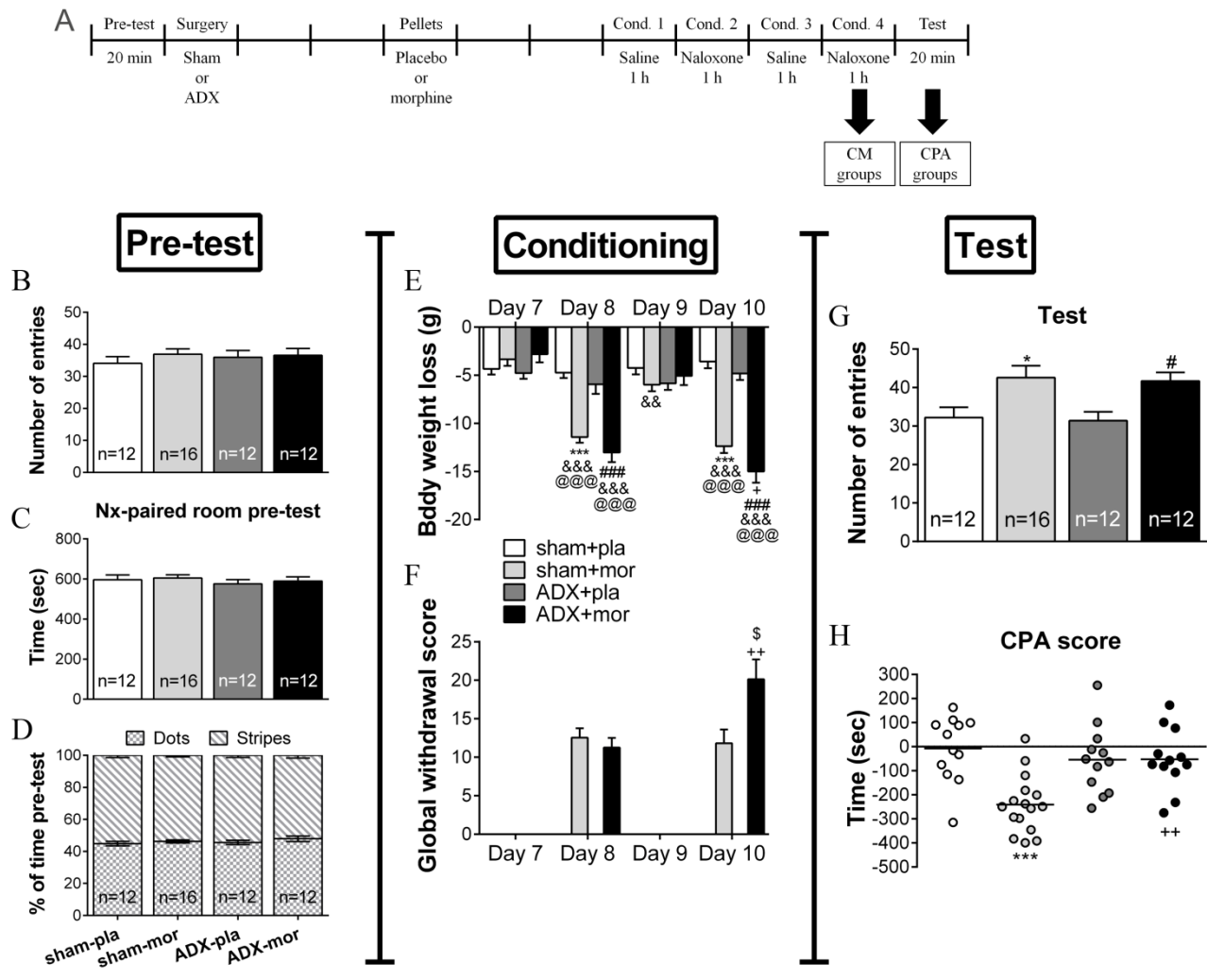
- Roozendaal B, Hahn EL, Nathan SV, de Quervain DJF, McGaugh JL (2004) Glucocorticoid Effects on Memory Retrieval Require Concurrent Noradrenergic Activity in the Hippocampus and Basolateral Amygdala. *The Journal of Neuroscience* 24:8161-8169
- Roozendaal B, McGaugh JL (1997) Glucocorticoid Receptor Agonist and Antagonist Administration into the Basolateral but Not Central Amygdala Modulates Memory Storage. *Neurobiology of Learning and Memory* 67:176-179
- Roozendaal B, McGaugh JL (2011) Memory modulation. *Behav Neurosci* 125:797-824
- Sanchez H, Quinn JJ, Torregrossa MM, Taylor JR (2010) Reconsolidation of a cocaine-associated stimulus requires amygdalar protein kinase A. *J Neurosci* 30:4401-4407
- Schratt GM, Tuebing F, Nigh EA, Kane CG, Sabatini ME, Kiebler M, Greenberg ME (2006) A brain-specific microRNA regulates dendritic spine development. *Nature* 439:283-289
- Schwabe L, Jo^{+1/2}ls M, Roozendaal B, Wolf OT, Oitzl MS (2012) Stress effects on memory: An update and integration. *Neuroscience & Biobehavioral Reviews* 36:1740-1749
- Shaywitz AJ, Greenberg ME (1999) CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. *Annu Rev Biochem* 68:821-861
- Smith GW, Aubry JM, Dellu F, Contarino A, Bilezikjian LM, Gold LH, Chen R, Marchuk Y, Hauser C, Bentley CA, Sawchenko PE, Koob GF, Vale W, Lee KF (1998) Corticotropin Releasing Factor Receptor 1G δ Deficient Mice Display Decreased Anxiety, Impaired Stress Response, and Aberrant Neuroendocrine Development. *Neuron* 20:1093-1102
- Stinus L, Caille S, Koob GF (2000) Opiate withdrawal-induced place aversion lasts for up to 16 weeks. *Psychopharmacology* 149:115-120
- Thomas KL, Arroyo M, Everitt BJ (2003) Induction of the learning and plasticity-associated gene Zif268 following exposure to a discrete cocaine-associated stimulus. *Eur J Neurosci* 17:1964-1972
- Timpl P, Spanagel R, Sillaber I, Kresse A, Reul JM, Stalla GK, Blanquet V, Steckler T, Holsboer F, Wurst W (1998) Impaired stress response and reduced anxiety in mice lacking a functional corticotropin-releasing hormone receptor 1. *Nat Genet* 19:162-166
- Tronson NC, Taylor JR (2007) Molecular mechanisms of memory reconsolidation. *Nat Rev Neurosci* 8:262-275
- Tronson NC, Wiseman SL, Neve RL, Nestler EJ, Olausson P, Taylor JR (2012) Distinctive roles for amygdalar CREB in reconsolidation and extinction of fear memory. *Learning & Memory* 19:178-181
- Tronson NC, Wiseman SL, Olausson P, Taylor JR (2006) Bidirectional behavioral plasticity of memory reconsolidation depends on amygdalar protein kinase A. *Nat Neurosci* 9:167-169
- White NM, Carr GD (1985) The conditioned place preference is affected by two independent reinforcement processes. *Pharmacol Biochem Behav* 23:37-42

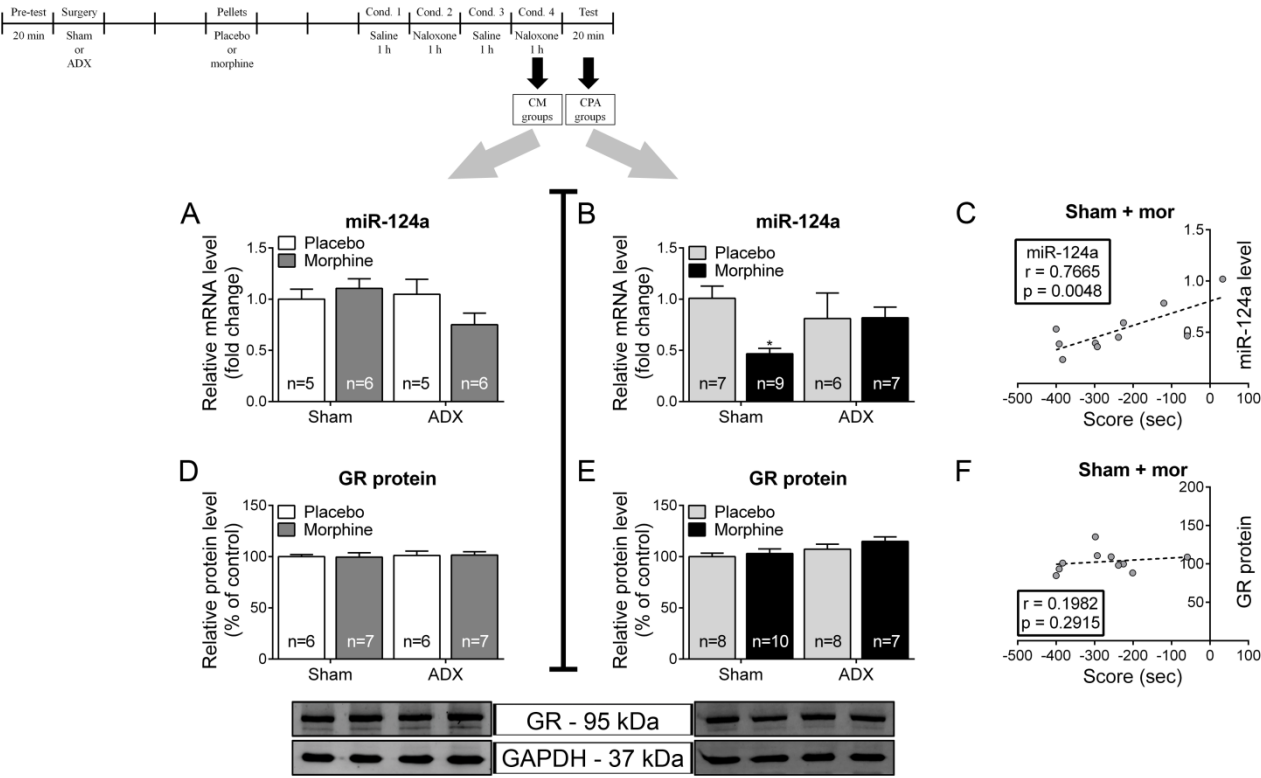
Worley PF, Bhat RV, Baraban JM, Erickson CA, McNaughton BL, Barnes CA (1993) Thresholds for synaptic activation of transcription factors in hippocampus: correlation with long-term enhancement. *The Journal of Neuroscience* 13:4776-4786

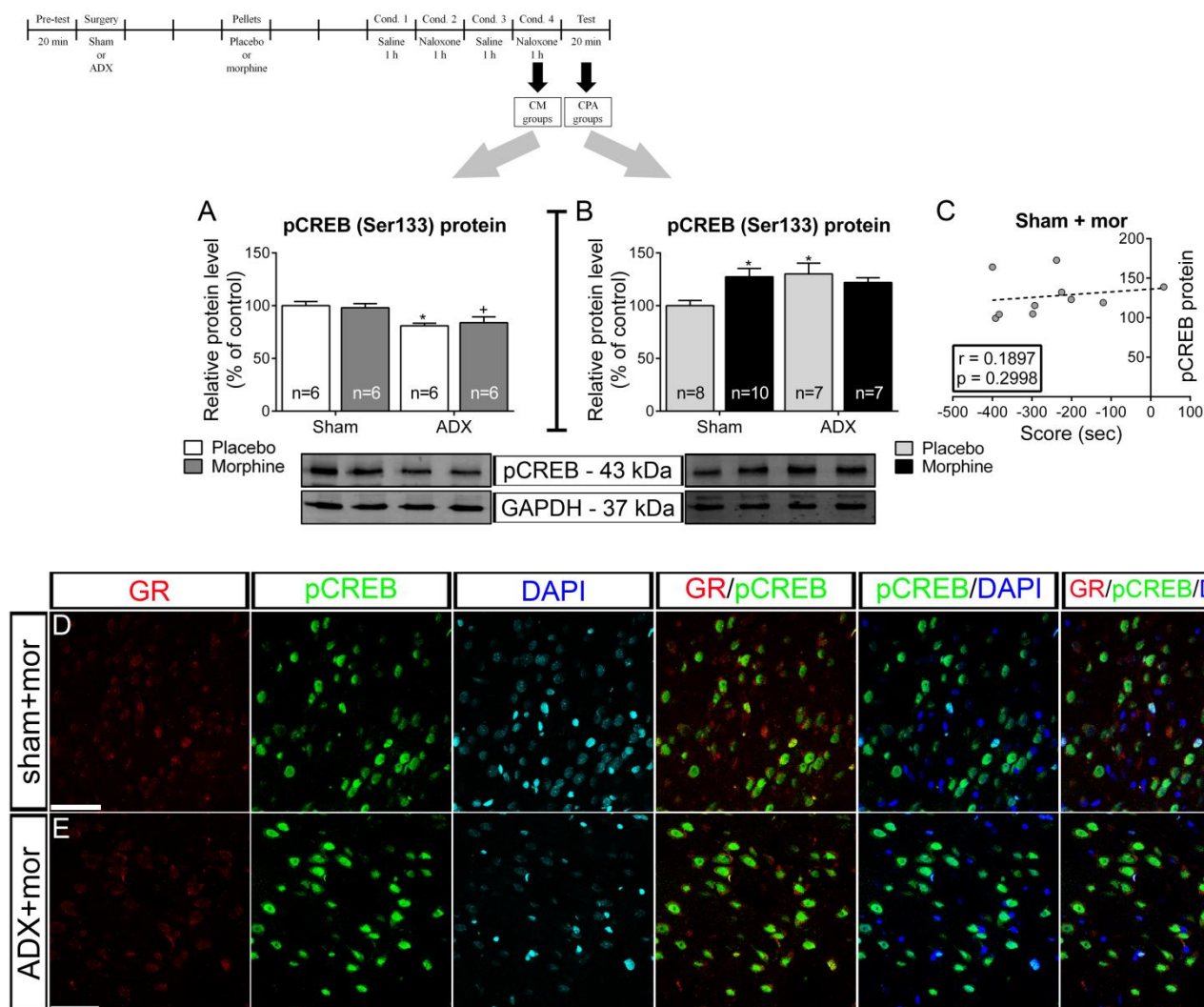
Yin JCP, Wallach JS, Del Vecchio M, Wilder EL, Zhou H, Quinn WG, Tully T (1994) Induction of a dominant negative CREB transgene specifically blocks long-term memory in *Drosophila*. *Cell* 79:49-58

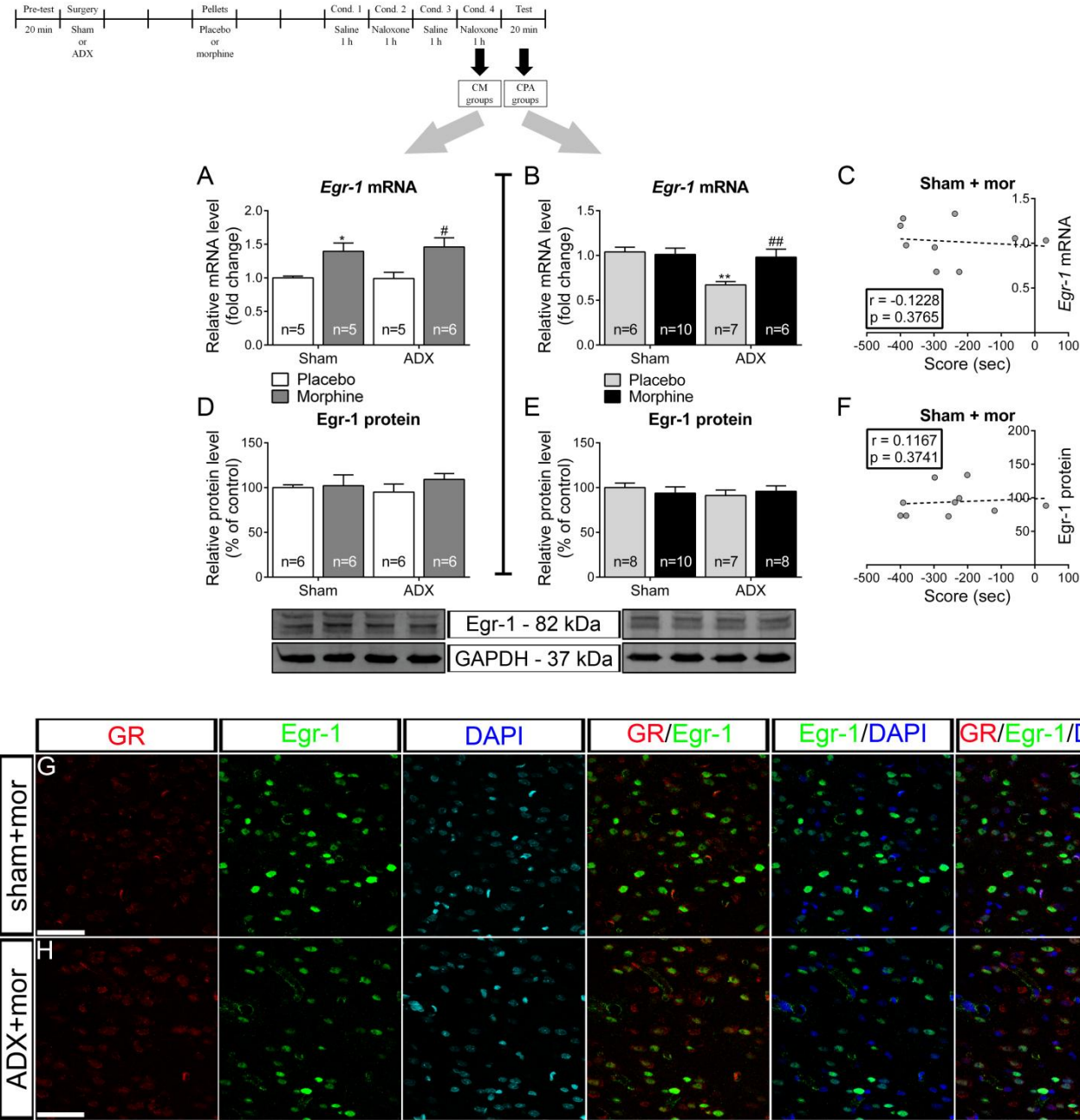
Zalachoras I, Houtman R, Meijer OC (2013) Understanding stress-effects in the brain via transcriptional signal transduction pathways. *Neuroscience* 242:97-109

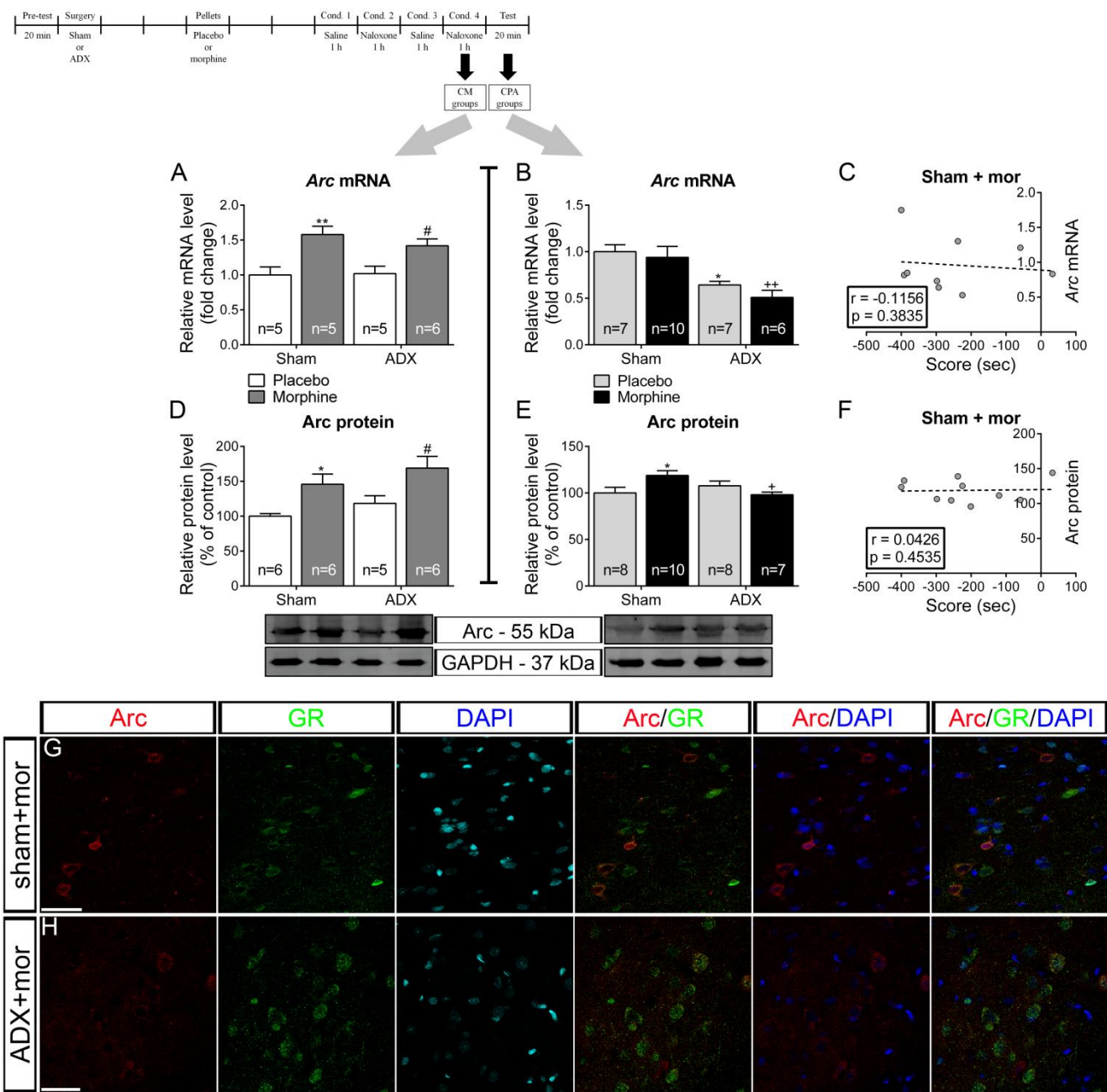
Zhang WP, Guzowski JF, Thomas SA (2005) Mapping neuronal activation and the influence of adrenergic signaling during contextual memory retrieval. *Learning & Memory* 12:239-247

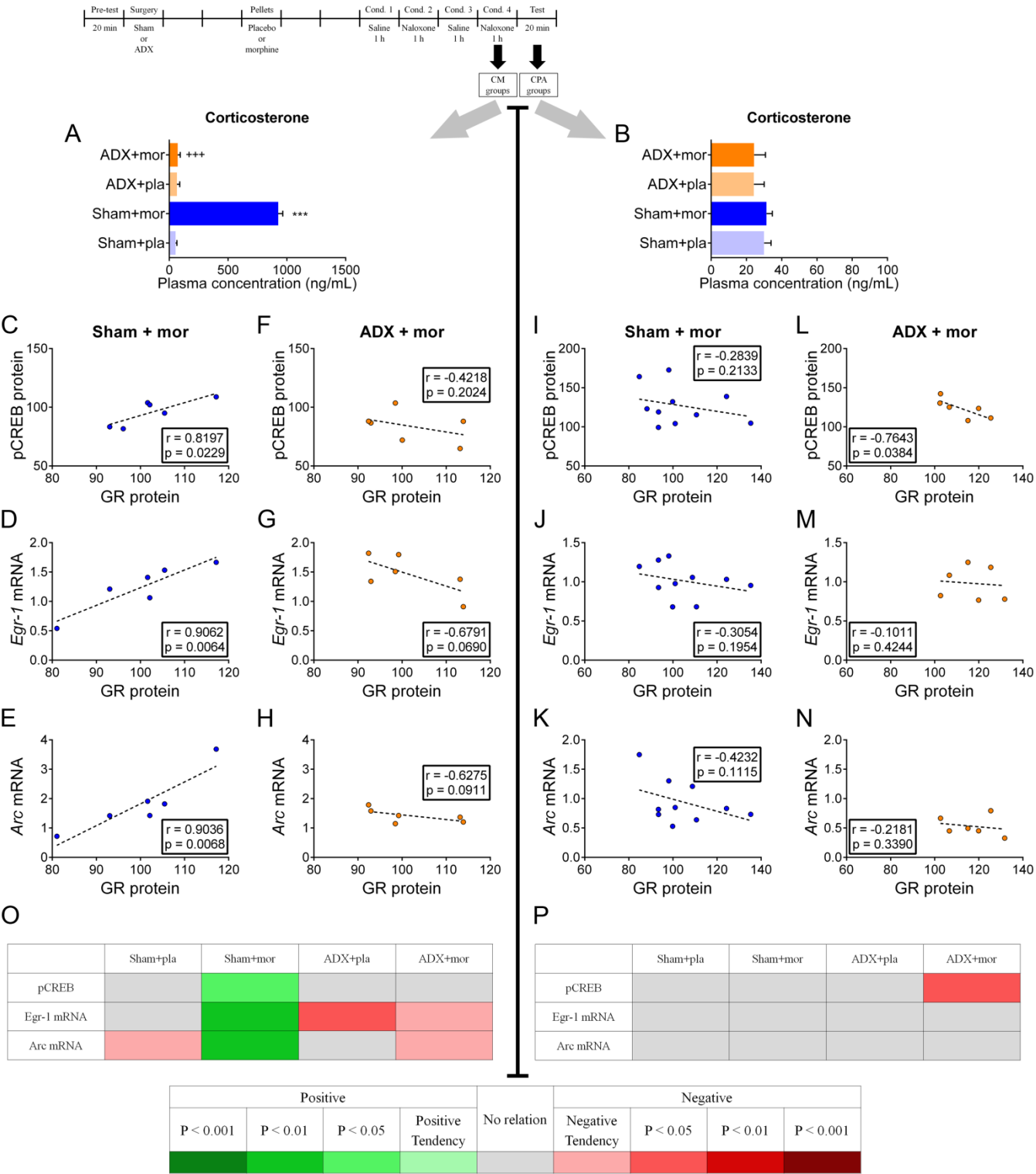


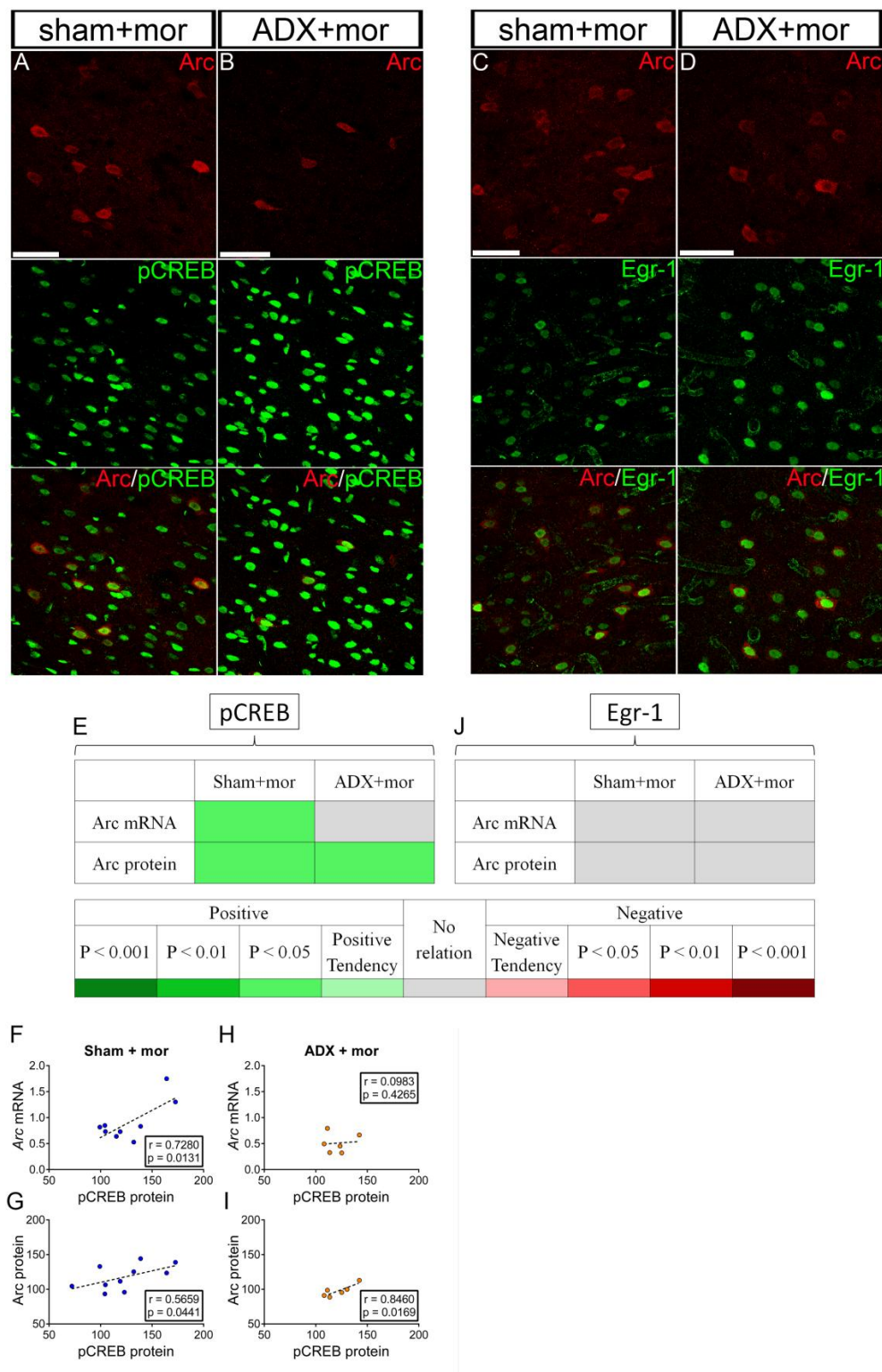


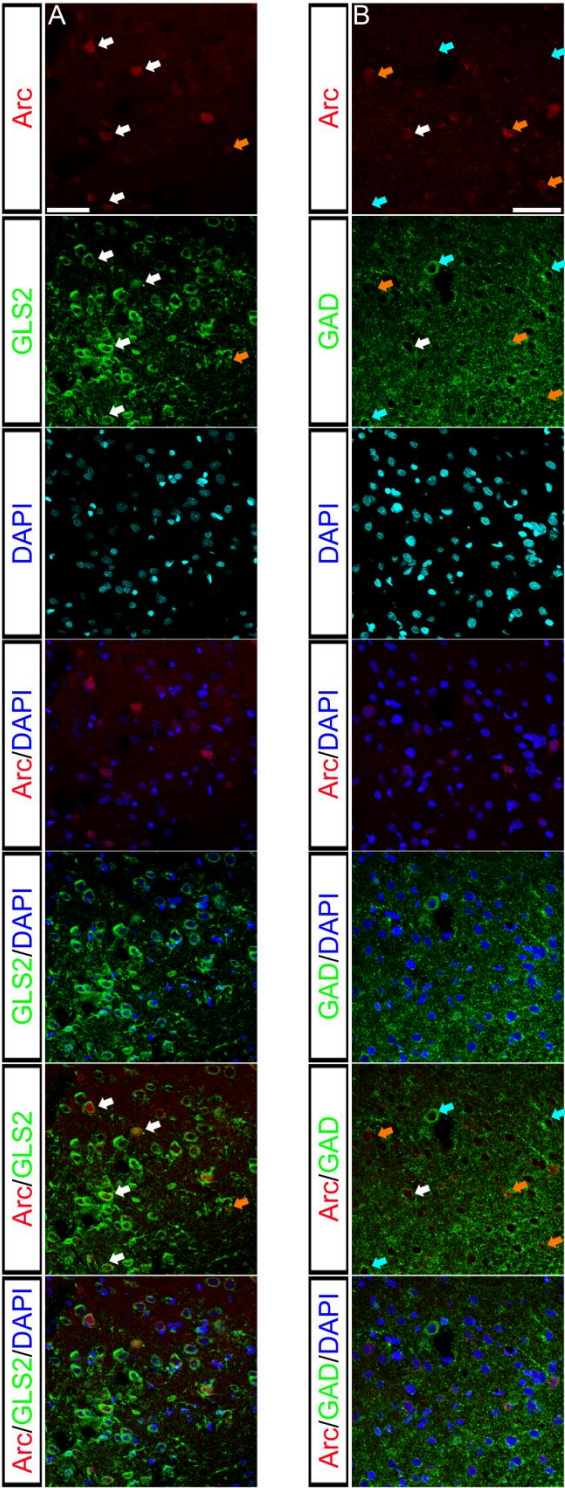












SUPPLEMENTAL INFORMATION

Supplemental materials and methods

Animals

All surgical and experimental procedures were performed in accordance with the European Community Council Directive (2010/63/UE), and were approved by the local Committees for animal research (REGA ES300305440012). Male Wistar rats ($n = 78$, Harlan, Barcelona, Spain) initially weighting 220–240 g were housed (2-3/cage) on arrival in a room with controlled temperature ($22 \pm 2^{\circ}\text{C}$) and humidity ($50 \pm 10\%$), with free access to water and food (Harlan Teklad standard rodent chow; Harlan Interfauna Ibérica, Barcelona, Spain). Animals were adapted to a standard 12 h light-dark cycle (lights on: 08:00 h – 20:00 h) for 7 days before the beginning of the experiments.

Adrenalectomy

Rats were bilaterally adrenalectomized (ADX) via a dorsal approach under 100 mg/kg ketamine chlorhydrate (Imalgene 1000, Merial, Lyon, France) and 8 mg/kg xylazin (Xilagesic® 2%, Lab. Calier S.A., Barcelona, Spain) intraperitoneally anaesthesia, and implanted s.c. with slow-release corticosterone pellets at surgery. The composition of steroid pellets (25 mg corticosterone plus 75 mg cholesterol) was chosen to provide stable corticosterone concentration corresponding to circadian nadir up to 20 days after implantation (Kovács et al. 2000). ADX rats with corticosterone replacement (ADX plus corticosterone) do not mount a challenge-induced increase of plasma corticosterone. After surgery, ADX plus corticosterone rats had free choice to drink isotonic saline (0.9% NaCl) to replace depleted

sodium secondary to the loss of aldosterone because of adrenalectomy. Control rats were subjected to the same surgical procedure (sham) without adrenal extirpation. Sham and ADX plus corticosterone rats were allowed to recover from surgery for 3 days before morphine or placebo pellet implantation. Successful bilateral adrenalectomy was confirmed by plasma concentration of corticosterone and ACTH and by post-mortem examination of the ADX animals.

Induction of Opiate Dependence

Morphine base was supplied from Alcaliber Laboratories (Madrid, Spain) in cooperation with the Área de Estupefacientes y Psicotropos, Agencia Española del Medicamento y de Productos Sanitarios (Madrid, Spain). Morphine dependence was induced by s.c. implantation (lower back) under light ether anaesthesia of two slow-release, morphine-containing pellets (each morphine pellet contains 75 mg of morphine base). Full dependence on morphine has been previously operationally defined in this model by using a complete naloxone dose effect on various behavioral parameters (spontaneous locomotor activity, operant responding for food, intracranial self-stimulation threshold, conditioned place aversion) (Schulteis et al. 1994), and the rating of abstinence signs showed that opiate dependence was achieved 24 h after implantation of the morphine pellets and remained constant for 15 d (Gold et al. 1994). There were four experimental groups: sham + placebo pellets, sham + morphine pellets, ADX + placebo pellets and ADX + morphine pellets.

Drugs

Naloxone hydrochloride (N-7758, Sigma Chemical Co, St Louis, MO, USA) was dissolved in sterile saline (0.9% NaCl; ERN Laboratories, Barcelona, Spain) and injected subcutaneously. Naloxone was administered at a dose of 0.1 mg/kg, 1 ml/kg body weight. Naloxone HCl doses are expressed as the weight of the salt. Sterile 0.9% saline was also injected subcutaneously at a dose of 1 ml/kg. Note that a slightly higher naloxone dose (0.120 mg/kg) has previously been shown to be without effect on place aversion conditioning in placebo control rats (Frenois et al. 2002).

Conditioned Place Aversion Apparatus

Briefly, the conditioned place aversion apparatus (Panlab, Barcelona, Spain) used to induce a reliable aversion consists in a box with two equally sized chambers (40 *L* x 34 *W* x 45 *H* cm) interconnected by a rectangular corridor (25 *L* x 13 *W* x 45 *H* cm). Distinctive visual and tactile cues distinguish the compartments: the motifs painted on the walls (either black dots or grey stripes), the floor colouring (black or grey) and the floor texture (smooth or rough). The sensory cues combination that produces a balanced choice are for walls and floor colouring and texture, respectively: (A) black dots, black smooth floor; (B) grey stripes, grey rough floor. Transparent walls are also used to minimize the time the animal spent in the corridor. The weight transducer technology (and PPCWIN software) allows for detection and analysis of animal position throughout the test and the number of entries in each compartment. The experimental protocol consists of three distinct phases: a preconditioning phase, a conditioning phase, and a testing phase. The weight gain of the rats was checked during the entire protocol to ensure that the morphine was liberated correctly from the pellets because it is known that chronic morphine treatment induces a decrease in body weight gain due to lower caloric intake (Houshyar et al. 2004; Núñez et al. 2009). In addition, the animals were

observed for body weight loss and opioid withdrawal behaviours for 15 min after the conditioning phase.

Conditioned Place Aversion Protocol

Pre-testing Phase

In the pre-testing phase (day 0), animals were placed in the central corridor and allowed to explore the apparatus freely for 20 min. Animals showing strong unconditioned aversion (less than 40% of the session time) or preference (more than 60% of the session time) for any compartment were discarded. For each rat, one room was randomly chosen to be paired with naloxone and the other chamber to vehicle. Importantly, after the compartment assignments were completed, there were no significant differences between time spent in the naloxone-paired and the vehicle-paired compartments during the preconditioning phase. This is an important step in the experimental procedure that avoids any preference bias prior to conditioning. Rats were adrenalectomized on day 1 and placebo or morphine pellets were implanted on day 4 according to the experimental protocol depicted on Figure 1A.

Conditioning Phase

In the second phase (conditioning), rats received injection of saline on days 7 and 9, prior to being confined to their preselected saline-paired compartment for 1 hour. On days 8 and 10, rats received 0.1 mg/kg s.c. of naloxone immediately prior to confinement in the naloxone-paired compartment for 1 hour. In addition, opioid withdrawal behaviours on sham or ADX animals receiving naloxone on days 8 and 10 were measured for 15 min after the conditioning phase. A cohort of animals, named conditioning memory rats (CM), were decapitated on day 10 (15 min after leaving the naloxone-paired compartment).

Testing Phase

The test was conducted on day 11, exactly as in the preconditioning phase (free access to each compartment for 20 min). The difference ($\Delta D = D - D0$) between the time spent in the naloxone-paired compartment after conditioning (D) minus the time spent in the same compartment before conditioning (preconditioning test $D0$) reflects the change of preference induced by opiate withdrawal. A negative score indicates a place aversion; a positive score indicates a place preference. Conditioned place aversion animals (CPA groups) were killed 1 hour after starting the testing phase.

Measurement of the withdrawal syndrome

Experiments were carried out in a quiet room. The observer was unaware of the drug combination used. Sham + mor and ADX + mor rats were individually placed into transparent plastic cages after the conditioning phase and observed continuously for the occurrence of somatic signs of opiate withdrawal up to 15 min on days 8 and 10 (naloxone injection). Subsequently, previously identified behavioural characteristics of the rat opiate abstinence syndrome (Lu et al. 2000) were evaluated, including jumping, wet-dog shakes, paw tremor, sniffing, writhing, tremor, ptosis, diarrhoea, mastication, piloerection, teeth chattering, salivation, rhinorrhea, chromodacryorrhea and scrabble. The number of jumping, wet-dog shakes, paw tremor, sniffing and writhing was counted as the number of events occurring during the total test time period (graded signs). Tremor, ptosis, diarrhoea, mastication, piloerection, teeth chattering, salivation, rhinorrhea, chromodacryorrhea and scrabble were scored 1 for appearance or 0 for non-appearance within each 5 min time. To obtain a comprehensive index of the severity of somatic opiate withdrawal including all the signs

examined, a global withdrawal score was calculated for each animal by giving each individual sign a relative weight as previously reported (Maldonado et al. 1996): jumping, x0.8; wet-dog shakes, x1; paw tremor, x0.35; sniffing, x0.5; writhing, x0.5; tremor, x1.5; ptosis, x1.5; diarrhoea, x1.5; mastication, x1.5; piloerection, x1.5; teeth chattering, x1.5; salivation, x1.5; rhinorrhea, x1.5; chromodacryorrhea, x1.5; and scrabble, x1.5. Body weight loss was determined as the difference between the weight determined immediately before naloxone injection and that determined 1 hour later, after the conditioning phase.

Preparation of tissue extract

Rats were decapitated on days 10 and 11 (between 10:00-12:00 h to avoid circadian variations in plasma levels of the hormones), the brains were rapidly removed, and stored immediately at -80°C until use for quantitative real-time PCR (qPCR) and Western blot analysis. Brains were sliced on a cryostat and kept at -20°C until each region of interest comes into the cutting plane. For BLA study, three consecutive 500-µm coronal slides were made corresponding to approximately -1.9 to -3.4 mm from bregma, according to the atlas of (Figure S1) (Paxinos and Watson 2007). The anatomical locations and boundaries of each region were determined using the rat brain Atlas of (Paxinos and Watson 2007). Bilateral punches of the BLA were collected into Eppendorf tubes, according to the method of (Leng et al. 2004).

RNA extraction and quantitative real-time PCR (qPCR)

One punch from the BLA was placed in an Eppendorf tube containing 30 µl of Trizol® reagent (Invitrogen Corp., Carlsbad, CA, USA) and rapidly stored at -80°C. Frozen brain

tissue samples were homogenized in Trizol® reagent (Invitrogen Corp., USA) and total RNA was isolated with QIAGEN miRNeasy Mini Kit (Qiagen, Valencia, CA, USA) according the manufacturer's instruction. To eliminate genomic DNA contamination DNase I treatment were used and 100 µl Rnase-free DNase I (1 unit DNase) (Thermo Scientific, USA) solution was added. Sample quality control and the quantitative analysis were carried out by NanoDrop (Thermo Scientific, USA). Amplification was not detected in the RT-minus controls. The cDNA synthesis was performed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Primers for the comparative Ct experiments were designed by Primer Express 3.0 Program. The primers (Microsynth, Balgach) were used in the Real-Time PCR reaction with Fast EvaGreen® qPCR Master Mix (Biotium, USA) on ABI StepOnePlus instrument was listed. The reverse transcription for microRNA assays were carried out by TaqMan MicroRNA Reverse Transcription Kit according the manufacturer's instruction. The microRNA expressions were detected using TaqMan MicroRNA assays.

Western Blotting

Four bilateral punches from VTA were placed in homogenization buffer. The Eppendorf tubes contained 100 µl of homogenization buffer. The tubes were frozen immediately on dry ice and stored at -80°C until assaying. Samples were sonicated, vortexed and sonicated again prior to centrifugation (6000× g; 10 minutes at 4°C). Samples containing equal quantities of total proteins (20–40 mg, depending on the protein of interest) were separated by 10% or 12% SDS-PAGE (depending on the molecular weight of the protein of interest) and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). Membranes were blocked in TBS containing 0.15 % Tween-20 (TBS-T), 1% BSA for 90 minutes at room temperature (RT), and incubated overnight at 4°C with the primary antibody diluted in 1% BSA in TBS-T. The primary antibodies used are referenced in Table S1. Blots were then washed and incubated for

90 min at RT in TBS-T with 1% BSA with appropriate horseradish peroxidase (HRP) conjugated secondary antibodies: goat anti-rabbit (1:5000; sc-2004, Santa Cruz Biotechnology) or goat anti-mouse (1:5000; sc-2005, Santa Cruz Biotechnology). After washing, immunoreactivity was detected with an enhanced chemiluminescent/chemifluorescent western blot detection system (ECL Plus, GE Healthcare, UK) and visualized by a Typhoon 9410 variable mode Imager (GE Healthcare). We used GAPDH as our loading control. Before reprobing, blots were stripped by incubation with stripping buffer (glycine 25 mM and SDS 1%, pH 2) for 1 h at 37°C. Blots were subsequently reblocked and probed with GAPDH. The ratios of protein of interest/GAPDH were plotted and analyzed. Protein levels were corrected for individual levels.

Table S1. Primary antibodies used in this study

| Antibody | Host | Supplier | Catalog Number | WB dilution | IF dilution |
|-----------|--------|--------------------------------|----------------|-------------|-------------|
| GR | Rabbit | Santa Cruz Biotechnology | sc-1004 | 1:500 | 1:600 |
| pCREB | Rabbit | Millipore | # 06-519 | 1:2,000 | 1:3,000 |
| Egr-1 | Rabbit | Santa Cruz Biotechnology | sc-189 | 1:500 | 1:400 |
| Arc | Mouse | Santa Cruz Biotechnology | sc-17839 | 1:500 | 1:400 |
| GAPDH | Rabbit | Cell Signaling Technology Inc. | #2118 | 1:5,000 | -- |
| GR | Mouse | Abcam | ab2768 | -- | 1:600 |
| GLS2 | Rabbit | Abcam | ab113509 | -- | 1:1,000 |
| GAD-65/67 | Goat | Santa Cruz Biotechnology | sc-7513 | -- | 1:200 |
| CRF | Rabbit | Provided by Wylie W. Vale | -- | -- | 1:500 |

Immunofluorescence study

Another set of rats was deeply anaesthetised with an overdose of pentobarbital (100 mg/kg i.p.) and perfused transcardially with 250 ml 0.9% saline followed by 500 ml cold fixative solution containing paraformaldehyde (4% paraformaldehyde in 0.1 M borate buffer, pH 9.5). After removal of the perfused brains, they were post fixed in the same fixative solution containing sucrose (30%) for 3 h and stored at 4°C in PBS containing 30% sucrose until coronal sections (30-µm thickness) were cut rostrocaudally on a freezing microtome (Leica, Nussloch, Germany). The atlas of Paxinos and Watson (2007) (Paxinos and Watson 2007) was used to identify different brain regions. The sections were cryoprotected and stored at -20°C until use.

Brain sections were rinsed in PBS and an antigen retrieval procedure was applied by treating sections with citrate buffer (10 mM citric acid in 0.05% Tween 20, pH 6.0) at 60°C for 20 min. Non-specific Fc binding sites were blocked with 3% normal horse serum/0.3% Triton-X-100 in PBS for 1 h at RT, and the sections were incubated for 72 h (4°C, constant shaking) with primary antibodies described in Table S1. Alexa Fluor 488 donkey anti-rabbit IgG (1:1000; A-21206, Invitrogen, Eugene, OR, USA), Alexa Fluor 488 donkey anti-goat IgG (1:1000; A-11055, Invitrogen), Alexa Fluor 594 donkey anti-goat IgG (1:1000; A-11058, Invitrogen) and Alexa Fluor 594 donkey anti-mouse IgG (1:1000; A-21203, Invitrogen) labelled secondary antibodies were applied for 4 h. After washing, sections were incubated in 4, 6-diamino-2-phenylindole (DAPI, 1:100,000) for 1 min and the sections were mounted in ProLong® Gold antifade reagent (Invitrogen).

Confocal analysis

The brain sections were examined using a Leica DMIRE2 confocal microscope and Leica Confocal Software (Leica Microsystems). Images from the BLA were captured from low magnification to high magnification (40X to 63X oil objective). Confocal images were obtained using 405-nm excitation for DAPI, 488-nm excitation for Alexa Fluor 488 and 594-nm excitation for Alexa Fluor 594. Emitted light was detected in the range of 450-460 nm for DAPI, 515-530 nm for Alexa Fluor 488 and 610-630 nm for Alexa Fluor 594. Every channel was captured separately to avoid spectral crosstalk. Series of optical sections were performed determining an upper and lower threshold using the Z/Y position for Spatial Image Series setting. The optical series covered 20 μ m of thickness through the tissue. The confocal microscope settings were established and maintained by Leica and local technicians for optimal resolution.

Radioimmunoassay

Blood was collected on days 10 and 11 into ice-cooled tubes containing 5% EDTA and was then centrifuged (500 g; 4°C; 15 min). Plasma was separated, divided into two aliquots and stored at -80°C until assayed for corticosterone or ACTH. Plasma concentration of corticosterone and ACTH were quantified using specific corticosterone and ACTH antibodies for rats ([¹²⁵I]-CORT and [¹²⁵I]-ACTH RIA; MP Biomedicals, Orangeburg, NY, USA). The sensitivity of the assay was 7.7 ng/mL for corticosterone and 5.7 pg/mL for ACTH.

Reagents

Protease inhibitors were purchased from Boehringer Mannheim, (Mannheim, Germany); phosphatase inhibitor Cocktail Set was purchased from Calbiochem (Darmstadt, Germany); HPLC reagents were purchased from Sigma Chemical Co.

Data Analysis

Data are presented as mean \pm standard error of the mean (SEM). Data were analyzed using one-way or two-way analysis of variance (ANOVA) followed by a *post hoc* Newman–Keuls test to determine specific group differences. Student's *t*-test was used when comparisons were restricted to two experimental groups. Correlations between different parameters were assessed using linear regression. Differences with a $p < 0.05$ were considered significant. Statistical analyses were performed with GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA).

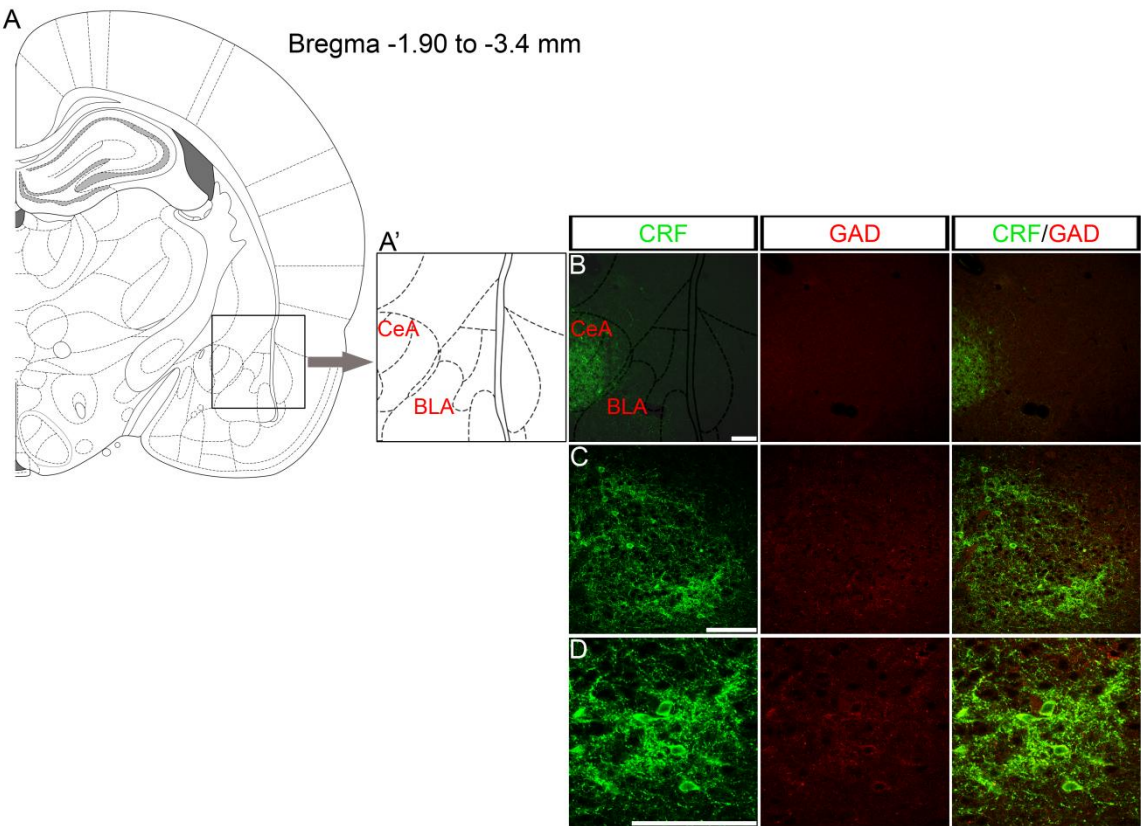
SUPPLEMENTAL REFERENCES

- Bajo M, Cruz MT, Siggins GR, Messing R, Roberto M (2008). Protein kinase C epsilon mediation of CRF- and ethanol-induced GABA release in central amygdala. *Proc Natl Acad Sci U S A* 105: 8410-8415.
- Day HEW, Curran EJ, Watson SJ, Akil H (1999). Distinct neurochemical populations in the rat central nucleus of the amygdala and bed nucleus of the stria terminalis: Evidence for their selective activation by interleukin-1 β . *J Comp Neurol* 413: 113-128.
- Frenois F, Cador M, Caille S, Stinus L, Le Moine C (2002). Neural correlates of the motivational and somatic components of naloxone-precipitated morphine withdrawal. *Eur J Neurosci* 16: 1377-1389.
- Gold LH, Stinus L, Inturrisi CE, Koob GF (1994). Prolonged tolerance, dependence and abstinence following subcutaneous morphine pellet implantation in the rat. *Eur J Pharmacol* 253: 45-51.
- Houshyar H, Manalo S, Dallman MF (2004). Time-Dependent Alterations in mRNA Expression of Brain Neuropeptides Regulating Energy Balance and Hypothalamo-Pituitary-Adrenal Activity after Withdrawal from Intermittent Morphine Treatment. *J Neurosci* 24: 9414-9424.
- Kovács KJ, Földes A, Sawchenko PE (2000). Glucocorticoid negative feedback selectively targets vasopressin transcription in parvocellular neurosecretory neurons. *J Neurosci* 20: 3843-3852.
- Leng A, Feldon J, Ferger B (2004). Long-term social isolation and medial prefrontal cortex: dopaminergic and cholinergic neurotransmission. *Pharmacol Biochem Behav* 77: 371-379.
- Lu L, Liu D, Ceng X, Ma L (2000). Differential role of corticotropin-releasing factor receptor subtypes 1 and 2 in opiate withdrawal and in relapse to opiate dependence. *Eur J Neurosci* 12: 4398-4404.
- Maldonado R, Blendy JA, Tzavara E, Gass P, Roques BP, Haoune J, *et al.* (1996). Reduction of morphine abstinence in mice with a mutation in the gene encoding CREB. *Science* 273: 657-659.
- Meister B, Hökfelt T, Geffard M, Oertel W (1988). Glutamic acid decarboxylase- and gamma-aminobutyric acid-like immunoreactivities in corticotropin-releasing factor-containing parvocellular neurons of the hypothalamic paraventricular nucleus. *Neuroendocrinology* 48: 516-526.
- Núñez C, Földes A, Pérez-Flores D, García-Borrón JC, Laorden ML, Kovács KJ, *et al.* (2009). Elevated glucocorticoid levels are responsible for induction of tyrosine hydroxylase (TH) mRNA expression, phosphorylation and enzyme activity in the nucleus of the solitary tract (NTS-A2) during morphine withdrawal. *Endocrinology* 150: 3118-3127.
- Paxinos G, Watson C (2007): *The rat brain in stereotaxic coordinates*. Academic Press: Amsterdam.

Schulteis G, Markou A, Gold LH, Stinus L, Koob GF (1994). Relative sensitivity to naloxone of multiple indices of opiate withdrawal: a quantitative dose-response analysis. *J Pharmacol Exp Ther* 271: 1391-1398.

Legends to supplemental figures

Figure S1: (A-A') The analysed region within BLA is schematically illustrated (diagram modified from Paxinos and Watson, 2007); coordinate is mm from Bregma. **(B-D)** Example of low and high magnification micrographs showing coronal section of rats immunostained for CRF (green) and glutamic acid decarboxylase 65/67 (GAD-65/67; red); scale bar, 100 μ m. Merged images are also depicted. CRF neurons, which are selectively expressed in the CeA, were immunolabelled to serve as a guide to study the BLA (BLA is located lateral to the CeA). There are two forms of GADs that are found in the brain: GAD-65 and GAD-67. GAD-65 and GAD-67 are members of the group II decarboxylase family of proteins and are responsible for catalyzing the rate limiting step in the production of GABA from L-glutamic acid. GAD-67 is responsible for the basal levels of GABA synthesis. In the case of a heightened demand for GABA in neurotransmission, GAD-65 will transiently activate to assist in GABA production. The antibody that we used is recommended for detection of GAD-65 and GAD-67 of rat origin, thus is a good marker for GABAergic neurons. In addition, we observed that CRF⁺ neurons co-expressed GAD, as previously described in the literature (Meister et al. 1988; Day et al. 1999; Bajo et al. 2008).



VIII. REFERENCES

1. Corbett AD, Henderson G, McKnight AT, Paterson SJ (2006): 75 years of opioid research: the exciting but vain quest for the Holy Grail. *Br J Pharmacol* 147:S153-S162.
2. Piazza P, Deroche-Gamonet V (2013): A multistep general theory of transition to addiction. *Psychopharmacology* 229:387-413.
3. De Vries TJ, Shippenberg TS (2002): Neural Systems Underlying Opiate Addiction. *The Journal of Neuroscience* 22:3321-3325.
4. APA (1994): DSM-IV. Diagnostic and Statistical Manual of Mental Disorders American Psychiatric Association
5. APA (2013): DSM-V. Diagnostic and Statistical Manual of Mental Disorders American Psychiatric Association
6. Hyman SE, Malenka RC (2001): Addiction and the brain: The neurobiology of compulsion and its persistence. *Nat Rev Neurosci* 2:695-703.
7. Leshner AI (1997): Addiction Is a Brain Disease, and It Matters. *Science* 278:45-47.
8. Koob GF, Le Moal M (1997): Drug Abuse: Hedonic Homeostatic Dysregulation. *Science* 278:52-58.
9. Koob GF, Le Moal M (2001): Drug addiction, dysregulation of reward and allostasis. *Neuropsychopharmacology* 24:97-129.
10. Koob GF, Le Moal M (2005): Plasticity of reward neurocircuitry and the 'dark side' of drug addiction. *Nat Neurosci* 8:1442-1444.
11. de Kloet ER, de Kock S, Schild V, Idhuis HD (1988): Antigluco-corticoid RU 38486 attenuates retention of a behaviour and disinhibits the hypothalamic-pituitary adrenal axis at different brain sites. *Neuroendocrinology* 47:109-115.
12. Roberts DCS, Brebner K, Vincler M, Lynch WJ (2002): Patterns of cocaine self-administration in rats produced by various access conditions under a discrete trials procedure. *Drug and Alcohol Dependence* 67:291-299.
13. Wise RA, Bozarth MA (1987): A psychomotor stimulant theory of addiction. *Psychological Review* 94:469-492.
14. Robinson TE, Berridge KC (2008): The incentive sensitization theory of addiction: some current issues. *Philosophical Transactions of the Royal Society B: Biological Sciences* 363:3137-3146.
15. Koob GF, Le Moal M (2007): Addiction and the Brain Antireward System. *Annu Rev Psychol* 59:29-53.
16. Koob GF, Le Moal M (2008): Neurobiological mechanisms for opponent motivational processes in addiction. *Philosophical Transactions of the Royal Society B: Biological Sciences* 363:3113-3123.

17. Jentsch JD, Taylor JR (1999): Impulsivity resulting from frontostriatal dysfunction in drug abuse: implications for the control of behavior by reward-related stimuli. *Psychopharmacology (Berl)* 146:373-390.
18. Bechara A (2001): Neurobiology of decision-making: risk and reward. *Semin Clin Neuropsychiatry* 6:205-216.
19. Bechara A (2005): Decision making, impulse control and loss of willpower to resist drugs: a neurocognitive perspective. *Nat Neurosci* 8:1458-1463.
20. Robbins TW, Ersche KD, Everitt BJ (2008): Drug Addiction and the Memory Systems of the Brain. *Ann NY Acad Sci* 1141:1-21.
21. Nestler EJ (1992): Molecular mechanism of drug addiction. *J Neurosci* 12:2439-2450.
22. Nestler EJ, Hope BT, Widnell KL (1993): Drug addiction: a model for molecular basis of neural plasticity. *Neuron* 11:995-1006.
23. Nestler EJ, Aghajanian GK (1997): Molecular and cellular basis of addiction. *Science* 278:58-63.
24. Nestler EJ (2000): Genes and addiction. *Nature Genetics* 26:277-281.
25. Hyman SE, Malenka RC, Nestler EJ (2006): Neural Mechanisms of Addiction: The Role of Reward-Related Learning and Memory. *Annu Rev Neurosci* 29:565-598.
26. Kauer JA, Malenka RC (2007): Synaptic plasticity and addiction. *Nat Rev Neurosci* 8:844-858.
27. Wolf ME (1998): The role of excitatory amino acids in behavioral sensitization to psychomotor stimulants. *Progress in Neurobiology* 54:679-720.
28. Everitt BJ, Wolf ME (2002): Psychomotor Stimulant Addiction: A Neural Systems Perspective. *The Journal of Neuroscience* 22:3312-3320.
29. Wolf ME (2010): The Bermuda Triangle of cocaine-induced neuroadaptations. *Trends in Neurosciences* 33:391-398.
30. Wolf M (2010): Regulation of AMPA Receptor Trafficking in the Nucleus Accumbens by Dopamine and Cocaine. *Neurotox Res* 18:393-409.
31. Piazza PV, Moal ML (1996): Pathophysiological Basis of Vulnerability to Drug Abuse: Role of an Interaction Between Stress, Glucocorticoids, and Dopaminergic Neurons. *Ann Rev Pharmacol Toxicol* 36:359-378.
32. de Wit H, Uhlenhuth EH, Johanson CE (1986): Individual differences in the reinforcing and subjective effects of amphetamine and diazepam. *Drug Alcohol Depend* 16:341-360.
33. Crowley TJ, Mikulich SK, MacDonald M, Young SE, Zerbe GO (1998): Substance-dependent, conduct-disordered adolescent males: severity of diagnosis predicts 2-year outcome. *Drug and Alcohol Dependence* 49:225-237.
34. Crabbe JC, Belknap JK, Buck KJ (1994): Genetic animal models of alcohol and drug abuse. *Science* 264:1715-1723.

35. Piazza PV, Deroche-Gamonet V, Rouge-Pont F, Le Moal M (2000): Vertical Shifts in Self-Administration Dose-Response Functions Predict a Drug-Vulnerable Phenotype Predisposed to Addiction. *The Journal of Neuroscience* 20:4226-4232.
36. Piazza PV, Deminière JM, Le Moal M, Simon H (1989): Factors that predict individual vulnerability to amphetamine self-administration. *Science* 245:1511-1513.
37. Piazza PV, Rouge-Pont F, Deminière JM, Kharoubi M, Le Moal M, Simon H (1991): Dopaminergic activity is reduced in the prefrontal cortex and increased in the nucleus accumbens of rats predisposed to develop amphetamine self-administration. *Brain Res* 567:169-174.
38. Hooks MS, Colvin AC, Juncos JL, Justice JBJr (1992): Individual differences in basal and cocaine-stimulated extracellular dopamine in the nucleus accumbens using quantitative microdialysis. *Brain Res* 587:306-312.
39. Rouge-Pont F, Piazza PV, Kharoubi M, Le Moal M, Simon H (1993): Higher and longer stress-induced increase in dopamine concentrations in the nucleus accumbens of animals predisposed to amphetamine self-administration. A microdialysis study. *Brain Res* 602:169-174.
40. Piazza PV, Maccari S, Deminière JM, Le Moal M, Mormède P, Simon H (1991): Corticosterone levels determine individual vulnerability to amphetamine self-administration. *Proc Natl Acad Sci U S A* 88:2088-2092.
41. Deroche-Gamonet V, Belin D, Piazza PV (2004): Evidence for Addiction-like Behavior in the Rat. *Science* 305:1014-1017.
42. Belin D, Mar AC, Dalley JW, Robbins TW, Everitt BJ (2008): High Impulsivity Predicts the Switch to Compulsive Cocaine Taking. *Science* 320:1352-1355.
43. Kasanetz F, Deroche-Gamonet V, Berson N, Balado E, Lafourcade M, Manzoni O, Piazza PV (2010): Transition to Addiction Is Associated with a Persistent Impairment in Synaptic Plasticity. *Science* 328:1709-1712.
44. Kasanetz F, Lafourcade M, Deroche-Gamonet V, Revest JM, Berson N, Balado E, Fiancette JF, Renault P, Piazza PV, Manzoni OJ (2013): Prefrontal synaptic markers of cocaine addiction-like behavior in rats. *Mol Psychiatry* 18:729-737.
45. Crabbe JC, Phillips TJ, Belknap JK (2010): The Complexity of Alcohol Drinking: Studies in Rodent Genetic Models. *Behav Genet* 40:737-750.
46. Ellenbroek BA, van der Kam EL, van der Elst MCJ, Cools AR (2005): Individual differences in drug dependence in rats: The role of genetic factors and life events. *European Journal of Pharmacology* 526:251-258.
47. Foroud T, Edenberg HJ, Crabbe JC (2010): Genetic Research: Who Is At Risk for Alcoholism? *Alcohol Res Health* 33:64-75.
48. Meyer AC, Rahman S, Charnigo RJ, Dwoskin LP, Crabbe JC, Bardo MT (2010): Genetics of novelty seeking, amphetamine self-administration and reinstatement using inbred rats. *Genes Brain Behav* 9:790-798.
49. Goeders NE (2002): Stress and Cocaine Addiction. *J Pharmacol Exp Ther* 301:785-789.

50. Bardo MT, Neisewander JL, Kelly TH (2013): Individual Differences and Social Influences on the Neurobehavioral Pharmacology of Abused Drugs. *Pharmacol Rev* 65:255-290.
51. Koehl M, Lemaire V, Mayo W, Abrous D, Maccari S, Piazza P, Le Moal M, Vallée M (2002): Individual vulnerability to substance abuse and affective disorders: Role of early environmental influences. *Neurotox Res* 4:281-296.
52. Barron S, White A, Swartzwelder HS, Bell RL, Rodd ZA, Slawecki CJ, Ehlers CL, Levin ED, Rezvani AH, Spear LP (2005): Adolescent Vulnerabilities to Chronic Alcohol or Nicotine Exposure: Findings From Rodent Models. *Alcoholism: Clinical and Experimental Research* 29:1720-1725.
53. Doremus-Fitzwater TL, Varlinskaya EI, Spear LP (2010): Motivational systems in adolescence: Possible implications for age differences in substance abuse and other risk-taking behaviors. *Brain and Cognition* 72:114-123.
54. Cabib S, Orsini C, Moal ML, Piazza PV (2000): Abolition and Reversal of Strain Differences in Behavioral Responses to Drugs of Abuse After a Brief Experience. *Science* 289:463-465.
55. van der Kam EL, Ellenbroek BA, Cools AR (2005): Gene - environment interactions determine the individual variability in cocaine self-administration. *Neuropharmacology* 48:685-695.
56. Caspi A, Moffitt TE (2006): Gene-environment interactions in psychiatry: joining forces with neuroscience. *Nat Rev Neurosci* 7:583-590.
57. Spanagel R, Weiss F (1999): The dopamine hypothesis of reward: past and current status. *Trends Neurosci* 22:521-527.
58. Di Chiara G, Imperato A (1988): Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *PNAS* 85:5274-5278.
59. Ikemoto S (2007): Dopamine reward circuitry: Two projection systems from the ventral midbrain to the nucleus accumbens-olfactory tubercle complex. *Brain Res Brain Res Rev* 56:27-78.
60. Koob GF, Volkow ND (2010): Neurocircuitry of addiction. *Neuropsychopharmacology* 35:217-238.
61. Nader K, Van Der Kooy D (1997): Deprivation State Switches the Neurobiological Substrates Mediating Opiate Reward in the Ventral Tegmental Area. *The Journal of Neuroscience* 17:383-390.
62. Laviolette SR, Van Der Kooy D (2004): The neurobiology of nicotine addiction: bridging the gap from molecules to behaviour. *Nat Rev Neurosci* 5:55-65.
63. Pierce RC, Kumaresan V (2006): The mesolimbic dopamine system: The final common pathway for the reinforcing effect of drugs of abuse? *Neuroscience & Biobehavioral Reviews* 30:215-238.
64. Gianoulakis C (2009): Endogenous opioids and addiction to alcohol and other drugs of abuse. *Curr Top Med Chem* 9:999-1015.

65. Gieryk A, Ziolkowska B, Solecki W, Kubik J, Przewlocki R (2010): Forebrain PENK and PDYN gene expression levels in three inbred strains of mice and their relationship to genotype-dependent morphine reward sensitivity. *Psychopharmacology* 208:291-300.
66. Bassareo V, Di Chiara G (1997): Differential Influence of Associative and Nonassociative Learning Mechanisms on the Responsiveness of Prefrontal and Accumbal Dopamine Transmission to Food Stimuli in Rats Fed Ad Libitum. *The Journal of Neuroscience* 17:851-861.
67. Di Chiara G (1998): A motivational learning hypothesis of the role of mesolimbic dopamine in compulsive drug use. *J Psychopharmacol* 12:54-67.
68. Berridge KC, Robinson TE (1998): What is the role of dopamine in reward: hedonic impact, reward learning, or incentive salience? *Brain Research Reviews* 28:309-369.
69. Schultz W (2004): Neural coding of basic reward terms of animal learning theory, game theory, microeconomics and behavioural ecology. *Current Opinion in Neurobiology* 14:139-147.
70. Wanat MJ, Willuhn I, Clark JJ, Phillips PE (2009): Phasic dopamine release in appetitive behaviors and drug addiction. *Curr Drug Abuse Rev* 2:195-213.
71. Schultz W, Dayan P, Montague PR (1997): A Neural Substrate of Prediction and Reward. *Science* 275:1593-1599.
72. Smith RJ, Lobo MK, Spencer S, Kalivas PW (2013): Cocaine-induced adaptations in D1 and D2 accumbens projection neurons (a dichotomy not necessarily synonymous with direct and indirect pathways). *Curr Opin Neurobiol* 23:546-552.
73. Dearry A, Gingrich JA, Falardeau P, Freneau RT, Bates MD, Caron MG (1990): Molecular cloning and expression of the gene for a human D1 dopamine receptor. *Nature* 347:72-76.
74. Zhou QY, Grandy DK, Thambi L, Kushner JA, Tol HHMV, Cone R, Pribnow D, Salon J, Bunzow JR, Civelli O (1990): Cloning and expression of human and rat D1 dopamine receptors. *Nature* 347:76-80.
75. Grandy DK, Zhang YA, Bouvier C, Zhou QY, Johnson RA, Allen L, Buck K, Bunzow JR, Salon J, Civelli O (1991): Multiple human D5 dopamine receptor genes: a functional receptor and two pseudogenes. *Proc Natl Acad Sci U S A* 88:9175-9179.
76. Sunahara RK, Guan HC, O'Dowd BF, Seeman P, Laurier LG, Ng G, George SR, Torchia J, Van Tol HHM, Niznik HB (1991): Cloning of the gene for a human dopamine D5 receptor with higher affinity for dopamine than D1. *Nature* 350:614-619.
77. Bunzow JR, Tol HHMV, Grandy DK, Albert P, Salon J, Christie M, Machida CA, Neve KA, Civelli O (1988): Cloning and expression of a rat D2 dopamine receptor cDNA. *Nature* 336:783-787.
78. Dal Toso R, Sommer B, Ewert M, Herb A, Pritchett DB, Bach A, Shivers BD, Seeburg PH (1989): The dopamine D2 receptor: two molecular forms generated by alternative splicing. *EMBO J* 8:4025-4034.
79. Sokoloff P, Giros B, Martres MP, Marie L, Schwartz JC (1990): Molecular cloning and characterization of a novel dopamine receptor (D3) as a target for neuroleptics. *Nature* 347:146-151.

80. Van Tol HHM, Bunzow JR, Guan HC, Sunahara RK, Seeman P, Niznik HB, Civelli O (1991): Cloning of the gene for a human dopamine D4 receptor with high affinity for the antipsychotic clozapine. *Nature* 350:610-614.
81. Baik JH (2013): Dopamine Signaling in reward-related behaviors. *Front Neural Circuits* 7:152-
82. Anzalone A, Lizardi-Ortiz JE, Ramos M, De Mei C, Hopf FW, Iaccarino C, Halbout B, Jacobsen J, Kinoshita C, Welter M, Caron MG, Bonci A, Sulzer D, Borrelli E (2012): Dual Control of Dopamine Synthesis and Release by Presynaptic and Postsynaptic Dopamine D2 Receptors. *The Journal of Neuroscience* 32:9023-9034.
83. Goto Y, Grace AA (2005): Dopaminergic modulation of limbic and cortical drive of nucleus accumbens in goal-directed behavior. *Nat Neurosci* 8:805-812.
84. Grace AA, Floresco SB, Goto Y, Lodge DJ (2007): Regulation of firing of dopaminergic neurons and control of goal-directed behaviors. *Trends Neurosci* 30:220-227.
85. Goto Y, Otani S, Grace AA (2007): The Yin and Yang of Dopamine Release: A New Perspective. *Neuropharmacology* 53:583-587.
86. Yawata S, Yamaguchi T, Danjo T, Hikida T, Nakanishi S (2012): Pathway-specific control of reward learning and its flexibility via selective dopamine receptors in the nucleus accumbens. *Proc Natl Acad Sci U S A* 109:12764-12769.
87. Kravitz AV, Tye LD, Kreitzer AC (2012): Distinct roles for direct and indirect pathway striatal neurons in reinforcement. *Nat Neurosci* 15:816-818.
88. Lobo MK, Covington HE, Chaudhury D, Friedman AK, Sun HS, Damez-Werno D, Dietz DM, Zaman S, Koo J-W, Kennedy PJ, Mouzon E, Mogri M, Neve RL, Deisseroth K, Han MH, Nestler EJ (2010): Cell Type-Specific Loss of BDNF Signaling Mimics Optogenetic Control of Cocaine Reward. *Science* 330:385-390.
89. Hikida T, Kimura K, Wada N, Funabiki K, Nakanishi S Distinct Roles of Synaptic Transmission in Direct and Indirect Striatal Pathways to Reward and Aversive Behavior. *Neuron* 66:896-907.
90. Durieux PF, Bearzatto B, Guiducci S, Buch T, Waisman A, Zoli M, Schiffmann SN, de Kerchove d'Exaerde A (2009): D2R striatopallidal neurons inhibit both locomotor and drug reward processes. *Nat Neurosci* 12:393-395.
91. Ferguson SM, Eskenazi D, Ishikawa M, Wanat MJ, Phillips PEM, Dong Y, Roth BL, Neumaier JF (2011): Transient neuronal inhibition reveals opposing roles of indirect and direct pathways in sensitization. *Nat Neurosci* 14:22-24.
92. Keller-Wood ME, Dallman MF (1984): Corticosteroid Inhibition of ACTH Secretion. *Endocrine Reviews* 5:1-24.
93. Sawchenko PE, Swanson LW (1985): Localization, colocalization, and plasticity of corticotropin-releasing factor immunoreactivity in rat brain. *Fed Proc* 44:221-227.
94. Antoni FA (1986): Hypothalamic regulation of adrenocorticotropin secretion: advances since the discovery of 41-residue corticotropin-releasing factor. *Endocrine Rev* 7:351-378.

95. Swanson LW, Simmons DM (1989): Differential steroid hormone and neural influences on peptide mRNA levels in CRH cells of the paraventricular nucleus: a hybridization histochemical study in the rat. *J Comp Neurol* 285:413-435.
96. de Kloet ER (1991): Brain corticosteroid receptor balance and homeostatic control. *Frontiers in Neuroendocrinology* 12:95-164.
97. McEwen BS, Weiss JM, Schwartz LS (1968): Selective retention of corticosterone by limbic structures in rat brain. *Nature* 220:911-912.
98. Reul JM, deKloet ER (1985): Two Receptor Systems for Corticosterone in Rat Brain: Microdistribution and Differential Occupation. *Endocrinology* 117:2505-2511.
99. Reul JM, de Kloet ER, van Sluijs FJ, Rijnberk A, Rothuizen J (1990): Binding Characteristics of Mineralocorticoid and Glucocorticoid Receptors in Dog Brain and Pituitary. *Endocrinology* 127:907-915.
100. Kreek MJ, Koob GF (1998): Drug dependence: stress and dysregulation of brain reward pathways. *Drug Alcohol Depend* 51:23-47.
101. Ignar DM, Kuhn CM (1990): Effects of specific mu and kappa opiate tolerance and abstinence on hypothalamo-pituitary-adrenal axis secretion in the rat. *J Pharmacol Exp Ther* 255:1287-1295.
102. Borowsky B, Kuhn CM (1991): Chronic cocaine administration sensitizes behavioral but not neuroendocrine responses. *Brain Res* 543:301-306.
103. Benwell ME, Balfour DJ (1979): Effects of nicotine administration and its withdrawal on plasma corticosterone and brain 5-hydroxyindoles. *Psychopharmacology (Berl)* 63:7-11.
104. Spencer RL, McEwen BS (1990): Adaptation of the hypothalamic-pituitary-adrenal axis to chronic ethanol stress. *Neuroendocrinology* 52:481-489.
105. Heesch CM, Negus BH, Keffer JH, Snyder RW, Risser RC, Eichhorn EJ (1995): Effects of cocaine on cortisol secretion in humans. *Am J Med Sci* 310:61-64.
106. Mendelson JH, Ogata M, Mello NK (1971): Adrenal Function and Alcoholism: I. Serum Cortisol. *Psychosomatic Medicine* 33
107. Kirschbaum C, Wüst S, Strasburger CJ (1992): 'Normal' cigarette smoking increases free cortisol in habitual smokers. *Life Sci* 50:435-442.
108. Gosselin RE, Blankstein J, Dent DW, Hobson WC, Fuller GB, Reyes FI, Winter JS, Faiman C (1983): Effects of Naloxone and an Enkephalin Analog on Serum Prolactin, Cortisol, and Gonadotropins in the Chimpanzee. *Endocrinology* 112:2168-2173.
109. Allolio B, Schulte HM, Deuss U, Kallabis D, Hamel E, Winkelmann W (1987): Effect of oral morphine and naloxone on pituitary-adrenal response in man induced by human corticotropin-releasing hormone. *Acta Endocrinologica* 114:509-514.
110. Baumann MH, Gendron TM, Becketts KM, Henningfield JE, Gorelick DA, Rothman RB (1995): Effects of intravenous cocaine on plasma cortisol and prolactin in human cocaine abusers. *Biological Psychiatry* 38:751-755.

111. Kreek MJ (2000): Methadone-Related Opioid Agonist Pharmacotherapy for Heroin Addiction: History, Recent Molecular and Neurochemical Research and Future in Mainstream Medicine. *Ann NY Acad Sci* 909:186-216.
112. Kant GJ, Bunnell BN, Mougey EH, Pennington LL, Meyerhoff JL (1983): Effects of repeated stress on pituitary cyclic AMP, and plasma prolactin, corticosterone and growth hormone in male rats. *Pharmacol Biochem Behav* 18:967-971.
113. Kant GJ, Eggleston T, Landman-Roberts L, Kenion CC, Driver GC, Meyerhoff JL (1985): Habituation to repeated stress is stressor specific. *Pharmacol Biochem Behav* 22:631-634.
114. Cleck JN, Blendy JA (2008): Making a bad thing worse: adverse effects of stress on drug addiction. *J Clin Invest* 118:454-461.
115. Ziegler DR, Herman JP (2000): Local Integration of Glutamate Signaling in the Hypothalamic Paraventricular Region: Regulation of Glucocorticoid Stress Responses. *Endocrinology* 141:4801-4804.
116. Herman JP, Cullinan WE, Ziegler DR, Tasker JG (2002): Role of the paraventricular nucleus microenvironment in stress integration. *Eur J Neurosci* 16:381-385.
117. Sarnyai Z, Shaham Y, Heinrichs SC (2001): The Role of Corticotropin-Releasing Factor in Drug Addiction. *Pharmacol Rev* 53:209-244.
118. Logrip ML, Koob GF, Zorrilla EP (2011): Role of Corticotropin-Releasing Factor in Drug Addiction: Potential for Pharmacological Intervention. *CNS Drugs* 25:271-287.
119. Hyman SE (1996): Addiction to Cocaine and Amphetamine. *Neuron* 16:901-904.
120. Walker DL, Toufexis DJ, Davis M (2003): Role of the bed nucleus of the stria terminalis versus the amygdala in fear, stress, and anxiety. *European Journal of Pharmacology* 463:199-216.
121. Heinrichs SC, Menzaghi FR, Merlo Pich E, Britton KT, Koob GF (1995): The Role of CRF in Behavioral Aspects of Stress. *Ann NY Acad Sci* 771:92-104.
122. Zhou Y, Spangler R, LaForge KS, Maggos CE, Ho A, Kreek MJ (1996): Corticotropin-releasing factor and type 1 corticotropin-releasing factor receptor messenger RNAs in rat brain and pituitary during "binge"-pattern cocaine administration and chronic withdrawal. *J Pharmacol Exp Ther* 279:351-358.
123. Rivier C, Lee S (1994): Stimulatory Effect of Cocaine on ACTH Secretion: Role of the Hypothalamus. *Molecular and Cellular Neuroscience* 5:189-195.
124. Suemaru S, Hashimoto K, Ota Z (1985): Effects of morphine on hypothalamic corticotropin-releasing factor (CRF), norepinephrine and dopamine in non-stressed and stressed rats. *Acta Med Okayama* 39:463-470.
125. Milanés MV, Laorden ML, Chapleur-Chateau M, Burlet A (1997): Differential regulation of corticotropin-releasing factor and vasopressin in discrete brain regions after morphine administration: correlations with hypothalamic noradrenergic activity and pituitary-adrenal response. *Naunyn-Schmiedeberg's Arch Pharmacol* 356:603-610.
126. Ogilvie KM, Rivier C (1997): Gender difference in hypothalamic-pituitary-adrenal axis response to alcohol in the rat: activational role of gonadal steroids. *Brain Res* 766:19-28.

127. Auclair A, Blanc G, Glowinski J, Tassin JP (2004): Role of serotonin_{2A} receptors in the D-amphetamine-induced release of dopamine: comparison with previous data on α_1b -adrenergic receptors. *Journal of Neurochemistry* 91:318-326.
128. Grenhoff J, Svensson TH (1993): Prazosin modulates the firing pattern of dopamine neurons in rat ventral tegmental area. *European Journal of Pharmacology* 233:79-84.
129. Devoto P, Flore G, Saba P, Fa M, Gessa GL (2005): Stimulation of the locus coeruleus elicits noradrenaline and dopamine release in the medial prefrontal and parietal cortex. *Journal of Neurochemistry* 92:368-374.
130. Sauvage M, Steckler T (2001): Detection of corticotropin-releasing hormone receptor 1 immunoreactivity in cholinergic, dopaminergic and noradrenergic neurons of the murine basal forebrain and brainstem nuclei - potential implication for arousal and attention. *Neuroscience* 104:643-652.
131. Van Pett K, Viau V, Bittencourt JC, Li HY, Arias C, Prins GS, Perrin M, Vale W, Sawchenko PE (2000): Distribution of mRNAs encoding CRF receptors in brain and pituitary of rat and mouse. *J Comp Neurol* 428:191-212.
132. Koob GF, Heinrichs SC (1999): A role for corticotropin releasing factor and urocortin in behavioral responses to stressors. *Brain Res* 848:141-152.
133. Bangasser DA, Reyes BA, Piel D, Garachh V, Zhang XY, Plona ZM, Van Bockstaele EJ, Beck SG, Valentino RJ (2013): Increased vulnerability of the brain norepinephrine system of females to corticotropin-releasing factor overexpression. *Mol Psychiatry* 18:166-173.
134. Lavicky J, Dunn AJ (1993): Corticotropin-releasing factor stimulates catecholamine release in hypothalamus and prefrontal cortex in freely moving rats as assessed by microdialysis. *J Neurochem* 60:602-612.
135. Delfs JM, Zhu Y, Druhan JP, Aston-Jones G (2000): Noradrenaline in the ventral forebrain is critical for opiate withdrawal-induced aversion. *Nature* 403:430-434.
136. Koob GF (2009): Dynamics of neuronal circuits in addiction: reward, antireward, and emotional memory. *Pharmacopsychiatry* 42:S32-S41.
137. Núñez C, Foldes A, Laorden ML, Milanes MV, Kovács KJ (2007): Activation of stress-related hypothalamic neuropeptide gene expression during morphine withdrawal. *J Neurochem* 101:1060-1071.
138. Núñez C, Földes A, Pérez-Flores D, García-Borrón JC, Laorden ML, Kovács KJ, Milanés MV (2009): Elevated glucocorticoid levels are responsible for induction of tyrosine hydroxylase (TH) mRNA expression, phosphorylation and enzyme activity in the nucleus of the solitary tract (NTS-A2) during morphine withdrawal. *Endocrinology* 150:3118-3127.
139. Laorden ML, Núñez C, Almela P, Milanés MV (2002): Morphine withdrawal-induced c-fos expression in the hypothalamic paraventricular nucleus is dependent on the activation of catecholaminergic neurones. *J Neurochem* 83:132-140.
140. Martin F, Nunez C, Marin MT, Laorden ML, Kovacs KJ, Milanes MV (2012): Involvement of Noradrenergic Transmission in the PVN on CREB Activation, TORC1 Levels, and Pituitary-Adrenal Axis Activity during Morphine Withdrawal. *PLoS ONE* 7:e31119-

141. Laorden ML, Fuertes G, González-Cuello A, Milanés MV (2000): Changes in catecholaminergic pathways innervating paraventricular nucleus and pituitary-adrenal axis response during morphine dependence: implication of α_1 - and α_2 -adrenoceptors. *J Pharmacol Exp Ther* 293:578-584.
142. Nestler EJ (2013): Cellular basis of memory for addiction. *Dialogues Clin Neurosci* 15:431-443.
143. Kalivas PW, O'Brien C (2008): Drug Addiction as a Pathology of Staged Neuroplasticity. *Neuropsychopharmacology* 33:166-180.
144. Nestler EJ (2004): Molecular mechanisms of drug addiction. *Neuropharmacology* 47:24-32.
145. Nestler EJ (2012): Transcriptional Mechanisms of Drug Addiction. *Clin Psychopharmacol Neurosci* 10:136-143.
146. Mayr B, Montminy M (2001): Transcriptional regulation by the phosphorylation-dependent factor CREB. *Nat Rev Mol Cell Biol* 2:599-609.
147. Carlezon JR, Duman RS, Nestler EJ (2005): The many faces of CREB. *Trends in Neurosciences* 28:436-445.
148. Graham DL, Edwards S, Bachtell RK, DiLeone RJ, Rios M, Self DW (2007): Dynamic BDNF activity in nucleus accumbens with cocaine use increases self-administration and relapse. *Nat Neurosci* 10:1029-1037.
149. Briand LA, Blendy JA (2010): Molecular and genetic substrates linking stress and addiction. *Brain Res* 1314:219-234.
150. Carlezon WA, Jr., Thome J, Olson VG, Lane-Ladd SB, Brodtkin ES, Hiroi N, Duman RS, Neve RL, Nestler EJ (1998): Regulation of Cocaine Reward by CREB. *Science* 282:2272-2275.
151. Barrot M, Olivier JDA, Perrotti LI, DiLeone RJ, Berton O, Eisch AJ, Impey S, Storm DR, Neve RL, Yin JC, Zachariou V, Nestler EJ (2002): CREB activity in the nucleus accumbens shell controls gating of behavioral responses to emotional stimuli. *Proc Natl Acad Sci U S A* 99:11435-11440.
152. DiNieri JA, Nemeth C, Parsegian A, Carle T, Gurevich VV, Gurevich E, Neve RL, Nestler EJ, Carlezon WA (2009): Altered sensitivity to rewarding and aversive drugs in mice with inducible disruption of CREB function within the nucleus accumbens. *J Neurosci* 29:1855-1859.
153. Larson EB, Graham DL, Arzaga RR, Buzin N, Webb J, Green TA, Bass CE, Neve RL, Terwilliger EF, Nestler EJ, Self DW (2011): Over-Expression of CREB in the Nucleus Accumbens Shell Increases Cocaine Reinforcement in Self-Administering Rats. *J Neurosci* 31:16447-16457.
154. Jin SH, Blendy JA, Thomas SA (2005): Cyclic AMP response element-binding protein is required for normal maternal nurturing behavior. *Neuroscience* 133:647-655.
155. Walters CL, Cleck JN, Kuo YC, Blendy JA (2005): μ -Opioid Receptor and CREB Activation Are Required for Nicotine Reward. *Neuron* 46:933-943.

156. Choi KH, Whisler K, Graham DL, Self DW (2006): Antisense-induced reduction in nucleus accumbens cyclic AMP response element binding protein attenuates cocaine reinforcement. *Neuroscience* 137:373-383.
157. Dong Y, Green T, Saal D, Marie H, Neve R, Nestler EJ, Malenka RC (2006): CREB modulates excitability of nucleus accumbens neurons. *Nat Neurosci* 9:475-477.
158. Josselyn SA, Nguyen PV (2005): CREB, synapses and memory disorders: past progress and future challenges. *Curr Drug Targets CNS Neurol Disord* 4:481-497.
159. Kandel ER (2012): The molecular biology of memory: cAMP, PKA, CRE, CREB-1, CREB-2, and CPEB. *Mol Brain* 5:14-14.
160. Tully T, Bourtchouladze R, Scott R, Tallman J (2003): Targeting the CREB pathway for memory enhancers. *Nat Rev Drug Discov* 2:267-277.
161. McClung CA, Nestler EJ (2003): Regulation of gene expression and cocaine reward by CREB and Δ FosB. *Nat Neurosci* 6:1208-1215.
162. Renthall W, Kumar A, Xiao G, Wilkinson M, Covington HE, Maze I, Sikder D, Robison AJ, LaPlant Q, Dietz DM, Russo SJ, Vialou V, Chakravarty S, Kodadek TJ, Stack A, Kabbaj M, Nestler EJ (2009): Genome Wide Analysis of Chromatin Regulation by Cocaine Reveals a Novel Role for Sirtuins. *Neuron* 62:335-348.
163. Huang YH, Lin Y, Brown TE, Han MH, Saal DB, Neve RL, Zukin RS, Sorg BA, Nestler EJ, Malenka RC, Dong Y (2008): CREB modulates the functional output of nucleus accumbens neurons: a critical role of N-methyl-D-aspartate glutamate receptor (NMDAR) receptors. *J Biol Chem* 283:2751-2760.
164. Brown TE, Lee BR, Mu P, Ferguson D, Dietz D, Ohnishi YN, Lin Y, Suska A, Ishikawa M, Huang YH, Shen H, Kalivas PW, Sorg BA, Zukin RS, Nestler E, Dong Y, Schlatter OM (2011): A Silent Synapse-based Mechanism for Cocaine-induced Locomotor Sensitization. *J Neurosci* 31:8163-8174.
165. Hiroi N, Brown JR, Haile CN, Ye H, Greenberg ME, Nestler EJ (1997): FosB mutant mice: Loss of chronic cocaine induction of Fos-related proteins and heightened sensitivity to cocaine's psychomotor and rewarding effects. *Proc Natl Acad Sci U S A* 94:10397-10402.
166. Chen J, Kelz MB, Hope BT, Nakabeppu Y, Nestler EJ (1997): Chronic Fos-Related Antigens: Stable Variants of Δ FosB Induced in Brain by Chronic Treatments. *The Journal of Neuroscience* 17:4933-4941.
167. Renthall W, Carle TL, Maze I, Covington HE, Truong HT, Alibhai I, Kumar A, Montgomery RL, Olson EN, Nestler EJ (2008): Δ FosB mediates epigenetic desensitization of the c-fos gene after chronic amphetamine exposure. *J Neurosci* 28:7344-7349.
168. Alibhai IN, Green TA, Potashkin JA, Nestler EJ (2007): Regulation of fosB and Δ fosB mRNA Expression: In Vivo and In Vitro Studies. *Brain Res* 1143:22-33.
169. Nestler EJ (2001): Molecular basis of long-term plasticity underlying addiction. *Nat Rev Neurosci* 2:119-128.
170. Nestler EJ (2008): Transcriptional mechanisms of addiction: role of Δ FosB. *Phil Trans R Soc B* 363:3245-3255.

171. Kelz MB, Chen J, Carlezon WA, Whisler K, Gilden L, Beckmann AM, Steffen C, Zhang YJ, Marotti L, Self DW, Tkatch T, Baranaukas G, Surmeier DJ, Neve RL, Duman RS, Picciotto MR, Nestler EJ (1999): Expression of the transcription factor Δ FosB in the brain controls sensitivity to cocaine. *Nature* 401:272-276.
172. Zachariou V, Bolanos CA, Selley DE, Theobald D, Cassidy MP, Kelz MB, Shaw-Lutchman T, Berton O, Sim-Selley LJ, DiLeone RJ, Kumar A, Nestler EJ (2006): An essential role for Δ FosB in the nucleus accumbens in morphine action. *Nat Neurosci* 9:205-211.
173. Colby CR, Whisler K, Steffen C, Nestler EJ, Self DW (2003): Striatal Cell Type-Specific Overexpression of Δ FosB Enhances Incentive for Cocaine. *The Journal of Neuroscience* 23:2488-2493.
174. Núñez C, Martín F, Földes A, Laorden ML, Kovács KJ, Milanés MV (2010): Induction of FosB/ Δ FosB in the brain stress system-related structures during morphine dependence and withdrawal. *Journal of Neurochemistry* 114:475-487.
175. Shippenberg TS, Rea W (1997): Sensitization to the Behavioral Effects of Cocaine: Modulation by Dynorphin and κ -Opioid Receptor Agonists. *Pharmacology Biochemistry and Behavior* 57:449-455.
176. Bruchas MR, Land BB, Chavkin C (2010): The Dynorphin-Kappa Opioid System as a Modulator of Stress-induced and Pro-addictive Behaviors. *Brain Res* 1314C:44-
177. Hazel TG, Nathans D, Lau LF (1988): A gene inducible by serum growth factors encodes a member of the steroid and thyroid hormone receptor superfamily. *Proc Natl Acad Sci U S A* 85:8444-8448.
178. Milbrandt J (1988): Nerve growth factor induces a gene homologous to the glucocorticoid receptor gene. *Neuron* 1:183-188.
179. Law SW, Conneely OM, DeMayo FJ, O'Malley BW (1992): Identification of a new brain-specific transcription factor, NURR1. *Mol Endocrinol* 6:2129-2135.
180. Ohkura N, Ito M, Tsukada T, Sasaki K, Yamaguchi K, Miki K (1996): Structure, mapping and expression of a human NOR-1 gene, the third member of the Nur77/NGFI-B family. *Biochim Biophys Acta* 1308:205-214.
181. Giguère V (1999): Orphan Nuclear Receptors: From Gene to Function. *Endocrine Reviews* 20:689-725.
182. Campos-Melo D, Galleguillos D, SÁnchez N, Gysling K, Andrés ME (2013): Nur transcription factors in stress and addiction. *Frontiers in Molecular Neuroscience* 6:44-
183. Zetterström RH, Williams R, Perlmann T, Olson L (1996): Cellular expression of the immediate early transcription factors Nurr1 and NGFI-B suggests a gene regulatory role in several brain regions including the nigrostriatal dopamine system. *Brain Res Mol Brain Res* 41:111-120.
184. Williams GT, Lau LF (1993): Activation of the inducible orphan receptor gene nur77 by serum growth factors: dissociation of immediate-early and delayed-early responses. *Mol Cell Biol* 13:6124-6136.

185. Maruyama K, Tsukada T, Bandoh S, Sasaki K, Ohkura N, Yamaguchi K (1995): Expression of NOR-1 and its closely related members of the steroid/thyroid hormone receptor superfamily in human neuroblastoma cell lines. *Cancer Letters* 96:117-122.
186. Satoh Ji, Kuroda Y (2002): The constitutive and inducible expression of Nurr1, a key regulator of dopaminergic neuronal differentiation, in human neural and non-neural cell lines. *Neuropathology* 22:219-232.
187. Maxwell MA, Muscat GE (2006): The NR4A subgroup: immediate early response genes with pleiotropic physiological roles. *Nucl Recept Signal* 4:e002-
188. McMorro JP, Murphy EP (2011): Inflammation: a role for NR4A orphan nuclear receptors? *Biochem Soc Trans* 39:688-693.
189. van Tiel CM, de Vries CJM (2012): NR4A1 in the vessel wall. *The Journal of Steroid Biochemistry and Molecular Biology* 130:186-193.
190. Mohan HM, Aherne CM, Rogers AC, Baird AW, Winter DC, Murphy EP (2012): Molecular Pathways: The Role of NR4A Orphan Nuclear Receptors in Cancer. *Clinical Cancer Research* 18:3223-3228.
191. Xiao Q, Castillo SO, Nikodem VM (1996): Distribution of messenger RNAs for the orphan nuclear receptors Nurr1 and Nur77 (NGFI-B) in adult rat brain using in situ hybridization. *Neuroscience* 75:221-230.
192. Zetterström RH, Solomin L, Mitsiadis T, Olson L, Perlmann T (1996): Retinoid X receptor heterodimerization and developmental expression distinguish the orphan nuclear receptors NGFI-B, Nurr1, and Nor1. *Mol Endocrinol* 10:1656-1666.
193. Bäckman C, Perlmann T, Wallén A, Hoffer BJ, Morales M (1999): A selective group of dopaminergic neurons express Nurr1 in the adult mouse brain. *Brain Res* 851:125-132.
194. Ojeda V, Fuentealba JA, Galleguillos D, Andrés ME (2003): Rapid increase of Nurr1 expression in the substantia nigra after 6-hydroxydopamine lesion in the striatum of the rat. *J Neurosci Res* 73:686-697.
195. Zetterström RH, Solomin L, Jansson L, Hoffer BJ, Olson L, Perlmann T (1997): Dopamine Neuron Agenesis in Nurr1-Deficient Mice. *Science* 276:248-250.
196. Castillo SO, Baffi JS, Palkovits Ms, Goldstein DS, Kopin IJ, Witta J, Magnuson MA, Nikodem VM (1998): Dopamine Biosynthesis Is Selectively Abolished in Substantia Nigra/Ventral Tegmental Area but Not in Hypothalamic Neurons in Mice with Targeted Disruption of the Nurr1 Gene. *Molecular and Cellular Neuroscience* 11:36-46.
197. Saucedo-Cardenas O, Quintana-Hau JD, Le WD, Smidt MP, Cox JJ, De Mayo F, Burbach JP, Conneely OM (1998): Nurr1 is essential for the induction of the dopaminergic phenotype and the survival of ventral mesencephalic late dopaminergic precursor neurons. *Proc Natl Acad Sci U S A* 95:4013-4018.
198. Smidt MP, Smits SM, Burbach JP (2003): Molecular mechanisms underlying midbrain dopamine neuron development and function. *European Journal of Pharmacology* 480:75-88.
199. Perlmann T, Wallén-Mackenzie A (2004): Nurr1, an orphan nuclear receptor with essential functions in developing dopamine cells. *Cell Tissue Res* 318:45-52.

200. Kadkhodaei B, Ito T, Joodmardi E, Mattsson B, Rouillard C, Carta M, Muramatsu SI, Sumi-Ichinose C, Nomura T, Metzger D, Chambon P, Lindqvist E, Larsson NG, Olson L, Björklund A, Ichinose H, Perlmann T (2009): Nurr1 Is Required for Maintenance of Maturing and Adult Midbrain Dopamine Neurons. *The Journal of Neuroscience* 29:15923-15932.
201. Kadkhodaei B, Alvarsson A, Schintu N, Ramsköld D, Volakakis N, Joodmardi E, Yoshitake T, Kehr J, Decressac M, Björklund A, Sandberg R, Svenningsson P, Perlmann T (2013): Transcription factor Nurr1 maintains fiber integrity and nuclear-encoded mitochondrial gene expression in dopamine neurons. *Proc Natl Acad Sci U S A* 110:2360-2365.
202. Galleguillos D, Fuentealba JA, Gómez LM, Saver M, Gómez A, Nash K, Burger C, Gysling K, Andrés ME (2010): Nurr1 regulates RET expression in dopamine neurons of adult rat midbrain. *Journal of Neurochemistry* 114:1158-1167.
203. Jiang C, Wan X, He Y, Pan T, Jankovic J, Le W (2005): Age-dependent dopaminergic dysfunction in Nurr1 knockout mice. *Experimental Neurology* 191:154-162.
204. Zhang L, Le W, Xie W, Dani JA (2012): Age-related changes in dopamine signaling in Nurr1 deficient mice as a model of Parkinson's disease. *Neurobiol Aging* 33:1001.e7-1001.e16.
205. Rojas P, Joodmardi E, Hong Y, Perlmann T, Ogren SO (2007): Adult mice with reduced Nurr1 expression: an animal model for schizophrenia. *Mol Psychiatry* 12:756-766.
206. Vuillermot S, Joodmardi E, Perlmann T, Ove Ögren S, Feldon J, Meyer U (2011): Schizophrenia-relevant behaviors in a genetic mouse model of constitutive Nurr1 deficiency. *Genes, Brain and Behavior* 10:589-603.
207. Johnson MM, Michelhaugh SK, Bouhamdan M, Schmidt CJ, Bannon MJ (2011): The transcription factor NURR1 exerts concentration-dependent effects on target genes mediating distinct biological processes. *Frontiers in Neuroscience* 5:135-
208. Tseng KY, Roubert C, Do L, Rubinstein M, Kelly MA, Grandy DK, Low MJ, Gershanik OS, Murer MG, Giros B, Raisman-Vozari R (2000): Selective increase of Nurr1 mRNA expression in mesencephalic dopaminergic neurons of D2 dopamine receptor-deficient mice. *Molecular Brain Research* 80:1-6.
209. Nielsen DA, Ji F, Yuferov V, Ho A, Chen A, Levran O, Ott J, Kreek MJ (2008): Genotype patterns that contribute to increased risk for or protection from developing heroin addiction. *Mol Psychiatry* 13:10-
210. Leo D, di Porzio U, Racagni G, Riva MA, Fumagalli F, Perrone-Capano C (2007): Chronic cocaine administration modulates the expression of transcription factors involved in midbrain dopaminergic neuron function. *Experimental Neurology* 203:472-480.
211. Horvath MC, Kovacs GG, Kovari V, Majtenyi K, Hurd YL, Keller E (2007): Heroin Abuse Is Characterized by Discrete Mesolimbic Dopamine and Opioid Abnormalities and Exaggerated Nuclear Receptor-Related 1 Transcriptional Decline with Age. *J Neurosci* 27:13371-13375.
212. Kim J, Inoue K, Ishii J, Vanti WB, Voronov SV, Murchison E, Hannon G, Abeliovich A (2007): A microRNA feedback circuit in midbrain dopamine neurons. *Science* 317:1220-1224.
213. Hwang DY, Ardayfio P, Kang UJ, Semina EV, Kim KS (2003): Selective loss of dopaminergic neurons in the substantia nigra of Pitx3-deficient aphakia mice. *Molecular Brain Research* 114:123-131.

214. Nunes I, Tovmasian LT, Silva RM, Burke RE, Goff SP (2003): Pitx3 is required for development of substantia nigra dopaminergic neurons. *Proc Natl Acad Sci U S A* 100:4245-4250.
215. Smits SM, Mathon DS, Burbach JP, Ramakers GMJ, Smidt MP (2005): Molecular and cellular alterations in the Pitx3-deficient midbrain dopaminergic system. *Molecular and Cellular Neuroscience* 30:352-363.
216. van den Munckhof P, Luk KC, Ste-Marie L, Montgomery J, Blanchet PJ, Sadikot AF, Drouin J (2003): Pitx3 is required for motor activity and for survival of a subset of midbrain dopaminergic neurons. *Development* 130:2535-2542.
217. Kas MJH, van der Linden AJA, Oppelaar H, von Oerthel L, Ramakers GMJ, Smidt MP (2008): Phenotypic segregation of aphakia and Pitx3-null mutants reveals that Pitx3 deficiency increases consolidation of specific movement components. *Behavioural Brain Research* 186:208-214.
218. Maxwell SL, Ho HY, Kuehner E, Zhao S, Li M (2005): Pitx3 regulates tyrosine hydroxylase expression in the substantia nigra and identifies a subgroup of mesencephalic dopaminergic progenitor neurons during mouse development. *Developmental Biology* 282:467-479.
219. Hwang DY, Hong S, Jeong JW, hoi S, Kim H, Kim J, Kim KS (2009): Vesicular monoamine transporter 2 and dopamine transporter are molecular targets of Pitx3 in the ventral midbrain dopamine neurons. *Journal of Neurochemistry* 111:1202-1212.
220. Reddy SD, Rayala SK, Ohshiro K, Pakala SB, Kobori N, Dash P, Yun S, Qin J, O'Malley BW (2011): Multiple coregulatory control of tyrosine hydroxylase gene transcription. *PNAS* 108:4200-4205.
221. Jacobs FMJ, van Erp S, van der Linden AJA, von Oerthel L, Burbach JP, Smidt MP (2009): Pitx3 potentiates Nurr1 in dopamine neuron terminal differentiation through release of SMRT-mediated repression. *Development* 136:531-540.
222. Chao MV (2003): Neurotrophins and their receptors: A convergence point for many signalling pathways. *Nat Rev Neurosci* 4:299-309.
223. Hallböök F, Wilson K, Thorndyke M, Olinski RP (2006): Formation and Evolution of the Chordate Neurotrophin and Trk Receptor Genes. *Brain, Behavior and Evolution* 68:133-144.
224. Cohen S, Levi-Montalcini R, Hamburger V (1954): A nerve growth-stimulating factor isolated from sarcomas 37 and 38. *Proc Natl Acad Sci U S A* 40:1014-1018.
225. Barde YA, Edgar D, Thoenen H (1982): Purification of a new neurotrophic factor from mammalian brain. *EMBO J* 1:549-553.
226. Leibrock J, Lottspeich F, Hohn A, Hofer M, Hengerer B, Masiakowski P, Thoenen H, Barde YA (1989): Molecular cloning and expression of brain-derived neurotrophic factor. *Nature* 341:149-152.
227. Hohn A, Leibrock J, Bailey K, Barde YA (1990): Identification and characterization of a novel member of the nerve growth factor/brain-derived neurotrophic factor family. *Nature* 344:339-341.
228. Berkemeier LR, Winslow JW, Kaplan DR, Nikolics K, Goeddel DV, Rosenthal A (1991): Neurotrophin-5: A novel neurotrophic factor that activates trk and trkB. *Neuron* 7:857-866.

229. Fahnstock M, Michalski B, Xu B, Coughlin MD (2001): The Precursor Pro-Nerve Growth Factor Is the Predominant Form of Nerve Growth Factor in Brain and Is Increased in Alzheimer's Disease. *Molecular and Cellular Neuroscience* 18:210-220.
230. Lee R, Kermani P, Teng KK, Hempstead BL (2001): Regulation of Cell Survival by Secreted Proneurotrophins. *Science* 294:1945-1948.
231. Rodriguez-Tebar A, Dechant G, Barde YA (1990): Binding of brain-derived neurotrophic factor to the nerve growth factor receptor. *Neuron* 4:487-492.
232. Rodriguez-Tebar A, Dechant G, Götz R, Barde YA (1992): Binding of neurotrophin-3 to its neuronal receptors and interactions with nerve growth factor and brain-derived neurotrophic factor. *EMBO J* 11:917-922.
233. Klein R, Jing SQ, Nanduri V, O'Rourke E, Barbacid M (1991): The trk proto-oncogene encodes a receptor for nerve growth factor. *Cell* 65:189-197.
234. Russo SJ, Mazei-Robison MS, Ables JL, Nestler EJ (2009): Neurotrophic factors and structural plasticity in addiction. *Neuropharmacology* 56, Supplement 1:73-82.
235. Chakravarthy S, Saiepour MH, Bence M, Perry S, Hartman R, Couey JJ, Mansvelder HD, Levelt CN (2006): Postsynaptic TrkB signaling has distinct roles in spine maintenance in adult visual cortex and hippocampus. *Proc Natl Acad Sci U S A* 103:1071-1076.
236. Danzer SC, Kotloski RJ, Walter C, Hughes M, McNamara JO (2008): Altered Morphology of Hippocampal Dentate Granule Cell Presynaptic and Postsynaptic Terminals Following Conditional Deletion of TrkB. *Hippocampus* 18:668-678.
237. Tanaka J, Horiike Y, Matsuzaki M, Miyazaki T, Ellis-Davies GCR, Kasai H (2008): Protein Synthesis and Neurotrophin-Dependent Structural Plasticity of Single Dendritic Spines. *Science* 319:1683-1687.
238. von Bohlen und Halbach O, Minichiello L, Unsicker K (2008): TrkB but not trkC receptors are necessary for postnatal maintenance of hippocampal spines. *Neurobiology of Aging* 29:1247-1255.
239. Grimm JW, Lu L, Hayashi T, Hope BT, Su TP, Shaham Y (2003): Time-Dependent Increases in Brain-Derived Neurotrophic Factor Protein Levels within the Mesolimbic Dopamine System after Withdrawal from Cocaine: Implications for Incubation of Cocaine Craving. *The Journal of Neuroscience* 23:742-747.
240. Le Foll B, Diaz J, Sokoloff P (2005): A single cocaine exposure increases BDNF and D3 receptor expression: implications for drug-conditioning. *NeuroReport* 16:175-178.
241. Chu NN, Zuo YF, Meng L, Lee DY, Han JS, Cui CL (2007): Peripheral electrical stimulation reversed the cell size reduction and increased BDNF level in the ventral tegmental area in chronic morphine-treated rats. *Brain Res* 1182:90-98.
242. Jenab S, Festa ED, Nazarian A, Wu HB, Sun WL, Hazim R, Russo SJ, Quinones-Jenab V (2005): Cocaine induction of ERK proteins in dorsal striatum of Fischer rats. *Molecular Brain Research* 142:134-138.
243. Shi X, McGinty JF (2006): Extracellular signal-regulated mitogen-activated protein kinase inhibitors decrease amphetamine-induced behavior and neuropeptide gene expression in the striatum. *Neuroscience* 138:1289-1298.

244. Shi X, McGinty JF (2007): Repeated amphetamine treatment increases phosphorylation of extracellular signal-regulated kinase, protein kinase B, and cyclase response element-binding protein in the rat striatum. *Journal of Neurochemistry* 103:706-713.
245. Sun WL, Zhou L, Hazim R, Quinones-Jenab V, Jenab S (2007): Effects of acute cocaine on ERK and DARPP-32 phosphorylation pathways in the caudate-putamen of Fischer rats. *Brain Res* 1178:12-19.
246. Valjent E, Pagès C, Hervé D, Girault JA, Caboche J (2004): Addictive and non-addictive drugs induce distinct and specific patterns of ERK activation in mouse brain. *Eur J Neurosci* 19:1826-1836.
247. Valjent E, Pascoli V, Svenningsson P, Paul S, Enslen H, Corvol JC, Stipanovich A, Caboche J, Lombroso PJ, Nairn AC, Greengard P, Hervé D, Girault JA (2005): Regulation of a protein phosphatase cascade allows convergent dopamine and glutamate signals to activate ERK in the striatum. *Proc Natl Acad Sci U S A* 102:491-496.
248. Muller DL, Unterwald EM (2004): In vivo regulation of extracellular signal-regulated protein kinase (ERK) and protein kinase B (Akt) phosphorylation by acute and chronic morphine. *J Pharmacol Exp Ther* 3010:774-782.
249. Ferrer-Alcón M, García-Fuster J, La Harpe R, García-Sevilla JA (2004): Long-term regulation of signalling components of adenylyl cyclase and mitogen-activated protein kinase in the pre-frontal cortex of human opiate addicts. *Journal of Neurochemistry* 90:220-230.
250. Berhow MT, Hiroi N, Nestler EJ (1996): Regulation of ERK (Extracellular Signal Regulated Kinase), Part of the Neurotrophin Signal Transduction Cascade, in the Rat Mesolimbic Dopamine System by Chronic Exposure to Morphine or Cocaine. *J Neurosci* 16:4707-4715.
251. Liu Y, Wang Y, Jiang Z, Wan C, Zhou W, Wang Z (2007): The extracellular signal-regulated kinase signaling pathway is involved in the modulation of morphine-induced reward by mPer1. *Neuroscience* 146:265-271.
252. Ortiz J, Harris HW, Guitart X, Terwilliger RZ, Haycock JW, Nestler EJ (1995): Extracellular signal-regulated protein kinases (ERKs) and ERK kinase (MEK) in brain: regional distribution and regulation by chronic morphine. *J Neurosci* 15:1285-1297.
253. Hall FS, Drzonova J, Goeb M, Uhl GR (2003): Reduced Behavioral Effects of Cocaine in Heterozygous Brain-Derived Neurotrophic Factor (BDNF) Knockout Mice. *Neuropsychopharmacology* 28:1485-1490.
254. Horger BA, Iyasere CA, Berhow MT, Messer CJ, Nestler EJ, Taylor JR (1999): Enhancement of Locomotor Activity and Conditioned Reward to Cocaine by Brain-Derived Neurotrophic Factor. *The Journal of Neuroscience* 19:4110-4122.
255. Lu L, Dempsey J, Liu SY, Bossert JM, Shaham Y (2004): A Single Infusion of Brain-Derived Neurotrophic Factor into the Ventral Tegmental Area Induces Long-Lasting Potentiation of Cocaine Seeking after Withdrawal. *The Journal of Neuroscience* 24:1604-1611.
256. Berglind WJ, See RE, Fuchs RA, Ghee SM, Whitfield TW, Miller SW, McGinty JF (2007): A BDNF infusion into the medial prefrontal cortex suppresses cocaine seeking in rats. *Eur J Neurosci* 26:757-766.

257. Guillin O, Diaz J, Carroll P, Griffon N, Schwartz JC, Sokoloff P (2001): BDNF controls dopamine D3 receptor expression and triggers behavioural sensitization. *Nature* 411:86-89.
258. Pu L, Liu Qs, Poo Mm (2006): BDNF-dependent synaptic sensitization in midbrain dopamine neurons after cocaine withdrawal. *Nat Neurosci* 9:605-607.
259. Berton O, McClung CA, DiLeone RJ, Krishnan V, Renthal W, Russo SJ, Graham D, Tsankova NM, Bolanos CA, Rios M, Monteggia LM, Self DW, Nestler EJ (2006): Essential Role of BDNF in the Mesolimbic Dopamine Pathway in Social Defeat Stress. *Science* 311:864-868.
260. Eisch AJ, Bolaños CA, de Wit J, Simonak RD, Pudiak CM, Barrot M, Verhaagen J, Nestler EJ (2003): Brain-derived neurotrophic factor in the ventral midbrain-nucleus accumbens pathway: a role in depression. *Biological Psychiatry* 54:994-1005.
261. Shirayama Y, Chen ACH, Nakagawa S, Russell DS, Duman RS (2002): Brain-Derived Neurotrophic Factor Produces Antidepressant Effects in Behavioral Models of Depression. *The Journal of Neuroscience* 22:3251-3261.
262. Dacher M, Nugent FS (2011): Opiates and plasticity. *Neuropharmacology* 61:1088-1096.
263. Luscher C, Malenka RC (2011): Drug-evoked synaptic plasticity in addiction: from molecular changes to circuit remodeling. *Neuron* 69:650-663.
264. Kalivas PW (2009): The glutamate homeostasis hypothesis of addiction. *Nat Rev Neurosci* 10:561-572.
265. Goldstein RZ, Volkow ND (2002): Drug Addiction and Its Underlying Neurobiological Basis: Neuroimaging Evidence for the Involvement of the Frontal Cortex. *Am J Psychiatry* 159:1642-1652.
266. Kolb B, Pellis S, Robinson TE (2004): Plasticity and functions of the orbital frontal cortex. *Brain and Cognition* 55:104-115.
267. Franklin TR, Acton PD, Maldjian JA, Gray JD, Croft JR, Dackis CA, O'Brien CP, Childress AR (2002): Decreased gray matter concentration in the insular, orbitofrontal, cingulate, and temporal cortices of cocaine patients. *Biological Psychiatry* 51:134-142.
268. Wilson SJ, Sayette MA, Fiez JA (2004): Prefrontal responses to drug cues: a neurocognitive analysis. *Nat Neurosci* 7:211-214.
269. Kalivas PW, Volkow ND (2005): The Neural Basis of Addiction: A Pathology of Motivation and Choice. *Am J Psychiatry* 162:1403-1413.
270. Garavan H, Pankiewicz J, Bloom A, Cho JK, Sperry L, Ross TJ, Salmeron BJ, Risinger R, Kelley D, Stein EA (2000): Cue-induced cocaine craving: neuroanatomical specificity for drug users and drug stimuli. *Am J Psychiatry* 157:1789-1798.
271. Volkow ND, Fowler JS, Wang GJ, Swanson JM (2004): Dopamine in drug abuse and addiction: results from imaging studies and treatment implications. *Mol Psychiatry* 9:557-569.
272. Turrigiano GG (1999): Homeostatic plasticity in neuronal networks: the more things change, the more they stay the same. *Trends Neurosci* 22:221-227.

273. Kogan JH, Nestler EJ, Aghajanian GK (1992): Elevated basal firing rates and enhanced responses to 8-Br-cAMP in locus coeruleus neurons in brain slices from opiate-dependent rats. *European Journal of Pharmacology* 211:47-53.
274. Lane-Ladd SB, Pineda J, Boundy VA, Pfeuffer T, Krupinski J, Aghajanian GK, Nestler EJ (1997): CREB (cAMP Response Element-Binding Protein) in the Locus Coeruleus: Biochemical, Physiological, and Behavioral Evidence for a Role in Opiate Dependence. *The Journal of Neuroscience* 17:7890-7901.
275. Han MH, Bolaños CA, Green TA, Olson VG, Neve RL, Liu RJ, Aghajanian GK, Nestler EJ (2006): Role of cAMP Response Element-Binding Protein in the Rat Locus Coeruleus: Regulation of Neuronal Activity and Opiate Withdrawal Behaviors. *The Journal of Neuroscience* 26:4624-4629.
276. Cao JL, Vialou VF, Lobo MK, Robison AJ, Neve RL, Cooper DC, Nestler EJ, Han MH (2010): Essential role of the cAMP-cAMP response-element binding protein pathway in opiate-induced homeostatic adaptations of locus coeruleus neurons. *Proc Natl Acad Sci U S A* 107:17011-17016.
277. Mazei-Robison MS, Koo JW, Friedman AK, Lansink CS, Robinson AJ, Vinish M, Krishnan V, Kim S, Siuta MA, Galli A (2011): Role for mTOR Signaling and Neuronal Activity in Morphine-Induced Adaptations in Ventral Tegmental Area Dopamine Neurons. *Neuron* 72:977-990.
278. Koo JW, Mazei-Robison MS, Chaudhury D, Juarez B, LaPlant Q, Ferguson D, Feng J, Sun H, Scobie KN, Damez-Werno D, Crumiller M, Ohnishi YN, Ohnishi YH, Mouzon E, Dietz DM, Lobo MK, Neve RL, Russo SJ, Han MH, Nestler EJ (2012): BDNF is a Negative Modulator of Morphine Action. *Science* 338:124-128.
279. Russo SJ, Dietz DM, Dumitriu D, Morrison JH, Malenka RC, Nestler EJ (2010): The addicted synapse: mechanisms of synaptic and structural plasticity in nucleus accumbens. *Trends in Neurosci* 33:267-276.
280. Carlisle HJ, Kennedy MB (2005): Spine architecture and synaptic plasticity. *Trends Neurosci* 28:182-187.
281. Bosch M, Hayashi Y (2012): Structural plasticity of dendritic spines. *Curr Opin Neurobiol* 22:383-388.
282. Grueter BA, Robison AJ, Neve RL, Nestler EJ, Malenka RC (2013): Δ FosB differentially modulates nucleus accumbens direct and indirect pathway function. *Proc Natl Acad Sci U S A* 110:1923-1928.
283. Robinson TE, Kolb B (2004): Structural plasticity associated with exposure to drugs of abuse. *Neuropharmacology* 47, Supplement 1:33-46.
284. Shen HW, Toda S, Moussawi K, Bouknight A, Zahm DS, Kalivas PW (2009): Altered Dendritic Spine Plasticity in Cocaine Withdrawn Rats. *J Neurosci* 29:2876-2884.
285. Gipson CD, Kupchik YM, Shen H, Reissner KJ, Thomas CA, Kalivas PW (2013): Relapse induced by cues predicting cocaine depends on rapid, transient synaptic potentiation. *Neuron* 77:867-872.

286. Dumitriu D, LaPlant Q, Grossman YS, Dias C, Janssen WG, Russo SJ, Morrison JH, Nestler EJ (2012): Subregional, dendritic compartment, and spine subtype specificity in cocaine-regulation of dendritic spines in the nucleus accumbens. *J Neurosci* 32:6957-6966.
287. Lee KW, Kim Y, Kim AM, Helmin K, Nairn AC, Greengard P (2006): Cocaine-induced dendritic spine formation in D1 and D2 dopamine receptor-containing medium spiny neurons in nucleus accumbens. *Proc Natl Acad Sci U S A* 103:3399-3404.
288. Kim Y, Teylan MA, Baron M, Sands A, Nairn AC, Greengard P (2009): Methylphenidate-induced dendritic spine formation and Δ FosB expression in nucleus accumbens. *Proc Natl Acad Sci U S A* 106:2915-2920.
289. Robison AJ, Vialou V, Mazei-Robison M, Feng J, Kourrich S, Collins M, Wee S, Koob G, Turecki G, Neve R, Thomas M, Nestler EJ (2013): Behavioral and Structural Responses to Chronic Cocaine Require a Feed-Forward Loop Involving Δ FosB and CaMKII in the Nucleus Accumbens Shell. *J Neurosci* 33:4295-4307.
290. Maze I, Covington HE, Dietz DM, LaPlant Q, Renthall W, Russo SJ, Mechanic M, Mouzon E, Neve RL, Haggarty SJ, Ren Y, Sampath SC, Hurd YL, Greengard P, Tarakhovskiy A, Schaefer A, Nestler EJ (2010): Essential Role of the Histone Methyltransferase G9a in Cocaine-induced Plasticity. *Science* 327:213-
291. Hope BT, Nye HE, Kelz MB, Self DW, Iadarola MJ, Nakabeppu Y, Duman RS, Nestler EJ (1994): Induction of a long-lasting AP-1 complex composed of altered Fos-like proteins in brain by chronic cocaine and other chronic treatments. *Neuron* 13:1235-1244.
292. Russo SJ, Wilkinson M, Mazei-Robison M, Dietz DM, Maze I, Krishnan V, Renthall W, Graham A, Birnbaum SG, Green TA, Robison B, Lesselyong A, Perrotti LI, Bolanos CA, Kumar A, Clark MS, Neumaier JF, Neve RL, Bhakar AL, Barker PA, Nestler EJ (2009): Nuclear factor kappa B Signaling Regulates Neuronal Morphology and Cocaine Reward. *J Neurosci* 29:3529-3537.
293. Bibb JA, Chen J, Taylor JR, Svenningsson P, Nishi A, Snyder GL, Yan Z, Sagawa ZK, Ouimet CC, Nairn AC, Nestler EJ, Greengard P (2001): Effects of chronic exposure to cocaine are regulated by the neuronal protein Cdk5. *Nature* 410:376-380.
294. Sklair-Tavron L, Shi WX, Lane SB, Harris HW, Bunney BS, Nestler EJ (1996): Chronic morphine induces visible changes in the morphology of mesolimbic dopamine neurons. *Proc Natl Acad Sci U S A* 93:11202-11207.
295. Russo SJ, Bolanos CA, Theobald DE, DeCarolis NA, Renthall W, Kumar A, Winstanley CA, Renthall NE, Wiley MD, Self DW, Russell DS, Neve RL, Eisch AJ, Nestler EJ (2007): IRS2-Akt pathway in midbrain dopamine neurons regulates behavioral and cellular responses to opiates. *Nat Neurosci* 10:93-99.
296. Rao A, Craig AM (2000): Signaling between the actin cytoskeleton and the postsynaptic density of dendritic spines. *Hippocampus* 10:527-541.
297. Blanpied TA, Ehlers MD (2004): Microanatomy of dendritic spines: emerging principles of synaptic pathology in psychiatric and neurological disease. *Biological Psychiatry* 55:1121-1127.
298. Matus A (2005): Growth of dendritic spines: a continuing story. *Current Opinion in Neurobiology* 15:67-72.

299. Toda S, Shen HW, Peters J, Cagle S, Kalivas PW (2006): Cocaine Increases Actin Cycling: Effects in the Reinstatement Model of Drug Seeking. *The Journal of Neuroscience* 26:1579-1587.
300. Kasai H, Matsuzaki M, Noguchi J, Yasumatsu N, Nakahara H (2003): Structure-stability-function relationships of dendritic spines. *Trends Neurosci* 26:360-368.
301. Mangiavacchi S, Wolf ME (2004): D1 dopamine receptor stimulation increases the rate of AMPA receptor insertion onto the surface of cultured nucleus accumbens neurons through a pathway dependent on protein kinase A. *Journal of Neurochemistry* 88:1261-1271.
302. Boudreau AC, Wolf ME (2005): Behavioral Sensitization to Cocaine Is Associated with Increased AMPA Receptor Surface Expression in the Nucleus Accumbens. *The Journal of Neuroscience* 25:9144-9151.
303. Sun X, Zhao Y, Wolf ME (2005): Dopamine Receptor Stimulation Modulates AMPA Receptor Synaptic Insertion in Prefrontal Cortex Neurons. *The Journal of Neuroscience* 25:7342-7351.
304. Sinha R (2007): The role of stress in addiction relapse. *Curr Psych Report* 9:388-395.
305. Koob G, Kreek MJ (2007): Stress, Dysregulation of Drug Reward Pathways, and the Transition to Drug Dependence. *Am J Psychiatry* 164:1149-1159.
306. Kosten TA, Miserendino MJD, Kehoe P (2000): Enhanced acquisition of cocaine self-administration in adult rats with neonatal isolation stress experience. *Brain Res* 875:44-50.
307. Piazza PV, Deminiere JM, Le Moal M, Simon H (1990): Stress- and pharmacologically-induced behavioral sensitization increases vulnerability to acquisition of amphetamine self-administration. *Brain Res* 514:22-26.
308. Goeders NE, Guerin GF (1994): Non-contingent electric footshock facilitates the acquisition of intravenous cocaine self-administration in rats. *Psychopharmacology (Berl)* 114:63-70.
309. McLaughlin JP, Marton-Popovici M, Chavkin C (2003): κ Opioid Receptor Antagonism and Prodynorphin Gene Disruption Block Stress-Induced Behavioral Responses. *J Neurosci* 23:5674-5683.
310. Goeders NE, Guerin GF (1996): Effects of surgical and pharmacological adrenalectomy on the initiation and maintenance of intravenous cocaine self-administration in rats. *Brain Res* 722:145-152.
311. Mantsch JR, Saphier D, Goeders NE (1998): Corticosterone Facilitates the Acquisition of Cocaine Self-Administration in Rats: Opposite Effects of the Type II Glucocorticoid Receptor Agonist Dexamethasone. *J Pharmacol Exp Ther* 287:72-80.
312. Goeders NE, Guerin GF (1996): Role of corticosterone in intravenous cocaine self-administration in rats. *Neuroendocrinology* 64:337-348.
313. Härfstrand A, Fuxe K, Cintra A, Agnati LF, Zini I, Wikström AC, Okret S, Yu ZY, Goldstein M, Steinbusch H (1986): Glucocorticoid receptor immunoreactivity in monoaminergic neurons of rat brain. *Proc Natl Acad Sci U S A* 83:9779-9783.

314. Barrot M, Marinelli M, Abrous DN, Rougé-Pont F, Le Moal M, Piazza PV (2000): The dopaminergic hyper-responsiveness of the shell of the nucleus accumbens is hormone-dependent. *Eur J Neurosci* 12:973-979.
315. Rougé-Pont F, Deroche V, Moal ML, Piazza PV (1998): Individual differences in stress-induced dopamine release in the nucleus accumbens are influenced by corticosterone. *Eur J Neurosci* 10:3903-3907.
316. Marinelli M, Aouizerate B, Barrot M, Le Moal M, Piazza PV (1998): Dopamine-dependent responses to morphine depend on glucocorticoid receptors. *Proc Natl Acad Sci U S A* 95:7742-7747.
317. Deroche-Gamonet V, Sillaber I, Aouizerate B, Izawa R, Jaber M, Ghozland S, Kellendonk C, Le Moal M, Spanagel R, Schutz G, Tronche F, Piazza PV (2003): The Glucocorticoid Receptor as a Potential Target to Reduce Cocaine Abuse. *J Neurosci* 23:4785-4790.
318. Lu L, Liu Z, Huang M, Zhang Z (2003): Dopamine-dependent responses to cocaine depend on corticotropin-releasing factor receptor subtypes. *Journal of Neurochemistry* 84:1378-1386.
319. Przegalinski E, Filip M, Frankowska M, Zaniewska M, Papla I (2005): Effects of CP 154,526, a CRF1 receptor antagonist, on behavioral responses to cocaine in rats. *Neuropeptides* 39:525-533.
320. Schluger JH, Borg L, Ho A, Kreek MJ (2001): Altered HPA Axis Responsivity to Metyrapone Testing in Methadone Maintained Former Heroin Addicts with Ongoing Cocaine Addiction. *Neuropsychopharmacology* 24:568-575.
321. Kreek MJ, Ragunath J, Hamer D, Schneider B, Hartman N (1984): ACTH, cortisol and beta-endorphin response to metyrapone testing during chronic methadone maintenance treatment in humans. *Neuropeptides* 5:277-278.
322. Navarro-Zaragoza J, Hidalgo JM, Laorden ML, Milanés MV (2012): Glucocorticoid receptors participate in the opiate withdrawal-induced stimulation of rats NTS noradrenergic activity and in the somatic signs of morphine withdrawal. *Br J Pharmacol* 166:2136-2147.
323. Navarro-Zaragoza J, Laorden ML, Milanés MV (2014): Spironolactone decreases the somatic signs of opiate withdrawal by blocking the mineralocorticoid receptors (MR). *Toxicology* 326:36-43.
324. Sarnyai Z, Bíró E, Gardi J, Vecsernyés M, Julesz J, Telegdy G (1995): Brain corticotropin-releasing factor mediates 'anxiety-like' behavior induced by cocaine withdrawal in rats. *Brain Res* 675:89-97.
325. Rassnick S, Heinrichs SC, Britton KT, Koob GF (1993): Microinjection of a corticotropin-releasing factor antagonist into the central nucleus of the amygdala reverses anxiogenic-like effects of ethanol withdrawal. *Brain Res* 605:25-32.
326. Skelton KH, Oren D, Gutman DA, Easterling K, Holtzman SG, Nemeroff CB, Owens MJ (2007): The CRF1 receptor antagonist, R121919, attenuates the severity of precipitated morphine withdrawal. *European Journal of Pharmacology* 571:17-24.
327. Navarro-Zaragoza J, Núñez C, Laorden ML, Milanés MV (2010): Effects of corticotropin-releasing factor receptor-1 (CRF1R) antagonists on the brain stress system responses to morphine withdrawal. *Mol Pharmacol* 77:864-873.

328. Navarro-Zaragoza J, Núñez C, Ruiz-Medina J, Laorden ML, Valverde O, Milanés MV (2012): CRF(2) mediates the increased noradrenergic activity in the hypothalamic paraventricular nucleus and the negative state of morphine withdrawal in rats. *Br J Pharmacol* 162:851-862.
329. Wang J, Fang Q, Liu Z, Lu L (2006): Region-specific effects of brain corticotropin-releasing factor receptor type 1 blockade on footshock-stress- or drug-priming-induced reinstatement of morphine conditioned place preference in rats. *Psychopharmacology* 185:19-28.
330. Gass JT, Olive MF (2007): Reinstatement of Ethanol-Seeking Behavior Following Intravenous Self-Administration in Wistar Rats. *Alcoholism: Clinical and Experimental Research* 31:1441-1445.
331. Kreibich AS, Blendy JA (2004): cAMP Response Element-Binding Protein Is Required for Stress But Not Cocaine-Induced Reinstatement. *The Journal of Neuroscience* 24:6686-6692.
332. Zislis G, Desai TV, Prado M, Shah HP, Bruijnzeel AW (2007): Effects of the CRF receptor antagonist D-Phe CRF₍₁₂₋₄₁₎ and the α 2-adrenergic receptor agonist clonidine on stress-induced reinstatement of nicotine-seeking behavior in rats. *Neuropharmacology* 53:958-966.
333. Shaham Y, Funk D, Erb S, Brown TJ, Walker CD, Stewart J (1997): Corticotropin-Releasing Factor, But Not Corticosterone, Is Involved in Stress-Induced Relapse to Heroin-Seeking in Rats. *The Journal of Neuroscience* 17:2605-2614.
334. Erb S, Salmaso N, Rodaros D, Stewart J (2001): A role for the CRF-containing pathway from central nucleus of the amygdala to bed nucleus of the stria terminalis in the stress-induced reinstatement of cocaine seeking in rats. *Psychopharmacology* 158:360-365.
335. Erb S, Stewart J (1999): A role for the bed nucleus of the stria terminalis, but not the amygdala, in the effects of corticotropin-releasing factor on stress-induced reinstatement of cocaine seeking. *J Neurosci* 19:RC35-
336. Lu L, Liu D, Ceng X (2001): Corticotropin-releasing factor receptor type 1 mediates stress-induced relapse to cocaine-conditioned place preference in rats. *European Journal of Pharmacology* 415:203-208.
337. Shaham Y, Erb S, Leung S, Buczek Y, Stewart J (1998): CP-154,526, a selective, non-peptide antagonist of the corticotropin-releasing factor1 receptor attenuates stress-induced relapse to drug seeking in cocaine- and heroin-trained rats. *Psychopharmacology (Berl)* 137:184-190.
338. Kalivas PW, Duffy P (1995): Selective activation of dopamine transmission in the shell of the nucleus accumbens by stress. *Brain Res* 675:325-328.
339. Saal D, Dong Y, Bonci A, Malenka RC (2003): Drugs of Abuse and Stress Trigger a Common Synaptic Adaptation in Dopamine Neurons. *Neuron* 37:577-582.
340. Robinson TE, Kolb B (1999): Morphine alters the structure of neurons in the nucleus accumbens and neocortex of rats. *Synapse* 33:160-162.
341. Robinson TE, Kolb B (1999): Alterations in the morphology of dendrites and dendritic spines in the nucleus accumbens and prefrontal cortex following repeated treatment with amphetamine or cocaine. *Eur J Neurosci* 11:1598-1604.
342. Liston C, Miller MM, Goldwater DS, Radley JJ, Rocher AB, Hof PR, Morrison JH, McEwen BS (2006): Stress-Induced Alterations in Prefrontal Cortical Dendritic Morphology Predict

- Selective Impairments in Perceptual Attentional Set-Shifting. *The Journal of Neuroscience* 26:7870-7874.
343. Perrotti LI, Hadeishi Y, Ulery PG, Barrot M, Monteggia L, Duman RS, Nestler EJ (2004): Induction of Δ FosB in Reward-Related Brain Structures after Chronic Stress. *The Journal of Neuroscience* 24:10594-10602.
 344. Walters CL, Kuo YC, Blendy JA (2003): Differential distribution of CREB in the mesolimbic dopamine reward pathway. *Journal of Neurochemistry* 87:1237-1244.
 345. Bilang-Bleuel A, Rech J, De Carli S, Holsboer F, Reul JM (2002): Forced swimming evokes a biphasic response in CREB phosphorylation in extrahypothalamic limbic and neocortical brain structures in the rat. *Eur J Neurosci* 15:1048-1060.
 346. Wang B, Shaham Y, Zitzman D, Azari S, Wise RA, You ZB (2005): Cocaine Experience Establishes Control of Midbrain Glutamate and Dopamine by Corticotropin-Releasing Factor: A Role in Stress-Induced Relapse to Drug Seeking. *The Journal of Neuroscience* 25:5389-5396.
 347. Wang B, You ZB, Rice K, Wise R (2007): Stress-induced relapse to cocaine seeking: roles for the CRF2 receptor and CRF-binding protein in the ventral tegmental area of the rat. *Psychopharmacology* 193:283-294.
 348. Cox S, Alcorn R (1995): Lofexidine and opioid withdrawal. *The Lancet* 345:1385-1386.
 349. Erb S, Hitchcott PK, Rajabi H, Mueller D, Shaham Y, Stewart J (2000): Alpha-2 Adrenergic Receptor Agonists Block Stress-Induced Reinstatement of Cocaine Seeking. *Neuropsychopharmacology* 23:138-150.
 350. Shaham Y, Highfield D, Delfs J, Leung S, Stewart J (2000): Clonidine blocks stress-induced reinstatement of heroin seeking in rats: an effect independent of locus coeruleus noradrenergic neurons. *Eur J Neurosci* 12:292-302.
 351. Leri F, Flores J, Rodaros D, Stewart J (2002): Blockade of Stress-Induced But Not Cocaine-Induced Reinstatement by Infusion of Noradrenergic Antagonists into the Bed Nucleus of the Stria Terminalis or the Central Nucleus of the Amygdala. *The Journal of Neuroscience* 22:5713-5718.
 352. Murphy EP, Conneely OM (1997): Neuroendocrine Regulation of the Hypothalamic Pituitary Adrenal Axis by the nurr1/nur77 Subfamily of Nuclear Receptors. *Mol Endocrinol* 11:39-47.
 353. Drouin J, Maira M, Philips A (1998): Novel mechanism of action for Nur77 and antagonism by glucocorticoids: a convergent mechanism for CRH activation and glucocorticoid repression of POMC gene transcription. *J Steroid Biochem Mol Biol* 65:59-63.
 354. Wilson TE, Mouw AR, Weaver CA, Milbrandt J, Parker KL (1993): The orphan nuclear receptor NGFI-B regulates expression of the gene encoding steroid 21-hydroxylase. *Mol Cell Biol* 13:861-868.
 355. Fernandez PM, Brunel F, Jimenez MaA, Saez JM, Cereghini S, Zakin MM (2000): Nuclear Receptors Nor1 and NGFI-B/Nur77 Play Similar, Albeit Distinct, Roles in the Hypothalamo-Pituitary-Adrenal Axis. *Endocrinology* 141:2392-2400.
 356. Bassett MH, Suzuki T, Sasano H, de Vries CJM, Jimenez PT, Carr BR, Rainey WE (2004): The Orphan Nuclear Receptor NGFI-B Regulates Transcription of 3 β -Hydroxysteroid

- Dehydrogenase: implications for the control of adrenal functional zonation. *J Biol Chem* 279:37622-37630.
357. Sun W, Choi SH, Park SK, Kim SJ, Noh MR, Kim EH, Kim HJ, Kim H (2007): Identification and characterization of novel activity-dependent transcription factors in rat cortical neurons. *Journal of Neurochemistry* 100:269-278.
 358. Schiltz CA, Kelley AE, Landry CF (2007): Acute Stress and Nicotine Cues Interact to Unveil Locomotor Arousal and Activity-Dependent Gene Expression in the Prefrontal Cortex. *Biol Psychiatry* 61:127-135.
 359. Katunar MR, Saez T, Brusco A, Antonelli MC (2009): Immunocytochemical expression of dopamine-related transcription factors Pitx3 and Nurr1 in prenatally stressed adult rats. *J Neurosci Res* 87:1014-1022.
 360. Katunar M, Saez T, Brusco A, Antonelli M (2010): Ontogenetic Expression of Dopamine-Related Transcription Factors and Tyrosine Hydroxylase in Prenatally Stressed Rats. *Neurotoxicity Research* 18:69-81.
 361. Rojas P, Joodmardi E, Perlmann T, Ogren SO (2010): Rapid increase of Nurr1 mRNA expression in limbic and cortical brain structures related to coping with depression-like behavior in mice. *J Neurosci Res* 88:2284-2293.
 362. Moore TM, Brown T, Cade M, Eells JB (2008): Alterations in amphetamine-stimulated dopamine overflow due to the Nurr1-null heterozygous genotype and postweaning isolation. *Synapse* 62:764-774.
 363. Nestler EJ (2014): Epigenetic Mechanisms of Drug Addiction. *Neuropharmacology* 76:259-268.
 364. Kumar A, Choi KH, Renthal W, Tsankova NM, Theobald DEH, Truong HT, Russo SJ, LaPlant Q, Sasaki TS, Whistler KN, Neve RL, Self DW, Nestler EJ (2005): Chromatin Remodeling Is a Key Mechanism Underlying Cocaine-Induced Plasticity in Striatum. *Neuron* 48:303-314.
 365. Schroeder FA, Penta KL, Matevossian A, Jones SR, Konradi C, Tapper AR, Akbarian S (2008): Drug-induced activation of dopamine D(1) receptor signaling and inhibition of class I/II histone deacetylase induces chromatin remodeling in reward circuitry and modulates cocaine-related behaviors. *Neuropsychopharmacology* 33:2981-2992.
 366. Shen HY, Kalda A, Yu L, Ferrara J, Zhu J, Chen JF (2008): Additive effects of histone deacetylase inhibitors and amphetamine on histone H4 acetylation, cAMP responsive element binding protein phosphorylation and Δ FosB expression in the striatum and locomotor sensitization in mice. *Neuroscience* 157:644-655.
 367. Sun H, Maze I, Dietz DM, Scobie KN, Kennedy PJ, Damez-Werno D, Neve RL, Zachariou V, Shen L, Nestler EJ (2012): Morphine epigenomically regulates behavior through alterations in histone H3 lysine 9 dimethylation in the nucleus accumbens. *J Neurosci* 32:17454-17464.
 368. Anier K, Malinovskaja K, Aonurm-Helm A, Zharkovsky A, Kalda A (2010): DNA Methylation Regulates Cocaine-Induced Behavioral Sensitization in Mice. *Neuropsychopharmacology* 35:2450-2461.

369. LaPlant Q, Vialou V, Covington HE, Dumitriu D, Feng J, Warren B, Maze I, Dietz DM, Watts EL, Iñiguez SD, Koo JW, Mouzon E, Renthal W, Hollis F, Wang H, Noonan MA, Ren Y, Eisch AJ, Bolaños CA, Kabbaj M, Xiao G, Neve RL, Hurd YL, Oosting RS, Fan G, Morrison JH, Nestler EJ (2010): Dnmt3a regulates emotional behavior and spine plasticity in the nucleus accumbens. *Nat Neurosci* 13:1137-1143.
370. The ENCODE Project Consortium (2012): An Integrated Encyclopedia of DNA Elements in the Human Genome. *Nature* 489:57-74.
371. Rinn JL, Chang HY (2012): Genome regulation by long noncoding RNAs. *Annu Rev Biochem* 81:145-166.
372. Valencia-Sanchez MA, Liu J, Hannon GJ, Parker R (2006): Control of translation and mRNA degradation by miRNAs and siRNAs. *Genes & Development* 20:515-524.
373. Moller HG, Rasmussen AP, Andersen HH, Johnsen KB, Henriksen M, Duroux M (2013): A Systematic Review of MicroRNA in Glioblastoma Multiforme: Micro-modulators in the Mesenchymal Mode of Migration and Invasion. *Mol Neurobiol* 47:131-144.
374. Kloosterman WP, Wienholds E, Ketting RF, Plasterk RH (2004): Substrate requirements for let-7 function in the developing zebrafish embryo. *Nucleic Acids Res* 32:6284-6291.
375. Easow G, Teleman AA, Cohen SM (2007): Isolation of microRNA targets by miRNP immunopurification. *RNA* 13:1198-1204.
376. Schaefer A, Im HI, Veno MT, Fowler CD, Min A, Intrator A, Kjems J, Kenny PJ, O'Carroll D, Greengard P (2010): Argonaute 2 in dopamine 2 receptor-expressing neurons regulates cocaine addiction. *J Exp Med* 207:1843-1851.
377. He Y, Yang C, Kirkmire CM, Wang ZJ (2010): Regulation of opioid tolerance by let-7 family microRNA targeting the mu opioid receptor. *J Neurosci* 30:10251-10258.
378. Zheng H, Chu J, Zeng Y, Loh HH, Law PY (2010): Yin Yang 1 Phosphorylation Contributes to the Differential Effects of mu-Opioid Receptor Agonists on MicroRNA-190 Expression. *J Biol Chem* 285:21994-22002.
379. Chandrasekar V, Dreyer JL (2009): microRNAs miR-124, let-7d and miR-181a regulate Cocaine-induced Plasticity. *Molecular and Cellular Neuroscience* 42:350-362.
380. Chandrasekar V, Dreyer JL (2011): Regulation of MiR-124, Let-7d, and MiR-181a in the Accumbens Affects the Expression, Extinction, and Reinstatement of Cocaine-Induced Conditioned Place Preference. *Neuropsychopharmacology* 36:1149-1164.
381. Hwang CK, Wagley Y, Law PY, Wei LN, Loh HH (2012): microRNAs in opioid pharmacology. *J Neuroimmune Pharmacol* 7:808-819.
382. Sartor GC, St-Laurent G, Wahlestedt C (2012): The Emerging Role of Non-Coding RNAs in Drug Addiction. *Front Genet* 3:106-
383. Hollander JA, Im HI, Amelio AL, Kocerha J, Bali P, Lu Q, Willoughby D, Wahlestedt C, Conkright MD, Kenny PJ (2010): Striatal microRNA controls cocaine intake through CREB signaling. *Nature* 466:197-202.
384. Robison AJ, Nestler EJ (2011): Transcriptional and epigenetic mechanisms of addiction. *Nat Rev Neurosci* 12:623-637.

385. Pietrzykowski AZ, Friesen RM, Martin GE, Puig SI, Nowak CL, Wynne PM, Siegelmann HT, Treisman SN (2008): Post-transcriptional regulation of BK channel splice variant stability by miR-9 underlies neuroadaptation to alcohol. *Neuron* 59:274-287.
386. Saba R, Störchel PH, Aksoy-Aksel A, Kepura F, Lippi G, Plant TD, Schrott GM (2012): Dopamine-Regulated MicroRNA MiR-181a Controls GluA2 Surface Expression in Hippocampal Neurons. *Mol Cell Biol* 32:619-632.
387. Wu Q, Law PY, Wei LN, Loh HH (2008): Post-transcriptional regulation of mouse μ opioid receptor (MOR1) via its 3' untranslated region: a role for microRNA23b. *FASEB J* 22:4085-4095.
388. Hui Z, Zeng Y, Chu J, Kam AY, Loh HH, Law PY (2010): Modulations of NeuroD activity contribute to the differential effects of morphine and fentanyl on dendritic spine stability. *J Neurosci* 30:8102-8110.
389. Zheng H, Zeng Y, Zhang X, Chu J, Loh HH, Law PY (2010): μ -Opioid Receptor Agonists Differentially Regulate the Expression of miR-190 and NeuroD. *Mol Pharmacol* 77:102-109.
390. Hébert SS, De Strooper B (2009): Alterations of the microRNA network cause neurodegenerative disease. *Trends Neurosci* 32:199-206.
391. Sanchez-Simon FM, Zhang XX, Loh HH, Law PY, Rodriguez RE (2010): Morphine Regulates Dopaminergic Neuron Differentiation via miR-133b. *Mol Pharmacol* 78:935-942.
392. Rodriguez RE (2012): Morphine and microRNA activity: is there a relation with addiction? *Front Genet* 3:223-
393. Aguilar-Valles A, Sánchez E, de Gortari P, Balderas I, Ramírez-Amaya V, Bermúdez-Rattoni F, Joseph-Bravo P (2005): Analysis of the Stress Response in Rats Trained in the Water-Maze: Differential Expression of Corticotropin-Releasing Hormone, CRH-R1, Glucocorticoid Receptors and Brain-Derived Neurotrophic Factor in Limbic Regions. *Neuroendocrinology* 82:306-319.
394. Joëls M, Baram TZ (2009): The neuro-symphony of stress. *Nat Rev Neurosci* 10:459-466.
395. McCarty R, Gold PE (1981): Plasma catecholamines: Effects of footshock level and hormonal modulators of memory storage. *Hormones and Behavior* 15:168-182.
396. Roozendaal B, McEwen BS, Chattarji S (2009): Stress, memory and the amygdala. *Nat Rev Neurosci* 10:423-433.
397. McGaugh JL, Roozendaal B (2002): Role of adrenal stress hormones in forming lasting memories in the brain. *Current Opinion in Neurobiology* 12:205-210.
398. Roozendaal B, Brunson KL, Holloway BL, McGaugh JL, Baram TZ (2002): Involvement of stress-released corticotropin-releasing hormone in the basolateral amygdala in regulating memory consolidation. *Proc Natl Acad Sci U S A* 99:13908-13913.
399. Gold PE, Van Buskirk R (1976): Enhancement and impairment of memory processes with post-trial injections of adrenocorticotrophic hormone. *Behav Biol* 16:387-400.
400. Schreurs J, Seelig T, Schulman H (1986): Beta 2-adrenergic receptors on peripheral nerves. *J Neurochem* 46:294-296.

401. Gold PE, Van Buskirk R (1978): Posttraining brain norepinephrine concentrations: correlation with retention performance of avoidance training and with peripheral epinephrine modulation of memory processing. *Behav Biol* 23:509-520.
402. Nakamura S, Tepper JM, Young SJ, Ling N, Groves PM (1982): Noradrenergic terminal excitability: Effects of opioids. *Neuroscience Letters* 30:57-62.
403. Hatfield T, Spanis C, McGaugh JL (1999): Response of amygdalar norepinephrine to footshock and GABAergic drugs using in vivo microdialysis and HPLC. *Brain Res* 835:340-345.
404. Quirarte GL, Galvez R, Roozendaal B, McGaugh JL (1998): Norepinephrine release in the amygdala in response to footshock and opioid peptidergic drugs. *Brain Res* 808:134-140.
405. Okuda S, Roozendaal B, McGaugh JL (2004): Glucocorticoid effects on object recognition memory require training-associated emotional arousal. *Proc Natl Acad Sci U S A* 101:853-858.
406. Roozendaal B, McGaugh JL (1996): Amygdaloid Nuclei Lesions Differentially Affect Glucocorticoid-Induced Memory Enhancement in an Inhibitory Avoidance Task. *Neurobiology of Learning and Memory* 65:1-8.
407. Zorawski M, Killcross S (2002): Posttraining Glucocorticoid Receptor Agonist Enhances Memory in Appetitive and Aversive Pavlovian Discrete-Cue Conditioning Paradigms. *Neurobiology of Learning and Memory* 78:458-464.
408. Lupien SJ, McEwen BS (1997): The acute effects of corticosteroids on cognition: integration of animal and human model studies. *Brain Research Reviews* 24:1-27.
409. Oitzl MS, de Kloet ER (1992): Selective corticosteroid antagonists modulate specific aspects of spatial orientation learning. *Behav Neurosci* 106:62-71.
410. Roozendaal B, Portillo-Marquez G, McGaugh JL (1996): Basolateral amygdala lesions block glucocorticoid-induced modulation of memory for spatial learning. *Behavioral Neuroscience* 110:1074-1083.
411. Beato M, Herrlich P, Schütz G (1995): Steroid hormone receptors: many actors in search of a plot. *Cell* 83:851-857.
412. Datson NA, Van Der Perk J, de Kloet ER, Vreugdenhil E (2001): Identification of corticosteroid-responsive genes in rat hippocampus using serial analysis of gene expression. *Eur J Neurosci* 14:675-689.
413. Heck S, Kullmann M, Gast A, Ponta H, Rahmsdorf HJ, Herrlich P, Cato AC (1994): A distinct modulating domain in glucocorticoid receptor monomers in the repression of activity of the transcription factor AP-1. *EMBO J* 13:4087-4095.
414. Barsegyan A, Mackenzie SM, Kurose BD, McGaugh JL, Roozendaal B (2010): Glucocorticoids in the prefrontal cortex enhance memory consolidation and impair working memory by a common neural mechanism. *Proc Natl Acad Sci U S A* 107:16655-16660.
415. Dallman MF (2005): Fast glucocorticoid actions on brain: Back to the future. *Frontiers in Neuroendocrinology* 26:103-108.

416. Karst H, Berger S, Erdmann G, Schütz G, Joëls M (2010): Metaplasticity of amygdalar responses to the stress hormone corticosterone. *Proc Natl Acad Sci U S A* 107:14449-14454.
417. Roozendaal B, Okuda S, Van der Zee EA, McGaugh JL (2006): Glucocorticoid enhancement of memory requires arousal-induced noradrenergic activation in the basolateral amygdala. *Proc Natl Acad Sci U S A* 103:6741-6746.
418. McGaugh JL (2000): Memory--a Century of Consolidation. *Science* 287:248-251.
419. McGaugh JL (2004): The amygdala modulates the consolidation of memories of emotionally arousing experiences. *Annu Rev Neurosci* 27:1-28.
420. Introini-Collison IB, Miyazaki B, McGaugh JL (1991): Involvement of the amygdala in the memory-enhancing effects of clenbuterol. *Psychopharmacology (Berl)* 104:541-544.
421. Ferry B, Roozendaal B, McGaugh JL (1999): Basolateral Amygdala Noradrenergic Influences on Memory Storage Are Mediated by an Interaction between β - and α 1-Adrenoceptors. *The Journal of Neuroscience* 19:5119-5123.
422. LaLumiere RT, Buen TV, McGaugh JL (2003): Post-Training Intra-Basolateral Amygdala Infusions of Norepinephrine Enhance Consolidation of Memory for Contextual Fear Conditioning. *The Journal of Neuroscience* 23:6754-6758.
423. Huff NC, Wright-Hardesty KJ, Higgins EA, Matus-Amat P, Rudy JW (2005): Context pre-exposure obscures amygdala modulation of contextual-fear conditioning. *Learning & Memory* 12:456-460.
424. Miranda MI, LaLumiere RT, Buen TV, Bermudez-Rattoni F, McGaugh JL (2003): Blockade of noradrenergic receptors in the basolateral amygdala impairs taste memory. *Eur J Neurosci* 18:2605-2610.
425. Barseguyan A, McGaugh JL, Roozendaal B (2014): Noradrenergic activation of the basolateral amygdala modulates the consolidation of object-in-context recognition memory. *Front Behav Neurosci* 8:160-
426. Liang KC, Chen LL, Huang TE (1995): The role of amygdala norepinephrine in memory formation: involvement in the memory enhancing effect of peripheral epinephrine. *Chin J Physiol* 38:81-91.
427. Roozendaal B, McGaugh JL (1997): Glucocorticoid Receptor Agonist and Antagonist Administration into the Basolateral but Not Central Amygdala Modulates Memory Storage. *Neurobiology of Learning and Memory* 67:176-179.
428. Roozendaal B, Quirarte GL, McGaugh JL (2002): Glucocorticoids interact with the basolateral amygdala β -adrenoceptor-cAMP/cAMP/PKA system in influencing memory consolidation. *Eur J Neurosci* 15:553-560.
429. McReynolds JR, Donowho K, Abdi A, McGaugh JL, Roozendaal B, McIntyre CK (2010): Memory-enhancing corticosterone treatment increases amygdala norepinephrine and Arc protein expression in hippocampal synaptic fractions. *Neurobiology of Learning and Memory* 93:312-321.
430. Roozendaal B, McGaugh JL (2011): Memory modulation. *Behav Neurosci* 125:797-824.

431. Roozendaal B, Williams CL, McGaugh JL (1999): Glucocorticoid receptor activation in the rat nucleus of the solitary tract facilitates memory consolidation: involvement of the basolateral amygdala. *Eur J Neurosci* 11:1317-1323.
432. Miyashita T, Williams CL (2002): Glutamatergic transmission in the nucleus of the solitary tract modulates memory through influences on amygdala noradrenergic systems. *Behav Neurosci* 116:13-21.
433. Winocur G, Moscovitch M, Bontempi B (2010): Memory formation and long-term retention in humans and animals: Convergence towards a transformation account of hippocampal-neocortical interactions. *Neuropsychologia* 48:2339-2356.
434. Petrovich GD, Canteras NS, Swanson LW (2001): Combinatorial amygdalar inputs to hippocampal domains and hypothalamic behavior systems. *Brain Research Reviews* 38:247-289.
435. Pitkänen A (2000): Connectivity of the rat amygdaloid complex. In: Aggleton JP, editor. *The amygdala*. London: Oxford University Press;31-117.
436. Roozendaal B, McGaugh JL (1997): Basolateral amygdala lesions block the memory-enhancing effect of glucocorticoid administration in the dorsal hippocampus of rats. *Eur J Neurosci* 9:76-83.
437. Almaguer-Melian W, Martínez-Martí L, Frey JU, Bergado JA (2003): The amygdala is part of the behavioural reinforcement system modulating long-term potentiation in rat hippocampus. *Neuroscience* 119:319-322.
438. Ikegaya Y, Saito H, Abe K (1995): High-frequency stimulation of the basolateral amygdala facilitates the induction of long-term potentiation in the dentate gyrus in vivo. *Neuroscience Research* 22:203-207.
439. Ikegaya Y, Saito H, Abe K (1995): Requirement of basolateral amygdala neuron activity for the induction of long-term potentiation in the dentate gyrus in vivo. *Brain Res* 671:351-354.
440. Ikegaya Y, Nakanishi K, Saito H, Abe K (1997): Amygdala beta-noradrenergic influence on hippocampal longterm potentiation in vivo. *NeuroReport* 8:3143-3146.
441. Akirav I, Richter-Levin G (2002): Mechanisms of Amygdala Modulation of Hippocampal Plasticity. *The Journal of Neuroscience* 22:9912-9921.
442. Vouimba RM, Yaniv D, Richter-Levin G (2007): Glucocorticoid receptors and β -adrenoceptors in basolateral amygdala modulate synaptic plasticity in hippocampal dentate gyrus, but not in area CA1. *Neuropharmacology* 52:244-252.
443. Cai WH, Blundell J, Han J, Greene RW, Powell CM (2006): Postreactivation Glucocorticoids Impair Recall of Established Fear Memory. *J Neurosci* 26:9560-9566.
444. Pakdel R, Rashidy-Pour A (2006): Glucocorticoid-induced impairment of long-term memory retrieval in rats: An interaction with dopamine D2 receptors. *Neurobiology of Learning and Memory* 85:300-306.
445. Yang Y, Cao J, Xiong W, Zhang J, Zhou Q, Wei H, Liang C, Deng J, Li T, Yang S, Xu L, Xu L (2003): Both stress experience and age determine the impairment or enhancement effect of stress on spatial memory retrieval. *J Endocrinol* 178:45-54.

446. Roozendaal B, de Quervain DJF, Schelling G, McGaugh JL (2004): A systemically administered beta-adrenoceptor antagonist blocks corticosterone-induced impairment of contextual memory retrieval in rats. *Neurobiology of Learning and Memory* 81:150-154.
447. Roozendaal B, Hahn EL, Nathan SV, de Quervain DJF, McGaugh JL (2004): Glucocorticoid Effects on Memory Retrieval Require Concurrent Noradrenergic Activity in the Hippocampus and Basolateral Amygdala. *The Journal of Neuroscience* 24:8161-8169.
448. Rashidy-Pour A, Sadeghi H, Taherain AA, Vafaei AA, Fathollahi Y (2004): The effects of acute restraint stress and dexamethasone on retrieval of long-term memory in rats: an interaction with opiate system. *Behavioural Brain Research* 154:193-198.
449. Hirsh R (1974): The hippocampus and contextual retrieval of information from memory: a theory. *Behav Biol* 12:421-444.
450. Squire LR, Clark RE, Knowlton BJ (2001): Retrograde amnesia. *Hippocampus* 11:50-55.
451. Holt W, Maren S (1999): Muscimol Inactivation of the Dorsal Hippocampus Impairs Contextual Retrieval of Fear Memory. *The Journal of Neuroscience* 19:9054-9062.
452. Riedel G, Micheau J, Lam AGM, Roloff E, Martin SJ, Bridge H, Hoz Ld, Poeschel B, McCulloch J, Morris RGM (1999): Reversible neural inactivation reveals hippocampal participation in several memory processes. *Nat Neurosci* 2:898-905.
453. Roozendaal B, Griffith QK, Buranday J, de Quervain DJF, McGaugh JL (2003): The hippocampus mediates glucocorticoid-induced impairment of spatial memory retrieval: Dependence on the basolateral amygdala. *Proc Natl Acad Sci U S A* 100:1328-1333.
454. Sajadi AA, Samaei SA, Rashidy-Pour A (2006): Intra-hippocampal microinjections of anisomycin did not block glucocorticoid-induced impairment of memory retrieval in rats: An evidence for non-genomic effects of glucocorticoids. *Behavioural Brain Research* 173:158-162.
455. Boujabit M, Bontempi B, Destrade C, Gisquet-Verrier P (2003): Exposure to a retrieval cue in rats induces changes in regional brain glucose metabolism in the amygdala and other related brain structures. *Neurobiology of Learning and Memory* 79:57-71.
456. Hall J, Thomas KL, Everitt BJ (2001): Cellular Imaging of zif268 Expression in the Hippocampus and Amygdala during Contextual and Cued Fear Memory Retrieval: Selective Activation of Hippocampal CA1 Neurons during the Recall of Contextual Memories. *The Journal of Neuroscience* 21:2186-2193.
457. Barros DM, Mello e Souza Tu, De David T, Choi H, Aguzzoli A, Madche C, Ardenghi P, Medina JH, Izquierdo I (2001): Simultaneous modulation of retrieval by dopaminergic D1, β -noradrenergic, serotonergic-1A and cholinergic muscarinic receptors in cortical structures of the rat. *Behavioural Brain Research* 124:1-7.
458. Liang KC, Hu SJ, Chang SC (1996): Formation and retrieval of inhibitory avoidance memory: differential roles of glutamate receptors in the amygdala and medial prefrontal cortex. *Chin J Physiol* 39:155-166.
459. Abraham WC, Dragunow M, Tate WP (1991): The role of immediate early genes in the stabilization of long-term potentiation. *Mol Neurobiol* 5:297-314.

460. Bozon B, Davis S, Laroche S (2002): Regulated transcription of the immediate-early gene Zif268: Mechanisms and gene dosage-dependent function in synaptic plasticity and memory formation. *Hippocampus* 12:570-577.
461. Beckmann AM, Wilce PA (1997): Egr transcription factors in the nervous system. *Neurochem Int* 31:477-510.
462. O'Donovan KJ, Tourtellotte WG, Millbrandt J, Baraban JM (1999): The EGR family of transcription-regulatory factors: progress at the interface of molecular and systems neuroscience. *Trends Neurosci* 22:167-173.
463. Davis S, Bozon B, Laroche S (2003): How necessary is the activation of the immediate early gene zif268 in synaptic plasticity and learning? *Behav Brain Res* 142:17-30.
464. Knapska E, Kaczmarek L (2004): A gene for neuronal plasticity in the mammalian brain: Zif268/Egr-1/NGFI-A/Krox-24/TIS8/ZENK? *Prog Neurobiol* 74:183-211.
465. Cole AJ, Saffen DW, Baraban JM, Worley PF (1989): Rapid increase of an immediate early gene messenger RNA in hippocampal neurons by synaptic NMDA receptor activation. *Nature* 340:474-476.
466. Wisden W, Errington ML, Williams S, Dunnett SB, Waters C, Hitchcock D, Evan G, Bliss TVP, Hunt SP (1990): Differential expression of immediate early genes in the hippocampus and spinal cord. *Neuron* 4:603-614.
467. Jones MW, Errington ML, French PJ, Fine A, Bliss TVP, Garel S, Charnay P, Bozon B, Laroche S, Davis S (2001): A requirement for the immediate early gene Zif268 in the expression of late LTP and long-term memories. *Nat Neurosci* 4:289-296.
468. Bozon B, Kelly A, Josselyn SA, Silva AJ, Davis S, Laroche S (2003): MAPK, CREB and zif268 are all required for the consolidation of recognition memory. *Philos Trans R Soc Lond B Biol Sci* 358:805-814.
469. Malkani S, Wallace KJ, Donley MP, Rosen JB (2004): An egr-1 (zif268) Antisense Oligodeoxynucleotide Infused Into the Amygdala Disrupts Fear Conditioning. *Learn Mem* 11:617-624.
470. Bozon B, Davis S, Laroche S (2003): A requirement for the immediate early gene zif268 in reconsolidation of recognition memory after retrieval. *Neuron* 40:695-701.
471. Lee JLC, Everitt BJ, Thomas KL (2004): Independent Cellular Processes for Hippocampal Memory Consolidation and Reconsolidation. *Science* 304:839-843.
472. Baumgartel K, Genoux D, Welzl H, Tweedie-Cullen RY, Koshibu K, Livingstone-Zatchej M, Mamie C, Mansuy IM (2008): Control of the establishment of aversive memory by calcineurin and Zif268. *Nat Neurosci* 11:572-578.
473. Gass P, Herdegen T, Bravo R, Kiessling M (1993): Induction and suppression of immediate early genes in specific rat brain regions by the non-competitive N-methyl-D-aspartate receptor antagonist MK-801. *Neuroscience* 53:749-758.
474. Davis S, Vanhoutte P, Pages C, Caboche J, Laroche S (2000): The MAPK/ERK cascade targets both Elk-1 and cAMP response element-binding protein to controllong-term potentiation-dependent gene expression in the dentate gyrus in vivo. *J Neurosci* 20:4563-4572.

475. Sarrazin N, Di Blasi F, Roullot-Lacarrière V, Rougé-Pont F, Le Roux A, Costet P, Revest JM, Piazza PV (2009): Transcriptional Effects of Glucocorticoid Receptors in the Dentate Gyrus Increase Anxiety-Related Behaviors. *PLoS ONE* 4:e7704-
476. Revest JM, Kaouane N, Mondin M, Le Roux A, Rougé-Pont F, Vallée M, Barik J, Tronche F, Desmedt A, Piazza PV (2010): The enhancement of stress-related memory by glucocorticoids depends on synapsin-Ia/Ib. *Mol Psychiatry* 15:1140-1151.
477. Segal M (2005): Dendritic spines and long-term plasticity. *Nat Rev Neurosci* 6:277-284.
478. Matus A, Ackermann M, Pehling G, Byers HR, Fujiwara K (1982): High actin concentrations in brain dendritic spines and postsynaptic densities. *PNAS* 79:7590-7594.
479. Fukuzawa Y, Saitoh Y, Ozawa F, Ohta Y, Mizuno K, Inokuchi K (2003): Hippocampal LTP is accompanied by enhanced F-actin content within the dendritic spine that is essential for late LTP maintenance in vivo. *Neuron* 38:447-460.
480. Zhou Q, Homma KJ, Poo MM (2004): Shrinkage of dendritic spines associated with long-term depression of hippocampal synapses. *Neuron* 44:749-757.
481. Zito K, Knott G, Shepherd GM, Shenolikar S, Svoboda K (2004): Induction of spine growth and synapse formation by regulation of the spine actin cytoskeleton. *Neuron* 44:321-334.
482. Fischer A, Sananbenesi F, Schrick C, Spiess J, Radulovic J (2004): Distinct Roles of Hippocampal De Novo Protein Synthesis and Actin Rearrangement in Extinction of Contextual Fear. *The Journal of Neuroscience* 24:1962-1966.
483. Lisman J (2003): Actin's actions in LTP-induced synapse growth. *Neuron* 38:361-362.
484. Offenhäuser N, Castelletti D, Mapelli L, Soppo BE, Regondi MC, Rossi P, D'Angelo E, Frassoni C, Amadeo A, Tocchetti A, Pozzi B, Disanza A, Guarnieri D, Betsholtz C, Scita G, Heberlein U, Di Fiore PP (2006): Increased ethanol resistance and consumption in Eps8 knockout mice correlates with altered actin dynamics. *Cell* 127:213-226.
485. Hou YY, Lu B, Li M, Liu Y, Chen J, Chi ZQ, Liu JG (2009): Involvement of Actin Rearrangements within the Amygdala and the Dorsal Hippocampus in Aversive Memories of Drug Withdrawal in Acute Morphine-Dependent Rats. *The Journal of Neuroscience* 29:12244-12254.
486. Lyford GL, Yamagata K, Kaufmann WE, Barnes CA, Sanders LK, Copeland NG, Gilbert DJ, Jenkins NA, Lanahan AA, Worley PF (1995): Arc, a growth factor and activity-regulated gene, encodes a novel cytoskeleton-associated protein that is enriched in neuronal dendrites. *Neuron* 14:433-445.
487. Steward O, Wallace CS, Lyford GL, Worley PF (1998): Synaptic Activation Causes the mRNA for the IEG Arc to Localize Selectively near Activated Postsynaptic Sites on Dendrites. *Neuron* 21:741-751.
488. Messaoudi E, Kanhema T, Soulé J, Tiron A, Dągryte G, da Silva B, Bramham CR (2007): Sustained Arc/Arg3.1 synthesis controls long-term potentiation consolidation through regulation of local actin polymerization in the dentate gyrus in vivo. *J Neurosci* 27:10445-10455.
489. Plath N, Ohana O, Dammermann B, Errington ML, Schmitz D, Gross C, Mao X, Engelsberg A, Mahlke C, Welzl H, Kobalz U, Stawrakakis A, Fernandez E, Waltereit R, Bick-Sander A,

- Therstappen E, Cooke S, Blanquet V, Wurst W, Salmen B, Bösl M, Lipp HP, Grant S, Bliss TVP, Wolfer DP, Kuhl D (2006): Arc/Arg3.1 Is Essential for the Consolidation of Synaptic Plasticity and Memories. *Neuron* 52:437-444.
490. Link W, Konietzko U, Kauselmann G, Krug M, Schwanke B, Frey U, Kuhl D (1995): Somatodendritic expression of an immediate early gene is regulated by synaptic activity. *Proc Natl Acad Sci U S A* 92:5734-5738.
 491. Rial Verde EM, Lee-Osbourne J, Worley PF, Malinow R, Cline HT (2006): Increased Expression of the Immediate-Early Gene Arc/Arg3.1 Reduces AMPA Receptor-Mediated Synaptic Transmission. *Neuron* 52:461-474.
 492. Shepherd JD, Rumbaugh G, Wu J, Chowdhury S, Plath N, Kuhl D, Huganir RL, Worley PF (2006): Arc Mediates Homeostatic Synaptic Scaling of AMPA Receptors. *Neuron* 52:475-484.
 493. Pintchovski SA, Peebles CL, Kim HJ, Verdin E, Finkbeiner S (2009): The Serum Response Factor and a Putative Novel Transcription Factor Regulate Expression of the Immediate-Early Gene Arc/Arg3.1 in Cultured Cortical Neurons. *J Neurosci* 29:1525-1537.
 494. Wallace CS, Lyford GL, Worley PF, Steward O (1998): Differential intracellular sorting of immediate early gene mRNAs depends on signals in the mRNA sequence. *J Neurosci* 18:26-35.
 495. Li L, Carter J, Gao X, Whitehead J, Tourtellotte WG (2005): The Neuroplasticity-Associated Arc Gene Is a Direct Transcriptional Target of Early Growth Response (Egr) Transcription Factors. *Mol Cell Biol* 25:10286-10300.
 496. Penke Z, Chagneau C, Laroche S (2011): Contribution of Egr1/zif268 to Activity-Dependent Arc/Arg3.1 Transcription in the Dentate Gyrus and Area CA1 of the Hippocampus. *Front Behav Neurosci* 5:48-
 497. McIntyre CK, Miyashita T, Setlow B, Marjon KD, Steward O, Guzowski JF, McGaugh JL (2005): Memory-influencing intra-basolateral amygdala drug infusions modulate expression of Arc protein in the hippocampus. *Proc Natl Acad Sci U S A* 102:10718-10723.
 498. Huff NC, Frank M, Wright-Hardesty K, Sprunger D, Matus-Amat P, Higgins E, Rudy JW (2006): Amygdala Regulation of Immediate-Early Gene Expression in the Hippocampus Induced by Contextual Fear Conditioning. *The Journal of Neuroscience* 26:1616-1623.
 499. Bramham CR, Alme MN, Bittins M, Kuipers SD, Nair RR, Pai B, Panja D, Schubert M, Soule J, Tiron A, Wibrand K (2010): The Arc of synaptic memory. *Exp Brain Res* 200:125-140.
 500. Stinus L, Le Moal M, Koob GF (1990): Nucleus accumbens and amygdala are possible substrates for the aversive stimulus effects of opiate withdrawal. *Neuroscience* 37:767-773.
 501. Azar M, Jones B, Schulteis G (2003): Conditioned place aversion is a highly sensitive index of acute opioid dependence and withdrawal. *Psychopharmacology* 170:42-50.
 502. Mucha RF, Walker MJ (1987): Aversive property of opioid receptor blockade in drug-naive mice. *Psychopharmacology (Berl)* 93:483-488.
 503. Parpura V, Verkhratsky A (2012): Astrocytes revisited: concise historic outlook on glutamate homeostasis and signaling. *Croat Med J* 53:518-528.

504. Turner DA, Adamson DC (2011): Neuronal-Astrocyte Metabolic Interactions: Understanding the Transition into Abnormal Astrocytoma Metabolism. *J Neuropathol Exp Neurol* 70:167-176.
505. Williams SM, Sullivan RKP, Scott HL, Finkelstein DI, Colditz PB, Lingwood BE, Dodd PR, Pow DV (2005): Glial glutamate transporter expression patterns in brains from multiple mammalian species. *Glia* 49:520-541.
506. Albrecht J, Sidoryk-Wegrzynowicz M, Zielinska M, Aschner M (2010): Roles of glutamine in neurotransmission. *Neuron Glia Biology* 6:263-276.
507. Zorec R, Araque A, Carmignoto G, Haydon PG, Verkhratsky A, Parpura V (2012): Astroglial excitability and gliotransmission: an appraisal of Ca^{2+} as a signalling route. *ASN Neuro* 4:e00080-
508. Poskanzer KE, Yuste R (2011): Astrocytic regulation of cortical UP states. *Proc Natl Acad Sci U S A* 108:18453-18458.
509. Santello M, Calì C, Bezzi P (2012): Gliotransmission and the Tripartite Synapse. *Adv Exp Med Biol* 970:307-331.
510. Fellin T, Carmignoto G (2004): Neurone-to-astrocyte signalling in the brain represents a distinct multifunctional unit. *The Journal of Physiology* 559:3-15.
511. Bohn MC (2004): Motoneurons crave glial cell line-derived neurotrophic factor. *Experimental Neurology* 190:263-275.
512. Vesce S, Bezzi P, Volterra A (2001): Synaptic Transmission with the Glia. *Physiology* 16:178-184.
513. Huang D, Han Y, Rani MRS, Glabinski A, Trebst C, Sorensen T, Tani M, Wang J, Chien P, O'Bryan S, Bielecki B, Zhou ZL, Majumder S, Ransohoff RM (2000): Chemokines and chemokine receptors in inflammation of the nervous system: manifold roles and exquisite regulation. *Immunological Reviews* 177:52-67.
514. Ransohoff RM, Brown MA (2012): Innate immunity in the central nervous system. *J Clin Invest* 122:1164-1171.
515. Beitner-Johnson D, Guitart X, Nestler EJ (1993): Glial fibrillary acidic protein and the mesolimbic dopamine system: regulation by chronic morphine and Lewis-Fischer strain differences in the rat ventral tegmental area. *J Neurochem* 61:1766-1773.
516. Hutchinson MR, Northcutt AL, Chao LW, Kearney JJ, Zhang Y, Berkelhammer DL, Loram LC, Rozeske RR, Bland ST, Maier SF, Gleeson TT, Watkins LR (2008): Minocycline suppresses morphine-induced respiratory depression, suppresses morphine-induced reward, and enhances systemic morphine-induced analgesia. *Brain, Behavior, and Immunity* 22:1248-1256.
517. Hutchinson MR, Lewis SS, Coats BD, Skyba DA, Crysedale NY, Berkelhammer DL, Brzeski A, Northcutt A, Vietz CM, Judd CM, Maier SF, Watkins LR, Johnson KW (2009): Reduction of opioid withdrawal and potentiation of acute opioid analgesia by systemic AV411 (ibudilast). *Brain, Behavior, and Immunity* 23:240-250.

518. Watkins LR, Hutchinson MR, Rice KC, Maier SF (2009): The "toll" of Opioid-Induced Glial Activation: Improving the Clinical Efficacy of Opioids by Targeting Glia. *Trends in Pharmacological Sciences* 30:581-591.
519. Johnston IN, Milligan ED, Wieseler-Frank J, Frank MG, Zapata V, Campisi J, Langer S, Martin D, Green P, Fleshner M, Leinwand L, Maier SF, Watkins LR (2004): A Role for Proinflammatory Cytokines and Fractalkine in Analgesia, Tolerance, and Subsequent Pain Facilitation Induced by Chronic Intrathecal Morphine. *The Journal of Neuroscience* 24:7353-7365.
520. Schwarz J, Smith S, Bilbo S (2013): FACS analysis of neuronal-glial interactions in the nucleus accumbens following morphine administration. *Psychopharmacology* 230:525-535.
521. Adler MW, Rogers TJ (2005): Are chemokines the third major system in the brain? *Journal of Leukocyte Biology* 78:1204-1209.
522. Rostene W, Kitabgi P, Parsadaniantz SM (2007): Chemokines: a new class of neuromodulator? *Nat Rev Neurosci* 8:895-903.
523. Schwarz JM, Hutchinson MR, Bilbo SD (2011): Early-Life Experience Decreases Drug-Induced Reinstatement of Morphine CPP in Adulthood via Microglial-Specific Epigenetic Programming of Anti-Inflammatory IL-10 Expression. *The Journal of Neuroscience* 31:17835-17847.
524. Hutchinson MR, Watkins LR (2013): Why is Neuroimmunopharmacology crucial for the future of addiction research? *Neuropharmacology* 76 Pt B:218-227.
525. Moussawi K, Riegel A, Nair S, Kalivas PW (2011): Extracellular Glutamate: Functional Compartments Operate in Different Concentration Ranges. *Front Syst Neurosci* 5:94-
526. Moussawi K, Kalivas PW (2010): Group II metabotropic glutamate receptors (mGlu2/3) in drug addiction. *European Journal of Pharmacology* 639:115-122.
527. Mitrano DA, Smith Y (2007): Comparative analysis of the subcellular and subsynaptic localization of mGluR1a and mGluR5 metabotropic glutamate receptors in the shell and core of the nucleus accumbens in rat and monkey. *J Comp Neurol* 500:788-806.
528. Simonyi A, Schachtman TR, Christoffersen GR (2005): The role of metabotropic glutamate receptor 5 in learning and memory processes. *Drug News Perspect* 18:353-361.
529. Schotanus SM, Chergui K (2008): Dopamine D1 receptors and group I metabotropic glutamate receptors contribute to the induction of long-term potentiation in the nucleus accumbens. *Neuropharmacology* 54:837-844.
530. Liu L, Wong TP, Pozza MF, Lingenhoehl K, Wang Y, Sheng M, Auberson YP, Wang YT (2004): Role of NMDA Receptor Subtypes in Governing the Direction of Hippocampal Synaptic Plasticity. *Science* 304:1021-1024.
531. Shen H, Moussawi K, Zhou W, Toda S, Kalivas PW (2011): Heroin relapse requires long-term potentiation-like plasticity mediated by NMDA2b-containing receptors. *Proc Natl Acad Sci U S A* 108:19407-19412.
532. Gipson CD, Reissner KJ, Kupchik YM, Smith ACW, Stankeviciute N, Hensley-Simon ME, Kalivas PW (2013): Reinstatement of nicotine seeking is mediated by glutamatergic plasticity. *Proc Natl Acad Sci U S A* 110:9124-9129.

533. Wang J, Fe Lanfranco M, Gibb SL, Yowell QV, Carnicella S, Ron D (2010): Long-Lasting Adaptations of the NR2B-containing NMDA Receptors in the Dorsomedial Striatum Play a Crucial Role in Alcohol Consumption and Relapse. *J Neurosci* 30:10187-10198.
534. Kadomatsu K, Tomomura M, Muramatsu T (1988): cDNA cloning and sequencing of a new gene intensely expressed in early differentiation stages of embryonal carcinoma cells and in mid-gestation period of mouse embryogenesis. *Biochemical and Biophysical Research Communications* 151:1312-1318.
535. Muramatsu H, Yokoi K, Chen L, Ichihara-Tanaka K, Kimura T, Muramatsu T (2011): Midkine as a factor to counteract the deposition of amyloid β -peptide plaques: in vitro analysis and examination in knockout mice. *Int Arch Med* 4:1-
536. Kadomatsu K, Muramatsu T (2004): Midkine and pleiotrophin in neural development and cancer. *Cancer Letters* 204:127-143.
537. Muramatsu T (2010): Midkine, a heparin-binding cytokine with multiple roles in development, repair and diseases. *Proc Jpn Acad Ser B Phys Biol Sci* 86:410-425.
538. Jin L, Jianghai C, Juan L, Hao K (2009): Pleiotrophin and peripheral nerve injury. *Neurosurg Rev* 32:387-393.
539. Muramatsu T (2011): Midkine: A Promising Molecule for Drug Development to Treat Diseases of the Central Nervous System. *Curr Pharm Des* 17:410-423.
540. Martin YB, Herradón G, Ezquerra L (2011): Uncovering new pharmacological targets to treat neuropathic pain by understanding how the organism reacts to nerve injury. *Curr Pharm Des* 17:434-448.
541. Le Grevès P (2005): Pleiotrophin gene transcription in the rat nucleus accumbens is stimulated by an acute dose of amphetamine. *Brain Research Bulletin* 65:529-532.
542. Mailleux P, Preud'homme X, Albala N, Vanderwinden JM, Vanderhaeghen JJ (1994): delta-9-Tetrahydrocannabinol regulates gene expression of the growth factor pleiotrophin in the forebrain. *Neurosci Lett* 175:25-27.
543. Ezquerra L, Pérez-García C, Garrido E, Díez-Fernández C, Deuel TF, Alguacil LF, Herradón G (2007): Morphine and yohimbine regulate midkine gene expression in the rat hippocampus. *European Journal of Pharmacology* 557:147-150.
544. Flatscher-Bader T, Wilce PA (2008): Impact of Alcohol Abuse on Protein Expression of Midkine and Excitatory Amino Acid Transporter 1 in the Human Prefrontal Cortex. *Alcoholism: Clinical and Experimental Research* 32:1849-1858.
545. Herradón G, Pérez-García C (2013): Targeting midkine and pleiotrophin signaling pathways in addiction and neurodegenerative disorders: Recent progress and perspectives. *Br J Pharmacol* 171:837-848.
546. Muramatsu T (2002): Midkine and Pleiotrophin: Two Related Proteins Involved in Development, Survival, Inflammation and Tumorigenesis. *Journal of Biochemistry* 132:359-371.
547. Deuel TF, Zhang N, Yeh HJ, Silos-Santiago I, Wang ZY (2002): Pleiotrophin: A Cytokine with Diverse Functions and a Novel Signaling Pathway. *Archives of Biochemistry and Biophysics* 397:162-171.

548. Polykratis A, Katsoris P, Courty J, Papadimitriou E (2005): Characterization of Heparin Affin Regulatory Peptide Signaling in Human Endothelial Cells. *J Biol Chem* 280:22454-22461.
549. Qi M, Ikematsu S, Maeda N, Ichihara-Tanaka K, Sakuma S, Noda M, Muramatsu T, Kadomatsu K (2001): Haptotactic migration induced by midkine. Involvement of protein-tyrosine phosphatase zeta. Mitogen-activated protein kinase, and phosphatidylinositol 3-kinase. *J Biol Chem* 276:15868-15875.
550. Volkow ND, Skolnick P (2012): New Medications for Substance Use Disorders: Challenges and Opportunities. *Neuropsychopharmacology* 37:290-292.
551. Li MD, van der Vaart AD (2011): MicroRNAs in addiction: adaptation's middlemen? *Mol Psychiatry* 16:1159-1168.
552. Narita M, Suzuki M, Kuzumaki N, Miyatake M, Suzuki T (2008): Implication of Activated Astrocytes in the Development of Drug Dependence. *Ann NY Acad Sci* 1141:96-104.
553. Maze I, Nestler EJ (2011): The epigenetic landscape of addiction. *Ann N Y Acad Sci* 1216:99-113.
554. Hutvagner G, Simard MJ (2008): Argonaute proteins: key players in RNA silencing. *Nat Rev Mol Cell Biol* 9:22-32.
555. Jacobs FMJ, van der Linden AJA, Wang Y, von Oerthel L, Sul HS, Burbach JPH, Smidt MP (2009): Identification of *Dlk1*, *Ptpru* and *Klhl1* as novel *Nurr1* target genes in meso-diencephalic dopamine neurons. *Development* 136:2363-2373.
556. Korb E, Finkbeiner S (2011): Arc in synaptic plasticity: from gene to behavior. *Trends in Neurosciences* 34:591-598.
557. Sanchis-Segura C, Spanagel R (2006): Behavioural assessment of drug reinforcement and addictive features in rodents: an overview. *Addiction Biology* 11:2-38.