THERAPEUTIC POTENTIAL OF PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE AND EPIGALLOCATECHIN GALLATE IN MOTOR AND COGNITIVE DEFICITS OF HUNTINGTON'S DISEASE MODELS

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Universitat de Girona

Doctoral Thesis

Therapeutic potential of pituitary adenylate cyclase-activating polypeptide and epigallocatechin gallate in motor and cognitive deficits in Huntington's disease models

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“Work hard in silence, let your success be your noise”
Frank Ocean

“It always seems impossible until it's done”
Nelson Mandela

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Abbreviations

A	Adenine
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma 2
BDNF	brain-derived neurotrophic factor
C	Cysteine
CA	Cornus Ammonis
cAMP	Cyclic-adenosine monophosphate
CBP	CREB-binding protein
CNS	Central nervous system
CREB	cAMP Response Element Binding Protein
DIV	Days in vitro
ECGC	Epigallocatechin-3-gallate
ERK	Extracellular signal-regulated kinase
FASN	Fatty Acid Synthase
fEPSPs	Excitatory post-synaptic potentials
G	Guanine
GABA	Gamma-aminobutyric acid
GFP	Green-fluorescent protein
GluA1	AMPA receptor subunit GluR1
GP	Globus pallidus
GPe	External globus pallidus
GPi	Internal globus pallidus
h	hours
HAP1	Huntingtin-associated protein I
HAT	Histone acetyltransferases
HD	Huntington's Disease
HDAC	Histone deacetylase
HIP14	Huntingtin interacting proteins
HEAT	huntingtin, elongation factor 3, protein phosphatase 2A and TOR1
htt	Huntingtin
IP3	Inositol triphosphate
IT15	Interesting Transcript 15
Kb	Kilobase
kDa	KiloDalton
LTD	Long-term depression
LTM	Long-term memory
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
mhtt	Mutant huntingtin
min	minutes
MSNs	Medium-sized spiny neurons
NES	Nuclear export signal
NGF	Nerve growth factor
NLS	Nuclear localization signal

NMDA	N-methyl-D-aspartate acid
NMDAR	N-methyl-D-aspartate acid receptor
PACAP	Pituitary adenylate cyclase-activating polypeptide
PAT	Palmitoyl acyltransferases
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
polyP	Polyproline
polyQ	Polyglutamine
PSD95	Post-synaptic density 95 protein
REST1/NRSE	Repressor element 1 transcription/neuron restrictive silencer factor
rpm	Revolutions per min
SN	Substantia nigra
SNc	Substantia nigra pars compacta
SNr	Substantia nigra pars reticulata
TPR	Translocated promoter region
VGlut1	Vesicular glutamate transporter 1
VIP	Vasoactive intestinal polypeptide
YAC	Yeast artificial chromosome

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SUMMARY

Resum

La malaltia de Huntington (MH) és un trastorn neurodegeneratiu progressiu que es caracteritza per una alteració del control motor, per dèficits cognitius i símptomes psiquiàtrics. La MH és hereditària, causada per una expansió anòmala del codó CAG en l'exó 1 del gen IT15 que codifica per la proteïna anomenada huntingtina (htt). En individus sans el nombre de repeticions oscil·la de 6 a 35; quan el nombre del triplet CAG és superior a 40, l'individu desenvoluparà la malaltia. L'edat mitjana de les primeres manifestacions és entre els 35 i els 44 anys amb una esperança de vida de 15-20 anys després de l'aparició de la simptomatologia. A dia d'avui no existeix cap tractament terapèutic efectiu, essent indispensable la recerca de compostos amb capacitat terapèutica per tal d'oferir alguna solució a les persones que pateixen la malaltia, així com el seu entorn.

A nivell neuropatològic, es produeix una pèrdua selectiva de neurones GABAèrgiques de projecció en el nucli estriat i una important afectació de l'escorça cerebral o l'hipocamp, entre d'altres àrees cerebrals. S'ha descrit que els trastorns cognitius i emocionals estan associats a la funció cortical i hipocampal i els símptomes motors amb la funció cortico-estriatal. S'ha proposat que restablir la funció hipocampal i estriatal seria clau per recuperar la funció cognitiva i motora, respectivament. Fisiopatològicament, s'ha descrit que en primer terme es produeix un procés de disfunció neuronal caracteritzat per una pèrdua de la plasticitat sinàptica. Posteriorment, apareixerà la mort neuronal. A nivell de l'hipocamp però, no s'ha detectat presència de mort neuronal, essent la disfunció neuronal l'únic procés fisiopatològic. Per això, al llarg d'aquesta tesi doctoral proposem un estudi dual que ens permeti definir diferents compostos terapèutics i dianes moleculars amb capacitat de restablir la plasticitat sinàptica en l'hipocamp i de protegir enfront de la disfunció i mort neuronal a l'estriat per tal de lluitar contra les simptomatologies cognitiva i motora.

L'expansió del codó CAG dóna com a resultat l'allargament de la cua de poliglutamines (poliQ) a l'extrem N-terminal de la htt, generant la htt mutada (httm). La presència de la httm causa una activació de processos cel·lulars patològics tals com, la deprivació tròfica, l'alteració de l'activitat de les caspases, la desregulació transcripcional,

l'agregació proteica, la desregulació de l'activitat dels receptors cel·lulars, entre d'altres. Aquests mecanismes condueixen a la pèrdua de plasticitat sinàptica i a la mort neuronal. No obstant, a dia d'avui encara no estan completament definits i és per això que es continuen estudiant. En aquesta tesi analitzarem en l'hipocamp i estriat a través de quins mecanismes interactuen les dianes terapèutiques i els compostos utilitzats per tal de tenir efectes beneficiosos.

Primerament, hem estudiat el possible paper terapèutic del polipèptid activador de l'adenilat ciclasa (PACAP), un neuropèptid de la família pèptid vasoactiu intestinal (VIP). El PACAP té afinitat per tres receptors: VPAC1, VPAC2 i PAC1. S'ha descrit en diferents models neuronals la capacitat neuroprotectora del PACAP però no existeixen dades en la MH. A més a més, la capacitat del PACAP en promoure la plasticitat sinàptica i millorar els dèficits cognitius és poc coneguda. Hem volgut analitzar si el PACAP té la capacitat de promoure plasticitat sinàptica i neuroprotecció en neurones hipocampals i estriatals en models experimentals de la MH conduint a una millora en la funció motora i cognitiva. També ens interessava saber a través de quin receptor exerceix els seus efectes en la MH.

Els nostres resultats mostren que es produeix una reducció dels receptors del PACAP en l'hipocamp de dos models murins de la MH a partir de l'edat que manifesten dèficits cognitius. De manera interessant, l'anàlisi de mostres humanes post-mortem revela nivells inferiors de PAC1 en l'hipocamp de persones que han patit MH, sense que es detecti canvis en els nivells de VPAC1 i VPAC2. Es va realitzar un tractament intranasal amb PACAP durant una setmana en ratolins R6/1 a l'edat on s'inicien els dèficits cognitius per tal de veure si l'activació dels receptors que es troben reduïts pot revertir la simptomatologia cognitiva. L'estudi de la capacitat d'aprenentatge i de memòria a llarg termini mostra que els ratolins R6/1 tractats amb PACAP milloren cognitivament. Aquesta millora estava associada a una recuperació dels nivells proteics del PAC1 i a un augment de marcadors sinàptics, com la proteïna de la densitat sinàptica 95 (PSD95) i del transportador vesicular de glutamat tipus 1 (VGluT1), de gens vinculats a la plasticitat sinàptica, com c-fos i CBP, i del factor neurotròfic derivat del cervell (BDNF). A més, l'administració de PACAP en els animals R6/1 reduïa la formació d'agregats de la httm en l'hipocamp, marca histopatològica de la malaltia. Per tant, en un primer terme,

el nostre treball mostra que la reducció dels nivells PAC1 en l'hipocamp és una característica que es repeteix en models animals i mostres humanes, suggerint un paper fisiopatològic de PAC1 en els dèficits cognitius de la MH. Suggerim que l'efecte de PACAP seria a través del restabliment dels nivells de PAC1 en l'hipocamp, que fomentaria l'activitat de gens relacionats amb la plasticitat sinàptica, promovent la síntesi del BDNF i de proteïnes sinàptiques. La recuperació de l'activitat neuronal permetria disminuir la formació dels agregats de httm, afavorint encara més una correcta funció neuronal.

En segon lloc, vàrem estudiar si PACAP tenia la capacitat de protegir les neurones estriatals enfront la toxicitat de la httm i millorar la funció motora. En aquesta tesi s'ha caracteritzat que l'expressió de la httm produeix una reducció específica del receptor PAC1 en estriat i escorça cerebral dels ratolins R6/1 en fases d'inici de simptomatologia motora, sense observar canvis importants en els nivells VPAC1 i VPAC2. El tractament amb PACAP en un model cel·lular estriatal (STHdh) protegeix de la toxicitat de la httm, reduint l'apoptosi, a través de la inhibició de la caspasa-3. La transfecció d'aquestes cèl·lules amb un siRNA contra PAC1 ens ha mostrat que aquest efecte protector del PACAP és a través de PAC1. Per saber si aquest efecte neuroprotector era capaç de produir una millora de l'estat motor, vàrem tractar durant una setmana animals R6/1 en una edat d'inici de dèficits motors. Aquests animals van ser subjectes a proves conductuals mostrant una clara millora de la seva capacitat motriu. Hem observat que aquesta capacitat del PACAP està associada a una recuperació dels nivells de PAC1 en l'estriat, a un increment de factors de transcripció associats a supervivència i a un augment dels nivells de BDNF. Així doncs, en aquest segon treball hem determinat que el receptor PAC1 també es troba reduït en l'estriat i escorça en models de la MH. Suggerim que l'efecte terapèutic del PACAP també seria a través de l'augment dels nivells de PAC1, ara en l'estriat, afavorint l'expressió de gens i factors tròfics que promourien la inhibició de la caspasa-3 i la reducció de l'apotosi.

Finalment, l'última part d'aquesta tesi ha consistit en avaluar el possible paper terapèutic de l'epigalocatequina gal·lat (EGCG) en la MH. El EGCG és una catequina molt present en el te verd. En models experimentals de la malaltia d'Alzheimer i de Parkinson, s'ha mostrat que l'EGCG protegeix de la mort neuronal i millora la capacitat

cognitiva. El seu mecanisme d'acció no està ben establert, ja que pot interactuar amb múltiples dianes que promouen una correcta funció neuronal. En els darrers anys però, en models experimentals relacionats amb processos tumorals, s'ha observat que l'EGCG exerciria la seva funció terapèutica mitjançant la inhibició de l'activitat de l'enzim Sintasa d'Àcids Grassos (FASN). Aquest enzim és clau en la síntesi d'àcids grassos de cadena llarga com el palmitat. Tot i aquesta funció tan important, no existeix cap treball que avaluï el paper de FASN en les malalties neurodegeneratives. Amb aquest context, en aquesta tesi s'ha investigat si EGCG tindria un paper neuroprotector en el estriat promovent una major funcionalitat motora, utilitzant els mateixos models de la MH de l'apartat anterior. Tanmateix, hem estudiat si el possible efecte del EGCG seria a través de la seva capacitat d'inhibir l'enzim FASN.

Amb aquests objectius, primer hem tractat amb EGCG les cèl·lules STHdh. Hem observat que el EGCG protegia de la toxicitat provocada per la presència de la httm, i que aquest efecte neuroprotector era a través de la inhibició de l'apoptosi mitjançant la reducció de l'activació de la caspasa-3. Per saber si aquest efecte protector podia induir una millora fenotípica, vàrem administrar intraperitonealment EGCG durant 15 dies en animals R6/1 en una edat en la que s'inicia la simptomatologia motora. L'anàlisi de la funció motora ens mostra que el tractament amb EGCG millora la funció motora dels ratolins R6/1. A més a més, també vàrem analitzar la funció cognitiva, observant una augment de la capacitat d'aprenentatge i de memòria dels ratolins R6/1 tractats amb EGCG. En un segon terme, vàrem estudiar si aquest efecte beneficiós del EGCG era a través d'inhibir FASN. Primer vàrem mesurar els nivells proteics de FASN en les dues principals àrees associades a la simptomatologia motora en la MH, l'estriat i l'escorça cerebral. Vàrem observar que existia un patró dual de l'expressió de FASN; en l'inici de la simptomatologia motora (edat en la que s'ha tractat els animals amb EGCG) els nivells de FASN eren elevats i a fases avançades de la malaltia FASN es trobava reduït respecte els animals control. Utilitzant un assaig d'activitat hem comprovat que l'augment dels nivells de FASN es correlaciona amb un increment de la seva activitat enzimàtica. A més, també hem detectat un augment dels nivells de FASN en el putamen i escorça de mostres post-mortem de persones que han patit la MH i en el model cel·lular STHdh, indicant que és un fenòmen general de la MH. Per saber si els efectes observats amb

l'EGCG eren a través de la inhibició de FASN, en un primer terme vàrem transfectar les cèl·lules STHdh amb un siRNA contra FASN. Semblant als tractaments amb EGCG, la reducció dels nivells de FASN induïa la supervivència de les cèl·lules que expressen la httm a través de la inhibició de la caspasa-3. A més, els tractaments de EGCG en els ratolins R6/1 provoquen una reducció de l'activitat de FASN. Aquests resultats suggereixen una capacitat terapèutica del EGCG en la MH. Hem mostrat que a nivell de la funció motora, aquest efecte beneficiós podria ser a través del seu efecte protector en les cèl·lules estriatals que expressen la httm. L'augment dels nivells proteics i d'activitat de FASN observada, l'efecte protector observat al reduir la seva expressió i la capacitat d'EGCG d'inhibir aquest enzim indica que FASN podria ser una nova diana terapèutica en la MH.

En conjunt, en aquesta tesi hem descrit dos nous compostos amb capacitat terapèutica en models experimentals de la MH, el PACAP i el EGCG; i hem proposat dues noves dianes terapèutiques relacionades amb els seus efectes, el receptor PAC1 i l'enzim FASN respectivament. Hem demostrat que l'administració de PACAP en un model animal de la MH promou una millora cognitiva i motora. Aquests efectes estan associats a una recuperació dels nivells proteics del seu receptor PAC1 en l'hipocamp i estriat, receptor que es troba reduït en diferents models de la MH. Tanmateix, el tractament amb PACAP indueix un augment de l'activitat transcripcional i dels nivells de BDNF tant en neurones hipocampals com en neurones estriatals. Aquesta acció similar en ambdues àrees tindria dos efectes beneficiosos diferents: un augment de proteïnes sinàptiques que millorarien la plasticitat sinàptica en l'hipocamp i una inhibició de l'apoptosi que protegiria a les neurones estriatals. Pel que fa a l'EGCG, hem determinat que protegeix a les cèl·lules estriatals de la toxicitat per la httm i que aquest efecte neuroprotector està associat a una millora conductual quan s'administra EGCG. Els nostres resultats suggereixen que aquesta acció del EGCG seria a través de la inhibició de l'enzim FASN en l'estriat, activitat del qual es troba incrementada en la MH. Amb tot, els resultats obtinguts amb aquesta tesi obren dues noves portes pel tractament dels símptomes cognitius i motors de la MH.

Resumen

La enfermedad de Huntington (EH) es un trastorno neurodegenerativo progresivo que se caracteriza por una alteración del control motor, por déficits cognitivos y síntomas psiquiátricos. La EH es hereditaria, causada por una expansión anómala del codón CAG en el exón 1 del gen IT15 que codifica para la proteína llamada huntingtina (htt). En individuos sanos el número de repeticiones oscila de 6 a 35; cuando el número del triplete CAG es superior a 40, el individuo desarrollará la enfermedad. La edad media de las primeras manifestaciones es entre los 35 y los 44 años con una esperanza de vida de 15-20 años después de la aparición de la sintomatología. A día de hoy no existe ningún tratamiento terapéutico efectivo, siendo indispensable la búsqueda de compuestos con capacidad terapéutica para ofrecer alguna solución a las personas que padecen la enfermedad, así como a su entorno.

A nivel neuropatológico, se produce una pérdida selectiva de neuronas GABAérgicas de proyección en el núcleo estriado y una importante afectación de la corteza cerebral o del hipocampo, entre otras áreas cerebrales. Se ha descrito que los trastornos cognitivos y emocionales están asociados a la función cortical e hipocampal y los síntomas motores con la función cortico-estriatal. Se ha propuesto que restablecer la función hipocampal y estriatal sería clave para recuperar la función cognitiva y motora respectivamente. Fisiopatológicamente, se ha descrito que en primer término se produce un proceso de disfunción neuronal caracterizado por una pérdida de la plasticidad sináptica. Posteriormente, aparecerá la muerte neuronal. A nivel del hipocampo sin embargo, no se ha detectado presencia de muerte neuronal, siendo la disfunción neuronal el único proceso fisiopatológico. Por ello, a lo largo de esta tesis doctoral proponemos un estudio dual que nos permita definir diferentes compuestos terapéuticos y dianas moleculares con capacidad de restablecer la plasticidad sináptica en el hipocampo y de proteger frente a la muerte neuronal en el estriado para luchar contra las sintomatologías cognitiva y motora.

La expansión del codón CAG da como resultado el alargamiento de la cola de poliglutaminas (poliQ) en el extremo N-terminal de la htt, generando la htt mutada (httm). La presencia de la httm causa una activación de procesos celulares patológicos

tales como, la deprivación trófica, la alteración de la actividad de las caspasas, la desregulación transcripcional, la agregación proteica, la desregulación de la actividad de los receptores celulares, entre otros. Estos mecanismos conducen a la pérdida de plasticidad sináptica y la muerte neuronal. Sin embargo, a día de hoy todavía no están completamente definidos y es por eso que se siguen estudiando. En esta tesis analizaremos en el hipocampo y en el estriado a través de qué mecanismos interactúan las dianas terapéuticas y los compuestos testados para tener efectos beneficiosos.

Primeramente, hemos estudiado el posible papel terapéutico del polipéptido activador de la adenilato ciclasa (PACAP), un neuropéptido de la famila péptido vasoactivo intestinal (VIP). El PACAP tiene afinidad por tres receptores: VPAC1, VPAC2 y PEC1. Se ha descrito en diferentes modelos neuronales la capacidad neuroprotectora del PACAP pero no existen datos en la EH. Además, la capacidad del PACAP en promover la plasticidad sináptica y mejorar los déficits cognitivos es poco conocida. Hemos querido analizar si el PACAP tiene la capacidad de promover plasticidad sináptica y neuroprotección en neuronas hipocampales y estriatales en modelos experimentales de la EH conduciendo a una mejora en la función motora y cognitiva. También nos interesaba saber a través de qué receptor ejerce sus efectos en la EH.

Nuestros resultados muestran que se produce una reducción de los receptores del PACAP en el hipocampo de dos modelos murinos de la EH a partir de la edad que manifiestan déficits cognitivos. De manera interesante, el análisis de muestras humanas *post-mortem* revela niveles inferiores de PAC1 en el hipocampo de personas que han sufrido EH, sin que se detecten cambios en los niveles de VPAC1 y VPAC2. Se realizó un tratamiento intranasal con PACAP durante una semana en ratones R6/1 a la edad donde se inician los déficits cognitivos para ver si la activación de los receptores que se encuentran reducidos puede revertir la sintomatología cognitiva. El estudio de la capacidad de aprendizaje y de memoria a largo plazo muestra que los ratones R6/1 tratados con PACAP mejoran cognitivamente. Esta mejora estaba asociada a una recuperación de los niveles proteicos del PAC1 y a un aumento de marcadores sinápticos, como la proteína de la densidad sináptica 95 (PSD95) y del transportador vesicular de glutamato tipo 1 (VGluT1), de genes vinculados a la plasticidad sináptica, como c-fos y CBP, y del factor neurotrófico derivado del cerebro (BDNF). Además, la

administración de PACAP en los animales R6/1 reducía la formación de agregados de la httm en el hipocampo, marca histopatológica de la enfermedad. Por tanto, en un primer término, nuestro trabajo muestra que la reducción de los niveles PAC1 en el hipocampo es una característica que se repite en modelos animales y muestras humanas, sugiriendo un papel fisiopatológico de PAC1 en los déficits cognitivos de la MH. Sugerimos que el efecto de PACAP sería a través del restablecimiento de los niveles de PAC1 en el hipocampo, que fomentaría la actividad de genes relacionados con la plasticidad sináptica, promoviendo la síntesis del BDNF y de proteínas sinápticas. La recuperación de la actividad neuronal permitiría disminuir la formación de los agregados de httm, favoreciendo aún más una correcta función neuronal.

En segundo lugar, estudiamos si PACAP tenía la capacidad de proteger las neuronas estriatales frente la toxicidad de la httm y mejorar la función motora. En esta tesis se ha caracterizado que la expresión de la httm produce una reducción específica del receptor PAC1 en estriado y corteza cerebral de los ratones R6/1 en fases de inicio de sintomatología motora, sin observar cambios importantes en los niveles VPAC1 y VPAC2. El tratamiento con PACAP en un modelo celular estriatal (STHdh) protege de la toxicidad de la httm, reduciendo la apoptosis, a través de la inhibición de la caspasa-3. La transfección de estas células con un siRNA contra PAC1 nos ha mostrado que este efecto protector del PACAP es a través de PAC1. Para saber si este efecto neuroprotector era capaz de producir una mejora del estado motor, tratamos durante una semana animales R6/1 en una edad de inicio de déficits motores. Estos animales fueron sujetos a pruebas conductuales mostrando una clara mejora de su capacidad motriz. Hemos observado que esta capacidad del PACAP está asociada a una recuperación de los niveles de PAC1 en el estriado, a un incremento de factores de transcripción asociados a supervivencia y a un aumento de los niveles de BDNF. Así pues, en este segundo trabajo hemos determinado que el receptor PAC1 también se encuentra reducido en el estriado y corteza en modelos de la EH. Sugerimos que este efecto terapéutico de PACAP también sería a través del aumento de los niveles de PAC1, ahora en el estriado, favoreciendo la expresión de genes y factores tróficos que promoverían la inhibición de la caspasa-3 y la reducción de la apotosi.

Finalmente, la última parte de esta tesis ha consistido en evaluar el posible papel terapéutico de la epigalocatequina galato (EGCG) en la EH. La EGCG es una catequina muy presente en el té verde. En modelos experimentales de la enfermedad de Alzheimer y de Parkinson, se ha mostrado que la EGCG protege de la muerte neuronal y mejora la capacidad cognitiva. Su mecanismo de acción no está bien establecido, ya que puede interactuar con múltiples dianas que promueven una correcta función neuronal. En los últimos años pero, en modelos experimentales relacionados con procesos tumorales, se ha observado que la EGCG ejercería su función terapéutica mediante la inhibición de la actividad de la enzima Sintasa de Ácidos Grasos (FASN). Esta enzima es clave en la síntesis de ácidos grasos de cadena larga como el palmitato. A pesar de esta función tan importante, no existe ningún trabajo que evalúe el papel de FASN en las enfermedades neurodegenerativas. Con este contexto, en esta tesis se ha investigado si EGCG tendría un papel neuroprotector en el estriado promoviendo una mayor funcionalidad motora, utilizando los mismos modelos de la EH del apartado anterior. Además, hemos estudiado si el posible efecto del EGCG sería a través de su capacidad de inhibir la enzima FASN.

Con estos objetivos, primero hemos tratado con EGCG las células STHdh. Hemos observado que el EGCG protegía de la toxicidad provocada por la presencia de la httm, y que este efecto neuroprotector era a través de la inhibición de la apoptosis mediante la reducción de la activación de la caspasa-3. Para saber si este efecto protector podía inducir una mejora fenotípica, se administró intraperitonealment EGCG durante 15 días en animales R6/1 en una edad en la que se inicia la sintomatología motora. El análisis de la función motora muestra que el tratamiento con EGCG mejora la función motora de los ratones R6/1. Además, también analizamos la función cognitiva, observando un aumento de la capacidad de aprendizaje y de memoria de los ratones R6/1 tratados con EGCG. En un segundo término, estudiamos si este efecto beneficioso del EGCG era a través de inhibir FASN. Primero medimos los niveles proteicos de FASN en las dos principales áreas asociadas a la sintomatología motora de la EH, el estriado y la corteza cerebral. Observamos que existía un patrón dual de la expresión de FASN; en el inicio de la sintomatología motora (edad en la que se ha tratado a los animales con EGCG) los niveles de FASN eran elevados y en fases avanzadas de la enfermedad FASN se

encontraba reducido respecto a los animales control. Utilizando un ensayo de actividad se ha comprobado que el aumento de los niveles de FASN se correlaciona con un incremento de su actividad enzimática. Además, también hemos detectado un aumento de los niveles de FASN en el putamen y corteza de muestras *post-mortem* de personas que han sufrido la EH y en el modelo celular STHdh, indicando que es un fenómeno general de la EH. Para saber si los efectos observados con el EGCG eran a través de la inhibición de FASN, en un primer término transfectamos las células STHdh con un siRNA contra FASN. Semejante a los tratamientos con EGCG, la reducción de los niveles de FASN inducía la supervivencia de las células que expresan la httm a través de la inhibición de la caspasa-3. Además, los tratamientos de EGCG en los ratones R6/1 provocan una reducción de la actividad de FASN. Estos resultados sugieren una capacidad terapéutica del EGCG en la EH. Hemos mostrado que a nivel de la función motora, este efecto beneficioso podría ser a través de su efecto protector en las células estriatales que expresan la httm. El aumento de los niveles proteicos y de actividad de FASN observada, el efecto protector observado al reducir su expresión y la capacidad de EGCG de inhibir esta enzima indica que FASN podría ser una nueva diana terapéutica en la MH.

En conjunto, en esta tesis hemos descrito dos nuevos compuestos con capacidad terapéutica en modelos experimentales de la MH, el PACAP y el EGCG; y hemos propuesto dos nuevas dianas terapéuticas relacionadas con sus efectos, el receptor PAC1 y la enzima FASN respectivamente. Hemos demostrado que la administración de PACAP en un modelo animal de la MH promueve una mejora cognitiva y motora. Estos efectos están asociados a una recuperación de los niveles proteicos de su receptor PAC1 en el hipocampo y estriado, receptor que se encuentra reducido en diferentes modelos de la MH. Además, el tratamiento con PACAP induce un aumento de la actividad transcripcional y de los niveles de BDNF tanto en neuronas hipocampales como en neuronas estriatales. Esta acción similar en ambas áreas tendría dos efectos beneficiosos diferentes: un aumento de proteínas sinápticas que mejorarían la plasticidad sináptica en el hipocampo y una inhibición de la apoptosis que protegería a las neuronas estriatales. En cuanto a la EGCG, hemos determinado que protege a las células estriatales de la toxicidad por la httm y que este efecto neuroprotector está

asociado a una mejora conductual cuando se administra EGCG. Nuestros resultados sugieren que esta acción del EGCG sería a través de la inhibición de la enzima FASN en el estriado, actividad que se encuentra incrementada en la MH. Con todo, los resultados obtenidos con esta tesis abren dos nuevas puertas para el tratamiento de los síntomas cognitivos y motores de la MH.

Abstract

Huntington's disease (HD) is a progressive neurodegenerative disorder characterised by motor cognitive and psychiatric dysfunction. HD is hereditary, caused by an abnormal expansion of the CAG codon in exon 1 of the IT15 gene that encodes for the protein called huntingtin (htt). In healthy individuals, the number of CAG repetitions ranges from 6 to 35; when the CAG length overtakes 40, the patients will manifest the disease. The initial symptoms appear between 35 and 44 years of age with a lifespan of 15-20 years after the onset of symptoms. Nowadays, there is no effective therapeutic treatment, being essential the study of new compounds with therapeutic potential to help people who suffer from the disease, as well as their immediate family circle.

At a neuropathological level, there is a selective loss of GABAergic projection neurons in the striatum and an important involvement of the cerebral cortex and the hippocampus, among other brain areas. It has been described that cognitive and emotional disorders are associated with cortical and hippocampal function and motor symptoms with cortico-striatal function. It has been proposed that restoring hippocampal and striatal function would be the key to recover cognitive and motor function, respectively. It has been described that symptomatology involves neuronal dysfunction characterised by a loss of synaptic plasticity. Subsequently, neuronal death will appear. However, no neuronal death has been detected in the hippocampus, with neuronal dysfunction being the only physiopathological process. Therefore, throughout this doctoral thesis we propose a dual study that allows us to define different therapeutic compounds and molecular targets with the ability to restore synaptic plasticity in the hippocampus and protect against neuronal death in the striatum to stop cognitive and motor symptoms of HD.

CAG codon expansion leads to an expansion of a polyglutamine domain (polyQ) at the amino terminal of the htt protein generating the mutated htt (mhtt). The presence of nhtt causes an activation of cellular pathological processes such as trophic deprivation, alteration of caspase activity, transcriptional dysregulation, protein aggregation, dysregulation of cellular receptor activity, among others. These mechanisms lead to synaptic plasticity disruption and neuronal death. However, currently all the molecular

mechanisms altered are not completely known. For this reason, in this thesis we want understand the mechanism involved the therapeutic targets and compounds beneficial actions in the hippocampus and stiatum.

Firstly, we have studied the possible therapeutic role of the adenylate cyclase activating polypeptide (PACAP), a neuropeptide of the intestinal vasoactive intestinal (VIP) family. PACAP has affinity for three receptors: VPAC1, VPAC2 and PAC1. The neuroprotective capacity of PACAP has been described in different neuronal models, but no data of its role in HD. In addition, the ability of PACAP to promote synaptic plasticity and improve cognitive deficits is poorly understood. We wanted to analyse if PACAP has the ability to promote synaptic plasticity and neuroprotection in hippocampal and striatal neurons in HD models leading to a motor and cognitive improvement. We were also interested in understanding through which receptor PACAP exerts its effects in HD.

Our results show that there is a reduction of PACAP receptors in the hippocampus of two murine models of HD from the onset cognitive deficits. Interestingly, the analysis of human *post-mortem* samples reveals reduced levels of PAC1 in the hippocampus of patients who have suffered from HD, with no changes in VPAC1 and VPAC2. Intranasal treatment with PACAP was carried out for a week in R6/1 mice at the onset of cognitive deficits to study if PACAP receptors activation can revert cognitive symptoms. The study of long-term learning and memory shows that R6/1 mice treated with PACAP improve cognitively. This improvement was associated with a recovery of PAC1 protein levels and an increase of synaptic markers, such as the synaptic density protein 95 (PSD95) and the vesicular glutamate transporter type 1 (VGlut1), genes linked to synaptic plasticity, such as c-fos and CBP, and brain-derived neurotrophic factor (BDNF). In addition, the administration of PACAP in the R6/1 mice reduced mhtt aggregates formation in the hippocampus, the histopathological hallmark of the disease. Therefore, our work shows that PAC1 reduced levels in the hippocampus is a parameter that is reproduced in animal models and human samples, suggesting a physiopathological role of PAC1 in the cognitive deficits of HD. We suggest that the effect of PACAP would be through the restoration of PAC1 levels in the hippocampus, which would induce synaptic plasticity genes transcription, promoting the synthesis of BDNF and other synaptic proteins. The

recovery of the neuronal activity would allow diminishing the formation of mhtt aggregates, leading to a correct neuronal function.

Secondly, we studied whether PACAP had the ability to protect striatal neurons against the toxicity of mhtt and improve motor function. In this thesis, it has been characterised that mhtt expression produces a specific reduction on PAC1 receptor in the striatum and cerebral cortex of the R6/1 mice in the onset of motor symptoms, without observing important changes in VPAC1 and VPAC2 levels. Treatment with PACAP in a striatal cell model (STHdh) protects from mhtt toxicity, reducing apoptosis through the inhibition of caspase-3. Cells transfection with a siRNA against PAC1 has shown that the protective effect of PACAP is through PAC1 activation. In order to know if this neuroprotective effect was able to reduce motor phenotype, R6/1 animals were treated for a week at the onset of motor deficits. These animals were subjected to behavioural tests showing a decrease in motor deficits. We have observed that PACAP effects are associated with PAC1 levels recovery in the striatum, with an increase in transcription factors associated and with survival and an increase in BDNF levels. Thus, in this second work we have concluded that the PAC1 receptor is also reduced in striatum and cortex in HD models. We suggest that PACAP therapeutic effects would also be through the increase in PAC1 levels, now in the striatum, inducing the expression of genes and trophic factors that would promote the inhibition of caspase-3 and apoptosis.

Finally, the last part of this thesis the possible therapeutic role of epigallocatechin gallate (EGCG) in HD has been evaluated. EGCG is a catechin highly present in green tea. In experimental models of Alzheimer's and Parkinson's disease, EGCG has been shown to protect against neuronal death and improve cognition. Its mechanism of action is not well-established, since it can interact through multiple targets that promote a correct neuronal function. Recently, in experimental models related to tumor processes, it has been observed that EGCG exerts its therapeutic function by inhibiting the activity of the Fatty Acid Synthase (FASN) enzyme. This enzyme has a key role in the synthesis of long chain fatty acids such as palmitate. Despite this important role, there is no work evaluating the role of FASN in neurodegenerative diseases. Thus, in this thesis has been studied whether EGCG would have a neuroprotective role in the striatum promoting greater motor functionality, using the same models of HD as in the previous section. In

addition, we have studied whether the possible effect of EGCG would be through its ability to inhibit FASN enzyme.

We have first treated the STHdh cells with EGCG. We have observed that EGCG protects from mhtt toxicity, and it has seen that this neuroprotective effect was through the inhibition of apoptosis by reducing the activation of caspase-3. To know if this protective effect could induce a phenotypic improvement, EGCG was administered intraperitoneally for 15 days in R6/1 mice at the onset of motor signs. The analysis of the motor function shows that EGCG treatment improves the motor function of R6/1 mice. In addition, cognitive function has been also analysed, observing an increase in learning and memory function of R6/1 mice treated with EGCG. Secondly, we studied whether this beneficial effect of EGCG was through FASN inhibition. First, we measured FASN protein levels in the two main responsible areas related to motor symptoms in HD, striatum and cerebral cortex. We observed a dual pattern of FASN expression; At the onset of motor symptoms (age at which animals were treated with EGCG) there are higher levels of FASN whereas it was reduced in advanced stages compared to control animals. Using an activity assay, it has been found that the increase in FASN levels correlates with an increase in enzymatic activity. In addition, it was also detected an increase of FASN levels in the putamen and cortex of *post-mortem* human samples from HD and in the STHdh cell model, indicating that it is a general phenomenon of HD. To know if the effects observed with the EGCG were through the inhibition of FASN, STHdh cells were transfected with a siRNA against FASN. Similar to EGCG treatments, the reduction of FASN levels induced cells viability through caspase-3 inhibition. In addition, EGCG treatments in R6/1 mice cause a reduction in FASN activity. These results suggest a therapeutic potential of EGCG in HD. Increased in protein levels and in FASN activity, the protective effect observed when its levels were reduced and the capacity of EGCG to inhibit this enzyme, suggest that FASN could be a new therapeutic targets in HD.

Altogether, in this thesis we have described two new compounds with therapeutic potential in HD models, PACAP and EGCG; and we have proposed two new therapeutic targets, PAC1 receptor and the FASN enzyme, respectively. We have demonstrated that PACAP administration in an animal model of HD promotes cognitive and motor improvement. These effects are associated with a recovery in PAC1 protein levels in the

hippocampus and striatum, a receptor which is reduced in different HD models. In addition, PACAP treatment induces an increase in transcriptional activity and in BDNF levels in both, hippocampal and striatal neurons. This similar action in both areas would have two different beneficial effects: an increase in synaptic proteins that would improve synaptic plasticity in the hippocampus and an inhibition of apoptosis that would protect striatal neurons. Regarding EGCG, we have determined that it protects striatal cells from mhtt toxicity and that this neuroprotective effect is associated with a behavioural improvement when EGCG is administered. Our results suggest that this action of EGCG would be through the inhibition of FASN enzyme in the striatum, which activity is increased in the HD. Thus, the results obtained with this thesis open two new doors for the treatment of HD cognitive and motor symptoms.

I. INTRODUCTION

1. Huntington's disease

Neurodegenerative disorders are considered a group of diseases with a progressive impairment of the nervous system function due to selective neuronal vulnerability of specific brain regions, synaptic abnormalities and progressive cell death. Huntington's disease (HD) is a progressive, fatal, autosomal dominant neurodegenerative disorder characterised by cognitive, motor and psychiatric dysfunction. The earliest mention to HD dates back to the first half of the XIX century when symptoms, progression and heredity of the disease started to be described and it was popularly known as "San Vitus" dance. However, the first accurate description of the disease came by the American physician George Huntington (Huntington, 1872), who named it Chorea due to the characteristic and involuntary movements in those who are affected with the disease. Nowadays, the prevalence of the mutation is approximately 9.71 in 10,000 individuals in most populations of Caucasian descendant (Rawlins et al., 2016). Physical symptoms of HD usually begin between 35 and 44 years of age; however, about 6% of HD patients develop juvenile forms (Foroud et al., 1999). The patients usually die within 10 to 20 years after the first symptoms appear, as there is no treatment to prevent or delay the progression (Ambrose et al., 1994). The main cause of death is infection in about 45% of patients, being pneumonia the most common one for around 25% of deaths in HD (Sturrock & Leavitt, 2010).

The causal gene, already mapped in human chromosome 4 in 1983 (Gusella et al., 1983) was isolated in 1993 by the Huntington's Disease Collaborative Research Group (HDCRG, 1993). The mutation was isolated in *Interesting Transcript* 15 (IT15) gene that codifies for a protein called huntingtin (htt). The gene comprised 67 exons, its length is 200 Kb and it is located on chromosome 4p16.3. The mutation is located in the first exon (which is about 88 amino acids long) of the gene that results in an expansion of a polyglutamine (polyQ) domain at the amino terminal of the htt protein. This expansion consists of three nucleotides: C (cysteine), A (adenine) and G (guanine) and it is known as CAG repeat (Figure 1). The polyQ expansion is cleaved off proteolitically and the cleaved fragments increase the probability of protein misfolding events leading to protein aggregation and a gain-of-function toxicity (Sieradzan et al., 1999; DiFiglia et

al., 1997). Mutant huntingtin (mhtt) is highly aggregation prone and the formation of cytoplasmatic aggregates and nuclear inclusions throughout the brain is one of the hallmarks of HD (DiFiglia et al., 1997, Davies et al., 1997). PolyQ domains include amyloid fibres with high β-sheet content and low detergent solubility that have the capacity to sequester numerous proteins thereby contributing to their role in cellular dysfunction and to the complex loss-of-function phenotype (Soto et al., 2003).

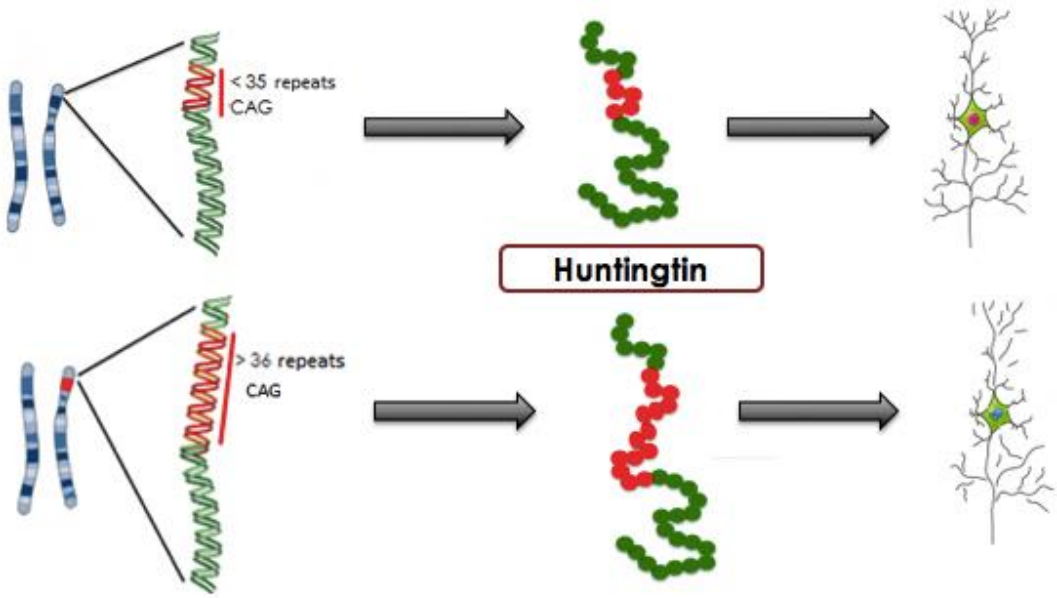


Figure 1: Genetic of HD. Huntingtin (htt) gene, located in chromosome 4, contains of CAG triplet repetitions. Individuals with less than 35 CAG repeats do not manifest HD. However, when the CAG length overtakes 36, patients can manifest the disease. Adapted from EHDN, 2016. Available: www.ehdn.org

As mentioned, HD is a hereditary autosomal dominant disorder, so that, descendants of an HD patient have a probability of 50% to develop the disease. However, HD shows a direct correlation between the number of CAG repetitions and the disease penetration (Squitieri et al., 2012). The number of CAG repeats varies from 6 to 35 within the normal population range. Nevertheless, repeats containing 29-35 triplets are considered of intermediate length, prone to expansion, not sufficient to produce the phenotype, but are associated with risk of transmitting the disease to the offspring (Ranen et al., 1995). This expanded trinucleotide is unstable and tends to expand from generation to generation, a phenomenon called genetic anticipation (Pearson, 2003). Patients with 36 to 39 repeats have an incomplete penetrance of the disease, thus they can manifest it or not (Nopoulos et al. 2016, European Huntington's disease

Network, 2016). When the CAG length overtakes 40, there is a full penetrance and patients manifest the disease. The onset of symptoms has been correlated with the lengthening of the mutation (Table 1) (Rubinsztein et al., 1996).

Table 1: Classification of the huntingtin trinucleotide repeat ranges. Relation between the number of trinucleotides CAG in the exon 1 of the htt gene, the resulting disease status and the risk to the offspring. Disease status depends on the number of CAG repeats.

Repetitions	Classification	Disease status	Risk to the offspring
<28	Normal	Not affected	None
29-35	Intermediate	Not affected	<50%
36-39	Reduced Penetrance	May or may not be affected	50%
>40	Full Penetrance	Affected	50%

HD progression can be divided into two different periods; premanifest and manifest (Figure 2). First, a premanifest period divided into the presymtomatic and the prodromal phase. At presymtomatic phase no signs or symptoms are present. After a variable presymptomatic period, a prodromal phase emerges. This period is portrayed of being the precursor of the complete manifestation of the disease and is characterised by behavioural changes, motor dysfunction and cognitive decline even though in an almost imperceptible way (EHDH, 2016; Folstein et al., 1986).

Then, manifest period appears and is characterised by a slow progression of cognitive and motor dysfunctions. In general, the progression of the disease can be divided into three stages: early, moderate and advanced. In the early stage the person remains employable but at a reduced capacity they can still be able to manage daily affairs despite some difficulties. In the moderate stage HD patients can no longer work or manage household responsibilities. They need considerable help or supervision to handle daily financial affairs. Other daily activities may pose slight difficulties but they usually only need minor help. Finally, in the advanced stage the person requires complete support in daily activities and professional nursing care is usually needed (Nopoulos, 2016; Ghosh & Tabrizi, 2015; Shoulson et al., 1979)

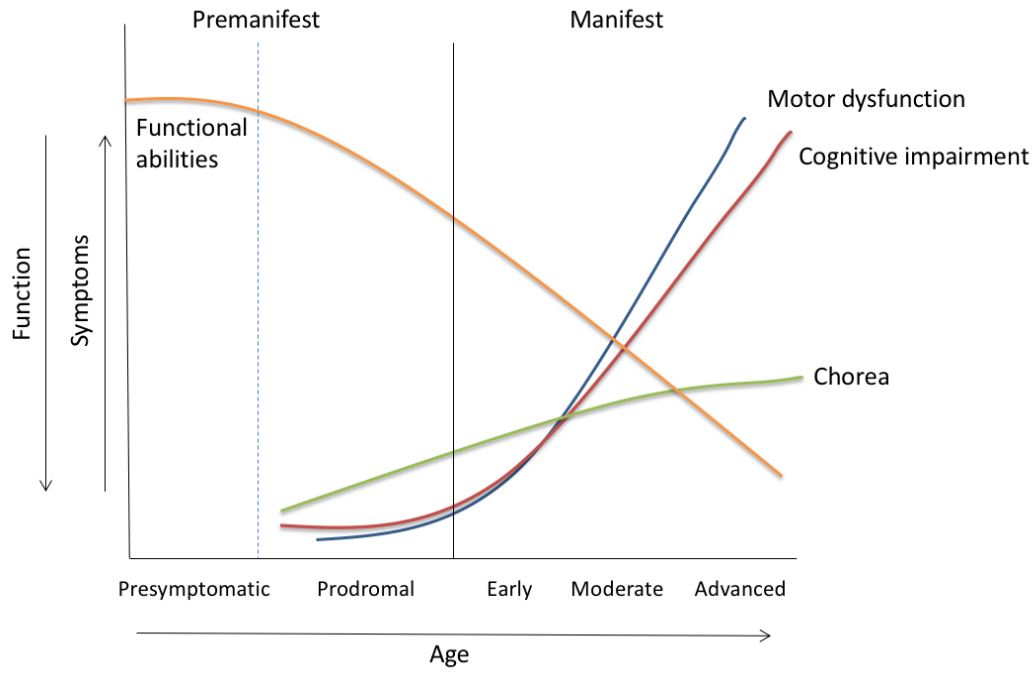


Figure 2: HD stages. HD progression is divided into different stages associated with symptomatology. HD starts with the premanifest stage, which is divided into presymptomatic and prodromal. Then, manifest period appears, which consist in three different stages; early, moderate and advanced. Manifest period is characterised by slow progression of motor and cognitive symptoms and chorea is often prominent early but then plateaus. Adapted from Ross et al., 2014.

As far as symptomatology is concerned, motor discoordination in HD has a biphasic pattern; added involuntary movements and impaired voluntary movements. Initial features are characterised by involuntary choreic movements of the distal extremities and face. They then spread more proximally and the patient suffers from, what is called, choreiform movements that are short and can seem to be purposeful. These movements can bother the patients as chorea progresses because it can cause writing and eating problems, and also it can contribute to fallings (Ghosh & Tabrizi, 2015).

With the disease progression, dystonia is observed. Slowness in movement and rigidity become more prominent rather than choreiform movements (Novak & Tabrizi, 2011). Most HD patients eventually succumb due to aspiration pneumonia because of swallowing difficulties (Folstein et al., 1986).

Cognitive symptoms begin at prodromal phase thus, appear before the onset of motor symptoms (Figure 2) (Tabrizi et al., 2013). Cognitive deficits are usually related to executive functioning affecting daily functional ability such as impaired organizational skills, planning inability, lack of attention capacity, among others (Ghosh & Tabrizi,

2015; Peavy et al., 2010; Paulsen et al., 1995). As cognitive dysfunction progresses, patients have impairments of short-term memory and sometimes visuospatial skills can be also affected (Craufurd & Snowden et al., 2002).

Regarding psychiatric symptoms, these are very variable but they are common in prodromal and manifest stages of HD (Craufurd & Snowden et al., 2002; Paulsen et al., 2001). The most common condition is depression, followed by anxiety and apathy (Craufurd & Snowden, 2002). These features often lead to irritability (Thompson et al., 2012).

2. Huntingtin protein

Huntingtin is encoded by a single gene, which is 200 Kb long and possesses 67 exons. The translated htt is a 384 KDa protein with 3144 amino acids (HDCRG, 1993) containing a polymorphic stretch of between 6 and 35 glutamines residues in its N-terminal domain (exon-1) known as polyQ region. It has been known that polyQ tract is a key regulator of htt binding to its partners and that htt interacts with a large number of partners (Harjes & Wanker, 2003). People who express mhtt contain more than 36 glutamines residues in the polyQ. This expansion generates mhtt fragments due to conformational change. Some of these fragments contain the polyQ expansion produced through proteolysis (Chiu & Alexander, 1982). N-terminal mhtt fragments present a strong propensity to misfold and self-associate, giving rise to oligomers and the characteristic aggregates of the disease (Landles et al., 2010; Hazeki et al., 1999).

Htt protein shows ubiquitous expression, being the brain and testis the organs where the protein is highly expressed (Marques Sousa et al., 2013). Within the brain, htt is expressed in whole neurons and glial cells of the central nervous system (CNS) (Landwehrmeyer et al., 1995). Neocortex, cerebellar cortex, striatum and hippocampus are the main expression sites within the brain (Borrell-Pages et al., 2006). Subcellular location of the htt is complex and changeable and its conformation can vary depending on the cellular location (Harjes & Wanker, 2003). Although a small proportion is found in the nucleus where it interacts with transcription factors and splicesome related proteins (Kegel et al., 2002; Takano & Gusella, 2002), htt is basically a cytoplasmatic protein known to be associated with the plasma membrane, endocytic

and autophagic vesicles, endosomal compartments, the endoplasmic reticulum, the Golgi apparatus, mitochondria and microtubules (Saudou & Humbert, 2016; Imarisio et al., 2008; Bhide et al., 1996).

2.1 Huntingtin structure

The more studied region of htt is the polyQ region where the mutation is located (Figure 3). First 17 N-terminal amino acids have the N17 domain (Arndt, 2015). This domain associates in a reversible manner with lipid membranes of the endoplasmic reticulum, autophagic vesicles and endosomes and it also acts as a nuclear exportation signal by interacting with TPR (translocated promoter region) protein (Rockabrand, 2007) suggesting a key role of this region in the regulation, activity and location of htt protein.

Downstream of this N-terminal region, htt is also enriched in consensus sequences called HEAT (huntingtin, elongation factor 3, protein phosphatase 2A and TOR1) repeats that are 40 amino acid-long sequences organized into protein domains important for protein-protein interactions (Andrade & Bork, 1995). Three HEAT clusters have been identified (MacDonald, 2003) and their presence suggests that htt may exert its physiological function by using different protein partners.

Next to the polyQ region a polyproline (polyP) tract is found. It is suggested to act as a stabilizator of the polyQ by keeping it soluble (Steffan et al., 2004). Htt also contains well-characterised cleavage sites for caspases and calpains (Hermel et al., 2004; Kim et al., 2001), although the function of htt proteolysis to cell function is unclear. These sites are enriched with different amino acids: proline (P), glutamic acid (E), serine (S) and threonine (T) and act as a proteolytic motif. They are responsible of the posttranslational modifications of the protein. Huntingtin is subject to different posttranslational modifications such as phosphorylation, palmitoylation, acetylation, sumoylation, ubiquitination and protein cleavage (Cong et al., 2011; Yanai et al., 2006; Luo et al., 2005; Steffan et al., 2004). All these modifications play a key role in htt function, localization, stability and homeostasis. The polyQ expansion alters the posttranslational modifications increasing mhtt toxicity (Saudou & Humbert, 2016;

Arndt et al, 2015; Cong et al., 2011; Yanai et al., 2006; Luo et al., 2005: Steffan et al., 2004).

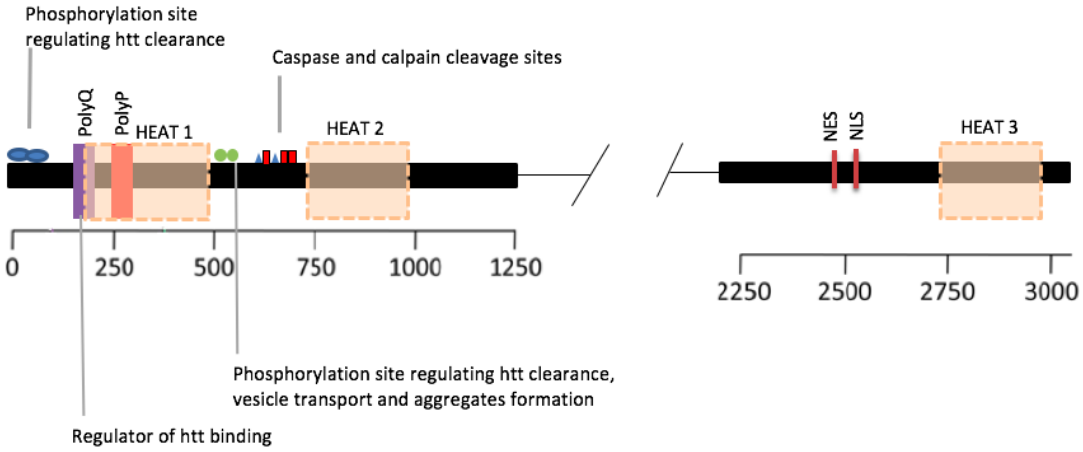


Figure 3: Structure of htt. Huntingtin (htt) amino acids sequence are represented in the diagram with the poly glutamine (PolyQ) and the polyproline (PolyP) tracts, HEAT domains, nuclear export signal (NES) and the nuclear localization signal (NLS). Different posttraduccional modifications are stated: phosphorylation sites regulating huntingtin clearance, cleavage by caspases and calpains, huntingtin proteolysis, aggregates formation and vesicle transport.

Additionally, an active COOH-terminal nuclear export signal (NES) sequence and a less active nuclear localization signal (NLS) are present in htt, which might indicate that the protein is involved in transporting molecules from the nucleus to the cytoplasm (Xia et al., 2003; Bessert et al., 1995).

2.2 Huntingtin function

The gene that codifies for htt was discovered in 1993 and since then a lot of studies tried to understand its cellular role. However, the physiological role of the protein only has begun to be understood. Not only it is difficult to study this protein due to its high molecular weight but also for its ubiquitous expression, expressed in both, nucleus and cytoplasm and its interaction with a high number of proteins coordinating numerous cellular processes.

It is well-known the key role that htt plays in the early developing embryo on several processes such as cell differentiation and neuronal survival. Inactivation of the gene in a mouse model results in an embryonic lethality at E7.5 (Duyao et al. 1995; Nasir et al., 1995; Zeitlin et al., 1995). Moreover, neuronal inactivation of htt gene in adult mice

causes apoptosis in hippocampal, cortical and striatal neurons (Mao et al., 2006; Dragatsis et al., 1998).

Htt highest levels in the brain are found in neurons (Ferrante et al., 1997; Trottier et al., 1995) being specially enriched in cortical pyramidal neurons in layers III and V that project to striatal neurons (Fusco et al., 1999). In these neurons htt is found in neurites, in the cytoplasm, at the synapses and can be also detected in the nucleus (Kegel et al., 2002). It can bind to organelles and structures such as endosomal and endoplasmic compartments, mitochondria, microtubules and plasma membranes (Kegel et al., 2005; DiFiglia et al., 1997). It is known that htt is involved in gene expression regulation interacting with a large number of transcriptional factors, nuclear receptors, proteins in charge of protein remodelation, coactivators and transcriptional repressors (Futter et al., 2009; Seong et al., 2009; Harjes & Wanker, 2003; Zuccato et al., 2003). Htt may also act as a general facilitator of neuronal gene transcription. A well-characterised transcriptional regulation mechanism is the one related to brain-derived neurotrophic factor protein (BDNF), a neurotrophin required for survival of striatal neurons and for the activity of the corticostriatal synapsis (Zucato & Cattaneo, 2009; Charrin et al., 2005; Zuccato et al., 2003; Canals et al., 2001).

In the cytoplasm htt is associated with vesicular structures and microtubules (DiFiglia et al., 1995; Trottier et al., 1995) and it is known that htt plays key role in intracellular trafficking (Kaltenbach et al., 2007; Harjes & Wanker, 2003). In order to facilitate protein trafficking, htt interacts with dynein protein or in an indirect way through Huntingtin-Associated Protein I (HAP1) (Caviston et al., 2011; Gauthier et al., 2004). HAP1 can carry protein complexes through axons, cellular vesicles, lysosomes and endosomes (Wong et al., 2014; Caviston et al., 2011; Gauthier et al., 2004).

Furthermore, htt may also take part of the synaptic communication through its interaction with proteins in the pre-synaptic terminal (Parker et al., 2007) and with proteins that play a key role in synaptic transmission, as well as in receptor recycling and synaptic vesicle (Smith et al., 2005; Sun et al., 2002). At the post-synaptic level, htt interacts with post-synaptic density 95 protein (PSD95), which is essential in the post-

synaptic density organization and dendrites morphology (Parsons et al., 2014; Zheng et al., 2011). PSD95 regulates the anchoring of N-methyl-D-aspartate acid (NMDA) and kainate receptors to the post-synaptic membrane (Sun et al., 2002). Htt protects against neurotoxicity promoted by activation of these receptors (Parsons et al., 2014).

Among all its functions, htt has a pro-survival role due to its antiapoptotic properties by protecting cell against toxic and apoptotic stimuli (Imarisio et al., 2008; Leavitt et al., 2006; Rigamonti et al., 2001). This antiapoptotic role of htt occurs downstream mitochondrial cytochrome c release, blocking caspase 3, 8 and 9 activation (Zhang et al., 2006; Gervais et al., 2002; Rigamonti et al., 2001)

3. Neuropathology

Htt is expressed in all tissues; however, some brain areas are more susceptible to the presence of the mutated protein compared to others, and to some other tissues in the body. Brain alterations are progressive and appear before the onset of symptomatology (Vonsattel et al., 1998; Vonsattel et al., 1985). HD patients can have a brain mass loss of about 25%. Striatal (caudate and putamen nucleus) and cortical atrophies are the most significant neuropathological abnormalities (Figure 4A). However, atrophy also occurs in other brain areas such as hippocampus, thalamus, hypothalamus, globus pallidus, subthalamic nuclei and cerebellum (Vonsattel et al., 1998; Vonsattel et al., 1985). A scale to grade the striatal neuropathology in HD was developed in 1985 by a neuropathologist called Jean Paul Vonsattel and is the most commonly used grading system to assess the severity of HD degeneration. This classification consists of 5 grades from 0 to 4 (Vonsattel et al., 1985). As seen in figure 4B, no cerebral atrophy is observed in grade 0, which is indistinguishable from normal brains after gross examination. Initial atrophy is seen at grade 1 with an approximately 30-40% of neuronal loss in caudate nucleus. This cerebral atrophy becomes more evident from grade 2 when lateral ventricles begin to enlarge. Neural loss, astrogliosis in the tail and, sometimes, in the body of caudate nucleus are also observed. A severe atrophy with a 90% of neuronal loss is seen in grade 3. At a stage 4 of the disease, the most severe, striatal efferent projections were almost depleted.

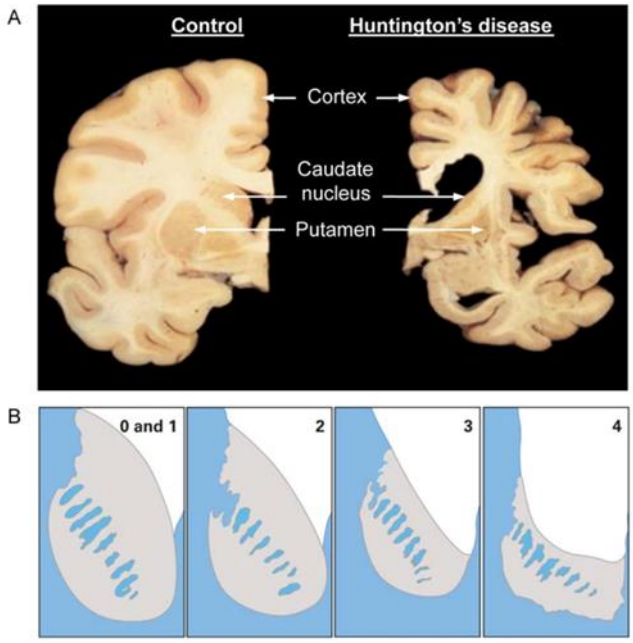


Figure 4: HD pathology. (A) Post-mortem human brain coronal sections that show the degeneration that occurs in the caudate nucleus and putamen as well as the cortical atrophy in Huntington's disease. Image adapted from http://hdroster.iu.edu. (B) Scheme that represents the degrees of the striatal neuropathology in the Vonsattel grade scale. A brain in grade 0 appears macroscopically indistinguishable from normal brains, in grade 1 there is already a 30–40% neuronal loss in the striatum. The neuropathology progresses from grade 2 until grade 4, with progressive atrophy, increasing astrogliosis and neuronal loss. At grade 4 there is up to 95% neuronal loss in the striatum. Figure adapted from Glass et al., 2000.

A part from atrophy, mhtt aggregates are also a hallmark of the neuropathology of HD. Intranuclear and cytoplasmatic inclusions are observed in all brain regions and these inclusions have been used as a pathological marker because they appear before clinical symptoms (Gutekunst et al., 1999; Davies et al., 1997).

4. Hippocampal neuropathology in HD

Hippocampus, together with the amygdala and the nucleus accumbens, form the central axis of the limbic system playing a central role in the memory consolidation, with emotional processes. The nucleus accumbens is involved in reward, pleasure and addiction whereas the amygdala, located deep within the temporal lobes, are related with a number of emotional processes. The thalamus has also an important role in mediating sensory information to cortical areas and is part of the olfactory and visual system. It is also implicated in regulating sleep and wakefulness. Entorhinal cortex is a structure also related with memory and associative components. Mammillary bodies,

part of the hypothalamus that receives signals from the hippocampus via the fornix and projects them to the thalamus. Cingulate gyrus is involved in emotion and pain regulation (Figure 5) (Schultz & Engelhardt, 2014).

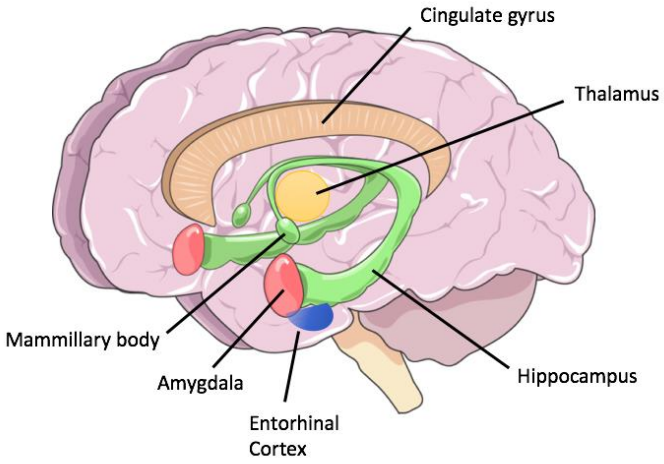


Figure 5: Part of the anatomical components of the limbic system. Cingulate gyrus, Thalamus, Hippocampus, Entorhinal Cortex, Amygdala and Mammillary bodies. The hippocampal formation, which is made up of the entorhinal cortex and hippocampus extends over the anterior-to-posterior axis of the brain.

This system is a critical regional for the formation of episodic/event memory, spatial learning and awareness, navigation, object recognition and visual memory among others (Kandel et al., 2001; Milner et al., 1998; Bliss & Collingridge, 1993). Furthermore, hippocampo-fronto-striatal pathway is involved in higher cognitive tasks such as executive functions (Thierry et al., 2000).

Regarding the hippocampus, either for its anatomy, its connectivity and/or its functionality, is one of the most studied structures in the brain. It is crucially involved in the formation of episodic memories and their recollection, and is believed to be, at least temporarily, the site of episodic memory storage (Andersen et al., 2007). The principal forms of neuronal plasticity known as long-term depression (LTD) and long-term potentiation (LTP) were first discovered in 1973. LTP occurs in the hippocampus and is one of the main mechanisms by which memory is stored in the brain (Lynch, 2004; Bliss & Collingridge, 1993).

From an anatomically point of view, hippocampus is divided in different cell areas. It has the shape of a curved tube called *Cornus Ammonis* (CA). This area is divided into three subfields: CA1, CA2 and CA3 formed by pyramidal neurons. On the other hand,

there is the dentate gyrus, which is a separate structure formed by a tighly packed layer of granule cells (Figure 6) (O'Mara, 2005; Somogyi & Klausberger, 2005).

The perforant path is the principal input to the hippocampus and is a closed circuit that has the dentate gyrus as the start point. The axons of the perforant path arise in layers II and III of the entorhinal cortex. Axons from layer II project to granule cells of the dentate gyrus and pyramidal neurons of CA3, whereas axons from layer III of the perforant path project to pyramidal neurons of CA1 and to the subiculum. Granule cells of the dentate gyrus project their axons through mossy fibres to CA3. Axons of CA3 pyramidal neurons send connections within the region, project to pyramidal cells of CA1 through a set of fibres called Schaffer collateral. At the same time, pyramidal cells of CA1 project to the subiculum. The subiculum is the final step in this pathway, combining and processing information from CA1 and from the layer III of the entorhinal cortex to send this information along the output pathways of the hippocampus (Schultz & Engelhardt, 2014).

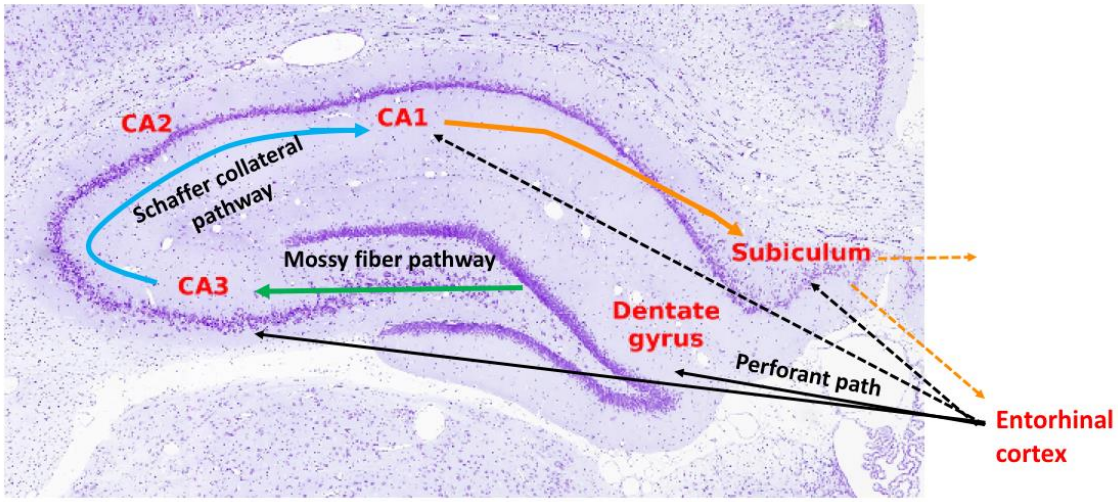


Figure 6: Hippocampal circuits. The hippocampus forms a principally uni-directional network, with inputs from the entorhinal cortex that forms connections with the Dentate gyrus and CA3 pyramidal neurons via the layer II perforant path. CA3 neurons also receive inputs from the Dentate gyrus via the mossy fibers. They send axons to CA1 pyramidal cells via the Schaffer collateral pathway. CA1 neurons also receive inputs directly from the layer II perforant path and send axons to the Subiculum. These neurons in turn send the main hippocampal output back to the Entorhinal Cortex, forming a loop.

Most of the studies of HD have been focused on neurodegeneration, specifically on cortico-striatal pathway (see section 5) (Montoya et al. 2006) and less frequently on the hippocampal implication. Although changes in hippocampal connectivity due to HD

one still poorly adressed, there is strong evidence of morphological alterations and hippocampal atrophy with a clear hippocampal area and volume reduction, as well as a neuronal loss, astrogliosis and presence of intranuclear inclusions in primary stages of the disease (Rosas et al., 2003; Murphy et al., 2000; Vonsattel et al., 1998; Spargo et al., 1993).

Cognitive deficits have been observed in different HD mouse models (Giralt et al., 2012; Zuccato et al., 2010; Ramaswamy et al., 2007) and these deficits seem to be due to the accumulation of mhtt aggregates in the hippocampus (Ramaswamy et al., 2007). Moreover, aberrant synaptic plasticity has been found in mouse model of HD (McBride et al., 2006; Milnerwood et al., 2006) supporting the key role of hippocampus impairment in cognitive dysfunctions of HD.

Several studies identified cognitive deficits linked with hippocampal dysregulation, before the onset of motor symptoms in many HD mouse models (Miguez et al., 2015; Brito et al., 2014; Giralt et al., 2012). Several tests involving cortical and hippocampal structures such as novel object recognition (Broadbent et al., 2004) or Morris water maze (Morris et al., 1986) have been used to evaluate memory in mouse models of HD.

Regarding studies in HD patients, neuroimaging allowed to corroborate the presence of hippocampal atrophy in initial stages of the disease (Rosas et al., 2003). Interestingly, a recent study has used functional tests in human equivalents to the ones used in mouse models to evaluate the hippocampal function. HD patients had difficulties to execute the cognitive tasks even before the onset of motor symptoms. Moreover, results showed a direct relation between cognitive deficits and hippocampal dysfunction (Begeti et al., 2016).

5. Basal ganglia neuropathology in HD

Basal ganglia are associated with automatic voluntary motor control, learning habits, eye movements and cognitive and emotional functions (Brown et al., 1997). Basal ganglia are composed by different subcortical nuclei located in the forebrain interconnected between them: the striatum (caudate nucleus and putamen), the

globus pallidus (GP; including internal (GPi) and external segment (GPe)) and the amygdala (Figure 7) (Graybiel, 2000). An alteration in one nucleus can generate serious problems to the others. Despite the fact that, anatomically, the substantia nigra (SN) and the subthalamic nuclei are not part of the basal ganglia, due to their physiological function and the high connectivity with the nucleus they are considered a part of the basal ganglia. The SN is divided in two main regions: SN pars reticulata (SNr) and SN pars compacta (SNc) (Waldvogel et al., 2014; Balleine et al., 2009).

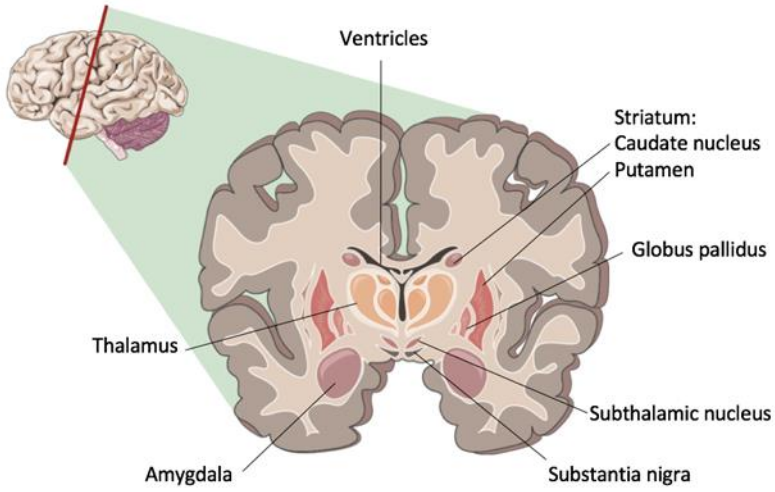


Figure 7: Components and location of the basal ganglia. Localisation of the basal ganglia, as well as associated structures such as the subthalamic nucleus, subtantia nigra, the thalamus and the ventricles. The main nuclei of the basal ganglia, striatum, globus pallidus and amygdala are also represented. Adapted figure from Waldvogel et al., 2014.

In the striatum, almost all neurons (90-95%) are projecting neurons known as medium-sized spiny neurons (MSNs) and the remaining 5-10% are interneurons. MSNs excitability is modulated by interneurons (Kreitzer, 2009). There are two major subtypes of striatal MSNs based on their protein expression and their axonal projections. First, striatonigral MSNs express substance P and D1-like dopamine receptors. Striatopallidal MSNs express encephalin and D2-like dopamine receptors (Kreitzer & Malenka, 2008).

Both subtypes of MSNs are GABAergic striatal efferent neurons and connect with GPe and GPi by two different pathways, the “direct” and the “indirect” (Figure 8). In the

direct pathway, striatonigral MSNs project directly from the striatum to the basal ganglia output nuclei: GPi and SNr. On the other hand, neurons from the indirect pathway project to GPe, which in turn projects to GPi (Gerfen & Surmeier, 2011; Obeso et al., 2008).

These routes make parallel circuits with opposite functions but they work close and highly connected in order to control motor function (Figure 8) (Calabresi et al., 2014). The direct pathway facilitates movement (DeLong, 1990). Striatonigral MSNs receive excitatory, glutamatergic projections mostly from the cortex but also from the thalamus resulting in the excitation of striatal GABAergic MSNs, which in turn inhibits GABAergic projections in the GPi/SNr. The inhibition of inhibitory GABAergic neurons reduces the inhibition of the thalamic glutamatergic neurons, leading to the excitation of the motor cortex and initiation of movement. Taking everything into account, a dysregulation in these projection neurons may result in bradykinesias (Bunner & Rebec, 2016).

On the other hand, the indirect pathway seems to inhibit movement (Raymond, 2017). Striatopallidal MSNs inhibit GABAergic neurons in the GPe which in turns, inhibits the subthalamic nuclei (Figure 8). The indirect pathway leads to less inhibition on subthalamic nuclei. The glutamatergic projections from the subthalamic nuclei excite GPi/SNr resulting in a major inhibition of thalamus. A dysregulation in these projection neurons may result in uncontrollable voluntary movements, such as chorea and tremor (Bateup et al., 2010).

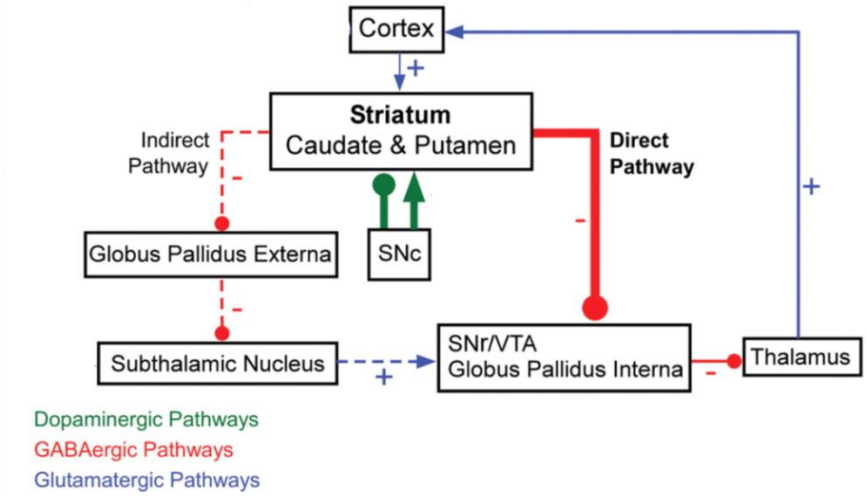


Figure 8: Basal ganglia circuits. The striatum receives glutamatergic inputs from the cortex and projects to the GPe and GPi/SNr by two different GABAergic pathways, the 'indirect' and 'direct', respectively. In HD the 'indirect' pathway is affected earlier than the 'direct' pathway. Striatum also receives dopaminergic inputs from the SNc.

Cerebral cortex and striatum are the main areas affected in HD, MSNs are the most affected neuronal population and suffer a progressive degeneration whereas interneurons are relatively spared (Ferrante et al., 1991). At early and middle stages of HD the indirect pathway is more affected than the direct one. Cortex receives too much stimulation causing the indirect pathway degeneration and generating the characteristic hyperkinetic choreic movements of HD. Evaluation of indirect striatopallidal MSNs not only show neuronal loss in symptomatic HD patients but also in presymptomatic brains of HD mouse models. In contrast, direct striatonigral projections are not as affected as striatopallidal ones till advanced stages of the disease (Menalled et al., 2003).

In a more advanced stage of the disease, almost all striatal efferent projection was depleted, only last some projection to the GPi/SNc. In this stage, cortex does not receive enough stimulation generating hypokinetic and rigid movements (Reiner et al., 1988).

A new pathway that connects cortex directly to the subthalamic nuclei has been recently described. Nowadays, several studies are trying to understand the role of this pathway and to know its relation with the motor symptomatology of HD (Waldvogel, 2014).

Interneurons also play a role in corticostriatal dysfunction. Control of GABAergic pathways comes from inhibition of inhibitory interneurons (Tepper & Bolam, 2004). Some interneurons receive strong cortical innervation and respond faster than MSNs (Mallet et al., 2005). In recent studies atrophy in interneurons was observed in post-mortem HD patients that may result in disrupted direct pathway MSN communication to the GPi (Reiner et al., 2013).

Although the striatum is highly affected in HD, the disease is far from being only characterised by motor symptoms. It is well known that the degeneration is a widespread phenomenon within the brain, which explains the complexity of the disease. Almost all brain regions show reduced volumes at mid-stages of HD: cerebral cortex, striatum, amygdala, hippocampus, brainstem, globus pallidus and even the cerebellum (Rosas et al., 2003).

6. Animal models of HD

HD is caused by a mutation in a single gene, so that, the introduction of the mutant gene into non-human primate, mouse, fish, worm or sheep has permitted the generation of accurate models that have helped to study and understand the pathologic mechanisms and the progression of HD. Not all models are suitable for particular applications and, as result, it is crucial to understand at the outset which is the most appropriate model to use depending on the purpose of the study.

However, genetic knowledge of the disease is recent. Initially, toxic models of HD were used. These models were induced by excitotoxic lesioning or mitochondrial dysfunction and they mimic the striatal degeneration of the disease (Coyle, 1979). Serveral toxic models were developed; these insults induced in rats a selective degeneration of medium-sized spiny neurons, while sparing interneurons (Beal et al., 1986; Coyle & Schwarcz, 1976). The first toxic model which induced this selective degeneration consisted on intrastriatal administration of kainic acid (Coyle & Schwarcz, 1976) or quinolinic acid (an NMDA receptor agonist) (Beal et al., 1986) or by systemic administration of 3-nitropropionic acid which is an inhibitor of the mitochondrial respiratory chain (Beal et al., 1986). Although these models are good models to study

HD since neuropathological characteristics are well-reproduced and it helped to understand striatal vulnerability, mhtt was lacking. Therefore, the progression of the disease and the molecular mechanism altered cannot be well-understood with these models.

The generation of transgenic models has allowed to better understand the progression of the pathogenesis that underlies HD and to evaluate the potential of new therapeutic approaches. Even though a wide array of animals has been generated, the more extensively specie used for HD studies is the mouse, and many different mouse models have been created.

Mouse models differ from each other with regard to the CAG repetitions expressed, portion of the protein included in the transgene, promoter employed, expression levels of mutant protein and even background strain, making each of them unique (Alberch et al., 2008). While some strains display early neuropathology and mortality, others progress so slowly that visible phenotype is not appreciated until mice get very old. This permits the study of different progression degrees of the human pathology. Importantly, none of these models do recapitulate with total reliability the phenotypic aspects of human pathology.

Mouse models can be classified into categories based on the genetic introduction of the mutation, (1) mice that express only a N-terminal fragment of the mhtt gene, and (2) mice that express the full-length mhtt gene, being the first group the one that usually expresses a more severe phenotype of the disease.

6.1 Exon 1 mouse models: R6 mice and N171-82Q

R6 model was the first transgenic model developed by Bates' group in 1996. R6 models are characterised by the presence of the most aggressive phenotype with very clear neuropathological and behavioural characteristics. The mice contain only exon 1 fragment of human htt gene into the mouse genome, the promoter and the first 262 base pair of human htt intron Q sequence (Mangiarini et al., 1996). There are two R6 lines, the difference between them relies in the transgene copies that have been integrated in the genome and the expression level of the transgene. R6/1 mice have

116 CAG repeats and integrate one single copy of the transgenic fragment leading to an expression of 31% of mhtt compared to htt expression. On the other hand, R6/2 mice express 145 CAG repeats and integrate three transgene copies with a 75% of mhtt expression (Mangiarini et al., 1996).

R6/2 mice develop an early onset and have an aggressive behavioural phenotype, exhibit weigh loss at 7 weeks of age and average age at death is 14 weeks (Mangiarini et al., 1996). R6/2 mice show impaired motor function as early as 6-8 weeks of age (Giralt et al., 2011a; Stack et al., 2005; Carter et al., 1999; Mangiarini et al., 1996) and cognitive decline at 4 weeks of age (Lione et al., 1999), preceding the onset of motor symptoms. R6/2 mice also develop astrogliosis at 12 weeks of age (Giralt et al., 2011a).

The fact that R6/1 mice express less number of CAG repeats makes their behavioural phenotype relatively mild, they exhibit weight loss at 14 weeks of age and their lifepan is around 32-40 weeks of age (Bates et al., 1997; Davies et al., 1997; Mangiarini et al., 1996). R6/1 mice show a later affection of motor and cognitive function, according with the expression of less CAG repeats, compared to R6/2 mice. Thus, motor deficits have been detected at 14 weeks of age while cognitive decline starts at 12 weeks of age (Giralt et al., 2011a; Giralt et al., 2009; Mangiarini et al., 1996).

Moreover, in both transgenic mice neuronal nuclear inclusions were found in different brain areas along with dense accumulation of mhtt in the cytoplasm (Davies et al., 1997). Importantly, neuronal atrophy and the presence of cellular aggregates in transgenic mice occur prior to the initial manifestations of symptomathology (Davies et al., 1997).

The latest transgenic mouse line named N171-82Q was generated by inserting 171 amino acids of the N-terminal fragment of the human htt gene with 82, 44 or 16 glutamines into the gene (Schilling et al., 1999). Mice with 82Q have a shortened lifespan compared to 16Q mice, dying at 16-24 weeks of age (Schilling et al., 1999). During their life, these animals show motor alteration like tremors, hypokinesis, hind limb clasping followed by weight loss at 11 weeks of age (McBride et al., 2006).

6.2 Full-length mutant huntingtin models

Mice that express full-length mhtt gene can be grouped in those in which mhtt is delivered in a yeast artificial chromosome (YAC) and those in which a knock-in mutation modifies the endogenous murine huntingtin.

Several YAC models with different repeat length have been created. Although all the YAC lines suffer from brain atrophy, the one with 128 CAG repeats presents the most severe pathology (Slow et al., 2003). It is the only model that develops quantitative striatal neuronal loss at 12 months of age (15-18%) (Slow et al., 2003).

Finally, knock-in models are more faithful genetic models of the human condition because they carry the mutation in its appropriate genomic context. These animals present a late onset of the disease and a mild progression of the pathology. None of the knock-in lines generated develops neuronal loss (Menalled, 2005; Yu et al., 2003; Wheeler et al., 2000), however reactive gliosis can be detected in 111 and 150 CAGs knock-in models (Wheeler et al., 2000; Lin et al., 2001). Moreover, at 4.5 months of age knock-in mice show translocation of mhtt protein to the nucleus but nuclear aggregates do not appear until 10 months of age (Wheeler et al., 2000). Additionally, an early pathological onset manifested by significant deficits in implicit learning tasks at 4 months of age was seen in 92 CAGs knock-in mice (Trueman et al., 2007). Although knock-in mice are considered one of the most representative models of HD there are few studies regarding behavioural dysfunction.

7. Molecular mechanisms involved in HD

Mhtt can trigger cellular stress and even death, inducing alteration in signalling pathways. The presence of mhtt results in multiple physiological alterations such as aggregate formation, transcriptional dysregulation, trophic dysfunction, disruption of calcium homeostasis and caspase-3 activation, alterations in cellular receptors expression and cell signalling, among others (Saudou & Humbert, 2016; Bates et al., 2015; Borrell-Pagès et al., 2006; Harjes & Wanker, 2003). Altogether, these mechanisms contribute to neuronal and synaptic dysfunction leading to the

neurodegeneration observed in HD. It is suggested that the HD pathogenesis is due to the gain of toxic properties of mhtt and the loss of normal htt function (Zuccato & Cattaneo, 2009; Cattaneo et al., 2005).

Although it is well-known that the main cause of the disease is the expanded polyQ above 35 and that the polyQ length accounts for the onset of the disease (HRCRG, 1993), the pathological mechanisms hereby mhtt results in disease symptoms and progression are not fully understood yet. Thus, efforts to describe the molecular pathways involved in HD could offer new targets for the development of new effective treatments. The molecular mechanisms studied in this thesis are described in the following sections.

7.1 Synaptic dysfunction

Nowadays, it is suggested that synaptic dysfunction is crucial in HD pathogenesis. Furthermore, cognitive deficits that appear even before the onset of motor signs, show alterations in neuronal synaptic function in cortical and hippocampal neurons and occur long before in the absence of cell death (Lichter & Hershey, 2010; Milnerwood et al., 2007). It is important to note that 85% of synapsis received by the striatum are excitatory and hippocampal pyramidal neurons can receive 25,000 excitatory synapsis (Cepeda et al., 2007). That is the reason why synaptic impairment and, in particular, alterations in excitatory synapsis are becoming of great interest to find new therapeutic targets for HD.

Several studies have been conducted in order to understand neuronal dysfunction seen in HD and to know how important changes in the synaptic information processing are. Mhtt seems to directly impair the cellular machinery involved in synaptic transmission. For example, cognitive symptoms could be explained by synaptic dysfunctions in cells affected by mhtt (Milnerwood et al., 2007). Moreover, it has been shown that HD mouse models and HD patients present a lower number of dendrites (DiFiglia et al, 1997; Graveland et al., 1985) which seems to be correlated with the aberrant synaptic plasticity in HD models that show LTP and LTD impairment in hippocampus, striatum or cortex (Milnerwood et al., 2006; Cummings et at., 2006;

Klapstein et al., 2001). Some candidates have been suggested to contribute to synaptic dysfunction in HD such as the α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate receptor (AMPAR) subunit GluA1 (Giralt et al., 2009; Nithianantharajah et al., 2008), the dopamine receptors (Hodges et al., 2006; Jay, 2003) or changes in the expression of some trophic factors (Zuccato & Cattaneo, 2007; del Toro et al., 2006) and these can be suitable therapeutic targets.

As far as mhtt role in neuronal dysfunction is concerned, its presence alters synaptic transmission by altering cellular machinery both, presynaptically and extrasynaptically. Presynaptically, mhtt modifies synaptic function by altering the expression of proteins involved in vesicular trafficking, excitotoxic processes and endocytosis. For example, SNARE is a protein complex that plays a key role in exocytosis. Complexin II, a membrane protein, interacts with SNARE regulating fusion processes between synaptic vesicles and the plasma membrane. It has been shown that this protein is decreased in the cortex, striatum and hippocampus of HD models suggesting that complexin II reduction might have a role in synaptic impairment. Moreover, presynaptic receptors are also affected in HD (DiProspero et al., 2004; Morton et al., 2001). Presynaptic area contains at least three membrane proteins: calcium channels, transsynaptic cell-adhesion molecules and presynaptic neurotransmitter receptors (Zhai et al., 2004). The presence of mhtt alters glutamate receptors. For example, in R6/2 mice, metabotropic glutamate receptor 2 (mGluR2) was found reduced (Cha et al., 1998). In addition to glutamate receptors, the vesicular glutamate transporter 1 (VGlut1) also contributes to the HD glutamatergic system imbalance in HD. A significant reduction in VGlut1 protein levels has been detected in the striatum of R6/2 mice (Giralt et al., 2011a). The expression of VGlut1 in the presynaptic terminal correlates with greater expression of synaptic proteins and the reduction of VGlut1 protein levels can reduce synaptic density (Berry et al., 2012) and disrupts corticostriatal excitatory terminals in R6/2 (Giralt et al., 2011a).

Postsynaptically, aberrant glutamatergic signalling leads to excitotoxicity. The overactivation of glutamate receptors is associated with sustained neuronal membrane depolarization, calcium overload, mitochondrial energy failure and subsequent cell death. Although glutamate binds a variety of metabotropic and

ionotropic receptors, N-methyl-D-aspartate (NMDA) ionotropic glutamate receptors (NMDAR) play a larger role than others in mediating neuronal toxicity due to high calcium permeability and slow deactivation and desensitization.

NMDAR are altered in HD; their proper location is crucial for synaptic plasticity. Some receptors are located to synaptic sites while others are located extrasynaptically (Figure 9). The balance between both sites can affect neuronal function. Synaptic NMDAR activation induces suppression of cell death pathways, and the preservation of mitochondrial function (Hardingham & Bading, 2003), whereas calcium influx though extrasynaptic NMDARs promotes prodeath signalling pathways (Hardingham, 2009). This is due to post-translational modifications on signalling proteins or variation in gene expression. Calcium activates cAMP Response Element Binding Protein (CREB) through synaptic NMDARs and by not the extrasynaptic NMDARs (Wu et al., 2001).

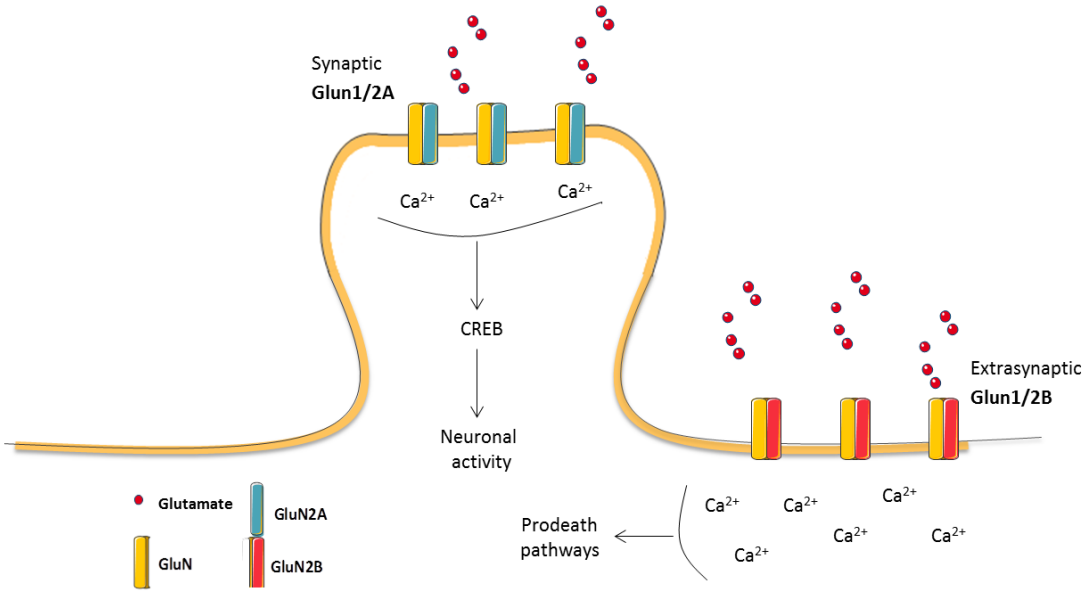


Figure 9: Extrasynaptic location of NMDAR in HD produces excitotoxicity. Stimulation of synaptic NMDARs activates prosurvival signalling and inhibits prodeath signalling, whereas extrasynaptic NMDARs induce excitotoxicity and are thought to contribute to the cell death. Figure from Hughes, 2017.

7.2 Transcriptional dysregulation

Transcriptional dysregulation represents one of the earliest mechanisms involved in HD pathogenesis (Cha, 2007; Dunah et al., 2002; Luthi-Carter et al., 2000). First studies showed downregulation at some genes in R6/2 mice before the onset of

symptomatology (Cha et al., 1998). Several mechanisms have been proposed to explain the role of mhtt in transcriptional alterations (Figure 10). One mechanism could be the sequestration of transcriptional regulators such as TATA-binding protein (Schaffar et al., 2004) or CREB-binding protein (CBP) (Schaffar et al., 2004; Steffan et al., 2000) into htt aggregates. On the other hand, mhtt can lose interaction with negative transcriptional regulators such as the repressor element 1 transcription/neuron restrictive silencer factor (REST1/NRSE) complex leading to nuclear translocation and, consequently, a transcriptional repression of several neuronal-specific genes, including BDNF (Zuccato et al., 2003). Htt can also interfere to chromatin structure increasing the activity of histone methylation and ubiquitination and decreased histone acetylation. Histone acetyltransferases (HAT) favor gene transcription through the opening of chromatin whereas histone deacetyltransferases (HDAC) repress gene transcription through chromatin condensation. Mhtt binds to the HAT domain of some factors such as CBP and blocks its activity, (Cong et al., 2005; McCampbell et al., 2000; Steffan et al., 2001).

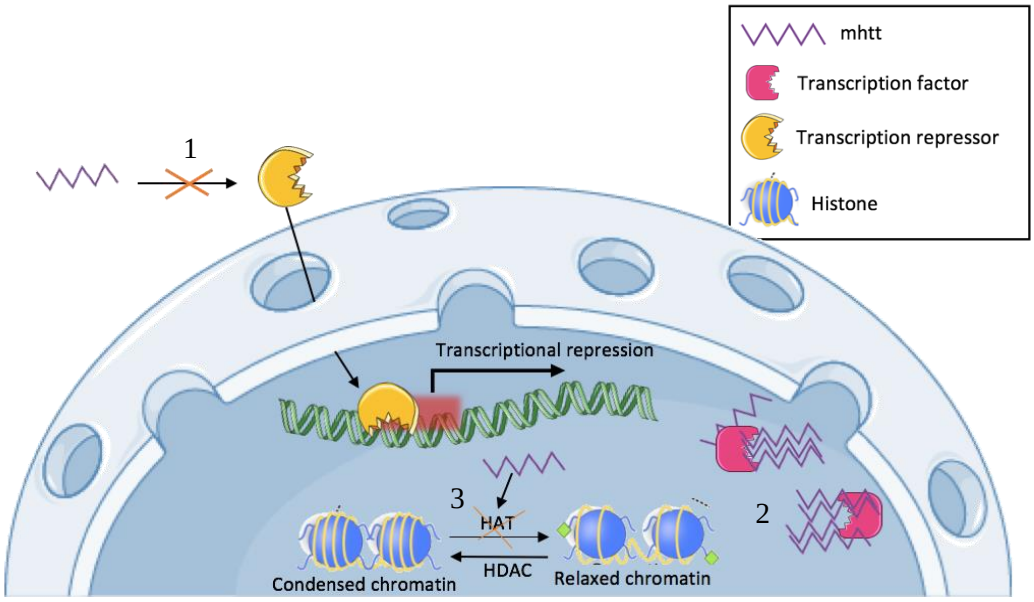


Figure 10: Mechanisms of transcriptional dysregulation in HD. Mhtt interferes with transcriptional machinery at different levels: (1) mhtt loses the capacity to bind to transcriptional repressors, allowing them to translocate into the nucleus and repress transcription; (2) mhtt recruits transcription factors into intranuclear inclusions; (3) mhtt interacts with HATs resulting in the inhibition of proper histone acetylation and repressing transcription.

Overall, transcriptional machinery modulation could be a key mechanism to achieve novel treatment approaches (Cha, 2007; Beal & Ferrante, 2004; Luthi-Carter et al.,

2003).

One of the best well-known transcription factor altered in HD is CREB. CREB is known to mediate transcription of some genes critical for neuronal survival, plasticity and growth such as c-fos, egr-1, BDNF, among others (Lee et al., 2005a; Lee et al., 2005b; Lonze & Ginty, 2002). Activation of CREB is also necessary to increase synaptic transmission and its expression is enough for survival of multiple neurons (Bonni et al., 1999; Davis et al., 1996).

CREB is activated through phosphorylation of Serine 133 (Ser133). Nevertheless, its activity can be regulated by additional sites or by its association with CREB co-activators (Rosenfeld & Glass, 2001). Thus, CREB phosphorylation facilitates the binding to the co-activator CBP (Radhakrishnan et al., 1997; Parker et al., 1996; Kwok et al., 1994; Chrivia et al., 1993). CREB and CBP, together with the other members of the transcriptional machinery, facilitate gene expression (Parker et al., 1996; Ferreri et al., 1994).

Htt function in transcription is well-established. The presence of mhtt is known to downregulate CBP expression (Nucifora et al., 2001). Importantly, altered CREB regulation is also observed in knock-in cells and R6/2 mouse model (Sugars et al., 2004; Gines et al., 2003). Furthermore, it has been shown that mhtt expression downregulates CRE-mediated transcription of numerous genes (Zuccato et al., 2010; Luthi-Carter et al., 2000). One of the genes regulated by CREB-mediated transcriptional activity is BDNF (Zuccato et al., 2010). BDNF is a key regulator in many processes such as synaptic potentiation, dendrites formation and in memory formation (Beckinschtein et al., 2008; Lu et al, 2005).

Decreased levels of BDNF in the striatum and cortex of different models of HD has been extensively studied (Zuccato et al., 2007; Gines et al., 2003; Gorski et al., 2003) suggesting an important role of this neurotrophin in the pathology.

Taking everything into account, CREB-mediated transcription to regulate gene expression has become a promising target for pharmaceutical therapeutic intervention in HD.

8. New therapeutic targets

Several mechanisms are responsible for neuronal dysfunction and neurodegeneration in HD and some have been explained in the previous sections. Nevertheless, no effective treatment has been found in order to stop or slow down HD progression. Therefore, study and characterise new therapeutic targets with ability to revert neuronal mechanisms affected in HD has become a priority for researchers. In the following sections the molecular targets studied in this thesis are exposed.

8.1 Pituitary adenylate cyclase-activating polypeptide (PACAP)

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide, primarily isolated from an ovine hypothalamus extract (Miyata et al., 1989) that belongs to the vasoactive intestinal polypeptide (VIP)/secretin/glucagon superfamily and is broadly expressed in the CNS and periphery (Arimura et al., 1991; Miyata et al., 1989). It has the capacity to activate adenylate cyclase to produce cyclic AMP (Vaudry et al., 2009; Miyata et al., 1990; Miyata et al., 1989). PACAP exists in two amidated forms from the same precursor, PACAP38, with 38 amino acid residues and PACAP27. PACAP38 is 10-100-fold more abundant within the CNS (Arimura, 1998). PACAP is expressed in all vertebrates studied and is one of the most highly conserved neuropeptides even almost preserved during evolution, from fish to mammals (Sherwood et al., 2000), indicating that it has an important and essential role in biological functions. PACAP and VIP, concretely share a 68% homology. They have two common G protein coupled receptors, VPAC1 and VPAC2, whereas PACAP also has affinity to an additional specific receptor called PAC1 (Figure 11) (Harmar et al., 1998). PAC1 belongs to the class II family of G protein-coupled receptors that trigger mainly adenylate cyclase activation through Gαs protein subunits (Dickson et al., 2009) and it is also capable to activate the phospholipase C (PLC) pathways leading to increased inositol triphosphate (IP3) turnover and a rise in intracellular calcium concentrations (McCulloch et al., 2001, Vaudry et al., 2000). Moreover, alternative splicing of PAC1 receptor gene generates multiples isoforms that display different ligand binding and signal transduction properties (Blechman & Levkowitz, 2013; Vaudry et al., 2009). Interestingly, within the

hippocampus, PAC1 is located postsynaptically on CA1-CA3 pyramidal cells (Gupte et al., 2015, Joo et al., 2004), on the granule cells of the dentate gyrus and recently evidenced on glial cells (Gupte et al., 2015). Moreover, PAC1 is localised presynaptically in hippocampal mossy fiber terminals (Otto et al., 1999). There is, thus, a remarkable coincidence of the presynaptic expression of PAC1 and the well-established role of calcium and cAMP in synaptic transmission and long-term potentiation (LTP) at hippocampal mossy fiber terminals (Huang et al., 1993).

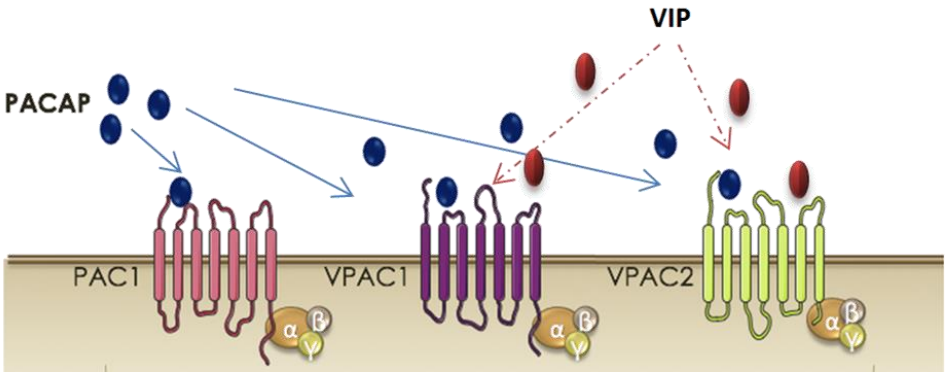


Figure 11: PACAP receptors. While VPAC1 and VPAC1 can be activated by both VIP and PACAP, PAC1 can only interact with PACAP. G protein-coupled receptors stimulate G proteins resulting in the activation of AC that activates of multiple pathways leading to the regulation of several cytoplasmic proteins and activate transcription factors.

PACAP is characterised by its widely distribution giving to the peptide the capacity to exert its pleiotropic physiological functions. Regarding the presence of PACAP in peripheral tissues, it is found in different lobes of the pituitary gland (Arimura, 1998). PACAP immunoreactive elements are also found in the gonads (Hannibal & Fahrenkrug, 1995), adrenal gland (Arimura et al., 1991), parathyroid (Luts & Sundler, 1994) and endocrine pancreas (Love & Szebeni, 1999). However, the highest amounts of the neuropeptide are found in the testis which concentration exceeds the whole concentration found in the brain. The peptide has also been detected in exocrine glands such as parotid and submandibular glands, exocrine pancreas or in the liver (Luts & Sundler, 1994; Moller et al., 1993; Fridolf et al., 1992; Arimura et al., 1991). In urinary tract (Zvarova et al., 2005) or innervating muscles bundles and blood vessels in the trachea or in the lung (Shigyo et al., 1998; Hauser-Kronberger et al., 1996). In the immune system, PACAP is expressed in lymphoid tissues, like thymus, spleen and duodenal mucosa (Abad et al., 2002; Gaytan et al., 1994) and in peritoneal

macrophages (Pozo et al., 1997). To sum up, PACAP highest concentrations are found in testis together with the adrenal gland, gastrointestinal tract and lymphoid tissues (Arimura et al., 1991).

8.1.1. PACAP distribution in the CNS

PACAP is a multifunctional neuropeptide that can act as a neurotransmitter, neuromodulator or as a neurotrophic factor (Shioda et al, 1996; Vigh et al., 1991). Although PACAP was isolated from a hypothalamus extract, the neuropeptide and its receptors are widely expressed in numerous extra-hypothalamic regions. These regions include the hippocampus, cerebral cortex, amygdala, substantia nigra and nucleus accumbems (Table 2) (Kivipelto et al, 1992; Vigh et al., 1991; Köves et al., 1991) suggesting a role of PACAP in neuronal functions (Dejda et al., 2008).

Table 2: Relative abundance of PACAP in the rat brain. mRNA in the rat brain by *in situ* hybridization as denoted by: high (+++), moderate (++), low (+), very low (-) expression. PACAP mRNA levels in the rat brain assessed by immunocytochemistry (Kivipelto et al, 1992; Vigh et al., 1991; Köves et al., 1991).

Brain structures	mRNA expression
Olfactory bulb	++
Cerebral cortex	++
Amygdala	++
Hippocampus	+
Thalamus	++
Hypothalamus	
Arcute nucleus	++
Mediobasal hypothalamus	++
Ventromedial nuclei	+++
Paraventricular nucleus	++
Cerebellum	-/++
Brainstream	-/++

Distributions of PACAP and VIP in the CNS are quite different (Masuo et al., 1993). For example, whereas PACAP is found as a dense network of fibres in the thalamus, a very

few VIP-positives fibres are found (Köves et al., 1991). In the brainstem, VIP-positive cells can be found in the mesencephalic periaqueductal gray and the dorsal and linear raphe nuclei, whereas neurons containing PACAP are more abundant in the parvoventricular nuclei and in the dorsal vagal complex.

Regarding distribution of PACAP receptors, PAC1 is much more expressed in the whole CNS than VPAC1 and VPAC2 transcripts (Basille et al., 2000). PAC1 is highly expressed in the dentate gyrus of the hippocampus, the supraoptic nucleus of the hypothalamus, the cerebral cortex, the olfactory bulb and the area postrema (Zhou et al., 2000; Hashimoto et al., 1996, Nomura et al., 1996). PAC1 mRNA was also observed to be highly expressed in the cingulate, entorhinal and piriform cortices; pyramidal and nonpyramidal cells of the hippocampal formation; the amygdaloid nuclei, the centromedial, mediodorsal, and ventromedial nuclei of the thalamus, the hypothalamus, the central gray, the raphe nuclei, and the superior colliculus (Zhou et al., 2000; Shioda et al., 1997; Hashimoto et al., 1996).

VPAC1 and VPAC2 mRNA localisation studies indicate a different, but also complementary distribution throughout the brain. VPAC1 is basically expressed in the hippocampus and in the cerebral cortex, while VPAC2 highest expression is found in the thalamus, the SNc, the amygdala and the pontine nucleus. In the cerebral cortex, VPAC1 is abundant in layers III and V, on the other hand, VPAC2 is located in layer VI (Usdin et al., 1994). Hippocampus is the only region where both receptors are expressed. They are very abundant postsynaptically on pyramidal cells of the CA1-CA3 regions and on granule cells of the dentate gyrus (Joo et al., 2004).

Taking altogether, PAC1 is highly and more abundantly expressed in the CNS than VPAC1 and VPAC2. Moreover, PAC1 expression is especially high in neurogenic areas such as dentate gyrus of the hippocampus.

8.1.2. PACAP signalling pathways

It is well-known that PACAP activates adenylate cyclase leading to the activation of CREB pathway that can induce neuroplasticity (Carlezon et al., 2005). Moreover, PACAP can activate multiple signal transduction pathways, including PKA, PKC (protein

kinase C) and MAPK (mitogen-activated protein kinase) pathways (Vaudry et al., 2009; Agarwal et al., 2005). PACAP can also stimulate the expression of BDNF, a neurotrophic factor related to neuronal growth (Hammack et al., 2009; Bramham & Messaoudi, 2005). Altogether, PACAP affect several pathways that can induce neurotransmitters release, regulate neuronal plasticity or may have rapid effects on cell excitability.

Most of PACAP actions are mediated through PAC1 receptor stimulating cAMP production (Figure 12) (Villalba et al., 1997), while the effects on peripheral organs usually involve VPAC receptors (Botia et al., 2007). Downstream of PKA, PACAP induces ERK (extracellular signal-regulated kinase) phosphorylation (Obara et al., 2007; Villalba et al., 1997). Activation of this pathways is important for inhibition of caspase-3 activity (Falluel-Morel et al., 2004; Vaudry et al., 2000) contributing to the neuroprotective effect exerts by the peptide (Vaudry et al., 2003). It is also well-known that PACAP has the ability to regulates c-fos expression through PKA activation leading to B-cell lymphoma 2 (Bcl-2) stimulation (Botia et al., 2007; Aubert et al., 2006). This signalling pathway brings to the inhibition of caspase-9 that in turn regulates caspase-3 expression (Figure 12).

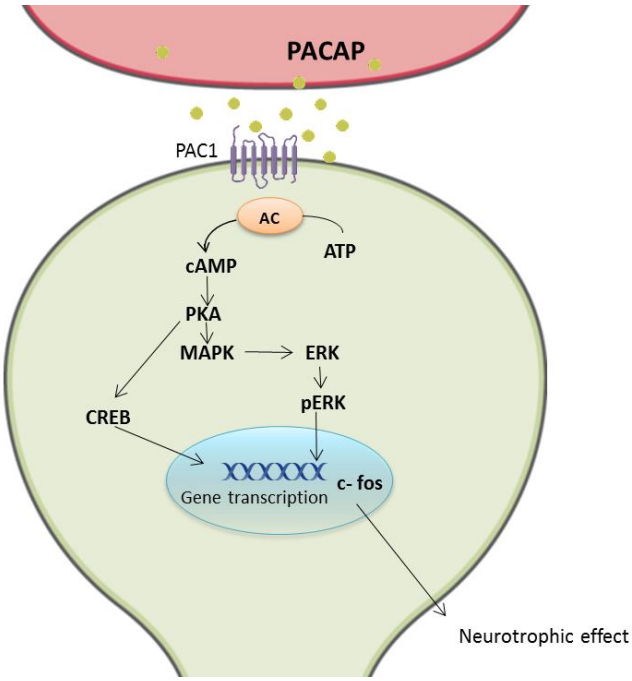


Figure 12: Schematic representation of the intracellular major mechanisms of the neuroprotective effect of PACAP. Neurotrophic effect of PACAP is mediated through activation of cAMP, activating PKA cascade signaling leading to ERK phosphorylation which induce the transcription of genes contributing to the neuroprotective effect of PACAP. Adapted from Vaudry et al., 2000.

On the other hand, VPAC1 and VPAC2 are also coupled to Gαs protein subunits associated with activation of adenylate cyclase through cAMP and activate PKA which in turn may activate the ERK signalling pathway to promote proliferation (Pechon-Vallee et al., 2000) and neuroendocrine cell differentiation (Gutierrez-Canas et al., 2005). VIP-induced PKA activation is responsible for most of the anti-inflammatory activity of VIP by regulating several signalling pathways and transcription factors increasing anti-inflammatory cytokines and reducing pro-inflammatory cytokines production (Gonzalez-Rey et al., 2007).

8.1.3. PACAP and its role in the CNS

As just said, the widespread distribution of PACAP and its receptors indicates that it can be characterised by the capacity to exert pleiotropic functions. As a matter of fact, PACAP is known to act as a neurotransmitter, neuromodulator, as a neurotrophic factor or even as a hormone and neurohormone (Shioda et al, 1996; Vigh et al., 1991).

A neuroprotective effect of PACAP in different models of neurodegenerative disease is now well established (Reglodi et al., 2017; Seaborn et al., 2011). It has been shown that PACAP concentration is increased in neuronal cells suggesting an implication of the peptide in protective mechanisms (Waschek, 2002). For example, intravenous injection of PACAP significantly reduced the infarct size (Reglodi et al., 2002). Moreover, in a rat model of Parkinson's disease, PACAP protects dopaminergic neurons from apoptosis and improves cognitive impairments (Reglodi et al., 2004) and PACAP has shown to amelioreate cognitive deficits in a mouse model of Alzheimer's disease (Rat et al., 2011).

On the other hand, PACAP has the ability to enhance glutamate release by granule cell depolarization (Aoyagi & Takahashi, 2001). The calcium influx activation by PACAP also induces VIP expression (Fukuchi et al., 2004). Interestingly, PACAP has the abilibty to increase its own expression turning short-term PACAP exposure to a long-term action and promoting cerebellar granule cell survival (Vaudry et al., 2005). This neuroprotective effect of PACAP might be indirectly mediated by BDNF release (Shintani et al., 2005) upon activation of the NMDAR complex (Yaka et al., 2003).

To sum up, PACAP promotes neuronal survival by inhibiting apoptosis in both physiological and pathological conditions. Several signal transduction pathways activated by PACAP which lead to neuroprotection have been identified. However, a better understanding of PACAP signalling and its disruption in many neurodegenerative diseases will undoubtedly help finding new ways to use this prominent anti-apoptotic factor as a potential treatment for several pathologies such as HD.

Regarding the role of PACAP in cognition, among the brain structures playing a key role in learning and memory, the hippocampus contains PACAP-expressing cells and nerve fibres, as shown in the rat by immunohistochemistry (Hannibal, 2002; Piggins et al., 1996; Koves et al., 1991) and in situ hybridization (Hannibal, 2002; Jaworski & Proctor, 2000). Some studies show that the presence of high levels of PACAP and PAC1 in limbic system areas such as the hippocampus and amygdala have a role in modulating neural activity related to stress and emotional states (Hammack & May, 2014; Joo et al., 2004; Hannibal, 2002). Several studies show evidence for the functional role of PACAP in anxiety-like behaviours. However, very few studies have been conducted regarding its role in synaptic plasticity.

Related to PACAP actions in the hippocampus, some studies revealed that the neuropeptide has the ability to modulate the major components of glutamatergic transmission mediated by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) and NMDAR for glutamate. PACAP enhances NMDAR-mediated synaptic plasticity responses in CA1 region through PAC1 (Macdonald et al., 2005). On the other side, the effects on AMPA receptor-mediated are less clear (Yang et al., 2010). Rats treated with 0.5 nM of PACAP should enhanced AMPA expression but, antagonistically, a 10 nM concentration inhibited its expression (Costa et al., 2009). In the same way, PACAP inversely regulates the phosphorylation of the two sites on GluA1 subunit of AMPAR (Toda & Huganir, 2015).

Interestingly, PACAP also has the ability to induce LTD through CA1 and CA3 synapse (Ster et al., 2009; Roberto et al., 2001; Kondo et al., 1997). PACAP-deficient mice exhibit LTP impairment in the dentate gyrus and learning deficiency (Takuma et al.,

2014; Matsuyama et al., 2003). Other studies demonstrate that PACAP infusion in the hippocampus and in the basolateral amygdala have an impact on memory consolidation (Schmidt et al., 2015). Taken altogether, PACAP has several roles in relation to synaptic plasticity as glutamatergic transmission, long-term synaptic plasticity, learning and memory.

Moreover, PACAP seems to have the capacity to also stimulate acethylcholine (ACh) release leading to a partially reduction of glutamatergic transmission (Figure 12) (Roberto & Brunelli, 2000). As mention above, PACAP effect on hippocampus, specifically on CA1 and CA3 synapse, occurs in a dose-dependent manner. This variability of PACAP actions seems to be related to its different modulation of AMPAR and NMDAR, its multiple sites of action and also its effect in the cholinergic system (Pecoraro et al., 2017).

Therefore, the ability of PACAP to modulate glutamate release in hippocampal synaptic transmission seems to be correlated to PACAP concentration and the experimental conditions. PACAP enhances the ACh release in the dorsal hippocampus in a dose-dependent manner (Masuo et al., 1993). A low concentration of PACAP (0.05 nM) enhanced excitatory post-synaptic potentials (fEPSPs) in CA1 region. In addition, a high concentration of PACAP (1-3 μM) induced long-term inhibition of fEPSPs at hippocampal CA1 synapses (Kondo et al., 1997). Intermediate doses (100-500 nM) produce biphasic inhibitory/excitatory effects (Roberto et al., 2001).

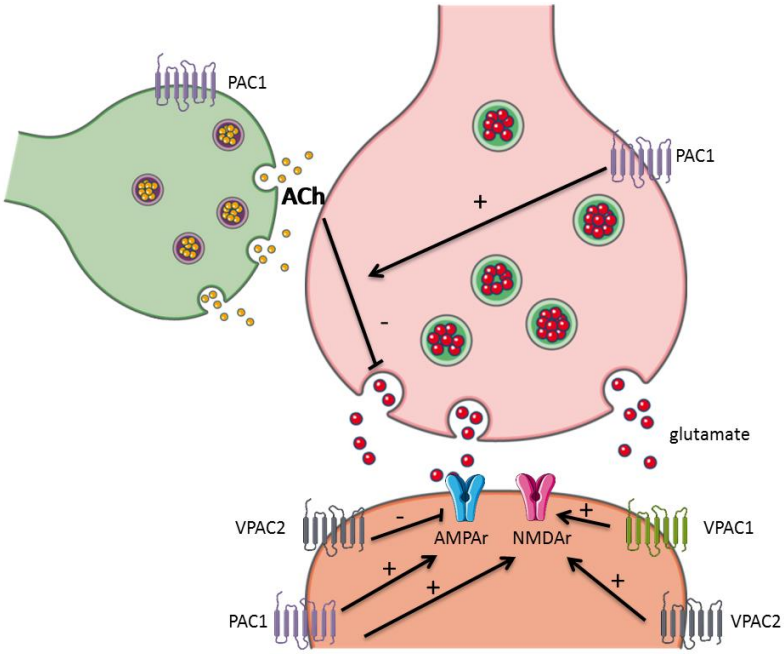


Figure 13: PACAP and glutamatergic transmission in CA1 and CA3 hippocampal regions. PACAP modulates AMPAR and NMDAR in a different manner. At a post-synaptic level, pyramidal neurons found in CA1 express PAC1, VPAC1 and VPAC2. All enhance NMDAR pathways, whereas PACAP exerts different effects on AMPAR. Activation of PAC1 enhances AMPA-excitatory transmission while VPAC2 inhibits it. At subnanomolar concentrations, PACAP modulates AMPAR at the presynaptic level and requires Ach release. Adapted from Pecoraro et al.,2017.

8.2 Epigallocatechin-3-gallate (EGCG)

In lasts years, interest in study natural products to treat several diseases has been raised. Particularly, green tea and more specifically it's main and more active catechin EGCG has gained a lot of attention as a potent therapeutic molecule.

Catechins account for 25-35% of the green tea dry extract and consist of eight polyphenolic flavonoid-type compounds. EGCG accounts for about 10% of the extract (Khokhar et al., 2002) and it is estimated that a cup of green tea may contain 90 mg of EGCG and thus, contribute to the beneficial effects attributed to green tea including its role in neuroprotection (Lin et al., 2003). Several studies indicated that polyphenols can produce neuroprotective effects simply by drinking (Rezai-Zadeh et al., 2008; Levites et al., 2001). However, polyphenols are excreted within 24 h and, particularly, EGCG exhibit a poorer oral bioavailability with a half-life of about 5 h possibly due to its inability to pass through the gut (Landis-Piwowar et al., 2013). Furthermore, the EGCG is unstable because the hydroxyl groups could be modified reducing its biological activity. To resolve these limitations, new polyphenolic compounds related to EGCG

have been developed, showing higher therapeutic activity than EGCG (Landis-Piwowar et al., 2013; Puig et al., 2008).

EGCG is known to have therapeutic properties in many systems, even in the nervous system, for example, its potential in inflammatory diseases (Singh et al., 2010) and in cancer chemoprevention (Lambert et al., 2010). This ability is attributed to their radical scavenging (Weinreb et al., 2004), metal chelating (Lambert et al., 2010; Weinreb et al., 2004), anti-apoptotic (Guo et al., 2005; Weinreb et al., 2004; Nie et al., 2002), anti-inflammatory (Kuang et al., 2012; Singh et al., 2010), antioxidant (Guo et al., 2005; Weinreb et al., 2004) and anti-carcinogenic (Lambert et al., 2010) properties. Due to the hopefully results of green tea catechins and their low toxicity, researchers want to study their effectiveness in neurodegenerative diseases.

Green tea polyphenols are known to possess neuroprotective properties. In particular, EGCG has shown to decrease reactive oxygen species (Zhang et al., 2014; Choi et al., 2012), anti-apoptotic markers (Han et al., 2014; Yao et al., 2014) and increase cell viability (Zhang et al., 2014). It also prevents against brain inflammation, neuronal damage (Herges et al., 2011; Aktas et al., 2004) and preserves mitochondrial energetics (Sutherland et al., 2005).

Extensive research on EGCG have brought into light their potential to supress or to reduce the motor and cognitive alterations that occurs in neurodegenerative disease (Weinreb et al., 2009; Koh et al., 2006). Epidemiological studies showed that higher consumption of green tea is associated with a lower prevalence of cognitive impairment (Kuriyama et al., 2006) and with a reduced risk of Parkinson's disease (Hu et al., 2007). Although evidence in human studies is limited, the beneficial effects of green tea in cognitive and motor dysfunction have been associated to EGCG using different animal models of neurodegenerative diseases. Administration of EGCG has improved cognitive deficits in mice models of Alzheimer's disease (Rezai-Zadeh et al., 2008) and Down syndrome (De la Torre et al., 2014), significantly prolonged motor symptom onset and life span of amyotrophic lateral sclerosis transgenic mice (Koh et al., 2006) and in a mouse model of Parkinson's disease, a recent study demonstrates the ability of EGCG to reduce oxidative stress and its neurorescue effect (Xu et al.,

2017). Moreover, the neuroprotective effects have also been seen in a toxic mouse model of Parkinson's disease (Levites et al., 2001). On the other hand, the EGCG capacity to prevent protein aggregates was seen in Alzheimer transgenic mice (Rezai-Zadeh et al., 2008) and in a study in transgenic flies expressing mhtt it was concluded that EGCG is a potent inhibitor of polyQ aggregation (Ehrnhoefer et al., 2006). However, the mechanism by which protein aggregates are reduced by EGCG is unknown. In addition, related to HD, a multicentre trial evaluating the efficiency and tolerability of EGCG in HD patients is currently ongoing (ClinicalTrials.gov identifier: NCT01357681).

8.2.1. EGCG signalling pathways

EGCG promotes neuroprotective effects not only through its antioxidant potential but also through the modulation of signalling pathways, cell survival and cell death genes. A number of intracellular signalling pathways have been described to exert key function in EGCG-promoted neuronal protection (Figure 14). The catechin interacts with a neurotransmitter receptor, downstream protein kinases or signalling cascades such as PI3K-Akt, MAPK and PKC signalling pathways. It is known that EGCG effectively induces PKC signalling pathway bringing neuroprotection against amyloid beta neurotoxicity in Alzheimer's disease (Mandel et al., 2004) and against 6-hydroxydopamine in Parkinson's disease (Kalfon et al., 2007). In addition, PKC signalling upregulation has also been noted with ERK activation with a reduction of proapoptotic factors (Mandel et al., 2004). Moreover, *in vitro* treatment with EGCG has shown to stimulate PI3K/Akt pathway to reduce cell death (Shen et al., 2014). Moreover, previous studies demonstrated EGCG potency to induce MAPKs pathways leading to the activation of many regulators such as ERK 1/2 or p38 (Chen et al., 2000)

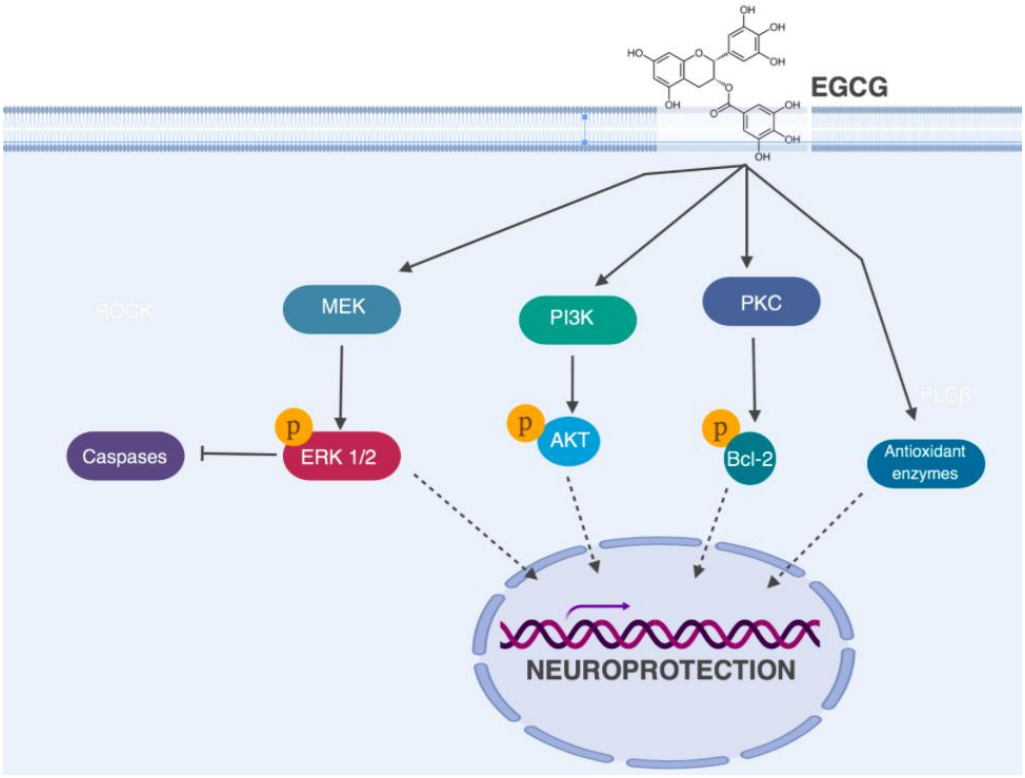


Figure 14: Schematic model of main neuroprotective mechanisms of action of EGCG. The diagram demonstrates the potential molecular pathways involved in the multifunctional effects of EGCG in neuronal tissues.

The fact that EGCG has the ability to activate protein kinase pathway promoting a survival effect (Levites et al., 2002) makes it of great interest in the field of HD. Moreover, more studies have been revealed other molecular targets of this catechin encouraging its implication in HD. For example, it has been shown in non-neuronal cells that EGCG inhibits HDAC1 (Levites et al., 2001) and by the inhibition of dryk1a, EGCG rescues cognitive deficits in Down syndrome mouse model and in humans by the promotion of synaptic plasticity (De la Torre et al., 2014). Finally, it has been observed in non-neuronal cells that EGCG inhibits fatty acid synthase (FASN) (Puig et al., 2008). Nevertheless, therapeutic targets underlying neuroprotective effects are not fully understood as they can act through several targets and, moreover, its possible effect on HD has not been studied yet.

8.2.2. FASN

As said above, EGCG is found to be a natural inhibitor of FASN. It has been observed that the effect of EGCG on thermal hyperalgesia is strongly correlated with the

inhibition of FASN activity in the dorsal horn of the spinal cord (Xifro et al., 2015). However, no data about the role of FASN in neurodegenerative diseases has been published. We only know that FASN activity has the capacity to modulate the membranous levels of NMDA receptors subunits and that elevated plasma levels of FASN have been associated with the severity of autism (Zakareia & Al-Ayadhi, 2013). Although normal tissues have low levels of fatty acid synthesis, a number of recent studies have demonstrated surprisingly high levels of fatty acid synthase expression in a wide variety of human malignancies and their precursor lesions including carcinoma of the colon, prostate, ovary, endometrium and breast (Zaytseva et al., 2015; Witkiewicz et al., 2008; Shah et al., 2006). It is also known that FASN confers an advantage for tumor growth (Kuhajda et al., 2000).

Fatty acid synthase (FASN) is a multifunctional enzyme that catalyses the *de novo* synthesis of the fatty acid palmitate from acetyl-CoA, malonyl-CoA and NADPH and also has a key role in lipid biosynthesis (Figure 15) (Chirala & Wakil, 2004).

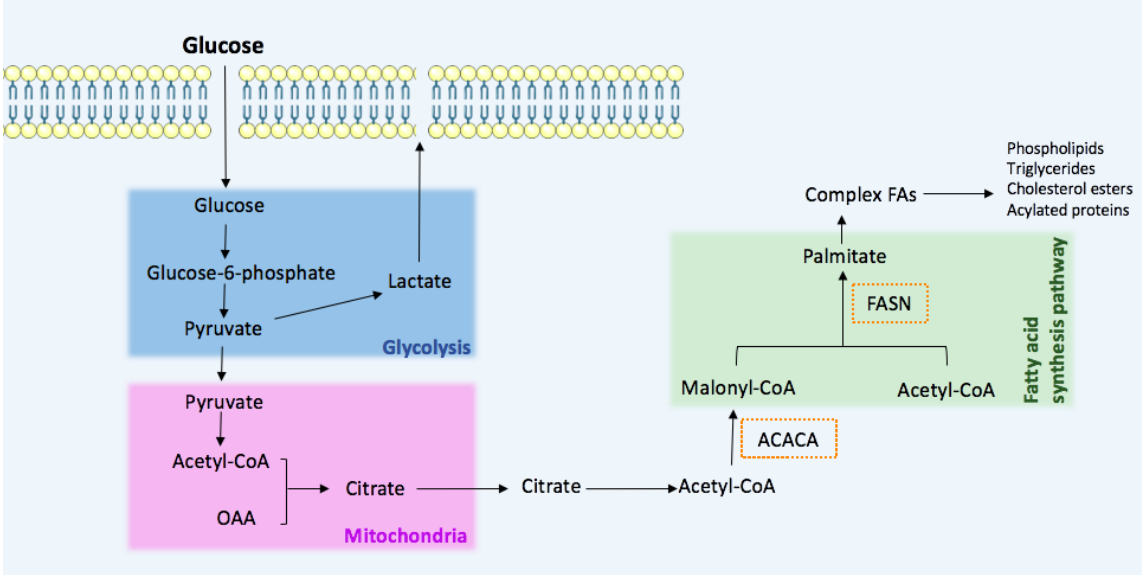


Figure 15: Connecting glucose metabolism with fatty acid biosynthesis pathways. Glucose is phosphorylated (glucpse-6-phosphate) generating pyruvate. Pyruvate can be converted in lactate, under anaerobic conditions, and then be secreted from the cell or can be converted to acetyl coenzyme A (CoA) which together with oxaloacetate (OAA) enter to citric acid cycle in the mitochondria producing citrate. Citrate can be transported to the cytoplasm where it is converted back to Acetyl-CoA which is carboxylated to Malonyl-CoA by Acetyl-CoA carbocyclase (ACACA). Then, fatty acid synthase (FASN), the main biosynthetic enzyme, conducts the condensation of Acetyl-CoA and Malonyl-Coa to produce the 16-carbon fatty acid, palmitate. Palmitat can be further modified by elongases or desaturases to form

more complex fatty acids used for the synthesis of different cellular lipids such as phospholipids, triglycerides and cholesterol esters, or for the acylation of proteins. Adapted from Zaidi et al., 2012

FASN is a saturated fatty acid with 16 carbons that contain seven catalytic subunits divided in three different domains (Wakil, 1989). It is the major component of cell membranes and is stored into triglycerides for energy metabolism. Palmitate is also a substrate in the palmitoylation of membrane proteins, known to be disrupted in HD, and a precursor in the synthesis of complex lipids such as glycerophospholipids and cholesterol (Chirala & Wakil, 2004).

In normal tissue, fatty acid synthesis occurs when there is an excess of calories, and the carbohydrates are stored as triglyceride. However, in a pathological condition it has seen that most of the fatty acids are synthesize de novo mainly as phospholipids, acting as structural blocks for membrane biosynthesis, protein modification, or signalling molecules (Ventura et al., 2015; Jones & Infante, 2015; Menendez & Lupu, 2007).

Considering this background, could be interesting to find a FASN inhibitor such as EGCG with therapeutic potential and evaluate its implication in neurodegenerative diseases, HD in this case, as there is nothing described before.

Altogether, it is therefore necessary to look for drugs capable of simultaneously manipulate multiple targets exerting therapeutic effectiveness. Thus, as EGCG has a broad spectrum of biological and pharmacological actions, it can be studies as a therapeutic agent in the treatment of neurodegenerative diseases. In this context, we found interesting to see if EGCG can be a good therapeutic molecule by inhibiting FASN in neurodegenerative diseases, HD specifically, as well as it is in other human malignancies.

II. AIMS

HD is a progressive neurodegenerative disorder characterised by cognitive, motor and psychiatric dysfunction without good candidates to be suitable therapeutic targets to contribute in mitigating the disease progression. Symptomatology caused by a progressive neuronal dysfunction in several brain areas such as striatum, hippocampus and cerebral cortex related to the activation of complex pathogenic mechanisms by mhtt that, finally, lead to neuronal death in the striatum. For this reason, study of molecules that can have direct effect on toxic processes activated in HD or enhance synaptic plasticity and neuronal survival has become of great interest. Several studies in different neuronal models have postulated that the neuropeptide PACAP and the polyphenol EGCG are able to promote both synaptic plasticity and neuroprotection. However, their possible therapeutic effect and the underlying molecular targets remain to be elucidated in the context of HD.

Therefore, the main objective of this thesis was to understand the therapeutic capacity of PACAP and EGCG in HD and identify by which therapeutic target they might exert its beneficial effects for the management of the disease.

This thesis was divided into two different studies with the following aims stated:

1. **Study the role of PACAP and its receptors in HD models**
    - **1.1** Evaluate the therapeutic capacity of PACAP in the hippocampus
        - 1.1.1 Analyse PACAP receptors levels in the hippocampus of HD mice models
        - 1.1.2 Test the therapeutic effect of PACAP on cognitive deficits in a HD mice
        - 1.1.3 Study whether PACAP can promote synaptic plasticity in the hippocampus of HD mice models
        - 1.1.4 Investigate whether therapeutic effect of PACAP is mediated by PAC1 receptor
    - **1.2** Evaluate the neuroprotective capacity of PACAP against mhtt-mediated toxicity
        - 1.2.1 Study the protein levels of PACAP receptors in the striatum of HD models

1.2.2 Evaluate PACAP capacity to protect striatal cells from toxicity caused by mhtt

1.2.3 Determine whether PACAP administration improves motor deficits in HD mice

1.2.4 Investigate whether PAC1 would be the therapeutic target for which PACAP protects striatal cells

2. Study the effect of EGCG in HD models

2.1 Evaluate the neuroprotective capacity of EGCG toxicity caused by mhtt

2.2 Analyse EGCG potential to improve motor and cognitive phenotype in a mouse model of HD

2.3 Study the contribution of FASN in the pysiopathology of HD

2.4 Determine whether EGCG administration in HD mice has the capacity to inhibit FASN

III. METHODS

1. HD mouse models

R6/1 mouse model of Huntington's disease were obtained from Jackson Laboratory (Bar Harbor, ME, USA). R6/1 mice express an N-terminal exon 1 fragment of mhtt, originally containing a stretch of 115 CAG repeats (Mangiarini et al., 1996). Nevertheless, the CAG repeat sequence is unstable, and CAG repeat-length determination revealed a longer CAG stretch (Giralt et al., 2011b). Thus, our R6/1 mouse colony expresses the exon 1 mhtt with 145 CAG repeats. Mice were genotyped and CAG repeat-length was determined by PCR amplification of the previously described by the Huntington's Disease Collaborative Research Group (HDCRG, 1993). Male R6/1 mice were used for all the experiments, and results were compared to non-transgenic wild-type littermate mice.

Hdh^{Q111} knock-in mice, with targeted insertion of 109 CAG repeats that extends the glutamine segment in murine htt to 111 residues (Lloret et al., 2006). Male and female $Hdh^{Q7/Q111}$ heterozygous mice were intercrossed to generate $Hdh^{Q7/Q111}$ heterozygous and $Hdh^{Q7/Q7}$ wild-type littermates (Giralt et al., 2012). Only males were used for all experiments.

Animals were housed with access to food and water ad libitum in a colony room kept at a 19-22ºC and 40-60% humidity, under a 12:12 h light/dark cycle. Litters containing distinct genotypes were being housed together. Microchips were implanted on the back subcutaneously, and were used to number all mice. All procedures were performed in compliance with the National Institute of Health Guide for the care and use of laboratory animals, and approved by the local animal care committee of the Universitat de Barcelona (99/01), and the Generalitat de Catalunya (99/1094), in accordance with the Directive 86/609/EU of the European Commission.

2. Human brain samples

All human brain samples were obtained from the Neurological Tissue Bank of the Biobanc-Hospital Clinic-IDIBAPS, Barcelona (NTB-Biobanc-HC-IDIBAPS). All the ethical guidelines contained within the latest Declaration of Helsinki were taken into

consideration and informed consent was obtained for all subjects under study. Samples were obtained from control subjects (mean ± SEM; age 53.5 ± 6.8 years; *post-mortem* intervals of 4-18 h), and HD brains Vonsattel grades III and IV (mean ± SEM; age 54.5 ± 6.5 years; *post-mortem* intervals of 4-17 h).

3. Cell culture

3.1 STHdh cells

WT STHdh$^{Q7/Q7}$ cells which express htt with 7 repetitions and mutant STHdh$^{Q111/Q111}$ cells with 111 CAG repetitions were used. This cell line is an immortalized striatal neuronal progenitor cell line which was derived from striatal precursors isolated from Hdh$^{Q7/Q7}$ and Hdh$^{Q111/Q111}$ mice immortalized with the SV40 Large T antigen (Trettel et al., 2000). This striatal cell model faithfully represents htt mutation carried by patients because elongated polyglutamine tracts are placed within the correct context of the murine HdH gene. Thereby, immortalized cells accurately express normal and mhtt and do not exhibit amino-terminal inclusion which allow us to stud changes involved in early HD pathogenesis (Trettel et al., 2000). Cells were maintained at 33ºC in *Dulbecco's modified Eagle's medium* (DMEM; Sigma-Aldrich, Saint Louis, MO, USA), supplemented with 10% of fetal bovine serum, 1% streptomycin-penicillin, 2 mM L-glutamine, 1 mM sodium pyruvate.

3.2 Primary hippocampal cultures

Primary hippocampal cultures were obtained from 18-days old embryos from WT and R6/1 mouse as previously described (Anglada-Huguet et al., 2016). Briefly, hippocampus was dissected and cells were seeded (100,000 cells/cm^2) on plates pre-coated with 0.1 mg/mL poly-D-lysine (Sigma Chemical Co., St. Louis, MO) and cultured in Neurobasal medium (Gibco-BRL, Renfrewshire, Scotland, UK) supplemented with B27 and GlutaMAX (both Gibco-BRL, Renfrewshire, Scotland, UK) at 37°C in a humidified atmosphere containing 5% CO_2.

4. Pharmacological treatments

4.1 STHdh cells

STHdh$^{Q7/Q7}$ and STHdh$^{Q111/Q111}$ were treated with different drugs in a different time-course and concentration depending on the aim of the experiment. Cells were treated at 80% confluence with PBS, as vehicle, or PACAP (10^{-7}M), which were kindly supplied by Dr. Vaudry, and harvest at different time points (between 30 min to 24 h). Concentration was decided based on previous studies (Vaudry et al., 2000). Cells were treated with PBS, as vehicle, and EGCG (Sigma Chemical Co., St. Louis, MO) at 50% confluence at different concentrations (30, 40, 50 and 100 μM) for 48 h treatment.

4.2 Primary hippocampal cultures

Primary hippocampal cultures from WT and R6/1 mouse were treated after 7 DIV with PBS, as vehicle, or PACAP (10^{-7}M). Immunocytochemical staining was performed four days later.

4.3 R6/1 mice

For PACAP testing, wild-type or R6/1 mice were treated intranasally (i.n.) with PBS as vehicle (VEH) or PACAP (30 μg/kg) at 13-week-old and 18-week-old. The administration was daily for 7 days, then behavioural assessment is performed and finally animals were sacrificed by cervical dislocation and the hippocampus, striatum and cortex were rapidly removed. Fifteen animals were used for every condition.

For EGCG testing, wild-type or R6/1 mice were treated intraperitoneally (i.p.) with PBS as vehicle (VEH) or EGCG (40 mg/kg) at 14-week-old and 16-week-old. The administration was daily for 15 days, then behavioural assessment is performed and finally animals were sacrificed by cervical dislocation and the hippocampus, striatum and cortex were rapidly removed. Ten animals were used for every condition.

5. Immunocytochemical staining and branching analysis

Fixed cells from primary hippocampal cultures were permeabilised in 0.1% saponin for 10 min. Blocking was performed with 1% BSA in PBS for 1 h. Cells were incubated overnight with the primary antibody anti-MAP2 (1:500; Sigma-Aldrich). Afterwards, cells were incubated with fluorescent secondary antibody, Cyanine 3 anti-mouse (1:150; Jackson ImmunoReseach, West Grove, PA). After washing twice with PBS, the coverslips were mounted with mowiol. Hippocampal neuron staining was observed with a fluorescence microscope (Olympus). At least 30 pyramidal neurons were randomly selected for each embryo and each condition. Dendrite analysis was performed with the ImageJ plugin NeuronJ (NIH, USA) to determine the number of dendrites per neuron (Sanchez-Danes et al., 2012). All the analysis was performed in a blinded fashion.

6. Plasmids and siRNA transfections

6.1 Plasmids

Exon-1 htt plasmids, expressing either 16 or 94 polyQ repeats and tagged with green-fluorescent protein (GFP), were kindly supplied by Dr. George M. Lawless (Cure HD Initiative; Reagent Resource Bank of the Hereditary Disease Foundation, New York, USA).

E. coli cells (Subcloning Efficiency™ DH5α™ Competent Cells, Invitrogen, Varlsbad, CA, USA), transformed by thermal shock, were used to expand the plasmid. Plasmid (250 ng) was added to 20 μl cultured-bacteria and left 15 min on ice. In order to induce transformation, the mixture was incubated for 1.5 min at 42ºC and quickly placed on ice for 2 min. After the thermic shock, *E. coli* cells growth is proceeding. LB media (Lysogeny broth media; 800 μl) was added and it was incubated for 1h at 37ºC at 250 revolutions per min (rpm). Finally, 200 μl of transformed cells were spread on an agar plate containing 30 μg/ml of Kanamicine or 500 μg/ml Ampicillin. Colonies were left growing overnight at 37ºC.

Plasmid isolation was performed from 5 ml bacterial culture using the Wizard Plus SV Minipreps and Maxipreps DNA purification system from Promega (Madison, WI, USA).

Transfection procedures were carried out using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) as instructed by the manufacturer. STHdh$^{Q7/Q7}$ and STHdh$^{Q111/Q111}$ cells at 50% confluence were transfected with 1µg of plasmid in Opti-MEM media (Sigma Chemical Co., St. Louis, MO). Media was replaced 6 h later by serum-supplemented DMEM media (Sigma Chemical Co., St. Louis, MO). Cells were harvest for Western blot analysis 72 h post-transfection.

6.2 siRNA transfection

To suppress PAC1 or FASN expression, STHdh $^{Q7/Q7}$ and STHdh $^{Q111/Q111}$ cells were transfected using Lipofectamine RNAiMAX reagent (Invitrogen, Life Technologies) with the appropriate antisense oligonucleotides sc-39005 or sc-41516 respectively (Santa Cruz Biotechnology, Santa Cruz, CA) or with scramble control (sc-37007; Santa Cruz Biotechnology, Santa Cruz, CA) and as instructed by the manufacturer. Briefly, STHdh$^{Q7/Q7}$ and STHdh$^{Q111/Q111}$ cells at 50% confluence were transfected with 30 or 50pmol of siRNA. Cells were harvest for Western blot analysis 72 h post-transfection.

7. Behavioural assessment

7.1 Clasping

Clasping was measured weekly in R6/1 mice from 11 to 18 weeks of age by suspending mice from their tails at least 1 foot above the surface for 1 min. A clasping event was defined by the retraction of either or both hindlimbs into the body and toward the midline.

7.2 Cognitive assessment

7.2.1 Novel object recognition test (NORT)

Mice were tested at 14 weeks of age in an open field (40 cm diameter and 35 cm high) in a room with dim lighting. Mice were habituated to the open field in the absence of objects for 10 min over 2 days. On the third day, during testing, two identical objects

were placed in the field for 10 min to each mouse (training session), after which they were returned to their home cage. The retention test was performed 24 h after by placing them back in the open field for 10 min and by randomly exchanging one of the familiar objects with a novel one (retrieval session). The objects varied in colour, shape and size. To avoid olfactory cues, chamber and objects were thoroughly cleaned between trials. The object preference was measured as the time exploring novel object × 100/time exploring novel and familiar objects. Animals were tracked and recorded with SMART Junior software (Panlab, Spain).

7.2.2 Novel object location test (NOLT)

NOLT test is performed similarly as NORT. Mice were tested in the open field at 14 weeks-old (40 cm diameter and 35 cm high) in a room with dim lighting. Mice were habituated to the open field in the absence of objects for 10 min over 2 days. On the third day, during testing, two identical objects were placed in the field for 10 min to each mouse (training session), after which they were returned to their home cage. The retention test was performed 24 h after by placing them back in the open field for 10 min. The difference lies in the retrieval session where one object is returned immediately to its former location, while the other object is placed in a new location. The object preference was measured as the time exploring novel object × 100/time exploring novel and familiar objects. Animals were tracked and recorded with SMART Junior software.

7.2.3 T-maze spontaneous alternation task (T-SAT)

Mice were tested at 14 weeks of age in T-maze apparatus used for the T-SAT is a wooden maze consisting of 3 arms; 2 of them are situated at 180ºC from each other, and the third is positioned at 90ºC of the others, thus giving a T-shape. All three arms are 45 cm long and 8 cm wide, with walls of 20 cm high. A starting area was located at the end of the stem arm and closed by a wooden guillotine door. In the training session, one arm was closed (new arm) and mice were placed in the stem arm of the T and allowed to explore this arm and the other available arm (old arm) for 10 min. After intervals of 4 h, mice were placed in the stem arm of the T-maze and allowed to explore the three arms for 10 min. The arm preference was determined by calculation

of the time spent in new arm × 100/time spent in both arms (new and old). Animals were tracked and recorded with SMART Junior software.

7.3 Motor assessment

7.3.1 Rotarod

Motor coordination and balance were evaluated at 18 weeks-old using the rotarod apparatus at 16, 24 and 32 rpm. Animals were trained at 16 weeks of age and evaluated at 18 weeks of age. The number of falls in a total of 60 seconds was recorded.

7.3.2 Balance beam

The motor coordination and balance of mice were also assessed at 18 weeks of age by measuring their ability to traverse a narrow beam. The beam consisted of a long steel cylinder (50 cm) with a 15 mm-round diameter. The beam was placed horizontally, above the bench surface, with each end mounted on a narrow support. The beam was divided in 5 cm frames. Animals were allowed to walk for 2 min along the beam, and the number of slips, the distance covered and the latency to cover 30 frames were measured.

8. Protein extraction

Biological samples were processed to obtain the protein fraction. Each type of sample was processed following a specific protocol that is detailed below.

8.1 Mouse and human brain tissue

Animals were killed by decapitation at different weeks representing different phases of the disease. Then, the striatum, cortex and hippocampus were quickly dissected out and immediately frozen upon dissection. Total proteins were extracted by the mechanical disruption in lysis buffer containing 1% Triton X-100, 10% Glicerol, 50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 150 mM NaCl, protease inhibitors (2 mM phenylmethylsulphonyl fluoride, 10 µg/µl aprotinin, 1 µg/µl leupeptin) and phosphatase inhibitors (2 mM Na_3VO_4, 100 mM NaF).

For human samples, total proteins were extracted by the sonication in the same lysis buffer.

Finally, in both cases, samples were centrifuged at 16,100 x g for 20 min at 4ºC, and supernatants were collected. Protein concentration was determined using the Dc protein assay kit (Bio- Rad Laboratories, Hercules, CA, USA).

8.2 Cell culture

$STHdh^{Q7/Q7}$ and $STHdh^{Q111/Q111}$ cells were washed once with PBS (phosphate-buffered saline) and total cellular proteins were extracted by incubating cells in lysis buffer containing 1% Triton X-100, 50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 150 mM NaCl, protease inhibitors (2 mM phenylmethylsulphonyl fluoride, 10 µg/µl aprotinin, 1 µg/µl leupeptin) and phosphatase inhibitors (2 mM Na_3VO_4).

Samples were centrifuged at 16,100 x g for 20 min at 4ºC, and supernatants were collected. Protein concentration was determined using the Dc protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

9. Western Blot Analysis

Western blot analysis was performed following a standard protocol. Proteins were denatured in 62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS (*Sodium Dodecyl Sulfate)*, 10% glycerol, 140 mM β- mercaptoethanol and 0.1% (w/v) bromophenol blue and heated at 100ºC for 5 min. In order to separate proteins by their molecular weight, samples were resolved in denaturing polyacrylamide gels (*SDS Polyacrylamide Gel Electrophoresis*, SDS-PAGE) at different polyacrylamide concentrations depending on its molecular weight; at 35 mA during 1 h. Proteins were then transferred to a nitrocellulose membrane (Whatman Schleicher&Schuell; Dassel, Germany) during 1.5 h at 90 V and at 4ºC to avoid excessive warming. Nitrocellulose membranes were blocked in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) solution plus 5% bovine serum albumin or 5% skimmed milk during 1 h at room temperature. Membranes were washed twice in TBS-T and blotted overnight at 4ºC with the following primary antibodies (Table 3).

Table 3: Primary antibodies for Western blot. A list of primary antibodies is provided, as well as their source and the dilution that was used for Western blot.

ANTIGEN	MOLECULAR WEIGHT (kDa)	HOST SPECIE	DILUTION	SOURCE
BDNF	14	Rabbit	1:1000	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
Cleaved Caspase-3	17, 19	Rabbit	1:1000	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
CBP	265	Rabbit	1:250	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
c-fos	62	Rabbit	1:250	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
FASN	273	Rabbit	1:1500	Enzo Life Sciences (Farmingdale, NY, USA)
egr-1	82	Rabbit	1:250	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
Htt (181-810aa)	350	Mouse	1:1000	Merk Millipore (Billerica, MA, USA)
Mhtt (clone mEM48	350	Mouse	1:1000	Merk Millipore (Billerica, MA, USA)
PAC1	60	Mouse	1:200	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
VPAC1	47,58	Rabbit	1:200	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
VPAC2	65	Rabbit	1:200	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
α-actin	45	Mouse	1:2000	MP Biomedicals (Aurora, OH, USA) Lamin
α-tubulin	55	Mouse	1:50000	Sigma-Aldrich (Sant Louise, MO, USA)

After primary antibody incubation, membranes were washed three times for 10 min with TBS-T and incubated for 1h at room temperature with the appropriated horseradish peroxidase-conjugated secondary antibody (Table 4).

Table 4: Secondary antibodies for Western blot. A list of secondary antibodies is provided, as well as their source and the dilution that was used for western blot. All antibodies are conjugated to the horseradish peroxidase.

SECONDARY ANTIBODY	DILUTION	SOURCE
Donkey Anti-Goat IgG	1:2000	Promega (Madison, WI, USA)
Anti-Mouse IgG	1:2000	Promega (Madison, WI, USA)
Anti-Rabbit IgG	1:2000	Promega (Madison, WI, USA)

Membranes were washed again three times for 10 min with TBS-T to remove secondary antibody remains and the reaction was finally visualized with the Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Western blot replicates were scanned and densitometries were quantified using the GelPro analyser program version 4.0.

For protein loading control, membranes were incubated with an antibody against α-tubulin or α-actin.

10. Quantitative real-time PCR analysis

10.1 RNA extraction

Cells were PBS washed, and then 1mL of Qiazol (Qiagen, Venlo, Netherlands) was added. Total-RNA was isolated using RNeasy mini kit (Qiagen, Venlo, Netherlands) following the instructions provided by the manufacturer. 200µl of chloroform was added to each sample and mix gently. Centrifuge at 14000 rpm for 15 min at 4 °C. Recover the supernatant into a new eppendorf. Add 350 µl of ethanol 70% to each sample, mix. Transfer all the solution into the column (QIAGEN RNeasy mini Kit): 700 µl at a time. Centrifuge at 6000 rpm for 15 seconds at room temperature, discard the flow-through. Add 350 µl of RW1 Buffer. Centrifuge at 14000 rpm for 30 seconds at room temperature, discard the flow-through. For each sample, 10 µl of DNase (RNase-free DNase Set-QIAGEN) were mixed with 70 µl RDD Buffer, add 80 µl of this mixture to each. Wait for 15 min at room temperature. 350 µl of RW1 Buffer were added to the column and then centrifuged at 14000 rpm for 30 seconds, discard the flow-through. 500 µl of RPE Buffer was added. Centrifuge at 14000 rpm for 30 seconds, discard the flow-through, and change to Collection Tube (2mL, without caps).

Centrifuge at 14000 rpm for 1 min, change to Collection Tube (1.5 mL, with caps). Add 30-50µl of RNase-free water were added, centrifuge at 14000 rpm for 30 seconds. Total-RNA concentration was quantified using Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

10.2 Reverse transcription

RNA was reverse-transcribed into complementary DNA (cDNA) using High Capacity cDNA Archive Kit (Applied Biosystems, Foster, USA). The cDNA synthesis was performed according to manufacturer's instructions. 20 µl of Reverse transcription reaction mix were added into each reaction tube. Program used consisted in a first cycle at 25°C for 10 min followed by a cycle at 37°C for 120 min and a final cycle at 85°C for 5 min. Negative controls in order to exclude DNA contamination, reverse transcriptase enzyme was not added in the controls.

10.3 Real time PCR (q-PCR)

Gene expression levels of c-fos, egr-1 and PAC1 were assessed using LightCycler® 480 Real-time PCR System (Roche Basel, Switzerland) with LightCycler® 480 SYBR Green I Master (Roche). Primers used are described in Table 5. RT-PCR analyses were performed at least three times and each gene was run in triplicate. GADPH was used for normalization. Both control and experimental reactions were placed in a thermal-cycler to undergo the following thermal-cycling program: 40 cycles of a two-step PCR; 95°C for 15 seconds and 60°C for 30 seconds, after initial denaturing at 95°C for 10 min.

Table 5: Primers sequence

GENE	FORWARD PRIMER	REVERSE PRIMER
c-fos	GATGTTCTCGGGTTTCAACGCG	ACGTCGGTAGAATAAGGAAAGGG
egr-1	GGGAGCCGAGCGAACAA	GTCTCCACCAGCGCCTTCT
PAC1	CCCTGACTGCTCTCCTCCTGCTGCCTAT	CAGGGCAGCTCACAAGGACCATCTCACC
GAPDH	*ATCATCCCTGCCTCTACTGG*	*GTCAGGTCCACCACTGACAC*

11. Cell viability assays

11.1 MTT assay

In this work, MTT (Thiazolyl Blue Tetrazolium Blue, Sigma Chemical Co., St. Louis, MO) test is used to study cell viability. This colorimetric technique measures mitochondrial activity as an indicator of the cell state, that is, lower levels of mitochondrial activity reflect worse conditions than if levels are high. STHdh$^{Q7/Q7}$ and STHdh$^{Q111/Q111}$ cells were seeded in 96-well plates in their corresponding growth medium. After 24 h, growth medium was removed and 100µL of fresh medium containing the corresponding concentration of PACAP for 24 h or EGCG for 48 h was added. MTT is added to each well (0.5mg/ml) and it is allowed to incubate an hour at 37ºC. Subsequently the medium is sucked and 100µl of DMSO is added to dissolve the formazan, a product that results from mitochondrial MTT metabolism. Finally, the absorbance is measured in a microplate spectrophotometer (Benchmark Plus, BioRad, California, USA) at 570nm

11.2 Quantification of apoptotic cell death

STHdh$^{Q7/Q7}$ and STHdh$^{Q111/Q111}$ cells were grown on cover glasses and fixed in 4% paraformaldehyde in PBS for 10 min after different treatments. Cells were incubated with 0.2 M glycine during 20 min to block paraformaldehyde. After that, cells were permeated in PBS with 1% bovine serum albumin and 0.1% saponin for 10 min. Blocking was performed with 1% bovine serum albumin in PBS for 1 h before incubating specimens overnight at 4ºC with the primary antibody diluted in the same blocking buffer. Remaining primary antibody was removed in three consecutive washes with PBS and specimens were incubated with the subtype-specific fluorescent secondary antibody. To stain nuclei, cells were incubated during 5 min at room temperature with Hoechst 33258 (1:10,000; Invitrogen, Carlsbad, CA, USA) and washed afterwards with PBS. Finally cover glasses were mounted with Mowiol-mounting media (Merck, Darmstadt, Germany).

12. Immunohistofluorescence

Animals were deeply anesthetized with pentobarbital (60 mg/kg) and intracardially perfused with a 4% paraformaldehyde in 0.1 mM phosphate buffer. Brains were removed and post-fixed for 2h in the same solution, cryoprotected with 30% sucrose in PBS with 0.02% sodium azide and frozen in dry-ice cooled isopentane. Serial coronal cryostate 30 µm-thick sections were collected in PBS as free-floating sections and processed for immunohistofluorescence.

Sections were washed twice with PBS and incubated with NH_4Cl 50 mM during 30 min to block free fixation-remaining aldehydes and stop aldehyde-induced fluorescence. Tissue was permeated along 20 min by treatment with PBS containing 0.5% Triton X-100 and blocked during 1h at room temperature in PBS plus 0.2% bovine serum albumin, 0.2% lysine, 0.2% glycine, 0.2% sodium azide, 0.5% Triton X- 100 and 5% normal horse serum (Pierce Biotechnology, Rockford, IL, USA). Slices were incubated overnight at 4°C with the corresponding primary antibodies in a buffer containing PBS plus 0.3% Triton X-100, 0.2% bovine serum albumin and 0.2% sodium azide (Table 6).

Table 6 : Primary antibodies for immunofluorescence. A list of primary antibodies is provided, as well as their source and the dilution that was used.

ANTIGEN	*HOST SPECIE*	*DILUTION*	*SOURCE*
MAP2	Mouse	1:500	Sigma-Aldrich (Sant Louise, MO, USA)
VGlut1	Rabbit	1:300	Synaptic Systems (Göttingen, Germany)
PSD95	Rabbit	1:300	Affinity BioReagents (Golden, CO)

After primary antibody incubation, slices were washed in PBS twice for 10 min and incubated 2h at room temperature with subtype-specific fluorescent secondary antibodies (Table 7). After two consecutive 10 min-washes, slices were incubated 10 min at room temperature with Hoechst 33258 (1:4000; Invitrogen, Carlsbad, CA, USA) for nuclear staining and washed again twice with PBS before being mounted with Mowiol (Merck, Darmstadt, Germany) on silane-coated slides. No signal was detected

in controls incubated in the absence of the primary antibody.

Table 7: Secondary antibodies for immunofluoresence. A list of secondary antibodies is provided, as well as their source and the dilution that was used.

SECONDARY ANTIBODY	*DILUTION*	*SOURCE*
Cy™3 AffiniPure Donkey anti-Mouse IgG (H+L)	1:150	*Jackson ImmunoResearch (West Grove, PA, USA)*
Cy™3 AffiniPure Donkey anti-Rabbit IgG (H+L)	1:300	*Jackson ImmunoResearch (West Grove, PA, USA)*

13. Confocal microscopy analysis

Blinded examination to genotype and treatment of fluorescently labelled coronal sections was conducted with a Leica TCS SL laser scanning confocal spectral microscope (Mannheim, Germany) equipped with argon and helium-neon lasers. Images were taken with a 63× oil-immersion objective using an additional electronic zoom factor between 1.5 and 4× and standard (one Airy disc) pinhole. For each mouse, at least 4 hippocampal tissue slices were analysed, and three representative images from each slice were taken. The number of PSD95- and VGlut1-positive particles was counted as previously described using freeware NIH ImageJ software (Giralt et al., 2011a).

14. FASN activity assay

FASN activity was assayed in particle-free supernatants by recording spectrophotometrically at 37°C (Lambda Bio 20, Perkin Elmer Boston, MA, USA, EUA) and measuring the decrease of A340 nm due to oxidation of NADPH as previously described (Puig et al., 2008). Briefly, mice (n = 4 per group) were sacrificed by cervical dislocation and striatum and motor cortex were quickly removed and snap frozen. Tissues were homogenized in lysis buffer (250mM sucrose, 20 mM HEPES pH 7.6, 2 mM MgCl 2, 1 mM DTT, 1 mM EDTA, 50 mM NaF, 2 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg/µL aprotinin, 1 µg/µL leupeptin, 2 mM sodium orthovanadate). Homogenized samples were centrifuged at 3,500 x g for 10 min, and then supernatant was collected and centrifuged at 100,000 x g for 30 min to obtain supernatants

particle-free. The protein concentrations were determined using the Dc protein assay kit. A 90 µg amount of protein was used for the reaction. A pre-incubation during 15 min at 37°C in 0.2 M of potassium phosphate buffer, pH = 7.0 was kept for temperature equilibration. Then, samples were added to the reaction mixture (200 mM potassium phosphate buffer, pH 7.0; 1 mM EDTA; 1 mM dithiothreitol, 30 µM Acetyl-CoA and 0.24 mM NADPH) and then were monitored at 340 nm for 3 min to measure background NADPH oxidation. After the addition of 50 µM of malonyl-CoA, the reaction was assayed for 10 min to determine FASN- dependent oxidation of NADPH. Rates were corrected with the background rate of NADPH oxidation.

15. Statistical analysis

All data are expressed as mean ± SEM. All graphs were created with GraphPad Prism 6. Statistical analysis was performed using two-way analysis of variance (Two-way ANOVA) followed by the post hoc Bonferroni comparison test when the effect of more than one factor was simultaneously analysed or by Student's t-test, when only one parameter was analysed. Values of $p<0.05$ were considered statistically significant.

IV. RESULTS

1. PACAP and memory performance in HD

1.1 PACAP receptors are decreased in the hippocampus of HD mouse models from the onset of cognitive dysfunction

We first studied the protein levels of PACAP receptors (PAC1, VPAC1 and VPAC2) in the hippocampus of WT and R6/1 mice at 8, 12, 20 and 30 weeks of age. Concerning the PAC1 receptor, the immunoblot analysis revealed an important reduction of its protein levels in R6/1 mice compared to WT mice from 12 weeks of age (Figure 16A), an age that represents the onset of cognitive dysfunctions. This decrease was maintained during the progression of the disease, at 20 and 30 weeks of age (Figure 16A). In contrast, no changes were detected at 8 weeks of age between WT and R6/1 mice (Figure 16A). A similar pattern of expression was observed for VPAC1 (Figure 16B) and VPAC2 (Figure 16C) receptors with a reduction of the protein levels of the receptors in the hippocampus of R6/1 mice compared to WT mice from 12 weeks of age.

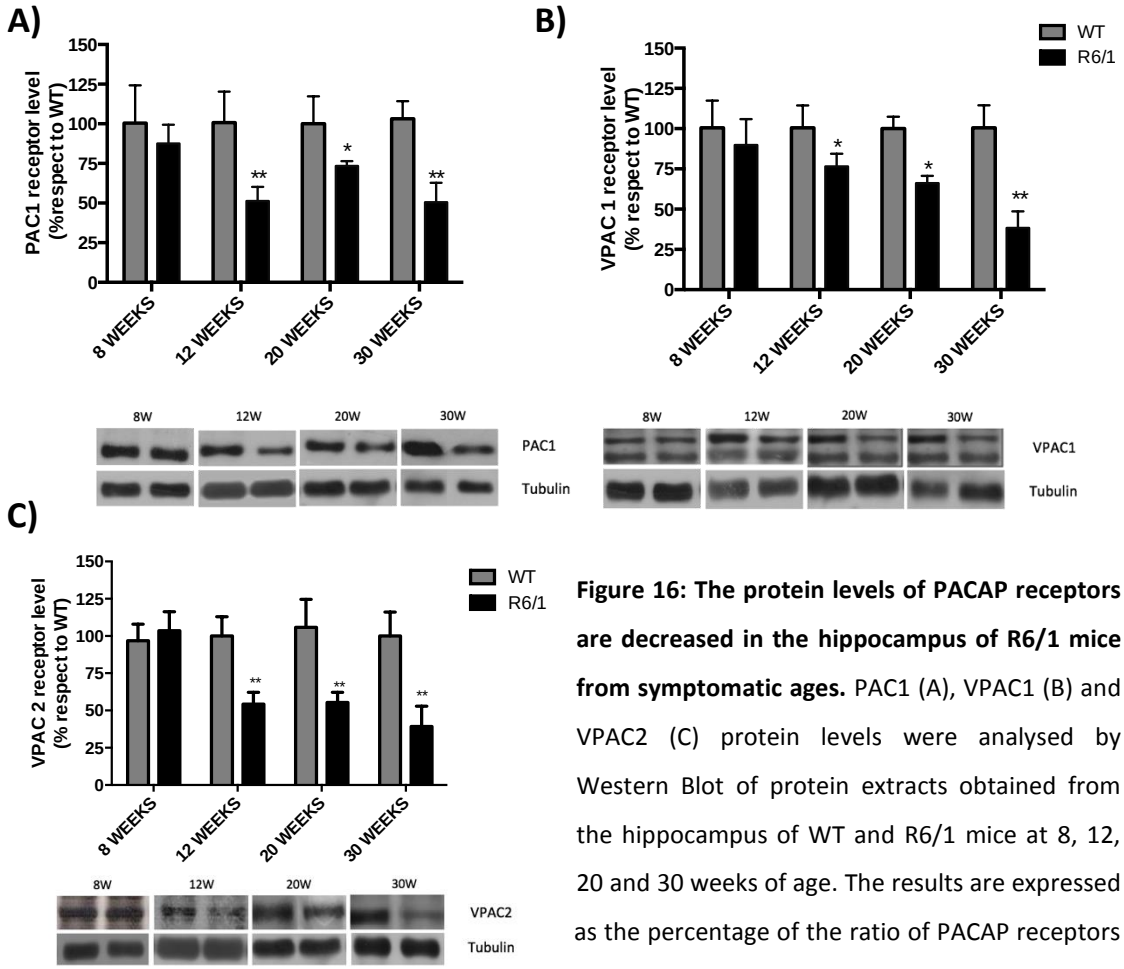
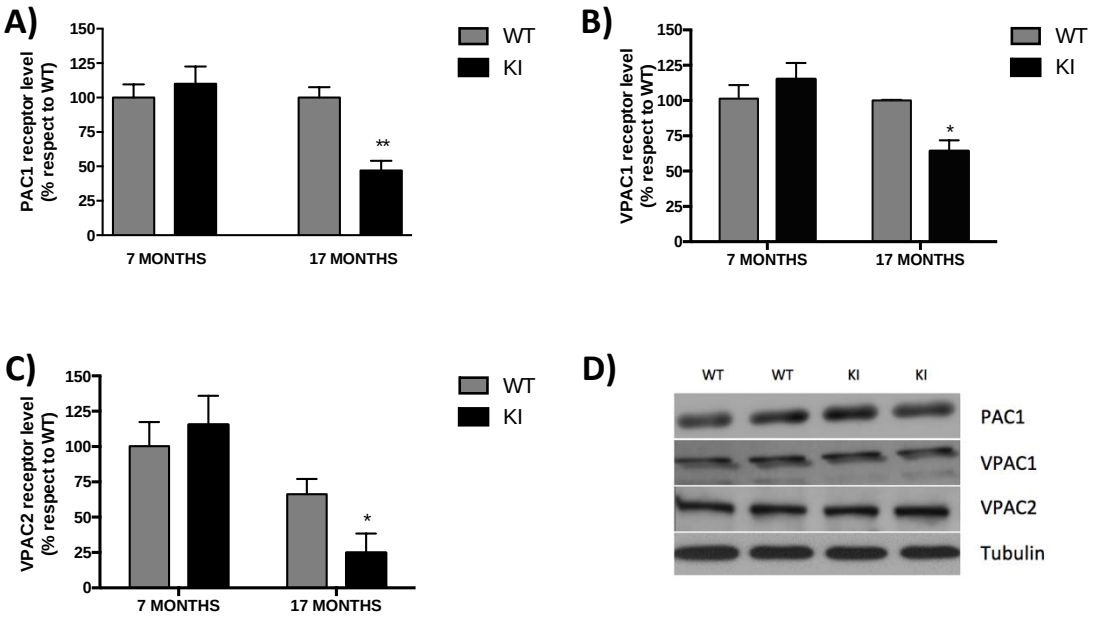


Figure 16: The protein levels of PACAP receptors are decreased in the hippocampus of R6/1 mice from symptomatic ages. PAC1 (A), VPAC1 (B) and VPAC2 (C) protein levels were analysed by Western Blot of protein extracts obtained from the hippocampus of WT and R6/1 mice at 8, 12, 20 and 30 weeks of age. The results are expressed as the percentage of the ratio of PACAP receptors vs tubulin levels at each age. Data shown are the

mean ± SEM (6 animals/group). Results were analysed by unpaired Student's t-test. *p<0.05 and **p<0.01 as compared to WT mice at each age. Representative immunoblots are shown.

The level of PACAP receptors was then also analysed by Western blot in knock-in mice with targeted insertion of 5 or 109 CAG repeats that extends the glutamine segment in murine htt to 7 ($Hdh^{Q7/Q7}$) or 111 ($Hdh^{Q7/Q111}$) residues. As in R6/1 mice, the protein expression level of PAC1, VPAC1 and VPAC2 receptors was similar between $Hdh^{Q7/Q7}$ and $Hdh^{Q7/Q111}$ at presymptomatic stages (7 months), but was significantly reduced in $Hdh^{Q7/Q111}$ at symptomatic stages (17 months) (Figure 17). Therefore, reduction of PACAP receptors is a general hallmark of HD mouse models.



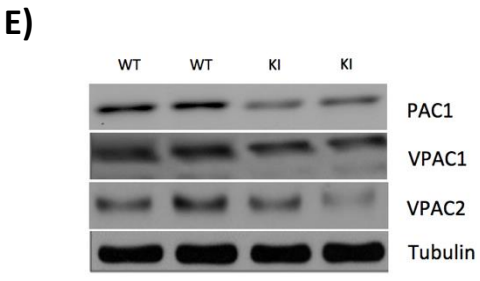


Figure 17: The protein levels of PACAP receptors are decreased in the hippocampus of Hdh knock-in mice at symptomatic ages. PAC1 (A), VPAC1 (B) and VPAC2 (C) protein levels were analysed by Western Blot of protein extracts obtained from the hippocampus of $Hdh^{Q7/Q7}$ and $Hdh^{Q111/Q111}$ mice at 7 and 17 months of age. The results are expressed as the percentage of the ratio of PACAP receptors vs tubulin levels at each age. Data shown are the mean ± SEM (6 animals/group). Results were analysed by unpaired Student's t-test. *p<0.05 as compared to $Hdh^{Q7/Q7}$ mice at each age. Representative immunoblots are shown for 7 months (D) and 17 months (E).

1.2 PAC1 receptor expression is reduced in the hippocampus of HD patients

To establish whether the reduction of PACAP receptors observed in HD mouse models occurs in humans, the levels of PAC1, VPAC1 and VPAC2 were investigated in *post-mortem* samples from the hippocampus of HD patients. In accordance with the results found in HD mice, Western blot analysis revealed a down-regulation of PAC1 receptor levels in the hippocampus of HD patients compared to the hippocampus of control individuals (Figure 18). However, no differences were observed in the protein levels of VPAC1 and VPAC2 between the hippocampal tissues from control individuals and HD patients (Figure 18).

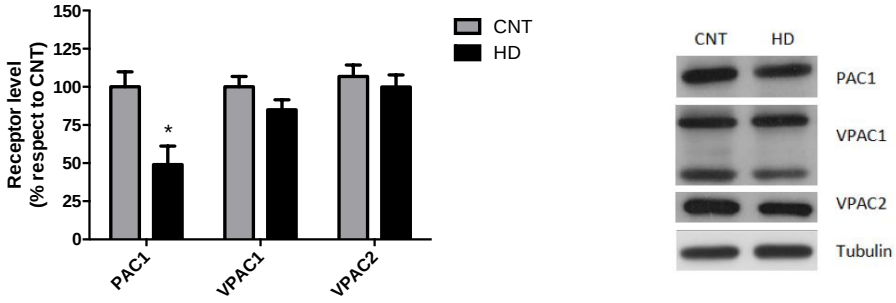


Figure 18: Hippocampus of HD patients shows specific decrease of PAC1 receptor protein levels. PAC1, VPAC1 and VPAC2 protein levels were analysed by Western Blot of protein extracts obtained from the hippocampus of control (CNT) and HD *post-mortem* brains. The results are expressed as the percentage of the ratio of PACAP receptors vs tubulin levels. Data shown are the mean ± SEM (5 samples/group). Results were analysed by unpaired Student's t-test. *p<0.05 as compared to CNT samples. Representative immunoblots are shown.

1.3 Intranasal administration of PACAP ameliorates memory deficits in R6/1 mice

The cognitive impairment observed in HD models has been related to deficits in hippocampal function (Brito et al., 2014; Nithianantharajah et al., 2008). As PACAP receptors levels were reduced in the hippocampus of HD mouse models, we investigated the ability of PACAP receptor activation to improve cognitive functions in HD. To this aim, 13-week-old WT and R6/1 mice were treated intranasally with PBS as vehicle or PACAP (30 µg/kg) for 7 days. Then, animals were subjected to NORT and T-SAT to study long-term memory (LTM). In the NORT, no differences were observed between vehicle and PACAP-treated WT mice as both groups exhibited the same preference for the novel object (Figure 19A). In contrast, vehicle-treated R6/1 mice did not recognize the old object resulting in a lower recognition index when compared to

vehicle-treated WT mice (Figure 19A). Importantly, the recognition index of PACAP-treated R6/1 mice was significantly improved compared to vehicle-treated R6/1 mice and was similar to vehicle- or PACAP-treated WT animals (Figure 19A).

To confirm the results obtained in the NORT, we next studied LTM using the T-SAT. The results obtained with T-maze were quite similar to those found in the NORT. The recognition index of vehicle-treated R6/1 mice was lower than in vehicle-treated WT group (Figure 19B) but treatment of R6/1 mice with PACAP induced a significant increase in new arm recognition index compared to vehicle-treated R6/1 mice and similar to vehicle-treated WT animals (Figure 19B).

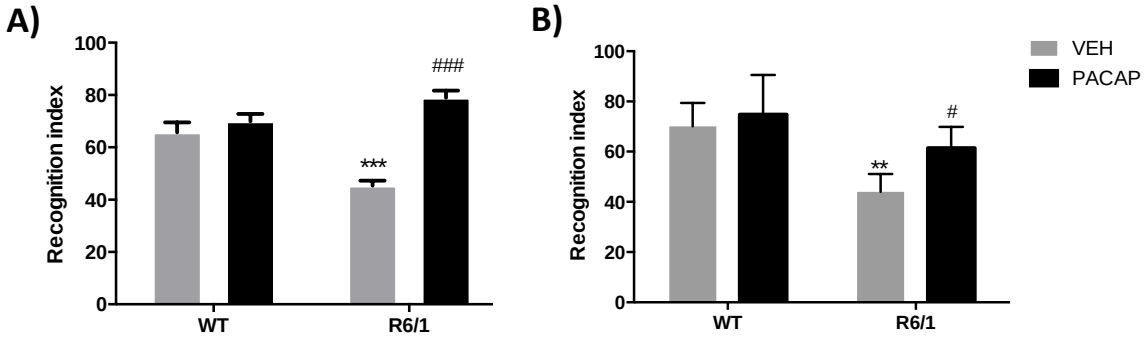
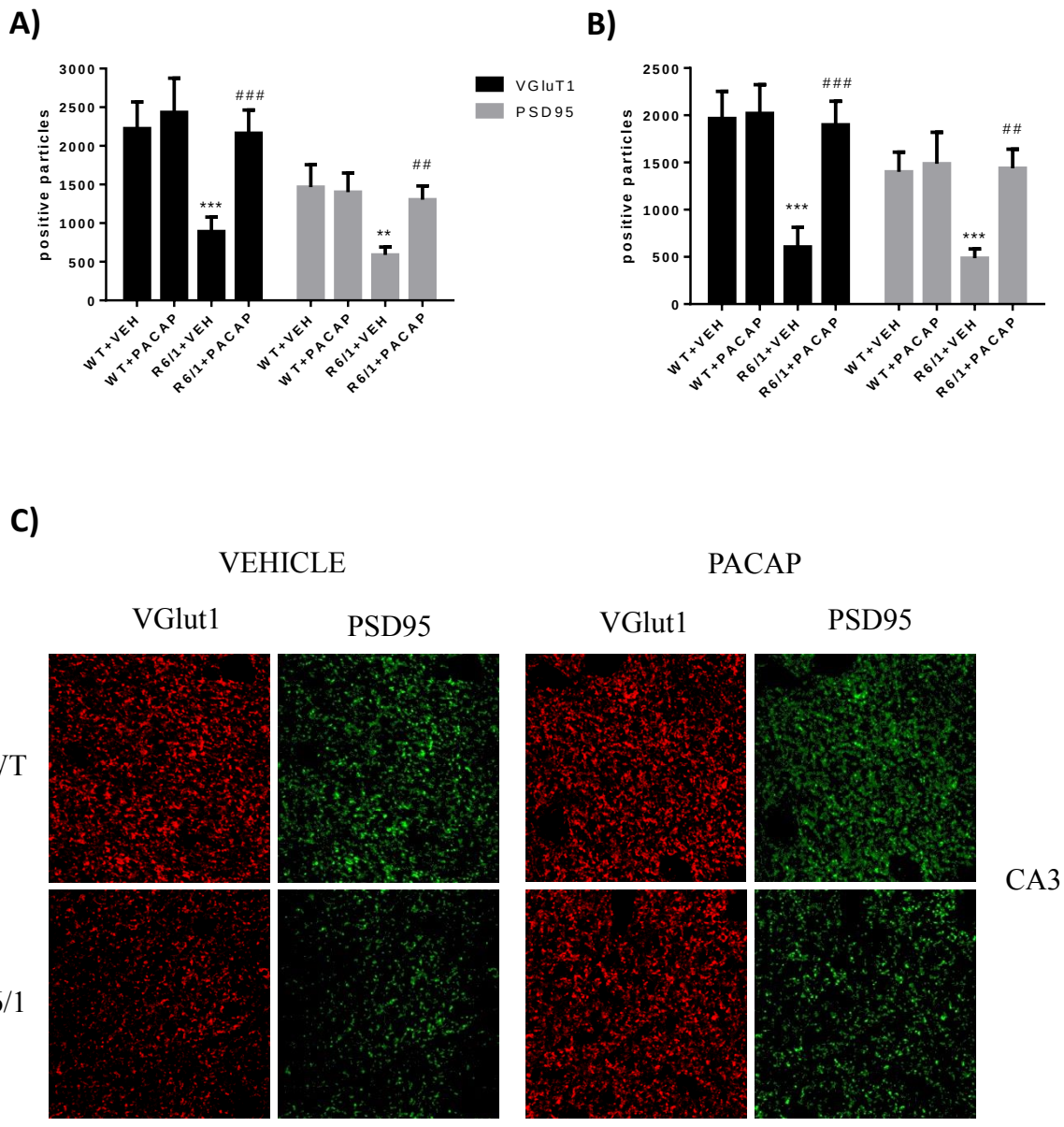


Figure 19: Intranasal administration of PACAP improves LTM capacity in R6/1 mice. WT and R6/1 mice were intranasally treated with PACAP (30 µg/kg/day) or vehicle from 13 weeks of age. Seven days later, mice were subjected to the NORT (A) and T-maze (B). (A) In the NORT task, all mice spent similar time exploring both objects during the training session. Recognition index represents the percentage of new object preference. Data shown are the mean ± SEM of 10-12 animals/group. Statistical analysis was performed using two-way ANOVA followed by Bonferroni as post-hoc test. ***p<0.001 as compared to WT-vehicle mice and ###p<0.001 as compared to R6/1-vehicle mice. (B) The arm exploration time 4 h after training session of four groups. Recognition index represents the percentage of the preference for new arm compared to old arm and is shown as mean ± SEM of 10-12 animals/group. Statistical analysis was performed using two-way ANOVA followed by Bonferroni as post-hoc test. **p<0.01 and ***p<0.001 as compared to WT-vehicle mice and #p<0.05 and #p<0.001 as compared to R6/1-vehicle mice

1.4 PACAP treatment significantly increases the number of VGluT1 and PSD95 synaptic positive particles in the hippocampus of R6/1 mice

To correlate memory improvements in R6/1 PACAP-treated mice with changes in synaptic plasticity we analysed the expression of VGlut1 and PSD95, two synaptic markers that are known to be reduced in the hippocampus of R6/1 animals (Anglada-Huguet et al., 2012; Milnerwood et al., 2006). Confocal image analysis of CA3 (Figure 20A,C) and dentate gyrus (DG; Figure 20B, C) after 7 days of PACAP administration

showed that only vehicle-treated R6/1 mice had a significant decrease in both VGlut1 and PSD95-positive particle density in comparison with WT animals. Importantly, PACAP-treated R6/1 mice showed a significant improvement in VGlut1 and PSD95 positive particles in both hippocampal regions analysed, compared to vehicle-treated R6/1 mice (Figure 20). No differences were observed between the two treatment groups in WT mice (Figure 20).



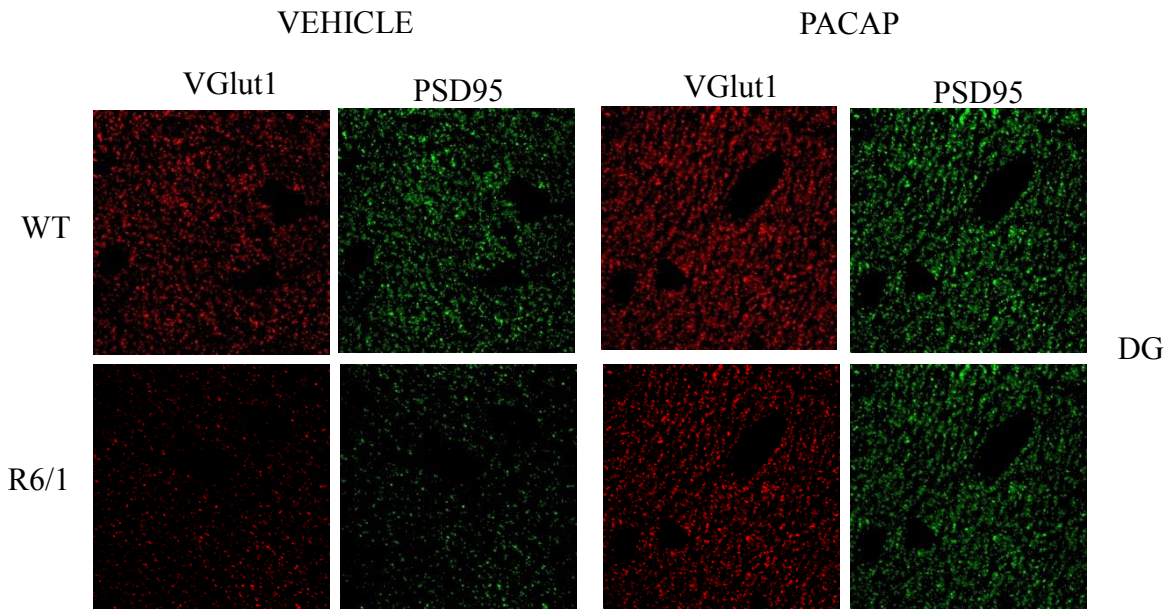


Figure 20: Preservation of VGlut1 and PSD95 positive particles in PACAP-treated R6/1 mice. Immunohistochemical staining against VGlut1 and PSD95 was performed in the CA3 and dentate gyrus (DG) of the hippocampus of vehicle-WT, PACAP-treated WT, vehicle-R6/1 and PACAP-treated R6/1 mice. The images were taken by confocal microscopy. VGlut1 and PSD95-positive particles were counted in the (A) CA3 and (B) DG by ImageJ software and represented in a graph as number of particles/field. Values are expressed as mean ± SEM (n = 6/group). Data were analysed by two-way ANOVA followed by Bonferroni as a post-hoc. **p<0.01 and ***p<0.001 as compared to vehicle- WT mice; ## p<0.01 and ### p<0.001 as compared to vehicle-R6/1 mice. Scale bar 10 µm. (C) Representative images for each hippocampal region are shown.

To determine whether the increase of synaptic markers observed in HD mice model resulted in morphologic improvement, the ability of PACAP to modulate neuritic structure in *in vitro* hippocampal neurons was investigated. To this aim, primary hippocampal cultures from WT and R6/1 animals were treated after 7 DIV with 10^{-7}M PACAP. After 4 days of treatment, immunofluorescence against MAP2 was performed (Figure 21A) and the number and length of neurites were quantified. Data analysis revealed a decrease in the number (Figure 21B) and length (Figure 21C) of neurites in vehicle-treated R6/1 neurons compared to vehicle-treated WT neurons and an increase in number and length of neurites in R6/1 neurons treated with 10^{-7} M PACAP, compared to vehicle-treated cells (Figure 21B,C). Of note, PACAP had no effect per se on WT neurons (Figure 21B,C).

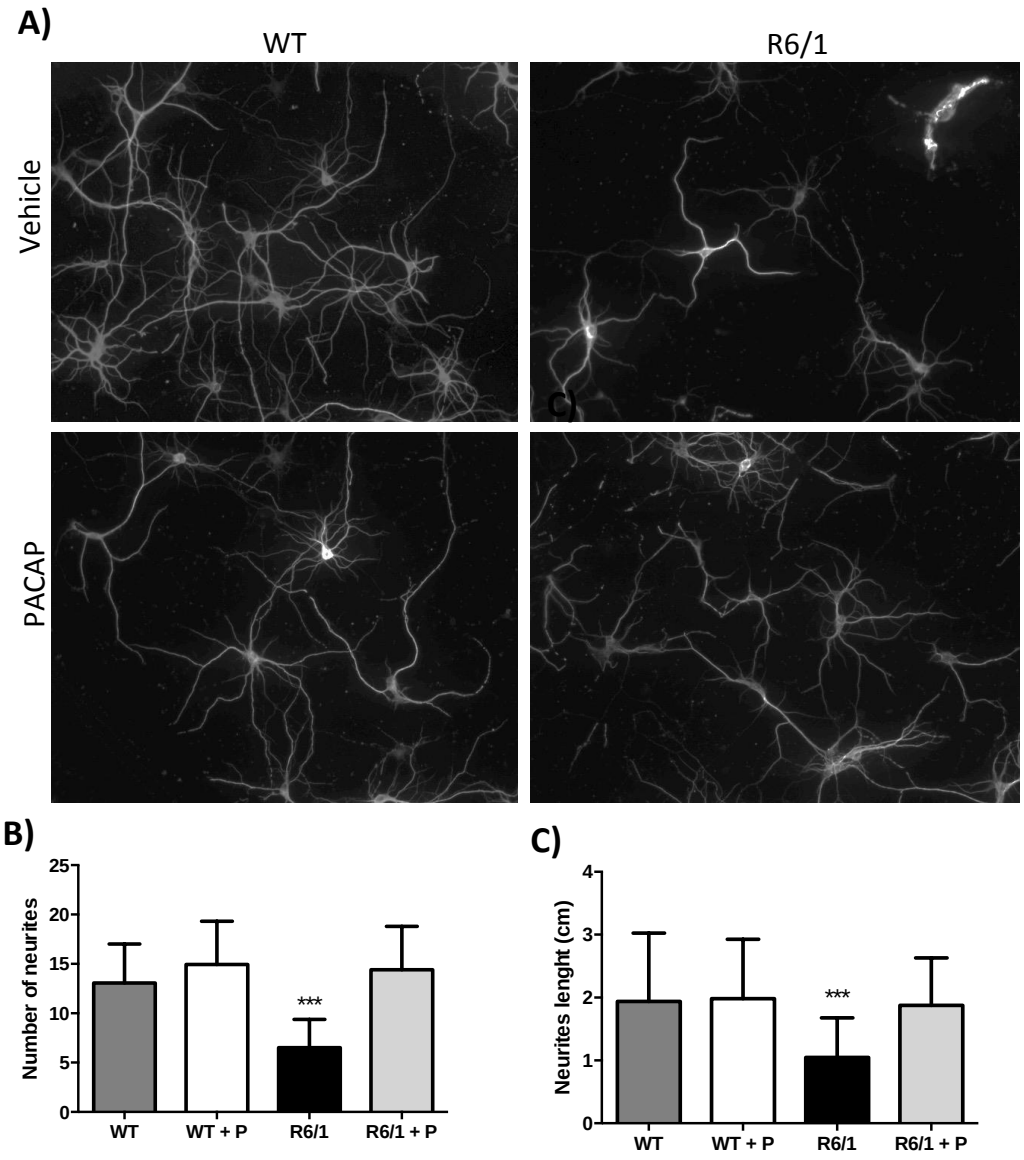


Figure 21: Administration of PACAP induces neuritic branching in R6/1 hippocampal primary cultures. Hippocampal primary cultures from 18-day-old WT and R6/1 mouse embryos were treated at 7 DIV with vehicle or PACAP (10^{-7}M). Four days later, neuronal cultures were fixed and immunolabelled against MAP2. (A) Representative microphotographs of WT and R6/1 hippocampal neurons in cultures treated or not with PACAP. Scale bar = 30 µm. (B) Graph showing the quantification of the number of neurites counted in each condition. (C) Graph showing the length of neurites in cm measured in each condition. In B and C data were analysed by two-way ANOVA followed by Bonferroni as a post-hoc. ***p<0.001 when compared to vehicle-treated WT primary cultures.

1.5 The administration of PACAP restores PAC1 receptor levels in the hippocampus of R6/1 mice

To investigate how PACAP could improve memory tasks and hippocampal synaptic markers in R6/1 mice, we next investigated by Western blot the levels of PAC1 receptor in WT and R6/1 mice after PACAP treatment. In accordance with Figure 16,

the protein levels of PAC1 were reduced in vehicle-treated R6/1 mice compared to vehicle treated WT mice (Figure 22). Interestingly, the administration of PACAP in R6/1 mice restored PAC1 to WT expression levels (Figure 22). PACAP had no effect on hippocampal PAC1 levels in WT mice, indicating the specific effect of PACAP in R6/1 mice (Figure 22).

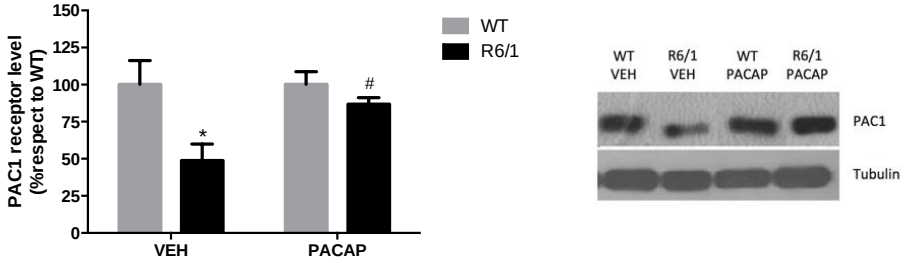


Figure 22: Intranasal administration of PACAP restores PAC1 protein levels in the hippocampus of R6/1 mice. WT and R6/1 mice were intranasally treated from 13 weeks of age with PACAP (30 µg/kg/day) or vehicle (VEH) for 7 days. Then, protein extracts from the hippocampus of all groups were subjected to Western blot to study PAC1 protein levels. Results are expressed as the percentage of the ratio between PAC1 and tubulin levels and represent the mean ± SEM of 6 animals per group. Data were analysed by two-way ANOVA followed by Bonferroni as a post-hoc. *$p<0.05$ as compared to WT-vehicle mice and #$p<0.05$ as compared to R6/1 vehicle mice. Representative immunoblots are presented.

1.6 PACAP stimulates the expression of genes involved in synaptic plasticity in the hippocampus of R6/1 mice

To identify some mechanisms that could contribute to the effects of PACAP, the levels of c-fos and CBP, two proteins associated with synaptic activity and known to be regulated by PACAP were evaluated in the hippocampus of WT and R6/1 mice treated with vehicle or PACAP (Aubert et al., 2006). Western blot analysis revealed a reduction in c-fos and CBP in vehicle-treated R6/1 mice compared to vehicle WT-treated mice (Figure 23) while R6/1 mice treated with PACAP displayed an increase of both proteins compared to vehicle-treated R6/1 mice. In WT mice, PACAP treatment did not significantly affect c-fos level but promoted CBP expression (Figure 23).

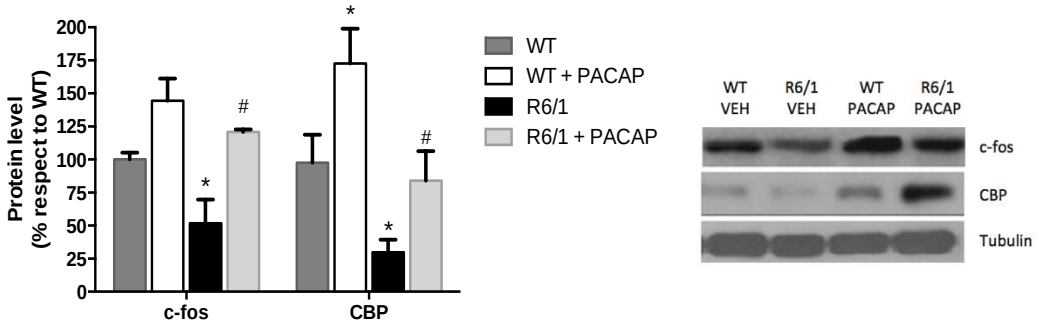


Figure 23: Intranasal administration of PACAP restores c-fos and CBP protein levels in the hippocampus of R6/1 mice. WT and R6/1 mice were intranasally treated from 13 weeks of age with PACAP (30 µg/kg/day) or vehicle (VEH) for 7 days. Then, c-fos and CBP protein levels were analysed by Western blot of protein extracts obtained from the hippocampus. Results are expressed as the percentage of the ratio between c-fos or CBP and tubulin levels and represent the mean ± SEM of 6 animals per group. Statistical analysis was performed using two-way ANOVA followed by Bonferroni as post-hoc test. *p<0.05 when compared to WT; #p<0.05 when compared to R6/1. Representative Western blots are shown.

1.7 Treatment with PACAP induces the expression of BDNF in the hippocampus of R6/1 mice

It has been described that a decrease in BDNF level is a key element of HD neuronal dysfunction (Baydyuk & Xu, 2014; Gorski et al., 2003). Furthermore, the expression of BDNF requires the presence of CBP as a co-activator of CREB (Zuccato et al., 2010). As PACAP induces the expression of CBP in R6/1 mice, we determined BDNF after PACAP treatment. The vehicle-treated R6/1 mice group showed reduced BDNF levels as described previously (Spires et al., 2004). Interestingly, the intranasal administration of PACAP rescued BDNF levels in the hippocampus of R6/1 mice without inducing changes in WT mice (Figure 24).

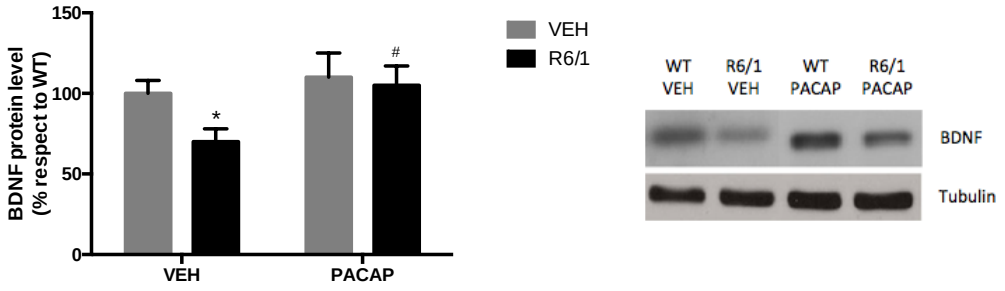


Figure 24: Intranasal administration of PACAP restores BDNF protein levels in the hippocampus of R6/1 mice. WT and R6/1 mice were intranasally treated from 13 weeks of age with PACAP (30 µg/kg/day) or vehicle (VEH) for 7 days. Then, protein extracts from the hippocampus of all groups were subjected to Western blot to study BDNF protein levels. Results are expressed as the percentage of the ratio between BDNF and tubulin levels and represent the mean ± SEM of 6 animals per group. Data were analysed by two-way ANOVA followed by Bonferroni as post-hoc test. *p<0.05 as compared to WT-

vehicle mice and #p<0.05 as compared to R6/1 vehicle mice. Representative immunoblots are presented.

1.8 Mhtt aggregates are reduced in the hippocampus of PACAP-treated R6/1 mice

Finally, the ability of PACAP treatment to prevent or reduce mhtt aggregation in the hippocampus of R6/1 mice was investigated using htt EM48 antibody that reacts with htt expressing more than 82 CAG repeats. Due to the difficulty to disaggregate mhtt inclusions, mhtt protein remained in the stacking gel of the Western blot. As shown in Figure 25, EM48 immunoreactivity was detected in R6/1 mice, but not in WT animals. Quantification of immunobands in vehicle and PACAP-treated R6/1 mice revealed that 7 days of PACAP treatment significantly reduced the formation of mhtt aggregates (Figure 25).

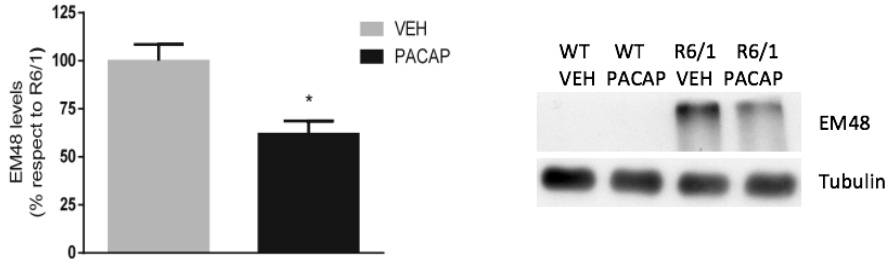
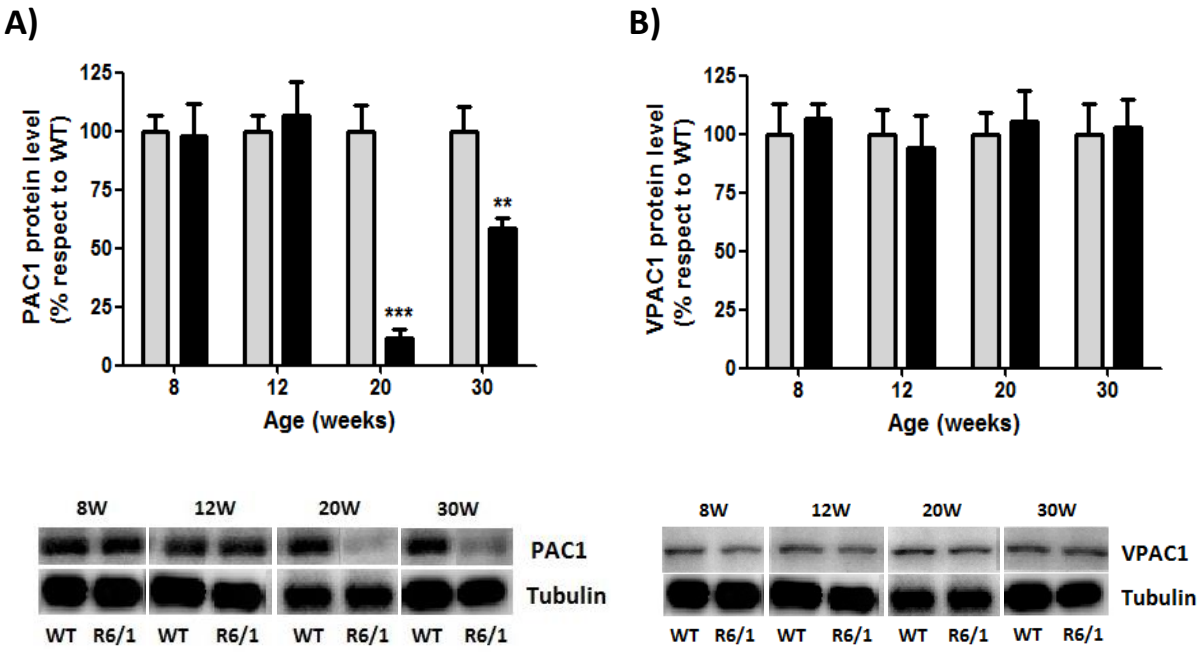


Figure 25: Administration of PACAP reduces the formation of mhtt aggregates in the hippocampus of R6/1 mice. WT and R6/1 mice were intranasally treated from 13 weeks of age with PACAP (30 µg/kg/day) or vehicle (VEH) for 7 days. Then, mhtt protein levels were analysed by Western blot of protein extracts obtained from the hippocampus. Quantification of the immunoreactivity in the stacking gel was performed by densitometric analysis. The results are expressed as the percentage of the ratio of mhtt vs tubulin levels. Data shown are the mean ± SEM of 6 animals per group. Results were analysed by unpaired Student's t-test. *p<0.05 as compared to vehicle-treated R6/1 mice. Representative immunoblots are shown.

2. Role of PACAP in motor impairment in HD

2.1 PAC1 receptor levels are reduced in the striatum and in the cortex of R6/1 mice from week 12

Protein levels of PACAP receptors (PAC1, VPAC1 and VPAC2) were characterised in the striatum and cortex of WT and R6/1 mice at 8, 12, 20 and 30 weeks of age. Regarding PAC1 receptor in the striatum, Western Blot analysis revealed an important reduction of its protein level in R6/1 mice compared to WT mice from 20 weeks of age (Figure 26A) which corresponds to a symptomatic phase of the disease progression. No changes were detected nor at 8 weeks of age neither at 12 weeks between WT and R6/1 mice (Figure 26A). No significant changes were detected in VPAC1 levels at any age analysed whereas for VPAC2 a significant reduction of its protein level was detected in R6/1 mice at 30 weeks of age (Figure 26B,C).



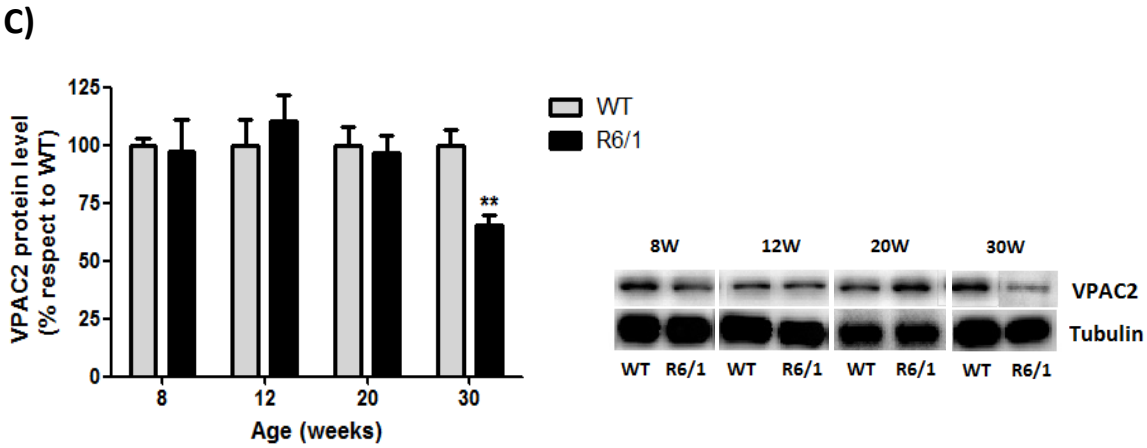
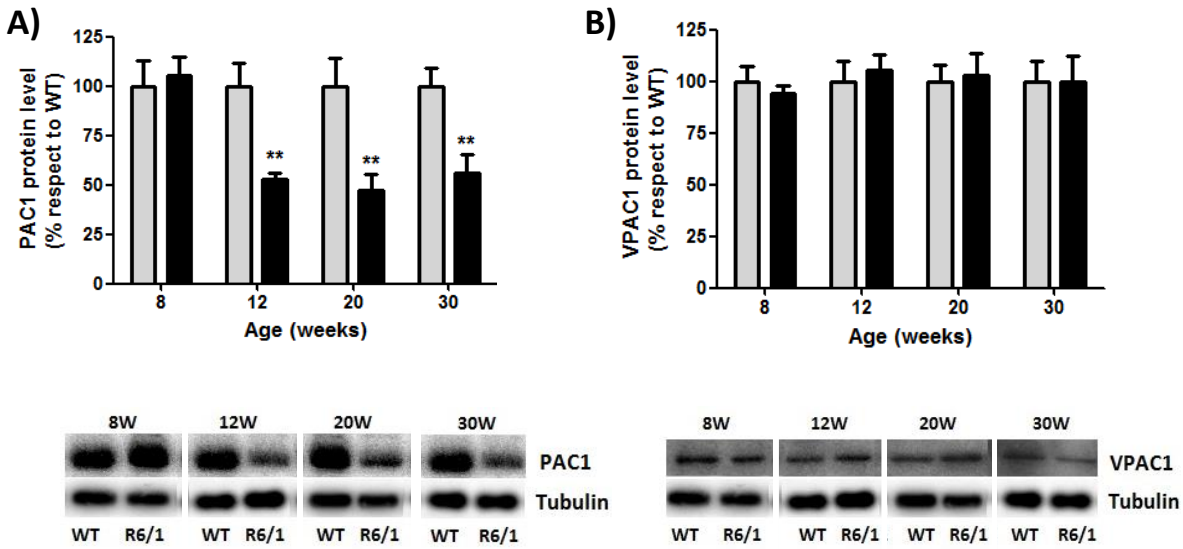


Figure 26: PAC1 receptor levels are reduced in the striatum of R6/1 mice from week 12. Western Blot analysis of PACAP receptors PAC1 (A), VPAC1 (B) and VPAC2 (C) in striatal samples of WT and R6/1 mice at different ages. Statistical analysis was performed using unpaired Student's t-test. Data are the mean ± SEM (n=8). **p<0.01 and ***p<0.001 as compared to WT mice.

Concerning cortical samples, the immunoblot analysis showed a notable reduction of PAC1 at 12 weeks of age that is maintained during the progression of the disease (Figure 27A). No changes were detected in VPAC1 and VPAC2 in any age analysed (Figure 27B,C).



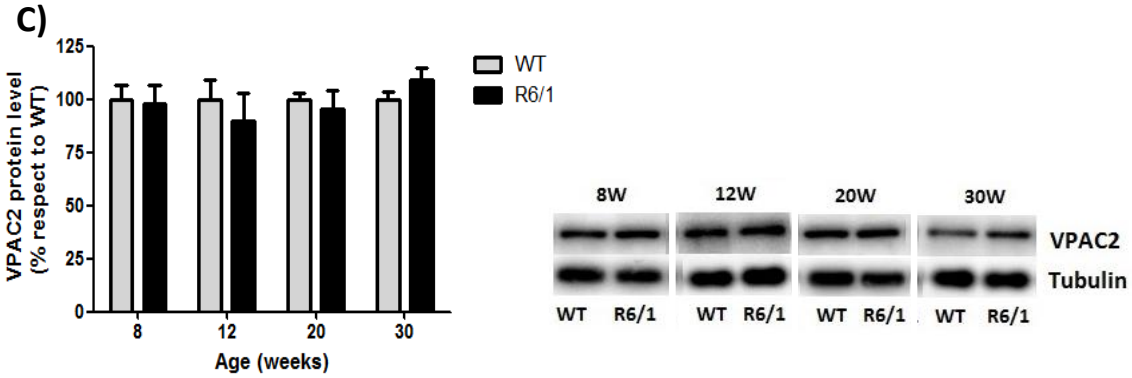


Figure 27: PAC1 receptor level are reduced in the cortex of R6/1 mice from 12 weeks of age. Western Blot analysis of PACAP receptors PAC1 (A), VPAC1 (B) and VPAC2 (C) in cortical samples of WT and R6/1 mice at different ages. Statistical analysis was performed using unpaired Student's t-test Data are the mean ± SEM (n=6). **p<0.01, as compared to WT mice.

2.2 PAC1 receptor levels are reduced due to mhtt

As PAC1 levels were decreased in all brain regions that play a key role in HD we wanted to examine if the formation of mhtt aggregates is enough to produce the reduction of PACAP receptors observed in the mouse model. First, we characterised the expression of PACAP receptors *in vitro*. PAC1, VPAC1 and VPAC2 protein levels were studied in the striatal STHdh$^{Q7/Q7}$ cell line, which expresses 7 CAG repeats and in the striatal STHdh$^{Q111/Q111}$ cell line, which expresses 111 CAG repeats. STHdh$^{Q111/Q111}$ cells showed a significant decrease in PAC1 receptor levels. In contrast, no differences were detected in VPAC1 and VPAC2 levels between cells lines. (Figure 28).

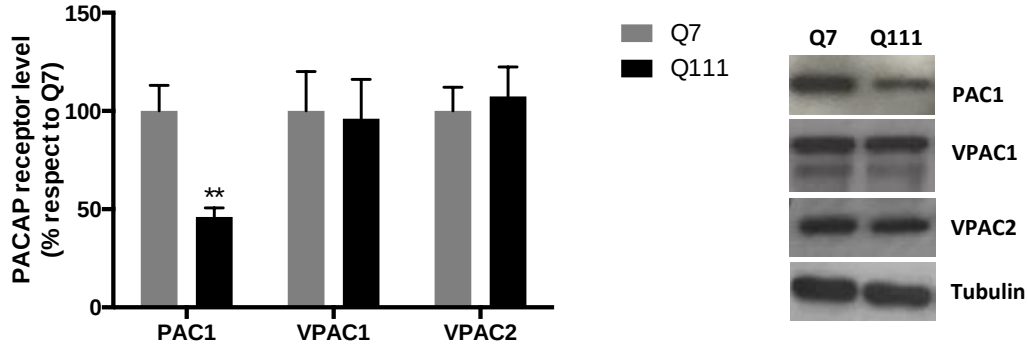


Figure 28: PAC1 receptor levels are reduced in STHdh$^{Q111/Q111}$ cells. PAC1, VPAC1 and VPAC2 protein levels were analysed by Western Blot of protein extracts obtained from STHdh$^{Q7/Q7}$ cells (Q7) and STHdh$^{Q111/Q111}$ cells (Q111). The results are expressed as the percentage of the ratio of PACAP receptors vs tubulin level. Statistical analysis was performed using Student's t-test Data are the mean ± SEM (n=5). **p<0.01; as compared to control cells. Representative immunoblots are shown.

Then, STHdh$^{Q7/Q7}$ cells were transfected with exon-1 htt plasmids, expressing either 16 or 94 polyQ repeats and tagged with GFP. As seen in Figure 29A, in cells transfected with GFP-exon-1-16Q htt presented a diffuse pattern with no aggregates formation, whereas cells transfected with GFP-exon-1-94Q showed the characteristic aggregates when exon-1 of mhtt is expressed. In parallel, protein level of PACAP receptors were analysed 48 h after transfection. PAC1, VPAC1 and VPAC2 receptors levels were reduced in cells transfected with GFP-exon-1-94Q plasmid respect to cells transfected with GFP-exon-1-16Q (Figure 29B). In the representative immunoblots, it is observed that the detection of htt by Western Blot showed the expected electrophoretic retardation in cells transfected with the GFP-exon-1-94Q respect to the cells transfected with GFP-exon-1-16Q due to the increase in the number of CAG repetitions.

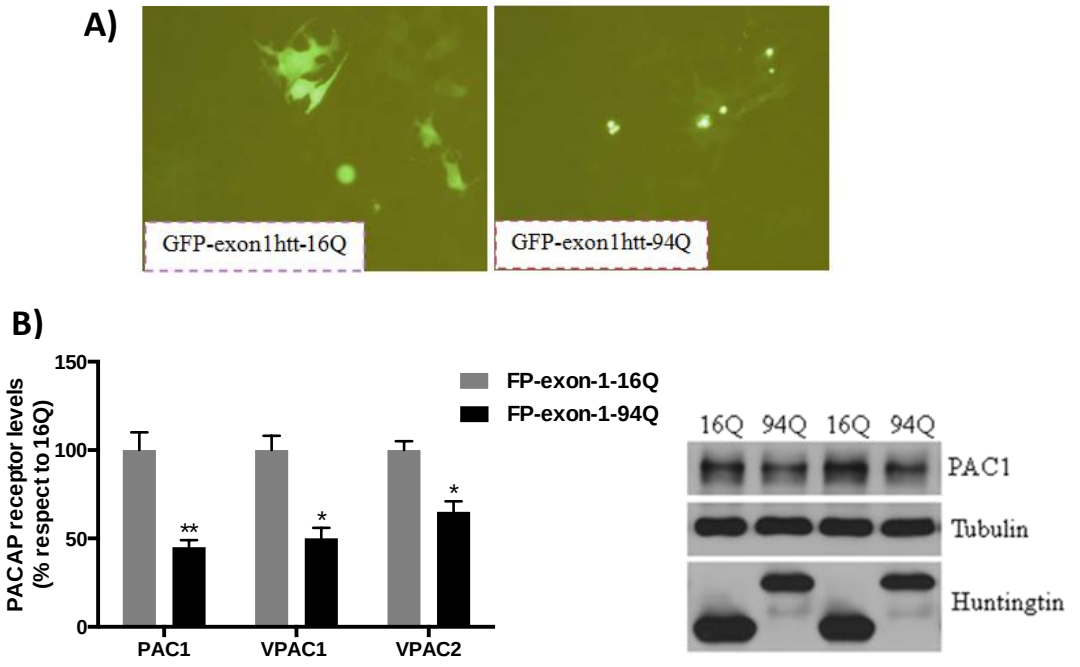


Figure 29: Expression of GFP-exon-1-94Q plasmid promotes a reduction in PACAP receptors level in STHdh$^{Q7/Q7}$ cells. A) Representative microphotograph of STHdh$^{Q7/Q7}$ cells transfected with GFP-exon-1-16Q or GFP-exon-1-94Q plasmid. B) Protein extracts were analysed by Western Blot to quantify PAC1, VPAC1 and VPAC2 levels. The results are expressed as the percentage of the ratio of PACAP receptors vs tubulin level. Data are the mean ± SEM (n=3). Statistical analysis was performed using Student's t-test. *p<0,05 and **p<0.01, as compared to GFP-exon1-16Q. Representative immunoblots are shown.

2.3 PACAP protects striatal cells against mhtt toxicity

To study the PACAP potential to exert a neuroprotector effect against mhtt toxicity, STHdh$^{Q7/Q7}$ and STHdh$^{Q111/Q111}$ cells were treated with PACAP at a concentration of 10^{-7}M according to previous data (Vaudry et al., 2000) and incubated for 24 h.

Assessment of cell viability study using MTT test showed that STHdhQ$^{111/Q111}$ cells present approximately a 50% reduction in cell viability compared to STHdh$^{Q7/Q7}$ cells (Figure 30). This significant decrease in cell viability is likely associated with the presence of mhtt. However, PACAP treatment (10^{-7}M) promoted STHdh$^{Q111/Q111}$ cell viability with an increase of 65% respect to non-treated STHdh$^{Q111/Q111}$ cells, with no effect on STHdh$^{Q7/Q7}$ cells (Figure 30).

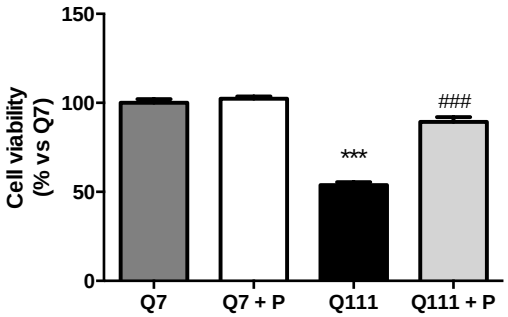


Figure 30: Effect of PACAP on cell viability in STHdh$^{Q7/Q7}$ (Q7) and STHdh$^{Q111/Q111}$ (Q111) cells. Statistical analysis was performed using two-way ANOVA followed by Bonferroni as post-hoc test. Data are the mean ± SEM (n=3). ***p<0.001 as compared to Q7 cells; ###p<0.001, as compared to STHdh$^{Q111/Q111}$ cells.

To know if this protective effect observed was related to blockade of apoptosis, cells were incubated in the presence or in the absence of 10^{-7}M PACAP and then stained with Hoechst33258. This assay is used to stain the nuclei in a specific manner because this colorant joins adenine and timine nucleotides with high affinity. Analysis of fluorescence microscopy images from STHdh$^{Q7/Q7}$ and STHdh$^{Q111/Q111}$ cells after treatment to quantify apoptotic cell death showed an increased of 35% in apoptotic cell death in STHdh$^{Q111/Q111}$ cells respect to STHdh$^{Q7/Q7}$. Nevertheless, PACAP treatment promotes a reduction in both, STHdh$^{Q7/Q7}$ and STHdh$^{Q111/Q111}$ cells (Figure 31B). Specifically, treated- STHdh$^{Q111/Q111}$ cells reduces in an approximately 35% respect to non-treated- STHdh$^{Q111/Q111}$ cells. This suggests that PACAP treatment can revert cytotoxicity caused by mhtt, making cells have a similar cell death as STHdh$^{Q7/Q7}$.

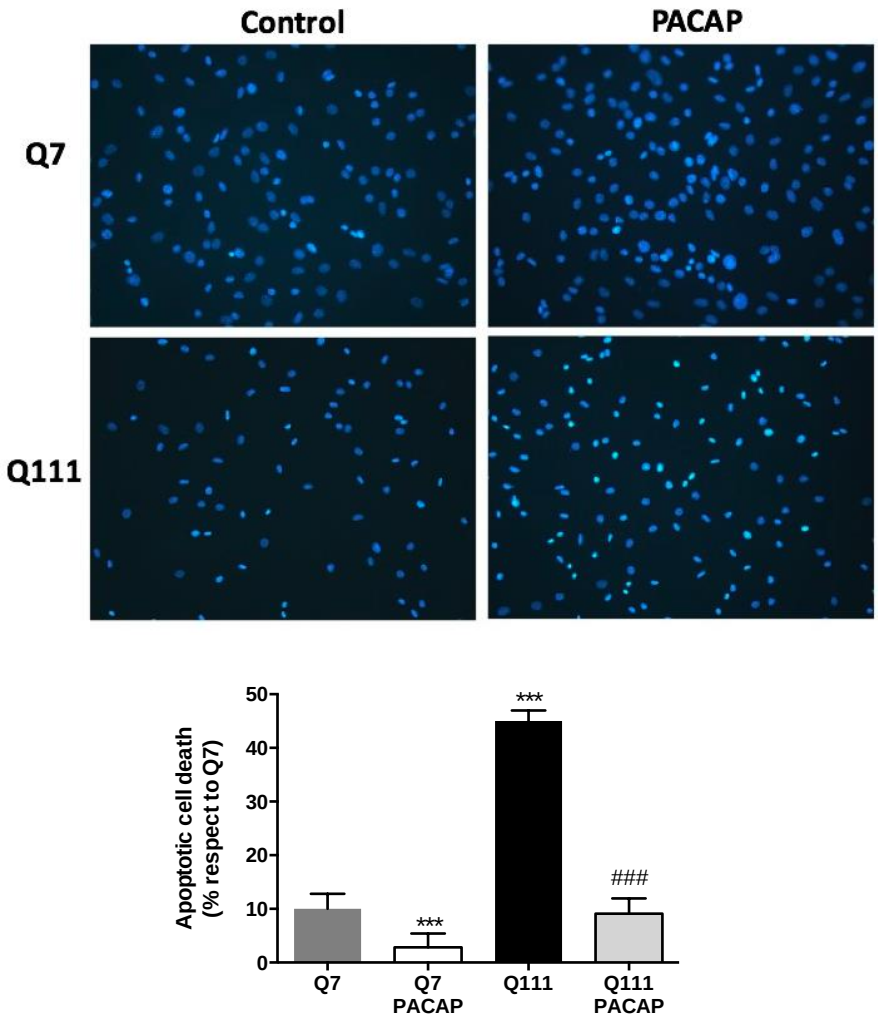


Figure 31: Effect of PACAP on cell survival in STHdh$^{Q7/Q7}$ (Q7) i STHdh$^{Q111/Q111}$ (Q111) cells wih Hoechst assay. Statistical analysis was performed using two-way ANOVA followed by Bonferroni as post-hoc test. Data are the mean ± SEM (n=3). Scale bar 10μm. ***p<0.001 as compared to STHdh$^{Q7/Q7}$ cells; ###p<0.001, as compared to STHdh$^{Q111/Q111}$ cells.

Caspases are crucial mediators of programmed cell death through apoptosis. Among them, caspase-3 is a protease that catalyses the specific cleavage of many cellular proteins. So, to corroborate the PACAP potential as a neuroprotector cleaved caspase-3 protein level was evaluated in STHdh$^{Q7/Q7}$ and STHdh$^{Q111/Q111}$ cells (Figure 32).

Cleaved caspase-3 levels in STHdh$^{Q111/Q111}$ cells were 15 fold higher than in STHdh$^{Q7/Q7}$ cells. Upon PACAP treatment, there was an 88% reduction of cleaved caspase-3 levels in in STHdh$^{Q111/Q111}$ cells respect to non-treated cultures. In STHdh$^{Q7/Q7}$ cells PACAP treatment reduced cleaved caspase-3 levels by 63% compared to control cultures (Figure 32).

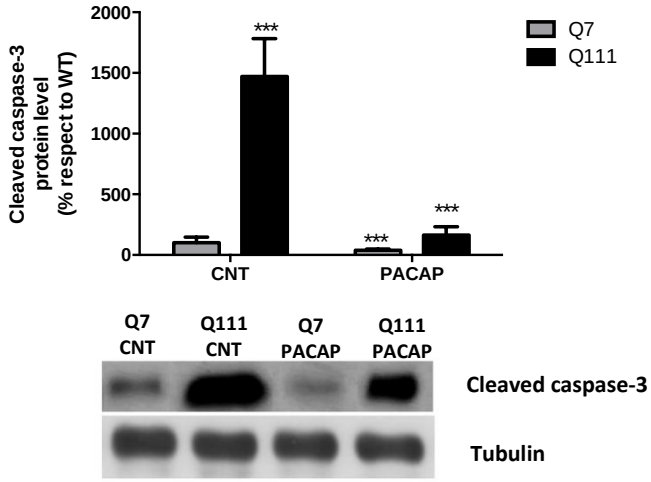


Figure 32: PACAP treatment reduces cleaved caspase-3 protein levels in STHdh cells. Quantification of Western Blot data was performed by densiometric analysis. The results are expressed as the percentage of ratio of each protein vs tubulin levels. Statistical analysis was performed using two-way ANOVA followed by Bonferroni as post-hoc test. Data are the mean ± SEM (n = 3). ***p<0.001 as compared to $STHdh^{Q7/Q7}$ cells; ###p<0.001, as compared to STHdhQ111/Q111 cells. Representative immunoblots are shown.

2.4 PACAP stimulates the expression of genes related to neuronal survival in cells expressing mhtt

PACAP is now considered to be a potent neuroprotective and general cytoprotective peptide with potential therapeutic use in numerous diseases. PACAP regulates the activation of transcription activating signalling pathways leading to the phosphorylation of many early growth-response genes such as c-fos and egr-1 (Vaudry et al., 2000). The activation of these transcription factors is known to induce pathways tightly related to neuronal survival (Lee et al., 2005).

For that reason, our next objective was to study if PACAP induced the expression of these genes in cells expressing mhtt. Well-known immediate early genes whose expression is induced by PACAP were analysed to establish the best treatment conditions. We found that treatment with 1 h induce the maxium effect on immediate early genes level.

Here, we analysed c-fos and egr-1 expression in $STHdh^{Q7/Q7}$ and $STHdh^{Q111/Q111}$ cells treated with vehicle or PACAP (10^{-7}M) to assess the transcriptional activity of PACAP in the presence of mhtt. After 1 h treatment c-fos and egr-1 protein levels were also

analysed in the same conditions and similar results were obtained. As shown in Figure 33, protein and mRNA c-fos and egr levels were reduced in non-treated STHdh$^{Q111/Q111}$ cells respect to non-treated STHdh$^{Q7/Q7}$ cells. Moreover, both cell lines presented increased levels of c-fos and egr-1 when treated with PACAP.

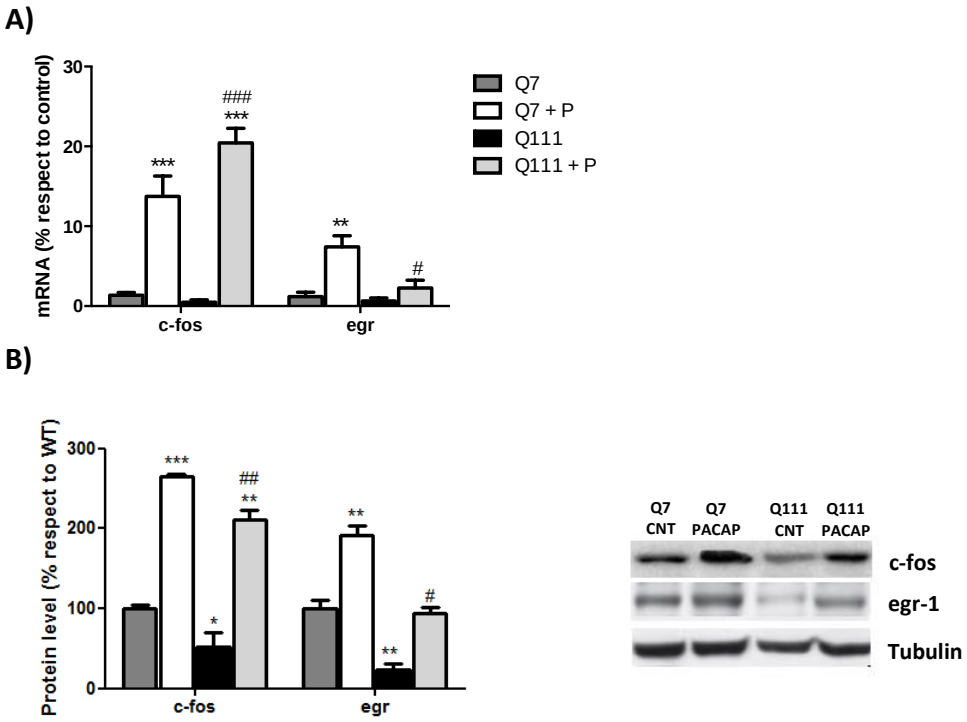


Figure 33: PACAP treatment increased c-fos and egr-1 expression in STHdh$^{Q7/Q7}$ and STHdh$^{Q111/Q111}$ cells. RT-PCR (A) and Western Blot (B) analysis performed in STHdhQ7/Q7 (Q7) and STHdhQ111/Q111 (Q111) treated or not with PACAP (10^{-7}M). mRNA quantifications were performed through RT-PCR. The results are expressed as the percentage of ratio of each protein vs GADPDH or tubulin levels. Statistical analysis was performed using two-way ANOVA followed by Bonferroni as post-hoc test. Data are the mean ± SEM. *p<0.05, **p<0.01 and ***p<0.001 when compared to WT; cells #p<0.05, ## p>0.01 and ###p<0.001 when compared to KI cells. Representative immunoblots are shown (B).

2.5 PACAP protective effects are PAC1-dependent

It has been described that PACAP has the ability to stimulate its own expression in an autocrine manner (Hashimoto et al., 2000). Moreover, PACAP treatment also influence PAC1 receptor stimulation in the mouse brain (Rat et al., 2011). Therefore, we examined *in vitro* whether PACAP treatment can affect PAC1 receptor expression. Our results confirmed previous finding. First, STHdh$^{Q111/Q111}$ cells expressed reduced levels of PAC1 mRNA respect to STHdh$^{Q7/Q7}$ (Figure 34A). Moreover, PAC1 mRNA level increased significantly after PACAP treatment in both STHdh$^{Q7/Q7}$ and STHdh$^{Q111/Q111}$ cells respect to their control. On the other hand, PACAP-treated STHdh$^{Q111/Q111}$ cells

showed increased protein receptor levels. Nevertheless, no differences were observed in receptor level between control and PACAP-treated STHdh$^{Q7/Q7}$ cells (Figure 34B).

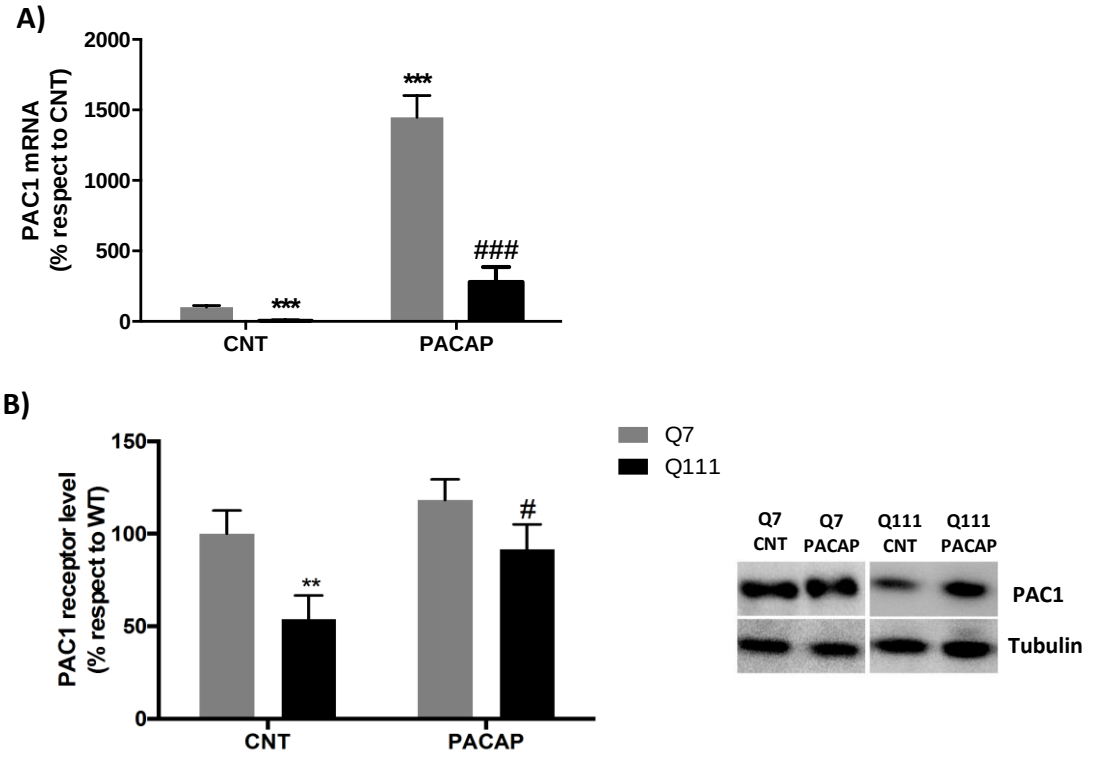


Figure 34: PACAP administration increased PAC1 mRNA and proten levels in STHdh$^{Q7/Q7}$ (Q7) and STHdh$^{Q111/Q111}$ (Q111) cells. mRNA quantifications were performed through RT-PCR. Quantification of Western Blot data was performed by densiometric analysis. The results are expressed as the percentage of ratio of each protein vs GADPDH or tubulin levels. Statistical analysis was performed using two-way ANOVA followed by Bonferroni as post-hoc test. Data are the mean ± SEM. **p<0.01 and ***p<0.001 as compared to WT cells; #<0.05 and ###p<0.001 as compared to KI cells. . Representative immunoblots are shown.

To confirm the involvement of PAC1 receptor in the PACAP neuroprotective effect, gene silencing was used. STHdh cells were transfected with siRNA specific for PAC1 (50 pmol) during 72 h. 24 h before transfection finished, cells were treated with PACAP (10^{-7}M). PAC1-specific siRNA treatment marked decrease PAC1 protein level (Figure 35) in both STHdhQ$^{111/Q111}$ and STHdh$^{Q7/Q7}$ cells. In addition, this reduction of PAC1 protein level was not recovered by PACAP treatment after siRNA treatment while the neuropeptide has the capacity to increase the expression of its own receptor.

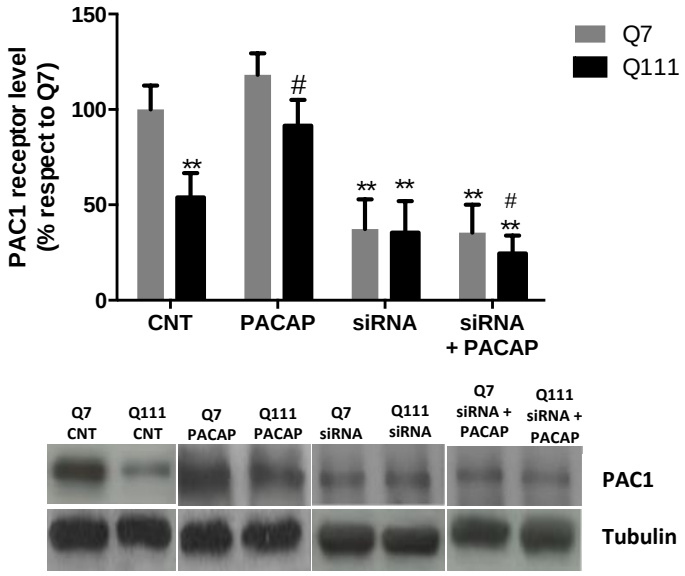


Figure 35: PAC1 receptor expression is increased in STHdh$^{Q111/Q111}$ after PACAP administration but not after siRNA specific for PAC1. STHdh$^{Q7/Q7}$ (Q7) and STHdh$^{Q111/Q111}$ (Q111) are transfected with siRNA (50 pmol) for 72 h. Then, treated with PACAP (10^{-7}M) for 24 h. Protein levels of PAC1 were measured by Western Blot in each group. The results are expressed as percentage of the ratio of PAC1 vs tubulin levels. Data shown are the mean mean ± SEM (n=3). Data were analysed by two-way ANOVA followed by Bonferroni as post-hoc test. **p<0.01 as compared to Q7 CNT and #p<0.05 as compared to Q111 CNT. Representative immunoblots are shown (B).

In addition, as PACAP abolished cleaved caspase-3 expression in both cell types, PAC1-specific siRNA treatment also decreases significantly cleaved caspase-3 expression in PACAP-treated STHdhQ$^{111/Q111}$ cells but not with the same potential as PACAP (Figure 36). On the other hand, PAC1-specific siRNA increases the expression of cleaved caspase-3 in STHdh$^{Q7/Q7}$ cells. Moreover, and consistent with figure 35 results, PAC1-specific siRNA treatment marked increase cleaved caspase-3 levels in STHdh$^{Q7/Q7}$ cells.

As said above, PACAP has affinity not only for PAC1 but also for VPAC1 and VPAC2 receptors which are more correlated to exert beneficial effects on inflammation and in immune responses (Abad et al., 2002; Delgado et al., 2003); nevertheless, some studies have shown the ability of this peptide to prevent neuronal death (Dejda et al., 2005).

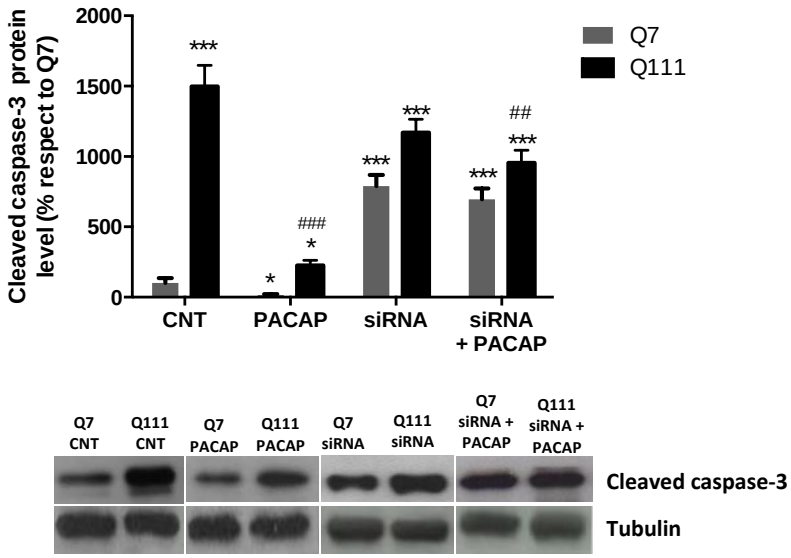


Figure 36: Cleaved caspase-3 protein level is reduced by PACAP administration but not after siRNA specific for PAC1. STHdh$^{Q7/Q7}$ (WT) and STHdh$^{Q111/Q111}$ (KI) were transfected with siRNA (50 pmol) for 72 h. Then, treated with PACAP (10^{-7}M) for 24 h. Protein levels of cleaved caspase-3 were measured by Western Blot in each group. The results are expressed as percentage of the ratio of cleaved caspase-3 vs tubulin levels. Data shown are the mean mean ± SEM (n=3). Data were analysed by two-way ANOVA followed by Bonferroni as post-hoc test. *p<0.05, ***p<0.001 as compared to Q7 CNT and ##p<0.05 and ##0.01 as compared to Q111 CNT. Representative immunoblots are shown.

2.6 Intranasal administration of PACAP improve motor performance in R6/1 mice

The motor dysfunction seen in HD has been linked to corticostriatal signalling pathways disruption (Ferrante et al., 1991). As PAC1 receptor was reduced in the striatum of R6/1 mice, and PACAP treatment increased transcriptional activity in striatal cells *in vitro*, we sought to analyse the ability of PACAP activation to ameliorate motor symptoms in HD. Thus, 18-week-old WT and R6/1 mice were treated intranasally with PBS as vehicle or PACAP (30 µg/kg) for 7 days. Then, animals were subjected to rotarod performance test and balance beam to study motor balance and coordination. The performance of all groups on the balance beam task at 18 weeks of age by measuring the covered distance and the number of slips were studied. Our data showed that PACAP-treated R6/1 mice performed significantly better than the vehicle-treated R6/1 group in all the parameters analysed (Figure 37). No changes were observed between vehicle and PACAP-treated WT mice.

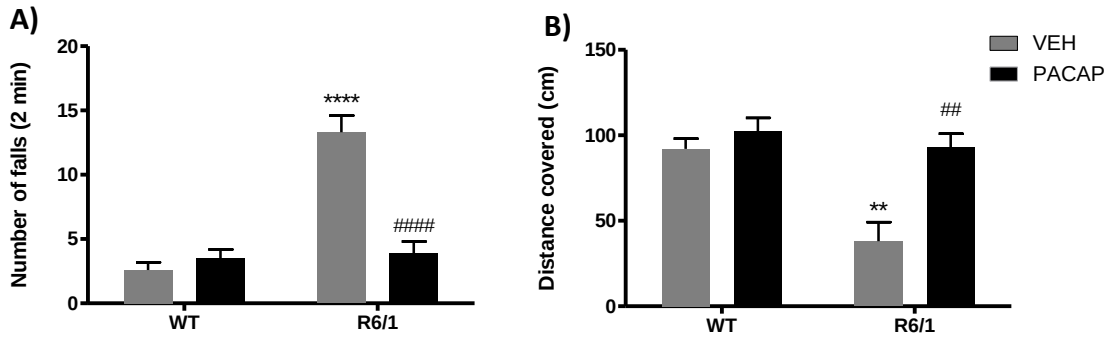


Figure 37: PACAP treatment decreases R6/1 mice deficits in motor coordination and balance. At 18 weeks of age, WT and R6/1 mice were treated with PACAP (30µg/kg) or vehicle (PBS). The balance beam task was performed after 7 days treatment. The following measurements were recorded: A) number of falls and B) distance covered. Data are expressed as mean ± SEM (n=5-7/group). Data were analysed by two-way ANOVA followed by Bonferroni as post-hoc test. **p<0.01 and ****p<0.0001 as compared to WT vehicle-mice and ##p<0.01 and ####p<0.0001 as compared to R6/1-vehicle mice.

To further characterise the motor improvement induced by PACAP, we evaluated that rotarod performance at 16 and 24 rpm by determing the number of falls in all experimental group. Interestingly, treatment with PACAP significantly ameliorated motor coordination in R6/1 mice assessed at 16 rpm and 24 rpm. No significant changes were observed between vehicle and PACAP-treated WT mice at any speed. Altogether, we conclude that the intranasal administration of PACAP improves motor function in R6/1 mice (Figure 38).

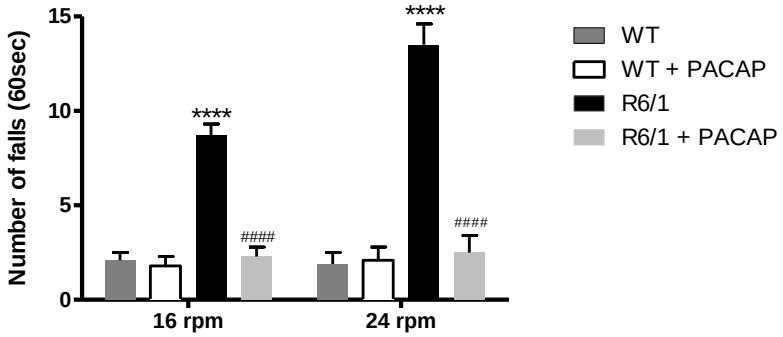


Figure 38 : PACAP administration in R6/1 mice improves the performance in the rotarod task. At 18 weeks of age, WT and R6/1 mice were treated with PACAP (30 µg/kg) or vehicle (PBS). The rotarod task was performed after 7 days treatment. Values represent the number of falls within 60 seconds. Data are expressed as mean ± SEM (n=5-7/group). Data were analysed by two-way ANOVA followed by Bonferroni as post-hoc test. ****p<0.0001 as compared to WT vehicle-mice and ####p<0.0001 as compared to R6/1-vehicle mice.

2.7 PACAP administration improves PAC1 levels and reduces cleaved caspase-3 cleavage in the striatum of R6/1 mice

To know if PACAP has the ability to promote the expression of its own receptor also in the striatum, PAC1 receptor protein levels were analysed in WT and R6/1 mice after 7 days of PACAP treatment. In accordance with Figure 39, the protein levels of PAC1 were reduced in vehicle-treated R6/1 mice compared to vehicle-treated WT mice. On the other hand, PACAP treatment induced the expression of its own receptor in R6/1 mice but not in WT mice, indicating, as in the hippocampus, a specific effect of PACAP in R6/1 mice (Figure 39).

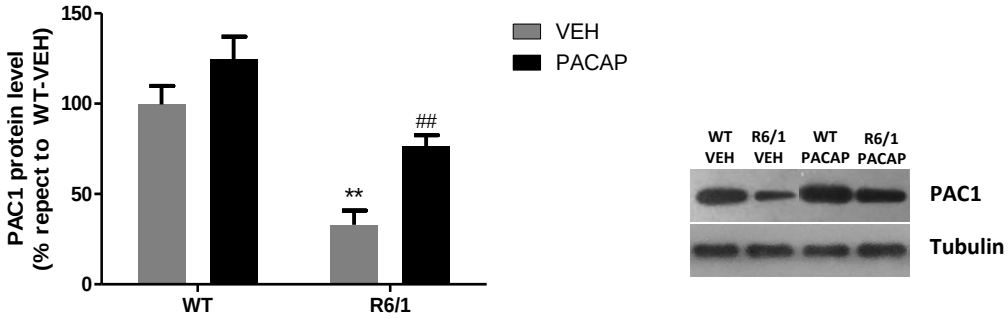


Figure 39: PAC1 receptor expression is increased by PACAP administration. At 18 weeks of age, WT and R6/1 mice were treated with PACAP (30 µg/kg) or vehicle (PBS) for 7 days. Then, protein extracts from the striatum were subjected to Western Blot to analyse PAC1 receptor level. Representative immunoblots are presented. Data are expressed as a percentage with respect to WT vehicle-treated mice. Results are mean ± SEM (n=5-7/group) and represent the ratio between PAC1 and tubulin levels Data were analysed by two-way ANOVA followed by Bonferroni as post-hoc test. **p<0.01 as compared to WT vehicle-mice and ##p<0.01 as compared to R6/1-vehicle mice.

No cell death in R6/1 mice striatum, however, to verify the neuroprotective role of PACAP in R6/1 mice model, we studied the levels of cleaved caspase-3, a pro-apoptotic protein known to be regulated by PACAP. These results agree with the results obtained in STHdh model (Figure 32). PACAP treatment significantly reduced cleaved caspase-3 levels in both genotypes (Figure 40).

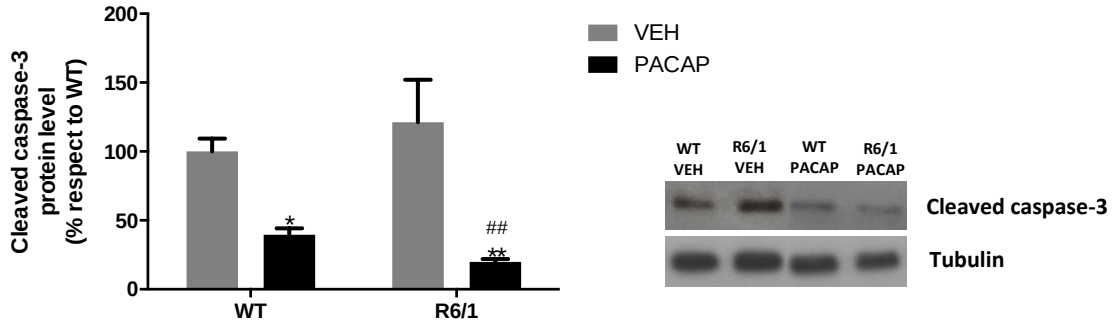


Figure 40: Cleaved caspase-3 receptor expression are decreased by PACAP administration. At 18 weeks of age, WT and R6/1 mice were treated with PACAP (30 μg/kg) or vehicle (PBS) for 7 days. Then, protein extracts from the striatum of WT and R6/1 mice treated with vehicle or PACAP were subjected to Western Blot to analyse cleaved caspase-3 protein level. Representative immunoblots are presented. Data are expressed as a percentage with respect to WT vehicle-treated mice. Results are mean ±SEM (n=5-7/group) and represents the ratio between PAC1 and tubulin levels. Data were analysed by two-way ANOVA followed by Bonferroni as post-hoc test. ***p<0.001 and **p<0.01 as compared to WT vehicle-mice and ###p<0.0001 as compared to R6/1-vehicle mice.

2.8 Intranasal administration of PACAP increases CBP-BDNF protein levels in the striatum of WT and R6/1 mice.

In human samples, BDNF protein and mRNA levels in fronto-paritetal cortex are decreased (Zuccato & Cattaneo, 2009). Reduced levels of cortical and striatal BDNF have also been reported in different mouse models of HD (Zuccato & Cattaneo, 2007; Gines et al., 2003; Luthi-Carter et al., 2003).

Since the neuroprotective effect of PACAP is indirectly mediated by BDNF release (Shintani et al., 2005), we next analysed whether improvements seen in R6/1 mice treated with PACAP correlated with changes in striatal BDNF expression. Figure 41 shows BDNF levels at 18 weeks in striatum of WT and R6/1 mice after PACAP treatment. The vehicle-treated R6/1 mice showed reduced BDNF. Interestingly, the intranasal administration of PACAP increased BDNF levels in the striatum of WT mice and rescued BDNF levels in R6/1 mice.

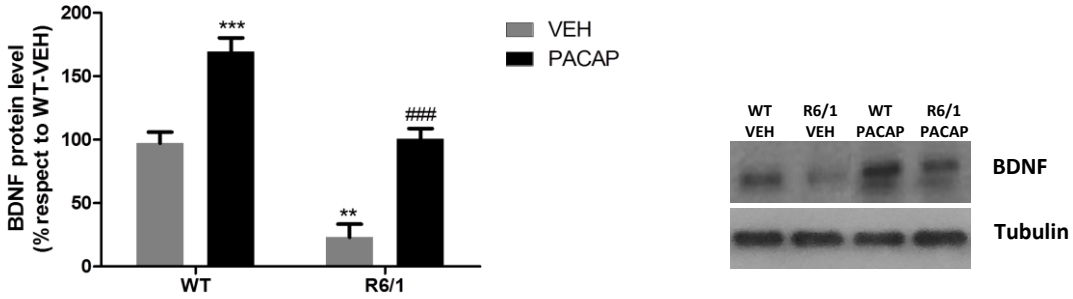


Figure 41: Effect of PACAP treatment on CBP-BDNF expression in the brain of R6/1 mice. WT and R6/1 mice were intranasally treated from 18 weeks of age with PACAP (30 µg/kg/day) or vehicle (VEH) for 7 days. Then, protein extracts from the striatum of all groups were subjected to Western blot to study BDNF protein levels. Results are expressed as the percentage of the ratio between BDNF and tubulin levels and represent the mean ± SEM (n=6/group). Data were analysed by two-way ANOVA followed by Bonferroni as post-hoc test. **p<0.01 and ***p<0.001 as compared to WT vehicle-mice and ###p<0.001 as compared to R6/1-vehicle mice.

BDNF expression is regulated by CREB-dependent transcription factor. CBP/CREB signalling is crucial for long-lasting improvements in HD (Zuccato et al., 2010; Gines et al., 2003). To study if BDNF levels are increased due to CBP transcription and reinforce the idea of a beneficial effect of increasing gene expression mediated by CBP/CREB, CBP protein levels were analysed in the striatum of PACAP-treated WT and R6/1 mice. The vehicle-treated R6/1 mice showed reduced CBP protein levels. Interestingly, the intranasal administration of PACAP rescued CBP levels in the striatum of R6/1 mice. No changes in CBP levels were observed in WT mice treated with PACAP when compared to vehicle-treated WT mice (Figure 42).

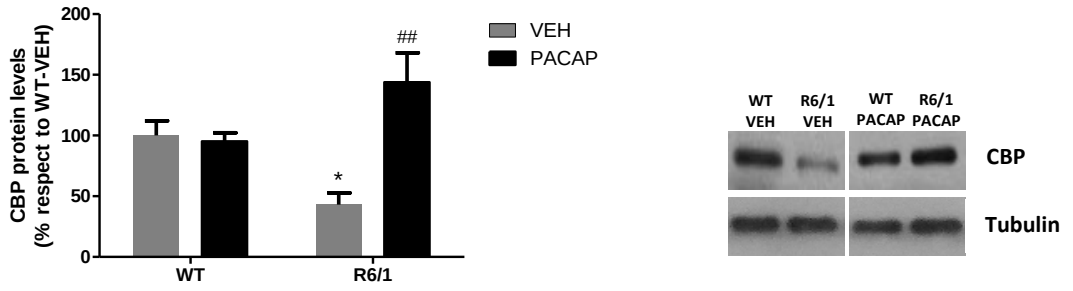


Figure 42: Intranasal administration of PACAP increases CBP protein levels in the striatum of R6/1 mice. WT and R6/1 mice were intranasally treated from 18 weeks of age with PACAP (30 µg/kg/day) or vehicle (VEH) for 7 days. Then, protein extracts from the striatum of all groups were subjected to Western blot to study CBP protein levels. Results are expressed as the percentage of the ratio between CBP and tubulin levels and represent the mean ± SEM (n=6/group). Data were analysed by two-way ANOVA followed by Bonferroni as post-hoc test. *p<0.05 as compared to WT-vehicle mice and ##p<0.01 as compared to R6/1-vehicle mice. Representative immunoblots are presented.

3. EGCG improves motor and cognitive deficits in HD. Possible role of FASN

3.1 EGCG administration protects against neuronal degeneration in STHdh cells

To evaluate the neuroprotective effect of EGCG *in vitro*, in the presence of mhtt, we performed a concentration-response study and analysed viability using the MTT assay. A range of concentration of EGCG between 0 and 100 μM were used to state the best EGCG concentration (Table 8). Cultures were incubated with EGCG for 48 h, followed by analysis of cell viability. Viability percentage has been normalised with data obtained of $STHdh^{Q7/Q7}$ cells. $STHdh^{Q111/Q111}$ cells showed a significant reduction of viability (52.55% ± 9.44) respect to $STHdh^{Q7/Q7}$ cells (P = 0.037), which reinforce mhtt toxicity. EGCG treatment increases viability in $STHdh^{Q111/Q111}$ cells with a maximum at 40 μM. In $STHdh^{Q7/Q7}$ cells, difference between 50 and 100 μM is only 3.14% and the viability remained above 100% survival. In $STHdh^{Q111/Q111}$ cells the difference is 7.76% representing an 80% of viabity. These results corroborate the studies that have shown that green tea polyphenols display a concentration-dependent window of neuroprotective action. At low micromolar concentrations polyphenols protect, whereas they become proapoptotic when increasing their concentrations (Levites et al., 2002). To discard a toxic effect of EGCG already describes in previous studies at the highest con centrations (Weinreb et al., 2003), 40 μM EGCG was considered as the optimal concentration.

Table 8: Concentration-response study with EGCG in $STHdh^{Q7/Q7}$ and $STHdh^{Q111/Q111}$ cells.

EGCG concentration	Cell viability $STHdh^{Q7/Q7}$ cells (%)	Cell Viability $STHdh^{Q111/Q111}$ cells (%)
Control	100 ± 2.33	52.55 ± 9.44
30 μM	121.59 ± 1.75	133.33 ± 7.93
40 μM	110.44 ± 9.61	137.96 ± 9.44
50 μM	109.44 ± 7.31	84.02 ± 10.01
100 μM	106.30 ± 8.31	76.26 ± 6.33

In Figure 43 is shown cell viability at 40 μM. $STHdh^{Q111/Q111}$ cells present approximately 50% less viability. However, EGCG treatment (40 μM) causes an increase of cell

viability compared to control STHdh$^{Q111/Q111}$ cells, and showing similar level to control STHdh$^{Q7/Q7}$ cells. No significant changes were seen in STHdh$^{Q7/Q7}$ cells.

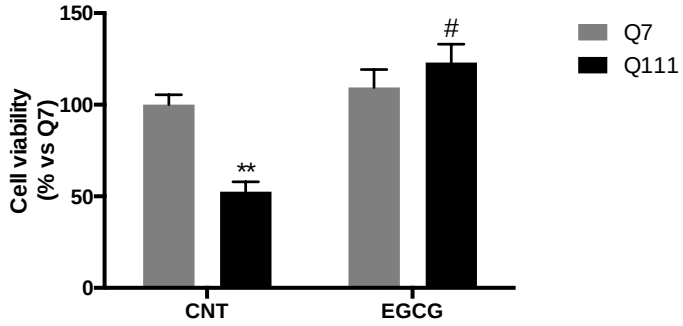
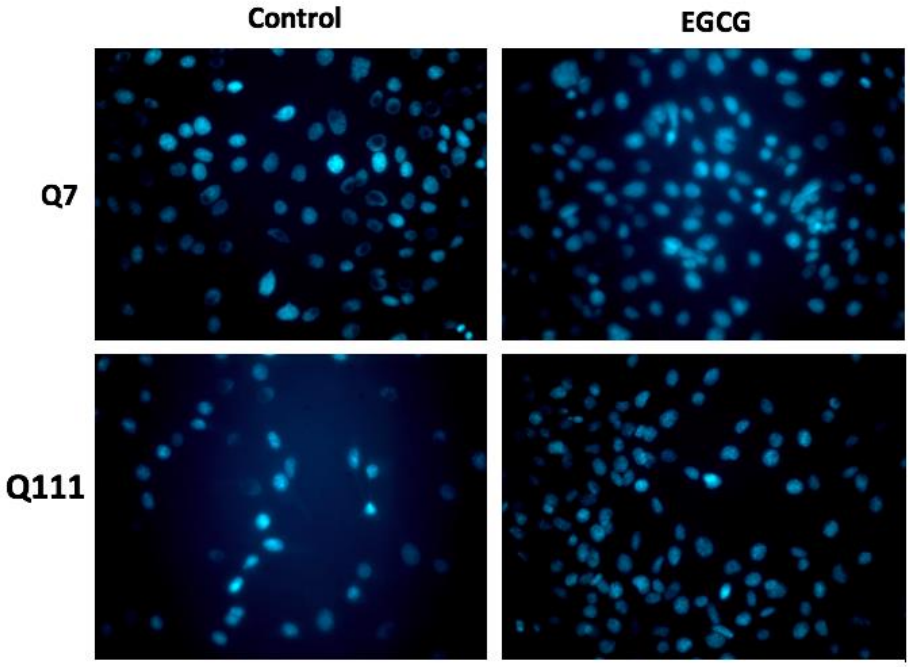


Figure 43: Effect on EGCG on STHdh$^{Q7/Q7}$ (Q7) and STHdh$^{Q111/Q111}$ (Q111) cells at 40 µM. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's as post-hoc test. Data are the mean ± SEM (n=3). **p<0.01 as compared to Q7 CNT cells; #p<0.05, as compared to Q111 CNT cells.

To know if the protective effect of EGCG was through inhibition of apoptosis, the cells were incubated with Hoechst33258 after treatment with 40 µM EGCG during 48 h. Analysis of fluorescence microscopy images of control and EGCG-treated cells showed an increase in the cell survival of STHdh$^{Q111/Q111}$ cells respect to STHdh$^{Q7/Q7}$ that was promoted by EGCG treatment. (Figure 44). This suggests that EGCG treatment can reduce the toxicity seen in HD, making cells have a similar viability as STHdh$^{Q7/Q7}$.



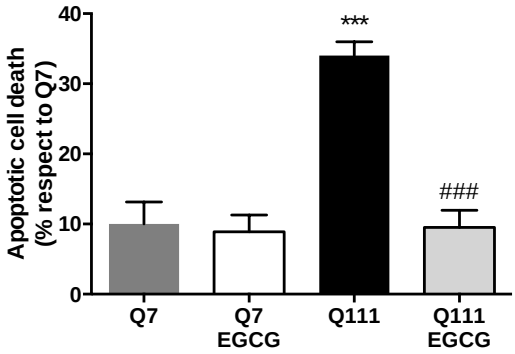


Figure 44: Effect of EGCG on cell survival in STHdh$^{Q7/Q7}$ (Q7) and STHdh$^{Q111/Q111}$ (Q111). Statistical analysis was performed using two-way ANOVA followed by Bonferroni's as post-hoc test. Scale bar 10μm. Data are the mean ± SEM. ***p<0.001 as compared to Q7 cells; ###p<0.001, as compared to Q111 cells.

Cleaved caspase-3 protein levels were also examined due to its key role in cell death. The study was performed in STHdh$^{Q7/Q7}$ and STHdh$^{Q111/Q111}$ cells with 40 μM EGCG for 48 h treatment.

As seen in figure 32, cleaved caspase-3 levels were significantly higher in STHdh$^{Q111/Q111}$ cells compared to STHdh$^{Q7/Q7}$ cells. After EGCG treatment (40 μM, 48 h), cleaved caspase-3 levels were notable reduced in STHdh$^{Q7/Q7}$ cells (approximately 60%) and in STHdh$^{Q111/Q111}$ cells (approximately 50%) respect to controls (Figure 45).

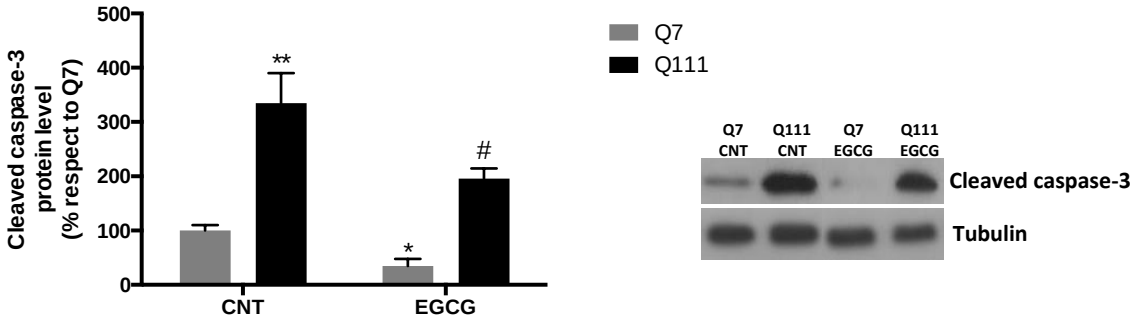


Figure 45: EGCG treatment reduces cleaved caspase-3 protein levels in STHdh$^{Q7/Q7}$ (Q7) and STHdh$^{Q111/Q111}$ (Q111) cells. The results are expressed as the percentage of ratio of each protein vs tubulin levels. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's as post-hoc test. Data are the mean ± SEM (n=3). p*<0.05 and **p<0.01 as compared to Q7 CNT cells; #p<0.05, as compared to Q111 CNT cells. Representative immunoblots are shown.

3.2 FASN protein levels are increased in STHdhQ$^{111/Q111}$ cells and EGCG treatment reduces FASN levels.

Inhibition of FASN has been implicated in EGCG protective effects in non-neuronal cells (Xifró et al., 2015). Thus, we investigated FASN levels in STHdh cells treated with EGCG for 48 h. Results show a significant increase in FASN levels in STHdh$^{Q111/Q111}$ cells respect to STHdh$^{Q7/Q7}$ cells (Figure 46).

In order to verify that EGCG protective effects in STHdh cells were due to blocking FASN enzyme, gene silencing against FASN was used. STHdh cells were transfected with siRNA specific for FASN (30 pmol) and 72 h later FASN-specific siRNA treatment marked decrease FASN protein level (Figure 46) in both, STHdhQ$^{111/Q111}$ and STHdh$^{Q7/Q7}$ cells. In addition, this reduction of FASN protein level was similar to the effect of EGCG to this enzyme. Combined treatment produced the same effect.

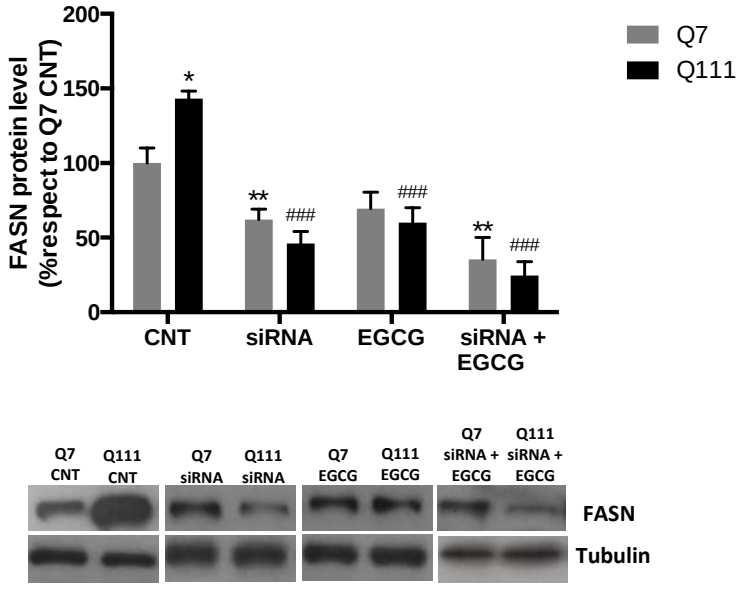


Figure 46: FASN expression is reduced after siRNA specific for FASN and after EGCG treatment. STHdh$^{Q7/Q7}$ (Q7) and STHdh$^{Q111/Q111}$ (Q111) are transfected with siRNA (30 pmol) for 72 h or treated with EGCG for 48 h. Protein levels of FASN were measured by Western Blot in each group. The results are expressed as percentage of the ratio between FASN and tubulin levels. Data shown are the mean mean ± SEM (n=3). Data were analysed by two-way ANOVA followed by Bonferroni as post-hoc test. *<p0.05 and **p<0.01 as compared to Q7 CNT and ###p<0.001 as compared to Q111 CNT. Representative immunoblots are shown.

In addition, EGCG abolished cleaved caspase-3 expression in STHdhQ$^{111/Q111}$ cells (Figure 47), thus, we want to know if this effect may be done through FASN inhibition. FASN-specific siRNA treatment also decreases significantly cleaved caspase-3

expression in STHdhQ$^{111/Q111}$ cells but not with the same potential as EGCG. As said above, EGCG act not only through FASN inhibition, it has lot of targets that can give the capacity of EGCG to exert its pleiotropic actions.

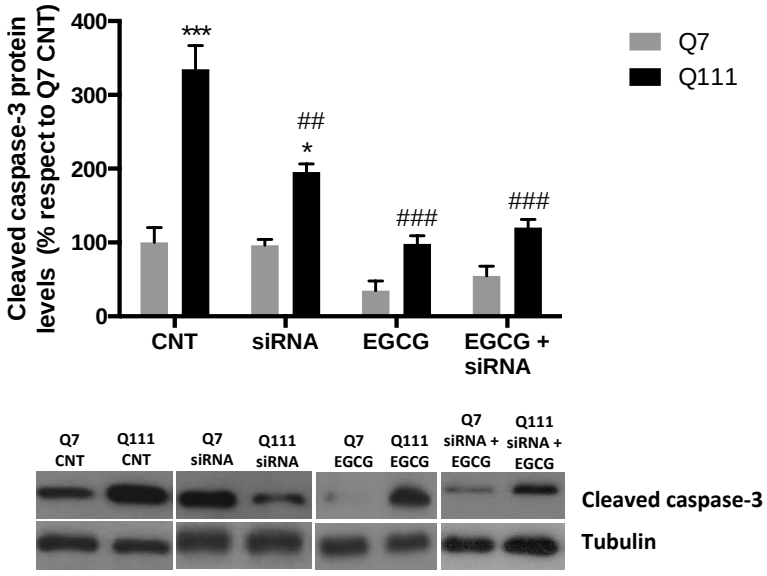


Figure 47: Cleaved caspase-3 protein level is reduced after siRNA specific for FASN. STHdh$^{Q7/Q7}$ (Q7) and STHdh$^{Q111/Q111}$ (Q111) are transfected with siRNA (30 pmol) for 72 h. Protein levels of cleaved caspase-3 were measured by Western Blot in each group. The results are expressed as percentage of the ratio obetween cleaved caspase-3 and tubulin levels. Data shown are the mean mean ± SEM (n=3). Data were analysed by two-way ANOVA followed by Bonferroni as post-hoc test. *p<0.05 and ***p<0.001 as compared to Q7 CNT and ###p<0.001 as compared to Q111 CNT. Representative immunoblots are shown.

3.3 EGCG improves motor coordination in the R6/1 mouse model

To address whether EGCG treatment could improve the motor phenotype of R6/1 mice PBS, as vehicle, or EGCG (40 mg/kg) were daily intraperitoneally administrated at 14 weeks of age during two weeks. Motor coordination was assessed at 16 weeks of age by using the balance beam task measuring the distance covered on the beam (Figure 48A) and the number of slips (Figure 48B). The vehicle R6/1 mice showed a reduction on the distance covered and an increase in the number of slips compared to WT mice. The administration of EGCG induced a significant amelioration in motor coordination in R6/1 mice, increasing the distance covered and reducing the number of slips. No differences were observed between vehicle-WT and EGCG-WT groups (Figure 48A,B). Then, we evaluated the clasping phenotype. The treatment with EGCG drastically reduced the clasping phenotype in R6/1 mice (Figure 48C).

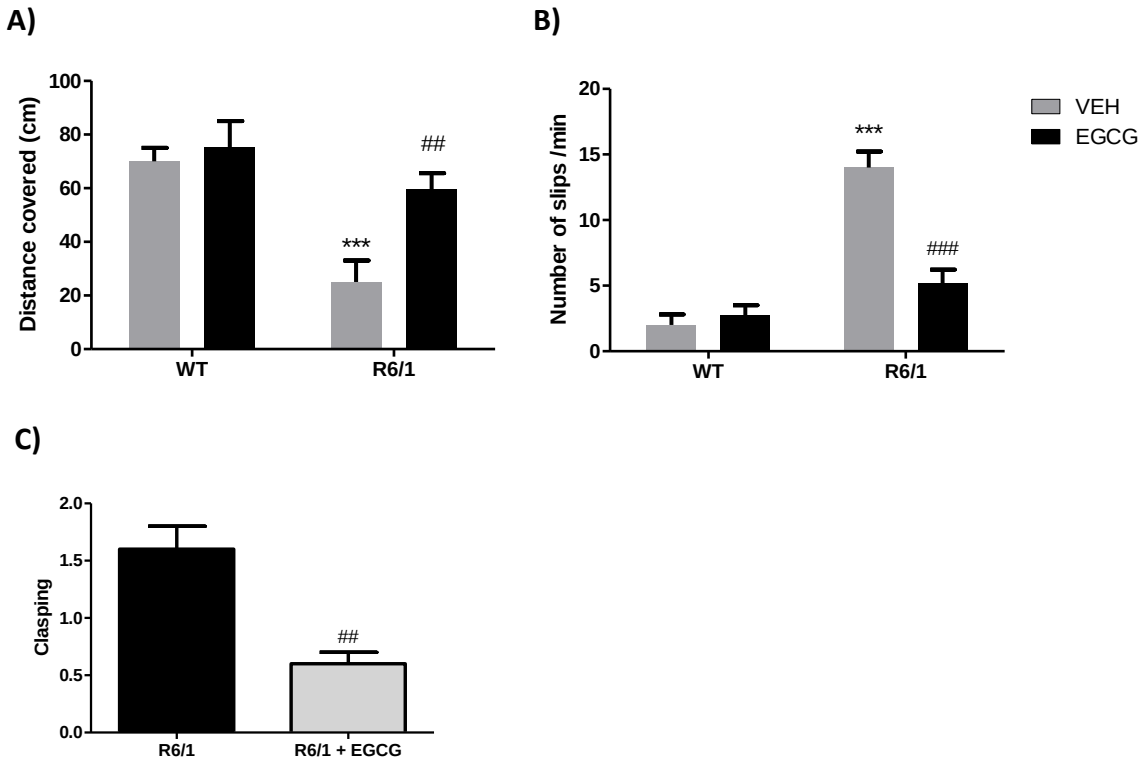


Figure 48: Motor coordination improvement in R6/1 mice treated with EGCG. WT and R6/1 mice were daily intraperitoneally injected with vehicle or EGCG starting at 14 weeks of age during 2 weeks. At 16 weeks of age, the four groups of animals were evaluated by balance beam measuring the distance covered (A) and the number of slips (B). At the same age clasping was evaluated in vehicle-mice and EGCG-treated R6/1 mice (C), as indicated in methods. The graphs represent the mean ± SEM (n=5). In balance beam task, statistical analysis was performed using two-way ANOVA followed by Bonferroni's as post-hoc test. ***p<0.001 when comparing to vehicle-WT, ##p<0.01 and ###p<0.001 when comparing to vehicle-R6/1 mice. In clasping statistical analysis was performed using Student t-test ##p< 0.01 as compared to vehicle-R6/1 mice.

3.4 Intraperitoneal administration of EGCG ameliorates memory deficits in R6/1 mice

Then, we want to study if EGCG has also the ability to improve cognitive functions in R6/1 mice. To this aim, WT and R6/1 mice were treated intraperitoneally with PBS as vehicle or EGCG (40 mg/kg) at 12 weeks of age for 15 days. Then, animals were subjected to NORT and NOLT to evaluate LTM. In the NORT, no differences were observed between vehicle and EGCG-treated WT mice as both groups exhibited preference for the novel object without differencies between them (Figure 49A). In contrast, vehicle-treated R6/1 mice did not recognize the old respect to the new object resulting in a lower object recognition index when compared to vehicle-treated WT mice (Figure 49A). Remarkably, the recognition index of EGCG-treated R6/1 mice was

significantly improved compared to vehicle-treated R6/1 mice and similar to WT animals (Figure 49A).

Additionaly, we next studied the LTM using the NOLT. Interestingly, the results obtained with NOLT were similar as in NORT. The recognition index of vehicle-treated R6/1 mice was lower than the one of vehicle-treated WT mice and treatment of R6/1 mice with EGCG induced an increase of recognition index compared to vehicle-treated R6/1 mice (Figure 49B).

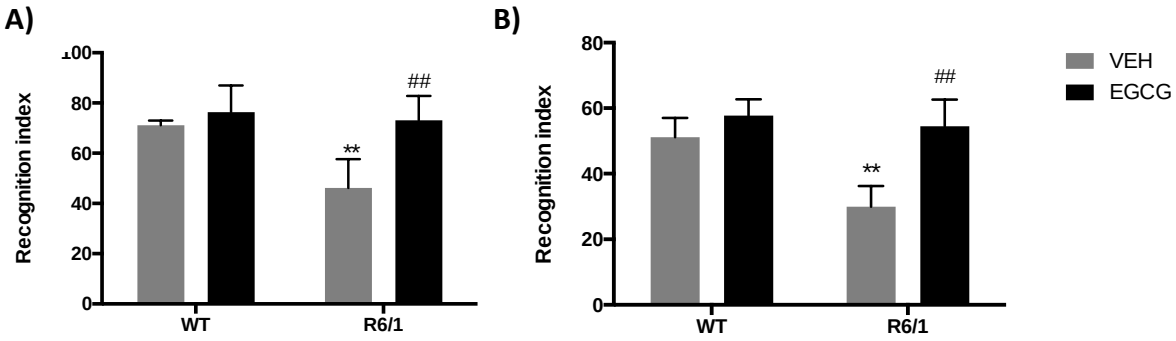


Figure 49: Intraperitoneal administration of EGCG improves the LTM in R6/1 mice. WT and R6/1 mice were intraperitoneally treated with EGCG (40 mg/kg) or vehicle from 12 weeks of age. Fifteen days later, mice were subjected to the NORT (A) and NOLT (B). (A) In the both tasks, all mice spent similar time exploring both objects during the training session. LTM was assessed 24 h later. Bars are shown as mean ± SEM of 10-12 animals/group. Recognition index represents the percentage of new object preference. Statistical analysis was performed using two-way ANOVA followed by Bonferroni as post-hoc test. **p<0.01 as compared to WT-vehicle mice and ##p<0.01 as compared to R6/1-vehicle mice**.**

3.5 FASN is up-regulated in striatum and cortex of HD mouse model

Since EGCG has been shown to inhibit FASN in non-neuronal cells, we wanted to asses whether the beneficial effects were through inhibition of FASN. First, we characterised FASN expression in the bran regions implicated in motor coordination and affected in HD. Western Blot analysis revealed a significant increase in FASN protein levels in the striatum of R6/1 mice compared to WT mice at 8 weeks of age (asymptomatic stage) and this up-regulation was maintained at 12 weeks of age (presymptomatic stage). No changes were detected at 20 weeks of age, whereas a decrease in FASN levels was observed at 30 weeks of age (Figure 50A). Similarly, a byphasic pattern of FASN expression was also found in the cortex of HD mice compared to WT animals. There

were no differencies at 8 weeks, higher levels of FASN were detected at 12 and 20 weeks of age followed by a reduction at 30 weeks of age. (Figure 50B)

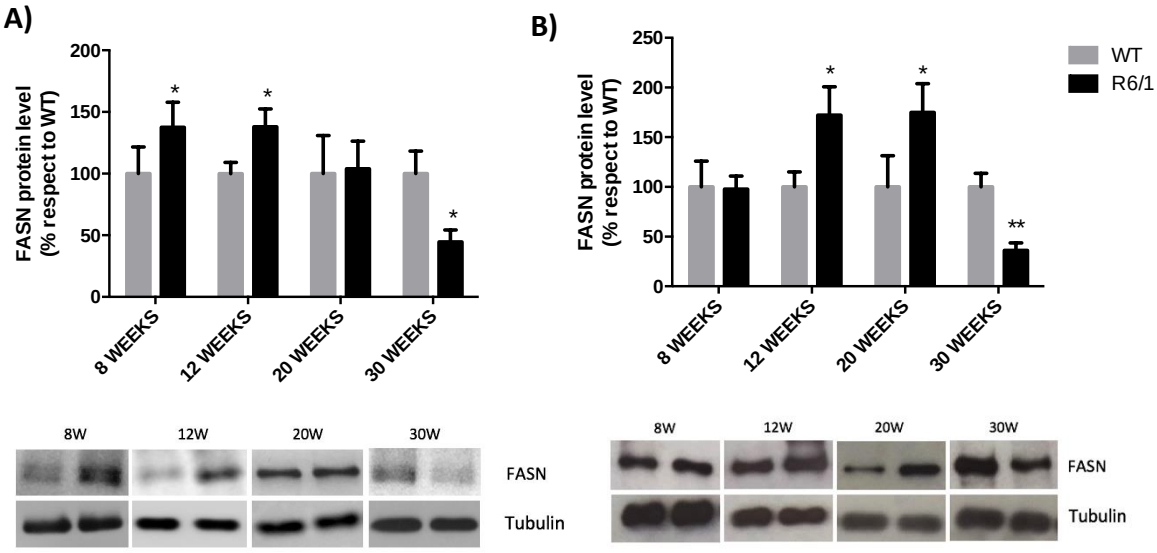


Figure 50: Upregulation of FASN in the striatum and cortex of R6/1 mice at key stages of HD. Striatal (A) and cortical (B) samples from WT and R6/1 mice at different ages were processed for Western Blot analysis of FASN. The results are expressed as the percentage of the ratio between FASN and tubulin levels at each age. Data shown are the mean ± SEM (6 animals/group). Results were analysed by unpaired Student's t-test. *p<0.05 and **p<0.01 as compared to age-matched WT mice. Representative immunoblots are shown.

3.6 Striatal and cortical FASN activity is increased in R6/1 mice.

To know whether increased levels of FASN were accompained by enhanced activity, we performed a FASN activity assay in the striatum and cerebral cortex of 12 week-old WT and R6/1 mice. As shown in figure 51, we found increased levels of FASN activity in the striatum and cerebral cortex of R6/1 mice compared to WT mice.

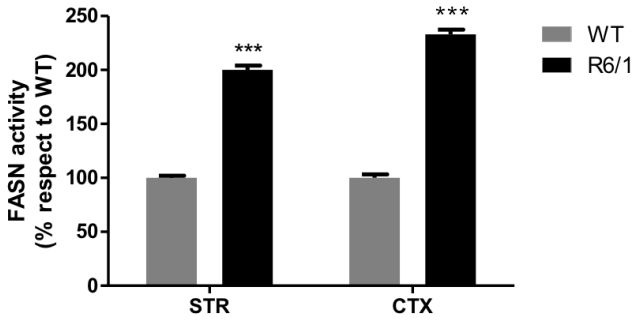


Figure 51: FASN activity is increased in the striatum and cortex of R6/1 mice. The FASN activity was analysed in the striatum (STR) and cortex (CTX) of WT and R6/1 mice at 12 weeks of age. Data were expressed as a percentage with respect to WT mice and represent the mean ± SEM (n = 4). Data were analysed by Student t-test. ***p<0.001 compared to WT.

3.7 FASN levels are also increased in the putamen and cerebral cortex of human *post-mortem* HD samples

FASN levels were also analysed in *post-mortem* human brain samples from the putamen and cerebral cortex of control and HD patients. The results revealed increased FASN protein levels in the putamen (Figure 52A) and cortex (Figure 52B) of HD patients compared to samples from control individuals.

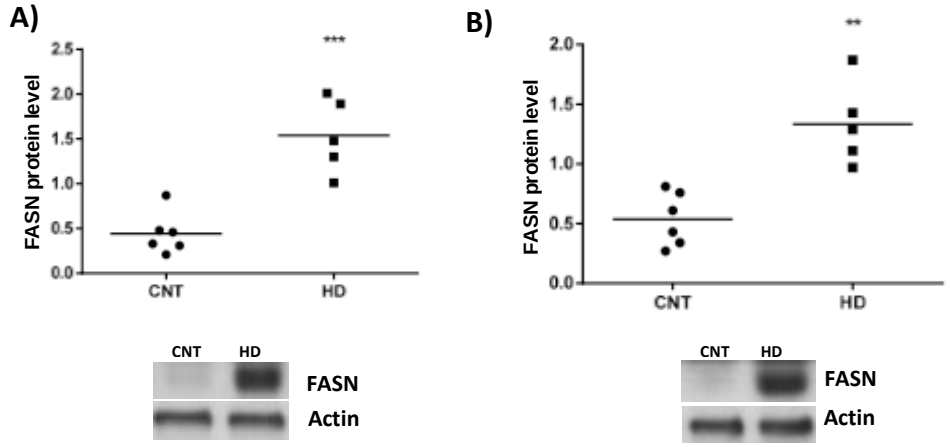


Figure 52: Putamen and cortex of HD patients show increased FASN levels. FASN levels were analysed by Western blot of protein extracts obtained from the putamen (A), and cortex (B) of control (CNT) and HD *post-mortem* brains. The results are expressed as the ratio between FASN and actin levels. Data were analysed by unpaired Student's *t*-test. **p<0.01 and ***p<0.001 compared with CNT. Representative immunoblots are shown.

3.8 Intraperitoneal injection of EGCG reduces FASN activity in striatum and cortex of R6/1 mice.

Finally, we investigated whether the improvement seen in motor coordination in R6/1 EGCG-treated mice was accompanied by inhibition of FASN. To attain this objective, striatal and cortical samples from 16-week-old WT and R6/1 mice treated with EGCG for two weeks were processed for FASN activity assay.

There was an increase in FASN activity in the striatum of vehicle-treated R6/1 mice respect the WT group (Figure 53A). The administration of EGCG inhibited striatal FASN activity in both WT and R6/1 mice (Figure 53A). Similar results were observed in the cerebral cortex (Figure 53B).

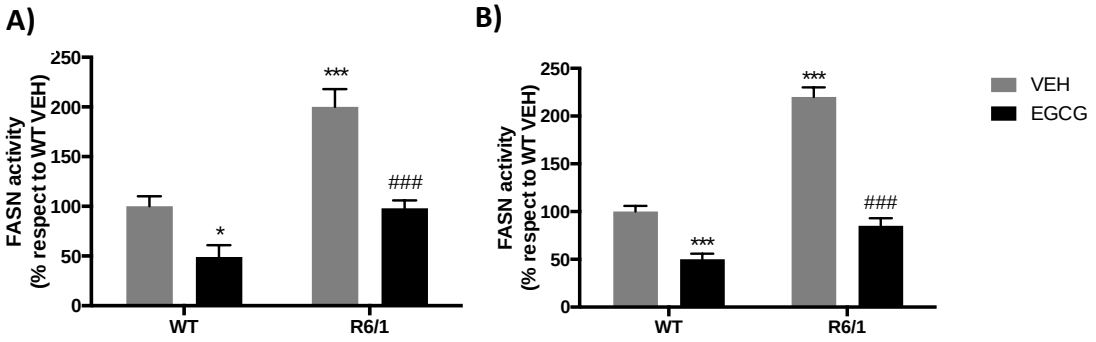


Figure 53: Administration of EGCG decreases FASN activity in the striatum and cerebral cortex of WT and R6/1 mice. Vehicle and EGCG were daily administrated during two weeks to WT and R6/1 mice and the FASN activity was analysed in the striatum (A) and cerebral cortex (B) at 18 weeks of age. Data are expressed as a percentage with respect to vehicle-treated WT mice and represent the mean ± SEM (n = 4). Statistical analysis was performed using two-way ANOVA followed by Bonferroni's as post-hoc test. *p<0.05 and ***p<0.001 when comparing to WT-vehicle group and ###p<0.001 when comparing to R6/-vehicle group.

V. DISCUSSION

Huntington's disease remains a progressive neurodegenerative disorder for which therapies are woefully inadequate and do not prevent the progression of the disease leading to death. Currently approved therapies are primarily aimed at treating chorea, they have some benefit but not enough to stop motor, behavioural and cognitive features of HD. Although none of them assured to cure the disease, it is necessary to continue studying the mechanisms to identify relevant therapeutic targets that can slow down or stop its progression contributing to the pathology of the disease due to mhtt presence.

The degeneration of striatal MSNs is classically associated to HD, however, dysfunction of other cerebral areas, such as cortex and hippocampus, is described (Hadzi et al., 2012; Rosas et al., 2003; Vonsattel et al., 1998). Nowadays, it is established that neuronal death is not the most important issue as it occurs at advanced stages of the disease. Nowadays, it has been described that neuronal and synaptic dysfunction precedes cell death in humans and animal models (Levine et al., 2004; Cepeda et al., 2001). Taking this into account, research to find new therapeutic targets focus in restoring neuronal and synaptic function and to prevent neuronal death in late stages of the disease. Moreover, the identification of new therapeutic targets in HD pathology that play a key role in the affected molecular pathways due to mhtt expression is a priority to design proper pharmacological treatments for HD patients. In this thesis, we have mainly focused on two principal aims: (1) study if two interesting molecules can reduce motor and cognitive deficits as well as improve synaptic plasticity and prevent cell death in HD models and (2) identify the molecular target by which they exert its actions. We analysed two important areas related to the HD symptomatology, striatum for motor discoordination and hippocampus for cognitive dysfunction.

Specifically, our results have helped to understand the capacity of PACAP and EGCG to increase synaptic plasticity, which is disrupted in HD pathology and their role on neuronal death in the context of mhtt expression. We have shown preclinical results of both compounds supporting the therapeutic potential for treating the clinical hallmarks of HD pathology.

When PACAP was discovered in 1989, Akira Arimura and his colleagues, did not know the high interest that this neuropeptide was going to arouse (Arimura, 2007). It was discovered its ability to exert pleiotropic effects due to its widespread distribution through the CNS and in most peripheral organs. So, the crucial roles played by PACAP are well-studied. However, its implication in pathological conditions and the molecular mechanisms by which PACAP exerts its effect are not completely understood. Thus, the aim of our work was to understand the role of PACAP in HD and identify if its receptors can be a new therapeutic target for the management of the disease. Here, we demonstrate for the first time, a therapeutic potential of PACAP in HD. Interestingly, results of the current study extend the growing body of evidence for a role of PACAP in modulating not only neuroprotective effects but also enhancing learning and memory deficits.

The first published study on EGCG as main polyphenol in green tea is dated 1985 (Chen et al., 2011b). From these publications, research provides clear support for hypotheses on the use of EGCG to prevent or even cure several diseases. Even though green tea benefits are well-known since a thousands of years, basic research findings on its main active component, EGCG, have been studied in the last decades becoming a promising compound. It is well documented that EGCG acts through different pathways, however, as for its large number of actions, there are still several important EGCG targets that remain unclear and need to be addressed in future research. Thus, our work was focused to understand the role of EGCG in HD and identify if FASN, a recent identified target of EGCG, can be a possible therapeutic target of the disease. Results show that modulation of FASN by EGCG promotes neuroprotection ameliorating behavioural impairments in HD models.

1. PACAP enhances hippocampal synaptic plasticity and improves memory performance in HD

The present results indicate that activation of hippocampal PACAP receptors, and more specifically the PAC1 receptor, promotes cognitive improvements in the context of HD. Importantly, PACAP receptors start to decline in the hippocampus at the onset of cognitive deficits in both an exon-1 and a full-length mhtt mouse model. In a

previous study using R6/2 mice to investigate the circadian clock disruption in HD, it has been shown that VPAC2 receptor mRNA levels are reduced in the suprachiasmatic nucleus (Fahrenkrug et al., 2007). Interestingly, a specific decrease of PAC1 receptor was detected in hippocampal *post-mortem* samples from HD patients which takes to us to consider a PAC1 specific role in HD pathology than the other PACAP receptors. Moreover, PAC1-deficient mice show deficits in hippocampus-dependent associative learning (Otto et al., 2001). Thus, to determine whether decreased PACAP receptor levels in the hippocampus could be responsible for cognitive disturbances in HD, WT and R6/1 mice were intranasally treated with PACAP for 7 days at 13 weeks of age, when memory decline was already present. We showed for the first time that administration of PACAP decreases the hippocampal-dependent cognitive deficits in the R6/1 mouse model of HD. These results are in agreement with the improvement of learning and memory induced by PACAP and previously reported under physiological conditions in rats (Roberto & Brunelli, 2000) and in an Alzheimer's disease transgenic mice (Rat et al., 2011). In agreement, studies in humans revealed that a decrease of PACAP levels is associated with severity (Han et al., 2014) and cognitive impairment (Han et al., 2015) in Alzheimer's disease. We found that daily administration of PACAP to R6/1 mice enhanced the expression of PAC1 receptor, suggesting an activation of the PACAP/PAC1-dependent signalling pathway. This capacity of PACAP to promote the transcription of its own receptor was previously observed in PC12 cells (Hashimoto et al., 2000) and in a transgenic mouse model of Alzheimer's disease (Rat et al., 2011). PAC1 is the specific receptor of PACAP (Seaborn et al., 2011) and it is most abundant in the brain when compared to VPAC1 and VPAC2 (Jolivel et al., 2009). The observation of the specific reduction of PAC1 in the hippocampus of HD patients together with the behavioural results obtained in rodent suggest a key role of the PACAP/PAC1 system in the regulation of cognitive function in HD.

Reduced levels of VGlut1 and PSD95 are associated with disruption of synaptic signalling and plasticity, causing cognitive dysfunction (Giralt et al., 2011a; Xu, 2011; Cha, 2000). Synaptic dysfunction is related to the onset of memory and learning impairment in HD (Milnerwood et al., 2006), and it has been suggested that recovery of the synaptic markers improves cognitive function in R6/1 mice (Anglada-Huguet,

2016; Cha, 2000). The present study revealed that administration of PACAP rescued the number of spine-like structures, as shown by recovery of VGlut1 and PSD95 synaptic markers in the hippocampus of R6/1 mice. This is the first report showing such an effect of PACAP on hippocampal synaptic markers *in vivo*. Importantly, PACAP was effective in different hippocampal CA1, CA3 and dentate gyrus, indicating a general beneficial effect of the peptide on hippocampal synaptic function. Furthermore, PACAP did not affect number of the spine-like structures in WT mice, exerting a specific pro-synaptic effect in the context of HD. Moreover, it has been reported that the stimulation of slices from CA1 hippocampus of Wistar rats by PACAP enhances synaptic transmission (Roberto & Brunelli, 2001). We also provided evidence that PACAP treatment has the capacity to increase the number and length of neurites in R6/1 primary hippocampal cultures. Similar to the results obtained *in vivo*, no effect on neurite development was observed in WT PACAP-treated hippocampal cultures, suggesting a specific action of PACAP in the context of HD. This does not correlate with the results from Ogata et. al who showed that PACAP increases the number of neurites in cultured hippocampal neurons (Ogata et al., 2015). A possible explanation to this discrepancy is the maturation status of hippocampal neurons and the duration of the PACAP treatment. Indeed, in that study, PACAP was added to the cells for only 24 h at 3 DIV, while our analysis was performed in more mature neurons, at 11 DIV, after 4 days of PACAP treatment. Taken together, these *in vitro* and *in vivo* data suggest that the behavioural improvement of PACAP-treated R6/1 mice is due to the stimulation of synaptic function by the activation of synaptic markers expression.

To test the functionality of the PACAP/PAC1 system after intranasal administration of PACAP in R6/1 mice, we evaluated c-fos levels in the hippocampus, as a marker of neuronal activation. We observed that c-fos protein level was reduced in the hippocampus of R6/1 mice and that intranasal administration of PACAP blocked this loss. It has been shown that inhibition of hippocampal c-fos by the intrahippocampal administration of antisense oligonucleotides in mice impairs memory consolidation (Guzowski, 2002). In HD, the decrease in c-fos in the hippocampus and its association with impaired memory function was also observed in heterozygous knock-in mice (Lynch et al., 2007). PACAP induced of c-fos expression has also been shown to

promote cultured cerebellar granule cell survival (Aubert et al., 2006; Vaudry et al., 1998). Thus, in HD, our results allowed to demonstrate that PACAP stimulate c-fos expression to promote both synaptic plasticity and cell survival.

There is also a recovery of CBP levels in the hippocampus of R6/1 mice after 7 days of PACAP treatment. The role of CBP as a transcriptional co-activator is crucial for the transcription of genes associated with learning and memory processes (Wood et al., 2006; Hardingham et al., 1999), in particular its role as a co-activator of CREB (Altarejos & Montminy, 2011). Deficits of CBP in the hippocampus have been related to disruption of synaptic plasticity and memory formation (Vecsey et al., 2007; Korzus et al., 2004). In the context of HD, CBP is reduced in the hippocampus of HD mice. Hence, the stimulation of its production could diminish memory and synaptic plasticity deficits (Lynch et al., 2007; Oliveira et al., 2006). The reduction of CBP was also observed in HD hippoccampal *post-mortem* human brain tissues (Lynch et al., 2007) and, in agreement with our behavioural data, increased CBP hippocampal levels were shown to improve spatial and object recognition memories in R6/1 mice (Giralt et al., 2012). Importantly, we described for the first time the ability of PACAP to increase CBP protein levels in the context of neurodegenerative diseases.

The key role of BDNF in synaptic function, plasticity and cognition has been extensively described (Lu et al., 2013; Giralt et al., 2009; Binder & Scharfmann, 2004). A reduction in BDNF levels occurs in HD (Lu et al., 2013) and this decrease contributes to the onset and severity of the symptoms (Giralt et al., 2009; Canals et al., 2004; Arimura et al., 1994). Thus, based on the present results, the decrease in BDNF levels seen in R6/1 mice could be due to the decrease of PAC1 receptor expression in HD. However, recovery of BDNF after PACAP treatment could be sufficient to improve synaptic plasticity and cognitive functions in HD mouse models (Giralt et al., 2011a; Simmons et al., 2009). It is worth to mention that PACAP stimulates BDNF expression in rat hippocampal neuronal cultures (Yaka et al., 2003) and that BDNF expression is significantly reduced in the hippocampal CA3 and dentate gyrus of PAC1-receptor-deficient mice (Zink et al., 2004). Consistent with our results in a HD model, intranasal treatment with PACAP has also been shown to enhance mRNA and protein levels of BDNF in the brain of Alzheimer's disease transgenic mice (Rat et al. 2011). So, taking

together all our results, it is tempting to propose that improvement of cognition and synaptic structures in the hippocampus of PACAP-treated R6/1 mice is mediated by an increase of CBP levels via PAC1, which in turn promotes the protein expression of BDNF. Accordingly, the expression of BDNF requires the presence of CBP as a co-activator of CREB (Zuccato et al., 2010).

Mhtt aggregates are the histopathological hallmark of HD (Nithianantharajah et al. 2008) and some studies associated the reduction of mhtt aggregates with a behavioural improvement in different HD mouse models (Chen et al., 2011a; Yamamoto et al., 2000). Importantly, we showed that 7 days of intranasal administration of PACAP reduced mhtt aggregation in the hippocampus. Similarly, intranasal administration of PACAP reduced amyloid deposition and improved the β-amyloid clearance in Alzheimer's disease mouse model (Rat et al., 2011). The increase of mhtt aggregates in the HD brain could be induced by the caspase-mediated cleavage of mhtt, thereby inducing the formation of truncated mhtt fragments (Wellington et al., 2000; Hodgson et al., 1999; DiFiglia et al., 1997) and causing disturbances in the ubiquitin-proteasome system (Wang et al., 2008; Glickman & Cienchanover, 2002; Waelter et al., 2001). The inhibition of caspases by PACAP has been extensively described, commonly associated with the antiapoptotic activity mediated by PAC1 (Reglodi et al., 2017). In addition, PACAP receptors are G protein-coupled receptors that stimulate the release of cAMP and the activation of PKA (Seaborn et al. 2011), and it is known that the stimulation of the cAMP/PKA pathway activates the ubiquitin-proteasome system (Chiang et al., 2009; Zhang et al., 2009). Thus, PACAP may delay the formation or enhance the clearance of the mhtt aggregates through stimulation of the ubiquitin-proteasome system.

In summary, we provide for the first time evidence of the beneficial role of PACAP receptor activation to improve cognitive function in HD. Our results show that reduction of PAC1 receptor in the hippocampus is a general mechanism observed at the onset of cognitive deficits in HD mice, and in HD human tissues. We also demonstrate that intranasal administration of PACAP in R6/1 mice results in a recovery of the memory function. We suggest that the recovery of memory-related features is mediated by the restoration of PAC1 levels. This effect is associated with increased

levels of neuronal markers and up-regulation of proteins involved in neuronal activity and synaptic function, as well as reduced formation of mhtt aggregates. These results clearly show a preclinical therapeutic effect of PACAP through the stimulation of hippocampal activity and improvement of memory decline in HD mice. Accordingly, we propose that activation of PAC1 receptor is a suitable strategy for treating neurodegenerative diseases, and that this strategy should be the focus of intense studies.

2. PACAP protects from mtt-mediated cell death

The presence of two main groups of receptors (specific PAC1 and VPAC1, VPAC2) and the splice variants of PAC1 receptor could explain the wide range of functions of PACAP in different organs and tissues (Moody et al., 2016; Blachman & Levkowitz, 2013; Vaudry et al., 2009). Its effects are unique as its closest analogue, VIP, is demonstrated to be unlikely to be as potent as PACAP. The exceptional combination of effects of PACAP has focused us on study this neuropeptide in the neuropathology of HD.

First, results indicate that PAC1 receptor levels decline in the striatum of R6/1 mice at 20 weeks of age and in the cortex the reduction starts at 12 weeks of age. Interestingly, this reduction appears with motor symptomatology in HD (Mangiarini et al., 1996) and it is maintained during the progression of the disease. Moreover, VPAC1 and VPAC2 levels were unaltered in the striatum and cortex of R6/1 mice. A specific reduction of PAC1 receptor was also detected in STHdh$^{Q111/Q111}$ cells. This pattern suggests a correlation between the reduction of PAC1-dependent signalling and the onset of symptomatology. Reinforcing this hypothesis, PACAP receptors were also characterised in STHdh cells.

In contrast, in STHdh$^{Q7/Q7}$ cells transfected with exon-1 htt plasmids, expressing 94 polyQ repeats and tagged with green-fluorescent protein (GFP) all three PACAP receptors were down-regulated. These results showed a direct relation between the presence of mhtt and the receptors protein levels reduction. Taking into account the low transfection efficacy, thus, not all cells have incorporated the plasmid; we can

assume that the effect may be higher. Mutant htt plasmid transfection generate intracellular aggregates (as seen in Figure 28A), these aggregates are highly linked to the transcriptional dysregulation (Roze et al., 2011). Altogether allowed us to suggest that PACAP receptors reduction appears when aggregates are already found in striatum and cortex of HD patients. These results agree with studies performed in HD mice models. In R6/1 mice, aggregate formation was described at 9 weeks of age (Mangiarini et al., 1996), before PACAP receptor reduction was produced.

Our results showed a significant reduction of STHdh$^{Q111/Q111}$ cells viability compared to STHdh$^{Q111/Q111}$ cells confirming that mhtt toxicity was basically due to apoptosis. After PACAP treatment STHdh$^{Q111/Q111}$ cells do not showed cytotoxicity induced by mhtt, having similar viability levels to STHdh$^{Q7/Q7}$ cells. Mutant htt induced apoptosis, is a quite complex cellular process which involved the action of numerous caspases. These proteins are divided into three categories: the ones which participate in inflammatory processes, initiator and executioner. Caspase-3 takes part of the last group and it is considered as the principal executor of the apoptosis signalling pathway (Seaborn et al., 2011). Caspase-3 is highly used in HD models because htt inhibits caspase-3 activation protecting cells from apoptosis (Zhang et al., 2006). The presence of mhtt increase caspase-3 activation leading to the activation of pro-apoptosis signalling pathways (Wellington et al., 2000). In this line, cleaved-caspase-3 levels were significantly increased in STHdh$^{Q111/Q111}$ respect to STHdh$^{Q7/Q7}$ cells. In this work, it has been observed an important reduction of cleaved caspase-3 levels after PACAP administration in both, cells and mice model.

The neuroprotective effect of PACAP was highly studied in many neurodegenerative diseases and results showed that PACAP could be implicated in different protective mechanisms (Baxter et al., 2011; Seaborn et al., 2011; Reglodi et al., 2011). For example, administration of PACAP in rats after artery occlusion significantly reduces infarct size (Reglodi et al., 2002). Interesting to know, the blood-brain barrier is temporarily more permeable after ischemia, facilitating the effect of PACAP after intravenous injection (Somogyvari-Vigh et al., 2000). Moreover, in a Parkinson's disease model, PACAP protects dopaminergic cells from apoptosis and improves behavioural impairments (Maasz et al., 2017; Reglodi et al., 2017). *In vitro*, PACAP

inhibits apoptosis induce by β-amyloid peptide (Rat et al., 2011). These and our results revealed a promising role of PACAP in treating neurodegenerative diseases.

PAC1 receptor has been associated to antiapoptotic role in brain (Vaudry et al., 2009). From now on, we want to study if the whole effects of PACAP seen in HD models are mediated through PAC1 activation. PACAP treatment seems to influence PAC1-receptor activation in the mouse brain (Rat et al., 2011). Thus, we first examine if PACAP treatment can affect PAC1 receptor expression. Results showed an increase of PAC1 levels in treated-STHdh$^{Q111/Q111}$ cells. The capacity of PACAP to promote the expression of its own receptors is reinforced with the daily administration of PACAP to R6/1 mice. PAC1 receptor enhancement suggests an activation of PACAP/PAC1-dependent signalling pathway. PAC1 receptor activates PKA and ERK signalling pathways inducing the transcription of key genes in HD such as BDNF leading to the inhibition of Bcl-2 associated X protein (Bax) and stimulating other anti-apoptotic proteins such as Bcl-2. The fact that PACAP has the ability to increase anti-apoptotic proteins level, avoids cytocrom c release from the mitochondria to the cytosol, thus, inhibiting the activation of caspase-3 (Lee & Seo, 2014; Dejda et al., 2008). We show for the first time that PACAP has the ability to protect from apoptosis induced by mhtt presence. Therefore, it's temptative to speculate that the protective effect of PACAP observed in our HD models may be through the increased protein levels of its receptor and the subsequent signalling pathways overactivation.

Importantly, we observed that transfection with siRNA against PAC1 receptor is enough to increase cleaved caspase-3 level in STHdh$^{Q7/Q7}$ cells suggesting that PAC1 receptor is necessary to maintain cell viability in our *in vitro* striatal model. It is well-known that there is a tight regulation between cell survival and cell death. This balance is basically regulated at the level of phosphorylation and transcription. Promoting the expression of specific genes is under the control of transcription factors. Then, these mechanisms could be altered in the presence of stress insults, such as mhtt, in order to swing the balance to cell death or cell survival. In addition, PACAP treatment in siRNA-transfected cells did not revert the activation of caspase-3 confirming that effect of PACAP on caspase-3 levels was through PAC1 receptor.

PACAP capacity to regulate gene expression is well studied in different cell lines (Vaudry et al., 2002). In PC12 cells and in granular neurons from the cerebellum it has been described the capacity of PACAP to promote the expression of early growth-response genes highly linked to cell survival such as c-fos and egr (Vaudry et al., 1998). In this thesis, we have demonstrated the mechanisms activated by PACAP in the presence of mhtt that plays a key role in trying to tip the balance to cell survival: the immediate early genes c-fos and egr-1 and CBP transcription factor which is considered, together with CREB, the major transcriptional regulator in striatal projection neurons (Vanhoutte et al., 1999). The activation of these factors has revealed the neuroprotective role of PACAP in the paradigm of mhtt-induced neuronal toxicity in different mouse and cellular models of HD pathology. The activation of these transcription factors is known to induce the expression of genes tightly related to neuronal survival (Lee et al., 2005a; Lee et al., 2005b; Lonze and Ginty, 2002). Moreover, analysis of c-fos and egr-1 expression in the striatum of R6/1 mice agree with studies of other authors showing a reduction of the immediate early genes in the straitum of R6/2 mice (Roze et al., 2008).

To determine whether PACAP has also a motor behavioural effect in HD, WT and R6/1 mice were intranasally treated with PACAP for 7 days at 18 weeks of age, when motor dysfunction is present. We show stimulation of PACAP receptor by PACAP decreases motor deficits in R6/1 mouse model of HD. These results are in agreement with the neuroprotective effects induced by PACAP (Rat et al., 2011; Deguil et al., 2010; Wang et al., 2008; Reglodi et al., 2004). Altogether, these data suggest that the reduction of PACAP receptor expression in the striatum is a key element for the progression of motor symptomatology. The results of the present work show a biological relevance of PACAP in the pathophysiology of HD and agree with the results obtained in STHdh cell line.

BDNF is a neurotophic factor highly regulated by CBP/CREB (Tao et al., 1998; Shieh et a., 1998) and its reduced levels in HD are well-described (Giralt et al., 2009; Zuccato & Cattaneo, 2007; Lynch et al., 2007; Canals et al., 2004). In addition, PACAP stimulates the release of other trophic factors, such as BDNF and nerve growth factor (NGF) (Reglodi et al., 2011; May et al., 2010; Vaudry et al., 2009; Shioda et al., 2006). In the

same way, our results in R6/1 mice showed that the improvements seen in motor coordination after PACAP treatment are highly linked to BDNF protein levels in striatum, which were also enhanced. BDNF has a key role in synaptic plasticity (West & Greenberg, 2011; Cohen & Greenberg, 2008) and its potent action not only improves cognition but also motor functions is different HD mouse models (Giralt et al., 2011, Simmons et al., 2009). Therefore, the motor improvements seen in R6/1 mice after PACAP administration could be associated to the recovery of BDNF levels.

The next question is whether PACAP could be used or not as a therapeutic tool to treat neurodegenerative diseases in humans. A case study was reported showing the short half-life of PACAP in human blood, ranged from 5 to 10 min (Li et al., 2007). However, in our study, we used the intranasal administration, a procedure that avoids rapid degradation and directly targets the brain. We observed specific changes in the hippocampus and in the striatum of R6/1 mice following PACAP treatment, such as an increase of PAC1 receptor expression, as well as stimulation of c-fos, CBP and BDNF protein production. In addition, the improvements in cognitive and motor performance and in recovery of synaptic structures observed in PACAP-treated-R6/1 mice indicate that intranasal administration of the peptide may be a good therapeutic alternative, as already reported in an Alzheimer's disease transgenic mice model (Rat et al., 2011). Also, it has been shown that inhaled PACAP is well tolerated in humans (Doberer et al., 2007; Born et al., 2002). Moreover, a synthetic PACAP analogue was shown to exert potent neuroprotective effects in a Parkinson's disease model with reduced cardiovascular side-effects when compared to the native peptide (Lamine et al., 2016). Therefore, although the peptide can still be modified to enhance its activity and/or specificity, our and other results show the therapeutic applicability of PACAP receptor activation to treat symptoms in the context of neurodegenerative diseases.

Combined, all these data indicate an important role of PACAP and its PAC1 receptor in HD. Our results seem to be indicate that PACAP improves motor and memory function through the reestablishement of PAC1 receptor levels, providing the first link between PAC1 receptor and HD. We suggest that that PAC1 receptor modulation lead to the recovery of PAC1-mediated signalling in the hippocampus and striatum, promoting the

transcriptional machinery and up-regulating the levels of BDNF. The activation of this machinery promotes synaptic plasticity in the hippocampus and protects from mhtt-mediated cell death in the striatum of HD (Figure 54). Altogether, these findings point out PAC1 receptor as a promising new therapeutic target in the CNS for HD.

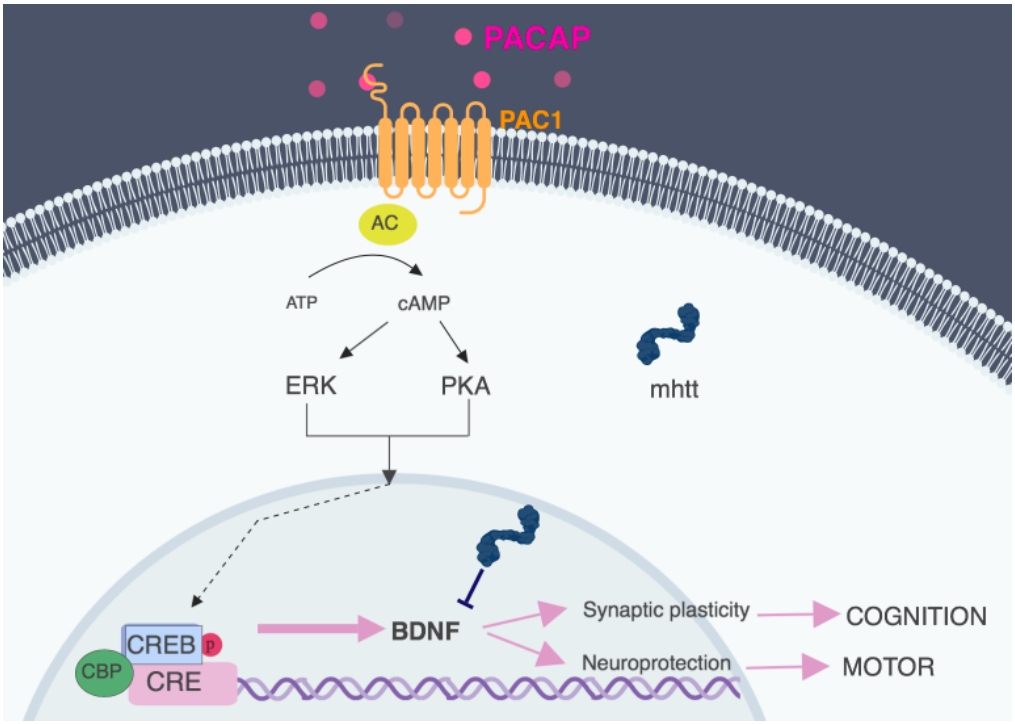


Figure 54: Schematic representation of beneficial effect of PACAP treatment in the presence of mhtt. The presence of mhtt alters the transcriptional machinery inducing a reduction of synaptic plasticity and an increase in neuronal death leading to the cognitive and motor symptoms of HD. PAC1 modulation due to PACAP treatment improves transcriptional activity, such as CREB activation, and restores the levels of BDNF improving synaptic plasticity in the hippocampus and neuroprotection in the striatum leading to cognitive and and motor function.

3. EGCG treatment improves motor and cognitive deficits in R6/1 mice model. Possible role of FASN inhibition.

The encouraging results of green tea catechins in cancer chemoprevention or cardiovascular diseases, have led to explore their effectiveness in neurodegenerative diseases. Several studies using *in vivo* models have been shown a beneficial effect of EGCG, the main active cathecin of green tea, mainly through its antioxidant and metal chelator activity (Han et al., 2014; Rezai-Zadeh et al., 2008; Weinreb et al., 2004; Levites et al., 2002). Furthermore, an increasing number of *in vitro* works have shown

that EGCG can act on many molecular targets in the nervous system (Zhang et el., 2014; Yao et al., 2014; Guo et al., 2005). However, the therapeutic mechanism of action of EGCG has not been yet elucidated, and well-known molecular targets of EGCG such as FASN have not been explored in the nervous system. Hence, the administration of EGCG, the in vivo effectiveness and the identification of its target sites remain a challenging task.

We wanted to verify *in vitro* the neuroprotective role of EGCG in the context of HD. Several studies have shown that polyphenols, including EGCG, display a dose-dependent action, for example, the proapoptotic activity was exerted at high doses whereas at micromolar concentration a neuroprotective effect was seen (Levites et al., 2002). Therefore, we tested a wide concentration range in order to state the best dose to produce a neuroprotective effect in our HD model. The highest neuroprotective effect of EGCG was seen at 40 µM. Interestingly, treated-STHdh$^{Q111/Q111}$ showed a significant increase of the viability respect to the non-treated ones. These data demonstrate that EGCG can rescue neuronal injury *in vitro*. Surprisingly, relative high-dose EGCG (100 µM) has no different effect compared to the lower dose. Nevertheless, taking into account previous studies (Ding et al., 2012; Levites et al., 2002), high dose of EGCG is not the best choice as could exert negative effects.

It has been demonstrated that EGCG behaved as a potent neuroprotective agent, decreasing not only the expression of proapoptotic genes such as *fas* ligand but also increasing the expression of cell survival genes (Mandel et al., 2004). In parallel with these findings, STHdh$^{Q111/Q111}$ present higher quantity of apoptotic nucleus leading to a reduced viability. We studied the effect of EGCG treatment on cleaved caspase-3 levels, an apoptotic marker used through this entire thesis, in STHdh$^{Q7/Q7}$ and STHdh$^{Q111/Q111}$ cells. The presence of EGCG reduced in both cell types cleaved caspase-3 levels. In parallel with these findings, STHdh$^{Q111/Q111}$ treated cells present less quantity of apoptotic nucleus. These results are in accordance with a recent study where protective effects of EGCG against sevoflurane-induced neurotoxicity in neonatal mice were assessed (Ding et al., 2017). The observations of this study suggest that decreased apoptotic cell counts observed on EGCG treatment could be due to down-regulated caspase-3 activation. Modulation of cell death and cellular survival

produce biological modulations which are important to understand drugs with multipharmacological actions, specifically, the different pharmacological and toxicological actions of EGCG. This could allow to design a more specific therapy.

EGCG has many beneficial effects and different targets and thus, at this point, we cannot rule out that its effect on FASN is indirect. One of the possibilities to influence on FASN was to generate knock-out animals for the protein. The idea was discarded as Chirala and Wakil in 2004 proved that FASN -/- null mutant embryos died before their implantation in the uterus, and also that due to haploid insufficiency most of the FASN+/- heterozygotes also died at various stages of their embryonic development (Chirala & Wakil, 2004). RNA interference could address the key questions on the fundamental aspects of FASN pathophysiological role in HD and to assess therapeutic potential benefits on silencing the protein (Fernando et al., 2017). First, we used siRNA against FASN in STHdh cells. STHdh cells treatment with siRNA against FASN significantly reduced FASN and cleaved caspase-3 levels in both $STHdh^{Q7/Q7}$ and $STHdh^{Q111/Q111}$ cells showing a similar effect as cells treated with EGCG. Our results suggest that FASN overexpression in the presence of mhtt is linked to neuronal degeneration in STHdh cells.

We also investigated whether administration of EGCG could induce motor performance in R6/1 mice. EGCG treatment in R6/1 mice increased the distance covered whereas the number of slips were reduced drastically respect to the non-treated ones. When evaluating clasping phenotype, non-treated R6/1 mice showed a highly clasping phenotype that appeared significant reduced after EGCG treatment. These results indicate that EGCG ameliorates motor discoordination in R6/1 mice and reinforce the neuroprotective effect of EGCG already described in different neurodegenerative diseases (Levites et al., 2002).

This EGCG neuroprotective activity also involves the intracellular signalling mediator PKC (Levites el al., 2003), a family of serine/threonine kinases. It is thought to mediate a key role in the regulation of cell survival, programmed cell death and long-term potentiation (Maher, 2001). The induction of PKC in neurons by this polyphenol seems to be the responsible for neuroprotection against neurotoxins such as the prevention

of the amyloid-beta peptide in Alzheimer's disease (Levites et al., 2003) or to stop the striatal dopamine depletion in Parkinson's disease models (Levites et al., 2001). Few number of compounds have been reported to prevent the decline in PKC activity, some examples are BDNF or VIP. On the other hand, it has been also demonstrated that EGCG has the ability to increase ERK1/2 activity in a Parkinson's disease model (Levites et al., 2002). ERK1/2 is well-studied in HD for its role in neuroprotection against NMDA toxicity (Hetman et al., 2006).

The administration of EGCG in R6/1 mice also revealed the capacity of EGCG to improve the R6/1 memory performance. It is known that not only does EGCG exert neuroprotective effect, it also induces neurorescue activity by promoting neurite growth. However, the connection between EGCG and neural activity in learning and memory are not fully understood. There are some studies which demonstrate the potential of EGCG to induce LTP in CA1 after ischemia injury (Ding et al., 2012), thus we also evaluated cognition after EGCG treated in R6/1 mice. The present results indicate that EGCG treatment, and more specifically inhibition of FASN, promote cognitive improvements in the context of HD. We showed for the first time that administration of EGCG decreases the hippocampal-dependent cognitive deficits in R6/1 mouse model of HD. Altogether, these data suggest that the administration of EGCG to ameliorate cognitive symptoms in HD patient would be useful at the onset of learning and memory impairment and indicating that EGCG has the ability to facilitate the efficiency of synaptic transmission alleviating the hippocampal LTP deficiency of R6/1 mice.

As EGCG was effective neuroprotection against mhtt toxicity and in ameliorating motor and cognitive symptoms, the question remains by which target EGCG exerts these beneficial effects. Then, we focused our work on study FASN and its role in HD. FASN is a constitutive protein that plays vital roles like membrane lipid biosynthesis and palmitoylation (Gonzalez & Visentin, 2016; Chirala & Wakil, 2004).

In this work we have demonstrated a biphasic alteration of FASN levels in the striatum and cortex of R6/1 mice. Increases in FASN levels are associated with early stages of the disease and accompanied by enhanced FASN activity and reduced levels were

detected at advanced stages of the disease in both cerebral areas. Moreover, increased protein levels of FASN in human putamen and cerebral cortex and in the cellular model were also observed. Altogether, our results suggest that an up-regulation of FASN protein levels and activity are highly linked to HD phenotype. To the best of our knowledge, only one report relates altered FASN activity with a neuropathological condition. This study is related to its expression in pre-frontal cortex in autism (Chen et al., 2016). They suggest that the upregulation of FASN could occur as a consequence of dysregulation of intracellular signalling pathways.

No data is published about the pathophysiological role of increased FASN in neurons. Interestingly, FASN is responsible to synthesize plasmatic membrane, thus, it has a direct effect on important elements such as membrane receptors. For our interest, in HD high levels of excitotoxicity are known to cause damage leading to neuronal dysfunction and death. Some studies found how NMDAR subunits can regulate pro-survival or pro-apoptosis signalling (Hardingham & Bading, 2010). Therefore, it could be interesting to relate high levels of protein and activity with increased excitotoxicity affecting also membrane stability. Moreover, palmitate, the immediate product of FASN goes through a process called palmitoylation (Gonzalez & Visentin, 2016). Palmitoylation of GluN2B subunit of NMDA receptor can modulate its trafficking. Severe consequences as excitotoxicity can appear if the process is dysregulated as GluN2B-mediated excitotoxicity agrees with striatal vulnerability for neurodegeneration in HD (Hayashi, 2009). In agreement, results obtained in our laboratory in the spinal cord of a neuropathic mice model revealed that increased FASN activity is associated with higher GluN2B subunit levels in synaptic membranes (Xifró et al., 2015).

To know whether the effect of EGCG could be mediated by FASN activity inhibition we studied FASN activity in EGCG-treated R6/1 mice. Our results showed, for the first time in a model of neurodegenerative diseases that injection of EGCG induces an inhibition of FASN activity in the brain that was paralleled by behavioural improvement in R6/1 mice. These results suggest a therapeutic capacity of inhibition of FASN by EGCG in HD. However, the human applicability of EGCG presents some limitations. Importantly, EGCG exhibits a low oral bioavailability and low brain uptake due to extensive

oxidation of hydroxyl groups reducing its stability and potency (Landis-Piwowrar et al., 2013). Interestingly, in neuropathic mice models, EGCG derivatives with high capacity to inhibit FASN have been revealed as therapeutic synthetic molecules (Xifró et al., 2015).

Combined, all these data indicate an important role for FASN in HD. We provide the first link between FASN overactivation and HD. We suggest that behavioural performance of R6/1 mice induced by the administration of EGCG could be mediated by the decrease of FASN activity, enzyme up-regulated at the onset of HD (Figure 55). In agreement, we previously described in a neuropathic mice model that EGCG reduces neuropathic pain by the inhibition of FASN activity (Xifró et al., 2015). More investigations aimed to describe the pathophysiological role of FASN in HD and its inhibition will elucidate whether FASN could be a promising therapeutic target in HD.

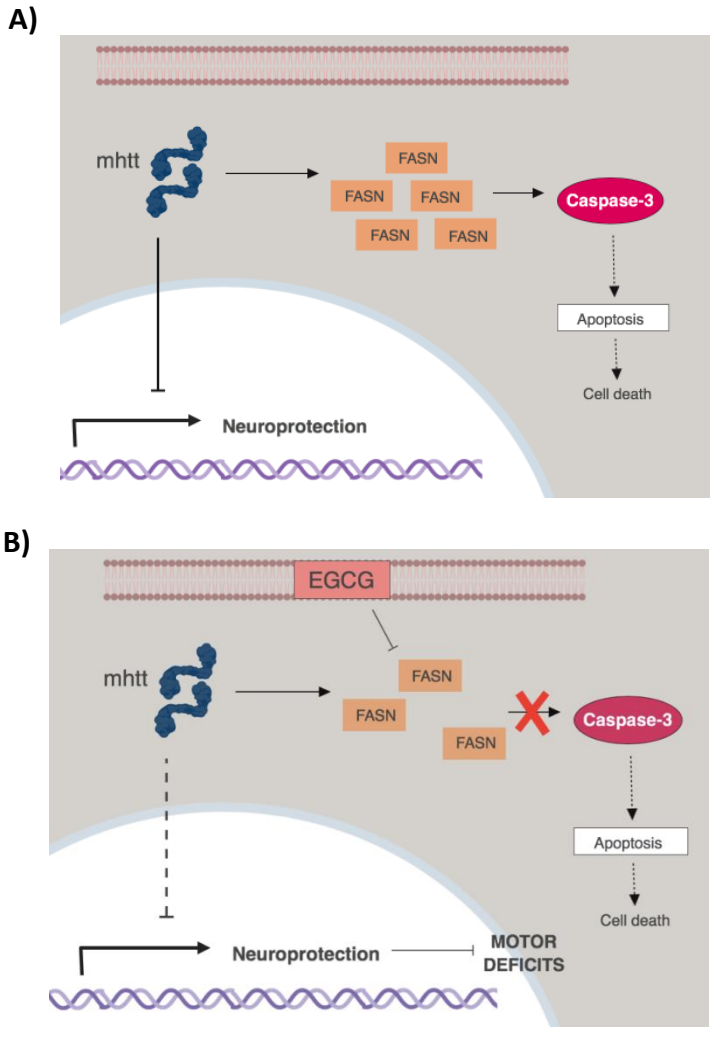


Figure 55: Schematic representation of beneficial effect of EGCG treatment in the presence of mhtt. A) In the presence of mhtt, the transcriptional machinery is altered which reduced the expression of genes related to synaptic plasticity and neuroprotection. There is also an increase in FASN levels increasing the activity of pro-apoptotic factors leading to neuronal death. B) FASN modulation by EGCG treatment reduces the expression of caspase-3 levels ameliorating behavioural deficits observed in R6/1 mice.

Altogether in this thesis, we proposed that reduced PAC1 receptor levels and increase FASN activity could be related to HD physiopathology. Alteration in both targets may be caused due to transcriptional machinery impairment. Moreover, PAC1 and FASN modulation with PACAP and EGCG respectively, have a strong therapeutic effect leading to the partial recovery of some biochemical and histopathological markers in striatum and hippocampus of R6/1 mice and point out PACAP and EGCG as promising new therapeutic molecules in the CNS for HD and other neurological disease.

VI. CONCLUSIONS

1. PAC1 reduction is a feature of HD from the onset of symptomatology. We observed decreased levels in different HD models and cerebral areas.

2. Intranasal administration of PACAP has a strong therapeutic effect reducing cognitive and motor phenotype in a HD mice model.

3. The therapeutic target of PACAP seems to be PAC1 as the administration of this neuropeptide restores PAC1 receptor level in the hippocampus and striatum of HD mice model.

4. Behavioural recovery of HD mice treated with PACAP is associated with higher expression of genes related to synaptic plasticity and survival and the production of the neurotrophic factor BDNF in the hippocampus and striatum.

5. In the hippocampus PACAP promotes the expression of synaptic markers, whereas in the striatum PACAP blocks mhtt-mediated apoptosis, suggesting a dual effect depending on the site of action.

6. PACAP has the capacity to reduce mhtt aggregates, the histopathological hallmark of the disease.

7. EGCG protects against mhtt mediated-apoptosis in a cellular model of HD.

8. In a HD mouse model the administration of EGCG ameliorates motor and memory deficits.

9. FASN seems to have a physiopathological role in the onset of motor deficits as increased activation was observed in the striatum and cortex of HD models.

10. The beneficial effect of EGCG observed in HD models is associated with decreased FASN activity, suggesting that the therapeutic role of this polyphenol is mediated at least, in part, by the inhibition of FASN.

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