Characterization of mechanisms underlying neuronal survival and plasticity in Huntington's disease

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CHARACTERIZATION OF MECHANISMS UNDERLYING NEURONAL SURVIVAL AND PLASTICITY IN HUNTINGTON'S DISEASE

Doctoral degree of Biomedicine in the Facultat de Medicina de la Universitat de Barcelona.
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This work was performed at Departament de Biologia Cel·lular, Immunologia i Neurociències de la Facultat de Medicina de la Universitat de Barcelona, under the supervision of Dr. Jordi Alberch Vié and Dr. Xavier Xifró Collsamata.

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Juliol 2013

Learn from yesterday,

live for today,

hope for tomorrow.

The important thing is to not stop questioning.

Albert Einstein

A totes les persones que han estat al

meu costat durant aquest temps.

Sense vosaltres res d'això no hagués estat possible.

Als meus pares i amics.

AGRAÏMENTS

La tesi és aquella etapa de la teva vida que quan la comences, 4 anys abans, et penses que no arribarà mai el moment de llegir-la, que encara queda molt; però un dia et despertes i ja està, ja han passat 4 anys i quasi no te n'has adonat. En aquests 4 anys hem passat per molts moments, alguns de molt bons, de dolents i d'altres encara més dolents, però tota la gent que ens envolta ha fet que tot fos fantàstic. Realment hem creat una petita família que ens ha ajudat el dia a dia a saltar els entrebancs i a fer-nos tira endavant en els moments difícils. Però no tot ha estat dolent, també ens ho hem passat molt bé. Fer la tesi és un experiència vital increïble, t'ajuda a créixer com ha persona, a ser autosuficient i fins i tot ja gent que pensa que et canvia.

Primer de tot m'agradaria agrair al Jordi i al Xavi que m'hagin donat la oportunitat de realitzar la meva tesi doctoral en aquest laboratori. La veritat és que en el moment en el que em va sortir la oportunitat no me'n vaig adonar, però mica en mica quan va anar passant el temps em vaig adonar de la sort que havia tingut, tan a nivell professional pel laboratori, com a nivell personal per tota la gent que he tingut la oportunitat de conèixer aquí. Gràcies Jordi per donar-me la llibertat de pensar per mi mateixa, per la confiança i el suport que has dipositat en totes les meves idees i projectes. I sobretot, gràcies per tenir aquest punt de vista pràctic de la vida i per la teva sinceritat que sempre fa que les coses siguin molt més fàcil. A tu Xavi, moltes gràcies per estar sempre al meu costat i trobar sempre el cantó positiu de les coses (encara que a vegades no existeixi,jaja), així m'has ajudat a contrarestar la meva negativitat i per fer-me veure que les coses sempre són més fàcils del que semblen. Gràcies per ser, a part del meu jefe, el meu company, ajudar-me en tot el que he necessitat, per poder-te dir les coses sempre com les penso, per creure en les meves idees i intentar potenciar-me al màxim. Per aquelles reunions last minute fent un frankfurt i una birra i per portar-me sempre la contrària,jaja, es que t'encanta fer-me rabiar i ja saps que no em costa gaire,jaja! Bueno espero que vagi tot molt bé per Girona!

També volia agrair a l'Esther tot el suport durant aquesta tesis. Per solucionar els meus 50.000 dubtes i per poder estar tant discutint aferrissadament sobre resultats a un seminari com fent una birra a la terrasseta d'un hotel parlant de cotilleos i intentant que els veïns no s'ofeguin a la piscina mentre estem sent el buffet lliure de tots els mosquits de New Orleans. I tu Sílvia, gràcies per ser sempre tant positiva i amb aquest caràcter tant espontani que sembla que tot es pugui fer si hi posem una mica d'alegria. I finalment, agrair al Pep per ensenyar-me el món de les cèl·lules mare i el desenvolupament i per la teva enorme capacitat d'organització i divulgació de la ciència. Tot i que no hem treballat en el mateix grup, agrair també al Gustavo i a la Bet aquest punt de serenitat que sempre despreneu i la calma a l'hora d'intentar

solucionar qualsevol conflicte. Molta sort en el nou món de Marfan en el que us heu endinsat. No em puc oblidar d'agrair a la Núria i a la Carme; ja sé que sóc una pesada, però sempre heu sabut solucionar tots els problemes i mal de caps amb els que us he atabalat.

En aquests anys que he passat aquí el flux de gent ha estat constant. Les meves companyes inicials de taula Olga, Empar i Paola! Olga, sempre em va sorprendre el dia que em vas dir que eres basca, amb el Català tan perfecte que parlaves! Tu ets la serenitat personificada que me la vas transmetre els pocs mesos que vam ser companyes de taula abans de que baixessis a la tercera. I tu Paola també! Em vareu ajudar a neutralitzar la meva hiperactivitat i nerviosisme. Algun dia m'heu d'explicar el secret per poder estar sempre tan tranquil·les. Gràcies Paola per tenir tanta paciència amb mi amb les preguntes constants que no parava de fer-te. Crec que és de les poques persones que has estat escrivint la tesis al meu costat i no he notat en cap moment ni mica d'estrès ni d'inseguretat, seràs una supermami!! I si parlo de la Paola, no em puc deixar a la Ingrid. Que bien que nos lo hemos pasado de fiesta en esos sitios chungos que nos llevabas, jaja, el Merlin no se llamaba...dios!!! La calidad de la música del lab ya no es la misma desde que te fuiste...por suerte,jajaja, viva MAXIMA. I finalment tu Amparituuu! Que t'he de dir. Et vaig trobar molt a faltar quan te'n vas anar, ja no sabia amb qui discutir ;-)! Saps que al principi quan em van assentar al teu costat em pensava que ens mataríem, però has estat la meva companya de taula ideal, sempre m'has ajudat en tot, tan a nivell professional com personal, tot i que no sempre (o quasi mai) ens posàvem d'acord. M'encanta la teva manera de viure les coses, amb tanta passió, la teva capacitat d'ajuntar a tothom per fer el cafè del matí, el del berenar, la birra de la tarda, els videos de tesis i infinitat d'activitats més. Tu si que ets una persona emprenedora, Empar for president!!i visca esquerra! No et preocupis que segur que Catalunya algun dia serà lliure! Però abans que vosaltres encara va marxar més gent. Dani tot hi que sembla que faci infinit, et recordo com si fos ahir assentat a la teva cadira davant de les milions d'imatges del confocal. Dani ets un crack! Ets d'aquelles persones que sempre que les necessites esta disposada a donar-te un cop de mà, a explicar-te el que sigui necessari i a part ho fas amb passió, com dient això de la ciència està fet per mi. Començar la tesi a prop teu m'ha fet il·lusionar-me molt més per la ciència. Mai oblidaré aquells confocals fins les 2 del matí on jo ja no pillava de res, aquells seminaris eterns, aquells "voy voy" escrivint de peu davant l'ordinardor, les borratxeres amb el "Jo mai mai", el brake dance en el sofa de l'oshum i l'ampolla de cava amb pots d'orina escoltant pont aeri! D'altres heu marxat més recentment, com tu Miriam. Gràcies per se com ets, sempre sincera, decidida i apuntant-te a tot la primera. Estic molt contenta de que et vagi tot bé per Munich i a veure si aviat ens veiem tots per allà Alemanya!

Tota la feina que fem aquí al lab no seria possible sense l'ajuda de la Maite, la nostra jefa de la cinquena. No se com t'ho fas, però sempre aconsegueixes solucionar tots els problemes. Sempre que passa algo és...i la Maite..ahhh...salva'ns! Ets una peça fonamental d'aquest laboratori i fas que tot funcioni com ha de funcionar. I després estàs tu Ana L. No sé que hagués fet durant la meva tesi sense la teva ajuda, ets una superwoman, puedes con todo. Sempre ho tens tot tan organitzat, ets tan eficient i penses en tot. Qualsevol dels meus experiments els he pogut fer gràcies a la tu. Ets d'aquell tipus de persones que en queden poques, atemporal, sempre jove i amb empenta, m'alegro molt d'haver-te conegut. També agrair a la Lara, per tota l'ajuda a l'estabulari de psico i per compartir tants dinars amb mi quan estàvem avorrides de només interaccionar amb ratolins. Molta sort amb la tesi!

Gràcies a tota la gent de l'Stem cell lab. Desitjar-vos sort Raquel i Cris amb el nou projecte de la sala blanca! Que segur que us ho passeu molt bé allà amb l'alegria que despreneu sempre. També a les més recents incorporacions, Marco, Phil, Jordi, Georgina, Inés i Clara. Moltíssima sort amb el superprojecte de la HighQ que esteu portant a terme. Mònica, no sé com t'ho fas, però sempre desprens felicitat! Sé que tindràs molt sort amb tot el projecte dels microarrays i les microdisseccions, perquè és xulíssim, ens vas deixar a tots flipant el primer cop que ens ho vas explicar! I finalmente a tu Andy! No faltas a ninguna fiesta! Ahora ya no podemos decir "Andy no entiende nada", porque ya casi hablas catalán. Nunca te han dicho que eres un Alemán atípico? Lo único de Alemán es que bebes cerveza y eres rubio,jaja! Pero como puedes estar siempre tan moren. Bueno aun que hay leído la tesis todavía me debes un par de barbacoas eh, que no te escapas! Obwohl die Straße ist schwierig, immer bis zum Ende erhalten! Ein Tag auch uns zu erklären sind Ihre private Feiern;-)! Mucha suerte y „all in".

Per més que passi el temps sempre sereu els nostres nens, Jordiet, Rafa, Roser i Laura. Ja fa dos anyets que vas entre Jordiet, com passa el temps, mira si t'han sortit canes i tot,jaja. M'encanta la teva empenta, la teva il.lusió i motivació per tot lo que envolta la ciència, ets un crack com a investigador i com a persona; segueix així i arribaràs molt lluny, ja quasi podràs llegir el treball de màster per articles! Bueno Rafa, ja t'has fet un lloc al mini-lab, però pensar que abans d'acabar la tesi haurem d'aconseguir que parlis català amb algú més que no sigui l'Esther. Roser, Rosereta, que faran sense tu al laboratori, crec que per fi ens podrem plantejar el tema de l'operación bikini. Estic molt contenta que hagis trobat el teu futur a Bellvitge que sé que et feia molta il·lusió, però espero que igualment portis alguna ensiamada i avarques. Ets d'aquelles persones que te la mires i penses: „Mira si es buena persona". Espero que aquesta alegria que et caracteritza et duri per sempre. I tu Laureta, estàs a punt de començar una nova etapa de la teva vida aquí al lab, però no tinguis por que com suposo que has pogut comprovar,

no t'has equivocat! Ara si, que sàpigues que quan me'n vagi et tocarà a tu obrir la porta. Bueno a veure quan ens convides a una paella d'aquestes tan bones que feu pel delta. Molta sort!

La meva taula privilegiada m'ha permès conèixer, a banda de tota la fantàstica gent del laboratori, la gran quantitat de transportistes que ens vénen a portar paquets, jaja, ja saben com em dic i tot. Aquest és un món que la gent del mini-lab mai podrà gaudir. Bueno no sé si criticar-ho, perquè en certa part jo també sóc del club del mini-lab on estàs tu Albert. Ets una de les persones que més m'ha ajudat al llarg de la tesi. M'has ensenyat tot el que te demanat i més i gràcies a tu, a les teves aportacions, a les teves idees i a les nostres reflexions he pogut tira endavant una part molt important d'aquesta tesi. Em sembla que m'hauràs de passar el teu número de París perquè pugui seguir molestant-te un cop ens en haguem anat. No puc contar amb els dits de la mà la infinitat de tècniques que m'ha ensenyat i la paciència que has tingut, sempre disposat a ajudar. No perdís mai aquesta empenta i decisió que et caracteritza, però tot i ser un Nature Medicine guy a data d'avui encara no saps com s'ha de fer per obrir la porta o recollir un paquet,jaja. Dins del mini-lab també et trobem a tu Ana, sempre tant respectuosa i tranquil·la. No sé com ho fas però crec que ets la persona més ordenada i estructurada que conec. Sempre disposada a ajudar quan et necessiten i sempre interessada per com van les coses. Tu ets un gran exemple de com ser una bona científica i muntar una família. Espero que et vagi tot molt bé. And here you are Shiraz!I hope some day you will speak better catalan than spanish, and you have to teach as how to dance bollywood! I am really happy that finally your wife is here, but meeeennnn you'll never be completely happy until you try jamón de jabugo!I per últim moltes gràcies Gari, espero que acabis de disfrutar del temps que et queda aquí al lab. Zorte Ona!

Aquest temps també he compartit els meus moments al laboratori amb molta altra gent. La Vero, espero que tengas mucha suerte con todos los proyectos de p75 y disfrutes de tu estada en el CELLEX. Molta sort Gerardo amb tots els teus projectes, ets la primera persona que conec que quan li vaig preguntar com és que parlava català em va contestar: „un dia tenia una calçotada a Valls i vaig pensar que si no parlava català no entendria a ningú". Ets com un pubmed amb potes, jaja. Espero que vagi molt bé la tesis. A ti Andrés, muchas gracias por ser tan servicial, siempre tan buena persona y por hacernos reír.

Dins del laboratori s'ha creat un nou grup, alias Marfanitos. Javi, el superpapi, espero que tots els projectes que tens els puguis dur a terme i et desitjo molta sort. Moltes gràcies per tots els teus consells, ets com un superpapi a casa i a la feina; gràcies pel teu humors, que tot s'ha de dir, és molt negre,jaja i vista Sarrià-Sant Gervasi. Jèssica, tu has passat i ja has marxat, però no

ens has deixat indiferents. Hem disfrutat molt de la teva companyia al lab i espero que et vagi tot molt bé per Bèlgica, la boda, els projectes...Bueno Henry nosaltres hem viscut a la vegada el procés d'escriure la tesis, em sembla que hi havien apostes a veure qui llegia primer,jajaj, però al final me avançat. A veure si puc estar aquí quan et toqui llegir la tesi. Ara ja ets un marfanito! També desitjar-vos sort a les noves incorporacions Yolanda, Thayna, Rocio y Dasha, que aun que seas del Real Madrid, lo disimulas muy bien, jaja.

I he deixat el millor pel final, a tots vosaltres, als que no heu estat només els meus companys, sinó també els meus amics. Als que cada cop que he tingut algun problema, alguna il·lusió, alguna xorrada tonta que m'ha passat pel cap, he tingut ganes d'arribar a la feina per poder-la compartir amb vosaltres. Mar, Cheru, Carla, Laura, Laia i Adrià, heu fet que a part de sentir-ho com una feina fos alguna cosa més especial, gràcies a tots vosaltres pels moments que hem compartit junts. A les dos maries, que us he de dir! Sabeu que no hi ha paraules, que cabrones m'ha tocat a mi primera escriure els agraïments! Sempre heu estat al meu costat, tant en els bons moments com en els dolents. Des del primer moment que us vaig conèixer ja vaig notar aquesta connexió especial. Gràcies Zipi per fer-me veure el cantó més maco les coses, per el teu positivisme, per ser tant bona persona i per fer que el Zape ho fos una mica més. I a tu Carla, gràcies per les nostres divagacions a l'ascot fins que ens tancaven el bar o ja no podíem veure més birra,jaja, quants dies me n'he anat al llit sense sopar...Pels nostres pitis de desconnexió i perquè sempre heu estat la vàlvula d'escapament, no sé com ho feu, però sempre que hem passa alguna cosa i no vull explicar al cap de 5 minuts ja ho sabeu tot. Laureta, joooo et trobo molt a faltar. Realment tu vas ser la meva primera companya de laboratori, em vaig enganxar a tu i hem vas ensenyar infinitat de coses. Per mi has estat una de les persones més especials de la meva tesi i las pogut viure quasi tota fins al final. M'ha ajudat tant poder parla amb tu tots els problemes i explicar-nos la vida constant-me a la mínima oportunitat. Sempre has sabut com donar-me suport i fer-me sentir millor, tant a nivell professional com a nivell personal. Gràcies Adrià, ets el meu freak preferit, però no veure ni tenis ni el tour de França amb tu! Que hagués estat d'aquest tesis sense els nostres chistes verds i la nostra font de videos i imatges suggerents, el que no tinguis tu al mòbil no ho té ningú. Ets la nostra princeseta del club de les sirenes. Has estat aquí des del principi de la meva tesi fins al final on les hem vist de tots colors! No podrem deixar de fer les nostres classes magistrals a l'ascot o per skype sinó,jaja. De tu he aprés lo important que és el bacon per subsistir i que sense ell podem arribar a posar-nos malalts. Ets el meu compi de trobar el segon sentit a les coses i de reeducar aquesta gent amb la ment tant pura, crec que hem fet una bona feina,jaja, fins i tot hem fet caure a la Roser...Espero que ens cuidis el T-Rex i us trobaré molt a faltar! I tu Laieta,

que te n'has anat tan lluny, amb qui parlaré de roba interior de color carn i criticaré a la gent. Ets d'aquelles persones que quan les coneixes sembla que t'hagis d'acostar i t'hagin de punxar, però després només tindries ganes de donar-li abraçades. Molta sort per Thailandia! Cheru,joooo te has ido a mordor i me has abandonado, es desolador, tengo una mesa para mi sola, nadie me insulta, creo que no puedo vivir así ;-)....daiiiiiiiii!Me gusto haber conocido a tu hermana, nos tenia a todos engañados, pero después apareciste tu y pensamos vanffanculo esta tia es la ostia. Siempre me haces reír, eres espontania y me encanta tu manera de ser rancia! No cambies nunca piccola fiammiferaia! I no hem puc oblidar de tu Gerard, el xispas, que encara que no formis part del lab és com si ho fossis. M'encanta perquè sempre coincidim quan intentem convèncer la Mar d'alguna cosa.

A banda de l'increïble companyia del laboratori, també ha estat essencial tot el suport que he rebut de tots els meus amics i família. Gràcies als meus pares i al meu germà per aguantar-me en tots els moments difícils, perquè ja se sap, la confiança a vegades fa fàstic. Heu estat sempre allà per ajudar-me a guanyar confiança en mi mateixa. A tu Borja, que has estat al meu costat tota la tesi, gràcies per aguantar les meves rallades quan les coses no sortien bé, però també per compartir amb mi les alegries. Gràcies a tu Helena, Ester, Laures, per els dinars dels dijous (bueno o quan ho aconseguim), que ens ajuden una mica a desconnectar de la rutina i també a tota l'altre gent de la uni que després de 10 anys que fa que ens coneixem ho hem de celebrar! També vull agrair les meves amigues, Merx, Diana, Clàudia, Carla, Eula, Núria, Patty, Deyi, per la paciència que han tingut amb mi, arriba divendres i comentaris com "qué??! Has d'anar a treballar dissabte pel matí?"; "No pots sortir de festa perquè tens ratolins", jaja no entenc res. Però tot i així heu tingut paciència i m'heu seguit trucant, és que ja són molts anys eh....! Finalment donar les gràcies a tots els amics que he fet al Barça, Fiona i Marina sou genial i en la meva vida ja no hi ha espai sense vosaltres; a tu Moncho, que aun que seas de Pucela sabes que te quiero; i a l'Oier, l'Ander i el Diego que las fiestas sin vosotros no serían lo mismo.

Moltes gràcies a tots per aquest anys que heu compartit amb mi!

RESUM

INTRODUCCIÓ

La malaltia de Huntington (MH) és una malaltia neurodegenerativa d'herència autosòmica dominant causada per l'expansió anormal del codó CAG en l'exó 1 del gen IT15, el qual codifica per la proteïna huntingtina (HDCRG, 1993). L'expansió del codó CAG dóna com a resultat l'aparició d'una cua de poliglutamines les quals generen una complexa resposta patogènica en les cèl·lules neuronals, el mecanisme exacte de la qual encara esdevé una incògnita (Bossy-Wetzel et al., 2004; Ross, 2004; Tobin and Signer, 2000). En individus sans, el nombre de repeticions oscil·la de 6 a 35; quan el nombre de repeticions d'aquest triplet és superior a 40, l'individu desenvoluparà la malaltia. Tot i que la huntingtina s'expressa per tot l'organisme, la neurodegeneració afecta de manera selectiva les neurones GABA-èrgiques de projecció estriatals (caudat-putamen) i l'escorça. També trobem afectació en altres regions del cervell com l'hipocamp, l'hipotàlam i l'amígdala (Graveland et al., 1985; Reiner et al., 1988; Vonsattel et al., 1985; Vonsattel and DiFiglia, 1998). Tot i que la malaltia es caracteritza per alteracions motores (Vonsattel and DiFiglia, 1998), un nombre creixent d'estudies en pacients (HDCRG, 1993; Lawrence et al., 1998; Lemiere et al., 2004) i en models murins (Giralt et al., 2012; Murphy et al., 2000) ens mostren que els dèficits cognitius són un altre fenotip característic de la malaltia i sovint apareixen abans que els símptomes motors.

La identificació de les rutes moleculars alterades a causa de l'expressió de la huntingtina mutada és crucial per entendre els processos cel·lulars afectats en la malaltia així com pel desenvolupament de tractaments farmacològics eficaços. En aquesta tesi ens centrarem per una banda, en l'estudi de proteïnes involucrades en la vulnerabilitat estriatal i en el procés de supervivència davant l'expressió de la huntingtina mutada i per altre banda en la identificació de tractaments farmacològics eficaços per reduir o pal·liar els dèficits motors i cognitius característics de la MH com la modulació dels receptors de prostaglandina E2.

Diferents mecanismes moleculars es poden trobar alterats en presència de la huntingtina mutada. En les cèl·lules, existeix un balanç entre l'activació de vies que fomenten la supervivència i vies que promouen la mort neuronal. Moltes d'aquestes vies es troben controlades a nivell de fosforilació o de transcripció. La transcripció gènica està controlada per un seguit de factors de transcripció que a la vegada es troben regulats per un gran nombre de quinases. Dins dels mecanismes moleculars pels quals la huntingtina mutada és capaç d'induir la mort cel·lular es troba críticament involucrada la desregulació del sistema transcripcional (Sugars and Rubinsztein, 2003). A més, en presència de huntingtina mutada també es poden trobar alterades les vies d'activació dels factors de transcripció i això a la vegada pot provocar canvis en la regulació transcripcional. Aquest és el cas, per exemple, de la via de les MAPK (de l'anglès *mitogen-activated protein kinase*) (Apostol et al., 2006).

La proteïna Rsk (de l'anglès *90-kDa ribosomal S6 kinase*) es troba regulada, en part, per la via de les MAPK (Chen et al., 1992). Investigacions prèvies han demostrat una desregulació de les dues quinases

que s'encarreguen del control de l'activitat de la proteïna Rsk, la proteïna quinasa ERK (de l'anglès *Extracellular signal-Regulated Kinase*) i la proteïna quinasa PDK1 (de l'anglès *3-Phosphoinositide-Dependent protein Kinase-1)*, en línies cel·lulars models de la MH (Gines et al., 2003; Gines et al., 2010). Rsk és una família constituïda per 4 isoformes (Rsk1-4) de serina/threonina quinases àmpliament expressades en el cervell que regulen importants funcions cel·lulars, incloent la supervivència (Anjum and Blenis, 2008). Una vegada activada per fosforilacions seqüencials (Anjum and Blenis, 2008; Chen et al., 1992; Frodin et al., 2000; Frodin et al., 2002; Jensen et al., 1999), Rsk promou la fosforilació i conseqüent inactivació de moltes proteïnes citosòliques pro-apoptòtiques com Bad (Tan et al., 1999) i la glucogen sintasa quinasa 3β (GSK-3β) (Sutherland et al., 1993); així com també l'activació de proteïnes anti-apoptòtiques nuclears com CREB (de l'anglès *cAMP response element binding protein*) (Xing et al., 1996) i el factor de resposta a sèrum (SRF) (Rivera et al., 1993). En aquest treball ens hem proposat estudiar el paper de la proteïna quinasa Rsk en la MH, així com el seu paper protector davant l'excitotoxicitat per huntingtina mutada. Per tal de dur a terme aquest estudi hem utilitzat un model *knock-in* de ratolí que expressa 7 (en el cas dels ratolins control; HdhQ7/Q7) i 111 (en el cas dels ratolins de la MH; HdhQ111/Q111) repeticions del triplet CAG sota el control del propi promotor murí (Wheeler et al., 1999), així com una línia cel·lular derivada d'aquests ratolins (Trettel et al., 2000). També hem utilitzat un model de ratolí transgènic de la MH, el ratolí R6/1. Aquest model expressa l'exó 1 del gen de la huntingtina mutada, el qual té una expansió del nombre de triplets CAGs de 115 (Mangiarini et al., 1996). Aquests animals desenvolupen alguns símptomes i alteracions específiques similars a les observades en la MH, incloent disfunció motora i canvis neuropatològics com la formació d'agregats (Petersen et al., 2002; Smith et al., 2005).

Per altra banda, el factor de transcripció Elk-1 representa la major diana nuclear de les MAPK (Gille et al., 1995b; Janknecht et al., 1993). Aquesta proteïna pertany a la família de factors de transcripció de complex ternari (TCFs) amb domini ETS (Buchwalter et al., 2004). La fosforilació del TCF promou ràpidament la transcripció dels gens d'expressió primerenca (iEG) a través d'un complex ternari format amb un dímer del SRF en el lloc d'unió al DNA on es troba l'element de resposta a sèrum (SRE) (Sharrocks, 2000). Elk-1 s'ha relacionat amb processos com la diferenciació neuronal (Vanhoutte et al., 2001), la proliferació cel·lular i supervivència (Sharrocks, 2002; Vickers et al., 2004). En la segona part d'aquesta tesi estudiem els canvis en l'expressió i l'activitat del factor de transcripció Elk-1 i el seu possible paper en la fisiopatologia de la MH. Per assolir aquest objectiu hem utilitzat dos models de ratolins transgènics de la MH, els ratolins R6/1 i R6/2. En aquest cas, el model R6/2 expressa l'exó 1 del gen de la huntingtina mutada, el qual té una expansió del nombre de triplets CAGs de 150 repeticions (Mangiarini et al., 1996). També s'utilitzarà una línia cel·lular estriatal prèviament descrita. Aquest model cel·lular resulta un bon model per estudiar fidelment la pròpia mutació que expressen els pacients de MH ja que l'expressió de la huntingtina mutada en aquestes cèl·lules està regulada pel propi promotor intrínsec de la cèl·lula.

Finalment, recentment s'ha donat importància als efectes beneficiosos de la modulació dels receptors de la prostaglandina E2 (PGE2) en models d'isquèmia (Ahmad et al., 2006a; Ahmad et al., 2010; Akaike et al., 1994; Liu et al., 2005; Taniguchi et al., 2011) o davant d'estímuls tòxics (Kawano et al., 2006; Zhou et al., 2008). No obstant, l'actuació terapèutica en aquests receptors no ha estat investigada en el camp de les malalties neurodegeneratives. PGE2 s'uneix a receptors acoblats a proteïnes G anomenats EP (de l'anglès *E-prostanoid receptor*) (Breyer et al., 2001): EP1, EP2, EP3 i EP4. Els diferents receptors EP es troben acoblats a diferents proteïnes G produint efectes diversos com canvis en els nivells d'AMP cíclic (pels receptors EP2-EP4) i/o fosfoinositol i canvis en els nivell intracel·lulars de calci (pel receptor EP1). Els receptors de EP més estudiats a nivell de sistema nerviós són el receptor EP1 i EP2. L'activació del receptor EP1 s'ha relacionat amb el paper neurotòxic dels receptors de prostaglandines. La inhibició o deleció genètica del receptor EP1 contraresta la desregulació de calci induïda per la sobre-activació dels receptors NMDA (*N-Methyl-D-aspartate*) i indueix neuroprotecció (Kawano et al., 2006); senyalant aquest receptor com a bona diana terapèutica ja que s'ha descrit una desregulació dels nivells de calci en models de la MH (Hansson et al., 2001) així com una excitotoxicitat produïda per una sobre-activació dels receptors NMDA (DiFiglia, 1990). L'antagonisme del receptor EP1 també protegeix de la mort induïda per la deprivació d'oxigen i glucosa (Zhou et al., 2008) o per la 6-OHDA (de l'anglès *6-Hydroxydopamine*) (Carrasco et al., 2005; Carrasco et al., 2007). Per altra banda, l'activació del receptor EP2 estimula la formació d'AMP cíclic que promou l'activació de PKA (*Gs-cAMP/protein kinase*) i del factor de transcripció CREB (Regan, 2003). L'activació de CREB promou l'expressió de gens que contenen la seqüència CRE (de l'anglès *CREB Response Element*), incloent molts gens que bloquegen la mort neuronal (Dawson and Ginty, 2002), com la neurotrofina BDNF (de l'anglès *brain-derived neurotrophic factor*) (Tabuchi et al., 2002; Tao et al., 1998). Estudis recents han demostrat un paper neuroprotector de l'activació del receptor EP2 en paradigmes de toxicitat i neurodegeneració (Ahmad et al., 2006b; Ahmad et al., 2010; Bilak et al., 2004; Carrasco et al., 2008; Echeverria et al., 2005; Liu et al., 2005; McCullough et al., 2004). A més a més, l'activació del receptor EP2 indueix l'alliberació de BDNF de micròglia i d'astròcits d'origen humans en cultiu gràcies a l'activació de la via cAMP/PKA/CREB (Hutchinson et al., 2009). Dèficits en el suport neurotròfic causats per una reducció dels nivells de BDNF s'han implicat en la vulnerabilitat estriatal en la MH (Zuccato and Cattaneo, 2007). Aquest dèficit neurotròfic no és exclusiu de la regió estriatal sinó que també s'ha observat en la regió hipocampal i cortical d'individus i en models animals de la MH (Ferrer et al., 2000; Gines et al., 2003; Giralt et al., 2009; Lynch et al., 2007; Spires et al., 2004; Zuccato and Cattaneo, 2007). En ratolins, els receptors EP1 i EP2 es troben altament expressats a l'escorça cerebral, l'estriat i l'hipocamp (Ahmad et al., 2006a; Andreasson, 2010; Kitaoka et al., 2007; Zhang and Rivest, 1999), regions afectades en la MH (Vonsattel and DiFiglia, 1998). Aquests antecedents ens han fet plantejar-nos la modulació dels receptors EP1 i EP2 com a possible diana terapèutica en la MH.

En resum, l'objectiu principal d'aquesta tesis doctoral és descriure i validar estratègies terapèutiques per contrarestar la vulnerabilitat estriatal a la presència de la huntingtina mutada i els dèficits motors i cognitius característics de la MH.

RESULTATS I DISCUSSIÓ

1. ESTUDI DELS MECANISMES COMPENSATORIS ACTIVATS DURANT LA PROGRESSIÓ DE LA MALALTIA DE HUNTINGTON:

1.1. Paper de la proteïna quinasa Rsk en la prevenció de la mort induïda per la presència de la huntingtina mutada.

La família de proteïnes Rsk està involucrada en funcions de supervivència cel·lular (Anjum and Blenis, 2008). L'activació de Rsk està regulada per fosforilacions seqüencials controlades per ERK 1/2 i per PDK1 (Chen et al., 1992). Aquestes dos quinases es troben alterades en models de la MH (Apostol et al., 2006; Gines et al., 2003; Gines et al., 2006; Saavedra et al., 2010); tot i així, el paper de la proteïna Rsk en aquesta malaltia neurodegenerativa és una incògnita. L'objectiu d'aquest treball ha estat analitzar els nivells, l'activitat i el paper de Rsk en models *in vivo* i *in vitro* de la MH. D'altra banda, mitjançant la transfecció de formes mutants de la proteïna Rsk vam observar que l'augment de la seva activitat en cèl·lules STHdhQ111/Q111 era degut principalment a l'activitat PDK1. Aquest augment en l'activitat de Rsk es donava tant en el citosol com en el nucli, la qual cosa augmentava la fosforilació de substrats citosòlics i nuclears de Rsk. Finalment, els experiments d'inhibició farmacològica de Rsk, el silenciament i la seva sobre-expressió ens han mostrat que l'activitat de Rsk exerceix un paper protector davant la mort induïda per la huntingtina mutada en cèl·lules STHdhQ7/Q7 transfectades amb huntingtina mutada. Gràcies als resultats anteriors podem concloure que l'augment observat en els nivells i l'activitat de Rsk actuaria com a mecanisme compensatori amb la capacitat de prevenir la mort induïda per la presència de la huntingtina mutada. Proposem Rsk com a bona diana terapèutica en la MH.

1.2. Possible paper del factor de transcripció Elk-1 en la mort neuronal selectiva que es produeix en la MH.

Elk-1 és un factor de transcripció que regula la l'expressió dels iEGs i s'ha relacionat amb efectes neuroprotectors davant d'estímuls tòxics (Sharrocks, 2001; Wasylyk et al., 1998). La seva activitat neuroprotectora depèn de la seva fosforilació en la serina 383 (pElk-1^{Ser383}) (Janknecht et al., 1993). L'objectiu d'aquest estudi ha estat examinar el possible paper neuroprotector del factor de transcripció Elk-1 en la MH.

Per assolir aquest objectiu hem utilitzat dos models murins que expressen l'exó 1 de la huntingtina mutada, els ratolins R6/1 i R6/2, amb 145 i 115 repeticions del triplet CAG (Giralt et al., 2009)

respectivament i una línia cel·lular estriatal que expressa la forma completa de la huntingtina mutada (STHdhQ111/Q111). Hem observat un augment dels nivells d'Elk-1 i pElk-1^{Ser383} a l'estriat dels ratolins R6 durant la progressió de la malaltia. De manera similar també hem observant un increment en els nivells i la fosforilació d'Elk-1 en les cèl·lules STHdhQ111/Q111 comparat amb les cèl·lules *wild-type* (STHdhQ7/Q7). A més, aquest augment d'Elk-1 té una localització nuclear però, tot i així, aquest factor de transcripció no es troba segrestat en els agregats de huntingtina mutada, suggerint una major activitat transcripcional d'Elk-1. Tot i que s'ha descrit una regulació a la baixa de molts iEGs en l'estriat de ratolins R6 i en cèl·lules STHdhQ111/Q111, el silenciament d'Elk-1 amb RNA d'interferència en disminueix l'expressió d'aquests gens, però només en aquelles cèl·lules que presenten huntingtina mutada. Aquest resultat suggereix que els augments en l'expressió d'Elk-1 són necessaris en presència de la huntingtina mutada. Per tal de determinar si l'augment en l'activitat d'Elk-1 podria jugar un paper protector en la MH, vam estudiar la mort per apoptosi de les cèl·lules STHdh després de la transfecció amb RNA d'interferència per d'Elk-1. De manera interessant, la reducció en els nivells d'Elk-1 promou la mort cel·lular i l'activació de la proteïna pro-apoptòtica caspasa-3 en les cèl·lules STHdhQ111/Q111, però no en les cèl·lules *wild-type*. En resum, es proposa que l'augment en els nivells de proteïna, de la seva fosforilació i de la localització nuclear d'Elk-1 observats en models de la MH que expressen exó 1 de la huntingtina mutada i en línies cel·lulars que expressen la forma completa de la huntingtina mutada pot suposar un mecanisme compensatori activat per les cèl·lules estriatals en resposta a la huntingtina mutada el qual contribueix a la neuroprotecció d'aquestes cèl·lules.

2. CARACTERITZACIÓ DE LA MODULACIÓ DELS RECEPTORS DE PROSTAGLANDINA E2 EN LA MALALTIA DE HUNTINGTON

2.1. Definir el paper del receptor EP1 com a diana terapèutica per a reduir el fenotip de la malaltia de Huntington en ratolins R6/1.

En el sistema nerviós central, el receptor EP1 de prostaglandines s'expressa en condicions basals en l'escorça cerebral, l'hipocamp i l'estriat entre d'altres zones (Andreasson, 2010). Aquest receptor es troba acoblat a una proteïna Gαq. L'activació d'aquest receptor promou la hidròlisi del fosfatidil inositol i un augment en els nivells intracel·lulars de calci. En el cervell, la inhibició farmacològica del receptor EP1 té un efecte neuroprotector en models de deprivació d'oxigen i glucosa (Zhou et al., 2008), davant d'excitotoxicitat produïda per NMDA i en isquèmia cerebral, identificant-se aquest receptor com a principal transductor de neurotoxicitat mitjançada per COX-2 (Ahmad et al., 2006a; Kawano et al., 2006). Efectes directes de l'excitotoxicitat mitjançada per EP1 s'han demostrat en models de la malaltia de Parkinson (Carrasco et al., 2007) i en models de la malaltia d'Alzheimer (Li et al., 2013; Zhen et al., 2012). En aquest treball ens volem centrar en estudiar els efectes a nivell motor, cognitiu, bioquímic i

histològic de la inhibició crònica del receptor EP1 amb l'antagonista SC-51089 (SC) en animals R6/1. El nostre mètode d'administració es basa en la implantació intraperitoneal d'un dispositiu de difusió osmòtica que allibera una dosi constant de fàrmac en fases inicials de la simptomatologia. Primer hem analitzat l'efecte de l'antagonista EP1 en un model excitotoxic de la MH (Schwarcz et al., 2010). Hem observat que l'antagonista SC-51089 aconsegueix disminuir considerablement el volum de la lesió després d'una injecció estriatal d'àcid quinolínic, corroborant l'efecte neuroprotector de l'antagonisme del receptor EP1 i validant el nostre sistema d'administració. Posteriorment, hem procedit a implantar el sistema de perfusió durant 4 setmanes en ratolins R6/1, des de la setmana 14 a la setmana 18. L'administració de l'antagonista del receptor EP1 s'ha mostrat eficaç en la millora de les disfuncions motores i cognitives en els nostres models murins de la MH. Els resultats mostren una millora en el *clasping*, fenotip neurològic d'aquests models (Pang et al., 2006). Per analitzar les millores a nivell motor s'han realitzat diferents proves: *rotarod*, *balance beam* i *vertical pole*. S'ha pogut observar una millora en totes les tasques en aquells animals R6/1 tractats amb SC-51089 comparat amb els R6/1 vehicle. Posteriorment s'ha procedit a analitzar l'estat cognitiu d'aquests ratolins mitjançant el *novel object recognition test* (NORT) i el *T-spontaneous alternation task* (T-SAT). S'observa una reversió dels dèficits cognitius en els animals R6/1 tractats amb l'antagonista del receptor EP1. En aquesta línia, els animals R6/1 tractats amb SC-51089 presenten un increment de la potenciació a llarg termini (LTP) a l'hipocamp.

Un cop analitzades les millores en conducta dels animals R6/1 tractats amb SC-51089 durant 4 setmanes hem procedit a analitzar canvis a nivell bioquímic i histològic. Al observar millores cognitives molt més notòries que les millores motores ens hem proposat comparar els nivells del receptor entre diferents àrees del cervell. Hem comparat l'expressió d'EP1 en estriat i escorça (regions més relacionades amb la conducta motora) i hipocamp (regió més relacionada amb respostes cognitives). Com pronosticàvem, hem observat una expressió molt més elevada d'EP1 en l'hipocamp comparat amb les altres dos regions. Després hem procedit a analitzar diferents marcadors sinàptics els quals s'ha descrit una reducció en models de la MH. El tractament amb SC-51089 millora significativament l'expressió de GluR1 i PSD-95 a l'hipocamp i VGluT1 a l'estriat de ratolins R6/1. En models R6/1 que expressen l'exó 1 de la huntingtina mutada s'observa l'aparició d'agregats de huntingtina mutada entre les 4 i les 12 setmanes (Milnerwood et al., 2006) que són una característica histopatològica de la malaltia. L'anàlisi dels nombre d'agregats en el cervell dels ratolins R6/1 després de 4 setmanes de tractament ens mostra una reducció d'aquest marcador histopatològic tan a l'hipocamp com a l'estriat.

En el present treball, hem demostrat la relació entre el receptor EP1 i la MH. Consistent amb les millores motores i cognitives observades, el fàrmac SC-51089 produeix una recuperació parcial de diferents marcadors bioquímics i histopatològics tan a l'estriat com a l'hipocamp de ratolins R6/1, així com millores en la plasticitat sinàptica i reducció en el nombre d'agregats de huntingtina mutada al nucli de les neurones estriatals i hipocampals. Així, es demostra que l'antagonisme del receptor EP1 té un efecte terapèutic en aquests animals, i senyala el receptor EP1 com a nova diana terapèutica en el sistema nerviós central per moltes malalties neurològiques, com en el cas de la MH.

2.2. Estudiar el mecanisme del receptor EP2 en la regulació dels dèficits cognitius i tròfics en ratolins R6/1 models de la malaltia de Huntington.

El receptor EP2 de prostaglandines està acoblat a la producció d'AMP cíclic i es troba altament expressat al cervell (Andreasson, 2010). A nivell de sistema nerviós central, s'ha descrit una important implicació del receptor EP2 en plasticitat sinàptica. A l'hipocamp la potenciació i la depressió a llarg termini (LTP i LTD) es veuen afectades per la deleció el receptor EP2 provocant importants dèficits cognitius (Savonenko et al., 2009; Yang et al., 2009). A nivell de neurotoxicitat existeix important literatura fent referència a la seva acció de pro-supervivència. Estudis *in vitro* demostren que el receptor EP2 protegeix de paradigmes excitotòxics com l'NMDA o la deprivació d'oxigen i glucosa (Ahmad et al., 2010; Liu et al., 2005; McCullough et al., 2004). Aquest efecte neuroprotector es reverteix al inhibir PKA, suggerint que la neuroprotecció es dependent de la senyalització per cAMP. En models d'isquèmia *in vivo*, la deleció del receptor EP2 augmenta el volum del infart a nivell cortical (Liu et al., 2005; McCullough et al., 2004); també exerceix un paper neuroprotector en models d'exicitotoxicitat estriatal (Ahmad et al., 2010). Finalment, l'estimulació del receptor EP2 té un efecte de pro-supervivència en models *in vitro* de diferents malalties neurodegeneratives (Ahmad et al., 2006a; Bilak et al., 2004; Carrasco et al., 2008; Echeverria et al., 2005).

L'activació d'EP2 promou la fosforilació de CREB que activarà gens regulats sota el promotor CRE, com és el cas del gen *bdnf* (Fukuchi et al., 2005). BDNF és un factor neurotròfic estretament implicat en la regulació de la plasticitat sinàptica, la supervivència neuronal i la diferenciació; convertint-lo així en una diana terapèutica clau en molts desordres neurològics (Binder and Scharfman, 2004). Diferents estudis han demostrat que l'activació del receptor EP2 pot induir l'alliberació de BDNF i diferents neurotrofines (Hutchinson et al., 2009; Toyomoto et al., 2004). Al ser la disminució dels nivells de BDNF un fenomen característic de la MH (Zuccato and Cattaneo, 2007; Zuccato et al., 2008) vam decidir utilitzar agonistes de receptor EP2 en un model murí R6/1 de la MH.

Primer de tot hem realitzar un estudi *in vitro* amb cultius primaris hipocampals per analitzar l'efecte d'un agonista del receptor EP2, el misoprostol, sobre el creixement dendrític. Hem observat que el tractament amb misoprostol augmenta el nombre de dendrites i que aquest efecte es bloquejat al afegir anticossos de BDNF al medi de cultiu, indicant que l'efecte del misoprostol és dependent de BDNF. Posteriorment, en estudis *in vivo*, hem observat una activació del factor de transcripció CREB després d'una injecció intraperitoneal amb l'agonista del receptor EP2, misoprostol. Després hem procedir a tractar els animals R6/1 amb el mateix sistema de bombes osmòtiques utilitzat en els tractaments amb l'antagonista del receptor EP1 i diferent paradigmes cognitius van ser analitzats. Hem observat un retard en l'aparició del *clasping* en els animals tractats amb misoprostol així com una reversió dels dèficits cognitius a les 17 setmanes (després de 3 setmanes de tractament amb misoprostol) en el T-SAT i el NORT. En l'anàlisi bioquímic posterior al tractament ha revelat un augment dels nivells de BDNF tant en els animals WT com en els R6/1 tractats amb misoprostol. Aquest resultats són la primera evidència del

paper beneficiós de l'activació del receptor EP2 en la MH. Consistent amb les millores observades en la memòria, els tractaments amb misoprostol provoquen una recuperació dels nivells de BDNF a l'hipocamp de ratolins R6/1. A més a més, aquesta regulació positiva dels nivells de BDNF per l'activació del receptor EP2 s'ha observat també en cultius de neurones hipocampals on el misoprostol provoca un augment del creixement dendrític de manera dependent de l'acció del BDNF. Conjuntament aquests resultats ens demostren que l'agonisme del receptor EP2 té un fort efecte terapèutic reduint els dèficits cognitius produïts per l'expressió de la huntingtina mutada en ratolins R6/1 i potser una diana útil per tal de revertir els la disminució del suport tròfic que es produeix durant la MH.

ABBREVIATIONS

6-OHDA	6-Hydroxydopamine
A	Adenine
AA	Arachidonic acid
AD	Alzheimer's disease
Akt	serine/threonine protein kinase B
AMPAR	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
C	Cytosine
CA	*Cornus Ammonis*
Ca^{2+}	Calcium
CAMKs	Ca^{2+}/calmodulin-dependent protein kinases
cAMP	Cyclic-adenosine monophosphate
CBP	CREB-binding protein
COX	Cyclooxygenase
CREB	cAMP-response element protein
CTKD	C-terminal kinase domain
DAG	diacylglycerol
DG	Dentate gyrus
DLG	PDZ-domain scaffolding protein Discs-large
EC	Entorhinal cortex
Elk-1	Ets-like Gene 1
EP	E-prostanoid
ER	Endoplasmatic reticulum
ERK 1/2	Extracellular signal-regulated kinase ½
ETS	E- twenty six
G	Guanine
GABA	Gamma-aminobutyric acid
GluA1	AMPAR subunit GluR1
GPCRs	G protein-coupled receptors
GSK-3β	glycogen synthase kinase-3β
HATs	Histone acetyl transferases
HD	Huntington's disease
HDACs	Histone desacetylases
iEGs	Immediate early genes
IP3	inositol-1,4,5-triphosphate
IP3R	inositol (1,4,5)-triphosphate receptor
JNK	c-Jun-N terminal kinase
KID	kinase inducible domain
LTP	Long-term potentiation
M	Months
MAGUKs	Membrane-associated guanylate kinases
MAPKs	*mitogen-activated protein kinases*
MCAO	Middle cerebral arteria occlusion
Mhtt	Mhtt
MSK	stress-activated protein kinases
NES	Nuclear export signal
NLS	Nuclear localization signal
NMDA	N-methyl-D-aspartate acid
NTKD	N-terminal kinase domain
OGD	oxygen and glucose deprivation
PD	Parkinson's disease

PDK1	3'-phosphoinositide-dependent kinase-1
PGE2	Prostaglandin E2
PGES	Prostaglandin E2 synthase
PIP2	phosphatidylinositol-4,5-bisphosphate
PKA	cAMP-dependent protein kinase
PKC	Protein kinase C
PolyQ	Polyglutamine
PolyP	Polyproline
PP	Perforant path
PSD-93	Post-synaptic density 93
PSD-95	Post-synaptic density 95
Rsk	90-kDa ribosomal S6 kinases
SC	Schaffer collateral
Ser	Serine
SRE	Serum response element
SRF	Serum response factor
Sub	Subiculum
TCF	Ternary complex factor
TFs	Transcription factors
Thr	threonine
VGluT1	Vesicular glutamate transporter 1
W	Weeks
YAC	Yeast articifial chromosome

SUMMARY

I. INTRODUCTION

1. HUNTINGTON'S DISEASE

Huntington's disease (HD) is a progressive, fatal, dominant inherited neurodegenerative disorder (Wexler et al., 1987) characterized by motor, cognitive, behavioral, and psychological dysfunction. This disorder was popularly known as "San Vitus" dance and the first accurate description of the disease came in 1872, by George Huntington, who named it Chorea (from the Greek *choreia* that means dance) due to the marked and characteristic feature of involuntary movements in those who are affected (Huntington, 1872). The prevalence of the mutation is 4-10 cases per 100,000 in populations of Western European descent. 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 patient usually dies within 10 to 20 years after the first symptoms appear, as there is currently no treatment to prevent or delay disease progression (Ambrose et al., 1994). The early onset is associated with increased severity as well as with a more rapid disease progression (Beighton and Hayden, 1981; Conneally, 1984).

The Huntington's Disease Collaborative Research Group discovered (1993) that in the first exon of the IT15 gene, that encodes for a protein called huntingtin, there was a repetitive DNA element consisting of three nucleotides: C (cytosine), A (adenine), and G (guanine). In non-HD controls, the number of CAG repeats varied from 6 to 35 ("instability of the trinucleotide repeat"). On the other hand, the same region in individuals with HD always showed 40 or more CAG repeats and they conclude that the trinucleotide repeat expansion was the responsible for HD (Table 1). But then, there were some individuals with no symptoms who show "intermediate-sized" CAG repeats from 27 to 35 (Semaka et al., 2010) whose are at risk of transmitting the disease to their children, due to the phenomenon known as "genetic anticipation" (Ranen et al., 1995). This expanded CAG repeats are not stable and tend to expand from generation to generation specifically when the disease gene is inherited from the father (Pearson, 2003). The onset of symptoms has been inversely correlated with the lengthening of the mutation (Rubinsztein et al., 1996).

HD symptoms comprise adult-onset personality changes, generalized motor dysfunctions, and cognitive decline. In the early stages, HD is classically associated with progressive emotional, psychiatric, and cognitive disturbances (Folstein et al., 1986). Commonly reported symptoms in HD include progressive weight loss, alterations in sexual behavior, and disturbances in the

Repeat count	Classification	Disease status	Risk to offspring
< 26	Normal	Not affected	None
27 - 35	Intermediate	Not affected	Elevated but <<50%*
36 - 39	Reduced Penetrance	May or may not be affected	50%
> 40	Full Penetrance	Affected	50%

* "genetic anticipation"

Table 1. Classification of the huntingtin trinucleotide repeat. Table showing the relation between the number of trinucleotides CAG in the exon 1 of the huntingtin gene and the resulting disease status. Disease status depends on the number of CAG repeats.

wake-sleep cycle that occur very early in the course of the disease and may partly be explained by hypothalamic dysfunction (Politis et al., 2008). The progression of the disease is characterized by motor signs, accelerating dementia, or gradual impairment of the mental processes involved incomprehension, verbal fluency, reasoning, judgment, and memory (Bachoud-Levi et al., 2001). Due to increasingly severe dementia and progressive motor dysfunction, patients with advanced HD may become unable to walk, have poor dietary intake, eventually cease to talk, and are not able to care for themselves, therefore potentially requiring long-term institutional care. Life-threatening complications may result from injuries related to serious falls, poor nutrition, infection, choking, and inflammation. Most HD patients eventually succumb due to aspiration pneumonia because of swallowing difficulties (Folstein et al., 1986).

1.1. HUNTINGTIN PROTEIN

1.1.1. Huntingtin structure

Huntingtin is a 348-kDa protein and it is encode by a single gene, the huntingtin gene (also called IT15 for Interesting Transcript), which is 200 Kb long, possesses 67 exons and it is localized on the chromosome 4p16.3 (Gilliam et al., 1987; HDCRG, 1993; Wexler et al., 1987). Huntingtin is ubiquitous expressed, being brain and testis the organs that express it at highest levels, and cerebral cortex, striatum and hippocampus the main expression sites within the brain (Borrell-Pages et al., 2006). Huntingtin protein contains a polymorphic stretch of 6 to 35 glutamine (CAG) residues in its N-terminal domain called polyQ region and begins at the 18th amino acid (HDCRG, 1993) (Figure 1). It has been shown that polyQ tract is a key regulator of huntingtin binding to its partners and that huntingtin interacts with a large number of partners (Harjes and Wanker, 2003). Downstream of the polyQ, huntingtin 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 and Bork, 1995). Three main clusters of HEATs have been identified (MacDonald, 2003) and their presence suggests that huntingtin may exert its physiological function by using different protein partners. In higher vertebrates, the polyQ region is followed by a polyproline (polyP) tract that is suggested to act promoting the stabilization of the polyQ tract by keeping it soluble (Steffan et al., 2004). Huntingtin contains well-characterized consensus cleavage sites for caspases and calpains (Hermel et al., 2004; Kim et al., 2001; Wellington et al., 1998) (Figure 1), although the contribution of huntingtin proteolysis to cell functioning is unclear. Additionally, an active COOH-terminal nuclear export signal (NES) sequence and a less active nuclear localization signal (NLS) are present in huntingtin, which might indicate that the protein (or a portion of it) is involved in transporting molecules from the nucleus to the cytoplasm (Xia et al., 2003).

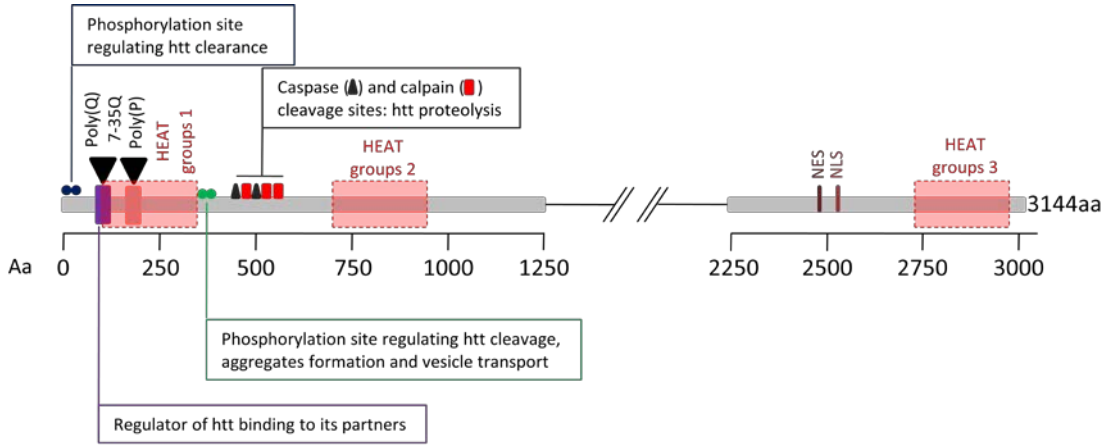

Figure 1. Structure of the huntingtin gene. The diagram represents the huntingtin (htt) amino acids (Aa) sequence with the polyglutamine (PolyQ) and the polyproline (PolyP) tracts, the HEAT repeats domains, the nuclear export signal (NES) and the nuclear localization signal (NLS). The following post-translational modifications are indicated: phosphorylation sites regulating huntingtin clearance, cleavage by caspases and calpains, huntingtin proteolysis, aggregate formation and vesicle transport.

1.1.2. Huntingtin function

Although the gene was discovered some years ago, the physiological role of the protein only has just begun to be understood. Huntingtin is ubiquitously expressed, with highest levels in the neurons of the central nervous system (Ferrante et al., 1997; Trottier et al., 1995). Particularly, huntingtin is enriched in cortical pyramidal neurons in layers III and V that project

to the striatal neurons (Fusco et al., 1999).Within neurons huntingtin is found in the cytoplasm, in neurites and at the synapses; but it can be also detected in the nucleus (Kegel et al., 2002). It associates with various organelles and structures, such as clathrin-coated vesicles, endosomal and endoplasmatic compartments, mitochondria, microtubules and plasma membrane (DiFiglia et al., 1995; Gutekunst et al., 1995; Kegel et al., 2005; Trottier et al., 1995). Given its subcellular localization, huntingtin appears to contribute to various cellular functions in the cytoplasm and the nucleus. Consistent with this, huntingtin interacts with proteins that covers diverse cellular roles including clathrin-mediated endocytosis, apoptosis, vesicle transport, cell signaling, morphogenesis, and transcriptional regulation (Harjes and Wanker, 2003).

Huntingtin is widely expressed in the early developing embryo where it plays an essential role on several processes including cell differentiation and neuronal survival. Inactivation of the mouse gene results in developmental retardation and embryonic lethality at E7.5 (Duyao et al., 1995; Nasir et al., 1995; Zeitlin et al., 1995). Scott Zeitlin's group also showed that neuronal inactivation of huntingtin gene in adult mice causes apoptotic cells in the hippocampus, cortex and striatum and lack of axon fibers (Dragatsis et al., 1998; Mao et al., 2006). These mice also showed progressive motor abnormalities such as those observed in transgenic HD mouse; suggesting that loss of huntingtin function may contribute to the disease phenotype (Dragatsis et al., 1998).

Huntingtin functions in transcription are well established. Huntingtin has been shown to interact with a large number of transcription factors (Holbert et al., 2001; Kegel et al., 2002; McCampbell et al., 2000; Steffan et al., 2000), indicating a role of the protein in the control of gene transcription (Cha, 2000). On one hand, huntingtin acts as an activator of transcription (Dunah et al., 2002) or binds to transcriptional repressors maintaining them in the cytoplasm and allowing the transcription (Zuccato et al., 2003; Zuccato and Cattaneo, 2007); and on the other hand huntingtin can also promote the repression of gene transcription (Boutell et al., 1999; Luthi-Carter et al., 2000). Huntingtin may therefore act in the nervous system as a general facilitator of neuronal gene transcription. Particularly, huntingtin regulates the production of brain-derived neurotrophic factor protein (BDNF), a neurotrophin required for the survival of striatal neurons and for the activity of the cortico-striatal synapses (Canals et al., 2001; Charrin et al., 2005; Zuccato et al., 2001; Zuccato et al., 2003; Zuccato and Cattaneo, 2007), and promotes its vesicular transport along the microtubules (del Toro et al., 2006; Gauthier et al., 2004).

Actually, huntingtin is predominantly found in the cytoplasm where it associates with vesicular structures and microtubules (DiFiglia et al., 1995; Gutekunst et al., 1995; Trottier et al., 1995). Indeed, huntingtin associates with various proteins that play a role in intracellular trafficking (Harjes and Wanker, 2003; Kaltenbach et al., 2007). Huntingtin interacts with many proteins that regulate exo- and endocytosis (Engqvist-Goldstein et al., 2001; Kalchman et al., 1997; Li et al., 1995; Modregger et al., 2002; Singaraja et al., 2002; Wanker et al., 1997). Furthermore, huntingtin may also take part to the pre-synaptic complex through its interaction with different proteins in the pre-synaptic terminal (Parker et al., 2007) and proteins that play a key role in synaptic transmission, as well as in synaptic vesicle and receptor recycling (Smith et al., 2005; Sun et al., 2002). Finally, huntingtin interacts with post-synaptic density 95 protein (PSD-95) regulating the anchoring of *N*-methyl-D-aspartate acid (NMDA) and kainate receptors to the post-synaptic membrane (Sun et al., 2002).

Among all its functions described, huntingtin is believed to have a pro-survival role. Several *in vitro* and *in vivo* studies have demonstrated that expression of the full-length protein protected from a variety of apoptotic stimuli (Imarisio et al., 2008; Leavitt et al., 2001; Leavitt et al., 2006; Rigamonti et al., 2000; Rigamonti et al., 2001; Zuccato et al., 2001). Then, several molecular mechanisms underlying the pro-survival activities of huntingtin have been elucidated: huntingtin appeared to act downstream of mitochondrial cytochrome c release, preventing the activation of caspase-9 (Rigamonti et al., 2001) and caspase-3 (Rigamonti et al., 2000) and it can also prevent the activation of caspase-8 (Gervais et al., 2002).

1.2. PATHOPHYSIOLOGY OF HUNTINGTON'S DISEASE

Neuropathologically, HD is primarily characterized by neuronal loss in the striatum and cortex (Vonsattel and DiFiglia, 1998) (Figure 2a). However, many other nuclei including the globus pallidus, thalamus, hypothalamus, subthalamic nucleus, hippocampus, substantia nigra, and cerebellum also are affected (Heinsen et al., 1996; Kassubek et al., 2004a; Kassubek et al., 2004b; Kassubek et al., 2004c; Kremer et al., 1990; Lemiere et al., 2002; Lemiere et al., 2004; Petersen et al., 2002; Rosas et al., 2003). The most commonly used grading system to assess the severity of HD degeneration was developed by the neuropathologist Jean Paul Vonsattel in 1985 (Vonsattel et al., 1985). It is based on the pattern of striatal degeneration in post mortem tissues and classifies HD cases into five different severity grades (0–4) (Figure 2b). Grade 0 appears indistinguishable from normal brains after gross examination. However, 30–40% neuronal loss can be detected in the head of the caudate nucleus, which is a nucleus located

within the basal ganglia. Grade 1 shows atrophy, neuronal loss, and astrogliosis in the tail and, in some cases, the body of the caudate nucleus. Grades 2 and 3 are characterized by progressive severe gross striatal atrophy. Grade 4 includes the most severe HD cases with atrophy of the striatum and up to 95% neuronal loss (Vonsattel et al., 1985).

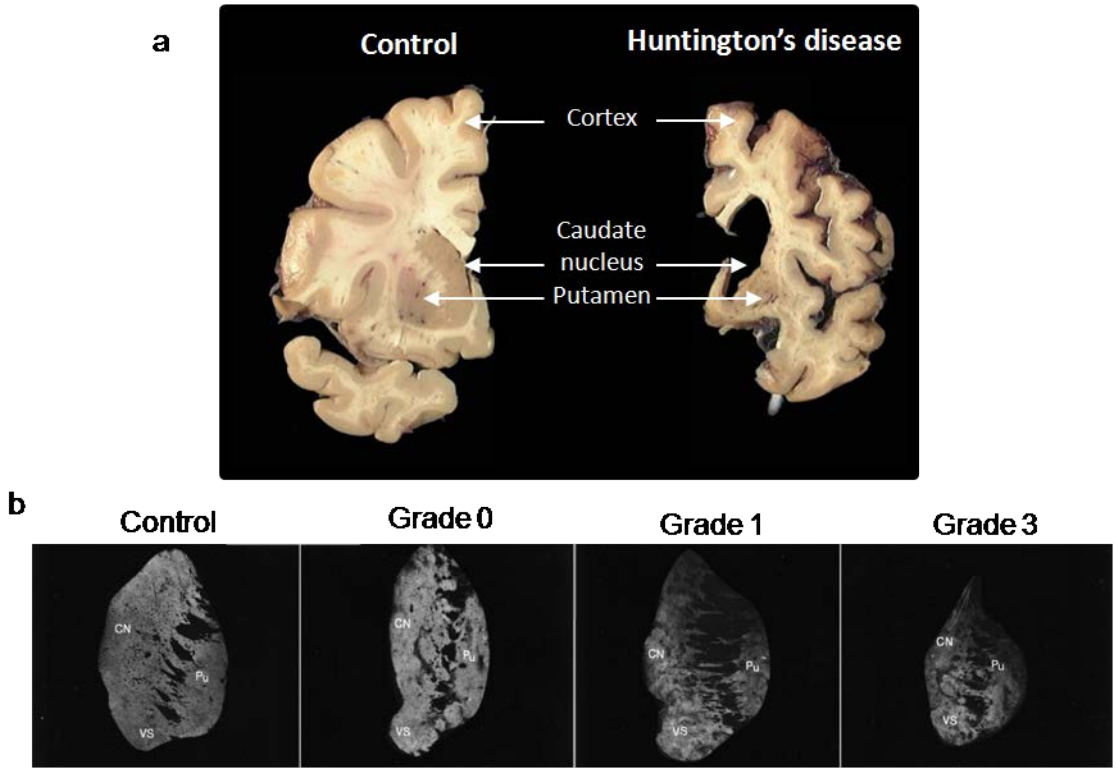

Figure 2. Huntington's disease pathology. a) Human brain coronal sections that shows the degeneration that occurs in the caudate nucleus and putamen as well as the cortical atrophy in Huntington's disease. b) Autoradiograms showing the binding of [^{3}H]SCH23390 to dopamine D_1 receptors in the caudate nucleus and putamen of: control; grade 0 Huntington's disease; grade 1 Huntington's disease; and grade 3 Huntington's disease brains. There was an increasing loss of D1 receptor binding at more advanced grades of Huntington's disease (Glass et al., 2000).

1.2.1. Basal ganglia pathophysiology and motor alterations

The basal ganglia are associated with a variety of functions, including voluntary motor control, procedural learning relating to routine behaviors or "habits", eye movements and cognitive and emotional functions (Brown et al., 1997). The main components of the basal ganglia are the striatum (caudate nucleus and putamen), the globus pallidus, and the subthalamic nucleus (Graybiel, 2000). Not anatomically, but for it physiological function substantia nigra is also considered part of the basal ganglia.

In the striatum, medium-sized spiny neurons constitute 90–95% of all neurons and use GABA as their principal neurotransmitter (Kita and Kitai, 1988), as well as colocalizing specific neuropeptides. Neuropathologically, HD primarily affects the striatum (Vonsattel et al., 1985), as it is evident from the abundant accumulation of mutant huntingtin inclusion formation and cell loss in the medium-sized spiny neurons from the striatum.

There are two populations of GABA-ergic striatal neurons and neuronal death does not occur at the same time in them (Figure 3) (Reiner et al., 1988). In early stages of the disease, neurons which co-express enkephalin and D2 dopamine receptors (Gerfen et al., 1990) and project to the external segment of the globus pallidus via the indirect pathway are preferentially vulnerable. In normal basal ganglia circuitry, the indirect pathway is involved in the inhibition of neurons in the motor cortex and hence the inhibition of voluntary movements (Albin et al., 1989; Alexander and Crutcher, 1990). Death of these enkephalin-containing medium-sized spiny neurons causes the hallmark hyperkinetic, choreaform (dance-like) movements typical of HD. In late stages of the disease, death occurs in medium-sized spiny neurons that co-express substance P and D1 dopamine receptors (Gerfen et al., 1990) and project to the globus pallidus via the direct circuit. This pathway is normally involved in the initiation of voluntary movements (Albin et al., 1989; Alexander and Crutcher, 1990), and so the death of these GABA-ergic/substance P neurons produces hypokinetic and parkinsonian symptoms. Degeneration in the striatum does not occur in all types of neurons, as interneurons are not affected (Dawbarn et al., 1985; Ferrante et al., 1986; Ferrante et al., 1987a; Ferrante et al., 1987b).

One of the major questions in the field of HD is why medium-sized spiny neurons of the indirect pathway are more susceptible to neurodegeneration. There is a different innervation between the indirect and the direct pathway (Lei et al., 2004). A possible functional consequence of this differential innervation is that indirect pathway cells would be subjected to increased glutamate release from cortico-striatal terminals (Ballion et al., 2008; Reiner et al., 2003), which could make them more susceptible to excitotoxicity in HD as the major glutamate input comes from the cortex. This specific striatal degeneration and neuronal dysfunction produces in HD patients, at pre-symptomatic stages, alterations in executive functions, verbal fluency in favor of perseverance in learned responses as emotional and psychiatric (Hahn-Barma et al., 1998; Paulsen et al., 2001), procedural learning, planning, and explicit motor learning arguing (Lawrence et al., 1998; Rosenberg et al., 1995; Schneider et al., 2010). In early-stage symptomatic HD patients, there is also a deficit in discrimination and

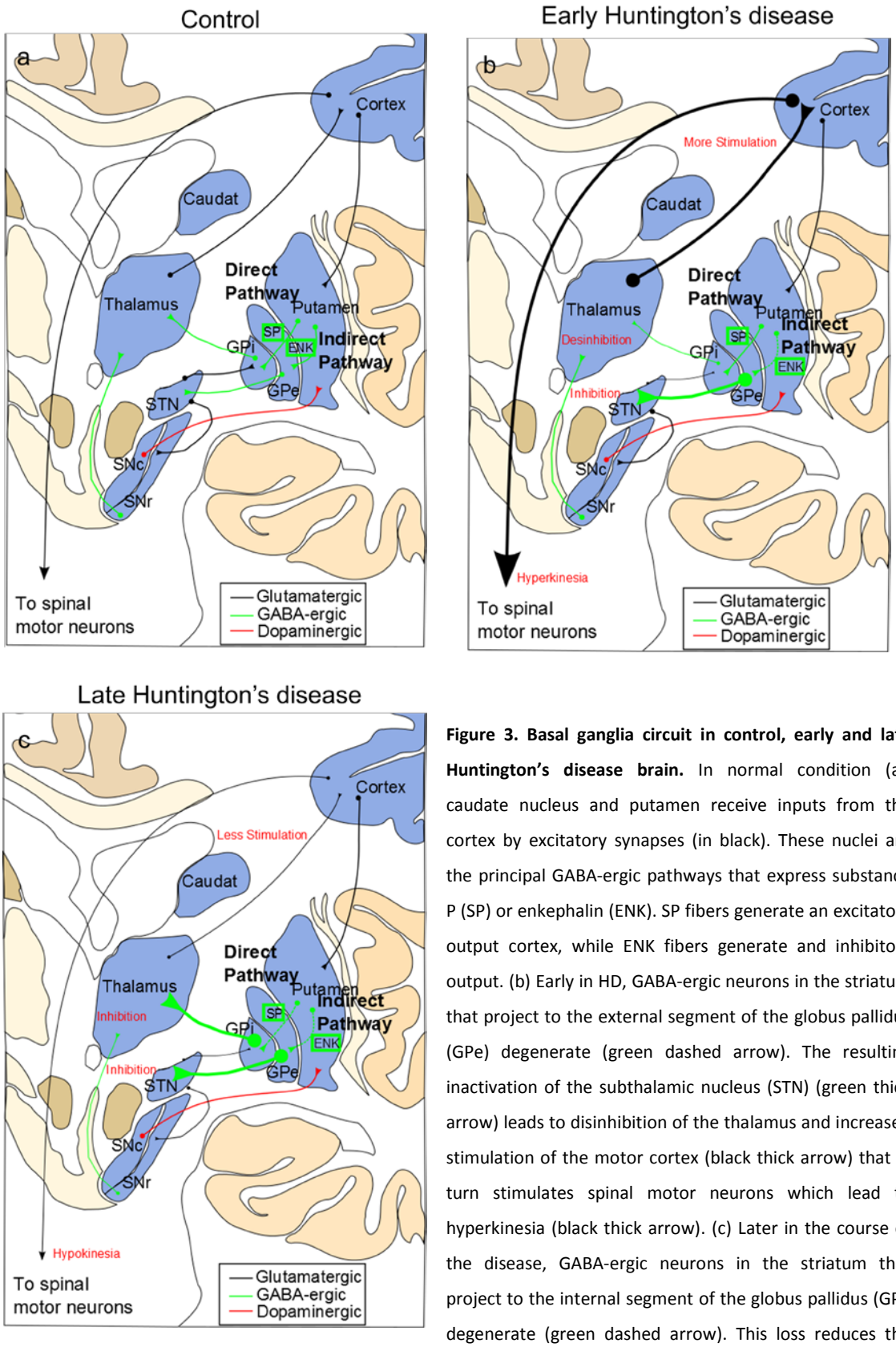

Figure 3. Basal ganglia circuit in control, early and late Huntington's disease brain. In normal condition (a), caudate nucleus and putamen receive inputs from the cortex by excitatory synapses (in black). These nuclei are the principal GABA-ergic pathways that express substance P (SP) or enkephalin (ENK). SP fibers generate an excitatory output cortex, while ENK fibers generate and inhibitory output. (b) Early in HD, GABA-ergic neurons in the striatum that project to the external segment of the globus pallidus (GPe) degenerate (green dashed arrow). The resulting inactivation of the subthalamic nucleus (STN) (green thick arrow) leads to disinhibition of the thalamus and increased stimulation of the motor cortex (black thick arrow) that in turn stimulates spinal motor neurons which lead to hyperkinesia (black thick arrow). (c) Later in the course of the disease, GABA-ergic neurons in the striatum that project to the internal segment of the globus pallidus (GPi) degenerate (green dashed arrow). This loss reduces the inhibition on GPi neurons and increases their firing (green thick arrow), which results in an enhanced inhibition of the thalamus and decreased stimulation of the motor cortex (black thin arrow) that reduces stimulation to spinal motor neurons and produces hypokinesia (black thin arrow).

reversal learning (Lawrence et al., 1999). Moreover, attention, acquisition of psychomotor skills, planning, executive functions and memory progressively decline (Heindel et al., 1988; Ho et al., 2003; Watkins et al., 2000), are also observed. In middle clinically symptomatic HD patients a widespread cognitive decline is observed in which executive function, verbal fluency, perceptual speed and reasoning are strongly affected (Backman et al., 1997; Lemiere et al., 2004). Finally, at more advanced stages, a sub-cortical dementia gradually develops, with alterations in several simple and complex cognitive functions involving slow information processing, decreased motivation, depression, apathy and personality changes (Paulsen et al., 1995; Zakzanis, 1998). Behavioral and anatomical symptoms have also been described in mouse models of HD. Most of the HD mouse models present striatal neurodegeneration and mutant huntingtin nuclear inclusions accompanied by motor deficits analyzed by simple locomotor paradigms as the rotarod and the balance beam (See chapter 1.3. Animal models of HD) (Brooks et al., 2012; Davies et al., 1997; Mangiarini et al., 1996; Menalled and Chesselet, 2002).

1.2.2. Hippocampal pathophysiology and cognitive deficits

The hippocampus is perhaps the most studied structure in the brain; together with the amygdale and the nucleus accumbens, it forms the central axis of the limbic system. This brain region is known to be critical to the formation of episodic/event memory, spatial learning and awareness, navigation, object recognition and visual memory between others (Bliss and Collingridge, 1993; Eichenbaum, 2001; Kandel, 2001; Milner et al., 1998; O'Keefe et al., 1979), and the hippocampo-fronto-striatal pathway is involved in higher cognitive tasks such as goal-directed behavior executive function (Thierry et al., 2000). Moreover, the forms of neuronal plasticity known as long-term potentiation (LTP) and long-term depression were first discovered to occur in the hippocampus and have often been studied in this structure (Bliss and Collingridge, 1993; Lynch, 2004). LTP is widely believed to be one of the main neural mechanisms by which memory is stored in the brain.

Anatomically, the hippocampus is divided in different cell areas called *Cornus Ammonis* 1, 2 and 3 (CA1-CA3) and dentate gyrus (Figure 4) (O'Mara, 2005; Somogyi and Klausberger, 2005; Witter et al., 2000). The CA areas are filled with densely packed pyramidal cells and the dentate gyrus is actually a separate structure form by a tightly packed layer of granule cells. The perforant path is the major input to the hippocampus. The axons of the perforant path arise principally in layers II and III of the entorhinal cortex, with minor contributions from the

deeper layers IV and V. Axons from layers II project to the granule cells of the dentate gyrus and pyramidal cells of the CA3 region, while those from layers III project to the pyramidal cells of the CA1 and the subiculum. Granule cells of the dentate gyrus send their axons, called mossy fibers, to CA3. Region CA3 combines this input with signals from entorhinal cortex layer II and sends extensive connections within the region and also sends connections to region CA1 through a set of fibers called the Schaffer collateral. Region CA1 also receives input from entorhinal cortex layer III. In turn, pyramidal cells of CA1 projects to the subiculum and deep layers of the entorhinal cortex. The subiculum is the final stage in the pathway, combining information from the CA1 projection and entorhinal cortex layer III to also send information along the output pathways of the hippocampus.

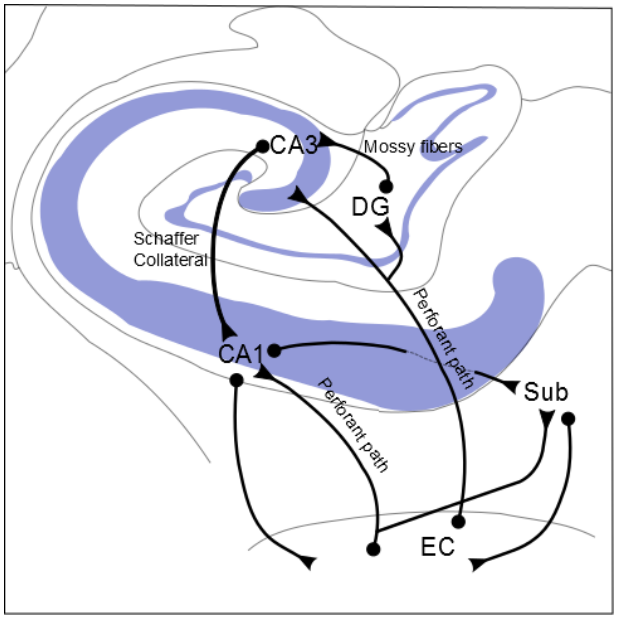

Figure 4. Hippocampal circuits. The hippocampus forms a principally uni-directional network, with inputs from the Entorhinal Cortex (EC) that forms connections with the Dentate Gyrus (DG) and CA3 pyramidal neurons via the layer II perforant path. CA3 neurons also receive inputs from the DG via the mossy 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 (Sub). These neurons in turn send the main hippocampal output back to the Entorhinal Cortex, forming a loop.

The primary sites of neurodegeneration in HD are the striatum and cerebral cortex; however, in later stages of the disease, neuronal cell loss is also evident in other brain regions, including the hippocampus (Folstein et al., 1986; Hedreen et al., 1991; Spargo et al., 1993; Utal et al., 1998; Vonsattel et al., 1985). Although hippocampal pathways and interaction between different areas and cell types are highly described, changes in the connectivity due to HD are still poorly studied in comparison with cortico-striatal pathway (see chapter 1.2.1. and figure 3).

Several studies in patients and in mouse models of HD have been carried out to identify cognitive deficits and evaluate their progression. Unfortunately, studies in humans have mainly focused on the cortico-striatal function, and less frequently on the hippocampal formation (Montoya et al., 2006). However, some cognitive tests/abilities involve hippocampal integrity

in addition to cortico-striatal function. There is now considerable evidence that a range of alterations in cognitive function, including problems in attention, planning, memory and visuo-spatial abilities, can appear prior to the onset of motor abnormalities in patients (Foroud et al., 1995; Lange et al., 1995; Lawrence et al., 1996; Lawrence et al., 1998; Lawrence et al., 1999; Lemiere et al., 2002; Lemiere et al., 2004; Mohr et al., 1991), as well as, evidences of clear morphological alterations and hippocampal atrophy in these HD patients (Rosas et al., 2003) that correlates with the punctuation in The Unified Huntington's disease Rating Scale (Jech et al., 2007).

Cognitive deficits typically hippocampal dependent have been observed in many HD mouse models (Giralt et al., 2012; Ramaswamy et al., 2007; Van Raamsdonk et al., 2005; Zuccato et al., 2010). These deficits may be due in part to accumulation of mutant huntingtin aggregates in the hippocampus of these animals (Ramaswamy et al., 2007) as hippocampal neuronal intranuclear inclusions have been described in different mouse models of HD (McBride et al., 2006; Menalled et al., 2003; Milnerwood et al., 2006; Wheeler et al., 2000). Similarly, studies carried out in mouse models show that synaptic plasticity alterations are found, not only in striatum but also in the hippocampus (Milnerwood et al., 2006), further supporting an important role for hippocampal impairment in cognitive dysfunction in HD. Several tasks involving hippocampal and cortical structure such as the Morris water maze (Morris et al., 1986), spontaneous alternation task (Aggleton et al., 1989; Gerlai, 1998) and novel object recognition (Broadbent et al., 2004) have been used to evaluate learning and memory in HD mouse models. Alterations in spatial learning and navigation have been observed in different pre-symptomatic HD mouse models (Brooks et al., 2012; Giralt et al., 2011b; Luesse et al., 2001; Nithianantharajah et al., 2008). Moreover, object and spatial recognition memories are also impaired (Giralt et al., 2011b; Giralt et al., 2012; Nithianantharajah et al., 2008). These specific cognitive abnormalities (Brooks et al., 2006; Grote et al., 2005; Lione et al., 1999; Mazarakis et al., 2005; Murphy et al., 2000; Pang et al., 2006; Van Raamsdonk et al., 2005; von Horsten et al., 2003) also precede motor changes in adult-onset of these mouse models of HD (Mazarakis et al., 2005; Van Raamsdonk et al., 2005).

1.3. HUNTINGTON'S DISEASE ANIMAL MODELS

Since the discovery of HD gene (HDCRG, 1993) various research groups have aimed to discover the molecular mechanisms underlying this neurodegenerative pathology. One major advance in HD research was the generation of various genetic models of HD. The most widely studied

animal models employ rodents, but animal models of HD exist in non-mammalian species as well and more recently in non-human primates. Invertebrate models like *Caenorhabditis elegans* (Segalat and Neri, 2003) and *Drosophila melanogaster* (Gunawardena et al., 2003) allow a rapid and high-throughput testing of specific hypotheses and novel therapeutic strategies (Brignull et al., 2006). However, through evaluation of disease processes and research to develop novel therapeutics ultimately require models with more complexity. 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 to match the time course, mechanism, and purpose of the study.

Mouse models of HD can be classified into two broad categories, genetic and toxic models. Initially, toxic models of HD were broadly used as the actual genetic mutation responsible for the disease was not discovered since 1993 (HDCRG, 1993). The transgenic mouse models express either a truncated or full-length form of the mutant huntingtin gene inserted either randomly into the genome (transgenic models) or specifically into the rodent huntingtin gene locus (knock-in models) (Figure 5).

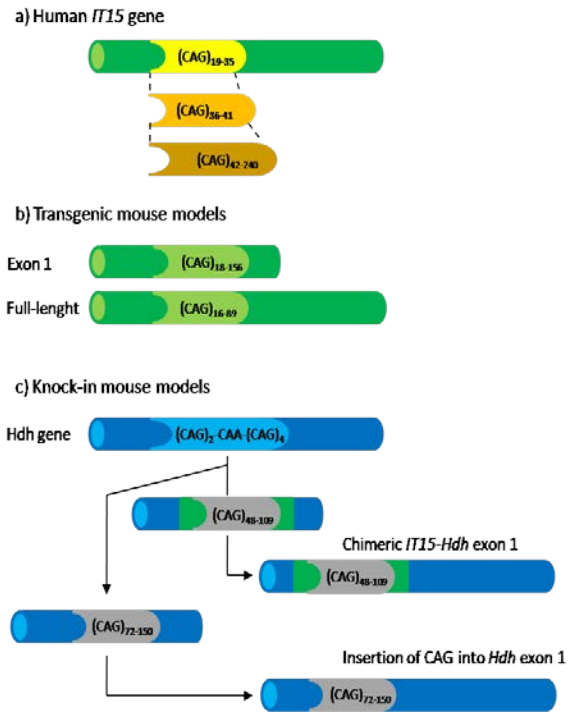

Figure 5. Schematic representation of the gene encoding huntingtin in humans (IT15), and constructs used for the generation of mouse models of Huntington's disease. a) The CAG repeats in exon 1 of IT15. The normal range of repeats 19-35, the range of low penetrance 36-41, and disease range of more than 41 repeats. b) In transgenic mouse models either a fragment or full-length of IT15 with an expanded CAG repeat region is randomly inserted into the genome. c) Knock-in mouse models were generated by gene targeting of the endogenous mouse Hdh gene (mouse homolog of human IT15). These mice have either a chimeric IT15-Hdh exon 1 with an expanded CAG repeat or insertion of an expanded CAG repeats.

1.3.1. Quinolinic acid models.

Historically, non-genetic models have dominated the field of HD research. Non-genetic models typically induce cell death either by excitotoxic mechanisms or by disruption of mitochondrial machinery, which are both mechanisms of degeneration seen in the HD brain. Quinolinic acid, an NMDA receptor agonist, has been one of the most commonly used excitotoxic agents in both rodent and primate models of HD.

Normal levels of quinolinic acid do not cause damage, but only small increases in quinolinic acid levels cause toxicity. Quinolinic acid is incapable of crossing the blood-brain barrier and is therefore experimentally administered directly to the striatum (Foster et al., 1984). It causes striatal neurodegeneration in rats (Bordelon et al., 1997; Ribeiro et al., 2006), mice (McLin et al., 2006), and primates (Emerich et al., 2006; Kendall et al., 2000). Striatal quinolinic acid lesions cause symptoms that often mimic deficits seen in early (but not later) stages of HD. Importantly, the quinolinic acid model influences cognitive function (Furtado and Mazurek, 1996), such as mutant huntingtin. There are several similarities between pathology observed in the HD brain and in the quinolinic acid model. Similarly to HD, intrastriatal injection of quinolinic acid-induced GABA-ergic cell death which involves degeneration of enkephalin-positive and substance P-positive medium-sized spiny neurons (Beal et al., 1986). Moreover, quinolinic acid-induced cell death may mimic the mechanism of neuronal death seen in HD brains (Beal et al., 1991; Ferrante et al., 1993; Roberts et al., 1993). Although the exact mechanism of cell death in HD is unknown, glutamate-induced excitotoxic cell death has been postulated. Administration of quinolinic acid to the NMDA-receptor-rich striatum causes an increase in Ca^{2+} influx, a decrease in adenosine triphosphate (ATP) production, and corresponding excitotoxic cell death in a manner that mimics certain aspects of neurodegeneration in human HD (Bordelon et al., 1997).

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

Then, emerging molecular technology has enabled the development of genetic murine to capture the hereditary nature of HD (Table 2). With respect to the different constructs, the original R6 mouse lines were the first mutant mouse models of HD (Mangiarini et al., 1996). Bates and colleagues, created these transgenic murine line by inserting a 1.9-kB fragment derived from the 5-end of the human huntingtin gene into the mouse genome (Mangiarini et al., 1996). This fragment contains only exon 1 of the human huntingtin gene and expresses approximately 115 CAG repeats for the R6/1 mice and 145 CAG repeats for the R6/2 mice.

Mouse model	Promoter	CAG repeats	Aggregates	Motor symptoms	Cognitive deficits	Lifespan	References
Exon 1 models							
R6/1	*IT15* (Human *Huntingtin)*	145	9 w	14 w	12 w	32-40 w	(Mangiarini et al., 1996) (Canals et al., 2004) (Giralt et al., 2011b)
R6/2	*IT15* (Human *Huntingtin)*	115	3-4 w	6-8 w	4-8 w	13-16 w	(Carter et al., 1999) (Davies et al., 1997) (Mangiarini et al., 1996)
N171-82Q	Mouse prion protein	82	6.5 m	12 w	14 w	24-30 w	(Mievis et al., 2011) (Schilling et al., 1999)
HD94-tet off	CamKIIα-tTA Bi Tet-O-CMV minimal	94	12 w	12 w	Not reported	Normal	(Martin-Aparicio et al., 2001) (Yamamoto et al., 2000)
Full-length models							
YAC72	*IT15* (Human *Huntingtin)*	72	No	16 m	Not reported	Normal	(Hodgson et al., 1999)
YAC128	*IT15* (Human *Huntingtin)*	128	14 m	4 m	7 m	Normal	(Brooks et al., 2012) (Southwell et al., 2009)
Knock-in models							
Hdh^{Q94}	Mouse Huntingtin (Chimeric Hdh-IT15 exon 1)	94	14 m	4 m	4 m	Normal	(Trueman et al., 2007) (Brooks et al., 2012)
Hdh^{Q111}	Mouse Huntingtin (Chimeric Hdh-IT15 exon 1)	109	10 m	24 m	8 m in $Hdh^{Q7/Q111}$	Normal	(Giralt et al., 2012) (Wheeler et al., 2000)
Hdh^{Q150}	Mouse Huntingtin (Insertion of CAG repeats in to exon 1 *Hdh* gene)	150	10 m	10 m	4 m	Normal	(Brooks et al., 2012)

Table 2. Genetically modified mouse models of Huntington's disease. The table includes information about the promoter under which the mutation is expressed; the CAG repeat number, the onset of mutant huntingtin aggregation, motor and cognitive symptoms, and lifespan. Weeks (w); months (m).

Expression of the mutant huntingtin gene is driven by the human huntingtin promoter. The mutant gene is expressed in all cells of R6/1 and R6/2 mice at 31% and 75% of the level of the wild-type gene, respectively (Mangiarini et al., 1996). The R6/2 mice have a very aggressive behavioral phenotype and average age at death is 14 weeks. R6/2 mice show deficits in motor and cognitive function. Motor symptoms, that can be measured in these mice as early as 6-8 weeks of ages (Carter et al., 1999), include resting tremor, chorea-like movements, stereotypic involuntary grooming movements, and dystonia of the limbs when suspended by the tail (clasping behavior) (Mangiarini et al., 1996; Stack et al., 2005). As well, these mice also show cognitive deficits (Lione et al., 1999) as early as 4 weeks, before the onset of overt motor symptoms. R6/2 mice also suffer from epileptic seizures, spontaneous shuddering movements and reduced body weight (Mangiarini et al., 1996). R6/1 contains less CAG repeats, making their behavioral phenotype relatively mild and average age at death is about 32-40 weeks (Mangiarini et al., 1996). Mice show cognitive decline that starts at early as 12 weeks and share the same motor deficits than R6/2 mice but starting at 14 weeks of age (del Toro et al., 2006; Giralt et al., 2011a; Mangiarini et al., 1996).

R6 mice develop progressive mutant huntingtin-positive inclusions in the brain. Indeed, R6/2 immunostaining detected conspicuous inclusions in the nucleus of most brain neurons as early as 7 weeks of age and nuclear inclusions were preceded by an abnormal location of huntingtin in the nucleus, which was observed at 4 weeks (Davies et al., 1997). In R6/1 cellular inclusions start appearing throughout the brain at 9 weeks and their numbers increase with age (Mangiarini et al., 1996) (Figure 6). Neuronal atrophy is a general event within the brain in both mice strains that curses together with ventricular enlargement but minimal neuronal loss (Canals et al., 2004; Mangiarini et al., 1996; Turmaine et al., 2000).

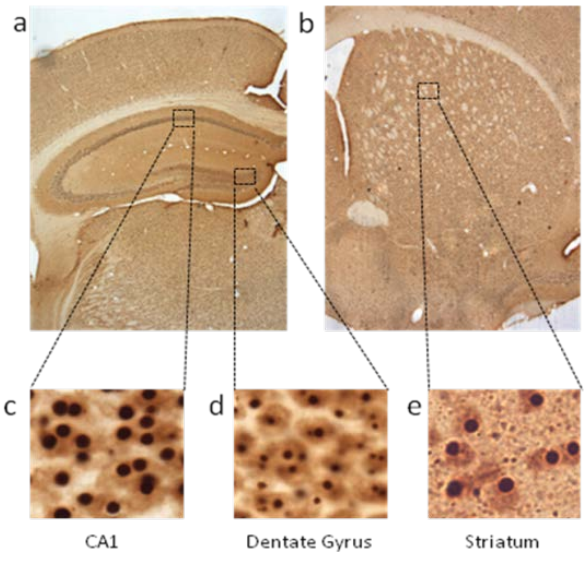

Figure 6. Formation of neuronal intranuclear inclusions of mutant huntingtin in the hippocampus and the striatum of R6/1 mouse model of Huntington's disease. Representative microphotography of striatal (a) and hippocampal (b) brain coronal sections stained with EM48 were obtained from 18-weeks-old R6/1 mice. Amplified sections of the CA1 (c) and the dentate gyrus (d) of the hippocampus, and the striatum where clear intranuclear inclusions of mutant huntingtin can be observed. Photos were realized by Marta Anglada.

On the other hand, Borchelt and colleagues generated the N171-82Q transgenic mouse model by inserting the first 171 amino acids from the N-terminal of the human huntingtin gene into the mouse genome (Schilling et al., 1999). Expression of the N-terminal fragment of huntingtin in this model is driven by the mouse prion promoter and its expression is restricted to neurons. This model has a later onset of motor symptoms, beginning at week 12 (McBride et al., 2006). With respect to cognition, the N171-82Q mice show deficits on working and reference memory at 14 weeks (Ramaswamy et al., 2007).

1.3.3. Full-length mouse models: YAC and Knock-in

Mice that express the full-length mutant huntingtin gene can be classified in those in which mutant huntingtin is delivered in a yeast artificial chromosome (YAC) and those in which a knock-in mutation has been performed to modify the endogenous murine huntingtin (Table 2). YAC transgenic mice are a creation of Hayden and colleagues, who used a YAC vector system to express the entire human huntingtin gene under the control of the human huntingtin promoter (Hodgson et al., 1999). YAC mouse strains contain either 72 or 128 CAG repeats. Both show neuronal loss, preferentially in the lateral striatum (Hodgson et al., 1999; Slow et al., 2003). YAC 72 mice show reduced body weight and motor abnormalities at 16 month of age, while YAC 128 mice have a more severe phenotype showing a progressive motor decline that starts at 4 month of age. Studies of cognitive dysfunction in the YAC 128 model show severe deficits at 7 month of age (Van Raamsdonk et al., 2005). Only the YAC 128 strain striatum shows positive staining for inclusion bodies at 14 months (Brooks et al., 2012). These mice exhibit normal life span.

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, however, present a late onset of the disease and a mild progression of the pathology. Furthermore, although currently expanding studies are being done, nowadays these models are poorly studied. Motor deficits have been observed in the different strains, which an age of onset depending on the number of CAG repeats and the testing methods used (Brooks et al., 2012; Menalled et al., 2000). Similar happens in terms of cognitive decline. Memory deficits has been reported in knock-in mice, starting at 4 months of ages for Hdh^{Q94} and Hdh^{Q150} and at 8 months in mouse heterozygous for Hdh^{Q111} (Brooks et al., 2012; Giralt et al., 2012).

2. CONSEQUENCES OF POLYGLUTAMINE EXPANSION IN HUNTINGTON'S DISEASE

Although it is well established that the cause of the disease is the expanded polyQ above 35 and that the polyQ length accounts for the disease onset (HDCRG, 1993; Snell et al., 1993), the pathological mechanisms are not fully understood yet.

The presence of mutant huntingtin results multiple pathophysiological changes among which the most severe include protein aggregation, transcriptional deregulation and chromatin remodeling, decreased trophic support, alteration in signaling pathways, disruption of calcium homeostasis, excitotoxicity, and caspase activation. Although the presence of mutant huntingtin could have terrible consequences for neurons, it is also suggested that in addition to the gain of toxic properties, the loss of physiological activities of the normal protein could also contribute to disease pathogenesis (Zuccato and Cattaneo, 2009; Cattaneo et al., 2005).

2.1. MUTANT HUNTINGTIN AGGREGATES FORMATION

Several caspases and calpains cleave mutant huntingtin (Gafni and Ellerby, 2002; Gafni et al., 2004; Goldberg et al., 1996; Hermel et al., 2004; Kim et al., 2001; Wellington et al., 2000; Wellington and Hayden, 2000; Wellington et al., 2002) and produce N-terminal mutant fragments that are more toxic and more susceptible to aggregation (Figure 1) (Kim et al., 2001; Ratovitski et al., 2009). Abnormal activation of these proteases could result from various insults received by neurons in HD such as excessive levels of cytosolic Ca^{2+}, reduced trophic support and activation of the apoptotic machinery.

The proteolytic cleavage of mutant huntingtin into N-terminal fragments containing the polyQ stretch is sufficient to reproduce HD pathology in animal models of the disease (Davies et al., 1997; Palfi et al., 2007; Schilling et al., 1999), and it is required to induce neurodegeneration (Saudou et al., 1998; Wellington et al., 2000). Reducing polyQ huntingtin cleavage decreases its toxicity and slows disease progression (Gafni et al., 2004; Wellington and Hayden, 2000). In addition, expression of exon 1 of mutant huntingtin in animal models (see chapter 1.3) results in an increased toxicity and more acute progression of the disease compare to expression of full-length mutant huntingtin with the same polyQ expansion (Figure 5) (Hackam et al., 1998). Once cleaved, N-terminal fragments of mutant huntingtin trends to translocate from the

cytosol into the nucleus increasing its nuclear accumulation (Cornett et al., 2005). In the nucleus of neurons, N-terminal fragments of mutant huntingtin form neuronal intranuclear inclusions (DiFiglia et al., 1997; Goldberg et al., 1996), which may interfere with normal transcriptional control (Davies et al., 1997; DiFiglia et al., 1997), and aggregates can be found also in the cytoplasm and the dendrites of affected neurons (DiFiglia et al., 1997). It is well established that the nuclear localization of mutant huntingtin is required for neuronal death, but the role of aggregate formation in the pathology of HD is widely debated (Saudou et al., 1998; Scherzinger et al., 1999). Although some evidence suggests that mutant huntingtin-associated toxicity is linked to soluble mutant huntingtin (Saudou et al., 1998), inclusions are a clinical hallmark of HD pathology and have been reported in several transgenic mouse models (Brooks et al., 2012). Increasing evidences suggest that reduction in mutant huntingtin aggregates formation is directly linked with alleviation in behavioral dysfunction in HD mouse models (Chen et al., 2011; Giralt et al., 2011a; Yamamoto et al., 2000). Over the course of the disease, the accumulation of mutant huntingtin aggregates may overload the proteasome degradation system promoting a progressive accumulation of misfolded and abnormal proteins, further increasing the rate of protein aggregation (Jana et al., 2001; Waelter et al., 2001).

2.2. EXCITOTOXICITY AND CALCIUM ALTERATIONS

Excitotoxicity contributes to neuronal degeneration in many acute as well as chronic diseases of the central nervous system (Mehta et al., 2013). The presence of mutant huntingtin engenders multiple cellular dysfunctions including an increase of pathological excitotoxicity. PolyQ expansion produces an hyper-activation of NMDA receptors (and kainate receptors) (Song et al., 2003; Sun et al., 2001); stabilizes NMDA receptors in the post-synaptic membrane (Li et al., 2002a; Roche et al., 2001; Vissel et al., 2001); inhibits the uptake and release of glutamate at the synapses (Li et al., 2003); and can also sensitize the inositol (1,4,5)-triphosphate receptor type 1 (IP3R) located in the membrane of the endoplasmatic reticulum (Tang et al., 2003). In addition, mutant huntingtin can contribute to excitotoxicity by decreasing the expression of the major astroglial glutamate transporter (Lievens et al., 2001), which reduces the glutamate uptake (Shin et al., 2005). All these alterations promote glutamate-mediated excitotoxicity by a massive increase of intracellular Ca^{2+}, which affect calcium homeostatic mechanism (Chen et al., 2000) and lead to deleterious consequences. Imbalance in the calcium homeostasis has been previously reported in different HD mice (Chen et al., 2011; Hansson et al., 2001; Hodgson et al., 1999; Tang et al., 2005) that it is in

agreement with consistent changes in the expression levels of many Ca^{2+} signaling proteins (Kuhn et al., 2007). Moreover, different proteins involved in neuronal Ca^{2+} signaling have been proposed as attractive targets for developing therapies for HD (Bezprozvanny, 2009).

2.3. TRANSCRIPTIONAL DYSREGULATION

Many studies have provided evidences that transcription may be a major target of mutant huntingtin (Dunah et al., 2002; Luthi-Carter et al., 2002; McCampbell et al., 2000; Steffan et al., 2000), as gene dysregulation occurs before the onset of symptoms (Cha, 2007). Subsequently, a large number of studies showed transcriptional abnormalities in HD (Arzberger et al., 1997; Cha, 2007; Luthi-Carter et al., 2002). These discoveries include demonstration of consistent changes in mRNA levels, direct interaction between mutant huntingtin and proteins of the transcriptional machinery, and inhibition of enzymes involved in chromatin remodeling (Figure 7).

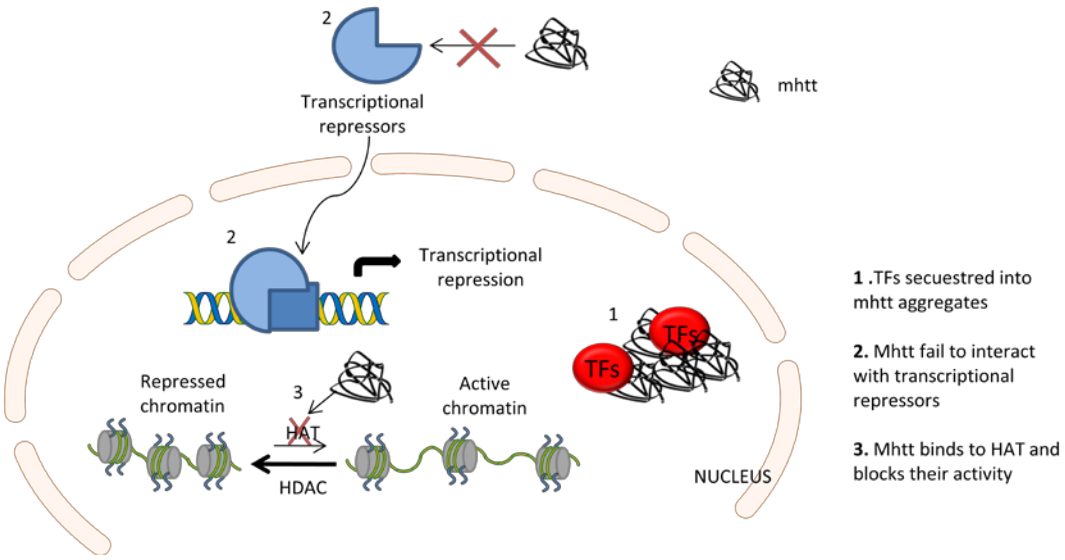

Figure 7. Potential mechanisms of transcriptional dysregulation in Huntington's disease. Different mechanisms by which mutant huntingtin (mhtt) disrupt normal transcriptional activity: (1) Mutant huntingtin can bind transcription factors (TFs) and sequestered them into intranuclear inclusions. (2) Mutant huntingtin loss the capacity to bind to transcriptional repressors allowing them to get into the nucleus and repress transcription. (3) Transcription depends on the acetylation status of histones, regulated by activities of histone acetyl transferases (HATs) and histone deacetylases (HDACs). Mutant huntingtin interaction with HATS inhibits proper histone acetylation and causes repression of the transcription.

Initially it was shown that mutant huntingtin establishes abnormal protein-protein interactions with several nuclear proteins and transcription factors, recruiting them into the aggregates and inhibiting their transcriptional activity (Figure 7). Some examples include TATA-binding

protein (Schaffar et al., 2004), CREB (cyclic-adenosine monophosphate (cAMP) response element (CRE) binding protein)-binding protein (CBP) (Schaffar et al., 2004; Steffan et al., 2000), specificity protein-1 (Li et al., 2002b) and the TATA-binding protein-associated factor II130 (Dunah et al., 2002).

On the other hand, mutant huntingtin can also fail to interact with other transcription factors (Figure 7), as in the case of neuron restrictive silencer element-binding transcription factor. Mutant huntingtin fails to interact with repressor element-1 transcription/neuron restrictive silencer factor (NRSE), so then the complex can translocate from the cytoplasm into the nucleus and bind NRSE repressing a large cohort of neuronal-specific genes, including the *bdnf* (Zuccato et al., 2003). Moreover, huntingtin can also interfere in chromatin structure. The regulation of gene expression depends not only on transcription factors, but also on enzymes that modify chromatin structure (Figure 7). Histone acetyltransferases favors gene transcription through the opening of chromatin whereas histone deacetyltransferases repress gene transcription through chromatin condensation. Mutant huntingtin binds to the acetyltransferase domain of some factors, as CBP and p300/CBP associated factor, blocking their activity (Cong et al., 2005; Steffan et al., 2001).

2.4. SYNAPTIC DYSFUNCTION

Mutant huntingtin has been shown to impair directly the cellular machinery involved in synaptic transmission. Huntingtin is a cytoplasmatic protein closely associated with vesicle membranes and microtubules, suggesting it may have a role in vesicle trafficking, exocytosis and endocytosis (DiFiglia et al., 1995). In addition, it is shown to be associated with various proteins involved in synaptic function (Li et al., 2007; Parker et al., 2007; Sun et al., 2002). Thus, it is probable that mutant huntingtin causes abnormal synaptic transmission in HD (Li et al., 2003; Smith et al., 2005). While many mechanisms have been proposed to explain the neuronal degeneration that occurs in HD (Reddy and Shirendeb, 2012), there are now considerable evidence that synaptic dysfunction is associated with the onset of symptoms. Some good candidates have been suggested: the α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor (AMPAR) subunit GluA1 (Giralt et al., 2009; Nithianantharajah et al., 2008), the dopamine receptors (Augood et al., 1997; Cha et al., 1998; Hodges et al., 2006; Jay, 2003; Luthi-Carter et al., 2000; Pavese et al., 2003; Weeks et al., 1996), alterations in scaffold proteins such as PSD-95 (Nithianantharajah et al., 2008) and changes in the expression of some trophic factors (del Toro et al., 2006; Gauthier et al., 2004; Zuccato et al., 2001; Zuccato and Cattaneo, 2007) are suitable to contribue to synaptic dysfunction in HD.

2.4.1. Alterations in glutamate receptors and transporters

Alterations in proteins involved in glutamatergic signaling have been reported in mouse models of HD (Luthi-Carter et al., 2003; Nithianantharajah et al., 2008)(Figure 8). Since the main hypothesis underlying striatal neurodegeneration in HD has been excitotoxicity, due in part to increase in glutamate release, NMDA receptors were the first glutamate receptors studied. At early stages of the disease, when cognitive and plasticity alterations are detected, no changes in the protein levels of any NMDAR subunit are observed in the striatum and

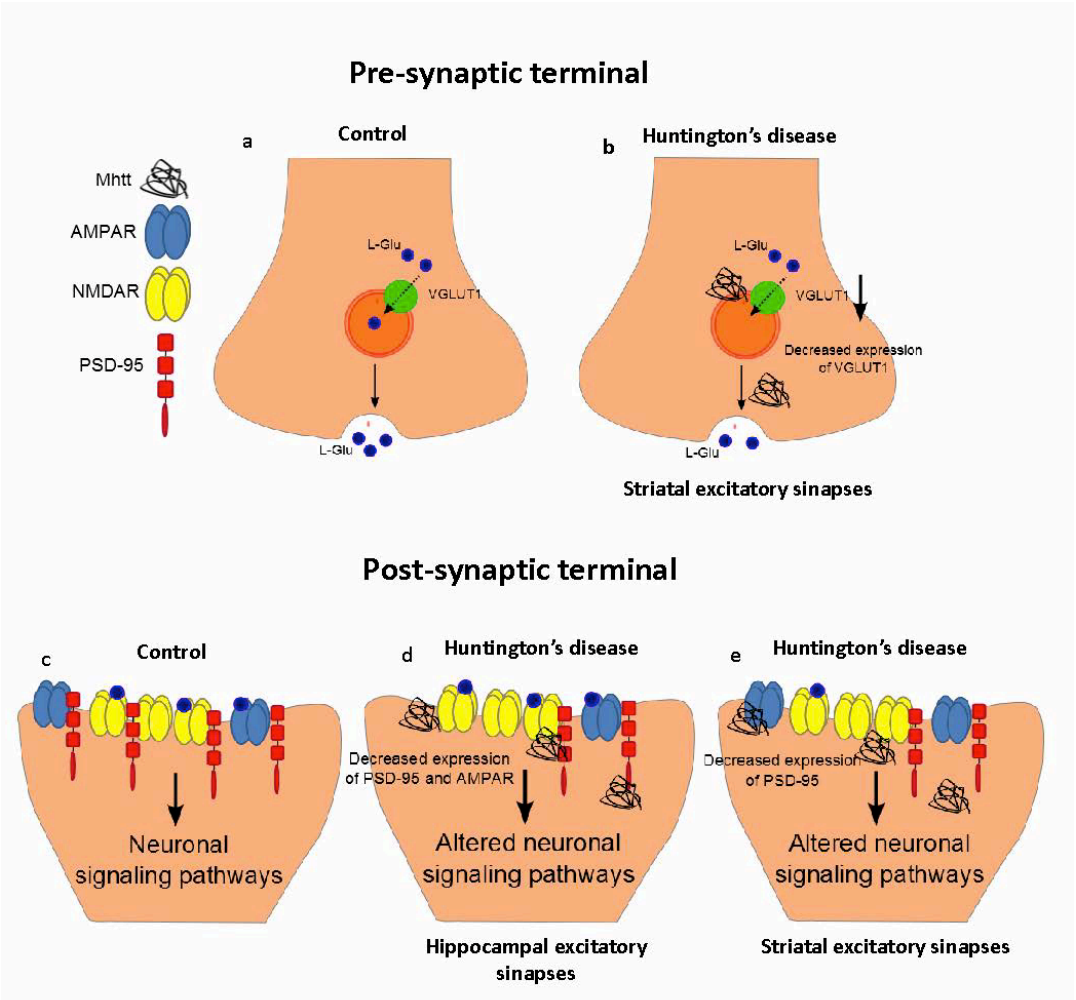

Figure 8. Alteration in synaptic proteins in Huntington's disease. Simplified scheme of a typical dendritic spine and associated structures. a) In control conditions, there are normal levels of vesicular glutamate transporter 1 (VGLUT1) in the pre-synaptic terminal and c) normal levels of α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor (AMPAR), *N*-methyl-d-aspartate (NMDA) and the post-synaptic density- 95 (PSD-95) in the post-synaptic terminal. b) In striatal medium spiny neurons in the presence of mutant huntingtin (mhtt), there is a reduction of VGLUT1 protein levels in the pre-synaptic terminal and e) a reduction of PSD-95 in the post-synpatic terminal. d) In hippocampal excitatory synapses in HD there is a reduction of AMPAR and PSD-95. d and e) Changes in striatal and hippocampal synaptic composition in HD models and patients produces alterations in neuronal signaling pathways.

hippocampus of HD mouse models (Giralt et al., 2009; Jarabek et al., 2004; Torres-Peraza et al., 2008). Conversely, HD mouse models do not respond to intra-striatal NMDAR agonists (Hansson et al., 1999; Hansson et al., 2001; Jarabek et al., 2004), which supports the idea that signaling downstream the receptor is affected in HD (Cepeda et al., 2001) and contributes to synaptic plasticity impairment.

It is noteworthy that in addition to NMDARs other glutamate receptors could play an important role in synaptic dysfunction in HD. A good candidate is GluA1 receptor since it is early down-regulated in the hippocampus of HD mouse models (Giralt et al., 2009; Simmons et al., 2011; Torres-Peraza et al., 2008) and this down-regulation is likely to induce alterations in synaptic signaling and plasticity(Jarabek et al., 2004; Nithianantharajah et al., 2008; Torres-Peraza et al., 2008). In addition to hippocampal reduction of GluA1, abnormal AMPAR function in striatal medium spiny neurons occurs in pre-symptomatic and early symptomatic phases in R6/2 mice (Cepeda et al., 2007). Interestingly, treatment with drugs that enhance AMPARs' function, rescues LTP and object recognition memory in a knock-in mouse model of HD (Simmons et al., 2009) and reduces neuropathology and locomotor dysfunction in R6/2 mice (Simmons et al., 2011).

On the other hand, not only glutamate receptors are altered in HD, but also glutamate transporters, such as the vesicular glutamate transporter 1 (VGluT1) (Giralt et al., 2011a) which contributes to the imbalance of glutamate in neurons and could play a role in cell dysfunction in HD. The expression of VGluT1 in the pre-synaptic terminal correlates with greater expression of synaptic proteins and the reduction of VGluT1 protein levels , as happens in the striatum of R6/2 mice (Giralt et al., 2011a), can reduce synaptic density (Berry et al., 2012) and disrupts cortico-striatal excitatory terminals (Giralt et al., 2011a).

2.4.2. Alterations in membrane-associated guanylate kinase proteins.

In addition to glutamate receptors and transporters, alterations in scaffolding proteins are suitable to contribute to synaptic dysfunction in HD. In this line, the first point where we stopped is the PSD. Many scaffold proteins in the PSD are involved in the regulation of synaptic function, among which the super-family of membrane-associated guanylate kinases (MAGUKs) (Montgomery et al., 2004) and specifically the subfamily of PDZ-domain scaffolding protein Discs-large (DLG) are the most known. MAGUKs are proteins that act as key scaffolds in surface complexes containing receptors, adhesion proteins, and various signaling molecules (Zheng et

al., 2011). DLG subfamily is composed by the PSD-95, synapse-associated protein 97 and 102 and PSD-93 (Garner and Kindler, 1996). PSD-95, the most studied MAGUKs protein, stabilizes and modulate the function of NMDARs and AMPARs (Elias et al., 2006; Funke et al., 2005; Xu, 2011) and it is crucial for signaling downstream NMDARs (Kim and Sheng, 2004; Sheng and Sala, 2001). PSD-95 protein levels are strongly down-regulated in human HD brain, and in the striatum and hippocampus of HD mouse models from very early stages (Figure 8) (Nithianantharajah et al., 2008; Torres-Peraza et al., 2008). Importantly, PSD-95 directly interacts with huntingtin and polyQ expansions increase this interaction removing it from the synapse and altering neurotransmission (Sun et al., 2001).

2.4.3. Abnormal synaptic transmission and plasticity

A number of studies concluded that synaptic plasticity is altered in HD (Cepeda et al., 2007; Milnerwood et al., 2006; Milnerwood and Raymond, 2010; Murphy et al., 2000; Raymond et al., 2011; Usdin et al., 1999). Alterations in cortico-striatal and hippocampal plasticity have been described in HD mice. Electrophysiological studies carried out in cortico-striatal slices from HD mouse models showed severe alterations in these connections (Cepeda et al., 2007; Milnerwood et al., 2006; Milnerwood and Raymond, 2007). Evoked synaptic responses and spontaneous synaptic currents are both altered in the cortico-striatal pathway, and these alterations get worse as the neurological phenotype advances in HD mice (Cepeda et al., 2007). Exon-1 mouse models display deficits in plasticity of primary somato-sensory cortex, impaired cortico-striatal LTP, and progressive loss of cell capacitance of medium spiny neurons (Cepeda et al., 2001; Cybulska-Klosowicz et al., 2004; Kung et al., 2007; Levine et al., 1999). This correlates with a loss of cortico-striatal synaptic markers, reduction of dendritic spines, reduced synaptic transmission, and with an inability to express cortico-striatal LTP in the dorsal striatum (Giralt et al., 2011a; Guidetti et al., 2001; Klapstein et al., 2001; Kung et al., 2007).

In the hippocampus, basal neurotransmission (CA3-CA1 field excitatory post-synaptic potentials) appears normal, whereas LTP is reduced in transgenic (Giralt et al., 2009; Hodgson et al., 1999; Murphy et al., 2000) and knock-in (Usdin et al., 1999) mouse models of HD suggesting that neuronal plasticity dysfunction is synaptic in origin. Abnormalities in hippocampal long-term depression are also observed in different exon-1 (Milnerwood et al., 2006; Murphy et al., 2000) and YAC HD mouse models (Hodgson et al., 1999).

2.5. NEUROTROPHIC DYSFUNCTION: ROLE OF BDNF

Neurotrophins are molecules that promote the development, health and survival of neurons. These signaling molecules exert considerable control over the switch between life and death pathways in cells (Bibel and Barde, 2000). Among neurotrophins, BDNF has emerged as a major regulator of synaptic and neuronal survival (Arancio and Chao, 2007; Liu et al., 2008).

BDNF is a member of the neurotrophin family, which includes the nerve growth factor, the neurotrophin-3 and the neurotrophin 4/5 (Thoenen, 1991). Within this family BDNF has been used as a key molecular target for drug development in HD (Binder and Scharfman, 2004). A deficiency in BDNF-mediated signaling alone is sufficient to cause dendritic abnormalities and neuronal loss in the cerebral cortex and striatum (Baquet et al., 2004; Gorski et al., 2003). Interestingly, BDNF is highly expressed in the regions with highest dysfunction rates in HD (cortex, striatum and hippocampus), and striatal cells are highly dependent on BDNF for function and survival (Altar et al., 1997; Baquet et al., 2004; Gavalda et al., 2004; Perez-Navarro et al., 2000). Moreover, BDNF down-regulation is a widespread phenomenon in different brain regions of HD mouse models as well as in human HD patients (Zuccato and Cattaneo, 2007; Zuccato et al., 2008).

Therefore, deficient BDNF signaling likely plays an important role in synaptic dysfunction, and learning and memory defects in HD. Actually, BDNF levels regulate the onset and severity of cognitive and motor symptoms, and synaptic plasticity deficits in HD mouse models (Canals et al., 2004; Giralt et al., 2009; Lynch et al., 2007). Interestingly, treatments focused on the recovery of BDNF levels successfully improve synaptic plasticity, and motor and cognitive functions in HD mouse models (Gharami et al., 2008; Giralt et al., 2009; Simmons et al., 2009; Simmons et al., 2011).

2.6. ALTERATIONS IN INTRACELLULAR SIGNALING PATHWAYS

Many mechanisms of the cell are controlled by attachment/removal of phosphate groups through the action of protein kinases and phosphatases, respectively. Therefore, alterations in their levels/activity in the presence of mutant huntingtin can impact on cell survival and function. In fact, several kinases and phosphatases have been reported to be altered in HD patients and animal models. Some of these kinases altered in HD are closely related to plasticity phenomena and cell survival, such as cAMP-dependent protein kinase (PKA) (Abel and Nguyen, 2008; Giralt et al., 2011b; Kandel and Pittenger, 1999), the serine/threonine

protein kinase B (also known as Akt) (Gines et al., 2003; Manning and Cantley, 2007; Saavedra et al., 2010), and the mitogen-activated protein kinases (MAPKs): the extracellular signal-regulated kinase 1/2 (ERK1/2), the c-Jun-N terminal kinase (JNK) and p38 MAPK (Adams and Sweatt, 2002; Apostol et al., 2006; Fan et al., 2012; Gines et al., 2010; Roze et al., 2008; Saavedra et al., 2011).

Furthermore, several phosphatases are altered in HD mouse models (Saavedra et al., 2012). In mouse models, most of them are decreased, which seems to be a compensatory mechanism induced in response to mutant huntingtin; but changes in the expression of these phosphatases varies between different HD models. Some examples are the phosphatase calcineurin (Xifro et al., 2008; Xifro et al., 2009), the protein phosphatase 1 and 2A (Ehrnhoefer et al., 2011), the PH domain and leucine rich repeat protein phosphatases (Saavedra et al., 2010) and the striatal-enriched protein tyrosine phosphatase (Saavedra et al., 2011).

3. MOLECULAR MECHANISMS INVOLVED IN NEURONAL SURVIVAL

As reviewed in chapter 2, many mechanisms are altered in the presence of mutant huntingtin. Understanding altered mechanisms due to mutant huntingtin expression in order to find new therapeutic targets to reduce neuronal dysfunction/death in HD, must be a priority. It is believed that there is a balance between positive signaling for cell survives or dies. Many of the pathways involved in these processes are controlled at the level of phosphorylation and transcription. Transcription of target genes is controlled by a series of transcription factors, which are, in turn regulated by number of kinases. One family of such kinases is the MAPKs family.

3.1. INTRACELLULAR SIGNALING PATHWAYS: mitogen-activated protein kinases and 90-kDa ribosomal S6 kinases

MAPKs are a family of serine/threonin protein kinases that play a critical role in transducing multiple signals from the cell surface to the nucleus in all eukaryotic species (Krishna and Narang, 2008). The members of the MAPK family encompass the ERKs and the stress-activated protein kinases, which include both JNKs and p38 kinases (Krishna and Narang, 2008). Historically, the ERK pathway has been implicated in signal transduction pathways initiated by

multitude of growth factors and mitogens that eventually lead to effects on cellular growth and differentiation (Volmat and Pouyssegur, 2001), whereas the JNK and p38 pathways have been implicated in stress-induced signaling pathways leading to apoptotic and inflammatory responses (Xia et al., 2003). Following phosphorylation and activation, each individual MAPK is capable of phosphorylate and regulate a number of specific substrates that include transcription factors, cytoskeletal elements and other protein kinases (Yang et al., 2003).

Among the pathways implicated in HD, those involving ERK signaling cascade are of particular interest (Apostol et al., 2006; Gines et al., 2006; Lievens et al., 2002; Saavedra et al., 2010). ERK 1/2 is a strong anti-apoptotic and pro-survival mediator. Moreover, ERK 1/2 down-regulation is linked to neurodegenerative conditions such as ischemia (Liebelt et al., 2010) and traumatic spinal cord injury (Yu et al., 2010). Recent studies using HD mouse and cellular models provide strong evidence that activation of ERK has neuroprotective effect, while specific inhibition of ERK activation enhances cell death (Apostol et al., 2006; Maher et al., 2011; Saavedra et al., 2010). These studies suggest that pharmacological intervention in MAPK pathways, particularly at the level of ERK activation or downstream ERK, may be an appropriate approach to HD therapy.

ERK 1/2 can phosphorylate different kinases. Most common kinases phosphorylated by ERK1/2 include 90-kDa ribosomal S6 kinases (Rsk) and the mitogen- and stress-activated protein kinases (MSK) (Chen et al., 1992; Deak et al., 1998). In this context, alterations in the phosphorylation of MSK-1 has been previously reported in R6/2 mouse models of HD (Roze et al., 2008); but no data exist about the possible role of Rsk in the context of neurodegenerative disease. This fact, together with previous studies demonstrating alterations in the phosphorylation of the other kinase involved in Rsk activation, 3'-phosphoinositide-dependent kinase-1 (PDK1) (Gines et al., 2003), makes Rsk an interesting target to study in HD.

Rsk is a family constituted by four isoforms (Rsk1-4) of serine/threonine (Ser/Thr) kinases broadly expressed in the brain that regulate important cellular functions, including cell survival (Anjum and Blenis, 2008). Rsk structure contains distinct kinase domain: an N-terminal kinase domain that phosphorylates the substrates of Rsk and a C-terminal kinase domain involved in the activation mechanism of Rsk by autophosphorylation (Chen et al., 1992) (Figure 9). Six different phosphorylation sites have been mapped in Rsk1/2 (Figure 9).

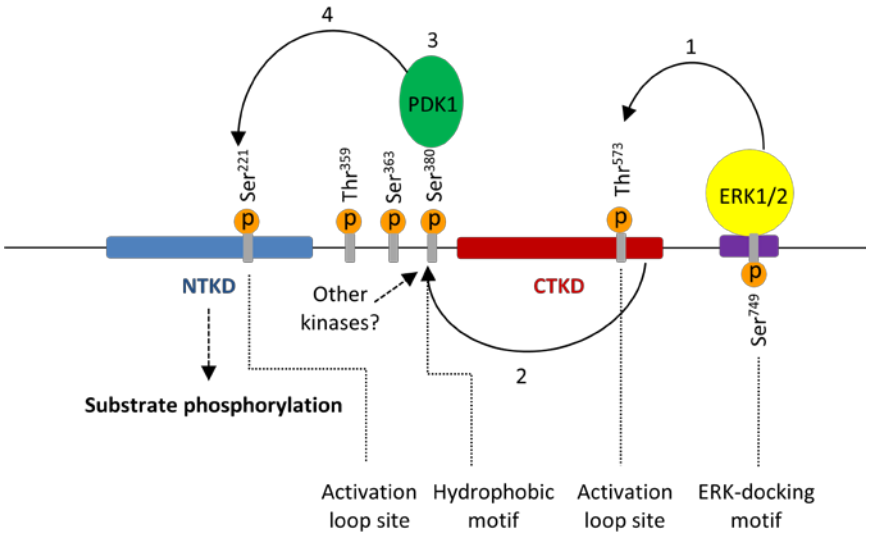

Figure 9. The domain structure and current model of activation of Rsk. 90 kDa ribosomal S6 kinases (Rsks) are characterized by the presence of two functional domains, the N-terminal kinases (NTKD) and the C-terminal kinase domain (CTKD), which are connected by a linker region, and the C-terminal end contains an extracellular signal-regulated kinase-1/2 (ERK1/2)-docking domain. (1) ERK activation and docking to Rsk results in the phosphorylation of Thr573 in the CTKD. (2) The activated CTKD autophosphorylates Rsk at the hydrophobic motif, Ser380. (3) 3'-phosphoinositide-dependent kinase-1 (PDK1) docks at the phosphorylated hydrophobic motif and (4) phosphorylates Ser221 in the NTKD activation loop site, which results in complete Rsk activation. The activation of Rsk phosphorylates substrates throughout the cell. Amino-acid numbering refers to human Rsk1.

Briefly, sequential phosphorylations are initiated by ERK1/2 at Thr-573 of C-terminal kinase domain leading to the autophosphorylation of Rsk at Ser380 (Figure 9). This phosphorylation allows the dockage of PDK1, a constitutively active Ser/Thr kinase, to the hydrophobic motif and enables PDK1-dependent phosphorylation in the N-terminal kinase domain of Rsk at Ser221, resulting in its maximal activation (Figure 9 and 10) (Anjum and Blenis, 2008; Frodin and Gammeltoft, 1999). Despite this described sequential activation, other factors have been shown to be involved in the activation of Rsk (Zaru et al., 2007).

When activated, Rsk promotes the phosphorylation of many cytosolic and nuclear targets, which have a role in transcription, cell-cycle regulation and survival (Roux and Blenis, 2004). In the nucleus, Rsk directly phosphorylates transcription factors that are involved in the synthesis of anti-apoptotic proteins (Figure 10). Some examples include the transcription factor CREB, the serum response factor (SRF) and the nuclear factor-κβ (Rivera et al., 1993; Xing et al., 1996). Rsk also phosphorylates a number of immediate early gene (iEGs) products, including FOS, JuN and NuR77 (Chen et al., 1992; Chen et al., 1993; Wingate et al., 2006). In the cytosol, Rsk induces the inactivation of certain pro-apoptotic proteins by phosphorylation, such as Bad, glycogen synthase kinase-3β (GSK-3β) or death-associated protein kinase (Anjum et al., 2005;

Sutherland et al., 1993; Tan et al., 1999). Rsk can also indirectly control the expression of the neurotrophic/neuroprotective protein Bcl-2 (Creson et al., 2009). The activation and the inhibition of anti- and pro-apoptotic proteins, respectively, point out Rsk as a new target in controlling cell survival (Ballif and Blenis, 2001).

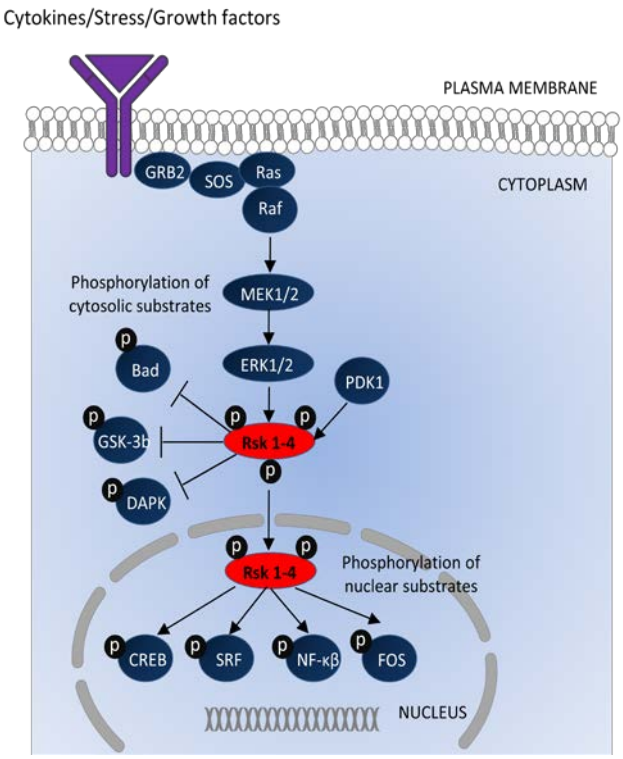

Figure 10. Schematic model of Rsk pathway. 90kDa ribosomal S6 kinase (Rsk) is activated following the activation of the Ras-mitogen-activated protein kinasa (MAPK) cascade upon exposure of cells to different external stimuli. After sequential activation of Ras-Raf- MAPK and extracelular signal-regulated kinase (ERK)-1/2 (MEK1/2), ERK1/2 is activated. Rsk1-4 is directly activated by ERK1/2 and also by 3'-phosphoinositide-dependent kinase-1 (PDK1). Activated Rsk remain in the cytosol or translocate to the nucleus, where it can phosphorylate a large variety of substrates.

3.2. TRANSCRIPTION FACTORS

3.2.1. cAMP response element binding protein (CREB)

ERK 1/2 cannot only phosphorylate different kinases, but also some transcription factors such as CREB. CREB belongs to the bZIP superfamily of transcription factors, and within this superfamily, CREB and the closely related factors cAMP response element modulator and activation transcription factor 1 comprise a subcategory referred as the CREB family. CREB contains a C-terminal basic domain that mediates DNA binding, and a leucine zipper domain that facilitates the formation of homo- and heterodimers (De Cesare et al., 1999; Mayr and Montminy, 2001; Shaywitz and Greenberg, 1999). This transcription factor binds to DNA in the cAMP response element (CRE). The other domains of CREB serve to facilitate interactions with co-activators and components of the transcriptional machinery (Foulkes et al., 1991; Gonzalez et al., 1989; Hai et al., 1989; Hoeffler et al., 1988). The functional domains of CREB are represented in Figure 13. CREB contains two glutamine rich domains (constitutive active domains) that are separated by the kinase inducible domain.

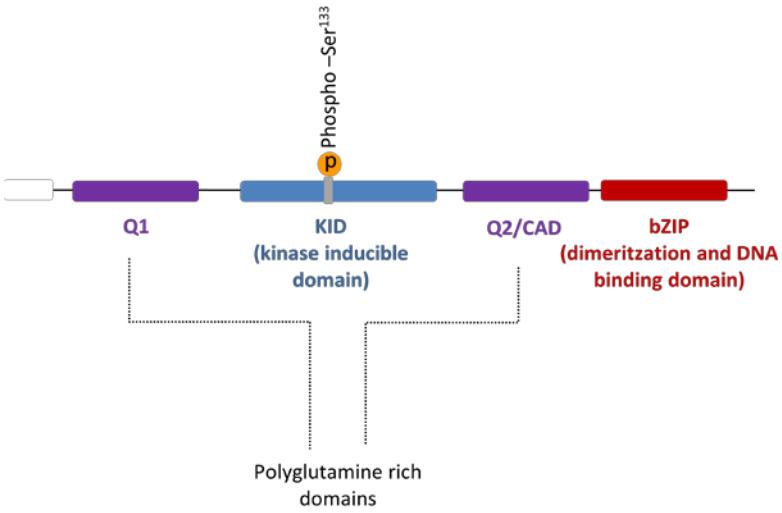

Figure 13. Structure of CREB transcription factor. The glutamine-rich domains (Q1 and Q2) and the bZIP region are indicated in addition to the kinase inducible domain (KID). Phosphorylation at the Ser 133 in the KID turns CREB into activator through the interaction with the co-activatior CBP. Ser133 could be phosphorylated by various kinases.

Within the kinase inducible domain resides the critical residue, Ser133, which when phosphorylated makes the KID domain a binding target for the transcriptional co-activator CBP (Chrivia et al., 1993; Kwok et al., 1994). After interaction between CREB and CBP, together with other members of the transcriptional machinery, the cAMP response element-mediated gene expression is induced (Ferreri et al., 1994; Parker et al., 1996).

CREB is a widely expressed transcription factor known to mediate stimulus-dependent expression of genes critical for plasticity, growth, and survival of neurons (Lee et al., 2005a; Lee et al., 2005b; Lonze and Ginty, 2002). Activation of CREB has also been found to be necessary for functional increases in synaptic transmission (Davis et al., 1996) and CREB-mediated gene expression is sufficient for survival of multiple neuronal subtypes (Bonni et al., 1999; Riccio et al., 1999; Walton et al., 1999). CREB may exert this pro-survival effect by regulating the transcription of pro-survival factors, including *bcl-2* and *bdnf* (Riccio et al., 1999; Zuccato et al., 2010).

CREB is activated by a vast array of physiological stimuli (Figure 14). CREB phosphorylation occurs in response to mitogens, neurotrophins, and other neuronal growth factors. The first kinase known to phosphorylate CREB was the PKA (Gonzalez et al., 1989). PKA activity is regulated by cAMP levels, and therefore by molecules that regulate adenylate ciclase activity, as G protein-coupled receptors (GPCRs). cAMP is formed from ATP by adenylyl cyclase (Figure 14) (Rall and Sutherland, 1958). Adenylate cyclase is a membrane-bound enzyme that can be

activated by the Gα subunit, as well as the βγ subunit of the G-protein family and by Ca^{2+} (Iyengar, 1993; Tang and Ziboh, 1991). Once formed, cAMP activates PKA that is a tetrameric protein composed of two catalytic subunits and two regulatory subunits (Johnson et al., 2001; Johnson and Jameson, 2000).

Two cAMP molecules bind to each regulatory subunit, which results in the release of the active catalytic subunits. PKA enters the nucleus where it phosphorylates CREB protein (Delghandi et al., 2005). On the other hand, CREB can be also activated in a Ca^{2+}-dependent manner (Dash et al., 1991; Shaywitz and Greenberg, 1999; Sheng et al., 1991). In neurons, Ca^{2+} interacts with large number of intracellular proteins as Ca^{2+}-binding protein calmodulin which in turn activates a signaling cascade that has the capacity to phosphorylate CREB (Dash et al., 1991; Sheng et al., 1991). In addition to the Ca^{2+}/calmodulin-dependent protein kinases pathway,

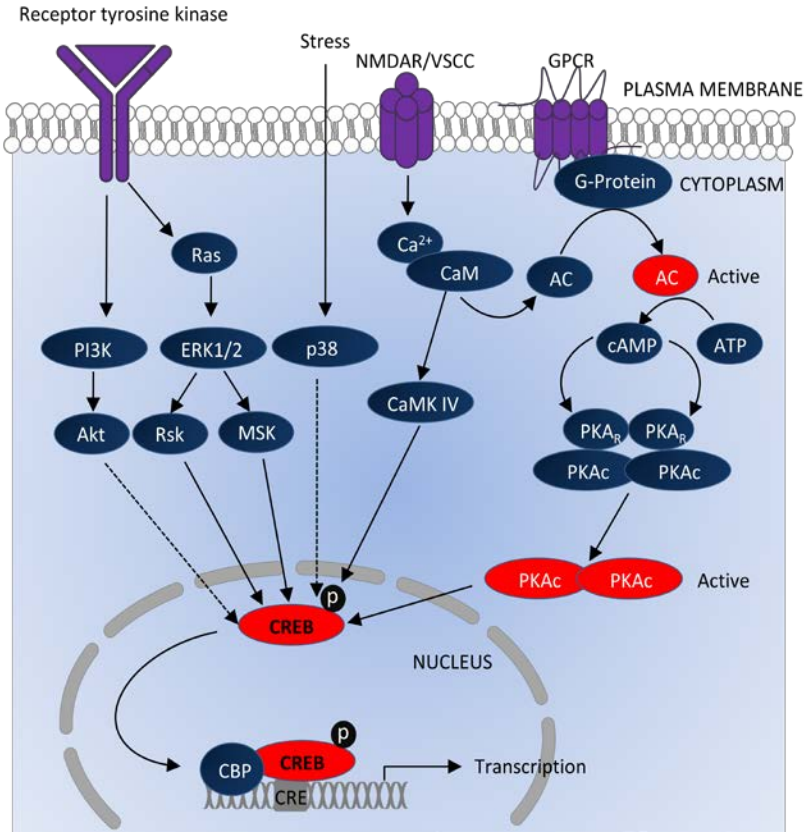

Figure 14. Signaling pathways that activates CREB transcription factor. Excitatory neurotransmitters acting through *N-Methyl-D-aspartate* receptors (NMDAR), ligands for G-protein coupled receptors (GPCR), neuronal growth factors acting through tyrosine kinase receptors, and stress inducers are among the stimuli that activate signaling pathways that converge upon cAMP response element binding protein (CREB). Multiple stimulus-dependent protein kinases have been implicated as CREB kinases in neurons. Stimulus-dependent CREB kinases include protein kinase A (PKA), Calcium-calmodulin dependent kinase type IV (CaM IV), MAPK as extracellular signal-regulated kinases (ERK 1/2) and p38, and members of the ribosomal S6 kinase (Rsk) and mitogen and stress activated protein kinase (MSK).

CREB can also be activated by ERK (Bading and Greenberg, 1991; Davis et al., 2000). The ERK-dependent phosphorylation of CREB can be carried out by several different kinases, among which are members of the Rsk family (De Cesare et al., 1999; Xing et al., 1996; Xing et al., 1998). Moreover, receptor tyrosine kinases can also activate CREB by a second major signaling pathway, the phosphoinositide 3-kinase/Akt pathway (Cantley, 2002), but the direct link between CREB and Akt is still unknown. While CREB activation occurs in response to pro-growth and pro-survival stimuli, it is also phosphorylated in response to harmful or stressful stimuli, including irradiation and hypoxia by the action of stress-activated protein kinases (Deak et al., 1998; Tan et al., 1996; Wiggin et al., 2002), suggesting that the activation of CREB-dependent survival program in response to stressful stimuli might represent a cellular form of defense. Finally, protein phosphatase 1, protein phosphatase 2A, and calcineurin are thought to be capable of directly dephosphorylate CREB (Alberts et al., 1994; Hagiwara et al., 1992; Mukherjee and Soto, 2011; Wadzinski et al., 1993).

Huntingtin functions in transcription are well established. Huntingtin has been shown to interact with a large number of transcription factors such as the CBP (McCampbell et al., 2000; Steffan et al., 2000). Different studies observed that CREB signaling is compromised in different mouse and cellular models of HD and in human samples, where the expression of mutant huntingtin induces aggregation of the co-activator CBP (Nucifora, Jr. et al., 2001; Steffan et al., 2000; Steffan et al., 2001), reduces the levels of cAMP (Gines et al., 2003) and down-regulates CRE-mediated transcription of numerous genes (Augood et al., 1997; Luthi-Carter et al., 2000; Zuccato et al., 2010), which is believed to contribute to HD pathogenesis. One of the genes regulated by CREB-mediated transcriptional activity is BDNF (Zuccato et al., 2010). Reduced CREB- dependent transcription of BDNF is a robust feature of HD pathology. In human samples, BDNF protein and mRNA levels in the fronto-parietal cortex are decreased (Ferrer et al., 2000; Zuccato et al., 2001; Zuccato and Cattaneo, 2009). Reduced levels of cortical and striatal BDNF have also been reported in multiple mouse models of HD, including R6/2, N171-82Q, Hdh and YAC-72 lines(Gines et al., 2003; Luthi-Carter et al., 2000; Luthi-Carter et al., 2003; Zuccato et al., 2001). Decreased BDNF is thought to contribute to decreased cell survival and gross atrophy during HD progression (Zuccato et al., 2010).

Use of drugs that maintain CREB phosphorylated, like specific phosphodiesterases inhibitors, rolipram and TP10, decreases striatal cell loss after the injection of quinolinic acid in an excitotoxic model of HD (DeMarch et al., 2007), reduces gross brain atrophy, increases the number of surviving striatal neurons and the expression of BDNF (DeMarch et al., 2008;

Giampa et al., 2006; Giampa et al., 2009). These treatments also improve motor deficits, decrease neurodegeneration and the number of neuronal intranuclear inclusions, and increase phosphorylated CREB and the levels of BDNF in the striatum and cortex in HD mouse models (Giampa et al., 2009; Giampa et al., 2010). Moreover, CREB over-expression is sufficient to rescue polyQ-dependent lethality in *Drosophila* (Iijima-Ando et al., 2005). These data suggest that mutant huntingtin might lead to alterations of CRE-mediated gene transcription and reinforce the idea of a beneficial effect of increasing gene expression mediated by CREB could be a good therapeutic approach in HD.

3.2.2. Ets-like Gene 1 (Elk-1)

ERK1/2 can not only phosphorylate the transcription factor CREB, but also other transcription factors such as Ets-like Gene 1 (Elk-1), which is considered one of the more important transcription factors in neurons (Sgambato et al., 1998b; Vanhoutte et al., 1999). Elk-1 is one of the most thoroughly studied targets of the ERK1/2 cascade (Yoon and Seger, 2006), and it can also be phosphorylated by the other MAPKs, JNK and p38 (Janknecht et al., 1994) (Figure 11).

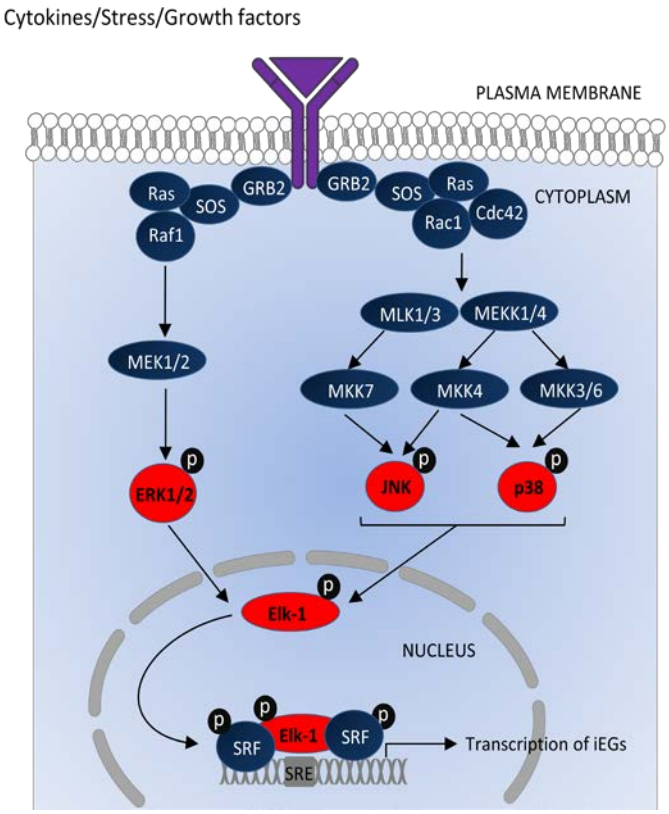

Figure 11. Representation of Elk-1 activation pathway. MAPKs respond to external signals including cytokines, stress and growth factors. Signals are transduced via small G-proteins Ras to multiple tiers of protein kinases that amplify them and/or regulate each other. Finally, the kinase cascade leads to an activation of the three major mitogen-activated protein kinases (MAPKs)*: the* Extracellular signal-regulated kinases (ERK), the c-Jun N-terminal kinases (JNK) and p38 MAPK. These kinases are able to phosphorylate the transcription factor Ets-like Gene 1 (Elk-1), which in turn forms a ternary complex with a dimer of serum response factor (SRF) and promotes the transcription of different immediate early genes regulated under the serum response element (SRE) promoter.

The transcription factor Elk-1, together with SRF accessory protein-1 and SRF accessory protein-2/Net, are member of the ternary complex factor (TCF), which is the most studied subfamily of ETS (E- twenty six) domain proteins (Buchwalter et al., 2004). Each TCF contains four conserved domains, named A-D (Figure 12). The A domain, localized in the N-terminal region, contains a nuclear localization signal as well as a nuclear export signal (Besnard et al., 2011). The B domain enables TCFs to interact with the SRF, promoting the ternary complex formation (Hill et al., 1993; Treisman et al., 1992). The D domain contains the consensus sequence that permits the binding to MAPKs (Figure 11), which in turn will phosphorylate the transcriptional activation domain, or C domain, in the C-terminal part of the protein (Figure 12) (Janknecht et al., 1994).

The phosphorylation of Elk-1 at serine 383 (pElk-1^{Ser383}) residue is particularly important for the transactivation of Elk-1 (Janknecht et al., 1993), induces the formation of the TCF with a dimer of SRF (Gille et al., 1995b; Gille et al., 1995a) (Figure 11). This complex promotes the transcription of many iEGs, such as c-fos, egr-1, egr-2 and mcl-1, via the serum response element (SRE) DNA consensus site (Figure 11) (Wasylyk et al., 1998).

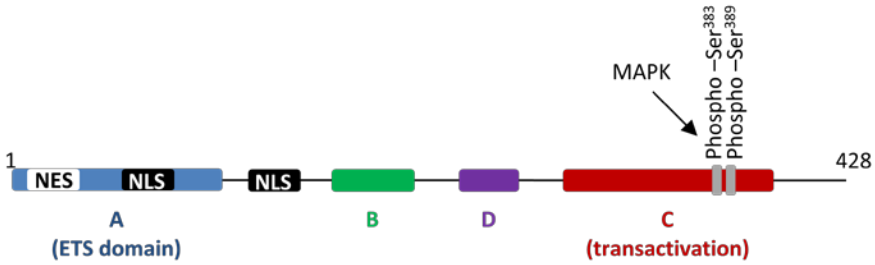

Figure 12. Functional domains and major post-translational modifications of Elk-1. The ETS (or A) domain in the N-terminal end of the protein is responsible for Elk-1 binding to DNA. The B domain is involved in the binding of Elk-1 to a dimer of its cofactor, the SRF. The C (or transactivation) domain contains the amino acids that are phosphorylated by MAP kinases. The D domain is responsible for the binding of Elk-1 to activated MAP kinases. The nuclear export signals (NES) and the nuclear localization signal (NLS) motifs are involved in nuclear export and import of Elk-1, respectively.

Elk-1 transcriptional activity is involved in many biological processes, such as cell growth, differentiation and survival (Sharrocks, 2001). The products of Elk-1-mediated transcription, the iEGs, are genes which are activated transiently and rapidly in response to a wide variety of cellular stimuli. They represent a standing response mechanism that is activated before any new proteins are synthesized (Wasylyk et al., 1998). Many iEGs are naturally transcription factors or DNA-binding proteins; however, iEGs products include secreted proteins, cytoskeletal proteins and receptor subunits (Boros et al., 2009). The best characterized iEGs

include c-fos, c-myc and c-jun which are early regulators of cell growth and differentiation (Boros et al., 2009; Sharrocks, 2001). Moreover, some iEGs such as egr-1, egr-2, Arc and also c-fos have been implicated in learning and memory and LTP processes (DeSteno and Schmauss, 2008; Feldman et al., 2010), as iEGs are highly responsive to neurotransmitter signals, and their expression correlates closely with neuronal activity (Sheng and Greenberg, 1990). These findings suggest roles for iEGs in many cellular processes and so it is Elk-1.

Elk-1 is strongly expressed in the central nervous system (Price et al., 1995), where it is restricted to neuronal cells, and has been found in nuclear, somatic and dendritic compartments (Sgambato et al., 1998b; Sgambato et al., 1998a). In striatal projection neurons, Elk-1 is considered the major transcriptional regulator together with the CREB (Sgambato et al., 1998b; Vanhoutte et al., 1999). In the cortex, Elk-1 is activated after quinolinic acid-induced lesion and has the capacity to prevent excitotoxic cell death (Ferrer et al., 2001). Moreover, Tong et al. (Tong et al., 2004) showed that sublethal concentrations of β-amyloid interfere with BDNF-induced activation of Elk-1 in cultured cortical neurons and results in an altered SRE-driven gene regulation, which is likely to account for increased neuronal vulnerability. Additionally, Elk-1 activity has been linked with increased synaptic plasticity, as iEGs regulated by Elk-1 are critical for this neuronal plasticity (DeSteno and Schmauss, 2008; Feldman et al., 2010; Sheng and Greenberg, 1990).

In the case of HD, different studies demonstrate that the N-terminal huntingtin fragment can have dramatic effects on gene expression (Cha, 2000; Cha, 2007). Changes in mRNA expression seems to appear early in the disease process (Murphy et al., 2000) and alterations in the expression of different iEGs have been previously reported in different models of HD (Luthi-Carter et al., 2000; Roze et al., 2008; Spektor et al., 2002), which correlates with hypo-activity of striatal neurons in R6/2 mice (Cepeda et al., 2001). Accompanying these alterations, increased phosphorylation of Elk-1 has been observed in the striatum of R6/2 mice models of HD (Roze et al., 2008). Although Elk-1 phosphorylation has been shown to be modulated in various CNS diseases (Besnard et al., 2011), the role for Elk-1 in HD remains elusive.

4. SYNAPTIC DYSFUNCTION: ROLE OF PROSTAGLANDIN E2 RECEPTORS IN THE CENTRAL NERVOUS SYSTEM

The prevention of cell death is an important point for neurodegenerative diseases; even so, it is unlikely that cellular machinery works well until the death of the cells. In many

neurodegenerative diseases, such as HD, neuronal and synaptic dysfunction precedes cell death and occurs long before, or sometimes in absence of cell death (Lynch et al., 2007; Milnerwood et al., 2006; Orth et al., 2010; Schilling et al., 1999; Usdin et al., 1999). Recent studies suggest that targeting early pathophysiological disturbances in models of HD can reverse neuronal dysfunction (Milnerwood and Raymond, 2010; Simmons et al., 2009) and delay progression to neurodegeneration (Okamoto et al., 2009). This section is focused on the importance of Prostaglandin E2 (PGE2) EP receptors, and its possible role as new therapeutic targets that can be used to enhance synaptic dysfunction that occurs in HD.

4.1. PROSTAGLANDIN E2 AND PROSTAGLANDIN E2 RECEPTORS

PGs are derived from the metabolism of the arachidonic acid by cyclooxygenase (COX)-1 and COX-2. When tissues are exposed to diverse physiological and pathological stimuli, arachidonic acid is liberated from membrane phospholipids and is converted to an unstable intermediate, PGH2 (Bonvalet et al., 1987; Smith, 1992). PGH2 then servers as the substrate for the generation of PGs (PGE2, PGF2α, PGD2 and PGI2) and thromboxane A2. Each prostanoid is synthesized in specific compartments within the body via the action of specific synthases. These prostanoids binds to specific GPCRs designated EP (for E-prostanoid receptors), FP, DP, IP and TP, respectively (Narumiya et al., 1999). The PG receptors have the characteristic seven-hydrophobic-transmembrane segment typical of GPCRs and belong to the family GPCRs (Kolakowski, Jr., 1994).

PGE2 is the most widely produced PG in the body and is formed by the action of PGE synthase. PGE2 binds to four receptors subtypes, namely EP1, EP2, EP3 and EP4 (Figure 15) (Breyer et al., 2001); having different and even opposite effects depending on the type of receptor that it interacts. EP1 receptors mediate signaling events by inducing the activation of Gq protein and phospholipase C that leads to an elevation of cytoplasmatic signaling intermediates including inositol triphosphate, diacylglycerol and Ca^{2+}. While EP2 and EP4 receptors are linked to the activation of Gs protein and to the stimulation of adenylate cyclase and PKA (Honda et al., 1993), signaling via EP3 receptors is unique, as it has three different splicing variants that couple different G proteins and its activation can change cAMP levels or Ca^{2+} levels (Narumiya et al., 1999) (Figure 15). EP receptors are differentially expressed in almost all organs, including within the central nervous system. Their expressions are also located in specific regional and cellular areas in the brain (Cimino et al., 2008). EP receptors are found in endotelial cells,

microglia, astrocytes and neurons (Carlson et al., 2009; Cimino et al., 2008). EP4 expression in neurons is restricted to hypothalamic nuclei, while neuronal EP1, EP2 and EP3 are expressed in multiple brain regions including the hippocampus, striatum and cortex (Andreasson, 2010; Bhattacharya et al., 1998; Kawano et al., 2006; Nakamura et al., 2000; Oka et al., 2000; Sugimoto et al., 1994).

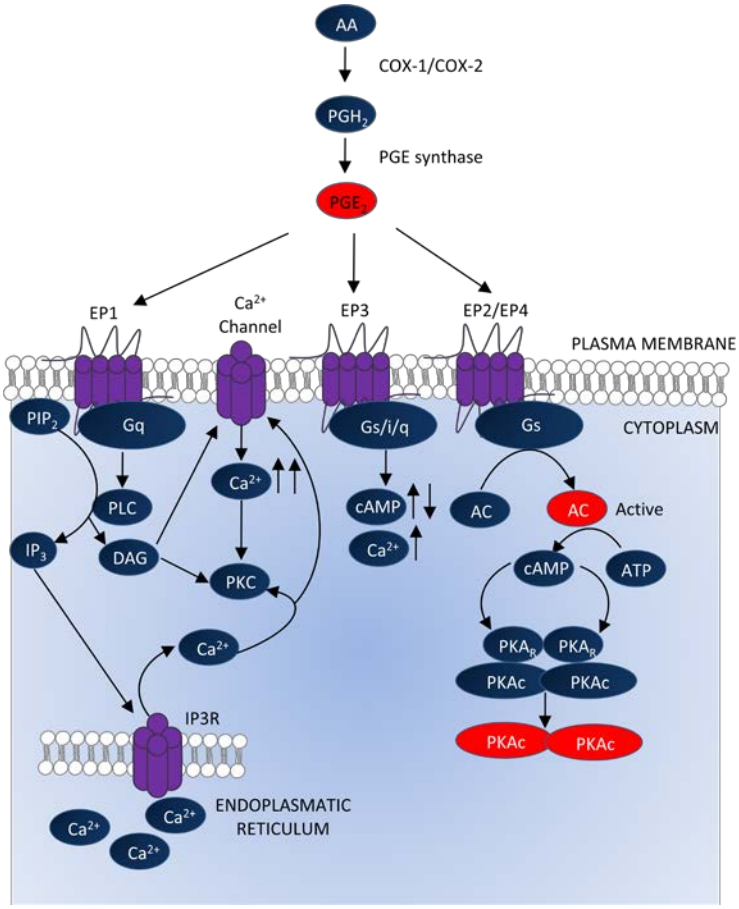

Figure 15. Signaling through prostaglandin E2 receptors. The four GPCRs that respond to prostaglandin E2 (PGE_2) are called E-prostanoid receptors (EP), of which there are four subtypes (EP1-4). EP1 signals through Gq, which produces a transient rise in intracellular calcium (Ca^{2+}). EP1 activates Gq, which in turn activates PLC that hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP_2) to generate diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3). IP_3 binds to IP_3 receptors (IP_3R) on the endoplasmatic reticulum (ER), which act as Ca^{2+} channel, allowing the release of Ca^{2+} from intracellular stores. DAG and Ca^{2+} activate cell-specific Ca^{2+} channels at the plasma membrane, producing further increase in cytoplasmatic Ca^{2+}. Ca^{2+} and DAG could activate kinases as PKC (protein kinase C). EP_2 and EP_4 are coupled to G_s, which directs the synthesis of cAMP, while EP_3 is $G_{s/i/q}$-linked and acts to inhibit or increasing cAMP production or increasing intracellular Ca^{2+}. The principle enzyme, adenylate cyclase (AC), converts ATP to cAMP, which in turn activates PKA.

The study of the possible effects of EP receptor activation in the brain has yielded with conflicting results due to their biological heterogeneity (Andreasson, 2010; Gendron et al., 2005). While different studies have linked the activation of EP receptors with toxicity (Ahmad

et al., 2006a; Kawano et al., 2006; Zhen et al., 2012), effects on neuronal survival have also been observed after EP receptor activation (Ahmad et al., 2005; Ahmad et al., 2006b; Ahmad et al., 2010; Bilak et al., 2004; Jiang et al., 2010; McCullough et al., 2004). Therefore, the pro-death and pro-survival effect of PGE2 depends on the EP receptor that it activates.

4.2. EP1 RECEPTOR IN NEURONAL INJURY

As explained above, the primary effect observed after EP1 receptor activation is an increase in intracellular levels of Ca^{2+} (Breyer et al., 2001). EP1 signaling has been identified as a major transducer of COX-2 neurotoxicity in the central nervous system, both in acute neuronal injury (Ahmad et al., 2006a; Kawano et al., 2006) and in neurodegenerative processes (Carrasco et al., 2007; Zhen et al., 2012).

4.2.1. Role of EP1 in excitotoxicity

Many *in vitro* and *in vivo* studies have been done in order to elucidate the role of EP1 receptor in neuronal excitotoxicity (Table 3). Neuroprotective effects of EP1 receptor inhibition/deletion have been reported in different model of acute neuronal damage.

EP1 receptor involvement in the neurotoxicity has been supported by experiments where its pharmacological inhibition or gene inactivation ameliorates brain injury and rescues behavioral effects in *in vivo* models of NMDA excitotoxicity and cerebral ischemia (Abe et al., 2009; Ahmad et al., 2006a; Kawano et al., 2006). Moreover, EP1 receptor agonist administration into neocortex reversed the protection against NMDA excitotoxicity elicited by COX-2 inhibition (Manabe et al., 2004).

Gendron et al. (2005) demonstrated that in a model of oxygen-glucose deprivation, the neuroprotection elicited by COX-2 inhibition was reversed by administration of PGE2, and this effect was mediated by EP1 receptor. Trying to explain this EP1 receptor inhibition-mediated neuroprotection in oxygen-glucose deprivation, Zhou and colleagues (2008) discovered that EP1 receptor blockade increases Akt phosphorylation in this excitotoxic condition, where has been proposed as an important neuroprotective pathway in distinct acute (Endo et al., 2006; Noshita et al., 2001; Owada et al., 1997; Zhang et al., 2006) and chronic (Gines et al., 2003; Hashimoto et al., 2004; Saavedra et al., 2010) models of neurodegeneration. Furthermore, inhibition of EP1 receptor signaling significantly reduced hypoxic-ischemic encephalopathy

cerebral injury (Taniguchi et al., 2011) and increases survival rate in hypoxic-treated cortical neurons (Liu et al., 2012).

Model	Transgenic mice or pharmacologycal treatments	Brain region/Cell type	Effects	References
Models of excitotoxicity				
NMDA	$EP^{-/-}$	Neocortex	↓lesion	Kawano et al., 2006
				Anrather et al., 2011
		Striatum	↓lesion	Ahmad et al., 2006
	EP1 antagonist	Neocortex	↓lesion	Kawano et al., 2006
		Striatum	↓lesion	Ahmad et al., 2006
		Hippocampal slices	↓cell death	Zhou et al., 2008
	EP1 agonist	Striatum	↑lesion	Ahmad et al., 2006
MCAO	$EP^{-/-}$		↓lesion	Kawano et al., 2006
				Ahmad et al., 2006
	EP1 antagonist		↓lesion	Kawano et al., 2006
			improved motor deficits	
Oxidative stress	$EP^{-/-}$	Cortical neurons	↑survival	Saleem et al., 2007
	EP1 antagonist	Cortical neurons	↑survival	Saleem et al., 2007
OGD	EP1 antagonist	Hippocampal slices	↓cell death	Zhou et al., 2008
			↑pAkt	
			↓Bad translocation	
		Cortical neurons	↑survival	Grendron et al., 2005
		Hippocampal neurons	↑survival	Taniguchi et al., 2011
			↓Caspase 3 activation	
Hypoxia	EP1 antagonist	Cortical neurons	↑survival	Liu et al., 2012
			↓Caspase 3 activation	
			↓lesion	Taniguchi et al., 2011
Models of neurodegeneration				
AD	$EP^{-/-}$	Hippocampus	↓neuronal damage	Zhen et al., 2012
			↓amiloid-paques	
			↓memory loss	
	EP1 antagonist	MC65 cells	↓cell death	Li et al., 2013
		Cortical neurons		
PD (6-OHDA)	$EP^{-/-}$	Medial forebrain bunc	↓cell death	Ahmad et al., 2013
			↓behavioral deficits	
	EP1 antagonist	Dopaminergic neuron:	↓cell death	Carrasco et al., 2007

Table 3. EP1 receptor antagonism or depletion in excitotoxic and neurodegenerative conditions. Table includes information about the effect of EP1 receptor depletion or inhibition in different models of excitotoxic insults and neurodegeneration. *N*-methyl-D-aspartate (NMDA); Middle cerebral arteria occlusion (MCAO); OGD (oxygen and glucose deprivation); AD (Alzheimer's disease); PD (Parkinson's disease); 6-OHDA (6-Hydroxydopamine).

4.2.2. Role of EP1 in neurodegenerative diseases

In the context of neurodegeneration, levels of PGE2 in the central nervous system are up-regulated in various neurological disorders including Alzheimer's disease, Parkinson's disease,

multiple sclerosis, Creutzfeldt-Jakob disease and amyotrophic lateral sclerosis (Almer et al., 2002; Andreasson, 2010; Cimino et al., 2008; Ilzecka, 2003; Jiang et al., 2010; Minghetti et al., 2002; Montine et al., 2002). Part of the toxic effect of PGE2 in the central nervous system has been reported to be through the EP1 receptor. Direct effects of EP1-mediated neurotoxicity have been demonstrated in models of neurodegenerative diseases, such as models of Parkinson's disease and Alzheimer's disease (Table 3).

Increasing evidences show that EP1 receptor blockade has the capacity to protect dopaminergic neurons from 6-Hydroxydopamine (6-OHDA)-induced cell death in an *in vitro* model of Parkinson's disease (Carrasco et al., 2007). This effect was not only observed in cultured neurons but also in mice, where EP1 receptor depletion protects dopaminergic neurons in the substantia nigra *pars compacta* and reduces behavioral deficits after 6-OHDA lesion (Ahmad et al., 2013). In the context of Alzheimer's disease, it has been demonstrated that β-amiloid-mediated neurotoxicity *in vitro* was partially EP1 receptor-dependent (Li et al., 2013). In the former, EP1 receptor antagonism has a neuroprotective effect on both intracellular β-amiloid 1-40 aggregates cytotoxicity, and extracellular β-amiloid 1-42 neurotoxicity. In this regard, recent results show reduced β-amiloid peptid accumulation in EP1 deficient mice (Zhen et al., 2012). Moreover, genetic deletion of EP1 attenuates amyloid-induced hippocampal neuronal damages and reduces memory loss in a transgenic mouse model of Alzheimer's disease (Zhen et al., 2012).

4.3. EP2 RECEPTOR

In contrast to EP1 receptor, previous reports have shown that EP2 receptor activation is associated to neuroprotection and hippocampal-dependent synaptic plasticity (Andreasson, 2010).

4.3.1. EP2 receptor: activation of PKA/CREB/BDNF pathway

The EP2 receptor is positively coupled to Gs protein and is widely expressed in neurons, microglia and astrocytes in forebrain (Cimino et al., 2008). Activation of the EP2 prostanoid receptor is known to stimulate cyclic AMP-dependent intracellular signaling that involves the activation of PKA and consequent phosphorylation and activation of the transcription factor CREB (Figure 15) (Regan, 2003). Activation of CREB leads to the expression of genes under the control of promoters that contain the cAMP response element sequence, including many that block neuronal death (Dawson and Ginty, 2002). The *bdnf* gene is known to contain multiple

cAMP response element sequences in its 5'-upstream promoter region and expression of this gene is increased by cAMP signaling and CREB activation (Fukuchi et al., 2005; Tabuchi et al., 2002; Tao et al., 1998). Some studies have shown that PGE2 activates a cyclic AMP/PKA/CREB signal transduction pathway in cultured human microglia and astrocytes by EP2 receptor activation and stimulation of these receptor leads to the induction of BDNF release (Hutchinson et al., 2009). These findings are consistent with reports that show prostaglandin stimulated release of neurotrophins from mouse astrocytes (Toyomoto et al., 2004) and studies that show correlations between PGE2 and BDNF levels (Ajmone-Cat et al., 2006; Shaw et al., 2003).

In mice, the EP2 receptor has been reported to be highly expressed in cerebral cortex, striatum, and hippocampus (Ahmad et al., 2006b; Zhang and Rivest, 1999), regions affected in HD (Vonsattel and DiFiglia, 1998). In this context, BDNF is of particular interest because of its neurotrophic actions on neuronal populations involved in several neurodegenerative disease, including amytrophic lateral sclerosis (Askanas, 1995), Parkinson's disease, Alzheimer's disease (Siegel and Chauhan, 2000), and HD (Alberch et al., 2002; Alberch et al., 2004; Zuccato and Cattaneo, 2007; Zuccato and Cattaneo, 2009). As suggested above, reduced levels of BDNF has been point out as one of the mechanisms important for the specific neurodegeneration and dysfunction observed in HD pathology (Zuccato and Cattaneo, 2009).

4.3.2. Neuroprotective role of EP2 activation

PGE2 may exert functionally opposing effects. In case of EP2 receptor, neuroprotective effect of its activation in models of brain injury has been widely reported. *In vitro* studies in hippocampal cultured neurons and slices demonstrated that activation of the EP2 receptor is neuroprotective in paradigms of NMDA toxicity and oxygen-glucose deprivation (Ahmad et al., 2010; Liu et al., 2005; McCullough et al., 2004). This neuroprotective effect is blocked by inhibition of PKA, suggesting that EP2-mediated neuroprotection is dependent on cAMP signaling. In the middle cerebral arterial occlusion, model of transient forebrain ischemia, genetic deletion of EP2 receptor significantly increased cerebral infarction in cerebral cortex and subcortical structures (Liu et al., 2005; McCullough et al., 2004). EP2 receptor stimulation was also protective in a model of striatal excitotoxicity (Ahmad et al., 2006b).

Stimulation of the EP2 receptor rescues neurons in additional *in vitro* models of neurodegenerative disease, including the threo-hydroxyaspartate model of glutamate-induced motor neurotoxicity (Bilak et al., 2004), a model of human amyotrophic lateral sclerosis where

chronic glutamate toxicity is induced by blocking astrocyte glutamate transporters. In the 6-OHDA model of dopaminergic neuronal degeneration, a model of Parkinson's disease, EP2 signaling was also neuroprotective (Carrasco et al., 2008). Moreover, EP2 receptor agonist protects cortical cultures from β-amiloid 1-42-mediated neurotoxicity, an *in vitro* model of Alzheimer's disease (Echeverria et al., 2005). In all cases, as in hippocampal neuroprotection, the EP2-dependent protective effects are dependent on cAMP/PKA activation. These studies indicate that activation of the PGE2-EP2 receptor can protect against excitotoxic and anoxic injury in a cAMP-dependent manner.

Although EP2 signaling mediates significant neuroprotection in acute models of cerebral ischemia and excitotoxicity, a neurotoxic effect of EP2 receptor activation has been suggested in models of chronic inflammation and neurodegeneration (Jin et al., 2007; Liang et al., 2005; Liang et al., 2008; Shie et al., 2005; Wu et al., 2007). Thus, the effect of EP2 signaling on neuronal viability in neurodegenerative disorders will depend on the context of the stimulus and the degree of inflammatory response versus excitotoxicity in the specific injury model.

4.3.3. EP2 receptor and synaptic plasticity

PGE2 regulates illness-induced impairment of memory formation, but under physiological conditions, the development of knock-out mice has provided evidence that PGE2 signaling has unexpected actions in brain functions, such as the role of EP2 in hippocampus-dependent learning and memory formation and synaptic plasticity (Akaneya and Tsumoto, 2006; Furuyashiki and Narumiya, 2011; Sang et al., 2005; Savonenko et al., 2009; Yang et al., 2009).

EP2 is localized at the pre-synaptic structure of primary neurons in the hippocampus (Sang et al., 2005). PGE2 released from the post-synaptic site was proposed to act on pre-synaptic EP2 receptor as a retrograde messenger, which mediates signaling from a post-synaptic structure to an adjacent pre-synaptic structure in the hippocampus (Sang et al., 2005) (Figure 16).

In terms of its physiologic function in brain, a role of EP2 receptor signaling in activity-dependent synaptic plasticity is supported by an emerging literature in widely differing models. PGE2, acting via the EP2 receptor increases dendritic spines by an AMPAR-dependent mechanism (Figure 16) (Burks et al., 2007) and regulates levels of spinophilin (Ahmadi et al., 2002). Moreover, EP2 deficient mice show various memory disturbances (Savonenko et al., 2009; Yang et al., 2009). For example, EP2 deficient mice have impaired spatial memory studied by the Morris water task (Yang et al., 2009) and deficits in pre-pulse inhibition

(suppression of sensory-evoked motor response because of a prior weak sensory stimulus (Savonenko et al., 2009).

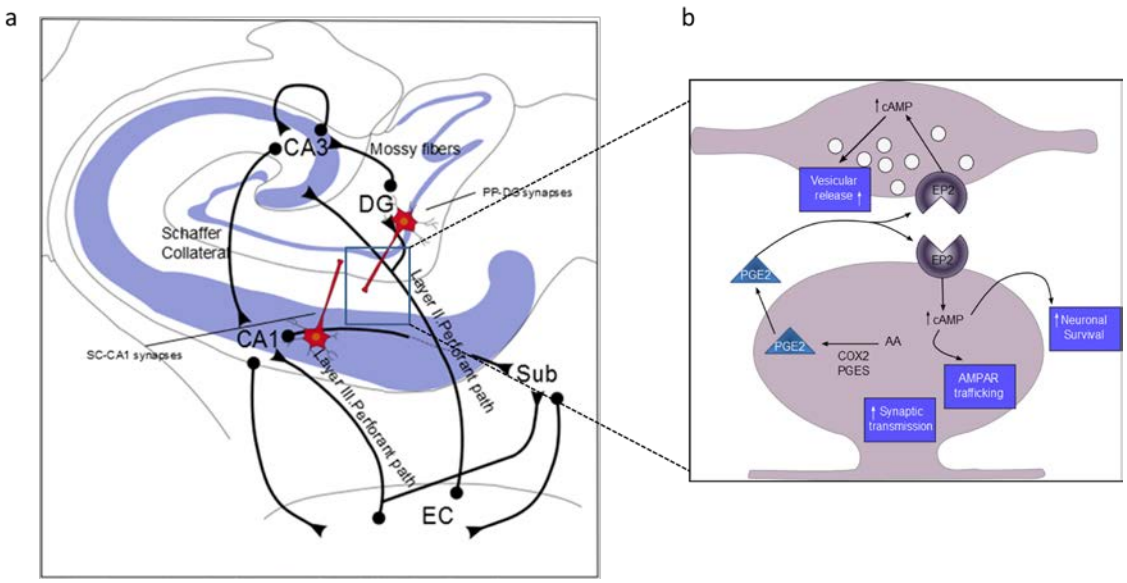

Figure 16. Proposed mechanism for EP2 activation in hippocampus. a) In the hippocampus, EP2 is suggested to function at the perforant path (PP)-dentate gyrus (DG) synapses for long-term potentiation and at the SC-CA1 synapse for long-term depression of responses. b) Proposed mechanism for EP2 actions on LTP of neuronal circuits. Released PGE2 is suggested to act on synaptic EP2. To cause LTP, increased levels of cAMP downstream of EP2 could increase the probability of neurotransmitter release and/or the trafficking of AMPAR, as well as improve neuronal survival. AA, arachidonic acid; AMPAR, AMPA-type glutamate receptors; CA, cornus ammonis; DG, dentate gyrus; EC, entorhinal cortex, EP, prostaglandin E receptor; PGES, prostaglandin E synthase; PP perforhant path; Sub, subiculum; SC, Schaffer collateral.

Results from electrophysiological studies have also demonstrated the role of PGE2 in excitatory synaptic transmission and long-term plasticity (Akaneya and Tsumoto, 2006; Chen et al., 2002; Cowley et al., 2008; Le et al., 2010; Murray and O'Connor, 2003; Sang et al., 2005; Savonenko et al., 2009; Shaw et al., 2003; Yang et al., 2009). In this context, addition of COX-2 inhibitors blocks the induction of hippocampal LTP at the perforant path-dentate gyrus excitatory synapse, deficits that are reversed by exogenous application of PGE2 (Chen et al., 2002; Cowley et al., 2008; Shaw et al., 2003). LTP at this synapse was consistently impaired in EP2 deficient mice (Yang et al., 2009); thus, PGE2-EP2 signaling seems to be crucial in LTP at the perforant path-dentate gyrus synapse in the hippocampus via pre-synaptic EP2 signaling in a PKA-dependent manner (Figure 16) (Sang et al., 2005). Moreover, EP2 deficiency impaired long-term depression at the Schaffer Collateral-CA1 synapse (Figure 16) (Savonenko et al., 2009); further supporting the role of this PGE2 receptor in the hippocampus.

II. AIMS

Huntington's disease is a progressive neurodegenerative disorder with an onset that depends on the CAG repeat length. Mutant huntingtin induces a large amount of toxic effects that trigger cell dysfunction and consequently, behavioral alterations. However, before the onset of symptoms individuals are healthy. Thus, it is plausible that compensatory mechanisms may be activated to regulate a balance between cell death and survival (Butler et al., 2006). This compensatory mechanism might modulate the progression of Huntington's disease. We would focus on the study of different mechanisms, which could enhance cell survival and delay cell dysfunction, along the progression of the disease in different Huntington's disease mouse and cellular models. We would study mechanism of transcription and protein activation, which are considered one of the main causes that trigger to mutant huntingtin-induced neuronal dysfunction (Cha, 2007). Moreover, modulation of pathways that could directly regulate potential targets involved in the pathology is also essential.

AIM 1. To study molecular compensatory mechanism activated during the progression of Huntington's disease.

1.1. To analyze the possible role of the protein kinase Rsk in preventing mutant huntingtin-induced cell death.

1.2. To analyze the role of the transcription factor Elk-1 in the selective neuronal death that is produced in Huntington's disease.

AIM 2. To characterize the modulation of Prostaglandin E2 EP receptors in Huntington's disease:

2.1. To define the role of EP1 receptor as a therapeutic tool to reduce Huntington's disease phenotype in R6/1 mouse models of Huntington's disease.

2.2. To study the mechanism of EP2 receptor in the regulation of cognitive and trophic deficits in R6/1 mouse models of Huntington's disease.

III. RESULTS

Increased 90-kDa ribosomal S6 kinase (Rsk) activity is protective against mutant huntingtin toxicity

This paper has been published in Molecular Neurodegeneration

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AIM 1. TO STUDY MOLECULAR COMPENSATORY MECHANISM ACTIVATED DURING THE PROGRESSION OF HUNTINGTON'S DISEASE.

1.1. To analyze the possible role of the protein kinase Rsk in preventing mutant huntingtin-induced cell death.

La família de quinases ribosomals S6 90-kDa (Rsk) es troba estretament involucrada en processos de supervivència. L'activació de Rsk està regulada per fosforilació seqüencial de diferents residus controlada per la quinasa ERK (de l'anglès *extracellular signal-regulated kinase*) 1/2 i per la proteïna quinasa PDK1 (de l'anglès *3-phosphoinositide-dependent protein kinase 1*). Alteracions en l'activació de ERK1/2 i PDK1 han estat descrites en la malaltia de Huntington, malaltia caracteritzada per l'expressió d'una forma mutant de la proteïna huntingtina que promou una degeneració principalment estriatal. Tot i així, el paper de la quinasa Rsk en aquesta malaltia neurodegenerativa encara esdevé una incògnita. En aquest treball hem analitzat els nivells proteics, l'activitat i el paper de Rsk en models *in vivo* i *in vitro* de la malaltia de Huntington. Els resultats ens han mostrat un augment dels nivells proteics de dos isoformes de Rsk, Rsk1 i Rsk2, a l'estriat de ratolins que expressen la forma completa de la huntingtina mutada (ratolins HdhQ111/Q111), a l'estriat de ratolins que expressen l'exó 1 de la huntingtina mutada (ratolins R6/1) i en una línia cel·lular estriatal que expressa també la forma completa de la huntingtina mutada (STHdhQ111/Q111). L'anàlisi de la fosforilació de p90Rsk en ratolins Hdh i cèl·lules STHdh ens ha revelat una reducció de la fosforilació dependent d'ERK1/2 (Ser380) i un augment de la fosforilació derivada de l'activitat de PDK1 (Ser221). A més a més, s'ha demostrat que aquest augment en fosforilació de Rsk correlacionava amb un augment de l'activitat d'aquesta quinasa en cèl·lules STHdhQ111/Q111. Mitjançant la transfecció de diferents constructes mutants de la proteïna Rsk, s'ha pogut veure que l'augment en l'activitat de risc era principalment degut a l'activitat PDK1. L'augment dels nivells proteics de p90Rsk es produïa tan al citosol com al nucli de les neurones provocant un augment en la fosforilació de substrats de Rsk tan nuclears com citosòlics. Finalment, hem pogut comprovar mitjançant experiments d'inhibició farmacològica, bloqueig i sobre-expressió de Rsk, que l'augment d'activitat aquesta quinasa té un paper protector davant la mort induïda per la presència de la huntingtina mutada. En resum, aquest treball ens mostra que l'augment de l'activitat de Rsk estaria actuant com a mecanisme compensatori amb capacitat per prevenir la mort neuronal induïda per la huntingtina mutada. Proposem Rsk com a bona diana terapèutica in la malaltia de Huntington.

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MOLECULAR NEURODEGENERATION

RESEARCH ARTICLE Open Access

Increased 90-kDa ribosomal S6 kinase (Rsk) activity is protective against mutant huntingtin toxicity

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Abstract

Background: The 90-kDa ribosomal S6 kinase (Rsk) family is involved in cell survival. Rsk activation is regulated by sequential phosphorylations controlled by extracellular signal-regulated kinase (ERK) 1/2 and 3-phosphoinositide-dependent protein kinase 1 (PDK1). Altered ERK1/2 and PDK1 phosphorylation have been described in Huntington's disease (HD), characterized by the expression of mutant huntingtin (mhtt) and striatal degeneration. However, the role of Rsk in this neurodegenerative disease remains unknown. Here, we analyzed the protein levels, activity and role of Rsk in *in vivo* and *in vitro* HD models.

Results: We observed increased protein levels of Rsk1 and Rsk2 in the striatum of Hdh$^{Q111/Q111}$ and R6/1 mice, STHdh$^{Q111/Q111}$ cells and striatal cells transfected with full-length mhtt. Analysis of the phosphorylation of Rsk in Hdh mice and STHdh cells showed reduced levels of phospho Ser-380 (dependent on ERK1/2), whereas phosphorylation at Ser-221 (dependent on PDK1) was increased. Moreover, we found that elevated Rsk activity in STHdh$^{Q111/Q111}$ cells was mainly due to PDK1 activity, as assessed by transfection with Rsk mutant constructs. The increase of Rsk in STHdh$^{Q111/Q111}$ cells occurred in the cytosol and in the nucleus, which results in enhanced phosphorylation of both cytosolic and nuclear Rsk targets. Finally, pharmacological inhibition of Rsk, knock-down and overexpression experiments indicated that Rsk activity exerts a protective effect against mhtt-induced cell death in STHdh$^{Q7/Q7}$ cells transfected with mhtt.

Conclusion: The increase of Rsk levels and activity would act as a compensatory mechanism with capacity to prevent mhtt-mediated cell death. We propose Rsk as a good target for neuroprotective therapies in HD.

Keywords: cell death, ERK, Huntington's disease, knock-in mouse, neuroprotection, PDK1, R6/1 mouse, striatum

Background

The 90-kDa ribosomal S6 kinase (Rsk) is a family constituted by four isoforms (Rsk1-4) of serine/threonine kinases broadly expressed in the brain that regulate important cellular functions, including cell survival [1]. Rsk is activated by extracellular signal-regulated protein kinase (ERK) 1/2 [2] and 3-phosphoinositide-dependent protein kinase 1 (PDK1) [3] by sequential phosphorylations in the C-terminal kinase domain (CTKD) and N-terminal kinase domain (NTKD) [1,4], respectively. Briefly, sequential phosphorylations are initiated by ERK1/2 at Thr-573/574 of CTKD leading to the autophosphorylation of Rsk at Ser-380. This phosphorylation allows the dockage of PDK1 to the hydrophobic motif and enables PDK1-dependent phosphorylation in the NTKD of Rsk at Ser-221, resulting in its maximal activation [1,4]. When activated, Rsk promotes the phosphorylation of many cytosolic and nuclear targets. In the cytosol, Rsk induces the inactivation of certain pro-apoptotic proteins, such as Bad [5], glycogen synthase kinase 3β (GSK-3β) [6] or death-associated protein kinase (DAPK) [7], whereas in the nucleus it activates transcription factors involved in the synthesis of anti-apoptotic proteins, namely cAMP response element

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binding protein (CREB) [8], serum response factor (SRF) [9], and IκBα [10,11]. Although the function and the mechanism of Rsk activation have been well studied in non-neural cells, in neurons there are few studies about Rsk, and they associate its activity with the anti-apoptotic effect of trophic factors [12-14]. However, no data exists about the possible role of Rsk in neurodegenerative diseases.

Huntington's disease (HD) is a neurodegenerative disorder caused by a dominantly heritable expansion of a trinucleotide CAG repeat in the *huntingtin* (htt) gene [15], and characterized by the preferential neurodegeneration of striatal medium-sized spiny neurons [16]. Although the brain areas affected by the disease are well established, the mechanisms by which neural dysfunction and neurodegeneration occurs are not well defined yet. Interestingly, previous data from a HD cellular model show a de-regulation of both kinases that control Rsk activity. Knock-in striatal cells expressing full-length mutant huntingtin (mhtt) ($STHdh^{Q111/Q111}$) show increased levels of active PDK1 [17] and reduced levels of ERK1/2 activity [18] compared with striatal cells expressing wild-type htt ($STHdh^{Q7/Q7}$). Moreover, stimulation of these kinases and their pathways has been proposed as good therapeutic approaches for HD [19-21]. These results suggest a de-regulation of Rsk activity in HD models and that modulation of its activity could be a good therapeutic strategy. Therefore, here we studied whether the protein levels and activity of Rsk1 and Rsk2, the two isoforms with higher expression levels [1], are modified in the presence of mhtt. To this end, we analyzed striatal protein levels and activity of Rsk in knock-in mhtt mouse and cellular models. In addition, we studied the contribution of ERK1/2 and PDK1 to the activation of Rsk in the presence of mhtt. Finally, we evaluated the potential protective role of Rsk against mhtt toxicity.

Results

Rsk1 and Rsk2 protein levels are increased in knock-in and R6/1 models of HD

First of all, we analyzed by western blot whether the protein levels of the two major Rsk isoforms, Rsk1 and Rsk2, were altered in the striatum of 6- and 10-month old wild-type ($Hdh^{Q7/Q7}$) and mutant ($Hdh^{Q111/Q111}$) knock-in mice. We observed elevated Rsk1 and Rsk2 protein levels in the striatum of $Hdh^{Q111/Q111}$ mice compared to $Hdh^{Q7/Q7}$ mice at both ages (Figure 1A and 1B). In addition, the levels of these proteins were also augmented in striatal cells expressing full-length mhtt ($STHdh^{Q111/Q111}$) when compared to those in wild-type cells ($STHdh^{Q7/Q7}$; Figure 1C and 1D). These changes in Rsk1 and Rsk2 protein levels were not dependent on mhtt protein levels since we observed increased levels in Hdh mouse striatum expressing similar levels of mhtt (Figure 1A and 1B; $Hdh^{Q7/Q7}$ mice: 100 ± 9.8%; $Hdh^{Q111/Q111}$ mice: 89 ± 10.3%; Student's *t*-test: $p = 0.4164$) and in $STHdh^{Q111/Q111}$ cells, which express lower levels of mhtt compared with $STHdh^{Q7/Q7}$ (Figure 1C and 1D; $STHdh^{Q7/Q7}$ cells: 100 ± 11.6%; $STHdh^{Q111/Q111}$ cells: 36 ± 6.9%; Student's *t*-test: $p < 0.001$).

To know whether increased Rsk1 and Rsk2 protein levels also occur in exon-1 mhtt mice we analyzed by western blot these proteins in the striatum of R6/1 mice at 8- and 12-week of age, when they do not show motor symptoms [22]. Similar to that observed in the striatum of knock-in mice, R6/1 mouse striatum displayed higher Rsk1 and Rsk2 levels compared to wild-type (WT) mice at both ages (Figure 1E and 1F). Altogether, these results indicate that an increase of Rsk1 and Rsk2 protein levels is an event that occurs in full-length and exon-1 models of HD at presymptomatic stages. In addition, changes in Rsk1 and Rsk2 are not dependent on mhtt levels since we observed a similar response in striatal cells expressing low ($STHdh^{Q111/Q111}$ cells), normal (knock-in mice striatum) or very high (R6/1 mouse striatum) levels of mhtt.

Overexpression of full-length mhtt increases Rsk protein levels

In order to confirm that increased levels of Rsk were dependent on mhtt expression, we looked at the protein levels of Rsk1 and Rsk2 in $STHdh^{Q7/Q7}$ cells, M213 cells and striatal primary neurons transfected with a plasmid expressing full-length wild-type (FL-17Q htt) or mutant (FL-75Q htt) htt. The quantification of Rsk1 and Rsk2 levels was performed by confocal microscopy due to the low efficiency of transfection (15-20% approximately). In all cell types examined, transfection with FL-75Q htt increased Rsk1 and Rsk2 protein levels compared to those registered in cells expressing FL-17Q htt (Figure 2), indicating that the increase in Rsk1 and Rsk2 protein levels are due to the presence of mhtt.

Rsk phosphorylation in HD knock-in models: ERK-dependent residues *versus* PDK1-dependent residues

To study whether the phosphorylation levels of Rsk were altered by changes in total Rsk protein levels, we analyzed its phosphorylation at Ser-380 (dependent on ERK1/2) and at Ser-221 (dependent on PDK1), in the striatum of 10-month old $Hdh^{Q7/Q7}$ and $Hdh^{Q111/Q111}$ mice. We detected reduced levels of phospho-Rsk (Ser-380; reduction of 63 ± 13%; Figure 3A) and increased levels of phospho-Rsk (Ser-221; increase of 190 + 19%; Figure 3A) in $Hdh^{Q111/Q111}$ respect to $Hdh^{Q7/Q7}$ mice. Similar results were obtained in $STHdh^{Q111/Q111}$ cells (Figure 3B). These results indicate that in the presence

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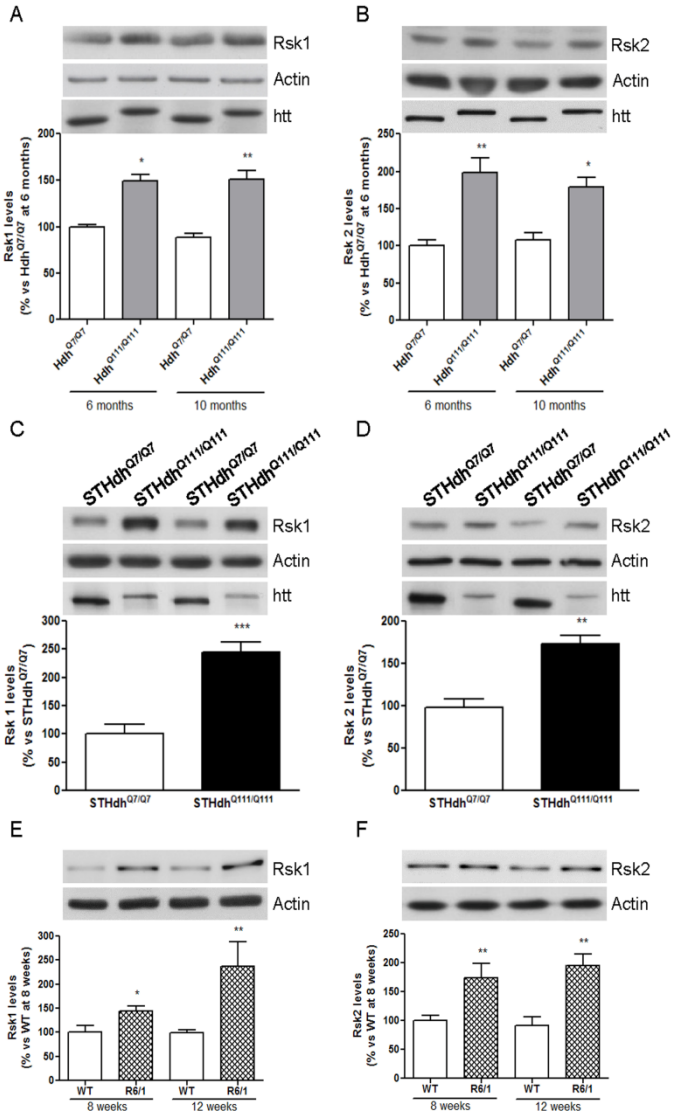

Figure 1 Rsk levels are elevated in HD mouse and cellular models. Rsk1 (A, C and E) and Rsk2 (B, D and F) protein levels were analyzed by western blot of protein extracts obtained from the striatum of 6- and 10-month old wild-type ($Hdh^{Q7/Q7}$) and knock-in ($Hdh^{Q111/Q111}$) mice (A and B), from wild-type ($STHdh^{Q7/Q7}$) and mutant ($STHdh^{Q111/Q111}$) htt knock-in striatal cells (C and D), and from the striatum of 8- and 12-week old WT and R6/1 mice (E and F). Htt protein levels were also analyzed by western blot in knock-in models (A-D). Results (mean ± SEM; n = 4-6) represent the ratio between Rsk and actin levels obtained by densitometric analysis of western blot data, and are expressed as a percentage of $Hdh^{Q7/Q7}$ levels at 6 months (A and B), as a percentage of protein levels in $STHdh^{Q7/Q7}$ cells (C and D), or as a percentage of protein levels in WT mice at 8 weeks (E and F). Data were analyzed by two-way ANOVA followed by Bonferroni's *post hoc* test (A, B, E and F) or by Student's t-test (C and D). *p < 0.05 and **p < 0.01 as compared with $Hdh^{Q7/Q7}$ mice (A and B), **p < 0.01 and ***p < 0.001 as compared with $STHdh^{Q7/Q7}$ cells (C and D), and *p < 0.05 and **p < 0.01 as compared with WT mice (E and F). Representative immunoblots are presented.

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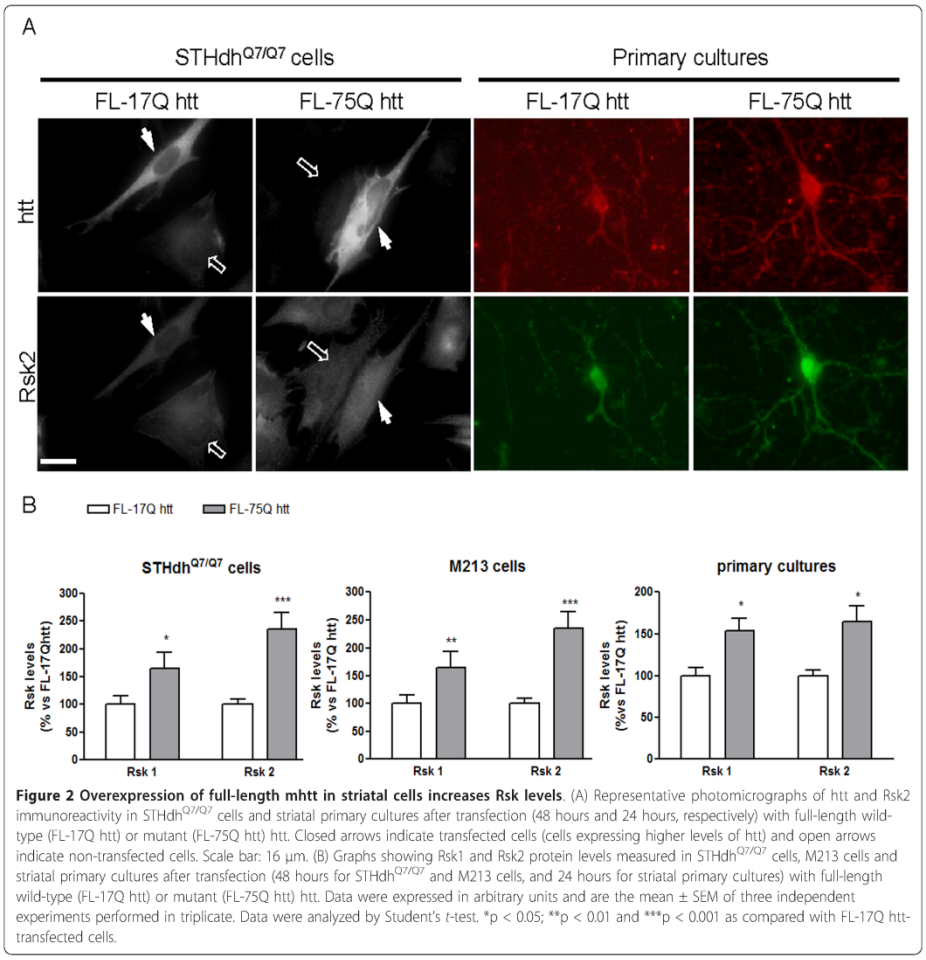

Figure 2 Overexpression of full-length mhtt in striatal cells increases Rsk levels. (A) Representative photomicrographs of htt and Rsk2 immunoreactivity in STHdh$^{Q7/Q7}$ cells and striatal primary cultures after transfection (48 hours and 24 hours, respectively) with full-length wild-type (FL-17Q htt) or mutant (FL-75Q htt) htt. Closed arrows indicate transfected cells (cells expressing higher levels of htt) and open arrows indicate non-transfected cells. Scale bar: 16 μm. (B) Graphs showing Rsk1 and Rsk2 protein levels measured in STHdh$^{Q7/Q7}$ cells, M213 cells and striatal primary cultures after transfection (48 hours for STHdh$^{Q7/Q7}$ and M213 cells, and 24 hours for striatal primary cultures) with full-length wild-type (FL-17Q htt) or mutant (FL-75Q htt) htt. Data were expressed in arbitrary units and are the mean ± SEM of three independent experiments performed in triplicate. Data were analyzed by Student's *t*-test. *p < 0.05; **p < 0.01 and ***p < 0.001 as compared with FL-17Q htt-transfected cells.

of mhtt the phosphorylation of Rsk at ERK- and PDK1-dependent residues is altered in an opposite way.

STHdh$^{Q111/Q111}$ cells show increased Rsk activity that is mainly regulated by PDK1

Our next goal was to know whether increased Rsk1 and Rsk2 protein and phosphorylation levels were associated with elevated Rsk activity. To this end, we analyzed Rsk activity in knock-in cells by using an *in vitro* activity assay. We observed that Rsk activity was higher in STHdh$^{Q111/Q111}$ than in STHdh$^{Q7/Q7}$ cells (270 ± 15%; Figure 4A). Moreover, overexpression of Rsk by transfection of HA-Rsk1 in STHdh$^{Q7/Q7}$ cells increased Rsk activity (STHdh$^{Q7/Q7}$ cells: 100 ± 9%; STHdh$^{Q7/Q7}$ + HA-Rsk: 222 ± 13%; p < 0.0002; Student's *t*-test) indicating that one of the parameters that regulates Rsk activity is its protein levels.

To address the importance of phosphorylation by ERK1/2 and PDK1 on elevated Rsk activation in STHdh$^{Q111/Q111}$ cells, we measured Rsk activity in knock-in cells transfected with two mutant forms of Rsk: HA-RskT574A and HA-RskS380E, which cannot be phosphorylated by ERK1/2 and PDK1, respectively. Transfection with HA-RskT574A or HA-RskS380E similarly reduced Rsk activity in STHdh$^{Q7/Q7}$ cells (reduction of 28% and 33% respectively; Figure 4B). Interestingly, and supporting a main

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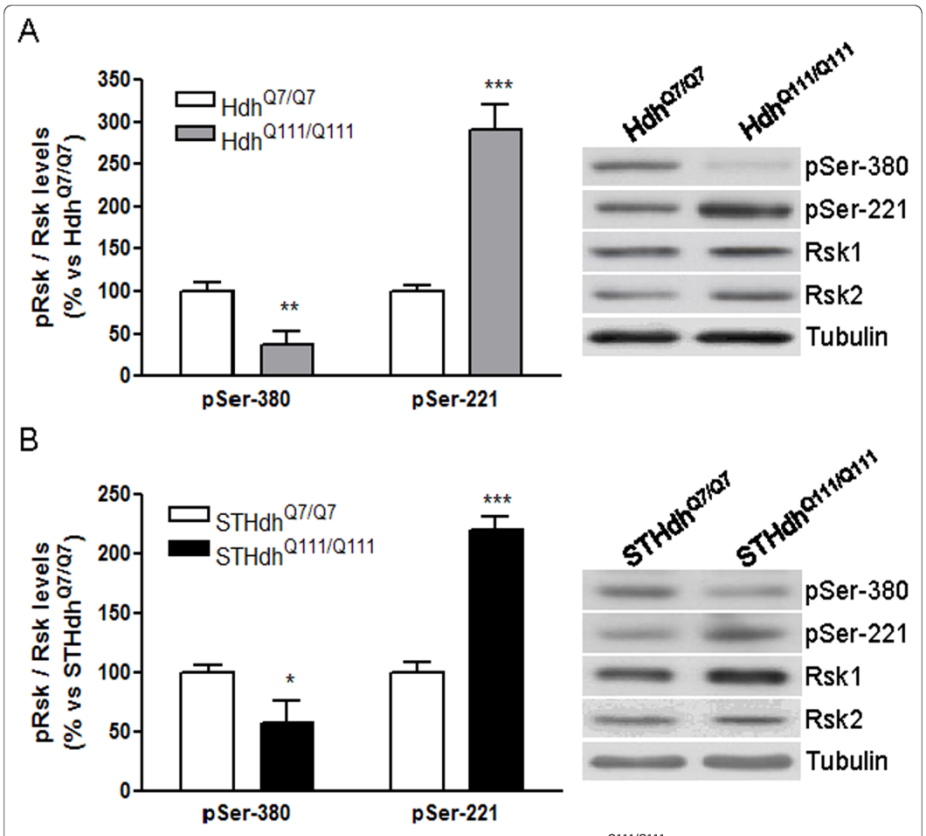

Figure 3 Different regulation of phospho-Rsk residues in HD knock-in mice and STHdh$^{Q111/Q111}$ cells. Lysates from the striatum of 10-month old wild-type (Hdh$^{Q7/Q7}$) and knock-in (Hdh$^{Q111/Q111}$) mice (A) or from wild-type (STHdh$^{Q7/Q7}$) and mutant (STHdh$^{Q111/Q111}$) htt knock-in striatal cells (B) were subjected to western blot to analyze phosphor-Rsk (Ser-380; Ser-221), Rsk1, Rsk2 and tubulin protein levels. Results (mean ± SEM; n = 4-6) represent the ratio between phospho-Rsk levels and Rsk1 plus Rsk2 levels obtained by densitometric analysis of western blot data, and are expressed as a percentage of Hdh$^{Q7/Q7}$ (A) or STHdh$^{Q7/Q7}$ (B) levels. Representative immunoblots are presented. All data were analyzed by Student's *t*-test. *p < 0.05, **p < 0.01 and ***p < 0.001 as compared with Hdh$^{Q7/Q7}$ mice (A) or STHdh$^{Q7/Q7}$ cells (B).

role for PDK1 in the increased Rsk activity observed in STHdh$^{Q111/Q111}$ cells, transfection with HA-RskS380E induced a stronger decrease of Rsk activity (47%; Figure 4B) compared with the transfection with HA-RskT574A (19%; Figure 4B). Note that co-transfection with both mutant forms reduced the activity of Rsk only by 30-40% probably because the efficiency of transfection was not maximal (Figure 4B).

Rsk levels are increased in both cytosol and nucleus of STHdh$^{Q111/Q111}$ cells

Phosphorylated and activated Rsk can translocate from the cytosol to the nucleus. In these compartments, it regulates different targets [1]. Thus, we studied Rsk1 and Rsk2 levels in cytosolic and nuclear fractions of knock-in cells by western blot. When compared with control cells, STHdh$^{Q111/Q111}$ cells displayed enhanced levels of Rsk1 and Rsk2 in both compartments, with a more pronounced effect in the nucleus (Figure 5A). To confirm these data, we analyzed the localization of Rsk by immunocytochemistry. We detected three different patterns of expression: homogeneous expression, and exclusive cytosolic or nuclear localization (Figure 5B). Analysis of STHdh$^{Q7/Q7}$ cells revealed a predominant homogeneous distribution of Rsk1, whereas Rsk2 was mainly located in the nucleus. In STHdh$^{Q111/Q111}$ cells,

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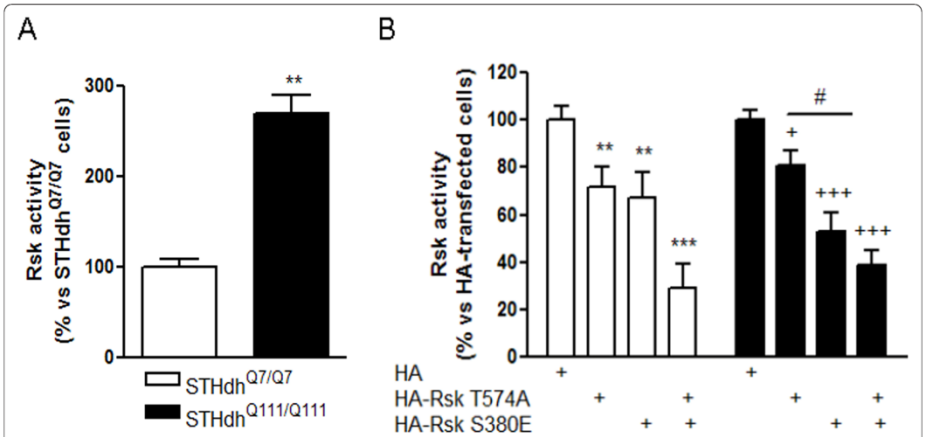

Figure 4 Increased Rsk activity in STHdh$^{Q111/Q111}$ cells is mainly regulated by PDK1. (A) Lysates from STHdh$^{Q7/Q7}$ and STHdh$^{Q111/Q111}$ cells were subjected to immunoprecipitation with anti-Rsk1 and anti-Rsk2 antibodies and the resulting immunocomplexes were used to determine Rsk activity as indicated in material and methods. Kinase activity was expressed as percentage of Rsk activity in STHdh$^{Q7/Q7}$ cells and data are the mean ± SEM of three independent experiments. Results were analyzed by Student's *t*-test. **$p < 0.01$ as compared with STHdh$^{Q7/Q7}$ cells. (B) STHdh$^{Q7/Q7}$ and STHdh$^{Q111/Q111}$ cells were transfected with the following constructs: HA-RskT574A (to study the role of ERK1/2 on Rsk activity) and HA-RskS380E (to analyze the role of PDK1 on Rsk activity). Twenty-four hours after transfection, both STHdh$^{Q7/Q7}$ and STHdh$^{Q111/Q111}$ cells were subjected to Rsk activity assay. Results are the mean ± SEM of three independent experiments and are expressed as percentage of control cells (cells transfected with HA alone). Data were analyzed by two-way ANOVA followed by Bonferroni's *post hoc* test. **$p < 0.01$ and ***$p < 0.001$ as compared with STHdh$^{Q7/Q7}$ control cells, $^{+}p < 0.05$ and $^{+++}p < 0.001$ as compared with STHdh$^{Q111/Q111}$ control cells and $^{\#}p < 0.05$ as compared with STHdh$^{Q111/Q111}$ cells transfected with HA-Rsk T574A.

Rsk1 changed its distribution as it was located only in the nucleus, while the nuclear expression of Rsk2 was even more evident than in STHdh$^{Q7/Q7}$ cells. Note that we did not observe exclusive cytosolic localization of either Rsk isoforms in STHdh$^{Q111/Q111}$ cells (Figure 5B). Altogether, these results indicate that although the increase of Rsk protein levels in STHdh$^{Q111/Q111}$ cells occurs in both compartments, this increase is more pronounced in the nucleus.

Increased activity of Rsk in STHdh$^{Q111/Q111}$ cells enhances the phosphorylation of both cytosolic and nuclear targets

Rsk plays its protective role through the inactivation of cytosolic pro-apoptotic proteins and/or the activation of transcription factors that mediate the synthesis of anti-apoptotic proteins. Thus, we determined in knock-in cells expressing wild-type or mhtt the phospho-levels of two Rsk substrates, Bad at Ser-112 (cytosolic target), and SRF at Ser-103 (nuclear target). According with the elevated Rsk activity observed in STHdh$^{Q111/Q111}$ cells, we found increased levels of phospho-Bad (Figure 6A) and phospho-SRF (Figure 6B) respect to STHdh$^{Q7/Q7}$ cells. Then, to determine whether the increased phosphorylation of Bad and SRF was due to the action of Rsk, we treated knock-in cells with a pharmacological and specific inhibitor of Rsk, BI-D1870 (0.1 μM) [23]. The presence of BI-D1870 reduced the phosphorylation levels of both Bad and SRF in STHdh$^{Q111/Q111}$ cells (Figure 6A and 6B). In STHdh$^{Q7/Q7}$ cells, we did not observe changes in phospho-Bad levels in the presence of BI-D1870 (Figure 6A), whereas phospho-SRF levels were slightly decreased (Figure 6B). To corroborate that BI-D1870 efficiently inhibited Rsk, we tested the activity of Rsk in both cell lines after treatment with Rsk inhibitor. Addition of BI-D1870 (0.1 μM) completely inhibited Rsk activity in STHdh cells (STHdh$^{Q7/Q7}$ cells: 100 ± 14%; STHdh$^{Q7/Q7}$ + BI: 7 ± 3%; STHdh$^{Q111/Q111}$ cells: 286 ± 22%; STHdh$^{Q111/Q111}$ + BI: 13 ± 5%). These results show that increased Rsk activity in STHdh$^{Q111/Q111}$ cells results in augmented phosphorylation of both cytosolic and nuclear targets.

Increased Rsk activity contributes to prevent mhtt-induced cell death

To evaluate whether elevated Rsk activity could exert a protective effect against mhtt-induced cell death, we study its protective capacity against mhtt-induced toxicity using pharmacological inhibition, knock-down and overexpression approaches. The expression of endogenous mhtt in immortalized STHdh$^{Q111/Q111}$ cells does not

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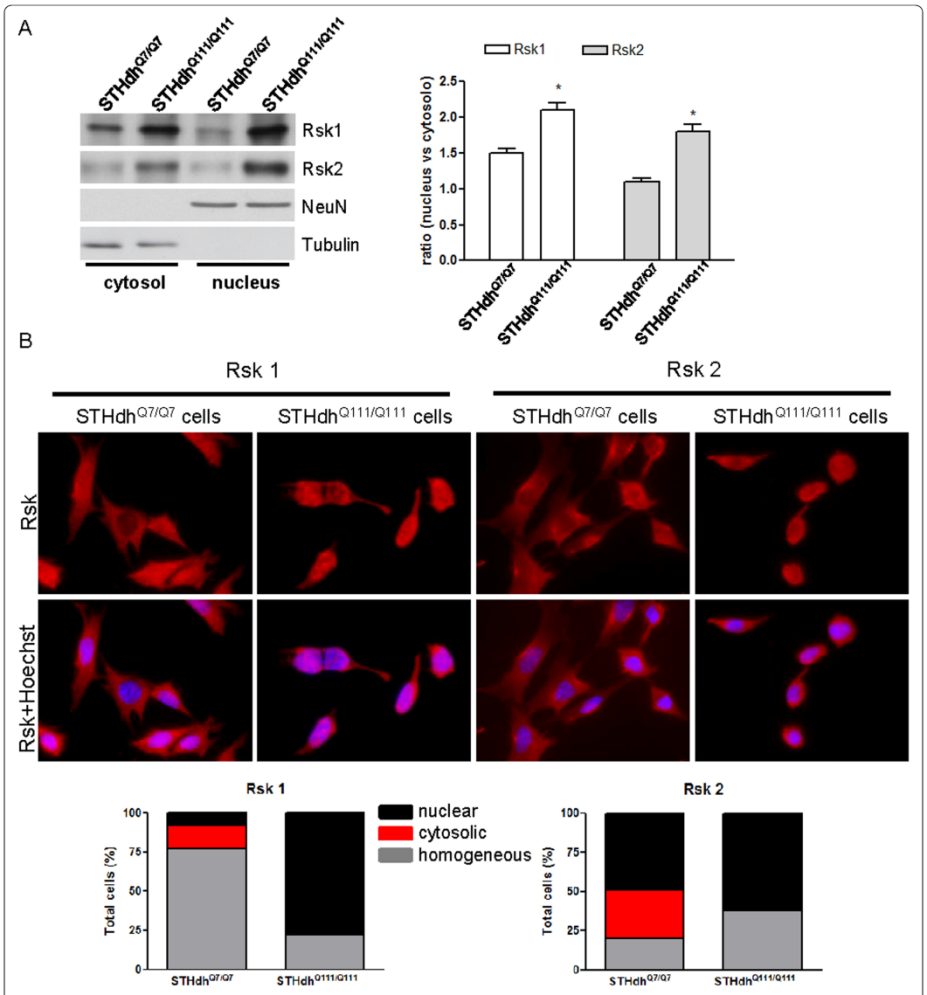

Figure 5 Striatal cells expressing full-length mhtt show increased levels of Rsk in nuclear and cytosolic compartments. (A) Rsk1, Rsk2, tubulin and NeuN were analyzed by western blot in cytosolic and nuclear fractions of STHdh$^{Q7/Q7}$ and STHdh$^{Q111/Q111}$ cells. Four independent experiments were performed in duplicate. Representative immunoblots are shown. We quantified the levels of Rsk1 and Rsk2 in both types of cells and analyzed the ratio between the nuclear and the cytosolic levels for each case. Data were analyzed by two-way ANOVA followed by Bonferroni's *post hoc* test. *$p < 0.05$ as compared with STHdh$^{Q7/Q7}$ cells. (B) Representative photomicrographs showing Rsk1 and Rsk2 immunoreactivity in STHdh$^{Q7/Q7}$ and STHdh$^{Q111/Q111}$ cells. The number of cells was quantified according to three different patterns of Rsk1 and Rsk2 localization: homogeneous localization (cytosolic and nuclear), exclusive cytosolic and exclusive nuclear localization. Nuclear localization was determined by co-staining with Hoechst 33258. Results are expressed as percentage of total cell number. Data was analyzed by chi-square (χ^2). For Rsk1; χ square df: 102.5, 2 and $p < 0.001$. For Rsk2: χ square df: 38.11, 2 and $p < 0.001$.

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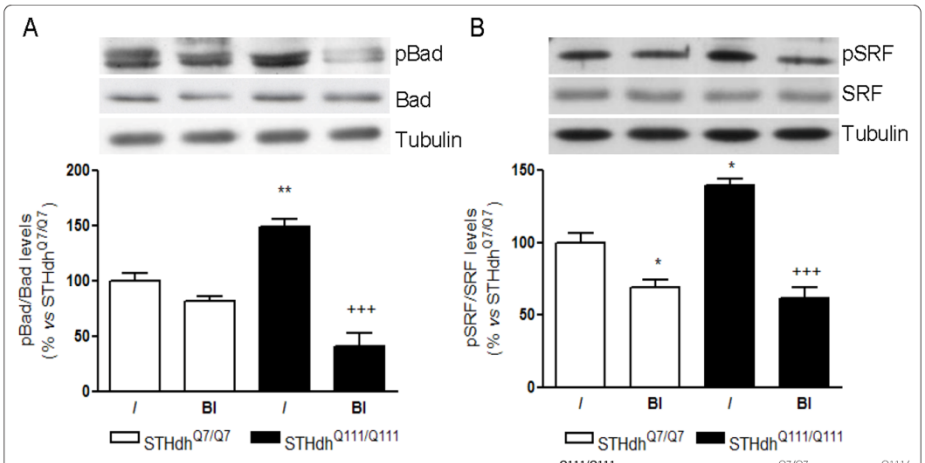

Figure 6 Inhibition of Rsk induces a reduction of pBAD and pSRF levels in STHdh$^{Q111/Q111}$ cells. Lysates from STHdh$^{Q7/Q7}$ and STHdh$^{Q111/Q111}$ cells treated with or without BI-D1870 (0.1 μM; BI) were subjected to western blot to analyze pBAD (Ser-112)/Bad (A) and pSRF (Ser-103)/SRF (B) and tubulin protein levels. Results are expressed as the ratio between phospho and total Bad (A) and SRF (B) levels. Data are the mean ± SEM of four independent experiments performed in duplicate. Representative immunoblots are presented. Results are expressed in percentage respect to STHdh$^{Q7/Q7}$ cells. Data were analyzed using two-way ANOVA followed by Bonferroni's *post hoc* test. *$p < 0.05$ and **$p < 0.01$ as compared with control STHdh$^{Q7/Q7}$ cells and$^{+++}p < 0.001$ as compared with control STHdh$^{Q111/Q111}$ cells.

produce cell death. Thus, to induce mhtt toxicity, STHdh$^{Q7/Q7}$ cells were transfected with wild-type (FL-17Q htt) or mutant (FL-75Q htt) htt and cell death was assessed by Hoechst 33258 staining 72 hours after transfection. Overexpression of FL-75Q htt induced 16 ± 3% apoptotic cell death *versus* 6 ± 2% apoptotic cell death observed in FL-17Q htt-transfected cells (Figure 7A). In parallel experiments we treated transfected cells with the Rsk inhibitor BI-D1870 (0.1 μM). The inhibition of Rsk exacerbated the toxic effect of FL-75Q htt expression and increased apoptotic cell death to 28 ± 4% (Figure 7A). In contrast, addition of BI-D1870 to FL-17Q htt-transfected cells did not alter cell death (Figure 7A). Our next goal was to know whether the protective role of Rsk was mediated by Rsk1, Rsk2, or by both isoforms. To address this issue, STHdh$^{Q7/Q7}$ cells were co-transfected with FL-75Q htt and with siRNAs against Rsk1 (siRsk1), Rsk2 (siRsk2) or both (siRsk1 + siRsk2). First, we checked that transfection with siRsk1 or siRsk2 separately decreased the protein levels of each isoform, and that the co-transfection with siRsk1 and siRsk2 reduced the expression of both isoforms (Figure 7B). The analysis of cell death showed that inhibition of Rsk1 or Rsk2 separately was not enough to increase the toxic effect of mhtt (Figure 7C). In contrast, the knock-down of both isoforms enhanced FL-75Q htt-mediated cell death (Figure 7C), similar to that observed after treatment with BI-D1870 (Figure 7A). To confirm the beneficial effect of Rsk in cells expressing mhtt, we overexpressed Rsk in cells transfected with FL-75Q htt by the co-transfection with HA-Rsk. The analysis of cell death 72 hours later revealed that Rsk overexpression reduced two-fold the cell death induced by mhtt (Figure 7D). Thus, we conclude that Rsk activity exerts a protective effect against mhtt-induced toxicity, and that both Rsk1 and Rsk2 isoforms are involved in this protective effect.

Discussion

In this work, we provide for the first time evidence for a role of Rsk in HD. Within Rsk family, we analyzed Rsk1 and Rsk2, two isoforms that are broadly expressed in the brain, including the striatum, and whose expression levels are higher respect to other Rsk isoforms [1]. We observed increased protein levels of both Rsk1 and Rsk2 in the striatum of Hdh$^{Q111/Q111}$ and R6/1 mice, and in STHdh$^{Q111/Q111}$ cells, which are not dependent on mhtt protein levels as these HD models express different levels of mhtt. Thus, increased Rsk1 and Rsk2 protein levels is the result of the presence of mhtt, and we confirmed this hypothesis by showing that transfection of FL-75Q htt in STHdh$^{Q7/Q7}$ cells, M213 cells or striatal primary cultures elevated the levels of both Rsk isoforms. In addition, increased Rsk1-2 protein levels correlated with higher basal Rsk activity in STHdh$^{Q111/Q111}$ cells than in cells expressing wild-type htt. Interestingly, the inhibition of Rsk activity enhanced striatal cell death

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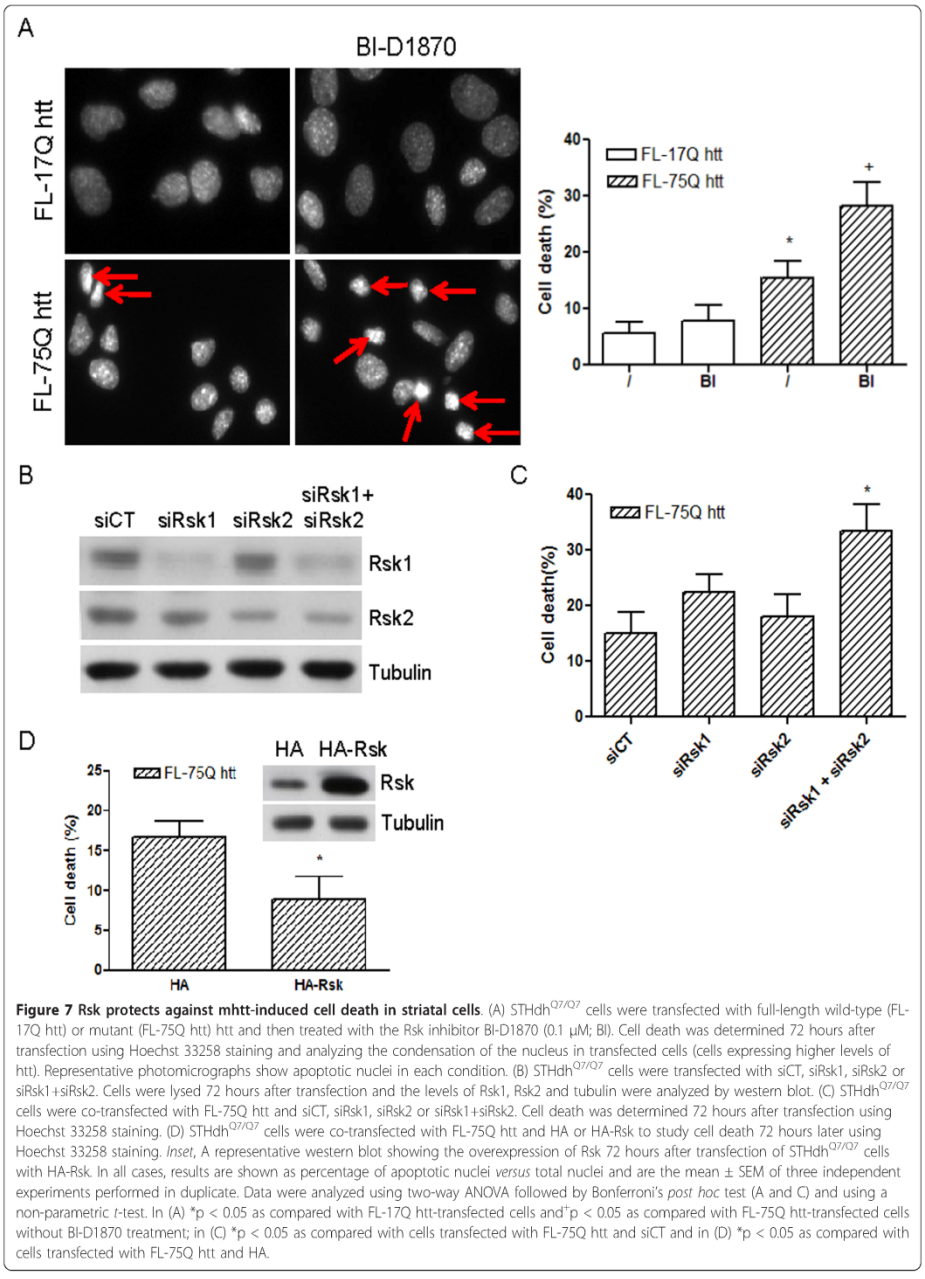

Figure 7 Rsk protects against mhtt-induced cell death in striatal cells. (A) STHdh$^{Q7/Q7}$ cells were transfected with full-length wild-type (FL-17Q htt) or mutant (FL-75Q htt) htt and then treated with the Rsk inhibitor BI-D1870 (0.1 μM; BI). Cell death was determined 72 hours after transfection using Hoechst 33258 staining and analyzing the condensation of the nucleus in transfected cells (cells expressing higher levels of htt). Representative photomicrographs show apoptotic nuclei in each condition. (B) STHdh$^{Q7/Q7}$ cells were transfected with siCT, siRsk1, siRsk2 or siRsk1+siRsk2. Cells were lysed 72 hours after transfection and the levels of Rsk1, Rsk2 and tubulin were analyzed by western blot. (C) STHdh$^{Q7/Q7}$ cells were co-transfected with FL-75Q htt and siCT, siRsk1, siRsk2 or siRsk1+siRsk2. Cell death was determined 72 hours after transfection using Hoechst 33258 staining. (D) STHdh$^{Q7/Q7}$ cells were co-transfected with FL-75Q htt and HA or HA-Rsk to study cell death 72 hours later using Hoechst 33258 staining. *Inset*, A representative western blot showing the overexpression of Rsk 72 hours after transfection of STHdh$^{Q7/Q7}$ cells with HA-Rsk. In all cases, results are shown as percentage of apoptotic nuclei *versus* total nuclei and are the mean ± SEM of three independent experiments performed in duplicate. Data were analyzed using two-way ANOVA followed by Bonferroni's *post hoc* test (A and C) and using a non-parametric *t*-test. In (A) *$p < 0.05$ as compared with FL-17Q htt-transfected cells and $^{+}p < 0.05$ as compared with FL-75Q htt-transfected cells without BI-D1870 treatment; in (C) *$p < 0.05$ as compared with cells transfected with FL-75Q htt and siCT and in (D) *$p < 0.05$ as compared with cells transfected with FL-75Q htt and HA.

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induced by transfection of mhtt (FL-75Q htt) in STHdh$^{Q7/Q7}$ cells. Moreover, we show that the overexpression of Rsk reduces considerably cell death in STHdh$^{Q7/Q7}$ cells transfected with FL-75Q htt. Altogether, these results indicate that elevated Rsk1-2 activity is an efficient mechanism to protect cells against mhtt toxicity. Thus, increased activity of Rsk1-2 could be a compensatory mechanism occurring in HD striatum.

Compensatory responses activated at early phases of HD are considered interesting targets to design neuroprotective therapies to inhibit the progression of neurodegeneration. Here, we analyzed Hdh$^{Q111/Q111}$ mice at 6 and 10 months of age, and R6/1 at 8 and 12 weeks of age, when they do not show motor dysfunction, but express cellular and molecular markers of HD pathology [22,24-28]. In addition, we studied the activity and the neuroprotective role of Rsk in STHdh cells, which derive from Hdh$^{Q111/Q111}$ embryos [29], and reproduce early events in the disease cascade [17]. We observed that increased Rsk activity is neuroprotective against mhtt-induced cell death since inhibition of its activity in FL-75Q htt-transfected STHdh$^{Q7/Q7}$ cells increased cell death. Moreover, we determined that this neuroprotective effect is due to an increase of Rsk activity in striatal cells expressing mhtt, since the inhibition of Rsk did not affect the viability of FL-17Q htt-transfected STHdh$^{Q7/Q7}$ cells. Confirming the protective role of Rsk against mhtt, we overexpressed Rsk in STHdh$^{Q7/Q7}$ cells and showed a protective effect against mhtt-mediated cell death. In addition, we show that both Rsk1 and Rsk2 are necessary to exert this protective role. Different studies in HD models have shown the regulation of other kinases as compensatory mechanisms activated in response of mhtt toxicity. These include Akt [17,30,31] and proteins closely related to Akt, such as the serum- and glucocorticoid-induced kinase [32]. Here, we show that in addition to these kinases, Rsk activity is also up-regulated in the presence of mhtt, and more importantly, that Rsk activity is neuroprotective against mhtt-induced cell death.

Present results also show that the increased activity of Rsk in striatal neurons expressing mhtt is mainly due to the action of PDK1, a kinase whose activity is independent of extracellular factors [3]. We detected increased phosphorylation of Rsk at Ser-221 (dependent on PDK1) and reduced phosphorylation of Rsk at Ser-380 (indirectly dependent on ERK1/2) in Hdh$^{Q111/Q111}$ mice striatum and STHdh$^{Q111/Q111}$ cells. In accordance with our results, STHdh$^{Q111/Q111}$ cells display elevated levels of phospho-PDK1 [17] and reduced levels of phospho-ERK1/2 [18] respect to STHdh$^{Q7/Q7}$ cells. In addition, using mutant forms of Rsk, we show that the activity of Rsk in STHdh$^{Q111/Q111}$ cells was considerably inhibited when PDK1-regulated, but not when ERK1/2-regulated residues, were mutated. Consistent with our observations, it has been suggested that PDK1 has the capacity to activate Rsk in an ERK1/2 independent manner [33]. Furthermore, and supporting the important role of PDK1-mediated phosphorylation to Rsk activity, PDK1 deficiency results in Rsk inactivation [34]. Although the neuroprotective role of Rsk has been classically associated with extracellular stimuli induced by trophic factors such as brain-derived neurotrophic factor [13] or epidermal growth factor [12] through the activation of ERK1/2, Rsk phosphorylation by PDK1 increases its activity to a higher extend than ERK1/2-dependent phosphorylation [3]. In HD, neurotrophic deprivation has been proposed as one of the mechanisms involved in the preferential loss of striatal neurons [28,35,36]. Thus, in the absence of trophic support, activation of Rsk through the basal activity of PDK1 could be a crucial mechanism to prevent cell death in HD.

The neuroprotective activity of Rsk is basically due to the wide range of proteins that it regulates. In the nucleus, Rsk phosphorylates and activates several transcription factors, some of them implicated in neuronal survival such as SRF [9], CREB [37] or NFκB [10,11]. Studies in non-neural cell lines showed that in the cytosol Rsk phosphorylates and inactivates pro-apoptotic proteins such as Bad [5], GSK-3β [6] or DAPK [7]. In this way, our data indicate that Rsk1-2 activity is elevated in the cytosol and in the nucleus of STHdh$^{Q111/Q111}$ cells. In the cytosol, increased Rsk activity correlated with an enhancement of phosphorylated Bad, whereas in the nucleus we observed increased levels of phospho-SRF. Changes in phospho-Bad and phospho-SRF were due, at least in part, to Rsk activation, since inhibition of Rsk significantly reduced the phosphorylation levels of both proteins in STHdh$^{Q111/Q111}$ cells. In STHdh$^{Q7/Q7}$ cells, the inhibition of Rsk produced a slight effect on SRF phosphorylation levels, and we did not detect an effect on Bad phosphorylation. Probably, this lack of effect on Bad phosphorylation is due the predominant nuclear activity of Rsk in unstimulated cells [1,2]. Overall, we propose that the neuroprotective effect of Rsk observed in the models studied here could be mediated by the inactivation of pro-apoptotic factors in addition to the activation of transcription factors that regulate the expression of anti-apoptotic proteins.

Conclusions

In conclusion, here we provide evidences that the increase of Rsk1-2 levels is an early event taking place in striatal cells expressing full-length mhtt. Increased Rsk1-2 levels contribute to enhance Rsk activity. Interestingly, our results strongly support that increased Rsk activity in the presence of mhtt is mainly regulated by

the basal activity of PDK1 and not by ERK1/2. Moreover, we show that the increase of Rsk1-2 activity observed in cells expressing mhtt could contribute to prevent mhtt-induced cell death. This is the first work showing a role for Rsk in HD, and we propose that therapies targeted to maintain Rsk activity would be a good approach for neuroprotection in HD.

Methods

HD mouse models

Homozygous mutant $Hdh^{Q111/Q111}$ and wild-type $Hdh^{Q7/Q7}$ knock-in mice were obtained from mating between male and female $Hdh^{Q111/Q7}$ heterozygotes as described previously [23]. We also used R6/1 mice (B6CBA background) expressing the exon-1 of mhtt with 145 CAG repeats [38]. Mouse genotype was determined as described elsewhere [22]. CAG repeat length was determined by PCR amplification of the repeat using HD1 and HD2 fluorescently labeled primers as previously describe by the Huntington's Disease Collaborative Research Group [15], and subsequent size determination in an ABI 3100 analyzer. These results were double checked by Laragen, Inc. (Los Angeles, CA). All mice used in the present study were housed together in numerical birth order in groups of mixed genotypes, and data were recorded for analysis by microchip mouse number. Experiments were conducted in a blind-coded manner respect to genotype. Mice were genotyped by polymerase chain reaction as described previously [23]. The animals were housed with access to food and water *ad libitum* in a colony room kept at 19-22°C and 40-60% humidity, under a 12:12 hours light/dark cycle. All procedures were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approved by the Local Animal Care Committee of *Universitat de Barcelona* (99/01), and *Generalitat de Catalunya* (99/1094), in accordance with the Directive 86/609/EU of the European Commission.

Cell cultures and pharmacological treatments

Conditionally immortalized striatal neuronal progenitor cell lines expressing endogenous levels of wild-type ($STHdh^{Q7/Q7}$) or mutant ($STHdh^{Q111/Q111}$) full-length htt with 7 and 111 glutamines, respectively, have been described elsewhere [26]. Cells were grown at 33°C in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co. St. Louis, MO) supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 2 mM L-glutamine, and 400 μg/ml G418 (Geneticin; Invitrogen, Carlsbad, CA). M213 cells (conditionally immortalized striatal-derived neural stem cells) were grown as previously described [39]. Primary striatal cultures were obtained from 18-day old C57BL6 mouse embryos (Charles River, France). Striata were dissected as previously described [40,41]. Cells (50,000 cells/cm^2) were seeded on plates pre-coated with 0.1 mg/mL poly-D-lysine (Sigma Chemical Co.) and cultured in Neurobasal medium supplemented with B27 (Gibco, Paisley, Scotland, UK) and glutamax at 37°C in a humidified atmosphere containing 5% CO_2.

To measure Rsk activity and phosphorylated levels of Bad and SRF, the Rsk inhibitor BI-D1870 (0.1 μM, Boehringer Ingelheim Pharma GmbH & Co) was added during 4 hours to $STHdh^{Q7/Q7}$ and $STHdh^{Q111/Q111}$ cell cultures. For quantification of apoptosis, $STHdh^{Q7/Q7}$ and $STHdh^{Q111/Q111}$ cells were treated with BI-D1870 (0.1 μM) for 72 hours.

DNA constructs and transfection

Full-length wild-type (FL-17Q htt) and mutant (FL-75Q htt) htt constructs were a gift from Drs. Frederic Saudou and Sandrine Humbert (Institut Curie, Orsay, France). HA-Rsk, HA-Rsk T574A and HA-Rsk S381E were kindly provided by Dr. Dario Alessi (MRC Protein Phosphorylation Unit, Dundee, Scotland, UK). All DNA constructs were transfected using Lipofectamine 2000 (Invitrogen) as instructed by the manufacturer. Both $STHdh^{Q7/Q7}$ and M213 cells were transfected at 50% confluence, whereas primary striatal cultures were transfected at 5 days *in vitro*.

$STHdh^{Q7/Q7}$ cells were transfected using 7.5 pmol Rsk1 siRNA (Silencer® Pre-designed siRNA, s73163 and s73164, Ambion, Applied Biosystems, Foster City, CA) and/or Rsk2 siRNA (Silencer® Pre-designed siRNA, s99855 and s99857, Ambion) with Lipofectamine 2000 as instructed by the manufacturer and incubated during 4 hours. A non-targeting control siRNA (7.5 pmol) (Silencer® Negative Control 1 siRNA, Ambion) was used to assess non-specific gene silencing effects. Cells were lysed or fixed 72 hours after transfection.

Protein extraction and subcellular fractionation

$STHdh^{Q7/Q7}$ and $STHdh^{Q111/Q111}$ cells, with or without BI-D1870 treatment or Rsk knock-down, were washed once with phosphate-buffered saline (PBS), 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 EGTA, 150 mM NaCl, protease inhibitors (2 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/μL aprotinin, 1 μg/μL leupeptin) and phosphatase inhibitor sodium orthovanadate (2 mM). $Hdh^{Q7/Q7}$ and $Hdh^{Q111/Q111}$ mice were deeply anesthetized and killed by decapitation at the age of 6 and 10 months and wt and R6/1 mice at 8 and 12 weeks of age. The brain was quickly removed and the striatum was dissected out and homogenized in lysis buffer (as above). All samples were centrifuged at 16 100 × *g* for 20 minutes at 4°C, the

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supernatants were collected and protein concentration was measured using the *Dc* protein assay kit (Bio-Rad Laboratories, Hercules, CA).

For subcellular fractionation, STHdh$^{Q7/Q7}$ and STHdh$^{Q111/Q111}$ cells were rinsed once with PBS and centrifuged at 800 *g* for 5 minutes. Pellets were homogenized in lysis buffer (10 mM Tris-HCl pH 7.5, 0.25 M sucrose, 2 mM PMSF, 10 μg/μL aprotinin, 1 μg/μL leupeptin, 2 mM sodium orthovanadate) and centrifuged at 3000 × *g* for 10 minutes. The resulting supernatant was centrifuged at 10 000 × *g* for 15 minutes to obtain a cytosol/light membrane supernatant that was further centrifuged at 100 000 × *g* for 15 minutes to obtain the cytosolic fraction (supernatant). The pellet resulting from the initial centrifugation was resuspended in lysis buffer and centrifuged at 800 × *g* for 10 minutes. The pellet, containing washed nuclear fraction, was then resuspended in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 10 mM EGTA, 2 mM PMSF, 10 μg/μL aprotinin, 1 μg/μL leupeptin, 2 mM sodium orthovanadate) and incubated for 30 minutes at 4°C in a tube rotator. Finally, after centrifuging for 15 minutes at 16 100 × *g*, the supernatant was collected and stored. Protein concentrations were determined as above.

Western blot

Western blot was performed as described elsewhere [41]. The following primary antibodies were used: anti-Rsk1, anti-Rsk2, anti-phospho-Rsk (Ser-221) and anti-phospho-Rsk (Ser-380) (all 1:500; Santa Cruz Biotechnology, Santa Cruz, CA), anti-htt monoclonal 2166 (1:1000; Millipore Bioscience Research reagents, Temecula, CA), anti-phospho-SRF (Ser-103), anti-SRF, anti-phospho-Bad (Ser-112) and anti-Bad (all 1:1000; Cell Signaling Technology, Beverly, MA), and anti-HA (1:1000; Sigma-Aldrich, Saint Louis, MO). Loading control was performed by reprobing the membranes with anti-NeuN (1:1000; Chemicon, Temecula, CA), anti-α-tubulin (1:100.000; Sigma-Aldrich) or anti-actin (1:10 000; MP Biochemicals, Aurora, OH).

Immunocytochemical staining, confocal microscopy analysis, and Rsk localization

Cells were fixed in 4% paraformaldehyde for 10 minutes, incubated with 0.2 M glycine for 20 minutes and permeabilized in 0.1% saponin for 10 minutes. Blocking was performed with 1% BSA in PBS for 1 hour. Specimens were incubated overnight with the primary antibodies (all 1:100): anti-htt monoclonal 2166, anti-Rsk1 and anti-Rsk2. Afterwards, specimens were incubated with subtype-specific fluorescent secondary antibodies: Cyanine 3 anti-rabbit (1:200; Invitrogen), rhodamine-conjugated anti-mouse (1:200; Jackson ImmunoResearch, West Grove, PA) and Alexa 647 anti-mouse (1:150; Invitrogen).

For quantification of Rsk in htt-transfected cells, immunocytochemistry was performed 24 (striatal primary cultures) or 48 (striatal knock-in and M213 cells) hours after transfection. Quantification of Rsk1 and Rsk2 was performed by confocal microscopy (Leica, Mannheim, Germany) as previously described [42]. Values were expressed as a ratio between the sums of Rsk1 or Rsk2 positive pixels *versus* cell area. For each condition, 30-40 cells were randomly selected. To study the localization of Rsk1 and Rsk2, STHdh$^{Q7/Q7}$ and STHdh$^{Q111/Q111}$ cells were fixed at 80% confluence and processed for immunocytochemistry against Rsk1 or Rsk2. At least 250 cells were evaluated for each condition.

Rsk activity assay

To measure Rsk activity, the assay was performed in STHdh wild-type or mutant cells at 80% confluence or 24 hours after transfection with Rsk constructs, as described previously [23]. Briefly, immunoprecipitation of Rsk was performed by incubation of total protein extracts (100 μg) with anti-Rsk1 and anti-Rsk2 antibodies, 1 μg each. Then, immunoprecipitates were incubated for 15 minutes at 30°C under continuous agitation with the assay mixture buffer containing the substrate peptide and the mixture of ATP and [γ-^{32}P] ATP (PerkinElmer, Boston, MA). Reactions were terminated and analyzed as described elsewhere [43]. Incubation with BI-D1870 was used to assess the specificity of Rsk activity assay.

Quantification of apoptosis

STHdh$^{Q7/Q7}$ cells transfected with FL-17Q htt or FL-75Q htt, with or without Rsk siRNAs, or Rsk DNA plasmid transfection or BI-D1870 treatment were processed for immunocytochemistry against htt as described above. Finally, cells were washed twice in PBS and stained with Hoechst 33258 (1 μg/mL; Molecular Probes, Inc, Eugene, OR) for 5 minutes. After washing twice with PBS the coverslips were mounted with mowiol. Nuclear DNA staining was observed with a fluorescence microscope (Olympus). Transfected cells were detected by the overexpression of htt respect to non-transfected cells. Condensed or fragmented nuclei were counted as apoptotic. At least 100 cells were evaluated for each condition in each independent experiment.

Statistical analysis

Statistical analysis was performed by using the one- or two-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test, or the unpaired Student's *t*-test, as appropriate and indicated in the figure legends.

The analysis of Rsk distribution by immunocytochemical staining was performed using the chi square. A value of $p < 0.05$ was accepted as denoting statistical significance.

Abbreviations

The abbreviations used are: CREB: cAMP response element binding protein; CTKD: C-terminal kinase domain; DAPK: death-associated protein kinase; ERK: extracellular signal-regulated kinase; GSK: glycogen synthase kinase; HD: Huntington's disease; htt: huntingtin; $Hdh^{Q7/Q7}$: wild-type huntingtin mouse; $Hdh^{Q111/Q111}$: mutant huntingtin mouse; mhtt: mutant huntingtin; NTKD: N-terminal kinase domain; PDK1: 3-phosphoinositide-dependent protein kinase-1; Rsk: 90-kDa ribosomal S6 kinase; SRF: serum response factor; $STHdh^{Q7/Q7}$: striatal wild-type huntingtin cells; $STHdh^{Q111/Q111}$: striatal mutant huntingtin cells; WT: wild-type.

Acknowledgements

We thank Dr. M. MacDonald (Massachusetts General Hospital, Boston, MA) for the knock-in striatal cell lines and mice, Dr. W. J. Freed (NIMH-Neuroscience Center, Washington, DC) for the M213 cells, Drs. S. Humbert and F. Saudou (Institut Curie, Orsay, France) for the full-length wild-type and mhtt plasmids, and Dr. D. R. Alessi (MRC Protein Phosphorylation Unit, University of Dundee, Scotland, UK) for the generous gift of HA-Rsk mutant plasmids. We also thank Maria Teresa Muñoz and Ana López for technical assistance. Financial support was obtained from Ministerio de Ciencia e Innovación (SAF2008-0436) and Fondo de Investigaciones Sanitarias (Instituto de Salud Carlos III, PI071183; RETICS, R006/0010/0006). MAH and LR are fellows of Ministerio de Ciencia e Innovación, Spain (BES-2009-026742 and AP2007-01066, respectively) and AS was supported Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED, CB06/05/0054), Spain.

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Authors' contributions

XX, and JA conceptualized the study. XX, EPN and JA wrote the manuscript. XX, MAH, LR and AS carried out experiments. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 28 July 2011 Accepted: 31 October 2011
Published: 31 October 2011

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doi:10.1186/1750-1326-6-74
Cite this article as: Xifró *et al.*: Increased 90-kDa ribosomal S6 kinase (Rsk) activity is protective against mutant huntingtin toxicity. *Molecular Neurodegeneration* 2011 **6**:74.

Activation of Elk-1 participates as a neuroprotective compensatory mechanism in models of Huntington's disease

This paper has been published in Journal of Neurochemistry

Anglada-Huguet et al. J. Neurochem. 2012, 121, 639–648.

http://onlinelibrary.wiley.com/doi/10.1111/j.1471-4159.2012.07711.x/full

AIM 1. TO STUDY MOLECULAR COMPENSATORY MECHANISM ACTIVATED DURING THE PROGRESSION OF HUNTINGTON'S DISEASE.

1.2. To analyze the role of the transcription factor Elk-1 in the selective neuronal death that is produced in Huntington's disease.

El factor de transcripció Elk-1 té un paper protector davant estímuls tòxics. En aquest estudi hem explorat l'efecte neuroprotector d'Elk-1 en la malaltia de Huntington. Per assolir aquest objectiu, hem utilitzat dos models de ratolí que expressen l'exó 1 de la huntingtina mutada (els ratolins R6/1 i R6/2) i una línia cel·lular que expressa la forma completa de la huntingtina mutada (STHdhQ111/Q111). Hem pogut observar que es produeix un augment en els nivells proteics i la fosforilació (Ser383) del factor de transcripció Elk-1 a l'estriat dels ratolins R6 durant la progressió de la malaltia, així com també en les cèl·lules STHdhQ111/Q111. A més a més, vam observar una localització preferencialment nuclear d'Elk-1 en les cèl·lules STHdhQ111/Q111 i a l'estriat del ratolins R6/1 a les 30 setmanes d'edat. En el nucli, Elk-1 no mostrava colocalització amb els agregats de huntingtina mutada, suggerint que era possible que l'augment en la seva fosforilació i localització nuclear correlacionés amb una major activitat transcripcional. D'acord amb aquests resultats, el bloqueig d'Elk-1 provocava una disminució dels gens d'expressió immediata en les cèl·lules STHdhQ111/Q111, però no en aquelles cèl·lules que no expressaven huntingtina mutada. De manera interessant, la reducció dels nivells d'Elk-1 per la transfecció d'RNA d'interferència promovia la mort cel·lular i el clivatge de la proteïna caspasa 3 (marcador de mort neuronal), només en aquelles cèl·lules que expressaven la huntingtina mutada. En resum, proposem que l'augment en els nivells proteics, la fosforilació i la localització nuclear del factor de transcripció Elk-1 en models de la malaltia de Huntington que expressen la forma truncada o la forma completa de la huntingtina mutada podria actuar com a mecanisme compensatori activat en les cèl·lules estriatals en resposta a la presència de la huntingtina mutada i contribuint així a un efecte neuroproctetor.

Journal of Neurochemistry JNC

JOURNAL OF NEUROCHEMISTRY | 2012 | 121 | 639–648 doi: 10.1111/j.1471-4159.2012.07711.x

ORIGINAL ARTICLE

Activation of Elk-1 participates as a neuroprotective compensatory mechanism in models of Huntington's disease

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Abstract

The transcription factor Elk-1 has been revealed as neuroprotective against toxic stimuli. In this study, we explored the neuroprotective capacity of Elk-1 in Huntington's disease. To this aim, we used two exon-1 mutant huntingtin (mhtt) mouse models (R6/1 and R6/2), and a full-length mhtt striatal cell model (STHdh$^{Q111/Q111}$). Analysis of Elk-1 and pElk-1^{Ser383} in the striatum of R6 mice revealed increased levels during the disease progression. Similarly, Elk-1 and pElk-1^{Ser383} levels were increased in STHdh$^{Q111/Q111}$ cells when compared with wild-type cells. In addition, we observed a predominant nuclear localization of Elk-1 in STHdh$^{Q111/Q111}$ cells, and in the striatum of 30-week-old R6/1 mice. Nuclear Elk-1 did not colocalize with mhtt aggregates, suggesting a higher transcriptional activity. In agreement, the knock-down of Elk-1 decreased immediate early genes expression in STHdh$^{Q111/Q111}$ cells, but not in wild-type cells. Interestingly, reduction of Elk-1 levels by siRNAs transfection promoted cell death and caspase 3 cleavage in STHdh$^{Q111/Q111}$ cells, but not in wild-type cells. In summary, we propose that increased protein levels, phosphorylation and nuclear localization of Elk-1 observed in exon-1 and full-length Huntington's disease models could be a compensatory mechanism activated by striatal cells in response to the presence of mhtt that contributes to neuroprotection.

Keywords: huntingtin, immediate early genes, knock-in cells, neuroprotection, R6 mouse, transcription.

J. Neurochem. (2012) **121**, 639–648.

The transcription factor Elk-1 belongs to the ternary complex factor family of erythroblast transformation specific (ETS)-domain transcription factors (Buchwalter *et al.* 2004) and represents a major transcription factor target of mitogen-activated protein kinases (MAPKs): extracellular signal-regulated kinases, c-Jun N-terminal kinases and p38 (Janknecht *et al.* 1993; Gille *et al.* 1995a). The phosphorylation of Elk-1 at serine 383 (pElk-1^{Ser383}) (Janknecht *et al.* 1993) induces the formation of a ternary complex factor with a dimer of serum response factor (SRF) (Gille *et al.* 1995a,b). This complex promotes the transcription of many immediate early genes (iEGs), such as c-fos, egr-1, egr-2 and mcl-1 (Wasylyk *et al.* 1998), by interacting with their serum response element DNA regulatory site (Sharrocks *et al.* 2000). The activation of Elk-1 is involved in important cellular functions, such as proliferation, differentiation and survival (Sharrocks 2002).

Elk-1 is strongly expressed in the central nervous system (Price *et al.* 1995), where it is restricted to neuronal cells (Sgambato *et al.* 1998a). In striatal projection neurons, Elk-1

Received November 20, 2011; revised manuscript received February 13, 2012; accepted February 14, 2012.

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Abbreviations used: ETS, erythroblast transformation specific; HD, Huntington's disease; iEG, immediately early gene; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; SRF, serum response factor.

is considered the major transcriptional regulator together with the cyclic AMP response element-binding protein (Sgambato *et al.* 1998b; Vanhoutte *et al.* 1999). In the cortex, Elk-1 is activated after quinolinic acid-induced lesion and has the capacity to prevent excitotoxic cell death (Ferrer *et al.* 2001). Although Elk-1 has been recently associated with neurologic diseases (Besnard *et al.* 2011), the neuroprotective role of Elk-1 in the brain is poorly studied.

Huntington's disease (HD) is a devastating late-onset neurodegenerative disorder induced by an expansion of a CAG repeat in the exon-1 of the huntingtin (htt) gene (The Huntington's Disease Collaborative Research Group 1993) that results in a mutant huntingtin (mhtt) protein. This mutation originates motor dysfunction, cognitive decline and psychological disturbances (Vonsattel and DiFiglia 1998). Neurodegeneration occurs most severely in the GABA-ergic medium-sized spiny neurons of the striatum (Reiner *et al.* 1988). Interestingly, some data show that proteins regulating Elk-1 activation, like MAPKs and calcineurin (Sugimoto *et al.* 1997; Tian and Karin 1999; Shaw and Saxton 2003), are modified in HD models (Liu 1998; Apostol *et al.* 2006; Roze *et al.* 2008; Xifró *et al.* 2008, 2009; Ginés *et al.* 2010; Saavedra *et al.* 2011) suggesting that Elk-1 activity could be altered. In this line, the expression of some Elk-1 related iEGs is dysregulated in HD models (Spektor *et al.* 2002; Roze *et al.* 2008), indicating that downstream targets of Elk-1 are also modified during the disease. In addition, R6/2 mice striatum displays increased levels of phospho-Elk-1-positive cells (Roze *et al.* 2008).

In this study, we analyzed the role of Elk-1 in the pathology of HD, by studying total and phosphorylated Elk-1 protein levels and its subcellular localization in the striatum of R6 mice at different stages of the disease progression. Moreover, using a knock-in striatal cell model of HD we investigated the possible neuroprotective role of Elk-1.

Materials and methods

HD mouse and cell models

Male R6/1 and R6/2 transgenic mice expressing exon-1 of mhtt were obtained from Jackson Laboratory (Bar Harbor, ME, USA). Mice were genotyped by polymerase chain reaction (PCR) as described previously (Mangiarini *et al.* 1996). CAG repeat length was determined by PCR amplification of the repeat using HD1 and HD2 fluorescently labeled primers and subsequent size determination in an ABI 3100 analyzer as previously described (The Huntington's Disease Collaborative Research Group 1993). Our R6/1 and R6/2 colonies have 145 and 115 CAG repeats, respectively (Giralt *et al.* 2009; Saavedra *et al.* 2011). These results were corroborated by Laragen, Inc. (Los Angeles, CA, USA). All mice used in the present study were housed together in numerical birth order in groups of mixed genotypes, and data were recorded for analysis by microchip mouse number. Experiments were conducted in a blind-coded manner respect to genotype. Animals were housed with access to food and water *ad libitum* in a colony room kept at 19–22°C and 40–60% humidity, under a 12 : 12 hours light/dark cycle. All procedures were performed in compliance with the National Institutes 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 *Generalitat de Catalunya* (99/1094), in accordance with the Directive 86/609/EU of the European Commission.

Conditionally, immortalized wild-type ($STHdh^{Q7/Q7}$) and mutant ($STHdh^{Q111/Q111}$) striatal neuronal progenitor cell lines expressing endogenous levels of normal and mhtt with 7 and 111 glutamines, respectively, were grown as previously described (Gines *et al.* 2003).

Protein extraction and subcellular fractionation

Animals were killed by decapitation at the age of 12, 20 and 30 weeks (wild-type and R6/1) and 4, 8 and 12 weeks (wild-type and R6/2). The brain was quickly removed and the striatum was dissected out and homogenized in the following lysis buffer: 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 100 mM NaF, 5 μM $ZnCl_2$, 10 mM EGTA and protease inhibitors (phenylmethylsulphonyl fluoride (2 mM), aprotinin (1 μg/mL), leupeptin (1 μg/mL) and sodium orthovanadate (1 mM)). $STHdh^{Q7/Q7}$ and $STHdh^{Q111/Q111}$ cells were washed once with phosphate-buffered saline (PBS) and total cellular proteins were extracted by incubating cells in the lysis buffer described above. All samples were centrifuged at 16 100 *g* for 20 min at 4°C, the supernatants were collected and protein concentration was measured using the *Dc* protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

To obtain striatal cytosolic and nuclear fractions of R6/1 mice at 30 weeks, the striatum was homogenized in lysis buffer (4 mM HEPES, 0.32 M sucrose, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/mL aprotinin, 1 μg/mL leupeptin, 2 mM sodium orthovanadate, 0.1 mg/mL benzamidine) with a Teflon-glass potter and centrifuged at 3000 *g* for 10 min to obtain the cytosolic (supernatant) and the nuclear (pellet) fractions. The nuclear fraction was resuspended with lysis buffer (10 mM Tris–HCl (pH 7.5), 0.25 M sucrose, 2 mM PMSF, 10 μg/μL aprotinin, 1 μg/μL leupeptin, 2 mM Na_3VO_4) and sonicated. Protein concentration was measured as above.

For subcellular fractionation, $STHdh^{Q7/Q7}$ and $STHdh^{Q111/Q111}$ cells were rinsed once with PBS and centrifuged at 800 *g* for 5 min. Pellets were homogenized in lysis buffer (10 mM Tris–HCl pH 7.5, 0.25 M sucrose, 2 mM PMSF, 10 μg/μL aprotinin, 1 μg/μL leupeptin, 2 mM sodium orthovanadate) and centrifuged at 3000 *g* for 10 min. The resulting supernatant was centrifuged at 10 000 *g* for 15 min to obtain a cytosol/light membrane supernatant that was further centrifuged at 100 000 *g* for 15 min to obtain the cytosolic fraction (supernatant). The pellet resulting from the initial centrifugation was resuspended in lysis buffer and centrifuged at 800 *g* for 10 min. The pellet, containing the nuclear fraction, was then resuspended in lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 10 mM EGTA, 2 mM PMSF, 10 μg/μL aprotinin, 1 μg/μL leupeptin, 2 mM sodium orthovanadate) and incubated for 30 min at 4°C in a tube rotator. Finally, after centrifugation for 15 min at 16 100 *g*, the supernatant was collected and protein concentration was determined as above.

Western blot

Western blotting was performed as described elsewhere (Xifró *et al.* 2009). The following primary antibodies were used: anti-Elk-1, anti-pElk-1^{Ser383} (both at 1 : 500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-cleaved caspase 3 (Asp175) (1 : 1000; Cell Signaling, Beverly, MA, USA). Loading control was performed by reprobing the membranes with anti-NeuN for the nuclear fractions (1 : 1000; Chemicon, Temecula, CA, USA), anti-α-tubulin (1 : 100 000; Sigma-Aldrich, St Louis, MO, USA) or anti-actin (1 : 10 000; MP Biochemicals, Aurora, OH, USA) for the cytosolic fraction. Membranes were incubated with the corresponding horseradish peroxidase-conjugated antibody (1 : 2000; Promega, Madison, WI, USA) for 1 h at 20°C. Immunoreactive bands were visualized using the Western Blotting Luminol Reagent (Santa Cruz Biotechnology) and quantified by a computer-assisted densitometer (Gel-Pro Analyzer, version 4; Media Cybernetics, Bethesda, MD, USA).

Immunohistochemistry, confocal microscopy and quantification of nuclear localization

Animals were deeply anesthetized with pentobarbital (60 mg/kg) and intracardially perfused with a 4% paraformaldehyde solution in 0.1 M sodium phosphate, pH 7.2. Brains were removed and post-fixed for 2 h in the same solution, cryoprotected with 30% sucrose in PBS with 0.02% sodium azide and frozen in dry-ice cooled isopentane. Serial coronal sections (30 µm), obtained with a cryostate, were processed for immunohistochemistry as free floating. The sections were washed three times in PBS, permeabilized 15 min at 20°C with PBS containing 0.3% Triton X-100 and 3% normal goat serum (Pierce Biotechnology, Rockford, IL, USA). After three washes, brain slices were incubated overnight at 4°C with PBS with 0.02% sodium azide plus anti-Elk-1 (1 : 250; Santa Cruz Biotechnology) and anti-htt EM48, which reacts with htt that express more than 82 CAG repeats (1 : 100; Millipore, Temecula, CA, USA). After primary antibody incubation, slices were washed three times, and then incubated 2 h at 20°C with Cy3 goat anti-rabbit (1 : 100; Jackson ImmunoResearch, West Grove, PA, USA). Then, slices were washed three times and incubated with Hoescht 33258 for 5 min (1 µg/mL; Molecular Probes, Inc.).

Double-stained slices were examined with a Leica TCS SP5 laser scanning spectral confocal microscope (Leica Microsystems, Heidelberg GmbH, Manheim, Germany) with Argon and HeNe lasers attached to a Leica DMI6000 inverted microscope. Confocal images were taken using a 63× numerical aperture objective with standard (one Airy disc) pinhole. For each slice, the entire three-dimensional stack of images from the ventral surface to the top of the slice was obtained by using the Z drive in the Leica TCS SP5 microscope. The size of the optical image was 0.5 µm. Colocalization between Elk-1 and Hoescht 33258 was measured by using the 'colocalization' plug-in of the freeware ImageJ v1.33 by Wayne Rasband (National Institutes of Health, Bethesda, MD, USA).

Transfection of siRNAs

STHdh$^{Q7/Q7}$ and STHdh$^{Q111/Q111}$ cells were seeded 24 h before transfection. Cells were transfected using 7.5 pmol of Elk-1 siRNA (Silencer® Pre-designed siRNA *Elk-1* s65447 and s65449, Ambion; Applied Biosystems, Foster City, CA, USA) with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) as instructed by the manufacturer, and incubated during 4 h. A non-targeting control siRNA (7.5 pmol) (Silencer® Negative Control 1 siRNA, Ambion; Applied Biosystems) was used to assess non-specific gene silencing effects. Cells were lysed or fixed 72 h after transfection.

Quantification of apoptosis

STHdh$^{Q7/Q7}$ and STHdh$^{Q111/Q111}$ transfected cells were washed twice with PBS, fixed with 4% paraformaldehyde in PBS for 10 min, washed twice in PBS and stained with Hoechst 33258 (1 µg/mL; Molecular Probes, Inc.) for 5 min. After washing twice with PBS, the coverslips were mounted with mowiol. Nuclear DNA staining was observed with a fluorescence microscope (Olympus). Condensed or fragmented nuclei were considered apoptotic. At least 300 cells were evaluated for each condition in each independent experiment. All the analysis was performed in a blinded fashion.

Quantitative (Q)-PCR assays

Total RNA and cDNA from R6/1 mice at 12 and 30 weeks-of-age and STHdh$^{Q7/Q7}$ and STHdh$^{Q111/Q111}$ cells were obtained as previously described (Ginés *et al.* 2010). The cDNA was then analyzed by Q-PCR using the following TaqMan® Gene Expression Assays (Applied Biosystems): 18S (Hs99999901_s1), egr-2 (Mm00456650_m1), c-fos (Mm00487425_m1) and the following Prime Time™ Mini qPCR Assay (IDT): 18S and Elk-1 (N007922.pt.Elk-1). Reverse-transcriptase (RT)-polymerase chain reaction (PCR) was performed as previously described (Ginés *et al.* 2010). All Q-PCR assays were performed in duplicate and repeated for at least five independent experiments.

Statistical analysis

Statistical analysis was performed using the unpaired Student's *t*-test. A value of $p < 0.05$ was considered as denoting statistical significance.

Results

Increased protein and phosphorylation levels of Elk-1 in the striatum of R6 mice during the progression of the disease

Elk-1 protein levels were analyzed in the striatum of two transgenic exon-1 mhtt mice models, the R6/1 and R6/2. No changes in Elk-1 protein levels were detected at 12 and 20 weeks of age in R6/1 mice, nor at 4 weeks of age in R6/2 mice compared with wild-type mice (Fig. 1a and b). Interestingly, during the progression of the disease, Elk-1 protein levels were elevated in the striatum of both HD mouse models when compared with wild-type mice. In the striatum of R6/1 mice, we observed an increase of Elk-1 levels only at 30 weeks of age, whereas in R6/2 mice striatum we detected the increase at 8 and 12 weeks of age (Fig. 1a and b).

To investigate whether increased Elk-1 protein levels observed in R6 mice striatum were associated with an increase of its phosphorylation, we analyzed pElk-1^{Ser383} levels by Western blot in the striatum of R6/1 and R6/2 mice

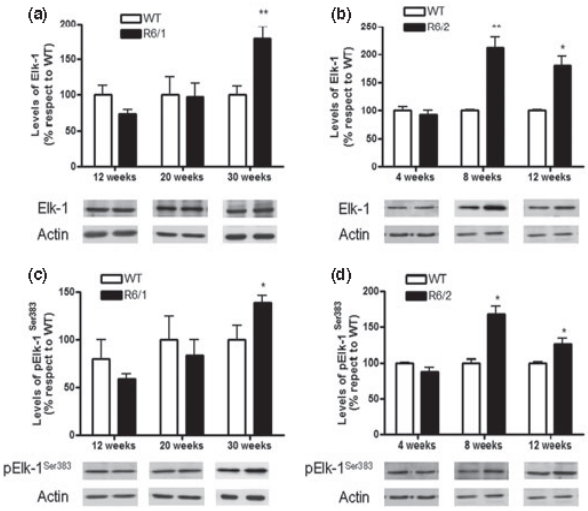

Fig. 1 Elk-1 protein levels and phosphorylation are increased in the striatum of R6 mice. (a) Protein extracts from the striatum of R6/1 mice at 12, 20 and 30 weeks of age, and (b) of R6/2 mice at 4, 8 and 12 weeks of age were subjected to western blot to analyze Elk-1 levels. (c, d) pElk-1^{Ser383} protein levels were analyzed in the same samples by western blot. Representative immunoblots are presented. Results (mean ± SEM; $n = 5–7$) represent the ratio between Elk-1 or pElk-1^{Ser383} and actin levels, obtained by densitometric analysis of western blot. Data were expressed as a percentage respect to the corresponding wild-type (WT), and were analyzed by Student's *t*-test. $^{*}p < 0.05$ and $^{**}p < 0.01$ as compared with WT mice.

at the same ages studied before. In correlation with elevated Elk-1 protein levels (Fig. 1a and b), we observed increased pElk-1^{Ser383} levels at 30 weeks of age in R6/1mice (Fig. 1c), and at 8 and 12 weeks of age in R6/2 mice (Fig. 1d), whereas no changes were detected at the other ages analyzed (Fig. 1c and d). No significant differences were observed between wild-type and R6/1 mice when comparing the pElk-1^{Ser383}/Elk-1 ratio (pElk-1^{Ser383}/Elk-1 in R6/1 compared with wt: 78.3 ± 14.9%, 100.5 ± 22.3% and 77.4 ± 13.3% at 12, 20 and 30 weeks of age, respectively; pElk-1^{Ser383} /Elk-1 in R6/2 compared with wt: 90.5 ± 20%, 78 ± 5% and 79.2 ± 10% at 4, 8 and 12 weeks of age respectively, in all cases $p > 0.05$). Altogether, these results show that increased Elk-1 protein and phosphorylation levels is an event that occurs during the progression of the disease, and that pElk-1^{Ser383} protein levels are elevated only when Elk-1 protein levels are increased too.

STHdh$^{Q111/Q111}$ cells exhibit elevated Elk-1 and pElk-1^{Ser383} levels

To analyze whether changes observed in the striatum of R6 mice models were also occurring in a cellular full-length mhtt

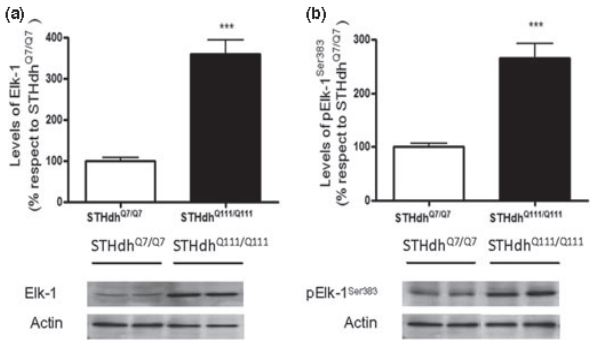

Fig. 2 Elk-1 protein levels and phosphorylation are increased in striatal cells expressing full-length mutant huntingtin. Lysates from wild-type (STHdh$^{Q7/Q7}$) and knock-in (STHdh$^{Q111/Q111}$) striatal cells were analyzed by western blot to study Elk-1 protein levels (a) and phosphorylation (b) of Elk-1. Quantification was performed by densitometric analysis. Representative immunoblots are presented. Results (mean ± SEM; $n = 6$) were normalized to the loading control actin, and expressed as a percentage of levels in STHdh$^{Q7/Q7}$ cells. Data were analyzed by Student's *t*-test. $^{***}p < 0.001$ as compared with STHdh$^{Q7/Q7}$ cells.

model, we studied Elk-1 and pElk-1^{Ser383} levels in neural cells expressing full-length wild-type (STHdh$^{Q7/Q7}$) or mhtt (STHdh$^{Q111/Q111}$). In agreement with the results obtained in R6 mice striatum (Fig. 1), immunoblot analysis revealed a significant increase of total Elk-1 protein and phosphorylation levels in STHdh$^{Q111/Q111}$ cells when compared with STHdh$^{Q7/Q7}$ cells (Fig. 2a and b). These results indicate that the increase of Elk-1 and pElk-1^{Ser383} levels is a common event in *in vivo* and *in vitro* HD models, and occurs in models of HD expressing different forms of mhtt.

Increased nuclear Elk-1 levels in the striatum of R6/1 mice and in knock-in striatal cells

In neurons, Elk-1 is localized in the cytosol and in the nucleus (Sgambato *et al.* 1998a; b). Thus, we next analyzed Elk-1 protein levels in nuclear and cytosolic fractions obtained from 30-week-old R6/1 striatum. We found elevated nuclear protein levels of Elk-1 in R6/1 mice striatum compared with wild-type mice striatum, whereas no differences were detected in the cytosolic levels (Fig. 3a). To further characterize Elk-1 localization, we

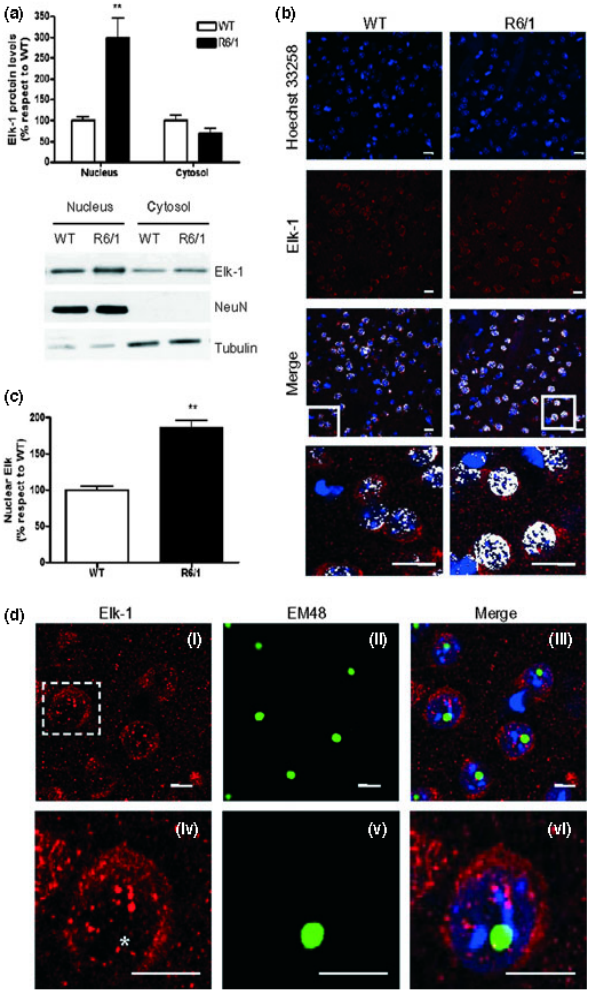

Fig. 3 R6/1 mice striatum displays increased Elk-1 levels in the nuclear compartment. (a) Protein extracts from the striatum of R6/1 mice at 30 weeks of age were subjected to subcellular fractionation, and the levels of Elk-1, NeuN and tubulin were analyzed in cytosolic and nuclear fractions by western blot. Results (mean ± SEM; $n = 4$–5) represent the ratio between Elk-1 and tubulin for the cytosolic fraction and Elk-1 and NeuN for the nuclear fraction, obtained by densitometric analysis of western blot. For the statistical analysis, Student's *t*-test was used. $^{**}p < 0.01$ respect to wild-type. (b) Representative section of 30-week-old R6/1 mice striatum stained with Elk-1 antibody and Hoescht 33258. Scale bar, 20μm. (c) Bar graph represent the percentage of nuclear Elk-1 (mean ± SEM; $n = 4$) determined by the colocalization analysis. For the statistical analysis, Student's *t*-test was used. $^{**}p < 0.01$ respect to WT nuclear Elk-1. (d) Representative section of the striatum of R6/1 mice at 30 weeks of age stained with Elk-1 antibody (i, iii, iv and vi), htt antibody (EM48, which recognizes polyglutamine tract of the first exon of htt) (ii, iii, v and vi) and Hoescht 33258 (iii and vi). Scale bar, 10μm. Asterisk shows exon-1 mhtt aggregate.

performed an immunohistochemistry in 30-week-old R6/1 mice brain sections by using an antibody against Elk-1, and Hoescht 33258 to stain the nuclei. The colocalization analysis showed that Elk-1 had a preferential nuclear localization in striatal neurons from R6/1 mice compared with wild-type animals (Fig. 3b and c). Some transcription factors are sequestered in nuclear mhtt aggregates, which block their transcriptional activity (Li and Li 2004). To investigate the association of Elk-1 with mhtt aggregates, we analyzed their colocalization by confocal microscopy in the striatum of R6/1 mice at 30 weeks of age. We observed that Elk-1 was not sequestered in intranuclear mhtt inclusions (Fig. 3d).

To determine whether the increased nuclear localization of Elk-1 was replicated in STHdh$^{Q111/Q111}$ cells, we studied its subcellular localization by western blot. We observed very low levels of cytosolic Elk-1 in both STHdh$^{Q7/Q7}$ and STHdh$^{Q111/Q111}$ cells (Fig. 4). In contrast, the nuclear fraction of STHdh$^{Q111/Q111}$ cells was enriched in Elk-1 compared with STHdh$^{Q7/Q7}$ cells nuclear fraction (Fig. 4). Altogether, these results show that in cells expressing mhtt Elk-1 protein levels are exclusively increased in the nuclear fraction.

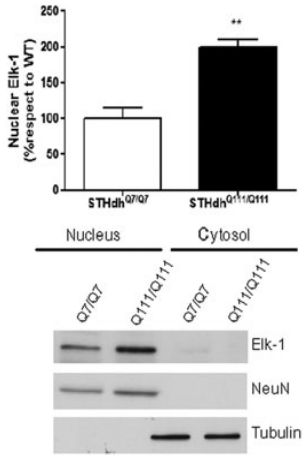

Fig. 4 Elk-1 protein levels are increased in the nucleus of mutant knock-in striatal cells. Lysates from STHdh$^{Q7/Q7}$ and STHdh$^{Q111/Q111}$ cells were subjected to subcellular fractionation, and Elk-1, NeuN and tubulin were analyzed in cytosolic and nuclear fractions by western blot. Representative immunoblots are shown. Results (mean ± SEM; $n = 4–5$) represent the ratio between Elk-1 and NeuN for the nuclear fraction obtained by densitometric analysis of western blot. Cytosolic fraction was not represented in the bar graph as Elk-1 was expressed at very low levels. Data were analyzed by Student's *t*-test. $^{**}p < 0.01$ as compared with STHdh$^{Q7/Q7}$ cells.

Elk-1 regulates the expression of c-fos and egr-2 in striatal cells expressing mhtt

In the nucleus, Elk-1 becomes a transactivator of several iEGs (Treisman 1995). However, reduced iEGs expression has been described in the striatum of R6/2 mice at symptomatic ages (Luthi-Carter *et al.* 2000; Spektor *et al.* 2002; Roze *et al.* 2008). Thus, we analyzed whether these changes also occur in R6/1 mice striatum and in striatal cells expressing mhtt by studying c-fos and egr-2 mRNA levels. In R6/1 mice striatum, no changes were detected at 12 weeks of age, whereas a drastic reduction of both c-fos and egr-2 mRNA levels were observed at 30 weeks of age when compared with their respective controls (Fig. 5a and b). In contrast, in STHdh$^{Q111/Q111}$ cells, c-fos mRNA levels were not modified whereas egr-2 expression was decreased compared with its expression levels in STHdh$^{Q7/Q7}$ cells (Fig. 5c). To further analyze the possible contribution of increased nuclear localization of Elk-1 to the control of iEGs transcriptional activity in striatal cells expressing mhtt, we transfected siRNAs to knock-down Elk-1 in wild-type and in mutant STHdh cells. The transfection with two different siRNAs against Elk-1 induced an important decrease of Elk-1 protein (Fig. 5d) and mRNA levels, 72 h after siRNAs transfection (STHdh$^{Q7/Q7}$ cells siElk-1: 25.1 ± 4.1%, compared with STHdh$^{Q7/Q7}$ cells si-; STHdh$^{Q111/Q111}$ cells siElk-1: 45.1 ± 10.8%, compared with STHdh$^{Q111/Q111}$ cells si-). In both cases $^{***}p < 0.001$).We also studied the mRNA levels of c-fos and egr-2 after Elk-1 siRNAs transfection. We observed a decrease of both c-fos and egr-2 mRNA levels in STHdh$^{Q111/Q111}$ transfected cells, but not in STHdh$^{Q7/Q7}$ transfected cells (Fig. 5e and f). Our data suggest that increased Elk-1 nuclear levels observed in STHdh$^{Q111/Q111}$ cells could participate in maintaining Elk-1-induced iEGs transcription.

A reduction of Elk-1 levels induces cell death in cells expressing mhtt

As the knock-down of Elk-1 had a differential effect on Elk-1-induced iEGs transcription in STHdh$^{Q111/Q111}$cells compared with STHdh$^{Q7/Q7}$ cells (Fig. 5e and f), we studied whether elevated Elk-1 levels in striatal cells expressing mhtt contributed to cell survival. For this purpose, we analyzed cell death in STHdh cells 72 h after Elk-1 siRNA transfection using Hoechst 33258 nuclear staining to identify apoptotic nuclei. We observed that Elk-1 knock-down induced a 3-fold increase in cell death in STHdh$^{Q111/Q111}$ cells when compared with the control condition, whereas we did not detect changes in STHdh$^{Q7/Q7}$ cells (Fig. 6a and b). This finding was corroborated by the increase of cleaved caspase 3 levels only in STHdh$^{Q111/Q111}$ cells transfected with siElk-1 (Fig. 6c). These results showed that the increase of Elk-1 levels observed in HD models may contribute to protect striatal neurons from mhtt toxicity.

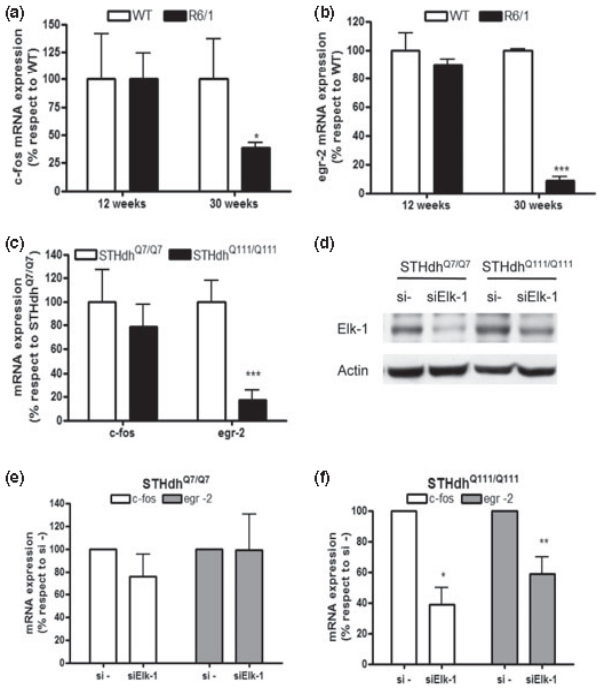

Fig. 5 Down-regulation of c-fos and egr-2 in STHdh$^{Q111/Q111}$ cells after Elk-1 inhibition. (a, b) mRNA levels of c-fos and egr-2 in R6/1 mice at 12 and 30 weeks of age. The results (mean ± SEM; $n = 4$) are expressed as a percentage respect to 12-week-old WT mice values. Data were analyzed using Student's *t*-test. $^{*}p < 0.05$ respect to WT mice. (c) mRNA levels of c-fos and egr-2 in STHdh cells. The results (mean ± SEM; $n = 5$) are expressed as a percentage respect to STHdh$^{Q7/Q7}$ cells. Data were analyzed by using Student's *t*-test. $^{***}p < 0.001$ respect to STHdh$^{Q7/Q7}$ cells. (d) STHdh$^{Q7/Q7}$ and STHdh$^{Q111/Q111}$ cells were transfected with two different siRNAs targeting Elk-1 or with a non-targeting control RNA. After 72 h cells were lysed and protein levels of Elk-1 were analyzed by western blot. A representative immunoblot is shown. (e, f) mRNA of transfected cells was extracted and c-fos and egr-2 expression was analyzed using quantitative RT-PCR. The results (mean ± SEM; $n = 5$) are expressed as a percentage respect to the corresponding control, STHdh$^{Q7/Q7}$ and STHdh$^{Q111/Q111}$ cells with non-targeting control RNA (si-) for each gene. Data were analyzed by using Student's *t*-test. $^{*}p < 0.05$ and $^{**}p < 0.01$ respect to STHdh$^{Q111/Q111}$ cells with non-targeting control RNA.

Discussion

The results of the present work show a biological relevance of Elk-1 in the pathophysiology of HD. We observed increased Elk-1 protein levels in the striatum of two HD models, R6/1 and R6/2 mice. Interestingly, this increase occurs during the progression of the disease (R6/1: 30 weeks; R6/2: 8 and 12 weeks). Similarly, higher Elk-1 levels were also detected in striatal cells expressing full-length mhtt (STHdh$^{Q111/Q111}$), indicating that the increase of Elk-1 occurs in striatal cells as a response to the expression of either truncated (R6 mouse striatal cells) or full-length mhtt (STHdh$^{Q111/Q111}$). The phosphorylation of Elk-1 at serine 383 residue is a key event for the transactivating properties of this transcription factor (Gille *et al.* 1995a), and is due to a balance between MAPKs-dependent phosphorylation (Shaw and Saxton 2003) and calcineurin-dependent dephosphorylation (Sugimoto *et al.* 1997; Tian and Karin 1999). Striatal cells expressing mhtt show a dysregulation of MAPKs and calcineurin activity (Liu 1998; Gianfriddo *et al.* 2004; Apostol *et al.* 2006; Roze *et al.* 2008; Perrin *et al.* 2009; Xifró *et al.* 2009, 2011; Ginés *et al.* 2010; Saavedra *et al.* 2011). Here, we observed an increase of pElk-1^{Ser383} levels only when Elk-1 protein levels were also elevated. Thus, our results show that phosphorylation levels would directly depend on protein levels, and suggest that the altered balance between MAPKs-dependent phosphorylation and calcineurin-dependent dephosphorylation favors the increase

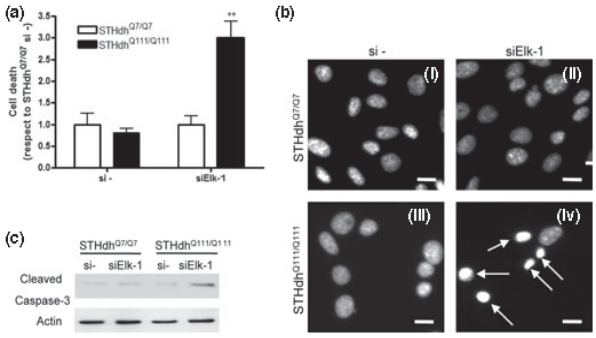

Fig. 6 Knockdown of Elk-1 increases cell death in STHdh$^{Q111/Q111}$ cells. Cell death and caspase 3 cleavage were analyzed 72 h after Elk-1 siRNA transfection. (a) Cell death was determined using Hoescht 33258 staining and analyzing nuclei condensation. Results are expressed as a percentage of apoptotic nuclei versus total nuclei and are represented as fold increase respect to STHdh$^{Q7/Q7}$ cells transfected with the control RNA (si-). Data are the mean ± SEM of five independent experiments performed in triplicate. Data were analyzed by using Student's *t*-test. $^{**}p < 0.01$ respect to STHdh$^{Q7/Q7}$ cells. (b) Representative images showing Hoescht 33258 staining to visualize the nuclei of STHdh$^{Q7/Q7}$ (i and ii) and STHdh$^{Q111/Q111}$ (iii and iv). Both cell types were transfected with Elk-1 siRNAs (ii and iv) or with non-targeting control RNA (i and iii) during 72 h and cell viability was assessed by counting nuclear condensation (iv, arrows). Scale bar, 10 μm. (c) Representative immunoblots of cleaved caspase 3 obtained from the lysates of STHdh$^{Q7/Q7}$ and STHdh$^{Q111/Q111}$ cells transfected with siElk-1 or control RNA are showed.

of pElk-1^{Ser383} levels. Our results are in agreement with those reported by Roze *et al.* (2008), who showed an increase of pElk-1^{Ser383}-positive neurons in the dorsal striatum of R6/2 mice at 12 weeks of age.

Previous reports have shown an association of Elk-1 with inclusions in cases of human Lewy body disease, Alzheimer's disease and HD (Sharma *et al.* 2010). The interaction between mhtt aggregates and transcription factors has been shown to reduce their transcriptional activity (Li and Li 2004). Our results show that nuclear Elk-1 does not colocalize with mhtt inclusions in R6/1 mouse striatum, discarding the association between Elk-1 and mhtt and suggesting an elevated transcriptional capacity of Elk-1 in striatal neurons expressing mhtt. Moreover, we observed unchanged Elk-1 protein levels in R6 mouse striatum at ages in which striatal cells present mhtt nuclear inclusions (DiFiglia *et al.* 1997) confirming that Elk-1 protein levels are not increased due to a sequestration in mhtt aggregates.

Elk-1 anti-apoptotic action is mediated through the transcription of some iEGs (Wartiovaara *et al.* 2002; Nijhawan *et al.* 2003; Knapska and Kaczmarek 2004; Boros *et al.* 2009; Lawson *et al.* 2010). Here, we analyzed c-fos and egr-2 expression in the striatum of R6/1 mice and in the striatal cell line to assess the transcriptional activity of Elk-1 in the presence of mhtt. The mRNA levels of these two iEGs were decreased in the striatum of R6/1 mice at late stages of the disease. Moreover, STHdh$^{Q111/Q111}$ cells presented decreased egr-2 mRNA levels and no variations of c-fos mRNA levels when compared with STHdh$^{Q7/Q7}$ cells. Therefore, our data agree with studies of other authors showing a reduction of some iEGs, such as c-fos, jun B, egr-1 and N10, in the striatum of R6/2 mice at late stages of the disease (Luthi-Carter *et al.* 2000; Spektor *et al.* 2002; Roze *et al.* 2008). Despite of these findings, we hypothesized that decreased levels of iEGs detected in R6/1 mice striatum and in STHdh$^{Q111/Q111}$ cells are not due to a disruption of Elk-1 pathway. Reinforcing our hypothesis, the inhibition of Elk-1 in STHdh$^{Q111/Q111}$, but not in STHdh$^{Q7/Q7}$ cells, resulted in decreased of c-fos and egr-2 mRNA levels. In addition, the transcriptional activity of Elk-1 is SRF-dependent (Gille *et al.* 1995a,b), and we have detected increased levels of phospho-SRF in STHdh$^{Q111/Q111}$ compared with STHdh$^{Q7/Q7}$ cells (Xifró *et al.* 2011). Finally, as we mentioned before, Elk-1 is not interacting with mhtt aggregates, showing that nuclear Elk-1 could be functional. Altogether, our findings indicate that increased Elk-1 levels in striatal cells expressing mhtt are capable of maintaining the expression of some Elk-1-dependent iEGs because in its absence iEGs mRNA levels are more down-regulated. Moreover, as Elk-1 is considered a key transcriptional mediator of Brain derived neurotrophic factor (BDNF)-dependent striatal functions (Gokce *et al.* 2009), increased nuclear Elk-1 levels would compensate for the reduction of BDNF, which is an important regulator of HD pathophysiology (Canals *et al.* 2001). Finally, Elk-1 could also replace the function of other factors that control the expression of iEGs and are inhibited in HD such as cyclic AMP response element-binding protein (Steffan *et al.* 2000; Nucifora *et al.* 2001) and Sp1 (Luthi-Carter *et al.* 2002).

Here, we show that inhibition of Elk-1 induces cell death in striatal cells expressing mhtt, showing for the first time a protective role of Elk-1 in HD. Similarly, it has been recently demonstrated that inhibition of Elk-1 causes apoptosis in neuronal cultures stimulated with nerve growth factor (NGF) (Demir *et al.* 2011). In addition, the capacity of Elk-1 to regulate apoptosis is higher than other ETS transcription factors (Boros *et al.* 2009). Importantly, we show that the inhibition of Elk-1 did not affect the viability of cells expressing wild-type htt. This lack of effect could be explained by the overlap with other ETS transcription factors indicating that cellular environment, in HD the presence of mhtt, has a key role in dictating the functional specificity of Elk-1 as has been previously described (Shore and Sharrocks 1995; Boros *et al.* 2009). Altogether, we show that the stress environment induced by the presence of mhtt promotes an efficient pro-survival compensatory mechanism, increasing Elk-1 levels, phosphorylation and nuclear localization. In agreement, activation of Elk-1 after toxic stimuli, in our case the expression of mhtt, has been revealed as neuroprotective (Ferrer *et al.* 2001). Therefore, we propose the regulation of Elk-1 pathway as a therapeutical tool to prevent the neurodegeneration that occurs in HD. In accordance with our proposal, a neuroprotective role of Elk-1 has been recently suggested for other neurodegenerative diseases (Besnard *et al.* 2011).

In conclusion, we provide evidences of an increase of Elk-1 protein and phosphorylation levels in R6 mouse striatum, as well as in striatal knock-in cells, with a preferential nuclear localitzation. Interestingly, inhibition of Elk-1 selectively affects iEGs expression and viability in striatal cells expressing mhtt. Taken together, these findings support a pro-survival function for Elk-1 in the presence of mhtt and situate it as a putative target for future therapeutical studies.

Acknowledgements

We thank Dr M. MacDonald (Massachusetts General Hospital, Boston, MA, USA) for providing us the knock-in striatal cell lines. We also thank Maria Teresa Muñoz and Ana López for their technical assistance, the staff of the animal care facility for their help and Dr Maria Calvo from Confocal Microscopy Unit of Serveis Científico-Tècnics (Facultat de Medicina, Universitat de Barcelona) and Dr Daniel del Toro for their support and advice with all confocal techniques. Financial support was obtained from Ministerio de Ciencia e Innovación (SAF2008-0436 and SAF2011-29507) Fondo de Investigaciones Sanitarias (Instituto de Salud Carlos III, PI10/01072; RETICS, R006/0010/0006) and Generalitat de Catalunya (2009SGR-00326). MA-H is a fellow of Ministerio de Ciencia e Innovación, Spain (BES-2009-026742), AG has a contract from Generalitat de Catalunya (2009SGR-00326). The authors declare that they have no competing interests. MA-H, XX, and JA conceptualized the study. MA-H, XX, and AG carried out experiments. MA-H, XX, EPN and JA wrote the manuscript. All authors read and approved the final manuscript.

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Prostaglandin E2 EP1 receptor antagonist improves motor deficits and rescues memory decline in R6/1 mouse del of Huntington's disease

This draft is submitted

AIM 2. TO CHARACTERIZE THE MODULATION OF PROSTAGLANDIN E2 EP RECEPTORS IN HUNTINGTON'S DISEASE.

2.1. To define the role of EP1 receptor as a therapeutic tool to reduce Huntington's disease phenotype in R6/1 mouse models of Huntington's disease.

En aquest estudi hem avaluat el potencial efecte terapèutic d'antagonitzar el receptor de la prostaglandina E2 (PGE2) EP1 per reduir els dèficits motors i cognitius que es produeixen en la malaltia de Huntington. Per tal d'assolir aquest objectiu, vam administrar de manera crònica un antagonista del receptor EP1 (SC-51089) en ratolins de la malaltia de HuntingtonR6/1 mitjançant la implantació d'un sistema de bombes osmòtiques. El tractament va ser realitzat entre les 13 i les 18 setmanes d'edat d'aquests animals i van ser utilitzats diferents paradigmes per avaluar la seva funció motora i cognitiva. Vam observar que l'administració del fàrmac SC-51089 millorava la coordinació motora i l'equilibri dels ratolins R6/1 en la realització de les tasques del *rotarod*, el *balance beam* i el *vertical pole*. A més a més, l'antagonisme del receptor EP1 restitueix els dèficits en la memòria a llarg termini analitzada mitjançant el laberint en T d'alternança espontània i el test de reconeixement d'objectes nous. Així mateix, el tractament amb el fàrmac SC-51089 millora l'expressió de marcadors sinàptics i redueix el nombre d'inclusions nuclears de huntingtina mutada a l'estriat i l'hipocamp dels ratolins R6/1 a les 18 setmanes d'edat. En aquesta línia, estudis electrofisiològics demostren que la potenciació a llarg termini es trobava significativament millorada er ratolins R6/1 després del tractament amb l'antagonista del receptor EP1. El conjunt dels nostres resultats ens mostra que l'antagonisme del receptor EP1 de PGE2 produeix una millora motora i cognitiva important en els ratolins R6/1 i senyala aquest receptor com a nou candidat terapèutic per a tractar aquests dèficits que es produeixen durant el desenvolupament de la malaltia de Huntington.

PROSTAGLANDIN E2 EP1 RECEPTOR ANTAGONIST IMPROVES MOTOR DEFICITS AND RESCUES MEMORY DECLINE IN R6/1 MOUSE MODEL OF HUNTINGTON'S DISEASE

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ABSTRACT

In this study, we evaluated the potential beneficial effects of antagonizing prostaglandin E2 (PGE2) EP1 receptor on motor and memory deficits in Huntington's disease (HD). To this aim, we implanted an osmotic mini-pump system to chronically administrate an EP1 receptor antagonist (SC-51089) in the R6/1 mouse model of HD, from 13 to 18 weeks of age, and used different paradigms to assess motor and memory function. SC-51089 administration ameliorated motor coordination and balance dysfunction in R6/1 mice as analyzed by rotarod, balance beam and vertical pole tasks. Long term memory deficit was also rescued after EP1 receptor antagonism as assessed by the T-maze spontaneous alternation and the novel object recognition tests. Additionally, treatment with SC-51089 improved the expression of specific synaptic markers and reduced the number of huntingtin nuclear inclusions in the striatum and hippocampus of 18 week-old R6/1 mice. Moreover, electrophysiological studies showed that hippocampal long-term potentation was significantly recovered in R6/1 mice after EP1 receptor antagonism. Altogether, these results show that the antagonism of PGE2 EP1 receptor has a strong therapeutic effect on R6/1 mice, and point out a new therapeutic candidate to treat motor and memory deficits in HD.

Keywords: long-term potentiation, hippocampus, striatum, huntingtin, PSD-95, GluA1

INTRODUCTION

Huntington's disease (HD) is an hereditary neurodegenerative disorder caused by the expansion of CAG tract in exon-1 of the huntingtin (htt) gene [1]. The clinical hallmark of HD is motor dysfunction [2], but increasing evidence in patients [1,3,4] and mice models [5,6] shows that cognitive impairment is another clinical feature of HD that often appears before the onset of motor symptoms. These alterations are due to a preferential degeneration of striatal medium spiny neurons [7,8], as well as hippocampal and cortical dysfunction [9,10]. While many mechanisms have been proposed to explain the neuronal degeneration that occurs in HD [11], there is now considerable evidence that synaptic dysfunction is associated with the onset of symptoms [12]. Mutant htt (mhtt) is expressed in dendrites and synapses, where it interacts with several proteins of the synaptic machinery leading to altered synaptic plasticity [12]. Increasing evidence shows early deficits in synaptic plasticity in different mouse models of HD, not only in striatum but also in the hippocampus [6,13,14]. Therefore, identification of new targets, strategies for drug discovery, and therapeutic approaches focused in the prevention of neuronal dysfunction are now reaching an important turning point in HD [15].

Several studies have postulated that prostaglandin E2 (PGE2) and its receptors participate in the mechanism involved to propagate neurotoxicity in brain injury [16,17]. PGE2 G-protein-coupled receptors are broadly expressed in the central nervous system, and have been associated with pro-death to pro-survival functions in the brain [16,17], including modulation of synaptic plasticity [18-20]. PGE2 is derived from the metabolism of arachidonic acid mainly by the action of cyclooxygenase 2 and binds to four different receptors (EP1-EP4) [21]. Interestingly, EP1 receptor activation is related to intracellular calcium mobilizations through the action of phospholipase C/inositol trisphosphate, while EP2, EP3 and EP4 receptor activation leads to changes in cAMP levels [21]. Therefore, the activation of different EP receptors can have opposite effects. Whereas the blockade of EP2-EP4 receptors can aggravate neurodegeneration [22-24], antagonizing EP1 receptor has neuroprotective effects

[25-27]. EP1 receptor depletion/inhibition significantly attenuates focal ischemic and excitotoxic brain damage in the striatum [16,26]. Additionally, EP1 antagonism successfully rescues dopaminergic neurons from 6-hydroxydopamine-induced toxicity in an *in vitro* model of Parkinson's disease [25]. Genetic deletion of EP1 reduces amyloid plaques, attenuates amyloid-induced hippocampal neuronal damage and reduces memory loss in a transgenic mouse model of Alzheimer's disease [27]. Although EP1 receptor is highly expressed in GABAergic pre-synaptic terminals derived from striatal neurons, as well as in the cortex and hippocampus [28], the most affected brain regions in HD pathology [2], the possible beneficial effect of EP1 receptor modulation has not been investigated in the field of HD yet. Thus, our study focuses on delineating the possible therapeutic role of EP1 modulation in R6/1 mice, using SC-51089, a selective antagonist of EP1.

MATERIALS AND METHODS

HD mouse model

Male R6/1 transgenic mice expressing exon-1 of mhtt were obtained from Jackson Laboratory (Bar Harbor, ME), and maintained in a B6CBA background. Mice were genotyped by polymerase chain reaction (PCR) as described previously [29]. CAG repeat length was determined as previously described [1]. Our R6/1 colony has 145 CAG repeats [30]. Wild type (WT) littermate animals were used as the control group. All mice used in the present study were housed together in numerical birth order in groups of mixed genotypes, and data were recorded for analysis by microchip mouse number. Experiments were conducted in a blind-coded manner with respect to genotype and treatment. All mice used in the present study were housed together in numerical birth order in groups of mixed genotypes, and data were recorded for analysis by microchip mouse number. Animals were housed with access to food and water ad libitum in a colony room kept at 19–22ºC and 40–60% humidity, under a 12 : 12

hours light/dark cycle. All procedures were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approved by the local animal care committee of *Universitat de Barcelona* (99/01), and *Generalitat de Catalunya* (99/1094), in accordance with the Directive 86/609/EU of the European Commission.

Pharmacological treatments

At 13 weeks of age, WT and R6/1 mice were deeply anesthetized with pentobarbital (40 mg/kg) and an osmotic minipump was intraperitoneally (i.p.) implanted (model 1004; Alzet, Palo Alto, CA). Vehicle (water) or the EP1 receptor antagonist, 8-chlorodibenz[*b,f*][1,4]oxazepine-10(11*H*)-carboxylic acid, 2-[1-oxo-3-(4-pyridinyl)propyl] hydrazide hydrochloride (SC-51089; Santa Cruz Biotechnology, Santa Cruz, CA), were infused i.p. at a rate of 0.11 µl/h during 28 days for the behavioral analysis, resulting in a dose of 40 µg/kg/day. Mice were allowed to recover for 3-5 days before starting behavioral tests.

Striatal lesions

Osmotic mini-pump (model 1002; Alzet) was i.p. implanted to 10-week-old WT mice (*n* = 5 per group**)** with SC-51089 (40 µg/kg/day) or vehicle (water), during 10 days. Eight days after osmotic mini-pump implantation, animals received vehicle or 15 nmol quinolinic acid (QUIN) (Sigma Chemicals Co., St. Louis, MO) in 0.5 µl sterile phosphate-buffered saline (PBS) stereotaxically into the striatum (coordinates + 0.6 mm, 2.0 mm left, 2.7 mm ventral from bregma) using a 10-µl Hamilton syringe. QUIN was injected over 2 min and the cannula was left in place for further 3 min. Animals were deeply anesthetized and immediately perfused transcardially 48 hours after QUIN injection.

Behavioral assessment

Clasping and weight

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. Mice were scored according to the following criteria: 0 = no clasping, 1 = clasping 2 paws and 2 = clasping all paws. Animals were weighted weekly.

Rotarod

Motor coordination and balance were evaluated using the rotarod apparatus at distinct rotations per minute (rpm), as described elsewhere [31,32]. Animals were trained at 9 weeks of age and evaluated once a week at 16, 24 and 32 rpm as described elsewhere [32]. The number of falls in a total of 60 sec was recorded. The curves representing the behavioral pattern were compared, and the percentage of motor coordination impairment was calculated as described elsewhere [33].

Balance beam

The motor coordination and balance of mice were also assessed between 13 and 18 weeks of age by measuring their ability to traverse a narrow beam [31]. 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.

Vertical pole

In the vertical pole test each mouse at 14 and 16 weeks of age was placed in the center of the pole, which was held in a horizontal position. The pole was then gradually lifted to a vertical position. Falls during the first 10 seconds in the vertical position were counted.

Novel object recognition test (NORT)

Mice were tested in a circular open field (40 cm diameter) located in a room with dim lighting. Briefly, 15- and 17-week-old mice were habituated to the open field in the absence of the

objects for 10 min/day over 2 days. During the training period, mice were placed in the open field with two identical objects for 10 min. The retention test was performed 24 hours after training (long-term memory) by placing the mice back to the open field for a 5 min session, and by randomly exchanging one of the familiar objects with a novel one. Results were analyzed as previously described [5].

T-maze spontaneous alternation task (T-SAT)

T-SAT was used to analyze hippocampal-dependent memory in 15- and 17-week-old mice. The T-maze apparatus used and the light conditions were previously described elsewhere [34]. In the training session, one arm was closed (new arm) and mice were placed in the stem arm of the T (home arm) and allowed to explore this arm and the other available arm (familiar arm) for 10 min, after which they were returned to the home cage. To assess long-term memory, after inter-trial intervals of 4 hours mice were placed in the stem arm of the T-maze and allowed to freely explore all three arms for 5 min. Arm preference was determined by calculating the time spent in each arm × 100/time spent in both arms (old and new).

Protein extraction and western blot analysis

Animals were sacrificed by cervical dislocation and the striatum, cortex and hippocampus were rapidly removed. Total protein was extracted as previously described [35]. Western blotting was performed as described elsewhere [36]. The following primary antibodies were used: anti-EP1 (1:1,000; Cayman Chemical, Ann Harbor, MI), anti-PSD-95 (1:2,000, Affinity BioReagents, Golden, CO), anti-GluA1 (1:1,000, Upstate Biotechnology, NY) and anti-vesicular glutamate transporter 1 (VGluT1; 1:10,000; Synaptic Systems, Göttingen, Germany). Loading control was performed by reprobing the membranes with anti-α-tubulin (1:50,000; Sigma-Aldrich). Membranes were incubated with the corresponding horseradish peroxidase-conjugated antibody (1:2,000; Promega, Madison, WI). Immunoreactive bands were visualized

using the Western Blotting Luminol Reagent (Santa Cruz Biotechnology), and quantified by a computer-assisted densitometer (Gel-Pro Analyzer, version 4, Media Cybernetics).

Immunohistochemistry

For immunohistochemical analysis, coronal sections of the whole brain were obtained from 18-week-old WT and R6/1 mice treated with SC-51089 or vehicle as described elsewhere [35]. Diaminobenzidine immunohistochemistry was performed as previously described [37]. Tissue was incubated with the following primary antibodies: anti-DARPP32 (1:500, BD Bioscience, NJ) or anti-EM48 (1:500; Millipore, MA). Sections were washed three times in PBS, incubated with the corresponding biotinylated secondary antibody (1:200; Thermo Fisher, Rockford, IL), and developed as previously described [32]. Cresyl violet staining was performed as previously described [34].

Stereology

Striatal and hippocampal volume estimations were performed in 18-week-old WT and R6/1 mice treated with SC-51089 or vehicle as described elsewhere [37]. Unbiased counting for genotype and treatment was performed with Computer-Assisted Stereology Toolbox (CAST) software (Olympus Danmark A/S). To determine the number of neuronal intra-nuclear inclusions (NIIs) in the striatum and hippocampus, we used the dissector counting procedure in coronal sections spaced 240 μm apart, as described elsewhere [32].

Electrophysiology

Transverse mice brain slices (400 μm) were prepared using conventional methods and incubated with artificial cerebrospinal fluid (aCSF) as previously described [38]. Excitatory postsynaptic potentials were recorded as described elsewhere [30]. Evoked fEPSPs were elicited by stimulation of the Schaeffer collateral fibers with an extracellular bipolar nichrome electrode via a 2100 isolated pulse stimulator (A-M Systems, Carlsborg, WA). The stimulation intensity was adjusted to give fEPSP amplitude that was approximately 50 % of maximal fEPSP

sizes. Long-term potentiation (LTP) was induced by applying 4 trains (1 s at 100 Hz) spaced 20 s, and potentiation was measured for 1 hour after LTP induction at 0.1 Hz. For each experiment, fEPSP slopes were expressed as a percentage of average pre-tetanus baseline slope values. Data were filtered (highpass, 0.1 Hz; lowpass 3 kHz) and digitized using a PowerLab 4/26 acquisition system (AD Instruments). The software Scope (AD Instruments) was used to display fEPSPs and measurements of the slopes of fEPSPs.

Statistical analysis

All data are expressed as mean±SEM. All graphs were created with GraphPad Prism 4 version 4.02. Different statistical analyses were performed as appropriate, and indicated in the figure legends. Values of $p<0.05$ were considered statistically significant.

RESULTS

SC-51089 delivery by osmotic mini-pump protects striatal neurons in an excitotoxic model of HD

We first evaluated the neuroprotective effect of SC-51089 against the intrastriatal injection of QUIN, an agonist of N-methyl-D-aspartate receptor, that induces selective neuronal death and it has been extensively used as an acute model of HD [39]. The osmotic mini-pump with SC-51089 (40 μg/kg/day) was implanted at 10-week-old WT mice. After 8 days of treatment, QUIN (15 nmol) was intra-striatally injected in the striatum). SC-51089 treatment reduced drastically the volume of striatal lesion induced by QUIN-um (62.3%; Fig.1), demonstrating that SC-51089 mini-osmotic pump deliver system exerts a neuroprotective role (Fig. 1).

Chronic administration of SC-51089 ameliorates clasping reflex, improved motor coordination and balance in R6/1 mice

Osmotic pumps were implanted in 13-week-old WT and R6/1 mice to deliver chronically SC-51089 (40 µg/kg/day) during 28 days. We weekly evaluated the clasping reflex, a neurological phenotype of HD mice, and the body weight from 11 to 18 weeks of age. As shown in Fig. 2a, the administration of SC-51089 resulted in a significant delay of clasping phenotype progression. The typical loss of body weight was detected in R6/1 mice at 18 weeks of age (Genotype; $F_{(1,45)}$=95.51, p<0.001), but no differences were observed between vehicle- and SC-51089-treated mice in any genotype (Treatment; $F_{(1,45)}$=0.168, p=0.6833). Then, we evaluated in vehicle- and SC-51089-treated WT and R6/1 mice the latency to fall off in the rotarod test at 16, 24 and 32 rpm. Interestingly, treatment with SC-51089 significantly ameliorated motor coordination in R6/1 mice assessed at 16 rpm (Fig. 2b), 24 rpm (Fig. 2c) and 32 rpm (Fig. 2d). No significant differences were observed between vehicle- and SC-51089-treated WT mice at any speed tested.

To further characterize the motor amelioration induced by SC-51089, we also studied the performance of all groups on the balance beam task from 13 to 18 weeks of age by measuring the mean speed, the covered distance and the number of slips. Our data showed that SC-51089-treated R6/1 mice performed significantly better than the R6/1 vehicle-treated group in the entire parameters analyzed (Fig. 3 and Online Resource 1). We next performed the vertical pole task to analyze motor coordination and muscular abnormalities. We studied the percentage of falls off the pole in WT and R6/1 vehicle- and SC-51089-treated mice. At 14 and 16 weeks of age both groups of WT animals accomplished properly the task, while 35% R6/1 mice fell off the vertical pole (Fig. 3d). The administration of SC-51089 to R6/1 mice clearly reduced the number of animals falling off the vertical pole task at 14 weeks, and at 16 weeks of age no SC-51089-treated mice fell (Fig. 3d). Altogether, we conclude that the systemic administration of SC-51089 improves motor function in R6/1 mice.

Increased expression of VGluT1 in the striatum in R6/1 mice after SC-51089 treatment

The improvement in motor function in SC-51089-treated R6/1 mice correlated with changes in the expression of some synaptic markers in the striatum. PSD-95 and VGluT1 are strongly down-regulated in human HD brain and in the striatum of HD mouse models [32,40,41]. The levels of PSD-95 and VGluT1 in the striatum of WT and R6/1 mice after 28 days of treatment with vehicle or SC-51089 were analyzed by western blot. SC-51089-treated R6/1 mice showed significant improvement in VGluT1 protein levels, compared to the vehicle-treated R6/1 group (Fig. 3f), but not in PSD-95 protein levels (Fig. 3e).

The effect of chronic SC-51089 treatment onEP1 receptor protein levels was at 18 weeks of age in the striatum of WT and R6/1 mice. No significant changes were observed in EP1 receptor protein levels in any condition (SC-51089-treated WT mice 94.85±14.12%; vehicle-treated R6/1 mice 105.7±24.99% and SC-51089-treated R6/1 mice 112±14.2%, as compared with WT vehicle-treated mice; in all cases $p>0.05$).

EP1 antagonism rescues long-term memory deficits in R6/1 mice

To determine whether EP1 blockade improves LTM deficits in R6/1 mice, we performed the T-SAT and the NORT to all groups at 15 and 17 weeks of age. In the T-SAT we observed that WT mice spent more time in the new arm than in the old arm at both ages, but R6/1 mice did not show preference for the new arm either at 15 or 17 weeks of age (Fig. 4a). Interestingly, SC-51089-treated R6/1 mice explored the new arm similarly to WT animals (Fig. 4a). Then, we subjected all groups of animals to the NORT at the same ages. In 15-week-old mice, all groups explored more the new object respect to the old one. However, the preference of R6/1 mice for the new object was significantly reduced compared with WT mice, but SC-51089-treated R6/1 mice explored the new object as much as control littermates (Fig. 4b). Interestingly, when we checked long-term object recognition memory at 17 weeks of age, we observed that SC-51089-treated R6/1 mice preferred significantly more the novel object compared with vehicle-treated R6/1 animals (Fig. 4b). During NORT habituation, no differences were observed

between vehicle- and SC-51089-treated R6/1 mice in the distance covered and the exploration of the center, either at 15 or 17 weeks of age (Online Resource 2), suggesting that the improvement in LTM was not due to SC-51089-induced changes in motivation, locomotor activity or anxiety.

SC-51089 treatment improves hippocampal long-term potentiation in R6/1 mice

In order to provide a cellular mechanism to explain our behavioral data, we performed electrophysiological analysis of the Schaffer collateral-CA1 synapse in the hippocampus of mice chronically treated with vehicle or SC-51089. fEPSPs were recorded in the stratum radiatum of CA1 region to analyze basal synaptic transmission. Extracellular field recordings and fiber volley amplitude showed that input/output curves were not significantly different between groups (data not shown). We next investigated synaptic plasticity in the CA1 region of hippocampal slices using a high-frequency conditioning stimulus (HFS) to induce LTP. After HFS, the responses from R6/1 mice hippocampi showed an absence of potentiation, whereas WT mice displayed a potentiated response (Fig. 4c). Interestingly, the electrophysiological recordings from SC-51089-treated R6/1 mice showed significant potentiation, whereas no significant differences were detected between vehicle- and SC-51089-treated WT mice (Fig. 4c). The data indicate that EP1 antagonism improves the LTP in R6/1 mice.

SC-51089 increases the expression of synaptic markers the hippocampus of R6/1 mice

We next analyzed whether improvements in behavior and synaptic plasticity correlated with changes in the expression of PSD-95 and GluA1, which have been shown to be down-regulated in the hippocampus of R6/1 animals [40,41]. SC-51089-treated R6/1 mice showed a significant improvement in hippocampal PSD-95 (Fig. 4d) and GluA1 (Fig. 4e) protein levels, compared to R6/1 receiving vehicle. Similar to the striatum, no differences were observed in the levels of

EP1 receptor in the hippocampus of WT and R6/1 animals after 4 weeks of SC-51089 treatment (SC-51089-treated WT mice 119.8±22%, vehicle-treated R6/1 mice 123±19.6% and SC-51089-treated-R6/1 mice 97.64±15.7%, as compared with WT vehicle-treated mice; in all cases $p>0.05$).

SC-51089 treatment has higher impact in the hippocampal-dependent behavioral test and in the expression of synaptic markers in that brain region. Therefore, we wanted to know whether protein levels of this receptor were different comparing distinct brain regions involved in motor and memory function and if these differences correlate with the effect of SC-51089 observed in R6/1 mice. We then analyzed the protein levels of EP1 receptor in the striatum, hippocampus and cortex in 12-week-old WT and R6/1 mice. Interestingly, we observed that hippocampal expression of EP1 was higher than in the striatum and cortex in both genotypes (Online Resource 3).

Formation of neuronal intranuclear inclusions is reduced in the striatum and hippocampus of R6/1 mice after EP1 chronic antagonism

We next evaluated whether SC-51089 treatment improved some neuropathological features in R6/1 mice. First, we analyzed the volume of the striatum and hippocampus. Stereological estimation of volumes revealed a reduction in striatal volume in R6/1 mice that was not prevented by SC-51089 treatment (Fig. 5a). In contrast, no differences were observed in the hippocampal volume between any groups (Fig. 5b).

Given that the appearance of mhtt aggregates is a hallmark of HD neuropathology [8,42,43], we wanted to determine whether SC-51089 treatment could prevent or reduce mhtt aggregation. NIIs of mhtt were observed throughout the striatum and the hippocampus of both groups of R6/1 mice (Fig. 5c and d). Interestingly, SC-51089-treated R6/1 mice showed a reduction in the number of NIIs of mhtt in medium spiny neurons of the striatum (Fig. 5c), in

the pyramidal cells of the CA1 (where LTP improvements were observed) and in the granular cells of the dentate gyrus (Fig. 5d) of the hippocampus.

DISCUSSION

In the present work, we provide the first evidence that chronic pharmacological antagonism of EP1 receptor has a beneficial effect in motor coordination and cognitive alterations in R6/1 mice. The performance in the rotarod, the balance beam and the vertical pole tests was improved after SC-5089 treatment. We also observed a recovery of LTM deficits in the T-SAT and the NORT that correlates with the improvement in hippocampal synaptic plasticity in SC-51089-treated R6/1 mice. Additionally, the antagonism of EP1 receptor promotes a rescue of specific synaptic protein, and a decrease in mhtt nuclear aggregation in the striatum and hippocampus of R6/1 mice.

Previous studies have demonstrated that acute EP1 receptor antagonism protects against over-activation of NMDA receptor [16,26]. Here, we also demonstrated that administration of EP1 receptor antagonist using osmotic mini-pump protects striatal cells in an excitotoxic model of HD. Moreover, in agreement with our results, EP1 can mediate not only toxic effects in models of acute neuronal damage, such as ischemic and excitotoxic insults [16,26], but also in models of chronic neurodegeneration [25,27,44]. Although some pharmacological treatments have been done in order to study the neuroprotective role of EP1 deletion/antagonism, only an improvement in the memory latency in the passive avoidance in mouse models of Alzheimer's disease with a deletion of EP1 gene [27]. In the present study, we go further into the possible role of EP1 receptor in modulating neuronal plasticity showing that chronic pharmacological EP1 antagonism improves synaptic markers. Interestingly, EP1 activation results in increased phosphatidyl inositol hydrolysis and elevation of the intracellular Ca^{2+} concentration [21]. Neuronal Ca^{2+} signaling regulates multiple neuronal functions, including

synaptic transmission, plasticity and cell survival [45,46]. An imbalance in Ca^{2+} homeostasis has been reported in different transgenic HD mice [47,48], which can disturb neuronal signaling leading to cellular dysfunction [47] and possibly contributing to the behavioral deficits found in these animals. *In vivo* and *in vitro* studies demonstrate that antagonism/deletion of EP1 results in a normalization of intracellular Ca^{2+} concentration [26,49]. We found that blockade of EP1 significantly improved R6/1 phenotype; reinforcing the hypothesis that disturbed intracellular Ca^{2+} signaling plays a significant role in the pathogenesis of HD.

Changes in synaptic composition and function may also contribute to behavioral alterations in HD models [40,50]. PSD-95 protein levels are strongly down-regulated in human HD brain, and in the striatum and hippocampus of HD mouse models [40,41]. Importantly, the down-regulation in PSD-95 was partially reverted in the hippocampus, but not in the striatum, of SC-51089-treated R6/1 mice, Moreover, we observed a clear recovery from hippocampal-related cognitive deficits, while the amelioration of striatal-related motor dysfunction in SC-51089-treated R6/1 mice was slighter, which suggests a greater effect of SC-51089 in the hippocampus than the striatum. This region-specific effect is in agreement with the levels of EP1 receptor that are more expressed in the hippocampus than in the striatum. On the other hand, alterations in proteins involved in glutamatergic signaling have also been reported in R6 mice [40,50]. After EP1 chronic antagonism, GluA1 and VGluT1 protein levels were improved in the hippocampus and striatum of R6/1 mice, respectively. The increased expression of VGluT1 observed in the striatum of SC-51089-treated R6/1 mice suggests that cortico-striatal excitatory terminals are more preserved [32], which correlates with an improvement in the performance of all the motor tasks analyzed. Moreover, altered levels of PSD-95 and GluA1 in the hippocampus are likely to induce alterations in synaptic signaling and plasticity, consistent with that observed in HD mice. Previous studies already showed that the down-regulation of PSD-95 and GluA1 are involved in HD pathogenesis [40,41,51]. Amelioration of these protein deficits may contribute to the beneficial effects of EP1 receptor blockade, and could be related

to the rescue of LTM deficits observed in R6/1 mice after SC-51089 treatment. Basal neurotransmission at hippocampal synapses (CA3-CA1 field excitatory post-synaptic potentials, fEPSPs) was normal in R6/1 mice compared to WT, whereas LTP was strongly reduced in these transgenic mice. This result indicates that such deficits are synaptic, as previously described in several HD mouse models [6,30,52]. Importantly, hippocampal synaptic plasticity in SC-51089-treated R6/1 mice was significantly improved, according with the hippocampal-related cognitive improvements and the recovery of synaptic markers after SC-51089 treatment.

Inclusions, a clinical hallmark of HD pathology, can reflect pathologically relevant processes and have been reported in several transgenic mouse models [53]. Increasing evidence suggest that reduction in mhtt aggregates is directly linked to alleviation in behavioral dysfunction in HD mice [32,54,55]. Murphy and colleagues (2000) showed in R6/2 mice that hippocampal cells with inclusions are dysfunctional. In addition, a number of compounds that are neuroprotective in HD mice significantly suppress mhtt aggregates [33,54]. Here, we report a decrease in NIIs of mhtt in the striatum and hippocampus, regions where marked alterations in synaptic plasticity have been found [6,32]. These results correlate with improvements in motor dysfunction, and a clear reversion of LTM deficits, together with increased hippocampal plasticity in R6/1 mice. Therefore, the antagonism of EP1 decreases neuronal dysfunction induced by mhtt expression, and this improvement leads to a partial reduction of the number of NIIs, which could be due to an increase in mhtt clearance, changes between synthesis and degradation or decreased impairment of the proteasomal system.

Conclusions

These results show a link between PGE2-EP1 receptor and HD. Consistent with motor and memory improvements, SC-51089 treatment leads to the partial recovery of some biochemical and histopathological markers in the striatum and hippocampus of R6/1 mice, as well as to improved hippocampal synaptic plasticity and reduced mhtt nuclear aggregation. Taken

together, these findings show that antagonism of EP1 has a strong therapeutic effect on these mice, and point out EP1 as promising new therapeutic target for neurological diseases, such as HD.

Conflict of Interest

The authors declare that they have no conflict of interest

Acknowledgments

We are very grateful to Ana Lopez and Maria Teresa Muñoz for technical assistance and to Dr Teresa Rodrigo, Amèrica Jiménez and the staff of the animal care facility (Facultat de Psicologia and Facultat de Medicina, Universitat de Barcelona) for their help. We thank members of our laboratory for helpful discussion. Financial support was obtained from Ministerio de Economía y Competitividad (SAF2011-29507 and BFU2011-26339), Instituto de Salud Carlos III (PI10/01072), RETICS (CIBERNED, R006/0010/0006), Junta de Comunidades de Castilla-La Mancha, JCCM (PEII10-0095-8727), Generalitat de Catalunya (2009SGR-00326), INCRECyT project from European Social Fund and JCCM to E.D.M.

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FIGURE LEGENDS

Fig. 1 Antagonism of EP1 receptor prevents striatal QUIN-induced cell death Osmotic pumps with vehicle or EP1 receptor antagonist (SC; SC-51089; 40 µg/kg/day) were implanted intraperitoneally in 10 weeks-old WT mice during 8 days before intrastriatal QUIN (15 nmol) injection. To examine cell death, Fluoro-Jade staining was performed 48 hours after lesion. (a) Representative photomicrographs showing striatal area occupied by Fluoro-Jade-positive cells in vehicle plus QUIN and SC-51089 treatment plus QUIN-injected mice. (b) Graph showing the quantification of the volume of the lesion measured in Fluoro-Jade stained sections expressed in mm^3. Results are the mean±SEM of five animals per condition. Data were analyzed by Student's t-test. ***p<0.001 compared with vehicle plus QUIN-injected mice.

Fig. 2 Chronic administration of the EP1 antagonist, SC-51089, in R6/1 mice reduces clasping behavior and improves the performance in the rotarod task WT and R6/1 mice were treated with SC-51089 (SC; 40 µg/kg/day) or vehicle using an osmotic pump system implanted i.p. at 13 weeks of age (discontinuous vertical line). (a) Clasping reflex of R6/1 vehicle- or SC-51089-treated mice was analyzed weekly from 11 to 18 weeks of age. (b-d) The rotarod task was performed weekly at 16 (b), 24 (c) and 32 rpm (d) in all groups from 10 to 18 weeks of age. Values represent the number of falls within 60 seconds. Values are expressed as a mean±SEM (n=10-13/group). Data were analyzed by two-way ANOVA from 13 to 18 weeks of age. ***p<0.001, as compared to WT vehicle-treated mice, $$$$p$<0.001 and $$p$<0.05, as compared to R6/1 vehicle-treated mice.

Fig. 3 R6/1 mice motor coordination, balance and muscular strength impairments are reduced, and striatal VGLUT1 expression is increased by chronic SC-51089 administration At 13 weeks of age, WT and R6/1 mice were treated with SC-51089 (SC; 40 µg/kg/day) or vehicle. The balance beam task was performed weekly from 13 to 18 weeks of age. The following measurements were monitored for each animal: (a) the mean speed between 14 to 18 weeks;

(b) the mean time taken to cross the beam between 14 to 18 weeks; (c) the mean number of slips between 14 to 18 weeks. (d) The vertical pole task was performed at 14 and 16 weeks of age in WT and R6/1 treated with vehicle or SC-51089. White bars represent the percentages of animals that stand in the vertical pole and black bars the percentage of animals that fall; (e and f) At 18 weeks of age, protein extracts from the striatum of WT and R6/1 mice treated with vehicle or SC-51089 were subjected to western blot to analyze PSD-95 (e) and VGluT1 (f). Representative immunoblots are presented. Data were expressed as a percentage with respect to WT vehicle-treated mice. Results mean±SEM (n=6-7/group), and represent the ratio between each protein and tubulin levels, obtained by densitometric analysis of western blot. Data were analyzed for a, b, c, e and f by one-way ANOVA with Student t-test as a *post-hoc*, and by Fisher's exact test for d. For a-d values are expressed as a mean±SEM (n = 10-13/group). ***p< 0.001, **p< 0.01 and *p<0.05 as compared to WT vehicle-treated mice; $$$$p$<0.001, $$$p$<0.01 and $$p$<0.05, as compared to R6/1 vehicle-treated mice.

Fig. 4 Chronic treatment with SC-51089 reverses R6/1 mice long-term memory deficits, improves hippocampal LTP and increases PSD-95 and GluA1 protein levels in the hippocampus WT vehicle-, WT SC-51089-, R6/1 vehicle- and R6/1 SC-51089-treated mice were subjected to the T-SAT and NORT tasks at 15 and 17 weeks of age. The chance level for exploration was represented as a discontinue line in all graphs. (a) The arm exploration time 4 hours after the training session of all groups during the T-SAT task is shown as a mean±SEM (n=11-13/group) in percentage of the preference for new arm (open bars) or old arm (closed bars) at 15 and 17 weeks of age. Statistical analysis was performed using two-way ANOVA followed by Bonferroni as *post-hoc* test. ***p<0.001 and **p<0.01, when compared with the percentage of preference for the new arm. (b) In the NORT task, all mice spent similar time exploring both objects during the training session. Long-term memory was assessed 24 hours later introducing a new object instead of an old object. Bars are shown as mean±SEM (n=10-13/group), and represent the percentage of new object preference at 15 and 17 weeks of age

in all groups. Statistical analysis was performed by one-way ANOVA followed by Student t-test as *post-hoc* test. ** $p<0.01$ and * $p<0.05$, when compared with the percentage of preference for the new object of WT vehicle-treated mice; $ $p<0.05$ when compared with the percentage of preference for the new object of R6/1 vehicle-treated mice. (c) Time-course of mean fEPSPs slope in basal condition (discontinuous line) and after LTP induction (tetanization pulse is shown by an arrow). The insert depicts a representative superimposed recording taken before and after LTP induction in different groups of mice. The data were normalized for each slice with respect to the average baseline slope recorded. From 9 to 12 slices from 4 different mice were used per group and data was analyzed by two-way ANOVA. *** $p<0.001$, when compared to WT vehicle-treated mice; $$$ $p<0.001$, when compared to R6/1 vehicle-treated mice. (d and e) At 18 weeks of age, protein extracts from the hippocampus of WT and R6/1 mice treated with vehicle or SC-51089 were subjected to western blot to analyze PSD-95 (d) and GluA1 (e) protein levels. Results are expressed as mean±SEM (n=6-7/group), and represent the ratio between each protein and tubulin levels, obtained by densitometric analysis of western blot. Data were expressed as a percentage respect to WT vehicle-treated mice, and were analyzed by one-way ANOVA, followed by Student t-test as a *post-hoc*. *** $p<0.001$ and ** $p<0.01$, as compared to WT vehicle-treated mice; $ $p<0.05$, as compared to R6/1 vehicle-treated mice. Representative immunoblots are presented.

Fig. 5 The SC-51089 chronic treatment reduced the formation of neuronal intranuclear inclusion in the striatum and the hippocampus of R6/1 mice Striatal (a) and hippocampal (b) volumes of coronal sections stained for DARPP32 and Nissl, respectively, were stereologically measured in 18-week-old WT and R6/1 vehicle- and SC-51089- (SC) treated mice. (c) Representative immunohistochemistry revealed htt aggregates in the striatum of R6/1 treated with vehicle or SC-51089. Graph shows mean±SEM (n=6-8/group), and represents total number of EM48-positive neuronal intranuclear inclusions (NIIs) stereologically counted in R6/1 vehicle- and SC-51089 treated mice. (d) Representative microphotography of NIIs of mhtt

in the CA1 and dentate gyrus of the hippocampus of R6/1 vehicle- and SC-51089-treated group. Graph shows mean±SEM (n=4-6/group) and represent CA1 and dentate gyrus total number of EM48-positive NIIs stereologically counted in R6/1 control and SC-51089-treated mice. Scale bar: 10 µm. Data were analyzed by one-way ANOVA followed by Student's t-test as a *post-hoc* for a and b, and by Student t-test for c and d. *$p<0.05$ and **p<0.01 as compared to WT vehicle-treated mice; $$p<0.05$, as compared to R6/1 vehicle-treated mice.

Online Resource 1 Chronic treatment with SC-51089 in R6/1 mice decreases deficits in motor coordination and balance. At 13 weeks of age, WT and R6/1 mice were treated with SC-51089 (SC; 40 µg/kg/day) or vehicle (discontinuous vertical line). The balance beam task was performed weekly from 13 to 18 weeks of age. The following measurements were recorded: (A) the mean speed; (B) the time taken to cross the beam and (C) the number of slips. Values are expressed as a mean±SEM (n=10-13/group). Data were analyzed by two-way ANOVA. ***$p< 0.001$ as compared to WT vehicle-treated mice; $$$p<0.001$, $$p<0.01$ and $p<0.05$, as compared to R6/1 vehicle-treated mice.

Online Resource 2 No changes in anxiety or motivation in R6/1 SC-51089-treated mice Table showing the covered distance expressed in centimeters (cm) and the percentage of time spent in the center of the open field arena in R6/1 treated with vehicle or SC-51089. Results are expressed as mean±SEM (n=10-13/group), and were analyzed by Student t-test. No significant differences were observed between groups.

Online Resource 3 EP1 expression is higher in hippocampus than in other brain regions in WT and R6/1 mice Protein extracts from the striatum (STR), the hippocampus (HIP) and the cortex (CTX) of 12-week-old WT (A) and R6/1 (B) mice were subjected to western blot to analyze EP1 protein levels. Results are shown as mean±SEM (n=5-7), and represent the ratio between EP1 and tubulin levels, obtained by densitometric analysis of western blot. Data were expressed as a percentage respect to striatal EP1 protein levels, and were analyzed by one-way ANOVA,

followed by Student t-test as a *post-hoc* test. ***p*<0.01, as compared to striatal EP1 protein levels and $$*p*<0.01 and $*p*<0.05, as compared to cortical EP1 protein levels. Representative immunoblots are presented.

Figure 1

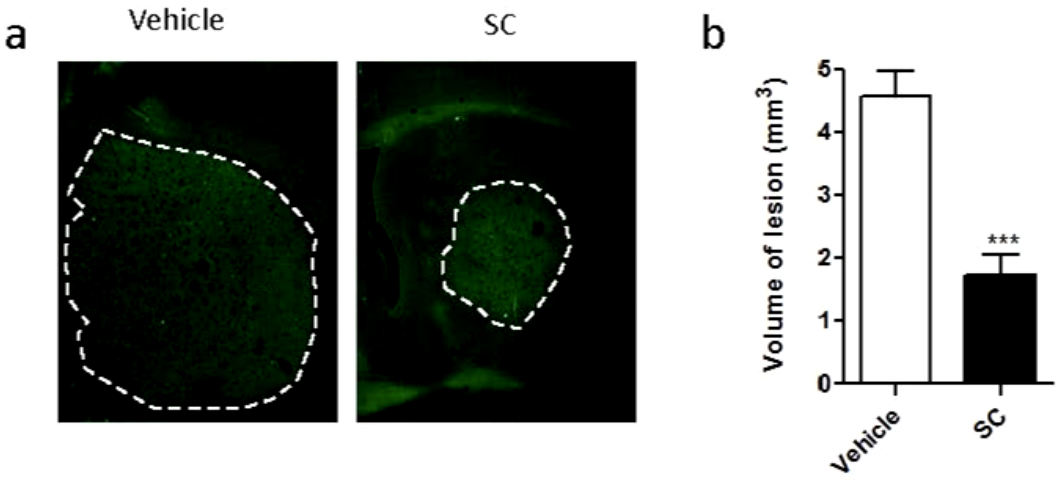


Figure 2

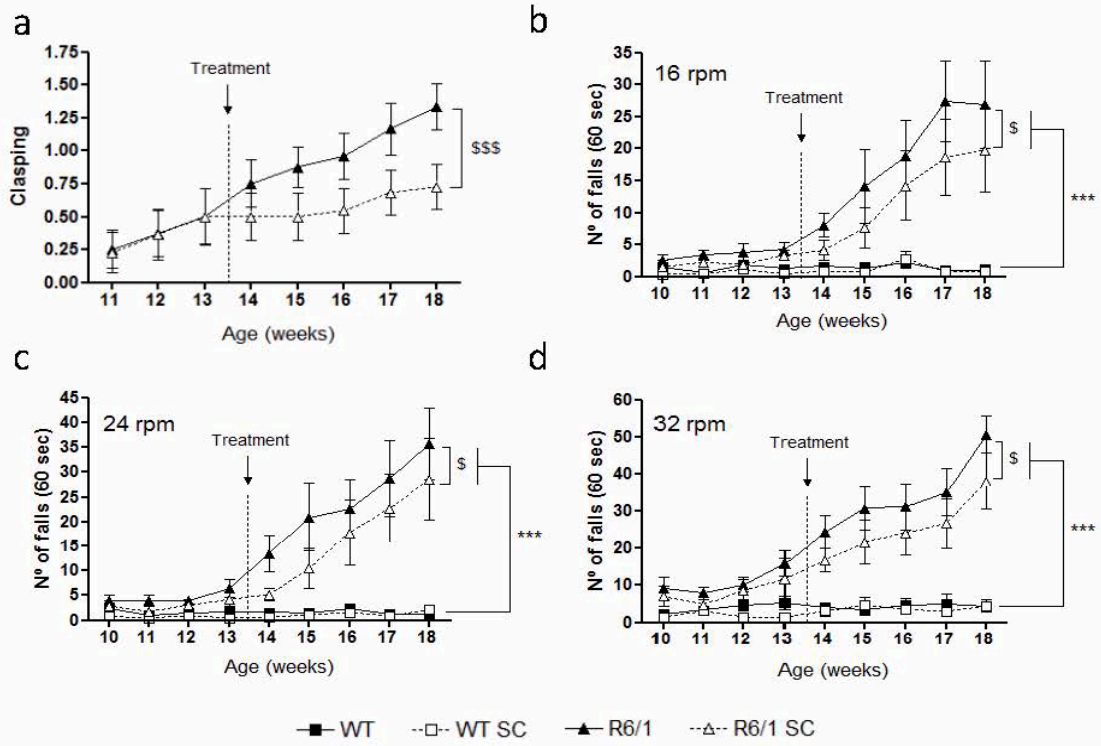

a
Clasping
Treatment
$$$
Age (weeks)
b
16 rpm
Treatment
Nº of falls (60 sec)
$
***
Age (weeks)
c
24 rpm
Treatment
Nº of falls (60 sec)
$
***
Age (weeks)
d
32 rpm
Treatment
Nº of falls (60 sec)
$
***
Age (weeks)
WT
WT SC
R6/1
R6/1 SC

Figure 3

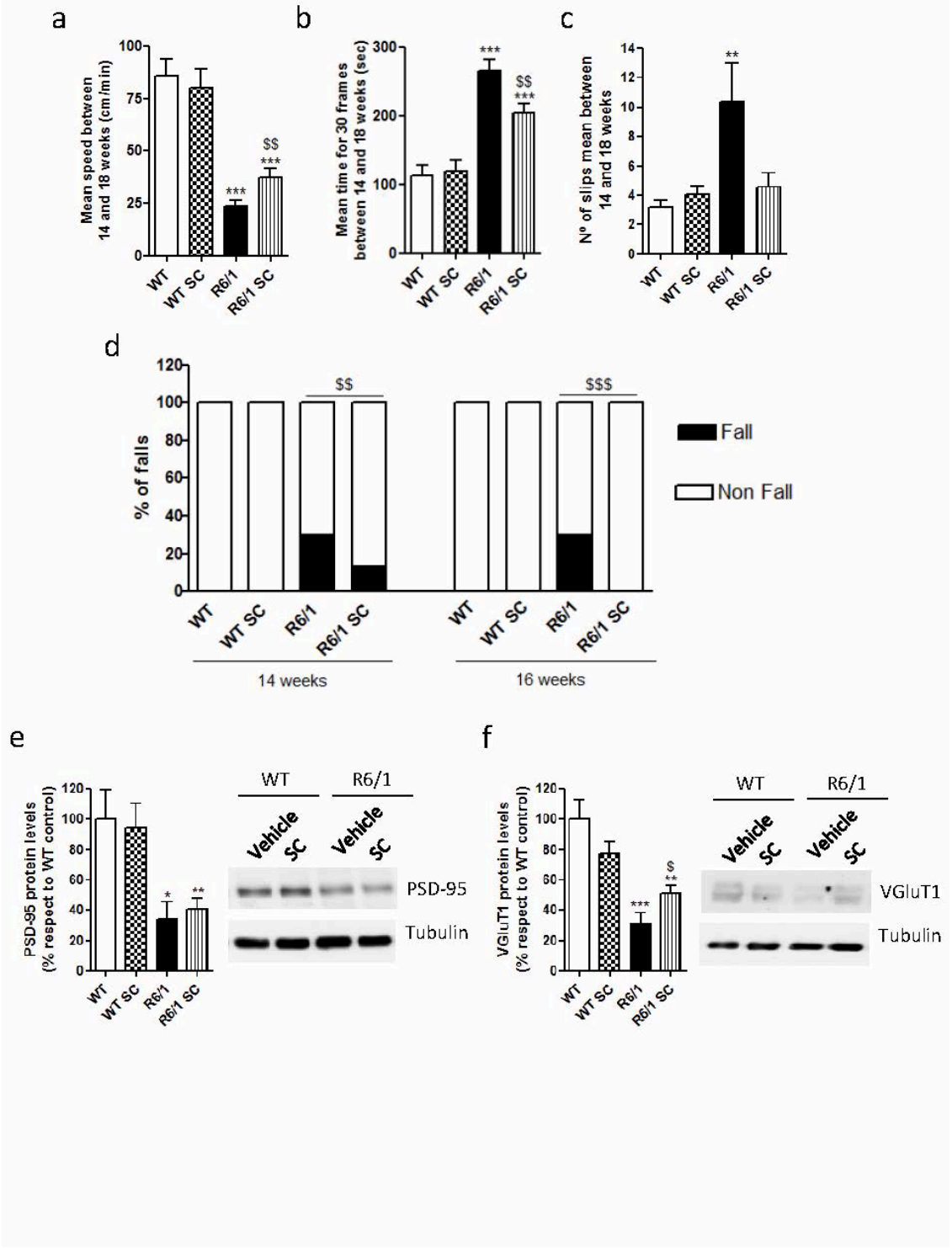

a
Mean speed between 14 and 18 weeks (cm/min)
100
75
50
25
0
***
$$
***
WT
WT SC
R6/1
R6/1 SC
b
Mean time for 30 frames between 14 and 18 weeks (sec)
300
200
100
0
***
$$
***
WT
WT SC
R6/1
R6/1 SC
c
Nº of slips mean between 14 and 18 weeks
14
12
10
8
6
4
2
0
**
WT
WT SC
R6/1
R6/1 SC
d
% of falls
120
100
80
60
40
20
0
$$
$$$
Fall
Non Fall
WT
WT SC
R6/1
R6/1 SC
14 weeks
16 weeks
e
PSD-95 protein levels (% respect to WT control)
120
100
80
60
40
20
0
*
**
WT
WT SC
R6/1
R6/1 SC
WT
R6/1
Vehicle
SC
Vehicle
SC
PSD-95
Tubulin
f
VGluT1 protein levels (% respect to WT control)
120
100
80
60
40
20
0
***
$
WT
WT SC
R6/1
R6/1 SC
WT
R6/1
Vehicle
SC
Vehicle
SC
VGluT1
Tubulin

Figure 4

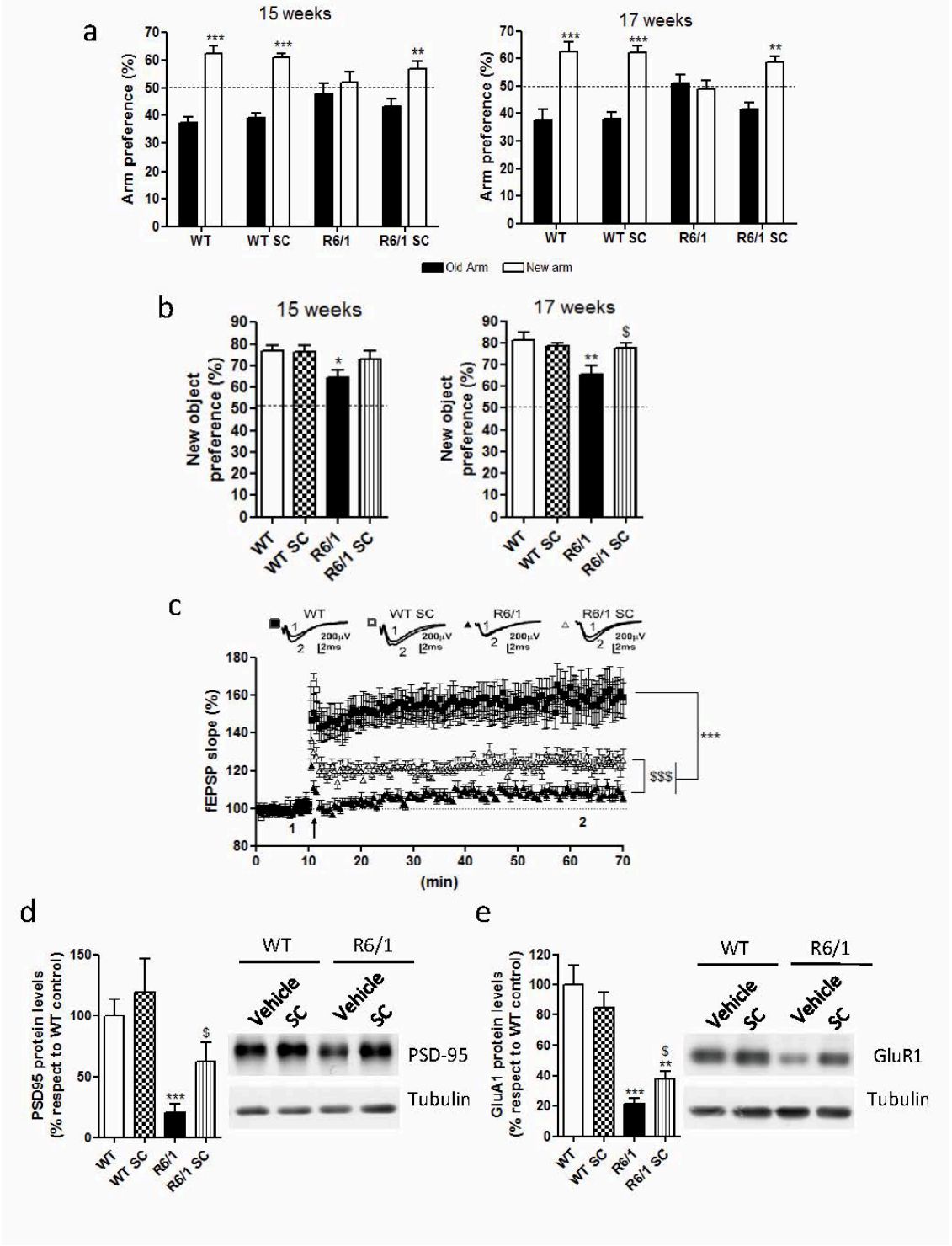
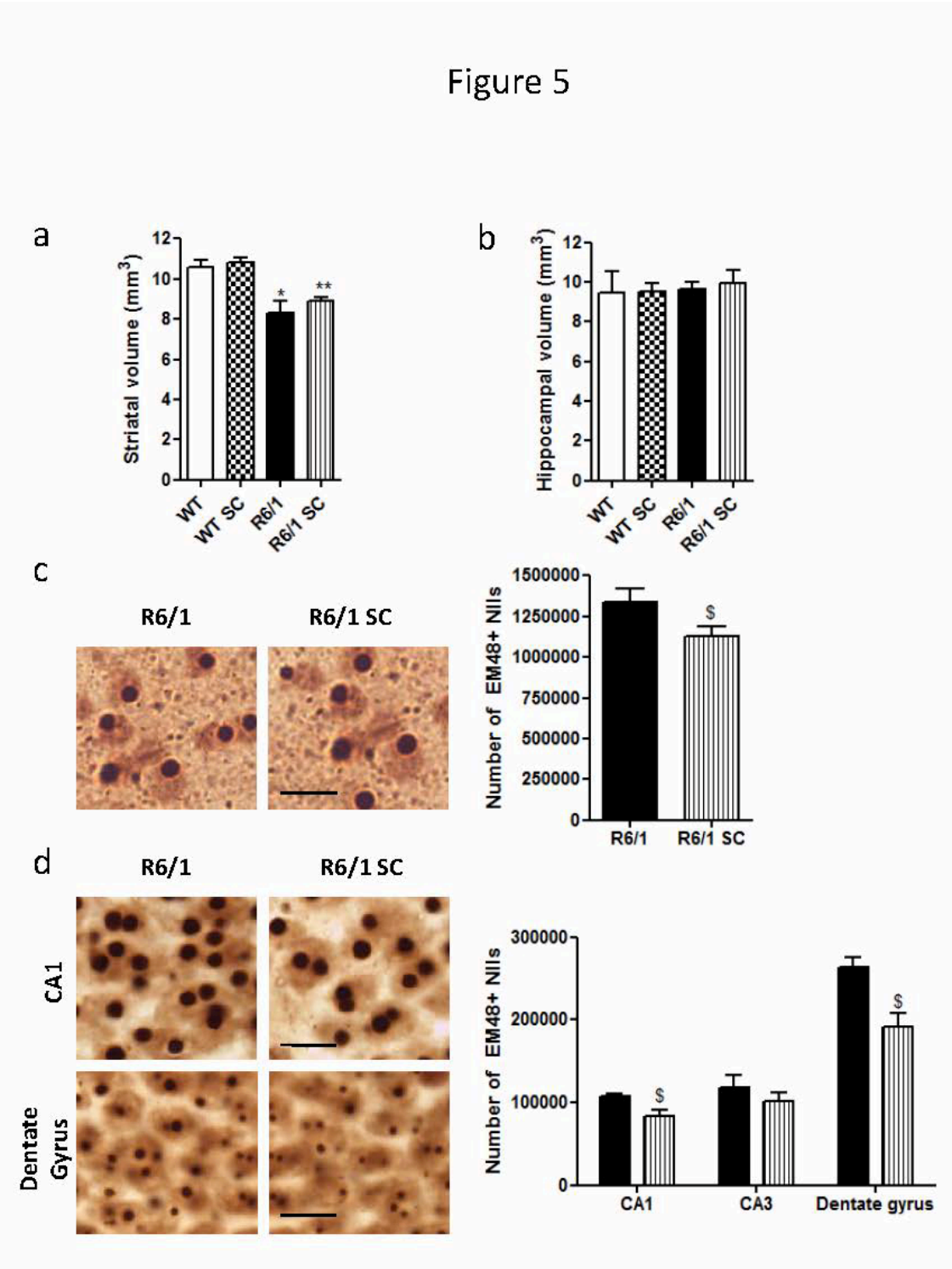
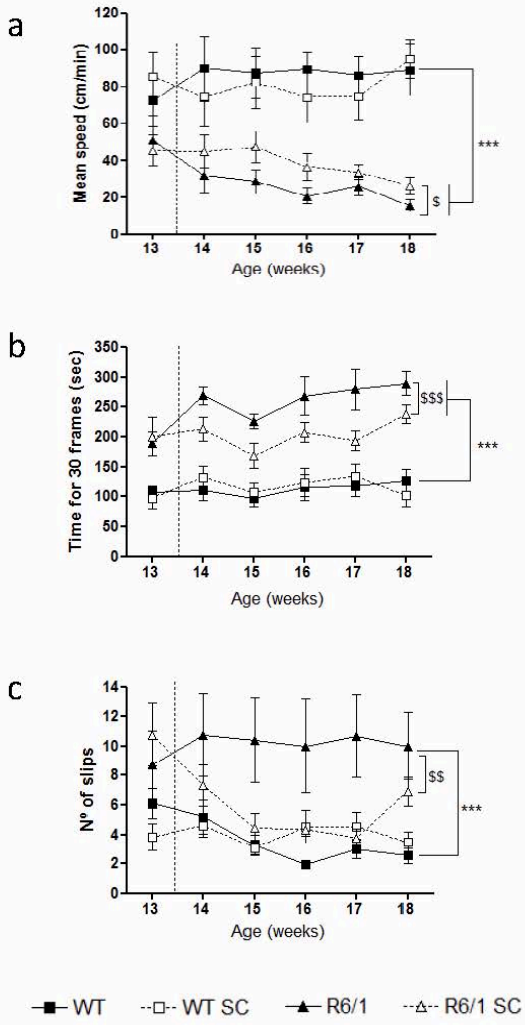

a
15 weeks
17 weeks
Arm preference (%)
WT
WT SC
R6/1
R6/1 SC
Old Arm
New arm
b
15 weeks
17 weeks
New object preference (%)
c
fEPSP slope (%)
(min)
d
PSD95 protein levels (% respect to WT control)
Vehicle
SC
PSD-95
Tubulin
e
GluA1 protein levels (% respect to WT control)
GluR1
Tubulin

Figure 5


a
Striatal volume (mm3)
12
10
8
6
4
2
0
*
**
WT
WT SC
R6/1
R6/1 SC
b
Hippocampal volume (mm3)
12
10
8
6
4
2
0
WT
WT SC
R6/1
R6/1 SC
c
R6/1
R6/1 SC
Number of EM48+ NIIs
1500000
1250000
1000000
750000
500000
250000
0
$
R6/1
R6/1 SC
d
R6/1
R6/1 SC
CA1
Dentate Gyrus
Number of EM48+ NIIs
300000
200000
100000
0
$
$
CA1
CA3
Dentate gyrus

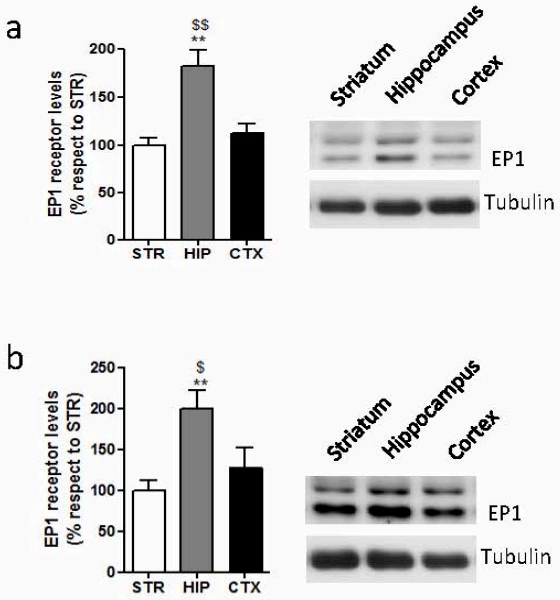
Online Resource 1



Online Resource 2

	15 weeks-old		17 weeks-old	
	Covered distance (cm)	Time in the center (%)	Covered distance(cm)	Time in the center (%)
R6/1+ vehicle	2670 ± 251.6	24.11 ± 3.4	1752 ± 217.4	28.71 ± 2.8
R6/1+SC-1089	2382 ± 225.1	33.26 ± 3.3	1825 ± 167.5	23.69 ± 4.9

Online Resource 3



Prostaglandin E2 EP2 activation improves synaptic plasticity and memory decline by increasing BDNF levels R6/1 mouse model of Huntington's disease

This manuscript is under preparation

AIM 2. TO CHARACTERIZE THE MODULATION OF PROSTAGLANDIN E2 EP RECEPTORS IN HUNTINGTON'S DISEASE.

2.2. To study the mechanism of EP2 receptor in the regulation of cognitive and trophic deficits in R6/1 mouse models of Huntington's disease.

Tot i que clàssicament la malaltia de Huntington es considera un desordre motor, avui en dia existeixen cada cop més evidències en pacients i models animals que ens mostren que els dèficits en aprenentatge i memòria apareixen fins i tot abans que els símptomes motors. Estudis recents suggereixen un paper del receptor EP2 de prostaglandina E2 en la modulació de la formació de la memòria i la plasticitat sinàptica. En aquest treball vam estudiar l'efecte de l'activació del receptor EP2 en cultius primaris hipocampals i el seu potencial terapèutic en un model de la malaltia de Huntington, el ratolí R6/1. Vam observar que l'agonista del receptor EP2, el misoprostol, augmentava el creixement dendrític en cultius de neurones hipocampals de manera dependent del factor neurotròfic derivat del cervell (BDNF). Per tal d'estudiar l'efecte del misoprostol en la malaltia de Huntington, vam administrar de manera crònica misoprostol en animals R6/1 mitjançant la implantació d'un sistema de bombes osmòtiques. El tractament va ser realitzat entre les 13 i les 18 setmanes d'edat d'aquest animals i van ser utilitzats diferents paradigmes per avaluar la seva funció cognitiva. L'administració de misoprostol millorava els dèficits en la memòria a llarg termini analitzada mitjançant el laberint en T d'alternança espontània i el test de reconeixement d'objectes nous. Així mateix, el misoprostol reduïa el reflex de *clasping*, un fenotip característic d'aquest ratolins. A més, el tractament amb l'agonista del receptor EP2 induïa un augment en l'expressió de BDNF a l'hipocamp tant de ratolins *wild-type* com en els ratolins R6/1. Aquests resultats reforcen la idea del paper del receptor EP2 en la modulació de la plasticitat sinàptica i suggereixen aquest receptor com a possible diana terapèutica per a restituir els dèficits cognitius que es produeixen en la malaltia de Huntington.

PROSTAGLANDIN E2 EP2 RECEPTOR ACTIVATION IMPROVES SYNAPTIC PLASTICITY AND MEMORY DECLINE BY INCREASING BDNF LEVELS IN R6/1 MICE MODELS OF HUNTINGTON'S DISEASE

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Abstract

Huntington's disease (HD) patients and mouse models show learning and memory impairment even before the onset of motor symptoms. However, the molecular events involved in this cognitive decline and decreased synaptic plasticity are still poorly understood. Recent studies suggest a role of prostaglandin EP2 receptor in the modulation of memory formation and synaptic plasticity. Here, we investigated the beneficial effect of EP2 receptor activation synaptic plasticity in hippocampal primary cultures and the potential therapeutic action of this receptor in a model of HD, the R6/1 mice. To this aim, we used misoprostol, an EP2 receptor agonist. We found that EP2 receptor agonist increases dendritic branching in cultured hippocampal neurons. This increase in dendritic outgrowth was blocked by the addition of a brain-derived neurotrophic factor (BDNF) antibody suggesting that the positive effect of misoprostol was BDNF-dependent. To study the beneficial effect of misoprostol in *vivo*, we implanted an osmotic mini-pump system to chronically administrate an EP2 receptor agonist to R6/1 mice from 13 to 18 weeks of age, and used different paradigms to assessed memory function. Misoprostol administration ameliorated R6/1 long-term memory deficits as analyzed by the T-maze spontaneous alternation and the novel object recognition test. Moreover, misoprostol reduced clasping reflex. Additionally, treatment with EP2 receptor agonist increased the expression of BDNF in the hippocampus of wild-type and R6/1 mice. Altogether, these results reinforce the role of EP2 receptor activation in synaptic plasticity modulation and suggest a putative therapeutic effect of this receptor in reducing cognitive deficits of R6/1 mice.

INTRODUCTION

Huntington's disease (HD) is a neurodegenerative disorder caused by an expanded CAG repeat in the exon-1 of the *huntingtin* (htt) gen (HDCRG, 1993). Although severe motor impairments characterize the disease, cognitive and memory deficits are an early clinical feature in HD that often appear before the onset of motor symptoms or neuronal loss (Lawrence et al., 1996; Montoya et al., 2006; Stout et al., 2011; Vonsattel and DiFiglia, 1998). Deficits in synaptic plasticity and memory have also been described in different mouse models of HD (Brooks et al., 2012; Giralt et al., 2009; Lione et al., 1999; Lynch et al., 2007; Simmons et al., 2009), although the precise molecular mechanisms underlying these memory deficits remain largely unknown. Different studies observed that cAMP-responsive element binding protein (CREB) signaling is compromised in different mouse and cellular models of HD, and in human samples, where the expression of mhtt induces aggregation of the co-activator CREB-binding protein (CBP) (Nucifora, Jr. et al., 2001; Steffan et al., 2000; Steffan et al., 2001), reduces the levels of cAMP (Gines et al., 2003) and down-regulates CRE-mediated transcription of numerous genes (Augood et al., 1996; Zuccato et al., 2010; Luthi-Carter et al., 2002). Activation of CREB has also been found to be necessary for functional increases in synaptic transmission (Davies et al., 2004) and CREB-mediated gene expression is sufficient for survival of multiple neuronal subtypes (Bonni et al., 1999; Riccio et al., 1999; Walton et al., 1999). CREB may exert this pro-survival effect by regulating the transcription of many proteins, including *bdnf* (brain-derived neurotrophic factor) (Dawson and Ginty, 2002; Fukuchi et al., 2005; Tabuchi et al., 2002; Tao et al., 1998), which is known to be down-regulated in different brain regions of HD mouse models as well as in human HD patients (Zuccato and Cattaneo, 2007; Zuccato et al., 2008). Therefore, the modulation of CREB can be a good therapeutic approach to ameliorate the cognitive deficits observed in HD.

Prostaglandin E2 (PGE2) is one of the molecules that activate CREB. Its receptor EP2, one of the four different receptor (EP1-EP4) activated by PGE2 (Breyer et al., 2001), is positively coupled to Gαs protein and is widely expressed in neurons, microglia and astrocytes in forebrain (Cimino et al., 2008). Activation of the EP2 receptor is known to stimulate cAMP-dependent intracellular signaling that involves the activation of cAMP-dependent protein kinase (PKA), and consequent phosphorylation and activation of the transcription factor CREB (Regan, 2003). Activation of EP2 receptor has revealed as neuroprotective in paradigms of NMDA toxicity and ischemia (Ahmad et al., 2006; Ahmad et al., 2010; Liu et al., 2005; McCullough et al., 2004), and in additional models of neurodegenerative diseases such as *in vitro* models of amyotrophic lateral sclerosis (Bilak et al., 2004), Parkinson's disease (Carrasco

et al., 2008) and Alzheimer's disease (Echeverria et al., 2005). In all cases, the EP2-dependent protective effects were associated to cAMP/PKA activation. Moreover, in terms of physiological conditions, emerging literature supported the role of EP2 receptor signaling in activity-dependent learning and memory formation and synaptic plasticity (Akaneya and Tsumoto, 2006; Furuyashiki and Narumiya, 2011; Sang et al., 2005; Savonenko et al., 2009; Yang et al., 2009). Results from electrophysiological studies have also demonstrated the role of PGE2 and EP2 receptor in excitatory synaptic transmission and long-term plasticity in the hippocampus(Akaneya and Tsumoto, 2006; Chen et al., 2002; Sang et al., 2005; Savonenko et al., 2009; Shaw et al., 2003; Yang et al., 2009). Moreover, EP2 deficient mice show various memory disturbances.

In mice, the EP2 receptor has been reported to be highly expressed in cerebral cortex, striatum, and hippocampus (Ahmad et al., 2006; Zhang and Rivest, 1999), regions affected in HD (Vonsattel and DiFiglia, 1998). In this context, some studies have shown that PGE2 activates BDNF release in cultured human microglia and astrocytes by EP2 receptor activation (Hutchinson et al., 2009). These findings are consistent with reports showing that PGE2 stimulates the release of neurotrophins from mouse astrocytes, and PGE2 levels correlates with BDNF levels (Ajmone-Cat et al., 2006; Shaw et al., 2003; Toyomoto et al., 2004). Since BDNF modulation participates in the specific neurodegeneration and dysfunction observed in HD pathology (Zuccato and Cattaneo, 2009), our study focuses on analyzing the possible therapeutic role of EP2 receptor stimulation in R6/1 mice model of HD and the implication of BDNF in EP2 receptor signaling.

METHODS

HD mouse models

Male R6/1 transgenic mice expressing exon-1 of mhtt were obtained from Jackson Laboratory (Bar Harbor, ME), and maintained in a B6CBA background. Mice were genotyped by polymerase chain reaction (PCR) as described previously (Mangiarini et al., 1996). Our R6/1 colony has 145 CAG repeats (Giralt et al., 2009). Wild type (WT) littermate animals were used as the control group. All mice used in the present study were housed together in numerical birth order in groups of mixed genotypes, and data were recorded for analysis by microchip mouse number. Experiments were conducted in a blind-coded manner with respect to genotype and treatment. All mice used in the present study were housed together in

numerical birth order in groups of mixed genotypes, and data were recorded for analysis by microchip mouse number. Animals were housed with access to food and water *ad libitum* in a colony room kept at 19–22ºC and 40–60% humidity, under a 12 : 12 hours light/dark cycle. All procedures were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approved by the local animal care committee of *Universitat de Barcelona* (99/01), and *Generalitat de Catalunya* (99/1094), in accordance with the Directive 86/609/EU of the European Commission.

Cell cultures

Primary hippocampal cultures were obtained from 18-days old B6CBA mouse embryos (Charles River, France). Hippocampi were dissected and cells (100,000 cells/cm2) were seeded on plates pre-coated with 0.1 mg/mL poly-D-lysine (Sigma Chemical Co., St. Louis, MO) and cultured in Neurobasal medium supplemented with B27 (Gibco, Paisley, Scotland, UK) and glutamax at 37ºC in a humidified atmosphere containing 5% CO2. To measure number of dendrites, cultures were treated at 3 days *in vitro* (DIV) with vehicle, EP2 receptor agonist (misoprostol; 10µM; Santa Cruz Biotechnology, Santa Cruz, CA) (Li et al., 2008), BDNF (10ng/mL; Peprotech EC Ltd., London, UK) (Gavalda et al., 2004) and/or BDNF antibody (10 ng/mL; Santa Cruz Biotechnology)(Xifro et al., 2005). Seven days after treatments (10 DIV) cultures were fixed as previously described (Xifro et al., 2011).

Immunocytochemical staining and branching analysis

Fixed cells were permeabilised in 0.1% saponin for 10 min. Blocking was performed with 1% BSA in PBS for 1h. 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.

Pharmacological treatments in R6/1 mice

For testing EP2 receptor agonist, misoprostol, 10-week-old WT mice were intraperitoneally (i.p.) injected with vehicle (PBS), misoprostol 50 µg/kg/day or misoprostol 500 µg/kg/day. One

hour after injection animals were sacrificed by cervical dislocation and the hippocampus was rapidly removed.

For behavioral analysis, 13-weeks-old WT and R6/1 mice were deeply anesthetized with pentobarbital (40 mg/kg) and an osmotic minipump was i.p. implanted (model 1004; Alzet, Palo Alto, CA). Vehicle or the EP2 receptor antagonist, misoprostol (Santa Cruz Biotechnology, Santa Cruz, CA), were infused i.p. at a rate of 0.11 µl/h during 28 days resulting in a dose of 50 µg/kg/day. Mice were allowed to recover for 3-5 days before starting behavioral tests.

Protein extraction and western blot analysis

Animals were sacrificed by cervical dislocation and the striatum, cortex and hippocampus were rapidly removed. Total protein was extracted as previously described (Anglada-Huguet et al., 2012). Western blotting was performed as described elsewhere (Xifro et al., 2009). The following primary antibodies (1:1000) were used: anti-phospho-CREB (Ser133) (Millipore, Temecula, CA) and anti-BDNF (Santa Cruz Biotechnology). Loading control was performed by reprobing the membranes with anti-α-tubulin (1:50000; Sigma-Aldrich). Membranes were incubated with the corresponding horseradish peroxidase-conjugated antibody (1:2000; Promega, Madison, WI). Immunoreactive bands were visualized using the Western Blotting Luminol Reagent (Santa Cruz Biotechnology), and quantified by a computer-assisted densitometer (Gel-Pro Analyzer, version 4, Media Cybernetics).

Behavioral assessment

Clasping and weight

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. Mice were scored according to the following criteria: 0 = no clasping, 1 = clasping 2 paws and 2 = clasping all paws. Animals were weighted weekly.

Novel object recognition test (NORT)

Mice were tested in a circular open field (40 cm diameter) located in a room with dim lighting. Briefly, 15- and 17-week-old mice were habituated to the open field in the absence of the objects for 10 min/day over 2 days. During the training period, mice were placed in the open field with two identical objects for 10 min. The retention test was performed 24 hours after training (long-term memory) by placing the mice back to the open field for a 5 min session, and by randomly exchanging one of the familiar objects with a novel one. Results were analyzed as previously described (Giralt et al., 2012) .

T-maze spontaneous alternation task (T-SAT)

T-SAT was used to analyze hippocampal-dependent memory in 15- and 17-week-old mice. The T-maze apparatus used and the light conditions were previously described elsewhere (Giralt et al., 2011). In the training session, one arm was closed (new arm) and mice were placed in the stem arm of the T (home arm) and allowed to explore this arm and the other available arm (familiar arm) for 10 min, after which they were returned to the home cage. To assess long-term memory, after inter-trial intervals of 4 hours mice were placed in the stem arm of the T-maze and allowed to freely explore all three arms for 5 min. Arm preference was determined by calculating the time spent in each arm × 100/time spent in both arms (old and new).

Results

EP2 receptor agonist increases dendritic complexity in a BDNF-dependent manner

We initially investigate the putative role of EP2 in synaptic plasticity (Furuyashiki and Narumiya, 2011; Sang et al., 2005; Savonenko et al., 2009; Yang et al., 2009) by assessing the effect of the EP2 receptor agonist, misoprostol, in the branching of hippocampal primary cultures. We treated the cultures at 3 DIV, fixed seven days after and dendritic complexity was assessed by quantifying the number of dendrites (Fig. 1). In cultures treated with misoprostol, we found that EP2 receptor activation increased the number of dendrites comparing with the control neurons (Fig. 1A and 1C i and ii). BDNF treatment was additionally used as a positive control of branching, as it is well known that BDNF causes dendritic growth in hippocampal neurons (Horch and Katz, 2002; Cheung et al., 2007; Takemoto-Kimura et al., 2007). As expected, we found that BDNF increased dendritic arborization in hippocampal primary cultures (Fig. 1A and 1C i and iii).

As activation of EP2 receptor has been associated with an increase of neurotrophins, such as BDNF (Hutchinson et al., 2009; Toyomoto et al., 2004), we wanted to validated if the increased arborization of hippocampal primary cultures observed after misoprostol treatment was in a BDNF-dependent manner. Therefore, we treated cultures with misoprostol and BDNF in the presence of BDNF antibody to block the putative effect of BDNF (Xifro et al., 2005). We observed that BDNF antibody completely abolished the misoprostol- and BDNF-dependent dendritic growth (Fig. 1A and 1C v and vi), and decreased the number of dendrites to the control situation (Fig. 1A). A reduction in the dendritic arborization was also observed in the control situation when the BDNF antibody was added into the media (Fig. 1A and 1C i and iv)

Misoprostol increases CREB phosphorylation in the hippocampus.

After observing a beneficial role of EP2 activation in the dendritic growth of neuronal cultures, we wonder to know whether treatments with misoprostol could also be beneficial *in vivo*. As it is know that EP2 receptor activates cAMP/PKA/CREB pathway (Regan, 2003); we therefore injected mice with 2 different concentrations of misoprostol (50 µg/kg and 500 µg/kg, i.p.) and we analyzed the phosphorylation of the transcription factor CREB after 1 hour (Fig.2). We could observe an increase in the levels of CREB phosphorylation in the hippocampus of mice in both concentration of misoprostol tested (Fig. 2). Thus, we decided to use a concentration of 50 µg/kg as it was the concentration with maximal increase in CREB phosphorylation observed.

EP2 agonism reduces clasping and long-term memory deficits in R6/1 mice

Then, we implanted i.p. an osmotic pump in 13-week-old WT and R6/1 mice to deliver chronically misoprostol (50 µg/kg/day) during 28 days. First, we weekly evaluated the clasping reflex and the body weight from 12 to 18 weeks of age. As we show in Fig. 3A, the administration of misoprostol resulted in a significant delay of clasping phenotype progression. Regarding to body weight, we observed the typical loss of body weight in R6/1 mice at 18 weeks of age, but no differences were observed between vehicle- and misoprostol-treated mice in any genotype (Fig. 3B).

EP2 receptor signaling is related to activity-dependent learning and memory formation and synaptic plasticity (Akaneya and Tsumoto, 2006; Sang et al., 2005; Savonenko et al., 2009; Shaw et al., 2005). Then, we wanted to determine whether EP2 activation improves LTM deficits in R6/1 mice by performing the T-SAT and the NORT to all groups of mice at 17 weeks of age. In the T-SAT we observed that WT mice spent more time in the new arm than in the old arm, but R6/1 mice did not show preference for the new arm (Fig. 3C). Interestingly, misoprostol-treated R6/1 mice explored the new arm similarly to WT animals (Fig. 3C). Then, we subjected all groups of animals to the NORT. In 17-week-old mice, all groups explored more the new object respect to the old one. However, the preference of R6/1 mice for the new object was significantly reduced compared with WT mice. Interestingly, misoprostol-treated R6/1 mice explored the novel object as much as vehicle-treated WT mice, and significantly more compared with vehicle-treated R6/1 animals (Fig. 3D).

During NORT habituation, no differences were observed between vehicle- and misoprostol-treated R6/1 mice in the exploration of the center (vehicle-treated WT mice 29±2.4%;

misoprostol-treated WT mice 24.3±2.6%; vehicle-treated R6/1 mice 26.1±3.2% and SC-51089-treated R6/1 mice 28±3.9%; in all cases $p>0.05$), suggesting that the improvement in LTM was not due to misoprostol-induced changes in anxiety.

Misoprostol chronic treatment restores the expression of BDNF protein levels

As we have previously demonstrated that EP2 receptor activation could mediate dendritic growth in hippocampal primary cultures in a BDNF-dependent manner (Fig.1), we wanted to analyze whether improvements in behavior correlated with changes in the expression of BDNF in the hippocampus of these animals. BDNF protein levels were reduced in the hippocampus of vehicle-treated R6/1 mice comparing to the vehicle-treated WT mice (Fig. 4). Interestingly, we observed that hippocampal expression of this neurotrophic factor was increased after misoprostol chronic treatment in WT and R6/1 mice comparing with their respective vehicle-treated groups (Fig. 4).

Discussion

In the present study, we identify PGE2 as a candidate factor to reduce cognitive deficits in HD. We observed that chronic EP2 receptor pharmacological activation with misoprostol decreased clasping and hippocampal-dependent memory in R6/1 mouse model of HD. These behavioral improvements correlated with increased levels of BDNF in R6/1-treated mice and an enhancement in dendritic complexity induced by BDNF in hippocampal primary cultures after misoprostol treatment. We suggest PGE2 EP2 receptor pathway as a candidate to block neuronal dysfunction.

Our results show that chronic treatment with the EP2 agonist misoprostol ameliorated LTM deficits in 17-week-old R6/1 mice. Treated animals performed better T-SAT and NORT than control ones. This improvement is related with the effect of EP2 receptor activation on dendritic outgrowth induced by BDNF. Moreover, the chronic treatment with misoprostol produced an increase in BDNF levels. This effect was independent of mutant huntingtin since the increase was observed in R6/1 and WT mice. Previous studies have already linked PGE2 EP2 receptor with the BDNF pathway. Prostaglandin stimulates the release of BDNF in cultured astrocytes and neurons (Hutchinson et al., 2009; Rage et al., 2006; Toyomoto et al., 2004), and in sensory ganglion explants (Cruz et al., 2012). Shaw et al. also showed the involvement of PGE2 in memory formation and synaptic plasticity (Shaw et al., 2003; Shaw et

al., 2005). All these results support the role of PGE2 EP2 receptor regulating trophic support in hippocampal neurons (Fig. 5).

It is well known that EP2 receptor is coupled to Gαs protein and its stimulation activates the cAMP/PKA/CREB pathway (Regan, 2003). In agreement, we observed an activation of the transcription factor CREB after treating mice with an EP2 receptor agonist. CREB is a transcription factor whose function is impaired by mhtt (Steffan et al., 2000; Sugars and Rubinsztein, 2003; Sugars et al., 2004), suggesting that inhibition of CREB-mediated gene transcription contributes to HD (Jiang et al., 2003; Mantamadiotis et al., 2002; Nucifora, Jr. et al., 2001; Steffan et al., 2000). Confirming these observcations, cAMP levels are reduced in the cerebrospinal fluid of patients with HD (Cramer et al., 1984) and transcription of CREB-regulated genes is decreased in transgenic mouse model of HD (Fig. 5) (Giralt et al., 2012; Luthi-Carter et al., 2000; Mantamadiotis et al., 2002; Nucifora, Jr. et al., 2001; Wyttenbach et al., 2001). Moreover, levels of activated CREB are related to the vulnerability to neuronal excitotoxicity (Giampa et al., 2006). Recently, it has been suggested that increased CREB phosphorylation exerts neuroprotective effects in both the quinolinic acid model of HD (DeMarch et al., 2007) and the R6/2 mouse (DeMarch et al., 2008; Giampa et al., 2009b). These beneficial effects included sparing of striatal neurons, increased expression of BDNF in the striatum, prevention of neuronal intranuclear inclusion formation, preventing CREB-binding protein sequestration and rescuing motor coordination and activity deficits in these mice (DeMarch et al., 2008; Giampa et al., 2009b). Our results further confirm the beneficial effect of CREB activation in HD. We show that treatments that increase CREB activation not only rescue striatal-dependent HD phenotype, as previously reported (Giampa et al., 2009), but also hippocampal-dependent deficits in R6/1 mice, reinforcing the suggested role of EP2 receptor in memory formation (Shaw et al., 2003). Many hypotheses about the selective degeneration and neuronal dysfunction induced by mhtt have been proposed, but increasing attention has been given to the lack of trophic support, specially the reduced levels of BDNF (Zuccato and Cattaneo, 2007; Zuccato et al., 2008). BDNF levels regulates the onset and severity of cognitive and motor symptoms, and synaptic plasticity deficits in HD mouse models (Canals et al., 2004; Giralt et al., 2009; Lynch et al., 2007). Interestingly, treatments focused on the recovery of BDNF levels successfully improve synaptic plasticity, and motor and cognitive functions in HD mouse models (Gharami et al., 2008; Giralt et al., 2009; Simmons et al., 2009; Simmons et al., 2011). Furthermore, BDNF has been shown to be important for memory consolidation (Bekinschtein et al., 2008; Binder and Scharfman, 2004). Therefore, increased levels of BDNF after misoprostol treatment is the most likely explanation for the cognitive improvements observed in R6/1-treated mice (Fig. 5).

Over the past years, the complex contributions of PGE2 in neuronal survival and death and in synaptic plasticity of neurons have been the subject of great interest. Much of this work has focused on the actions of PGE2 through the EP2 prostanoid receptor and, surprisingly, has linked the activation of this receptor to both neurotoxic and neuroprotective outcomes. Many studies show neuroprotection by EP2 receptor activation in paradigms of NMDA and glutamate neurotoxicity (Liu et al., 2005), oxygen and glucose deprivation (McCullough et al., 2004) and in additional models of ischemia (Ahmad et al., 2006), amyotrophic lateral sclerosis (Bilak et al., 2004) and Parkinson's disease (Carrasco et al., 2008). However, the role of EP2 receptor seems to elicit a very different response in the context of neuroinflammatory conditions. Accumulating evidence indicates a pro-inflammatory neurotoxic effect of EP2 receptor signaling in activated microglia (Milatovic et al., 2004; Montine et al., 2002; Shie et al., 2005) and in models of inflammatory neurodegeneration (Jin et al., 2007; Liang et al., 2005; Liang et al., 2008); thus, depending on the type of injury (acute excitotoxicity versus chronic inflammation) EP2 signaling mediates significant different effect. In the context of HD, we have observed a beneficial effect of EP2 receptor activation, which reduces HD cognitive deficits and increases the expression of BDNF. Although some studies suggest significant role of neuroinflamation in HD pathology (Moller, 2010; Tai et al., 2007), excitotoxicity is one of the main triggering mechanism of the disease (Zuccato et al., 2010; Mehta et al., 2013). Thus, the beneficial effect of EP2 receptor activation on neuronal dysfunction observed in the present study is in agreement with previous reports demonstrating EP2-mediated neuroprotection in the context of excitotoxicity.

We provide the first evidence of the beneficial role of chronic activation of PGE2 EP2 receptor in HD. Consistent with memory improvements, misoprostol treatment lead to the partial recovery of the levels of BDNF in the hippocampus of R6/1 mice. Moreover, this positive regulation of BDNF by EP2 receptor activation has been also observed in primary hippocampal cultures, where misoprostol increases dendritic growth in an EP2/BDNF-dependent manner. Taken together, these findings show that the activation of EP2 receptor has a strong therapeutic effect reducing mhtt-induced cognitive deficits in R6/1 mice, and could be a useful tool to reverse the decrease in trophic support characteristic of HD.

Acknowledgments

We are very grateful to Ana Lopez and Maria Teresa Muñoz for technical assistance and to Dr Teresa Rodrigo, Amèrica Jiménez and the staff of the animal care facility (Facultat de Psicologia and Facultat de Medicina, Universitat de Barcelona) for their help. Financial support was

obtained from Ministerio de Economía y Competitividad (SAF2011-29507), RETICS (CIBERNED, R006/0010/0006), Generalitat de Catalunya (2009SGR-00326).

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FIGURE LEGENDS

Figure 1. EP2 receptor agonism increases the number of dendrites in hippocampal primary cultures. Hippocampal primary cultures from 18-days old mouse embryos were treated at 3 DIV with the EP2 receptor agonist, misoprostol (10 µM; MIS) or BDNF (10 ng/mL) in the presence or abscense of BDNF antibody (10 ng/mL; aBDNF). Seven days after treatment cultures were fixed. (A) Graph showing the quantification of the number of dendrites analyzed for each condition. (B) Representative image of the quantification of the number of dendrites by ImageJ plugin NeuronJ. (C) Representative microphotographs showing hippocampal piramidal neurons in culture treated with vehicle (i and iv), misoprostol (ii and v) and BDNF (iii and vi) in the presence (iv-vi) or absence (i-iii) of BDNF antibody. Data were analyzed by Student's t-test. *** $p< 0.001$ i * $p<0.05$, comparing each condition in the presence or absence of BDNF antibody; $$ $p<0.01$ i $ $p<0.05$, as compared to control condition without BDNF antibody.

Figure 2. Misoprostol increases the actiation of the transcription factor CREB in mouse hippocampus. Teen-weeks-old WT mice were i.p. injected with vehicle or misoprostol (50 µg/kg/day or 500 µg/kg/day) during 1h. Protein extracts from the hippocampus of these mice were subjected to western blot to phospho-CREB. Representative immunoblots are presented. Data were expressed as a percentage with respect to vehicle-treated mice. Results mean±SEM (n=3-4/group), and represent the ratio between each protein and tubulin levels, obtained by densitometric analysis of western blot. Data were analyzed by Student's t-test. * $p<0.05$, when compared to vehicle-injected group.

igure 3. Chronic administration of the EP2 agonist, misoprostol, reduces clasping behavior and long-term memory deficits in R6/1 mice. WT and R6/1 mice were treated with misoprostol (MIS; 50 µg/kg/day) or vehicle using an osmotic pump system implanted i.p. at 13 weeks of age (discontinuous vertical line). (A) Clasping reflex and (B) weight of R6/1 vehicle- or misoprostol-treated mice was analyzed weekly from 12 to 18 weeks of age. Vertical discontinuous line represent the moment of the surgery. Values are expressed as a mean±SEM (n=10-15/group). Data were analyzed by two-way ANOVA. *** $p<0.001$, as compared to WT vehicle-treated mice, $$$ $p<0.001$, as compared to R6/1 vehicle-treated mice. (C and D) WT vehicle-, WT SC-51089-, R6/1 vehicle- and R6/1 SC-51089-treated mice were subjected to the T-SAT and NORT at 17 weeks of age. The chance level for exploration was represented as a discontinue line in all graphs. The arm exploration time 4 hours after the training session of all groups during the T-SAT task (C) is shown as a mean±SEM (n=10-15/group) in percentage of

the preference for new arm (open bars) or old arm (closed bars) at 17 weeks of age. Statistical analysis was performed using two-way ANOVA followed by Bonferroni as *post-hoc* test. ***p<0.001 and **p<0.01, when compared with the percentage of preference for the new arm; $$$p$<0.01, when compared with the percentage of preference for the new arm of R6/1 vehicle-treated mice. In the NORT task (D), all mice spent similar time exploring both objects during the training session. Long-term memory was assessed 24 hours later introducing a new object instead of an old object. Bars are shown as mean±SEM (n=10-15/group), and represent the percentage of new object preference at 17 weeks of age in all groups. Statistical analysis was performed by one-way ANOVA followed by Student t-test as *post-hoc* test. ***p<0.01, when compared with the percentage of preference for the new object of WT vehicle-treated mice; $$$p$<0.01 when compared with the percentage of preference for the new object of R6/1 vehicle-treated mice.

Figure 4. Chronic treatment with misoprostol increases BDNF protein levels in the hippocampus. At 18 weeks of age, protein extracts from the hippocampus of WT and R6/1 mice treated with vehicle or misoprostol were subjected to western blot to analyze BDNF protein levels. Results are expressed as mean±SEM (n=6-7/group), and represent the ratio between each protein and tubulin levels, obtained by densitometric analysis of western blot. Data were expressed as a percentage respect to WT vehicle-treated mice, and were analyzed by one-way ANOVA, followed by Student t-test as a *post-hoc*. ***p<0.001, as compared to WT vehicle-treated mice; $$p$<0.05, as compared to R6/1 vehicle-treated mice. Representative immunoblots are presented.

Figure 5. Hypothesis of EP2 receptor pharmacological activation in Huntington's disease. In control conditions, EP2 receptor (EP2R) activation increases the levels of cAMP, which in turn activates protein kinasa A (PKA) that phosphorylates cAMP-response element binding protein (CREB). CREB is a transcription factor that once in the nucleaus can promote the transcription of genes regulated by CRE promotor as *bdnf* gene. BDNF in turn can induce dendritic growth and synaptic plasticity. In Hungtinton's disease mutant huntingtin interferce with all these processes by altering levels of cAMP, sequestring proteins necessary for CREB-mediated transcription, as CREB-binding protein (CBP), reducing the levels of BDNF and its transport, which finally results in a reduction of dendritic growth and synaptic plasticity. In the presence of misoprostol, there is an increase in the levels of BDNF (1), that can affect dendritic growth and plasticity (2).

Figure 1

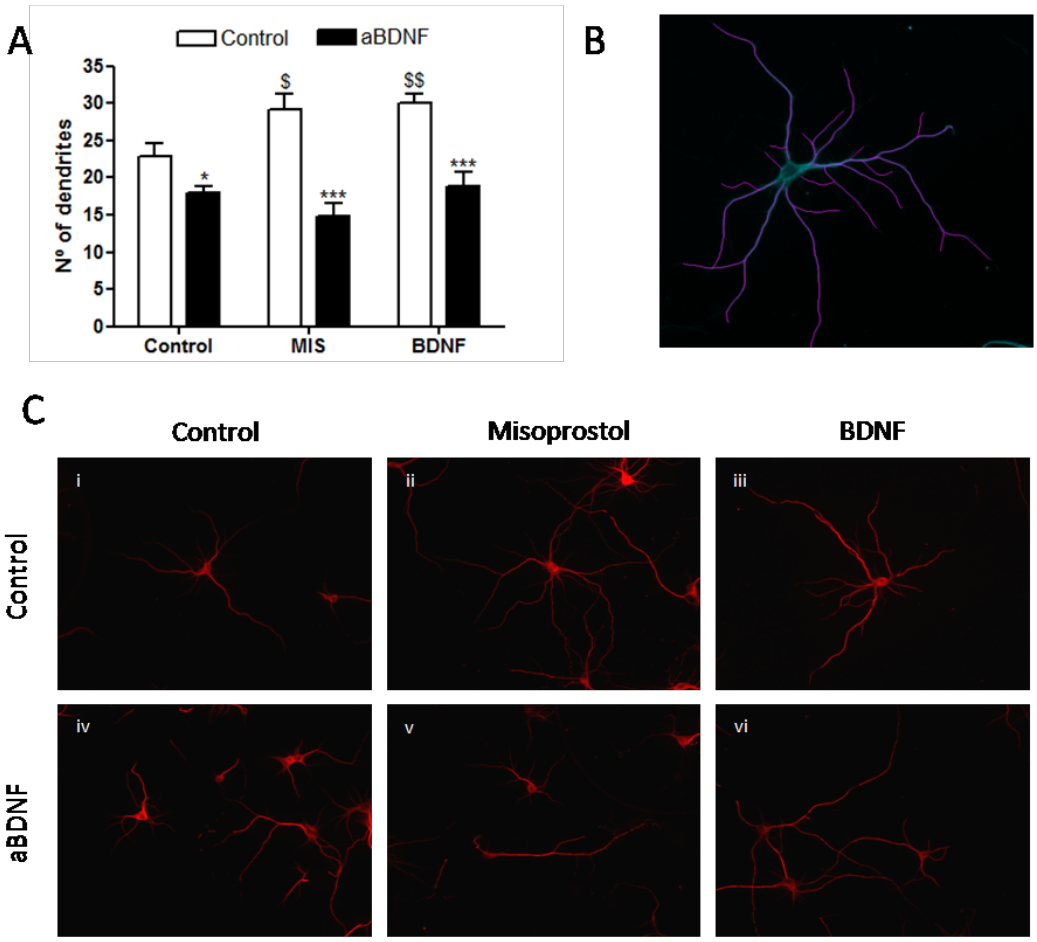
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Figure 2

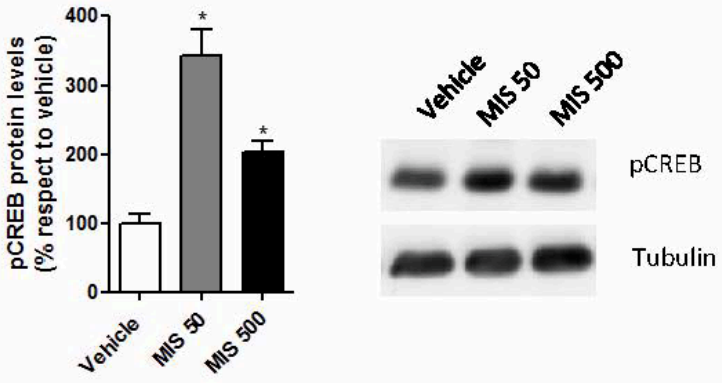
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Figure 3

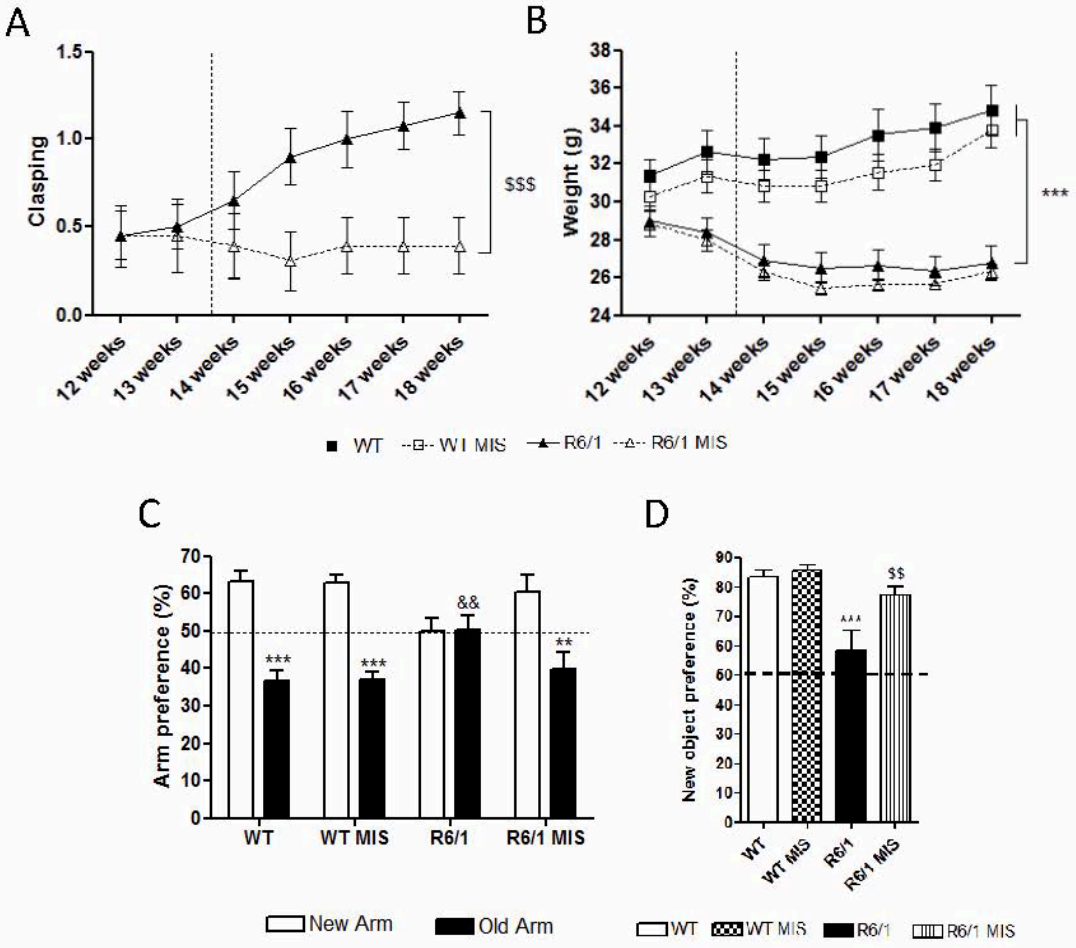
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Figure 4

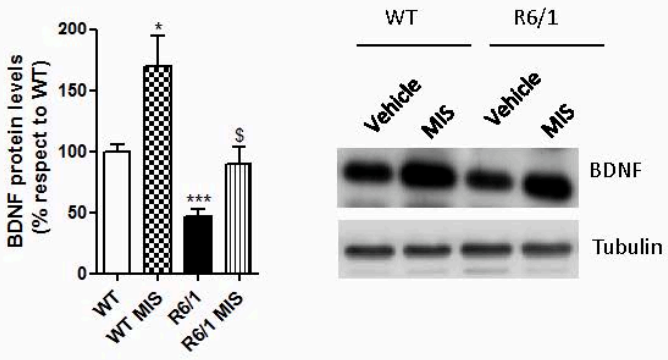
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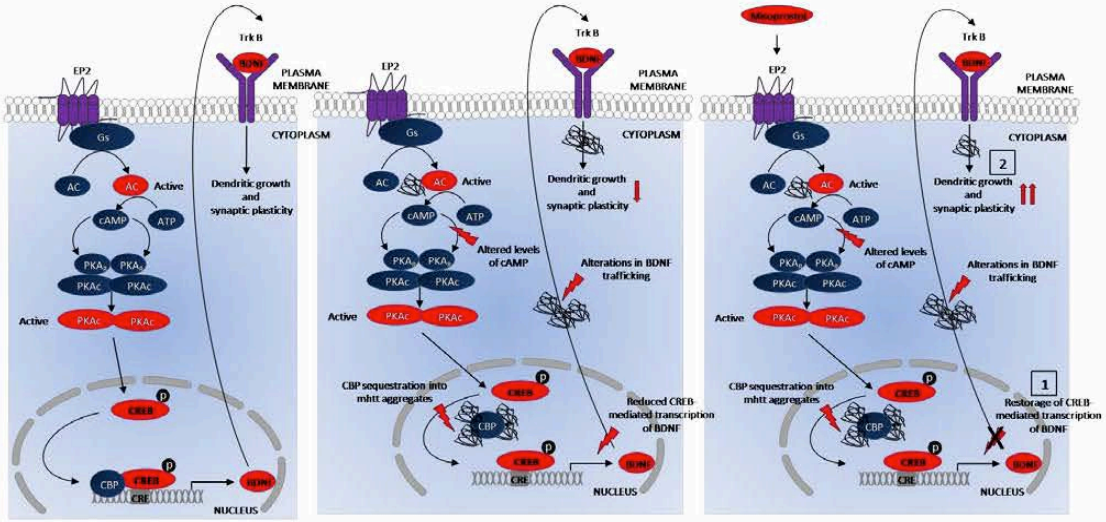
Figure 5



Control | Huntington's disease | EP2R activation in Huntington's disease

IV. DISCUSSION

The identification of molecular pathways altered due to mutant huntingtin expression is a priority to understand the cellular processes affected in HD pathology as well as for designing proper pharmacological treatments to palliate HD related symptoms (Figure 17). In this thesis, we have mainly focused on two principal aims: (1) the study of compensatory mechanism activated in the presence of mutant huntingtin in order to improve neuronal survival and (2) the identification of molecular targets to reduce motor and cognitive deficits as well as improve synaptic plasticity in mouse models of HD.

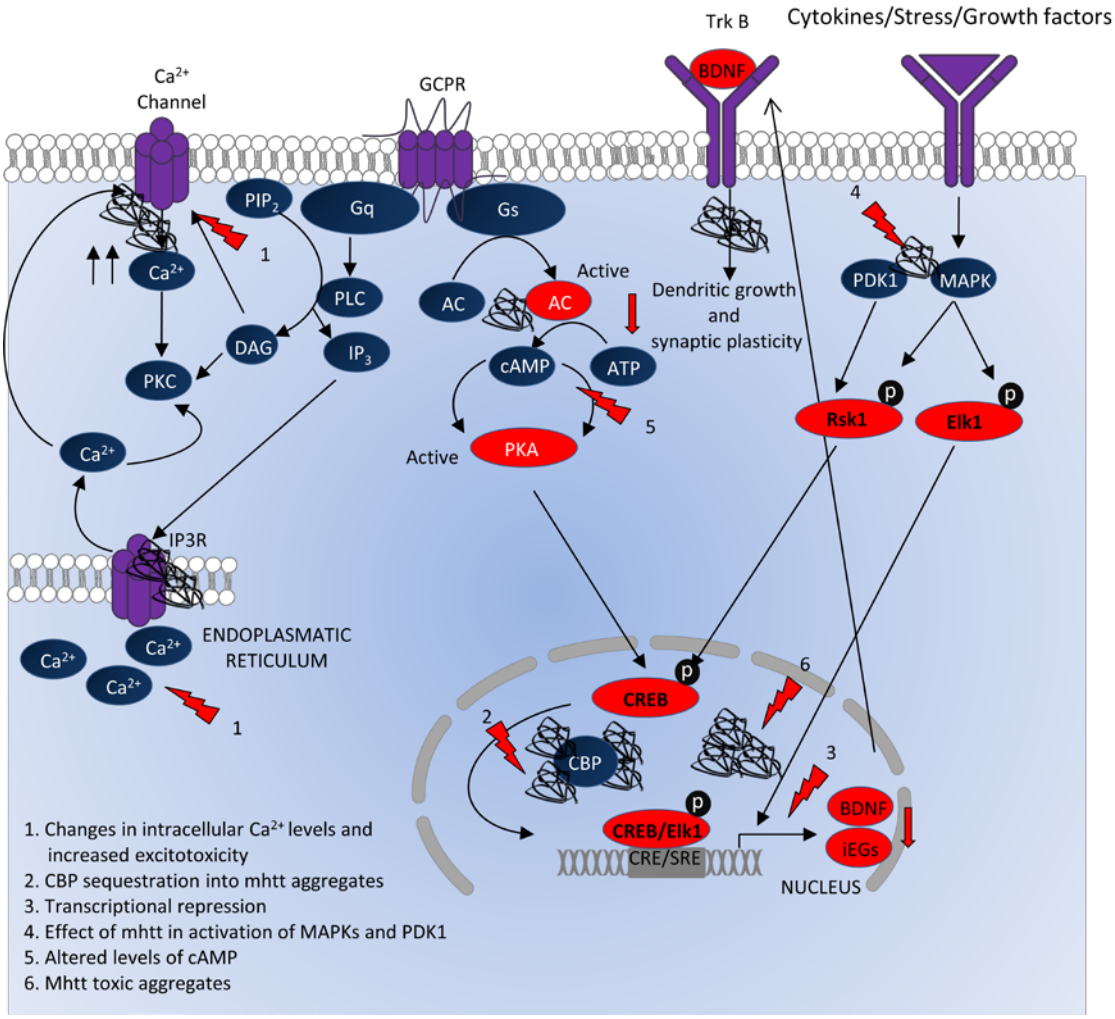


Figure 17. Altered mechanism in the presence of mutant huntingtin. The presence of mutant huntingtin (mhtt) engenders increased excitotoxicity and sensitize inositol (1,4,5)-triphosphote receptor type 1 (IP3R) promoting a massive increase in intracellular levels of Ca^{2+}. Mhtt also alters levels of cAMP, sequester proteins necessary for cAMP-response element binding protein (CREB)-mediated transcription, as CREB-binding protein (CBP), reducing the levels of brain-derived neurotrophic factor (BDNF), which finally results in a reduction of dendritic growth and synaptic plasticity. Moreover, the synthesis of other proteins, as immediate-early genes (iEGs), is also disrupted in the presence of mhtt. Finally, the activation or levels of several kinases are altered in the presence of mhtt, as 3'-phosphoinositide-dependent kinase-1 (PDK1) and mitogen-activated protein kinases (MAPKs).

Specifically, our results have helped to understand the role of the protein kinase Rsk and the transcription factor Elk-1 on the susceptibility of striatal neuronal death in the context of mutant huntingtin expression. Moreover, we show for the first time the therapeutic potential of PGE2 EP receptors for treating the clinical hallmarks of HD pathology.

1. NEUROPROTECTIVE COMPENSATORY MECHANISM ACTIVATED IN THE PRESENCE OF MUTANT HUNTINGTIN: The role of Rsk and Elk-1

It is known that the balance between cell death and cell survival is tightly regulated, also in pathological conditions. This balance is mostly regulated at the level of phosphorylation and transcription. Promoting the expression of specific genes is under the control of transcription factors, which are regulated by number of kinases. Then, these mechanisms could be altered in the presence of stress insults, such as mutant huntingtin, in order to swing the balance to cell death or cell survival. Several studies in HD models have shown the regulation of different proteins as compensatory mechanism activated in response of mutant huntingtin toxicity. This includes Akt (Gines et al., 2003; Humbert et al., 2002; Saavedra et al., 2010), ERK 1/2 (Gines et al., 2010; Saavedra et al., 2011) and some protein phosphatases as calcineurin (Xifro et al., 2008; Xifro et al., 2009) or striatal-enriched protein tyrosine phosphatase (Saavedra et al., 2011). In this thesis, we have first demonstrated two mechanisms activated in the presence of mutant huntingtin that play a role in this match trying to tip the balance to cell survival: the protein kinase Rsk and the transcription factor Elk-1. The over-activation of these two proteins has revealed as neuroprotective in the paradigm of mutant huntingtin-induced neuronal toxicity in different mouse and cellular models of HD pathology.

Both Rsk and Elk-1 are proteins related to transcription and survival. Rsk is broadly expressed in the brain where regulates cell survival (Anjum and Blenis, 2008) by inducing the inactivation of pro-apoptotic proteins (Ballif and Blenis, 2001), and the regulation of transcription directly phosphorylating pro-survival transcription factors such as CREB, SRF and c-fos (Figure 10) (Rivera et al., 1993; Xing et al., 1996). In the same context, Elk-1, together with CREB transcription factor, are considered the major transcriptional regulators in striatal projection neurons (Sgambato et al., 1998a; Vanhoutte et al., 1999). 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; Sharrocks, 2001).

Within Rsk family, we have analyzed Rsk1 and Rsk2, Rsk isoforms that are expressed in the brain, including the striatum, and whose expression levels are higher than to other Rsk isoforms (Anjum and Blenis, 2008). We observed increased levels of Rsk1, Rsk2 and Elk-1 in different *in vivo* and *in vitro* model of HD. Rsk 1 and 2 protein levels were increased in animals expressing a truncated form of mutant huntingtin (R6/1 mice) and a full-length form of mutant huntingtin (HdhQ111/Q111 mice). Increased levels of Elk-1 were also observed in R6/1 mice and further confirmed in R6/2 mice. Similarly, all proteins were elevated in a striatal cell line expressing a full-length form of mutant huntingtin (STHdhQ111/Q111). These results indicate that increased Rsk and Elk-1 protein levels are neither dependent on mutant huntingtin protein levels nor in the form of mutant huntingtin expressed, as these HD models express different levels and forms of mutant huntingtin. Thus, increased protein levels of Rsk 1, Rsk 2 and Elk-1 are the result of the presence of mutant huntingtin. This hypothesis was confirmed, in the case of Rsk, by showing that transfection of FL-75Q huntingtin in STHdhQ7/Q7 cells, M213 cells and striatal primary cultures elevated the levels of both Rsk isoforms. While the enhancement in Rsk protein levels is observed at more early stages of the disease (HdhQ111/Q111: 6 and 10 months; R6/1: 8 and 12 weeks), the levels of Elk-1 increase during the progression of the disease (R6/1: 30 weeks; R6/2: 8 and 12 weeks), suggesting that increased Rsk levels would be an early mechanism activated in the pathology.

The phosphorylation of Elk-1 at serine 383 residue is a key event for the transactivating properties of this transcription factor (Gille et al., 1995a), suggesting that incremented Elk-1 phosphorylation at this residue directly correlates with an increase in its activity. Here, in addition to raised Elk-1 protein levels, we observed an increase in Elk-1 phosphorylation at serine 383 in R6 models at the same ages where protein levels where elevated, and in the HD cellular model (Figure 18). Our results are in agreement with those reported by Roze et al. (2008), who showed an increase of pElk-1 Ser383-positive neurons in the dorsal striatum of R6/2 mice at 12 weeks of age. The phosphorylation status of Elk-1 is due to a balance between MAPKs-dependent phosphorylation (Shaw and Saxton, 2003) and calcineurin-dependent dephosphorylation (Sugimoto et al., 1994; Tian and Karin, 1999). Abnormal MAPK signaling and calcineurin activity has been reported in different models of HD. Specifically, in polyQ-truncated models (such as R6 mice), increased levels of phospho-ERK1/2, phospho-JNK and phospho-p38 (Apostol et al., 2006; Apostol et al., 2008; Gianfriddo et al., 2004; Lievens et al., 2002; Liu, 1998; Perrin et al., 2009; Saavedra et al., 2011), and decreased activation of calcineurin (Xifro et al., 2009) have been reported (Figure 18). These observations correlate with increased levels of phopho-Elk-1 observed in R6 mice in this thesis. Whereas

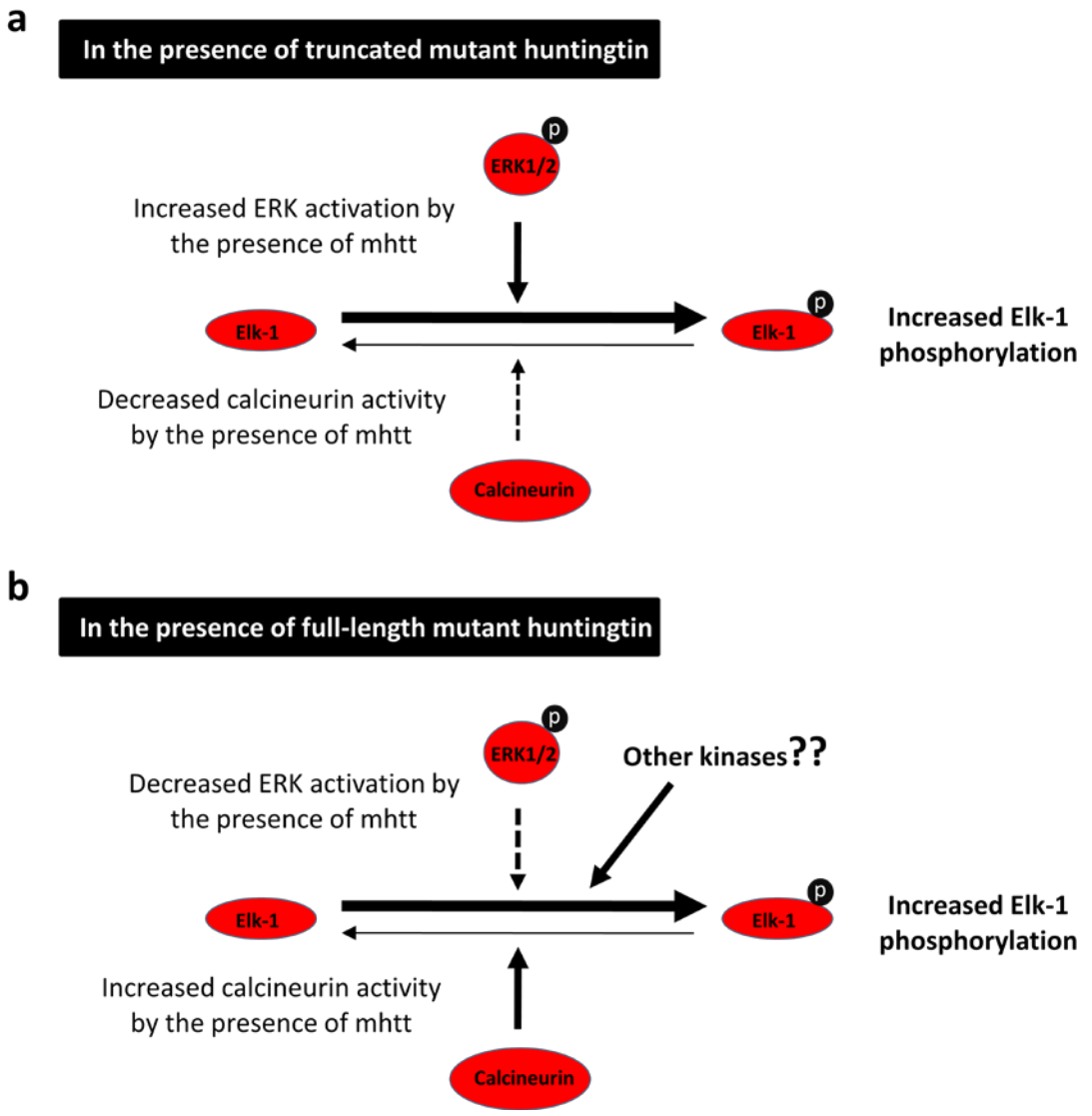


Figure 18. Putative mechanism of Elk-1 activation in the presence of different forms of mutant huntingtin. a) In the presence of a truncated form of mutant huntingtin (mhtt) there is an increase in phospho-ERK1/2 and phospho-Elk-1, and a decrease in calcineurin activity. b) In the presence of full-length mutant huntingtin there is a decrease inf ERK 1/2 activation, an increase in Elk-1 phosphorylation and calcineurin activity.

activation of ERKs, JNKs, and p38s can occur in response to mutant huntingtin toxicity, JNK- and p38-mediated pathways have generally been regarded as contributing to HD pathogenesis and ERK activation has been ascribed a compensatory, pro-survival role (Gokce et al., 2009; Lee et al., 2008; Scotter et al., 2010; Varma et al., 2007; Varma et al., 2010); suggesting that increased Elk-1 phosphorylation in exon 1 HD models may be due to phospho-ERK1/2 over-activation and calcineurin activity down-regulation. In the case of full-length mutant huntingtin models data are more controversial. Although some studies suggested increased activation of ERK1/2 in these models (Apostol et al., 2006; Ribeiro et al., 2010; Taylor et al., 2013),

decreased phospho-ERK1/2 has been observed in STHdhQ111/Q111 cells (Gines et al., 2010). Moreover, increased calcineurin activity has been reported in STHdhQ111/Q111 cells compared to STHdhQ7/Q7 cells (Figure 18) (Xifro et al., 2008). Instead of these data, we observed an increase of phospho-Elk-1 that correlated with increased neuronal survival. One possibility, not addressed by the present thesis, is to extent the regulation of Elk-1 to other MAPKs, for example, little is known about ERK 5 activation in models of neurodegenerative diseases.

In the case of Rsk1-2, although we observed that increased protein levels correlated with higher basal Rsk activity in STHdhQ111/Q111 cells than in cells expressing wild-type huntingtin, the regulation of Rsk phosphorylation is more complex. The study of Rsk phosphorylation at the Ser380, indirectly regulated by ERK1/2, and at the Ser221, regulated by PDK-1 (Anjum and Blenis, 2008; Frodin and Gammeltoft, 1999), revealed an increase in the phosphorylation of Rsk at the Ser221, but a decrease in the Ser380 both in knock-in mice and STHdhQ111/Q111 cells (Figure 19). This result, together with the finding that Rsk activity is increased in STHdhQ111/Q111 cells comparing to STHdhQ7/Q7 cells and that mutation of PDK-1-dependent phosphorylation site induces a stronger decrease of Rsk activity, support the idea that increased Rsk activity in striatal neurons expressing mutant huntingtin is mainly due to the action of PDK1. In accordance with our results, STHdhQ111/Q111 cells display elevated levels of phopho-PDK1 (Gines et al., 2003) and reduced levels of phospho-ERK1/2 (Gines et al., 2010) respect to STHdhQ7/Q7 cells (Figure 19) and it has been suggested that PDK1 has the capacity to activate Rsk in an ERK1/2-independent manner (Cohen et al., 2007). Furthermore, and supporting the important role of PDK1-mediated activation of Rsk, PDK1 deficiency results in Rsk inactivation (Williams et al., 2000). Our results show, contrary to what was usually thought that the phosphorylation of Rsk by ERK is not a limiting step for PDK1 activation of Rsk.

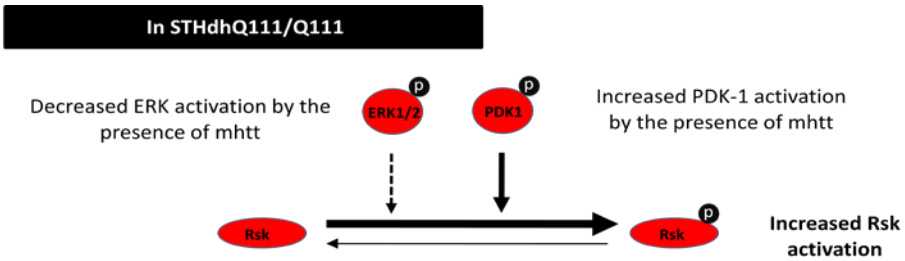
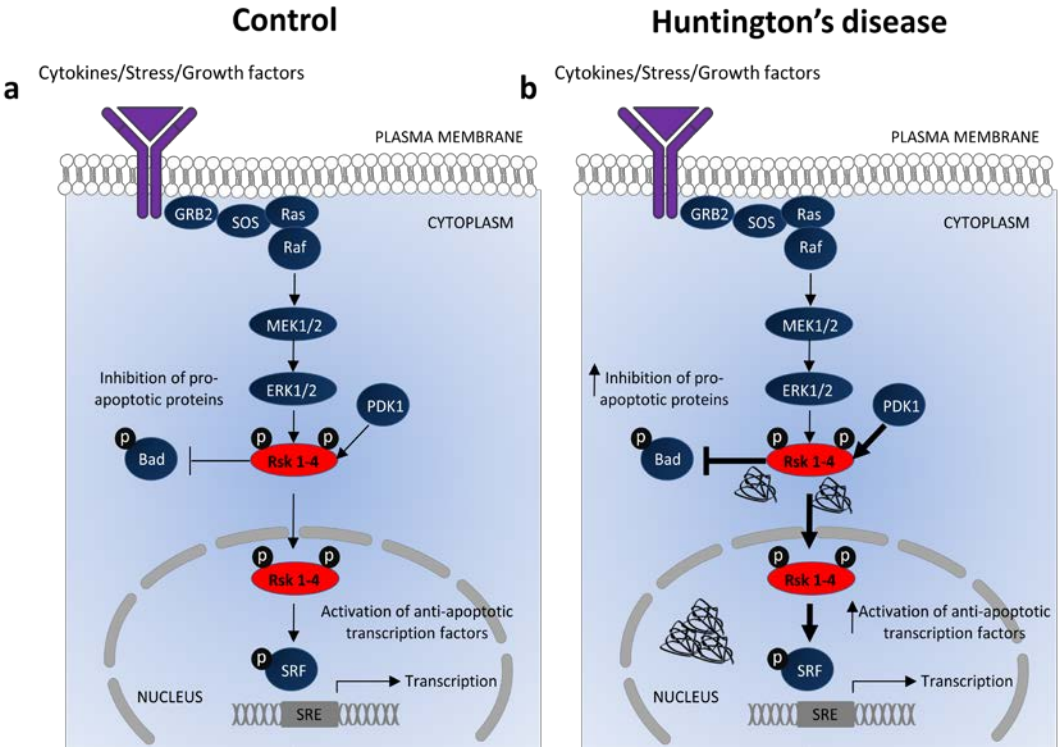


Figure 19. Putative mechanism of Rsk activation in the presence of mutant huntingtin. In STHdhQ111/Q111 cells, Rsk over-activation is mainly dependent on PDK1 activity. Mutant huntingtin, mhtt.

The localization of Elk-1 is very important for carrying out their function. As a transcription factor, Elk-1 needs to be in the nucleus to promote the transcription of target genes. Despite it is clear Elk-1 nuclear function, its activation in the brain has been linked with different functions, some derived from Elk-1-dependent transcriptional activity and others from recently suggested extranuclear functions of Elk-1. Studies from Eberwine and colleagues demonstrated that Elk-1 binds to the mitochondrial permeability transition pore complex, triggering apoptosis of cortical neurons (Barrett et al., 2006b). Although they observed that over-expression of Elk-1 in distal dendrites was toxic, when Elk-1 presence was restricted to cell body did not trigger neuronal death (Barrett et al., 2006a). Despite these studies linking Elk-1 with neuronal damage (Barrett et al., 2006a; Barrett et al., 2006b), we observed that the over-activation of this transcription factor in HD pathology was restricted to the nucleus in R6/1 mice and in STHdhQ111/Q111 cells, suggesting that increased expression and activation of Elk-1 in this context would not be toxic. Following non-transcriptional functions of Elk-1, another group found phospho-Elk-1 (Thr417) associated with several types of inclusions present in cases of human Lewy body Disease, Alzheimer's disease and HD (Sharma et al., 2010), and they suggested a pro-apoptotic function of Elk-1 once phosphorylated in this residue. They suggested that inclusions may sequester phospho-Elk-1 (Thr417) to prevent its transport to other subcellular regions and its association with other components of cell death pathway. This observation could be in some part contradictory since interaction between mutant huntingtin aggregates and transcription factors has been shown to reduce their transcriptional activity (Li and Li, 2004) and many studies have provide evidence that transcriptional dysregulation, in part due to this sequestration into mutant huntingtin aggregates, may be one of the major consequences that triggers to HD pathology (Cha, 2007; Dunah et al., 2002; Luthi-Carter et al., 2002; McCampbell et al., 2000; Steffan et al., 2000). Our results show that nuclear Elk-1 does not colocalize with mutant huntingtin inclusions in R6/1 mouse striatum. Discarding the association between Elk-1 and mutant huntingtin aggregates, we can suggest that increased nuclear Elk-1 has the capacity to be functional and could trigger to elevate Elk-1-dependent transcriptional machinery in striatal neurons expressing mutant huntingtin. Moreover, we observed unchanged Elk-1 protein levels in R6 mouse striatum at ages in which striatal cells present mutant huntingtin nuclear inclusions (DiFiglia et al., 1997) confirming that Elk-1 protein levels are not increased due to a sequestration in mutant huntingtin aggregates, and further discarding Elk-1 sequestration as a common mechanism in HD pathology.



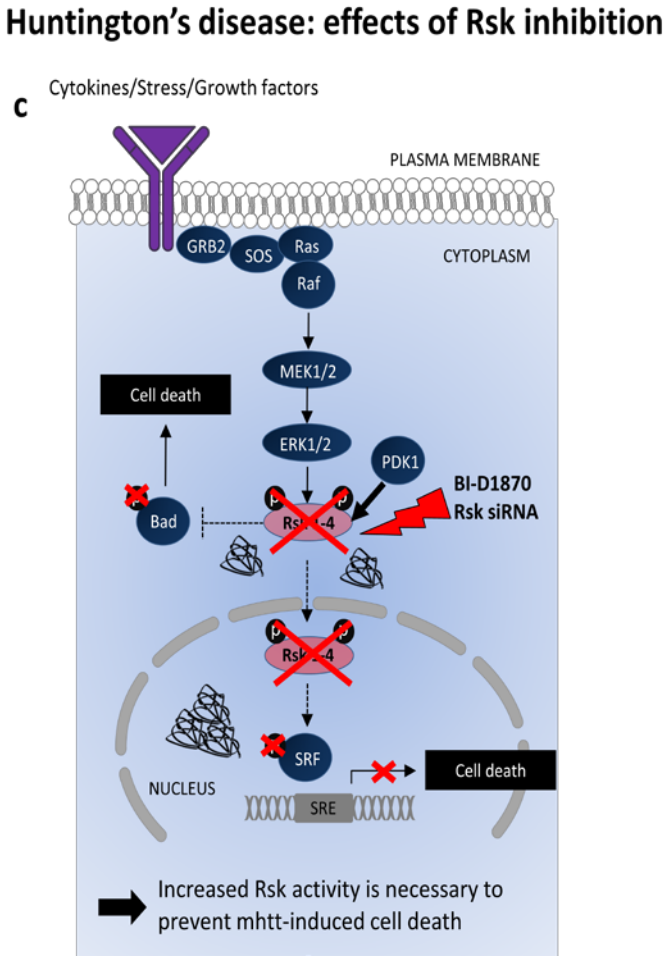


Figure 20. Schematic representation of beneficial effect of Rsk over-activation in against mutant huntingtin. a) In control conditions, once activated, Rsk inhibits pro-apoptotic proteins in the cytoplasm and activates pro-survival transcription factors in the nucleus. b) In the presence of mutant huntingtin (mhtt) increased Rsk activation promotes the increase in the phosphorylation of Bad (the inactive form of Bad) and SRF. c) Inhibition of Rsk promotes a decrease in phospho-Bad and phosphor-SRF protein levels, inducing cellular death due to mutant huntingtin expression.

The neuroprotective activity of Rsk is basically due to the wide range of proteins that it phosphorylates. Its function is important in both cytosolic and nuclear compartments, where it promotes the phosphorylation of a large number of substrates. In the cytoplasm Rsk induces the inactivation of certain pro-apoptotic proteins by phosphorylation, such as Bad, GSK-3β or DAPK (Anjum et al., 2005; Sutherland et al., 1993; Tan et al., 1999), while in the nucleus Rsk directly phosphorylate transcription factors that are involved in the synthesis of anti-apoptotic proteins, like SRF and CREB (De Cesare et al., 1999; Rivera et al., 1993). Supporting our hypothesis of the beneficial role of Rsk over-activation in HD, we observed increased levels of Rsk 1 and 2 in the cytoplasm of STHdhQ111/Q111 cells comparing with STHdhQ7/Q7 that correlates with an enhancement in the phosphorylation of the pro-apoptotic protein Bad, inhibiting its activity (Figure 20b). Even much higher was the increase of Rsk 1 and 2 observed in the nucleus of STHdhQ111/Q111 cells which leads to increase the phosphorylation of the transcription factor SRF (Figure 20b).Changes in phospho-Bad and phospho-SRF were due, at least in part, to Rsk activation, since inhibition of Rsk significantly reduces the phosphorylation of both proteins in STHdhQ111/Q111 cells (Figure 20c). In STHdhQ7/Q7 cells, the inhibition of Rsk produced a slight effect on SRF phosphorylation levels, and we did not detect an effect on Bad phosphorylation. Probably, this lack of effect on Bad phosphorylation is due to the predominant nuclear activity of Rsk in unstimulated cells (Anjum and Blenis, 2008; Chen et al., 1992) or by the fact that in control situation the lack of Rsk could be compensated by other proteins but under cellular stress, in this case the presence of mutant huntingtin, could not. Regarding these results, we propose that the neuroprotective effect of Rsk observed in the models of HD analyzed here could be mediated by the inactivation of pro-apoptotic factors in addition to the activation of transcription factors that regulate the expression of anti-apoptotic proteins.

Transcriptional regulation is essential in neuronal survival. In this context, Rsk exerts its neuroprotective role by activating transcription factors that regulate neuronal survival, while Elk-1 exerts its anti-apoptotic action (Sharrocks, 2001) directly mediating the expression of many iEGs, such as c-fos, egr-1, egr-2 and mcl-1 (Boros et al., 2009; Knapska and Kaczmarek, 2004; Lawson et al., 2010; Nijhawan et al., 2003; Wartiovaara et al., 2002). Here, we analyzed c-fos and egr-2 expression in the striatum of R6/1 mice and in the striatal cell line to assess the transcriptional activity of Elk-1 in the presence of mutant huntingtin. Although we expected increased levels of these two iEGs due to increased Elk-1 activation and nuclear localization, the mRNA levels of c-fos and egr-2 were decreased in the striatum of R6/1 mice at 30 weeks of age (Figure 21b). Moreover, STHdhQ111/Q111 cells presented decreased egr-2 mRNA levels

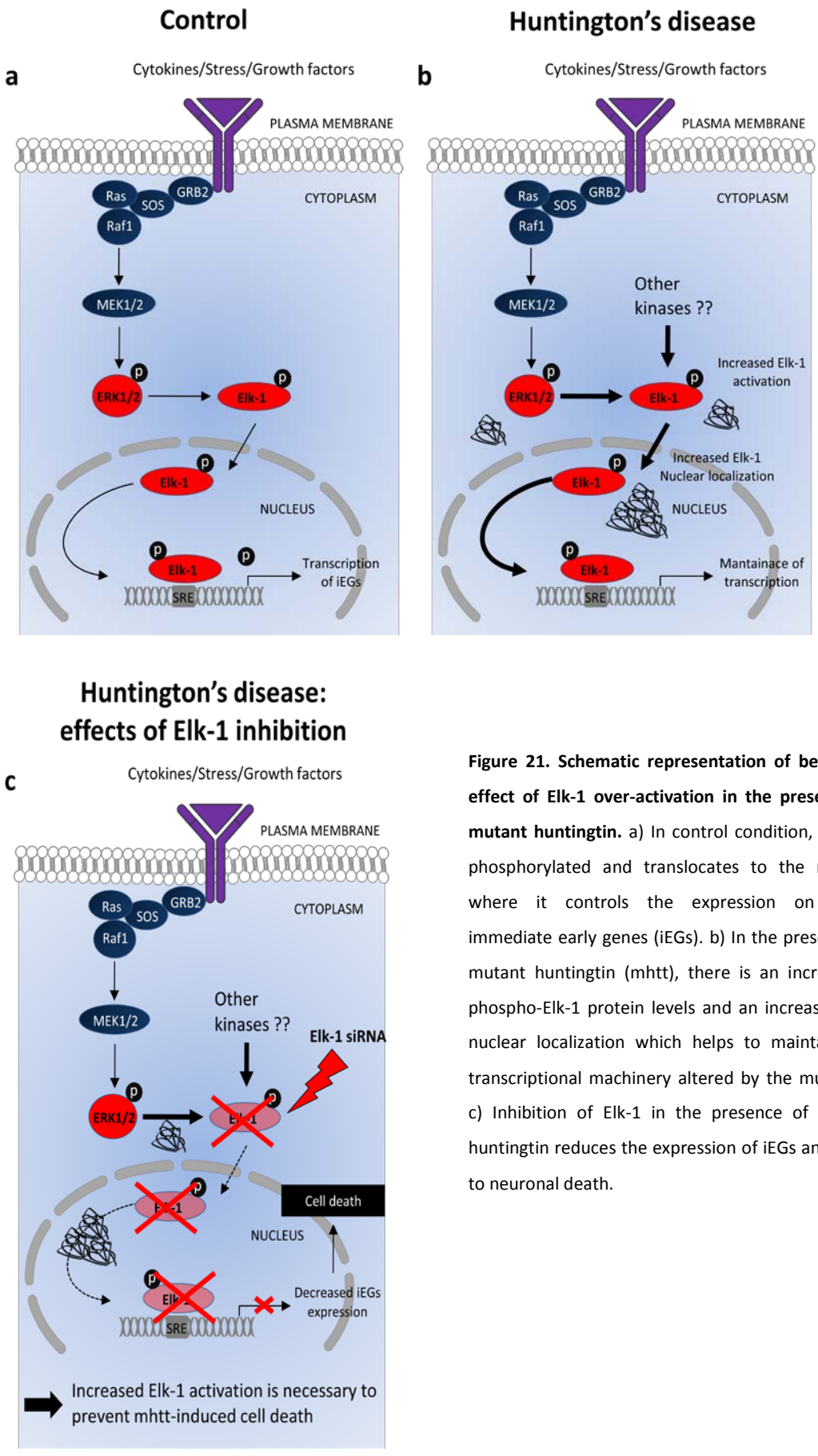


Figure 21. Schematic representation of beneficial effect of Elk-1 over-activation in the presence of mutant huntingtin. a) In control condition, Elk-1 is phosphorylated and translocates to the nucleus where it controls the expression on many immediate early genes (iEGs). b) In the presence of mutant huntingtin (mhtt), there is an increase in phospho-Elk-1 protein levels and an increase in its nuclear localization which helps to maintain the transcriptional machinery altered by the mutation. c) Inhibition of Elk-1 in the presence of mutant huntingtin reduces the expression of iEGs and leads to neuronal death.

and no variations of c-fos mRNA levels when compared with STHdhQ7/Q7 cells. Although expressions of these genes do not correlate with the increased levels of Elk-1, our data are in agreement with previous studies showing a reduction of some iEGs in the striatum of R6/2 mice at late stages of the disease (Luthi-Carter et al., 2000; Roze et al., 2008; Spektor et al., 2002). Moreover, abnormalities in transcriptional activity in HD have been reported by others (Arzberger et al., 1997; Cha, 2000; Cha, 2007; Luthi-Carter et al., 2002), which include changes in mRNA levels, direct interaction between mutant huntingtin and proteins of the transcriptional machinery, and inhibition of enzymes involved in chromatin remodeling (Figure 17). These mechanisms could mask, in part, the effect of Elk-1 in transcriptional activity, that is why we hypothesized that decreased levels of iEGs detected in R6/1 mice striatum and in STHdhQ111/Q111 cells are not due to a disruption of Elk-1 pathway. Reinforcing our hypothesis, the inhibition of Elk-1 in STHdhQ111/Q111, but not in STHdhQ7/Q7 cells, resulted in decreased of c-fos and egr-2 mRNA levels (Figure 21c). In addition, the transcriptional activity of Elk-1 is SRF-dependent (Gille et al., 1995b; Gille et al., 1995a), and we have observed analyzing Rsk substrates that phospho-SRF protein levels are increased in STHdhQ111/Q111 compared to STHdhQ7/Q7 (Figure 20b). This observation, together with the lack of interaction between Elk-1 and mutant huntingtin aggregates and the preferential nuclear localization of the transcription factor, further enhance the idea that nuclear Elk-1 is functional and can increase transcription in HD. Altogether, our findings indicate that increased Elk-1 levels in striatal cells expressing mutant huntingtin are capable of maintaining the expression of some Elk-1-dependent iEGs because in its absence iEGs mRNA levels are more down-regulated (Figure 21). This is a very interesting mechanism, as increasing the levels of Elk-1 in response to mutant huntingtin neurons can restore in part the levels of iEGs. Even though we did not check the effect of Rsk inhibition on the gene expression, it would be likely that inhibiting Rsk we could observed a similar effect to Elk-1 in the gene expression as Rsk regulates the phosphorylation of CREB (Xing et al., 1996), and CREB is an essential transcription factor in medium spiny neurons. Moreover, we risk hypothesizing that part of the cell death observed after Rsk inhibition in the presence of mutant huntingtin could be due to a reduction in CREB-mediated gene expression. Curiously, in the same way than results observed in Rsk substrates, when we inhibited Elk-1 in STHdhQ7/Q7 no changes in the c-fos and egr-2 mRNA levels were observed. This lack of effect could be explained by the overlap with other ETS transcription factors indicating that cellular environment, in the presence of mutant huntingtin, has a key role in dictating the functional specificity of Elk-1 as has been previously described (Boros et al., 2009; Shore and Sharrocks, 1995). Similarly, mice deficient for the *elk-1* gene present a mild alteration in their phenotype when compared to wt littermate (Cesari et al., 2004), but a

decrease in c-fos and egr-1 expression was observed in the hippocampus and cortex after kainate-induced seizure, suggesting different relevance of Elk-1 depending on the context, such as in case of the presence of mutant huntingtin. In accordance with our hypothesis, Nordheim and colleagues (Cesari et al., 2004) suggested that this mild phenotype could be attributed to a functional redundancy between Elk-1 and other TCF members.

Here, we show that Rsk 1-2 pharmacological inactivation and Elk-1 and Rsk inhibition by siRNA transfection induces cell death in striatal cells expressing mutant huntingtin, although both isoforms of Rsk (Rsk1 and Rsk2) had to be inhibited. These results show for the first time a protective role of Rsk and Elk-1 in HD (Figure 20c and 21c). Confirming the protective role of Rsk against mutant huntingtin, when we over-expressed Rsk in STHdhQ7/Q7 cells transfected with FL-75Q huntingtin, cell death was considerably reduced. In both cases, inhibition of Rsk and Elk-1 did not affect the viability of cells expressing wild-type huntingtin. Importantly, this lack of effect was similar as the previously observed when phospho-Bad levels were checked in the presence of Rsk pharmacological inhibitor or when levels of c-fos and egr-2 were analyzed after Elk-1 inhibition in STHdhQ7/Q7 cells, suggesting different importance or relevance of these proteins in physiological conditions or with mutant huntingtin expression. Altogether, we show that the stress environment induced by the presence of mutant huntingtin promotes efficient pro-survival compensatory mechanisms, increasing Elk-1 and Rsk protein levels, activation and nuclear localization (mainly in the case of Elk-1). In agreement, the activation of Elk-1 after toxic stimuli has been revealed as neuroprotective effects (Ferrer et al., 2001), and Elk-1 inhibition causes apoptosis in neuronal cultures stimulated with NGF (Demir et al., 2011). In addition to these observations, the capacity of Elk-1 to regulate apoptosis is higher than other ETS transcription factors (Boros et al., 2009). Similarly, studies in *Rsk2-/-* mice showed that Rsk2 induces c-fos transcription by activating both Elk-1/SRF complex and CREB (Bruning et al., 2000; Doehn et al., 2009) and participates in the post-translational modification of many iEGs products (Chen et al., 1993; Davis et al., 1993; Fisher and Blenis, 1996; Rivera et al., 1993; Wingate et al., 2006) tightly link to neuronal survival.

Neurons, either as individual cells or as a part of an integrated nervous system, continually attempt to compensate for disruptive and damaging influences and maintain a dynamic equilibrium. In mouse models, different compensatory mechanisms are induced in response to mutant huntingtin expression in order to prevent neuronal cell death. These compensatory mechanisms are considered interesting targets to design neuroprotective therapies in HD. Early activated compensatory mechanism, as the case of Rsk, which expression is increased in HdhQ111/Q111 mice at 6 and 10 month of age, and in R6/1 mice at 8 and 12 weeks of age,

when they do not present motor dysfunction (Brooks et al., 2012; Fossale et al., 2002; Menalled, 2005; Wheeler et al., 2000), could be a useful target to inhibit the progression of neurodegeneration. Other compensatory mechanisms are activated more lately during the progression of the disease, as in the case of Elk-1, which increases at 30 weeks in R6/1 mice and at 8 and 12 weeks in R6/2 mice, suggesting that perhaps an earlier stimulation of the activity of these proteins could result in a delay or a reduction of HD pathology. Different studies in HD models have shown the regulation of other proteins as compensatory mechanism activated in response of mutant huntingtin toxicity as exposed in the first part of this discussion (Gines et al., 2003; Gines et al., 2010; Humbert et al., 2002; Saavedra et al., 2010; Saavedra et al., 2011). Here, we show that in addition to as ERK1/2 and Akt, Elk-1 and Rsk are also up-regulated in the presence of mutant huntingtin, and more importantly, their activity is neuroprotective against mutant huntingtin induced cell death, suggesting them as an efficient compensatory mechanism occurring in HD striatum. Therefore, we propose the regulation of Elk-1 and Rsk pathways as a therapeutic tool to prevent neurodegeneration that occurs in HD (Figure 22). In accordance with our proposal, a neuroprotective role of Elk-1 has been recently suggested for other neurodegenerative disease (Besnard et al., 2011) and also a role of Rsk in brain disease is clearly observed in the Coffin-Lowry Syndrome (Trivier et al., 1996), where the lack of Rsk2 causes an X-linked mental retardation syndrome.

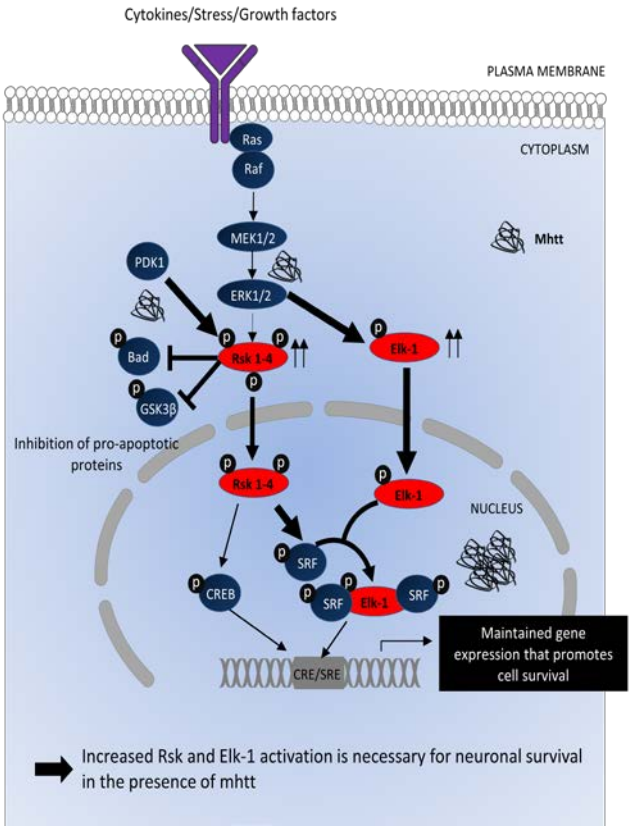


Figure 22. Activation of pro-survival compensatory mechanism in HD. In the presence of mutant huntingtin (mhtt) there is an over-activation of protein 90 kDa ribosomal S6 kinases (Rsk) and the transcription factor Elk-1. The increased Rsk phosphorylation is mainly due to phosphoinositide-dependent kinase-1 (PDK1). While Elk-1 increases only in the nuclear compartment, increases of Rsk are observed in both nuclear and cytosol compartments, where it promotes the inhibition of pro-apoptotic proteins by phosphorylation and activates proteins involved in the transcriptional machinery. In the nucleus, both Elk-1 and Rsk contribute in the maintenance of transcription impaired by the presence of mhtt. CRE, cAMP response element; SRE, serum response element; CREB, cAMP response element binding protein; SRF, serum response factor.

We provide evidences of an increase of Elk-1, Rsk 1 and Rsk 2 protein and phosphorylation levels in the striatum of different transgenic mouse models, as well as in striatal knock-in cells, with a preferential nuclear localization. Interestingly, increased levels of Rsk and Elk-1 in the presence of mutant huntingtin contributes to enhance their activity, either by increasing the phosphorylation of its substrates (Rsk) or helping to maintain its activity transcripcional activity (Elk-1). Moreover, increased activity of these two proteins observed in cells expressing mutant huntingtin contributes to prevent mutant huntingtin-induced cell death. Taken together, these findings support a pro-survival function for Rsk and Elk-1 in the presence of mutant huntingtin, and we propose that therapies targeted to maintain their activity would be a good approach for neuroprotection in HD (Figure 22).

2. PROSTAGLANDIN RECEPTORS: NEW THERAPEUTIC STRATEGIES TO REDUCE MOTOR AND COGNITIVE DEFICITS IN HUNTINGTON'S DISEASE.

Another therapeutic approach could be the modulation of the PG system. PG signaling plays a central role in physiologic and pathophysiologic responses in almost all organs, including brain. Emerging data indicate that PGs, and more specifically PGE2, play a central role in brain diseases including ischemic injury and several neurodegenerative diseases (Andreasson et al., 2010). Attention is now focused on modulating PGE2 signaling that is mediated by interaction with four distinct G protein-coupled receptors, EP1-4 (Breyer et al., 2001), which are differentially expressed in neuronal and glial cells throughout the central nervous system. The complex dual neuroprotective and neurotoxic role of PGE2 receptor activation in human neurological diseases leaves many pathogenic issues to be resolved. In this thesis, we have demonstrated the utility and promise of therapeutically targeting the PGE2 signaling pathway, specifically EP1 and EP2 receptors, in the case of the neurodegenerative process of HD. Here, we observed improvements in the behavior and reduction of the clinical hallmarks of HD after chronically treating R6/1 mice with and EP1 receptor antagonist or and EP2 receptor agonist.

In the present work, we provide the first evidence that chronic pharmacological blockade of EP1 with SC-51089 and activation of EP2 with misoprostol reduces clasping reflex. Moreover, the treatment with EP1 receptor antagonist improves striatal-dependent motor coordination in R6/1 mice evaluated by the rotarod, the balance beam and the vertical pole tests. We also observed that both EP1 antagonist and EP2 receptor agonist recovery LTM deficits in the T-SAT and the NORT. Accordingly, we observed an improvement in hippocampal synaptic plasticity in SC-51089- treated R6/1 mice, and an increased in dendritic complexity in hippocampal primary neurons treated with misoprostol. Additionally, chronic treatment with SC-51089 promotes an

increase of specific synaptic protein deficits, and misoprostol treatment recovery deficits in the expression of the trophic factor BDNF. Finally, there was a decrease in mutant huntingtin nuclear aggregation in the striatum and hippocampus of R6/1 mice after SC-51089 treatment.

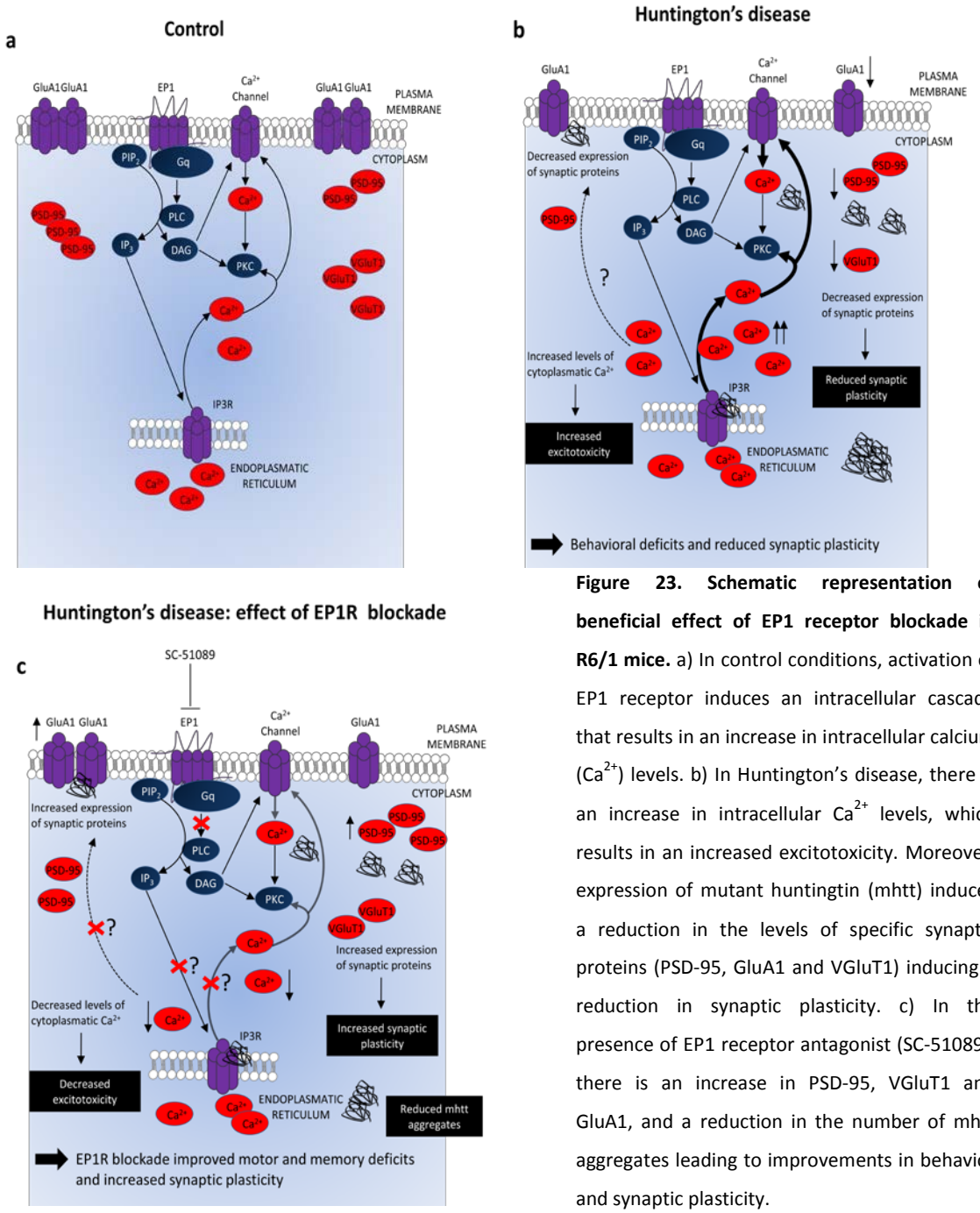


Figure 23. Schematic representation of beneficial effect of EP1 receptor blockade in R6/1 mice. a) In control conditions, activation of EP1 receptor induces an intracellular cascade that results in an increase in intracellular calcium (Ca^{2+}) levels. b) In Huntington's disease, there is an increase in intracellular Ca^{2+} levels, which results in an increased excitotoxicity. Moreover, expression of mutant huntingtin (mhtt) induces a reduction in the levels of specific synaptic proteins (PSD-95, GluA1 and VGluT1) inducing a reduction in synaptic plasticity. c) In the presence of EP1 receptor antagonist (SC-51089), there is an increase in PSD-95, VGluT1 and GluA1, and a reduction in the number of mhtt aggregates leading to improvements in behavior and synaptic plasticity.

These observations further support the dual role of PGE2 prostaglandin receptors, which activated different intracellular pathways that triggers even to opposite effects, as we can see a beneficial effect in HD antagonizing EP1 and agonizing EP2.

Previous studies have demonstrated that acute treatment with an EP1 receptor antagonist protects against over-activation of NMDA receptor (Ahmad et al., 2006a; Kawano et al., 2006). Here we demonstrated that administration of EP1 receptor antagonist by osmotic mini-pump system protects striatal cells in an excitotoxic model of HD (Figure 23b). Moreover, in agreement with our results, EP1 can mediate not only toxic effects in models of neuronal damage, such as ischemic and excitotoxic insults (Ahmad et al., 2006a; Kawano et al., 2006), but also in models of neurodegeneration (Carrasco et al., 2007; Li et al., 2013; Zhen et al., 2012). Interestingly, the EP1 activation results in increased phosphatidyl inositol hydrolysis and elevation of the intracellular Ca^{2+} concentration (Breyer et al., 2001). Neuronal Ca^{2+} signaling regulates multiple neuronal functions, including synaptic transmission, plasticity and cell survival (Berridge et al., 1998; Wojda et al., 2008). Therefore, an imbalance in this calcium homeostasis, as happens in different transgenic HD mice (Hansson et al., 2001; Hodgson et al., 1999), can disturb neuronal signaling producing cellular dysfunction (Hansson et al., 2001) and could result in behavioral deficits found in these animals (Figure 23b). For this reason, different Ca^{2+} signaling stabilizers have recently been used as potential therapeutics for the treatment of HD (Chen et al., 2011; Tang et al., 2005). *In vitro* and *in vivo* studies demonstrated that pharmacologic antagonism/deletion of EP1 resulted in a normalization of intracellular Ca^{2+} concentration (Kawano et al., 2006; Zhang et al., 2007) (Figure 23). We found that blockade of EP1 significantly improved R6/1 phenotype (Figure 23), reinforcing the hypothesis that disturbed intracellular Ca^{2+} signaling plays a significant role in the pathogenesis of HD and offering a potential and clinically relevant, drug for the treatment of this and other diseases where excitotoxic and neuronal dysfunction processes are related to calcium increases.

Controversially, EP2 receptor activation demonstrates a neuroprotective effect during excitotoxic conditions (Ahmad et al., 2006b; Ahmad et al., 2010; Liu et al., 2005; McCullough et al., 2004), and in additional models of neurodegeneration (Bilak et al., 2004; Carrasco et al., 2008; Echeverria et al., 2005). Although the modulation of both receptors appears to be beneficial in R6/1 mouse model of HD, the mechanisms that trigger to this positive effect are completely different. The protective effect of EP2 receptor stimulation is promoted by the activation of cAMP/PKA/CREB pathway (Figure 24) (Regan, 2003). In this context, we observed an increased CREB phosphorylation after treating mice during 1h with EP2 receptor agonist (Figure 24). With these results, we checked that drugs and doses chosen for the experiments can reach the brain and have the desired effect. Increasing studies showed that CREB-mediated gene expression is both necessary and sufficient for survival of multiple neuronal subtypes (Bonni et al., 1999; Riccio et al., 1999; Walton et al., 1999). This finding, in

conjunction with the observation that the loss of CREB imparts a BAX-dependent form of apoptosis (Lonze and Ginty, 2002) and that CREB phosphorylation under toxic conditions has revealed as neuroprotective (Hu et al., 1999; Mabuchi et al., 2001; Tanaka et al., 1999; Walton et al., 1999), suggested a model in which the activation of CREB shift the intracellular balance

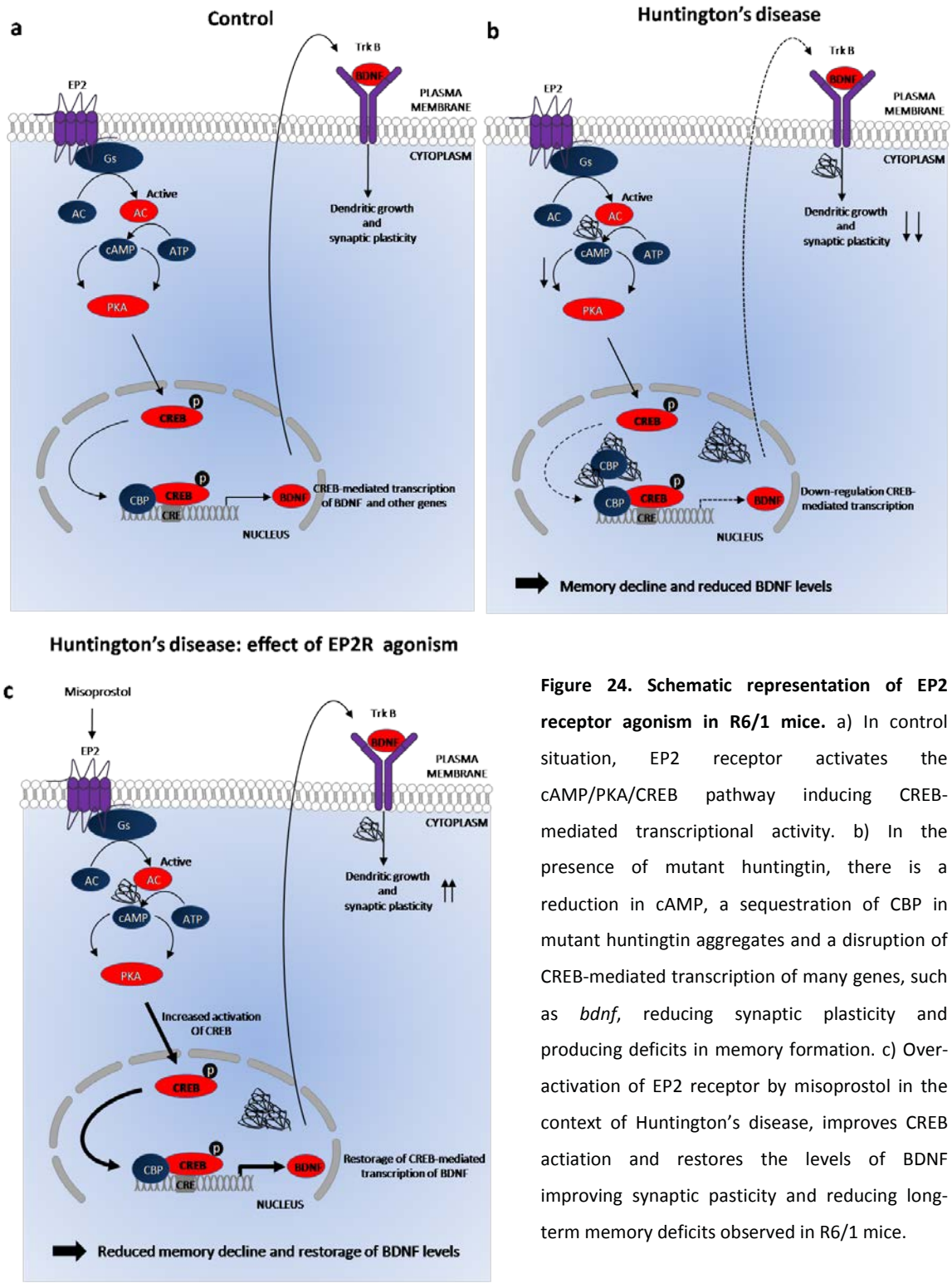


Figure 24. Schematic representation of EP2 receptor agonism in R6/1 mice. a) In control situation, EP2 receptor activates the cAMP/PKA/CREB pathway inducing CREB-mediated transcriptional activity. b) In the presence of mutant huntingtin, there is a reduction in cAMP, a sequestration of CBP in mutant huntingtin aggregates and a disruption of CREB-mediated transcription of many genes, such as *bdnf*, reducing synaptic plasticity and producing deficits in memory formation. c) Over-activation of EP2 receptor by misoprostol in the context of Huntington's disease, improves CREB actiation and restores the levels of BDNF improving synaptic pasticity and reducing long-term memory deficits observed in R6/1 mice.

between survival- and death-promoting factors in favor of those that support survival (Lonze and Ginty, 2002). CREB is central not only to cell survival but also to many other physiological processes, so it is not surprising that the consequences of disruption CREB function in vivo are quite severe. Complete disruption of CREB is lethal in mice (Rudolph et al., 1998). Incomplete or subtly disrupted CREB function can cause genetic disorders, for example, mutation of Rsk-2, the putative CREB kinase, produces the Coffin-Lowry syndrome as we have previously explained, which is a complex disorder characterized by multiple physical abnormalities and mental retardation (Trivier et al., 1996). In addition, CBP mutation produces Rubenstein-Taybi Syndrome, which is similarly characterized by multiple deficits including mental retardation (Petrij et al., 1995). Similarly, CREB disruption has been implicated as a central factor to the pathophysiology of HD (Figure 24) (Dawson and Ginty, 2002). CREB function is impaired by mutant huntingtin (Steffan et al., 2000; Sugars and Rubinsztein, 2003; Sugars et al., 2004), suggesting that inhibition of CREB-mediated gene transcription contributes to HD (Jiang et al., 2003; Kazantsev et al., 1999; Mantamadiotis et al., 2002; Nucifora, Jr. et al., 2001; Steffan et al., 2000). In agreement with this, cAMP levels are reduced in the cerebrospinal fluid of patients with HD (Cramer et al., 1984) and CREB signaling is compromised in different mouse and cellular models of HD and in human samples, where the expression of mutant huntingtin induces aggregation of the CBP (Nucifora, Jr. et al., 2001; Steffan et al., 2000; Steffan et al., 2001), reduces the levels of cAMP (Gines et al., 2003) and down-regulates CRE-mediated transcription of numerous genes (Augood et al., 1997; Luthi-Carter et al., 2002; Zuccato et al., 2010) (Figure 24). Recently, it has been suggested that increased CREB phosphorylation exerts neuroprotective and beneficial effects in both excitotoxic and genetic mouse models of HD (DeMarch et al., 2007; DeMarch et al., 2008; Giampa et al., 2009). Our results further confirm the beneficial effect of CREB activation in HD, suggesting EP2 receptor as a novel target to induce CREB activation. We show that misoprostol treatment reduces hippocampal-dependent deficits in R6/1 mice, reinforcing the idea of the beneficial role of CREB activation in HD and the suggested role of EP2 receptor in memory formation (Figure 24) (Shaw et al., 2003).

Changes in synaptic composition and lack of trophic support may also contribute to behavioral alterations in HD models (Canals et al., 2004; Giralt et al., 2009; Luthi-Carter et al., 2003; Lynch et al., 2007; Nithianantharajah et al., 2008; Zuccato and Cattaneo, 2007; Zuccato et al., 2008). Down-regulation of proteins involved in the synaptic machinery, such as PSD-95, GluA1 and VGluT1, has been previously reported in human HD brain, and in the striatum and hippocampus of HD mouse models (Luthi-Carter et al., 2003; Nithianantharajah et al., 2008; Torres-Peraza et al., 2008). Importantly, the down-regulation of VGluT1 in the striatum and

PSD-95 and GluA1 in the hippocampus was partially reverted in SC-51089-treated R6/1 mice (Figure 19). Increased expression of these synaptic markers in R6/1 mice after EP1 blockade correlates with behavioral improvements observed in these animals, as previously suggested (Giralt et al., 2011a; Jarabek et al., 2004; Nithianantharajah et al., 2008; Torres-Peraza et al., 2008). Specifically, altered levels of PSD-95, GluA1 and VGluT1 are likely to induce alterations in synaptic signaling and plasticity, which in turn leads to learning impairment, consistent with that observed in HD mice and suggesting the down-regulation of these proteins as a molecular mechanism implicated in HD pathogenesis (Figure 23) (Giralt et al., 2011a; Jarabek et al., 2004; Nithianantharajah et al., 2008; Torres-Peraza et al., 2008). In this context, increased expression of VGluT1 in the striatum of SC-51089-treated mice suggests that cortico-striatal excitatory terminals are more preserved, which correlates with an improvement in the performance of all the motor tasks analyzed; while amelioration of GluA1 and PSD-95 protein deficits in the hippocampus after EP1 blockade could be related to the rescue of LTM deficits observed in R6/1 mice (Figure 23). Checking neurotransmission at hippocampal synapses, we observed that LTP was strongly reduced in R6/1 mice. Importantly, hippocampal synaptic plasticity in SC-51089-treated R6/1 mice was significantly improved, consistent with the hippocampal-related cognitive improvements and the recovery of synaptic markers seen after SC-51089 treatment (Figure 23). Observing these results, we have to note that the down-regulation in PSD-95 was partially reverted in the hippocampus, but not in the striatum, of SC-51089-treated R6/1 mice. Moreover, we observed a clear recovery from hippocampal-related cognitive deficits, while the amelioration of striatal-related motor dysfunction in SC-51089-treated R6/1 mice was slighter, suggesting a greater effect of SC-51089 in the hippocampus than the striatum. In agreement, we show that EP1 receptor is much more expressed in the hippocampus than in the striatum.

Although deficits in synaptic plasticity could be related to the expression of synaptic proteins, other mechanisms, as trophic factors, also play a pivotal role in this phenomenon. BDNF has emerged as a major regulator of synaptic plasticity, neuronal survival and differentiation, and also as a key molecular target for drug development in neurological disorders (Binder and Scharfman, 2004). Down-regulation of BDNF in HD (Zuccato and Cattaneo, 2007; Zuccato et al., 2008) seems to regulate the onset and severity of symptoms, and synaptic plasticity deficits in HD mouse models (Canals et al., 2004; Giralt et al., 2009; Lynch et al., 2007). The neurotrophin BDNF has been widely put forward as a possible therapeutic molecule for HD treatment (Alberch et al., 2004; Zuccato and Cattaneo, 2009). However, the key challenge in the field of growth factor therapy is drug delivery to the central nervous system, requiring efficacious and

safe strategies and appropriate amount of BDNF delivery. Another important consideration in the design of growth factor therapies for neurodegenerative disorders is the need for extended periods of treatment (Nagahara and Tuszynski, 2011). To date, there are no successful systems for delivering BDNF, because it does not cross the blood-brain barrier via peripheral administration (Zhang and Pardridge, 2001; Zhang and Pardridge, 2006). On the other hand, approaches such as infection/lipotransfection and cell therapy are not accessible yet, because of their collateral toxicity effects and the likelihood of their inducing aberrant proliferations, respectively. In addition, they are invasive procedures (Lindvall et al., 2004). In this thesis, we proposed a new approach to increase the levels of BDNF. EP2 receptor chronic stimulation in R6/1 mice significantly increases the levels of BDNF (Figure 24), which is the most likely explanation for the hippocampal-dependent cognitive improvements observed in misoprostol-treated in these mice. Our results are in agreement with previous studies showing that treatments focused on the recovery of BDNF levels successfully improve synaptic plasticity, and motor and cognitive functions in HD mouse models (Gharami et al., 2008; Giralt et al., 2009; Simmons et al., 2009; Simmons et al., 2011). Moreover, in physiological conditions, we observed that EP2 stimulation could increase dendritic branching in hippocampal primary cultures, extending the benefits of EP2 receptor stimulation. In this case, we can strongly affirm that misoprostol effect in dendritic complexity was directly related to BDNF expression, as when we blocked BDNF by adding BDNF antibody in the media, the increase in branching was completely abolished. A physiological action of EP2 stimulation was also observed in the case of WT-treated mice, where chronic misoprostol administration also increases BDNF protein levels comparing to WT vehicle-treated group.

Although some pharmacological treatments have been done in order to study the neuroprotective role of EP1 receptor delection/antagonism and EP2 receptor activation, most of them are focus on their protective effect against acute excitotoxicity. While many studies show neuroprotection by modulating EP1 and EP2 receptor in paradigms of NMDA and glutamate neurotoxicity, and in additional models of ischemia (Liu et al., 2005) limited literature study the modulation of these receptors in the context of neurodegeneration (Ahmad et al., 2013; Bilak et al., 2004; Carrasco et al., 2008; Li et al., 2013; Zhen et al., 2012) and almost no data about the role of EP1 receptor blockade or EP2 receptor stimulation in synaptic plasticity processes in neurodegenerative conditions. Only an improvement in the memory latency in the passive avoidance has been reported in Alzheimer's disease mouse models with a deletion of EP1 gene (Zhen et al., 2012); and studies focus of EP2 receptor and signaling in activity-dependent memory formation and synaptic plasticity have only been done

in physiological conditions (Akaneya and Tsumoto, 2006; Furuyashiki and Narumiya, 2011; Sang et al., 2005; Savonenko et al., 2009; Yang et al., 2009). In this work we go further into the possible role of EP1 and EP2 receptor in modulating synaptic plasticity, namely this is the first time showing that chronic pharmacological EP1 antagonism and EP2 activation improves LTM and synaptic plasticity in R6/1 mouse model of HD. This discovery is of great interest as opens a world of possibilities for the treatment of synaptic dysfunction in different brain diseases.

Even though most literature agrees on the neuroprotective effect of EP1 blockade, the role of EP2 in the context of neurodegeneration seems to be more debated. EP2 receptor elicits a very different response in the context of neuroinflammatory conditions, where accumulating evidence indicates a pro-inflammatory neurotoxic effect of EP2 receptor signaling in activated microglia (Milatovic et al., 2004; Montine et al., 2002; Shie et al., 2005) and in models of inflammatory neurodegeneration (Jin et al., 2007; Liang et al., 2005; Liang et al., 2008). In the context of HD, we have observed a beneficial effect of EP2 receptor activation, which reduces HD cognitive deficits and increases the expression of BDNF, suggesting that, although a role of neuroinflamation in HD pathology has been reported (Moller, 2010; Tai et al., 2007), excitotoxicity is one of the main triggering mechanism of the disease (Mehta et al., 2013; Zuccato et al., 2010). Thus, although it has to be further confirm, the beneficial effect of EP2 receptor activation on neuronal dysfunction observed in this work is in accordance with previous bibliography demonstrating EP2-mediated neuroprotection in the context of excitotoxicity.

Finally, and to further confirm the potential therapeutic importance of EP receptors, we studied mutant huntingtin inclusions, which are a clinical hallmark of HD pathology, can reflect pathologically relevant processes and have been reported in several transgenic mouse models (Brooks et al., 2012). Increasing evidence suggest that reduction in mutant huntingtin aggregates is directly linked to alleviation in behavioral dysfunction in HD mice(Chen et al., 2011; Giralt et al., 2011a; Yamamoto et al., 2000). In addition, a number of compounds that are neuroprotective in HD mice significantly suppress mutant huntingtin aggregates (Chen et al., 2011; Ferrante et al., 2002). Here, we report a decrease in the number of intranuclear inclusions of mutant huntingtin in the striatum and hippocampus of R6/1 SC-51089-treated mice, regions where marked alterations in synaptic plasticity have been found (Giralt et al., 2011a; Murphy et al., 2000), which correlate with improvements in motor dysfunction, and a clear reversion of LTM deficits, together with increased hippocampal plasticity in R6/1 mice. We suggest that antagonism of EP1 decreases neuronal dysfunction induced by mutant huntingtin expression, and this improvement leads to a partial reduction of the number of

intranuclear inclusions of mutant huntingtin which could be due to an increase in mutant huntingtin clearance, changes between synthesis and degradation or decreased impairment of the proteasomal system. It would be also very interesting to check the number of intranuclear inclusions of mutant huntingtin in the hippocampus of misoprostol-treated R6/1 mice as there has been shown that treatment of cortical primary cultures with an EP2 receptor agonist suppresses aggregated amyloid-β peptide-mediated neurotoxicity (Echeverria et al., 2005).

Combined, all these data indicate an important role for PGE2 and its EP receptors in HD. We provide the first link between PGE2-EP receptor and HD. Consistent with motor and memory improvements, EP receptor modulation lead to the partial recovery of some biochemical and histopathological markers in the striatum and hippocampus of R6/1 mice, as well as to improved hippocampal synaptic plasticity and reduced mutant huntingtin nuclear aggregation. Moreover, misoprostol treatment also has an effect in physiological conditions increasing branching in hippocampal primary cultured neurons and up-regulating the levels of BDNF in misoprostol-treated WT animals. Taken together, these findings show that modulation of EP receptors has a strong therapeutic effect on R6/1 mice, and point out EP receptor as a promising new therapeutic target in the central nervous system for HD and other neurological diseases. We also propose treatments with both EP1 receptor antagonist and EP2 receptor agonist to further increase the beneficial effect of EP receptor modulation in HD pathology.

V.CONCLUSIONS

1. Increased activation of Rsk, and the consequent pro-survival signaling, is necessary to protect striatal neuronal cells from mutant huntingtin-induced neuronal death, and could represent a promising compensatory mechanism to delay neurodegeneration in Huntington's disease.

2. The nuclear over-activation of Elk-1, combined with the activation of its related transcriptional machinery, is necessary for maintaining the expression of immediate early genes and for promoting the survival of striatal neurons expressing mutant huntingtin.

3. Prostaglandin E2 EP receptors could play a relevant role in the control of neuronal dysfunction in HD.

4. Modulation of EP receptors in the early onset of Huntington's disease has a strong therapeutic effect reducing behavioral phenotype in R6/1 mice.

5. Prostaglandin E2 EP2 receptor stimulation modulates synaptic plasticity in a BDNF-manner, reinforcing the link between EP2 receptor and the PKA/CREB/BDNF pathway.

6. Focusing on the development of experimental strategies to increase BDNF levels, misoprostol activation of prostaglandin E2 EP2 receptor represents a good therapeutic approach in physiological conditions and in the context of Huntington's disease pathology.

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