Estudio de los procesos activadores de la patología en la enfermedad de Huntington: Alteraciones en la plasticidad sináptica y perspectivas terapéuticas

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ESTUDIO DE LOS PROCESOS ACTIVADORES DE LA PATOLOGÍA EN LA ENFERMEDAD DE HUNTINGTON: ALTERACIONES EN LA PLASTICIDAD SINÁPTICA Y PERSPECTIVAS TERAPÉUTICAS

Tesis presentada por Albert Giralt Torroella
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Esta tesis ha sido realizada bajo la dirección del Dr. Jordi Alberch Vié, en el Departamento de Biología Celular, Inmunología y Neurociencias de la Facultad de Medicina de la Universidad de Barcelona.

Dr. Jordi Alberch Vié

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Tot i que correm el risc que es barregin èxits amb fracassos, és millor atrevir-se a començar nous projectes que no pas unir-se a les files dels qui ni pateixen ni gaudiexen gaire, perquè viuen en una zona grisa on no es coneixen la victòria ni el fracàs.

Theodore Roosvelt

A la meva familia
A l'Elisabet.

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Com que la secció d'agraïments és especial i amb força càrrega emocional, com a mínim, per mi, preferiria fer-la diferent, una mica biogràfica, amb dedicatòries i en català.

Quan vaig començar a treballar al laboratori de psicologia animal de la facultat de psicologia de la UB, entre el 2004 i el 2005, va ser sota supervisió de la catedràtica Victoria Díez Chamizo. Ella em va transmetre molta de la seva energia i motivació per la recerca. Es podria dir que ella va ser la primera "porta" i li estaré eternament agraït. A més, allà em vaig trobar, afortunadament, amb una gran persona que sempre va estar (i encara ho està) al costat, va ser propera i comprensiva, en definitiva, una bellíssima persona. Gràcies per tot Tere. No vull oblidar també a la Justina, la afable tècnic d'aquell laboratori, amb ella hem tingut i espero continuar tenint, grans converses sobre la vida en general. També allà hi vaig conèixer belles persones l'Antonio i el Joan Sansa i, com no, al Pep Marimón. Espero que tot et vagi bé a l'estabulari de la facultat de medicina Pep.

Al poc temps d'estar al laboratori de psicologia animal, un dia em va venir la Dra. Chamizo i em digué, "*hi ha una gent de la facultat de medicina allà all clínic que està interessada en col·laborar amb nosaltres perquè volen mirar el què mirem nosaltres en rates en els seus ratolins transgènics. Jo t'he recomanat a tu, estaries interessat en fer una tesi doctoral en neurociències?"*. Que si estaria jo interessat?? La investigació directa del funcionament del cervell al meu abast? A l'abast d'un psicòleg? Ni ho podria haver ni somiat! Un psicòleg ho estudia a la carrera per tenir "*culturilla*" però sap que potser mai podrà tocar aquella matèria. Així, vaig conèixer els Drs. Jordi Alberch i el Dr. Josep Maria Canals. Els hi tenia tant respecte el principi (i ara també, és clar!). A ells els vull agrair, especialment, al Jordi, el haver confiat en mi. No sé si és conscient amb el què m'ha obsequiat respecte a la oportunitat que em van donar

aleshores. Amb el temps crec que ell m'ha entès i ha vist com funciono, i crec que jo també a ell.

Va arribar el moment d'entrar al laboratori. No coneixia a ningú, suposo que us sonarà aquesta sensació. No obstant, no podria haver millor fada madrina que l'Ana López. Crec que ella es va sentir identificada amb mi, arribar amb molta il·lusió però no tenir ni idea de gairebé res. Va fer ús de la seva gran empatia i em va ajudar en tot i més. L'Ana, és una de les persones més belles i interessants que he conegut en el laboratori, tot i que posseeix un món interior molt gran al qual és difícil d'accedir-hi de vegades.

A part del Jordi i el Pep, en el laboratori hi havia també la Sílvia i l'Esther. Gràcies Sílvia, sobre tot al principi, perquè et tenia al costat i m'aguantaves totes les parides que et llençava. Gràcies per les discussions apassionades que hem mantingut i per força bons moments, com alguns a Amsterdam. I a tu Esther, també gràcies i especials! M'has ajudat en tot i també m'has aguantat en tot. M'has donat molta confiança i jo m'he aprofitat, potser massa de vegades! Ens hem entès molt bé dins i fora del laboratori i, treballar i viure així, és genial!

Durant aquell temps vaig anar coneixent el que jo anomeno la primera generació. Jo els mirava des de sota. Alguns que s'anaven com el Miquel Bosch, la Sònia Marco i la Núria Gavaldà. Llàstima no haver-los conegut millor. Hi havia per allà una persona singular, el JR. Exemple de llibre de persona treballadora. Varem tenir força converses interessants. Ei JR, un dia intentaré de fer el què deies tu que eres capaç de fer amb els ulls tancats en el *criostat*. El Juan Manuel (JuanMa), una màquina d'ordre i de treballar (l'antítesi del prototip andalús, de veritat!), esportista i, sobre tot, veure'l et transmetia alegria i confiança. La Raquel, que encara està al grup. Sempre hi haurà un abans i un després a partir de la teva marxa de la 5ª planta. Les teves rialles

encara es poden sentir en algunes de les parets de tant potents que eren. Ànims Raquel, t'esperen grans coses. Però, d'aquella generació, sens dubte, recordaré sempre de forma especial a una persona: a Jesús (Chucho). Paciència il·limitada (fins i tot amb psicòlegs jugant a ser biòlegs), savi, proper, amb humor, filòsof, gran amic, etc. Crec que no li faltava res. El que em va transmetre ell era peça clau per poder moure'm mínimament bé en ciència. Vas marxar a Venezuela però ara has tornat a Espanya. Estàs a Madrid i, fins i tot des d'allà, puc sentir de nou les bones vibracions que emets.

La que considero la segona generació (la meva), va ser la millor (com no): Noelia, Dani i Xavi. Noelia, des de Sant Pol et dic: els bons moments que hem passat no s'obliden. Tu sí que tens valor, no el perdis. Recordes quan es va acabar la bateria del meu portàtil quan el Bliss donava una xerrada a Soria? Per cert, recorda que segones parts mai foren bones. A tu Dani, quantes coses junts! Port Aventura i quan vaig tocar el cul a la teva nòvia, el pub de les *viejas* a València, caps d'any, etc. Però, per altra banda, sempre atent, sempre disposat a ajudar i a discutir des del tema més inversemblant fins al més transcendental. I el Xavi (o Xavito), crec que mai he vist a una persona madurar (al principi només ho feies veure) i créixer tant ràpid en tant poc temps. La vida es xunga però tu ets un bon model a seguir de com afrontar-la. Sempre disposat a portar la contrària, cosa que he gaudit enormement. Tots aquells cafès plens de "*maruejos*", en conjunt, impossible d'oblidar tot allò.

Durant la següent generació, va ser el primer cop que em vaig sentir veterà. L'arribada de persones com l'Empar, la Paola (i la seva annexa Íngrid ;-)) o la Laura una mica després. Empar, l'enciclopèdia vivent, catalanista, "*marujil*", ho té tot per ser la col·lega ideal dins i fora de la feina. Una bellíssima persona que encara no sap que estar de morros massa sovint envelleix. Quan, en un futur, treballis al meu (hipotètic) laboratori segur que estaràs sempre rient. Gràcies a tu també Paola per la teva

companyia. Amb tu Íngrid va ser tot de *pu** mare* a partir de la segona meitat de la teva estança aquí, no creus Íngrid? A tu Laura he d'agrair-te, sobre totes les coses, la possibilitat de participar en la creació d'un reportatge sobre Huntington que m'ha injectat una nova dosi de realisme que encara m'empenta més a la ciència. Pensa que, a més, aquesta creació farà que no ens oblidem mai entre nosaltres. Finalment, Olga, et considero d'aquesta generació tot i que, malauradament, has arribat tard al laboratori. Estant al meu costat, no solsament m'has ajudat laboralment sinó personalment. Crec que madurar juntament amb persones com tu millora la personalitat. Sempre crítica (de vegades massa), amb visions obertes, però estrictes (i tossudes de vegades, segur que aquesta paraula t'agrada ;-)). *Friendship foreveranever*!

Crec que ara toca fer dedicació a la última generació. Em refereixo a la Mònica, a la Mar i a la Marta. Mònica, no ens coneixem massa però durant el proper any segur que ho haurem de fer i segur que serà una experiència maca. A la Marta i a la Mar, les noves generacions de la 5ª planta, si no fuméssiu tant serieu encara més màquines del què ja sou (per cert, guardeu-ne sempre un "*piti*" per mi, porsiaca). Crec que us espera un futur prometedor en tots els sentits, com a científiques i persones, no cal ser molt àvid per veure-ho davant l'alegria i la iniciativa que mostreu i transmeteu.

No vull oblidar els post docs del grup que han anat passant. Tot i que ja n'he esmentat alguns abans, em refereixo a persones com l'Ana Saavedra. Sempre discreta però amb una gran intel·ligència i professionalitat. Sense oblidar la grandesa de la seva amistat quan te l'ofereix. A la Maria Rifé, sempre tranquil·la, pacient i serena. Espero que et vagi bé en el teu nou món professional (i el no-professional també!). A la Verónica també li vull agrair la seva companyia. I a la Bego, finalment, també agrair-li la seva passada pel laboratori per permetre'm conèixer a tan bella persona. Una amant dels gossos, sí senyor! I dels animals en general. No et preocupis que jo cuido bé als

ratolins. Segur que els teus pacients deuen millorar a tots els nivells amb les teves dietes (segur que alguna vegada receptaràs la del "*cucurucho*").

També m'agradaria agrair i de pas mantenir en el meu record a persones com l'Eva Moreno i l'Eduard, "alumnes" meus durant dos estius diferents els quals van aprendre de mi i jo d'ells. M'han ajudat a ser més atent i pacient.

En aquesta estrofa m'agradaria també dedicar unes paraules de sincer agraïment a tots els membres del grup del Gustavo que han anat passant i que he anat coneixent començant pel Gustavo mateix i seguint amb persones com el Frank (inhòspit i tot un personatge). La Inés, sempre aplicada. La Cecilia o "Pileta", o l'Ari o Aristóteles, aquest últim una persona amb un carisma especial. El Yován, sempre alegre i tant obert i expressiu. El Sergi, que ni anant-se del grup l'hem perdut de vista, per sort, és clar! Encara em pregunto si la teva Elisabet no serà la meva... Tu ja m'entens, oi Sergi? La Carla, nova incorporació, aportant frescor al lab i la Laia, prudent i reservada però amb geni eh! Repetirem l'any que ve a l'observatori Fabra? També et recordaré Adrià, espero que no siguis tu qui em vegi penjant les botes de futbol! La teva companyia al lab fa que tot sigui més fàcil, crees molt bon rotllo. Finalment dir-te Javi, que jo sempre havia pensat que la persona que es amiga de tothom divideix tant la seva estima que acaba no fent sentir a ningú especial. Tu m'has demostrat el contrari, la teva amistat és infinita. Falten persones com tu en aquest món.

Per altra banda, tampoc puc oblidar a persones com la Maite, la supertècnic sempre pendents de tots nosaltres. Crec que si per ella fos, de vegades ens posaria penyals a tots. I també la Cristina. Recordo el primer dia que vares venir com si fos ahir. Una cara de mala baba que no vegis. Sort que la primera impressió realment no compta perquè, encara que ho neguis, tens molta dolçor a dins. Llàstima que també t'anessis a la 3ª, però sempre ens quedaran els primers mesos, no?. També vull endur-me grans

records de les nostres super-secres, la Núria (i col·lega psicòloga), la Carme (més catalanista però no més pagesa que jo) i l'Eva. Ho heu fet tot més fàcil. Tampoc vull oblidar persones que han anat passant o encara hi són i que m'han deixat un bon record com el Martí de veterinària, la Meri de l'estabulari, l'Adam Kassan, la Solene que ara ja té una criatura i tot! La Lídia Sabater (gadjet del nostre laboratori), a tu també et recordaré. A tots vosaltres infinites gràcies.

ÍNDICE

ABREVIATURAS

αCaMKII	Cinasa activada por calcio/calmodulina II tipo α
AMPAR	Receptores del ácido α-amino-3-hidroxi-5-metil-4-isoxazolepropionic
AMPc	Monofosfato de adenosina cíclico
BDNF	Factor neurotrófico derivado del cerebro
DARPP-32	Fosfoproteína regulada por dopamina y AMPc de 32 KDa
ERK ½	Cinasa regulada externamente 1 y 2
GFAP	Proteina asociada a fibras gliales
GluR1	Subunidad 1 del receptor de glutamato tipo AMPA
htt	Huntingtina normal
LTD	Depresión a largo plazo
LTP	Potenciación a largo plazo
mhtt	Huntingtina mutada
MSNs	Neuronas de proyección GABAérgicas estriatales
NR1	Subunidad tipo 1 de los receptores NMDA
NR2A-B	Subunidades tipo 2A-B de los receptores NMDA
NMDAR	Receptores de glutamato tipo N-metil-D-aspartato
PDE4	Fosfodiesterasa 4
PDE10A	Fosfodiesterasa 10A
PKA	Proteína cinasa dependiente de AMPc
PLCγ	Fosfolipasa C gamma
PSD-95	Densidad post-sináptica de 95 KDa
PSD-93	Densidad post-sináptica de 93 KDa
QUIN	Ácido quinolínico o quinolinato
Ras-GRF1	Factor de liberación de guanina dependiente de Ras
SAP102	Proteína asociada a sinapsis de 102 KDa
SAP97	Proteína asociada a sinapsis de 97 KDa
STEP	Proteína fosfatasa enriquecida del estriado
TrkB	Cinasa relacionada a tropomiosina tipo B
VGLUT1	Transportador de glutamato asociado a vesícula tipo 1
YAC	Cromosoma artificial de levadura

I Introducción

Las enfermedades neurodegenerativas se caracterizan por ser patologías que alteran la función cerebral induciendo síntomas psiquiátricos y neurológicos que progresan de forma continuada y sin remisión. Estas enfermedades producen una enorme discapacidad con el sufrimiento familiar asociado debido a la carga física y psíquica que suponen. Estas alteraciones inducen progresivamente a una imposibilidad por realizar un trabajo, a relacionarse socialmente, a tener una familia y a unas alteraciones cognitivas de tal magnitud que con frecuencia acaban por una pérdida de identidad y personalidad. Por consiguiente, estas enfermedades crónicas producen un impacto social enorme y también un importante gasto económico que conlleva la atención social y sanitaria de los pacientes afectados.

Las enfermedades neurodegenerativas se pueden distinguir por las regiones cerebrales afectadas y las manifestaciones clínicas asociadas. El estudio de la vulnerabilidad celular de estas regiones conduciría al desarrollo de estrategias terapéuticas neuroprotectoras capaces de desacelerar, detener el proceso neurodegenerativo o incluso lograr la total remisión de las manifestaciones clínicas. Sin embargo, en la actualidad no existe cura efectiva para tales entidades y los tratamientos tan solo poseen efectos paliativos sobre la sintomatología.

En la presente tesis nos hemos centrado en una de estas enfermedades, la enfermedad de Huntington. En esta enfermedad los síntomas se caracterizan por una afectación clara y progresiva sobre el control motor. Estas manifestaciones clínicas se deben a la afectación selectiva de los ganglios basales. Sin embargo, existen otros síntomas más precoces como las alteraciones cognitivas que podrían ser debidos a alteraciones en otras regiones como el hipocampo. Es importante resaltar que para el estudio de esta enfermedad se han generado modelos animales que mimetizan la

sintomatología humana. Además, estos han permitido estudiar en detalle algunos de los mecanismos celulares y moleculares implicados en su fisiopatología.

Así pues, esta tesis se ha centrado, en la caracterización de los mecanismos moleculares implicados en la fisiopatología de la enfermedad de Huntington para el desarrollo de posibles nuevos tratamientos. Los correspondientes estudios se han realizado desde un abordaje precoz con especial interés en los mecanismos moleculares que se pueden situar como eventos primarios de la enfermedad de Huntington. Este abordaje nos ha llevado a estudiar, mediante el uso de modelos murinos, alteraciones tales como las cognitivas, procesos excitotóxicos y mecanismos moleculares implicados en la plasticidad sináptica. El siguiente paso fue diseñar en los mismos modelos, posibles estrategias terapéuticas con el objetivo de enlentecer, atenuar o incluso detener la progresión de la enfermedad.

1. La enfermedad de Huntington

1.1. Etiología

La enfermedad de Huntington es un trastorno neurodegenerativo de penentrancia autosómica dominante completa (Wexler y col., 1987) causada por una mutación localizada en el gen que codifica la proteína llamada huntingtina (htt) (HDCRG, 1993). Su nombre se debe al médico George Huntington quién realizó las primeras observaciones detalladas sobre la sintomatología (1850-1916). Los síntomas clínicos clásicos se manifiestan hacia la cuarta década de vida y progresa de forma inexorable con un pronóstico de vida de unos 15-20 años desde su aparición. Sin embargo se han detectado formas “juveniles” con una edad de inicio a los 20 años (Papapetropoulos y

Mash, 2005). La incidencia de la enfermedad es de unos 5-7 por 100.000 habitantes (Walker, 2007). A nivel sintomático, la enfermedad de Huntington se define como un trastorno de alteraciones motoras a veces llamados corea (que significa baile). Sin embargo, siendo más amplios, la sintomatología consta además de alteraciones motoras, de declive cognitivo y trastornos psiquiátricos asociados (Walker, 2007).

El gen de la proteína htt, llamado IT15, se localiza en el brazo corto del cromosoma 4 (Gusella y col., 1983). Este gen tiene 67 exones llegando a un total de unos 170.000 pares de bases. Sin embargo, la mutación del gen se halla en el exón-1 caracterizada por una elongación aberrante de tripletes CAG en un segmento codificador de poliglutaminas (poliQ). En individuos normales, los tripletes CAG no superan los 35, en cambio, si estas repeticiones alcanzan las 40 o más se produce la enfermedad (Lin y col., 1995). Es interesante que cuantas más repeticiones padezca la mutación, mayor será la severidad de la enfermedad (Snell y col., 1993).

Como se ha comentado, el gen IT15 codifica la proteína htt (Figura 1). Esta se expresa principalmente en testículos y en cerebro (Trottier y col., 1995). En el cerebro, se expresa principalmente en la neocorteza, en la corteza cerebelosa,

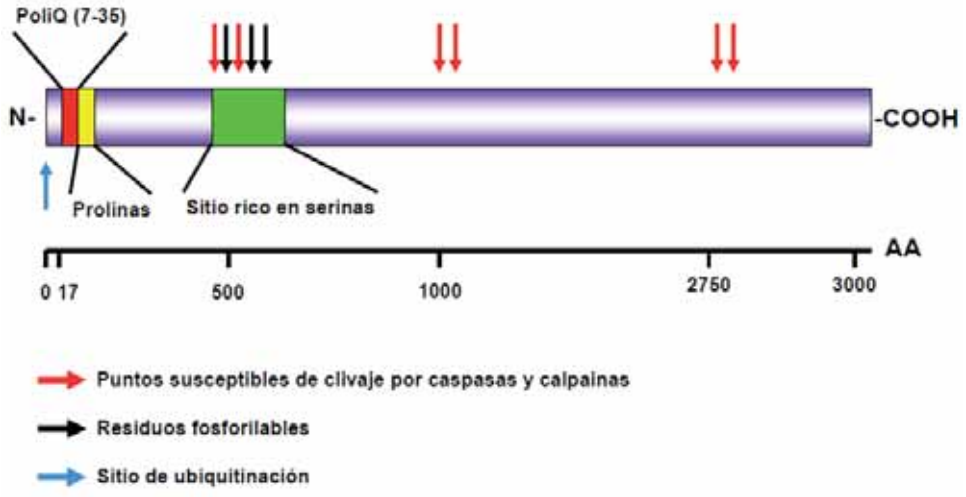


Figura 1. **La proteína htt.** La figura muestra esquemáticamente la estructura de la htt. La escala representa el número de aminoácidos que contiene la proteína. La región poliQ (que contiene normalmente entre 7 y 35 glutaminas aproximadamente) está representada en rojo seguida de la región rica en prolinas representada en amarillo. En verde residen las zonas fosforilables (concretamente señaladas en flechas negras). Las flechas rojas indican zonas donde la htt puede ser cortada por

caspasas y/o calpaínas. Finalmente, la flecha azul indica por donde la htt puede ser ubiquitinada (Adaptado de Cattaneo y col., 2005).

en la formación hipocampal y en el núcleo estriado (Li y col., 1993; DiFiglia y col., 1995; Sharp y col., 1995), localizándose tanto en neuronas como en neuroglía (Lievens y col., 2001; Shin y col., 2005). Como se indica en el esquema (Figura 1), esta proteína posee unos 3136 aminoácidos y un peso aproximado de ~348 kDa. El extremo N-terminal contiene un segmento poliQ que empieza en el residuo 17 seguido de una región rica en prolinas. Además, la htt posee una región rica en serinas y múltiples sitios susceptibles de ser cortada (Figura 1) (Cattaneo y col., 2005). Cuando la proteína huntingtina mutante (mhtt) se corta produce unos fragmentos que han demostrado ser tóxicos cuando son solubles (Hackam y col., 1998; Hodgson y col., 1999) y capaces de formar agregados con distintos grados de complejidad tanto citoplasmáticos como nucleares (DiFiglia y col., 1997; Ross y col., 2004) (Figura 2).

La función específica de la htt no se conoce bien y menos cuando esa está mutada. Sin embargo, la htt se la ha asociado con importantes y numerosas funciones. Algunos ejemplos son: participación en la trascripción génica (Cha y col., 2000), desarrollo normal del cerebro (Auerbach y col., 2001), funciones mitocondriales (Browne y col., 1997), tráfico intra-celular (Gauthier y col., 2004), modulación de la excitotoxicidad (Fan y Raymond, 2007) y regulación de la función trófica (Alberch y col., 2004) entre otras. Dada esta amplia variedad de funciones en que la htt está implicada, el estudio de la fisiopatología de la enfermedad de Huntington es altamente complejo. Esto supone que la identificación de los procesos alterados por la mhtt en las primeras etapas de la enfermedad es de interés primordial para la subsiguiente propuesta de estrategias terapéuticas.

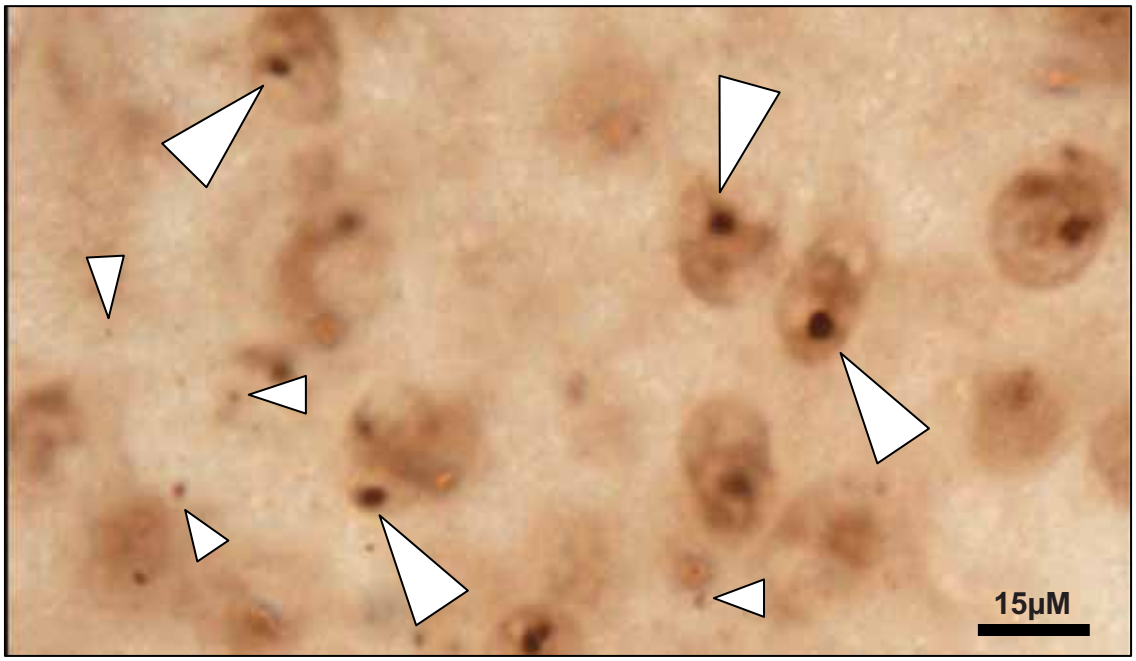


Figura 2. Agregados nucleares (flechas grandes) (NII), y agregados citoplasmáticos (flechas pequeñas). Fotografía obtenida de sección fijada y procesada para inmunohistoquímica usando el anticuerpo contra expansiones de poliglutaminas llamado EM48. Fotografía realizada en el laboratorio propio.

1.2. La enfermedad de Huntington: Neuropatología de los ganglios basales y alteraciones motoras asociadas

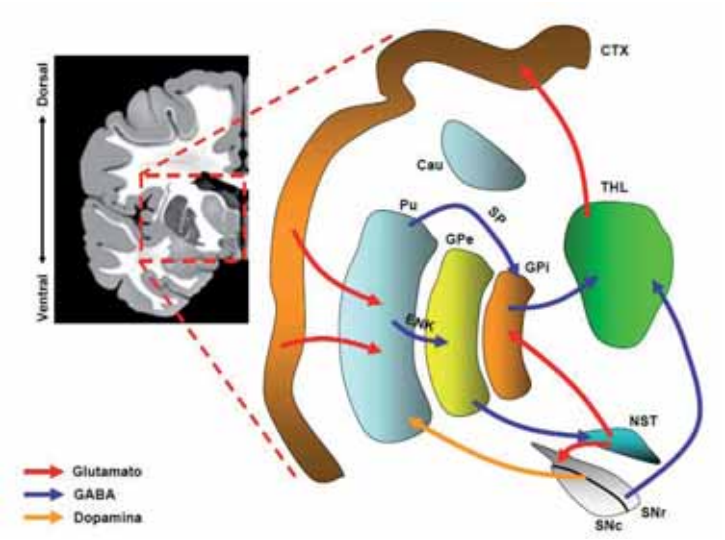
En el progreso de la enfermedad, es conocido que son los núcleos caudado y putamen, pertenecientes a los ganglios basales, las regiones cerebrales más afectadas (Vonsattel y col., 1985). Adicionalmente, existe una neurodegeneración cortical significativa con muerte neuronal asociada (Vonsattel y col., 1985; Rosas y col., 2003). Sin embargo, esta neurodegeneración cortical es más secundaria y se produce en fases más tardías de la enfermedad (Mann y col., 1993), por lo que nos centraremos más en la patología de los ganglios basales. Al ser los núcleos caudado y putamen (en conjunto también llamados núcleo estriado) los más afectados, es imperativo conocer las funciones de estas regiones cerebrales. Así, los ganglios basales tienen diversas funciones que van desde el control del movimiento, procesos de atención, percepción visual y aprendizaje (Brown y col., 1997; Walker y col., 2007).

A nivel del control motor, es conocido que las lesiones en los ganglios basales pueden producir síntomas hipercinéticos (movimientos involuntarios exagerados) o hipocinéticos (pérdida de capacidad motora) (Galvan y Wichmann, 2007). Esta aparente contradicción funcional llevó a postular e identificar que los ganglios basales constan de dos vías distintas principales; la directa (excitadora) y la indirecta (inhibidora) (Alexander y Crutcher, 1990; Gerfen, 1992). De hecho, la sintomatología motora en la enfermedad de Huntington tiene un curso primero hipercinético con corea, movimientos exagerados y descontrolados no estereotipados, principalmente en extremidades, pero también presentes en el tronco y, adicionalmente, con trastornos oculomotores asociados (Van Vugt y col., 2001). Posteriormente, la sintomatología progresa hacia una fase hipocinética con perdida de capacidad de generar movimiento, rigidez y distonía (Berardelli, 1999). Esto sugiere que la vía principalmente afectada sería la indirecta (pérdida de inhibición del movimiento) a la que más tarde se añadiría la degeneración de la vía directa (pérdida de excitación o producción del movimiento). Estos procesos nos llevan a la necesidad de definir la circuitería de los ganglios basales para una mayor comprensión de las alteraciones descritas.

Los ganglios basales constan de diversos núcleos (Figura 3A). El núcleo estriado (que consta del conjunto putamen, caudado y acumbens) es la puerta de entrada de todos los inputs hacia los ganglios basales (Graybiel, 2000; Gerfen, 1992). Casi toda la neocorteza envía proyecciones, principalmente desde las neuronas piramidales de la capa V (glutamatérgicas), hacia el núcleo estriado pero tan solo los lóbulos frontales reciben su *output*. Existen cuatro circuitos principales que atraviesan los ganglios basales, los circuitos esqueletomotor, oculomotor, asociativo y límbico (Martin, 1998). El nombre de estos circuitos nos puede sugerir algunas de las funciones potencialmente alteradas en la enfermedad de Huntington. A nivel de microcircuitería, el núcleo

estriado establece proyecciones (GABAérgicas) hacia el segmento interno del globo pálido (GPi) y la parte reticular de la sustancia negra (SNr) (vía directa) pero también proyecta hacia el segmento externo del globo pálido (GPe) (vía indirecta) (Figura 3A).

A



B

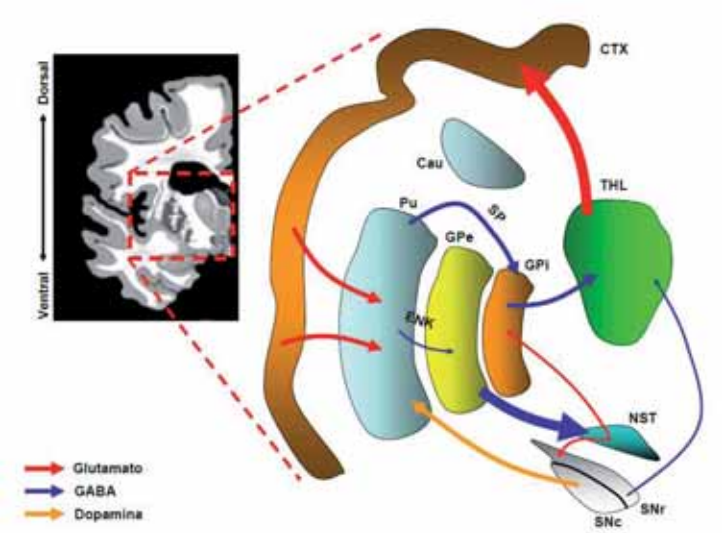


Figura 3. Neuroanatomía funcional de los ganglios basales en condiciones normales y en cerebros con la enfermedad de Huntington. En condiciones normales (A), los núcleos caudado (Cau) y putamen (Pu) reciben su *input* de prácticamente toda la neocorteza cerebral (CTX) mediante sinapsis excitatorias (en rojo). De los núcleos caudado y putamen surgen las vías GABAérgicas principales pudiendo expresar o sustancia P (SP) o encefalina (ENK). Las vías GABAérgicas originadas en las fibras SP generan hacia el CTX un *output* excitatorio, mientras que las GABAérgicas originadas en las fibras ENK generan un *output* inhibitorio. En la condición con la enfermedad de Huntington (B), al estar mayormente alterada la vía originada en las fibras ENK, se genera una falta de *output* inhibitorio y, por lo tanto, un exceso de *output* excitatorio explicando así la sintomatología motora hipercinética. Finalmente (B), en estadios tardíos, la degeneración es tal que se ven alteradas las dos vías induciendo la sintomatología tardía hipocinética y falta de generación de movimientos.

A su vez, el GPi y la SNr proyectan (axones GABAérgicos y dopaminérgicos respectivamente) hacia los núcleos talámicos lateral ventral, anterior ventral y dorsal medial los cuales devuelven algunas proyecciones (glutamatérgicas) de vuelta al núcleo estriado y, principalmente, a áreas del lóbulo frontal cerrando así el circuito (Graybiel, 2000). Por otra parte, el GPe proyecta (axones GABAérgicos) hacia el núcleo subtalámico el cuál envía proyecciones (glutamatérgicas) hacia la SNr y el GPi. Adicionalmente, el área tegmental ventral y la parte compacta de la sustancia negra (SNc) proyectan (axones dopaminérgicos) directamente hacia el núcleo estriado (Figura 3A).

Como hemos comentado anteriormente, la enfermedad de Huntington se caracteriza, a nivel histopatológico, por una neurodegeneración severa del núcleo estriado (Figura 3B) debido a una atrofia del neurópilo en general y, sobre todo, debido a la muerte selectiva de las neuronas de proyección GABAérgicas principales llamadas MSNs (del inglés *Medium Spiny Neurons*) además de ir asociada a una gliosis reactiva asociada (DiFiglia y col., 1997; Ferrante y col., 1991; Vonsattel y col., 1985). Sin embargo, las diversas interneuronas existentes en el núcleo estriado resultan mucho menos afectadas y solamente en fases tardías (Graveland y col., 1985; Kowall y col., 1993; Beal y col., 1990; Ferrante y col., 1987; 1991). Las MSNs representan el ~90-95% del total de neuronas estriatales (Graveland y col., 1985), utilizan GABA como neurotransmisor e inervan gran cantidad de aferencias glutamatérgicas provinentes de la corteza cerebral (vía cortico-estriatal) (Nakano y col., 2000). Esta alta inervación glutamatérgica cortical por parte de las MSNs las convierte en uno de los tipos neuronales con mayor densidad de espinas dendríticas (sinapsis excitatorias) del sistema nervioso central (DiFiglia y col., 1976; Wilson y Groves, 1980). A nivel de marcaje, las MSNs pueden ser específicamente inmuno-reactivas para la proteína quelante de calcio

Calbindina y también para DARPP-32 (del inglés *dopamine- and cyclicAMP- regulated phosphoprotein of 32 KDa*) (Fienberg y Greengard, 2000). Es interesante considerar que las MSNs pueden ser clasificadas bioquímicamente pertenecientes a la vía directa (expresando el neuropéptido sustancia P (SP) y dinorfina (DYN)) o pertenecientes a la vía indirecta (expresando el neuropéptido encefalina (ENK)) (Martin, 1998). Gracias a estos métodos de marcaje, se ha podido corroborar que las MSNs pertenecientes a la vía indirecta y que expresan ENK son las que estan principalmente afectadas y de forma más temprana (Reiner y col., 1988, Albin y col., 1992, Richfield y col., 1995, Mitchell y col., 1999; Glass y col., 2000). Estos hallazgos explican de forma satisfactoria la sintomatología motora hipercinética en las fases iniciales de la enfermedad de Huntington (Figura 3B).

A nivel cognitivo, al núcleo estriado también se le asocia a fuertes componentes reguladores de los procesos de aprendizaje y memoria basados en secuencias estímulo-respuesta (condicionamiento instrumental), aprendizaje de destrezas (también llamado aprendizaje de procedimiento) y en secuencias de movimientos (Brown y col., 1997; White, 1997; Packard y Knowlton, 2002). Debido a este rol en los procesos cognitivos superiores regulados por el núcleo estriado, numerosos estudios han caracterizado ya alteraciones de aprendizaje y memoria dependientes de esta región cerebral en pacientes con la enfermedad de Huntington incluso mucho antes de la aparición de los síntomas hipercinéticos motores (Foroud y col., 1995; Lawrence y col., 1996, 1998; Lemiere y col., 2004). De forma interesante, estas alteraciones se han basado en una disfunción en las vías cortico-estriatales mediando funciones ejecutivas, de memoria de trabajo y velocidad de procesamiento (para una revisión ver Montoya y col., 2006). Lo que es también importante, es que esos hallazgos también se han replicado en todos los modelos animales de la enfermedad de Huntington testados hasta el momento (Van

Raamsdonk y col., 2005a; Lione y col., 1999; Trueman y col., 2007). En conjunto, esta amplia bibliografía también demuestra que los procesos cognitivos superiores, en este caso dependientes del núcleo estriado, resultan afectados con mayor precocidad ante los efectos de la mhtt que los procesos cerebrales implicados en el control motor *per se*.

1.3. La enfermedad de Huntington: Neuropatología de la formación hipocampal y alteraciones cognitivas asociadas

Dado que las alteraciones cognitivas en pacientes pertenecen al conjunto de síntomas más tempranos en su aparición en la enfermedad de Huntington (Lemiere y col., 2002; Ho y col., 2003; Kirkwood y col., 2000), es de vital importancia el estudio de las estructuras cerebrales subyacentes a tales procesos. Hasta ahora hemos descrito alteraciones cognitivas que podrían estar reguladas por las disfunciones existentes en las conexiones entre la neocorteza cerebral y el núcleo estriado. Muchos de los trabajos realizados en pacientes con la enfermedad de Huntington que analizan funciones cognitivas superiores podrían estar reguladas por la formación hipocampal. Está ampliamente demostrada la importancia del hipocampo en funciones cognitivas superiores como la formación de memorias declarativas, reconocimiento de objetos y memoria visuoespacial entre otras (Kandel y col., 2001; Eichenbaum, 2001; Dere, 2007; O'Kefe y Nadel, 1979). Existen pocos estudios en pacientes que evalúan la función hipocampal a nivel neuroanatómico, de neuroimagen y/o neuropsicológico en pacientes con la enfermedad de Huntington. Sin embargo, existen suficientes evidencias tanto en pacientes con la enfermedad de Huntington como en modelos animales de la enfermedad que sugieren una fuerte implicación de la formación hipocampal en la

regulación de muchas de las alteraciones cognitivas observadas. Para entender tales alteraciones es necesario un análisis de esta región cerebral.

Siguiendo el esquema propuesto por varios autores (Cotterill, 2001; Witter, 2000; O'Mara, 2005; Somogyi, 2005) (Figura 4), el hipocampo recibe la mayoría de sus *inputs* de la corteza entorinal mientras que la mayoría de sus *outputs* se proyectan hacia el subículo. El hipocampo además, se subdivide en varias subregiones llamadas Cuernos de Amón 1-3 (CA1-CA3) y Giro Dentado (GD). Esta región cerebral se caracteriza por estar altamente organizada y poseer unas conexiones secuenciales que han permitido

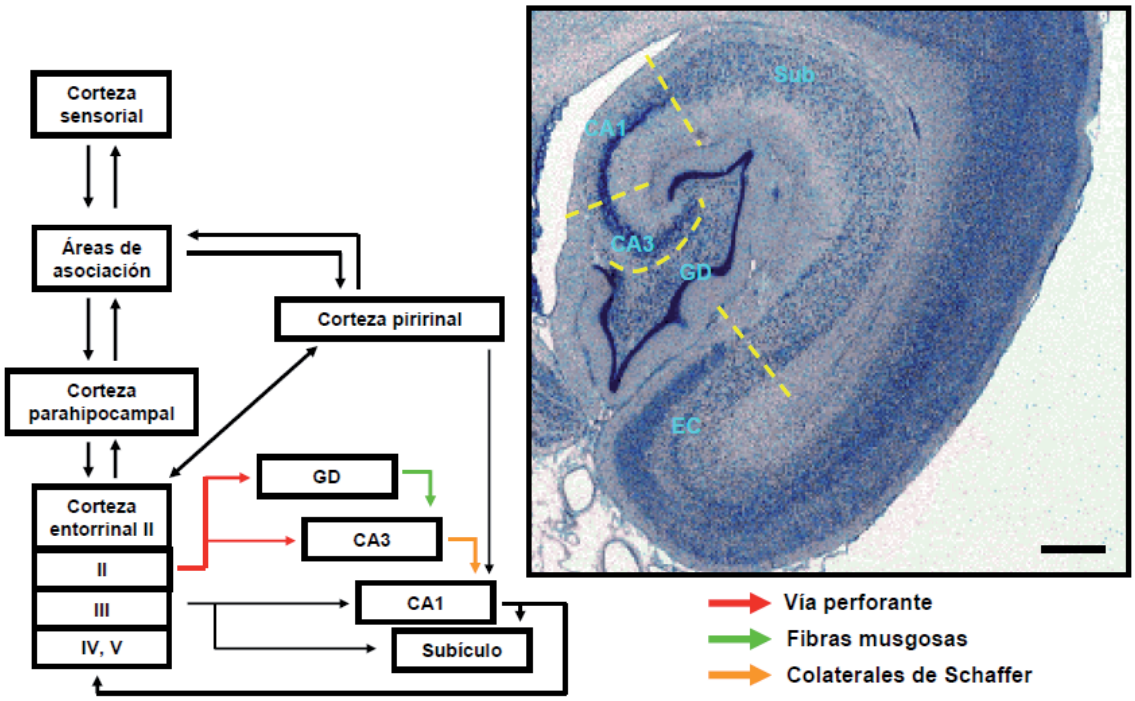


Figura 4. Neuroanatomía funcional de la formación hipocampal. El esquema muestra la organización de la formación hipocampal. La puerta de entrada del hipocampo es la corteza entorrinal. De ella surge la vía perforante (en rojo) hacia el giro dentado (GD) y hacia el cuerno de Amón 3 (CA3). Del GD surgen las fibras musgosas (en verde) hacia la CA3. Luego, desde la CA3 surgen las vías llamadas colaterales de Schaffer (en naranja). Foto representativa de formación hipocampal de primate y teñida mediante el método de Nissl. Sub: subículo, EC: corteza entorinal. Esquema modificado de O'Mara, 2005.

gran accesibilidad para su estudio. Se conocen tres vías principales en el hipocampo, la primera, con el nombre de vía perforante, nace de las neuronas piramidales de la corteza entorrinal y proyecta hacia las dendritas de las neuronas granulares del GD (Figura 4). Asimismo, estas células granulares formarían los característicos haces neuronales no

mielinizados llamados fibras musgosas que conectarían con las células principales piramidales de la CA3. Estas mismas células piramidales forman el haz de axones llamado colaterales de Schaffer que proyectan hacia la región CA1 y hacia el subículo (que sería la puerta de salida principal del hipocampo). La misma CA1 también proyecta hacia el subículo. Cabe mencionar que existen otras vías menos estudiadas dentro de la formación hipocampal tal y como quedan representadas en el esquema (Figura 4) y que conectan distintas subregiones entre sí. Aunque el hipocampo se considera una corteza cerebral primitiva (con menos de seis capas), este posee una organización estratificada en diversas capas con diferentes características citológicas y funcionales. Finalmente mencionar que, además de las neuronas principales piramidales y granulares, existen múltiples tipos de interneuronas en el hipocampo modulando la actividad y funcionalidad de estas células principales (Maccaferri y Lacaille, 2003).

Las funciones hipocampales se han estudiado largamente y se han relacionado casi todas con funciones cognitivas superiores como la formación de nuevas memorias. Brevemente, el hipocampo (normalmente en coordinación con otras estructuras del lóbulo temporal) está altamente relacionado con formaciones de nuevas memorias declarativas (Eichenbaum, 2001), reconocimientos de objetos (Dere, 2007), memoria visuoespacial (Kandel, 2001), navegación espacial (O'Kefe y Nadel, 1978), varios tipos de condicionamiento (Shaún y col., 2007) y formación de nuevas memorias en general (Corkin, 2002). Además, en la formación hipocampal es donde más se han caracterizado los fenómenos como la LTP (del inglés *long-term potentiation*) y LTD (del inglés *long term depression*), siendo ambos actualmente los modelos celulares y neurofisiológicos más aceptados para explicar la formación de memorias (Bliss y Collingridge, 1993; Lynch, 2004).

En el contexto de la enfermedad de Huntington, existen pocos pero suficientes trabajos que han aportado evidencias de alteraciones morfológicas y funcionales de la formación hipocampal en pacientes con la enfermedad. Rosas y colegas demostraron la existencia de atrofia en la sustancia gris del hipocampo en pacientes con sintomatología temprana (Rosas y col., 2003). Este estudio fue corroborado por el grupo de Roth (Jech y col., 2007) encontrando además, una correlación entre atrofia hipocampal en pacientes con enfermedad de Huntington y su puntuación en la UHDRS (una escala para evaluar la gravedad de la sintomatología en estos pacientes). Además, estudios *post-mortem* demuestran pérdida neuronal en el hipocampo de estos mismos pacientes (Spargo y col., 1993). A nivel funcional, diversos estudios neuropsicológicos han puesto de manifiesto alteraciones de aprendizaje y memoria que son regulados por la formación hipocampal como lo son la memoria de reconocimiento espacial, reconocimiento de figuras, aprendizaje asociativo (Lawrence y col., 2000; Redondo-Vergé, 2001; Bylsma y col., 1991) y memoria declarativa en general (Sprengelmeyer y col., 1995; Ghilardi y col., 2008). Por otra parte, los estudios con modelos animales transgénicos de la enfermedad han permitido mayor entendimiento de estos procesos patológicos. En todos estos modelos se han demostrado los mismos (o equivalentes) déficits de memoria y aprendizaje que en humanos tanto dependientes de las vías cortico-estriatales como de la formación hipocampal y, además, con una aparición anterior a los síntomas motores (Lione y col., 1999; Murphy y col., 2000; Trueman y col., 2007; Van Raamsdonk y col., 2005a). Adicionalmente, estudios en estos modelos evaluando los procesos implicados en la formación de la LTP y la LTD hipocampales también han demostrado alteraciones funcionales específicas de las vías que, presumiblemente, modulan la formación de estos procesos de memoria (Murphy y col., 2000; Lynch y col., 2007; Milnerwood, 2006). En conjunto, todos estos estudios indican que, a pesar del bajo grado de

afectación anatómica a *grosso modo* de la formación hipocampal en la enfermedad de Huntington, existen múltiples alteraciones (principalmente funcionales) en esta región debido a la presencia de la mhtt mutada modulando su correcta actividad. Esto se traduciría en numerosos y tempranos déficits cognitivos detectables en estos pacientes y desde las fases más tempranas de la enfermedad.

2. Modelos de la enfermedad de Huntington

Hasta ahora, hemos ido definiendo la etiología, la sintomatología y la neuropatología de la enfermedad de Huntington. En estos apartados repetidas veces hemos hecho referencia a trabajos basados en modelos animales (principalmente en roedores) de la enfermedad. Eso refleja la importancia que han cobrado estas "herramientas" para un mejor entendimiento del objeto de estudio. Poco después de descubrirse la localización de la mutación en el gen de la htt en la enfermedad de Huntington, tres grupos distintos crearon ratones *knock-outs* para esta proteína (Nasir y col., 1995; Duyao y col., 1995; Zeitlin y col., 1995). En todos los casos, la ausencia total de la proteína era letal para el embrión y en dos de los casos, los heterocigotos no mostraban patología clara. Esto demuestra que la enfermedad de Huntington se debe principalmente a una ganancia de función tóxica debido a la mutación y no tanto a una pérdida de función (Brouillet y col., 1999). De forma subsiguiente, la clave para desentrañar la fisiopatología de la enfermedad y para probar nuevos fármacos residía en mimetizar la mutación de la mhtt propia de la enfermedad de Huntington en ratones, más que en eliminar la expresión de la proteína en sí. Aquí explicaremos los primeros modelos de la enfermedad de Huntington, los modelos tóxicos agudos, debido a su utilidad y pragmatismo. También repasaremos varios de los modelos transgénicos y

Knock-in que se han creado y, finalmente, también haremos hincapié en los nuevos modelos emergentes de la enfermedad, los cuáles han resultado ser muy útiles para estudiar aspectos más concretos.

2.1. Modelos tóxicos agudos: Ácido quinolínico (QUIN) y ácido 3-nitropropiónico (3-NP)

Los modelos agudos de la enfermedad de Huntington en roedores fueron los primeros en aparecer y se consiguieron mediante el uso de varios tóxicos que inducían cambios bioquímicos y neuropatológicos imitando algunos de los observados en la enfermedad de Huntington (Wang y Qin, 2006). El grupo de Beal descubrió que la inyección intra-estriatal del agonista de receptores de glutamato NMDA llamado ácido quinolínico (QUIN) induce una muerte selectiva de las MSNs mientras que las interneuronas permanecían relativamente intactas (Beal y col., 1986). Estas lesiones además pueden provocar en los animales hiperquinesia, trastornos motores y alteraciones de aprendizaje pero, sin embargo, éstos no muestran signos de corea ni de disquinesias (Sanberg y Coyle, 1984).

Existe también el ácido 3-nitropropiónico (3-NP) que produce una lesión específicamente en los ganglios basales mediante la inhibición del succinato deshidrogenasa localizado en la membrana interna de la mitocondria y responsable de la oxidación del succinato y el fumarato (Beal y col., 1993; Brouillet y col., 1995). Aunque el modelo animal de 3-NP comparta varias caracerísticas histoquímicas, de comportamiento y patológicas con la enfermedad de Huntington, la muerte neuronal estriatal es mucho más acentuada que en otros modelos y con poca especificidad hacia las MSNs (Hamilton y Gould, 1987; Guyot y col., 1997; Brouillet y col., 1999).

2.2. Modelos transgénicos exón-1: Líneas R6 y los N171-82Q

Los modelos murinos (ratones) de la enfermedad de Huntington exón-1 más conocidos son las líneas R6 (R6/1 y R6/2) y los N171-82Q (Figura 5). Las líneas de ratones R6/1 y R6/2 expresan el exón-1 del gen humano de la proteína mhtt con unas ~115 y ~150 repeticiones CAG respectivamente y con unos niveles de expresión respecto de la htt endógena de un 33% y un 75% respectivamente (Mangiarini y col., 1996). Estos ratones muestran hiperactividad ya a las 3 semanas de edad (Luesse y col., 2001), alteraciones cognitivas posteriores (Lione y col., 1999) hasta presentar alteraciones de coordinación motora a partir de las 7-8 semanas de edad empeorando

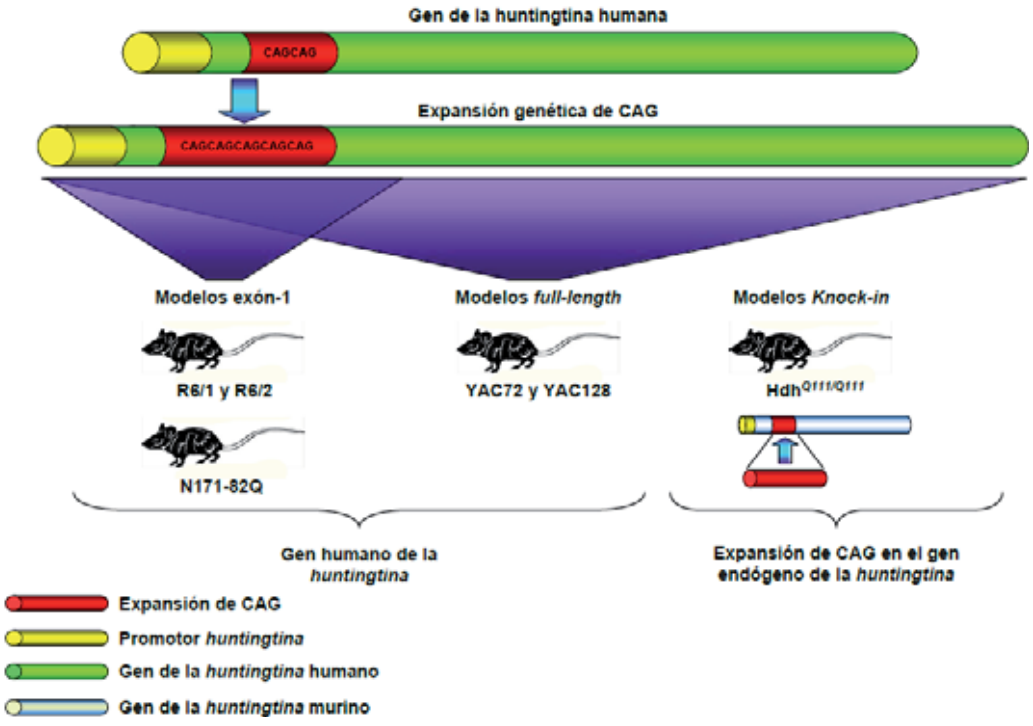


Figura 5. Modelos animales transgénicos y *Knock-in* de la enfermedad de Huntington. En el esquema se pueden contemplar los modelos animales genéticos de la enfermedad de Huntington más estudiados. Por una parte, existen los transgénicos exón-1 (tan sólo expresan una porción N-terminal de la mhtt mutante humana) y los transgénicos *full-length* (que expresan la mhtt humana entera). Ejemplos de los primeros son los modelos R6/1, R6/2 y N171-82Q. Ejemplos de los segundos son los YAC72 y YAC128. Finalmente, existen los modelos *Knock-in* (como por ejemplo los $Hdh^{Q111/Q111}$) los cuáles se les ha insertado una expansión de CAG en el gen murino endógneo de la htt.

progresivamente (Carter y col., 1999) hasta llegar a una hipoactividad clara (Luesse y col., 2001). Estos animales también presentan progresivamente alteraciones de equilibrio, pérdida de peso, movimientos involuntarios, ataques epilépticos, atrofia muscular, cierta incidencia de diabetes y muerte alrededor de las 12-16 semanas de edad (para una revisión ver Li, 2005).

A nivel neuropatológico, los R6/2 padecen una pérdida del 20% del peso cerebral al final de su vida (Davies y col., 1997), aunque la muerte de MSNs en estos ratones es sutil comparado con humanos afectados de la enfermedad de Huntington (Turmaine y col., 2000). Sin embargo, estas células padecen una reducción del 20% del tamaño del soma (Klapstein y col., 2001). Además, estos animales también expresan agregados en muchas regiones cerebrales ya a las 3-4 semanas de edad (Morton y col., 2000; Meade y col., 2002). Cabe señalar que los ratones R6/1 padecen casi los mismos síntomas pero con un retraso de varias semanas o incluso meses.

Los ratones N171-82 fueron generados en 1999 por el grupo del Dr. Borchelt (Schilling y col., 1999). Estos ratones expresan un cDNA con los 171 primeros aminoácidos de la N-terminal de la mhtt humana con 82 repeticiones CAG y con el promotor murino PrP (Schilling y col., 1999) (Figura 5). El desarrollo de la sintomatología empieza a las 4-6 semanas de edad con pérdida de peso y con alteraciones motoras a partir de las 12 semanas de edad. A la vez, van apareciendo agregados nucleares en varias regiones cerebrales, muerte neuronal estriatal y cortical, una astrogliosis marcada y una esperanza de vida de unas 24 semanas (Schilling y col., 1999; Yu y col., 2003).

2.3. Modelos *full-length*: YAC y *Knock-in*

El grupo de M. Hayden generó los ratones full-length llamados YAC (Hodgson y col., 1999). Estos ratones expresan la mhtt humana y regiones reguladoras circundantes en un YAC (del inglés *Yeast Artifical Chromosome*) (Figura 5) con un 75% de expresión respecto a la endógena. Diferentes líneas con diferentes longitudes de repeticiones CAG (YAC46, YAC72 y YAC128) fueron creadas pero aquí nos centraremos tan sólo en la línea YAC128 debido a su fenotipo más marcado (Hodgson y col., 1999; Slow y col., 2005).

La patología en los ratones YAC128 es bastante más lenta y progresiva que la observada en los modelos exón-1. A los 2 meses de edad, estos animales presentan agregados nucleares en el núcleo estriado, hiperactividad locomotora y alteraciones cognitivas leves (Van Raamsdonk y col., 2007a; 2007b; 2005a). La patología avanza progresivamente hasta llegar a los 9-12 meses de edad donde alteraciones en el volumen de diferentes regiones cerebrales, degeneración y muerte neuronal de las MSNs, pérdida de peso corporal y cerebral e hipoactividad locomotora son ya evidentes (Slow y col., 2003; 2005; Van Raamsdonk y col., 2007a; 2007b; 2005b).

Los ratones *Knock-in* se han generado por recombinación homóloga de los tripletes CAG expandidos en el gen endógeno de la htt del ratón (Figura 5) y, por lo tanto serían los modelos más fidedignos de la enfermedad de Huntington (Wang y Qin, 2006; Rubinsztein, 2002; Levine y col., 2004). Aunque se han generado un gran número de líneas de ratones *Knock-in* con diferentes repeticiones (Menalled, 2005), su fenotipo es en general discreto y su evolución lenta (Wang y Qin, 2006; Rubinsztein, 2002; Levine y col., 2004). Una de estas líneas son los ratones *Knock-in* llamados HdhQ111 con 109 repeticiones CAG (Wheeler y col., 2000). Estos ratones no padecen alteraciones motoras claras, no empiezan a mostrar presencia de agregados intranucleares hasta los ~10 meses aproximadamente y cierto grado de degeneración y

gliosis no se observan hasta casi los 20-24 meses de edad (Wheeler y col., 2000; 2002). Otros modelos *Knock-in* como los HdhQ140 y los HdhQ(CAG)150 (de 140 y 150 repecticiones CAG respectivamente) muestran un poco más de fenotipo que los HdhQ111 pero aún así, éste es bastante más ténue que el que muestran los modelos exón-1 (Menalled y col., 2003; Heng y col., 2007; Lin y col., 2001; Tallaksen-Greene y col., 2005).

2.4. Modelos animales emergentes para el estudio de la fisiopatología de la enfermedad de Huntington y terapias potenciales

Con la creación de todos estos modelos animales transgénicos, *Knock-in* y tóxicos descritos anteriormente muchos de los mecanismos basales de la fisiotpaología de la enfermedad fueron caracterizados. Sin embargo, muchos de estos trabajos con estos modelos eran descriptivos y no aislaban de forma satisfactoria los procesos fisiopatológicos entre sí para discernir la importancia de cada uno en el desarrollo de la fisiopatología. Eso limitaba un mayor entendimiento de la enfermedad y la realización de enfoques terapéuticos más adecuados. De ahí la necesidad de la generación de nuevos modelos animales de la enfermedad de Huntington para satisfacer este punto.

Un buen ejemplo ha sido la generación de un animal transgénico que expresa el exón-1 de la mhtt con 94 repeticiones CAG de forma condicional mediante el sistema doxiciclina tTA (Yamamoto y col., 2000). Con este animal se ha podido demostrar que muchos de los elementos fenotípicos de la enfermedad son reversibles tan sólo con la inhibición de la expresión de la mhtt (Yamamoto y col., 2000; Díaz-Hernandez y col., 2005) dando así esperanzas para el enfoque terapéutico. Otro ejemplo ha sido la generación de un ratón que expresa la mhtt con el promotor que se expresa

específicamente en astrocitos llamado GFAP (del inglés *Glial Fibrillary Acidic Protein*) (Bradford y col., 2009; 2010). De esa forma se han podido aislar los efectos que tiene la expresión de esta mutación en los astrocitos y qué implicaciones tienen estas células en el progreso de la enfermedad. También existen modelos donde la mhtt tan sólo se expresa en poblaciones neuronales concretas como, por ejemplo, en neuronas piramidales corticales de la capa V o en interneuronas o en todas ellas (Gu y col., 2005, 2007). Dado que en este trabajo la patología sólo ocurría de forma clara cuando la mutación se encontraba en todos los tipos neuronales, se demostraba así la trascendencia de las alteraciones entre las conexiones neuronales en el transcurso de la fisiopatología de le enfermedad. Previamente, nuestro laboratorio también ha generado un nuevo modelo murino de la enfermedad de Huntington para poder estudiar como los niveles de la neurotrofina llamada BDNF (del inglés *Brain Derived Neurotrophic Factor*) puede regular la severidad de la enfermedad (Canals y col., 2004). Para ello se cruzaron ratones R6/1 (uno de los modelos R6 explicados anteriormente) con animales heterocigotos para la neurotrofina llamada BDNF (Erforns y col., 1994) para poder evaluar esta cuestión (Canals y col., 2004; Pineda y col., 2005). Esos modelos y otros más no citados aquí revelan la necesidad del uso de estas tecnologías para generar nuevos modelos de la enfermedad de Huntington. Estos nos deberían ayudar a obtener una mayor comprensión de la fisiopatología de la enfermedad de Huntington y para un mejor diseño de intervenciones terapéuticas potenciales.

3. La disfunción sináptica excitadora como evento primario en la fisiopatología de la enfermedad de Huntington

Como ya hemos visto, la enfermedad de Huntington es una enfermedad neurodegenerativa progresiva que empieza en la segunda mitad de la edad adulta. Sin embargo, muchos otros síntomas pueden ser detectados antes del inicio de las características alteraciones motoras y coreicas. En esta línea, es muy importante definir y caracterizar los primeros cambios moleculares más sutiles (presumiblemente asociados a los síntomas pre-motores más tempranos) para detectar donde afecta primero y con mayor afinidad la mhtt. Esto nos permitirá centrarnos en las alteraciones con mayor probabilidad de ser iniciadoras de los procesos fisiopatológicos de la enfermedad y no en las que la poseen menor para un mejor diseño de futuras estrategias terapéuticas (Figura 6).

En la presente tesis, postulamos que las alteraciones funcionales en sinapsis excitadoras podrían explicar los primeros síntomas de la enfermedad de Huntington debido a varias razones. Principalmente porqué son unos de los procesos moleculares detectados con mayor precocidad en modelos animales (Cepeda y col., 2007; Fan y

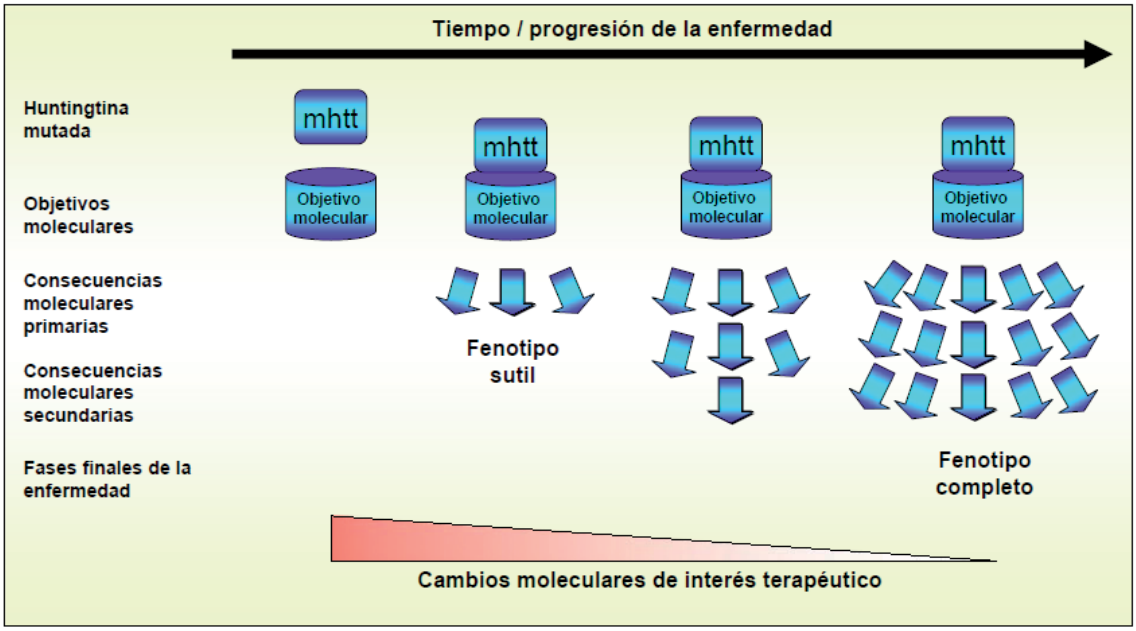


Figura 6. Cascada de eventos moleculares en la enfermedad de Huntington. Esquema hipotético de los eventos progresivos iniciándose con una expansión de poliglutaminas en la proteína htt. Esta expansión dará lugar a una interacción aberrante con los objetivos moleculares intracelulares más susceptibles en presencia de la mhtt. Estas interacciones darán lugar a procesos patológicos secundarios adicionales que se sumarán hasta el desarrollo del fenotipo completo de la enfermedad. Con este esquema en mente, la intervención terapéutica debería centrarse en los procesos con mayor probabilidad de iniciar

la fisiopatología que, a su vez, coincidirían con ser los más tempranos (esquema adaptado de MacDonald y col., 2003).

Raymond, 2007; Lynch 2008). En segundo lugar, porqué muchas de estas alteraciones funcionales sinápticas pueden explicar varios síntomas cognitivos y de memoria (que se cuentan entre los más precoces) tal y como ocurre tanto en pacientes humanos como en modelos animales (Lione y col., 1999; Trueman y col., 2007; van Raamsdonk y col., 2005a; Montoya y col., 2006). También nos pueden explicar gran parte de las alteraciones en la trascripción de genes ampliamente descritos en la enfermedad (Cha, 2007; Sugars y Rubinsztein, 2003; Thomas, 2006). Otro punto muy importante es que la mhtt se expresa en las membranas sinápticas de las sinapsis excitadoras e incluso forma micro-agregados en ellas en edades muy jóvenes en modelos animales (Suopanki y col., 2006). En esta línea, la htt se asocia a gránulos de ARNm dendrítico neuronal modulando su tráfico y su estabilidad (Savas y col., 2010), por lo que tiene potencial de afectar la trascripción *in situ* en sinapsis excitadoras. Adicionalmente, los procesos patológicos sinápticos pueden también explicar otros fenómenos observados en la enfermedad de Huntington como la excitotoxicidad (Fan y Raymond, 2007; Estrada-Sánchez y col., 2008; Dong y col., 2009). En los procesos excitotóxicos, el incremento de Ca^{2+} sostenido iniciaría una cascada de eventos y procesos asociados como la conocida mal función de las mitocondrias en la enfermedad (Panov y col., 2002; Choo y col., 2004; Browne y col., 2004; Tabrizi y col., 1999) y la regulación al alza de caspasas y calpaínas también observadas en la fisiopatología de la enfermedad de Huntington y que podrían resultar en procesos apoptóticos (Hickey y Chesselet, 2003; Bezprozvanny y Hayden, 2004). De forma interesante, el área de investigación que estudia el posible papel del sistema UPS (del inglés *ubiquitin proteasome system*) en la fisiopatología de la enfermedad de Huntington, se encuentra en un punto de amplia controversia (Davies y col., 2007; Ortega y col., 2007). Esta situación ha llevado a apuntar a algunos autores

que, posiblemente, la alteración en el UPS está principalmente localizada en sinapsis (Wang y col., 2008). Así pues, es de vital importancia conocer de manera lo más detallada y amplia posible esas micro-estructuras (sinapsis) primero y luego, analizar dónde la mhtt podría realizar sus efectos perjudiciales.

3.1. Sinapsis excitadoras

La sinapsis se podría entender como la unidad básica de la organización de la circuitería neural (Shepherd y col, 1998). Existen dos categorías principales de sinapsis, las asimétricas (o tipo 1) y las simétricas (o tipo 2). Normalmente, las asimétricas se asocian a sinapsis excitadoras (glutamatérgicas). Como en esta tesis nos hemos centrado en las alteraciones sinápticas plásticas excitadoras y procesos excitotóxicos asociados a la enfermedad de Huntington, revisaremos tan solo las de tipo 1 / excitadoras (Figura 7).

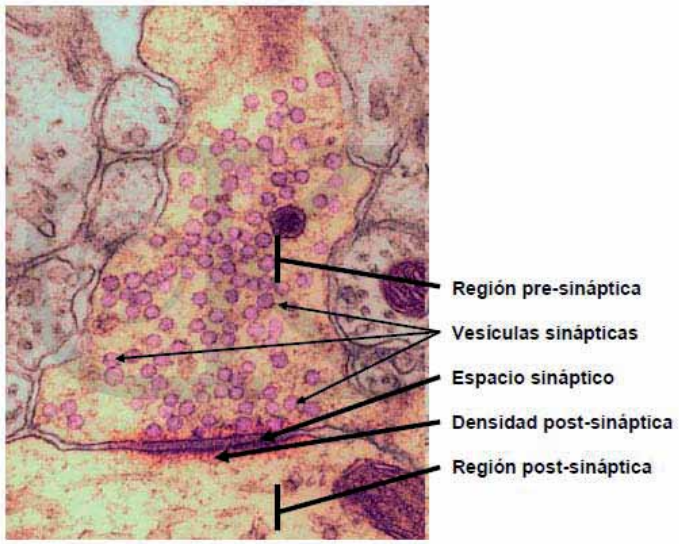


Figura 7. Sinapsis excitatoria. La figura muestra la microfotografía de una sinapsis excitadora del sistema nervioso central pseudo-coloreada (TEM x27,360). Imagen modificada de Dennis Kunkel Microscopy. (en www.DennisKunkel.com).

Precisamente, las MSNs (las neuronas que más degeneran en la enfermedad de Huntington) y las neuronas del hipocampo (la otra región que hemos estudiado en esta tesis) están enriquecidas de tales microestructuras (Shepherd y col, 1998). De hecho, el

85% de todas las sinapsis en el núcleo estriado son excitadoras o de tipo 1 y con espina dendrítica asociada (Kemp y Powell, 1971), mientras que una neurona piramidal típica del hipocampo recibe unas 25.000 sinapsis excitadoras también con su espina dendrítica asociada (Ishizuka y col., 1995). Por lo tanto, es importante ser conscientes de la importancia potencial que tienen estas estructuras en tales regiones cerebrales (Tabla 1). A nivel molecular, la organización de la sinapsis excitadora es altamente compleja, especialmente la densidad post-sináptica o PSD (del inglés *Post-Synaptic Density*) (Figura 8). Así, en la membrana sináptica existen numerosos receptores que responden al ligando liberado por el terminal pre-sináptico o por la astroglía circundante (Volterra y Steinhäuser; 2004; Haydon y Carmignoto, 2006) que inician, a su vez, múltiples cascadas de señalización (Kennedy, 2005). Estas cascadas de señalización ayudarán a iniciar el potencial excitador postsináptico o activarán vías implicadas en múltiples procesos neuronales (Shepherd y col., 1998; Kandel y col., 2001). Como cabe esperar, es de vital importancia que la mayoría de estos receptores y vías de señalización permanezcan en un correcto balance en su actividad (Kennedy, 2005).

Propiedades de superficie de membrana
Es diana de la migración electroforética de membrana
Incrementa la capacitancia de la membrana dendrítica.
Incrementa la distancia inter-sináptica
Compartimiento bioquímico
Absorbe nutrientes
Representa un aislante bioquímico relacionado a una sola sinapsis
Es sitio de síntesis local de proteínas (mediante poliribosomas)
Es sitio de incremento local de calcio
Es neuroprotectivo (aísla la dendrita de niveles tóxicos de calcio)
Unidad de procesamiento temporal
Actúa como detector de coincidencia
Unidad de plasticidad sináptica
Posee capacidad de cambio mecánico rápido
Sitio de LTP/LTD
Modulación de amplitud de PEPSs (Potenciales Excitatorios Post-Sinápticos)

Tabla 1. Múltiples funciones de las espinas dendríticas / sinapsis excitadoras. La figura muestra resumidamente algunos de los roles y propiedades más importantes de las sinapsis excitadoras para la correcta funcionalidad neuronal (adaptado de Shepherd, 1996).

3.2. Receptores de glutamato ionotrópicos

Los receptores de glutamato ionotrópicos son canales transmembrana activados por glutamato enriquecidos en sinapsis excitadoras y que modifican rápidamente las propiedades eléctricas de la membrana mediando los efectos rápidos del glutamato como, por ejemplo, la inducción de corrientes excitadoras post-sinápticas (Mori y col., 1994). Existen dos tipos, los de tipo N-Metil-D-Aspartato (NMDA) y los no-NMDA, cada uno con diferentes subunidades y propiedades estructurales y funcionales.

Los de tipo NMDA son tetrámeros compuestos por al menos una subunidad NR1 que une a glicina, dos o más subunidades NR2A-D que unen a glutamato y NMDA (Cull-Candy, 2001; Mayer y Armstrong, 2004) o alguna subunidad NR3A-B que también unen a glicina (Chatterton y col., 2002). Dependiendo de las subunidades que conformen el receptor NMDA, las propiedades del canal como la permeabilidad

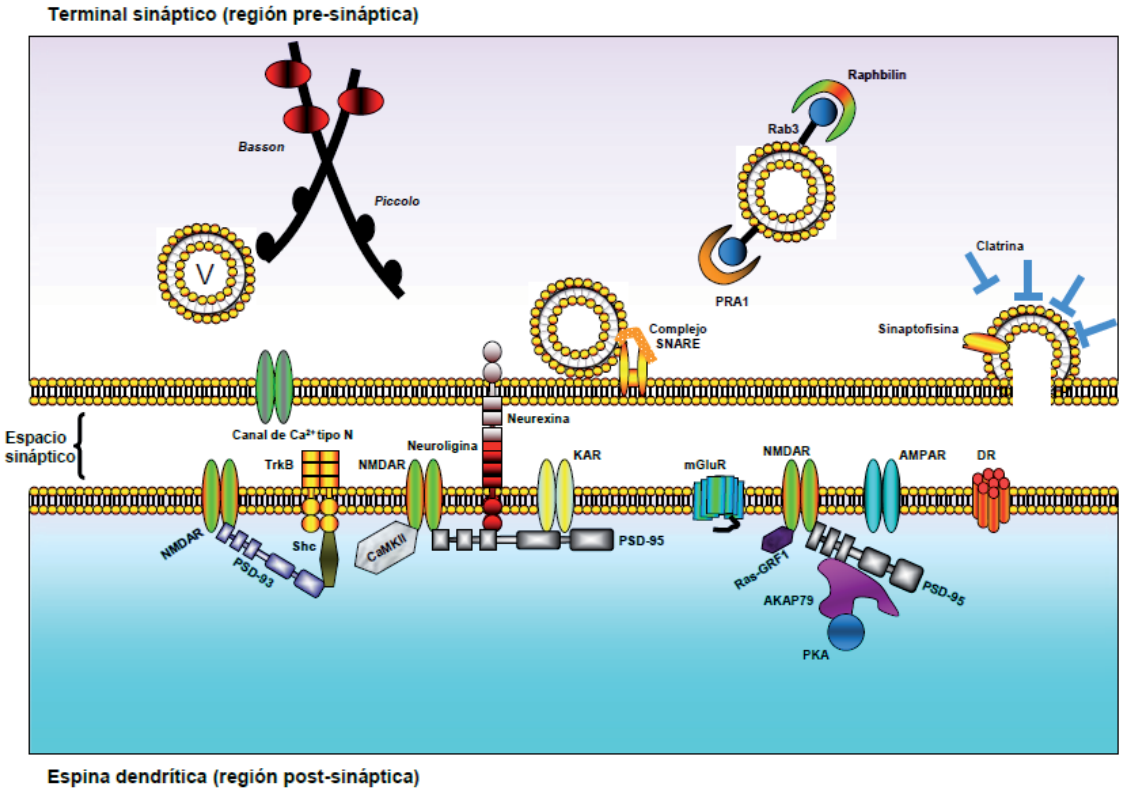


Figura 8. Complejidad molecular de la sinapsis excitadora. La figura muestra esquemáticamente la estructura molecular de una sinapsis excitadora con algunos de sus elementos proteicos más importantes y representativos. TrkB: Tropomyosin-related kinase; PSD-93; PSD-95: Post-synaptic density 93 and 95; NMDAR: N-methyl-D-aspartate receptor; CaMKII: Ca^{2+}/Calmodulin-dependent protein kinase II; KAR: Kainate receptor; mGluR: Metabotropic glutamate receptor; AKAP79/150: *A-kinase associated protein*; PKA: c*AMP-dependent protein kinase*; AMPAR: Alfa-amino-3-hidroxi-5-metil-4-isoxazolepropionato receptor; DR: Dopamine receptor ; SNARE: *Soluble NSF Attachment Protein*; PRA1: prenylated Rab acceptor 1 (adaptado de: Jessel y Kandel, 1993, Kennedy, 2005, Smith y col., 2005).

al Ca^{2+}, la localización en membrana sináptica, la interacción con proteínas intracelulares en su C-terminal y activación de vías de señalización subsiguientes varían en alto grado (Pérez-Otaño y col., 2001, 2006; Cull-Candy y col., 2001; Waxman y Lynch, 2005; Kornau y col., 1995; Dingledine y col., 1999). Los receptores NMDA tienen muchas funciones en la inducción y mantenimiento de la actividad sináptica, la regulación de la expresión de genes (Rao y Finkbeiner, 2007) y de la expresión de fenómenos plásticos como la LTP y la LTD (Bliss y Collingridge, 1993). Sin embargo, una desregulación (en ese caso, una sobre-activación) de estos receptores, al ser altamente permeables al Ca^{2+} (Ozawa y col., 1998), pueden inducir a procesos excitotóxicos (Lipton y Rosenberg, 1994).

Los receptores de glutamato ionotrópicos de tipo no-NMDA se subdividen en receptores AMPA (Alfa-amino-3-hidroxi-5-metil-4-isoxazolepropionato) y kainato. Los receptores AMPA tienen al AMPA como ligando y pueden ser compuestos de 4 subunidades distintas (GluR1-4) con distintas propiedades cada una para formar tretrámeros funcionales (Palmer y col., 2005). Estos receptores permiten el flujo de iones de potasio y sodio a través de la membrana produciendo así el potencial excitatorio post-sináptico (PEPS) (Kennedy, 2000), lo que los sitúa como iniciadores de la transmisión sináptica rápida. Una sobre-activación de los receptores AMPA puede llevar al desarrollo de procesos de excitotoxicidad neuronal debido a su regulación de la permeabilidad al Ca^{2+} (Jayakar y Dikshit, 2004). Finalmente, los receptores kainato con el kainato como su ligando específico, pueden poseer subunidades GluR5-7 y KA1-2 siendo necesarias las primeras para formar canales funcionales (Safferling y col., 2001; Pinheiro and Mulle, 2006). También esos receptores son capaces de modular en alto grado procesos de excitotoxicidad (Wang y col., 2005).

3.3. Receptores acoplados a proteína G

La superfamilia de los receptores asociados a proteína G o GPCRs (del inglés *G Protein Coupled Receptors*) son proteínas transmembrana que, cuando se les une su ligando específico, traducen la señalización mediante la producción de segundos mensajeros intracelulares. De entre todos los GPCRs, en la enfermedad de Huntington reciben especial interés los receptores de glutamato metabotrópicos (mGluR1-mGluR5) y los receptores de dopamina (tipo D1 y tipo D2) debido al gran número de investigaciones que los implican en la fisiopatología de la enfermedad de Huntington (Calabresi y col., 1999; Schiefer y col., 2004; Anborgh y col., 2005; Ribeiro y col., 2010; Charvin y col., 2005; Benchoua y col., 2008; Cyr y col., 2006). Los GPCRs pueden estar acoplados a proteínas G activadoras o inhibidoras del adenilato ciclasa (AC) la cuál regula positiva o negativamente los niveles de cAMP (Gainetdinov y col., 2004). Por otra parte, los GPCRs también pueden activar la vía del IP3/DAG vía fosfolipasa C gamma (o PLCγ del inglés *phospholipase C gamma*) y regular la liberación del calcio intracelular (Gainetdinov y col., 2004). De esta forma, en sinapsis excitadoras, los GPCRs, especialmente los mGluRs y también los receptores de dopamina, pueden funcionar como potentes neuromoduladores de la señalización sináptica, plasticidad neuronal y procesos excitotóxicos (Tang y col., 2007; Calabresi y col., 1999; Ribeiro y col., 2010; Ronesi y Huber, 2008; Surmeier y col., 2007; Wickens y col., 2007; Benchoua y col., 2008).

3.4. Proteínas de anclaje a la densidad post-sináptica: Las MAGUKs

Los primeros objetivos potenciales del calcio que entra por los canales de los receptores NMDA o activadas las cascadas de los GPCRs son las proteínas localizadas en la densidad post-sináptica o PSD (Kennedy, 2000). La organización estructural y funcional de la PSD depende de varias familias de proteínas de andamiaje de las cuáles, la superfamilia de las MAGUKs (del inglés *membrane-associated guanylate kinases*) es la más conocida (Montgomery y col., 2004). De la superfamilia de las MAGUKs, la subfamilia DLG es la más estudiada (Kim y Sheng, 2004). Esta subfamilia está compuesta por las proteínas PSD-95 (del inglés *post-synaptic density 95*), SAP102, SAP97 (del inglés *synaptic associated protein 102 y 97*) y Chapsyn-110/PSD-93 (del inglés *post-synaptic density 93*) (Garner y Kindler, 1996) (Figura 9).

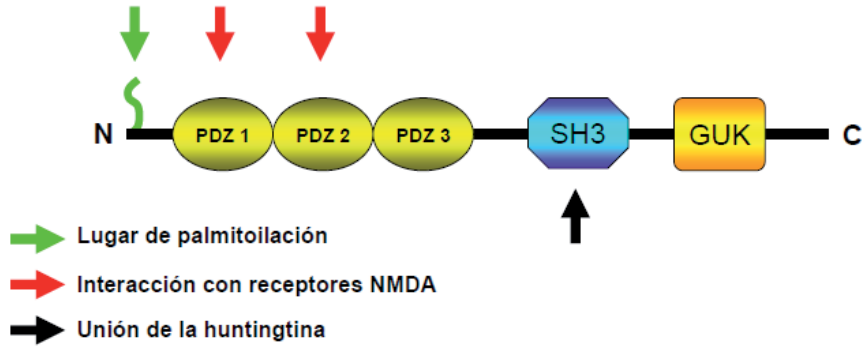


Figura 9. Las MAGUK. La figura muestra esquemáticamente la estructura principal de las MAGUK (de la subfamilia DLG). Desde el extremo N-terminal se distinguen tres dominios PDZ, un dominio SH3 y un dominio GUK catalíticamente inactivo. La flecha negra indica el sitio de unión a la htt y las rojas los sitios de unión de los receptores NMDA (esquema adaptado de Montgomery y col., 2004; Kim y Sheng, 2004).

Siguiendo el esquema de la Figura 9, cada una de ellas posee tres dominios PDZ que permiten interaccionar con moléculas como los receptores NMDA (Kornau y col., 1995; Muller y col., 1996) o proteínas como nNOS (del inglés *neural nitric oxide synthease*) (Brenman y col., 1996), synGAP (del inglés s*ynaptic Ras GTPase activating protein*) (Kim y col., 1998) o a la subunidad GluR1 (Cai y col., 2002). Las MAGUKs también poseen un dominio SH3 (del inglés *Src homology domain 3*) y un dominio

catalíticamente inactivo GUK (del inglés *guanylate kinase*) que es capaz de anclar diversas proteínas estructurales (Brenman y col., 1998; Colledge y col., 2000). Las MAGUKs PSD-95 y PSD-93 son las más abundantes en la PSD mientras que SAP97 y SAP102 son más citosólicas e implicadas en tráfico (El-Husseini y col., 2000; Sans y col., 2001; Rumbaugh y col., 2003; Valtschanoff y col., 2000). Las presencia de MAGUKs como la PSD-95 en la PSD influye en procesos como el promedio de apertura del canal de receptores NMDA (Lin y col., 2004), modulan fenómenos plásticos como la LTP (Migaud y col., 1998) y regulan la vía de señalización de muerte celular NMDA-nNOS-p38 (Sattler y col., 1999; Cao y col., 2005) además de muchos otros pocesos neuronales (para revisiones ver Kennedy, 2005; Calabrese y col., 2006; Yamauchi, 2002).

3.5. Vías efectoras de receptores NMDA y GPCRs en sinapsis excitadoras

En las sinapsis excitadoras y, más concretamente, en su localización o microdominios llamados espinas dendríticas, existe una amplia riqueza de vías de señalización. De hecho, existe una amplia variedad de unos ~200 tipos de proteínas en este espacio de 1-2 μm^3 (Grant y Husi, 2001; Husi y col., 2000). En este espacio existe un equilibrio entre las actividades fosfatasas y cinasas que, debido a su complejidad, la mínima alteración de una de ellas podría conllevar desde alteraciones en la funcionalidad a la degeneración y muerte de la neurona (Kennedy, 2005; Waxman y Lynch, 2005; Lynch y Guttmann, 2002). Los receptores NMDA junto con la correcta funcionalidad de las proteínas de andamiaje como las MAGUKS regulan un sinfín de vías de señalización moleculares en las sinapsis excitadoras como ERK1/2 vía Ras-GRF1 (Oh y col., 2004), p38 (Sattler y col., 1999; y Cao y col., 2005) y αCaMKII (del

inglés *α Ca^{2+}/Calmodulin-dependent protein kinase II*) (Colbran, 2004). Los receptores NMDA también activan fosfatasas como la calcineurina vía Ca^{2+}/Calmodulina (Guerini y col., 1997). Por otra parte, los GPCRs como los receptores de dopamina (tipo 1 ó 2) o los mGluR pueden activar o inhibir vías como la de la PKA, la PLCγ (Mao y col., 2008; Bergson y col., 2003; Jay, 2003) y, recientemente descrita, la vía de la fosfatasa STEP (del inglés *STriatal Enriched tyrosine phosphatase Protein*) (Braithwaite y col., 2006). Debido al enriquecimiento de STEP en el núcleo estriado y al estar regulada por vías alteradas en la enfermedad de Huntington, su estudio podría ser de alto interés. Esta fosfatasa puede ser activada por defosforilación mediada por la calcineurina (previamente activada por receptores NMDA) (Paul y col., 2003) o regulada por GPCRs (principalemente receptores de dopamina) debido a que puede ser fosforilada por PKA y así pasar a ser inactiva (Paul y col., 2000). STEP puede controlar procesos de aprendizaje, de plasticidad sináptica y distintos estímulos neurotóxicos como por ejemplo, neurotoxicidad inducida por estados convulsivos en neuronas del hilo en el hipocampo (Paul y col., 2007, Choi y col., 2007).

3.6. Alteraciones en sinapsis excitadoras en la enfermedad de Huntington: Disfunciones en la plasticidad sináptica y procesos excitotóxicos

Las investigaciones sobre las alteraciones sinápticas en la enfermedad de Huntington son amplias, realizadas en distintas regiones, e implican tanto procesos de excitotoxicidad como de plasticidad neuronal. En esta línea, se han descrito niveles alterados de distintas proteínas enriquecidas en sinapsis tanto cortico-estriatales como hipocampales (Tabla 2). Sin embargo, una de las formas más usadas y validadas de analizar el estado y funcionalidad de las sinapsis es mediante su estudio a nivel

electrofisiológico. Esta ha sido la metodología principalmente empleada. De estos estudios, lo que parece claro es que son las sinapsis excitadoras las que parecen padecer mayores alteraciones (Fan y Raymond, 2007; Cepeda y col., 2007; Li y col., 2003) y que existen dos líneas principales, las que estudian las sinapsis cortico-estriatales y las hipocampales.

Proteínas sináptica	Nombre completo del inglés	Región cerebral estudiada	Referencia
D1R	*Dopamine receptor type-1*	Estriado	Ariano y col., 2002
D2R	*Dopamine receptor type-2*	Estriado	Ariano y col., 2002
NR2A-B	*N-metyl-D-aspartate receptor subunit 2A-B*	Hipocampo	Luthi-Carter y col., 2002
mGluR1, 3 y 5	*Metabotropic glutamate receptor 1, 3 and 5*	Estriado	Cha y col., 1998
Rabphilin 3A	*Rabphilin 3A*	Estriado	Smith y col., 2007; Smith y col., 2005
PSD-95	*Post-synaptic density-95*	Estriado, hipocampo	Jarabek y col., 2004; Nithianantharajah y col., 2008
TrkB	*Tropomyosin kinase receptor*	Estriado	Ginés y col., 2006
STEP	*STriatal-Enriched tyrosine Phosphatase*	Estriado	Desplats y col., 2006
Complexina II	*Complexin II*	Hipocampo	Morton y col., 2001
GluR5-6	*Glutamate receptor 5-6*	Estriado	Cha y col., 1998
PACSIN1	*Protein kinase C and casein kinase substrate in neurons protein 1*	Estriado	Modregger y col., 2002
GluR1	*α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor subunit 1*	Hipocampo	Nithianantharajah y col., 2008
SNAP25	*Synaptosomal-assoicated protein 25*	Estriado	Smith y col., 2007

Tabla 2. Alteraciones en la expresión de proteínas específicas de sinapsis excitadoras en la enfermedad de Huntington.

A nivel de conexiones cortico-estriatales, se ha descrito que en modelos murinos de la enfermedad de Huntington se produce una progresión bifásica en lo que concierne a las conductancias de calcio siendo durante las primeras etapas incrementadas y en las subsiguientes progresivamente reducidas (Cepeda y col., 2001; Klapstein y col., 2001, Levine y col., 1999). Los receptores de glutamato ionotrópicos regularían esas disfunciones siendo los NMDA los más representativos (Levine y col., 1999; Starling y

col., 2005) mientras que los AMPA no padecerían tantas alteraciones funcionales (Andre y col., 2006).

Estas alteraciones electrofisiológicas cortico-estriatales se han asociado a fenómenos relacionados con la susceptibilidad a la excitotoxicidad que intentarían explicar la muerte selectiva de las MSNs. La excitotoxicidad implica una sobre-activación de receptores glutamatérgicos que inducen la muerte neuronal (Arundine y col., 2003). Aunque la excitotoxicidad vía receptores NMDA ha sido considerada de los procesos principales en la fisiopatología de la enfermedad de Huntington (Fan y Raymond, 2007), múltiples modelos animales y celulares han aportado conclusiones contradictorias (Zuchner y Brundin, 2007). No ha sido hasta años recientes en que se ha empezado a diseñar un modelo aceptable y que sea integrador. Así, dependiendo de la edad en que se estudien los procesos excitotóxicos y del modelo animal o celular más o menos severo (exón-1 o *full-length* respectivamente), los investigadores se han encontrado con mayor, menor o ausencia de susceptibilidad ante procesos excitotóxicos (Xifró y col., 2008; Zeron y col., 2002; Shehadeh y col., 2006; Hansson y col., 1999; Jarabek y col., 2004). Recientemente, el grupo de M. Hayden demostró que en los modelos murinos *full-length* llamados YAC128, éstos eran susceptibles a la excitotoxicidad en edades primerizas mientras que, conforme envejecían, se volvían resistentes a dicho fenómeno (Graham. y col., 2009). Esto coincide con una sobre-activación o hiper-función de los receptores NMDA en modelos celulares y en ratones *full-length* jóvenes y también coincide con la hipo-función y respuesta reducida ante agentes excitotóxicos en modelos exón-1 o full-length adultos (Xifró y col., 2008; Zeron y col., 2002; Shehadeh y col., 2006; Hansson y col., 1999; Jarabek y col., 2004). Así, el modelo más aceptable es que sólo durante un breve período del inicio de la vida

en portadores de la mhtt imperaría una susceptibilidad aumentada a la excitotoxicidad pero que, rápidamente, la susceptibilidad se transformaría en resistencia.

Se postula que estos fenómenos se deberían a una desconexión progresiva cortico-estriatal pero no se sabe exactamente cómo (Cepeda y col., 2007; Spires y col., 2004; Guidetti y col., 2001). Aunque en todos estos procesos comentados hasta ahora se ha implantado un modelo en que los receptores NMDA parecen ser el eje del esquema, Diversos trabajos indican que su localización y niveles de expresión están poco alterados (Hansson y col., 1999; Jarabek y col., 2004). Así, aunque sus propiedades bioeléctricas sí parecen estar alteradas (Cepeda y col., 2007; Fan y Raymond, 2007), se ha especulado ampliamente sobre un posible papel potencial en las vías moleculares situadas justo por debajo de su activación e incluso de vías moduladoras de estos receptores. En este sentido han cobrado interés proteínas enriquecidas en sinapsis excitadoras como PSD-95, PACSIN1, calcineurina, Cdk5 y p53 entre otras (Jarabek y col., 2004; Sun y col., 2001; Xifró y col., 2008; Hernández-Espinosa y Morton, 2006; Pardo y col., 2006; Paoletti y col., 2008; Steffan y col., 2000; Modregger y col., 2002).

A nivel hipocampal, también se han realizado múltiples estudios electrofisiológicos en las sinapsis excitadoras y los procesos plásticos siendo ambos el foco de interés. Así, se ha demostrado que los fenómenos plásticos más aceptados como modelos celulares del aprendizaje y la memoria como la LTP y la LTD (Bliss y Collingridge, 1993; Lynch, 2004; Gladding y col., 2009; Behr y col., 2009) están alterados en diversos modelos animales de la enfermedad de Huntington, en diferentes sub-regiones de la formación hipocampal y a edades muy tempranas (Milnerwood y col., 2006; Cummings y col., 2006, 2007; Murphy y col., 2000; Lynch y col., 2007; Hodgson y col., 1999; Kung y col., 2007). Estos resultados se han replicado incluso en el hipocampo de ratones *Knock-in* jóvenes sugiriendo la existencia de alteraciones en la

formación y motilidad de las espinas dendríticas (Lynch y col., 2007). De forma coincidente, las disfunciones en los procesos de aprendizaje y memoria dependientes del hipocampo en estos modelos también se pueden observar de forma similarmente temprana (Trueman y col., 2007; Simmons y col., 2009; Van Ramsdoonk y col., 2005; Lione y col., 1999; Mazarakis y col., 2005; Nithianantharajah y col., 2008).

Adicionalmente, las disfunciones sinápticas expuestas aquí también podrían ser reguladas por un funcionamiento aberrante de la astroglía circundante, ya sea debido a una mala regulación de los niveles de glutamato en sinapsis y/o por una liberación deficiente de factores tróficos (Chou y col., 2008; Shin y col., 2005; Bradford y col., 2009; 2010).

Es tentativo sugerir que los mecanismos implicados en las alteraciones de plasticidad en sinapsis excitatorias también median los procesos excitotóxicos y posterior desconexión sináptica. Sin embargo, no existe un modelo que explique e integre los fenómenos excitotóxicos desde la susceptibilidad hasta la resistencia y desconexión sináptica (Zuchner y Brundin, 2007). Menos aún, que también explique e integre al mismo tiempo las alteraciones de plasticidad neuronal observadas en los modelos animales y en pacientes de la enfermedad de Huntington. Ante tal disyuntiva, es lógico proponer el estudio de moléculas que se encuentren enriquecidas en sinapsis excitatorias y que sean importantes para su correcta funcionalidad. También es imprescindible que las regiones cerebrales más afectadas en la enfermedad de Huntington (núcleo estriado, corteza cerebral e hipocampo) estén enriquecidas de tales moléculas o sean dependientes de su presencia. Estas moléculas, además, deben controlar vías de señalización tanto de plasticidad neuronal, memoria y aprendizaje como de supervivencia *versus* la muerte neuronal. Finalmente, su alteración debe ser temprana para explicar los primeros síntomas de la enfermedad de Huntington. Estos

argumentos nos llevan a proponer a una neurotrofina como uno de los principales candidatos, nos referimos al factor neurotrófico derivado del cerebro o BDNF.

4. Disfunción neurotrófica en la enfermedad de Huntington: Papel del BDNF

Durante los últimos años, en el intento de explicar la fisiopatología de la enfermedad de Huntington ha cobrado mucha fuerza el paradigma de la alteración en el rol de los factores neurotróficos (Alberch y col., 2002). En concreto, el interés lo ha recibido un miembro de la familia de las neurotrofinas, el BDNF (Zucatto y Cattaneo, 2007). El hecho de que BDNF esté involucrado en múltiples procesos neuronales imprescindibles como la supervivencia neuronal y la plasticidad sináptica (Lu y col., 2000; Reichardt, 2006) y que en la enfermedad de Huntington se hayan propuesto estos procesos como fundamentales en su fisiopatología (Zuccato y Cattaneo, 2007, 2009), hacen de la neurotrofina uno de los candidatos más importantes en el estudio de la enfermedad y posibles tratamientos.

4.1. BDNF, miembro de la familia de las neurotrofinas

BDNF pertenece a la familia de las neurotrofinas. Esta familia está compuesta por el NGF (del inglés *nerve grow factor*), BDNF, NT-3 (*nerotrophin-3*) y NT-4/5 (*neurotrophin-4/5*) (Barbacid y col., 1993). Estas neurotrofinas, cuando son liberadas al medio extracelular, pueden unirse a sus respectivos receptores de alta afinidad llamados Trk (del inglés *tropomyosin-related kinase*). Así, NGF se uniría con alta afinidad a TrkA, BDNF a TrkB i NT-3 a TrkC (revisado en Ibáñez, 1998; Barbacid y col., 1993). Alternativamente, algunas de las neurotrofinas pueden mostrar promiscuidad en su unión a más de un tipo de receptor (Ibáñez, 1998). Además, existe el receptor p75 al

cual se unen con baja afinidad a todas las neurotrofinas cuando se hallan sin procesar (Chao y col., 1992) activando procesos totalmente diferentes a los que realizarían las formas maduras como, por ejemplo, vías de muerte neuronal y depresión sináptica (Dechant, y Barde, 1997; Lu y col., 2003; Woo y col., 2005; Teng y col., 2005). Una vez activados los receptores, en su extremo intracelular, que están enriquecidos de dominios tirosina quinasa, éstos dimerizan y se autofosforilan dando lugar a la activación de tres cascadas de señalización principales: La vía, PI(3)K-Akt, la vía fosfolipasa C gamma (PLCy) y la vía Ras-Raf-ERK1/2 (de las siglas *extracelular regulated kinase 1/2*) (revisado en: Reichardt, 2006; Ernfors y Bramham, 2003) (Figura 10). Cada una de ellas está involucrada de forma vital en la supervivencia neuronal y/o plasticidad sináptica (Kalcheim, 1996; Huang y Reichardt, 2001; Reichardt, 2006;

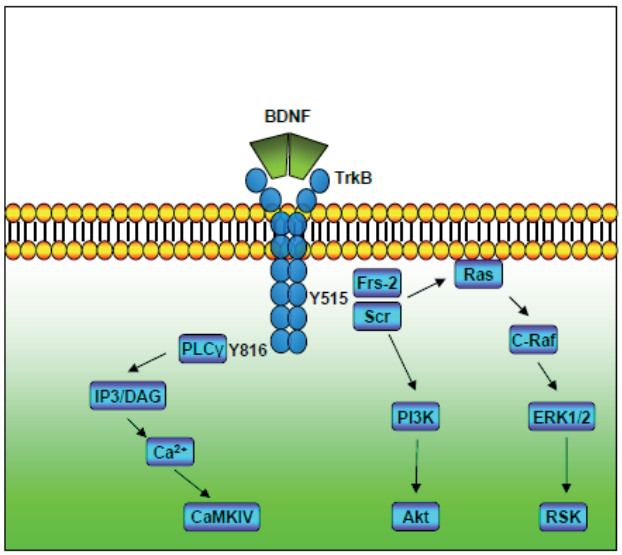


Figura 10. Vías de señalización activadas por BDNF. Cuando dos moléculas de BDNF dimerizan se vuelven biológicamente activas y se unen a su receptor específico TrkB (también dimerizado) en el extremo N-terminal extracelular. Ante esa interacción, se activan tres vías principales debido a las múltiples fosforilaciones de tirosinas localizadas en el extremo C-terminal intracelular. Las tres vías son: la vía de la PLCγ-IP3/DAG-CaMKIV, otra es la de PI3K-Akt y la tercera corresponde a la vía Ras-Raf-ERK1/2-RSK. Cada una de ellas está implicada en múltiples procesos, entre ellos, de plasticidad neuronal y/o supervivencia. Abreviaturas (del inglés): BDNF (*brain derived neurotrophic factor*), TrkB (*tropomyosin-related kinase B*), Y515 (*tyrosin 515*), Y816 (*tyrosin 816*), ERK1/2 (*external regulated kinase 1/2*), RSK (*ribosomal S6 kinase*), PI3K (*phosphoinositaide-3 kinase*), PLCγ (*phospholipase C gamma*), IP3 (*inositol (1,4,5)-triphosphate*), DAG (*diacylglycerol*), CaMKIV (Ca^{2+}*-calmodulin dependent kinase IV*) (esquema adaptado de Ernfors y Bramham (2003)).

Ernfors y Bramham, 2003). De esta manera, la señalización de BDNF, vía su receptor específico TrkB, ha sido largamente descrita como una de las más importantes vías en plasticidad sináptica y de supervivencia en el sistema nervioso central (Reichardt, 2006; Ernfors y Bramham, 2003).

4.2. Regulación, localización y función del BDNF

El gen del BDNF (de los roedores) contiene al menos 9 exones, algunos de ellos con distintos promotores que llevan a la trascripción de ARN con diferentes regiones no codificantes 5' y dos distintas 3' que difieren en longitud a causa de la existencia de otros dos sitios diferentes de poliadenilación (Aid y col., 2007; Timmusk y col., 1993). Esta complejidad conlleva a la producción de cómo mínimo 18 productos transcritos de BDNF distintos capaces de codificar la misma proteína (Aid y col., 2007). Así pues, en una misma neurona, diferentes vías de señalización podrían activar la producción de BDNF de una forma también distinta. El objetivo de esta forma de regulación podría ser, con mucha probabilidad, el de activar la producción de la proteína de formas distintas dependiendo de la vía de señalización molecular activada que la inicia (Lu y col., 2003). A nivel de trascripción de BDNF, su regulación puede depender de dos vías principales. El factor de transcripción CREB (de las siglas en inglés *CRE Response Element Binding*) mediatiza la regulación dependiente de actividad de los promotores I y IV (Chen y col., 2003; Tabuchi y col., 2002; Tao y col., 1998; Shieh y col., 1998), mientras que el complejo REST/NRSF (del inglés *RE-1 silencing transcription factor/neuron-restrictive silencer factor*) reprime la expresión del BDNF desde el promotor II (Zuccato y col., 2003).

Una vez el ARNm de BDNF llega al proceso de traducción, éste es sintetizado como un precursor de la proteína aún inmadura (Seidah y col., 1996). Después de su

ensamblaje dentro de las vesículas del aparato de Golgi, la neurotrofina es introducida en las vesículas y estas se dirigiren hacia la vía de secreción regulada o la vía de secreción constitutiva (Farhadi y col., 2000; Mowla y col., 1999) (Figura 10). Al menos en neuronas hipocampales la vía regulada parece el origen principal de la secreción total de BDNF en neuronas, al contrario de lo que ocurre con otras neurotrofinas como la NT-3 y el NGF las cuales son secretadas predominantemente por la vía constitutiva de secreción celular (Mowla y col., 1999; Farhadi y col., 2000). El proBDNF puede ser procesado a su forma madura (y bioactivo) disociando el domino pro del dominio propio de BDNF intracelularmente mediante la pro hormona convertasa furina (Seidah y col., 1996) o, principalmente, en el espacio extracelular por la plasmina previamente activada por la tPA (del inglés *tissue plasminogen activator*) (Pang y col., 2004).

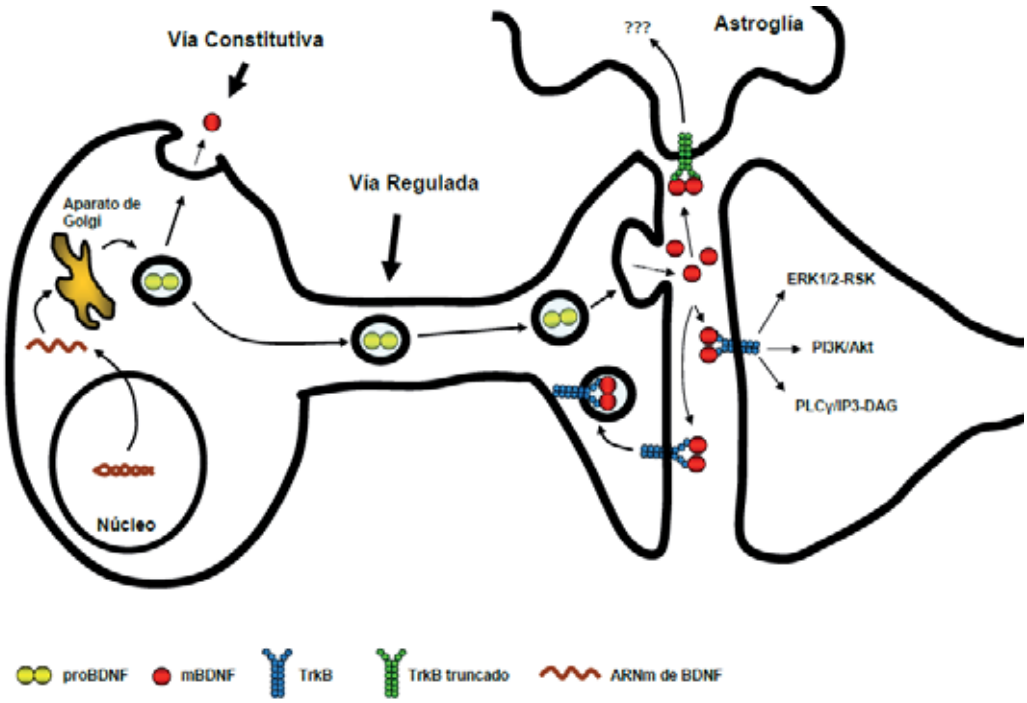


Figura 11. Regulación y mecanismos de acción del BDNF. La figura muestra esquemáticamente la regulación a todos los niveles de BDNF. Después de una compleja modulación de la trascripción de BDNF (ver texto), éste es introducido en vesículas dentro del aparato de Golgi en un estado inmaduro (proBDNF). Posteriormente, es trasladado tanto por la vía constitutiva y liberado de forma contínua o, por lo contrario, viajará a través de las prolongaciones neuronales hasta llegar a su destino y será liberado de forma regulada bajo el efecto de algún tipo de estímulo neural. Al ser liberado, BDNF será procesado enzimáticamente llegando así, a su forma madura (mBDNF) y se unirá a su receptor de alta afinidad TrkB (ya sea la isoforma completa o truncada). De esta forma, BDNF mediará sus efectos principales al regular las vías PI3K-Akt, PLCγ-IP3/DAG o Ras-Raf-ERK1/2. ERK1/2 (*external*

regulated kinase 1/2), PI3K (*phosphoinositaide-3 kinase*), PLCγ (*phospholipase C gamma*), IP3 (*inositol (1,4,5)-triphosphate*), DAG (*diacylglycerol*), RSK (*ribosomal S6 kinase*).

BDNF es la neurotrofina más expresada en el sistema nervioso central y la más estudiada durante los últimos años (Murer, 2001). Se expresa en sus máximos niveles en el cerebro anterior siendo el hipocampo y la corteza cerebral las principales regiones y siguiéndoles desde relativamente cerca el núcleo estriado, el cerebelo, el cerebro medio y el hipotálamo (Murer y col., 2001; Hofer y col., 1990). En la corteza cerebral, las neuronas piramidales de la capa V son las que presentan mayores niveles de expresión de BDNF (Pitts y col., 2000; Pitts y col., 2000; Murer y col., 1999), que curiosamente es de donde surgen la mayoría de proyecciones que recibirá el núcleo estriado (Gerfen, 1992). Por otro lado, aunque haya altos niveles de la neurotrofina en todo el hipocampo son las neuronas granulares del hilo del giro dentado y las piramidales de la CA3 las que más expresan BDNF (Conner y col., 1997; Yan y col., 1997; Yang y col., 2009; Pitts y col., 2000; Murer y col., 1999; Conner y col., 1997). Contrariamente, en el núcleo estriado, debido a la baja expresión local de la neurotrofina (deduciéndolo de los niveles de mRNA), su aporte principal viene proporcionado anterógradamente desde la corteza cerebral (Altar y col., 1997; Baquet y col., 2004). Finalmente, aunque BDNF se expresa mayoritariamente en neuronas, también se expresa, aunque a bajos niveles, en células de Schwann (Acheson y col., 1991), microglía (Elkabes y col., 1996), oligodendrocitos (Riley y col., 2004) y astrocitos (Dougherty y col., 2000; Condorelli y col., 1994).

A nivel subcelular en neuronas, BDNF se expresa en soma y dendritas pero no en el núcleo (Furukawa y col., 1998; Kawamoto y col., 1999; Ferrer y col., 1999; Murer y col., 1999). BDNF también se localiza en sinapsis excitadoras, tanto pre- como postsinápticamente (Hartmann y col., 2001; Kohara y col., 2001; Kojima y col., 2001). A su vez, el BDNF colocaliza con su receptor TrkB en tales sinapsis excitadoras

(Carvalho y col., 2008), lo que sugiere que posee funciones como modulador bidireccional en tales estructuras. De forma similar, el BDNF también se expresa en sinapsis inhibidoras (o GABAérgicas) tanto a nivel pre- como postsináptico (Wardle y Poo, 2003).

4.3. Función del BDNF en procesos de memoria, aprendizaje y plasticidad sináptica

En los últimos años se ha relacionado el BDNF con un importante papel en la plasticidad sináptica, concretamente, en fenómenos como la potenciación sináptica, la formación y la dinámica de las espinas dendríticas y con la formación y el mantenimiento de nuevas memorias y procesos de aprendizaje (Lu, 2003; Genoud y col., 2004; Kuipers y Bramham, 2006; Bramham y Messaoudi, 2005; Bekinschtein y col., 2008). En ese sentido, ratones heterocigotos para BDNF muestran alteraciones en aprendizaje espacial en el laberinto de Morris (Linnarson y col., 1997). Ratones que sobre-expresan TrkB muestran un aprendizaje facilitado en el laberinto de Morris y en el condicionamiento de aversión al sabor (Koponen y col., 2004). Además, la inhibición amigdalar de BDNF impide a los ratones a aprender tareas asociativas al miedo (Alonso y col., 2005). Por otra parte, diferentes tipos de aprendizaje motor, espacial y el ejercicio físico intenso aumentan BDNF y su receptor específico en las regiones cerebrales correspondientes (Gomez-Pinilla y col., 2001; Hall y col., 2000; Johnson y col., 2003; Klintsova y col., 2004). En humanos, polimorfismos en el gen de BDNF como el de la substitución de la metionina por valina en el codón 66 (Val66Met), mutación que altera la secreción dependiente de actividad de BDNF (Chen y col., 2004, 2005), podría contribuir a algunos aspectos fisiopatológicos de la depresión y ansiedad e induciría a una reducción en el volumen hipocampal y a tareas cognitivas asociadas

(Chen y col., 2006; Egan y col., 2003; Bath y Lee, 2006; Hariri y col., 2003; Cheeran y col., 2008).

¿Cómo regula BDNF la plasticidad y los procesos de aprendizaje subyacentes? Previamente hay que recalcar que, por una parte, el fenómeno llamado LTP y por otra, que la motilidad y proliferación de espinas dendríticas neuronales son ambos dos de los paradigmas funcionales y morfológicos con mayores evidencias que sirven para explicar la adquisición, formación y mantenimiento de memorias (Bliss y Collingridge, 1993; Lynch, 2004; Lynch y col., 2008; Bourne y Harris, 2008). En las líneas siguientes veremos que BDNF tiene un papel muy importante en estos dos procesos plásticos.

En paradigmas de LTP, se han descrito un déficit en la inducción y el mantenimiento de tal fenómeno en preparaciones hipocampales de ratones deficientes para el BDNF (Korte y col., 1998; Patterson y col., 1996). También con el uso de proteínas de fusión como la TrkB-imunoglobulina G se ha demostrado atenuación de LTP (Minichiello y col., 1999; Xu y col., 2000). En sentido contrario, la aplicación de BDNF en rodajas de hipocampo hacía más robusta la LTP en la región CA1 (Kang y col., 1997). Por otra parte, para la inducción de la LTP, la señalización BDNF-TrkB es tan importante a nivel pre-sináptico como post-sináptico (Gruart y col., 2007; Gartner y col., 2006). A nivel presináptico, el BDNF ayudaría al mantenimiento de la liberación continuada de glutamato (Yano y col., 2006). A nivel post-sináptico se han descrito aún más posibles mecanismos de BDNF en la plasticidad sináptica como el de facilitar la inserción de GluR1 en la membrana sináptica (Caldeira y col., 2007a), de iniciar la activación de PLCγ (Minichielo y col., 2002) y regular y potenciar la síntesis de proteínas *in situ* (Aakalu y col., 2001) como αCaMKII, NR1 y Arc, posiblemente, vía activación de la cascada mTOR-PI3K (Takei y col., 2004; Schratt y col., 2004). En los últimos años, múltiples aproximaciones farmacológicas y genéticas han permitido

distinguir entre una función *permisiva* y otra *instructiva* de BDNF en la función sináptica excitadora (para revisiones más extendidas ver Schinder y Poo, 2000; Bramham y Messaoudi, 2005). El concepto permisivo se refiere al rol de BDNF en habilitar a las sinapsis para expresar LTP sin estar directamente implicado en su generación (por ejemplo, la facilitación en la acumulación de vesículas pre-sinápticas) y el rol instructivo se refiere a los efectos de BDNF al iniciar una señalización vía TrkB en respuesta a una estimulación de alta frecuencia como la LTP (por ejemplo, la movilización de calcio vía PLCγ al estimular la sinapsis a altas frecuencias).

BDNF también juega un papel muy importante en el mantenimiento, dinámica y número de las espinas dendríticas en varias regiones del cerebro anterior (Alonso y col., 2005; Rex y col., 2007; Whitford y col., 2002; Baquet y col., 2004). Los mecanismos potenciales en esta modulación podrían depender por ejemplo (I) del control dependiente de actividad que ejerce BDNF en la síntesis de nuevas proteínas (como Arc y CaMKII) en las mismas dendritas estimuladas (Yin y col., 2002; Kelleher y col., 2004). Por otra parte (II), BDNF también regularía la morfología y densidad de espinas dendríticas mediante el control de la polimerización de actina (Rex y col., 2007; Lynch y col., 2007).

4.4. Modulación en sinapsis excitadoras y función neuroprotectora del BDNF

La neurotrofina BDNF y su receptor específico TrkB se encuentran ambos enriquecidos en sinapsis excitadoras / espinas dendríticas (Wu y col., 1996; Drake y col., 1999). Es de importancia saber qué implicaciones tiene esta neurotrofina en la modulación de la función de tales micro-estructuras. Los primeros estudios en enfocar estas cuestiones indicaron que la aplicación de BDNF de forma exógena incrementa las

corrientes glutamatérgicas (Li y col., 1998), pero disminuye las GABAérgicas (Tanaka y col., 1997), aunque los efectos pueden ser contrarios dependiendo del tipo de conexiones implicadas (Gottmann y col., 2009). Además, BDNF también modula el fortalecimiento sináptico excitador a largo plazo dependiente de síntesis de nuevas proteínas (Tanaka y col., 2008). Así, la neurotrofina está implicada en el fortalecimiento sináptico excitador principalmente a través de mecanismos tanto pre-sinápticos (para una revisión ver Lessmann, 1998) como post-sinápticos (para una revisión ver Carvalho y col., 2008). A nivel estructural de fortalecimiento sináptico, BDNF no tan sólo lo modularía a nivel de aumento de corrientes glutamatérgicas sino que también la haría mediante la formación de nuevas sinapsis (Tyler y Pozzo-Miller, 2003; Rex y col., 2007). A nivel molecular esto se podría explicar mediante la inducción, por parte de BDNF, del tráfico de receptores AMPA hacia la membrana sináptica vía señalización por PLCγ (Nakata y Nakamura, 2007; Pereira y col., 2006) y el aumento de sus niveles de expresión (Caldeira y col., 2007). También BDNF podría estar implicado en la mayor presencia de distintas subunidades de receptores NMDA en membrana (Caldeira y col., 2007).

4.5. Alteraciones del BDNF en la enfermedad de Huntington y su relevancia

En la enfermedad de Huntingon, el BDNF parece jugar un papel muy importante por muchas razones. (I) El BDNF y la htt colocalizan en un 95% de neuronas corticales y en un 75% de neuronas estriatales (Fusco y col., 2003). (II) La htt regula la expresión de BDNF directamente vía regulación del complejo REST/NRSF (Zuccato y col., 2001; 2003). (III) El BDNF está disminuído en la mayoría de las regiones analizadas como la corteza cerebral, el núcleo estriado y el hipocampo tanto en modelos animales como en

humanos (para una revisión ver Zuccato y Cattaneo 2007). (IV) No sólo está alterada su expresión, sino también su transporte vesicular (del Toro y col., 2006; Gauthier y col., 2004). (V) BDNF parece ejercer un papel neuroprotector en neuronas estriatales al sobre-expresarlo en modelos transgénicos de la enfermedad de Huntington (Gharami y col., 2008) o en modelos excitotóxicos de la enfermedad (Pérez-Navarro y col., 1999, 2000; Kells y col., 2008). (VI) Las neuronas estriatales, las más afectadas en la enfermedad de Huntington (Alberch y col., 2002; Pérez-Navarro y col., 2006), son altamente dependientes del BDNF (Altar y col., 1997, Baquet y col., 2004; Altar y DiStefano, 1998). En esta línea, se ha sugerido que esa dependencia llega hasta el punto de que un ratón donde se le anulan genéticamente (*Knock-out)* los niveles de BDNF en el cerebro anterior desarrolla algunos aspectos fenotípicos similares a ciertos modelos animales de la enfermedad, aunque de aparición mucho más tardía (Baquet y col., 2004; Strand y col., 2007). Incluso inhibiendo la expresión de BDNF tiene efectos morfológicos y bioquímicos muy similares a los de la inhibición de la expresión de la htt en un modelo de la enfermedad de Huntington generado en el pez zebra (Diekmann y col., 2009). Adicionalmente, no sólo parece verse afectada la neurotrofina sino que los niveles de su receptor específico, TrkB, también están alterados en el núcleo estriado de varios modelos animales de la enfermedad (Ginés y col., 2006). Sin embargo, su actividad y función se encuentra bastante preservada (Canals y col., 2004; Ginés y col., 2010). Finalmente, a nivel de las alteraciones de plasticidad neuronal hipocampal en la enfermedad de Huntington, se ha demostrado que el déficit de niveles de BDNF hipocampal es uno de los mayores contribuidores a las alteraciones en la expresión de LTP y formación de espinas dendríticas en modelos *Knock-in* (Lynch y col., 2007).

5. Propuestas terapéuticas en la enfermedad de Huntington

Desde que se conocen muchos de los procesos fisiopatológicos de la enfermedad de Huntington, al mismo tiempo se han ido realizando investigaciones en relación con las posibilidades terapéuticas para su tratamiento. El cuerpo de investigación con respecto al intento de diseñar estrategias terapéuticas para la enfermedad de Huntington es grande, prometedor pero aún con muchos aspectos a mejorar. Una vez pasados los ensayos en roedores, algunos fármacos o tratamientos han conseguido algunos efectos paliativos (revisado en Bonelli y Wenning, 2006). En este apartado revisaremos varias de las perspectivas terapéuticas más estudiadas hasta el momento donde el BDNF ha cobrado un papel significativo dada su importancia en la fisiopatología de la enfermedad (Zuccato y Cattaneo, 2007; 2009).

5.1. Terapia génica

En un trabajo previo se demostró de forma elegante en un modelo transgénico murino condicional de la enfermedad de Huntington que la enfermedad es casi totalmente reversible mediante la inhibición total de la trascripción de la mhtt (Yamamoto y col., 2000). Adicionalmente, la inhibición de la trascripción del transgen revierte y hace desaparecer los agregados de poliglutaminas a la vez que revierte la atrofia estriatal (Martin-Aparicio, 2001). Esto significa que si existiera la posibilidad de inhibir la mhtt, ésta sería, a nivel conceptual, la mejor estrategia terapéutica.

Recientemente se han intentado realizar los primeros estudios en modelos animales intentando inhibir la expresión de la mhtt. El método empleado ha sido el uso de la tecnología de interferencia de ARN como los shARN o siARN en distintos modelos animales de la enfermedad de Huntington como los R6/1, R6/2 o N171-82Q (Harper y col., 2005; Rodríguez-Lebron y col., 2005; DiFiglia y col., 2007). Aunque

estos trabajos han mostrado mejoras en los susodichos modelos, la metodología, por desgracia, aún se sitúa lejos de ser segura para los pacientes, por lo que aun falta desarrollarla más.

En la terapia génica para la enfermedad de Huntington también ha cobrado mucho interés la administración de neurotrofinas como BDNF (Alberch y col., 2002; Zuccato y Cattaneo, 2007) debido a su papel pivote en la fisiopatología de la enfermedad de Huntington (Canals y col., 2004; Fusco y col., 2003; Ferrer y col., 2000; del Toro y col., 2006; Gauthier y col., 2004; Zuccato y col., 2001). Sin embargo, esa molécula no es capaz de traspasar la barrera hematoencefálica de forma eficiente (Poduslo y Curran, 1996; Thorne y Frey, 2001) y los estudios donde administran BDNF de forma intra-estriatal directa padecen de adicionales problemas discutidos más abajo. En esa línea, se están intentando diseñar nuevas terapias para sortear ese problema. Una de las más interesantes es el uso de los llamados "caballos de Troya moleculares" (Pardridge y col., 2007; Boado y col., 2007). Estos fármacos harían un efecto mimético de BDNF mediante la fusión de moléculas de la neurotrofina sintéticas con anticuerpos monoclonales que se unen a receptores específicos cuando llegan a la barrera hematoencefálica. Estos los endocitarían y los liberarían intracerebralmente para ejercer sus efectos ante diversos estímulos neurotóxicos (Wu y Pardridge, 1999; Zhang y col., 2001). Por desgracia, estos estudios se encuentran, de momento, en estadios muy iniciales.

5.2. Terapia farmacológica

La terapia farmacológica es la más recurrida en lo referente a posibles tratamientos para la enfermedad de Huntington (Tabla 3). Distintos fármacos con

distintos objetivos moleculares han sido testados en ensayos pre-clínicos con animales e incluso algunos se han probado en pacientes. Aunque existen otros tipos de fármacos interesantes que también presentaremos aquí, muchos de estos intentan modular la funcionalidad excitadora o la agregación de la mhtt. Fármacos como el Riluzole, que inhibe la liberación de glutamato (Palfi y col., 1997), se han probado en animales R6/2 mejorando algunos de los aspectos de su fenotipo (Schiefer y col., 2002) y, posteriormente, también en pacientes con algunas mejorías motoras aunque a corto plazo (Seppi y col., 2001).

También se han utilizado otros tipos de fármacos para el tratamiento de la enfermedad de Huntington con objetivos moleculares distintos. Uno de ellos es la coenzima Q10, potente anti-oxidante y modulador de la cadena respiratoria mitocondrial que se ha utilizado para intentar paliar la sintomatología de la enfermedad de Huntington tanto en ratones como en humanos (Beal y col., 1999; Ferrante y col., 2002; Huntington Study Group, 2001). A pesar de los buenos resultados con este agente antioxidante en modelos animales, los datos en humanos no siguieron la misma línea. Otro fármaco interesante es el Congo Red que, administrado en ratones transgénicos, es capaz de disgregar agregados, mejorar el peso corporal, funciones motoras y supervivencia (Sánchez y col., 2003).

Por otra parte, también se han testado fármacos para incrementar la expresión génica debido a la grave alteración de tales procesos en la enfermedad (Cha, 2007). Algunos de ellos, como los inhibidores de las deacetilasas de histonas, como el ácido valproico, el fenilbutirato o el ácido hidroxámico suberoilanilido (SAHA), han revelado ciertas mejoras en modelos animales y/o pacientes (Saft y col., 2006; Gardian y col., 2005; Hockly y col., 2003; McCampbell y col., 2001). Finalmente, ya que en varias enfermedades neurodegenerativas como la enfermedad de Huntington existe una

hiperactividad transglutaminasa (Gentile y Cooper, 2004), se han probado inhibidores de tal actividad como la Cistamina y la Cisteamina con resultados paliativos prometedores en modelos animales (Van Ramsdoonk y col., 2005b; Dedeoglu y col., 2002; Karpuj y col., 2002) mediados, como mínimo en parte, por un restablecimiento de la función y expresión de BDNF (Borrell-Pagès y col., 2006).

Tratamiento	Modelo	Efectos	Referencia
Butirato sódico	R6/2	Mejora síntomas motores, peso y neuropatología	Ferante et al., 2003
Rapamicina	*Drosophila*, N171-N82Q	Mejora sintomatología, disminuye toxicidad y acumulación de agregados	Ravikumar et al., 2004
Inhibidores de la transglutaminasa	bDM y R6/1	Mejora supervivencia, neuropatología y niveles de BDNF	Borrell-Pagès et al., (2006)
Congo red	R6/2	Mejora supervivencia, comportamiento motor y peso	Sanchez et al., 2003
Riluzole	R6/2	Mejora motora, supervivencia y peso	Schiefer et al., 2002
SAHA	R6/2	Mejora motora	Hockly et al., 2003
Minociclina	R6/2	Inhibición de la formación de agregados y supervivencia	Wang et al., 2003
Ácidos Grasos esenciales	R6/1	Mejora supervivencia y *clasping*	Clifford et al., 2002
Coenzima Q10	R6/2	Disminuye formación de agregados, supervivencia, comportamiento motor i peso	Ferrante et al., 2002

Tabla 3. Estudios pre-clínicos farmacológicos para el tratamiento de la enfermedad de Huntington.

En esta línea, es interesante recalcar que muchos de los efectos terapéuticos beneficiosos de distintos tratamientos farmacológicos usados para el tratamiento de la enfermedad de Huntington (por ejemplo, con litio, cistamina, ampaquinas o inhibidores de la recaptación de serotonina) van asociados a un aumento de la expresión o función de BDNF (Chuang et al., 2004; Borrell-Pagès et al., 2006; Simmons y col., 2009).

5.3. Terapia celular

El uso de trasplantes de células precursoras neuronales, ya sean células neuronales neonatales estriatales (Kendall y col., 2000) o provinentes de fetos (Chen y col., 2002) o precursores embriónicos de células madre (Dihne y col., 2006) o precursores neurales (Lepore y col., 2004) están siendo utilizados en ensayos pre-clínicos con modelos murinos de la enfermedad de Huntington con ciertos resultados satisfactorios en la sintomatología motora y neurofisiológica (Dunnett y White, 2006; McBride y col., 2004). Actualmente, se han realizado ya algunos ensayos clínicos en pacientes con la enfermedad de Huntington usando la terapia celular mediante células obtenidas de fetos humanos con resultados discretos pero esperanzadores (Bachoud-Levi y col., 2000; Lindvall y Bjorklund, 2000; Rosser y col., 2002).

Estudios en pacientes han mostrado que implantes en el núcleo putamen con células disgregadas en suspensión provinentes de tejido fetal muestran buena integración tisular y expresión de marcadores neuronales como DARPP-32 (Watts y Dunnett, 2000) e incluso mejoras cognitivas en modelos primates de la enfermedad de Huntington (Palfi y col., 1998). También se han realizado transplantes de precursores neuronales de origen fetal en modelos murinos excitotóxicos con ciertas mejoras funcionales (Chen y col., 2002), pero cuando esos transplantes se han trasladado a modelos animales transgénicos no se han obtenido, de momento, resultados satisfactorios (Dunnett y col., 1998).

En los últimos años ha crecido el interés hacia el uso de las células madre por varios motivos. En general, esas células sobreviven y se integran bien en el cerebro una vez trasplantadas, migran en cierta medida pero permanecen en estado quiescente indiferenciado o, como mucho, se diferencian a tipos celulares gliales y,

adicionalmente, son manipulables genéticamente (Akerud y col., 2001 y 2002; Arenas y col., 2002). De hecho, se han realizado ya algunos estudios previos en modelos excitotóxicos de la enfermedad de Huntington mediante el uso de células madre liberadoras de NGF o BDNF con ciertos resultados de mejora motora (Kordower y col., 1997; Martínez-Serrano y col., 1996).

5.4. Terapia génica/celular

Combinando la terapia génica con la celular, el objetivo es el de trasplantar células modificadas genéticamente con los genes terapéuticos de interés insertados e incapaces de atravesar la barrera hematoencefálica para que los produzcan y liberen una vez dentro del tejido objetivo (Alberch y col., 2004; Arenas y col., 2005). En modelos animales de la enfermedad de Huntington, hace tiempo ya se probó de administrar CNTF (del inglés *ciliary neurotrophic factor*) mediante trasplante estriatal de estos tipos de células con ciertos efectos protectores (Kordower y col., 1999). Sin embargo, los ensayos clínicos posteriores con el trasplante de células encapsuladas y liberadoras de CNTF no han reportado demasiados efectos beneficiosos (Bloch y col., 2004; Emerich y col., 2004). Por otra parte, se han realizado estudios con implantes de fibroblastos capaces de liberar grandes cantidades de neurotrofinas y otros factores tróficos en modelos excitotóxicos agudos de la enfermedad de Huntington con evidentes efectos neuroprotectores a nivel estructural e histológico (Pérez-Navarro y col., 1999; 2000).

5.5. Limitaciones de las terapias

Aunque sean muchas las estrategias terapéuticas que se han realizado para el tratamiento de la enfermedad de Huntington, son múltiples los inconvenientes que padecen cada una de ellas. Ello nos demuestra que el tratamiento de la enfermedad de Huntington es muy complejo y que posee muchas variables moduladoras que se deben tener en cuenta. Sin embargo, la situación actual también nos indica que existe mucha actividad científica en progreso para el diseño de estrategias terapéuticas, que muchas de ellas son propuestas interesantes y con potencial, pero que aún falta desarrollarlas mucho más.

A nivel de terapia génica, estrategias como la infección viral o la lipotransfección para la expresión de moléculas neuroprotectoras ha demostrado ser muy invasivo e incluso tóxico para las células endógenas además de carecer de suficiente difusión (Zuccato y Cattaneo, 2007; Lindvall y col., 2004; Dunnett y Rosser, 2004). En el uso de tecnología reguladora del ARN (mediante ARN de interferencia), de momento es difícil controlar aspectos como la modulación de los niveles de producción del ARN de interferencia y el nivel de inhibición del gen objetivo. También existen complicaciones debido a efectos inespecíficos / colaterales, falta de especificidad de efectos en tejidos específicos, déficits al cruzar la barrera hemotencefálica y su propia inestabilidad molecular intrínseca (revisado en Prakash y col., 2010). Así, aunque el silenciamiento de la mutación parece ser una de las estrategias terapéuticas con mayor potencial, aún se encuentra en un estado muy inmaduro para su uso fiable y con seguridad en humanos. Respecto al uso de herramientas como la transducción de vectores virales, éstos pueden inducir a la aparición de tumores debido a mutagénesis de inserción accidental (Hacein-Bey-Abina y col., 2008), lo que indica que es un aspecto importante a mejorar. Por lo que respecta a los "caballos de Troya moleculares" todavía no se ha demostrado su fiabilidad y seguridad al cien por cien. Además, la generación

de BDNF suficiente (aunque sintético) para uso terapéutico por si mismo resulta, actualmente, difícilmente viable a nivel económico. Por lo tanto, aunque prometedora, esta herramienta aún requiere mucha investigación y desarrollo.

Con frecuencia, el éxito de los tratamientos farmacológicos en modelos murinos no funcionan cuando se intentan extrapolar en humanos, lo que demuestra una vez más la complejidad de la enfermedad y algunas de las limitaciones del uso de modelos animales (Ferrante y col., 2002; Schilling y col., 2001). Adicionalmente, algunas drogas conllevan dificultad de administración como el Congo Red que muestra complejas curvas dosis-respuesta (Smith y col., 2001). Finalmente, los tratamientos farmacológicos difícilmente son específicos y, muchas veces, modulan múltiples dianas moleculares además de crear algunos efectos adversos cuando éstos son crónicos (Stahl, 2002). Sin embargo, muchos de los tratamientos estudiados han aportado, además de algunas mejoras, nuevos conocimientos sobre la fisiopatología de la enfermedad, lo que ha generado la necesidad del estudio para la modificiación de nuevas vías moleculares o mejorar los mecanismos de acción de las ya existentes.

Respecto a la terapia celular con células fetales, con ella van asociados algunos problemas éticos y burocráticos además de conllevar problemas técnicos como la dificultad de obtención del material a trasplantar. Además, es difícil plantear estos estudios con los controles adecuados y suficientes y, algunas veces, los pacientes, después de mejorar durante un tiempo, recaen. Lo que es realmente esperanzador, sin embargo, es que estos estudios se cuentan entre los que más beneficios han aportado a los pacientes, lo que indica la necesidad de invertir esfuerzos en esta dirección. Algunos de ellos podrían ir dirigidos a algunos aspectos a mejorar como los que siguen: Mayor difusión del transplante para un mayor efecto sistémico. También debería investigarse para obtener una mayor diferenciación, durabilidad y, a la vez, funcionalidad del

transplante, objetivo que no siempre se logra (Lindvall y col., 2004). Finalmente, el implante de precursores neuronales provinente de fetos en el núcleo estriado, aunque son capaces de sobrevivir, de adaptarse y de expresar marcadores neuronales como DARPP-32 (Watts y Dunnett, 2000), con cierta frecuencia no producen buenos contactos sinápticos y su arborización es a veces caótica (Armstrong y col., 2000), por lo tanto, este aspecto también debería mejorarse.

El uso de líneas celulares modificadas genéticamente como terapia celular, a pesar de sus efectos protectores, puede ser causa de la formación de tumores (Hoffman y col., 1993), por lo que el control de la proliferación aberrante de estas células debe ser de interés primordial. Los trasplantes de células (u otros sistemas) secretoras de factores tróficos como por ejemplo, la neurotrofina BDNF, deberían ser capaces de regular la administración de la molécula terapéutica en cuestión (Martínez-Serrano y col., 1996; Rubio y col., 1999). Eso es así porqué, en ese caso, el exceso de expresión de neurotrofinas también pueden conllevar efectos neurotóxicos o disfunciones de procesos cognitivos (Kells y col., 2008; Croll y col., 1999).

Finalmente, las células madre aparecen como futuras herramientas terapéuticas potenciales. De hecho, existe algún estudio en modelos animales transgénicos de la enfermedad de Huntington trasplantados con células madre mesenquimales, los cuáles muestran cierto grado de mejoría (Lee y col., 2009). Sin embargo, los conocimientos actuales sobre las células madre aún son insuficientes para conseguir su diferenciación y poseen, además, un potencial teratogénico significiativo para poder enfocarlas aún hacia su uso clínico. Sin embargo, su potencial terapéutico es muy alto por lo que requieren aún mucha investigación.

II Objetivos

1. Descripción del papel de los receptores NMDA, de sus proteínas de andamiaje específicas llamadas MAGUK y de la posible mediación del BDNF en la regulación de los procesos excitotóxicos y alteraciones sinápticas cortico-estriatales asociadas en modelos animales transgénicos de la enfermedad de Huntington.

2. Identificación y caracterización de las vías de señalización afectadas específicamente por debajo de la funcionalidad de los receptores NMDA en procesos de excitotoxicidad y disfunción sináptica en modelos animales transgénicos y agudos de la enfermedad de Huntington.

3. Estudio y caracterización de las vías moleculares implicadas en el inicio de las alteraciones cognitivas superiores afectadas en modelos animales de la enfermedad de Huntington.

4. Estudio de la implicación del BDNF en las disfunciones cognitivas de la enfermedad de Huntington.

5. Generación y caracterización de un modelo condicional de liberación de BDNF en condiciones patológicas: un ratón transgénico que sobre-exprese BDNF bajo el promotor GFAP.

6. Estudio del efecto neuroprotector del uso de astrocitos que liberen BDNF de forma condicional en diferentes modelos de la enfermedad de Huntington.

III Resultados

Primer trabajo: ***"Disruption of striatal glutamatergic transmission induced by mutant huntingtin involves remodeling of both postsynaptic density and NMDA receptor signalling".***

(Publicado en la revista Neurobiology of Disease)

Objetivo 1: Descripción del papel de los receptores NMDA, de sus proteínas de andamiaje específicas llamadas MAGUK y de la posible mediación del BDNF en la regulación de los procesos excitotóxicos y alteraciones sinápticas cortico-estriatales asociadas en modelos animales transgénicos de la enfermedad de Huntington.

Existe un largo cuerpo de investigaciones apuntando a los procesos excitotóxicos como uno de los ejes centrales en la fisiopatología de la enfermedad de Huntington. Su importancia radica, en parte, a su temprana aparición en todos los modelos testados. Sin embargo, existe controversia debido a que algunos modelos son susceptibles mientras que otros son resistentes a tales procesos. Lo que sí queda claro es que la transmisión glutamatérgica está gravemente comprometida. Esto se traduce en disfunciones progresivamente peores durante el transcurso de la enfermedad en lo que respecta a la funcionalidad neuronal hasta terminar con la neurodegeneración y muerte celular. Debido a que los receptores NMDA se han propuesto como principales mediadores de los procesos excitotóxicos en neuronas estriatales en la enfermedad de Huntington, nuestro objetivo ha sido evaluar *in vivo* qué relevancia tiene su activación en tales alteraciones a nivel de muerte y degeneración neuronal y cómo podrían estar influenciando en estos procesos las proteínas de andamiaje de los mismos receptores llamadas MAGUK. Finalmente, analizamos el papel de BDNF en este esquema debido a su relevancia en la enfermedad de Huntington y de su importancia en la funcionalidad de las sinapsis excitatorias. Todo esto lo estudiamos en modelos R6/1 y R6/1:BDNF+/- en fases tempranas de la enfermedad.

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Disruption of striatal glutamatergic transmission induced by mutant huntingtin involves remodeling of both postsynaptic density and NMDA receptor signaling

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We study the striatal susceptibility to NMDA receptor (NMDAR)-mediated injury of two Huntington's disease (HD) transgenic mice: R6/1 and R6/1:BDNF$^{+/-}$. We found that R6/1:BDNF$^{+/-}$ mice – which express reduced levels of BDNF – were more resistant than R6/1 mice to intrastriatal injection of quinolinate. This increased resistance is related to a differential reduction in expression of NMDAR scaffolding proteins, MAGUKs (PSD-95, PSD-93, SAP-102 and SAP-97) but not to altered levels or synaptic location of NMDAR. A robust reorganization of postsynaptic density (PSD) was detected in HD transgenic mice, shown by a switch of PSD-93 by PSD-95 in PSD. Furthermore, NMDAR signaling pathways were affected by different BDNF levels in HD mice; we found a reduction of synaptic αCaMKII (but not of nNOS) in R6/1:BDNF$^{+/-}$ compared to R6/1 mice. The specific regulation of MAGUKs and αCaMKII in striatal neurons may reflect a protective mechanism against expression of mutant huntingtin exon-1.

Keywords: Brain-derived neurotrophic factor; Excitotoxicity; Postsynaptic density; Quinolinic acid; Synaptic dysfunction; Huntington's disease

Introduction

Huntington's disease (HD) is due to an abnormal expansion of CAG codon in exon-1 of the huntingtin (*htt*) gene, resulting in a devastating cognitive and motor disorder (HDCRG, 1993) which is mainly attributable to a selective degeneration of striatal projection neurons (Reiner et al., 1988). Growing evidence links NMDA receptor (NMDAR)-mediated excitotoxicity with the selective degeneration in HD (Perez-Navarro et al., 2006). Presymptomatic HD transgenic mice display increased NMDAR-mediated current densities (Starling et al., 2005) and higher intracellular Ca^{2+} overload after glutamate application (Tang et al., 2005). These characteristics are concomitant with enhanced susceptibility to NMDAR-mediated excitotoxicity of striatal neurons induced by mutant htt *in vitro* (Zeron et al., 2002). However, in spite of sustained NMDAR overactivity, the loss of striatal neurons is a late event in some HD transgenic mice (Mangiarini et al., 1996; Canals et al., 2004; Diaz-Hernandez et al., 2005). It has therefore been suggested that the chronic excitotoxic stress initiated by mutant htt could be modulated by compensatory mechanisms that involve changes in NMDAR-mediated neurotoxic pathways (Jarabek et al., 2004).

Synaptic targeting and activity of NMDAR are modulated by members of the membrane-associated guanylate kinase (MAGUK) family of proteins (PSD-95, PSD-93, SAP-102 and SAP-97; Kim and Sheng, 2004). MAGUKs also mediate the interaction of NMDAR with signaling proteins, such as nNOS (Brenman et al., 1996; Sattler et al., 1999). This interaction facilitates the NMDAR-mediated Ca^{2+} influx activation of nNOS (Sattler et al., 1999), which in turn activates the p38-kinase neurotoxic pathway (Cao et al., 2005). Altered striatal MAGUK expression has been related to the pathophysiology of neurodegenerative disorders of the basal ganglia (Gardoni et al., 2006; Nash et al., 2005) including HD (Jarabek et al., 2004; Luthi-Carter et al., 2002). Moreover, mutant htt increases NMDAR phosphorylation via PSD-95 (Song et al., 2003). On the other hand, αCaMKII also modulates NMDAR function and participates in NMDAR-mediated neurotoxicity (Hajimohammadreza et al., 1995; Colbran and Brown, 2004). Following glutamate treatment, αCaMKII translocates to the postsynaptic density (PSD) (Dosemeci et al., 2001) where it phosphorylates NMDAR (Gardoni et al., 1998) and inhibits the pro-survival RAS/ERK pathway (Oh et al., 2004). Thus, pretreat-

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ment with a CaMKII inhibitor prevents neuronal death following ischemia (Hajimohammadreza et al., 1995).

Another mechanism involved in the specific vulnerability of striatal neurons in HD is the regulation of the activity of neurotrophic factors (Zuccato et al., 2001). BDNF is the most potent trophic factor for striatal projection neurons in the excitotoxic model (Perez-Navarro et al., 2000) and in transgenic mice (Canals et al., 2004). Furthermore, reduced levels of endogenous BDNF increase mutant htt-induced degeneration of striatal neurons inducing advanced onset and increased severity of motor abnormalities (Canals et al., 2004). However, the mechanism by which BDNF levels modulate mutant htt-induced striatal neuropathology remains unclear. We therefore studied whether impaired BDNF trophic support in HD modulates abnormalities of NMDAR in striatal neurons, and the possible mechanism involved in these changes. Our results show that the reduction of BDNF levels in mice expressing mutant htt exon-1 increases the disruption of NMDAR signaling in striatal neurons by selectively modulating scaffolding and signaling proteins of NMDAR in the PSD.

Experimental procedures

Animal model

We used R6/1 mice (Mangiarini et al., 1996) expressing transgenic exon-1 of mutant htt with 115 CAG repeats and normal levels of BDNF (Canals et al., 2004) and R6/1 mice with the same transgene but with reduced levels of BDNF (R6/1:BDNF$^{+/-}$) (Canals et al., 2004). To obtain R6/1:BDNF$^{+/-}$, we cross-mated R6/1 mice (Mangiarini et al., 1996) with BDNF heterozygous (BDNF$^{+/-}$) mice (Ernfors et al., 1994). We used wild-type (wt) and BDNF$^{+/-}$ littermates as controls. Male littermates from the F3 population were used for all experiments to avoid strain and sex differences. Mice were housed together in numerical birth order in groups of mixed genotypes with access to food and water *ad libitum* in a colony room kept at 19–22 °C and 40–50% relative humidity on a 12-h light/dark cycle. All experiments were conducted in a blind-coded manner with respect to genotype, and data were recorded for analysis using a microchip mouse number. All procedures conformed to the European Community guidelines for the care and use of laboratory animals, and were approved by the animal care committee of the University of Barcelona and by the autonomous government (Generalitat de Catalunya). Every effort was made to minimize the suffering of the animals. For genotyping, DNA was obtained from a tail biopsy and processed for PCR. The primers used for DNA amplification are described in the literature (Mangiarini et al., 1996; Agerman et al., 2003). PCR fragments were resolved in agarose gels of 2% and 1% for BDNF and mutant htt, respectively.

Striatal lesions

12-Week-old mice (n=3–5 per genotype) were anesthetized with pentobarbital (40 mg/kg). Thirty nanomoles of quinolinate (QUIN) (Sigma Chemicals Co., St. Louis, MO, USA) in 0.5 μl of sterile phosphate-buffered saline (PBS) was stereotaxically injected into the striatum (coordinates −0.6 mm posterior, 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 a further 3 min. The animals were monitored for 2 h post-injection and then returned to the housing facility for 7 days.

Immunohistochemistry

This assay was performed on paraformaldehyde-fixed material as described elsewhere (Torres-Peraza et al., 2007). The primary antibody was anti-Neu-N (1:100, MAB377, Chemicon, Temecula, CA, USA). No signal was detected in controls incubated in the absence of the primary antibody.

Cell counting

Unbiased stereological counts of striatal volume and neurons were performed blind with respect to genotype and obtained using the Computer Assisted Stereology Toolbox (CAST) software (Olympus Denmark A/S, Ballerup, Denmark) as described (Torres-Peraza et al., 2007). The dissector counting method was used to determine neuronal density (neurons/mm^3) in coronal sections spaced 150 μm apart in the area of the lesion and the contralateral striata 1.05 to 0.15 mm from the bregma, following Franklin and Paxinos (1997).

Striatal homogenates and subcellular fractionation

To obtain proteins from total striatal extracts, animals (n=4 to 6 per genotype and time point) were deeply anesthetized in a CO_2 chamber, their brains were quickly removed and the striatum was dissected out. The tissue was frozen using CO_2 pellets and stored at −80 °C until use. Samples were sonicated in 200 μl of lysis buffer (PBS, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 2 mM sodium orthovanadate) centrifuged at 13,400×g for 20 min, and the supernatants were collected and frozen. Synaptic plasma membrane (SPM) fraction was obtained by differential centrifugation as described (Perez-Otano et al., 2006). Three striata of the same genotype were pooled per each experiment and homogenized in lysis buffer (4 mM HEPES, 0.32 M sucrose, 1 mM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 2 mM sodium orthovanadate, 0.1 mg/ml benzamidine) and centrifuged at 1000×g for 10 min. The resulting supernatant was centrifuged at 10,000×g for 15 min to obtain a crude membrane fraction and cytosol/light membrane supernatant (S2). The pellet was resuspended in deionized H_2O (1 mM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 2 mM sodium orthovanadate, 0.1 mg/ml benzamidine) in a Teflon-glass potter and centrifuged at 25,000×g for 20 min. Next, the pellet was resuspended in lysis buffer and centrifuged on a discontinuous sucrose gradient (0.8 M, 1 M and 1.2 M) at 150,000×g for 2 h. SPM was collected from the 1:1.2 M interface and resuspended in 50 mM HEPES (2 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 2 mM sodium orthovanadate, 0.1 mg/ml benzamidine). S2 was centrifuged at 100,000×g for 15 min to obtain cytosolic (supernatant) and membrane fractions (pellet). All subfragmentation were repeated three times for each genotype (n=3).

Postmortem brain tissue

Samples of caudate nucleus from four patients with HD (65, 68 and 71 years old, and a 28-year-old juvenile-onset HD patient) at postmortem intervals of 4–15 h, and from three control cases (42, 74 and 77 years old) at postmortem intervals of 4–23 h were supplied by the neurological tissue bank (University of Barcelona, Spain), in compliance with all the ethical guidelines of the latest Declaration of Helsinki.

Western blot analysis

Non-heat-denatured proteins, from total striatal extracts (15 µg) or from striatal SPM (1–5 µg) were loaded in a 7.5% SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, MA, USA). Blots were blocked in TBS-T (150 mM NaCl, 20 mM Tris–HCl, pH 7.5, 0.05% Tween 20) with 5% non-fat dry milk and 5% BSA. Immunoblots were probed with the appropriate primary antibodies: anti-NR1, anti-NR2A and anti-NR2B (Chemicon, Temecula, CA, USA); anti-GluR1, anti-GluR2/3, anti-GluR4, anti-GluR5, anti-GluR6/7 (UPSTATE Biotechnology, NY, USA); anti-PSD-95/SAP-90 and anti-SAP-97/hdlg (Affinity BioReagents, Golden, CO, USA); anti-SAP-102 (Synaptic System, Germany); anti-PSD-93 (Alomone Labs, Jerusalem, Israel), anti-nNOS (BD Transduction Laboratories, San Diego, CA, USA), anti-CaMKinaseII (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-phospho-NR1 serine^{896} that recognizes NR1 phosphorylated at ser^{896} and anti-phospho-NR1 serine^{897} that recognizes NR1 phosphorylated at ser^{897} and dually phosphorylated at ser^{896} and ser^{897} (UPSTATE Biotechnology, NY, USA); anti-p38-kinase and anti-panERK (BD Transduction Laboratories), anti-phospho-p38-kinase and anti-phospho-ERK (Cell Signalling Technology, Danvers, MA, USA). All blots were incubated with the primary antibody overnight at 4 °C in PBS with 0.02% sodium azide. After several washes in TBS-T, blots were incubated with anti-mouse or anti-rabbit IgG HRP-conjugated (Promega, Madison, WI, USA) and developed using the ECL Western blotting analysis system (Bioscience Europe, Freiburg, Germany). A monoclonal anti-β-tubulin antibody (Sigma, St. Louis, MO, USA) was used as a loading control.

Stripping blots

Blots were stripped by incubating at 55 °C for 30 min in stripping buffer (62.5 mM Tris pH 6.7, 2% SDS, 100 mM β-mercaptoethanol). Stripped blots were washed 3 times for 5 min in TBS-T and incubated for 1 h with secondary antibody as described above, to check whether the stripping was complete. Each membrane was immunoblotted first with anti-phospho-NR1ser^{896} or anti-phospho-NR1scr^{897}, stripped and probed with anti-NR1 antibody.

All results are expressed as the mean±SEM of different genotypes and time points. Statistical analysis was performed using ANOVA followed by the LSD post hoc test.

Results

R6/1:BDNF$^{+/-}$ were more resistant than R6/1 mice to intrastriatal injection of QUIN

To study the involvement of mutant htt and endogenous BDNF levels in the vulnerability of striatal neurons to excitotoxicity, intrastriatal injection of QUIN was performed on 12-week-old R6/1 (with normal BDNF levels) and R6/1:BDNF$^{+/-}$ mice (with reduced BDNF levels) and 7 days later striatal volume and neuronal density were measured in the lesion area. Intrastriatal injection of QUIN induced a similar reduction of neuronal density in wt and BDNF heterozygous mice (wt: 83±4% and BDNF$^{+/-}$: 82±7% with respect to contralateral striatum; Figs. 1A, B). However, this reduction was lower in animals expressing mutant htt exon-1 (R6/1: 43±13% with respect to contralateral striatum; Figs. 1A, B). HD mice with decreased BDNF levels were more resistant to QUIN-induced excitotoxic damage than R6/1 mice with normal BDNF levels, since reduction in neuronal density after QUIN-induced injury was lower (R6/1:BDNF$^{+/-}$: 14±4% with respect to contralateral striatum; Figs. 1A, B). Since an alteration in striatal volume and neuronal density was previously described in this model at 30 weeks of age (Canals et al., 2004) we analyzed the density of striatal neurons in non-lesioned striata in 12-week-old mice. However, striatal volume and cell density were similar in R6/1 and R6/1:BDNF$^{+/-}$ mice at 12 weeks of age (data not shown).

Excitotoxic resistance did not depend on abnormal expression of NMDAR or AMPA receptor (AMPAR) subunits

Differential expression of NMDAR subunits is known to control NMDA activity (Koles et al., 2001) and neuronal

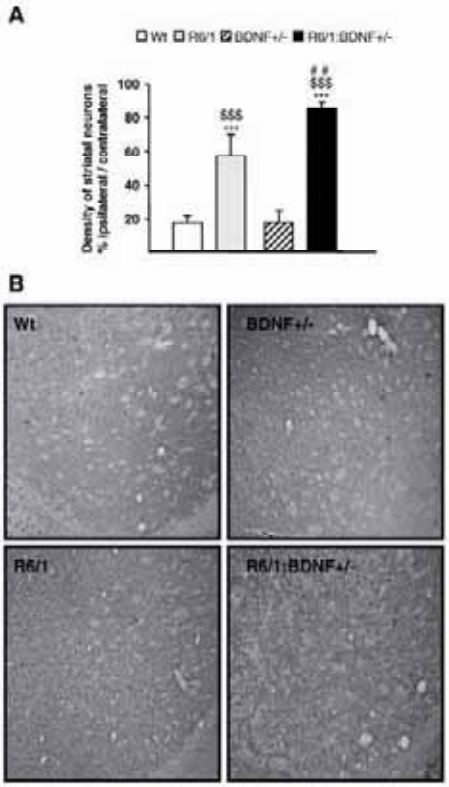


Fig. 1. R6/1:BDNF$^{+/-}$ mice are more resistant to QUIN-induced lesion. (A) Density of striatal NeuN-positive cells was determined stereologically 7 days after unilateral intrastriatal injection of QUIN. Graph shows neuronal density of injected striatum (percent with respect to non-injected striatum). Bars represent mean±SEM; $n=3–5$ per genotype. ***$p<0.005$ with respect to wt; $^{\$\$\$}p<0.005$ with respect to BDNF $^{+/-}$; $^{\#\#}p<0.01$ with respect to R6/1. (B) Representative microphotograph of striatal NeuN immunohistochemistry 7 days after QUIN injection at 12 weeks of age.

susceptibility to excitotoxicity (Kovacs et al., 2001; Torres-Peraza et al., 2007). To study whether the reduced NMDAR-mediated injury exhibited by R6/1 and R6/1:BDNF$^{+/-}$ mice depends on altered expression of NMDAR subunits, we measured striatal levels of NMDAR subunits in presymptomatic and symptomatic stages of disease. There were no differences in NR1, NR2A or NR2B protein levels in wt, BDNF$^{+/-}$ or R6/1 mice at 8, 12 or 30 weeks of age (Fig. 2). However, NR2B protein levels decreased in R6/1:BDNF$^{+/-}$ mice at 30 weeks of age. Levels were normal at presymptomatic stages (8 and 12 weeks) but reduced (35±7% compared with wt) in 30-week-old animals (Figs. 2E, F). This is a late symptomatic stage that shows loss of striatal projection neurons (Canals et al., 2004).

There were no differences in total levels of AMPAR subunits (GluR1, wt: 100±4%, R6/1: 87±2%, BDNF$^{+/-}$: 90±0% and R6/1:BDNF$^{+/-}$: 93±8% with respect to wt; GluR2/3, wt: 100±5%, R6/1: 89±9%, BDNF$^{+/-}$: 94±6% and R6/1:BDNF$^{+/-}$: 87±2% with respect to wt; and GluR4, wt: 100±8%, R6/1: 112±13%, BDNF$^{+/-}$: 117±13% and R6/1:BDNF$^{+/-}$: 102±4% with respect to wt; n=4–6).

We analyzed the expression of NMDAR and AMPAR subunits in the striatal SPM fraction of 12-week-old mice. There were no differences in synaptic expression of NR1 (Figs. 3A and D), NR2A (Figs. 3B and D) or of NR2B (Figs. 3C, D). Likewise, AMPAR subunits showed no changes in the expression in SPM preparations (Figs. 3E–H). Taken together, these results demonstrate that the striatal resistance to QUIN-induced injury observed in R6/1 and R6/1:BDNF$^{+/-}$ mice at 12 weeks of age does not depend on differences in the levels and/or the synaptic location of NMDAR or AMPAR subunits.

We also studied phosphorylation levels of NR1 subunits of NMDAR. In striata from mice expressing mutant htt exon-1, both ser^{896} and ser^{897} of the NR1 subunit were more phosphorylated, but only phospho-NR1ser^{897} levels were selectively modulated by BDNF (Fig. 4). At 12 weeks of age, R6/1 mice showed an increase of 46±12% in phospho-NR1ser^{897} levels with respect to wt (Figs. 4A and B) whereas age-matched R6/1:BDNF$^{+/-}$ mice showed a 99±13% increase in phospho-NR1ser^{897} levels with respect to wt (Figs. 4A and B). In addition, both R6/1 and R6/1:BDNF$^{+/-}$ mice showed increased levels of phospho-NR1ser^{896} (Figs. 4A, B) but – in contrast to phospho-NR1ser^{897} – mutant htt exon-1-induced phosphorylation of NR1 ser^{896} was not affected by BNDF levels: there were no significant differences in the levels of phospho-NR1 ser^{896} between R6/1 and R6/1:BDNF$^{+/-}$ mice (Figs. 4A, B). Phospho-NR1 levels were not increased in BDNF$^{+/-}$ mice (Figs. 4A and B), demonstrating that reduced levels of BDNF increase phospho-NR1 levels only in mice expressing mutant htt exon-1. This effect was transient, since this large increase was not observed in older animals (data not shown).

Mutant htt exon-1-induced abnormalities in striatal expression of MAGUKs are differentially modulated by BDNF levels

MAGUK proteins interact with NMDAR modulating their traffic and surface expression, and linking NMDAR to intracellular signaling pathways. Therefore, we quantified striatal levels of PSD-95, SAP-97, SAP-102 and PSD-93 in R6/1 and R6/1:BDNF$^{+/-}$ mice at 8, 12 and 30 weeks of age. In agreement with the increased resistance found in mutant htt exon-1-expressing mice, we observed differences in the levels of MAGUKs in striata from

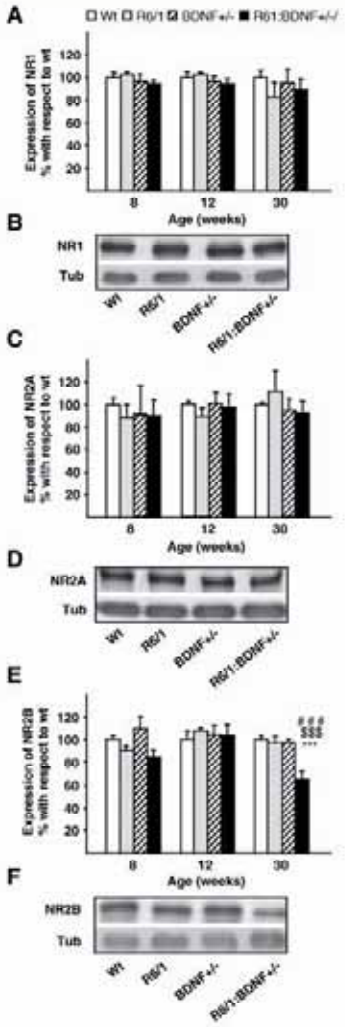


Fig. 2. Reduced susceptibility to QUIN-induced lesion of R6/1 and R6/1:BDNF$^{+/-}$ mice does not depend on NMDAR subunit expression. Striatal expression of NR1, NR2A and NR2B subunits was determined by Western blot in total protein extract at 8, 12 and 30 weeks of age. (A, C, E) Graphs show quantification of striatal expression of NR1 (A), NR2A (C) and NR2B (E) at different ages. Representative blots of total NR1 (B), NR2A (D) and NR2B (F) subunits at 30 weeks of age. The NR2B subunit is down-regulated in R6/1:BDNF$^{+/-}$ mice at 30 weeks of age. Bars represent mean±SEM; n=4–6 per genotype. ***$p<0.005$ with respect to wt; $^{\$\$\$}p<0.005$ with respect to BDNF $^{-/-}$; $^{\#\#\#}p<0.005$ with respect to R6/1.

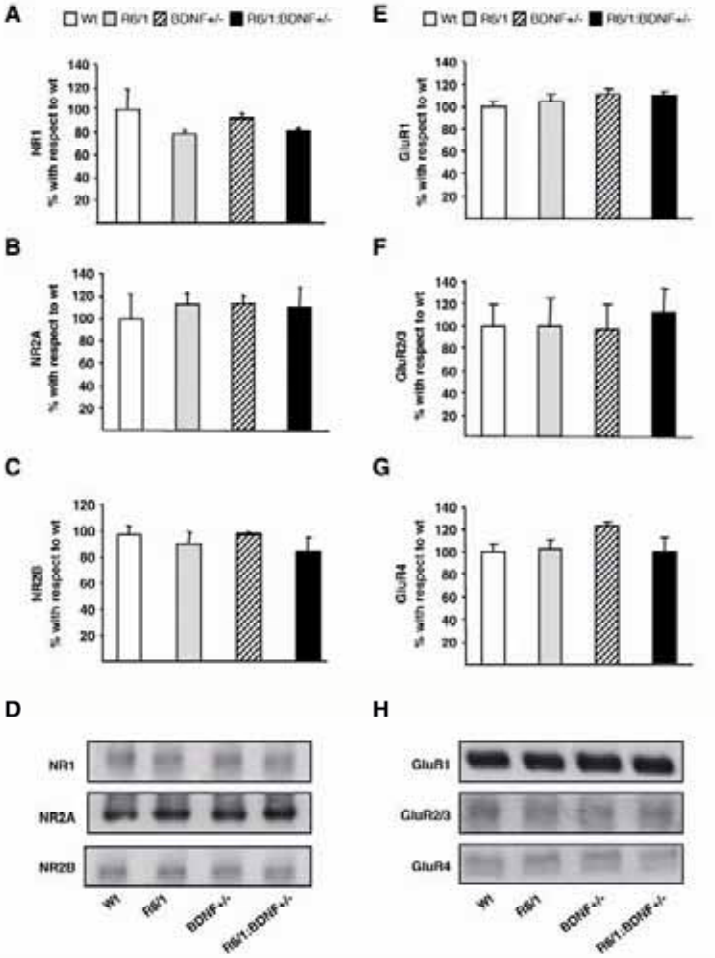


Fig. 3. Synaptic expression of NMDAR and AMPAR is not altered in mutant htt exon-1-expressing mice. Synaptic expression of NMDAR and AMPAR subunits in striatum was determined by Western blots of SPM fractions (1 μg of SPM protein per lane) at 12 weeks of age. (A–C) Graphs show quantification of synaptic NR1 (A), NR2A (B) and NR2B (C). (D) Representative blots of synaptic NR1, NR2A and NR2B subunits at 12 weeks of age. (E–G) Graphs show quantification of synaptic GluR1 (E), GluR2/3 (F) and GluR4 (G). (H) Representative blots of synaptic GluR1, GluR2/3 and GluR4 subunits at 12 weeks of age. For each SPM preparation 3 striata from mice sharing the same genotype were pooled together, and SPM preparations were repeated three times for each genotype ($n=3$ per genotype). Bars represent mean±SEM.

R6/1 and R6/1:BDNF$^{+/-}$ mice (Fig. 5). Mutant htt expression corresponded to a reduction of PSD-95 protein levels in all the ages analyzed (Figs. 5A, B). However, at the late stage (30 weeks) R6/1 mice with low levels of BDNF showed a greater reduction of PSD-95 levels (54%) than R6/1 mice with normal levels of BDNF (32%). SAP-97 protein levels began to decrease at 12 weeks of age in mutant htt exon-1-expressing mice (Figs. 5C, D) and showed no differences between R6/1 and R6/1:BDNF$^{+/-}$ mice. BDNF$^{+/-}$ mice did not have different levels of PSD-95 or SAP-97 with respect to wt mice, at any of the ages analyzed (Figs. 5A–D).

Decreased BDNF levels in the presence of mutant htt exon-1 produced a selective reduction of PSD-93 (Figs. 5E, F) and SAP-102 levels (Figs. 5G, H). Thus, R6/1:BDNF$^{+/-}$ mice showed a slight, but not significant, decrease of PSD-93 from 12 weeks of

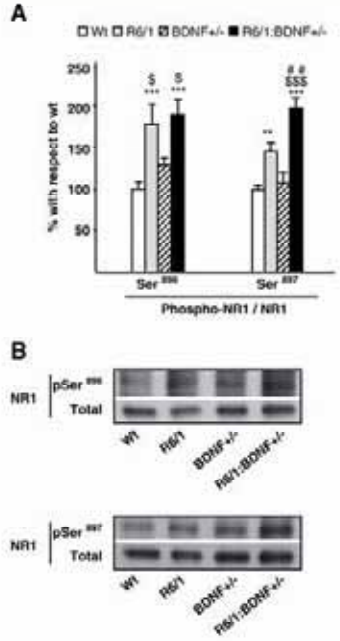


Fig. 4. BDNF modulates hyper-phosphorylation of NMDAR subunits induced by mutant htt exon-1. Levels of phospho-NR1 subunit were determined by Western blot using specific antibodies against NR1 phospho-serine^{896} (ser^{896}) and NR1 phospho-serine^{897} (ser^{897}). (A) Quantification of phosphorylation ratio of NR1 subunit at ser^{896} and ser^{897} in striatum from 12-week-old mice. (B) Representative blots of phospho-NR1 ser^{896}, phospho-NR1 ser^{897} and total NR1. Bars represent mean ± SEM; $n = 4–6$ per genotype. $^{**}p<0.01$ and $^{***}p<0.005$ with respect to wt; $^{\$}p<0.05$, $^{\$\$\$}p<0.005$ with respect to BDNF $^{+/-}$; $^{\#\#}p<0.01$ with respect to R6/1.

age (Figs. 5E, F). This specific down-regulation of total PSD-93 was significant in 30-week-old R6/1:BDNF$^{+/-}$ mice. Similarly, total SAP-102 levels were decreased in R6/1:BDNF$^{+/-}$ mice from 8 weeks of age (Figs. 5G, H).

Both R6/1 and BDNF$^{+/-}$ mice had the same levels of SAP-102 and PSD-93 as wt mice at all the ages analyzed (Figs. 5E, H). This shows that changes in the expression of these two MAGUK proteins do not depend on the expression of mutant htt exon-1 or on reduced levels of BDNF alone. However, there is a synergistic effect when these two situations are together, following the disease progression.

MAGUK expression is altered in the caudate nucleus of HD patients

Levels of PSD-95 were decreased in caudate nuclei from HD patients (Fig. 6). Levels of SAP-102 and PSD-93 were also significantly decreased in caudate nuclei from HD patients (Fig. 6).

Differential regulation of synaptic MAGUKs in mutant htt exon-1-expressing mice

We determined synaptic expression of MAGUKs in SPM and cytosol fractions from 12-week-old mice. Only synaptic expression of PSD-95 was reduced in R6/1 and R6/1:BDNF$^{+/-}$ mice. Mutant htt exon-1 produced a significant decrease of PSD-95 levels in both SPM (Figs. 7A and E) and cytosol (Figs. 7F and J). We observed a switch between synaptic PSD-95 and PSD-93, since R6/1 and R6/1:BDNF$^{+/-}$ mice showed a significant increase in synaptic levels of PSD-93 (Figs. 7B and E) whereas their cytosolic levels of PSD-93 were reduced in both R6/1 and R6/1:BDNF$^{+/-}$ mice (Figs. 7G and J). These findings suggest that the pool of cytosolic PSD-93 moved to the synaptic membranes without affecting the total levels of this MAGUK. This redistribution of PSD-93 may be a mechanism to compensate the reduction of PSD-95, and it helps to explain normal synaptic expression of NMDAR and AMPAR subunits.

However, synaptic levels of SAP-102 tended to increase in R6/1 and R6/1:BDNF$^{+/-}$ mice (Figs. 7C and E) while cytosolic levels of SAP-102 were reduced only in R6/1:BDNF$^{+/-}$ mice (Figs. 7H and J). This result is consistent with the decreased expression found in total extracts (Figs. 5G and H). In the same way, in the SPM fraction, there were no significant differences in SAP-97 for the different genotypes (Figs. 7D, E) while R6/1 and R6/1:BDNF$^{+/-}$ mice had reduced cytosolic levels (Figs. 7I, J), which is consistent with the decreased expression found in total extracts.

Specific alteration of αCaMKII in R6/1:BDNF$^{+/-}$ mice

To test whether the aforementioned changes in synaptic expression of MAGUKs affect NMDA signaling pathway, we studied the integrity of the NMDAR:nNOS complex and αCaMKII. Therefore, we quantified nNOS expression in both total extract and SPM fraction. There were no differences between genotypes in total extracts (Figs. 8A, B) or in SPM (Figs. 8C, D). We also analyzed the phosphorylation levels of p38, a protein kinase activated by phosphorylation downstream of nNOS (Cao et al., 2005). There were no differences in levels of phospho-p38 between genotypes (Figs. 8E, F).

We next studied the striatal expression of αCaMKII, an NMDAR signaling protein involved in both NMDAR- and AMPAR-mediated neurotoxic pathways (Colbran, 2004). Total levels of αCaMKII showed a slight decrease in R6/1:BDNF$^{+/-}$ mice (Figs. 8G, H). However, synaptic expression of αCaMKII was decreased in R6/1:BDNF$^{+/-}$ mice (Figs. 8I, J). It has recently been reported that αCaMKII modulates RAS/ERK signaling through activation of a RAS inhibitor, synGAP (Oh et al., 2004). Since correct αCaMKII function depends on its synaptic location (reviewed in Colbran and Brown, 2004) we expected R6/1:BDNF$^{+/-}$ mice to show impaired inhibition of RAS/ERK signaling. Thus, we analyzed the phosphorylation levels of ERK42/44 and found that mice with reduced synaptic expression of αCaMKII showed a two-fold increase in phospho-ERK42/44 levels (Figs. 8K, L) which corresponds to the increased resistance to NMDAR-mediated injury found in R6/1:BDNF$^{+/-}$ mice.

Discussion

This study shows that striatal neurons respond to the expression of mutant htt exon-1 by activating protective compensatory

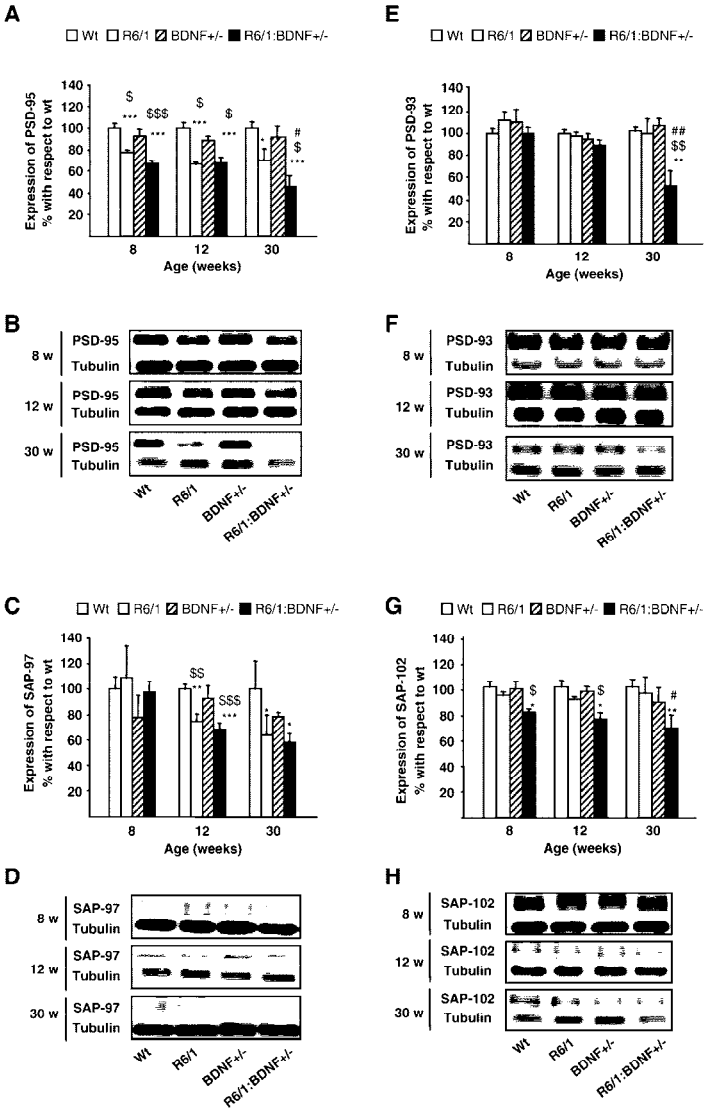


Fig. 5. BDNF differentially modulates total MAGUK expression in striatum of mutant htt exon-1-expressing mice. Graphs show quantification by Western blot of striatal PSD-95 (A), SAP-97 (C), PSD-93 (E) and SAP-102 (G) at 8, 12 and 30 weeks of age. Mutant htt-expressing mice (R6/1 and R6/1:BDNF$^{+/-}$) have reduced levels of PSD-95 (A) and SAP-97 (C) in striatum. However, total extracts from R6/1:BDNF$^{+/-}$ mice have down-regulations of PSD-93 (E) and SAP-102 (G) from 12 and 8 weeks of age, respectively. Panels B, D, F and H are representative blots of PSD-95 (B), SAP-97 (D), PSD-93 (F) and SAP-102 (H) at the ages indicated. Bars represent mean ± SEM; $n=4$–6 mice per age and genotype. $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.005$ with respect to wt; $^{\$}p<0.05$, $^{\$\$}p<0.01$, $^{\$\$\$}p<0.005$ with respect to BDNF $^{+/-}$; $^{\#}p<0.05$ and $^{\#\#}p<0.01$ with respect to R6/1.

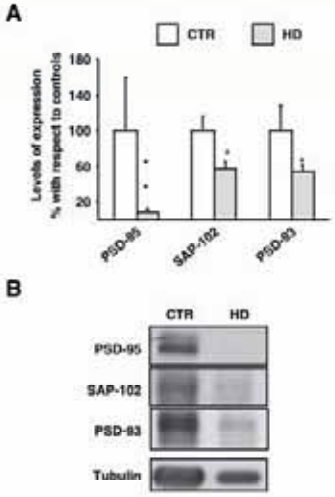


Fig. 6. Levels of MAGUK proteins in caudate nucleus from HD patients were determined by Western blot. (A, B) Levels of PSD-95, SAP-102 and PSD-93 were decreased compared to controls. (C) Representative blots of MAGUKs from caudate nuclei from controls and HD patients. CTR: controls ($n=3$); HD: HD patients ($n=4$). $^{*}p<0.5$; $^{**}p<0.005$.

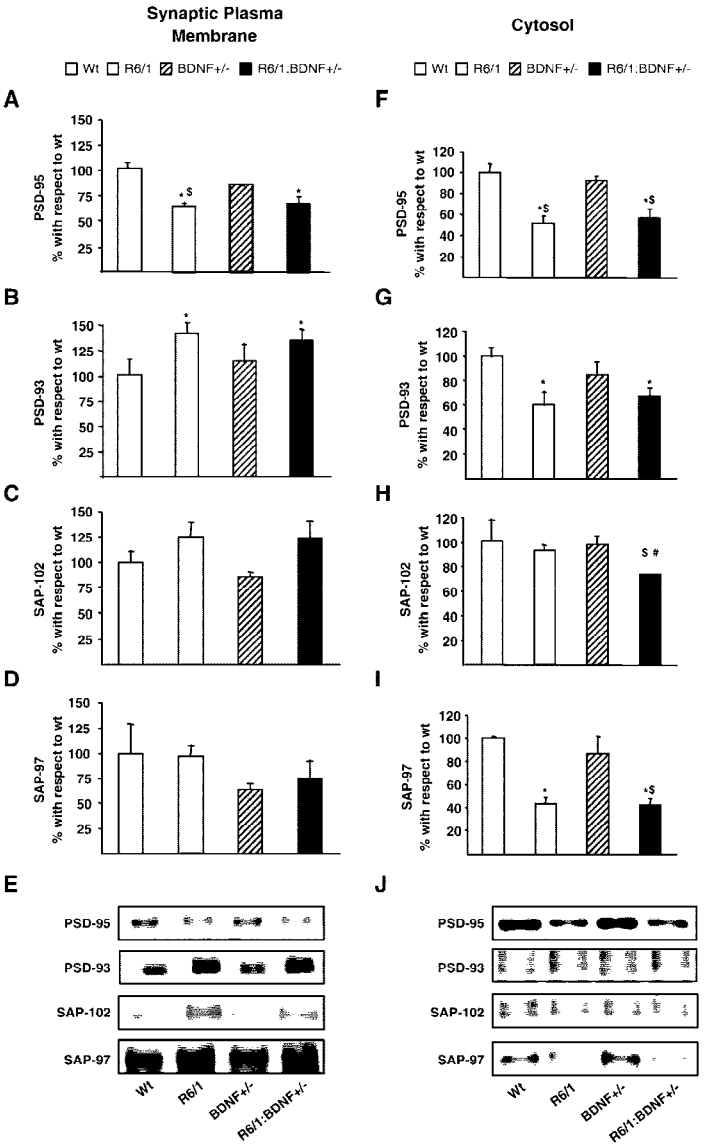
mechanisms that modify NMDAR-mediated excitotoxic damage. Thus, R6/1 mice are resistant to intrastriatal injection of the NMDAR agonist, QUIN. BDNF levels can modulate this resistance: R6/1 mice with reduced BDNF expression (R6/1:BDNF$^{+/-}$) are more resistant to QUIN lesion than those with normal BDNF expression. Levels of NMDAR subunits were not affected in the striata of mice expressing mutant htt exon-1, except for NR2B, which was lower in R6/1:BDNF$^{+/-}$ mice at late stages. However, NMDAR signaling pathways were greatly altered in both HD models. Mutant htt exon-1 produced a disorganization of NMDAR scaffolding proteins. An age-dependent decrease in the levels of PSD-95, PSD-93, SAP-97 and SAP-102 was observed in total protein extracts from striatum. However, in SPM only PSD-95 and PSD-93 were altered, in opposite directions: a decrease in PSD-95 and an increase in PSD-93. These changes were similar in R6/1 and R6/1:BDNF$^{+/-}$ mice but a reduction in αCaMKII levels in SPM were only observed in animals with a severe pathology (R6/1:BDNF$^{+/-}$).

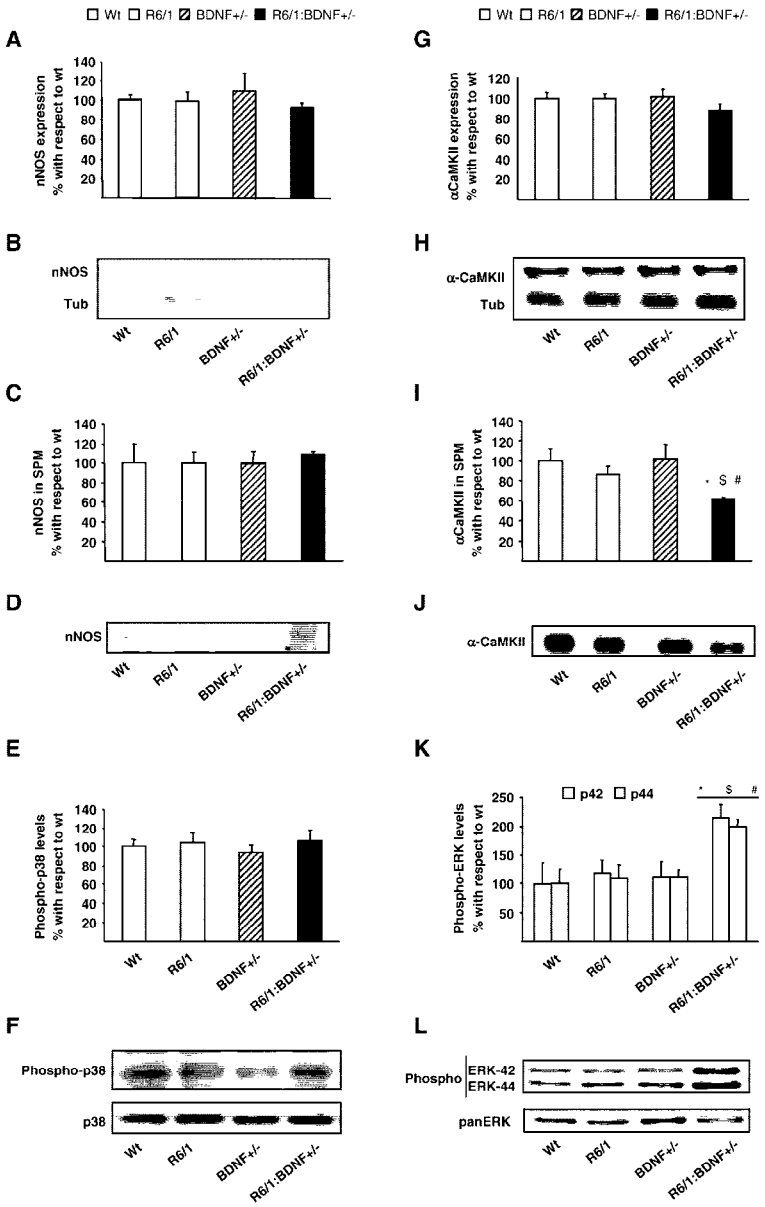
Abnormal glutamate receptor function can underlie some of the motor and cognitive impairments found in transgenic models of HD. Thus, the excitotoxic hypothesis is based on evidence that mutant htt increases NMDAR function from the presymptomatic stage of the disease (Cepeda et al., 2001), producing an increased NMDAR-dependent Ca^{2+} influx (Hansson et al., 2001), which in turn leads to neuronal degeneration (Zeron et al., 2002). Here, we show that presymptomatic R6/1 mice have transient increased levels of the phosphorylated NR1 subunit of NMDAR, especially those that express reduced levels of BDNF. This increase is in agreement with immunohistochemical studies showing enhanced NR1-p897 levels in striatal projection neurons in R6/2 mice (Ariano et al., 2005). However, decreased levels have been observed in symptomatic N171-82Q mice (Jarabek et al., 2004). These results may suggest a biphasic effect of corticostriatal activity during disease progression. In fact, transient electrophysiological alterations have been described in mouse models of HD (Cepeda et al., 2003). Nevertheless, R6 and N171-82Q mice exhibit striatal resistance to QUIN (Hansson et al., 1999, 2001; Jarabek et al., 2004, and present results), which progresses as the symptoms worsen (Hansson et al., 2001). Our present results demonstrate that this resistance is directly related to severity, since the reduction of BDNF levels in R6/1 enhances the severity of motor dysfunction (Canals et al., 2004) and reduces QUIN-induced striatal damage (present results). A similar relationship between the severity of the disease and striatal resistance was observed when striatal susceptibility to QUIN of R6/1 and R6/2 was compared (Hansson et al., 2001). However, increased susceptibility to NMDAR-mediated striatal neurotoxicity has been observed in HD transgenic mice expressing mutant full-length htt (YAC72 and YAC128), which show slower progression of motor impairment than R6 mice (Zeron et al., 2002; Tang et al., 2005). All these results support the idea that expression of mutant htt exon-1 causes conditioning changes which result in an up-regulation of defence mechanisms against excitotoxic stress (Hansson et al., 2001; Gines et al., 2003; Jarabek et al., 2004). These mechanisms may be related to neuronal pre-conditioning, whereby non-toxic overactivation of NMDAR provides tolerance against damage produced by a major insult (Llado et al., 1999; Ogita et al., 2003; Tarabal et al., 2005).

A reduction in NR2B levels, without changes in other NMDAR subunits, was only observed at late stages in R6/1:BDNF$^{+/-}$ mice. These results suggest that those neurons that express the NR2B subunit, such as striatal projection neurons, are specifically affected (Standaert et al., 1999; Landwehrmeyer et al., 1995). Similarly, NMDAR expression is reduced in postmortem samples of human HD patients (Young et al., 1988; Dure et al., 1991). In agreement with these results, NR2B subunit receptors have been implicated in the initial degeneration of striatal projection neurons in the YAC72 mouse (Zeron et al., 2002). However, our results demonstrate that decreased levels of NR2B do not contribute to striatal resistance to NMDA, since affectation of this subunit receptor was not observed

Fig. 7. HD transgenic mice show specific alterations in synaptic and cytosolic MAGUK expression. Synaptic and cytosolic MAGUK expression in striatum was determined by Western blots employing SPM (1 μg of protein per lane) and cytosolic fractions from 12-week-old mice. (A–D) Graphs show that synaptic levels of PSD-95 (A) are significantly reduced in both R6/1 and R6/1:BDNF$^{+/-}$ mice. By contrast, synaptic levels of PSD-93 (B) are increased in these mice with respect to controls. Other MAGUKs, like SAP-102 (C) and SAP-97 (D), present no statistic differences in their synaptic expression with respect to controls. (E) Representative blots of synaptic PSD-95, PSD-93, SAP-102 and SAP-97. (F–I) Cytosolic MAGUK expression is different in R6/1 and R6/1:BDNF$^{+/-}$ mice. Western blots of cytosolic fractions show that R6/1 mice have reduced levels of PSD-95 (F), PSD-93 (G) and SAP-97 (I), while cytosolic SAP-102 (H) is reduced only in R6/1:BDNF$^{+/-}$ mice. (J) Representative blots of cytosolic PSD-95, PSD-93, SAP-102 and SAP-97. Tubulin was employed as a load control in cytosolic fractions. For each SPM preparation 3 striata from mice sharing the same genotype were pooled together, and SPM preparations were repeated three times for each genotype ($n=3$ per genotype). Bars represent mean ± SEM. $^{*}p<0.05$ with respect to wt; $^{\$}p<0.05$ with respect to BDNF$^{+/-}$; $^{\#}p<0.05$ with respect to R6/1. For SPM and cytosolic fraction, striata from 3 mice were used ($n=3$).

in total or in SPM levels in presymptomatic mice. Therefore, other mechanisms must be invoked to explain the resistance to excitotoxicity induced by mutant htt exon-1. We observed that total levels of MAGUKs are reduced in young mice when they show resistance to QUIN. These findings suggest that MAGUKs may be more relevant than NMDAR per se in the vulnerability of striatal neurons. Thus, the decrease in MAGUKS levels may participate in the compensatory mechanisms activated by mutant




Wt R6/1 BDNF+/- R6/1:BDNF+/-
A
nNOS expression % with respect to wt
B
nNOS
Tub
C
nNOS in SPM % with respect to wt
D
nNOS
E
Phospho-p38 levels % with respect to wt
F
Phospho-p38
p38
G
αCaMKII expression % with respect to wt
H
α-CaMKII
Tub
I
αCaMKII in SPM % with respect to wt
J
α-CaMKII
K
p42 p44
Phospho-ERK levels % with respect to wt
L
Phospho
ERK-42
ERK-44
panERK
Wt
R6/1
BDNF+/-
R6/1:BDNF+/-

htt exon-1 toxicity. This view is supported by the fact that decreased levels of both PSD-95 and PSD-93 reduce both AMPA and NMDAR excitatory postsynaptic currents (Elias et al., 2006). Similarly, a recent paper using RNAi show that cortical neurons lacking PSD-95 but not PSD-93, SAP97 or SAP102 exhibit reduced NMDA toxicity, suggesting specific excitotoxic signaling through MAGUKs (Cui et al., 2007). In addition, down-regulation of PSD-95 by PSD-95 antisense *in vitro* (Sattler et al., 1999) and *in vivo* (Hou et al., 2005) confers increased resistance to excitotoxicity and ischemia, respectively. Although other MAGUKs are also decreased in R6/1 mice, PSD-95 may have a relevant role in HD pathophysiology, since it is reduced in both cytosol and SPM. This agrees with previous observations of a decrease in PSD-95 levels in different transgenic mouse models of HD (Luthi-Carter et al., 2002; Jarabek et al., 2004). We also observed that total levels of MAGUKs are also reduced in human postmortem HD samples. Decreased levels of MAGUKs may be a consequence of overactivation of NMDAR. Thus, electrophysiologic studies have described transient and progressive changes in spontaneous synaptic currents in transgenic R6 mice (Cepeda et al., 2003). These initial dysfunctions of striatal and cortical circuits during the development of the HD phenotype produce a corticostriatal disconnection (for a review, see Cepeda et al., 2007). Therefore, the reduction in the levels of MAGUKs may play an important role in the corticostriatal dysfunction, which can trigger compensatory mechanisms as the consequence of the progression of the disease. In fact, reduced glutamatergic synaptic currents are correlated with a marked reduction in the expression of synaptic markers synaptophysin and PSD-95 (Cepeda et al., 2003). Furthermore, early studies already showed that in order to produce an excitotoxic lesion in the striatum the integrity of the excitatory cortical projection is required (Biziere and Coyle, 1979; McGeer et al., 1978; Orlando et al., 2001).

MAGUKs interact with different NR2 subunits and are involved in trafficking, synaptic targeting and clustering of NMDAR (Kim and Sheng, 2004). However, synaptic levels of NMDAR subunits remain unchanged in spite of altered levels of MAGUKs. Thus, the normal synaptic levels of NMDAR found in HD mice reinforce the hypothesis that synaptic expression of NMDAR is affected only when the levels of MAGUKs fall below a critical threshold. This is supported by the observation that knockdown of SAP-102 *in vivo* does not alter NMDAR-dependent synaptic transmission but reduces NMDAR excitatory postsynaptic currents only when both PSD-95 and PSD-93 are lacking (Elias et al., 2006). Therefore, the kinetics of MAGUK-dependent traffic and/or synaptic expression of NMDAR may be affected in more advanced stages of the disease, when the greatest reduction in SAP-102, PSD-93 and PSD-95 levels occurs in R6/1:BDNF$^{+/-}$ mice. Furthermore, we observed MAGUK compensatory mechanisms against htt-induced toxicity. Thus, synaptic levels of PSD-95 were reduced in the SPM of HD transgenic mice, while synaptic expression of PSD-93 increased and its cytosolic levels decreased, indicating that PSD-93 translocates from cytosol to SPM. Functional compensation by molecular redundancy among MAGUKs has been demonstrated, as mutant mice with a simultaneous lack of PSD-95 and PSD-93 (Elias et al., 2006) or those expressing mutant PSD-95 (Vickers et al., 2006) show a compensatory increase of SAP-102.

These changes in MAGUKs may be involved in the resistance of excitotoxicity in R6/1 through modification of different signaling pathways. PSD-95 imparts signaling and neurotoxic specificity to NMDARs through the coupling of receptor activity to critical second messenger pathways. Thus, MAGUK regulation of NMDAR pathways may depend, at least in part, on the possibility of binding both NMDAR and nNOS in a ternary complex (Sattler et al., 1999; Dawson and Dawson, 1996; Aarts et al., 2002; Cao et al., 2005). However, our results demonstrate that synaptic levels of nNOS and phospho-p38-kinase remain unaltered in spite of reduced synaptic levels of PSD-95. This is in agreement with previous *in vitro* studies showing that suppressing PSD-95 blocks excitotoxicity without affecting nNOS expression or function (Sattler et al., 1999). Other second messenger involved in NMDA-MAGUK signaling is αCaMKII. Our results show that, in R6/1:BDNF$^{+/-}$ mice, αCaMKII-dependent signaling was altered, since the mice displayed reduced synaptic expression of αCaMKII and increased levels of phospho-ERK42/44. These findings correspond with an increased striatal resistance to QUIN found in R6/1:BDNF$^{+/-}$ mice. Dysfunction of αCaMKII has been implicated in some neurodegenerative disorders such as Alzheimer disease (Dewachter et al., in press) and Parkinson's disease (Picconi et al., 2004). αCaMKII has also been implicated in excitotoxic pathways (Hajimohammadreza et al., 1995; Gardoni et al., 2002). Thus, synaptic expression of αCaMKII increases after brain ischemia (Yan et al., 2004) and traumatic brain injury (Atkins et al., 2006) and previous treatment with a specific CaMKII inhibitor provides neuroprotection against NMDA induced excitotoxicity and hypoxia/hypoglycemia-induced neuronal injury (Hajimohammadreza et al., 1995). In addition, recent findings show that activation of αCaMKII strengthens NMDA (Gardoni et al., 2007) and AMPA receptor-mediated Ca^{+2} influx (Gardoni et al., 2002). Furthermore, αCaMKII activates synGAP; a negative modulator of the pro-survival RAS/ERK pathway (Oh et al., 2004). Our results also suggest that BDNF modulates changes in NMDAR signaling in PSD induced by mutant htt exon-1, as reduced synaptic levels of αCaMKII and increased levels of phospho-ERK42/44 occur in R6/1:BDNF$^{+/-}$ mice, but not in BDNF$^{+/-}$ or R6/1 mice.

In conclusion, the present results provide molecular evidence that reorganization of NMDAR signaling proteins occurs in PSD of HD transgenic mice. These changes may reflect an important basis underlying dysfunctions of NMDAR and their intracellular signaling in HD neuropathology.

Fig. 8. Signaling of NMDAR is selectively impaired in R6/1:BDNF$^{+/-}$ mice. (A–F) Striatal expression of nNOS and αCaMKII was determined by Western blot in total protein extract and synaptic plasma membrane preparation (1 and 5 μg of protein from SPM per lane for αCaMKII and nNOS, respectively) from 12-week-old mice. There are no differences in striatal nNOS expression in total extract (A, B) or SPM (C, D). Therefore, there are no differences in phospho-p38 levels between genotypes (E, F). Striatal αCaMKII expression (G, H) shows no differences between genotypes. By contrast, only R6/1:BDNF$^{+/-}$ mice have reduced levels of αCaMKII in SPM (I, J) which correlates with their increased levels of phospho-ERK (K, L). For each SPM preparation 3 striata from mice sharing the same genotype were pooled together, and SPM preparations were repeated three times for each genotype. Bars represent mean±SEM; total extracts, $n=4–6$; SPM preparation, $n=3$ per genotype. $^{*}p<0.05$ with respect to wt; $^{\$}p<0.05$ with respect to BDNF $^{+/-}$; $^{\#}p<0.05$ with respect to R6/1.

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Segundo trabajo: ***"Reduced calcineurin protein levels and activity in exon-1 mouse models of Huntington's disease: Role in excitotoxicity."***

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Objetivo 2: Identificación y caracterización de las vías de señalización afectadas específicamente por debajo de la funcionalidad de los receptores NMDA en procesos de excitotoxicidad y disfunción sináptica en modelos animales transgénicos y agudos de la enfermedad de Huntington.

Aunque la excitotoxicidad ha sido hasta el momento, uno de los procesos más estudiados y aceptados como moduladores de la fisiopatología de la enfermedad de Huntington y, aunque los receptores NMDA han sido el foco de interés dentro de ellos, no existe consenso de cuáles son las vías moleculares implicadas. Distintos grupos han propuesto múltiples vías de señalización mediando estos procesos sin embargo, el hecho de que dependiendo del modelo utilizado los procesos excitotóxicos están disminuidos o incrementados ha hecho, hasta ahora, imposible la propuesta de una explicación integrada. Trabajos previos en nuestro grupo indican que la activación de la fosfatasa calcineurina regula ampliamente los procesos de excitotóxicos en modelos celulares de la enfermedad de Huntington. Así, en el presente estudio, nuestra intención era analizar qué papel juega la expresión y actividad de la fosfatasa *in vivo* en tales procesos mediante el uso de ratones R6/1 (exón-1), los cuáles presentan resistencia ante estímulos excitotóxicos. Adicionalmente, también se analizó si BDNF podría estar mediando estos efectos mediante el uso de los ratones R6/1:BDNF+/-.

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Reduced calcineurin protein levels and activity in exon-1 mouse models of Huntington's disease: Role in excitotoxicity

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ABSTRACT

Calcineurin is a serine/threonine phosphatase involved in the regulation of glutamate receptors signaling. Here, we analyzed whether the regulation of calcineurin protein levels and activity modulates the susceptibility of striatal neurons to excitotoxicity in R6/1 and R6/1:BDNF+/− mouse models of Huntington's disease. We show that calcineurin inhibition in wild-type mice drastically reduced quinolinic acid-induced striatal cell death. Moreover, calcineurin A and B were differentially regulated during disease progression with a specific reduction of calcineurin A protein levels and calcineurin activity at the onset of the disease in R6/1:BDNF+/− mice. Analysis of the conditional mouse model Tet/HD94 showed that mutant huntingtin specifically controls calcineurin A protein levels. Finally, calcineurin activation induced by intrastriatal quinolinic acid injection in R6/1 mouse was lower than in wild-type mice. Therefore, reduction of calcineurin activity by alteration of calcineurin A expression participates in the pathophysiology of Huntington's disease and contributes to the excitotoxic resistance observed in exon-1 mouse models.

Introduction

Huntington's disease (HD) is a genetic neurodegenerative disorder due to an abnormal expansion of a CAG codon in exon-1 of the huntingtin (htt) gene (The Huntington's Disease Collaborative Group, 1993). The hallmark of HD neuropathology is the striatal atrophy caused by a selective degeneration of medium-sized spiny neurons (Reiner et al., 1988). It has been proposed that excitotoxicity through the over-activation of NMDA receptors (NMDARs) contributes to the selective loss of these neurons (for review see Perez-Navarro et al., 2006; Fan and Raymond 2007). This suggestion firstly came from studies showing that selective loss of striatal neurons occurring in HD can be reproduced by intrastriatal injection of quinolinic acid (QUIN), a NMDAR agonist (Beal et al., 1986). The development of transgenic mouse models of HD has provided a good tool to study whether excitotoxicity participates in neuronal dysfunction and death. Different results have been obtained after intrastriatal QUIN injection in these models, which seem to correlate with the severity of the disease. Transgenic mice expressing full-length mutant htt (mhtt), such as YAC72, with a mild phenotype and late striatal neurodegeneration (Hodgson et al., 1999) show increased sensitivity to intrastriatal QUIN injection (Zeron et al., 2002), whereas YAC128 mice display enhanced sensitivity to QUIN in asymptomatic stages and resistance when disease manifests (Graham et al., 2009). Furthermore, mouse models expressing a truncated form of mhtt, such as R6/1, R6/1:BDNF+/−, R6/2 and N171-82Q that show an accelerated progression of the disease (Mangiarini et al., 1996; Yu et al., 2003; Canals et al., 2004), develop resistance to intrastriatal QUIN injection gradually over time (Hansson et al., 1999, 2001; Jarabek et al., 2004; Torres-Peraza et al., 2008). It has been suggested that the development of resistance to QUIN is a consequence of an adaptative response to counteract mhtt toxicity but the molecular mechanisms underlying this phenomenon are not clear yet.

Calcineurin is a calcium (Ca^{2+})- and calmodulin-dependent serine/threonine phosphatase composed by a catalytic subunit (calcineurin A) and a regulatory subunit (calcineurin B), and particularly enriched in the hippocampus and striatum (Rusnak and Mertz, 2000). Stimulation of NMDAR activates calcineurin (Asai et al., 1999; Xifro et al., 2008) by Ca^{2+} and calmodulin binding (Stewart et al., 1982; Klee et al., 1998), and by calpain-dependent proteolysis of calcineurin A (Tallant et al., 1988). The pro-apoptotic function of calcineurin is associated with dephosphorylation of selected

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substrates (Wang et al., 1999; Springer et al., 2000; Shamloo et al., 2005; Xifro et al., 2008). Furthermore, calcineurin also dephosphorylates mhtt promoting its toxic effects (Pardo et al., 2006). Pharmacological blockade of calcineurin activity prevents excitotoxic-induced cell death through inhibition of the mitochondrial apoptotic pathway (Uchino et al., 2002; Almeida et al., 2004; Xifro et al., 2008), and caspase-3 and calpain proteases (Asai et al., 1999; Ferrand-Drake et al., 2003; Xifro et al., 2008). We have recently reported that mhtt knock-in striatal cells show increased vulnerability to NMDAR stimulation that is associated with high calcineurin A protein levels and calcineurin activity (Xifro et al., 2008). We therefore analyzed whether changes in calcineurin activity and protein levels participate in the resistance to excitotoxicity in several N-terminal exon-1 mhtt transgenic mice.

Materials and methods

HD mouse models

R6/1 heterozygous transgenic mice expressing the exon-1 mhtt with 145 CAG repeats (Giralt et al., 2009) were cross-mated with BDNF heterozygous mice (BDNF+/−; Ernfors et al., 1994) to obtain R6/1:BDNF+/− mice, as previously described (Canals et al., 2004). Wild-type (WT) and BDNF+/− littermates were used as controls. Conditional Tet/HD94 mice express a chimeric mouse/human exon-1 mhtt with 94 CAG repeats under the control of the bidirectional tetO responsive promoter (Yamamoto et al., 2000). To turn off mhtt expression (gene-off group), 17-month-old WT and Tet/HD94 mice were treated with doxycycline in drinking water during 5 months (2 mg/ml for 4 months followed by 0.5 mg/ml for 1 month; Diaz-Hernandez et al., 2005). Some animals were left without intervention (gene-on 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. Mice were genotyped by polymerase chain reaction as described previously (Mangiarini et al., 1996; Yamamoto et al., 2000; Canals et al., 2004). 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 h light/dark cycle. All procedures were performed in compliance with the National Institute of Health Guide for the care and use of laboratory animals, and approved by the local animal care committee of the Universitat de Barcelona (99/01), and the Generalitat de Catalunya (99/1094).

Striatal lesions

WT and R6/1 mice (12-week-old) were anesthetized with pentobarbital (50 mg/kg) and QUIN [10 nmol, Sigma Chemical Co., St Louis, MO; dissolved in phosphate-buffered saline (PBS)] was intrastriatally injected at the following coordinates relative to bregma: AP+0.6 mm, ML+2 mm and 2.7 mm below the dural surface with the incisor bar at 3 mm above the interaural line. The contralateral striatum was injected with vehicle (PBS) at the following coordinates relative to bregma: AP+0.6 mm, ML−2 mm and 2.7 mm below the dural surface with the incisor bar at 3 mm above the interaural line. QUIN or PBS was injected over 2 min and the cannula was left in place for additional 5 min. To analyze the effect of a calcineurin inhibitor on excitotoxic-induced cell death in WT mice, FK-506 (5 mg/kg; dissolved in Cremophor, Sigma) or vehicle (Cremophor) was injected intraperitoneally 30 min prior to QUIN injection in the striatum. Animals were sacrificed at different time points for morphological (Fluoro-Jade staining and *in situ* detection of DNA fragmentation; 48 h) or biochemical (calcineurin activity and protein levels; 1, 2 and 4 h) analysis.

Fluoro-Jade staining and in situ detection of DNA fragmentation

Forty-eight hours after intrastriatal QUIN injection, with or without FK-506 treatment, mice ($n=7$ for each condition) were deeply anesthetized and immediately perfused transcardially with saline followed by 4% paraformaldehyde/phosphate buffer. Brains were removed and post-fixed for 1–2 h in the same solution, cryoprotected by immersion in 30% sucrose/PBS and then frozen in dry ice-cooled isopentane. Serial coronal cryostat sections (30 μm) through the whole striatum were collected on silane-coated slides. Striatal sections were processed for Fluoro-Jade staining (Histo-Chem, Inc., Jefferson, AR) as described elsewhere (Schmued et al., 1997). Sections stained with Fluoro-Jade were visualized on a computer, and the border of the lesion was outlined using the Computer-Assisted Stereology Toolbox (CAST) software (Olympus). The volume of the lesion was estimated by multiplying the sum of all the sectional areas (μm^2) by the distance between successive sections (240 μm), as described previously (Perez-Navarro et al., 2000). DNA fragmentation was examined using the *in situ* Apoptosis detection system (Fluorescein, Promega, Madison, WI) as described elsewhere (Perez-Navarro et al., 2000).

Calcineurin activity

Under deep anesthesia, the animals were killed by decapitation and the striatum was quickly dissected and frozen at −80 °C. Calcineurin activity was measured using a calcineurin assay kit (Calbiochem, La Jolla, CA) as described elsewhere (Xifro et al., 2008). Striatal proteins (10 μg) obtained by homogenization in lysis buffer containing 50 mM Tris–HCl, pH 7.5, 1 mM dithiothreitol, 100 μM EDTA, 100 μM EGTA and 0.2% Nonidet P-40 were incubated with a well-known calcineurin substrate, RII phosphopeptide. The assay was performed according to the protocol suggested by the manufacturer. Reactions were terminated by adding GREEN™ reagent (Calbiochem), and colorimetric intensity was measured at 620 nm in a μQuant™ Microplate Spectrophotometer (BioTek Instruments, Winooski, UT).

Post-mortem brain tissues

Brain tissues were obtained from the University of Barcelona and Institute of Neuropathology Brain Banks (Barcelona, Spain) following the guidelines of the local ethics committees. Samples from control subjects and HD patients were processed using the same protocol. Calcineurin A, calcineurin B and tubulin were analyzed in samples from three HD patients [post-mortem intervals 4–13 h, with death at end-stage disease at 28 (juvenile onset HD patient), 59 and 60 years (both Vonsattel grade IV)] and five control subjects that did not suffer any neurological disease (post-mortem intervals 3–9 h, with death at 39, 56, 64, 68 and 71 years).

Total protein extraction

Animals were deeply anesthetized and killed by decapitation at 8, 12, 16 or 30 weeks (R6/1 and R6/1:BDNF+/− mice) or 22 months (Tet/HD94 mice) of age. Striatum, cortex and hippocampus were quickly removed, homogenized in lysis buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 100 mM NaF, 5 μM $ZnCl_2$ and 10 mM EGTA] plus protease inhibitors [phenylmethylsulphonyl fluoride, PMSF (2 mM), aprotinin (1 μg/ml), leupeptin (1 μg/ml) and sodium orthovanadate (1 mM)] and centrifuged at 15,000×*g* for 20 min. The supernatants were collected and protein concentration was measured using the Dc protein assay kit (Bio-Rad, Hercules, CA).

Western blot analysis

To analyze the protein levels of calcineurin A and B, Western blotting was performed as described elsewhere (Perez-Navarro et

al., 2005). Protein extracts were resolved in denaturing polyacrilamide gels using the Mini-Protean system (Bio-Rad). After separation, proteins were transferred to a nitrocellulose membrane (Whatman®, Schleicher and Schuell, Dassel, Germany) and washed with Tris-buffered saline containing 0.1% Tween-20 (TBS-T). After incubation with blocking buffer (5% non-fat dry milk in TBS-T) at room temperature for 1 h, membranes were incubated overnight at 4 °C with the antibodies against calcineurin A (1:500; BD Biosciences, San Jose, CA) or B (1:1000; Calbiochem). Loading control was performed by reprobing the membranes with an anti-actin (1:10,000; MP Biomedicals, Aurora, OH) or an anti-tubulin (1:10,000; Sigma) antibody during 45 min at room temperature. Then, membranes were washed twice with TBS-T, incubated for 1 h at room temperature with the corresponding horseradish peroxidase-conjugated antibody (Promega, 1:2000 dilution for all primary antibodies with the exception of actin and tubulin detection, where the secondary antibody was diluted at 1:3000) and washed again twice with TBS-T. Immunoreactive bands were visualized using the Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Santa Cruz, CA) and quantified by a computer-assisted densitometer.

Statistical analysis

Statistical analysis was performed 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. A value of $p<0.05$ was accepted as denoting statistical significance.

Results

Inhibition of calcineurin activity prevents QUIN-induced cell death in the striatum

To investigate whether calcineurin activity contributes to excitotoxic-induced cell death *in vivo*, FK-506, a calcineurin inhibitor, was injected 30 min prior to intrastriatal QUIN injection in WT mice. Treatment with

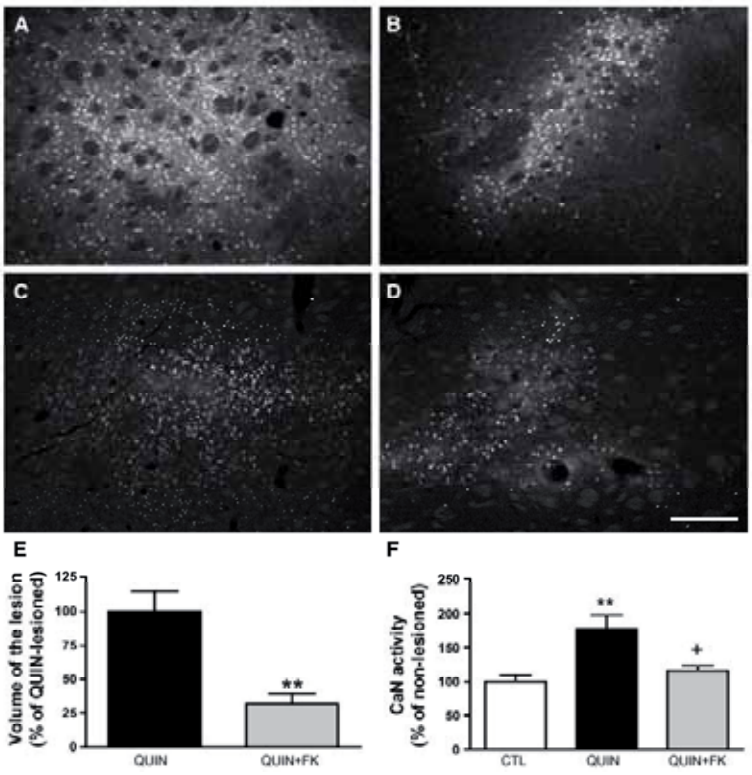


Fig. 1. FK-506 treatment prevents QUIN-induced cell death by blocking calcineurin activity. FK-506 (5 mg/kg) or vehicle was intraperitoneally injected in WT mice 30 min before intrastriatal QUIN (10 nmol) injection. To examine cell death, Fluoro-Jade staining and the TUNEL assay were performed 48 h after lesion. Photomicrographs show the striatal area occupied by Fluoro-Jade-positive cells (A and B) and TUNEL-positive nuclei (C and D) in vehicle plus QUIN (A and C) and in FK-506 plus QUIN (B and D) injected WT mice. Scale bar, 200 μm. (E) Graph showing the quantification of the volume of the lesion measured in Fluoro-Jade stained sections expressed as a percentage of vehicle plus QUIN. Results are the mean ± SEM for seven animals per condition. Data were analyzed by unpaired Student's t test. $^{**}p<0.01$ compared with vehicle plus QUIN-injected mice. (F) Graph shows calcineurin activity 2 h after intrastriatal QUIN injection in mice intraperitoneally injected with vehicle (QUIN) or with FK-506 (QUIN+ FK). Control (CTL) values were obtained from the striatum contralateral to the lesion in vehicle-injected mice. Results are expressed as a percentage of CTL and represent the mean ± SEM ($n=5$ for each condition). Results were analyzed by one-way ANOVA followed by Bonferroni's post-hoc test. $^{**}p<0.01$ compared with CTL; $^{+}p<0.05$ compared QUIN.

FK-506 produced a decrease in the number of degenerating neurons 48 h after the excitotoxic lesion as assessed by Fluoro-Jade staining (Figs. 1A and B) and TUNEL assay (Figs. 1C and D). Analysis of the striatal volume occupied by Fluoro-Jade-positive cells showed that FK-506 treatment reduced the volume of the lesion by 68 ± 18% when compared to QUIN-injected striatum (Fig. 1E). To confirm that the reduction of the lesion volume was due to the inhibition of calcineurin activity in the striatum, we analyzed the activity of this phosphatase 2 h after intrastriatal QUIN injection in mice with or without FK-506 pre-treatment. As shown in Fig. 1F, intraperitoneal injection of FK-506 prevented the increase in calcineurin activity induced by intrastriatal QUIN injection.

R6/1 and R6/1:BDNF+/− striatum show reduced levels of calcineurin A and B at different stages of the disease progression

To examine whether calcineurin is involved in the resistance to excitotoxicity in exon-1 mhtt mouse models (Zuchner and Brundin, 2008), we first analyzed calcineurin protein levels in the striatum of R6/1 and R6/1:BDNF+/−, two transgenic mouse models that show different sensibility to striatal-induced excitotoxicity (Torres-Peraza et al., 2008), and distinct temporal pattern of disease progression (Canals et al., 2004). Calcineurin A and B protein levels were analyzed by Western blot at several stages of the disease progression (8, 12, 16 and 30 weeks). Analysis of calcineurin A protein levels disclosed a different regulation in R6/1 and R6/1:BDNF+/− striatum. No changes in calcineurin A protein levels were detected at 8 weeks of age in any of the genotypes analyzed (Figs. 2A and E). Interestingly, at 12 weeks of age decreased levels of calcineurin A were detected in R6/1:BDNF+/− (by 24 ± 2%; Figs. 2B and E), but not in R6/1 animals. These data are consistent with R6/1:BDNF+/− mice being more resistant to intrastriatal QUIN injection than R6/1 mice at 12 weeks of age (Torres-Peraza et al., 2008). At older ages, 16 and 30 weeks, R6/1 and R6/1:BDNF+/− striatum showed a similar reduction in calcineurin A protein levels (about 30%; Figs. 2C–E).

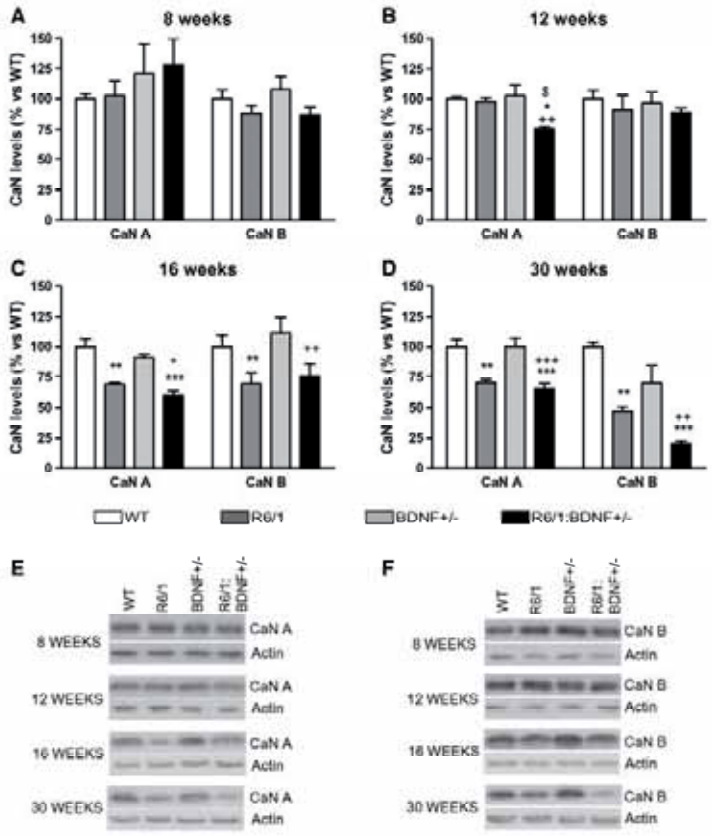


Fig. 2. Calcineurin A and B protein levels are differentially regulated during disease progression in R6/1 and R6/1:BDNF+/− mouse striatum. Calcineurin A (CaN A) and B (CaN B) protein levels were analyzed by Western blot in the striatum of WT, R6/1, BDNF+/− and R6/1:BDNF+/− mice at 8 (A), 12 (B), 16 (C) and 30 (D) weeks of age. Quantification of Western blot was performed by densitometric analysis. The results are expressed as the percentage of the ratio of CaN A or CaN B levels vs. actin levels in WT, and data shown are the mean ± SEM ($n = 4–7$). Data were analyzed by one-way ANOVA followed by Bonferroni's post-hoc test. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared to WT mice; $^{+}p<0.05$, $^{++}p<0.01$ and $^{+++}p<0.001$ compared to BDNF+/− mice; $^{\$}p<0.05$ compared to R6/1 mice. Representative immunoblots showing protein levels of actin and CaN A (E) or B (F) in WT, R6/1, BDNF+/− and R6/1:BDNF+/− mice at different ages.

Determination of calcineurin B protein levels revealed a similar pattern of protein expression in R6/1 and R6/1:BDNF+/− striatum with reduced levels at 16 and 30 weeks of age when compared with their littermate controls (Fig. 2). This decrease was comparable at 16 weeks of age (about 30%) whereas in 30-week-old mice calcineurin B protein levels were more affected in R6/1:BDNF+/− (75±5% reduction) than in R6/1 striatum (50±5% reduction; Figs. 2D and F).

Calcineurin A is reduced in the cortex, but not in the hippocampus, of R6/1 and R6/1:BDNF+/− mice

In order to analyze whether the reduction of calcineurin A and B protein levels in R6/1 and R6/1:BDNF+/− mice occurs specifically in the striatum, their protein levels were also determined in the cortex (the second main area degenerating in HD; Vonsattel and DiFiglia, 1998) and hippocampus at 30 weeks of age. Calcineurin A protein levels were significantly reduced in the cortex of both R6/1 and R6/1:BDNF+/− mice (WT: 100±18%; R6/1: 47±12%*; BDNF+/−: 88±24%; R6/1:BDNF+/−: 53±6%; *$p<0.05$ compared to WT values) whereas no changes were observed in the hippocampus (WT: 100±7%; R6/1: 108±10%; BDNF+/−: 107±14%; R6/1:BDNF+/−: 110±6%). In contrast, calcineurin B protein levels were not modified in any of the genotypes analyzed, either in the cortex (WT: 100±14%; R6/1: 76±11%; BDNF+/−: 90±8%; R6/1:BDNF+/−: 85±13%) or in the hippocampus (WT: 100±6%; R6/1: 99±13%; BDNF+/−: 97±10%; R6/1:BDNF+/−: 104±11%).

Reduction of striatal calcineurin A, but not B, is reverted by suppressing transgene expression in Tet/HD94 mice

We next used the conditional model of HD, Tet/HD94 mice, to determine whether the decrease of calcineurin A and B protein levels observed in R6/1 and R6/1:BDNF+/− mice depends on the stress induced by continuous mhtt expression. Calcineurin A and B were examined at 22 months of age in three different groups: WT, Tet/HD94 with no pharmacological intervention (gene-on) and Tet/HD94 after 5 months of doxycycline administration that turns off the mhtt transgene expression (gene-off). Consistent with the results from the striatum of R6/1 and R6/1:BDNF+/− mice, we detected a reduction of calcineurin A (by 70%) and B (by 65%) protein levels in Tet/HD94 gene-on group compared to WT mice (Fig. 3). Interestingly, suppression of transgene expression (Tet/HD94 gene-off) blocked the reduction of calcineurin A (Figs. 3A and C), but not that of calcineurin B (Figs. 3B and C).

The reduction of calcineurin A levels results in lower calcineurin activity

To investigate whether decreased calcineurin A and B protein levels were associated with a reduction in basal calcineurin activity, we analyzed this parameter in the striatum at 12 weeks (when calcineurin A protein levels are reduced only in R6/1:BDNF+/− mice; Fig 2B) and at 30 weeks of age (when both subunits are down-regulated in R6/1 and R6/1:BDNF+/− mice; Fig. 2D). Concomitant with lower calcineurin A levels, we detected a decrease of calcineurin activity at 12 weeks of age only in the R6/1:BDNF+/− striatum (Fig. 4A), whereas at 30 weeks of age both genotypes displayed reduced calcineurin activity when compared to their littermate controls (Fig. 4B).

Calcineurin activity is differentially regulated in WT and R6/1 mice in response to intrastriatal QUIN injection

The striatum of 12-week-old R6/1 mice showed levels of calcineurin protein and activity similar to WT mice. However, they

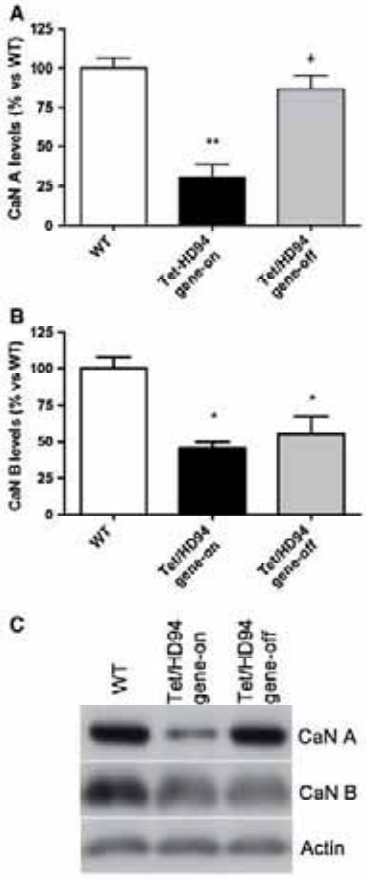


Fig. 3. Regulation of calcineurin A protein levels is mediated by the expression of mhtt in the conditional Tet/HD94 mouse model of HD. Calcineurin A (CaN A) (A) and calcineurin B (CaN B) (B) were analyzed by Western blot in the striatum of WT and Tet/HD94 mice either with no pharmacological intervention (gene-on) or after 5 months of transgene shut-down by doxycycline administration (gene-off). Quantification of Western blot was performed by densitometric analysis. The results are expressed as the percentage of the ratio of CaN A levels vs. actin levels (A), and the ratio of CaN B levels vs. actin levels (B) in WT mice, and values shown are the mean±SEM ($n=3$–4). Data were analyzed by one-way ANOVA followed by Bonferroni's post-hoc test. *$p<0.05$ and **$p<0.01$ compared to WT mice and $^{+}p<0.05$ compared to Tet/HD94 gene-on mice. (C) Representative immunoblots showing protein levels of CaN A, CaN B and actin in the striatum of WT, Tet/HD94 gene-on and Tet/HD94 gene-off mice.

are resistant to intrastriatal QUIN injection (Hansson et al., 2001; Torres-Peraza et al., 2008). Thus, we examined whether the activation of calcineurin in response to intrastriatal QUIN injection was different in R6/1 and WT mice. To this end, calcineurin activity was analyzed at different time points (from 1 to 4 h) after QUIN injection. In WT mice, intrastriatal QUIN injection induced an increase in calcineurin activity with maximal levels at 1 and 2 h after injection (a two-fold increase compared with vehicle-injected side; Fig. 5A). In the R6/1 striatum the increase of calcineurin activity after QUIN injection was lower (a 1.5-fold increase) than in WT mice and sustained between 1 and 4 h

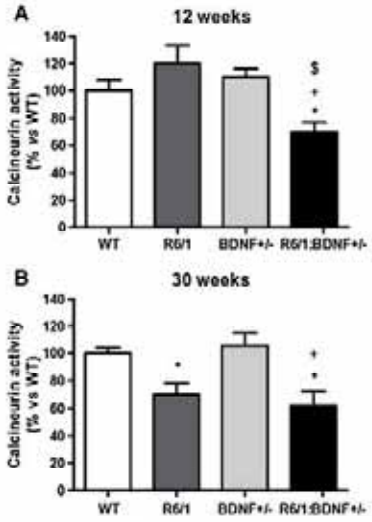


Fig. 4. Calcineurin activity is differentially regulated at different stages of disease progression in R6/1 and R6/1:BDNF+/− striatum. Calcineurin activity was measured in the striatum of WT, R6/1, BDNF+/− and R6/1:BDNF+/− mice at 12 (A) and 30 (B) weeks of age by a colorimetric assay. Values were obtained as arbitrary units and expressed as a percentage of calcineurin activity in WT in mice. Data are the mean ± SEM ($n = 4$). Data were analyzed by one-way ANOVA followed by Bonferroni's post-hoc test. *$p<0.05$ compared to WT mice, $^{+}p<0.05$ compared to BDNF+/− mice and $^{\$}p<0.05$ compared to R6/1 mice.

(Fig. 5A). Changes in calcineurin activity after intrastriatal QUIN injection were not accompanied by changes in calcineurin A or B protein levels either in WT or R6/1 mice (Fig. 5B).

Calcineurin protein levels are reduced in the putamen of HD patients

We finally analyzed the protein levels of both calcineurin A and B in the putamen of HD patients. As observed in the striatum of R6/1, R6/1:BDNF+/− and Tet/HD94 (gene-on) mice, calcineurin A and B protein levels were also decreased in human HD samples compared to control cases (Fig. 6). Interestingly, we detected a statistically significant down-regulation of calcineurin A (by 60%; Fig. 6A) but not of calcineurin B (by 35%; Fig. 6B). Note that cleaved products of calcineurin A, attributed to post-mortem intervals (Sorimachi et al., 1996), were observed in some samples (Figs. 6C (lane 4) and D).

Discussion

Transcriptional deregulation has been proposed as an important early pathogenic mechanism in HD (Sugars and Rubinsztein, 2003; Cha, 2007). Here, we show that expression of exon-1 mhtt specifically controls calcineurin A protein levels. Reduced calcineurin A protein levels were detected earlier in R6/1:BDNF+/− (12 weeks) than in R6/1 (16 weeks) mice, in good agreement with the development of motor symptoms (Mangiarini et al., 1996; Canals et al., 2004). Calcineurin modulates many important functions not only in striatal neurons but also in other neuronal types (Halpain et al., 1990; Klee et al., 1998). Present results, together with the finding that treatment with calcineurin inhibitors accelerates the progression of the deleterious phenotype in R6/2 mice (Hernandez-Espinosa and Morton, 2006), suggest that decreased levels of calcineurin could result in striatal neuronal dysfunction affecting the onset of motor alterations. Furthermore, our data from Tet/HD94 mice showed that striatal calcineurin A protein levels return to control levels when these animals fully recover from their motor deficit (gene-off group; Diaz-Hernandez et al., 2005).

Our data indicate that mhtt expression controls calcineurin A protein levels with consequent changes of basal calcineurin activity. (i) Along with the reduction of basal calcineurin activity in 12-week-old R6/1:BDNF+/− mice we show a decrease of calcineurin A protein levels, without changes in calcineurin B. (ii) In the cortex, which also degenerates in HD (Vonsattel and DiFiglia, 1998), we detected a specific decrease of calcineurin A protein levels both in R6/1 and R6/1:BDNF+/− mice at 30 weeks of age. The reduction of calcineurin A protein levels may be due to increased protein degradation or decreased transcription. Calcineurin A is degraded by calpain (Tallant et al., 1988), which has been shown to be activated in HD (Gafni and Ellerby, 2002), and after excitotoxicity (Wu et al., 2004; Xifro et al., 2008). However, we observed a similar amount of cleaved calcineurin A products in the striatum of wild-type and N-terminal exon-1 mhtt mice in basal conditions and after QUIN injection (data not shown). Furthermore, we did not observe increased levels of calcineurin A fragments in the putamen of HD patients thus suggesting that mhtt induces a deregulation of calcineurin A expression. In agreement, calcineurin A mRNA levels are decreased in human HD samples (Hodges et al., 2006). In addition, we show that endogenous levels of

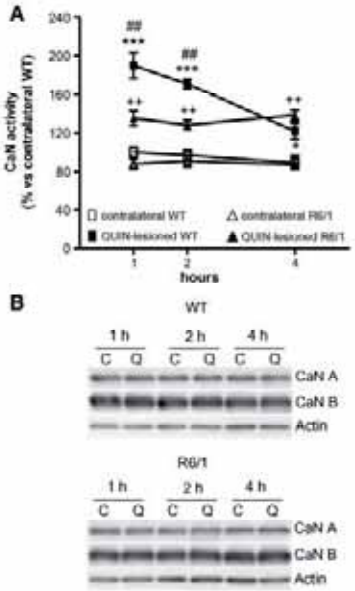
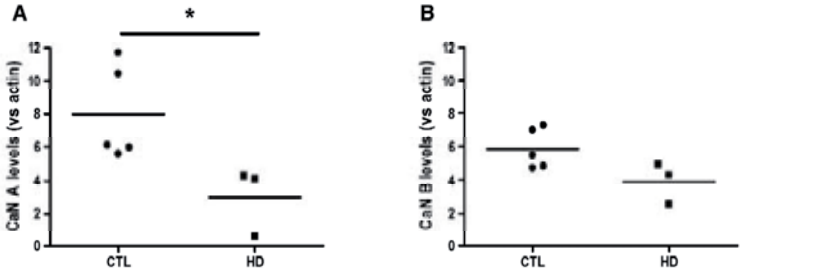


Fig. 5. Intrastriatal injection of QUIN differentially regulates calcineurin activity in WT and R6/1 mice. Calcineurin activity, and calcineurin A (CaN A) and B (Can B) protein levels were analyzed at different time points (1, 2 and 4 h) after QUIN (10 nmol; QUIN-lesioned) or vehicle (contralateral) injection in the striatum of 12-week-old WT and R6/1 animals. (A) Calcineurin activity results were expressed as a percentage of contralateral WT striatum, and represent the mean ± SEM ($n = 5$ for each condition). Data were analyzed by two-way ANOVA followed by Bonferroni's post-hoc test. *$p<0.05$ and ***$p<0.001$ with respect to WT striatum contralateral to the lesion; $^{++}p<0.01$ with respect to R6/1 striatum contralateral to the lesion; $^{\#\#}p<0.01$ with respect to QUIN-injected R6/1 striatum. (B) Representative immunoblots showing protein levels of CaN A, CaN B and actin in the striatum of WT and R6/1 mice at 1, 2 and 4 h after QUIN (Q) or vehicle (C) injection.



D

Case	Age (years)	Sex	PMI (hrs)
HD1	28	F	4,25
CTL1	64	M	3,5
HD2	59	M	5,5
CTL2	71	F	8,5

Fig. 6. The putamen of HD patients shows decreased calcineurin A and B protein levels. Calcineurin A (CaN A) and B (CaN B) protein levels were analyzed by Western blot in samples from the putamen of control (CTL) and HD post-mortem brains. Quantification of Western blot was performed by densitometric analysis. The results are expressed as the ratio of CaN A vs. tubulin levels (A) and CaN B levels vs. tubulin levels (B) in arbitrary units. Data were analyzed by unpaired Student's t test. *$p<0.05$ compared with CTL. (C) Representative immunoblots of CaN A, CaN B and tubulin in CTL and HD samples are shown. (D) Table showing age, sex and post-mortem intervals (PMI) of the CTL subjects and HD patients represented in (C).

BDNF do not directly regulate calcineurin A expression since wild-type and BDNF+/− mice displayed similar protein levels at all ages analyzed. However, in the presence of mhtt decreased calcineurin A levels were detected earlier in animals expressing lower levels of BDNF (R6/1:BDNF+/− mice) suggesting that BDNF deficit could exacerbate the effects of mhtt on calcineurin A expression. We have previously observed that, in contrast to N-terminal exon-1 mhtt, full-length mhtt up-regulates calcineurin A mRNA levels (Xifro et al., unpublished results). The opposite results on calcineurin A protein regulation observed in full-length (Xifro et al., 2008) and N-terminal exon-1 mhtt models (present results; Lievens et al., 2002; Hernandez-Espinosa and Morton, 2006) could be related to the presence of aggregates. It has been proposed that proteins interacting with exon-1 mhtt, such as transcription factors, can be sequestered in the aggregates, which impede their function (Li and Li, 2004). Supporting this hypothesis, the total number of mhtt-containing inclusions is reverted in gene-off Tet/HD94 mice striatum (Diaz-Hernandez et al., 2005), and these animals recover normal striatal calcineurin A protein levels (present results).

In addition to decreased calcineurin A protein levels, we also observed a reduction in calcineurin B protein levels in the striatum of R6/1, R6/1:BDNF+/− and Tet/HD94 mice at symptomatic stages. However, in contrast to calcineurin A, calcineurin B protein levels were not modified in the cortex of R6/1 and R6/1:BDNF+/− mice nor in the putamen of HD patients. Furthermore, calcineurin B protein levels did not return to wild-type values after mhtt shut-down in Tet/HD94 animals suggesting that different mechanisms regulate calcineurin A and B protein levels in the presence of mhtt. In agreement, we have previously shown that calcineurin A, but not calcineurin B, protein levels are modified in striatal cells expressing full-length mhtt (Xifro et al., 2008). There are no data on the mechanisms that regulate calcineurin A and B expression making it difficult to explain these differences. One explanation could be that mhtt alters the function of transcription factors controlling calcineurin A, but not B, expression.

Previous studies show that inhibition of calcineurin protects several types of neurons from glutamate-induced cell death *in vitro* (Dawson et al., 1993; Ankarcrona et al., 1996; Ruiz et al., 2000; Terada et al., 2003), and that calcineurin is involved in cell death induced by ischemic injury *in vivo* (Bochelen et al., 1999; Uchino et al., 2002). Similarly, we have previously shown that activation of calcineurin by NMDAR stimulation is involved in both caspase- and calpain-mediated striatal cell death *in vitro* (Xifro et al., 2008). Here, we show that calcineurin is quickly activated in an *in vivo* model of excitotoxicity, and that this activation is important to QUIN-induced lesion since treatment with the calcineurin inhibitor FK-506 decreased cell death. Thus, the reduced calcineurin levels detected in the striatum of R6/1 and R6/1:BDNF+/− mice could participate in the excitotoxic resistance that these animals develop over time (Hansson et al., 2001; Torres-Peraza et al., 2008). It is interesting to notice that calcineurin A protein levels were reduced in the striatum of R6/1:BDNF+/−, but not R6/1, mice at 12 weeks of age, when these animals are more resistant to excitotoxicity than R6/1 (Torres-Peraza et al., 2008). Present results, together with our previous observations showing increased calcineurin A protein levels in striatal cells expressing full-length mhtt, suggest a dual regulation of calcineurin A expression during the progression of the disease, with high levels at early stages that could result in high susceptibility to excitotoxicity (Xifro et al., 2008), and low levels at end stages that could participate in the resistance to excitotoxic-induced cell death (present results). However, in the R6/1 and R6/1:BDNF+/− striatum we did not detect up-regulated calcineurin A protein levels prior to the onset of HD-related symptoms. Therefore, as previously suggested for NMDAR-mediated cell death in HD mouse models (Zeron et al., 2002), it is possible that the full-length context of mhtt is required to increase calcineurin activity and calcineurin A protein levels, and that these initial steps of the pathogenesis are skipped in R6/1 mice. It would be very interesting to study whether this dual calcineurin regulation

occurs in YAC128 mice since they are more sensitive to excitotoxicity than controls at presymptomatic stages but resistant to intrastriatal QUIN injection when signs of HD are obvious (Graham et al., 2009).

Although calcineurin A protein levels in R6/1 mouse striatum at excitotoxic resistant age (12 weeks) were similar to those in wild-type mice, after intrastriatal QUIN injection we observed lower calcineurin activation than in wild-type mice. These results indicate that reduced levels of calcineurin are not the unique mechanism leading to the development of resistance to excitotoxicity in the presence of mhtt. Excitotoxicity can be modulated by changes in the responsiveness of glutamate receptors either due to alterations in receptor density, number, subunit composition or signaling properties. Striatal levels of NMDAR are not modified in R6/1 mice (Cha et al., 1998; Hansson et al., 1999) but the expression of MAGUKs, NMDAR scaffolding proteins, is reduced in the striatum of R6/1, R6/1:BDNF+/− and N171-82Q mice leading to alterations in NMDAR-dependent signaling pathways (Jarabek et al., 2004; Torres-Peraza et al., 2008). In addition, calmodulin, which together with Ca^{2+} activates calcineurin (Stewart et al., 1982; Klee et al., 1998), has been shown to interact with mhtt (Bao et al., 1996), and to be sequestered in aggregates, which can hamper its normal activity (Zainelli et al., 2004). Collectively, these observations suggest that mhtt expression in striatal cells regulates basal calcineurin activity through a reduction of calcineurin A protein levels that together with altered calcineurin activation in response to excitotoxicity by changes in NMDAR and calmodulin function leads to the development of resistance to excitotoxicity.

In conclusion, our data show that expression of exon-1 mhtt alters basal calcineurin activity by down-regulation of calcineurin A protein levels that could participate in striatal neuronal dysfunction. Furthermore, the resistance to excitotoxicity in the R6/1 and R6/1:BDNF+/− mouse striatum is mediated, at least in part, by a decrease of calcineurin activity in response to NMDAR stimulation. We suggest that a therapy targeted to maintain calcineurin A protein levels in a normal range will be a good approach to delay neuronal dysfunction in HD.

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Tercer trabajo: ***"Brain-derived neurotrophic factor modulates the severity of cognitive alterations induced by mutant huntingtin: involvement of phospholipaseCgamma activity and glutamate receptor expression."***

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Objetivo 3: Estudio y caracterización de las vías moleculares implicadas en el inicio de las alteraciones cognitivas superiores afectadas en modelos animales de la enfermedad de Huntington.

Objetivo 4: Estudio de la implicación del BDNF en las disfunciones cognitivas de la enfermedad de Huntington.

Según algunos trabajos previos en humanos y modelos animales, los déficits cognitivos en la enfermedad de Huntington son de los primeros en aparecer. BDNF es una de las proteínas que más se ha estudiado en los últimos años como potente mediador de la plasticidad neuronal, de las sinapsis excitatorias y de los fenómenos de aprendizaje y memoria. Sabiendo, por los trabajos expuestos en esta tesis y de otros publicados anteriormente, que BDNF está gravemente alterado en la enfermedad de Huntington, el siguiente objetivo fue caracterizar cómo la neurotrofina regula las alteraciones cognitivas de la enfermedad de Huntington. Para ello utilizamos el modelo murino de la enfermedad de Huntington llamado R6/1:BDNF+/-. Realizamos una batería de pruebas de aprendizaje y memoria dependientes de distintas regiones cerebrales, además de registros electrofisiológicos hipocampales. Los resultados obtenidos nos llevan a proponer que BDNF (y una de las vías moleculares que activa directamente) también juega un papel muy importante en el desarrollo de los déficits de aprendizaje y memoria en la enfermedad de Huntington.

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BRAIN-DERIVED NEUROTROPHIC FACTOR MODULATES THE SEVERITY OF COGNITIVE ALTERATIONS INDUCED BY MUTANT huntingtin: INVOLVEMENT OF PHOSPHOLIPASECγ ACTIVITY AND GLUTAMATE RECEPTOR EXPRESSION

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Abstract—The involvement of brain-derived neurotrophic factor (BDNF) in cognitive processes and the decrease in its expression in Huntington's disease suggest that this neurotrophin may play a role in learning impairment during the disease progression. We therefore analyzed the onset and severity of cognitive deficits in two different mouse models with the same mutant huntingtin but with different levels of BDNF (R6/1 and R6/1:BDNF+/− mice). We observed that BDNF modulates cognitive function in different learning tasks, even before the onset of motor symptoms. R6/1:BDNF+/− mice showed earlier and more accentuated cognitive impairment than R6/1 mice at 5 weeks of age in discrimination learning; at 5 weeks of age in procedural learning; and at 9 weeks of age in alternation learning. At the earliest age at which cognitive impairment was detected, electrophysiological analysis was performed in the hippocampus. All mutant genotypes showed reduced hippocampal long term potentiation (LTP) with respect to wild type but did not show differences between them. Thus, we evaluated the involvement of BDNF-trkB signaling and glutamate receptor expression in the hippocampus of these mice. We observed a decrease in phospholipaseCγ activity, but not ERK, in R61, BDNF+/− and R6/1:BDNF+/− hippocampus at the age when LTP was altered. However, a specific decrease in the expression of glutamate receptors NR1, NR2A and GluR1 was detected only in R6/1:BDNF+/− hippocampus. Therefore, these results show that BDNF modulates the learning and memory alterations induced by mutant huntingtin. This interaction leads to intracellular changes, such as specific changes in glutamate receptors and in BDNF-trkB signaling through phospholipaseCγ.

Key words: NMDA receptor, hippocampus, learning, neurotrophin, plasticity, R6/1 mice.

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Abbreviations: aCSF, artificial cerebrospinal fluid; BDNF, brain-derived neurotrophic factor; fEPSP, field excitatory postsynaptic potential; HD, Huntington's disease; HFS, high-frequency conditioning stimulus; LTP, long term potentiation; PLCγ, phospholipaseCγ; Q-PCR, quantitative PCR; RT, reverse-transcriptase; TBS-T, 150 mM NaCl, 20 mM Tris–HCl, pH 7.5, 0.05% Tween 20.

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Huntington's disease (HD) is due to an abnormal expansion of a CAG codon in exon 1 of the huntingtin gene (Huntington Disease Collaborative Research Group, 1993), resulting in devastating cognitive, psychological and motor disturbances (Vonsattel and DiFiglia, 1998). The primary sites of neurodegeneration are the striatum and cerebral cortex (Vonsattel et al., 1985), but other structures involved in cognition, including the hippocampus, are also affected in early stages of the disease (Rosas et al., 2003). Cognitive impairment can appear in patients before the onset of the motor symptoms (Foroud et al., 1995; Lawrence et al., 1998). Furthermore, initial symptoms of the disease precede neuronal loss (Vonsattel et al., 1985), suggesting that neurological symptoms may reflect an underlying neuronal dysfunction rather than being a consequence of neuronal death.

Impaired learning has also been described in different mouse models of HD (Lione et al., 1999; Mazarakis et al., 2005; Van Raamsdonk et al., 2005). The R6 mouse lines (R6/1 and R6/2) express exon 1 of the mutant huntingtin gene with different polyQ lengths (Mangiarini et al., 1996). R6/2 mice display progressive deterioration in specific aspects of learning in the Morris water maze, two-choice swim tank and T-maze tasks before they show an overt motor phenotype (Lione et al., 1999). These cognitive deficits are related to abnormal synaptic plasticity including a selective impairment of long term potentiation (LTP) (Murphy et al., 2000; Milnerwood et al., 2006).

Loss of neuronal plasticity can be due to deficient trophic support. brain-derived neurotrophic factor (BDNF) is a potent, positive modulator of LTP (Bramham and Messaoudi, 2005); activity-induced BDNF expression gen-

erates sustained structural and functional changes at hippocampal synapses that underlie some forms of long-term memory (Lu et al., 2007). Therefore, changes in BDNF levels may be related to cognitive deficits.

There are strong evidences that link BDNF with the pathophysiology of motor dysfunctions in HD. It has been reported that BDNF expression is decreased in humans (Ferrer et al., 2000; Zuccato et al., 2001, 2008) and mice (Lynch et al., 2007; Zuccato et al., 2001) with HD, and that changes in endogenous BDNF levels modulate the onset and severity of motor symptoms in R6/1 mice (Canals et al., 2004). A recent study, analyzing the differential gene expression between several models of HD, shows that the BDNF knock-out model (Baquet et al., 2004) profiles are very similar to human HD (Strand et al., 2007). However, the specific mechanisms that modulate cognitive alterations in HD remain to be elucidated, although the involvement of BDNF has already been suggested, since a reduction in BDNF levels has been described in the hippocampus of R6/1 mice (Spires et al., 2004) and knock-in mice (Lynch et al., 2007). Furthermore, this neurotrophin rescues LTP in slices from HD knock-in mice (Lynch et al., 2007).

We therefore studied the putative role of BDNF in the regulation of learning and memory impairment seen in HD. To examine the effect of BDNF on severity and onset in different memory tasks, we compared two different mouse models with the same mutant huntingtin but with different levels of BDNF (R6/1 vs. R6/1:BDNF+/−; Canals et al., 2004). We performed a variety of learning paradigms before motor symptoms appeared: a simple swimming test, a T-maze and a discrimination swimming test to examine procedural, alternation and discrimination learning respectively. Besides the results of this behavioral study, we also provide electrophysiological and biochemical data related to hippocampal synaptic plasticity.

EXPERIMENTAL PROCEDURES

Animals

Male R6/1 mice, expressing transgenic exon-1 of mutant huntingtin (Mangiarini et al., 1996) were compared with R6/1 mice with lower BDNF levels (R6/1:BDNF+/−; Canals et al., 2004). To obtain these mice we cross-mated R6/1 (B6CBA) mice with BDNF heterozygous (BDNF+/−; BALB/c) mice (Ernfors et al., 1994). To reduce strain background effects, we maintained the BDNF+/− colony with a B6CBA host inbred strain for at least six generations. It is considered that after five generations of backcrossing (F5), a congenic strain is more than a 95% identical to the host inbred strain at all loci except those linked to the transferred gene of interest. Thus, we only used males from littermates for all genotypes with a B6CBAxBALB/c hybrid background for wild type, BDNF+/−, R6/1 and R6/1:BDNF+/− genotypes for the experimental groups.

All experiments were conducted in a blind-coded manner with respect to genotype, and data were recorded for analysis by microchip mouse number. Animals were maintained on *ad libitum* food and water, except for mice used in the alternation task (see below), and were kept in a colony room at constant temperature (20–22 °C) and relative humidity (40–60%) on a 12-h light/dark cycle. To discern the possible effects caused by the different genetic background of the BDNF+/− (BALB/c) and the R6/1 (B6CBA) strains on learning tasks, preliminary behavioral experiments were performed with animals from the two strains.

We used a total number of 20 BALB/c mice and 19 B6CBA mice for the preliminary experiments, and 26 wild type, 35 BDNF+/−, 39 R6/1 and 48 R6/1:BDNF+/− mice with B6CBAxBALB/c genetic background for the experimental groups. All procedures met the European Community guidelines for the care and use of laboratory animals (86/609//EEC), and were approved by the animal care committee of the University of Barcelona and by the regional autonomous government (Generalitat de Catalunya), to minimize the number of animals used and suffering.

For genotyping, DNA was obtained from tail biopsy and processed for PCR. The primers used for DNA amplifications have been described previously (Agerman et al., 2003; Ernfors et al., 1994; Mangiarini et al., 1996). PCR fragments were resolved in agarose gels: 2% for BDNF amplification and 1.5% for huntingtin analysis.

CAG repeat length was measured in R6/1 and R6/1:BDNF+/− mice. The size of the expansion was determined by PCR amplification of the repeat using HD1 and HD2 fluorescently labeled primers previously described in Huntington Disease Collaborative Research Group (1993), and subsequent size determination in an ABI 3100 analyzer. The peak sizes for R6/1 and R6/1:BDNF+/− mice were the same, 142–144 repeat units. These results were double checked by analyzing other samples by the company Laragen, Inc. (Los Angeles, CA, USA).

Simple swimming test

We used the learning paradigm described previously (Van Raamsdonk et al., 2005) to test procedural learning. The apparatus consisted of an extended swimming tank (100 cm long, 15 cm wide with walls measuring 30 cm high). Water level was 15 cm from floor to surface, and it was maintained at 26 °C (±1) in order to avoid hypothermia (Iivonen et al., 2003). A blue screen surrounded the tank to avoid the vision of distal cues. A watt lamp was situated 60 cm above the water surface and positioned over the middle of the apparatus providing a light intensity of 15 lux throughout the water surface.

The mice were placed in the middle of the swimming tank facing away from a visible escape platform (11 cm wide, 6 cm long and 10 cm high with the top surface 0.5 cm above the water level) at one end of the tank. The first direction taken and the time required to reach the platform (escape latencies) in these trials were recorded by a researcher blinded to genotype. The animals had to find the visible platform by turning around and swimming directly to it, while any other action was considered an incorrect performance. A correct choice was given a score of 0 and an incorrect choice a score of 1. For the analysis of correct choices, we assessed the probability of error for each animal. Three pairs of two trials with an inter-trial interval of 2 h were performed. The experiment was run over 2 days, obtaining a total of 12 trials. The different age intervals for background strains BALB/c and B6CBA on one hand (21 weeks) and for wild type, BDNF+/−, R6/1 and R6/1:BDNF+/− mice on the other hand (5, 10, 15 and 20 weeks) were examined in separate groups of different animals. As described previously (Van Raamsdonk et al., 2005), this task consisted of only one acquisition session of procedural learning with no habituation or pre-training sessions.

Alternation in the T-maze

Alternation learning was assessed in a T-maze task similar to that used by Lione et al. (1999) with slight modifications. The apparatus was a wooden maze consisting of three arms, two of them situated at 180° from each other and the third situated at 90° with respect to the other ones representing the stem arm of the T. All three arms were 45 cm long, 8 cm wide and enclosed by a 20 cm wall. The maze was thoroughly painted with waterproof gray paint. Light intensity was five lux throughout the maze. A 10 cm start

area was situated at the end of the stem arm and closed by a wooden guillotine door. Another two identical guillotine doors provided entry in the arms situated at 180°. The maze was elevated 60 cm above the floor and a blue screen was placed around the maze in order to prevent the use of distal cues. The reinforcement used was a 20 mg sweet pellet (Bio-serve, Frenchtown, NJ, USA) placed in a silvered container at the end of the two opposite arms. All the mice had been habituated to sweet pellets three consecutive days before the beginning of the experiment. Prior to the experiment, all mice were submitted to 7 days of food restriction and received a 3.5 g±0.5 fixed quantity of food 1 h after each experimental session. Water was available *ad libitum* and mice were weighed daily, maintaining body weights between 80 and 85% of free-feeding weight throughout the experimental procedure.

As described previously (Lione et al., 1999), the experiment was divided into three phases. The first one was a habituation phase consisting of three consecutive daily sessions in which animals were placed individually in the maze for 6 min in order to accustom them to the new environment. The guillotine doors of the arms were opened and baited with five free access pellets each. The number of baited arms visited, pellets eaten, fecal boli and time remaining in the start area were recorded in order to evaluate anxiety, motivational or locomotor components that could influence the performance in this task. In the second phase, mice were administered 10 consecutive forced alternation trials for 3 days before training phase began. As several days were needed to obtain the appropriate weight and to habituate animals to sweet pellets, the maze and the forced alternations, the mice were older at the time of the alternation learning assessment than at the time of the simple swimming and discrimination tasks.

In the third phase, when alternation learning was measured, mice were situated in the start area for 10 s. In the first trial, both arms were baited and opened. After 10 s the animal was allowed to visit one of them. Entry was considered when all four paws were allocated inside the arm. Then, the guillotine door was closed and the mice received their food reward. The first choice was free and was not recorded. Mice remained in the arm for 20 s and were then placed again in the start area for the second trial. In this second trial, mice had to turn to the opposite arm in order to find the sweet pellet. Thus, from the second trial onwards, mice had to alternate 10 times before the end of the experimental session. A significant level of alternation was considered when animals produced 80% of alternated choices on the first 10 trials. Additional trials were given in order to ensure that all the animals received 10 food rewards. The maze was thoroughly cleaned between subjects, controlling odors. Because BALB/c mice learned slower than B6CBA in this task, for statistical analysis we divided the trials in two blocks of 3 days each, the first comprising days 1–3 and the second comprising days 4–6.

Visual discrimination swimming test

A visual discrimination learning task was assessed using a blue swimming tank 50 cm long, 30 cm wide and enclosed by a 30 cm wall filled with water at 26 °C±1 dyed with blue non-toxic paint. The tank was elevated 60 cm from the floor. A 10 cm diameter platform was placed 1 cm below the water level and situated in front of one of two circular visual stimuli of 15 cm diameter with two opposite black/white patterns. The stimuli were situated at one of the ends separated by a perpendicular plastic wall in order to ensure that they were not visible at the same time. Light intensity was 15 lux throughout the water surface and a blue screen was situated surrounding the swimming tank to avoid the use of distal visual cues.

The procedure was divided into three phases: pretraining, acquisition training, and reversal training. The pretraining phase was run over 2 days with four daily trials and without visual stimuli. Mice could freely turn left or right in order to find the submerged platform. Left or right preferences were controlled. When mice found the submerged platform, they were kept there for 10 s.

In the training acquisition phase both discrimination cues were present. The submerged platform was situated in front of one visual cue for half of the subjects of each group and in front of the other visual cue for the other half. Twelve counterbalanced trials were performed for each mouse per day. When an animal found the platform it remained there for 10 s and the trial was counted as a correct choice. The animal was then removed. If a mouse swam toward the wrong visual cue it was counted as an error trial. The time to reach the platform (escape latencies) was also recorded to evaluate motor, motivational or anxiety components. When the wild type produced 80% of correct choices, the reversal phase started. Visual cues were then inverted and mice had to swim to the previously unreinforced cue in order to find the submerged platform following the same procedure as in the acquisition phase. Moreover, statistical analysis of escape latencies of the acquisition phase and reversal learning phase was separated in two blocks in both control strains and experimental groups because statistically significant day of training/groups interactions were observed.

Western blot analysis

Animals (n=4–7 per genotype) at 5 weeks of age were deeply anesthetized in a CO_2 chamber and their brains quickly removed. The hippocampus was dissected out, frozen using CO_2 pellets and stored at −80 °C until use. Briefly, hippocampus was homogenated by sonication in 250 μl of lysis buffer (PBS, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM phenylmethylsulphonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 2 μg/ml sodium orthovanadate). After homogenization, samples were centrifuged at 12,000 rpm for 20 min. Supernatant proteins (15 μg) without head denaturation proteins from total hippocampal extracts were loaded in 7.5% SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA). Blots were blocked in TBS-T (150 mM NaCl, 20 mM Tris–HCl, pH 7.5, 0.05% Tween 20) with 5% nonfat dry milk and 5% BSA. Immunoblots were probed with the appropriate antibodies: anti-NR1 1:500, anti-NR2A 1:1000 and anti-NR2B 1:1000 (Chemicon, Temecula, USA); anti-BDNF 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-phosphoERK 1:1000, phospholipaseCγ (PLCγ) 1:1000, $pPLC\gamma^{Tyr783}$ 1:1000 (Cell Signaling Technology, Danvers, MA, USA), anti-GluR1 1:1000, anti-GluR2/3 1:2000 (Upstate Biotechnology, NY, USA), anti-panERK 1:5000, anti-TrkB (BD Transduction Laboratories, San Diego, CA, USA). All blots were incubated with the primary antibody overnight at 4 °C by shaking in PBS 0.02% sodium azide buffer. After several washes in TBS-T, blots were incubated with anti-mouse or anti-rabbit IgG HRP-conjugated (Promega, Madison, WI, USA) and developed by ECL Western blotting analysis system (Bioscience Europe, Freiburg, Germany). For loading control a monoclonal anti-β-tubulin antibody (Sigma, St. Louis, MO, USA) was used. The specificity of anti-BDNF antibody was checked in membranes from KO BDNF mice (Ernfors et al., 1994).

Quantitative (Q)-PCR assays

Total RNA from hippocampus at 5 weeks of age (n=4–6 per genotype) was extracted using the Total RNA Isolation Nucleospin® RNA II Kit (Macherey-Nagel, Düren, Germany). Total RNA (500 ng) was used to synthesize cDNA using random primers with the StrataScript® First Strand cDNA Synthesis System (Stratagene, La Jolla, CA, USA). The cDNA synthesis was performed at 42 °C for 60 min in a final volume of 20 μl according to manufacturer's instructions. The cDNA was then analyzed by Q-PCR using the following TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA, USA): 18S (Hs99999901_s1) and BDNF (Mm00432069_m1). Reverse-transcriptase (RT) polymerase

chain reaction was performed in 25-μl volumes on 96-well plates, in a reaction buffer containing 12.5 μl Brilliant® Q-PCR Master Mix (Stratagene), 1.25 μl TaqMan® Gene Expression Assays, and 10–20 ng of cDNA. Reactions were as follows: 40 cycles of a two-step PCR; 95 °C for 30 s and 60 °C for 1 min, after initial denaturation at 95 °C for 10 min. All RT-PCR assays were performed in duplicate and repeated for at least three independent experiments. To provide negative controls and exclude contamination by genomic DNA, the RT was omitted in the cDNA synthesis step, and the samples were subjected to the PCR reaction in the same manner with each TaqMan® Gene Expression Assay.

The RT-PCR data were analyzed using the MxPro™ Q-PCR analysis software version 3.0 (Stratagene). Quantification was performed with the Comparative Quantitation Analysis program of the mentioned software and using the 18S gene expression as internal loading control.

Electrophysiology

Transverse brain slices (400 μm thickness) were prepared from mice (n=3–4 per genotype) at 5 weeks of age as described previously (Martín and Buño, 2003), and incubated for >1 h at room temperature (21–24 °C) in artificial cerebrospinal fluid (aCSF). The aCSF contained (in mM): NaCl 124, KCl 2.69, KH_2PO_4 1.25, $MgSO_4$ 2, $NaHCO_3$ 26, $CaCl_2$ 2 and glucose 10, and was gassed with 95% O_2 and 5% CO_2. Slices were transferred to an immersion recording chamber and placed in an Olympus BX50WI microscope (Olympus Optical, Tokyo, Japan) and superfused (2.5 ml/min) with gassed aCSF. Extracellular field excitatory postsynaptic potentials (fEPSPs) were recorded with a glass microelectrode (impedances: 2–3 MΩ; filled with 1 M NaCl) positioned in stratum radiatum area CA1. Evoked fEPSPs were elicited by stimulation of the Schaeffer collateral fibers with an extracellular bipolar nichrome electrode via an S-900 stimulator and S-910 isolation unit (Dagan Corporation, MN, USA). The stimulation intensity was adjusted to give fEPSP amplitude that was approximately 50% of maximal fEPSP sizes. LTP was induced by applying four trains (1 s at 100 Hz) spaced 20 s, and potentiation was measured for 1 h after LTP induction at 0.06 Hz. Data were transferred to the hard disk of a Pentium-based computer using a DigiData 1322 A interface and the pCLAMP 9.0 software (Axon Instruments). For each experiment, fEPSP slopes were expressed as percentages of average pre-tetanus baseline slope values. Student's t-test was used for all statistical comparisons of mean fEPSP slopes and all data are expressed as mean±S.E.M.

Statistical analysis

All results were expressed as the mean±S.E.M for each genotype. The behavioral analyses except for the simple swimming test were conducted using ANOVA (R package) with one between-subject factor (genotype) and with repeated measures with one within-subject factor (day of training/block of days/block of trials). The unpaired Student's t-test was used as a post hoc. In case of significant interactions the unpaired t-test was also used. Time reaching the platform in learning tasks (escape latencies) and Western blotting experiments was analyzed using one-way ANOVA and unpaired Student's t-test as a post hoc. For the simple swimming test the logistic regression models with random effects were used to model the probability of error including genotype and block as independent variables for each mouse as a random effect of model observed heterogeneity among mice. Odds ratios and their 95% confidence intervals were computed for each genotype to measure the association between genotype and the error probability.

RESULTS

Reduction of BDNF levels induces earlier cognitive deficits in R6/1 mice in a simple swimming test

Control strains. BALB/c and B6CBA mice were trained at 21 weeks of age (Fig. 1a) to ensure that the results were not due to an age effect. Both strains quickly learned the task, showing a progressive decrease in error probability without significant differences in the performance of the two groups (Fig. 1a). However, time to reach the platform differed between control strains (P<0.01) (Fig. 1b), suggesting a possible effect of adaptation due to anxiety which has been widely described in BALB/c mice (Belzung and Berton, 1997; Tang et al., 2005), or motor impairments. However, the error probability showed that both strains were able to learn the task correctly and that there were no significant differences between their performances.

Experimental groups. Simple swimming test was performed in wild type, R6/1, BDNF+/− and R6/1: BDNF+/− mice at different ages, using separate groups of animals for each time point to examine acquisition.

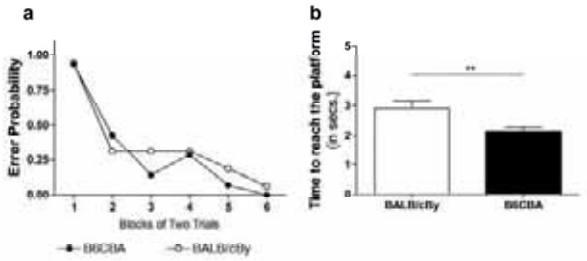

Fig. 1. BALB/c and B6CBA mice learn the simple swimming test equally well. (a) Probability of error shown by BALB/c (n=8) and B6CBA (n=7) mice. The two groups present similar degrees of improvement on this task. (b) The time to reach the platform was measured in order to evaluate likely influences in escape latencies due to motor and/or anxiety effects. The task was performed at 21 weeks of age. Bars represent means±S.E.M. ** P<0.01 with respect to B6CBA. For probability of error, logistic regression with random effects was used. For time to reach the platform analysis the one-way ANOVA with Student t-test as a post hoc was used.

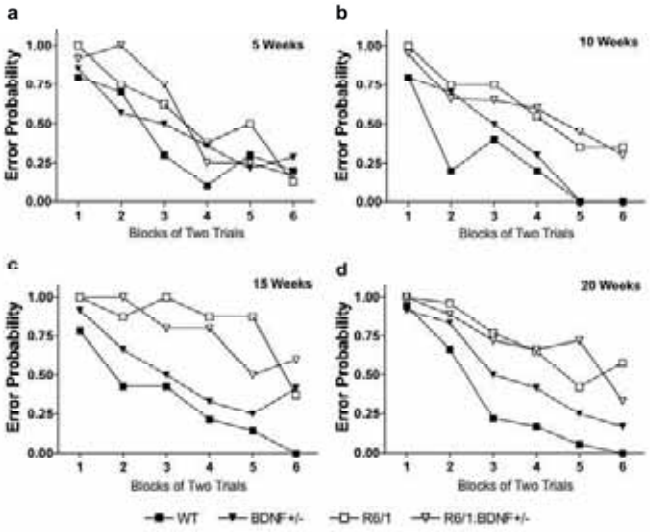

Fig. 2. R6/1:BDNF+/− mice show earlier cognitive deficits in a simple swimming test. Note the progressive age- and genotype-dependent learning alteration. The first to show increased error probability were R6/1:BDNF+/− mice (a–d) at 5 weeks of age, the second R6/1 mice (b–d) at 10 weeks of age and finally, BDNF+/− mice (c, d) at 15 weeks of age, which showed a later and smaller increase in error probability. Separate groups of wild type, BDNF+/−, R6/1 and R6/1:BDNF+/− were tested at 5, 10, 15 and 20 weeks of age (n=5–7 per genotype in each condition). For probability of error, logistic regression with random effects was used.

At 5 weeks of age (Fig. 2a), all groups showed a progressive decrease of probability of errors during the training sessions. But only R6/1:BDNF+/− mice presented a higher probability of error than wild type mice at this age (P<0.05); while R6/1 and BDNF+/− mice performed the task correctly without any differences with respect to wild type mice. At 10 weeks of age, R6/1 mice started to present a higher probability of error than wild type mice (Fig. 2b, P<0.001), approaching the rate found in R6/1:BDNF+/− mice (P<0.001). BDNF+/− mice did not show any differences with respect to wild type, but they started to make more errors from the age of 15 weeks onwards (Fig. 2c, P<0.01). Therefore, R6/1:BDNF+/−, R6/1 and BDNF+/− mice performed the task with a higher error probability than wild type mice at 15 (Fig. 2c) and 20 weeks of age, although the animals with mutant huntingtin showed the highest error probability (Fig. 2d). These results suggest severe learning impairments in R6/1:BDNF+/− and R6/1 mice and moderate impairments in BDNF+/− mice.

In order to analyze possible motor deficits or anxiogenic effects, we also measured the time to reach the platform. All animals reached the platform within a similar time at 5 and 10 weeks (Fig. 3a, b). After 15 weeks of age, R6/1 and R6/1:BDNF+/− mice took longer (15 weeks: $F_{(3,18)}$=11.74 P<0.001; 20 weeks: $F_{(3,34)}$=7.929 P<0.001) (Fig. 3c, d). Thus, R6/1:BDNF+/− and R6/1 mice showed significantly increased escape latencies with respect to wild type mice (P<0.01; P<0.05 respectively). This sustained motor impairment would be due to mutant huntingtin since a decline in cognitive ability was already different in the early ages as presented above. This is in accordance with previous data from our laboratory where we showed that motor impairment is not observed at 5 and 10 weeks of age in R6/1 and R6/1:BDNF+/− mice (Canals et al., 2004). Interestingly, the performance of wild type mice at this age is very similar to that of control strains at the same age.

Mutant huntingtin expression and low levels of BDNF modulate alternation learning impairment shown by R6/1 mice in a T-maze

Control strains. We used BALB/c and B6CBA mice at 14 weeks of age (see Experimental Procedures). We found that initial and final levels of performance in the habituation phase (Fig. 4a–c) did not differ between groups in any variable measured (baited arms visited, time remaining in the start box, fecal boli and pellets eaten). In addition, from day 1 to day 3 both strains showed a significant reduction in the time spent inside the start box from day 1 to day 3 ($F_{(2,20)}$=6.49 P<0.01), suggesting a decrease in the novelty-related anxiety-like behavior.

The statistical analysis of the training phase data was performed after dividing the experiment into two blocks (as described in Experimental Procedures), the first between

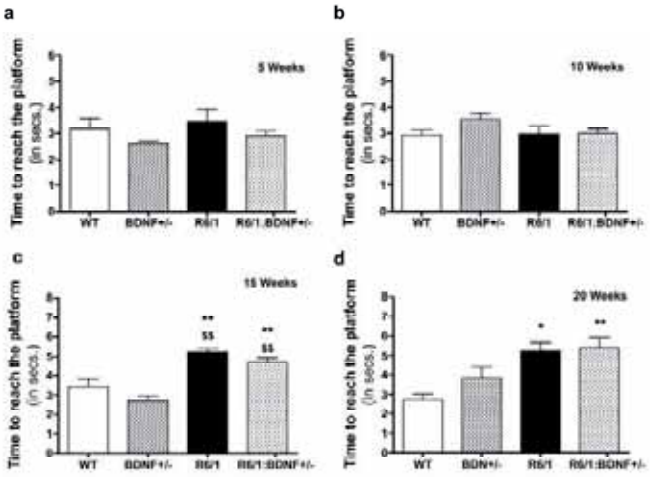

Fig. 3. Progressive age-dependent escape latency impairments showed by R6/1 and R6/1:BDNF+/− mice in a simple swimming test. (a, b) At 5 and 10 weeks of age there were no significant differences between groups in time to reach the platform. (c, d) On the other hand, impairments in time to reach the platform in R6/1 and R6/1:BDNF+/− mice were found at 15 and 20 weeks of age with the same time of onset. Bars represent means±S.E.M. (n=5–7 per genotype in each condition). * P<0.05 with respect to wild type; ** P<0.01 with respect to wild type; ^{ss} P<0.01 with respect to BDNF+/−. For time to reach the platform analysis the one-way ANOVA with Student t-test as a post hoc was used.

days 1–3 and the second between days 4–6. In the first block BALB/c showed fewer alternations than B6CBA mice ($F_{(1,20)}$=6.10 P<0.05), an effect that was lost in the second block. This result suggests that the higher anxiety levels reported for BALB/c mice (Belzung and Berton, 1997; Tang et al., 2005) or strain-specific motor differences may initially affect the performance. However, at the end of the experiment this strain reached a similar level of alternation learning to B6CBA mice (a rate of alternations of 80%). In addition, both strains showed a significant improvement in alternation learning from day 1 to day 6 ($F_{(5,50)}$=7.48 P<0.001). In order to check for differences between strains in the second block, we performed an average of the total alternation from each animal and per group (Fig. 4e). This analysis showed no differences between strains in the second block of alternation (Fig. 4e). These results indicate that control strains can learn to alternate significantly and reach a similar average of correct choices.

Experimental groups. Regarding the experimental groups, at 9 weeks of age (see Experimental Procedures) there were no significant differences between groups in any variable measured in the habituation phase: baited arms visited, time remaining in the start box, fecal boli (data not shown) or pellets eaten (Fig. 5a–c). All groups showed a significant decrease in visits to baited arms from day 1 to day 3 ($F_{(2,40)}$=14.6 P<0.001). Further, all groups showed a significant increase in pellets eaten from day 1 to day 3 ($F_{(2,40)}$=4.89 P<0.05) suggesting a decrease of anxiety levels and a higher motivation to eat the sweet pellets (reinforcement). Moreover, from day 1 to day 3, all groups also spent substantially less time spent in the start box ($F_{(2,40)}$=15,84 P<0.001), which can be interpreted as a progressive reduction in anxiety, as observed for control strains.

In the training phase (Fig. 5d), BDNF+/− and wild type mice showed a significant increase in alternations (80%) ($F_{(5,50)}$=2.80 P<0.05). However, R6/1 and R6/1:BDNF+/− mice both did not show significant improvement in this learning task and the average of alternation was lower. In accordance with the results obtained from control strains (see above and Experimental Procedures), we divided the experiment into two blocks of days, but we only evaluated the second block (from day 4 to day 6) since the first might have been affected by a strain-dependent factor (see above). There was a significant effect of genotype in this learning task ($F_{(4,38)}$=5.92 P<0.01) and a post hoc test showed that R6/1:BDNF+/− performed significantly fewer alternations than R6/1 (P<0.05), BDNF+/− (P<0.01) and wild type (P<0.01) with no differences between the other genotypes. However, although R6/1 mice did not show significant differences with respect to wild type mice, they never reached a significant level of alternations (80% of correct choices) suggesting slight cognitive impairments in these mice at 9 weeks of age. To further check this possibility, we performed an average of total alternations in each mouse per genotype from the second block of trials (Fig. 5e). One-way ANOVA ($F_{(4,59)}$=13,59 P<0.001) showed that R6/1:BDNF+/− mice differed with respect to wild type and BDNF+/− mice

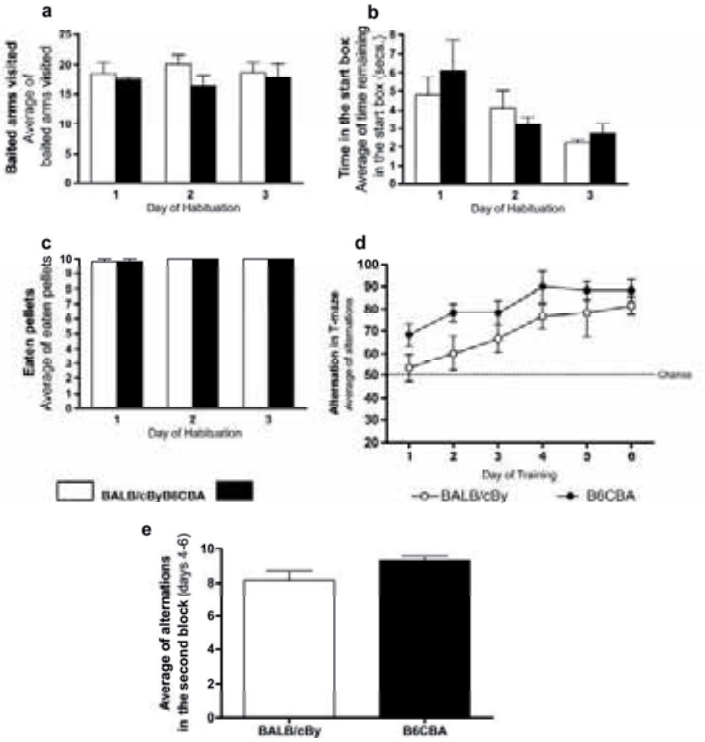

Fig. 4. BALB/c and B6CBA mice perform equally in alternation learning in a T-maze. (a–c) To assess the possible influences of motivation, locomotor activity and fear states we measured the number of baited arms visited, time remaining in the start box and pellets eaten during the habituation phase. No significant differences were observed between groups in any measure. (d) In the training phase, when the number of alternations was evaluated, both groups progressively reached a significant level of alternation by the sixth day of training, showing that both strains reach similar number of alternations. (e) An average of total alternations in the second block of trials (days 4–6) of the training phase in each animal per genotype. The task was performed at 14 weeks of age. Bars represent means±S.E.M. (BALB/c n=6, B6CBA n=6). The two-way ANOVA with repeated measures analysis with Student t-test as a post hoc was used in all experiments except for the average of alternations in the second block where the one-way ANOVA with Student t-test as a post hoc was used.

($P<0.001$) and compared with R6/1 ($P<0.05$). Moreover, R6/1 mice were different with respect to wild type and BDNF+/− mice ($P<0.05$) showing slight cognitive impairments in R6/1 mice. Additionally, it should be considered that performance of wild type mice at this age is very similar to that of control strains at the same age.

Severe learning deficits in a visual discrimination swimming task are caused by mutant huntingtin and low levels of BDNF

Control strains. BALB/c and B6CBA mice at 10 weeks of age were evaluated in a visual discrimination swimming task (Fig. 6a). In the acquisition phase, both groups started the experiment at the same level of chance of performing only ~50% of correct choices. However, they increased their correct choices equally from day 1 to day 13 ($F_{(1,156)}$=18,003 $P<0.001$) with no strain-dependent differences, suggesting adequate discrimination learning in both groups. We also evaluated the time taken to reach the platform in order to assess motor, anxiety and motivation features (Fig. 6c). For the analysis of the time taken to reach the platform we analyzed two equal blocks of the acquisition phase separately (see Experimental Procedures): days 1–6 and days 7–13. We found that BALB/c swam slower than B6CBA from day 1 to day 6 ($F_{(1,110)}$=13.17 $P<0.01$) but not from day 7 to day 13. They both attained similar escape latencies in the second half of the phase (Fig. 6c).

When both control strains reached a significant number of correct choices (80%) (Fig. 6a), the next step was to

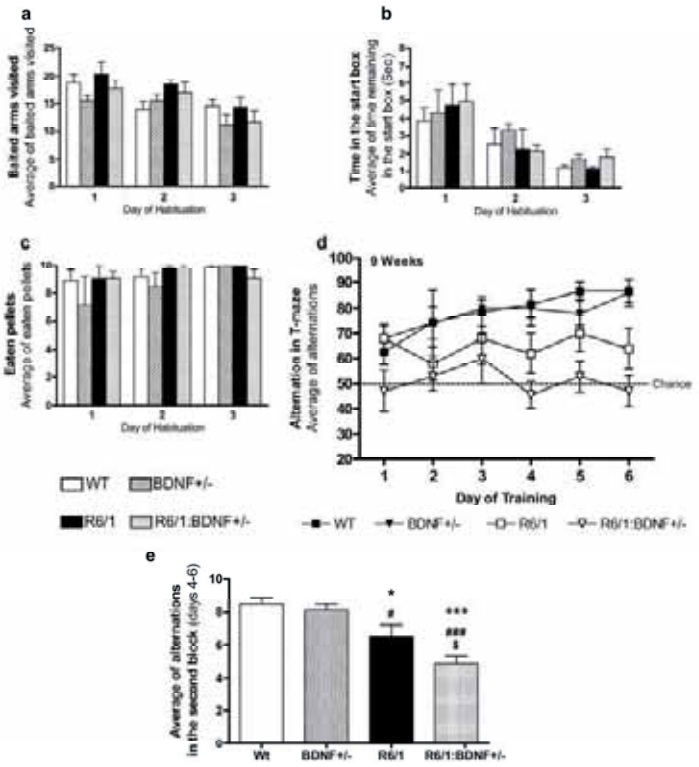

Fig. 5. Mutant huntingtin and low levels of BDNF modulate the severity of cognitive impairments shown by mice models of HD in an alternation learning task in a T-maze. (a–c) To assess the likely influences of motivation, locomotor activity and fear states we measured the number of baited arms visited, time remaining in the start box and pellets eaten during the habituation phase. No significant differences were observed between groups. (d) In the training phase the number of alternations was evaluated and was shown to be different in a genotype-dependent manner; the R6/1:BDNF+/− mice were the worst learners. (e) An average of total alternations in the second block of trials (days 4–6) of the training phase in each animal per genotype. The task was performed at 9 weeks of age. Bars represent means+S.E.M. * $P<0.05$ and *** $P<0.001$ with respect to wild type; # $P<0.05$ ### $P<0.001$ respect to BDNF+/− and $ $P<0.05$ with respect to R6/1 (wild type $n=5$, BDNF+/− $n=7$, R6/1 $n=5$ and R6/1:BDNF+/− $n=7$). The two-way ANOVA with repeated measures analysis with Student *t*-test as a post hoc was used in all experiments except for the average of alternations in the second block where the one-way ANOVA with Student *t*-test as a post hoc was used.

initiate the reversal phase (Fig. 6b). On the first day of the reversal phase, both strains showed a significant decrease in the number of correct choices (they performed only 20–30% of correct choices) when comparing the last day of the acquisition phase to the first day of the reversal phase for each strain separately, using the Student-*t* test (BALB/c $P<0.05$; B6CBA $P<0.05$). In the reversal phase, we again divided the experiment into two blocks of days for statistical analysis: the first block was the block of days 1–4 and the second the block of days 5–9 (see Experimental Procedures). There were no significant differences between strains in either block, but there were important effects of time in the first block ($F_{(4,52)}=29.38$ $P<0.001$) and in the second block ($F_{(8,104)}=71.05$ $P<0.001$), suggesting a good performance of both strains with no differences in the time taken to reach the platform in this phase of the task (Fig. 6d). Taken together, these results suggest that both strains have good discrimination learning.

Experimental groups. R6/1:BDNF+/−, R6/1, BDNF+/− and wild type mice were subjected to the visual discrimination learning task at 5 weeks of age (Fig. 7a). The results obtained showed that all four groups started the experiment at a similar chance level of number of correct choices (around 50%) but the groups' performance was markedly different. There was a significant improvement in learning

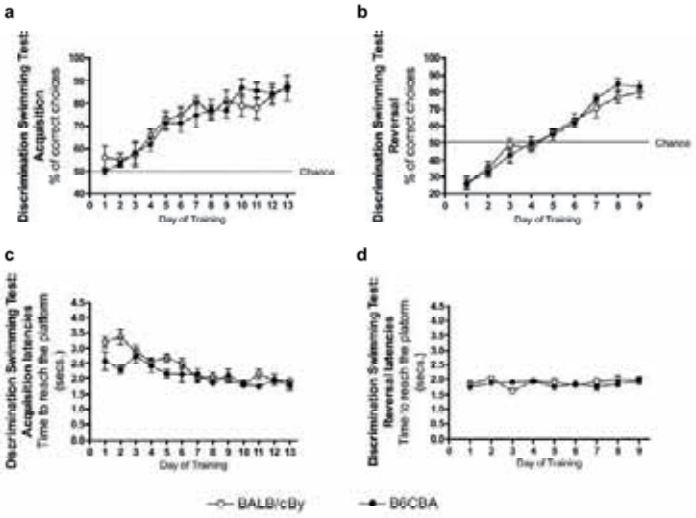

Fig. 6. BALB/c and B6CBA mice performed equally in a visual discrimination swimming task. This learning task was divided into two phases: acquisition phase (a) and reversal phase (b), with their respective escape latencies measuring the time to reach the platform (c, d). (a) In the acquisition phase both groups reached a correct choice score of 80%; no differences were found between them. In the reversal phase (b) BALB/c and B6CBA mice progressively reached a significant number of correct choices (80%); no differences were found between them. (c, d) Time to reach the platform was measured in order to evaluate whether motor or anxiety effects could modulate the task performance either in the acquisition phase (c) or in the reversal phase (d). The task was performed at 10 weeks of age. Bars represent means ± S.E.M. (BALB/c $n=8$, B6CBA $n=7$). For statistical analysis, the two-way ANOVA with repeated measures analysis with Student *t*-test as a post hoc was used.

from day 1 to day 8 ($F_{(7,133)}=30.72$ $P<0.001$) in all genotypes, but only wild type mice reached a significant level of number of correct choices (80% of correct choices or above). R6/1:BDNF+/− performed fewer correct choices than R6/1 ($P<0.001$), BDNF+/− ($P<0.001$), and wild type ($P<0.0001$); on the other hand, R6/1 performed fewer correct choices than wild type ($P<0.05$), and finally, BDNF+/− mice also made fewer correct choices than wild type mice ($P<0.05$). However, the performance of the R6/1 and BDNF+/− mice on the discrimination task was similar. Additionally, we detected a significant genotype/day interaction in training ($F_{(21,133)}=2.28$ $P<0.01$) and an additional analysis per day was needed. These results suggest that R6/1:BDNF+/− mice showed worse visual discrimination learning impairments than BDNF+/− and R6/1 mice although these last two groups also showed moderate, but significant, cognitive alterations with respect to wild type mice in this task. We also analyzed the latencies, dividing the acquisition phase in two blocks (Fig. 7c). Neither in the first (days 1–4) nor in the second block (days 5–8) were significant differences between genotypes. In addition, in both blocks there was a significant effect of the training day ($F_{(3,57)}=7.15$ $P<0.001$; $F_{(3,57)}=4.83$ $P<0.01$ respectively), suggesting a progressive improvement of escape latencies in the acquisition phase with no apparent motor and anxiety abnormalities in any genotype.

The next step was started when wild type mice reached 80% of correct choices in the acquisition phase. All groups were then subjected to the reversal phase (Fig. 7b). In this phase, the number of correct choices fell in all groups. We compared the last day of the acquisition phase and the first day of the reversal phase in each genotype, finding that all groups performed significantly worse in the reversal phase. The order of the decrease was as follows (from best to worst): wild type ($P<0.001$) >R6/1 ($P<0.001$) >BDNF+/− ($P<0.05$) >R6/1:BDNF+/− ($P<0.05$), showing that wild type mice performed best during the acquisition phase. However, in the reversal phase the number of correct choices increased in all groups from day 1 to day 6, indicating a progressive improvement on the task (Fig. 7b). For the statistical analysis we analyzed the two blocks of the reversal phase separately (see Experimental Procedures): days 1–3 and days 4–6. In the first block there was a significant improvement in the number of correct choices from day 1 to day 3 ($F_{(2,38)}=27.38$ $P<0.001$) but with a significant day-of-training/genotype interaction ($F_{(6,38)}=6.83$ $P<0.001$) and no significant differences between genotypes. Therefore, we analyzed only the second block, which showed no day-of-training/genotype interaction. This second block presented a significant time effect, indicating that the rate of correct choices increased significantly in all groups ($F_{(2,38)}=23.83$

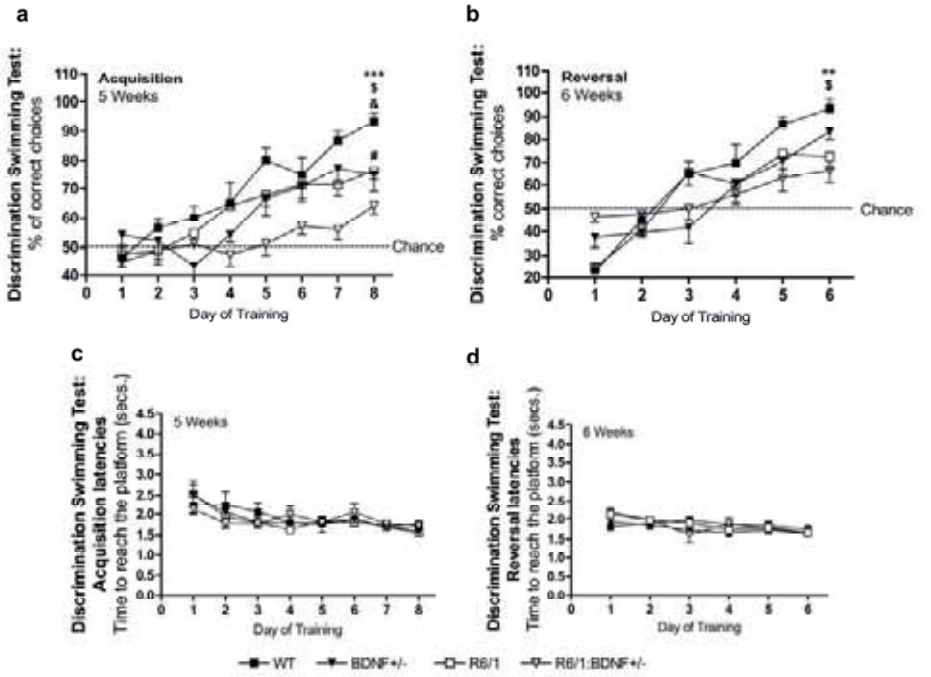

Fig. 7. Summative effects in learning deficits due to low levels of BDNF and the presence of mutant huntingtin in a visual discrimination swimming task. This learning task was divided into two phases: the acquisition phase (a) and the reversal phase (b). The time to reach the platform was also assessed (c, d). (a) Graph shows how the number of correct choices improved in all groups. In the acquisition phase (a) R6/1:BDNF+/− mice showed the worst performance. In the reversal phase (b) all groups improved their performance and progressively increased the number of correct choices. In this phase, R6/1:BDNF+/− mice were the worst learners (b). Time to reach the platform for all genotypes (wild type, BDNF+/−, R6/1, R6/1:BDNF+/−) in the acquisition phase (c) and in the reversal phase (d) was measured in order to evaluate whether motor or anxiety disturbances could influence the learning task. No differences between genotypes were detected at this age in escape latencies (c, d). The task was performed at 5 weeks of age. Bars represent means±S.E.M. ** $P<0.01$ and *** $P<0.001$ comparing wild type with R6/1:BDNF+/−; $^{\$}$ $P<0.05$ comparing wild type with R6/1; $^{\&}$ $P<0.05$ comparing wild type with BDNF+/−; $^{\#}$ $P<0.05$ comparing R6/1 with R6/1:BDNF+/− (wild type $n=5$, BDNF+/− $n=7$, R6/1 $n=4$ and R6/1:BDNF+/− $n=7$). For statistical analysis, the two-way ANOVA with repeated measures analysis with Student *t*-test as a post hoc was used.

$P<0.001$). Moreover, there were significant differences between genotypes on the performance of this task ($F_{(3,38)}=3.23$ $P<0.05$). The post hoc test revealed that wild type mice made more correct choices than R6/1:BDNF+/− mice ($P<0.01$) and R6/1 ($P<0.05$) but not more than BDNF+/− mice ($P=0.076$). However, neither R6/1:BDNF+/− nor R6/1 nor BDNF+/− reached a significant level of discrimination (above 80%), suggesting a severe discrimination learning impairment in R6/1:BDNF+/− mice, a moderate learning alteration in R6/1 and a slight learning dysfunction in BDNF+/− mice for successful performance of the reversal phase. Moreover, the analysis of the time to reach the platform showed no significant differences between genotypes in the escape latencies in either block 1 (days 1–3) or block 2 (days 4–6) of the reversal phase (Fig. 7d), suggesting that there were no anxiogenic or motor effects.

Electrophysiological studies show substantial alterations in the hippocampal LTP of R6/1, BDNF+/− and R6/1:BDNF+/− mice

Having identified different degrees of severity in cognitive and learning impairments in R6/1, and R6/1:BDNF+/− mice compared with wild type, we next used electrophysiology to investigate synaptic plasticity at CA1 hippocampal synapses. We performed a high-frequency conditioning tetanus (see Experimental Procedures) to induce LTP in hippocampus of wild type, R6/1, BDNF+/− and R6/1:BDNF+/− mice at 5 weeks of age. LTP is the sustained increase in synaptic strength obtained after a high-frequency conditioning stimulus (HFS), and is a useful model for the study of synaptic mechanisms underlying certain forms of learning and memory. Baseline responses were monitored for 10–30 min before conditioning and were

found to be stable. At 60 min after tetanus, potentiation (as mean percentage of baseline) in wild-type mice was 160.8±4 (n=9 slices, three mice) versus 129.2±7 in R6/1 (P<0.01, n=8 slices, three mice), 131.9±7 in BDNF+/− (P<0.01, n=9 slices, three mice) and 121.6±11 in R6/1:BDNF+/− (P<0.01, n=7 slices, three mice) (Fig. 8a). These data indicate that all genotypes had a deficit in HFS-LTP with respect to wild type mice. Interestingly, HFS-LTP revealed no significant differences between slices from R6/1, BDNF+/− and R6/1:BDNF+/− mice (Fig. 8a).

Specific alterations in the BDNF-TrkB-PLCγ signaling in the hippocampus of R6/1, BDNF+/− and R6/1:BDNF+/− mice

Due to the severe learning deficits seen in R6/1:BDNF+/− mice and the moderate learning impairments observed in BDNF+/− and R6/1 mice, the BDNF-TrkB signaling pathway was studied in the hippocampus at 5 weeks of age which was the same age when behavioral and electrophysiological experiments were performed. We used Western blot and real time PCR to check hippocampal protein and mRNA BDNF levels. Real time PCR demonstrated a significant decrease in mRNA BDNF levels in BDNF+/− (P<0.01), R6/1 (P<0.05) and R6/1:BDNF+/− (P<0.001) with respect to wild type (Fig. 8b). Furthermore, R6/1:BDNF+/− mice showed a significant decrease of mRNA BDNF levels compared with BDNF+/− and R6/1 mice (P<0.05 and P<0.05 respectively) (Fig. 8b). Accordingly, Western blot analysis showed decreased levels of mature BDNF in BDNF+/− (P<0.001) and R6/1:BDNF+/− (P values <0.001) hippocampus with respect to wild type (Fig. 8c) and also a significant decrease in R6/1 with respect to wild type hippocampus (P<0.05) (Fig. 8c). Additionally, both BDNF+/− and R6/1:BDNF+/− hip-

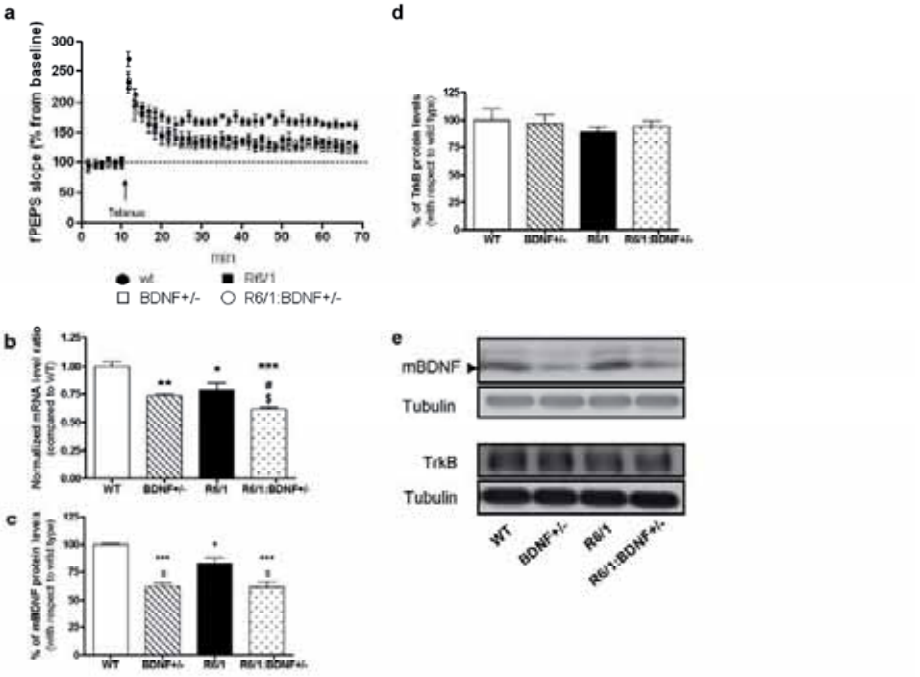

Fig. 8. LTP and BDNF levels were similarly altered in all genotypes with respect to wild type mice. Summary data showing the time course of mean fEPSPs slope in basal condition and following LTP induction (a). Data were normalized for each slice with respect to the average slope recorded during baseline and showed equal alterations in all genotypes with respect to wild type after tetanization. Hippocampal expression of BDNF mRNA (b) and protein (c) levels in wild type, BDNF+/−, R6/1 and R6/1:BDNF+/− mice at 5 weeks of age by quantitative PCR (b) and Western blot (c) analysis. Hippocampal TrkB protein levels from the same animals were also analyzed (d). Finally, representative blots for each antibody are depicted (e). The experiments were performed using mice at 5 weeks of age. Bars represent mean±S.E.M. * P<0.05: ** P<0.01: *** P<0.001 with respect to wild type; $^{\$}$ P<0.05 with respect to R6/1; $^{\#}$ P<0.05 with respect to BDNF+/− (wild type n=5, BDNF+/− n=4, R6/1 n=7 and R6/1:BDNF+/− n=6). For statistical analysis the one-way ANOVA analysis with Student t-test as a post hoc was used.

pocampi showed a significant decrease in mature BDNF ($P<0.05$) with respect to R6/1 (Fig. 8c).

Next, we quantified the total levels of the high affinity receptor for BDNF, TrkB, in the hippocampus (Fig. 8d). TrkB protein levels were normal in all genotypes compared with wild type hippocampus at the same age as the LTP experiment. In view of this result, we checked the downstream signaling of TrkB involved in LTP and plasticity processes by exploring the ERK and PLCγ signaling pathways. We determined the total and phosphorylated levels of ERK and PLCγ. We did not detect alterations in the ERK1/2 phosphorylation rate (Fig. 9a) with respect to total ERK1/2 (Fig. 9b) in the hippocampus of any genotype compared with wild type. In contrast, the PLCγ pathway results were quite different (Fig. 9c–d). We found normal levels in the total protein expression of PLCγ in all genotypes (Fig. 9d), but an identical decrease was seen in the ratio of phospho-PLCγ^{Tyr783}/total PLCγ (Fig. 9c) in BDNF+/− ($P<0.001$), R6/1 ($P<0.01$) and R6/1:BDNF+/− ($P<0.01$) hippocampus with respect to wild type.

Different levels of BDNF modulate the expression of different AMPAR and NMDAR subunits in mouse models of HD

We next evaluated the basal expression levels of NMDAR and AMPAR subunits in the hippocampus at 5 weeks of age. First, we performed immunoblots for total levels of AMPAR subunits expression (Fig. 10a–c). Thus, GluR1 was only significantly reduced in R6/1:BDNF+/− hippocampus ($P<0.05$) (Fig. 10a). In addition, GluR2/3 expression was not altered in any genotype (Fig. 10b). Next, we quantified NR1, NR2A and NR2B total expression levels (Fig. 10d–g). Only R6/1:BDNF+/− mice presented

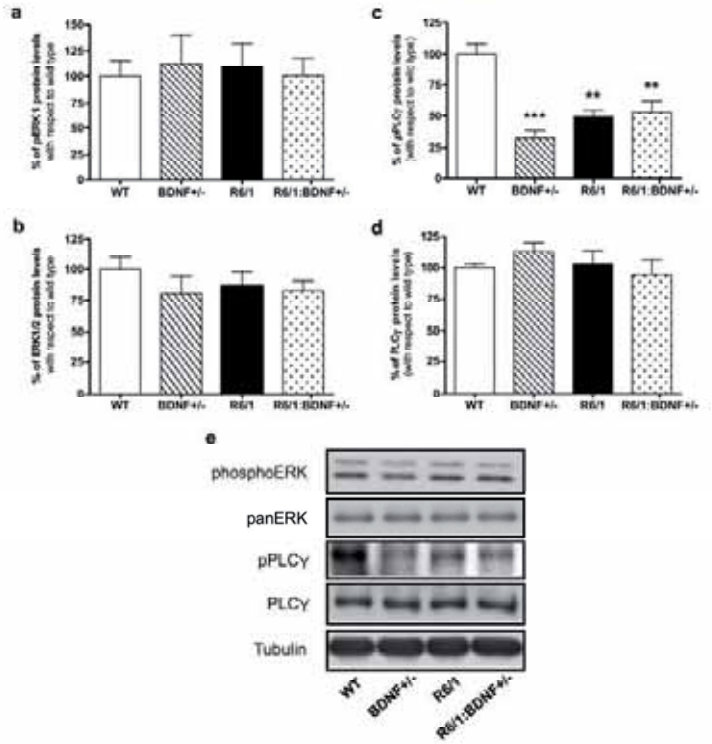

Fig. 9. The alteration on BDNF levels was associated to an specific alteration in PLCγ signaling which was equally altered in all genotypes. Total ERK and its phosphorylation levels are not altered in any group (a, b, e) but PLCγ phosphorylation levels fall dramatically in all genotypes with respect to wild type without alterations in basal levels (c, d, e). The experiments were performed using 5 week old mice. Bars represent mean±S.E.M. ** $P<0.01$ and *** $P<0.001$ with respect to wild type (wild type $n=5$, BDNF+/− $n=4$, R6/1 $n=7$ and R6/1:BDNF+/− $n=6$). For statistical analysis the one-way ANOVA analysis with Student *t*-test as a post hoc was used.

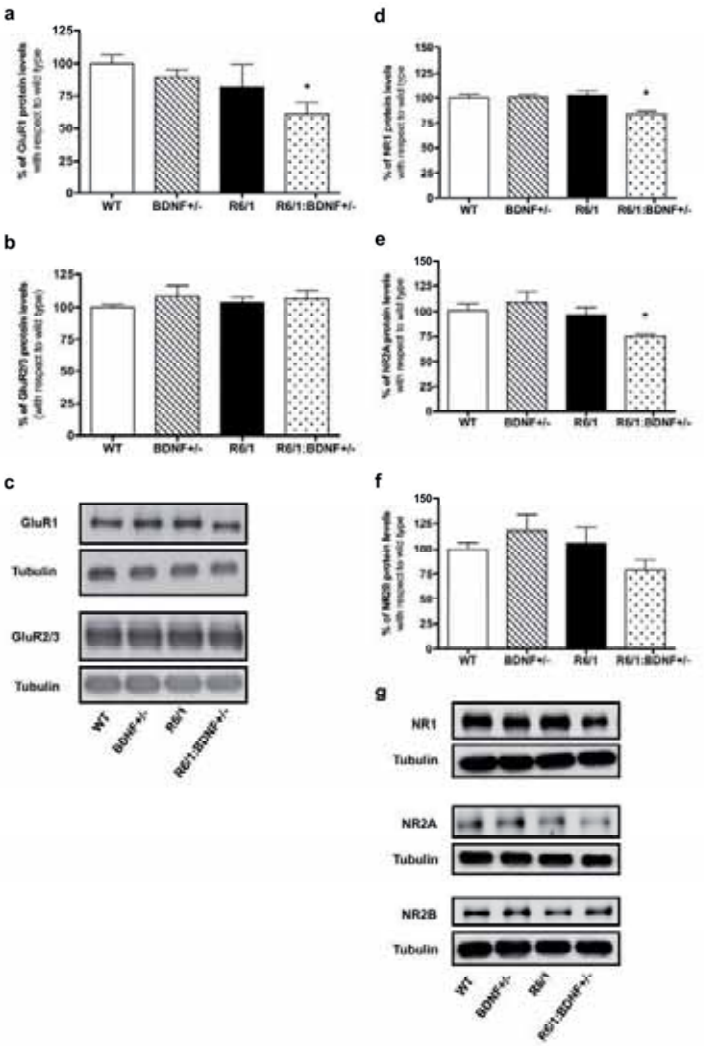

Fig. 10. R6/1:BDNF+/− mice show specific alterations in the expression of AMPAR and NMDAR subunits in the hippocampus. Total protein levels of AMPAR (a–c) and NMDAR (d–g) subunits were determined by Western blot in total protein extracts at 5 weeks of age. Representative blots of AMPAR subunits GluR1 and GluR2/3 are shown (c). Representative blots of NR1, NR2A and NR2B subunits are also shown (g). The experiments were performed using mice at 5 weeks of age. Bars represent mean±S.E.M. * $P<0.05$, with respect to wild type (wild type $n=5$, BDNF+/− $n=4$, R6/1 $n=7$ and R6/1:BDNF+/− $n=6$). For statistical analysis, the one-way ANOVA analysis with Student t-test as a post hoc was used.

significantly decreased levels in NR1 ($P<0.05$) (Fig. 10d) and NR2A ($P<0.05$) (Fig. 10e), but not in NR2B expression (Fig. 10f), with respect to wild type hippocampus. These specific alterations in glutamate receptors in R6/1:BDNF+/− mice correlate with more severe alterations in learning and memory observed in these mice.

DISCUSSION

In the present study we performed a battery of behavioral tests in wild type, R6/1, BDNF+/− and R6/1:BDNF+/− mice to evaluate the relationship of BDNF levels and mutant huntingtin in cognitive alterations of HD. We observed deficits in procedural, alternation and discrimination learning, but onset and severity differed according to genotype. Animals with mutant huntingtin and lower levels of BDNF (R6/1:BDNF+/−) were the worst learners. R6/1 and BDNF+/− mice showed moderate and slight cognitive impairments respectively. These learning deficits may be attributed to the decrease in BDNF levels observed in these genotypes. Moreover, we observed decreased levels of hippocampal LTP in BDNF+/−, R6/1 and R6/1:BDNF+/− mice, which could be explained by the alterations in levels of phospho-PLCγ. Interestingly, we found a specific reduction in levels of glutamate receptors, but only, in the R6/1:BDNF+/− hippocampus that correlates with enhanced severity in learning deficits observed in these mice.

First, the results obtained from control strains are of interest. It has been reported that BALB/c mice are poor learners in different learning tasks (Upchurch and Wehner, 1987; Francis et al., 1995; Roullet and Lassalle, 1995), probably due to anxiogenic components (Belzung and Berton, 1997; Tang et al., 2005). However, anxiety levels fall in these mice in dimly lit conditions (Avgustinovich and Korjakina, 2000). In agreement with these results, and after extensive pre-training (Sik et al., 2003), this strain can improve its performance in different learning paradigms (Chapillon and Debouzie, 2000; Klapdor and Van Der Staay, 1996; Royle et al., 1999; Sik et al., 2003; Wahlsten et al., 2005). Indeed, we obtained positive results with BALB/c mice in different learning tasks. On the other hand, B6CBA mice, a strain obtained by crossing CBA and C57BL/6 mice, have been described as good learners (Lione et al., 1999; Mazarakis et al., 2005; Murphy et al., 2000). Furthermore, we previously demonstrated no anxiety differences between wild type, BDNF+/−, R6/1 and R6/1:BDNF+/− mice by using the open field paradigm (Canals et al., 2004). Therefore, our results indicate that strains used to generate R6/1 and BDNF+/− mice learn the different tasks equally well. Moreover, visual acuity could not affect the performances, since all strains and substrains used in the present work have been previously demonstrated to be able to learn correctly several learning tasks which require the integrity of visual system (Lione et al., 1999; Canals et al., 2004; Chapillon and Debouzie, 2000). In addition, we used mice from, at least, F6 inbred littermates for all genotypes. Thus, the results obtained in the experimental groups could not be attributed to an effect of strain background, but changes in learning appear to be due to the effects of mutant huntingtin, levels of BDNF, and their interactions.

The present work shows very early cognitive alterations (5–8 weeks) that are not determined by motor impairment, anxiogenic or motivational components in a stage defined as presymptomatic in mouse models of HD, since R6/1 and R6/1:BDNF+/− mice show an overt motor phenotype from the ages of 18 and 14 weeks respectively (Canals et al., 2004). These results are in agreement with those obtained in R6/2 mice, which showed very early learning deficits mainly in discrimination and spatial learning (Lione et al., 1999), but also in somatosensory discrimination learning deficits in 10-week-old R6/1 mice (Mazarakis et al., 2005). Moreover, several studies have been performed in different mouse models with the complete huntingtin transgene. In fact, cognitive impairment has been demonstrated in YAC128 mice using procedural learning tasks (Van Raamsdonk et al., 2005), and slight implicit learning deficits in HdhQ92 mice (Trueman et al., 2007) both at stages without motor alterations. All these studies in animal models support the observation that patients with HD show learning and memory alterations even at preclinical stages (Foroud et al., 1995; Lawrence et al., 1996, 1998; Lemiere et al., 2004).

The molecular mechanism by which mutant huntingtin affects cognitive behavior has not been described yet. The impairment of learning and memory in mice expressing mutant huntingtin has been linked to abnormal plasticity (Milnerwood et al., 2006; Murphy et al., 2000). Here we observed that R6/1:BDNF+/− mice performed all cognitive tasks worse than R6/1 mice, suggesting that deficits induced by mutant huntingtin could be modulated by BDNF levels. To test this hypothesis we next tested the levels of BDNF and its high affinity receptor in the hippocampus of the HD mouse models studied at 5 weeks of age. Changes in BDNF levels in different models of HD have been contradictory depending on the techniques used (see review in Zuccato and Cattaneo, 2007). In the present study, we measured a specific band by Western blot that is not present in KO BDNF mice. Thus, a reduction in the levels of mature form of BDNF was observed in the hippocampus, and similar results were observed by quantitative PCR. These data are in agreement with previous studies showing decreased BDNF levels in the hippocampus of R6/1 (Spires et al., 2004) and knock-in mice (Lynch et al., 2007). Our results also show that TrkB protein levels were not altered in the hippocampus of R6/1, BDNF+/− and R6/1:BDNF+/− mice. However, TrkB signaling was impaired in all these mouse models that have reduced levels of BDNF. We found an identical and specific decrease in the levels of PLCγ phosphorylation, but not of ERK-1/2 phosphorylation, in R6/1, BDNF+/− and R6/1:BDNF+/− mice. These results are in agreement with previous studies which show that a targeted mutation in PLCγ docking sites of TrkB is sufficient to impair hippocampal LTP (Minichiello et al., 2002; Gruart et al., 2007), and that an over-expression of the same receptor induces increased PLCγ activity together with improved learning (Koponen et al., 2004). Similarly, our finding on altered PLCγ phosphorylation are in accordance with the CA3–CA1 LTP dysfunction that we observed in these mouse models. CA3–CA1 LTP is affected very early at 5 weeks of age in BDNF+/−, R6/1 and R6/1:BDNF+/− mice. In agreement with these results, previous studies already reported that mutant huntingtin

induces hippocampal LTP deficits before motor symptoms (Milnerwood et al., 2006).

Several proteins are involved in the regulation of synaptic plasticity. Among them, BDNF activates distinct mechanisms to regulate the induction, and early and late maintenance phases of LTP (Bramham and Messaoudi, 2005). In the present work, we demonstrate that BDNF, by itself, modulates learning deficits since BDNF+/− mice showed these deficits at late ages. However, evidences from previous reports are conflictive; some authors suggest learning alterations in BDNF+/− mice (Linnarsson et al., 1997) while others do not (Montkowski and Holsboer, 1997). These differences may be understood as an age-dependent effect. Young BDNF+/− mice performed the cognitive tasks successfully (Montkowski and Holsboer, 1997; and present results) while older animals showed moderate learning deficits (Linnarsson et al., 1997; and present results). Besides this important role of BDNF in synaptic plasticity of the hippocampal LTP, we did not observe additive effects in R6/1:BDNF+/− with respect to R6/1, suggesting that mutant huntingtin affects cognitive procedures through the decrease of this neurotrophin. In fact, it has been suggested that there is a threshold in BDNF levels to induce hippocampal LTP deficit since heterozygous and homozygous mice for BDNF show identical alterations in this paradigm (Patterson et al., 1996). The relevance of BDNF in plasticity alterations seen in HD mouse models has been reported in a recent study showing a decrease in actin polymerization in dendritic spines and deficits in LTP stabilization in HdhQ111 knock-in mice, which can be reverted by BDNF treatment (Lynch et al., 2007). Taken together, all these results suggest that the decrease of BDNF and the consequent affecting of BDNF-TrkB-PLCγ signaling integrity are involved in the learning impairment in HD.

Our present work also demonstrates that the affecting of CA3–CA1 LTP by BDNF in HD does not correlate with the severity of learning alterations between the different genotypes. This result suggests either the involvement of other molecular mechanisms that could be alterations in other hippocampal subregions or the implication of other brain regions, such as the striatum, which has been previously described with very early synaptic alterations (Torres-Peraza et al., 2008). In this line, there are clear evidences that the NMDA and AMPA receptor-dependent synaptic function is critical for the encoding storage of memory traces (Morris, 2006; Kennedy et al., 2005; Luscher and Frerking, 2001). Our results show a specific decrease in NR1, NR2A and GluR1 protein levels only in the hippocampus of R6/1:BDNF+/− mice at 5 weeks of age, which were the worst learners. Previous studies already show that R6/1:BDNF+/− mice have a downregulation of NMDA receptors and postsynaptic proteins in the striatum (Torres-Peraza et al., 2008). These results suggest a specific decrease of these proteins caused by the expression of mutant huntingtin and a severe reduction of BDNF levels inducing an early onset of cognitive dysfunction. Accordingly, a recent study has reported decreased levels of GluR1 and PSD-95 in the hippocampus of R6/1 mice at 14 weeks of age, which can be related to a deficit in short-term hippocampal-dependent memory (Nithianantharajah et al., 2008). These results suggest a specific decrease of these proteins caused by the expression of mutant huntingtin together with a severe reduction of BDNF levels inducing an early onset of cognitive dysfunction. An early and specific GluR1 decrease in R6/1:BDNF+/− mice compared with R6/1 could explain their poor performance in all learning tasks since GluR1 deletion impairs some forms of learning but not hippocampal LTP (Shimshek et al., 2006). Furthermore, it has been previously demonstrated that BDNF could modulate the GluR1 trafficking (Narisawa-Saito et al., 2002) and expression (Caldeira et al., 2007) in hippocampal neurons. Moreover, GluR1 is essential for non-spatial hippocampal dependent learning tasks (Reisel et al., 2005), which is in agreement with our results of non-spatial learning tasks. On the other hand, the NR1 subunit in dentate gyrus is essential for the correct performance of hippocampal dependent working memory tasks but not for some spatial learning tasks (Niewoehner et al., 2007), a finding that is in accordance with the specific deficits in alternation learning seen in our R6/1:BDNF+/− mice. Finally, mice lacking the NR2A subunit show only moderate hippocampal dependent learning deficits (Sakimura et al., 1995), which may help to explain the severe cognitive alterations seen in R6/1:BDNF+/− mice. Similarly to R6/1:BDNF+/− mice in the present study, R6/2 mice, which belong to a more severe line than R6/1, also show decreased expression of different NMDA subunits in the hippocampus in early stages of the disease (Luthi-Carter et al., 2003).

CONCLUSION

In conclusion, our results show evidences that mutant huntingtin alters BDNF function, which in turn modulates a major component of the dysfunction in learning and memory in mouse models of HD at presymptomatic ages. Additionally, we found decreased hippocampal CA3–CA1 LTP that correlated with a specific dysfunction of the BDNF-TrkB signaling via PLCγ regulated mainly by mutant huntingtin and its effects on BDNF function. These results are accompanied by a specific downregulation of AMPA and NMDA receptors in the hippocampus which depend on both mutant huntingtin and BDNF levels. Therefore, BDNF function is a key regulator factor for the cognitive deficits of HD.

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Cuarto trabajo: ***"BDNF regulation under GFAP promoter provides engineered astrocytes as a new approach for long-term protection in Huntington's disease".***

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Objetivo 5. Generación y caracterización de un modelo condicional de liberación de BDNF en condiciones patológicas: un ratón transgénico que sobre-exprese BDNF bajo el promotor GFAP.

Objetivo 6. Estudio del efecto neuroprotector del uso de astrocitos que liberen BDNF de forma condicional en diferentes modelos de la enfermedad de Huntington..

En esta tesis y en muchos otros trabajos previos se ha descrito que el núcleo estriado, la región más afectada en la enfermedad de Huntington, es altamente dependiente del aporte de BDNF desde otras regiones como, por ejemplo, desde la corteza cerebral. Por lo tanto, debido a su potencial terapéutico, es imperativo el diseño de terapias dirigidas a la administración de la neurotrofina. Sin embargo, aunque se han demostrado ya los efectos beneficiosos de BDNF como terapia neuroprotectora en la enfermedad de Huntington, existen muchos problemas en lo que concierne a su vía de administración y regulación de sus niveles y funciones. Estos conocimientos previos nos han llevado a diseñar un ratón transgénico que tenga insertado un transgen de BDNF bajo el promotor de GFAP. El diseño de este constructo nos indica que tan sólo las células que expresen tal proteína (la astroglía) será capaz de sobre-expresar la neurotrofina. Una vez conseguida la obtención de astrocitos en cultivo provinentes de estos ratones transgénicos, el siguiente objetivo fue el de utilizarlos como terapia celular neuroprotectora en modelos agudos y tóxicos de la enfermedad. En estos estudios es importante realizar un seguimiento a largo plazo de tales modelos experimentales y de analizar los cambios potenciales debido a los transplantes a todos los niveles: bioquímico, morfológico y conductual.

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SHORT COMMUNICATION

BDNF regulation under GFAP promoter provides engineered astrocytes as a new approach for long-term protection in Huntington's disease

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Brain-derived neurotrophic factor (BDNF) is the main candidate for neuroprotective therapeutic strategies for Huntington's disease. However, the administration system and the control over the dosage are still important problems to be solved. Here we generated transgenic mice over-expressing BDNF under the promoter of the glial fibrillary acidic protein (GFAP) (pGFAP-BDNF mice). These mice are viable and have a normal phenotype. However, intrastriatal administration of quinolinate increased the number of reactive astrocytes and enhanced the release of BDNF in pGFAP-BDNF mice compared with wild-type mice. Coincidentally, pGFAP-BDNF mice are more resistant to quinolinate than wild-type mice, suggesting a protective effect of astrocyte-derived BDNF. To verify this, we next cultured astrocytes from pGFAP-BDNF and wild-type mice for grafting. Wild-type and pGFAP-BDNF-derived astrocytes behave similarly in nonlesioned mice. However, pGFAP-BDNF-derived astrocytes showed higher levels of BDNF and larger neuroprotective effects than the wild-type ones when quinolinate was injected 30 days after grafting. Interestingly, mice grafted with pGFAP-BDNF astrocytes showed important and sustained behavioral improvements over time after quinolinate administration as compared with mice grafted with wild-type astrocytes. These findings show that astrocytes engineered to release BDNF can constitute a therapeutic approach for Huntington's disease.
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Keywords: *neurotrophin; cell therapy; excitotoxicity*

Introduction

Huntington's disease (HD) is due to an abnormal expansion of a CAG codon in exon 1 of the *huntingtin* (htt) gene, resulting in a devastating cognitive and motor disorder,[1] which is characterized by a selective degeneration of projection neurons in the striatum.[2,3] Many efforts have been made to develop treatments that counteract the symptomatology but an efficient therapy to alleviate or compensate the neural dysfunction or loss is not currently available.

Striatal neurons depend on brain-derived neurotrophic factor (BDNF) for function and survival,[4–7] which is mainly provided by anterograde transport from corticostriatal afferents.[8,9] Mutant huntingtin alters the BDNF function by an impairment in its transport, which results in deficient striatal BDNF levels.[10,11] Furthermore, mutant huntingtin also deregulates the BDNF transcription in the cerebral cortex which, in turn, affects striatal BDNF levels.[12,13] This reduction of BDNF levels affects the onset and severity of the disease in HD mouse models.[5] Moreover, upregulation of BDNF in different models of HD improves the disease symptomatology.[14–17] BDNF can also be relevant in the regulation of cognitive alterations observed in HD.[18,19] Thus, due to its prosurvival effects in striatal and cortical neuropathology, BDNF is the main candidate for neuroprotective therapies[20,21] as it has been tested after intrastriatal administration of quinolinate (QUIN), an *N*-methyl-D-aspartic acid receptor agonist used as an acute model of HD[22], and in transgenic mouse models.[5] However, the main problem to use neurotrophic factors as therapeutic agents for neurodegenerative disorders is the chronic delivery system.[21,23] Therefore, it is necessary to develop new systems for localized, conditional and safe delivery of neurotrophic factors.

The neuroprotective effects of BDNF have been shown by using different strategies such as transplantation of engineered cells,[24,25] direct administration[5] or gene transfer.[26,27] However, vector and virus toxicity, tumorigenesis and uncontrolled gene expression are often

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important problems to be solved.[21,25,28] Cell therapy has emerged as a putative tool to treat neurodegenerative diseases, genetically engineered cells being one of the most frequently considered strategies (for review see Dunnett and Rosser[29]). In particular, engineered astrocytes could be good candidates to release neurotrophic factors. In fact, genetically modified astrocytes have been used with positive results in some models of neurodegeneration.[24,30] This strategy could be relevant for HD as numbers of reactive astrocytes increase progressively concomitant to the disease progression.[3] Moreover, astrogliosis has been shown to be one of the specific hallmarks of disease progression in mouse models of HD.[31–33] Because of this increase in astrocytes in HD, and the fact that astrogliosis leads to increased glial fibrillary acidic protein (GFAP) promoter activation, one would predict that the use of this promoter in cell therapy would provide neurotrophic support at the time when it is critically needed.

In this work we show that transgenic astrocytes engineered to overexpress BDNF under control of the GFAP promoter release higher levels of BDNF than control astrocytes. This enhanced release of BDNF exerts neuroprotection of striatal neurons, which, in turn, results in long-term behavioral improvements.

Results

Generation and characterization of pGFAP-BDNF transgenic mice

We generated a new transgenic mouse line, pGFAP-BDNF mice, which overexpress BDNF regulated by the GFAP promoter. A visual scheme of the transgene construct is shown in Figure 1a. PCR analysis of different nervous and non-nervous system tissues showed transgene expression restricted to the central nervous system (Figure 1b). Note the different levels of expression in several brain regions such as cortex, hippocampus and striatum (Figure 1b). Data obtained from the SHIRPA protocol indicated that the general appearance shown by pGFAP-BDNF mice was well groomed with a pink coloration in the ear and footpads (Figure 1c). Neurosensorial items showed no gross neurological alterations in pGFAP-BDNF mice (Supplementary Figure 1A). Moreover, there were no differences between wild-type (wt) and pGFAP-BDNF mice performing the Rotarod task (Supplementary Figure 1B). Next, to analyze anxiety behavior and locomotor activity we used the open field. We observed that pGFAP-BDNF mice showed a similar locomotor behavior (Supplementary Figure 1C), and identical mean speed, number of defecations and center/periphery ratios (data not shown). Also, pGFAP-BDNF mice showed body weight (Figure 1d) and whole-brain weight (Figure 1e) similar to that of wt mice. Stereological volume measurements of brain regions such as motor cortex, hippocampus and striatum showed no differences between pGFAP-BDNF and wt mice (Figure 1f). Western blot analyses showed similar GFAP levels in the hippocampus, motor cortex, prefrontal cortex, and striatum of pGFAP-BDNF and wt mice (Figure 1g). BDNF enzyme-linked immunosorbent assay (ELISA) analysis indicated that the levels of this neurotrophin were not altered in pGFAP-BDNF mice (Figure 1h).

Astrocytes from pGFAP-BDNF mice are viable and release BDNF specifically under proinflammatory conditions

Tissue culture grown astrocytes from wt and pGFAP-BDNF mice (P1–P3 pups) capable of GFAP regulated overexpression of BDNF were shown to have equivalent astrocytic morphology (data not shown) and to be essentially pure by GFAP immunostaining (Figure 2a). In addition, during *in vitro* amplification viability and survival of astrocytes from the two sources were also similar (Trypan blue procedure) (data not shown).

To study the response of these cultures to proinflammatory conditions, we induced astrogliosis *in vitro*. To this end, we used the proinflammatory cytokine tumor necrosis factor-α (TNF-α), which has been previously shown to increase selectively the BDNF production and release in pure astrocyte cultures.[34] Results showed that the basal content of BDNF in pGFAP-BDNF astrocytes was higher than in wt astrocytes (Figure 2b). However, the amount of released neurotrophin was equal in both groups under control conditions but significantly higher levels of BDNF were secreted from pGFAP-BDNF than from wt astrocytes on TNF-α stimulation (Figure 2c). We also tested the effect of the *N*-methyl-D-aspartic acid agonist QUIN on BDNF release in astrocyte cultures. However, QUIN itself did not modify the levels of BDNF (Supplementary Figure 2). Taken together, these results show a normal morphology but increased BDNF protein expression in pGFAP-BDNF astrocytes, which was released in higher amounts only on inflammatory-like conditions.

pGFAP-BDNF mice show striatal neuroprotection against acute administration of QUIN

We next tested whether the release of BDNF after astrocyte stimulation *in vitro* would be translated into a neuroprotective effect after intrastriatal QUIN administration. To analyze astrogliosis *in vivo*, we first showed, by western blot analysis, that the expression level of GFAP in the striatum of wt and pGFAP-BDNF mice 1 and 7 days after QUIN administration was similar (Figures 3a and b). In these experiments GFAP was rapidly upregulated and the elevated level was maintained for at least 1 week in ipsilateral striata of both genotypes, in comparison with sham controls, suggesting that transgene expression did not affect the capacity for a glial response. Next, we analyzed the levels of BDNF in wt and pGFAP-BDNF mice 1 and 7 days after QUIN administration. BDNF levels were upregulated from day 1 post-QUIN administration in lesioned striata of both wt and pGFAP-BDNF mice in comparison with their respective sham controls (Figure 3c). This upregulation was still detectable after 1 week in both genotypes and, interestingly, BDNF levels were higher in the lesioned striata of pGFAP-BDNF mice than in that of wt mice (Figure 3c). It is noteworthy that QUIN did not induce the BDNF upregulation due to direct astroglial NMDAR activation (Supplementary Figure 2), suggesting it was essentially due to an astrocyte response to neuronal inflammation and injury. These results indicated a rapid and sustained induction in BDNF production by pGFAP-BDNF astrocytes after the excitotoxic insult. We then evaluated the extent of QUIN-induced lesion in both genotypes. At 7 days after

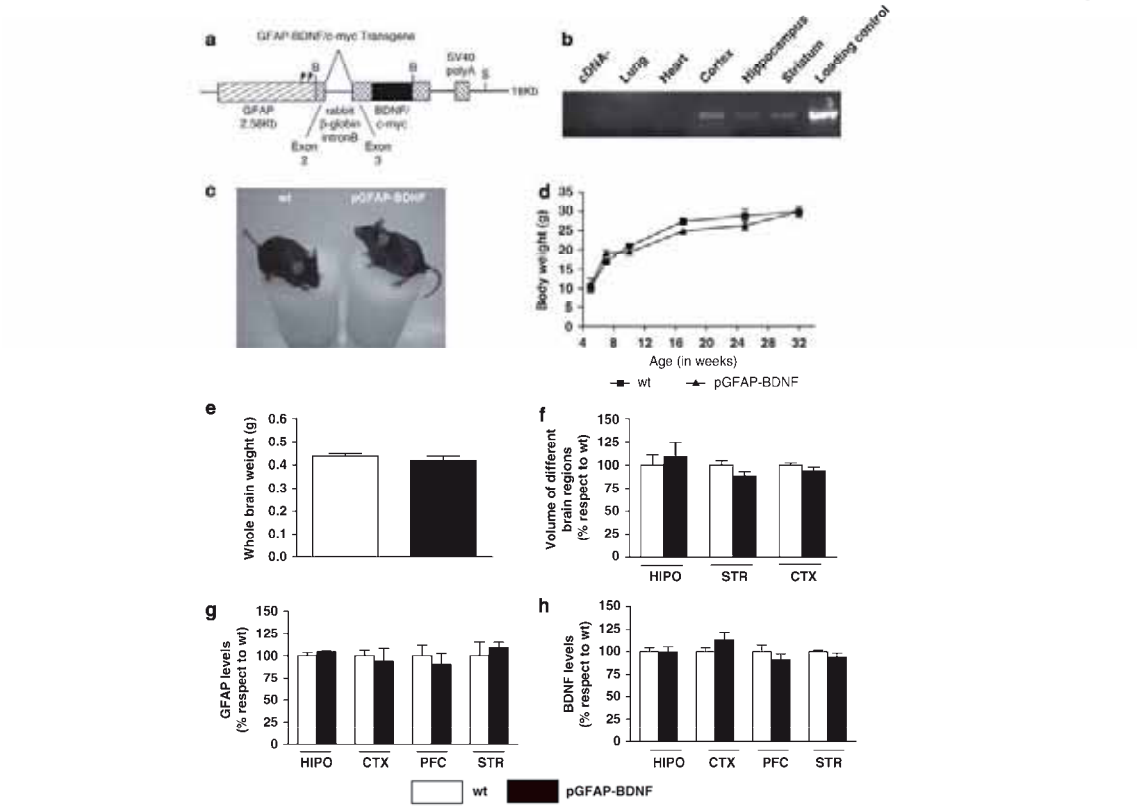

Figure 1 Generation and characterization of pGFAP-BDNF mice having normal viability and life span but no difference in phenotype in comparison with wt type mice. (**a**) A scheme of the construct used to generate the transgene is depicted. (**b**) RNA transgene expression levels for the different tissues analyzed. Note that non-nervous tissues such as heart and lungs show no RNA transgene expression due to GFAP specificity for neural populations. Appearance (**c**), body weight (**d**) and total brain weight (**e**) of wt and pGFAP-BDNF mice. Volume estimates for different brain regions (**f**). Analysis of GFAP (**g**) and BDNF (**h**) levels in different brain regions shows normal neurotrophin levels in both genotypes and no effect on GFAP protein expression in pGFAP-BDNF mice in comparison with wt mice. HIPO: hippocampus, CTX: motor cortex, STR: striatum, PFC: prefrontal cortex. Bars represent the mean ± s.e.m. ($n = 5$ per group at 6–8 weeks of age). Data were analyzed using the *t*-test when comparing two groups. To compare the body weight in both genotypes, we used the two-way ANOVA with Student's *t*-test as a *post hoc*.

administration, the volume of the striatal lesion was smaller in pGFAP-BDNF mice than in wt mice, suggesting neuroprotective effects of transgenic astrocytes in pGFAP-BDNF mice (Figures 3d and e). This protection was also observed by western blot analysis of dopamine and cyclic AMP-regulated phosphoprotein of 32 kDa (DARPP-32) levels (Figure 3f). Although wt mice intrastriatally injected with QUIN showed a decrease in the levels of DARPP-32 relative to wt sham-injected striata, the levels of DARPP-32 in the striata of QUIN injected pGFAP-BDNF mice were similar to those of their sham injected counterparts. In agreement with these findings, stereological cell counts showed a higher density of NeuN, DARPP-32 and parvalbumin-positive neurons in lesioned striata of pGFAP-BDNF mice than in lesioned striata of wt mice (Figures 4a–c). However, no neuroprotective effect was observed in the number of choline acetyltransferase (ChAT)-positive cells, indicating that these cells were not protected against excitotoxicity by astrocytes expressing the pGFAP-BDNF transgene (Figure 4d). These results indicated that in response to an intrastriatal excitotoxic insult, pGFAP-BDNF mice show regulated neuroprotection.

Wt and pGFAP-BDNF astrocytes grafted in Swiss nu-nu mice striata survive for long periods

We next tested whether grafting of pGFAP-BDNF mouse-derived astrocytes into Swiss nu-nu mice could be neuroprotective against intrastriatal QUIN lesion. We

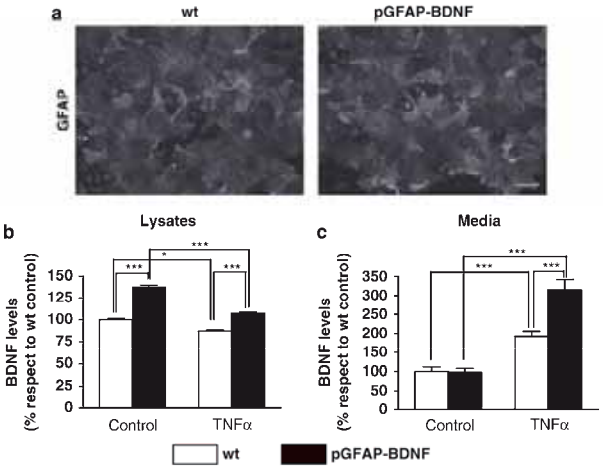

Figure 2 pGFAP-BDNF astrocyte cultures produced and released more BDNF than wt astrocytes without significant differences in morphology and growth between genotypes. (**a**) GFAP staining reveals no difference between wt and pGFAP-BDNF astrocytes. (**b**, **c**) ELISA experiments were performed to evaluate levels of released BDNF levels in control conditions and after stimulation with TNF-α. For that, cell lysates (**b**) and serum-free conditioned media (**c**) from both genotypes in control and stimulated conditions were obtained and compared. Bars represent means ± s.e.m. ($n = 5$ per group). Data were analyzed by one-way ANOVA with Student's *t*-test as a *post hoc* (*$P<0.05$ and ***$P<0.001$). Scale bar, 20 μm.

first characterized the survival and migration of grafted astrocytes from wt and pGFAP-BDNF mice in response to intrastriatal administration of phosphate-buffered saline (PBS) or QUIN. To track and characterize them after transplantation, we transduced cultured astrocytes with a retroviral vector for expression of enhanced green fluorescent protein (eGFP)[35] and grafted the resulting (an ~50% proportion of eGFP-positive/GFAP-positive cells; data not shown) astrocytes into ipsilateral striata of Swiss nu-nu mice (Figures 5b3–b6). Histological analysis showed that eGFP-positive astrocytes were detectable at days 7 and 30 after grafting, indicating a sustained persistence of the graft (Figures 5b3–b6). In sham-injected striata, astrocytes were mainly distributed in an elongated capsule-like structure surrounded by reactive astrocytes that formed a glial-like scar (Figures 5b3–b4). Conversely, in QUIN-injected striata, grafted astrocytes were more disperse in comparison with sham-injected striata, although they were also surrounded by a glial-like scar (Figures 5b5–b6). To rule out the possibility of a downregulation of the transduced eGFP in grafted astrocytes, we performed additional experiments using astrocytes isolated from transgenic mice expressing eGFP under control of the β-actin promoter.[36] In these experiments, we obtained similar results showing low dispersion of grafted astrocytes surrounded by a glial-like scar (Supplementary Figure 3).

Further morphological analysis of transplanted and endogenous astrocytes after QUIN administration showed a high density of reactive and stellate astrocytes with small and picnotic nuclei in the graft center and a considerably lower density of also reactive astrocytes outside the graft (Figure 6a). Grafts of wt and pGFAP-BDNF astrocytes showed similar characteristics and their volumes were stereologically quantified without significant differences (wt: 0.152 ± 0.022 mm^3, pGFAP-BDNF: 0.204 ± 0.053 mm^3, NS).

pGFAP-BDNF astrocytes grafted in the striatum protect neurons against QUIN administration by BDNF upregulation

Next, we examined whether pGFAP-BDNF grafted astrocytes provide neuroprotection to striatal cells against QUIN insult. At 7 or 30 days post-astrocyte transplantation (Figures 6b and e), QUIN or PBS were injected and the levels of BDNF in the grafted striata were examined (Figures 6d and g).

We found that in the sham conditions, BDNF levels were equal when comparing mice grafted with wt or pGFAP-BDNF astrocytes at both time points (Figures 6d and g). However, the levels of BDNF increased when grafted striata were lesioned with QUIN, at 7 or 30 days after grafting (Figures 6d and g). Interestingly, mice grafted with pGFAP-BDNF astrocytes showed a higher enhancement of this neurotrophin after QUIN administration than did mice grafted with wt astrocytes. These data correlated with a smaller lesion volume in mice grafted with pGFAP-BDNF astrocytes 7 and 30 days after grafting (Figures 6c and f). Furthermore, mice grafted with pGFAP-BDNF astrocytes showed a higher density of DARPP-32-positive neurons than mice grafted with wt astrocytes after QUIN administration (Figures 7a and d). Similar results were obtained for parvalbumin-positive cells (Figures 7b and e) but not for ChAT-positive cells (Figures 7c and f), which were not protected by the presence of pGFAP-BDNF astrocytes. These findings showed that pGFAP-BDNF-grafted astrocytes produced

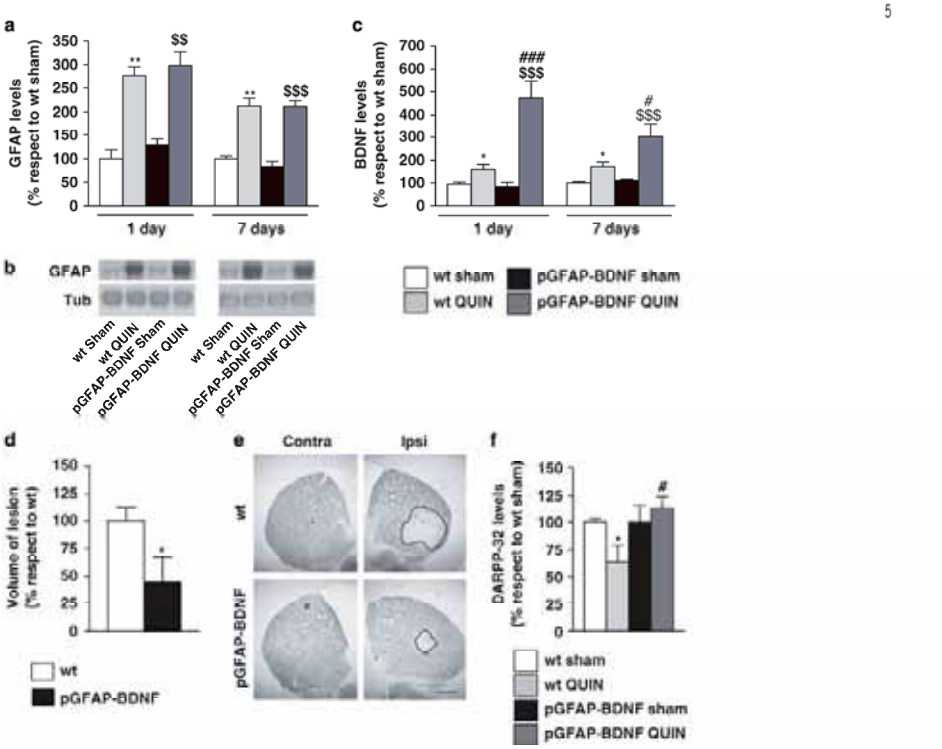

Figure 3 Upregulation of BDNF levels in pGFAP-BDNF transgenic mice results in increased resistance and neuroprotection against intrastriatal QUIN injection in comparison with wt mice. (**a**, **b**) Histogram showing GFAP levels from western blots and (**c**) ELISA BDNF levels at 1 and 7 days after QUIN injection. (**d**) Volumes of striatal lesions quantified using DARPP-32 immunohistochemistry. (**e**) Representative brain sections showing DARPP-32 immunostaining in contralateral (Contra) and ipsilateral (Ipsi) lesioned striata from both wt and pGFAP-BDNF lesioned animals. (**f**) DARPP-32 protein levels from sham- and QUIN-injected striata were quantified by western blot. Bars represent means ± s.e.m. ($n=7$ per group in histochemical experiments and $n=5$ per group in biochemical experiments). For statistical analysis one-way ANOVA with Student's *t*-test as a *post hoc* was used when more than two groups were compared (corresponding to figures **a**, **c** and **f**, $^{*}P<0.05$, $^{**}P<0.01$ with respect to wt sham; $^{\$\$}P<0.01$, $^{\$\$\$}P<0.001$ with respect to pGFAP-BDNF sham and $^{\#}P<0.05$, $^{\#\#\#}P<0.001$ with respect to wt QUIN). When only two groups were compared, Student's *t*-test was used (corresponding to figure **d**, $^{*}P<0.05$). Scale bar, 500 μm.

high BDNF levels which, in turn, protected striatal neurons even after long post-grafting periods.

Grafted pGFAP-BDNF astrocytes improve striatum-dependent behavioral tasks

To determine if the neuroprotection provided by pGFAP-BDNF grafted astrocytes against QUIN administration could produce behavioral improvements, we performed two tasks to check striatal function: the rotation test and the corridor task. Both tasks have been previously described in rodents to evaluate striatal function after unilateral QUIN administration.[35,37] We performed a longitudinal study for both tasks in animals lesioned 30 days after grafting (Figures 8a and c).

The rotation test was performed at 7, 15 and 30 days after lesion in unilaterally lesioned mice. As two-way repeated-measures analysis of variance (ANOVA) indicated ($P<0.001$), mice grafted with pGFAP-BDNF astrocytes showed less apomorphine-induced rotations as compared with mice implanted with wt astrocytes at all the time points analyzed (Figure 8b). To further analyze the effects of pGFAP-BDNF-grafted astrocytes on striatum-dependent behavior, we also performed the corridor task. This task involves bilateral spatial awareness and a strong element of choice due to direct competition for the food pellets placed on either side of the corridor.[37] To avoid group bias we first collected data in a baseline pregrafting phase. As we expected, no differences were observed between mice (Figure 8d). Thereafter, mice received a unilateral graft with wt or pGFAP-BDNF astrocytes and were examined to determine whether grafting *per se* could affect this task at two time points, 7 and 29 days after grafting. Neither wt nor

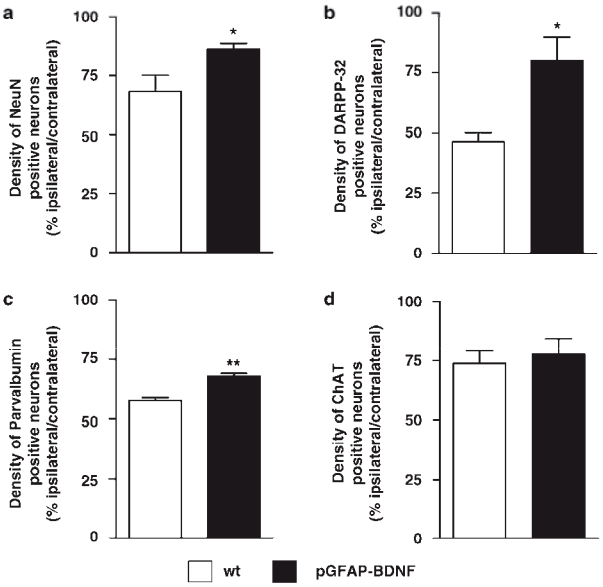

Figure 4 Different neuronal subpopulations are protected from intrastriatal QUIN lesion in pGFAP-BDNF mice. (**a**–**d**) Histograms show the density of different neuronal subpopulations immunohistochemically detected in the striatum of wt and pGFAP-BDNF mice 7 days after QUIN injection. (**a**) NeuN, (**b**) DARPP-32, (**c**) parvalbumin and (**d**) ChAT-positive neurons were counted in both contralateral and ipsilateral lesioned striata of wt and pGFAP-BDNF mice. The ratio of ipsilateral striatum to contralateral striatum values measured using a stereological procedure is represented. Bars: means ± s.e.m. ($n = 7$ per group). For statistical analysis the Student's *t*-test was used (*$P < 0.05$, **$P < 0.01$).

pGFAP-BDNF-grafted astrocytes affected this task when both groups were compared at 7 and 29 days after grafting. Next, sham or QUIN administration was performed 30 days after grafting and grafted mice were examined at 7 and 15 days after lesion. At 7 days after QUIN administration both wt and pGFAP-BDNF-grafted mice showed a slight but not significant increase in ipsilateral bias relative to their respective sham controls (Figure 8d). At 15 days after lesion, striatal pathology was exacerbated by the administration of the dopaminergic agonist apomorphine. Wt and pGFAP-BDNF-grafted mice showed a significant increase in ipsilateral bias compared with their respective sham controls (Figure 8d). In agreement with the results obtained in the rotation test, after the acute injection of apomorphine, mice grafted with pGFAP-BDNF astrocytes showed less ipsilateral bias relative to mice grafted with wt astrocytes after QUIN lesion. Taken together, these findings show that pGFAP-BDNF-grafted astrocytes were significantly more neuroprotective and induced larger behavioral improvements than wt grafted astrocytes even 60 days after transplantation.

Discussion

Here we show a novel neuroprotective therapy for HD using BDNF-engineered astrocytes. To this end, we generated and characterized a new transgenic mouse line that overexpresses BDNF under control of the GFAP promoter (pGFAP-BDNF mice). *In vitro* we show that only under inflammation-like conditions, pGFAP-BDNF mice-derived astrocytes expressed and released more BDNF than wt mouse-derived astrocytes. Accordingly, pGFAP-BDNF transgenic mice showed an upregulation of BDNF levels and neuroprotection of striatal neurons after intrastriatal QUIN administration in comparison with wt mice. To evaluate the long-term benefits of the pGFAP-BDNF-derived astrocytes, we performed striatal grafts of cultured astrocytes from wt and pGFAP-BDNF mice and compared their effect after intrastriatal administration of PBS or QUIN. pGFAP-BDNF-grafted astrocytes produced long-term neuroprotective effects when compared with wt grafted astrocytes which, in turn, also improved striatum-dependent behavioral tasks after intrastriatal QUIN administration.

Presently, there is no efficient treatment for HD patients.[38,39] A large number of clinical trials, testing pharmacological approaches, have so far produced discouraging results.[39,40] Neuroprotective strategies using several growth factors have also been proposed for HD.[41–43] Among them, BDNF, so far the best candidate, has been shown to modulate the onset and severity of motor and cognitive functions in HD mouse models[6,18,19,44] and to participate in the disruption of the cortico-striatal glutamatergic transmission that occurs in HD.[45,46] Furthermore, transgenic mice overexpressing BDNF in the cerebral cortex, when cross-mated with R6/1

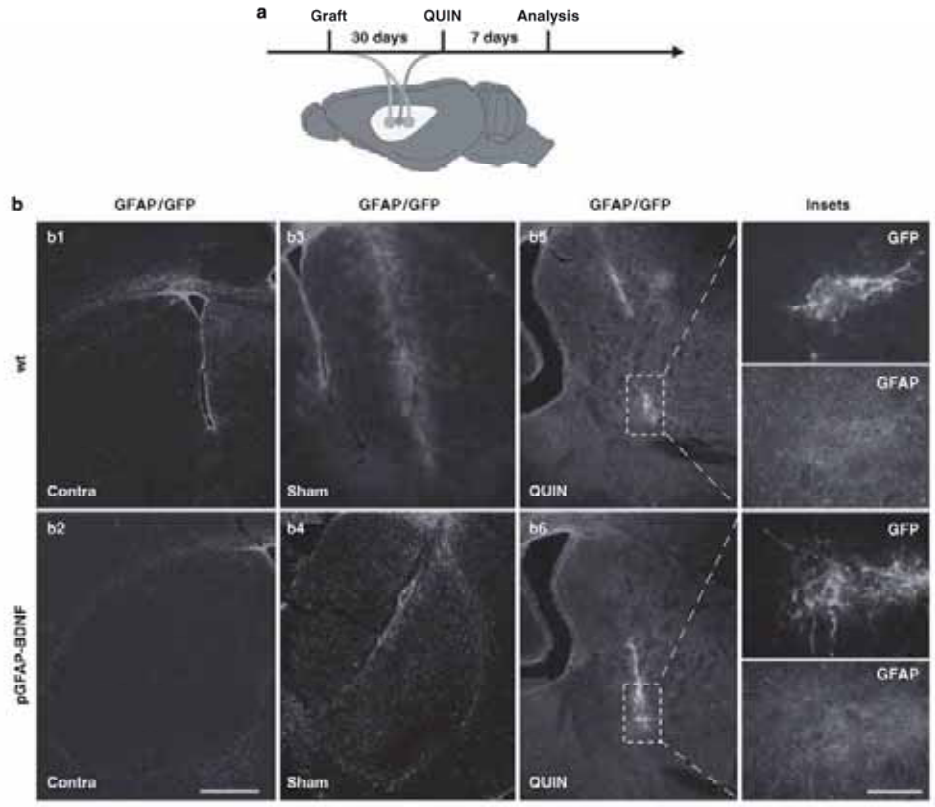

Figure 5 Characterization of wt and pGFAP-BDNF-grafted astrocytes used in sham and QUIN injection. (a) Schematic representation of the grafting procedures used to characterize wt and pGFAP-BDNF-transplanted astrocytes. (**b**) Double immunohistochemical analysis of wt (b1, b3 and b5) or pGFAP-BDNF (b2, b4 and b6) astrocytes contralaterally (b1 and b2) and unilaterally grafted in the striatum of SWISS nu-nu mice 30 days after unilateral intrastriatal vehicle (b3 and b4) or QUIN (b5 and b6) injection. Note the slightly greater migration rate of grafted astrocytes after QUIN injection with respect to vehicle injection ($n = 3$–4 per group). Scale bar, 500 μm.

mice, improve several morphological and behavioral symptoms.[15] As per these data, the development of a putative neuroprotective therapy based on BDNF could be a successful treatment strategy for the disease.[20,21]

Several methods have been tested to deliver neurotrophic factors in the central nervous system. Exogenous supply has been examined using viral infection and lipotransfection to promote the expression of protective molecules. However, this proved to be too invasive for the endogenous cells and not sufficiently diffusible in the striatum.[21,25,28] Grafting of engineered cells for the release of protective molecules has also been proposed as one of the most promising therapeutic tools for HD.[29] We have previously shown that BDNF-overexpressing cell lines prevent the death of striatal projecting neurons in a rat model of HD.[43] However, the methodology used in these studies cannot be used for chronic pathologies because of the likelihood of tumor generation by brain-implanted fibroblasts.[47] The use of stem cells in HD cell therapy has also emerged as a powerful tool for the release of striatal neuron protecting neurotrophic factors.[35,48,49] Owing to their teratogenic potential, embryonic stem cells are, so far, not useful for clinical use. Neural cells may be a better candidate source for the overexpression of BDNF. However, as an excessive amount of this neurotrophin is deleterious,[42] its release must be controlled.[48,49] To resolve this important issue, here we used engineered astrocytes that release high levels of BDNF only in pathological situations. Our study findings showed successful cell integration without aberrant proliferation. pGFAP-BDNF-grafted astrocytes were restricted to a scar-like structure mainly formed by endogenous reactive astrocytes, and showed low levels of migration as expected.[50,51] These findings indicate that transplanted astrocytes engineered for BDNF-secreting may be safe for gene therapy of not only HD but also for other neurological disorders where neuronal death is BDNF dependent and astrogliosis is associated.

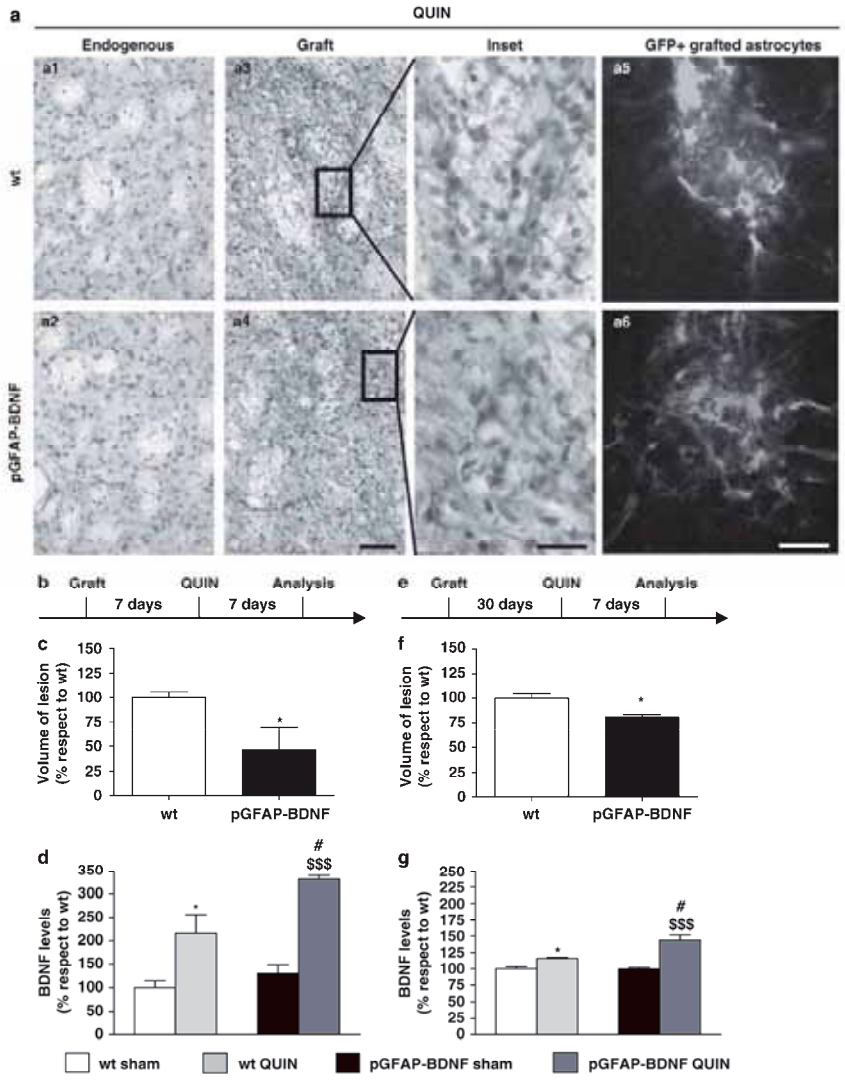

Figure 6 pGFAP-BDNF astrocytes grafted in SWISS nu-nu mice striata show higher levels of BDNF expression and neuroprotection than wt astrocytes when challenged by QUIN injection. (**a**, a1–4) Representative images showing immunohistochemical detection of Nissl and GFAP in QUIN-injected SWISS nu-nu mice (10–12 weeks old) grafted (a3–4) or not (a1–2; endogenous) with wt or pGFAP-BDNF astrocytes. (a5–6) Representative images were also fluorescently immunostained for GFP to further show the astrocyte identity and morphology. (**b**, **e**) Schematic representation of the experimental procedure to evaluate the temporal viability and functionality of the grafts. (**c**, **f**) Volumes of lesion or sham-injected striatum 7 (**c**) or 30 (**f**) days after grafting are also shown. (**d**, **g**) BDNF levels were measured by ELISA 1 day after QUIN or sham injections 7 days (**d**) or 30 days (**g**) after grafting. Bars represent means ± s.e.m. ($n = 5$–7 per group). For statistical analysis Student's *t*-test was used when comparing two groups, and one-way ANOVA with Student's *t*-test as a *post hoc* was used when comparing more than two groups (in figures **c** and **f**, $^{*}P<0.05$, with respect to wt; in **d** and **g**, $^{\$\$\$}P<0.001$ with respect to pGFAP-BDNF sham, $^{\#}P<0.05$, with respect to wt QUIN and $^{*}P<0.05$, with respect to wt sham). Scale bars, 100 and 20 μm.

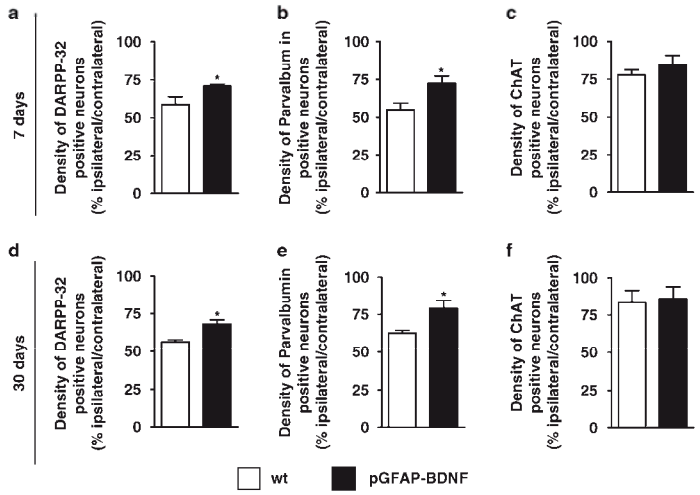

Figure 7 pGFAP-BDNF astrocytes grafted in Swiss nu-nu mice striatum protect several neuronal subpopulations more than wt astrocytes after excitotoxic intrastriatal QUIN injection. The histograms show immunostaining of DARPP-32 (**a**, **c**), parvalbumin (**b**, **e**) and ChAT (**c**, **f**)-positive neurons from mice grafted for 7 (**a–c**) or 30 (**d–f**) days with wt or pGFAP-BDNF astrocytes. Bars represent means ± s.e.m. ($n = 5–7$ per group). For statistical analysis Student's *t*-test was used (*$P < 0.05$, **$P < 0.01$ with respect to wt).

Our results also showed increased levels of migration when grafted astrocytes were subjected to QUIN administration, suggesting that they respond to endogenous stimuli. This is in agreement with previous works that showed a different behavior of grafted cells after excitotoxicity.[52] In addition, we show an increase in GFAP expression after QUIN administration. Thus, QUIN-induced excitotoxicity not only increases astrocyte mobility at the cellular level but also increases the expression of GFAP, indicating activation of these glial cells. We were able to take advantage of this reaction because in our construct BDNF production is controlled by the GFAP promoter. Importantly, HD patients show high levels of astrogliosis[3] and this pathological hallmark also shows a progressive increase in all tested animal models of HD in correlation with the degenerative process.[31–33,53] Interestingly, our study results show that pGFAP-BDNF transgenic astrocytes release high levels of this neurotrophin only on stimulation. We did not find differences in BDNF release between pGFAP-BDNF astrocytes and wt astrocytes in basal conditions. Moreover, although we show a similar pattern of BDNF production and release by wt astrocytes after inflammation-like conditions, to that previously described,[34,54] however, this inflammation-like stimulation resulted in a higher release of BDNF from pGFAP-BDNF astrocytes than from wt astrocytes. Similar to the *in vitro* situation studies, in our *in vivo* experiments, BDNF was only overexpressed when the striatum was subject to an excitotoxic insult. Altogether, these results indicate that GFAP-controlled BDNF release may constitute a self-regulated neuroprotective strategy for HD allowing BDNF release in a severity-dependent manner during disease progression. Such conditional regulation is highly desirable to prevent the undesired effects of uncontrolled BDNF release. In line with this hypothesis, similar strategies to release NGF have been proposed for other neurodegenerative diseases.[24]

Our long-term experiments showed that astrocytes grafted 30 days before QUIN injury can be induced to overexpress BDNF, which results in neuronal protection and behavioral improvements. The viability and functionality overtime of a graft is a critical issue that merits research efforts.[25] We have achieved this objective from a morphological, biochemical and behavioral point of view. pGFAP-BDNF grafted astrocytes remained functional for neuroprotection against QUIN administration even 1 month after grafting. Furthermore, this protection was sustained for, at least, four additional weeks after QUIN administration as we showed by different striatum-dependent behavioral tasks. Therefore, these genetically modified astrocytes could be a good tool for neuroprotection studies in genetic animal models of HD.

Although BDNF has a clear survival effect on striatal neurons,[6,43] we cannot rule out that the upregulated BDNF levels produced by pGFAP-BDNF-grafted astrocytes induced synergistic effects with other released molecules during inflammation and thereby exert more neuroprotection.[34] However, it is noteworthy that BDNF also increases neurogenesis in QUIN-lesioned striatum,[55] and the induction of neostriatal neurogenesis produces a functional improvement in R6/2 mice.[56] Thus, all these results together may suggest that the combined neuroprotective and cell replacement effects induced by BDNF could help to slow disease progression.

In conclusion, our data provide a putative therapeutic approach for HD through grafting stable and functional astrocytes with conditional upregulated BDNF

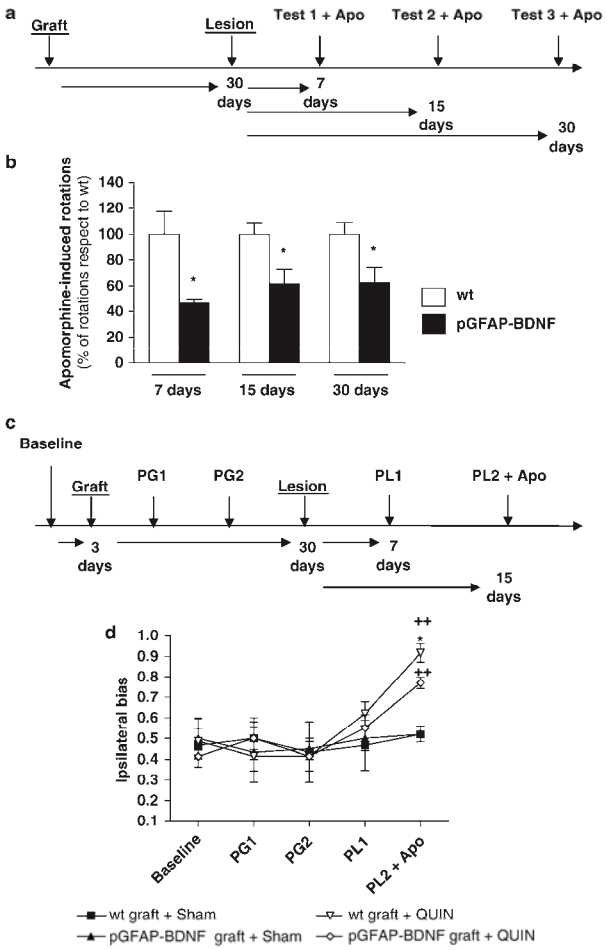

Figure 8 Swiss nu-nu mice grafted with pGFAP-BDNF astrocytes show better performances in striatum-dependent tasks than animals grafted with wt astrocytes after excitotoxic QUIN injection. (**a**, **c**) Schematic representation of the protocols used for testing rotation (**a**) and corridor task (**c**) behavior of wt and pGFAP-BDNF-grafted mice. (**b**) Apomorphine-induced rotation after QUIN injection. (**d**) Ipsilateral bias was determined by the corridor task under the conditions indicated in the figure. (**d**) In the corridor task, Swiss nu-nu mice grafted with wt or pGFAP-BDNF astrocytes were divided into the injected with vehicle (wt graft+sham and pGFAP-BDNF graft+sham) or QUIN (wt graft+QUIN and pGFAP-BDNF graft+QUIN) obtaining a total of four groups. Apo, apomorphine; PG1, post-graft 1; PG2, post-graft 2; PL1, post-lesion 1; PL2+Apo, post-lesion 2+apomorphine. Bars represent means ± s.e.m. ($n=7$ per group). For statistical analysis the two-way repeated-measures ANOVA with Student's *t*-test as a *post hoc* was used in the rotation test and one-way ANOVA with Student's *t*-test as a *post hoc* was used for each condition in the corridor task (in figure **b**, $^{*}P<0.05$ with respect to wt. In figure **d**, $^{*}P<0.05$ is the significance between wt graft+QUIN and pGFAP-BDNF graft+QUIN, $^{++}P<0.01$ is the significance between wt graft+QUIN and pGFAP-BDNF graft+QUIN with respect to their respective control sham groups).

expression. This therapeutic strategy not only protects striatal neurons against QUIN injury, but also produces behavioral improvements. We suggest that this approach should be considered for future treatment of HD patients.

Materials and methods

Generation of pGFAP-BDNF/c-myc transgenic mice

The rat GFAP 5′ flanking sequence spans bp −2570 to +27 relative to the transcriptional start site. This promoter is

fused to the last 18 bp of exon 2 of the rabbit β-globin gene followed by intron 2 and the first 51 bp of exon 3. The BDNF c-myc cDNA was cloned into the third exon of the rabbit β-globin gene, with approximately 50 bp of this exon at its 3′ end. The cDNA is followed by the SV40 polyadenylation sequence. The vector backbone (pSK+) was removed by *Xho*I digestion and a 5 kb fragment was injected into the male pronucleus to generate transgenic mice. A DNA segment encoding 10 amino acids of the c-myc protein was fused to the complete mouse BDNF protein sequence[57] to serve as a tag facilitating detection of the BDNF transgene. Both, RNA and protein transgene products are readily distinguishable from endogenous BDNF RNA and protein. This construct has been previously used[58] in the preparation of an adenovirus vector. The BDNF produced from this vector was clearly bioactive, supporting neuronal survival *in vitro* as well as *in vivo*. Thus the c-myc tag did not interfere with BDNF activity. The GFAP promoter was obtained from J Henderson. BDNF c-myc cDNA was kindly provided by Y Barde.

Animal care

We used 6- to 8-week-old male inbred C57/BL6 wt and pGFAP-BDNF mice, expressing the BDNF transgene under control of the GFAP promoter. For transplantation experiments we used adult nude mice, Swiss nu-nu; 10- to 12-week old (Charles River Laboratories, Les Oncis, France). Animals were housed in mixed genotype groups. All experiments were blind-coded regarding genotype, and data were recorded for analysis by microchip mouse number. Animals were maintained on *ad libitum* food and water diet in a colony room kept at a constant temperature (20–22 °C) and 40–60% relative humidity on a 12 h light/dark cycle. All procedures were according to European Community guidelines for the care and use of laboratory animals (86/609//EEC), and were approved by the animal care committee of the University of Barcelona, and by the regional autonomous government (*Generalitat de Catalunya*). For genotyping, DNA was obtained from tail biopsy and processed for PCR. The primers used for DNA amplifications were: 5′ globin (intron included in construct): 5′-GCTTGGAT CCTGAGAACTTCAGG-3′ and BDNF 3′2 (in BDNF): 5′-CT GTCACACACGCTCAGCTC-3′. PCR reactions were according to Invitrogen (El Prat del Llobregat, Barcelona, Spain) TaqMan gene expression assays instructions. PCR fragments were resolved in agarose gels.

Characterization of the pGFAP-BDNF transgene expression

Total RNA from lung, heart and several brain regions (cortex, hippocampus and striatum) from 6- to 8-week-old mice ($n=4$) was extracted using the Total RNA Isolation Nucleospin RNA II Kit (Macherey-Nagel, Düren, Germany). Total RNA (500 ng) was used to synthesize cDNA with random primers from the StrataScript First Strand cDNA Synthesis System (Stratagene, La Jolla, CA, USA). The cDNA synthesis was performed at 42 °C for 60 min in a final volume of 20 µl according to manufacturer's instructions. The cDNA was then analyzed by PCR, using the above-described primers, and PCR fragments were resolved in agarose gels.

Astrocyte cultures

Primary astrocyte cultures were obtained from P1 to P3 wt and pGFAP-BDNF mouse pups by cortical dissections, and removal of the meninges. Extracted tissue was dissociated and placed in 25 cm^2 flasks in a MEM 1 × conditioned media NM-15 (20% fetal bovine serum; Gibco-BRL, Renfrewshire, Scotland, UK; D-Glucose 90 mM; Buchs, Switzerland) with L-glutamine and Earle's salts (Gibco-BRL) and placed in an incubator at 37 °C with 5% CO_2. A tail biopsy was obtained from each pup for genotyping. After two passages cultures were purified by agitating in a shaker during 10 min at 400 r.p.m. Medium with undesired floating cells was replaced and flasks were placed in an incubator for 2 h at 37 °C. Next, flasks were agitated again for 16–18 h at 250 r.p.m. Finally, medium with floating cells was replaced with new medium.

BDNF release from astrocytes

At 24 h after purification, astrocyte cultures were trypsinized, seeded at 1×10^6 cells per P100 and allowed to reach 100% confluence. Next, cells were deprived of serum during a 3-day period following, and the medium was collected and stored at −80 °C. Astrocytes were then stimulated during 72 h in serum-free media containing 10 ng ml^{-1} of TNF-α (Sigma, St Louis, MO, USA) or only vehicle. Medium was collected and stored at −80 °C, until tested for induction of BDNF synthesis and release. Cell homogenates to evaluate intracellular BDNF levels were produced in PBS, 1% Nonidet P40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM PMSF, 10 µg ml^{-1} aprotinin, 1 µg ml^{-1} leupeptin, 2 µg ml^{-1} sodium orthovanadate lysis buffer. To reduce biological variance, we performed three to five independent experiments and each sample was measured in triplicates in ELISA assay.

Astrocytes transduction

Astrocyte cultures were transduced using retroviral vectors carrying the construct peGFP-CMVluc.[59] Briefly, cells in the proliferation stage, before astrocyte purification, were infected during 8 h in 3 consecutive days using viral supernatants from 293GPG packaging cells diluted 1:3 in fresh normal medium. Polybrene 8 µg ml^{-1} was added to enhance viral internalization. After the third day of infection, cells were passed normally and were used for cell transplantation, as described for nontransduced cells.

Striatal transplantations

After purification, astrocytes were grown to 100% confluence and then used for transplantation in adult (10- to 12-week-old) Swiss nu-nu mice (*n*: 5–7 per genotype). Wt and pGFAP-BDNF astrocytes were detached by trypsinization, collected by addition of 10 ml cold NM-15 medium to each flask, centrifuged at 1000 *g* for 5 min and the pellet was then resuspended in 1 ml of serum-free NM-15 medium. Finally, the cell suspension was centrifuged at 1000 *g* for 5 min and the pellet was diluted to a concentration of 4×10^5 cells per µl, the astrocyte concentration used for transplantation. In the last three steps, NM-15 medium was supplemented with 33 mM DNAase I (Sigma). To test viability of astrocytes, we stained cells with Trypan blue (Sigma) and counted alive cells before (~100% of cell viability) and after

transplantation (~95% of cell viability). Following anesthesia with pentobarbital (40 mg kg^{-1}), we grafted cells at four sites of the striatum of Swiss nu-nu mice at the following coordinates (millimeters); anteroposterior (AP), +0.8 and +0.33; lateral (L), +2.0 and +2.55 from bregma, and dorsoventral (DV), −2.25 and −2.75 from dura. Cells were injected over 2 min leaving the cannula in place for additional 5 min, and then slowly removing the cannula. The animals were monitored for 2 h after administration and then returned to the housing facility for 7 or 30 days. After these periods, animals from experimental groups were subjected to intrastriatal administration of QUIN or vehicle.

Striatal lesions

Anesthetized wt, pGFAP-BDNF and grafted SWISS nu-nu mice (*n*: 5–7 per genotype) were stereotaxically injected into the striatum with either a 0.5 µl of QUIN (15 nmol; Sigma) or vehicle at following coordinates (in millimeters): AP, +0.6, L, +2.0 from bregma and DV, −2.7 from dura. QUIN was injected more than 1 min after which the cannula was left in the place for a further 5 min period, and then slowly removed. The animals were monitored for 2 h after injection and then returned to the housing facility for 1, 3 or 7 days (wt and pGFAP-BDNF animals) and for 1, 7, 15 or 30 days for grafted Swiss nu-nu mice.

Enzyme-linked immunosorbent assay

Brain-derived neurotrophic factor content in different brain regions, cell homogenates and their corresponding culture media was determined using E_{max} Immunoassay System Kit (Promega, Madison, WI, USA), according to the manufacturer's instructions. Brains regions of deeply anesthetized mice (*n*: 5–7) were dissected, placed in ice and rapidly frozen in dry ice. Samples were then sonicated in lysis buffer (PBS, 1% Nonidet P40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM PMSF, 10 µg ml^{-1} aprotinin, 1 µg ml^{-1} leupeptin, 2 µg ml^{-1} sodium orthovanadate) and centrifuged (25 min × 13 200 r.p.m.). Supernatants were collected and the protein contents were measured by Detergent Compatible Protein Assay (Bio-Rad, Hercules, CA, USA). Total protein was analyzed by using 150 µg samples, for each point, diluted 1:3 in blocking or sample buffer. Duplicate serial dilutions of recombinant BDNF (0–500 pg ml^{-1}) were used to generate a standard curve. Each sample was analyzed three times.

Western blot analysis

Mice (5- to 6-week old; *n*: 5–7 per genotype) were deeply anesthetized in a CO_2 chamber, the brains quickly removed, brain regions dissected, frozen in dry ice and stored at −80 °C until use. Briefly, tissue was sonicated in 250 µl of lysis buffer (PBS, 1% Nonidet P40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM PMSF, 10 µg ml^{-1} aprotinin, 1 µg ml^{-1} leupeptin, 2 µg ml^{-1} sodium orthovanadate) and centrifuged at 12 000 r.p.m. for 20 min. Proteins (15 µg) from different brain regions were analyzed by 7.5% SDS–polyacrylamide gel electrophoresis and transferred to Immobilon-P membranes (Millipore, Bredford, MA, USA). Gel blots were blocked in TBS-T (150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 0.05% Tween 20) with 5% non-fat dry milk and 5% bovine serum albumin. Immunoblots were probed with anti-DARPP-32 1:10 000 (Chemicon, Temecula, USA) or Anti-GFAP 1:1000 (Dako A/S, Glostrup, Germany). All blots were incubated, with the primary antibody, overnight at 4 °C in PBS 0.02% sodium azide buffer with shaking. After several washes in TBS-T, blots were incubated with IgG HRP-conjugated anti-mouse or anti-rabbit antibodies (Promega) and developed using the ECL western blotting analysis system (Santa Cruz Biotechnology, Santa Cruz, CA, USA). A monoclonal anti-β-tubulin antibody (Sigma) was used as a loading control.

Immunohistochemistry

Animals were deeply anesthetized with pentobarbital (60 mg kg^{-1}) 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 by shaking at room temperature 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 by shaking at 4 °C with the corresponding primary antibodies in PBS with 0.02% sodium azide 1:200 buffer: anti-GFAP (Dako A/S), anti-GFP 1:2500 (Upstate Biotechnology, NY, USA) and/or anti-BDNF 1:1000 (Alomone, Jerusalem, Israel). After primary antibody incubation, slices were washed three times and then incubated 2 h shaking at room temperature with subtype-specific fluorescent secondary antibodies: Cy3 goat anti-rabbit (1:100) and Cy2 goat anti-mouse (1:100) (both from Jackson ImmunoResearch, West Grove, PA, USA). No signal was detected in controls incubated in the absence of the primary antibody.

For diaminobenzidine immunohistochemistry experiments, endogenous peroxidases were blocked for 30–45 min in PBS containing 10% methanol and 3% H_2O_2. Then, nonspecific protein interactions were blocked with normal serum or bovine serum albumin. Tissue was incubated overnight at 4 °C with the following primary antibodies: anti-NeuN (1:1000; Chemicon) anti-DARPP32 (1:10 000; Chemicon), anti-parvalbumin (1:1250; Sigma), anti-ChAT (1:500; Upstate) or anti-GFAP (1:1000; Sigma). Sections were washed three times in PBS and incubated with a biotinylated secondary antibody (1:200; Pierce) at room temperature for 2 h. The immunohistochemical reaction was developed using the ABC kit (Pierce) and visualized with diaminobenzidine. No signal was detected in controls in which the primary antibodies have been omitted. Cresyl violet staining was performed as described previously.[9]

Stereology

Volume estimates for striatum, hippocampus and cortex were performed as previously described.[5,46,59] Unbiased blind counting relative to genotype and condition was performed using the Computer Assisted Stereology Toolbox (CAST) software (Olympus Danmark A/S, Ballerup, Denmark). To determine the neuronal subpopulations densities (neurons per mm^3) in the lesion

area and the contralateral striatum (1.05–0.15 mm from bregma), we used the dissector counting procedure in coronal sections spaced 240 or 120 μm apart.

Adjacent version of the corridor task

Behavioral effects were analyzed using a previously described apparatus sensitive to striatal lesions adapted for mice[37] consisting of a rectangular 50 cm long × 5 cm wide × 15 cm deep black metacrylate box. Plastic dishes, 1 cm diameter × 0.5 cm deep, containing one sugar pellet (20 mg; Bioserve, Frenchtown, NJ, USA), were placed along the corridor floor touching either one or the other side walls. Ten plastic dishes were arranged in five pairs at 5 cm intervals. Mice were food-deprived to ~85% of their normal body weight. They were habituated to the corridor, and learned to search for, and retrieve pellets, over a 15 min period on the day before baseline testing. On testing days, trials began by releasing the mouse at one end of the baited corridor and allowing it to explore. A 'retrieval' involved a nose poke into a lid, regardless of whether a pellets was taken from it, that defined the side according to the mouse's body axis. Trials were completed after 10 retrievals, or a 5 min period.

Mice performance was tested in five conditions: at baseline, post-graft 1, post-graft 2, and post-lesion 1 and post-lesion+apomorphine (baseline was performed 2 days before graft, post-graft 1 and 2 were performed on days 7 and 29 post-astrocytes grafting and post-lesion 1, and finally, post-lesion+apomorphine were performed at 7 and 15 days post-QUIN administration). On post-lesion+apomorphine day, 5 min before testing, the animals were administered 2 mg kg^{-1} apomorphine hydrobromide (Sigma) buffered in 0.9% saline.

Rotation test

To further study the behavioral effects of intrastriatal grafted astrocytes, we used a previously described open field apparatus.[35] Behavioral data were recorded using a video capture system (Videotrack 512; Electronique Iyonnais, Lyon, France). After administration of 2 mg kg^{-1} apomorphine hydrobromide (Sigma) buffered in 0.9% saline, animals were placed into the register area for a 15 min period and their rotational behavior was recorded at 5 min intervals. We then compared the curves for each genotype.

Statistical analysis

All results were expressed as the mean ± s.e.m. for each genotype or group. For the statistical analysis of stereological, behavioral and biochemical experiments, the unpaired Student's *t*-test, one-way ANOVA, two-way ANOVA, two-way repeated-measures ANOVA and Fisher's exact test were used when appropriate and specified at the corresponding figure legend. $P < 0.05$ was considered significant.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Gene Therapy website (http://www.nature.com/gt)

IV Discusión

En esta tesis hemos creído de vital importancia centrarnos en el análisis de los procesos alterados debido a la presencia de la mhtt desde un abordaje precoz. Con esto se pretendía que fuera posible detectar los primeros cambios conductuales y moleculares para el diseño de un enfoque terapéutico más efectivo. Esta presuposición parte de la idea de que la mhtt está implicada en múltiples procesos (Aronin y col., 1999; Li y Li, 2004). De ahí que, a nuestro entender, uno de los retos más importantes en el estudio de los procesos fisiopatológicos de la enfermedad de Huntington es detectar los primeros fenómenos alterados para saber cómo la mhtt inicia la enfermedad (Figura 6, MacDonald y col., 2003). Así se intentaría responder la siguiente cuestión: ¿Cuáles serían los procesos iniciales que desencadenan una cascada que irá en aumento y que afectará a otros sistemas indirectamente causando al final, el fenotipo típico y completo de la enfermedad de Huntington? Muchos estudios interesantes y elegantes no se centran en los procesos iniciales que desencadenan la enfermedad. Algunos ejemplos son los realizados sobre la disfunción mitocondrial, o la actividad de caspasas y procesos apoptóticos (Guidetti y col., 2001; Wellington y col., 2000), fenómenos tardíos en el desarrollo de la enfermedad. Estos estudios se desvían de lo que debería ser el objetivo principal: describir y estudiar los fenómenos iniciales de le enfermedad. Es por eso que creemos, en parte, que muchos de los tratamientos son tan sólo paliativos o casi inefectivos, porqué no atacan los procesos iniciales de la enfermedad.

En esta tesis, creemos demostrar que nuestros resultados van en la siguiente línea que proponemos: A saber, que con la expresión de la mhtt, progresivamente se va acumulando su disfunción suficientemente como para que, finalmente, empiece a realizar sus primeros efectos nocivos detectables a edades denominadas pre-sintomáticas de la enfermedad. Además, estos fenómenos son acumulativos, es decir, que la dinámica del progreso de la enfermedad también se debe a la suma progresiva de

alteraciones debidas a la mhtt. En esa línea, creemos que entre los primeros efectos provocados por la mhtt son los de modular de forma aberrante los procesos sinápticos, en concreto, de las sinapsis excitadoras (Trabajos 1-3 y 5-6). Estas alteraciones de las sinapsis excitadoras se traducirían en una actividad anómala de los receptores ionotrópicos de glutamato (principalmente, los NMDA) y de las vías de señalización que se activan por ellos (Trabajos 1-3 y 5). A nivel funcional, los efectos de tales alteraciones se traducirían en dos consecuencias principales muy precoces. Nos referimos a una susceptibilidad alterada a procesos excitotóxicos (Trabajos 1-3 y 5) por una parte, y, por otra, a un mecanismo de deterioro progresivo de la plasticidad neuronal y de la transmisión sináptica traduciéndose todo esto, en un declive cognitivo (Trabajos 3 y 6).

Las alteraciones moleculares neuronales más primerizas y sutiles de carácter disfuncional que hemos encontrado a lo largo de la presente tesis son principalmente tres (Figura 12); Primero, una alteración en la vía de PKA aumentando su actividad de forma aberrante. Este aumento en su actividad resulta en una hiperactividad de múltiples de sus sustratos desequilibrando el sistema molecular intracelular neuronal (Trabajos 5 y 6). Segundo, encontramos que los receptores de glutamato ionotrópicos, principalmente los NMDA, son los que padecen más alteraciones funcionales a nivel de sinapsis excitadora con lo que esto supone para la funcionalidad sináptico-plástica (Trabajos 1-3 y 5). Sin embargo, parece ser que, tanto las proteínas de andamiaje (tipo PSD-95) de los propios receptores como las vías de señalización por debajo de estos (principalmente las vías CaMKII, Calcineurina y STEP) cobran una relevancia muy elevada y podrían explicar los efectos de la disfunción de los mismos receptores (Trabajos 1-2 y 5). Finalmente y la más importante, creemos demostrar que la alteración en la función y la expresión de BDNF mediada por la mhtt es el proceso mediador de la

fisiopatología de la enfermedad de Huntington más importante de todos los analizados debido a su implicación en múltiples de las disfunciones estudiadas (Trabajos 1-3 y 7).

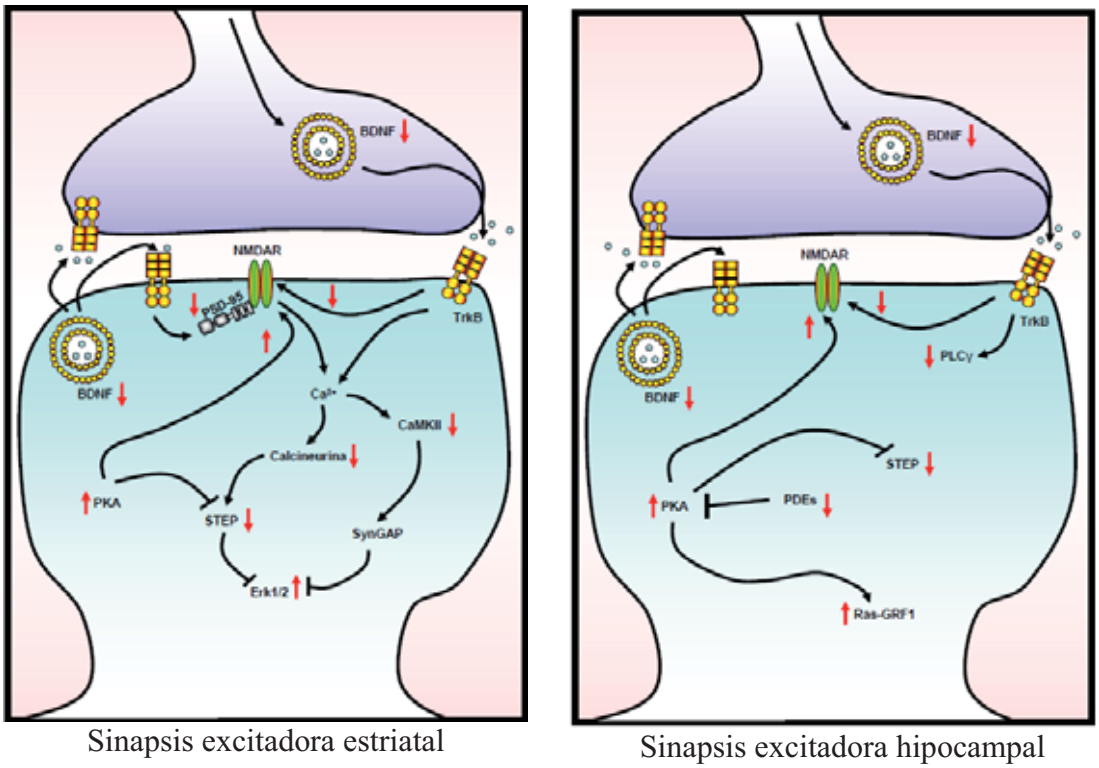

Figura 12. Mecanismos patológicos en sinapsis excitadoras estriatales e hipocampales en la enfermedad de Huntington. En la presente tesis proponemos que la enfermedad de Huntington puede ser iniciada, de forma muy temprana, por un defecto sináptico que podría ser producido por varios fenómenos complementarios. Desde las primeras edades, la mhtt ya se expresa en sinapsis excitadora (ver texto). En el esquema vemos las alteraciones estriatales e hipocampales descritas en la presente tesis que, como se puede apreciar, comparten muchos puntos. En el núcleo estriado, la mhtt puede ejercer efectos nocivos mediante su interacción aberrante con PSD-95 alterando así su anclaje con los receptores NMDA y, por consiguiente, la entrada de calcio y la señalización por debajo como la vía de la calcineurina, la de STEP y la de la cinasa CaMKII. En el hipocampo también los NMDAR están afectados, en gran medida, por la regulación alterada de la vía BDNF-TrkB-PLCγ. Sumándose a ese desequilibrio entre fosfatasas y cinasas, también encontramos una hiperactividad aberrante de PKA en tales regiones cerebrales debido a una alteración de la expresión de diversas fosfodiesterasas reguladoras de la cinasa. Finalmente, los niveles y funciones alterados del BDNF en las sinapsis excitadoras en la enfermedad de Huntington son capaces de regular múltiples de los procesos descritos.

Ya que BDNF parece cumplir con la mayoría de requisitos necesarios para entender la enfermedad de Huntington como una sinaptopatía (alteración en expresión y función en múltiples regiones cerebrales, de carácter tempro y mediando efectos en sinapsis), seguidamente sugerimos la neurotrofina como elemento terapéutico altamente potencial. Por eso mismo generamos unos ratones que sobre-expresan BDNF de forma

condicional (ratones pGFAP-BDNF). Así estudiamos su valor terapéutico localmente en la región más afectada de la enfermedad de Huntington, el núcleo estriado (Trabajos 4 y 7). El uso de estos ratones nos supuso corroborar los efectos beneficiosos de administrar BDNF en modelos agudos y transgénicos de la enfermedad de Huntington (Trabajos 4 y 7). Sin embargo, aportamos un método condicional (BDNF sólo se administraba a medida que la enfermedad se manifestaba debido al control del transgen de la neurotrofina bajo el promotor de GFAP) regulado endógenamente (la propia astrogliosis regulaba la producción y liberación), sin necesidad de atravesar la barrera hematoencefálica (ya que BDNF no atraviesa dicha barrera) y salvando muchos de los efectos negativos, colaterales y éticos de otros métodos de administración (como la toxicidad por infección, transducción y lipotransfección, tumorigénesis de líneas celulares y probabilidad de teratogénesis en el caso de células madre) (Trabajos 4 y 7). Seguidamente discutiremos con más detalle cada uno de los puntos expuestos.

1. La enfermedad de Huntington se inicia como una sinaptopatía que termina en una neurodegeneración general donde múltiples sistemas se ven afectados

Como ya mencionamos más arriba, creemos que la enfermedad de Huntington podría iniciarse, como una sinaptopatía clara y de inicio temprana. Esta es progresiva y sucede en todos los modelos animales, en diferentes regiones cerebrales y a edades muy tempranas.

A *grosso modo*, el supuesto teórico que proponemos es integrador y no excluye ninguno de los procesos fisiopatológicos comúnmente aceptados en la enfermedad de Huntington. Así pues, debido a que la mhtt se expresa desde el inicio de la vida del sujeto, esta puede actuar de muchas maneras como por ejemplo, alterando la tras-

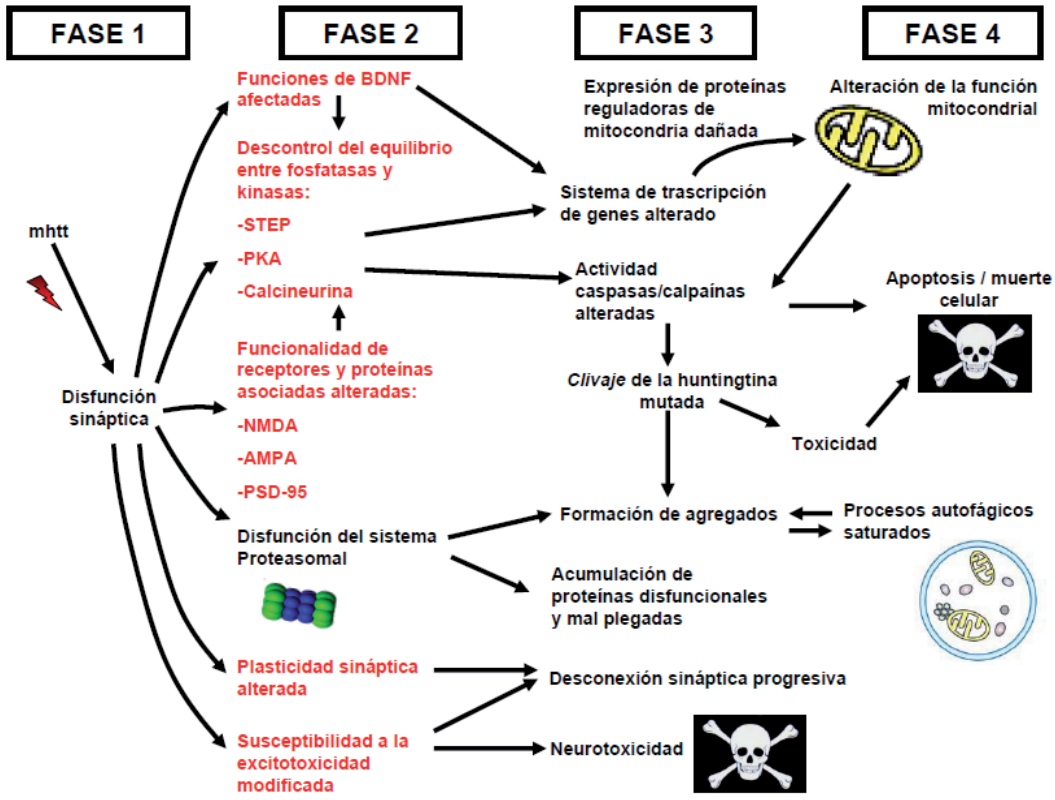

Figura 13. La sinaptopatía en la enfermedad de Huntington puede ser el elemento iniciador de la mayoría de los procesos fisiopatológicos asociados a la enfermedad. Tomando la sinaptopatía como elemento iniciador de la fisiopatología de la enfermedad de Huntington, a nivel molecular, la progresión de la enfermedad de Huntington podría dividirse en 4 posibles fases secuenciales. En una primera fase, encontramos la disfunción sináptica como el fenómeno más susceptible ante la presencia de la mhtt. En una segunda fase encontramos cómo esta disfunción sináptica se acompaña de, por ejemplo, una alteración en los procesos de susceptibilidad a la excitotoxicidad y una disminución de la plasticidad sináptica tal y como se detalla en los resultados de la presente tesis. Progresivamente, los fenómenos fisiopatológicos se van sumando e interaccionando entre sí de forma acumulativa induciendo el fenotipo completo y progresivo de la enfermedad. En la presente tesis es importante resaltar que las alteraciones que se han estudiado (en rojo) se desarrollan durante las primeras fases de la enfermedad.

cripción génica, el tráfico celular, etcétera. Puede que la situación no sea patológica al principio debido a su sutilidad, pero llega un momento en que su función tóxica o disfuncional llega a realizar sus primeros efectos. Estos se producirán primero en los sistemas más susceptibles a la presencia de la mhtt, es decir, la sinapsis. Esta podría ser una **primera fase** patológica en el progreso de la enfermedad (figura 13).

En **un posible segundo nivel o fase** (figura 13), las alteraciones en sinapsis se traducirían en varios efectos. Por un lado, tendríamos una disfunción en la producción, liberación y función del propio BDNF (Lynch y col., 2007; del Toro y col., 2006;

Gauthier y col., 2004; Zuccato y col., 2001) incluso a edades muy tempranas (Trabajo 3). Por otro, una disfunción de los receptores NMDA, AMPA, (Zeron y col., 2002; Xifró y col., 2008; Pineda y col., 2009) de sus proteínas de anclaje como PSD-95 (Sun y col., 2001; Trabajo 1) y de cascadas de señalización por debajo como la iniciada por Calcineurina, STEP o CaMKII (Trabajos 1-2 y 5-6). Estas alteraciones conjuntas llevarían a tal disfunción sináptica que se iniciarían procesos de susceptibilidad alterada a la excitotoxicidad. En este nivel, la disfunción sináptica también podría regular las alteraciones en el sistema UPS (del inglés *Ubitquitin-Proteasome-System*) ya que este es altamente regulable a nivel de actividad sináptica (Bingol y col., 2010; Rinetti y col., 2010).

En un posible tercer nivel o fase (figura 13), la alteración en cinasas y fosfatasas se transformaría en una actividad disfuncional en la trascripción de genes clara tal y como se ha descrito previamente en la enfermedad de Huntington (Cha, 2000; Sugars y Rubinsztein, 2003). Esta alteración en la trascripción induciría déficits en la producción de proteínas de supervivencia, neuroprotectoras, funcionales, o reguladoras de la actividad mitocondrial por poner algunos ejemplos. Paralelamente, se produciría una alteración del UPS que facilitaría la formación de agregados y acumulación de proteínas mal plegadas (Valera y col., 2007; Finkbeiner y Mitra, 2008).

En un cuarto nivel o fase (figura 13), la disfunción mitocondrial induciría a activación de caspasas/calpaínas, sistema apoyado por actividades aberrantes de proteínas como la Calcineurina (Han y col., 2008; Almeida y col., 2004). La actividad de caspasas y calpaínas facilitarían el recorte de la mhtt total produciendo los extremos N-terminal (tóxicos) (Gafni y Ellerby, 2002). Estos se acumularían formando agregados cada vez más grandes y saturando otros sistemas como el de autofagia (Ravikumar y col., 2004) el cuál ya no sería capaz de degradar las acumulaciones de fragmentos

tóxicos de la mhtt. Conjuntamente, todos estos procesos se transforman rápidamente hacia una desconexión sináptica (tal y como proponen varios autores para la desconnexión cortico-estriatal en la enfermedad de Huntington (Cepeda y col., 2007) haciendo la neurona cada vez más disfuncional hasta llegar a una falta de función total y, consecuentemente, llevándola a la muerte (especialmente presente en el núcleo estriado).

Nuestros resultados, junto con una amplia bibliografía previa, nos aportan múltiples argumentos a favor de tales presupuestos. A saber:

1.-Las alteraciones, primero de fosforilaciones y actividades de múltiples proteínas y receptores implicados en funcionalidad y plasticidad sináptica y, posteriormente en el tiempo, de sus niveles de expresión, ocurren desde edades muy tempranas (Trabajos 1-3 y 5-6).

2.-Las alteraciones electrofisiológicas medidoras de la funcionalidad sináptica están entre los fenómenos más tempranos en todos los modelos de la HD (principalmente, en sinapsis excitatorias) (Trabajo 3, Cummings y col., 2006; 2007; Milnerwood y col., 2006; Lynch y col., 2007; Cepeda y col., 2007).

3.-En modelos animales se detectan fragmentos de la N-terminal de la mhtt en la densidad postsináptica Petrasch-Parwez y col., 2007; Gutekunst y col., 1999; J Neurosci) a edades tan tempranas como, por ejemplo, a 4 semanas de edad en ratones R6/2 (Suopanki y col., 2006).

4.-Entre las primeras alteraciones en aparecer a nivel sintomático son las cognitivas tanto en todos los modelos animales (Lione y col., 1999; Van Raamsdonk y col., 2005a; Trueman y col., 2007 y Trabajos 3 y 6) como en humanos (Lawrence y col., 1996; Lemiere y col., 2004; Montoya y col., 2008). Estas alteraciones están

ampliamente relacionadas con los procesos de plasticidad anteriormente mencionados (Trabajos 3 y 6).

5.-Las funciones de BDNF, crucial para la funcionalidad sináptica, plasticidad neuronal, memoria y aprendizaje, están alteradas en la enfermedad de Huntington y desde edades muy tempranas coincidiendo en alto grado con stas alteraciones plásticas y cognitivas (Trabajo 3, Zuccato y col., 2001; Ginés y col., 2003; Lynch y col., 2007).

6.-Existe un abanico de alteraciones de expresión más o menos tempranas de múltiples proteínas específicas de sinapsis excitatorias (Trabajos 1-3 y 5-6, Li y col., 2003; Smith y Brundin, 2005).

7.-Entre los múltiples procesos fisiopatológicos en la progresión de la enfermedad de Huntington, la disfunción sináptica puede explicar:

-La disfunción mitocondrial ya que la actividad sináptica excitatoria la puede regular vía receptores NMDA por ejemplo (Hardingham, 2009). En esta línea varios autores proponen estudiar las vías por encima de la función mitocondrial (Pandey y col., 2010).

-La alteración de trascripción de genes, dado que la actividad sináptica la regula ampliamente (Loebrich y Nedivi, 2009).

-La activación aberrante de caspasas y calpaínas y, consecuentemente, la muerte celular debido a que estas vías pueden ser activadas por los receptores NMDA o fosfatasas enriquecidas en sinapsis excitatorias como la calcineurina (Xifró y col., 2008; Pineda y col., 2009).

-La alteración en el sistema UPS, el cuál se está proponiendo actualmente en la enfermedad de Huntington que su mayor disfunción se localiza en sinapsis (Wang y col., 2008). De acuerdo con esta idea, estas alteraciones podrían ser debidas por ejemplo, a las alteraciones en la expresión y

función de BDNF ya que la neurotrofina está también implicada en la funcionalidad del UPS (Jia y col., 2008)

-La disfunción trófica *per se* ya que la actividad neuronal induce expresión de BDNF del tipo *early-gene* (Castrén y col., 1998; Hardingham y col., 2002) y/o su transporte hacia las sinapsis (Greenberg y Lu, 2009) e incluso su misma transcripción *in situ* en tales micro-estructuras (Tongiorgi, 2008).

-La progresión de los procesos excitotóxicos propios de la enfermedad (Hansson y col., 1999, 2001; Jarabek y col., 2004; Zeron y col., 2002; Xifró y col., 2008) ya que *per se* se deben principalmente a alteraciones sinápticas.

8.-La comunicación neurona-astroglía también está alterada en modelos animales de la enfermedad de Huntington y también desde edades tempranas o en modelos muy precoces (Shin y col., 2005; Chou y col., 2008; Faideau y col., 2010). Por lo tanto, hay que considerar un déficit del aporte de nutrientes desde la astroglía, alteraciones en la producción de colesterol e incluso alteraciones de recaptación de glutamato. Incluso la importancia de la participación de la astroglía en la funcionalidad sináptica podría estar comprometida.

9.-La disfunción sináptica se encuentra en todos los modelos animales y pacientes mientras que otros procesos no cumplen tal requisito. Algunos ejemplos son la alteración en la producción de colesterol que es muy distinta según el modelo animal o la aproximación experimental (Valenza y col., 2005; Trushina y col., 2006; Del Toro y col., 2010). Por otra parte, la formación de agregados en algunos modelos se forman en todas las regiones (como en humanos) y en otros modelos tan solo en el núcleo estriado (Van Raamsdonk y col., 2005b, 2007a, 2007b Wheeler y col., 2000; 2002).

2. La neurotrofina BDNF es el gran modulador de la progresión de la fisiopatología y sintomatología en la enfermedad de Huntington: Regulación de las alteraciones cognitivas y de plasticidad sináptica

Ha sido largamente descrito que la neurotrofina BDNF padece graves alteraciones en sus niveles de expresión y función a lo largo de la progresión de la enfermedad (Zuccato y Cattaneo, 2007; 2009; Alberch y col., 2004; Lynch y col., 2008). Pero, ¿cómo modula la mhtt las alteraciones de BDNF? Existen trabajos previos implicando directamente a la mhtt en el transporte axonal de BDNF (Gauthier y col., 2004) que puede ser agravado por polimorfismos genéticos en la misma neurotrofina (del Toro y col., 2006). Estas alteraciones en su transporte se han descrito en modelos celulares, lo que sugiere su inicio temprano en la fisiopatología y podría ayudar a explicar la disfunción sináptica. Ya se ha descrito *in vitro* (en líneas celulares estables) que los niveles de BDNF pueden ser dependientes de los niveles de la proteína mhtt (Zuccato y col., 2001) y por la longitud de la expansión de repeticiones (Canals y col., 2004). Finalmente, el proceso más claro sobre BDNF que produce la mhtt es una alteración en los niveles de su expresión a nivel trascripcional debido a una relación alterada con los complejos REST/NRSF desde edades también tempranas (Zuccato y col., 2001; 2003).

Otros trabajos habían sugerido ya la importancia de BDNF a nivel funcional en la fisiopatología de la enfermedad. Estos lo habían hecho directamente mediante el uso de animales transgénicos de la enfermedad de Huntington con niveles de BDNF selectivamente alterados a nivel genético (Canals y col., 2004; Pineda y col., 2005) así como, indirectamente, mediante el estudio de similitudes fenotípicas entre animales con los niveles de BDNF reducidos comparados con animales transgénicos propios de la

enfermedad de Huntington (Baquet y col., 2004; Strand y col., 2007; Diekmann y col., 2009). Nosotros, además, ampliamos estos trabajos previos y demostramos que los niveles de BDNF y su función también son claves en la regulación de las alteraciones en los procesos plásticos y sinápticos y en distintos tipos de aprendizaje y memoria en la enfermedad de Huntington (Trabajo 3). En este trabajo aportamos evidencias de cómo BDNF y la mhtt conjuntamente modulan la expresión de diversas subunidades de receptores de glutamato ionotrópicos hipocampales como las subunidades NR1, NR2A y GluR1 (Trabajo 3). Además, enseñamos cómo la neurotrofina podría regular las alteraciones en la expresión de la LTP en estos modelos animales mediante la disrupción de la vía molecular de la PLCγ activada por TrkB (Trabajo 3). De nuevo, hay que recalcar la precocidad en que estas alteraciones fueron observadas y la relevancia potencial terapéutica que eso supone para la intención de la presente tesis. Por otra parte, hay que tener en cuenta que algunos de los tipos de aprendizaje y memoria testados en el trabajo 3 son también dependientes del núcleo estriado. Así pues, las alteraciones cognitivas pueden ser explicados de forma acumulativa junto con las alteraciones en los niveles de expresión de proteínas sinápticas estriatales cómo CaMKII y PSD-95 (Trabajo 1).

Yendo a nivel más molecular, vemos en nuestros trabajos que la alteración de los niveles y función de BDNF en la enfermedad de Huntington es capaz de regular muchas vías moleculares de las sinapsis excitadoras y procesos excitotóxicos asociados (Trabajos 1-2 y 7). Concretamente, hemos demostrado que las alteraciones en los niveles y función de BDNF pueden regular la susceptibilidad de los procesos excitotóxicos (Trabajo 1), vías moleculares implicadas como la expresión y función de proteínas sinápticas como CaMKII y PSD-95 (Trabajo 1) y los niveles y la actividad de la vía de la fosfatasa Calcineurina (Trabajo 2). No existen trabajos previos donde se

analiza directamente la implicación de BDNF en la regulación de estas vías moleculares o en la expresión de sus componentes moleculares en la enfermedad de Huntington, lo que resalta la novedad y potencialidad de nuestros hallazgos. Finalmente, tomado todo en conjunto, nuestros trabajos demuestran que BDNF funciona como pivote en múltiples de los efectos perjudiciales iniciados por la mhtt. Además, estas alteraciones pueden ser detectadas de forma muy precoz en todos los modelos estudiados y en diferentes regiones cerebrales.

3. Propuesta de la neurotrofina BDNF como candidato terapéutico para el tratamiento de la enfermedad de Huntington: Administración regulada, segura, funcional y a largo plazo

Los efectos beneficiosos debidos a la administración de BDNF ya se han demostrado en la enfermedad de Huntington: Tanto a nivel endógeno (Gharami y col., 2008) como exógeno (Kells y col., 2008, Pérez-Navarro y col., 1999; 2000; Canals y col., 2004). La cuestión es cómo administrarlo ya que su uso como herramienta terapéutica está lleno de dificultades técnicas como veremos más adelante.

El BDNF es uno de los mayores candidatos en el tratamiento de la enfermedad de Huntington (Alberch y col., 2004; Zuccato y Cattaneo, 2007; 2009). De ese modo, administrar la neurotrofina en el momento adecuado, en las regiones cerebrales adecuadas, durante el tiempo suficiente y en la cantidad adecuada puede ser una de las propuestas terapéuticas más prometedoras y a la vez desafiantes. En esta línea, existen múltiples limitaciones respecto a su administración: El BDNF no atraviesa la barrera hematoencefálica (Poduslo y Curran, 1996; Thorne y Frey, 2001) y niveles demasiados

altos de la neurotrofina son perjudiciales (Kells y col., 2008; Croll y col., 1999). Debido a estas dificultades, existen otros métodos. Sin embargo, tampoco están

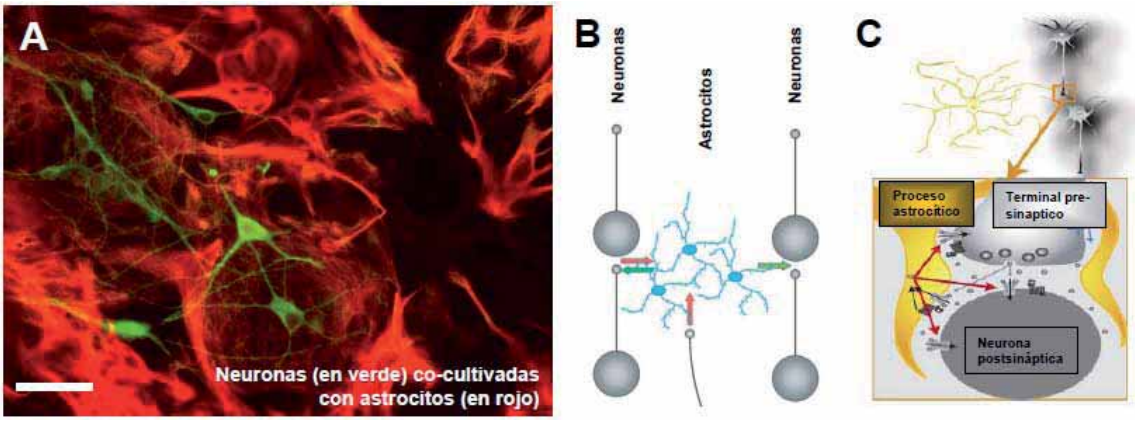

Figura 14. El uso de astrocitos diseñados para liberar más BDNF como una estrategia terapéutica potencial en la enfermedad de Huntington. De los resultados obtenidos en esta tesis creemos que el uso y trasplante de astrocitos que liberan específicamente más BDNF puede ser una buena estrategia terapéutica debido a múltiples razones. Son células de origen neural, realizan múltiples funciones de soporte a la función neuronal, se adaptan bien al tejido comparado con otros tipos o líneas celulares, sobreviven mucho tiempo y siguen siendo funcionales sin proliferar de forma aberrante. Además, dado que durante los últimos años se está proponiendo a la astroglía como gran moduladora de la sinapsis, su uso terapéutico cobra interés adicional en línea con la presente tesis. Los astrocitos son vitales para la funcionalidad neuronal (A), especialmente, de la función sináptica (B y C). (A: microfotografía propia; B y C, modelos de interacción neurona-glía-neurona.

exentos de importantes problemas metódicos y de seguridad para su uso terapéutico. Las transducciones virales y las lipotransfecciones son demasiado invasivas y se acompañan de demasiados efectos colaterales (Zuccato y Cattaneo, 2007; Lindavall y col., 2004; Dunnett y Rosser, 2004). Los transplantes de líneas celulares que lo sobre-expresen pueden causar tumores (Hoffman y col., 1993) mientras que el transplante de células madre que también lo liberen posee el potencial de causar teratomas.

En la presente tesis proponemos futuras propuestas para el tratamiento de la enfermedad ya que hemos sorteado a nivel experimental los importantes problemas planteados en el párrafo anterior (Trabajos 4 y 7). Además, esta producción de BDNF mejora la funcionalidad sináptica (Trabajo 3) recuperando niveles de distintos marcado-

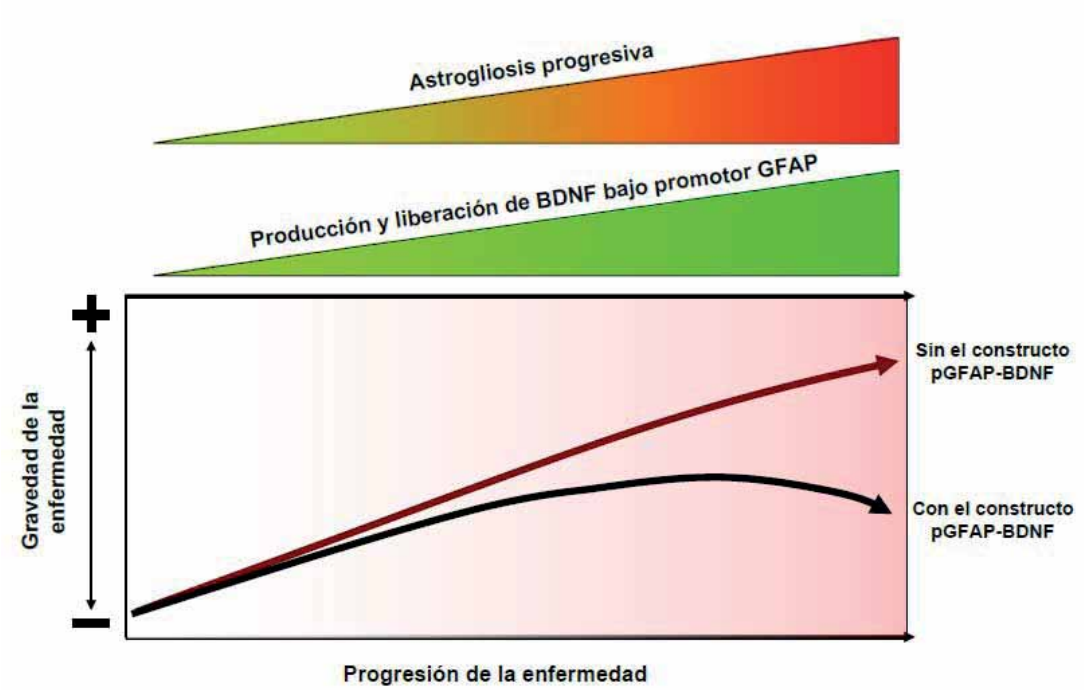

Figura 15. Modelo de auto-regulación de la expresión de BDNF según la progresión y severidad de la enfermedad. Dada la importancia de BDNF en la fisiopatología de la enfermedad de Huntington, un modelo terapéutico con potencial que proponemos en la presente tesis sería el de la administración de la neurotrofina de forma endógena y regulada de forma condicional. Usando el marcador astroglial GFAP como promotor del transgen de BDNF conseguimos que, a medida que avanza la enfermedad en modelos animales (astrogliosis progresiva), la neurotrofina se produce en mayor cantidad. Esta forma de producción y liberación de BDNF muestra todo su potencial terapéutico a nivel experimental cuando se administra de forma sistémica en todo el cerebro al cruzar el ratón transgénico pGFAP-BDNF con el ratón modelo de la enfermedad de Huntington llamado R6/2 (Trabajo 7).

res pre- y post-sinápticos cortico-estriatales como VGLUT1 y PSD-95 respectivamente. Teniendo en cuenta el papel de la astroglía en la funcionalidad sináptica (Haydon, 2001; Santello y Volterra, 2010), y de las alteraciones sinápticas que en esta tesis hemos descrito (Trabajos 1-3 y 5-6), el uso de astrocitos diseñados para liberar BDNF posee numerosos argumentos para su uso terapéutico en la enfermedad de Huntington (Figura 14). En nuestro modelo (Figura 15), vemos que una administración intracerebral, regulada endógenamente, en cantidad suficiente y a largo plazo es beneficiosa para el fenotipo huntingtoniano.

Finalmente, aunque de nuestros resultados se pueda concluir la posibilidad de una propuesta terapéutica potencial para el tratamiento de la enfermedad de Huntington, es evidente que aún faltan aspectos que mejorar. Aunque nostros y otros grupos

demostramos el importante papel que juega BDNF en la fisiopatología de la enfermedad, y que su uso terapéutico posee muchos puntos a favor, es importante recalcar que el tratamiento sólo con BDNF quizás no sería suficiente y debería ir acompañado. Esto es así desde que la neurotrofina no és la única alteración importante y temprana implicada en la función sináptica en el desarrollo de la enfermedad de Huntington. Por otra parte, el sistema que nosotros proponemos de regulación endógena y al alza de BDNF de forma condicional posee muchos puntos fuertes. Sin embargo, sería interesante estudiar sistemas similares pero con un inicio más inicial para comprobar si de esta manera las mejoras observadas en nuestros modelos podrían tener un inicio más temprano y más significativo. Finalmente, otro punto importante es que la terapia celular en la enfermedad de Huntington difícilmente deba basarse en el transplante de un solo tipo de célula. El trasplante de astrocitos diseñados para liberar BDNF podría ir acompañado de células precursoras neuronales con objetivos principalmente sustitutivos. Estos son los principales puntos que desde la presente tesis creemos que deberían plantearse para futuros estudios.

V Conclusiones

1. Las neuronas estriatales padecen las alteraciones en las vías de señalización excitadora principalmente vía receptores NMDA debido a una reorganización molecular en la Densidad Post-Sináptica (PSD) desde fases muy iniciales de la enfermedad. Los principales protagonistas de esta reorganización son las proteínas de andamiaje molecular sináptico llamadas MAGUKs y el BDNF.

2. La reorganización sináptica debida a la presencia de la mhtt también produce una señalización aberrante de la fosfatasa llamada Calcineurina dependiente de actividad de los receptores NMDA. Esta señalización además, depende de los niveles y función del BDNF.

3. Las alteraciones en la vía de señalización de la fosfatasa Calcineurina junto con un hiper-actividad aberrante de la proteína PKA inducen a una inhibición progresiva de la proteína fosfatasa enriquecida en el núcleo estriado llamada STEP. Esta inhibición se traduce en una respuesta progresivamente disminuida ante la estimulación de NMDA y correlaciona con la progresiva neurodegeneración específica de este núcleo.

4. En todos los modelos animales testados existe una alta correlación en la edad de aparición de los primeros síntomas cognitivos con las alteraciones de plasticidad sináptica tanto en el núcleo estriado como en el hipocampo. De entre los síntomas cognitivos descritos, la formación de memoria a largo plazo parece ser uno de los más susceptibles a la presencia de la mhtt.

Conclusiones

5. La activación crónica de PKA induce alteraciones específicas en la formación de memorias a largo plazo. Esta misma activación aberrante se encuentra en varios modelos animales de la enfermedad y en muestras *post-mortem* de pacientes.

6. El BDNF regula las cascadas de eventos fisiopatológicos en la enfermedad de Huntington involucrados en la edad de inicio y la severidad de los síntomas cognitivos y mnemónicos al modular la señalización de su receptor específico TrkB vía la PLCγ y alterando los niveles de diferentes subunidades de receptores NMDA y AMPA como NR1, NR2A y GluR1.

7. El uso de un promotor capaz de ser regulado endógenamente y de forma dependiente de la severidad de la enfermedad como GFAP para la producción de BDNF, induce a mejoras bioquímicas, histopatológicas y comportamentales en diferentes modelos de la enfermedad de Huntington.

8. Los astrocitos diseñados genéticamente podrían ser utilizados en la terapia celular para el tratamiento de la enfermedad de Huntington como fuente de factores neurotróficos. Este argumento se refuerza por el hecho de su alto índice de supervivencia, de su funcionalidad a largo plazo, de su origen neural y de su baja capacidad en producir proliferaciones aberrantes.

VI Bibliografía

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VII Annexos

Trabajo 5: ***"STriatal-Enriched protein tyrosine Phosphatase expression and activity in Huntington's disease – a STEP between calcineurin and ERK in R6/1 resistance to excitotoxicity".*** **(Manuscrito en revisión)**

Objetivo 2: Identificación de las vías de señalización afectadas específicamente por debajo de la funcionalidad de los receptores NMDA en procesos de excitotoxicidad y disfunción sináptica. Caracterización de tales efectos subyacentes en modelos animales transgénicos y agudos de la enfermedad de Huntington.

En el trabajo previo demostramos que en modelos *in vivo* exón-1 de la enfermedad de Huntington existe una bajada de niveles basales y actividad de la fosfatasa calcineurina. Además, demostramos que este fenómeno era uno de los principales mediando la resistencia a la excitotoxicidad y desconexión sináptica en dichos modelos. Ante estos resultados nos planteamos de qué forma la actividad a la baja de la fosfatasa calcineurina podría mediar esos efectos. Nos centramos en una fosfatasa llamada STEP (del inglés *STriatal-Enriched protein tyrosine Phosphatase*), que se encuentra enriquecida en el núcleo estriado y que puede ser regulada positivamente por calcineurina vía actividad de receptores NMDA y negativamente por PKA vía actividad los receptores dopaminérgicos D1. Además, STEP es una de las fosfatasas principales que median la actividad y función de ERK1/2 (substrato altamente conocido de supervivencia neuronal) en el estriado. En este trabajo analizamos los niveles y actividad de la fosfatasa en diferentes regiones afectadas en la enfermedad de Huntington y en diferentes modelos animales (tanto exón-1 como *Knock-in*). Adicionalmente manipulamos los niveles y actividad de STEP en animales *wild type* y modelos murinos de la enfermedad de Huntington y estudiamos sus efectos en la modulación de la susceptibilidad a la excitotoxicidad vía regulación de ERK1/2.

Neurobiology of Disease

STriatal-Enriched protein tyrosine Phosphatase expression and activity in Huntington's disease – a STEP in the resistance to excitotoxicity

Abbreviated title: STEP protein levels and activity in HD models

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Abstract

STriatal-Enriched protein tyrosine Phosphatase (STEP) is highly expressed in striatal medium-sized spiny neurons, the neuronal population most affected in Huntington's disease. Here, we examined STEP expression and phosphorylation, which regulates its activity, in N-terminal exon-1 and full-length mutant huntingtin mouse models. R6/1 mice displayed reduced STEP protein levels in the striatum and cortex whereas its phosphorylation was increased in the striatum, cortex and hippocampus. The early increase in striatal STEP phosphorylation levels correlated with a deregulation of the protein kinase A pathway, and decreased calcineurin activity at later stages further contributes to an enhancement of STEP phosphorylation and inactivation. Accordingly, we detected an accumulation of phosphorylated ERK2 and p38, two targets of STEP, in R6/1 mice striatum at advanced stages of the disease. Activation of STEP participates in excitotoxic-induced cell death. Since Huntington's disease mouse models develop resistance to excitotoxicity, we analyzed whether decreased STEP activity was involved in this process. After intrastriatal quinolinic acid (QUIN) injection, higher phosphorylated STEP levels in R6/1 mice correlated with a more sustained ERK2 signal suggesting that STEP inactivation could mediate neuroprotection in R6/1 striatum. In agreement, intrastriatal injection of TAT-STEP increased QUIN-induced cell death in R6/1 mice. R6/2, Tet/HD94 and $Hdh^{Q7/Q111}$ mice striatum also displayed decreased STEP protein and increased phosphorylation levels. In Tet/HD94 mice striatum mutant huntingtin transgene shut-down reestablished STEP expression. In conclusion, the STEP pathway is severely down-regulated in the presence of mutant huntingtin and participates in compensatory mechanisms activated by striatal neurons that lead to the resistance to excitotoxicity.

Introduction

STriatal-Enriched protein tyrosine Phosphatase (STEP), encoded by the *Ptpn5* gene, is a brain-specific phosphatase involved in neuronal signal transduction. STEP is enriched in the striatum (Lombroso et al., 1991), and expressed at lower levels in the cortex, hippocampus and amygdala (Boulanger et al., 1995). STEP mRNA is alternatively spliced into the membrane-associated $STEP_{61}$ and the cytosolic $STEP_{46}$ (Bult et al., 1997). Both isoforms are expressed in the striatum whereas the hippocampus and cortex only express $STEP_{61}$ (Boulanger et al., 1995).

STEP activity is regulated through phosphorylation/dephosphorylation of a serine residue within its kinase interacting motif domain. Stimulation of dopamine D1 receptors (D1Rs) activates the cAMP-dependent protein kinase A (PKA) (Stoof and Kebabian, 1981) which phosphorylates $STEP_{46}$ and $STEP_{61}$, thereby inactivating them (Paul et al., 2000). In contrast, glutamate stimulation of N-methyl-D-aspartate receptors (NMDARs) results in the dephosphorylation and activation of STEP through a calcineurin/PP1 pathway (Valjent et al., 2004; Paul et al., 2003). Once activated, STEP dephosphorylates the glutamate receptor subunits NR2B (Pelkey et al., 2002; Snyder et al., 2005; Braithwaite et al., 2006) and GluR2 (Zhang et al., 2008), leading to their endocytosis, and the kinases ERK1/2 (extracellular signal-regulated kinase 1/2), p38 and Fyn, thereby controlling the duration of their signal (Pulido et al., 1998; Nguyen et al., 2002; Munoz et al., 2003; Paul et al., 2003).

Striatal medium-sized spiny neurons are specially affected in Huntington's disease (HD) (Reiner et al., 1988), a dominantly inherited neurodegenerative disorder caused by an abnormal expansion of a CAG codon in exon-1 of the *huntingtin* (htt) gene (The Huntington's Disease Collaborative Research Group, 1993). The enrichment of STEP in

these neurons, together with its role in the regulation of key substrates implicated in neuronal function, suggest that STEP may play a role in the pathophysiology of HD. In fact, previous studies show decreased mRNA levels of STEP in the caudate nucleus and cortex of HD patients (Hodges et al., 2006), and in the striatum of R6/1 mice (Desplats et al., 2006). Moreover, both dopaminergic and glutamatergic systems, which regulate STEP activity, are affected in HD patients (Jakel and Maragos, 2000) and mouse models (Bibb et al., 2000; Pineda et al., 2005; Fan and Raymond, 2007).

Excitotoxicity has been proposed to contribute to the selective loss of striatal medium-sized spiny neurons in HD (reviewed by Perez-Navarro et al., 2006; Fan and Raymond, 2007). However, mouse models of HD develop resistance to excitotoxicity (Hansson et al., 1999, 2001; Jarabek et al., 2004; Torres-Peraza et al., 2008; Graham et al., 2009), and we have previously shown that reduced levels of calcineurin expression and activity could participate in this phenomenon (Xifro et al., 2009). Interestingly, disruption of STEP activity leads to the activation of ERK1/2 signaling and attenuates excitotoxic-induced cell death in the hippocampus (Choi et al., 2007). Thus, in the present study we sought to investigate STEP protein expression and activity in several mouse models of HD, and to analyze whether STEP acts downstream of calcineurin to regulate cell survival after a striatal excitotoxic lesion.

Materials and Methods

HD mouse models. Wild-type $Hdh^{Q7/Q7}$ and heterozygous mutant $Hdh^{Q7/Q111}$ knock-in mice were obtained from matings between male and female $Hdh^{Q7/Q111}$ heterozygotes as described previously (Wheeler et al., 1999). R6/1 and R6/2 heterozygous transgenic mice expressing exon-1 mutant huntingtin (mhtt) with 145 (Giralt et al., 2009) and 115

CAG repeats, respectively, were obtained from Jackson Laboratory (Bar Harbor, ME). Conditional Tet/HD94 mice express a chimeric mouse/human exon-1 mhtt with 94 CAG repeats under the control of the bidirectional tetO responsive promoter (Yamamoto et al., 2000). To turn-off mhtt expression, 17-month old wild-type and Tet/HD94 mice were treated with doxycycline in drinking water during 5 months (2 mg/ml for 4 months followed by 0.5 mg/ml for 1 month; gene-OFF group; Diaz-Hernandez et al., 2005). Some animals were left without intervention (gene-ON 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 respect to genotype. All mice were genotyped by polymerase chain reaction as described previously (Mangiarini et al., 1996; Wheeler et al., 1999; Yamamoto et al., 2000). The animals were housed with access to food and water *ad libitum* in a colony room kept at 19-22ºC and 40-50% humidity, under a 12:12 h light/dark cycle. All animal-related procedures were in accordance with the National Institute of Health Guide for the care and use of laboratory animals, and approved by the local animal care committee of the Universitat de Barcelona (99/01), and the Generalitat de Catalunya (99/1094).

Total protein extraction. The animals were deeply anesthetized and killed by decapitation at the age of 4, 8, 12, 20 or 30 weeks (wild-type and R6/1), 12 weeks (wild-type and R6/2), 8 months ($Hdh^{Q7/Q7}$ and $Hdh^{Q7/Q111}$ knock-in mice), or 22 months (wild-type, gene-ON and gene-OFF Tet/HD94 mice). QUIN-lesioned mice were sacrificed 1 or 4 h after QUIN injection. The brain was quickly removed and the striatum, cortex and hippocampus were dissected out and homogenized 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 phenylmethylsulphonyl fluoride (PMSF), 10 µg/µl aprotinin,

1 μg/μl leupeptin] and phosphatase inhibitors (2 mM Na_3VO_4, 100 mM NaF). Samples were centrifuged at 16,100 x g for 20 min at 4ºC, the supernatants were collected and the protein concentration was determined using the Dc protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Western blot analysis. Proteins were denatured in 62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% glycerol, 140 mM β-mercaptoethanol and 0.1% (w/v) bromophenol blue, heated at 100ºC for 5 min, and resolved in denaturing polyacrylamide gels. For pNR1 (Ser897) analysis, the samples were denatured in a 170 mM phosphate buffer (pH 7) with 2.5% (w/v) SDS, 10% glycerol, 3.2 mM dithiothreitol and 0.1% (w/v) bromophenol blue. Proteins were transferred to nitrocellulose membranes (Whatman® Schleicher&Schuell, Dassel, Germany), and washed twice in Tris-buffered saline containing 0.1% Tween-20 (TBS-T). After blocking (TBS-T solution plus 5% bovine serum albumin and 5% skimmed milk) at room temperature for 1 h, the membranes were blotted overnight at 4ºC with the following primary antibodies: anti-STEP (23E5; 1:1000) and anti-phosphoERK2 (pERK; Tyr204) (1:1000) from Santa Cruz Biotechnology (Santa Cruz, CA); anti-panERK (1:5000), anti-p-p38, anti-p38 and anti-phospho-dopamine- and cyclic AMP-regulated phosphoprotein-32 KDa (Thr34) (pDARPP-32 (Thr34) 1:1000) from Cell Signaling (Beverly, MA); anti-DARPP-32 (1:1000; BD Biosciences, San Jose, CA); anti-pNR1 (Ser897) (1:1000; Upstate, Lake Placid, NY) and anti-pSTEP (1:1000; Paul et al., 2003). Incubation with a mouse monoclonal antibody against α-tubulin (1:100,000; Sigma Chemical Co., St Louis, MO) was performed to obtain loading controls. After primary antibody incubation, the membranes were washed with TBS-T and incubated for 1 h at room temperature with the appropriated horseradish peroxidase-conjugated secondary antibody (1:2000; Promega, Madison, WI), and the reaction was finally visualized with the Western

Blotting Luminol Reagent (Santa Cruz Biotechnology). Western blot replicates were scanned and quantified using a computer-assisted densitometric analysis.

Quantitative (Q)-polymerase chain reaction (PCR) assay. Total RNA was extracted from 8- and 20-week old wild-type and R6/1 striatal samples and cDNA synthesized as described elsewhere (Garcia-Martinez et al., 2007). The cDNA was then analyzed by Q-PCR using the following TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA): 18S (Hs99999901_s1) and STEP (Mm00479063_m1) as previously described (Saavedra et al., 2010). The data were analyzed and quantified using the Comparative Quantitation Analysis program of the MxProTM Q-PCR analysis software version 3.0 (Stratagene) with the 18S gene expression as internal loading control. Results were expressed as percentage of wild-type values.

Pharmacological treatments and excitotoxic lesion. Wild-type mice (12-week old) were injected intraperitoneally (i.p.) with FK-506 [5 mg/kg; dissolved in cremophor (Sigma Chemical Co.)] or vehicle (cremophor) and sacrificed 2.5 h later. Papaverine (30 mg/kg; dissolved in water, Sigma Chemical Co.) or vehicle (water) was injected i.p. into 12-week old wild-type mice and animals were sacrificed by decapitation 10 min post-injection. The striatum was removed and stored at – 80ºC until processed for protein extraction and western blot analysis as above. The striatal excitotoxic lesion using quinolinic acid (QUIN) was performed in 12- (Fig. 5; 10 nmol) and 25-30-week old (Fig. 7; 20 nmol) mice as previously described (Xifro et al., 2009).

Intrastriatal injection of TAT-STEP or TAT-myc control peptide. We inserted the trans-activating transduction (TAT) nucleotide sequence (TAC-GGT-CGT-AAAAAA-CGT-CGT-CAG-CGT-CGT-CGT) at the N-terminal of the $STEP_{46}$ complementary DNA (cDNA), subcloned it in pTrcHis-TOPO expression vector, and transformed it into *Escherichia coli*, Top10 (Invitrogen, Carlsbad, CA). Six histidines and a

myelocytomatosis virus (myc) tag were added to the C-terminus to purify the fusion protein and to track it *in vivo*, respectively. Fusion proteins were induced with isopropyl-β-D-thiogalactopyranoside (IPTG) and affinity purified. Single bands on Westerns blotted with myc- and STEP-antibodies were used as an indication of purity. The peptides were synthesized by the core facility at Yale University (New Haven, CT). One hour prior to QUIN injection (20 nmol) in 25-30-week old R6/1 mice TAT-STEP or TAT-myc (both 4 μM in 0.5 μl) was intrastriatally injected, at the same coordinates as QUIN. To evaluate the possible toxicity of TAT-STEP, 25-30-week old wild-type mice received an intrastriatal injection of PBS alone or TAT-STEP (4 μM in 0.5 μl) 1 h prior to PBS injection.

Fluoro-Jade staining. Twenty-four hours after intrastriatal QUIN injection, with or without prior TAT-peptide infusion, mice (n = 4-5 for each condition) were deeply anesthetized and immediately perfused transcardially with saline followed by 4% paraformaldehyde/phosphate buffer. Brains were removed and post-fixed for 1-2 h in the same solution, cryoprotected by immersion in 30% sucrose/PBS and then frozen in dry ice-cooled isopentane. Serial coronal cryostat sections (30 μm) through the whole striatum were collected on silane-coated slides. Striatal sections were processed for Fluoro-Jade staining (Histo-Chem Inc., Jefferson, AR) as described elsewhere (Schmued et al., 1997). Sections stained with Fluoro-Jade were visualized on a computer, and the border of the lesion was outlined using the Computer-Assisted Stereology Toolbox (CAST) software (Olympus). The volume of the lesion was estimated by multiplying the sum of all the sectional areas (μm^2) by the distance between successive sections (240 μm), as described previously (Perez-Navarro et al., 2000).

Immunohistochemistry. Serial coronal sections (30 μm) were obtained as described for Fluoro-Jade staining and processed for free-floating immunohistochemistry for myc or pERK2. The sections were washed three times in PBS and permeabilized/blocked by incubating with PBS containing 0.3% Triton X-100 and 3% of normal goat serum (Pierce Biotechnology, Rockford, IL) for 15 min at room temperature. After three washes the brain slices were incubated overnight at 4ºC under continuous agitation with mouse anti-myc (1:200) or rabbit anti-pERK2 (1:250), both from Santa Cruz Biotechnology, in a PBS with 0.02% sodium azide buffer. After primary antibody incubation, slices were washed three times and then incubated 2 h under continuous agitation at room temperature with Cy3 goat anti-mouse or Cy3 goat anti-rabbit (both 1:300; Jackson ImmunoResearch, West Grove, PA), respectively. No signal was detected in controls incubated in the absence of the primary antibody.

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

Results

$STEP_{61}$ and $STEP_{46}$ levels are decreased in the striatum of R6/1 mice

We first analyzed the expression of $STEP_{61}$ and $STEP_{46}$ in the striatum of R6/1 mice to determine if STEP protein levels were changed in striatal cells expressing N-terminal exon 1 mhtt. To this end, we performed Western blot of striatal protein extracts obtained from 4-, 8-, 12-, 20- and 30-week old mice. $STEP_{61}$ and $STEP_{46}$ protein levels were unchanged in R6/1 mice at 4 weeks of age compared to wild-type animals (Fig. 1*A* and *B*). In contrast, $STEP_{61}$ (Fig. 1*A*) and $STEP_{46}$ (Fig. 1*B*) protein levels were significantly

decreased in the striatum of 8-week old R6/1 mice, a reduction that was maintained in 12-, 20- and 30-week old animals.

To determine whether the decrease in striatal STEP levels was associated with a down-regulation of mRNA expression, we performed Q-PCR. Consistent with reduced STEP protein levels at 8 weeks, Q-PCR analysis revealed a reduction in STEP mRNA levels in the striatum of R6/1 mice (50 ± 7% of wild-type levels; Fig. 1*C*). A similar decrease in striatal STEP mRNA levels was found in samples from 20-week old R6/1 mice (Fig. 1*C*). These data indicate that in the presence of mhtt both STEP mRNA and protein levels are reduced in the striatum, a decrease that is sustained during the disease progression.

Progressive increase of STEP phosphorylation in the striatum of R6/1 mice

Since STEP ability to bind to and dephosphorylate its substrates is regulated through phosphorylation, we looked at $pSTEP_{61}$ and $pSTEP_{46}$ levels to evaluate STEP activity in R6/1 mice striatum. As shown in Fig. 2*A*, 8- and 12-week old R6/1 mice showed around a 3-fold increase in striatal $pSTEP_{61}$ levels that progressively increased at later stages of the disease (Fig. 2*A*). In contrast, striatal $pSTEP_{46}$ levels were only increased in 30-week old R6/1 mice (Fig. 2*B*). These results indicate that STEP phosphorylation increases, and suggests that its activity progressively decreases in the striatum of R6/1 mice.

Alterations in STEP expression and phosphorylation in R6/1 mice are not restricted to the striatum

In order to determine whether the reduction of STEP protein levels and activation was a general mechanism triggered by neurons in response to mhtt expression, $STEP_{61}$ protein and phosphorylation levels were determined in the cortex and hippocampus of R6/1

mice at different stages of the disease. Similarly to what was observed in the striatum, $STEP_{61}$ levels were decreased in the cortex of R6/1 animals when compared with age-matched wild-type mice, although the time-course was delayed as a significant reduction was observed in 12-week old but not in 8-week old R6/1 mice (Table 1). In contrast, no changes were found in $STEP_{61}$ protein levels in the hippocampus of R6/1 mice at the ages analyzed (Table 1). As observed in the striatum, cortical and hippocampal $pSTEP_{61}$ levels were significantly increased from 8 to 30 weeks of age although the increase observed in the cortex was lower than that found in the striatum and hippocampus (Table 1 and Fig. 2*A*). Taken together, these data indicate that in the presence of mhtt STEP protein expression is regulated in a region-dependent manner while the effect on STEP phosphorylation is more widespread.

Increased STEP phosphorylation in the striatum of R6/1 mice correlates with increased pERK2 and p-p38 levels at late stages of disease progression

If the phosphorylation/inactivation of STEP in R6/1 mice is physiologically relevant, then STEP targets should be more phosphorylated in these animals. To address this question, we next examined pERK2 and p-p38 levels in the striatum of R6/1 mice from 8 to 30 weeks of age. Neither pERK2 nor p-p38 levels were significantly altered in 8- or 12-week old R6/1 mice (Fig. 3*A* and *B*). In contrast, there was a significant increase in both pERK2 and p-p38 levels in the striatum of 20- and 30-week old R6/1 mice compared with age-matched wild-type animals (Fig. 3*A* and *B*). These data support the idea that the loss of STEP activity is progressive in R6/1 striatum.

The accumulation of pSTEP correlates with a deregulation of PKA and calcineurin pathways in the striatum of R6/1 mice

The phosphorylation levels of STEP are regulated by PKA-dependent phosphorylation and calcineurin-dependent dephosphorylation (Paul et al., 2000, 2003). Since calcineurin activity in the striatum of R6/1 mice is not reduced until 16 weeks of age (Xifro et al., 2009), we examined whether the increase in $pSTEP_{61}$ levels observed in the striatum of 8- and 12-week old R6/1 mice (Fig. 2*A*) was due to altered PKA activity. To address this issue, we analyzed two additional PKA substrates: (i) the NMDAR subunit NR1 (Ser897; Tingley et al., 1997) and (ii) DARPP-32 (Thr34; Hemmings et al., 1984). The levels of pNR1 (Ser897) were not altered in the striatum of R6/1 mice at 4 weeks of age whereas we observed a comparable increase in pNR1 (Ser897) levels in 12- and 30-week old R6/1 mice striatum (Fig. 4*A*). Similarly to $pSTEP_{61}$ and pNR1 (Ser897), pDARPP-32 (Thr34) levels were not altered at 4 weeks of age whereas its levels were progressively enhanced in the striatum of R6/1 mice, with the highest increase observed at 30 weeks of age (Fig. 4*B*). Since calcineurin dephosphorylates pDARPP-32 (Halpain et al., 1990), but not pNR1 (Lee, 2006) these results suggest that the early changes in $pSTEP_{61}$, pNR1 and pDARPP-32 levels in the striatum of R6/1 mice are caused by a deregulation in the PKA pathway which at later stages of the disease progression operates together with reduced calcineurin activity to additionally enhance $STEP_{61}$ and DARPP-32 phosphorylation.

To explore this possibility, we examined $pSTEP_{61}$ and pNR1 (Ser897) levels in wild-type mice after acute modulation of PKA or calcineurin activity. To activate PKA we inhibited phosphodiesterases (PDEs), the enzymes responsible for cAMP hydrolysis. For that we used papaverine, an inhibitor of the PDE10A (Siuciak et al., 2006), an isoform that is enriched in striatal medium spiny neurons (Fujishige et al., 1999; Seeger et al., 2003; Xie et al., 2006). To inhibit calcineurin activity we used FK-506 (Xifro et al., 2009). Both papaverine and FK-506 treatment induced an increase in $pSTEP_{61}$

levels in the striatum of wild-type mice (Fig. 4*C*). We also observed increased pNR1 (Ser897) levels after papaverine injection while calcineurin inhibition had no effect on pNR1 (Ser897) levels (Fig. 4*D*). Altogether, these findings indicate that both PKA and calcineurin deregulation contribute to enhance STEP phosphorylation in the presence of mhtt.

STEP regulates ERK2 signaling after an excitotoxic stimulus

Intrastriatal injection of the NMDAR agonist QUIN has been extensively used as a model of HD (Schwarcz et al., 2010). We recently reported that activation of calcineurin promotes QUIN-induced excitotoxic cell death in the striatum (Xifro et al., 2009). Since the stimulation of NMDARs activates STEP in a calcineurin-dependent manner (Paul et al., 2003), we next examined whether STEP acts as a calcineurin target and regulates ERK signaling after an excitotoxic stimulus to the striatum. In wild-type mice, there was a reduction in $pSTEP_{61}$ and $pSTEP_{46}$ levels 1 h after intrastriatal QUIN injection that returned to control values at 4 h (Fig. 5*A* and *B*). These results are consistent with our previous results showing a 2-fold increase in calcineurin activity 1 h after intrastriatal QUIN injection with no differences from non-lesioned striatum at 4 h (Xifro et al., 2009). Since $STEP_{61}$ can be cleaved by calpain after a rapid influx of Ca^{2+} upon glutamate receptor stimulation leading to the appearance of a lower molecular weight inactive isoform, $STEP_{33}$ (Nguyen et al., 1999; Xu et al., 2009), we analyzed whether this process occurred in QUIN-injected striatum. We found no changes in $STEP_{61}$ or $STEP_{33}$ levels either 1 or 4 h after QUIN injection in wild-type mice striatum (supplemental Fig. 1). Consistent with ERK activation upon synaptic NMDAR stimulation (Ivanov et al., 2006; Leveille et al., 2008; Xu et al., 2009), 1 h after intrastriatal QUIN injection there was an increase in pERK2 levels that returned to basal

levels at 4 h post-injection (Fig. 5*C*). In accordance with STEP dephosphorylation and activation 1 h after QUIN injection we also observed a reduction in p-p38 levels (76 ± 7% compared to the vehicle-injected side; P = 0.0095) that returned to control levels 4 h after QUIN injection.

R6 mouse models gradually develop resistance to excitotoxicity induced by QUIN (Hansson et al., 2001), and reduced calcineurin activity accounts for R6/1 mice resistance to excitotoxicity (Xifro et al., 2009). Since our results show a dynamic regulation of STEP and ERK1/2 activity after intrastriatal QUIN injection in wild-type animals, we hypothesized that this signaling cascade could be modified in the presence of exon-1 mhtt and thereby account for the resistance to excitotoxicity reported in these animals. Thus, we analyzed pSTEP and pERK2 levels after QUIN injection in R6/1 mice at 12 weeks of age, when they are resistant to excitotoxicity. In contrast to that observed in wild-type animals, in R6/1 mice neither $pSTEP_{61}$ (Fig. 5*A*) nor $pSTEP_{46}$ (Fig. 5*B*) levels were altered 1 or 4 h after QUIN injection. These results are consistent with the previous finding that calcineurin activity after QUIN injection in R6/1 mice is significantly lower than that observed in wild-type mice, and constant between 1 and 4 h post-injection (Xifro et al., 2009). No changes in $STEP_{61}$ or $STEP_{33}$ levels were found upon intrastriatal QUIN injection in R6/1 mice either (supplemental Fig. 1).

We also observed increased pERK2 levels 1 h after intrastriatal QUIN injection in R6/1 mice, which were comparable to those registered in wild-type animals (296 ± 49% as compared with wild-type contralateral side). However, in contrast with wild-type mice, pERK2 levels remained significantly elevated 4 h after intrastriatal QUIN injection in R6/1 mice (Fig. 5*C*). These results indicate that, despite a similar activation of the ERK pro-survival pathway in wild-type and R6/1 mice after intrastriatal QUIN injection, ERK signaling is more prolonged in resistant R6/1 than in vulnerable wild-

type mice. In contrast, p-p38 levels were unchanged 1 h and 4 h after intrastriatal QUIN injection in R6/1 mice (data not shown), a result consistent with no significant changes in pSTEP levels (Fig. 5*A* and *B*).

Our hypothesis was that enhanced activity of the ERK pathway as a consequence of a lack of activation of STEP may participate in the reduced neuronal loss observed after QUIN injection in R6/1 mice. To address this possibility we analyzed pERK2 levels in wild-type mice after treatment with FK-506 or papaverine, as inhibition of calcineurin (Xifro et al., 2009) or PDE10A (Giampa et al., 2009) prevents QUIN-induced cell death in the striatum. Consistent with STEP inactivation (Fig. 4*C*), treatment with papaverine or FK-506 increased pERK2 levels in the striatum (Fig. 6), thus supporting the idea that increased STEP inactivation in R6/1 striatum can mediate resistance to excitotoxicity through the maintenance of ERK signaling.

Intrastriatal injection of TAT-STEP peptide increases QUIN-induced cell death in R6/1 mice

Older R6/1 mice are more resistant to excitotoxicity (Hansson et al., 2001) than younger animals and, interestingly, have significantly higher striatal levels of phosphorylated/inactive STEP (Fig. 2 and 3). Thus, in order to directly clarify the relationship between STEP activity and vulnerability *versus* resistance to excitotoxicity in HD mouse models we next infused TAT-STEP or TAT-myc (4 μM) in the striatum of 25-30-week old R6/1 mice 1 h before intrastriatal injection of PBS or QUIN (20 nmol). To confirm that TAT-STEP was taken up by striatal cells at the moment of QUIN injection, we performed a staining with anti-myc antibody 1 h post TAT-STEP intrastriatal infusion. As shown in supplemental Fig. 2*B*, TAT-STEP was detected in cells surrounding the site of injection. Intrastriatal injection of TAT-STEP plus vehicle

did not induce cell death in the striatum of wild-type mice as assessed by Fluoro-Jade staining (supplemental Fig. 2*C*). Cell death induced by QUIN injection in R6/1 mice striatum was not modified by the infusion of the control peptide TAT-myc (Fig. 7*a2* and *a4*). In contrast, infusion of TAT-STEP prior to intrastriatal QUIN injection increased the volume of the lesion compared to that registered in R6/1 mice receiving the control peptide plus QUIN injection (Fig. 7*a3* and *a4*). Immunohistochemistry against pERK2 in TAT-STEP+QUIN injected R6/1 mice striatum revealed that pERK levels were decreased in the striatal region where cells undergo cell death (Fig. 7*b3*). These data demonstrate that STEP increases neuronal vulnerability to excitotoxic stimuli, and further support the involvement of STEP reduced levels and activity in R6/1 resistance to excitotoxicity.

STEP and pSTEP levels are also modified in the striatum of R6/2 and Hdh$^{Q7/Q111}$ mice

To investigate whether changes in STEP and pSTEP levels are a common feature in striatal cells expressing mhtt, we analyzed them in mice expressing different forms of mhtt: R6/2 mice, which express N-terminal exon-1 mhtt and show earlier onset and more severe symptoms than R6/1 mice (Mangiarini et al., 1996), and knock-in Hdh$^{Q7/Q111}$ mice that show late onset and slow progression of the disease (Wheeler et al., 1999). As observed in the striatum of R6/1 mice, STEP$_{61}$ (Fig. 8*A*) and STEP$_{46}$ levels were reduced in the striatum of 12-week old R6/2 mice (58 ± 5% of wild-type values, P = 0.0005; Student's t-test). There was also a decrease of both STEP$_{61}$ (Fig. 8*B*) and STEP$_{46}$ levels in 8-month old Hdh$^{Q7/Q111}$ mice striatum (81 ± 2% compared with Hdh$^{Q7/Q7}$ mice striatum; P = 0.0006; Student's t-test). R6/2 mice also showed increased striatal levels of phosphorylated, inactive STEP$_{61}$ (Fig. 8*C*), whereas pSTEP$_{61}$ levels

were enhanced to 324 ± 41% in $Hdh^{Q7/Q111}$ mice striatum (Fig. 8*D*). These results indicate that the down-regulation of STEP protein and increased phosphorylation are found in the striatum of the three HD mouse models analyzed.

Changes in STEP expression are reverted by suppressing the mhtt transgene expression in Tet/HD94 mice striatum

To determine whether STEP down-regulation depends on the continuous expression of mhtt, we quantified STEP protein levels in the striatum of Tet/HD94 mice. Striatal STEP protein levels were examined in 22-month old mice from three different groups: wild-type, Tet/HD94 with no pharmacological intervention (gene-ON) and Tet/HD94 after 5 months of doxycycline administration that turns-off the expression of the mhtt transgene (gene-OFF). As observed in the striatum of the two other exon-1 models analyzed here (R6/1 and R6/2, Figs. 1 and 8*A*, respectively), $STEP_{61}$ and $STEP_{46}$ protein levels were significantly decreased in the Tet/HD94 gene-ON group when compared with wild-type animals (Fig. 9*A*). By suppressing transgene expression, $STEP_{61}$ and $STEP_{46}$ protein levels were no longer different from wild-type mice (Fig. 9*A*).

We also determined the levels of $pSTEP_{61}$ and $pSTEP_{46}$ in the striatum of these mice. Tet/HD94 mice expressing mhtt showed increased levels of $pSTEP_{61}$ (Fig. 9*B*), but not of $pSTEP_{46}$ (data not shown), when compared with wild-type values. In contrast to that observed for STEP protein levels, $pSTEP_{61}$ levels were only partially reverted to wild-type levels 5 months after mhtt shut-down (Fig. 9*B*).

Discussion

In the present study, we show that STEP protein and activity are reduced in the presence of mhtt *in vivo*. In the R6/1 mouse striatum STEP hyper-phosphorylation occurs early in the disease process and correlates with a deregulation of the PKA pathway, which at later stages cooperates with a reduction in calcineurin activity to further inactivate STEP. We found that increased STEP phosphorylation also occurs in the cortex and hippocampus of R6/1 mice. These observations were extended to the striatum of R6/2, Tet/HD94 and $Hdh^{Q7/Q111}$ mouse models of HD. In addition, we show that STEP acts downstream of calcineurin to control ERK signaling after an excitotoxic stimulus, and that inactivation of STEP is one of the mechanisms involved in neuronal dysfunction leading to resistance to excitotoxicity in R6/1 mice striatum.

In the presence of mhtt, STEP is regulated at two different levels, expression and phosphorylation, suggesting that both effects could lead to a synergistic reduction of STEP activity. The down-regulation of STEP is brain region-specific whereas changes in STEP phosphorylation occur in all brain regions examined. In R6/1 mice, reduced STEP levels were observed earlier in the striatum than in the cortex, the brain areas most affected in HD (Vonsattel and DiFiglia, 1998), while hippocampal STEP levels were unaltered. In agreement, it has been suggested that select combinations of trans-acting factors and co-activators could account for both gene- and tissue-specific effects of mhtt (Gomez et al., 2006). After mhtt shut-down in Tet/HD94 mice we observed a reestablishment of STEP protein to wild-type levels. This finding, together with the fact that R6/1 mice develop motor symptoms around 15-21 weeks of age (Mangiarini et al., 1996) and that STEP levels are already decreased at 8 weeks of age, suggest that altered transcription of the *STEP* gene is a direct result of mhtt expression and not due to

compensatory changes in response to cellular dysfunction. In agreement with our results, a previous report showed that STEP mRNA levels are decreased to a similar extent in 10- and 24-week old R6/1 mice striatum (Desplats et al., 2006). Furthermore, STEP mRNA levels are also decreased in the caudate nucleus and cortex of HD patients (Hodges et al., 2006), suggesting that altered expression of STEP could be involved in the pathogenesis of HD.

Our results indicate that STEP inactivation in R6/1 mice is a common feature to all brain regions analyzed. The levels of pSTEP are regulated by PKA-dependent phosphorylation and calcineurin/PP1-dependent dephosphorylation (Paul et al., 2000, 2003; Valjent et al., 2005). Calcineurin levels and activity are reduced in the striatum and cortex, but not in the hippocampus of R6/1 mice (Xifro et al., 2009). Furthermore, reduced calcineurin levels in the striatum were observed from 16 weeks of age thus suggesting that changes in $pSTEP_{61}$ levels at early stages could be linked to a deregulation of the PKA pathway. Accordingly, the phosphorylation level of other PKA substrates, DARPP-32 (Thr34; Hemmings et al., 1984) and NR1 (Ser897; Tingley et al., 1997), was significantly increased in the striatum of R6/1 mice at 12 weeks of age. Increased STEP phosphorylation occurs after stimulation of D1R (Paul et al., 2000), whose reduced expression is a well-characterized hallmark of HD (Cha et al., 1998; Jakel and Maragos, 2000; Ariano et al., 2002). However, despite D1R loss, D1R agonist-induced immediate early genes mRNA levels are significantly increased in the striatum of R6/2 mice, thus suggesting that mhtt may enhance the sensitivity of D1R-mediated signaling (Spektor et al., 2002). Accordingly, D1R-mediated toxicity is increased in striatal primary cultures from YAC128 mice (Tang et al., 2007) and in knock-in $STHdh^{Q111/Q111}$ cells (Paoletti et al., 2008). In addition, enhanced levels of D5R (positively coupled to adenylyl cyclase) and elevations in intracellular cAMP

staining have been shown in the striatum of several HD mouse models (Ariano et al., 2002, 2005). In the presence of mhtt, parallel changes in other components of the cAMP/PKA pathway, such as PDEs, can also be involved in a PKA-dependent increase in pSTEP levels. Indeed, the levels of PDE10A, which is enriched in striatal medium spiny neurons (Fujishige et al., 1999; Seeger et al., 2003; Xie et al., 2006), are reduced in the striatum of R6 mice (Hebb et al., 2004; Hu et al., 2004; Desplats et al., 2006), and in the caudate-putamen of HD patients (Hebb et al., 2004). Altogether, these changes could cooperate to enhance cAMP levels leading to increased PKA-dependent phosphorylation and inactivation of STEP.

We also provide data showing that the deregulation of the PKA pathway is accompanied at later stages by a reduction in calcineurin activity, as evidenced by an even more robust elevation of $pSTEP_{61}$ and pDARPP-32 (Thr34) levels, together with a significant increase in $pSTEP_{46}$ levels in the striatum of R6/1 mice. In agreement, the phosphorylation level of NR1 (Ser897), which is phosphorylated by PKA (Tingley et al., 1997) but not dephosphorylated by calcineurin (Lee, 2006; present results), was similarly increased in 12- and 30- week old R6/1 striatum, and increased in wild-type mice treated with papaverine, but not with FK-506. Previous reports also showed increased striatal levels of pNR1 (Ser897) in 12-week old R6/1 (Torres-Peraza et al., 2008) and R6/2 mice (Ariano et al. 2005).

The progressive inactivation of STEP in the striatum of R6/1 mice led to the accumulation of pERK2 and p-p38 at later stages of the disease, consistent with previous findings in R6 mice (Lievens et al., 2002; Gianfriddo et al., 2004; Roze et al., 2008; Torres-Peraza et al., 2008) and in the brain of STEP knockout mice (Venkitaramani et al., 2009; Xu et al., 2009). The absence of changes in pERK2 and p-p38 levels at early stages suggests that under baseline conditions a high inactivation of

STEP is necessary to impact on the phosphorylation level of its targets. This is supported by the observation that hippocampal pERK1/2 levels are increased in STEP knockout mice but not in STEP +/- mice (Venkitaramani et al., 2009). Inhibition of the ERK pathway increases mhtt-induced cell death in a cellular HD model (Apostol et al., 2006). Thus, despite higher p-p38 levels (present results; Gianfrido et al., 2004), increased ERK signaling in the striatum of R6/1 (present results) and R6/2 (Lievens et al., 2002; Roze et al., 2008) mice, in addition to high levels of activation of the PI3K/Akt pro-survival pathway (Saavedra et al., 2010), could participate in delaying striatal cell death as R6/1 and R6/2 mice show no significant cell loss (Mangiarini et al., 1996).

The fact that R6/1 mice develop resistance to NMDAR-induced excitotoxicity raises the possibility that activation of intracellular pathways upon NMDAR stimulation might be differentially regulated in the presence of mhtt. Actually, activation of the PI3K/Akt pathway may participate in the reduced neuronal loss observed after QUIN injection in R6/1 mice striatum (Saavedra et al., 2010). Here, we show that higher and unaltered pSTEP levels in R6/1 striatum lead to more sustained ERK signaling compared with QUIN-injected wild-type striatum. These results are consistent with lower calcineurin activation after intrastriatal QUIN injection in R6/1 than in wild-type mice which, importantly, correlates with reduced cell death (Xifro et al., 2009). Calpain-mediated proteolysis of $STEP_{61}$ has recently been associated with p38 activation and excitotoxic cell death (Xu et al., 2009). Consistent with no differences in calpain activity between wild-type and R6/1 mice after intrastriatal injection of QUIN (Xifro et al., 2009) we observed no differences in $STEP_{61}$ cleavage to $STEP_{33}$ and no p38 activation after QUIN injection. Thus, other mechanisms should regulate vulnerability/resistance to excitotoxicity in striatal neurons, and the present results point

out to an important role for STEP regulation of ERK activity. Accumulating evidence shows that increases in ERK activity are neuroprotective (Irving et al., 2000; Almeida et al., 2005; Jiang et al., 2005; Choi et al., 2007). In agreement, pharmacological inhibition of calcineurin or PDE10A, which lead to decreased STEP activity with subsequent increase in pERK2 levels (present results), is protective against QUIN-induced excitotoxicity in the striatum (Giampa et al., 2009; Xifro et al., 2009). In addition, a higher level of tonic STEP activity in hilar interneurons of the hippocampus renders them highly vulnerable to excitotoxic-induced cell death, while blockade of STEP activity with FK-506 allows ERK activation and confers protection against excitotoxicity (Choi et al., 2007). Therefore, our results showing lower STEP levels and activity in R6/1 than in wild-type mice striatum are in agreement with their distinct vulnerability to excitotoxicity. This hypothesis is clearly supported by the increased cell death observed in R6/1 striatum injected with TAT-STEP plus QUIN.

In conclusion, we show that mhtt regulates STEP expression in the brain regions particularly affected in HD, the striatum and cortex, and exerts a broader effect on the regulation of STEP phosphorylation. Increased STEP phosphorylation resulting from altered kinase (PKA) and phosphatase (calcineurin) activities may lead to increased ERK signaling in the striatum, which can contribute to delay striatal cell death, and to neuronal dysfunction leading to the development of resistance to excitotoxicity in HD mouse models (Supplemental Fig. 3).

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Figure legends

Figure 1. STEP protein and mRNA levels are decreased in the striatum of R6/1 mice. STEP protein levels (***A* and *B***) were analyzed by Western blot of protein extracts obtained from the striatum of wild-type (WT) and R6/1 mice at different stages of the disease progression (from 4 to 30 weeks of age). Representative immunoblots show the protein levels of $STEP_{61}$, $STEP_{46}$ and α-tubulin in WT and R6/1 mice at 4, 8 and 30 weeks of age. The graphs show the decrease in striatal $STEP_{61}$ (***A***) and $STEP_{46}$ (***B***) protein levels in R6/1 mice with respect to their littermate controls at different stages of the disease progression. Values (obtained by densitometric analysis of Western blot data) are expressed as percentage of WT mice ($STEP_{61}$ or $STEP_{46}$/α-tubulin ratio), and shown as mean ± SEM (n = 9). (***C***) Graph showing STEP mRNA levels analyzed by Q-PCR in the striatum of 8- and 20-week old WT and R6/1 mice. Results were normalized to the 18S gene expression, expressed as percentage of WT values, and shown as mean ± SEM (n = 5-7 for each genotype). Data was analyzed by Student's t-test. **P < 0.01 and ***P < 0.001 as compared with WT mice.

Figure 2. STEP phosphorylation is increased in the striatum of R6/1 mice. $pSTEP_{61}$ (***A***) and $pSTEP_{46}$ (***B***) levels were analyzed by Western blot of protein extracts obtained from the striatum of wild-type (WT) and R6/1 mice at different stages of the disease progression (from 4 to 30 weeks of age). Representative immunoblots show protein levels of $pSTEP_{61}$, $pSTEP_{46}$, $STEP_{61}$, $STEP_{46}$ and α-tubulin in WT and R6/1 mice at 4, 8 and 30 weeks of age. The graphs show striatal pSTEP levels in R6/1 mice with respect to their littermate controls at different stages of the disease progression. Values (obtained by densitometric analysis of Western blot data) are expressed as

percentage of age-matched WT mice ($pSTEP_{61}/STEP_{61}$ or $pSTEP_{46}/STEP_{46}$ after normalization with α-tubulin), and shown as mean ± SEM (n = 4-7). Data were analyzed by Student's t-test. *P < 0.05, **P < 0.01 and ***P < 0.001 as compared with WT mice; $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ as compared with 12-week old R6/1 mice and $^{+}P < 0.05$ as compared with 20-week old R6/1 mice.

Figure 3. pERK2 and p-p38 levels are increased in the striatum of R6/1 mice. pERK2 (***A***) and p-p38 (***B***) levels were analyzed by Western blot of protein extracts obtained from the striatum of 8 to 30-week old wild-type (WT) and R6/1 mice. Representative immunoblots show protein levels of pERK2, panERK, p-p38, p38 and α-tubulin in WT and R6/1 mice at 8, 20 and 30 weeks of age. The graphs show that the striatal levels of pERK2 and p-p38 are significantly increased in R6/1 mice with respect to their littermate controls at 20 and 30 weeks of age. Values (obtained by densitometric analysis of Western blot data) are expressed as percentage of age-matched WT mice (pERK2/panERK and p-p38/p38 after normalization with α-tubulin), and shown as mean ± SEM (n = 6). Data were analyzed by Student's t-test. *P < 0.05, **P < 0.01 and ***P < 0.001 as compared with WT mice.

Figure 4. Regulation of NR1, DARPP-32 and STEP phosphorylation in the striatum. The phosphorylation levels of a specific PKA residue on NR1 (Ser897) (***A***) and on DARPP-32 (Thr34) (***B***) were analyzed by Western blot of protein extracts obtained from the striatum of wild-type (WT) and R6/1 mice at 4, 12 and 30 weeks of age. Representative immunoblots are presented. The graphs show increased levels of pNR1 (Ser897) (***A***) and pDARPP-32 (Thr34) (***B***) in R6/1 mice with respect to their littermate controls at different stages of the disease progression. Values (obtained by

densitometric analysis of Western blot data) are expressed as percentage of age-matched WT mice (pNR1 (Ser897)/α-tubulin ratio or pDARPP-32 (Thr34)/DARPP-32 ratio after normalization with α-tubulin), and shown as mean ± SEM (n = 8). Data were analyzed by Student's t-test. *P < 0.05 and **P < 0.01 as compared with WT mice and #P < 0.05 as compared with 12-week old R6/1. (***C***) PKA activation or calcineurin inhibition increases $pSTEP_{61}$ levels in the striatum. Twelve-week old wild-type mice (n = 6 for each condition) received an i.p. injection of vehicle, papaverine (30 mg/Kg; Papav.) or FK-506 (5 mg/Kg) and striatal $pSTEP_{61}$ levels were analyzed by Western blot (10 min after papaverine and 2.5 h after FK-506). Representative immunoblots show protein levels of $pSTEP_{61}$, $STEP_{61}$ and α-tubulin in all conditions examined. Values (obtained by densitometric analysis of Western blot data) are expressed as percentage of vehicle-injected mice ($pSTEP_{61}/STEP_{61}$ ratio after normalization with α-tubulin) and shown as mean ± SEM. (***D***) PKA activation, but not calcineurin inhibition, increases pNR1 (Ser897) levels in the striatum. Twelve-week old wild-type mice (n = 6 for each condition) received an i.p. injection of vehicle, papaverine (30 mg/Kg; Papav.) or FK-506 (5 mg/Kg) and striatal pNR1 (Ser897) levels were analyzed by Western blot. Representative immunoblots show protein levels of pNR1 (Ser897) and α-tubulin in all conditions examined. Values (obtained by densitometric analysis of Western blot data) are expressed as percentage of vehicle-injected mice (pNR1 (Ser897)/α-tubulin ratio) and shown as mean ± SEM. Data were analyzed by Student's t-test. **P < 0.01 and ***P < 0.001 as compared with vehicle-injected mice.

Figure 5. **STEP regulates ERK signaling after an excitotoxic stimulus to the striatum.** $pSTEP_{61}$ (***A***) $pSTEP_{46}$ (***B***) and pERK2 (***C***) levels were analyzed by Western blot of protein extracts obtained from the striatum of 12-week old wild-type (WT) and

R6/1 mice 1 and 4 h after an intrastriatal injection of vehicle or QUIN (10 nmol). Representative immunoblots show protein levels of $pSTEP_{61}$, $STEP_{61}$ and α-tubulin (***A***), $pSTEP_{46}$, $STEP_{46}$ and α-tubulin (***B***) and pERK2, panERK and α-tubulin (***C***) in WT and R6/1 striatum, 1 and 4 h after QUIN injection. Values (obtained by densitometric analysis of Western blot data) are expressed as percentage of the contralateral vehicle-injected WT striatum ($pSTEP_{61}/STEP_{61}$ ratio, $pSTEP_{46}/STEP_{46}$ ratio or pERK2/panERK ratio, after normalization with α-tubulin), and data shown are the mean ± SEM (n = 7-9). Data were analyzed by two-way ANOVA followed by Bonferroni's post-hoc test. *P < 0.05; **P < 0.01 and ***P < 0.001 as compared with vehicle-injected contralateral WT striatum; WT: vehicle-injected WT striatum; WT + QUIN: QUIN-injected WT striatum; R6/1: vehicle-injected R6/1 striatum; R6/1 + QUIN: QUIN-injected R6/1 striatum.

Figure 6. Inhibition of PDE10A or calcineurin increases pERK2 levels in the striatum. Twelve-week old wild-type mice (n = 6 for each condition) received an i.p. injection of vehicle, papaverine (30 mg/Kg; Papav.) or FK-506 (5 mg/Kg) and striatal pERK2 levels were analyzed by Western blot (10 min after papaverine and 2.5 h after FK-506 injection). Representative immunoblots show protein levels of pERK2, panERK and α-tubulin in all the conditions examined. Values (obtained by densitometric analysis of Western blot data) are expressed as percentage of vehicle-injected mice (pERK2/panERK ratio after normalization with α-tubulin) and expressed as mean ± SEM. Data were analyzed by Student's t-test. *P < 0.05 and **P < 0.01 as compared with vehicle-injected mice.

Figure 7. **QUIN-induced cell death in R6/1 mice striatum is increased by TAT-STEP injection.** Control peptide (TAT-myc) or TAT-STEP (4 μM) was intrastriatally injected in R6/1 mice 1 h before intrastriatal QUIN (20 nmol) injection. Cell death was assessed by Fluoro-Jade staining 24 h after QUIN injection. (***A***) Representative photomicrographs showing the striatal area occupied by Fluoro-Jade-positive cells in R6/1 mice striatum injected with (***a1***) QUIN, (***a2***) TAT-myc plus QUIN or (***a3***) TAT-STEP plus QUIN. (***a4***) Graph showing the quantification of the volume of the lesion measured in Fluoro-Jade stained sections and expressed as a percentage of QUIN-injected R6/1 mice striatum. *P < 0.05 as compared with QUIN-injected R6/1 striatum. (***B***) Immunohistochemical staining was performed with anti-pERK2 antibody 24 h after QUIN injection in R6/1 mice striatum with or without previous injection of TAT-STEP. Representative images showing the striatum of R6/1 mice in all the conditions analyzed. Note the distinct pERK2 immunoreactivity in non-injured (1) and injured (2) striatal cells. Scale bar, 500 μm.

Figure 8. **$STEP_{61}$ and $pSTEP_{61}$ levels are also altered in the striatum of R6/2 and $Hdh^{Q7/Q111}$ mice.** $STEP_{61}$ (***A, B***) and $pSTEP_{61}$ (***C, D***) levels were analyzed by Western blot of protein extracts obtained from the striatum of wild-type (WT) and R6/2 mice at 12 weeks of age (***A, C***), and from 8-month old $Hdh^{Q7/Q7}$ and knock-in $Hdh^{Q7/Q111}$ mice (***B, D***). Representative immunoblots showing the protein levels of $pSTEP_{61}$, $STEP_{61}$ and α-tubulin in WT/$Hdh^{Q7/Q7}$, R6/2 and $Hdh^{Q7/Q111}$ mice are presented. Values (obtained by densitometric analysis of Western blot data) are expressed as percentage of WT/$Hdh^{Q7/Q7}$ mice ($STEP_{61}$/α-tubulin ratio in ***A*** and ***B***; $pSTEP_{61}/STEP_{61}$ ratio after normalization with α-tubulin in ***C*** and ***D***), and shown as mean ± SEM (n = 5-8). Data

were analyzed by Student's t-test. ***P < 0.001 as compared with WT or $Hdh^{Q7/Q7}$ mice, respectively.

Figure 9. **Changes in STEP expression but not in STEP phosphorylation are reverted by suppressing the mhtt transgene expression in Tet/HD94 mice striatum.** $pSTEP_{61}$, $STEP_{61}$ and $STEP_{46}$ protein levels were analyzed by Western blot in the striatum of 22-month old wild-type (WT) and Tet/HD94 mice, either with no pharmacological intervention (Gene-ON), or after 5 months of mhtt transgene shut-down by doxycycline administration (Gene-OFF). The graphs show the densitometric measures of $STEP_{61}$ and $STEP_{46}$ normalized to α-tubulin (***A***) and the $pSTEP_{61}$/STEP ratio after normalization with α-tubulin (***B***). Results are expressed as percentages of WT ± SEM. Data were analyzed by one-way ANOVA followed by Bonferroni's post-hoc test. *P < 0.05 as compared with WT mice; #P < 0.05 as compared with Tet/HD94 gene-ON mice.

Figure 1

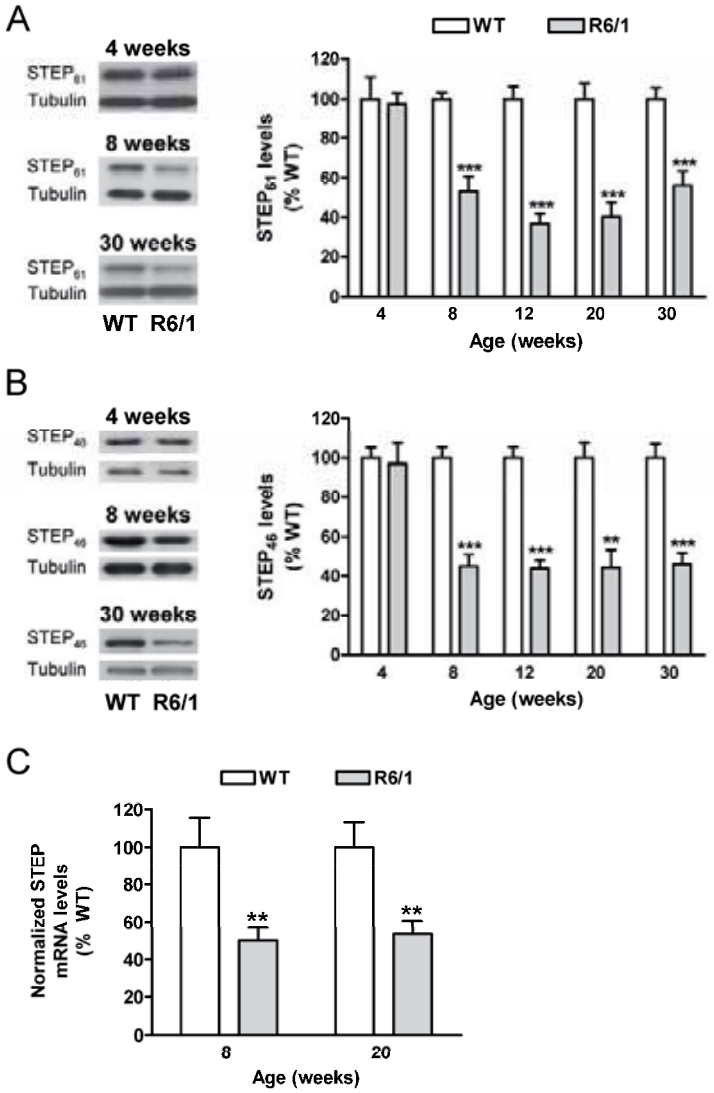
A
4 weeks
STEP61
Tubulin
8 weeks
STEP61
Tubulin
30 weeks
STEP61
Tubulin
WT R6/1
WT
R6/1
STEP61 levels (% WT)
120
100
80
60
40
20
0
***
4
8
12
20
30
Age (weeks)
B
4 weeks
STEP46
Tubulin
8 weeks
STEP46
Tubulin
30 weeks
STEP46
Tubulin
WT R6/1
STEP46 levels (% WT)
**
C
Normalized STEP mRNA levels (% WT)
Age (weeks)

Figure 2

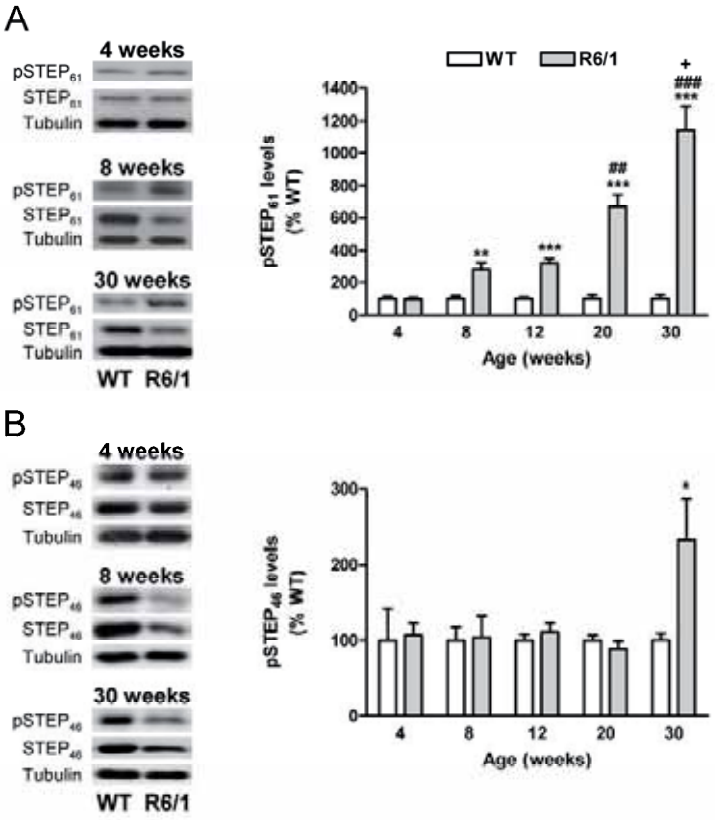
A
4 weeks
pSTEP61
STEP61
Tubulin
8 weeks
pSTEP61
STEP61
Tubulin
30 weeks
pSTEP61
STEP61
Tubulin
WT R6/1
WT
R6/1
pSTEP61 levels (% WT)
1400
1200
1000
800
600
400
200
0
**
***
##
***
+
###
***
4
8
12
20
30
Age (weeks)
B
4 weeks
pSTEP46
STEP46
Tubulin
8 weeks
pSTEP46
STEP46
Tubulin
30 weeks
pSTEP46
STEP46
Tubulin
WT R6/1
pSTEP46 levels (% WT)
300
200
100
0
*
4
8
12
20
30
Age (weeks)

Figure 3

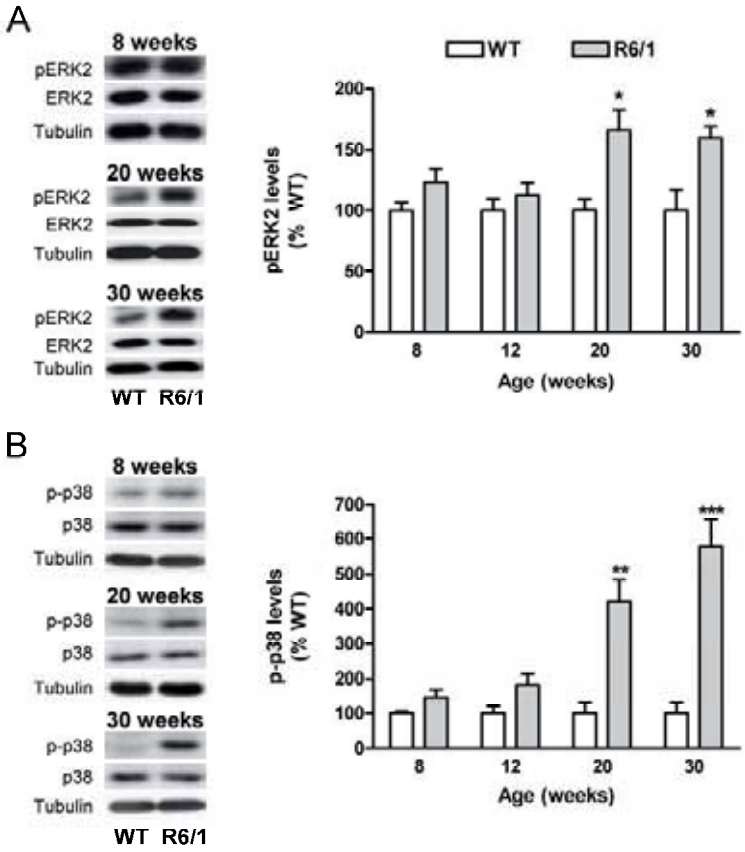
A
8 weeks
pERK2
ERK2
Tubulin
20 weeks
pERK2
ERK2
Tubulin
30 weeks
pERK2
ERK2
Tubulin
WT R6/1
WT
R6/1
pERK2 levels (% WT)
200
150
100
50
0
8
12
20
30
Age (weeks)
B
8 weeks
p-p38
p38
Tubulin
20 weeks
p-p38
p38
Tubulin
30 weeks
p-p38
p38
Tubulin
WT R6/1
p-p38 levels (% WT)
700
600
500
400
300
200
100
0
8
12
20
30
Age (weeks)

Figure 4

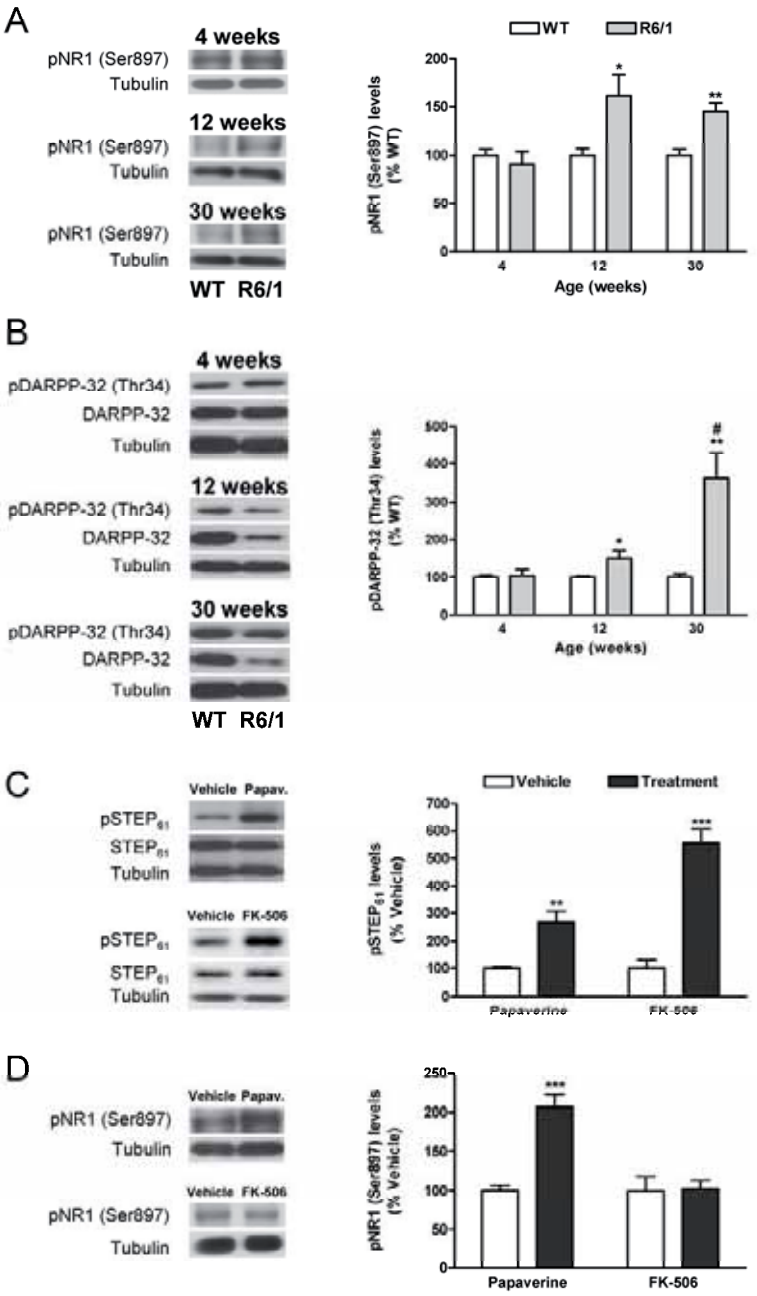
A
4 weeks
pNR1 (Ser897)
Tubulin
12 weeks
pNR1 (Ser897)
Tubulin
30 weeks
pNR1 (Ser897)
Tubulin
WT R6/1
WT
R6/1
pNR1 (Ser897) levels (% WT)
Age (weeks)
B
4 weeks
pDARPP-32 (Thr34)
DARPP-32
Tubulin
12 weeks
pDARPP-32 (Thr34)
DARPP-32
Tubulin
30 weeks
pDARPP-32 (Thr34)
DARPP-32
Tubulin
WT R6/1
pDARPP-32 (Thr34) levels (% WT)
Age (weeks)
C
Vehicle Papav.
pSTEP61
STEP61
Tubulin
Vehicle FK-506
pSTEP61
STEP61
Tubulin
Vehicle
Treatment
pSTEP61 levels (% Vehicle)
Papaverine
FK-506
D
Vehicle Papav.
pNR1 (Ser897)
Tubulin
Vehicle FK-506
pNR1 (Ser897)
Tubulin
pNR1 (Ser897) levels (% Vehicle)
Papaverine
FK-506

Figure 5

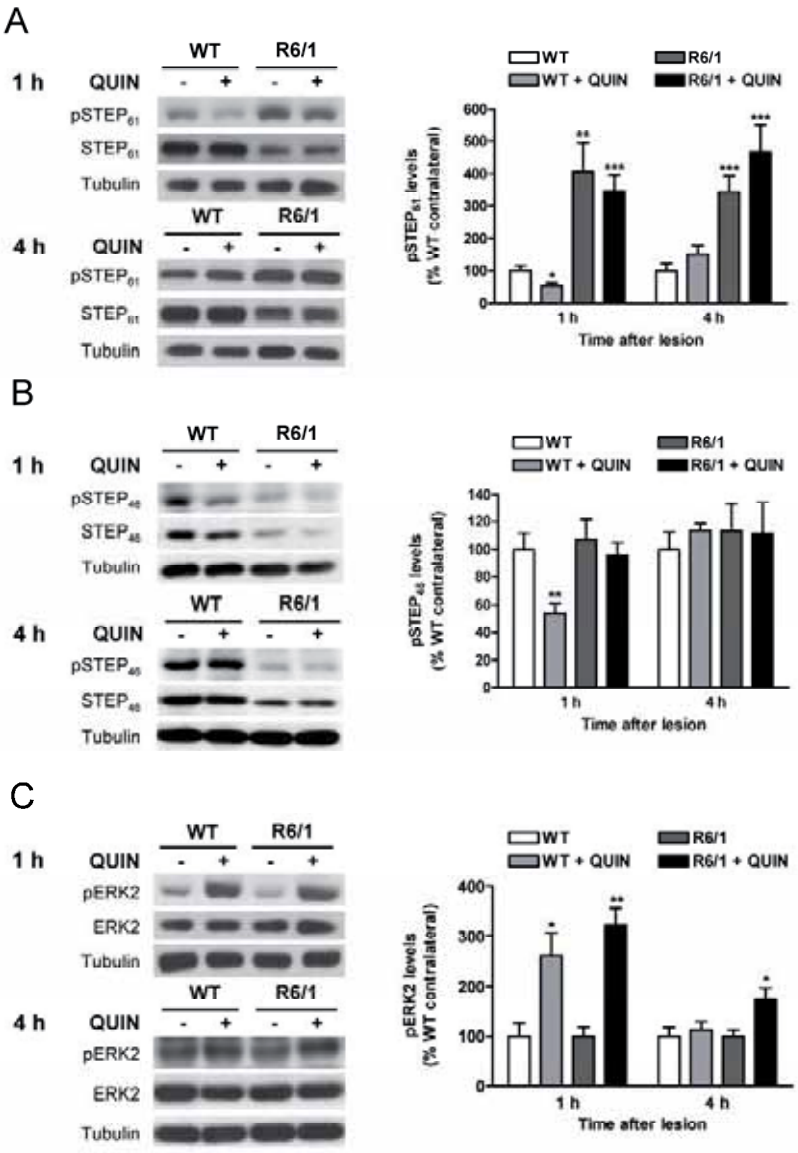
A
WT
R6/1
1 h
QUIN
- + - +
pSTEP61
STEP61
Tubulin
4 h
WT + QUIN
R6/1 + QUIN
pSTEP61 levels (% WT contralateral)
Time after lesion
B
pSTEP46
STEP46
pSTEP46 levels (% WT contralateral)
C
pERK2
ERK2
pERK2 levels (% WT contralateral)

Figure 6

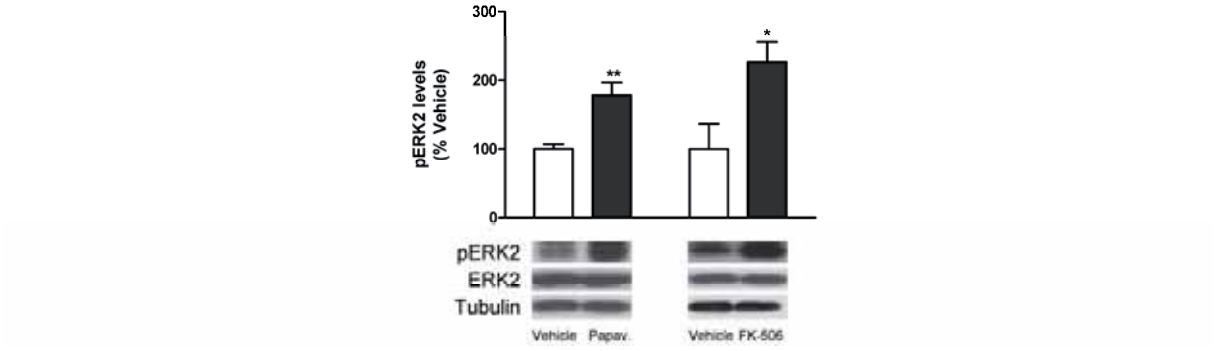
300
200
100
0
pERK2 levels
(% Vehicle)
**
*
pERK2
ERK2
Tubulin
Vehicle
Papav.
Vehicle
FK-506

Figure 7

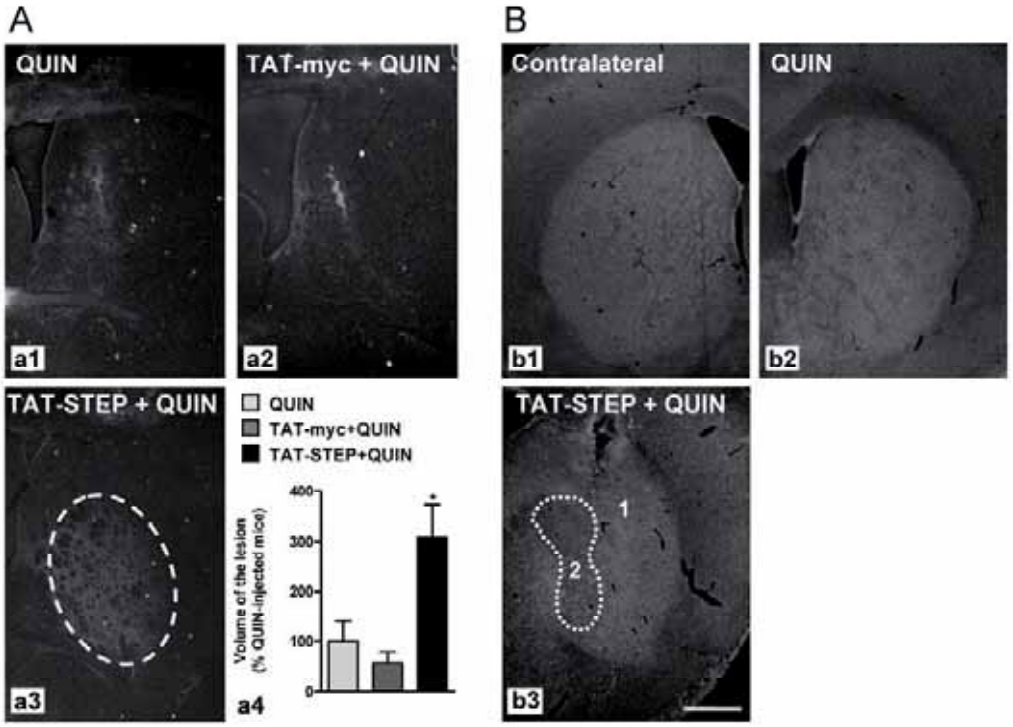
A
QUIN
TAT-myc + QUIN
a1
a2
TAT-STEP + QUIN
a3
QUIN
TAT-myc+QUIN
TAT-STEP+QUIN
Volume of the lesion (% QUIN-injected mice)
400
300
200
100
0
*
a4
B
Contralateral
QUIN
b1
b2
TAT-STEP + QUIN
1
2
b3

Figure 8

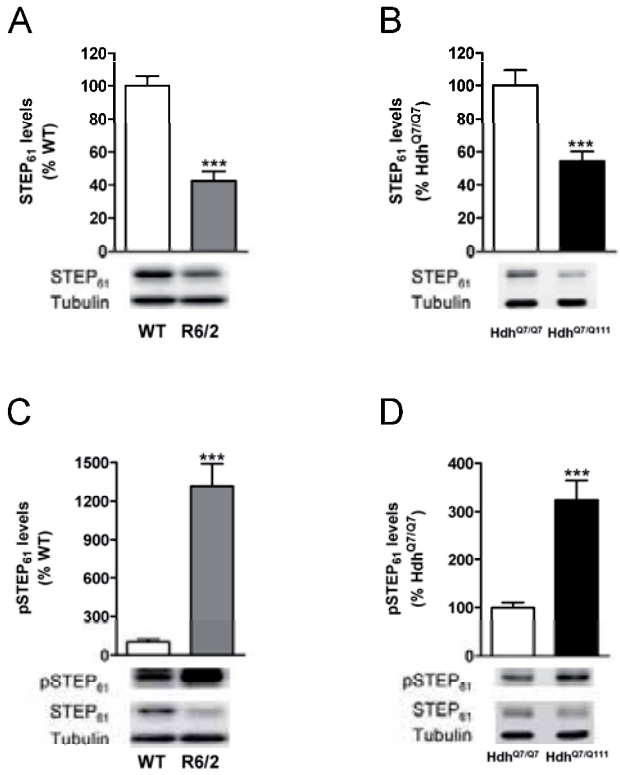
A
B
C
D
STEP61 levels (% WT)
STEP61 levels (% HdhQ7/Q7)
pSTEP61 levels (% WT)
pSTEP61 levels (% HdhQ7/Q7)
***
STEP61
Tubulin
pSTEP61
WT R6/2
HdhQ7/Q7 HdhQ7/Q111

Figure 9

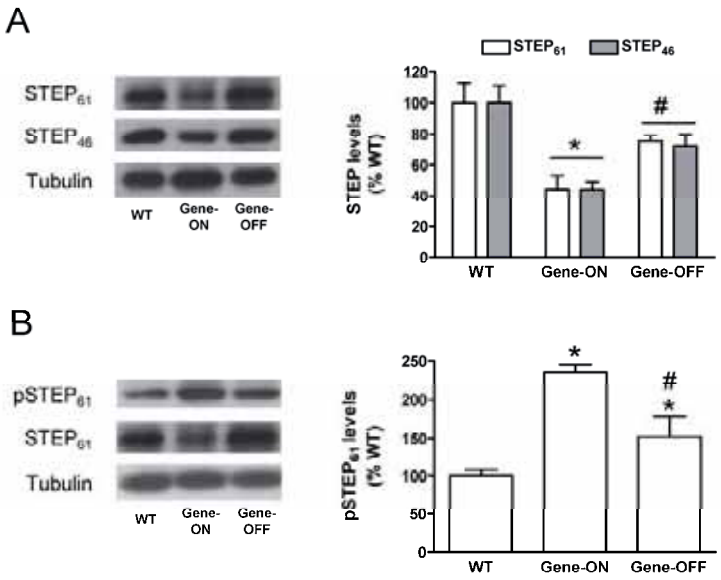
A
STEP61
STEP46
Tubulin
WT
Gene-ON
Gene-OFF
STEP61
STEP46
STEP levels (% WT)
120
100
80
60
40
20
0
*
#
WT
Gene-ON
Gene-OFF
B
pSTEP61
STEP61
Tubulin
WT
Gene-ON
Gene-OFF
pSTEP61 levels (% WT)
250
200
150
100
50
0
*
#
*
WT
Gene-ON
Gene-OFF

Table I – STEP protein expression and phosphorylation levels in the cortex and hippocampus of R6/1 mice at different ages.

		Cortex		Hippocampus	
Age (weeks)	**Genotype**	**$STEP_{61}$**	**$pSTEP_{61}$**	**$STEP_{61}$**	**$pSTEP_{61}$**
8	WT	100.0 ± 4.4	99.8 ± 8.7	100.0 ± 7.0	100.0 ± 12.8
	R6/1	96.2 ± 6.0	167.5 ± 7.8*	90.5 ± 13.8	260.4 ± 33.9**
12	WT	100.0 ± 13.1	99.9 ± 15.5	100.0 ± 5.5	100.0 ± 24.3
	R6/1	61.6 ± 7.7*	192.0 ± 0.1*	99.2 ± 5.3	516.0 ± 16.4**
30	WT	100.0 ± 8.5	99.8 ± 12.6	100.0 ± 13.0	100.0 ± 15.6
	R6/1	50.1 ± 5.3***	242.9 ± 23.8***	94.9 ± 14.9	614.2 ± 131.3*

$STEP_{61}$ and $pSTEP_{61}$ levels were analyzed by Western blot of protein extracts obtained from the cortex and hippocampus of wild-type (WT) and R6/1 mice at different stages of the disease progression. Values (obtained by densitometric analysis of Western blot data) are expressed as percentage of WT mice ($STEP_{61}$/α-tubulin and $pSTEP_{61}/STEP_{61}$ ratio after normalization with α-tubulin), and shown as mean ± SEM (n=5-8). Data were analyzed by Student's t test. $^{*}P < 0.05$; $^{**}P < 0.01$ and $^{***}P < 0.001$ as compared with wild-type mice.

Trabajo 6: ***"Increased PKA signaling is involved in the disruption of hippocampal long-term memory in Huntington's disease".*** **(Manuscrito en revisión)**

Objetivo 3: Estudio y caracterización de las vías moleculares implicadas en el inicio de las alteraciones cognitivas superiores afectadas en modelos animales de la enfermedad de Huntington.

En los trabajos previos de la presente tesis se apunta a alteraciones específicas de vías moleculares de sinapsis excitatorias en edades muy tempranas y distintos modelos animales de la enfermedad de Huntington. Estas alteraciones correlacionan en tiempo de aparición y progresión con los síntomas de aparición más temprana en la enfermedad de Huntington y en modelos murinos correspondientes. Nos referimos a los síntomas cognitivos. De ahí que nuestro siguiente objetivo fue el estudio y caracterización de las alteraciones cognitivas presentes en nuestros modelos animales de la enfermedad de Huntington y del estudio de las regiones cerebrales y vías moleculares posiblemente implicados. Para ello estudiamos el posible papel de varias moléculas que podrían mediar los déficits cognitivos detectados como ERK, calcineurina y PKA. Para ello utilizamos varios modelos de la enfermedad de Huntington exón-1, tratamientos farmacológicos en ratones *wild type* e incluso utilizamos muestras hipocampales post-mortem de enfermos con enfermedad de Huntington.

Increased PKA signaling disrupts hippocampal long-term memory in Huntington's disease

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Running title: Deregulation of PKA signaling in HD

Total number of words: 5193 (max 6000)

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 cognitive decline are still poorly understood. Here we assessed learning and memory deficits in the R6/1 mouse model of HD before the onset of motor symptoms using two different hippocampal-dependent tasks, the Novel Object Recognition Test and the spontaneous alternation in a T-maze. Neither long- nor short-term memory was affected in 4-week old animals, while 12-week old R6/1 mice showed long- but not short-term memory impairment. We next investigated the putative molecular pathways involved in these alterations. R6/1 mice hippocampus showed a PKA hyper-activation whereas ERK1/2 and calcineurin activity were not modified compared to wild-type mice. Increased PKA activity resulted in hyper-phosphorylation of its substrates, *N*-methyl-D-aspartate receptor subunit 1 (NR1), Ras-guanine nucleotide releasing factor-1 (Ras-GRF1) and STriatal-Enriched protein tyrosine Phosphatase (STEP). No changes were observed in dopamine receptor 1 (D1R), D2R, adenylyl cyclase I or V, $G_s\alpha 1$ or $G_i\alpha 1$ protein levels in the hippocampus of 12-week old R6/1 mice. In contrast, the over activation of the PKA pathway correlated with a down-regulation of the phosphodiesterase (PDE) 4 family, together with a reduction in PDE10A expression. Similar molecular changes were found in the hippocampus of R6/2 mice and HD patients. Chronic treatment of wild-type mice with the PDE inhibitors rolipram or papaverine, which lead to the up-regulation of PKA activity, induced learning and memory deficits similar to those observed in R6 mice. Thus, up-regulation of PKA signaling may occlude normal PKA-dependent processes and contribute to the early cognitive dysfunction in HD.

Keywords: NR1, Phosphodiesterases, Ras-GRF1, STEP

Abbreviations: CREB = cAMP-responsive element binding protein; D1R = dopamine receptor 1; D2R = dopamine receptor 2; ERK1/2 = extracellular signal-regulated kinase 1/2; LTM = long-term memory; mhtt = mutant huntingtin; NMDAR = *N*-methyl-D-aspartate receptor; NORT = Novel Object Recognition Test; NR1 = NMDAR subunit 1; PDE = phosphodiesterase; PKA = cAMP-dependent protein kinase A; Ras-GRF1 = Ras-guanine nucleotide-releasing factor 1; STEP = STriatal-Enriched protein-tyrosine-Phosphatase; STM = short-term memory; T-SAT = T-maze spontaneous alternation task

INTRODUCTION

Huntington's disease (HD) is a neurodegenerative disorder caused by an abnormal expansion of a CAG codon in exon-1 of the *huntingtin* gene (The Huntington's Disease Research Collaborative Group, 1993). Despite the preferential loss of medium spiny neurons from the striatum (Vonsattel *et al.,* 1985), other structures involved in cognition, like the hippocampus, are also affected at early stages of the disease (Rosas *et al.,* 2003).

HD patients show alterations in hippocampal- and striatal-dependent learning and memory, even before the onset of motor symptoms (Foroud *et al.,* 1995; Lawrence *et al.,* 1996, 1998; Lemiere *et al.,* 2004), which have been replicated in all mouse models of the disease analyzed (Giralt *et al.,* 2009; Lione *et al.,* 1999; Trueman *et al.,* 2007; Van Raamsdonk *et al.,* 2005). In this line, several electrophysiological studies carried out in different HD mouse models show aberrant hippocampal long-term potentiation (LTP) and long-term depression (LTD) (Lynch *et al.,* 2007; Milnerwood *et al.,* 2006; Murphy *et al.,* 2000). Moreover, these alterations are not restricted to the hippocampus (Cummings *et al.,* 2006, 2007; Kung *et al.,* 2007). Recent reports indicate that Brain-Derived Neurotrophic Factor (BDNF) and *N*-methyl-D-aspartate receptors (NMDARs) could be implicated in the deficiencies described in the hippocampus of HD mice (Giralt *et al.,* 2009; Lynch *et al.,* 2007). All these evidences show that cognitive processes are sensitive to the presence of mutant huntingtin (mhtt), but the molecular pathways involved in these alterations are poorly characterized.

Short- and long-term changes in the strength of synapses in neural networks underlie short-term memory (STM) and long-term memory (LTM) storage. These changes are regulated by many biochemical signaling pathways and it is necessary a balance between kinase and phosphatase activities to achieve normal memory and

plasticity processes (Kennedy *et al.,* 2005). In fact, several kinases and phosphatases play distinct roles depending on the type of memory, the time window and the plasticity phenomena (LTP or LTD) in question. Some examples are the cases of cAMP-dependent protein kinase A (PKA) (Abel and Nguyen, 2008), extracellular signal-regulated kinase 1/2 (ERK1/2) (Adams and Sweatt, 2002), calcium/calmodulin-dependent protein kinase II (CaMKII) (Colbran, 2004), protein phosphatase type-1 (PP1) (Colbran, 2004) and calcineurin (Malleret *et al.,* 2001).

Here, using the Novel Object Recognition Test (NORT) and the T-maze spontaneous alternation task (T-SAT) we show that hippocampal LTM but not STM is impaired in N-terminal exon-1 mhtt mouse models at early stages of the disease. Cognitive dysfunction correlates with increased PKA activity in the hippocampus, likely caused by the down-regulation of distinct phosphodiesterases (PDEs), and the hyper-phosphorylation of NMDAR subunit 1 (NR1), STriatal-Enriched protein-tyrosine-Phosphatase (STEP) and Ras-guanine nucleotide-releasing factor 1 (Ras-GRF1). Chronic treatment of wild-type (wt) mice with the PDE inhibitors rolipram or papaverine up-regulated PKA activity and caused learning and memory deficits similar to those observed in R6 mice. Furthermore, we report evidences showing that alterations in the PKA pathway also occur in the hippocampus of HD patients.

MATERIALS AND METHODS

HD mouse models

For this study we used male R6/1 and R6/2 heterozygous transgenic mice expressing the exon-1 of mhtt with 145 and 115 CAG repeats, respectively. Genotyping of mice and repeat length were determined as described elsewhere (Giralt *et al.,* 2009; Mangiarini *et al.,* 1996). All mice were housed together in numerical birth order in groups of mixed

genotypes, and data were recorded for analysis by microchip mouse number. The 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 h light/dark cycle. All procedures were performed in compliance with the National Institute of Health guide for the care and use of laboratory animals, and approved by the local animal care committee of *Universitat de Barcelona* (99/01), and *Generalitat de Catalunya* (99/1094).

Behavioral Analysis

NORT (Dere *et al.,* 2007) and T-SAT (Gerlai, 2001; Pang *et al.,* 2006) were used to analyze STM and LTM. For the NORT, the device consisted in a white circular arena with 40 cm diameter and 40 cm high. The light intensity was 40 lux throughout the arena and the room was kept at 19-22ºC and 40-60% humidity. Mice were first habituated to the arena in the absence of objects (3 days, 15 min/day). On the fourth day, two similar objects were presented to each mouse during 10 min (A'A" condition) after which they were returned to their home cage for 15 min. After that, the animals were placed in the arena where they were tested during 10 min with a familiar and a new object (A'B condition; STM) and then returned to the home cage. Twenty-four hours later the animals were re-tested for 10 min in the arena with a familiar and a new object (BC condition; LTM). The object preference was measured as the time exploring each object x 100/time exploring both objects. The arena was rigorously cleaned between animal trials in order to avoid odors.

The T-maze used for the T-SAT was described previously (Giralt *et al.,* 2009). In the training trial, one arm was closed (novel 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. After inter-

trial intervals of 15 min (STM) or 5 h (LTM), mice were placed in the stem arm of the T-maze and allowed to freely explore all three arms for 5 min. Different groups of wt and R6/1 mice were used for each inter-trial interval condition. The arm preference was determined by calculating the time spent in each arm x 100/time spent in both arms (familiar and novel). Animal tracking and recording was performed using the automated SMART junior software (Panlab, Spain). The effects of motivation, locomotor activity and anxiogenic components on the learning tasks were also evaluated.

Pharmacological treatments

Twenty-four B6CBA male mice were obtained from Charles River (Charles River Laboratories, Les Oncis, France). All animals were handled as described above. To allow accurate comparisons with the behavioral experiments conducted in R6/1 mice, all pharmacological treatments were performed between 10 and 13 weeks of age. Animals (n = 8/group) received a chronic treatment (23 days) with daily intraperitoneal (i.p.) injections of rolipram (Sigma, St. Louis, MO; 5 mg/kg), papaverine (Sigma; 20 mg/kg) or vehicle (distilled water). Mice performed the NORT between days 18-22 of treatment. To avoid the acute relaxant effects of the drugs animals were habituated, trained and tested 1.5-2 h post-injection. Mice received the last i.p. injection at day 23 and were sacrificed by decapitation 10 min later.

Total protein extraction

Mice were killed by decapitation at the age of 4, 12 or 30 weeks (wt and R6/1), 8 weeks (wt and R6/2) or 13 weeks (wt mice receiving pharmacological treatments) and the hippocampi were quickly removed. Hippocampal samples from control subjects (post-mortem intervals 3–16 h, with death at 39, 60, 64 and 71 years) and HD patients (post-

mortem intervals 4–17 h, with death at end-stage disease at 28 (juvenile onset HD patient), 59, 60 and 72 years (Vonsattel grade IV)) were obtained from the University of Barcelona and the Institute of Neuropathology Brain Banks (Barcelona, Spain) following the guidelines of the local ethics committees. Tissue was homogenized in lysis buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 100 mM NaF, 5 μM $ZnCl_2$ and 10 mM EGTA] plus protease inhibitors [phenylmethylsulphonyl fluoride, PMSF (2 mM), aprotinin (1 μg/ml), leupeptin (1 μg/ml) and sodium orthovanadate (1 mM)] and centrifuged at 16100 x *g* for 20 min. The supernatants were collected and the protein concentration was measured using the Dc protein assay kit (Bio-Rad, Hercules, CA).

Western blot analysis

Western blot analysis was performed as previously described (Saavedra *et al.,* 2010). The primary antibodies used were: anti-PKA catalytic subunit α (PKAc; 1:1000), anti-STEP (1:1000), anti-Ras-GRF1 (1:1000), anti-adenylyl cyclase (AC) I, anti-AC V, anti-dopamine receptor D1 and anti-D2 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA); anti-phospho(Ser/Thr) PKA substrates, anti-cAMP-responsive element binding protein (CREB), anti-pERK1/2 and anti-pRas-GRF1^{ser916} (1:1000; Cell Signaling, Beverly, MA); anti-PDE4 and anti-PDE10A (1:1000; Abcam, Cambridge, UK); anti-$G_s\alpha 1$ and anti-$G_i\alpha 1$ subunits (1:1000; Calbiochem, La Jolla, CA); anti-panERK1/2 (1:5000) and anti-calcineurin A (1:500; BD Transduction Laboratories, San Diego, CA); anti-pNR1^{ser897} (1:1000; Upstate, Lake Placid, NY); anti-NR1 (1:1000; Chemicon, Temecula, CA) and anti-pCREB^{ser133} (1:1000; Millipore, Temecula, CA). The anti-pSTEP antibody (1:1000) was a kind gift from Dr. Paul J. Lombroso (Yale University School of Medicine, New Haven, CT). Loading control was performed by reprobing the

membranes with an anti-α-tubulin (1:50000; Sigma) antibody during 20 min at room temperature. Then, membranes were washed with TBS-T, incubated for 1 h at room temperature with the corresponding horseradish peroxidase-conjugated antibody (1:2000; Promega, Madison, WI) and washed again twice with TBS-T. Immunoreactive bands were visualized using the Western Blotting Luminol Reagent (Santa Cruz Biotechnology) and quantified by a computer-assisted densitometer.

Calcineurin activity

Calcineurin activity was measured in 4-, 12- and 30-week old wt and R6/1 mice using protein extracts obtained from fresh hippocampi as described elsewhere (Xifro *et al.,* 2009).

Statistical analysis

All data are expressed as mean ± s.e.m. Statistical analyses were performed by using the unpaired Student's t-test (95% confidence) or one-way ANOVA with Student's t-test as a *post hoc* as appropriate and indicated in the figure legends. Values of $p < 0.05$ were considered as statistically significant.

RESULTS

Hippocampal-dependent LTM, but not STM, is altered in R6/1 mice

R6/1 mice were subjected, before the onset of motor symptoms, to two low-stressful tasks, the NORT and the T-SAT, which enable to distinguish between hippocampal STM and LTM (Dere *et al.,* 2007; Gerlai, 2001; Pang *et al.,* 2006; Wood *et al.,* 2000). In the NORT, we first habituated all mice to the open field arena and ambient conditions during 3 consecutive days by allowing them to freely explore the

environment during 15 min/day. After the habituation process, animals were subjected to a training session in the arena during 10 min in the presence of two similar objects (A' and A"). Four- (Fig. 1A) and 12- (Fig. 1B) week old wt and R6/1 mice similarly explored the object A' and A" indicating no object or place preferences. Animals assessed for STM explored with greater preference the new object without significant differences between genotypes indicating that hippocampal-dependent STM is not impaired in R6/1 mice at either 4 (Fig. 1A) or 12 (Fig. 1B) weeks of age. At 4 weeks of age, when assessing LTM we observed that both wt and R6/1 mice explored more the new object respect to the old one without differences between genotypes (Fig. 1A). In contrast, at 12 weeks of age, although both wt and R6/1 mice explored more the new object respect to the old one, the preference of R6/1 mice for the new object was significantly reduced compared to that registered in wt animals indicating specific LTM deficits in R6/1 mice at this age (Fig. 1B). Motivation, locomotor activity and anxiety levels were also analyzed by recording the number of defecations, distance traveled and centre/periphery ratio. No significant differences were observed in any condition or variable measured, but R6/1 mice tended to be less anxious (Supplementary Fig. 1A). Thus, motivational, locomotor or anxiogenic factors did not significantly affect the learning task.

To further validate these results, 12-week old mice were also subjected to the T-SAT. In this task one evaluates the time a mouse spends exploring a familiar and novel arm in a T-maze (Pang *et al.,* 2006). When STM was evaluated, we found that both wt and R6/1 mice spent more time in the novel than in the familiar arm (Fig. 1C). Similar results were obtained when assessing LTM in wt mice (Fig. 1C). In contrast, R6/1 mice did not show preference for the novel arm respect to the familiar one (Fig. 1C). Additionally, defecations and the number of arms entries were also quantified and no

significant differences were observed between wt and R6/1 mice indicating no effect of anxiogenic components on the task (Supplementary Fig. 1B). Thus, in the T-SAT 12-week-old R6/1 mice also showed LTM, but not STM, deficits.

PKA activity is increased in R6/1 mice hippocampus correlating with the presence of hippocampal-dependent LTM deficits

The specific hippocampal-dependent LTM alterations observed in R6/1 mice led us to hypothesize that the molecular pathways implicated in the formation and maintenance of hippocampal LTM could be altered in the presence of mhtt. ERK1/2, calcineurin and PKA are thought to play distinct roles in the formation and maintenance of LTM (Abel and Nguyen, 2008; Adams and Sweatt, 2002; Mansuy *et al.,* 1998; Riedel, 1999). Thus, we next studied their phosphorylation/activity levels in the hippocampus of R6/1 mice in a time course representative of the disease progression. The analysis of hippocampal phosphorylated (p)ERK1/2 levels revealed that R6/1 mice did not show alterations when compared to wt mice until 30 weeks of age (a late stage of the disease), when a significant decrease of ERK1/2 phosphorylation levels was detected (Fig. 2A). Calcineurin protein levels in the hippocampus of R6/1 mice were similar to those observed in wt animals at all ages analyzed (data not shown). Similarly, there were no significant differences between wt and R6/1 mice on calcineurin activity at any age either (Fig. 2B). Finally, PKA activity was evaluated by using an antibody that detects its serine/threonine substrates when phosphorylated at the PKA consensus region. At 4 weeks of age PKA activity was not altered in R6/1 mice hippocampus (Fig. 2C). However, at 12 (at the same age where LTM deficits were detected) and 30 weeks of age PKA activity was significantly increased in the hippocampus of R6/1 mice as compared to their littermate controls (Fig. 2C). Since the increased phosphorylation

level of PKA substrates was a phenomenon that correlated with the early cognitive deficits in R6/1 mice we further analyzed, at 12 weeks of age, the phosphorylation levels of specific PKA substrates involved in plasticity and memory processes, such as CREB (Abel and Nguyen, 2008), NR1 subunit of NMDAR (Chen and Roche, 2007; Niewoehner *et al.,* 2007; Tingley *et al.,* 1997), Ras-GRF1 (Brambilla *et al.,* 1997; Yang *et al.,* 2003) and STEP (Braithwaite *et al.,* 2006; Paul *et al.,* 2000). We detected increased levels of $pNR1^{ser897}$ (Fig. 2D), $pRas\text{-}GRF1^{ser916}$ (Fig. 2E) and $pSTEP_{61}{}^{ser221}$ (Fig. 2F) whereas total protein levels were not modified. Surprisingly, $pCREB^{ser133}$ levels were not altered in the hippocampus of 12-week old R6/1 mice ($p = 0.7426$). These results indicate that among the pathways examined only the basal PKA activity was modified in the hippocampus of 12-week old R6/1 mice, and the abnormal phosphorylation of the PKA substrates $NR1^{ser897}$, $Ras\text{-}GRF1^{ser916}$ and $STEP_{61}{}^{ser221}$ may contribute to R6/1 mice hippocampal LTM deficits.

Increased hippocampal PKA activity correlates with a decrease of PDE 4 and 10A protein levels in R6/1 mice

Our next aim was to determine the origin of increased PKA activity in R6/1 hippocampus. Alterations in striatal D1R and D2R expression are a well-characterized hallmark of HD (Cha *et al.,* 1998; Jakel and Maragos, 2000; Ariano *et al.,* 2002), and changes in adenylyl cyclase expression were also reported in the striatum of exon-1 mouse models (Luthi-Carter *et al.,* 2000). However, fewer data are available on the expression of these proteins in the hippocampus of HD mice. Here we measured the protein levels of dopamine receptors D1 and D2, ACs I and V, $G_i\alpha1$ and $G_s\alpha1$ in R6/1 mice at 12 weeks of age, when hippocampal-dependent LTM deficits were detected in these animals. Similar total protein levels of D1R, D2R, AC I, AC V, $G_s\alpha1$ and $G_i\alpha1$

were detected in 12-week old wt and R6/1 mice hippocampus (Supplementary Fig. 2). Since PDEs are important regulators of PKA activity through the regulation of cAMP levels we analyzed their expression focusing on PDE4 and PDE10A. Interestingly, the hippocampus of R6/1 mice exhibited a significant reduction in the protein levels of different PDE4 isoforms, namely PDE4AX, PDE4D1 and PDE4D3 whereas there were no significant changes in PDE4A5 or PDE4A1 expression (Fig. 3A). Similarly, the protein levels of PDE10A were also reduced in the R6/1 hippocampus (Fig. 3B). Thus, our results suggest that reduced PDE4 and PDE10A protein levels could result in subsequent hyper-activation of PKA in the hippocampus of R6/1 mice.

Chronic pharmacological inhibition of PDE4 or PDE10A results in LTM, but not STM, deficits

We hypothesized that the down-regulation of hippocampal PDE4 and PDE10A protein expression could underlie the LTM deficits observed in R6/1 mice in a PKA-dependent manner. To address our hypothesis, we mimicked in wt mice the PKA over-activation through the chronic injection (22 days) of the PDE4 and PDE10A inhibitors rolipram and papaverine, respectively, followed by assessment of STM and LTM using the NORT. During the training session vehicle-, rolipram- and papaverine-treated mice showed similar object preference (Fig. 4A). Neither rolipram nor papaverine treatment impaired hippocampal-dependent STM as all mice explored with greater preference the new object respect to the old one without significant differences between groups (Fig. 4A). Concerning LTM, we observed that all groups explored more the new object respect to the old one but the preference for the new object in rolipram- or papaverine-treated mice was significantly reduced compared to that registered in the vehicle group (Fig. 4A). In order to rule out the influence of rolipram- or papaverine-induced

alterations in motor activity and anxiety levels on the performance of the task, we also monitored the time spent in the centre, the distance traveled and the number of defecations. No differences were detected between groups during training, short- or long-term memory assessment, indicating that alterations in spontaneous locomotor activity or anxiety levels are unlikely to affect the performance of rolipram- or papaverine-treated mice in the NORT (Supplementary Fig. 3). Therefore, these results show that chronic treatment with the PDE inhibitors rolipram or papaverine induces in wt mice specific LTM deficits similar to those observed in R6/1 mice. and support the view that a reduction in PDE activity in the hippocampus of R6/1 mice may underlie a PKA-dependent LTM impairment.

We next checked if the hippocampal-dependent LTM deficits observed in mice treated with PDE inhibitors were associated with increased PKA activity and the consequent hyper-phosphorylation of its substrates. Treatment with rolipram or papaverine for 23 days increased PKA activity in the hippocampus (Fig. 4B). Both rolipram and papaverine increased $pNR1^{ser897}$ levels (Fig. 4C) whereas $pRas\text{-}GRF1^{ser916}$ and $pSTEP_{61}{}^{ser221}$ levels were only increased after papaverine (Fig. 4D) and rolipram (Fig. 4E) treatment, respectively. Furthermore, papaverine treatment also increased hippocampal NR1 expression (284 ± 44% respect to vehicle-injected mice; $p < 0.01$).

This set of results demonstrates that chronic inhibition of different PDEs is sufficient to induce hippocampal-dependent LTM deficits without effects on STM, as observed in R6/1 mice, and support the view that a reduction in PDE activity in the hippocampus of R6/1 mice may underlie a PKA-dependent LTM impairment.

R6/2 mice replicate the major hippocampal alterations observed in R6/1 mice

Next, we analyzed if our findings could be replicated in R6/2 mice, which show a more severe phenotype (Canals *et al.,* 2004; Carter *et al.,* 1999). STM and LTM were analyzed using the NORT in 8-week old R6/2 mice. At this age, our R6/2 colony is still pre-symptomatic and mice do not show clear motor impairment until 9-10 weeks of age (Giralt *et al.,* unpublished results). Similar to R6/1 mice, R6/2 animals suffered LTM but not STM alterations (Fig. 5A). The hippocampus of R6/2 mice also showed increased levels of PKA activity (Fig. 5B), pRas-GRF1^{ser916} (Fig. 5C) and pSTEP$_{61}$^{ser221} (Fig. 5D), without changes in pCREB^{ser133} (p = 0.7495). Furthermore, we found a prominent down-regulation of several PDE4 isoforms (Fig. 5E). These results lend further support to the idea that hippocampal-dependent LTM deficits induced by mhtt may be caused by PKA over-activation.

Human hippocampal HD samples also reveal an increase in PKA function

We finally analyzed if the hallmarks found in R6 mice hippocampus were also present in the hippocampus of HD patients. As observed in R6 mice, we detected an up-regulation of PKA activity in the hippocampus of HD patients when compared to control cases (Fig. 6A), which correlated with increased levels of pNR1^{ser897} (Fig. 6B). Similar to R6 mice, D1R (Supplementary Fig. 4A), D2R (Supplementary Fig. 4B) and AC V (Supplementary Fig. 4C) protein levels were unchanged in human HD post-mortem hippocampal samples compared to control cases, further validating the findings in mouse models.

DISCUSSION

In the present work we show that in R6 mouse models of HD hippocampal LTM is more prone to impairment than STM. This specific cognitive decline correlates with

hyper-activity of the PKA pathway that could be the result of reduced expression of several members of the PDE4 family and PDE10A. In accordance, chronic inhibition of PDE4 or PDE10A in wt mice leads to PKA hyper-activity and hippocampal LTM deficits. Finally, we show that some of the molecular alterations in this pathway are also present in the hippocampus of HD patients.

Our results show deficits in hippocampal-dependent learning and memory in two HD mouse models before the onset of motor symptoms. R6 mice showed a clear inability to identify a novel object in the NORT paradigm, as well as less new environment/context preference than wt mice in the T-SAT. Accordingly, previous studies in HD mouse models have shown deficits in hippocampal-dependent cognition before the onset of motor symptoms (Lione *et al.,* 1999; Murphy *et al.,* 2000; Simmons *et al.,* 2009). Here, we demonstrate that the molecular pathways involved in the induction and maintenance of LTM are more vulnerable to the presence of mhtt than those implicated in STM. Accordingly, R6/1 mice do not show STM alterations in the NORT until 14 weeks of age (Nithianantharajah *et al.,* 2008) whereas LTM deficits can be found at least at 12 weeks of age (present results).

The crucial role of PKA activation in the formation and maintenance of LTM dependent on the hippocampus is largely known (Abel and Nguyen, 2008). However, whereas the direct or indirect activation of PKA can enhance memory storage in a time window of 3–6 h after training (Bernabeu *et al.,* 1997), abnormally active basal PKA signaling can occlude normal PKA-dependent processes leading to cognitive impairment. We found increased hippocampal PKA signaling in naïve R6 mice, which suggests a putative role for this pathway in their cognitive decline. ERK1/2 and calcineurin are also classic mediators of hippocampal-dependent LTM processes (Adams and Sweatt, 2002; Mansuy *et al.,* 1998; Riedel, 1999), and we cannot rule out

that alterations in ERK1/2 or calcineurin activity in the hippocampus of R6/1 mice after training or in the STM to LTM transition phase might contribute to LTM deficiencies. Nevertheless, the absence of alterations in these pathways in naïve R6/1 animals when we first registered hippocampal-dependent LTM deficits excludes the possibility of their implication in an occlusion mechanism as we propose in the case of hyperactive PKA pathway. In agreement, recent evidences indicate that aberrant and sustained activation of cAMP-PKA signaling can lead to long-lasting plasticity and LTM deficits (Bourtchouladze *et al.,* 2006; Horiuchi *et al.,* 2008; Kelly *et al.,* 2008; Pineda *et al.,* 2004). In this context, it is worth mentioning that manipulations that increase cAMP signals lead to memory enhancements whereas increases in basal cAMP levels lead to memory impairments (Abel and Nguyen, 2008).

Increased levels of hippocampal PKA activity in HD mice could be related to the reduced expression of PDEs. In fact, PDEs expression is decreased in the striatum or/and cortex of N-terminal exon-1 mouse models of HD (Hebb *et al.,* 2004; Hu *et al.,* 2004), and here we extend these observations to the hippocampus. Reinforcing our hypothesis, we show that chronic treatment of wt mice with rolipram or papaverine, which up-regulated PKA activity, induced learning and memory deficits similar to those observed in R6 mice. Similarly, it has been previously shown in rodents that chronic inhibition of PDE4 (Giorgi *et al.,* 2004) or PDE10A (Hebb *et al.,* 2008) can induce LTM deficits. Furthermore, knock-out mice for PDE4D (Rutten *et al.,* 2008) or PDE10A (Piccart *et al.,* 2010) show learning and memory impairment. Thus, the severe down-regulation of several PDEs can participate in the chronic over-activation of the PKA pathway in the hippocampus of R6 mice, and help to explain their specific hippocampal LTM impairment. Although PDE inhibitors are proposed as a promising therapeutic tool for cognition enhancement (Reneerkens *et al.,* 2009), our results draw

attention to the risk of using chronic treatment with PDEs inhibitors as cognitive enhancers that would result in prolonged activation of PKA pathway.

Up-regulation of basal PKA activity in HD mouse models can produce learning and memory deficits by several mechanisms. Since hippocampal PKA can induce the phosphorylation and activity of both ERK and CREB (Nguyen and Woo, 2003), the lack of alterations in their activities when memory impairments were observed in R6/1 mice suggest the participation of other PKA substrates in an occlusion mechanism. The selective effect of increased PKA activity on specific substrates in the hippocampus of R6/1 mice could be explained by changes in PKA signaling being restricted to discrete cellular microdomains.

Here we propose that PKA-induced hyper-phosphorylation of NR1, Ras-GRF1 or/and STEP, which are involved in plasticity and memory processes (Braithwaite *et al.,* 2006; Brambilla *et al.,* 1997; Chen and Roche, 2007; Hopf and Bonci, 2009; Niewoehner *et al.,* 2007; Yang *et al.,* 2003), could be responsible for memory deficits in R6/1 mice. First, the PKA-dependent over-activation of NR1 (present results) could be a mechanism underlying the cognitive deficits in R6 mice since up-regulation of this NMDAR subunit has been associated with hippocampal learning and memory alterations (Sahun *et al.,* 2007; Shi *et al.,* 2006). Second, changes in Ras-GRF1 alter hippocampal LTM but not STM (Brambilla *et al.,* 1997), and phosphorylation of Ras-GRF1^{ser916} is involved in the induction of LTD (Li *et al.,* 2006). In accordance, PKA activation can induce LTD in the dentate gyrus and in the medial perforant pathway of the hippocampus (Huang *et al.,* 1999a,b), and inhibition of PDE4 with rolipram strengths hippocampal LTD (Navakkode *et al.,* 2005). Interestingly, R6 mice show an aberrant facilitation of LTD expression in the hippocampus (Milnerwood *et al.,* 2006; Murphy *et al.,* 2000). Additionally, STEP is thought to play a role in synaptic plasticity

(Braithwaite *et al.,* 2006; Hopf and Bonci, 2009). Since STEP phosphorylation inhibits its phosphatase activity (Paul *et al.,* 2000) this could be translated directly, or indirectly via the kinase Fyn, into increased activity of the NMDAR subunit 2B (Braithwaite *et al.,* 2006), possibly leading to the induction of LTD (Li *et al.,* 2006).

It is noteworthy that cAMP immunoreactivity (Ariano *et al.,* 2002, 2005) is increased in the striatum of R6 mouse models of HD. Furthermore, some PKA substrates as STEP and NR1 are hyper-phosphorylated in the striatum at pre-symptomatic stages in exon-1 and full-length mouse models of HD (Torres-Peraza *et al.,* 2008; Saavedra *et al.*, unpublished results). In this line, our group and others have also demonstrated cognitive deficits in striatal-dependent learning tasks in R6 mice (Giralt *et al.,* 2009; Lione *et al.,* 1999). Thus, it is tempting to speculate that aberrant PKA signaling is a global event in HD so that striatal-dependent cognitive deficiencies may also be affected by a deregulation in this molecular pathway. The possibility of a global alteration in PKA activity in HD might have therapeutic implications (Arnsten *et al.,* 2005).

In summary, we demonstrate specific hippocampal-dependent LTM deficits in R6 mice early during disease progression and we propose that PKA hyper-activation, likely due to the down-regulation of distinct PDEs, is implicated in this cognitive impairment (Fig. 7). Our proposal is further supported by the finding that chronic treatment of wt mice with PDE inhibitors caused learning and memory deficits similar to those observed in R6 mice. Our data indicate that aberrant basal PKA hyper-activity is sufficient to induce LTM impairment in HD mice likely due to occlusion of normal PKA-dependent learning. Thus, increased PKA activity in HD mice, together with other known mhtt-induced alterations affecting synaptic plasticity, can act as a major contributor to cognitive decline in HD. Finally, we report evidences showing that the

alteration in this pathway is a common feature in the hippocampus of mouse models and HD patients.

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FIGURE LEGENDS

Figure 1 Both the NORT and the T-SAT reveal deficits in hippocampal-dependent LTM but not STM in R6/1 mice. Wt and R6/1 mice were subjected to the NORT to evaluate hippocampal-dependent STM and LTM. To assess STM, preference for an original object (A') and a new object (B) was quantified 15 min after training. LTM was assessed 24 h after training (old object B and new object C). Graphs show the percentage of object preference in 4-week old (**A**) and in 12-week old (**B**) wt and R6/1 mice during STM and LTM assessment. (**C**) STM and LTM were also assessed by the T-SAT in two independent groups of 12-week old wt and R6/1 mice. Animals were tested for novel arm preference 15 min (STM) or 5 h (LTM) after the training trial. Bars represent mean ± s.e.m. (n = 12-17/group in the NORT and n = 7-8/group in the T-SAT). All data were analyzed by one-way ANOVA with Student's t-test as a *post hoc*. * $p < 0.05$ and *** $p < 0.001$ as compared with the percentage of preference for the familiar object/arm, and $$$ $p < 0.001$ as compared with the percentage of preference for the new object in wt mice.

Figure 2 PKA activity and the phosphorylation status of its substrates, but not ERK1/2 or calcineurin activities, are altered in R6/1 hippocampus. pERK1/2 (**A**) and p(Ser/Thr) PKA substrates (**C**) levels were analyzed by Western blot of hippocampal samples from wt and R6/1 mice at different ages. Calcineurin activity (**B**) was determined in hippocampal samples from wt and R6/1 mice at the same ages by using a calcineurin assay kit. The serine phosphorylation levels of NR1 (**D**), Ras-GRF1 (**E**) and $STEP_{61}$ (**F**) were analyzed by Western blot of protein extracts obtained from the hippocampus of 12-week old wt and R6/1 mice. Representative immunoblots from 12-week old wt and R6/1 mice are shown (**C, D, E, F**). Values (obtained by densitometric analysis of Western blot data, except in **B**) are expressed as percentage of wt mice and shown as mean ± s.e.m. (n = 4-7). Data were analyzed by Student's t-test. * $p < 0.05$ and ** $p < 0.01$ as compared with wt mice.

Figure 3 Several PDE4 isoforms and PDE10A protein levels are reduced in the hippocampus of R6/1 mice. The protein levels of different PDE4 isoforms (**A**) and PDE10A (**B**) were analyzed by Western blot of hippocampal samples from 12-week old wt and R6/1 mice. Representative immunoblots are shown. Values (obtained by densitometric analysis of Western blot data) are expressed as percentage of wt mice, and shown as mean ± s.e.m. (n = 4-6). Data were analyzed by Student's t-test. * $p < 0.05$ and ** $p < 0.01$ as compared with wt mice.

Figure 4 Chronic pharmacological inhibition of PDE4 or PDE10A in wt mice induces memory and biochemical alterations similar to those observed in R6/1 mice. (**A**) Ten-week old wt mice were treated during 22 days with i.p injections of rolipram (5 mg/kg), papaverine (20 mg/kg) or vehicle. At days 18-22 all groups were subjected to the NORT

to evaluate hippocampal-dependent STM and LTM. Preference for a new object respect to an old one was quantified 15 min (STM) or 24 h (LTM) after the training session. Bars represent mean ± s.e.m. (n = 8/group). All data were analyzed by one-way ANOVA with Student's t-test as a *post hoc* for each condition. * $p < 0.05$ as compared with the percentage of preference for the new object in the vehicle group. PKA activity (**B**), PKA-dependent phosphorylation levels of $NR1^{ser897}$ (**C**), Ras-$GRF1^{ser916}$ (**D**) and $STEP_{61}{}^{ser221}$ (**E**) after treatment with PDE inhibitors were analyzed by Western blot of hippocampal samples from vehicle-, rolipram- and papaverine-treated wt mice. Representative immunoblots are presented. Values (obtained by densitometric analysis of Western blot data) are expressed as mean ± s.e.m. Data were analyzed by one-way ANOVA with Student's t-test as a *post hoc*. * $p < 0.05$ and ** $p < 0.01$ as compared to the vehicle group.

Figure 5 Hippocampal alterations observed in R6/1 mice are replicated in R6/2 mice. (**A**) Eight-week old wt and R6/2 mice were subjected to the NORT to evaluate hippocampal-dependent STM and LTM. To assess STM the preference for an original object (A') and a new object (B) was quantified 15 min after training. LTM was assessed 24 h after training (old object B and new object C). Bars represent mean ± s.e.m. (n = 8/group). Data were analyzed by one-way ANOVA with Student's t-test as a *post hoc*. * $p < 0.05$ and *** $p < 0.001$ as compared with the percentage of preference for the familiar object, and $ $p < 0.05$ as compared with the percentage of preference for the new object in wt mice. (**B**) p(Ser/Thr)PKA substrates, (**C**) pRas-$GRF1^{ser916}$, (**D**) $pSTEP_{61}{}^{ser221}$ and (**E**) PDE4 isoforms levels were analyzed by Western blot in hippocampal samples obtained from wt and R6/2 mice at 8 weeks of age. Values (obtained by densitometric analysis of Western blot data) are expressed as percentage of

wt mice, and shown as mean ± s.e.m. (n = 4-7). Data were analyzed by Student's t-test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ as compared to wt mice.

Figure 6 The hippocampus of HD patients shows increased p(Ser/Thr)PKA substrates levels and pNR1^{ser897} levels. p(Ser/Thr)PKA substrates and pNR1^{ser897} levels were analyzed by Western blot of hippocampal samples from control (CTL) and HD post-mortem brains. Values (obtained by densitometric analysis of Western blot data) are expressed as percentage of control, and shown as mean ± s.e.m. Data were analyzed by Student's t-test. * $p < 0.05$ and ** $p < 0.01$ as compared to control subjects.

Figure 7 Molecular mechanisms proposed to underlie the hippocampal-dependent LTM impairment in R6 mice. We propose that in the hippocampus of R6 mice, in the absence of changes in the expression of dopamine receptors D1 and D2, AC I, AC V, $G_s\alpha1$ and $G_i\alpha1$, the reduced expression of PDE4 and PDE10A induced by mhtt results in an abnormal stimulation of the PKA activity. The down-regulation of PDE expression could be due to mhtt-induced transcriptional deregulation (Hebb *et al.,* 2004; Hu *et al.,* 2004). The increased PKA activity results in aberrant NR1, Ras-GRF1 and STEP phosphorylation, which may participate in occlusion mechanisms of PKA-dependent hippocampal-dependent LTM formation and/or maintenance. These biochemical alterations could explain the specific hippocampal-dependent LTM deficits in HD mouse models and possibly the altered expression of hippocampal LTD previously observed in R6/1 and R6/2 mice (see discussion).

Supplementary Figure 1 Spontaneous locomotor activity and anxiety levels do not significantly affect the performance of mice in either the NORT or the T-SAT. We

monitored covered the distance traveled, the number of defecations and the time spent in the centre of the open field in the NORT (**A**) and number of arm entries and defecations in the T-SAT (**B**). No significant differences were detected in any condition between genotypes indicating that neither locomotor activity nor anxiety levels influence the performance in either task. Bars represent mean ± s.e.m. (n = 12-17/group in the NORT and n = 7-8/group in the T-SAT). All data were analyzed by one-way ANOVA with Student's t-test as a *post hoc*.

Supplementary Figure 2 The expression of D1R, D2R, AC I, AC V, $G_s\alpha1$ and $G_i\alpha1$ subunits is unaltered in the hippocampus of R6/1 mice. The protein levels of D1R (**A**), D2R (**B**), AC I (**C**), AC V (**D**), $G_s\alpha1$ (**E**) and $G_i\alpha1$ (**F**) were analyzed by Western blot of hippocampal samples from 12-week old wt and R6/1 mice. Representative immunoblots are shown. Values (obtained by densitometric analysis of Western blot data) are expressed as percentage of wt mice, and shown as mean ± s.e.m. (n = 4-9).

Supplementary Figure 3 Spontaneous locomotor activity and anxiety levels do not significantly affect the performance of rolipram- or papaverine-treated mice in the NORT. We monitored the distance traveled, the number of defecations and the time spent in the centre of the open field in vehicle-, rolipram- and papaverine-treated mice. Note that no differences were detected between groups during training (Trai), STM or LTM assessment indicating that neither locomotor activity nor anxiety levels influence the performance of the task. It is noteworthy that mice were tested 1.5-2 h after drug injection. Bars represent mean ± s.e.m. (n = 8/group). All data were analyzed by one-way ANOVA with Student's t-test as a *post hoc* for each condition. * $p < 0.05$ as compared with the vehicle group.

Supplementary Figure 4 D1R, D2R and AC V protein levels are not altered in the hippocampus of HD patients. D1R, D2R and AC V levels were analyzed by Western blot of hippocampal samples from control (CTL) and HD post-mortem brains. Values (obtained by densitometric analysis of Western blot data) are expressed as percentage of control, and shown as mean ± s.e.m.

Figure 1

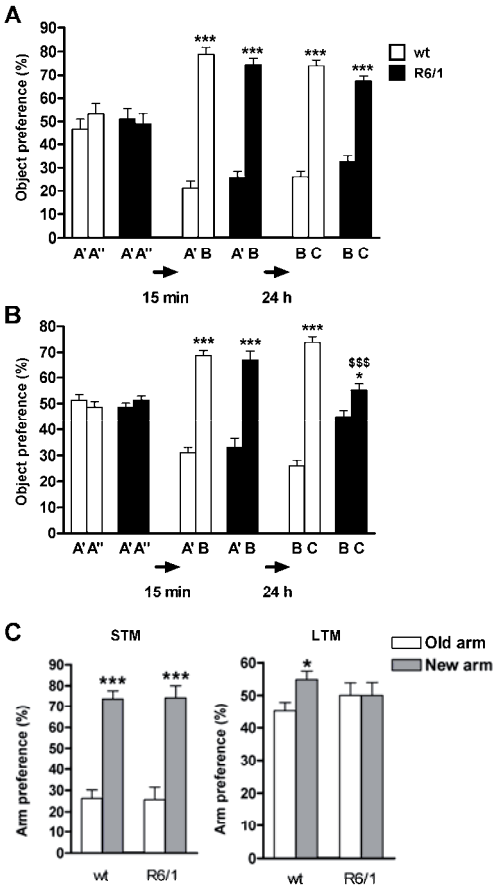

A
Object preference (%)
wt
R6/1
A' A''
A' A''
A' B
A' B
B C
B C
15 min
24 h
B
Object preference (%)
$$$
C
STM
LTM
Old arm
New arm
Arm preference (%)
wt
R6/1

Figure 2

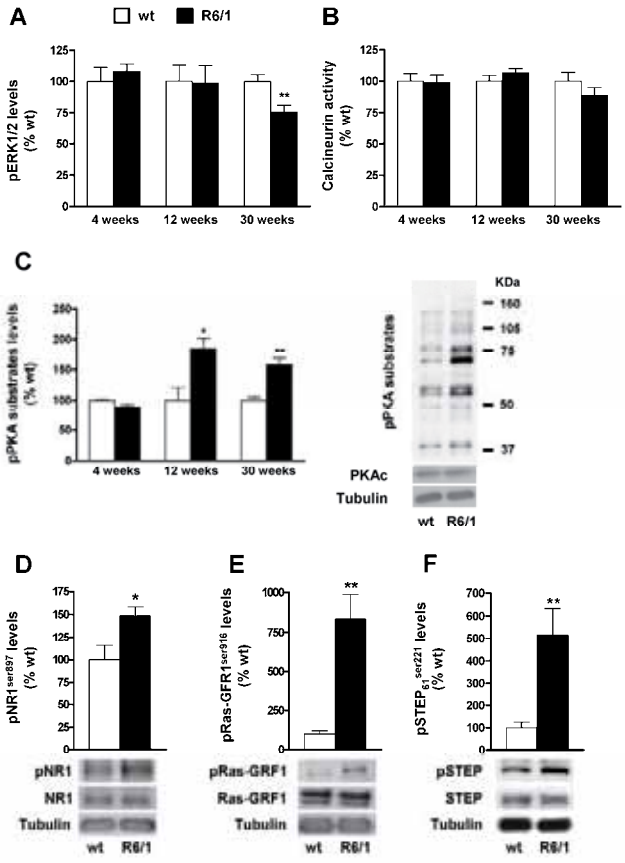

A
wt
R6/1
pERK1/2 levels (% wt)
4 weeks
12 weeks
30 weeks
B
Calcineurin activity (% wt)
C
pPKA substrates levels (% wt)
pPKA substrates
KDa
PKAc
Tubulin
D
pNR1ser897 levels (% wt)
pNR1
NR1
E
pRas-GFR1scr916 levels (% wt)
pRas-GRF1
Ras-GRF1
F
pSTEP61 ser221 levels (% wt)
pSTEP
STEP

Figure 3

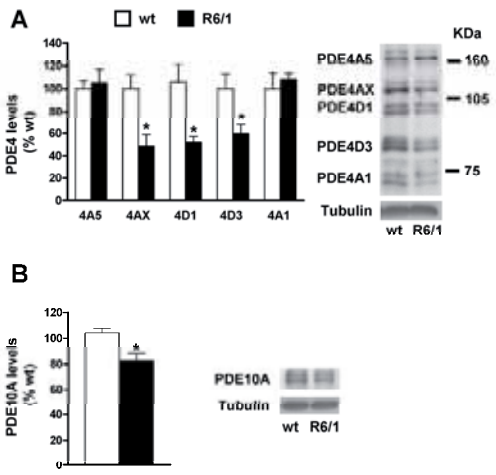

A
wt
R6/1
PDE4 levels (% wt)
140
120
100
80
60
40
20
0
4A5
4AX
4D1
4D3
4A1
KDa
PDE4A5
PDE4AX
PDE4D1
PDE4D3
PDE4A1
Tubulin
160
105
75
wt R6/1
B
PDE10A levels (% wt)
120
100
80
60
40
20
0
PDE10A
Tubulin
wt R6/1

Figure 4

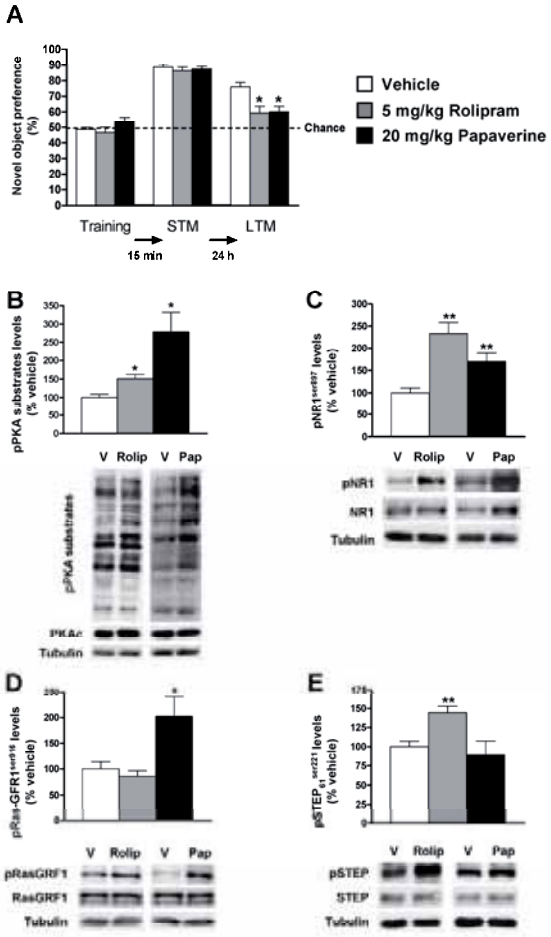

A
Novel object preference (%)
Vehicle
5 mg/kg Rolipram
20 mg/kg Papaverine
Chance
Training
STM
LTM
15 min
24 h
B
pPKA substrates levels (% vehicle)
V Rolip V Pap
pPKA substrates
PKAc
Tubulin
C
pNR1 levels (% vehicle)
V Rolip V Pap
pNR1
NR1
Tubulin
D
pRas-GFR1 levels (% vehicle)
V Rolip V Pap
pRasGRF1
RasGRF1
Tubulin
E
pSTEP levels (% vehicle)
V Rolip V Pap
pSTEP
STEP
Tubulin

Figure 5

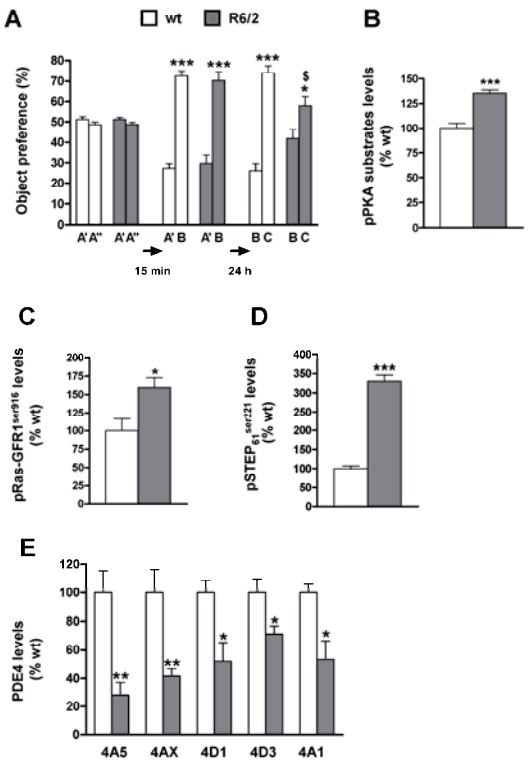

A
wt
R6/2
Object preference (%)
A'A"
A'A"
A'B
A'B
BC
BC
15 min
24 h
B
pPKA substrates levels (% wt)
C
pRas-GFR1 ser916 levels (% wt)
D
pSTEP 61 ser221 levels (% wt)
E
PDE4 levels (% wt)
4A5
4AX
4D1
4D3
4A1

Figure 6

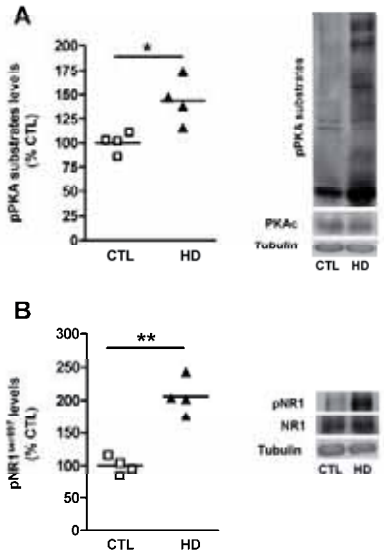
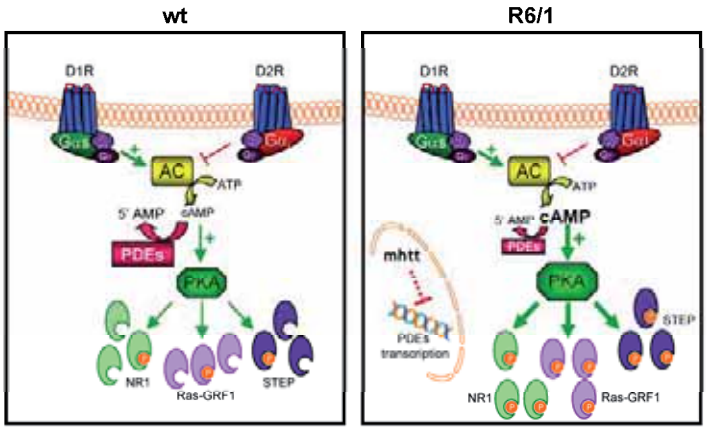
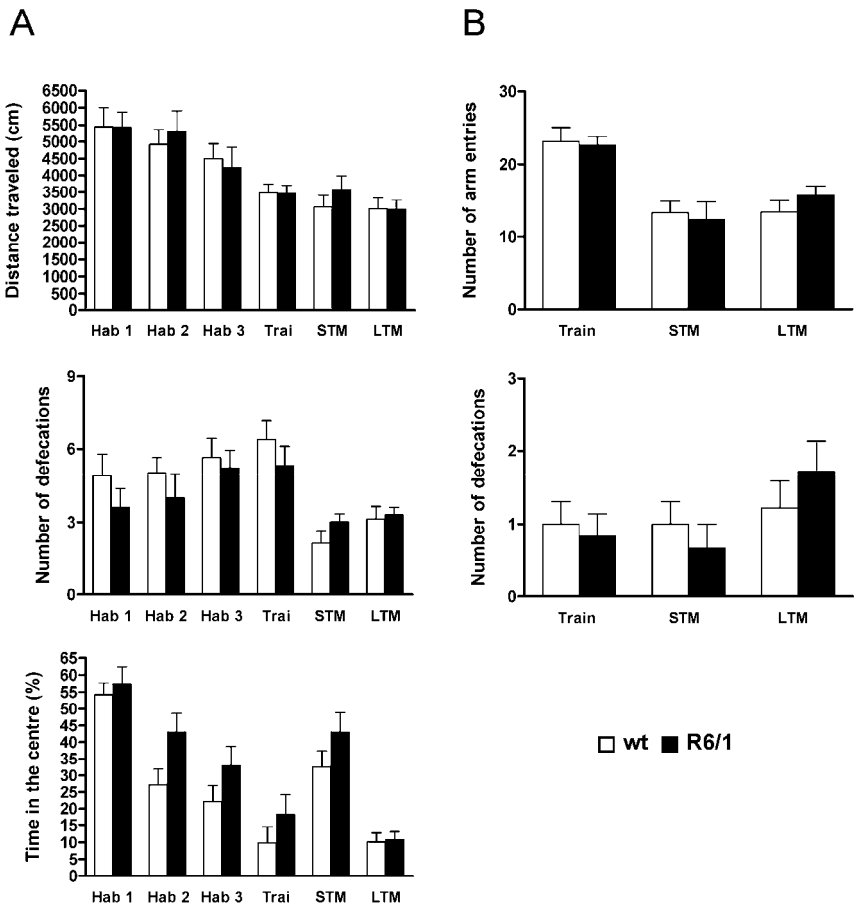

A
pPKA substrates levels (% CTL)
200
175
150
125
100
75
50
25
0
CTL
HD
*
pPKA substrates
PKAc
Tubulin
CTL HD
B
pNR1 levels (% CTL)
300
250
200
150
100
50
0
CTL
HD
**
pNR1
NR1
Tubulin
CTL HD

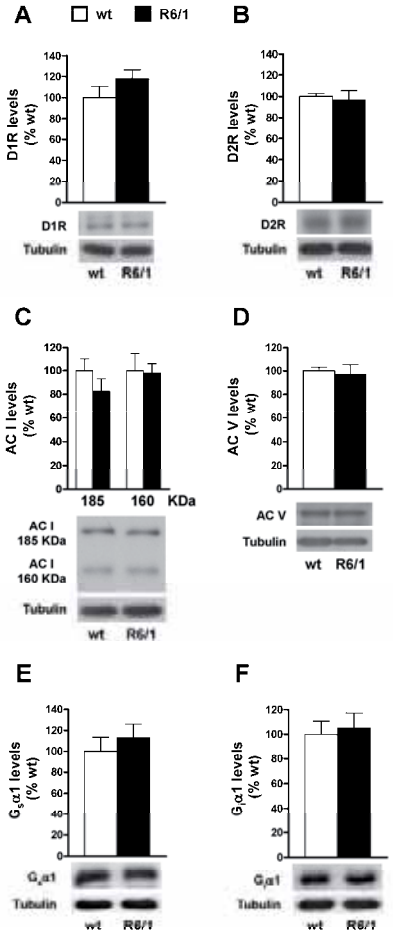
Figure 7


wt
R6/1
D1R
D2R
AC
ATP
5' AMP
cAMP
PDEs
PKA
mhtt
PDE1 transcription
NR1
Ras-GRF1
STEP

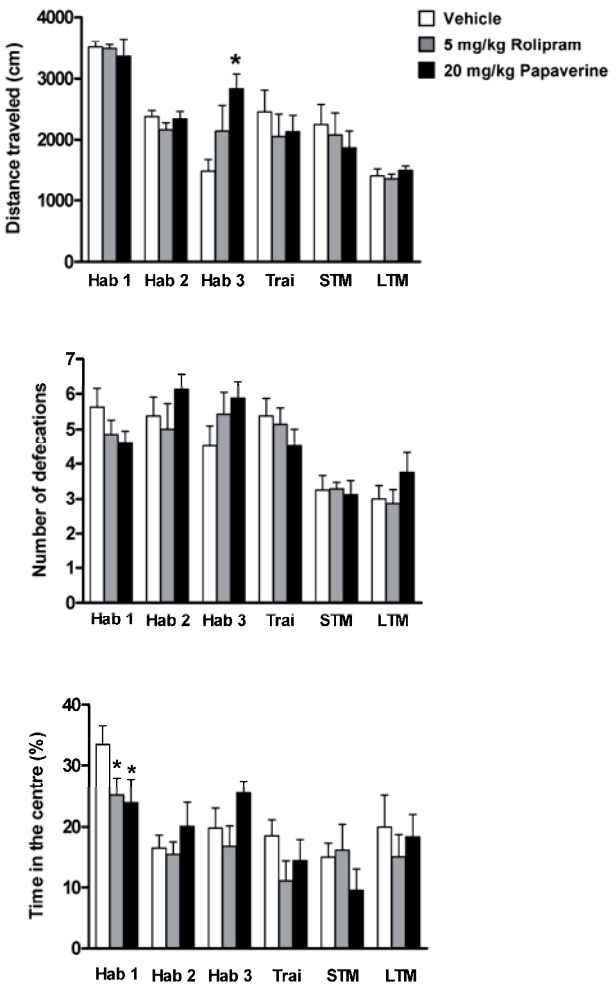
Supplementary Figure 1



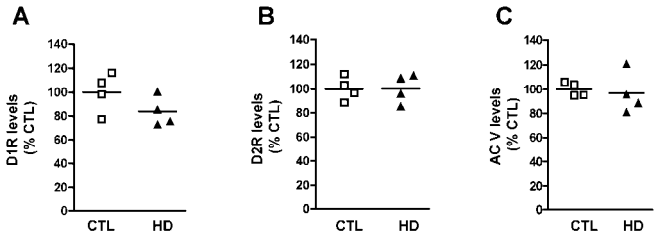
Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4



Trabajo 7: ***"Conditional BDNF release under the GFAP promoter prevents synaptic and neuronal atrophy and improves the behavioural phenotype in a mouse model of Huntington's Disease".*** **(Manuscrito en preparación)**

Objetivo 6. Estudio del efecto neuroprotector del uso de astrocitos que liberen BDNF de forma condicional en diferentes modelos de la enfermedad de Huntington..

Debido a los resultados beneficiosos y positivos del trabajo 4, nuestro siguiente objetivo fue estudiar el efecto de la liberación condicional del BDNF en un modelo murino de la enfermedad de Huntington. En los modelos animales de la enfermedad de Huntington, así como pasa en los pacientes humanos, se produce una reacción astroglial cerebral progresiva con el avance de la enfermedad y con un aumento de expresión de GFAP como principal marcador (de astrogliosis) neuropatológico asociado. Así, para valorar nuestra hipótesis, cruzamos los ratones generados en el trabajo anterior (pGFAP-BDNF) con los ratones modelos de la enfermedad de Huntington llamados R6/2. En ese nuevo modelo de ratones dobles mutantes (R6/2:pG-B) se predijo que, a medida que la enfermedad avanzase y GFAP también aumentase de expresión, debido a la regulación al alza del promotor del transgen (pGFAP-BDNF), los ratones R6/2:pG-B producirían más BDNF que los ratones R6/2, esperando una mejoría en su fenotipo.

Regular article

Conditional BDNF release under the GFAP promoter slows synaptic and neuronal atrophy and improves motor coordination in a transgenic mouse model of Huntington's Disease

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Short running title: Conditional BDNF administration in Huntington's Disease.

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ABSTRACT

Brain derived neurotrophic factor (BDNF) is the main candidate for neuroprotective therapeutic strategies for Huntington's disease (HD). However, the administration system and the control over the dosage are still important problems to be solved. Here we used transgenic mice which over-express BDNF under the GFAP promoter (pGFAP-BDNF mice) as a neuroprotective strategy for HD. We examined whether BDNF regulation by the GFAP promoter could induce improvements in a transgenic mouse model of HD. To this end, we cross-mated pGFAP-BDNF mice with R6/2 mice, a well characterized HD transgenic model. In these double mutant mice (R6/2:pG-B mice), we have performed a behavioral, morphological and biochemical characterization. First, levels of BDNF were reverted in R6/2:pG-B mice respect to R6/2 mice at 12 weeks of age, when astrogliosis is significantly increased in these transgenic mice. The recovery of BDNF levels prompted to R6/2:pG-B mice to improve their motor coordination alterations respect to R6/2 measured by the Rotarod. The behavioral improvements observed in the R6/2:pG-B mice were associated with a delay of the neuronal atrophy of striatal projection neurons exemplified by a partial reversal of striatal and neuronal soma volumes and a lesser loss of dendritic spine density than in R6/2 mice. Altogether indicates that the conditional administration of BDNF under the GFAP promoter could constitute a therapeutic strategy to release BDNF for HD.

Nº of words: 220

Key words: R6/2, synaptic plasticity, therapy, astrocytes, learning.

INTRODUCTION

Huntington's disease (HD) is an inherited neurodegenerative disorder. It results from the expansion of a CAG trinucleotide repeat region in the *huntingtin* gene, which lengthens a glutamine stretch in the huntingtin protein (htt) (Andrew et al., 1993). Mutant htt (mhtt) causes the death of neurons, particularly medium spiny neurons (MSNs), which account for ~90% of striatal neurons. The demise of MSNs causes motor, cognitive, and behavioral dysfunction (Reiner et al., 1988).

One possible mechanism leading to cell dysfunction and loss in HD is a reduction of the neurotrophic function (Zuccato & Cattaneo, 2007, 2009; Alberch et al., 2004). Patients with HD have reduced Brain Derived Neurotrophic Factor (BDNF) transcript in the cortex and decreased BDNF protein in cortex and striatum (Ferrer et al., 2000; Zuccato et al., 2001) and similar results have been found in different brain regions of several animal models (Ginés et al., 2003, Giralt et al., 2009; Gharami et al., 2008; Diekmann et al., 2009; Lynch et al., 2007). Furthermore, the transport and function of the neurotrophin is also disrupted (Gauthier et al., 2004; del Toro et al., 2006). Thus, due to its beneficial effects, BDNF is the main candidate for neuroprotective therapies as it has been tested in acute (Giralt et al., 2010; Pérez-Navarro et al., 2000) and in transgenic mouse models (Canals et al., 2004; Gharami et al., 2008) of HD. However, the main problem to use neurotrophic factors as therapeutic agents for neurodegenerative disorders is the chronic delivery system (Zuccato & Cattaneo, 2007; Lindvall et al., 2000; 2004). Therefore, it is necessary to develop new procedures for conditional and safe delivery of neurotrophic factors.

We previously demonstrated that transgenic astrocytes engineered to over-express BDNF under control of the GFAP promoter, when grafted in wild type mice, release higher levels of BDNF than control astrocytes in an excitotoxic model of HD

(Giralt et al., 2010). This enhanced release of BDNF exerted neuroprotection of striatal neurons, which in turn, resulted in long-term behavioral improvements. Here we tested if a conditional regulation and delivery of BDNF could be neuroprotective in a transgenic mouse model of HD. Thus, we cross-mated the R6/2 (Mangiarini et al., 1996) with pGFAP-BDNF mice (Giralt et al., 2010) to obtain a double mutant mouse called R6/2:pG-B. We performed in these mice a behavioral, biochemical and morphological characterization. We found a significant and progressive improvement of their striatal function due to a recovery of BDNF levels and a partial preservation of the neuronal morphology together with a reversal of synaptic alterations.

MATERIALS AND METHODS

Animal handling and care

To obtain double-mutant mice with mhtt and progressively increased levels of BDNF (R6/2:pG-B mice), we cross-mated R6/2 mice (Mangiarini et al., 1996) with pGFAP-BDNF mice (Giralt et al., 2010). Wild type (WT) littermate animals were used as the control group. In this study, the colony was maintained at B6CBA strain background with more than F6. Mice were housed together in numerical birth order in groups of mixed genotypes with access to food and water *ad libitum* in a colony room kept at a constant temperature (19-22°C) and humidity (40-50%) on a 12 hr light/dark cycle. All experiments were conducted in a blind-coded manner with respect to genotype, and data were recorded for analysis by microchip mouse number. All animal-related procedures were in accordance with the European Community guidelines for the care and use of laboratory animals (89/609//EEC) and approved by the local animal care committee of the Universitat de Barcelona (99/01) and by the Generalitat de Catalunya (99/1094).

Behavioral and health characterization of R6/2:pG-B mice

Behavioral characterization began at 5-7 weeks of age. Experiments like general health, body weight, clasping and rotarod were monitored weekly. Muscular strength evaluation was performed at 7, 10 and 12 weeks of age. Clasping was measured by suspending them from their tails at least 1 foot above a 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: 0 = no clasping, 1 = clasping 2 paws and 2 = clasping all paws. Animals that arrive at 13-16 weeks of age received euthanasia, just before they drastically lost weight and died. All experiments were performed on male littermates to avoid sex differences (n = 10-15 per group).

Hiring wire and muscular strength

Neuromuscular abnormalities were analyzed by the hiring wire test. Hence, a standard wire cage lid was used. To test balance and grip strength, mice were placed on the top of a wire cage lid. The investigator then did shake the lid lightly to cause the mouse to grip the wires and then turns the lid upside down and registered the time to fall down by the mouse. To further analyze neuromuscular function, the muscular strength was also tested as previously described (Canals et al., 2004; Gimenez-Llort et al., 2002).

Rotarod

Motor coordination and balance were evaluated on the rotarod apparatus at several rotations per minute as described previously (Canals et al., 2004; Carter et al., 1999). In brief, animals were trained at constant speed (16 rpm) for 60 sec at 5 weeks of age. We performed two trials per day for three consecutive days and the latency to fall and numbers of falls in 60 sec were recorded. No differences between groups were

detected at this period. After training, animals were evaluated once a week at 16 and 24 rpm starting at 7 weeks of age, and number of falls in a total of 60 sec was recorded. The animals were put on the rotarod several times until the addition of the latency to fall off reached the total time of 60 sec. The curves representing the behavioral pattern were compared and the percentage of motor coordination function impairment was calculated as previously described (Ferrante et al., 2002;).

Immunohistochemistry

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 (40 μm) obtained with a cryostate were processed for immunohistochemistry as free floating.

The sections were washed three times in PBS, permeabilized and blocked 15 min by shaking at room temperature 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 by shaking at 4ºC with the corresponding primary antibodies in PBS with 0.02% sodium azide buffer. Antibodies used were the anti-PSD-95 1:500 (Affinity BioReagents, Golden, CO, USA) and anti-VGLUT1 1:500 (Synaptic systems, Göttingen, Germany). After primary antibody incubation, slices were washed three times and then incubated 2 h shaking at room temperature with subtype-specific fluorescent secondary antibodies: Cy3 goat anti-rabbit (1:100) and Cy2 goat anti-mouse (1:100) (both from Jackson ImmunoResearch, West Grove, PA, USA). No signal was detected in controls incubated in the absence of the primary antibody. In order to obtain

a successful staining for the VGLUT1 immunohistochemistry, some specifications have been performed as previously described (Martin-Ibañez et al., 2006).

For diaminobenzidine immunohistochemistry experiments, endogenous peroxidases were blocked for 30–45 min in PBS containing 10% methanol and 3% H_2O_2. Then, nonspecific protein interactions were blocked with normal serum or bovine serum albumin. Tissue was incubated overnight at 4 1C with the following primary antibodies: anti-DARPP32 (1:500; Chemicon, Temecula, CA, USA), anti-GFAP (1:500; Dako A/S, Glostrup, Denmark) and anti-Calbindin (Sigma Chemical Co. St. Louis, MO). Sections were washed three times in PBS and incubated with a biotinylated secondary antibody (1:200; Pierce) at room temperature for 2 h. The immunohistochemical reaction was developed using the ABC kit (Pierce) and visualized with diaminobenzidine. No signal was detected in controls in which the primary antibodies have been omitted.

Stereology

Volume estimates for striatum, was performed as previously described (Canals et al., 2004). Unbiased blind counting relative to genotype and condition was performed using the Computer Assisted Stereology Toolbox (CAST) software (Olympus Danmark A/S, Ballerup, Denmark). For estimating mean cellular/perikaryal volumes of neurons (so-called local volumes) with design-based stereology, the “nucleator” method was used as previously described (Gundersen, 1988). To determine the neuronal subpopulations densities (neurons per mm^3) in the striatum, we used the dissector counting procedure in coronal sections spaced 240 mm apart as previously described (Torres-Peraza et al., 2007).

Confocal analysis and dendritic spine-like structures counting

Fluorescently stained coronal sections were examined by confocal microscopy using Leica (Mannheim, Germany) TCS SL lasser scanning confocal spectral microscope with argon and helium-neon lasers. Images were taken using 63x numerical aperture objective with 4x digital zoom and standard (one Airy disc) pinhole. For each mouse, at least 4 slices of 30 μm with striatal tissue represented were analyzed. Between 4 and 6 representative images were obtained from each slice. For each image, the entire three-dimensional stack of images was obtained by the use of the Z drive present in the Leica TCS SL microscope, and the size of the optical image was of 0.5 μm separated 4 μm between each one. The number of dendritic spine-like structures was counted by the freeware NHI ImageJ version 1.33 by Wayne Rasband (National Institutes of Health, Bethesda, MD) similarly to previously described (Lynch et al., 2007).

Statistical analysis

All data are expressed as mean ± s.e.m. Different statistical analyses were performed as appropriate and indicated in the figure legends. Values of $p < 0.05$ were considered as statistically significant

RESULTS

Striatal astrogliosis in R6/2:pG-B mice induces an up-regulation of the BDNF protein levels respect to R6/2 mice

Although BDNF could be a good candidate as a neuroprotective strategy for HD (Zuccato et al., 2007, 2009; Alberch et al., 2004), its administration is difficult to perform due to a several technical problems. Some of them are the dosage control and the inability to cross the blood brain barrier (reviewed in Zuccato et al., 2009). In order

to take advantage against these troubles we crossed mice, which over-express BDNF under the control of the GFAP promoter (pGFAP-BDNF mice) (Giralt et al., 2010) with R6/2 mice (Mangiarini et al., 1996) and we obtained the double mutant R6/2:pG-B mice. We chose this HD mouse model because of its striatal astrogliosis and GFAP up-regulation associated (Fig. 1A).

As previously predicted (Giralt et al., 2010), this neuropathological hallmark induced an up-regulation of the GFAP promoter function in R6/2:pG-B mice, which resulted in a significant increase of the striatal BDNF levels respect to R6/2 mice (Fig 1B).

Up-regulation of striatal BDNF levels in R6/2:pG-B mice induces a motor coordination improvement compared with R6/2 mice

With this recovery of BDNF levels in R6/2:pG-B mice compared with the R6/2 mice we study if this effect could induce a phenotype improvement. First, both, R6/2 and R6/2:pG-B mice developed a normal body weight compared with wt and pGFAP-BDNF mice until 10-11 weeks of age (Fig 2A). However, at this age R6/2 and R6/2:pG-B mice, both began to loss their body weight without significant differences between them (Fig 2A). Similarly, both R6/2 and R6/2:pG-B mice developed a progressive increase in the clasping behavior (Fig 2B). However, R6/2:pG-B mice showed a significant delay of these alterations compared with R6/2 mice (Fig 2B), suggesting neuronal function preservation due to increased BDNF levels. Muscular strength was also evaluated by the hiring wire test (Fig 2C) and the muscular strength (Fig 2D) tests. Both measurements showed that R6/2 and R6/2:pG-B mice have normal muscular strength respect to wt, and pGFAP-BNF mice until 12 weeks of age when both groups displayed alterations on this measurement (Fig 2C-D). Finally, we also evaluated motor

coordination in all groups by checking their rotarod performance at different speeds (Fig 2E-F). We found that motor coordination alterations began to be clear at 10 weeks of age in both speeds and progressed until 12 weeks of age in R6/2 compared to wt and pGFAP-BDNF mice (Fig 2E-F). However, motor coordination deficits in R6/2:pG-B mice began 1 week later and progressed with less severity compared with R6/2 mice (Fig 2E-F). These results show that increase of striatal BDNF levels by the pGFAP-BDNF transgene activity induced neurological and striatal-dependent behavior improvements in R6/2:pG-B mice.

Preservation of striatal-dependent behavior in R6/2:pG-B respect to R6/2 mice correlates with improvements of the neuropathology

Because R6/2:pG-B mice improved their striatal-dependent motor coordination alterations respect to R6/2 mice, we next evaluated if striatal neuropathology correlated with these behavioral results. Stereological estimation of the striatal volume revealed that R6/2:pG-B mice have a larger striatum than R6/2 mice (Fig 3A) suggesting morphological improvements in the double mutant mice. Nevertheless, R6/2 and R6/2:pG-B mice have smaller striatum than wt and pGFAP-BDNF mice (Fig 3A).

Next we checked if the striatal volume recovery observed in R6/2:pG-B mice was due to higher survival of their projection neurons. Stereological counting of the striatal calbindin-positive cells in all groups indicate that both R6/2 and R6/2:pG-B mice have a similar significant decrease in the number of these neurons respect to wt and pGFAP-BDNF mice (Fig 3B). However, when we measured the soma volume of calbindin-positive neurons, we found a significant volume recovery in R6/2:pG-B mice respect to R6/2 mice (Fig 3C). Taken together, these results suggest that the striatal-

dependent behavior improvement observed in R6/2:pG-B mice compared with R6/2 mice correlate with a recovery of the neuronal morphology.

The recovery of the striatal BDNF levels in R6/2:pG-B compared with R6/2 mice results on an increase of the cortico-striatal synaptic markers VGLUT1 and PSD-95

Since we observed a recovery of the volume of the striatal projection neurons but not in neuronal survival in R6/2:pG-B mice respect to R6/2 ones, we hypothesized that mainly subtle micro-structural changes should be involved. Hence, we additionally tested the state of cortico-striatal synapses in all groups by performing immuno-stainings against VGLUT1 and PSD-95.

Confocal image analysis of coronal sections from all groups reveal that only R6/2 mice at 12 weeks of age suffer of significant decrease of the VGLUT1 (Fig 4A) and PSD-95 (Fig 4B) positive particles density respect to wt control mice. On the other hand, R6/2:pG-B mice displayed a highly spread on VGLUT1 (Fig 4A) and PSD-95 (Fig 4B) positive synapses density respect to wt control mice. These results are in accordance to the volume preservation of the whole striatum and striatal calbdinin-positive neurons in R6/2:pG-B mice respect to R6/2 ones.

DISCUSSION

Here we show a new conditional delivery system in which BDNF is only delivered under pahological conditions. R6/2:pG-B mice display improvements in striatal-dependent behavior and clasping compared with R6/2 mice. In addition, they develop associated significant neuronal architecture preservation when also compared with the R6/2 mice.

The neurotrophin BDNF has been largely proposed as a highly putative therapeutic molecule for HD treatment (Zuccato et al., 2007; 2009; Alberch et al., 2004). Thereby, since its levels and function are both down-regulated in the disease, its administration and/or over-expression should be the method employed (Zuccato et al., 2007; 2009; Alberch et al., 2004). It is noteworthy that the neurotrophin over-expression in this mutant mouse is progressive and dependent of the disease severity. The BDNF transgene is activated under the GFAP promoter activity as previously demonstrated (Giralt et al., 2010). The GFAP molecule is an up-regulated marker in astrogliosis processes. In this line, the R6/2 mice (Kusakabe et al., 2001; present results), as occurs in HD human patients (Faideau et al., 2010), show a progressive increase of this pathological hallmark. In the present work, we take advantage of the progressive astrogliosis in R6/2 mice to produce and release BDNF in a conditional manner. Furthermore, our method overcomes several of the technical problems for the intracerebral BDNF administration. Thus, we solve the problem of the neurotrophin dosage (Kells et al., 2008), the inability from BDNF to cross the blood brain barrier (Zhang & Pardridge, 2001; 2006), the invasive rate of some methods as lipotransfection or infection (Lindvall et al., 2004) and the tumorigenesis or teratogenesis risk from stem cells grafting or other types of cell therapy (Hoffman et al., 1993). Our results point out that the GFAP promoter could be a key promoter capable to produce and release BDNF in a conditional way, with regulated mechanisms and only when it is pathologically required, thus being of high therapeutic interest.

In the HD context, the down-regulated striatal BDNF levels have been largely described to be produced by an alteration of the gene transcription (Zuccato et al., 2003), but also by a deficit of its transport and delivery (Gauthier et al; 2004; del Toro et al., 2006). Thus, the over-expression of BDNF from genetically modified striatal

astrocytes could help to compensate locally the problem of the BDNF trafficking from the cortical afferent projections.

Our results also reveal that astrocytes *per se* could be useful in the design of neuroprotective strategies for HD. This neuroprotection is related to the preservation of plasticity processes more than to neuronal survival since no changes were observed in this parameter between R6/2:pG-B and R6/2 mice when compared to wt and pGFAP-BDNF mice. However, synaptic markers and neuronal soma volume were both improved in R6/2:pG-B mice respect to R6/2 mice. These findings are in agreement with the recent and expanded idea that astrocytes are highly involved in neuro-plasticity phenomena like the regulation and strengthening of signals and connectivity of neuronal networks some of them implicated, at least, in cognitive functions (Haydon, 2001; Santello y Volterra, 2010). It is also important to point out that our results also suggest that astrocytes could modulate directly or indirectly several of these plasticity processes via production and delivery of BDNF, likely into the synapse. This is highly probable since astrocytes are capable to produce and release BDNF (Giralt et al., 2010; Riley et al., 2004; Dougherty et al., 2000) and they send very fine projections to the (excitatory) synapses in order to strengthen them (reviewed in Fellin, 2009). In this scenario, the neurotrophin could be released from astrocytes to perform their plasticity functions at these sites and inducing the recovery of different neuronal and behavioral functions in HD mice models. In conclusion, conditional BDNF delivery regulated by the GFAP promoter in astrocytes could be an interesting therapeutic strategy for HD treatment.

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FIGURE LEGENDS

Figure 1. Astrogliosis caused by the mutant huntingtin induces an up-regulation of the BDNF protein levels in R6/2:pG-B mice. The presence of the mutant huntingtin induces an increase of astroglia reactivity in R6/2 mice as showed by immunohistochemistry against GFAP (A). We cross-mated R6/2 with pGFAP-BDNF mice in order to obtain the double mutant mice called R6/2:pG-B mice (B). The BDNF levels in striatal samples from wt, pGFAP-BDNF, R6/2 and R6/2:pG-B mice were measured by western blot (B). Values are expressed as mean ± s.e.m. Data were analyzed by one-way ANOVA with Student's t-test as a *post hoc*. * $p < 0.05$ as compared to the wt group (n = 5-6/group).

Figure 2. The recovery of striatal BDNF levels in R6/2:pG-B mice improve their striatal-dependent motor deficits compared to R6/2 mice. We performed a general behavioral and healthy characterization of all groups in order to detect if the BDNF transgene expression improve neurological symptoms in the R6/2:pG-B mice. Body weight (A), clasping level (B), muscular strength (C-D) and motor coordination (E-F) were all analyzed. To monitor the body weight, animals were weighed weekly from 6 to

13 weeks of age (A). Clasping level was also monitored from 6 to 13 weeks of age (B). Muscular tone was measured by using the muscular strength (C) and hiring tests (D). Motor coordination and beam were both analyzed by performing the Rotarod task at 16 (E) and 24 (F) r.p.m. Symbols indicate ± s.e.m by mice of each group on each measure. Two-way ANOVA with repeated measures with the Student's *t*-test as a *post-hoc* was performed (* $p < 0.05$ indicates the difference of R6/2 and R6/2:pG-B mice respect to wt; $ $p < 0.05$ indicates the difference of R6/2 and R6/2:pG-B mice respect to pGFAP-BDNF. For clarity, in the rotarod test only statistical comparisons between R6/2 and R6/2:pG-B mice are depicted: * $p < 0.05$ and ** $p < 0.01$ are the statistical differences between R6/2 and R6/2:pG-B mice) (n = 10-12/group).

Figure 3. Striatal BDNF levels recovery in R6/2:pG-B mice induce a striatal and neuronal volumes improvement. Striatal volume from all genotypes was stereologically measured (A). A representative microphotograph immunostained for DARPP-32 of coronal sections from the four genotypes is depicted (A). The number of calbindin-positive projecting neurons was stereologically counted in all groups (B). The soma volume of calbindin-positive projecting neurons was stereologically estimated in all genotypes by the nucleator method (C). Values are expressed as mean ± s.e.m. Data were analyzed by one-way ANOVA with Student's *t*-test as a *post hoc* (* $p < 0.05$ and ** $p < 0.01$ as compared to wt group (n = 5-6/group)).

Figure 4. Striatal BDNF levels recovery in R6/2:pG-B mice promote a preservation of the cortico-striatal connectivity. Immunohistochemical staining in all genotypes against the pre-synaptic and post-synaptic markers VGLUT1 (A) and PSD-95 (B) respectively was performed in all groups. The images were taken by confocal microscopy. Counting

for VGLUT1 (A) and PSD-95 (B) positive particles were performed by the ImageJ software and represented in a graph. Values are expressed as mean ± s.e.m. Data were analyzed by one-way ANOVA with Student's *t*-test as a *post hoc* (* $p < 0.05$ as compared to wt group (n = 4/group)).

Figure 1

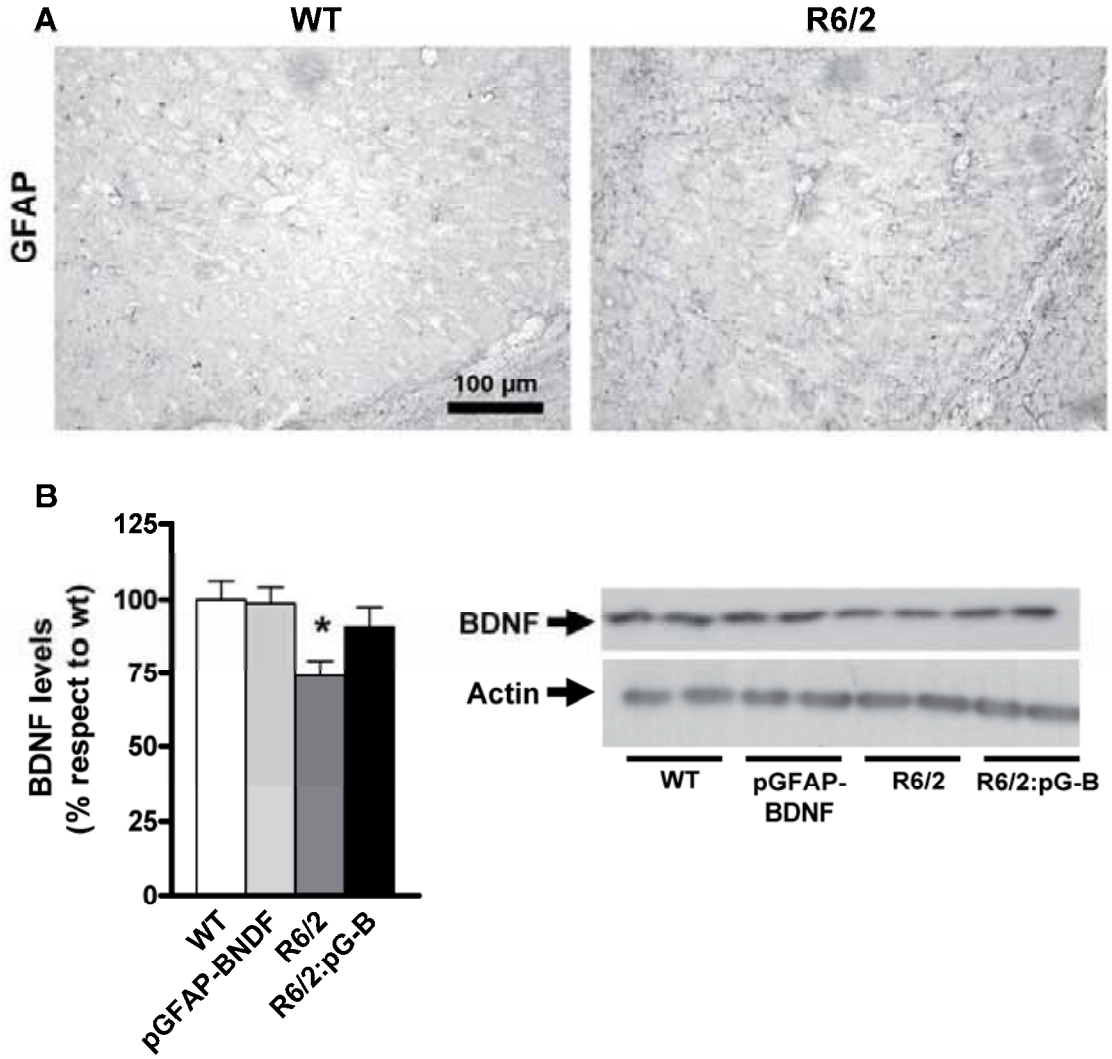

A
WT
R6/2
GFAP
100 µm
B
BDNF levels (% respect to wt)
125
100
75
50
25
0
*
WT
pGFAP-BNDF
R6/2
R6/2:pG-B
BDNF
Actin
WT
pGFAP-BDNF
R6/2
R6/2:pG-B

Figure 2

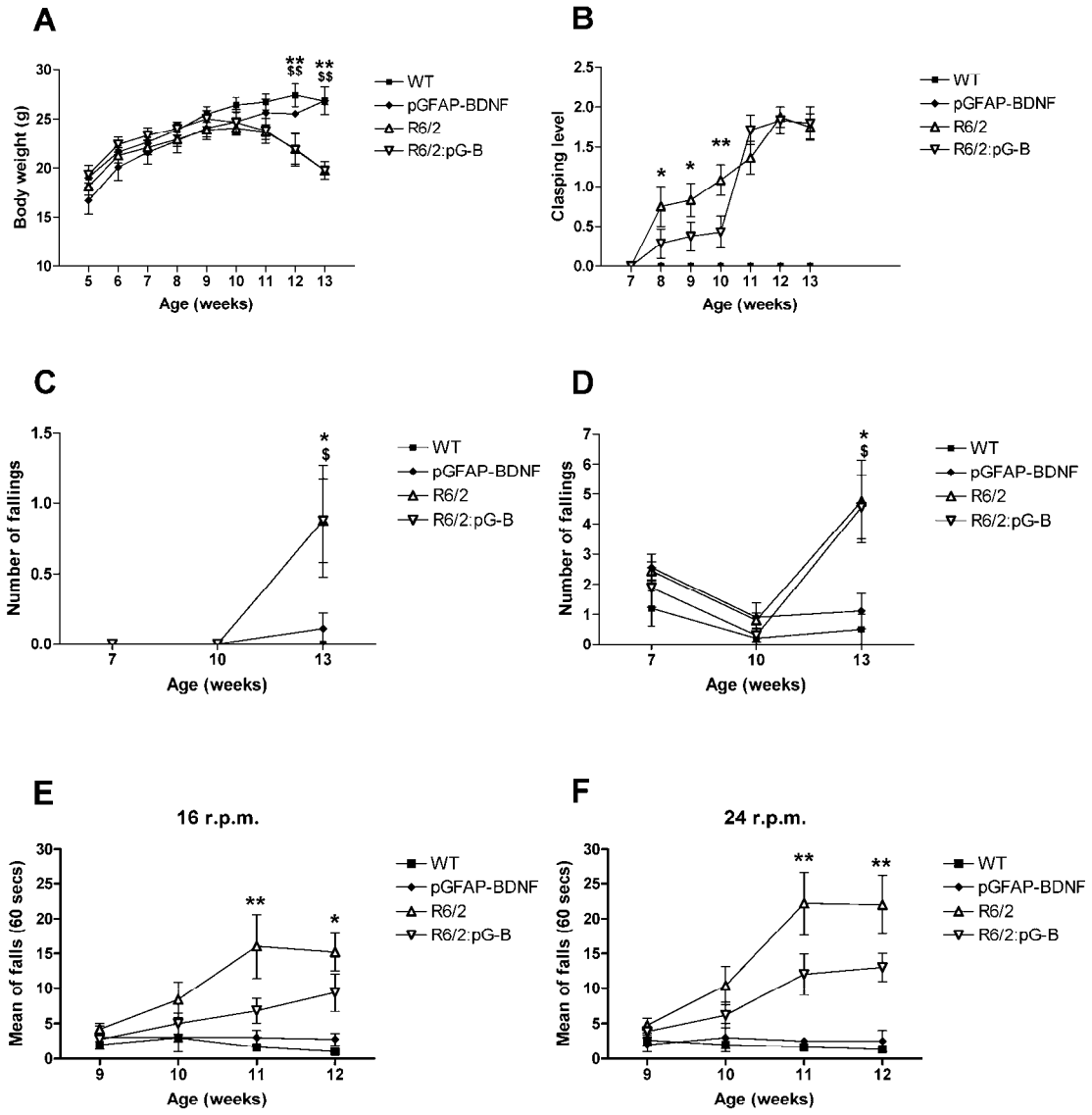

A
Body weight (g)
Age (weeks)
WT
pGFAP-BDNF
R6/2
R6/2:pG-B
B
Clasping level
Age (weeks)
C
Number of fallings
Age (weeks)
D
Number of fallings
Age (weeks)
E
16 r.p.m.
Mean of falls (60 secs)
Age (weeks)
F
24 r.p.m.
Mean of falls (60 secs)
Age (weeks)

Figure 3

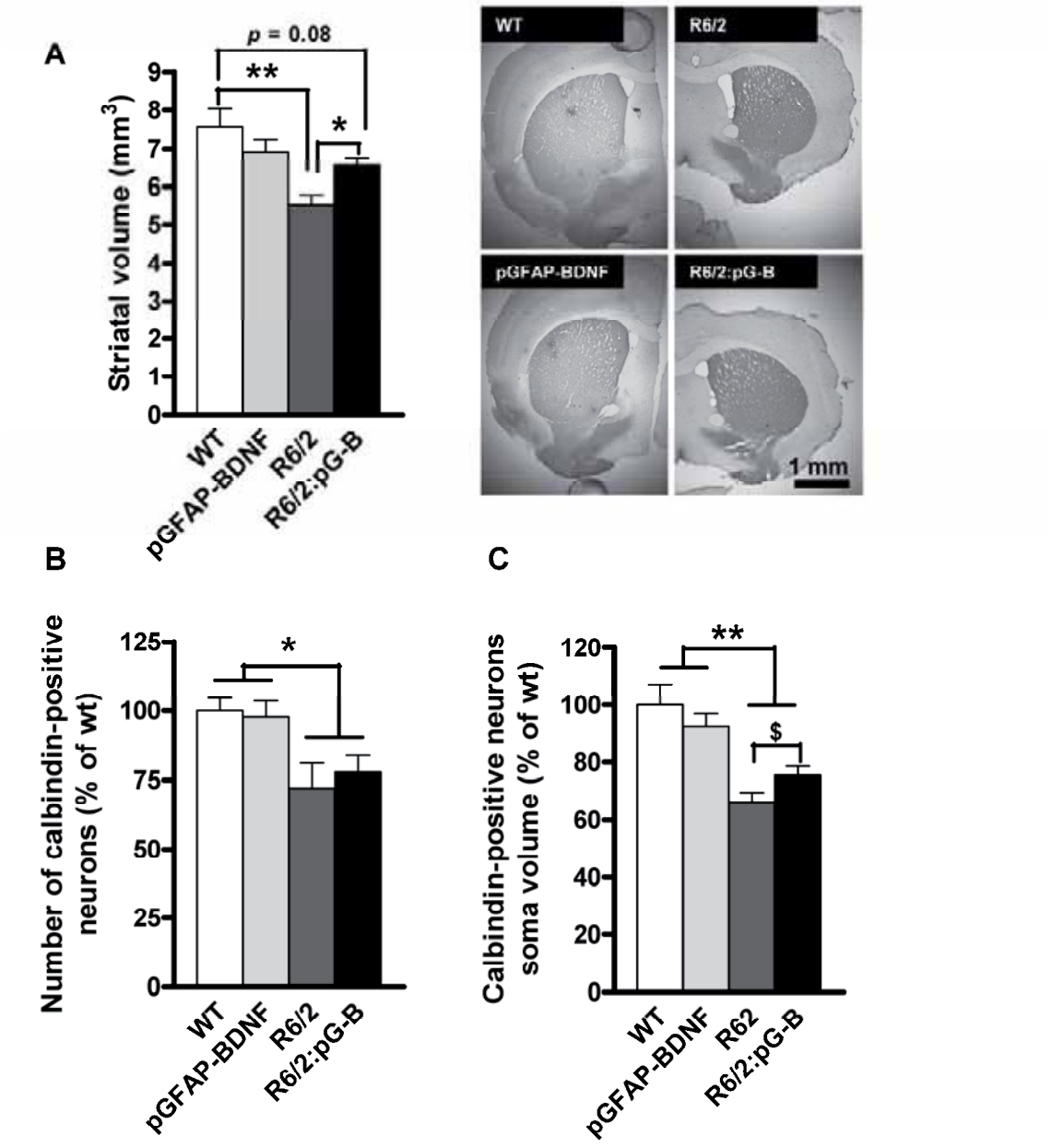
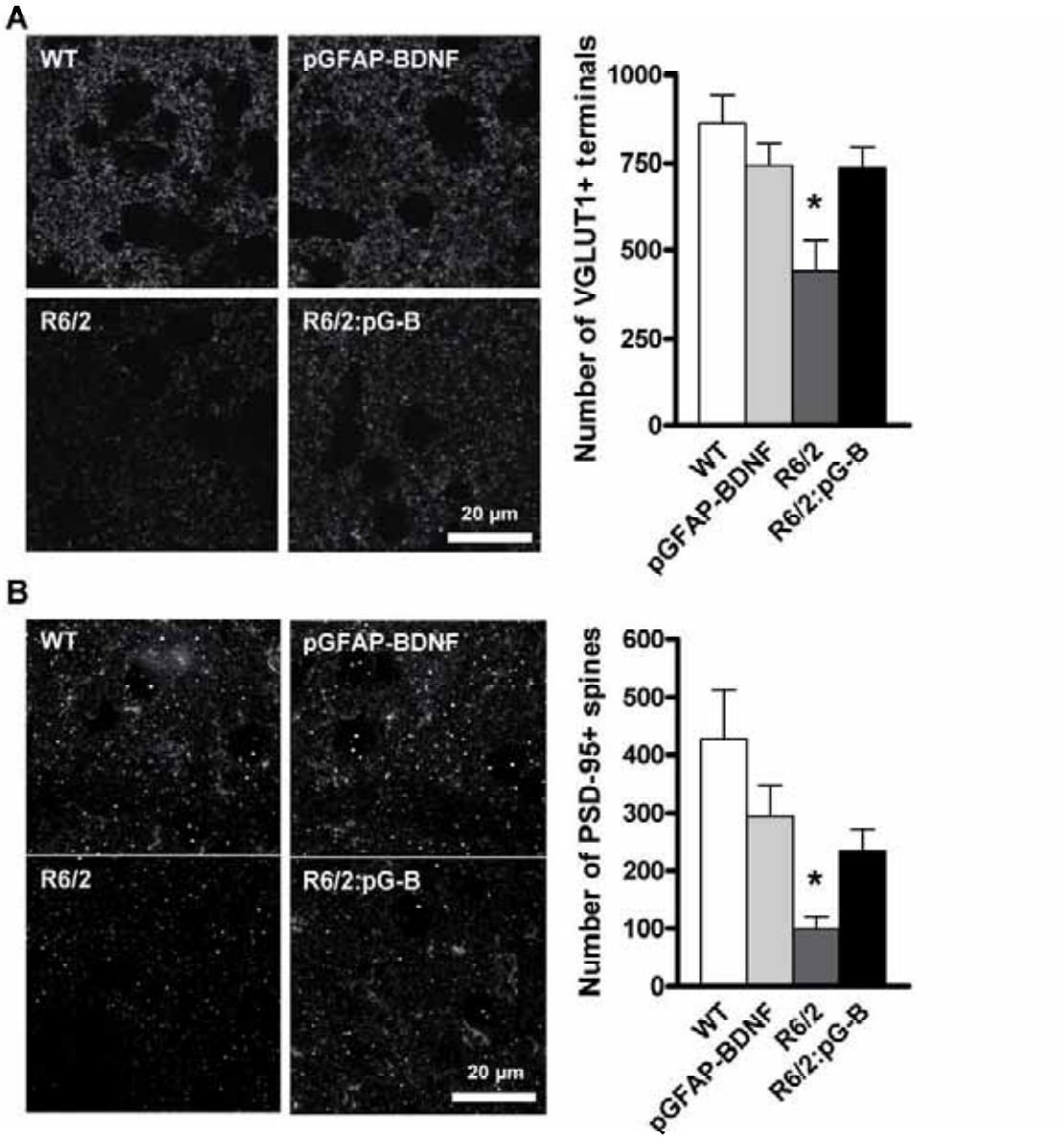

A
p = 0.08
**
*
Striatal volume (mm3)
0
1
2
3
4
5
6
7
8
9
WT
pGFAP-BDNF
R6/2
R6/2:pG-B
WT
R6/2
pGFAP-BDNF
R6/2:pG-B
1 mm
B
Number of calbindin-positive neurons (% of wt)
0
25
50
75
100
125
*
WT
pGFAP-BDNF
R6/2
R6/2:pG-B
C
Calbindin-positive neurons soma volume (% of wt)
0
20
40
60
80
100
120
**
$
WT
pGFAP-BDNF
R62
R6/2:pG-B

Figure 4


A
WT
pGFAP-BDNF
R6/2
R6/2:pG-B
20 µm
Number of VGLUT1+ terminals
1000
750
500
250
0
*
WT
pGFAP-BDNF
R6/2
R6/2:pG-B
B
WT
pGFAP-BDNF
R6/2
R6/2:pG-B
20 µm
Number of PSD-95+ spines
600
500
400
300
200
100
0
*
WT
pGFAP-BDNF
R6/2
R6/2:pG-B