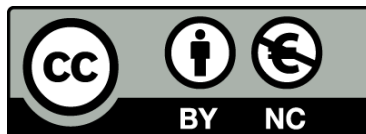




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Dual role of CDK5 on cognitive deficits and striatal vulnerability in Huntington's disease

Elena Alvarez Periel



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DUAL ROLE OF CDK5 ON COGNITIVE DEFICITS AND STRIATAL VULNERABILITY IN HUNTINGTON'S DISEASE

Doctoral degree of Biomedicine

Dissertation submitted by:

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This work was performed at the Departament de Biomedicina in the
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Elena Alvarez Periel

Silvia Ginés Padrós

Programa de Doctorat de Biomedicina

I'm no longer accepting things I cannot change.
I'm changing things I cannot accept.

Angela Davis

When the intellectual historians... look back on these decades,
they are likely to acknowledge that the deepest insights into
the nature of mental processes... will have come not from philosophy,
from the arts, or even from psychology or psychoanalysis,
but from biology.

*Kandel ER, Pittenger C.
The past, the future and the biology of memory storage.
Philos Trans R Soc Lond B Biol Sci. 1999;354(1392):2027-52*

Als meus pares
A la meva germana

Al meu avi

AGRAÏMENTS

És curiós que després d'haver pensat moltes vegades al llarg de la tesi en el que m'agradaria escriure en aquest moment, ara em trobo una mica en síndrome de pàgina en blanc. I és que, quan fa poc em van preguntar per què havia fet una tesi, vaig pensar que, tot i haver estat probablement l'etapa més enriquidora i intensa de la meva vida, tenia la sensació d'haver arribat aquí una mica per casualitat, i sobretot, gràcies a l'ajuda de molta gent. Per tant, intentaré començar pel principi i no deixar-me a ningú.

Si començo pel principi, he de començar per la meva família. Gràcies papa i mama per haver-me recolzat sempre en totes les decisions que he pres, per haver confiat en mi i per haver-me deixat la llibertat per escollir el que volia fer mantenint sempre tot el vostre suport. Sé que sempre puc contar amb vosaltres per qualsevol cosa i això és vital per a mi. Lidia, el petardo que ja s'ha fet gran i s'ha convertit en una personeta amb les idees molt clares a qui admiro molt. Encara que no ens veiem tant com abans, la complicitat no caduca i sempre estàs allà per passar l'estona, parlar de qualsevol tonteria, mirar vídeos xorra i inclús discutir una miqueta, que els costums no s'han de perdre. Gràcies per estar sempre disponibles per mi, ara i sempre.

Gràcies també a la resta de la meva família, als meus tiets Miquel, Carme, Montse, Manolo, als meus cosins, Xavier, Lourdes, Jordi, Gerard i també a la Maite i el Ramon. La família és molt important i en aquesta etapa tan intensa, una conversa telefònica o una tarda de tertúlia recordant anècdotes i parlant de tot i de res, és un remei infal·lible per desconnectar i carregar piles i ànims. Gràcies a tots pel vostre suport, els vostres consells i per fer-me sentir valorada.

Passem ara a un altre tipus de família, els amics. Començant pels més antics, els que portem compartint experiències des dels 6 anys. Lili, Anna, Miriam, Oscar, Alba, els anys passen i les vides es compliquen, i cada cop ens veiem menys, però amb vosaltres la connexió és instantània, fa masses anys que ens coneixem perquè es perdi. Gràcies per ajudar-me a desconnectar en totes les nostres quedades i per continuar compartint experiències! Gràcies també Anna per tots els moments compartits al pis i per totes les llargues converses i tertúlies parlant de tot i de res.

I va arribar la universitat, i amb ella, també noves amistats. Gràcies Cris, Jèssica, Mariona, Mila, Bego, Silvia, Mireia, Marta, Lara i Monica pels múltiples sopars, excursions, berenars i fins i tot algun viatge (l'escapadeta a Porto va ser una recarrega d'energia molt important). Amb la tonteria ja fa més de 10 anys que ens coneixem i quedar amb vosaltres sempre és sinònim de passar-ho bé i riure una bona estona. Tot i que hem agafat camins professionals diferents, enteneu perfectament totes les complicacions i problemes que acompanyen fer una tesi (sobretot vosaltres Mireia i Lara! Jejeje), i poder-me desfogar amb vosaltres amb un soparet i bailoteo ha estat molt important per a mi durant aquest temps. Gràcies a totes i per molts anys més, que encara tenim pendent el gran viatge!

I després de la universitat va venir l'Erasmus. Una mica per casualitat vaig anar a parar a un laboratori de neuro, i vaig descobrir que allò m'interessava molt. Va ser la meva primera experiència a un laboratori professional i vaig tenir la sort de trobar gent molt amable i

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desinteressada que em van ensenyar molt i em van obrir la porta al món de la recerca. Thanks Satyam, Robert and Anna for your time, for sharing your experience and especially for your encouraging, I might not be upon finishing a PhD in neuroscience if I hadn't had the experience of staying in your lab. Thank you!

I arribem a l'última etapa que m'ha dut fins aquí i que de passada també em va portar noves amistats. Núria, Angie, Inés i Laura, ha estat un plaer haver compartit aquesta etapa amb vosaltres. Amb tota la intensitat que va suposar l'any del màster (encara recordo els treballs de 20 a 22h, i les hores de dibuix durant el curs d'animals jejeje), i totes les quedades que han anat venint després, i és que totes vam emprendre el camí del doctorat i anar compartint les penes i alegries de cada una ha estat un plaer. Angie i Laura, em va fer molta il·lusió poder veure-us convertides en doctores, i malgrat la distància, espero poguem continuar compartint moments juntes. Núria, el fet de fer la tesi tan apropet ha fet que a més haguem compartit múltiples trobades en passadissos i ascensors, on només faltava un cafetó o una birra per acabar de millorar la conversa jejeje. Gràcies per estar sempre disposada a escoltar-me amb un somriure! Molta sort en la recta final (tot i que estic segura que no la necessites). Inés! Eres una de las personas más positivas que conozco y desprendes bondad. Ha sido un lujo compartir esta etapa contigo y tus sonrisas. Eres el claro ejemplo de que, con ganas y entusiasmo, todo es posible. Estoy segura de que, hagas lo que hagas, te irá de coña y, sobre todo, conseguirás contagiar tu energía y motivación a quién te rodee!

I amb el màster va arribar també el que, sense saber-ho, acabaria sent el lloc on passaria quasi els propers 6 anys, i on coneixeria persones meravelloses.

Gràcies Silvia, en primer lloc, per haver-me deixat fer les pràctiques al teu grup. Recordo el primer dia que vaig venir, molt estressada perquè estàvem a setembre i encara no tenia grup per fer les pràctiques del màster i amb molta calma i molta claredat em vas fer un esquema dels projectes del grup i em vas ensenyar el laboratori, casi com si ja fos una més del grup. Però sobretot, gràcies per donar-me l'oportunitat de fer la tesi amb tu. Gràcies per estar sempre disponible per resoldre dubtes de tot tipus i per tranquil·litzar-me en els moments de màxim estrès. Gràcies també per tenir en compte la meva opinió, per deixar-me llibertat per proposar coses noves i per totes les hores de discussió científica. Sempre tindrè un record especial de totes les reunions intenses, amb alguna que altre interrupció, i que fins i tot acabaven requerint pauses per anar al lavabo i obrir algun paquet de galetes. Ha estat una etapa extremadament enriquidora i he après molt més del que em podia imaginar, tant a nivell professional com a nivell personal, moltes gràcies per l'oportunitat!

Gràcies també a la resta de jefes del grup. Esther, les reunions amb la Silvia no serien el mateix sense els tetrís que hem de fer a vegades per entrar en aquell despatx, jejeje. Gràcies per ser tan propera, pels teus comentaris i opinions en els seminaris, per la teva ajuda a l'hora de preparar les pràctiques i per donar-nos l'oportunitat de fer les xerrades de la setmana del cervell,

sempre han sigut experiències molt boniques! Gràcies també Jordi pels teus comentaris i el teu interès sempre que hem coincidit, i per convidar-nos a casa teva, va ser un dia molt divertit!

Sense deixar la 4a planta, també vull donar les gràcies a les seces, Núria, Carme i Mercè, per la vostra eficàcia i sobretot per la vostra amabilitat a l'hora de solucionar tot tipus de problemes de comandes, congressos, targetes i tot el que calgui, sense perdre mai el somriure. Gràcies també als postdocs de la 4a, Phil i Mercè per totes les vostres aportacions i consells científics i per compartir la vostra experiència, tant sigui en seminaris, com fent un cafè.

Gràcies també a tota la gent de la 3a, tant les generacions passades com les noves. Gràcies, Andy, Mònica i Jordi, per la vostra amabilitat i predisposició sempre que he necessitat qualsevol cosa. Gràcies també a tots els estudiants que han passat durant aquest temps i els que encara hi són: la Cristina Vila, la Sandra, el Xavi i la Cristina Salado. Mireia, gràcies per estar sempre disponible i posar les coses fàcils quan he vingut a consultar-vos coses. Ana, nunca lo diremos lo suficiente, el trabajo que haces es admirable, y por si no fuera poco, siempre tienes predisposición absoluta para ayudarnos en cualquier cosa que necesitamos y con una sonrisa en los labios. Eres un sol, gracias por todo! Georgina, la meva veïna graciencia, després felicitat i energia! Gràcies per haver-me ajudat sempre que ho he necessitat, espero continuar gaudint de moltes més festes de barri amb tu! Andrea, ha sido un placer compartir esta etapa de doctorado contigo, con las tardes de birras y experiencias como la FENS de entre medio. Mucha suerte en esta etapa final, estoy segura de que llegarás donde te propongas!

Gràcies també a l'equip de la sala blanca. Gràcies Gemma i Vero per totes les converses de dinar i de cafè compartides. Cris, eres una persona super alegre, ha sido un placer compartir entre otras cosas la organización de eventos del lab y alguna que otra birra y bailoteo. Raquel, eres todo bondad, cargada de experiencia, siempre dispuesta a ayudar e interesándote por los demás. Te deseo lo mejor en esta nueva etapa, porque si alguien se lo merece, eres tú!

I una mica més avall de la sala blanca, arribem al Cellex, la casa nova que vam estrenar ja fa més de 5 anys, i on no podríem haver trobar uns millors companys de planta. Gràcies Manel, Cris, Paqui, Francesc i la resta de gent de la 3B per tenir sempre disponibilitat 100% per qualsevol consulta, per ser excel·lents companys de laboratori i per tenir sempre un somriure i alguna que altre broma, que s'agraeixen moltíssim en els dies de més estrès! Gràcies també a la Maite Cazorla per estar sempre disponible per solucionar qualsevol problema i per facilitar-nos les coses, sempre amb un somriure a la cara!

I arribant al Cellex, arribo al meu grup. Gracias Vero por haber compartido conmigo tus consejos y experiencia y por haberte interesado siempre por mis proyectos. Eres un ejemplo de cómo con perseverancia y esfuerzo, todo es posible! Albert, amb tu he coincidit una mica al principi i una mica al final i em sap greu que no hagi pogut ser més temps perquè admiro molt la teva visió científica. Molta sort en aquesta nova etapa! Gràcies també a tots els estudiants que han anat passant durant tots aquests anys pel lab: Lidia, Irina, Sergio, Mireia, Ainhoa i Aida. Alex, Anna i Esther, que us vagi genial en les noves etapes que vindran! Marc, és curiós com, ensenyant

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també s'aprèn, i en aquest sentit he après moltes coses en tot el temps que ja portes al lab. Gràcies pel teu interès en el projecte des del primer dia, sense la teva ajuda no hagués arribat fins a aquí. Molta sort a partir d'ara, amb esforç i dedicació arribaràs molt lluny! Anika, mucha suerte también en tu tesis, vales mucho y estoy segura que irá genial!

I és el torn de la 5a, on tot i físicament, hi vam estar poc temps, continua sent casa nostra. I és impossible pensar en la 5a sense pensar en la Maite. Ets l'ànima de la 5a i, si més no pels predocs, crec que una mica la nostra mama científica, que ens renya quan ens comportem com porquets però que sempre està disponible per solucionar qualsevol problema i resoldre qualsevol dubte, i ens mima portant-nos xocolata o galetes de tant en tant! Gràcies per totes les vegades que m'has ajudat, per estar sempre disposada a escoltar els nostres drames i per fer-ho sempre amb un somriure. Et trobaré molt a faltar!

I tenim la sort de compartir la 5a amb els Gustavos i, compartir lab amb un grup que estudia un tema totalment diferent pot ser molt enriquidor. Gràcies a les antigues generacions per tots els consells, tant necessaris quan arribes a un lloc nou, Adrià, Javi, Jessica, Enric. I a tota la gent que ha passat durant aquests anys, Thayna, Dasha, Yolanda, Nacho, Carla i Julia. Laura, gràcies per totes les converses de cafè o de tupper, ets un exemple de superació i espero que et vagi molt bé, perquè t'ho mereixes! Fabio, gracias por mantenernos siempre alimentados jejeje. Isaac i Cristina, molta sort en les vostres noves etapes!

I arribem ara al minilab! Ana, gracias por todos tus consejos y por las múltiples charlas sobre mil temas que hemos compartido. Eres un sol y he aprendido muchísimo de ti! Glòria, ha estat un plaer haver coincidit amb tu, després positivisme i tranquil·litat. Gràcies per ensenyar-me a veure les coses amb perspectiva en els moments complicats. Gari, aún echo de menos tus anécdotas a la hora del café, tienes las ideas super claras y te sobra energía. Gracias por todos los momentos compartidos.

A les antigues generacions d'estudiants, Sara, Silvia i Aina, vau entrar carregades d'energia i de ganes i ens ho vau encomanar. Ha estat un plaer veure-us construir la vostra carrera científica, no podia ser de cap altre manera. Raquel, tú también fuiste un torbellino de energía y de motivación que entró al lab! Muchísima suerte en tu doctorado, con tus ganas, estoy segura de que te irá genial! I a les noves generacions, Anna, Carla, Ened, Cynthia, teniu les idees clares i moltes ganes, i amb això arribareu molt lluny, molta sort!

I acabo amb l'equip regletón. Tenir companys de feina meravellosos és una sort, però quan aquests es converteixen en amics, i pràcticament en família, és un luxe. Hem compartit viatges, excursions, congressos, dinars, sopars, cafès, birres i festes, així com incomptables hores al laboratori (pot ser masses i tot, perquè coses com medir-nos el diàmetre del cap, demanar pizzes o tastar certes coses, no sé si són massa normals... tot i que tenir la confiança per fer-ho és de les coses que més trobaré a faltar).

Començo amb les antigues generacions, qui ens va transmetre el bon rollo i companyerisme que desprèn aquest grup. Mar! Vaig tenir la sort de començar a aquest lab amb tu, i per sobre de la part més tècnica, em vas transmetre la teva energia, positivisme i passió per la ciència. Encara recordo els intensius de 16 hores i els atacs de riure que no entenia ningú més (probablement perquè ens faltava glucosa o oxigen al cervell jejeje). Ha estat un plaer compartir amb tu aquest projecte (carai si ha costat!), i encara tenim pendent, com a mínim, un bikini juntes, per celebrar-ho! Gràcies per tot! Cheru, he aprendido tanto de ti! Tienes una determinación y una seguridad que siempre me han fascinado. Viéndote trabajar y gracias a tus consejos y experiencia, aprendí a perderle el miedo a probar cosas nuevas. Gracias por estar siempre ahí para resolver todas mis dudas y para aconsejarme siempre que lo he necesitado, tanto científica como personalmente, y sobretodo por seguir estándolo a día de hoy! Carla! Qui ens havia de dir que acabaríem compartint pis! De tu també vaig aprendre molt mentre estaves al lab, em vas transmetre la teva passió per la biologia cel·lular i la ciència bàsica, encara recordo la quantitat d'interrogatoris que et vaig fer per aprendre a fer fraccionaments! Gràcies també per tots els cafès i birres en els dies que feien més falta i per totes les converses d'aquests últims mesos. Havia dit moltes vegades que volia estar sola al pis per escriure la tesi, i m'alegro molt que no hagi estat així, perquè m'has ajudat molt! Gràcies!

Xavi, sembla mentida que només estesis al lab uns quants mesos! Amb tu vaig aprendre com pot arribar a ser d'enriquidor col·laborar amb persones d'altres especialitats. Gràcies per tots els teus consells i per compartir amb mi la teva experiència! Annemie! Nos unimos mucho durante mi breve estancia de escritura en la 5a y ya no nos hemos separado. Gracias por escucharme tanto y darme ánimo y abrazos siempre que lo he necesitado. Eres un amor y desprendes bondad por todos los lados. Por muchos más cafés y birras compartiendo dramas juntas! Rafa! Parece mentira que ya haga casi un año que eres doctor! Aunque cuando entré en el lab tú llevabas solo unos meses, fuiste de las personas a quién más veces consulté, supongo que porque nunca tienes un no para nadie y siempre tienes una sonrisa para todos. Gracias por hacerme sentir como en casa desde el primer día, por hacerme reír y por estar siempre ahí. Eres un sol y te mereces lo mejor!

Laura, vas ser una onada d'aire fresc que va entrar al cellex! Tot i que sempre et dic que tens l'edat de la meva germana petita, he après molt de tu i amb la teva serenor i tranquil·litat m'has ajudat molt en aquesta etapa final (ara qui em dirà que mengi quan estic marejada? jejeje). Ha estat un plaer veure't créixer com a científica i estic segura que només és el principi, amb la teva motivació i la teva manera de ser, tens un futur molt prometedor! Marta, costa conèixer-te en profunditat, però una cosa queda clara enseguida, ets una bellíssima persona i tens les idees super clares. I així amb més confiança, també tens una part bromista i casi una mica gamberrilla que m'encanta. Gràcies per ajudar-me sempre que ho he necessitat, i molta sort a la recta final de la tesi, tot i que sent com ets, no et farà falta!

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Andrés, me encanta meterme contigo (tú tampoco te quedas corto, eh?), pero sabes que viene desde la confianza y el cariño. Eres un excelente científico, pero eres aún mejor persona. Gracias por todos los consejos, por las horas y horas de charlas y sobretodo por darme ánimo y hacerme creer en mí cuando más falta me hacía, eres un gran amigo!

Ger, ha estat una sort compartir aquesta etapa final frenètica amb tu i posar en comú dubtes i preocupacions. Gràcies per les mil i una vegades que m'has ajudat durant la tesi, ja fos amb problemes del cell profiler, a l'hora de triar pcs o de revisar analítiques de sang jejeje Mai tens un no per ningú i ets un pou de sabiduria, però sobretot ets una gran persona. Gràcies per tots els sopars, birres i riures que hem compartit, i pels que encara han de venir!

Jordi, tens l'estranya capacitat de generar somriures i "ves a cagars" en proporcions bastant similars, però fins i tot aquests últims acaben destensant situacions d'estrès, cosa que sempre és d'agrair. Sempre m'has fet sentir molt respectada i això en els moments baixos és molt important. Gràcies per tots els moments compartits en congressos, birres i sopars que sempre acaben generant anècdotes, i també per totes les discussions científiques que espero poder continuar mantenint. Ets un bon científic, però sobretot, una bona persona, i com comentàvem després de la tercera birra a la nostra casa de Brooklyn, fa falta molta gent així en ciència.

Laura, ho hem dit moltes vegades, sembla mentida que amb el poc cas que ens vam fer durant el màster, avui ja siguis una amiga per tota la vida! Ja fa un any i mig que vas marxar del lab i encara et trobo a faltar, els nostres cafés i les nostres xerrades inacabables discutint experiments, papers i maneres d'entendre la ciència (i algun que altre cotilleo...). Tens els valors claríssims i una ètica que admiro molt i que fa molta falta en el nostre món. Ha estat un plaer créixer com a científiques juntes i, malgrat crec que amb tu la ciència ha perdut una gran investigadora, a la llarga en guanyarem molts, perquè aconseguiràs transmetre la teva vocació i la teva energia a tots els teus estudiants. Gràcies per haver-hi estat sempre durant aquests anys, i per recollir-me de terra i donar-me ànims per continuar sempre que ho he necessitat!

Sara! no hace falta decir mucho porque ya nos lo hemos dicho todo... Eres todo energía y positivismo y se lo pegas a cualquiera que tengas cerca. Compartimos el gusto por la ropa, el comer, el beber, las fotos y tantas otras cosas, y podemos hablar de cualquier mierda (a veces literal jejeje). Eres todo bondad y una de las personas más generosas que conozco, tienes una motivación envidiable y eres una currante como pocas. Por eso estoy segura de que vas a conseguir cualquier cosa que te propongas. Ha sido un lujo compartir esta etapa contigo y te has convertido en una de mis mejores amigas. Gracias por todas las sonrisas y lloros compartidos y por estar siempre ahí con un abrazo a punto cuando más falta me hacía.

Núria, vam començar juntes i acabarem juntes. Fa tant de temps que ens coneixem i hem passat tantes hores juntes, que moltes vegades sobren les paraules. Ens vam entendre des del primer moment i ens vam convertir en un pack (el que va costar que la gent aprengué a distingir-nos! jejeje). Ens hem fet costat des del primer dia, hem après juntes, i hem compartit bons i mals moments. Ets una gran persona, tens una força de voluntat tremenda i ets una gran científica,

molt més del que t'atribueixes! Gràcies per ser-hi sempre, per escoltar totes les meves preocupacions i anades d'olla i per tranquil·litzar-me, no saps fins a quin punt ha estat important per a mi durant aquests anys. Ha estat un privilegi i una sort compartir aquest viatge amb tu (i els que vindran!).

I podria continuar agraint a tantes persones que en un moment o altre m'han ajudat: l'Elisenda, l'Anna i la Maria del confocal, la Isa de citometria, la Lara i el Pep dels estabularis, la Nuri de la facultat que sempre et rep amb un somriure els dies que toca "trasnochar", i fins i tot al Manel de l'Ascot per tenir-nos preparada sempre una Estrella quan volem desconnectar. Però com que no acabaria mai, em limitaré a fer un agraïment final a tothom que m'ha ajudat a arribar fins aquí, perquè està clar que sola, no ho hagués aconseguit. Em serà impossible pensar en vosaltres i en tots els moments viscuts a la facultat, al cellex i a l'histolab, sense que se m'escapi un somriure. Gràcies a tots!

RESUM

INTRODUCCIÓ

La malaltia de Huntington (MH) és un desordre neurodegeneratiu letal causat per una mutació autosòmica dominant en el gen que codifica per la proteïna Huntingtina (HTT). Aquesta mutació consisteix en l'expansió anòmala (<40) del triplet CAG que codifica per l'aminoàcid glutamina (Q), cosa que es tradueix en l'aparició d'un fragment poliQ a l'extrem N-terminal de la proteïna HTT, amb efectes tòxics per les cèl·lules que l'expressen (DiFiglia *et al*, 1995; The Huntington's Disease Collaborative Research Group, 1993; Zuccato *et al*, 2010). La MH es caracteritza, per una banda, per la presència de símptomes motors consistents en l'aparició de corees, és a dir, moviments involuntaris; i per una altra banda, per la presència de símptomes cognitius, que es manifesten abans de l'aparició dels dèficits motors. Aquests consisteixen en problemes de memòria, aprenentatge i alteracions en funcions executives. Finalment, també hi ha presència de símptomes psiquiàtrics com apatia o depressió (Huntington, 1872; Paulsen *et al*, 2008; Roos, 2010; Walker, 2007). L'aparició dels símptomes motors està fortament associada a la degeneració específica de neurones estriatals espinoses de mida mitjana, el que representa una de les característiques principals de la malaltia (Reiner *et al*, 2011; Vonsattel *et al*, 1985). D'altra banda, l'aparició dels símptomes cognitius s'associa a una alteració en la connexió corticoestriatal i a una disfunció hipocampal (Begeti *et al*, 2016; Giralt *et al*, 2012b; Raymond *et al*, 2011).

A nivell molecular, s'han descrit diversos mecanismes que contribueixen a una major vulnerabilitat estriatal, com l'exposició a l'excitotoxicitat o la disfunció mitocondrial (Zuccato *et al*, 2010), tot i que els mecanismes que desencadenen la degeneració estriatal selectiva a causa de la presència de la HTT mutada (HTTm), encara es desconeixen. A més, s'ha descrit que la presència de la HTTm comporta una alteració de múltiples processos sinàptics, com ara els nivells de diferents neurotransmissors i els seus respectius receptors, o bé, la densitat d'espines dendrítiques. D'aquesta manera la HTTm altera la inducció de la plasticitat sinàptica, que és necessària per a l'aprenentatge i la formació de memòria (Li *et al*, 2003; Tyebji and Hannan, 2017). Per aquests motius, la identificació de dianes que participin simultàniament en la vulnerabilitat estriatal i en la disfunció sinàptica, permetria actuar tant sobre els dèficits motors, com sobre els dèficits cognitius de la MH, per tal de prevenir o frenar el desenvolupament de la patologia.

Degut a les seves propietats, una d'aquestes possibles dianes és la cinasa Cdk5 (Cyclin-dependent kinase 5). A diferència de la resta de membres de la seva família que participen en la regulació del cicle cel·lular en cèl·lules proliferatives, l'activitat de Cdk5 es restringeix principalment a les neurones, gràcies a l'expressió específica dels seus activadors p35 i p39 en aquest tipus cel·lular (Tsai *et al*, 1994). Així, s'han descrit múltiples funcions neuronals de Cdk5 que engloben des de la regulació de la migració neuronal durant el desenvolupament

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fins a la modulació de diversos processos implicats en la plasticitat sinàptica, com la regulació d'alguns receptors de neurotransmissors o de proteïnes del citoesquelet que permeten la remodelació d'espines dendrítiques (Su and Tsai, 2011). A part d'aquestes funcions i en contraposició al que s'ha descrit en cèl·lules en proliferació, Cdk5 sí que participa en la regulació del cicle cel·lular neuronal, però, en aquest cas, inhibint la seva progressió un cop s'han diferenciat (Zhang and Herrup, 2008). Donat doncs aquest gran nombre de funcions que Cdk5 duu a terme al sistema nerviós central (SNC), és lògic que alteracions en la seva activitat s'hagin associat a processos de neurodegeneració. En resposta a diferents estressors neuronals, la proteïna calpaina s'activa i processa p35 a la forma p25, que s'ha associat a efectes neurotòxics per la cèl·lula, alterant l'activitat de Cdk5. Donat que p25 té una vida mitjana més llarga que p35, s'incrementa el temps d'activació de la cinasa. A més, s'altera la seva distribució subcel·lular, ja que es perd el residu de miristoilació que li permetria ancorar-se a la membrana plasmàtica, alterant l'especificitat dels substrats de Cdk5 (Asada *et al*, 2008; Kusakawa *et al*, 2000; Patrick *et al*, 1998, 1999). Aquest tipus de desregulació s'ha descrit en múltiples processos neuropatològics, com la malaltia d'Alzheimer o la MH (Cheung and Ip, 2012; McLinden, 2012). En el cas concret de la MH, estudis previs del nostre grup han descrit que la desregulació de l'activitat de Cdk5 participa en la major vulnerabilitat estriatal davant d'estímuls glutamatèrgics i dopaminèrgics, i contribueix a la disfunció mitocondrial (Cherubini *et al*, 2015; Paoletti *et al*, 2008). Tot i així, la possible implicació d'una desregulació de Cdk5 en la disfunció sinàptica present en la MH i, conseqüentment, en l'aparició dels dèficits cognitius, no ha estat examinada.

Amb aquests antecedents, en aquesta Tesi ens hem plantejat, per una banda, estudiar el paper de Cdk5 en l'aparició dels dèficits cognitius en la MH, analitzant la seva participació en les alteracions sinàptiques; i per una altra, analitzar si la desregulació de Cdk5 podria contribuir a una reentrada aberrant al cicle cel·lular per part de neurones estriatals, contribuint a la major vulnerabilitat estriatal present en la MH.

RESULTATS I DISCUSSIÓ

1. Paper de Cdk5 en els dèficits cognitius de la malaltia de Huntington

Per tal de dur a terme el primer objectiu, vam crear un nou model murí doble mutant que expressa la proteïna Htt amb 111 repeticions CAG (knock-in o KI), i és heterozigot per la proteïna Cdk5 (Cdk5^{+/-}) específicament en neurones del SNC. Després de comprovar que tant els ratolins Cdk5^{+/-} com els ratolins dobles mutants o KI:Cdk5^{+/-}, presentaven aproximadament un 50% menys d'expressió de Cdk5 en les regions cerebrals de l'escorça, l'estriat i l'hipocamp, vam procedir a avaluar la seva funció cognitiva a l'edat de 6 mesos, quan els ratolins model de la MH (KI) presenten símptomes cognitius però no motors (Brito

et al, 2014; Puigdemívol *et al*, 2015). Vam utilitzar dues tasques per avaluar l'aprenentatge motor i la memòria de procediment, que depenen de la funció corticoestriatal; i dues tasques per avaluar la memòria de reconeixement i la memòria espacial, que són dependents de la funció hipocampal. Els nostres resultats van demostrar que la reducció genètica de Cdk5, revertia els dèficits cognitius, tant corticoestriatals com hipocampals, en ratolins KI, demostrant una implicació crítica de Cdk5 en l'aparició de les alteracions d'aprenentatge i memòria en la MH.

A continuació, per tal d'investigar quins mecanismes moleculars participaven en les millores funcionals observades, vam analitzar els nivells dels receptors glutamatèrgics NMDA (NMDARs). Els NMDARs tenen un paper fonamental en els processos de plasticitat sinàptica (Izquierdo, 1991) i diversos estudis han demostrat que Cdk5 és capaç de regular els seus nivells a través de diferents vies (Li *et al*, 2001a). En primer lloc, vam mesurar els nivells proteics de les tres subunitats principals dels NMDARs: NR1, NR2A i NR2B, a l'escorça, l'estriat i l'hipocamp de ratolins salvatges (WT), KI, Cdk5^{+/-} i KI:Cdk5^{+/-}. Els nostres resultats van revelar una disminució específica dels nivells de NR2B a l'escorça dels ratolins KI, sense mostrar alteracions en els nivells de NR1 ni de NR2A. Notablement, aquesta disminució era revertida en els ratolins KI:Cdk5^{+/-}. A l'estriat i a l'hipocamp, en canvi, no vam observar alteracions significatives entre genotips en els nivells de cap de les subunitats de NMDARs analitzades. Seguidament, per tal de comprovar si la disminució dels nivells totals de NR2B observada a l'escorça, correlacionava amb una alteració funcional, vam passar a analitzar específicament els nivells de membrana de NR2B. Amb aquesta finalitat vam realitzar un assaig de biotinitilació, amb el que vam detectar que els nivells de membrana de NR2B també estaven significativament disminuïts a l'escorça dels ratolins KI. Sorprenentment, tot i que els nivells totals de NR2B no mostraven cap alteració en l'estriat ni en l'hipocamp dels ratolins KI, sí que vam observar una reducció significativa en analitzar específicament els nivells de membrana de NR2B en aquestes regions. De forma destacable, la reducció genètica de Cdk5 en els ratolins KI:Cdk5^{+/-}, era capaç de recuperar els nivells de NR2B a la membrana plasmàtica tant en l'escorça com en l'estriat. En canvi, a l'hipocamp, els ratolins KI:Cdk5^{+/-} mostraven només una restauració parcial dels nivells de NR2B de membrana. Finalment, vam voler analitzar a través de quina via, la disminució genètica de Cdk5 podia estar recuperant els nivells de membrana de NR2B. S'ha descrit que Cdk5 és capaç de regular negativament la localització a la membrana de NR2B directament, a través de la fosforilació de NR2B (Plattner *et al*, 2014) i facilitant el seu processament i degradació per part de la proteïna calpaina (Hawasli *et al*, 2007), i indirectament, a través de la inhibició de la via pSrc/pNR2B (Zhang *et al*, 2008b). Curiosament, els nostres resultats van demostrar que els ratolins KI presenten una disminució específica de la via pSrc/pNR2B a l'escorça, sense que les altres dues vies prèviament mencionades es veiessin alterades. Aquesta via en qüestió evita la internalització de NR2B, a través de l'activació de la proteïna Src (pSrc) i la

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conseqüent fosforilació de NR2B al residu tirosina 1472 (pNR2B), cosa que evita la seva internalització. D'acord amb estudis previs on s'ha descrit que Cdk5 és capaç d'inhibir aquesta via (Zhang *et al*, 2008b), els nostres resultats van mostrar que la reducció de Cdk5 en els ratolins KI:Cdk5^{+/-}, revertia la disminució de la via pSrc/pNR2B observada en l'escorça dels ratolins KI. En contraposició, no vam trobar canvis significatius entre genotips d'aquesta via ni en l'estriat ni en l'hipocamp.

En conjunt, aquests resultats indiquen que la presència de Httm en els ratolins KI causa una disminució generalitzada dels nivells de membrana de NR2B tant a l'escorça, com a l'estriat, com a l'hipocamp. Ara bé, els mecanismes que porten a aquesta disminució varien segons la regió cerebral analitzada, ja que a l'escorça també s'observa una disminució dels nivells totals de NR2B, no detectable en l'estriat ni en l'hipocamp. De manera destacable, la reducció genètica de Cdk5 és capaç de revertir totalment les alteracions corticoestriatals, cosa que podria explicar la millora en les tasques cognitives dependents d'aquestes regions. En canvi, el fet que en l'hipocamp, la disminució de Cdk5 no recuperi els nivells de NR2B, tot i que els ratolins KI:Cdk5^{+/-} sí que mostrin una preservació de la funció cognitiva hipocampal, indica que la disminució genètica de Cdk5 probablement afecta altres vies involucrades en la regulació de la plasticitat sinàptica en aquesta regió.

Per tal de corroborar aquesta hipòtesi, vam analitzar la densitat d'espines dendrítiques en neurones piramidals de la regió CA1 de l'hipocamp, ja que Cdk5, a través de la modulació de diversos substrats implicats en la regulació del citoesquelet, participa en la remodelació d'espines dendrítiques (Jin *et al*, 2016; Kim *et al*, 2006a; Sala and Segal, 2014). D'aquesta manera vam observar que els ratolins KI presenten una menor densitat d'espines dendrítiques, i aquesta alteració és recuperada en els ratolins KI:Cdk5^{+/-}. Sorprenentment, a diferència dels mecanismes analitzats prèviament, els ratolins Cdk5^{+/-} mostraven un augment en el nombre d'espines dendrítiques en comparació amb els ratolins WT. Ja que estudis anteriors han descrit que un dels substrats a través dels quals Cdk5 regula la remodelació d'espines dendrítiques, és la RhoGTPasa Rac1 (Nakayama *et al*, 2000; Posada-Duque *et al*, 2015), també vam analitzar els nivells de la seva activitat. Els resultats obtinguts van mostrar que, tot i que ratolins KI presentaven nivells d'activitat Rac1 similars als ratolins WT, aquests estaven augmentats tant en els ratolins Cdk5^{+/-}, com en els KI:Cdk5^{+/-}. Això encaixaria amb estudis que descriuen que la inhibició de Cdk5 comporta un augment de l'activitat Rac1 (Posada-Duque *et al*, 2015), i explicaria la recuperació de la densitat d'espines dendrítiques hipocampals que presenten els ratolins KI:Cdk5^{+/-}.

Així doncs, els nostres resultats demostren que la prevenció dels dèficits cognitius corticoestriatals correlaciona amb una recuperació dels nivells de membrana de NR2B a l'escorça i a l'estriat, mentre que la prevenció dels dèficits cognitius hipocampals està

associada a una recuperació de la densitat d'espines dendrítiques a l'hipocamp. Ara bé, per tal de saber si la recuperació del nombre d'espines era un mecanisme específic de l'hipocamp, vam analitzar també aquest paràmetre a l'escorça. Els resultats obtinguts van determinar que els ratolins KI també mostraven una disminució significativa del nombre d'espines dendrítiques en neurones piramidals de la capa V de l'escorça, i de manera similar a l'hipocamp, els ratolins KI:Cdk5^{+/-}, recuperaven aquest dèficit. Tot i així, l'anàlisi d'activitat Rac1 va fer palesa l'existència de diferències entre les dues regions. Així doncs, en l'escorça, els ratolins KI presentaven uns nivells d'activitat Rac1 inferiors als ratolins WT, tot i que aquesta disminució no era recuperada totalment en els ratolins KI:Cdk5^{+/-}. En canvi, el fet que en l'hipocamp, els ratolins KI:Cdk5^{+/-} presentessin un augment d'activitat Rac1 respecte als ratolins WT i KI, indicaria que la recuperació d'espines dendrítiques hipocampals podria ser deguda a la pròpia reducció genètica de Cdk5, independentment de la presència de Httm. Aquesta idea encaixaria amb el fet que els ratolins Cdk5^{+/-} també mostraven un augment d'activitat Rac1 i d'espines dendrítiques respecte als ratolins WT, específicament en l'hipocamp.

Aquests resultats demostren que Cdk5 té un paper molt complex i diferenciat depenent de la regió cerebral en qüestió, en l'aparició dels símptomes cognitius en la MH, participant en la regulació de diversos processos implicats en la plasticitat sinàptica, necessària per l'aprenentatge i la formació de memòria. Particularment, les nostres observacions suggereixen que Cdk5 té un paper especialment important en l'escorça, on contribueix a la recuperació, tant dels nivells de NR2B de membrana, com del nombre d'espines dendrítiques.

Els resultats obtinguts en l'anàlisi dels nivells de fosforilació de diversos substrats de Cdk5, per tal de mesurar possibles canvis en l'activitat de la cinasa entre genotips, reforcen aquesta hipòtesi. Així doncs, els ratolins KI presentaven un augment significatiu en els nivells de fosforilació de Tau (pTau) en l'escorça, així com una tendència a l'alça en els nivells de fosforilació de l'inhibidor de la PP1 (pIPP1) i dels residus serina dels substrats de CDKs. En canvi, els ratolins KI no mostraven alteracions significatives d'aquests substrats ni en l'estriat ni en l'hipocamp, suggerint que l'activitat Cdk5 podria tenir una alteració preferent en l'escorça dels ratolins KI. Tot i així, la reducció genètica de Cdk5 no revertia l'increment observat en els nivells de pTau, tot i que sí que evitava la tendència a l'alça observada en els nivells de fosforilació dels substrats de CDKs. Aquests resultats suggereixen que els canvis en l'activitat Cdk5 també són específics de substrat, cosa que podria ser deguda a una regulació diferencial de l'activitat Cdk5 a nivell subcel·lular. Per corroborar aquesta hipòtesi, vam realitzar un fraccionament subcel·lular per analitzar específicament els nivells de Cdk5 i p35 a la fracció de membrana i a la fracció citosòlica. Els nostres resultats van mostrar que els nivells de p35 a la membrana plasmàtica estaven significativament reduïts en l'escorça

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dels ratolins KI, indicant que la presència de Httm estaria disminuint la localització de p35 a la membrana, i per tant podria estar alterant l'accés de Cdk5 a substrats localitzats en aquesta fracció cel·lular. De forma destacada, aquesta disminució era recuperada en els ratolins KI:Cdk5^{+/-}. A més, els nivells de p35 a la fracció citosòlica mostraven un augment en els ratolins Cdk5^{+/-} i els KI:Cdk5^{+/-}, cosa que podria explicar la major localització de p35 a la membrana plasmàtica en els ratolins KI:Cdk5^{+/-}. Donat que Cdk5 regula positivament la degradació de p35 (Patrick *et al*, 1998), el fet que hi hagi menys expressió de Cdk5 contribuiria a una major disponibilitat de p35, que podria localitzar-se a la membrana plasmàtica i, per tant, recuperar l'especificitat de Cdk5 pels seus substrats.

Sorprenentment, en l'estriat i en l'hipocamp no vam observar canvis en els nivells de p35 ni de Cdk5 entre genotips, ni en la fracció de membrana ni en la fracció citosòlica. Tot i així, el fet que en aquestes regions sí que haguem observat una recuperació dels dèficits cognitius i de determinats substrats de Cdk5 en els ratolins KI:Cdk5^{+/-}, suggeriria que altres mecanismes de regulació de l'activitat Cdk5, a part de p35, podrien estar alterats en aquestes regions. De fet, tot i que p35 és el principal activador de Cdk5, la seva activitat també està regulada per diferents modificacions post-traduccionals i per altres proteïnes com la CiclinaE o GSTP1 (Shah and Lahiri, 2014). Així doncs, tot i que no hem detectat p25 en les nostres condicions experimentals, no podem descartar la seva presència o la d'altres modificacions post-traduccionals, corroborant així la complexitat de la regulació de l'activitat Cdk5 i per tant de l'estudi de la seva possible alteració.

En general, els resultats obtinguts en el primer objectiu descriuen el complex paper de Cdk5 com a un nou regulador en l'aparició dels dèficits cognitius en la MH. Aquesta regulació es dona de manera diferencial segons la regió cerebral, amb un paper particularment rellevant en l'escorça, afectant diferents substrats implicats en diversos processos necessaris per la correcta modulació de la plasticitat sinàptica.

2. Paper de Cdk5 en la reentrada neuronal al cycle cel·lular com a mecanisme de vulnerabilitat estriatal

El segon objectiu d'aquesta Tesi pretenia analitzar si la desregulació de Cdk5 en l'estriat en la MH podria causar que les neurones reentressin al cycle cel·lular de forma aberrant, contribuint així a una major vulnerabilitat estriatal. Tot i que Cdk5 és considerada una CDK atípica per la seva funció principal en neurones diferenciades, també s'ha descrit que la presència nuclear de Cdk5 és necessària per mantenir la inhibició del cycle cel·lular neuronal (Zhang and Herrup, 2008). Una alteració d'aquesta funció pot conduir a una reentrada aberrant al cycle cel·lular. Notablement, aquest procés s'ha descrit en diferents malalties neurodegeneratives com la malaltia d'Alzheimer, i s'ha proposat que podria ser un mecanisme comú de neurodegeneració, ja que, tot i que un intent de divisió cel·lular per

part d'una neurona acabaria causant la seva mort, es creu que aquest procés podria trigar mesos i fins i tot anys (Herrup and Yang, 2007).

Pel que fa al paper de Cdk5, concretament, s'ha descrit que la cinasa forma un complex al nucli junt amb l'inhibidor del cicle cel·lular, p27, i el factor de transcripció, E2F1, evitant així que E2F1 s'uneixi al seu cofactor, DP1, i iniciï la transcripció de gens necessaris per a la progressió del cicle cel·lular (Zhang and Herrup, 2011). Per aquest motiu, vam realitzar una co-immunoprecipitació de Cdk5 i vam analitzar la seva interacció amb p27 en l'estriat de ratolins KI a l'edat en la que comencen els dèficits motors (8 mesos) i en una etapa més avançada de la patologia (13 mesos). Tot i que els nivells d'interacció de p27 amb Cdk5 tendien a estar reduïts en l'estriat dels ratolins KI a 8 mesos d'edat, aquests canvis no eren significatius i no s'observaven a 13 mesos d'edat. Tot i així, com que la interacció de Cdk5 i p27 es dona específicament al nucli, també vam realitzar un fraccionament subcel·lular per analitzar específicament els nivells nuclears de Cdk5 i p27 a 8 mesos d'edat. Els resultats obtinguts van revelar una reducció significativa dels nivells nuclears de Cdk5 i p27 en els ratolins KI, cosa que podria comportar una pèrdua de la funció inhibidora d'aquestes proteïnes sobre la progressió del cicle cel·lular neuronal.

Per tal de corroborar aquesta hipòtesi, vam analitzar els nivells de p27 i també de les proteïnes de la fase G₁ del cicle cel·lular, CiclinaD1 i Cdk4, a l'estriat de ratolins WT i KI a diferents etapes de la malaltia. Els resultats obtinguts van mostrar que, contràriament a les observacions fetes en la fracció nuclear, els nivells totals de p27 estaven significativament augmentats des dels 6 mesos d'edat, quan comencen els dèficits cognitius, fins a les etapes més avançades de la patologia (18 mesos). Aquestes dades, per tant, indicarien que la reducció nuclear de p27 probablement no és deguda a una alteració en la seva expressió o en la seva degradació, sinó a una alteració en el seu transport nuclear. De forma destacada, Cdk5 requereix unir-se a p27 per tal d'entrar al nucli, ja que no posseeix una seqüència de localització nuclear, per tant, una alteració de la localització nuclear de p27, també podria comportar una disminució dels nivells nuclears de Cdk5, i per tant una pèrdua de la inhibició de la progressió del cicle cel·lular. D'acord amb aquesta hipòtesi, vam observar que els nivells de CiclinaD1 en l'estriat dels ratolins KI, estaven significativament augmentats des dels 8 mesos d'edat fins a les etapes més avançades de la patologia. En canvi, els nivells de Cdk4 només presentaven una tendència a l'alça als 18 mesos d'edat, sense presentar alteracions en etapes prèvies de la malaltia. També vam analitzar els nivells d'aquestes proteïnes en mostres de putamen de pacients control i de la MH. Tot i això, els resultats obtinguts en mostres de pacients humans, variaven respecte als obtinguts en els ratolins KI. Així doncs, els pacients de la MH presentaven un augment significatiu dels nivells de Cdk4, mentre que els nivells de CiclinaD1 no estaven alterats. Els nivells totals de p27 i d'E2F1 tampoc canviaven, tot i que en mostres de pacients de la MH vam observar un increment

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d'una forma d'E2F1 amb un pes molecular més baix, el que podria correspondre a la seva forma processada. Per la seva banda, p27 mostrava alteracions en el seu pes molecular aparent en la separació electroforètica, específicament en les mostres de pacients de la MH, cosa que podria indicar la presència de modificacions post-traduccionals. Així doncs, d'acord amb estudis anteriors (Fernandez-Fernandez *et al*, 2011; Liu *et al*, 2015; Pelegrí *et al*, 2008), aquest resultat indiquen, que en el context de la MH, hi ha una alteració de proteïnes del cycle cel·lular, tot i que aquestes alteracions es donen de forma diferent en el model murí KI, que en mostres de pacients humans. Aquestes diferències podrien ser atribuïdes a canvis entre els dos contextos, com ara al fet que en el model KI no hi ha mort neuronal, mentre que en pacients humans aquesta sí que es dona. Tot i així, el fet que l'alteració de proteïnes del cycle cel·lular es doni en diferents contextos de la patologia, suggereix que la presència de Httm podria estar induint una reentrada aberrant al cycle cel·lular, contribuint a la disfunció i a la mort estriatal.

Per tal d'analitzar amb més profunditat aquesta hipòtesi, vam utilitzar cultius primaris neuronals estriatals i els vam tractar amb NMDA donat que, l'excitotoxicitat és un dels principals mecanismes proposats per explicar la vulnerabilitat estriatal específica que es dona en la MH (Fan and Raymond, 2007). A més, l'entrada de calci després de l'activació de NMDARs, és també un dels principals mecanismes que porten a l'activació de calpaina i per tant al processament de p35 a p25 (Miao *et al*, 2012; Patrick *et al*, 1999). Així doncs, després de determinar quina concentració de NMDA i quina duració del tractament era l'òptima per generar l'activació de calpaina, sense induir una mort apoptòtica significativa, vam analitzar l'efecte del tractament amb NMDA sobre les proteïnes del cycle cel·lular prèviament analitzades. Els nostres resultats van mostrar que el tractament amb NMDA en cultius estriatals WT causava una disminució significativa dels nivells proteics de p27, i una tendència a la baixa dels nivells de Cdk4 i de CiclinaD1 analitzats per Western blot. En el cas d'E2F1, tot i que els nivells totals no mostraven canvis significatius, el tractament amb NMDA causava l'aparició d'una banda de pes molecular inferior, probablement producte del processament d'E2F1. Per tal de confirmar aquesta hipòtesi vam fer un tractament previ amb un inhibidor de calpaina en cultius estriatals WT, abans de tractar-los amb NMDA. Així vam observar que la presència de l'inhibidor de calpaina prevenia l'aparició d'aquesta forma d'E2F1 de pes molecular inferior. Aquests resultats indiquen que, de manera similar al que han descrit estudis anteriors, l'activació de calpaina en resposta al tractament amb NMDA, causaria un processament d'E2F1. Notablement, un augment d'aquest tipus de processament d'E2F1 s'ha associat a efectes neurotòxics en un model neurodegeneratiu *in vitro* (Zyskind *et al*, 2015). De forma similar, els nostres resultats també van mostrar que la disminució de p27 després del tractament amb NMDA, també era revertida quan s'inhibia la calpaina. Tenint en compte que la calpaina també és la responsable de la hiperactivació de Cdk5, seria interessant en futurs estudis inhibir específicament Cdk5, per tal de

determinar si aquests efectes depenen directament de la calpaina, o si la desregulació de Cdk5 també hi està implicada.

Els nostres resultats també van mostrar que el tractament amb NMDA causava una alteració de la distribució subcel·lular de les proteïnes del cicle cel·lular. Així doncs, p27, que en condicions basals es trobava al nucli d'una alta proporció de neurones, perdia part de la seva localització nuclear, cosa que podria induir una desinhibició de la progressió del cicle cel·lular. D'altra banda, E2F1 que en condicions normals es trobava concentrada al citosol de la majoria de neurones, passava a tenir una distribució difosa per tota la neurona després del tractament amb NMDA, trobant-se també en el nucli en alguns casos. El tractament amb NMDA afectava de forma similar a Cdk4, que també mostrava una localització citosòlica en condicions basals. El fet que E2F1 i Cdk4 siguin proteïnes associades a l'inici de la fase G₁ del cicle cel·lular, fa que el seu canvi de localització, i especialment la seva localització en el nucli, pugui reflectir una reentrada neuronal al cicle cel·lular. Sorprenentment, la distribució de CiclinaD1, que s'associa a Cdk4 durant les primeres etapes de la fase G₁, presentava una distribució diferent a Cdk4. D'aquesta manera, en condicions basals, CiclinaD1 mostrava una baixa expressió neuronal, localitzant-se al nucli en les poques neurones on s'expressava, cosa que podria reflectir funcions addicionals de CiclinaD1. Després del tractament amb NMDA, la distribució de CiclinaD1 en les neurones on s'expressava passava a ser també citosòlica, a més de nuclear. Aquests resultats indiquen que el tractament amb NMDA altera de manera important la funció de les proteïnes del cicle cel·lular en neurones estriatals WT.

Pel que fa a l'efecte de la presència de Httm en aquestes alteracions, no vam observar canvis significatius en els nivells totals de p27, E2F1, Cdk4 o CiclinaD1 entre cultius WT i KI, ni en condicions basals ni després del tractament amb NMDA. Tot i així, l'anàlisi immunocitoquímica de l'expressió d'aquestes proteïnes específicament en neurones, van mostrar algunes alteracions entre genotips. Així doncs, els cultius KI en condicions basals mostraven més expressió neuronal de Cdk4, així com una tendència a l'alça pel que fa als nivells d'E2F1. En canvi l'expressió de p27 i de CiclinaD1 presentava una tendència a disminuir que també s'observava entre cultius WT i KI tractats amb NMDA. Futurs estudis corroborant aquests resultats i analitzant específicament els nivells nuclears i citosòlics d'aquestes proteïnes, permetrien una millor caracterització de l'efecte de la Httm sobre les proteïnes del cicle cel·lular. Ara bé, el fet que els cultius on s'expressa la Httm tendeixin a tenir menys p27, podria afavorir una pèrdua de la inhibició de la progressió del cicle cel·lular, i coincidiria amb les nostres observacions en l'estriat dels ratolins KI on els nivells nuclears de p27 es troben disminuïts. De manera similar, la tendència d'E2F1 i Cdk4 a presentar major expressió neuronal, podria ser un indicador d'una reentrada al cicle cel·lular en neurones que expressen la Httm. Tot i així, el fet que l'expressió i localització subcel·lular de CiclinaD1 sigui tan diferent a Cdk4, suggereix que aquestes proteïnes podrien tenir funcions

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addicionals a la seva participació al cicle cel·lular en neurones diferenciades. Això concordaria amb el fet que tant E2F1 com Cdk4 mostrin una forta expressió en la majoria de neurones en condicions basals, cosa que no seria d'esperar en cèl·lules diferenciades. En efecte, tot i que s'ha descrit que algunes proteïnes del cicle cel·lular estan implicades en funcions com la regulació del citoesquelet, la transcripció gènica o inclús amb funcions sinàptiques (Frank and Tsai, 2009; Lim and Kaldis, 2013; Odajima *et al*, 2011), encara es té poca informació sobre quines funcions addicionals tenen aquestes proteïnes en condicions basals en una neurona. Donat que l'alteració de proteïnes del cicle cel·lular s'ha descrit, no només en la MH, sinó també en altres contextos neuropatològics, continuar estudiant el paper d'aquestes proteïnes en neurones, contribuiria a entendre els efectes de la seva desregulació i per tant a descriure nous mecanismes implicats en la disfunció neuronal en aquests contextos.

En el cas de la MH, on l'excitotoxicitat glutamatèrgica representa un dels principals factors de vulnerabilitat estriatal, els nostres resultats suggereixen que l'alteració de proteïnes del cicle cel·lular, afavorint una reentrada neuronal al cicle cel·lular, podria ser un mecanisme important involucrat en aquest procés. En aquest context, Cdk5 podria actuar com un enllaç entre una excessiva senyalització glutamatèrgica i una desregulació de la inhibició del cicle cel·lular neuronal.

CONCLUSIONS

Els resultats obtinguts en aquesta Tesi demostren que Cdk5 té un paper important en l'aparició dels dèficits cognitius corticoestriatals i hipocampals presents en la MH. Aquesta implicació es dona a través de la regulació de diferents processos implicats en la plasticitat sinàptica, com la modulació dels nivells de superfície dels receptors NMDA i la regulació de proteïnes del citoesquelet implicades en la remodelació d'espines dendrítiques. De forma destacada, aquesta regulació es dona de forma diferent segons la regió cerebral, i les nostres observacions suggereixen que l'alteració d'especificitat de substrats per part de Cdk5 davant la presència de Httm, podria ser especialment rellevant a l'escorça.

D'altra banda, en aquesta Tesi també hem descrit que, tant en el context de la MH, com en resposta a l'activació de NMDARs, es dona una alteració de diverses proteïnes implicades en la regulació del cicle cel·lular, cosa que podria portar a una reentrada neuronal al cicle cel·lular, causant una disfunció i/o mort neuronal. A més, el fet que els nivells nuclears de Cdk5 estiguin disminuïts en l'estriat de ratolins simptomàtics de la MH, suggereix que el paper de Cdk5 com a inhibidor del cicle cel·lular podria estar alterat en neurones estriatals de la MH, contribuint a l'alteració de proteïnes del cicle cel·lular.

Així doncs, proposem que la desregulació de proteïnes del cicle cel·lular en resposta a la desregulació de Cdk5 i/o a una excessiva activació de NMDARs, podria ser un nou mecanisme molecular implicat en la vulnerabilitat estriatal en la MH. D'altre banda, el paper de Cdk5 en l'aparició dels dèficits cognitius i la disfunció sinàptica, atorga a Cdk5 una doble implicació, i per tant, el converteix en una possible diana terapèutica, tant dels dèficits motors com cognitius de la MH.

ABBREVIATIONS

ABBREVIATIONS

3-NP	3-nitropropionic acid
A ₁ R	adenosine A1 receptor
A _{2A} R	adenosine A2A receptor
ACSF	artificial cerebrospinal fluid
AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
AP2	adaptor protein 2
APC/C	anaphase-promoting complex/cyclosome
ARTP	accelerating rotarod training procedure
A-T	ataxia-telangiectasia
ATP	adenosine triphosphate
AU	airy unit
Aβ	beta amyloid
BAC	bacterial artificial chromosome
BDNF	brain-derived neurotrophic factor
BrdU	bromodeoxyuridine
C/Cys	cysteine
Ca ²⁺	calcium
CA1	cornu ammonis 1
CA3	cornu ammonis 3
CaMKII	Ca ²⁺ /calmodulin-dependent protein kinase II
CB	cerebellum
CBP	CREB binding protein
CDK	cyclin-dependent kinase
Cdk5	cyclin-dependent kinase 5
ChAT	choline acetyltransferase enzyme
CKI	cyclin-dependent kinase inhibitor
CNS	central nervous system
cm	centimetre
CREB	cAMP response element-binding protein
CRMP2	collapsin response mediator protein
CTX	cortex
D ₁ R	dopamine 1 receptor
D ₂ R	dopamine 2 receptor
DA	dopamine
DARPP32	dopamine- and cAMP-regulated neuronal phosphoprotein

ABBREVIATIONS

DG	dentate gyrus
DRPLA	dentatorubral-pallidoluysian atrophy
DNA	deoxyribonucleic acid
EC	entorhinal cortex
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGTA	egtazic acid
EphA4	ephrin type-A receptor 4
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinases
F-actin	filamentous actin
GABA	γ -Aminobutyric acid
GDP	guanosine diphosphate
GKAP	guanylate kinase-associated protein
GLT1	glutamate transporter 1
GPe	external globus pallidus
GPi	internal globus pallidus
GSTP1	glutathione S-transferase P
GTP	guanosine triphosphate
h	hours
HAT	histone acetyltransferase
HD	Huntington's disease
HDAC	histone deacetylases
HEAT	huntingtin, elongation factor 3, protein phosphatase 2A and TOR1
HIP	hippocampus
HIP14	huntingtin-interacting protein 14
<i>HTT</i>	human huntingtin gene
HTT	human huntingtin protein
<i>Htt</i>	mouse huntingtin gene
Htt	mouse huntingtin protein
iGluR	ionotropic glutamate receptor
IKK	I κ B kinase
JNK	c-Jun N-terminal kinase
K/Lys	lysine
kb	kilobase
kDa	kilodalton
KI	knock-in
KO	knock-out

ABBREVIATIONS

LTD	long-term depression
LTP	long-term potentiation
M	molar
MAPK	mitogen-activated protein kinases
MEF2	myocyte enhancer factor-2
MEK	MAPK/ERK kinase
mGluR	metabotropic glutamate receptor
mHTT	human mutant huntingtin
mHtt	mouse mutant huntingtin
min	minute
mm	millimetre
mM	millimolar
MMR	mismatch repair
mRNA	messenger ribonucleic acid
MSN	medium spiny neuron
MWM	Morris water maze
µg	microgram
µL	microlitre
NeuN	neuronal nuclear antigen
NES	nuclear export signal
NF	neurofilaments
NFκβ	nuclear factor kappa-light-chain-enhancer of activated B cells
NHEJ	non-homologous end joining
nm	nanometre
NMDA	<i>N</i> -Methyl-D-aspartate
NO	nitric oxide
NORT	novel object recognition test
p	phosphorylation
p75 ^{NTR}	p75 neurotrophin receptor
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffer saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PD	Parkinson's disease
PDE	phosphodiesterase
PFA	paraformaldehyde
polyP	poly proline
polyQ	poly glutamine

ABBREVIATIONS

PP1	protein phosphatase 1
<i>Prp</i>	mouse prion promoter
PSD	postsynaptic density
Rb	retinoblastoma protein
REST	RE1-silencing transcription factor
RhoGDI	Rho GDP-dissociation inhibitor
RNA	ribonucleic acid
rpm	revolutions per minute
S/Ser	serine
S6K	ribosomal protein S6 kinase
SBDP	spectrin breakdown product
SDS	sodium dodecyl sulphate
SEM	standard error of mean
sEPSC	spontaneous excitatory postsynaptic currents
SGK	serine/threonine-protein kinase
SNC	substantia nigra pars compacta
SNr	substantia nigra pars reticulata
Sp1	specificity protein 1
SPAR	spine-associated RapGAP
STAT3	signal transduced and activator of transcription 3
STEP	striatal-enriched protein tyrosine phosphatase
STN	subthalamic nucleus
STR	striatum
SUMO	small ubiquitin-like modifier
T/Thr	threonine
TBS-T	tris-buffered saline-Tween 20
TrkB	tropomyosin receptor kinase B
T-SAT	T-maze spontaneous alternation task
VAchT	vesicular acetylcholine transporter
VS	Vonsattel grade
WAVE-1	WASP-family verprolin-homologous protein
WT	wild-type
Y/Tyr	tyrosine
YAC	yeast artificial chromosome

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INTRODUCTION

Neurodegenerative disorders are characterized by a middle-late onset and by a progressive degeneration which starts selectively in a subset of neurons and expands to other brain regions as the pathology advances. Although affected neuronal populations are different in each disease, they all lead to the appearance of increasingly disabling motor, cognitive and/or psychiatric deficits and they all present shared molecular mechanisms such as presence of protein aggregates, altered autophagy or mitochondrial dysfunction. Despite many efforts, causes of specific neuronal degeneration and, more importantly, ways of stopping its progression, are still unknown. For these reasons, in a society with a continuously increasing life expectancy, it is crucial the maintained study of molecular mechanisms leading to the appearance of these disorders, to have a better understanding of their pathophysiology and to find new therapeutic targets able to delay their progression. Huntington's disease, despite being considered a rare pathology, is one of the most well-known neurodegenerative disorders along with Alzheimer's disease and Parkinson's disease. In this Thesis we have focused on exploring the dual role of the multifaceted cyclin-dependent kinase 5 (Cdk5) in the appearance of both cognitive and motor deficits in Huntington's disease.

1. Huntington's Disease

Huntington's disease (HD) is a rare fatal neurodegenerative disorder caused by the selective degeneration of striatal medium spiny neurons (MSNs) which leads to the appearance of choreas or involuntary movements. It is also characterized by the presence of cognitive and psychiatric deficits caused by corticostriatal disconnection and hippocampal dysfunction. It has a prevalence of 5-10 per 100,000 in most Caucasian populations, with reduced incidence in Asia and Africa, especially in Japan, and higher prevalence in specific populations originated from a reduced number of predecessors, such as Tasmania and the area around Lake Maracaibo (Walker, 2007). HD is classically characterized by the appearance of choreas (from the Ancient Greek word *choreia*, meaning dance) and first reports describing these symptoms date back to the mid-19th century (Vale and Cardoso, 2015). However, it was not until 1872 that George Huntington described more accurately the pathology, detailing its other main hallmarks besides chorea: its manifestation during adult life, the diseased tendency to insanity and suicide and its hereditary condition (Huntington, 1872). Despite this early knowledge of HD genetic transmission, another century passed before in 1983, chromosome 4 was identified as the region where the HD causing mutation was located (Gusella *et al*, 1983), and it was not until 1993 that the *IT15* gene was identified as the HD cause (The Huntington's Disease Collaborative Research Group, 1993) making a huge impact on HD research progress.

1.1. Aetiology

1.1.1. Genetics

Nowadays, it is known that HD is caused by an autosomal dominant mutation in the *IT15* or Huntingtin gene (*HTT*), located in the short arm of chromosome 4p16.3 and covering ~170 kb which include 67 exons. This mutation consists of an abnormal expansion of the CAG repeat sequence present in exon 1, which codifies for a polyglutamine (polyQ) stretch (The Huntington's Disease Collaborative Research Group, 1993). In normal conditions, the gene contains between 1-34 CAG repeats. A number of repetitions comprised between 35-39 causes incomplete penetrance of the disease, whereas expansions exceeding 40 CAG repeats, cause full penetrance of the pathology (Mcneil *et al*, 1997; Rubinsztein *et al*, 1996; Walker, 2007). It is also known that the number of CAG repeats inversely correlates with the onset of the pathology (Figure 1) accounting for 50-70% of the variance in age onset between individuals with the remaining variance being attributable to environmental factors (Ross and Tabrizi, 2011; Wexler, 2004). In this sense, it has been observed that expansions between 40-45 CAGs lead to an average onset at 45-60 years of age, whereas repetitions higher than 55 usually lead to a juvenile form of the disorder (Andrew *et al*, 1993; Roos, 2010).

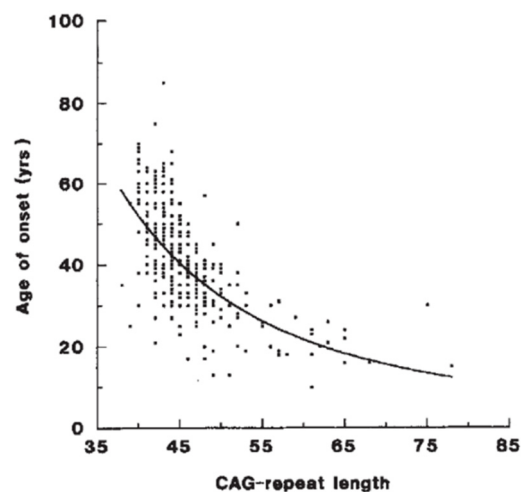


Figure 1. Inverse correlation between the number of CAG repeats and the age of onset in Huntington's disease. Adapted from (Andrew *et al*, 1993).

CAG expansions also present instability. Although instability can result both in a reduction or expansion of the CAG repeats number, expansions are more frequent, causing anticipation in the transmission of the disease. Anticipation consists of an aggravation both in the number of CAGs and in the onset of the pathology in the offspring. This phenomenon is particularly relevant when HD is paternally transmitted and is also responsible for the existence of sporadic cases of HD (Duyao *et al*, 1993; Ranen *et al*, 1995; Trottier *et al*, 1994). Instability has also been observed at a somatic level. Importantly brain areas which are particularly affected in HD, like basal ganglia and cerebral cortex, present higher levels of

mosaicism, correlating increased somatic instability with increased vulnerability in the pathology (Swami *et al*, 2009; Telenius *et al*, 1994).

1.1.2. Huntingtin protein

The product of the *IT-15* gene is Huntingtin (HTT), a 348 kDa protein whose function is still not fully understood due to several reasons. One of them, is its heterogeneous distribution. Indeed, HTT is ubiquitously expressed in all human and mammalian cells, with higher expression in central nervous system (CNS) neurons and testes. It is also broadly distributed at the subcellular level, as it can be localized in association with the nucleus, endoplasmic reticulum, Golgi complex and mitochondrion, and it is also present at synapses where it associates with structures like vesicles or microtubules (Difiglia *et al*, 1995; Zuccato *et al*, 2010).

Identifying HTT function from its structure has also been extremely complex. On one hand, because its high molecular weight complicates a proper determination of the structure and conformation and, on the other hand, due to the lack of known motifs with defined functions. HTT structure (Figure 2) consists primarily of 36 HEAT repeats, sequences of ~40 amino acids, believed to be associated with protein-protein interactions (Li *et al*, 2006). HTT also presents a NES (nuclear export signal) sequence near to its C-terminus, but the main region of interest is the N-terminus, where the polyQ stretch is localized. Along with a polyproline (polyP) sequence located next to it, this region has been suggested to mediate most of HTT's multiple protein-protein interactions (Ross and Tabrizi, 2011; Zuccato *et al*, 2010). HTT also contains several consensus cleavage sites for proteolytic enzymes such as caspases and calpains, which is relevant as cleavage of the mutant HTT protein (mHTT) has been associated with neurotoxicity (Cattaneo *et al*, 2005; Graham *et al*, 2006).

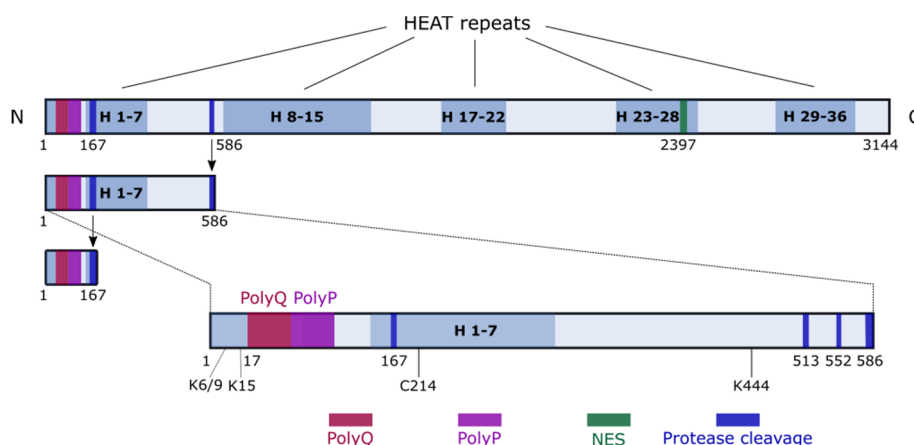


Figure 2. Huntingtin protein structure. Different colours and amino acid numbers indicate location of HTT's domains, including polyQ and polyP tracts, NES sequence and consensus protease cleavage sites (K, Lysine; C, Cysteine). Sequential cleavage of the N-terminal region leads to neurotoxicity. Magnification of the N-terminal region shows some of the post-translational modifications mentioned on the text. Adapted from (Ross and Tabrizi, 2011)

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The N-terminus fragment can also be subjected to different post-translational modifications, including ubiquitination, acetylation, sumoylation, palmitoylation and phosphorylation. These modifications regulate different HTT aspects like its stability, subcellular localization, trafficking or provide neuroprotection against caspase cleavage (Table 1).

Post-translational modification	Amino acid residue	Functional effect	Reference
Ubiquitination	K6, K9 and K15	Control of HTT stability, function and intracellular localization	(Kalchman <i>et al</i> , 1996)
Acetylation	K444	Targeting to the macroautophagy pathway	(Jeong <i>et al</i> , 2009)
Sumoylation	K6, K9 and K15	Control of HTT stability, function and intracellular localization	(Steffan <i>et al</i> , 2004)
Palmitoylation	C214 (by HIP14)	Control of HTT trafficking and function	(Yanai <i>et al</i> , 2006)
Phosphorylation	S421 (by Akt) S434, 1181, 1201 (by Cdk5)	Neuroprotection and vesicular transport	(Humbert <i>et al</i> , 2002; Luo <i>et al</i> , 2005; Schilling <i>et al</i> , 2006)

Table 1. HTT is subjected to different post-translational modifications, with various effects on its function. K, Lysine; C, Cysteine; S, Serine; HIP14, Huntingtin-interacting protein 14; Cdk5, Cyclin dependent kinase 5.

All this information, along with generation of animal models, has brought some light at the multiple functions in which HTT is involved. For instance, it is known that HTT has an essential role during embryonic development, as a full HTT knockout causes embryonic lethality before day 8.5 (Duyao *et al*, 1995; Nasir *et al*, 1995). It has also been described that HTT has an anti-apoptotic role, as its overexpression protects against apoptotic stressors such as serum deprivation in *in vitro* models or excitotoxicity in *in vivo* models (Leavitt *et al*, 2006; Rigamonti *et al*, 2000). HTT function is also involved in neuroprotection through regulation of the neurotrophin BDNF (brain-derived neurotrophic factor) transcription and trafficking (Gauthier *et al*, 2004; Zuccato *et al*, 2001, 2003), an important prosurvival factor for striatal neurons produced by cortical neurons (Baquet *et al*, 2004). Finally, HTT localizes in nerve terminals and it has been described to interact with several proteins involved in clathrin-mediated endocytosis, modulation of dendritic spine morphology and organization of the postsynaptic density (PSD), thus being involved in synaptic functions (Harjes and Wanker, 2003).

Despite lack of complete understanding of HTT function, it is assumable that loss of these roles caused by the presence of the HD mutation could greatly impair different neuronal processes. Nonetheless, gain of toxic function by the extended polyQ tract is believed to play a bigger role in HD neuropathology. One of the main arguments in favour of this hypothesis is that HD is part of nine other human genetic disorders caused by polyglutamine expansions (spinocerebellar ataxia types 1, 2, 3, 6, 7, 12 and 17; dentatorubropallidoluysian atrophy (DRPLA) and spinobulbar muscular atrophy (SBMA)), and even though

polyglutamine expansions are localized in other proteins, all diseases present a pattern of selective neurodegeneration, inverse correlation between length of the polyQ tract and onset of the disease, and neuronal inclusions of polyQ aggregates (Margolis and Ross, 2001; Walker, 2007).

1.2. Clinical aspects

HD symptoms consist of a triad of progressive motor, cognitive and psychiatric deficits. The disease onset is established as the moment when formal clinical diagnosis of motor symptoms takes place (Huntington Study Group, 1996) (Figure 3). Its average age is between 30 and 50 years, although it can range from 2 to 85 years (Myers, 2004). Before disease onset, there is a prodromal phase, characterized by subtle motor, cognitive and behavioural changes which can take place up to 15 years before the diagnosis. Prior to the prodromal phase, patients known to carry the mutation, but not presenting any symptoms, are referred to as presymptomatic (Bates *et al*, 2015; Ghosh and Tabrizi, 2015). After clinical diagnosis there is an increasingly disabling progression of symptoms until patients die 15 to 20 years after motor onset, the most common cause being secondary pneumonia followed by suicide (Roos, 2010; Roos *et al*, 1993).

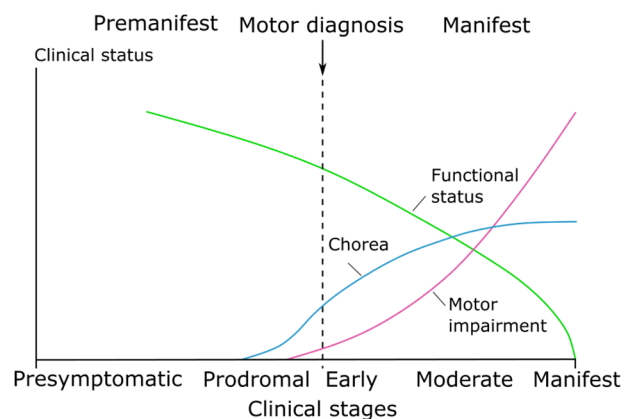


Figure 3. HD clinical progression. Average age onset determined by motor diagnosis is 45 years. The period before is termed premanifest and includes the presymptomatic and prodromal stages. Individuals functional abilities are affected during prodromal phases and rapidly decrease through the manifest period, as chorea appears and motor and cognitive impairment worsen. Adapted from (Bates *et al*, 2015)

1.2.1. Motor symptoms

Motor symptoms have two kinds of manifestations, on one hand appearance of involuntary movements and, on the other hand, impairment of voluntary movements. Early stages of the disease are characterized by appearance of chorea, which consists of involuntary, excessive and short-lived movements, which start in distal extremities and in the face, and then spread more proximally becoming larger in amplitude. Dystonia, which are slower movements caused by sustained muscle contractions, is another feature and leads to

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abnormal postures. In later disease stages, chorea plateaus and there is an increasing impairment of voluntary movements, which include bradykinesia, akinesia and rigidity. Bradykinesia refers to movement slowness and akinesia to difficulty in initiating movement. There is also gait affectation which leads to falls, and alteration in talking and swallowing which causes choking and difficulty to speak (Bates *et al*, 2015; Ghosh and Tabrizi, 2015; Roos, 2010). Globally, these alterations have an increasing impact on HD patient's life, affecting their ability to work and to perform everyday tasks such as eating or talking.

1.2.2. Cognitive deficits

Cognitive decline can be present years before motor onset (Paulsen *et al*, 2008; Stout *et al*, 2011) and presents variable severity depending on the person. Cognitive deficits in HD are similar to those present in disorders associated with striatal-subcortical brain pathologies and include problems in executive functioning, i.e. high-level cognitive processing, which leads to problems with attention, mental flexibility or planning of actions. There is a general slowing of thought processes and impairment in short-term memory, which causes reduction of learning and retrieval of new information (Bates *et al*, 2015; Ghosh and Tabrizi, 2015). Language is generally preserved, although speech can be affected because of motor impediments (Aretouli and Brandt, 2010). In later stages, cognitive decline can reach severe frontal and subcortical dementia, although not all patients are affected (Peavy *et al*, 2010). Other cognitive alterations such as lack of awareness of deficits and disinhibition are more closely related with psychiatric alterations, which have an impact not only on the patient's, but also on their caregivers' quality of life (Duff *et al*, 2010).

1.2.3. Psychiatric symptoms

Psychiatric and emotional disturbances in HD patients are also quite variable and, as mentioned, along with cognitive decline, can be more distressing for patients and their carers than motor symptoms. Psychiatric deficits can also appear before motor onset, but tend not to present a correlation with disease progression (Roos, 2010). Depression is one of the most common symptoms, being reported by half of HD patients and presenting important implications due to its involvement with increased suicidality, which, as mentioned before, is one of the main causes of death in HD patients (Hubers *et al*, 2012; Paulsen *et al*, 2005). Anxiety and irritability are also frequent in affected subjects, along with obsessive and compulsive thoughts and behaviours (Dale and van Duijn, 2015; Ghosh and Tabrizi, 2015; Roos, 2010; Thompson *et al*, 2012). Finally, apathy, characterized by a loss of interest and passive behaviour, is another frequent feature of the disease. Importantly, premanifest patients have been reported to already present apathy and in contrast to other psychiatric symptoms, apathy presents a correlation with disease progression and predicts functional decline (Tabrizi *et al*, 2013).

1.3. Neuropathology

The most characteristic neuropathological hallmark of HD is a marked bilateral atrophy, cell loss and astrogliosis of the striatum (caudate and putamen), which eventually leads to an enlargement of the lateral ventricles (Vonsattel and DiFiglia, 1998) (Figure 4). However, 80% of human HD brains also present an important atrophy of the cerebral cortex with a 21-29% of area loss (Vonsattel and DiFiglia, 1998) and a general brain alteration. Hence, human HD brains present a reduction in whole brain weight, a 29-34% loss of telencephalic white matter, and affectation of other brain regions including the globus pallidus, subthalamic nucleus, substantia nigra, thalamus, hypothalamus, cerebellum and hippocampus (Heinsen *et al*, 1996; Jeste *et al*, 1984; Kremer *et al*, 1991; Monte *et al*, 1988; Spargo *et al*, 1993; Vonsattel and DiFiglia, 1998).

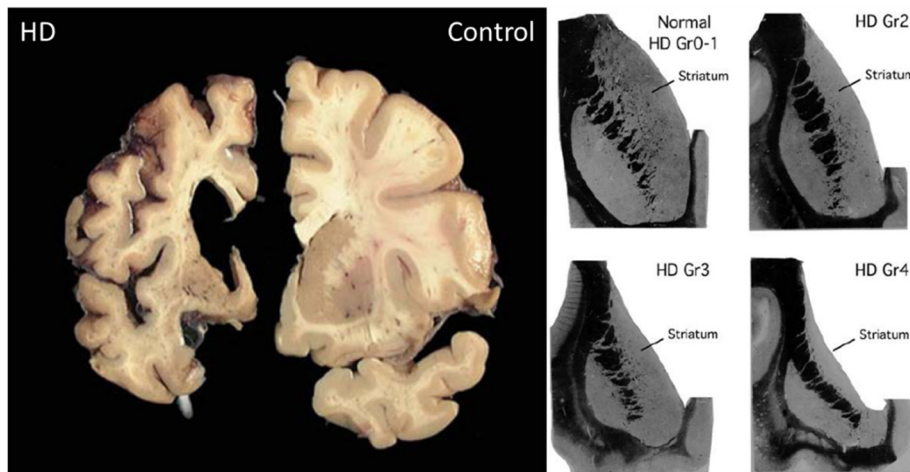


Figure 4. HD neuropathology. Coronal slices of human HD and control brains. Striatal reduction and ventricle enlargement, along with cerebral cortex atrophy can be observed in HD brains (left). Schematic illustrations of progressive striatal degeneration through Vonsattel grades, changes in striatal ventricular profile shape can be appreciated (right). Adapted from (Reiner *et al*, 2011)

Striatal degeneration presents an ordered progression starting caudally and progressing in a dorsomedial to ventrolateral direction (Reiner *et al*, 2011; Roos *et al*, 1985). Based on this neuropathological progression, a grading system consisting of five grades (0-4) was established to evaluate HD severity in correlation with clinical disability. Grade 0-1 includes prodromal patients with small alterations compared to controls, while in Grade 2 striatal atrophy is mild to moderate, with a reduced convex ventricular profile of the caudate, which becomes flat or concave in Grade 3-4 (Figure 4) (Reiner *et al*, 2011; Vonsattel, 2008; Vonsattel *et al*, 1985).

1.3.1. Corticostriatal pathology

The **striatum** is the main input of cortical projections to the basal ganglia, which are a group of subcortical nuclei comprised by the striatum (caudate and putamen), internal and external globus pallidus (GPi and GPe), subthalamic nucleus (STN) and substantia nigra pars

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compacta (SNc) and pars reticulata (SNr). Glutamatergic inputs from the cortex and dopaminergic inputs from the SNc are processed and relayed to downstream basal ganglia nuclei (Figure 5). From there, information is transmitted to the thalamus and back to the cortex, controlling voluntary movements, as well as cognitive and emotional processing (Obeso *et al*, 2014; Rothwell, 2011).

MSNs constitute 90-95% of striatal neurons, with the remaining 5-10% being GABAergic and cholinergic interneurons (Kita and Kitai, 1988). MSNs are GABAergic neurons that can be classically subdivided in two different populations depending on their projection destination (direct and indirect pathway) and their differential expression of neuropeptides and dopamine (DA) receptor subtypes. Hence, striatonigral MSNs express D₁ DA receptor (D₁R), the neuropeptides substance P and dynorphin and they project to the GPi and SNr forming the direct pathway. On the other hand, striatopallidal MSNs express D₂ DA receptor (D₂R), the neuropeptide enkephalin and they project to the GPe forming the indirect pathway (Gerfen *et al*, 1990; Kawaguchi *et al*, 1990). The direct and indirect pathways regulate movement initiation and inhibition, respectively, through excitatory (glutamatergic) and inhibitory (GABAergic) circuits. This classification is important as neurodegeneration in HD affects primarily MSNs from the indirect pathway. Moreover, dopaminergic inputs from the SNc to MSNs from both pathways also further modulate movement. DA projections to striatonigral MSNs are believed to further activate this pathway, whereas DA projections to striatopallidal MSNs are thought to further inhibit movement (Surmeier *et al*, 2007).

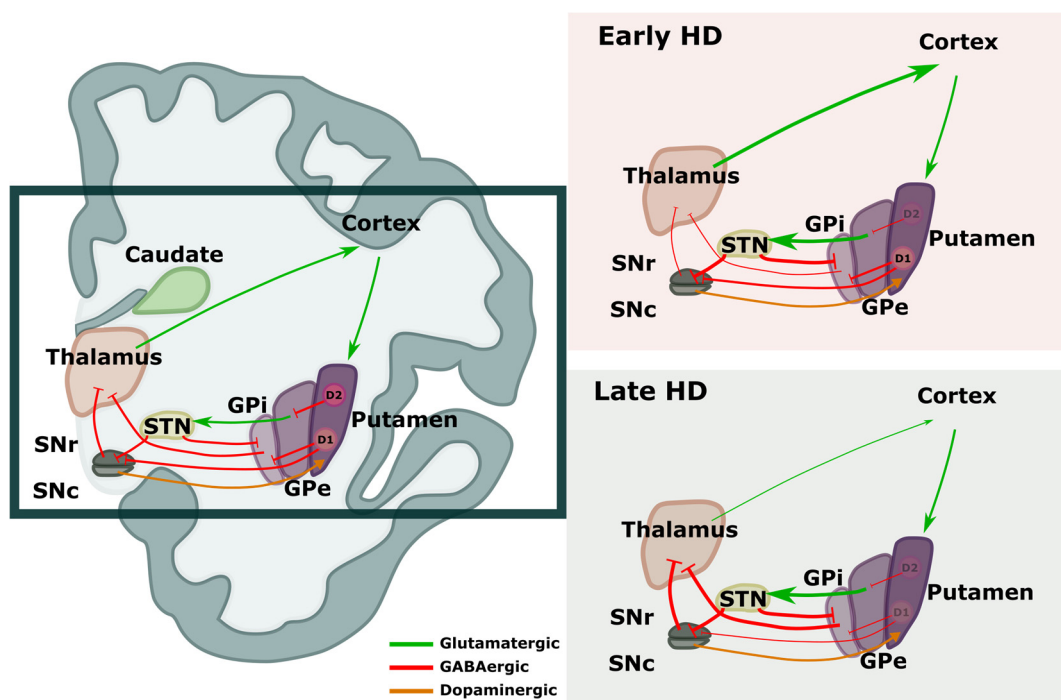


Figure 5. Schematic representation of the direct and indirect pathways in physiological conditions (left) and in early and late HD stages (right). Modified from (Calabresi *et al*, 2014).

As illustrated in Figure 5, during HD early phases, the specific loss of striatopallidal MSNs (D_2) from the indirect pathway causes reduced inhibitory signalling to the GPe, increasing excitatory signalling to the STN and subsequently, enhanced inhibitory signalling arrives to the GPi and SNr. This leads to reduced inhibitory signalling to the thalamus from these regions and to increased excitatory signalling to the motor cortex, which causes inability to control voluntary movements and appearance of hyperkinesia (choreas). In later stages, however, striatal affection extends to striatonigral MSNs (D_1) from the direct pathway as well, causing reduced inhibitory signalling to the GPi and SNr and subsequent augmented inhibitory signalling to the thalamus from these regions. This leads to reduced excitatory signalling to the motor cortex, causing inability to facilitate movement, which results in rigidity and bradykinesia (Bolam *et al*, 2000; Bunner and Rebec, 2016; Calabresi *et al*, 2014; Obeso *et al*, 2014; Rothwell, 2011).

The **cerebral cortex** is structured in six layers. Cortical neurons establish internal connections with neurons from other layers and outer connections with other brain areas. Pyramidal neurons from layers III, V and VI are the main inputs to the striatum (McGeorge and Faull, 1989). HD patients present approximately 30% of cortical global thinning, which is more pronounced in layers III and V, reaching up to a 43% in layer VI (Hedreen *et al*, 1991). This thinning has been associated with specific loss of pyramidal neurons from regions like the primary motor cortex (Macdonald and Halliday, 2002). Importantly, cortex atrophy begins during premanifest period in a region-specific manner, starting with thinning in primary cortical regions including sensory, motor and visual regions with no significant changes in whole brain volume. This atrophy then expands to posterior frontal and more occipital and parietal areas in early HD, reaching a general affection of most cortical regions by late stages of the disease (Rosas *et al*. 2008a; Rosas *et al*. 2008b). Correlation between affected cortical regions and differential nature of symptoms in patients has also been established, providing an explanation to the complexity and heterogeneity of HD symptoms (Raymond *et al*, 2011; Thu *et al*, 2010). Finally, the fact that alterations in the cortex can be observed as soon as 10-15 years before the onset of the disease, support the hypothesis that cortical cellular and synaptic dysfunction plays a key role in early pre-motor deficits in HD patients (Paulsen *et al*, 2008; Raymond *et al*, 2011).

1.3.2. Hippocampal pathology

The hippocampus is a key element of the limbic system which mediates encoding, consolidation and retrieval of memory, and participates in regulation of emotions, fear, anxiety, stress or depression (Bartsch and Wulff, 2015; Pittenger and Duman, 2008; Squire, 1992). As illustrated in Figure 6, the hippocampus receives inputs from the main cortical association areas through the entorhinal cortex (EC), and the information is relayed through the DG (dentate gyrus), and the CA3 (cornu ammonis 3) to the CA1 (cornu ammonis 1)

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pyramidal neurons, which are the major hippocampal output projections to deep layers of the EC (Bartsch and Wulff, 2015; Ghiglieri *et al*, 2011).

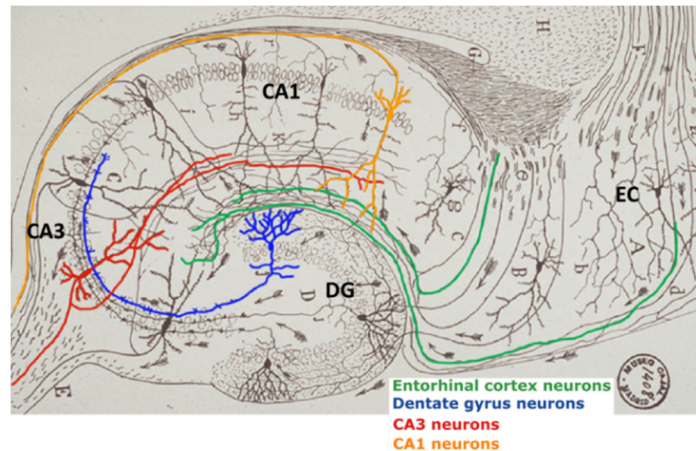


Figure 6. Hippocampal circuitry representation. Projection neurons from different hippocampal regions are marked with the stated colours. Adapted from (Saura *et al*, 2015).

HD patients present a 35% neuronal loss in the CA1 region (Spargo *et al*, 1993) and mHTT nuclear inclusions have been observed in several regions from the hippocampal formation (Herndon *et al*, 2009). Voxel-based morphometric studies have also shown that reduction of hippocampal grey matter correlates with impairment in negative emotion recognition in HD patients (Ille *et al*, 2011). However, most evidence of hippocampal early affection in HD comes from mice models studies, which have revealed that hippocampal-dependent functions, like navigation and spatial and recognition memories, are impaired in most HD models before motor onset (Brito *et al*, 2014; Giralto *et al*, 2011, 2012a, 2012b; Lione *et al*, 1999). In line with these results, a recent study revealed that early HD patients present similar deficits in hippocampal-specific tasks, which correlate with estimated years to diagnosis, indicating that hippocampal dysfunction is an important and early contributor to HD cognitive decline (Begeti *et al*, 2016).

1.4. HD mice models

Despite their limitations, animal models have been and still represent an essential tool in HD research. Problems arising from research with human patients, like high heterogeneity, reduced availability of patients or long disease progression, are minimized by inherent characteristics of animal models, such as shorter lifespans or the possibility to genetically manipulate them. Thus, use of animal models in HD research has greatly improved understanding of the molecular mechanisms underlying mHTT toxicity and has allowed identification of putative therapeutic targets which could delay disease progression.

First developed HD models took advantage of neurotoxins that induced specific striatal lesioning (Coyle and Schwarz, 1976; McGeer and McGeer, 1976). Glutamate agonists, like quinolinic acid (Beal *et al*, 1986), and mitochondrial toxins, like 3-nitropropionic acid (3-NP)

and malonate (Beal *et al*, 1993; Brouillet *et al*, 1993), were commonly used for their relatively specific toxic effect on MSNs. The discovery of the *IT15* gene allowed the generation of multiple genetic animal models. Even though, mice are the most extensively used, nowadays HD models from different species are available, including worms (*Caenorhabditis elegans*) (Faber *et al*, 1999), fruit flies (*Drosophila melanogaster*) (Jackson *et al*, 1998), rats (von Horsten *et al*, 2003) and more recently, sheeps (Jacobsen *et al*, 2010), pigs (Yang *et al*, 2010) and monkeys (Yang *et al*, 2008). Nonetheless, given HTT's large size, the differential effect of distinct CAG lengths or its genomic instability, design of HD models has been complex and diverse. Thus, several genetic approaches have been used regarding some of the following factors: use of full-length mHTT or of the N-terminal fragment, use of different CAG repeat lengths, use of human or mice Htt promoter and expression of mHTT from a transgene or by knocking the CAG mutation into the endogenous murine gene (Figure 7).

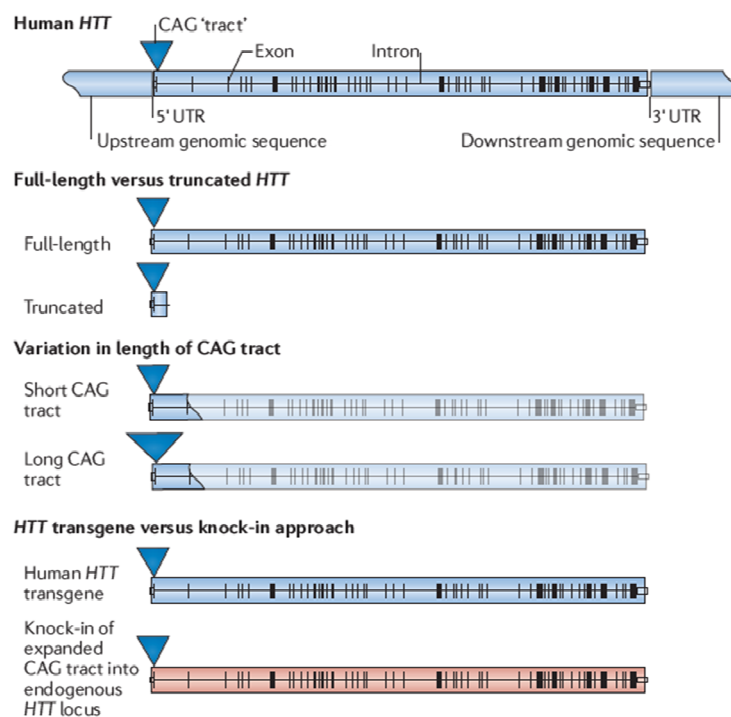


Figure 7. Different genetic approaches for HD mice generation. Illustrative scheme showing different genetic design features which differ between HD mice models. Adapted from (Pouladi *et al*, 2013).

1.4.1. Truncated N-terminal models

Mice models expressing the truncated N-terminal fragment of mHTT present a rapid onset of motor, cognitive and behavioural symptoms, weight loss, reduced lifespan and generalized degenerative phenotype. Despite being the less genetically accurate models, its rapid onset and progression makes them a recurrent choice for therapy screening studies (Ferrante, 2009; Figiel *et al*, 2012; Gil and Rego, 2009).

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R6/1 and R6/2 mice express, respectively, one and three randomly inserted copies of the 1.9 kb human genomic fragment containing promoter sequences and exon 1 from *HTT* gene. R6/1 mice, expressing 116 CAG repeats and R6/2 mice, expressing 144 CAG repeats, were the first ever generated HD genetic mice models (Mangiarini *et al*, 1996). **R6/1 mice** present cognitive deficits at 12 weeks of age and motor alterations, including limb clasping and impaired rotarod performance, at 14-20 weeks of age. They also present neuronal inclusions with no neuronal death and an average lifespan of 32-40 weeks (Brooks *et al*, 2012; Mangiarini *et al*, 1996; Naver *et al*, 2003). **R6/2 mice** present similar alterations but with an earlier onset with cognitive deficits at 3.5 weeks and motor symptoms at 8 weeks of age (Lione *et al*, 1999; Stack *et al*, 2005). Both lines present genomic instability, which can cause CAG tracts to expand up to 250 and even 400 (Mangiarini *et al*, 1996; Pouladi *et al*, 2013). They also show brain atrophy, even though it is believed to be caused by neuronal dysfunction rather than by neuronal death, as neuronal apoptosis is not detected in these models (Yu *et al*, 2003).

N171-Q82 mice are another truncated N-terminal model which differs from the R6 lines by its expression of a longer sequence including exon 1 and exon 2 with 82 CAG repeats, under the mouse prion promoter (*Prp*) regulation, which restricts its expression to neurons (Schilling *et al*, 1999). In addition they present loss of striatal neurons by apoptosis (Yu *et al*, 2003) and a more variable phenotype, which can be a downside as it might require larger sample sizes (Ferrante, 2009).

1.4.2. Full-length models

Full-length mHTT models can be generated by means of two main strategies: introducing a full-length human mutant *HTT* transgene (transgenic models) or knocking a human *HTT* exon 1 with expanded CAG repeats into the endogenous mouse *Htt* gene (knock-in models). They present a milder disease progression compared to the N-terminal fragment models, especially in the case of knock-in mice, making them a good choice for studying early stages of the disease.

1.4.2.1. Transgenic models

Transgenic models were created using the YAC (yeast artificial chromosome) and BAC (bacterial artificial chromosome) technologies, which allow expression of large DNA sequences. Therefore these models express human genomic mutant *HTT* transgenes, including all introns, exons and regulatory elements, thus presenting a more accurate tissue and temporal genetic expression of mHTT (Gray *et al*, 2008; Hodgson *et al*, 1999; Pouladi *et al*, 2013; Slow *et al*, 2003).

Several YAC lines with different CAG lengths (48, 72 and 128) have been created, although **YAC128 mice** are the most commonly used (Hodgson *et al*, 1999; Slow *et al*, 2003). They present learning deficits at 2 months, motor symptoms at 3 months and aggregates and neuronal loss at 12-18 months of age. They also present low inter-animal variability (Ferrante, 2009; Figiel *et al*, 2012; Van Raamsdonk *et al*, 2005; Slow *et al*, 2003). **BACHD mice** were generated more recently, containing 97 polyglutamine expansions, expressed from a mixed CAA-CAG repeat sequence, in order to avoid genomic instability (Gray *et al*, 2008).

1.4.2.2. Knock-in models

Knock-in models were established by insertion of the human *HTT* exon 1 with CAG lengths ranging from 50 to 200 into the endogenous mouse *Htt* gene (Wheeler *et al*, 1999; White *et al*, 1997). These mice represent the most genetically accurate models, not only allowing proper mHtt spatial and temporal expression, but avoiding possible effects arising from species-specific sequence differences (Ehrnhoefer *et al*, 2009; Pouladi *et al*, 2013). Knock-in mice also present a milder phenotype with, usually, a normal lifespan. Onset of symptoms and disease progression varies depending on the length of the CAG tract, the homozygosity or heterozygosity of the knock-in gene and their genetic background (Ferrante, 2009; Figiel *et al*, 2012; Lloret *et al*, 2006). HdhQ111, CAG140, HdhQ150 and zQ175 are some of the most frequently used knock-in lines.

In this Thesis we have used **Hdh^{Q7/Q111} mice** in a C57BL/6 strand. These mice present depressive-like behaviour at 2 months of age (unpublished results), corticostriatal and hippocampal cognitive deficits at 6 months of age, with motor alterations at 8 months and presence of striatal intranuclear mHTT inclusions starting at 6 months of age (Brito *et al*, 2014; Giralt *et al*, 2012a; Lloret *et al*, 2006; Puigdellívol *et al*, 2015). No overt neuronal loss or astrogliosis is detected at motor symptoms onset (Bragg *et al*, 2017; Brito *et al*, 2014).

2. Molecular mechanisms underlying striatal vulnerability and cognitive symptoms in HD

HTT's complexity, as well as its involvement in multiple cellular pathways are some of the reasons why 25 years after the discovery of the HD mutation, mechanisms underlying striatal degeneration and cognitive decline are still not fully understood. Nonetheless, generation of HD mice models has identified some of the molecular mechanisms affected by the presence of mHTT. Moreover, knowledge that models like the R6 lines display a severe phenotype without showing overt neuronal loss, has underlined the idea that neuronal dysfunction precedes neuronal death and, therefore, targeting of affected pathways in early disease stages could prevent or delay disease progression. In this section we will present the proposed molecular mechanisms underlying striatal vulnerability and what is known from synaptic dysfunction greatly responsible for cognitive decline.

2.1. Striatal vulnerability

Understanding why striatal MSNs present specific affectation in HD despite ubiquitous HTT expression in all body cells, has been and still is one of the main HD conundrums. Even though years of research have identified several mechanisms which could contribute to specific striatal dysfunction and degeneration (Figure 8), full comprehension of mHTT-induced striatal vulnerability is still lacking. Therefore, further study of the classically proposed mechanisms, as well as, characterization of new molecular pathways participating in this process is pivotal to complete our knowledge of striatal pathophysiology in HD.

2.1.1. Canonical mechanisms

2.1.1.1. BDNF

BDNF is a cortically produced neuroprotective factor for striatal neurons. As mentioned before, HTT participates in BDNF transcription and trafficking and presence of mHTT has been shown to alter both processes *in vitro*, in mice models and in human patients. Thus, a reduction in both BDNF mRNA and protein levels has been observed in the striatum and other brain regions of different HD models and in human patients (Ferrer *et al*, 2000; Zuccato *et al*, 2001, 2010). mHTT alters BDNF transcription through dysregulation of different transcription factors, e.g. REST (RE1-silencing transcription factor) (Zuccato *et al*, 2003), CREB (cAMP response element-binding protein), CBP (CREB binding protein) or Sp1 (specificity protein 1) (Cha, 2007; Zuccato *et al*, 2003), while also decreases anterograde axonal BDNF vesicle transport from the cortex to the striatum (Gauthier *et al*, 2004). In addition, changes in BDNF receptors have also been described in HD, further impairing BDNF signalling. Thus, levels of the BDNF receptor TrkB, which activates pro-survival pathways,

are reduced in the striatum of different HD mice models and in HD patients. Moreover, levels of p75^{NTR}, an additional BDNF receptor which can induce pro-apoptotic pathways, have been shown to be increased in HD (Brito *et al*, 2013; Ginés *et al*, 2006; Plotkin *et al*, 2014; Zuccato *et al*, 2008). Therefore, lack of BDNF trophic support along with alterations in its signalling pathways have been proposed to participate in increased striatal vulnerability in HD.

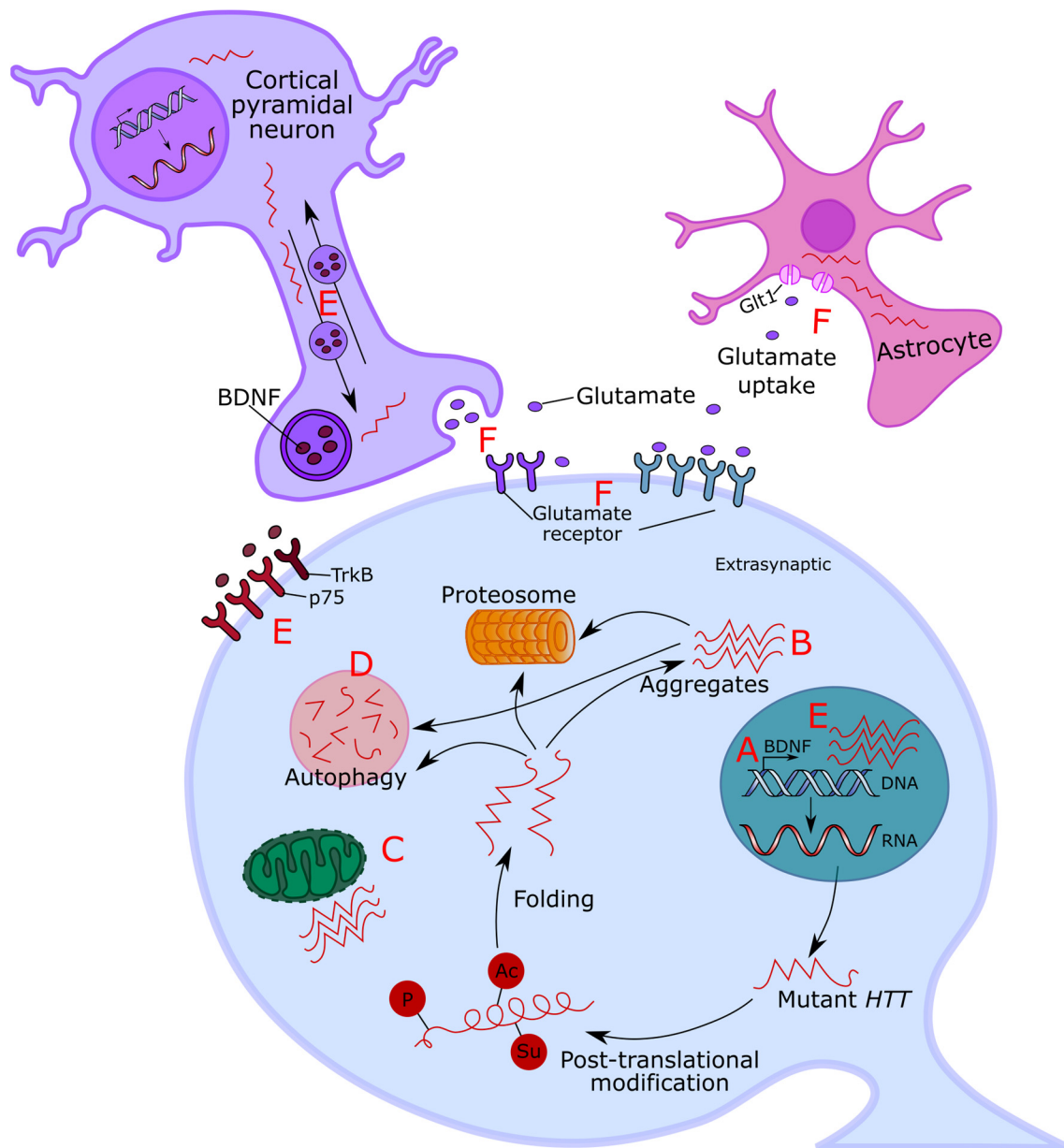


Figure 8. Canonical molecular pathways involved in striatal vulnerability in HD. Schematic representation of the principal cellular processes involved in mHTT-induced striatal vulnerability: transcriptional dysregulation (A), mHTT aggregate formation (B), mitochondrial dysfunction (C), alterations in autophagy and proteosomal degradation (D), loss of BDNF trophic function, associated with impairment of its transcription and transport and with an imbalance in TrkB and p75 receptors levels (E), and excitotoxicity, attributed to alterations in glutamate release and uptake and to changes in glutamate receptors levels and localization (F). Modified from (Bates *et al*, 2015).

2.1.1.2. Excitotoxicity

Excitotoxicity refers to excessive excitatory amino acids signalling, mainly glutamate, leading to toxic calcium overload which promotes neuronal death (Rothman and Olney, 1995). Observations that treatment with glutamate agonists led to preferential striatal degeneration causing an HD-like phenotype, suggested that excitotoxicity might play an important role in striatal vulnerability in HD. In addition, inherent characteristics of the striatum as a receiver of mainly excitatory inputs, place it in a vulnerable position to alterations in corticostriatal glutamate signalling, which have been described in HD. For instance, R6/1 mice show heightened activity-induced striatal glutamate release (Nicnocaill *et al*, 2001), while astroglial glutamate transporter (GLT1) mRNA and protein levels are decreased in R6/1 and R6/2 mice (Behrens *et al*, 2002; Liévens *et al*, 2001). GLT1 plays a major role in extracellular glutamate clearance, avoiding excitotoxicity or glutamate spill-over to extrasynaptic sites (Takahashi *et al*, 2015), the latest being associated with activation of apoptotic pathways (reviewed in (Hardingham and Bading, 2010; Wang *et al*, 2013)). Notably, increased localization of NMDA (*N*-Methyl-D-aspartate) receptors (NMDAR) at extrasynaptic sites and subsequent activation of extrasynaptic NMDARs downstream pathways has been described in the striatum of YAC128 mice at early disease stages prior to motor onset (Milnerwood *et al*, 2010). Therefore, mHTT contributes to striatal excitotoxicity by altering multiple steps of glutamatergic signalling, including glutamate release and clearance, glutamate receptors levels and localization and activation of their downstream signalling pathways (Fan and Raymond, 2007).

However, analysis of excitotoxicity responses in HD models have yielded diverse results depending on the disease stage and the model being studied (Table 2). Therefore, although enhanced sensitivity to excitotoxicity has been described at early stages in YAC128 and R6/1 and R6/2 mice, these animals present resistance to excitotoxicity at later disease stages (Graham *et al*, 2009; Hansson *et al*, 2001; Levine *et al*, 1999). Notably, different sensitivity to excitotoxicity has also been described in the absence of mHTT, depending on the mice strand, which could further contribute to different excitotoxicity responses between HD models (Schauwecker and Steward, 1997). Nonetheless, increased sensitivity to glutamatergic stimulation has been associated with alterations in intracellular calcium homeostasis (Fernandes *et al*, 2007) and with impaired mHTT and PSD-95 interaction (Sun *et al*, 2001). As a result of these impairment, increased NR2B and PSD-95 interaction is detected causing enhanced NMDARs surface localization, which plays a major role in excitotoxicity (Fan *et al*, 2009; Sun *et al*, 2001). On the other hand, resistance to excitotoxicity has been proposed as a compensatory mechanism, and it has been associated with reduction of synaptic proteins like PSD-95 and citron, which could lead to NMDARs hypofunction (Jarabek *et al*, 2004). Importantly, reduction of NMDARs levels as a protective

mechanism against excitotoxicity could impair NMDAR-dependent synaptic plasticity processes, highlighting the relevance of proper balance in NMDAR function.

HD model	Stage	Excitotoxicity response	Reference
YAC128	presymptomatic	enhanced excitotoxicity sensitivity	(Graham <i>et al</i> , 2009)
YAC128	late	resistance to excitotoxicity	(Graham <i>et al</i> , 2009)
R6/2	symptomatic	enhanced excitotoxicity sensitivity	(Levine <i>et al</i> , 1999)
R6/1 and R6/2	motor onset	resistance to excitotoxicity	(Hansson <i>et al</i> , 2001)
R6/2	Early symptomatic (10 weeks)	similar excitotoxicity response	(Estrada-Sánchez <i>et al</i> , 2009)
R6/2	Late symptomatic (14 weeks)	enhanced excitotoxicity sensitivity	(Estrada-Sánchez <i>et al</i> , 2009)
CAG94 (KI)	presymptomatic	enhanced excitotoxicity sensitivity	(Levine <i>et al</i> , 1999)
N171-82Q		resistance to excitotoxicity	(Jarabek <i>et al</i> , 2004)

Table 2. Differential excitotoxicity responses between HD mice models. Examples of reported cases showing contrasting responses to excitotoxic insults depending on the HD model, the disease stage or the mice strand.

2.1.1.3. Aggregate formation and protein homeostasis

HTT fragmentation is considered one of the earlier steps of HD pathogenesis, as it can be detected in most HD models and in HD human brains and it takes place before aggregate formation (Bates *et al*, 2015; Landles *et al*, 2010; Lunkes *et al*, 2002). Small N-terminus HTT fragments can be produced via proteolytic cleavage by different caspases (Wellington *et al*, 2002), calpains (Gafni and Ellerby, 2002; Kim *et al*, 2001) and other proteases (Lunkes *et al*, 2002). Post-translational modifications also affect mHTT cleavage, as it has been described that HTT phosphorylation at residue Ser434 by Cdk5 (Luo *et al*, 2005) and at residue Ser421 by Akt and SGK (Warby *et al*, 2009), are protective against caspase-mediated HTT cleavage.

Presence of polyglutamine expansions disrupts the balance of protein folding quality control formed mainly by chaperones and clearance machinery, which have been shown to progressively decrease in HD causing a global disruption of the proteome (Gidalevitz *et al*, 2006; Hay *et al*, 2004; Labbadia and Morimoto, 2013). Moreover, both cellular protein clearance pathways (autophagy and the ubiquitin-proteasome system) are also impaired in HD (reviewed in (Martin *et al*, 2015; Ortega and Lucas, 2014)). In this context, mHTT aggregates are formed, and even though there is some debate about aggregate formation being protective against free soluble N-terminal fragments, it is usually considered that aggregates are detrimental for neurons. One of the main reasons is that they sequester other polyQ tract-containing proteins, affecting different cellular processes. Hence,

intranuclear aggregates have been associated with sequestering of transcription factors and transcriptional regulators (Cha, 2007), whereas extranuclear aggregates impair functions like axonal transport (Gunawardena *et al*, 2003; Li *et al*, 2001b).

2.1.1.4. Mitochondrial dysfunction

Similar to glutamate agonists, mitochondrial toxins, like 3-NP and malonate, also induce an HD-like phenotype, thus pointing mitochondrial dysfunction as an HD striatal vulnerability factor. Long before the mHTT mutation was discovered, it had already been observed in HD brain biopsies, the presence of aberrant dense mitochondria with sparse cristae (Tellez-Nagel *et al*, 1974). Nowadays, it is known that several aspects of mitochondria physiology are altered. For instance, alterations in mitochondrial transport and biogenesis, resulting from impaired axonal transport and transcription have been reported, as well as aberrant mitochondrial dynamics, leading to fragmented mitochondria (Cherubini and Ginés, 2017; Dubinsky, 2017; Kim *et al*, 2010). Mitochondria from YAC72 mice and HD patients lymphoblasts also show reduced calcium uptake capacity (Panov *et al*, 2002). Importantly, striatal mitochondria are particularly sensitive to calcium and to permeability transition induction, a process contributing to mitochondrial dysfunction and activation of apoptotic pathways in response to calcium and oxidative stress, which is produced for instance after excitotoxic insults (Brustovetsky *et al*, 2003, 2005; Crompton, 1999). Therefore, inherent properties of striatal mitochondria could contribute to HD striatal vulnerability, particularly to aberrant excitotoxic responses.

2.1.1.5. Transcriptional dysregulation

Transcriptional dysregulation of genes associated with e.g., neurotransmitter receptors, synaptic transmission, cytoskeletal and structural proteins, intracellular signalling or calcium homeostasis, can be detected in HD since early stages (reviewed in (Cha, 2007; Zuccato *et al*, 2010)). As mentioned before, some of these alterations are caused by sequestering of transcription factors or their coactivators in mHTT aggregates. Some of these transcription factors include p53, CBP, which is involved in cell survival and memory consolidation, Sp1, which regulates expression levels of D₂R amongst others, and REST, which as mentioned before regulates BDNF transcription levels (Dunah *et al*, 2002; Giralto *et al*, 2012a; Nucifora *et al*, 2001; Steffan *et al*, 2000; Zuccato *et al*, 2003). In addition, alterations in histone post-translational modifications modulating chromatin structure, have also been described in different HD models (Glajch and Sadri-Vakili, 2015). Thus, sequestering in mHTT aggregates of CBP which displays HAT (histone acetyltransferase) activity, has been associated with increased chromatin condensation and reduced gene transcription (Steffan *et al*, 2001). Moreover, HDACs (histone deacetylases) activity has also been shown to be increased in HD,

further contributing to reduced gene transcription (reviewed in (Glajch and Sadri-Vakili, 2015; Sharma and Taliyan, 2015)).

2.1.1.6. Alteration of kinases and phosphatases

Protein phosphorylation participates in the modulation of most cellular processes, therefore alterations in the levels of kinases and phosphatases can impact multiple neuronal functions (Manning *et al*, 2002; Sacco *et al*, 2012). Presence of mHTT has been described to alter several kinases and phosphatases involved in processes such as regulation of neuronal survival or activation of inflammatory pathways. For instance, both pro-survival (MEK/ERK) and pro-apoptotic (p38 and JNK) pathways of the MAPK family (Keshet and Seger, 2010; Miloso *et al*, 2008), as well as, the Akt pathway, which has anti-apoptotic functions (Song *et al*, 2005), have been shown to be altered in HD. However, both excessive activation and inhibition of these pathways has been described depending on the HD model and disease progression state. These conflicting results have been attributed to the presence of compensatory mechanisms in some models at some disease stages, which probably also underlie differences in neuronal death and sensitivity or resistance to excitotoxicity between models. Other kinases described to be altered in HD include IKK and NF κ B, which are associated with increased inflammatory signalling in some HD models (Khoshnan *et al*, 2004; Reijonen *et al*, 2010), and Cdk5, which has been associated with increased striatal vulnerability, participates in multiple neuronal functions (Cheung *et al*, 2006; Paoletti *et al*, 2008; Su and Tsai, 2011) and will be discussed with further detail in later sections.

In addition, several phosphatases have also been described to be altered in HD, including PP1 (Branco-Santos *et al*, 2017; Metzler *et al*, 2010), calcineurin, a calcium-dependent phosphatase with high expression in the striatum and hippocampus (Gratuze *et al*, 2015; Xifró *et al*, 2009) and STEP, whose decreased activity has been associated with excitotoxicity resistance in R6/2 mice whereas increase in its activity has been involved with extrasynaptic localization of NMDARs (Gladding *et al*, 2012; Saavedra *et al*, 2011). In sum, there is a complex deregulation of kinases and phosphatases in HD, which further contribute to the previously described alterations in HD. Importantly, their function as downstream effectors place them in a fitting position to become therapeutic targets in order to minimize side effects.

2.1.2. Non-canonical mechanisms

2.1.2.1. Cell cycle re-entry as a new mechanism

Neurons are classically described as permanent postmitotic cells. For this reason, up until a few decades ago, little attention had been paid to proteins and checkpoints involved in cell cycle regulation in neurons (Fig. 9). Nowadays it is known that re-expression of cell cycle

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proteins and even DNA duplication can take place in several neurodegenerative disorders in response to different stressors, although not much is known about its possible role in HD.

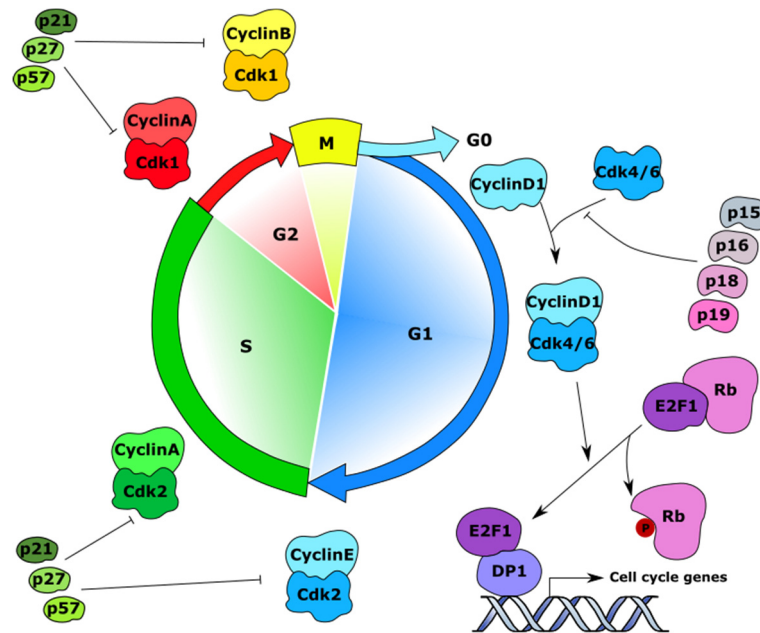


Figure 9. Cell cycle phases. Cyclins and CDKs (Cyclin-dependent kinases), along with CKIs (Cyclin-dependent kinase inhibitors) regulate transition between different cell cycle phases. During the G_1 phase CyclinD1 activates Cdk4/6 which phosphorylates Rb protein allowing E2F1 release. E2F1 binds to DP1 promoting transcription of cell cycle progression genes. CyclinE/Cdk2 regulate G_1/S transition and CyclinA/Cdk2 promote DNA replication activation during S phase. During G_2 phase CyclinA/Cdk1 modulate condensation of chromosomes. Finally, CyclinB/Cdk1 modulate G_2/M transition, after which the cell duplicates during the M phase, Rb becomes dephosphorylated and the cell cycle can begin again. CKIs from the Ink family (p15, p16, p18 and p19) and from the Cip/Kip family (p21, p27 and p57) also participate in cell cycle regulation by inhibiting CDKs activation.

Prove that aberrant neuronal cell cycle re-entry leads to cell death instead of cell division, was first described in the early 1990s when it was observed that, forcing cell cycle initiation by expressing the oncogene SV40 T-antigen under a neuronal promoter, lead to neuronal demise (Al-Ubaidi *et al*, 1992; Feddersen *et al*, 1992). Since then, it has been described that stressors such as neurotrophic factor deprivation (Freeman *et al*, 1994), DNA damage (Park *et al*, 1998), excitotoxicity (Park *et al*, 2000) or presence of amyloid β protein (Copani *et al*, 1999; Giovanni *et al*, 1999) induce expression of cell cycle proteins. Similarly, alteration of cell cycle proteins has been described in ischemia (Love, 2003), amyotrophic lateral sclerosis (ALS) (Ranganathan *et al*, 2001), ataxia-telangiectasia (A-T) (Yang and Herrup, 2005), Parkinson's disease (PD) (Jordan-Sciutto *et al*, 2003), and especially in Alzheimer's disease (AD) (reviewed in (Lee *et al*, 2009)). Some of these proteins include CyclinD1, Cdk4, hyperphosphorylated Retinoblastoma (Rb) and E2F1, which are crucial regulators of the G_1/S transition (Figure 9), but also proliferation markers like PCNA and Ki67. Incorporation of BrdU has also been described in mature neurons indicating DNA duplication (Herrup and Busser, 1995). Importantly, it is believed that once a neuron starts expressing cell cycle proteins and even after it duplicates its DNA, it can take up to months or even years before

its death (Frade and Ovejero-Benito, 2015; Herrup and Yang, 2007), thus contributing to a long process of neuronal dysfunction prior to neuronal degeneration, common in most neurodegenerative diseases. In fact, small subsets of aneuploid neurons can be found in physiological conditions in the brain without causing overt alterations (Arendt, 2012; Frade and Ovejero-Benito, 2015).

As previously mentioned, some cellular stressors associated with cell cycle re-entry, such as neurotrophic factor deprivation or excitotoxicity play an important role in HD striatal vulnerability. In addition, alterations in DNA damage, which also can lead to increased cell cycle re-entry, have also been described in HD. Impaired non-homologous end joining (NHEJ) DNA repair has been described to be impaired in HD due to mHTT interaction with Ku70 (Enokido *et al*, 2010), whereas proteins involved in mismatch repair (MMR), like Msh2 have been associated with increased CAG instability (Manley *et al*, 1999).

Thus, it is possible that cell cycle re-entry of striatal neurons may contribute to striatal dysfunction in HD. In accordance, brain samples from HD patients present increased levels of E2F1, CyclinD1 and Cdk4, which play a key role in G₁ phase progression (Fig. 9). Cdk4 levels have also been described to be increased in the striatum of R6/1 mice, as well as in rats treated with 3-NP, along with increased Cdk2, CyclinD1 and E2F1 levels (Fernandez-Fernandez *et al*, 2011; Pelegrí *et al*, 2008). Importantly, 3-NP treatment has also been shown to cause reduction of p27 and increased phosphorylated-Rb (pRB) levels in the striatum of treated mice, which can be prevented by CDK inhibitors and NMDARs antagonists in striatal primary cultures (Akashiba *et al*, 2008). Finally, differential expression of several microRNAs has also been shown to affect cell cycle progression of HD immortalized striatal cells (*STHdh^{Q111}*) (Das *et al*, 2013, 2015). Therefore, available data suggests that cell cycle proteins are altered by mHTT presence but little is known about their changes during disease progression and about how mHTT triggers their expression.

2.2. Synaptic dysfunction

Synaptic plasticity, i.e., activity-dependent strength modification of synaptic transmission in pre-existing synapses, is necessary and sufficient for the information storage underlying learning and memory formation (Citri and Malenka, 2008; Martin *et al*, 2000). The best characterized synaptic plasticity forms are LTP (long-term potentiation) and LTD (long-term depression), firstly described in the hippocampus. However, there are other forms of synaptic plasticity, such as an endocannabinoid-dependent LTD form which has been identified in different brain regions including D₂-MSNs (reviewed in (Citri and Malenka, 2008)). In any case, all kinds of synaptic plasticity induction rely on normal electrophysiological properties, proper function and localization of neurotransmitter receptors and synaptic proteins, and correct cytoskeleton regulation underlying dendritic

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spine remodelling. The fact that many HTT interactors include pre- and post-synaptic proteins has led to the idea that mHTT may alter these proteins, generating a complex synaptic dysfunction.

In accordance, analysis of HD patients brains have shown impairments in cortical and brainstem LTP-like plasticity (Crupi *et al*, 2008), while studies in HD mouse models have revealed alterations in corticostriatal LTP induction prior to motor onset in R6/2 (Kung *et al*, 2007) and Hdh^{Q111} mice (Puigdemívol *et al*, 2015). Changes in synaptic plasticity within the cortex have also been demonstrated, with R6/1 mice showing reduced LTP induction in the medial prefrontal cortex, and impaired LTD induction in the perirhinal cortex, alterations that have been associated with reduced dopamine signalling (Cummings *et al*, 2006, 2007; Dallérac *et al*, 2011). Finally, in the hippocampus, impaired LTP induction in the CA1 has been described in YAC mice (Hodgson *et al*, 1999) and in early disease stages in R6/2 mice, which also present alterations in the DG at later stages, correlating with chronological appearance of mHTT neuronal intranuclear inclusions in these regions (Murphy *et al*, 2000). Hippocampal synaptic plasticity impairments have been associated, in turn, with altered glutamate and BDNF signalling (Brito *et al*, 2014; Lynch *et al*, 2007; Milnerwood *et al*, 2006; Simmons *et al*, 2009). Altogether, even though functional alterations may vary depending on the studied model and the experimental procedure, aberrant corticostriatal and hippocampal synaptic plasticity are observed prior to overt motor phenotype and neuronal death in most HD models, supporting the idea that neuronal dysfunction rather than neurodegeneration is playing an important role in HD pathology. In accordance, several neuronal processes implicated in synaptic plasticity show alterations in HD.

2.2.1. Synaptic transmission

Several studies have pointed out that altered corticostriatal transmission is an early HD hallmark which, similar to motor symptoms progression, manifests in a biphasic way (reviewed in (Raymond *et al*, 2011)). It has been observed that in both YAC128 and R6/2 mice, at pre-motor disease stages, there is an increase in striatal sEPSCs (spontaneous excitatory postsynaptic currents) (Andre *et al*, 2011; Cepeda *et al*, 2003; Joshi *et al*, 2009). In addition, although basal glutamate levels are significantly reduced in the striatum and prefrontal cortex of R6/1 and knock-in Hdh^{CAG150} mice, respectively, as previously mentioned, activity-induced striatal glutamate release is significantly increased in R6/1 mice (Nicnocaill *et al*, 2001; Wolfram-Aduan *et al*, 2014). On the other hand, in later stages, there is a reduction in excitatory activity of MSNs, which is believed to arise from MSNs disconnection from their inputs, as it correlates with alteration of different synaptic markers and loss of dendritic spines (Cepeda *et al*, 2003). In agreement, it has been described that knock-in Q140 mice present loss of corticostriatal terminals prior to overt striatal dysfunction (Deng *et al*, 2013). In addition, at this stage, cortical sEPSCs occur at higher

frequencies (Cummings *et al*, 2009) and it has been described that reduction of cortical inputs to the striatum or mHTT removal from cortical neurons, is able to delay disease progression (Cepeda *et al*, 2003; Gu *et al*, 2007). In sum, these findings support the idea that aberrant cortical afferences and/or corticostriatal disconnection play a major role in impaired corticostriatal function.

2.2.2. Dendritic spines

Dendritic spines are dynamic and heterogeneous structures where most synapses occur. Remodelling of their shape, size and number is a key step in synaptic plasticity processes and it relies on cytoskeleton reorganization, which is mainly composed by filamentous actin (F-actin). Therefore, proteins involved in positive or negative actin polymerization will modulate growth or shrinkage of dendritic spines. These proteins include neurotransmitter receptors (e.g. NR1 and NR2B), neurotrophin receptors (e.g. TrkB and p75^{NTR}), kinases (e.g. CaMKII and Cdk5) and scaffold proteins (e.g. PSD95) (reviewed in (Sala and Segal, 2014)). However, downstream signalling of these proteins, usually converge on proteins from the small GTPases family, which act as molecular switches depending on their active (GTP-bound) or inactive (GDP-bound) state. Rac1 and RhoA are the best characterized members of this family for their opposite roles in dendritic spine remodelling, with Rac1 acting as a dendritic spine positive regulator (Nakayama *et al*, 2000), and RhoA as a negative dendritic spine modulator (Tashiro *et al*, 2000).

In this regard, initial studies from HD patients revealed early morphologic and quantitative alterations in dendritic arbours and spines in the striatum and cortex (Ferrante *et al*, 1991; Graveland *et al*, 1985; Sotrel *et al*, 1993), which could be associated with presence of mHTT aggregates on dendritic spines (Gutekunst *et al*, 1999). Additionally, dendritic spine alterations are also present in most HD mouse models, including YAC128 mice, which present reduced spines in MSNs at late disease stages (Xie *et al*, 2010), R6/2 and R6/1 mice, which have decreased dendritic spines density in the striatum, cortex and hippocampus (Bulley *et al*, 2012; Giralt *et al*, 2017; Klapstein *et al*, 2001; Murmu *et al*, 2013; Spires *et al*, 2004) and Hdh^{Q111} knock-in mice which present early decrease of cortical spines (Puigdemívol *et al*, 2015) and reduction of striatal and hippocampal dendritic spines (Brito *et al*, 2014; Lerner *et al*, 2012). Importantly, studies tracking individual dendritic spines through the disease progression have also shown increased spine formation, although their survival is greatly reduced, indicating aberrant dendritic spine neurogenesis and maintenance (Murmu *et al*, 2013).

Importantly, some of these modifications have been associated with alterations in cytoskeleton regulation. Thus, knock-in Hdh^{Q111} mice present reduced actin polymerization at dendritic spines which correlates with failing to stabilize CA1 LTP in presymptomatic

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stages (Lynch *et al*, 2007; Star *et al*, 2002). On the other hand, both Rac1 and RhoA have been described to be altered in knock-in Hdh^{Q111} mice. Specifically reduced Rac1 activity correlating with reduced dendritic spines was observed in the cortex (Puigdel·l·ivol *et al*, 2015), whereas increased RhoA activity was described in the hippocampus (Brito *et al*, 2014). Moreover, a protein interaction study identified the RhoGTPase signalling pathway for being enriched in HTT interacting proteins (Tourette *et al*, 2014). Thus, alterations in cytoskeleton proteins and their upstream regulators may underlie modifications in dendritic spines participating in synaptic dysfunction in HD.

2.2.3. Synaptic proteins

Excitatory synapses are characterized by the presence of a postsynaptic density, a specialised macromolecular complex where neurotransmitter receptors, scaffold proteins and cytoskeletal components are localized, and which receives inputs from a presynaptic site (Citri and Malenka, 2008; Sheng, 2001). Presence of mHTT modifies many of these proteins, contributing to changes in synaptic plasticity. Thus, alterations in presynaptic processes associated with exocytosis and endocytosis and with neurotransmitter release, such as reduced levels of acetylcholine metabolism enzymes ChAT (choline acetyltransferase enzyme) and VAChT (vesicular acetylcholine transporter) have been described in HD patients and in several HD models (reviewed in (Li *et al*, 2003; Rozas *et al*, 2010; Tyebji and Hannan, 2017)). Reduced levels of the endocannabinoid receptor CB₁, which is expressed presynaptically inhibiting neurotransmitter release, has also been observed at very early stages in HD patients (Glass *et al*, 2000; Van Laere *et al*, 2010) and in HD mice (Blázquez *et al*, 2011; Chiodi *et al*, 2012; Ruiz-Calvo *et al*, 2018).

However, alterations in the PSD have been more extensively analysed showing deficits in the expression of several neurotransmitter receptors, including **serotonin** receptors which are reduced in the hippocampus and cortex in HD models (reviewed in (Tyebji and Hannan, 2017)) and **adenosine** receptors (A₁R and A₂AR) which participate in bioenergetic homeostasis and are affected at early disease stages (Lee and Chern, 2014; Matusch *et al*, 2014). **GABA_A** receptors and **DA** receptors (D₁ and D₂) also present reduced levels since initial stages (Chen *et al*, 2013; Glass *et al*, 2000). Levels of DA, that are crucial for striatal LTP and LTD induction (Chen *et al*, 2013; Citri and Malenka, 2008), are also altered in a biphasic way, with an early increase, and a reduction at later disease stages (Dall·erac *et al*, 2015; Garrett and Soares-da-Silva, 1992; Kish *et al*, 1987). Finally, **glutamate** receptors have been extensively studied in HD, for their pivotal role in synaptic plasticity induction and because of the association between glutamate agonists treatment and induction of HD-like phenotypes in pharmacologic HD models (Coyle and Schwarz, 1976). Nonetheless, even though alterations in glutamate AMPARs and in mGluRs have been detected in HD (Benn *et*

al, 2007; Cha *et al*, 1998; Miller and Bezprozvanny, 2010; Wagster *et al*, 1994), most studies concerning glutamate receptors have focused on NMDARs.

2.2.3.1. NMDA receptors

Glutamate receptors can be divided in two classes: metabotropic receptors (mGluRs) which are G-protein coupled receptors regulating different effector systems (Conn and Pin, 1997), and ionotropic receptors (iGluRs) which form ion channel pores that allow cationic influx and include kainate, AMPA and NMDA receptors (Traynelis *et al*, 2010). Functional NMDARs are tetrameric structures generally composed by two NR1 subunits and two NR2 subunits (mostly NR2A and NR2B). Calcium entry following their activation is well known to be pivotal for development of new synapses and processes of synaptic plasticity underlying learning and memory formation (Maren and Baudry, 1995). Notably, NMDARs synaptic localization plays a role in functional outcome of NMDAR-dependent signalling. Thus, as mentioned before, while activation of synaptic NMDARs leads to neuroprotective pathways including CREB phosphorylation, BDNF transcription and ERK activation; activation of extra-synaptic NMDARs results in induction of apoptotic pathways (reviewed in (Hardingham and Bading, 2010; Wang *et al*, 2013)). Therefore, NMDARs signalling needs to be precisely regulated, as aberrant NMDAR activation can lead to excitotoxicity, highly associated with HD striatal vulnerability, but reduction of functional NMDARs can lead to impaired synaptic plasticity.

Although it has been observed that HD patients present reduced NMDAR binding and density in the putamen (Albin *et al*, 1990; Young *et al*, 1988), most NMDAR-associated alterations have been studied and described in HD models. Reported alterations of NMDAR subunits levels vary depending on the HD model, disease stage and subcellular localization being analysed (Table 3) (Cepeda *et al*, 2001; Cha *et al*, 1999; Cowan *et al*, 2008; Fan *et al*, 2007; Giralt *et al*, 2017; Jarabek *et al*, 2004; Luthi-Carter *et al*, 2003; Milnerwood *et al*, 2010). A general reduction of NMDARs mRNA and protein levels have been described in R6/2 mice across different brain regions (Luthi-Carter *et al*, 2003), and reduced striatal NR2B levels have also been reported in YAC128 mice (Cowan *et al*, 2008). Importantly, specific NMDAR subunits reduction at the plasma membrane has also been observed in R6/2 mice (Luthi-Carter *et al*, 2003). In accordance, alterations in NMDAR subunits trafficking kinetics have been described in cultured striatal MSNs from YAC72 mice (Fan *et al*, 2007). In general, evidence indicates a general deregulation of NMDARs, including their transcription, protein levels and subcellular localization, which could contribute to impaired synaptic plasticity in HD.

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HD model	Stage	Described alterations	Analysed sample	Brain region	Reference
R6/2	Symptomatic	Increased NR1 levels. Decreased NR2A and NR2B levels.	Immuno-histochemical signal	Striatum	(Cepeda <i>et al</i> , 2001)
R6/2	12 weeks	No changes in NR1 levels	Total protein levels	Brain	(Cha <i>et al</i> , 1999)
R6/2	4-8 weeks	Reduced NR2A	mRNA	Brain	(Luthi-Carter <i>et al</i> , 2003)
R6/2	8-12 weeks	Reduced NR2B	mRNA	Brain	(Luthi-Carter <i>et al</i> , 2003)
R6/2		Decreased NR2A, NR1 and NR2B	Plasma membrane protein levels	Striatum, cortex, hippocampus	(Luthi-Carter <i>et al</i> , 2003)
R6/1	4-5 months	Decreased NR2A levels with no changes in NR2B levels	Total protein levels	Hippocampus	(Giralt <i>et al</i> , 2017)
YAC72		Increased NR1 and NR2B trafficking to the plasma membrane	Biotinylated protein levels	Cultured MSNs	(Fan <i>et al</i> , 2007)
YAC128	1 month	Increased NR1 and NR2B extrasynaptic levels	Non-PSD protein levels	Striatum and cortex	(Milnerwood <i>et al</i> , 2010)
YAC128	12 months	Reduced NR2B levels	Total protein levels	Striatum	(Cowan <i>et al</i> , 2008)
YAC128	12 months	No alterations in NR2B levels	Total protein levels	Cortex and hippocampus	(Cowan <i>et al</i> , 2008)
N171-82Q	Symptomatic	No changes in NR1, NR2A and NR2B levels	Total protein levels	Striatum	(Jarabek <i>et al</i> , 2004)

Table 3. Alterations in NMDAR subunits in HD mice models. Multiple studies have reported alterations in NMDARs subunits levels, however specific changes differ depending on the HD model, the disease stage, the NMDAR subunit, the brain region or the technique being used.

3. Cdk5 implication in HD

Cdk5, like the others members of the CDK family, is a proline-directed protein kinase that phosphorylates Ser or Thr residues followed by a proline amino acid ((Kimura *et al*, 2014). However, unlike the rest of the CDK family members, Cdk5 activity does not rely on Cyclins nor on phosphorylation of its activation loop, but on the neuronal specific activator p35 (Tsai *et al*, 1994). Thus, although recent studies have described Cdk5 involvement in maintaining cell cycle suppression in post-mitotic neurons, its classical roles are associated with many crucial CNS functions, particularly synaptic plasticity processes (Angelo *et al*, 2006; Su and Tsai, 2011). Moreover, deregulation of Cdk5 activity, mainly caused by calpain-dependent cleavage of p35 to p25, is a hallmark of several neurodegenerative disorders and pathological conditions, including ischemia, Alzheimer's disease or Huntington's disease (McLinden, 2012; Patrick *et al*, 1998). For these reasons, Cdk5 is a promising putative target in neurodegenerative disorders and particularly as a dual contributor to both motor and cognitive dysfunction in HD.

3.1. Cdk5 regulation

Cdk5 is expressed in most tissues with particularly high levels in the brain. However, as its activators, p35 and p39, are expressed in neurons, its activity is mainly restricted to the CNS (Tsai *et al*, 1994; Wu *et al*, 2000). Levels of Cdk5 activation are also determined by its activators availability, as their levels are less abundant than Cdk5. Indeed, p35 and p39 present a short half-life due to its rapid proteosomal degradation (Patrick *et al*, 1998; Takasugi *et al*, 2016). In fact, phosphorylation of p35 by Cdk5 induces p35 proteosomal degradation and suppresses its calpain-dependent conversion to p25, thus regulating its own activity (Kamei *et al*, 2007; Patrick *et al*, 1998; Wei *et al*, 2005). Increased Cdk5-dependent p35 proteosomal degradation has been described to take place after calcium influx induced by glutamate receptors activation (Wei *et al*, 2005). However, calpain activation leads to p25 production, the p35 cleaved form, which in contrast to p35 is resistant to proteosomal degradation, thus constitutively activating Cdk5. In addition, the myristoylation residue which allows plasma membrane localization of Cdk5/p35 is lost in p25 leading to Cdk5 redistribution to the cytosol and alteration of its substrate specificity (Asada *et al*, 2008; Kusakawa *et al*, 2000; Patrick *et al*, 1999) (Figure 10). Production of p25 has been widely associated with neurodegenerative disorders (Camins *et al*, 2006).

Although p35 and p39 are the main regulators of Cdk5 activity, it can also be regulated by post-translational modifications and by interaction with other proteins. For instance, CyclinI has been described to activate Cdk5 (Brinkkoetter *et al*, 2009; Hagmann *et al*, 2015), whereas interaction with CyclinE, CyclinD1 or GSTP1 (glutathione S-transferase P1) has been

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associated to Cdk5 inhibition, as they prevent Cdk5 from interacting with p35 (De Falco *et al*, 2004; Modi *et al*, 2012; Odajima *et al*, 2011; Sun *et al*, 2011).

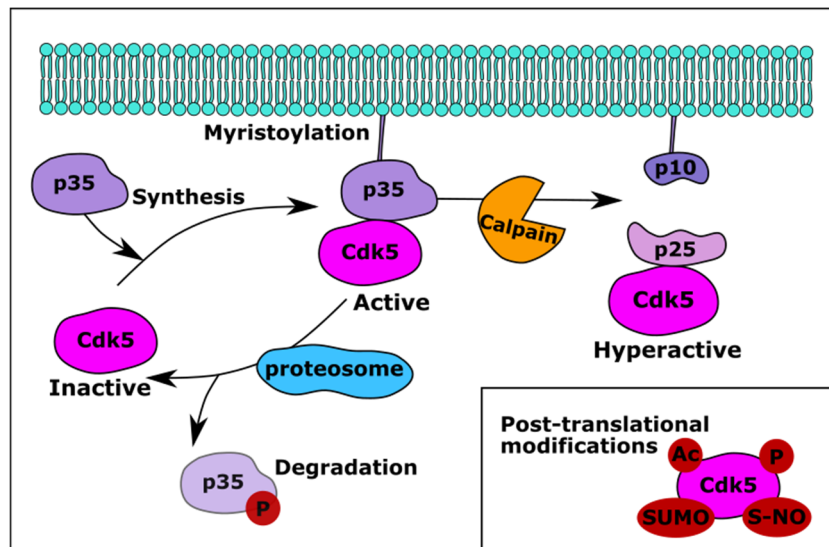


Figure 10. Cdk5 activity regulation. Cdk5 is activated by p35 which presents a myristoylation residue allowing its association with the plasma membrane. Calpain activation cleaves p35 to p25, altering duration of Cdk5 activation and its subcellular localization. Cdk5 can also be regulated by post-translational modifications including, acetylation (Ac), phosphorylation (P), sumoylation (SUMO) and S-nitrosylation (S-NO). Adapted from (Kimura *et al*, 2014; Shah and Lahiri, 2014)

Cdk5 activity can also be modulated by **phosphorylation**. Even though a recent study has stated the opposite, it is generally believed that phosphorylation of Cdk5 at Tyr15 leads to its activation (Kobayashi *et al*, 2014). Cables and c-Abl were the first proteins described to phosphorylate Cdk5 on this residue (Zukerberg *et al*, 2000), although later studies have shown that Cdk5 phosphorylation can also be positively regulated by TrkB (Cheung *et al*, 2007), Fyn (Sasaki *et al*, 2002) and EphA4 (Fu *et al*, 2007), while being negatively regulated by D₂R activation (Yamamura *et al*, 2013). Additional Cdk5/p35 post-translational modifications include **sumoylation**, i.e., the addition of a small ubiquitin-related modifier (SUMO), which enhances Cdk5/p35 activity in response to oxidative stress (Büchner *et al*, 2015), **acetylation** by the histone acetyltransferase GCN5 in the Cdk5 ATP binding domain (Lee *et al*, 2014) and **S-nitrosylation**. Intriguingly, Cdk5 S-nitrosylation, which consists in the covalent attachment of NO to a cysteine residue forming a S-nitrosothiol, has been reported to have opposite effects on its activity, with low concentrations of nitrosylating agents causing increased Cdk5 activity and high concentration of nitrosylating agents leading to decreased activity (Qu *et al*, 2011; Shah and Lahiri, 2014; Zhang *et al*, 2010c). In addition, a recent report has also described p35 S-nitrosylation leading to its ubiquitination and degradation, thus inhibiting Cdk5 activity (Zhang *et al*, 2015). In summary, Cdk5 presents a very complex regulation of its activity which is reasonable considering that Cdk5 is present in most cellular compartments and participates in a wide range of neuronal functions.

3.2. Cdk5 function

Since its first discovery after being cloned based on sequence homology with other CDKs (Hellmich *et al*, 1992; Lew *et al*, 1992; Meyerson *et al*, 1992), much has been learned about Cdk5 complex function. Dozens of Cdk5 substrates involved in multiple processes have been described, underlining Cdk5 importance in neuronal function. First hints at Cdk5 role came from observations that Cdk5 knockout mice resulted in perinatal death which correlated with lack of cortical laminar structure and impaired axonal transport of neurofilaments (NF) (Ohshima *et al*, 1996), thus implying an essential role for Cdk5 in developmental **neuronal migration**. In fact, recent studies in a ferret model have shown that inhibition of Cdk5

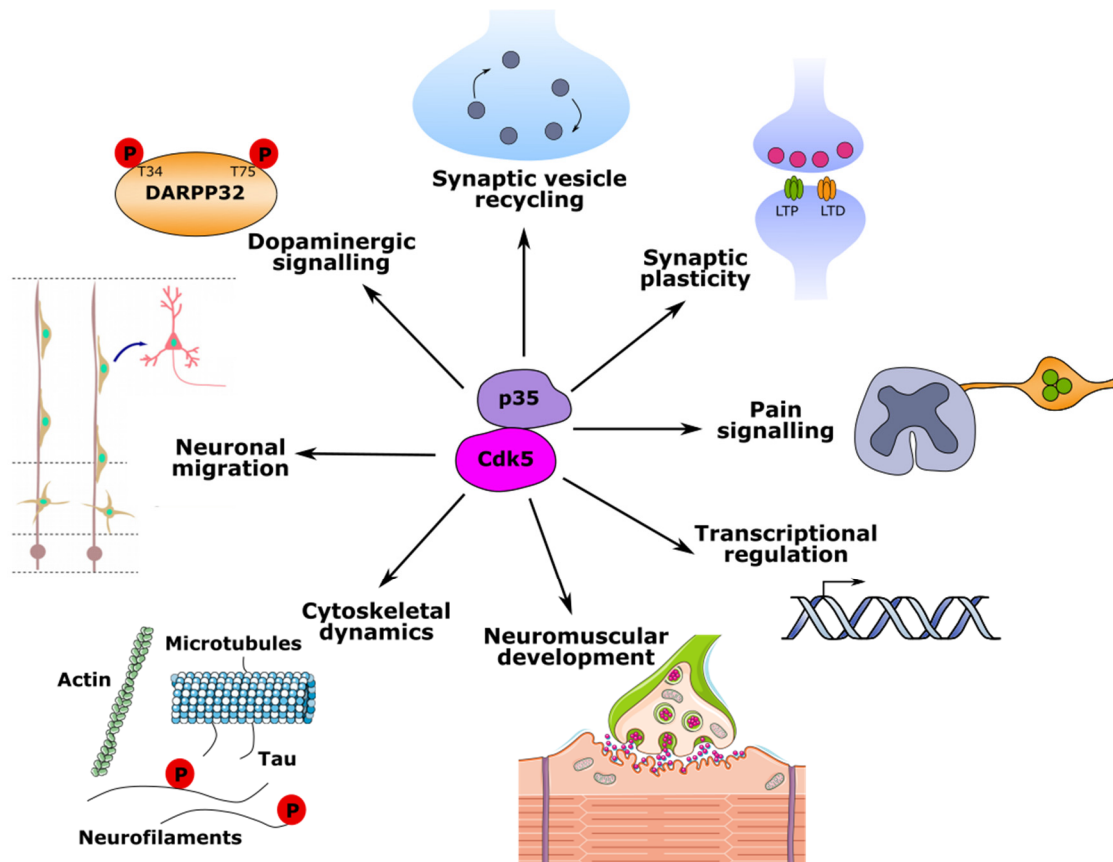


Figure 11. Cdk5 functions in the CNS. Modulation of multiple substrates allows Cdk5 to participate in the regulation of a plethora of essential processes in the CNS. Adapted from (Hori and Hoshino, 2017; Su and Tsai, 2011).

impairs normal cortical folding during development (Shinmyo *et al*, 2017). Nowadays, it is known that Cdk5 participates in numerous neuronal processes (Figure 11). Cdk5 phosphorylates several **transcription factors**, such as STAT3 (Fu *et al*, 2004) or MEF2, whose pro-survival signalling is inhibited upon Cdk5 phosphorylation (Gong *et al*, 2003), and participates in processes such as **pain signalling** (reviewed in (Utreras *et al*, 2009)) and in development of **neuromuscular junctions** by modulating clustering of acetylcholine receptors and neuregulin signalling (Fu *et al*, 2001, 2005; Lin *et al*, 2005). Finally, Cdk5 plays an important role in synaptic plasticity modulation. Hence, it modulates **dopamine**

signalling both by regulating dopamine synthesis and by inhibiting its signalling via phosphorylation of DARPP32, a striatal-enriched protein important for dopamine-dependent striatal LTP and LTD induction (Bibb *et al*, 1999; Calabresi *et al*, 2000; Moy and Tsai, 2004). This function is particularly relevant in the striatum where Cdk5-dependent dopamine signalling modulation has been associated with cocaine reinforcement effects (Benavides *et al*, 2007; Bibb *et al*, 2001a). Cdk5 also phosphorylates several **presynaptic** substrates, such as Munc18, dynamin-I or synapsin-I, modulating exocytosis and recycling of synaptic vesicles (reviewed in (Cheung *et al*, 2006; McLinden, 2012)), and several postsynaptic substrates which will be discussed in more detail hereafter.

3.2.1. Cdk5 role in synaptic plasticity

Cdk5 role in synaptic plasticity was firstly discovered after observations that pharmacological Cdk5 inhibition led to blocking of LTP induction in hippocampal slices and to impairment of context-dependent fear conditioning (Fischer *et al*, 2002, 2003; Li *et al*, 2001a). Since then, multiple studies have been performed using different genetic models to inhibit Cdk5 or its activator p35. Functional effects of these models differ greatly, showing both improvement and impairment of synaptic plasticity and learning and memory, depending on the brain region being targeted, on the duration of the inhibition period and even on the mouse strain being used (Table 4 and (Fischer *et al*, 2002, 2003)). For example, p35 knockout mice present lower threshold for seizure-induced lethality (Chae *et al*, 1997). Similar results have been described after genetic Cdk5 inhibition in the CNS for a long period of time, which leads to appearance of seizures associated with lower epileptiform activity threshold. In contrast, Cdk5 genetic deletion for a short period of time improves spatial learning which correlates with increased hippocampal LTP and NMDAR-mediated excitatory currents (Hawasli *et al*, 2007, 2009). Opposite effects depending on the duration of Cdk5 activity modification have also been found in mice models over-expressing p25. Even though increased levels of p25 have been associated with several neurodegenerative disorders, it has been reported that transient p25 over-expression facilitates LTP induction and hippocampal-dependent memory. In fact, it has been described that p25 is generated in physiological conditions upon neuronal activation in a NR2B- and CaMKII α -dependent manner (Seo *et al*, 2014). However, over-expression of p25 for longer periods leads to memory impairment and neuronal loss (Fischer *et al*, 2005). Importantly, prolonged p25 over-expression in the hippocampus and cortex causes neuronal loss, whereas p25 increase in the striatum causes synaptic alterations without neuronal death being detected (Cruz *et al*, 2003; Meyer *et al*, 2008). Differential brain region-specific effects have also been observed after Cdk5 inhibition. Thus, specific Cdk5 deletion in the hippocampal CA1 causes increased PDE levels leading to decreased cAMP-dependent signalling, conversely Cdk5 inhibition in the striatum decreases PDE activity leading to increased cAMP levels (Guan *et*

al, 2011; Plattner *et al*, 2015). This data illustrates the complex role of Cdk5 as a modulator of synaptic plasticity and its homeostasis. In this sense, Cdk5 has also been shown to regulate GKAP and SPAR, two proteins involved in synaptic scaling, this is, strength adjustment of all excitatory synapses in a neuron in order to stabilize its firing (Roselli *et al*, 2011; Seeburg *et al*, 2008; Turrigiano, 2008). Moreover, a recent study has shown that Cdk5 represses NMDAR- and AMPAR-dependent signalling in a particularly rapid response to altered synaptic transmission (Sheng *et al*, 2016). Hence, Cdk5 fine-tuned function appears necessary for proper balance between enough synaptic activation, to ensure learning and memory processes, and excessive neuronal activity, responsible for excitotoxicity.

Action	Method	Tissue	Effect on synaptic plasticity	Reference
Cdk5 inhibition	Pharmacological: Roscovitine	Hippocampal slices	Blocking of LTP induction and NMDA-evoked currents in CA1 hippocampal neurons	(Li <i>et al</i> , 2001a)
Cdk5 inhibition	Pharmacological: Roscovitine and butyrolactone I	Brain slices	No effect on LTP induction	(Wang <i>et al</i> , 2004)
Cdk5 inhibition	Pharmacological: Roscovitine	Brain slices	Increased percentage of population spikes that exhibit LTP	(Miranda-Barrientos <i>et al</i> , 2014)
Cdk5 inhibition	Pharmacological: Butyrolactone I microinjection	Septum and hippocampus	Impairment of context-dependent fear conditioning and of its facilitation in response to stress	(Fischer <i>et al</i> , 2002, 2003)
Cdk5 inhibition	Pharmacological: Butyrolactone I microinjection	Hippocampus	Facilitation of extinction of aversive freezing behaviour	(Sananbenesi <i>et al</i> , 2007)
Cdk5 inhibition	Genetic by shRNA-miR microinjection	Hippocampus	Increased LTP	(Posada-Duque <i>et al</i> , 2016)
Cdk5 inhibition	Short genetic KO (2-4 weeks)	CNS	Improved spatial learning, enhanced hippocampal LTP and NMDAR-mediated excitatory postsynaptic currents	(Hawasli <i>et al</i> , 2007)
Cdk5 inhibition	Chronic genetic KO (8 weeks)	CNS	Lower threshold for epileptiform activity and seizures	(Hawasli <i>et al</i> , 2009)
Cdk5 inhibition	Genetic KO	Forebrain	Hyperactivity in the open field, reduced anxiety, reduced behavioural despair, impaired spatial learning in the MWM and in contextual fear memory	(Su <i>et al</i> , 2013)
Cdk5 inhibition	Genetic KO	Forebrain	Altered behavioural responses to acute or chronic stress	(Plattner <i>et al</i> , 2015)
Cdk5 inhibition	Genetic KO	Hippocampus	Impairment in memory formation and retrieval	(Guan <i>et al</i> , 2011)
Cdk5 inhibition	Genetic KO	Forebrain and dorsolateral striatum	Impairment of dopamine-facilitated LTP, increased locomotor activity and attenuated motor learning	(Hernandez <i>et al</i> , 2016)
p35 inhibition	Transient genetic KO	General	Impairment in spatial learning and memory, reduced anxiety-like behaviour and defective LTD induction in the hippocampus	(Mishiba <i>et al</i> , 2014)
p35 inhibition	Genetic KO	General	Lower threshold for LTP induction in hippocampal slices	(Wei <i>et al</i> , 2005)
p35 inhibition	Genetic KO	General	Deficits in spatial learning and memory, impaired LTD and depotentiation of hippocampal LTP	(Ohshima <i>et al</i> , 2005)

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p25 over-expression	Transient genetic over-expression (1-2 weeks)	General	Enhancement of LTP and facilitation of hippocampal-dependent memory	(Fischer <i>et al</i> , 2005)
p25 over-expression	Prolonged genetic over-expression (6 weeks)	General	Impairment of learning in fear conditioning and in the water maze paradigm	(Fischer <i>et al</i> , 2005)
p25 over-expression	Genetic over-expression	Forebrain	No alterations in the learning phase of the MWM but improved performance in the reversal phase. Reduced contextual conditioning and enhanced tone fear conditioning	(Angelo <i>et al</i> , 2003)
p25 over-expression	Genetic over-expression	Forebrain	Impaired LTP in male mice. Faster spatial learning in females in the MWM	(Ris <i>et al</i> , 2005)
p25 over-expression	Genetic over-expression	Forebrain	Impaired contextual fear conditioning in female, but not male	(Mizuno <i>et al</i> , 2006)

Table 4. Functional effects of Cdk5 activity modulation on synaptic plasticity. Inhibition of Cdk5 or p35 and over-expression of p25 have been described to have both positive and negative effects on synaptic plasticity induction, depending on the method used to modulate Cdk5 activity, the duration of this modulation or the brain region being targeted. KO (knock-ou), MWM (Morris Water Maze).

3.2.1.1. Cdk5 regulation of NMDA receptors

One of the Cdk5-dependent mechanisms most frequently associated with its role in synaptic plasticity regulation, is modulation of NMDARs. As mentioned before, they play an important role in LTP and LTD processes and Cdk5 has been shown to regulate their surface levels through different pathways. Thus, Cdk5 has been described to negatively regulate phosphorylation levels of **NR1** (Ser897), as Cdk5 inhibition leads to increased phospho-NR1 levels and increased NMDAR activity (Chergui *et al*, 2004). **NR2A** total and phosphorylation (Ser1232) levels have also been described to be positively regulated by Cdk5 in models of ischemia, neuropathic pain and glaucoma (Chen *et al*, 2011; Wang *et al*, 2003; Yang *et al*, 2014). However, most studies describing Cdk5-dependent NMDAR regulation focus on the **NR2B** subunit (Figure 12). Accordingly, Cdk5 has been shown to phosphorylate NR2B at residues Ser1284 and Ser1116. Ser1284 phosphorylation levels are increased after hippocampal ischemic insults without being affected by neuronal activity, whereas Ser1116 phosphorylation levels, which lead to reduced NR2B surface levels, become reduced after neuronal activation (Lu *et al*, 2015; Plattner *et al*, 2014). Cdk5 can also regulate NR2B surface levels by physically interacting with NR2B and calpain, contributing to calpain-dependent NR2B degradation and consequent reduction of total and NR2B surface levels (Hawasli *et al*, 2007). Finally, Cdk5 is able to indirectly modulate NR2B surface levels by promoting its internalization via the clathrin adaptor AP-2. Phosphorylation of PSD-95 by Cdk5 reduces Src colocalization with PSD-95 and its auto-activation (phosphor-Src (Tyr416)), leading to reduced Src-dependent NR2B phosphorylation at Tyr1472, which allows AP-2 binding and subsequent internalization (Zhang *et al*, 2008b). Direct Cdk5 phosphorylation of PSD-95 has also been shown to negatively regulate PSD-95 clustering, which could also affect NMDARs

and other proteins which rely on PSD-95 to localize at the PSD (Morabito *et al*, 2004). In general, most of these mechanisms lead to a Cdk5-dependent reduction of NMDARs subunit levels, which could act as a “brake” for excessive NMDAR activation, but could also lead to synaptic plasticity impairments if these mechanisms were not properly regulated.

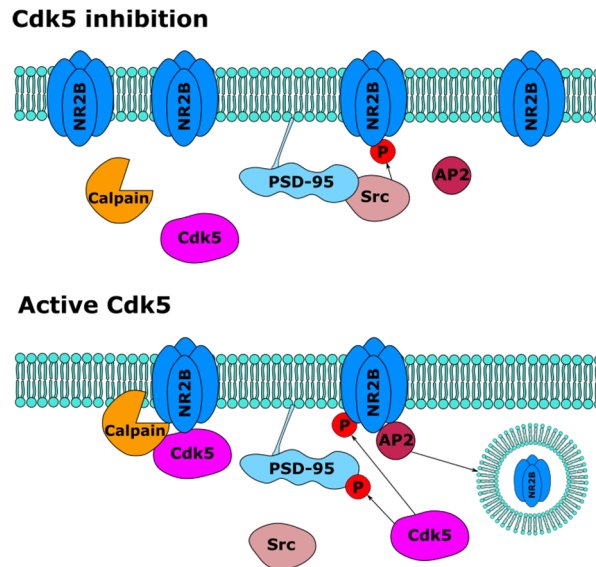


Figure 12. Cdk5 regulation of NMDAR. Schematic representation of the Cdk5-dependent pathways described to modulate NR2B surface levels.

3.2.1.2. Cdk5 modulation of dendritic spines

In adult neurons Cdk5 also plays an important role in cytoskeleton dynamics regulation, by directly modulating cytoskeletal proteins, such as Tau, which is phosphorylated by Cdk5 at different residues (Paudel *et al*, 1993) and F-actin, which is stabilized by direct interaction with p35 (He *et al*, 2011). Cdk5 also phosphorylates several substrates involved in cytoskeletal and dendritic spine remodelling, such as S6K or CRMP2 (Jin *et al*, 2016; Lai *et al*, 2015), and multiple substrates which positively and negatively modulate the members of the small GTPases family, RhoA and Rac1. Specifically, it has been described that Cdk5 positively activates RhoA by phosphorylating ephexin1 and CaMKv (Fu *et al*, 2007; Liang *et al*, 2016), while negatively regulating it during development through phosphorylation and stabilization of p27 (Kawauchi *et al*, 2006). On the other hand, Cdk5 positively modulates Rac1 by phosphorylating Kalirin-7 (Xin *et al*, 2008), whereas Cdk5 phosphorylation of RasGRF2, neurabin-1, WAVE-1 and Rac1 downstream Pak1, negatively regulate Rac1-dependent dendritic spine outgrowth (Causeret *et al*, 2007; Kesavapany *et al*, 2004; Kim *et al*, 2006b; Nikolic *et al*, 1998). Thus, although Cdk5 is able to both promote and inhibit dendritic spine density, it has been described that, in agreement with its positive regulation of several Rac1 inhibitors, inhibition of hippocampal Cdk5 *in vivo* leads to increased Rac1 activity (Posada-Duque *et al*, 2015). Once more, this function allows Cdk5 to modulate synaptic strength on another level.

3.2.2. Cdk5 role in cell cycle

Even though Cdk5 is classically considered an atypical CDK for its main functions in postmitotic neurons and for its independence of cyclins for its activation, in the last decade different studies have shown that Cdk5 does have a role in neuronal cell cycle regulation. First hints at this idea came from observations of Cdk5 knockout mice, which showed alterations in neuronal differentiation with higher expression of proliferation markers and reduced expression of mature neuronal markers during development. Cdk5^{-/-} neurons continued to incorporate BrdU *in vitro*, in contrast with wild type or Cdk5^{+/-} neurons, indicating lack of cell cycle inhibition in the absence of Cdk5 (Cicero, 2005). It was later observed that induction of cell cycle re-entry both in cycling cells and in neurons after exposure to stressors, was accompanied by reduction of nuclear Cdk5, which relies on binding to p27 as Cdk5 does not present a nuclear localization signal (NLS) (Zhang *et al*, 2008a, 2010a). Two main Cdk5-dependent cell cycle suppression mechanisms have been described (Figure 13).

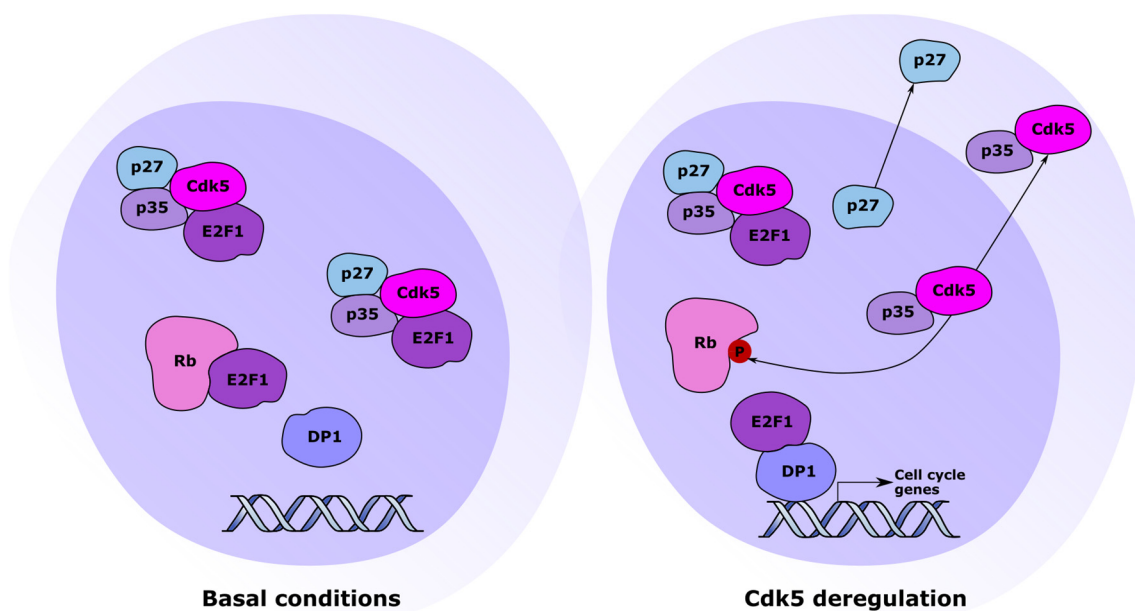


Figure 13. Cdk5 role in neuronal cell cycle regulation. Schematic representation of the main molecular Cdk5-dependent mechanisms maintaining cell cycle inhibition in differentiated neurons. Adapted from (Zhang and Herrup, 2011)

In the first place, it has been shown that Cdk5, along with p35, forms a complex with p27 and the transcription factor E2F1, which is independent of its kinase activity, sequestering E2F1 from its cofactor DP1. Given that E2F1/DP1 regulate transcription of cell cycle progression genes, Cdk5-dependent E2F1 sequestering inhibits cell cycle progression (Zhang *et al*, 2010b; Zhang and Herrup, 2011). On the other hand, it has been described that increased nuclear Cdk5 activity is able to phosphorylate Rb, similarly to Cdk4 and Cdk2, causing increased E2F1 activity leading to cell cycle re-entry (Futatsugi *et al*, 2012). Therefore, both reduction of Cdk5 nuclear localization and increase in nuclear Cdk5 activity

could cause loss of cell cycle inhibition in postmitotic neurons. Finally, it has been described that glutamate-dependent calpain activation, leads to Cdk5 dependent Cdh1 phosphorylation and inhibition. As Cdh1 is a cofactor of the E3 ubiquitin ligase APC/C (anaphase-promoting complex/cyclosome), APC/C activity is reduced, causing accumulation of CyclinB1, reduction of p27 and activation of Cyclin D1/Cdk4 (Cataldo *et al*, 2013; Maestre *et al*, 2008; Veas-Pérez de Tudela *et al*, 2015). Calpain-dependent p25 production has also been described to inhibit HDAC1 activity which leads to increased DNA damage and cell cycle proteins re-expression (Kim *et al*, 2008). These findings indicate that deregulation of Cdk5 could lead to neuronal cell cycle re-entry through different mechanisms.

3.3. Cdk5 alteration in neurodegenerative disorders and neuropathological conditions

As reviewed so far, due to its relevant role in so many neuronal processes, deregulation of Cdk5 can have a big impact in neuronal function. Hence, deregulation of Cdk5 has been described in many pathological conditions, especially in Alzheimer's disease (AD). In fact, first description of p35 conversion to p25 was detected in brains from AD patients, where it was described to play a key role in Tau hyperphosphorylation (Patrick *et al*, 1999). Since then, deregulation of Cdk5 has been associated to other AD hallmarks such as A β formation, alterations in synaptic plasticity, cell cycle re-entry or mitochondrial dysfunction (reviewed in (Liu *et al*, 2016)). Moreover, several studies have shown that inhibition of Cdk5 is able to ameliorate Tau pathology and cognitive deficits in AD mice models (reviewed in (Bhounsule *et al*, 2017; López-Tobón *et al*, 2011)).

Cdk5 deregulation associated to p25 production, as well as neurodegeneration amelioration following Cdk5/p25 inhibition, has also been described in models of Parkinson's disease and ischemic injury (Gutiérrez-Vargas *et al*, 2015; Meyer *et al*, 2014; Smith *et al*, 2003; Zhang *et al*, 2016). In addition, aberrant Cdk5 activity has been associated with other neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) and prion disease (Nguyen *et al*, 2001; Rouget *et al*, 2015) and even with psychiatric disorders like major depression and schizophrenia (Engmann *et al*, 2011; Ramos-Miguel *et al*, 2013; Zhu *et al*, 2012).

3.3.1. Cdk5 deregulation in Huntington's disease

In HD, Cdk5 has been associated to several processes involved in striatal vulnerability (Figure 14). For instance, it has been described that physiological Cdk5 phosphorylation of HTT at Ser434 impairs caspase-mediated HTT cleavage and therefore its aggregation, while phosphorylation at Ser1181 and Ser1201 confers cell protection against polyQ-induced toxicity (Anne *et al*, 2007; Luo *et al*, 2005). Interestingly, Htt phosphorylation at S1181/1201,

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which is induced by DNA damage, has also been associated with BDNF axonal transport and release (Ben M'Barek *et al*, 2013).

Importantly, reduced Cdk5 and p35 levels have been reported in the striatum of HD patients and in knock-in Hdh^{Q111} mice (Anne *et al*, 2007; Paoletti *et al*, 2008), whereas increased p25 levels have been observed in the striatum of YAC128 after quinolinic acid injections, and in human HD patients and in knock-in Hdh^{Q111} mice, which also present increased phospho-Tau levels, thus indicating aberrant Cdk5 activation in HD (Paoletti *et al*, 2008; Park *et al*, 2012). Increased p25 levels have also been described in HD-like pharmacological models using kainic acid or 3-NP (Crespo-Biel *et al*, 2007; Putkonen *et al*, 2011). Moreover, aberrant Cdk5 activation has been shown to contribute to mHTT-mediated striatal vulnerability to dopaminergic and glutamatergic activation (Paoletti *et al*, 2008) and in increased mitochondrial fragmentation (Cherubini *et al*, 2015). Thus, these studies indicate that Cdk5 dysfunction participates in HD striatal vulnerability pathways, like excitotoxicity and mitochondrial dysfunction, however its role in other mechanisms such as cell cycle re-entry, have not been analysed. Importantly, despite Cdk5 pivotal function as a synaptic plasticity modulator, no studies have determined its possible involvement in HD cognitive deficits.

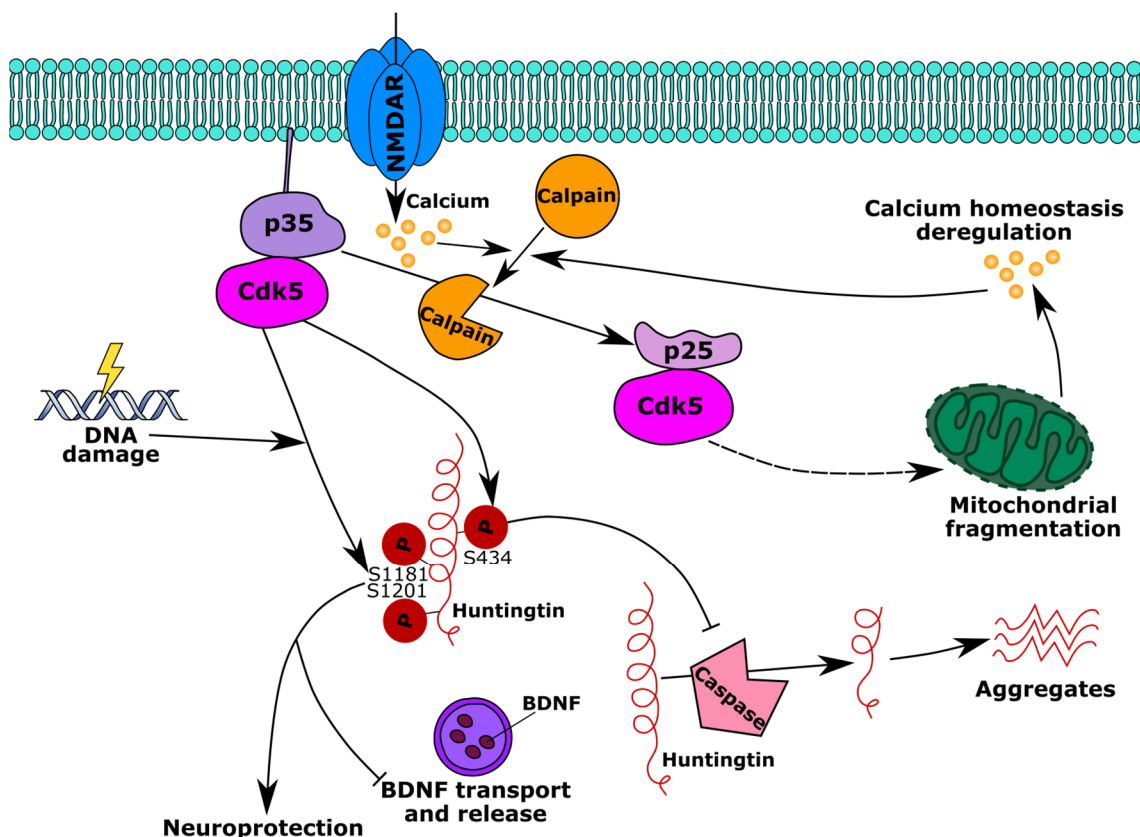


Figure 14. Cdk5 association with processes involved in HD striatal vulnerability. Relationship between Cdk5 and processes associated with HD striatal vulnerability. Cdk5 has been described to contribute to striatal vulnerability factors, such as mitochondrial fragmentation, mHTT aggregate formation or BDNF transport and release, whereas some mechanisms described to be altered in HD, like deregulation of calcium homeostasis or DNA damage, modify Cdk5 activity.

AIMS

Huntington's disease is characterized by the presence of motor deficits, mostly associated to striatal vulnerability, and by cognitive impairments, caused by aberrant corticostriatal connectivity and hippocampal dysfunction, which are related with alterations in synaptic plasticity. For this reason, characterization of molecular mechanisms involved both in neuronal degeneration and synaptic plasticity dysfunction, could help to design new therapeutic approaches to target all together motor and cognitive deficits in HD. In this Thesis, we have focused on Cdk5 as one of these molecular targets.

On one hand, we aimed to study Cdk5 contribution to cognitive decline in HD. Cdk5 has a pivotal role in synaptic plasticity regulation, and deregulation of Cdk5 activity has been described in HD. Therefore, we hypothesized that Cdk5 alterations could support cognitive deficits appearance in HD. On the other hand, Cdk5 deregulation has been associated with aberrant cell cycle re-entry of differentiated neurons in response to several neuronal stressors, and Cdk5 activity deregulation has been described to heighten striatal vulnerability in HD. Hence, we aimed to study whether neuronal cell cycle re-entry resulting from Cdk5 deregulation could contribute to striatal vulnerability in HD. Therefore, the aims of this Thesis are:

1. TO ANALYSE THE ROLE OF CDK5 IN COGNITIVE DEFICITS IN HD.

- 1.1 To generate and validate a new transgenic mouse model that expresses mHtt and has genetically reduced Cdk5 levels.
- 1.2 To analyse the effects of Cdk5 genetic reduction on cognitive deficits development in mHtt KI mice.
- 1.3 To analyse Cdk5 role in synaptic plasticity alterations underlying cognitive impairment in mHtt KI mice.

2. TO ANALYSE NEURONAL CELL CYCLE RE-ENTRY AS A PUTATIVE MECHANISM FOR CDK5-DEPENDENT STRIATAL VULNERABILITY IN HD.

- 2.1 To analyse whether Cdk5 function as a cell cycle modulator is altered in mHtt KI mice.
- 2.2 To analyse if mHtt presence affects cell cycle proteins expression in HD.
- 2.3 To determine whether alteration of cell cycle proteins contributes to increased NMDA excitotoxic response in mHtt KI striatal primary cultures.

METHODS

1. MICE MODELS

All mice 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 12h light/dark cycle. All mice used were males, in order to avoid estrus hormonal alterations, and were housed together in numerical birth order in groups of mixed genotypes. Data was recorded for analysis by microchip mouse number and genotype was determined by PCR analysis. All procedures were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approved by the local animal care committee of the Universitat de Barcelona (76/15) and Generalitat de Catalunya (00/1094), in accordance with the Directive 2010/63/EU of the European Commission.

1.1. Huntington's disease model

Knock-in Hdh^{Q7/Q111} mice, maintained on a C57BL/6 genetic background, and originally obtained from Dr. M. MacDonald's lab (Lloret *et al*, 2006), were used as HD mice model for all experiments. These mice present cognitive deficits at 6 months of age, motor deficits at 8 months of age and a normal life span (Brito *et al*, 2014; Puigdemívol *et al*, 2015).

1.2. Double mutant KI:Cdk5^{+/-} model

Newly generated transgenic double mutant KI:Cdk5^{+/-} mice were obtained from knock-in Hdh^{Q7/Q111} mice and homozygous floxed Cdk5 mice (Cdk5^{flox/flox}) as shown in Figure 15. Cdk5^{flox/flox} mice were obtained from Dr. P. Greengard's lab and were generated by flanking encoding Cdk5 catalytic domain regions with *loxP* sequences (floxed) using homologous recombination (Hawasli *et al*, 2007). Cre-CaMKII^{+/-} mice were obtained from Dr. C. Saura's lab and they express Cre recombinase protein under the α -CaMKII promoter, restricting Cre recombinase expression to adult forebrain neurons (Dragatsis and Zeitlin, 2000).

We crossed homozygous Cdk5 floxed and homozygous knock-in Hdh^{Q111/Q111} mice to obtain heterozygous Cdk5 floxed and heterozygous knock-in mice (Cdk5^{flox/+}; Hdh^{Q7/Q111} mice). These mice were crossed again with homozygous Cdk5 floxed mice to obtain homozygous Cdk5 floxed and heterozygous knock-in mice (Cdk5^{flox/flox}; Hdh^{Q7/Q111} mice). We crossed homozygous Cdk5 floxed and heterozygous knock-in mice with Cre-expressing mice to obtain the final genotypes: Cdk5^{flox/+}, Hdh^{Q7/Q7}, Cre^{-/-} (Hdh^{Q7/Q7};Cdk5^{+/+} or wild-type (WT)), Cdk5^{flox/+}, Hdh^{Q7/Q111}, Cre^{-/-} (Hdh^{Q7/Q111};Cdk5^{+/+} or mHtt knock-in (KI)), Cdk5^{flox/+}, Hdh^{Q7/Q7}, Cre^{+/-} (Hdh^{Q7/Q7};Cdk5^{+/-} or Cdk5 heterozygous (Cdk5^{+/-})), Cdk5^{flox/+}, Hdh^{Q7/Q111}, Cre^{+/-} (Hdh^{Q7/Q111};Cdk5^{+/-} or double mutant (KI:Cdk5^{+/-})). All mice were maintained on a C57BL/6 genetic background.

METHODS

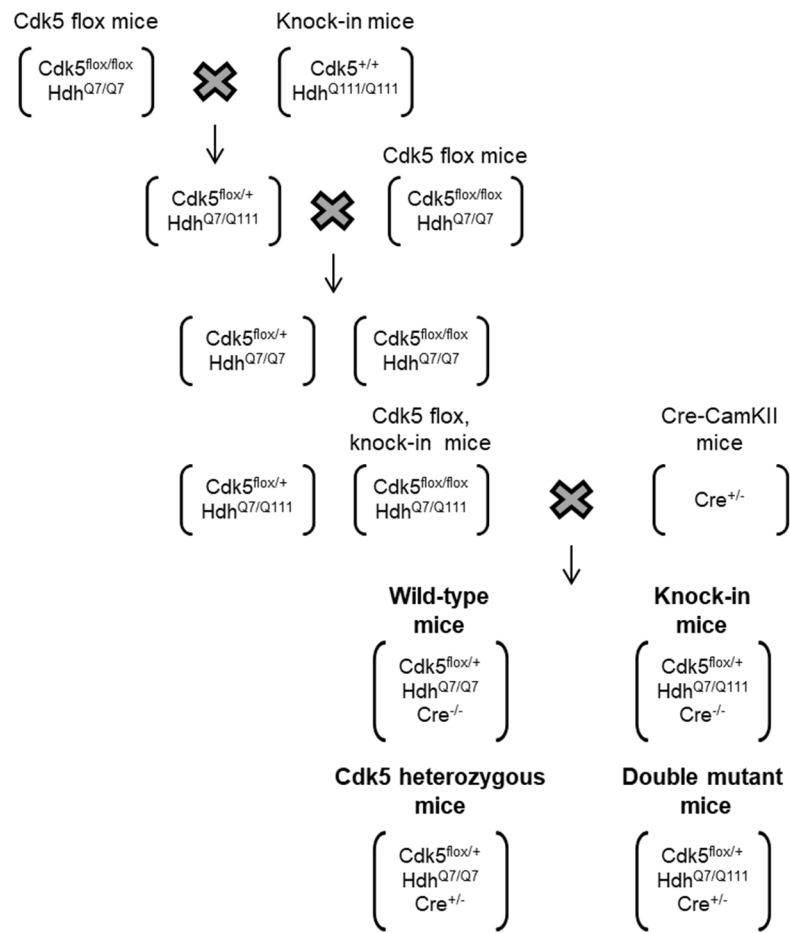


Figure 15. Generation of double mutant KI:Cdk5^{+/-} mice. The stated crossing were performed to obtained the final four used genotypes: wild-type (WT), knock-in (KI), Cdk5^{+/-} and double mutant KI:Cdk5^{+/-} mice.

2. HUMAN SAMPLES

Putamen samples from HD patients and control individuals were obtained from the Neurological Tissue Bank of the Biobank-Hospital Clínic-Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS) following the guidelines and approval of the local ethics committee (Hospital Clínic de Barcelona's Clinical Research Ethics Committee). Details from control and HD patients are provided in Table 5.

Registration number	Clinical diagnosis	Sex	Age
CS-0810	Control	Female	81
CS-1491	Control	Male	83
CS-1563	Control	Male	79
CS-1570	Control	Female	86
CS-1679	Control	Female	90
CS-1697	Control	Male	78
CS-1733	Control	Male	76
CS-1774	Control	Female	74

CS-1334	HD - VS 1, 40 CAG repeats	Male	73
CS-1630	HD - VS 2, 20/40 CAG repeats	Male	76
CS-1638	HD - VS 2	Male	72
CS-1758	HD - VS 2-3, 42 CAG repeats	Male	68
CS-1120	HD - VS 3, 48 CAG repeats	Male	55
CS-1294	HD - VS 3, 45 CAG repeats	Male	53
CS-1438	HD - VS 3, 40 CAG repeats	Male	85
CS-1193	HD - VS 3-4	Male	55

Table 5. Human post-mortem brain samples. Information relative to human control and HD patients brain samples indicating sex, age, Vonsattel grades and number of CAG repeats when available.

3. STRIATAL PRIMARY CULTURES

Striatums from E18 WT and mHtt KI mice embryos were dissected and mechanically dissociated. Densities of 160,000 cells and 500,000 cells were seeded onto 12 mm coverslips in 24-well plates for immunocytochemistry, and onto 12-well plates for Western blot analysis, respectively. Plates were pre-coated with 0.1 mg/mL poly-D-lysine (P0899, Sigma) and dissociated cells were cultured with Neurobasal Medium (21103-049, Gibco) supplemented with 1% Glutamax (35050-038, Gibco) and 2% B27 (17504-044, Gibco) for 21 days *in vitro* (DIV). Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂. Embryos genotype was determined by PCR analysis.

When stated, 21 DIV cultures were treated with NMDA (M3262, Sigma) at 10µM, 50µM or 100µM for 1h, 3h or 24h as specified in each experiment. To inhibit calpain activity, cultures were pre-treated with 10µM ALLN (Calbiochem) for 1 hour prior to NMDA treatment. Cultures were fixed or harvested immediately after treatment.

4. BEHAVIOURAL ASSESSMENT

Separate cohorts of 6-month old WT, mHtt KI, Cdk5^{+/-} and mHtt KI:Cdk5^{+/-} mice, were used to evaluate **corticostriatal** function using the accelerating rotarod training procedure (ARTP) and the swimming T-Maze of strategy shifting and **hippocampal**-dependent memory using the T-maze spontaneous alternation task (T-SAT) and the novel object recognition test (NORT), respectively. To reduce the effect of stress and anxiety on subsequent tasks, the least stressful and anxiety-inducing tasks were performed first as indicated: ARTP, T-SAT, NORT, swimming T-Maze of strategy shifting. All experiments were performed blind to the experimental condition.

4.1. Corticostriatal-dependent tasks

METHODS

The **ARTP** was performed as previously described (Puigdemívol *et al*, 2015) (Figure 16A). Briefly, animals were placed on a motorized rod (30 mm diameter). The rotation speed increased gradually from 4 to 40 rpm over the course of 5 min. Latency refers to the time the animal was able to keep up with increasing speed before falling. Rotarod training/testing was performed 4 times per day for 3 consecutive days.

The **swimming T-Maze test of strategy shifting** was performed as previously described (Puigdemívol *et al*, 2015) (Figure 16B). Briefly, a T-maze apparatus consisting of a glass maze with three arms, two situated at 180° from each other, and the third representing the stem arm of the T, at 90° respect to the other two, was used. All arms were 45 cm long, 8 cm wide and enclosed by a transparent 20 cm wall. Mice were trained to swim from the stem arm of the T to the other arms. In one of them, there was a transparent escape platform, covered by water. During the acquisition phase (two consecutive days: first day: 8 trials; second day: 4 trials), each mouse was placed in the water, allowed to swim until they reached the platform and the latency and errors (1: error, 0: non-error) to reach the platform were recorded. Mice had to be able to find the platform and learn the strategy faster across trials. After the acquisition phase, location of the platform was changed to the opposite arm. During the reversal phase of (two consecutive days: second day: 4 trials; third day: 8 trials) each mouse was placed in the water and the latency and errors to reach the platform were recorded.

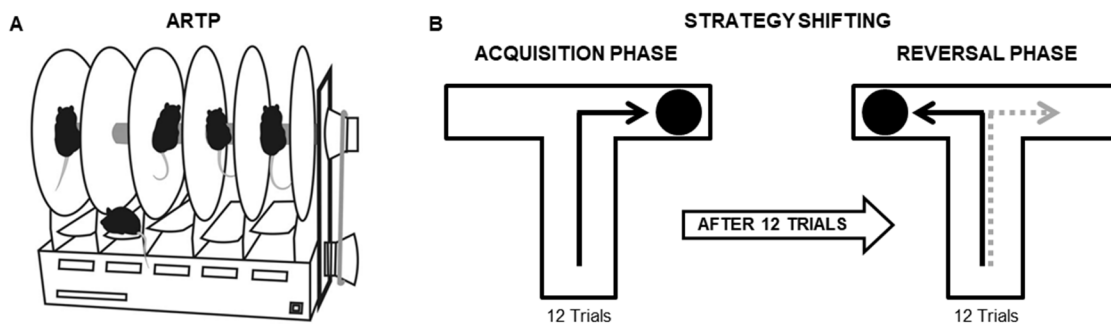


Figure 16. Schematic representations of the accelerating rotarod (ARTP) (from (Madróñal *et al*, 2010)) and the acquisition and reversal phases of the Strategy shifting test.

4.2. Hippocampal-dependent tasks

The **T-SAT** was performed as previously described (Brito *et al*, 2014) (Figure 17A). Briefly, a T-maze apparatus consisting of a wooden maze with three arms, two of them situated at 180° from each other, and the third, representing the stem arm of the T, situated at 90° respect to the other, was used. All arms were 45 cm long, 8 cm wide and enclosed by a 20 cm wall. Two identical guillotine doors were placed at the entry of the arms situated at 180°. During 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 5 h (long-term memory), mice were placed in the stem arm of the T-maze and allowed to freely explore all three arms for 5 min. The arm preference was measured as the percentage of time exploring the novel arm.

The **NORT** was performed as previously described (Brito *et al*, 2014) (Figure 17B). Briefly, a 40 cm diameter and 40 cm high white circular arena, was used. First, mice were habituated to the arena in the absence of objects (2 days, 10 min/day). On the third day, two similar objects were presented to each mouse for 10 min. Later, they were returned to their home cage. 24 hours later, the same animals were re-tested for 5 min in the arena with a familiar and a new object. The object preference was measured as the percentage of time exploring the new object.

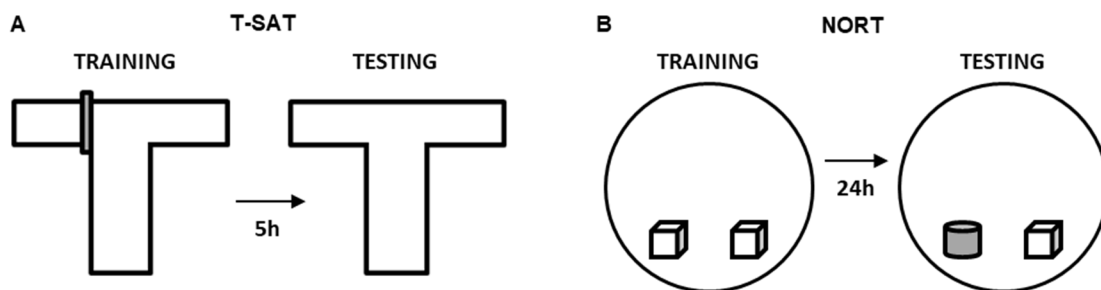


Figure 17. Schematic representations of the T-maze spontaneous alternation task (T-SAT) and the novel object recognition test (NORT).

5. PROTEIN EXTRACTION

5.1. From mice and human brain tissue

WT, mHtt KI, Cdk5^{+/-} and mHtt KI:Cdk5^{+/-} mice were killed by cervical dislocation at the stated age in each experiment. Brains were quickly removed, desired regions were dissected, frozen in dry ice and stored at -80°C until use.

In Aim 1, same sets of samples were used for Western blot analysis and immunoprecipitation studies simultaneously. To this end, brain tissue was homogenized in cold lysis buffer (140 mM NaCl, 10 mM HEPES (pH 7.4), 1 mM EGTA, 0.1 mM MgCl₂, 1% X-100 Triton, 0.5% NP-40 supplemented with 1 mM sodium orthovanadate and protease inhibitor cocktail (Sigma-Aldrich)) with a dounce homogenizer, left 1 hour at 4°C in ice and cleared by centrifugation at 13,000g for 15 min at 4°C to collect the supernatants.

In Aim 2, different sets of samples were used for Western blot analysis and immunoprecipitation studies. Therefore, brain tissue used solely for Western blot studies, was sonicated in cold lysis buffer (150 mM NaCl, 20 mM Tris, 50 mM NaF, 1% NP-40, 10%

METHODS

glycerol supplemented with 1 mM sodium orthovanadate and protease inhibitor cocktail (Sigma-Aldrich)) and centrifuged at 16,000g for 15 minutes at 4°C to collect the supernatants.

5.2. From primary cultures

Striatal primary cultures grown in 12-well plates at DIV21 were rinsed with PBS, harvested in Lysis Buffer (50 mM Tris-HCl (pH = 7.5), 150 mM NaCl, 2 mM EDTA, 1% NP-40 supplemented with 1 mM sodium orthovanadate and protease inhibitor cocktail (Sigma-Aldrich)) and centrifuged at 16,100g, for 10 minutes at 4°C to collect the supernatants.

5.3. Protein quantification

Protein concentration was assessed with a colorimetric assay following detergent solubilization similar to the Lowry assay (Lowry *et al*, 1951) using the Detergent-Compatible Protein Assay kit (Bio-Rad Laboratories) as indicated by the manufacturer's instructions. 15 minutes after adding the provided reagents to the samples, absorbance at 650-750 nm was measured in a Synergy 2™ Multi-Mode Multiplate Reader (BioTek). A standard curve obtained from a 16 µg/µL BSA (bovine serum albumin) stock was used as reference, duplicates were analysed per each sample and only samples with a standard deviation lower than 0.5 were used for further experiments.

6. WESTERN BLOTTING

Protein extracts (10-20 µg) were mixed with 5X SDS sample buffer, boiled for 5 min, resolved on 6-10% SDS-PAGE and transferred to nitrocellulose membranes (Whatman Schleicher & Schuell). After 1h incubation in blocking buffer containing 10% non-fat powdered milk in TBS-T (50 mM Tris-HCl, 150 mM NaCl, pH 7.4, 0.05% Tween 20), membranes were blotted overnight at 4°C with the indicated primary antibodies:

Antigen	Dilution	Molecular Weight (kDa)	Source	Reference
Cdk5 (J-3)	1:1000	30	Santa Cruz Biotechnologies	sc-6247
p35 (C-19)	1:500	35	Santa Cruz Biotechnologies	sc-820
NR1	1:1000	150	Chemicon	MAB363
NR2A	1:1000	180	Millipore	AB1555
NR2B	1:1000	180	Cell Signaling Technology	4207
µ-calpain	1:1000	80	Sigma	C5736
pNR2B (S1116)		180	James A. Bibb Lab	
pSrc (Y416)	1:1000	60	Cell Signaling Technology	2101

Src	1:2000	60	Cell Signaling Technology	2110
pNR2B (Y1472)	1:1000	180	Cell Signaling Technology	4208
Rac1	1:2000	20	BD Biosciences	612652
(PHF)-1	1:1000	50	Peter Davis Lab	
pCDK substrates	1:2000	50-250	Cell Signaling Technology	9477
pIPP1 (S67)	1:500	19	Santa Cruz Biotechnologies	sc-14268
Lamin B1	1:1000	67	Abcam	
RhoGDIα	1:1000	30	Santa Cruz Biotechnologies	sc-359
p27	1:1000	27	BD Biosciences	610241
E2F1	1:500	60	Santa Cruz Biotechnologies	sc-251
Cyclin D1	1:1000	30	Abcam	ab134175
Cdk4	1:1000	30	Santa Cruz Biotechnologies	sc-260
spectrin	1:2000	250/150	Chemicon	MAB1622
Cleaved caspase 3	1:1000	17	Cell Signaling Technology	9661

Table 6. Primary antibodies used for Western blot. Dilution, molecular weight, source and reference for each used primary antibody are indicated.

Membranes were then rinsed three times with TBS-T and incubated with horseradish peroxidase-conjugated mouse or rabbit secondary antibody (1:3000; Promega) for 1h at room temperature. After washing for 30 min with TBS-T, membranes were developed using the enhanced chemiluminescence ECL kit (Santa Cruz Biotechnology).

Loading control was performed by reprobing the membranes with the following antibodies depending on the sample being analysed:

Antigen	Sample	Molecular Weight (kDa)	Source	Reference
α-actin	Total lysate and cytosolic fraction	42	MP Biomedicals	69100
Transferrin receptor	Plasma membrane fraction	95	Invitrogen	13-6800
Lamin A/C	Nuclear fraction	50-60	Santa Cruz Biotechnologies	sc-20681

Table 7. Primary antibodies used as loading control. Sample, molecular weight, source and reference for each used primary antibody are indicated.

ImageJ software was used to quantify the different densitometry immunoreactive bands relative to the intensity of the loading control band in the same membranes within a linear range of detection for the ECL reagent. Data is expressed as the mean \pm SEM of band density.

7. SUBCELLULAR FRACTIONATION

Subcellular fractionation was performed based on (Baghirova *et al*, 2015; Benito *et al*, 2015; Giralt *et al*, 2013). Dissected striatums, cerebral cortex or hippocampus were homogenized in Buffer A (0.32 M sucrose, 10 mM HEPES pH=7.4 and 5 mM MgCl₂) by repetitive pipetting and by passing through syringe and needle. At this point 30 μ L were obtained as a total lysate fraction. Remaining sample was centrifuged for 10 min, 800g, 4°C. On one hand, supernatant was further centrifuged for 15 min, 10000g, 4°C. The newly obtained supernatant was ultracentrifuged in a Sorvall MX150 ultracentrifuge using the fix rotor S140-AT for 2 h, 200,000g, 4°C. The resulting supernatant represents the **cytosolic fraction**. The resulting pellet was resuspended in 40 μ L Lysis Buffer (150 mM NaCl, 20 mM Tris pH=8.0, 50 mM NaF, 1% NP-40, 10% Glycerol), sonicated and further centrifuged for 15 min, 16,100g, 4°C to obtain a new supernatant corresponding to the **plasma membrane fraction**. When nuclear fractions were also desired, the first obtained pellet was further processed by resuspending it in 100 μ L Low Sucrose Buffer (0.32 M sucrose, 5 mM Mg(Ac)₂, 0.1 mM EDTA, 50 mM HEPES pH=8.0, 1% IGEPAL), mechanically homogenizing it and centrifuging it for 10 min, 4000g, 4°C. The resulting pellet was newly resuspended in 150 μ L Low Sucrose Buffer, layered on top of 350 μ L High Sucrose Buffer (1 M sucrose, 3 mM Mg(Ac)₂, 10 mM HEPES pH=8.0) and centrifuged for 10 min, 4000g, 4°C. The resulting pellet was resuspended in 50 μ L Lysis Buffer, sonicated and further centrifuged for 15 min, 16,100g, 4°C to obtain the **nuclear fraction**. Proper purification of each sample was assessed by specific presence of the following markers: Transferrin receptor for plasma membrane fraction, Lamin B1 for nuclear fraction and RhoGDI for cytosolic fraction (Figure 18).

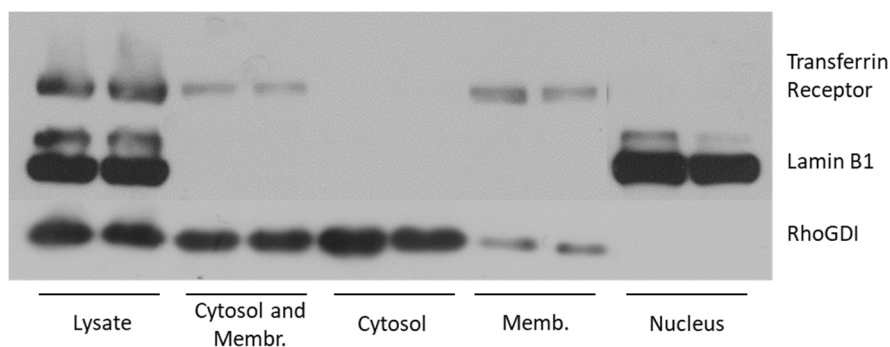


Figure 18. Distribution of subcellular markers in each obtained fraction. Representative bands for each subcellular fraction showing proper distribution of each fraction markers.

8. CO-IMMUNOPRECIPITATIONS

8.1. Calpain immunoprecipitation

300 μ g protein from cortical lysates of WT and mHtt KI mice, obtained as previously described, were incubated in 300 μ L IP Buffer (50 mM Tris-HCl pH=8.0, 150 mM NaCl, 1% IGEPAL, 1 % NP-40, 2 mM EDTA supplemented with 1 mM sodium orthovanadate and

protease inhibitor cocktail (Sigma-Aldrich)) with 5 μ L of calpain primary antibody or anti-mouse IgG (Jackson ImmunoResearch) for the negative control, overnight at 4°C with rotation. The following day 40 μ L protein A-sepharose (sc-2001, Santa Cruz Biotechnology) were added in each tube and further incubated overnight at 4°C with rotation. Samples were centrifuged for 5 minutes, at 1500g at 4°C and rinsed three times in decreasing dilutions of IP Buffer in PBS. Finally, beads were incubated in 10 μ L SDS loading buffer with 10% β -mercaptoethanol, boiled at 100°C for 10 minutes, spun down and analysed by Western blotting as previously described. HRP-conjugated protein A/G (32490, Thermo) was used to detect the primary antibodies avoiding IgG light and heavy chains detection.

8.2. Cdk5 immunoprecipitation

200 μ g protein from striatal lysates from WT and mHtt KI mice, obtained as previously described, were incubated in 300 μ L IP Lysis Buffer (140 mM NaCl, 10 mM HEPES (pH 7.4), 1 mM EGTA, 0.1 mM MgCl₂, 1% X-100 Triton, 0.5% NP-40) with 2 μ L agarose-conjugated Cdk5 primary antibody (sc-173, Santa Cruz Biotechnology) or with 2 μ L protein A-sepharose (sc-2001, Santa Cruz Biotechnology) for negative controls, overnight at 4°C with rotation. Samples were centrifuged for 5 minutes, at 1500g at 4°C and rinsed three times in decreasing dilutions of IP Lysis Buffer in PBS. Finally, beads were incubated in 10 μ L SDS loading buffer with 10% β -mercaptoethanol, boiled at 100°C for 10 minutes, spun down and analysed by Western blotting as previously described.

9. RAC1 ACTIVITY ASSAY

Rac1 activity assay was performed as previously described (Puigdemívol *et al*, 2015). Briefly, hippocampus and cortex from WT, mHtt KI, Cdk5^{+/-} and mHtt KI:Cdk5^{+/-} mice were lysed in Pull-down lysis buffer (25 mM HEPES, pH=7.5, 150 mM NaCl, 10 mM MgCl₂, 1 mM EDTA, 1% NP-40, 2% Glycerol) and centrifuged for 15 minutes at 16,000 g at 4°C. Cleared lysates were quantified and 600 μ g of total protein were incubated with 10 μ g PAK-GST protein beads (Cytoskeleton Inc.) for 1 hour at 4°C in rotation. Then, PAK-GST beads were pelleted by centrifugation at 5000 g, for 1 minute at 4°C, washed 3 times with Pull-down lysis buffer, re-suspended with 5X SDS sample buffer with β -mercaptoethanol and boiled for 5 minutes. The resulting samples were loaded to perform Western blotting analysis as previously described and levels of GTP-bound Rac1 were determined. Controls were incubated with 10 μ M EDTA and 200 μ M GTP γ S (positive control) or 1 mM GDP (negative control) at 30°C and the reaction was stopped with 60 mM MgCl₂ after 30 minutes.

10. BIOTINYLATION ASSAY

Biotinylation assay was performed as previously described (Gabriel *et al*, 2014). Briefly, WT, mHtt KI, Cdk5^{+/-} and mHtt KI:Cdk5^{+/-} mice were killed by cervical dislocation at 8 months of age. Brains were quickly removed, placed in oxygenated ice-cold ACSF buffer (125 mM NaCl, 2.5 mM KCl, 1.2 mM NaH₂PO₄, 1.2 mM MgCl₂, 2.4 mM CaCl₂, 26 mM NaHCO₃ and 11 mM glucose) and processed in a vibratome to obtain 400 μm brain slices. The slices were incubated for 30 minutes with 0.5 mg/ml sulfo-NHS-SS-Biotin (Sigma), rinsed with ice-cold ACSF and with quench buffer (ACSF with 100mM glycine) 3-6 times. Then, slices were incubated with quench buffer for 20 minutes and rinsed 3 times with ACSF buffer. Striatum, cerebral cortex and hippocampus from each slice were dissected, and lysed with lysis buffer (150 mM NaCl, 20 mM Tris (pH = 8.0), 50 mM NaF, 1% NP-40, 10% glycerol supplemented with 1 mM sodium orthovanadate and protease inhibitor cocktail (Sigma-Aldrich)). Samples were centrifuged for 15 minutes, at 16000g at 4°C and supernatants were collected. After protein quantification, 150 μg of protein were incubated in Binding Buffer (100 mM K₂PO₄, 150 mM NaCl and 1% NP-40 (pH=7.2)) with 40 μL of NeutrAvidin beads (Thermo Scientific) at 4°C in overnight rotation. Then, samples were centrifuged 2 minutes at 2500g at 4°C and rinsed 4 times with decreasing amounts of Binding Buffer in PBS. Finally, pellets were re-suspended with 5X SDS sample buffer with β-mercaptoethanol and boiled for 10 minutes. Biotinylated samples were loaded to perform Western blotting analysis as previously described and NR2B surface levels were determined.

11. IMMUNOCYTOCHEMISTRY

Striatal primary cultures were grown in coverslips and fixed at DIV21 with 4% paraformaldehyde (PFA) for 10 minutes, rinsed with PBS and incubated with 0.1 M Glycine for 15 minutes. Coverslips were rinsed 3 times in PBS, incubated in blocking solution (0.3% Triton X-100, 1% BSA and 1% Normal Donkey Serum in PBS) for 45 minutes at room temperature and incubated overnight at 4°C with blocking solution containing the corresponding primary antibodies:

Antigen	Dilution	Host Species	Source	Reference
p27	1:500	mouse	BD Biosciences	610241
E2F1	1:100	mouse	Santa Cruz Biotechnologies	sc-251
Cdk4	1:100	rabbit	Santa Cruz Biotechnologies	sc-260
CyclinD1	1:100	rabbit	Abcam	ab134175

NeuN	1:500	rabbit	Cell Signaling Technology	24307
NeuN	1:500	mouse	Chemicon	MAB377

Table 8. Primary antibodies used for immunocytochemistry. Dilution, host species, source and reference for each used primary antibody are indicated.

Then, coverslips were rinsed 3 times in PBS and incubated for 2 hours at room temperature with blocking solution containing the corresponding secondary antibodies:

Antigen	Dilution	Source	Reference
CyTM3 AffiniPure Donkey Anti-Rabbit IgG	1:200	Jackson ImmunoResearch	711-165-152
CyTM3 AffiniPure Donkey Anti-Mouse IgG	1:200	Jackson ImmunoResearch	715-165-150
Alexa Fluor[®] 488 AffiniPure Donkey Anti-Rabbit IgG	1:200	Jackson ImmunoResearch	711-545-152
Alexa Fluor[®] 488 AffiniPure Donkey Anti-Mouse IgG	1:200	Jackson ImmunoResearch	715-545-150

Table 9. Secondary antibodies used for immunocytochemistry. Dilution, source and reference for each used secondary antibody are indicated.

Coverslips were rinsed 3 times in PBS, mounted using DAPI Fluoromount-G[®] (0100-20, Southern Biotech), which contains the fluorescent DNA-binding DAPI, and left to dry.

12. FLUORESCENCE MICROSCOPY ANALYSIS

After immunocytochemistry, 10-15 images per coverslip were obtained in an optical microscope (Olympus BX60 Microscope) equipped with an ORCA-ER digital camera (Hamamatsu), using the HoKaWO (Hamamatsu) image acquisition software. Images were obtained randomly using a 20X objective and maintaining the same acquisition settings for each channel. Analysis of these images was performed using the free open-source CellProfiler 2.0.0 software (<http://cellprofiler.org/>). Using the “IdentifyPrimaryObjects” function, the number of DAPIs, NeuN, p27, Cdk4, CyclinD1 and E2F1 positive cells were quantified. Specific parameters such as diameter, threshold correction factor or method to distinguish clumped objects, were corrected according to different staining patterns of each protein, in order to minimize false positive counting. Finally, using the “MaskObjects” function, colocalization of DAPI, NeuN and each analysed cell cycle protein was determined according to a minimum overlapping region between channels (Figure 19).

METHODS

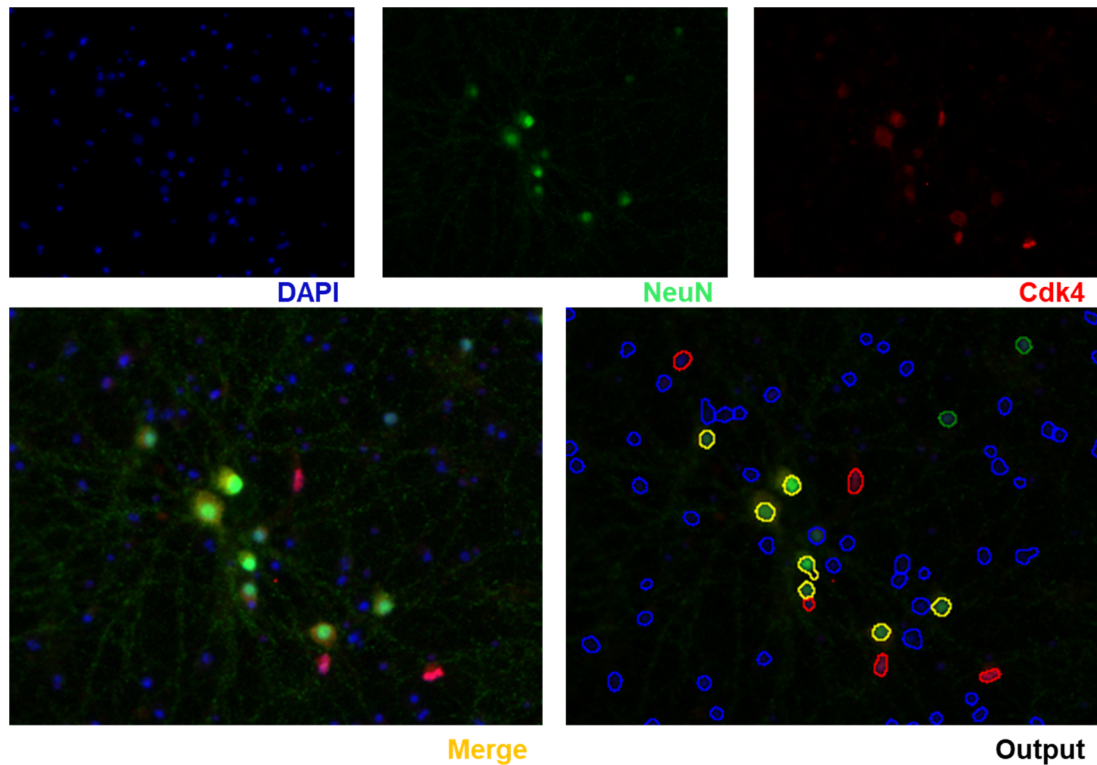


Figure 19. Quantification of striatal primary cultures using Cell Profiler software. Representative images of nuclei (DAPI), NeuN and Cdk4 positive cells and their colocalization. Output image represents Cell Profiler quantification of DAPI (blue circles), DAPI and NeuN colocalization (green circles), DAPI and Cdk4 colocalization (red circles) and DAPI, NeuN and Cdk4 colocalization (yellow circles).

13. GOLGI STAINING AND CONFOCAL ANALYSIS

We performed the Golgi-Cox impregnation using the Rapid Golgistain Kit (FD Neurotechnologies) following the manufacturer's instructions. Briefly, WT, mHtt KI, Cdk5^{+/-} and mHtt KI:Cdk5^{+/-} mice were killed by cervical dislocation at the age of 7-8 months of age, their brains were quickly removed, briefly rinsed and both hemispheres were separated and incubated in a mix of solution A/B for 2 weeks. Then, solution A/B was changed to solution C and after 3 days, brains were embedded in 4% agarose to be processed using a vibratome to obtain 100 μm sections that were mounted in gelatine-coated slides and left to dry for 24 hours. Then sections were stained with the kit's provided solutions and finally sections were dehydrated and mounted with DPX. For dendritic spine analysis, we obtained bright field images with the differential interference contrast (DIC) technique using a Leica SP5 laser scanning confocal microscope (Leica) with a x63 numerical aperture objective. Conditions such as pinhole size (1 AU) and frame averaging (four frames per z-step) were held constant throughout the study. Confocal z-stacks were taken with a digital zoom of 5, a z-step of 0.5 μm and 1024 \times 1024 pixel resolution, yielding an image with pixel dimensions of 49.25 \times 49.25 μm . Segments of apical dendrites from hippocampal CA1 and cortical layer

V pyramidal neurons with no overlap with other branches were selected for analysis of spine density. Distal spines to the soma of the neuron were included in the study, no distinction between dendritic spine types was made. Spine density was measured manually in the stacks using the ImageJ Plugin Cell Counter. Spines were marked in the appropriate focal plane preventing any double counting of spines. Spines were counted in dendritic segments range from 15 to 40 μm of length. Z-Stacks were deconvolved using the ImageJ Parallel Iterative Deconvolution plugin to improve voxel resolution and reduce optical aberration along the z-axis.

14. STATISTICAL ANALYSIS

All data are expressed as mean \pm S.E.M. Statistical analysis was performed by using the unpaired Student's t-test (95% confidence), Wald's test, One-way ANOVA, Two-way ANOVA, and the appropriate post-hoc tests as indicated in the figure legends. Values of $p < 0.05$ were considered as statistically significant.

RESULTS

1. ROLE OF CDK5 IN COGNITIVE DEFICITS APPEARANCE IN HD

Cdk5 is an important modulator of synaptic plasticity, learning and memory processes, and accordingly, reduction or inhibition of either Cdk5 or its activator p35 impacts these crucial functions (Angelo *et al*, 2006; Barnett and Bibb, 2011; Lai and Ip, 2009). In agreement, in neurodegenerative diseases, such as AD, deregulation of Cdk5 function contributes to several neuropathological hallmarks, while its inhibition improves cognitive deficits in AD mice models (Castro-Alvarez *et al*, 2014; Lopes and Agostinho, 2011; McLinden, 2012). Cognitive impairment is an important feature of Huntington's disease, as it deeply affects the patient's quality of life. Surprisingly, although Cdk5 has been shown to be altered in HD, no studies have analysed its involvement in HD cognitive disturbances. For this reason, we have generated new transgenic mice expressing mutant huntingtin but with reduced Cdk5 expression, in order to analyse their cognitive performance and to determine Cdk5 role in HD synaptic plasticity alterations.

1.1 GENERATION AND VALIDATION OF A NEW TRANSGENIC MOUSE MODEL: mHTT KI:CDK5^{+/-} MICE

Double mutant mHtt KI:Cdk5^{+/-} mice express one copy of mHtt with 111Q in the murine *Htt* gene and are conditionally heterozygous for Cdk5 (Figure 15). This is achieved by means of the Cre recombinase system. Cdk5 heterozygous mice have loxP sequences in its catalytic domain and express Cre recombinase under regulation of the α CaMKII promoter, thus restricting its expression mainly to postnatal forebrain neurons (Dragatsis and Zeitlin, 2000).

In order to validate this new transgenic mice, we analysed total protein levels of Cdk5 in the cortex, striatum and hippocampus of WT, mHtt KI, Cdk5^{+/-} and mHtt KI:Cdk5^{+/-} mice. Our results showed that Cdk5^{+/-} and mHtt KI:Cdk5^{+/-} mice presented approximately 50% reduction of Cdk5 levels in all analysed brain regions (Fig. 20A). Importantly, no alterations between genotypes were observed in the cerebellum, where α CaMKII has especially low expression (Fig. 20A). Given that Cdk5 is able to regulate degradation of its own activator p35, we also analysed p35 protein levels. No significant changes were observed between genotypes in any of the analysed brain regions, thus indicating that 50% reduction of Cdk5 expression does not have a major effect in p35 levels (Fig. 20B). These results validate our new generated mouse model to study the role of Cdk5 in knock-in HD mice.

RESULTS

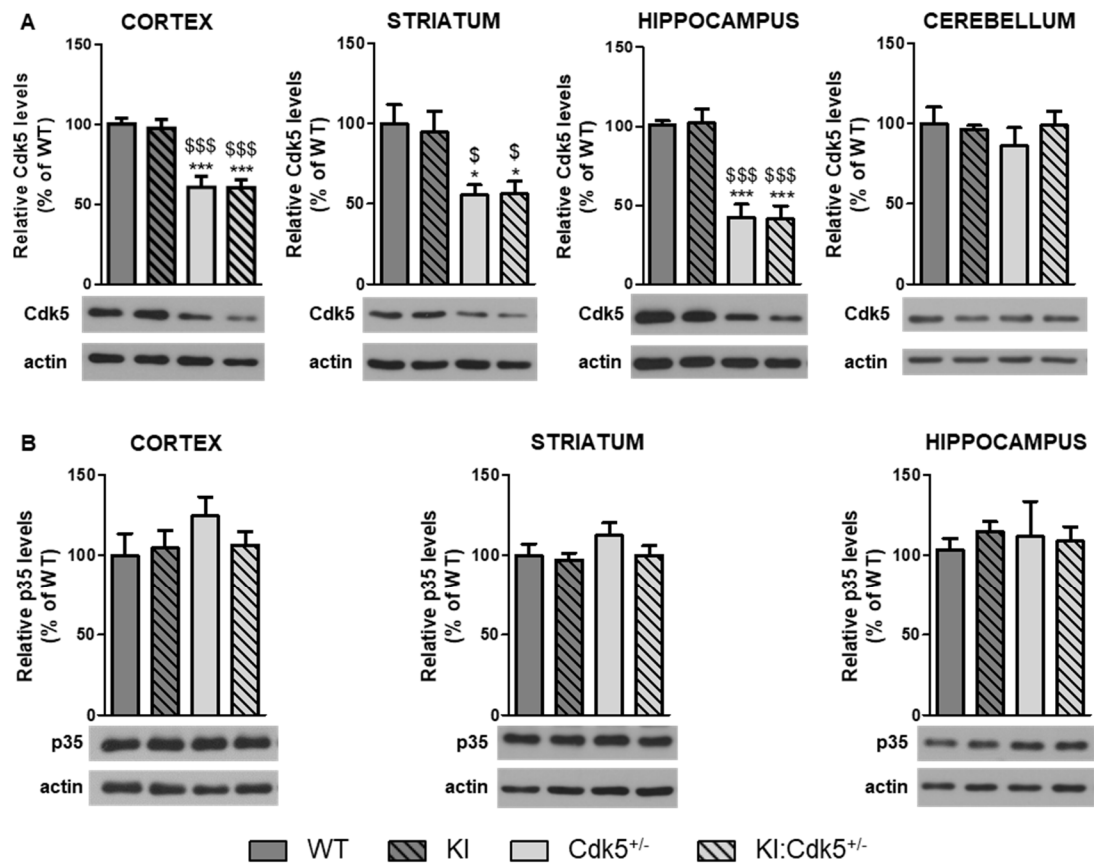


Fig. 20. Total Cdk5 levels are significantly reduced in the cortex, striatum and hippocampus of Cdk5^{+/-} and KI:Cdk5^{+/-} mice. Representative Western blots from cortical, striatal, hippocampal and cerebellar lysates probed for Cdk5 (A) and p35 (B) in WT ($n = 3-7$), mHtt KI ($n = 3-8$), Cdk5^{+/-} ($n = 3-7$) and mHtt KI:Cdk5^{+/-} ($n = 3-7$) mice. Histograms represent the mean \pm SEM and are normalized to WT values. Statistical analysis was performed using One-way ANOVA with Bonferroni post hoc comparisons. * $p < 0.05$, *** $p < 0.001$ compared to WT mice. \$ $p < 0.05$, \$\$\$ $p < 0.001$ compared to mHtt KI mice.

1.2 BEHAVIOURAL ASSESSMENT OF mHTT KI:CDK5^{+/-} MICE

In order to analyse Cdk5 contribution to the appearance of HD cognitive deficits, we analysed corticostriatal and hippocampal-dependent behavioural tasks in 6 months old WT, mHtt KI, Cdk5^{+/-} and KI:Cdk5^{+/-} mice, an age in which previous studies from our group have shown that mHtt KI mice exhibit cognitive decline without presenting motor coordination alterations (Brito *et al*, 2014; Puigdel·l·ivol *et al*, 2015).

1.2.1 Cdk5 genetic reduction prevents corticostriatal deficits in mHtt KI mice

Motor learning and procedural memory were evaluated as indicators of corticostriatal cognitive function, using the accelerating rotarod (ARTP) and the swimming T-maze test of strategy shifting, respectively. In the ARTP, all genotypes were able to learn the rotarod task as evidenced by an increased latency to fall across trials (Fig. 21A). However, mHtt KI mice

exhibited a significant reduction in the latency to fall compared to WT and $Cdk5^{+/-}$ mice, denoting an impairment in motor skill learning. In contrast, mHtt KI: $Cdk5^{+/-}$ mice showed a comparable performance to WT mice, indicating that reduced $Cdk5$ expression can counteract mHtt-induced deficits. To exclude implication of motor coordination deficits in ARTP impairment in mHtt KI mice, we analysed spontaneous locomotor activity in the open field. No differences in the distance travelled were observed between genotypes (Fig. 21B).

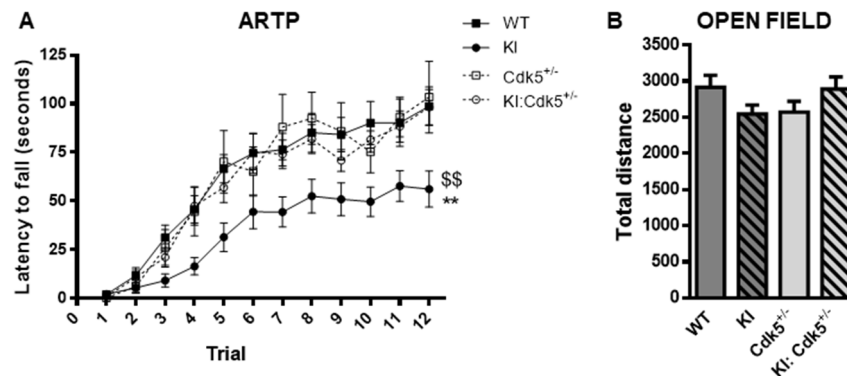


Figure 21. Motor learning deficits in mHtt KI mice are ameliorated by genetic reduction of $Cdk5$ expression. **A.** Latency to fall in the accelerating rotarod task for WT ($n = 17$), mHtt KI ($n = 26$), $Cdk5^{+/-}$ ($n = 9$) and mHtt KI: $Cdk5^{+/-}$ ($n = 12$) mice. Data represents the mean \pm SEM. Statistical analysis was performed using Two-way ANOVA with repeated measures. ** $p < 0.01$ compared to WT mice and \$\$ $p < 0.01$ compared to mHtt KI mice. **B.** Distance travelled in the open field arena during 15 minutes for WT ($n = 12$), mHtt KI ($n = 16$), $Cdk5^{+/-}$ ($n = 8$) and mHtt KI: $Cdk5^{+/-}$ ($n = 12$) mice. Histograms represent the mean \pm SEM. Statistical analysis was performed using One-way ANOVA.

Next, procedural memory was analysed using the Swimming T-Maze test. During the acquisition phase the escape platform was localized in the T-maze right arm. Latency to reach the platform and number of “errant” trials were scored (Fig. 22A, C). All genotypes showed a similar decrease in the latency to reach the platform after the first trial and a constant level of performance (~ 10 s to reach the platform; Fig. 22A) by the last trial. Next, the ability to change strategy was assessed in the reversal phase, by switching the platform from the T-maze right arm to the left one. Latency to reach the platform was significantly higher in mHtt KI mice compared to WT mice (Fig. 22B) and correlated with increased number of errant trials (Fig. 22D). Similar to the ARTP results, mHtt KI: $Cdk5^{+/-}$ mice showed a similar performance in the reversal phase of the swimming T-Maze test compared to WT mice (Fig. 22B, D), indicating preserved procedural learning function. Together, these data demonstrate that genetic reduction of $Cdk5$ expression in mHtt KI mice improves corticostriatal-dependent learning deficits.

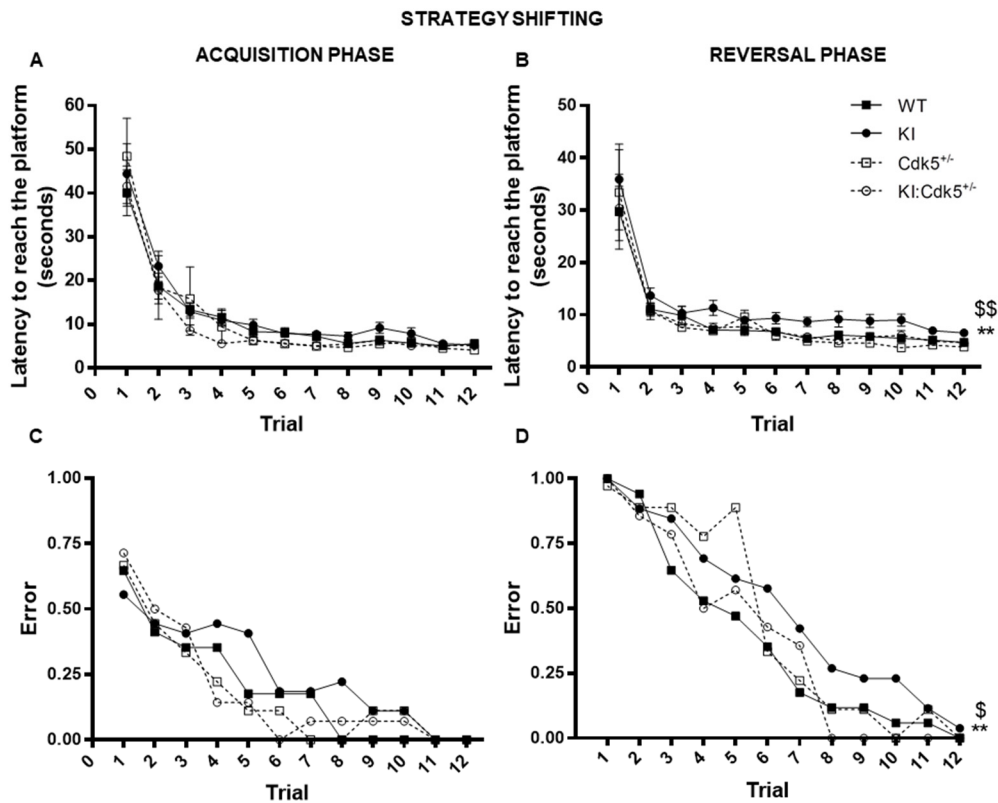


Figure 22. Procedural memory impairments in mHtt KI mice are ameliorated by genetic reduction of Cdk5 expression. Latency to reach the platform (A and B) and error trials (C and D) in the Swimming T-maze test of Strategy shifting in WT ($n = 17$), mHtt KI ($n = 27$), Cdk5^{+/-} ($n = 9$) and mHtt KI:Cdk5^{+/-} ($n = 14$) mice during the acquisition phase (A and C) and the reversal phase (B and D). Data represent the mean \pm SEM. Statistical analysis was performed using Two-way ANOVA with repeated measures to analyse the latency to reach the platform. Logistic regression analysis using the Wald statistical test from IBM SPSS Statistics was used to analyse the error probability to reach the platform in the correct arm. ** $p < 0.01$ compared to WT mice. \$ $p < 0.05$, \$\$ $p < 0.01$ compared to mHtt KI mice.

1.2.2 Reduction of Cdk5 rescues hippocampal memory deficits on mHtt KI mice

To determine Cdk5 role in hippocampal-dependent deficits, spatial and recognition memories were evaluated using the T-maze spontaneous alternation task (T-SAT) and the Novel Object Recognition task (NORT), respectively, which are both based on mice natural exploratory tendency. During the training trial of the T-SAT, all genotypes spent similar time in the open or familiar arm (Fig. 23A). During the testing trial, however, mHtt KI mice exhibited significantly reduced exploration time of the novel arm, indicating spatial memory impairments (Fig. 23B). Genetic reduction of Cdk5 in mHtt KI:Cdk5^{+/-} mice attenuated this spatial memory decline as shown by higher preference to explore the novel *versus* the familiar arm (Fig. 23B).

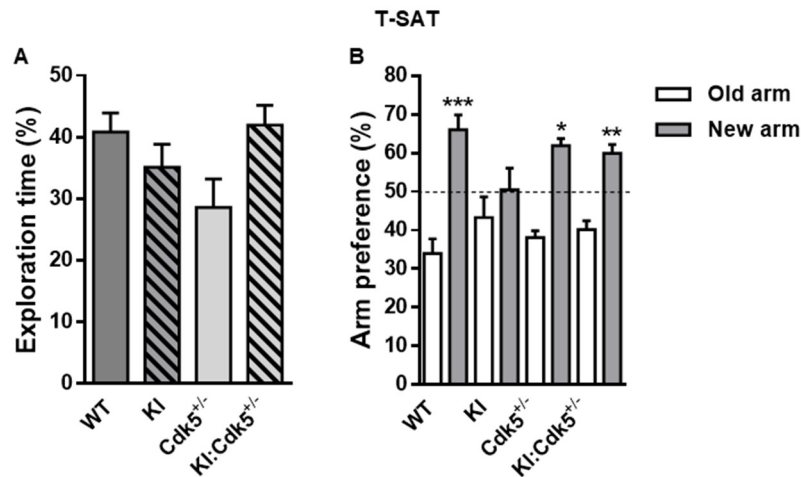


Figure 23. Spatial long-term memory impairments are ameliorated in mHtt KI:Cdk5^{+/-} mice. Percentage of time spent exploring the open arm during the training session (A) and percentage of time spent in arms (old *versus* new) during the testing session (B) from WT ($n = 12$), mHtt KI ($n = 16$), Cdk5^{+/-} ($n = 8$) and mHtt KI:Cdk5^{+/-} ($n = 12$) mice. Dashed line indicates chance level for exploration. Data represents mean \pm SEM. Statistical analysis was performed using One-way ANOVA with Bonferroni post hoc comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the old arm.

Then, recognition memory was evaluated using the NORT. During the training session, all genotypes showed similar exploration time for each object with no detectable object preference (Fig 24A). However, during the testing session after 24 hours to evaluate long-term memory, mHtt KI mice demonstrated similar exploration time of the novel *versus* the familiar object (Fig. 24B), indicating impaired recognition memory. Consistent with the results in the T-SAT, we found that mHtt KI:Cdk5^{+/-} mice exhibited a comparable performance in the NORT as WT mice (Fig. 24B), denoting that genetic reduction of Cdk5 expression attenuated recognition memory deficits. In conclusion, these results demonstrate that the deficits in hippocampal-dependent spatial and recognition memory in mHtt KI mice can be ameliorated by genetic reduction of Cdk5 expression.

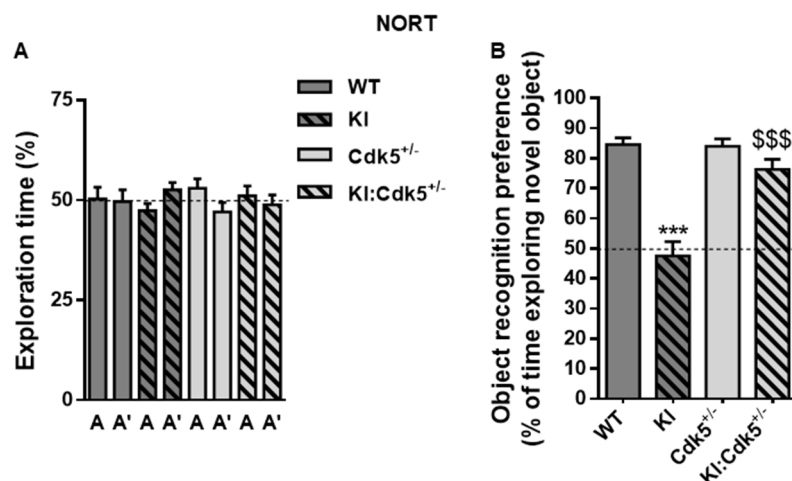


Figure 24. Recognition long-term memory impairments are rescued by Cdk5 genetic reduction. A. Percentage of time spent exploring similar objects during the training session (A) and percentage of time spent exploring the new object during the testing session (B) from WT ($n = 12$), mHtt KI ($n = 16$), Cdk5^{+/-} ($n = 8$) and mHtt KI:Cdk5^{+/-} ($n = 12$) mice. Dashed line indicates chance level for exploration. Data

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represents mean \pm SEM. Statistical analysis was performed using One-way ANOVA with Bonferroni post hoc comparisons. *** $p < 0.001$ compared to WT mice. \$\$\$ $p < 0.001$ compared to mHtt KI mice.

1.3 ANALYSIS OF CDK5-DEPENDENT MECHANISMS UNDERLYING COGNITIVE IMPROVEMENT IN mHtt KI:CDK5^{+/-} MICE

In view of our results showing that Cdk5 genetic reduction can restore corticostriatal and hippocampal function in mHtt KI mice, we aimed to identify molecular mechanisms that could underlie this recovery. Therefore, we analysed synaptic markers known to be altered in HD and, at the same time, known to be regulated by Cdk5, such as NMDA receptor levels and dendritic spine density. In addition, as little information is known about Cdk5 alterations in HD in the cortex and hippocampus, we further analysed alterations in Cdk5 activity in mHtt KI mice, as well as, the effect of Cdk5 genetic reduction on Cdk5 activity regulation.

1.3.1 ANALYSIS OF NMDA RECEPTORS

In the first place, we focused on NMDARs since many studies have shown their alteration in HD (Luthi-Carter *et al*, 2003), they play a pivotal role in synaptic plasticity (Izquierdo, 1991; Tang *et al*, 1999) and, Cdk5 is known to regulate NMDARs levels and function through different pathways (Plattner *et al*, 2014; Sahin *et al*, 2008; Zhang *et al*, 2008b).

1.3.1.1 Cortical NR2B levels are decreased in mHtt KI mice and are restored by genetic reduction of Cdk5

We analysed total levels of the main NMDARs subunits: NR1, NR2A and NR2B in the cortex, striatum and hippocampus of WT, mHtt KI, Cdk5^{+/-} and mHtt KI:Cdk5^{+/-} mice. No significant changes between genotypes were detected neither in the striatum nor in the hippocampus in the levels of any of the analysed NMDAR subunits (Fig. 25B, C). However, a significant reduction of NR2B was found in the cortex of mHtt KI mice. Importantly, this decrease was prevented in mHtt KI:Cdk5^{+/-} mice as a result of Cdk5 genetic reduction (Fig. 25A). No significant changes were detected in cortical NR1 and NR2A levels between genotypes.

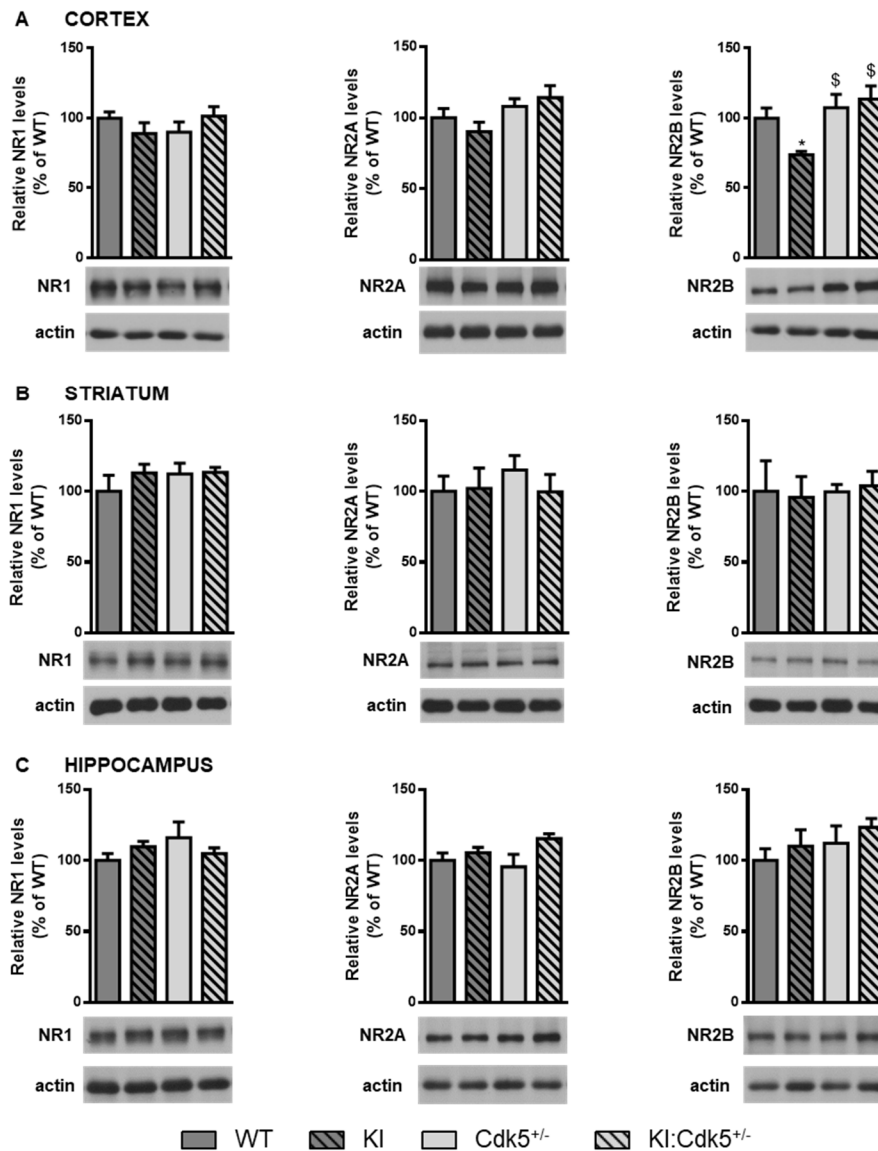


Figure 25. Reduced cortical NR2B levels in mHtt KI mice are recovered by Cdk5 genetic reduction. Representative Western blots showing total NR1, NR2A and NR2B levels in the cortex (A), striatum (B) and hippocampus (C) of WT ($n = 5-7$), mHtt KI ($n = 5-7$), Cdk5^{+/-} ($n = 6-7$) and mHtt KI:Cdk5^{+/-} ($n = 6-7$) mice. All data represent mean \pm SEM and are normalized to WT levels. Statistical analysis was performed using One-way ANOVA with Newman-Keuls post-hoc comparisons. * $p < 0.05$ compared to WT mice. \$ $p < 0.05$ compared to mHtt KI mice.

1.3.1.2 Corticostriatal NR2B surface levels are reduced in mHtt KI mice and restored by genetic reduction of Cdk5

In order to corroborate whether alterations in total NR2B levels correlated with a functional outcome, we analysed specifically NR2B surface levels. To this end, we performed a biotinylation assay and we analysed NR2B surface levels in the cortex, striatum and hippocampus of WT, mHtt KI, Cdk5^{+/-} and mHtt KI:Cdk5^{+/-} mice. We also observed a significant reduction of cortical NR2B surface levels in mHtt KI mice, which was restored in mHtt KI:Cdk5^{+/-} mice (Fig. 26A). Surprisingly, even though no changes in NR2B total levels were detected in the striatum, we did find a significant reduction of NR2B surface levels in

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mHtt KI mice. Similarly, genetic reduction of Cdk5 was also able to recover striatal NR2B surface levels decrease (Fig. 26B). Finally, analysis of hippocampal NR2B surface levels also yielded a significant reduction in mHtt KI mice, although in this case, mHtt KI:Cdk5^{+/-} mice only presented a partial recovery of NR2B surface levels (Fig. 26C). These results indicate that genetic reduction of Cdk5 can completely restore reduced corticostriatal NR2B surface levels in mHtt KI mice, thus providing a molecular mechanism for corticostriatal cognitive improvement in mHtt KI:Cdk5^{+/-} mice. On the other hand, NR2B surface levels partial recovery in the hippocampus suggests that other Cdk5-dependent mechanisms may be responsible for hippocampal cognitive restoration.

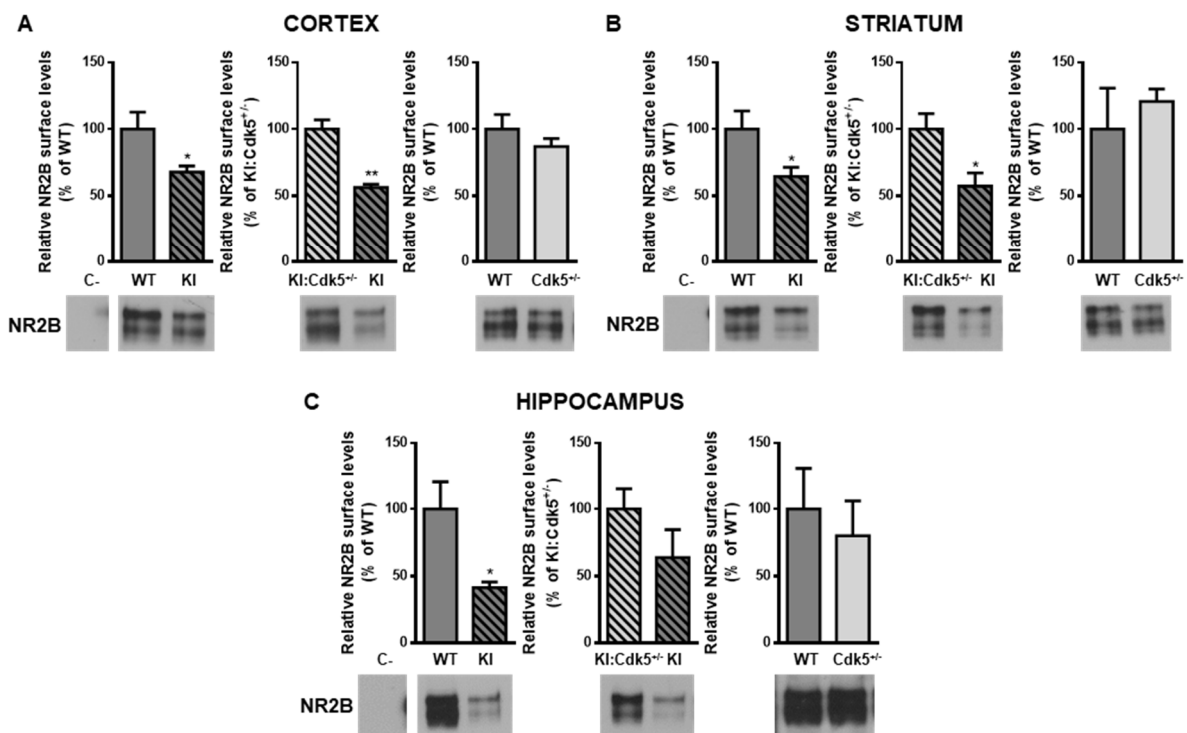


Figure 26. Decreased corticostriatal NR2B surface levels in mHtt KI mice are recovered by genetic reduction of Cdk5. Representative Western blots showing surface levels of NR2B in the cortex (A), striatum (B) and hippocampus (C) of WT ($n = 4$), mHtt KI ($n = 5$), Cdk5^{+/-} ($n = 3-4$) and mHtt KI:Cdk5^{+/-} ($n = 4$) mice. All data represent mean \pm SEM and are normalized to WT or mHtt KI:Cdk5^{+/-} levels. Statistical analysis was performed using Student's two tailed t -test. * $p < 0.05$, ** $p < 0.01$ compared to WT or mHtt KI:Cdk5^{+/-} mice.

1.3.1.3 Analysis of Cdk5-dependent pathways regulating NR2B surface levels

Several Cdk5-dependent pathways have been described to modulate NR2B surface levels (Hawasli *et al*, 2007; Plattner *et al*, 2014; Zhang *et al*, 2008b). For this reason, we analysed whether these pathways were altered in mHtt KI mice, and therefore, could be restored by Cdk5 genetic reduction, providing a link between Cdk5 reduction and restoration of NR2B surface levels.

1.3.1.3.1 mHtt KI mice do not show alterations in NR2B/Cdk5/calpain interaction

Previous studies using *Cdk5^{+/-}* mice, have described that genetic reduction of *Cdk5* is able to increase NR2B surface levels by reducing NR2B degradation via decreased formation of the NR2B/*Cdk5*/calpain complex. *Cdk5* interaction with calpain and NR2B facilitates NR2B cleavage by calpain and its subsequent internalization and degradation. Reduction of *Cdk5*, therefore, leads to increased NR2B total and surface levels (Hawasli *et al*, 2007). In order to determine whether this pathway could be responsible for our previous observations, we analysed the interaction between *Cdk5*, calpain and NR2B in the cortex, where we have observed changes of NR2B both in total and surface levels. However, analysis of neither NR2B and calpain levels after *Cdk5* immunoprecipitation nor of NR2B and *Cdk5* levels after calpain immunoprecipitation, showed significant changes between WT and mHtt KI mice (Fig. 27). Hence, our results indicate that other molecular pathways probably underlie reduction of cortical NR2B total and surface levels in mHtt KI mice.

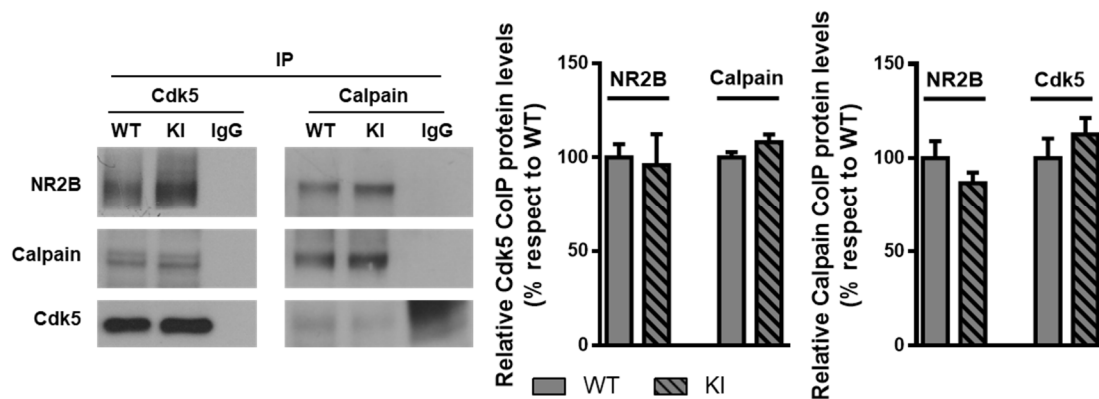


Figure 27. NR2B/*Cdk5*/calpain interaction levels are not altered in the cortex of mHtt KI mice. *Cdk5* and calpain co-immunoprecipitation was performed in the cortex of WT ($n = 5-6$) and mHtt KI ($n = 4-6$) mice and NR2B and calpain levels and NR2B and *Cdk5* levels, respectively, were analysed by Western blot. Represented protein levels are normalized to the levels of each immunoprecipitated protein. All data represent mean \pm SEM and are normalized to WT levels. Statistical analysis was performed using Student's two tailed *t*-test.

1.3.1.3.2 NR2B phosphorylation levels at Ser1116 are not altered in mHtt KI mice

Cdk5 has also been described to modulate NR2B surface levels via direct phosphorylation at the serine 1116 residue (pNR2B S1116). Reduction of *Cdk5* leads to reduced pNR2B (S1116) levels and increased NR2B surface levels (Plattner *et al*, 2014). Therefore, if increased *Cdk5* activity in mHtt KI mice were affecting this pathway we would expect to find an increase in pNR2B (S1116) levels which would correlate with reduced NR2B surface levels in mHtt KI mice. Thus, we determined pNR2B (Ser1116) levels in the cortex, striatum and hippocampus of WT and mHtt KI mice. However no significant changes were observed in the striatum nor the hippocampus, and surprisingly we found a reduction of pNR2B (S1116) levels in the cortex of mHtt KI mice (Fig. 28). Nonetheless, this reduction probably accounts for the

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decrease in total NR2B levels observed specifically in the cortex. In sum, these results indicate that deregulation of pNR2B (S1116) is probably not associated with reduced NR2B surface levels in mHtt KI mice.

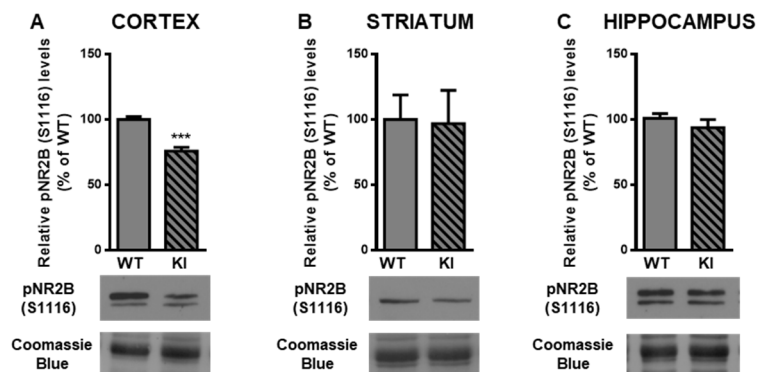


Figure 28. Cortical pNR2B (S1116) levels are decreased in mHtt KI mice. Representative Western blots of cortical, striatal and hippocampal lysates probed for pNR2B (S1116) in WT ($n = 6-7$) and mHtt KI ($n = 5-7$) mice. Histograms represent the mean \pm SEM and are normalized to WT levels. Statistical analysis was performed using Student's two tailed t -test. *** $p < 0.001$ compared to WT.

1.3.1.3.3 pSrc and pNR2B (Y1472) levels are significantly decreased in the cortex of mHtt KI mice and are restored by Cdk5 genetic reduction

Finally, it has also been described that Cdk5 can indirectly regulate NR2B surface levels through phosphorylation of PSD95 which reduces Src/PSD95 interaction, necessary for Src auto-activation. Reduced Src activity (pSrc Y416) leads to reduced NR2B phosphorylation at the tyrosine 1472 residue (pNR2B Y1472), which allows AP2 binding and subsequent NR2B internalization (Zhang *et al*, 2008b). In order to analyse if alterations in this pathway could be responsible for reduced NR2B surface levels in mHtt KI mice, we analysed levels of pSrc (Y416) and pNR2B (Y1472) in the cortex, striatum and hippocampus of WT and mHtt KI mice. We found that mHtt KI mice present reduced levels of pSrc (Y416) and pNR2B (Y1472) in the cortex, without showing alterations in total Src levels (Fig. 28A). Therefore, we also analysed pSrc (Y416) and pNR2B (Y1472) levels in Cdk5^{+/-} and mHtt KI:Cdk5^{+/-} mice. Importantly, we observed that reduced pSrc (Y416) and pNR2B (Y1472) levels were recovered as a result of Cdk5 genetic reduction in mHtt KI:Cdk5^{+/-} mice (Fig. 29A). However, no significant changes were detected neither in the striatum nor the hippocampus between genotypes (Fig. 29B, C). These findings indicate that Cdk5-dependent pSrc/pNR2B pathway restoration in the cortex could account for the recovery of cortical NR2B surface levels and thus, for restored corticostriatal cognitive performance in mHtt KI:Cdk5^{+/-} mice. Nonetheless, other Cdk5-dependent mechanisms are probably involved in the recovery of striatal NR2B surface levels in mHtt KI:Cdk5^{+/-} mice and in the recovery of hippocampal-dependent cognitive decline.

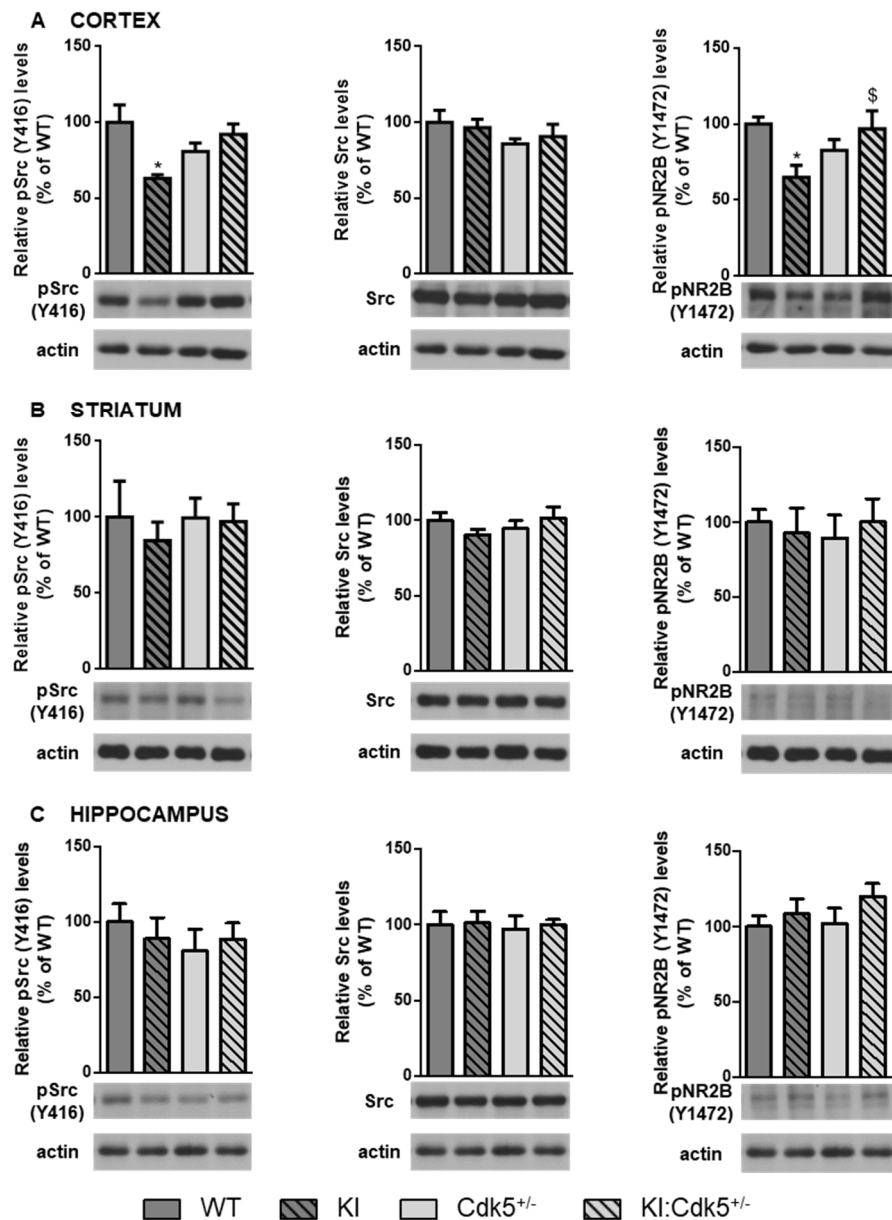


Figure 29. Reduced cortical pSrc (Y416) and pNR2B (Y1472) phosphorylation levels in mHtt KI mice are recovered in mHtt KI:Cdk5^{+/-} mice. Representative Western blots showing levels of pSrc (Y416), total Src and pNR2B (Y1472) in the cortex (A), striatum (B) and hippocampus (C) of WT ($n = 5-7$), mHtt KI ($n = 6-7$), Cdk5^{+/-} ($n = 5-7$) and mHtt KI:Cdk5^{+/-} ($n = 5-7$) mice. All data represent mean \pm SEM and are normalized to WT levels. Statistical analysis was performed using One-way ANOVA with Newman-Keuls post hoc comparisons. * $p < 0.05$ compared to WT mice. § $p < 0.05$ compared to mHtt KI mice.

1.3.2 ANALYSIS OF DENDRITIC SPINE DENSITY

Cdk5 is also an important modulator of several cytoskeleton associated proteins, and therefore of dendritic spine remodelling (Kim *et al*, 2006b; Posada-Duque *et al*, 2015). Given that previous studies from our group have described that mHtt KI mice present deficits in dendritic spine density in the cortex since 2 months of age and in the hippocampus at 8 months of age (Brito *et al*, 2014; Puigdemívol *et al*, 2015), we decided to analyse if Cdk5

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reduction could prevent this alteration, providing an underlying mechanism for improved hippocampal cognitive impairment.

1.3.2.1 Reduction of Cdk5 in mHtt KI mice attenuates decreased hippocampal dendritic spines and increases Rac1 activity

We performed Golgi staining on hippocampal slices from WT, mHtt KI, Cdk5^{+/-} and mHtt KI:Cdk5^{+/-} mice in order to analyse dendritic spine density. We measured apical dendrites from CA1 pyramidal neurons, and we found that mHtt KI mice displayed reduced dendritic spine density (Fig. 30A). Importantly this reduction was rescued in mHtt KI:Cdk5^{+/-} mice and Cdk5^{+/-} mice presented slightly increased dendritic spine density when compared to WT mice (Fig. 30A), suggesting that Cdk5 negatively regulates CA1 dendritic spines. Next, in order to address which Cdk5 substrate could be responsible for this recovery, we analysed the levels of Rac1 activity, as it can be regulated by Cdk5 and previous studies from our group have described its alteration in the cortex of mHtt KI mice (Posada-Duque *et al*, 2015; Puigdemívol *et al*, 2015). Although no changes were observed in Rac1 activity levels between WT and mHtt KI mice, a significant increase was detected in mHtt KI:Cdk5^{+/-} mice, and an increasing trend was also detected in Cdk5^{+/-} compared to mHtt KI mice (Fig. 30B). These results indicate that hippocampal-dependent cognitive improvement in mHtt KI:Cdk5^{+/-} mice could be associated to restoration of dendritic spine density, which in turn could result from increased Rac1 activity.

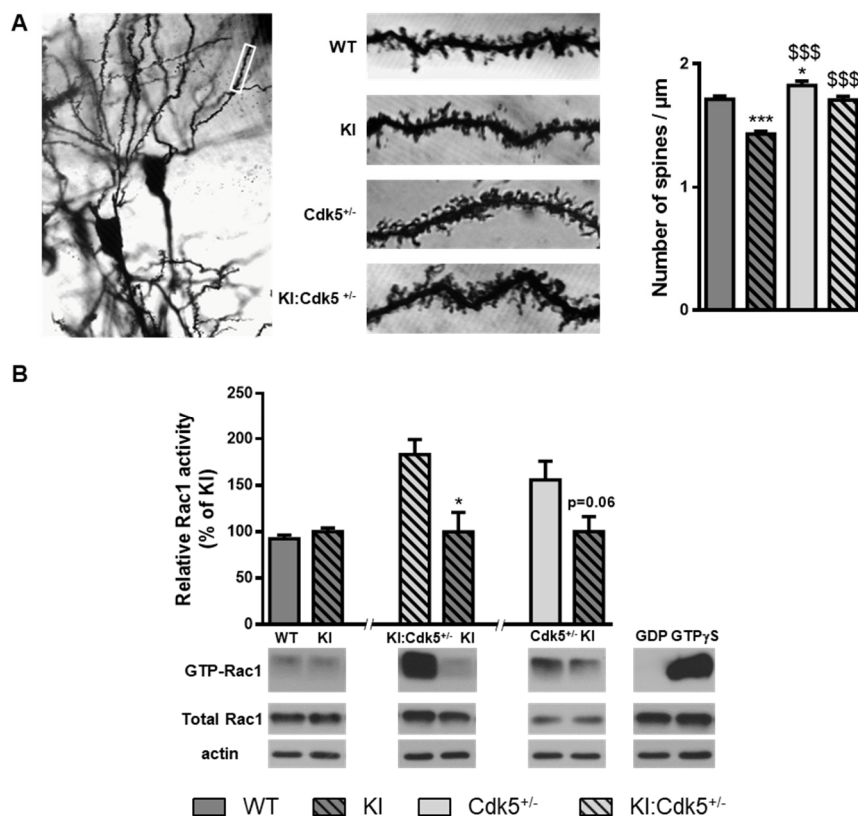


Figure 30. Decreased hippocampal dendritic spine density is attenuated by genetic reduction of Cdk5.

A. Representative images of a Golgi-impregnated CA1 hippocampal neuron and of apical dendrites of CA1 pyramidal neurons from WT, mHtt KI, Cdk5^{+/-} and mHtt KI:Cdk5^{+/-} mice. Histogram showing quantitative analysis of dendritic spine density per micrometre of dendritic length. Statistical analysis was performed using One-way ANOVA with Newman-Keuls post hoc comparisons (30 dendrites from 3-5 animals per genotype were analysed). * $p < 0.05$, *** $p < 0.001$ compared to WT mice. \$\$\$ $p < 0.001$ compared to mHtt KI mice. **B.** Representative Western blots showing levels of GTP-bound Rac1 immunoprecipitated from hippocampal lysates, indicating Rac1 activity. Histograms represent the mean \pm SEM and are normalized to mHtt KI values (n=4-5 mice per genotype). Statistical analysis was performed using Student's two tailed t -test. * $p < 0.05$ compared to mHtt KI.

1.3.2.2 Reduced cortical dendritic spines are recovered by Cdk5 reduction

We have observed that recovery of corticostriatal-dependent deficits correlates with restoration of NR2B surface levels and that restoration of hippocampal-dependent decline correlates with recovery of dendritic spine density, suggesting a brain region specificity in Cdk5-dependent substrate regulation. For this reason, in order to determine if restoration of dendritic spine density was a hippocampus-specific mechanism, we also performed Golgi staining of corticostriatal slices and we measured dendritic spine density in cortical layer V pyramidal neurons. Similar to our observations in the hippocampus, mHtt KI mice presented a significant reduction of cortical dendritic spines which was recovered in mHtt KI:Cdk5^{+/-} mice as a result of Cdk5 genetic reduction (Fig. 31A). However, we did not find any significant changes between WT and Cdk5^{+/-} mice (Fig. 31A), indicating that genetic reduction of Cdk5 *per se*, does not influence cortical dendritic spine density. We also analysed Rac1 activity in cortical lysates and, in accordance with previous studies (Puigdellívol *et al*, 2015), we found a significant reduction of Rac1 between WT and mHtt KI mice (Fig. 31B). However, no significant changes were found between mHtt KI and mHtt KI:Cdk5^{+/-} mice or Cdk5^{+/-} mice (Fig. 31B). These results indicate that in contrast to the hippocampus, mHtt KI mice present a reduction of Rac1 activity in the cortex, however Cdk5 reduction does not affect cortical Rac1 activity, suggesting that other Cdk5 substrates might be responsible for recovery of dendritic spine density in the cortex of mHtt KI:Cdk5^{+/-} mice, reinforcing our hypothesis of a brain region-dependent modulation of Cdk5 substrates.

RESULTS

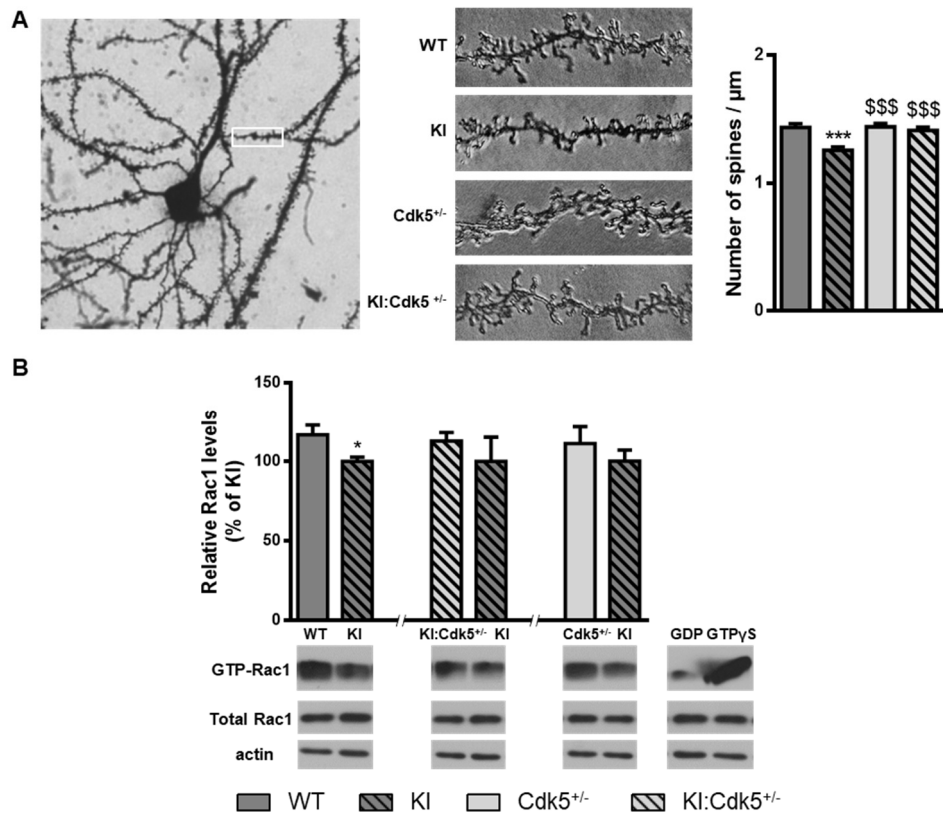


Figure 31. Decreased cortical dendritic spine density in mHtt KI mice is attenuated in mHtt KI:Cdk5^{+/-} mice. **A.** Representative images of a Golgi-impregnated cortical pyramidal neuron and of apical dendrites of layer V pyramidal neurons from WT, mHtt KI, Cdk5^{+/-} and mHtt KI:Cdk5^{+/-} mice. Histogram showing quantitative analysis of dendritic spine density per micrometre of dendritic length. Statistical analysis was performed using One-way ANOVA with Newman-Keuls post hoc comparisons (30 dendrites from 3-5 animals per genotype were analysed). *** $p < 0.001$ compared to WT mice. \$\$\$ $p < 0.001$ compared to mHtt KI mice. **B.** Representative Western blots showing levels of GTP-bound Rac1 immunoprecipitated from cortical lysates indicating Rac1 activity. Histograms represent the mean \pm SEM and are normalized to mHtt KI values ($n=4-5$ mice per genotype). Statistical analysis was performed using Student's two tailed t -test. * $p < 0.05$ compared to mHtt KI.

1.3.3 ANALYSIS OF CDK5 ACTIVITY IN mHtt KI:CDK5^{+/-} MICE

Our results indicate that genetic reduction of Cdk5 restores cognitive decline in mHtt KI mice, via normalization of NMDARs levels and dendritic spine density in a brain region-specific manner. Since Cdk5 activity remains unexplored in the cortex and hippocampus of HD mice, we wanted to analyse its activity to further correlate Cdk5-dependent modulation of synaptic substrates presented up to this point with alterations in Cdk5 activity.

1.3.3.1 Phosphorylation levels of Cdk5 substrates are altered preferentially in the cortex of mHtt KI mice.

As a readout of putative changes in Cdk5 activity between genotypes, we analysed phosphorylation levels of different Cdk5 substrates. Cdk5 plays an important role in Tau

hyperphosphorylation in AD, and Tau hyperphosphorylation has also been described in HD (Gratuze *et al*, 2015; Patrick *et al*, 1999). For this reason, amongst the plethora of Cdk5 substrates, we first analysed phosphorylation levels of Tau at the PHF1 epitope, which has been described to be phosphorylated by Cdk5 (Kimura *et al*, 2014). Our results showed that mHtt KI mice presented increased levels of PHF1 in the cortex, although genetic reduction of Cdk5 in mHtt KI mice did not recover this increase (Fig. 32A). On the other hand, analysis of PHF1 levels in the striatum and hippocampus showed no significant changes between genotypes in these regions, although PHF1 levels presented an increasing trend in the striatum of mHtt KI mice, which was not recovered either by Cdk5 genetic reduction (Fig. 32B, C).

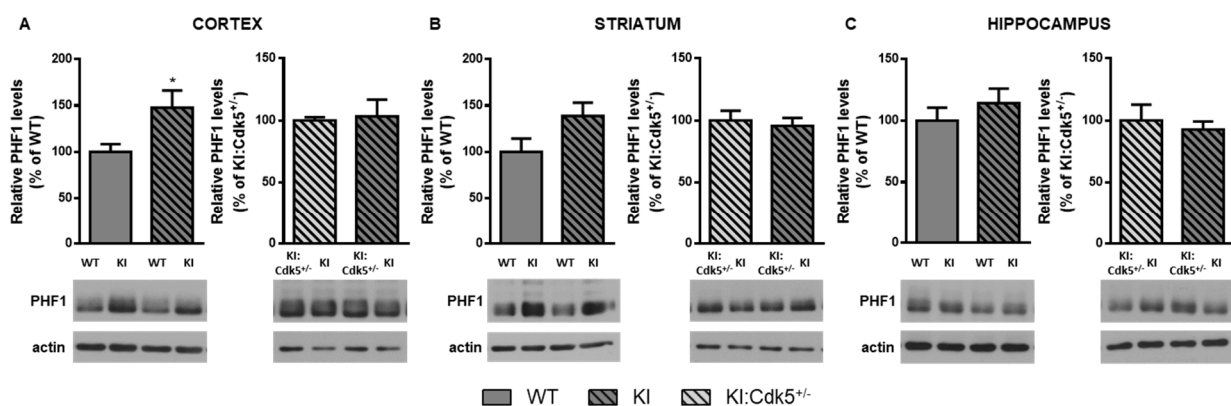


Figure 32. PHF1 levels are significantly increased in the cortex and present an increasing trend in the striatum of mHtt KI mice. Representative Western blots of cortical (A), striatal (B) and hippocampal (C) lysates probed for PHF1 in WT ($n = 4-6$), mHtt KI ($n = 4-7$) and mHtt KI:Cdk5^{+/-} (4-6) mice. Histograms represent the mean \pm SEM and are normalized to WT or mHtt KI:Cdk5^{+/-} levels. Statistical analysis was performed using Student's two tailed t -test. * $p < 0.05$ compared to WT.

We also analysed phosphorylation levels of the protein phosphatase 1 (PP1) inhibitor or IPP1, which has also been described to be phosphorylated by Cdk5 at the serine 67 residue (pIPP S67) (Bibb *et al*, 2001b). Similar to PHF1 levels, mHtt KI mice presented an increasing trend in pIPP1 (S67) levels in the cortex, which was not recovered after Cdk5 genetic reduction in mHtt KI:Cdk5^{+/-} mice (Fig. 33A). No changes were observed either in pIPP1 levels between genotypes in the striatum nor the hippocampus (Fig. 33B, C). These results suggest that reduction of Cdk5 is not sufficient to restore alteration of PHF1 or pIPP1 resulting from mHtt presence in mHtt KI mice.

RESULTS

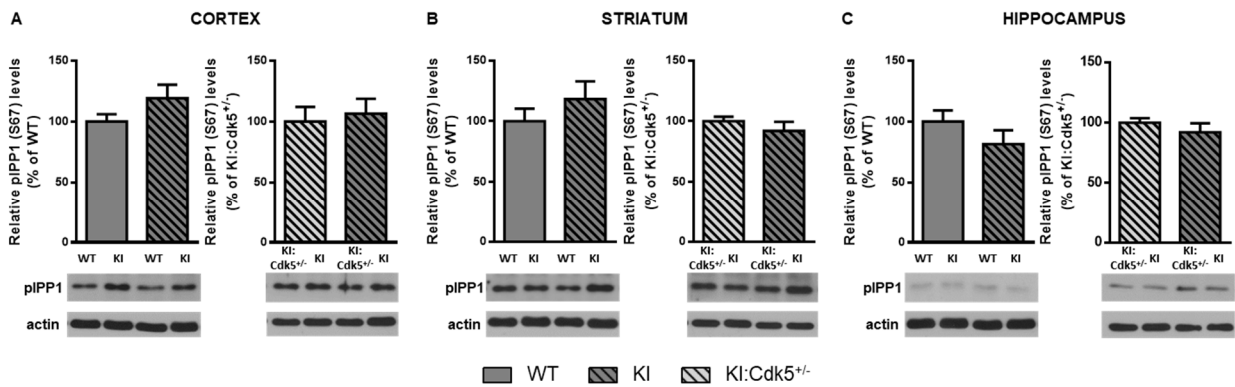


Figure 33. pPPP1 (S67) levels show no significant alterations between genotypes. Representative Western blots of cortical (A), striatal (B) and hippocampal (C) lysates probed for pPPP1 (Ser67) in WT ($n = 5-6$), mHtt KI ($n = 5-6$) and mHtt KI:Cdk5^{+/-} (6-7) mice. Histograms represent the mean \pm SEM and are normalized to WT or mHtt KI:Cdk5^{+/-} levels. Statistical analysis was performed using Student's two tailed

Finally, we checked the general phosphorylation state of CDK-dependent serine residues (pCDK substrates) in WT, mHtt KI and mHtt KI:Cdk5^{+/-} mice. In this case we also found that in the cortex, mHtt KI mice presented an increasing trend in pCDK (Ser) substrates when compared to WT mice. Importantly, this trend was also observed when comparing mHtt KI with mHtt KI:Cdk5^{+/-} mice, indicating that increased phosphorylation of pCDK (Ser) substrates might be partially reverted as a result of genetic reduction of Cdk5 (Fig. 34A). However, no changes were observed neither in the striatum nor the hippocampus between genotypes (Fig. 34B,C). Notably, intensity changes in certain molecular weight bands could be observed between genotypes in all regions in pCDK (Ser) substrates Western blot analysis (Fig. 34), which might reflect changes in phosphorylation levels of specific Cdk5 substrates.

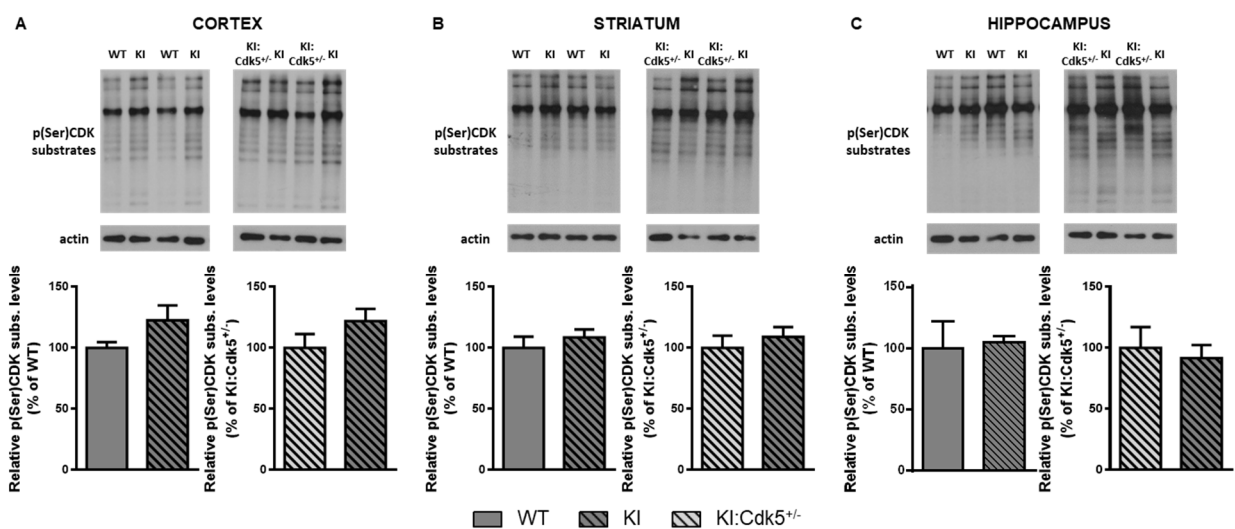


Figure 34. pCDK (Ser) substrates levels present an increasing trend in the cortex of mHtt KI mice. Representative Western blots of cortical (A), striatal (B) and hippocampal (C) lysates probed for pCDK (Ser) substrates in WT ($n = 4-6$) mHtt KI ($n = 4-6$) and mHtt KI:Cdk5^{+/-} (6-7) mice. Histograms represent

the mean \pm SEM and are normalized to WT or mHtt KI:Cdk5^{+/-} levels. Statistical analysis was performed using Student's two tailed *t*-test.

These results indicate that alteration of Cdk5 substrates phosphorylation levels in mHtt KI mice occurs preferentially in the cortex and to a lesser extent in the striatum, suggesting a preferential dysfunction of Cdk5 activity in the cortex over other analysed brain regions. However, even within the cortex, Cdk5 activity presents differential regulation depending on the substrate.

1.3.3.2 p35 plasma membrane levels are significantly reduced in the cortex of mHtt KI which is recovered by Cdk5 genetic reduction

Given that we have observed differential alteration of Cdk5 substrates, we wondered whether this might result from modifications in subcellular distribution of Cdk5 and its activator, as correct subcellular localization of Cdk5 and p35 is pivotal for its proper function (Asada *et al*, 2008; Patrick *et al*, 1999). To this end, we performed subcellular fractionation to obtain plasma membrane and cytosolic fractions from the cortex, striatum and hippocampus of WT, mHtt KI, Cdk5^{+/-} and mHtt KI:Cdk5^{+/-} mice.

Our results showed that p35 levels were significantly reduced in the cortical plasma membrane fraction of mHtt KI mice (Fig. 35A), and that cortical Cdk5 plasma membrane levels also presented a decreasing trend (Fig. 35B), indicating that normal Cdk5/p35 function might be altered in the cortex of mHtt KI mice. Importantly, this reduction in p35 plasma membrane levels was recovered to levels similar to WT in mHtt KI:Cdk5^{+/-} mice. Nonetheless, besides Cdk5 genetic reduction, no significant changes were detected in the striatal or hippocampal plasma membrane fractions between genotypes (Fig. 35). These results fit with our prior observations of preferential changes in Cdk5 substrates phosphorylation in the cortex of mHtt KI mice.

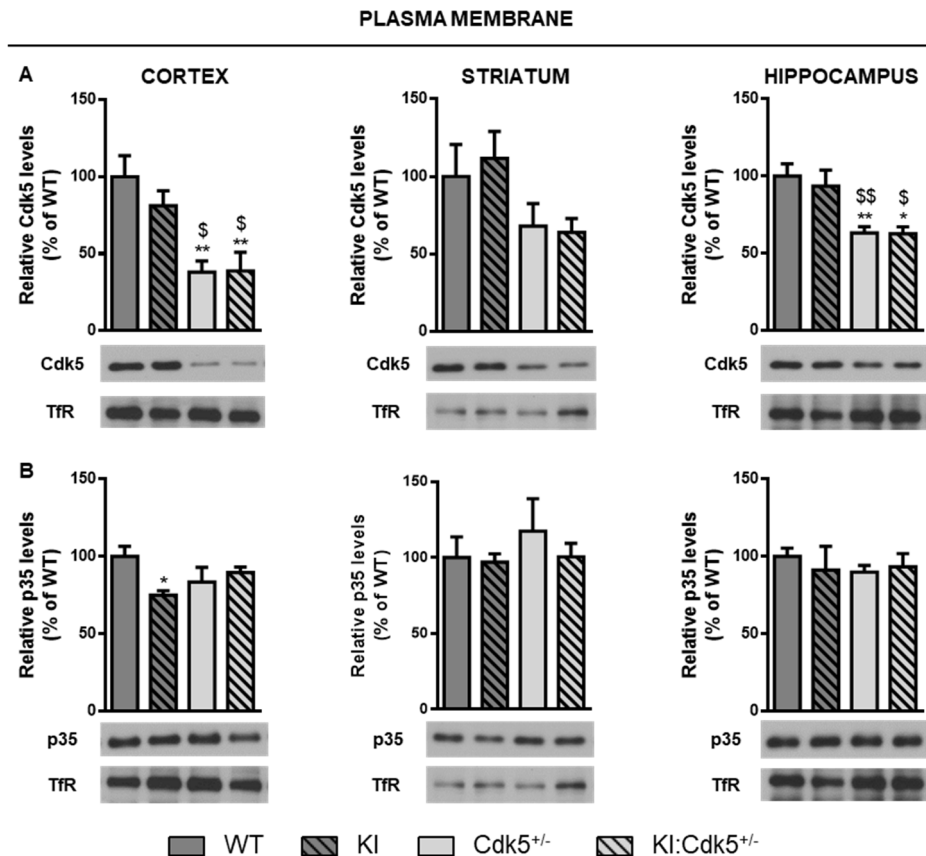


Figure 35. Cortical p35 plasma membrane levels are significantly reduced in mHtt KI mice.

Representative Western blots of cortical, striatal and hippocampal plasma membrane fractions probed for Cdk5 (A) and p35 (B) in WT ($n = 5-6$), mHtt KI ($n = 4-7$), Cdk5^{+/-} ($n = 4-5$) and mHtt KI:Cdk5^{+/-} ($n = 4-6$) mice. Histograms represent the mean \pm SEM and are normalized to WT levels. One-way ANOVA with Newman-Keuls post hoc comparisons. * $p < 0.05$ compared to WT. \$ $p < 0.05$, \$\$ $p < 0.01$ compared to mHtt KI mice.

We also analysed cytosolic Cdk5 and p35 levels, and although we did not find any significant differences between WT and mHtt KI mice, we detected a significant increase of cytosolic p35 levels in the cortex of Cdk5^{+/-} mice when compared to mHtt KI mice. Cytosolic p35 levels also presented an increasing trend in mHtt KI:Cdk5^{+/-} mice (Fig. 36B). Similar to data obtained in plasma membrane fraction, no changes were observed in the striatal and hippocampal cytosolic fractions (Fig. 36), suggesting that additional Cdk5 activity regulation mechanisms such as post-translational modifications, could underlie differential regulation of Cdk5 substrates in these regions. Levels of the cleaved p35 form, p25, could not be detected neither in the plasma membrane nor in the cytosolic fraction in our experimental conditions.

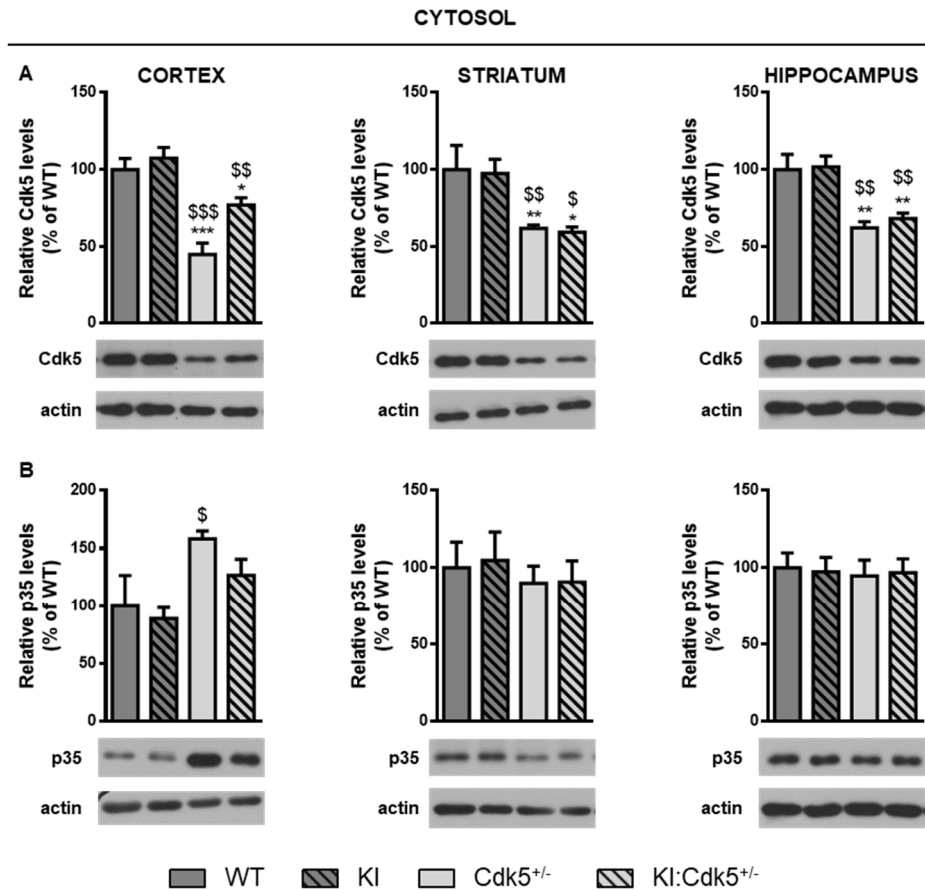


Figure 36. Cortical p35 cytosol levels are increased in Cdk5^{+/-} mice. Representative Western blots of cortical, striatal and hippocampal cytosol fractions probed for Cdk5 (A) and p35 (B) in WT ($n = 4-6$), mHtt KI ($n = 5-7$), Cdk5^{+/-} ($n = 4-5$) and mHtt KI:Cdk5^{+/-} ($n = 4-5$) mice. Histograms represent the mean \pm SEM and are normalized to WT levels. One-way ANOVA with Newman-Keuls post hoc comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to WT. \$ $p < 0.05$, \$\$ $p < 0.01$, \$\$\$ $p < 0.001$ compared to mHtt KI mice.

2. CELL CYCLE REENTRY AS A POTENTIAL UNDERLYING MECHANISM FOR CDK5-DEPENDENT STRIATAL VULNERABILITY IN HD

Cdk5 deregulation contributing to neuronal death, has been described in several neurodegenerative disorders, including HD. Particularly, alteration of Cdk5 in HD has been associated with increased striatal vulnerability to dopaminergic and glutamatergic activation (Paoletti *et al*, 2008). Nonetheless, not much is known about the underlying mechanisms linking Cdk5 deregulation with increased striatal vulnerability. For this reason, we decided to study whether Cdk5 deregulation in HD might alter Cdk5 role in the maintenance of cell cycle inhibition in postmitotic neurons. Cell cycle re-entry of differentiated neurons has been proposed as a possible common mechanism in several neurodegenerative disorders. It has been described, especially in Alzheimer's disease, that deregulation of cell cycle inhibition in neurons, leads to expression of cell cycle proteins and to cell cycle re-entry, which leads, eventually, to neuronal dysfunction and death (Herrup and Yang, 2007). Coincidentally, some articles have described alteration of some cell cycle proteins in HD human samples and in HD mice models (Fernandez-Fernandez *et al*, 2011; Liu *et al*, 2015; Pelegrí *et al*, 2008). However, little is known about the molecular mechanisms leading to these alterations or the possible involvement of Cdk5 in cell cycle deregulation in HD.

2.1 ANALYSIS OF CDK5 FUNCTION AS A CELL CYCLE MODULATOR IN HD

Cdk5 has been described to maintain cell cycle progression inhibition in the nucleus of differentiated neurons. Specifically, it is known that Cdk5 along with p35 forms a complex with the CDKs inhibitor p27 and the transcription factor E2F1, preventing E2F1 interaction with its cofactor DP1, and therefore avoiding transcription of cell cycle proliferation genes (Zhang and Herrup, 2011). For this reason, we analysed interaction and nuclear levels of these proteins in the striatum of WT and KI mice at early symptomatic motor stages (8 months of age).

2.1.1 Cdk5 and p27 interaction is not significantly altered in the striatum of symptomatic mHtt KI mice

To analyse if presence of mHtt alters the formation of the Cdk5/p27/E2F1 complex, we performed Cdk5 immunoprecipitation and we analysed its interaction with p27 as we were not able to detect E2F1 after Cdk5 immunoprecipitation. Our results showed a decreasing trend, although not significant, in Cdk5/p27 interaction in the striatum of 8-month-old mHtt KI mice (Fig. 37A). We also analysed this interaction at later symptomatic stages (13 months) and we found no significant changes either (Fig. 37B). These results suggest that Cdk5/p27 interaction is not significantly altered in the striatum of mHtt KI mice. However, as

immunoprecipitation was performed on total lysates and this complex is formed in the nucleus, we decided to analyse specifically nuclear levels of these proteins.

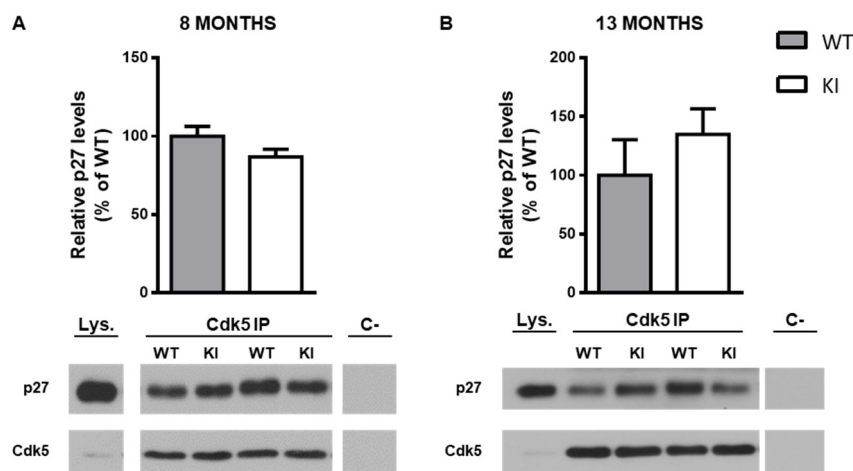
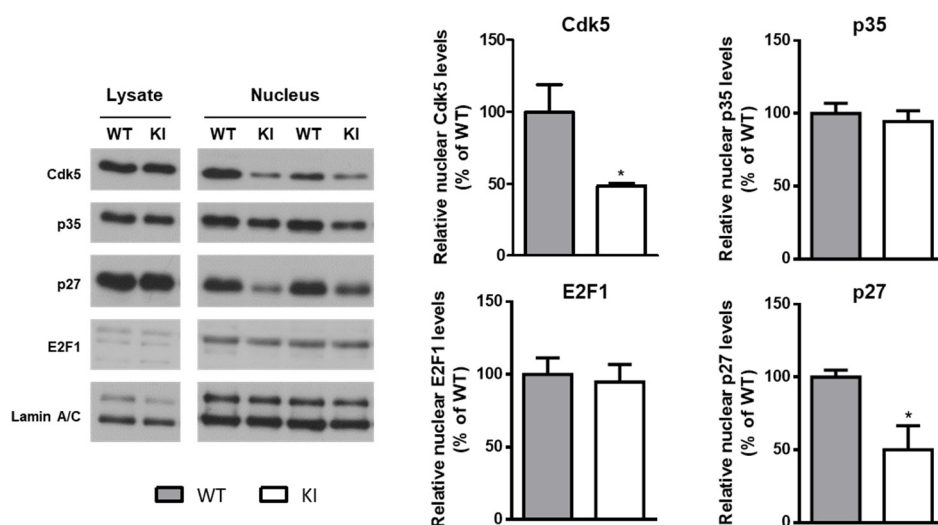


Figure 37. Cdk5/p27 interaction is not significantly altered in the striatum of 8 and 13-month-old mHtt KI mice. Cdk5 immunoprecipitated was performed in samples from the striatum of 8 (A) and 13-month-old (B) WT ($n = 5-6$) and mHtt KI ($n = 5-6$) mice. Co-immunoprecipitated p27 levels were analysed by Western blot and normalised to Cdk5 immunoprecipitated levels. Histograms represent the mean \pm SEM and are normalized to WT levels. Statistical analysis was performed using Student's two tailed t -test.

2.1.2 Cdk5 and p27 nuclear levels are significantly reduced in the striatum of 8-month-old mHtt KI mice

We performed subcellular fractionation in order to specifically analyse nuclear levels of Cdk5, p35, p27 and E2F1. Our results showed that nuclear levels of Cdk5 and p27 were significantly reduced in the striatum of 8-month-old mHtt KI mice, whereas E2F1 and p35 levels were not altered (Fig. 38). These results suggest that, although total Cdk5/p27 interaction is not significantly altered in the striatum of mHtt KI mice, reduced Cdk5 and p27 nuclear localization could lead to aberrant cell cycle re-entry as both proteins act as cell cycle inhibitors when localized in the nucleus (Akashiba *et al*, 2006a; Zhang *et al*, 2008a).



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Figure 38. Nuclear Cdk5 and p27 levels are significantly reduced in the striatum of mHtt KI mice. Cdk5, p35, p27 and E2F1 levels were determined in nuclear fractions from the striatum of 8-month-old WT ($n = 3-4$) and mHtt KI ($n = 3-4$) mice. Lamin A/C was used as loading control. Histograms represent the mean \pm SEM and are normalized to WT levels. Statistical analysis was performed using Student's two tailed t -test. * $p < 0.05$ compared to WT.

2.2 ANALYSIS OF CELL CYCLE PROTEINS EXPRESSION IN HD

Previous studies have described alteration of some cell cycle proteins in human HD brains and in R6/1 mice (Fernandez-Fernandez *et al*, 2011; Pelegrí *et al*, 2008). However, little is known about their alteration in the less severe knock-in HD mouse model. For this reason, in order to determine if reduction of Cdk5 and p27 nuclear levels could also involve aberrant expression of other cell cycle proteins, we analysed the levels of p27, CyclinD1 and Cdk4, which are expressed during early G₁ cell cycle phase, at different disease stages in the striatum of mHtt KI mice.

2.2.1 p27 and CyclinD1 levels are increased in the striatum of mHtt KI mice since early symptomatic stages

We analysed the levels of p27, CyclinD1 and Cdk4 in striatal lysates from motor asymptomatic (2-4 months), presymptomatic (6 months) and early (8 months) and late (13-18 months) symptomatic stages in mHtt KI mice. In contrast with our observations in nuclear p27 levels, our results showed that p27 presented a slight but significant increase at 6 months of age in mHtt KI mice. This increase became more apparent with increasing disease progression stages until 18 months of age (Fig. 39A), indicating that reduced nuclear p27 levels are probably caused by altered subcellular distribution rather than by total decrease of p27 levels.

Analysis of G₁ phase proteins Cdk4 and CyclinD1, showed that even though Cdk4 protein levels only displayed an increasing trend in mHtt KI mice at late disease stages with no significant changes in previous phases (Fig. 39B), CyclinD1 levels were increased since early stages. Thus, CyclinD1 levels presented an increasing trend at 6 months. This increase became significant at 8 months and was maintained at 13 and 18 months of age, when CyclinD1 levels presented almost a 50% increase in mHtt KI mice when compared to WT mice (Figure 39C). These results indicate that expression of cell cycle proteins present an alteration in the striatum of mHtt KI mice at ages that correlate with disease motor onset, which could lead to aberrant cell cycle re-entry.

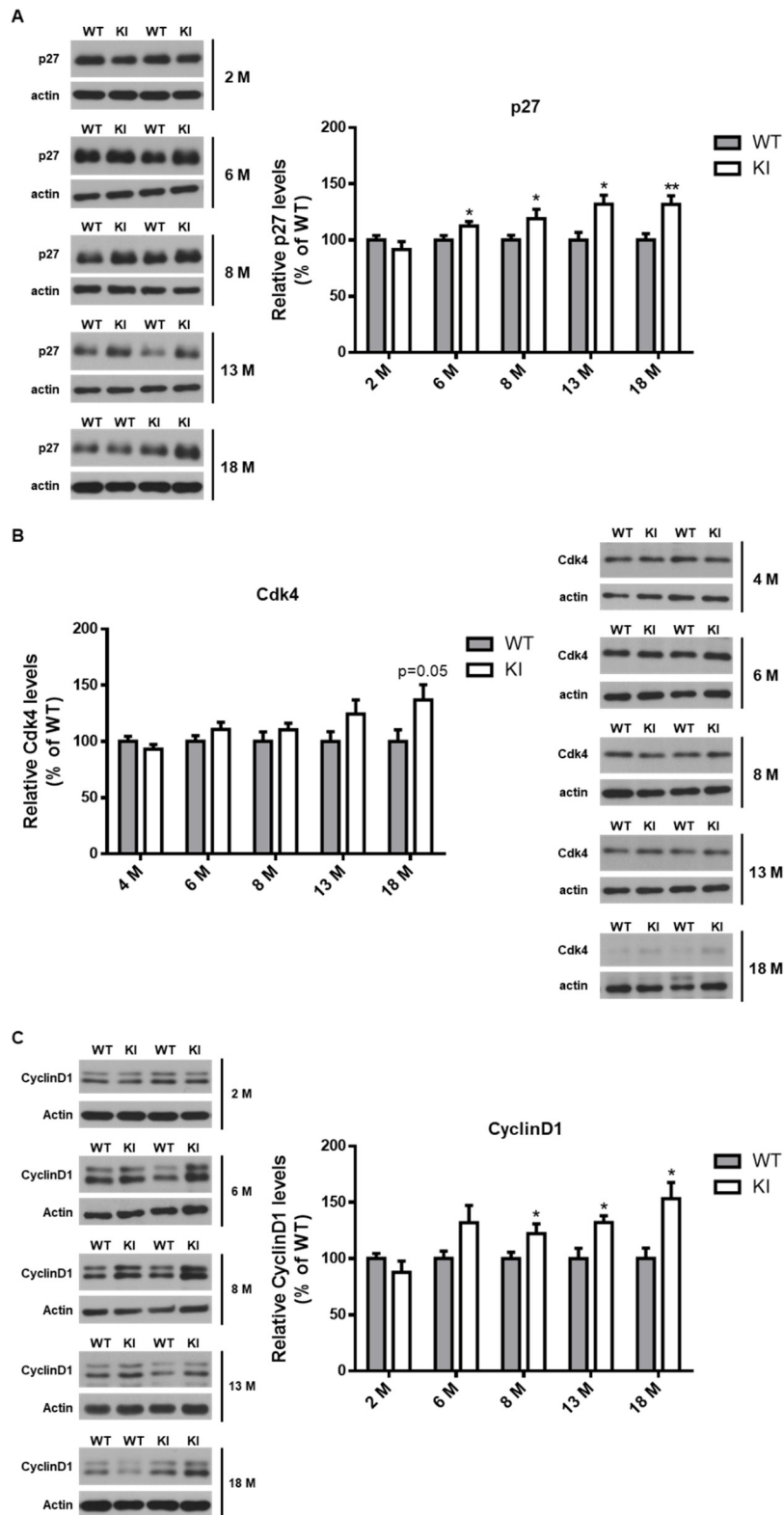
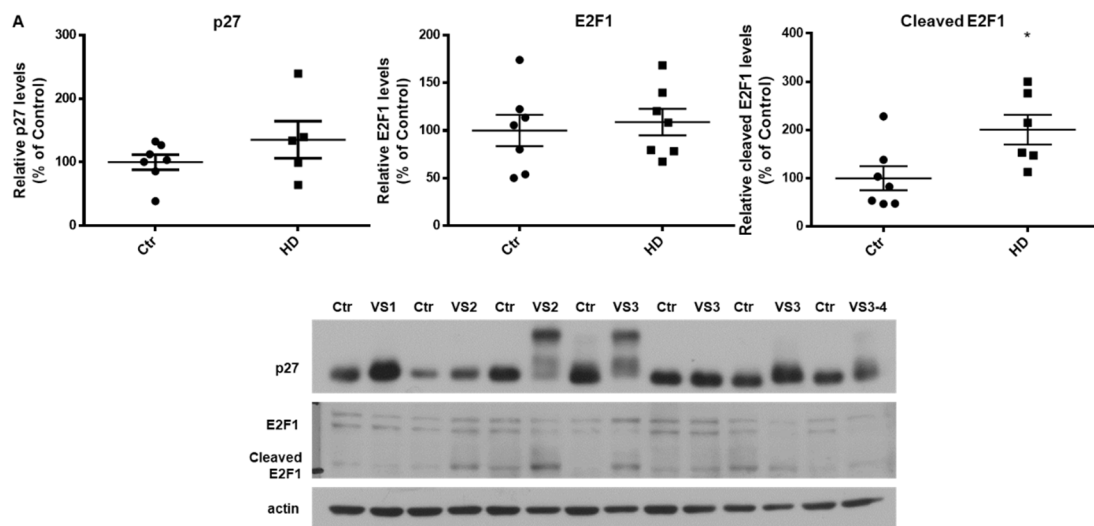


Figure 39. p27 and CyclinD1 levels are significantly increased in the striatum of mHtt KI mice since early symptomatic disease stages. Representative Western blots of striatal lysates probed for p27 (A), Cdk4 (B) and CyclinD1 (C) in WT ($n = 5-13$) and mHtt KI ($n = 5-12$) mice. Histograms represent the mean \pm SEM and are normalized to WT levels. Statistical analysis was performed using Student's two tailed t -test. * $p < 0.05$, ** $p < 0.01$ compared to WT.

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2.2.2 Cdk4 levels are significantly increased in the putamen of HD patients

Finally, we also determined the levels of these proteins in putamen samples from HD patients. In contrast with our observations in HD mice models, p27 levels were not significantly increased in putamen samples from HD patients, although p27 blot bands showed notable alterations in their pattern (Fig. 40A). These alterations were reproducible and only observed for p27 suggesting possible presence of post-translational p27 modifications in samples from HD patients leading to increased molecular weight. Similarly, even though we did not find any significant changes in total E2F1 levels, we detected a significant increase of a lower molecular weight band (Fig. 40A). Interestingly, it has been described that E2F1 can be cleaved by calpains in neurotoxic conditions, producing a 50 kDa band (Zyskind *et al*, 2015), suggesting that E2F1 in the putamen of HD patients could experience increased cleavage compared to controls. On the other hand, in contrast with our results in HD models, analysis of CyclinD1 levels in HD patients yielded no significant alterations respect to control samples. However, Cdk4 levels did show a significant increase in the putamen of HD patients (Fig. 40B). These results suggest that cell cycle proteins also present alterations in the putamen of HD patients. However, these alterations differ from the ones observed in HD models, which could be attributed to the fact that mHtt KI mice do not present neuronal death, in contrast with HD human brains.



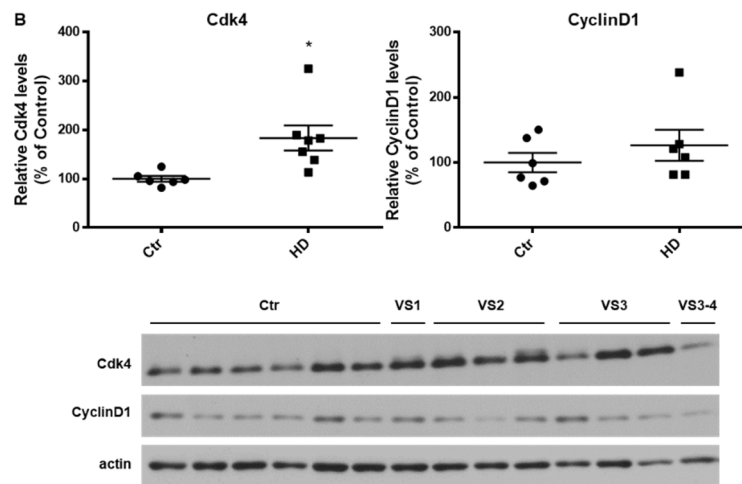


Figure 40. Cdk4 levels are significantly increased in the putamen of HD patients. Representative Western blots of putamen lysates probed for p27 and E2F1 (A), and Cdk4 and CyclinD1 (B) in Control ($n = 6-7$) and HD ($n = 5-7$) patients. Histograms represent the mean \pm SEM and are normalized to Control levels. Statistical analysis was performed using Student's two tailed t -test. * $p < 0.05$ compared to Control.

2.3 ANALYSIS OF CELL CYCLE PROTEINS FOLLOWING NMDAR ACTIVATION IN WT AND KI STRIATAL PRIMARY CULTURES

Our results show that mHtt induces alteration of cell cycle proteins levels in HD mice and in HD patients, which might be associated with decreased Cdk5 nuclear levels. One of the pathways leading to Cdk5 deregulation is calpain-dependent p35 cleavage following NMDARs activation (Miao *et al*, 2012; Patrick *et al*, 1999). Incidentally, NMDARs activation also plays a major role in excitotoxicity in HD (Fan and Raymond, 2007) and, particularly, deregulation of Cdk5 has been associated with increased striatal vulnerability in HD following glutamatergic activation (Paoletti *et al*, 2008). Therefore, we aimed to analyse whether NMDA treatment, leading to Cdk5 hyperactivation, might participate in cell cycle proteins alteration in striatal primary cultures from WT and mHtt KI mice.

2.3.1 NMDA treatment alters expression levels of cell cycle proteins

We tested different treatment durations and different NMDA concentrations, in DIV21 WT striatal primary cultures, in order to determine appropriate conditions leading to p35 cleavage to p25. We performed treatments consisting on 100 μ M NMDA for 1h, 50 μ M NMDA for 3h and 10 μ M NMDA for 24h. We analysed the levels of spectrin breakdown products (SBDP) which are commonly used as calpain activation indicators (Vanderklish and Bahr, 2000). Our results showed that all NMDA treatments led to increased SBDP levels (Fig. 41A), indicating NMDA activation and subsequent calpain activation in our treatment conditions. Next, we analysed the levels of p35 cleaved product, p25. Western blot analysis showed that 50 μ M NMDA for 3h, and particularly 100 μ M NMDA for 1h, caused p25

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production. This effect, however, was lost in the 10 μM NMDA for 24h treatment (Fig. 41A). Finally, we analysed the levels of cleaved caspase 3, as an apoptotic marker, after 1h 100 μM NMDA treatment, which produced the most significant response in the previously measured parameters. We did not detect any significant changes between control and NMDA treated cultures (Fig. 41B), suggesting that this treatment does not lead to overt neuronal apoptosis.

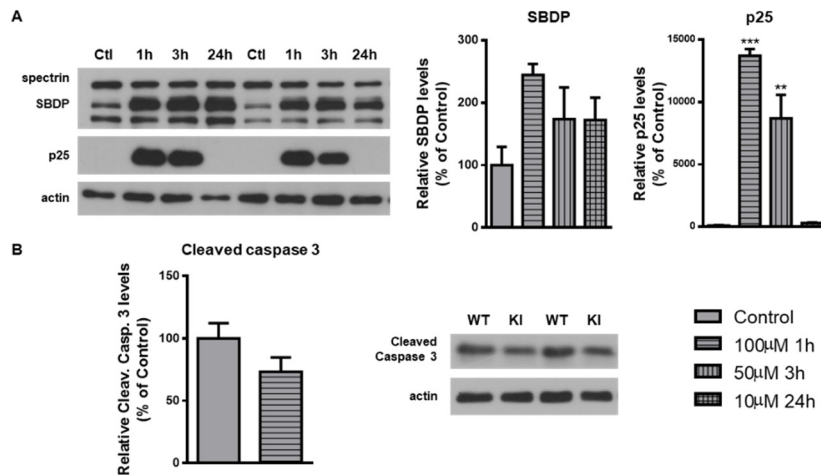


Figure 41. Efficiency evaluation of different NMDA treatments conditions. Representative Western blots of WT striatal primary cultures lysates probed for spectrin and p25 (A) and cleaved caspase 3 (B) in Control ($n = 3$), 100 μM NMDA for 1h ($n = 2$), 50 μM NMDA for 3h ($n = 4$) and 10 μM NMDA for 24h ($n = 4$) conditions. Histograms represent the mean \pm SEM and are normalized to Control levels. Statistical analysis was performed using One-way ANOVA with Newman-Keuls post hoc comparisons. ** $p < 0.01$, *** $p < 0.001$ compared to Control.

Next, we also analysed the effect of these different NMDA treatments on the levels of the cell cycle proteins, p27, E2F1, CyclinD1 and Cdk4 in WT striatal cultures. Western blot analysis, showed that 50 μM NMDA treatment for 3h, and particularly, 100 μM NMDA treatment for 1h, induced significant p27 reduction. A decreasing trend in Cdk4 and CyclinD1 protein levels could also be observed after 100 μM NMDA for 1h (Fig. 42). Finally, even though total E2F1 levels did not present alterations in response to NMDA treatment, we observed an additional E2F1 lower molecular weight band in cultures treated with NMDA, indicating that, according to previous studies (Zyskind *et al*, 2015), E2F1 could be cleaved under these conditions.

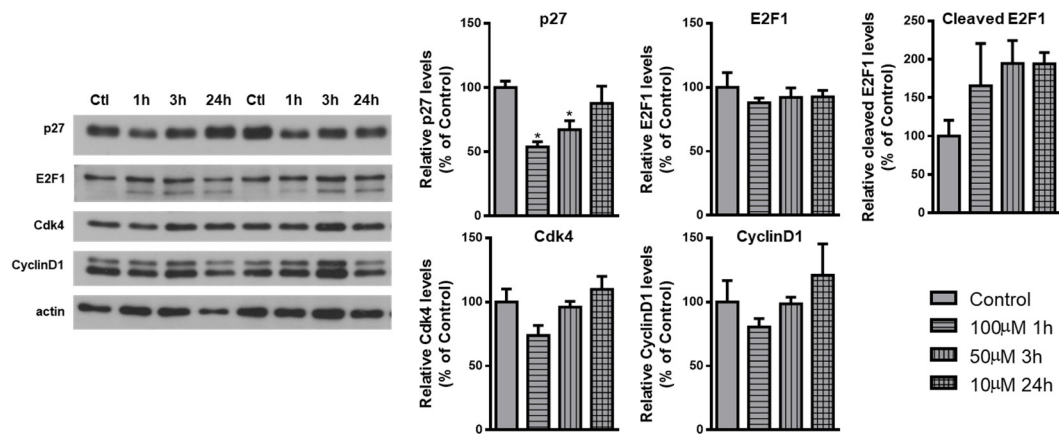


Figure 42. Cell cycle proteins response to different NMDA treatment conditions. Representative Western blots of WT striatal primary cultures lysates probed for p27, E2F1, Cdk4 and CyclinD1 in Control ($n = 3$), 100 μ M NMDA for 1h ($n = 2$), 50 μ M NMDA for 3h ($n = 4$) and 10 μ M NMDA for 24h ($n = 4$) conditions. Histograms represent the mean \pm SEM and are normalized to Control levels. Statistical analysis was performed using One-way ANOVA with Newman-Keuls post hoc comparisons. * $p < 0.05$ compared to Control.

To analyse the potential role of calpain in mediating alterations of cell cycle proteins, WT striatal primary cultures were pre-treated with the calpain inhibitor ALLN for 1 hour. Similarly to previous works (Akashiba *et al*, 2006b; Zyskind *et al*, 2015) we observed that, p27 reduction and presence of lower molecular weight E2F1 band after NMDA treatment, relied on calpain activation, given that ALLN pre-treatment, prevented these changes (Fig. 43). Efficiency of ALLN pre-treatment was confirmed by reduction of SBDP and p25 production (Fig. 43). In addition, Cdk4 and CyclinD1 levels showed an increasing trend in their levels in cultures pre-treated with calpain inhibitor, although these changes were not significant. These results confirm that NMDA treatment leads to p27 reduction and E2F1 cleavage in a calpain-dependent manner. Considering that calpain activation also leads to Cdk5 hyperactivation, Cdk5 might also participate in these calpain-dependent alterations in cell cycle proteins.

RESULTS

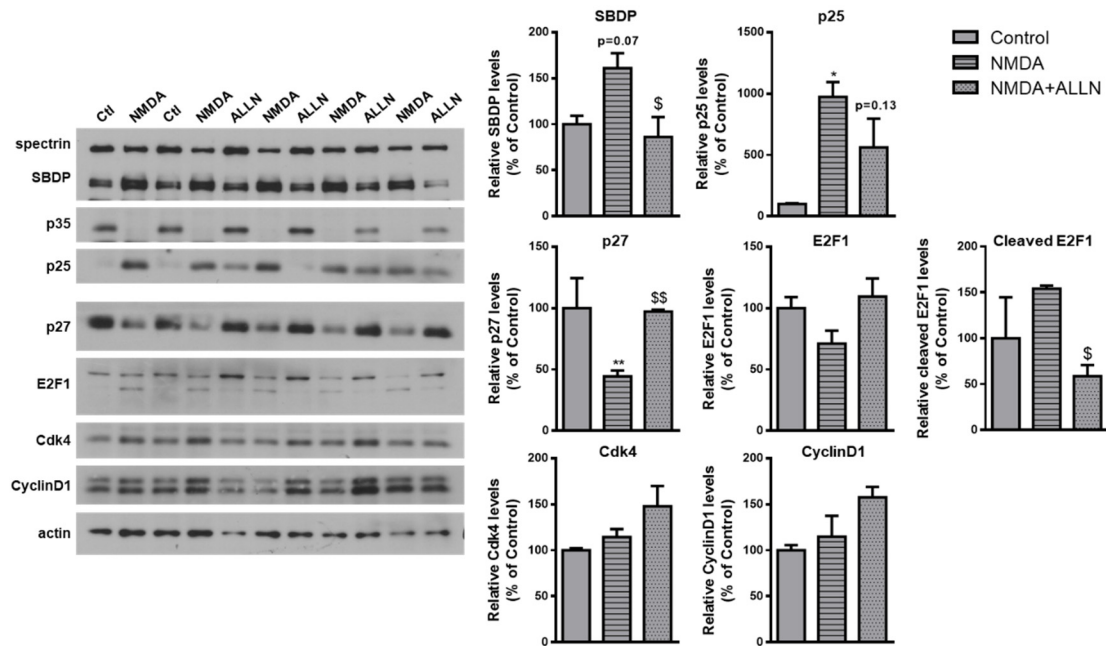


Figure 43. Cell cycle proteins response to calpain inhibition. Representative Western blots of WT striatal primary cultures lysates probed for spectrin, p25, p27, E2F1, Cdk4 and CyclinD1 in Control ($n = 2$), 100 μM NMDA for 1h ($n = 5$) and 100 μM NMDA for 1h + ALLN ($n = 4$) conditions. Histograms represent the mean \pm SEM and are normalized to Control levels. Statistical analysis was performed using One-way ANOVA with Newman-Keuls post hoc comparisons. * $p < 0.05$, ** $p < 0.01$ compared to Control. § $p < 0.05$, §§ $p < 0.01$ compared to 100 μM NMDA for 1h.

2.3.2 Total protein levels of cell cycle proteins are not altered in mHtt KI striatal cultures

In view of our previous results, we decided to use 100 μM NMDA for 1h treatment to analyse whether presence of mHtt differently altered the levels of cell cycle proteins. First, we determined in basal conditions whether the levels of p27, E2F1, CyclinD1 and Cdk4 were modified in mHtt KI cultures compared to WT cultures. However, western blot analysis yielded no significant alterations in the levels of analysed cell cycle proteins, neither in basal conditions (Fig. 44A), nor after NMDA treatment (Fig. 44B). These results suggest that presence of mHtt does not overtly affect total protein levels of cell cycle proteins in striatal primary cultures.

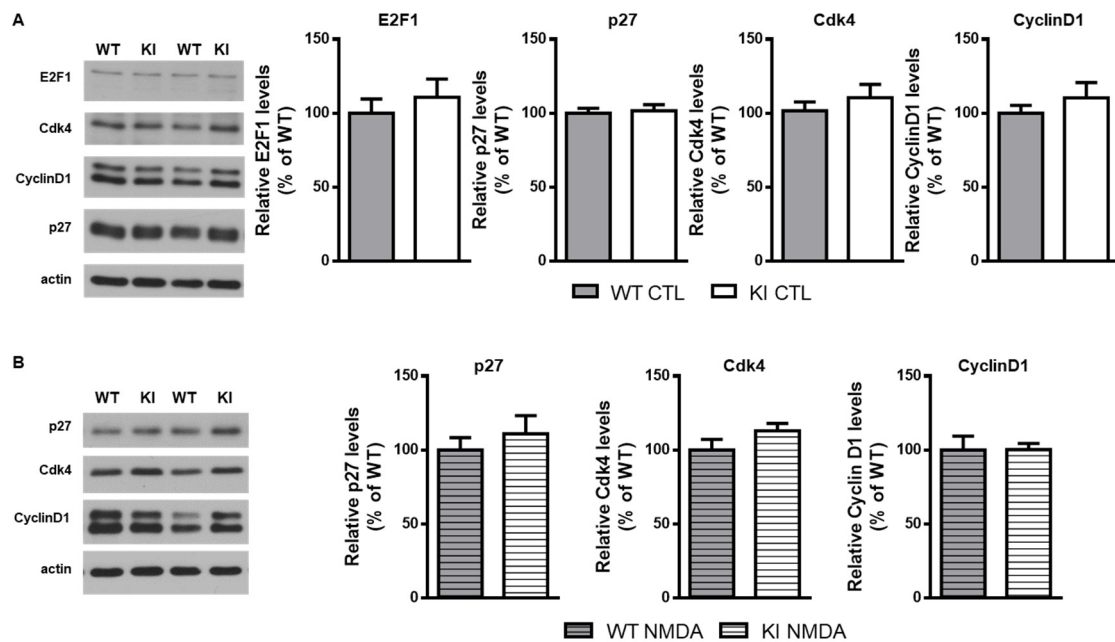


Figure 44. Cell cycle proteins levels are not significantly altered in mHtt KI cultures. Representative Western blots of lysates from WT and mHtt KI striatal primary cultures under basal conditions (A) or after 100uM NMDA for 1h treatment (B), probed for E2F1, p27, Cdk4 and CyclinD1 in WT Control ($n = 10-13$), KI Control ($n = 10-13$), WT NMDA ($n = 6-7$) and KI NMDA ($n = 6-7$) conditions. Histograms represent the mean \pm SEM and are normalized to Control levels. Statistical analysis was performed using Student's two tailed t -test.

2.3.3 Analysis of putative alterations in cell cycle proteins subcellular distribution in striatal primary cultures

Our results show that mHtt does not alter total protein levels of cell cycle proteins. However, we wondered whether it might affect their subcellular distribution. To this end, and to analyse the levels of cell cycle proteins specifically in neuronal cells, we performed immunocytochemical analysis of the previously studied cell cycle proteins along with the neuronal marker NeuN.

2.3.3.1 Distribution of cell cycle proteins in cultured striatal neurons

Subcellular localization of cell cycle proteins is well characterized in proliferating cells, however not much is known about their distribution in differentiated neurons. For this reason, prior to studying possible alterations between WT and mHtt KI striatal cultures, we analysed neuronal presence and subcellular distribution of p27, E2F1, Cdk4 and CyclinD1 in WT mature neuronal cultures by immunocytochemical analysis (Fig. 45).

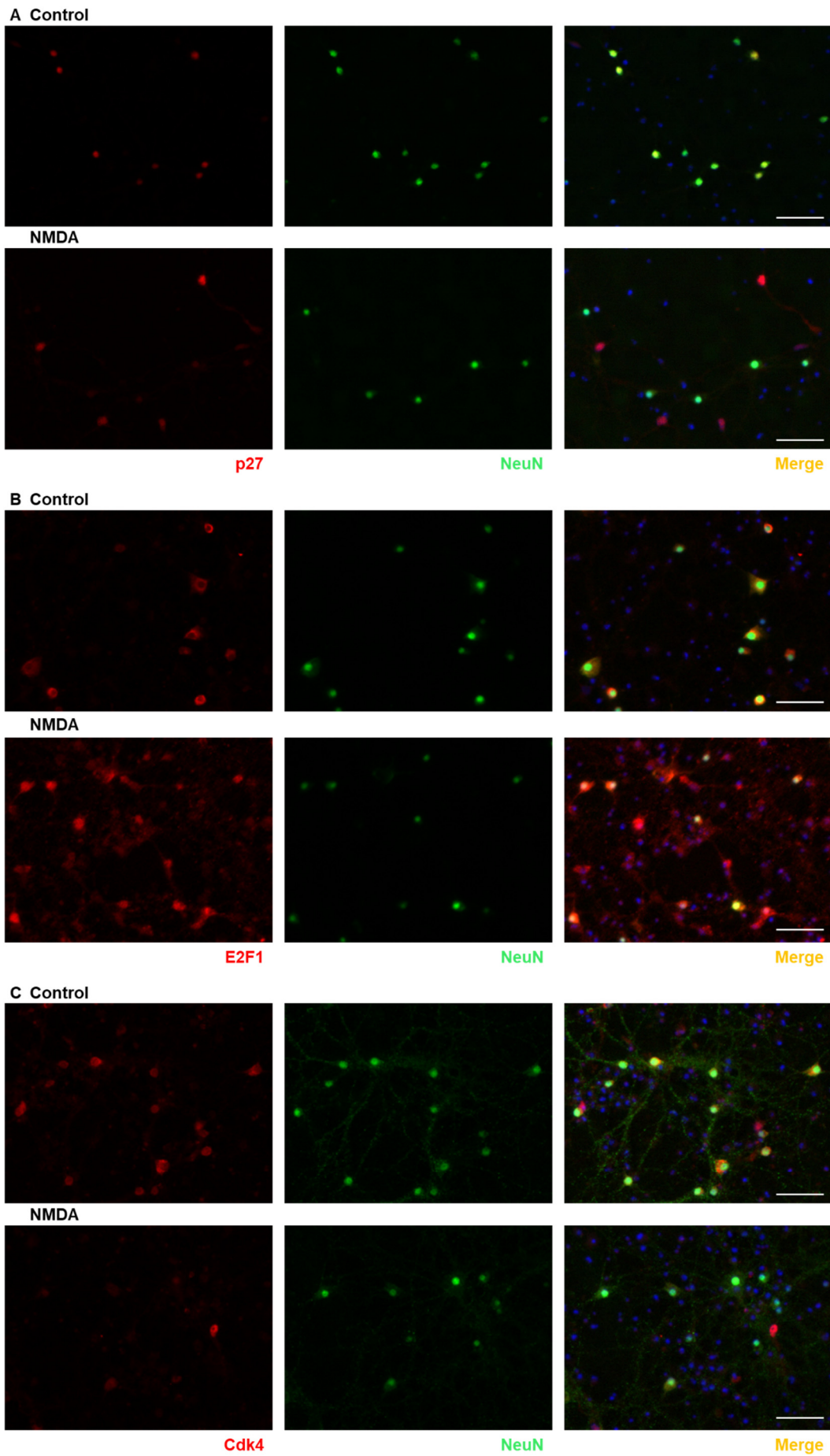
According to its function as a cell cycle inhibitor, most neurons (NeuN positive cells) expressed p27 in the nucleus ($76.02\% \pm 0.24$) (Fig. 45A). Notably, p27 was also present in glial cells (NeuN negative cells), as only $47.43\% \pm 6.02$ of p27 positive cells colocalized with NeuN.

RESULTS

E2F1 distribution showed cytosolic localization surrounding the nuclei of almost all neurons (94.38 ± 1.74) (Fig. 45B), with low presence in glial cells, as 68.79 ± 5.81 of E2F1 positive cells colocalized with NeuN. G₁ phase proteins, CyclinD1 and Cdk4, showed different distribution with Cdk4 presenting cytosolic distribution similar to E2F1 in 66.1 ± 10.07 of neurons (Fig. 45C), whereas CyclinD1 was absent in most neurons, being present in the nucleus of only 32.50 ± 8.23 of NeuN positive cells (Fig. 45D). In fact, only 38.96 ± 10.35 of CyclinD1 positive cells colocalized with NeuN, indicating that CyclinD1 presents higher expression in glial cells than in neurons. Cdk4 was also present in glial cells as only 55.06 ± 6.99 of Cdk4 positive cells colocalized with NeuN. In sum, according to our observations p27 presents neuronal nuclear localization, whereas E2F1 and Cdk4 present cytosolic distribution in most postmitotic neurons. CyclinD1 is absent in most neurons but when present, it shows nuclear localization.

2.3.3.2 NMDA treatment alters subcellular localization of cell cycle proteins

Next, we analysed the effects of NMDA treatment on the subcellular distribution of cell cycle proteins. Representative images show that NMDA treatment led to reduction of p27 intensity in the nucleus, mainly in NeuN positive cells (Fig. 45A), indicating that NMDA treatment could affect cell cycle suppressor role of p27 in the nucleus of neurons. E2F1 distribution was also altered, losing its cytosolic localization to a more diffuse pattern along the entire neuron, and being present in the nucleus of some NeuN positive cells (Fig. 45B). Similarly, Cdk4 showed reduced intensity, however it presented nuclear localization in some neurons (Fig. 45C). This could indicate a change of function of E2F1 and Cdk4, as a delocalization from the cytosol to the nucleus could be related with neuronal cell cycle re-entry. Finally, CyclinD1 was also present in the nucleus and cytosol of some neurons after NMDA treatment (Fig. 45D). Thus, reduction of p27 nuclear localization and presence of E2F1, Cdk4 and CyclinD1 in the nucleus of NeuN positive cells could reflect NMDA-induced cell cycle re-entry.



RESULTS

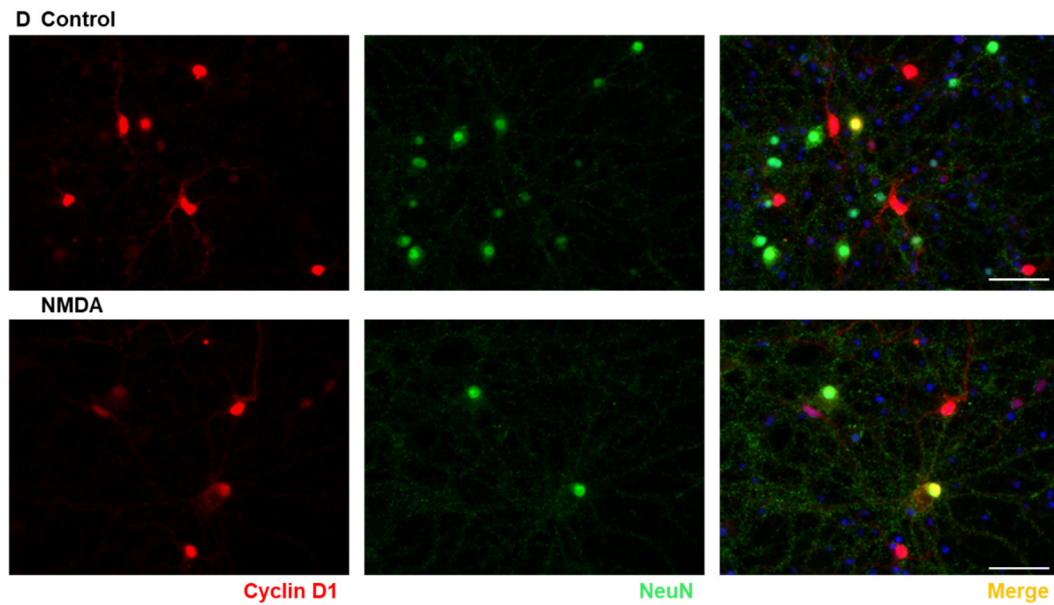


Figure 45. Cell cycle proteins distribution in striatal neuronal cultures under basal conditions and after 100 μ M NMDA for 1h treatment. Representative images of p27 (A), E2F1 (B), Cdk4 (C) and CyclinD1 (D) (red) in colocalization with the neuronal marker NeuN (green) and the nuclear marker Hoescht (blue) in WT striatal primary cultures under basal conditions or after 100uM NMDA for 1h treatment. Scale bar, 25 μ m.

2.3.3.3 mHtt KI striatal cultures present increased Cdk4 neuronal expression

Finally, we analysed whether presence of mHtt affected neuronal expression of cell cycle proteins both under basal conditions and after NMDA treatment. We observed no overt differences in subcellular distribution between WT and mHtt KI mice neither in control conditions nor after NMDA treatment. Quantification of cell cycle proteins colocalization with NeuN showed significant changes between WT and mHtt KI cultures in basal conditions concerning Cdk4 neuronal expression, which was significantly increased in mHtt KI cultures (Fig. 46). E2F1 expression also showed an increasing trend between WT and mHtt KI cultures in basal conditions, whereas p27 and CyclinD1 showed a decreasing trend in mHtt KI compared to WT cultures under the same conditions (Fig. 46). No significant changes were detected either between WT and mHtt KI cultures after NMDA treatment, even though a decreasing trend could also be observed in p27 and CyclinD1 neuronal expression in mHtt KI compared to WT cultures (Fig. 46). Altogether, these results suggest that mHtt might induce increased expression of Cdk4 and E2F1, while decreasing p27 expression, which could contribute to increased cell cycle re-entry, given their respective roles as inducers and inhibitor of cell cycle progression. Reduced CyclinD1 expression is puzzling given its known function as a cell cycle progression inductor. However, its differential expression compared to its cell cycle partner, Cdk4, suggests that CyclinD1 might have additional functions in neuronal cells. Future studies increasing the number of analysed samples and analysing

specifically nuclear and cytosolic levels of cell cycle proteins in WT and mHtt KI cultures, might shed some light to mHtt-induced cell cycle proteins alterations.

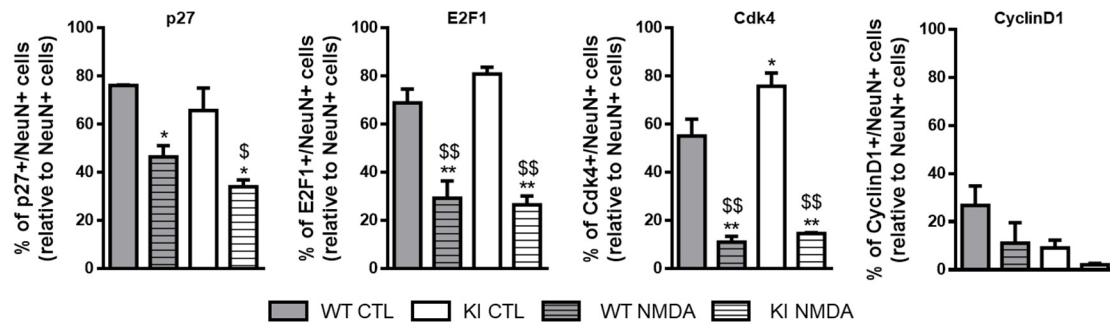


Figure 46. mHtt KI cultures present higher Cdk4 neuronal expression in Control conditions. Percentage of neurons expressing Cdk4, E2F1, p27 or CyclinD1 in WT cultures in control conditions or after 1h 100 μ M NMDA treatment. 10-20 photos each containing an average of 30 neurons were analysed per embryo, 2 embryos per condition are represented. * $p < 0.05$, ** $p < 0.01$ compared to WT Control. § $p < 0.05$, §§ $p < 0.01$ compared to KI Control.

DISCUSSION

Cdk5 is a multifaceted kinase with essential roles in the CNS participating in learning and memory processes (Angelo *et al*, 2006; Cheung *et al*, 2006; Lai and Ip, 2009). Extensive studies have shown Cdk5 contribution to neuronal dysfunction or death in response to several cell stressors such as A β , excitotoxicity or oxidative stress (Mushtaq *et al*, 2016; Patrick *et al*, 1999; Shelton and Johnson, 2004). Accordingly, deregulation of Cdk5 has been described in several neuropathological conditions (Cheung and Ip, 2012; McLinden, 2012; Su and Tsai, 2011). Based on these data and given that Huntington's disease is characterized by striatal neurodegeneration and by corticostriatal and hippocampal dysfunction associated with both motor and cognitive impairments, Cdk5 appears as a fitting target to simultaneously ameliorate both types of symptoms.

For this reason, in this Thesis, we have evaluated Cdk5 role in cognitive impairment and in synaptic alterations and we have further analysed its role in striatal vulnerability by studying cell cycle re-entry of striatal neurons in HD.

1. Role of Cdk5 in HD cognitive impairment

Appropriate learning and memory formation rely on precise regulation of multiple neuronal substrates that modulate processes such as, neurotransmitter release, neurotransmitter receptors levels or cytoskeletal remodelling. Because of its multiple synaptic substrates, Cdk5 acts as a hub in the regulation of these synaptic events. In accordance, inhibition of Cdk5 or its activator, p35, have a deep impact on learning and memory. Importantly, both impaired and enhanced cognitive performance have been reported as a result of Cdk5 inhibition, indicating that Cdk5 activity must be precisely regulated for proper synaptic plasticity regulation (reviewed in (Hawasli and Bibb, 2007)). Despite Cdk5 deregulation has been largely studied in several neuropathological conditions (Barnett and Bibb, 2011; Cruz and Tsai, 2004; McLinden, 2012), its involvement in cognitive decline in neurodegenerative disorders has not been broadly explored. Notwithstanding, it has been described that inhibition of Cdk5 in a mice model of Alzheimer's disease or in ischemic injury, ameliorates cognitive deficits present in both models (Gutiérrez-Vargas *et al*, 2015; Posada-Duque *et al*, 2015).

One of the earliest pathological hallmarks of HD is cognitive impairment, which has a huge impact on the patients' life (Li *et al*, 2003; Nithianantharajah and Hannan, 2013; Smith *et al*, 2005). Additionally, given that these deficits are more associated with altered synaptic transmission and neuronal dysfunction rather than with neuronal death, therapeutic treatments aiming to restore these early disturbances may present superior benefits than those targeting late disease events, when neuronal death is already apparent. Though previous studies from our group have demonstrated Cdk5 deregulation in the striatum of HD models and patients (Paoletti *et al*, 2008), no studies have examined its involvement in

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HD cognitive decline. In order to address this issue, we generated a new transgenic mice model, which expresses one copy of Htt with 111 CAGs and is conditionally heterozygous for Cdk5. We found that these new mice have preserved corticostriatal and hippocampal-dependent cognitive performance evaluated at 8 months of age, when their mHtt knock-in (KI) littermates present deficits in these functions, which denotes a critical role of Cdk5 dysfunction on cognitive disturbances in mHtt KI mice.

Cdk5 regulates multiple synaptic substrates that may contribute to preserve cognitive function in mHtt KI:Cdk5^{+/-} mice. Therefore, we focused on studying those Cdk5 molecular targets known to be altered in HD. First, we concentrated on NMDARs, since several studies have reported Cdk5-dependent NMDAR regulation associated with synaptic plasticity and memory (Hawasli *et al*, 2007; Li *et al*, 2001a; Plattner *et al*, 2014; Zhang *et al*, 2008b), while alterations on NMDARs expression and function have been largely reported in HD (Fan and Raymond, 2007; Luthi-Carter *et al*, 2003; Tyebji and Hannan, 2017). We found a significant reduction of NR2B surface levels in the cortex, striatum and hippocampus of mHtt KI mice, in accordance with previous studies showing a decrease of NR2A and NR2B mRNA and plasma membrane levels in the striatum, cortex and hippocampus of exon-1 transgenic R6/2 mice (Cepeda *et al*, 2001; Luthi-Carter *et al*, 2003). Similarly, reduced NR2B total levels have also been described in the striatum of full-length YAC128 mice, while no significant changes were found in the cortex or the hippocampus (Cowan *et al*, 2008). In this sense, we also detected brain region-dependent reduction of NR2B total levels, although our observations show that this decrease is specific for the cortex in mHtt KI mice. These results suggest that reduced NR2B surface levels in the striatum and hippocampus of mHtt KI mice probably involve impaired NMDAR trafficking, while decreased cortical NR2B surface levels may also include, altered subunit transcription and/or degradation. In fact, it has been described that NMDARs present altered trafficking to the plasma membrane in MSNs from YAC72 mice even though total levels of NR1 and NR2B are not significantly altered. This alteration in NMDARs trafficking involves increased rate of both insertion to the plasma membrane and internalization, which correlates with enhanced NMDAR current (Fan *et al*, 2007). Therefore, our results are in line with the idea that alteration of NMDARs is a common feature of HD models, affecting different aspects of NMDARs regulation (transcription, trafficking or degradation) in a different way depending on the HD model and the brain region analysed.

Notably, we observed that genetic reduction of Cdk5 was able to partially or completely restore these synaptic alterations via brain region-dependent pathways. Previous studies have described that Cdk5 indirectly regulates NR2B surface levels through phosphorylation of PSD-95, leading to reduced activation of Src (pSrc (Y416)), reduced pNR2B (Y1472) levels and increased NR2B internalization (Zhang *et al*, 2008b). Even though we were not able to

directly analyse phosphorylation levels of PSD-95 on the Cdk5-specific phosphorylation residues, mHtt KI mice showed reduced pSrc (Y416), pNR2B (Y1472) and reduced NR2B surface levels in the cortex. The fact that these alterations were completely recovered in mHtt KI:Cdk5^{+/-} mice, strongly indicates Cdk5 involvement in aberrant NR2B function/expression in mHtt KI mice. Conspicuously, we found no alteration of the other described Cdk5-dependent NMDAR regulation mechanisms, i.e., phosphorylation of NR2B at S1116 and promotion of NR2B degradation via calpain (Hawasli *et al*, 2007; Plattner *et al*, 2014), which highlights the specificity of the pSrc/pNR2B pathway in Cdk5-dependent cortical NR2B reduction in HD. Interestingly, we detected no significant changes in any of these Cdk5-dependent NMDARs modulatory pathways in the striatum, despite recovery of NR2B surface levels was observed in this region in mHtt KI:Cdk5^{+/-} mice. Therefore, it is possible that genetic reduction of Cdk5 in the striatum contributes to restore NR2B surface levels via recovery of other synaptic proteins involved in regulation of NMDARs trafficking or in their surface localization, such as PSD95. Nevertheless, changes in glutamatergic signalling from the cortex to the striatum have been proposed to correlate with alterations in striatal postsynaptic proteins in HD, implying an important role for cortical modifications in striatal dysfunction (Cepeda *et al*, 2003). Therefore, it can be hypothesized that restoration of cortical NR2B levels could contribute to normalize cortical function and therefore, cortical inputs to the striatum, which might prevent or delay alterations of striatal synaptic proteins. Finally, the partial recovery of NR2B surface levels in the hippocampus of mHtt KI mice following genetic reduction of Cdk5, suggests that Cdk5 deregulation might not play a major role in modulating hippocampal NR2B surface levels in mHtt KI mice. Additionally, this result also indicates that despite NMDARs pivotal role in synaptic plasticity, other Cdk5-dependent mechanisms likely participate in cognitive decline restoration in mHtt KI:Cdk5^{+/-} mice.

For this reason, we also analysed dendritic spine density, another process tightly associated with synaptic plasticity modulation. Our findings corroborated previous results from our group showing that in 8 months-old mHtt KI mice, CA1 hippocampal pyramidal neurons exhibit reduced dendritic spine density (Brito *et al*, 2014). Similarly, we also observed reduced dendritic spines in layer V cortical neurons, in agreement with previous data from our group (Puigdemívol *et al*, 2015). Notably, Cdk5 genetic reduction prevented dendritic spine density decrease in CA1 pyramidal neurons and in cortical layer V pyramidal neurons. Similar to our results on NMDARs regulation, the molecular mechanisms underlying normalization of hippocampal and cortical dendritic spine density also differed depending on the brain region analysed. Thus, even though Rac1 activity, known to be modulated by Cdk5, was reduced in the cortex of mHtt KI mice, Cdk5 genetic reduction did not completely prevent this decrease. These results suggest that, besides Rac1, other Cdk5 substrates, such as Kalirin or S6K (Lai *et al*, 2015; Xin *et al*, 2008), would be involved in restoration of cortical

DISCUSSION

dendritic spines in mHtt KI:Cdk5^{+/-} mice. On the other hand, despite similar hippocampal Rac1 activity was found in WT and mHtt KI mice, genetic reduction of Cdk5 led to increased levels of hippocampal Rac1 activity, both in Cdk5^{+/-} and in mHtt KI:Cdk5^{+/-} mice. These results are in agreement with previous studies describing increased Rac1 activity after Cdk5 inhibition in the hippocampus of an AD mouse model (Posada-Duque *et al*, 2015), and also with our results showing that Cdk5^{+/-} mice present increased dendritic spine density specifically in the hippocampus. Altogether, these data suggest that the recovery of cortical dendritic spines following Cdk5 reduction may involve prevention of mHtt-induced alterations in Cdk5 substrates that modulate dendritic spine density. In contrast, the reversion of hippocampal dendritic spines loss is likely a consequence of Cdk5 genetic reduction *per se*.

In line with our observations showing brain region-dependent modulation of Cdk5 substrates involved in synaptic plasticity and memory, previous studies have shown similar findings. For instance, it has been described that Cdk5 inhibition in the hippocampal CA1 region leads to decreased cAMP signalling due to increased PDE activity (Guan *et al*, 2011), whereas, inhibition of Cdk5 in the striatum causes increased cAMP levels attributed to Cdk5 phosphorylation of PDE4 (Plattner *et al*, 2015). Moreover, even though p25-overexpressing mice present a general neurodegenerative phenotype, neuronal loss is only observed in the hippocampus and the cortex, while striatal neurons only present morphological alterations without showing overt neuronal death (Cruz *et al*, 2003; Meyer *et al*, 2008).

Particularly, our findings showed preferential modification of Cdk5 substrates in the cortex of mHtt KI mice. Assessment of possible brain region-specific alterations of Cdk5 activity further corroborated these results. Therefore, the analysis of phosphorylation levels of Cdk5 substrates showed no significant changes between WT and mHtt KI in the hippocampus nor in the striatum, although Tau phosphorylation (pTau) showed an increasing trend in the striatum of mHtt KI mice. Conversely, phosphorylation levels of Tau were significantly increased in the cortex of mHtt KI mice compared with WT mice, while phosphorylation levels of IPP1 (pIPP1) and CDK substrates at serine residues (pCDK substrates) also showed an increasing trend. Importantly, while the increase in pTau and pIPP1 levels in mHtt KI was not modified following genetic reduction of Cdk5, levels of pCDK substrates showed a decreasing trend. The fact that changes in pCDK substrates between genotypes were more apparent in specific molecular weight bands, while others remained similar, suggests different Cdk5 substrate modulation between genotypes. Accordingly, previous studies have shown that in the striatum of p25-overexpressing mice, phosphorylation levels of Tau and IPP1 are increased, whereas Cdk5-dependent DARPP32 phosphorylation is decreased (Meyer *et al*, 2008). This differential regulation might result from changes in Cdk5 subcellular localization, and therefore of substrates availability.

In this line, our analysis of Cdk5/p35 subcellular distribution revealed specific alterations in Cdk5/p35 location in the cortex of mHtt KI mice, with no significant changes between genotypes in the striatum and the hippocampus. Thus, mHtt KI mice, presented significantly reduced plasma membrane p35 levels when compared with WT mice. This alteration was prevented in mHtt KI:Cdk5^{+/-} mice, suggesting that reduction of Cdk5 contributes to the recovery of proper p35 subcellular localization, and therefore to normalization of Cdk5 substrate specificity. Increased p35 plasma membrane localization in response to Cdk5 genetic reduction could be partially attributed to increased p35 levels, given that it is known that Cdk5 is one of the main promoters of p35 degradation (Patrick *et al*, 1998). In fact, our results showed that, while we detected no changes in total p35 levels between genotypes, both Cdk5^{+/-} and mHtt KI:Cdk5^{+/-} mice presented augmented p35 levels in the cytosol. This increase in the cytosol could facilitate p35 availability for plasma membrane localization. In addition, it has been described that inhibition of Cdk5 leads to increased p35 levels and Rac1 activity (Posada-Duque *et al*, 2015), and in turn, it has been shown that specific plasma membrane Rac1 activity regulates p35 and Cdk5 plasma membrane localization (Sananbenesi *et al*, 2007). Therefore, it is possible that, even though we did not find changes in total Rac1 activity in the cortex of Cdk5^{+/-} and mHtt KI:Cdk5^{+/-} mice, reduction of Cdk5 could promote specific increase of plasma membrane Rac1 activity, thus facilitating p35 localization to this compartment. Moreover, it has also been described that localization of p35 to the plasma membrane is associated with its dephosphorylation by PP1 (Sato *et al*, 2007), which is inhibited by Cdk5 phosphorylation (Hou *et al*, 2013). Hence, reduction of Cdk5 could lead to increased PP1 activity and reduced p35 phosphorylation, contributing to its localization to the plasma membrane (Fig. 47). Anyhow, restoration of p35 plasma membrane levels in the cortex of mHtt KI mice could underlie normalization of Cdk5 substrates highlighting the relevance of proper Cdk5/p35 subcellular localization for normal Cdk5 function.

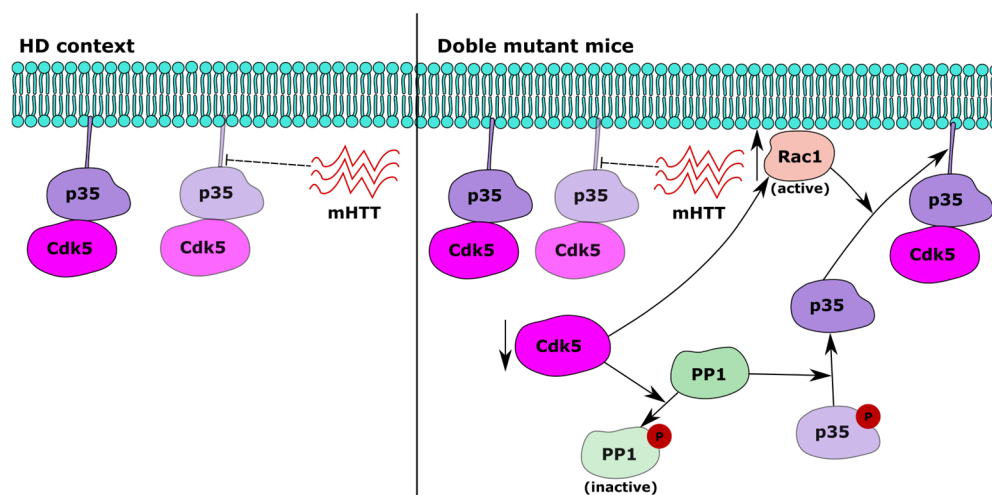


Figure 47. Proposed mechanisms underlying restoration of p35 plasma membrane localization following Cdk5 genetic reduction in the cortex of mHtt KI:Cdk5^{+/-} mice. Reduction of Cdk5 could increase active PP1 and plasma membrane Rac1 activity, favouring p35 plasma membrane localization.

DISCUSSION

Even though previous studies from our group showed that mHtt KI mice exhibit increased p25 levels in the striatum, we were not able to detect presence of p25 in our conditions. This could be attributed to different technical conditions or to the fact that previous studies were performed in homozygous mHtt KI mice at 9 months of age, which might manifest a more severe phenotype. Nonetheless, it is important to remember that, despite Cdk5 activity is usually assessed based on p35 and p25 levels, its regulation is much more complex. Thus, proteins like CyclinI, GSTP1 and CyclinE have also been described to modulate Cdk5 activity, as well as several posttranslational modifications including phosphorylation, acetylation and S-nitrosylation (Shah and Lahiri, 2014). Therefore, we cannot rule out that some of these regulatory mechanisms may participate in Cdk5 alteration in HD and, subsequently, in functional recovery in mHtt KI:Cdk5^{+/-} mice. For instance, aberrant S-nitrosylation has been described in several neurodegenerative disorders such as AD, and has been associated to excessive NMDAR activation and to mitochondrial dysfunction (Nakamura *et al*, 2015; Nakamura and Lipton, 2017), which are also present in HD (Zuccato *et al*, 2010). Given that both Cdk5 and p35 have been described to be S-nitrosylated (Qu *et al*, 2011; Zhang *et al*, 2015), it is possible that changes in Cdk5 and/or p35 S-nitrosylation might be additional regulatory pathways contributing to Cdk5 deregulation in HD. In sum, the complex regulation of Cdk5 at the molecular level, adds up to the already intricate Cdk5 function on a subcellular level and between different brain regions, which, altogether, complicates the analysis of Cdk5 deregulation in pathological conditions and reveals the need of further studying Cdk5 function on a physiological context.

Indeed, most studies analysing the physiological brain function of Cdk5 have concentrated mainly in the striatum and hippocampus, while reports in the cortex are mostly restricted to Cdk5 role in neuronal migration during neurodevelopment, with few studies in the adult brain. In this sense, our observations of increased cytosolic p35 as a result of Cdk5 genetic reduction, specifically in the cortex, might reflect differential regulatory feedback between Cdk5 and p35 in this brain region compared to the striatum or the hippocampus. This hypothesis could explain our results showing that, at least in an HD context, Cdk5 genetic reduction modifies Cdk5 substrates preferentially in the cortex (Table 10). Given that cortical alterations are common in different neurodegenerative disorders including AD, a deeper study of Cdk5 physiological function in the cortex, might be critical to understand neuropathologies involving Cdk5 deregulation in this region.

Importantly, cortex affectation in HD starts years prior to disease onset (Paulsen *et al*, 2008; Rosas *et al*, 2008a), and, for instance, specific mHtt expression in the striatum does not reproduce some HD hallmarks like locomotor deficits (Gu *et al*, 2007), indicating the relevance of aberrant cortical inputs for mHtt-induced striatal vulnerability. In fact, previous studies from our group have demonstrated synaptic disturbances in the cortex prior to

striatal alteration, correlating with impaired corticostriatal cognitive performance prior to motor onset (Puigdemívol *et al*, 2015). Even though we cannot rule out implication of other non-analysed Cdk5-dependent mechanisms in cognitive restoration in the striatum and hippocampus of mHtt KI:Cdk5^{+/-} mice, our current results are in line with the hypothesis that cortical dysfunction might play a key role in early pathogenesis of HD. Particularly, our observations suggest that Cdk5 might be an important player in HD's cortical dysfunction.

CORTEX		STRIATUM		HIPPOCAMPUS	
mHtt KI	mHtt KI:Cdk5 ^{+/-}	mHtt KI	mHtt KI:Cdk5 ^{+/-}	mHtt KI	mHtt KI:Cdk5 ^{+/-}
↓ NR2B total	↑ NR2B total	= NR2B total	= NR2B total	= NR2B total	= NR2B total
↓ NR2B surface	↑ NR2B surface	↓ NR2B surface	↑ NR2B surface	↓ NR2B surface	(↑) NR2B surface
↓ pSrc / pNR2B	↑ pSrc / pNR2B	= pSrc / pNR2B	= pSrc / pNR2B	= pSrc / pNR2B	= pSrc / pNR2B
↓ dendritic spines	↑ dendritic spines			↓ dendritic spines	↑ dendritic spines
↓ Rac1 activity	= Rac1 activity			= Rac1 activity	↑ Rac1 activity
↑ pTau	= pTau	(↑) pTau	= pTau	= pTau	= pTau
(↑) pIPP1	= pIPP1	= pIPP1	= pIPP1	= pIPP1	= pIPP1
(↑) pCDK substrates	(↓) pCDK substrates	= pCDK substrates	= pCDK substrates	= pCDK substrates	= pCDK substrates
↓ p35 plasma membrane	(↑) p35 plasma membrane	= p35 plasma membrane	= p35 plasma membrane	= p35 plasma membrane	= p35 plasma membrane
= p35 cytosol	(↑) p35 cytosol	= p35 cytosol	= p35 cytosol	= p35 cytosol	= p35 cytosol

Table 10. Brain region-dependent modification of Cdk5 substrates. Summary of observed alterations in the stated substrates in the cortex, striatum and hippocampus of mHtt KI and mHtt KI:Cdk5^{+/-} compared to WT and mHtt KI mice, respectively. Arrows indicate significant changes between genotypes, while arrows in parentheses indicate no significant trends.

In sum, our results highlight the relevance of Cdk5 deregulation in HD, not only as a contributor to neuronal degeneration, but also as an intricate player of neuronal dysfunction and synaptic plasticity alterations. These results are relevant to understand the molecular mechanisms underlying mHtt-induced cognitive alterations in HD, and might also provide clues for other neuropathological conditions involving synaptic alterations and Cdk5 deregulation.

2. Cell cycle role in HD striatal vulnerability

Cdk5 deregulation has been associated with neuronal dysfunction and/or death in multiple neuropathological conditions (Liu *et al*, 2016; Lopes and Agostinho, 2011; Patrick *et al*, 1999). In HD, Cdk5 has been involved in several known striatal vulnerability factors, such as mitochondrial dysfunction (Cherubini *et al*, 2015) or increased sensitivity to glutamatergic and dopaminergic signalling (Paoletti *et al*, 2008; Park *et al*, 2012). On the other hand, Cdk5 has been implicated in neuronal cell cycle re-entry in response to neurotoxic factors such as β -amyloid and prion peptides (Lopes *et al*, 2009; Niu *et al*, 2012). Coincidentally, cell cycle dysregulation has also been associated with alterations present in HD such as excitotoxicity and DNA damage (Kruman *et al*, 2004; Park *et al*, 2000). For these reasons, we wanted to assess whether Cdk5 deregulation could also lead to cell cycle deregulation in HD, and the implication of neuronal cell cycle re-entry in HD striatal vulnerability.

To act as a cell cycle inhibitor, it has been described that, Cdk5 needs to be located in the nucleus of differentiated neurons (Zhang *et al*, 2008a). Interestingly, our results showed that nuclear Cdk5 levels were significantly reduced in the striatum of 8-month-old mHtt KI mice, suggesting that cell cycle inhibition by Cdk5 could be altered by mHtt presence. We also observed reduction of nuclear p27 levels in mHtt KI mice, which could underlie reduction of Cdk5 levels, given that Cdk5 nuclear localization relies on p27 interaction, as Cdk5 does not possess a NLS (Zhang *et al*, 2010a). Reduced p27 nuclear levels could be caused, in turn, by impaired p27 nuclear import as a result of mHtt presence, which is in agreement with reports describing alterations in nucleocytoplasmic trafficking in HD (Grima *et al*, 2017; Truant *et al*, 2007). In contrast, analysis of total p27 levels in striatal lysates revealed a significant increase in mHtt KI mice from 6 months of age onwards. These results indicate that reduced p27 nuclear levels are likely not caused by alterations in p27 transcription or degradation and therefore, correlate with the hypothesis that reduced p27 nuclear levels might be associated with altered nucleocytoplasmic transport. On the other hand, increase in total p27 levels might be related to reduced p27 degradation, as it has been described that the transcriptional coactivator Jab1 which degrades p27, is sequestered in mHtt aggregates in HD patient's brains (Cong *et al*, 2012). Given that mHtt KI mice already present mHtt aggregates at 6 months of age (Lloret *et al*, 2006), the same mechanism might underlie our observations.

Regardless of the underlying mechanisms, reduced Cdk5 and p27 nuclear localization might affect formation of the nuclear Cdk5/p27/E2F1 complex. It has been described that formation of this complex prevents cell cycle progression by sequestering the transcription factor E2F1 from activating necessary genes for cell cycle initiation (Zhang and Herrup, 2011). Hence, even though we found no significant changes in global Cdk5 and p27

interaction, reduced Cdk5 and p27 nuclear localization might result in reduced formation of the Cdk5/p27/E2F1 complex, and subsequently, it could lead to aberrant cell cycle re-entry of HD striatal neurons (Fig. 48).

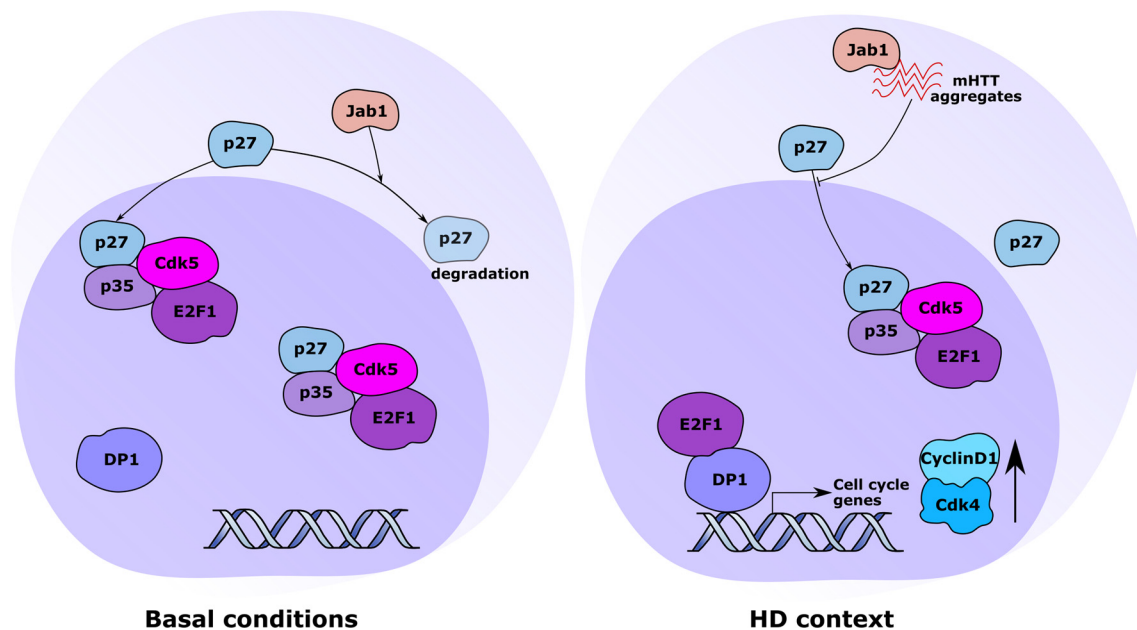


Figure 48. Proposed mechanism underlying Cdk5-dependent cell cycle proteins alteration in HD. Presence of mHtt aggregates in mHtt KI mice could cause sequestering of Jab1 contributing to increased p27 levels. mHtt could also impair p27, and consequently Cdk5, nuclear localization, disrupting formation of the Cdk5/p27/E2F1 complex, which could allow E2F1 to activate transcription of cell cycle genes.

In this regard, we observed that the early G₁ phase protein, CyclinD1 presented a trend towards increased protein levels in the striatum of 6-month-old mHtt KI mice. This increase became significant at motor onset (8 months) and was maintained at later disease stages (13 and 18 months), indicating a sustained alteration of CyclinD1 levels by the presence of mHtt. At 18 months of age, levels of the CyclinD1 partner, Cdk4, also presented an increasing trend. Accordingly, previous studies have found increased Cdk4 protein levels as well as increased Cdk4 and CyclinE neuronal expression in the striatum of 7-month-old R6/1 mice (Fernandez-Fernandez *et al*, 2011). CyclinB1 has also been detected in the striatum of R6/2 mice in contrast with WT mice which show no expression of this protein (Liu *et al*, 2015). Therefore, this data suggests that alteration of cell cycle proteins expression might be a common mechanism resulting from mHtt presence in both full-length and N-terminal fragment HD mice models.

Importantly, we also observed alteration of cell cycle proteins in the putamen of HD human patients. Specifically, we detected a significant increase of Cdk4 protein levels, whereas CyclinD1 levels were not significantly altered. Human HD putamen samples also presented p27 electrophoretic mobility alterations, which might reflect specific post-translational modifications leading to increased molecular weight, such as sumoylation or ubiquitination

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(Hilgarth and Sarge, 2005; Xu and Jaffrey, 2013). In addition, E2F1 exhibited an increase in a lower molecular weight band, which could be associated with increased E2F1 cleavage, as it has been described that in NMDAR-mediated toxicity conditions, E2F1 is cleaved to a stable 55-kDa band (Zyskind *et al*, 2015). Previous studies had already described alteration of cell cycle proteins in samples from HD patients brains (Fernandez-Fernandez *et al*, 2011; Pelegrí *et al*, 2008), further corroborating the hypothesis that mHtt presence causes aberrant expression of cell cycle proteins. Nonetheless, the fact that alteration of specific proteins differs between HD mice models and human patients, suggests that neuropathological differences such as presence or absence of neuronal death or of mHtt aggregates, might lead to differential deregulation of cell cycle proteins.

In fact, deregulation of cell cycle proteins has been directly or indirectly associated in previous studies with mHtt aggregate formation. For instance, presence of perinuclear mHtt aggregates leading to nuclear envelope disruption has been described to cause cell cycle re-entry (Liu *et al*, 2015). Moreover, alteration of ER-stress response in HD, which is activated in response to misfolded proteins, has also been associated with alteration of cell cycle proteins in HD models (Fernandez-Fernandez *et al*, 2011). On the other hand, 3-NP treatment has been described to induce alteration of cell cycle proteins both *in vivo* and *in vitro* (Akashiba *et al*, 2008; Pelegrí *et al*, 2008). Importantly, even though 3-NP treatment causes death of both striatal and cortical neurons, only striatal neurons show p27 reduction and increased calpain activity after 3-NP treatment, and are partially neuroprotected by CDKs inhibitors (Akashiba *et al*, 2008). These data indicate that cell cycle re-entry might be an additional mechanism resulting from inherent striatal susceptibility properties, and therefore could play a role in HD increased striatal vulnerability.

In this sense, excitotoxicity represents one of the main striatal vulnerability factors in HD (Fan and Raymond, 2007) and, it has also been associated with aberrant expression of cell cycle proteins (Park *et al*, 2000). Excitotoxic responses caused by excessive NMDARs activation is also one of the mechanisms leading to Cdk5 hyperactivation via p35 cleavage to p25 (Lee *et al*, 2000; Patrick *et al*, 1998). For these reasons, we explored whether alteration of cell cycle proteins in HD, might be associated with NMDA activation and/or with subsequent Cdk5 hyperactivation. Our results showed that treatment of mature striatal primary cultures with 100 μ M NMDA for 1h, led to a general reduction of cell cycle proteins expression in neurons. These data contrast with the hypothesis that excitotoxic stress might lead to re-expression of cell cycle proteins. However, we also observed important alterations in the subcellular distribution of the analysed cell cycle proteins. Thus, NMDA treatment led to a reduction of nuclear levels of p27, which could prevent its role as a cell cycle suppressor contributing to cell cycle initiation. Cytosolic localization of the cell cycle promoter E2F1 in basal striatal cultures was also altered following NMDA treatment. A

diffuse distribution along the entire neuron, including presence in the nucleus of some neurons, was observed, which might reflect an activation of E2F1 function as a transcription factor of cell cycle progression genes. Similar distribution alterations were observed for the early G₁ phase kinase Cdk4, which could also reflect cell cycle initiation of striatal neurons after NMDA treatment. On the other hand, CyclinD1 distribution, which showed mostly nuclear localization in basal conditions, presented cytosolic, in addition to nuclear localization, after NMDA treatment. Therefore, our results indicate that, although NMDA treatment causes a general reduction of cell cycle proteins expression, it also leads to a significant alteration of their subcellular distribution, which undoubtedly affects its physiological function and could lead to cell cycle re-entry.

Nonetheless, the fact that CyclinD1 was expressed only in the nucleus of a small subset of neurons under basal conditions, might reflect additional functions of CyclinD1 on certain striatal neurons. In fact, not much is known about cell cycle proteins function in differentiated neurons, given that, with the exception of cell cycle inhibitors, they would be expected to show little expression in non-proliferating cells. Therefore, the fact that most cell cycle proteins are expressed in neurons, sometimes in cellular compartments other than the nucleus, indicates their implication in alternative functions, complicating the extrapolation of functional effects from changes in their levels and localization. For instance, increase of E2F1 levels has been associated with neuronal death in neurodegeneration in *in vitro* models. Importantly, this function has been attributed to cytosolic localization of E2F1, without alteration of some of the main E2F1 transcription products, suggesting a cell cycle-independent function for E2F1 in a neurodegenerative context (Wang *et al*, 2010). In this sense, our results show that under basal conditions, in contrast with other analysed cell cycle progression proteins, like Cdk4 and CyclinD1, E2F1 was expressed in the cytosol of 94% of striatal neurons, suggesting that E2F1 cytosolic expression is normal in striatal neurons. Other functions like regulation of cell motility and migration, DNA repair, transcription modulation or even synaptic plasticity and memory formation, have also been ascribed to cell cycle proteins like p27, CyclinD1 or CyclinE (Frank and Tsai, 2009; Larrea *et al*, 2009; Lim and Kaldis, 2013; Odajima *et al*, 2011). In this sense, it is also possible that some of the alterations we have observed in cell cycle proteins levels and distribution after NMDA treatment may affect some of these cell cycle-independent functions, leading to neuronal dysfunction through additional mechanisms besides neuronal cell cycle re-entry.

We also observed that some of the NMDA-dependent alterations in cell cycle proteins were dependent on calpain activation. Thus, in accordance with previous studies (Akashiba *et al*, 2006b; Zyskind *et al*, 2015) we observed that reduced p27 levels and E2F1 cleavage after NMDA treatment were prevented when calpain was inhibited. It would be interesting to analyse if calpain inhibition also prevents changes in subcellular localization of these

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proteins. Additionally, given that calpain inhibition also avoids p25 production and subsequent Cdk5 hyperactivation, future studies specifically inhibiting Cdk5, might reveal if NMDA-dependent cell cycle proteins alterations are directly dependent on calpain or if Cdk5 hyperactivation is also involved. As calpain is activated by intracellular calcium (Khorchid and Ikura, 2002), and deregulation of calcium homeostasis is an HD hallmark (Giacomello *et al*, 2013), aberrant calpain activation might contribute to calpain-dependent cell cycle proteins alteration in HD.

In this sense, our preliminary results regarding mHtt effect on cell cycle proteins indicate that mHtt led to higher expression of cytosolic Cdk4 and E2F1 in neuronal cells under basal conditions, while the small subset of neurons expressing CyclinD1 showed a decreasing trend in mHtt KI cultures. In contrast, NMDA treated mHtt KI cultures showed similar alterations when compared with NMDA treated WT cultures, with the exception of p27, which showed a decreasing trend in mHtt KI cultures, indicating that mHtt presence could further reduce NMDA-induced p27 decrease, contributing to cell cycle re-entry. Given that p27 localization in striatal neurons is mainly nuclear, this data would fit with our observations in the striatum of mHtt KI mice which also show decreased p27 nuclear levels. Therefore, future studies contrasting these results and analysing whether mHtt affects subcellular distribution of cell cycle proteins, might shed some light to the role of neuronal cell cycle re-entry in HD.

Notwithstanding, it is noteworthy that, even though re-expression of cell cycle proteins has been associated with aberrant cell cycle re-entry, it has also been proposed to play a role in neuronal DNA repair responses. Neurons are particularly vulnerable to DNA damage caused by toxic species resulting from their high metabolic rate. Given that most DNA repair mechanisms rely on cell cycle progression, activation of certain cell cycle phases has been proposed to be necessary for activation of certain DNA repair responses (Schwartz *et al*, 2007). Importantly, altered DNA repair responses have been described in HD (Enokido *et al*, 2010) and have been associated with increased genomic instability, which shows high correlation with neuronal dysfunction in HD (Manley *et al*, 1999). Thus, altered cell cycle proteins expression in HD might also be associated with DNA repair responses and therefore with genomic instability.

Altogether, alteration of cell cycle proteins has been described in several neuropathological conditions and has been associated with cell cycle re-entry of differentiated neurons contributing to neuronal dysfunction or death. Nonetheless, it is evident that a lot of information is still lacking regarding physiological functions of cell cycle proteins in differentiated neurons and therefore interpretation of functional effects resulting from their alteration can be complex. Alteration of cell cycle proteins has received little attention, particularly in HD, even though several molecular mechanisms involved with cell cycle re-

entry are associated with increased striatal vulnerability. Therefore, further study of this mechanism might contribute to understanding, not only molecular pathways contributing to striatal affectation in HD, but to neuronal dysfunction processes in other neurodegenerative disorders, therefore providing new therapeutic targets for their treatment.

3. Cdk5 as a dual player in neuronal vulnerability and synaptic plasticity

Over the last decades Cdk5 has become a pivotal player in synaptic plasticity modulation and in neurodegenerative processes associated to several pathological conditions. However, its relevant function goes in hand with its complexity making difficult full understanding of its regulation both under physiological and pathological conditions.

The results obtained in this Thesis reflect this intricacy. Particularly, we propose that involvement of Cdk5 deregulation in striatal vulnerability in HD might be preferentially associated to calpain activation and p35 cleavage to p25. This situation would be favoured by calcium homeostasis dysregulation caused, in part, by excitotoxicity and mitochondrial dysfunction, present in HD (Cherubini and Ginés, 2017; Fan and Raymond, 2007; Giacomello *et al*, 2011). Our observations suggest that alteration of cell cycle proteins might be an additional mechanism participating in this scenario. On the other hand, deregulation of Cdk5 associated to reduced p35/Cdk5 plasma membrane localization might have a more prevalent role in altered Cdk5 synaptic substrates availability, and therefore to synaptic plasticity alterations. Importantly, this deregulation on a subcellular level might not be necessarily associated with production of p25 and therefore could go undetected when analysing total levels of Cdk5/p35 or some of the substrates commonly used as Cdk5 activity indicators. We propose that this kind of deregulation is particularly relevant in cortical dysfunction in HD, and therefore could also play an important role in other pathological processes affecting this region (Fig. 49).

In conclusion, our results point Cdk5 as a potential target for simultaneous treatment of synaptic plasticity alterations and neuronal vulnerability present in HD, but also highlight the relevance of further studying Cdk5 intricate function which could contribute to obtaining therapeutic targets for other neuropathological conditions involving Cdk5 deregulation.

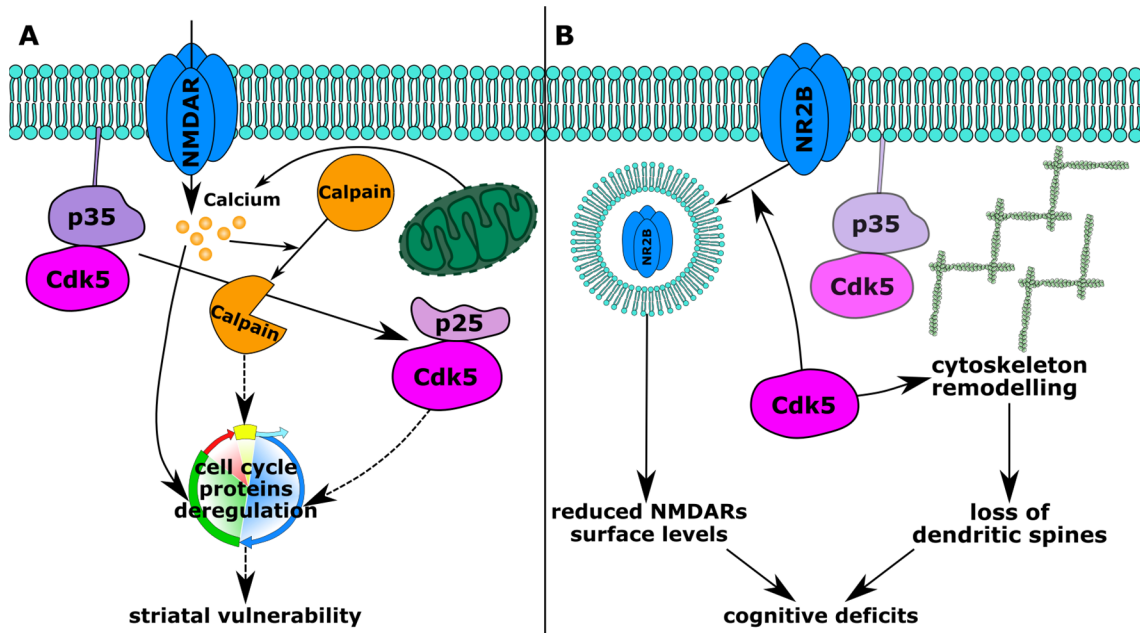


Figure 49. Proposed Cdk5 deregulation mechanisms involved in striatal vulnerability and cognitive decline, respectively, in HD. A. Excessive NMDAR activation and calcium dysregulation in the striatum might favour calpain activation and p25 production. In this context, deregulation of cell cycle proteins might contribute to striatal vulnerability. **B.** Delocalization of Cdk5/p35 from the plasma membrane in the cortex, might alter regulation of Cdk5 substrates leading to reduced NMDARs surface levels and to dendritic spine loss, contributing to cognitive decline in HD.

CONCLUSIONS

As a result of the presented studies, the main conclusions of this Thesis are:

1. Cdk5 is a new important contributor to corticostriatal and hippocampal-dependent cognitive deficits in mHtt KI mice.
2. Prevention of cognitive deficits by Cdk5 genetic reduction in mHtt KI mice is associated with brain region-specific modulation of synaptic plasticity substrates.
3. Rescue of corticostriatal deficits in double mutant mHtt KI:Cdk5^{+/-} mice is associated with restoration of mHtt induced-synaptic alterations in the cortex. This recovery is mediated by normalization of dendritic spine density and NR2B surface levels through the pSrc/pNR2B pathway. In contrast, prevention of hippocampal deficits associates with restoration of dendritic spine density which correlates with increased Rac1 activity, resulting from Cdk5 genetic reduction.
4. mHtt alters Cdk5/p35 subcellular localization specifically in the cortex of mHtt KI mice, which correlates with preferential alteration of Cdk5 substrates in this region.
5. Symptomatic mHtt KI mice exhibit reduced Cdk5 and p27 nuclear levels in the striatum, suggesting that mHtt may induce cell cycle re-entry by impairing their function as cell cycle inhibitors.
6. mHtt causes alteration of G₁ phase cell cycle proteins expression in the striatum of mHtt KI mice and in the putamen of HD patients, which could further contribute to aberrant cell cycle re-entry.
7. Alteration of cell cycle proteins in response to Cdk5 deregulation and/or excessive NMDARs activation, might be a new molecular mechanism involved in striatal vulnerability in HD.

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ANNEX



Cdk5 Contributes to Huntington's Disease Learning and Memory Deficits via Modulation of Brain Region-Specific Substrates

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Abstract

Cognitive deficits are a major hallmark of Huntington's disease (HD) with a great impact on the quality of patient's life. Gaining a better understanding of the molecular mechanisms underlying learning and memory impairments in HD is, therefore, of critical importance. Cdk5 is a proline-directed Ser/Thr kinase involved in the regulation of synaptic plasticity and memory processes that has been associated with several neurodegenerative disorders. In this study, we aim to investigate the role of Cdk5 in learning and memory impairments in HD using a novel animal model that expresses mutant huntingtin (mHtt) and has genetically reduced Cdk5 levels. Genetic reduction of Cdk5 in mHtt knock-in mice attenuated both corticostriatal learning deficits as well as hippocampal-dependent memory decline. Moreover, the molecular mechanisms by which Cdk5 counteracts the mHtt-induced learning and memory impairments appeared to be differentially regulated in a brain region-specific manner. While the corticostriatal learning deficits are attenuated through compensatory regulation of NR2B surface levels, the rescue of hippocampal-dependent memory was likely due to restoration of hippocampal dendritic spine density along with an increase in Rac1 activity. This work identifies Cdk5 as a critical contributor to mHtt-induced learning and memory deficits. Furthermore, we show that the Cdk5 downstream targets involved in memory and learning decline differ depending on the brain region analyzed suggesting that distinct Cdk5 effectors could be involved in cognitive impairments in HD.

Keywords Huntingtin · Cdk5 · Cognition · NR2B · Rac1 · Dendritic spines

The authors wish it to be known that, in their opinion, Elena Alvarez-Periel and Mar Puigdemívol should be regarded as joint first authors.

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Introduction

Huntington's disease (HD) is a genetic neurodegenerative disorder caused by a polyglutamine expansion in the huntingtin protein (Htt) [1]. HD is classically characterized by the appearance of motor symptoms caused by the selective degeneration of striatal medium spiny neurons [2, 3]. However, learning and memory disturbances, attributed to altered corticostriatal connectivity and hippocampal dysfunction, manifest years before the onset of motor impairments and notably affect the quality of life of HD patients and their caregivers [4–9].

Although the exact molecular mechanisms linking mutant Htt (mHtt) expression to cognitive deficits are not completely understood, it is known that processes such as synaptic dysfunction and dendritic spine loss occur prior to striatal degeneration (reviewed in [10–13]). Consistent with this idea, altered expression of both presynaptic and postsynaptic proteins in the striatum, cortex, and hippocampus has been reported in various HD mouse models [10, 14–18]. Furthermore, HD

mouse models feature structural alterations at dendritic spines and loss of synaptic function [19–23].

Cyclin-dependent kinase 5 (Cdk5) is a proline-directed serine/threonine protein kinase that shows high level of activity in the central nervous system (CNS) due to the expression of its activators p35 and p39 [24, 25]. An extensive body of research has implicated Cdk5 as a crucial regulator of a plethora of pre- and postsynaptic substrates, allowing Cdk5 to modulate processes such as vesicle cycling, neurotransmitter receptor clustering and function, neurotransmission, and remodeling of dendritic spines (reviewed in [26–28]). In particular, it has been reported that Cdk5 regulates the levels of the NMDA receptor (NMDAR) subunits NR2A and NR2B [29–31] as well as several proteins involved in dendritic spine remodeling, such as WAVE1 [32], ephexin1 [33], Kalirin [34], S6K [35], and Rac1 [36, 37]. Moreover, Cdk5 has been involved in several neurodegenerative disorders, including Alzheimer's disease (AD) and HD (reviewed in [38–40]).

Consistent with a role of Cdk5 in HD, we previously found that Cdk5 contributes to increased vulnerability of striatal cells to dopaminergic and glutamatergic inputs in the presence of mHtt [41, 42]. Based on these findings, we hypothesize that Cdk5 might play a role, not only in striatal vulnerability but also in cognitive deficits in HD. To test this idea, we have generated a new double mutant mouse heterozygous for Cdk5 (Cdk5^{+/−}) and expressing full-length mHtt (Hdh^{Q7/Q111}). In contrast to the mHtt KI mouse line (Hdh^{Q7/Q111}) that exhibits deficits in corticostriatal and hippocampal-dependent learning and memory, we found that the behavioral performance in double mutant mice (Cdk5^{+/−};Hdh^{Q7/Q111}) was preserved. Moreover, we uncovered that the molecular mechanisms by which Cdk5 contributes to motor and procedural learning impairment partially differs from those involved in hippocampal memory dysfunction. Together these data suggest a complex role for Cdk5 in learning and memory processes and in its contribution to cognitive deficits in HD.

Materials and Methods

Animals

Hdh^{Q111} knock-in mice, with targeted insertion of 109 CAG repeats that extends the glutamine segment in murine huntingtin to 111 residues, were maintained on a C57BL/6 genetic background. Female Hdh^{Q7/Q7} mice were crossed with male Hdh^{Q7/Q111} mice to generate age-matched Hdh^{Q7/Q7} wild-type (WT) and Hdh^{Q7/Q111} knock-in (mHtt KI) littermates, determined by PCR analysis. The average CAG repeat length in our colony measured by DNA sequencing technique was 111 ± 1.3. All mice used in the present study were males and 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 19–22 °C and 40–60% humidity, under a 12-h light/dark cycle. All procedures were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the local animal care committee of the Universitat de Barcelona (76/15) and Generalitat de Catalunya (00/1094), in accordance with the Directive 2010/63/EU of the European Commission.

Generation of New Transgenic mHtt KI:Cdk5^{+/−} Mice

Homozygous floxed Cdk5 mice were generated as previously described [30]. Using homologous recombination, exons encoding Cdk5 catalytic domain regions were flanked with *LoxP* elements (floxed). We crossed homozygous Cdk5 floxed (generously provided by Dr. P. Greengard, Laboratory of Molecular and Cellular Neuroscience, Rockefeller University, USA) and homozygous knock-in Hdh^{Q111/Q111} mice to obtain heterozygous Cdk5 floxed and heterozygous knock-in mice (Cdk5^{fllox/+}; Hdh^{Q7/Q111} mice). These mice were crossed again with homozygous Cdk5 floxed mice to obtain homozygous Cdk5 floxed and heterozygous knock-in mice (Cdk5^{fllox/fllox}; Hdh^{Q7/Q111} mice). In collaboration with Dr. Carlos A. Saura (Institute of Neuroscience, Department of Biochemistry and Molecular Biology, Universitat Autònoma de Barcelona, Spain), we obtained the Cre-CaMKII^{+/−} line. These mice express Cre recombinase protein under the CaMKII alpha promoter. We crossed homozygous Cdk5 floxed and heterozygous knock-in mice with Cre-expressing mice to obtain the final genotypes: Cdk5^{fllox/+}; Hdh^{Q7/Q7}; Cre^{+/−} (Hdh^{Q7/Q7}; Cdk5^{+/+} or WT), Cdk5^{fllox/+}; Hdh^{Q7/Q111}; Cre^{+/−} (Hdh^{Q7/Q111}; Cdk5^{+/+} or mHtt KI), Cdk5^{fllox/+}; Hdh^{Q7/Q7}; Cre^{+/−} (Hdh^{Q7/Q7}; Cdk5^{+/−} or Cdk5 heterozygous mice), Cdk5^{fllox/+}; Hdh^{Q7/Q111}; Cre^{+/−} (Hdh^{Q7/Q111}; Cdk5^{+/−} or double mutant mice). All mice were on a C57BL/6 genetic background. For more information, see Supplementary Fig. 1. Primers for allele genotyping had the following nucleotide sequence: (1) Cdk5: 5'-GCTGCAATGGTGACCTGGAC-3' and 5'-CCTCAGCCTTATGATGCTC-3', (2) Cre: 5'-GCCTGCATTAACGGTCGATGCAACGA-3' and 5'-GTGGCAGATGGCGCGCAACACCATT-3', and (3) mHtt: 5'-ATGAAGGCCTTCGATCCCTCAAGTCTTTC-3' and 5'-GGCGGCTGAGGAAGCTGAGGA-3'.

Western Blot Analysis

Wild-type (Hdh^{Q7/Q7}), mHtt KI (Hdh^{Q7/Q111}), Cdk5 heterozygous (Cdk5^{+/−}), and double mutant (mHtt KI:Cdk5^{+/−}) mice

were killed by cervical dislocation at the age of 7–8 months. The brain was quickly removed, dissected, frozen in dry ice, and stored at -80°C until use. Brain tissue was homogenized in cold lysis buffer (140 mM NaCl, 10 mM HEPES (pH 7.4), 1 mM EGTA, 0.1 mM MgCl_2 , 1% X-100 Triton, 0.5% NP-40 supplemented with 1 mM sodium orthovanadate and protease inhibitor cocktail (Sigma-Aldrich)) with a dounce homogenizer, left 1 h at 4°C in ice, and cleared by centrifugation at 13,000g for 15 min at 4°C to collect the supernatants. Following determination of protein contents by Detergent-Compatible Protein Assay (Bio-Rad), protein extracts (20 μg) were mixed with 5 \times SDS sample buffer, boiled for 5 min, resolved on 6–10% SDS-PAGE, and transferred to nitrocellulose membranes (Whatman Schleicher & Schuell). After incubation (1 h) in blocking buffer containing 10% non-fat powdered milk in TBS-T (50 mM Tris-HCl, 150 mM NaCl, pH 7.4, 0.05% Tween 20), membranes were blotted overnight at 4°C with primary antibodies: Cdk5 (1:1000; Santa Cruz Biotechnology), p35 (1:500; Santa Cruz Biotechnology), transferrin receptor (1:5000; Invitrogen), NR1 (1:1000; Chemicon), NR2A (1:1000; Millipore), NR2B (1:1000; Cell Signaling Technology), phospho-NR2B (Tyr1472) (1:1000; Cell Signaling Technology), phospho-NR2B (Ser1116) (1:1000 provided by Dr. J.A. Bibb), phospho-Src (Tyr416) (1:1000; Cell Signaling Technology), Src (1:2000; Cell Signaling Technology), and Rac1 (1:2000; BD Biosciences). Loading control was performed by re-probing the membranes with anti-actin (1:20,000; MP Biochemicals) during 30 min at room temperature. The membranes were then rinsed three times with TBS-T and incubated with horseradish peroxidase-conjugated secondary antibody (1:3000; Promega) for 1 h at room temperature. After washing for 30 min with TBS-T, the membranes were developed using the enhanced chemiluminescence ECL kit (Santa Cruz Biotechnology). ImageJ software was used to quantify the different densitometry immunoreactive bands relative to the intensity of the loading control band in the same membranes within a linear range of detection for the ECL reagent. Data is expressed as the mean \pm SEM of band density.

Behavioral Analysis

Separate cohorts of 6-month old wild-type ($\text{Hdh}^{\text{Q7/Q7}}$), mHtt KI ($\text{Hdh}^{\text{Q7/Q111}}$), Cdk5 heterozygous ($\text{Cdk5}^{+/+}$), and double mutant ($\text{mHtt KI:Cdk5}^{+/+}$) mice were used to evaluate (1) corticostriatal function by using the accelerating rotarod training procedure (ARTP) and the swimming T-maze of strategy shifting and (2) spatial and recognition memory by using the T-maze spontaneous alternation task (T-SAT) and the novel object recognition test (NORT), respectively. To reduce the effect of stress and anxiety on subsequent tasks, we performed first the least stressful and anxiety-inducing tasks as follows: ARTP, T-SAT, NORT, and swimming T-maze of strategy

shifting. All experiments were performed blind to the experimental condition.

ARTP

The ARTP was performed as previously described [22]. Briefly, animals were placed on a motorized rod (30 mm diameter). The rotation speed gradually increased from 4 to 40 rpm over the course of 5 min. The latency corresponds to the time the animal was able to keep up with the increasing speed before it fell. Rotarod training/testing was performed 4 times per day for 3 consecutive days.

Swimming T-Maze Test of Strategy Shifting

The swimming T-maze test of strategy shifting was performed as previously described [22]. Briefly, the T-maze apparatus used was a glass maze consisting of three arms, two of them situated at 180° from each other, and the third, representing the stem arm of the T, situated at 90° with respect to the other two. All arms were 45 cm long, 8 cm wide and enclosed by a transparent 20-cm wall. Six-month-old male mice were trained to swim from the stem arm of the T to the other arms. In one of them, there was a transparent escape platform, covered by water. During the acquisition phase of the swimming T-maze of strategy shifting (two consecutive days: first day, 8 trials; second day, 4 trials), each mice was placed in the water and allowed to swim until they reached the platform, and the latency and errors (1: error, 0: nonerror) to reach the platform were recorded. Mice had to be able to find the platform and learn the strategy faster across trials. After the acquisition phase, we changed the location of the platform to the opposite arm. During the reversal phase of swimming T-maze of strategy shifting (two consecutive days: second day, 4 trials; third day, 8 trials), each mice was placed in the water and the latency and errors to reach the platform were recorded.

T-SAT

The T-SAT was performed as previously described [21]. Briefly, the T-maze apparatus used was a wooden maze consisting of three arms, two of them situated at 180° from each other, and the third, representing the stem arm of the T, situated at 90° with respect to the other two. All arms were 45 cm long, 8 cm wide and enclosed by a 20-cm wall. Two identical guillotine doors were placed in the entry of the arms situated at 180° . 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 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.

NORT

The NORT was performed as previously described [21, 43]. Briefly, the device consisted of a 40-cm diameter and 40-cm high white circular arena. Mice were first habituated to the arena in the absence of objects (2 days, 10 min/day). On the third day, two similar objects were presented to each mouse for 10 min (A and A' condition) after which they were returned to their home cage. Twenty-four hours later, the same animals were retested for 5 min in the arena with a familiar (A) and a new (B) object (A, B condition; LTM). The object preference was measured as the time exploring each object \times 100/time exploring both objects.

Subcellular Fractionation

The striatum, cortex, and hippocampus from wild-type (Hdh^{Q7/Q7}), mHtt KI (Hdh^{Q7/Q111}), Cdk5 heterozygous (Cdk5^{+/-}), and double mutant (mHtt KI:Cdk5^{+/-}) mice were homogenized in buffer A (0.32 M sucrose, 10 mM HEPES pH = 7.4, and 5 mM MgCl₂) by repetitive pipetting and by passing through a syringe and needle. Homogenized samples were centrifuged for 10 min at 800g at 4 °C, and the supernatant was further centrifuged for 15 min at 10,000g at 4 °C. The newly obtained supernatant was ultracentrifuged in a fix rotor for 2 h at 200,000g at 4 °C. The resulting supernatant represents the cytosolic fraction. The pellet was resuspended in lysis buffer (150 mM NaCl, 20 mM Tris pH = 8.0, 50 mM NaF, 1% NP-40, 10% glycerol), sonicated and further centrifuged for 15 min at 16,100g and at 4 °C to obtain the plasma membrane fraction.

Biotinylation Assay

Biotinylation assay was performed as previously described [44]. Briefly, wild-type (Hdh^{Q7/Q7}), mHtt KI (Hdh^{Q7/Q111}), Cdk5 heterozygous (Cdk5^{+/-}), and double mutant (mHtt KI:Cdk5^{+/-}) mice were killed by cervical dislocation at 8 months of age. Brains were quickly removed, placed in oxygenated ice-cold ACSF buffer (125 mM NaCl, 2.5 mM KCl, 1.2 mM NaH₂PO₄, 1.2 mM MgCl₂, 2.4 mM CaCl₂, 26 mM NaHCO₃, and 11 mM glucose), and processed in a vibratome to obtain 400 μ m brain slices. The slices were incubated for 30 min with 0.5 mg/ml sulfo-NHS-SS-biotin (Sigma) and rinsed with ice-cold ACSF and with quench buffer (ACSF with 100 mM glycine) 3–6 times. Then, slices were incubated with the quench buffer for 20 min and rinsed 3 times with ACSF buffer. The striatum, cortex, and hippocampus from each slice were dissected and lysed with lysis buffer (150 mM NaCl, 20 mM Tris (pH = 8.0), 50 mM NaF, 1% NP-40, 10% glycerol supplemented with 1 mM sodium orthovanadate, and protease inhibitor cocktail (Sigma-

Aldrich)). Samples were centrifuged for 15 min at 16,000g at 4 °C and the supernatants were collected. After protein quantification, 150 μ g of protein was incubated in binding buffer (100 mM K₂PO₄, 150 mM NaCl, and 1% NP-40 (pH = 7.2)) with 40 μ l of NeutrAvidin beads (Thermo Scientific) at 4 °C in overnight rotation. Then, the samples were centrifuged for 2 min at 2500g at 4 °C and rinsed 4 times with decreasing amounts of binding buffer in PBS. Finally, the pellets were resuspended with 5 \times SDS sample buffer with β -mercaptoethanol and boiled for 10 min. Biotinylated samples were loaded to perform the western blot as previously described and NR2B surface levels were determined.

Rac1 Activity Assay

Rac1 activity assay was performed as previously described [22]. Briefly, the hippocampus and cortex from wild-type (Hdh^{Q7/Q7}), mHtt KI (Hdh^{Q7/Q111}), Cdk5 heterozygous (Cdk5^{+/-}), and double mutant (mHtt KI:Cdk5^{+/-}) mice were lysed in pull-down lysis buffer (25 mM HEPES, pH = 7.5, 150 mM NaCl, 10 mM MgCl₂, 1 mM EDTA, 1% NP-40, 2% glycerol) and centrifuged for 15 min at 16,000g at 4 °C. Cleared lysates were quantified and 600 μ g of total protein was incubated with 10 μ g PAK-GST protein beads (Cytoskeleton Inc.) for 1 h at 4 °C in rotation. Then, PAK-GST beads were pelleted by centrifugation at 5000g, for 1 min at 4 °C, washed 3 times with pull-down lysis buffer, resuspended with 5 \times SDS sample buffer with β -mercaptoethanol, and boiled for 5 min. The resulting samples were loaded to perform the western blot as previously described, and the levels of GTP-bound Rac1 were determined. Controls were incubated with 10 μ M EDTA and 200 μ M GTP γ S (positive control) or 1 mM GDP (negative control) at 30 °C, and the reaction was stopped with 60 mM MgCl₂ after 30 min.

Golgi Stain and Dendritic Spine Measurement

We performed the Golgi-Cox impregnation using the Rapid Golgistain Kit (FD Neurotechnologies) following the manufacturer's instructions. Briefly, wild-type (Hdh^{Q7/Q7}), mHtt KI (Hdh^{Q7/Q111}), Cdk5 heterozygous (Cdk5^{+/-}), and double mutant (mHtt KI:Cdk5^{+/-}) mice were killed by cervical dislocation at 7–8 months of age, their brains were quickly removed and briefly rinsed, and both hemispheres were separated and incubated in a mix of solution A/B for 2 weeks. Then, solution A/B was changed to solution C, and after 3 days, brains were embedded in 4% agarose to be processed using a vibratome to obtain 100- μ m sections that were mounted in gelatine-coated slides and left to dry for 24 h. Then, sections were stained with the kit's provided solutions, and finally, sections were

dehydrated and mounted with DPX. For dendritic spine analysis, we obtained bright field images with the differential interference contrast (DIC) technique using a Leica SP5 laser scanning confocal microscope (Leica) with a $\times 63$ numerical aperture objective. Conditions such as pinhole size (1 AU) and frame averaging (four frames per z-step) were held constant throughout the study. Confocal z-stacks were taken with a digital zoom of 5, a z-step of 0.5 μm , and 1024×1024 pixel resolution, yielding an image with pixel dimensions of $49.25 \times 49.25 \mu\text{m}$. Segments of apical dendrites from hippocampal CA1 pyramidal neurons and from cortical pyramidal neurons from motor cortex (M1) with no overlap with other branches were selected for analysis of spine density. Distal spines to the soma of the neuron were included in the study. Spine density was measured manually in the stacks using the ImageJ Plugin Cell Counter. Spines were marked in the appropriate focal plane preventing any double counting of spines. Spines were counted in dendritic segments ranging from 15 to 40 μm of length. All visible spines of the selected dendritic segment were counted. Z-stacks were deconvolved using the ImageJ Parallel Iterative Deconvolution plugin to improve voxel resolution and reduce optical aberration along the z-axis.

Brain Processing and Immunohistochemistry

Wild-type ($Hdh^{Q7/Q7}$) ($n = 5$), mHtt KI ($Hdh^{Q7/Q111}$) ($n = 5$), *Cdk5* heterozygous ($Cdk5^{+/-}$) ($n = 3$), and double mutant (mHtt KI: $Cdk5^{+/-}$) ($n = 5$) mice were deeply anesthetized and immediately perfused transcardially with saline followed by 4% paraformaldehyde (PFA)/phosphate buffer. Brains were removed and postfixed overnight in the same solution, cryoprotected by immersion in 30% sucrose, and then frozen in dry ice-cooled methylbutane. Serial coronal cryostat sections (30 μm) through the whole brain were collected in PBS as free-floating sections. Sections were rinsed three times in PBS and permeabilized and blocked in PBS containing 0.3% Triton X-100 and 5% normal goat serum (Pierce Biotechnology, Rockford, IL) for 1 h at room temperature. The sections were then washed in PBS and incubated overnight at 4 $^{\circ}\text{C}$ with spinophilin (1:250, Millipore) antibody that was detected with anti-rabbit Alexa Fluor 488 secondary antibody (1:200, Jackson ImmunoResearch, West Grove, PA). As negative controls, some sections were processed as described in the absence of primary antibody and no signal was detected.

Confocal Microscopy Analysis and Immunofluorescence-Positive Puncta Counting

Spinophilin-positive spine-like structures were examined as previously described [22]. The analysis was performed using an Olympus IX81 FV1000 laser scanning confocal

microscope equipped with argon and helium-neon lasers. Images were taken using a $\times 60$ numerical aperture objective with $\times 4$ digital zoom and 1.42 numerical aperture. Three to 4 coronal sections (30 μm thick) per animal spaced 0.24 mm apart containing the hippocampus were used. For each slice, we obtained three fields/stratum radiatum and three fields/stratum oriens of the CA1 hippocampal area. In each field, an entire z-stack was obtained and optical sections (2–5 per field) of 0.5 μm were collected separately (4 μm) in order to avoid biased counting. The number of spinophilin-positive puncta was measured using NIH ImageJ version 1.33 by Wayne Rasband (National Institutes of Health, Bethesda, MD). To analyze spinophilin immunolabeling, brightness and contrast of fluorescence images were adjusted so that only punctate fluorescence but no weak diffuse background labeling was visible. In the article, we use the terms “puncta” and “cluster” interchangeably to refer to discrete points of protein at the fluorescence microscope. Positive puncta/cluster within a specific field was recognized by identifying the presence of overlapping 10–300 pixels.

Statistical Analysis

All data are expressed as mean \pm SEM. Statistical analysis was performed by using the unpaired Student's *t* test (95% confidence), Wald's test, one-way ANOVA, two-way ANOVA, and the appropriate post hoc tests as indicated in the figure legends. Values of $p < 0.05$ were considered as statistically significant.

Results

Genetic Reduction of *Cdk5* in Adult mHtt KI Mice

Our previous studies uncovered an involvement of *Cdk5* in increased vulnerability of striatal cells to dopaminergic and glutamatergic inputs in the presence of mHtt [41, 42].

To further characterize the role of *Cdk5* in pathophysiological processes underlying HD and its associated learning and memory deficits, we have generated a new transgenic double mutant mouse line by breeding full-length mHtt mice ($Hdh^{Q7/Q111}$) with conditional *Cdk5* heterozygous mice ($Cdk5^{+/-}$) (Supplementary Fig. 1). In these conditional *Cdk5* heterozygous mice, *Cdk5* catalytic-domain regions are flanked by loxP sequences, and the expression of Cre recombinase is under the control of the CaMKII alpha promoter. This strategy allows conditional loss of *Cdk5* in the adult forebrain preventing major development-related deleterious effects. Western blot analysis of total protein lysates obtained from the cortex, striatum, and hippocampus of wild-type (WT),

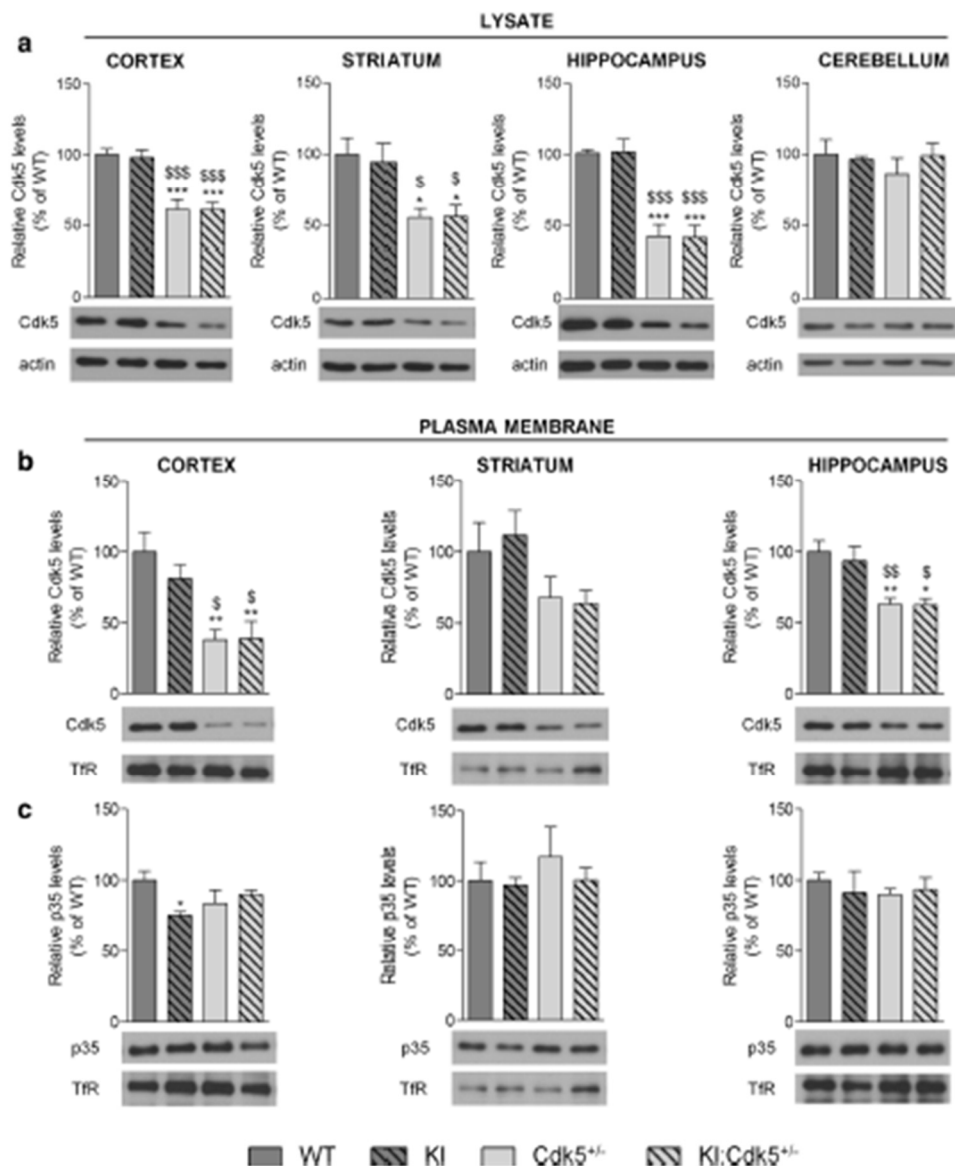


Fig. 1 Total Cdk5 levels are decreased in Cdk5^{+/-} and mHtt KI:Cdk5^{+/-} mice. Representative Western blots of cortical, striatal, hippocampal, and cerebellar total lysates probed for Cdk5 (a) in WT ($n=3-7$), mHtt KI ($n=3-8$), Cdk5^{+/-} ($n=3-7$), and mHtt KI:Cdk5^{+/-} ($n=3-7$) mice. A reduction of Cdk5 levels in cortical, striatal, and hippocampal lysates was observed in Cdk5^{+/-} and mHtt KI:Cdk5^{+/-} mice. Representative Western blots of cortical, striatal, and hippocampal plasma membrane fractions probed for Cdk5 (b) and p35 (c) in WT ($n=5-6$), mHtt KI ($n=4-7$), Cdk5^{+/-} ($n=4-5$), and mHtt KI:Cdk5^{+/-} ($n=4-6$) mice. A

reduction of cortical p35 plasma membrane levels was observed in mHtt KI mice with no changes in mHtt KI:Cdk5^{+/-} mice compared to WT. Histograms represent the mean \pm SEM and are normalized to WT values. Actin and transferrin receptor were used as loading controls in lysate and plasma membrane fractions, respectively. Statistical analysis was performed using one-way ANOVA with Bonferroni post hoc comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to WT mice. \$ $p < 0.05$, \$\$ $p < 0.01$, \$\$\$ $p < 0.001$ compared to mHtt KI mice

mHtt knock-in (KI), Cdk5 heterozygous (Cdk5^{+/-}), and double mutant mice (KI:Cdk5^{+/-}) demonstrated a 50% reduction in Cdk5 protein levels in both Cdk5 heterozygous

and double mutant KI:Cdk5^{+/-} mice (Fig. 1a). Importantly, this reduction was not detected in the cerebellum, where CaMKII alpha expression is especially low. Next, the

levels of the Cdk5 activator p35 were determined. No significant changes between genotypes and brain regions were found indicating that reduction of Cdk5 expression did not alter total levels of p35 (Supplementary Fig. 2). Given that Cdk5/p35 and Cdk5/p25 complexes display distinct subcellular distribution, with Cdk5/p35 being more abundant at the plasma membrane and Cdk5/p25 largely cytosolic (reviewed in [45]), subcellular distribution of p35 and p25 was determined in the cortex, striatum, and hippocampus of WT, mHtt KI, Cdk5^{+/-}, and mHtt KI:Cdk5^{+/-} mice. A significant reduction of p35 and a decreasing trend for Cdk5 were observed in the plasma membrane fraction obtained from the cortex but not the striatum or hippocampus of mHtt KI mice compared with WT mice (Fig. 1b, c), suggesting that in the cortex of mHtt KI mice, Cdk5/p35 complexes could be reduced at the level of the plasma membrane. Importantly, no differences between double mutant mHtt KI:Cdk5^{+/-}, heterozygous Cdk5^{+/-} and WT mice were found when p35 was analyzed in the plasma membrane fraction (Fig. 1b). Unfortunately, no specific bands for p25 were detected neither in the plasma membrane fraction nor in the cytosolic fraction. These results suggest that Cdk5 deregulation in mHtt KI mice as well as the effect of reducing Cdk5 levels might take place on a subcellular level.

Genetic Reduction of Cdk5 Attenuates Corticostriatal-Dependent Learning Deficits in mHtt KI Mice

Cdk5 plays a key role in modulating synaptic plasticity, learning, and memory processes [27, 46, 47]. Our previous studies have shown that mHtt KI mice exhibit altered Cdk5 activity [41] and age-related corticostriatal learning deficits [22]. Therefore, we explored whether reduction of Cdk5 expression in mHtt KI mice could improve corticostriatal cognitive dysfunction. Motor learning was evaluated in 6-month-old WT, mHtt KI, Cdk5^{+/-}, and mHtt KI:Cdk5^{+/-} mice using the accelerating rotarod and the swimming T-maze test of strategy shifting paradigm. All genotypes were able to learn the rotarod task as evidenced by an increased latency to fall across trials (Fig. 2). However, mHtt KI mice exhibited a significant reduction in the latency to fall as compared to WT and Cdk5^{+/-} mice, indicating an impairment in motor skill learning. In contrast, mHtt KI:Cdk5^{+/-} mice showed a comparable level of rotarod performance as WT mice, suggesting that a reduction in Cdk5 expression can counteract the deficits induced by mHtt. To exclude the possibility that deficits in motor coordination contributed to the rotarod impairment, we analyzed spontaneous locomotor activity as measured in the open field in 6-month-

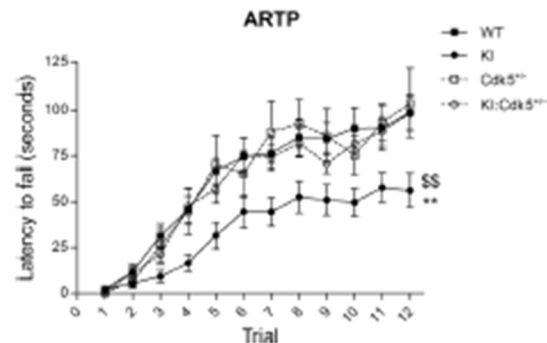


Fig. 2 Motor learning deficits in mHtt KI mice are ameliorated by genetic reduction of Cdk5 expression. Latency to fall in the accelerating rotarod task for WT ($n = 17$), mHtt KI ($n = 26$), Cdk5^{+/-} ($n = 9$), and mHtt KI:Cdk5^{+/-} ($n = 12$) mice. mHtt KI mice exhibited a significant decrease in motor learning performance. Amelioration of motor learning performance was observed in mHtt KI mice by genetic reduction of Cdk5 expression. Data represents the mean \pm SEM. Statistical analysis was performed using two-way ANOVA with repeated measures. Analysis between WT and mHtt KI mice revealed significant differences in genotype ($F_{(1, 451)} = 11.11$; $p = 0.0018$), an effect of time ($F_{(11, 451)} = 51.02$; $p < 0.0001$) and an interaction between genotype and time ($F_{(11, 451)} = 2.935$; $p = 0.0009$). Analysis between WT and Cdk5^{+/-} mice revealed no significant differences in genotype ($F_{(1, 264)} = 0.0119$; $p = 0.9656$), an effect of time ($F_{(11, 264)} = 46.70$; $p < 0.0001$), although no interaction between genotype and time ($F_{(11, 264)} = 0.5621$; $p = 0.8586$). Similarly, analysis between WT and mHtt KI:Cdk5^{+/-} revealed no significant differences in genotype ($F_{(1, 297)} = 0.2102$; $p = 0.6503$), an effect of time ($F_{(11, 297)} = 63.56$; $p < 0.0001$), although no interaction between genotype and time ($F_{(11, 297)} = 0.3760$; $p = 0.9647$). Analysis between mHtt KI and mHtt KI:Cdk5^{+/-} mice revealed significant differences in genotype ($F_{(1, 296)} = 7.709$; $p = 0.0087$), an effect of time ($F_{(11, 296)} = 46.70$; $p < 0.0001$) and an interaction between genotype and time ($F_{(11, 296)} = 2.648$; $p = 0.0028$). ** $p < 0.01$ compared to WT mice and \$\$\$ $p < 0.01$ compared to mHtt KI mice

old WT, mHtt KI, Cdk5^{+/-}, and mHtt KI:Cdk5^{+/-} mice. No differences in the distance traveled in the open field were observed between groups (Supplementary Fig. 3). Next, procedural memory was analyzed using the swimming T-maze test (Fig. 3a). During the acquisition phase, the escape platform was located in the right arm of the T-maze, and the latency to reach the platform (Fig. 3b) and the number of "errant" trials (Fig. 3d) were scored. After the first trial, all genotypes showed a similar decrease in the latency to reach the platform, showing by the last trial a constant level of performance (~ 10 s to reach the platform; Fig. 3b). As all genotypes including the mHtt KI mice learned the location of the escape platform, we next assessed the ability to change the strategy by switching the platform from the right arm to the left arm of the T-maze (reversal phase) (Fig. 3c, e). The latency to reach the platform was significantly higher in mHtt KI mice compared to WT mice (Fig. 3c) and correlated with an increased number of errant trials (Fig. 3e).

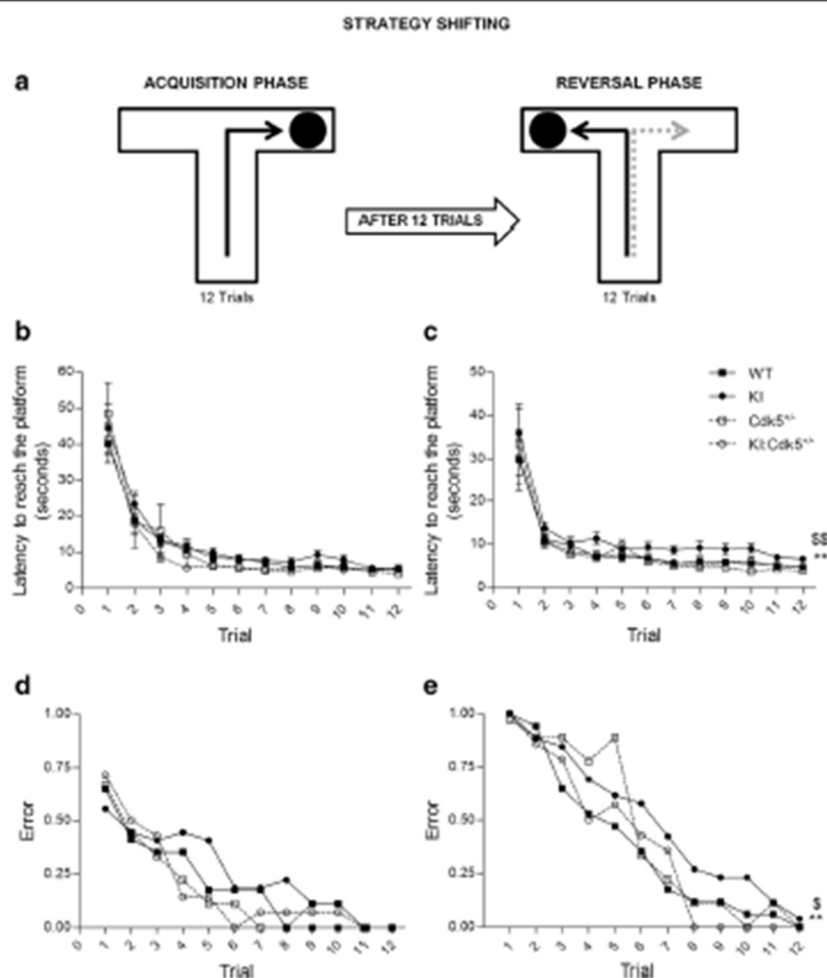


Fig. 3 Procedural memory impairments in mHtt KI mice are ameliorated by genetic reduction of Cdk5 expression. **a** Schematic representation of the acquisition and reversal phase of the strategy shifting test. Latency to reach the platform and error trials in the swimming T-maze test of strategy shifting in WT ($n=17$), mHtt KI ($n=27$), $Cdk5^{-/-}$ ($n=9$), and mHtt KI: $Cdk5^{-/-}$ ($n=14$) mice during the acquisition phase (**b** and **d**) and the reversal phase (**c** and **e**). mHtt KI mice showed procedural memory deficits in the reversal phase of the swimming T-maze test that were prevented by genetic reduction of Cdk5 expression. Data represent the mean \pm SEM. Statistical analysis was performed using two-way ANOVA with repeated measures to analyze the latency to reach the platform. In the acquisition phase, no significant differences between genotypes but an effect of time ($p < 0.0001$) was observed. Reversal phase: analysis between WT and mHtt KI mice revealed significant differences in genotype ($F_{(1, 462)} = 8.476$; $p = 0.0057$), an effect of time ($F_{(11, 462)} = 24.21$; $p < 0.0001$), although no interaction between genotype and time ($F_{(11,$

$462) = 0.2228$; $p = 0.9961$). Analysis between WT and $Cdk5^{-/-}$ mice revealed no significant differences in genotype ($F_{(1, 264)} = 0.1153$; $p = 0.7372$), an effect of time ($F_{(11, 264)} = 17.10$; $p < 0.0001$) and no interaction between genotype and time ($F_{(11, 264)} = 0.2322$; $p = 0.9952$). Analysis between WT and mHtt KI: $Cdk5^{-/-}$ revealed no significant differences in genotype ($F_{(1, 319)} = 0.0039$; $p = 0.9503$), an effect of time ($F_{(11, 319)} = 26.16$; $p < 0.0001$), and no interaction between genotype and time ($F_{(11, 319)} = 0.0660$; $p = 1.0$). Analysis between mHtt KI and mHtt KI: $Cdk5^{-/-}$ mice revealed significant differences in genotype ($F_{(1, 429)} = 8.831$; $p = 0.0051$), an effect of time ($F_{(11, 429)} = 26.99$; $p < 0.0001$), and no interaction between genotype and time ($F_{(11, 429)} = 0.1603$; $p = 0.9991$). Logistic regression analysis using the Wald statistical test from IBM SPSS Statistics was used to analyze the error probability to reach the platform in the correct arm. ** $p < 0.01$ compared to WT mice. \$ $p < 0.05$, \$\$\$ $p < 0.001$ compared to mHtt KI mice

Comparable to the results in the accelerating rotarod, genetic reduction of Cdk5 attenuated procedural learning deficits in the swimming T-maze test. Thus, mHtt KI: $Cdk5^{-/-}$ mice showed a similar performance in the

reversal phase of the swimming T-maze test as WT mice (Fig. 3). Together, these data demonstrate that genetic reduction of Cdk5 expression in mHtt KI mice improves corticostriatal-dependent learning deficits.

Cortico-striatal NR2B Surface Expression Is Reduced in mHtt KI Mice and Restored by Genetic Reduction of Cdk5

Synaptic plasticity and memory processes are dependent on the proper expression, location, and function of NMDARs [48–50]. Importantly, regulation of NMDARs at synapses relies on accurate Cdk5 activity [29, 31, 51, 52]. To examine whether the effect of Cdk5 reduction on mHtt-induced learning impairments was associated to modulation of NMDARs, we analyzed the levels of the different NMDAR subunits (NR1, NR2A, and NR2B) in the cortex (Fig. 4a) and striatum (Fig. 4b) of WT, mHtt KI, Cdk5^{+/-}, and mHtt KI:Cdk5^{+/-} mice. A significant reduction of NR2B, but not NR1 or NR2A levels, was observed in the cortex of mHtt KI compared to WT mice (Fig. 4a). Next, we investigated whether this decrease in NR2B expression could be altered by genetic reduction of Cdk5. Interestingly, mHtt KI:Cdk5^{+/-} mice exhibited similar levels of NR2B compared to WT mice (Fig. 4a) suggesting a detrimental role of Cdk5 in cortical NR2B expression. No significant differences between genotypes were detected when NR1, NR2A, and NR2B levels were analyzed in the striatum (Fig. 4b). Next, to correlate NR2B cortical changes with a functional outcome, cell surface expression of NR2B was determined by using a brain slice biotinylation assay and Western blot analysis. A significant reduction of NR2B surface levels was found in the cortex of mHtt KI mice compared to WT mice, a decrease that was attenuated by genetic reduction of Cdk5 in mHtt KI:Cdk5^{+/-} mice (Fig. 4c). Surprisingly, NR2B cell surface levels were also diminished in the striatum of mHtt KI mice, though no significant changes were detected in total levels. This reduction was also reversed in mHtt KI:Cdk5^{+/-} mice (Fig. 4d). Together, these findings reveal altered levels and/or localization of NR2B in the cortex and striatum of mHtt KI mice that can be reversed by reduction of Cdk5 expression.

Src-Mediated Phosphorylation of NR2B at Y1472 Is Reduced in the Cortex of mHtt KI Mice

It has been reported that Cdk5 can affect Src kinase activation, which in turn leads to decreased levels of NR2B phosphorylation at tyrosine 1472 (Y1472) allowing AP2 binding to NR2B and its subsequent internalization [53]. Therefore, we next tested whether this pathway could contribute to Cdk5-dependent reduction of cortical and striatal NR2B surface levels in mHtt KI mice. First, we determined whether Src function, measured as phosphorylation at tyrosine 416 of Src [54], was deregulated in mHtt KI mice. A significant decrease in pSrc (Y416) levels was detected in the cortex of mHtt KI mice with no significant changes in total Src levels (Fig. 5a). Accordingly, phosphorylation of NR2B subunit at Y1472 was also found significantly decreased in the cortex of mHtt KI

mice (Fig. 5a). If diminished Src-mediated phosphorylation of NR2B was associated with altered Cdk5 activity [53], we might assume that genetic reduction of Cdk5 in mHtt KI mice would prevent such decrease. Consistent with this hypothesis, we found that both pSrc (Y416) and pNR2B (Y1472) cortical levels showed comparable levels in mHtt KI:Cdk5^{+/-} mice and WT (Fig. 5a). Surprisingly, no significant changes in pSrc (Y416) and pNR2B (Y1472) levels were found in the striatum of mHtt KI mice (Fig. 5b), suggesting that other signaling pathways must be involved in the regulation of striatal NR2B localization. In this view, recent studies have demonstrated that phosphorylation of NR2B at Ser1116 is critical to target NR2B to the plasma membrane by inducing its translocation from intracellular stores [31]. To address whether Cdk5 could also modulate NR2B in mHtt KI mice by acting on Ser1116, Western blot analysis was performed in total lysates obtained from the cortex and striatum of WT, mHtt KI, and mHtt KI:Cdk5^{+/-} mice. A significant decrease in pS1116 NR2B levels was found in the cortex but not in the striatum of mHtt KI mice (Supplementary Fig. 4). Though apparently contradictory with the role of pS1116 as a negative regulator of NR2B translocation to the plasma membrane, we can reason that this reduction is a consequence of decreased total cortical NR2B levels. Indeed, no differences in pS1116 levels were found in the striatum where total NR2B levels are unchanged (Supplementary Fig. 4). Together, these findings suggest that Cdk5 regulation of total NR2B levels takes place through modulation of pSrc (Y416) and subsequent reduction of pNR2B (Y1472) levels.

Genetic Reduction of Cdk5 Attenuates Spatial and Recognition Memory Deficits in mHtt KI Mice

Our previous work uncovered hippocampal-dependent memory deficits in mHtt KI mice [21, 43]. Given the critical role of Cdk5 in hippocampal synaptic plasticity and memory functions [30, 55, 56], we next explored whether genetic reduction of Cdk5 expression in mHtt KI mice could also attenuate memory impairments. For this purpose, we evaluated spatial and recognition memories using the T-SAT and the NORT, respectively (Fig. 6). In the T-SAT, we evaluated the time a mouse spends exploring a familiar versus a novel arm in a T-maze. During the training trial, all genotypes spent similar time in the open arm (familiar) (Supplementary Fig. 5a). In contrast, during the test trial, mHtt KI mice exhibited significantly reduced exploration time in the novel arm, indicating spatial memory impairments (Fig. 6a). Interestingly, genetic reduction of Cdk5 in mHtt KI:Cdk5^{+/-} mice attenuated this spatial memory decline as indicated by a higher preference to explore the novel versus the familiar arm (Fig. 6a). We then evaluated recognition memory using the NORT. This test is based on the natural tendency of mice to spend more time exploring novel objects than familiar ones. In the training

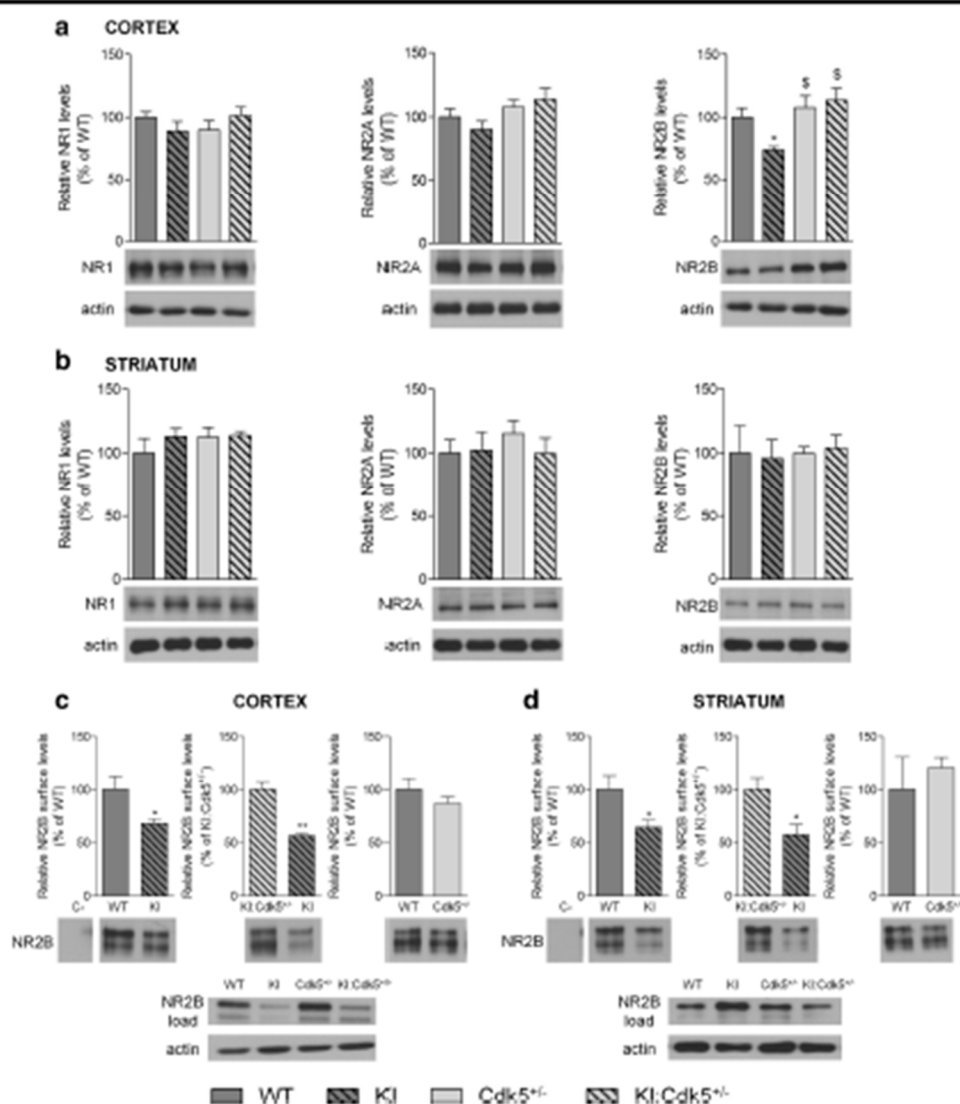


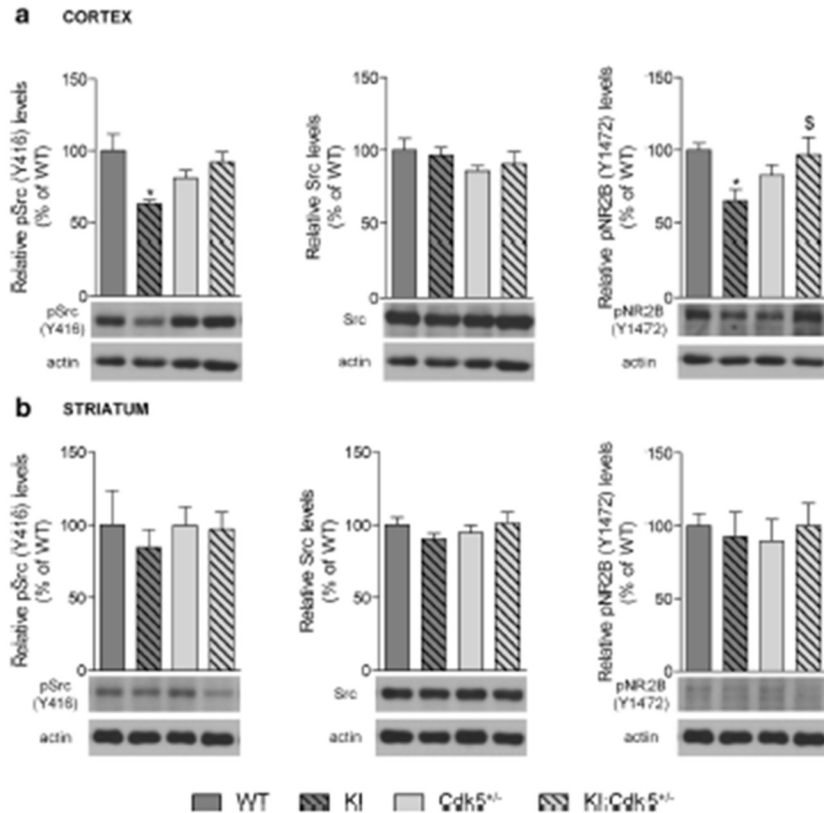
Fig. 4 Decreased corticostriatal NR2B surface levels in mHtt KI mice are recovered by genetic reduction of Cdk5. Representative Western blots showing total NR1, NR2A, and NR2B levels in the cortex (a) and striatum (b) of WT ($n = 5-7$), mHtt KI ($n = 6-7$), Cdk5^{-/-} ($n = 6$), and mHtt KI:Cdk5^{-/-} ($n = 6-7$) mice. The levels of NR2B in the cortex of WT or mHtt KI:Cdk5^{-/-} mice were significantly higher than those in mHtt KI mice. Representative Western blots showing surface levels of NR2B in the cortex (c) and striatum (d) of WT ($n = 4$), mHtt KI ($n = 5$), Cdk5^{-/-} ($n = 3-4$), and mHtt KI:Cdk5^{-/-} ($n = 4$) mice. Prior to pull-down, lysates

were tested for NR2B load (bottom). A significant decrease in cortical and striatal NR2B surface levels was observed in mHtt KI mice compared to either WT mice or mHtt KI:Cdk5^{-/-} mice. All data represent mean \pm SEM and are normalized to WT or mHtt KI:Cdk5^{-/-} levels. Statistical analysis was performed using one-way ANOVA with Newman-Keuls post hoc comparisons (a and b) and Student's two-tailed t test (c and d). * $p < 0.05$, ** $p < 0.01$ compared to WT or mHtt KI:Cdk5^{-/-} mice. $\$p < 0.05$ compared to mHtt KI mice

session, all genotypes showed similar exploration time for each object (A and A') with no detectable object preference (Supplementary Fig. 5b). However, when long-term memory was assessed 24 h after training, mHtt KI mice demonstrated impaired recognition memory as manifested by similar

exploration time of the novel versus the familiar object (Fig. 6b). Consistent with the results in the T-SAT, we found that mHtt KI:Cdk5^{-/-} mice exhibited a comparable performance in the NORT test as WT mice (Fig. 6b), indicating that genetic reduction of Cdk5 expression attenuated recognition memory

Fig. 5 Reduced cortical Y416 Src and Y1472 NR2B phosphorylation levels in mHtt KI and Y1472 NR2B phosphorylation levels in mHtt KI mice are recovered in mHtt KI:Cdk5^{+/-} mice. Representative Western blots showing levels of pSrc (Y416), total Src, and pNR2B (Y1472) in the cortex (a) and striatum (b) of WT (*n* = 5–7), mHtt KI (*n* = 6–7), Cdk5^{+/-} (*n* = 5–7), and mHtt KI:Cdk5^{+/-} (*n* = 5–7) mice. A significant reduction of cortical pSrc and pNR2B was observed in mHtt KI mice but not in mHtt KI:Cdk5^{+/-} mice. No significant changes in the phosphorylation levels of Src and NR2B were observed in the striatum. All data represent mean ± SEM and are normalized to WT levels. Statistical analysis was performed using one-way ANOVA with Newman-Keuls post hoc comparisons. **p* < 0.05 compared to WT mice. *Sp* < 0.05 compared to mHtt KI mice



deficits. In the open field paradigm, 6-month-old WT, mHtt KI, Cdk5^{+/-}, and mHtt KI:Cdk5^{+/-} mice showed no significant differences between genotypes in the number of entries, the time spent, or the distance traveled in the center, demonstrating that these mice do not exhibit an anxiety-like phenotype (Supplementary Fig. 6). In conclusion, these results demonstrate that the deficits in spatial and recognition memory in mHtt KI mice can be attenuated by genetic reduction of Cdk5 expression.

Attenuation of Hippocampal Memory Deficits in mHtt KI Mice Does not Correlate with Restoration of NR2B Surface Levels

In this study, we have shown that decreased NR2B cell surface levels correlate with corticostriatal-dependent behavioral deficits in mHtt KI mice and that genetic reduction of Cdk5 expression can overcome both alterations (Fig. 4). To determine whether Cdk5 also contributes to hippocampal-dependent memory impairments in mHtt KI mice by altering NMDAR subunit expression, protein levels of NR1, NR2A, and NR2B were examined in hippocampal lysates. No significant changes in total NMDAR

subunit levels were found between genotypes (Fig. 7a). In contrast, NR2B surface levels were significantly lower in mHtt KI mice compared to those in WT mice (Fig. 7b). Genetic reduction of Cdk5 expression in mHtt KI:Cdk5^{+/-} mice led to a partial but not significant increase in NR2B surface levels as compared to mHtt KI mice (mHtt KI:Cdk5^{+/-} 100 ± 15.8% and mHtt KI 63 ± 20.8%; *p* = 0.2). As we found that reduced NR2B surface levels in the cortex of mHtt KI mice correlated with reduced Src phosphorylation, the levels of pSrc (Y416) and pNR2B (Y1472) were analyzed in hippocampal lysates. No significant changes between genotypes were found neither in pSrc (Y416) nor in pNR2B (Y1472) levels (Fig. 7c). Next, the levels of pNR2B at Ser1116 were evaluated. Similar results as those found in the striatum could be observed with no significant differences between genotypes (Supplementary Fig. 4). Altogether, these results indicate that decreased NR2B surface levels in the hippocampus of mHtt KI mice are not dependent on Src signaling or direct phosphorylation by Cdk5. Moreover, the lack of a complete recovery of NR2B surface levels by Cdk5 genetic reduction indicates that Cdk5 contribution to hippocampal memory improvement must involve other downstream targets besides NR2B.

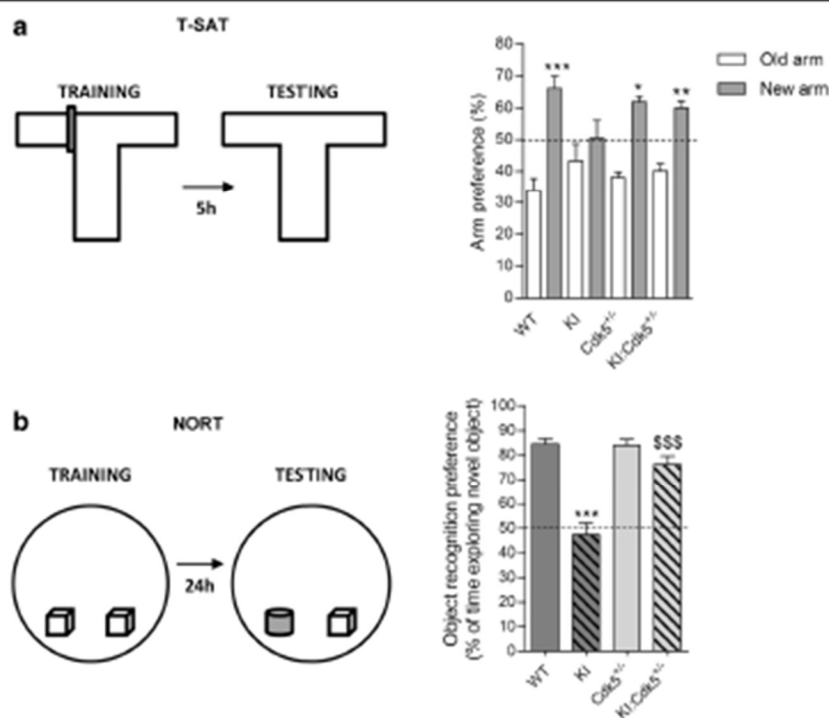


Fig. 6 Spatial and recognition long-term memory impairments are ameliorated in mHtt KI mice. **a** Schematic representation of the T-maze spontaneous alternation task (T-SAT) (left). Percentage of time spent in arms (old versus new) from WT ($n = 12$), mHtt KI ($n = 16$), Cdk5^{+/-} ($n = 8$), and mHtt KI.Cdk5^{+/-} ($n = 12$) mice. Dashed line indicates chance level for exploration (right). mHtt KI mice exhibited no preference for the new arm of the T-maze, whereas mHtt KI.Cdk5^{+/-} showed a preference comparable to WT mice. **b** Schematic representation of the novel object recognition test (NORT) (left). Percentage of time spent exploring the new

object from WT ($n = 12$), mHtt KI ($n = 16$), Cdk5^{+/-} ($n = 8$), and mHtt KI.Cdk5^{+/-} ($n = 12$) mice. Dashed line indicates chance level for exploration (right). mHtt KI mice display no preference for the new object, whereas mHtt KI.Cdk5^{+/-} mice showed similar preference to WT mice. Data represents mean \pm SEM. Statistical analysis was performed using one-way ANOVA with Bonferroni post hoc comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the old arm (in **a**) or to WT mice (in **b**). \$\$\$ $p < 0.001$ compared to mHtt KI mice

Genetic Reduction of Cdk5 in mHtt KI Mice Attenuates Loss of Hippocampal Dendritic Spine Number and Increases Rac1 Activity

Several studies indicate that Cdk5 is necessary for dendritic spine formation and remodeling through phosphorylation of various substrates [32–36]. Dendritic spine alterations have been reported to mediate hippocampal memory deficits in mHtt KI mice [21]. Thus, we investigated whether genetic reduction of Cdk5 in mHtt KI mice could restore dendritic spine abnormalities. For this purpose, Golgi impregnation was performed on hippocampal slices from 8-month-old WT, mHtt KI, Cdk5^{+/-}, and mHtt KI.Cdk5^{+/-} mice, and dendritic spine density of CA1 pyramidal neurons was analyzed. Consistent with a previous work [21], mHtt KI mice exhibited lower number of spines per dendrite as compared to WT mice (Fig. 8a, b). A small but significant increase in spine number was detected in Cdk5^{+/-} mice. Interestingly, reduction of Cdk5 expression in mHtt KI mice ameliorated the loss of

dendritic spines, showing that Cdk5 negatively regulates spine number. Moreover, these results also suggest that cognitive improvements in mHtt KI.Cdk5^{+/-} mice could be linked to increased dendritic spine density in the hippocampus. To determine whether the restoration of dendritic spines in mHtt KI.Cdk5^{+/-} mice associates with recovery of potential synaptic sites, spinophilin immunostaining was performed in CA1 hippocampal slices obtained from WT, mHtt KI, Cdk5^{+/-}, and mHtt KI.Cdk5^{+/-} mice, as previously described [22]. Confocal microscopy analysis revealed a significant reduction in spinophilin-immunoreactive puncta in the *stratum radiatum* (apical dendrites of CA1 pyramidal neurons) and *stratum oriens* (basal dendrites of CA1 pyramidal neurons) of mHtt KI mice compared to WT mice (Supplementary Fig. 7). Importantly, this decrease was attenuated by genetic reduction of Cdk5 in both *stratum radiatum* and *stratum oriens* layers of mHtt KI.Cdk5^{+/-} mice. Altogether, this data confirms that the reduction in the number of dendritic spines in the CA1 hippocampus of mHtt KI mice correlates with a

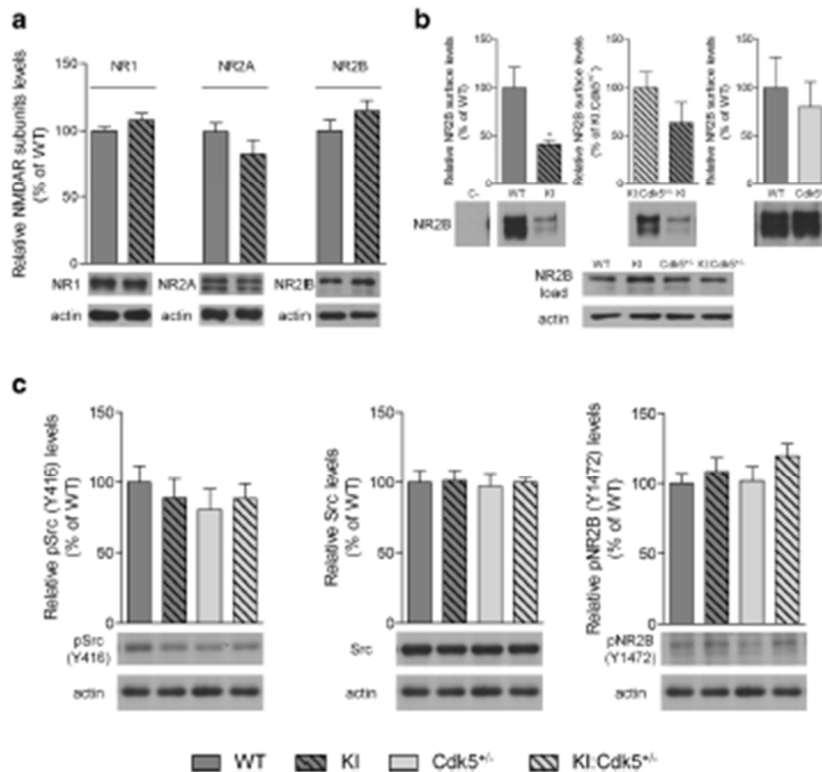


Fig. 7 Levels of NMDAR subunits are not changed in the hippocampus of mHtt KI mice. **a** Representative Western blots of NR1, NR2A, and NR2B from hippocampal lysates of WT and mHtt KI mice. **b** Representative Western blots showing surface NR2B levels in the hippocampus of WT, mHtt KI, Cdk5^{-/-}, and mHtt KI:Cdk5^{-/-} mice. Prior to pull-down, lysates were tested for NR2B load (bottom). **c** Representative Western blots showing levels of pSrc (Y416), total Src, and pNR2B (Y1472) in hippocampal lysates of WT, mHtt KI, Cdk5^{-/-},

and mHtt KI:Cdk5^{-/-} mice. All data represent mean \pm SEM and are normalized to WT or mHtt KI:Cdk5^{-/-} levels. Statistical analysis was performed using Student's two-tailed *t* test (**a** and **b**) and one-way ANOVA with Newman-Keuls post hoc comparisons (**c**). WT ($n=5-7$), mHtt KI ($n=5-6$), Cdk5^{-/-} ($n=6-7$), and mHtt KI:Cdk5^{-/-} ($n=6-7$) mice for total protein analysis and WT ($n=4$), mHtt KI ($n=5$), Cdk5^{-/-} ($n=4$), and mHtt KI:Cdk5^{-/-} ($n=4$) mice for surface protein analysis

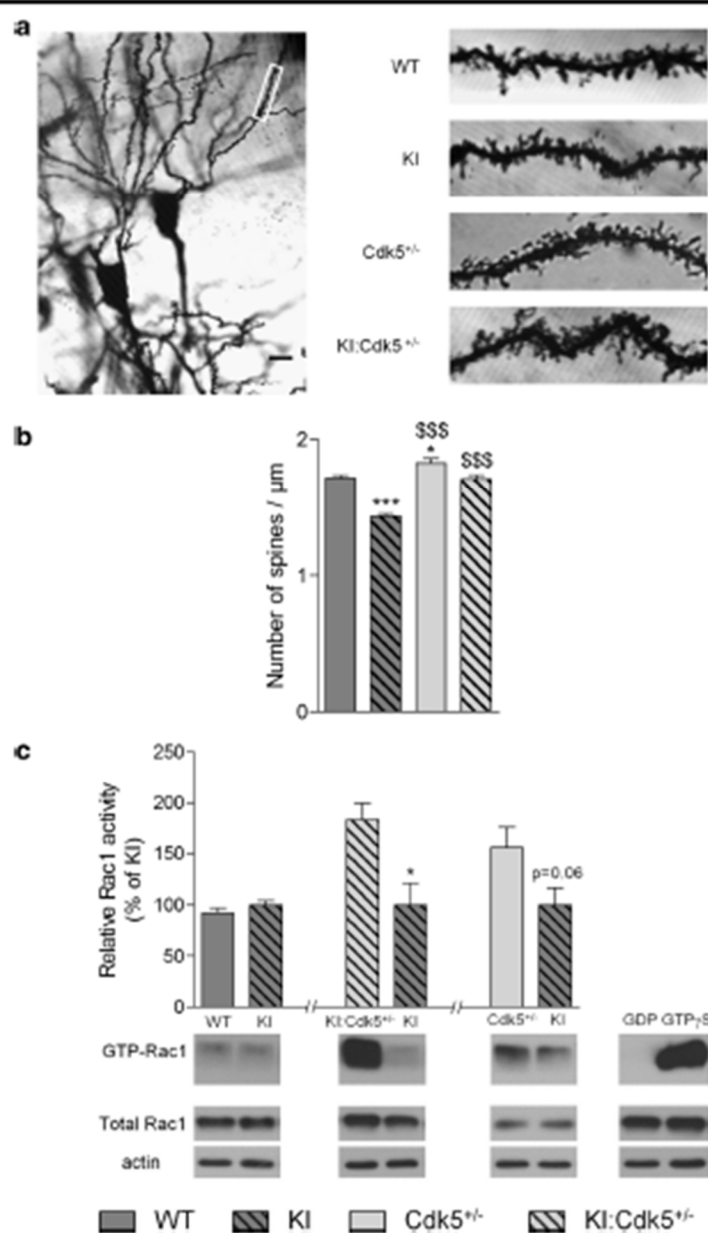
decrease in the number of postsynaptic clusters and that genetic reduction of Cdk5 attenuates such decrease in the mHtt KI:Cdk5^{-/-} mice.

To elucidate the Cdk5-dependent mechanism responsible for the rescue of dendritic spine number, we next analyzed the activity of the small Rho-GTPase Rac1. It has been previously reported that Rac1 can induce dendritic spine formation [57] and its activity is increased following Cdk5 inhibition [37]. We found that Rac1 activity in the hippocampus, determined as GTP-bound Rac1 levels, was similar between WT and mHtt KI mice (Fig. 8c). However, genetic reduction of Cdk5 either in Cdk5^{-/-} or mHtt KI:Cdk5^{-/-} mice resulted in a significant increase in Rac1 GTPase activity (Fig. 8c). No changes in total Rac1 protein levels were detected between genotypes. Together, these data indicate that reduction of hippocampal Cdk5 expression in mHtt KI mice leads to the recovery of dendritic spine density along with an increase in Rac1

activity, providing a plausible mechanism underlying the attenuation of memory deficits in mHtt KI:Cdk5^{-/-} mice.

Genetic Reduction of Cdk5 in mHtt KI Mice Attenuates Loss of Cortical Dendritic Spines but Does not Modify Rac1 Activity

Our results have shown that reversion of corticostriatal-dependent deficits in mHtt KI mice correlates with the recovery of surface NR2B levels, a mechanism not found in the hippocampus. In contrast, we have demonstrated that increased Rac1 activity and normalization of dendritic spine density in the hippocampus likely contribute to preserve memory function. Based on these data, we wondered whether Cdk5 was improving corticostriatal performance in mHtt KI mice not only by acting on NR2B levels but also through regulation of Rac1 and dendritic spines. To this aim, Golgi staining and



analysis of dendritic spines was performed in motor cortex slices from 8-month-old WT, mHtt KI, Cdk5^{+/-}, and mHtt KI:Cdk5^{+/-} mice (Fig. 9). A significant decrease in the number of spines per micrometer of dendrite length was found in the motor cortex of mHtt KI mice compared to WT mice, a reduction that was prevented in mHtt KI:Cdk5^{+/-} mice (Fig. 9b).

Contrary to the hippocampus, no significant differences between WT and Cdk5^{+/-} mice were observed indicating that in the cortex the reduction of Cdk5 per se does not induce an increase in spine density. Next, Rac1 activity was analyzed by pull-down assays on cortical extracts obtained from all four genotypes. A significant decrease in Rac1 activity evidenced

Fig. 8 Decreased hippocampal dendritic spine density is attenuated by genetic reduction of Cdk5. **a** Representative images of a Golgi-impregnated CA1 hippocampal neuron (left) and of apical dendrites of CA1 pyramidal neurons from WT, mHtt KI, Cdk5^{+/−}, and mHtt KI:Cdk5^{+/−} mice (right). Scale bar, 10 μm. **b** Histogram showing quantitative analysis of dendritic spine density per micrometer of dendritic length. Significant reduction of dendritic spine density in mHtt KI mice was attenuated in mHtt KI:Cdk5^{+/−} mice. Statistical analysis was performed using one-way ANOVA with Newman-Keuls post hoc comparisons (30 dendrites from 3 to 5 animals per genotype were analyzed). **p* < 0.05, ***p* < 0.001 compared to WT mice. ****p* < 0.001 compared to mHtt KI mice. **c** Representative Western blots showing levels of GTP-bound Rac1 immunoprecipitated from hippocampal lysates, indicating Rac1 activity. A significant increase in Rac1 activity was found in Cdk5^{+/−} and mHtt KI:Cdk5^{+/−} mice when compared to mHtt KI mice. Histograms represent the mean ± SEM and are normalized to mHtt KI values (*n* = 4–5 mice per genotype). Statistical analysis was performed using Student's two-tailed *t* test. **p* < 0.05 compared to mHtt KI

by a decrease in the levels of Rac1-GTP was found in mHtt KI mice compared to WT mice. However, no significant differences between mHtt KI:Cdk5^{+/−} and Cdk5^{+/−} were found when compared to mHtt KI mice (Fig. 9c).

Altogether, these results demonstrate a reduction in the number of dendritic spines not only in the hippocampus but also in the cortex of mHtt KI mice and that genetic reduction of Cdk5 prevents this decrease. In contrast, our findings suggest that Rac1 activity is differently modulated by Cdk5 in the cortex compared to the hippocampus reinforcing the idea of a brain region-dependent role of Cdk5 in learning and memory.

Discussion

Synaptic dysfunction is an early event in the pathogenesis of HD that involves an array of alterations in different signaling pathways [10, 11, 14]. The identification of key proteins commonly involved in these pathways may reveal new therapeutic targets for the treatment of cognitive deficits in HD. One of the prime candidates contributing to the signaling disturbances underlying synaptic deficits is Cdk5 due to its role in regulating the expression and function of numerous synaptic proteins [27, 46, 58] and its deregulation in different neurodegenerative disorders [38–40]. In fact, altered Cdk5 activity has already been proposed to contribute to striatal dysfunction and neurodegeneration in HD [41, 42, 59, 60]. However, no studies have addressed the role of Cdk5 in HD memory decline. Interestingly, in pathological conditions, such as Alzheimer's disease [37], Parkinson's disease [61], or ischemic injury [62], inhibition of Cdk5 prevents learning and memory impairments. Based on these data, we hypothesized that cognitive decline in HD mutant mice could be ameliorated or prevented by reducing Cdk5 expression. For this purpose, we generated a new full-length KI mutant mouse model, in which one copy of the Cdk5 gene has been deleted in forebrain neurons and we

evaluated learning and memory performance. Our experiments demonstrate that genetic reduction of Cdk5 in these mHtt KI:Cdk5^{+/−} mice attenuates motor learning and procedural memory decline as well as spatial and recognition memory impairments. These results reveal a critical role for Cdk5 in both corticostriatal and hippocampal-dependent learning and memory deficits associated with HD.

To understand the molecular mechanism underlying Cdk5-dependent disturbances in memory functions, we focused on NMDARs, as Cdk5 has been implicated in the control of synaptic plasticity and memory via modulation of NMDAR levels and membrane location [29–31, 51, 52]. We found a significant decrease in NR2B cell surface levels in the striatum, cortex, and hippocampus of mHtt KI mice without changes in NR1 or NR2A total levels. Previous studies in other HD mouse models have already demonstrated altered expression of NMDAR subunits. Thus, in exon-1 transgenic R6/2 mice, membrane-associated NR2A and NR2B levels were reduced in the hippocampus [63], while in full-length YAC72 mice, altered NMDARs trafficking in the striatum were associated with increased levels of NR1 and NR2B at the cell surface with no significant changes in total expression [64]. Interestingly, the reduction of NR2B surface levels that we observed in the striatum and hippocampus of mHtt KI mice was not related with a decrease in NR2B total levels suggesting that in these brain areas, disturbances in NMDAR trafficking likely account for such decrease. In contrast, in the cortex given the reduction in both NR2B total and surface levels, we can speculate that multiple mechanisms, such as trafficking, expression, or degradation, may participate in NR2B deregulation. Notably, Cdk5 may attenuate NR2B trafficking to the cell membrane by direct or indirect phosphorylation [31, 53], while facilitating NR2B degradation by enhancing its calpain-mediated proteolysis [30]. Consistent with this detrimental role of Cdk5 on NR2B levels, we found that in mHtt KI:Cdk5^{+/−} mice, NR2B surface levels were completely recovered in the cortex and striatum, while only partially in the hippocampus. These findings suggest that Cdk5 contributes to reducing NR2B surface levels in mHtt KI mice but in a different manner depending on the brain area. In support of this idea, we found that the signaling pathways acting downstream of Cdk5 that modulate NR2B surface levels also differ between the cortex, striatum, and hippocampus. Thus, in the cortex of mHtt KI mice, we identified the Cdk5/pSrc/pNR2B pathway [53] as one of the putative underlying mechanism by which Cdk5 alters NR2B surface expression. However, no contribution of this pathway was observed neither in the striatum nor in the hippocampus. For this reason, the levels of p-S1116 NR2B, known to modulate NR2B translocation to the plasma membrane [31], were also analyzed. No changes between genotypes neither in the striatum nor in the hippocampus were found. However, a significant decrease in pS1116 NR2B levels was detected in the cortex of mHtt KI mice. This result was unexpected given that

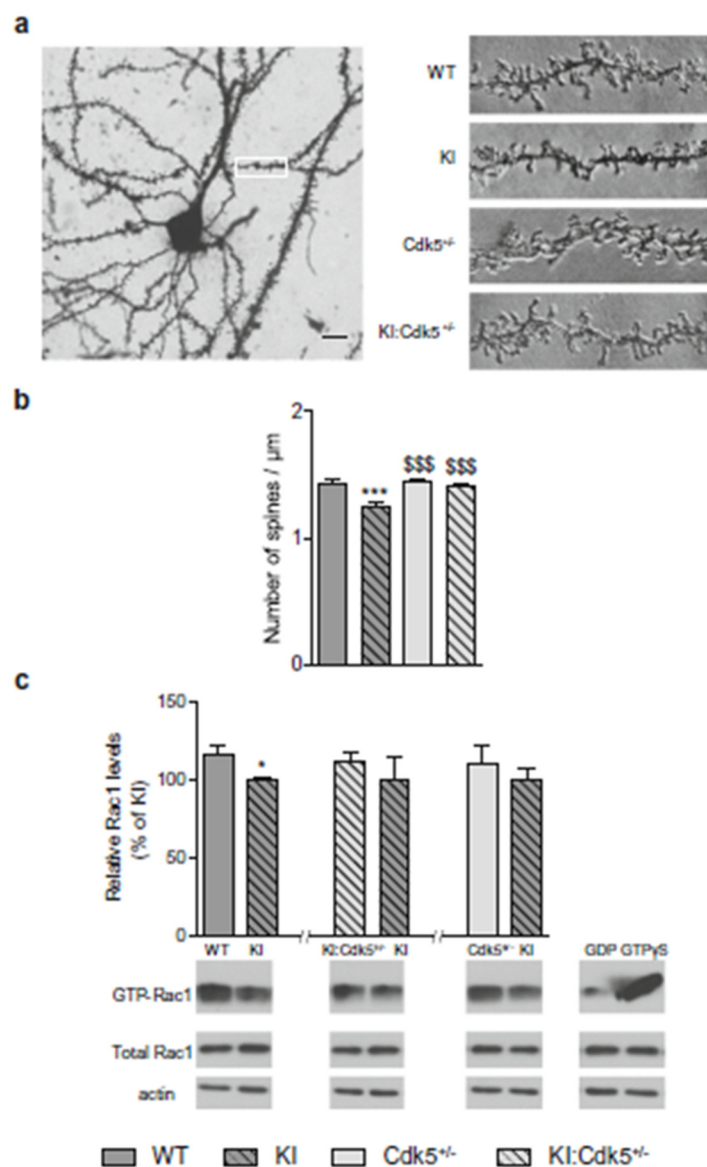
Fig. 9 Decreased cortical dendritic spine density in mHtt KI mice is attenuated in mHtt KI:Cdk5^{-/-} mice. **a**

Representative images of a Golgi-impregnated cortical pyramidal neuron (left) and of apical dendrites of layer V pyramidal neurons from WT, mHtt KI, Cdk5^{-/-}, and mHtt KI:Cdk5^{-/-} mice (right).

b Histogram showing quantitative analysis of dendritic spine density per micrometer of dendritic length. Significant reduction of dendritic spine density in mHtt KI mice was attenuated in mHtt KI:Cdk5^{-/-} mice. Statistical analysis was performed using one-way ANOVA with Newman-Keuls post hoc comparisons (30 dendrites from 3 to 5 animals per genotype were analyzed).

****p* < 0.001 compared to WT mice. \$\$\$*p* < 0.001 compared to mHtt KI mice. **c** Representative Western blots showing levels of GTP-bound Rac1 immunoprecipitated from cortical lysates indicating Rac1 activity. A significant decrease in Rac1 activity was found in mHtt KI mice when compared to WT mice.

Histograms represent the mean ± SEM and are normalized to mHtt KI values (*n* = 4–5 mice per genotype). Statistical analysis was performed using Student's two-tailed *t* test. **p* < 0.05 compared to mHtt KI.



reduced pS1116 phosphorylation correlates with increased surface NR2B levels [31]. However, it is important to remark that we have only found a decrease of pS1116 NR2B levels in the cortex, where total NR2B levels are also reduced. Thus, we can speculate that reduction in total levels may account for the decrease in pS1116 phosphorylation levels. In our view, all these data indicate that, at least in the cortex, Cdk5 is acting as a negative regulator of Src activity facilitating NR2B internalization and its subsequent degradation and support our hypothesis that it is not a global increase in Cdk5 activity but an

aberrant phosphorylation of specific substrates, the mechanism responsible for altered cognition in HD mice. Indeed, although we have not found significant differences in Cdk5 cortical activity between WT and mHtt KI mice (data not shown), a significant reduction of p35 at the plasma membrane and a decreasing trend for Cdk5 were observed in the cortex but not in the striatum or the hippocampus of mHtt KI mice compared to WT mice. In contrast, similar levels of p35 at the plasma membrane were found between mHtt KI:Cdk5^{-/-}, Cdk5^{-/-}, and WT mice. Based on these results, it is possible

to hypothesize that in the cortex of mHtt KI mice, the levels of Cdk5/p35 complexes at the plasma membrane are reduced leading to altered phosphorylation of physiological Cdk5 substrates such as NR2B. These results are in line with previous studies showing a protective role for Cdk5/p35 complexes in HD [65–67].

Nevertheless, it is important to emphasize that in the hippocampus contrary to the cortex or the striatum, the recovery of NR2B surface levels by genetic reduction of Cdk5 was only partial, indicating that Cdk5 acts on hippocampal memory deficits by modulating other substrates besides NR2B. Since different Cdk5 substrates have been reported to modulate dendritic spine formation and maintenance, we analyzed whether normalization of dendritic spine density could underlie hippocampal memory improvements [32–36]. In accordance with previous findings [21], we found a reduced number of dendritic spines in CA1 pyramidal neurons in mHtt KI mice compared to WT mice, which correlates with a decrease in the number of postsynaptic clusters. Among the different Cdk5 substrates, we focused on the Rho-GTPase Rac1 as (i) several lines of evidence demonstrate its involvement in structural and functional spine plasticity and therefore learning and memory [68–70] and (ii) inhibition or downregulation of Cdk5 activates Rac1 function [37, 71, 72]. Interestingly, both Cdk5^{+/−} and mHtt KI:Cdk5^{+/−} mice present a significant increase in hippocampal Rac1 activity which correlates with increased dendritic spine density in Cdk5^{+/−} mice and with a complete recovery of spine numbers in mHtt KI:Cdk5^{+/−} mice. Thus, although mHtt is not altering dendritic spine density by decreasing Rac1 function, prevention of dendritic spine loss in the hippocampus can be achieved in mHtt KI mice by reducing Cdk5 expression, which leads to increased Rac1 activity. Consistently, it has been reported that silencing Cdk5 in an Alzheimer's disease mouse model induces an increase in Rac1 activity that correlates with an amelioration of spatial memory deficits [37]. However, we cannot rule out that other Cdk5 substrates, whose phosphorylation is involved in spine retraction [32, 33], could also contribute to the recovery of hippocampal dendritic spines and memory improvement. Interestingly, we have also reported that Rac1 activity and dendritic spine density are reduced in the cortex of mHtt KI mice [22]. Therefore, we wondered whether Cdk5 dysregulation by acting on both cortical NR2B and Rac1 activity would underlie motor learning and procedural memory deficits in mHtt KI mice. Interestingly, our results revealed a similar reduction in the number of cortical dendritic spines in mHtt KI mice that was prevented by genetic reduction of Cdk5. In contrast, the decrease in Rac1 activity found was not recovered by genetic reduction of Cdk5 in mHtt KI:Cdk5^{+/−} mice supporting again a brain region-dependent role of Cdk5 in learning and memory.

Taken together, our study shows the complex involvement of Cdk5 in corticostriatal and hippocampal learning and memory deficits induced by mHtt. Moreover, it indicates that

Cdk5-dependent regulation of substrates and neuronal processes is controlled differentially within brain regions. Future studies are necessary to extend our knowledge on region-dependent functions of Cdk5 and its precise molecular interplay with mHtt. Gaining a better understanding of the role of Cdk5 in HD and other neurodegenerative disorders with Cdk5 involvement may reveal novel therapeutic targets for these pathologies.

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Compliance with Ethical Standards All procedures were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the local animal care committee of the Universitat de Barcelona (76/15) and Generalitat de Catalunya (00/1094), in accordance with the Directive 2010/63/EU of the European Commission.

Competing interests The authors declare that they have no competing interests.

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