UNIVERSITAT DE BARCELONA

# Evaluation of therapeutic targets for the treatment of behavioral alterations and neuropathology in Huntington's disease

## The role of histone deacetylase 3 and p75 neurotrophin receptor

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# Evaluation of therapeutic targets for the treatment of behavioral alterations and neuropathology in Huntington's disease

## The role of histone deacetylase 3 and p75 neurotrophin receptor

Doctoral degree of Biomedicine in the Facultat de Medicina de la Universitat de Barcelona.
Dissertation submitted by:

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Núria Suelves Caballol Sílvia Ginés Padrós

Programa de Doctorat en Biomedicina

*Als meus pares,*

*Al Marc,*

*"[...] la bellesa intel·lectual és autosuficient, i és per ella, més que pel bé futur de la humanitat, que el científic es condemna a un treball llarg i dolorós"*

Henri Poincaré

# AGRAÏMENTS

*Un seguit de casualitats i el resultat de la feina feta durant anys m'han fet arribar al final d'aquesta etapa, però són tots aquells amb qui he compartit el camí els que han fet que l'acabi sense perdre la il·lusió, tot ajudant a formar-me com a científica i com a persona. Sense vosaltres no hauria pogut escriure aquesta tesi, així que espero poder trobar les paraules correctes per expressar-vos el meu agraïment més sincer.*

*La meva primera incursió a la vida d'un laboratori va ser durant l'estiu de tercer de biotecnologia, quan vaig interessar-me pel grup de neuro del Carles, qui amablement va confiar en mi per formar-ne part i va guiar el meus inicis en recerca. Allà vaig tenir la sort de trobar uns companys de laboratori fantàstics. L'Arnaldo, que amb paciència i calma exemplars em va ensenyar a fer les meves primeres Real times, el Sergi, l'Elsa i el conjunt del grup, que van fer-me sentir una més i em van encomanar les ganes de treballar.*

*Vaig acabar aquesta etapa més o menys al mateix temps en què posava fi als quatre anys de carrera. Però per sort, ara puc dir que no posava fi a l'amistat d'un grup de persones amb qui havia compartit tants bons (a vegades també estressants) moments, tant a la universitat com a fora. A la Feilah, al Raül, a l'Alba, voldria agrair-vos especialment que no hagueu perdut les ganes d'organitzar i trobar temps per les quedades, amb aquest desig sincer de conèixer els camins diversos que hem triat, a la vegada que vosaltres mateixos heu seguit uns passos dels que em sento ben orgullosa. Gràcies també, Feilah, per fer possible el nostre viatge de fi de curs a casa de la teva tant nombrosa família (sin que decirlo sea un mal de ojo eh!), amb sobredosi de té però també d'afecte i hospitalitat.*

*I així, seguint els passos de la investigació en neurociència vaig demanar a la Sílvia que em deixés formar part del seu grup a l'hospital clínic per realitzar les pràctiques del màster. Amb maneres didàctiques i a la vegada amb un humor i simpatia que se'm contagiaven i em feien sentir còmode, vaig conèixer els detalls de la feina que hi feien i vaig pensar que havia triat un molt bon lloc. Al cap dels anys, he pogut confirmar aquesta primera sensació. Recordo amb un somriure quan escrivíem/revisàvem juntes al teu despatx alguns fragments del paper de la HDAC3 i m'explicaves que tu havies començat de la mateixa manera. Gràcies Sílvia, per creure en mi i transmetre'm aquella seguretat que a vegades em falta, per donar-me l'empenta necessària en el moment precís i estar sempre disponible per escoltar-me amb paciència i ensenyar-me tot el que saps. Compartint despatx amb la Sílvia, he d'agrair també a l'Esther el seu interès i aportacions a la meva feina. Gràcies per aquesta personalitat tant propera, tant vital, pel bon rotllo i optimisme. Les comissions de doctorat amb tu i la Cristina van ser molt amenes i divertides, amb alguns nervis al principi que se m'oblidaven al cap de cinc minuts. Aprofitant que parlo de vosaltres, també voldria mencionar a la resta de PIs, el Jordi, el Pep, el Gustavo, l'Eulàlia, per destacar la meva admiració cap la vostra feina, que feu amb tanta dedicació malgrat que la manca de finançament públic no us posen les coses gens fàcils.*

*I així com el treball dels caps de grups és important, també és clau la feina de les nostres secretaries, Núria, Carme i Mercè, que fan possible i faciliten totes les gestions administratives amb la seva llarga experiència i enorme professionalitat, sense perdre mai l'amabilitat ni oblidar-se d'un somriure.*

*Vaig passar a formar part del grup de l'histolab, doncs, amb l'excusa del màster de Biomedicina. Però el que havia de ser un curs relativament breu i que entenia sobretot com un tràmit em va sorprendre amb unes amistats que no m'esperava que agafessin un pes tant important a la meva vida. Júlia, Gian, Lluís, Laila, Guillem (malgrat el temps i la distància), i les incorporacions externes de l'Edu i el Lluís (el nou). Si d'alguna cosa estic especialment orgullosa i satisfeta he de dir que és d'haver-me acostat a vosaltres i ser part de la família biomed. Gràcies nois, per tantes i tantes coses, fins i tots pels sopars depriments de diumenge. Laila, amb aquesta pau interior i amabilitat que m'encanten, i a la vegada amb una passió energètica per tot el que et fa feliç. Gian, com enyoro les teves classes*

*d'escalada setmanals. Recordo com xerràvem mentre pujàvem a la foixarda i, un cop allà, m'insisties perquè obrís jo la via fins que amb les cames tremolant arribava a dalt de tot sentint que havia superat un gran repte. Lluís, amb el coneixement i la voluntat que tens sé que aconseguiràs fer tot el que et proposis, de fet tinc sort que no hagis començat abans a curar el Huntington amb el CRISPR perquè potser aquesta tesi ja no tindria cap interès... jejeje. Júlia, quantes coses hem viscut juntes i quants secrets que ja ho són una mica menys. Gràcies per la teva confiança i per voler sempre la meva companyia, ets millor del que em mereixo. I a tots en general, hem creat records meravellosos junts i m'heu fet molt feliç, espero que compteu amb mi sempre, tant pel bons com pels mals moments, i que no s'acabin mai les nostres aventures.*

*Un cop acabat el màster vaig quedar-me al mateix lloc per començar el projecte que després d'uns cinc anys per fi queda recollit en aquesta tesi. I, lògicament, al llarg de tot aquest temps he compartit moments amb moltes persones. Tot i que no vaig tenir-hi cadira gaire temps, el laboratori de la cinquena s'ha mantingut com a lloc clau de trobada amb la resta de companys del grup i, per sort, he tingut sempre motius per passar-hi llargues estones, ja sigui revelant, "saquejant" (de menjar i productes de lab), o fent les sempre tant necessàries pauses a la sala de cafès. Una de les persones imprescindibles de la cinquena és la Maite, la "mami" de l'histolab. Voldria remarcar la gran sort que té aquest grup de comptar amb tu. Amb la teva feina organitzada i metòdica i amb la valuosa ajuda que ofereixes a qualsevol que te la demana fas que tot funcioni correctament. Tens aquest balanç perfecte per saber renyar un miqueta quan cal (sempre amb raó, clar!), mentre segueixes sent una persona propera que troba temps per parlar de "cotilleos" i riure una estona. Gràcies per ser com ets i per haver-me ajudat tantes vegades.*

*En aquest laboratori, per tant, és on vaig començar els primers passos per acabar finalment escrivint aquestes línies, i no podria haver-ho fet sense ajuda. Vero, me gustaría empezar por transmitirte mi más sincero agradecimiento. Han sido muchas (y muy importantes para mí) las oportunidades que me has dado y aunque a veces sea una persona de pocas palabras creo que ya sabes que soy consciente de ello. Gracias a ti he tenido la oportunidad de participar en proyectos que terminaron en éxito y tengo la suerte de que me has hecho sentir siempre partícipe de ellos. Espero haber estado a la altura. Y aunque somos bastante distintas, admiro de verdad tu espontaneidad y esa fuerza y perseverancia que pones para llegar a conseguir tus metas, siempre complementado con trabajo duro. Gracias de corazón por todo lo que has hecho por mí.*

*I si d'algú vaig aprendre especialment, tant a nivell personal com professional, és de les companyes (ara a terres internacionals) amb qui vam exiliar-nos al Cellex. Mar, quina canya de persona! Com m'agrada aquest caràcter tant alegre i optimista, combinat amb una passió i professionalitat increïbles per fer la feina ben feta. Mai vas deixar de trobar temps per mi ni vas negar-me res, sempre amb un somriure, i vas ensenyar-me tantes coses que crec que no podré agrair-t'ho del tot... Cheru, no quería parecer repetitiva, pero es que realmente tú también me ayudaste muchísimo. Fue una suerte sentarme a tu lado y que poco a poco conociéramos más una de la otra. Gracias por tu amabilidad, por la enorme cantidad de experiencia transmitida y por ser una persona tan cercana con quien sé que siempre podré contar. A les dues regletoneras, que encara que us mencioni una mica apartades de la resta perquè representeu un punt clau en el meu creixement personal i professional, espero que sapigueu que sou pilars essencials en aquest família. Moltíssima sort en tots els vostres projectes i sobretot que la distància i el temps no ens facin perdre el contacte.*

*Als estudiants que han passat pel Cellex: Irina, Sergio, Mireia, Ainhoa, Aida, Àlex, gràcies per ser tant atents i treballadors però sobretot grans persones. I als que encara hi són. Anika, que aunque me hayas robado el sitio (es broma eh!) te deseo muchísima suerte en estos proyectos que ahora empiezas, que estoy convencida de que lo harás*

*muy bien. Sort també Ana, que hem coincidit poquet però sé que ets una treballadora nata i mostres un interès sincer per la ciència. Marc, estic contenta d'haver-te vist madurar i espero que segueixis aprenent amb aquest bon ritme i arribis fins on et proposis. I Esther, moltíssima sort i força allà on vagis!*

*També del Cellex m'agradaria destacar els meravellosos companys de planta: el Manel (on són els meus xipirons??), la Paqui, la Cristina, el Francesc i tota la resta, amb aquests riures que se m'encomanen sense voler-ho des de l'altra punta del lab i amb una disponibilitat permanent a ajudar-me sempre que ho necessitava. A la Maite (Cazorla), una persona imprescindible pel laboratori que no només és una professional exemplar (a part d'un nervi!) sinó que també té un tracte càlid i proper amb tothom. Gràcies per tota l'ajuda prestada i per ser una persona tant excel·lent. I també als amagats als despatxos del fons, Mario i Rubén, gràcies per l'interès i per l'intercanvi científic, us desitjo el millor en els vostre futur personal i professional.*

*Per canviar de zona, m'agradaria parlar ara de la gent de la tercera planta, al laboratori del Pep. Des dels primers amb qui vaig coincidir. Inés guapa! Que persona tan especial y que suerte tengo de haberte conocido. Las ganas y el empeño que pones en cualquier proyecto que empiezas me generan la más profunda admiración. He echado de menos nuestros encuentros en la tercera cuando venía a poner Real times y siempre me encontraba con tu sonrisa, tus bromas y tu calidez, y tengo que decir que todas las excursiones para hacer el papeleo de la FI no hubieran sido lo mismo sin ti. Mònica, sempre amb bon rollo i una personalitat tant oberta que em despertava molta confiança, com si et conegués de feia temps. Jordi, tant amable i disposat a ajudar-me amb tot. Andy, con esa simpatía y ese carácter tan poco alemán, recuerdo que las primeras clases de histología que me tocó dar fueron más amenas y divertidas contigo. Georgina, quina persona més encantadora! Crec que la tercera no seria el mateix sense el teu caràcter alegre i positiu. La veritat és que tinc ganes de provar aquest bingo musical que té tan bona pinta. A l'Ana, no sé quantes vegades t'ho hauran dit, però és que realment no podríem tirar endavant cap projecte sense la teva feina, que fas tant bé i de manera tant eficient, gracias por la inmensa ayuda prestada. A l'Andrea, amb aquest caràcter inquiet i ple d'entusiasme, admiro mucho tu interés apasionado para la investigación y estoy segura de que llegarás muy lejos. Mireia, ens hem conegut poquet però sé que fas molt bona feina i et desitjo el millor en tot. Phil, gracias por tus numerosas y más que valiosas contribuciones durante los seminarios y por ese sentido del humor inesperado y genial que siempre me saca una sonrisa. I als estudiants més jovenets, tant els que van passar ja fa temps pel lab com els que he conegut més recentment: les dues Cris, la Sandra i el Xavi, que sé que treballen molt dur a part de ser increïblement simpàtics, moltíssima sort en cadascun dels vostres camins. A tots vosaltres de la tercera, aquells dies que vaig "instal·lar-me" al vostre microscopi vaig sentir una mica d'enveja de no formar part d'aquest grup tant maco. Seguiu així!*

*I per canviar un altre cop de zona aprofitaré parlant primer de dues persones que van començar a la tercera però vaig veure entrar a la sala blanca, aquest búnquer que de moment no he aconseguit visitar tot i que em queda pendent. Raquel, quina superwoman! Quanta energia, saviesa i optimisme, quin tracte tant agradable. Tant de bo et vagi tot molt bé i ens veiem algun dia aviat! I Cristina, des del primer dia vaig veure que havies de ser una persona molt divertida i així m'ho has demostrat. Ha sigut molt agradable compartir estones amb tu. A la resta d'integrants del grup: Gemma, des del primer moment em vas fer sentir que còmode per explicar-te qualsevol cosa i vaig veure que eres una persona preciosa. Verónica, gràcies per tanta calidesa i amabilitat. A l'Unai, al Felipe, molta sort amb la feina i amb tot el que vingui en un futur.*

*M'agradaria ara fer una breu intrusió al laboratori de la Cristina, on hi treballa la Núria, una persona que admiro per la qualitat de la seva feina però també per l'amabilitat i el bon tracte que m'ha demostrat sempre. Sé que no et cal pas, però moltíssima sort amb la tesi, espero no perdre-me-la!*

*Torno per fi al lloc on va començar tot, la cinquena. Dels companys de la cinquena que ja no hi són, m'agradaria començar pels que van marxar abans, però que tot i la breu coincidència de temps van deixar una forta empremta. A l'Adrià, la Laura, la Marta (Anglada), la Laia, el Javi, l'Enric, el Shiraz. Em vau demostrar que la solidaritat amb els companys i el bon tracte són marca de la casa, i que no hi ha cap feina, per rutinària que sigui, que no pugui fer-se amb passió i un toc d'alegria. Albert, que vas marxar però has fet un retorn ben merescut. Sempre he admirat la teva dedicació per la ciència i la teva energia inesgotable, però sobretot la dosi d'humor que poses en tot el que fas i que s'encomana a la gent del teu voltant.*

*Als Gustavos de "segona generació", Yolanda, Dasha, Thayna, Júlia, i Fabio, i també als estudiants Nacho i Carla, dir-los que ha sigut un plaer compartir estones amb vosaltres. A la Laura, que sempre m'has demostrat ser una persona plena de comprensió i tendresa. T'envio tots els meus desitjos perquè estiguis sempre rodejada de bones persones que apreciïn les teves ganes de treballar i la bona feina que fas. I a l'Isaac i la Cristina, ens hem conegut poc però us desitjo molta sort en els projectes que comenceu.*

*A Ened, que sé que podrás superar todos los obstáculos del doctorado con esa fuerza que tienes, mucha suerte y asegúrate de disfrutar tanto como puedas del trayecto. Y a Cynthia, que aunque no te conozca mucho me han comentado que eres una persona genial, suerte y ánimos!*

*Si parlem d'estudiants exemplars, és de requisit mencionar l'Aina, la Sílvia i la Sara. Amb una il·lusió i interès que costen de trobar, el seu pas pel laboratori va deixar un rastre inesborrable. Encara que fa molt que no ens veiem, vaig passar molt bones estones amb vosaltres i em fa molt feliç saber que heu tingut èxit i aneu fent camí. Aina, em quedarà sempre gravat, entre d'altres coses, el moment que vas venir-me a buscar al Cellex per demanar-me ajuda per l'entrevista de Skype (i això que jo no tenia ni idea d'electrofisiologia) i Sílvia, vaig sentir complicitat amb tu des del primer dia i recordo amb tristesa quan vas passar a acomiadar-te i pensava que se m'escapava una gran persona. Tant de bo ens tornem a trobar.*

*Ha sigut molt important pel lab també el pas de la Raquel. Hasta el último momento he podido contar contigo, ¡no sabes cómo te lo agradezco guapísima! Tienes un corazón gigante y sé que cosechas amistades duraderas por cualquier lugar que pisas. ¡Mucha suerte en todo! Sort també a les noves adquisicions del minilab. Anna i Carla, demostreu una gran autonomia i feu un treball impecable, tant de bo sempre us valorin com us mereixeu.*

*Ja que ens trobem al minilab, allà treballa una persona a qui admiro i respecto, l'Ana. Gracias Ana por nuestras charlas y tu compañía, por toda la ayuda que me has ofrecido tantas veces e incluso por confiar en mi criterio y hacerme crecer. Este laboratorio ha tenido una suerte increíble de contar contigo todos estos años, y aunque será muy extraño que ya no estés en unos días, estoy segura de que vas a encontrar un buen sitio que te aportará nuevas experiencias y alegrías. I tu Gari, que ja fa temps que vas marxar però et tenim sempre molt present i per sort ens trobem de tant en tant. Gràcies per ser tant divertit i encantador. Glòria, buff que dir-te. Des del principi vaig saber que eres una tia molt guai! La de vegades que una petita conversa amb tu m'ha fet rebaixar el pes de les preocupacions i afrontar-les amb optimisme. Has sigut una persona que ràpidament es va fer molt propera i per això vaig viure els teus primers passos cap a la maternitat amb gran il·lusió i nervis. Que disfrutis ara d'aquest temps de descans,*

*i molta sort en les teves properes etapes. I parlant de postdocs, tot i que amb despatx a la quarta. Mercè, tens unes capacitats didàctiques envejables, amb una manera sempre calmada i educativa de transmetre el coneixement. Gràcies pel teu tracte proper i amable i per l'ajuda que m'has proporcionat tantes vegades.*

*Finalment, passarem a revisar la resta d'integrants d'aquesta família tant bonica que hem creat a partir d'incomptables hores de feina i estrès, però també de sortides, viatges o simples "afterworks" a l'Ascot, sempre plens de bons moments que puc assegurar que han ajudat a que aquest projecte tirés endavant.*

*Començaré per una de les persones més "currantes" del grup regletonero. Andrés!! Quanta pasión para tantas aficiones diferentes... y que estilo y buen gusto para todo. Eres la mejor compañía que cualquiera podría tener, tan divertido y alegre. He de confesar que siempre me he reído de tus chistes malos, aunque también es verdad que soy de risa fácil…jejeje. Gracias por tus incontables muestras de amistad y cariño, pero sobre todo por ser la persona tan especial que eres.*

*Carleta, que ara ja fa temps que vas marxar però recordo molt bé quan va quedar buida la teva cadira i se'm va fer tan estrany. M'agrada molt com ets! Tant apassionada per les coses que t'agraden i tan atenta amb els teus amics. Gràcies per l'ajuda que m'has donat sempre, i també perquè malgrat els anys passin no haguem perdut el contacte.*

*Annemie, que sorpresa me diste el otro día con el catalán, ¡aún estoy que alucino! ¿Como eres capaz de ser tan perfecta y a la vez tan loca? Recuerdo como si fuera ayer cuando te escuchaba durante la defensa de tu tesis, llena de admiración y orgullo. Ojalá sigamos compartiendo como hasta ahora tantas risas y buenos momentos. No hace falta que te diga que estaré siempre disponible para ti, dispuesta a ayudarte o a poner el hombro y escuchar cualquier drama que necesites contarme.*

*Xavi, guardo molts bons records de la teva breu però intensa estada al lab. No sé com però vas injectar d'energia el grup i vas transmetre'ns les ganes de sortir i celebrar l'amistat que teníem. Gràcies per les llargues xerrades sobre "sentadilles", excursions i altres passions compartides mentre tornàvem a casa els divendres i em sorprenia de que em poguessis fer sentir tan còmode.*

*Sara, que honor haberte conocido. Siempre he admirado esa personalidad que tienes, tan extrovertida, fuerte y decidida. Gracias por hacerme reír tanto, por contagiarme tu energía y por ser esa persona altruista, capaz de echar una mano a un amigo y alegrarse con sinceridad de los logros de los demás. Tus abrazos y muestras de afecto han sido un gran apoyo, gracias por tanto cariño.*

*Jordi, tot i que a vegades m'has posat una mica nerviosa, com quan em vas dir sense venir al cas que estava com un llum (tot i que els dos sabem que tampoc estaves equivocat...), sé perfectament que ets una persona impecable i aprecies i cuides els teus amics com ningú. A més, sempre he admirat la teva gran capacitat de treball, per mi tot un model a seguir. Gràcies per ajudar-me a entendre que la qualitat científica no està renyida amb la qualitat humana, i gràcies pels moments divertits que hem compartit.*

*Marta, ets una caixa de sorpreses. D'entrada sembles tímida i reservada, però realment ets una persona que sempre ve de gust escoltar, plena d'energia i amb un sentit de l'humor envejable. Sé també que ets una treballadora infatigable i perseverant, i tot i els obstacles dels teus projectes sé que aconseguiràs tirar-los endavant i acabar-los amb èxit. Ànims i sort en tot!*

*Laureta (Vidal), quina persona més meravellosa. Tant decidida i valenta, amb les idees clares i una voluntat constant de superació. Des del començament vas obrir-te amb una confiança cap a mi sempre total i sincera i vas ajudar-me moltíssim a mostrar-me tal com era. M'has fet sentir valorada i estimada, i aprendre i créixer al teu costat ha sigut un privilegi. En les nostres xerrades durant la setmana del cervell ja vaig veure que això d'ensenyar t'agradava i que volies aprendre a fer-ho cada cop millor. Ets una crack i estic segura que t'anirà genial i podràs superar tots els reptes.*

*Laura (López), quantes coses boniques m'agradaria i podria dir-te...Has fet de la meva estada al Cellex un veritable plaer (sobretot quan l'Elena em va abandonar per culpa de l'internet jejeje). Potser perquè trobo que tenim uns caràcters similars sempre he sentit molta connexió amb tu i he notat que podia explicar-te qualsevol cosa i que sempre m'entendries. Espero haver-te ajudat quan ho necessitaves, tot i que jo també he requerit la teva ajuda en incomptables ocasions. Ets fantàstica, gràcies per la teva amistat.*

*Rafa, no sé porque tengo grabado en el cerebro la imagen de la primera vez que te vi, sentado delante del ordenador de la sala de cafés. Fue una sorpresa descubrir que los dos habíamos hecho biotec en la autónoma, y desde entonces he ido descubriendo que nos unían muchas más cosas. Gracias por las charlas interminables, por ayudarme a escoger un móvil (no nos costó ni nada decidirnos eh jejeje), por explicarme con fascinante pasión todos tus proyectos e incluso por pedirme consejo y valorar mi opinión siempre. Creo que no voy a poder compensarte del todo el que sacaras siempre tiempo para ayudarme, casi sin pedirlo. Eres la persona más buena que he conocido, espero que todos te traten siempre como te mereces.*

*Gerardo, tens tantes bones qualitats i el cervell tant ple de coneixement que a vegades es fa difícil pensar que algú com tu és real i és, a més, una persona tan propera. Recordo amb carinyo les "shishes" compartides i les hores a l'ascot (on per sort ets dels que no hi falten mai) escoltant les teves "batalletes" amb fascinació i interès. Gràcies també per tota la teva ajuda durant tants anys, he après moltíssim de tu.*

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# RESUM

## RESUM

La malaltia de Huntington (MH) és un trastorn neurològic progressiu i incurable que es caracteritza clínicament per la presència d'alteracions motrius, cognitives i psiquiàtriques, les quals generalment es manifesten en l'edat adulta i acaben ocasionant la mort del pacient en 15-20 anys (Walker, 2007). L'evident simptomatologia motora, que consisteix en moviments involuntaris, ràpids i irregulars del tronc i extremitats (moviments coreics), segueix sent un factor clau per a la seva diagnosi clínica. No obstant, dècades abans de l'aparició dels primers símptomes motors, s'ha observat que els pacients poden presentar un ampli espectre de disfuncions en l'àmbit cognitiu, on destaquen les alteracions en l'aprenentatge de noves habilitats i en diferents tipus de memòria, i també en l'àmbit psiquiàtric, com són la depressió, l'apatia i els canvis bruscs de personalitat. Aquests símptomes primerencs poden arribar a ser greument incapacitants i suposen una gran inquietud tant pel malalt com pels seus familiars (Ready et al., 2008; Williams et al., 2010). La MH és un desordre genètic, d'herència autosòmica dominant, causat per una mutació situada a l'exó 1 del gen anomenat *IT15* o *Htt,* el qual codifica per la proteïna anomenada huntingtina (Htt) i es localitza al braç curt del cromosoma 4 (Gusella et al., 1983). El tipus d'alteració genètica va definir-se l'any 1993 com un increment exacerbat del número de repeticions consecutives del codó CAG (citosina, guanina, adenina), que codifica per l'aminoàcid glutamina (HDCRG, 1993). En individus no afectats, el tram de CAGs és polimòrfic i mostra una variació d'entre 9 a 35 repeticions, però a partir de 36 repeticions la proteïna Htt adquireix propietats tòxiques i la malaltia pot manifestar-se amb una severitat i edat d'inici que correlacionen de forma inversa amb el número de repeticions (Andrew et al., 1993; Duyao et al., 1993; Snell et al., 1993). La neuropatologia de la MH es caracteritza per una progressiva mort neuronal i/o atròfia en determinades zones cerebrals (Vonsattel et al., 1985). La regió estriatal, que en els humans està formada pels nuclis caudat i putamen, és a nivell macroscòpic la més afectada des d'etapes inicials de la malaltia, amb una pèrdua específica de les neurones de projecció estriatals que pot arribar a ser gairebé completa i que es creu especialment lligada a la progressió dels símptomes motors (Albin et al., 1990). No obstant, altres regions com l'hipocamp o l'escorça cerebral presenten una evident i precoç disfunció neuronal que podria contribuir de forma important a l'aparició de les primeres alteracions cognitives (Giralt et al., 2012b). Des de la descoberta del gen i la mutació causants de la MH fa més de vint anys, la recerca científica ha destinat grans esforços a caracteritzar els diferents mecanismes moleculars subjacents. Malgrat això, es desconeix la visió global que explicaria l'específica vulnerabilitat de la regió estriatal i la concreta aparició dels símptomes associats (Zuccato et al., 2010). Entre d'altres, la desregulació transcripcional, l'expansió somàtica del triplet CAG i l'alteració de la senyalització neurotròfica tenen un inici temprà en la patologia, s'han observat en pacients i en múltiples models animals de la MH, i es creu que podrien desenvolupar un paper clau en la progressió de les característiques patològiques i els símptomes de la malaltia. Per això, la identificació de proteïnes que intervinguin en aquests processos és crucial pel descobriment de prometedores dianes terapèutiques per la MH que puguin ser modulades farmacològicament.

Per una banda, les evidències experimentals recopilades durant els darrers anys, especialment gràcies als anàlisis d'expressió a gran escala (bioxips) i eines de seqüenciació massiva, han demostrat que existeix una alteració progressiva de la maquinària transcripcional, amb gens que majoritàriament presenten una disminució en la seva expressió (Seredenina and Luthi-Carter, 2012;

Francelle et al., 2017). Aquesta alteració podria ser causada per la interacció aberrant de la forma mutada de la Htt (mHtt) amb nombrosos reguladors transcripcionals o amb reguladors de la conformació de la cromatina. En relació amb el segon supòsit, es coneix que l'acetilació de les histones, proteïnes empaquetadores de l'ADN, juga un important paper en la regulació de la transcripció gènica. En concret, s'ha definit que uns nivells d'acetilació elevats proporcionen una conformació més relaxada de l'ADN, promovent la transcripció de gens, mentre que nivells baixos d'acetilació contrauen l'ADN i reprimeixen l'expressió gènica (Eberharter and Becker, 2002). Les proteïnes encarregades de modular els nivells d'acetilació de les histones són les anomenades histones acetiltransferases (HATs), que incorporen grups acetil a les lisines de les cues de les histones, i les histones deacetilases (HDACs), involucrades en l'eliminació d'aquests grups acetil. En la MH, s'ha descrit que les histones associades a regions gèniques que mostren una repressió transcripcional presenten nivells d'acetilació disminuïts (Sadri-Vakili et al., 2007). És per això que en els últims anys el desenvolupament terapèutic de la MH s'ha centrant en l'ús de petites molècules inhibidores de l'activitat de les HDACs, amb la finalitat d'incrementar els nivells d'acetilació de les histones i promoure l'expressió gènica. Els primers fàrmacs generats consistien en inhibidors d'ampli espectre que, malgrat demostrar beneficis terapèutics en diferents models animals de la MH (Ferrante et al., 2003; Hockly et al., 2003; Gardian et al., 2005), podien donar lloc a efectes secundaris nocius, especialment en tractaments de llarga durada, deguts a la inhibició general de diverses HDACs. Per aquest motiu, en els últims anys s'ha impulsat la identificació d'isoformes específiques de HDAC que contribueixin de forma majoritària en la progressió de la MH i el desenvolupament simultani d'inhibidors selectius d'aquestes isoformes. Recentment, diferents estudis han assenyalat la isoforma 3 de la HDAC (HDAC3) com a una prometedora diana per la MH. En concret, l'administració d'inhibidors selectius de la HDAC3 a models animals de la MH ha demostrat millorar les anomalies conductuals, especialment els desordres motors, i la neuropatologia corticoestriatal associada a la MH, tot demostrant la seva eficàcia corregint les alteracions transcripcionals (Jia et al., 2012b, 2015, 2016). La important implicació de la HDAC3 com a regulador transcripcional negatiu dels processos de formació de memòria (McQuown et al., 2011) fa prometedora la seva inhibició per millorar les alteracions cognitives que ocorren de forma primerenca en la MH i que contribueixen de forma significativa al deteriorament de la qualitat de vida del malalt. Per aquest motiu, en aquesta tesi hem volgut analitzar el paper de la HDAC3 en l'aparició d'aquests símptomes i els mecanismes moleculars implicats. Per a dur a terme aquest primer objectiu, vàrem realitzar un tractament crònic amb injeccions subcutànies periòdiques d'un inhibidor altament selectiu de la HDAC3, el RGFP966, a ratolins *wild-type* (WT) i ratolins model de la MH, anomenats $Hdh^{Q7/Q111}$ (KI), i en vam avaluar els efectes terapèutics gràcies a la realització de proves de comportament durant el transcurs del tractament i a l'avaluació bioquímica i molecular dels teixits cerebrals dels animals un cop finalitzat aquest. Així, els nostres resultats han demostrat per primer cop que la reducció selectiva de l'activitat de la HDAC3 és capaç de prevenir el deteriorament de diverses funcions cognitives en els ratolins KI, com és l'alteració en l'aprenentatge de noves habilitats motores o en la formació de memòria espacial i reconeixement a llarg termini. A nivell molecular, hem associat aquestes millores a una recuperació de l'activació transcripcional dels gens *Arc* i *Nr4a2*, els quals s'han implicat positivament en processos de potenciació a llarg termini i de formació de memòria (Guzowski et al., 2000; Peña de Ortiz et al.,

2000; Colón-Cesario et al., 2006; Plath et al., 2006; Shepherd and Bear, 2011; Hawk et al., 2012; Bridi et al., 2017) i aprenentatge (Kleim et al., 1996; Jin and Clayton, 1997; Hosp et al., 2013; Cao et al., 2015). En estudiar els mecanismes subjacents a aquesta recuperació transcripcional, hem descobert que la inhibició selectiva de la HDAC3 podria promoure la transcripció gènica per dos mecanismes no excloents. Per una banda, la reducció de l'activitat de la HDAC3 pot provocar directament un increment dels nivells d'acetilació de les histones, la qual cosa afavoreix una conformació més oberta i relaxada de la cromatina que facilitaria l'expressió gènica. D'altra banda, els nostres resultats confirmen, tal i com suggerien estudis previs (Chuang et al., 2006), la capacitat de la HDAC3 d'utilitzar substrats no histònics, com la proteïna CBP. D'aquesta manera, en administrar l'inhibidor RGFP966 els nivells d'acetilació de CBP augmenten, probablement incrementant la seva activitat transcripcional i, per tant, promovent l'expressió dels seus gens diana, els quals s'ha demostrat que participen en processos de memòria i plasticitat sinàptica (Alarcón et al., 2004; Korzus et al., 2004; Wood et al., 2005, 2006). Aquests resultats destaquen la importància de regular l'activitat de la HDAC3 en la MH, ja que una extensa bibliografia científica ha demostrat que les alteracions en l'activitat de CBP causats per la presència de la proteïna mHtt contribueixen a la neuropatologia de la MH i en concret a l'aparició dels dèficits cognitius (Steffan et al., 2000, 2001; Nucifora et al., 2001; Jiang et al., 2003; Taylor et al., 2003; Giralt et al., 2012a).

Un altre dels mecanismes que podrien promoure la neuropatologia de la MH és la progressiva expansió del tram de repeticions CAG en el gen de la huntingtina mutada (m*Htt*), que s'observa sobretot en regions cerebrals especialment afectades per la malaltia, com és la regió estriatal (Telenius et al., 1994; Mangiarini et al., 1997; Wheeler et al., 1999; Ishiguro et al., 2001). Aquest procés, a més, s'ha pogut determinar que és més freqüent en neurones, el tipus cel·lular més vulnerable, que no pas en cèl·lules de la glia (Shelbourne et al., 2007; Gonitel et al., 2008). Diferents estudis han demostrat que l'expansió somàtica de repeticions CAG és deguda a l'acció de diferents proteïnes implicades en processos de reparació de l'ADN. Així, tot i que la funció fisiològica d'aquestes proteïnes és la de detectar i reparar alteracions en la seqüència de nucleòtids de l'ADN, en situacions concretes en què una seqüència repetitiva de trinucleòtids supera un llindar de repeticions determinat i es troba en un context cel·lular específic, aquesta activitat reparadora es torna mutagènica i pot donar lloc a la incorporació aberrant de noves repeticions en la seqüència esmentada. Implicades en gran mesura en aquest procés es troben les proteïnes Msh2 i Msh3, les quals formen el complex proteic anomenat MutSβ, que participa en el reconeixement d'errors d'aparellament de l'ADN (Manley et al., 1999; Wheeler et al., 2003; Dragileva et al., 2009; Tomé et al., 2009). D'altra banda, estudis recents han descobert que l'expansió de triplets de nucleòtids podria ser modulada per HATs o HDACs concretes (Debacker et al., 2012; Gannon et al., 2012). En concret, es va observar que la inhibició farmacològica o genètica de la HDAC3 en una línia cel·lular d'astròcits humans reduïa la freqüència d'expansió del triplet CAG. A més, es va determinar que aquest efecte era específic per la HDAC3, ja que la inhibició de la HDAC1 o la HDAC2 no modulava la freqüència d'expansions. Aquests resultats ens van portar a formular la hipòtesi de que la utilització d'un inhibidor selectiu de la HDAC3 podria tenir un benefici terapèutic dual, no només incrementant la transcripció de gens que promouen funcions cognitives sinó també reduint l'expansió somàtica del triplet CAG i, per tant, aturant la progressió de la MH.

## RESUM

Per tal d'avaluar la contribució de la HDAC3 en el fenomen d'expansió del triplet CAG a l'estriat dels ratolins KI, vam analitzar la distribució de la mida del tram de repeticions CAG en l'estriat dels ratolins tractats de forma crònica amb el fàrmac RGFP966. Les nostres dades han demostrat que la inhibició sostinguda en el temps de la HDAC3 permet reduir la freqüència d'expansions, fet que es tradueix en la producció de molècules (d'ARN o proteiques) probablement menys tòxiques que, per tant, alteraren en menor mesura el correcte funcionament de les neurones estriatals. De fet, els nostres resultats mostren que l'estabilitat del triplet CAG en l'estriat és acompanyada per una recuperació en els marcadors bioquímics estriatals DARPP-32, PDE10A i A2AR i una disminució en l'acumulació de formes oligomèriques de la proteïna mHtt. El mecanisme associat a la supressió d'expansions CAG per mitjà de la inhibició de la HDAC3, però, es desconeix. En aquest sentit, es va descobrir que la HDAC3 podria participar en la mateixa via molecular per la qual opera el complex MutSβ en la incorporació de repeticions. Degut a que la Msh2 conté diversos residus acetilables i interacciona amb nombroses HDACs (d'entre les quals la HDAC3), ens vàrem preguntar si la inhibició de la HDAC3 podria afectar l'estat d'acetilació de la proteïna Msh2. En aquesta tesi hem demostrat que el tractament de cultius neuronals de ratolí amb el fàrmac RGFP966 incrementa l'acetilació de la proteïna Msh2 en el residu de lisina 73 (Radhakrishnan et al., 2015), la qual cosa podria afectar la seva activitat reparadora, dificultant així la prolongació del tram de repeticions CAG. Els nostres resultats són de gran rellevància no només per al desenvolupament d'estratègies terapèutiques per a la MH sinó també per al d'altres malalties genètiques causades per una expansió aberrant de repeticions consecutives de trinucleòtids.

En demostrar-se el múltiple benefici terapèutic resultant de la inhibició de la HDAC3 en ratolins model de la MH, ens vàrem plantejar fer un estudi exhaustiu dels nivells proteics i l'estat de fosforilació de la HDAC3, el qual és indicatiu de la seva activitat deacetilasa, en la MH. En concret, nivells incrementats de fosforilació en el residu de serina 424 li confereixen una major activitat enzimàtica (Zhang et al., 2005). D'acord amb estudis previs (Valor and Guiretti, 2014), els nivells proteics totals de la HDAC3 en les regions de l'hipocamp i l'estriat de ratolins KI no es van mostrar alterats a cap de les edats simptomàtiques avaluades. En canvi, les nostres dades indiquen que la HDAC3 es troba hiperactivada selectivament a l'estriat d'aquest model a partir dels 8 mesos d'edat, correlacionant amb l'inici dels primers símptomes motors, els quals depenen majoritàriament d'aquesta regió cerebral. Seguidament, vam voler corroborar aquests resultats en pacients clínicament diagnosticats per la MH. Anàlisis realitzats en mostres *post-mortem* de putamen humà han confirmat una hiperactivació significativa de la HDAC3 en pacients de la MH, mentre que els nivells proteics totals resten inalterats. Així, malgrat desconeixem els mecanismes que incrementen selectivament l'activitat de la HDAC3 a l'estriat, podem associar aquests canvis amb l'inici dels símptomes motors. Degut a l'important paper neurotòxic descrit per a la HDAC3 (Bardai and D'Mello, 2011; Bardai et al., 2013), suggerim que l'increment d'activitat de la HDAC3 observat a la regió estriatal en el context de la MH pot contribuir a la disfunció i degeneració de les neurones estriatals. D'aquesta manera, la inhibició selectiva de la HDAC3 representa una teràpia neuroprotectora prometedora per a la MH.

Finalment, s'ha demostrat que en la MH existeix una important alteració de la senyalització neurotròfica, que en un primer moment va definir-se com una reducció en els nivells del factor

neurotròfic derivat del cervell (BDNF) (Ferrer et al., 2000; Zuccato et al., 2001; Ginés et al., 2003b). El BDNF es considera un factor clau per a la supervivència i el correcte funcionament neuronal, especialment pel de les neurones estriatals (Baquet et al., 2004). Així, diferents estudis van demostrar que la deficiència del BDNF en diferents models animals de la MH contribueix a l'aparició i severitat dels símptomes motors i cognitius (Canals et al., 2004; Lynch et al., 2007; Giralt et al., 2009). Les teràpies farmacològiques destinades a incrementar els nivells del BDNF en cervell, malgrat mostrar resultats prometedors en animals, afronten nombrosos obstacles degut a les complexes propietats farmacocinètiques d'aquest factor. Les accions biològiques del BDNF són regulades a traves de l'activació dels seus receptors transmembranals, anomenats TrkB i $p75^{NTR}$. En general, l'estimulació del receptor TrkB promou l'activació de vies tròfiques, com la supervivència, diferenciació i plasticitat neuronal, mentre que el receptor $p75^{NTR}$, segons el context cel·lular, pot activar o inhibir la senyalització a través de TrkB o promoure, de forma independent de TrkB, l'activació de vies neurodegeneratives o tròfiques (Roux and Barker, 2002). Malgrat no es coneixen amb exactitud les condicions cel·lulars que promouen l'activació de fenòmens d'apoptosi a traves del receptor $p75^{NTR}$, s'ha estipulat que podrien ser induïts en el cervell adult quan existeixen, per exemple, deficiències en els nivells proteics de BDNF i TrkB (Brito et al., 2013). De fet, en models animals i pacients de la MH s'han observat reduccions en l'expressió del receptor TrkB, increments en el receptor $p75^{NTR}$, i fins i tot senyalitzacions aberrants independentment dels nivells dels receptors (Ginés et al., 2006; Zuccato et al., 2008; Brito et al., 2013; Simmons et al., 2013; Plotkin et al., 2014). D'aquesta manera, les teràpies basades únicament en la sobreexpressió del BDNF podrien resultar fortament limitades per les alteracions en la senyalització del factor. Una de les opcions per tal d'atenuar les vies de neurodegeneració activades en la MH és la modulació del receptor $p75^{NTR}$. Estudis previs del nostre grup varen demostrar que la reducció dels nivells aberrants de $p75^{NTR}$ en l'hipocamp dels ratolins KI millorava els dèficits de memòria depenents de forma majoritària de l'hipocamp, alhora que revertia les alteracions en plasticitat sinàptica d'aquesta regió (Brito et al., 2014). En vista d'aquests fets, ens vam proposar estudiar la contribució dels nivells incrementats de $p75^{NTR}$ estriatals en l'aparició i progressió de la neuropatologia estriatal i les anomalies motores associades. Per tal d'assolir el nostre objectiu, vàrem creuar ratolins KI amb ratolins que presentaven una sola còpia funcional del gen del receptor $p75^{NTR}$ ($p75^{+/-}$), generant així un model doble mutant, el KI:$p75^{+/-}$, el qual presentava nivells de $p75^{NTR}$ normalitzats en el context de la MH. Primerament, en avaluar longitudinalment l'habilitat motriu dels animals hem pogut comprovar que la normalització del receptor $p75^{NTR}$ en els ratolins KI endarrereix l'aparició (de 8 a 10 mesos d'edat) de les primeres alteracions en la coordinació motora i en l'activitat locomotora espontània, la qual cosa suggereix que els nivells aberrants del receptor tenen un paper important en l'inici d'aquests dèficits. D'acord amb aquesta millora conductual, els animals KI:$p75^{+/-}$ mostren, fins als 10 mesos d'edat, una recuperació de diverses característiques neuropatològiques, com són la reducció de marcadors estriatals, els agregats de mHtt i la pèrdua de densitat d'espines dendrítiques. A més, a les mateixes edats, els ratolins KI:$p75^{+/-}$ mostren una normalització de l'aberrant senyalització neurotròfica, amb una recuperació dels nivells proteics de BDNF i TrkB, els quals es trobaven disminuïts a partir dels 8 mesos d'edat en els ratolins KI, i de la senyalització a través de TrkB de la via PLCγ, atenuada a partir dels 6 mesos d'edat. És important destacar que la via PLCγ juga un paper fonamental en la

regulació de l'activitat sinàptica i s'ha descrit que modula positivament la densitat d'espines dendrítiques, de tal manera que la seva restauració podria estar implicada en les millores observades en els ratolins doble mutants (Kim et al., 2010; Jang et al., 2013). Per altra banda, els nostres resultats han demostrat una hiperactivació de la via pro-apoptòtica JNK en els ratolins KI, que es coneix que pot ser activada independentment pel receptor $p75^{NTR}$. De forma coherent, la reducció dels nivells de $p75^{NTR}$ mostren una atenuació d'aquesta via, fet que podria contribuir en les millores observades. A partir dels 10 mesos d'edat, però, els nostres resultats mostren que la normalització dels nivells de $p75^{NTR}$ no és suficient per a revertir la reducció dels biomarcadors estriatals, el procés d'agregació o la densitat d'espines dendrítiques, a la vegada que no aconsegueix recuperar els nivells de BDNF o TrkB ni l'activació de PLCγ. Malgrat en desconeixem les causes, les nostres dades suggereixen que l'acció d'altres mecanismes patogènics que ocorren de forma progressiva en la MH acaben anul·lant els efectes positius de la reducció en els nivells de $p75^{NTR}$. Aquests resultats són especialment rellevants per al desenvolupament d'estratègies terapèutiques destinades a modular els nivells o l'activitat del receptor $p75^{NTR}$ en la MH, ja que demostren que els possibles beneficis obtinguts en etapes inicials i intermèdies de la malaltia podrien ser anul·lats en etapes més avançades. Destaquem, per tant, la importància d'investigar altres estratègies que promoguin la senyalització de la via BDNF-TrkB-PLCγ en etapes avançades de la MH.

En conclusió, els resultats presentats en aquesta tesi han proporcionat un coneixement detallat de la contribució de les proteïnes HDAC3 i $p75^{NTR}$ en els processos de desregulació transcripcional, inestabilitat del triplet CAG i desequilibri de la senyalització neurotròfica i han assenyalat la important participació d'aquests mecanismes en la fisiopatologia de la MH. Les evidències experimentals recopilades permeten concloure que les proteïnes HDAC3 i $p75^{NTR}$ participen en l'aparició de mecanismes patològics clau per a la correcta funció neuronal en diferents regions cerebrals, com en l'estriat i l'hipocamp, i per tant representen prometedores dianes terapèutiques per tractar de forma comuna la simptomatologia motora i cognitiva de la MH.

# ABBREVIATIONS

## ABBREVIATIONS

| | |
|---|---|
| A2AR | Adenosine receptor type 2A |
| Arc | Activity-regulated cytoskeleton-associated protein |
| ARTP | Accelerating rotarod task procedure |
| BAC | Bacterial artificial chromosome |
| BDNF | Brain-derived neurotrophic factor |
| BER | Base excision repair |
| CA | Cornu ammonis |
| $Ca^{2+}$ | Calcium |
| CAG | Cytosine, Adenine, Guanine |
| CBP | CREB-binding protein |
| cDNA | Complementary DNA |
| CREB | Cyclic AMP-responsive element-binding protein |
| DARPP-32 | Dopamine- cAMP-regulated phosphoprotein of molecular weight 32 kDa |
| DG | Dentate gyrus |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| ENK | Enkephalin |
| ERK | Extracellular signal regulated kinase |
| GABA | γ-aminobutyric acid |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| GFP | Green fluorescent protein |
| GPe | External globus pallidus |
| GPi | Internal globus pallidus |
| HAP | Huntingtin-associated protein |
| HAT | Histone acetyltransferase |
| HD | Huntington's disease |
| HDAC | Histone deacetylase |

## ABBREVIATIONS

| | |
|---|---|
| HDACi | Histone deacetylase inhibitor |
| Hdh | Huntington's disease homolog |
| HEAT | Huntingtin, elongation factor 3, regulatory subunit A of protein phosphatase 2A, and TOR1 |
| Htt | Huntingtin |
| IEG | Immediate early gene |
| JNK | c-Jun N-terminal kinase |
| K | Lysine |
| kDa | Kilodalton |
| KI | Knock-in |
| LTD | Long-term depression |
| LTP | Long-term potentiation |
| mHtt | Mutant huntingtin |
| Mlh | MutL homolog |
| MMR | Mismatch repair |
| mRNA | Messenger ribonucleic acid |
| Msh | MutS homolog |
| MSN | Medium spiny neuron |
| N-CoR | Nuclear receptor corepressor |
| NES | Nuclear export signal |
| NGF | Nerve growth factor |
| NII | Neuronal intranuclear inclusion |
| NLS | Nuclear localization signal |
| NMDA | N-Methyl-D-aspartic acid |
| NORT | Novel object recognition task |
| OLT | Object location task |
| $p75^{NTR}$ | p75 neurotrophin receptor |
| PCR | Polymerase chain reaction |

| | |
|---|---|
| PDE10A | Phosphodiesterase 10A |
| PHLPP | PH domain leucine-rich repeat protein phosphatase |
| PLCγ | Phospholipase Cγ |
| PolyQ | Polyglutamine |
| REST/NRSF | Repressor element-1 transcription factor/neuron restrictive silencer factor |
| RhoA | Ras homolog gene family, member A |
| RNA | Ribonucleic acid |
| ROCK | Rho-associated protein kinase |
| rpm | Rotations per minute |
| RT-qPCR | Quantitative reverse transcription polymerase chain reaction |
| S | Serine |
| SNc | Substantia nigra pars compacta |
| SNr | Substantia nigra pars reticulata |
| SP1 | Specificity protein-1 |
| STN | Subthalamic nucleus |
| SV40 | Simian virus 40 |
| Trk | Tropomyosin receptor kinase |
| TrkB-FL/TrkB-T1 | TrkB full length/TrkB truncated isoform |
| TSA | Trichostatin A |
| WT | Wild-type |
| Y | Tyrosine |
| YAC | Yeast artificial chromosome |

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# INTRODUCTION

# 1. Overview of Huntington's disease

Huntington's disease (HD) is a rare but devastating genetic disorder characterized by progressive neurodegeneration in specific regions within the central nervous system, which causes a triad of symptoms including motor, cognitive and psychiatric features. Although it is generally acknowledged that Dr. Charles Waters made a written account of the illness in 1841 (Bates et al., 2014), the first complete description was made in 1872 by Dr. George Huntington, who recognized its hereditary pattern and named the disease Chorea (derived from the ancient Greek word *Choreia*, meaning dance) due to the observed dancing propensities of the affected patients (Huntington, 1872). The name remained unchanged until the 1980s, when a large amount of evidence showing other non-motor alterations in patients of the disease caused a renaming to Huntington's disease. To date, there is no effective cure available for this devastating disorder and current treatment only reduce the symptoms without altering neuropathological progression.

## 1.1. Clinical aspects

Worldwide, HD has an incidence of 0.38 per 100,000 per year and a prevalence of 2.71 per 100,000, as shown by a recent meta-analysis (Pringsheim et al., 2012). However, several reports have demonstrated more than tenfold variation in HD prevalence between different populations (Warby et al., 2011; Pringsheim et al., 2012; Rawlins et al., 2016), describing a higher prevalence in Europe, North America, and Australia (5-10 per 100,000) than in Asia (0.1-0.5 per 100,000). More data about African population are needed, since previous studies have used small sample sizes and the HD diagnosis was not confirmed by molecular testing. Due to the evolutionary event called the founder effect, a very high prevalence can be eventually identified in isolated populations, such as in a community living in Lake Maracaibo, Venezuela, where the prevalence is about 700 per 100,000 (Okun and Thommi, 2004).

Generally, HD is diagnosed based on the presence of involuntary choreic movements and a positive genetic test. Symptoms of HD typically develop in adulthood, usually between 30 and 50 years of age, but with a variability ranging from 2 to 85 years. Disease duration from symptom onset until death is about 15-20 years. Clinical manifestations comprise motor, cognitive and psychiatric disturbances, although other less-known features include unintended weight loss, sleep and circadian rhythm impairments, and autonomic nervous system dysfunction (Walker, 2007). First motor symptoms are mostly characterized by chorea, which is defined as rapid and irregular involuntary movements. These symptoms begin of a small degree in distal extremities (fingers and toes) and face, progressively increasing in amplitude and becoming more proximal and axial. At later stages of the disease, motor symptoms shift from hyperkinetic choreic movements to hypokinetic movements, with bradykinesia, akinesia, dystonia and rigidity being more prominent. Other classic symptoms include motor impersistence and abnormal eye movements (Novak and Tabrizi, 2010; Roos, 2010). Cognitive symptoms have shown to appear in HD carriers decades before the onset of motor dysfunction, during the prodromal phase (Paulsen et al., 2008; Harrington et al., 2012). These cognitive manifestations include alterations in attention, cognitive flexibility, working memory, verbal fluency and spatial memory, which gradually worsen to severe

dementia at late disease stages (Davis et al., 2003; Lemiere et al., 2004; Paulsen et al., 2008; Beglinger et al., 2010; Paulsen, 2011; Stout et al., 2011; Begeti et al., 2016). Finally, psychiatric impairments also precede the onset of motor symptoms by decades (Julien et al., 2007) and depression is the alteration most commonly observed, followed by anxiety. Patients also show impulsivity, apathy and personality changes and can sometimes present irritability and aggression although they rarely manifest physical violence. Obsessive, compulsive thoughts and behaviors can also be present (Naarding et al., 2001; Paulsen et al., 2001, 2005). Importantly, suicide seems to be more prevalent in HD patients than in the general population (Baliko et al., 2004). Both cognitive and psychiatric impairments cause the most distress and early quality of life impact in HD patients and their families (Helder et al., 2001; Ready et al., 2008; Williams et al., 2010). Because of severe dementia and motor dysfunction, patients in late HD stages may become unable to walk, have poor dietary intake and eventually stop to talk, therefore becoming less autonomous in daily life (Walker, 2007; Novak and Tabrizi, 2010). Aspiration pneumonia, probably due to difficulty swallowing (dysphagia), has been reported as the most common cause of death (Heemskerk and Roos, 2012).

In about 5% of HD patients (Quarrell et al., 2012) the disease begins before age 21 and is then referred to as juvenile HD (JHD) (van Dijk et al., 1986). In these patients, disease progression is more rapid and severe than in the adult form and has some clinically distinct treats. Motor features are characterized by rigidity, dystonia and bradykinesia, rather than chorea, probably as a result of a more widespread brain atrophy. Slowed saccadic eye movements are usually prominent in these patients. In addition, child patients may also present cerebellar dysfunction, epilepsy, myoclonus (brief, involuntary twitching), spasticity, developmental delay and autism (van Dijk et al., 1986; HDCRG, 1993; Ribaï et al., 2007).

## 1.2. Genetics

Huntington's disease is a single genetic disorder with an autosomal dominant inheritance. In 1983, the causative gene was mapped to the short arm of human chromosome 4, at the 4p16.3 region (Gusella et al., 1983), but it was not until 10 years later that the disease underlying mutation was found to be an aberrant expansion of a trinucleotide repeat formed by cysteine, adenosine and guanine (CAG) in a gene that was termed *IT15* (interesting transcript 15) (HDCRG, 1993). The gene was shown to span 180 kb and contain 67 exons (Ambrose et al., 1994), being the mutation in the first exon. Currently, the official name for *IT15* is *huntingtin (Htt)*, because of the name assigned to the protein. The *Htt* gene is poorly conserved or absent in invertebrates, but it has great homology among vertebrates (Baxendale et al., 1995; Gissi et al., 2006; Tartari et al., 2008). Its mouse homologous was localized in murine chromosome 5 (Barnes et al., 1994; Grosson et al., 1994; Nasir et al., 1994), a finding that would be crucial in the generation of knock-in HD mouse models.

In non-HD individuals, the CAG trinucleotide in the HD gene can be repeated 9 to 35 times, with an average median range between 17 and 20 repeats (Kremer et al., 1994). Individuals presenting the so called "intermediate allele" with 27 to 35 repeats are not going to develop the disease, but

because of gametic meiotic instability of the CAG tract they might pass an allele in the HD range to their offspring (Kelly et al., 1999; Semaka et al., 2006). Therefore, sporadic or de novo HD cases are thought to be a consequence of intergenerational instability of an intermediate allele (Goldberg et al., 1993; Myers et al., 1993). HD patients always present at least one allele with ≥ 36 CAG repeats (Kremer et al., 1994). However, the range of 36-39 repeats leads to an incomplete penetrance of the disease, occasionally resulting to a lack of HD symptoms in elderly individuals (Rubinsztein et al., 1996; McNeil et al., 1997), while 40 repeats or more lead to a disease manifestation with full penetrance (Nance et al., 1998) (Figure 1).

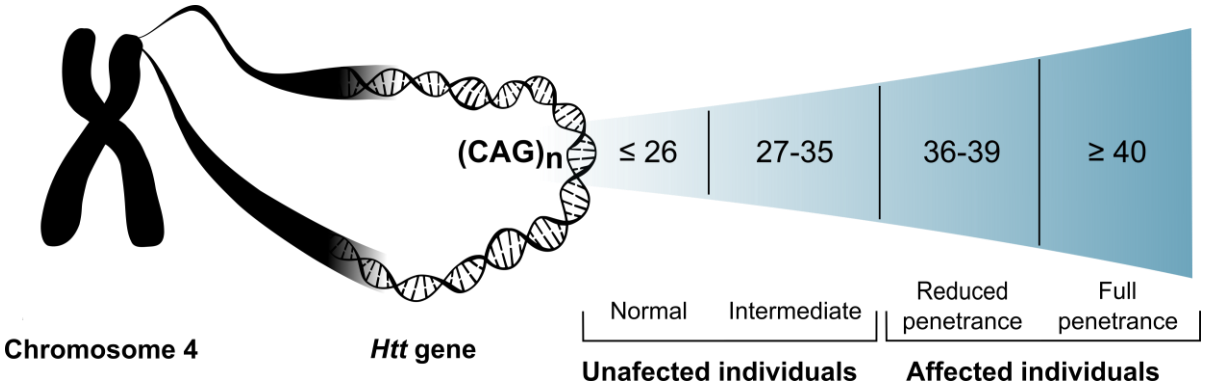



**Figure 1. Localization of human *Htt* gene and CAG repeat length categories.** *Htt* gene is located on chromosome 4 and presents a polymorphic CAG tract in the exon 1. Based on clinical manifestation of HD and tendency to pass an affected allele to the progeny, length of the tract can be classified in normal, intermediate, reduced penetrance and full penetrance.

HD patients presenting the HD mutation in both *Htt* copies are very rare (Kremer et al., 1994; Durr et al., 1999; Alonso et al., 2002), which reflects the low probability of finding both parents affected. Given the small amount of cases, more extensive studies are needed to confirm the effect of HD homozygosity on disease onset and severity, although some studies suggest that while age on onset might not be modified, disease is more severe in homozygous patients (Alonso et al., 2002; Squitieri et al., 2003; Lee et al., 2012).

As mentioned before, HD presents a high variability in age of onset, classically determined by first motor manifestations. This variability has been shown to inversely correlate with the length of the CAG repeat tract in m*Htt* gene (Andrew et al., 1993; Duyao et al., 1993; Snell et al., 1993; Stine et al., 1993), which means that individuals with longer CAG repeats typically present earlier age of onset. Supporting this idea, alleles with ~60 repeats are typically associated with juvenile HD (Telenius et al., 1993). However, a great amount of variability in onset age cannot be explained solely by repeat size, especially for repeats below 60 (which are the majority of the cases) and it has been demonstrated that genetic and environmental factors can influence this parameter (Wexler et al., 2004; GeM-HD Consortium, 2015). This has important ethical implications in predictive genetic testing since it is still not possible to accurately predict when an individual with an affected HD allele is going to develop symptoms.

As in other trinucleotide repeat disorders, HD presents genetic anticipation, which is the observation of earlier disease onset in successive generations. This feature is consequence of the

gametic CAG repeat instability observed in parents of either sex, with expansions being more prominent in the male germline (Duyao et al., 1993; Wheeler et al., 2007). Since paternal transmission undergoes larger increases in size, most juvenile cases of HD have affected fathers (Telenius et al., 1993; Trottier et al., 1994; Ranen et al., 1995). Interestingly, and as well as in other trinucleotide repeat disorders, the expanded CAG repeat tract in m*Htt* gene is also unstable in specific somatic tissues, which is thought to contribute to disease progression. This important phenomenon will be further explained in Introduction Section 2.3.

# 1.3. Characteristics of HD gene products

## 1.3.1. Huntingtin protein

The important discovery of the causative gene in HD enabled the characterization of its protein product, which was termed huntingtin (Htt) (HDCRG, 1993). Knowledge obtained about its structure, localization and interactome has helped to define its function in order to provide clues about the molecular mechanisms altered by the mutation.

Htt is a very large protein, generally presenting 3144 amino acids and a molecular mass of about 350 kDa (depending on the size of the CAG repeat region), and is highly conserved among vertebrates with no considerable homology with any other protein (Saudou and Humbert, 2016). The analysis of Htt structure reveals several sequence motifs that would help to understand its function (Figure 2). Because of the CAG repeat sequence encoded in its gene, the protein contains a polyglutamine (polyQ) tract beginning at the amino acid 18th, which is polymorphic in humans and might contain up to 35 residues in non-disease conditions (Jou and Myers, 1995). Although the consequences of its length variability are not completely understood, this tract has been found to regulate autophagy and the binding of Htt with several other proteins (Harjes and Wanker, 2003; Zheng et al., 2010). The N-terminal 17 amino acids (N17 domain) that precede the polyQ, form an amphipathic α-helix structure that has shown to be important for protein degradation and subcellular localization to the endoplasmic reticulum, vesicle structures and the nucleus, where it functions as a CRM1-dependent nuclear export signal (NES) (Atwal et al., 2007; Rockabrand et al., 2007; Maiuri et al., 2013). Only in mammals, the polyQ is followed by a proline-rich domain (PRD), which is also polymorphic in humans and mediates protein-protein interactions and protein solubility (Liu et al., 1997; Faber et al., 1998; Darnell et al., 2007). Next to the polyQ, and distributed between positions 69 and 3,144, the protein contains several tandem clusters of a repeat of ~40 amino acids that was called HEAT, after the four proteins in which it was first detected: Htt, elongation factor 3, regulatory subunit A of protein phosphatase 2A, and TOR1. Between 16 and 36 repeats have been found, and these could be organized into two to five domains. Each repeat unit consists of two hydrophobic α-helices connected by a short loop that are considered to be involved in intra- and inter-protein interactions, leading to a remarkable conformational flexibility (Andrade and Bork, 1995; Li et al., 2006; Palidwor et al., 2009; Seong et al., 2010; Guo et al., 2018). Indeed, this conformational heterogeneity has hampered the analysis of Htt tridimensional structure for years, although recent studies have provided solid prove for the protein to adopt an spherical α-helical solenoid structure, with the expanded polyQ tract affecting

its intramolecular interactions and therefore the overall structure (Vijayvargia et al., 2016; Guo et al., 2018). A non-classical karyopherin β1/β2 proline-tyrosine nuclear localization signal (PY-NLS) has been recently found between the amino acids 174 and 207 (Desmond et al., 2012). Apart from the observed NES in the N17 domain, another one is present near Htt C-terminus, between the amino acids 2,397 and 2,406 (Xia et al., 2003). Several proteolytic sites have also been found throughout the protein in what are termed PEST domains, which are regions enriched in the amino acids proline (P), glutamic acid (E), serine (S), and threonine (T) (Warby et al., 2008; Saudou and Humbert, 2016). Different caspases, calpains, cathepsins, and the metalloproteinase MMP10 have been demonstrated to cleave Htt protein (Goldberg et al., 1996; Wellington et al., 1998; Ona et al., 1999; Gafni et al., 2004). Although the consequences of wild-type Htt cleavage are not known, increased levels of N-ter and C-ter fragments has been found in HD (Mende-Mueller et al., 2001) and it is established that they are negatively involved in disease onset and progression (Ona et al., 1999; Wellington et al., 2000; Gafni et al., 2004; El-Daher et al., 2015). This has been particularly well demonstrated for N-ter fragments containing the polyQ stretch (Landles et al., 2010; Tebbenkamp et al., 2011). Additionally, huntingtin is subjected to several posttranslational modifications, such as ubiquitination, phosphorylation, sumoylation, palmitoylation and acetylation, that are predominantly located in PEST domains and have important effects in the stability, function and localization of the protein. Some of these modifications are altered when the expanded polyQ tract is present, potentially modifying important cellular functions (Steffan et al., 2004; Luo et al., 2005; Yanai et al., 2006; Thompson et al., 2009; Cong et al., 2011). Importantly, apart from classical translation of *Htt* RNA, a recent study has reported a non-canonical sense and antisense translation, termed repeat-associated non-ATG (RAN) translation, that occurs in HD vulnerable brain regions and generates different Htt peptides that might also confer neurotoxicity (Bañez-Coronel et al., 2015).

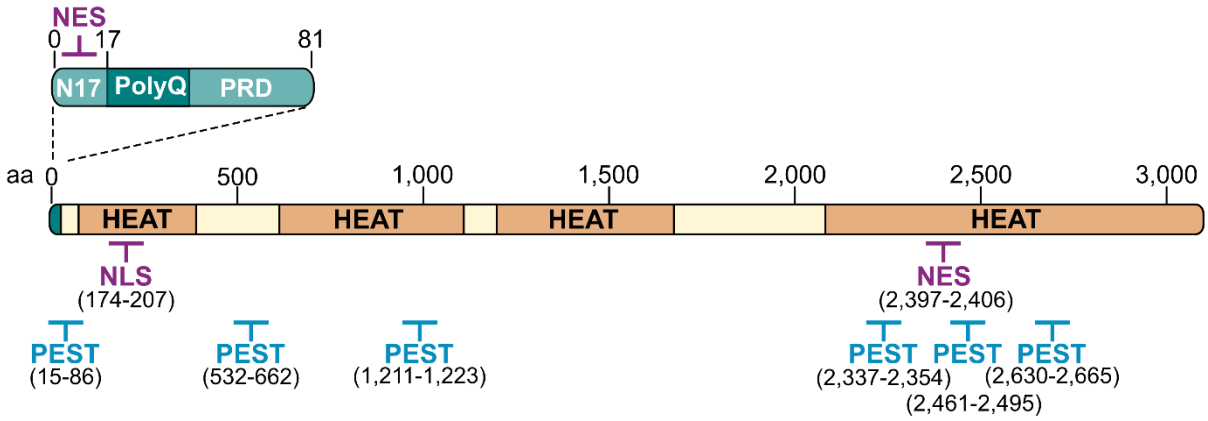



**Figure 2. Htt amino acid sequence.** Schematic diagram of Htt showing amino acid (aa) positions for: N17 domain, polyglutamine tract (PolyQ), proline-rich domain (PRD), nuclear export signals (NES), nuclear localization signal (NLS), PEST domains and HEAT domains. Adapted from (Saudou and Humbert, 2016). HEAT regions have been drawn as established in (Guo et al., 2018).

Regarding its expression, Htt is found ubiquitously and is detected at the highest levels in brain and testis. In brain, it has most expression in the neocortex, cerebellar cortex and hippocampus (with intermediate levels in the striatum) and it can be present in all types of neurons and glia (Li

et al., 1993; Strong et al., 1993; Gutekunst et al., 1995; Landwehrmeyer et al., 1995). Although the specific Htt subcellular localization varies between tissues, it has been found in multiple cellular compartments, where it can adopt different conformations (Ko et al., 2001). In neurons, Htt is predominantly cytoplasmic (observed throughout the soma, dendrites, axons and terminals) and it has been associated with the plasma membrane, microtubules, endocytic and phagocytic vesicles, endosomes, synaptosomes, mitochondria, the endoplasmic reticulum and the Golgi apparatus (DiFiglia et al., 1995; Schilling et al., 1995; Sharp et al., 1995; Sapp et al., 1997). However, small but important amounts of Htt can be found in the nucleus (Hoogeveen et al., 1993; Kegel et al., 2002). Htt is also expressed in several stages of embryonic and postnatal development, adding another layer of complexity to define its function (Bhide et al., 1996). Importantly, the mutated form of the protein shows similar regional and temporal patterns of expression (Aronin et al., 1995; Schilling et al., 1995; Bhide et al., 1996).

Hundreds of proteins have been found to interact with Htt (or its fragments) within its many protein-interaction domains and the expanded polyQ tract has shown to modify such interactions by increasing or reducing the efficiency of binding (Harjes and Wanker, 2003; Li and Li, 2004; Shirasaki et al., 2012). Although many of these Htt-interacting proteins remain to be further validated by binding assays and co-localization experiments, they have been correlated with a wide range of cellular functions. The large size and conformational flexibility of Htt, its widespread temporal and spatial expression and its many partners described, all together support the role of Htt as an important scaffolding protein that tethers multiple interactors into large complexes to coordinate a great variety of cellular processes, some of which are still being defined.

A well characterized function of Htt is the modulation of gene transcription. Interestingly, the polyQ tract is a common motif in many transcription factors, acting as an activation domain by allowing the interaction with other transcriptional regulators. Htt has shown to interact with a great amount of proteins involved in the regulation of RNA expression, such as transcription factors (activators, repressors), transcriptional coregulators (coactivators, corepressors), nuclear receptors and chromatin remodelers. In the nucleus, Htt can promote or hamper the activity of these transcriptional regulators and it has also been suggested to act as a transcription factor itself, binding to promoter regions (Benn et al., 2008). In the cytoplasm, Htt could also bind to some of these factors and alter their transport to the nucleus. This transcriptional regulation could modulate a wide range of cellular outcomes, with some of them having an important role in neuronal function and viability (Saudou and Humbert, 2016). Htt has also shown to mediate intracellular trafficking along microtubules by interacting directly or indirectly with the cellular motor machinery, which was further supported by the subcellular location of Htt into vesicles and organelles. Specifically, Htt interacts with dynein (Caviston et al., 2007) and with Huntingtin-associated protein 1 (HAP1), which in turn interacts with dynactin and kinesin (Engelender et al., 1997; McGuire et al., 2006). These interactions might facilitate the anterograde and retrograde transport within axons and dendrites of a variety of cargo, including synaptic and other type of vesicles (i.e. BDNF-containing vesicles), autophagosomes, endosomes and lysosomes (Trushina et al., 2004; Caviston et al., 2007, 2011; Colin et al., 2008; Twelvetrees et al., 2010). Additionally, Htt could mediate the short-range transport along the actin cytoskeleton (Caviston and Holzbaur,

2009) and it has been directly involved in the modulation of the endocytic pathway (Engqvist-Goldstein et al., 1999; Pal et al., 2006). Related to its role in intracellular transport, another described function for Htt is the regulation of synaptic transmission. Thus, by controlling the transport and release of synaptic vesicles (Twelvetrees et al., 2010) and the endocytosis of neurotransmitter receptors (Metzler et al., 2003), it could modulate synaptic communication. Additionally, it can directly interact with important structural proteins at the synaptic level, such as the postsynaptic density protein 95 (PSD95), influencing its localization and activity (Sun et al., 2001; Parsons et al., 2014). Indeed, a recent study of Htt interactome has revealed a great amount of interactors involved in neurotransmitter transport as well as pre- and postsynaptic organization and function (Shirasaki et al., 2012). Finally, Htt regulates intracellular signals related to apoptosis and neuroprotection, since it was found that Htt inhibits the pro-apoptotic proteins caspase-3, -8 and -9 (Rigamonti et al., 2001; Gervais et al., 2002; Zhang et al., 2006). The presence of an aberrant expanded polyQ tract in mHtt leads to alterations in all these cellular pathways and even the appearance of new pathological mechanisms, which eventually alter the proper function and compromise the viability of specific neuronal populations. These alterations will be addressed at Introduction Section 2.

Translating the molecular pathways regulated by Htt to a general function in organism physiology, studies have shown that it plays a key role during development, since the complete ablation of *Htt* gene in mice results in embryonic death before the emergence of the nervous system, correlating with poor organization and massive cell death of extra-embryonic tissue (Duyao et al., 1995; Nasir et al., 1995; Zeitlin et al., 1995). Engineered mice with reduced Htt expression have also uncovered the important role of the protein in the later formation of the central nervous system (Nasir et al., 1995; White et al., 1997; O'Kusky et al., 1999; Tong et al., 2011). However, the fact that the CAG-expanded huntingtin rescued the lethality or developmental deficits of the mice and that homozygous HD patients have no overt developmental defects support the idea that the mutation does not effectively impair these functions (Wexler et al., 1987; White et al., 1997; Leavitt et al., 2001). In the adult, Htt might play a specific role in neuronal survival, since its reduction in mice after birth has shown to produce apoptotic cell death in the striatum, cortex and hippocampus (Dragatsis et al., 2000) and confer vulnerability to toxic stimuli (Rigamonti et al., 2001; Zhang et al., 2006), while its overexpression has shown to protect neurons from pro-apoptotic stimuli (Leavitt et al., 2006; Zhang et al., 2006).

Some studies suggest that Htt loss of function effects could contribute to the adult neuropathology, since reductions in trophic functions only present in the wild-type protein might render specific neurons more vulnerable to the environmental stress caused by the mutant one (Cattaneo et al., 2001). However, the autosomal dominant inheritance of the disease, the lack of HD symptoms in rare human cases expressing just one copy of *Htt* gene and experimental data collected from HD patients and animal models provide strong evidence for a predominant gain-of-function effect of mHtt (Imarisio et al., 2008). One of the most obvious effects gained by the mutation is the tendency of the protein to form aggregates both in the nucleus and the cytoplasm of neurons (Figure 3) (DiFiglia et al., 1997; Scherzinger et al., 1997), which correlates with the length of the polyQ expansion (Scherzinger et al., 1999). Mainly involved in this process are the

N-terminal fragments of mHtt generated by increased proteolytic cleavage, since they are abundant inside the aggregates (DiFiglia et al., 1997; Sieradzan et al., 1999; Hoffner et al., 2005) and it has been demonstrated that the inhibition of cleavage with specific protease inhibitors as well as mutation of consensus Htt proteolytic sites reduces aggregate formation (Wellington et al., 2000; Lunkes et al., 2002; Hoffner et al., 2005). Apart from being originated by proteolytic cleavage, alternative splicing has been recently shown to generate the exon-1 N-ter mHtt fragment (Sathasivam et al., 2013; Neueder et al., 2017). The aggregation process comprises a complex series of events. Briefly, mHtt monomers stick together forming intermediate oligomeric species gradually increasing in size and eventually turning into progressively larger inclusions with low detergent solubility, which have been shown to present a high β-sheet content structured into ordered amyloid fibers (Scherzinger et al., 1999; McGowan et al., 2000; Poirier et al., 2002; Legleiter et al., 2010). The great diversity of oligomeric species, coexisting with monomeric and large inclusion forms, hinders the study of the specific contribution of each specie to HD neuropathology. While many essential proteins have been found sequestered within the large inclusions, potentially compromising important cellular functions (Nucifora et al., 2001; Hay et al., 2004; Qin et al., 2004), other studies have shown that such inclusions are protective, as they might reduce the availability of more toxic mHtt forms (Saudou et al., 1998; Arrasate et al., 2004; Steffan et al., 2004; Miller et al., 2010). In this line, some studies suggest that small oligomeric forms of mHtt may represent the most toxic species (Takahashi et al., 2008; Lajoie and Snapp, 2010; Miller et al., 2011). Nowadays it is still unclear whether the presence of these large aggregates or any of its intermediates is pathogenic, protective or incidental, but it is established that they provide useful hallmarks of regional HD neuropathology.

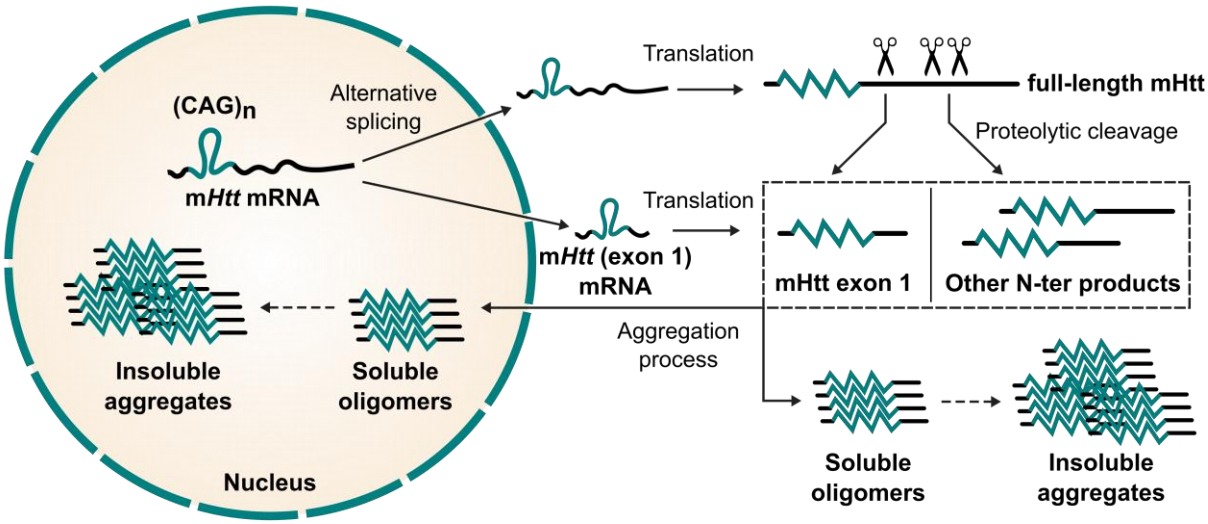



**Figure 3. Generation of mHtt aggregates.** Schematic diagram showing the aggregation process of mHtt. Alternative splicing of m*Htt* RNA and proteolytic processing of mHtt protein leads to the formation of multiple N-ter fragments that are prone to stick together and undergo a complex multi-step aggregation process. Small mHtt fragments travel easily to the nucleus, where they can also initiate the aggregation process.

## 1.3.2. Huntingtin RNA

A considerable amount of research into HD has been confined to the study of mHtt protein, since there is strong evidence to support a role for the protein itself (or its fragments) in the pathogenesis

of the disease (Marsh et al., 2000; Sakahira et al., 2002; Cattaneo et al., 2005). However, recent investigations point out the existence of toxic mHtt RNA forms that could also contribute to HD pathology (Bañez-Coronel et al., 2012; Rué et al., 2016; Zhang et al., 2018), as it have been described for other trinucleotide diseases (Li et al., 2008; Fiszer and Krzyzosiak, 2013; Nalavade et al., 2013; Zhang et al., 2018). Therefore, the study of huntingtin transcripts would also be important to understand HD pathology and in order to develop therapeutic strategies. Generally, RNA CAG-repeat sequences have been shown to form semi-stable hairpin structures, allowing their interaction with many RNA-binding proteins probably involved in gene expression (McLaughlin et al., 1996). When the CAG-repeat is expanded beyond a threshold, however, these mRNAs tend to aggregate forming liquid-like nuclear depositions (analogous to mHtt protein inclusions) called nuclear foci (de Mezer et al., 2011; Jain and Vale, 2017), sequestering proteins with affinity for the hairpin structure and therefore impairing cellular functions. In HD, the sequestration of nucleolin has been linked to the activation of nucleolar stress (Tsoi et al., 2012; Tsoi and Chan, 2013), while the sequestration of muscleblind-like 1 correlates with alterations in alternative splicing (de Mezer et al., 2011; Mykowska et al., 2011). Additionally, hairpins formed by CAG repeat expanded RNAs can resemble structures formed by dsRNA, thereby acting as substrates for Dicer. Indeed, the generation of small neurotoxic fragments (about 21 nucleotides) of CAG-repeat containing RNA (sCAG) via Dicer cleavage, which can interact and silence the expression of genes, has been reported (Krol et al., 2007; Bañez-Coronel et al., 2012). Interestingly, the toxicity of Htt mRNAs, the number of RNA foci and the magnitude of sCAG formation correlate with the length of the CAG repeat tract (Bañez-Coronel et al., 2012).

## 1.4. Neuropathology

Although mHtt is expressed in all tissues of the body, HD is fundamentally characterized by progressive neuronal death, with some brain regions being earlier and more severely affected than others. The most prominent alterations occurs within the striatum, a region comprising the nucleus caudate and putamen (in humans), in which there is evident gross atrophy and neuronal loss associated with astrocytic gliosis that eventually leads to a visible enlargement of the lateral ventricles (Figure 4A) (Vonsattel et al., 1985). These brain changes can be detected even at preclinical stages (Aylward et al., 2004; Kipps et al., 2005), increasing during disease progression and extending to many other regions, until brains of HD patients at advanced stages weigh about 25-30% less than normal (Sharp and Ross, 1996). The cerebral cortex is also visually affected, with marked neuronal loss and shrinkage particularly in layers III, V and VI (Sotrel et al., 1991). Other regions, including the globus pallidus, hippocampus, amygdala, thalamus, subthalamic nucleus, substantia nigra, and cerebellum, can also show varying degrees of atrophy and neuronal loss, depending on disease stage (Vonsattel et al., 1985; de la Monte et al., 1988; Spargo et al., 1993; Vonsattel and DiFiglia, 1998; Rosas et al., 2003). The neuronal loss has been linked to apoptotic cell death, since some neurons show nuclear fragmentation and expression of apoptotic markers (Thomas et al., 1995; Vis et al., 2005). Immunohistochemical analysis of HD brains have also revealed the presence of mHtt intranuclear aggregates (commonly termed neuronal intranuclear inclusions or NII), more predominant in juvenile HD cases, and somatic and neuropil extranuclear

aggregates. These aggregates colocalize with ubiquitin and are mostly observed in striatal and cortical regions, but they can appear also in the hippocampus and subiculum, more so in advanced and juvenile HD (DiFiglia et al., 1997; Sapp et al., 1997; Reiner et al., 2011). Importantly, data obtained particularly from research with HD mouse models have shown that the appearance of some disease-related behavioral changes often precedes neuronal loss, providing evidence for an early neuronal dysfunction that significantly contributes to the onset of symptoms (Mangiarini et al., 1996; Guidetti et al., 2001; Luthi-Carter et al., 2002; Rubinsztein, 2002; Menalled et al., 2003; Levine et al., 2004).

Due to the progressive nature of HD neuropathology and in order to rate the different disease stages, a grading system was developed in 1995 by the neuropathologist Jean-Paul Vonsattel. After analyzing the striatal morphology of several post-mortem clinically diagnosed HD brains, he designated 5 different grades (from 0 to 4) in an ascending order of severity, based on macroscopic and microscopic criteria (Figure 4B) (Vonsattel et al., 1985). Grade 0 defines cases without gross morphological changes together with 30-40% neuronal loss in the head of the caudate nucleus. In grade 1, cell counts in the head of the caudate nucleus are reduced by 50% and there is also atrophy and neuronal loss in the tail and sometimes in the body of the caudate nucleus. In grade 2 and 3, atrophy and neuronal loss is progressively more severe until in grade 4 the striatum presents 95% of neuronal loss and the ventricular surface of caudate nucleus has changed from convex to concave (Vonsattel et al., 1985; Vonsattel and DiFiglia, 1998).

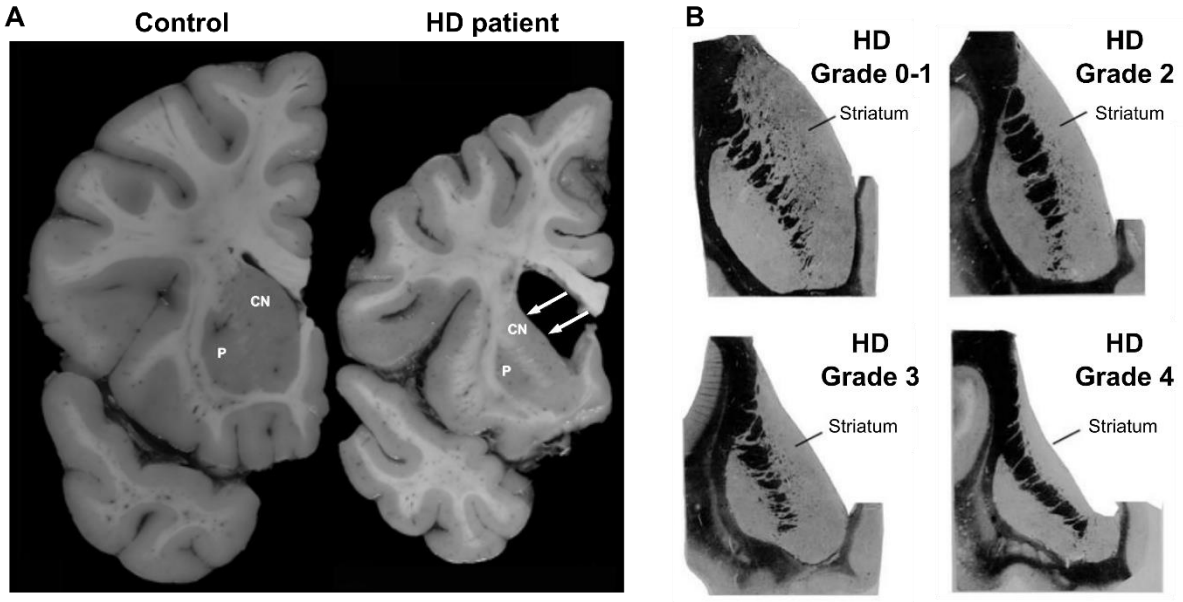



**Figure 4. Macroscopic HD neuropathology.** (**A**) Human coronal brain sections from a control case (left) and a Grade 3/4 HD patient (right). CN: caudate nucleus. P: putamen. Arrows point to the massive atrophy of the caudate nucleus and putamen. Cortical atrophy and enlargement of the lateral ventricle can also be observed. Modified from (Waldvogel et al., 2015). (**B**) Schematic illustrations showing Vonsattel grading system of striatal neurodegeneration (see text for explanation). The change in caudate nucleus surface is appreciated. Modified from (Reiner et al., 2011).

Although multiple brain regions have been found affected in HD, we will further discuss the neuropathology of two structures, the striatum and the hippocampus, that mainly contribute to the HD-related behavioral impairments investigated in this thesis.

### 1.4.1. Striatal pathology

As mentioned, the caudate nucleus and putamen are the most severely affected structures in HD. They are the main hub of an interconnected subcortical structure called the basal ganglia system, which was classically considered to play a key role in the control of movement (Crossman, 1987; Albin et al., 1989), but more recently has also been involved in cognition and emotions (Thorn et al., 2010; Obeso et al., 2014). Thus, striatal neuropathology in HD patients is considered to underlie not just motor alterations (chorea, dystonia, bradykinesia), but also cognitive disturbances (impaired motor learning, set-shifting, planning and decision-making) and loss of emotional control (impulsiveness). Because of the close anatomical connectivity and pathophysiological implications, the basal ganglia include, apart from the striatum, the globus pallidus (GP), which can be divided into internal (GPi) and external (GPe) segments, the subthalamic nucleus (STN) and the substantia nigra (SN), divided in pars compacta (SNc) and pars reticulata (SNr) (Graybiel, 2000; Obeso et al., 2014) (Figure 5A). The majority of afferent connections to the basal ganglia are received by the striatum, with most inputs coming from all regions of the cortex (glutamatergic), although predominantly ipsilateral and sensorimotor, and to a lesser extent from other regions like the thalamus (glutamatergic) and the SNc (dopaminergic) (Bolam et al., 2000; Kreitzer, 2009; Waldvogel et al., 2015). Thus, the large excitatory glutamate pool is responsible for striatal activation, while the dopaminergic input from SNc modulates this response (Gerfen and Surmeier, 2011). Striatal neurons can be divided into medium-sized spiny projection neurons called medium spiny neurons (MSNs), accounting for 90-95% of the population, and aspiny interneurons. Although there are few striatal interneurons, they form a morphologically and neurochemically heterogeneous group that plays an important role in the local circuit controlling the excitability of MSNs (Kawaguchi et al., 1995). MSNs receive external inputs into their dendritic spines and use the inhibitory neurotransmitter γ-aminobutyric acid (GABA) to send outputs by two different pathways, the direct and indirect pathway. Hence, MSNs can be further divided into two subpopulations, regarding their neurochemical content and target area. Neurons of the direct pathway, also called striatonigral neurons, project directly to the GPi/SNr and specifically express substance P and the dopamine D1 receptor subtype. Neurons in the indirect pathway, or striatopallidal neurons, project to the GPe, which in turn projects to STN and this to the GPi/SNr, and express exclusively enkephalin and the D2 subtype of dopamine receptor (Kreitzer, 2009). Classically, these two pathways appear to have opposite effects on the basal ganglia output involved in movement control (Figure 5B) (Alexander and Crutcher, 1990). Posterior to the Vonsattel classification, new neuropathological analysis performed in HD human brains showed a specific loss of MSNs while most striatal interneurons were spared (Ferrante et al., 1985, 1987) and, later on, more precise immunohistochemical studies revealed a sequential degeneration of distinct MSNs, which seemed to correlate well with the evolution of motor abnormalities in HD patients (Reiner et al., 1988; Albin et al., 1990; Sapp et al., 1995; Glass et al., 2000; Deng et al., 2004). Thus, in early and middle stages of HD, neurons of the indirect pathway are more selectively depleted than those of the direct one, ultimately producing a disinhibition of the thalamus, which leads to an overactivation of the cortex that will cause the hyperkinetic movements characteristic of the first stages of the disease. However, at late stages, both pathways are strongly affected,

eventually leading to an overall inhibition of the thalamus and the cortex, producing the hypokinetic symptoms of HD (Figure 5B).

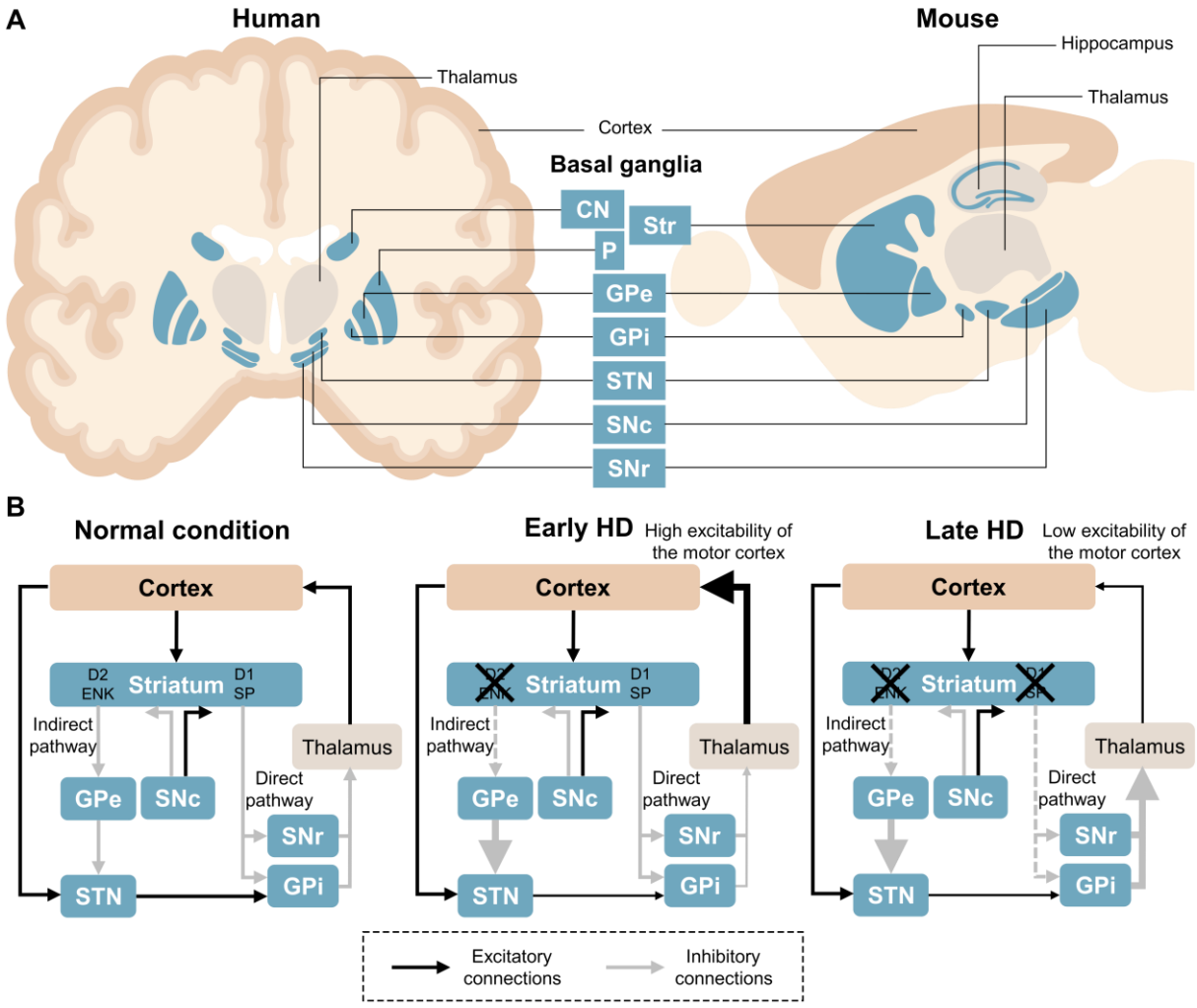



**Figure 5. Functional anatomy of the striatum and its alteration in HD.** (**A**) Localization of the striatum and other basal ganglia components in a coronal section of the human brain (left) or a sagittal section of the mouse brain (right). (**B**) Simplified diagram showing basal ganglia circuitry in normal conditions and in early or late stages of HD. See main text for information. Changes in the thickness of the arrows indicate an increase (larger arrow) or decrease (thinner arrow) in the activity of specific connections. Discontinued arrows indicate decreased number of connections as a result of neuronal loss. Additional transverse loops might be present, most of them with a putative modulatory role. CN: caudate nucleus. P: putamen. Str: striatum. GPe: external globus pallidus. GPi: internal globus pallidus. STN: subthalamic nucleus. SNc: substantia nigra pars compacta. SNr: substantia nigra pars reticulata.

Although intensive efforts have been put to analyze the molecular mechanisms underlying the selective vulnerability of MSNs (and especially those of the indirect pathway) in HD, with many hypothesis being formulated, the complete picture is still not clear (Zuccato et al., 2010). Besides, it has been postulated that cognitive and mood changes that occur early in HD patients may reflect an important neuronal dysfunction occurring in the striatum (and in other brain regions) prior the observation of neuronal death (Raymond et al., 2011). In this regard, HD mouse models exhibit striatal-dependent behavioral abnormalities before any observation of neuronal loss (Mangiarini et al., 1996; Guidetti et al., 2001; Menalled et al., 2003; Puigdellívol et al., 2015), while presenting an evident striatal neuronal dysfunction, which includes structural and functional synaptic changes as

well as severe proteomic and transcriptomic alterations potentially underlying such alterations (Bibb et al., 2000; Klapstein et al., 2001; Luthi-Carter et al., 2002; Menalled et al., 2003; Raymond et al., 2011; Marco et al., 2013; Simmons et al., 2013; Wu et al., 2016). Importantly, even earlier defects in the connections between the cortex and the striatum have been found in these models, correlating with first behavioral symptoms (Sapp et al., 1999; Laforet et al., 2001; Cepeda et al., 2003; Marangoni et al., 2014; Gatto et al., 2015; Puigdellívol et al., 2015; Veldman and Yang, 2018).

### 1.4.2. Hippocampal pathology

The hippocampus is an important component of the limbic system and is suggested to be indispensable for a wide variety of cognitive functions, particularly acquisition and consolidation of different forms of memory and spatial navigation (Morris et al., 1982; Squire, 1992; Maguire et al., 1998; Strange et al., 1999; Manns et al., 2003). This region has a striking capacity for structural reorganization, by changing the dendritic arborization and number of spines of preexisting neural connections or by establishing new ones through neurogenesis (Leuner and Gould, 2010). Additionally, activity-dependent alterations in synaptic efficacy (known as synaptic plasticity), such as long-term potentiation (LTP) and long-term depression (LTD), were first discovered in the hippocampus and have been most thoroughly studied in this region since then, as they represent the cellular mechanisms that underlie information processing and storage in the brain (Bliss and Collingridge, 1993). Although there is no consensus as to what parts are included, the hippocampus typically comprises two major regions: the Cornu Ammonis (CA), which is further divided in different subregions, and the dentate gyrus (DG) (Figure 6A). The CA is packed with pyramidal neurons and the dentate gyrus mostly contain granule neurons, although a small but highly heterogeneous population of interneurons have also been described (Freund and Buzsáki, 1996; Klausberger, 2009). The information flow within the hippocampus is largely unidirectional (Figure 6B) (Neves et al., 2008; Deng et al., 2010).

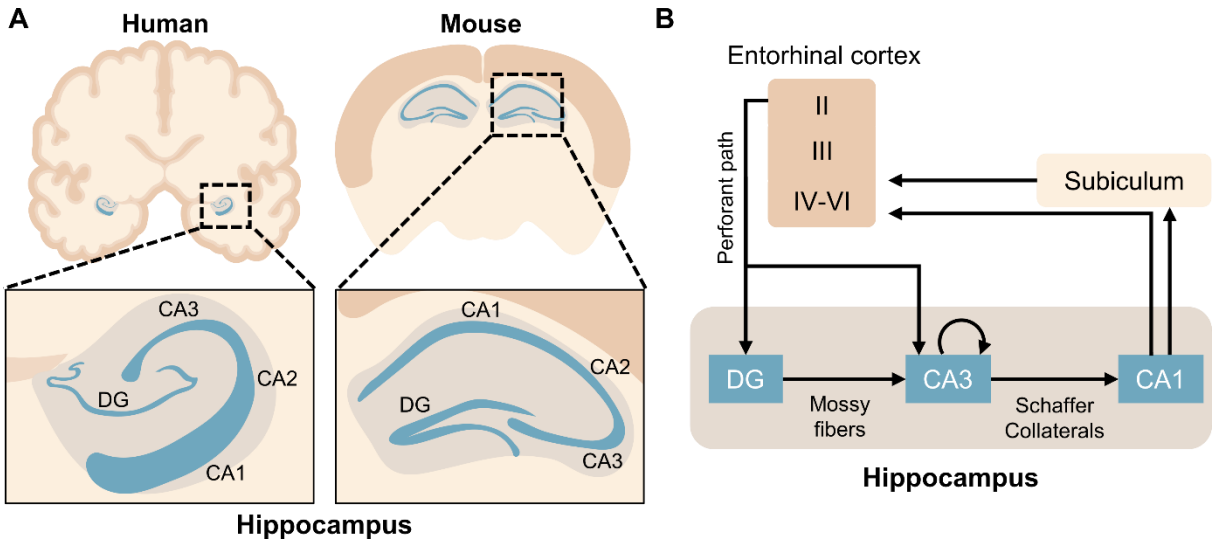



**Figure 6. Functional anatomy of the hippocampus.** (**A**) Localization and structure of the hippocampus in coronal sections from human and mouse brains. In humans, the hippocampus is buried within the medial temporal lobe and, in rodents, it is a substantially larger structure (proportionally) lying just beneath the neocortex. (**B**) Simplified diagram showing central pathways involved in hippocampal circuitry. The major input is provided by the entorhinal cortex (layer II) via the perforant path, projecting mostly to the DG and CA3. DG granule neurons send unmyelinated axons

called mossy fibers to the CA3 region and, in turn, CA3 neurons project to the CA1 via the Schaffer collaterals. CA3 neurons also show recurrent connections. Finally, the CA1 region projects directly or indirectly (via subiculum) to the entorhinal cortex (layer IV-VI).

Although little attention has classically been paid to the hippocampus in HD, some studies have provided evidence on gross morphological changes of this region. Hippocampal volume has been found slightly reduced from early disease stages (de la Monte et al., 1988; Rosas et al., 2003; van den Bogaard et al., 2011), and a 35% neuronal loss specifically in the CA1 region has been observed in brains that already presented massive atrophy in the striatum (Spargo et al., 1993). Importantly, several studies have found that HD patients present alterations in learning and memory tasks (including recognition, spatial and spatial working memories) that are known to be modulated, at least in part, by the hippocampus (Lawrence et al., 2000; Berrios et al., 2002; Lemiere et al., 2004; Montoya et al., 2006; Paulsen, 2011; Begeti et al., 2016). Some of these alterations occur early in the disease, years before the appearance of neuronal death, and therefore might be a consequence of an altered hippocampal function. Unfortunately, analysis of hippocampal connectivity in humans HD patients are scarce. In this regard, the use of genetic HD mouse models has been crucial to clarify how cognitive dysfunction takes place. Studies have revealed early hippocampal-dependent cognitive defects (Lione et al., 1999; Murphy et al., 2000; Lüesse et al., 2001; Nithianantharajah et al., 2008; Giralt et al., 2011b, 2012a; Brooks et al., 2012a; Brito et al., 2014) and the presence, in the hippocampal region, of structural and functional synaptic alterations as well as biochemical and transcriptional abnormalities that could underlie such alterations, preceding neuronal loss (Usdin et al., 1999; Murphy et al., 2000; Phillips et al., 2005; Lazic et al., 2006; Milnerwood et al., 2006; Lynch et al., 2007; Giralt et al., 2009, 2011b, 2012a; Simpson et al., 2011; Brito et al., 2014; Dargaei et al., 2018). Overall, the data collected so far indicate that the presence of mHtt causes an important hippocampal dysfunction that might impair cognitive processes early in the disease.

## 1.5. Genetic HD mouse models

The development of appropriate systems in which to study HD is crucial to understand the pathophysiology and find effective pharmacological treatments. Many cellular and animal models have been generated through years, providing great amount of valuable knowledge. Even so, the exact pathogenic molecular mechanisms caused by the HD mutation are not completely understood and no feasible treatment to cure or delay the disease has been ever obtained.

Before knowing the genetic etiology of the disease, first animal models were produced by infusing the striatum of rodents with excitotoxic glutamate analogs, such as kainic acid (a kainate receptor agonist) (Coyle and Schwarcz, 1976; McGeer and McGeer, 1976; Divac et al., 1978) or quinolinic acid (an NMDA receptor agonist) (Schwarcz et al., 1983; Beal et al., 1986; Sanberg et al., 1989), or with the mitochondrial toxin 3-nitropropionic acid (an inhibitor of the complex II of the mitochondrial respiratory chain) (Borlongan et al., 1995). Interestingly, these toxin-induced models are relatively easy to generate and replicate some region-specific lesions, neurochemical changes and behavioral abnormalities similar to those observed in HD patients. However, since there is no

expression of the ubiquitous mHtt transcript and protein, the study of HD pathogenesis in these models is limited.

The discovery of the human HD gene in 1993 enabled the generation of accurate genetic models of the disease. Given that HD is caused by a single mutation in a single gene, the introduction of the mutation into *in vivo* and *in vitro* systems was relatively simple and has been a powerful tool to understand the underlying pathological mechanisms. Over the years, the disease has been modeled in several species (mouse, rat, worm, fly, fish, minipig, sheep and non-human primates), although mice have been extensively used due to its many advantages, such as their small size, high reproductive rates and short time life, which make their cost relatively low. Additionally, since it is the first specie to be genetically modified with recombinant DNA technology (Jaenisch and Mintz, 1974), significant knowledge has been accumulated over the last decades about this specie. The murine *Htt* gene shares 86% identity with the human homolog and its CAG repeat tract encoding for only 7 consecutive glutamines is not polymorphic, which is consistent with the lack of a spontaneous mouse model of HD (Barnes et al., 1994).

Genetically-modified mouse models of HD can be classified into three different groups according to the technique used in their generation. In the first two categories, mice present a truncated or entire m*Htt* gene randomly inserted into their genome, being therefore classified into fragmented or full-length transgenic models. The third category consists of knock-in models, created by the addition of the HD mutation in the murine *Htt* locus (Table 1).

**Table 1. Commonly used genetic mouse models of HD**. For each model, information about the altered coding region, promoter type, polyQ length and original strain is provided.

| MOUSE MODEL | ALTERED CODING REGION | PROMOTER | POLYQ LENGTH | ORIGINAL STRAIN |
|---|---|---|---|---|
| | | **Fragmented transgenic mice** | | |
| **R6/1** | Human *Htt* exon 1 randomly inserted into genome | Human *Htt* | 116 | CBA x C57BL/6 |
| **R6/2** | Human *Htt* exon 1 (3 copies) randomly inserted into genome | Human *Htt* | 144 | CBA x C57BL/6 |
| **N171-82Q** | Human *Htt* exon 1, 2, part of 3 (first 171 amino acids) randomly inserted into genome | Murine prion protein | 82 | C3H/HEJ x C57BL/6JF1 |
| | | **Full-length transgenic mice** | | |
| **YAC128** | Full-length human *Htt* randomly inserted into genome | Human *Htt* | 128 | FVB/N |
| **BACHD** | Full-length human *Htt* randomly inserted into genome | Human *Htt* | 97 (CAG/CAA mix) | FVB/NJ |
| | | **Knock-in mice** | | |
| **HdhQ92** | Replacement of endogenous *Htt* exon 1 with chimeric human/mouse exon 1 | Murine *Htt* | 92 | 129 x CD1 |

| | | | | |
|---|---|---|---|---|
| **HdhQ111** | Replacement of endogenous *Htt* exon 1 with chimeric human/mouse exon 1 | Murine *Htt* | 111 | 129 x CD1 |
| **CAG140** | Replacement of endogenous *Htt* exon 1 with chimeric human/mouse exon 1 | Murine *Htt* | 140 | 129/Sv x C57BL/6 |
| **zQ175** | Replacement of endogenous *Htt* exon 1 with chimeric human/mouse exon 1 | Murine *Htt* | 175 | 129/Sv x C57BL/6 |
| **HdhQ150** | Targeted insertion of CAG repeats into endogenous *Htt* | Murine *Htt* | 150 | 129/Ola x C57BL/6J |
| **HdhQ200** | Targeted insertion of CAG repeats into endogenous *Htt* | Murine *Htt* | 200 | 129/Ola x C57BL/6J |
| **HdhQ250** | Targeted insertion of CAG repeats into endogenous *Htt* | Murine *Htt* | 260 | 129/Ola x C57BL/6J |

The length of the polyglutamine tract, the integrity and levels of expression of m*Htt* and the genetic background used are factors that can influence the great phenotypic variability observed between models, ranging from an early, fast and aggressive pathology to a late and less severe one. Additionally, none of these models can fully replicate the human HD pathology. Understanding the advantages or drawbacks of each model is necessary to choose the most suitable for a specific study.

Briefly, special mention should be made of cell culture models, which are cheap and useful systems to deeply characterize the molecular mechanisms altered by mHtt and to perform high-throughput screenings to uncover promising drug targets. Cells transiently or stably expressing mHtt with various polyQ repeat lengths have been used. One important example is the striatal cell line STHdhQ111, created from striatal neural progenitors of knock-in HdhQ111 mice at embryonic day 14, which were immortalized with the simian vacuolating virus 40 large T antigen (Trettel et al., 2000). Unfortunately, they might not faithfully recapitulate all aspects of neuronal biology. In this regard, primary neuronal cultures obtained from genetic animal models or human HD patient-derived iPSCs (Zhang et al., 2010), although more difficult to obtain, are also useful HD models.

## 1.5.1. Fragmented transgenic models

Transgenic HD models in which a N-terminal fragment of m*Htt* is randomly inserted into the genome have demonstrated that the expression of these fragments is enough to cause disease symptoms. These models have in common a short lifespan, evident weight loss and brain atrophy with little neuronal loss, mHtt aggregation in several brain regions and the exhibition of the most severe phenotype among all genetic animal models. The early onset and fast progression of symptoms in these models makes them extremely useful for therapeutic screening, although they are unfit to study the earliest HD symptoms and their underlying molecular mechanisms.

The first successful genetic model of HD was the R6 mouse line, developed in 1996. This included the transgenic R6/1 and R6/2 models, which were created by randomly inserting a 1.9 kb genomic fragment derived from the 5' end of the human *Htt* gene into the mouse genome. Specifically, this fragment contains ~1kb of the 5' UTR region (with promoter regions), the exon-1 (with an expanded CAG tract), and the first 262 bp of intron 1 (Mangiarini et al., 1996). The R6/1 has 1 copy of the fragment with 115 CAG repeats and an average expression of 31% of the endogenous huntingtin, while the R6/2 has 3 copies with 145 CAG repeats and a 75% of expression. However, CAG repeats may vary because of germline instability (Mangiarini et al., 1997). Although both lines show similar phenotypes, R6/2 mice exhibit an earlier and faster course of the disease due to the larger CAG tract and increased expression of the transgene. A reduction in total brain weight and in the volumes of several brain structures has been observed as a result of massive neuronal atrophy with little neuronal loss (Mangiarini et al., 1996; Van Raamsdonk et al., 2005a). Progressive cognitive and motor abnormalities are also observed (Carter et al., 1999; Lione et al., 1999; Lüesse et al., 2001; Stack et al., 2005; Hodges et al., 2008; Giralt et al., 2009, 2011b).

The R6 model was followed by the N171-82Q model, which was generated a few years later by the insertion of a larger cDNA fragment encoding the N-terminal region (171 amino acids) of human m*Htt* (with 82 glutamines) under the expression of the mouse prion protein promoter (Schilling et al., 1999). Protein expression of the transgene was found to be 10-20% of endogenous Htt (Schilling et al., 1999). While cognitive deficits have been poorly studied, progressive motor symptoms are similar to R6 mice (Schilling et al., 1999; McBride et al., 2006).

### 1.5.2. Full length transgenic models

Given the large size of the HD gene and in order to embedded it entirely into the mouse genome, the use of yeast artificial chromosome (YAC) or bacterial artificial chromosome (BAC) cloning systems was necessary. Several YAC mouse models expressing different repeat length were first generated, but the best known and most commonly used is the YAC128, which expresses the entire human m*Htt* locus containing 128 CAGs (Hodgson et al., 1999; Slow et al., 2003). By using BAC technology, the BACHD model was developed later on, carrying the entire m*Htt* with a mixture of 97 CAG/CAA repeats (Gray et al., 2008). Importantly, this mixed tract was shown to be stable in meiotic and somatic tissues, including the cortex and striatum, which exhibit high levels of instability in all other models. The two models also present flanking *Htt* segments that might contain genome regulatory elements, allowing an appropriate expression of the transgene. Lifespan is slightly decreased in YAC128 mice and unaltered in BACHD mice (Van Raamsdonk et al., 2005b). Surprisingly, and unlike other genetic models, these mice present an increase in total body weight compared to its WT littermates (Van Raamsdonk et al., 2005a; Gray et al., 2008). They also show an important striatal and cortical atrophy accompanied by neuronal loss (for YAC128) or degenerating neurons (for BACHD), and intranuclear huntingtin inclusions have been observed in several brain regions (Slow et al., 2003; Van Raamsdonk et al., 2005b; Gray et al., 2008). These mice exhibit a more accurate sequential progression of psychiatric, cognitive and motor impairments than the fragmented transgenic models (Slow et al., 2003; Van Raamsdonk et al., 2005b; Gray et al., 2008; Brooks et al., 2012a).

## 1.5.3. Knock-in models

Unlike classic HD transgenic mice, in which the transgene is randomly inserted and might interfere with other genes, knock-in mouse models are produced by directed gene targeting and display expanded CAG repeats into the murine *Htt* locus, being homozygous or heterozygous for the mutation. Therefore, a great advantage of these models is that they faithfully reproduce the HD mutation in its appropriate genomic context. Full length mHtt is expressed in its physiological levels and in mice heterozygous for the mutation, as happens in most human HD patients, the expression of wild-type Htt is reduced by half, which could help to understand the physiological effects caused by reduced normal huntingtin activity. Importantly, intrinsic characteristics of the HD mutation, such as somatic and intergenerational instability will be modulated in the appropriate genomic context. Since most knock-in models present a late disease onset and attenuated phenotype compared to the transgenic models, they are more expensive and unfit for high-throughput pharmacological screening, but really useful to study the earliest signs of HD pathology and its accurate progression.

Two different strategies have been used to generate knock-in models. The first consists in the homologous recombination of the endogenous *Htt* gene with a chimeric human/mouse fragment containing an expanded CAG repeat tract. In this group we find the HdhQ92 (Wheeler et al., 1999), HdhQ111 (Wheeler et al., 1999), CAG140 (Menalled et al., 2003) and zQ175 (Menalled et al., 2012) models, among others. The second strategy, which has appeared later, consists in inserting only an expanded CAG repeat tract in the CAG repeat region of the murine *Htt* gene, also by homologous recombination. In this group we can find the HdhQ150 (Lin et al., 2001), HdhQ200 (Heng et al., 2010), and HdhQ250 (Jin et al., 2015) models, among others. Differing in the length of the CAG repeat but also in the genetic background and the genetic construct, a great phenotypic variability is observed among these models. In general, larger CAG repeat stretches in these mice confer an earlier, quicker and more severe symptomatology, although most of them present a normal lifespan with body weight loss, brain atrophy, neuronal death and reactive gliosis only observed in models with larger CAG repeats (> 140 repeats) (Wheeler et al., 2000; Lin et al., 2001; Hickey et al., 2008; Phan et al., 2009; Heng et al., 2010; Menalled et al., 2012; Hölter et al., 2013; Jin et al., 2015). Interestingly, due to the slow progression of the disease, the regional and temporal pattern of mHtt aggregation process can be dissected. Interestingly, models with increased CAG repeats show decreased striatal specificity of this process (Wheeler et al., 2000, 2002; Menalled et al., 2003; Heng et al., 2010; Jin et al., 2015). At a behavioral level, progressive motor impairments have been extensively studied (Lin et al., 2001; Menalled et al., 2003, 2012; Hickey et al., 2008; Heng et al., 2010; Hölter et al., 2013; Jin et al., 2015) and learning and memory abnormalities have also been found (Hickey et al., 2008; Trueman et al., 2008; Simmons et al., 2009; Giralt et al., 2011b; Brooks et al., 2012b; Heikkinen et al., 2012; Smith et al., 2014; Curtin et al., 2015; Puigdellívol et al., 2015). Homozygous mice for the same mutation have frequently shown more severe or faster progression of neuropathological features (Wheeler et al., 2002; Marcellin et al., 2012; Menalled et al., 2012; Young et al., 2013).

### The $Hdh^{Q7/Q111}$ model

In this thesis, we have evaluated the phenotypic outcome resulting from the modulation of two potential therapeutic targets in heterozygous HdhQ111 mice ($Hdh^{Q7/Q111}$), and therefore we will further explain this model. HdhQ111 mice were created by replacing the majority of the endogenous *Htt* exon 1 and part of intron 1 with human DNA containing a stretch with 109 CAG repeats, although the final polyglutamine stretch is two residues longer (111) due to the glutamine codons CAA-CAG that follow the 109 CAGs (White et al., 1997; Wheeler et al., 1999). Given the intergenerational CAG instability (although it appears less pronounced than in transgenic mice), the actual CAG repeat length may vary. Importantly, although the original background of the model was a 129Sv/CD1 mixture, mice used in this thesis are breed in a C57BL/6 background (Lloret et al., 2006). Studies have shown that the genetic background greatly modifies disease progression, even within the same HdhQ111 model, showing C57BL/6 mice the most rapid phenotypic progression while 129 mice the slowest (Lloret et al., 2006; Ament et al., 2017). Therefore, we will review the neuropathological and behavioral abnormalities of heterozygous $Hdh^{Q7/Q111}$ mice in the C57BL/6 strain (Figure 7).

$Hdh^{Q7/Q111}$ mice present a normal lifespan and body weight for up to 1 year (Hölter et al., 2013; Bragg et al., 2017). Importantly, besides intergenerational instability of the CAG tract, progressive somatic CAG instability with an expansion-biased length distribution has been observed in the striatum of these mice beginning as early as 2 months of age (Lloret et al., 2006; Pinto et al., 2013; Ament et al., 2017). By using immunostaining techniques, it is possible to analyze the aggregation process of mHtt throughout the disease. With the EM48 antibody, that selectively labels mHtt in an aggregated form, previous studies have found diffuse immunostaining in the nucleus of a portion of striatal population as early as 2.5 months (Lloret et al., 2006; Kovalenko et al., 2012; Ament et al., 2017). The intensity and the number of stained nucleus increases with age, reflecting a disease-specific event (Kovalenko et al., 2012; Ament et al., 2017). Small punctate nuclear inclusions (microaggregates) have been observed at 4 months (Ament et al., 2017) but at 6 months there are already few visible neuronal intranuclear inclusions (NIIs), which appear as a single strongly immunostained big circular inclusion in each neuronal nucleus (more than one inclusion per cell is rarely observed) (Lloret et al., 2006). These NIIs increase in size and density and, at 10 months, almost 25% of total cells display a NII (Kovalenko et al., 2012). Although the aggregation process is highly restricted to the striatum, NIIs have been observed at 24 months of age in non-striatal brain areas, such as the olfactory bulbs and the piriform cortex (Agostoni et al., 2016). Recently, RNAseq (Langfelder et al., 2016; Bragg et al., 2017) and microarray (Ament et al., 2017) analysis in these mice have revealed early striatal-specific transcriptional alterations, starting at 4-5 months and progressively increasing along disease progression, with an overall downregulation of striatal MSN marker genes. Same analysis in the cortex or cerebellum did not revealed notable transcriptional changes, at least up to 9-10 months (Langfelder et al., 2016; Bragg et al., 2017). Importantly, transcriptional changes are not due to reduced number or size of striatal neurons, which has been found unaltered for up to 18 months (Bragg et al., 2017; Kovalenko et al., 2018). Striatal astrocytic and microglial density were also found unchanged at 12 months (Bragg et al., 2017), although at 24 months some astrogliosis has been reported (Agostoni et al., 2016). Early

synaptic changes have been detected in Hdh$^{Q7/Q111}$ mice, with reduced density of cortical dendritic spines at 2 months of age that might contribute to altered cortico-striatal synaptic transmission observed as LTP impairments (Puigdellívol et al., 2015). Decreased dendritic spine density has also been found in the hippocampus of these mice at 6 months, accompanied by LTP impairments (Brito et al., 2014). Regarding the behavioral phenotype, cognitive deficits have shown to precede motor abnormalities, as in human HD patients. In particular, Hdh$^{Q7/Q111}$ mice show motor learning and procedural memory deficits as early as 2 months, progressively worsening thereafter (Puigdellívol et al., 2015), and long-term memory impairments, including spatial, recognition and associative memories, at 6 months of age (Brito et al., 2014). Motor coordination deficits are not observed until 8 months of age (Puigdellívol et al., 2015).

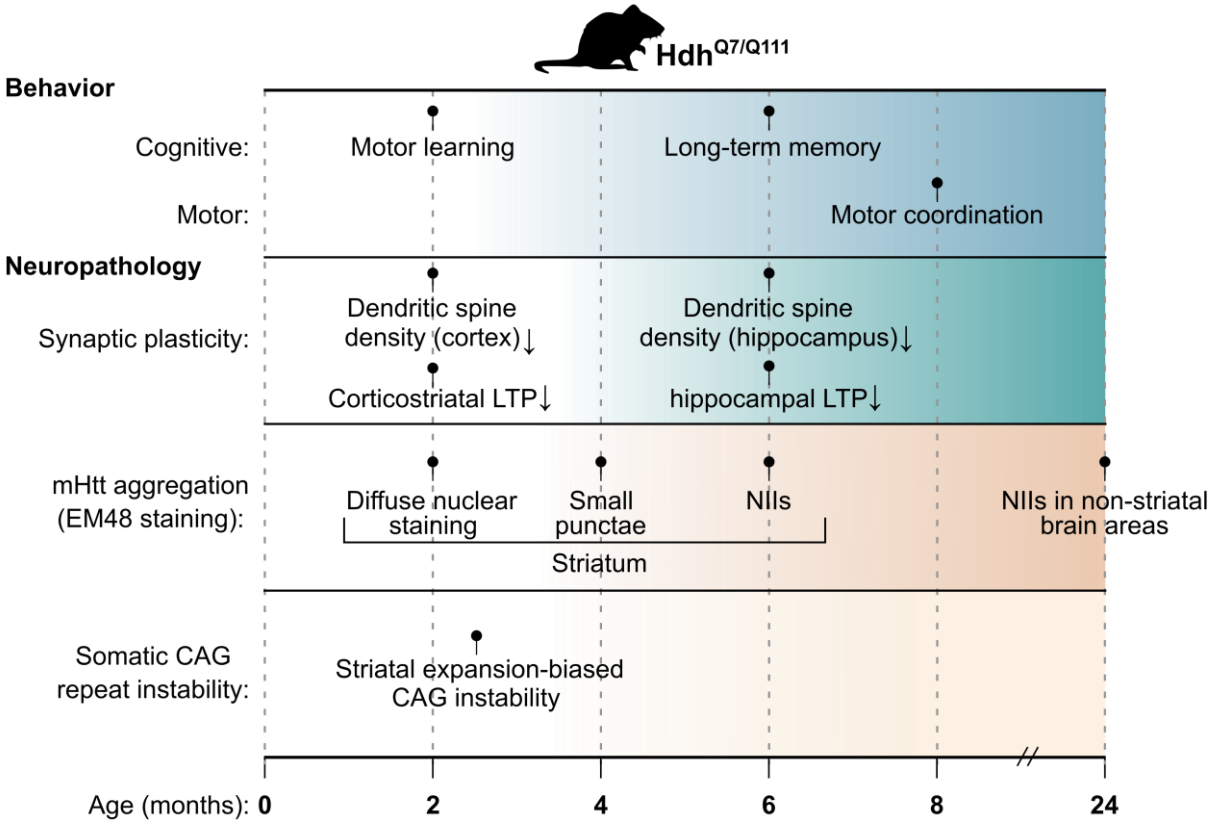



**Figure 7. Timeline of behavioral and neuropathological features of the Hdh$^{Q7/Q111}$ HD mouse model used in this thesis.** The picture depicts age of onset (in months) of motor and cognitive deficits, alterations in structural and functional synaptic plasticity, mHtt aggregates (measured by EM48 immunostaining) and CAG instability (in the brain). See main text for references.

# 2. From molecular pathogenic mechanisms to potential therapeutic strategies in HD

HD shares common pathological features with the two most prevalent neurodegenerative disorders: Alzheimer's disease and Parkinson's disease. All of them present an age-dependent degeneration of particular neuronal subsets and display misfolded proteins that, despite being widely distributed, form aggregates in specific brain regions, with cumulative evidence showing that the soluble oligomeric forms are likely the most toxic species (Haass and Selkoe, 2007). Therefore, the study of the pathophysiology of HD, which is relatively easy to model due to its simple genetic etiology, could led to the discovery of common therapeutic approaches for most neurodegenerative diseases, conferring crucial benefits to the entire world population. Additionally, such study could also provide insights about the mechanisms shared with other trinucleotide repeat diseases, which are less prevalent but also devastating, with many of them being severe neurological disorders that present aggregates of the mutated protein (Ross, 2002).

Despite being caused by a single mutation, the pathogenesis of HD is incredibly complex and, to date, how the expanded CAG tract triggers the diverse symptomatology and the exact cause of the great vulnerability of MSNs is still largely unknown. Nonetheless, after the identification of *Htt* gene in 1993 intensive research efforts have been made and many potential underlying mechanisms in a wide variety of cellular and molecular levels have been described (Figure 8).

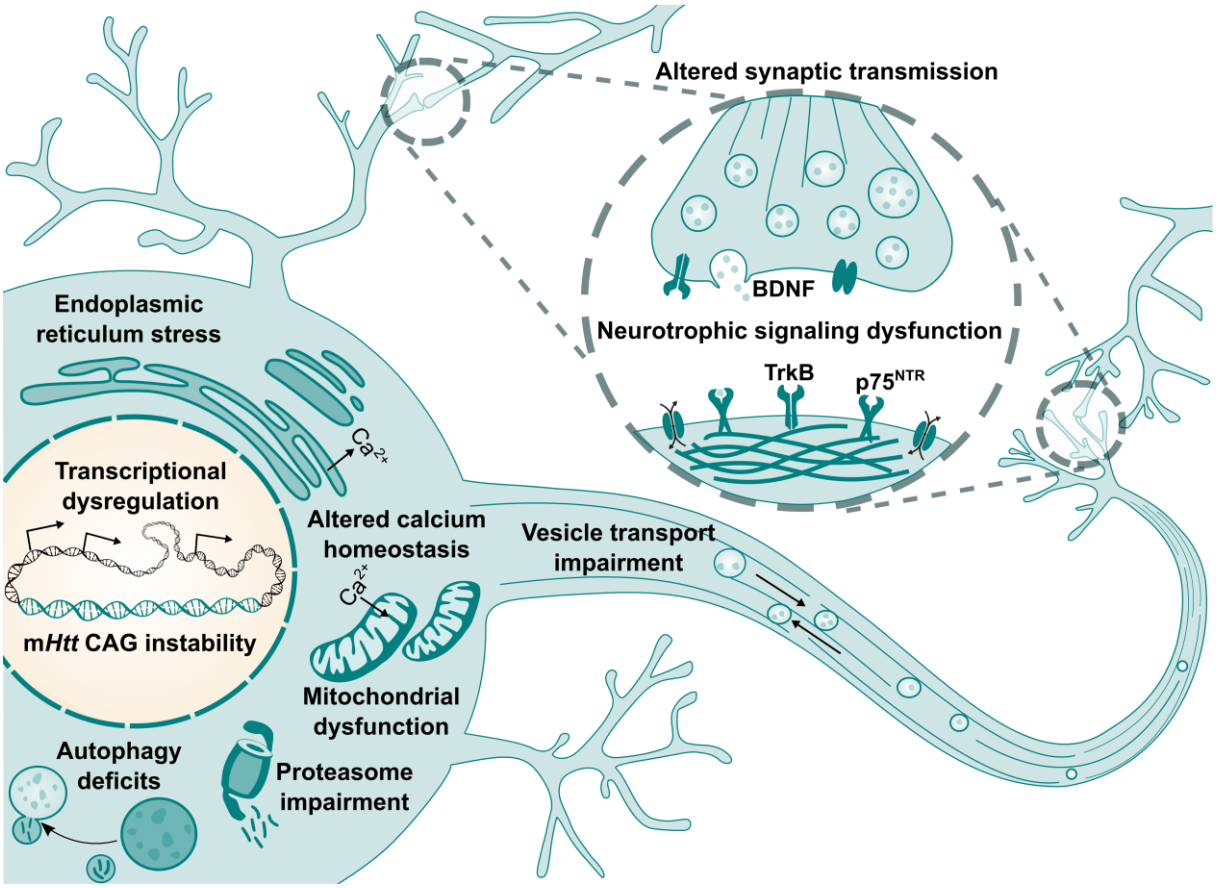


**Figure 8. Postulated molecular pathogenic mechanisms in HD.** Schematic diagram showing proposed molecular mechanisms altered by the presence of mHtt products that are thought to underlie HD pathology.

Some important examples of these pathogenic mechanisms include: somatic CAG repeat instability, transcriptional dysfunction, neurotrophic signaling dysfunction, altered synaptic transmission, vesicle transport impairment, mitochondrial dysfunction, altered calcium homeostasis, endoplasmic reticulum stress, autophagy deficits and proteasomal impairment (Swami et al., 2009; Zuccato et al., 2010; Ross and Tabrizi, 2011; Bates et al., 2015; Tyebji and Hannan, 2017). Such alterations could arise by the presence of one or more of the different mHtt products, either in RNA or protein forms and in monomeric or oligomeric conformations (see Introduction Section 1.2.), and they provide the basis for neuronal dysfunction, which could eventually compromise the viability of specific neuronal populations. Additionally, each proposed molecular mechanism could be differentially altered depending on the brain region with distinct impact on the overall cellular function resulting in a set of specific signs and clinical manifestations in the HD patient.

In order to discover targets with great therapeutic potential for drug development it is important to decipher which of the proposed pathogenic mechanisms are key first-step events that strongly contribute to the overall HD neuronal dysfunction and symptomatology. In this section we will explain in further detail some molecular pathways that appear disrupted early in HD and are thought to be important for the progression of the disease. We will also review feasible therapeutic approaches that could suppress or delay its downstream negative effects.

## 2.1. Transcriptional dysregulation

First evidence for early transcriptional dysregulation in HD was obtained by *in situ* hybridization analysis in post-mortem caudate-putamen samples from HD patients at early stages, which showed downregulated mRNA expression of certain signaling neuropeptides and neurotransmitter receptors (Augood et al., 1996, 1997). These studies were followed by analysis in the striatum of HD mouse models that confirmed the downregulation of several genes encoding neurotransmitter receptors, even before neuronal death and disease symptomatology (Cha et al., 1998). After the first groundbreaking study of Luthi-Carter and colleagues using DNA microarrays to measure gene expression in R6/2 mice (Luthi-Carter et al., 2000), the extensive implementation of these analysis and, later on, of massive sequencing techniques (with improved precision and a higher coverage), both in human HD and model systems, allowed a global overview of transcriptional changes in HD. These studies concluded that HD mice faithfully recapitulate the gene expression signature of the human disorder (Seredenina and Luthi-Carter, 2012; Francelle et al., 2017). Besides, transcriptional profiles from HD mouse models obtained at different diseases stages and brain regions showed that differentially expressed genes increase in number during disease progression, depend on CAG repeat length, and are prominent in affected regions, with most changes present in the striatum (followed by the cortex) (Kuhn et al., 2007; Becanovic et al., 2010; Langfelder et al., 2016; Ament et al., 2017). While genes can be found downregulated and upregulated, these studies show a preponderance of decreased transcripts, especially at earlier time points. Most

downregulated genes are neuronal-enriched and, particularly for the striatum, define neuronal and tissue identity and function. Examples of these striatal-enriched genes include those encoding for cAMP-regulated phosphoprotein of molecular weight 32 kDa (DARPP-32), phosphodiesterase 10A (PDE10A) and adenosine receptor type 2A (A2AR) (Desplats et al., 2006; Hodges et al., 2006; Kuhn et al., 2007; Vashishtha et al., 2013; Achour et al., 2015; Langfelder et al., 2016; Le Gras et al., 2017). Conversely, upregulated genes, particularly in HD patients, have been mostly correlated to inflammation processes (Hodges et al., 2006; Neueder and Bates, 2014; Labadorf et al., 2015). Laser capture microdissection studies in HD human brain (Hodges et al., 2006) and in mouse brain (Zucker et al., 2005; Sadri-Vakili et al., 2006) have demonstrated that gene expression disruption is neuronal specific while ruling out cell loss as a cause for the observed decrease in transcription. Additionally, transcriptional profiles obtained in cellular models and in HD mouse models at disease stages showing preserved neuronal density have also ruled out neuronal loss as a confounding variable (Seredenina and Luthi-Carter, 2012).

As a conclusion of the collected data, transcriptional dysregulation appears to be an early and important disease-contributor event in HD. Interestingly, the observed transcriptional decrease of genes that confer neuronal identity might result in an early and important neuronal dysfunction that, apart from potentially contribute to striatal vulnerability, could also explain the aberrant neuronal activity that underlies HD learning and memory deficits.

### 2.1.1. Mechanisms of transcriptional dysregulation

Although the process by which mHtt contributes to the dysregulation of a specific subset of genes is not completely understood, a variety of molecular mechanisms have been proposed (Figure 9). Many studies have shown that wild-type Htt plays an important role in the modulation of gene expression by interacting with several gene regulatory proteins (Saudou and Humbert, 2016). Thus, with the incorporation of an expanded polyQ tract in the mutant protein, some of these interactions might be tightened or loosened and even new ones with other proteins involved in transcription could be formed (Li and Li, 2004; Seredenina and Luthi-Carter, 2012). Additionally, cleaved mHtt fragments can easily enter and accumulate in the nucleus, affecting such interactions (DiFiglia et al., 1997; Wheeler et al., 2000; Van Raamsdonk et al., 2005a; Havel et al., 2009), and also form insoluble inclusions that might sequester important transcriptional regulators, many of them containing polyQ domains (Kazantsev et al., 1999; Steffan et al., 2000; Nucifora et al., 2001). However, it seems that gene expression changes appear prior and do not depend on mHtt aggregation, suggesting that soluble forms of mHtt are the ones primarily responsible for transcriptional impairments (Dunah et al., 2002; Yu et al., 2002; Sadri-Vakili et al., 2006). Among the transcriptional regulators known to be altered in HD, we can find several transcription factors, which activate or repress gene transcription by binding directly to the DNA, and also transcriptional, coregulators, which indirectly regulate transcription by interacting with transcription factors. Generally, the presence of mHtt seems to impair the activity of positive transcriptional regulators and liberate transcriptional repressors. The most extensively studied are the cAMP-response element (CREB)-binding protein (CBP), the specificity protein-1 (SP1), and the repressor element-1 transcription factor/neuron restrictive silencer factor (REST/NRSF)

(Zuccato et al., 2010). CBP is a transcriptional coactivator (with additional histone acetyltransferase activity) whose transcriptional activity has been described to be impaired in the presence of mHtt by three potential mechanisms: sequestration in mHtt aggregates, aberrant interaction with soluble forms of mHtt and/or abnormal degradation through the proteasome (Kazantsev et al., 1999; Steffan et al., 2000, 2001; Nucifora et al., 2001; Jiang et al., 2003). Importantly, CBP regulates the transcription factor CREB, which controls the expression of important genes for neuronal survival, synaptic plasticity and growth (Lonze and Ginty, 2002) and loss of CBP function has been linked to several neuropathologies, apart from HD (Rouaux et al., 2004). Sp1 is a transcriptional activator that, similarly to CBP, interacts with mHtt with higher affinity than with wild-type Htt, likely causing a disruption of Sp1 function (Dunah et al., 2002; Li et al., 2002). Despite this alteration, however, increased Sp1 levels have been found in multiple HD models, as well as in HD patients, and suppression of Sp1 was suggested to be beneficial for HD pathology (Qiu et al., 2006; Brito et al., 2014). Finally, REST/NRSF is a transcriptional repressor that in normal conditions is sequestered into the cytosol thanks to its interaction with wild-type Htt. Because of the expanded polyQ in mHtt, the interaction is lost and accumulation of REST/NRSF is observed in the nucleus of HD neurons, where it represses its target genes, like BDNF (Zuccato et al., 2003). Interestingly, REST/NRSF was described to mediate transcriptional responses associated with neural plasticity and it is thought to confer neuronal identity (Coulson, 2005).

Another proposed mechanism for the transcriptional dysregulation observed in HD is the alteration of chromatin structure. Indeed, transcriptional activity depends not only on transcription factors and coregulators, but also on the architecture of chromatin, which is modulated by covalent modifications of the DNA (methylation) and histones (acetylation, methylation, phosphorylation, ubiquitination and sumoylation). Acetylation at histone tails, one of the most studied epigenetic marks, is modulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs), that add or remove acetyl groups from their protein substrates, respectively. Although the regulation of gene expression is complex and dynamic, it is generally believed that increased histone acetylation loosens DNA wrapping along nucleosomes, eventually facilitating transcription of genes, while decreased histone acetylation leads to a tightly packed chromatin configuration, which subsequently repress transcription (Eberharter and Becker, 2002). The first evidence of an altered histone acetylation in HD was provided when Steffan and colleagues demonstrated, *in vitro*, that mHtt exon 1 decreases the acetyltransferase enzymatic activity of three proteins: CBP, p300 and PCAF (p300/CBP-associated factor) (Steffan et al., 2001). Moreover, as mentioned before, multiple studies have shown that CBP function is impaired by mHtt (Kazantsev et al., 1999; Steffan et al., 2000, 2001; Nucifora et al., 2001; Jiang et al., 2003). On the other hand, various groups have evaluated the levels of expression and subcellular distribution of different HDACs in several HD systems, finding subtle changes that correlated with gene repression. However, since some contradictory results among different studies and HD models are observed, more detailed analysis are needed to confirm an alteration of HDAC activity in HD (Valor and Guiretti, 2014). Interestingly, it has been recently described that mHtt disrupts the association of HDAC3 with wild type Htt, potentially leading to an increase in the repressive transcriptional activity of HDAC3 (Bardai et al., 2013). In accordance with a decreased HAT activity and a potential increase in

HDAC activity in HD, some studies have shown reduced levels of global histone acetylation in cellular systems overexpressing mHtt (Steffan et al., 2001; Igarashi et al., 2003), in some HD models (Gardian et al., 2005; Giralt et al., 2012a) and even in HD patients (Yeh et al., 2013). Conversely, many other studies, performed in a great variety of HD mouse and cellular models, have provided extensive evidence that reductions in histone acetylation are confined to specific gene loci, namely regions associated with downregulated genes, with an absence of bulk changes (Sadri-Vakili et al., 2007; Thomas et al., 2008; McFarland et al., 2012; Valor et al., 2013; Achour et al., 2015; Guiretti et al., 2016). Other epigenetic marks, such as histone methylation, ubiquitylation and phosphorylation and DNA methylation, have also been found altered in HD, probably as a result of an aberrant modulation of different chromatin remodeler enzymes by mHtt, which could also play a role in the overall HD transcriptional dysregulation (Valor and Guiretti, 2014; Francelle et al., 2017). Importantly, massive and dynamic epigenetic changes in neurons, like rapid increases in histone acetylation, are correlated with bursts in synaptic activity that are known to underlie learning and memory processes, suggesting that chromatin alterations could play an important role in HD cognitive deficits (Riccio, 2010; Gräff and Tsai, 2013).

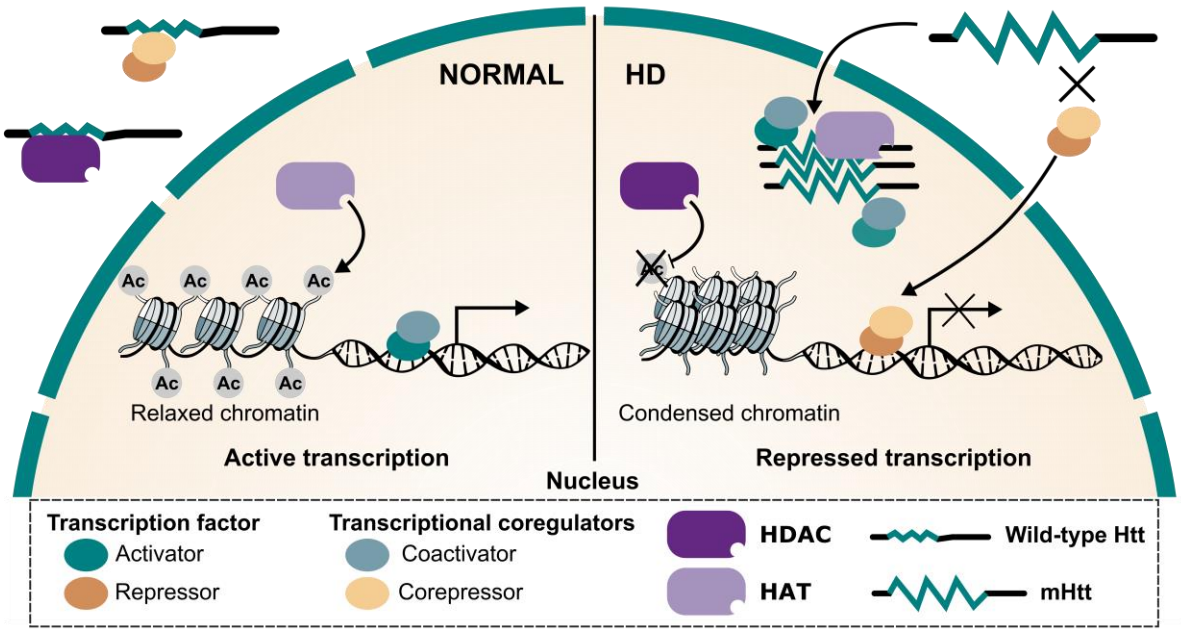



**Figure 9. Mechanisms of transcriptional dysregulation in HD.** Schematic diagram showing transcriptional processes potentially altered by mHtt that could lead to a repressive state of gene expression. Briefly, mHtt might impair the activity of positive transcriptional regulators (by increased interaction or sequestration inside NIIs) or can potentiate the activity of negative transcriptional regulators (by decreased interaction). Additionally, mHtt can alter the activity of acetyltransferases (HATs) or deacetylases (HDACs), changing chromatin structure to a more condensed configuration.

Finally, other mechanisms could also contribute to dysregulate important transcriptional programs, such as the action of toxic RNAs transcribed from m*Htt* (Martí, 2016) and the recent discovery of a disruption of the nuclear pore in HD (Gasset-Rosa et al., 2017; Grima et al., 2017). Gaining more knowledge about the mechanisms underlying transcriptional dysfunction in HD will be extremely helpful in order to identify key targets for therapeutic development.

## 2.1.2. Epigenetic-based therapies for HD

Scientific evidence showing altered chromatin structure (and particularly histone hypoacetylation) as an important underlying cause for transcriptional disruption in HD have prompted the use of small-molecule inhibitors targeting HDACs (HDACis) in order to increase histone acetylation and therefore favor gene transcription. Interestingly, these HDACis have been extensively employed as anticancer drugs, mostly due to their role in cell division, so a great amount of knowledge is available (West and Johnstone, 2014). Besides, since transcriptional dysregulation was likewise described as a key neuropathogenic mechanism for other polyglutamine and neurodegenerative diseases, HDACis are also being developed as therapeutic interventions for these disorders (Butler and Bates, 2006; Kazantsev and Thompson, 2008; Coppedè, 2014). The superfamily of HDACs comprises at least 18 members that are classified into five main subtypes, based on primary sequence homology and domain structure similarity: class I (HDACs 1, 2, 3 and 8), IIa (HDACs 4, 5, 7, and 9), IIb (HDACs 6 and 10), and IV (HDAC11), which comprise the classic HDAC family, and the structurally distinct class III (SIRTs 1-7), which is also known as the sirtuin family (Seto and Yoshida, 2014). The classic HDAC family shares a general catalytic mechanism that requires $Zn^{2+}$ for its enzymatic activity while sirtuins require $NAD^{+}$ as a cofactor. The structure and subcellular localization of each subtype is described in Figure 10.

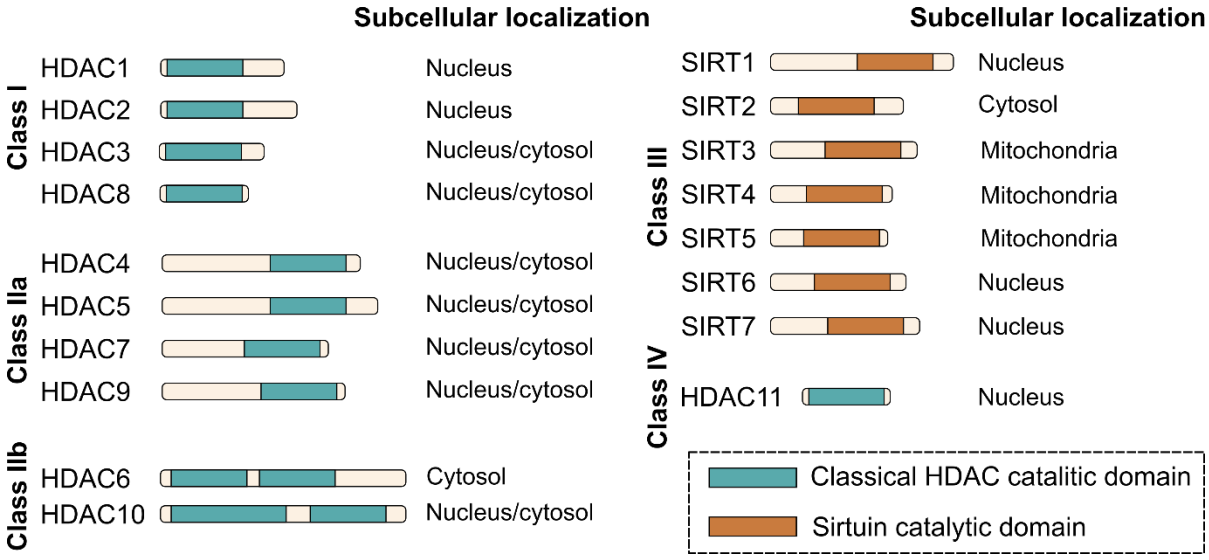



**Figure 10. The HDAC superfamily.** Schematic representation showing structure domains and subcellular localization of each HDAC isotype. Adapted from (Seto and Yoshida, 2014).

Broad-based HDACis, such as suberoylanilide hydroxamic acid, sodium butyrate, phenylbutyrate and trichostatin A, have provided benefits in cell (Nucifora et al., 2001), *Drosophila* (Steffan et al., 2001), and mouse models of HD (Ferrante et al., 2003; Hockly et al., 2003; Gardian et al., 2005; Sadri-Vakili et al., 2007; Giralt et al., 2012a; Valor et al., 2013). Particularly for HD mice, the inhibitors reversed transcriptional impairments and ameliorated neuropathological features and motor function. Nonetheless, the effectivity of these compounds might be counteracted by their toxicity, probably due to the non-selective inhibition against multiple HDACs (Li and Seto, 2016), so therapeutic research has recently focused on developing isoform-selective HDACis.

Accordingly, studies have tried to identify which HDAC subtypes could contribute to a greater extent to the transcriptional disruption underlying HD pathology. Several lines of evidence suggest that the HDAC3 isotype is of particular interest (Thomas, 2014).

HDAC3 is present in many tissues, including the brain (Mahlknecht et al., 1999), where is the most highly expressed class I HDAC (Broide et al., 2007). Within the brain, expression is particularly high in the hippocampus, cortex and cerebellum, followed by the striatum, amygdala and hypothalamus (Broide et al., 2007; Thomas, 2009), and is predominant in neurons, although it has also been observed in microglia, astrocytes and oligodendrocytes (Shen et al., 2005; Broide et al., 2007; Debacker et al., 2012; Yu et al., 2018). Moreover, HDAC3 can shuttle between the nucleus and the cytosol thanks to the presence of a nuclear localization signal and a nuclear export signal (Yang et al., 2002), suggesting that it might have different roles in each compartment. Importantly, HDAC3 forms a stable multiprotein complex with nuclear receptor corepressor (N-CoR) and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) in order to regulate gene transcription (Karagianni and Wong, 2007). It is also worth noting that many non-histone proteins in the nucleus or cytosol could be deacetylated by HDAC3 (McQuown and Wood, 2011; Yao and Yang, 2011; Alessandro Didonna, 2015).

Interestingly, selective HDACi treatments in HD models revealed an important role for HDAC3 in the pathology. First, an inhibitor named 4b, which has considerable selectivity for HDAC3, followed by HDAC1, showed low toxicity and great therapeutic efficacy in the R6/2 and N171-82Q transgenic mouse models of HD, ameliorating gene expression abnormalities and some aspects of disease phenotype (Thomas et al., 2008; Jia et al., 2012a,b). More recently, inhibitors with great selectivity for HDAC3, such as RGFP136 and RGFP966, have been tested: RGFP136 has shown to prevent the downregulation of a subset of genes in the striatum of R6/2 mice (Jia et al., 2012b) while RGFP966 was found to ameliorate some behavioral abnormalities and neuropathological features (mostly in the striatum and the cortex) of the N171-82Q model (Jia et al., 2015, 2016). However, restoration of HD-related cognitive deficits upon HDAC3 inhibition has not been extensively evaluated. In order to study individual HDAC involvement in HD by a different approach, the group of Bates et al. crossed heterozygous mice for HDAC3, 4 and 7 (Benn et al., 2009; Moumné et al., 2012; Mielcarek et al., 2013) or complete KO mice for HDAC6 and SIRT2 (Bobrowska et al., 2011, 2012) with R6/2 mice, and resulting animals were evaluated. Surprisingly, the downregulation of any of these proteins did not rescue global HD transcriptional impairments and only the reduction of HDAC4 elicited phenotypic benefits, although probably as a result of cytoplasmic effects and not of changes in chromatin remodeling (Mielcarek et al., 2013). Despite these results, the above-mentioned amelioration in transcription after HDAC inhibition in HD mice suggest that, at least for some HDAC isotypes, more than 50% reduction in their activity or levels might be needed to observe transcriptional consequences. Moreover, compensatory effects elicited by other HDACs with redundant activity could not be discarded in genetic ablation studies. Indeed, the specific histone substrates of some of the HDACs analyzed in the genetic studies appeared unchanged or were simply not shown (Bobrowska et al., 2012; Moumné et al., 2012; Mielcarek et al., 2013). Additionally, in the brain of double mutant HDAC3$^{+/-}$:R6/2 mice, cytoplasmic HDAC3 levels were unchanged whereas nuclear levels were at 60% of

normal protein levels, probably resulting in a suboptimal HDAC3 downregulation (Moumné et al., 2012). Supporting its pathological role in HD, HDAC3 has been identified as an important HD genetic modifier based on a bioinformatic study that compared data from the HD Research Crossroads database with genome-wide linkage analysis (Kalathur et al., 2012). Additionally, and as previously mentioned, a recent study has shown that Htt normally interacts with HDAC3, probably sequestering the protein to repress its activity, but, upon the addition of several apoptotic stimuli or mHtt *in vitro*, their interaction is decreased, releasing HDAC3 (Bardai et al., 2013). *In vivo*, the interaction was found specifically decreased in the striatum of R6/2 mice (Bardai et al., 2013). Thus, and given that HDAC3 was observed to cause selective neuronal toxicity (Bardai and D'Mello, 2011; Bardai et al., 2013), it was suggested that the dissociated HDAC3 might repress specific neuronal genes that would eventually cause HD neuronal vulnerability. These results are in accordance with the previously commented benefits of HDA3-selective inhibitors in striatal neuropathological features and in behavioral impairments mostly dependent on the striatal function (Jia et al., 2012b, 2015, 2016).

Importantly, besides its role in neuronal survival, HDAC3 has shown to be an important negative regulator of hippocampal-dependent and other types of memory (McQuown and Wood, 2011; McQuown et al., 2011; Malvaez et al., 2013; Rogge et al., 2013; Bieszczad et al., 2015; Kwapis et al., 2017). Particularly, it was demonstrated that the genetic or pharmacological inhibition of HDAC3 in the hippocampus of wild-type mice significantly enhanced long-term memory after a subthreshold training, probably by increasing histone acetylation and facilitating the expression of key IEGs (McQuown et al., 2011). In this context, it is important to keep in mind that neuronal dysfunction in non-striatal brain areas such as the hippocampus is receiving increasing interest in HD research since cognitive alterations depending on these regions are especially invalidating for the patients. In fact, alterations in hippocampal synaptic plasticity and function have been described in HD mouse models, which could be related to an altered chromatin regulation of transcriptional programs (Giralt et al., 2012b; Puigdellívol et al., 2016). Supporting this idea, histone hypoacetylation in multiple genomic loci has been found in the hippocampus of N171-82Q mice (Valor et al., 2013) while global hippocampal transcriptional impairments were demonstrated in N171-82Q (Valor et al., 2013), zQ175 (Langfelder et al., 2016) and hdhQ111 (Bragg et al., 2017) mice, although expression changes were modest compared with other more affected regions such as the striatum. Additionally, downregulated expression of plasticity-related IEGs has been confirmed in the hippocampus of the N171-82Q (Valor et al., 2013) and HdhQ111 (Giralt et al., 2012a) mouse models. Consistently, treatment of HdhQ111 mice with trichostatin A, which targets HDAC 1, 2, 3, 6, 10 and 11, has shown to reverse the expression of these key memory genes, preventing the appearance of hippocampal-dependent long-term memory deficits (Giralt et al., 2012a).

In conclusion, the central role of HDAC3 in memory formation and the altered chromatin regulation of transcriptional programs in the HD hippocampus have led us to investigate if HDAC3 inhibition could be a key pharmacological strategy to ameliorate or prevent HD cognitive deficits.

## 2.2. Somatic CAG repeat instability

Expansions in different trinucleotide repeats (TNRs), including CAG, CTG, CGG and GAA triplets, cause more than fifteen neurodegenerative, neurological and neuromuscular genetic diseases, including HD, several spinocerebellar ataxias (SCAs), myotonic dystrophy 1 (DM1), Friedreich ataxia (FRDA) and fragile X syndrome (FXS) (Figure 11) (López Castel et al., 2010; McMurray, 2010). It has long been established that all of the disease-causing TNRs, once above a threshold of 30 to 50 repeats, are likely to present intergenerational repeat size variations, with a preponderance of length increases. This phenomenon explains the worsening and earlier presentation of disease symptoms in each subsequent generation (genetic anticipation). Evidence of a common threshold for such instability might reflect an intrinsic property of the DNA involved in the switching from a stable to an unstable state. Intergenerational instability takes place in the germline and, in the case of HD, predominant length increases are only observed during spermatogenesis, explaining the paternal bias for inheritance of expanded alleles (Leeflang et al., 1999; Yoon et al., 2003; Wheeler et al., 2007). Although less known, other genetic diseases have been found to be caused by repeats of more than three units, such as tetranucleotides, pentanucleotides, and even larger repeats (López Castel et al., 2010).

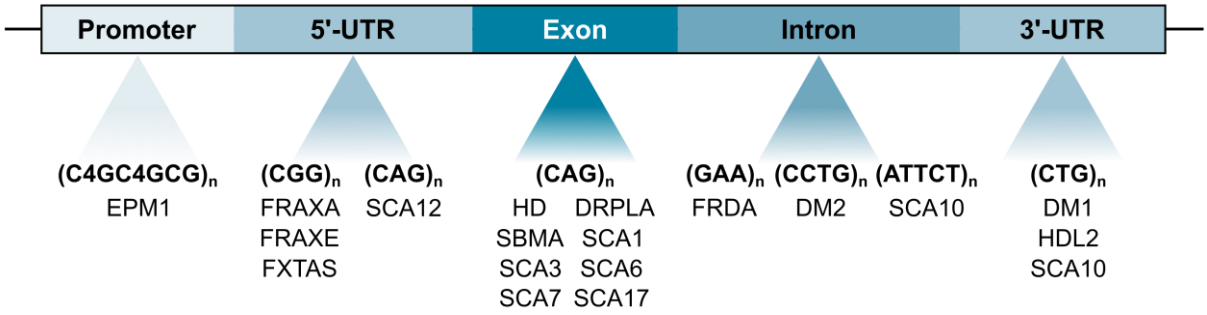



**Figure 11. Human diseases caused by dynamic repeat expansions.** Schematic diagram showing, within a generic gene, the location and sequence of expandable repeats that cause human diseases. EPM1: progressive myoclonic epilepsy 1; FRAXA: fragile X syndrome; FRAXE: fragile X mental retardation associated with FRAXE site; FXTAS: fragile X tremor and ataxia syndrome; DRPLA: dentatorubral-pallidoluysian atrophy; SBMA: spinal and bulbar muscular atrophy; SCA: spinocerebellar ataxia; FRDA: Friedreich's ataxia; DM: myotonic dystrophy; HDL2: Huntington's-disease-like 2.

In addition to germline instability, some expandable repeats are unstable in specific somatic tissues, undergoing progressive length increases over time (Telenius et al., 1994; Thornton et al., 1994; Wong et al., 1995; Lopes-Cendes et al., 1996). HD somatic CAG instability was first described in human patients (Telenius et al., 1994) and since then it has also been observed in multiple HD mouse models, such as R6 transgenic lines (Mangiarini et al., 1997; Shelbourne et al., 2007), and knock-in models (Wheeler et al., 1999; Kennedy and Shelbourne, 2000; Ishiguro et al., 2001; Kennedy et al., 2003). Additionally, longitudinal studies in these mice have shown that CAG repeat gains occur early in the disease and progressively accumulate thereafter. Importantly, the BACHD mouse model of HD presents a mix of CAG/CAA tandem repeats that is stable in both the germline and somatic tissues (Gray et al., 2008). The fact that these mice show HD phenotype demonstrates that somatic instability is not the only driving force of the disease, but several lines of evidence suggest that it could have an important contribution to HD progression.

Data obtained by examining and comparing a great diversity of tissues in HD patients and mouse models have revealed that somatic CAG instability is predominant in brain regions with most marked neuropathological changes, such as the striatum and the cortex. Consistently, a relatively unaffected region, the cerebellum, exhibits the lowest degree of instability (Telenius et al., 1994; Mangiarini et al., 1997; Wheeler et al., 1999; Kennedy and Shelbourne, 2000; Kennedy et al., 2003). Besides, studies performed in end-stage HD patients presenting almost non-existent caudate and putamen regions might have masked some specific instability rates. Indeed, larger (up to 1,000 CAGs) and more specific (limited to the striatum) expansions have been observed in HD brains at early stages, prior to neurodegeneration (Kennedy et al., 2003), suggesting that somatic instability might underlie the specific neuronal damage observed in HD. The same study also suggested that longer inherited CAG tracts could lead to a less specific and more generalized somatic instability throughout the brain, which adequately correlates with the decreased neuropathological specificity and increased clinical spectrum in mice and HD patients with very long CAG repeats. Strikingly, analysis in HD mouse models have also revealed great CAG instability in the liver, which shows a bimodal distribution of repeat sizes that might indicate the presence of two distinct populations of cells with significantly different levels of instability (Mangiarini et al., 1997; Kennedy et al., 2003; Lee et al., 2010, 2011). Somatic CAG instability has also been found in some other TNR diseases, with different tissue-specific patterns that likewise seems to partially correlate with disease-target regions. Thus, somatic CTG instability in DM1 is evident in muscle cells, the primarily affected tissue (Anvret et al., 1993; Wong et al., 1995), while GAA instability in FRDA is extensive in dorsal root ganglia and cerebellum, two important affected tissues in this disorder (Clark et al., 2007; De Biase et al., 2007).

Several studies provided evidence for the groundbreaking idea that genomic DNA polymorphisms, in the form of CAG repeat expansions, can also occur in post-mitotic differentiated neurons on specific conditions. First, analysis from micro-dissected tissues and individual laser-dissected cells obtained from different brain regions of human HD cases and knock-in HD mice indicated that neuronal alleles presented longer CAG tracts in m*Htt* that glial alleles (Shelbourne et al., 2007). Next, deeper and more accurate analysis with laser microdissection in the striatum of R6 and knock-in mice and in human HD patients confirmed that unstable CAG repeats were prominent in non-replicating terminally differentiated neurons (Gonitel et al., 2008). Therefore, non-replicative expansion mechanisms are likely to cause this type of expansion, which could differ from the ones involved in germline instability. Supporting this idea, previous experiments demonstrated that immortalized striatal cells derived from knock-in HdhQ111 mice (STHdhQ111 cells) present a CAG repeat tract in *Htt* gene that is stable over passages but, when cell cycle is arrested by maintaining the culture at a restrictive temperature for several weeks, significant instability can be observed (Lee et al., 2010).

The noticeable relationship between inherited CAG repeat length and age of onset and severity of the disease, together with the observation of prominent CAG repeat expansions in tissues and cells that are disease targets, which could result in the production of increasingly toxic mutant entities, have led to the hypothesis that somatic instability contributes to the progression of HD. Studies in HD models and patients have provided substantial proofs to support this idea. In

humans, somatic instability was found to be a significant contributor of onset age (Swami et al., 2009). Particularly, in individuals with the same number of repeats within the inherited m*Htt* allele but different rates of instability in the brain, the ones with larger repeat length gains will be prone to exhibit earlier disease onset. Recently, several genome-wide association studies have identified genes involved in repeat instability as potential modifiers of HD onset and progression (GeM-HD Consortium, 2015; Hensman Moss et al., 2017; Lee et al., 2017). Other studies in HD mice have used genetic or pharmacological approaches to effectively reduce CAG instability, resulting in a delay of HD-related phenotypic measures (Wheeler et al., 2003; Dragileva et al., 2009; Kovalenko et al., 2012; Pinto et al., 2013; Budworth et al., 2015). In view of all this, studies aimed to decipher the molecular pathways involved in this process will be key to reveal potential targets for therapeutic development that are able to modulate disease pathogenesis.

### 2.2.1. Mechanisms of DNA repeat instability

In recent years, a growing number of studies have suggested many different mechanisms underlying repeat instability. One prevalent feature of the proposed models is the formation of a mutagenic intermediate that consists in an unusual secondary structure in the repeat tract. Indeed, expandable C-G-rich TNRs, such as CAG, CTG, CCG and CGG, can form intra-strand hairpin structures that comprise both complementary base pairs and mismatched base pairs (Figure 12A). These have been observed *in vitro* (Gacy et al., 1995; Wells, 1996) and indirectly demonstrated *in vivo* (Moore et al., 1999; Liu et al., 2010). Importantly, when two strands with the same number of TNRs reanneal after a secondary structure is formed in one strand, they form a slipped stranded structure, with hairpins on both strands (Figure 12B) (Pearson et al., 2002). The formation of these structures might occur whenever the DNA is single-stranded, which could transiently happen during DNA replication, repair, recombination or transcription, all molecular processes that have shown to be involved in repeat instability (López Castel et al., 2010; Usdin et al., 2015). Importantly, the tendency of large CAG repeats to exhibit repeat instability correlates with their ability to form stable hairpins, suggesting that such structures might be responsible for initiating the expansion process (Petruska et al., 1996; Miret et al., 1998; McMurray, 1999; Rolfsmeier and Lahue, 2000). Supporting this idea, it has been demonstrated that CAG tracts interrupted with CAA repeats are unable to form hairpin structures in the same manner as pure CAG tracts (Figura et al., 2015) and BACHD mice present a mixed CAG/CAA repeat tract in the inserted m*Htt* transgene that does not exhibit instability in any tissue examined (Gray et al., 2008). Another uncommon structure that has been proposed for expandable CAG repeats is the R-loop, which consists in a stable and persistent RNA/DNA hybrid that occurs during transcription when the newly synthesized RNA transcript pairs with the single-stranded DNA in the transcription bubble (Figure 12C). The formation of this structure could also stimulate CAG repeat instability (Lin et al., 2010). Additionally, it is worth noting that a variety of different secondary structures have been observed for other TNR (not CAG) and larger repeats, such as G-quadruplexes and triplexes, which could also be involved in promoting DNA instability (Mirkin, 2007; Usdin et al., 2015).

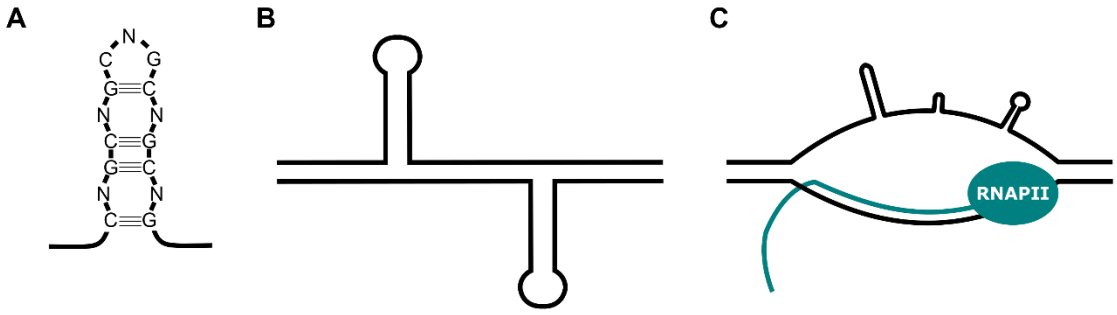



**Figure 12. Potential non-conventional DNA secondary structures formed by expandable $(CAG)_n \cdot (CTG)_n$ repeats in m*Htt*.** (**A**) Hairpin structure, where "N" could be A or T. (**B**) Slipped-stranded DNA structure with hairpins formed on both strands. (**C**) R-loop, consisting in a RNA/DNA hybrid that forms during transcription when the newly synthesized RNA transcript stably pairs with single-stranded DNA in the transcription bubble. Hairpins could additionally be formed in the single DNA strand. Adapted from (Usdin et al., 2015).

Despite the variety of non-conventional structures observed for different repeats, it seems that they share common ground when it comes to impede correct DNA metabolism within the repetitive tract, either in DNA replication or repair. The intergenerational instability, shared by all repeat diseases, prompted early studies aimed to elucidate the mechanisms of repeat instability to focus on germ cells and replication processes. Indeed, first models considered polymerase slippage during replication as a key phenomenon, since a misalignment between the two repetitive strands, further stabilized by unusual conformations, would result in a different number of bases in the newly formed strand. Thus, after a second round of replication, deletions would occur if a hairpin was formed on the template DNA strand and insertions when such structure was on the nascent strand (Figure 13) (Kunkel, 1993; Petruska et al., 1998).

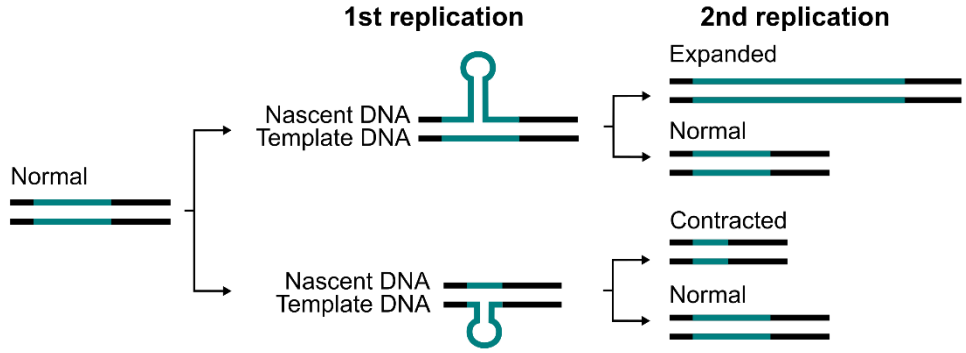



**Figure 13. Simplified replication slippage model for CAG repeat instability.** Sections in green within the DNA refer to $(CAG)_n \cdot (CTG)_n$ repeats.

After this simple idea, many other models for TNR expansions were proposed, further expanding the details of the mechanism but also assuming a replication-dependent process (Mirkin, 2007; McMurray, 2010; Neil et al., 2017). However, while these might hold true for instability in dividing cells, mechanisms operating in replication-independent pathways must explain mutability of the CAG tract in post-mitotic neurons. Indeed, any pathway involving the synthesis of new DNA may generate expansions of TNRs. It is now understood that the addition or deletion of repeats can occur during various types of DNA repair pathways in the absence of genome replication. Several mechanistic models for somatic instability have been proposed, providing a complex picture with

many potential targets (López Castel et al., 2010; Usdin et al., 2015; Jones et al., 2017; Massey and Jones, 2018). In general terms, the hypothesis is that these normally protective pathways, in specific contexts (such as the appearance of uncommon DNA structures), might have their normal biochemical functions replaced by new mutagenic ones. Therefore, the activity of these pathways is not defective in general, since that would mean an increased propensity to develop cancer, which has not been described in TNR disease patients. Genetic knock-down studies in TNR disease mouse models have proved the role of DNA repair in TNR instability. Particularly for HD, proteins involved in mismatch repair (MMR) (Manley et al., 1999; Wheeler et al., 2003; Dragileva et al., 2009; Tomé et al., 2009; Pinto et al., 2013) and base-excision repair (BER) (Kovtun et al., 2007; Budworth et al., 2015) have been implicated in this process.

MMR has attracted particular attention due to its great implication in repeat instability, not only in HD but also in other TNRs (Schmidt and Pearson, 2016). MMR plays a key role in genome maintenance by repairing both base-base mismatches and insertion-deletion loops. The whole process can be divided into several stages: mismatch recognition, excision, resynthesis and ligation. Interestingly, in humans, the initial recognition of mispairs is performed by two heterodimeric protein complexes: MutSα, formed by Msh2 and Msh6, and MutSβ, comprising Msh2 and Msh3. While MutSα preferentially binds single base-base mismatches and small insertion/deletion loops, MutSβ predominantly binds to larger insertion/deletion loops (Jiricny, 2006). The observation of decreased CAG repeat expansion rates after ablating *Msh2* and *Msh3* genes, but not *Msh6*, in HdhQ111 and R6/1 mice has outlined the preferential and positive involvement of MutSβ in somatic and even intergenerational instability (Manley et al., 1999; Kovtun and McMurray, 2001; Wheeler et al., 2003; Dragileva et al., 2009; Kovalenko et al., 2012). *In vitro* studies also showed that MutSβ stimulates CAG repeat instability in human cell extracts, suggesting a direct contribution to the process (Stevens et al., 2013). Regarding the mechanism, *in vitro* studies have shown that MutSβ specifically binds to the $(CAG)_n$ hairpin structure (Owen et al., 2005; Tian et al., 2009), and it requires its ATPase activity to promote instability (Tomé et al., 2009). Other proteins of the MMR pathway, such as Mlh1 and Mlh3 (which conform the heterodimer MutlLγ), also play a role in this process (Pinto et al., 2013) and it has been suggested that Mutlγ interacts with MutSβ but not with MutSα (Charbonneau et al., 2009). However, the detailed mechanism through which mismatch repair mediates CAG instability remains largely unknown. The picture is even more complex, since apart from correcting mismatches, MMR proteins also participate in other repair processes, including BER, nucleotide excision repair (NER), double strand break (DSB) repair and transcription-coupled repair (Stojic et al., 2004).

Furthermore, an attractive mechanism to promote repeat expansions in non-dividing cells has involved oxidative DNA damage and the action of BER proteins (Kovtun et al., 2007). BER typically senses, removes and correct small, non-helix-distorting base lesions in DNA (Akbari et al., 2015). The heavy oxygen demand of neurons (especially within the central nervous system) cause their constant exposure to reactive oxygen species (ROS) due to normal mitochondrial metabolism, which can cause many different types of DNA base damage (Cooke et al., 2003). Interestingly, several oxidative DNA lesions further accumulate in the brains of R6/1 and R6/2 animals (Bogdanov et al., 2002; Kovtun et al., 2007) and HD patients (Browne et al., 1997). One

abundant lesion, the 8-oxoguanine (8-oxoG), has been found preferentially accumulated at repeat loops (Jarem et al., 2009) and, importantly, excision of 8-oxo-G within CAG repeats by the glycosylase Ogg1 (8-oxoguanine DNA glycosylase 1) during BER has been reported to initiate strand displacement *in vitro* and mediate somatic age-dependent expansions *in vivo* (Kovtun et al., 2007). Neil1 (nei like DNA glycosylase 1), another BER protein that predominantly removes oxidized pyrimidines but also 8-oxoG, has likewise been implicated into the initiation of somatic repeat instability (Møllersen et al., 2012). Indeed, a recent study has proposed a model for instability with a MMR-BER crosstalk acting in a "toxic oxidation" cycle (Lai et al., 2016). Finally, it is also worth noting that stabilized R loops formed during transcription of repeat tracts, as mentioned earlier, could lead to a persistent single-stranded state of the DNA that allows the formation of hairpin structures and even the action of DNA damaging agents, all mechanisms involved in repeat instability (Freudenreich, 2018). The integrated model, therefore, might involve many different processes acting in coordination (Figure 14).

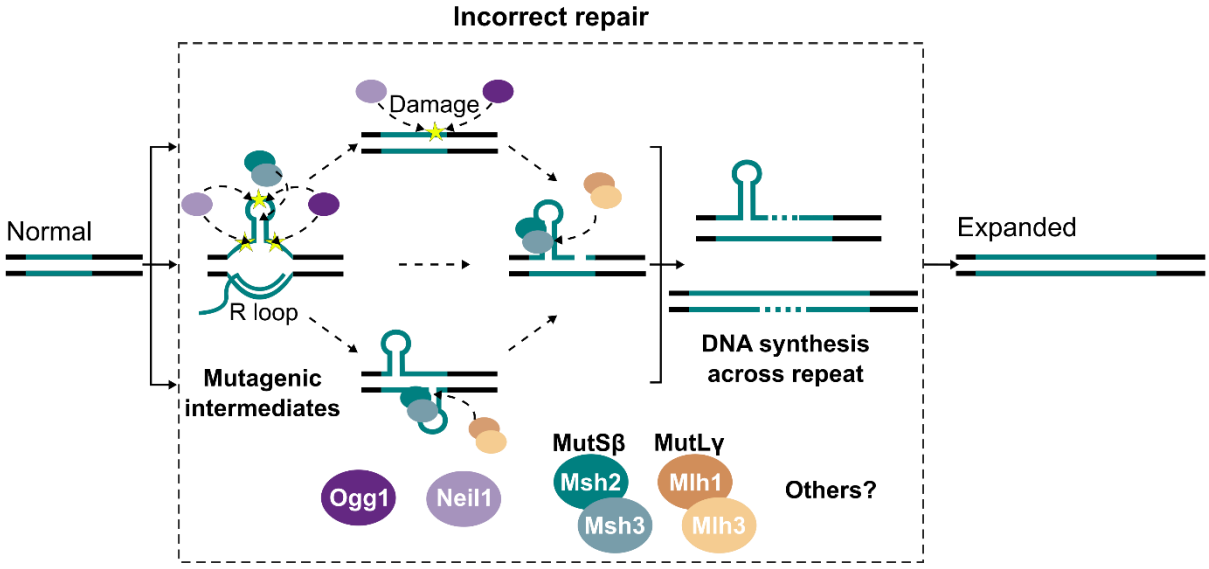



**Figure 14. Simplified model of somatic CAG repeat instability.** Sections in green within the DNA refer to $(CAG)_n \cdot (CTG)_n$ repeats. Mismatch repair proteins (Msh2, Msh3, Mlh1 and Mlh3) could bind and stabilize DNA hairpins or are involved by any other mechanism to induce incorrect repair. R-loops maintain DNA in a single-stranded form, which make it more vulnerable to toxic elements and allow hairpin formation. Base-excision repair (BER) proteins (Ogg1 and Neil1) attempt to repair oxidized DNA but eventually produce mutagenesis. Detailed models explaining CAG instability processes are shown in (Polyzos and McMurray, 2017).

Importantly, several hypotheses have been formulated in order to provide an explanation for the tissue-selective profile of somatic TNT instability, although it still remains a largely unanswered question (Dion, 2014).

## 2.2.2. Modifiers of CAG repeat instability as potential therapeutic targets for HD

As we have seen, the growing interest in exploring DNA repair pathways in the process of TNR instability have uncovered a great variety of proteins that could modulate this process in specific cellular contexts. However, important caution must be taken when evaluating such targets for HD

therapeutic development. Patients with different mutations in MMR proteins, particularly those that inactivate Msh2, Msh6, and Mlh1, present genome-wide instability and increased risk of colorectal, stomach, endometrial, pancreatic, and urinary tract cancers (Hsieh and Yamane, 2008; Dion, 2014). Besides, mouse lines in which specific MMR proteins have been knocked out exhibit increased incidence of cancer, mostly gastrointestinal, lymphoma, and skin tumors, and decreased lifespan (Hsieh and Yamane, 2008). Indeed, MMR is of great importance in dividing cells in order to correct mismatches naturally occurring at the replication fork. In view of that, increased cancer predisposition could arise from reducing the activity of mismatch repair proteins as a HD therapeutic strategy. The identification of new factors involved in CAG repeat addition/deletion that do not cause general genome instability would be of great importance.

Several lines of evidence have pointed to a role for HAT/HDAC proteins in DNA repeat instability (Figure 15). In 2007, one promising study demonstrated that the heterozygous depletion of the Drosophila CBP (dCBP) in a SCA3 fly model (CTG repeats) increased intergenerational repeat instability (Jung and Bonini, 2007). Conversely, treatment with the broad-spectrum HDAC inhibitor trichostatin A (TSA), that targets HDAC 1, 2, 3, 6, 10 and 11, resulted in a decrease in CTG repeat expansions. Furthermore, the downregulation of CBP levels in HD transgenic flies confirmed an exacerbation of CAG repeat instability in the progeny. Although expansions in disease-affected tissues were not evaluated, given that some repair pathways are involved both in germline and somatic instability, we could speculate that CBP and HDACs (or specific isoforms) could also modulate somatic instability in opposing ways. Later on, in a large-scale yeast mutant screen study, yeast cells carrying a CTG·CAG repeat tract near the threshold length were used to identify factors that could modulate TNR instability. Surprisingly, 3 of 11 hits were components of HDAC complexes, namely SIN3, PHO23, and HDA3, and the genetic or chemical inhibition of these factors significantly suppressed expansions (Debacker et al., 2012). In view of this, the same group proceeded to test the human homologues of these proteins (highly conserved from yeast to humans) in the SVG-A cell line, which is an immortalized human astrocytic line carrying an unstable CTG·CAG repeat tract (Debacker et al., 2012; Gannon et al., 2012). Importantly, this cell line was previously shown to be prone to expansions (Claassen and Lahue, 2007) and it has been demonstrated that, although post-mitotic neurons are predominantly unstable in HD patients, glial cells also display some expansion activity (Shelbourne et al., 2007). By using SVG-A cells, the authors of the aforementioned studies demonstrated that the genetic and/or pharmacological inhibition of class I HDAC3, but not HDAC1 or HDAC2, effectively reduced the frequency of expansions. Additionally, the deficiency of class II HDAC5 also suppressed expansions while, surprisingly, the class II HDAC9 promoted TNR length increases. siRNA treatment against the histone acetyltransferases CBP/p300 demonstrated an increase in expansions. Furthermore, the authors confirmed the contribution of MutSβ (while ruling out MutSα) in promoting such expansions and demonstrated that the double knockdown of Msh2 and HDAC3 or Msh2 and HDAC5 in SVG-A cells had the same effect in suppressing CAG repeat expansions than the single knockdown of Msh2, Msh3, HDAC3, or HDAC5, indicating that all these proteins might operate in a shared pathway to modulate TNR instability. After trying to decipher the molecular details of these interactions, authors found that the genetic HDAC3

downregulation did not affect occupancy of Msh2, Msh3 or Msh6 at CTG·CAG repeat tracts, or protein expression of Msh2 or Msh3 in SVG-A cells. They proposed other explanations yet to be elucidated, such as that HDAC3 regulates MutSβ function or the TNR occupancy or function of other unknown factors that functionally interact with MutSβ to enable expansions. Supporting this hypothesis, the initial description for HATs and HDACs as enzymes that modify histone acetylation to regulate chromosome dynamics and transcription has been greatly expanded with the discovery of their many non-histone proteins substrates (Glozak et al., 2005) and it has been suggested that the acetylation of key DNA repair proteins could influence the DNA damage response (Choudhary et al., 2009). Particularly, since Msh2 has shown to be acetylated on different residues and interact with several HDACs (Choudhary et al., 2009; Zhang et al., 2014; Radhakrishnan et al., 2015), it is of great interest to explore the regulation of this post-translational modification.

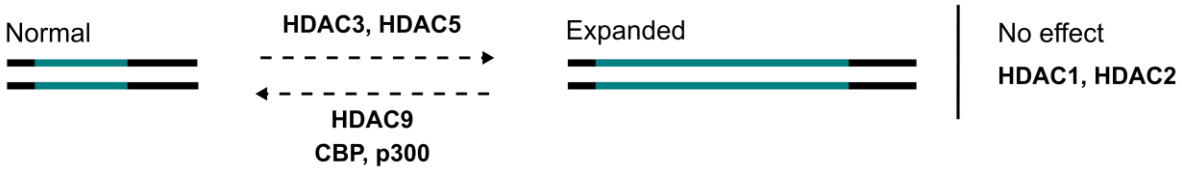



**Figure 15. Schematic diagram showing HAT/HDAC proteins tested for TNR repeat instability.** The participation of each protein in the instability process is inferred from several studies (Jung and Bonini, 2007; Debacker et al., 2012; Gannon et al., 2012). See main text for further information.

Overall, these promising data suggest that, while some HATs could function to stabilize repeats, specific HDACs might act in an opposing manner to promote expansions. Importantly, as mentioned previously, HDAC inhibitors have been extensively used in cancer as chemotherapeutic agents (West and Johnstone, 2014) and now are currently being developed as a potential therapeutic strategy to alleviate symptoms of TNR and neurodegenerative diseases (Kazantsev and Thompson, 2008), based on the rationale that transcriptional dysregulation is a key pathological feature in these disorders. The potential role for HDACs in repeat instability underscores new therapeutic applications and highlight the importance of examining the contribution of specific HDAC isoforms in this process. Based on the promising benefits of HDAC3-selective inhibitors in HD, one aim of this thesis was to investigate the potential contribution of HDAC3 in striatal CAG repeat instability.

## 2.3. Neurotrophic signaling dysfunction

Neurotrophins are key regulatory factors for the development and maintenance of the nervous system as they modulate the differentiation, survival, and function of neurons, including several forms of synaptic plasticity (Lewin and Barde, 1996; Huang and Reichardt, 2001). Four different types have been described in the mammalian brain: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3), and neurotrophin 4 (NT4, also named NT5 or NT4/5). All of them can be transcribed in neuronal and glial cells and are synthesized as precursors forms (pro-neurotrophins) that will be proteolytically cleaved to generate their mature form (Mowla et al., 2001). The biological effects of both neurotrophin forms are mediated by two types of cell surface receptor: the tropomyosin related kinase (Trk) receptor, which includes TrkA, TrkB and TrkC, and the p75 neurotrophin receptor ($p75^{NTR}$) (Chao, 2003). Whereas all mature neurotrophins and pro-neurotrophins (with higher affinity) can interact with $p75^{NTR}$, each Trk receptor has specific binding affinity for particular neurotrophins in its mature form: NGF is the preferred ligand for TrkA, BDNF and NT4 for TrkB, and NT3 for TrkC (although it can also interact with TrkA and TrkB) (Figure 16). Signaling through Trk receptors has generally been associated with intracellular pathways that elicit trophic functions, whereas $p75^{NTR}$ might play a dual and opposing role, interacting with Trks to modulate their signaling or independently triggering trophic or degenerative signals, depending on multiple factors (Roux and Barker, 2002). Thus, the specific spatio-temporal expression and trafficking of neurotrophins and their receptors in different types of neurons are key to fine-tune a wide range of neuronal functions.

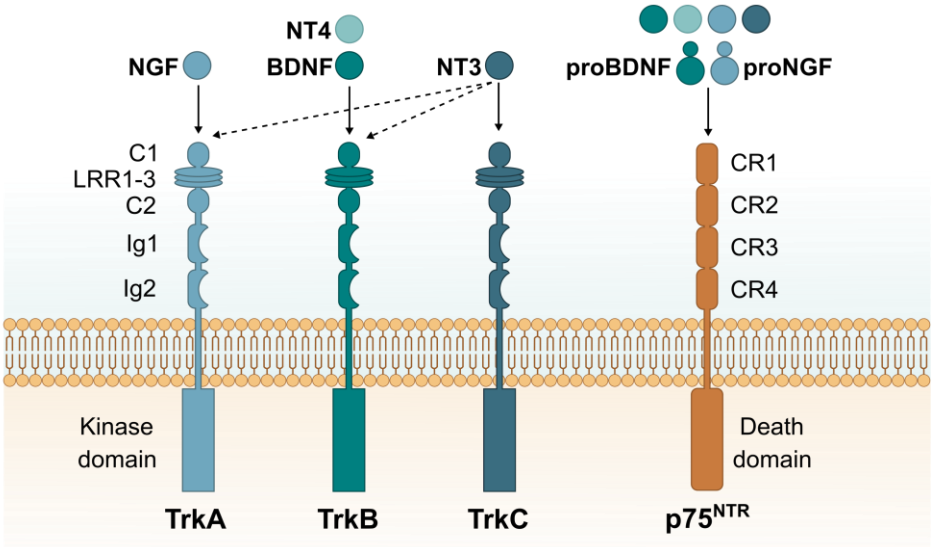



**Figure 16. Neurotrophins and their receptors.** Schematic diagram showing all types of mature and immature neurotrophins and their receptor binding. NGF binds with high affinity to TrkA, NT4 and BDNF to TrkB and NT3 to TrkC. Additionally, NT3 could bind with low affinity to TrkA or TrkB, based on cellular context. All mature neurotrophins bind with low affinity to $p75^{NTR}$, while proBDNF and proNGF bind with high affinity. Domain structure of neurotrophin receptors is pictured as in (Huang and Reichardt, 2003). C: cysteine-rich cluster. LRR: leucine-rich repeat. Ig: immunoglobulin-like domain. CR: cysteine-rich repeat.

Importantly, the presence of mHtt has shown to disrupt neurotrophin signaling in several brain regions, probably due to alterations at different cellular levels, such as transcriptional, vesicle

transport and synaptic transmission deficits. The resulting neurotrophin signaling dysfunction is considered a critical contributor to the global defects in neuroplasticity and to the specific vulnerability of MSNs in HD (Zuccato and Cattaneo, 2007; Brito et al., 2013; Simmons, 2017). Essentially, the mechanisms causing this impairment can be divided into those that affect the availability of neurotrophins and those that alter the activity or expression of its receptors

## 2.3.1. Mechanisms of neurotrophin signaling dysfunction

**Lack of neurotrophic support: role of BDNF**

Like other neurotrophic factors, BDNF plays a key role in regulating neuronal survival (Huang and Reichardt, 2001). Additionally, BDNF is the only neurotrophin certainly secreted in an activity-dependent manner and therefore has been recognized as a key regulator of synapse formation and plasticity (Poo, 2001; Lu, 2003). Although also expressed in peripheral tissues (Lommatzsch et al., 1999), it is widely distributed within the brain, with the cortex, hippocampus and cerebellum presenting highest levels of the transcript and the protein (Hofer et al., 1990; Salin et al., 1995). Conversely, BDNF mRNA in the striatum is almost absent, although protein levels are high (Salin et al., 1995; Spires et al., 2004b). Thus, it has been demonstrated that most striatal BDNF content comes by anterograde transport from cortical afferents and, to a lesser extent, from dopaminergic neurons of the substantia nigra (Altar et al., 1997; Altar and DiStefano, 1998; Baquet et al., 2004).

Importantly, reductions in BDNF mRNA or protein levels have been observed in the brains of HD patients and HD models. In HD patients, protein levels were found decreased in the caudate nucleus, putamen, and cortex and mRNA levels were also reduced in the cortex (Ferrer et al., 2000; Zuccato et al., 2001, 2008), while in genetic HD mouse models, decreases in mRNA or protein levels of BDNF have been found in cortex, striatum, hippocampus and cerebellum (Zuccato et al., 2001, 2005; Luthi-Carter et al., 2002; Duan et al., 2003; Ginés et al., 2003b; Hermel et al., 2004; Spires et al., 2004b; Ma et al., 2015). These reductions have been attributed to transcriptional and vesicular transport impairments elicited by mHtt (Zuccato et al., 2001; Gauthier et al., 2004). As previously mentioned, studies have shown that wild-type Htt, but not the mutant form, facilitates BDNF mRNA production by retaining the REST/NRSF repressor complex in the cytoplasm (Zuccato et al., 2003, 2007). Thus, the presence of a mutated *Htt* allele in HD leads to the nuclear accumulation of REST/NRSF, which represses BDNF transcription (among other genes). Indeed, increased DNA binding of REST/NRSF has been confirmed in HD cells, animal models, and post-mortem HD brains (Zuccato et al., 2007). Additionally, mHtt could also impair BDNF expression by altering the activity of other transcription factors that bind to BDNF promoter, such as CREB or CBP, or by altering chromatin structure (Zuccato et al., 2010). Importantly, the reduction in BDNF mRNA levels in the cortex of HD patients (Zuccato et al., 2008) would cause a lack of supply to striatal neurons, affecting their function and viability. Apart from modulating BDNF transcription, it was demonstrated that wild-type Htt interacts with dynein (Caviston et al., 2007) and with Huntingtin-associated protein 1 (HAP1), which in turn interacts with dynactin and kinesin (Engelender et al., 1997; McGuire et al., 2006), thus controlling vesicular transport along axons. The presence of mHtt in cortical neurons might prevent an efficient axonal transport of

BDNF-containing vesicles, strongly affecting cortical BDNF supply to the striatum (Gauthier et al., 2004).

**Neurotrophin receptor imbalance: role of TrkB/p75$^{NTR}$**

The biological effects of BDNF can be mediated by its interaction with the transmembrane receptors TrkB and p75$^{NTR}$. In the striatum, TrkB is highly expressed in MSNs, together with TrkC (Merlio et al., 1992). A recent study has shown that MSNs in the indirect pathway have preferred expression of TrkB than those in the direct pathway (Baydyuk et al., 2011). By contrast, TrkA mRNA is mainly expressed by large cholinergic striatal interneurons, which would depend on NGF signaling (Holtzman et al., 1992). Classically, three major complex signaling cascades are described to be activated upon BDNF binding to TrkB (and upon other types of neurotrophin-Trk binding), which include the PI3K/Akt, MAPK/ERK (also known as the Ras-Raf-MEK-ERK pathway), and PLCγ/PKC pathways (Figure 17) (Huang and Reichardt, 2003; Minichiello, 2009). Particularly, BDNF homodimers bind to pre-formed or neurotrophin-induced dimeric TrkB (Shen and Maruyama, 2012), triggering the autophosphorylation of the receptor on tyrosine residues located in the intracellular kinase domain. This autophosphorylation, in turn, leads to the phosphorylation of tyrosine residues in the juxtamembrane or C-terminal sites that would interact with adaptor molecules. On one hand, the phosphorylation on tyrosine residue 515, located in the juxtamembrane region of TrkB, recruits Shc adaptor molecules that would activate the PI3K/Akt and MAPK/ERK signaling pathways, promoting neuronal differentiation, survival and growth. On the other hand, the phosphorylation on tyrosine residue 816 (in mice, tyrosine 817 in humans) in C-terminal region causes PLCγ binding, triggering its activation by phosphorylation, promoting cell survival and synaptic plasticity (Minichiello, 2009). BDNF signaling through TrkB could also be modulated by the action of p75$^{NTR}$, which has been shown to interact with all Trks (Bibel et al., 1999), although the molecular details and functional consequences of these interactions in each specific cellular context are still being defined (Roux and Barker, 2002). Studies have shown that activation of TrkA by NGF can be enhanced by p75$^{NTR}$ (Barker and Shooter, 1994; Verdi et al., 1994), while controversial results are observed regarding p75$^{NTR}$ modulation of BDNF-mediated TrkB signaling (Bibel et al., 1999; Vesa et al., 2000).

In contrast to Trks, which are greatly expressed in the adult nervous system, p75$^{NTR}$ is widely downregulated after development, expressed at low but functional levels in multiple brain regions, including the striatum (Henry et al., 1994; Brito et al., 2013). However, its transcription appears to be upregulated upon several pathological conditions, often promoting neuronal death and dysfunction (Dechant and Barde, 2002; Longo and Massa, 2008; Ibáñez and Simi, 2012; Meeker and Williams, 2014). All mature neurotrophins, including BDNF, bind p75$^{NTR}$ with low affinity, while their respective pro-forms bind with high affinity (Chao, 2003). Additionally, p75$^{NTR}$ can interact with many transmembrane proteins, apart from Trks, being sortilin and Nogo receptors the most widely studied (Wang et al., 2002; Lu et al., 2005; Teng et al., 2005). p75$^{NTR}$ lacks known intrinsic enzymatic activity for autophosphorylation, but upon ligand binding through dimeric p75$^{NTR}$, which can be either preformed or induced by neurotrophin binding (Vilar et al., 2009), signaling occurs through association with many cytoplasmic adaptor proteins that initiate different

signaling cascades, which include: ceramide, NF-kB, JNK, PTEN, and RhoA/ROCK pathways (Figure 17) (Dechant and Barde, 2002; Pramanik et al., 2017). As a result, $p75^{NTR}$ is able to modulate a wide variety of functions, including survival, apoptosis, neuroinflamation or synaptic failure (among others), depending on multiple factors, such as the availability of co-receptors and adaptor molecules. Interestingly, specific conditions in the adult brain or in cell cultures have been shown to activate $p75^{NTR}$ neurodegenerative pathways, such as a deficiency in neurotrophins and/or Trks accompanied by increased $p75^{NTR}$ levels (Yoon et al., 1998; Fortress et al., 2011; Brito et al., 2013).

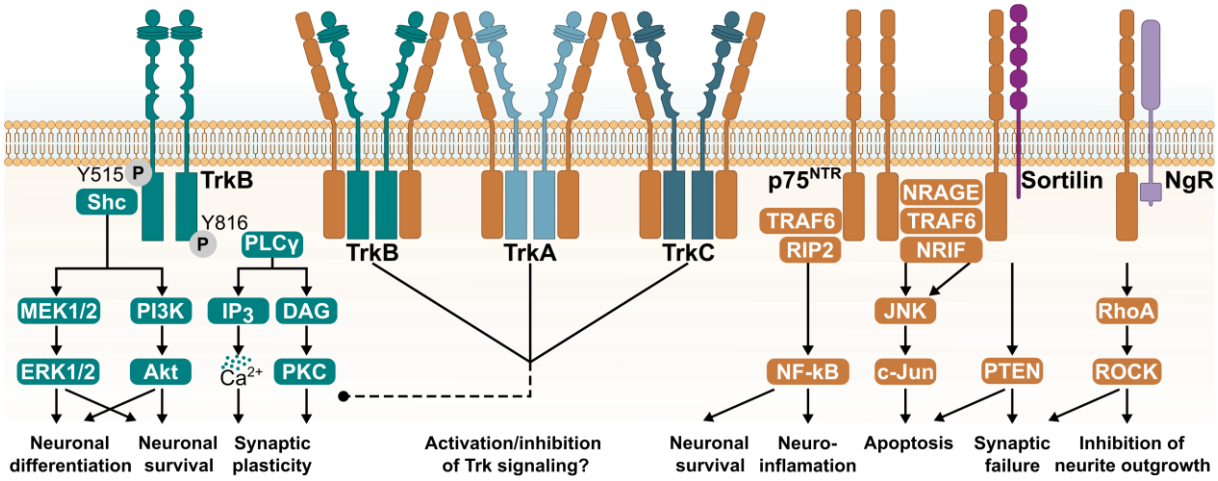



**Figure 17. Signal transduction pathways activated by TrkB and $p75^{NTR}$ receptors.** Simplified diagram showing main signaling pathways triggered upon ligand binding to TrkB or $p75^{NTR}$ (or $p75^{NTR}$ co-receptors). NgR: nogo receptor. Adapted from (Pramanik et al., 2017).

In the context of HD, total protein and mRNA levels of TrkB have been found reduced in the striatum of HD patients and in different mouse and cellular models (Ginés et al., 2006, 2010; Zuccato et al., 2008; Brito et al., 2013; Simmons et al., 2013). Moreover, additional studies have uncovered an important impairment in TrkB signaling that is suggested to occur independently from alterations in BDNF or TrkB levels (Ginés et al., 2010; Liot et al., 2013; Plotkin et al., 2014; Nguyen et al., 2016). Conversely, analysis of $p75^{NTR}$ expression in HD patients have found increased mRNA expression in the caudate nucleus (Zuccato et al., 2008) and increased protein levels in the hippocampus (Brito et al., 2014). Additionally, previous studies from our group demonstrated increased $p75^{NTR}$ protein levels in the striatum and increased $p75^{NTR}$ mRNA and protein levels in the hippocampus of transgenic and knock-in mouse models (Brito et al., 2013, 2014). Signaling through $p75^{NTR}$ was also found increased in the indirect pathway MSNs of the BACHD model (Plotkin et al., 2014). These results indicate that, in addition to the reduction in BDNF levels, there is also an unbalanced neurotrophic receptor signaling in HD. Few studies have tried to decipher the molecular mechanisms responsible for these alterations. In this regard, mHtt has been implicated in an impairment of the retrograde TrkB transport from striatal dendrites to cell bodies, affecting TrkB signaling upon BDNF stimulation (Liot et al., 2013), and enhanced Sp1 transcription has been suggested to contribute to increased $p75^{NTR}$ mRNA levels in the hippocampus of HdhQ111 mice and HD patients (Brito et al., 2014).

### 2.3.2. Neurotrophin receptor signaling modulation as a therapeutic strategy for HD

It is established that MSNs strongly depend on BDNF neurotrophic support for their survival and correct activity (Ivkovic and Ehrlich, 1999; Baquet et al., 2004). Particularly, one study demonstrated that genetically modified mice with a chronic reduction of forebrain BDNF levels displayed HD-like motor abnormalities after 4 months and presented striatal dendritic spine alterations as early as 1 month, with some degree of neuronal loss after 1 year (Baquet et al., 2004). Moreover, the overexpression of BDNF, as well as NT3 and NT4, have shown to be neuroprotective in the striatum of a murine excitotoxic model of HD (Pérez-Navarro et al., 1999, 2000), and experiments in cultured cells showed that BDNF confers neuroprotection against mutant huntingtin-induced apoptosis (Saudou et al., 1998; Zala et al., 2005). Additionally, the genetic downregulation of BDNF in transgenic R6/1 mice has shown to advance the onset of motor dysfunction and produce more severe motor coordination deficits, worsening neuropathological features (Canals et al., 2004) while genetic overexpression of BDNF or its delivery via pumps, gene therapy, stereotaxic injections or stem cell vectors partially improved these outcomes in several HD models (Cho et al., 2007; Gharami et al., 2008; Xie et al., 2010; Arregui et al., 2011; Giralt et al., 2011a; Giampà et al., 2013; Silva et al., 2015). Besides its contribution to neuronal function and survival within the striatum, BDNF is known to play an essential role in synaptic plasticity and memory processes that depend on the hippocampus. Indeed, reduction of hippocampal BDNF levels in HD mice has been associated with alterations in functional and structural synaptic plasticity that underlie learning and memory impairments occurring early in the disease (Lynch et al., 2007; Giralt et al., 2009). All these results clearly indicate that lack of BDNF support plays a pivotal role in the pathogenesis of HD and that increasing BDNF levels might provide important benefits. Interestingly, low brain BDNF levels have also been found in other more prevalent neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease and multiple sclerosis, although the mechanism underlying this reduction are not clear (Murer et al., 2001; Sohrabji and Lewis, 2006; Zuccato and Cattaneo, 2009). All these findings have stimulated an increasing interest for the development of experimental approaches aimed to boost BDNF levels in the brain. Unfortunately, therapeutic strategies based on BDNF-delivery are strongly limited by the complex pharmacokinetic properties of the protein. Particularly, BDNF presents a low propensity to cross the blood-brain barrier following systemic treatment, has a short *in vivo* half-life and displays limited diffusion in the brain parenchyma following intraventricular infusion (Zuccato and Cattaneo, 2009).

The serious limitations of BDNF protein-based therapies prompted the evaluation of small-molecule ligands for TrkB. A compound termed 7,8-Dihydroxyflavone (7,8-DHF) was identified as a putative TrkB agonist and was shown to ameliorate motor and cognitive impairments and some neuropathological features in HD mouse models (Jiang et al., 2013; Garcia-Diaz Barriga et al., 2017), while another TrkB agonist, themed LM22A-4, further confirmed similar benefits in HD mice (Simmons et al., 2013). However, when designing therapeutic strategies in HD based on BDNF-TrkB signaling, it is of great importance to take into consideration the overall TrkB/$p75^{NTR}$ signaling imbalance since, as previously mentioned, neurodegenerative pathways could be activated

by $p75^{NTR}$ in conditions in which Trk levels are low and $p75^{NTR}$ levels are elevated. Indeed, studies from our group showed that the imbalance between TrkB and $p75^{NTR}$ receptors disrupt BDNF neurotrophic effects in striatal neurons *in vitro* (Brito et al., 2013). Consistently, beneficial effects have been obtained when $p75^{NTR}$ was modulated in HD. In BACHD mice, the gene silencing or pharmacological inhibition of $p75^{NTR}$ (with the peptide called pep5) or the inhibition of its downstream signaling molecules PTEN or ROCK were able to rescue corticostriatal synaptic plasticity (LTP impairments), particularly at the indirect pathway MSNs (Plotkin et al., 2014). Additionally, our group has previously demonstrated that the genetic normalization of $p75^{NTR}$ in HD mice hippocampus prevented memory deficits and ameliorated structural and functional synaptic plasticity alterations in this brain region (Brito et al., 2014). Very recently, a small molecule $p75^{NTR}$ ligand, themed LM11A-31, was shown to downregulate $p75^{NTR}$-related degenerative signaling and up-regulate trophic signaling, ameliorating some neuropathological features in R6/2 and BACHD mice (Simmons et al., 2016). However, the contribution of the drug to HD-related motor behavior was not clear. In R6/2 mice treated with LM11A-31 from 4 to ~11 weeks, distance travelled, velocity and rearing duration in an activity chamber was significantly increased when measured at 7 weeks of age, but motor balance and coordination deficits measured in the accelerating rotarod at 10 weeks were not altered. Additionally, when LM11A-31 was administered to BACHD mice from 2 to 9 months, it was shown to improve motor performance at the accelerating rotarod at 3 months of age but not at 5 or 7 months. Thus, despite promising results, the precise contribution of $p75^{NTR}$ increased signaling to the onset and progression of motor deficits need to be explored in further detail. For this reason, in this thesis we have evaluated the effect of $p75^{NTR}$ reduction in HD striatal pathology and motor-behavior abnormalities along disease progression.

# AIMS

Despite the simple genetic etiology of HD, the fundamental aspects of the molecular processes leading to neuronal dysfunction and death are incredibly complex and remain largely unknown, which has hindered the development of effective disease-modifying therapies for years. Thus, current treatments only alleviate specific signs and symptoms without preventing the inevitable neuropathological progression of this severely debilitating disease.

In this context, the general goal of this thesis is to provide a better understanding of the therapeutic potential of HDAC3 and $p75^{NTR}$ as targets for pharmacological intervention in HD. To fulfill this, we aim to accomplish the following aims:

1. To analyze the contribution of HDAC3 to the transcriptional impairments underlying cognitive deficits and to the somatic CAG repeat expansions involved in the increased striatal vulnerability in HD.

2. To analyze the contribution of increased $p75^{NTR}$ levels to the onset and progression of motor abnormalities and the underlying striatal neuropathology in HD.

# METHODS

# 1. Animals

HdhQ111 knock-in mice (Lloret et al., 2006), presenting a targeted insertion of 109 CAG repeats that extends the glutamine segment in murine huntingtin to 111 residues, were used as a model of HD (see Introduction Section 1.4.3.). $Hdh^{Q7/Q7}$ wild type (WT) mice were cross-mated with $Hdh^{Q7/Q111}$ knock-in (KI) mice to generate age-matched WT and KI littermates. In order to study $p75^{NTR}$ function in HD, heterozygous $p75^{NTR}$/Exon III ($p75^{+/-}$) mice, which were originally obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and present one functional copy of *p75*$^{NTR}$ allele, were cross-mated with KI mice to obtain WT, KI, $p75^{+/-}$ and KI:$p75^{+/-}$ littermates. All mice were maintained on a C57BL/6 genetic background. Different genotypes were determined by PCR analysis. After genotyped, microchips were implanted into all mice to identify them by a reference number that provided information about their birth, location and genotype data. For all experiments, only males from each genotype were used in order to avoid oestrus hormonal alterations. Mice were housed with access to food and water ad libitum in a colony room kept at 19-22 ºC and 40-60% humidity, under a 12:12 hours light/dark cycle.

All procedures involving animals were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approved by the local animal care committee of the Universitat de Barcelona (99/01) and the Generalitat de Catalunya (00/1094), in accordance with the European (2010/63/EU) and Spanish (RD53/2013) regulations for the care and use of laboratory animals.

## 1.1. Pharmacological treatments *in vivo*

The HDAC3 inhibitor RGFP966 was generously provided by BioMarin Pharmaceutical Inc. (San Rafael, CA, USA). For evaluation of behavior, biochemical parameters and CAG repeat expansions, WT and KI mice were subcutaneously injected with 50 mg/kg of RGFP966 or vehicle (70% polyethylene glycol 200; 30% acetate buffer) three times per week from 3 to 6.5 months (chronic treatment). At 6 months of age (during treatment), mice performed motor learning (accelerating rotarod) and memory (OLT and NORT) tests. Habituation days of the OLT/NORT tasks were used as an open field test to confirm the lack of motor alterations. At about 6.5 months of age, mice were sacrificed by cervical dislocation 1 hour after final injection and brains were removed and dissected for Western blot and CAG repeat instability analysis (Figure 18). Animal body weight was recorded along treatment in order to identify any possible side effect.

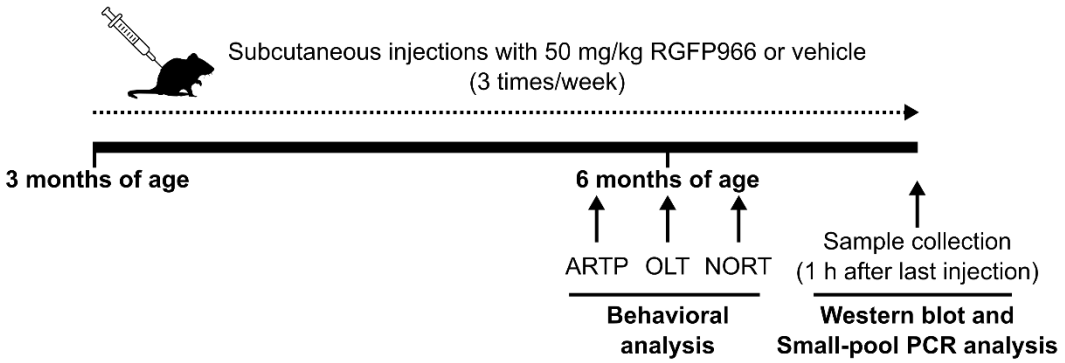


**Figure 18. Schematic of experimental plan for chronic treatment.** RGFP966 or vehicle was administered subcutaneously to WT and KI mice three times per week for 13 weeks starting at three months of age, prior to development of cognitive defects. At 6 months of age mice were analyzed for cognitive behavior. ARTP: accelerating rotarod task procedure; OLT: object location task; NORT: novel object recognition task. Mice were sacrificed at the end of treatment, 1 hour (h) after last injection and brains were removed and dissected for Western blot and CAG repeat (Small-pool PCR) analysis.

For analysis of activity-dependent genes, 7-month-old WT and KI mice were subcutaneously injected once a day with 25 mg/kg of RGFP966 or vehicle for 3 consecutive days (acute treatment). On day 3, mice received a 5-minute training in an environment with two identical objects to induce expression of activity-dependent memory genes. Training was immediately followed by last injection of RGFP966. Mice were sacrificed 2 hours later and their brains removed. One hemisphere of each brain was dissected for gene expression analysis and the other one was fixed in 4% paraformaldehyde (PFA) for immunohistochemistry analysis (Figure 19).

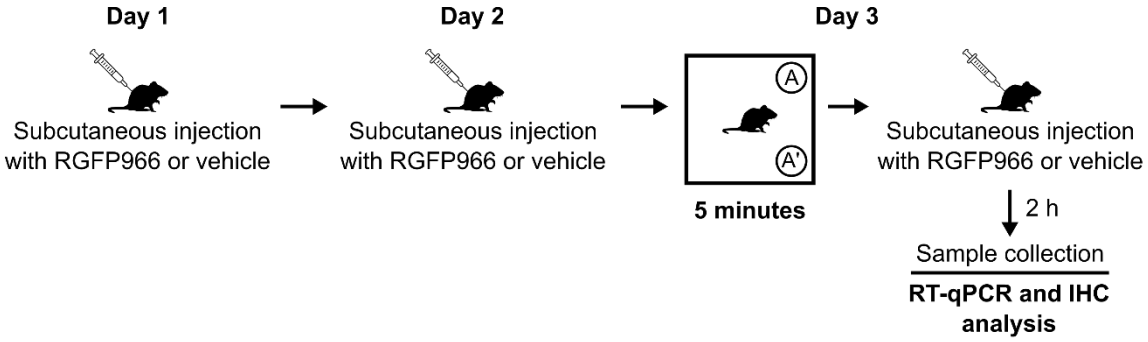



**Figure 19. Schematic of experimental plan for acute treatment.** RGFP966 or vehicle was administered subcutaneously to WT and KI mice once a day for 3 consecutive days at 7 months of age. On the last day, mice were trained for 5 minutes in an environment with two identical objects, followed immediately by last injection. 2 hours (h) later, mice were sacrificed and brains were removed. One hemisphere of each brain was dissected for gene expression analysis (RT-qPCR) and the other one was fixed for immunohistochemistry (IHC) analysis.

# 1.2. Behavioral assessment

## 1.2.1. Evaluation of cognitive functions

As previously mentioned, cognitive functions were evaluated in 6-months-old WT and KI mice chronically treated with RGFP966 or vehicle. Motor learning was assessed by the accelerating rotarod task while spatial and recognition long-term memory was assessed by the object location task (OLT) and the novel object recognition task (NORT), respectively. Protocols were performed as previously described (Brito et al., 2014).

### Accelerating rotarod training procedure

Animals were placed on a motorized rod (30 mm diameter) and the rotation speed was gradually increased from 4 to 40 rpm over the course of 5 minutes. Latency to fall, which corresponds to the time the animal was able to keep up with the increasing speed before it fell, was recorded. Accelerating rotarod training procedure training/testing was performed four times per day for three consecutive days. Animals rested a minimum of 1 hour between trials to avoid fatigue. The apparatus was rigorously cleaned between animal trials in order to avoid odors.

**OLT/NORT**

Exploration took place in a white open-top arena with a quadrangular form (45 × 45 cm sides, 40 cm high). The light intensity was 40 lux throughout the arena, and the room temperature was kept at 19-22 ºC, with a humidity of 40-60%. Mice were first habituated to the arena in the absence of objects (2 days, 15 minutes/day). During habituation, spontaneous locomotor activity and time/distance in the periphery/center were measured in order to ensure an absence of stress before starting the task. On the third day (OLT training), spatial clues were attached to the walls of the arena and 2 duplicate objects (A and A') were placed in two adjacent corners of the arena. Mice were allowed to explore for 10 min. 24 hours later (OLT testing), mice were placed in the experimental apparatus for 10 minutes, where one copy of the familiar object (A) was placed in the same location as during the training trial, and one copy of the familiar object (A'') was placed in the corner diagonally opposite. 48 hours after OLT testing, spatial clues were eliminated and 2 duplicate objects completely different from OLT test (B and B') were placed in two adjacent corners of the arena. Mice were allowed to explore for 10 min (NORT training). 24 hours later (NORT testing), mice were placed in the experimental apparatus for 10 minutes, where one copy of the familiar object (B) and a new object (C) were placed in the same location as during the training trial. The arena was rigorously cleaned between animal trials in order to avoid odors. Animals were tracked and recorded with SMART Junior software. Exploration times were recorded and used to calculate the Discrimination index as follows:

$$\frac{\textit{time exploring novel or relocated object} - \textit{time exploring familiar object}}{\textit{total time exploring both objects}} \times 100$$

Discrimination indices of 0 indicate equal exploration of both objects.

## 1.2.2. Evaluation of motor function

To evaluate motor function, motor coordination and balance were assessed by the fixed rotarod test and spontaneous locomotor activity was measured by the open field test.

**Fixed rotarod**

The fixed rotarod test was performed on a motorized rod (30 mm diameter) as described elsewhere (Canals et al., 2004). WT, $p75^{+/-}$, KI and KI:$p75^{+/-}$ mice (5-6 months old) were trained at constant speed (10 rpm) for 60 seconds. Two trials per day for three consecutive days were performed, and the latency to fall and the number of falls during 60 seconds was recorded to ensure complete learning of the task. After training, and starting at 7 months of age, mice were evaluated once a month at 10 and 24 rpm until 12 months of age, and the number of falls in a total of 60 seconds was recorded. Mice were put on the rotarod several times until the total latency to fall reached 60 seconds.

**Open field**

A white open-top arena with a quadrangular (45 × 45 cm sides, 40 cm high; for Results Section 1.2.1.) or circular (40 cm diameter, 40 cm high; for Results Section 2.2.1.) form was used. The light intensity was 40 lux throughout the arena, and the room temperature was kept at 19-22 ºC, with a

humidity of 40-60%. 6-months-old WT and KI mice chronically treated with RGFP966 (or vehicle) or 8-/10-months-old WT, $p75^{+/-}$, KI and KI:$p75^{+/-}$ mice were placed into the arena during two consecutive days (15 min/day), and spontaneous locomotor activity was measured as total distance traveled. The arena was rigorously cleaned between animals in order to avoid odors. Animals were tracked and recorded with SMART Junior Software.

# 2. Human samples

Post-mortem putamen samples from control and confirmed HD cases were provided by Neurological Tissue Bank of the Biobank-Hospital Clínic-Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS; Barcelona, Catalonia) following the guidelines and approval of the local ethics committee (Hospital Clínic of Barcelona's Clinical Research Ethics Committee). Details from human subjects is provided in Table 2.

**Table 2. Characteristics of the HD cases and controls included in analysis**. For each individual, a codified registration number is provided as well as information about the gender, age (years) and post-mortem delay (PMD, hours). For HD patients, the number of CAG repeats is also provided. Among all individuals, 8 are histopathologically non-related to HD (control) and 8 are HD cases.

| REGISTRATION NUMBER | TYPE | CAG REPEATS | GENDER | AGE | PMD |
|---|---|---|---|---|---|
| **CS-0810** | Control | - | Female | 81 | 23.5 |
| **CS-1491** | Control | - | Male | 83 | 13 |
| **CS-1563** | Control | - | Male | 79 | 5 |
| **CS-1570** | Control | - | Female | 86 | 4 |
| **CS-1679** | Control | - | Female | 90 | 12.3 |
| **CS-1697** | Control | - | Male | 78 | 6 |
| **CS-1733** | Control | - | Male | 76 | 11.5 |
| **CS-1774** | Control | - | Female | 74 | 5 |
| **CS-1120** | HD Vonsattel grade 3 | 48 | Male | 55 | 15 |
| **CS-1193** | HD Vonsattel grade 3-4 | - | Male | 55 | 7 |
| **CS-1294** | HD Vonsattel grade 3 | 45+/- 2 | Male | 53 | 7 |

| | | | | | |
|---|---|---|---|---|---|
| **CS-1334** | HD Vonsattel grade 1 | 40+/- 2 | Male | 73 | 7 |
| **CS-1438** | HD Vonsattel grade 3 | 40 | Male | 85 | 5.5 |
| **CS-1630** | HD Vonsattel grade 2 | 41 | Male | 76 | 6 |
| **CS-1638** | HD Vonsattel grade 2 | - | Male | 72 | 13.15 |
| **CS-1758** | HD Vonsattel grade 2-3 | 42+/- 2 | Male | 68 | 6.15 |

# 3. Neuronal cultures

## 3.1. Primary neuronal cultures

Primary hippocampal and striatal cultures were obtained from WT and KI embryos at day 18.5 of embryonic development. Genotypes of embryos were determined by PCR analysis by using tail DNA. Dissociated cultures were plated at a density of 400,000 neurons/well onto 6-well plates precoated with 0.1 mg/ml poly-D-lysine (Sigma-Aldrich; St. Louis, MO, USA). Neurons were cultured in Neurobasal medium (Gibco-BRL; Renfrewshire, Scotland, UK), supplemented with B27 (Gibco-BRL) and Glutamax™ (Gibco-BRL) and were maintained at 37 ºC in a humidified atmosphere containing 5% CO2.

## 3.2. Immortalized striatal cultures

Conditionally immortalized wild-type STHdh$^{Q7/Q7}$ and mutant STHdh$^{Q111/Q111}$ striatal neuronal progenitor cell lines expressing endogenous levels of normal and mutant huntingtin with 7 and 111 glutamines, respectively, were used (Trettel et al., 2000). Cells were grown at 33 ºC in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate and 400 μg/ml G418 (Geneticin; Gibco-BRL, Renfrewshire, Scotland, UK).

## 3.3. Pharmacological treatments *in vitro*

After 10-14 DIV, primary hippocampal or striatal cultures from WT or KI embryos were treated with vehicle (DMSO) or RGFP966 (10 μM) for 6 hours. Additionally, primary hippocampal cultures were treated with 50 mM KCl for the last 2 hours in order to induce neuronal stimulation. Subsequently, cells were harvested for Western blot analysis.

Wild-type STHdh$^{Q7/Q7}$ and mutant STHdh$^{Q111/Q111}$ striatal cells were treated with vehicle (DMSO) or RGFP966 (10μM or 1 μM) for 6 hours or 24 hours prior to harvesting for Western blot or immunoprecipitation analysis.

## 3.4. AAV transduction

For $p75^{NTR}$ overexpression, the plasmid pEGFP-N3-p75 obtained from E. Formaggio (Department of Medicine and Public Health, Pharmacology Section, University of Verona, Verona, Italy) was cloned into a rAAV2/8-GFP adenoviral vector (BamHI site at 5' and AgeI at 3'). Infectious AAV viral particles containing GFP expression cassette alone (AAV-GFP) or fused to the $p75^{NTR}$ sequence (AAV-GFP-p75) were generated by the Unitat de Producció de Vectors from the Center of Animal Biotechnology and Gene Therapy at the Universitat Autònoma de Barcelona. Primary striatal cultures from WT or KI embryos at 3 DIV were transduced with AAV-GFP or AAV-GFP-p75 at 50,000 GC/cell. At DIV14, expression of GFP was visualized with an inverted microscope to confirm efficient transduction and neurons were subsequently harvested for Western blot analysis.

# 4. Protein extraction

## 4.1. Extraction from mouse and human brain samples

Immediately after the sacrifice of the mice by cervical dislocation, brains were harvested and tissue samples were dissected on a thick glass slide placed on ice and quickly stored in dry ice and then at -80ºC. To obtain protein extracts, frozen mouse and human brain tissues were homogenized by sonication in ice-cold lysis buffer containing: 20 mM Tris base (pH 8.0), 150 mM NaCl, 50 mM NaF, 1% NP-40, 10% glycerol (for mouse samples) or 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100 and 10 mM EGTA (for human samples), supplemented with 1mM sodium orthovanadate and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Samples were centrifuged at 16,100 g for 15 minutes at 4 ºC, and supernatants were collected.

## 4.2. Extraction from neuronal cultures

To obtain protein extracts, cultures were rinsed once with PBS and then incubated with ice-cold lysis buffer (50 mM Tris base [pH 7.5], 150 mM NaCl, 2 mM EDTA, 1% NP-40, supplemented with 1mM sodium orthovanadate and protease inhibitor cocktail). Cellular lysates were collected from the wells by using a scraper and further disaggregated by pipetting the samples up and down using an insulin syringe. Then, all samples were centrifuged at 16,100 g for 10 min at 4 ºC, and supernatants were collected.

# 5. Subcellular fractionation

To obtain cytosolic and nuclear fractions, mouse striatal tissue was homogenized in a fractionation buffer (4 mM HEPES [pH 7.4], 0.32 M sucrose, 0.5 mM EDTA, supplemented with 1mM sodium orthovanadate and protease inhibitor cocktail) by pipetting the samples up and down first with a P1000 pipette, then with a P200 pipette and finally with an insulin syringe until achieving complete homogenization. A small fraction of the suspension was stored at -80 ºC and designated as the

whole cell extract and the rest was centrifuged at 800 g for 10 minutes at 4 ºC. The supernatant was collected and centrifuged again at 10,000 g for 15 minutes at 4 ºC, and the new generated supernatant was designated as the cytosolic fraction and was kept at -80 ºC until use. The pellet from first centrifugation, containing the nuclei, was resuspended in fractionation buffer and centrifuged at 800 g for 10 minutes at 4 ºC. The resulting pellet, containing washed nuclear fraction, was then resuspended in lysis buffer (20 mM Tris base [pH 8.0], 150 mM NaCl, 50 mM NaF, 1% NP-40, 10% glycerol, supplemented with 1mM sodium orthovanadate and protease inhibitor cocktail) and homogenized by sonication. Finally, after centrifugation at 16,100 g for 10 minutes at 4 ºC, the supernatant was designated as the nuclear fraction and was collected and stored at -80 °C until use.

# 6. Western blot

Determination of the protein content was performed using the Detergent-Compatible Protein Assay (Bio-Rad, Hercules, CA, USA). Protein extracts (typically 20 μg) were mixed with SDS sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% glycerol, 140 mM β-mercaptoethanol and 0.1% (w/v) bromophenol blue, and boiled at 100 ºC for 5 minutes. Then, samples were resolved on denaturing polyacrylamide gels (SDS-PAGE) with different polyacrylamide concentrations, depending on the protein of interest (6-12%), at 30 mA/gel during approximately 1 hour. The Precision Plus Protein™ Dual Color ladder (Bio-Rad) was used to ensure the correct size of the proteins of study. Proteins were then transferred to nitrocellulose membranes (Whatman Schleicher & Schuell, Keene, NH, USA) during 1-1.5 hours at 100 V at 4 ºC. Membranes were reversibly stained with Ponceau S reagent for ~30 seconds to confirm protein transfer, and after de-staining with Tris-buffered saline, 0.1% Tween 20 (TBS-T), nonspecific protein binding sites were blocked in 10% non-fat powdered milk in TBS-T during 1 hour at room temperature. Membranes were washed 3 times for 10 min each with TBS-T and incubated overnight at 4 °C with primary antibodies (Table 3). Actin, histone H3, lamin A/C and α-tubulin antibodies were incubated at room temperature for 0.5-1 hour.

**Table 3. Primary antibodies used for Western blot.** Information about the molecular weight (MW), host specie, dilution used and source is provided.

| ANTIGEN | MW (kDa) | HOST SPECIE | DILUTION | SOURCE |
|---|---|---|---|---|
| 1C2 (Anti-Polyglutamine-expansion) | - | Mouse | 1:1000 | Merck Millipore (Billerica, MA, USA) |
| A2AR | 45 | Mouse | 1:500 | Santa Cruz Biotechnology (Santa Cruz, CA, USA) |
| Acetyl-histone H3 (K9) | 17 | Rabbit | 1:1000 | Cell Signaling Technology (Beverly, MA, USA) |
| Acetyl-lysine | - | Mouse | 1:500 | Cytoskeleton (Denver, CO, USA) |
| Acetyl-Msh2 (K73) | 105 | Rabbit | 1:500 | Gift from Dr. Edward Seto (George Washington |

| | | | | |
|---|---|---|---|---|
| | | | | University, Washington DC, USA) |
| Actin | 42 | Mouse | 1:20,000 | MP Biochemicals (Aurora, OH, USA) |
| Akt | 60 | Mouse | 1:1000 | Cell Signaling Technology (Beverly, MA, USA) |
| Arc | 55 | Mouse | 1:500 | Santa Cruz Biotechnology (Santa Cruz, CA, USA) |
| BDNF | 14 (mature form) | Rabbit | 1:1000 | Santa Cruz Biotechnology (Santa Cruz, CA, USA) |
| BDNF | 14 (mature form) | Mouse | 1:1000 | Icosagen (Tartu, Estonia) |
| c-Fos | 62 | Rabbit | 1:1000 | Santa Cruz Biotechnology (Santa Cruz, CA, USA) |
| c-Jun | 39 | Mouse | 1:1000 | Santa Cruz Biotechnology (Santa Cruz, CA, USA) |
| CBP (A-22) | 265 | Rabbit | 1:1000 | Santa Cruz Biotechnology (Santa Cruz, CA, USA) |
| CREB | 43 | Rabbit | 1:1000 | Cell Signaling Technology (Beverly, MA, USA) |
| DARPP-32 | 32 | Mouse | 1:1000 | BD Biosciences (Franklin Lakes, NJ, USA) |
| Egr1 | 75 | Rabbit | 1:1000 | Cell Signaling Technology (Beverly, MA, USA) |
| ERK1/2 | 44 (ERK1), 42 (ERK2) | Mouse | 1:1000 | BD Biosciences (Franklin Lakes, NJ, USA) |
| GFP | 27 | Mouse | 1:25,000 | Molecular probes (Eugene, OR, USA) |
| HDAC3 | 49 | Rabbit | 1:1000 | Abcam (Cambridge, UK) |
| Histone H3 | 17 | Rabbit | 1:1000 | Cell Signaling Technology (Beverly, MA, USA) |
| Lamin A/C | 69 (Lamin A), 62 (Lamin C) | Rabbit | 1:2000 | Santa Cruz Biotechnology (Santa Cruz, CA, USA) |
| Msh2 | 105 | Rabbit | 1:2000 | Abcam (Cambridge, UK) |
| $p75^{NTR}$ | > 75 | Rabbit | 1:1000 | Promega (Madison, WI, USA) |
| PDE10A | 90-93 | Rabbit | 1:1000 | Abcam (Cambridge, UK) |
| Phospho-Akt (S473) | 60 | Rabbit | 1:1000 | Cell Signaling Technology (Beverly, MA, USA) |
| Phospho-c-Jun (S63) | 39 | Mouse | 1:1000 | Santa Cruz Biotechnology (Santa Cruz, CA, USA) |

| | | | | |
|---|---|---|---|---|
| Phospho-CREB (S133) | 43 | Rabbit | 1:1000 | Merck Millipore (Billerica, MA, USA) |
| Phospho-ERK1/2 (T202/Y204) | 44 (ERK1), 42 (ERK2) | Rabbit | 1:1000 | Cell Signaling Technology (Beverly, MA, USA) |
| Phospho-HDAC3 (S424) | 49 | Rabbit | 1:1000 | Cell Signaling Technology (Beverly, MA, USA) |
| Phospho-PLCγ1 (Y783) | 155 | Rabbit | 1:1000 | Cell Signaling Technology (Beverly, MA, USA) |
| Phospho-SAPK/JNK (T183/Y185) | 54, 46 | Rabbit | 1:1000 | Cell Signaling Technology (Beverly, MA, USA) |
| Phospho-TrkB (Y515) | 145 | Rabbit | 1:1000 | Abcam (Cambridge, UK) |
| Phospho-TrkB (Y816) | 145 | Rabbit | 1:1000 | Abcam (Cambridge, UK) |
| PLCγ1 | 155 | Rabbit | 1:1000 | Cell Signaling Technology (Beverly, MA, USA) |
| SAPK/JNK | 54, 46 | Rabbit | 1:1000 | Cell Signaling Technology (Beverly, MA, USA) |
| Spectrin | 120 (breakdown product 120) | Mouse | 1:1000 | Merck Millipore (Billerica, MA, USA) |
| TrkB | 145 (full-length), 95 (truncated) | Rabbit | 1:1000 | BD Biosciences (Franklin Lakes, NJ, USA) |
| α-Tubulin | 55 | Mouse | 1:50,000 | Sigma-Aldrich (St. Louis, MO, USA) |

Membranes were then rinsed 3 times for 10 min each with TBS-T and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature (Table 4).

**Table 4. Secondary antibodies used for Western blot.** All secondary antibodies were conjugated with the horseradish peroxidase. Information about the dilution used and source is shown.

| SECONDARY ANTIBODY | DILUTION | SOURCE |
|---|---|---|
| Anti-mouse IgG | 1:3000 | Promega (Madison, WI, USA) |
| Anti-rabbit IgG | 1:3000 | Promega (Madison, WI, USA) |

Membranes were washed 3 times for 10 min each with TBS-T and developed by using the Western blotting Luminol reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Super RX Fujifilm photographic films. These films were posteriorly scanned and the Gel-Pro densitometry program (Gel-Pro Analyzer for Windows- version 4.0.00.001) was used to quantify the different immunoreactive bands relative to the intensity of loading controls. Particularly, incubation with

antibodies against actin, histone H3, lamin A/C or α-tubulin were used as loading controls as specified in figure legends.

# 7. Immunoprecipitation

For immunoprecipitation analysis, STHdh$^{Q111/Q111}$ striatal cells treated with DMSO or RGFP966 (10 μM) for 6 hours were homogenized using an insulin syringe in ice-cold immunoprecipitation (IP) buffer containing 50 mM Tris-HCl (pH 7.5), 10% glicerol, 1% Triton X-100, 150 mM NaCl, 100 mM NaF, 5 μM $ZnCl_2$, 10 mM EDTA, and supplemented with 1mM sodium orthovanadate and protease inhibitor cocktail. Samples were centrifuged at 16,100 g for 15 minutes at 4 ºC, and supernatants were collected. After quantifying protein content, 300 μg of protein extracts were incubated overnight at 4 ºC on a rotating wheel with 3 μg of anti-CBP antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or rabbit IgGs (Jackson ImmunoResearch, West Grove, PA, USA) as a negative control. Then, 40 μl of protein A-Sepharose (Santa Cruz Biotechnology; Santa Cruz, CA, USA) was added to the samples, followed by an overnight incubation at 4°C with rotation. Beads were collected by centrifugation (5 minutes, 1500 g, 4 ºC) and sequentially washed with IP buffer, IP buffer-Phosphate buffered saline (PBS) (1:1) and PBS. Samples were boiled at 100 ºC for 5 minutes in SDS sample buffer and analyzed by Western blot (6% SDS-PAGE) as described above. Specifically, nitrocellulose membranes were sequentially incubated with anti-acetyl-lysine antibody (to visualize acetyl-CBP) and with anti-CBP antibody.

# 8. Enzyme-linked immunosorbent assay (ELISA)

BDNF quantification of striatal protein samples was performed using an ELISA as previously described (Kolbeck et al., 1999), with minor modifications. 96-well white polystyrene plates (Nunc, Roskilde, Denmark) were coated with 1 μg of anti-BDNF #1 antibody (Developmental Studies Hybridoma Bank) in 100 μl carbonate buffer (pH 9.7) per well overnight at 25 °C. Plates were blocked with 4% BSA in PBS and washed three times with TBS-T. Samples and standards (1-1024 pg of recombinant purified BDNF per well) supplemented with 1% BSA and 1% NP-40 were incubated for 3 hours at 30 °C together with BDNF #2 antibody coupled to HRP (Developmental Studies Hybridoma Bank) at 12 ng/well. Plates were washed three times with TBS-T. SuperSignal ELISA Femto Substrate (Life Technologies), diluted 50% in H2O, was used as the substrate. With these modifications to the assay, we improved its sensitivity to detect 1 pg of BDNF per well. In addition, using a range of standards with different amounts of recombinant BDNF (1-1024 pg/well), we generated standard curves for each experiment performed, which allowed us to precisely quantify the BDNF present in striatal tissue.

# 9. Immunohistochemistry

Half-brains from vehicle or RGFP966 acutely treated mice were immersed for 72 hours in a fixation solution containing 4% PFA (Sigma-Aldrich, St. Louis, MO, USA) and 0.1 mM phosphate

buffer (PB). During the next days, a gradient of sucrose (from 10% to 30%) in PBS and 0.02% sodium azide was performed to cryoprotect the brains. Brains were preserved in this buffer at 4 ºC until their posterior processing. Alternatively, KI and KI:p75$^{+/-}$ mice were deeply and irreversibly anesthetized with pentobarbital at 60 mg/kg and immediately perfused transcardially with 10 ml PBS followed by 50 ml of 4% PFA/PB. Brains were removed, postfixed overnight in the same solution and cryoprotected by immersion in a gradient of PBS/sucrose 10-30% with 0.02% sodium azide. All brains were frozen in dry ice-cooled methyl-butane (Sigma-Aldrich, St. Louis, MO, USA) and serial coronal sections (30 μm) through the whole brain were obtained using a cryostat (Microm) and kept in PBS with 0.02% sodium azide as free-floating sections.

## 9.1. Immunofluorescence staining

For immunofluorescence staining, free-floating sections were rinsed twice in PBS for 5 minutes and incubated 2 times for 15 minutes each with 50 mM $NH_4Cl$ to block unreacted aldehyde groups coming from fixation solution and thus reduce aldehyde-induced tissue autofluorescence. Then, sections were permeabilized 2 times for 10 minutes each with PBS containing 0.5% Triton X-100 and consecutively blocked with PBS containing 0.5% Triton X-100, 0.2% sodium azide, 0.2% bovine serum albumin and 5% normal goat serum (Pierce Biotechnology, Rockford, IL, USA) for 2 hours at room temperature. Sections were then incubated overnight at 4°C with mouse anti-Arc (1:200, Santa Cruz Biotechnology) or rabbit anti-Egr1 (1:200, Cell Signaling Technology), diluted in a solution of PBS containing 0.3% Triton X-100, 0.2% sodium azide, 0.2% bovine serum albumin and 5% normal goat serum. After primary antibody incubation, slices were washed three times for 10 minutes each and then incubated 1.5 hours at room temperature with secondary antibodies: anti-rabbit Alexa Fluor 488 (1:100; Jackson ImmunoResearch, West Grove, PA, USA) or anti-mouse Cy3 (1:100; Jackson ImmunoResearch, West Grove, PA, USA), diluted in PBS. From this step, tissues were protected from the light to avoid fluorescent bleaching. After washing 3 times for 10 min each with PBS, nuclei were stained with Hoechst 33258 (1:10,000; Invitrogen, Carlsbad, CA, USA) for 10 minutes. Three more washes of 10 minutes each in PBS were performed and slices were mounted in the slides and left for 4 hours at room temperature to dry completely. Next, mounting media (Mowiol; Merck, Darmstadt, Germany) was added to incorporate the coverslip. As negative controls, some sections were processed as described in the absence of primary antibody and no signal was detected.

Tissue sections were examined using an Olympus BX60 (Olympus, Tokyo, Japan) epifluorescence microscope coupled to an Orca-ER cooled CCD camera (Hamamatsu Photonics, Hamamatsu, Japan) or a Leica TCS SP5 laser scanning confocal microscope (Leica Microsystems Heidelberg GmbH, Manheim, Germany) with Argon and HeNe lasers coupled to a Leica DMI6000 inverted microscope at different magnifications. For each mouse, at least four slices of 30 μm spaced 240 μm apart and containing hippocampal tissue were analyzed. Arc immunoreactive-positive positive cells in the dentate gyrus were counted at 10x magnification with the Olympus BX60 microscope and Egr1 immunoreactivity in the CA1 or CA3 area of the hippocampus was quantified (at 10x magnification, Olympus BX60 microscope) by analysis of integrated optical density with ImageJ software, as previously described (Giralt et al., 2013).

## 9.2. Immunoperoxidase staining

For EM48 immunohistochemistry, sections were first rinsed twice in PBS for 5 minutes and then pre-treated with an antigen retrieval method by incubation for 30 min with 0.01 M sodium citrate buffer (pH 6) in a water bath preheated to and maintained at 80 °C. This method was adapted from a protocol previously described (Bayram-Weston et al., 2016). After washing twice in PBS for 5 minutes, endogenous peroxidases were blocked for 45 minutes in PBS containing 5% $H_2O_2$. 3 washes with PBS for 5 minutes each were performed and nonspecific protein interactions were blocked with PBS containing 3% normal horse serum. Tissue was incubated overnight at 4°C with the primary antibody anti-EM48 (1:500, Merck Millipore, Billerica, MA, USA), diluted in PBS containing 0.02% sodium azide and 3% normal horse serum. Then, sections were washed three times in PBS for 5 minutes each and incubated at room temperature for 2 hours with a biotinylated anti-mouse antibody (1:200; Pierce; Thermo Fisher Scientific, Waltham, MA, USA), diluted in PBS containing 3% normal horse serum. Sections were washed three times in PBS for 5 minutes each and incubated for 1.5 hours at room temperature in Reagent A (Avidin) and Reagent B (Biotinylated HRP), each of them diluted at 1:56 in PBS, from the ABC kit (Pierce, Thermo Fisher Scientific). Then, samples were rinsed again three times in PBS for 5 minutes each and two times in PB 0.1 M for 5 minutes each. The immunohistochemical reaction was developed by incubating the samples for 7 minutes in diaminobenzidine (DAB), diluted at 1x in PB 0.1 M and 5% $H_2O_2$. After washing two times in PB 0.1 M for 5 minutes each and three times in PBS for 5 minutes each, sections were mounted in gelatinized slides and left for 4 hours at room temperature to dry completely. Following dehydration (1 x 5 minutes EtOH 70%, 1 x 5 minutes EtOH 90%, 2 x 5 minutes EtOH 100% and 2 x 5 minutes xylene), slides were kept in xylene until coverslipped with DPX. As negative controls, some sections were processed as described in the absence of primary antibody and no signal was detected.

Automated quantification of the number of nuclear huntingtin aggregates and the number and intensity of EM48-stained nuclei within the striatum were performed using CellProfiler v2.8 software (a specific pipeline file was created). Three coronal striatal sections per animal spaced 240 μm apart were chosen for the analysis. Bright field images from the 100% of the dorsal striatum were acquired at 40x magnification with a BX51 Olympus microscope with the cast system software (Olympus Danmark A/S, Ballerup, Denmark). Diffuse EM48 immunostaining was quantified as a "staining index" that captures both the nuclear staining intensity and the number of immunostained nuclei, as described elsewhere (Lloret et al., 2006; Pinto et al., 2013).

# 10. Dendritic spine analysis

For DiOlistic labeling of neurons, the Helios Gene Gun System (Bio-Rad, Hercules, CA, USA) was used as previously described (Grutzendler et al., 2003). A suspension containing 3 mg of DiI (Molecular Probes, Invitrogen) dissolved in 100 μl of methylene chloride (Sigma-Aldrich, St. Louis, MO, USA) and mixed with 50 mg of tungsten particles (1.7 mm diameter, Bio-Rad) was spread on a glass slide and air-dried. The mixture was resuspended in 3.5 ml distilled water and sonicated.

Subsequently, the mixture was drawn into Tefzel tubing (Bio-Rad) and then removed to let tube dry during 5 minutes under a nitrogen flow gas. Then, the tube was cut into 13 mm pieces to be used as bio gun's cartridges. Dye-coated particles were delivered in the striatum using the following protocol. Shooting was performed over 150 μm coronal sections at 80 psi through a membrane filter of 3 μm pore size and 8 × 10 pores/cm$^2$ (Merck Millipore, Billerica, MA, USA). Sections were stored at room temperature in PBS for 3 hours protected from light, then incubated with DAPI, and finally mounted with Mowiol to be analyzed. DiI-labeled medium spiny neurons (MSNs) of the dorsal striatum were imaged using a Leica TCS SP5 laser scanning confocal microscope with a 63x oil-immersion objective. Conditions such as pinhole size (1 AU) and frame averaging (4 frames/z-step) were held constant throughout the study. Confocal z-stacks were taken with a digital zoom of 5, a z-step of 0.2 μm, and a resolution of 1024 × 1024 pixel, yielding an image with pixel dimensions of 49.25 × 49.25 μm. Z-stacks were deconvolved using the Acoloma plugins from ImageJ to improve voxel resolution and reduce optical aberration along the z-axis. Segments from MSN dendrites were selected for the analysis of spine density according the following criteria: (1) segments with no overlap with other branches that would obscure visualization of spines and (2) segments either "parallel to" or "at acute angles" relative to the coronal surface of the section to avoid unambiguous identification of spines. Only spines arising from the lateral surfaces of the dendrites were included in the study, ignoring spines located on the top or bottom of the dendrite surface. Given that spine density increases as a function of the distance from the soma, reaching a plateau 45 μm away from the soma, we selected dendritic segments 45 μm away from the cell body.

# 11. Quantitative reverse transcription PCR (RT-qPCR)

Immediately after the sacrifice of the mice by cervical dislocation, brains were harvested and tissue samples were dissected on a thick glass slide placed on ice and quickly stored in dry ice and then at -80 ºC. Total RNA from frozen tissue samples was isolated using the Nucleospin RNA II Kit (Macherey-Nagel, Düren, Germany), following manufacter's instructions. RNA quantification was performed using the Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). 500 ng of purified RNA were reverse transcribed using the PrimeScript RT Reagent Kit (Perfect Real Time; Takara Biotechnology Inc., Kusatsu, Shiga, Japan). To exclude contamination by genomic DNA, the PrimeScript RT Enzyme was omitted in negative controls. The cDNA synthesis was performed at 37 ºC for 15 minutes with a final step at 85 ºC for 5 seconds in a total volume of 20 μl, according to manufacturer's instructions. The cDNA was then analyzed by quantitative RT-PCR using PrimeTime qPCR Assays (Integrated DNA Technologies, Coralville, IA, USA) (Table 5). Quantitative PCR was performed in 12 μl of final volume on 96-well plates using the Premix Ex Taq (Probe qPCR; Takara Biotechnology Inc., Kusatsu, Shiga, Japan). Reactions included Segment 1: 1 cycle of 30 seconds at 95 ºC and Segment 2: 40 cycles of 5 seconds at 95 ºC and 20 seconds at 60 ºC. All quantitative PCR assays were performed in duplicate. The qPCR data were analyzed with the comparative quantitation analysis program of

MxProTM quantitative PCR analysis software version 3.0 (Stratagene), using 18S and Actinβ gene expression as housekeeping genes.

**Table 5. Probes used for gene expression analysis.** For each gene, information about the reference assay and source is shown.

| GENE | ASSAY | SOURCE |
|---|---|---|
| 18S | Hs.PT.39a.22214856.g | Integrated DNA Technologies (Coralville, IA, USA) |
| Actinβ | Mm.PT.39a.22214843.g | Integrated DNA Technologies (Coralville, IA, USA) |
| Arc | Mm.PT.56a.16160059 | Integrated DNA Technologies (Coralville, IA, USA) |
| Egr-1 | Mm.PT.58.29064929 | Integrated DNA Technologies (Coralville, IA, USA) |
| Htt | Mm.PT.58.12088552 | Integrated DNA Technologies (Coralville, IA, USA) |
| Nr4a2 | Mm.PT.58.16021564 | Integrated DNA Technologies (Coralville, IA, USA) |
| c-Fos | Mm.PT.58.29977214 | Integrated DNA Technologies (Coralville, IA, USA) |

# 12. Small-pool PCR

To analyze CAG repeat expansions, small pool PCR (SP-PCR) experiments were performed on striatal, cerebellar and tail tissue from chronically vehicle-injected and RGFP966-treated mice, using primers specific for the Htt allele (Dragileva et al., 2009). Genomic DNA was prepared from mouse tissue using Nucleospin Tissue Kit (Macherey-Nagel, Düren, Germany), following the manufacturer's protocol. 1 μg of genomic DNA was digested with 20 U EcoRV-HF at 37 °C for 1.5 hours then heat inactivated at 65°C for 10 minutes. Digested DNA was serially diluted in TE buffer (pH 8) containing 0.1 μM carrier primer (CAG1_HdhQ111_F) to give a final concentration of 20 pg/μl. CAG repeat sizes were determined by PCR using forward primer CAG1_HdhQ111_F (5'-atg aag gcc ttc gag tcc ctc aag tcc ttc-3') and reverse primer HU3_HdhQ111_R (5'-ggc ggc tga gga agc tga gga-3'). The PCR reaction mixture contained 10 pg template DNA, 1X buffer (67 mM Tris-HCl [pH 8.8], 16.6 mM $NH_4SO_4$, 2.0 mM $MgCl_2$, 10 mM 2-mercaptoethanol) 10% DMSO, 0.17 mg/ml BSA, 0.2 mM each dATP, dCTP, dGTP and dTTP, 4 ng/μl primers and 0.5 U Taq polymerase (Fisher BioReagents). PCR reactions were performed in multiplex (20-30 reactions per sample) with at least four negative control reactions. PCR conditions were one cycle of 90 sec at 94 °C, 34 cycles of (30 seconds at 94 °C, 30 seconds at 65 °C and 90 seconds at 72 °C) and one final cycle of 10 minutes at 72 °C. PCR products were denatured in formamide loading buffer (0.05% w/v Bromophenol Blue, 0.05% w/v Xylene cyanol, 96% v/v deionized formamide, 20 mM EDTA, pH 8) at 95 °C for 8 minutes then resolved,

alongside DIG labeled size markers, on a 6% denaturing sequencing gel in 1X TBE buffer at 60W for ~4 hours. Fragments were transferred to positively charged nylon membrane by electroblotting at 30 V overnight at 4 °C and fixed by UV cross-linking. Southern blot hybridization was performed using a 5' digoxigenin (DIG) labeled locked nucleic acid (LNA) probe GC*T G*CT GC*T G*CT GC*T GCT (Eurogentec, where C* indicates LNA cytosine and G* indicates LNA guanine). Detection was performed using DIG High Prime DNA labeling and Detection Starter Kit II (Roche Applied Science, Mannheim, Germany) following the manufacturer's protocol. Multiple exposures to X-ray film ensured detection of faint signals and separation of closely spaced bands. Starting tract size was deduced from the most common product length in the tail or cerebellum of each animal.

# 13. Statistical analysis

All data are expressed as mean ± SEM. Statistical analysis were performed using the unpaired Student's t-test, one-way ANOVA or two-way ANOVA, and the appropriate post-hoc tests as indicated in the figure legends. Values for n and p are also specified in each figure legend. A 95% confidence interval was used and values of $P < 0.05$ were considered as statistically significant.

# RESULTS

# 1. Benefits of HDAC3 modulation in HD

Transcriptional dysregulation has been proposed to play a key and early role in HD pathophysiology (Landles and Bates, 2004; Cha, 2007), although the exact molecular basis is not well understood. Some studies suggest that the expanded glutamine tract in mHtt changes its conformation, and confers new properties to the protein. Among them, aberrant interactions with proteins involved in chromatin maintenance and gene expression could be responsible for the altered genetic program. Accordingly, small-molecule inhibitors of HDACs (HDACis) have proved to be effective in upregulating gene transcription and ameliorating HD symptoms (Ferrante et al., 2003; Hockly et al., 2003; Gardian et al., 2005). However, the potential toxicity of broad-based HDACi, especially in chronic treatment, has led to the development of new isoform-selective HDACis.

Recent studies have already shown that HDAC3-selective pharmacological inhibition improves motor impairments and some neuropathological phenotypes in different HD models (Jia et al., 2012b, 2015, 2016), likely by ameliorating transcriptional deficits. Additionally, HDAC3 has shown to play a critical negative role in the molecular mechanisms underlying memory formation (McQuown et al., 2011), although still remains to be elucidated whether HDAC3 inhibition could ameliorate early cognitive HD symptoms. On the other hand, the mutant CAG repeat track in *huntingtin* gene has shown to undergo progressive, expansion-biased and tissue/cell type-specific somatic instability that are predicted to accelerate HD progression (Kennedy et al., 2003; Shelbourne et al., 2007; Gonitel et al., 2008; Swami et al., 2009). HDAC3 has been recently found to promote such expansions in cultured human astrocytes, although the underlying molecular mechanisms have not been deciphered (Debacker et al., 2012; Gannon et al., 2012).

Overall, the aim of this study is to examine the selective contribution of HDAC3 activity to specific HD molecular pathogenic mechanisms.

## 1.1. Safety and efficacy of chronic HDAC3 inhibition

RGFP966 is a potent HDAC3-selective inhibitor with an $IC_{50}$ of 0.06-0.21μM and with 10- to 200-fold selectivity over other HDACs, depending on the assay (Malvaez et al., 2013; Rumbaugh et al., 2015; Jia et al., 2016; Leus et al., 2016). This compound is a N-(o-aminophenyl)carboxamide that acts as a slow-on/slow-off, tight-binding competitive inhibitor, leading to a long-lasting histone hyperacetylation. Previous results have already demonstrated that RGFP966 reach the brain rapidly after a systemic injection, reaching maximum concentrations after 30 minutes post-injection (Malvaez et al., 2013).

To analyze toxicity and efficacy of this drug in a chronic treatment paradigm, subcutaneous injections of RGFP966 or vehicle were periodically administered (three times per week for 13 weeks) to wild-type (WT) and knock-in $Hdh^{Q7/Q111}$ (KI) mice, starting at three months of age. The inhibitor was well tolerated, without evident toxicity nor gross morphology abnormalities. Additionally, no significant differences in body weight were found between genotypes or treatment conditions (Figure 20).

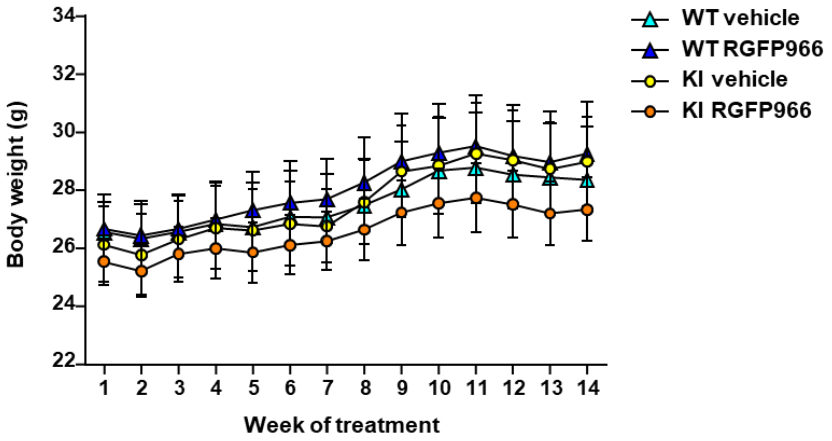



**Figure 20. Chronic administration of the HDAC3-selective inhibitor RGFP966 does not affect body weight.** Histogram of weekly body weight measurements of vehicle and RGFP966 chronically treated WT and KI mice along pharmacological treatment. Data represent the mean ± SEM ($n$ = 6-10 mice/group).

After chronic treatment, all animals were sacrificed and brain tissues were dissected and collected for posterior analysis. Cognitive behavioral tasks described in the next section are strongly dependent on the hippocampus and striatum, so it was important to demonstrate drug activity in these brain regions. Examination of histone H3 acetylation at lysine position 9 (AcH3K9) by Western blot analysis in hippocampal and striatal extracts showed drug-dependent increases in both WT and KI animals (Figure 21A), consistent with a decrease in HDAC3 activity. Conversely, despite previous results from our group showing decreased histone H3 acetylation in the hippocampus of KI mice at 8 months of age, there were no differences between genotypes at 6 months of age. Reduced HDAC3 activity was likely due to enzyme inhibition, since the abundance of HDAC3 protein was unaltered by RGFP966 treatment (Figure 21B). Besides, HDAC3 protein levels were found unchanged between genotypes, consistent with previous reports in different HD models (Valor and Guiretti, 2014).

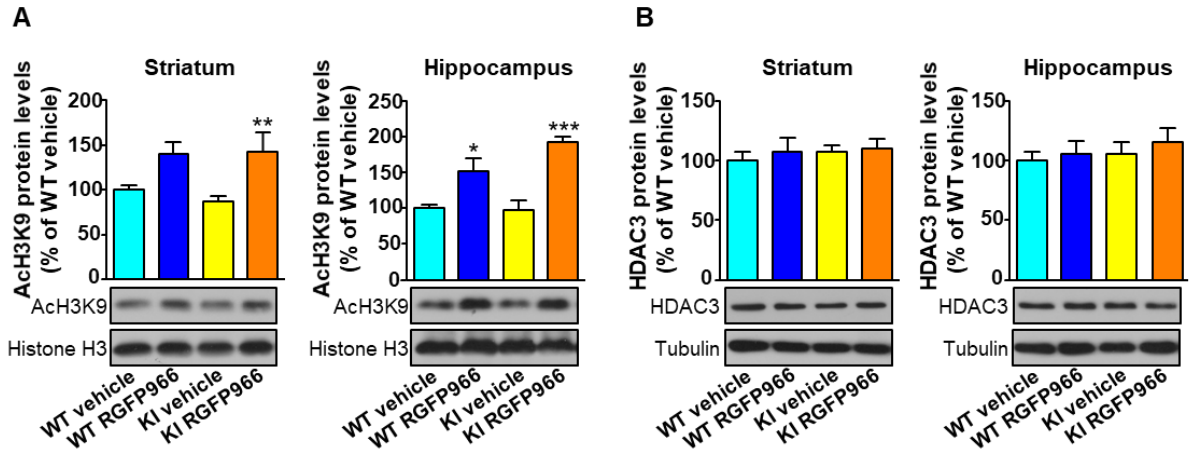



**Figure 21. Systemic RGFP966 treatment efficiently inhibits hippocampal and striatal HDAC activity.** Representative immunoblots and densitometric analysis showing protein levels of acetylated histone H3 at position lysine 9 (AcH3K9) with total histone H3 as loading control (**A**) and HDAC3 with tubulin as loading control (**B**) in striatal and hippocampal extracts from vehicle and RGFP966 chronically treated WT and KI mice. *$P$ < 0.05; **$P$ < 0.01 and ***$P$ < 0.001 compared to vehicle-treated mice by two-way ANOVA with Bonferroni post-hoc analysis. Histograms represent the relative protein levels expressed as percentage of WT vehicle values. All data represent the mean ± SEM ($n$ = 4-7 mice/group).

Importantly, a recent study showed that the pharmacological or genetic inhibition of HDAC3 in the dorsal hippocampus of WT mice significantly decreased HDAC4 protein levels (McQuown et al., 2011). HDAC4 has been shown to participate in memory formation (Sando et al., 2012) and in the pathophysiology of HD (Mielcarek et al., 2013; Yue et al., 2015), which may suggest that phenotypic changes due to HDAC3 modulation could be accountable for HDAC4. However, after evaluating HDAC4 levels by Western blot in the hippocampus of chronically treated mice we could not detect significant differences between experimental groups (Figure 22).

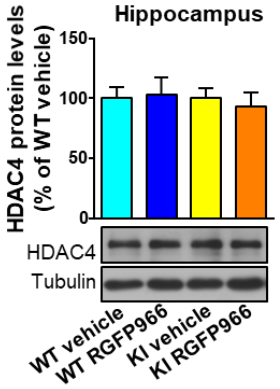



**Figure 22. Systemic RGFP966 treatment does not alter hippocampal HDAC4 levels.** Representative immunoblots and densitometric analysis showing HDAC4 protein levels with tubulin as loading control in hippocampal extracts from vehicle and RGFP966 chronically treated WT and KI mice. Histograms represent the relative protein levels expressed as percentage of WT vehicle values. All data represent the mean ± SEM ($n$ = 4-7 mice/group).

Overall, these findings ensured safety and efficacy of long-term systemic RGFP966 administration in target key areas.

## 1.2. HDAC3 activity negatively contributes to HD cognitive impairments

Cognitive decline is an early clinical hallmark that appears in pre-motor stages of HD and has shown to be the most disturbing concern for HD patients and their relatives. In this regard, HDAC3 has shown to be an important negative contributor of memory formation (McQuown et al., 2011). To study the contribution of HDAC3 to HD memory deficits, our mouse model represents a useful tool, since it presents a slow disease progression and an accurate timing of cognitive and motor impairments.

### 1.2.1. Chronic treatment with RGFP966 prevents motor learning impairments in KI mice

Impaired acquisition of new skills has been reported in HD patients (Martone et al., 1984; Rosenberg et al., 1995; Lawrence et al., 1996, 1998; Paulsen et al., 2008; Schneider et al., 2010) and mouse models (Mazarakis et al., 2005; Van Raamsdonk et al., 2005b; Puigdellívol et al., 2015) at early disease stages. Therefore, we first determined whether RGFP966 chronic treatment could improve the ability of KI mice to learn new motor skills, which is known to depend on

corticostriatal circuits. Performance on a gradually accelerating rotarod was evaluated in vehicle-treated and RGFP966-treated WT and KI mice at 6 months of age (Figure 23A). Both groups of WT mice exhibited good motor learning ability over the trials. In contrast, vehicle-treated KI mice performed significantly worse with shorter latency to fall. Importantly, RGFP966-treated KI mice were able to learn to stay on the rotarod as well as vehicle- or RGFP966-treated WT mice, indicating prevention of motor learning deficits upon treatment with the HDAC3-selective inhibitor. Since poor performance of vehicle-treated KI animals in the accelerating rotarod could be due to motor deficits, spontaneous locomotor activity in the open field was also evaluated. No significant differences in the distance traveled were found between groups (Figure 23B). These results are in accordance with previous data from our group, showing no alterations of KI mice in the fixed rotarod or the open field until 8 months of age (Puigdellívol et al., 2015). Thus, we can assume that differences in the accelerating rotarod task were due to motor learning and not motor coordination deficits.

Overall, these findings demonstrate that impaired ability to learn new corticostriatal-dependent motor skills can be prevented in KI mice by early treatment with RGFP966.

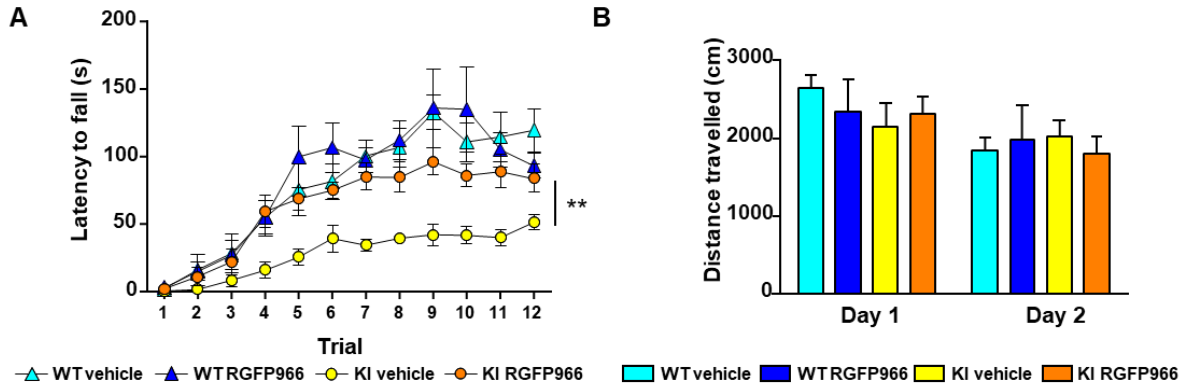



**Figure 23. RGFP966 treatment prevents motor learning impairment in KI mice.** Behavior analysis in vehicle and RGFP966 chronically treated WT and KI mice. (**A**) Latency to fall in the accelerating rotarod task. $^{**}P < 0.01$: vehicle-treated KI mice compared to vehicle-treated WT mice by two-way ANOVA with repeated measurements. (**B**) Spontaneous locomotor activity measured by total distance traveled during two consecutive days (15 min/day). Data represent the mean ± SEM ($n$ = 5-9 mice/group).

## 1.2.2. Chronic treatment with RGFP966 prevents long-term memory impairments in KI mice

Spatial and recognition memory impairments, especially dependent on the hippocampus, have also been reported in HD patients (Lawrence et al., 2000; Berrios et al., 2002; Brandt et al., 2005; Begeti et al., 2016) and mouse models (Lione et al., 1999; Nithianantharajah et al., 2008; Simmons et al., 2009; Giralt et al., 2011b, 2012a; Zeef et al., 2012; Brito et al., 2014). To evaluate this behavior in WT and KI mice chronically-treated with RGFP966 or vehicle, we assessed performance of the mice at the object location task (OLT), which evaluates long-term spatial memory, and at the novel object recognition task (NORT), which evaluates long-term recognition memory. These tests were performed at 6 months of age, an age at which KI mice start to exhibit long-term memory impairments (Brito et al., 2014). Mice were first subjected to a training session in the presence of

two similar objects and showed similar time exploring each object (Figure 24, left), indicating no detectable preference for object or location. When long-term memory was assessed 24 hours after training, WT mice exhibited a clear increase in time exploring both the novel location and object regardless whether the animals were treated with RFGP966 or vehicle alone. In contrast, vehicle-treated KI mice demonstrated a similar time exploring familiar and novel objects, with no preference for either spatial or object novelty. Interestingly, treatment of KI mice with RGFP966 prevented the loss of spatial and recognition memories, as shown by increasing time exploring the novel versus the familiar object and a significantly higher discrimination index compared to vehicle-treated KI animals (Figure 24, center and right). These findings indicate that RGFP966 treatment prevents spatial and recognition long-term memory decline in HD mice.

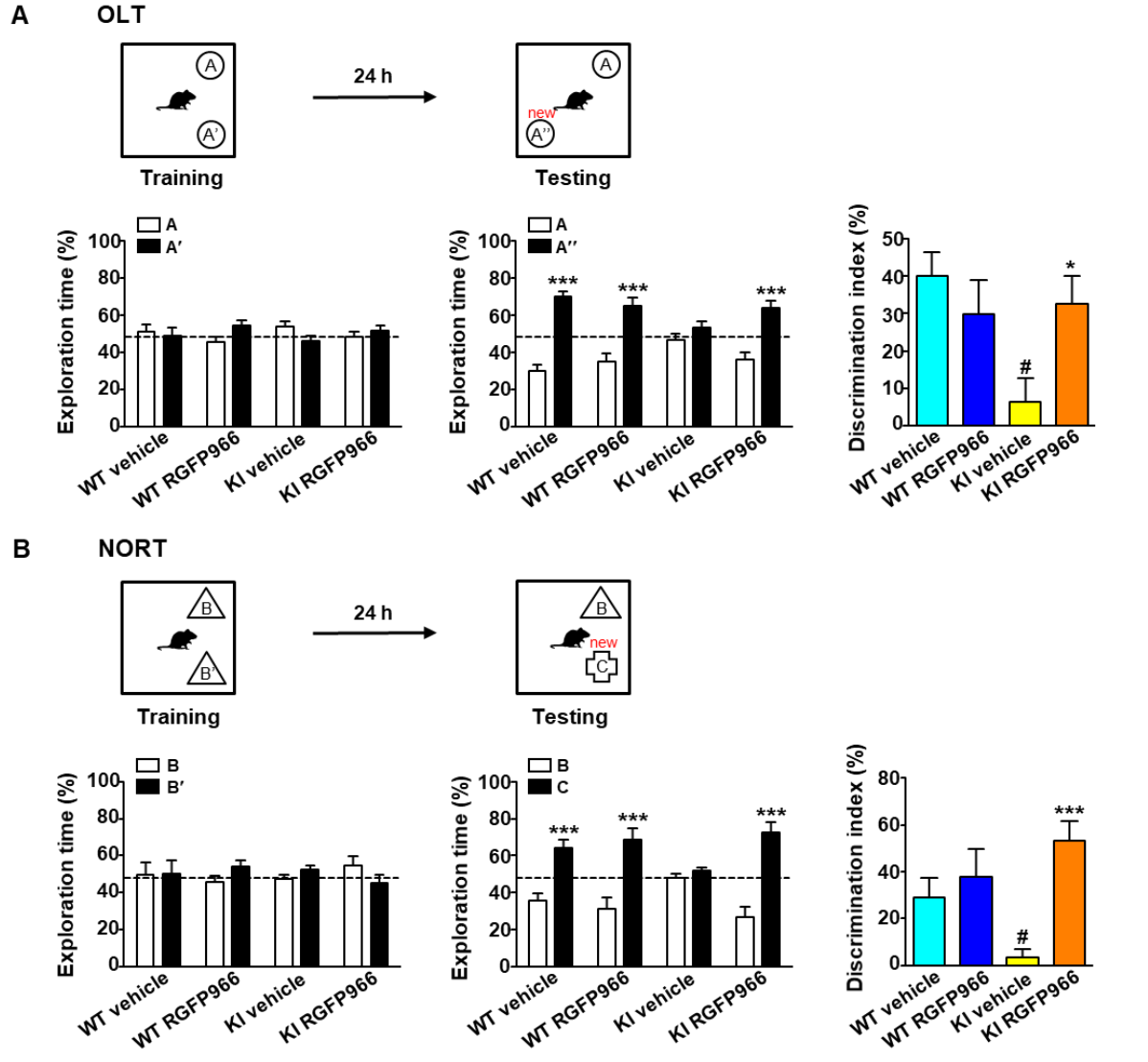



**Figure 24. RGFP966 treatment prevents impairment of long-term memories in KI mice.** Behavior analysis in vehicle and RGFP966 chronically treated WT and KI mice. Performance in spatial (OLT) (**A**) and recognition (NORT) (**B**) memory tests. Exploration time during training and testing session (left and center panels). Dashed line shows chance level for exploration. ***$P < 0.001$ compared to the percentage of time exploring the familiar object by two-way ANOVA with Bonferroni post-hoc analysis. Discrimination index (right panel). *$P < 0.05$, ***$P < 0.001$ compared to vehicle-treated KI mice; #$P < 0.05$ compared to vehicle-treated WT mice by two-way ANOVA with Bonferroni post-hoc analysis. Data represent the mean ± SEM ($n$ = 5-9 mice/group).

To exclude anxiety-like behaviors that could led us to misinterpret these results, we measured the distance travelled and percentage of time spend in the center of the arena during the habituation phases. No differences were detected between genotypes (Figure 25), which is consistent with previous reports from our group demonstrating lack of anxiety-like behaviors in KI mice at the same age (Brito et al., 2014; Alvarez-Periel et al., 2017). We also did not detect differences between treatment conditions.

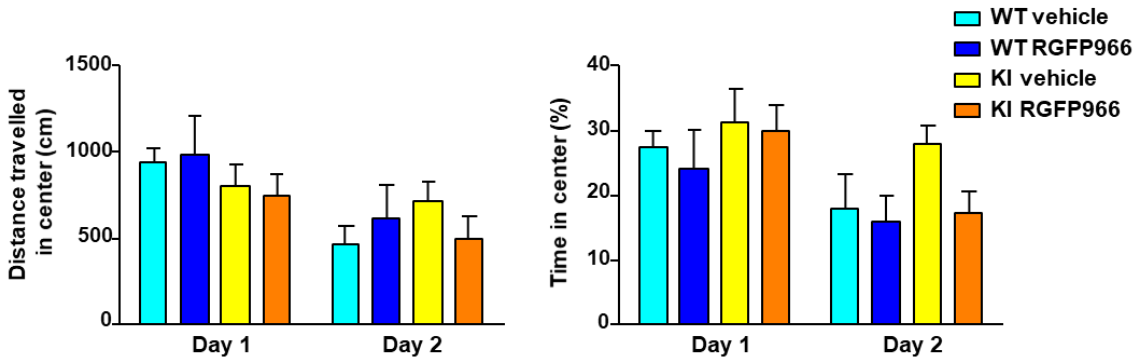



**Figure 25. Control data for memory tests.** Anxiety-like behavior in vehicle and RGFP966 chronically treated WT and KI mice measured by total distance traveled or time spend in the center of the open-field arena during two consecutive days (15 min/day). Data represent the mean ± SEM (*n* = 6-9 mice/group).

## 1.3. HDAC3 represses the expression of activity-induced memory genes

Recognition and spatial long-term memory deficits in KI mice have been associated with altered expression of activity-dependent, memory-related genes (Giralt et al., 2012a). Moreover, transcriptional activation of these genes is suggested to be required for the acquisition of new motor skills (Kleim et al., 1996; Jin and Clayton, 1997; Hosp et al., 2013; Cao et al., 2015). Thus, we have explored their potential regulation by HDAC3, accounting for the benefits in cognitive behavior observed in KI mice after RGFP966 treatment.

### 1.3.1. Acute treatment with RGFP966 restores the altered expression of memory-related genes in KI mice

WT and KI mice were acutely treated (once daily for 3 days) with either RGFP966 or vehicle and quantitative RT-PCR analysis were performed to examine hippocampal mRNA expression of the immediate early genes (IEGs) *Arc*, *Nr4a2*, *Egr1* and *c-Fos*. These are key neuronal activity-dependent genes whose expression is rapidly increased in subsets of neurons after a memory training event, therefore enabling long-term memory storage and synaptic plasticity (Minatohara et al., 2015). In order to detect activity-dependent transcriptional impairments in our HD model, mice also received a 5-minute training in an environment with two identical objects, immediately after last injection and 2 hours prior collection of samples. *Arc* and *Nr4a2* transcripts were found significantly reduced in vehicle-treated KI mice compared to WT mice, while no changes were found in *Egr1* and *c-Fos* expression. Interestingly, treatment of KI mice with RGFP966 led to *Arc* and *Nr4a2* expression at levels indistinguishable from WT mice (Figure 26).

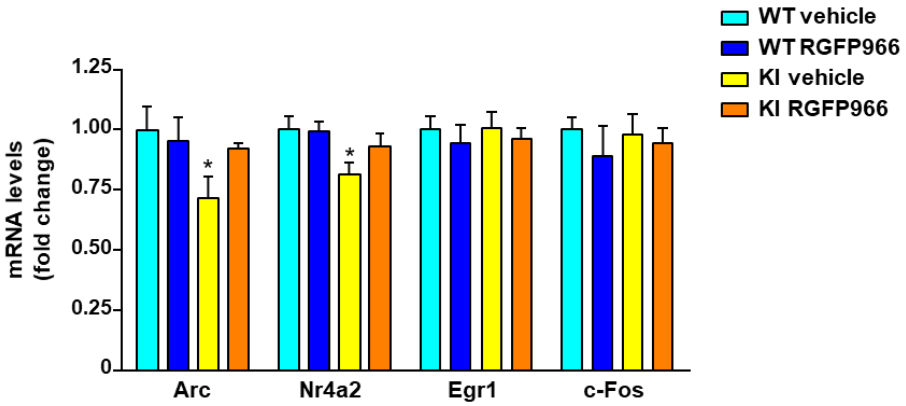
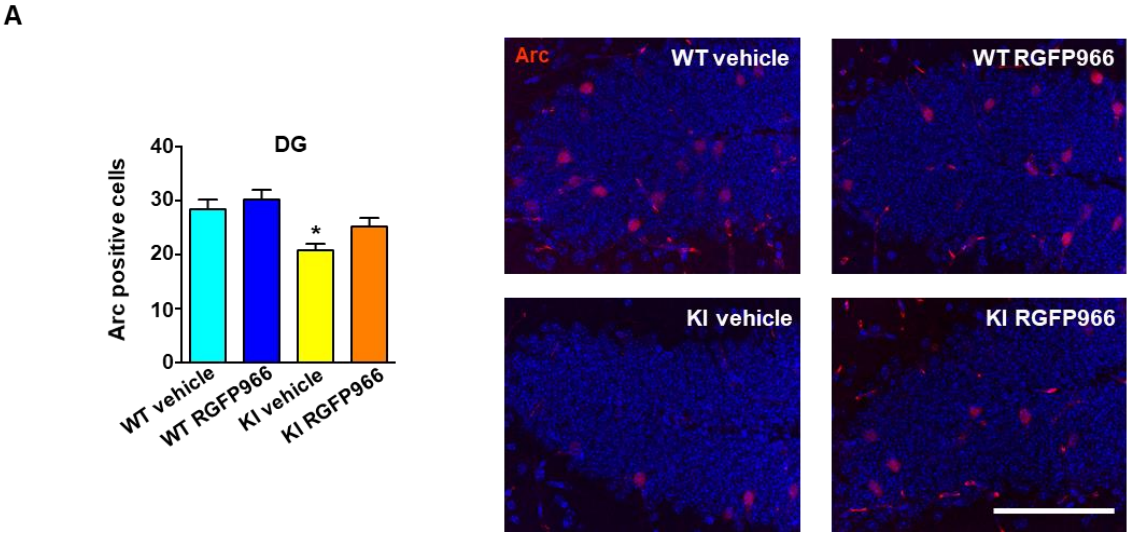



**Figure 26. RGFP966 treatment restores gene expression in the hippocampus of KI mice.** Quantitative RT-PCR analysis of memory-related genes in the hippocampus of vehicle and RGFP966 acutely treated WT and KI mice. Histogram represents relative mRNA abundance of *Arc*, *Nr4a2*, *Egr1* and *c-Fos* expressed as fold change relative to WT vehicle. Levels of mRNA were normalized to 18S and Actinβ. $*P < 0.05$ compared to vehicle-treated WT mice by two-way ANOVA with Bonferroni post-hoc analysis. Data represent the mean ± SEM ($n$ = 5-8 mice/group).

These results were confirmed by immunohistochemical analysis of Arc and Egr1 in hippocampal slices. A significant decrease in the number of Arc-positive cells was found in the dentate gyrus of vehicle-treated KI mice compared to WT mice, which was not observed in RGFP966-treated KI mice (Figure 27A). Arc-positive neurons were not evident in other hippocampal regions apart from the dentate gyrus, consistent with previous reports (Lonergan et al., 2010). In contrast, no significant differences between genotypes or treatment conditions were found in Egr1 immunoreactivity (Figure 27B), in accordance with our mRNA data (Figure 26). These findings demonstrate that HDAC3 inhibition restores the expression of memory-related genes in KI mice in response to a training.



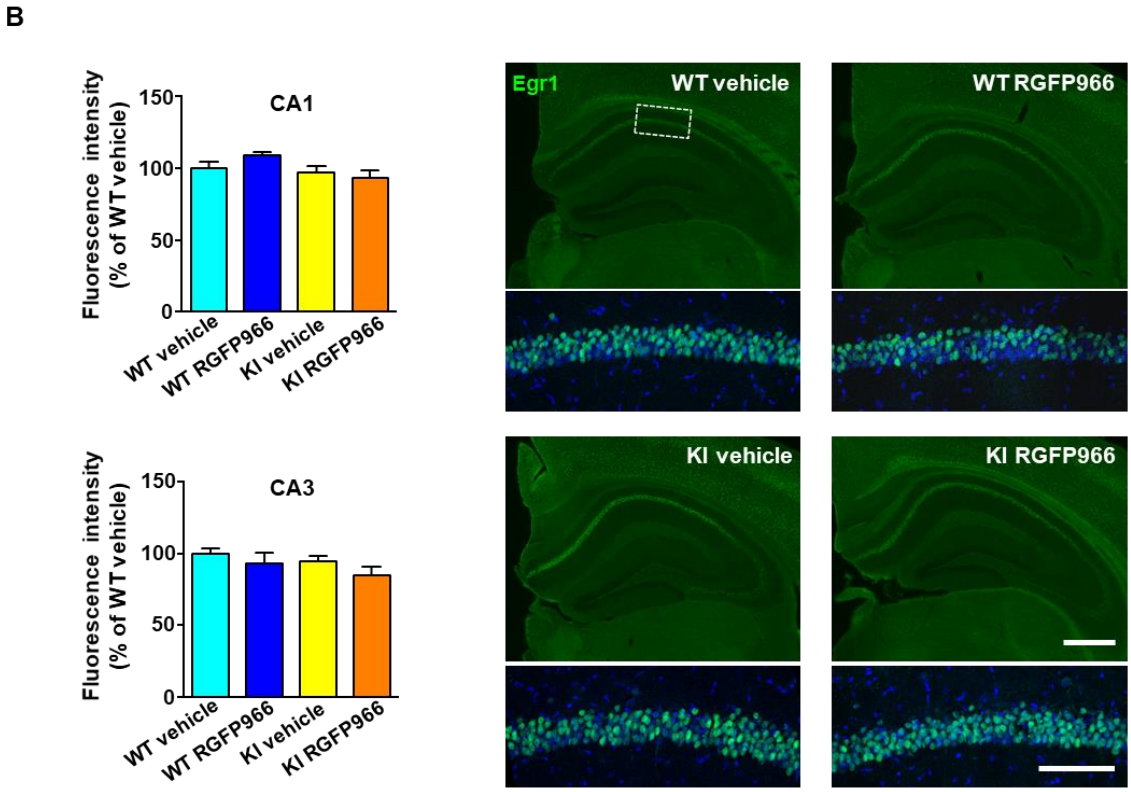



**Figure 27. RGFP966 treatment restores Arc protein expression in the hippocampus of KI mice.** (**A**) Representative photomicrographs (high magnification) showing Arc immunostaining in the hippocampal dentate gyrus of acutely treated WT and KI mice. Scale Bar: 100 μm. Histogram shows quantification of the average number of Arc-positive neurons in the dentate gyrus. $*P < 0.05$ compared to vehicle-treated WT mice by two-way ANOVA with Bonferroni post-hoc analysis. (**B**) Representative photomicrographs showing Egr1 immunostaining in the whole hippocampus, with magnification insets of the CA1 region, of acutely treated WT and KI mice. Scale Bar: 500 μm (low magnification) or 100 μm (high magnification). Histogram shows quantification of the average intensity of Egr1 immunoreactivity in the CA1 or the CA3 region, expressed as percentage of WT vehicle values. Data represent the mean ± SEM ($n$ = 5-8 mice/group).

## 1.3.2. RGFP966 treatment upregulates the expression of memory-related genes in neuronal cultures

To better explore the selective contribution of HDAC3 in the regulation of a subset of memory genes, we performed pharmacological *in vitro* analysis. As in the adult mice, primary neuronal cultures exhibit low basal expression of activity-dependent IEGs. To better detect differences upon RGFP966 treatment and to mimic brain conditions after a training session, primary hippocampal cultures at 10-14 DIV were subjected to depolarizing levels of KCl, which leads to increased intracellular $Ca^{2+}$ influx that induces IEGs expression (Bading et al., 1993; Zheng et al., 2009). As shown in Figure 28A, Western blot analysis revealed that KCl treatment at 50 mM for 2 hours triggered a robust increase in Arc, Egr1 and c-Fos proteins levels, compared to control conditions. Then, KCl-activated primary hippocampal cultures from WT and KI embryos at 10-14 DIV were incubated with vehicle or RGFP966 for 6 hours, followed by Western blot analysis. Consistent with HDAC3 inhibition, acetylation of H3K9 was found increased after RGFP966 treatment. Interestingly, Arc but not Egr1 or c-Fos protein levels were found increased after

HDAC3 inhibition, regardless of genotype, thus confirming the specific regulation of Arc by HDAC3 activity (Figure 28B).

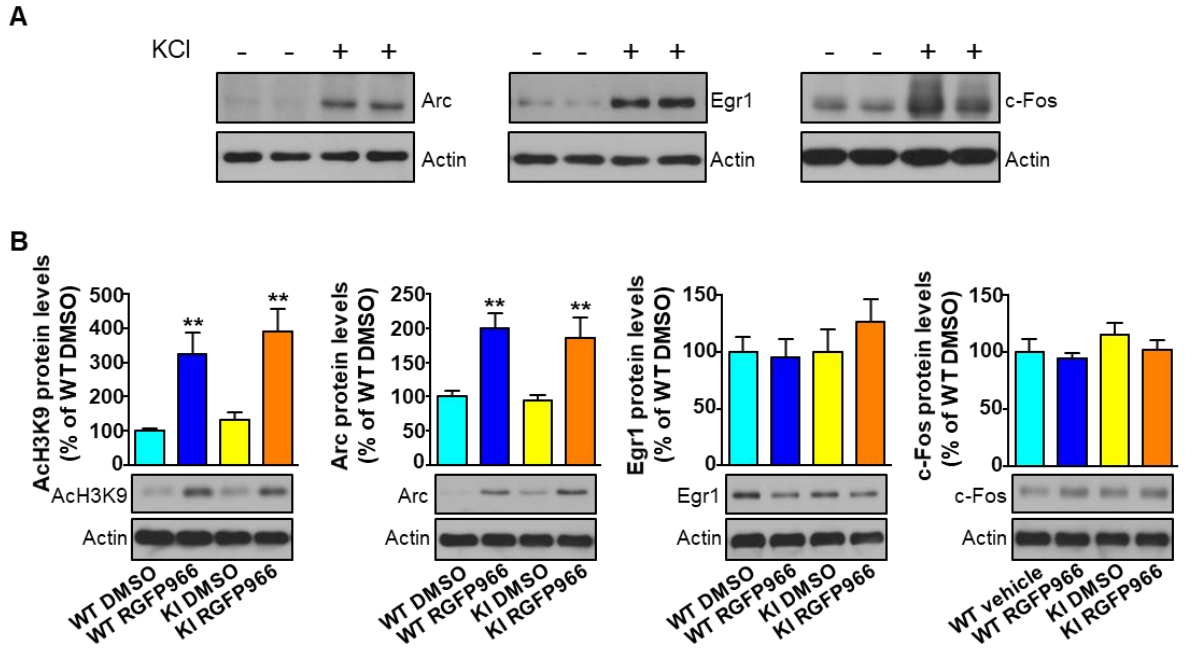
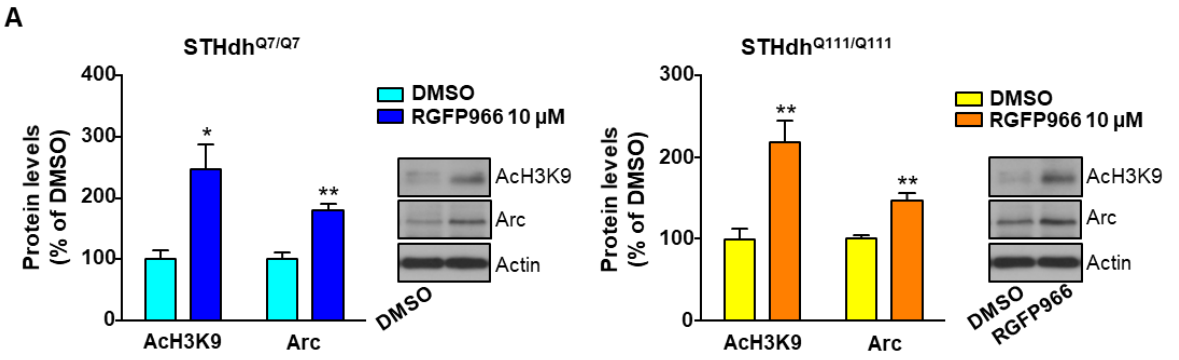



**Figure 28. RGFP966 treatment induces Arc but not Egr1 or c-Fos protein levels in activated primary hippocampal cultures.** (**A**) Representative immunoblots showing Arc, Egr1 and c-Fos protein levels with actin as loading control in primary hippocampal cultures treated or not with 50 mM KCl for 2 hours. (**B**) Representative immunoblots and densitometric analysis showing protein levels of acetylated histone H3 at lysine 9 (AcH3K9), Arc, Egr1 and c-Fos with actin as loading control in activated primary hippocampal cultures obtained from WT and KI embryos treated with 10 µM RGFP96 or DMSO for 6h. $^{**}P < 0.01$ compared to DMSO-treated cultures by two-way ANOVA with Bonferroni post-hoc analysis. Histograms represent the relative protein levels expressed as percentage of WT DMSO values. All data represent the mean ± SEM ($n$ = 4-5 cultures/group).

Since HDAC1 and/or HDAC2 might also be affected at the inhibitor dose used according to some assays (Jia et al., 2016; Leus et al., 2016), we tested a lower RGFP966 concentration in striatal-derived neuronal-like cell cultures expressing endogenous levels of either wild-type ($STHdh^{Q7/Q7}$), or mutant ($STHdh^{Q111/Q111}$) huntingtin. Treatment with RGFP966 at either 10 µM (Figure 29A) or 1 µM (Figure 29B), a dose below the $IC_{50}$ for HDAC1 and HDAC2, for 6 hours led to drug-dependent increases of AcH3K9 and Arc as measured by Western blot.



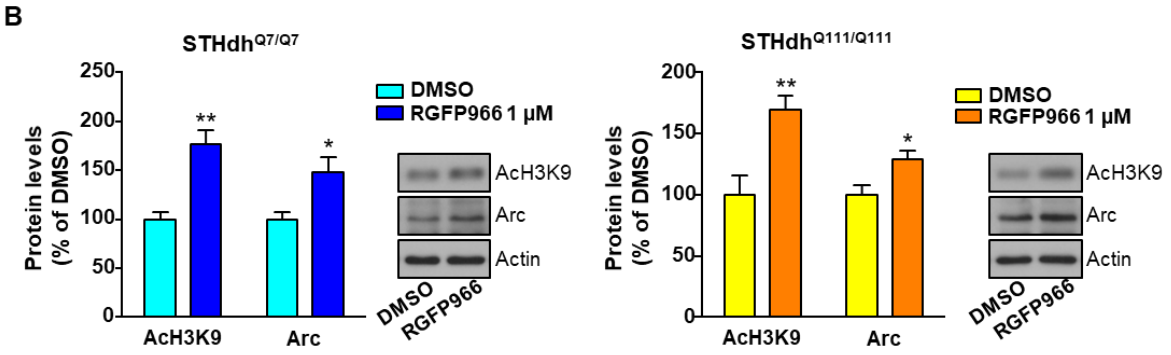



**Figure 29. RGFP966 treatment at different doses increases AcH3K9 and Arc protein levels in immortalized striatal cells.** Representative immunoblots and densitometric analysis showing protein levels of Arc and acetylated histone H3 at lysine 9 (AcH3K9) with actin as loading control in STHdh$^{Q7/Q7}$ or STHdh$^{Q111/Q111}$ striatal cells treated with DMSO or RGFP966 at 10 µM (**A**) or 1 µM (**B**) for 6 hours. *$P$ < 0.05 and **$P$ < 0.01 compared to DMSO-treated cultures by Student's t-test. Histograms represent the relative protein levels expressed as percentage of DMSO values. All data represent the mean ± SEM ($n$ = 4-5 cultures/ group).

These data demonstrate that doses of RGFP966 with low activity for HDAC1 and HDAC2 elicit similar results than those at higher doses, suggesting a major contribution of HDAC3 to the observed improvements in HD pathology.

### 1.3.3. RGFP966 treatment increases CBP acetylation in neuronal cultures

The transcriptional coactivator and acetyltransferase CBP has shown to play a key role in learning and memory processes (Alarcón et al., 2004; Barrett and Wood, 2008; Chen et al., 2010). Importantly, previous results from our group and others have demonstrated reduced activity of CBP in HD due to its aberrant interaction with mHtt (Steffan et al., 2000, 2001; Nucifora et al., 2001; Giralt et al., 2012a), which was associated with long-term memory impairments in the HdhQ111 mouse model (Giralt et al., 2012a). Recently, some studies have suggested that HDAC3 directly interacts and deacetylates CBP, repressing its function and therefore downregulating the expression of its target genes (Chuang et al., 2006; Grégoire et al., 2007).

To study the modulation of CBP acetylation by RGFP966 in the context of HD, mutant huntingtin striatal cells (STHdh$^{Q111/Q111}$) were treated with 10 µM RGFP966 or DMSO for 6 hours and subsequently harvested for Western blot analysis. First, we confirmed that total CBP levels were not changed upon treatment (Figure 30A). Next, protein samples were immunoprecipitated with an anti-CBP antibody and the immunoprecipitated proteins were immunoblotted with an anti-acetyl-lysine antibody, in order to measure acetyl-CBP protein levels. Interestingly, RGFP966 treatment significantly increased CBP acetylation *in vitro* (Figure 30B), suggesting that cognitive improvements in HD mice following HDAC3 inhibition could be related to enhanced CBP-mediated transcription of memory-related genes.

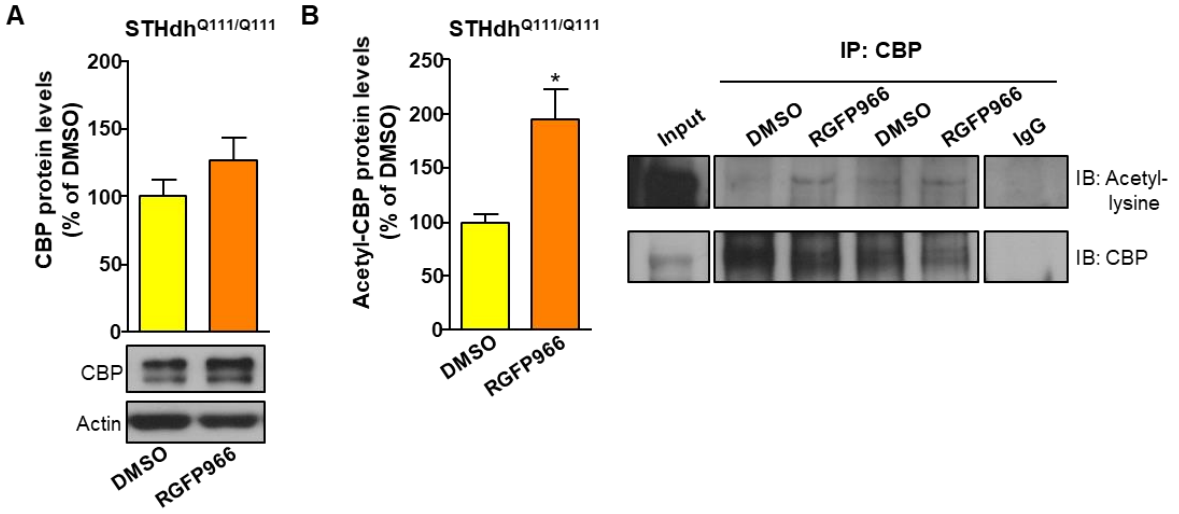



**Figure 30. RGFP966 treatment increases CBP acetylation in immortalized striatal cells.** (**A**) Representative immunoblots and densitometric analysis showing protein levels of CBP with actin as loading control in STHdh$^{Q111/Q111}$ striatal cells treated with DMSO or 10 μM RGFP966 for 6 hours. (**B**) Lysates from STHdh$^{Q111/Q111}$ striatal cells treated with DMSO or 10 μM RGFP966 for 6 hours were immunoprecipitated with an anti-CBP antibody, and levels of acetyl-CBP were evaluated after Western blot with an anti-acetyl-lysine antibody, followed by reprobing of the blot with the CBP antibody. Representative immunoblots and densitometric analysis are shown. $*P < 0.05$ compared to DMSO-treated cultures by Student's t-test. Histograms represent the relative protein levels expressed as percentage of DMSO values. All data represent the mean ± SEM ($n$ = 4-6 cultures/group).

## 1.4. HDAC3 contributes to striatal neuropathology in HD

Although mHtt is found throughout the HD brain, the striatum is classically considered the most severely affected structure in this disorder, playing a crucial role in the overt motor symptoms that characterize the disease. Importantly, the striatum has shown to exhibit the strongest transcriptional changes, as suggested by a comparative study that analyzed different tissues from a knock-in HD mouse model (Langfelder et al., 2016). Notably, some studies have shown the ability of selective HDAC3 inhibitors to improve the disease motor phenotype and some striatal transcriptional abnormalities (Jia et al., 2012b, 2015, 2016). In this context, the present thesis has further investigated the effects of RGFP966 in the striatal neuropathology of KI mice.

### 1.4.1. Chronic treatment with RGFP966 ameliorates expression of striatal markers in KI mice

Even at presymptomatic stages, reductions in mRNA and protein levels of dopamine- and cAMP-regulated phosphoprotein of molecular weight 32 kDa (DARPP-32), phosphodiesterase 10A (PDE10A) and adenosine receptor type 2A (A2AR) have been demonstrated as biomarkers of dysfunctional striatal neurons (Cha et al., 1999; Bibb et al., 2000; Glass et al., 2000; van Dellen et al., 2000; Hebb et al., 2004; Villar-Menéndez et al., 2013; Niccolini et al., 2015).

To evaluate whether a long-term treatment with RGFP966 was able to recover these proteins, we performed Western blot analysis in striatal extracts from chronically treated mice. As expected, levels of DARPP-32, PDE10A and A2AR were significantly reduced in vehicle-treated KI mice

compared to WT mice (Figure 31). Interestingly, chronic treatment with RGFP966 partially prevented the reduction of these striatal markers, restoring them closer to WT levels.

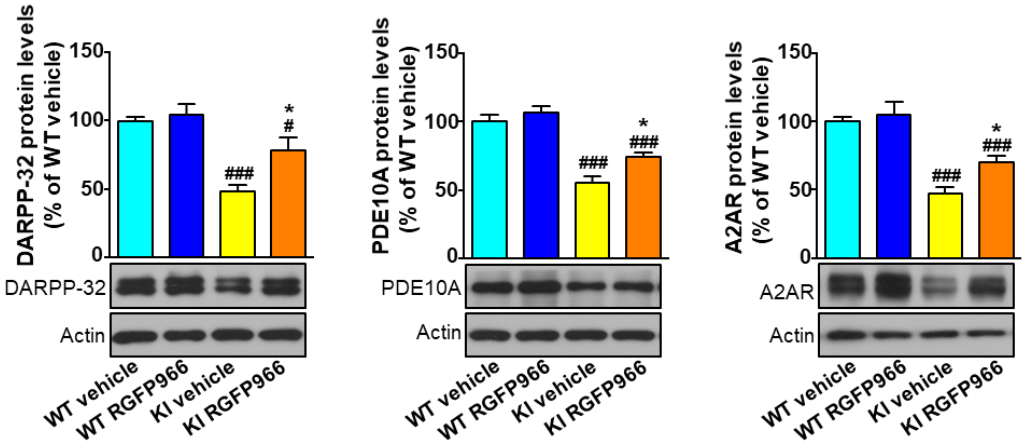



**Figure 31. RGFP966 treatment partially prevents the reduction of striatal markers in KI mice.** Representative immunoblots and densitometric analysis showing striatal protein levels of DARPP-32, PDE10A and A2AR, with actin as loading control, in vehicle and RGFP966 chronically treated WT and KI mice. $*P < 0.05$ compared to vehicle-treated KI mice; $\#P < 0.05$; $\#\#\#P < 0.001$ compared to vehicle-treated WT mice by two-way ANOVA with Bonferroni post-hoc analysis. Histograms represent the relative protein levels expressed as percentage of WT vehicle values. All data represent the mean ± SEM ($n$ = 6-11 mice/group).

## 1.4.2. Acute treatment with RGFP966 increases DARPP-32 expression in neuronal cultures

To elucidate whether the recovery of striatal biochemical markers was a direct effect of treatment with the HDAC3 inhibitor RGFP966 or a consequence of a generalized improvement in striatal pathology, primary striatal cultures at 10-14 DIV from WT and KI embryos were incubated with DMSO or 10 μM RGFP966 for 6 hours and levels of DARPP-32, PDE10A and A2AR were subsequently analyzed by Western blot. Though no significant differences in the expression of these striatal markers were found between DMSO-treated WT and KI striatal cultures, RGFP966 treatment led to a significant increase in DARPP-32 levels in both genotypes. Inhibitor treatment was without any significant effect in PDE10A or A2AR protein levels (Figure 32).

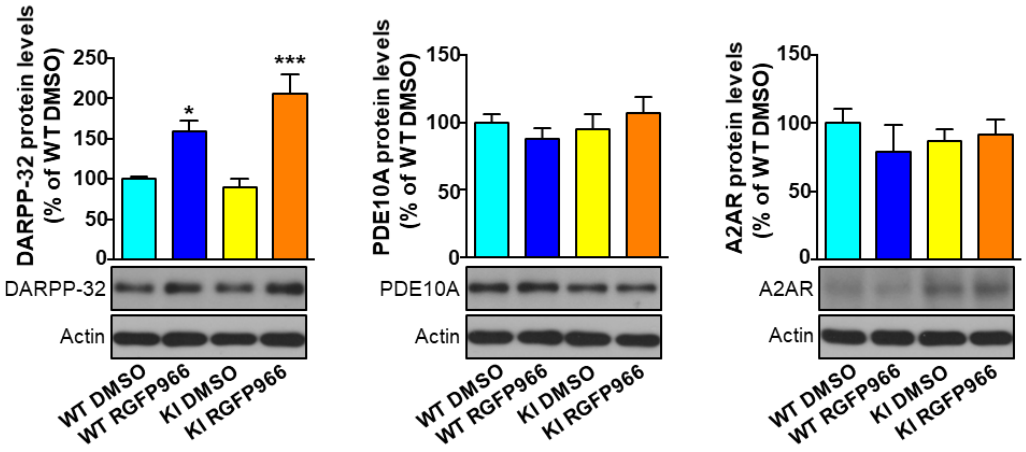



**Figure 32. RGFP966 treatment induces DARPP-32 expression in primary neurons.** Representative immunoblots and densitometric analysis showing protein levels of DARPP-32, PDE10A and A2AR with actin as

loading control in primary striatal cultures obtained from WT and KI embryos treated with DMSO or 10 μM RGFP966 for 6 hours. *$P < 0.05$ and ***$P < 0.001$ compared to DMSO-treated cultures by two-way ANOVA with Bonferroni post-hoc analysis. Histograms represent the relative protein levels expressed as percentage of WT DMSO values. All data represent the mean ± SEM ($n$ = 4-5 cultures/group).

These results suggest a direct induction of DARPP-32 expression because of RGFP966 treatment that could contribute to ameliorate mHtt-induced striatal damage.

### 1.4.3. Chronic treatment with RGFP966 reduces mutant huntingtin oligomerization

An important histopathological feature of HD is the property of mutant huntingtin to stick together, forming small oligomers that progressively develop into insoluble bigger aggregates (Vonsattel et al., 1985; DiFiglia et al., 1997; Bates, 2003). Given the positive effects of RGFP966 in the expression of protein striatal biomarkers, we analyzed the specific contribution of HDAC3 in the early aggregation process. To accomplish this, detergent-soluble fractions of striatal extracts from chronically treated KI mice were separated by SDS-PAGE. Using an antibody that specifically recognizes polyglutamine expanded proteins (1C2 antibody), we could visualize mHtt oligomers, trapped in the stacking gel, and the monomeric forms, which enter the resolving portion of the gel. Substantial accumulation of striatal mHtt oligomers was found in vehicle-treated KI mice, while treatment with RGFP966 prevented about one-half the accumulation of the oligomeric forms. In contrast, monomeric mHtt levels were unchanged by inhibitor treatment (Figure 33A). It is important to notice that the time of exposure to visualize the band corresponding to oligomeric mHtt forms was about 10 times higher than the one for monomeric mHtt, which could indicate that the second form is much more abundant than the first. To evaluate the effect of HDAC3 inhibition on wild-type Htt protein expression, we performed Western blot analysis in striatal extracts from chronically treated WT and KI mice using an antibody that recognizes both normal and aberrantly expanded huntingtin (2166 antibody), since there is a lack of specific commercial antibodies for the unexpanded form. Due to the difference in weight conferred by the polyglutamine tract, both wild-type and mutant proteins can be identified individually in an immunoblot. Thus, samples from WT mice show a single band corresponding to wild-type Htt while KI mice, which are heterozygous for the CAG-expanded tract in *Htt* gene, present a double band corresponding to the wild-type and the mutant protein. As expected, KI mice showed a ~50% reduction of wild-type Htt protein expression compared to WT mice. However, no differences in wild-type Htt levels were observed when comparing treatment conditions within the same genotype (Figure 33B). Finally, we evaluated whether RGFP966 might alter the transcriptional expression of the murine *Htt* gene by using a quantitative RT-PCR assay that measures both wild-type and mutant *Htt* mRNA. Consistent with a previous RNA-seq study on RGFP966-treated mice (Rumbaugh et al., 2015), we found no differences in total *Htt* mRNA levels between acutely RGFP966- and vehicle-treated mice within the same genotype (Figure 33C). Importantly, *Htt* mRNA levels from KI mice were slightly but significantly reduced compared to WT mice, as previously reported in other studies (Dixon et al., 2004; Lloret et al., 2006; Ament et al., 2017).

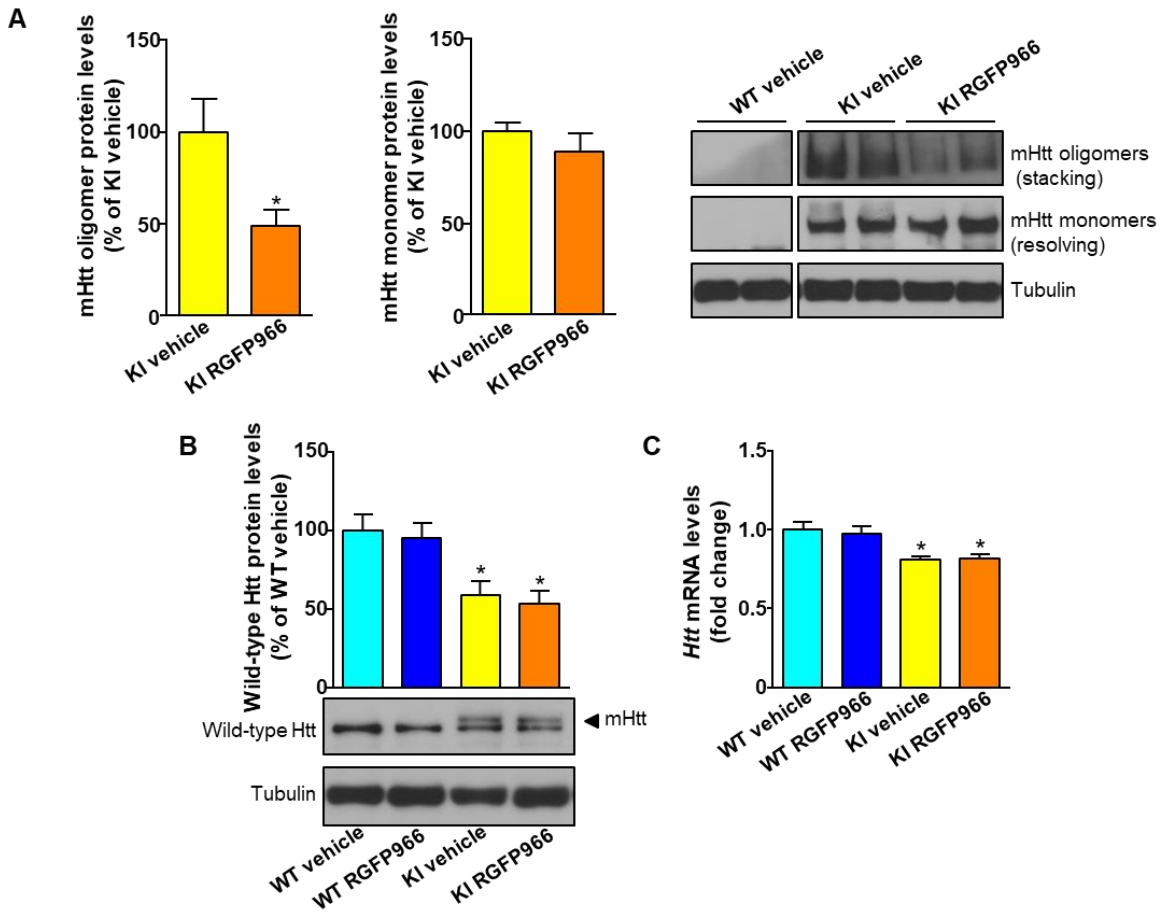



**Figure 33. RGFP966 treatment decreases mHtt oligomeric forms without altering wild-type huntingtin.** (**A**) Representative immunoblots and densitometric analysis showing protein levels of oligomeric (stacking gel) and monomeric (resolving gel) mutant huntingtin (1C2 antibody) with tubulin as loading control in vehicle and RGFP966 chronically treated KI mice. Representative immunoblots from vehicle-treated WT mice are also shown as a control. Histograms represent the relative protein levels expressed as percentage of KI vehicle values. *$P < 0.05$ compared with vehicle-treated KI mice by Student's t-test. (**B**) Representative immunoblots and densitometric analysis showing protein levels of wild-type huntingtin (2166 antibody) with tubulin as loading control in vehicle and RGFP966 chronically treated WT and KI mice. Histogram represents the relative protein levels expressed as percentage of WT vehicle values. *$P < 0.05$ compared to vehicle-treated WT mice by two-way ANOVA with Bonferroni post-hoc analysis. (**C**) Quantitative RT-PCR analysis of *Htt* in the striatum of vehicle and RGFP966 acutely treated WT and KI mice. Histogram represents relative *Htt* mRNA abundance expressed as fold change relative to WT vehicle. Levels of mRNA were normalized to 18S and Actinβ. *$P < 0.05$ compared to vehicle-treated WT mice by two-way ANOVA with Bonferroni post-hoc analysis. All data represent the mean ± SEM ($n$ = 6-10 mice/group).

Overall, these results suggest that RGFP966 reduces mHtt oligomerization process without altering m*Htt* transcription.

## 1.5. HDAC3 activity drives somatic CAG repeat expansions

Since HDAC3 has shown to mediate CAG repeat expansions *in vitro* (Debacker et al., 2012; Gannon et al., 2012), we were interested in examining if a chronic RGFP966 treatment could modulate trinucleotide instability *in vivo* and in evaluating the molecular mechanisms involved.

## 1.5.1. RGFP966 chronic treatment suppresses early somatic CAG repeat instability in KI mice

As mentioned before, the expanded CAG tract in m*Htt* gene has been reported to undergo progressive somatic instability with a preponderance of length increases, both in HD patients and HD mice. Greatest levels of instability are shown in brain regions that are most vulnerable in the disease (Wheeler et al., 1999; Kennedy and Shelbourne, 2000; Kennedy et al., 2003). Thus, the longest expansion lengths are observed in the striatum, followed by the cortex, while little expansion is present in the cerebellum, a region that is mildly affected in HD. Since heterozygous KI mice carry an expanded polyglutamine tract in one copy of its *Htt* gene, they provide an ideal model in which to study the instability of the CAG repeat tract in its appropriate genomic context. In order to confirm tissue-specific trinucleotide instability in this model, small-pool PCR analysis were first performed in striatal and cerebellar genomic extracts of 6-months-old KI mice. The allele length in tail at time of sacrifice was used as a control. As expected, there were many expansions present in the striatum of KI mice, judged by the increased molecular weight of the PCR products while little evidence was observed in cerebellar samples (Figure 34).

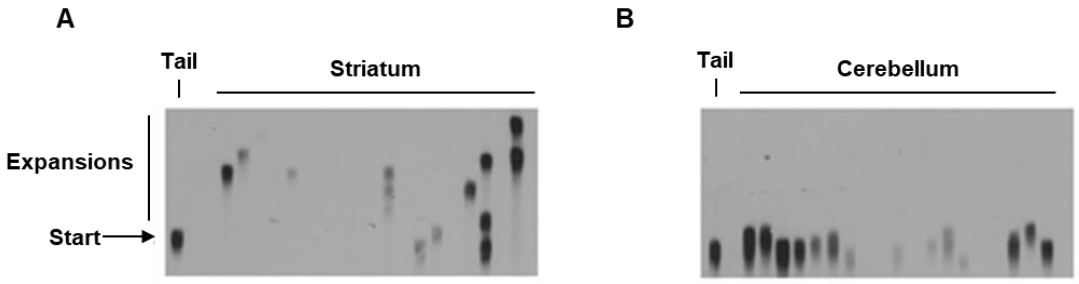



**Figure 34. KI mice exhibit striatal CAG repeat expansions**. Representative display of somatic CAG repeat lengths obtained by small-pool PCR from 6-months-old KI mice striatum (**A**) or cerebellum (**B**). Size markers correspond to CAG repeat number. Arrow indicates size of CAG repeat size in tail.

Next, small pool PCR analysis were performed in striatal and cerebellar genomic extracts from chronically treated KI mice with either RGFP966 or vehicle (Figure 35A). The starting tract size was deduced from the most common product length in the cerebellum of each animal. Since this number was similar for all mice examined, differences in initial CAG repeat length were eliminated as a confounding variable. We found predominant expansions in the striatum of vehicle-treated KI animals (72%) compared to unchanged alleles (21%) and a few contractions (7%) (Figure 35B). Interestingly, treatment with RGFP966 significantly reduced the frequency of expansions (44%), while it also led to a striking increase in the frequency of contractions (32%). The frequency data were corroborated by analysis of CAG repeat length changes (Figure 35C). Striatal changes in vehicle-treated KI animals spanned the range from -10 to +60 repeats with a weight-averaged length change of +13.3 repeats. RGFP966 treatment caused a leftward shift towards smaller size changes, with a weight-average repeat change of +8.1 repeats.

Overall, our results show that RGFP966 treatment substantially suppressed striatal CAG repeat expansions in KI mice.

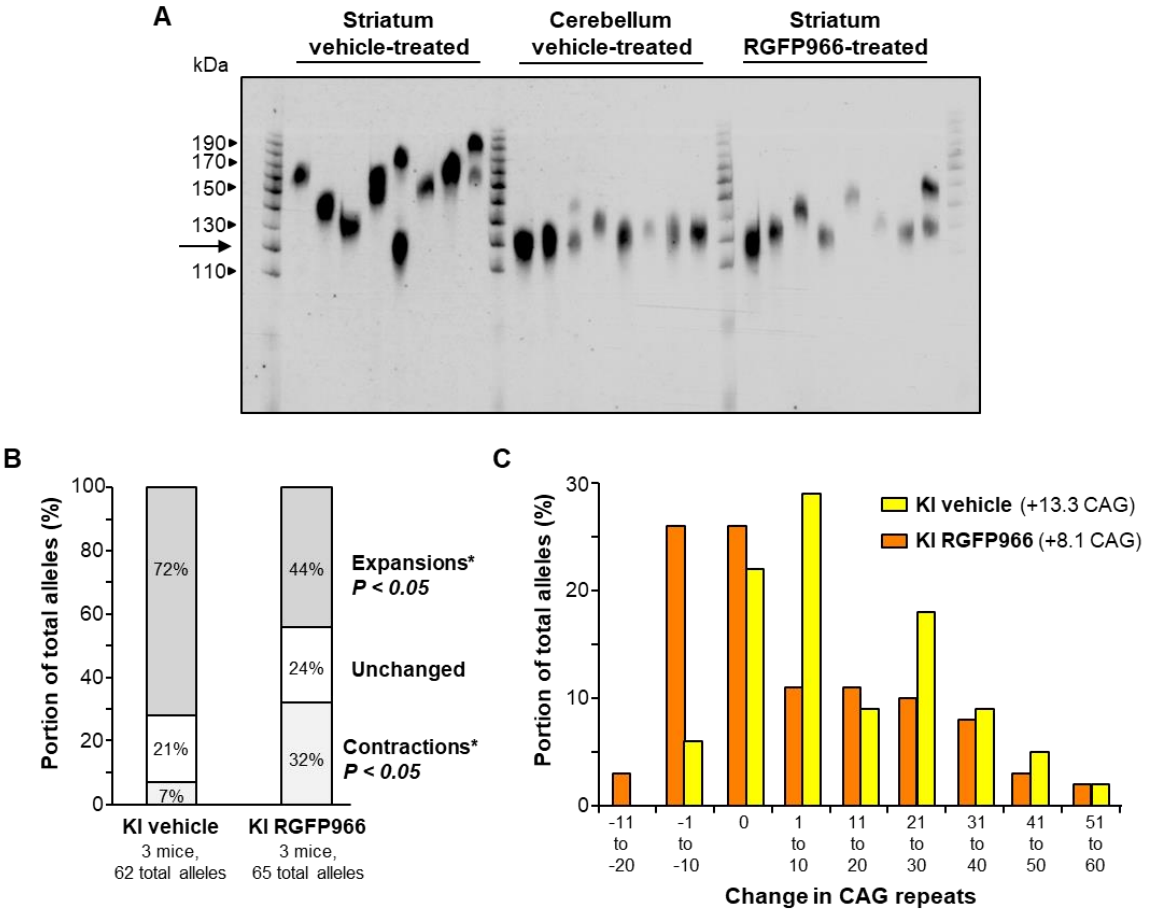



**Figure 35. RGFP966 chronic treatment suppresses striatal CAG repeat expansions in KI mice**. (**A**) Representative display of somatic CAG repeat lengths obtained by small-pool PCR from KI mice chronically treated with vehicle or RGFP966. Size markers correspond to CAG repeat number. The examples shown represent the positive signals from one analysis. Arrow indicates size of initial CAG tract. (**B**) Summary of genetic changes. Starting tract sizes, estimated from the most common allele size in cerebellum and stated as repeat units, were 110-119, 120-129 and 120-129 for vehicle-treated mice; and 120-129, 120-129 and 130-139 for RGFP966-treated mice. *$P < 0.05$ compared to vehicle-treated KI mice by Student's t-test. (**C**) Histogram of expansion sizes from the alleles tested in B, binned into 10-repeat size groups. Starting tract lengths were deduced from the most common allele size in the cerebellum of the same animals. For each group, $n = 3$ mice/group, 62-65 independent events.

## 1.5.2. HDAC3 inhibition increases Msh2 acetylation in neuronal cultures

Since our previous results indicated that HDAC3 activity could play a role in somatic CAG repeat expansions, we aimed to explore the molecular underlying mechanisms. Double knockdown analysis from a recent study showed that HDAC3 and MutSβ might act through a common pathway to promote expansions (Gannon et al., 2012). The Msh2 and Msh3 proteins conform the MutSβ heterodimer and both have been shown to contribute to somatic CAG repeat expansions in HD mouse models (Manley et al., 1999; Dragileva et al., 2009). Interestingly, it has been reported that Msh2 has many acetylation sites (Choudhary et al., 2009; Zhang et al., 2014; Radhakrishnan et al., 2015) and can interact with several HDACs, among them HDAC3 (Radhakrishnan et al., 2015). However, different acetylation sites can elicit distinct effects in cellular mismatch repair activity (MMR). For example, HDAC6 has shown to favor decreased MMR activity by promoting Msh2 degradation upon sequential deacetylation and ubiquitination (Zhang et al., 2014). In

contrast, Msh2 deacetylation by HDAC10 stimulates its activity and, accordingly, HDAC10 pharmacological inhibition reduces Msh2 function (Radhakrishnan et al., 2015). Since decreased MMR activity would be in accordance with suppression of CAG-repeat expansions in RGFP966-treated mice, we tested if HDAC3 could also modulate Msh2 at the same lysine residue deacetylated by HDAC10, lysine 73. As shown in Figure 36 (A and B), primary striatal neurons from WT or KI embryos or immortalized striatal STHdh cells treated with RGFP966 for 6 hours showed drug dependent increases in acetyl-Msh2 levels. Moreover, RGFP966 treatment for up to 24 hours did not change total Msh2 protein levels (Figure 36C). Unfortunately, the anti-acetyl-Msh2 antibody seemed to be unsuitable for tissue samples, since no clear band was detected at the corresponding weight when striatal extracts from chronically treated mice were used (data not shown).

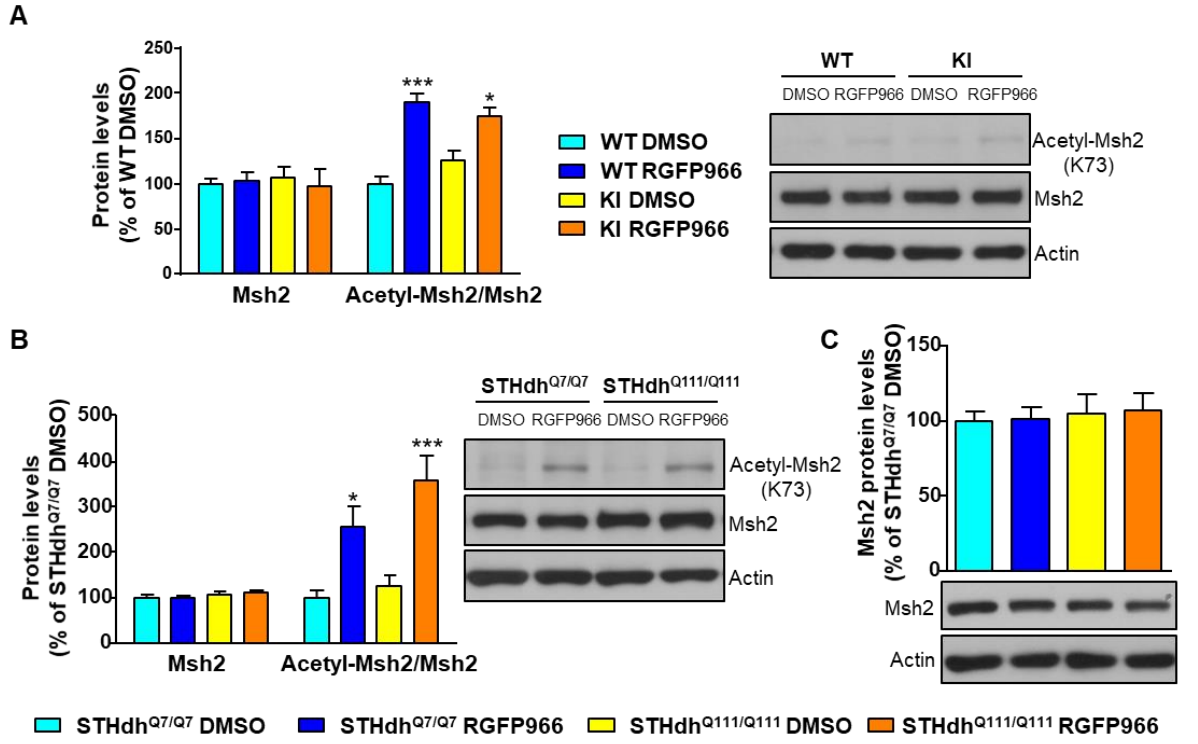



**Figure 36. Msh2 is acetylated at lysine 73 upon RGFP966 treatment.** Representative immunoblots and densitometric analysis showing protein levels of total Msh2 and acetylated Msh2 at lysine 73 with actin as loading control in primary striatal cultures obtained from WT and KI embryos (**A**) and in STHdh$^{Q7/Q7}$ and STHdh$^{Q111/Q111}$ striatal cells (**B**) treated with DMSO or 10 μM RGFP966 for 6 hours. *$P$ < 0.05 and ***$P$ < 0.001 compared to DMSO-treated cultures by two-way ANOVA with Bonferroni post-hoc analysis. (**C**) Representative immunoblots and densitometric analysis showing protein levels of total Msh2 with actin as loading control in STHdh$^{Q7/Q7}$ and STHdh$^{Q111/Q111}$ striatal cells treated with DMSO or 10 μM RGFP966 for 24 hours. Histograms represent the relative Msh2 protein levels or the relative Acetyl-Msh2/Msh2 ratios expressed as percentage of WT DMSO or STHdh$^{Q7/Q7}$ DMSO values. All data represent the mean ± SEM ($n$ = 3-6 cultures/group).

Overall, these results indicate that HDAC3 inhibition increases Msh2 acetylation at lysine 73, suggesting that RGFP966 treatment may impair MutSβ function without altering Msh2 protein stability.

## 1.6. HDAC3 protein levels and phosphorylation status in HD

Given that multiple benefits are observed upon HDAC3-selective inhibition in KI mice, we wondered if HDAC3 activity or subcellular distribution could be altered in HD, since our results and previous published reports have demonstrated unaltered HDAC3 total protein levels in HD mouse models (Valor and Guiretti, 2014). Interestingly, a recent report has demonstrated increased HDAC3 nuclear versus cytoplasmic distribution in the cortex of late stage N171-82Q transgenic HD mice, suggesting that nuclear HDAC3 could contribute to a repressive chromatin environment (Jia et al., 2012b). To analyze if the subcellular HDAC3 location was also altered in KI mice at the age at which we performed the *in vivo* RGFP966 treatments, nuclear and cytosolic fractions from the striatum of WT and KI mice were obtained at 6 months of age. HDAC3 was visualized in both fractions, but was notably enriched in the cytosolic compartment. However, no differences were detected between WT and KI animals in any of the fractions (Figure 37).

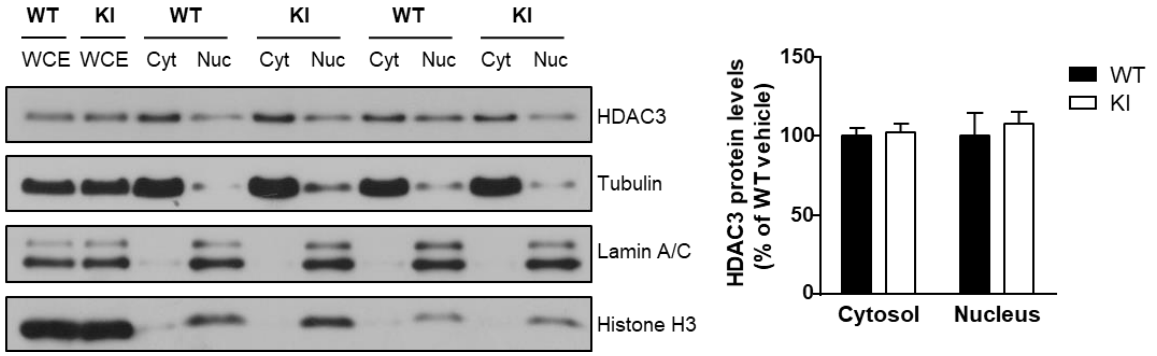



**Figure 37. Subcellular distribution of HDAC3 in KI mice.** Representative immunoblots and densitometric analysis showing HDAC3 protein levels in whole cell extracts (WCE) and in nuclear (Nuc) and cytosolic (Cyt) fractions from striatal extracts of WT and KI mice at 6 months of age. Tubulin was used as loading control for cytosolic fractions and lamin A/C and histone H3 for nuclear fractions. Histograms represent the relative protein levels expressed as percentage of WT values. All data represent the mean ± SEM ($n$ = 6-9 animals/group).

Another way to measure HDAC3 activity is evaluating its phosphorylated levels, as it was discovered in 2005 that phosphorylation of HDAC3 at serine 424 yields an increase in its deacetylase enzymatic activity (Zhang et al., 2005). Therefore, to evaluate HDAC3 activity, total and phosphorylated levels of HDAC3 were analyzed by Western blot in striatal and hippocampal extracts from WT and KI mice along disease progression. Interestingly, we found increased phospho-HDAC3 levels in the striatum of KI mice compared to WT mice starting at 8 months of age (Figure 38A), while the hippocampal region was spared of any alteration (Figure 38B). Interestingly, at 8 months of age KI mice start to present motor coordination deficits (Puigdellívol et al., 2015), which are strongly dependent on striatal function.

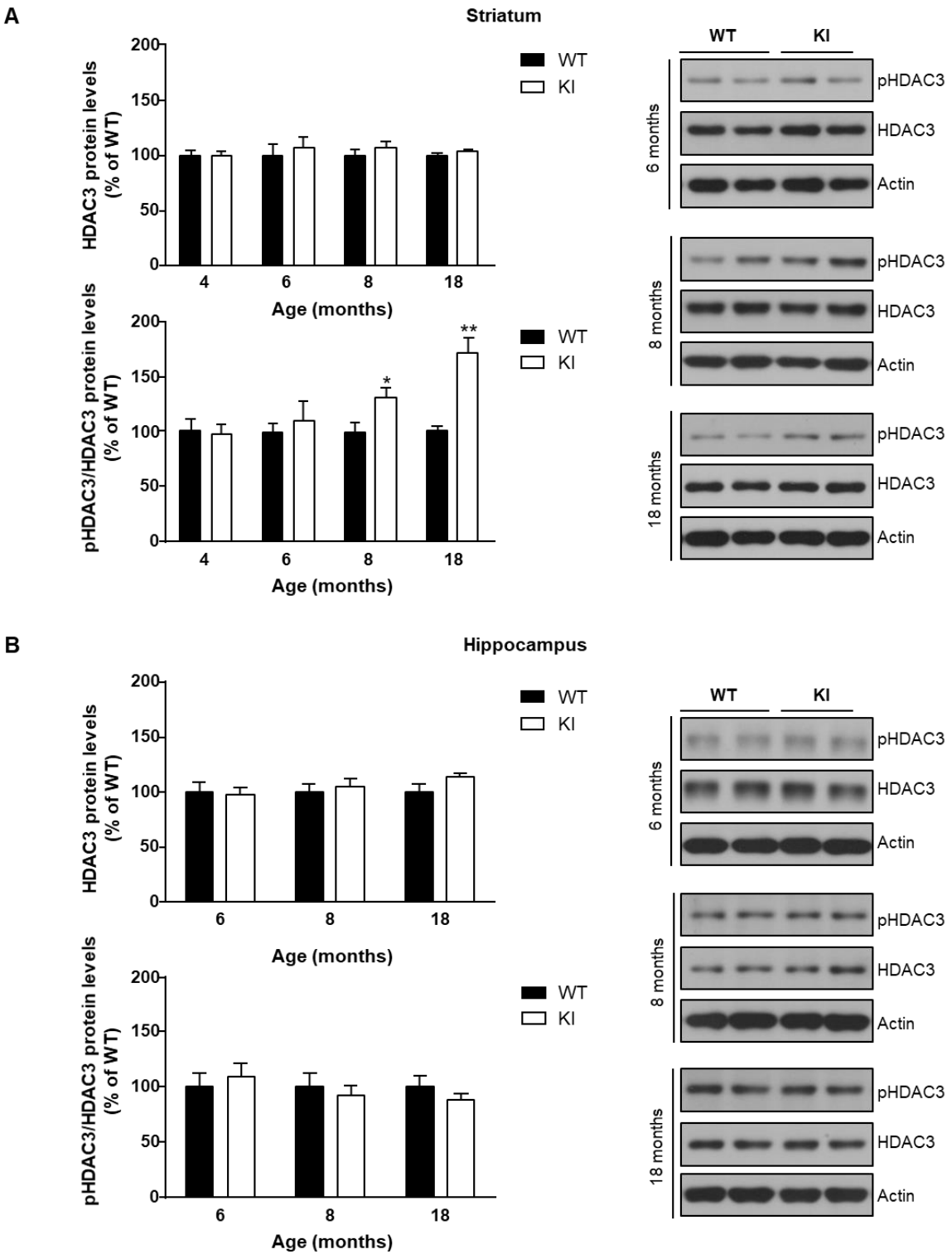



**Figure 38. HDAC3 phosphorylation is increased in a region-specific manner in the KI mouse brain.** Representative immunoblots and densitometric analysis showing protein levels of HDAC3 and phospho-HDAC3 (pHDAC3) with actin as loading control in WT and KI striatum at 4, 6, 8, and 18 months (**A**) or hippocampus at 6, 8, and 18 months (**B**). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with WT mice by Student's t-test. Histograms represent the relative HDAC3 protein levels or the relative ratios of pHDAC3/HDAC3 expressed as percentage of WT values. All data represent the mean ± SEM ($n$ = 6-9 mice/group).

To evaluate if these alterations were also present in HD patients, we performed Western blot analysis in post-mortem putamen samples from clinically diagnosed HD patients and unaffected controls. Interestingly, as in the striatum of KI mice, phosphorylated HDAC3 levels were significantly increased in HD putamens, while total HDAC3 protein levels remained unchanged (Figure 39).

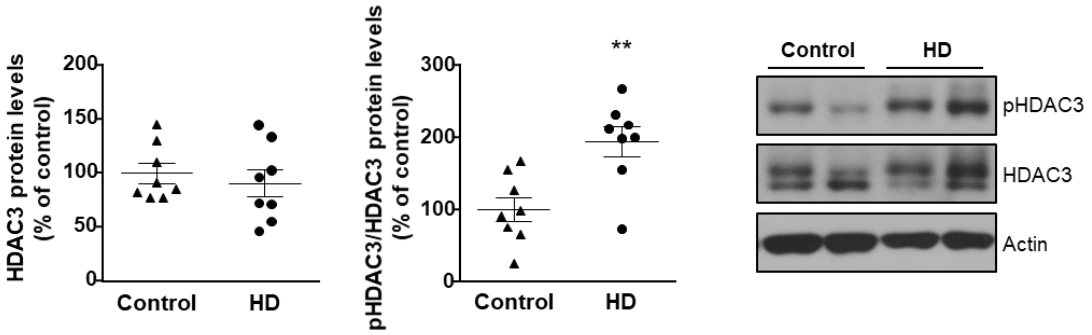



**Figure 39. HDAC3 phosphorylation is increased in the putamen of HD human patients.** Representative immunoblots and densitometric analysis showing protein levels of HDAC3 and phospho-HDAC3 (pHDAC3) with actin as loading control in the putamen of HD patients and control subjects. $^{**}P < 0.01$ compared with control subjects by Student's t-test. Histograms represent the relative HDAC3 protein levels or the relative ratios of pHDAC3/HDAC3 expressed as percentage of control values. All data represent the mean ± SEM ($n$ = 8 patients/group).

Overall, these results indicate that HDAC3 activity is aberrantly increased in the most affected HD region, the striatum, at a time of illness in which striatal-dependent motor disturbances are present. Additionally, since HDAC3 has been linked to neuronal toxicity (Bardai and D'Mello, 2011; Schmitt et al., 2014), this could have important implications for neuroprotective therapies in HD.

# 2. Benefits of p75$^{NTR}$ modulation in HD

Lack of neurotrophic support due to reduced BDNF levels has shown to contribute to the selective vulnerability of medium spiny neurons (MSN) in HD (Zuccato et al., 2001; Gauthier et al., 2004). However, therapeutic strategies aimed to raise BDNF levels only partially improved some disease phenotypes in different HD mouse models (Gharami et al., 2008; Giralt et al., 2010; Xie et al., 2010; Simmons et al., 2013). BDNF function has been linked to the optimal expression and activity of its receptors, TrkB and p75$^{NTR}$, through which it will promote the appropriate stimulus for neuronal maintenance or death. Reduced TrkB levels and signaling have been observed in the striatum of mouse and cellular HD models and in HD patients (Ginés et al., 2006, 2010; Zuccato et al., 2008; Brito et al., 2013; Liot et al., 2013; Simmons et al., 2013; Plotkin et al., 2014; Nguyen et al., 2016) along with increased p75$^{NTR}$ levels (Zuccato et al., 2008; Brito et al., 2013; Simmons et al., 2016). This imbalance between receptors is thought to increase the vulnerability of striatal neurons to excitotoxic insults. Additionally, previous studies from our group demonstrated an increase of p75$^{NTR}$ levels in the hippocampus of Hdh$^{Q7/Q111}$ and R6/1 mice, early in the disease process, which was associated with HD early cognitive impairments and dendritic spine loss (Brito et al., 2014; Miguez et al., 2015).

Overall, the present thesis aims to deeply explore the contribution of p75$^{NTR}$ protein levels to different neuropathological features and to the onset and progression of behavioral abnormalities in a HD mouse model.

## 2.1. Genetic manipulation of p75$^{NTR}$ in mice

Several p75$^{NTR}$ deficient mouse strains have been used to study the role of this receptor in its physiological context. The first p75$^{NTR}$ mutant mouse was generated with a targeted deletion of exon III, resulting in a protein that lacks the extracellular domain responsible for neurotrophin binding (Lee et al., 1992). By cross-mating Hdh$^{Q7/Q111}$ (KI) mice with heterozygous p75$^{NTR}$/Exon III (p75$^{+/-}$) mice we successfully generated double mutant (KI:p75$^{+/-}$) mice, carrying one copy of m*Htt* while a single functional *p75$^{NTR}$* allele (Brito et al., 2014), allowing the study of p75$^{NTR}$ function in HD.

### 2.1.1. p75$^{NTR}$ protein levels are normalized in the striatum of KI:p75$^{+/-}$ mice

p75$^{NTR}$ mRNA expression has been found increased in the caudate nucleus of human HD patients (Zuccato et al., 2008) and previous studies from our group have demonstrated increased protein levels in the striatum of Hdh$^{Q111/Q111}$, Hdh$^{Q7/Q111}$ and R6/1 mice (Brito et al., 2013, 2014). In order to explore the contribution of p75$^{NTR}$ to HD pathology along the disease progression, we first characterized our double KI:p75$^{+/-}$ mice by evaluating striatal p75$^{NTR}$ protein levels at 6, 8 and 10 months by Western blot. As expected, levels were found aberrantly increased in KI mice and consistently normalized in KI:p75$^{+/-}$ mice, compared to WT mice, at all ages evaluated (Figure 40A). Additionally, we corroborated unaltered p75$^{NTR}$ protein levels in cortical regions from KI mice at 6 months of age. Thus, both p75$^{+/-}$ and KI:p75$^{+/-}$ mice presented reduced cortical p75$^{NTR}$ levels compared to WT mice (Figure 40B).

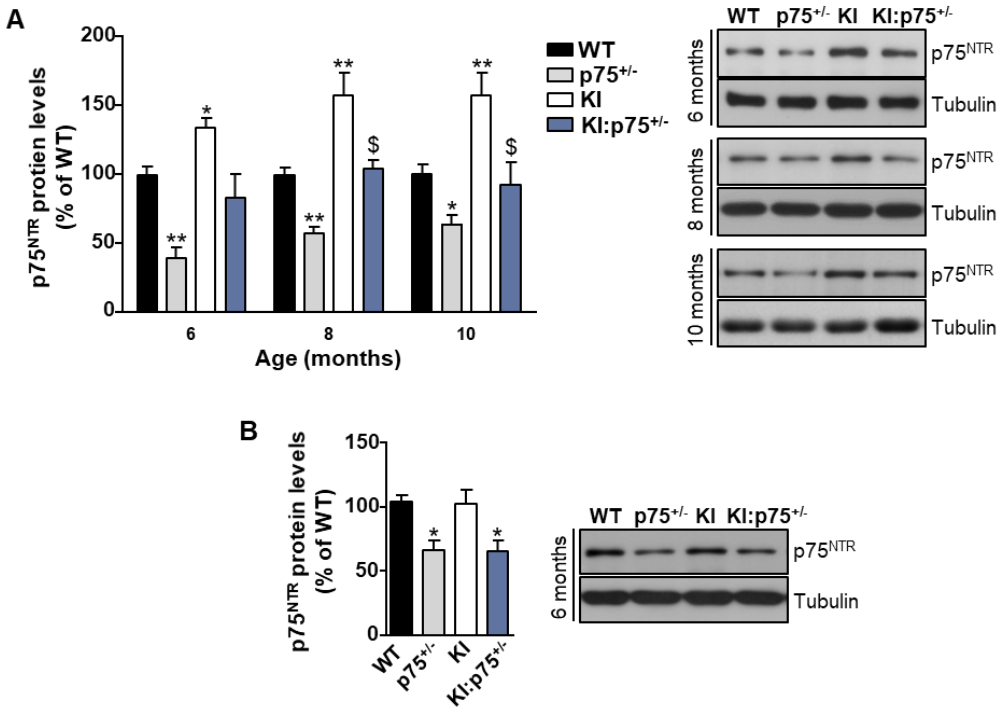



**Figure 40. Genetic normalization of p75$^{NTR}$ protein levels in the striatum of HD mice.** Representative immunoblots and densitometric analysis showing protein levels of p75$^{NTR}$ with tubulin as loading control in striatal extracts obtained from WT, p75$^{+/-}$, KI and KI:p75$^{+/-}$ mice at 6, 8 and 10 months of age (**A**) and in cortical extracts obtained from WT, p75$^{+/-}$, KI and KI:p75$^{+/-}$ mice at 6 months of age (**B**). *$P < 0.05$ and **$P < 0.01$ compared with WT; \$$P < 0.05$ compared with KI by one-way ANOVA with Tukey's post-hoc analysis. Histograms represent the relative protein levels expressed as percentage of WT values. All data are shown as the mean ± SEM ($n$ = 5-7 mice/group).

## 2.2. Increased striatal p75$^{NTR}$ levels contribute to motor deficits in HD

Although BDNF/TrkB/p75$^{NTR}$ alterations have been reported in HD striatum, the brain region most severely affected in the disease and mainly responsible for motor abnormalities, the specific contribution of neurotrophic signaling imbalance to the onset of HD motor symptoms remains to be elucidated.

### 2.2.1. p75$^{NTR}$ genetic normalization delays the onset of motor coordination deficits in KI mice

To evaluate motor behavior, WT, KI, p75$^{+/-}$ and KI:p75$^{+/-}$ mice were first trained on the rotarod at a fixed-speed of 10 rpm during three consecutive days, in which all genotypes achieved proper learning of the task (Figure 41A, left). Then, performance in the rotarod at 10 and 24 rpm was assessed longitudinally from 7 to 12 months of age (Figure 41A, right). Motor coordination was severely impaired in 8-months-old KI mice worsening thereafter, with a number of falls from the rotarod significantly higher than WT mice along trials. Interestingly, KI:p75$^{+/-}$ mice exhibited a significant reduction in the number of falls compared with KI mice at 8 months (when evaluated at 24 rpm) and at 9 months (when evaluated at 10 and 24 rpm).

Similar results were obtained when spontaneous locomotor activity was measured in the open field (Figure 41B). KI mice displayed a significant reduction in the total distance traveled compared with WT mice at 8 and 10 months of age, while KI:p75$^{+/-}$ mice only displayed significant differences at 10 months of age. At 8 months, performance in open field by KI:p75$^{+/-}$ mice was comparable to WT mice, traveling more distance than KI mice, although these differences were not statistically significant ($P = 0.053$).

Altogether, these data indicate that normalization of striatal p75$^{NTR}$ levels in KI mice delays the onset of motor coordination impairments.

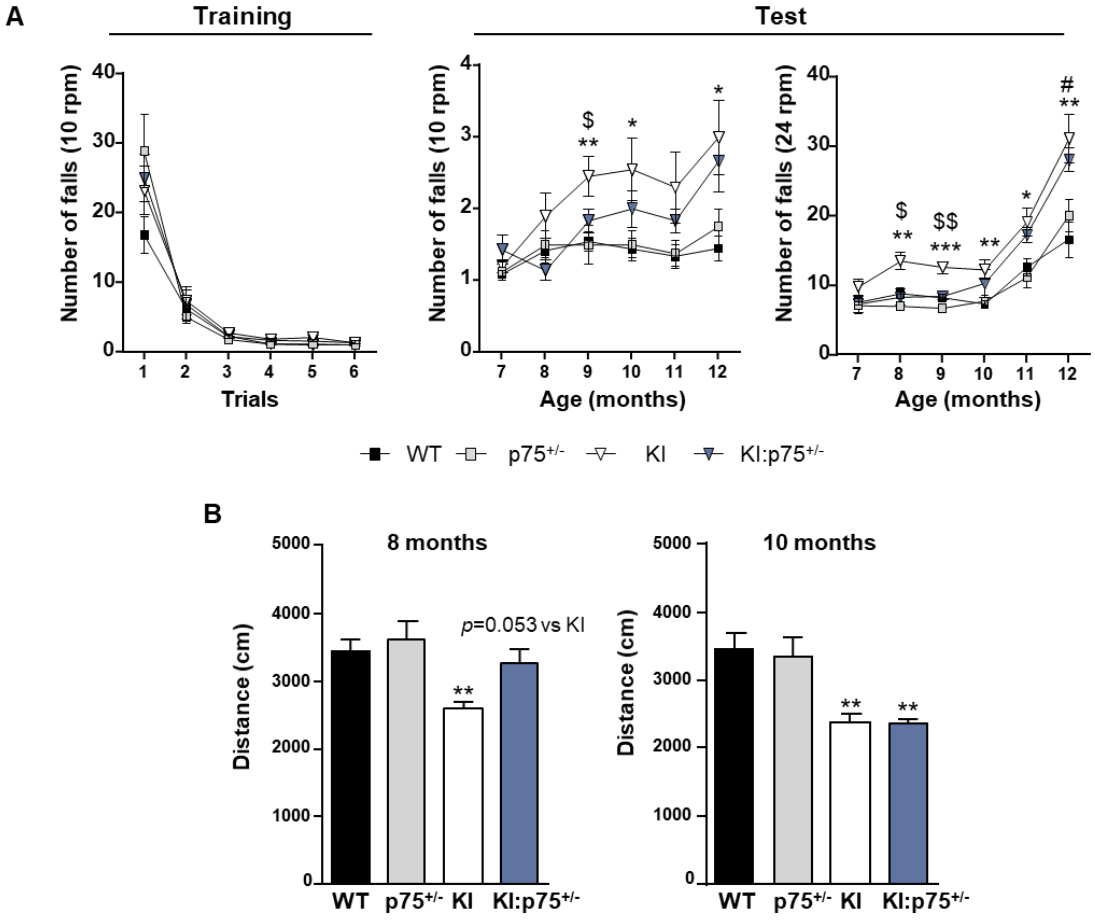


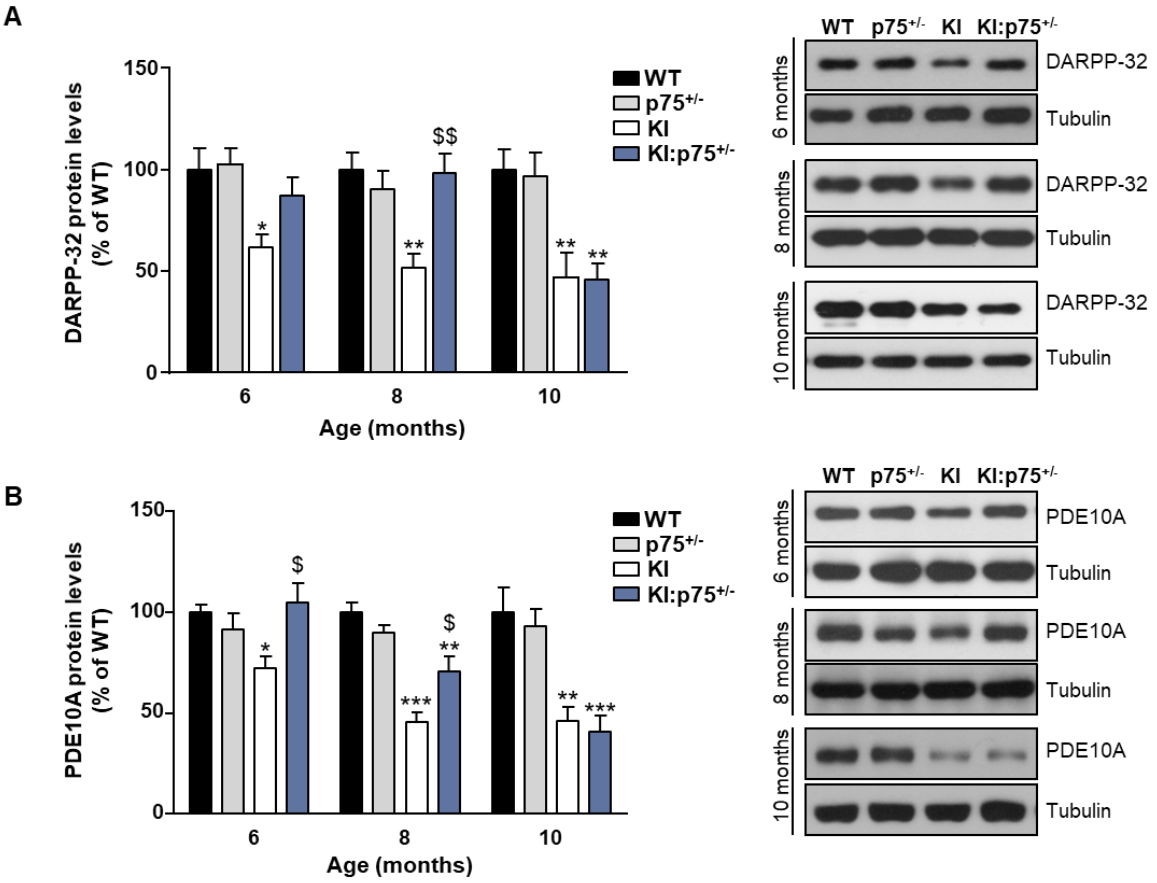

**Figure 41. Normalization of p75$^{NTR}$ expression in KI mice delays the onset of motor deficits.** Motor behavior was analyzed in WT, p75$^{+/-}$, KI and KI:p75$^{+/-}$ mice by performing the rotarod task (**A)** and the open field test (**B**). (**A**) During training (left), at the age of 5-6 months, number of falls from a fixed-speed rotarod at 10 rpm for 60 seconds was recorded. During testing (right), at 7 to 12 months of age, number of falls from a fixed-speed rotarod at 10 rpm and 24 rpm for 60 seconds was recorded. At different stages of the disease, progression data were analyzed by one-way ANOVA followed by Tukey's test. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$: KI compared with WT; \$$P < 0.05$ and \$\$$P < 0.01$: KI:p75$^{+/-}$ compared with KI mice; #$P <0.05$: KI:p75$^{+/-}$ compared with WT mice. (**B**) Spontaneous locomotor activity in the open-field test at 8 and 10 months of age. **$P <0.01$ compared with WT by one-way ANOVA with post-hoc analysis. All data are shown as the mean ± SEM ($n$ = 10-13 animals/genotype).

## 2.3. Increased p75$^{NTR}$ levels contribute to striatal neuropathology in HD

Given that HD motor impairments mostly depend on striatum and p75$^{NTR}$ normalization in KI mice delayed such impairments, we aimed to study the contribution of aberrant p75$^{NTR}$ levels to striatal pathology.

### 2.3.1. Normalization of p75$^{NTR}$ delays the reduction of striatal markers in KI mice

As previously commented, reduced levels of DARPP-32, PDE10A or A2AR have been found at early stages in Hdh$^{Q7/Q111}$ mice, as well as in other HD models and HD patients, and have been associated with neuronal dysfunction (Cha et al., 1999; Bibb et al., 2000; Glass et al., 2000; van Dellen et al., 2000; Hebb et al., 2004; Villar-Menéndez et al., 2013; Niccolini et al., 2015). As expected, when levels of these three proteins were analyzed in striatal extracts from KI mice at 6, 8 and 10 months of age they were found significantly reduced. Interestingly, in KI:p75$^{+/-}$ mice these striatal markers were preserved until 10 months of age, when a similar reduction to KI mice was observed (Figure 42).



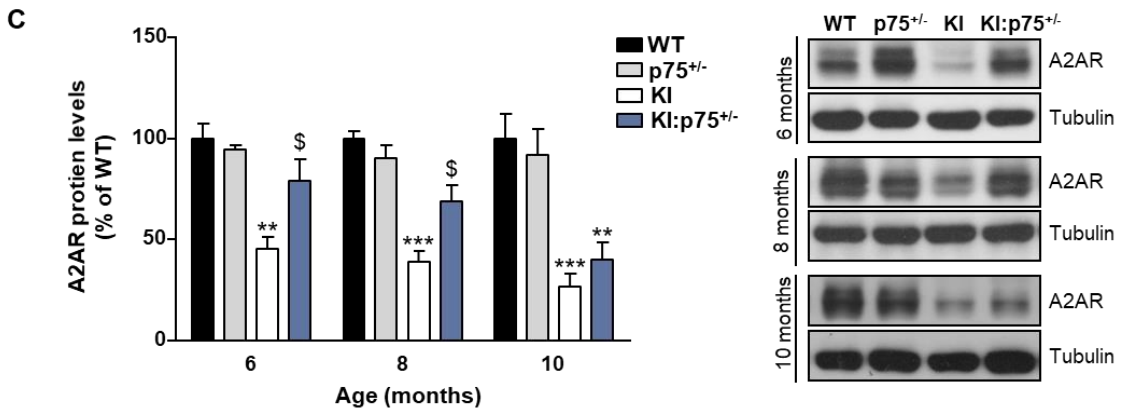



**Figure 42. Normalization of p75$^{NTR}$ expression in KI mice delays the reduction in striatal markers.** Representative immunoblots and densitometric analysis showing protein levels of DARPP-32 (**A**), PDE10A (**B**) and A2AR (**C**) with tubulin as loading control in striatal extracts obtained from WT, p75$^{+/-}$, KI and KI:p75$^{+/-}$ mice at 6, 8 and 10 months of age. *$P$ < 0.05 and **$P$ < 0.01 compared with WT; \$$P$ < 0.05 compared with KI by one-way ANOVA with Tukey's post-hoc analysis. Histograms represent the relative protein levels expressed as percentage of WT values. All data are shown as the mean ± SEM ($n$ = 5-7 mice/group).

## 2.3.2. Normalization of p75$^{NTR}$ delays mHtt aggregation in KI mice

The presence of mHtt aggregates is another neuropathological hallmark of HD (Vonsattel et al., 1985; DiFiglia et al., 1997; Bates, 2003). A great tool to evaluate aggregate formation in our HD mouse model at middle/late disease stages is the monoclonal EM48 antibody. This antibody was raised against the first 256 amino acids of human Htt lacking the polyglutamine tract, but it has shown to specifically recognize aggregated forms of the protein, probably because of conformational changes during the aggregation process that expose the epitope. By using this antibody, previous reports have found that Hdh$^{Q7/Q111}$ mice (in the C57BL/6J genetic background) exhibit a striatal population with early (2.5-5 months) diffuse nuclear staining that progressively increase in intensity and number. At 6 months, there are already few visible neuronal intranuclear inclusions (NIIs), increasing in size and density as disease progresses (Lloret et al., 2006; Kovalenko et al., 2012; Ament et al., 2017). Therefore, we evaluated the specificity of the EM48 antibody performing DAB (3,3'-diaminobenzidine) immunohistochemical analysis in coronal corticostriatal and hippocampal slices from WT and KI mice at 8-9 months of age. As expected, we found specific nuclear diffuse staining and strong immunolabelled circular inclusions (NII) in the striatum of KI mice, while other regions (such as the cortex or the hippocampus) did not show visible staining (Figure 43).

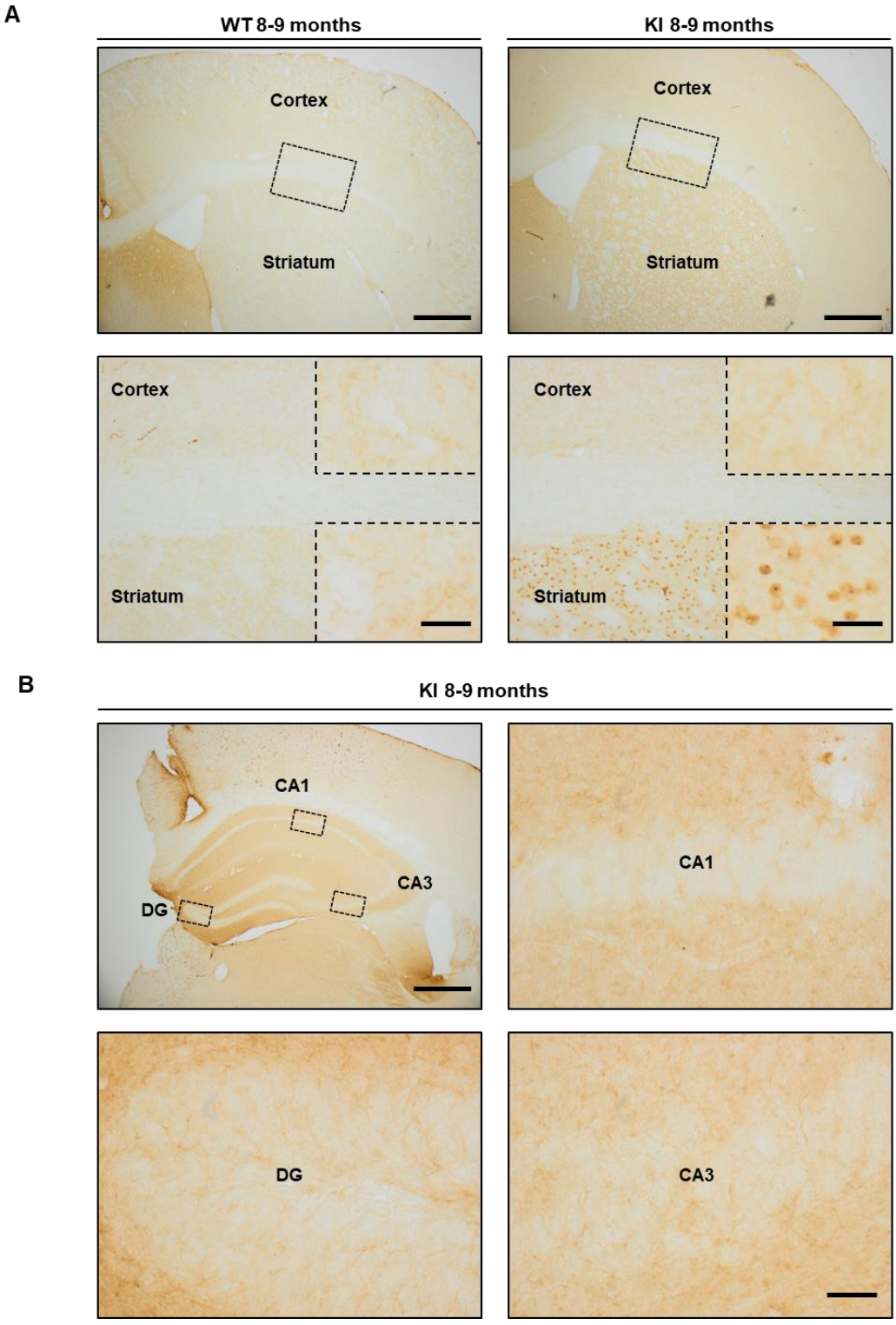



**Figure 43. Region-specific EM48 immunoreactivity. (A)** Representative photomicrographs showing EM48 immunostaining in corticostriatal regions from WT or KI mice at 8-9 months of age, with medium and high magnification insets. Scale Bar: 500 μm (low magnification) or 20 μm (high magnification). **(B)** Representative photomicrographs showing EM48 immunostaining in hippocampal regions from KI mice at 8-9 months of age, with high magnification insets. Scale Bar: 500 μm (low magnification) or 20 μm (high magnification).

Next, to address whether $p75^{NTR}$ levels could modulate mHtt aggregate formation, we performed the same analysis in corticostriatal slices from KI and KI:$p75^{+/-}$ mice at 8-9 and 10 months of age. Importantly, a significant reduction in both the number of positive nuclear inclusions and diffuse EM48 labeling intensity (staining index) was found in KI:$p75^{+/-}$ compared with KI mice at 8-9 months (Figure 44A), while similar aggregate density and staining index was observed at 10 months in both KI and KI:$p75^{+/-}$ mice (Figure 44B).

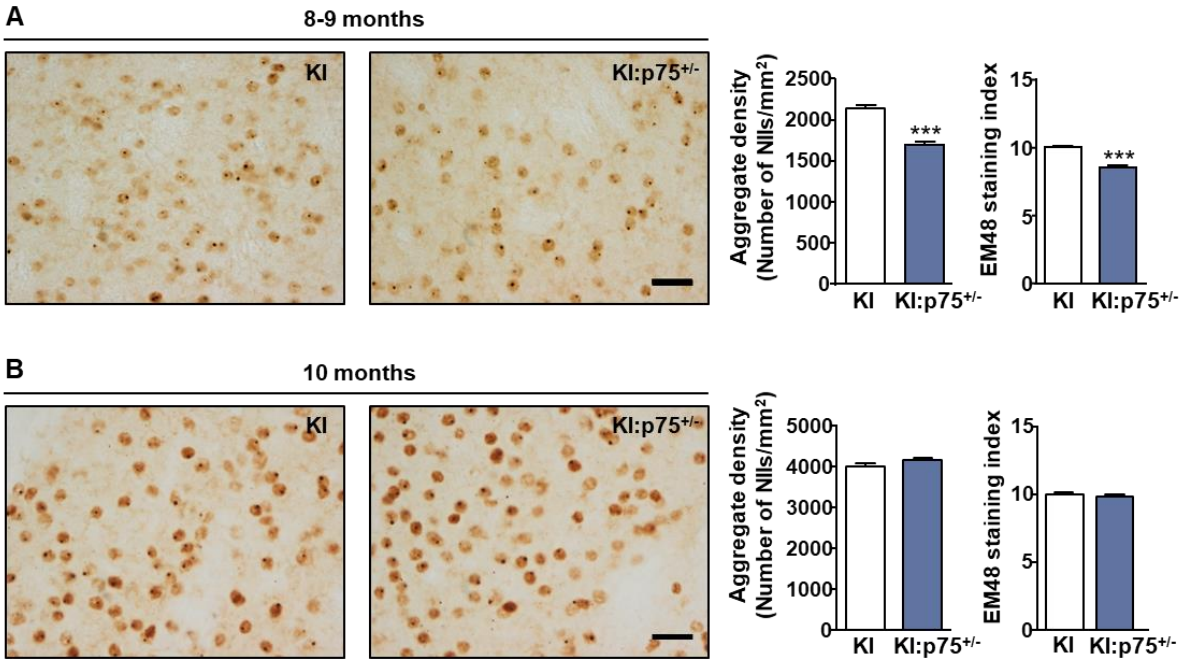



**Figure 44. Normalization of $p75^{NTR}$ expression in KI mice reduces early mutant huntingtin aggregates.** Representative photomicrographs showing nuclear EM48 immunostaining in the dorsal striatum of 8-9- (**A**) and 10-months-old (**B**) KI and KI:$p75^{+/-}$ mice. Scale Bar: 20 μm. Histograms represent density of EM48 positive neuronal intranuclear inclusions (NIIs) and "staining index", which captures both the nuclear staining intensity and the number of immunostained nuclei. Diffuse huntingtin immunostaining was lighter and surrounded the huntingtin aggregates. ***$P < 0.001$ compared with KI mice by Student's t-test. Data are shown as the mean ± SEM ($n$ = 600-900 images from 3-4 mice).

## 2.3.3. Normalization of $p75^{NTR}$ delays dendritic spine pathology in KI mice

Dendritic spine loss in MSNs has been reported in HD patients (Graveland et al., 1985; Ferrante et al., 1991) and mouse models (Guidetti et al., 2001; Spires et al., 2004a; Xie et al., 2010; Murmu et al., 2013). To analyze if $p75^{NTR}$ levels could modulate structural synaptic changes in HD striatum, DiOlistic labeling was performed in fixed brain slices from WT, $p75^{+/-}$, KI and KI:$p75^{+/-}$ mice at 3, 7-8 and 10 months of age and dendritic spine density was measured as the number of spines per micrometer of dendrite length (Figure 45). At 3 months (Figure 45A), there were no differences between groups. Consistently, previous data from our group reported a lack of dendritic spine alterations in the striatum of 2-months-old KI mice (Puigdellívol et al., 2015). By contrast, we found significant fewer spines in KI mice compared to WT mice at either 7-8 months (Figure 45B) or 10 months of age (Figure 45C). Genetic $p75^{NTR}$ downregulation significantly prevented spine density loss in KI mice, with a complete restoration at 7-8 months or a partial one at 10 months of age. Surprisingly, $p75^{+/-}$ mice exhibited a significant increase in the number dendritic spines

compared to WT mice at 7-8 months of age, while an increasing trend was observed at 10 months of age. These results are in accordance with the negative role of $p75^{NTR}$ in dendritic spine density previously reported by our group and others (Zagrebelsky et al., 2005; Chapleau and Pozzo-Miller, 2012; Brito et al., 2014).

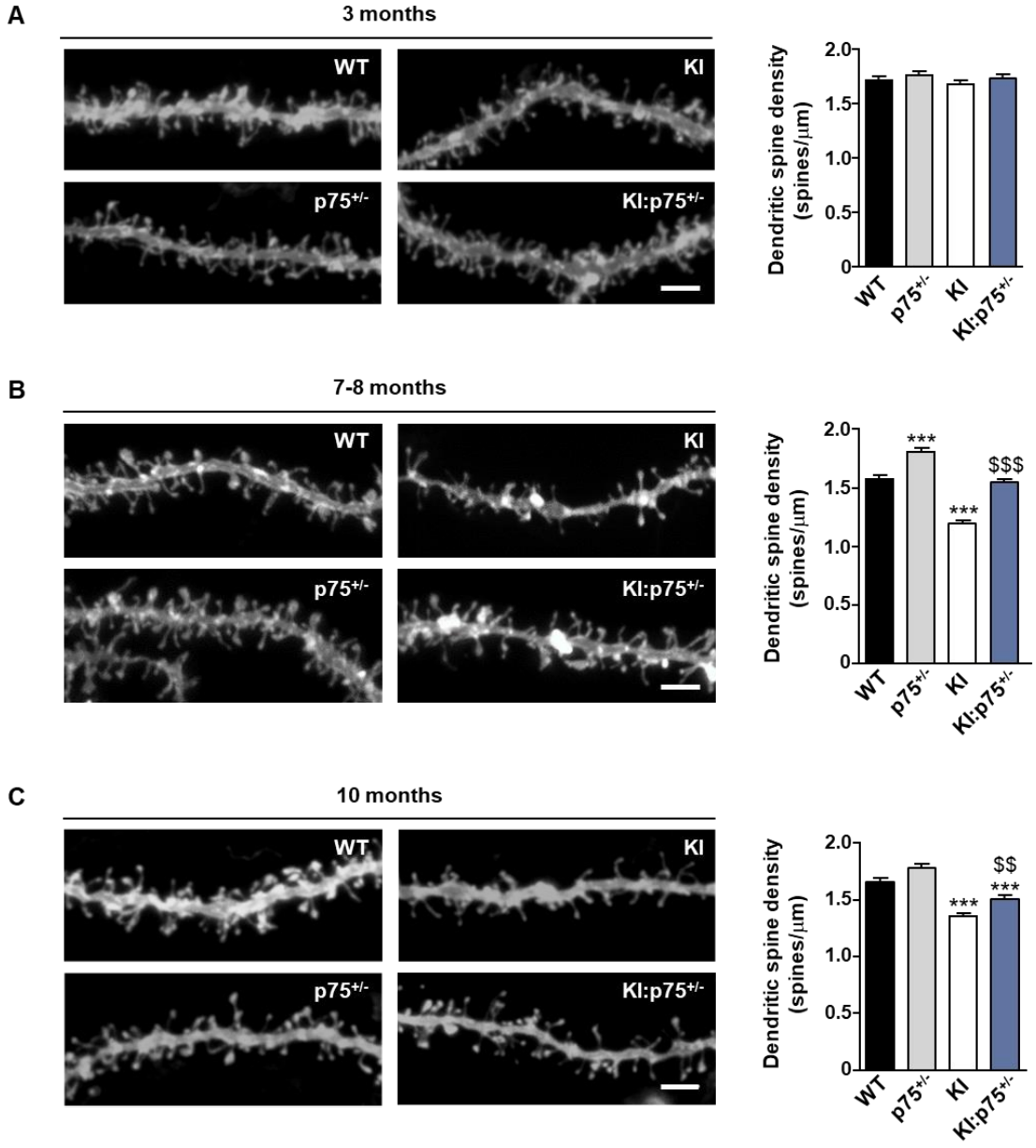



**Figure 45. Normalization of $p75^{NTR}$ expression in KI mice delays the onset of dendritic spine pathology.** Representative photomicrographs showing dendrites of medium spiny neurons from WT, $p75^{+/-}$, KI, and KI:$p75^{+/-}$ mice at 3 (**A**), 7-8 (**B**) and 10 months of age (**C**). Scale Bar: 3 μm. Histograms show quantitative analysis of dendritic spine density per micrometer of dendritic length. ***$P < 0.001$ compared with WT mice; $^{\$\$}P < 0.01$ and $^{\$\$\$}P < 0.001$ compared with KI mice by one-way ANOVA with Tukey's post-hoc analysis. Data are shown as the mean ± SEM (90-100 dendrites; $n$ = 4-5 mice/group).

## 2.4. Increased $p75^{NTR}$ levels contribute to BDNF/TrkB/$p75^{NTR}$ signaling imbalance in HD

Several studies have shown that the survival and maintenance of striatal MSNs are especially dependent on the BDNF-TrkB signaling pathway (Ivkovic and Ehrlich, 1999; Baquet et al., 2004; Baydyuk et al., 2011). Moreover, our group has demonstrated an imbalance of $p75^{NTR}$ and TrkB expression in mouse and human HD brains associated with increased striatal susceptibility (Brito et al., 2013). Given that our previous results have shown a delay in striatal-dependent behavioral deficits and striatal neuropathology upon genetic $p75^{NTR}$ downregulation in KI mice, we hypothesize that normalization of $p75^{NTR}$ levels may also contribute to restore striatal BDNF/TrkB/$p75^{NTR}$ signaling, at least at early/middle disease stages.

### 2.4.1. Normalization of $p75^{NTR}$ delays specific BDNF/TrkB signaling impairments in KI mice

Reduced BDNF protein levels have been detected in the striatum of HD patients and animal models (Ferrer et al., 2000; Zuccato et al., 2001; Spires et al., 2004b; Gharami et al., 2008; Ma et al., 2015). Thus, we have examined whether normalization of striatal $p75^{NTR}$ expression could affect BDNF levels. First, we performed an ELISA assay to measure absolute protein levels of total BDNF (tBDNF), including the precursor (proBDNF) and the mature (mBDNF) forms, as we were unable to specifically distinguish each isoform due to limited BDNF antibody specificity (Figure 46A). As expected, 8- and 10-month-old KI mice exhibited a significant reduction in striatal tBDNF levels. Importantly, a significant decrease could not be observed in KI:$p75^{+/-}$ mice until the age of 10 months, with comparable tBDNF levels between KI:$p75^{+/-}$ and WT mice at 6 and 8 months of age. It is noteworthy that all genotypes exhibit a trend toward an age-dependent reduction of tBDNF levels, which is consistent with previous studies that have shown loss of BDNF gene transcription with aging (Zuccato et al., 2005; Yang et al., 2009; Chapman et al., 2012; Calabrese et al., 2013; Ma et al., 2015). Since proBDNF is barely detectable in WT and HD striatum (Ma et al., 2015), these changes might be mostly ascribed to mBDNF levels. Consistent with this idea, mBDNF protein levels analyzed by Western blot were also reduced in 8-months-old KI mice (Figure 46B). Interestingly, mBDNF levels were significantly increased in KI:$p75^{+/-}$ mice compared to KI mice at 8 months of age, being equal to those observed in WT mice. At 10 months of age, a trend towards decreased mBDNF levels was observed in both KI and KI:$p75^{+/-}$ mice. Given that cortical afferents are the main source of striatal BDNF (Altar et al., 1997), we evaluated mBDNF levels in cortical extracts from the same mice (Figure 46C). Unaltered mBDNF protein levels were observed between genotypes at any of the evaluated ages, suggesting that cortical mBDNF production is not affected neither by mHtt expression nor by $p75^{NTR}$ modulation.

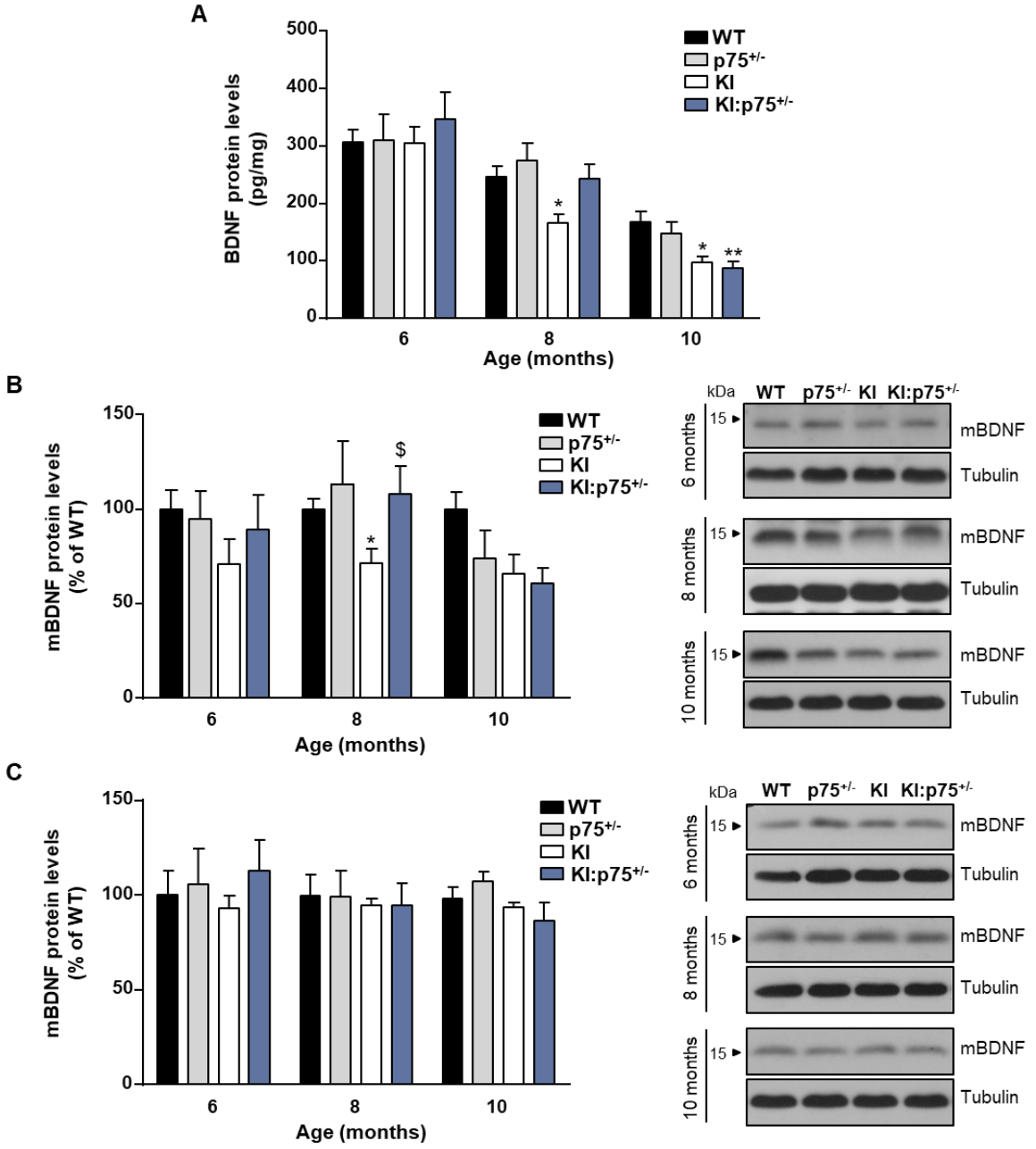



**Figure 46. Delayed reduction of striatal BDNF levels by normalization of p75$^{NTR}$ expression in KI mice.** BDNF quantification was performed using striatal (**A** and **B**) and cortical (**C**) tissue obtained from WT, p75$^{+/-}$, KI and KI:p75$^{+/-}$ mice at 6, 8 and 10 months of age. (**A**) Histogram represents total BDNF protein levels expressed as pg/mg of protein obtained by ELISA. *$P$ < 0.05 and **$P$ < 0.01 compared with WT mice by one-way ANOVA with Tukey's post-hoc analysis. (**B**) Representative immunoblots and densitometric analysis showing the levels of mature BDNF (mBDNF) and tubulin as loading control in striatal extracts. (**C**) Representative immunoblots and densitometric analysis showing the levels of mature BDNF (mBDNF) and tubulin as loading control in cortical extracts. Histograms in **B** and **C** represent the relative protein levels expressed as percentage of WT values. *$P$ < 0.05 compared with WT mice; $^{\$}P$ <0.05 compared with KI mice by one-way ANOVA with Tukey's post-hoc analysis. All data are shown as the mean ± SEM ($n$ = 5-7 mice/group).

Next, we evaluated if the genetic p75$^{NTR}$ modulation in KI mice could alter TrkB expression or activity, since such impairments have been shown in HD (Ginés et al., 2006, 2010; Zuccato et al., 2008; Brito et al., 2013; Liot et al., 2013; Simmons et al., 2013; Plotkin et al., 2014; Nguyen et al.,

2016). Similarly to striatal BDNF, TrkB levels were reduced in KI mice from 8 months of age while a significant decrease was detected in KI:p75$^{+/-}$ mice at 10 months (Figure 47B). No changes in the levels of a truncated TrkB isoform (TrkB-T1) were observed (Figure 47C). Next, activation of TrkB was analyzed by quantification of TrkB phosphorylation at tyrosine residues 816 (Y816), which binds phospholipase C-gamma (PLCγ), and 515 (Y515), which mediates SHC binding (Minichiello et al., 2002). Regarding Y816 phosphorylation, a decreasing trend at 6 months of age and a significant decrease at 8 and 10 months were found in KI mice, which could not be detected in KI:p75$^{+/-}$ mice until the age of 10 months, although not significant (Figure 47D). Importantly, levels of phosphorylated TrkB at the Y816 site in KI:p75$^{+/-}$ were significantly higher in comparison with KI mice at the age of 6 and 8 months. In contrast, no significant differences on Y515 phosphorylation were found between genotypes (Figure 47E).

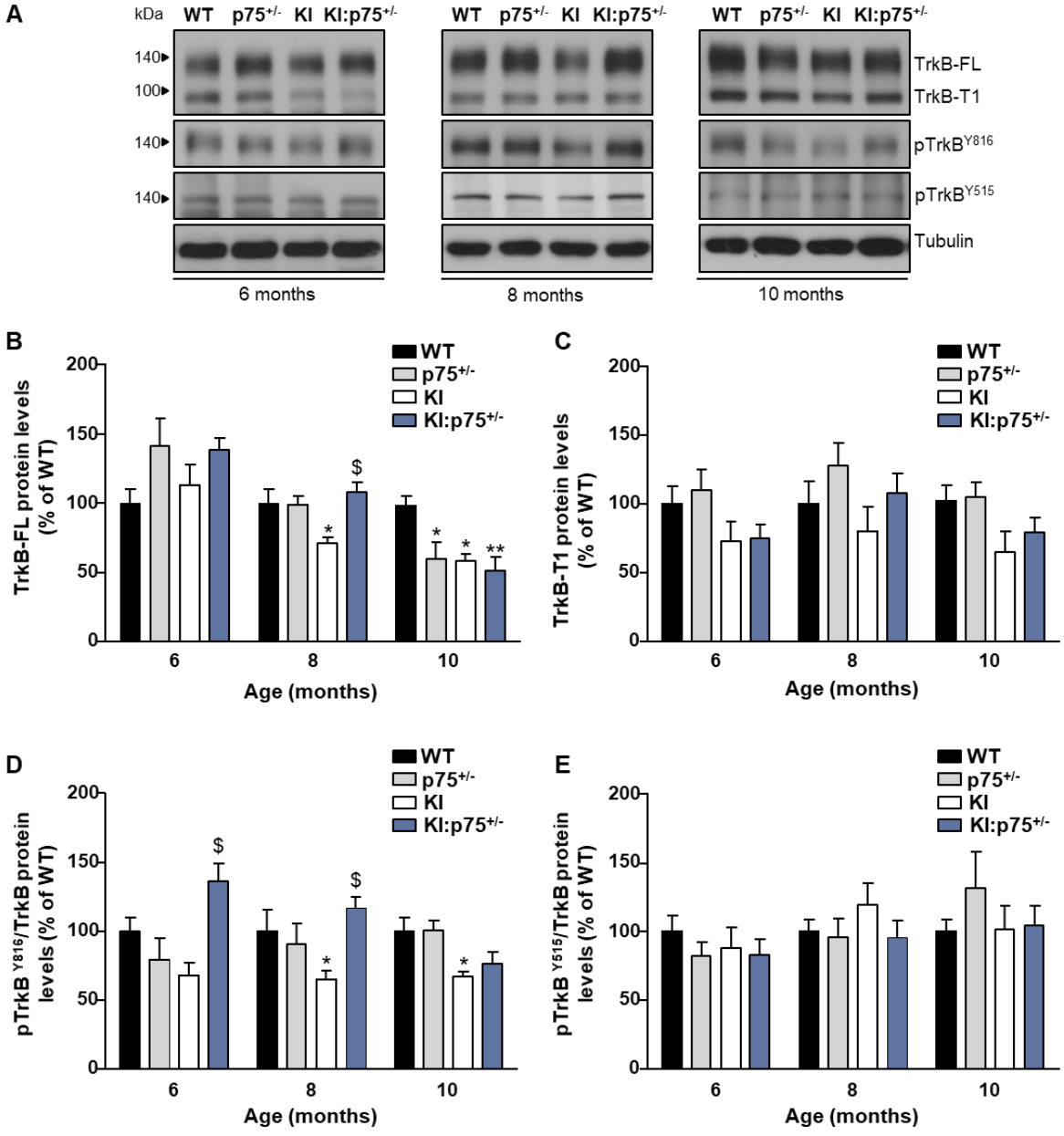
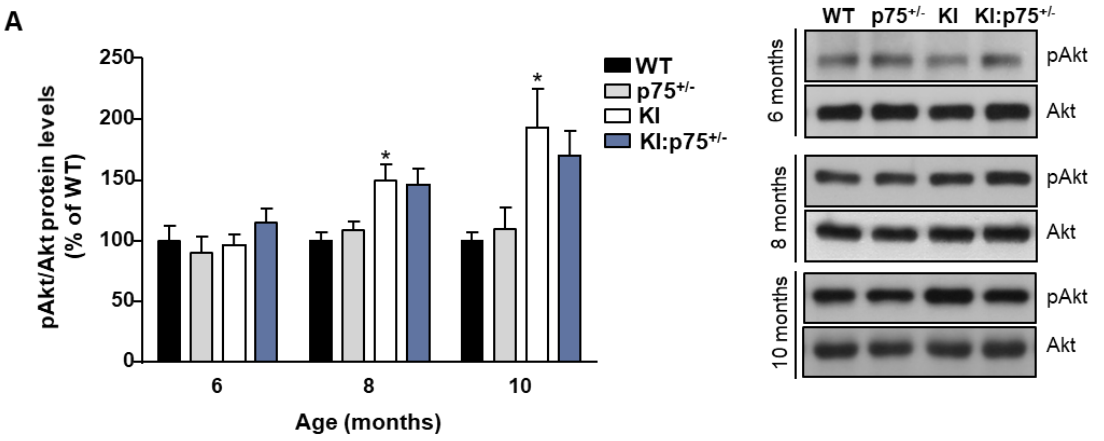


**Figure 47. Reduced expression and activation of TrkB can be delayed by normalization of p75$^{NTR}$ levels in KI mice.** Representative immunoblots (**A**) and densitometric analysis (**B**-**E**) showing protein levels of TrkB full-length (TrkB-FL) (**A** and **B**), TrkB truncated isoform (TrkB-T1) (**A** and **C**), phospho-TrkB at tyrosine 816 (pTrkB$^{Y816}$) (**A** and **D**) and phospho-TrkB at tyrosine 515 (pTrkB$^{Y515}$) (**A** and **D**) with tubulin as loading control in striatal extracts obtained from WT, p75$^{+/-}$, KI and KI:p75$^{+/-}$ mice at 6, 8 and 10 months of age. Histograms represent the relative TrkB-FL and TrkB-T1 protein levels or the relatives phospho-TrkB (Y816)/TrkB and phospho-TrkB (Y515)/TrkB ratios expressed as percentage of WT values. *$P$ < 0.05 and **$P$ < 0.01 compared with WT mice; \$$P$ < 0.05 compared with KI mice by one-way ANOVA with Tukey's post-hoc analysis. All data are shown as percentage of WT values and represented as the mean ± SEM ($n$ = 5-7 mice/genotype/age).

BDNF/TrkB activation triggers three major downstream signaling pathways: PI3K/Akt, MAPK/ERK, and PLCγ/PKC (Huang and Reichardt, 2003; Minichiello, 2009). Western blot analysis of striatal tissue at different ages revealed an increased activation of Akt at 8 and 10 months of age in KI mice (Figure 48A). This sustained Akt activation is consistent with previous data in knock-in and transgenic HD models (Bowles and Jones, 2014) and it has been considered a pro-survival mechanism associated with reduced levels of PHLPP1 phosphatase (Saavedra et al., 2010) and to an abnormal NMDA receptor activation (Ginés et al., 2003a). No changes in ERK1/2 activation were observed in the striatum of KI mice until 10 months of age, when a slight but not significant decrease was observed (Figure 48B). Importantly, a significant reduction in PLCγ activation was present from 6 months of age in advance, in accordance to the impaired TrkB phosphorylation at Y816 (Figure 48C). Given that aberrant levels or activation of p75$^{NTR}$ can alter TrkB signaling (Roux and Barker, 2002), we also investigated these pathways in KI:p75$^{+/-}$ mice. pAkt and pERK levels where comparable between KI and KI:p75$^{+/-}$ mice, indicating that these pathways are not affected by normalization of p75$^{NTR}$ levels (Figure 48A and B). In contrast, reduced pPLCγ levels were restored in KI:p75$^{+/-}$ mice at 6 and 8 months but not at 10 months of age, when levels were comparable to KI mice (Figure 48C).

Since Akt, ERK, and PLCγ pathways via TrkB signaling have been shown to phosphorylate CREB promoting transcription of its target genes, such as BDNF, we also evaluated CREB phosphorylation levels. However, no differences were observed between genotypes at none of the evaluated ages (Figure 48D).



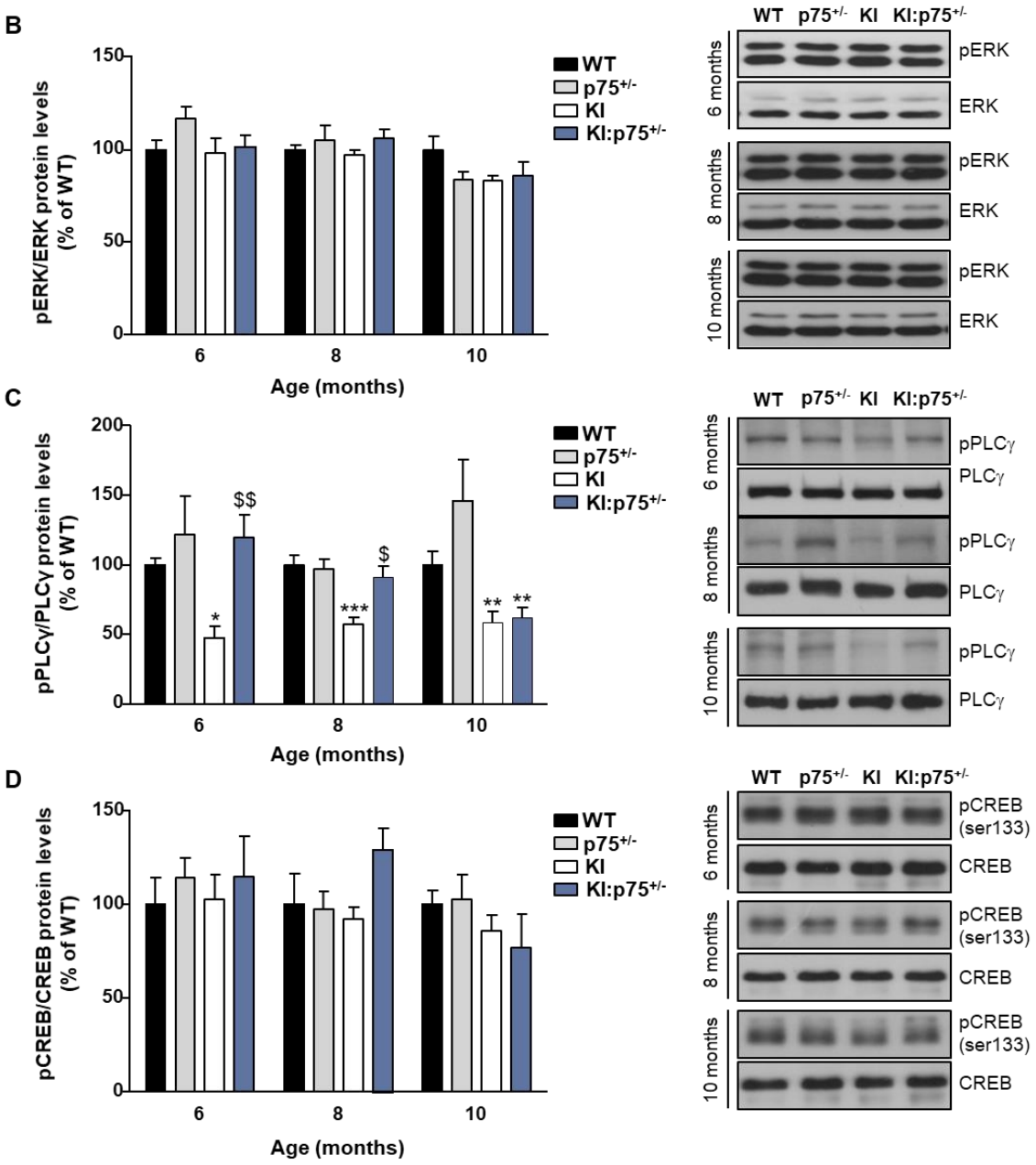



**Figure 48. Impaired TrkB-induced phosphorylation of PLCγ can be delayed in KI mice by normalization of striatal p75$^{NTR}$ levels.** Representative immunoblots and densitometric analysis showing the protein levels of pAkt and Akt (**A**), pERK and ERK (**B**), pPLCγ and PLCγ (**C**) and pCREB and CREB (**D**) in striatal extracts obtained from WT, p75$^{+/-}$, KI and KI:p75$^{+/-}$ mice at 6, 8 and 10 months of age. The histograms represent the relative pAkt/Akt, pERK/ERK, pPLCγ/PLCγ and pCREB/CREB ratios expressed as percentage of WT values. *$P$ < 0.05, **$P$ < 0.01 and ***$P$ < 0.001 compared with WT mice; \$$P$ < 0.05 and \$\$$P$ < 0.01 compared with KI mice by one-way ANOVA with Tukey's post-hoc analysis. All data are shown as the mean ± SEM ($n$ = 5-7 mice/group).

To further confirm the contribution of increased p75$^{NTR}$ protein levels to aberrant PLCγ signaling in KI mice, we used an *in vitro* approximation. First, we evaluated p75$^{NTR}$ protein levels in striatal primary cultures from WT and KI embryos at 14 DIV and found no significant differences (Figure 49A). Therefore, and in order to mimic the aberrant increase in p75$^{NTR}$ levels observed in the striatum of adult KI mice, we used infectious adeno-associated viral particles (AAV2/8) containing a GFP expression cassette alone (AAV-GFP) or fused to the p75$^{NTR}$ sequence (AAV-GFP-p75). Previous results from our group have already demonstrated efficient transduction of neurons with

these viruses (Brito et al., 2014). Primary striatal cultures from WT or KI embryos were transduced with AAV-GFP or AAV-GFP-p75 at 3 DIV and subsequently analyzed at 14 DIV. We confirmed transduction of neurons and similar expression of the inserted genes by microscopic (Figure 49B) and Western blot (Figure 49C) analysis. We also confirmed increased $p75^{NTR}$ protein levels in AAV-GFP-p75 transduced cultures (Figure 49D). When analyzing PLCγ activation (Figure 49E), we found that cultures transduced with AAV-GFP-p75 showed significantly lower pPLCγ levels compared to the AAV-GFP-transduced groups, both in WT and KI cells, confirming the negative role of $p75^{NTR}$ in PLCγ signaling. No differences in PLCγ activation were detected between genotypes (Figure 49E), which was consistent with unaltered $p75^{NTR}$ protein levels in primary striatal neurons at 14 DIV (Figure 49A).

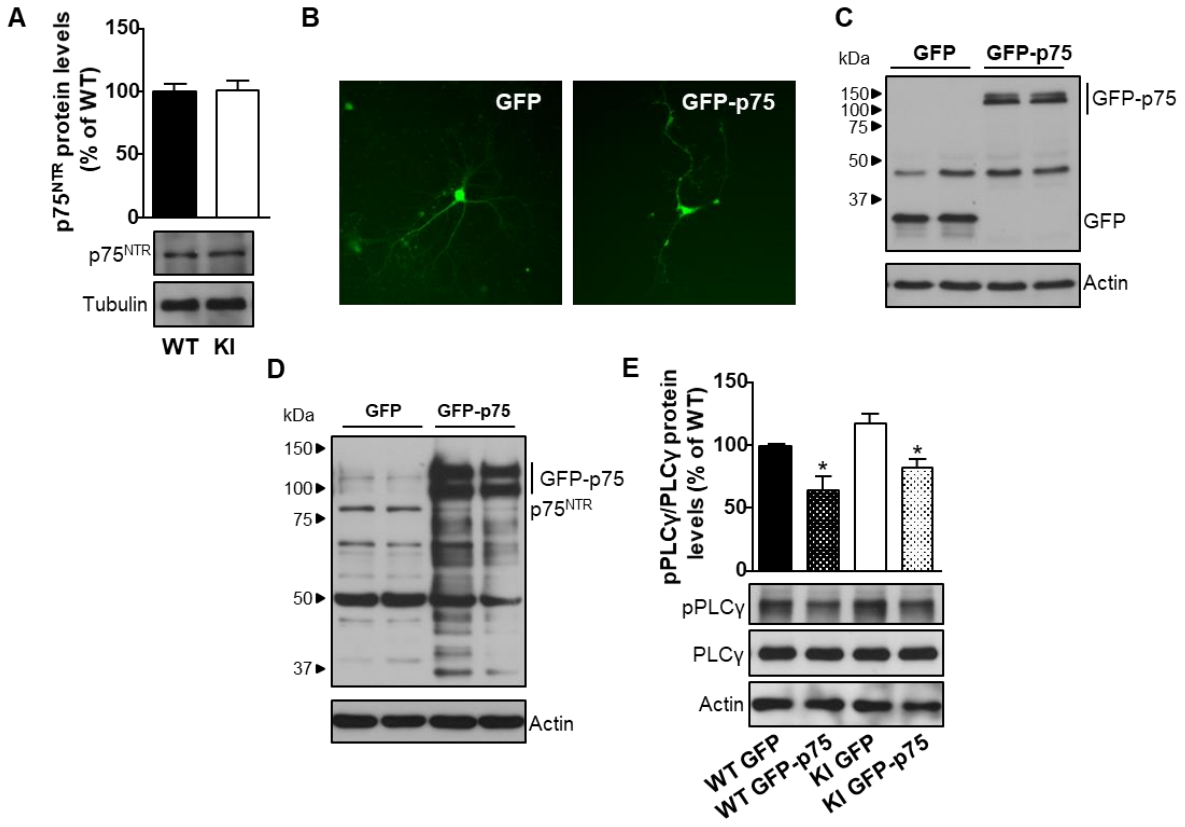
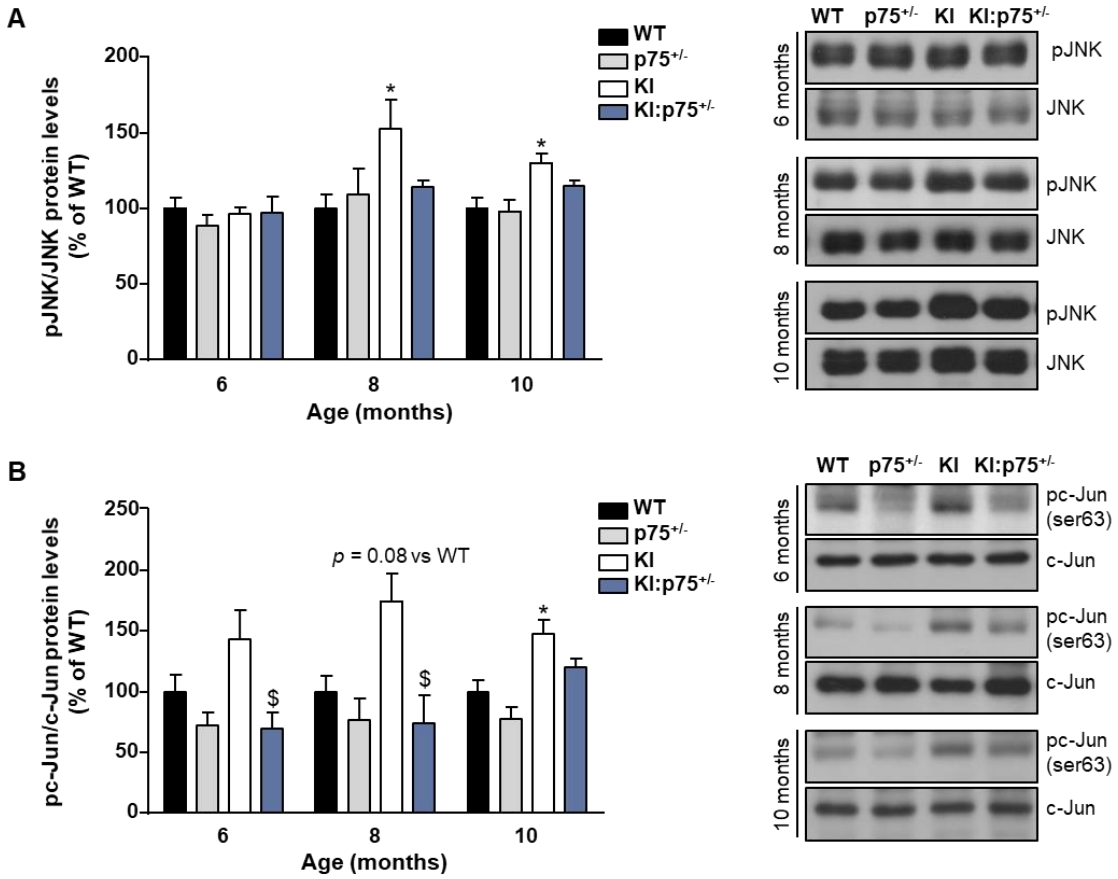



**Figure 49. *In vitro* overexpression of $p75^{NTR}$ impairs PLCγ activation. (A)** Representative immunoblots and densitometric analysis showing $p75^{NTR}$ levels and tubulin as loading control in primary striatal cultures from WT and KI embryos at 14 DIV. Histograms represent the relative $p75^{NTR}$ protein levels expressed as percentage of WT values. (**B-D**) Primary striatal cultures from WT and KI embryos were transfected with AAV-GFP or AAV-GFP-p75 at 3 DIV and evaluated at 14 DIV. (**B**) Representative photomicrographs showing transduced neurons expressing GFP or GFP fused to $p75^{NTR}$ (GFP-p75). (**C**) Representative immunoblots showing GFP or GFP-p75 protein expression (anti-GFP antibody), with actin as loading control, in transduced cultures. (**D**) Representative immunoblots showing $p75^{NTR}$ or GFP-p75 protein expression (anti-$p75^{NTR}$ antibody), with actin as loading control, in transduced cultures. (**E**) Representative immunoblots and densitometric analysis showing protein levels of pPLCγ and PLCγ with actin as loading control in GFP and GFP-p75 expressing neurons from WT and KI embryos. $*P < 0.05$ compared to GFP cultures by two-way ANOVA with Bonferroni post-hoc analysis Histograms represent the relative pPLCγ/PLCγ ratios expressed as percentage of WT GFP values. All data represent the mean ± SEM ($n$ = 3-7 cultures/group).

Taken together, these data indicate that BDNF-TrkB deficits along with deficient TrkB activation of PLCγ can be delayed in KI mice striatum through normalization of $p75^{NTR}$ levels.

## 2.4.2. Normalization of $p75^{NTR}$ delays the upregulation of neurodegenerative signaling in KI mice

Activation of cell death signaling pathways has previously been reported in different HD models (Apostol et al., 2008; Perrin et al., 2009; Taylor et al., 2013). Given that $p75^{NTR}$-mediated neurodegeneration has been associated with JNK activation (Roux and Barker, 2002), we next examined whether normalization of $p75^{NTR}$ levels in KI mice could modulate this pathway. Western blot analysis of striatal extracts from WT, $p75^{+/-}$, KI and KI:$p75^{+/-}$ mice revealed a significant increase in pJNK levels at 8 and 10 months of age in KI mice but not in KI:$p75^{+/-}$ animals, compared to WTs (Figure 50A). Since JNK activation mediates direct phosphorylation of its substrate c-Jun, we assessed levels of phospho-c-Jun at Serine 63 (pc-Jun). Compared to WT mice, a significant activation of this pro-apoptotic transcription factor was detected in KI mice at 10 months of age while an increasing trend was observed at 6 and 8 months (Figure 50B). Interestingly, pc-Jun was significantly reduced in KI:$p75^{+/-}$ mice in comparison with KI mice at 6 and 8 months. To determine whether JNK activation was also associated with increased caspase-3 activity, the caspase-3 cleaved spectrin fragment SBDP120 was analyzed by Western blot. No significant changes in SBDP120 levels were found between genotypes (Figure 50C).



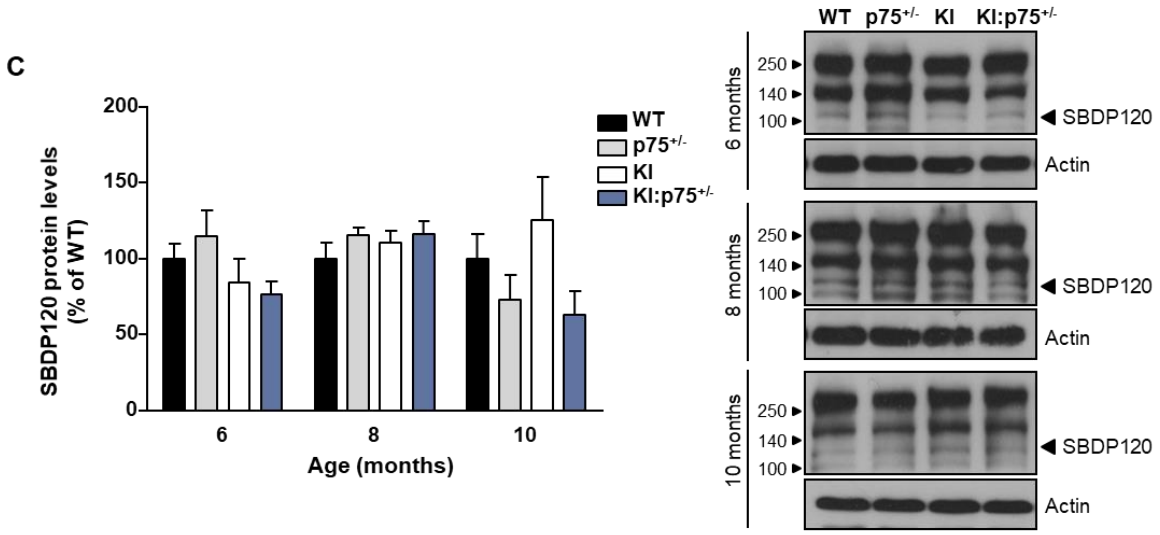



**Figure 50. Normalization of p75$^{NTR}$ in KI mice attenuates activation of JNK.** Representative immunoblots and densitometric analysis showing the levels of pJNK and JNK (**A**), pc-Jun and c-Jun (**B**), and αII-Spectrin breakdown product 120 (SBDP120) (**C**) with actin as loading control in striatal extracts obtained from WT, p75$^{+/-}$, KI and KI:p75$^{+/-}$ mice at 6, 8 and 10 months of age. Histograms represent the relative ratios of pJNK/JNK and pc-Jun/c-Jun and the relative levels of SBDP120 expressed as percentage of WT values. Data were analyzed by one-way ANOVA followed by Tukey's test. *$P < 0.05$ compared with WT mice; \$$P < 0.05$ compared with KI mice by one-way ANOVA with Tukey's post-hoc analysis. All data are shown as the mean ± SEM ($n$ = 5-7 mice/group).

These findings indicate that activation of JNK pro-apoptotic pathway can be partially prevented by normalization of p75$^{NTR}$ expression in KI mice. Despite the observed activation of cell death signaling pathways, it is widely accepted that the Hdh$^{Q7/Q111}$ mouse model is spared of evident neuronal loss. Indeed, we found no cleaved caspase-3 positive-stained cells in corticostriatal slices from WT and KI mice at 8 months of age (data not shown), indicating the absence of apoptotic neurons. This is consistent with previous studies showing unaltered NeuN-positive neuronal density in the striatum of Hdh$^{Q7/Q111}$ mice for up to 12 months (Bragg et al., 2017). Overall, these results suggest that the pro-apoptotic signaling activated by early increases in p75$^{NTR}$ levels could lead to neuronal dysfunction without affecting cell viability.

# DISCUSSION

Nowadays, HD is a devastating disorder, without any available treatment to stop or delay the massive neuronal death and dysfunction that contributes to the deterioration of both intellectual and physical capacities, proceeding inexorably to death in 10-20 years. Current treatments focus on the relief of specific signs and symptoms of the disease and on providing support and assistance to the patients and their relatives (Frank, 2014; Shannon and Fraint, 2015). Moreover, pharmacological interventions in HD mostly target hyperkinetic movements while treatments to improve cognition and psychiatric features are limited, even though recent studies have shown that these non-motor symptoms represent the most quality of life concern for patients and their families (Helder et al., 2001; Ready et al., 2008; Williams et al., 2010). Therefore, there is great need for finding new therapies that act at the root of the illness to prevent its advance and, therefore, alleviate the wide range of clinical manifestations. Since HD is mostly a gain-of-function genetic disorder, one obvious strategy relies in advanced experimental techniques that target m*Htt* DNA (like zinc-finger proteins or CRISPR/Cas9 strategies) or RNA (like antisense oligonucleotides or RNA interference compounds) (Wild and Tabrizi, 2017). However, the great amount of safety, cost and feasibility issues of these therapies could slow down their development for many years and, therefore, the discovery of other more feasible pharmacological targets is still crucial. In this regard, intensive research into the HD pathophysiology for more than two decades has provided broad knowledge about numerous cellular and molecular mechanisms that appear altered in HD from early stages and that might contribute to neuronal dysfunction and degeneration. Among them, transcriptional dysregulation, somatic CAG repeat instability and disrupted neurotrophin signaling are considered key underlying mechanisms and HDAC3 and $p75^{NTR}$ proteins have been described as important promoter factors. Consistently, the development of synthetic small molecules that specifically inhibit their activity is now being promoted (Thomas, 2014; Simmons, 2017). Given that HDAC3 and $p75^{NTR}$ could be promising and feasible therapeutic targets for HD, in this thesis we have evaluated the behavioral and neuropathological effects derived from the modulation of their activity/levels in a mouse model of HD, to help lay the groundwork for future clinical trials.

# 1. HDAC3 as a therapeutic target for HD

Important chromatin structure alterations, such as histone hypoacetylation, are present in HD brains and are thought to confer a repressive transcriptional state underlying numerous transcriptional deficits that might contribute to disease progression (Valor and Guiretti, 2014; Francelle et al., 2017). This idea has encouraged the use of small molecule HDAC inhibitors (HDACis) as a therapeutic approach for HD. However, the success of HDACis depends on the identification of individual HDACs that contribute the most to the HD pathology. Thus, selective HDACis that target such specific isotypes would avoid toxicity issues of broad-based HDACis, probably caused by their nonselective nature. In this regard, HDAC3 has emerged as an important candidate. A bioinformatic study that compared the HD Research Crossroads database with genome-wide linkage analysis identified HDAC3 as a key HD genetic modifier (Kalathur et al., 2012) and another study demonstrated that the presence of mHtt or different pro-apoptotic stimuli can liberate HDAC3 from its interaction with wild-type Htt, thus potentiating its activity (Bardai

et al., 2013). Additionally, selective HDAC3 inhibition has recently shown promise in improving HD motor impairments and corticostriatal neuropathology in a chronic treatment paradigm, although cognitive deficits were not extensively evaluated (Jia et al., 2012a, 2015, 2016; Thomas, 2014). Therefore, it is of great relevance for the HD research to evaluate the implication of HDAC3 inhibitors in a wider spectrum of HD symptoms and to study the molecular mechanisms associated with its preclinical efficacy.

Results of this thesis have confirmed the lack of toxicity of a potent and highly selective HDAC3 inhibitor, RGFP966, administered in a chronic paradigm in mice. We also confirmed the ability of RGFP966 to cross the blood-brain barrier after systemic injections and to efficiently diffuse within the brain, as observed by increased histone H3 acetylation in the striatum and the hippocampus of RGFP966-treated mice. In recent years, several studies have underscored the role of HDAC3 as an important negative regulator of hippocampal-dependent and other types of memory formation, suggesting that HDAC3 might decrease chromatin accessibility to repress important genes required for memory formation (McQuown and Wood, 2011; McQuown et al., 2011; Malvaez et al., 2013; Rogge et al., 2013; Bieszczad et al., 2015). These studies led us to formulate the hypothesis that HDAC3 inhibition could represent an ideal pharmacological strategy to restore cognitive abilities that appear deteriorated at early stages of HD. Consistently, we have demonstrated for the first time that the chronic administration of RGFP966, starting at presymptomatic stages, is able to prevent the appearance of motor-learning impairments and spatial and recognition memory deficits in the $Hdh^{Q7/Q111}$ (KI) mouse model of HD at 6 months of age, highlighting the contribution of HDAC3 to early HD cognitive deficits. These findings are in agreement with previous results from our group showing a recovery of long-term memory deficits in KI mice at 8 months of age after an acute treatment with the broad-based HDACi called TSA, which targets HDAC 1, 2, 3, 6, 10 and 11 (Giralt et al., 2012a). Importantly, impaired acquisition of new skills (Martone et al., 1984; Rosenberg et al., 1995; Lawrence et al., 1996, 1998; Paulsen et al., 2008; Schneider et al., 2010) and spatial and recognition memory deficits (Lawrence et al., 2000; Berrios et al., 2002; Brandt et al., 2005; Begeti et al., 2016) have also been reported in HD patients before the onset of motor symptoms, which reinforces the use of a selective HDAC3i treatment to treat early cognitive impairments in HD.

At the molecular level, it has long been known that the neuronal activity-dependent induction of gene transcription and *de novo* protein synthesis allows the modulation of neuroplastic mechanisms underlying long-term information storage in the brain (Davis and Squire, 1984). Additionally, growing evidence in both humans and HD animal models suggests that cognitive deficits in HD are strongly dependent on the hippocampal function (Giralt et al., 2012b; Puigdellívol et al., 2016). Consistent with the role of HDAC3 as a transcriptional repressor, our results correlate the positive effects of RGFP966 on cognition with the recovery of the activity-dependent hippocampal expression of the immediate early genes (IEGs) *Arc* and *Nr4a2* in 6-months-old KI mice (Figure 51). Reduced expression of these genes was previously observed by our group in the hippocampus of trained KI mice at 8 months, which was associated with decreased CBP expression, while a transcriptional recovery was also found after TSA treatment (Giralt et al., 2012a). Importantly, genetic deletion or pharmacological inhibition of HDAC3 has already been shown to increase

*Nr4a2* expression in mice hippocampus (McQuown et al., 2011; Malvaez et al., 2013; Rogge et al., 2013), while the relationship between HDAC3 and *Arc*, as far as we know, is not so well established. Like other IEGs, the expression of *Arc* (also called *Arg3.1*) and *Nr4a2* (also called *Nurr1*) is abruptly increased in subsets of neurons after a memory training event (or other relevant environmental experiences), thus enabling maintenance of long-term potentiation (LTP) and consolidation of long-term memory (Guzowski et al., 2000; Peña de Ortiz et al., 2000; Colón-Cesario et al., 2006; Plath et al., 2006; Shepherd and Bear, 2011; Hawk et al., 2012; Bridi et al., 2017). Related to their role in LTP maintenance, several studies that evaluated hippocampal neuronal function in HD mice with electrophysiological analysis have shown that LTP, but not basal neurotransmission, is reduced at hippocampal synapses (Usdin et al., 1999; Murphy et al., 2000; Lynch et al., 2007; Brito et al., 2014). Hence, we hypothesize that the recovery of *Arc* and *Nr4a2* transcription after a learning event in HD mice treated with RGFP966 might result in an improvement of synaptic plasticity. Indeed, HDAC3 inhibition with RGFP966 increased late-LTP and restored synaptic tagging and capture in hippocampal slices from aged rats (Ferrante et al., 2003) and recovered LTP impairments in rat hippocampal slices treated with the amyloid-$\beta_{42}$ oligomer (Krishna et al., 2016), suggesting that it could also ameliorate LTP impairments in HD mice. Interestingly, increased nuclear expression of HDAC3 has been found in the hippocampus of an AD mouse model (Zhu et al., 2017) and its inhibition was shown to attenuate spatial memory impairments (Zhu et al., 2017; Yu et al., 2018), indicating that HDAC3 could also play an important role in the altered cognitive phenotype of other neurodegenerative diseases.

In response to a training event that will produce long-term information storage, increased HAT and decreased HDAC activity act in coordination to allow rapid increases in histone acetylation that would make specific DNA regions more accessible to favor specific patterns of gene transcription (Korzus et al., 2004; Levenson et al., 2004). Although activity-dependent transcriptional impairments in KI mice were previously associated with decreased levels of histone H3 acetylation in the hippocampus of 8-months-old KI mice (Giralt et al., 2012a), results of this thesis show no differences in global histone acetylation at 6 months. However, 6-months-old KI mice could still present changes in specific genomic regions that might be undetectable with our analysis, since accumulating pieces of evidence demonstrate that histone hypoacetylation in HD models is confined to specific locus associated with downregulated genes, which is not detected by global analysis of histone acetylation levels (Sadri-Vakili et al., 2007; McFarland et al., 2012; Valor et al., 2013; Achour et al., 2015; Guiretti et al., 2016). Moreover, mHtt could also impair key transcriptional regulators that do not necessarily modify chromatin structure (Li and Li, 2004; Seredenina and Luthi-Carter, 2012). Another interesting idea that may be worth investigating is that KI mice exhibit a dysregulated induction of histone acetylation after a learning event, similar to what has been reported in aged mice with learning and memory alterations (Peleg et al., 2010). Importantly, the observed recovery of *Arc* and *Nr4a2* genes could be associated with the increase in histone H3 acetylation after RGFP966 treatment (Figure 51). Interestingly, although increased histone H3 acetylation was observed in both WT and KI mice treated with RGFP966, the upregulation of memory genes *in vivo* and the positive effect in cognitive behavior was only observed in KI mice, suggesting that RGFP966 treatment is not able to further potentiate

cognition when this is not compromised. Our results seem to contradict previous studies that have shown long-term memory improvements in young wild-type mice (evaluated in OLT or NORT tests) after pharmacological or genetic inhibition of HDACs (among them HDAC3) (Stefanko et al., 2009; Roozendaal et al., 2010; McQuown et al., 2011). In these studies, however, cognitive improvements were only observed in mice subjected to a subthreshold training event that consisted of 3 minutes of exploration, which does not elicit long-term memory formation in normal conditions, while mice trained for 10 min did not show any significant improvements.

Although HATs and HDACs were originally characterized for their ability to target histone tails in order to modulate chromosome dynamics, in posterior years they have been shown to recognize a great variety of non-histone proteins (Glozak et al., 2005; Alessandro Didonna, 2015). In this context, several studies have revealed that HDAC3 is able to deacetylate p65 (or RelA) (Chen et al., 2001), SRY (Thevenet et al., 2004), STAT3 (Yuan et al., 2005), CBP (Chuang et al., 2006; Grégoire et al., 2007), MEF2D (Grégoire et al., 2007), PCAF (Grégoire et al., 2007; Blanco-García et al., 2009), STAT1 (Krämer et al., 2009), and PPAR-γ (Jiang et al., 2014). CBP is of particular interest to our study due to its well-known contribution to HD pathology (Steffan et al., 2000, 2001; Nucifora et al., 2001; Jiang et al., 2003; Taylor et al., 2003; Giralt et al., 2012a) and its important involvement in long-term memory and specific forms of synaptic plasticity (Alarcón et al., 2004; Korzus et al., 2004; Wood et al., 2005, 2006; Chen et al., 2010; Barrett et al., 2011). Particularly, in human non-neuronal cell cultures, HDAC3 has been shown to directly interact with and deacetylate CBP, repressing its transcriptional activity (Chuang et al., 2006). Additionally, the positive effects of HDAC3 inhibition on long-term memory formation have been shown to depend on CBP activity (McQuown et al., 2011). Our results have confirmed the relationship between HDAC3 and CBP in immortalized neuronal cultures from KI mice, demonstrating the ability of RGFP966 to upregulate CBP acetylation. In view of this, we propose that the inhibition of HDAC3 in KI mice, apart from directly promoting histone acetylation and chromatin accessibility, drives an increase in CBP acetylation that likely results in an enhanced CBP-dependent transcriptional activity, contributing to the mRNA recovery of both *Arc* and *Nr4a2*, among other memory-related genes that are also CREB-CBP dependent (Figure 51) (Wood et al., 2006). In addition to CBP, some other non-histone HDAC3 substrates could also contribute to the observed cognitive improvement in KI mice (Merlo et al., 2005; Hsu et al., 2014; Jahrling et al., 2014; Rashid et al., 2014; Sharma et al., 2015). It is important to point out that HDAC3 can also repress transcription independently from its deacetylase enzymatic activity (Sun et al., 2013; Adikesavan et al., 2014) and that HDAC3is might not be able to affect this function, since they act by targeting the active site of the enzyme (Millard et al., 2017). However, our results and several other studies have demonstrated that HDAC3 enzymatic activity is necessary to impair memory processes (McQuown et al., 2011; Alaghband et al., 2017), which supports the use of HDAC3is as cognitive enhancers for HD.

The modulation of IEG expression by RGFP966 in other brain regions besides the hippocampus, although not evaluated, could also have an implication in the observed recovery of corticostriatal-dependent motor-learning deficits in KI mice. Indeed, *Arc* and *Nr4a2* genes have been involved in the process of motor-skill learning (Kleim et al., 1996; Jin and Clayton, 1997; Hosp et al., 2013;

Cao et al., 2015) and Arc expression (both in mRNA and/or protein forms) have been found consistently decreased in the caudate nucleus of HD patients and in the striatum of different knock-in and transgenic HD mouse models (Langfelder et al., 2016; Kusko et al., 2018). Additionally, CBP and CREB have been described to be critical for motor-skill learning (Oliveira et al., 2006), so the effect of RGFP966 on CBP acetylation could also provide a positive role on this behavior.

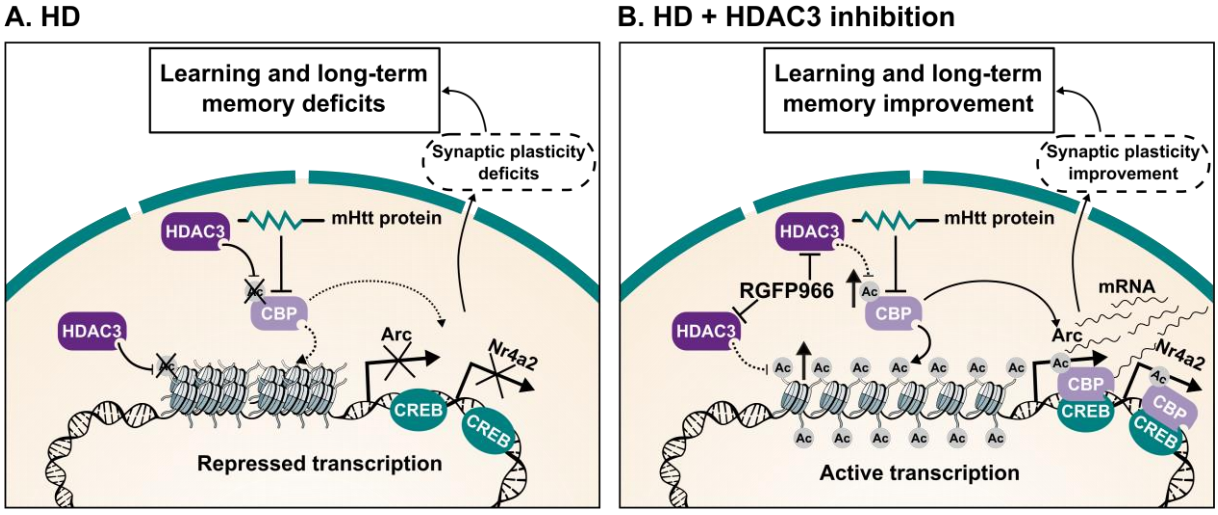



**Figure 51. HDAC3 inhibition restores the neuronal activity-dependent transcription of memory-related genes in HD at early disease stages.** (**A**) In the context of HD, the aberrant interaction of mHtt with CBP or with other transcriptional regulators/chromatin structure modifiers, could confer a repressive transcriptional state that impairs the activity-driven induction of important memory-related genes, such as *Arc* and *Nr4a2*. This transcriptional defect might underlie synaptic plasticity alterations that could impair the formation of learning and memory processes. (**B**) Pharmacological inhibition of HDAC3 activity with RGFP966 increases histone H3 acetylation, probably leading to a more relaxed chromatin configuration that allows transcriptional activity, and increases CBP acetylation, which likely facilitates its acetyltransferase and transcriptional activity. This allows the transcription of CBP:CREB-dependent genes that are important for synaptic plasticity and the formation of cognitive processes.

As mentioned, chronic HDAC3 inhibition in HD mice has shown to ameliorate some aspects of striatal pathology and the appearance of motor coordination abnormalities, which mostly relies on striatal integrity (Jia et al., 2012b, 2015, 2016). Consistently, we confirmed the beneficial effects of RGFP966 chronic treatment on early HD neuropathological hallmarks in KI mice striatum. First, we observed a partial normalization in protein levels of the striatal biomarkers DARPP-32, PDE10A and A2AR. Additionally, our results showed that DARPP-32 levels are directly increased after an acute HDAC3 inhibition, as it has been suggested in other studies (Jia et al., 2012b; Chandwani et al., 2013), while the recovery of the other striatal markers is expected to be only the result of a more indirect effect of a chronic HDAC3 inhibition producing a general relief in striatal pathology. Another concurrent benefit of RGFP966 in the striatum of KI mice was the decrease of the early accumulation of mHtt oligomeric forms. As mHtt oligomeric forms correspond (in protein content) to a very small fraction of the monomeric forms, decreased mHtt oligomerization was not followed by an increase in mHtt monomeric levels. Additionally, HDAC3 was not found to regulate *Htt* transcription, consistent with previous studies (Rumbaugh et al., 2015; Qu and D'Mello, 2018). Since smaller, soluble oligomeric forms of mutant huntingtin are thought to confer toxic effects and contribute to early cell dysfunction (Nucifora et al., 2012; Kim et al., 2016), a

reduction in their levels by HDAC3 inhibition is predicted to delay disease progression. Conversely, a recent study found no changes in the density of striatal mHtt bigger aggregates, measured by EM48 immunoreactivity, after chronic HDAC3 inhibition in a transgenic HD mouse model (Jia et al., 2016), while *in vitro* studies performed in non-neuronal cultured cells showed that HDAC3 inhibition increases the amount of insoluble mHtt aggregates (Mano et al., 2014). Our data suggest that HDAC3 could play a different role in the formation of soluble oligomeric versus insoluble inclusion forms of mHtt, although the mechanisms need to be further explored. Interestingly, CBP and HDAC1 have been shown to increase/decrease the acetylation status of mHtt to enhance/reduce its degradation by the autophagic-lysosomal pathway, probably affecting oligomeric but not monomeric forms (Jeong et al., 2009). Although in this study HDAC3 was found to play a minimal role in mHtt acetylation, we could not rule out that RGFP966 might affect some HDAC1 activity at the dose used in our *in vivo* experiments or that HDAC3 inhibition regulates this process by modulating CBP activity.

Age-dependent CAG-repeat length expansions in the HD locus of neuronal cells have been recently proposed as an important mechanism contributing to disease progression (Wheeler et al., 2003; Swami et al., 2009; Budworth et al., 2015). The proposed model is that fewer gains in the length of the glutamine tract, mostly occurring in the striatum, would reduce mHtt-induced toxicity, thereby leading to improvements in striatal function and recovery of striatal-dependent behavioral deficits. However, exactly how CAG instability occurs, why it is a cell-specific event and the molecular consequences in the disease are not completely understood. A wide variety of studies have demonstrated the contribution of different DNA repair proteins in the process of trinucleotide repeat (TNR) expansions. Msh2 and Msh3 proteins, which conform the MutSβ complex that participates in the initial recognition phase of the mismatch repair (MMR) pathway, seem particularly involved (Manley et al., 1999; Wheeler et al., 2003; Dragileva et al., 2009; Tomé et al., 2009; Pinto et al., 2013). This has led to the hypothesis that an incorrect DNA repair must contribute to the addition of new CAG repeats to the HD locus. Importantly, HDAC3 was identified as a novel factor promoting TNR expansions in cultured human cells, while the other class I isoforms, HDAC1 and HDAC2, were not found involved (Debacker et al., 2012; Gannon et al., 2012). Thus, we decided to evaluate the effect of HDAC3 inhibition on striatal CAG repeat instability *in vivo*. Small-pool PCR (SP-PCR) analysis of CAG repeat tracts offer enough suitable accuracy and confidence to reveal the full range of mutation lengths present in a specific tissue (Gomes-Pereira et al., 2004). Using the SP-PCR technique, we have provided first evidence that chronic treatment with RGFP966 suppresses somatic CAG repeat expansions in the striatum of KI mice. However, the molecular details by which HDAC3 promotes CAG instability are unclear. In cultured human cells, it has been suggested that HDAC3 might operate in the same pathway as Msh2 and Msh3, since the double knockdown of Msh2 and HDAC3 or Msh3 and HDAC3 had the same effect in suppressing CAG repeat expansions than the single knockdown of Msh2, Msh3 or HDAC3 (Gannon et al., 2012). Importantly, Msh2 can be acetylated on different residues and interact with several HDACs, including HDAC3 (Choudhary et al., 2009; Zhang et al., 2014; Radhakrishnan et al., 2015). Results of this thesis have shown that the inhibition of HDAC3 increases Msh2 acetylation at lysine 73 (K73) in striatal neuronal cultures. Additionally, we found

no effect on the expression/stability of the protein, consistent with previous studies showing that the genetic reduction of HDAC3 does not alter Msh2 protein levels (Gannon et al., 2012) and that the deacetylation/acetylation of Msh2 at lysine 73 does not affect half-life of the protein (Radhakrishnan et al., 2015). Since the acetylation of Msh2 at K73 after HDAC10 inhibition has been correlated with a decrease in MMR activity *in vitro* (Radhakrishnan et al., 2015), we could speculate that RGFP966 treatment in KI mice would reduce CAG-repeat expansions by modifying MMR activity (Figure 52). Strikingly, in the aforementioned study it was shown that the overexpression of a deacetylation mimetic form (K73R) of Msh2 did not alter DNA MMR activity when compared with that of wild type (Radhakrishnan et al., 2015). However, it is important to note that the acetylation status of Msh2 at this residue in basal conditions seems to be very low (as seen in our experiments and in Radhakrishnan et al.), so an increase in acetylation would be expected to cause a more pronounced effect in protein activity than a reduction in acetylation. Indeed, in the same study the effect of HDAC10 overexpression in MMR activity did not reach statistical significance, whereas the knockdown of HDAC10 produced a substantial and significant decrease in MMR activity. Therefore, more experiments would be necessary to confirm the specific effect of K73 acetylation on MMR activity and its contribution to somatic CAG repeat instability in HD. Interestingly, the K73 site of Msh2 is a highly conserved residue across multiple species and is located near the N terminal region, in the mismatch binding domain (or I domain), which is involved in DNA binding (Gupta et al., 2011; Radhakrishnan et al., 2015). One interesting hypothesis to explain the modulation of MMR activity by inhibition of HDAC3 or HDAC10 is that the acetylation of K73 in Msh2 reduces the positive charge in the mismatch binding domain, which might decrease the affinity of the protein to the DNA (negatively charged). Additionally, we cannot rule out that HDAC3 deacetylates Msh2 at other lysine residues. Since loss of either Msh2 or Msh3 in human cells and/or mouse models of DM1 (myotonic dystrophy type 1, CTG repeats) (van den Broek et al., 2002; Savouret et al., 2003; Foiry et al., 2006; Nakatani et al., 2015) or FXS (fragile X syndrome, CGG repeats) (Lokanga et al., 2014; Zhao et al., 2016), has also been shown to reduce somatic and intergenerational expansions, our study prompts future investigations into the promising role of HDAC3 as a potential therapeutic target for TNR diseases.

Results of this thesis showing the suppression of CAG-repeat expansions after RGFP966 treatment correlate with the observed benefits in striatal neuropathological features and motor learning abnormalities in KI mice, strengthening the link between somatic CAG expansions and HD progression (Figure 52). Although we have not evaluated the effect of RGFP966 in motor coordination function, since we focused our study on the improvement of early behavioral defects, the observed recovery of motor dysfunction in previous reports using HDAC3 inhibitors could be explained not only by the amelioration of HD-related transcriptional deficits, but also by the suppression of CAG-repeat length gains. This is consistent with previous reports showing that a pharmacological treatment aimed to inhibit the lengthening of the repeat track was able to rescue motor decline in a HD mouse model (Xun et al., 2012; Budworth et al., 2015).

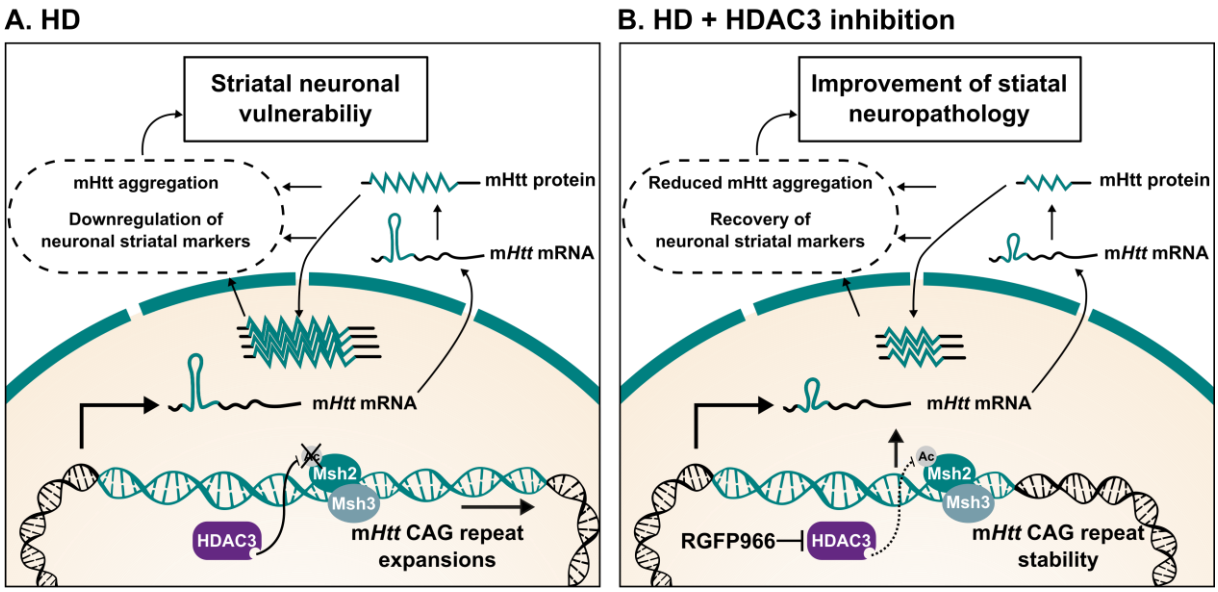



**Figure 52. HDAC3 inhibition suppresses somatic CAG repeat expansions and restores neuronal function in the HD striatum at early disease stages.** (**A**) The MutSβ complex, comprising Msh2 and Msh3, promote an aberrant expansion of the CAG repeat tract in m*Htt* gene, mostly in striatal neurons. The mutated gene would produce more toxic RNA and protein products that could accelerate neuropathological features in this region, such as mHtt aggregation or downregulation of striatal markers. (**B**) Chronic inhibition of HDAC3 with RGFP966 increases Msh2 acetylation, probably impairing its mismatch repair (MMR) activity, thus preventing the lengthening of the CAG repeat tract. The generation of less toxic molecules could produce an amelioration of the HD-related striatal neuropathology.

Given that multiple benefits were observed after HDAC3 inhibition in HD mice, it is reasonable to speculate that HDAC3 expression or activity might be altered in HD. Consistent with previous reports (Quinti et al., 2010; Mielcarek et al., 2011; Moumné et al., 2012; Valor and Guiretti, 2014), our results have revealed that total HDAC3 levels are not altered in HD. Additionally, unlike results from another group showing nuclear accumulation of HDAC3 in a transgenic HD mouse model at late disease stages (Jia et al., 2012b), our results show no differences in HDAC3 subcellular localization in the striatum of KI mice at early disease stages (6 months of age). However, no studies so far have evaluated whether mHtt dysregulates HDAC3 activity. The phosphorylation of HDAC3 at serine 424 (S424), a residue conserved between several species, is known to increase its deacetylase activity (Zhang et al., 2005; Choi et al., 2015; Wang et al., 2015; Hanigan et al., 2017). Importantly, we found significant increases in HDAC3 phosphorylation at S424 in KI mice striatum, but not in the hippocampus, from 8 months of age, which correlates with the onset of motor coordination deficits, as previously observed (Puigdellívol et al., 2015). Moreover, HDAC3 phosphorylation was found also increased in the putamen of HD patients with different Vonsattel grades (1 to 4) and all being clinically diagnosed. Therefore, the specific increase in striatal HDAC3 activity, measured by HDAC3 phosphorylation, goes in parallel with the presence of motor coordination abnormalities. Since we found no alteration of HDAC3 levels/activity in the striatum of KI mice at early diseases stages or in the hippocampus at any evaluated age, we hypothesize that the beneficial effects of HDAC3 inhibition on early cognitive abnormalities might be due to the amelioration of transcriptional deficits induced by the aberrant activity of other transcriptional regulatory proteins, such as CBP. Additionally, HDAC3 inhibition could also attenuate the striatal vulnerability exacerbated by the aberrant genomic expansion of

the CAG repeat tract. This instability process could be triggered by a repeat length above certain threshold combined with the specific cellular context present in the HD striatum, although this is still a subject of intense scientific research (Lee et al., 2010; Dion, 2014). As the diseases progresses, HDAC3 will become specifically hyperactivated in the striatum and will strongly contribute to the appearance of motor defects and striatal degeneration. Multiple kinases, including CK2 (Zhang et al., 2005), GSK3β (Bardai and D'Mello, 2011), PINK1 (Choi et al., 2015), mTOR (Wang et al., 2015), LRRK2 (Han et al., 2017) and JNK (Hanigan et al., 2017), have been shown to phosphorylate HDAC3, whereas the catalytic subunit of protein phosphatase 4 (PP4c) (Zhang et al., 2005) has shown to mediate its dephosphorylation. However, the effect of some of these proteins on HDAC3 phosphorylation was only evaluated in non-neuronal cells and their activity could differ depending on the cellular context. For example, inhibition of CK2 does not alter HDAC3 activity and phosphorylation in neuronal tissues (Choi et al., 2015). Nevertheless, we could speculate that the increase in JNK activity as a result of aberrant $p75^{NTR}$ signaling in KI mice striatum (results of this thesis) might have an impact on HDAC3 activity. Further studies will be necessary to understand the tissue-specific effect of mHtt on HDAC3 phosphorylation.

Importantly, HDAC3 has also been shown to cause selective neuronal damage (Bardai and D'Mello, 2011; Bardai et al., 2012, 2013; Schmitt et al., 2014; Yang et al., 2016; Han et al., 2017; Qu and D'Mello, 2018). First, experiments of Bardai et al. showed that the overexpression of HDAC3 reduced survival of primary neuronal cultures and immortalized neuronal cells but did not affect non-neuronal cultures, while suppression of HDAC3 was protective upon the addition of toxic stimulus in primary neurons (Bardai and D'Mello, 2011). It has also been demonstrated that the inhibition of HDAC3 deacetylase activity by RGFP966 was neuroprotective in primary neurons after exposure to injurious stimulus or in brain upon ischemic injury (Yang et al., 2016), while increased HDAC3 phosphorylation at Ser424 was suggested to promote 6-OHDA-induced neuronal death (Han et al., 2017). Although the downstream mediators of HDAC3 neurotoxicity have yet to be identified, a recent study has uncovered NPTX1 (neuronal pentraxin-1), HIP1R (huntingtin interacting protein 1 related) and HDAC9, previously implicated in the regulation of neurodegeneration, as potential candidates. Particularly, either mRNA or protein levels of these factors have been found altered following HDAC3 overexpression in neuronal cultures but not in non-neuronal cells, indicating that they could play a role in the specific neuronal death mediated by HDAC3 (Qu and D'Mello, 2018). Additionally, the direct effect of HDAC3 on CBP activity could also compromise neuronal viability, especially in the HD context, since loss of CBP function has been associated with increased striatal neurodegeneration in HD models (McCampbell et al., 2001; Nucifora et al., 2001; Taylor et al., 2003; Jiang et al., 2006). Overall, the specific alteration of HDAC3 activity in the striatum of HD patients and KI mice along with its important role in neurotoxicity support the idea that HDAC3 could mediate the observed increase in striatal vulnerability in HD, which makes the use of HDAC3is very promising as neuroprotective therapies in order to prevent the massive neuronal loss that occurs.

It is important to notice that our KI model, as well as other HD mice, exhibit neuronal dysfunction rather than substantial neuronal loss (Yu et al., 2003; Menalled, 2005; Bragg et al., 2017). In this context, several studies have proposed that the specific upregulation of different pro-survival

pathways in these HD models would act as anti-apoptotic signals (Bowles and Jones, 2014). One important example is the activation of the Akt pathway, observed as an increase in Akt phosphorylation in HD models (Ginés et al., 2003a; Saavedra et al., 2010; Bowles and Jones, 2014). Indeed, our results have shown that Akt is hyper-phosphorylated at serine 473 (S473) in the striatum of KI mice from 8 months of age in advance, which also correlates with an increase in HDAC3 phosphorylation. Consistently, a previous study has shown that the addition of IGF1, an activator of Akt signaling, or the overexpression of a constitutively active form of Akt, were able to suppress HDAC3-induced neurotoxicity in neuronal cultures (Bardai and D'Mello, 2011). Nonetheless, despite the absence of neuronal death in KI mice, the activation of neurodegenerative signals due to HDAC3 activation could still result in an altered neuronal function that would compromise behavioral outputs depending on the striatal region.

Our results, complemented with data from previous studies evaluating HDAC3 inhibition in HD mice, demonstrate that the selective inhibition of HDAC3 provides a common mechanism for treating the early and global neuronal dysfunction affecting learning and memory processes and the later HD striatal vulnerability that strongly contributes to motor coordination abnormalities.

# 2. $p75^{NTR}$ as a therapeutic target for HD

Striatal vulnerability in HD was first linked to a lack of neurotrophic support due to reduced BDNF levels (Ferrer et al., 2000; Zuccato et al., 2001, 2005, 2008; Duan et al., 2003; Ginés et al., 2003b; Hermel et al., 2004; Spires et al., 2004b), as MSNs strongly depend on this neurotrophin for their survival and correct activity (Ivkovic and Ehrlich, 1999; Baquet et al., 2004). However, although pharmacological strategies aimed to increase BDNF levels in the brain of HD patients seem promising, they also face serious limitations due to the negative pharmacokinetic characteristics of BDNF (Zuccato and Cattaneo, 2009). Besides, deficits of neurotrophic support have recently been associated not only with reduced levels of BDNF but also with altered signaling and expression of its receptors TrkB and $p75^{NTR}$ (Ginés et al., 2006, 2010; Zuccato et al., 2008; Brito et al., 2013, 2014; Liot et al., 2013; Simmons et al., 2013; Plotkin et al., 2014; Nguyen et al., 2016), through which BDNF promotes the appropriate stimulus for neuronal maintenance or death (Lu et al., 2005). Importantly, these alterations could explain why increasing BDNF levels in HD mouse models only partially improve the disease phenotype (Cho et al., 2007; Gharami et al., 2008; Xie et al., 2010; Arregui et al., 2011; Giralt et al., 2011a; Giampà et al., 2013; Silva et al., 2015). Particularly, the aberrant $p75^{NTR}$ increase in the HD striatum, together with the observed reduction in TrkB levels, could significantly contribute to striatal neuropathology and the associated motor alterations, since it has been shown that $p75^{NTR}$ can activate degenerative pathways, particularly when a deficiency of neurotrophins or Trks occurs (Yoon et al., 1998; Fortress et al., 2011; Brito et al., 2013). Therefore, in order to overcome the restricted benefits of BDNF-based therapies, one promising strategy would be to inhibit $p75^{NTR}$ signaling or expression.

Previous studies from our group have demonstrated that hippocampal-dependent memory and synaptic plasticity deficits can be prevented by the genetic downregulation of $p75^{NTR}$ in the hippocampus of KI mice (Brito et al., 2014). However, the precise contribution of the

BDNF/TrkB/p75$^{NTR}$ pathway to the onset of HD motor coordination deficits has not yet been elucidated. To address this question, we have evaluated double mutant KI:p75$^{+/-}$ mice carrying one copy of m*Htt* while a single functional *p75$^{NTR}$* allele, thus presenting a normalization of p75$^{NTR}$ levels in MSNs at disease-manifesting stages. Importantly, although it can be argued that the genetic normalization of p75$^{NTR}$ is not exclusively neuronal since expression of p75$^{NTR}$ in astrocytes has been reported following different brain injuries (Cragnolini and Friedman, 2008; Cragnolini et al., 2009), previous studies from our group have demonstrated that the aberrant p75$^{NTR}$ increase in the striatum and hippocampus of HD mice is neuron-specific (Brito et al., 2013, 2014). Longitudinal motor assessments revealed that genetic normalization of p75$^{NTR}$ expression in KI mice striatum delays the onset of motor coordination deficits (from 8 to 10 months). Conversely, previous studies from our group have shown that defects in the initial acquisition of motor skills were not prevented at 6 months of age by p75$^{NTR}$ normalization (Brito et al., 2014). This disparity might be explained by the fact that motor learning deficits in HD partially depend on cortical integrity (Puigdellívol et al., 2015). Since p75$^{NTR}$ levels have been found upregulated in the striatum and the hippocampus but not in the cerebral cortex of KI mice (Brito et al., 2014), the substantial decrease in cortical p75$^{NTR}$ levels in KI:p75$^{+/-}$ mice could play a negative role in the proper function of cortical neurons that might counteract any positive effect in the striatum. Additionally, we cannot rule out that other molecular pathological processes in the cortex could also contribute to this learning behavioral impairment.

In accordance with the delayed manifestation of motor abnormalities, KI:p75$^{+/-}$ mice exhibit a recovery of pathological hallmarks associated with degeneration of striatal neurons, but only until the age of 10 months. Particularly, we observed a restoration in the expression of the striatal protein markers DARPP-32, A2AR, and PDE10A, a decrease in mHtt aggregate load and a recovery of dendritic spine density. All these features could contribute to ameliorate neuronal dysfunction, which may be responsible of impaired control of motor function (Davies et al., 1997; Milnerwood and Raymond, 2010; Francelle et al., 2017). For instance, DARPP-32, A2AR, and PDE10A have been described to regulate striatonigral and striatopallidal downstream signaling cascades that are key for the fine-tuning of movement (Siuciak et al., 2006; Nishi et al., 2008; Girault, 2012; Cui et al., 2013). Moreover, A2AR facilitates BDNF-TrkB activation effects on synaptic transmission (Diogenes et al., 2004; Fontinha et al., 2008; Sebastiao et al., 2011) while DARPP-32 participates through interaction with adducin to modulate dendritic spine stability (Engmann et al., 2015).

The observed improvements in HD motor function and striatal pathology were also accompanied by a recovery of the overall neurotrophic signaling imbalance. First, our results demonstrated that the reduction of p75$^{NTR}$ levels normalized BDNF/TrkB levels in the striatum, which were found decreased in KI mice at 8 months of age, the age at which first motor coordination symptoms appear. Again, the normalization of p75$^{NTR}$ levels at 10 months of age was not enough to recover BDNF/TrkB expression. Nonetheless, it remains unclear which are the molecular mechanisms associated with the recovery at earlier stages. Our results showed that decreased BDNF levels in the striatum of KI mice were accompanied by unaltered cortical BDNF levels, suggesting that mHtt does not modify BDNF expression in the cortex (at least at the evaluated ages), in agreement

with results from other studies in different HD mouse models (Spires et al., 2004b; Gharami et al., 2008; Smith et al., 2014). Since most striatal BDNF content comes from the cortex, these data suggest that the anterograde transport of mBDNF and/or proBDNF from cortex to striatum could be impaired. Indeed, the presence of mHtt has been shown to prevent the efficient axonal transport of BDNF-containing vesicles along corticostriatal axons (Gauthier et al., 2004; Zala et al., 2008) and to cause progressive axonal degeneration in cortical neurons (Li and Conforti, 2013; Gatto et al., 2015). Additionally, our results showed that cortical BDNF levels were neither modified by the genetic modulation of $p75^{NTR}$ expression. In view of all these data, we hypothesize that $p75^{NTR}$ could influence BDNF delivery from the cortex. Consistent with this idea, $p75^{NTR}$ has been shown to play a role in axonal degeneration (Kraemer et al., 2014) so it is possible that $p75^{NTR}$ normalization in KI mice striatum contributes to attenuate the axonal pathology and restore BDNF delivery. Alternatively, $p75^{NTR}$ could directly modulate BDNF transport, since the presence of $p75^{NTR}$ has been shown to promote the retrograde transport of several neurotrophins, including BDNF (Curtis et al., 1995; von Bartheld et al., 1996; Harrison et al., 2000). Regarding the modulation of TrkB expression by $p75^{NTR}$, further studies are necessary to evaluate their relationship and the precise underlying mechanisms. However, it should be noted that TrkB recovery might not be related to BDNF normalization, since other studies have shown that genetic BDNF modulation in mice does not modify TrkB expression (Ginés et al., 2006; Gharami et al., 2008; Giralt et al., 2009; Xie et al., 2010).

Beyond the observed impairment in striatal BDNF/TrkB expression in KI mice, our results have shown an impaired activation of the TrkB-mediated PLCγ pathway starting early in the disease progression (6 months), which was delayed until the age of 10 months by normalizing $p75^{NTR}$ levels. In agreement with our data, previous studies in other HD mouse models have shown reduced phosphorylation of TrkB at tyrosine 816 (Y816), the tyrosine residue related to PLCγ activation, and/or reduced phosphorylation of PLCγ (Gharami et al., 2008; Simmons et al., 2013; Ma et al., 2015; Garcia-Diaz Barriga et al., 2017). Interestingly, an increasing body of evidence suggests that PLCγ plays a key role in the modulation of synaptic transmission and an abnormal activation of PLCγ has been detected in several brain disorders, resulting in impaired neuronal activity (Jang et al., 2013). By contrast, the phosphorylation ratio of TrkB at Y515, associated with the activation of Akt and ERK pathways, mostly involved in neuronal survival, was found unchanged in KI mice. In this regard, several studies have shown that the phosphorylation of different tyrosine residues in TrkB could be differentially regulated *in vivo* (Lucas et al., 2003; Saarelainen et al., 2003; Rantamäki et al., 2007; Di Lieto et al., 2012; Garcia-Diaz Barriga et al., 2017). Additionally, despite the lack of changes in the phosphorylation ratio of TrkB at Y515 and the decrease in TrkB and BDNF protein levels starting at 8 months of age, we found that Akt was hyper-phosphorylated at S473 without changes in ERK activation. As previously discussed, the upregulation of several pro-survival kinase signaling pathways, such as Akt and ERK, have been described in HD mouse models and are thought to contribute to the little neuronal loss observed in these mice (Bowles and Jones, 2014). Particularly, the sustained hyperphosphorylation of Akt (at S473) has been associated with reduced levels of PHLPP1 phosphatase, as both events occur simultaneously (Saavedra et al., 2010), and to an abnormal NMDA receptor activation mediated

by mHtt that in turns leads to a PI3K-dependent activation of Akt signaling (Ginés et al., 2003a). However, it is important to note that in the striatum of more aggressive HD mouse models (R6/1 and R6/2 mice), TrkB phosphorylation deficits were also observed in other tyrosine residues, together with decreased activation of Akt and/or ERK (Gharami et al., 2008; Simmons et al., 2013; Nguyen et al., 2016). Importantly, our results provide strong evidence for a specific modulation of the TrkB-dependent PLCγ pathway by $p75^{NTR}$ expression, without any effect on Akt or ERK. These results could be a consequence of BDNF normalization in KI:$p75^{+/-}$ mice, as genetic BDNF reduction in mice was shown to specifically decrease phospho-PLCγ levels (Giralt et al., 2009), and/or a direct effect of $p75^{NTR}$ on TrkB phosphorylation sites. In agreement with the second model, $p75^{NTR}$ has been shown to interact with all Trks (Bibel et al., 1999) and modulate TrkB phosphorylation, with some studies suggesting an inhibitory effect of $p75^{NTR}$ on TrkB phosphorylation (Vesa et al., 2000, Sakuragi et al., 2013). However, the molecular mechanisms by which $p75^{NTR}$ alters TrkB-PLCγ signaling need to be further explored.

Several pieces of evidence suggest a potential role for BDNF, TrkB or PLCγ expression/activity in the modulation of some of the striatal pathological hallmarks analyzed in our experiments. For instance, increased BDNF levels have been demonstrated to enhance DARPP-32 expression (Ivkovic et al., 1997; Ivkovic and Ehrlich, 1999), while BDNF (Baquet et al., 2004), TrkB (Li et al., 2012), and PLCγ (Zhou et al., 2007; Kim et al., 2010) have been shown to positively correlate with dendritic spine density. Additionally, the overexpression of BDNF or the specific activation of PLCγ with a small-molecule TrkB ligand was shown to reduce formation of neuronal intranuclear inclusions in the striatum of R6/1 mice (Gharami et al., 2008; Garcia-Diaz Barriga et al., 2017). Importantly, neuropathological features in our KI:$p75^{+/-}$ mice were preserved as long as the overall BDNF/TrkB/$p75^{NTR}$ imbalance was prevented. Thus, at 10 months of age, the normalization of striatal $p75^{NTR}$ levels in KI mice was not able to prevent the decrease in BDNF levels and in TrkB levels and signaling. This might be the result of the unstoppable advance of other pathological mechanisms triggered by mHtt and probably not modulated by $p75^{NTR}$ levels, such as the progressive dysregulation of transcription or the appearance of somatic CAG repeat expansions (Lloret et al., 2006; Bragg et al., 2017), among others (Zuccato et al., 2010; Ross and Tabrizi, 2011; Bates et al., 2015). Overall, these results have great implications for the development of pharmacological strategies in HD aimed to reduce only the expression or activity of $p75^{NTR}$, since they could provide some therapeutic benefits at early stages of the disease but as the pathogenic process progresses, such benefits would be lost. It is also important to take into consideration another study showing that the complete genetic ablation of $p75^{NTR}$ in the zQ175 knock-in mouse model of HD abolished pro-survival signaling pathways and exacerbated striatal atrophy (Wehner et al., 2016). Although this seem to contradict our results, it might indicate that downregulating $p75^{NTR}$ expression/signaling below threshold levels causes detrimental effects in the HD brain.

While $p75^{NTR}$ can potentiate or reduce Trk signaling depending on the cellular context, it can also act independently activating ceramide, NF-kB, JNK, PTEN, and RhoA/ROCK pathways (Dechant and Barde, 2002; Pramanik et al., 2017). In this line, our results demonstrated a correlation between increased $p75^{NTR}$ levels in the striatum of KI mice and an overactivation of the pro-apoptotic JNK pathway. Then, besides the recovery of trophic BDNF-TrkB signaling in

KI mice following normalization of $p75^{NTR}$ expression, an attenuation of JNK was also observed in these double mutant mice. It is noteworthy that even though in the $p75^{+/-}$ heterozygous mice a dose dependent effect of *p75*$^{NTR}$ gene copy number on pJNK levels would be expected, we observed that these mice mostly display phenotypes similar to WT mice. Therefore, we hypothesize that the activation or inhibition of specific pathways are dependent on an "optimal range" of $p75^{NTR}$ expression levels. Moreover, we cannot exclude that additional pathways could regulate JNK activation since $p75^{NTR}$ levels are increased at 6 months of age in KI mice striatum but JNK activity is not altered until the age of 8 months. Classically, JNK activation has been associated with cellular stress responses causing neuronal death in a wide range of pathological contexts (Coffey, 2014). Regarding HD, JNK activation was found increased in cultured cells after the overexpression of mHtt and in HD animal models, and inhibition of JNK was shown to protect from mHtt-induced apoptosis (Liu, 1998; Perrin et al., 2009; Taylor et al., 2013). However, our results showing lack of caspase-3 activation in KI mice indicate no effect on neuronal viability, in agreement with previous literature (Bragg et al., 2017). Despite this, the increased JNK signaling could still play an important role in HD striatal neuropathology. Indeed, as previously discussed, neuronal dysfunction is present in symptomatic HD patients and HD mouse models long before evidence of cell death. In this scenario of cellular dysfunction, inhibition of JNK signaling in cell cultures has been associated with a reduction in polyglutamine and mHtt protein aggregation (Cowan et al., 2003; Scappini et al., 2007). Similarly, treatment with a $p75^{NTR}$ ligand that enhances trophic downstream signals and inhibits JNK pathway has also been demonstrated to reduce mHtt aggregation in R6/2 mice (Simmons et al., 2016). Additionally, mHtt-mediated JNK activation has also been associated with an impairment of fast axonal transport (Morfini et al., 2009). Therefore, in view of all these findings, it can be speculated that the attenuation of JNK signaling at early/middle disease stages by normalization of $p75^{NTR}$ expression might contribute to decrease the number of intranuclear mHtt aggregates in the striatum and restore HD-related axonal pathology in our KI:$p75^{+/-}$ mice. Interestingly, the overactivation of JNK pathway mediated by increased $p75^{NTR}$ levels in HD might explain why a variety of therapies based on neurotrophic factors only partially improved mHtt-induced pathology in HD mouse models.

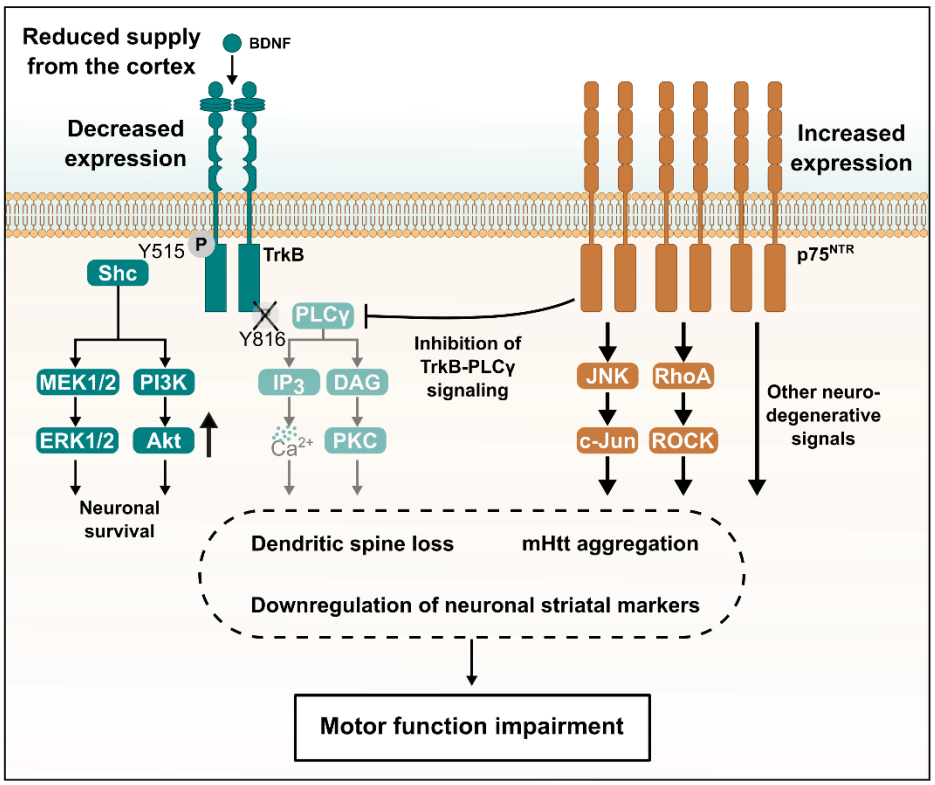
Although not evaluated in this thesis, we cannot rule out that the normalization of other pathways activated by $p75^{NTR}$ contributes to the observed behavioral and neuropathological benefits in KI:$p75^{+/-}$ mice. For example, treatment with an inhibitor of ROCK was shown to improve motor function in R6/2 mice (Li et al., 2009) and blocking of the RhoA/ROCK pathway has been demonstrated to enhance mHtt degradation and/or inhibit its aggregation status in cellular, Drosophila and mouse models of HD (Pollitt et al., 2003; Shao et al., 2008; Bauer and Nukina, 2009; Bauer et al., 2009; Li et al., 2009; Hensel et al., 2015). Additionally, it has been suggested that aberrant RhoA activation due to increased $p75^{NTR}$ levels in HD mice leads to impaired synaptic plasticity and decreased dendritic spine density (Brito et al., 2014; Plotkin et al., 2014; Miguez et al., 2015). Importantly, our results showing that $p75^{+/-}$ mice present an increase in dendritic spine density from 8 months of age suggest a direct and negative role of $p75^{NTR}$ on dendrite complexity and spine density even in WT animals, in accordance with previous studies (Zagrebelsky et al.,

2005; Chapleau and Pozzo-Miller, 2012), that cannot be explained by the signalling pathways evaluated in our study.

Overall, results of this thesis supporting the neuropathological role of $p75^{NTR}$ in HD suggest that potential benefits might be obtained from its inhibition in other prevalent neurodegenerative diseases, since several reports have described that $p75^{NTR}$ expression (in specific brain regions) is dramatically increased in aging and in patients of Alzheimer's disease, amyotrophic lateral sclerosis, multiple sclerosis and temporal lobe epilepsy (Meeker and Williams, 2014).

In summary, our findings shed new light on how the impaired BDNF/TrkB/$p75^{NTR}$ system, which leads to reduced TrkB-PLCγ pathway and increased JNK activation, can affect the onset of striatal neuropathology and motor behavioral alterations in HD. More importantly, the normalization of $p75^{NTR}$ expression was shown to delay such alterations (Figure 53). Results of this thesis, together with previous studies from our group showing cognitive improvements after $p75^{NTR}$ normalization in the hippocampus of KI mice, demonstrate that pharmacological treatments aimed to reduce $p75^{NTR}$ signaling could be beneficial for treating both cognitive and motor symptomatology at early/middle disease stages. However, restoring the overall BDNF/TrkB/$p75^{NTR}$ balance at later stages would be key to prevent the progression of HD pathology (Figure 53).

**A. Early/middle HD**



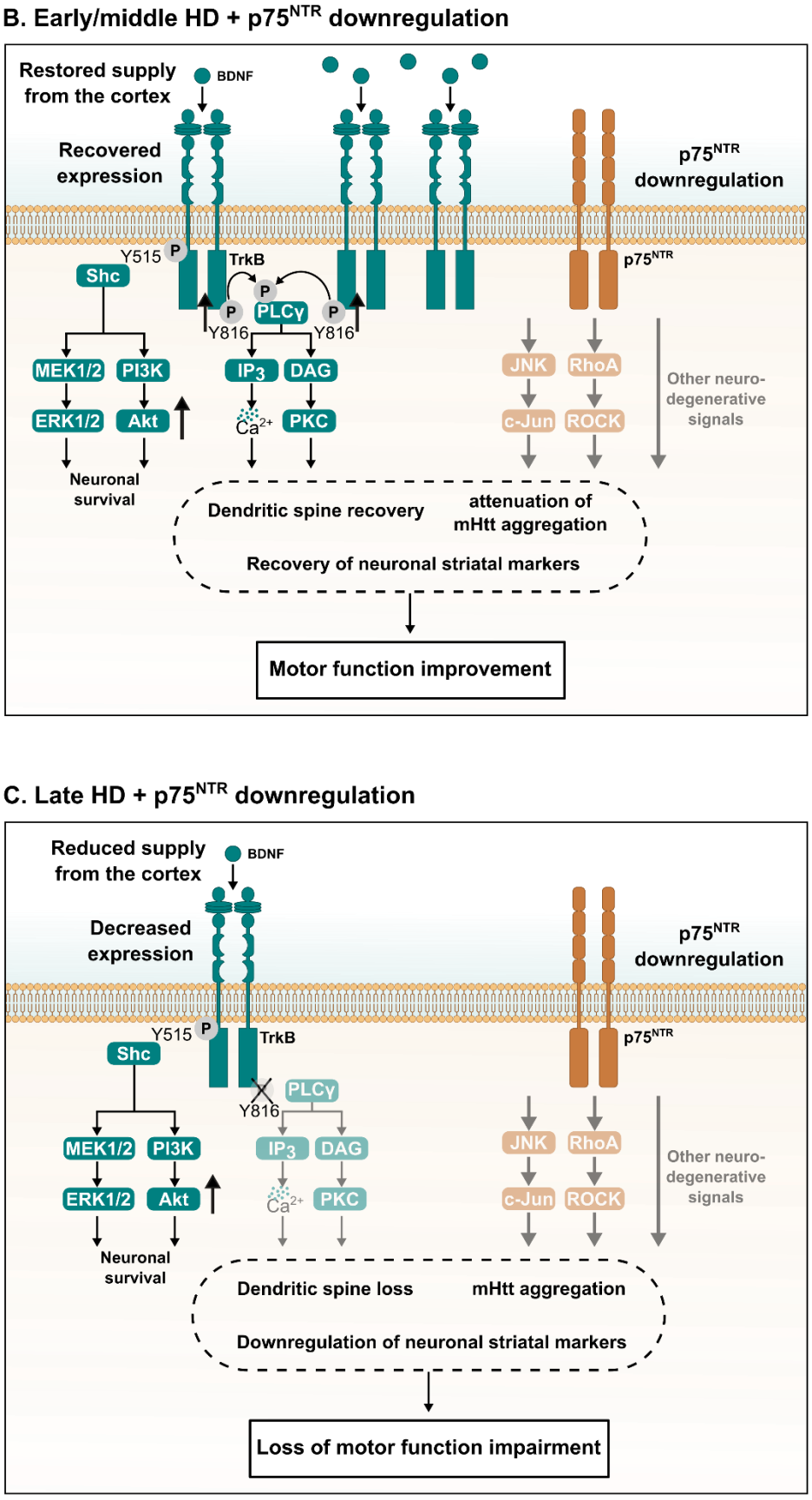



**Figure 53. Early downregulation of p75$^{NTR}$ delays the onset of motor deficits and striatal dysfunction in HD.** (**A**) In the HD striatum at early/middle stages the presence of mHtt impairs BDNF supply from the cortex and TrkB expression but increases p75$^{NTR}$ levels. The abundant p75$^{NTR}$ expression, together with TrkB and BDNF deficiency,

could contribute to reduce TrkB phosphorylation at Y816, which results in an attenuation of the PLCγ pathway, particularly involved in synaptic plasticity. Conversely, phosphorylation of Akt might be increased in some HD mouse models, likely by the presence of pro-survival mechanisms. Additionally, $p75^{NTR}$ receptors could increase the pro-apoptotic signal JNK or the RhoA/ROCK pathway, which compromise synaptic integrity, among other neurodegenerative pathways. Altered TrkB and $p75^{NTR}$ signaling results in striatal neuronal dysfunction that contributes to the onset of motor coordination abnormalities. (**B**) Genetic reduction of $p75^{NTR}$ levels at early/middle HD stages recovers BDNF and TrkB protein expression by an unknown mechanism and facilitates TrkB phosphorylation at Y816 to engage the PLCγ pathway. Besides, $p75^{NTR}$-dependent neurodegenerative signals are reduced. Overall, this contributes to a decreased striatal pathology and delays the onset of motor symptoms. (**C**). At late disease stages (10 months), reduced $p75^{NTR}$ levels are not enough to restore BDNF/TrkB expression or signaling, and observed improvements in neuropathological features are lost, as well as amelioration of motor behavior.

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Taken together, evidence from our findings and previous studies demonstrate that HDAC3 and $p75^{NTR}$ could affect key pathological mechanisms underlying neuronal dysfunction in multiple brain regions, such as the striatum and the hippocampus, and thus represent promising therapeutic targets for treating both cognitive and motor function impairments in HD.

# CONCLUSIONS

The main conclusions extracted from the results presented in this thesis can be summarized as follows:

1. Selective HDAC3 inhibition prevents cognitive deficits in $Hdh^{Q7/Q111}$ mice, likely by recovering the neuronal activity-dependent transcription of important memory-related genes, such as *Arc* and *Nr4a2*.

2. The effect of HDAC3 inhibition on gene transcription could be the result of a direct effect on histone acetylation and/or on CBP acetylation, potentially causing an increase in its activity.

3. Chronic inhibition of HDAC3 suppresses striatal CAG repeat expansions in $Hdh^{Q7/Q111}$ mice, likely by impairing Msh2 activity.

4. Increased HDAC3 phosphorylation in the striatum of $Hdh^{Q7/Q111}$ mice and in the putamen of HD patients correlates with the onset of motor symptoms and might reflect a tissue-specific increase in HDAC3-mediated neurotoxicity.

5. Aberrant $p75^{NTR}$ levels in the striatum of $Hdh^{Q7/Q111}$ mice impair the TrkB-dependent PLCγ pathway and increase JNK activation, triggering neurodegenerative signals that compromise neuronal function.

6. The normalization of striatal $p75^{NTR}$ levels in $Hdh^{Q7/Q111}$ mice delays the onset of motor coordination abnormalities and striatal neuropathological features, likely by slowing down the overall BDNF/TrkB/$p75^{NTR}$ imbalance.

7. Modulating $p75^{NTR}$ levels at late disease stages in $Hdh^{Q7/Q111}$ mice is not enough to bypass other mutant huntingtin-related pathological processes and earlier therapeutic benefits become abrogated, which highlights the importance of finding alternative therapeutic strategies to restore the overall neurotrophic imbalance.

Collectively, our results have provided further insight into the contribution of transcriptional dysregulation, somatic CAG instability and neurotrophin signaling disturbances to HD progression and highlight HDAC3 and $p75^{NTR}$ as promising therapeutic targets to correct these pathogenic mechanisms and ameliorate behavioral impairments and neuropathology in HD.

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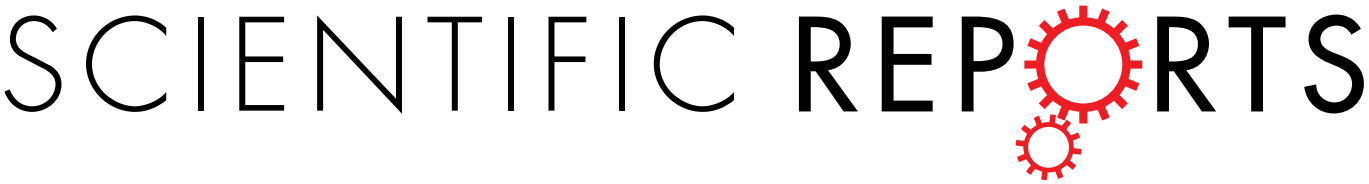
# ANNEX



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# A selective inhibitor of histone deacetylase 3 prevents cognitive deficits and suppresses striatal CAG repeat expansions in Huntington's disease mice

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**Huntington's disease (HD) is a neurodegenerative disorder whose major symptoms include progressive motor and cognitive dysfunction. Cognitive decline is a critical quality of life concern for HD patients and families. The enzyme histone deacetylase 3 (HDAC3) appears to be important in HD pathology by negatively regulating genes involved in cognitive functions. Furthermore, HDAC3 has been implicated in the aberrant transcriptional patterns that help cause disease symptoms in HD mice. HDAC3 also helps fuel CAG repeat expansions in human cells, suggesting that HDAC3 may power striatal expansions in the *HTT* gene thought to drive disease progression. This multifaceted role suggests that early HDAC3 inhibition offers an attractive mechanism to prevent HD cognitive decline and to suppress striatal expansions. This hypothesis was investigated by treating $Hdh^{Q111}$ knock-in mice with the HDAC3-selective inhibitor RGFP966. Chronic early treatment prevented long-term memory impairments and normalized specific memory-related gene expression in hippocampus. Additionally, RGFP966 prevented corticostriatal-dependent motor learning deficits, significantly suppressed striatal CAG repeat expansions, partially rescued striatal protein marker expression and reduced accumulation of mutant huntingtin oligomeric forms. These novel results highlight RGFP966 as an appealing multiple-benefit therapy in HD that concurrently prevents cognitive decline and suppresses striatal CAG repeat expansions.**

Huntington's disease (HD) is a fatal neurodegenerative disorder marked by progressive motor dysfunction, cognitive deficits and psychiatric disturbances. Currently available treatments help manage some of the symptoms but there is no cure, nor has disease progress be reversed or slowed. HD is caused by inheritance of an expanded CAG repeat in the huntingtin (*HTT*) gene, resulting in a mutant huntingtin (mHtt) protein containing extra glutamine residues[1,2]. Increasing evidence suggests that extending the glutamine tract confers mHtt with new toxic properties, including aberrant interactions with proteins necessary for chromatin maintenance and gene expression. In doing so, mHtt may induce transcriptional dysregulation and negatively impact expression of key genes for brain activity[3,4]. For example, the histone acetyltransferase CBP/p300 forms aggregates with mHtt, reducing acetyltransferase activity. In HD patients and mice, chromatin becomes hypoacetylated and transcriptional dysregulation occurs in many genes, including those involved in long-term memory[5–7].

Inhibition of histone deacetylases (HDACs) is predicted to increase histone acetylation and restore normal transcriptional patterns. Recent studies used inhibitors selective for HDAC isotypes to minimize toxicity. Treatment with inhibitors primarily targeting HDAC3, including some with additional activity on HDAC1, led to

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improvement in motor function and working memory in N-terminal transgenic HD mice[8–12]. These benefits were correlated with attenuation of striatal atrophy, reestablishment of normal striatal and cortical gene expression patterns and modulation of epigenetic DNA modifications. These encouraging findings are consistent with the discovery that wild type but not mHtt interacts with HDAC3 and represses its neurotoxic activity[13].

There is a gap in our understanding of potential benefits of HDAC3-selective inhibitors on cognitive functions, such as motor learning and long-term memory. HD patients and mice show significant declines in cognitive function before onset of motor symptoms[14–17]. Cognitive decline is a critical quality-of-life concern for HD patients and families[17]. Notably, HDAC3 is a negative regulator of gene expression required for long-term memory formation[18] and HDAC3 focal deletion or selective inhibition improves memory and neural plasticity in rodents[18–22]. Together, these findings highlight HDAC3 as a key player in enforcing transcriptional dysregulation that underlies cognitive symptoms of HD. In addition, HDAC3 also stimulates expansions of CAG repeats in human tissue culture cells[23, 24]. HDAC3 inhibition or knockdown suppressed most CAG repeat expansions. In contrast, inhibition of HDAC1/HDAC2 or knockdown of HDAC1 failed to suppress expansions in tissue culture[23]. There is significant evidence that the *HTT* CAG repeat undergoes progressive tissue- and cell type-specific expansions[25–32], which could contribute to disease progression and age of onset[33–37]. Therefore HDAC3 is also an attractive therapeutic target for inhibiting striatal expansions and delaying HD.

This study tested the idea that HDAC3 is important in HD pathology via its transcriptional repressive effects contributing to cognitive impairment and in fueling somatic CAG repeat expansions. We demonstrate that early intervention in $Hdh^{Q7/Q111}$ knock-in mice with RGFP966, a selective HDAC3 inhibitor with some activity on HDAC1/2, prevents motor learning and long-term memory deficits, reduces striatal CAG repeat expansions and improves hippocampal and striatal pathologies. These new findings, combined with beneficial effects of HDAC3 inhibition on motor function[8–12], indicate that RGFP966 simultaneously delivers multiple benefits in HD mice.

## Results

**Systemic RGFP966 administration efficiently inhibits HDAC activity in hippocampus and striatum.** The major goal of the project was to use an HDAC3-selective inhibitor in an early-intervention strategy to prevent or reduce key HD symptoms in mice. We focused on two important aspects of HD that have not been previously tested with HDAC3 inhibitors: preventing cognitive deficits, both in motor learning and long-term memory, and suppressing somatic CAG repeat expansions. RGFP966 was chosen as the inhibitor, as it is 30- to 200-fold selective for HDAC3, with reported $IC_{50}$ values of 64–80 nM[12, 19]. RGFP966 reaches the brain rapidly after systemic injection[19, 20]. The $Hdh^{Q7/Q111}$ knock-in (KI) mouse was utilized because, like most HD patients, these mice are heterozygous for the *HTT* mutation, expressing one normal mouse (Q7) and one expanded (Q111) allele. Importantly, the animals reproduce key features of human disease, including an accurate expression of huntingtin protein and a similar timing of emotional, cognitive and motor impairment[38–43]. The slow progression of HD pathology provides a suitable age window for early intervention with the HDAC3 inhibitor. Additionally, since these mice carry the human HD mutation, they also provide an ideal model in which to study the instability of the HD CAG repeat in its appropriate genomic context.

RGFP966 was administered subcutaneously to wild type ($Hdh^{Q7/Q7}$) and KI ($Hdh^{Q7/Q111}$) mice three times per week for 13 weeks starting at three months of age, prior to development of cognitive defects (Fig. 1a). The inhibitor was well tolerated, with no significant alteration in body weight compared to vehicle-treated animals (Fig. 1b). Mice were consequently analyzed for behavior at six months of age when cognitive but not motor symptoms start to appear (Fig. 1a). All animals were sacrificed subsequently and key brain tissues were examined by biochemical and genetic analysis.

Cognitive behavioral tasks described in the next section are mostly dependent on the hippocampus and striatum, so it was important to demonstrate drug activity in these tissues. Examination of histone H3 acetylation at lysine position 9 (AcH3K9) in hippocampal and striatal regions showed drug-dependent increases in both wild type and KI animals (Fig. 1c), consistent with a decrease in HDAC3 activity. Reduced HDAC3 activity was likely due to inhibition of enzyme activity, since the abundance of HDAC3 protein was unchanged between genotypes or by RGFP966 treatment (Fig. 1d). These findings indicate efficacy of systemic administration of RGFP966 in inhibiting HDAC activity in the hippocampus and striatum.

**RGFP966 prevents hippocampal-dependent long-term memory deficits.** Cognitive dysfunction is an early clinical hallmark of HD that precedes motor coordination deficits, both in KI mice[39, 40] and in HD patients[15–17]. Even though it is known that HDAC3 acts as a negative regulator of gene expression required for long-term memory formation[18], no data have been reported on the effect of HDAC3 inhibition on long-term memory impairments in HD. Therefore, we investigated whether RGFP966 treatment could block or delay impairments in hippocampal-dependent recognition and spatial memories.

Performance in the object location task (OLT) and the novel object recognition task (NORT) were evaluated in vehicle and RGFP966-treated mice (Fig. 2). In both tests, mice were first subjected to a training session in the presence of two similar objects. During training (Fig. 2a and b), the animals showed similar time exploring each object, indicating no detectable preference for object or location. When long-term memory was assessed 24 h after training (Fig. 2a and b), wild type mice demonstrated a clear increase in time exploring both the novel location and object regardless whether the animals were treated with RFGP966 or vehicle alone. In contrast, vehicle-treated KI mice demonstrated a similar time exploring familiar and novel objects, with minimal preference for either spatial or object novelty. This significant loss in recognition of object and spatial changes is consistent with previous studies of these KI mice[39]. Interestingly, treatment of KI mice with RGFP966 prevented the loss of spatial and recognition memories, as shown by increasing time exploring the novel versus the familiar object and a significantly

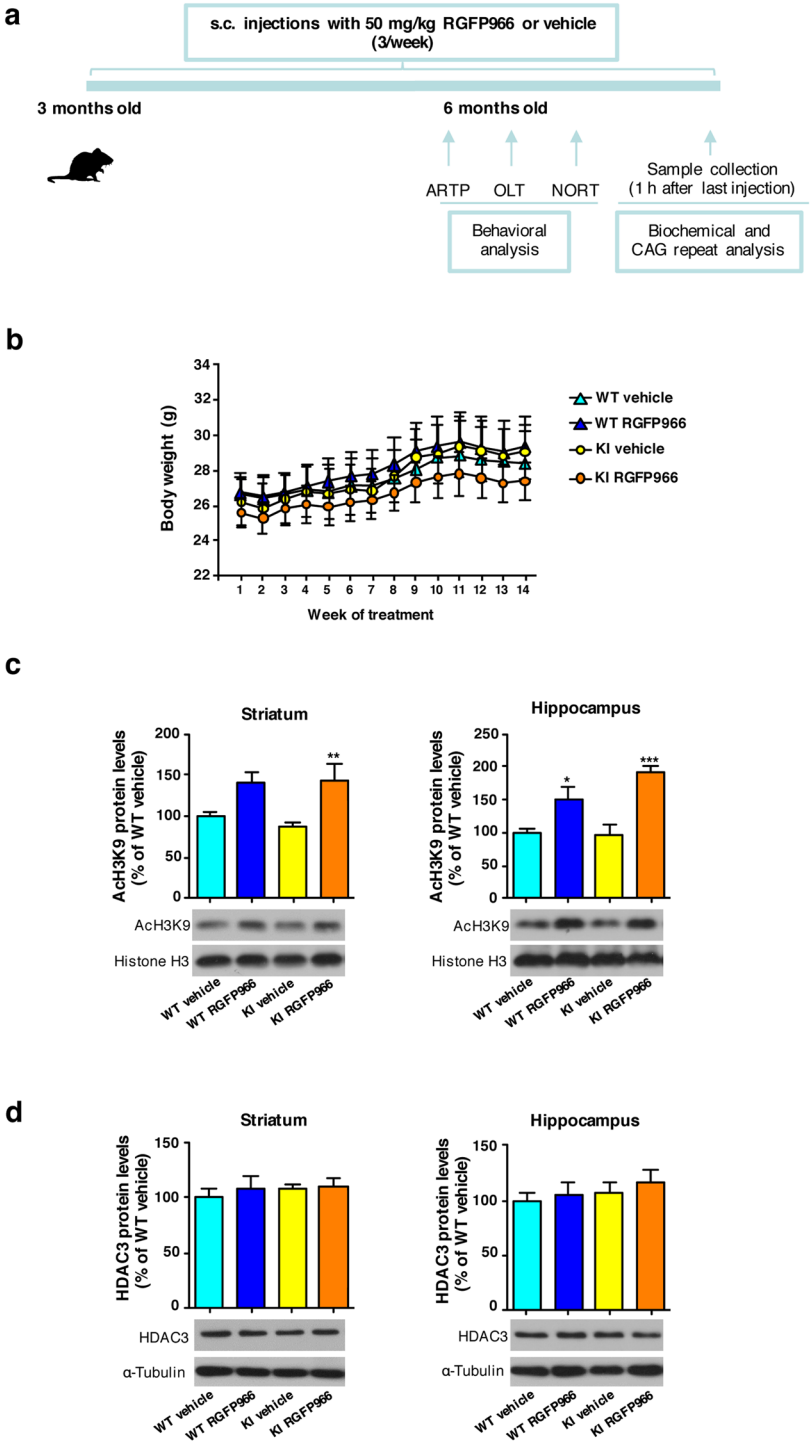


**Figure 1.** Systemic RGFP966 treatment efficiently inhibits hippocampal and striatal HDAC activity. **(a)** Schematic of chronic treatment and experimental plan. ARTP, accelerating rotarod task procedure; OLT, object location task; NORT, novel object recognition task. **(b)** Body weight measurements. Data represent the mean ± SEM (n = 6–10). **(c)** Representative immunoblots showing histone H3 acetylation levels at position lysine 9 (AcH3K9) normalized to total histone H3 and **(d)** HDAC3 levels normalized to α-tubulin in striatal and hippocampal extracts from vehicle and RGFP966 chronically treated wild type $Hdh^{Q7/Q7}$ (WT) and mutant $Hdh^{Q7/Q111}$ (KI) mice. The blots in **(c)** and **(d)** are cropped; full-length images are provided in Supplementary Figure 2. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared to vehicle-treated mice by two-way ANOVA with Bonferroni *post-hoc* analysis. Data represent the mean ± SEM (n = 4–7 per group).

higher discrimination index compared to vehicle-treated KI animals (Fig. 2a and b). These findings indicate that RGFP966 treatment prevents impairment in spatial and recognition long-term memory in HD mice.

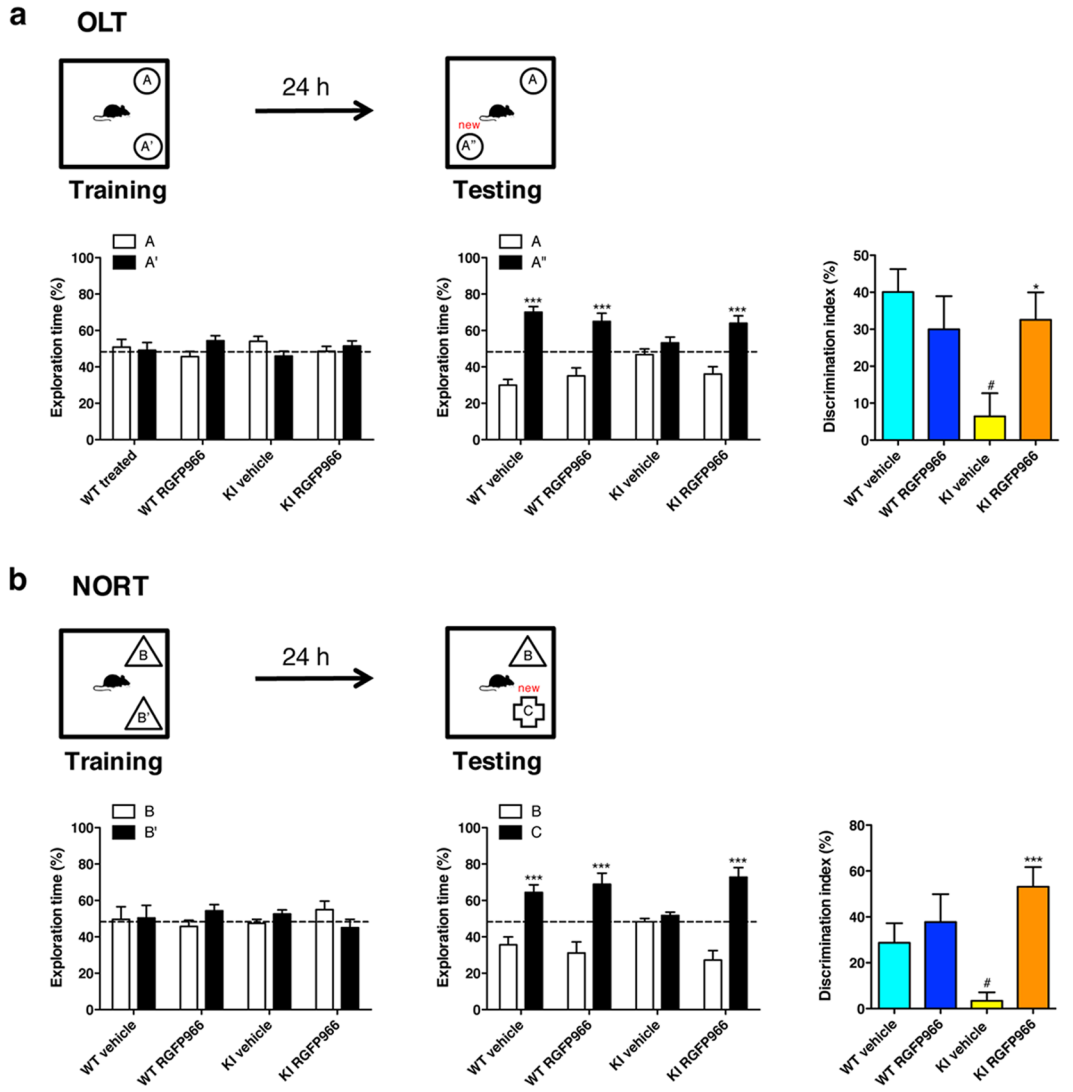


**Figure 2.** RGFP966 treatment prevents impairment of long-term memory in HD mice. Behavior analysis in vehicle and RGFP966 chronically treated $Hdh^{Q7/Q7}$ (WT) and $Hdh^{Q7/Q111}$ (KI) mice. Performance in **(a)** spatial (OLT) and **(b)** recognition (NORT) memory tests. Exploration time during training and testing session (left and center panels). Dashed line shows chance level for exploration. $^{***}p < 0.001$ compared to the percentage of time exploring the familiar object by two-way ANOVA with Bonferroni *post-hoc* analysis. Discrimination index (right panel). $^{*}p < 0.05$, $^{***}p < 0.001$ compared to vehicle-treated KI mice; $^{\#}p < 0.05$ compared to vehicle-treated WT mice by two-way ANOVA with Bonferroni *post-hoc* analysis. Data represent the mean ± SEM (n = 5–9 animals per group).

**RGFP966 normalizes hippocampal expression of memory-dependent genes.** Recognition and spatial long-term memory deficits in KI mice have been associated with altered expression of activity-dependent, memory-related genes[7]. Inhibiting HDAC3 in hippocampus is predicted to elevate transcription of key genes for long-term memory formation in response to training[18]. To test this hypothesis, RT-qPCR was used to examine hippocampal expression of *Arc*, *Nr4a2, Egr1* and *c-Fos* after acute treatment of mice with RGFP966 or vehicle and in trained conditions (Fig. 3a). These genes are key neuronal activity-dependent early genes whose expression is required for long-term memory and synaptic plasticity[7,44–46] and some of them are known to be regulated by HDAC3 in a brain-region dependent manner[18,19]. Arc and Nr4a2 transcript levels were significantly reduced in vehicle-treated KI mice compared to wild type animals while no changes were found in Egr1 and c-Fos expression. Interestingly, treatment of KI mice with RGFP966 led to Arc and Nr4a2 expression at levels indistinguishable from wild type mice (Fig. 3b). These results were confirmed by immunohistochemical analysis to detect Arc and Erg1 proteins. A significant decrease in the number of Arc-positive cells was detected in the dentate gyrus of vehicle-treated KI mice compared to wild type mice (Fig. 3c), and this decrease was blocked in RGFP966 treated KI mice. Arc-positive neurons were not evident in other hippocampal regions. In contrast, no significant differences between genotypes or treatment condition were found when Egr1 immunoreactivity was analyzed (Fig. 3d),

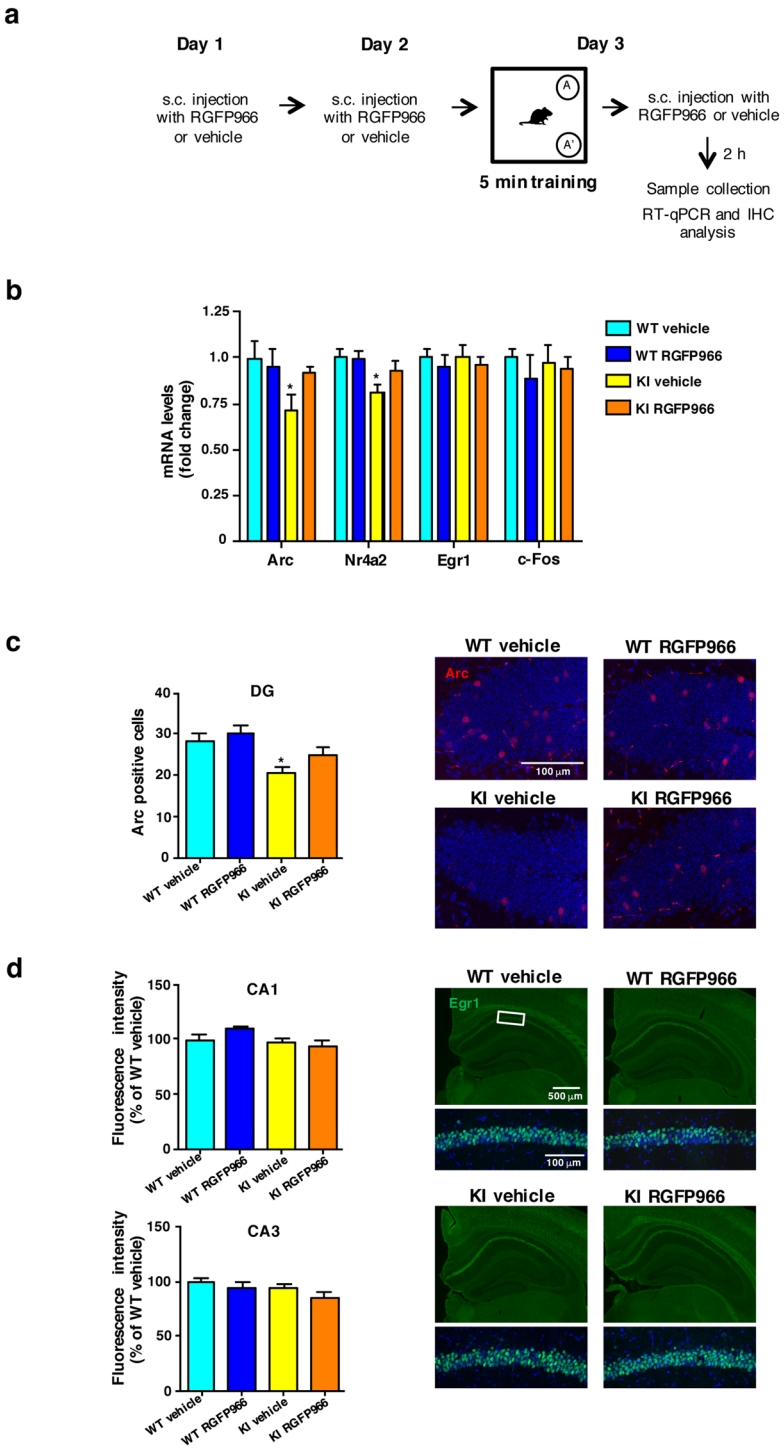


**Figure 3.** RGFP966 treatment restores gene expression in the hippocampus of HD mice. **(a)** Schematic time line of acute treatment. (**b)** Quantitative RT-PCR analysis of memory-related genes in the hippocampus of vehicle and RGFP966 acutely treated $Hdh^{Q7/Q7}$ (WT) and $Hdh^{Q7/Q111}$ (KI) mice. Histogram represents relative mRNA abundance of *Arc, Nr4a2, Egr1* and *c-Fos*. Levels of mRNA were normalized to 18S and Actinβ. $^{*}p < 0.05$ compared to vehicle-treated WT mice by two-way ANOVA with Bonferroni *post-hoc* analysis. **(c)** Representative images (high magnification) showing Arc immunostaining in the hippocampal dentate gyrus of acutely treated $Hdh^{Q7/Q7}$ (WT) and $Hdh^{Q7/Q111}$ (KI) mice. Histogram shows quantification of the average number of Arc-positive neurons in the dentate gyrus. $^{*}p < 0.05$ compared to vehicle-treated WT mice by two-way ANOVA with Bonferroni *post-hoc* analysis. **(d)** Representative images with magnification insets of the hippocampal CA1 region showing Egr1 immunostaining in acutely treated $Hdh^{Q7/Q7}$ (WT) and $Hdh^{Q7/Q111}$ (KI) mice. Histogram shows quantification of the average intensity of Egr1 immunoreactivity in the CA1 or the CA3 region. Data represent the mean ± SEM (n = 5–8 animals per group).

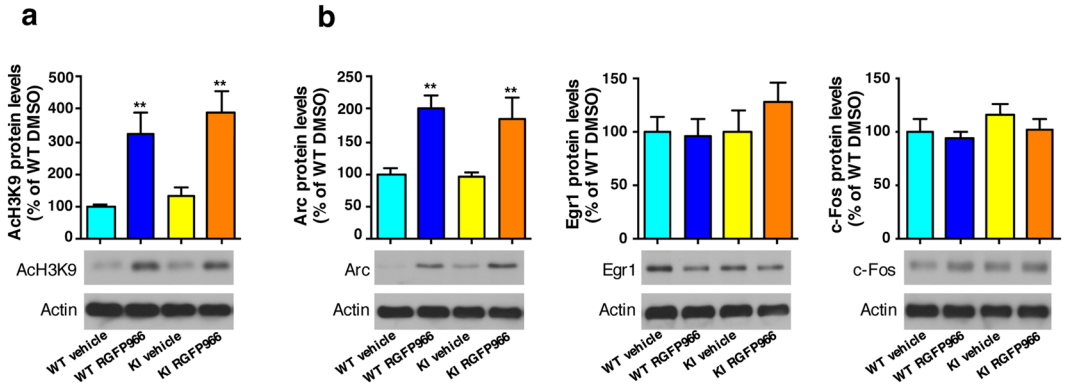


**Figure 4.** RGFP966 treatment induces Arc but not Egr1 or c-Fos protein levels in primary hippocampal cultures. **(a)** Representative immunoblots showing histone H3 acetylation levels at position lysine 9 (AcH3K9) with actin as loading control and **(b)** Arc, Egr1 and c-Fos protein levels normalized to actin levels in DMSO and RGFP966 treated primary hippocampal cultures obtained from $Hdh^{Q7/Q7}$ (WT) and $Hdh^{Q7/Q111}$ (KI) embryos. The blots in **(a** and **b)** are cropped; full-length images are provided in Supplementary Figure 3. $^{**}p < 0.01$ compared to DMSO-treated cultures by two-way ANOVA with Bonferroni *post-hoc* analysis. Data represent the mean ± SEM (n = 4–5 cultures per group).

in accordance with our data on mRNA gene expression (Fig. 3a). These findings suggest that HDAC3 inhibition affects the expression of certain memory-related genes.

To better explore this selective-expression hypothesis, primary hippocampal cultures from wild type and KI embryos were incubated with vehicle or RGFP966 for 6 h followed by immunoblot analysis of H3K9 acetylation and of Arc, Egr1 and c-Fos protein levels. RGFP966 efficacy was indicated by a significant increase in H3K9 acetylation levels, compared to DMSO-treated controls, both in wild type and KI hippocampal neurons (Fig. 4a). Arc, Egr1 and c-Fos protein levels showed a differential response to the HDAC3 inhibitor. The same extracts from wild type and KI primary cultures revealed that RGFP966 treatment increased Arc protein levels without affecting levels of Egr1 or c-Fos regardless of genotype (Fig. 4b). Finally, to test whether HDAC1 and/or HDAC2 might also be affected at the inhibitor dose used, concentrations of RGFP966 below the $IC_{50}$ of HDAC1/HDAC2[12, 19] were tested in wild type and mutant huntingtin neuronal-like cell lines. Acetylation of H3 at lysine 9 (AcH3K9) and Arc protein levels were detected by immunoblot (Supplementary Fig. 1). Treatment with either 10 μM (Supplementary Fig. 1a) or 1 μM (Supplementary Fig. 1b) of RGFP966 led to drug-dependent increases of AcH3K9 in both wild type and mutant huntingtin striatal cells. In accordance with HDAC3 inhibition, protein levels of Arc were found significantly increased in RGFP966-treated versus vehicle-treated striatal cells. These data demonstrate that doses of RGFP966 with low activity for HDAC1 and HDAC2 elicit similar results than those at higher doses, suggesting a major contribution of HDAC3 inhibition on improvements in HD mice disease phenotypes.

Consistently, the results of transcript levels, immunohistochemistry and immunoblot analysis all suggest that RGFP966 ameliorates hippocampal-dependent memory deficits in KI mice by preventing detrimental reductions in expression levels in a specific subset of memory-related genes.

**RGFP966 prevents deficits in corticostriatal-dependent motor learning.** Impaired acquisition of new skills involving corticostriatal circuits has been reported in HD mice[40, 47, 48] and HD patients[49–51]. Therefore, we next determined whether RGFP966 treatment of KI mice could also prevent the learning of new motor skills as an important example of corticostriatal-dependent behavior. Performance on an accelerating rotarod was evaluated in vehicle-treated and RGFP966-treated wild type and KI mice at 6 months of age (Fig. 5a). Both groups of wild type mice exhibited good motor learning ability over the trials. In contrast, vehicle-treated KI mice performed significantly worse with shorter latency to fall. Importantly, RGFP966-treated KI mice were able to learn to stay on the rotarod as well as vehicle- or RGFP966-treated wild type mice, indicating prevention of motor learning deficits upon treatment with the HDAC3-selective inhibitor. Since poor performance of vehicle-treated KI animals in the accelerating rotarod could be due to motor deficits, spontaneous locomotor activity in the open field was also evaluated. No significant differences in the distance traveled were found between genotypes and treatments (Fig. 5b). These results, along with previous data showing no deficits of KI mice in the fixed rotarod at six months of age[40], indicate that the results obtained with the accelerating rotarod task were due to motor learning and not motor coordination deficits. Overall, these findings demonstrate that impaired ability to learn new corticostriatal-dependent motor skills can be prevented in KI mice by early treatment with RGFP966.

**RGFP966 suppresses striatal CAG repeat expansions.** The molecular mechanisms underlying striatal pathology in HD are still being investigated. However, somatic *HTT* CAG expansions in striatum have been proposed as a mechanism that contributes to the pathogenic process[33–37]. We showed previously that HDAC3 stimulates CAG repeat expansions in cultured human astrocytes, and that a small molecule inhibitor of HDAC3 was

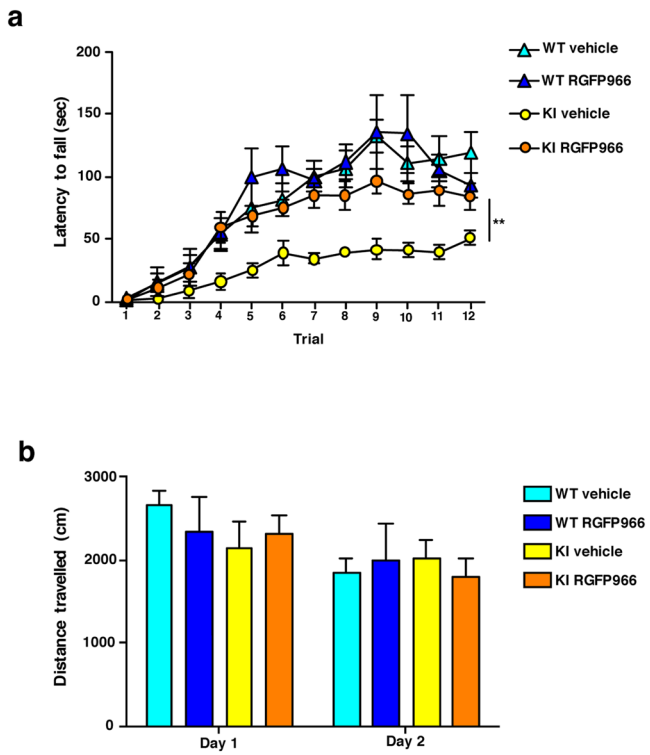


**Figure 5.** RGFP966 treatment prevents impairment of motor learning in HD mice. Behavior analysis in vehicle and RGFP966 chronically treated $Hdh^{Q7/Q7}$ (WT) and $Hdh^{Q7/Q111}$ (KI) mice. **(a)** Latency to fall in the accelerating rotarod task. $^{**}p < 0.01$ compared to vehicle-treated WT mice by two-way ANOVA with repeated measurements. **(b)** Spontaneous locomotor activity measured by total distance traveled during two consecutive days (15 min/day). Data represent the mean ± SEM (n = 5–9 animals per group).

as effective as RNAi knockdown at suppressing expansions[23,24]. If the same holds true *in vivo*, one would predict lower levels of striatal expansions in the *HTT* CAG repeat following RGFP966 treatment. Somatic CAG repeat expansions were evaluated by small pool PCR (SP-PCR). This analysis provides quantitative assessment of expansion frequency as well as the size of individual expansions[32,52]. Previous studies monitored expansions in the striatum, a tissue that shows substantial somatic instability and which is strongly affected in HD, and in cerebellum, where the CAG tract is relatively stable and mild pathology is found[25,32]. Consistent with these previous studies, we saw substantial expansion activity in the striatum of vehicle-treated KI animals, whereas the CAG repeat was mostly stable in the cerebellum of the same mice (Fig. 6a). RGFP966 treatment partially stabilized the CAG repeat in striatum, showing fewer expansions as well as smaller changes in tract length (Fig. 6a). Quantitative data from three mice in each group, totaling 62–65 independent alleles, illustrated the beneficial effects of the HDAC3-selective inhibitor (Fig. 6b). Expansions predominated in the striatum of vehicle-treated KI animals (72%) compared to unchanged alleles (21%) and a few contractions (7%). These quantitative data are fully consistent with previous reports for HD mice[25,32,53]. In contrast, treatment with RGFP966 reduced the frequency of expansions by 28%, from 72% to 44% (Fig. 6b). Interestingly, this inhibition of expansions was compensated by a striking increase in the frequency of contractions from 7% in vehicle-treated KI mice to 32% in RGFP966-treated animals. Since the starting tract sizes were similar for all mice examined (legend to Fig. 6), differences in initial CAG repeat length were eliminated as a confounding variable.

The frequency data were corroborated by analysis of changes to CAG repeat lengths (Fig. 6c). Striatal changes in vehicle-treated KI animals spanned the range from −10 to +60 repeats with a weight-averaged length change of +13.3 repeats. RGFP966-treatment caused a leftward shift towards smaller size changes. The decrease was about 40% to a weight-average repeat change of +8.1 repeats. (Fig. 6c). We conclude that RGFP966 treatment substantially suppressed striatal CAG repeat expansions in $Hdh^{Q7/Q111}$ mice.

**RGFP966 partially rescues expression of striatal protein markers and reduces accumulation of mutant huntingtin oligomeric forms.** The integrity of striatal medium spiny neurons can be monitored by the level of protein biomarkers. Even at presymptomatic stages, reductions of phosphoprotein DARPP-32, phosphodiesterase PDE10A and adenosine receptor $A_{2A}R$ have been demonstrated as markers of dysfunctional striatal neurons[54–60]. To evaluate whether striatal improvements in RGFP966-treated KI mice were also associated with a recovery of these protein markers, levels of DARPP-32, PDE10A and $A_{2A}R$ were analyzed by immunoblot. As expected, levels of these striatal proteins were significantly reduced in vehicle-treated KI mice compared to wild type animals (Fig. 7a). Notably, RGFP966 treatment of KI mice partially prevented the reduction of these striatal markers, restoring them closer to normal levels. No significant effect of RGFP966 was observed in wild

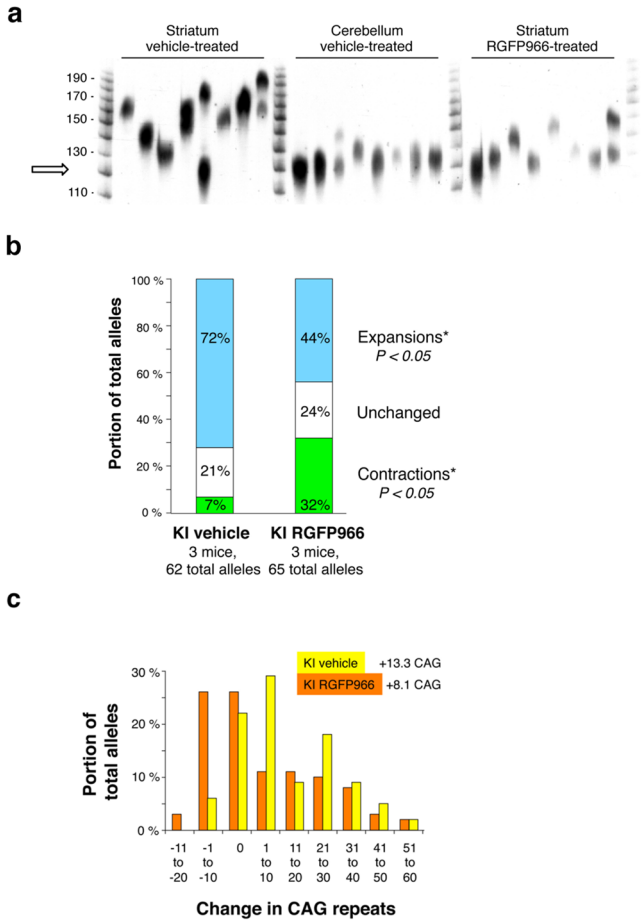


**Figure 6.** RGFP966 suppresses striatal CAG repeat expansions. **(a)** Representative display of small-pool PCR of somatic repeat lengths from single $Hdh^{Q7/Q111}$ (KI) animals, either chronically treated with vehicle or with RGFP966. Size markers correspond to CAG repeat number. The examples shown represent the positive signals from one analysis. Arrow indicates size of initial CAG tract. This image has been cropped; the original is provided in Supplementary Figure 4. (**b**) Summary of genetic changes. For each group, n = 3 mice, 62–65 independent events. Starting tract sizes, estimated from the most common allele size in cerebellum and stated as repeat units, were 110–119, 120–129 and 120–129 for vehicle-treated mice; and 120–129, 120–129 and 130–139 for RGFP966-treated mice. $*p < 0.05$ by Student's t-test. **(c)** Histogram of expansion sizes from the alleles tested in **b**. Allele sizes were binned into 10-repeat size groups, *e.g.* falling between +1 to +10 repeats compared to the starting allele size. Starting tract lengths were deduced from the most common allele size in the cerebellum of the same animals.

type mice. To elucidate whether the recovery of striatal expression of these protein markers was a direct effect of treatment with the HDAC3 inhibitor or a consequence of a general relief of striatal pathology, primary striatal cultures from wild type and KI embryos were incubated for 6 h with vehicle or RGFP966 and levels of DARPP-32, PDE10A and $A_{2A}R$ were subsequently analyzed by immunoblot (Fig. 7b). Though no significant differences in the expression of these striatal markers were found between DMSO-treated wild type and KI striatal cultures, RGFP966 treatment led to a significant increase in DARPP-32 levels in both genotypes (Fig. 7b). Inhibitor treatment was without any significant effect in PDE10A or $A_{2A}R$ protein levels. These results suggest a direct induction of DARPP32 expression as a result of RGFP966 treatment that contributes to ameliorating mHtt-induced striatal damage.

Oligomerization of mHtt in the striatum is another pathological hallmark of HD[61,62]. Given the positive benefits of RGFP966 in striatal CAG expansions and protein biomarkers, we tested if RGFP966 treatment also reduces formation of mHtt oligomers. Detergent-soluble fractions of striatal extracts from KI mice were separated by SDS-PAGE. Mutant huntingtin oligomers were trapped in the stacking gel (Fig. 7c, left) whereas monomeric forms entered the resolving portion of the gel (Fig. 7c, right). Substantial accumulation of striatal mHtt oligomers was found in vehicle-treated KI mice, while treatment with RGFP966 prevented about one-half the accumulation of these oligomeric forms of mHtt (Fig. 7c). Monomeric mHtt levels were unchanged by inhibitor treatment. Next, we tested if RGFP966 alters the expression of the *Htt* gene at the transcriptional level. Quantitative RT-PCR analysis revealed no differences in *Htt* mRNA levels between acutely RGFP966-treated KI mice and

vehicle-treated KI mice (Fig. 7d), suggesting that the reduction in mHtt oligomeric forms cannot be attributed to altered transcription of the *Htt* gene.

In sum, these observations demonstrate that the beneficial effects of RGFP966 were manifested both at the level of striatal protein markers and mHtt oligomerization.

## Discussion

This study shows that treating HD mice with an HDAC3-selective inhibitor provides multiple benefits. This conclusion is important because the success of HDAC inhibitors (HDACi) as a therapeutic approach for HD depends on the identification of key HDACs as targets. Selectively inhibiting one or a few key HDACs could provide therapeutic relief while avoiding toxicity issues that were encountered with broad-based HDACi. Selective HDAC3 inhibition has shown promise in improving HD motor impairments and neuropathology[8–12]. However, potential benefits of HDAC3 inhibition to cognitive function in HD have been largely unexplored. The current study demonstrates that early intervention with the HDAC3-selective inhibitor RGFP966 prevented deficits in hippocampal-dependent long-term memory likely by normalization of the expression in a specific subset of memory-related genes. In addition, RGFP966 suppressed striatal mHtt-induced protein toxicity by reducing both the frequency of somatic CAG expansions and the average change in repeat length which was associated with improvement of striatal pathology and prevention of motor skill learning deficits. While we cannot rigorously rule out some activity on HDAC1 or HDAC2 at the RGFP966 dose used here, these findings represent novel outcomes of inhibiting HDAC3 in HD mice and complement the work of Thomas and colleagues on motor function and working memory[9–11]. Our results and those of Thomas and colleagues indicate that early targeting with an HDAC3-selective inhibitor significantly prevents or delays cognitive deficits, somatic CAG repeat expansions and motor dysfunction in HD mice.

Our results are in contrast to those of Moumne *et al.*[63] who examined *Hdac3*$^{+/-}$ heterozygous R6/2 HD mice. They found no amelioration of physiological or behavioral phenotypes and no effect on molecular changes including dysregulated transcripts. Surprisingly, these heterozygous mice still express HDAC3 protein at 80% of the wild type levels. This modest effect on HDAC3 levels makes it possible that sufficient protein and enzymatic activity are present in the heterozygous animals to confer HDAC3 effects on symptoms and transcription.

**Gene expression and hippocampal-dependent memory.** Our results demonstrate that hippocampal-dependent memory decline was prevented by RGFP966. To our knowledge this is the first study involving the HDAC3 isotype in HD long-term memory impairment. These findings are consistent with our previous data showing restoration of long-term memory by trichostatin A (TSA), a broad-based HDACi[7], and they are in agreement with the role of HDAC3 as a negative regulator of long-term memory formation[18]. We found that treatment with RGFP966 improved memory performance and normalized hippocampal *Arc* and *Nr4a2* immediate early gene expression, likely by increasing histone acetylation. Accordingly, a significant increase in the hippocampal expression of *Nr4a2* associated with enhanced long-term memory has been reported in HDAC3 knockout mice or mice treated with broad-based or specific HDAC3 inhibitors[7,18]. Similarly, treatment with RGFP966 to inhibit HDAC3 enhances the memory processes involved in extinction of drug-seeking behaviors by enhancing *Nr4a2* and *c-Fos* expression[19]. Additionally, *Arc* expression has been widely involved in maintenance of long-term potentiation and consolidation of spatial long-term memory[64,65]. Interestingly, these genes are dependent on CREB:CBP-mediated gene expression and it has been demonstrated that HDAC3 can also repress CBP function by deacetylation[66,67]. Therefore, we speculate that inhibition of HDAC3 by RGFP966 may enhance CBP-mediated transcription of *Arc* and *Nr4a2* necessary for memory formation, which may help ameliorate memory decline in HD mice. It is also possible that other non-histone substrates of HDAC3 are modulated by RGFP966 treatment, contributing to memory improvement in HD mice.

**Striatal benefits of RGFP966 treatment.** In addition to improvements in hippocampal function, RGFP966 treatment also led to benefits in striatal function. To our knowledge, this is the first demonstration of an HDAC3 inhibitor preventing motor learning decline. This result is consistent with the opposing effects of CBP and HDAC3 in expression of striatal genes important for acquisition of new motor skills. For example, mice harboring either a mutation in the CREB binding domain of CBP or knockout for CREB exhibit impaired chromatin acetylation and motor learning deficits, suggesting that CBP-mediated transcription is important for the expression of genes involved in motor learning[68,69].

Our results also provide the first demonstration of an HDAC3-selective inhibitor suppressing striatal expansions. The parallels in suppression of CAG repeat expansions between cell culture[23] and the striatum of mice (Fig. 6) are consistent with the idea that suppression occurs primarily through RGFP966 inhibition of HDAC3, not off-target inhibition of HDAC1 or HDAC2. The changes in CAG repeat instability are similar to effects that were seen in Msh3$^{+/-}$ HdhQ$^{111}$ mice[70], suggesting a possible connection between HDAC3 and Msh3. Msh3 is a key subunit of the DNA mismatch repair protein complex MutSβ, which stimulates expansions[33,70–72]. Msh3 is a limiting factor for expansions in myotonic dystrophy type 1 mice[73] and for formation of MutSβ complex in human cells[74]. Msh3 has also been linked genetically to the expansion-driving activity of HDAC3 in immortalized human glial cells[24]. Thus RGFP966 inhibition of HDAC3 in KI mice phenocopies the Msh3$^{+/-}$ effect on striatal expansions, consistent with the model that mammalian HDAC3 and MutSβ work in the same pathway to drive CAG repeat expansions[24]. However, this interpretation is speculative and more experiments will be necessary to define the precise contribution of HDAC3 inhibition in MutSβ activity.

Consistent with inhibition of somatic CAG expansions, we report that RGFP966 treatment also prevented accumulation of mHtt oligomeric forms in the striatum of KI mice. Protein inclusions comprised of N-terminal fragments of mHtt are a characteristic hallmark of the disease, though whether they play a protective role or

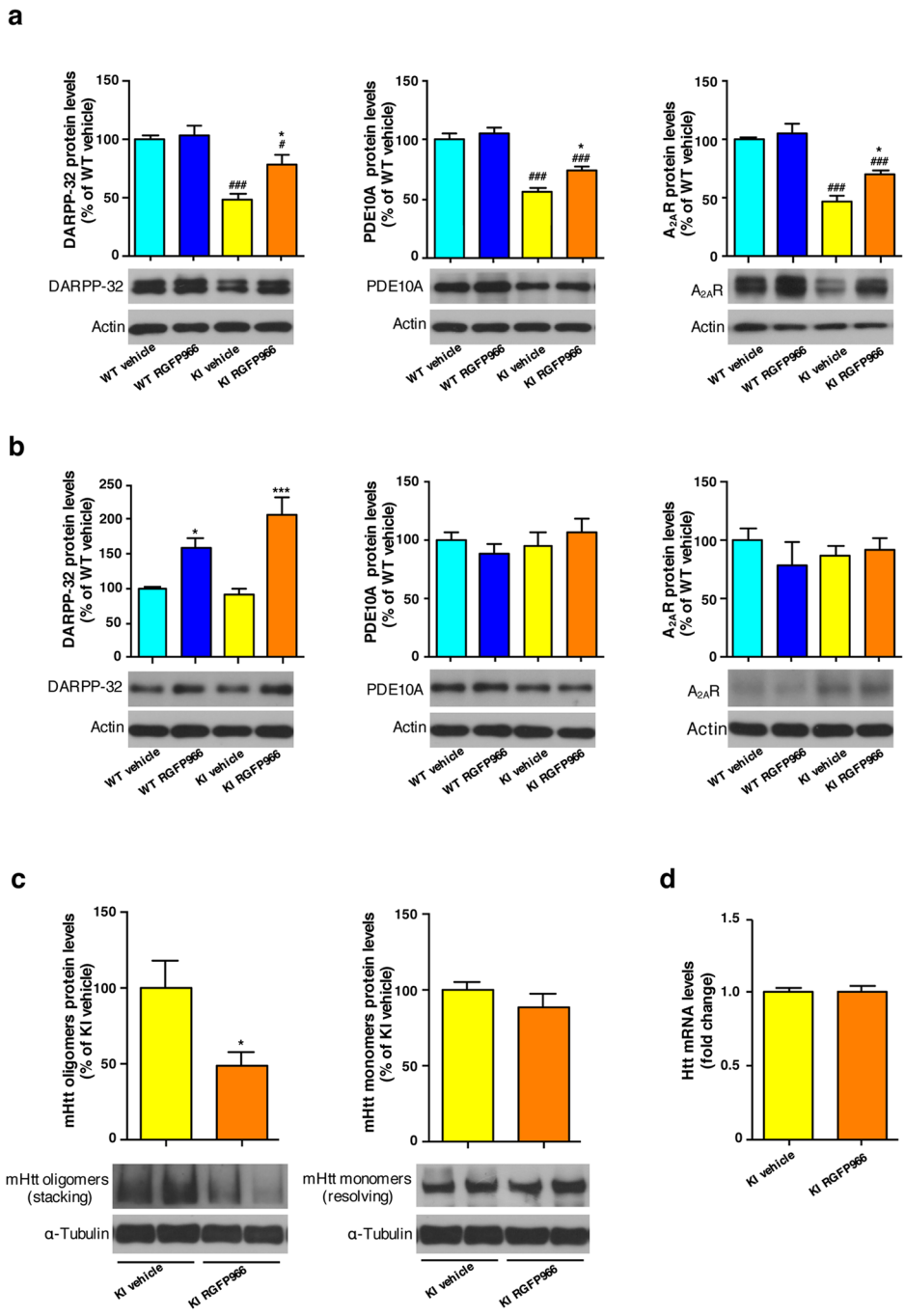


**Figure 7.** RGFP966 treatment ameliorates striatal pathology in HD mice. **(a)** Representative immunoblots showing striatal protein levels of DARPP-32, PDE10A and $A_{2A}R$, with actin as loading control in vehicle and RGFP966 chronically treated $Hdh^{Q7/Q7}$ (WT) and $Hdh^{Q7/Q111}$ (KI) mice. $^{*}p < 0.05$ compared to vehicle-treated KI mice; $^{\#}p < 0.05$; $^{\#\#\#}p < 0.001$ compared to vehicle treated WT mice by two-way ANOVA with Bonferroni *post-hoc* analysis. Data represent the mean ± SEM (n = 6–11 animals per group). **(b)** Representative immunoblots showing striatal protein levels of DARPP-32, PDE10A and $A_{2A}R$ with actin as loading control in DMSO and RGFP966 treated primary striatal cultures obtained from $Hdh^{Q7/Q7}$ (WT) and $Hdh^{Q7/Q111}$ (KI) embryos. $^{*}p < 0.05$ and $^{***}p < 0.001$ compared to DMSO-treated cultures by two-way ANOVA with Bonferroni *post-hoc* analysis. Data represent the mean ± SEM (n = 4–5 cultures per group). **(c)** Representative immunoblots showing oligomeric forms of mutant huntingtin (stacking gel), monomeric mutant huntingtin (resolving gel) and α-tubulin as loading control in vehicle and RGFP966 chronically treated $Hdh^{Q7/Q111}$ (KI) mice. Samples from two different mice are shown in adjoining lanes. All blots have been cropped; the original images are shown in Supplementary Figure 5. $^{*}p < 0.05$ by Student's t-test. Data represent the mean ± SEM (n = 6–10 animals per group). **(d)** Quantitative RT-PCR analysis of *Htt* mRNA in the striatum of vehicle and RGFP966 acutely treated $Hdh^{Q7/Q7}$ (WT) and $Hdh^{Q7/Q111}$ (KI) mice. Levels of mRNA were normalized to 18S and Actinβ. Data represent the mean ± SEM (n = 6–7 animals per group).

a causative one in neurodegeneration is controversial. Smaller, soluble oligomeric forms of huntingtin formed early in the aggregation process are thought to confer toxic effects and contribute to early cell dysfunction[75,76]. Therefore, a reduction in the level of mHtt toxic oligomers by HDAC3 inhibition is predicted to delay disease progression. Interestingly, a recent study found no changes in striatal levels of mhtt aggregates, measured by EM48 immunoreactivity, after chronic HDAC3 inhibition in an HD mouse model that overexpresses the N-terminal fragment of mhtt[12]. This suggests that HDAC3 inhibition could have a different effect on oligomeric versus aggregate/inclusion forms of mhtt.

Another concurrent benefit of RGFP966 in the striatum was the normalization of striatal biomarkers levels such as DARPP-32, PDE10A and $A_{2A}R$. Indeed, HDAC3 has been related to the transcriptional mechanisms that regulate striatal DARPP-32 expression. Thus, treatment of primary striatal cultures with the HDAC1/HDAC3 inhibitor 4b increased the overall levels of H3 and H4 histone acetylation as well as mRNA expression and protein levels of DARPP-32[77]. Similarly, inhibition of HDAC3 in R6/2 transgenic mice with HDACi-136 completely restored the expression of DARPP-32 mRNA levels[10]. Consistent with these reports, we have found that treatment of primary striatal cultures with RGFP966 significantly increased DARPP-32 protein levels suggesting that HDAC3 inhibition could elicit striatal benefits by directly inducing expression of DARPP-32.

Overall these findings strengthen the link between somatic CAG expansions and HD progression. One speculative model is that fewer expansions and shorter changes to CAG repeat length would limit gains in the length of the glutamine tract of mHtt, reducing mHtt-induced toxicity and thereby leading to improvements in striatal function and recovery of motor learning dysfunction. This is consistent with the work of McMurray and colleagues. They showed that treatment of HD mice with XJB-5-131, a bifunctional antioxidant, led to enhanced neuronal survival, suppression of motor decline and weight loss, improved mitochondrial function and inhibition of somatic CAG repeat expansions[37,78].

In summary, our study demonstrates that early targeting with an HDAC3-selective inhibitor provides multiple benefits in HD mice by preventing hippocampal-memory impairments and by suppressing striatal degeneration. The finding that somatic CAG repeat expansions were also suppressed by the inhibitor may represent an interesting new therapeutic approach not only for HD but also for other trinucleotide repeat disorders.

## 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[79]. 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 (KI) littermates, determined by PCR analysis. Only males were analyzed. 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 12:12 h light/dark cycle. All procedures involving animals were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approved by the local animal care committee of the Universitat de Barcelona (99/01) and Generalitat de Catalunya (99/1094), in accordance with the European (2010/63/EU) and Spanish (RD53/2013) regulations for the care and use of laboratory animals.

**RGFP966 mice treatment.** The HDAC3 inhibitor RGFP966 was generously provided by BioMarin Pharmaceutical Inc. (San Rafael, California, USA). For evaluation of behavior, biochemical parameters and CAG repeat expansions, WT and KI mice were subcutaneously injected with 50 mg/kg of RGFP966 or vehicle (70% polyethylene glycol 200; 30% acetate buffer) three times per week from 3 to 6.5 months (chronic treatment). Mice were sacrificed by cervical dislocation 1 hour after final injection and brains were removed for immunoblot and CAG repeat analysis. For analysis of activity-dependent genes, 7-month-old WT and KI mice were subcutaneously injected daily with 25 mg/kg of RGFP966 or vehicle for 3 days (acute treatment). On day 3, mice received 5 min training in an environment with two identical objects to induce expression of activity-dependent memory genes. Training was immediately followed by an injection of RGFP966. Mice were sacrificed 2 hours later and their brains removed for gene expression and histological analysis.

**Behavioral assessment.** Chronically treated animals were evaluated for behavior at 6 months of age. Motor learning was assessed by the accelerating rotarod task while spatial and recognition long-term memory was assessed by the object location task (OLT) and the novel object recognition task (NORT), respectively. Locomotor activity was evaluated by the open field test[39,40].

*Accelerating rotarod training procedure.* Animals were placed on a motorized rod (30 mm diameter) and the rotation speed was gradually increased from 4 to 40 rpm over the course of 5 minutes. Time latency was recorded when the animal was unable to keep up with the increasing speed and fell. Accelerating rotarod training procedure training/testing was performed four times per day for three consecutive days. Different trials during the same day were separated by 1 h. The apparatus was rigorously cleaned between animal trials in order to avoid odors.

*Open field.* The device consisted of a white open-top arena with quadrangular form (45 × 45 cm). The light intensity was 40 lux throughout the arena, and the room temperature was kept at 19–22 °C and 40–60% humidity. Mice were placed into the arena during two consecutive days (15 min/day) and spontaneous locomotor activity was measured as total distance traveled. The arena was rigorously cleaned between animals in order to avoid odors. Animals were tracked and recorded with SMART Junior Software.

*OLT/NORT.* Exploration took place in an open-top arena with quadrangular form (45 × 45 cm). The light intensity was 40 lux throughout the arena, and the room temperature was kept at 19–22 °C and 40–60% humidity.

Mice were first habituated to the arena in the absence of objects (2 days, 15 min/day). On the third day during the acquisition phase mice were allowed to explore 2 duplicate objects (A and A′ or B and B′), which were placed in two adjacent corners of the arena, for 10 min, after which they were returned to their home cage. During the 24 h retention test, mice were placed in the experimental apparatus for 10 min. For OLT, one copy of the familiar object (A) was placed in the same location as during the training trial, and one copy of the familiar object (A″) was placed in the corner diagonally opposite. For NORT, one copy of the familiar object (B) and a new object (C) were placed in the same location as during the training trial. The arena was rigorously cleaned between animal trials in order to avoid odors. Animals were tracked and recorded with SMART Junior software. Exploration times were recorded and used to calculate the Discrimination index = (time exploring novel or relocated object-time exploring familiar object)/(total time exploring both objects) × 100. Discrimination indices of 0 indicate equal exploration of both objects.

**Immunoblot analysis.** Brains from chronically treated mice were quickly removed, dissected, frozen in dry ice and stored at −80 °C until use. Brain tissue was homogenized in cold lysis buffer containing 20 mM Tris base (pH 8.0), 150 mM NaCl, 50 mM NaF, 1% NP-40, 10% glycerol and supplemented with 1 mM sodium orthovanadate and protease inhibitor cocktail (Sigma-Aldrich). Samples were centrifuged at 16,000 g for 20 min and the supernatants collected. For the analysis of cellular extracts, cells were collected in cold lysis buffer containing 50 mM Tris base (pH 7.4), 150 mM NaCl, 2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 1% NP-40 and supplemented with 1 mM sodium orthovanadate and protease inhibitor cocktail. Samples were centrifuged at 10,000 g for 10 min, and the supernatants collected. Following determination of the protein contents by Detergent-Compatible Protein Assay (Bio-Rad, Hercules, CA, USA), protein extracts (20–60 µg) were mixed with 5 × SDS sample buffer, boiled for 5 min, resolved on 6–10% SDS–PAGE and transferred to nitrocellulose membranes (Whatman Schleicher & Schuell, Keene, NH, USA). After incubation (30 min) in blocking buffer containing 10% non-fat powdered milk in Tris buffered saline-Tween (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. Antibodies used for immunoblot analysis were: Acetyl-histone H3 (Lys9) (1:1000, Cell Signaling Technology), Histone H3 (1:1000, Cell Signaling Technology), HDAC3 (1:1000; Abcam), Arc (1:500; Santa Cruz Biotechnology), Egr1 (1:1000; Cell Signaling Technology), c-Fos (1:1000, Santa Cruz Biotechnology), DARPP-32 (1:1000; BD Bioscience), PDE10A (1:1000; Abcam), $A_{2A}R$ (1:500; Santa Cruz Biotechnology), 1C2 (1:1000, Millipore), α-Tubulin (1:50,000; Sigma-Aldrich) and Actin (1:20,000; MP Biochemicals). The membranes were then rinsed three times with TBS-T and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After washing for 30 min with TBS-T, the membranes were developed using the enhanced chemilluminescence ECL kit (Santa Cruz Biotechnology). The Gel-Pro densitometry program (Gel-Pro Analyzer for Windows, version 4.0.00.001) was used to quantify the different immunoreactive bands relative to the intensity of the α-tubulin, actin or histone H3 band in the same membranes within a linear range of detection for the ECL reagent.

**Quantitative RT-PCR.** Brains from acutely-treated mice were quickly removed, dissected, frozen in dry ice and stored at −80 °C until use. Total RNA was isolated using the total RNA isolation Nucleospin RNA II Kit (Macherey-Nagel). Purified RNA (500 ng) was reverse transcribed using the PrimeScript RT Reagent Kit (Perfect Real Time, Takara Biotechnology Inc.). The cDNA synthesis was performed at 37 °C for 15 min and a final step at 85 °C for 5 s in a final volume of 20 µl according to the manufacturer's instructions. The cDNA was then analyzed by quantitative RT-PCR using the following PrimeTime qPCR Assays (Integrated DNA Technologies, Inc.): Arc (Mm.PT.56a.16160059), Nr4a2 (Mm.PT.58.16021564), Egr-1 (Mm.PT.58.29064929); c-Fos (Mm.PT.58.29977214), Htt (Mm.PT.58.12088552, 18S (Hs.PT.39a.22214856.g) and Actinβ (Mm.PT.39a.22214843.g). Quantitative PCR was performed in 12 µl of final volume on 96-well plates using the Premix Ex Taq (Probe qPCR) (TAKARA BIOTECHNOLOGY (Dalian) Co., LTD). Reactions included Segment 1:1 cycle of 30 s at 95 °C and Segment 2: 40 cycles of 5 s at 95 °C and 20 s at 60 °C. All quantitative PCR assays were performed in duplicate. To provide negative controls and exclude contamination by genomic DNA, the PrimeScript RTEnzyme was omitted in the cDNA synthesis step. The quantitative PCR data were quantified using the comparative quantitation analysis program of MxProTM quantitative PCR analysis software version 3.0 (Stratagene) using 18S and Actinβ gene expression as housekeeping genes.

**Immunohistochemical analysis.** Brains from acutely treated mice were fixed by immersion in paraformaldehyde (Sigma-Aldrich) 4% in PBS, cryoprotected in PBS/sucrose 30% with 0.02% sodium azide and frozen in methyl–butane (Sigma-Aldrich). Coronal sections (30 µm) of the brain were obtained using a cryostat (Microm) and kept in PBS. Free-floating sections were rinsed three times in PBS and permeabilized and blocked in PBS containing 0.3% Triton X-100 and 3% normal goat serum (Pierce Biotechnology, Rockford, IL) for 15 min at room temperature. Sections were then washed in PBS and incubated overnight at 4 °C with mouse anti-Arc (1:200, Santa Cruz Biotechnology) or rabbit anti-Egr1 (1:200, Cell Signaling Technology). After primary antibody incubation, slices were washed three times and then incubated 2 h shaking at room temperature with secondary antibodies: anti-rabbit Alexa Fluor 488 (1:100, Jackson ImmunoResearch, West Grove, PA, USA) or anti-mouse Cy3 (1:100, Jackson ImmunoResearch, West Grove, PA, USA). Nuclei were stained with Hoechst 33258 (1:10,000, Molecular Probes, Life Technologies) for 10 min. As negative controls, some sections were processed as described in the absence of primary antibody and no signal was detected. Images were acquired with a Leica SP5 laser scanning confocal microscope (Leica). For each mouse, at least four slices of 30 µm-containing hippocampal tissue were analyzed. Arc immunoreactive-positive positive cells in the dentate gyrus were counted and Egr1 immunoreactivity in the CA1 or CA3 area of the hippocampus was quantified by analysis of integrated optical density with ImageJ software, as previously described[80].

**Primary neuronal cultures.** Dissociated hippocampal and striatal cultures prepared from E18.5 WT and KI embryos were plated at a density of 400,000 neurons onto 60 mm culture dishes precoated with 0.1 mg/ml poly-d-lysine (Sigma Chemical Co., St. Louis, MO). Neurons were cultured in Neurobasal medium (Gibco-BRL, Renfrewshire, Scotland, UK), supplemented with B27 (Gibco-BRL) and Glutamax™ (Gibco-BRL) and were maintained at 37 °C in a humidified atmosphere containing 5% CO2. At 10–14 DIV, neurons were treated with vehicle (DMSO) or RGFP966 (10 μM) for 6 h. Additionally, primary hippocampal cultures were treated with 50 mM KCl for the last 2 hours in order to induce neuronal stimulation. Subsequently, cells were harvested for immunoblot analysis.

**Immortalized cell culture.** Conditionally immortalized wild-type STHdh$^{Q7/Q7}$ and mutant STHdh$^{Q111/Q111}$ striatal neuronal progenitor cell lines expressing endogenous levels of normal and mutant huntingtin with 7 and 111 glutamines, respectively, have been described previously[81]. Striatal cells were grown at 33 °C in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich; St. Louis, MO, USA), supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin, 2 mM l-glutamine, 1 mM sodium pyruvate and 400 μg/ml G418 (Geneticin; GIBCO-BRL, Life technologies; Gaithersburg, MD, USA). Cells were treated with vehicle (DMSO) or RGFP966 (10 μM or 1 μM) for 6 h prior to harvesting for immunoblot analysis.

**Striatal CAG repeat instability.** CAG repeat expansions were detected by small-pool PCR (SP-PCR) using primers specific for the *HTT* allele[70]. Genomic DNA was prepared from mouse tissue using Macherey-Nagel NucleoSpin Tissue Kit following the manufacturer's protocol. One μg of genomic DNA was digested with 20 U EcoRV-HF at 37 °C for 1.5 hours then heat inactivated at 65 °C for 10 minutes. Digested DNA was serially diluted in TE buffer (pH 8) containing 0.1 μM carrier primer (CAG1_HdhQ111_F) to give a final concentration of 20 pg/μl. CAG repeat sizes were determined by PCR using forward primer CAG1_HdhQ111_F (5′-atg aag gcc ttc gag tcc ctc aag tcc ttc-3′) and reverse primer HU3_HdhQ111_R (5′-ggc ggc tga gga agc tga gga-3′). The PCR reaction mixture contained 10 pg template DNA, 1X buffer (67 mM Tris-HCl pH 8.8, 16.6 mM $NH_4SO_4$, 2.0 mM $MgCl_2$, 10 mM 2-mercaptoethanol) 10% DMSO, 0.17 mg/ml BSA, 0.2 mM each dATP, dCTP, dGTP and dTTP, 4 ng/μl primers and 0.5 U Taq polymerase (Fisher BioReagents). PCR reactions were performed in multiplex (20–30 reactions per sample) with at least four negative control reactions. PCR conditions were one cycle of 90 sec at 94 °C, 34 cycles of (30 sec at 94 °C, 30 sec at 65 °C and 90 sec at 72 °C) and one final cycle of 10 min at 72 °C. PCR products were denatured in formamide loading buffer (0.05% w/v Bromophenol Blue, 0.05% w/v Xylene cyanol, 96% v/v deionized formamide, 20 mM EDTA pH 8) at 95 °C for 8 minutes then resolved, alongside DIG labeled size markers, on a 6% denaturing sequencing gel in 1X TBE buffer at 60 W for ~4 hours. Fragments were transferred to positively charged nylon membrane by electroblotting at 30 V overnight at 4 °C and fixed by UV cross-linking. Southern blot hybridization was performed using a 5′ digoxigenin (DIG) labeled locked nucleic acid (LNA) probe GC*T G*CT GC*T G*CT GC*T GCT (Eurogentec, where C* indicates LNA cytosine and G* indicates LNA guanine). Detection was performed using DIG High Prime DNA labeling and Detection Starter Kit II (#11 585 614 910; Roche Applied Science, Mannheim, Germany) following the manufacturer's protocol. Multiple exposures to X-ray film ensured detection of faint signals and separation of closely spaced bands. Starting tract size was deduced from the most common product length in the cerebellum of each animal.

**Statistical analysis.** All statistical comparisons were performed using either two-way ANOVA or Student's t-test, as indicated in figure legends. Values for n and $p$ are given in the legend to each figure. Differences with $p < 0.05$ were considered significant.

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## Author Contributions

N.S. contributed to the design and carried out the behavioral, biochemical and gene expression experiments, analyzed and interpreted data. L.K.-M. participated in the design and performed the CAG repeat expansion analysis. B.L. and S.G conceived the study and wrote the paper. All authors read and approved the final manuscript.

## Additional Information

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# Early Downregulation of p75$^{NTR}$ by Genetic and Pharmacological Approaches Delays the Onset of Motor Deficits and Striatal Dysfunction in Huntington's Disease Mice

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## Abstract

Deficits in striatal brain-derived neurotrophic factor (BDNF) delivery and/or BDNF/tropomyosin receptor kinase B (TrkB) signaling may contribute to neurotrophic support reduction and selective early degeneration of striatal medium spiny neurons in Huntington's disease (HD). Furthermore, we and others have demonstrated that TrkB/p75$^{NTR}$ imbalance in vitro increases the vulnerability of striatal neurons to excitotoxic insults and induces corticostriatal synaptic alterations. We have now expanded these studies by analyzing the consequences of BDNF/TrkB/p75$^{NTR}$ imbalance in the onset of motor behavior and striatal neuropathology in HD mice. Our findings demonstrate for the first time that the onset of motor coordination abnormalities, in a full-length knock-in HD mouse model (KI), correlates with the reduction of BDNF and TrkB levels, along with an increase in p75$^{NTR}$ expression. Genetic normalization of p75$^{NTR}$ expression in KI mutant mice delayed the onset of motor deficits and striatal neuropathology, as shown by restored levels of striatal-enriched proteins and dendritic spine density and reduced huntingtin aggregation. We found that the BDNF/TrkB/p75$^{NTR}$ imbalance led to abnormal BDNF signaling, manifested as a diminished activation of TrkB-phospholipase C-gamma pathway but upregulation of c-Jun kinase pathway. Moreover, we confirmed the contribution of the proper balance of BDNF/TrkB/p75$^{NTR}$ on HD pathology by a pharmacological approach using fingolimod. We observed that chronic infusion of fingolimod normalizes p75$^{NTR}$ levels, which is likely to improve motor coordination and striatal neuropathology in HD transgenic mice. We conclude that downregulation of p75$^{NTR}$ expression can delay disease progression suggesting that therapeutic approaches aimed to restore the balance between BDNF, TrkB, and p75$^{NTR}$ could be promising to prevent motor deficits in HD.

**Keywords** Huntington's disease · p75$^{NTR}$ · TrkB · BDNF · Motor deficits onset · Striatal pathology

## Introduction

Huntington's disease (HD) is a fatal neurodegenerative disorder with a characteristic phenotype including chorea and dystonia, incoordination, cognitive decline, and psychiatric symptoms [1]. It is caused by an abnormal expansion of a CAG codon in exon 1 of the *huntingtin* gene [2]. Neuropathological hallmarks include intranuclear aggregates of mutant huntingtin and selective neurodegeneration of striatal medium spiny neurons (MSNs) [3, 4], which are the principal projection neurons within the striatum. Survival and maintenance of MSNs are especially dependent on the brain-derived neurotrophic factor (BDNF)-tropomyosin receptor kinase B (TrkB) signaling pathway [5, 6], and the selective vulnerability in HD has been linked to reduced neurotrophic support caused by reduced levels of BDNF [7–10]. Therefore, exogenous

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BDNF administration or pharmacological treatments that raise BDNF levels have been proposed and extensively studied to slow or prevent the HD disease progression. However, these therapeutic strategies only ameliorate or partially improve morphological phenotypes or motor and cognitive behavior in different HD mouse models [11–14]. Several studies suggest that BDNF signaling deficits can be related not only to altered BDNF delivery but also to inappropriate levels of expression or activation of its receptors, TrkB and/or $p75^{NTR}$ [15–21]. TrkB is a member of the neurotrophin tyrosine receptor kinase family that activates cytosolic signaling cascades to promote survival and maintain neurochemical and morphological properties of neurons. On the other hand, BDNF binds also to $p75^{NTR}$, a member of the tumor necrosis factor receptor superfamily [22]. $p75^{NTR}$ can potentiate or reduce Trks signaling, promoting or hampering cell growth and survival depending on the cellular context and Trks or neurotrophins abundance, but it can also act independently activating NF-Kβ, c-Jun kinase (JNK), and RhoA/ROCK pathways [23–25]. $p75^{NTR}$, which is known to be induced in several disease conditions, can activate degenerative pathways, particularly in states in which deficiency of neurotrophins or Trks occurs [26, 27]. Importantly, it has been demonstrated that signaling via either TrkB or $p75^{NTR}$ receptors is compromised in HD. Downregulation of TrkB receptor levels or defects in TrkB signaling have been reported in HD cellular models and HD mice brain [14, 15, 17–21] while increased levels of $p75^{NTR}$ have been demonstrated in the striatum and hippocampus of different HD mouse models [19, 28–30]. These observations support the idea that a correctable defect in the response to BDNF underlies striatal dysfunction in HD. However, no studies have examined the precise contribution of the BDNF/TrkB/$p75^{NTR}$ pathway in the onset of HD motor coordination deficits. Based on these previous reports, we hypothesized that motor behavioral abnormalities in HD are directly associated with improper expression of $p75^{NTR}$, TrkB, and BDNF. Therefore, neuroprotective therapy for HD must involve the reestablishment of not only BDNF but also the complete BDNF/TrkB/$p75^{NTR}$ system. To test our hypothesis, motor behavior and striatal neuropathology were evaluated along the disease progression in double-mutant mice, expressing one copy of mutant huntingtin but heterozygous for $p75^{NTR}$ (KI:$p75^{+/-}$). Our results showed that genetic normalization of $p75^{NTR}$ expression in mutant $Hdh^{Q7/Q111}$ (KI) mice delayed by several months the onset of motor coordination deficits and striatal neuropathology. This improvement might be attributed to the prevention of BDNF/TrkB/$p75^{NTR}$ imbalance, the normalization of BDNF-TrkB-phospholipase C-gamma (PLCγ) signaling, and/or the attenuation of the $p75^{NTR}$-associated JNK pathway. Importantly, at 10 months of age, downregulation of $p75^{NTR}$ is not enough to achieve this improvement supporting the hypothesis that proper levels of TrkB and BDNF are also needed to prevent HD progression. We validated this hypothesis by showing that chronic infusion of fingolimod (FTY720) improved motor coordination behavior and striatal neuropathology in HD transgenic mice which were associated with normalization of $p75^{NTR}$ levels in the BDNF/TrkB/$p75^{NTR}$ system.

## Results

### Normalization of $p75^{NTR}$ Levels in the Striatum of KI Mice Delays the Onset of Motor Coordination Deficits

We have described that mutant KI mice manifest motor coordination impairments at the age of 8 months [31]. Given the critical role of $p75^{NTR}$ in HD pathology, we evaluated whether normalization of striatal $p75^{NTR}$ levels prevents these motor deficits. To address this question, levels of $p75^{NTR}$ were normalized in KI mice by crossmating of $Hdh^{Q7/Q111}$ with $p75^{NTR}$/Exon $III^{+/-}$ mice ($p75^{+/-}$ mice) to obtain double-mutant mice (KI:$p75^{+/-}$). First, $p75^{NTR}$ levels were analyzed by Western Blot in striatal extracts from wild type (WT), $p75^{+/-}$, KI, and KI:$p75^{+/-}$ mice. As expected, quantification of band intensities revealed a significant increase of $p75^{NTR}$ in KI mice, which was significantly reversed in KI:$p75^{+/-}$ mice at the three different ages evaluated (6, 8, and 10 months) (Fig. 1a). In contrast, no increased expression of $p75^{NTR}$ was observed in the cortex of HD mice according to previous published data by our group [28]. Therefore, both $p75^{+/-}$ and KI:$p75^{+/-}$ mice presented reduced cortical $p75^{NTR}$ levels compared with WT mice (Supplemental Fig. 1A). Next, motor coordination was evaluated. Mice were first trained at 10 rpm during three consecutive days (Fig. 1b), and performance in the rotarod at 10 and 24 rpm was assessed longitudinally in WT, KI, $p75^{+/-}$, and KI:$p75^{+/-}$ mice from 7 to 12 months of age (Fig. 1c). Motor coordination was severely impaired in KI mice starting at 8 months of age and worsening thereafter, with a number of falls significantly higher than WT mice along trials (Fig. 1c). We observed a significant reduction of number of falls in KI:$p75^{+/-}$ mice compared with KI mice at 8 and 9 months when evaluated at 24 rpm while at 10 rpm, these differences were significant only at 9 months. Similar results were obtained when spontaneous locomotor activity was measured in the open field (Fig. 1d). KI mice manifested a significant reduction in the distance traveled compared with WT mice at 8 and 10 month of age while KI:$p75^{+/-}$ mice only displayed significant differences at 10 months of age. At 8 months,

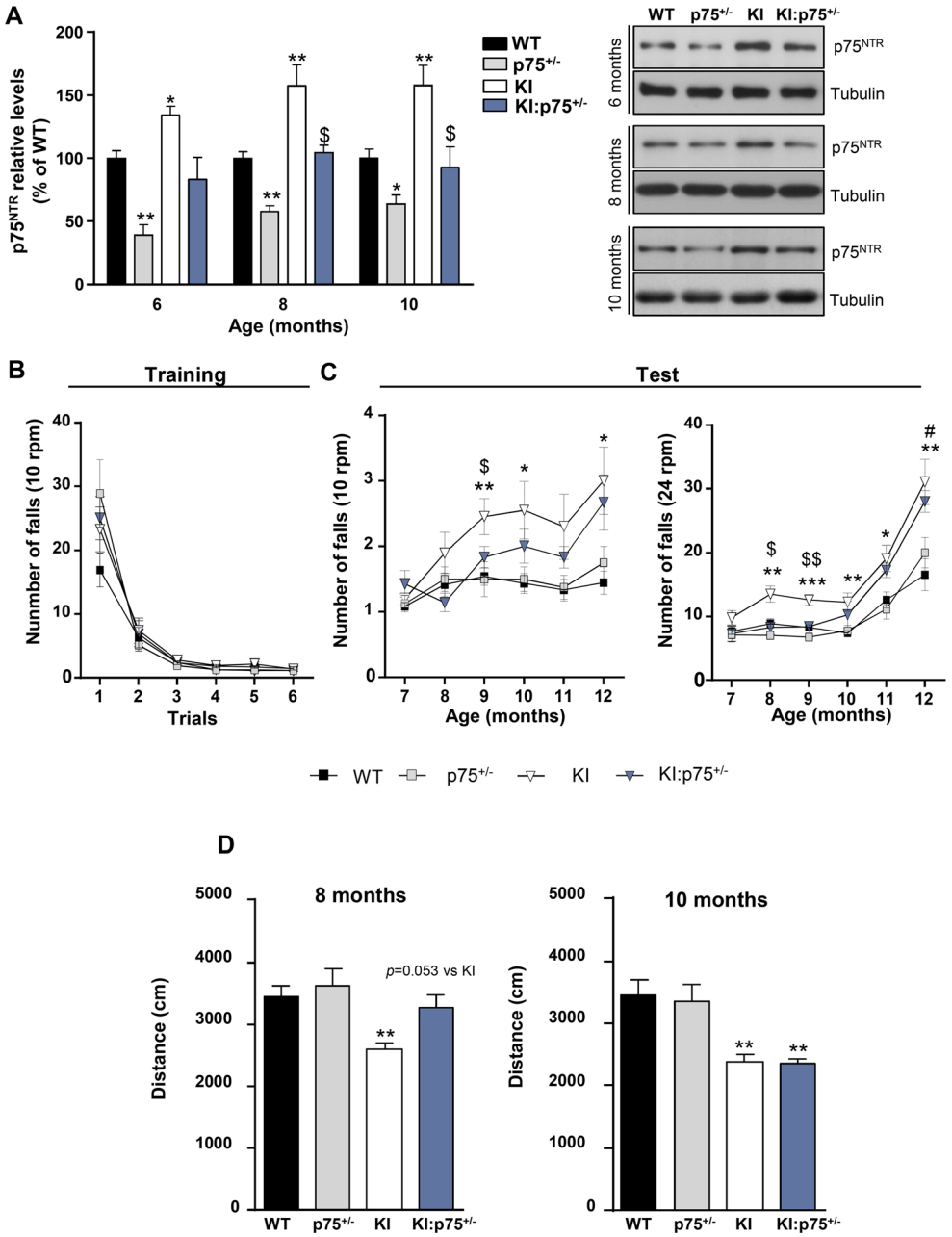


**Fig. 1** Normalization of $p75^{NTR}$ expression in KI mice delays the onset of motor deficits. **a** Representative immunoblots showing the levels of $p75^{NTR}$ and tubulin as loading control in striatal extracts obtained from WT, $p75^{+/-}$, KI, and KI:$p75^{+/-}$ mice at 6, 8, and 10 months of age. Histograms represent the relative protein levels expressed as percentage of WT values. All data are shown as the mean ± SEM ($n = 5$–7 mice/genotype/age). Data were analyzed by one-way ANOVA followed by Tukey's test. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with WT; $^{\$}P < 0.05$ compared with KI. Motor behavior was analyzed in WT, $p75^{+/-}$, KI, and KI:$p75^{+/-}$ by performing the rotarod task (**b**, **c**) and the open field (**d**). **b** The number of falls from a fixed speed rotarod was recorded during 60 s (s) at 10 rpm during training at the age of 5–6 months during three consecutive days. **c** The number of falls from a fixed speed rotarod was recorded during 60 s at 10 rpm and 24 rpm every 4 weeks from 7 to 12 months of age. Data are shown as the mean ± SEM ($n = 10$–13 animals/genotype). At different stages of the disease progression, data were analyzed by one-way ANOVA followed by Tukey's test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$: KI compared with WT; $^{\$}P < 0.05$ and $^{\$\$}P < 0.01$: KI:$p75^{+/-}$ compared with KI; $^{\#}P < 0.05$: KI:$p75^{+/-}$ compared with WT. **d** Spontaneous locomotor activity in the open field test at 8 and 10 months of age. Data were analyzed by one-way ANOVA followed by Tukey's test ($n = 10$–13 mice per genotype). $^{**}P < 0.01$ compared with WT

performance in open field by KI:$p75^{+/-}$ mice was comparable to WT mice, traveling more distance than KI mice, although these differences were not statistically significant ($P = 0.053$). Altogether, these data indicate that normalization of striatal $p75^{NTR}$ levels in KI mice delays the onset of motor coordination impairments.

## Normalization of p75$^{NTR}$ Levels in the Striatum of KI Mice Delays Striatal Neuropathology

Reduced levels of striatal proteins such as dopamine- and cAMP-regulated phosphoprotein Mr 32 kDa (DARPP32) [32], phosphodiesterase 10A (PDE10A) [33], or adenosine receptor type 2A (A2AR) [34] have been reported in several experimental models of HD and associated with striatal dysfunction. In accordance with these data, a significant decrease in these three proteins was found in KI striatal extracts at 6, 8, and 10 months of age (Fig. 2a–c). Interestingly, in KI:p75$^{+/-}$ mice, the levels of these striatal markers were preserved until 10 months of age, when a similar reduction was observed. Our results support the idea that striatal pathology can be delayed by normalization of p75$^{NTR}$ levels. To further confirm this hypothesis, two other neuropathological hallmarks of HD striatal dysfunction, such as mutant huntingtin (mHtt) aggregates [3, 4] and dendritic spine alterations [35, 36], were analyzed in mice aged between 7 and 10 months. First, mHtt aggregates were detected by EM48 diaminobenzidine staining in the striatum of KI and KI:p75$^{+/-}$ mice (Fig. 3a). A significant reduction in both the number of positive nuclear inclusions and EM48 labeling intensity (staining index) was found in KI:p75$^{+/-}$ compared with KI mice between 8 and 9 months (Fig. 3a), while similar aggregate density and staining index were observed at 10 months in both KI and KI:p75$^{+/-}$ mice (Fig. 3b). Next, DiOlistic labeling was performed to analyze dendritic spine density in fixed brain slices obtained from WT, p75$^{+/-}$, KI, and KI:p75$^{+/-}$ at 7–8 months (Fig. 3c) and 10 months of age (Fig. 3d). The number of spines per micrometer of dendrite length was significantly reduced in KI mice at the different ages evaluated, while a significant increase was observed in KI:p75$^{+/-}$ mice when compared with KI mice. Surprisingly, p75$^{+/-}$ presented a significant increase in spine density when compared with WT mice at 7–8 months, while only an increasing trend at 10 months of age. These results are in accordance with the negative role of p75$^{NTR}$ in dendritic spine density previously reported [37]. Interestingly, at earlier disease stages (3 months), no differences were observed between genotypes supporting the hypothesis that p75$^{NTR}$ downregulation in the striatum prevents the loss of dendritic spines during the course of the disease

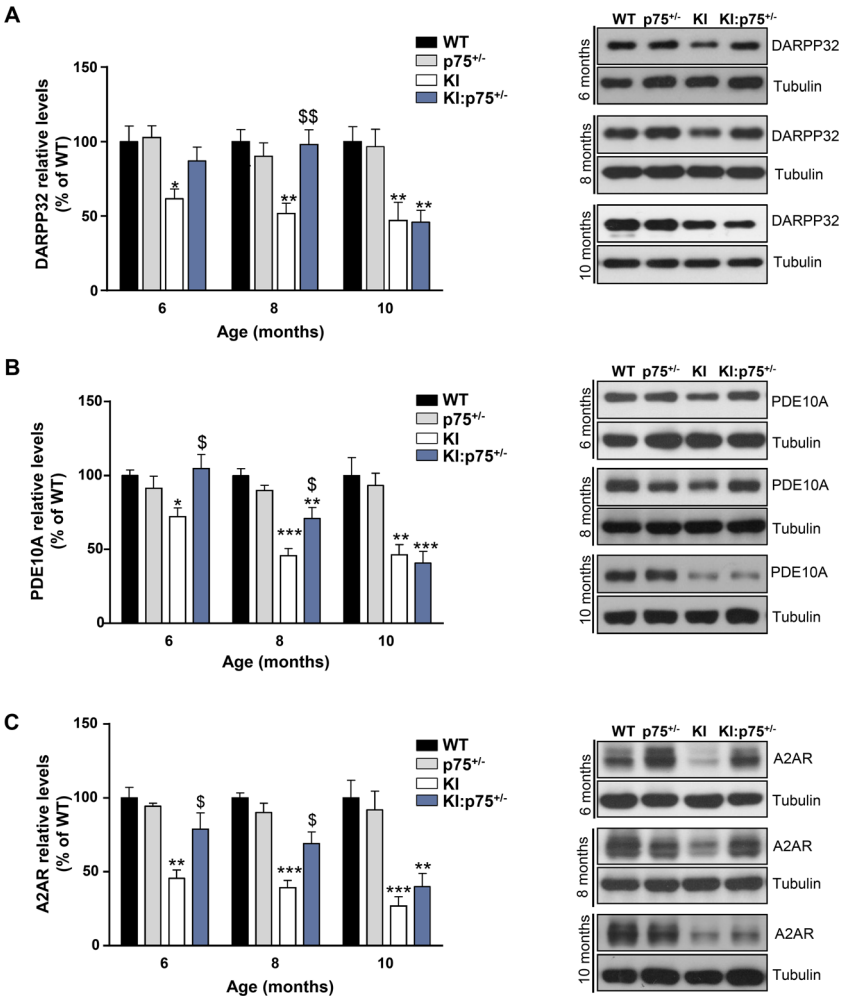


**Fig. 2** Normalization of p75$^{NTR}$ expression in KI mice delays the decrease of striatal-enriched proteins. Representative immunoblots showing the levels of DARPP32 (**a**), PDE10A (**b**), A2AR (**c**), and tubulin as loading control in striatal extracts obtained from WT, p75$^{+/-}$, KI, and KI:p75$^{+/-}$ mice at 6, 8, and 10 months of age. Histograms represent the relative protein levels expressed as percentage of WT values. All data are shown as the mean ± SEM ($n = 5$–7 mice/genotype/age). Data were analyzed by one-way ANOVA followed by Tukey's test. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared with WT; $^{\$}P < 0.05$ and $^{\$\$}P < 0.01$ compared with KI

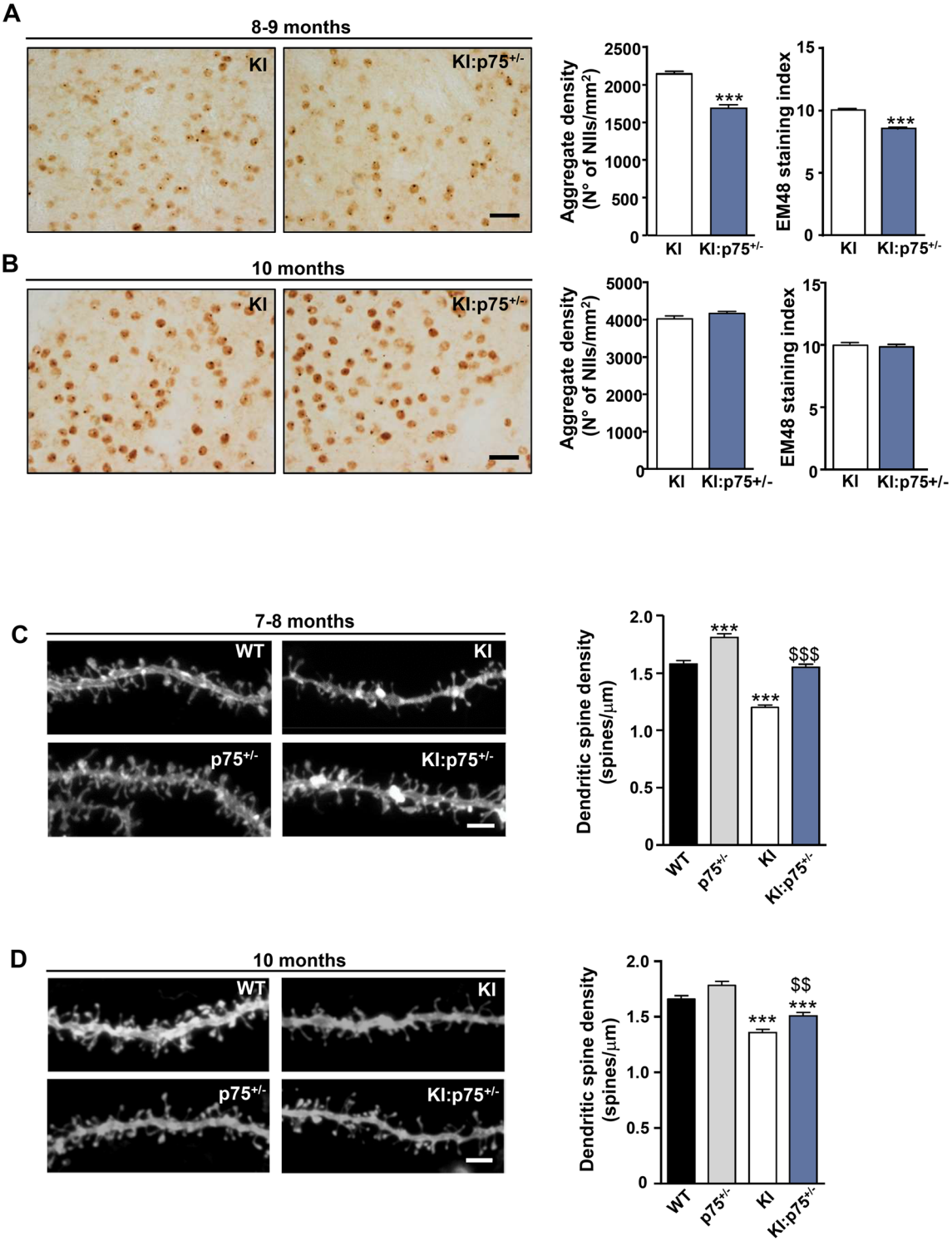


**Fig. 3** Normalization of $p75^{NTR}$ expression in KI mice reduces mutant huntingtin aggregation and dendritic spine loss. Representative photomicrographs showing nuclear EM48 immunostaining in the dorsal striatum of 8–9-month-old (**a**) and 10-month-old (**b**) KI and KI:$p75^{+/-}$ mice. Scale bar 20 μm. Histograms represent density of $EM48^{+}$ neuronal intranuclear inclusions (NIIs) and "staining index," which captures both the nuclear staining intensity and the number of immunostained nuclei. Diffuse huntingtin immunostaining was lighter and surrounded the huntingtin aggregates. Data are shown as the mean ± SEM ($n = 600$–900 images from 3 to 4 mice). Data were analyzed by a two-tailed Student's $t$ test. $^{***}P < 0.001$ compared with KI. Representative dendrites of medium spiny neurons from WT, $p75^{+/-}$, KI, and KI:$p75^{+/-}$ mice at 7–8 months (**c**) and 10 months of age (**d**). Scale bar 3 μm. Histograms show quantitative analysis of dendritic spine density per micrometer of dendritic length. Data are shown as the mean ± SEM (90–100 dendrites; $n = 4$–5 animals per genotype). Data were analyzed by one-way ANOVA followed by Tukey's test. $^{***}P < 0.001$ compared with WT; $^{\$\$}P < 0.01$ and $^{\$\$\$}P < 0.001$ compared with KI

(Supplemental Fig. 2). Altogether, these data suggest that normalization of $p75^{NTR}$ levels contributes to delay the onset of striatal neuropathology, in accordance with the delayed onset of motor deficits described above.

## Normalization of $p75^{NTR}$ in the Striatum of KI Mice Delays the Reduction of BDNF/TrkB Expression

Survival and maintenance of MSNs are especially dependent on the BDNF-TrkB signaling pathway [5, 6]. Low striatal BDNF protein levels have been reported in postmortem HD brain samples and in several HD mouse models [7–10]. Moreover, we have demonstrated an imbalance of $p75^{NTR}$ and TrkB expression in mouse and human HD brain associated with increased striatal susceptibility [19]. Therefore, we have examined whether normalization of striatal $p75^{NTR}$ expression could affect BDNF and TrkB levels in the striatum. Total levels of BDNF (tBDNF) including both BDNF isoforms, the precursor proBDNF and the mature (m)BDNF, were assessed by ELISA (Fig. 4a), while only mBDNF was measured by Western Blot analysis (Fig. 4b). The specificity of the commercial anti-BDNF antibodies used in the study was validated by Western Blot using brain lysates from homozygous and heterozygous BDNF mice and recombinant BDNF as a positive control (Supplemental Fig. 3). In line with previous published data in other HD mouse models [7, 10], 8- and 10-month-old KI mice exhibited a robust reduction in striatal tBDNF levels analyzed by ELISA (Fig. 4a). Although this reduction in tBDNF could be attributed to a reduction in both BDNF isoforms, it has previously been shown that proBDNF is barely detectable in WT and HD striatum [10]. Importantly, decreased tBDNF levels could not be observed in KI:$p75^{+/-}$ mice until the age of 10 months with comparable tBDNF levels between KI:$p75^{+/-}$ and WT mice at 6 and 8 months of age. However, no statistical differences were detected between 8-month-old KI:$p75^{+/-}$ and KI (Fig. 4a). Interestingly, the observed decrease in tBDNF levels by ELISA correlates well with the significant reduction of mBDNF protein revealed by Western Blot in 8-month-old KI mice (Fig. 4b). Furthermore, a significant increase in mBDNF levels was observed in KI:$p75^{+/-}$ at 8 months but not at 10 months of age compared to KI mice (Fig. 4b), indicating that normalization of $p75^{NTR}$ levels delays mBDNF downregulation in KI:$p75^{+/-}$ mice. It is noteworthy that we observed a trend toward an age-dependent reduction of tBDNF levels in the four genotypes which is consistent with previous studies that have shown loss of BDNF gene transcription with aging [10, 38–41]. Since cortical afferents are the main source of striatal BDNF [42], we further evaluated mBDNF levels in cortical extracts from WT,

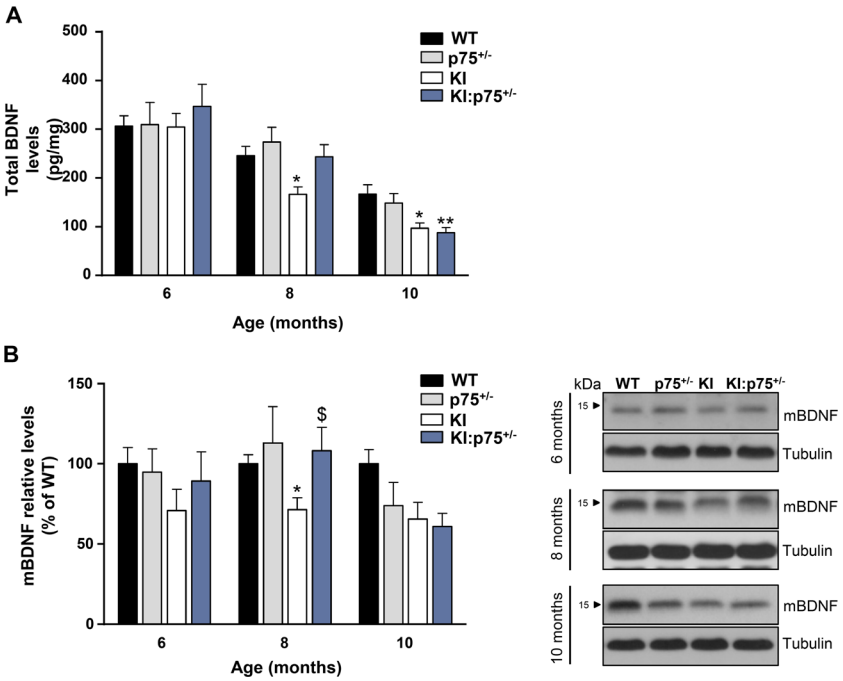


**Fig. 4** Delayed reduction of striatal BDNF levels by normalization of $p75^{NTR}$ expression in KI mice. BDNF quantification was performed using striatal tissue obtained from WT, $p75^{+/}$, KI, and KI:$p75^{+/-}$ mice at 6, 8, and 10 months of age. **a** Histogram represents total BDNF levels expressed as picograms per milligram of protein obtained by ELISA. Data are expressed as the mean ± SEM ($n = 5$–7 mice/genotype/age). Data were analyzed at each age by one-way ANOVA followed by Tukey's test. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with WT. **b** Representative immunoblots showing the levels of mature BDNF (mBDNF) and tubulin as loading control in striatal extracts. Histograms represent the relative protein levels expressed as percentage of WT values. All data are shown as the mean ± SEM ($n = 5$–7 mice/genotype/age). Data were analyzed by one-way ANOVA followed by Tukey's test. $^{*}P < 0.05$ compared with WT; $^{\$}P < 0.05$ compared with KI

p75$^{+/-}$, KI, and KI:p75$^{+/-}$ at 6, 8, and 10 months of age (Supplemental Fig. 1B). Our results demonstrated unaltered mBDNF levels between genotypes at any of the evaluated ages suggesting that cortical mBDNF production is not affected neither by upregulation of p75$^{NTR}$ in the striatum of KI mice nor by the genetic normalization of its levels in KI:p75$^{+/-}$. Similarly to striatal BDNF, a reduction in full-length TrkB levels was observed in KI mice from 8 months of age while such decrease could not be detected in KI:p75$^{+/-}$ mice until 10 months (Fig. 5a). No changes in the levels of the truncated TrkB isoform were observed (Fig. 5b). Next, activation of TrkB was analyzed by quantification of TrkB phosphorylation at tyrosine residues 816 (Y816), which lies within the C-terminus of TrkB-binding phospholipase C-gamma (PLC$\gamma$) and 515 (Y515), mediating SHC binding (Fig. 5c, d) [43]. A significant decrease in Y816 phosphorylation was found in KI mice at 8 months of age which could not be detected in KI:p75$^{+/-}$ mice until the age of 10 months (Fig. 5c). The levels of phosphorylated TrkB at the Y816 site in KI:p75$^{+/-}$ were significantly higher in comparison with KI mice at the age of 6 and 8 months. No significant differences on Y515 phosphorylation were found between genotypes (Fig. 5d). Taken together, these data indicate that BDNF-TrkB deficits along with altered TrkB phosphorylation status can be delayed in KI mice through normalization of p75$^{NTR}$ levels.

## Impaired TrkB-Induced Phosphorylation of PLCγ Can Be Delayed in KI Mice by Normalization of Striatal p75$^{NTR}$ Levels

Since levels of TrkB and BDNF are decreased in the striatum of KI mice from 8 months of age, we evaluated the three major downstream signaling pathways associated with TrkB

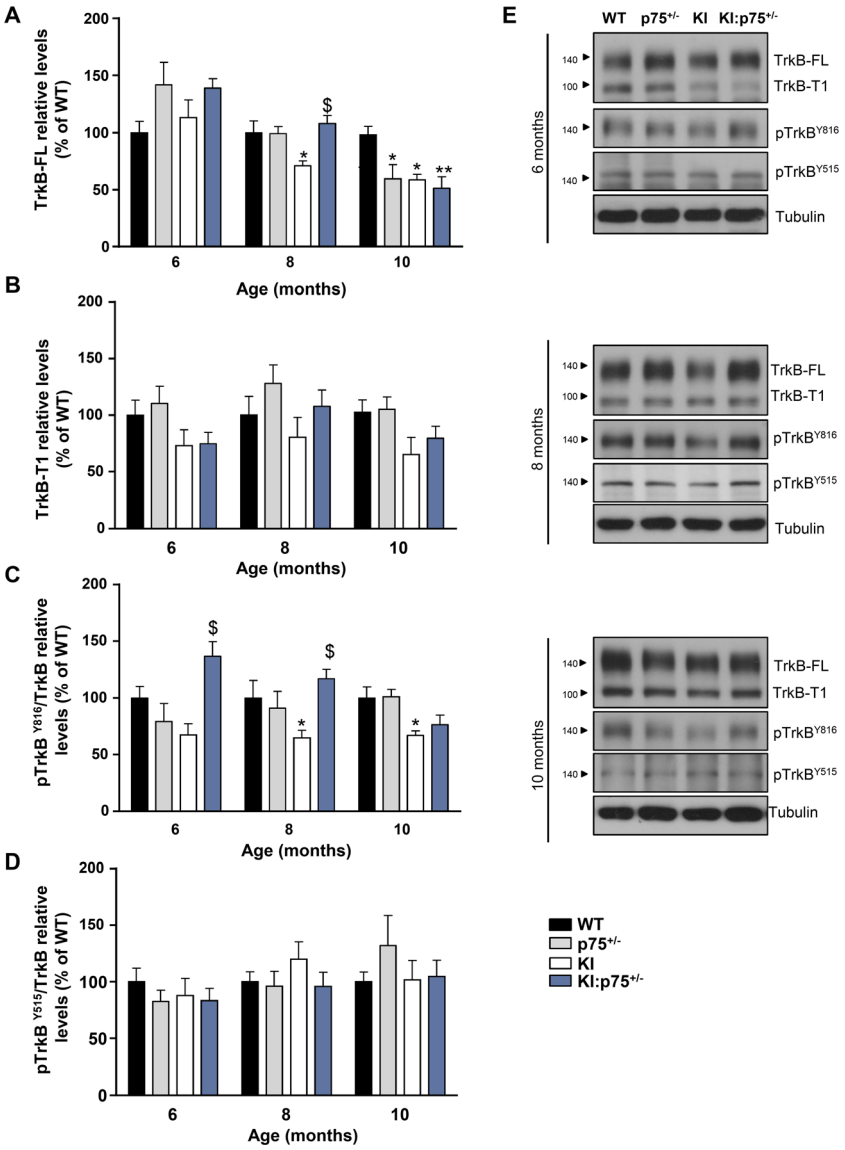


**Fig. 5** Reduced expression and activation of TrkB can be delayed by normalization of p75$^{NTR}$ expression in KI mice. Histograms represent the relative protein levels of TrkB full-length (TrkB-FL) (**a**) and TrkB truncated isoform (TrkB-T1) (**b**) with tubulin as loading control and the relatives ratios of phospho-TrkB (Y816)/TrkB (**c**) and phospho-TrkB (Y515)/TrkB (**d**) in striatal extracts obtained from WT, p75$^{+/-}$, KI, and KI:p75$^{+/-}$ mice at 6, 8, and 10 months of age. All data are shown as percentage of WT values and represented as the mean ± SEM ($n = 5$–7 mice/genotype/age). Data were analyzed by one-way ANOVA followed by Tukey's test. *$P < 0.05$ and **$P < 0.01$ compared with WT; $^{\$}P < 0.05$ compared with KI. **e** Representative immunoblots from **a** to **d**

activation: PI3K/Akt, MAP/ERK1/2, and PLC/PKC [44]. Western Blot analysis of striatal tissue at different ages revealed an increased activation of Akt at 8 and 10 months of age in KI mice (Fig. 6a). This sustained Akt activation is consistent with previous results shown in knock-in and transgenic HD mouse models [45] and has been considered a pro-survival mechanism associated to reduced levels of PHLPP1 phosphatase [46] and to an abnormal NMDA receptor activation [47]. No changes in ERK activation were observed in the striatum of KI mice until the age of 10 months, when a slight but not significant decrease was observed (Fig. 6b). Importantly, a significant reduction on PLCγ activation was present from 6 months of age (Fig. 6c). Because aberrant levels or activation of $p75^{NTR}$ can alter TrkB signaling [24], we also investigated these pathways in KI:$p75^{+/-}$ mice. pAkt and pERK levels where comparable between KI and KI:$p75^{+/-}$ mice, indicating that these pathways are not affected by normalization of $p75^{NTR}$ levels (Fig. 6a, b). In contrast, reduced pPLCγ levels were restored in KI:$p75^{+/-}$ mice at 6 and 8 months but not at 10 months of age, when phosphorylation of PLCγ was reduced to similar levels of KI mice (Fig. 6c). Since Akt, ERK, and PLCγ pathways via TrkB signaling have been shown to phosphorylate CREB, promoting transcription of its target genes, such as BDNF, we also evaluated phosphorylation levels of CREB. However, no differences were observed between genotypes at any age evaluated (Supplemental Fig. 4). Altogether, these data demonstrate that normalization of $p75^{NTR}$ levels in the striatum of KI mice delays the deficient activation of PLCγ induced by TrkB.

## Normalization of $p75^{NTR}$ in the Striatum of KI Mice Delays the Upregulation of JNK Activation

Activation of cell death signaling pathways, including JNK upregulation, has previously been reported in different HD models by our group and others [48–50]. Given that $p75^{NTR}$-mediated neurodegeneration has been associated with JNK activation [24], we next examined whether normalization of $p75^{NTR}$ levels in KI mice was accompanied by a reduction in JNK phosphorylation. Western Blot analysis of striatal extracts from WT, $p75^{+/-}$, KI, and KI:$p75^{+/-}$ mice revealed a significant increase in pJNK levels at 8 and 10 months of age in KI mice but not in KI:$p75^{+/-}$ animals, although no significant differences were observed between KI and

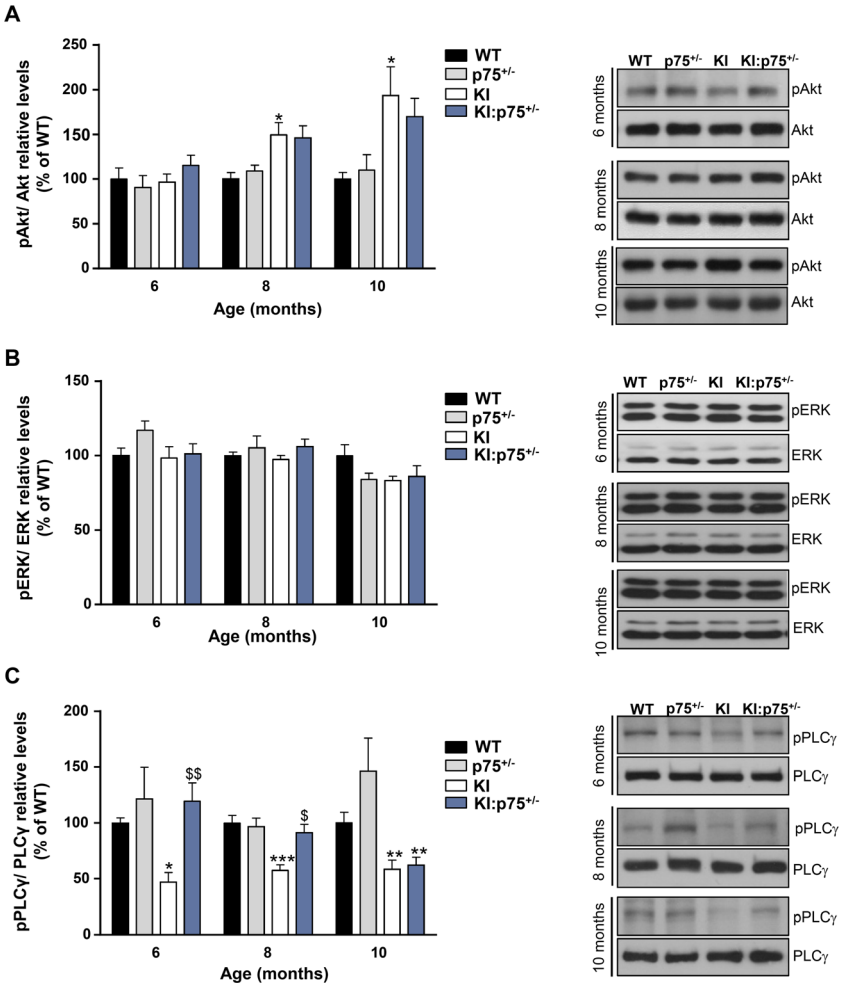


**Fig. 6** Impaired PLCγ activation can be delayed by normalization of $p75^{NTR}$ expression in KI mice. Representative immunoblots showing the levels of pAkt and Akt (**a**), pERK and ERK (**b**), and pPLCγ and PLC γ (**c**) in striatal extracts obtained from WT, $p75^{+/-}$, KI, and KI:$p75^{+/-}$ mice at 6, 8, and 10 months of age. The histograms represent the relative pAkt/Akt, pERK/ERK and pPLCγ/PLCγ ratios expressed as percentage of WT values. All data are shown as the mean ± SEM ($n = 5–7$ mice/genotype/age). Data were analyzed by one-way ANOVA followed by Tukey's test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with WT; $^{\$}P < 0.05$ and $^{\$\$}P < 0.01$ compared with KI

KI:p75$^{+/-}$ (Fig. 7a). Since JNK activation mediates direct phosphorylation of its substrate c-Jun, we assessed levels of phospho-c-Jun (Ser63) (pc-Jun) by Western Blot analysis. A significant increase in this pro-apoptotic transcription factor was detected at 10 months of age in KI mice while only a trend was observed at 6 and 8 months (Fig. 7b). Interestingly, pc-Jun was significantly reduced at 6 and 8 months in KI:p75$^{+/-}$ mice in comparison with KI mice. To determine whether reduced JNK activation was also associated with inhibition of caspase-3 activity, the caspase-3 cleaved spectrin fragment SBDP120 was analyzed by Western Blot. No significant changes in SBDP120 levels were found between genotypes (Fig. 7c). These findings indicate that activation of JNK apoptotic pathway can be partially prevented by normalization of p75$^{NTR}$ expression in KI mice. Despite the activation of these death signaling pathways, it is widely accepted that the Hdh$^{Q7/Q111}$ mouse model is spared of evident neuronal loss. Indeed, we found no cleaved caspase-3 positive-stained cells in corticostriatal slices from WT and KI mice (Supplemental Fig. 5), which confirms previous studies showing unaltered NeuN+ neuronal density in the striatum of Hdh$^{Q7/Q111}$ mice for up to 12 months [51]. Supporting the lack of neurodegeneration in this HD mouse model, no changes in cortical thickness were neither observed between genotypes (Supplemental Fig. 6). Overall, these results showing no neuronal death in our Hdh$^{Q7/Q111}$ HD model indicate that the early increase in p75$^{NTR}$ levels could lead to neuronal dysfunction without affecting cell viability.

## Chronic Administration of Fingolimod Prevents TrkB/p75$^{NTR}$ Imbalance and Ameliorates Neuropathology in the Striatum of R6/1 Mice

We have previously demonstrated that chronic treatment with fingolimod (FTY720), a sphingosine-1 phosphate receptor modulator, prevented the reduction of TrkB and BDNF levels

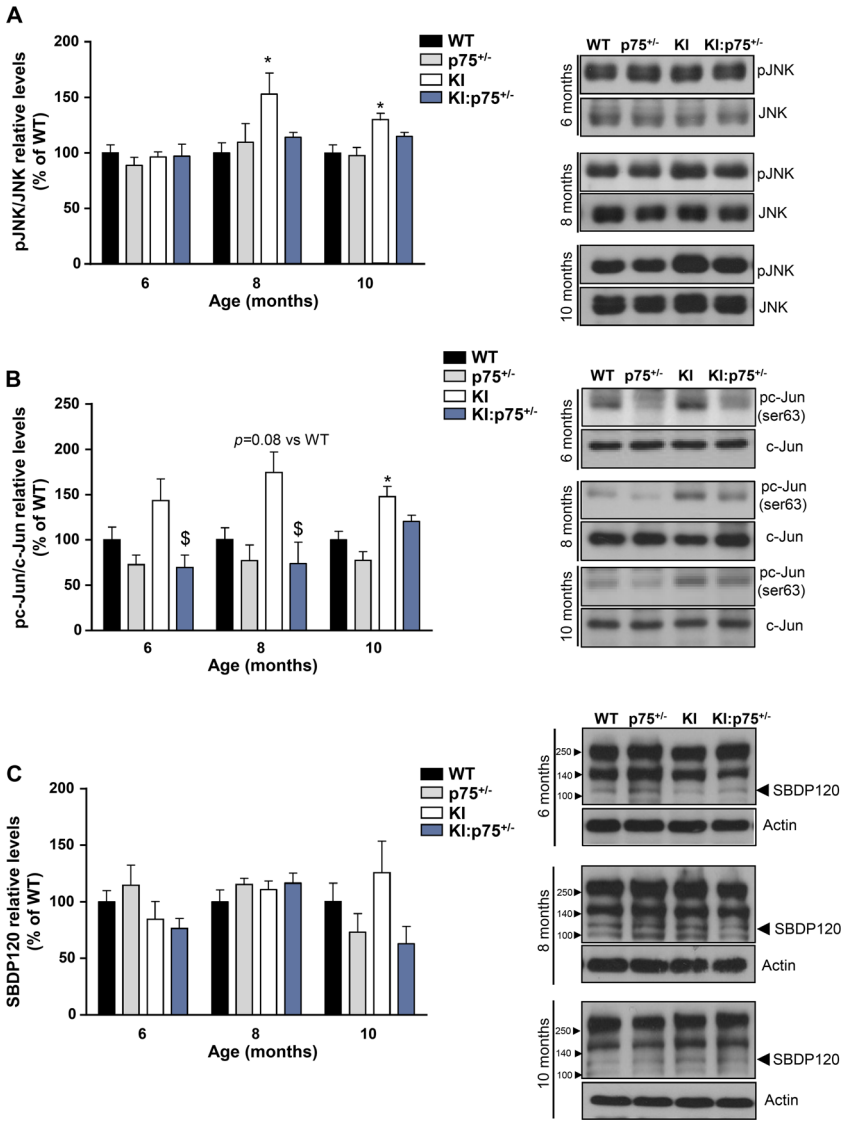


**Fig. 7** Normalization of p75$^{NTR}$ in KI mice attenuates activation of JNK. Representative immunoblots showing the levels of pJNK and JNK (**a**), pc-Jun and c-Jun (**b**), and αII-spectrin breakdown product 120 (SBDP120) (**c**) with tubulin as loading control in striatal extracts obtained from WT, p75$^{+/-}$, KI, and KI:p75$^{+/-}$ mice at 6, 8, and 10 months of age. Histograms represent the relative ratios of pJNK/JNK and pc-Jun/c-Jun and the relative levels of SBDP120 expressed as percentage of WT values. All data are shown as the mean ± SEM ($n = 5$–7 mice/genotype/age). Data were analyzed by one-way ANOVA followed by Tukey's test. *$P < 0.05$ compared with WT; $^{\$}P < 0.05$ compared with KI

and normalized the expression of $p75^{NTR}$ in the hippocampus of R6/1 transgenic mice [29]. Given that genetic normalization of striatal $p75^{NTR}$ expression delays BDNF/TrkB/$p75^{NTR}$ signaling alterations and improve neuropathology, we wondered whether FTY720 administration was also able to prevent striatal pathology in HD mice. To this aim, we used the R6/1 mouse model that displays earlier onset and faster disease progression than KI mice and presents a good correlation between the onset of motor impairments [52] and the upregulation of $p75^{NTR}$ in the striatum at 12 weeks of age [19]. Mice were treated with FTY720 from presymptomatic (8 weeks) to symptomatic (20 weeks) stages, when R6/1 mice manifest overt motor and cognitive behavioral deficits. In agreement with previous findings [11, 19, 53, 54], R6/1 mice at the age of 20 weeks did not show reduced levels of mature BDNF and full-length TrkB but a trend toward an increase in $p75^{NTR}$ protein levels ($P = 0.052$; Fig. 8a). Notably, treatment with FTY720 completely prevented $p75^{NTR}$ upregulation, showing R6/1-treated mice similar levels of $p75^{NTR}$ compared with WT mice. These results demonstrate that chronic administration of FTY720 prevents the TrkB/$p75^{NTR}$ imbalance in the striatum of HD mice. Next, we analyzed whether normalization of this imbalance could ameliorate striatal neuropathology. The presence of mHtt aggregates was evaluated by immunohistochemical analysis. Treatment with FTY720 significantly decreased the number of EM48 positive nuclear inclusions (Fig. 8b). Moreover, in accordance with the partial recovery of DARPP32 expression observed in the striatum of KI:$p75^{+/-}$ mice (Fig. 2a), FTY720 treatment also prevented DARPP32 reduction, as shown by the increase in protein levels exhibited by FTY720-treated R6/1 mice compared with vehicle-treated animals (Fig. 8c). Altogether, these data demonstrate that regulation of $p75^{NTR}$ levels by therapeutic strategies like FTY720 can maintain BDNF/TrkB/$p75^{NTR}$ balance preventing striatal neuropathology.

### Chronic Administration of Fingolimod Ameliorates Motor Coordination Deficits in R6/1 Mice

We have previously demonstrated that genetic normalization of $p75^{NTR}$ expression delays the onset of motor coordination deficits in KI mutant mice. Given that FTY720 treatment restores the balance between TrkB and $p75^{NTR}$ expression in R6/1 mice, we next assessed the potential of FTY720 to prevent or ameliorate motor abnormalities. Behavioral analysis using the rotarod at 24 rpm revealed that R6/1 mice start showing significant differences from WT mice at 14 weeks of age (Fig. 8d). Remarkably, the beneficial effects of the compound on motor function and coordination were first observed at this age, after 6 weeks of treatment (Fig. 8d). Chronic infusion of FTY720 significantly improved the rotarod performance of R6/1 manifested as a decrease in the number of falls compared with vehicle-treated R6/1 mice. No significant differences were observed between vehicle- and FTY720-treated WT mice. Overall, these results indicate that HD striatal neuropathology and motor coordination disturbances are dependent on the balance between TrkB and $p75^{NTR}$ expression.

## Discussion

The present study demonstrates the critical contribution of the BDNF/TrkB/$p75^{NTR}$ pathway to the onset of motor deficits and striatal neuropathology in HD mice. Longitudinal motor behavioral evaluation of full-length HD mice reveals a correlation between the onset of motor coordination abnormalities, the reduction in BDNF and TrkB levels, and the increase in $p75^{NTR}$ protein levels. Accordingly, genetic normalization of $p75^{NTR}$ expression in KI mutant mice delayed the onset of motor deficits and striatal dysfunction likely by restoring the balance between BDNF/TrkB/$p75^{NTR}$ expression and signaling. It is important to keep in mind that KI and R6/1 mice show increased levels of $p75^{NTR}$ in the striatum and hippocampus but not in the cortex [28]. This finding supports our hypothesis that the normalization of $p75^{NTR}$ in the striatum of KI mice is the responsible of the delay in motor deficits. Similarly, as previously reported by our group, hippocampal memory and synaptic plasticity deficits in KI mice can be prevented by the genetic downregulation of $p75^{NTR}$ in the hippocampus [28]. Furthermore, we also found that chronic pharmacological treatment with fingolimod improved motor behavior and striatal neuropathology in HD transgenic mice by normalizing $p75^{NTR}$ protein levels and therefore correcting the BDNF/TrkB/$p75^{NTR}$ imbalance.

Deficits of neurotrophic support to striatal neurons in HD have recently been associated not only to reduced levels of BDNF but also to impaired BDNF signaling and altered expression of BDNF receptors [17, 19–21, 30]. Besides these new pieces of evidence, no studies so far have explored the contribution of deficient BDNF signaling or impaired balance of BDNF receptors (TrkB and $p75^{NTR}$) to the onset of motor coordination deficits. To address this question, we have used double-mutant mice in which $p75^{NTR}$ expression has been normalized and we have evaluated many outcome measures such as motor coordination, striatal pathology and biochemical analysis along disease progression. Although in some measures 8-month-old KI:$p75^{+/-}$ mice did not perform significantly better than the KI, they did not show significant differences compared with WT neither. Then, our results show that normalization of $p75^{NTR}$ expression in KI mice delays from 6 to 10 months of age the onset of motor coordination deficits and striatal pathology, as well as the reduction in TrkB and BDNF protein levels. Interestingly, downregulation of $p75^{NTR}$ levels at 10 months of age fails to prevent striatal neuronal dysfunction and loss of motor coordination in KI mice most likely

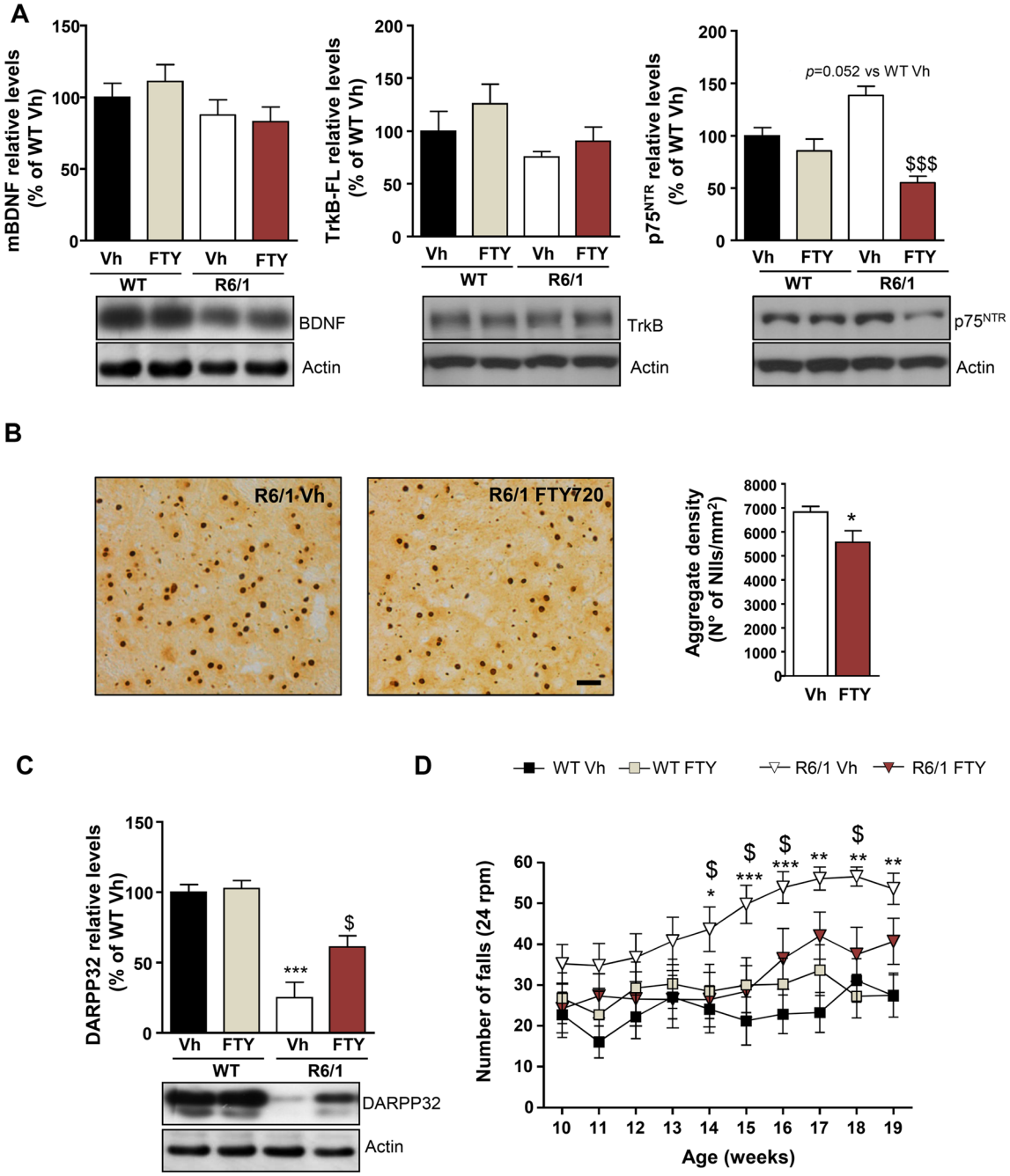


**Fig. 8** Fingolimod (FTY720) chronic treatment reduces $p75^{NTR}$ expression levels and prevents motor deficits and striatal pathology in R6/1 mice by **a** representative immunoblots showing the levels of mature BDNF (mBDNF), full-length TrkB (TrkB-FL), $p75^{NTR}$, and β-actin as loading control in striatal extracts of WT and R6/1 mice, after either vehicle or FTY720 chronic administration. Histograms represent protein levels expressed as percentage of WT vehicle. All data are shown as the mean ± SEM ($n = 5$–6 mice/genotype/treatment). Data were analyzed by one-way ANOVA followed by Tukey's test. $^{\$\$\$}P < 0.001$ compared with vehicle-treated R6/1 mice. **b** Representative photomicrographs showing nuclear EM48 immunostaining in the striatum of 20-week-old R6/1 mice treated with vehicle or FTY720. Scale bar 50 μm. Histograms represent the density of $EM48^+$ of neuronal intranuclear inclusions (NIIs) per square millimeter. Data are expressed as the mean ± SEM ($n = 6$ mice/treatment). Data were analyzed by a two-tailed Student's *t* test. $^{*}P < 0.05$ compared with vehicle-treated R6/1mice. **c** Western Blot analysis of DARPP32 and β-actin as loading control in striatal extracts from WT and R6/1 mice, after vehicle or FTY720 chronic administration. Histogram represents relative protein levels expressed as the percentage of WT vehicle. All data are shown as the mean ± SEM ($n = 5$–6 mice/genotype/treatment). Data were analyzed by one-way ANOVA followed by Tukey's test. $^{***}P < 0.001$ compared with vehicle-treated WT mice; $^{\$}P < 0.05$ compared with vehicle-treated R6/1 mice. **d** The number of falls from a fixed speed rotarod were recorded during 60 s at 24 rpm every week from 10 to 19 weeks of age. Data are expressed as the mean ± SEM ($n = 10$–12 animals/genotype/treatment). Data were analyzed by two-way ANOVA with repeated measures followed by Tukey's test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$: vehicle-treated R6/1 mice compared with vehicle-treated WT mice; $^{\$}P < 0.05$: FTY-treated R6/1 mice compared with vehicle-treated R6/1 mice

because the reduction in TrkB and BDNF levels can no longer be blocked. These results suggest that a correct balance in BDNF/TrkB/p75$^{NTR}$ levels is critical to support the neurotrophic signaling in medium spiny neurons (MSNs) needed to control motor function. Certainly, it is well known that the survival, maintenance, and function of MSNs are particularly dependent on the BDNF-TrkB signaling pathway [5, 6]. Consistently, key striatal-enriched proteins such as DARPP32, A2AR, and PDE10A whose expression is reduced in HD [32–34] were preserved as long as the BDNF/TrkB/p75$^{NTR}$ imbalance was prevented. Importantly, the function of these proteins is critical to regulate striatonigral and striatopallidal downstream signaling cascades that work for the fine-tuning of movement [55–57]. Thus, A2AR is required for most of the synaptic functions of BDNF/TrkB [58–60] while DARPP32, a key integrator of dopamine and glutamate neurotransmission in MSNs [61–63], participates through interaction with adducin to modulate dendritic spine stability [64]. Indeed, in our double-mutant KI:p75$^{+/-}$ mice, normalization of p75$^{NTR}$ levels prevented the loss of striatal dendritic spines. Altogether, these findings support the idea that abnormalities in the BDNF/TrkB/p75$^{NTR}$ signaling may contribute to reduce the expression of key striatal proteins leading to neuronal dysfunction and subsequent motor deficits in HD. As previously discussed, BDNF/TrkB pathway and striatal integrity cannot be restored by normalization of p75$^{NTR}$ at late disease stages in KI:p75$^{+/-}$, probably due to other pathological mechanisms triggered by mutant huntingtin, such as progressive transcriptional alterations, which have been already described in our Hdh$^{Q7/Q111}$ mouse model [51].

It remains unclear how the increment in p75$^{NTR}$ found in the striatum and the consequent restoration in the double-mutant mice can affect striatal mBDNF. In agreement with our results, several studies have described decreased striatal mBDNF levels without cortical BDNF alterations [11, 65, 66]. These results suggested that anterograde transport of mBDNF and/or proBDNF from cortex to striatum could be impaired [8, 67]. Since it is well known that p75$^{NTR}$ is involved in axonal degeneration [68], it is possible to hypothesize that normalization of p75$^{NTR}$ in HD mice could contribute to reduce the axonal pathology in corticostriatal connections present in HD mice [69, 70] and restore BDNF transport. Further studies would be necessary to elucidate this potential mechanism.

Beyond the observed alterations of BDNF and TrkB protein levels, we found that the onset of motor deficits and the reduction of striatal-enriched proteins in KI mice were also associated with a specific decrease in TrkB-PLCγ signaling. Thus, in line with previous studies in other HD mouse models [10, 53], reduced phosphorylation of PLCγ and TrkB at Y816, the tyrosine residue associated with PLCγ activation, was observed in KI mice along the disease progression. In contrast, TrkB phosphorylation at Y515 remained unchanged between genotypes. This differential phosphorylation of TrkB tyrosine residues could be the result of BDNF-independent molecular mechanisms already described in several studies [71–74]. Interestingly and consistent with a delay in motor dysfunction, impaired TrkB-PLCγ signaling was also delayed by normalization of p75$^{NTR}$ levels. This data might be explained by the restored BDNF levels observed in double-mutant KI:p75$^{+/-}$ mice and/or by a direct effect of p75$^{NTR}$ on TrkB phosphorylation sites. Indeed, previous studies have already reported attenuation of BDNF-mediated autophosphorylation of TrkB by p75$^{NTR}$ [75] and increased TrkB phosphorylation has been observed under p75$^{NTR}$ masking conditions in hippocampal neurons [76]. Evidence showing that p75$^{NTR}$ directly interacts with Trk receptors [77] leads to speculate that binding of BDNF to p75$^{NTR}$ directly affects conformation and, therefore, the affinity of TrkB for BDNF. However, this crosstalk between p75$^{NTR}$ and TrkB receptor affecting BDNF signaling in HD needs to be further explored.

Altered neuronal function in different brain disorders has been associated with abnormal activation of PLCγ resulting in impaired neuronal activity and ultimately cell death [78]. In our HD model, reduced PLCγ activity might contribute to striatal pathology by decreasing striatal protein markers such as DARPP32 [5, 79] and A2AR [80]. On the other hand, due to the role of PLCγ signaling in the functional changes of spine actin cytoskeleton [81–83], the decreased striatal spine density detected in mutant KI mice could be a consequence of PLCγ activity downregulation.

Besides the recovery of the pro-survival signaling pathway PLCγ following normalization of p75$^{NTR}$ expression, we also demonstrated reduced activation of the pro-apoptotic JNK pathway. Based in our previous published data showing that the increase in p75$^{NTR}$ levels is associated with DARPP32+ striatal neurons [19], we can hypothesize that the observed increase in JNK activation could also occur in these neurons. It is noteworthy that even though in the p75$^{+/-}$ heterozygous mice a dose-dependent effect of p75$^{NTR}$ gene copy number on pJNK levels would be expected, we observed that p75$^{+/-}$ mice mostly display phenotypes similar to WT. We hypothesize that the activation or inhibition of specific pathways are dependent on an "optimal range" of p75$^{NTR}$ expression levels. Moreover, we cannot exclude that additional pathways could regulate JNK activation since p75$^{NTR}$ levels are increased at 6 months of age in KI mice striatum but JNK activity is unaltered at this age. The involvement of JNK in mHtt-mediated striatal neurotoxicity is supported by a number of different in vitro and in vivo studies [48–50]. Activation of cellular stress response manifested as JNK phosphorylation has been reported in cells expressing polyglutamine aggregates while inhibition of JNK activation slowed and reduced the formation of such polyQ-protein aggregates [84–86]. In this view, we propose that attenuation of JNK signaling activation by normalization of p75$^{NTR}$ expression might also contribute to reduce the number of intranuclear mHtt aggregates observed in the striatum of

double-mutant KI:p75$^{+/-}$ mice. Indeed, it has been recently shown that modulation of p75$^{NTR}$ by small molecules that specifically enhances pro-survival downstream signals and inhibits pro-apoptotic JNK activation ameliorates HD striatal pathology [30]. Moreover, JNK activation has also been shown to mediate the inhibitory effect of mutant huntingtin on axonal transport [87]. Therefore, the present data showing JNK activity downregulation underlying the beneficial effect of p75$^{NTR}$ normalization on KI mice pathology might explain why a variety of therapies based on neurotrophic factors partially improve mutant huntingtin-induced pathology in other mouse HD models. Nevertheless, we cannot exclude that other pathways associated with p75$^{NTR}$ signaling, like RhoA/ROCK cascade [23, 25], could also be involved. Aberrant activity of p75$^{NTR}$ in HD mice increases RhoA activation leading to impaired synaptic plasticity [20, 28, 29], and importantly, inhibition of the ROCK pathway enhances mHtt degradation and reduces aggregation while improving motor function in R6/2 mice [88–91]. These findings suggest that the reduction of mHtt aggregates could be due to the inhibition of JNK, as well as RhoA/ROCK signaling, once levels of p75$^{NTR}$ are normalized.

We further confirmed the critical contribution of the proper balance of BDNF/TrkB/p75$^{NTR}$ to motor deficits in HD mice by using a pharmacological approach. Fingolimod (FTY720) is an immunomodulator known to improve BDNF release in the striatum, to rescue motor deficits in mouse models of Rett's syndrome and HD [93, 92], and to prevent cognitive deficits and imbalance of BDNF receptors in the hippocampus of R6/1 mice [29]. Chronic administration of fingolimod improved motor function in 20-week-old R6/1 mice, reduced mHtt aggregates and increased DARPP32 expression, in agreement with previous data in R6/2 mice [93]. Notably, FTY720 treatment also normalized p75$^{NTR}$ levels, aberrantly expressed in R6/1 mice between 12 and 30 weeks of age [19]. BDNF levels were not found significantly decreased in the striatum of R6/1 mice at 20 weeks of age. While this finding is consistent with previous reports [54], other studies have found decreased BDNF mRNA or protein levels at the same age analyzed [66, 94]. The addition of behavioral tasks prior to obtaining samples, the use of different genetic backgrounds, or the diversity in commercial antibodies against BDNF could account for the observed different results. No changes in TrkB levels at 20 weeks of age between WT and R6/1 mice were observed neither, consistent with previous studies reporting downregulation at 30 weeks of age [19]. We cannot rule out that other mechanisms modulated by FTY720 might contribute to the therapeutic benefits observed in R6/1 mice. Indeed, it has been demonstrated that FTY720 can increase phosphorylation of mutant huntingtin at serine 13/16 residues to attenuate protein toxicity [93], as well as modulate CREB expression [92] or inhibit class I histone deacetylases (HDACs) in neurons [95]. Nevertheless, based on our findings, we hypothesize that the improvement in motor coordination observed in FTY720-treated R6/1 mice can be mainly attributed to prevention of BDNF/TrkB/p75$^{NTR}$ imbalance.

It can be argued that the genetic or pharmacological normalization of p75$^{NTR}$ is not exclusively neuronal since expression of p75$^{NTR}$ in astrocytes has been reported following different brain injuries [96, 97]. However, we have previously demonstrated that the aberrant p75$^{NTR}$ increase in the striatum and the hippocampus of the Hdh$^{Q111}$ mice is neuron-specific [19, 28], supporting the idea that normalization of p75$^{NTR}$ contributes to restore BDNF/TrkB signaling in neurons.

In conclusion, this work shed new light on how the imbalance of the BDNF/TrkB/p75$^{NTR}$ system can affect striatal neuropathology and motor behavior in HD. It demonstrates that this imbalance leads to impaired BDNF signaling trough reduced TrkB-PLC$\gamma$ pathway and increased JNK activation. More importantly, we show that normalization of p75$^{NTR}$ expression delays such impairments that follow the well-described "dying back" pattern of neuronal degeneration in HD [98]. Our study further supports the role of p75$^{NTR}$ signaling in striatal pathophysiology highlighting the potential benefits of restoring BDNF/TrkB/p75$^{NTR}$ balance for preventing, delaying, or reversing the progression of HD pathology.

## Methods

**Animals** Heterozygous HdhQ111 knock-in mice (KI) [99] and p75$^{+/-}$ heterozygous mice (p75$^{NTR}$/ExonIII) from the Jackson Laboratory were bred. KI and p75$^{+/-}$ were C57BL/6 genetic background. Only males from each genotype, Hdh$^{Q7/Q7}$ (WT), Hdh$^{Q7/Q111}$ (KI), p75$^{+/-}$, and KI:p75$^{+/-}$, were used for all experiments. Male R6/1 transgenic mice (B6CBA background) expressing the exon-1 of mutant huntingtin with 145 repeats were used for pharmacological experiments [100]. Male C57BL/6J (WT) mice, BDNF$^{+/-}$ mice, and BDNF$^{-/-}$ from the Jackson Laboratory were bred and used to test specificity of BDNF antibodies. Experimental procedures were approved by the Local Ethical Committee of the University of Barcelona (99/01) and the Generalitat de Catalunya (00/1094), following European (2010/63/UE) and Spanish (RD 1201/2005) regulations for the care and use of laboratory animals.

**Drug Administration** FTY720 was obtained as a powder from Cayman Chemicals and dissolved in EtOH 10% in distilled water (vehicle). For chronic pharmacological treatment, intraperitoneal injections of FTY720 were given every 4 days at a dose of 0.3 mg/kg for 12 weeks, starting at 8 weeks of age. Animals were weighted weekly in order to determine the appropriate dose. Mice were distributed into four experimental groups ($n$ = 10–12 each): WT + FTY720, WT + vehicle, R6/1

+ FTY720, and R6/1 + vehicle. Last dose of FTY720 was administered 24 h before sacrificing the animals for histological and biochemical analysis.

## Behavioral Assessment

**Fixed Rotarod** Motor coordination and balance were evaluated on the rotarod apparatus at distinct rotations per minute (rpm), as described elsewhere [54]. In brief, KI mice (5–6 months old) and R6/1 mice (7 weeks old) were trained at constant speed (10 rpm) for 60 s. We performed two trials per day for three consecutive days, and the latency to fall and the number of falls during 60 s was recorded. No differences between groups were detected at this period. After training and starting at 7 months of age, KI mice were evaluated once a month at 10 and 24 rpm until 12 months of age, and the number of falls in a total of 60 s was recorded. R6/1 mice were evaluated at 24 rpm, once a week, from 10 to 19 weeks of age. The animals were put on the rotarod several times until the addition of the latency to fall off reached a total of 60 s.

**Open Field** Independent cohorts of 8- and 10-month-old WT, KI, $p75^{+/-}$, and KI:$p75^{+/-}$ animals (only males) were used. The device consisted of a white circular arena with 40 cm diameter and 40 cm high. The light intensity was 40 lx throughout the arena, and the room temperature was kept at 19–22 °C and 40–60% humidity. Mice were placed into the arena during two consecutive days (15 min/day), and spontaneous locomotor activity was measured as total distance traveled. The arena was rigorously cleaned between animals in order to avoid odors. Animals were tracked and recorded with SMART Junior Software.

**Western Blotting** WT and HD mice were killed by cervical dislocation, and the brains were quickly removed, dissected, frozen in dry ice, and stored at − 80 °C until use. Protein extracts were prepared from striatal brain samples by sonication on ice for 10 s in lysis buffer containing 50 mM Tris base (pH 7.4), 150 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, and 1% NP-40 supplemented with 1 mM sodium orthovanadate and protease inhibitor mixture (Sigma-Aldrich). Samples were centrifuged at 10,000×*g* for 10 min, and the protein contents determined by Detergent-Compatible Protein Assay (bicinchoninic acid, BCA; Bio-Rad). Protein extracts (30 μg) were mixed with 4× SDS sample buffer, boiled for 5 min, resolved by 8–12% SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes (Schleicher & Schuell). Blots were blocked in 10% non-fat powdered milk in Tris-buffered saline Tween-20 (50 mM Tris-HCl, 150 mM NaCl, pH 7.4, 0.05% Tween-20) for 1 h at room temperature. The membranes were then incubated overnight at 4 °C with primary antibodies: anti-DARPP32 (1:1000, BD Biosciences, cat. no. 611520), anti-PDE10A (1:1000, Abcam, cat. no. ab14622), anti-A2AR (1:1000, Santa Cruz, cat. no. sc-32261), anti-BDNF (1:1000, Santa Cruz, cat. no. sc-546), anti-BDNF (1:1000, Icosagen, cat. no. 327-100, Clone 3C11), anti-$p75^{NTR}$ (1:1000, Promega, cat. no. G3231), anti-phospho-TrkB (Tyr816) (1:1000, Abcam, cat. no. ab75173), anti-phospho-TrkB (Tyr515) (1:1000, Abcam, cat. no. ab131483), anti-TrkB (1:1000, BD Biosciences, cat. no. 610101), anti-phospho-Akt (Ser473) (1:1000, Cell Signaling, cat. no. 3787), anti-Akt (1:1000, Cell Signaling, cat. no. 2920), anti-phospho p44/42 ERK1/2 (Thr202/Tyr204) (1:1000, Cell Signaling, cat. no. 9101), anti-ERK1/2 (1:2500, BD Biosciences, cat. no. 610123), anti-phospho-PLCγ1 (Tyr783) (1:1000, Cell Signaling, cat. no. 2821), anti-PLCγ1 (1:1000, Cell Signaling. cat. no. 2822), anti-phospho-CREB (Ser133) (1:1000, Millipore, cat. no. 06-519), anti-CREB (1:1000, Cell Signaling, cat. no. 9197), anti-phospho-SAPK/JNK (Thr183/Tyr185) (1:1000, Cell Signaling, cat. no. 9251), anti-SAPK/JNK (1:1000, Cell Signaling, cat. no. 9252), anti-phospho-c-Jun (Ser63) (Santa Cruz, 1:1000, cat. no. sc-822), anti c-Jun (Santa Cruz, 1:1000, cat. no. sc-74,543), anti-spectrin (1:1000, Chemicon, cat. no. MAB1622), anti-α-tubulin (1:50,000, Sigma-Aldrich, cat. no. T9026), and anti-actin (1:20,000, MP Biomedicals, cat. no. 69100). Membranes were then rinsed three times with Tris-buffered saline Tween-20 (TBS-T) and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After washing for 30 min with TBS-T, membranes were developed using the enhanced chemiluminescence substrate kit (Santa Cruz Biotechnology). Gel-Pro densitometry program (Gel-Pro Analyzer for Windows version 4.0.00.001) was used to quantify the different immunoreactive bands relative to the intensity of the α-tubulin or actin band in the same membranes. Data are expressed as the mean ± SEM of band density obtained in independent experiments or sample.

**BDNF Immunoassay** BDNF quantification was performed using an ELISA as previously described [101], with minor modifications. In brief, 96-well white polystyrene plates (Nunc) were coated with 1 μg of anti-BDNF #1 antibody in 100 μl carbonate buffer (pH 9.7) per well overnight at 25 °C. Plates were blocked with 4% BSA in PBS and washed three times with TBST. Samples and standards (1–1024 pg of recombinant purified BDNF per well) supplemented with 1% BSA and 1% NP-40 were incubated for 3 h at 30 °C together with BDNF #2 antibody coupled to HRP. Plates were washed three times with TBST. SuperSignal ELISA Femto Substrate (Life Technologies), diluted 50% in $H_2O$, was used as the substrate. With these modifications to the assay, we improved its sensitivity to detect 1 pg of BDNF per well. In addition, using a range of standards with different amounts of

recombinant BDNF (1–1024 pg/well), we generated standard curves for each experiment performed, which allowed us to precisely quantify the BDNF present in striatal tissue. The following antibodies were used [101]: mouse BDNF monoclonal (Developmental Studies Hybridoma Bank) for ELISA, BDNF #1 at 1 μg/well for plate coating, and HRP-labeled BDNF #2 at 12 ng/well.

**Dendritic Spine Dying and Confocal Analysis** Neurons were labeled using the Helios Gene Gun System (Bio-Rad) as previously described [102]. Briefly, a suspension containing 3 mg of DiI (Molecular Probes, Invitrogen) dissolved in 100 μl of methylene chloride (Sigma) and mixed with 50 mg of tungsten particles (1.7 mm diameter, Bio-Rad) was spread on a glass slide and air-dried. The mixture was resuspended in 3.5 ml distilled water and sonicated. Subsequently, the mixture was drawn into Tefzel tubing (Bio-Rad) and then removed to let tube dry during 5 min under a nitrogen flow gas. Then, the tube was cut into 13 mm pieces to be used as bio gun's cartridges. Dye-coated particles were delivered in the striatum using the following protocol. Shooting was performed over 150 μm coronal sections at 80 psi through a membrane filter of 3 μm pore size and $8 \times 10$ pores/cm$^2$ (Millipore). Sections were stored at room temperature in PBS for 3 h protected from light, then incubated with DAPI, and finally mounted with Mowiol to be analyzed. DiI-labeled medium spiny neurons of the dorsal striatum were imaged using a Leica Confocal SP5 with a × 63 oil-immersion objective. Conditions such as pinhole size (1 AU) and frame averaging (4 frames/z-step) were held constant throughout the study. Confocal z-stacks were taken with a digital zoom of 5, a z-step of 0.2 μm, and a resolution of 1024 × 1024 pixel, yielding an image with pixel dimensions of 49.25 × 49.25 μm. Z-stacks were deconvolved using the Acoloma plugins from ImageJ to improve voxel resolution and reduce optical aberration along the *z*-axis. Segments from MSN dendrites were selected for the analysis of spine density according the following criteria: (1) segments with no overlap with other branches that would obscure visualization of spines and (2) segments either "parallel to" or "at acute angles" relative to the coronal surface of the section to avoid unambiguous identification of spines. Only spines arising from the lateral surfaces of the dendrites were included in the study, ignoring spines located on the top or bottom of the dendrite surface. Given that spine density increases as a function of the distance from the soma, reaching a plateau 45 μm away from the soma, we selected dendritic segments of basal dendrites 45 μm away from the cell body.

**Immunohistochemistry** For immunohistochemical analysis, mice were deeply anesthetized and immediately perfused transcardially with saline followed by 4% paraformaldehyde/phosphate buffer. The 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.

For EM48 immunohistochemistry, sections were first incubated for 30 min in a 0.01 M sodium citrate buffer (pH 6) preheated to and maintained at 80 °C in a water bath (just for KI and KI:p75$^{+/-}$ animals). Next, endogenous peroxidases were blocked for 45 min in PBS containing 3% $H_2O_2$. Non-specific protein interactions were blocked with 3% normal horse serum. Tissue was incubated overnight at 4 °C with the primary antibody anti-EM48 (1:500, Millipore). Sections were washed three times in PBS and incubated with a biotinylated anti-mouse antibody (1:200; Pierce) at room temperature for 2 h. The immunohistochemical reaction was developed using the ABC kit (Pierce) and made visible with diaminobenzidine. No signal was detected in controls in which the primary antibodies had been omitted. Automated quantification of the number of nuclear huntingtin aggregates and the number and intensity of EM48-stained nuclei within the striatum were performed using CellProfiler v2.8 software. Three coronal striatal sections per animal spaced 240 μm apart were chosen for the analysis. Bright field images from the 100% of the striatum were acquired at × 40 magnification with a BX51 Olympus microscope with the cast system software (Olympus Danmark A/S). The CellProfiler pipeline file containing the specific parameters of the automated quantification is available upon request. In particular, for KI and KI:p75$^{+/-}$ striatal sections, diffuse EM48 immunostaining was quantified as a "staining index" that captures both the nuclear staining intensity and the number of immunostained nuclei, as described previously [103, 104].

For cleaved caspase-3 immunohistochemistry, sections were rinsed two times in PBS, treated with 50 mM NH4Cl for 30 min, rinsed three more times in PBS, and permeabilized and blocked in PBS containing 0.3% Triton X-100 and 10% normal horse serum (Pierce Biotechnology) for 90 min at room temperature. Sections were then washed in PBS and incubated overnight at 4 °C with the primary antibody anti-cleaved caspase-3 (1:250, Cell Signaling). Slices were washed three times and then incubated 2 h shaking at room temperature with the secondary antibody anti-rabbit Cy3 555 (1:100, Jackson Immuno Research). Nuclei were stained with Hoechst 33258 (1:10,000, Molecular Probes, Life Technologies) for 10 min. No signal was detected in controls in which the primary antibodies had been omitted. As positive controls, sections were obtained from animals which had undergone physical lesion in the corticostriatal region causing cell apoptosis. Images were acquired with a BX60 epifluorescence microscope (Olympus) with an Orca-ER cooled CCD camera (Hamamatsu).

**Nissl Staining** Nissl staining was performed in floating sections (30 μm, three sections/mouse) after incubation with cresyl violet solution (0.1 g/L) for 45 min. Images were acquired at × 4 with a BX51 Olympus microscope, and thickness of motor cortex (M1) was measured using ImageJ.

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

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**Authors' Contributions** N.S contributed to the design and carried out the biochemical and immunohistochemical studies, analyzed, interpreted data, and participated in the manuscript draft. A.M contributed to the design and carried out the pharmacological studies in R6/1 mice, analyzed, and interpreted data. S.L.B contributed to the design and carried out the ELISA studies, as well as analyzed and interpreted data. G.G.D.B contributed to the design and carried out the biochemical studies in the R6/1 mice, as well as analyzed and interpreted data. A.G participated in behavioral studies in the KI:$p75^{+/-}$ mice, as well as analyzed and interpreted data. E.A.P carried out immunohistochemistry. J.C.A revised and commented the manuscript. J.A revised and commented the manuscript. S.G contributed to data interpretation, to experimental design, and to the manuscript draft. V.B conceived the study, contributed to the design and carried out behavioral and dendritic spine studies, analyzed and interpreted data, wrote the manuscript, and edited the document. All authors read and approved the final manuscript.

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## Compliance with Ethical Standards

Experimental procedures were approved by the Local Ethical Committee of the University of Barcelona (99/01) and the Generalitat de Catalunya (00/1094), following European (2010/63/UE) and Spanish (RD 1201/2005) regulations for the care and use of laboratory animals.

**Conflict of Interest** The authors declare that they have no conflict of interest.

**Abbreviations** A2AR, adenosine receptor type 2A; BDNF, brain-derived neurotrophic factor; DARPP32, dopamine- and cAMP-regulated phosphoprotein, Mr 32 kDa; HD, Huntington's disease; Htt, huntingtin; JNK, c-Jun kinase; KI, knock-in; PLCγ, phospholipase C gamma; PDE10A, phosphodiesterase 10A; mHtt, mutant huntingtin; MSN, medium spiny neuron; WT, wild type

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# Neurotrophin receptor p75$^{NTR}$ mediates Huntington's disease–associated synaptic and memory dysfunction

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**Learning and memory deficits are early clinical manifestations of Huntington's disease (HD). These cognitive impairments have been mainly associated with frontostriatal HD pathology; however, compelling evidence provided by several HD murine models suggests that the hippocampus may contribute to synaptic deficits and memory dysfunction in HD. The neurotrophin receptor p75$^{NTR}$ negatively regulates spine density, which is associated with learning and memory; therefore, we explored whether disturbed p75$^{NTR}$ function in the hippocampus could contribute to synaptic dysfunction and memory deficits in HD. Here, we determined that levels of p75$^{NTR}$ are markedly increased in the hippocampus of 2 distinct mouse models of HD and in HD patients. Normalization of p75$^{NTR}$ levels in HD mutant mice heterozygous for *p75$^{NTR}$* prevented memory and synaptic plasticity deficits and ameliorated dendritic spine abnormalities, likely through normalization of the activity of the GTPase RhoA. Moreover, viral-mediated overexpression of p75$^{NTR}$ in the hippocampus of WT mice reproduced HD learning and memory deficits, while knockdown of p75$^{NTR}$ in the hippocampus of HD mice prevented cognitive decline. Together, these findings provide evidence of hippocampus-associated memory deficits in HD and demonstrate that p75$^{NTR}$ mediates synaptic, learning, and memory dysfunction in HD.**

## Introduction

Evidence of cognitive deficits including altered acquisition of new motor skills, paired attention, planning, and memory has been demonstrated in Huntington's disease (HD) patients before the onset of motor symptoms (1–3). These clinical signs have been mainly attributed to corticostriatal dysfunction (4, 5). However, in recent years the idea has emerged that memory decline in HD is likely a reflection of a widespread brain circuitry defect and not exclusively a dysfunction of the basal ganglia (6–8). Indeed, besides the caudate and putamen, the volume of the hippocampus is reduced in premanifest HD individuals, while no changes in the amygdala, thalamus, or pallidum are observed (9). Interestingly, spatial and recognition memories have been reported to be altered in HD patients. Thus, in moderately advanced HD patients there is a simultaneous impairment of allocentric (hippocampal-dependent) and egocentric (striatal-dependent) spatial navigation, supporting the idea that beyond the atrophy of the striatum and cortex, a more general neurodegenerative process that involves the hippocampus could contribute to HD memory impairment (2, 10, 11). Actually, cytoplasmic and nuclear huntingtin aggregates within the hippocampus have been described in HD individuals (12). Importantly, such behavioral deficits, together with hippocampal long-term potentiation (LTP) disturbances, have been replicated in different HD mouse models (2, 13–15). Although altered synaptic plasticity and aberrant dendritic spine density and morphology have been proposed as underlying mechanisms (14, 16, 17), little is known about the precise molecular pathways involved in HD synaptic and memory disturbances.

In the adult brain, neurotrophins play a critical role in synaptic plasticity regulation. Among the different neurotrophins, brain-derived neurotrophic factor (BDNF) is the best characterized for its role in regulating LTP and long-term depression (LTD) through binding to TrkB and p75$^{NTR}$ receptors (18). It is generally accepted that BDNF via interaction with TrkB receptors modulates synaptic transmission and plasticity in adult synapses by regulating transcription, translation, and trafficking of distinct synaptic proteins (19, 20). Less is known about the role of p75$^{NTR}$ in synaptic plasticity. Null *p75$^{NTR}$* mice show improved spatial learning and enhanced LTP (21, 22) while impaired NMDA-dependent LTD (23), which points to an antagonistic role of p75$^{NTR}$ in synaptic plasticity. Moreover, it has been demonstrated that p75$^{NTR}$ is a negative modulator of spine-dendrite morphology and complexity (24), likely by regulation of RhoA activity (25). Consistently, while *p75$^{NTR-/-}$* mice exhibit increased hippocampal dendritic spine density, overexpression of p75$^{NTR}$ in hippocampal neurons decreases spine number and branching (24). Interestingly, upregulation of p75$^{NTR}$ levels has been reported in the cortex and hippocampus of Alzheimer's disease (AD) patients (26), while small-molecule p75$^{NTR}$ ligands prevent both cognitive

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ORIGINAL ARTICLE

# A role for Kalirin-7 in corticostriatal synaptic dysfunction in Huntington's disease

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## Abstract

Cognitive dysfunction is an early clinical hallmark of Huntington's disease (HD) preceding the appearance of motor symptoms by several years. Neuronal dysfunction and altered corticostriatal connectivity have been postulated to be fundamental to explain these early disturbances. However, no treatments to attenuate cognitive changes have been successful: the reason may rely on the idea that the temporal sequence of pathological changes is as critical as the changes *per se* when new therapies are in development. To this aim, it becomes critical to use HD mouse models in which cognitive impairments appear prior to motor symptoms. In this study, we demonstrate procedural memory and motor learning deficits in two different HD mice and at ages preceding motor disturbances. These impairments are associated with altered corticostriatal long-term potentiation (LTP) and specific reduction of dendritic spine density and postsynaptic density (PSD)-95 and spinophilin-positive clusters in the cortex of HD mice. As a potential mechanism, we described an early decrease of Kalirin-7 (Kal7), a guanine-nucleotide exchange factor for Rho-like small GTPases critical to maintain excitatory synapse, in the cortex of HD mice. Supporting a role for Kal7 in HD synaptic deficits, exogenous expression of Kal7 restores the reduction of excitatory synapses in HD cortical cultures. Altogether, our results suggest that cortical dysfunction precedes striatal disturbances in HD and underlie early corticostriatal LTP and cognitive defects. Moreover, we identified diminished Kal7 as a key contributor to HD cortical alterations, placing Kal7 as a molecular target for future therapies aimed to restore corticostriatal function in HD.

## Introduction

Basal ganglia dysfunction is a clear hallmark of Huntington's disease (HD) involved in the classical motor disturbances. However, it is patent that HD encompasses more than motor deficits, with evidence of cognitive dysfunction years before chorea symptoms appear. In this view, it has been suggested that functional and morphological changes in key brain areas involved in cognitive processes such as the neocortex could precede alterations in the striatum and be the initial trigger of striatal pathology and late-stage motor symptoms (1–3). Thus, cognitive deficits

[†]The authors wish it to be known that, in their opinion, the second two authors should be regarded as joint Second Authors.

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# Singular Location and Signaling Profile of Adenosine $A_{2A}$-Cannabinoid $CB_1$ Receptor Heteromers in the Dorsal Striatum

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The dorsal striatum is a key node for many neurobiological processes such as motor activity, cognitive functions, and affective processes. The proper functioning of striatal neurons relies critically on metabotropic receptors. Specifically, the main adenosine and endocannabinoid receptors present in the striatum, ie, adenosine $A_{2A}$ receptor ($A_{2A}R$) and cannabinoid $CB_1$ receptor ($CB_1R$), are of pivotal importance in the control of neuronal excitability. Facilitatory and inhibitory functional interactions between striatal $A_{2A}R$ and $CB_1R$ have been reported, and evidence supports that this cross-talk may rely, at least in part, on the formation of $A_{2A}R$-$CB_1R$ heteromeric complexes. However, the specific location and properties of these heteromers have remained largely unknown. Here, by using techniques that allowed a precise visualization of the heteromers *in situ* in combination with sophisticated genetically modified animal models, together with biochemical and pharmacological approaches, we provide a high-resolution expression map and a detailed functional characterization of $A_{2A}R$-$CB_1R$ heteromers in the dorsal striatum. Specifically, our data unveil that the $A_{2A}R$-$CB_1R$ heteromer (i) is essentially absent from corticostriatal projections and striatonigral neurons, and, instead, is largely present in striatopallidal neurons, (ii) displays a striking G protein-coupled signaling profile, where co-stimulation of both receptors leads to strongly reduced downstream signaling, and (iii) undergoes an unprecedented dysfunction in Huntington's disease, an archetypal disease that affects striatal neurons. Altogether, our findings may open a new conceptual framework to understand the role of coordinated adenosine-endocannabinoid signaling in the indirect striatal pathway, which may be relevant in motor function and neurodegenerative diseases.

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## INTRODUCTION

The dorsal striatum is a key node for many neurobiological processes such as motor activity, cognitive functions, and affective processes. The vast majority (~95%) of neurons within the striatum are GABAergic medium spiny neurons (MSNs), which receive glutamatergic inputs primarily from the cortex. MSNs differ in their neurochemical composition and form two major efferent pathways, the direct (striatonigral) pathway and the indirect (striatopallidal) pathway (Kreitzer, 2009). The proper functioning of MSNs relies critically on metabotropic receptor signaling. Many neurotransmitters and neuromodulators such as dopamine,