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Study of mitochondrial dysfunction mechanisms in Huntington's disease striatal degeneration

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STUDY OF MITOCHONDRIAL DYSFUNCTION MECHANISMS IN HUNTINGTON'S DISEASE STRIATAL DEGENERATION

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Programa de Doctorat de Biomedicina

"*Iniziare un nuovo cammino spaventa.*
Ma dopo ogni passo che percorriamo
ci rendiamo conto di come era
pericoloso rimanere fermi. "

Roberto Benigni

A mamma e papà

A Stefano

Alla nonna Rosi

A Álvaro

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RESUMEN

INTRODUCIÓN

La enfermedad de Huntington (EH) es un trastorno neurodegenerativo de herencia autosómica dominante causado por la expansión del trinucleótido CAG en el gen *IT15* que codifica para la proteína huntingtina (htt) (HDCRG, 1993). Los pacientes con EH desarrollan alteraciones neurológicas tales como trastornos psiquiátricos, motores y cognitivos (MacDonald, Gines, Gusella, & Wheeler, 2003; Perez-Navarro, Canals, Gines, & Alberch, 2006). Aunque varias áreas del cerebro muestran signos de neurodegeneración y/o atrofia, el sello neuropatológico más característico de este trastorno es la atrofia del cuerpo estriado (Martin & Gusella, 1986). Si bien se desconoce porque son estas neuronas las principalmente afectadas, distintos estudios apuntan a un exceso de liberación de glutamato desde las aferencias corticales y a una sobre-activación de los receptores NMDA como posible causa de esta selectiva muerte neuronal (Fan & Raymond, 2007; Perez-Navarro et al., 2006). Sin embargo, el estriado también recibe una densa inervación dopaminérgica. Esta evidencia, juntamente con el hecho de que la dopamina juega un papel central en el control de la función motora, sugiere que alteraciones del sistema dopaminérgico podrían contribuir a esta selectiva muerte estriatal (Jakel & Maragos, 2000). Acorde con esta hipótesis, distintos trabajos han sugerido una posible relación entre la vía dopaminérgica, las alteraciones estriatales y la mitocondria en la EH (Benchoua et al., 2008; Brouillet, Jacquard, Bizat, & Blum, 2005). Previamente, nuestro grupo ha demostrado que la htt mutada (mHtt) incrementa la susceptibilidad de las neuronas estriatales a la dopamina vía el receptor D1 de dopamina y mediante una aberrante activación de la proteína Cdk5 (Paoletti et al., 2008), una quinasa que normalmente participa en una amplia gama de funciones neuronales. Incrementos en la actividad de esta quinasa se han asociado a los procesos de muerte neuronal característicos de otras enfermedades neurodegenerativas tales como la enfermedad de Alzheimer o de Parkinson (Cruz & Tsai, 2004; Dhariwala & Rajadhyaksha, 2008). Sin embargo, los mecanismos moleculares por los que la desregulacion de la actividad Cdk5 conduce a muerte neuronal se desconocen. Evidencias recientes han sugerido que una alteración de la señalización de Cdk5 podría contribuir a la neurodegeneración mediante la modificación de los procesos de dinámica mitocondrial (Meuer et al., 2007; Sun, de Pablo, Vincent, & Shah, 2008). Por

todo ello, hipotetizamos que la desregulación de Cdk5 inducida por la huntingtina mutada podría participar en la muerte estriatal en la EH mediante alteraciones de la dinámica mitocondrial. Por otro lado, estudios recientes también han propuesto que una señalización aberrante de calcio (Ca^{2+}) podría estar implicada en la específica vulnerabilidad estriatal que caracteriza la EH. En particular, las neuronas estriatales son extremadamente sensibles a cambios en el Ca^{2+} intracelular y se ha demostrado que la expresión de la mHtt conlleva una desestabilización de la homeostasis del Ca^{2+} citosólico y mitocondrial (Bezprozvanny & Hayden, 2004). La captación de Ca^{2+} por la mitocondria es fundamental para el control de la señalización intracelular y para la regulación del metabolismo y la supervivencia celular (Rizzuto et al., 2012). Diferentes estudios han demostrado que en presencia de mHtt la mitocondria presenta una capacidad de re-captación de Ca^{2+} disminuida lo que podría contribuir a la alteración de la señalización del Ca^{2+} en la EH (Quintanilla, Jin, von Bernhardi, & Johnson, 2013; Ruan, Lesort, MacDonald, & Johnson, 2004) . Sin embargo, una alteración en las interconexiones entre la mitocondria y el retículo endoplasmico (RE) podrían representar otro mecanismo para explicar esta alteración, tal y como ya se ha observado en otra enfermedades neurodegenerativas (Cali, Ottolini, & Brini, 2013). Los puntos de contacto entre RE y mitocondria, definidos también como MAM (membranas mitocondriales asociadas), son componentes cruciales en la homeostasis del Ca^{2+} ya que tras la apertura de los canales de liberación de Ca^{2+} del RE se generan micro dominios de alta concentración de Ca^{2+} que pueden ser captados por la mitocondria (Patergnani et al., 2011). Dado que se ha demostrado que la presencia de la mHtt incrementa la activación del receptor inositol 1,4,5-trifosfato (IP3R) determinando una alteración en la liberación de Ca^{2+} desde el RE y que mHtt puede interactuar directamente con la membrana mitocondrial externa destabilizando la permeabilidad mitocondrial para el Ca^{2+} (Damiano, Galvan, Deglon, & Brouillet, 2010), nos preguntamos si podría también alterar la función de transferencia de Ca^{2+} entre mitocondria y RE afectando los niveles o la actividad de las proteínas implicadas en la formación de estos contactos.

RESULTADOS

Por todo ello, el primer objetivo de la Tesis ha sido determinar los mecanismos moleculares por los que Cdk5 incrementa la vulnerabilidad estriatal en modelos knock-in de la EH. En particular, (1) examinamos la morfología y la dinámica mitocondrial en modelos celulares y murinos de la EH, (2) analizamos cambios en los niveles de las proteínas implicadas en los procesos de fisión y fusión mitocondrial en modelos celulares y murinos de la EH y (3) estudiamos si la activación aberrante de Cdk5 inducida por la huntingtina mutada incrementa la susceptibilidad estriatal a activadores de receptores de dopamina D1 vía disfunción mitocondrial en modelos celulares y murinos de la EH. Para el análisis de la dinámica mitocondrial realizamos experimentos de inmunocitoquímica utilizando el anticuerpo Tom20 para marcar las mitocondrias de células estriatales wild-type (ST7/7Q) y knock-in (ST111/111Q) y de cultivos primarios estriatales de ratones huntingtonianos Hdh7/111Q y ratones control Hdh7/7Q en condiciones basales y después de activación dopaminérgica (tratamiento con el agonista del receptor D1, SKF-38393) y analizamos la población mitocondrial (distribución y procesos de fragmentación y/o fusión) mediante microscopía confocal y análisis de imágen mediante el programa ImageJ Software. Nuestros datos mostraron que la sola presencia de la mHtt inducía fragmentación mitocondrial. El análisis del Aspect Ratio (AR), un parámetro que describe el tamaño de los orgánulos, y del Form Factor (FF), un parámetro que describe la complejidad de la red de distribución mitocondrial, mostraban respectivamente una disminución en la longitud de las mitocondria y una reducción en la ramificación. Estas alteraciones concordaban con el aumento del número de mitocondrias por célula observado en las células ST111/111Q. Además, analizando el porcentaje de células que presentaban fragmentación mitocondrial constatamos que más del 40% de las células ST111/111Q estaban caracterizadas por una fisión mitocondrial incrementada. Analizamos, también, la población mitocondrial en cultivos primarios estriatales derivados de ratones huntingtonianos Hdh7/111Q y observamos la misma alteración en la dinámica mitocondrial. A continuación, analizamos el impacto de la activación del receptor D1 en la población mitocondrial. El tratamiento con el agonista D1R, SKF-38393, determinó una disminución significativa en el tamaño mitocondrial y una alteración en la distribución y formación de la red

mitocondrial tanto en células control como knock-in. La activación dopaminérgica produjo también un aumento en el número de orgánulos por célula en comparación con la condición control (tratamiento con vehículo). Por último, cuando analizamos la proporción de células con fragmentación mitocondrial observamos un incremento de la fisión mitocondrial significativamente mayor (~ 30% más) en las células que expresan la huntingtina mutada respecto a las wild-type. Nuestros datos indican que el efecto tóxico generado por estimulación de la vía dopaminérgica podría estar mediado por una alteración en la distribución y morfología de la población mitocondrial. Para relacionar la alteración de la morfología mitocondrial con la modulación de proteínas de fisión/fusión mitocondrial, analizamos en células estriatales wild-type y knock-in en condiciones basales y después de la activación dopaminérgica los niveles y distribución de las proteínas Opa1 (fusión) y Drp1 (fisión) en extractos totales y fracciones subcelulares (citosol/mitocondrial) mediante análisis por Western blot. No detectamos diferencias significativas entre genotipos en los niveles totales de Opa1 (fusión) pero en cambio, a nivel de la fracción mitocondrial se observó que las células mutadas presentaban niveles significativamente más bajos respecto a las células wild-type. Sorprendentemente, también observamos que las células que expresan la huntingtina mutada presentan niveles de Drp1 disminuidos respecto a las células wild-type y esta disminución es debida a una menor proporción de la proteína en la fracción mitocondrial. Para determinar si esta disminución es debida a una alteración de la transcripción génica, evaluamos la expresión de Drp1 por RT-PCR. Detectamos una disminución estadísticamente significativa en los niveles de Drp1 en las células mutadas STQ111/Q111 respeto a las wild-type, sugiriendo la implicación de la mHtt en la desregulación transcripcional de Drp1. Además, analizamos mediante un ensayo colorimétrico la actividad GTPasa de Drp1 ya que se ha descrito que la mHtt interactúa de manera aberrante con Drp1 alterando su estructura y aumentando su actividad enzimática (W. Song et al., 2011). A pesar de los bajos niveles de expresión de Drp1 presentes en las células mutadas, se observó un aumento significativo en la actividad enzimática de Drp1 sugiriendo que el aumento de la fragmentación mitocondrial en las células mutadas ST111/111Q podría estar relacionado con una mayor actividad de la proteína de fisión Drp1. Cuando examinamos la

distribución de Drp1 y Opa1 después del tratamiento con SKF-38393, vimos que la activación dopaminérgica no afectaba de manera significativa los niveles de Opa1 pero inducía un incremento de los niveles de Drp1. Además este aumento era distinto cuando se comparaban los niveles de esta proteína en las fracciones citosólicas y mitocondriales de células estriatales wild-type y mutadas. Así, mientras en las células wild-type el incremento de Drp1 era principalmente citosólico en las células mutadas era en la fracción mitocondrial. Seguidamente, investigamos si el estímulo dopaminérgico también podía afectar a la actividad enzimática de Drp1. Mientras en las células wild-type tratadas con SKF-38393 observamos una tendencia creciente, aunque no significativa, en la actividad de Drp1, en las células mutadas ST111/111Q el aumento fue mayor y significativo, sugiriendo que la activación de receptores D1 exacerba los eventos de fisión mitocondrial dependientes de Drp1 en presencia de la mHtt. Para determinar el papel de Cdk5 en la modulación de los procesos de dinámica mitocondrial, tratamos las células estriatales con el inhibidor de Cdk5 roscovitine y analizamos el efecto por microscopia confocal. El tratamiento con roscovitine mejoraba significativamente los defectos de la red tubular mitocondrial y reducía la fragmentación mitocondrial en las células mutadas ST111/111Q, lo que sugiere un papel importante de Cdk5 en la disfunción mitocondrial observada en las células estriatales que expresan la mHtt. Puesto que hemos demostrado una mayor actividad Drp1 en las células mutadas ST111/111Q, abordamos si la mejora en la fragmentación mitocondrial tras el tratamiento con roscovitine se asociaba a una reducción en la actividad Drp1. Efectivamente, la inhibición farmacológica de Cdk5 redujo significativamente la actividad de Drp1 en las células mutadas sin ningún efecto en las wild-type. Sorprendentemente, vimos que además de la actividad, Cdk5 también modulaba la transcripción del gen para Drp1. Es decir, observamos una disminución significativa en la expresión de Drp1 en ambos genotipos cuando la expresión de Cdk5 también se encontraba reducida, revelando un papel crítico para Cdk5 como regulador transcripcional de Drp1. Para investigar si Cdk5 también es mediador de la disfunción mitocondrial inducida por la activación dopaminérgica, tratamos las células estriatales con el inhibidor de Cdk5 roscovitine antes y durante la activación del receptor D1 de dopamina y analizamos el efecto por microscopía confocal. El tratamiento con roscovitine

rescató de las alteraciones en la distribución de la red mitocondrial expresada como alteración del FF tanto en células wild-type como en células mutadas. Además, la inhibición de la actividad de Cdk5 también bloqueó significativamente la fragmentación mitocondrial inducida por SKF-38393 en ambos tipos celulares. Estos datos, en conjunto, sugieren que Cdk5 participa en la aberrante dinámica mitocondrial inducida por la activación dopaminérgica. Para acabar de confirmar esta hipótesis, transfectamos células wild-type y mutadas con un siRNA específico para la quinasa Cdk5 con el fin de disminuir sus niveles de expresión. Los resultados del silenciamiento de Cdk5, mostrando que la reducción de los niveles de Cdk5 previene la disfunción mitocondrial observada tras el tratamiento con SKF-38393, confirmaron la implicación de la quinasa Cdk5 en la regulación de los procesos de fisión. Para determinar el mecanismo mediante el cual una aberrante activación de Cdk5 altera la fragmentación mitocondrial, analizamos si la inhibición de la quinasa rescataba el equilibrio en la dinámica mitocondrial modulando los niveles de la proteína de fisión Drp1. Realizamos análisis de Western blot de lisados celulares transfectados con siRNA para Cdk5 y tratados con SKF-38393 y vimos que cuando los niveles de Cdk5 están disminuidos, la activación del receptor D1 no modifica los niveles del regulador mitocondrial Drp1. Este resultado, nos indica que tras una estimulación dopaminergica, el aumento en la actividad de Cdk5 podría ser responsable del incremento en la expresión de Drp1. Puesto que previamente habíamos demostrado que la activación del receptor D1 induce un aumento de Drp1 específicamente en la fracción mitocondrial de las células mutadas, evaluamos si la actividad aberrante de Cdk5 podría ser responsable de esta translocación mitocondrial de Drp1. De acuerdo con esta hipótesis, observamos que la acumulación mitocondrial de Drp1 en la células mutadas ST111/111Q no se producía cuando Cdk5 estaba inhibida con roscovitine lo que indica que Cdk5 es mediador de la fragmentación mitocondrial inducida por la activación del receptor D1 mediante la modulación de los niveles y de la distribución de la proteína Drp1.

Nuestro segundo objetivo consistió en Investigar el papel de las MAM en la alteración de la señalización del Ca^{2+} que caracteriza la EH. En particular, (1) examinamos los sitios de contacto entre mitocondria y RE en neuronas estriatales de modelos murinos de la EH, (2) analizamos cambios en los

niveles de las proteínas implicadas en la formación de las MAM y en la homeostasis del Ca^{2+} y (3) examinamos el transporte de Ca^{2+} entre mitocondria y RE en neuronas estriatales de modelos murinos de la EH. Para investigar el papel de los contactos entre RE y mitocondria en la desregulación de la homeostasis del calcio en la EH, empezamos realizando análisis de co-localización entre los dos orgánulos en cultivos estriatales de ratones R6/1 y HdhQ111, modelos de la EH. El ratón R6/1 es un modelo transgénico de la EH que expresa el exón-1 de la huntingtina humana con 145 repeticiones CAG y que presenta un fenotipo más agresivo de la enfermedad respecto al modelo knock-in. Primero, co-transfectamos los cultivos estriatales con GFP-Sec61β, para marcar el retículo, y con pDsRed2-Mito, para marcar las mitocondria y sucesivamente analizamos los contactos mediante microscopía confocal y análisis de imágen mediante el programa ImageJ Software. Nuestros datos indican una significativa reducción en la co-localización entre los dos orgánulos en los cultivos estriatales de los dos modelos huntingtonianos, sugiriendo una posible ruptura de las membranas asociadas y una alteración de los mecanismos regulados por estos contactos. Para investigar si la desregulación de la homeostasis del calcio en la EH es debida a una alteración en el transporte del calcio entre retículo endoplasmico y mitocondria, analizamos las proteínas que están implicadas en este proceso y que constituyen los puentes moleculares que regulan los contactos entre los orgánulos. En particular, analizamos mediante Western blot en tejido estriatal de ratones modelos de la EH, los niveles del receptor inositol 1,4,5-trifosfato de tipo 3 (IP3R3), el principal transmisor de Ca^{2+} entre RE y mitocondria (Raturi & Simmen, 2013), y de Grp75, una chaperona mitocondrial que regula la señalización del Ca^{2+} y permite el acoplamiento físico entre los dos orgánulos (Szabadkai, Bianchi, et al., 2006). Nuestros resultados muestran una significativa disminución de los niveles de IP3R3 y Grp75 en estriado de ratones huntingtonianos R6/1 a partir de las 12 semanas, una edad caracterizada por déficits cognitivos. La disminución de IP3R3, se mantiene hasta las 30 semanas de edad, lo que indica una alteración que afecta hasta estadios avanzados de la enfermedad. En cambio, Grp75 solo se encuentra reducida a las 12 y a las 20 semanas, mientras que a las 30 semanas no se observan cambios de sus niveles en los ratones R6/1 respeto a los wild-type. Analizamos también los niveles en estriados de ratones wild-type Hdh7/7Q y

huntingtonianos Hdh111/111Q y observamos una disminución de IP3R3 a los 9 y 13 meses en los Hdh111/111Q. En estas edades los ratones Hdh^{Q111} presentan déficits cognitivos y motores. Cuando analizamos los niveles de Grp75 observamos una reducción en el estriado de los ratones Hdh111/111Q a los 9 meses de edad, mientras que a edades más tardías encontramos una recuperación de la proteína, observándose niveles parecidos a los de WT, tal y como habíamos observado en los ratones R6/1. Por último, quisimos comprobar si estas alteraciones se encontraban en tejido post-mortem de pacientes de la EH. Los resultados del análisis por Western blot mostraron una reducción en las dos proteínas en putamen derivado de pacientes con EH respecto a individuos control. Sin embargo, la disminución de IP3R3 no resultó estadísticamente significativa. En conjunto, nuestros datos indican que en presencia de la mHtt se genera una alteración en los niveles de proteínas cruciales para el mantenimiento de la homeostasis del Ca^{2+} y que este trastorno involucra estadios medianos y avanzados de la EH. Basándonos en la disminución de las proteínas IP3R3 y Grp75 y la reducción de los contactos entre RE y mitocondria, nos preguntamos si la perturbación de la homeostasis del Ca^{2+} en la enfermedad de Huntington podría ser debida a una alteración del intercambio de calcio entre RE y mitocondria. Para ello, examinamos los niveles de Ca^{2+} intracelular y del potencial de membrana mitocondrial antes y después de la inducción de la liberación de Ca^{2+} acumulado en los depósitos del RE. Para este experimento, incubamos cultivos primarios estriatales de ratones R6/1 con el marcador de Ca^{2+} citosolico, Fluo4, y con el marcador de potencial de membrana mitocondrial, TMRM, y estimulamos la salida de Ca^{2+} desde el retículo tratando con Thapsigargin (TG), un inhibidor de la ATPasa SERCA que incrementa la concentración de Ca^{2+} intracelular bloqueando su almacenamiento en el RE. Analizando los picos de Ca^{2+} creados por el tratamiento con TG, observamos que las neuronas estriatales de los cultivos R6/1 muestran un incremento de Ca^{2+} intracelular mayor respeto a las neuronas wild-type. Este fenómeno podría ser debido a una menor entrada de Ca^{2+} en la mitocondria, como indica la menor reducción del potencial de membrana de los cultivos R6/1 respecto a los wild-type obtenidos de las curvas de fluorescencia con TMRM. Para comprobar si realmente había una reducción en la captación de Ca^{2+} por parte de las mitocondrias en los cultivos R6/1, tratamos estos cultivos con

carbonilcianuro-p-trifluorometoxi-hidrazona (FCCP), un agente desacoplador de la fosforilación oxidativa de la cadena respiratoria que despolariza la membrana mitocondrial determinando la salida del Ca^{2+} al citosol. Nuestros resultados confirman la disminución en la captación del Ca^{2+} mitocondrial en las neuronas estriatales R6/1, ya que el tratamiento con FCCP provoca una salida de Ca^{2+} inferior respecto al de las mitocondria de los cultivos wild-type. Por lo tanto, nuestro datos demuestran una alteración del intercambio de Ca^{2+} entre RE y mitocondria en las neuronas estriatales de los ratones R6/1.

DISCUSIÓN Y CONCLUSIONES

Estudios previos de nuestro grupo de investigación han demostrado que el aumento de la vulnerabilidad de las células estriatales a la huntingtina mutada tras activación dopaminérgica implica la actividad aberrante de Cdk5 (Paoletti et al., 2008). Por otra parte, estudios recientes han involucrado también Cdk5 en la regulación de la fisión mitocondrial (Meuer et al., 2007). En este escenario hemos planteado la hipótesis de que la desregulación de Cdk5 inducida por la huntingtina mutada podría contribuir a la patología estriatal en la EH. Por ello el primer objetivo del presente proyecto de Tesis ha sido definir si el aumento de la actividad de Cdk5 inducida por la huntingtina mutada incrementa la vulnerabilidad estriatal a insultos excitotóxicos mediante la alteración de la dinámica mitocondrial. Nuestros datos revelan que Cdk5 es un mediador clave en la mayor vulnerabilidad de las células estriatales a la activación dopaminérgica induciendo fragmentación mitocondrial. La activación del receptor de dopamina D1 en presencia del agonista dopaminérgico SKF-38393, incrementa significativamente la fisión mitocondrial de las células estriatales que expresan huntingtina mutada. Este aumento está mediado por la actividad alterada de Cdk5 ya que la inhibición farmacológica de la quinasa con roscovitine o genéticamente mediante silenciamiento con siRNA previene la fragmentación aberrante y las alteraciones en la distribución de la red mitocondrial. Además nuestros datos demuestran que las proteínas de fisión y fusión Drp1 y Opa1, se expresan y se distribuyen diferencialmente en las células mutadas respecto a las células wild-type lo que podría explicar las alteraciones mitocondriales que se presentan ya en condiciones basales. La fragmentación aberrante que se observa después de la activación dopaminérgica podría ser debida a los

cambios significativos en los niveles y distribución de la proteína de fisión Drp1. El incremento de Drp1 y su localización a nivel de la mitocondria podría determinar la fragmentación aberrante que observamos en la células huntingtonianas. Nuestros datos también nos indican que Cdk5 tiene un papel crucial en la modulación de la actividad de Drp1 ya que la inhibición farmacológica de la quinasa impide el incremento de la actividad GTPasa de Drp1. Hipotetizamos además, que Cdk5 podría estar involucrada en la modulación de la expresión de Drp1 o en su reclutamiento en la membrana de la mitocondria que llevaría a iniciar el proceso de fisión ya que nuestros datos demuestran que la inhibición de Cdk5 impide el aumento transcripcional de Drp1y su translocación a la mitocondria tras la activación del receptor D1. En conjunto nuestros resultados avalan nuestra primera hipótesis según la cual Cdk5 juega un papel clave en la degeneración y atrofia estriatal propios de la enfermedad de Huntington mediante la modulación de los procesos de la dinámica mitocondrial. Recientemente se ha descrito que la regulación de la morfología y de la distribución mitocondrial es un mecanismo fundamental para garantizar el correcto funcionamiento de procesos de supervivencia celular como la homeostasis del calcio (Szabadkai, Simoni, et al., 2006). Un excesiva fragmentación mitocondrial podría alterar la dispersión de los orgánulos en el espacio intracelular alterando su capacidad de captación del Ca^{2+} y la interacción con otras membranas, tales como las del retículo endoplásmico. Estudios recientes, identifican una defectuosa captación del calcio mitocondrial como otro mecanismo responsable de la patogénesis y progresión de la enfermedad de Huntington (Giacomello, Hudec, & Lopreiato, 2011). La mayoría de estos estudios, muestran que en presencia de la mHtt las mitocondrias se despolarizan y son más sensibles al acúmulo de Ca^{2+} y esto se explicaría por un aumento de la susceptibilidad de la permeabilidad mitocondrial que a su vez conduciría a una mayor propensión a la muerte neuronal en el cuerpo estriado (Panov et al., 2002; Zhang, Tang, & Xu, 2003). Sin embargo, la aberrante fragmentación mitocondrial que caracteriza la EH y que hemos descrito y demostrado en nuestro primer objetivo de esta tesis, nos sugiere que la reducción en los contactos entre las membranas mitocondriales y del RE podría jugar un papel relevante en la alteración de la homeostasis del Ca^{2+}. En efecto, nuestros últimos datos avalan nuestra hipótesis ya que el análisis de co-localización en neuronas que expresan mHtt

nos reveló una reducción significativa de los contactos entre los dos orgánulos. Además, esta ruptura de las membranas asociadas explicaría los resultados de calcio obtenidos en los experimentos de modulación con TG y FCCP. Las neuronas que expresan el exón-1 de la htt mutada muestran una alteración en el intercambio de Ca^{2+} que impide la correcta captación de calcio por parte de la mitocondria determinando así una excesiva liberación citosólica desde las reservas del retículo. La alteración de la dinámica mitocondrial podría no ser la única responsable de la reducción de los puntos de contactos entre RE y mitocondria. Analizando los niveles de proteínas involucradas en la formación de estos puentes moleculares, encontramos una reducción significativa de IP3R3 y Grp75 en tejido estriatal de ratones huntingtonianos a edades que corresponden a estadios medios y avanzados de la EH. En particular, la disminución de IP3R3 podría indicar una desregulación en el trasporte de Ca^{2+} a la mitocondria mientras que una reducción de Grp75 impediría el acoplamiento conformacional entre el receptor y la maquinaria de captación mitocondrial. En conjunto, los resultados de este segundo objetivo, identifican a las MAM como otro importante mecanismo de control de la señalización del Ca^{2+} responsable de la progresión de la degeneración estriatal en la EH.

ABBREVIATIONS

3-NP	3-nitropropionic acid
AD	Alzheimer's disease
ADP	Adenosine diphosphate
ALS	Amyotrophic lateral sclerosis
AR	Aspect Ratio
ATP	Adenosine triphosphate
Aβ	Beta amyloid
BAC	Bacterial Artificial Chromosome
Bcl-2	B-cell lymphoma 2
BiP	Binding immunoglobulin Protein
Ca^{2+}	calcium
Ca_i^{2+}	intracellular calcium
CaMKIα	calcium/calmodulin-dependent protein kinase I alpha
Cdks	Cyclin-dependent kinases
CsA	Cyclosporin A
D1R	Dopamine 1 receptor
D2R	Dopamine 2 receptor
DA	Dopamine
DNA	Deoxyribonucleic acid
Drp1	Dynamine related protein 1
ER	Endoplasmic reticulum
Ero1α	Oxidoreductase 1 alpha
ERp44	Endoplasmic reticulum resident protein 44
FAD	Flavin adenine dinucleotide
FCCP	Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone
FF	Form Factor
Fis1	Mitochondrial fission 1 protein
GABA	gamma-Aminobutyric acid
GED	GTPase effector domain
GFP	Green fluorescent protein
GPe	External globus pallidus
GPi	Internal globus pallidus
Grp75	Glucose-regulated protein 75
GTP	Guanosine triphosphate
H^+	Hydrogen

ABBREVIATIONS

HAP1	Huntingtin-associated protein 1
HD	Huntington's disease
HIP1	Huntingtin-interacting protein 1
HR	Heptads repeat
Htt	Huntingtin
IEGs	Immediate early genes
IMM	Mitochondrial inner membrane
IMS	Intermembrane space
INSP3R	Inositol (1,4,5)-trisphosphate receptor
Kd	Dissociation constant
LRRK2	Leucine-rich repeat kinase 2
MAM	Mitochondria-associated membrane
MCU	Mitochondrial calcium uniporter
MEF2	Myocyte enhancer factor-2
Mfns	Mitofusins
mHtt	Mutant huntingtin
MIS	Mitochondrial import sequence
MSNs	Medium spiny neurons
mtDNA	Mitochondrial DNA
Na^+	Sodium
NMDA	N-Methyl-D-aspartic acid
OMM	Mitochondrial outer membrane
OXPHOS	Oxidative phosphorylation
PD	Parkinson's disease
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PH	Pleckstrin homology domain
Pi	Inorganic phosphorous
PINK1	PTEN-induced putative kinase 1
PKA	Protein kinase A
Pro	Proline
PSs	Presenilins
PTP	Permeability transition pore
PTPIP51	Protein tyrosine phosphatase-interacting protein 51
RCAN1-1L	Regulator Of Calcineurin 1

RNA	Ribonucleic acid
ROS	Reactive oxygen species
Ser	Serine
Sig-1R	Sigma 1 receptor
SiRNA	Small interfering RNA
SNc	Substancia nigra pars compacta
SNr	Substancia nigra pars reticulata
SOD1	Superoxide dismutase 1
STN	Subthalamic nucleus
TG	Thapsigargin
Thr	Threonine
TM	Transmembrane segment
TMRM	Tetramethylrhodamine methyl ester
TPR	Tetratricopeptide-like motifs
Tyr	Tyrosine
VAPB	Vesicle-associated membrane protein B
VDAC	Voltage-dependent anion channels
YAC	Yeast artificial chromosome
α-syn	Alpha-synuclein
$\Delta\Psi$	Membrane potential
$\Delta\Psi_m$	Mitochondrial membrane potential

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INTRODUCTION

Mitochondria mediate numerous processes in neurons from biogenesis to cell death and defects in mitochondrial function are a feature of almost all neurodegenerative diseases. Substantial evidence suggests that mitochondrial dysfunction plays a crucial role in Huntington's disease (HD) striatal degeneration. However, the question as to how mitochondrial defects could be involved in the region specific pattern of HD degeneration remains unclear. In this Thesis, we aim to characterize plausible mechanisms that may deregulate mitochondrial homeostasis and determine the selective striatal vulnerability in HD.

1. ROLE OF MITOCHONDRIA IN NEURONS

The adult human brain uses more energy than any other human organ, accounting for up to 20 percent of the body's total haul (Raichle & Gusnard, 2002). It normally relies almost exclusively on the metabolism of glucose to maintain its high metabolic rate and it owns small reserves of energy that have to be regularly supplied. Thus, there is a critical balance between a constant provide of nutrients and energy demands. Mitochondria sustain most of the energy for neurons through oxidative phosphorylation (OXPHOS) and constitute the site of many important biochemical pathways that are essential for neuronal functions (Chan, 2006a). Energized mitochondria, can synthesize ATP that fuels energy-dependent intracellular reactions, such as ion transport and neurotransmitter biosynthesis, and preserve critical mitochondrial function contributing to intracellular signalling (Fig. 1). Moreover, mitochondria dynamically undergo shape changes through regulated processes of fission and fusion and actively traffic between cell compartments (Frederick & Shaw, 2007; Hoppins, Lackner, & Nunnari, 2007). This is particularly important in specialized cells such as neurons. First, neurons present axons and multiple dendrites that extend away from the cell body and have distinct energy requiring functions (Z. Li, Okamoto, Hayashi, & Sheng, 2004). Second, the firing of a neuron is critically regulated by ionic flow while the maintaining of calcium (Ca^{2+}) homeostasis is essential in areas enriched with active channels such as the synapses. Mitochondria are capable of having an important effect on these processes because they can sequester large quantities of calcium reducing cytosolic Ca^{2+} overload (Duchen, 2000), are

present at high concentrations in presynaptic terminals and can be tethered to vesicle release sites (Gleichmann & Mattson, 2011; Nicholls & Budd, 2000). Thus, mitochondrial dynamics and Ca^{2+} buffering appear to play a crucial role in providing local energy and maintaining the functional heterogeneity in the complex morphology of the neurons.

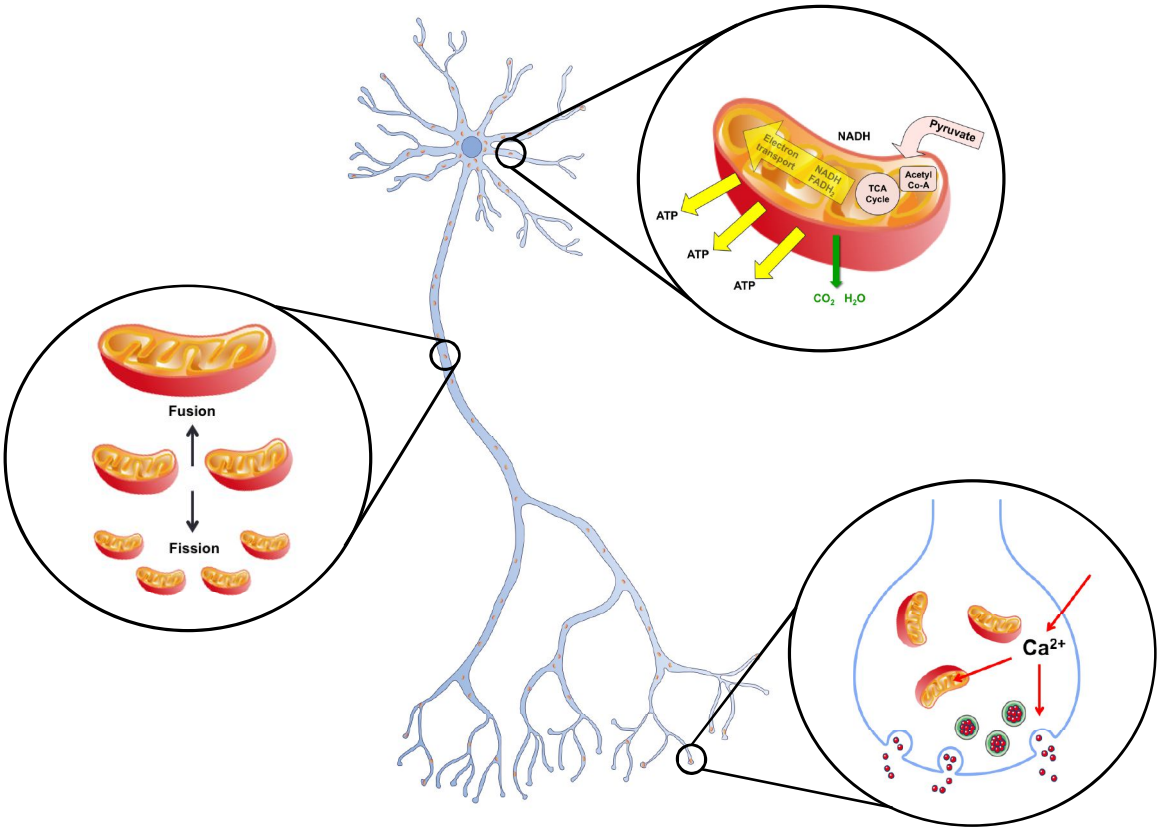

Figure 1. Role of mitochondria in neurons. Mitochondria travel long distances from the cell body to dendritic and axonal processes. Mitochondria are necessary for proper energy production trough respiratory chain process, and especially at the nerve terminal, they are important for calcium buffering and cycling of reserve pool synaptic vesicles. In addition, they dynamically undergo shape changes. Adapted from (Chan, 2006a).

1.1 Mitochondrial dynamics

Once thought to be solitary and rigidly structured, mitochondria are now appreciated to constitute a population of organelles that migrate throughout the cell, fuse and divide, and undergo regulated turnover (Chan, 2006b). Depending on physiological conditions and on the cell type, they may acquire different morphologies and, even in the same cell, mitochondria can form

giant tubule networks leading to a rapid exchange of mitochondrial content or divide into individual rod-like structures and allow deep penetration into slight neurites (Chan, 2006a). This morphological plasticity of mitochondria results from the equilibrium between fusion and fission events. Fusion and fission of mitochondria are important for many biological functions (Westermann, 2010). Division is required for inheritance and partitioning of organelles during cell division, for the release of pro-apoptotic factors from the mitochondrial intermembrane space (IMS), for intracellular distribution by cytoskeleton-mediated transport and for the turnover of damaged organelles by mitophagy. Fused mitochondrial networks are important for the dissipation of metabolic energy through the transmission of membrane potential along mitochondrial filaments and for the complementation of mitochondrial DNA (mtDNA) gene products. Normally, the frequencies of these two processes are coordinated to maintain the overall morphology of the mitochondrial population. When this balance is perturbed, dramatic transitions in mitochondrial shape can occur. Genetic studies in yeast and mammals indicate that cells with a high fusion-to-fission ratio have few mitochondria, and that these mitochondria are long and highly interconnected (Bleazard et al., 1999; H. Chen et al., 2003; Sesaki & Jensen, 1999). Conversely, cells with a low fusion-to-fission ratio have numerous mitochondria that are small spheres or short rods and these are often referred as "fragmented mitochondria". The molecular machineries behind mitochondrial fusion and fission events are relatively well known and the dynamic control of mitochondrial structure is performed by a growing set of "mitochondria-shaping" proteins that include both pro-fusion and pro-fission members (Liesa et al., 2009).

1.1.1 Machinery of mitochondrial fusion

Mitochondrial fusion provides a mechanism by which the organelle population is maintained homogeneously and facilitates inter-complementation of mtDNA (H. Chen, Chomyn, & Chan, 2005). Mitochondria consist of four distinct compartments: the mitochondrial outer (OMM) and inner (IMM) membranes serve as barriers for the maintenance and integrity of two soluble compartments, the IMS and mitochondrial matrix. The outer

membrane is permeable for ions and small molecules while the inner membrane is almost impermeable, thus allowing the formation of an electron gradient that is critical for mitochondrial function. Because mitochondria have double membranes, mitochondrial fusion is necessarily a well-coordinated and multistep process, where the outer and inner mitochondria membranes fuse by separable events (Fig. 2a) (Meeusen et al., 2006; Meeusen, McCaffery, & Nunnari, 2004; Sesaki, Southard, Yaffe, & Jensen, 2003; Z. Song, Ghochani, McCaffery, Frey, & Chan, 2009). This process can be explained by the finding that the most relevant proteins involved in mammalian mitochondrial fusion show distinct mitochondrial sublocalization. The mitofusins Mfn1 and Mfn2 are transmembrane proteins embedded in the OMM which function depends on GTPase activity (Rojo, Legros, Chateau, & Lombes, 2002; Santel & Fuller, 2001). In addition to the GTPase domain in the N-terminus, mitofusins structurally harbor a bipartite transmembrane domain and two heptads repeat (HR) coiled-coil domains in the middle and C-terminal regions (Fig.2b). This HR domain mediates the first step of mitochondrial fusion, namely, the tethering of the outer membranes of two adjacent mitochondria through a dimeric antiparallel coiled-coil structure (Koshiba et al., 2004). Diversely, Opa1 is localized in the IMS in soluble forms or tightly attached to the IMM, and is responsible for the inner membrane fusion (Alexander et al., 2000). On the basis of sequence analysis, Opa1 is a dynamin-related GTPase (Fig.2b). Consistent with this designation, recombinant Opa1 can interact with membranes and deform their structure. Opa1 has a low intrinsic rate of GTP hydrolysis that is enhanced by assembly into a higher-order structure. It contains an NH2-terminal mitochondrial import sequence (MIS; in the first 150 residues), characterized by enrichment in positively charged amino acids, which confers the mitochondrial localization of Opa1. The protein also contains a GTPase domain that is crucial for activity and a nearby middle domain, which is involved in tetramerization and higher order assembly of Opa1 (Ramachandran et al., 2007). The C-terminus harbors a coiled-coil region, which is also involved in oligomerization and activation of Opa1 (Praefcke & McMahon, 2004).

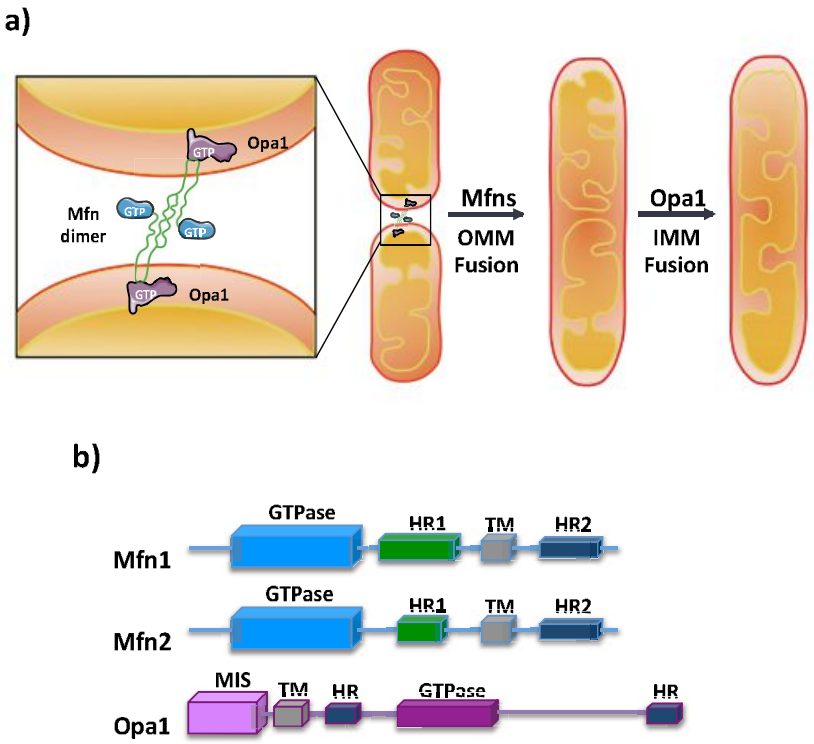

Figure 2. Molecules involved in mitochondrial fusion. a) Mitofusins and Opa1 act at sequential steps of mitochondrial fusion. Outer membrane (OMM) fusion requires mitofusins, whereas inner membrane (IMM) fusion requires Opa1. b) Schematic of the three central molecules, all large GTPases, necessary for mitochondrial fusion. Abbreviations: HR, heptad repeat; MIS, mitochondrial import sequence; TM, transmembrane segment. Adapted from (Chan, 2012).

Because of differential RNA splicing (Delettre et al., 2001) and protein processing, Opa1 is processed into 8 different isoforms and changes in the balance between long (L) and short (S) isoforms affects the fusion of mitochondria (Ishihara, Fujita, Oka, & Mihara, 2006). Under normal conditions, a combination of both long and short isoforms is required for membrane fusion. How the long and short isoforms of Opa1 work together to mediate fusion remains unknown. However, it has been described that increased processing of Opa1 leading to greater levels of soluble S-Opa1 isoforms occurs following loss of the mitochondrial membrane potential and/or the induction of apoptosis, resulting in extensive mitochondrial fragmentation and disruption of cristae. This fragmentation can be rescued by expression of L-Opa1 isoforms (Duvezin-Caubet et al., 2006; Z. Song, Chen, Fiket, Alexander, & Chan, 2007).

1.1.2 Machinery of mitochondrial fission

Genetic and cell biology studies have identified several molecules that are necessary for the fission of mitochondria. The central player appears to be Drp1, an evolutionarily conserved dynamin-related protein, member of the dynamin family of GTPases (Kageyama, Zhang, & Sesaki, 2011). Drp1 was found to be highly expressed in brain, skeletal muscle, and heart. However, the brain expresses a specific Drp1 isoform, generated by alternative splicing, that is absent or not detected in other tissues (Smirnova, Shurland, Ryazantsev, & van der Bliek, 1998; Yoon, Pitts, Dahan, & McNiven, 1998). The canonical dynamin domains present in human Drp1 (isoform 3, 699 residues) are the GTPase domain (residues 1-300), the middle domain (residues 301-508), and the GED domain (residues 599-699) (Fig.3a) (Smirnova et al., 1998). The middle and GED domains are known as "assembly domains" because they are important for dynamin dimerisation and further oligomerisation. They can both interact with the GTPase domains and the GED can also interact with itself. Some of these interactions will be intra- and some inter-molecular, therefore some will be important for dimerisation while others for further oligomerisation (Zhu et al., 2004). Analogous to the role of dynamin in endocytic vesicle fission, Drp1 assembles into spiral filaments on mitochondrial tubules and mediates constriction and scission through conformational changes, driven by GTP hydrolysis (Fig. 3b). The Pleckstrin homology domain (PH), a lipid-binding structure, mediates the interaction of dynamin with membranes. Nucleotide-dependent conformational changes cause rearrangement of the lipids within the membrane bilayer, leading to a hemifission intermediate followed by full scission (Schmid & Frolov, 2011). Unlike dynamin, Drp1 does not have an obvious PH domain. The GED domain of Drp1, in addition to being crucial for the regulation of GTPase activity and for the molecular associations, contains the information necessary for the mitochondrial targeting of the protein (Pitts, McNiven, & Yoon, 2004). Indeed, the localization of Drp1 is mainly cytosolic, and therefore receptor-like molecules on the mitochondrial membrane are necessary to efficiently recruit Drp1 for fission. Different outer-membrane proteins have been proposed as Drp1-receptor, including Fis1, Mff and the two homologous proteins Mid49 and Mid51 (Fig.3a) (Gandre-Babbe & van der Bliek, 2008; Palmer et al., 2011; Yoon, Krueger, Oswald, & McNiven, 2003). All these potential Drp1 receptors have a single transmembrane segment

anchored in the outer membrane, while the most of the protein protrudes into the cytosol. Only the cytosolic region of Fis1 is well characterized (Jofuku, Ishihara, & Mihara, 2005). It forms a six-helix bundle containing a pair of tetratricopeptide (TPR)-like motifs. These TRP-like domains are involved in protein-protein interactions. However, they are not required for Fis1 oligomerization, thereby suggesting that other proteins required for fission (such as Drp1, directly or not) interact with Fis1 through these domains.

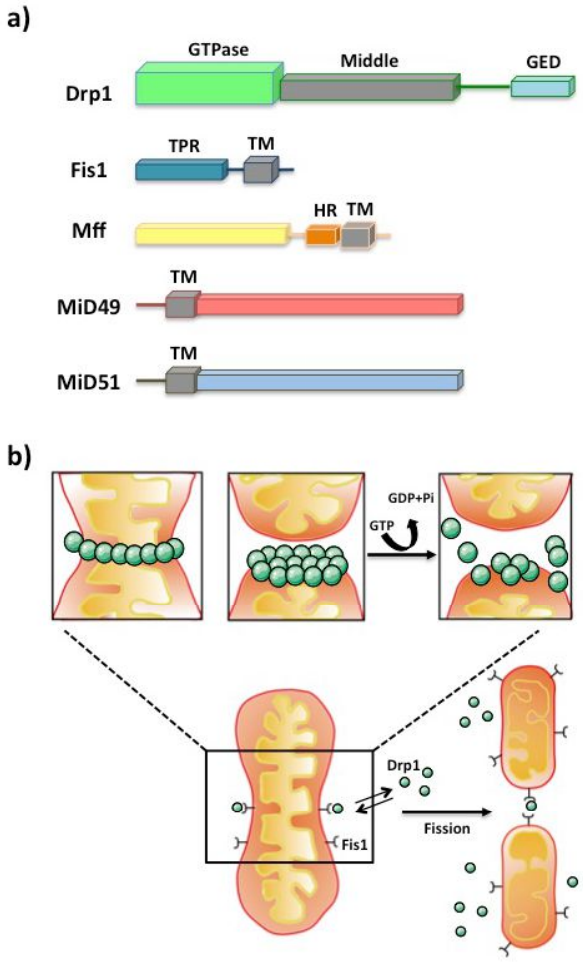

Figure 3. Molecules involved in mitochondrial fission. a) Schematic of Drp1 and the four potential Drp1 receptors: Fis1, Mff, MiD49, and MiD51. Drp1, as with other dynamin-related proteins, has a GTPase, middle, and GED (GTPase effector domain) domain. b) Recruitment of Drp1. Drp1 is cytosolic unless recruited by molecules on the mitochondrial surface. The candidate Drp1 receptor Fis1 is localized uniformly to the mitochondrial outer membrane. After recruitment, Drp1 assembles into a higher-order structure that further constricts the mitochondrial tubule. Abbreviations: HR, heptad repeat; TPR, tetratricopeptide-like motifs; TM, transmembrane segment. Image adapted from (Chan, 2012).

Drp1 is constantly changing localization from the cytosol to the mitochondria and it has been reported that this cycling activity and the formation of fission complexes are regulated through multiple post-translational modifications including S-nitrosylation, ubiquitination, and

sumoylation (D. H. Cho et al., 2009; Karbowski, Neutzner, & Youle, 2007; Zunino, Braschi, Xu, & McBride, 2009). A number of studies have also shown that Drp1 and its effects on mitochondrial dynamics are regulated by phosphorylation, thereby providing significant clues as to the intracellular signals that induce mitochondrial fission (Fig.4). The earliest reported phosphorylation was at Ser616 by Cdk1/cyclin B (Taguchi, Ishihara, Jofuku, Oka, & Mihara, 2007). This mitotic phosphorylation promotes Drp1-dependent mitochondrial fission, accounting for the increase in mitochondrial fragmentation that occurs in cells undergoing mitosis. Since this modification does not directly affect GTPase activity, the increase in fission may be mediated by alterations in Drp1 interactions with other proteins. A more widely studied phosphorylation site has been Drp1 Ser637. This was initially identified as a site for phosphorylation by protein kinase A (PKA), with dephosphorylation mediated by calcium-dependent protein phosphatase calcineurin. Three studies reported that modification of this site inhibits mitochondrial division through a reduction in GTPase activity and/or inhibition of Drp1 translocation to mitochondria (Cereghetti et al., 2008; Chang & Blackstone, 2007; Cribbs & Strack, 2007). Subsequently, Ser637 was also identified as a site for phosphorylation by Ca^{2+}/calmodulin-dependent protein kinase I alpha (CaMKIα). Indeed, a fourth study found that Ser637 phosphorylation by CaMKIα stimulates mitochondrial translocation of Drp1, perhaps through an increase in Drp1-binding affinity for Fis1, with an associated increase in mitochondrial fragmentation (Han, 2008). This apparent discrepancy highlights the importance of where and at what stage of the Drp1 activity cycle phosphorylation occurs in the cell as well as the possibility that other proteins are concurrently modified by these kinases.

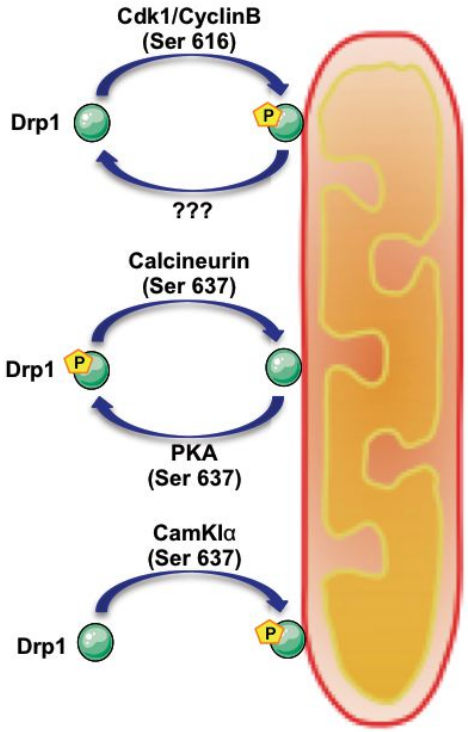

Figure 4. Regulation of fission by Drp1 phosphorylation. There are three kinases described to date that phosphorylate Drp1 (CaMKI, PKA, and Cdk1/Cyclin B) in different serine residues located in the divergent domain and/or near the GED domain. Of note, whereas phosphorylation in serine-616 enhances Drp1 mitochondrial fission activity, phosphorylation in serine-637 diminishes it, possibly because of a decrease in intramolecular interactions of Drp1, which could hypothetically affect mitochondrial recruitment or stabilization. However, in the case of serine-637 phosphorylation by CaMKI alpha DRP1 affinity for FIS1 increases and its mitochondrial recruitment and/or stabilization is enhanced. The phosphatases responsible for dephosphorylation of serine-616 residues are unknown. In the case of serine-637, calcineurin is the phosphatase responsible for its dephosphorylation. Adapted from (Liesa, Palacin, & Zorzano, 2009)

1.2 Mitochondria and calcium signalling

Ca^{2+} serves multiple complex and integrated functions in neurons, including the control of dendritic responses (morphological and functional) to neurotransmitters, signalling to the nucleus to regulate gene expression, and initiation of neurotransmitter release from presynaptic axon terminals (Greer & Greenberg, 2008; West et al., 2001; Zucker, 1999). Ca^{2+} is a messenger that transfers signals within the cell in response to membrane depolarization, thereby relaying information on neuronal activity status within the neuron. Ca^{2+} signals are decoded based on the characteristics of the intracellular changes in Ca^{2+} concentration (amplitude, duration, frequency and localization) and generate outputs as different as proliferation or death. Owing

to its vital importance, neurons use multiple ways of controlling intracellular Ca^{2+} concentration (Berridge, 1998), among those, mitochondria have an important role in shaping cytosolic Ca^{2+} signals. Most membrane-enclosed organelles are endowed with mechanisms that allow an energy-dependent Ca^{2+} accumulation and a release dependent on the cation concentration gradient between the organelle lumen and the cytosol. Mitochondrial Ca^{2+} accumulation and release are based on an opposite strategy since they do not need ATP for uptake and utilize gated channels for Ca^{2+} uptake and exchangers for release (Fig.5) (Nicholls, 2005). Several mechanisms or modes of transport have been described in the literature over the past 40 years (Bernardi, 1999; Gunter, Buntinas, Sparagna, Eliseev, & Gunter, 2000; Rizzuto, Bernardi, & Pozzan, 2000). It is widely accepted that mitochondria may sequester Ca^{2+} via mitochondrial uniporter (MCU). On the other hand, mitochondria release Ca^{2+} via either the Na^{+}/Ca^{2+} efflux mechanisms or in special circumstances via the permeability transition pore (PTP). This unique mitochondrial machinery ensures the maintenance of a low matrix Ca^{2+} concentration in resting cells and a rapid Ca^{2+} accumulation by the organelle when cytosolic Ca^{2+} is elevated during activation. In turn, mitochondrial Ca^{2+} uptake and release is central not only for the regulation of cellular Ca^{2+} homeostasis, but is vital also for the regulation of intramitochondrial enzymes (Rizzuto et al., 2012).

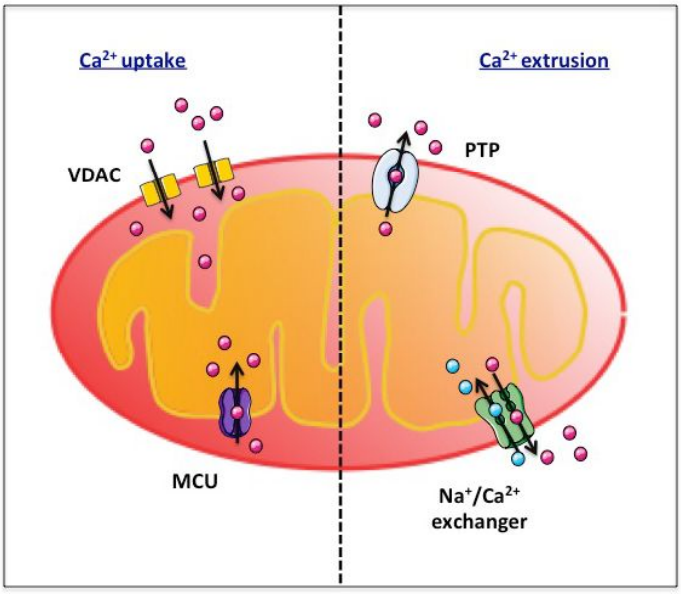

Figure 5. Schematic of calcium influx and efflux mechanisms in mitochondria. The figure shows the principle players of mitochondrial Ca^{2+} homeostasis.

1.2.1 Mitochondrial Ca^{2+} uptake

Evidence of mitochondria capacity to accumulate certain ions was first observed in the early 1960s, when was discovered that isolated mitochondria can efficiently take up Ca^{2+} from the medium and accumulate it in the matrix (Deluca & Engstrom, 1961; Vasington & Murphy, 1962). Later, the driving force for such Ca^{2+} accumulation was attributed to the membrane potential ($\Delta\Psi$) across the IMM (Mitchell & Moyle, 1967). Thus, Ca^{2+} enters the mitochondrial matrix down its electrochemical gradient, that can be generated either by the electron flow in the respiratory chain or by reversal of the ATP synthase. Ca^{2+} accumulation into the mitochondrial matrix requires the crossing of the outer and the immer membranes. OMM permeability is attributed to the abundant expression of voltage-dependent anion channels (VDACs) (Colombini, Blachly-Dyson, & Forte, 1996). Subsequently, accumulation of Ca^{2+} into the mitochondrial matrix through the ion impermeable IMM is mediated by the mitochondrial Ca^{2+} uniporter (MCU). The uniporter passes Ca^{2+} down the electrochemical gradient maintained across this membrane without direct coupling to ATP hydrolysis or transport of other ions (Kirichok, Krapivinsky, & Clapham, 2004). Despite the low affinity of the MCU (Kd around 10 μM in permeabilized cells) (Bragadin, Pozzan, & Azzone, 1979), the speed of mitochondrial Ca^{2+} uptake in living cells in response to relatively small increases in the concentration of cytosolic Ca^{2+}, is high. This paradox implied that mitochondria are located at privileged location inside cells, very close to Ca^{2+} release or Ca^{2+} entry channels. Indeed, functional and morphological evidences indicate that mitochondria are in close contacts with the endoplasmic reticulum (ER) and with plasma membrane channels (Rizzuto et al., 1998).

1.2.2 Mitochondrial Ca^{2+} release

The role of the efflux mechanisms is to remove the Ca^{2+} sequestered to maintain intramitochondrial Ca^{2+} homeostasis for repetitive physiological processes. While in non-excitable cells the Ca^{2+} efflux is essentially mediated by the H^+/Ca^{2+} exchange, the mitochondrial Na^+/Ca^{2+} exchanger is the main system extruding Ca^{2+} ions from the mitochondrial matrix in neurons and

other excitable cells (Castaldo et al., 2009). This antiporter extrudes Ca^{2+} ions from the mitochondrial matrix in exchange for cytosolic Na^{+}, which in turn enter the matrix and will later be cycled back to the cytosol by the Na^{+}/H^{+} exchanger in exchange with H^{+}. Ca^{2+} extrusion from the mitochondrial matrix accounts for the fact that steady-state intramitochondrial Ca^{2+} concentrations are significantly lower than those expected based on the driving force sustaining Ca^{2+} influx into these organelles. When the amount of Ca^{2+} accumulated inside the matrix is massive (Ca^{2+} overload), the transient opening of the PTP also contributes to the release of the ion to the cytosol. The mitochondrial PTP is a high-conductance channel that is located at the contact sites between the inner and outer mitochondrial membranes. This channel is responsible for the non-selective permeability state of the IMM and allows the passage of ions and molecules up to 1.5 kDa molecular weight, including Ca^{2+}. The molecular composition of the PTP is not yet clear, but several proteins have been shown to be components that participate in PTP activity, including VDAC channels (Szabo, De Pinto, & Zoratti, 1993), adenine nucleotide translocase (Beutner, Ruck, Riede, & Brdiczka, 1998), the inorganic phosphate carrier (Varanyuwatana & Halestrap, 2012) and several members of the Bcl-2 family (Vander Heiden et al., 2001; Zamzami et al., 2000). Ca^{2+} ions, pro-oxidant and pro-apoptotic proteins, a decrease in the mitochondrial membrane potential, pH variations and adenine nucleotides all sensitize the opening of the pore (Baumgartner et al., 2009; Kroemer, Galluzzi, & Brenner, 2007). Mitochondrial permeability transition resulting from PTP opening is usually considered a transducer event in between Ca^{2+} or oxidative signal and different type of cell death (Chinopoulos & Adam-Vizi, 2006; Giorgi et al., 2012). Nonetheless several observations have suggested that mitochondrial PTP is a component of the Ca^{2+} efflux mechanism proposing a physiological role for this ambiguous complex (Barsukova et al., 2011; De Marchi, Bonora, Giorgi, & Pinton, 2014; Smaili & Russell, 1999).

1.2.3 ER and mitochondrial structural association

Mitochondria are not solitary organelles, but they make contact with several other structures, among which the ER has obtained the most attention. The contact sites between the ER and mitochondria are specialized

regions where the two opposing membranes become tethered by specific proteins that maintain their distance in the range of 10–30 nm (Csordas et al., 2006; Friedman et al., 2011; Rizzuto et al., 1998). Mitochondria-ER interaction was originally found to determine the transfer of Ca^{2+} between the two organelles (Rizzuto, Brini, Murgia, & Pozzan, 1993; Rizzuto, Simpson, Brini, & Pozzan, 1992), and their existence was later proven when it was found that, during cell-fractionation experiments, subcompartmentalized ER membranes cosedimented with mitochondria, giving rise to the concept of mitochondria-associated membranes (MAMs) (Rusinol, Cui, Chen, & Vance, 1994). It is now well recognized that this fraction has distinct biochemical properties and it is involved in several important cellular functions ranging from lipid biosynthesis, mitochondrial division and dynamics regulation of the two organelles (Rowland & Voeltz, 2012). As for the role of MAMs in Ca^{2+} signalling, the monitoring of the mitochondrial Ca^{2+} transients in living intact cells demonstrated that InsP3R-induced Ca^{2+} release, that occurs at the sites of close contact with mitochondria, exposes the MCU to microdomains of high concentrations of Ca^{2+} that match its low Ca^{2+} affinity and make possible the Ca^{2+} uptake by mitochondria (Fig. 6) (Rizzuto et al., 1993).

Additional studies have directly measured the entity and the distribution

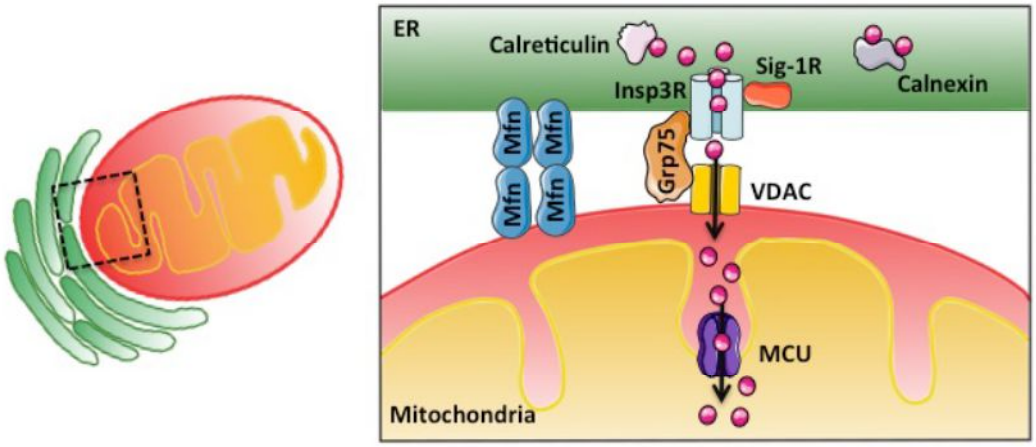

Figure 6. Ca^{2+} handling at the MAM. Close interactions between the endoplasmic reticulum (ER) and mitochondria are essential for rapid and sustained Ca^{2+} uptake by mitochondria. VDACs are responsible for the rapid transfer of Ca^{2+} from the ER–mitochondria apposition, and their function results in high Ca^{2+} microdomains in the mitochondria intermembrane space. Accumulation of Ca^{2+} into the mitochondrial matrix occurs via the MCU, which rapidly accumulates Ca^{2+} across the steep electrochemical gradient. A number of regulatory proteins, such as Mfns, control the formation of the ER–mitochondria junction. Chaperones modulate ER Ca^{2+} buffering (for example, calreticulin and calnexin) and control the stability or the sorting of signalling proteins. For example, sigma 1 receptor stabilizes Insp3Rs when ER Ca^{2+} stores are depleted, thereby ensuring proper Ca^{2+} fluxes from the ER to the mitochondria. Image adapted from (Rizzuto, De Stefani, Raffaello, & Mammucari, 2012).

of the Ca^{2+} hotspots by developing a probe targeted to the OMM (Giacomello et al., 2010) and showed that the spacing distance of the tethered bridge is important for the Ca^{2+} transfer efficiency (Csordas et al., 2006; Csordas et al., 2010). Thus, the physical link between ER and mitochondria is crucial to guarantee Ca^{2+} accumulation by mitochondria. Some factors, biochemically enriched in the MAMs, are essential to physically establish this interaction but, at the same time, they also regulate the Ca^{2+} transfer. The chaperone-mediated (Grp75/Mortalin) interaction between the mitochondrial outer membrane voltage-dependent anion-channel protein 1 (VDAC1) and the inositol 1,4,5 trisphosphate receptor (InsP3R) (Szabadkai, Bianchi, et al., 2006) favours the Ca^{2+} transfer, and Mfn2, partitioned between ER and mitochondria, is a key player in the tethering (de Brito & Scorrano, 2008). Interestingly, the type III isoform of the InsP3Rs (InsP3R3) are highly compartmentalized at MAMs, providing a direct and proper mitochondrial Ca^{2+} signalling from the ER to mitochondria (Mendes et al., 2005). Other components have been shown to operate at the ER-mitochondria interface and, interestingly, their function appears to be directly or indirectly influenced by Ca^{2+} (Hayashi, Rizzuto, Hajnoczky, & Su, 2009). From the mitochondrial side, the mitochondrial Rho GTPase Miro (Saotome et al., 2008) is essential to govern mitochondrial movements. Interestingly, Miro responds to Ca^{2+} over a concentration range that is never attained in the bulk cytosol of living cells (10–100 µM) (X. Wang & Schwarz, 2009), but that might be easily reached at the ER-mitochondria contact sites. Many MAMs-specific proteins are ER-resident oxidoreductases, Ca^{2+}-binding and glucose-regulated chaperones. Calnexin, calreticulin, and BiP are compartmentalized at the MAMs (Hayashi & Su, 2007; Myhill et al., 2008) and serve as high-capacity Ca^{2+} pools in the ER (Bastianutto et al., 1995; Hendershot, 2004). The oxidoreductase Ero1α and its co-chaperone ERp44 (Anelli et al., 2003) are enriched at the MAMs and regulate the activity of the InsP3R by a direct interaction (Higo et al., 2005; G. Li et al., 2009). Furthermore, Ero1α has been shown to directly regulate ER-mitochondria Ca^{2+} fluxes and to influence the activity of the mitochondrial transporters (Anelli et al., 2012). Analogously, the ER protein sigma-1 receptor (Sig-1R), a Ca^{2+}-sensitive chaperone, operates at MAMs forming a complex with the chaperone BiP. Upon ER Ca^{2+} depletion, Sig-1R dissociates

from BiP, leading to a prolonged Ca^{2+} signalling into mitochondria via InsP3Rs (Hayashi & Su, 2007).

1.3 Mitochondria defects in neurodegenerative diseases

Neurodegenerative diseases are characterized by gradually progressive, selective loss of anatomically or physiologically related neuronal systems. Over the past decade, numerous studies have linked mitochondrial dysfunction to neurodegenerative diseases (Kolesnikova, 2013). Neurons are particularly sensitive to changes in mitochondria function since, as we describe above, they are extremely energy dependent with many neuronal activities such as synaptic transmission, axonal and dendritic transport and maintenance of calcium homeostasis (Kann & Kovacs, 2007). Moreover, neurons are reliant particularly on the dynamic properties of mitochondria. Not surprisingly, mitochondrial injury can have severe consequences for neuronal function and survival. Indicators of mitochondrial dysfunction observed in neurodegenerative diseases include decreased ATP and free radical production, mtDNA deletions, respiratory complex inhibition and loss of mitochondrial membrane potential (M. T. Lin & Beal, 2006). Intriguingly, common elements such as defects in respiratory chain and oxidative stress have been recognized in almost all neurodegenerative disorders. However, they do not appear as the primary cause of the pathogenesis of neurodegenerative conditions. Compromised modulation of mitochondrial dynamics and Ca^{2+} buffering are instead suggested to be primary events. Therefore, we focus specifically on abnormal mitochondrial dynamics and Ca^{2+} mishandling, two phenomena that mediate and exacerbate mitochondrial impairment and neuronal dysfunction during the pathological process of neurodegeneration.

1.3.1 Mitochondrial fission and fusion in neurodegeneration

In a healthy neuron, tight regulation of fusion and fission enables the formation of continuous rearranging mitochondrial networks. This highly

dynamic balance not only controls mitochondrial morphology, length, size and number but also regulates mitochondrial function and distribution. Thus, defects in either fusion or fission can have severe consequences for the cell (Fig.7).

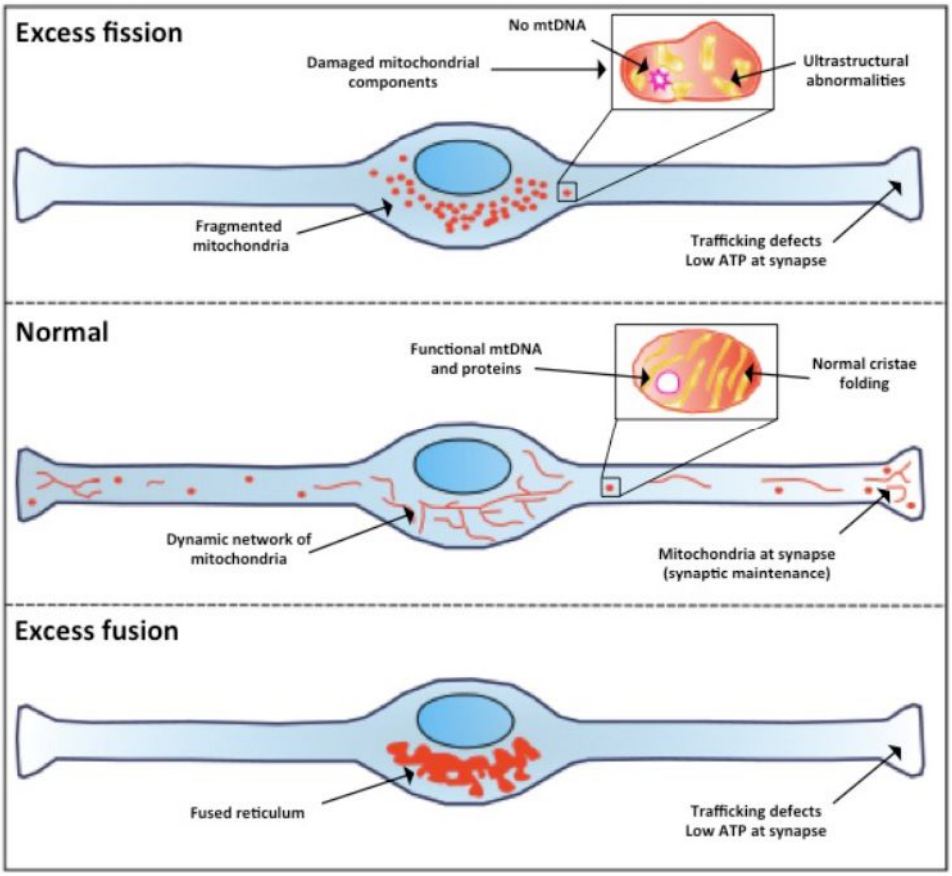

Figure 7. Effects of dysregulation of the fusion/fission machinery. A comparison of mitochondria in a healthy neuron with one in which either fusion or fission is impaired. Image adapted from (Burchell et al., 2010).

The prevalence of neuronal diseases associated with mutations in mitochondrial fission/fusion genes underscores the important relationship between mitochondrial dynamics and neuronal function. For instance, mutations in Opa1 cause autosomal dominant optic atrophy (Delettre, Lenaers, Pelloquin, Belenguer, & Hamel, 2002), a progressive degeneration of the optic nerve and retinal ganglion cells, while mutations in Mfn2 cause Charcot-Marie-Tooth disease A2, a peripheral neuropathy causing axonal degeneration of sensory and motor neurons (Zuchner et al., 2004). In these

cells, impaired fusion produces highly fragmented mitochondria, alteration of cristae structure, trafficking defects and impaired aerobic respiration. Diversely, mutations in the fission machinery are extremely rare because of the severity of the phenotype: a lethal mutation in Drp1 has been observed in a neonate with microcephaly, abnormal brain development and optic atrophy (Waterham et al., 2007). Skin fibroblasts isolated from the patient displayed elongated mitochondria, suggesting a defect in mitochondrial fission. Loss of either fusion or fission also results in damaged or absent mtDNA and bioenergetics defects, characterized by a reduction in oxygen consumption, ATP production and mitochondrial membrane potential (H. Chen, McCaffery, & Chan, 2007; Malena, Loro, Di Re, Holt, & Vergani, 2009; Parone et al., 2008).

Alterations in mitochondrial dynamics, not necessarily associated with genetic mutations of the dynamic machinery components, are also increasingly implicated in neurodegenerative diseases (Knott, Perkins, Schwarzenbacher, & Bossy-Wetzel, 2008; X. Wang, 2009). Fragmentation and clustering of mitochondria in the soma is observed in the brains of patients with sporadic Alzheimer's disease (AD), with reduced expression levels of Drp1, Opa1, Mfn1 and Mfn2 proteins (X. Wang et al., 2009). Although the mechanism leading to these changes are unclear, they may represent direct effect of increased amyloid β (Aβ), a peptide implicated in AD pathogenesis. Aβ localizes to mitochondria and specifically interacts with certain mitochondrial proteins, which potentiates mitochondrial, neuronal and synaptic stress (Manczak et al., 2006). Exposure of primary neurons to Aβ aggregates induces acute impairment in mitochondrial axonal transport (Rui, Tiwari, Xie, & Zheng, 2006) and aberrant mitochondrial fragmentation prior to neuronal death (Barsoum et al., 2006), through enhanced nitric-oxide production and increased S-nitrosylated Drp1 formation which activates its GTPase activity and mitochondrial fission (D. H. Cho et al., 2009).

Compelling evidences have also suggested that alterations of mitochondrial dynamics could represent a critical event in the pathogenesis of Parkinson's disease (PD). Neurons treated with rotenone, a toxin used to model PD, display a rapid Drp1-dependent mitochondrial fragmentation, which can be reversed by overexpressing Mfn1 or dominant negative Drp1

mutant form (Barsoum et al., 2006). Similarly, 6-Hydroxydopamine also induces Drp1-dependent mitochondrial fission, which precedes and mediates neuronal apoptosis (Gomez-Lazaro, Bonekamp, Galindo, Jordan, & Schrader, 2008), suggesting that excessive mitochondrial fission might be mediating neurotoxicity induced by complex I inhibition. Genetic factors associated with PD also cause changes in mitochondrial dynamics. For instance, mutation and/or overexpression of autosomal recessive PD proteins PINK1 and Parkin might affect mitochondrial dynamics. Mitochondria of fibroblasts from patients carrying Parkin mutations showed more branching network although no changes in mitochondrial length were noted (Yang et al., 2008). However, emerging evidence in mammalian cells has shown very different effect of these proteins on mitochondrial morphology: siRNA knockdown of PINK1 or Parkin in HeLa cells or neuroblastoma cells leads to mitochondrial fragmentation (Exner et al., 2007; Lutz et al., 2009) which is coordinated with enhanced mitochondrial turnover by mitophagy (Dagda et al., 2009). Interestingly, alterations of others autosomal recessive PD proteins, DJ-1, α-synuclein (α-syn) and LRRK2 (X. Wang, Petrie, et al., 2012; X. Wang, Yan, et al., 2012; Xie & Chung, 2012), can also affect mitochondrial dynamics revealing a crucial role of these PD-associated genes in the regulation of mitochondrial function.

1.3.2 Mitochondrial Ca^{2+} and neurodegeneration

Calcium overload is a major route of neurotoxicity. Mitochondria play important roles in Ca^{2+} deregulation, both as a means of buffering high cytosolic calcium levels and as a mediator of calcium-induced cell death. Indeed, moderate increases in mitochondrial Ca^{2+} concentration are necessary and sufficient to adjust ATP production to cell demand, but mitochondrial Ca^{2+} overload leads to disruption of mitochondrial membrane integrity, permeability transition, irreversible oxidative damage and loss of ATP production. A number of studies have suggested that the alteration of Ca^{2+} homeostasis is a hallmark of neurodegenerative diseases; in particular Ca^{2+}-dependent mitochondrial dysfunction may be critical to the degeneration of neurons in AD, PD, amyotrophic lateral sclerosis (ALS) and in demyelinating

diseases (Forte et al., 2007; Marambaud, Dreses-Werringloer, & Vingtdeux, 2009; Surmeier & Schumacker, 2013).

Disturbances in Ca^{2+} signalling have been found in both sporadic (Boada et al., 2010; Tolar et al., 1999) and familial cases of AD (Zatti et al., 2004). The development of a Ca^{2+} deregulation hypothesis was supported by the finding that Aβ aggregates can insert in the plasma membrane and form ion conducting channels, thus possibly mediating excitotoxicity by enhancing Ca^{2+} influx (Demuro et al., 2005). Aβ oligomers have been found to induce massive Ca^{2+} transfer between endoplasmic reticulum (ER) and mitochondria (Ferreiro, Oliveira, & Pereira, 2008) and mitochondrial Ca^{2+} overload (Sanz-Blasco, Valero, Rodriguez-Crespo, Villalobos, & Nunez, 2008). Aβ can also accumulate in mitochondria and interact with specific intra-mitochondrial targets promoting the opening of the mitochondrial PTP, thereby causing neuronal injury and a decline of cognitive functions (Du et al., 2008). Interestingly, it has been shown that mutated presenilins (PSs) may contribute to the deregulation of Ca^{2+} homeostasis observed in familial cases of AD (M. T. Lin & Beal, 2006). PS1 and PS2 are located in different intracellular compartments such as the ER and mitochondria and they have been proposed to function as low conductance Ca^{2+}-leak channels in the ER membrane, thus contributing to maintain physiological Ca^{2+} concentration within the ER. Mutations causing AD have been shown to abrogate this channel activity, and result in enhanced ER Ca^{2+} levels and increased cytosolic Ca^{2+} transients (Nelson et al., 2007; Tu et al., 2006).

Evidence for a possible role of mitochondrial Ca^{2+} dysfunction in the pathogenesis of PD comes from studies on the mitochondrial kinase PINK1. The first suggestion arose from the finding that the expression of mutant PINK1 exacerbates mitochondrial defects in a cellular model of PD. These defects, such as loss of mitochondrial $\Delta\Psi$ ($\Delta\Psi_m$), increased mitochondrial size with loss of cristae and reduced ATP levels, are fully rescued by the inhibition of MCU, suggesting that mitochondrial Ca^{2+} uptake is involved (Marongiu et al., 2009). Other studies have proposed that the absence of PINK1 induces mitochondrial Ca^{2+} accumulation, possibly as a consequence of the impairment of mitochondrial Ca^{2+} efflux through the mitochondrial Na^+/ Ca^{2+}

exchanger (Gandhi et al., 2009). Depletion of PINK1 could also impair mitochondrial Ca^{2+} uptake and consequently ATP production (Heeman et al., 2011). Moreover, increased sensitivity of mitochondria to Ca^{2+}-induced permeability has been shown to precede dopaminergic defects in PINK1-deficient mice, suggesting that mitochondrial Ca^{2+} alteration could be an early event in the pathogenesis of PD (Akundi et al., 2011).

Increasing evidence pointed out the importance of mitochondrial dysfunction in ALS, in particular defect in the electron transport chain and in the morphology of the organelles (Duffy, Chapman, Shaw, & Grierson, 2011). Familial cases of ALS are related to mutations in the mitochondrial superoxide dismutase (SOD1) gene, which result in reduced respiratory chain complex I and IV activity, and possibly in the abnormal structure and number of mitochondria in motor neurons and skeletal muscles (Faes & Callewaert, 2011). Although not numerous, the studies on mitochondria support a causative role for Ca^{2+} overload in neurons from individuals affected by sporadic and genetic ALS. Mitochondria at the synapse of motor neurons of mutant SOD1 mice display greater membrane potential depolarization after Ca^{2+} uptake, suggesting that Ca^{2+} buffering capacity of these organelles could be compromised (David, 1999). Accordingly, the overexpression of mutant SOD1 in neuroblastoma cells increases the cytosolic Ca^{2+} level (Carri et al., 1997). Finally, another candidate may relate mitochondrial Ca^{2+} dysfunction to ALS pathogenesis: the vesicle associated membrane protein B (VAPB) has been found to be mutated in familial ALS (Nishimura et al., 2004). VAPB is an ER membrane anchored-protein that exposed its C-terminal domain on the cytosolic side (De Vos et al., 2012) and has been reported to induce ER-stress and consequent Ca^{2+}-mediated death in motor neurons (Langou et al., 2010). It has been found that VAPB is enriched in the ER-mitochondrial associated membranes, where it is necessary to support ER-mitochondria Ca^{2+} transfer, since its siRNA-mediated silencing disrupt Ca^{2+} signalling between these two organelles (De Vos et al., 2012). Interestingly, a mutant form of VAPB induces mitochondria clustering, impaired mitochondrial Ca^{2+} uptake and increased cytosolic Ca^{2+} levels (Morotz et al., 2012).

1.3.3 ER-mitochondria contact sites and Ca^{2+} transfer in neurodegeneration

The vast implication of calcium signalling on various intracellular mechanisms emphasizes the importance of the MAMs for metabolism and cellular lifespan. Not surprisingly, MAMs have been proposed to be affected in neurodegenerative diseases (Cali, Ottolini, & Brini, 2013). In particular, alterations in proteins unequivocally linked to neurodegeneration, have been related to deregulation of ER-mitochondria cross-talk. For instance, it has been recently shown that siRNA-mediated downregulation of PD associated protein α-syn, Parkin and DJ-1, involved in the regulation of ER-mitochondria Ca^{2+} transfer and tether (Cali, Ottolini, Negro, & Brini, 2012, 2013; Ottolini, Cali, Negro, & Brini, 2013), impairs mitochondrial Ca^{2+} uptake and mitochondrial structure. DJ-1, Parkin, and α-syn have been shown to interact with Grp75/Mortalin (Davison et al., 2009; J. Jin et al., 2007; H. M. Li, Niki, Taira, Iguchi-Ariga, & Ariga, 2005), whose mutations have been reported in a limited number of PD patients as well (De Mena et al., 2009), and an association of α-syn with VDCA1 has been recently reported (Lu et al., 2013). Moreover, subcellular fractionation studies revealed the presence of DJ-1 (Ottolini et al., 2013) and α-syn (Guardia-Laguarta et al., 2014) in the MAMs fraction, and this localization may help explain the mitochondrial abnormalities associated with pathogenic mutations in these proteins.

The importance of MAMs-dependent regulation of Ca^{2+} transport in neurodegeneration is also underlined by the numerous data obtained in different animal and cell models of AD that have provided evidence for the ability of PSs to modulate the ER Ca^{2+} leak or releasing capacity. In 2009, Schon and coworkers showed that the PSs are enriched at MAMs (Area-Gomez et al., 2009), leading to the hypothesis that AD might be a MAMs-related dysfunction (Schon & Area-Gomez, 2010). Shortly after, others have reported that the PS2 action in Ca^{2+} signalling was dependent on the modulation of ER-mitochondria interactions and their Ca^{2+} cross talk (Kipanyula et al., 2012; Zampese, Fasolato, Pozzan, & Pizzo, 2011). The overexpression of a Familial Alzheimer's Disease (FAD) PS2 mutant favoured

the Ca^{2+} transfer by increasing the frequency of Ca^{2+} hotspots generated at the ER sites of Ca^{2+} release, thus predisposing the cells to Ca^{2+} overload and, eventually, imbalanced energetic metabolism. Increased ER-mitochondria connections and MAMs function have been reported both in FAD-mutant PS1 and Aβ precursor protein expressing cells and in fibroblasts from patients with the familial and sporadic forms of AD (Area-Gomez et al., 2012).

Interestingly, the link between ER-mitochondria connections and possible Ca^{2+} dyshomeostasis has been recently proposed in ALS as well. The ALS-related VAPB protein has been found to localize in the MAMs fraction and to interact with the mitochondrial outer membrane protein PTPIP51, suggesting a role in the maintenance of the contacts between these two organelles. The downregulation of both proteins impairs the mitochondrial Ca^{2+} uptake, and the mutated VAPB-P56S has been found particularly enriched, in respect with the wild-type form, in the MAMs fraction (De Vos et al., 2012). Mutant VAPB increases the mitochondrial Ca^{2+} uptake and disrupts anterograde axonal transport of mitochondria by perturbing the Ca^{2+}-regulated Miro1/kinesin-1 interaction with tubulin (Morotz et al., 2012).

Changes in mitochondrial dynamics and mitochondrial Ca^{2+} dysfunction have also been found in HD. However less is known about the mechanisms that lead to these alterations and how mitochondrial abnormalities cause the selective striatal neurodegeneration that characterize the disease. Therefore, in this Thesis, we focused on the study of mitochondrial alterations in HD and their contribution to the progression of the disorder. Molecular and pathological mechanisms involved in the pathogenesis of the disease will be described in detail in the following sections.

2. HUNTINGTON'S DISEASE

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder that occurs with a frequency of approximately 1 in 10,000 individuals in most populations of Caucasian descent (Hayden, Berkowicz, Beighton, &

Yiptong, 1981; Vonsattel & DiFiglia, 1998). The earliest mentions of HD date back to the first half of the XIX century when symptoms, progression and heredity of the disease started to be described. However, the first accurate description of the disease came in 1872 by the American physician George Huntington (Huntington, 1872). The precise causal gene, already mapped in human chromosome 4 in 1983 (Gusella et al., 1983), was isolated 10 years later and the mutation responsible for the onset of the disease was finally described (HDCRG, 1993). HD is characterized by involuntary movements (chorea) and cognitive and emotional disturbances. Onset usually occurs during the fourth or the fifth decade of life and the disease symptoms and signs progress with aging, with a mean survival of 15-20 years (Martin & Gusella, 1986). In the early stages, HD is classically associated with progressive emotional, psychiatric, and cognitive alterations. Commonly, patients show progressive weight loss, alterations in sexual behaviour, and disturbances in the wake-sleep cycle (Politis et al., 2008). As the disease progresses, the affected persons develop motor signs, dementia, or gradual impairment of the mental processes involved in comprehension, reasoning, judgment, and memory (Rosenblatt, 2007). In the late stages of the disease, patients become severely rigid, present severe dementia eventually ceasing to talk and becoming unable to care for themselves. As motor and cognitive deficits become severe, patients eventually die, usually from complications of falls, inanition, dysphagia, or aspiration (Folstein, Leigh, Parhad, & Folstein, 1986).

2.1 Genetics

Huntington's disease is caused by an abnormal expansion of CAG repeat in the IT15 gene which encodes for the Huntingtin (Htt) protein (HDCRG, 1993). The gene is comprised of 67 exons and it is located on chromosome 4p16.3. The mutation resides in the first exon (which is about 88 aminoacids long) of the gene and results in the expansion of a polyglutamine domain at the N-terminal of Htt protein. The poly-Q expansion is cleaved off proteolitically and the cleaved fragments increase probability of protein misfolding events showing propensity to aggregate and to form fibrils and oligomers (Fig. 8) (DiFiglia et al., 1997; Gutekunst et al., 1999; Martindale et

al., 1998; Sieradzan et al., 1999). Repeats with fewer than 29 triplets are within the normal range. The rare repeats with 29-35 triplets are considered of intermediate length, prone to expansion, but not of sufficient length to produce a phenotype. Repeats with 36 or more triplets generate the manifestation of the disease (Kremer et al., 1994).

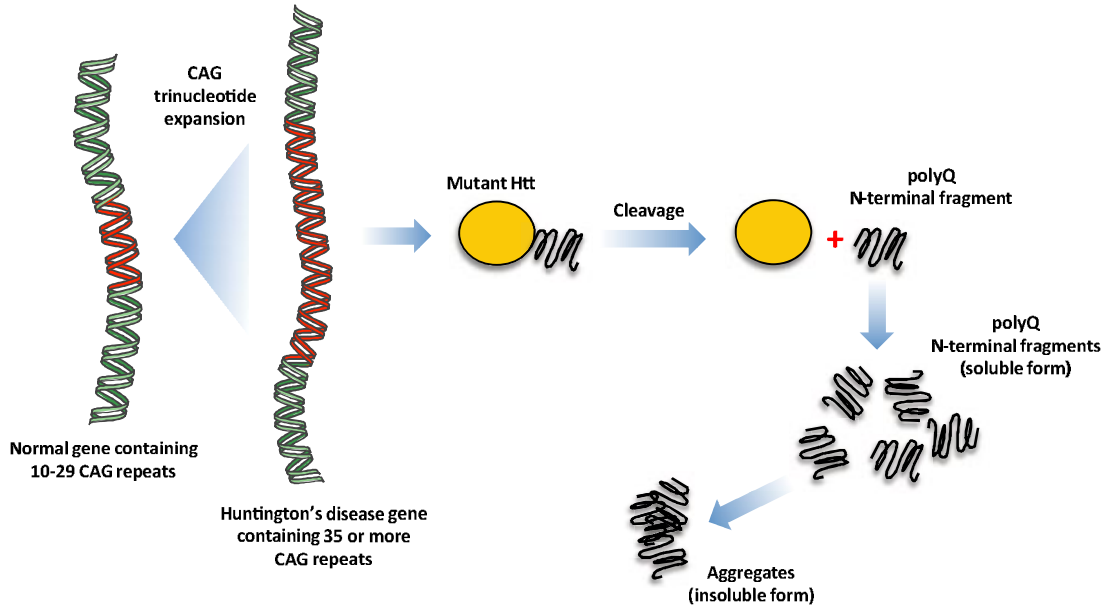

Figure 8. Generation of mutant Htt and its proteolitically cleaved fragments. Huntington's disease is caused by mutations in which an expanded CAG repeat (>35 repeats) tract results in long stretches of polyglutamine (polyQ) in the encoded protein Htt. Proteolytic processing of the mutated form of Htt lead to the formation of short N-terminal polyQ-containing fragments that have the propensity to aggregate. Toxicity might be elicited by mutant full-length Htt or by cleaved N-terminal fragments, which may form soluble monomers, oligomers or large insoluble aggregates.

Within the expanded range, the length of triplet repeats is inversely correlated with the age of disease onset (Andresen et al., 2007; Duyao et al., 1993). Therefore, more expanded CAG causes earlier onset, whereas the less expansion shows first symptoms in their late lifetime. Most patients with adult onset have repeat lengths of about 36-50 triplets and most patients with juvenile onset have 60 or more CAG triplets. Homozygous patients show, despite a similar age of onset to heterozygous patient, higher severity and faster progression of the disorder (Squitieri, Almqvist, Cannella, Cislaghi, & Hayden, 2003). Moreover, particularly when the mutation is inherited paternally, the age of onset of the disease decreases due to an increase in the number of repeats in successive generations, a phenomenon termed genetic anticipation (Andrew et al., 1993; Ranen et al., 1995).

2.2 Huntingtin protein

The translated wild-type Htt is a 350 kDa protein containing a polymorphic stretch of between 6 and 35 glutamine residues in its N-terminal domain (HDCRG, 1993). It is ubiquitously expressed with highest levels in brain, testis and to a lesser extent in heart, liver and lung (Schilling et al., 1995; Sharp et al., 1995). Within the brain, the main expression sites are the neocortex, the cerebellar cortex, the striatum and the hippocampus (DiFiglia et al., 1995; Gutekunst et al., 1995; Trottier et al., 1995). Within cells, the protein is found in the cytoplasm, in neurits and synapses where it associates with various vesicular structures, endoplasmic reticulum, the Golgi apparatus and microtubules (DiFiglia et al., 1995; Gutekunst et al., 1995; Metzler et al., 2001; Velier et al., 1998). Htt is also detected in the nucleus where it interacts with transcription factors and spliceosome related proteins (De Rooij, Dorsman, Smoor, Den Dunnen, & Van Ommen, 1996; Kegel et al., 2002; Takano & Gusella, 2002). Interestingly, the protein has also been observed directly associated with mitochondria (Choo, Johnson, MacDonald, Detloff, & Lesort, 2004; Gutekunst et al., 1998). According to its subcellular localization, Htt exerts different functions, from the regulation of organelle trafficking and vesicular endocytosis in the cytoplasm, to the control of the expression of specific target genes in the nucleus. A strong contribution to the understanding of the physiological functions of Htt comes from the identification of its numerous molecular interactors (Kaltenbach et al., 2007). Relevant examples include the Huntingtin associated protein 1 (HAP1) which interacts with the dynactin complex supporting a role for Htt in intracellular transport (X. J. Li & Li, 2005) and Huntingtin interacting protein 1 (HIP1) that binds alpha-adaptin and clathrin suggesting an involvement in endocytosis (Kalchman et al., 1997; Wanker et al., 1997). Finally, Htt is an indispensable protein that has anti-apoptotic properties. The generation of a KO mouse model for Htt revealed that the protein is required for embryonic development since the null embryos show massive cell death in the ectoderm at E7.5 (Zeitlin, Liu, Chapman, Papaioannou, & Efstratiadis, 1995). Interestingly, embryonic lethality can be rescued by the expression of a mutant form of Htt, suggesting that the protein function during development is independent from the length of the polyglutamine domain (Dragatsis, Levine, & Zeitlin, 2000).

Htt is also necessary for neurogenesis and normal hematopoiesis (Metzler et al., 2000; White et al., 1997).

2.3 Neuropathology

Brain abnormalities in HD develop well before evident symptoms, are progressive, and eventually involve the entire brain to a greater or lesser extent, resulting in about 25% brain weight loss in advanced HD (Halliday et al., 1998; Sharp & Ross, 1996). Nonetheless, the most prominent neuropathology in HD occurs within the striatal part of the basal ganglia (caudate nucleus and putamen), in which gross atrophy is accompanied by extensive neuronal loss and astrogliosis, both of which become more severe as the disease progresses, with the atrophy leading to marked dilatation of the lateral ventricles. Other areas of the basal ganglia, especially the globus pallidus and subthalamic nucleus, also become atrophic, though less than the striatum. Marked neuronal loss and shrinkage is also seen in deep layers of the cerebral cortex. Other regions, including hippocampus, amygdala, thalamus, substantia nigra, and cerebellum, show differently degrees of atrophy and/or neuronal loss, depending on disease stage (Rosas et al., 2003). Since the analysis of HD patients post-mortem tissue revealed different patterns of striatal degeneration, the neuropathologist Jean Paul Vonsattel developed a system for grading HD neuropathological severity based on macroscopic and microscopic criteria related to striatal morphology (Vonsattel et al., 1985) (Fig.9). This system recognizes five Grades (0–4), designated in the ascending order of severity, that correlate closely with the degree of clinical disability. Grade 0 appears indistinguishable from normal brains after gross examination. However, 30–40% neuronal loss can be detected in the head of the caudate nucleus upon histological examination. Grade 1 shows atrophy, neuronal loss, and astrogliosis in the tail and, in some cases, the body of the caudate nucleus. Grades 2 and 3 are characterized by progressive severe gross striatal atrophy. Grade 4 includes the most severe HD cases with atrophy of the striatum and up to 95% neuronal loss.

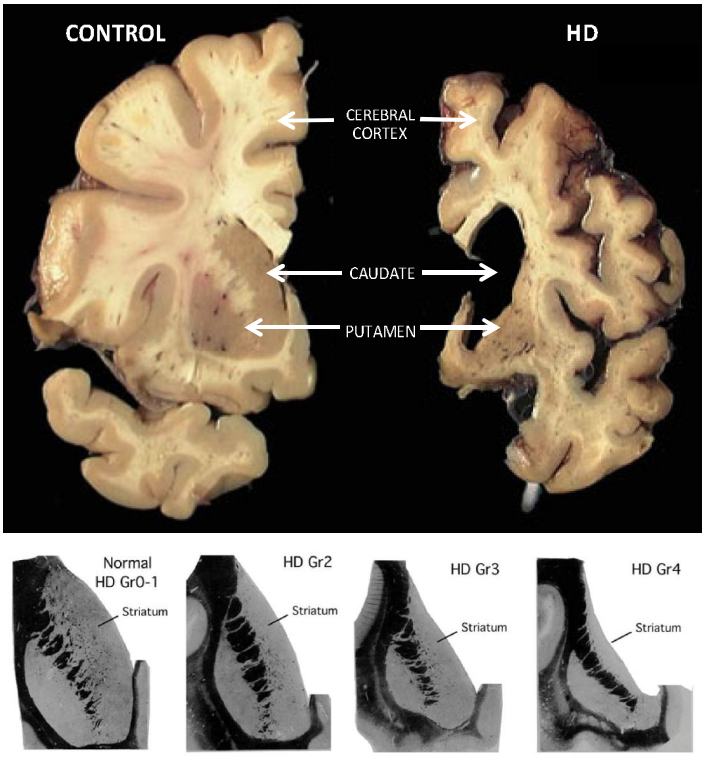

Figure 9. Human coronal slices, showing a normal brain on the left and an advanced HD brain on the right and representative illustrations of caudate at HD Grades 0 through 4 according to Vonsattel grading scale. The disease causes a profound shrinkage of cortex and a severe degeneration of caudate and putamen, resulting in a ventricular expansion in the HD brain. Image adapted from (Reiner, Dragatsis, & Dietrich, 2011).

As reviewed before, the major brain area of pathology in HD is the basal ganglia, a group of subcortical nuclei involved in a variety of processes including motor, associative, cognitive and mnemonic functions (Bolam, Hanley, Booth, & Bevan, 2000). The dorsal division of the basal ganglia consists of the striatum (or caudate-putamen), the external (GPe) and internal (GPi) segments of the globus pallidus, the subthalamic nucleus (STN) and the substantia nigra. The latter structure is divided into 2 main parts, the dorsal pars compacta (SNc) in which the dopaminergic nigrostriatal neurons are located and the more ventral pars reticulata (SNr). In addition to these structures, which are associated with motor and associative functions, there is a ventral division of the basal ganglia (nucleus accumbens and olfactory tubercle) that is associated with limbic functions. Essentially, the striatal complex receives glutamatergic excitatory inputs from cortical and thalamic structures, as well as dopaminergic innervation from SNc (Bolam et al., 2000) (Fig. 10). These two primary striatal afferent projections converge in the striatum and form excitatory synaptic connections with medium spiny neurons

(MSNs), the GABAergic output cells representing about 95% of striatal neurons (Dube, Smith, & Bolam, 1988), and provide the sole output of the striatum to other basal ganglia nuclei. MSNs can be divided into two populations based on their respective projection targets and neurochemical composition (Gerfen, 1992). One population constitutes the direct pathway that projects to the GPi and SNr and preferentially express, in addition to GABA, D1-type dopamine receptors and the neuropeptides substance P and dynorphin (Bolam et al., 2000; Y. Smith, Bevan, Shink, & Bolam, 1998). The second population, which express enkephalin and the D2-type dopamine receptors, constitutes the indirect pathway that projects almost exclusively to the GPe (Bolam et al., 2000; Y. Smith et al., 1998). Successively, GPe neurons project to the glutamatergic neurons of the subthalamic nucleus (STN) and to the output nuclei (GPi/SNr). STN neurons also project to both the GPi and SNr, forming a parallel pathway to the output nuclei.

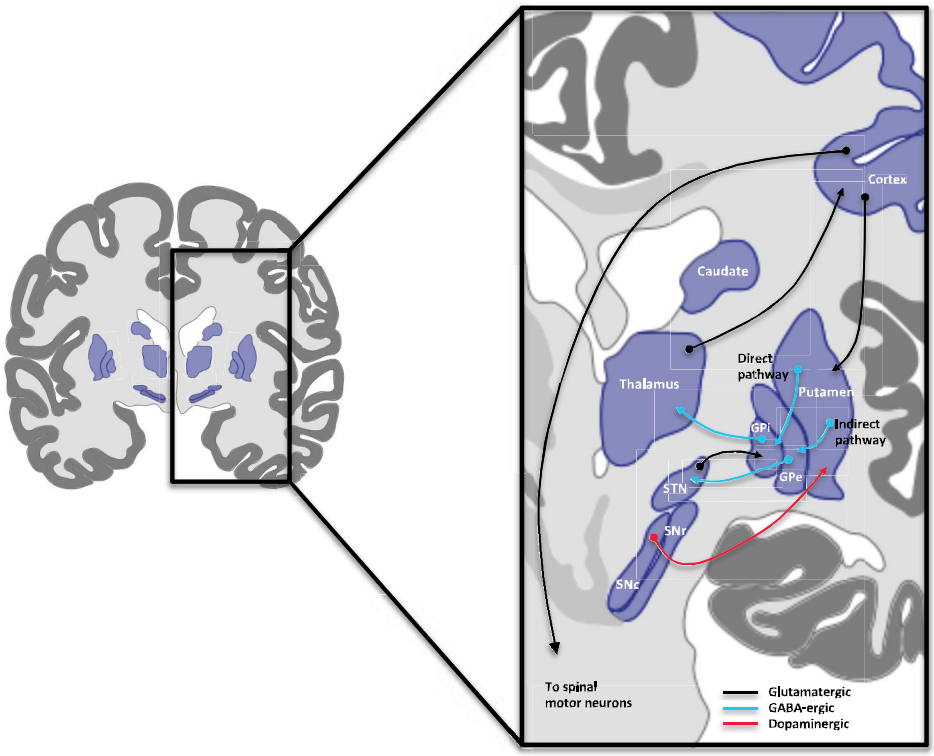

Figure 10. Shematic diagram of basal ganglia circuitry in human brain. The caudate and putamen (striatum) are the primary input structures for the basal ganglia circuit. These structures receive dopaminergic input from the substantia nigra pars compacta (SNc), as well as glutamatergic input from many regions of the cerebral cortex. Efferents from the putamen project to the internal globus pallidus (GPi) through two pathways: directly (direct pathway) and indirectly through the external globus pallidus (GPe) and subthalamic nucleus (STN) (indirect pathway). The GPi sends inhibitory fibres to the thalamus, and the thalamus, in turn, projects to sensorimotor cortex regions, conveying both the output of the basal ganglia (from ventral thalamic regions) and primary afferent information (from more dorsal regions of the thalamus). Finally, primary motor cortex neurons project through the brain stem to the spinal motor neurons, which themselves synapse on muscles to produce muscle contractions and movement. Image adapted from (Breakefield et al., 2008)

In the classical model of circuit function of the basal ganglia, the direct and indirect pathways act in opposing ways to control motor behaviour (Albin, Young, & Penney, 1989). Neuropathological analysis of the HD striatum revealed that in early and middle stages of the disease, neurons of the indirect pathway are preferentially vulnerable (Fig. 11) (Albin et al., 1992; Hedreen & Folstein, 1995). Since indirect pathway attenuates movement-related processing mediating the inhibition of neurons in the motor cortex, the death of these enkephalin-containing MSNs causes the hallmark hyperkinetic, choreaform movements typical of HD. At the most advanced stages of the disease, projections to all striatal target areas were depleted, including those that constitute the direct pathway (Deng et al., 2004; Reiner et al., 1988). This pathway is normally involved in the initiation of voluntary movements. Thus, degeneration of GABAergic/substance P neurons those project to the internal segment of the globus pallidus produces hypokinetic and parkinsonian symptoms in HD (Fig. 11).

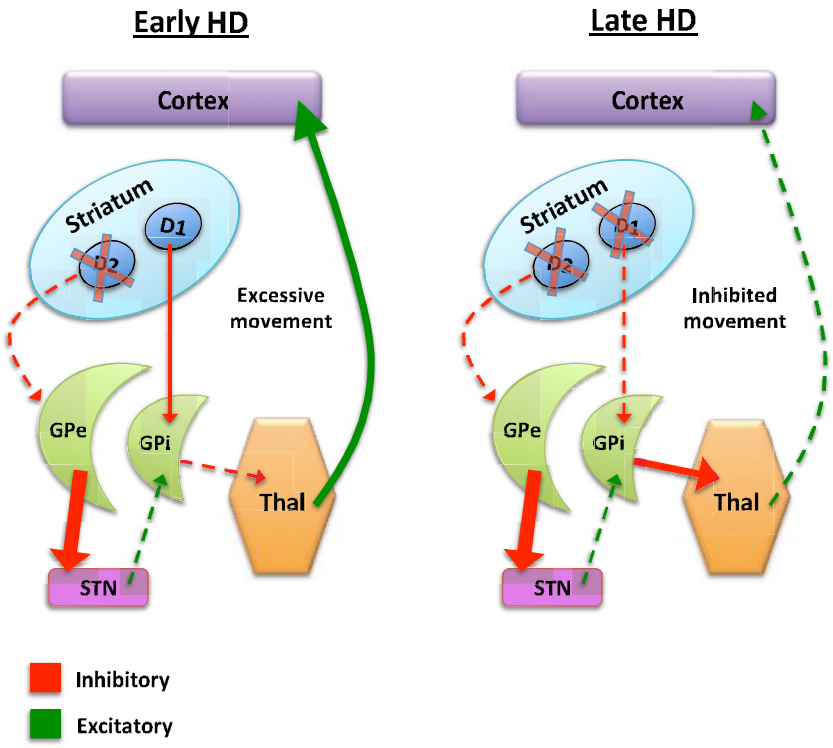

Figure 11. Basal ganglia circuitry in early and late stages of Huntington's disease. Early in HD, striatal GABAergic neurons that express D2 receptors and project to the external segment of globus pallidus (GPe) degenerate resulting in the inactivation of subthalamic nucleus (STN), the consequent dishinibition of the thalamus (Thal) and finally increased stimulation of the motor cortex, responsible for the excessive movement. Later in HD, striatal GABAergic neurons that express D1 receptors and project to the internal segment of globus pallidus (GPi) degenerate. This loss reduces the inhibition on GPi neurons and increases their firing, resulting in an enhanced inhibition of the thalamus and decreased stimulation of the motor cortex, responsible for the inhibition of the movement

2.4 Models for Huntington's disease

Research on HD largely depends on available animal and cellular models. Since it is caused by a single mutation, the introduction of the mutant gene into mammalian and nonmammalian species, non-human primate, and cells has allowed the generation of *in vivo* and *in vitro* disease models that have helped to investigate pathological pathways, molecular targets, and therapeutics in HD (M. Kim et al., 1999; Lunkes & Mandel, 1998; Ramaswamy, McBride, & Kordower, 2007). Before the discovery of the genetic ethiology of HD, models of this disease relied on neurotoxin-mediated striatal lesioning (Beal et al., 1993; Beal et al., 1986; Coyle & Schwarcz, 1976). The basis for these lesional models was the observation that the primary site of neurodegeneration in HD was the striatum while intrastriatal injections of glutamate receptor agonists resulted in selective loss of the GABAergic projection neurons the most severely affected neurons in this disorder. Nevertheless, these models have a number of limitations, including the acute nature of the lesions. Indeed, progressive and age-dependent pathogenic events cannot be represented in the acute lesion models. Furthermore, although striatal atrophy is the principal neuropathological feature of HD, mutant Htt (mHtt) is ubiquitously expressed and mHtt-dependent extrastriatal and peripheral pathologies are likely to contribute to the core pathological features of the disease. Although acute striatal lesion-based models do not contribute to the study of these aspects of the disease, they still remain good models to study neuroprotection and neurorestorative therapies in HD. Thanks to the generation of several genetic models of the disease, it is now possible to capture the hereditary nature of HD and monitor the actions of either normal or mutant Htt at tissue and subcellular levels at different time points. Rodents are by far the most commonly used animals for modelling HD. Indeed, over 20 different rodent models of this disease have been generated to date (Pouladi, Morton, & Hayden, 2013). Rodent models of HD fall into two broad categories characterized by different approaches in genetic design: truncated N-terminal and full-length models. Transgenic truncated N-terminal mice result from the random insertion of a portion of the human Htt gene, containing the polyglutamine repeats, in the mouse genome.

Alternatively, full-length mHtt rodent models were established either by the introduction of a mutant full-length human Htt transgene, or by knocking a human Htt exon 1 with an expanded CAG repeat tract into the endogenous mouse Htt gene locus. There are several truncated N-terminal and full-length rodent models (Table 1), and here, we focus on the most commonly used ones.

Model	Transgene product	Promoter	CAG repeat lenght	Lifespan
Truncated N-terminal fragment models				
R6/1 mice	67 amino acids of N-terminal fragment (human Htt)	Human Htt promoter	116	32-40W
R6/2 mice	67 amino acids of N-terminal fragment (human Htt)	Human Htt promoter	144	13-16W
N171-Q82 mice	171 amino acids of N-terminal fragment (human Htt cDNA)	Mouse Prp promoter	82	16-24W
Full-lenght HD models: transgenic models				
YAC128 mice	Full-length human HTT	Human HTT promoter and regulatory elements	128	Normal
BACHD mice	Full-length human HTT	Human HTT promoter and regulatory elements	97	Normal
BACHD rats	Full-length human HTT	Human HTT promoter and regulatory elements	97	Normal
Full-lenght HD models: knock-in models				
CAG140 mice	Full-length chimeric human HTT exon 1:mouse Htt	Endogenous mouse Htt promoter	140	Normal
HdhQ92 mice	Full-length chimeric human HTT exon 1:mouse Htt	Endogenous mouse Htt promoter	92	Normal
HdhQ111 mice	Full-length chimeric human HTT exon 1:mouse Htt	Endogenous mouse Htt promoter	111	Normal
HdhQ150 mice	Full-length chimeric human HTT exon 1:mouse Htt	Endogenous mouse Htt promoter	150	Normal

Table1. Genetically modified rodent models of Huntington's disease. The table includes information about the transgene product, the promoter under which the mutation is expressed, the CAG repeat number and the lifespan. Prp, prion protein; W, weeks. See the text for references.

2.4.1 Truncated N-terminal fragment rodent models

The R6/1, R6/2 and N171-Q82 mouse models of HD express truncated N-terminal fragments of mHtt. R6/1 and R6/2 mice express exon 1 of human Htt, originally with 116 and 144 CAG repeats respectively, under the control of the human Htt promoter (Mangiarini et al., 1996). R6/2 mouse line exhibits

early and severe behavioural symptoms. These mice develop cognitive deficits at 3,5 weeks of age, before the onset of the motor symptoms (5 weeks) (Carter et al., 1999; Lione et al., 1999; Stack et al., 2005). R6/2 mice exhibit neuroanatomical abnormalities including progressive reduction in brain and striatal volume by 5 weeks, substantially reduced striatal neuron number by 12 weeks, and death by 13–16 weeks. R6/1 mice show a phenotype less dramatic respect to R6/2. As with the R6/2 mice, the human htt promoter drives mutant Htt gene expression in all cells of the body, but it is at only 31% of the expression levels seen in the endogenous mouse gene (compared to 75% in the R6/2 line). The R6 lines are characterized by the presence of widespread nuclear inclusions of mutant Htt in brain neurons that increase steadily in number, size, and distribution as disease progresses (Davies et al., 1997; Morton, Lagan, Skepper, & Dunnett, 2000). Striatal dopamine D1 and D2 receptors, which are widely distributed on the dendrites of striatal projection neurons, are decreased as early as 8 weeks of age, consistent with both early striatal neuronal dysfunction and neurodegeneration (Cha et al., 1999; Cha et al., 1998). The early onset of symptoms and a fast progression of the disease make this mouse line particularly useful for therapy screening, but less suitable for the investigation of early disease mechanisms (Gil & Rego, 2009).

A similar neuropathological and behavioural phenotype characterize the transgenic line, N171–82Q. This model expresses a truncated Htt human cDNA with 82 CAG repeats under the control of the mouse prion protein promoter that restricts the expression of the mutant protein to brain neurons (Schilling et al., 1999). Intranuclear inclusions and neuritic aggregates were found in the brain of N171–82Q mice, resembling the human phenotype. Compared with the R6 mice, the N171–82Q model has fewer polyglutamine repeats resulting in a later onset of symptoms, which makes it an attractive model for the study of presymptomatic therapies.

2.4.2 Full-length rodent models

Transgenic mice expressing full-length Htt have in some cases been more successful than N-terminal fragment models in terms of neuronal loss

and capability to recapitulate more faithfully the sequence of events leading to HD. A group of full-length mHtt rodent models was created using yeast artificial chromosome (YAC) technology and bacterial artificial chromosome (BAC) technology (Gray et al., 2008; Hodgson et al., 1999; Slow et al., 2003; Yu-Taeger et al., 2012). Such models express human genomic mHtt transgenes, including all the introns and exons as well as the regulatory sequences, ensuring appropriate temporal and tissue-specific expression of mHtt. YAC128 HD mice express mHtt with 128 CAG repeats, whereas BACHD mice and rats express mHtt with 97 CAG repeats. The YAC128 and BACHD rodent models develop progressive motor, cognitive and psychiatric disturbances, as well as selective striatal and cortical atrophy (Gray et al., 2008; Pouladi et al., 2009; Van Raamsdonk, Murphy, Slow, Leavitt, & Hayden, 2005; Yu-Taeger et al., 2012).

Knock-in mice are considered the most HD accurate model from a genetic standpoint. Creation of these mice involves replacing a portion of the mouse Htt gene with a mutant human copy that contains an expanded CAG region. Thus, these models carry one or two copies of the mutant HD gene under control of the mouse Htt promoter, featuring temporally and spatially appropriate levels of mHtt expression. Initial studies in knock-in mice were disappointing because the mice did not show the overt neuropathology or symptoms seen in the transgenic mice. But closer examinations have revealed subtle changes in mice with larger numbers of repeat (Wheeler et al., 1999). The chimeric HdhQ lines were the first to be developed by replacing exon 1 of the mouse Htt gene with a mutated exon 1 containing either 111 or 92 CAG repeats in the HdhQ111 and HdhQ92 lines, respectively (Wheeler et al., 2000). Although there is no apparent striatal degeneration in either of these lines, striatal pathology is evident. Htt protein translocates to the nucleus at age of 4.5 months in both HdhQ111 and HdhQ92 lines and appears punctuate in the HdhQ111 mouse. Additionally, there is an increase in gliosis in the striatum at 24 months of age. These mice exhibit cognitive impairments (motor learning and procedural memory) as early as 2 months of age, getting worse with the disease progression. This deficiency is associated with altered cortico-striatal synaptic transmission and loss of dendritic spines (Puigdellivol et al., 2015). Moreover, impairments in spatial, recognition, and associative

memories together with decreased dendritic spine density in the hippocampus were found in HdhQ111 mice from 6 months of age, suggesting hippocampal dysfunction (Brito et al., 2014). Interestingly, motor coordination deficits do not appear until the age of 8 months (Puigdellivol et al., 2015).

More pronounced cellular dysfunction and progressive motor behavioural abnormalities were detected also in two other knock-in models characterized by the presence of longer CAG tracts (C. H. Lin et al., 2001; Menalled, Sison, Dragatsis, Zeitlin, & Chesselet, 2003). The CAG150 knock-in mice exhibit mutant Htt aggregates at 9 months of age (Yu et al., 2003) and weight loss, diminished activity, abnormal motor function as well as a clasping phenotype that is indicative of neurological deficits at 2 years of age (C. H. Lin et al., 2001). Similar phenotypes have been described in CAG140 knock-in mice (Menalled et al., 2003; Mende-Mueller, Toneff, Hwang, Chesselet, & Hook, 2001), which show that early behavioural abnormalities exist in a wide range of motor and nonmotor functions starting at 1–4 months of age followed by progressive gliosis (12 months) and loss of striatal neurons at 2 years of age (Hickey et al., 2008). These studies showed that knock-in mice reproduce canonical characteristics of HD, preceded by deficits that may correspond to the protracted premanifest phase of the disease in humans. Thus, knock-in models can be very important for the study of early and mild neuronal abnormalities that might be primarily responsible of early functional deficits.

2.4.3 Cell models

Research into the pathogenesis of HD has been greatly facilitated by the development of cell models. Engineered cell models, containing variable poly-Q lengths, provide an excellent platform to gain better insight into the disease pathogenesis and to explore treatment options. Two approaches have been used. In the first, polyQ-expanded Htt constructs are introduced into cells through transient transfection; in the other, cell lines are engineered with a stably expression of mHtt (Lunkes & Mandel, 1998; Moulder, Onodera, Burke, Strittmatter, & Johnson, 1999; Peters et al., 1999; Saudou, Finkbeiner, Devys, & Greenberg, 1998). In general, short truncations of Htt containing the expanded polyglutamine appear to be much more toxic than the full-

length Htt, and more liable to aggregate (Cooper et al., 1998; Hackam et al., 1998; Martindale et al., 1998). However, aggregate formation and cellular toxicity can be dissociated, suggesting that cell death is not related in a simple way to aggregation (M. Kim et al., 1999; H. Li et al., 1999; Saudou et al., 1998).

In this Thesis, we analysed the *in vitro* mHtt effect in a stable cell line generated from the knock-in HdhQ111 mouse model (Trettel et al., 2000). Striatal precursors at embryonic day 14 were immortalized with the simian vacuolating virus 40 Large T antigen. The generated cell lines are the wild-type ST7/7Q and the mutant ST111/111Q cellular model, expressing full-length endogenous normal or mutant huntingtin, respectively. These cells reproduce characteristic features of the disease. For example, mutant cells exhibit impaired energy metabolism and reduced ATP and ATP/ADP ratio (Gines, Ivanova, Seong, Saura, & MacDonald, 2003; Seong et al., 2005; Trettel et al., 2000). In addition, these cells show respiratory chain defects (Milakovic & Johnson, 2005), impaired ubiquitin-proteasome system (Hunter, Lesort, & Johnson, 2007) and mitochondrial Ca^{2+} handling defects (Milakovic, Quintanilla, & Johnson, 2006; Oliveira et al., 2006).

2.5 Pathological mechanisms participating in the striatal degeneration in Huntington's disease

Most studies to date postulates that MSNs degeneration results from a toxic "gain and/or loss of function" of mHtt, including altered gene transcription, enhanced protease activity, protein misfolding, disruption of axonal transport, changes in electrophysiological properties and induction of apoptotic mechanisms (Bauer & Nukina, 2009; Gil & Rego, 2008). However, most of these hypotheses do not explain the selective vulnerability of MSNs in HD. Increasing evidence points to a more complicated picture of HD pathogenesis, whereby different cascades of neuronal dysfunction occur early in disease progression and contribute to symptomatology. Indeed, various mechanisms such as excitotoxicity, dopamine toxicity, metabolic impairment, mitochondrial dysfunction, oxidative stress and autophagy have been implicated in HD pathology (Gil & Rego, 2008). In this section, we describe

those mechanisms that represented the background for the design of the aims of this Thesis.

2.5.1 Dopamine toxicity

Dopamine (DA) is a crucial neurotransmitter in brain, involved in numerous functions, including motor activity, cognition, motivation, and emotion (Jaber, Robinson, Missale, & Caron, 1996). The striatum receives massive DA input from SNc that contributes to modulate activity of the direct and indirect pathways facilitating movement or inhibiting unwanted movements. Therefore, due to this function, it has been proposed that aberrant DA metabolism might participate in HD pathology. The first evidence of the involvement of the dopaminergic system in HD came from asymptomatic offspring of affected individuals in which levodopa (L-DOPA) administration, a precursor substance of DA, developed dyskinesias (Klawans, Paulson, & Barbeau, 1970). Accordingly, later studies reported increased levels of DA in post-mortem brains of HD patients and showed that DA-depleting agents and DA receptor antagonists can be used with therapeutic benefit (Bird, 1980; Spokes, 1980). Moreover, rise in DA levels and dopaminergic hyperactivation occur at early stages of the disease (Chen et al., 2013a; Garret et al., 1992; Jakel and Maragos, 2000), resulting in an imbalance in striatal neurotransmission initiating signalling cascades that may contribute to striatal cell death (Paoletti et al., 2008; Ross and Tabrizi, 2011). Indeed, DA can generate neurotoxicity via products of its metabolism or via autoxidation (Graham, Tiffany, Bell, & Gutknecht, 1978; Sulzer & Zecca, 2000). Possible mechanisms include the generation of radical oxygen species (ROS) and interaction with excitatory amino acids (Jakel & Maragos, 2000). For instance, elevated levels of DA can inhibit glutamate uptake (Kerkerian, Dusticier, & Nieoullon, 1987), perhaps via the action of DA-derived ROS (Berman & Hastings, 1997). This could result in an elevation of synaptic glutamate levels, which could over activate NMDA receptors, and lead to cell death, via stimulation of excitotoxic pathways. However, little is known about how the action of DA in the striatum is altered by mHtt. *In vitro* and *in vivo* studies have shown that DA itself can enhance polyglutamine toxicity. Using primary cultures of striatal neurons transiently expressing exon 1 of mHtt, it

has been shown that low doses of DA, concurrently with mHtt, activates the proapoptotic transcription factor c-Jun through a mechanism that is thought to involve the production of ROS and the activation of the c-Jun N-terminal kinase pathway (Charvin, Vanhoutte, Pages, Borrelli, & Caboche, 2005). Importantly, DA also increases mHtt aggregate formation in this *in vitro* model, which could be reversed by a selective D2 receptor antagonist. In another recent study, double-mutant mice, generated by crossing the dopamine transporter knockout mouse with a knock-in HD mouse model expressing huntingtin with 92 CAG repeats, exhibited increased stereotypic activity at 6 months of age, followed by a progressive decline of their locomotor hyperactivity. In this hyperdopaminergic mouse model of HD the appearance of mHtt aggregates was shown to occur much earlier and to a greater extent in the striatum and other dopaminergic brain regions as compared to control HD mice (Cyr, Sotnikova, Gainetdinov, & Caron, 2006). Thus, DA exacerbates mHtt aggregation both *in vitro* and *in vivo*, although the relationship between DA-mediated aggregation and striatal neurodegeneration needs to be clarified. Finally, DA might play an important role in the preferential striatal vulnerability in HD through a functional interaction with mitochondria. Indeed, it has been observed that primary cultures of striatal neurons expressing mHtt are vulnerable to DA when compared to normal neurons, and this is correlated with a reduction in the levels of complex II activity (Benchoua et al., 2008).

Receptor-binding studies have also provided clues that the dopaminergic system might be involved in HD. Decreases in the levels of both D1 and D2 receptors in the post mortem HD caudate and putamen have been found using positron emission tomography, autoradiography and markers for pre and post synaptic neurons (Joyce, Lexow, Bird, & Winokur, 1988; Richfield, O'Brien, Eskin, & Shoulson, 1991; Suzuki, Desmond, Albin, & Frey, 2001; Turjanski, Weeks, Dolan, Harding, & Brooks, 1995). Reduction in the levels of striatal D1 and D2 receptors have also been quantified in asymptomatic patients carrying the HD mutation, further indicating that DA signalling is disrupted early in HD (Antonini et al., 1996; Weeks, Piccini, Harding, & Brooks, 1996). In agreement with analyses of HD patients, striatal D1 and D2 receptors are also compromised in HD mouse models (Andre, Fisher, &

Levine, 2011; Ariano et al., 2002; Bibb et al., 2000; Cha et al., 1998; Petersen et al., 2002). Striatal D1 and D2 receptor binding is reduced early, with deficiencies in DA signalling seen in R6/2 and R6/1 mice. Significant reductions also are seen in mRNA levels of striatal D1 and D2 receptors in late stage YAC128 mice, but not in BACHD mice (Pouladi et al., 2012). Because D1 and D2 receptors exist on GABA-ergic striatal interneurons (Gerfen et al., 1990), it could be argued that loss of receptor binding simply reflects degeneration of this cell population. However, it should be noted that the early selective vulnerability of neurons expressing D2 receptors (D2R) produces an imbalance between the direct and indirect pathway leading to an over-activation of D1 receptors (D1R) containing neurons (J. Y. Chen, Wang, Cepeda, & Levine, 2013). In line with this, besides the decrease in D1R levels in R6/1 mice, mHtt enhances the sensitivity of D1R-mediated signals as evidenced by similar induction of immediate early genes (IEGs) transcription compared with wild-type mice (Spektor et al., 2002). Moreover, experimental evidence from our group and others suggests that in HD, glutamate and DA signalling pathways may act synergistically to enhance the sensitivity of striatal neurons to mHtt toxicity via deregulated Cycline-dependent kinase 5 signalling (Paoletti et al., 2008) and disturbed Ca^{2+} homeostasis (Tang, Chen, Liu, & Bezprozvanny, 2007). This deleterious process occurs through D1 but not D2 receptor activation and is in agreement with previous studies demonstrating that DA and D1R agonists enhance excitotoxicity (Cepeda & Levine, 1998; McLaughlin, Nelson, Erecinska, & Chesselet, 1998). Interestingly, it has been shown that reducing DA or antagonizing D1R in an animal model of HD, the inhibitory and excitatory transmission is normalized in direct pathway neurons (Andre, Cepeda, et al., 2011; Andre, Fisher, et al., 2011). By contrast, activation of D2R is thought to be neuroprotective (Bozzi & Borrelli, 2006; Lee et al., 2002). For example, activation of D2R by quinpirole reduces the toxicity of both NMDA in rat striatal neurons (Cepeda & Levine, 1998), as well as in mesencephalic and cortical neurons (Kihara et al., 2002; Sawada et al., 1998). However, a role for D2R in mediating MSN degeneration has been put forward (Charvin et al., 2008; Charvin et al., 2005) thus raising the hypothesis that both activation of D1R and D2R might contribute to glutamate/DA dependent toxicity.

2.5.2 Mitochondrial dysfunction

Evidence for mitochondrial abnormalities in HD was first observed over three decades ago in ultrastructural studies on brain biopsies of HD patients, which revealed aberrant dense mitochondria with sparse cristae (Tellez-Nagel, Johnson, & Terry, 1974). Consistent with these findings, electron transport chain complex subunits have been reported to be involved in the selective degeneration of the basal ganglia. Indeed, significant reductions have been found in mitochondrial complex II–III and IV activities in the neostriatum from HD patients (Brennan, Bird, & Aprille, 1985; Browne et al., 1997; Gu et al., 1996), although these abnormalities were not observed in premanifest and early-stage HD patients (Guidetti et al., 2001). Reduced activity of other enzymes of oxidative metabolism in the striatum was also reported. In particular, massive loss of aconitase activity (Sorolla et al., 2008; Tabrizi et al., 1999) and reduced pyruvate dehydrogenase activity (Butterworth, Yates, & Reynolds, 1985; Sorbi, Bird, & Blass, 1983) has been found in caudate and putamen from post-mortem HD human brains. Similarly, impaired mitochondrial respiratory complexes and decreased aconitase and pyruvate dehydrogenase activity were found in R6/2 mice, suggesting that mitochondria are somehow involved in HD pathogenesis (Tabrizi et al., 2000; Zourlidou et al., 2007). These abnormalities in mitochondrial proteins related to energy metabolism not simply result from the loss of mitochondria due to neuronal loss. Experiments using the mitochondrial toxin 3-nitropropionic acid (3-NP) and studies on mitochondrial complex II support the hypothesis that complex II dysfunction in conjunction with oxidative stress damage to mitochondrial proteins might be causal in HD-linked striatal cell death (Benchoua et al., 2006; Brouillet et al., 2005). Moreover, others studies of HD knock-in striatal cells and lymphoblasts from HD patients also revealed expanded polyglutamine repeats associated with low levels of mitochondrial ATP and decreased mitochondrial ADP-uptake, suggesting that HD mutation is associated with mitochondrial functional defects (Seong et al., 2005). One of the mechanism proposed is that mHtt indirectly interferes with energy metabolism by transcriptional repression of PGC-1α (peroxisome proliferator-activated receptor gamma coactivator-1 α), a transcriptional coactivator that regulates several metabolic processes, including mitochondrial biogenesis and

oxidative phosphorylation. Consistent with this, PGC-1α levels have been found to be reduced in striatum of HD patients and mice models and its transcriptional repression has been shown to lead mitochondrial dysfunction and neurodegeneration (Cui et al., 2006; J. Lin et al., 2004). Overall, findings from these studies suggest that defective mitochondrial bioenergetics plays a large role in the progression and pathogenesis of HD.

More recently, alterations in mitochondrial dynamics have also been found in HD. For instance, in HD patients and animal models of the disease, mitochondria are excessively fragmented and show decreased motility (Bossy-Wetzel, Petrilli, & Knott, 2008), suggesting a relationship between mHtt and impaired mitochondrial dynamics and dysfunction in HD. Interestingly, mHtt can associate with the surface of mitochondria (Orr et al., 2008; Panov et al., 2002; Yu et al., 2003), although it is unclear if this interaction mediates the effects of Htt on mitochondrial morphology. Recent studies have identified Drp1 as a potential target of mHtt. It has been shown that excessive activation of Drp1 occurs in HD, although several distinct mechanisms have been proposed to underlie these findings. For instance, Htt aggregates directly bind to Drp1 *in vitro* and stimulate its GTPase activity (W. Song, 2011). In neurons expressing mHtt, Drp1 colocalizes with Htt aggregates on mitochondria and this interaction could facilitate abnormal assembly of Drp1 oligomers on the surface of mitochondria, thereby activating fission. The mitochondrial fragmentation and increased cell death that are induced by mHtt can be rescued by introducing a dominant negative form of Drp1 (W. Song, 2011; H. Wang, Lim, Karbowski, & Monteiro, 2009), supporting the idea that Drp1 activation is a major target of mHtt. Although the above studies observed normal levels of Drp1 in HD models, increased amounts of Drp1 and decreased levels of Mfns and Opa1 are found in HD patients, suggesting that mitochondrial dynamics are shifted toward fission over fusion (U. P. Shirendeb et al., 2012). Finally, increase of cytosolic Ca^{2+} levels could also aberrantly activate Drp1 through dephosphorylation by calcineurin (Costa et al., 2010). Indeed, the reduction in the levels of the calcineurin inhibitor RCAN1-1L (Ermak, Hench, Chang, Sachdev, & Davies, 2009) and the deregulation of cytosolic Ca^{2+}, the proximal activator of calcineurin (Tang et al., 2005), cooperate to increase the activity of the phosphatase in HD. This

hyperactivation produces an aberrant dephosphorylation of Drp1 increasing its mitochondrial translocation and activation, ultimately leading to fragmentation of the organelle.

Further studies on impaired Ca^{2+} homeostasis in HD have provided additional evidence of mitochondrial dysfunction. Mitochondria isolated from lymphoblasts of HD patients and from the brain of HD mice models manifest impaired Ca^{2+} uptake ability. This mitochondrial Ca^{2+} mishandling could result in an increased opening susceptibility of the PTP that would in turn lead to higher propensity to spontaneous apoptosis in the striatum and/or other affected brain regions. Interestingly, the addition of purified mHtt to isolated mitochondria has been found to increase the sensitivity to Ca^{2+} loads. The effect was corrected by cyclosporine A (CsA), a known inhibitor of PTP (Choo et al., 2004). These findings have been confirmed also in cultured neuronal cells (Milakovic et al., 2006). Particularly, in studies performed on clonal cells derived from knock-in mice (ST111/111Q), the expression of mHtt made mitochondria unable to handle large Ca^{2+} loads, very likely because of induced mitochondrial PTP opening. The observed decrease of mitochondrial Ca^{2+} appears to be a compensatory attempt to prevent the mitochondrial Ca^{2+} stress that would irreversibly damage the organelles and eventually lead to cell death (Lim et al., 2008). However, the ablation of cyclophilin D (the CsA receptor) in HD mouse models failed to induce any improvement in the disease progression, despite the increased Ca^{2+}-buffering capacity of their mitochondria (Brustovetsky et al., 2005; Oliveira et al., 2007; Perry et al., 2010). These apparently controversial findings may depend on the experimental procedure adopted and on the possible differences in the mitochondrial preparations. For instance, striatal mitochondria appear more sensitive to Ca^{2+} than the cortical counterparts (Brustovetsky et al., 2005). Further, isolated mitochondria are extracted form their physiological context loosing, for example, their native interaction with ER compartments. Indeed, a number of reports have described the involvement of ER Ca^{2+} handling in HD cellular models. Mutant Htt was shown to influence intracellular Ca^{2+} signalling by acting on the InsP3R activity and expression (Lim et al., 2008; Tang et al., 2003; H. Zhang et al., 2008), and striatal precursors of HD

neurons display increased Ca^{2+} content in the ER (Costa et al., 2010) that could compromise mitochondria Ca^{2+} handling.

Altogether, these data suggest that mHtt can impair directly or indirectly mitochondrial functions, compromising cytosolic and mitochondrial calcium homeostasis, and contributing to neuronal dysfunction and death in HD. However, the mechanisms underlying these impairments remain to be elucidated. Deregulation of signalling pathways could be critical for the pathological neuronal cell death and, especially, aberrant activity of specific kinases have been identified in multiple cell and mouse models of HD, as well as in human HD brain (Bowles & Jones, 2014). Among these, Cyclin-dependent kinase 5 (Cdk5) represents a potential mediator of the striatal degeneration in HD.

2.5.3 Cyclin-dependent kinase 5 aberrant activity

Cyclin-dependent kinases (Cdks) are Ser/Thr protein kinases that associate with specific cyclin subunits in order to be activated. This family currently includes 11 classic Cdks (Cdk1–Cdk11) and two new family members (Cdk12, Cdk13) (Malumbres et al., 2009). All Cdks phosphorylate Ser-Pro or Thr-Pro sites, with a preference for the basic residues Lys and Arg at proximal upstream and downstream positions. Cdks are highly expressed in proliferating cells and play indispensable roles in specific phases of cell cycle. However, not all CDKs have primary roles in cell cycle control. Cdk5, although highly homologous to its relatives, is a unique serine/threonine Cdk family member (Dhavan & Tsai, 2001). It is highly expressed in the brain and associated kinase activity is detected in the nervous system, where other Cdks are not expressed or active (Hellmich, Pant, Wada, & Battey, 1992; Ino, Ishizuka, Chiba, & Tatibana, 1994; Tsai, Takahashi, Caviness, & Harlow, 1993). Cdk5 neuron-specific activity is conferred based on neuron-specific expression of its activators, p35 and p39 (Humbert, Dhavan, & Tsai, 2000; Zheng, Leung, & Liem, 1998). Indeed, these two regulatory partners modulate the spatial and temporal expression of active Cdk5 and restrict its activity primarily to post-mitotic neurons. Cdk5 is involved in various cellular events, such as microtubule and actin cytoskeletal organization, cell–cell and

cell–extracellular matrix adhesions, and membrane trafficking (D. Smith, 2003; D. S. Smith & Tsai, 2002). Moreover, Cdk5 activity has been found to be crucial for neuronal migration, neuronal differentiation, axon elongation and dendrite arborisation, synapse development and synaptic functions (Cheung, Fu, & Ip, 2006). Cdk5 is also implicated in the control of neuronal survival during development and in disease; either too much or too little Cdk5 activity has been implicated in cell death induced by oxidative stress and neurotoxicity (Cheung & Ip, 2004). Recent advances in the field have provided additional mechanistic insights into the role of Cdk5 in the maintenance of neuronal survival. Identification of antiapoptotic protein Bcl-2 as a Cdk5 substrate revealed that Cdk5-mediated phosphorylation of Bcl-2 is crucial for its antiapoptotic function, which contributes to the maintenance of neuronal survival during development (Cheung, Gong, & Ip, 2008). Furthermore, Cdk5 acts early as a regulator of mitochondrial fission in the cell death cascade before the onset of mitochondrial dysfunction, and Cdk5 inhibition prevents the decline of the mitochondrial transmembrane potential (Meuer et al., 2007; Weishaupt et al., 2003). Although it has been assumed that Cdk5 exerts a minimal role in cell cycle control, several studies have also revealed that nuclear Cdk5 is crucial for the suppression of cell cycle entry, which induces neuronal death (J. Zhang et al., 2008). Thus, these findings reveal that a certain level of Cdk5 activity is necessary for maintaining neuronal survival in postmitotic neurons.

Aberrant Cdk5 activation has long been associated with the pathophysiology of numerous neurodegenerative conditions (Cheung & Ip, 2012). Excessive Cdk5 activity has been implicated in neuronal loss triggered by oxidative stress, excitotoxicity and ischemia, and in animal models of neurodegenerative diseases (Cheung & Ip, 2004). The first evidence for a degenerative role of Cdk5 came from *in vitro* studies showing that under select conditions such as with β-amyloid treatment of cultured neurons in vitro, the activator of Cdk5, p35 can be cleaved to a smaller more stable p25 form (Patrick et al., 1999). This formation of p25 is dependent upon activation of the Ca^{2+}-regulated protease, calpain and results in a mislocalisation and an aberrant prolonged activation of Cdk5. Indeed, overexpression of p25 has been reported to induce neuronal death (Ahlijanian

et al., 2000; Cruz, Tseng, Goldman, Shih, & Tsai, 2003). Cdk5 activity can be further tweaked by post-translational modifications such as phosphorylation. The kinases Abl, ephrin receptor A (EphA) and Fyn phosphorylate Cdk5 at Tyr15, leading to its activation, thereby promoting neuronal migration, neurite outgrowth and synaptogenesis (Cheng et al., 2003; Sasaki et al., 2002; Zukerberg et al., 2000). Considerably, it has been reported that Abl-mediated phosphorylation of Tyr15 can also hyperactivate Cdk5, resulting in neurodegeneration (Cancino et al., 2011; H. Lin, Lin, & Juang, 2007). Although it has been well established that Cdk5–p25 activation exerts a detrimental role in Alzheimer and Parkinson's diseases, less is known on the Cdk5 involvement in HD pathology. Indeed, literature supporting a role for Cdk5 in HD is quite recent. Immunoprecipitation experiments demonstrated that Htt interacts and co-localizes with Cdk5 in cellular membrane fractions, but not with its co-activator p35. Cdk5 lowers Htt aggregation by phosphorylating the protein at Ser434, which reduces its cleavage by caspases. Cdk5 activity is reduced in the brain of N171-82Q HD transgenic mice compared with controls, probably because polyQ-expanded Htt fragment interferes with Cdk5–p35 association. Therefore, these data predict that the ability of Cdk5 phosphorylation to protect against Htt cleavage, aggregation, and toxicity is compromised in cells expressing toxic fragments of Htt (Luo, Vacher, Davies, & Rubinsztein, 2005). Another study revealed that Htt can be phosphorylated by Cdk5 at serines 1181 and 1201 (Anne, Saudou, & Humbert, 2007). Importantly, whereas the absence of Htt phosphorylation at these residues confers toxic properties to wild-type-Htt in a p53-dependent manner, these phosphorylations protects against polyQ-induced toxicity. These phosphorylations are crucial to regulate neuronal cell death through the p53 pathway. Moreover, the authors demonstrated that sustained DNA damage in late stages of HD associates with a decrease in Cdk5/p35 levels, causing a reduction in htt phosphorylation at Ser1181 and 1201 and accelerating p53-mediated neuronal death (Anne et al., 2007). Additionally, it has been reported that Cdk5 inhibits aggregation of mHtt by disrupting the microtubule network (Kaminosono et al., 2008). These findings collectively suggest that Cdk5 activity is required to limit mHtt toxicity and may serve a protective role in HD. However, emerging evidence also associate the deregulation of Cdk5 activity with the striatal degeneration process of the

disease. A study has demonstrated that intraperitoneal injection of 3-NP in rats, as an experimental model of HD, induces calpain activation that in turns increases the levels of p25 and therefore increases the activity of Cdk5 in the striatum. Moreover, the strong activation of Cdk5 by calpain reduces the neuroprotective effect mediated by MEF2 (Crespo-Biel et al., 2007). Interestingly, lithium reduces 3-NP mediated striatal neurodegeneration in rats inhibiting calpain activation, thereby avoiding p25 generation and Cdk5 aberrant activity (Crespo-Biel, Camins, Pallas, & Canudas, 2009). Finally, a study from our group identifies p25/Cdk5 as an important mediator of dopamine and glutamate neurotoxicity associated to HD (Paoletti et al., 2008) (Fig. 12). We have reported an elevated p25/p35 ratio in striatal ST111/111Q cells and in human brain samples of HD patients, suggesting enhanced activation of this kinase. This was attributed to substantially higher p25 expression and increased phosphorylation of Tyr15 in these models, which may be pushing Cdk5 activity towards a pro-apoptotic function rather than a neuroprotective one. Increased Cdk5 activity also sensitised neuronal cells to stimulation with D1R agonist, therefore enhancing vulnerability to excitotoxicity. However, the mechanism by which this kinase mediates the striatal vulnerability remains to be clarified. It has been proposed that p25/Cdk5-mediated hyperphosphorylation of Tau may be involved in the vesicular trafficking deficiencies associate to HD pathology (Paoletti et al., 2008).

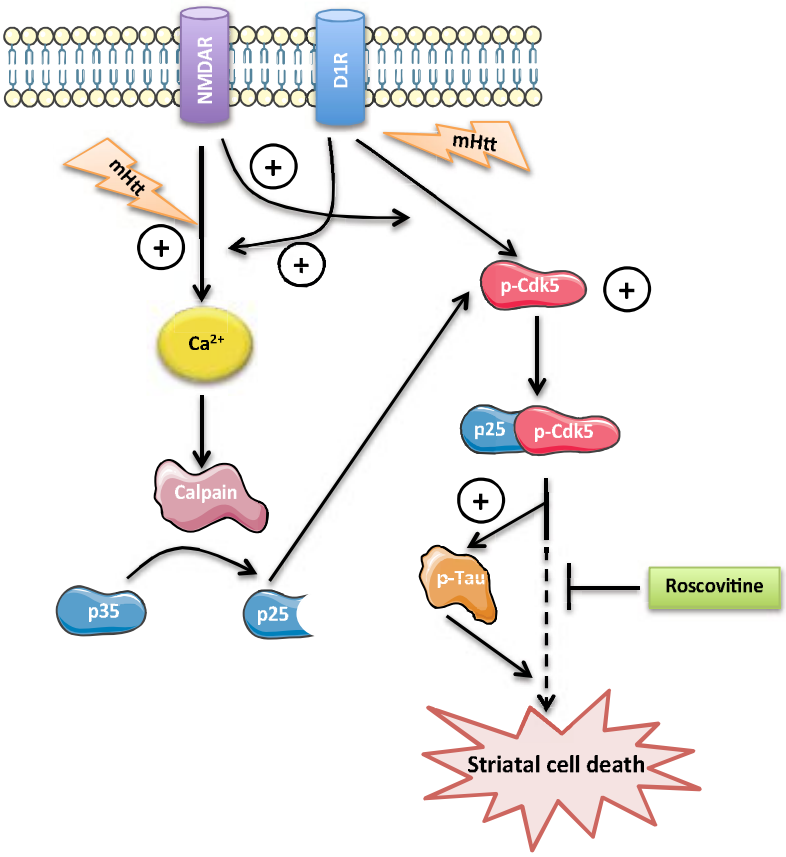

Figure 12. Increased sensitivity of mHtt striatal cells to glutamate and dopamine receptor activation involves enhanced Cdk5 activity. Enhanced activation of NMDAR and D1R induced by mHtt leads to deregulation of calcium homeostasis. Increased intracellular calcium in turn leads to higher calpain activity that results in enhanced cleavage of p35 into p25. In addition, activation of NMDAR may potentiate D1R-mediated phosphorylation of Cdk5 at Tyr 15, which has been shown to regulate Cdk5-induced neurotoxicity. The generation of p-Cdk5/p25 complexes implies Cdk5 hyperactivation that is responsible for the phosphorylation of new substrates such as tau protein. Hyperphosphorylation of tau could contribute to striatal cell death and dysfunction and therefore to HD neuropathology. Roscovitine, a Cdk5 inhibitor, prevent cellular death induced by NMDAR and D1R activation. Adapted from (Paoletti et al., 2008)

The reasons for the specific loss of medium spiny striatal neurons in HD remain obscure. Compelling evidence suggest that mitochondrial defects may play a crucial role in the disease, however, it is still debated whether mitochondrial dysfunction represents just an epiphenomenon of the cellular degeneration or it has an actual pathogenic role. In this Thesis, the general purpose was to identify possible mitochondrial impaired mechanisms and to investigate their role in the selective striatal vulnerability of HD, in order to provide important insights for the development of new therapeutic strategies.

The first aim was to clarify whether mitochondrial injuries perpetrate the dopaminergic neurotoxicity in HD striatal degeneration. Since Cdk5 has been proposed as a critical regulator of mitochondrial fission (Meuer et al., 2007) and as a deleterious player in the striatal vulnerability upon dopamine signalling (Paoletti et al., 2008), we hypothesize a new detrimental role for Cdk5 in HD pathology by mediating dopaminergic neurotoxicity through deregulation of mitochondrial dynamic processes.

On the other hand, several studies have proposed mitochondrial Ca^{2+} mishandling as a component of Ca^{2+} dyshomeostasis in HD. However, the results appear controversial and a conclusive cause remains uncertain. We propose that the propensity of mitochondria to undergo fragmentation in HD could result in the disruption of ER-mitochondria contacts, which are essential for the proper buffering of Ca^{2+} by mitochondria. Therefore, the second aim of this study was to investigate whether defects in ER-mitochondria associated membranes could be responsible for the alteration of mitochondrial Ca^{2+} handling in HD.

The main aims of this Thesis are:

1. To identify the molecular mechanisms by which Cdk5 increases striatal vulnerability in knock-in models of HD.

1.1 To analyse mitochondrial morphology and distribution in cellular and murine models of HD.

1.2 To analyse changes in the levels and activity of proteins involved in mitochondrial fission/fusion processes in cellular and murine models of HD.

1.3 To explore the role of mHtt-induced aberrant Cdk5 activity in the regulation of mitochondrial dynamics processes in cellular models of HD.

1.4 To determine whether mHtt-induced aberrant Cdk5 activity increases striatal susceptibility to dopamine through mitochondrial dysfunction in cellular models of HD.

2. To investigate the role of mitochondria associated membranes (MAMs) in altered Ca^{2+} signalling in HD.

2.1 To analyse the contact sites between mitochondria and ER in striatal primary neurons of mouse models of HD.

2.2 To analyse changes in the levels of proteins involved in the formation of MAMs and in Ca^{2+} homeostasis in striatal tissue from murine models of HD and from post-mortem brain of HD patients.

2.3 To assess the ER-mitochondria Ca^{2+} transport in striatal primary neurons of mouse models of HD.

METHODS

1. Huntington's disease mouse models

For the development of the objectives of this Thesis, we have used two different mouse models of HD. The first one is the HdhQ111 knock-in mouse model. These mice were maintained on a C57BL/6 genetic background and present targeted insertion of 109 CAG repeats that extends the glutamine segment in murine huntingtin to 111 residues. Male and female Hdh7/111Q heterozygous mice were intercrossed to generate age-matched Hdh7/111Q heterozygous and Hdh7/7Q wild-type littermates. The second one is the R6/1 mouse model. These mice expressing the exon 1 of mHtt with 145 CAG repeats (Giralt et al., 2011), were originally obtained from Jackson Laboratory (Bar Harbor, ME, USA) and maintained in a B6CBA background. No transgenic wild-type littermates animals were used as the control group. All mice used in the present Thesis were males and were housed together in numerical birth order in groups of mixed genotypes, and data were recorder for analysis by microchip mouse number. The animals were housed with access to food and water *ad libitum* in a colony room kept at 19-22°C and 40-60% humidity, under a 12:12 hours light/dark cycle. All animal-related procedures were performed in compliance with the National Institute of Health Guide for the care and use of laboratory animals, and approved by the local animal care committee of the Universitat de Barcelona (99/01), and the Generalitat de Catalunya (00/1094), in accordance with the Directive 2010/63/EU of the European Commission.

2. Cell cultures

Conditionally immortalized wild-type STQ7/Q7 and mutant ST111/111Q striatal neuronal progenitor cell line expressing endogenous levels of normal and mutant Htt with 7 and 111 glutamines, respectively, were used. These cells, derived from striatal precursors isolated from HdhQ7 and HdhQ111 mice at embryonic day 14, were immortalized with the SV40 Large T antigen containing the tsA58/U19 temperature-sensitive mutations and the neomycin resistance gene (Trettel et al., 2000), and were grown at 33°C in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co, St. Louis, MO, USA)

supplemented with 10% fetal bovine serum, 1% streptomycin-penicillin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 400 µg/ml neomycin analogue G418 (Geneticin; Gibco, Life Technologies; Carlsbad, CA, USA).

3. Primary cultures of mouse striatal neurons

Dissociated striatal cultures prepared from E.18 Hdh7/7Q, Hdh111/111Q and wild-type and R6/1 embryos were plated at different densities, depending on the type of experiment, onto plates pre-coated with 0.1 mg/ml poly-D-lysine (Sigma Chemical Co., St. Louis, MO, USA). Neurons were cultured in Neurobasal medium (Gibco-BRL, Renfrewshire, Scotland, UK), supplemented with B27 (Gibco-BRL) and GlutamaxTM (Gibco-BRL). Cultures were maintained at 37ºC in a humidified atmosphere containing 5 % CO2.

4. Human brain tissue

Samples of human putamen (six controls and four HD patients) were obtained from the Neurological Tissue Bank of the Biobanc-Hospital Clinic-IDIBAPS, Barcelona (NTB-Biobanc-HC-IDIBAPS) following the guidelines of the local ethics committees (Table 2). All the ethical guidelines contained within the latest Declaration of Helsinki were taken into consideration and informed consent was obtained for all the subjects under study.

Case n°	Pathological diagnosis	CAG repeats	Gender	Age (years)
1	Normal	-	Male	31
2	Normal	-	Female	68
3	Normal	-	Male	56
4	Normal	-	Male	64

5	Normal	-	Female	71
6	Normal	-	Female	60
7	HD,Vonsattel grade 4	62	Female	28
8	HD,Vonsattel grade 4	44	Male	59
9	HD,Vonsattel grade 4	43	Male	60
10	HD,Vonsattel grade 3-4	-	Male	55

Table 2. Human post-mortem Huntington's disease (HD) brains. Among the post-mortem brains that were provided by the NTB-Biobanc-HC-IDIBAPS, 6 were histopathologically non-related to Huntington's disease, 3 were histopathologically diagnosed with Vonsattel grade 4 and 1 with Vonsattel grade 3-4.

5. Drug treatment

Cells were treated with various drugs at different concentrations and times depending on the planned experiment. In order to activate dopaminergic signalling through D1 receptor (D1R), wild-type ST7/7Q and mutant ST111/111Q striatal cells were serum depleted (2,5% FBS) for 24h and then exposed to Locke's solution (154 mM NaCl, 5.6 mM KCl, 2.3 mM CaCl2, 3.6 mM NaHCO3, 5 mM HEPES, 5.6 mM glucose, and 10 μM glycine) for 30 min before the addition of D1R agonist SKF 38393, 30 or 60 μM (Sigma Aldrich) in fresh DMEM medium (2.5% FBS). To prevent SKF 38393 effect, cultures were treated with the D1R antagonist SCH 23390 10 μM (Sigma Aldrich) 1 h before SKF 38393 treatments. Total extracts were obtained 60 min after SKF 38393 treatment for later analysis. To inhibit Cdk5 activity, cultures were treated with the Cdk5 inhibitor roscovitine, 20 μM (Sigma-Aldrich) 1 h before SKF 38393 treatments. Cells were then fixed or harvested for later analysis.

6. Plasmids and transfection

6.1 Striatal primary culture

Fluorescent labeling of mitochondria and endoplasmic structures was obtained by transfection of striatal primary cultures with pDsRed2-Mito and GFP-Sec61-β constructs, respectively. The pDsRed2-Mito vector (Clontech Laboratories Inc, Mountain View, CA, USA; no. 632421) encodes a fusion of *Discosoma sp.* red fluorescent protein (DsRed2) and a mitochondrial targeting sequence of human cytochrome c oxidase subunit VIII (Mito). The GFP-Sec61-β vector, expressing the ER membrane protein Sec61β tagged with the green-fluorescent protein (GFP), was kindly supplied by Dr. Rapoport through the Addgene platform (Addgene, Cambridge, MA, USA; no. 15108). To expand the plasmids, E. Coli (Subcloning Efficiency™ DH5α™ Competent Cells; Invitrogen, Life Technologies, Carlsbad, CA, USA) was transformed by thermal shock. Each plasmid (250 ng) was added to 50 µl-cultured bacteria and left incubating on ice for 15 min. To induce transformation, the mixture was incubated for 1.5 min at 42ºC and quickly placed again on ice for 2 min. Lysogeny broth media (LB; 800 µl) was added, and was incubated for 1h at 37ºC at 250 rpm to let transformed E. Coli grow. Finally, the transformation (200 µl) was spread on a pre-warmed agar plate containing 20 µg/ml kanamycin. Colonies were left growing overnight at 37ºC. Plasmid isolation was performed from 5 ml bacterial culture using the Wizard Plus SV Minipreps and Maxipreps DNA purification system from Promega (Madison, WI, USA). Transfection procedures were carried out using Lipofectamine 3000 (Invitrogen, Life Technologies) as instructed by the manufacturer. Striatal primary cultures were transfected with 1µg of both plasmids at day 0 and later fixed and analysed at day 14.

6.2 Cell culture

To suppress Cdk5 expression, striatal cells were transfected using Lipofectamine 2000 (Invitrogen, Life Technologies) with the appropriate antisense oligonucleotides (sc-35047; Santa Cruz Biotechnology, Santa Cruz, CA) or with a scramble control (sc-37007; Santa Cruz Biotechnology) and harvested for Western blot or fixed for immunocytochemistry analysis 48 hours post-transfection.

7. Protein extraction

7.1 Mouse and human brain tissue

Animals were killed by cervical dislocation at different steps of the disease. The brain was quickly removed and the striatum was quickly dissected out. All mouse and human samples were homogenized by sonication in lysis buffer containing 1% Triton X-100, 50 mM Tris-HCl (pH 7.5), 10 mM EGTA, 150 mM NaCl, protease inhibitor mixture (Sigma-Aldrich) and phosphatase inhibitor 1 mM sodium othovanadate.

7.2 Cell culture

STQ7/Q7 and ST111/111Q cells were washed once with phosphate-buffered saline (PBS; 140 mM NaCl, 2 mM KCl, 1.5 KH2PO4, 8 mM NaH2PO4, pH 7.4) and total cellular proteins were extracted by harvesting cells in 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 mixture (Sigma-Aldrich).

All the lisates were centrifuged at 10,000 x g for 10 min at 4ºC, and supernatants were collected. Protein concentration was determined using the Detergent-Compatible Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

8. Subcellular fractionation

For subcellular fractionation, cells or fresh tissue were washed twice with ice cold PBS and homogenized with a Potter-Elvehjem homogenizer in isolation buffer (IB; 250 mM sucrose, 20 mM HEPES-KOH pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA) with protease and phosphatase inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 mg/ml aprotinin and 10 mg/ml leupeptin). Homogenates were then centrifuged at 800 ×g for 10 min at 4 °C to remove nuclei and unbroken cells. The

supernatants were centrifuged again at 10,000 ×g for 20 min at 4°C to pellet the heavy membrane fractions containing mitochondria. The pellet fraction was washed, spun down again at 10,000 ×g, resuspended in IB containing 1% Triton X-100 and finally saved at−80 °C for later analysis. The cytosolic fraction was obtained as supernatants by further centrifugation at 16,000 ×g for 20 min at 4 °C to remove residual mitochondria and saved at −80 °C for later analysis.

9. Western blot analysis

Western blot analysis was performed following a standard protocol. Protein extracts (10–20 µg) were denatured in 62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% glycerol, 140 mM β-mercaptoethanol and 0.1% (w/v) bromophenol blue and heated at 100ºC for 5 min. They were resolved in denaturing polyacrylamide gels (SDS-PAGE) at different polyacrylamide concentrations; at 35 mA during 1 h. Proteins were then transferred to a nitrocellulose membrane (Whatman Schleicher & Schuell, Keene, NH, USA) during 1.5 hours at 100 V and at 4ºC to avoid excessive warming. Nitrocellulose membranes were blocked in 10% non-fat powdered milk in Tris-buffered saline Tween-20 (TBS-T; 50 mM Tris-HCl, 150 mM NaCl, pH 7.4, 0.05% Tween 20) during 1h at room temperature. Membranes were washed twice in TBS-T and blotted overnight at 4ºC with the following primary antibodies (Table 3):

Antigen	Molecular weight	Host species	Dilution	Source
Cdk5-J3	35 kDa	Mouse	1:1000	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
Drp1	79-84 kDa	Mouse	1:1000	BD Transduction Laboratories (San Jose, CA, USA)
Drp1 (pSer616)	79-84 kDa	Rabbit	1:1000	Cell Signaling (Beverly, MA, USA)
Grp75	75 kDa	Rabbit	1:500	Cell Signaling (Beverly, MA, USA)

IP3R3	300 kDa	Mouse	1:500	BD Transduction Laboratories (San Jose, CA, USA)
Mnf2	86 kDa	Mouse	1:2000	Abcam (Cambridge,UK)
Opa1	80-100 kDa	Mouse	1:8000	BD Transduction Laboratories (San Jose, CA, USA)

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

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

Secondary antibody	Dilution	Source
Anti-Mouse IgG	1:3000	Promega (Madison, WI, USA)
Anti-Rabbit IgG	1:3000	Promega (Madison, WI, USA)

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

Membranes were washed again three times for 10 min to remove secondary antibody remains and the reaction was finally visualized using the enhanced chemiluminescence ECL kit (Santa Cruz Biotechnology). Western blot replicates were scanned and the intensity of immunoreactive bands was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA). For protein loading control, membranes were incubated 15 min with an antibody against α-tubulin or α-actin. CoxV (incubation of 1 h) was used for loading control in mitochondrial fraction (Table 5).

Antigen	Molecular weight	Host species	Dilution	Source
α-Actin	45 kDa	Mouse	1:20000	MP Biomedicals (Irvine, CA, USA)
α-Tubulin	55 kDa	Mouse	1:50000	Sigma Chemical Co (St. Louis, MO, USA)
CoxV	17 kDa	Mouse	1:3000	Molecular Probes Inc. (Eugene, OR, USA)

Table 5. Primary antibodies used for loading controls in Western blot. A list of primary antibodies is provided, as well as their source and the dilution that was used for Western blot. All these antibodies were used to detect loading control proteins.

10. Immunocytochemistry

Striatal cells were grown on cover glasses and fixed in 4% paraformaldehyde (ElectronMicroscopy Science EMS, Hatfield, PA, USA) for 10 min, rinsed in PBS, treated with 0.1 M Glycine for 20 min, and then permeabilized in 0.1% saponin for 10 min. Notably, for detection of D1 receptor on cell surface, the permeabilization with saponin was omitted. Blocking was done in 1% bovine serum albumin in phosphate-buffered saline for 1 h. Specimens were incubated with primary antibody diluted in the same blocking buffer for 2 h at room temperature or overnight at 4°C (Table 6). For immunocytochemical experiments in primary cultures of Hdh7/7Q and Hdh111/111Q striatal neurons, eight days after plating, neurons were fixed with 4% PFA/phosphate buffer for 10 min, rinsed in PBS, blocked in PBS containing 0.1 M glycine for 10 min and permeabilized in PBS containing 0.1% saponin for 10 min and blocked in PBS containing Normal Horse Serum 15% for 30 min at room temperature. Neurons were then washed in PBS and incubated with primary antibodies (Table 6).

Antigen	Incubation time	Host species	Dilution	Source
Dopamine receptor D1	o/n at 4°C	Rabbit	1:500	Abcam (Cambridge,UK)
MAP2	o/n at 4°C	Mouse	1:500	Sigma Chemical Co (St. Louis, MO, USA)

TOM20	2 h at RT	Rabbit	1:250	Santa Cruz Biotechnology (Santa Cruz, CA, USA)

Table 6. Secondary antibodies for immunofluorescence. A list of secondary antibodies is provided, as well as their source, the incubation time and temperature and the dilution that were used for immunofluorescence.

Remaining primary antibody was removed in three consecutive washes with PBS and specimens were incubated during 1 h at room temperature with the subtype-specific fluorescent secondary antibody and Phalloidin-conjugated TRITC to stain cell structure (Table 7). To stain nuclei, cells were incubated during 5 min at room temperature with Hoechst 33258 (1:10,000; Invitrogen, Carlsbad, CA, USA) and washed afterwards with PBS. Finally cover glasses were mounted with Mowiol-mounting media (Merck, Darmstadt, Germany).

Antigen	Dilution	Source
Alexa Fluor® 488 AffiniPure Donkey Anti-Rabbit IgG (H+L)	1:100	Jackson ImmunoResearch (West Grove, PA, USA)
Cy™3 AffiniPure Donkey Anti-Mouse IgG (H+L)	1:100	Jackson ImmunoResearch (West Grove, PA, USA)
Phalloidin-TRITC	1:1000	Sigma Chemical Co (St. Louis, MO, USA)

Table 7. Primary antibodies used for immunofluorescence. A list of primary antibodies is provided, as well as their source and the dilution that was used for immunofluorescence.

11. Confocal image uptake and analysis

Immunofluorescence was analysed by confocal microscopy using a Leica TCS SP5 laser scanning spectral confocal microscope (Leica Microsystems Heidelberg GmbH, Manheim, Germany). Confocal images were taken using a HCX PL APO lambda blue 63.0x 1.40 OIL UV objective with a standard (one Airy disk) pinhole.

11.1 Mitochondrial morphology

For the analysis of mitochondrial morphology in striatal cells and primary striatal cultures, images were taken using a 63× numerical aperture objective with a 3.0 digital zoom and standard pinhole. For each cell/neuron, the entire three-dimensional stack of images from the ventral surface to the top of the cell was obtained by using the Z drive in the Leica TCS SP5 microscope. The size of the optical image was 0.4 µm. For the quantitative analysis, digital images were processed through a convolve filter to obtain isolated and equalized fluorescent pixels and then to a thresholding step using ImageJ software. This procedure yields a binary image containing black mitochondrial structures on a white background (Fig. 13).

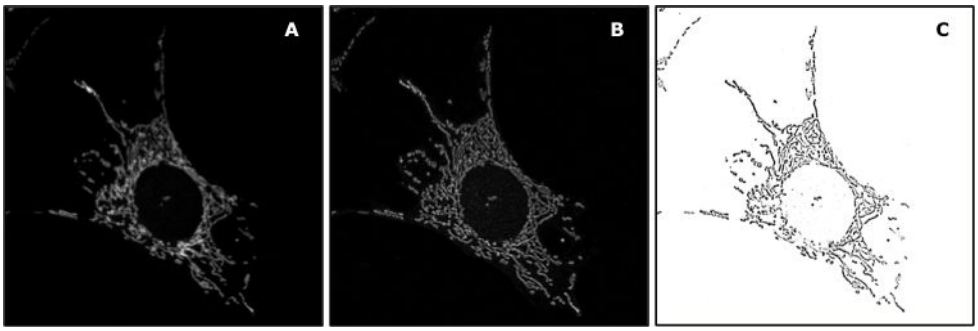

Figure 13. Digital confocal image processing. Original digital images (A) were subjected to convolve filters (B) and tresholding (C) to isolate and equalize fluorescent pixels in the image and subsequently quantify and analyse single particles.

From this binary image, individual mitochondria (particles) were subjected to particle analyses to acquire Form Factor (FF, (4π × Am/Pm2) where Pm is the length of mitochondrial outline and Am is the area of mitochondrion) and Aspect Ratio values (AR, the ratio between the major and minor axis of the ellipse equivalent to the mitochondrion) as well as the number of mitochondria per cell. An AR value of 1 indicates a perfect circle, and as mitochondria elongate and become more elliptical, AR increases. A FF value of 1 corresponds to a circular, unbranched mitochondrion, and higher FF values indicate increase of mitochondrial complexity (length and branching). For determination of the percentage of cells with fragmented mitochondria, a cell was determined to have fragmented mitochondria if it had ≥50% of its mitochondria with length/width (axis) ratios <2.5. In average 25–30

cells/genotype were analysed from 3 to 6 independent experiments. For the analysis in neurons, the number of mitochondria per micron of axon was measured using ImageJ software.

11.2 ER-mitochondria co-localization

Images for investigating ER–mitochondria contact sites were taken using a 63× numerical aperture objective with a 2.0 digital zoom and standard pinhole. For each pDsRed2-Mito and GFP-Sec61-β transfected neuron, the entire three-dimensional stack of images from the ventral surface to the top of the neuron was obtained by using the Z drive in the Leica TCS SP5 microscope. The size of the optical image was 0.2 µm. Stacks were automatically thresholded, deconvolved and the background was subtracted using ImageJ software. Interactions between organelles were quantified by Manders' co-localization coefficient obtained from ImageJ plugin JACoP.

12. Drp1 immunoprecipitation and GTPase enzymatic activity assay

Wild-type ST7/7Q and mutant ST111/111Q cells were washed with cold PBS and incubated on ice in lysis buffer (20 mM Tris [pH 7.5], 0.6% CHAPS, 10% glycerol, 1mMsodium orthovanadate and protease inhibitor cocktail) for 15 min. Cells were scraped and then disrupted 10 times by repeated aspiration through a 25-gauge needle. Samples were centrifuged at 10,000 ×g for 10 min and the supernatant was saved as the whole cell extract. To determine GTPase activity of Drp1, a total of 400 µg of whole-cell extract was immunoprecipitated overnight with 25 µg of anti-Drp1 antibody (BD Bioscience) and 40 µl of protein A/G agarose (Santa Cruz Biotechnology). For the analysis of Drp1 enzymatic activity in Hdh mice, frozen striatal tissues from heterozygous mutant Hdh7/111Q and wild type Hdh7/7Q mice at the age of 8 months were washed with cold PBS and incubated on ice in lysis buffer (20 mM Tris [pH 7.5], 0,6% CHAPS, 10% glycerol, 1mMsodium orthovanadate and protease inhibitor cocktail) for 15 min. Tissue was homogenized with a Potter-Elvehjem homogenizer and the lysate was centrifuged at 10,000 ×g for 10 min and the supernatant was saved as the

whole tissue extract. To determine GTPase activity of Drp1, a total of 400 µg of whole-tissue extract was immunoprecipitated overnight with 25 µg of anti-Drp1 antibody (BD Bioscience) and 40 µl of protein A/G-agarose (Santa Cruz Biotechnology). GTPase activity of the protein was determined using a GTPase assay kit (Novus Biologicals, Littleton, CO, USA) according to manufacturer's instructions. After three washes with lysis buffer and three washes with GTPase buffer (50 mM Tris [pH 7.5], 2.5 mM MgCl2, and 0.02% 2-mercaptoethanol), the beads were incubated with 0.5 mM GTP at 30 °C for 1 h. Drp1 hydrolyses GTP to GDP and inorganic phosphorous (Pi) and we measured GTPase activity, based on the amount of inorganic phosphorous that the GTP produces. By adding the ColorLock Gold (orange) substrate to the Pi that is generated from GTP, we assessed GTP activity, based on the inorganic complex solution (green). Colorimetric measurements (green) were read in the wavelength range of 650 nm using a Synergy 2Multi-ModeMicroplate Reader (BioTek Instruments, Inc.; Winooski, VT, USA).

13. Real-time quantitative RT-PCR

Total RNA was isolated from the wild-type ST7/7Q and mutant knock-in ST111/111Q striatal cell lines using the total RNA isolation Nucleospin RNA II Kit (Macherey-Nagel, Düren, Germany). 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 analysed by quantitative RT-PCR using PrimeTime qPCR Assays from Integrated DNA Technologies (Coralville, IA, USA) (Table 8). RT-PCR was performed in 12 µl of final volume on 96-well plates using the Premix Ex Taq (Probe qPCR) (Takara Bio Inc., Shiga, JP). 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. Results were normalized to cDNAs of wild-type ST7/7Q cells and expressed as a fold change of these data.

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)
Dnm1l	Mm.PT.56a.16160059	Integrated DNA Technologies (Coralville, IA, USA)

Table 8. Gene expression probes. A list of probes, together with their source, is provided.

14. Monitoring dynamic changes in Ca_i^{2+} and $\Delta\Psi_m$

To detect cytosolic Ca^{2+} changes (Ca_i^{2+}), wild-type and R6/1 primary striatal neurons, cultured on glass coverslips during 14 days, were washed with Krebs buffer (145 mM NaCl, 5 mM KCl, 10 mM HEPES, 1 mM MgCl2, 1 mM CaCl2, 5.6 mM glucose and pH 7.4/NaOH) and loaded with 5 µm Fluo-4 AM/0.02% Pluronic F-127 (Molecular Probes, Invitrogen) at room temperatute in the dark for 30 min. After rinsing with fresh buffer, cells were loaded with 20 nM tetramethylrhodamine methyl ester (TMRM; Molecular Probes, Invitrogen) at room temperature in the dark for 20 min in order to detect changes in mitochondrial membrane potential ($\Delta\Psi_m$). This low concentration of the dye (nonquenching mode) leads that depolarized (less negative) mitochondria will have lower cationic dye concentrations and lower fluorescence, and hyperpolarized (more negative) mitochondria will have higher dye concentrations and fluorescence. Then, cells were washed and coverslip were assembled in a chamber filled with 500 µl of buffer containing TMRM on the stage of the Leica confocal microscope described previously equipped with an incubation system with temperature and CO_2 control. After a 5 min equilibration time, neurons were exposed to 0.5 µM Thapsigargin (TG; Sigma-Aldrich), a SERCA ATPase inhibitor that increases the concentration of

intracellular Ca^{2+} by blocking its storage in the ER. Simultaneously, TMRM was excited at 543 nm and the emission collected with a 560 nm long-pass filter, and Fluo-4 was excited at 488 nm and the emission was collected through a 505–550 nm barrier filter. Experiments were terminated inducing a maximal mitochondrial depolarization by addition of 2 μM Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP; Sigma-Aldrich), an uncoupler of respiratory chain oxidative phosphorylation that depolarizes mitochondrial membrane determining Ca^{2+} release into the cytosol. Images were captured every 2 sec throughout a 10 min experiment. The quantification of fluorescence intensities was calculated using ImageJ software and the values were normalized to the baseline images. Fluo 4 and TMRM fluorescence changes are presented as fold change of normalized response F1/F0 (F0 is the fluorescence intensity at time zero; F1 is the corrected fluorescence intensity at a given time point).

15. Statistical analysis

All the data were analyzed with the program GraphPad Prism version 5.0a (Graph Pad Software). Results were expressed as the mean ± SEM. Statistical analysis was performed using the Student's t test or the one-way ANOVA, followed by Newman's Keuls post hoc test as appropriate and indicated in the figure legends. A 95% confidence interval was used and values of $p < 0.05$ were considered as statistically significant.

RESULTS

1. MOLECULAR MECHANISMS BY WHICH CDK5 INCREASES STRIATAL VULNERABILITY IN KNOCK-IN MODELS OF HD

Huntington's disease (HD) is associated with progressive degeneration of the striatum. This pathological feature of HD have recently been linked to the deregulation of Cdk5. Although, it has been reported that aberrant activity of this kinase is neurotoxic, the molecular basis underlying the contribution of Cdk5 to neurodegeneration in HD remain to be elucidated. Here, we wondered whether Cdk5 could lead to striatal degeneration by altering mitochondrial function. To this aim, we have examined the role of Cdk5 in mitochondrial dysfunction in cellular and murine HD models. Especially, we have explored how altered Cdk5 activity may participate in HD pathology by increasing striatal susceptibility to D1R activation through deregulation of mitochondrial dynamic processes.

1.1 Mitochondrial morphology and distribution in cellular and murine models of HD

1.1.1 Mutant ST111/111Q striatal cells exhibit aberrant mitochondrial dynamics

Abnormalities in mitochondrial dynamics have been suggested to occur as early events during the pathogenesis of HD (Bossy-Wetzel et al., 2008) with mitochondrial fragmentation and dysfunction observed in different HD models (Costa et al., 2010; Oliveira et al., 2007). To validate these antecedents in our HD striatal cell model, mitochondrial morphology was evaluated. Wild-type ST7/7Q and mutant ST111/111Q huntingtin striatal cells were stained with TOM20 and phalloidin-TRITC and mitochondrial population was analysed by confocal microscopy (Fig. 14). Morphometric analysis revealed that mutant ST111/111Q striatal cells display significant differences in the mitochondrial morphology compared to wild-type ST7/7Q cells. Accordingly, the values of the Aspect Ratio and Form Factor demonstrated reduced mitochondrial length (~10% decrease; $p<0.05$. Fig. 15A) and lower mitochondrial complexity and decreased branching, respectively (~14%; $p<0.05$. Fig.15B). These alterations correlated in mutant cells with a

significant increase in the number of mitochondria per cell (~15%; p<0.01. Fig. 15C) Moreover, when the percentage of cell population showing mitochondrial fragmentation was analysed more than 40% of mutant ST111/111Q striatal cells exhibited fragmented mitochondria whereas only 20% of wild-type ST7/7Q cells showed this mitochondrial fission morphology (p<0.01; Fig. 15D). Altogether, these findings suggest a toxic effect of mHtt on mitochondrial dynamics.

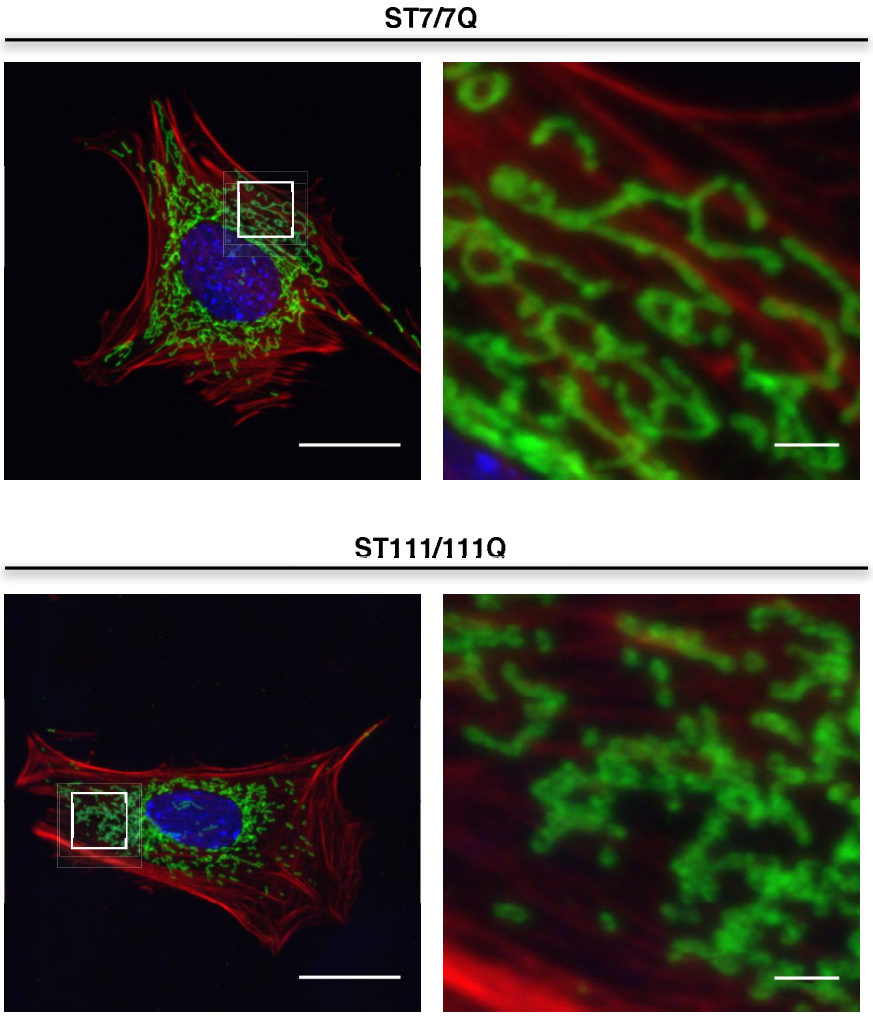

Figure 14. ST111/111Q mutant cells show increased mitochondrial fragmentation. Representative confocal images showing mitochondrial morphology in wild-type ST7/7Q and mutant ST111/111Q cells immunostained with anti-TOM20 (green), anti-Phalloidin-TRITC (red) and Hoechst stain (blue); scale bar 20 μm. The boxed areas are enlarged in the panels on the right; scale bar 2 μm.

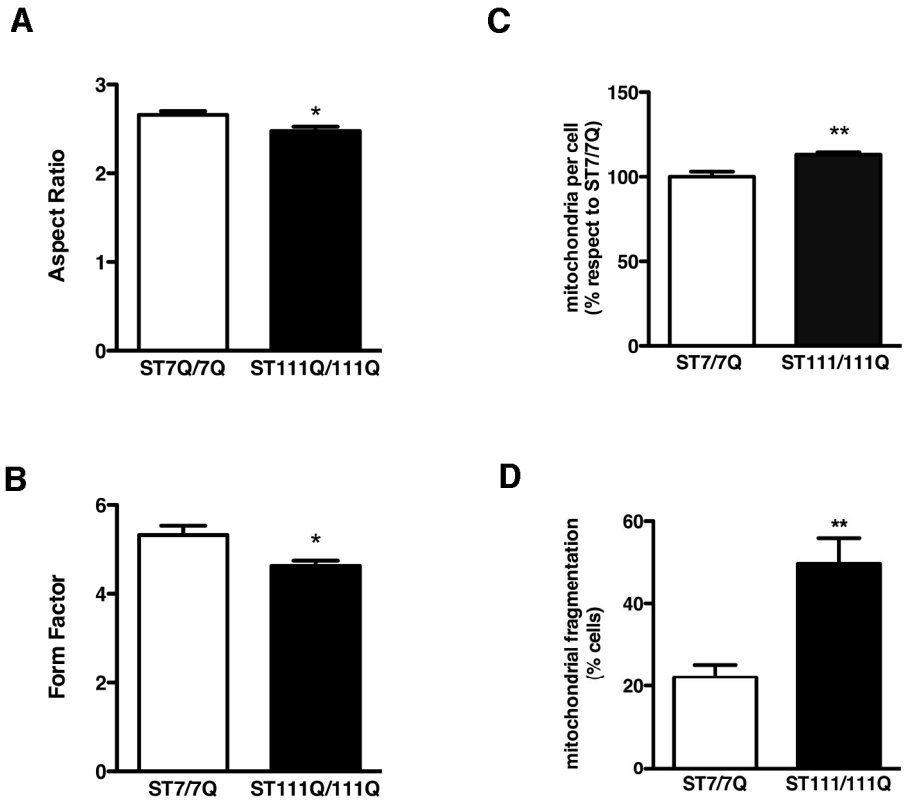

Figure 15. Mitochondrial morphometric analysis reveals smaller and unbranched mitochondria in ST111/111Q respect to those from ST7/7Q cells. (A, B) Bar histogram showing the Aspect Ratio and Form Factor values. *p<0.05 as determined by unpaired Student's t-test. (C) Bar histogram showing the number of mitochondria per cell. *p<0.05 as determined by unpaired Student's t-test. (D) Bar histogram showing the percentage of cells with fragmented mitochondria relative to the total number of cells. **p <0.01 as determined by unpaired Student's t-test. Data represent mean ±SEM of 6 independent experiments in which 25–30 cells/genotype were analysed with ImageJ software.

1.1.2 Hdh7/111Q knock-in mice display impaired mitochondrial dynamics

To extend our mitochondrial findings in an *in vivo* HD model, we next analysed whether alterations in mitochondrial dynamics were also detected in primary striatal neurons from Hdh7/111Q knock-in mutant mice. Wild-type Hdh7/7Q and mutant Hdh7/111Q primary striatal neurons were stained with TOM20 and MAP2 in order to label mitochondria and neuronal structure, respectively. Similarly to the data observed in striatal cells, confocal microscopy analysis (Fig. 16A) revealed that mitochondria from mutant

Hdh7/111Q primary striatal neurons were more fragmented respect to those in wild-type Hdh7/7Q mice (~30%; p <0.05. Fig. 16B).

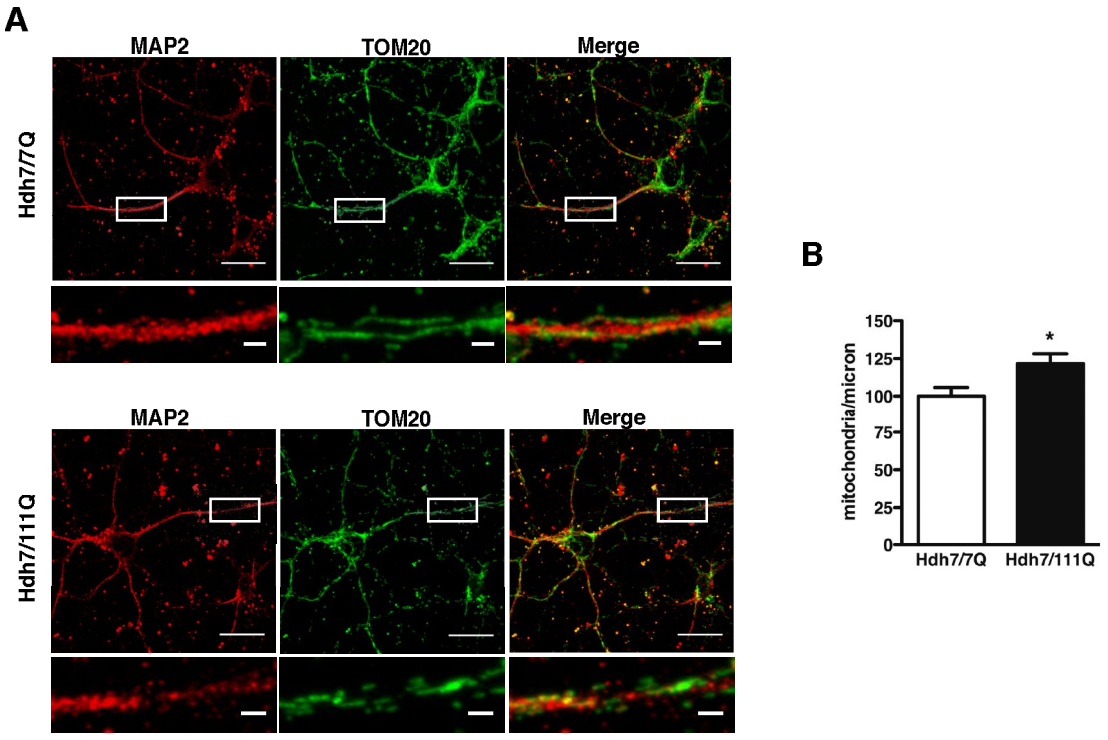

Figure 16. Mutant Hdh7/111Q primary striatal cultures exhibit impaired mitochondrial morphology. (A) Representative confocal images of striatal primary cultures from wild-type Hdh7/7Q and mutant Hdh7/111Q mice immunostained with anti-TOM20 (green) and anti-MAP2 (red); scale bar 20 µm. The boxed areas are enlarged in the panels on the bottom; scale bar 4 µm. (B) The bar graph provides the percentage of mitochondria per axonal micron. Data represent mean ± SEM of 4 independent experiments in which 20 neurons/genotype were analysed with ImageJ software; *p<0.05 vs. wild-type Hdh7/7Q mice as determined by unpaired Student's t-test.

1.2 Levels and activity of mitochondrial fission/fusion proteins in cellular and murine models of HD

1.2.1 mHtt deregulates the levels and activity of mitochondrial fission/fusion proteins in ST111/111Q striatal cells

Modifications of mitochondrial morphology depend on the balance of opposing fission and fusion events (Bereiter-Hahn & Voth, 1994). Interestingly, mHtt has been reported to induce changes in the expression of different pro-fission and pro-fusion mitochondrial mediators (Y. N. Jin et al., 2013). To understand the molecular basis of the observed abnormal

mitochondrial fragmentation we determined the levels of mitochondria-shaping proteins in total and subcellular fractions derived from striatal cells by Western blot analysis (Fig. 17). No significant differences in the total levels of the fusion protein Opa1 were observed between genotypes (Fig. 17A). However, when the mitochondrial fraction was analysed, mutant ST111/111Q cells displayed significantly lower levels of Opa1 compared to those in wild-type ST7/7Q cells (20% decrease; $p<0.05$). We also assessed the levels of another essential fission protein, Mfn2. No significant changes either between subcellular fractions or between genotypes were observed (Fig. 17B).

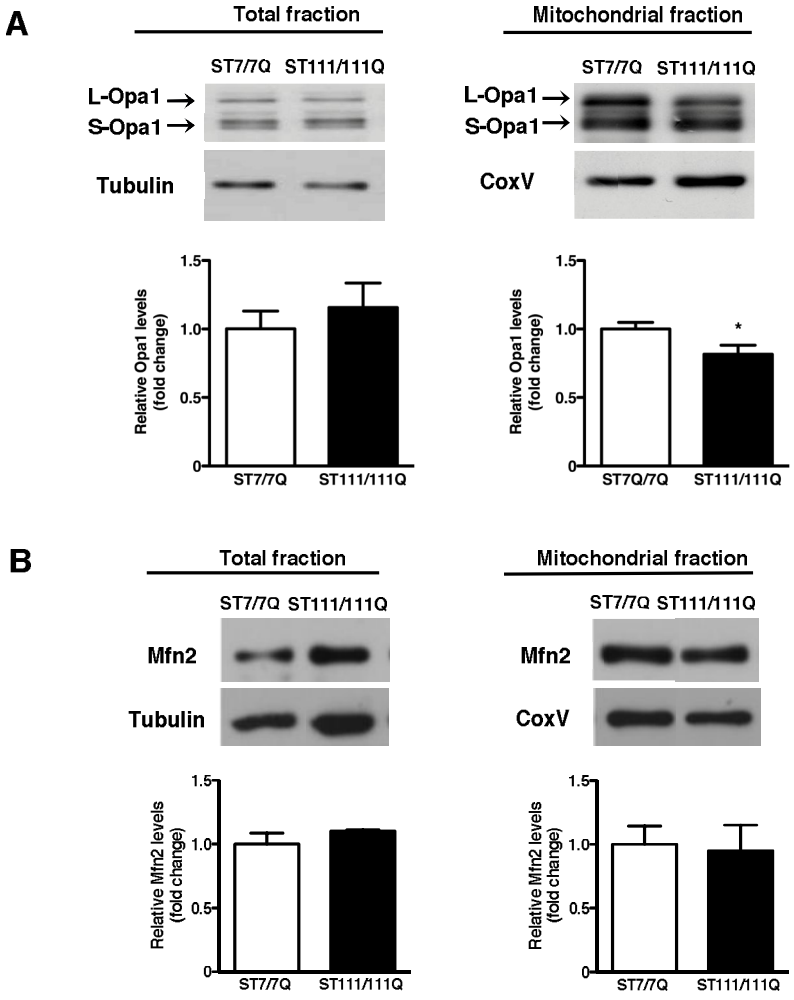

Figure 17. Levels of fusion protein Opa 1 and Mfn2 in wild-type ST7/7Q and mutant ST111/111Q cells. Representative Western blots showing the levels of the fusion proteins Opa1 (A) and Mfn2 (B) in total and mitochondrial fractions from wild-type ST7/7Q and mutant ST111/111Q cells. α-Tubulin (total) or CoxV (mitochondria) were used as loading controls. Letters confer to the different isoforms recognized by the respective antibodies (L-Opa1 and S-Opa-1). Bar histograms indicate the relative fold change ± SEM of 6-9 independent experiments; *$p<0.05$ vs. wild-type ST7/7Q cells as determined by unpaired Student's t-test.

Surprisingly, we also found a significant decrease (30% decrease; $p<0.05$. Fig. 18) in the total levels of the fission protein Drp1 in mutant compared to wild-type striatal cells, a reduction that was even higher when mitochondrial subcellular fractions were evaluated (50% decrease; $p<0.01$) without changes in the cytosolic fraction.

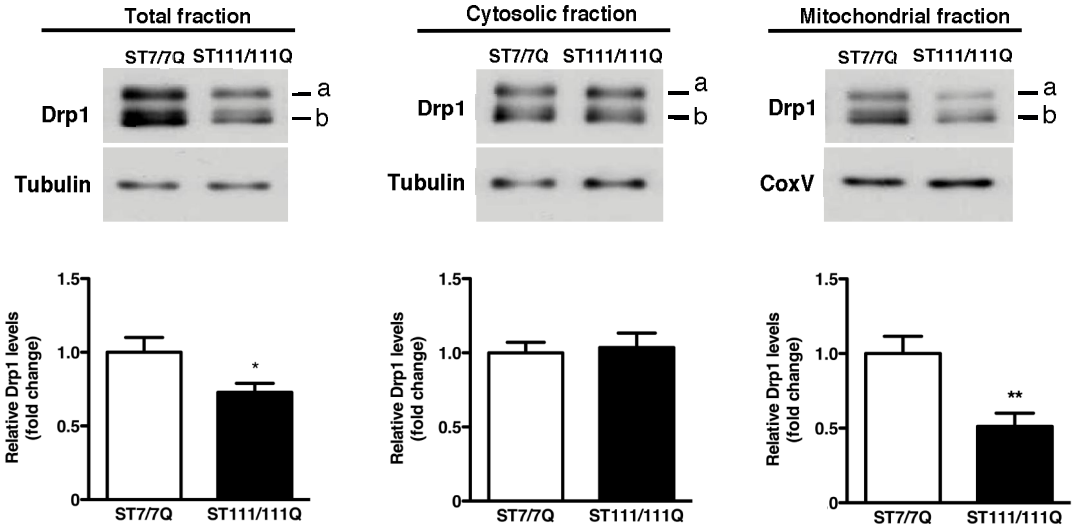

Figure 18. Levels of fission protein Drp1 in wild-type ST7/7Q and mutant ST111/111Q cells. Representative Western blots showing the levels of the fission protein Drp1 in total, cytosolic and mitochondrial fractions from wild-type ST7/7Q and mutant ST111/111Q cells. α-Tubulin (total and cytosol) or CoxV (mitochondria) were used as loading controls. Letters confer to the different isoforms recognized by the respective antibodies (DRP1: a–b.). Bar histograms indicate the relative fold change ± SEM of 9 independent experiments; **p<0.01, *p<0.05 vs. wild-type ST7/7Q cells as determined by unpaired Student's t-test.

To determine if this decrease was due to altered gene transcription, Drp1 mRNA expression was evaluated. A statistically significant decrease in Drp1 mRNA levels was detected in mutant ST111/111Q compared to wild-type ST7/7Q striatal cells (20% decrease; $p<0.05$. Fig. 19A) suggesting an involvement of mHtt in the transcriptional deregulation of Drp1. Since mHtt abnormally interacts with Drp1 altering its structure and increasing its enzymatic activity (W. Song et al., 2011), we then evaluated Drp1-GTPase activity in striatal cells (Fig. 19B). Despite the low levels of Drp1 expression found in mutant huntingtin cells, a significant increase (~3.5-fold increase; $p<0.01$. Fig. 19B) in the GTP-binding activity of Drp1 was observed in mutant compared to wild-type cells suggesting that enhanced mitochondrial

fragmentation in mutant huntingtin cells could be related with higher Drp1 activity.

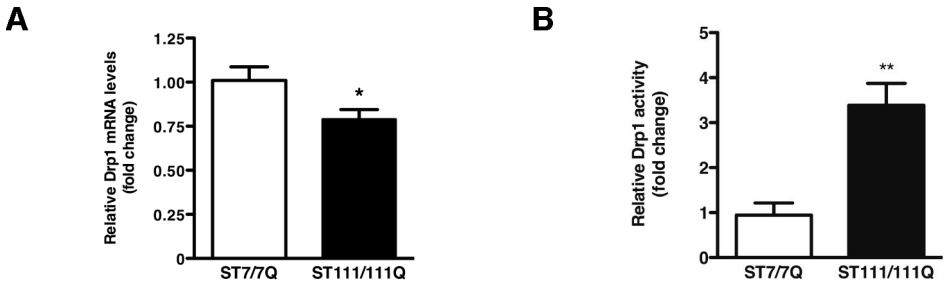

Figure 19. ST111/111Q mutant cells exhibit altered expression and activity of Drp1. (A) Histogram showing Drp1 mRNA expression analysed by RT-PCR in wild-type ST7/7Q and mutant ST111/111Q cells. Data were normalized to 18S and actinβ gene expression. Bar histogram represents the relative fold change ± SEM of 7 independent experiments. *p<0.05 vs. wild-type ST7/7Q cells as determined by unpaired Student's t-test. (B) Histogram showing GTPase Drp1 enzymatic activity in striatal extracts obtained from wild-type ST7/7Q and mutant ST111/111Q cells. Bar diagram represents the relative fold change ± SEM of 5 independent experiments. **p<0.01 vs. wild-type ST7/7Q cells as determined by unpaired Student's t-test.

1.2.2 Mitochondrial fragmentation in Hdh7/111Q knock-in mice is associated with increased activity but not with alteration of Drp1 levels

We next investigated whether alterations in Drp1 levels and activity, could be responsible for the increased mitochondrial fragmentation previously observed in Hdh7/111Q, similarly as those founded in mutant ST111/111Q striatal cells. Considering that abnormalities in the morphology and function of mitochondria persist along the disease progression (U. Shirendeb et al., 2011), alterations in the levels of the fission protein Drp1 were analysed in the striatum of Hdh7/111Q knock-in mutant mice at 8 months of age. Immunoblot analysis revealed no changes in striatal Drp1 protein levels between mutant Hdh7/111Q and wild-type Hdh7/7Q mice in any of the subcellular fractions analysed (Fig. 20A). However and in agreement with our results in mutant ST111/111Q striatal cells, the enzymatic activity of Drp1 was significantly increased (~2-fold increase; $p<0.05$. Fig. 20B) in mutant Hdh7/111Q mice compared to wild-type Hdh7/7Q mice. These data support the idea that mitochondrial abnormalities in HD models are related with enhanced Drp1 enzymatic activity induced by mHtt.

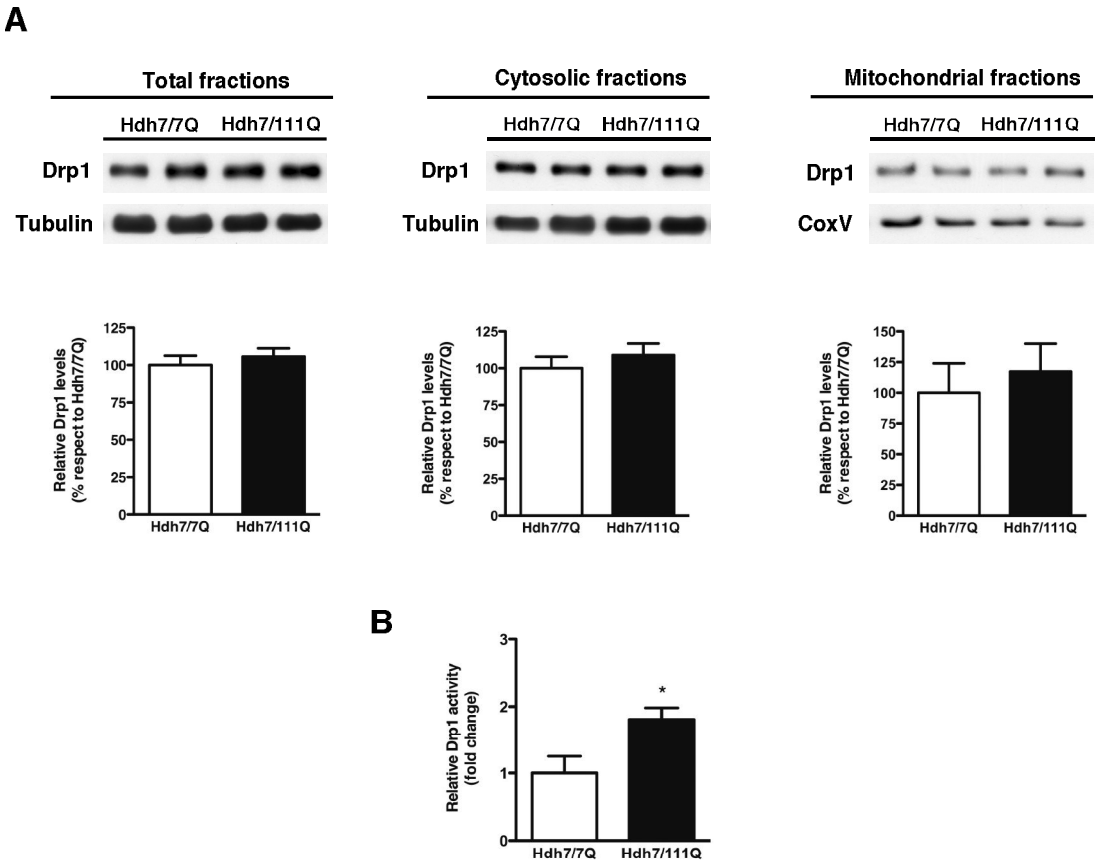

Figure 20. Levels and activity of fission protein Drp1 in wild-type Hdh7/7Q and mutant Hdh7/111Q knock-in mice. (A) Representative Western blots showing the levels of the fission protein Drp1 in total, cytosolic and mitochondrial fractions obtained from the striatum of 8-month-old wild-type Hdh7/7Q and mutant Hdh7/111Q mice. α-Tubulin (total and cytosol) or CoxV (mitochondria) were used as loading controls. Bar histograms indicate the relative levels of Drp1 ± SEM of 7 independent experiments. (B) Histogram showing GTPase Drp1 enzymatic activity in striatal extracts obtained from 8-month-old wild-type Hdh7/7Q and mutant Hdh7/111Q mice. Bar histogram indicates the relative fold change ± SEM of 6 independent experiments; *p<0.05 vs. Hdh7/7Q as determined by unpaired Student's *t*-test.

1.3 Role of mHtt-induced aberrant Cdk5 activity in the regulation of mitochondrial dynamics in cellular models of HD

Cdk5 may promote mitochondrial dysfunction (Sun et al., 2008) acting as an upstream regulator of mitochondrial fission during neuronal apoptosis (Meuer et al., 2007). Since our group previously demonstrated an aberrant increase in Cdk5 activity in mutant ST111/111Q cells (Paoletti et al., 2008), we next aimed to determine the relevance of Cdk5 in mitochondrial fission. Wild-type ST7/7Q (Fig. 21A) and mutant ST111/111Q cells (Fig. 21E) were

treated with the Cdk5 inhibitor roscovitine and mitochondrial morphology was analysed by confocal microscopy. Roscovitine treatment significantly improved mitochondrial tubular network defects (~10% increase in the Form Factor value; $p<0.05$. Fig. 21F) and reduced mitochondrial fragmentation (~20%; $p<0.05$. Fig. 21G and 21H) in mutant but not wild-type huntingtin cells (Fig. 21A–D) suggesting an important role of Cdk5 in mitochondrial dysfunction in HD striatal cells.

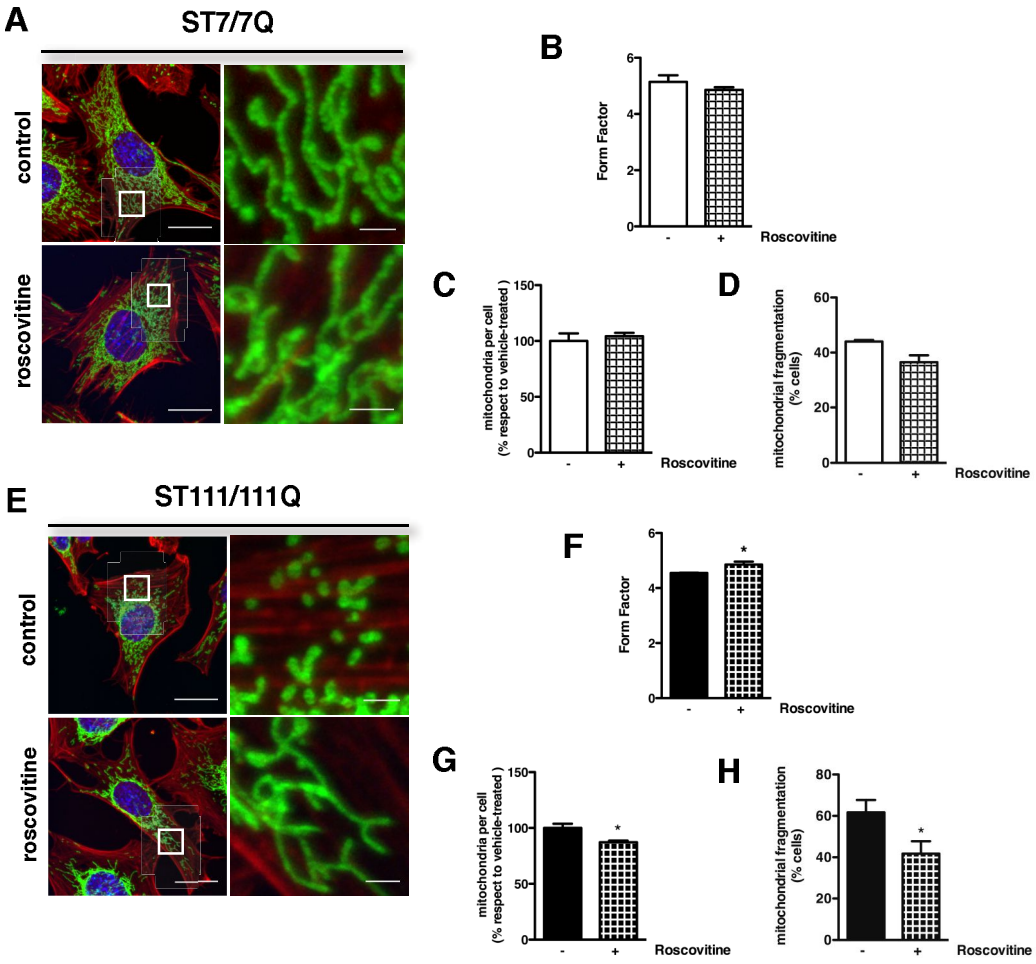

Figure 21. Aberrant Cdk5 activity in ST111/111Q mutant cells induces mitochondrial fragmentation. (A, E) Representative confocal images showing mitochondrial morphology in wild-type ST7/7Q and mutant ST111/111Q cells treated with roscovitine (20 μM). Cells were stained with anti-TOM20 (green), anti-Phalloidin-TRITC (red) and Hoechst stain (blue); scale bar 20 μm. Panels on the right show enlargement of the boxed areas; scale bar 2 μm. Bar histograms showing: (B, F) the relative Form Factor (FF) value, (C, G) the percentage of number of mitochondria per cell and (D, H) the percentage of cells with fragmented mitochondria relative to the total number of cells. Data represent mean ± SEM of 5 independent experiments in which 25–30 cells/condition were analysed with ImageJ software. *$p<0.05$ vs. vehicle treated condition as determined by unpaired Student's *t*-test.

Since we have demonstrated increased Drp1 activity in mutant huntingtin striatal cells, we next addressed whether the improvement in mitochondrial fragmentation following roscovitine treatment was associated with a reduction in Drp1 activity. According to a role of Cdk5 in mitochondrial impairments, pharmacological inhibition of Cdk5 significantly reduced (~50% decrease; $p<0.01$. Fig. 22A) Drp1 activity in mutant ST111/111Q cells without any effect in wild-type ST7/7Q cells. Interestingly, we also found that in addition to Drp1 activity, Cdk5 modulated Drp1 gene transcription as well. Thus, a significant decrease in Drp1 mRNA expression was observed in wild-type (~40% decrease; $p<0.01$) and mutant (~70% decrease; $p<0.001$) huntingtin striatal cells when expression of Cdk5 was reduced by Cdk5 siRNAs (Fig. 22B) revealing a critical role for Cdk5 as a transcriptional regulator of Drp1.

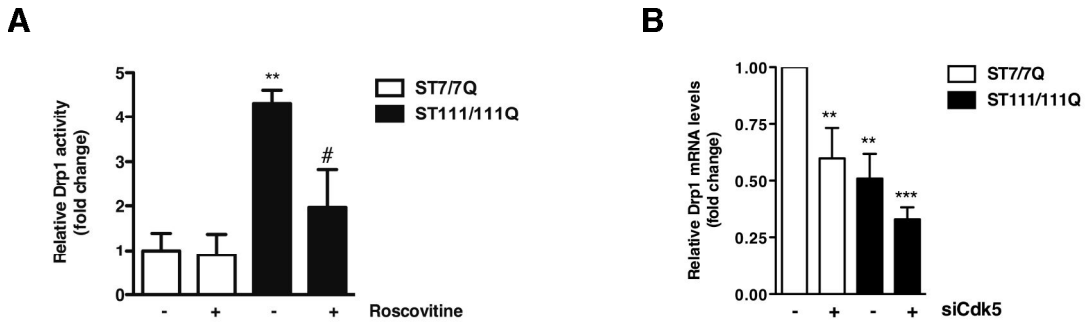

Figure 22. Cdk5 alters Drp1 activity in Hdh7/111Q knock-in mice and regulates Drp1 expression. (A) Histogram showing GTPase Drp1 enzymatic activity in striatal extracts obtained from vehicle or roscovitine treated wild-type ST7/7Q and mutant ST111/111Q cells. Bar histogram indicates the relative fold change ± SEM of 4 independent experiments; **p<0.01 vs. wild-type ST7/7Q cells and #p<0.05 vs. vehicle-treated ST111/111Q cells as determined by One-way ANOVA with Newman–Keuls post hoc analysis. (B) Histogram showing Drp1 mRNA expression analysed by RT-PCR in wild-type ST7/7Q and mutant ST111/111Q cells transfected with siRNA or siCdk5. Data were normalized to 18S and actinβ gene expression. Bar histogram indicates the relative fold change ± SEM of 7 independent experiments. ***p<0.001 and **p<0.01 vs. siRNA transfected ST7/7Q cells as determined by One-way ANOVA with Newman–Keuls post hoc analysis.

1.4 mHtt-induced aberrant Cdk5 activity increases striatal susceptibility to dopamine through mitochondrial dysfunction in cellular models of HD

1.4.1 Activation of dopaminergic D1 receptors increases mitochondrial dysfunction in mutant ST111/111Q striatal cells

We have previously demonstrated that mutant ST111/111Q cells are more susceptible to cell death induced by D1R activation than wild-type ST7/7Q cells (Paoletti et al., 2008). Interestingly, it has been reported that dopamine could negatively influence mitochondrial function (Hastings, 2009; Jana et al., 2011). Therefore, we wondered whether the enhanced vulnerability of mutant ST111/111Q cells to dopamine-induced cell death was mediated by mitochondrial impairments. First, we analysed whether our striatal cell line properly express D1R at the plasma membrane (Fig. 23). Cell surface D1R expression examined with an antibody against the extracellular domain of D1R in non-permeable cell conditions was similar between wild type and mutant huntingtin striatal cells.

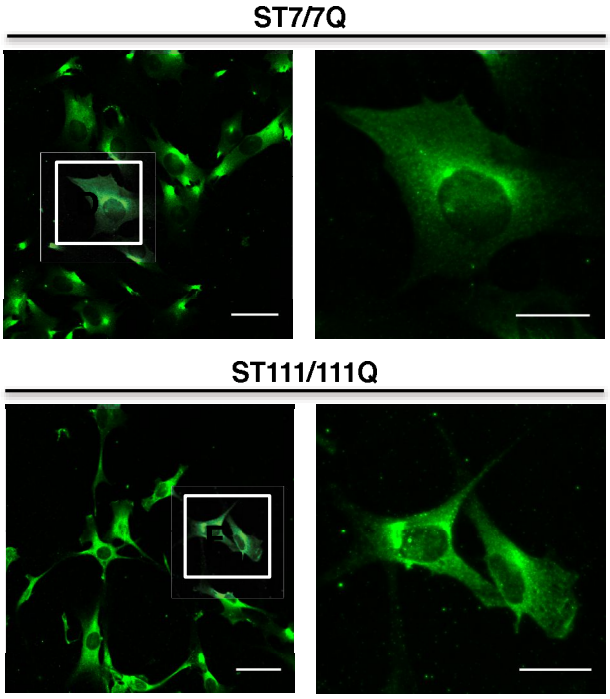

Figure 23. Wild-type ST7/7Q and mutant ST111/111Q cells equally express surface dopamine D1 receptor. Representative confocal images showing D1R on membrane in non-permeabilized wild-type ST7/7Q and mutant ST111/111Q cells immunostained with anti-D1R. Scale bar 20 μm. The boxed areas are enlarged in the panels on the right; scale bar 10 μm.analysis.

Successively, wild-type ST7/7Q and mutant ST111/111Q cells were treated with the D1R agonist SKF 38393 (60 μM) and mitochondrial morphology was analysed by confocal microscopy (Fig 24A).

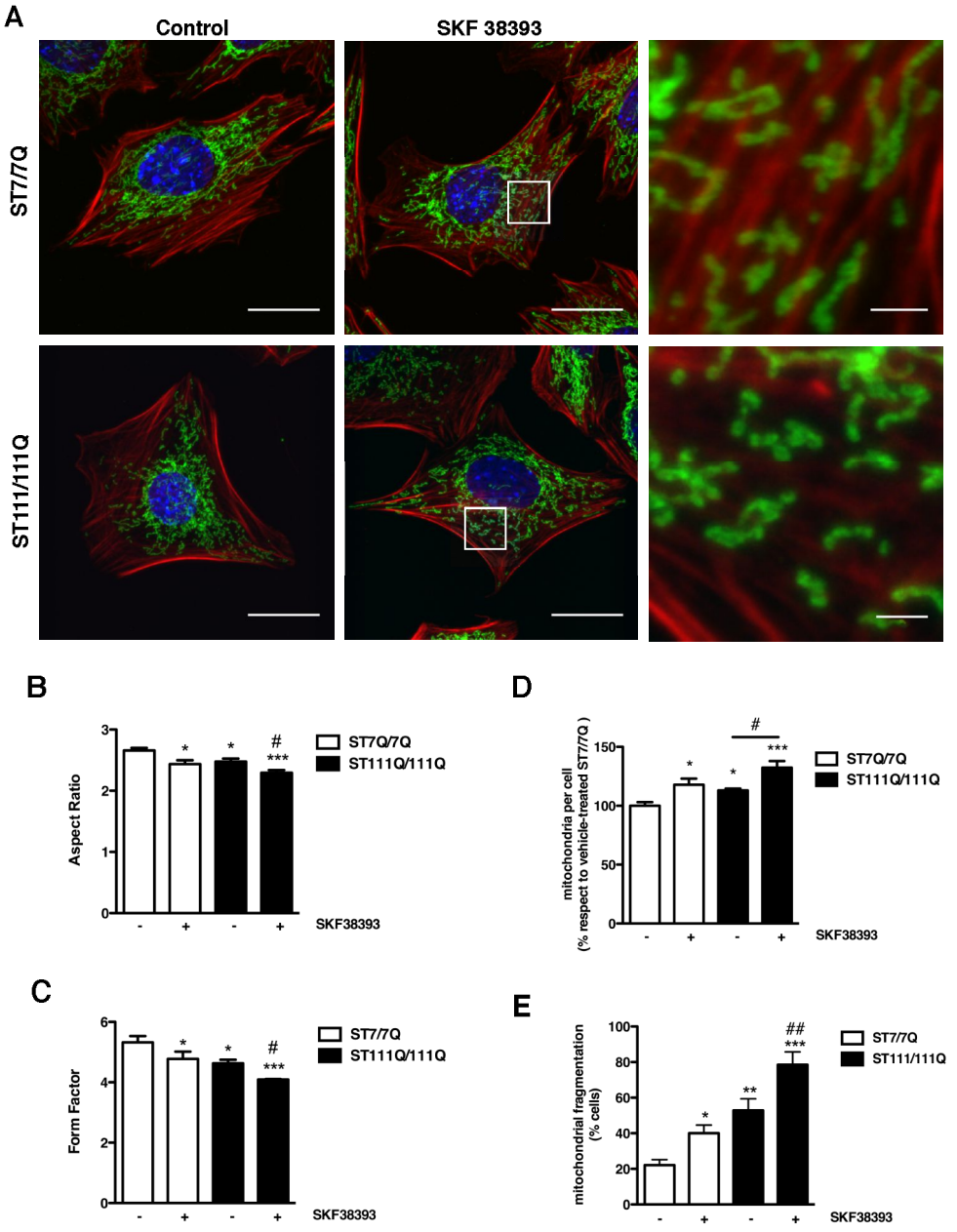

Figure 24. Dopaminergic D1 receptor activation increases mitochondrial fission events and mitochondrial branching defects in striatal cells. (A) Representative confocal images showing mitochondrial morphology in wild-type ST7/7Q and mutant ST111/111Q cells treated with vehicle or the dopaminergic agonist SKF 38393 (60 µM). Cells were stained with anti-TOM20 (green), anti-Phalloidin-TRITC (red) and Hoechst stain (blue); scale bar 20 µm. Last panels on the right show enlargement of the boxed areas; scale bar 2 µm. (B, C) Bar histograms showing mitochondrial size by Aspect Ratio and Form Factor values in SKF 38393-treated and vehicle-treated wild-type ST7/7Q and mutant ST111/111Q cells. Data represent mean ± SEM of 6 independent experiments in which 25–30 cells/condition were analyzed with ImageJ software. ***p<0.001, *p<0.05 vs. vehicle-treated ST7/7Q cells and $^{\#}$p<0.05 vs. vehicle-treated ST111/111Q cells as determined by One-way ANOVA with Newman–Keuls post hoc analysis. (D) Bar histogram showing percentage of number of mitochondria per cell. (E) Bar histogram showing the percentage of vehicle or SKF 38393-treated wild-type ST7/7Q and mutant ST111/111Q cells with fragmented mitochondria relative to the total number of cells. Data represent mean ± SEM of 6 independent experiments in which 25–30 cells/condition were analysed with ImageJ software. ***p<0.001, **p<0.01 and *p<0.05 vs. vehicle-treated ST7/7Q cells and $^{\#\#}$p<0.01 and $^{\#}$p<0.05 vs. vehicle-treated ST111/111Q cells as determined by one-way ANOVA with Newman–Keuls post hoc analysis.

Our findings demonstrated that mitochondrial fragmentation occurs quickly in response to D1 receptor activation leading to a significant decrease in the size (Fig. 24B) and mitochondrial reticular network distribution (Fig. 24C) in both cell genotypes. It is important to notice that dopaminergic stimulation was able to further decrease FF and AR values in mutant huntingtin cells compared to vehicle conditions (~10%; $p<0.05$). The alterations in mitochondrial morphology induced by SKF 38393 treatment were also detected by an increase in the number of organelles per cell showing both genotypes an increment of 20% ($p<0.05$; Fig. 24D) compared to vehicle conditions. Finally, when the percentage of cells with mitochondrial fragmentation was analysed, we found that, in wild-type cells D1R activation raised the percentage from 20% to 40% ($p<0.05$ Fig. 24E) while the transition in mutant cells was from 50% to 80% ($p<0.01$). These mitochondrial alterations were dose-dependent since the treatment with lower concentration of SKF 38393 (30 µM) did not alter mitochondrial conformation in both cell types (Fig. 25A-D).

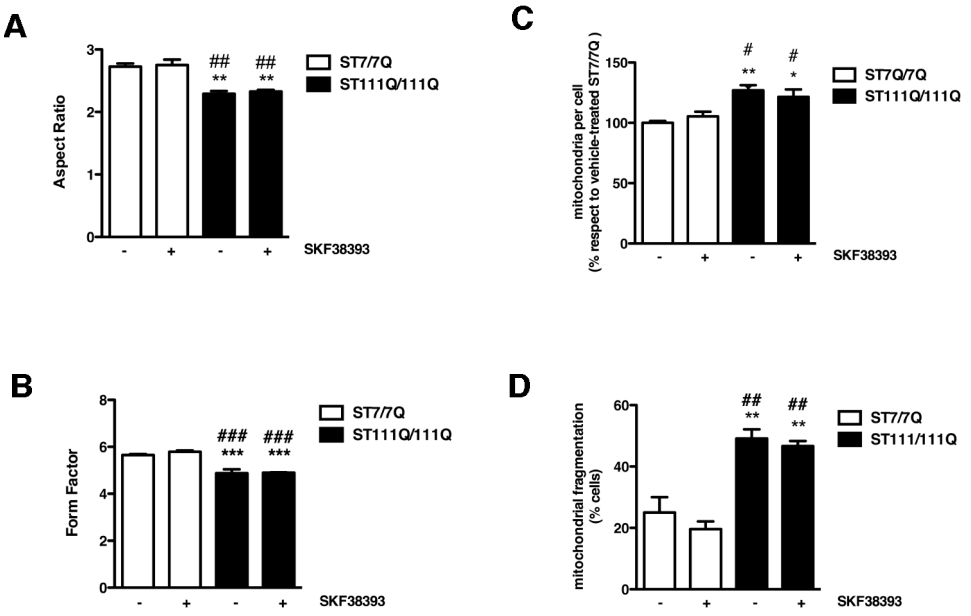

Figure 25. SKF 38393 treatment at 30 µM does not alter mitochondrial morphology. Bar histograms showing (A, B) the relative Aspect Ratio and Form Factor value, (C) the percentage of number of mitochondria per cell and (D) the percentage of cells with fragmented mitochondria relative to the total number of cells in SKF 38393-treated (30 µM) and vehicle-treated wild-type ST7/7Q and mutant ST111/111Q cells. Data represent mean ± SEM of 3 independent experiments in which 25–30 cells/condition were analysed with ImageJ software. ***p<0.001, **p<0.01, *p<0.05 vs. vehicle-treated ST7/7Q cells and $^{\#\#\#}p<0.001$, $^{\#\#}p<0.01$, $^{\#}p<0.05$ vs. SKF 38393-treated ST7/7Q cells as determined by One-way ANOVA with Newman–Keuls post hoc analysis.

Finally, to validate that SKF 38393 effects on mitochondria morphology were D1R-dependent, wild-type and mutant huntingtin striatal cells were treated with the D1R antagonist SCH 23390 prior to D1R activation and mitochondrial fission was analysed (Fig. 26A-H). Importantly, co-incubation with SCH 23390 completely abrogated SKF 38393-induced alterations in mitochondrial morphology in both cell genotypes.

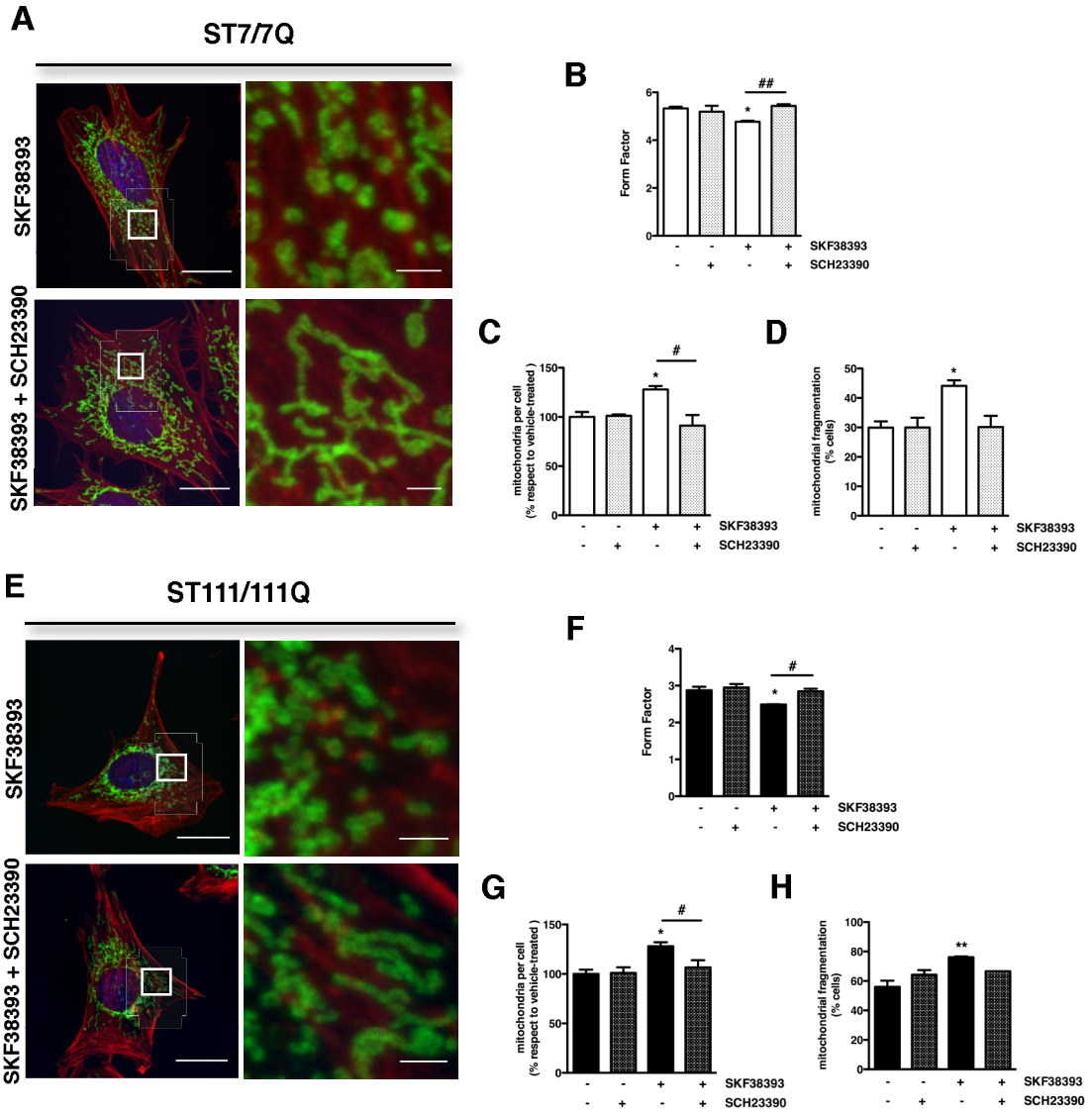

Figure 26. Mitochondrial fission induced by SKF 38393 is D1R-dependent. (A, E) Representative confocal images showing mitochondrial morphology in wild-type ST7/7Q and mutant ST111/111Q cells treated with SKF 38393 (60 µM) and SCH 23390 (10 µM) + SKF 38393. Cells were stained with anti-TOM20 (green), anti-Phalloidin-TRITC (red) and Hoechst stain (blue); scale bar 20 µm. Panels on the right show enlargement of the boxed areas; scale bar 2 µm. Bar histograms showing (B, F) the relative Form Factor value, (C, G) the percentage of number of mitochondria per cell and (D, H) the percentage of cells with fragmented mitochondria relative to the total number of cells. Data represent mean ± SEM of 5 independent experiments in which 25–30 cells/condition were analysed with ImageJ software. **p<0.01 and *p<0.05 vs. vehicle treated and $^{\#\#}$p<0.01 and $^{\#}$p<0.05 vs. SKF 38393 treated cells as determined by One-way ANOVA with Newman–Keuls post hoc analysis.

Overall these results indicate that the increased susceptibility of mutant huntingtin striatal cells to dopaminergic activation could be mediated by alterations in the distribution and morphology of the mitochondrial population.

1.4.2 Activation of dopaminergic D1 receptors alters levels, distribution and activity of the fission protein Drp1 in ST111/111Q striatal cells

Confocal microscopy analysis has shown that SKF 38393 treatment induced higher mitochondrial fragmentation in mutant compared to wild-type huntingtin cells. To correlate these morphological alterations with changes in mitochondrial fission/fusion proteins, the levels of Opa1, Mfn2 and Drp1 were analysed in total, cytosolic and mitochondrial fractions obtained from vehicle and SKF 38393 treated wild-type and mutant huntingtin striatal cells. SKF 38393 treatment did not affect the expression of the pro-fusion proteins Opa1 (Fig. 27) and Mfn2 (Fig. 28) neither in wild-type nor in mutant huntingtin striatal cells.

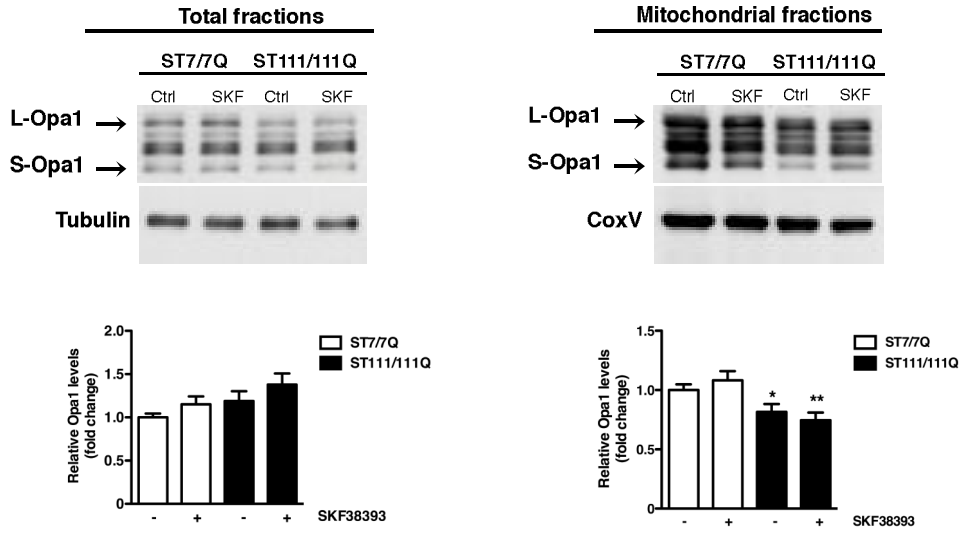

Figure 27. Levels of fusion protein Opa 1 in wild-type ST7/7Q and mutant ST111/111Q cells after D1R activation. Representative Western blots showing the levels of the fusion protein Opa1 in total and mitochondrial fractions obtained from vehicle and SKF 38393 (60 µM) treated wild-type ST7/7Q and mutant ST111/111Q cells. α-Tubulin (total) or CoxV (mitochondria) were used as loading controls. Letters indicate the different isoforms recognized by the respective antibodies (L-Opa1 and S-Opa-1). Bar histograms indicate the relative fold change ± SEM of 12 independent experiments. **p<0.01, *p<0.05 vs. vehicle-treated ST7/7Q cells and $^{\#}$p < 0.05 vs. vehicle-treated ST111/111Q cells as determined by One-way ANOVA with Newman–Keuls post hoc analysis.

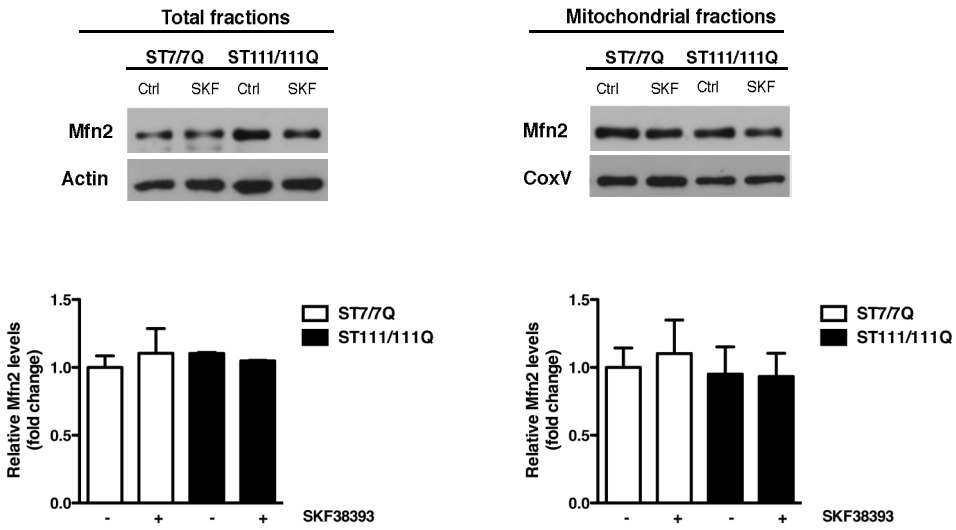

Figure 28. Levels of fusion protein Mfn2 in wild-type ST7/7Q and mutant ST111/111Q cells after D1R activation. Representative Western blots showing the levels of the fusion protein Mfn2 in total and mitochondrial fractions obtained from vehicle and SKF 38393 (60 µM) treated wild-type ST7/7Q and mutant ST111/111Q cells. α-Tubulin (total) or CoxV (mitochondria) were used as loading controls. Bar histograms indicate the relative fold change ± SEM of 6 independent experiments.

However, SKF 38393 treatment induced a significant increase in total Drp1 levels (~ 1.3-fold increase and ~ 1.5-fold increase, respectively; $p<0.05$. Fig. 29A). Interestingly, the distribution of Drp1 within the subcellular compartments following dopaminergic activation was different between cell genotypes. Whereas in wild-type cells the increase in Drp1 levels was found in the cytosolic fraction, that in mutant huntingtin cells was located in the mitochondrial fraction. Given the requirement of Drp1 translocation to the mitochondria for the fission of this organelle (Labrousse, Zappaterra, Rube, & van der Bliek, 1999; Smirnova, Griparic, Shurland, & van der Bliek, 2001), the aberrant distribution of Drp1 in mutant huntingtin striatal cells may explain the increased mitochondrial fragmentation observed in these cells compared to wild-type cells. Next, we investigated whether dopaminergic stimuli could also affect Drp1 enzymatic activity. An increasing trend, although not significant, in Drp1 activity was found in wild-type cells after SKF 38393 treatment (Fig. 29B). By contrast, a significant increase (~ 8-fold increase; $p<0.01$. Fig. 29B) was detected in SKF 38393-treated mutant huntingtin cells suggesting that D1 receptor activation exacerbates Drp1-dependent mitochondrial fission events in ST111/111Q mutant cells.

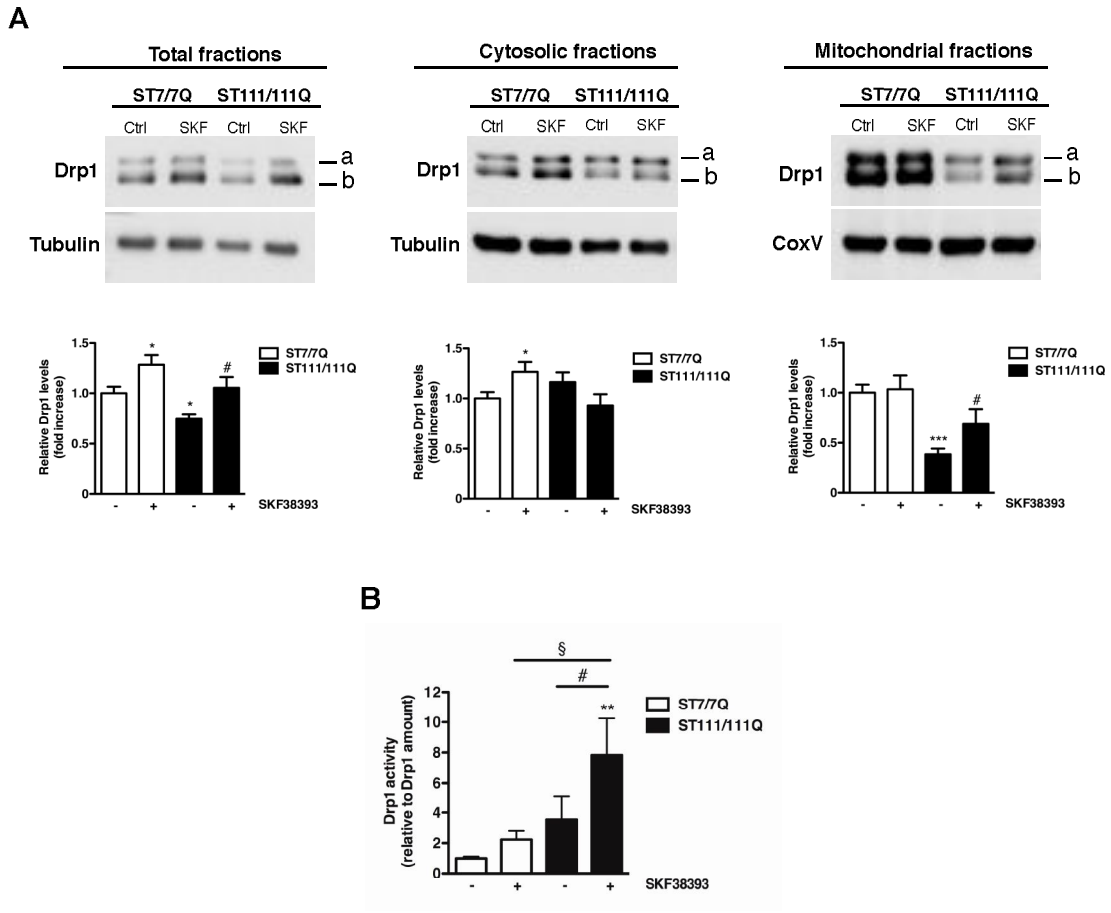

Figure 29. Aberrant mitochondrial Drp1 activity exacerbates D1R-induced mitochondrial fission in ST111/111Q mutant cells. (A) Representative Western blots showing the levels the fission protein Drp1 in total, cytosolic and mitochondrial fractions obtained from vehicle and SKF 38393 (60 μM) treated wild-type ST7/7Q and mutant ST111/111Q cells. α-Tubulin (total and cytosol) or CoxV (mitochondria) were used as loading controls. Letters indicate the different isoforms recognized by the respective antibodies (DRP1: a–b). Bar histograms indicate the relative fold change ± SEM of 12 independent experiments. ***p<0.001, **p<0.01, *p<0.05 vs. vehicle-treated ST7/7Q cells and $^{\#}$p<0.05 vs. vehicle-treated ST111/111Q cells as determined by One-way ANOVA with Newman–Keuls post hoc analysis. (B) Histogram showing GTPase Drp1 enzymatic activity in striatal extracts obtained from vehicle or SKF 38393-treated mutant ST111/111Q striatal cells. Bar histogram represents the relative fold change ± SEM of 6 independent experiments; **p<0.01 vs. vehicle-treated ST7/7Q cells, $^{\#}$p<0.05 vs. vehicle treated ST111/111Q cells and §p<0.05 vs. SKF 38393 treated ST7/7Q cells as determined by One-way ANOVA with Newman–Keuls post hoc analysis.

1.4.3 Inhibition of Cdk5 prevents D1R-induced mitochondrial fission and mitochondrial branching alterations in striatal cells

We have shown that Cdk5 contributes to mitochondrial fragmentation in mutant ST111/111Q cells while D1R activation increases mitochondrial fission. Interestingly, we have previously reported that aberrant Cdk5 activity mediates D1R-induced neurotoxicity in mutant ST111/111Q striatal cells (Paoletti et al., 2008). Altogether, it prompted us to investigate whether Cdk5 was also mediating the mitochondrial dysfunction induced by dopaminergic

D1R activation. To this aim, wild-type and mutant huntingtin striatal cells were treated with roscovitine prior to incubation with SKF 38393 and mitochondrial morphology was analysed by confocal microscopy (Fig. 30).

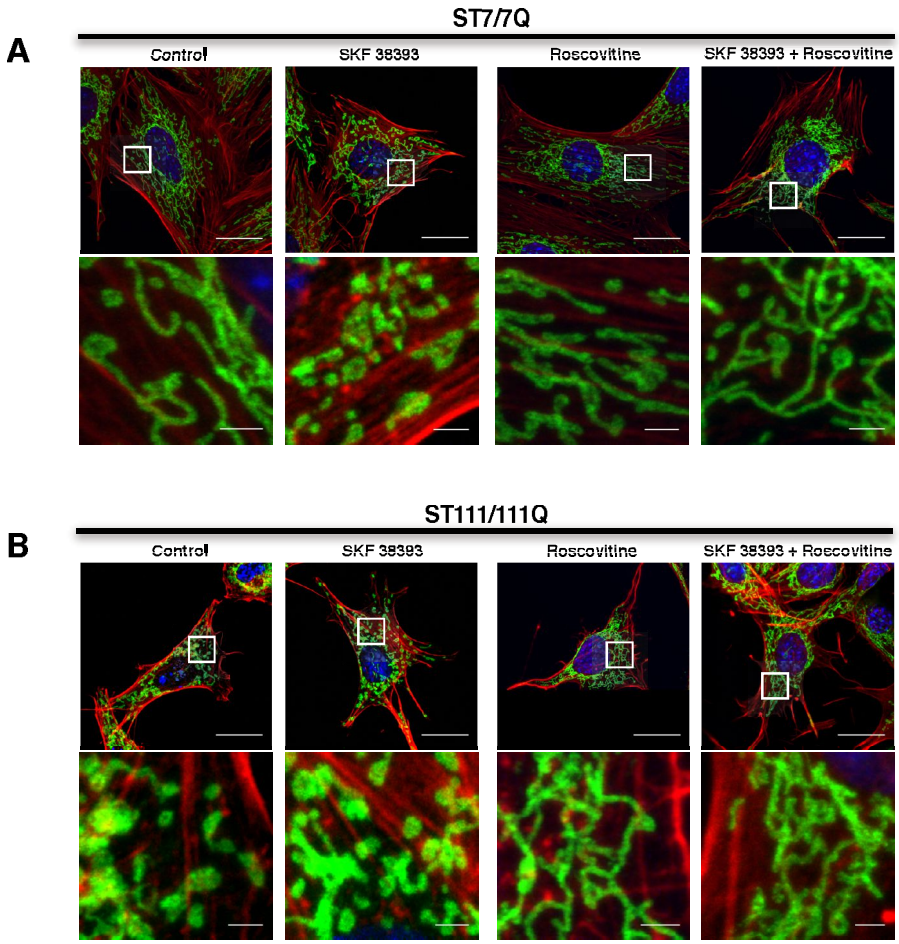

Figure 30. Cdk5 inhibition restores the mitochondrial network in SKF 38393 treated ST7/7Q wild-type and ST111/111Q mutant cells. Representative confocal images showing the mitochondrial morphology in wild-type ST7/7Q and mutant ST111/111Q cells treated with vehicle, SKF 38393 (60 μM), roscovitine (20 μM) or roscovitine + SKF 38393. Cells were stained with anti-TOM20 (green), anti-phalloidin-TRITC (red) and Hoechst stain (blue); scale bar 20 μm. Bottom panels show enlargement of the boxed areas; scale bar 2 μm.

Roscovitine treatment completely recovered mitochondrial branching alterations (Fig. 31A and 31D) and prevented mitochondrial fragmentation, either mitochondrial number (Fig. 31B and 31E) or percentage of cells with mitochondria fragmentation (Fig. 31C and 31F) in both cell genotypes, pointing Cdk5 as a mediator of D1R-induced mitochondrial fission.

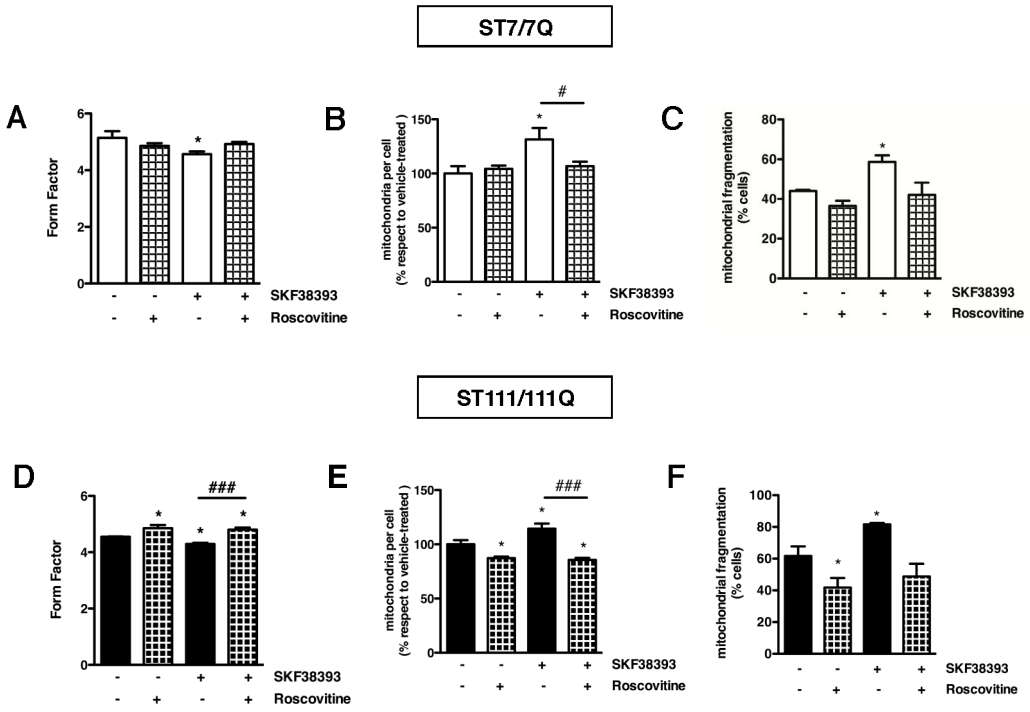

Figure 31. Cdk5 inhibition prevents mitochondrial morphological changes in SKF 38393 treated ST7/7Q wild-type and ST111/111Q mutant cells. (A and D) Bar histogram showing mitochondrial branching changes determined by the analysis of the Form Factor value in vehicle or treated wild-type ST7/7Q and mutant ST111/111Q cells. Data represent mean ± SEM of 5 independent experiments in which 25–30 cells/condition were analysed with ImageJ software. *p<0.05 vs. vehicle-treated striatal cells and ###p<0.001 vs. SKF 38393 treated cells as determined by One-way ANOVA with Newman–Keuls post hoc analysis. (B and E) Bar histograms showing the percentage of number of mitochondria per cell. Data represent mean ± SEM of 5 independent experiments in which 25–30 cells/condition were analysed with ImageJ software. *p<0.05 vs. vehicle-treated striatal cells and ###p<0.001 and #p<0.05 vs. SKF 38393 treated cells as determined by One-way ANOVA with Newman–Keuls post hoc analysis. (C and F) Bar histograms showing the percentage of vehicle or treated wild-type ST7/7Q and mutant ST111/111Q cells with fragmented mitochondria relative to the total number of cells. Data represent mean ± SEM of 5 independent experiments in which 25–30 cells/condition were analysed with ImageJ software. *p<0.05 vs. vehicle-treated striatal cells as determined by One-way ANOVA with Newman–Keuls post hoc analysis.

To further corroborate these results, Cdk5 protein levels were knocked-down in wild-type and mutant huntingtin striatal cells by using specific Cdk5 siRNAs and mitochondria fragmentation was analysed following D1R activation. First, the efficiency of the Cdk5 siRNA to knock-down Cdk5 levels was determined by Western blot analysis. A similar and a significant decrease in Cdk5 levels was found in Cdk5 siRNA transfected cells compared to those transfected with scramble siRNA (~ 50% decrease; p<0.01. Fig. 32).

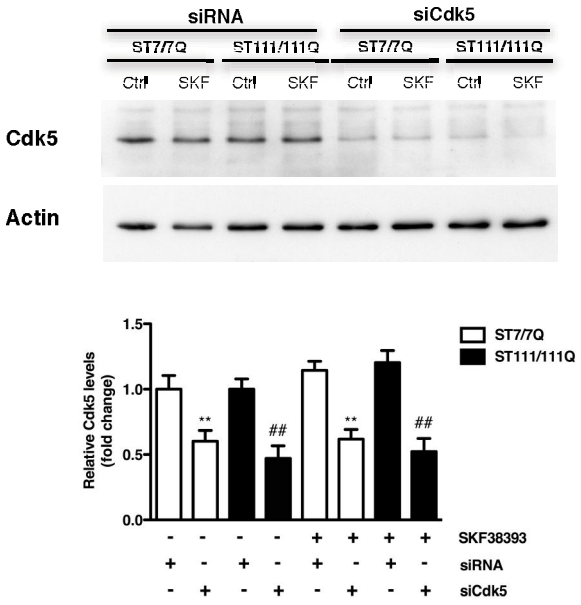

Figure 32. Genetic knockdown of Cdk5 in wild-type ST7/7Q and mutant ST111/111Q cells. Representative immunoblots showing the levels of Cdk5 in vehicle or SKF 38393 treated wild-type ST7/7Q and mutant ST111/111Q cells transfected with a siRNA or siCdk5. Histogram showing relative Cdk5 fold change ± SEM of 6 independent experiments. **p<0.01 vs vehicle treated siRNA ST7/7Q cells, ##p ≤ 0.01 vs vehicle treated siRNA ST111/111Q cells as determined by One-way ANOVA with Newman–Keuls post hoc analysis.

Next, mitochondrial fission was analysed by confocal microscopy (Fig. 33).

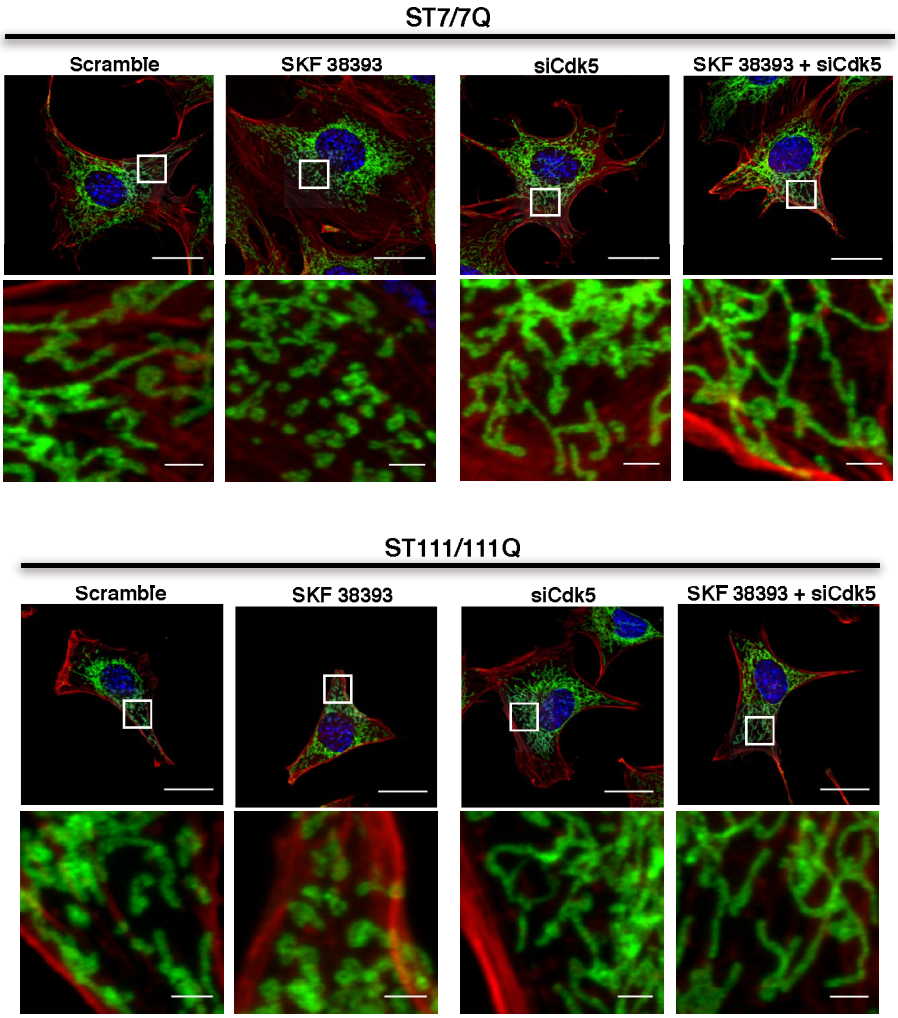

Figure 33. Inhibition of Cdk5 by Cdk5 siRNA prevents aberrant mitochondrial fission and altered branching induced by D1R activation in ST7/7Q wild-type and ST111/111Q mutant striatal cells. Representative confocal images showing the mitochondrial morphology in wild-type ST7/7Q and mutant ST111/111Q cells treated with vehicle or SKF 38393 (60 μM) and transfected with siRNA or siCdk5 RNA. Cells were stained with anti-TOM20 (green), anti-Phalloidin-TRITC (red) and Hoechst stain (blue); scale bar 20 μm. Bottom panels show enlargement of the boxed areas; scale bar 2 μm

Genetic knock-down of Cdk5 completely prevented the decrease in mitochondrial branching (Fig. 34A and 34D) and the increase in mitochondrial fragmentation induced by SKF 38393 treatment in both wild-type ST7/7Q and mutant ST111/111Q huntingtin striatal cells (Fig. 34B, 34C, 34F and 34G),

strongly supporting a role for Cdk5 in D1R-induced mitochondrial dysfunction in striatal cells.

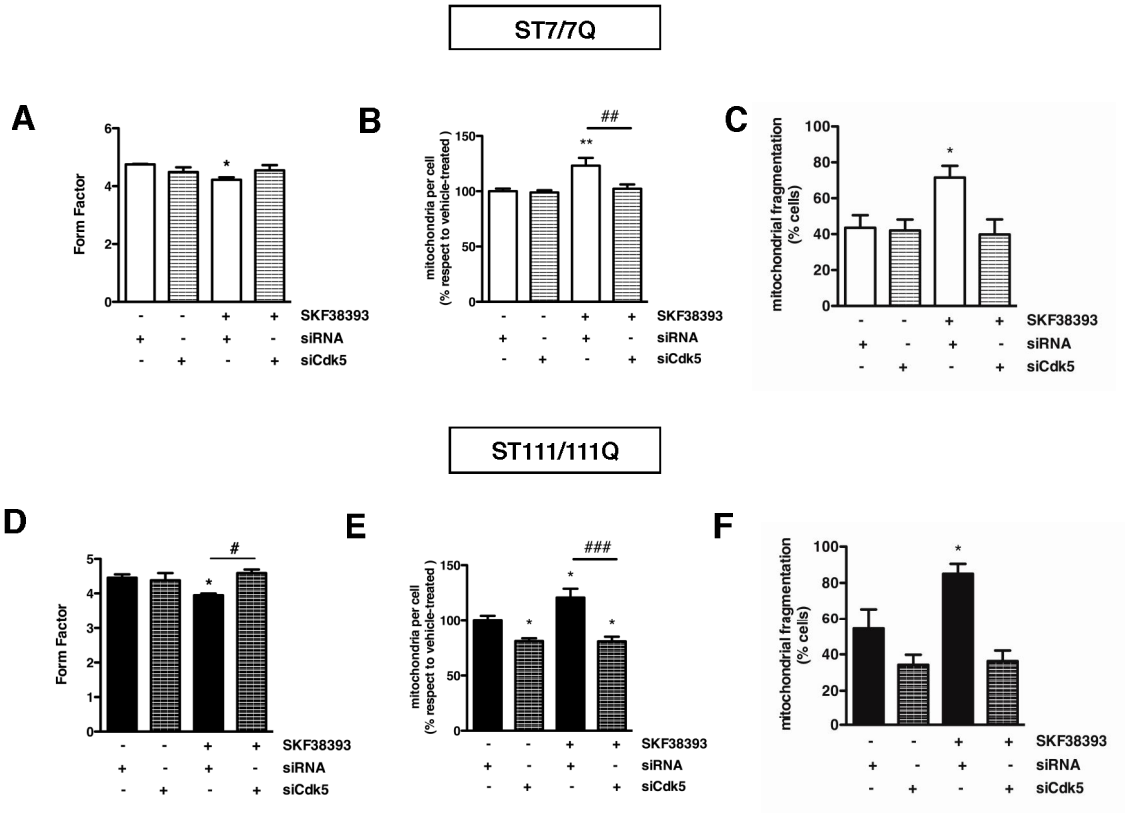

Figure 34. Cdk5 down-regulation prevents mitochondrial morphological changes in SKF 38393 treated ST7/7Q wild-type and ST111/111Q mutant cells. (A and D) Bar histogram showing mitochondrial branching changes determined by the analysis of the Form Factor value in vehicle or treated wild-type ST7/7Q and mutant ST111/111Q cells. Data represent mean ± SEM of 5 independent experiments in which 25–30 cells/condition were analysed with ImageJ software. *p<0.05 vs. vehicle-treated striatal cells and $^{###}$p<0.001 vs. SKF 38393 treated cells as determined by One-way ANOVA with Newman–Keuls post hoc analysis. (B and E) Bar histograms showing the percentage of number of mitochondria per cell. Data represent mean ± SEM of 5 independent experiments in which 25–30 cells/condition were analysed with ImageJ software. *p<0.05 vs. vehicle-treated striatal cells and $^{###}$p<0.001 and $^{#}$p<0.05 vs. SKF 38393 treated cells as determined by One-way ANOVA with Newman–Keuls post hoc analysis. (C and F) Bar histograms showing the percentage of vehicle or treated wild-type ST7/7Q and mutant ST111/111Q cells with fragmented mitochondria relative to the total number of cells. Data represent mean ± SEM of 5 independent experiments in which 25–30 cells/condition were analysed with ImageJ software. *p<0.05 vs. vehicle-treated striatal cells as determined by One-way ANOVA with Newman–Keuls post hoc analysis.

1.4.4 Inhibition of Cdk5 abrogates the D1R-induced increase and the mitochondrial translocation of Drp1 in ST111/111Q striatal cells

We have demonstrated that mitochondrial fragmentation induced by D1R activation was prevented by down-regulation of Cdk5 while activation of D1R induced an increase in the levels of the fission protein Drp1. Hence, we analysed whether mitochondrial fission induced by D1R activation was

mediated by a Cdk5-dependent increase in Drp1 function. Wild-type ST7/7Q and mutant ST111/111Q cells were transfected with scramble or Cdk5 siRNA, treated with vehicle or SKF 38393 and levels of Drp1 were determined by Western blot analysis (Fig. 35A). We found that reduction of Cdk5 expression prevented the abnormal increase of Drp1 caused by dopaminergic activation in both cell genotypes (Fig. 35B).

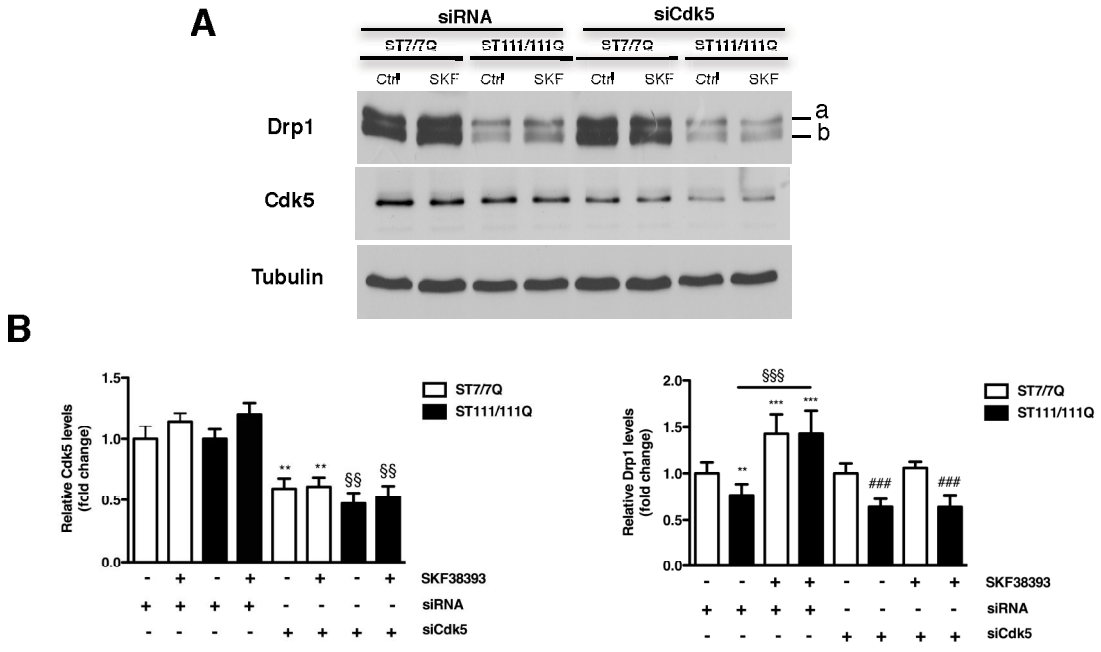

Figure 35. Cdk5 promotes mitochondrial fission by altering the levels of Drp1 in SKF 38393 treated ST111/111Q mutant cells. (A) Representative Western blots showing the levels of the fission protein Drp1 and Cdk5 in total extracts obtained from siRNA or siCdk5 transfected wild-type ST7/7Q and mutant ST111/111Q cells. α-Tubulin was used as loading control. When indicated, cells were treated with SKF 38393 (60 μM) during 1 h. Letters confer to the different isoforms recognized by the respective antibody (DRP1: a–b). (B) Bar histograms showing the relative fold change of Cdk5 and Drp1 protein levels in wild-type ST7/7Q and mutant ST111/111Q cells. Data represent mean ± SEM of 6 independent experiments. ***p<0.001, **p<0.01 vs. vehicle-treated siRNA transfected ST7/7Q cells, $^{###}$p<0.001 vs. vehicle-treated siCdk5 transfected ST7/7Q cells, §§§p<0.001 and §§p<0.01 vs. vehicle-treated siRNA transfected ST111/111Q cells as determined by One-way ANOVA with Newman–Keuls post hoc analysis.

Since we previously demonstrated in mutant huntingtin cells (Fig. 29) that D1R activation induces an increase in mitochondrial Drp1, we evaluated whether aberrant Cdk5 activity could be responsible of SKF 38393-induced Drp1 mitochondrial enrichment. Consistent with this idea, we found that mitochondrial Drp1 accumulation in mutant huntingtin striatal cells was completely prevented by inhibition of Cdk5 with roscovitine (Fig. 36),

suggesting that Cdk5 mediates D1R-induced mitochondrial fragmentation by modulation of Drp1 protein levels and distribution.

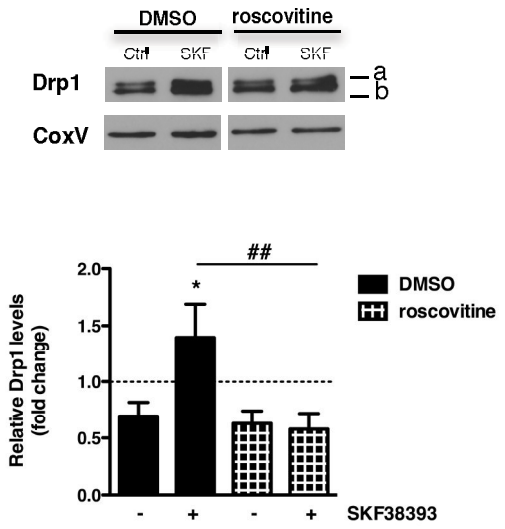

Figure 36. Cdk5 promotes mitochondrial fission by altering the distribution of Drp1 in SKF 38393 treated ST111/111Q mutant cells. Representative Western blots showing the levels of the fission protein Drp1 in mitochondrial fractions obtained from vehicle or roscovitine treated mutant ST111/111Q cells. When indicated, cells were treated with SKF 38393 (60 μM) during 1 h. CoxV was used as loading control. Letters indicate the different isoforms recognized by the respective antibody (DRP1: a–b). Bar histogram indicates the relative fold change ± SEM of 6 independent experiments; *p<0.05 vs. vehicle-treated ST111/111Q cells and $^{\#\#}$p<0.01 vs. roscovitine-treated ST111/111Q cells as determined by One-way ANOVA with Newman–Keuls post hoc analysis.

Recent reports have highlighted the role of phosphorylation in controlling Drp1 mitochondrial fission activities (Santel & Frank, 2008). In particular, it has been reported that phosphorylation at residue Ser616 is associated with Drp1 displacement to the mitochondria (Qi, Disatnik, Shen, Sobel, & Mochly-Rosen, 2011). In order to determine how Cdk5 modulates Drp1 distribution and translocation to the mitochondria, we determined by Western blot analysis, the levels of phosphorylated Drp1 at Ser616 in total and mitochondrial fraction of wild-type ST7/7Q and mutant ST111/111Q cells treated with vehicle or SKF 38393 (Fig. 37).

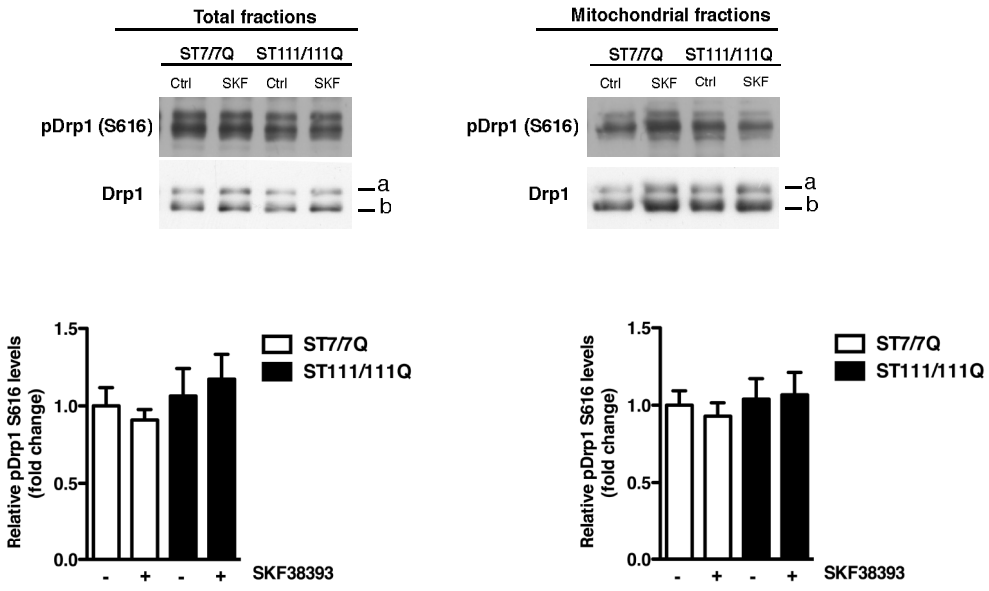

Figure 37. Wild-type ST7/7Q and mutant ST111/111Q cells exhibit unaltered levels of phospho-Drp1 S616. Representative Western blots showing the levels of pDrp1 S616 in total and mitochondrial fractions obtained from vehicle and SKF 38393 treated wild-type ST7/7Q and mutant ST111/111Q cells. Drp1 levels were used to relativize pDrp1 S616 amount. Letters indicate the different isoforms recognized by the respective antibody (DRP1: a–b). Bar histograms show the relative fold change ± SEM of 6 independent experiments.

No significant differences on the phosphorylation at Ser616 of Drp1 protein were observed between genotypes, neither at the total nor at the mitochondrial fraction. Moreover, also D1R activation induced by SKF 38393 did not affect the phosphorylation of Drp1, suggesting that the increase of mitochondrial fragmentation mediated by Cdk5 does not depend on Drp1 phosphorylation on this residue.

2. ROLE OF MITOCHONDRIA ASSOCIATED MEMBRANES (MAMs) IN ALTERED CA^{2+} SIGNALLING IN HD

Aberrant calcium signalling may represent another mechanism involved in the specific HD striatal vulnerability. In particular, striatal neurons are extremely sensitive to changes in intracellular Ca^{2+} and mHtt has been postulated to be involved in the destabilization of cytosolic and mitochondrial Ca^{2+} homeostasis (Bezprozvanny & Hayden, 2004). Since the distance between ER and mitochondria tightly regulates the cross talk between both organelles, including Ca^{2+} transfer, we wonder if the mitochondrial pathology previously described could also alter these contacts and generate Ca^{2+} dyshomeostasis in HD. To validate our second aim, we investigated ER-mitochondria contact sites and related Ca^{2+} transport in murine models of HD along the disease progression. We analysed co-localization between the organelles, the levels of the MAMs proteins involved in Ca^{2+} handling and Ca^{2+} transfer between the two compartments.

2.1 Contact sites between mitochondria and ER in striatal primary neurons of mouse models of HD

To determine the effect of mHtt on MAM behaviour, we examined ER-mitochondria contact sites in primary striatal neurons from two different HD mouse models, mutant Hdh7/111Q knock-in mice and R6/1 mice. These two HD models differ in the onset and progression of HD pathology, with R6/1 mice showing earlier onset and faster disease progression than Hdh7/111Q knock-in mice (J. Kim, Bordiuk, & Ferrante, 2011). First, we transfected primary striatal neurons from wild-type Hdh7/7Q and mutant Hdh7/111Q knock-in mice with pDsRed2-Mito to detect mitochondria (in red) and with GFP-Sec61β to detect ER (in green), and regions where the two signals were in close apposition were analysed and quantitated (Fig. 38). We found that the degree of ER–mitochondrial contact in mutant Hdh7/111Q knock-in striatal neurons was lower compared with wild-type Hdh7/7Q neurons (~ 10% decrease; $p<0.05$;Fig. 38A). Next, the same experiment was conducted in transfected primary striatal neurons from wild-type and R6/1 mice.

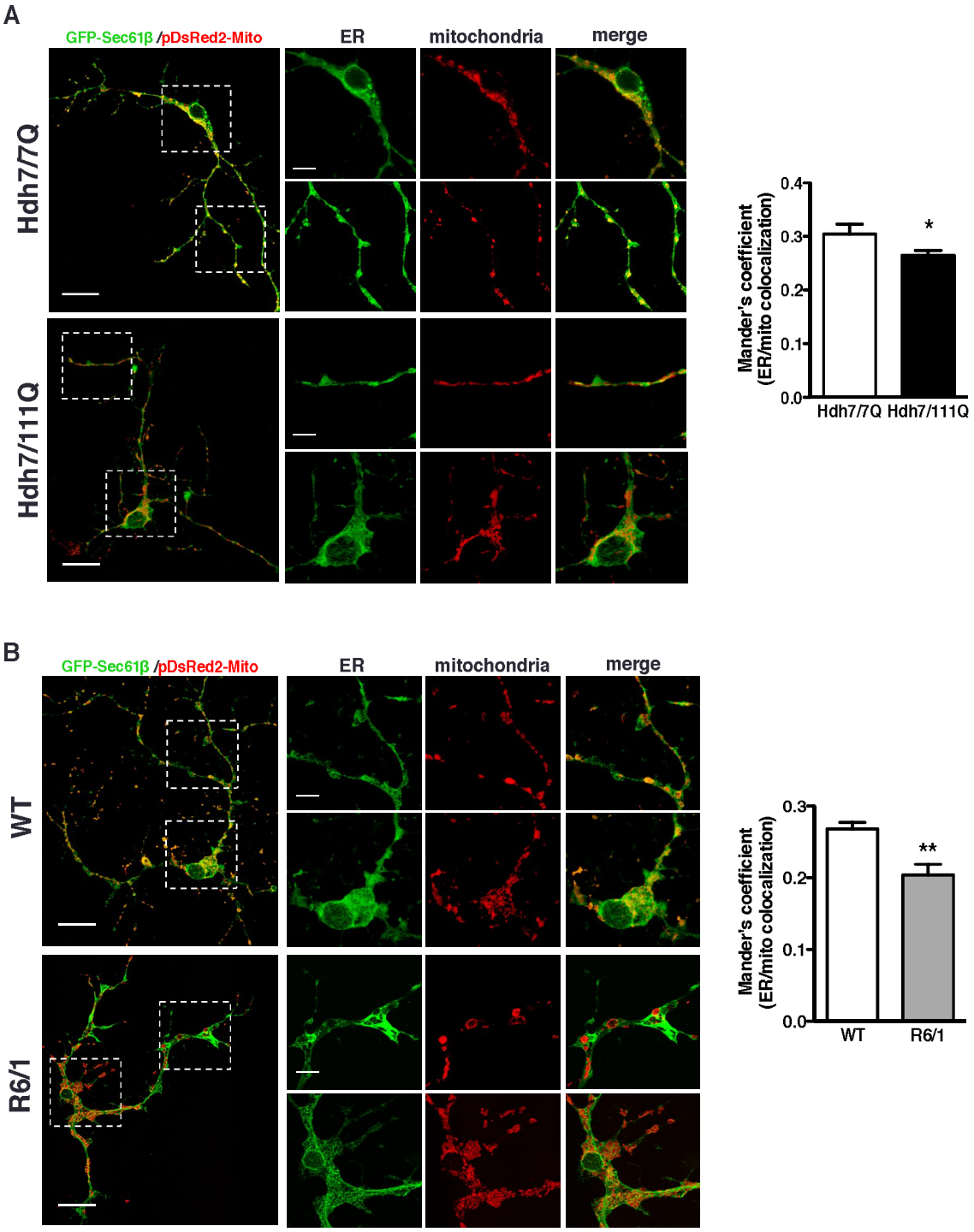

Figure 38. Hdh7/111Q and R6/1 primary striatal neurons exhibit decreased ER-mitochondria contacts. Representative confocal images showing ER and mitochondrial apposition in (A) knock-in and (B) R6/1 primary striatal neurons labeled with GFP-Sec61-β (green) and pDsRed2-mito (red); scale bar 20 μm. Panels on the right show splitted or merged channel magnifications of the boxed areas; scale bar 10 μm. Bar histogram indicates quantitation of ER/mito co-localization represented by normalized Manders' coefficient values calculated from z-axis confocal stacks. Data represent mean ± SEM of 4 independent experiments in which 15–20 neurons were analysed with ImageJ software. **p<0.01, *p<0.05 vs. wild-type striatal neurons as determined by unpaired Student's t-test.

Similar changes in ER–mitochondrial apposition with a significant decreased in R6/1 respect to wild-type striatal neurons was observed (~ 20% decrease; $p<0.05$;Fig. 38B). These data suggest a possible rupture between ER-mitochondria associated membranes and therefore an alteration of mechanisms regulated by these contacts, such as Ca^{2+} homeostasis.

2.2 Levels of proteins involved in the formation of MAMs and in Ca^{2+} homeostasis in striatal tissue from murine models of HD and from post-mortem brain of HD patients

Mutation or ablation of some MAMs-resident proteins can alter the distance between ER and mitochondrial compartments (de Brito & Scorrano, 2008). Since this distance tightly regulates the cross talk between both organelles, including Ca^{2+} transfer (Hayashi et al., 2009), we examined by Western blot the levels of MAM proteins involved in Ca^{2+} transport in the striatum of HD mice. In particular, we have analysed the levels of inositol 1,4,5-triphosphate receptor type 3 (IP3R3), the main transmitter of Ca^{2+} between ER and mitochondria (Mendes et al., 2005), and of Grp75, a mitochondrial chaperone that regulates Ca^{2+} signalling and allows the physical link between the two organelles (Szabadkai, Bianchi, et al., 2006). First, we analysed the levels of these proteins in the striatum of wild-type Hdh7/7Q and mutant Hdh7/111Q knock-in mice at different ages. Our results showed that IP3R3 protein levels were decreased in mutant Hdh7/111Q mice compared to wild-type mice (~ 50% decrease; $p<0.05$; Fig. 39) at 9 and 13 months of age, while a similar tendency was found at 18 months, although it did not reach the significance.

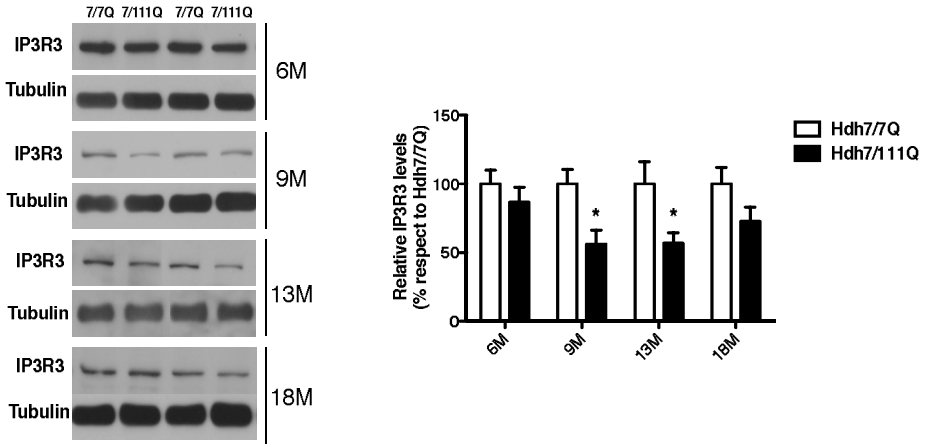

Figure 39. Deregulation of IP3R3 in Hdh7/111Q knock-in mice striatum during the progression of the disease. Representative Western blots showing the levels of IP3R3 in striatum obtained from wild-type Hdh7/7Q and Hdh7/111Q knock-in mice at different stages (M, months) of the disease progression. α-Tubulin was used as loading control. Bar histogram showing the relative IP3R3 protein levels in wild-type Hdh7/7Q and mutant Hdh7/111Q mice. Data represent mean ± SEM (n=7). *p<0.05 vs. wild-type Hdh7/7Q mice as determined by unpaired Student's t-test.

The analysis of Grp75 revelead a significant reduction in mutant Hdh7/111Q mice at 9 months of age (~ 40% decrease; p<0.05; Fig.40), whereas at later disease stages no significant changes were detected.

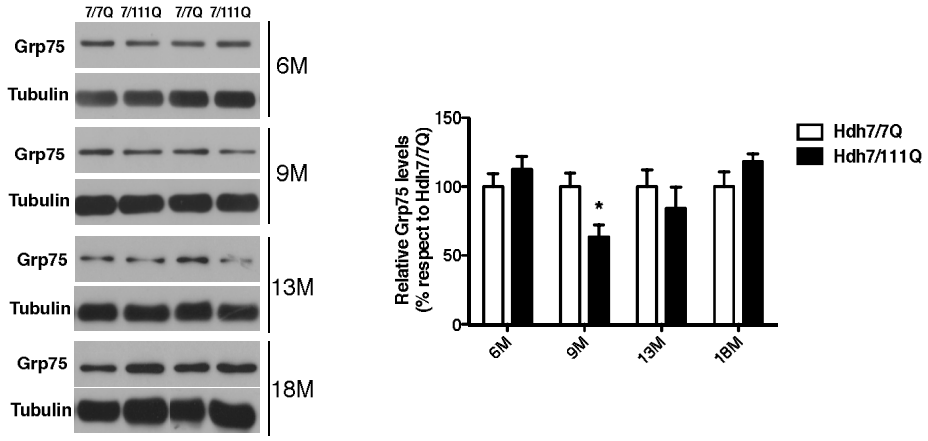

Figure 40. Grp75 levels in Hdh7/111Q knock-in mice striatum during the progression of the disease. Representative Western blots showing the levels of Grp75 in striatum obtained from wild-type Hdh7/7Q and Hdh7/111Q knock-in mice at different stages (M, months) of the disease progression. α-Tubulin was used as loading control. Bar histogram showing the relative Grp75 protein levels in wild-type Hdh7/7Q and mutant Hdh7/111Q mice. Data represent mean ± SEM (n=7). *p<0.05 vs. wild-type Hdh7/7Q mice as determined by unpaired Student's t-test.

Afterwards, we also investigated the levels of IP3R3 and Grp75 in the striatum of R6/1 mice. Western blot analysis revealed a significant decrease in IP3R3 protein levels in R6/1 mice at 12 weeks that was maintained along the disease progression, showing at 20 and 30 weeks of age a similar decline (~ 50% decrease; $p<0.05$; Fig. 41).

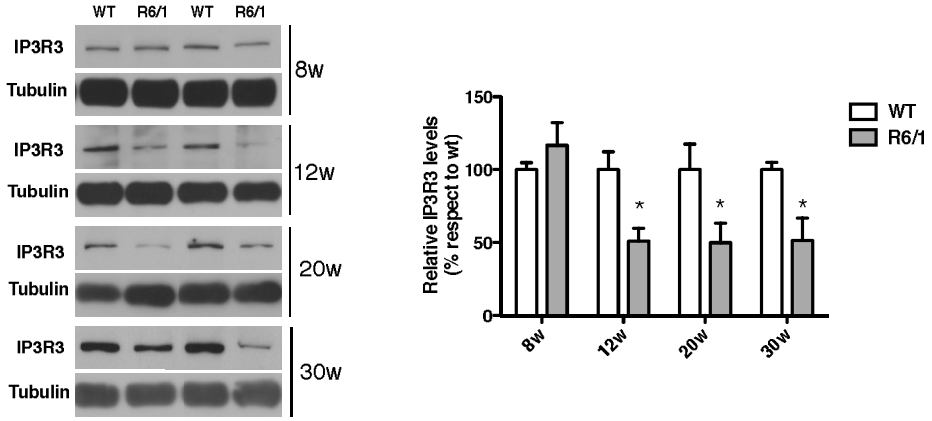

Figure 41. Decrease of IP3R3 levels in R6/1 knock-in mice striatum during the progression of the disease. Representative Western blots showing the levels of IP3R3 in striatum obtained from wild-type and R6/1 mice at different stages (W, weeks) of the disease progression. α-Tubulin was used as loading control. Bar histogram showing the relative IP3R3 protein levels in wild-type and R6/1 mice. Data represent mean ± SEM (n=7). *p<0.05 vs. wild-type Hdh7/7Q mice as determined by unpaired Student's t-test.

As previously observed in Hdh7/111Q mice, Grp75 reduction was observed at middle but not at late disease stages. In fact, Grp75 levels started to decline at 12 weeks (~ 50% decrease; $p<0.05<$; Fig. 40) that was maintained at 20 weeks in R6/1 mice (~ 20% decrease; $p<0.001$; Fig. 40), whereas no changes were observed at 30 weeks between genotypes (Fig. 42).

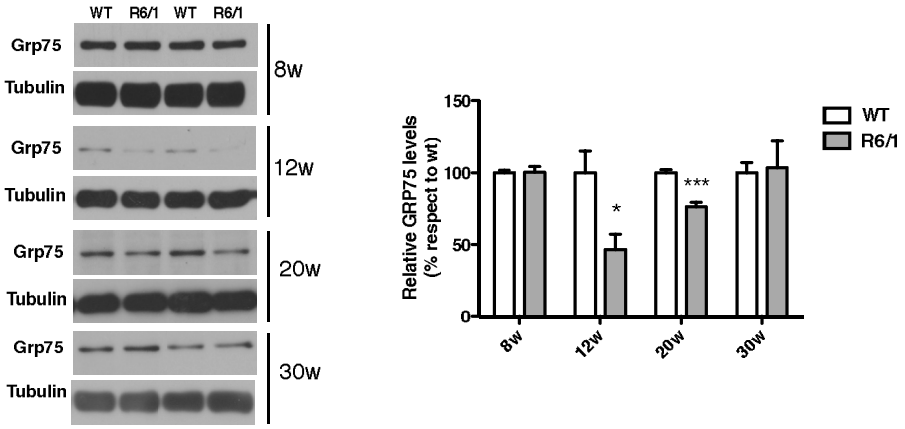

Figure 42. Grp75 levels in R6/1 knock-in mice striatum during the progression of the disease. Representative Western blots showing the levels of Grp75 in striatum obtained from wild-type and R6/1 mice at different stages (W, weeks) of the disease progression. α-Tubulin was used as loading control. Bar histogram showing the relative IP3R3 protein levels in wild-type and R6/1 mice. Data represent mean ± SEM (n=7). ***p<0.001, *p<0.05 vs. wild-type Hdh7/7Q mice as determined by unpaired Student's t-test.

Finally, we analysed the levels of these proteins in the putamen of HD patients (Fig. 43). According to the results obtained in HD mice, IP3R3 and Grp75 (~ 40% decrease; p<0.05) protein levels were decreased in the putamen of HD patients compared with levels in the putamen of non-affected individuals.

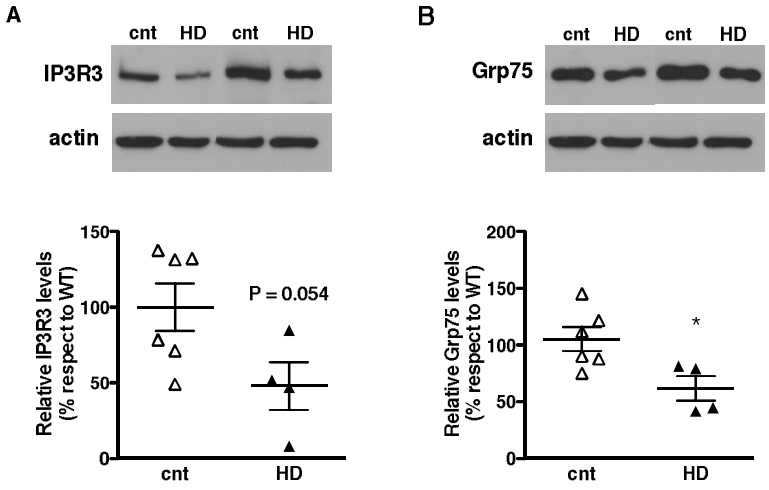

Figure 43. Alteration of IP3R3 and Grp75 levels in human HD putamen. Representative Western blots showing the levels of IP3R3 and Grp75 in post-mortem putamen obtained from control and HD patients. α-Actin was used as loading control. Histogram showing the relative IP3R3 and GRP75 protein levels in control and HD patients. Data represent mean ± SEM (n=4-6). *p<0.05 vs. control as determined by unpaired Student's t-test.

Altogheter, our data indicate that the expression of mHtt induces alterations in the levels of MAM proteins crucials for maintaining Ca^{2+}

homeostasis and that this perturbation is manifested principally at middle HD stages.

2.3 ER-mitochondria Ca^{2+} transport in striatal primary neurons of mouse models of HD

Considering the decline in IP3R3 and Grp75 protein levels and the reduction of contacts between ER and mitochondria, we wonder if the disruption of Ca^{2+} homeostasis in HD could be due to altered Ca^{2+} exchange between the two compartments. To this aim, we simultaneously monitored the levels of intracellular Ca^{2+} (Ca_i^{2+}) with Fluo4 and the potential of mitochondrial membrane ($\Delta\Psi_m$) with TMRM before and after the induction of Ca^{2+} release from the ER using Thapsigargin (TG), which depletes ER Ca^{2+} stores inhibiting SERCA activity (Representative traces in Fig. 44A I-IV). Our results show that TG causes a higher increase in Ca_i^{2+} concentration in R6/1 striatal neurons compared to wild-type neurons (~ 50% increase; $p<0.05$; Fig. 44B II). This phenomenon could be due to reduced mitochondria Ca^{2+} uptake in R6/1 neurons, since the robust Ca_i^{2+} increase was associated with a slighter depolarization of the $\Delta\Psi_m$ compared to wild-type neurons (~ 20% less decrease; $p<0.05$; Fig. 44B V), as indicated by TMRM fluorescence curves (Fig. 44A IV). Importantly, differences between genotypes in Ca_i^{2+} and in $\Delta\Psi_m$ before the initial stimuli were not detected (Fig. 44B I and IV). In order to demonstrate that mitochondria Ca^{2+} uptake in R6/1 striatal neurons is decreased, we induced a maximal depolarization treating the primary cultures with FCCP, an uncoupler of oxidative phosphorylation that collapses the mitochondrial membrane potential, thus inducing the release of Ca^{2+}. Mitochondrial depolarization in R6/1 neurons induced lower increase of Ca_i^{2+} compared to wild-type neurons (~ 25% less increase; $p<0.001$; Fig. 44B III). Interestingly, these differences between genotypes could be associated with a lower mitochondrial Ca^{2+} accumulation in R6/1 respect to wild-type neurons, as represented by the slight decrease in mitochondrial FCCP-releasable Ca^{2+} pool (~ 25% less decrease; $p<0.001$; Fig. 44B VI). Thus, our results suggest that the expression of mHtt induces a deregulation of Ca^{2+} homeostasis associated with reduced mitochondrial Ca^{2+} retention, probably due to alteration in the ER-mitochondrial Ca^{2+} transfer mechanism.

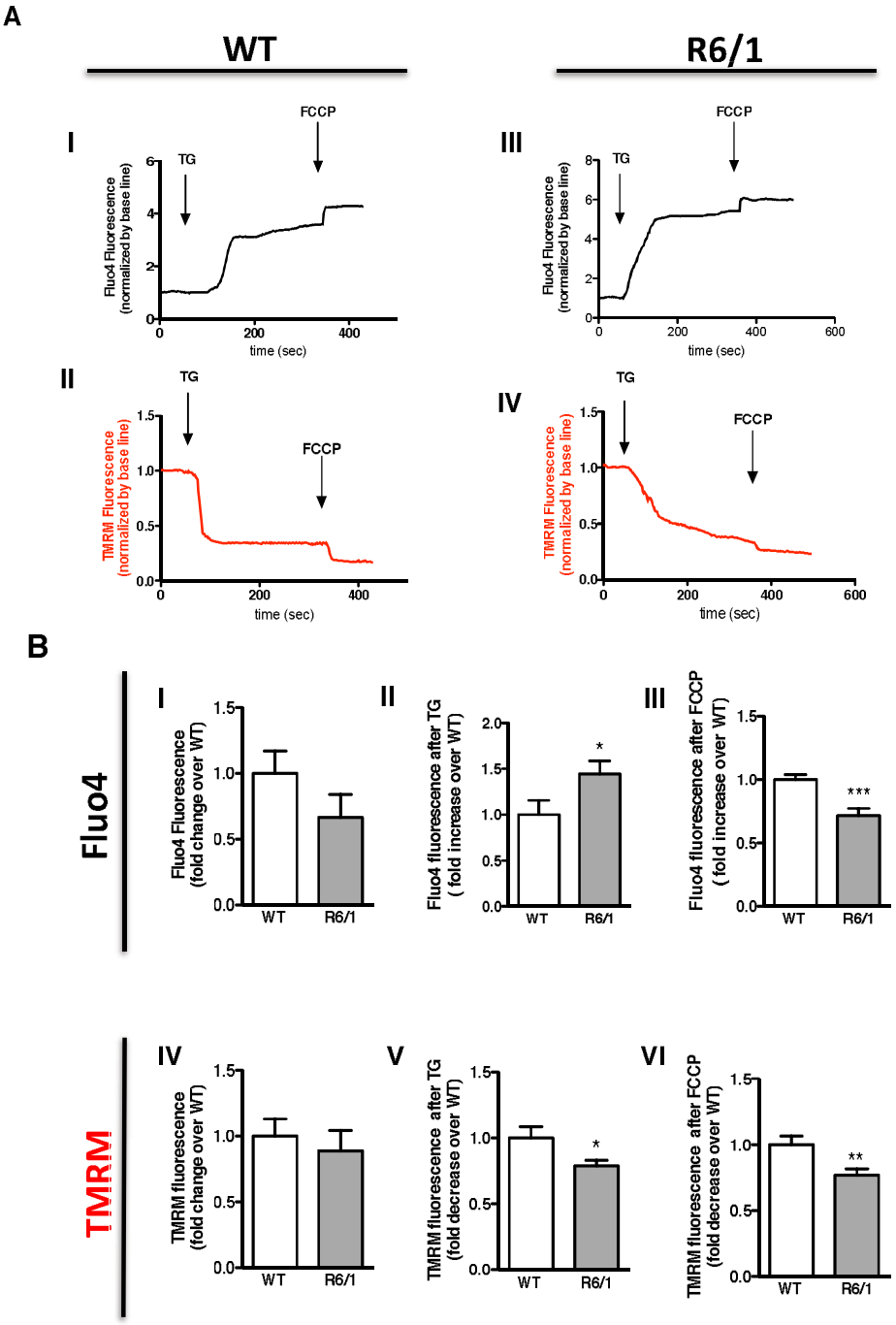

Figure 44. ER-mitochondrial Ca^{2+} transfer is altered in R6/1 primary striatal neurons. Primary striatal neurons loaded with Fluo4 (5 μM) and TMRM (20 nM) were stimulated with TG (0.5 μM) and FCCP (2 μM). (A) Representative Fluo4 (I-III) or TMRM (II-IV) fluorescence traces from individual cells. (B) Fluorescence folds change of Fluo4 (I, II, III) or TMRM (IV, V, VI) before and after TG or FCCP exposure. For single-cell imaging studies, data represent mean ± SEM of 6 independent experiments. ***p<0.001, **p<0.01 *p<0.05 vs. wild-type mice as determined by unpaired Student's t-test.

DISCUSSION

Mitochondrial dysfunction is a well-documented feature of Huntington's disease (HD) pathogenesis and other neurodegenerative disorders (Browne, 2008; Lezi & Swerdlow, 2012). Several mechanisms have been proposed to explain how mitochondrial defects could activate cell death pathways in HD. However, the outstanding question is whether these defects are linked or not to the preferential striatal degeneration in HD. In this Thesis, we provide evidence showing that deregulation of mitochondrial dynamics may have a detrimental role in HD striatal pathology, by enacting dopamine neurotoxicity and inducing alterations in intracellular Ca^{2+} homeostasis.

Mitochondrial dynamics is a crucial event that determines mitochondrial morphology and size, but also regulates mitochondrial distribution and function (Chan, 2006b). Several studies reported that this process is disrupted in HD revealing increased mitochondrial fragmentation, and therefore an imbalance towards mitochondrial fission events (Guo et al., 2013; Reddy, 2014). In line with this evidence, our results have shown in a precise genetic HD striatal cell line, impaired mitochondrial dynamics manifested as higher mitochondrial fragmentation and disrupted mitochondrial network compared to wild-type striatal cells. These changes in mitochondrial morphology are not related to cell cycle variations between cell genotypes, although it is known that cell cycle progression may influence mitochondrial fission/fusion (Mitra, Wunder, Roysam, Lin, & Lippincott-Schwartz, 2009). Indeed, the analysis of mitochondrial size in amitotic striatal neurons obtained from Hdh7/111Q mice, showed the same patter of alteration, indicating that the impairment in mitochondrial dynamics exclusively depends on the effect of mHtt toxicity. Importantly, excessive mitochondrial fragmentation in HD has been associated with alterations in levels and distribution of pro-fission and pro-fusion mitochondria-shaping proteins (Costa et al., 2010; U. Shirendeb et al., 2011). Generally, these studies have correlated the aberrant mitochondrial fragmentation with increased levels and mitochondrial translocation of fission protein Drp1 and with decreased levels of fusion components Opa1 and Mfns. Accordingly, we found lower levels of Opa1 in the mitochondrial fraction of mutant ST111/111Q striatal cells compared to wild-type, indicating a possible decrease of mitochondrial fusion events. By contrast, enhanced mitochondrial

fragmentation in mutant ST111/111Q striatal cells was not associated with an increase in the levels of Drp1, neither at the total nor at the mitochondrial fraction, but with a decrease. Notably, Drp1 mRNA expression was also decreased in mutant ST111/111Q striatal cells suggesting that mHtt may alter transcriptional regulation of Drp1. In this view, it has been described that p53 can induce mitochondrial fission by transcriptional upregulation of Drp1 expression (J. Li et al., 2010). Interestingly, previous works have demonstrated reduced p53-mediated gene transcription in mHtt expressing cells suggesting that the decrease in Drp1 mRNA levels found in mutant striatal cells could be related with a defective p53 transcriptional function (Steffan et al., 2000). Since the levels and expression of Drp1 are low in mutant ST111/111Q striatal cells, one compelling question is how mitochondrial fragmentation can be enhanced in these cells. A possible explanation is provided by the results obtained from the analysis of enzymatic activity of the fission protein. As reviewed before, Drp1 is the main mediator of mitochondrial fission, forming constricting spirals that split both outer and inner mitochondrial membranes through a GTP hydrolysis-dependent mechanism (Smirnova et al., 2001). Interestingly, we found that the GTPase activity of Drp1 was significantly higher in mutant ST111/111Q striatal cell lines and in the striatum of Hdh7/111Q knock-in mutant mice compared to wild-type mice indicating that in the presence of mHtt, the activity of Drp1 is aberrantly activated. These findings are in agreement with previous studies showing elevated Drp1 activity in the striatum and cortex of BACHD mice and in the cortex of HD patients (U. P. Shirendeb et al., 2012). Thus, altogether these data suggest that the imbalance in the levels and activity of proteins involved in the mitochondrial dynamic machinery could be responsible for the abnormal mitochondrial morphology observed in mutant striatal cells.

As largely discussed in the previous sections, striatum is the principal target in HD pathogenesis with preferential neurodegeneration of MSNs. This type of neurons may fire at high frequencies with consequent increased energy utilization, thus making the striatum particularly vulnerable to alteration in energy production. The evidence that mitochondria differ across tissues, cell-types and even sub-cellular compartments within neurons (Dubinsky, 2009; Kuznetsov, Hermann, Saks, Hengster, & Margreiter, 2009)

could provide one possible explanation of this selective vulnerability. Indeed, striatal mitochondria have intrinsically higher oxidative phosphorylation (OXPHOS) function and a higher basal $\Delta\Psi_m$ respect to mitochondria from other brain regions, suggesting that this increased dependence on OXPHOS could make the striatum more sensitive to partial defects (Pickrell, Fukui, Wang, Pinto, & Moraes, 2011). Importantly, it has been reported that striatal mitochondria may also be more sensitive than cortical ones when challenged with different stressors. For instance, hypoxia produced a decrease in striatal but not cortical succinate dehydrogenase and cytochrome oxidase mitochondrial enzyme activity (Dagani et al., 1984). Moreover, mitochondria from dorsolateral striatum, but not paramedian cortex, exhibited decreased cytochrome oxidase and lactate dehydrogenase activity 24 hours after an ischemic insult (Zaidan & Sims, 1997). Others studies using primary cultures showed also an increased Ca^{2+} vulnerability of striatal versus cortical-derived neurons, thus revealing that mitochondria from different regions react differently to Ca^{2+} stress (Oliveira & Goncalves, 2009). Taking account the intrinsic property of striatal mitochondria it should not surprise that perturbation of mitochondrial function consequent to mHtt production could adversely affect the function of MSNs over other neuronal cells. However, the precise mechanism by which mHtt induces this selective degeneration remains uncertain. On the basis that striatum receives a dense dopaminergic innervation and aberrant dopamine (DA) metabolism occurs in HD (Jakel & Maragos, 2000), we converged our efforts in the study of the possible causes of dopamine neurotoxicity and the consequent striatal degeneration, especially looking for alteration in mitochondrial function. So far, little has been described on how dopaminergic system directly affects the control of mitochondrial biology. Oxidative stress as a consequence of DA metabolism results in the formation of reactive oxidative species and quinones that may alter several mitochondrial processes such as ATP production, membrane permeability or fission/fusion mitochondrial events (Berman & Hastings, 1999; Frank et al., 2012; Wu, Zhou, Zhang, & Xing, 2011). Indeed, mitochondrial respiration and ATP production were found significantly reduced in our HD striatal cell line along with an increase in mitochondrial-generated reactive-oxidative species and a decrease in the mitochondrial membrane potential (Milakovic & Johnson, 2005; Quintanilla, Jin, Fuenzalida, Bronfman,

& Johnson, 2008; Ribeiro et al., 2012; Seong et al., 2005). Moreover, DA besides being an inductor of oxidative stress can also induce mitochondrial dysfunction through activation of DA receptors. Actually, activation of D2 receptors in striatal neurons expressing mHtt alters mitochondrial function by down-regulating mitochondrial complex II expression (Benchoua et al., 2008) while mitochondrial axonal transport can be regulated by integration of the opposite effects of D1R and D2R activation (S. Chen, Owens, & Edelman, 2008). Notably, there is no data on the potential role of DA receptors in mitochondrial fission-fusion events. In this Thesis, we have described for the first time that direct D1R activation induced mitochondrial dynamics defects, leading to a percentage of striatal cells displaying mitochondrial fragmentation close to 80% when mHtt is expressed and to 40% in wild type conditions. Importantly, D1R-induced mitochondrial fragmentation either in wild-type or mutant ST111/111Q striatal cells is associated with an increase in Drp1 protein levels. However, two major differences can be noticed when mutant and wild-type cells are compared. First, in wild-type cells the increase in Drp1 levels was located in the cytosolic fraction while that in mutant ST111/111Q striatal cells was found in the mitochondria and second, in mutant but not wild-type huntingtin cells the increase in Drp1 protein levels was accompanied by an increase in the respective GTPase activity, which agrees with the data on mitochondrial Drp1 localization. Overall, these data strengthen previous findings reported from our group in which D1R activation induces higher neuronal death of mutant ST111/111Q striatal cells compared to ST7/7Q cells (Paoletti et al., 2008). Thus, we propose that D1R activation increases mHtt-induced mitochondrial fragmentation by altering Drp1 activity, which may be related with the higher susceptibility of mutant ST111/111Q striatal cells to neurotoxicity.

How D1R activation induces such changes in Drp1 levels, distribution and activity is a crucial question. Research in the literature did not provide any evidence on a potential regulation of the mitochondrial fission proteins through activation of dopaminergic signalling. However, we previously found that the increase in cell death induced by D1R activation in mutant ST111/111Q striatal cells was related with an aberrant increase in the activity of the kinase Cdk5 (Paoletti et al., 2008). Interestingly, this kinase has been

involved in mitochondrial dysfunction (Sun et al., 2008) by increasing oxidative stress or acting as an upstream regulator of mitochondrial fission (Meuer et al., 2007). In agreement with these findings, in this Thesis we have demonstrated that pharmacological inhibition of Cdk5 in mutant ST111/111Q striatal cells completely restores mitochondrial morphology and mitochondrial network arrangement. Consistently, we found that this recovery is attributed to a reduction in the activity and expression of Drp1, mediated by the reduction of Cdk5 function (Fig. 45). Thus, an appropriate Cdk5 activity seems to be necessary for the modulation of fission events and for the preservation of functional mitochondrial structures. These data prompted us to explore the hypothesis that in mutant ST111/111Q striatal cells the increase in Cdk5 activity induced by D1R activation could be responsible for the increase in mitochondrial fragmentation. Our results validate this hypothesis since we have demonstrated that either pharmacological inhibition or the knockdown of Cdk5 expression repressed abnormal fission events induced by D1R activation. In line with these findings, we also observed that inhibition of Cdk5 prevents in mutant ST111/111Q striatal cells the D1R-mediated increase of Drp1 protein levels and their translocation to the mitochondria.

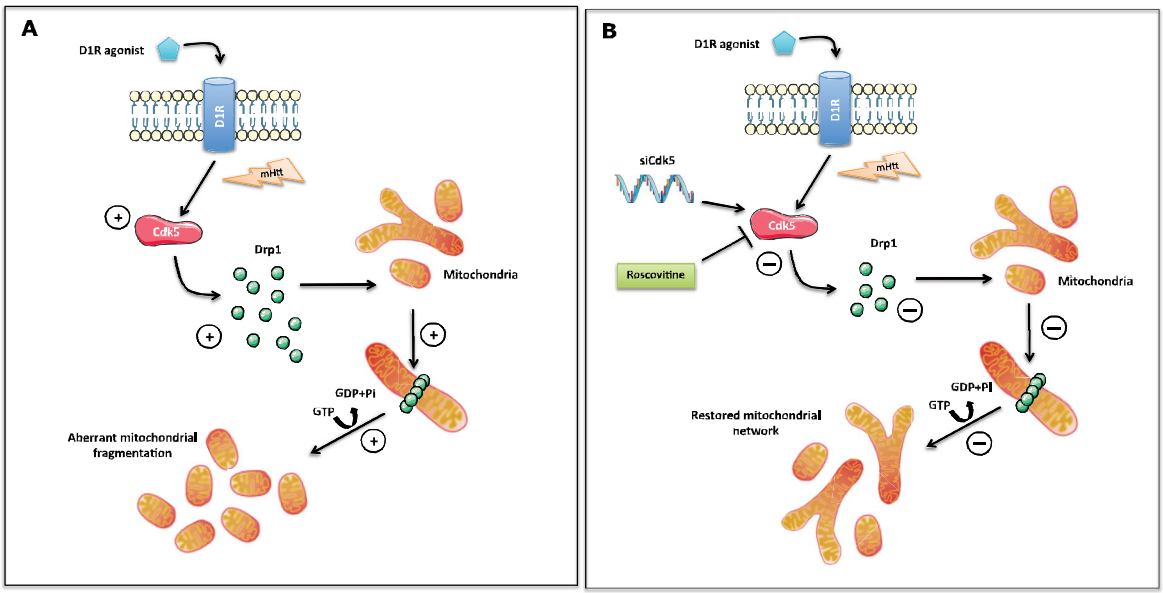

Figure 45. Aberrant Cdk5 activity mediates D1R-induced mitochondrial fragmentation in HD. Schematic representation of the results obtained in the first part of the Thesis. (A) As previously described, D1R agonist enhances CDk5 activity in striatal cells expressing mHtt (Paoletti et al., 2008). This alteration induces an increase in fission protein Drp1 levels and its recruitment to the mitochondrial membrane. Finally, enhanced Drp1 activity leads to an aberrant mitochondrial fragmentation. (B) D1R-induced mitochondrial fragmentation is mediated by Cdk5 in HD. Indeed, Cdk5 pharmacological inhibition with roscovitine or its down-regulation using a specific siRNA, completely restore the mitochondrial network by decreasing Drp1 activity and subsequently reducing fission events.

Recruitment of Drp1 to the mitochondria is a critical event in mitochondrial fission since represents the starting point of the constriction and further fragmentation of the organelle (Pon, 2013). Even though the precise mechanisms underlying the recruitment of Drp1 to the mitochondria surface are not well understood several studies stress the role of Drp1 post-translational modifications (Chang & Blackstone, 2010). Regarding regulation of Drp1 activity by phosphorylation the published data can be rather confusing, since phosphorylation at the same residue may result in induction or prevention of mitochondrial fission depending on cell type, physiological/pathological condition or even the kinase involved (B. Cho, Choi, Cho, Kim, & Sun, 2013). This is the case for Ser616, a residue that can be phosphorylated by Cdk5. While in post-mitotic mature neurons and in physiological conditions phosphorylation at Ser616 by Cdk5 inhibits Drp1 activity and therefore mitochondrial fission (B. Cho et al., 2014), under pathological conditions, Ser616 (or Ser579 in Drp1 isoform 3) promotes mitochondrial fission either in mitotic or neuronal cells (Jahani-Asl et al., 2015; Qi et al., 2011; Taguchi et al., 2007). In this Thesis, we have not observed any increase in phosphorylation at Ser616 in HD striatal cells compared to wild type cells although Drp1 activity has been found to be increased suggesting that phosphorylation in other residues or other post-translational modifications could be involved. In another view, several studies have pointed out the role of the actin and microtubule cytoskeleton in Drp1-induced mitochondrial fission (De Vos, Allan, Grierson, & Sheetz, 2005; Pon, 2013; Varadi et al., 2004). Interestingly, Cdk5 can phosphorylate a plethora of different proteins involved in cytoskeleton dynamics (Contreras-Vallejos et al., 2014; Kawauchi, 2014). Indeed, increased Tau phosphorylation has been previously reported in mutant ST111/111Q striatal cells by our group (Paoletti et al., 2008) suggesting that an aberrant phosphorylation of cytoskeleton-related proteins could be involved in the accumulation of Drp1 on the mitochondria membranes leading to mitochondrial fission.

Excessive mitochondrial fragmentation could have important negative effects on the neuronal homeostasis of Ca^{2+}. Indeed, recent studies have described a mutual interaction between the determinants of mitochondrial morphology and functions like Ca^{2+} accumulation (Szabadkai, Simoni, et al.,

2006). Increase of cytoplasmic and mitochondrial Ca^{2+} levels was shown to induce mitochondrial division and transient break of mitochondrial movements (Rintoul, Filiano, Brocard, Kress, & Reynolds, 2003; Yi, Weaver, & Hajnóczky, 2004). On the other hand, mitochondrial shape and its distribution in the intracellular space modifies mitochondrial Ca^{2+} uptake properties, partly by changing the interaction with other cellular membranes such as the ER membrane (Szabadkai et al., 2004). Importantly, the impairment of mitochondria to handle Ca^{2+} has been recently linked to the pathogenesis of neurodegenerative diseases (Giacomello, Drago, Pizzo, & Pozzan, 2007), and abnormalities in mitochondrial Ca^{2+} buffering have been described in many HD models, both in early and late stages (Giacomello et al., 2011). However, data in the literature are somewhat controversial and offer no conclusive molecular mechanism behind the mitochondrial Ca^{2+} mishandling in HD. In this Thesis, we have explored whether the propensity of mitochondria to undergo fragmentation in HD could result in the disruption of ER-mitochondria contacts, which are essential for the proper handling of Ca^{2+} by mitochondria. To this aim, we first analysed ER-mitochondrial apposition in striatal primary neurons from HD mice. We observed a decrease in the co-localization between the two organelles in striatal neurons expressing mHtt, indicating an important loss of contacts. This abnormality could be due to the aberrant mitochondrial fission that we have previously described, since the alteration of mitochondrial network leads to the positioning of mitochondrial particles far from ER membranes.

Besides disruption of mitochondrial dynamics, another mechanism could underlie the reduction of contact points between ER and mitochondria and for altering mitochondrial Ca^{2+} buffering. Mitochondria-associated membrane (MAMs) contains key Ca^{2+} handling proteins and Ca^{2+} sensing ER chaperones that may participate in the fine-tuning of cellular Ca^{2+} signals (Gilady et al., 2010; Hayashi & Su, 2007; Simpson, Mehotra, Lange, & Russell, 1997; Simpson & Russell, 1997; Szabadkai, Bianchi, et al., 2006). Interestingly, the deletion/mutation of some of these proteins has been shown to impair Ca^{2+} homeostasis leading to a reduction of Ca^{2+} mitochondrial uptake as well as ER Ca^{2+} overload (Bernard-Marissal, Medard, Azzedine, & Chrast, 2015; de Brito & Scorrano, 2008). In this view, we explored possible candidates which could

be responsible for the reduction of ER-mitochondria contacts as well as for Ca^{2+} dyshomeostasis in HD. Our results have revealed significant changes in two regulatory Ca^{2+} proteins that are localized in the MAMs: IP3R3 and Grp75. IP3R3 constitutes the principal isoform of the IP3Rs ER intracellular Ca^{2+} release channels that highly co-localizes with mitochondria where modulates mitochondrial Ca^{2+} concentration since its down-regulation significantly decreases mitochondrial Ca^{2+} uptake (Mendes et al., 2005). On the other hand, Grp75 is a mitochondrial chaperone that regulates IP3R-mediated mitochondrial Ca^{2+} signalling (Szabadkai, Bianchi, et al., 2006). In particular, Grp75 leads the physical interaction of VDAC with the ER Ca^{2+}-release channels IP3Rs, thus mediating conformational coupling between the IP3Rs and the mitochondrial Ca^{2+} uptake machinery. In this Thesis, we have demonstrated that mHtt induces an important reduction in IP3R3 and Grp75 protein levels in the striatum of two HD mouse models Hdh7/111Q and R6/1 mice at middle stages of the disease, suggesting that the decrease of IP3R3 and Grp75 protein levels is a general hallmark of HD condition. Although there is no evidence linking the role of IP3R3 and Grp75 to the ER-mitochondria Ca^{2+} mishandling in HD, these previous findings suggest that mHtt may deregulate the levels of these protein at MAMs, leading to altered Ca^{2+} homeostasis. However, we only have demonstrated a general reduction of these proteins and we cannot assume that the decrease of IP3R3 and Grp75 involves exclusively the MAMs portion. Therefore, a more accurate analysis of the proteins levels in this fraction needs to be performed.

So far, our findings suggest that direct communication between mitochondria and ER is critically compromised in HD, therefore affecting Ca^{2+} homeostasis. In agreement with this hypothesis, we found that mitochondrial Ca^{2+} handling is altered in striatal neurons obtained from R6/1 mice. In particular, we have analysed the transfer of Ca^{2+} from ER to the mitochondria by using *in situ* single-cell simultaneous measurement of Ca_i^{2+} and $\Delta\Psi_m$. We have demonstrated that neurons expressing mHtt show an alteration in the Ca^{2+} exchange between organelles preventing proper mitochondrial Ca^{2+} uptake thereby determining excessive cytosolic Ca^{2+} release from the ER stores. Indeed, the induction of ER Ca^{2+} release in HD striatal neurons produces an immediate increase in Ca_i^{2+} associated with a low mitochondrial

depolarization, suggesting a defective mitochondrial Ca^{2+} uptake probably due to the disruption of the MAMs (Fig. 46).

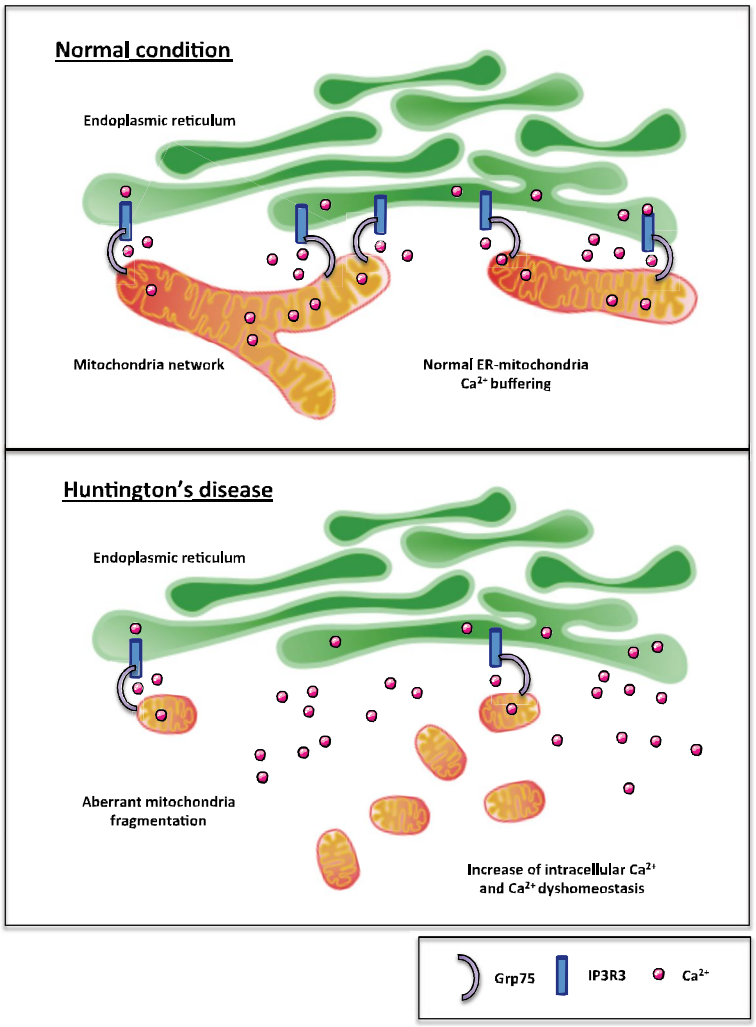

Figure 46. ER-mitochondrial Ca^{2+} transfer is altered in HD. Model proposed for the mechanism underlying Ca^{2+} dyshomeostasis in HD. In normal conditions mitochondria load Ca^{2+} released by ER, thus maintaining intracellular Ca^{2+} homeostasis. ER-mitochondria Ca^{2+} cross-talk is mediated by normal expression of Grp75 and IP3R3 proteins that physically interact and regulate the transport of the ion into the mitochondria. In Huntington's disease, mitochondrial Ca^{2+} uptake is reduced due to the loss of tethers between the two organelles. Based on our results, we propose that aberrant mitochondrial fragmentation and the reduction of Grp75 and IP3R3 protein levels trigger mitochondrial Ca^{2+} mishandling that finally leads to an aberrant increase of cytosolic Ca^{2+}.

These results prompted us to advance a new but not exclusive mechanism for mitochondrial Ca^{2+}mishandling in HD, which differs from studies previously proposed (Choo et al., 2004; Milakovic et al., 2006). These studies attribute the aberrant increase of intracellular Ca^{2+} in HD to the enhanced mitochondrial sensitivity to Ca^{2+} loads. However, the recovery of the mitochondrial Ca^{2+} capacity and the inhibition of mitochondrial PTP induction

failed to ameliorate either the behavioural and neuropathological features of the disease (Perry et al., 2010). Moreover, it has been reported an age-dependent increase in the Ca^{2+} loading capacity of striatal mitochondria (Brustovetsky et al., 2005). Thus, striatal mitochondria isolated from HD mice were more sensitive to Ca^{2+} at 8 weeks of age becoming more resistant to Ca^{2+} several months before the onset of motor symptoms and behavioral phenotype. Similarly, others did not detect a reduced Ca^{2+} capacity in forebrain mitochondria isolated from three different transgenic HD mouse models (Oliveira et al., 2007). The discrepancy between these results and prior studies indicating decreased HD mitochondrial Ca^{2+} capacity may be due to differences in the disease stages of the HD mouse model used or to variations in experimental conditions to examine the mitochondrial Ca^{2+} handling. Further, isolation of mitochondria from brain tissue inevitably results in a mixture of mitochondria from different cell types and with different functional properties, complicating the interpretation of studies using this approach. Our results derived from analysis performed in an appropriate physiological context, proclaimed that Ca^{2+} dishomeostasis in HD could not only depends on intrinsic mitochondrial Ca^{2+} buffering capacity. The gap between the organelles generated by aberrant mitochondrial fragmentation could be sufficiently wide to isolate mitochondria from the Ca^{2+} leakage from the ER, impeding properly mitochondrial Ca^{2+} handling. Indeed, compelling evidence highlights the importance of the distance between the ER and mitochondria in the efficient transfer of Ca^{2+}. It has been reported that increasing the ER–mitochondria distance enhances the efficiency of Ca^{2+} transfer from the ER to the mitochondria, whereas limiting the junctional gap reduces the transfer efficiency (Csordas et al., 2010; Shin & Muallem, 2010). Thus, tightening of the MAMs coupling invokes mitochondria in the handling of Ca^{2+} under resting conditions, sensitizing mitochondria to Ca^{2+} overloading committing the cells to apoptosis, while decreased ER–mitochondria coupling suppresses the Ca^{2+} signal propagation to the mitochondria, putting at risk the Ca^{2+}-dependent control of mitochondrial metabolism (Csordas et al., 2006). Considering the critical role played by mitochondrial Ca^{2+} in the modulation of numerous physiological responses, overall findings reveal a crucial dependence of cell function and survival on the maintenance of a proper distance between the ER and mitochondria.

In conclusion, in this Thesis we have provided new evidences that can explain the preferential striatal neurotoxicity in HD. In particular, we propose a model in which increased Cdk5 activity induced by both mHtt expression and aberrant dopaminergic signalling would contribute to increasing striatal susceptibility in HD by altering the expression, distribution and activity of Drp1 leading to mitochondrial fission events. Moreover, we propose that aberrant ER-mitochondria tethering play a crucial role in the striatal Ca^{2+} dyshomeostasis. Although the mechanism by which mHtt deregulates these contacts remains to be clarified, we hypothesize that excessive mitochondrial fragmentation and reduction of some regulatory Ca^{2+} MAMs-resident proteins could contribute to the dysfunction of the ER–mitochondria architecture. Thus, it is tempting to speculate that by targeting MAMs Ca^{2+} deregulation in HD beneficial therapeutic effects could be achieved.

CONCLUSIONS

The main conclusions obtained as result of the studies presented in this Thesis are:

1. Increased mitochondrial fragmentation in mutant ST111/111Q striatal cells is associated with altered Cdk5 function and increased GTPase activity of mitochondrial fission protein Drp1.

2. Activation of dopaminergic signalling via D1R enhances mitochondrial fission events and disrupts mitochondrial branching network in mutant ST111/111Q striatal cells by increasing Drp1 protein levels and activity.

3. Increased Cdk5 activity mediates D1R-induced aberrant mitochondrial fragmentation in mutant ST111/111Q striatal cells, suggesting that Cdk5 is a crucial player in the regulation of dopamine-induced mitochondrial fission in HD.

4. Increased Cdk5 function in mutant ST111/111Q striatal cells mediates D1R-induced mitochondrial fission by increasing the expression and mitochondrial translocation of Drp1

5. ER-mitochondria contact sites are decreased in striatal neurons of HD mice. As a potential underlying mechanism we propose an excessive mitochondrial fragmentation that will cause an aberrant distribution of these organelles far away from ER membranes.

6. Regulation of intracellular Ca^{2+} homeostasis mediated by ER-mitochondria cross-talk is altered in striatal neurons of HD mice. Loss of membrane tethering between these two organelles could underlie mitochondrial Ca^{2+} mishandling leading to an aberrant increase of cytosolic Ca^{2+}in mutant striatal neurons

7. The decrease in the levels of IP3R3 and Grp75, two crucial MAM-resident Ca^{2+} regulatory proteins in striatum of HD mice may lie behind the alterations in Ca^{2+} homeostasis observed in HD striatal neurons.

8. Our results provide a new detrimental role for the aberrant mitochondrial fragmentation in the selective striatal vulnerability in HD, emerging as an important mediator of dopamine neurotoxicity and a critical event in the disruption of Ca^{2+} homeostasis.

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ANNEX

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Cdk5-mediated mitochondrial fission: A key player in dopaminergic toxicity in Huntington's disease

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ABSTRACT

The molecular mechanisms underlying striatal vulnerability in Huntington's disease (HD) are still unknown. However, growing evidence suggest that mitochondrial dysfunction could play a major role. In searching for a potential link between striatal neurodegeneration and mitochondrial defects we focused on cyclin-dependent kinase 5 (Cdk5). Here, we demonstrate that increased mitochondrial fission in mutant huntingtin striatal cells can be a consequence of Cdk5-mediated alterations in Drp1 subcellular distribution and activity since pharmacological or genetic inhibition of Cdk5 normalizes Drp1 function ameliorating mitochondrial fragmentation. Interestingly, mitochondrial defects in mutant huntingtin striatal cells can be worsened by D1 receptor activation a process also mediated by Cdk5 as down-regulation of Cdk5 activity abrogates the increase in mitochondrial fission, the translocation of Drp1 to the mitochondria and the raise of Drp1 activity induced by dopaminergic stimulation. In sum, we have demonstrated a new role for Cdk5 in HD pathology by mediating dopaminergic neurotoxicity through modulation of Drp1-induced mitochondrial fragmentation, which underscores the relevance for pharmacologic interference of Cdk5 signaling to prevent or ameliorate striatal neurodegeneration in HD.

1. Materials and methods

1.1. Cell cultures

Conditionally immortalized wild-type ST7/7Q and mutant ST111/111Q striatal neuronal progenitor cell lines expressing endogenous levels of normal and mutant huntingtin with 7 and 111 glutamines, respectively, have been described previously [1]. 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).

1.2. Primary cultures of mouse striatal neurons

Dissociated striatal cultures prepared from E.18 Hdh7/7Q and Hdh7/111Q embryos were plated at a density of 40,000 neurons onto 24-well plates pre-coated 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 GlutamaxTM (Gibco-BRL). Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

1.3. HD mouse models

HdhQ111 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. Male and female Hdh7/111Q heterozygous mice were intercrossed to generate age-matched Hdh7/111Q heterozygous and Hdh7/7Q wild-type littermates. The animals were housed with access to food and water ad libitum in a colony room kept at 19–22 °C and 40–60% humidity, under a 12:12 h light/dark cycle. Only males were used to perform all the experiments. All procedures were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approved by the local animal care committee of the Universitat de Barcelona (99/01) and Generalitat de Catalunya (00/1094), in accordance with the Directive 2010/63/EU of the European Commission.

1.4. Drug treatment

To assess the effect of dopamine receptor activation on mitochondrial population, striatal cell lines were treated as previously described [2].

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Briefly, wild-type ST7/7Q and mutant ST111/111Q striatal cells were serum depleted (2.5% FBS) for 24 h and then exposed to Locke's solution (154 mM NaCl, 5.6 mM KCl, 2.3 mM CaCl2, 3.6 mM NaHCO3, 5 mM HEPES, 5.6 mM glucose, and 10 μM glycine) for 30 min before the addition of SKF 38393, 30 or 60 μM (Sigma Aldrich) in fresh DMEM medium (2.5% FBS). To prevent SKF 38393 effect, cultures were treated with the D1 receptor antagonist SCH 23390 10 μM (Sigma Aldrich) 1 h before SKF 38393 treatments. Total extracts were obtained 60 min after SKF 38393 treatment for later analysis. To inhibit Cdk5 activity, cultures were treated with the Cdk5 inhibitor roscovitine, 20 μM (Sigma-Aldrich) 1 h before SKF 38393 treatments. Cells were then fixed for later analysis.

1.5. Cell transfection

Cells were transiently transfected using Lipofectamine 2000 (Invitrogen, Life Technologies) according to the manufacturer's instructions and incubated for 48 h before use. To suppress Cdk5 expression, cells were transfected with the appropriate antisense oligonucleotides (sc-35047; Santa Cruz Biotechnology, Santa Cruz, CA) or with a scramble control (sc-37007; Santa Cruz Biotechnology) and harvested for Western blot or fixed for immunocytochemistry analysis 48 h post-transfection.

1.6. Subcellular fractionation

Mitochondria from cell lines have been isolated as described by Yang and colleagues [3] with slight modifications. Briefly, wild-type ST7/7Q and mutant ST111/111Q cells were washed twice, harvested in ice cold phosphate-buffered saline (PBS; 140 mM NaCl, 2 mM KCl, 1.5 KH_2PO_4, 8 mM NaH_2PO_4, pH 7.4) and resuspended in isolation buffer (IB; 250 mM sucrose, 20 mM HEPES-KOH pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA) with protease and phosphatase inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 mg/ml aprotinin and 10 mg/ml leupeptin). Cells were homogenized with a Dounce homogenizer and the suspension was then centrifuged at 800 ×g for 10 min at 4 °C to remove nuclei and unbroken cells. The supernatants were centrifuged again at 10,000×g for 20 min at 4 °C to pellet the heavy membrane fractions containing mitochondria. The pellet fraction was washed, spun down again at 10,000 ×g, resuspended in IB containing 1% Triton X-100 and finally saved at −80 °C for later analysis. The cytosolic fraction was obtained as supernatants by further centrifugation at 16,000 ×g for 20 min at 4 °C to remove residual mitochondria and saved at −80 °C for later analysis.

1.7. Western blot analysis

Total cellular extracts were collected in 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 mixture (Sigma-Aldrich). Samples were centrifuged at 10,000×g for 10 min and the protein contents determined by Detergent-Compatible Protein Assay (Bio-Rad, Hercules, CA, USA). For the analysis of the striatum of Hdh mice, heterozygous mutant Hdh7/111Q and wild type Hdh7/7Q mice were killed by cervical dislocation at the age of 8 months. The brain was quickly removed, dissected, frozen in dry ice and stored at −80 °C until use. Protein extraction from striatal tissue and Western blot analysis were performed as previously described [4]. Protein extracts (10–20 μg) were mixed with 5×-SDS sample buffer, boiled for 5 min, resolved on 8–10% SDS-PAGE, and transferred to nitrocellulose membranes (Whatman Schleicher & Schuell, Keene, NH, USA). Blots were blocked in 10% non-fat powdered milk in Tris-buffered saline Tween-20 (TBS-T; 50 mM Tris-HCl, 150 mM NaCl, pH 7.4, 0.05% Tween 20) for 60 min at room temperature. The membranes were then incubated overnight at 4 °C with primary antibodies: Drp1 (DLP1; 1:1000; BD Bioscience, San Jose, CA, USA), phospho-Drp1 (S616) (1:1000, Cell Signaling Technology, Beverly, MA, USA), OPA1 (1:8000, BD Bioscience), Mfn2 (1:2000, Abcam, Cambridge, UK), and Cdk5 J-3 (1:1000, Santa Cruz Biotechnology). Loading control was performed by reproving the membranes with anti-tubulin (1:50,000, Sigma Aldrich) or anti-actin (1:20,000; MP Biochemicals, Irvine, CA, USA) and with anti-OxPhos Complex V (CoxV; 1:3000; Molecular Probes Inc., Eugene, OR, USA) for mitochondrial fractions. The membranes were then rinsed three times with TBS-T and incubated with horseradish peroxidase-conjugated secondary antibody (1:3000; Promega Madison, WI, USA) for 1 h at room temperature. After washing for 30 min with TBS-T, the membranes were developed using the enhanced chemiluminescence ECL kit (Santa Cruz Biotechnology). The intensity of immunoreactive bands was quantified by using Image J software (National Institutes of Health, Bethesda, MD, USA). Data are expressed as the mean ± SEM of band density.

1.8. Immunocytochemistry and confocal analysis

Striatal cells were fixed in 4% paraformaldehyde (Electron Microscopy Science EMS, Hatfield, PA, USA) for 10 min, rinsed in PBS, treated with 0.1 M Glycine for 20 min, and then permeabilized in 0.1% saponin for 10 min. Blocking was done in 1% bovine serum albumin in phosphate-buffered saline for 1 h. Specimens were incubated with primary antibody TOM20 (1:250, Santa Cruz Biotechnology) for 2 h at room temperature. Thereafter, samples were incubated with the following secondary antibodies: AlexaFluo 488 anti-rabbit (1:100; Jackson Immunoresearch, West Grove, PA) and Phalloidin-conjugated with TRITC (1:1000, Sigma Chemicals). Nuclei were stained with the Hoechst 33258 (1:10,000, Molecular Probes, Life Technologies) for 5 min. For detection of D1 receptor on cell surface, the permeabilization with saponin was omitted and samples were incubated with primary antibody anti-Dopamine Receptor D1 (1:500, Abcam) overnight. Afterwards, samples were incubated with the secondary antibody AlexaFluo 488 anti-rabbit (1:100; Jackson Immunoresearch). For immunocytochemical experiments in primary cultures, eight days after plating, neurons were fixed with 4% PFA/phosphate buffer for 10 min, rinsed in PBS, blocked in PBS containing 0.1 M glycine for 10 min and permeabilized in PBS containing 0.1% saponin for 10 min and blocked in PBS containing Normal Horse Serum 15% for 30 min at room temperature. Neurons were then washed in PBS and incubated overnight at 4 °C with primary antibodies TOM20 (1:250, Santa Cruz Biotechnology) and MAP2 (1:500, Sigma-Aldrich) and detected with AlexaFluo 488 anti-rabbit and Cy3 anti-mouse secondary antibodies (1:100, Jackson ImmunoResearch). As negative controls, some neurons were processed as described in the absence of primary antibody and no signal was detected. Nuclei were stained with the Hoechst 33258 (1:10,000, Molecular Probes, Life Technologies) for 5 min. Stained cells and neurons were then washed twice with PBS and mounted under glass coverslips with Mowiol. Immunofluorescence was analyzed by confocal microscopy using a Leica TCS SP5 laser scanning spectral confocal microscope (Leica Microsystems Heidelberg GmbH). Confocal images were taken using a 63× numerical aperture objective with a 3× digital zoom and standard pinhole. For each cell, the entire three-dimensional stack of images from the ventral surface to the top of the cell was obtained by using the Z drive in the Leica TCS SP5 microscope. The size of the optical image was 0.4 μm.

1.9. Analysis of mitochondrial morphology

Quantitative analyses of mitochondrial morphology were performed as previously described [5,6]. Briefly, digital images were processed through a convolve filter to obtain isolated and equalized fluorescent pixels and then to a thresholding step using the NIH-developed ImageJ software (National Institutes of Health, Bethesda, MD). This procedure yields a binary image containing black mitochondrial structures on a white background (Supplementary Fig. 1). From this binary image, individual mitochondria (particles) were subjected to particle analyses to

acquire Form Factor (FF, ($4\pi \times Am/Pm^2$) where Pm is the length of mitochondrial outline and Am is the area of mitochondrion) and Aspect Ratio values (AR, the ratio between the major and minor axis of the ellipse equivalent to the mitochondrion) as well as the number of mitochondria per cell. An AR value of 1 indicates a perfect circle, and as mitochondria elongate and become more elliptical, AR increases. A FF value of 1 corresponds to a circular, unbranched mitochondrion, and higher FF values indicate increase of mitochondrial complexity (length and branching). For determination of the percentage of cells with fragmented mitochondria, a cell was determined to have fragmented mitochondria if it had ≥50% of its mitochondria with length/width (axis) ratios <2.5. In average 25–30 cells/genotype were analyzed from 3 to 6 independent experiments. For the analysis in neurons, the number of mitochondria per micron of axon was measured using NIH ImageJ software.

1.10. Drp1 immunoprecipitation and GTPase enzymatic activity assay

Wild-type ST7/7Q and mutant ST111/111Q cells were washed with cold PBS and incubated on ice in lysis buffer (20 mM Tris [pH 7.5], 0.6% CHAPS, 10% glycerol, 1 mM sodium orthovanadate and protease inhibitor cocktail) for 15 min. Cells were scraped and then disrupted 10 times by repeated aspiration through a 25-gauge needle. Samples were centrifuged at 10,000 ×g for 10 min and the supernatant was saved as the whole cell extract. To determine GTPase activity of Drp1, a total of 400 μg of whole-cell extract was immunoprecipitated overnight with 25 μg of anti-Drp1 antibody (BD Bioscience) and 40 μl of protein A/G-agarose (Santa Cruz Biotechnology). For the analysis of Drp1 enzymatic activity in Hdh mice, frozen striatal tissues from heterozygous mutant Hdh7/111Q and wild type Hdh7/7Q mice at the age of 8 months were washed with cold PBS and incubated on ice in lysis buffer (20 mM Tris [pH 7.5], 0,6% CHAPS, 10% glycerol, 1 mM sodium orthovanadate and protease inhibitor cocktail) for 15 min. Tissue was homogenized with a Dounce homogenizer and the lysate was centrifuged at 10,000 ×g for 10 min and the supernatant was saved as the whole tissue extract. To determine GTPase activity of Drp1, a total of 400 μg of whole-tissue extract was immunoprecipitated overnight with 25 μg of anti-Drp1 antibody (BD Bioscience) and 40 μl of protein A/G-agarose (Santa Cruz Biotechnology). GTPase activity of the protein was determined using a GTPase assay kit (Novus Biologicals, Littleton, CO, USA) according to manufacturer's instructions. After three washes with lysis buffer and three washes with GTPase buffer (50 mM Tris [pH 7.5], 2.5 mM $MgCl_2$, and 0.02% 2-mercaptoethanol), the beads were incubated with 0.5 mM GTP at 30 °C for 1 h. Drp1 hydrolyzes GTP to GDP and inorganic phosphorous (Pi) and we measured GTPase activity, based on the amount of inorganic phosphorous that the GTP produces. By adding the ColorLock Gold (orange) substrate to the inorganic phosphorous that is generated from GTP, we assessed GTP activity, based on the inorganic complex solution (green). Colorimetric measurements (green) were read in the wavelength range of 650 nm using a Synergy 2 Multi-Mode Microplate Reader (BioTek Instruments, Inc.; Winooski, VT, USA).

1.11. Real-time quantitative RT-PCR

Total RNA was isolated from the wild-type ST7/7Q and mutant knock-in ST111/111Q striatal cell lines 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.): Dnm1l (Mm.PT.56a.16160059); 18S (Hs.PT.39a.22214856.g) and Actinβ (Mm.PT.39a.22214843.g). RT-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.

1.12. Statistical analysis

All the data were analyzed with the program GraphPad Prism version 5.0a (Graph Pad Software). Results are expressed as mean ± SEM. Unpaired Student's *t*-test for differences between two groups or One-way ANOVA followed by the post hoc Newman–Keuls multiple comparison test were used to assess significance ($p<0.05$), as indicated in figure legends.

2. Introduction

Huntington's disease (HD) is an autosomal-dominant inherited neurodegenerative disorder, characterized by progressive behavioral, motor and cognitive deficits [7,8]. The predominant neuropathological hallmark of HD is the selective loss of medium spiny neurons within the striatum that extends to other brain regions with the progression of the disease [9]. Although mutant huntingtin (mHtt) represents a key factor in the pathogenesis of the disease, the molecular mechanisms underlying the preferential vulnerability of the striatum to mHtt toxicity remain unclear. From last years, compelling evidence argues in favor of a role of mitochondrial dysfunction in HD neuropathology [10–12]. Thus, the expression of mHtt leads to deficits in energy metabolism [13], alterations in mitochondrial calcium handling [14,15] and severe changes in mitochondrial structure integrity [16,17]. However, given the ubiquitous expression of mHtt within the brain other factors should contribute to alter mitochondrial function in the striatum. One hypothesis is that dopamine (DA), which is present at high concentrations in the striatum compared to other brain areas, might increase the sensitivity of mitochondria to mHtt toxicity inducing mitochondrial dysfunction and neurodegeneration. Actually, by using primary striatal cultures expressing the N-terminal mHtt fragment it has been reported that DA acting via D2 receptors reduces the levels of the mitochondrial Complex II (mCII) increasing the vulnerability of striatal cells to mHtt-induced cell death [18]. Besides D2 receptors, we and others have also demonstrated a critical role for D1 receptors in striatal neurodegeneration in HD [2,19]. Thus, we reported that activation of D1 receptors induces an increase in the susceptibility of mutant huntingtin striatal cells to cell death, an effect that was mediated by Cyclin-dependent kinase-5 (Cdk5) [2]. Importantly, Cdk5 has been identified as an upstream kinase that regulates mitochondrial fission during neuronal apoptosis while its suppression attenuates apoptotic mitochondrial fragmentation [20,21]. In this view, we hypothesized that the increased susceptibility of mutant huntingtin striatal cells to D1R activation could be mediated by Cdk5-induced disturbances in mitochondrial function. To validate this theory we used precise genetic HD models expressing endogenous levels of full-length wild-type or mutant huntingtin [1,2]. Our results reveal a new role for Cdk5 in HD pathology by playing a key role in regulating mitochondrial fission events involved in striatal neurodegeneration and highlight Cdk5 as a therapeutic target to treat mitochondrial dysfunction in HD and other neurodegenerative disorders.

3. Results

3.1. Mutant ST111/111Q striatal cells exhibit aberrant mitochondrial dynamics

Abnormalities in mitochondrial morphology and dynamics have been reported in HD as early pathological events [22–24]. To determine

whether mitochondrial morphology was altered in our HD striatal cell model, wild-type ST7/7Q and mutant ST111/111Q huntingtin striatal cells were stained with TOM20 and phalloidin and mitochondrial population was analyzed by confocal microscopy (Fig. 1A). Morphometric analysis revealed that mutant ST111/111Q striatal cells display significant differences in the mitochondrial morphology compared to wild-type ST7/7Q cells. Thus, the values of the Aspect Ratio (AR) and Form factor (FF) demonstrated reduced mitochondrial length (~10% decrease; $p < 0.05$. Fig. 1B) and lower mitochondrial complexity and decreased branching (~14%; $p < 0.05$. Fig. 1C), respectively. These alterations correlated in mutant cells with a significant increase in the number of mitochondria per cell (~15%; $p < 0.01$. Fig. 1D) Moreover, when the percentage of cell population showing mitochondrial fragmentation was analyzed more than 40% of mutant ST111/111Q striatal cells exhibited fragmented mitochondria whereas only 20% of wild-type ST7/7Q cells showed this mitochondrial fission morphology ($p < 0.01$; Fig. 1E). Altogether, these findings suggest a toxic effect of mHtt on mitochondrial dynamics.

3.2. Mutant huntingtin deregulates the levels and activity of mitochondrial fission/fusion proteins in ST111/111Q striatal cells

Changes in mitochondrial morphology depend on the balance of opposing fission and fusion events [25]. Interestingly, mHtt has been reported to induce changes in the expression of different pro-fission and pro-fusion mitochondrial proteins [26]. To understand the molecular basis of the observed abnormal mitochondrial fragmentation we determined the levels of mitochondria-shaping proteins in our striatal cell lines by Western blot analysis (Fig. 2). No significant differences in the total levels of the fusion protein Opa1 were observed between genotypes (Fig. 2A). However, when the mitochondrial fraction was analyzed, mutant ST111/111Q cells displayed significantly lower levels of Opa1 compared to those in wild-type ST7/7Q cells (20% decrease; $p < 0.05$). Next the levels of another essential fission protein, Mfn2 were investigated. No significant changes either between subcellular fractions or between genotypes were observed (Supplementary Fig. 2). Surprisingly, we also found a significant decrease (30% decrease; $p < 0.05$. Fig. 2B) in the total levels of the fission protein Drp1 in mutant compared to wild-type striatal cells, a reduction that was even higher when mitochondrial subcellular fractions were evaluated (50% decrease; $p < 0.01$) without changes in the cytosolic fraction. To determine if this decrease was due to altered gene transcription, Drp1 mRNA expression was evaluated. A statistically significant decrease in Drp1 mRNA levels was detected in mutant ST111/111Q compared to wild-type ST7/7Q striatal cells (20% decrease; $p < 0.05$. Fig. 2C) suggesting an involvement of mHtt in the transcriptional deregulation of Drp1. Since mHtt abnormally interacts with Drp1 altering its structure and increasing its enzymatic activity [27], we then evaluated Drp1-GTPase activity in striatal cells (Fig. 2D). Despite the low levels of Drp1 expression found in mutant huntingtin cells, a significant increase (~3.5-fold increase; $p < 0.01$. Fig. 2D) in the GTP-binding activity of Drp1 was observed in mutant compared to wild-type cells suggesting that enhanced mitochondrial fragmentation in mutant huntingtin cells could be related with higher Drp1 activity.

3.3. Hdh7/111Q knock-in mutant mice display impaired mitochondrial dynamics and increased Drp1 activity

To extend our mitochondrial findings in an in vivo HD model, we next analyzed whether alterations in mitochondrial dynamics were also detected in primary striatal neurons from Hdh7/111Q knock-in mutant mice. Confocal microscopy analysis (Fig. 3A) revealed that mitochondria from mutant Hdh7/111Q primary striatal neurons were more fragmented with respect to those in wild-type Hdh7/7Q mice (~30%; $p < 0.05$. Fig. 3B). Considering that abnormalities in the morphology and function of mitochondria persist along the disease progression

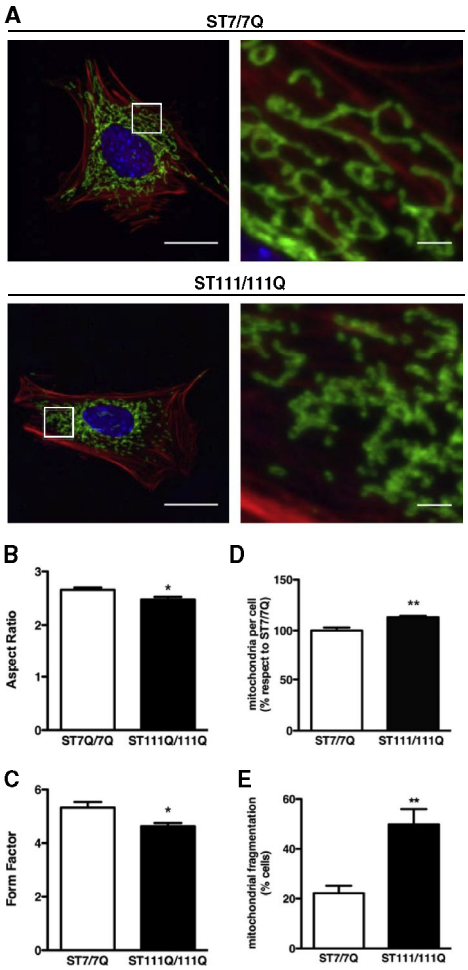

Fig. 1. ST111/111Q mutant cells show increased mitochondrial fragmentation. (A) Representative confocal images showing mitochondrial morphology in wild-type ST7/7Q and mutant ST111/111Q cells immunostained with anti-TOM20 (green), anti-Phalloidin-TRITC (red) and Hoechst stain (blue); scale bar 20 μm. The boxed areas are enlarged in the panels on the right; scale bar 2 μm. (B, C) Bar histogram showing the Aspect Ratio (AR) and Form Factor (FF) value. *$p < 0.05$ as determined by unpaired Student's *t*-test. (D) Bar histogram showing the number of mitochondria per cell. *$p < 0.05$ as determined by unpaired Student's *t*-test. (E) Bar histogram showing the percentage of cells with fragmented mitochondria relative to the total number of cells. **$p < 0.01$ as determined by unpaired Student's *t*-test. Data represent mean ± SEM of 6 independent experiments in which 25–30 cells/genotype were analyzed with ImageJ software.

[17], alterations in the levels of the fission protein Drp1 were analyzed in the striatum of Hdh7/111Q knock-in mutant mice at 8 months of age. Immunoblot analysis revealed no changes in striatal Drp1 protein

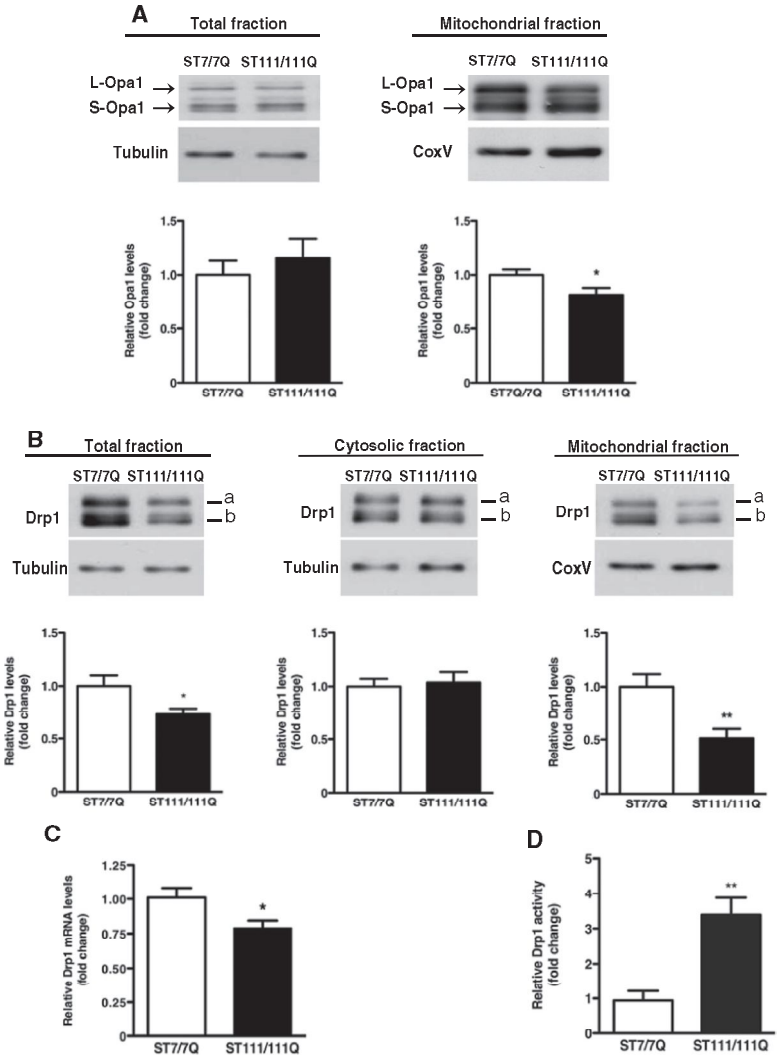

Fig. 2. ST111/111Q mutant cells exhibit altered expression, subcellular distribution and activity of Drp1. (A, B) Representative Western blots showing the levels of the fusion protein Opa1 in total and mitochondrial fractions and the fission protein Drp1 in total, cytosolic and mitochondrial fractions from wild-type ST7/7Q and mutant ST111/111Q cells. α-Tubulin (total and cytosol) or CoxV (mitochondria) were used as loading controls. Letters confer to the different isoforms recognized by the respective antibodies (L-Opa1 and S-Opa-1; DRP1: a–b). Bar histograms indicate the relative fold change ± SEM of 9 independent experiments; $^{**}p < 0.01$, $^{*}p < 0.05$ vs. wild-type ST7/7Q cells as determined by unpaired Student's *t*-test. (C) Histogram showing Drp1 mRNA expression analyzed by RT-PCR in wild-type ST7/7Q and mutant ST111/111Q cells. Data were normalized to 18S and actinβ gene expression. Bar histogram represents the relative fold change ± SEM of 7 independent experiments. $^{*}p < 0.05$ vs. wild-type ST7/7Q cells as determined by unpaired Student's *t*-test. (D) Histogram showing GTPase Drp1 enzymatic activity in striatal extracts obtained from wild-type ST7/7Q and mutant ST111/111Q cells. Bar diagram represents the relative fold change ± SEM of 5 independent experiments. $^{**}p < 0.01$ vs. wild-type ST7/7Q cells as determined by unpaired Student's *t*-test.

levels between mutant Hdh7/111Q and wild-type Hdh7/7Q mice in any of the subcellular fractions analyzed (Fig. 3C). However and in agreement with our results in mutant ST111/111Q striatal cells, the enzymatic activity of Drp1 was significantly increased (~2-fold increase; $p < 0.05$. Fig. 3D) in mutant Hdh7/111Q mice compared to wild-type Hdh7/7Q mice. These data support the idea that

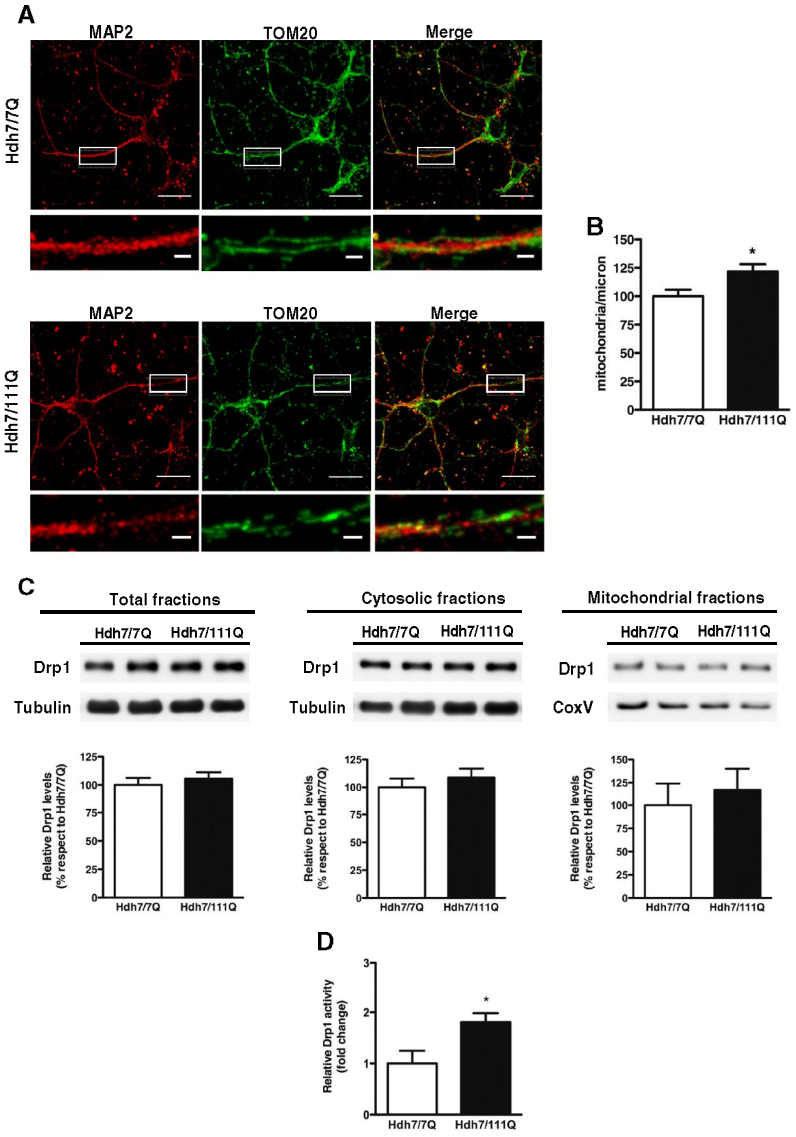

Fig. 3. Mutant Hdh7/111Q mice exhibit impaired mitochondrial fragmentation associated with increased Drp1 activity. (A, B) Representative confocal images of striatal primary culture from wild-type Hdh7/7Q and mutant Hdh7/111Q mice immunostained with anti-TOM20 (green) and anti-MAP2 (red); scale bar 20 μm. The boxed areas are enlarged in the panels on the bottom; scale bar 4 μm. The bar graph provides the percentage of mitochondria per axonal micron. Data represent mean ± SEM of 4 independent experiments in which 20 neurons/genotype were analyzed with ImageJ software; $^{*}p < 0.05$ vs. wild-type Hdh7/7Q mice as determined by unpaired Student's *t*-test. (C) Representative Western blots showing the levels of the fission protein Drp1 in total, cytosolic and mitochondrial fractions obtained from the striatum of wild-type Hdh7/7Q and mutant Hdh7/111Q mice. α-Tubulin (total and cytosol) or CoxV (mitochondria) were used as loading controls. Bar histograms indicate the relative levels of Drp1 ± SEM of 7 independent experiments. (D) Histogram showing GTPase Drp1 enzymatic activity in striatal extracts obtained from 8-month-old wild-type Hdh7/7Q and mutant Hdh7/111Q mice. Bar histogram indicates the relative fold change ± SEM of 6 independent experiments; $^{*}p < 0.05$ vs. Hdh7/7Q as determined by unpaired Student's *t*-test.

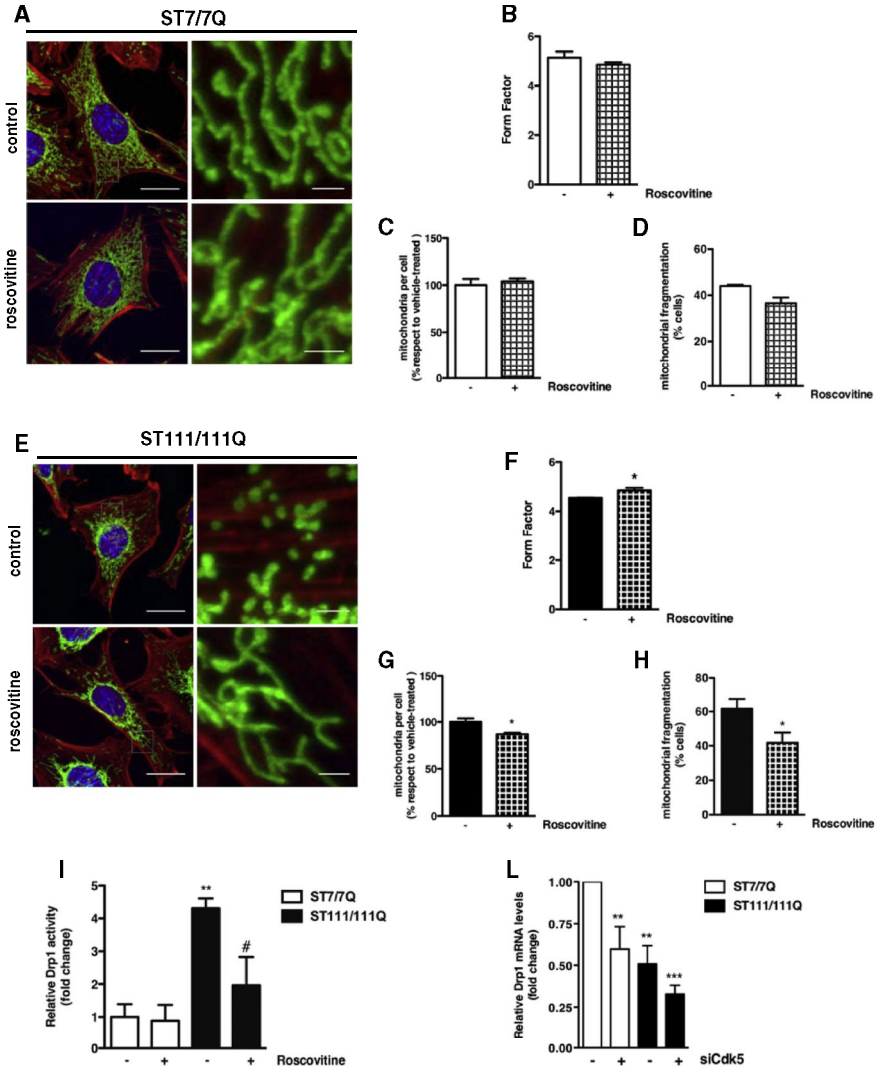

Fig. 4. Aberrant Cdk5 activity in ST111/111Q mutant cells induces mitochondrial fragmentation. (A, E) Representative confocal images showing mitochondrial morphology in wild-type ST7/7Q and mutant ST111/111Q cells treated with roscovitine (20 μM). Cells were stained with anti-TOM20 (green), anti-Phalloidin-TRITC (red) and Hoechst stain (blue); scale bar 20 μm. Panels on the right show enlargement of the boxed areas; scale bar 2 μm. Bar histograms showing: (B, F) the relative Form Factor (FF) value, (C, G) the percentage of number of mitochondria per cell and (D, H) the percentage of cells with fragmented mitochondria relative to the total number of cells. Data represent mean ± SEM of 5 independent experiments in which 25–30 cells/condition were analyzed with ImageJ software. *$p < 0.05$ vs. vehicle treated condition as determined by unpaired Student's *t*-test. (I) Histogram showing GTPase Drp1 enzymatic activity in striatal extracts obtained from vehicle or roscovitine treated wild-type ST7/7Q and mutant ST111/111Q cells. Bar histogram indicates the relative fold change ± SEM of 4 independent experiments; **$p < 0.01$ vs. wild-type ST7/7Q cells and $^{\#}p < 0.05$ vs. vehicle-treated ST111/111Q cells as determined by One-way ANOVA with Newman–Keuls post hoc analysis. (L) Histogram showing Drp1 mRNA expression analyzed by RT-PCR in wild-type ST7/7Q and mutant ST111/111Q cells transfected with siRNA or siCdk5. Data were normalized to 18S and actinβ3 gene expression. Bar histogram indicates the relative fold change ± SEM of 7 independent experiments. ***$p < 0.001$ and **$p < 0.01$ vs. siRNA transfected ST7/7Q cells as determined by One-way ANOVA with Newman–Keuls post hoc analysis.

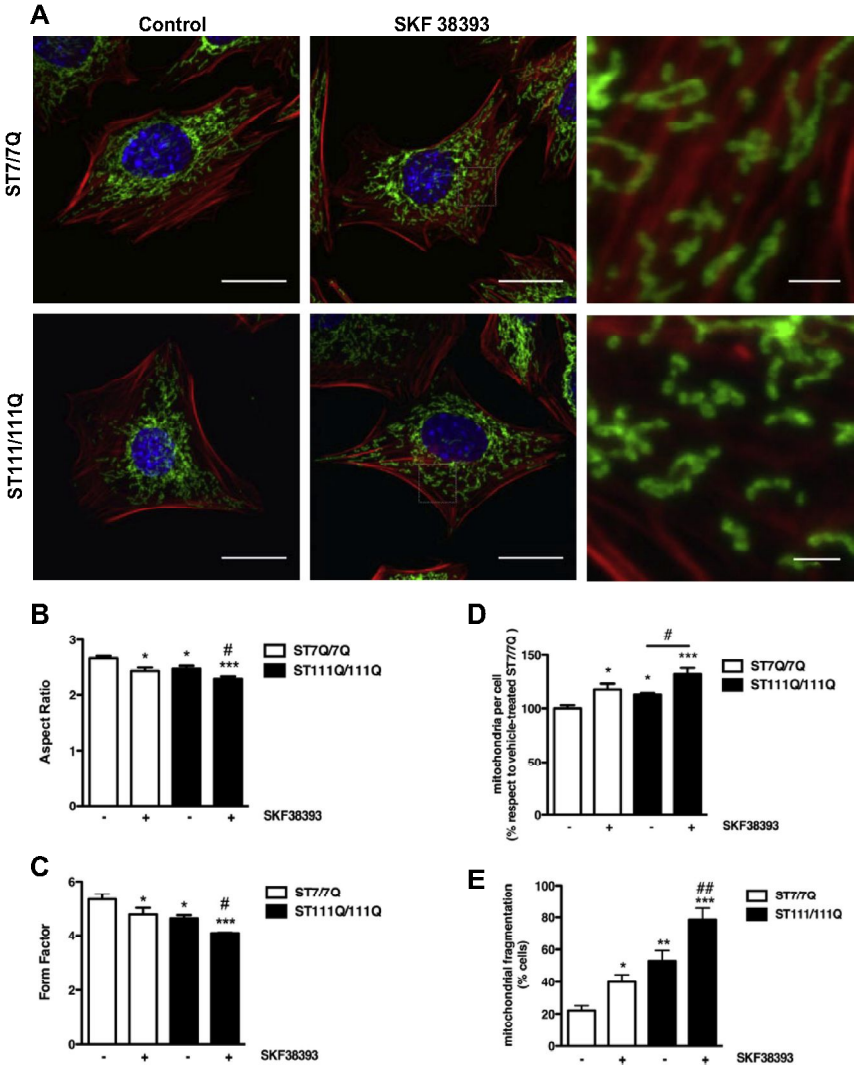

Fig. 5. Dopaminergic D1 receptor activation increases mitochondrial fission events and mitochondrial branching defects in striatal cells. (A) Representative confocal images showing mitochondrial morphology in wild-type ST7/7Q and mutant ST111/111Q cells treated with vehicle or the dopaminergic agonist SKF 38393 (60 μM). Cells were stained with anti-TOM20 (green), anti-Phalloidin-TRITC (red) and Hoechst stain (blue); scale bar 20 μm. Last panels on the right show enlargement of the boxed areas; scale bar 2 μm. (B, C) Bar histograms showing mitochondrial size by Aspect Ratio (AR) values and mitochondrial branching changes determined by the analysis of the Form Factor (FF) value in SKF 38393-treated and vehicle-treated wild-type ST7/7Q and mutant ST111/111Q cells. Data represent mean ± SEM of 6 independent experiments in which 25–30 cells/condition were analyzed with ImageJ software. ***$p < 0.001$, *$p < 0.05$ vs. vehicle-treated ST7/7Q cells and #$p < 0.05$ vs. vehicle-treated ST111/111Q cells as determined by One-way ANOVA with Newman–Keuls post hoc analysis. (D) Bar histogram showing percentage of number of mitochondria per cell. (E) Bar histogram showing the percentage of vehicle or SKF 38393-treated wild-type ST7/7Q and mutant ST111/111Q cells with fragmented mitochondria relative to the total number of cells. Data represent mean ± SEM of 6 independent experiments in which 25–30 cells/condition were analyzed with ImageJ software. ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$ vs. vehicle-treated ST7/7Q cells and ##$p < 0.01$ and #$p < 0.05$ vs. vehicle-treated ST111/111Q cells as determined by one-way ANOVA with Newman–Keuls post hoc analysis.

mitochondrial abnormalities in HD models are related with enhanced Drp1 enzymatic activity induced by mHtt.

3.4. Cdk5 contributes to Drp1-induced mitochondrial dysfunction in mutant ST111/111Q striatal cells

Cdk5 may promote mitochondrial dysfunction [28] acting as an upstream regulator of mitochondrial fission during neuronal apoptosis [21]. Since we have previously demonstrated an aberrant increase in Cdk5 activity in mutant ST111/111Q cells [2] we next aimed to determine the relevance of Cdk5 in mitochondrial fission. Wild-type ST7/7Q (Fig. 4A) and mutant ST111/111Q cells (Fig. 4E) were treated with the Cdk5 inhibitor roscovitine and mitochondrial morphology was analyzed by confocal microscopy. Roscovitine treatment significantly improved mitochondrial tubular network defects (~10% increase in the Form Factor value; $p < 0.05$. Fig. 4F) and reduced

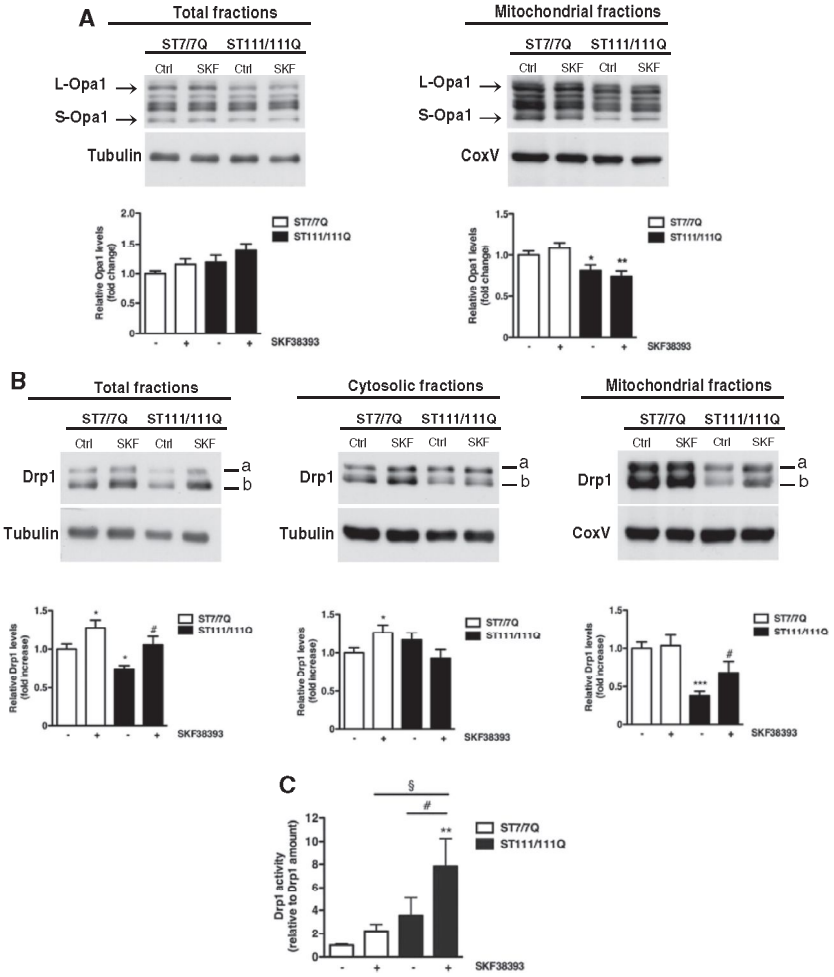

Fig. 6. Aberrant mitochondrial Drp1 activity exacerbates D1R-induced mitochondrial fission in ST111/111Q mutant cells. (A, B) Representative Western blots showing the levels of the fusion protein Opa1 in total and mitochondrial fractions and the fission protein Drp1 in total, cytosolic and mitochondrial fractions obtained from vehicle and SKF 38393 (60 μM) treated wild-type ST7/7Q and mutant ST111/111Q cells. α-Tubulin (total and cytosol) or CoxV (mitochondria) were used as loading controls. Letters indicate the different isoforms recognized by the respective antibodies (L-Opa1 and S-Opa-1; DRP1: a–b). Bar histograms indicate the relative fold change ± SEM of 12 independent experiments. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ vs. vehicle-treated ST7/7Q cells and #$p < 0.05$ vs. vehicle-treated ST111/111Q cells as determined by One-way ANOVA with Newman–Keuls post hoc analysis. (C) Histogram showing GTPase Drp1 enzymatic activity in striatal extracts obtained from vehicle or SKF 38393-treated mutant ST111/111Q striatal cells. Bar histogram represents the relative fold change ± SEM of 6 independent experiments; **$p < 0.01$ vs. vehicle-treated ST7/7Q cells, #$p < 0.05$ vs. vehicle treated ST111/111Q cells and §$p < 0.05$ vs. SKF 38393 treated ST7/7Q cells as determined by One-way ANOVA with Newman–Keuls post hoc analysis.

mitochondrial fragmentation (~20%; $p < 0.05$. Fig. 4G and H) in mutant but not wild-type huntingtin cells (Fig. 4A–D) suggesting an important role of Cdk5 in mitochondrial dysfunction in HD striatal cells. Since we have demonstrated increased Drp1 activity in mutant huntingtin striatal cells, we next addressed whether the improvement in mitochondrial fragmentation following roscovitine treatment was associated with a reduction in Drp1 activity. According to a role of Cdk5 in mitochondrial impairments, pharmacological inhibition of Cdk5 significantly reduced (~50% decrease; $p < 0.01$. Fig. 4I) Drp1 activity in mutant ST111/111Q cells without any effect in wild-type ST7/7Q cells. Interestingly, we also found that in addition to Drp1 activity Drp1 gene transcription was also modulated by Cdk5. Thus, a significant decrease in Drp1 mRNA expression was observed in wild-type (~40% decrease; $p < 0.01$) and mutant (~70% decrease; $p < 0.001$) huntingtin striatal cells when expression of Cdk5 was reduced by Cdk5 siRNAs (Fig. 4L) revealing a critical role for Cdk5 as a transcriptional regulator of Drp1.

3.5. Activation of dopaminergic D1 receptors increases mitochondrial dysfunction in mutant ST111/111Q striatal cells

We have previously demonstrated that mutant ST111/111Q cells are more susceptible to cell death induced by D1R activation than wild-type ST7/7Q cells [2]. Interestingly, it has been reported that dopamine could negatively influence mitochondrial function [29,30]. Therefore, to determine whether the enhanced vulnerability of mutant ST111/111Q cells to dopamine-induced cell death was mediated by mitochondrial impairments wild-type ST7/7Q and mutant ST111/111Q cells were treated with the D1 receptor agonist SKF 38393 (60 μM) and mitochondrial morphology was analyzed by confocal microscopy (Fig. 5A). First, we analyze whether these striatal cells properly express D1R at the plasma membrane (Supplementary Fig. 3). Cell surface D1R expression examined with an antibody against the extracellular domain of D1R in non-permeable cell conditions was similar between wild type and mutant huntingtin striatal cells. Then mitochondrial fission was examined. Our findings demonstrated that mitochondrial fragmentation occurs quickly in response to D1 receptor activation leading to a significant decrease in the size (Fig. 5B) and mitochondrial reticular network distribution (Fig. 5C) in both cell genotypes. It is important to notice that dopaminergic stimulation was able to further decrease FF and AR values in mutant huntingtin cells compared to vehicle conditions (~10%; $p < 0.05$). The alterations in mitochondrial morphology induced by SKF 38393 treatment were also detected by an increase in the number of organelles per cell (Fig. 5D) showing both genotypes an increment of 20% ($p < 0.05$; Fig. 5E) compared to vehicle conditions. Finally, when the percentage of cells with mitochondrial fragmentation was analyzed, we found that, in wild-type cells D1R activation raised the percentage from 20% to 40% ($p < 0.05$ Fig. 5E) while the transition in mutant cells was from 50% to 80% ($p < 0.01$). These mitochondrial alterations were dose-dependent since the treatment with lower concentration of SKF 38393 (30 μM) did not alter mitochondrial conformation in both cell types (Supplementary Fig. 4). Finally, to validate that SKF 38393 effects on mitochondria morphology were D1R-dependent, wild-type and mutant huntingtin striatal cells were treated with the D1R antagonist SCH 23390 prior to D1R activation and mitochondrial fission was analyzed (Supplementary Fig. 5). Importantly, co-incubation with SCH 23390 completely abrogates SKF 38393-induced alterations in mitochondrial morphology in both cell genotypes. Overall these results indicate that the increased susceptibility of mutant huntingtin striatal cells to dopaminergic activation could be mediated by alterations in the distribution and morphology of the mitochondrial population.

3.6. Activation of dopaminergic D1 receptors alters the levels, distribution and activity of the fission protein Drp1 in ST111/111Q striatal cells

Confocal microscopy analysis has shown that SKF 38393 treatment induces higher mitochondrial fragmentation in mutant compared to wild-type huntingtin cells. To correlate this morphological alterations with changes in mitochondrial fission/fusion proteins, the levels of Opa1 and Drp1 were analyzed in total, cytosolic and mitochondrial fractions obtained from vehicle and SKF 38393 treated wild-type and mutant huntingtin striatal cells. SKF 38393 treatment did not affect the expression of the pro-fusion protein Opa1 neither in wild-type nor in mutant huntingtin striatal cells (Fig. 6A) but induced a significant increase in total Drp1 levels (~1.3-fold increase and ~1.5-fold increase, respectively; $p < 0.05$. Fig. 6B). Interestingly, the distribution of Drp1 within the subcellular compartments following dopaminergic activation was different between cell genotypes. Whereas in wild-type cells the increase in Drp1 levels was found in the cytosolic fraction, that in mutant huntingtin cells was located in the mitochondrial fraction. Given the requirement of Drp1 translocation to the mitochondria for the fission of this organelle [31,32], the aberrant distribution of Drp1 in mutant huntingtin striatal cells may explain the increased mitochondrial fragmentation observed in these cells compared to wild-type cells. Next, we investigated whether dopaminergic stimuli could also affect Drp1 enzymatic activity. An increasing trend, although not significant, in Drp1 activity was found in wild-type cells after SKF 38393 treatment (Fig. 6C). By contrast, a significant increase (~8-fold increase; $p < 0.01$. Fig. 6C) was detected in SKF 38393-treated mutant huntingtin cells suggesting that D1 receptor activation exacerbates Drp1-dependent mitochondrial fission events in ST111/111Q mutant cells.

3.7. Inhibition of Cdk5 prevents D1R-induced mitochondrial fission and mitochondrial branching alterations in striatal cells

We have shown that Cdk5 contributes to mitochondrial fragmentation in mutant ST111/111Q cells while D1R activation increases mitochondrial fission. Interestingly, we have previously reported [2] that aberrant Cdk5 activity mediates D1R-induced neurotoxicity in mutant ST111/111Q striatal cells. Altogether, it prompted us to investigate whether Cdk5 was also mediating the mitochondrial dysfunction induced by dopaminergic D1R activation. To this aim, wild-type and mutant huntingtin striatal cells were treated with roscovitine prior to incubation with SKF 38393 and mitochondrial morphology was analyzed by confocal microscopy (Fig. 7A and E). Roscovitine treatment completely recovered SKF 38393-induced mitochondrial branching alterations (Fig. 7B and F) and prevented mitochondrial fragmentation, either mitochondrial number (Fig. 7C and G) or percentage of cells with mitochondria fragmentation (Fig. 7D and H) in both cell genotypes, pointing Cdk5 as a mediator of D1R-induced mitochondrial fission. To further corroborate these results, Cdk5 protein levels were knocked-down in wild-type and mutant huntingtin striatal cells by using specific Cdk5 siRNAs and mitochondria fragmentation was analyzed following D1R activation (Fig. 8A and E). First, the efficiency of the Cdk5 siRNA to knock-down Cdk5 levels was determined by Western blot analysis. A similar and a significant decrease in Cdk5 levels was found in Cdk5 siRNA transfected cells compared to those transfected with scramble siRNA (~50% decrease; $p < 0.01$. Supplementary Fig. 6). Then, mitochondrial fission was analyzed. Genetic knock-down of Cdk5 completely prevented the decrease in mitochondrial branching (Fig. 8B and F) and the increase in mitochondrial fragmentation induced by SKF 38393 treatment in both wild-type ST7/7Q and mutant ST111/111Q huntingtin striatal cells (Fig. 8C, D, G and H), strongly supporting a role for Cdk5 in D1R-induced mitochondrial dysfunction in striatal cells.

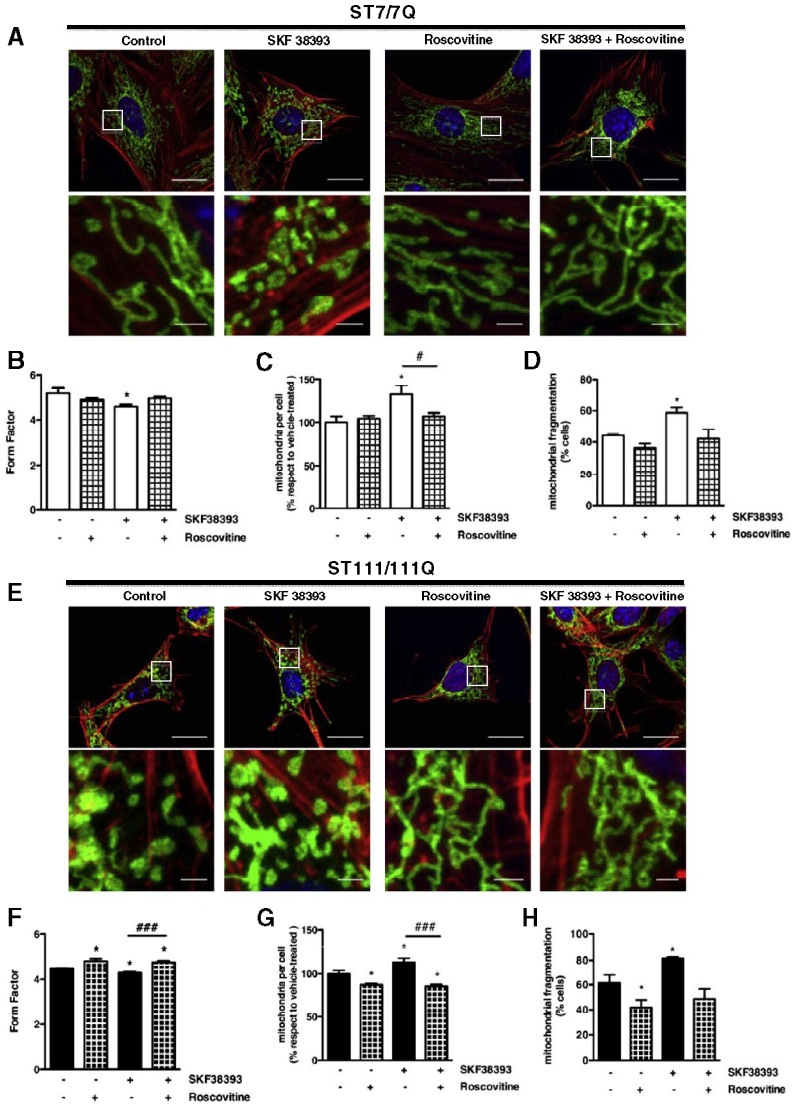

Fig. 7. Cdk5 inhibition restores the mitochondrial network in SKF 38393 treated ST7/7Q wild-type and ST111/111Q mutant cells. (A, E) Representative confocal images showing the mitochondrial morphology in wild-type ST7/7Q and mutant ST111/111Q cells treated with vehicle, SKF 38393 (60 μM), roscovitine (20 μM) or roscovitine + SKF 38393. Cells were stained with anti-TOM20 (green), anti-phalloidin-TRITC (red) and Hoechst stain (blue); scale bar 20 μm. Bottom panels show enlargement of the boxed areas; scale bar 2 μm. (B and F) Bar histogram showing mitochondrial branching changes determined by the analysis of the Form Factor (FF) value in vehicle or treated wild-type ST7/7Q and mutant ST111/111Q cells. Data represent mean ± SEM of 5 independent experiments in which 25–30 cells/condition were analyzed with ImageJ software. $^{*}p < 0.05$ vs. vehicle-treated striatal cells and $^{###}p < 0.001$ vs. SKF 38393 treated cells as determined by One-way ANOVA with Newman–Keuls post hoc analysis. (C and G) Bar histograms showing the percentage of number of mitochondria per cell. Data represent mean ± SEM of 5 independent experiments in which 25–30 cells/condition were analyzed with ImageJ software. $^{*}p < 0.05$ vs. vehicle-treated striatal cells and $^{###}p < 0.001$ and $^{\#}p < 0.05$ vs. SKF 38393 treated cells as determined by One-way ANOVA with Newman–Keuls post hoc analysis. (D and H) Bar histograms showing the percentage of vehicle or treated wild-type ST7/7Q and mutant ST111/111Q cells with fragmented mitochondria relative to the total number of cells. Data represent mean ± SEM of 5 independent experiments in which 25–30 cells/condition were analyzed with ImageJ software. $^{*}p < 0.05$ vs. vehicle-treated striatal cells as determined by One-way ANOVA with Newman–Keuls post hoc analysis.

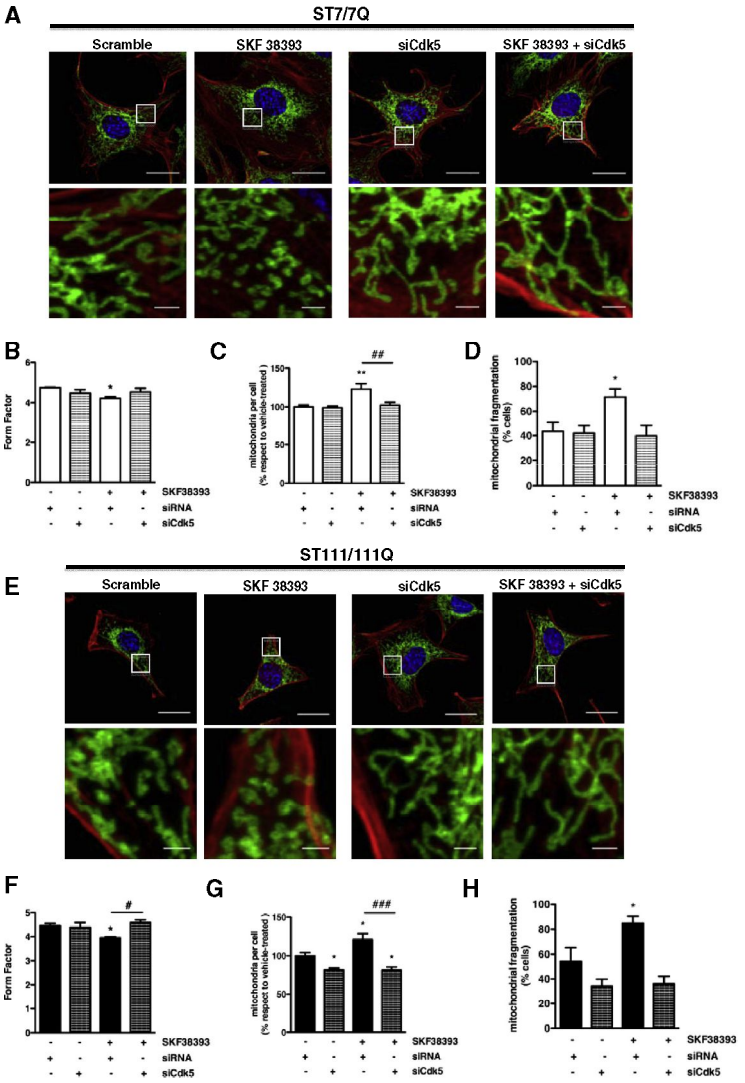

Fig. 8. Inhibition of Cdk5 by Cdk5 siRNA prevents aberrant mitochondrial fission and altered branching induced by D1R activation in ST7/7Q wild-type and ST111/111Q mutant striatal cells. (A and E) Representative confocal images showing the mitochondrial morphology in wild-type ST7/7Q and mutant ST111/111Q cells treated with vehicle or SKF 38393 (60 μM) and transfected with siRNA or siCdk5 RNA. Cells were stained with anti-TOM20 (green), anti-Phalloidin-TRITC (red) and Hoechst stain (blue); scale bar 20 μm. Bottom panels show enlargement of the boxed areas; scale bar 2 μm. (B and F) Bar histogram showing mitochondrial branching changes determined by the analysis of the Form Factor (FF) value in vehicle or SKF 38393 treated wild-type ST7/7Q and mutant ST111/111Q cells. Data represent mean ± SEM of 5 independent experiments in which 25–30 cells/condition were analyzed with ImageJ software. $^{*}p < 0.05$ vs. vehicle-treated siRNA-transfected striatal cells and $^{\#}p < 0.05$ vs. SKF 38393-treated siRNA-transfected cells as determined by One-way ANOVA with Newman–Keuls post hoc analysis. (C and G) Bar histograms showing the percentage of number of mitochondria per cell. Data represent mean ± SEM of 5 independent experiments in which 25–30 cells/condition were analyzed with ImageJ software. $^{**}p < 0.01$ and $^{*}p < 0.05$ vs. vehicle-treated siRNA-transfected striatal cells and $^{\#\#\#}p < 0.001$ and $^{\#\#}p < 0.01$ vs. SKF 38393-treated siRNA-transfected cells as determined by One-way ANOVA with Newman–Keuls post hoc analysis. (D and H) Bar histograms showing the percentage of vehicle or SKF 38393 treated wild-type ST7/7Q and mutant ST111/111Q cells with fragmented mitochondria relative to the total number of cells. Data represent mean ± SEM of 5 independent experiments in which 25–30 cells/condition were analyzed with ImageJ software. $^{*}p < 0.05$ vs. vehicle-treated striatal cells as determined by One-way ANOVA with Newman–Keuls post hoc analysis.

3.8. Inhibition of Cdk5 abrogates the D1R-induced increase and the mitochondrial translocation of Drp1 in ST111/111Q striatal cells

We have demonstrated that mitochondrial fragmentation induced by D1R activation was prevented by down-regulation of Cdk5 while activation of D1R induced an increase in the levels of the fission protein Drp1. Hence, we analyzed whether mitochondrial fission induced by D1R activation was mediated by a Cdk5-dependent increase in Drp1 function. Wild-type ST7/7Q and mutant ST111/111Q cells were transfected with scramble or Cdk5 siRNA, treated with vehicle or SKF 38393 and levels of Drp1 were determined by Western blot analysis (Fig. 9A). We found that reduction of Cdk5 expression prevented the abnormal increase of Drp1 caused by dopaminergic activation in both cell genotypes (Fig. 9B). Since we previously demonstrated in mutant huntingtin cells (Fig. 6) that D1R activation induces an increase in mitochondrial Drp1, we evaluated whether aberrant Cdk5 activity could be responsible of SKF 38393-induced Drp1 mitochondrial enrichment. Consistent with this idea, we found that mitochondrial Drp1 accumulation in mutant huntingtin striatal cells was completely prevented by inhibition of Cdk5 with roscovitine (Fig. 9C), suggesting that Cdk5 mediates D1 receptor-induced mitochondrial fragmentation by modulation of Drp1 protein levels and distribution.

4. Discussion

Mitochondria are essential organelles for neuronal function and survival given the prominent dependence of neuronal cells on mitochondrial ATP production to support different functions including membrane potential maintenance, neurotransmitter release and uptake or transportation of synaptic vesicles [33]. In HD the most important neuropathological alteration is the preferential loss of medium spiny neurons within the striatum [9]. Though the precise molecular mechanisms leading to this specific cell death are unknown, growing evidence have emerged for impaired mitochondrial function as a causative factor [34,35]. In this view, here, we have reported in a precise genetic HD striatal cell line and in HD striatal cell cultures impaired mitochondrial dynamics manifested as higher mitochondrial fragmentation and decreased mitochondrial branching compared to wild-type striatal cells, which is consistent with previous studies reporting, in different HD models, altered levels of fission/fusion proteins and increased mitochondrial fission [17,24,36]. Surprisingly, enhanced mitochondrial fragmentation in mutant huntingtin striatal cells was not associated with an increase in the levels of the fission protein Drp1, neither at the total nor at the mitochondrial level, but with a decrease. Importantly, the decrease in Drp1 protein levels in mutant huntingtin cells was associated with lower Drp1 mRNA expression suggesting that mHtt may alter mitochondrial dynamics by acting on transcriptional regulation of Drp1. In this view, it has been described that p53 is an important modulator of mitochondrial fission by transcriptional regulation of Drp1 expression [37]. Interestingly, previous works have demonstrated reduced p53-mediated gene transcription in mutant huntingtin expressing cells suggesting that the decrease in Drp1 mRNA levels found in mutant striatal cells could be related with a deficient p53 transcriptional function [38].

If the levels and expression of Drp1 are low in mutant huntingtin striatal cells, one intriguing question is how mitochondrial fragmentation can be enhanced in these cells. Interestingly, we found that the GTPase activity of Drp1, that is critical for mitochondrial fragmentation, was significantly higher in mutant huntingtin striatal cell lines and in the striatum of HD knock-in mutant mice indicating that in the presence of mHtt, the activity of Drp1 is aberrantly activated. These findings are in agreement with previous studies showing elevated Drp1 activity in the striatum and cortex of BACHD mice and in the cortex of HD patients [39]. Importantly, besides an increase in Drp1 activity a decrease in the levels of the fusion protein Opa1 was also observed suggesting altogether, that an imbalance in the levels and activity of proteins involved in the mitochondrial dynamic machinery could be responsible for the abnormal mitochondrial morphology observed in mutant huntingtin cells.

How these changes in mitochondrial dynamics may contribute to increasing susceptibility of mutant huntingtin striatal cells to neurodegeneration? Oxidative stress as a consequence of dopamine metabolism results in the formation of reactive oxidative species and quinones that may affect several mitochondrial processes such as ATP production, membrane permeability or fission/fusion mitochondrial events [40–42]. Indeed, mitochondrial respiration and ATP production are significantly reduced in our HD striatal cell lines along with an increase in mitochondrial-generated reactive-oxidative species and a decrease in the mitochondrial membrane potential [43–46]. However, dopamine besides being an inductor of oxidative stress can also induce mitochondrial dysfunction through activation of dopamine receptors. Actually, activation of dopamine D2 receptors in mutant huntingtin striatal neurons alters mitochondrial function by down-regulating mitochondrial complex II expression [18] while mitochondrial axonal transport can be regulated by integration of the opposite effects of D1R and D2R activation [47]. Notably, there is no data on the potential role of dopamine receptors in mitochondrial fission-fusion events. In the present manuscript, we describe for the first time that D1R activation induces mitochondrial dynamics defects, leading to a percentage of striatal cells displaying mitochondrial fragmentation close to 80% when mHtt is expressed and to 40% in wild type conditions. The molecular mechanism underlying D1R-induced mitochondrial fragmentation either in wild-type or mutant huntingtin striatal cells involves an increase in Drp1 protein levels. However, two major differences can be noticed when mutant and wild-type cells are compared. First, in wild-type cells the increase in Drp1 levels was located in the cytosolic fraction while that in mutant huntingtin cells was found in the mitochondria and second, in mutant but not wild-type huntingtin cells the increase in Drp1 protein levels was accompanied by an increase in their GTPase activity, which agrees with the data on mitochondrial Drp1 localization. Altogether, these data suggest that D1R activation increases mHtt-induced mitochondrial fragmentation by altering Drp1 activity, which may be related with the higher susceptibility of mutant huntingtin striatal cells to D1R activation.

How D1R activation induces such changes in Drp1 levels, distribution and activity is a crucial question. Previous studies from our group have demonstrated that the increase in cell death induced by D1R activation in mutant huntingtin striatal cells is related with an increase in the activity of the Ser/Thr kinase Cdk5 [2]. Interestingly, Cdk5 has been involved in mitochondrial dysfunction by increasing oxidative stress [28] or acting as an upstream regulator of mitochondrial fragmentation [21]. In this scenario, we tried to integrate both pathways hypothesizing that in mutant huntingtin striatal cells the increase in Cdk5 activity induced by D1R activation was responsible for the increase in mitochondrial fragmentation. Supporting this hypothesis our data demonstrates that (1) inhibition of Cdk5 reduces Drp1 activity in mutant cells to levels similar to those found in wild-type cells, which was associated with a significant decrease in mitochondrial fragmentation and (2) prevents in mutant huntingtin cells the D1R-mediated increase of Drp1 protein levels and their translocation to the mitochondria. Translocation of Drp1 to the mitochondria is a critical event in mitochondrial fragmentation [48]. Even though the precise mechanisms underlying the recruitment of Drp1 to the mitochondria surface are not well understood several studies stress the role of Drp1 post-translational modifications [49]. Regarding regulation of Drp1 activity by phosphorylation the published data can be rather confusing, since phosphorylation at the same residue may result in induction or prevention of mitochondrial fission depending on cell type, physiological/pathological condition or even the kinase involved [50]. This is the case for Ser616. While in post-mitotic mature neurons and in physiological conditions phosphorylation at Ser616 by Cdk5 inhibits Drp1 activity and therefore mitochondrial fission [51], under pathological conditions, Ser616 (or Ser579 in Drp1 isoform

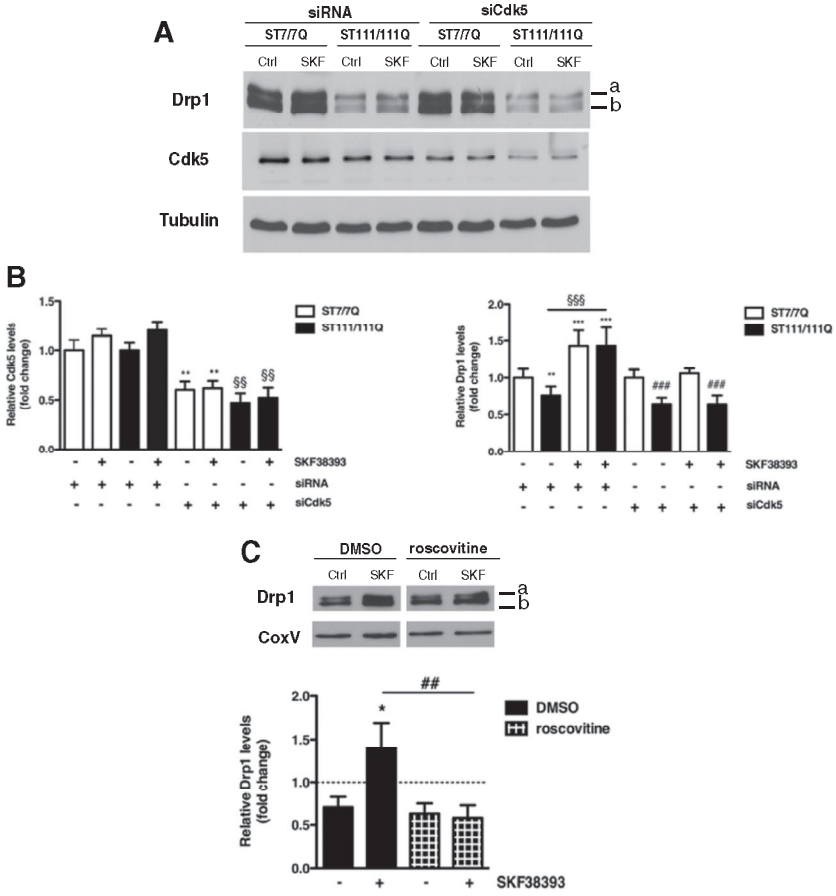

Fig. 9. Cdk5 promotes mitochondrial fission by altering the levels and the distribution of Drp1 in SKF 38393 treated ST111/111Q mutant cells. (A) Representative Western blots showing the levels of the fission protein Drp1 and Cdk5 in total extracts obtained from siRNA or siCdk5 transfected wild-type ST7/7Q and mutant ST111/111Q cells. α-Tubulin was used as loading control. When indicated, cells were treated with SKF 38393 (60 μM) during 1 h. Letters confer to the different isoforms recognized by the respective antibody (DRP1: a–b). (B) Bar histograms showing the relative fold change of Cdk5 and Drp1 protein levels in wild-type ST7/7Q and mutant ST111/111Q cells. Data represent mean ± SEM of 6 independent experiments. $^{***}p<0.001$, $^{**}p<0.01$ vs. vehicle-treated siRNA transfected ST7/7Q cells, $^{\#\#\#}p<0.001$ vs. vehicle-treated siCdk5 transfected ST7/7Q cells, $^{\S\S\S}p<0.001$ and $^{\S\S}p<0.01$ vs. vehicle-treated siRNA transfected ST111/111Q cells as determined by One-way ANOVA with Newman–Keuls post hoc analysis. (C) Representative Western blots showing the levels of the fission protein Drp1 in mitochondrial fractions obtained from vehicle or roscovitine treated mutant ST111/111Q cells. When indicated, cells were treated with SKF 38393 (60 μM) during 1 h. CoxV was used as loading control. Letters indicate the different isoforms recognized by the respective antibody (DRP1: a–b). Bar histogram indicates the relative fold change ± SEM of 6 independent experiments; $^{*}p<0.05$ vs. vehicle-treated ST111/111Q cells and $^{\#\#}p<0.01$ vs. roscovitine-treated ST111/111Q cells as determined by One-way ANOVA with Newman–Keuls post hoc analysis.

3) promotes mitochondrial fission either in mitotic or neuronal cells [52–54]. In our study, however, we have not observed any increase in phosphorylation at Ser616 in HD striatal cells compared to wild type cells (Supplementary Fig. 7) although Drp1 activity has been found to be increased suggesting that phosphorylation in other residues or other post-translational modifications could be involved. In this view, several studies have pointed out the role of the actin and microtubule cytoskeleton in Drp1-induced mitochondrial fission [48,55,56]. Interestingly, Cdk5 can phosphorylate a plethora of different proteins involved in cytoskeleton dynamics [57,58]. Indeed, increased Tau phosphorylation has been previously reported in mutant huntingtin striatal cells by our group [2] suggesting that an aberrant phosphorylation of cytoskeleton-related proteins could be involved in the accumulation of Drp1 in the mitochondria of mutant huntingtin striatal cells leading to mitochondrial fission.

5. Conclusion

In conclusion, we propose a model in which increased Cdk5 activity induced by both mHtt expression and aberrant dopaminergic signaling would contribute to increasing striatal susceptibility in HD by altering the expression, distribution and activity of Drp1 leading to mitochondrial fission events. Thus, our study contributes to the understanding of

the molecular mechanisms underlying striatal neurotoxicity in HD suggesting an important link between Cdk5 and Drp1 as mediators of dopaminergic-induced mitochondrial fragmentation in neurodegenerative diseases.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2015.06.025.

Transparency Document

The Transparency Document associated with this article can be found, in the online version.

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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

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Neurobehavioral characterization of Endonuclease G *knockout* mice reveals a new putative molecular player in the regulation of anxiety

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ABSTRACT

Endonuclease G (EndoG) has been largely related with a role in the modulation of a caspase-independent cell death pathway in many cellular systems. However, whether this protein plays a specific role in the brain remains to be elucidated. Here we have characterized the behavioral phenotype of EndoG$^{-/-}$ null mice and the expression of the nuclease among brain regions. EndoG$^{-/-}$ mice showed normal neurological function, learning, motor coordination and spontaneous behaviors. However, these animals displayed lower activity in a running wheel and, strikingly, they were consistently less anxious compared to EndoG$^{+/+}$ mice in different tests for anxiety such as plus maze and dark–light test. We next evaluated the expression of EndoG in different brain regions of wild type mice and found that it was expressed in all over but specially enriched in the striatum. Further, subcellular biochemical experiments in neocortical samples from wild type mice revealed that EndoG is localized in pre-synaptic compartments but not in post-synaptic compartments. Altogether these findings suggest that EndoG could play a highly specific role in the regulation of anxiety by modulating synaptic components.

Introduction

Despite the efforts of many groups, human genetic studies aimed to identify novel susceptibility genes for anxiety disorders have not been very successful, and few replicate findings exist (Hovatta and Barlow, 2008). Although environment is important for the development of anxiety traits in an individual, genetic background plays also a crucial role (Hettema et al., 2005; Tambs et al., 2012). Nowadays, the most promising candidate genes for anxiety disorders include Dopamine receptor D2 (DRD2) (Wacker et al., 2005), Catechol-Omethyltransferase (COMT) (Montag et al., 2012), Monoamine oxidase A (MAOA) (Deckert et al., 1999), 5-hydroxytryptamine receptor 2A (5-HTR2A) (Aloyo et al., 2009), Adenosine A2 receptor (ADORA2A) (Lopes et al., 2011) and Brain-derived neurotrophic factor (BDNF) (Hall et al., 2003), among others. However, many of these genes involved in anxiety modulation share molecular mechanisms with other brain systems involved with other neurological or cognitive functions (Harvey, 2003; Nonkes et al., 2012; Papaleo et al., 2011; Wall et al., 2003) increasing the complexity of the research in the anxiety field. Thus, the search for more specific genetic candidates is still open.

Endonuclease G (or EndoG) has a classical role in the regulation of cell death by activation of caspase-independent pathways (Li et al., 2001). This protein was early described to be localized in the mitochondria (Cummings et al., 1987) and in the nucleus of non-neural cells (Ruiz-Carrillo and Renaud, 1987). However, new features of this protein have been described, as for example, it plays an essential role in the regulation of left ventricular mass (LVM) and blood-pressure-independent related cardiac hypertrophy (McDermott-Roe et al., 2011). Thus, EndoG could play important roles in cell physiology other than DNA fragmentation and cell death. In this line, it has been recently described that EndoG is also expressed in the brain (Zhang et al., 2011). Unfortunately, only few studies have addressed the role of EndoG in neuronal function and all of them have focused only in cell death processes (Zhang et al., 2007; Zhao et al., 2009). Thus, if EndoG plays a role in specific neural functions still remains to be explored.

Here we characterize the behavior of a previously generated knockout mice for EndoG (EndoG $^{/}$ mice) (Irvine et al., 2005) for the first time. We found no changes in many behavioral paradigms except for

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