Universidade Federal de Minas Gerais (UFMG)

Instituto de Ciências Biológicas (ICB)

Programa de Pós-graduação em Bioquímica e Imunologia

Tese de Doutorado

Investigação do papel dos canais para Ca^{2+} sensíveis à voltagem, $Ca_v2.2$ e $Ca_v1.2$, e dos receptores de NMDA em um camundongo modelo da doença de Huntington, o BACHD

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Orientadora: Fabíola Mara Ribeiro

Co-orientadora: Luciene Bruno Vieira

Belo Horizonte, 04 de Maio de 2017

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Tese de Doutorado apresentada ao programa de Pós-graduação em Bioquímica e Imunologia, como parte dos requisitos para obtenção do título de Doutora em Ciências, área de concentração em Bioquímica, sob orientação da Profª Fabíola Mara Ribeiro.

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Sumário

Introdução

1.1 A Doença de Huntington

A doença de Huntington (DH) foi descrita inicialmente por George Huntington em abril de 1872, na revista norte-americana *"Medical and Surgical Reporter"*, com o nome de coréia hereditária (Huntington, 1872). Trata-se de uma desordem neurodegenerativa de caráter autossômico dominante, caracterizada por sintomas de movimento involuntário do corpo, perda da função cognitiva, distúrbios psiquiátricos, levando o paciente inevitavelmente à morte (Young, 2003, Li and Li, 2004). A proteína huntingtina (Htt), apresentando uma expansão de mais de 37 poliglutaminas na região amino-terminal, é apontada como fator determinante para desenvolvimento desta doença (Group, 1993). Portanto, pacientes que apresentam a forma mutada da Htt irão, invariavelmente, desenvolver a DH (Group, 1993). A prevalência da DH é de 5 a 10 casos em cada 100.000 indivíduos (Smith *et al.*, 2005). Em geral, a doença se manifesta na meia idade, sendo que a severidade da doença aumenta com o número de glutaminas, ou seja, quanto maior o número de expansões de poliglutaminas, mais precoces e mais severos serão os sintomas (Jason *et al.*, 1997, Kirkwood *et al.*, 1999). Observa-se inicialmente na DH a incapacidade de executar movimentos simples, como por exemplo, dificuldades na execução da marcha, que se tornam mais evidentes com o avanço da doença (Poe and Seifert, 1997). Bradicinesia e rigidez são pouco observadas nas fases iniciais e surgem no estágio final da doença, quando o paciente

torna-se rígido e acinético (Folstein *et al.*, 1986, Penney *et al.*, 1990). Em estágios avançados, a rigidez muscular, bradicinesia e distonia, incapacitam a realização de movimentos voluntários do paciente (Ribchester *et al.*, 2004). A proteína Htt selvagem exibe ampla distribuição no organismo e está presente em diversos tipos celulares, podendo estar associada ao núcleo, retículo endoplasmático, complexo de Golgi e às mitocôndrias, apresentando papel importante para a homeostase celular (Li and Li, 2004). Além disso, ela exerce também um papel importante no transporte vesicular e nas sinapses, sendo capaz de interagir com proteínas da vesícula sináptica, transporte axonal e microtúbulos (Smith *et al.*, 2005).

A razão pela qual a Htt mutante afeta neurônios ainda não está completamente elucidada, mas sugere-se que, além da perda da função normal da Htt selvagem, também ocorra um ganho de função da Htt mutante, resultando em toxicidade e consequente morte neuronal (Di Prospero and Fischbeck, 2005). Já é bem estabelecido que a Htt mutante promove alterações na transcrição gênica, função mitocondrial e homeostase de Ca^{2+} (Smith *et al.*, 2005, Rozas *et al.*, 2010). Embora ainda não esteja claro como a Htt mutante promove a morte celular, é certo que a progressão da DH é determinada pela morte neuronal progressiva que ocorre no neocortex e no corpo estriado destes pacientes (Group, 1993).

1.2 Modelos animais para a DH

Existe uma constante busca pelo desenvolvimento de modelos animais que possam refletir o padrão da DH. Os primeiros modelos transgênicos que foram

desenvolvidos para o estudo dessa doença foram as linhagens de camundongos R6/1 e R6/2 (Mangiarini *et al.*, 1996). Estes modelos foram seguidos por várias outras linhagens transgênicas de camundongos que diferem quanto à extensão da expansão de poliglutaminas, a porção do gene inserido, o promotor e o nível de expressão da proteína mutante (Li *et al.*, 2005). A linhagem R6/2 apresenta um fenótipo de déficit motor, incluindo falta de coordenação motora, marcha anormal, hipoatividade e dificuldade de aprendizagem, com idade de início por volta de quatro semanas (Carter *et al.*, 1999, Lione *et al.*, 1999). A formação de agregados é muito acentuada nesses animais e eles apresentam sintomas neurológicos graves e morte precoce, entre 3 e 6 meses de idade (Mangiarini *et al.*, 1996). No entanto, apesar de apresentarem um fenótipo robusto, esses animais não representam um modelo preciso para o estudo da DH, pois expressam somente a região N-terminal da proteína huntingtina, a qual é composta principalmente por poliglutaminas.

O modelo YAC128 expressa a Htt humana inteira contendo uma expansão de 128 glutaminas (Slow *et al.*, 2003). Esse modelo da DH foi criado utilizando-se cromossomos artificiais de levedura (Davies *et al.*, 1997, Schilling *et al.*, 1999). Já os camundongos knock-in, $Hdh^{Q97/Q97}$, $Hdh^{Q111/Q111}$ e $Hdh^{Q150/Q150}$, foram gerados pela substituição do primeiro éxon do gene da Htt murina pelo primeiro éxon do gene humano, contendo a expansão CAG (Wheeler *et al.*, 2000). Os animais $Hdh^{Q111/Q111}$ apresentam um fenótipo muito leve, exibindo menos anomalias comportamentais que o R6/2, BACHD e YAC128 (Menalled *et al.*, 2014).

O modelo BACHD foi criado utilizando-se um cromossomo artificial de bactéria contendo a Htt humana inteira, onde foram inseridas 97 repetições CAA-CAG no éxon

1 da Htt (Gray *et al.*, 2008). Este modelo é bastante robusto por apresentar progressão da DH semelhante ao que ocorre em pacientes. Assim, camundongos BACHD apresentam déficits mais suaves quando comparado aos demais modelos e exibem formação de agregados de Htt e perda de células neuronais apenas aos 12 meses de idade (Gray *et al.*, 2008, Doria *et al.*, 2015). Camundongos BACHD apresentam alterações motoras relativamente precoces e também exibem déficit cognitivo a partir dos 6 meses de idade (Gray et al. 2008, Doria et al 2015, Menalled). Nesse sentido, o BACHD é considerado como um dos melhores modelos até o momento para o desenvolvimento de estudos relativos à DH (Ribeiro *et al.*, 2013).

1.3 Transmissão glutamatérgica e sinalização de Ca^{2+} neuronal

O glutamato é o principal neurotransmissor das sinapses excitatórias no sistema nervoso central (SNC) de mamíferos, desempenhando um papel importante na plasticidade sináptica, aprendizado, memória e outras funções cognitivas (Hayashi, 1954, Hollmann and Heinemann, 1994, Humeau *et al.*, 2003, Yu *et al.*, 2008). Os receptores para glutamato são divididos em duas classes principais: metabotrópicos e ionotrópicos (Hollmann and Heinemann, 1994, Magistretti, 2009, Traynelis *et al.*, 2010, Nicoletti *et al.*, 2011). Existem oito receptores metabotrópicos (mGluRs) (O'Brien *et al.*, 2003, Conn *et al.*, 2009) os quais são subdivididos em três subgrupos (I, II e III) de acordo com homologia de sequência, farmacologia e mecanismo de transmissão de sinais (O'Hara *et al.*, 1993, Conn and Pin, 1997, Bockaert and Pin, 1999, O'Brien *et al.*, 2003, Anborgh *et al.*, 2005, Conn *et al.*, 2005). O grupo I compreende os receptores

mGluR1 e mGluR5, os quais são acoplados à proteína $G\alpha_{q/11}$, sendo localizados principalmente na região pós-sináptica (Conn and Pin, 1997, O'Brien *et al.*, 2004). A ativação do mGluR1 e mGluR5 pelo agonista promove a ativação da proteína $G_{\alpha q}$, fazendo com que a subunidade α da proteína G seja desacoplada das subunidades β/γ, desencadeando a ativação da fosfolipase C (PLC), a qual catalisa a hidrólise do lipídeo de membrana fosfatidilinositol 4,5 - bifosfato (PIP_2) em diacilglicerol (DAG) e inositol 1,4,5-trifosfato ($InsP_3$). O $InsP_3$ associa-se aos receptores presentes na membrana do retículo endoplasmático (RE) promovendo sua ativação e ocasionando um influxo de Ca^{2+} proveniente do RE para o citoplasma (Masu *et al.*, 1991, Ritzen *et al.*, 2005, Xu *et al.*, 2009, Ribeiro *et al.*, 2010). Os grupos II (mGluR2 e mGluR3), e III (mGluR4, mGluR6, mGluR7 e mGluR8) são acoplados à proteína $G\alpha_{i/0}$ e estão associados à redução da produção de adenosina monofosfato cíclico (AMPc). Esses receptores de glutamato estão localizados principalmente na região pré-sináptica, onde são capazes de regular a liberação de glutamato através da interação das subunidades β/γ da proteína $G\alpha_{i/0}$ com os canais para Ca^{2+} sensíveis à voltagem (CCSV), os quais regulam a liberação de neurotransmissores **(Fig. 1)** (Conn and Pin, 1997, Zamponi, 2013).

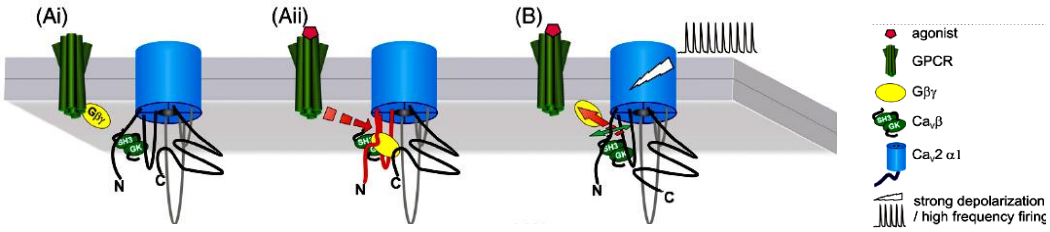

Figura 1: Ilustração das interações moleculares que ocorrem durante a inibição mediada pelas subunidades $G_{\beta\gamma}$ sobre os canais Ca_V2. A e B representam um canal Ca_V2. A ligação da $G_{\beta\gamma}$ ocorre provavelmente em múltiplos sítios: na porção amino-terminal, no loop I-II e no domínio C-terminal do canal. A ligação de $G_{\beta\gamma}$ provoca uma alteração conformacional nas regiões amino-terminal e no loop I-II do canal (painel Aii), levando a alteração do *gating* para potenciais mais despolarizados, além do desacoplamento do sensor de voltagem para ativação do canal. A forte despolarização da membrana (painel B) leva a alterações conformacionais que resultam no desacoplamento da $G_{\beta\gamma}$ e à perda de sua interação com o canal, restabelecendo a conformação das regiões amino-terminal e do loop I-II. Fonte: (Zamponi and Currie, 2013) (Adaptado).

Além de ativar receptores metabotrópicos, o glutamato liberado a partir de terminais pré-sinápticos também age nos receptores ionotrópicos pós-sinápticos, promovendo excitação. Os receptores ionotrópicos ativados por glutamato foram nomeados através dos seus agonistas seletivos: N-metil-d-aspartato (NMDA), ácido α-amino-3-hidroxi-5-metil-4-isoxazol propiónico (AMPA) e cainato (MacDermott *et al.*, 1986, Hawkins, 2009, Traynelis *et al.*, 2010). Os receptores de NMDA (NMDARs) são proteínas transmembranares que formam um poro permeável ao Na^+, K^+ e Ca^{2+}. Sete subunidades dos NMDARs já foram identificadas até o momento: GluN1, GluN2A-GluN2D e GluN3A-GluN3B, sendo que a maioria dos NMDARs são complexos heterotetraméricos compostos tipicamente por duas subunidades GluN1 e duas GluN2 (Stephenson *et al.*, 2008). As subunidades do NMDAR contem um domínio amino-terminal extracelular longo, um domínio de ligação ao ligante (o glutamato liga-se às subunidades GluN2 e o co-agonista glicina liga-se às subunidades GluN1 e GluN3), um domínio transmembranar (quatro domínios transmembrana ligados por laços curtos) e um domínio intracelular C-terminal (Mayer and Armstrong, 2004, Paoletti, 2011).

O tipo de subunidade que compõe o NMDAR é importante para as propriedades funcionais desse canal. Por exemplo, se a subunidade GluN3 for incorporada ao canal funcional além das subunidades GluN1 e GluN2, as correntes do NMDAR são reduzidas e o receptor se torna menos sensível ao bloqueio de Mg^{2+} (Cavara and Hollmann, 2008, Henson *et al.*, 2010). Além disso, os receptores contendo as subunidades GluN2A e GluN2B apresentam uma maior permeabilidade ao Ca^{2+}, enquanto que a presença de uma subunidade GluN3 na composição do receptor resulta numa baixa permeabilidade ao Ca^{2+} (Burton-Bradley, 1973).

O Ca^{2+} é um sinalizador intracelular que é responsável por regular diferentes processos fisiológicos, como a liberação de neurotransmissores, transcrição gênica, proliferação celular e apoptose (Berridge *et al.*, 2003). O aumento da concentração intracelular de Ca^{2+} pode ser oriundo dos estoques internos ou do meio extracelular. O aporte de Ca^{2+} a partir da região extracelular ocorre através do influxo desse íon por diversos canais presentes na membrana, em resposta a estímulos como despolarização ou ativação por ligantes (Carafoli and Brini, 2000, Parekh, 2003). Diversos tipos de canais estão envolvidos na sinalização de Ca^{2+} neuronal, como por exemplo, os CCSV, os receptores para $InsP_3$ ($InsP_3R$) e os NMDARs. A liberação de Ca^{2+} a partir dos estoques intracelulares, como o RE, é mediada por $InsP_3Rs$. A ativação desses $InsP_3Rs$ pode ocorrer pela estimulação de mGluR1 e mGluR5, como descrito anteriormente, bem como pela estimulação de outros receptores metabotrópicos acoplados à proteína $G_{\alpha q}$. Além disso, tanto a mitocôndria possui um papel muito importante na sinalização citosólica de Ca^{2+}, como também proteínas ligadoras de Ca^{2+} participam na manutenção dos seus níveis citosólicos, por exemplo, calbindina-D28 e parvalbumina, e dentro do RE, a calreticulina e calnexina em neurônios (Bezprozvanny, 2010).

O influxo de Ca^{2+} através do NMDAR inicia eventos criticamente envolvidos em mudanças duradouras da atividade sináptica, como a potencialização de longo prazo (LTP) e a depressão de longo prazo (LTD) (Bliss and Collingridge, 1993, Traynelis *et al.*, 2010). Dessa forma, alterações nas concentrações intracelulares de Ca^{2+} são importantes para eventos de plasticidade sináptica, os quais são essenciais para a cognição e memória. Por outro lado, a superativação do receptor pode ser

prejudicial ao estimular as vias de morte celular dependentes de Ca^{2+}, resultando em neurodegeneração (Lipton and Rosenberg, 1994). Por exemplo, a elevação exacerbada da concentração de Ca^{2+} intracelular pode iniciar uma série de eventos citoplasmáticos e nucleares, promovendo ativação de enzimas proteolíticas, endonucleases e lipases, e, consequentemente, morte celular (Mitani *et al.*, 1998). Além disso, pode ocorrer produção de radicais livres e esgotamento dos estoques de antioxidantes, com consequente aumento do dano por peroxidação lipídica (Iadecola, 1997).

Assim, distúrbios da sinalização de Ca^{2+} estão intimamente associados a processos de morte neuronal e é possível que processos excitotóxicos associados a um aumento dos níveis de Ca^{2+} citosólicos sejam os principais eventos desencadeadores da morte neuronal que ocorre na DH (Hajnoczky *et al.*, 2003, Orrenius *et al.*, 2003). Vários estudos indicam que a Htt mutada pode promover distúrbios na sinalização de Ca^{2+} que estariam associados à morte de neurônios estriatais. O nosso grupo e outros demonstraram que tanto a ativação de NMDAR quanto a ativação de mGluR **(Fig. 2)** podem estar envolvidas no distúrbio da sinalização de Ca^{2+} que ocorre na DH (Zeron *et al.*, 2002, Ribeiro *et al.*, 2010).

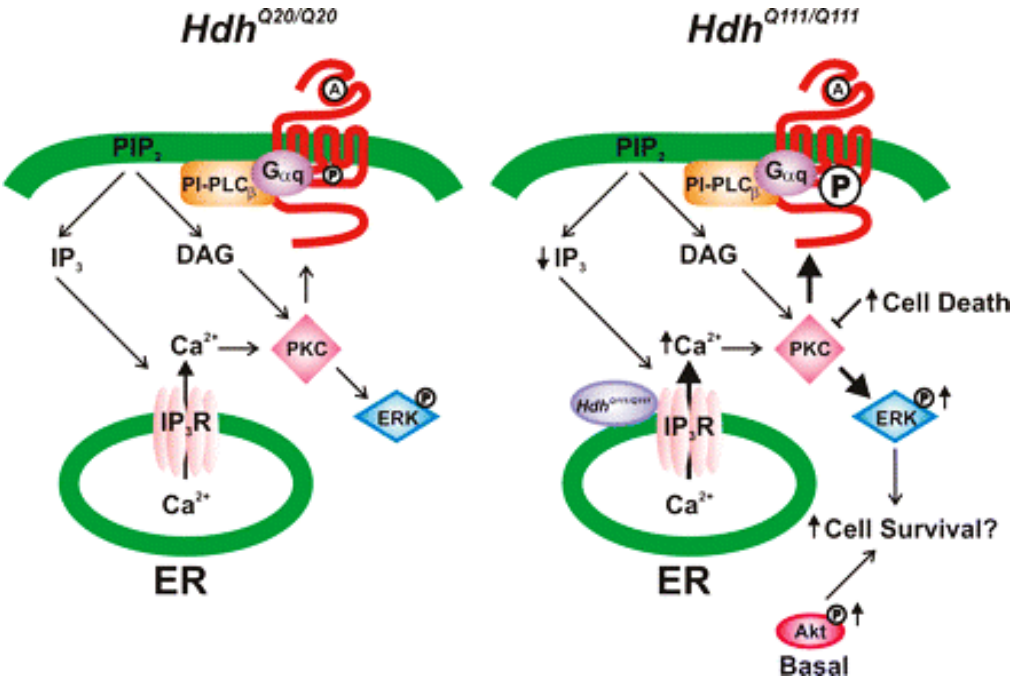

Figura 2: Ilustração do modelo proposto para alterações de sinalização de mGluR1/5 na DH em camundongos Hdh$^{Q20/Q20}$ e Hdh$^{Q111/Q111}$. mGluR1/5 são acoplados às proteínas $G_{\alpha q/11}$, e sua ativação resulta na formação de diacilglicerol (DAG) e IP_3, liberação de Ca^{2+} dos estoques intracelulares e ativação de PKC, bem como ativação de ERK e AKT. Em camundongos Hdh$^{Q111/Q111}$, a formação de IP_3 mediada por mGluR1/5 é diminuída devido ao aumento da dessensibilização de mGluR1/5 mediada por PKC. Apesar da diminuição da formação de InsP, a liberação de Ca^{2+} mediada por mGluR1/5, assim como a ativação de ERK e AKT estão aumentadas em camundongos Hdh$^{Q111/Q111}$, em comparação aos Hdh$^{Q20/Q20}$. Assim, esta diminuição da formação de InsP através do mGluR5 é provavelmente a consequência de uma resposta adaptativa numa tentativa de manter os neurônios Hdh$^{Q111/Q111}$ vivos na fase pré-sintomática da doença. Fonte: (Ribeiro, FM *et al*. The Journal of Neuroscience, 2010).

Foram identificados pelo menos três mecanismos diferentes que são responsáveis pelo aumento dos níveis citosólicos de Ca^{2+} causado pela Htt mutada **(Fig.3)**: 1) A proteína Htt mutada é capaz de sensibilizar NMDAR, aumentando sua permeabilidade ao Ca^{2+}, o que promove o aumento dos níveis citosólicos desse íon em neurônios estriatais (Chen *et al.*, 1999, Sun *et al.*, 2001); 2) A proteína Htt mutada promove uma desestabilização da mitocôndria, diminuindo a capacidade dessa organela de regular os níveis de Ca^{2+} (Panov *et al.*, 2002, Choo *et al.*, 2004) e 3) A Htt mutada é capaz de sensibilizar o $InsP_3R$, aumentando a liberação de Ca^{2+} a partir dos estoques intracelulares (Tang *et al.*, 2003, Zuccato *et al.*, 2010).

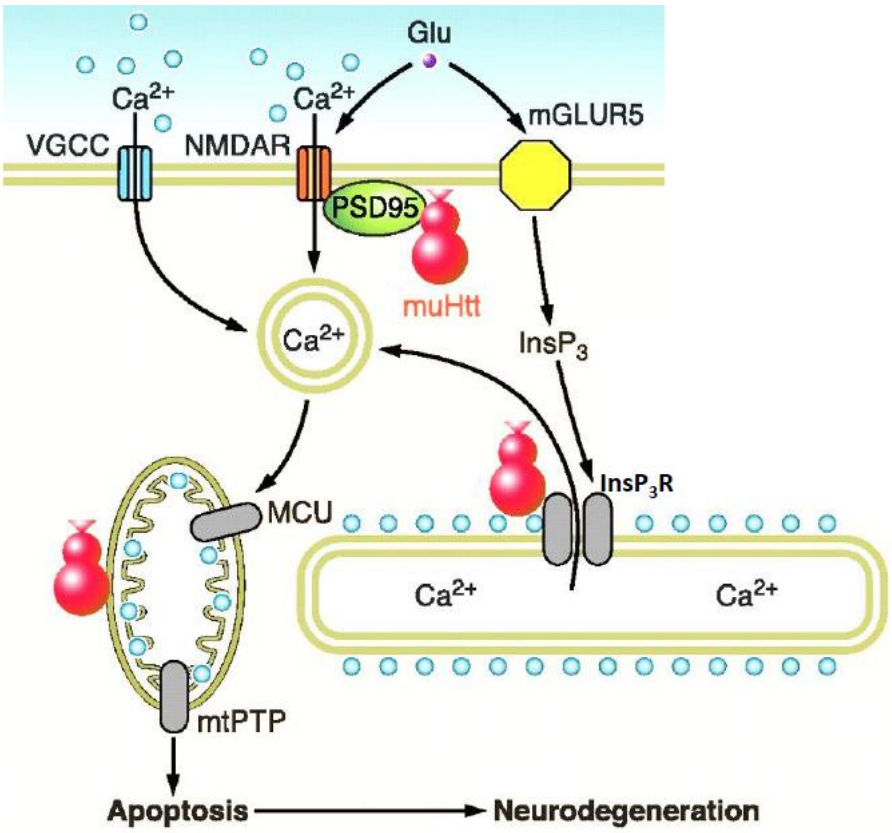

Figura 3: Disfunção da sinalização de Ca^{2+} na DH. A Htt mutante provoca um aumento de Ca^{2+} citosólico, mitocondrial e apoptose nos neurônios espinhosos médios (MSN). A Htt mutante perturba a sinalização de Ca^{2+} promovendo uma hiperfunção dos NMDARs, possivelmente através de uma diminuição da interação entre o complexo PSD95-NR1A/NR2B. A Htt mutante leva ainda a uma hiper sensibilização dos $InsP_3Rs$, facilitando a liberação de Ca^{2+} a partir do retículo endoplasmático. O aumento exacerbado de Ca^{2+} ativam calpaínas, que clivam a Htt. Este aumento citosólico excessivo de Ca^{2+} resulta também na captação mitocondrial de Ca^{2+}, que desencadeia a abertura de mtPTP e consequente apoptose. O controle mitocondrial de Ca^{2+} é ainda desestabilizado pela associação direta de Htt mutante com as mitocôndrias. **MuHtt, huntingtina mutante; MCU, transportador uniporte de Ca^{2+} mitocondrial; MtPTP, poro de transição de permeabilidade mitocondrial; VGCC, canal para Ca^{2+} abertos por voltagem**. Fonte: (Zuccato *et al.*, 2010) (Adaptado).

1.4 Canais para Ca^{2+} Sensíveis à voltagem

Os CCSV são ativados pela despolarização celular, sendo cruciais para o processo de excitabilidade em neurônios e células musculares (Reuter, 1979, McCleskey *et al.*, 1986, Takahashi and Momiyama, 1993, Bers, 2002, Nowycky and Thomas, 2002). Os CCSV apresentam ampla distribuição no corpo e são divididos em

três famílias **(Tab. 1)**: Ca_v1 ($Ca_v1.1$, $Ca_v1.2$, $Ca_v1.3$ e $Ca_v1.4$), Ca_v2 ($Ca_v2.1$, $Ca_v2.2$ e $Ca_v2.3$) e Ca_v3 ($Ca_v3.1$, $Ca_v3.2$ e $Ca_v3.3$) (McCleskey *et al.*, 1986, Miller, 2001), cuja classificação é baseada em suas propriedades biofísicas e farmacológicas (Bean, 1989, Hess, 1990, Zhang *et al.*, 1993, Randall and Tsien, 1995). Os CCSV são proteínas multiméricas cujas propriedades são determinadas pela sua subunidade formadora do poro, a α_1 (Hofmann *et al.*, 1999). Esta subunidade é composta por quatro domínios homólogos contendo seis segmentos transmembrana que incluem o sensor de voltagem S4 e a região P de revestimento do poro. Esses canais ainda são compostos por subunidades denominadas auxiliares β, γ e $\alpha_2\delta$, as quais exercem um papel modulatório, regulando as propriedades biofísicas do canal, bem como seu tráfego membranar (Gerster *et al.*, 1999, Klugbauer *et al.*, 1999, Klugbauer *et al.*, 2000).

Gene(nome atual)	**Nome anterior**	**Tipo**	**Localização**	**Bloqueador**
$Ca_v1.1$–$Ca_v1.4$	α1S, α1C, α1D, α1F	L	Músculos e neurônios	Diidropiridinas
$Ca_v2.1$	α1A	P/Q	neurônios	ω-agatoxinas (veneno de aranha)
$Ca_v2.2$	α1B	N	neurônios	ω-conotoxinas (veneno de molusco marinho)
$Ca_v2.3$	α1E	R	neurônios	SNX-482 (veneno de tarântula)
$Ca_v3.1$-$Ca_v3.3$	α1G, α1H, α1I	T	Músculos e neurônios	Mibefradil

Tabela 1: Nomenclatura e distribuição dos CCSV.

Os CCSV exercem um papel importante nos processos funcionais do sistema nervoso, onde pelo menos dois desses subtipos ($Ca_v2.2$ e $Ca_v2.1$) foram relacionados ao controle da liberação de neurotransmissores nas sinapses (Catterall, 2000). O aumento da concentração de Ca^{2+} no terminal nervoso é necessário para que ocorra a exocitose de neurotransmissores via proteínas do complexo SNARE (*Soluble NSF Attachment Receptor)* (Araque *et al.*, 2000). Além de regular as concentrações de Ca^{2+} no terminal pré-sináptico, já foi demonstrado que $Ca_v2.2$ interage com as proteínas do complexo SNARE, sintaxina 1A e SNAP25, bem como com a sinaptotagmina, a qual é a proteína sensora de Ca^{2+} associada ao complexo SNARE (Westenbroek *et al.*, 1992, Sheng *et al.*, 1994, Rettig *et al.*, 1996, Sheng *et al.*, 1996, Jarvis and Zamponi, 2001). Essa interação entre o CCSV e as proteínas do complexo SNARE é de grande importância para a regulação das correntes de Ca^{2+} e para a modulação da exocitose de neurotransmissores. Por exemplo, já foi demonstrado que a sintaxina 1A é capaz de regular o $Ca_v2.2$ (Zamponi GW1, 1997, Zamponi *et al.*, 1997, Zamponi and Snutch, 1998). Além disso, o $Ca_v2.2$ é capaz de interagir com as subunidades $\beta\gamma$ da proteína G e essa interação leva a uma inibição das correntes de Ca^{2+} (abertura do canal em potenciais mais hiperpolarizados) e a uma diminuição da liberação de neurotransmissores (Zhang *et al.*, 1996, Herlitze *et al.*, 1997, Qin *et al.*, 1997, Zamponi *et al.*, 1997, Agler *et al.*, 2003).

Este trabalho tem como foco o estudo dos canais dos subtipos $Ca_v2.2$ e $Ca_v1.2$, uma vez que esses canais são altamente expressos no cérebro (Schlick *et al.*, 2010). Além disso, como descrito anteriormente, o $Ca_v2.2$ tem um papel importante no controle da liberação de neurotransmissores nas sinapses (Catterall, 2000). Já, os

canais $Ca_v1.2$, que representam 90% da expressão dos Ca_v1 no cérebro (Hell *et al.*, 1993, Sinnegger-Brauns *et al.*, 2009), são localizados pós-sinapticamente, no corpo celular e em dendritos dos neurônios (Di Biase *et al.*, 2008, Jenkins *et al.*, 2010). Os $Ca_v1.2$ participam de vias envolvidas no controle da expressão gênica, além de modularem a plasticidade neuronal, aprendizado e memória (Ma *et al.*, 2012, Striessnig *et al.*, 2014).

1.5 Bloqueadores de canais para Ca^{2+}

Uma relação entre CCSV e doenças neurodegenerativas já foi demonstrada, uma vez que dados publicados anteriormente indicam que o peptídeo β-amilóide é capaz de interagir com $Ca_v1.2$, alterando a atividade do canal e promovendo um aumento da expressão desse canal para Ca^{2+} na membrana plasmática (Scragg *et al.*, 2005, Kim and Rhim, 2011). Além disso, $Ca_v1.2$ parece ter função relevante na morte neuronal que ocorre em doenças neurodegenerativas, tais como a doença de Alzheimer (DA), uma vez que bloqueadores de $Ca_v1.2$, especialmente a isradipina, apresentam função neuroprotetora contra a morte induzida pelo oligômero Aβ (Anekonda *et al.*, 2011). Alguns estudos epidemiológicos também suportam a hipótese de que bloqueadores de $Ca_v1.2$ são capazes de prevenir ou retardar a progressão da DA (Tollefson, 1990, Fritze and Walden, 1995, Forette *et al.*, 1998). Isradipina é um bloqueador de $Ca_v1.2$ que é bem tolerado por pacientes e é aprovado para o uso em humanos para o tratamento da hipertensão. Já foi demonstrado que injeções subcutâneas de isradipina (2.5 mg/kg por 7 dias) antagoniza o dano hipocampal e o

déficit de memória causado por hipóxia em ratos, indicando que essa droga apresenta efeito neuroprotetor *in vivo* (Barhwal *et al.*, 2009). Uma dose de isradipina de 3 mg/kg também foi capaz de proteger neurônios dopaminérgicos *in vivo* em camundongos modelo da doença de Parkinson (DP) (Chan *et al.*, 2007, Ilijic *et al.*, 2011). Atualmente a isradipina está sendo avaliada em ensaios clínicos para o tratamento da DP (Simuni *et al.*, 2010). Apesar desses vários estudos que relacionam o $Ca_v1.2$ e doenças neurodegenerativas, não existem estudos que investiguem se o $Ca_v1.2$ poderia ter uma função no distúrbio de Ca^{2+} que ocorre em pacientes com DH. Além disso, não existem pesquisas que investiguem se o $Ca_v2.2$ poderia contribuir para a alteração de liberação de glutamato observada em camundongos modelo da DH (Lievens *et al.*, 2001, Behrens *et al.*, 2002, Lee *et al.*, 2013, Valencia *et al.*, 2013).

1.6 Toxinas de *Phoneutria nigriventer*

As toxinas de aranhas *Phoneutria nigriventer*, denominadas Phoneutriatoxinas, têm sido extensamente estudadas e estão se tornando ferramentas farmacológicas de grande importância (Escoubas *et al.*, 2000). Estas Phoneutriatoxinas oferecem potencial farmacológico por atuarem em receptores neuronais e em sua maioria em canais iônicos (Diniz *et al.*, 1990, Romano-Silva *et al.*, 1993, Cassola *et al.*, 1998, Grishin, 1999, Gomez *et al.*, 2002, Rajendra *et al.*, 2004). Vários trabalhos descrevem as ações destas toxinas em modular canais para Ca^{2+}, sendo capazes de inibir as correntes de Ca^{2+} por bloquearem o poro do canal (Cassola *et al.*, 1998, Leao *et al.*, 2000, Dos Santos *et al.*, 2002, Vieira *et al.*, 2005).

Inicialmente, quatro grupos ou frações diferentes de polipeptídeos neurotóxicos (PhTx1, PhTx2, PhTx3 e PhTx4) foram purificados a partir do veneno de *Phoneutria nigriventer*. A PhTx1 contém apenas um peptídeo (Diniz *et al.*, 1990), enquanto PhTx2 e PhTx3 são uma coleção de pelo menos nove e seis isotoxinas, respectivamente (Cordeiro Mdo *et al.*, 1992, Cordeiro Mdo *et al.*, 1993). Duas toxinas, PnTx4-6-1 e PnTx4-5-5, foram purificadas a partir de PhTx4 e as suas sequências de aminoácidos completas foram determinadas. Ambas possuem alta atividade inseticida (Figueiredo *et al.*, 1995). Contudo, experimentos demonstraram que a PnTx4-5-5 inibe seletivamente NMDAR em neurônios hipocampais de ratos (de Figueiredo *et al.*, 2001). Como os NMDARs estão intimamente envolvidos nos processos de morte neuronal que ocorrem em várias doenças neurodegenerativas (Lipton and Rosenberg, 1994, Milnerwood *et al.*, 2010, Wang *et al.*, 2013, Parsons and Raymond, 2014), levantamos a hipótese que essa toxina seria uma droga neuroprotetora potencial.

2. Hipótese do trabalho

A nossa hipótese é que a Htt mutante possa alterar a função de CCSV, contribuindo para a morte neuronal e para as alterações da plasticidade sináptica observadas na DH.

As alterações promovidas na função do $Ca_V1.2$ poderiam contribuir para o distúrbio da sinalização de Ca^{2+}, facilitando assim a morte de neurônios que expressam a Htt mutante. Já as alterações da função do canal $Ca_V2.2$ pela proteína Htt mutante poderiam contribuir para a perturbação da liberação de glutamato, promovendo, portanto, alterações na plasticidade sináptica.

3. Discussão

Com o objetivo de elucidar as alterações promovidas pela Htt mutante sobre a regulação dos níveis de Ca^{2+} intracelular, buscamos nos artigos que compõem essa tese, determinar se os CCSV, $Ca_v2.2$ e $Ca_v1.2$, apresentariam alterações em suas funções em um camundongo modelo da DH, o BACHD. Observamos que a expressão desses CCSV, bem como suas propriedades eletrofisiológicas apresentavam-se alteradas em camundongos BACHD. Entretanto, as consequências das alterações nesses canais se mostraram bastante diversas quando são comparados $Ca_v2.2$ e $Ca_v1.2$. É sabido que alterações nos níveis de Ca^{2+} pré e pós-sinápticos podem influenciar tanto processos de neurotransmissão, quanto sobrevivência/morte neuronal. Assim, um primeiro artigo publicado mostra que as alterações observadas nos canais $Ca_v2.2$ em animais BACHD parecem estar relacionadas às alterações de liberação de glutamato observada nesses animais, uma vez que $Ca_v2.2$ é expresso principalmente nos terminais pré-sinápticos e tem função importante na liberação de neurotransmissores. Já num segundo artigo, as alterações observadas em canais $Ca_v1.2$, cuja localização é principalmente pós-sináptica, estão intimamente relacionadas a um aumento da entrada de Ca^{2+} e morte neuronal. Dessa forma, tendo por base os resultados obtidos neste trabalho, propomos que bloqueadores dos canais $Ca_v1.2$ apresentam potencial neuroprotetor. Além disso, analisamos ainda em um terceiro artigo publicado, a importância de uma outra droga com potencial neuroprotetor, a PnTx4-5-5, a qual é bloqueadora de NMDARs. Os dados apresentados

nesses artigos indicam que tanto bloqueadores dos canais $Ca_v1.2$ quanto a toxina bloqueadora de NMDARs (PnTx4-5-5) apresentam atividade neuroprotetora *in vitro*.

O trabalho apresentado no **Apêndice 1**, "***N-type Ca^{2+} channels are affected by full-length mutant huntingtin expression in a mouse model of Huntington's disease",*** demonstra que o canal $Ca_v2.2$ contribui para as alterações de liberação de glutamato observadas em animais BACHD e que essas alterações variam de acordo com a idade dos animais. Embora animais BACHD de 3 meses de idade apresentem um aumento da liberação de glutamato, animais aos 12 meses de idade apresentam uma diminuição dessa liberação. Essas alterações na liberação de glutamato já haviam sido relatadas em trabalhos anteriores (Joshi *et al.*, 2009, Valencia *et al.*, 2013). Entretanto, nenhum desses trabalhos havia relacionado as alterações na liberação de glutamato a uma disfunção do $Ca_v2.2$, o que é claramente demonstrado em nossos resultados, visto que o bloqueio dos canais $Ca_v2.2$ pela ω-CgTxGVIA, bloqueadora seletiva de $Ca_v2.2$, aboliu o aumento da liberação de glutamato apresentado por camundongos BACHD jovens. Uma vez que alguns sintomas relacionados à DH aparecem mesmo antes que níveis detectáveis de perda neuronal possam ser detectados, alterações na neurotransmissão poderiam explicar os sintomas iniciais desta doença (Diamond *et al.*, 1992, Hahn-Barma *et al.*, 1998). Em vista disso, um estudo pormenorizado dos mecanismos envolvendo as alterações do $Ca_v2.2$ em camundongos BACHD em muito poderia contribuir para a elucidação das alterações da neurotransmissão e da plasticidade sináptica observadas na DH. O trabalho apresentado no Apêndice 1 deixa claro que camundongos BACHD aos 3 meses de idade apresentam um aumento da expressão de $Ca_v2.2$ na membrana plasmática e um

aumento das correntes de Ca^{2+}. Além disso, a Htt mutante parece aumentar os níveis de interação entre $Ca_v2.2$ e sintaxina 1A e diminuir a interação do canal com as subunidades βγ da proteína G. Tanto o aumento da expressão do $Ca_v2.2$ na membrana plasmática quanto a diminuição da interação do canal com as subunidades βγ da proteína G poderiam explicar a redução das correntes de Ca^{2+} via $Ca_v2.2$ e, em consequência, o aumento da liberação de glutamato observado nesses camundongos jovens. Interessantemente, os dados desse trabalho indicam que em camundongos BACHD com 12 meses de idade, ocorre uma diminuição da liberação de glutamato e uma redução na expressão total e na membrana plasmática do $Ca_v2.2$. Além disso, observa-se uma diminuição da interação do $Ca_v2.2$ com a sintaxina 1A e um aumento de sua interação com as subunidades βγ da proteína G. Assim, as alterações da função e expressão do $Ca_v2.2$ se alteram com a idade e estão intimamente relacionadas às alterações da liberação de glutamato.

O aumento da liberação de glutamato que ocorre no estriado de camundongos BACHD com 3 meses de idade poderia levar a um aumento da ativação de receptores de glutamato, incluindo NMDARs, os quais poderiam modular a excitabilidade neuronal e a transmissão sináptica, bem como desencadear excitotoxicidade (Parsons and Raymond, 2014, Banerjee *et al.*, 2016). O estriado é a primeira e principal região cerebral afetada na DH e os NMDARs desempenham um papel importante na perda neuronal relacionada à doença (Vonsattel and DiFiglia, 1998, Zeron *et al.*, 2002). Assim, o aumento da liberação de glutamato estriatal observado neste estudo poderia contribuir para a excitotoxicidade mediada por NMDARs. No entanto, como citado anteriormente na introdução, a morte de neurônios do estriado e do córtex só pode ser

detectada em camundongos BACHD com 12 meses de idade (Gray *et al.*, 2008, Doria *et al.*, 2015). Apesar do aumento da liberação de glutamato aos 3 meses de idade, mostramos no nosso estudo que aos 12 meses de idade camundongos BACHD apresentam uma diminuição da liberação de glutamato. Portanto, é possível que esse aumento da liberação de glutamato aos 3 meses de idade não contribua para a morte neuronal, mas que possa modular outros processos relacionados ao glutamato, incluindo a neurotransmissão. O glutamato é o principal neurotransmissor do cérebro de mamíferos e tem uma função essencial na excitabilidade neuronal (Banerjee *et al.*, 2016). Portanto, uma hipótese que levantamos é a de que esse aumento da liberação de glutamato em camundongos BACHD jovens possa exercer influência na neurotransmissão, a qual poderia influenciar os sintomas que se apresentam antes mesmo que sejam observados níveis detectáveis de morte neuronal. Experimentos futuros serão importantes para determinar se esse aumento da liberação de glutamato em camundongos BACHD jovens contribui para a evolução da doença ou constitui um mecanismo compensatório presente nas fases iniciais da doença, mas que é perdido em camundongos mais velhos.

Diferentemente do observado no caso do $Ca_v2.2$, houve um aumento da expressão total e na membrana plasmática de $Ca_v1.2$ no córtex de camundongos BACHD nas idades de 3 e 12 meses de idade, quando comparados a camundongos WT. Os dados relacionados ao trabalho sobre o $Ca_v1.2$ constam no **Apêndice 2, *"Alterations on calcium channels in a mouse model of Huntington´s disease and neuroprotection by blockage of* Ca_v*1.2 channel".*** Esses achados são bastante interessantes, tendo em vista que estudos anteriores já haviam demonstrado que a Htt

mutante é capaz de promover alterações nas concentrações intracelulares de Ca^{2+}, envolvendo uma sensibilização dos NMDARs e dos $InsP_3Rs$ e uma desestabilização mitocondrial (Chen *et al.*, 1999, Sun *et al.*, 2001, Panov *et al.*, 2002, Zeron *et al.*, 2002, Tang *et al.*, 2003, Choo *et al.*, 2004, Ribeiro *et al.*, 2010). Esses estudos são de grande relevância, visto que esse aumento da concentração intracelular de Ca^{2+} está intimamente associado à morte neuronal. Assim, os resultados apresentados no Apêndice 2 representam um novo mecanismo através do qual a Htt mutante é capaz de promover um aumento da concentração intracelular de Ca^{2+}. Os resultados apresentandos nesse trabalho demonstram que camundongos BACHD apresentam um aumento da expressão de $Ca_v1.2$ e um aumento das corrente de Ca^{2+} sensíveis à Nifedipina, a qual é um bloqueador seletivo desse canal. Além disso, bloqueadores dos canais $Ca_v1.2$ se mostraram neuroprotetores contra o insulto de glutamato em neurônios BACHD, demonstrando que o aumento da atividade desse canal poderia contribuir para os processos de morte neuronal observados na DH.

Vários dos resultados obtidos nesses trabalhos nos indicam que as alterações relacionadas ao canal $Ca_v1.2$, e não ao $Ca_v2.2$, estão envolvidas em processos de morte neuronal. Como exposto anteriormente, a expressão do $Ca_v2.2$ encontra-se reduzida na idade onde foram detectadas perda neuronal (12 meses de idade). Já a expressão de $Ca_v1.2$ encontra-se aumentada tanto em camundongos jovens quanto em camundongos de 12 meses de idade. Além disso, o bloqueio de canais $Ca_v1.2$ com nifedipina e isradipina se mostrou neuroprotetor. Dessa forma, os nossos dados favorecem a hipótese que bloqueadores de $Ca_v1.2$, mas não de $Ca_v2.2$, constituem drogas potenciais para o tratamento da neurodegeneração que ocorre na DH. Como

descrito na introdução, drogas capazes de bloquear $Ca_v1.2$ já estão sendo testadas em outras doenças neurodegenerativas (E. Ilijic, 2011, Parkinson Study, 2013). Assim, nosso trabalho contribui para incluir a DH nessas pesquisas.

Afim de identificar alterações nas correntes dos canais $Ca_v2.2$ e $Ca_v1.2$, realizamos experimentos de eletrofisiologia utilizando a técnica *Patch clamp* na configuração *Whole cell* em neurônios corticais em cultura primária de embriões BACHD. Os resultados desses experimentos indicam que os neurônios BACHD apresentam maior corrente de Ca^{2+} total comparado aos WT. Utilizando-se da ω-CgTxGVIA e Nifedipina foi possível identificar que correntes $Ca_v2.2$ e $Ca_v1.2$ apresentam-se aumentadas em camundongos BACHD (Apêndices 1 e 2). Entretanto, nem a ω-CgTxGVIA nem a Nifedipina isoladamente foram capazes de eliminar a diferença da corrente de Ca^{2+} observada entre camundongos BACHD e WT. Em vista disso, estudos posteriores serão importantes para determinar se a combinação desses dois compostos seria suficiente para eliminar o aumento da corrente de Ca^{2+} observada em camundongos BACHD. É bem descrito na literatura que os CCSV do subtipo $Ca_v2.1$ também são importantes para a entrada de Ca^{2+}, principalmente em terminais pré-sinápicos (Takahashi and Momiyama, 1993, Regehr and Mintz, 1994, Wheeler *et al.*, 1994; 1996, Iwasaki and Takahashi, 1998, Qian and Noebels, 2001). Assim, estudos posteriores envolvendo esses canais também poderiam ser importantes para elucidar a desregulação da corrente de Ca^{2+} observada em camundongos BACHD.

Outro resultado relevante que pode ser apreciado ao se comparar os resultados obtidos nos trabalhos onde buscamos analisar as alterações de $Ca_v2.2$ (Apêndice 1) e $Ca_v1.2$ (Apêndice 2) na DH diz respeito às diferenças observadas nas

regiões cerebrais analisadas. Nos dois trabalhos, analisamos as regiões do córtex e estriado, as quais estão intimamente relacionadas ao desenvolvimento da DH (Group, 1993, Young, 2003, Li and Li, 2004). A maioria das alterações observadas em relação ao $Ca_v2.2$ ocorreram no estriado. Por exemplo, camundongos BACHD de 3 meses de idade apresentaram diminuição da expressão de $Ca_v2.2$ na membrana plasmática, diminuição da liberação de glutamato e alterações nos níveis de interação do CCSV com as proteínas reguladoras (sintaxina 1A e $\beta\gamma$) quando analisada a região do estriado, mas não do córtex cerebral. Já camundongos BACHD de 12 meses de idade apresentaram tais alteração tanto no estriado quanto no córtex. Interessantemente, as alterações observadas com relação à atividade e expressão de $Ca_v1.2$ ocorreram no córtex de camundongos BACHD, mas não no estriado, tanto no caso de camundongos com 3 quanto com 12 meses de idade. Esses dados deixam claro que a investigação das alterações observadas na DH relativas a CCSV devem levar em conta não só a idade do camundongo, como também a região cerebral analisada.

Como mencionado na introdução, a superestimulação de NDMARs pode levar a excitotoxicidade (Waxman and Lynch, 2005, Parsons and Raymond, 2014). Consequentemente, alterações na expressão e/ou função de NMDARs foram implicadas em várias desordens neurológicas e alterações patológicas, incluindo esquizofrenia, isquemia, DA, DH e DP (Lipton and Rosenberg, 1994, Milnerwood *et al.*, 2010, Wang *et al.*, 2013, Parsons and Raymond, 2014). Também já foi demonstrado que a inibição farmacológica do NMDAR exerce um efeito neuroprotetor em um modelo animal para a DH, utilizando-se cultura primária de neurônios estriatais de camundongos YAC128 (Tang *et al.*, 2005, Shehadeh *et al.*, 2006). Dessa maneira,

drogas capazes de bloquear NMDARs constituem ferramentas importantes com potencial neuroprotetor. Dados anteriores demonstraram que uma toxina obtida a partir da aranha *Phoneutria nigriventer*, a PnTx4-5-5, seria capaz de inibir os NMDARs em cultura de neurônios hipocampais (de Figueiredo *et al.*, 2001). O trabalho constante no **Apêndice 3, "*The Phoneutria nigriventer spider toxin, PnTx4-5-5, promotes neuronal survival by blocking NMDA receptors*",** corroborando esse dado anteriormente publicado, mostra que houve um bloqueio eficiente dos NMDARs pela PnTx4-5-5 em fatias hipocampais de camundongos. Este trabalho mostra ainda que a PnTx4-4-5 exerce efeito neuroprotetor frente à morte induzida por glutamato e por peptídeo β-amilóide em neurônios cortico-estriatais. Além disso, essa toxina também foi eficiente em promover a neuroproteção de neurônios cortico-estriatais de camundongos BACHD contra o insulto glutamatérgico. Sendo assim, esse trabalho traz uma nova opção de bloqueador de NMDARs com ação neuroprotetora. Embora outros bloqueadores de NMDARs existam, nenhuma dessas drogas se mostrou capaz de modificar o curso de doenças neurodegenerativas quando utilizadas em pacientes. Esse é o caso da memantina, que, embora seja uma droga aprovada para o tratamento da DA, não é capaz de parar a progressão da doença e não produz efeitos positivos em longo prazo (Ehret and Chamberlin, 2015, Geldenhuys and Darvesh, 2015). Dessa forma, novas classes de drogas capazes de bloquear NMDARs através de um novo mecanismo são uma necessidade premente para o tratamento de doenças neurodegenerativas. Embora a PnTx4-5-5 também seja um bloqueador de NMDARs, essa toxina tem uma estrutura química completamente diferente da memantina e, portanto, deve promover o bloqueio do receptor e a neuroproteção através de um

mecanismo diferente dos bloqueadores de NMDARs desenvolvidos até o momento. Assim, mais estudos envolvendo PnTx4-5-5 poderiam levar ao desenvolvimento de uma nova classe de drogas capazes de bloquear NMDARs, tendo o potencial de serem mais eficazes em modificar o curso da doença.

4. Conclusão

Os CCSV $Ca_V2.2$ e $Ca_V1.2$ apresentam alterações em sua expressão, em sua localização subcelular e em suas correntes de Ca^{2+} em camundongos BACHD, quando comparado a camundongos selvagens. As alterações envolvendo o $Ca_V2.2$ variam de acordo com a idade e promovem alterações na liberação de glutamato, podendo contribuir para as alterações na neurotransmissão observadas na DH. Já o aumento da expressão e das correntes de Ca^{2+} via $Ca_V1.2$ ocorrem em camundongos de todas as idades, inclusive em camundongos mais velhos, o que poderia contribuir para os processos de excitotoxicidade observados na DH. Interessantemente, bloqueadores tanto de $Ca_V1.2$ (nifedipina e isradipina) quanto de NMDARs (PnTx4-5-5) são capazes de promover neuroproteção.

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Apêndice 1

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N-type Ca^{2+} channels are affected by full-length mutant huntingtin expression in a mouse model of Huntington's disease

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ABSTRACT

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder caused by a polyglutamine expansion in the amino-terminal region of the huntingtin (htt) protein. In addition to facilitating neurodegeneration, mutant htt is implicated in HD-related alterations of neurotransmission. Previous data showed that htt can modulate N-type voltage-gated Ca^{2+} channels ($Ca_V2.2$), which are essential for presynaptic neurotransmitter release. Thus, to elucidate the mechanism underlying mutant htt-mediated alterations in neurotransmission, we investigated how $Ca_V2.2$ is affected by full-length mutant htt expression in a mouse model of HD (BACHD). Our data indicate that young BACHD mice exhibit increased striatal glutamate release, which is reduced to wild type levels following $Ca_V2.2$ block. $Ca_V2.2$ Ca^{2+} current-density and plasma membrane expression are increased in BACHD mice, which could account for increased glutamate release. Moreover, mutant htt affects the interaction between $Ca_V2.2$ and 2 major channel regulators, namely syntaxin 1A and $G_{\beta\gamma}$ protein. Notably, 12-month old BACHD mice exhibit decreased Cav2.2 cell surface expression and glutamate release, suggesting that Cav2.2 alterations vary according to disease stage.

1. Introduction

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder caused by a polyglutamine expansion in the amino-terminal region of the huntingtin (htt) protein (Group, 1993). HD patients exhibit neurodegeneration in the caudate-putamen and neocortical regions of the brain, as well as symptoms such as motor alterations, cognitive decline, psychiatric disturbances, and inevitable death (Piira et al., 2013; Ross and Tabrizi, 2011). Although neurodegeneration is considered the main cause of HD symptoms, cognitive decline can be observed even before detectable levels of neuronal cell loss (Diamond et al., 1992; Hahn-Barma et al., 1998). In addition, a number of reports indicate that alterations in neurotransmission including decreased long-term potentiation and diminished excitatory post-synaptic current frequency can be observed before overt HD-related neuronal cell death (Cepeda et al., 2003; Cha et al., 1998; Klapstein et al., 2001; Usdin et al., 1999). Thus, it is possible that earlier pathological events causing dysfunction of neurotransmission may contribute to phase I HD symptoms. The htt can directly interact with synaptic vesicle proteins and alter vesicle trafficking and exocytosis (DiFiglia et al., 1995; Lievens et al., 2002; Morton et al., 2001). Moreover, an increase in glutamate release has been observed in synaptosomes from 6-month old $Hdh^{140Q/140Q}$ knock-in HD mice (Valencia et al., 2013). Therefore, 1 compelling hypothesis is that HD-related cognitive deficit involves altered synaptic vesicle release. However, the mechanism underlying these alterations in synaptic vesicle release is still unknown.

It has been demonstrated that wild-type htt can interact with and modulate N-type voltage-gated Ca^{2+} channels ($Ca_V2.2$) (Swayne et al., 2005), which are crucial for presynaptic neurotransmitter release (Turner et al., 1993). $Ca_V2.2$ channels are heteromultimers composed of the pore-forming $Ca_V2.2$ subunit, associated with auxiliary $Ca_V\beta$- and $Ca_V\alpha_2\delta$-subunits (Dolphin, 2012; Simms and Zamponi, 2014). The intracellular loop between domains II and III of the $Ca_V2.2$ subunit interacts with presynaptic proteins that are part of the SNARE complex and are important for

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synaptic vesicle exocytosis, including syntaxin 1A, SNAP25 and synaptotagmin (Leveque et al., 1992, 1994; Sheng et al., 1994). Notably, interaction of presynaptic Ca^{2+} channels with SNARE proteins is a Ca^{2+}-sensitive process, which may be important to couple Ca^{2+} influx with fast synaptic vesicle exocytosis (Sheng et al., 1996). In addition, syntaxin 1A causes a negative shift in steady-state inactivation of $Ca_v2.2$ and enhances channel inhibition by G-protein βγ subunits (Bezprozvanny et al., 1995, 2000; Degtiar et al., 2000; Jarvis and Zamponi, 2001; Jarvis et al., 2000; Stanley and Mirotznik, 1997). Interestingly, this effect of syntaxin 1A on N-type Ca^{2+} channels is antagonized by overexpression of htt exon 1, which was shown to compete with syntaxin 1A for binding to the channel and to increase Ca^{2+} currents (Swayne et al., 2005). However, it is not known how N-type Ca^{2+} channels are affected by full-length mutant htt expression in a mouse model of HD and whether it could account for HD-related altered neurotransmission. Our main results in this study indicate that young adult BACHD mice exhibit increased striatal glutamate release, which can be reduced to wild type (WT) levels following N-type Ca^{2+} channel block. $Ca_v2.2$ plasma membrane expression and channel activity are increased in 3-month old BACHD mice, which could account for increased glutamate release. Furthermore, we found evidence that mutated htt can affect the interaction between $Ca_v2.2$ and 2 major channel regulators, syntaxin 1A and the Gβγ protein. Moreover, $Ca_v2.2$ plasma membrane expression levels and glutamate release are decreased in 12-month old BACHD mice. Therefore, we show that Cav2.2 alterations are brain region-specific and vary according to mouse age and, consequently, disease stage.

2. Materials and methods

2.1. Mouse model

FVB/NJ WT and FVB/N-Tg (HTT*97Q) IXwy/J (BACHD) (Gray et al., 2008) were purchased from The Jackson Laboratory. Mice were housed in an animal care facility at 23 °C on a 12-hour light/12-hour dark cycle with food and water provided ad libitum. Forty-three WT and 43 BACHD adult mice and 8 WT and 9 BACHD embryos were used in this study. Animal care was in accordance with the Universidade Federal de Minas Gerais Ethics Committee on Animal Experimentation, CETEA.

2.2. Purification of synaptosomes

Synaptosomal preparations were obtained from the cortex and striatum of 3 or 12-month old WT and BACHD mice. Cortex and striatum were dissected and homogenized 1:10 (w/v) in 0.32 M sucrose solution containing 0.25 mM dithiothreitol and 2 mM ethylenediaminetetraacetic acid (EDTA). Then, homogenates were submitted to low-speed centrifugation (1000 g for 10 minutes) and synaptosomes were purified from the supernatant by discontinuous Percoll-density gradient centrifugation (Dunkley et al., 1988). The isolated nerve terminals were resuspended in Krebs–Ringer 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) solution (124 mM NaCl, 4 mM KCl, 1.2 mM $MgSO_4$, 10 mM glucose, 25 mM HEPES, pH 7.4) with no $CaCl_2$ and kept on ice until measurement of continuous glutamate release.

2.3. Measurement of continuous glutamate release

Glutamate release was assessed by a continuous fluorimetric assay described in the study by Nicholls et al. (1987). Synaptosomes were incubated for 30 minutes, washed with Krebs-Ringer HEPES buffer medium, and transferred to an Eppendorf tube (final synaptosomal concentration of 1 mg/mL) at 37 °C with constant stirring. At the beginning of each assay, $CaCl_2$, $NADP^+$ and glutamate dehydrogenase were added to synaptosomes. Fluorescence emission was recorded using a spectrofluorometer (Synergy 2, BioTek Instruments) at 450 nm and the excitation wavelength was set at 360 nm. Glutamate release was evoked by depolarizing stimuli of 33 mM KCl.

2.4. Neuronal primary culture preparation

Neuronal cultures were prepared from the cortical region of either WT or BACHD E15 mouse embryo brains, as described previously (Doria et al., 2013). After dissection, cortical tissue was subjected to trypsin digestion followed by cell dissociation using a fire-polished Pasteur pipette. Cells were plated on poly-L-ornithine coated dishes in Neurobasal medium (Thermo Scientific) supplemented with N2 and B27 supplements (Thermo Scientific), 2 mM GlutaMAX (Thermo Scientific), 50 μg/mL penicillin, and 50 μg/mL streptomycin (Thermo Scientific). Cells were incubated at 37 °C and 5% CO_2 in a humidified incubator and cultured for 12–15 days in vitro with medium replenishment every 4 days.

2.5. Electrophysiology

Whole-cell voltage clamp recordings were obtained using an EPC-10 patch clamp amplifier (HEKA) at room temperature (22 °C–25 °C). Current recordings were filtered at 2.9 kHz and digitally sampled at 10 kHz. Patch pipette resistance was 2.5–3.0 MΩ and were filled with an internal solution composed of (120 mM CsCl, 1 mM $MgCl_2.6H_2O$, 10 mM HEPES, 10 mM ethylene-bis (oxyethylenenitrilo)tetraacetic acid, 2 mM Mg-adenosine triphosphate, pH 7.2). Primary cultured cortical neurons with series resistance over 8.0 MΩ were excluded from the analysis. During whole-cell experiments neurons were bathed in Tyrode solution (140 mM NaCl, 5.4 mM KCl, 0.5 mM $MgCl_2$, 0.33 mM NaH_2PO_4, 1.8 mM $CaCl_2$, 5 mM HEPES, 11 mM glucose, pH 7.4). After the establishment of the whole-cell configuration, the plate-containing cells were perfused with control solution (130 mM TEA-CL, 2 mM $MgCl_2.6H_2O$, 10 mM $BaCl_2$, 10 mM HEPES, 10 mM Glucose, pH 7.2) for 5 minutes followed by 5 minutes exposure to ω-CTx-GVIA (Sigma-Aldrich) 50 nM added to the same solution. Cortical neurons were hyperpolarized to −100 mV for 50 ms from a holding potential of −80 mV, followed by a ramp protocol from −100 mV to +50 mV at a rate of 1.5 V/s, with a frequency of 0.1 Hz. N-type Ca^{2+} currents were determined by the digital subtraction between Ca^{2+} currents before and after the effects of ω-conotoxin GVIA (ω-CTx-GVIA). Only cells with stable Ca^{2+} current densities (i.e., without noticeable rundown of the current) were used in the analysis.

2.6. Cell surface biotinylation

The striatum of 3- and 12-month old WT and BACHD mice were sliced (300 μm) using a McIlwain tissue chopper. Slices were recovered in artificial cerebrospinal fluid (ACSF) (127 mM NaCl, 2 mM KCl, 10 mM glucose, 1.2 mM KH_2PO_4, 26 mM NaH_2CO_3, 1 mM $MgSO_4$, 1 mM $CaCl_2$, pH 7.4), gassed with 95% O_2/5% CO_2, and incubated in a shaking bath at 37 °C for 30 minutes. Plasma membrane proteins of striatal slices were biotinylated with 1 mg/mL sulfo-NHS-SS-biotin (Thermo Scientific) for 1 hour on ice, as described previously (Ribeiro et al., 2010). To quench the biotinylation reaction, slices were washed and incubated for 30 minutes with cold 100 mM glycine in ACSF, followed by 3 washes with cold ACSF. Slices were then lysed in radioimmunoprecipitation assay buffer (0.15 M NaCl, 0.05 M tris-HCl, pH 7.2, 0.05 M EDTA, 1% Nonidet P40, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate) containing protease inhibitors (1 mM AEBSF and 10 g/mL of both leupeptin and aprotinin, Sigma-Aldrich). Biotinylated proteins were

separated from nonbiotinylated proteins by NeutrAvidin bead (Thermo Scientific) pull-down from equivalent amounts of total cellular protein from each sample. Twenty microliters of supernatant was saved to determine intracellular $Ca_V2.2$ levels. Total $Ca_V2.2$ expression (input) was determined using 100 μg of protein obtained from whole-cell lysate. Proteins were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by electroblotting onto nitrocellulose membranes and immunoblot to determine plasma membrane, intracellular and total $Ca_V2.2$ protein levels.

2.7. Coimmunoprecipitation

The cortex and striatum of 3- and 12-month old BACHD and WT mice were dissected on ice and lysed in ice cold lysis buffer (0.5 M HEPES, 2.5 M NaCl, 0.5 M $MgCl_2$, 0.5 M EDTA, 0.2% Triton X-100, pH 7.4) containing protease inhibitors. Lysates were rotated for 1 hour at 4 °C and centrifuged to pellet insoluble material. Precleared supernatant was incubated for 30 minutes with 1 μL rabbit anti-$Ca_V2.2$ (Merck Millipore) or rabbit anti-htt (DB Biotech) monoclonal antibodies to immunoprecipitate $Ca_V2.2$ or htt, respectively. Following this incubation, 100 μL of freshly washed protein G-sepharose beads (GE Healthcare) were added to lysate/antibody mixture and samples were incubated under rotation for 2 hours at 4 °C. Following incubation, beads were washed twice with phosphate buffered saline. Proteins were subjected to SDS-PAGE, followed by electroblotting onto nitrocellulose membranes.

2.8. Immunoblotting

The cortex and striatum of 3- and 12-month old BACHD and WT mice were dissected and lysed in radioimmunoprecipitation assay buffer containing protease inhibitors. Hundred micrograms of total cellular protein for each sample was subjected to SDS-PAGE, followed by electroblotting onto nitrocellulose membranes. Membranes were blocked with 10% skim milk in wash buffer (150 mM NaCl, 10 mM Tris-HCl, and 0.075% Triton X-100, pH 7.4) for 1 hour and then incubated with rabbit anti-$Ca_V2.2$ (1:200), rabbit anti-Htt (1:1.000), rabbit anti-Gβγ (1:200, Santa Cruz Laboratories), mouse anti-syntaxin 1A (1:1.000, Santa Cruz Laboratories) or mouse anti-βactin (1:1.000, Sigma-Aldrich) antibodies in wash buffer containing 3% skim milk overnight at 4 °C. Membranes were rinsed 3 times for 5 minutes with wash buffer and then incubated with either secondary horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:5.000, BioRad) or secondary horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (1:5.000, Merck Millipore) in wash buffer containing 3% skim milk for 1 hour at room temperature. Membranes were rinsed 3 times for 10 minutes with wash buffer and incubated with enhanced chemiluminescence luminol Prime (GE Healthcare). Nonsaturated, immunoreactive bands were quantified by scanning densitometry using Image Quant LAS software (GE Healthcare). Immuno-band intensity was obtained by ImageJ software. In the case of total cell lysate expression, $Ca_V2.2$ expression was normalized to actin expression levels.

2.9. Data analysis

Means ± standard error of the mean values are shown for the number of independent experiments indicated in the figure legends. GraphPad Prism software was used to analyze data for statistical significance determined by either unpaired *t* test (for comparing 2 groups) or 2-way analysis of variance testing followed by Bonferroni post-hoc multiple comparison testing.

3. Results

3.1. Increased glutamate release in BACHD striatum is abrogated by $Ca_V2.2$ block

It has been demonstrated that htt plays an important role in neurotransmission (DiFiglia et al., 1995; Morton et al., 2001; Valencia et al., 2013). Thus, we investigated whether glutamate release could be altered in BACHD mice, a transgenic mouse model of HD. We submitted cortical and striatal synaptosomes from 3-month old BACHD and WT mice to high potassium depolarization and measured glutamate release levels. Glutamate release by cortical synaptosomes obtained from BACHD mice was not significantly different than that of WT mice (Fig. 1A). However, levels of glutamate release exhibited by synaptosomes obtained from BACHD striatum were higher than those of WT ($t_4 = 3.79$, $p = 0.0006$; Fig. 1A). It has been demonstrated that synaptosomal preparations express high levels of $Ca_V2.2$ and that this Ca^{2+} channel is important for glutamate release from synaptosomes (Gardezi et al., 2010; Turner and Dunlap, 1995). Thus, we determined whether $Ca_V2.2$ channel could be involved in the augmented levels of glutamate release observed in BACHD striatal synaptosomes. We found that increased glutamate release observed in BACHD was abolished when striatal synaptosomes were incubated with the selective $Ca_V2.2$ channel blocker ω-CTx-GVIA, reaching values of glutamate release that were not different when comparing BACHD and WT mice (genotype: $F_{1,6} = 7.31$, $p = 0.0354$; drug: $F_{1,6} = 42.84$,

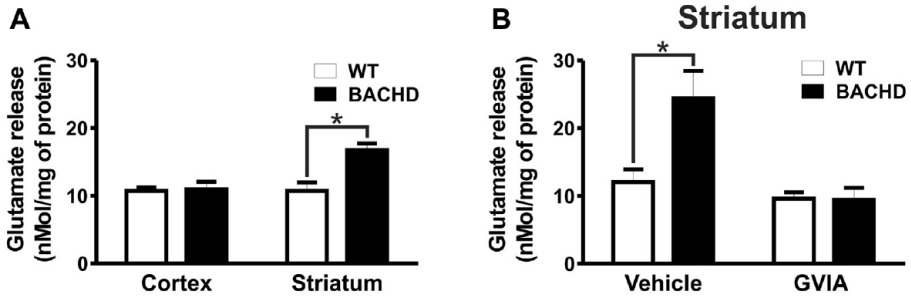

Fig. 1. Increased glutamate release in 3-month old BACHD striatum is abrogated by $Ca_V2.2$ inhibition with ω-CTx-GVIA toxin. (A) Graph shows total glutamate released by high K^+-depolarized synaptosomes from the cortex and striatum of either wild-type (WT) or BACHD mice at 3 months of age. (B) Graph shows total glutamate released by high K^+-depolarized synaptosomes from the striatum of either WT or BACHD mice at 3 months of age in the presence or absence of ω-CTx-GVIA toxin. Data represent the means ± SEM of 3 independent experiments. *Indicates significant difference as compared to WT glutamate release ($p < 0.05$). Abbreviations: $Ca_V2.2$, N-type voltage-gated Ca^{2+} channels; SEM, standard error of the mean; ω-CTx-GVIA, ω-conotoxin GVIA.

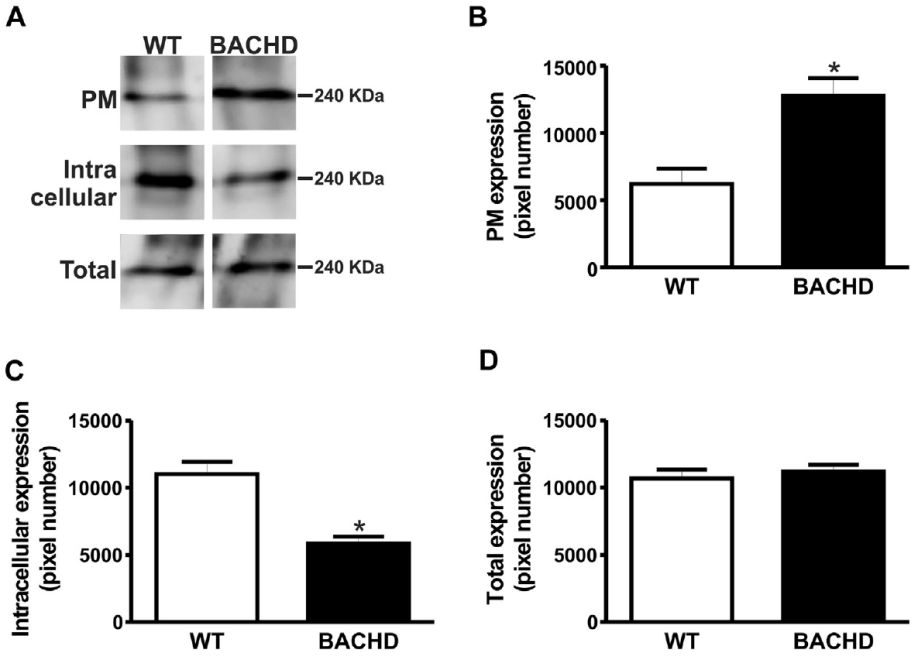

Fig. 2. $Ca_v2.2$ plasma membrane expression is increased in the striatum of 3-month old BACHD mice. (A) Shown are representative immunoblots for $Ca_v2.2$ cell surface (upper panel), intracellular (middle panel), and total cell lysate expression (lower panel) in the striatum of either wild type (WT) or BACHD mice at 3 months of age. Graphs show the densitometric analysis of cell surface (B) intracellular (C) and total cell lysate (D) expression of $Ca_v2.2$ in the striatum of either WT or BACHD mice at 3 months of age. Data represent the mean ± SEM of 4 independent experiments. *Indicate significant differences as compared to WT ($p < 0.05$). Abbreviations: $Ca_v2.2$, N-type voltage-gated Ca^{2+} channels; SEM, standard error of the mean.

$p = 0.0006$; interaction: $F_{1,6} = 8.05$, $p = 0.0297$; Fig. 1B). These data indicate that the increase in glutamate release observed in BACHD striatal synaptosomes is mainly due to $Ca_v2.2$ channels.

3.2. $Ca_v2.2$ plasma membrane expression is increased in the striatum of BACHD mice

To investigate the mechanism underlying the involvement of $Ca_v2.2$ channels in increased glutamate release in BACHD striatum, we evaluated the total and cell surface expression of $Ca_v2.2$ protein in striatal slices from 3-month old BACHD and WT mice. Total cell lysate expression of $Ca_v2.2$ was not different when comparing BACHD and WT striatal slices (Fig. 2A and D). However, plasma membrane expression of $Ca_v2.2$ was increased in BACHD striatal slices, as compared to WT slices ($t_6 = 3.75$, $p = 0.0096$; Fig. 2A and B). The levels of $Ca_v2.2$ present in intracellular compartments were also determined. Levels of intracellular $Ca_v2.2$ protein were significantly decreased in BACHD striatum, as compared to that of WT. Thus, the changes in $Ca_v2.2$ cell surface expression were accompanied by opposing changes in $Ca_v2.2$ intracellular protein levels ($t_6 = 4.96$, $p = 0.0026$; Fig. 2A and C). As most $Ca_v2.2$ are located at synapses (Bahls et al., 1998; Maximov and Bezprozvanny, 2002), this increase in $Ca_v2.2$ plasma membrane expression in BACHD striatal slices may underlie the increase in glutamate release observed in BACHD striatal synaptosomes.

3.3. BACHD neurons display increased $Ca_v2.2$ N-type calcium currents

In order to further address the mechanisms by which BACHD striatum displays increased glutamate release, whole-cell patch clamp experiments were performed to evaluate whether increased plasma membrane expression of Cav2.2 is reflected in an increase in N-type Ca^{2+} current-density. Patch clamp experiments were conducted in primary cultured cortical neurons, as most glutamatergic inputs into the striatum emerges from the cortex and, thus, the increase in glutamate release observed in the striatum is due to increased glutamate released by cortical terminals (Reiner et al., 2010). Fig. 3A display representative records of the current–voltage relationship of WT and BACHD cortical neurons before and after the exposure to ω-CTx-GVIA. BACHD neurons have increased total voltage-gated Ca^{2+} current densities compared to WT neurons. ω-CTx-GVIA could efficiently block $Ca_v2.2$-mediated currents from both WT and BACHD neurons in a fast and irreversible manner, as seen from the time course in representative experiments (Fig. 3B). The N-type Ca^{2+} current was determined by digital subtraction of the total Ca^{2+} current before and after exposure to ω-CTx-GVIA (ω-CTx-GVIA-sensitive current). From Fig. 3C, it is evident that both total Ca^{2+} peak current-density (−12.4 ± 2.4 vs. −31.6 ± 5.7; WT vs. BACHD; $t_{15} = 3.27$, $p = 0.0051$) and N-type Ca^{2+} peak current-density (−5.5 ± 1.0 vs. −11.0 ± 2.1; WT vs. BACHD; $t_{15} = 2.51$, $p = 0.0242$) are augmented in BACHD cortical neurons, compared to WT cortical neurons. These data strongly indicate that there is more $Ca_v2.2$ activity in BACHD cortical neurons, which could underlie increased glutamate release into the striatum. However, as $Ca_v2.2$ block did not completely abrogate the increase in total Ca^{2+} current observe in BACHD neurons, these data also suggest that other Ca^{2+} channels might play a role in HD-related Ca^{2+} current dysregulation.

3.4. Mutated huntingtin affects the interaction between $Ca_v2.2$ and its partners, syntaxin 1A and $G_{\beta\gamma}$

It has been shown previously that htt can interact with $Ca_v2.2$ (Swayne et al., 2005) and we have confirmed that

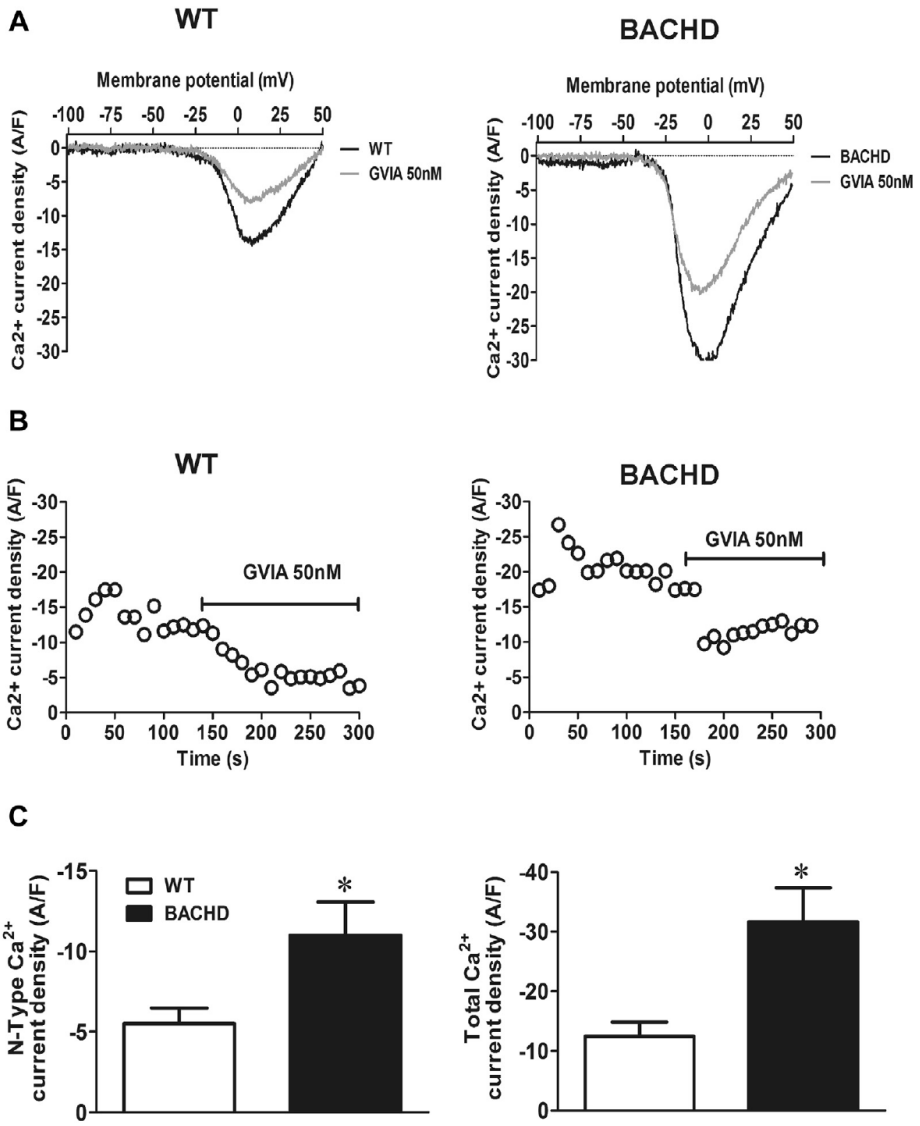

Fig. 3. BACHD cortical neurons display increased voltage-dependent Ca^{2+} currents. (A) Shown are representative records of the Ca^{2+} current–voltage relationship from WT (left panel) and BACHD (right panel) in the absence (black) or after exposure to GVIA 50 nM (gray). (B) Shown are representative time courses of GVIA 50-nM effects on total Ca^{2+} current-density from WT (left) and BACHD (right) cultured neurons. (C) Graphs show peak N-Type Ca^{2+} current-density (left) and total Ca^{2+} current-density (right) from WT ($n = 8$) and BACHD ($n = 9$) mice. *Indicate significant differences as compared to matched WT ($p < 0.05$). Abbreviation: WT, wild type.

immunoprecipitated htt can coimmunoprecipitate $Ca_v2.2$ (Supplementary Fig. 1). To determine whether the interaction between $Ca_v2.2$ and htt could be altered by htt polyglutamine expansion, we immunoprecipitated $Ca_v2.2$ and determined the levels of htt interacting with the channel in striatal and cortical lysates from 3-month old WT and BACHD mice. Similar levels of $Ca_v2.2$ were immunoprecipitated by anti-$Ca_v2.2$ antibody from cortical and striatal lysates from WT and BACHD mice (Fig. 4A). However, the amount of htt protein that was coimmunoprecipitated with $Ca_v2.2$ was reduced in the striatum of BACHD mice, as compared to that of WT mice ($t_4 = 6.97$, $p = 0.0022$; Fig. 4A and B). Moreover, the levels of interaction between htt and $Ca_v2.2$ in the cortex were not different when comparing BACHD and WT mice (Fig. 4A and B). Previously published data indicate that htt and syntaxin 1A bind to the same site in $Ca_v2.2$ channels and that these 2 proteins compete for the interaction with the channel (Swayne et al., 2005). Thus, we determined whether decreased interaction between htt and $Ca_v2.2$ could facilitate syntaxin 1A binding to the Ca^{2+} channel. Supporting this hypothesis, coimmunoprecipitation experiments indicated that more syntaxin 1A proteins interact with $Ca_v2.2$ in BACHD mouse striatum ($t_4 = 3.36$, $p = 0.0283$; Fig. 4C and D). The levels of interaction between $Ca_v2.2$ and syntaxin 1A in the cortex were not different when comparing BACHD and WT mice (Fig. 4C and D). Moreover, syntaxin 1A total lysate expression was not different when comparing BACHD and WT cortex and striatum (Fig. 4C). It has been demonstrated that syntaxin 1A can modulate both the voltage dependence of steady-state inactivation and the inhibition of $Ca_v2.2$ by G-protein $\beta\gamma$ subunits (Jarvis et al., 2000;

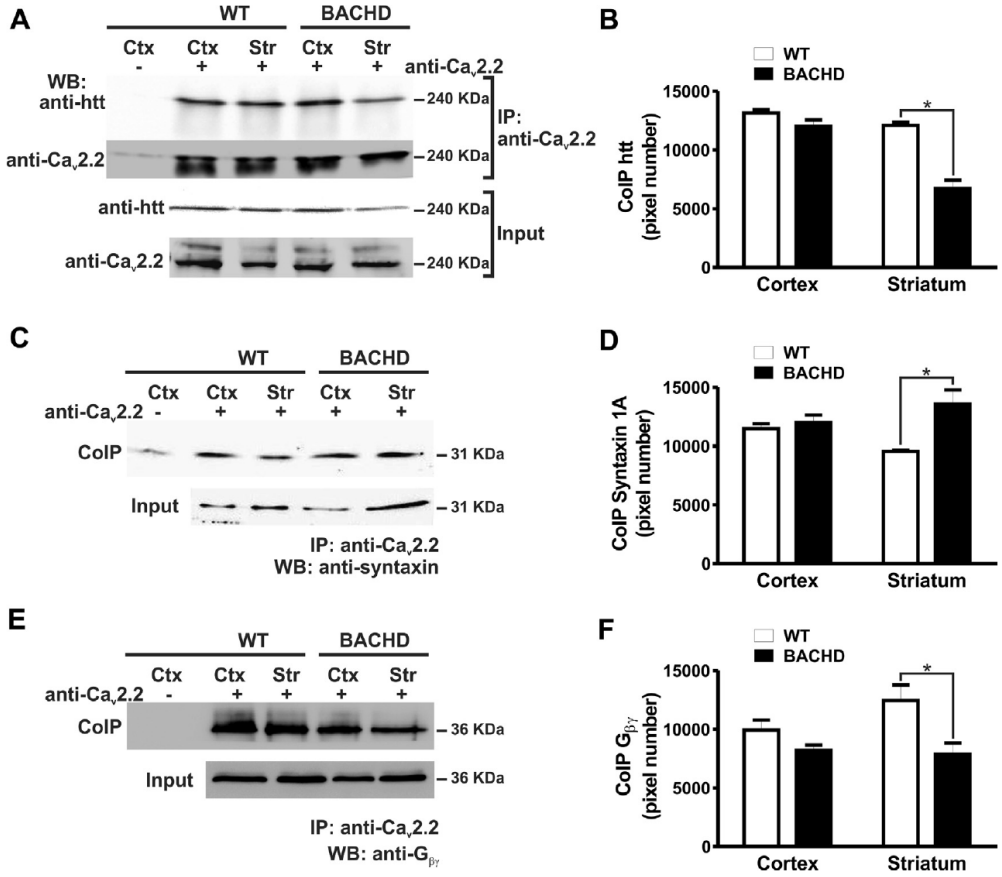

Fig. 4. Mutated huntingtin affects the interaction between $Ca_v2.2$ and its partners, syntaxin 1A and $G_{\beta\gamma}$. (A) Shown are representative immunoblots for coimmunoprecipitation (CoIP) of htt with $Ca_v2.2$ (first panel) and immunoprecipitated $Ca_v2.2$ (second panel) in the cortex and striatum of either wild-type (WT) or BACHD mice at 3 months of age. Lower panels show htt (third panel) and $Ca_v2.2$ (fourth panel) expression in corresponding 100 μg total cell lysate (Input). (B) Graph shows the densitometric analysis of coimmunoprecipitated htt with $Ca_v2.2$ in the cortex and striatum of either WT or BACHD mice at 3 months of age. Upper panels show representative immunoblots for coimmunoprecipitated syntaxin 1A (C) and $G_{\beta\gamma}$ (E) with $Ca_v2.2$ in the cortex and striatum of either WT or BACHD mice at 3 months of age. Lower panels show syntaxin 1A (C) and $G_{\beta\gamma}$ (E) expression in corresponding 100 μg total cell lysate (Input). Graphs show the densitometric analysis of coimmunoprecipitated syntaxin 1A (D) and $G_{\beta\gamma}$ (F) with $Ca_v2.2$ in the cortex and striatum of either WT or BACHD mice at 3 months of age. Data represent the means ± SEM of 3 independent experiments. *Indicate significant differences as compared to matched WT ($p < 0.05$). Abbreviations: $Ca_v2.2$, N-type voltage-gated Ca^{2+} channels; htt, huntingtin; SEM, standard error of the mean.

Sheng et al., 1994). As G-protein βγ subunits interact and modulate $Ca_v2.2$ via a mechanism that can be regulated by syntaxin 1A, we investigated whether $Ca_v2.2$ interaction with Gβγ subunits were altered in BACHD mice. Total cell lysate expression of Gβγ subunits was not different when comparing BACHD and WT cortex and striatum (Fig. 4E). In addition, the levels of Gβγ subunits coimmunoprecipitated with $Ca_v2.2$ in the cortex were not different when comparing BACHD and WT mice (Fig. 4E and F). However, reduced levels of G-protein βγ subunits were coimmunoprecipitated with $Ca_v2.2$ in the striatum of BACHD mice, as compared to that of WT mice ($t_4 = 2.82$, $p = 0.0477$; Fig. 4E and F). These data indicate that mutated htt can affect the interaction between $Ca_v2.2$ and its protein partners and that this effect may contribute to the $Ca_v2.2$ channel activity alterations observed in BACHD neurons.

3.5. *Twelve-month old BACHD mice exhibit decreased expression of $Ca_v2.2$ and reduced glutamate release*

Increased glutamate release, as well as $Ca_v2.2$ alterations observed in 3-month old BACHD mice could either contribute to the progression of HD-related phenotype or be part of an adaptive response present in the early stages of the disease. To determine whether these alterations were retained during disease progression, we investigated $Ca_v2.2$ total and plasma membrane expression, as well as $Ca_v2.2$ interaction with its protein partners, htt, syntaxin 1A and G-protein βγ subunits, in 12-month old BACHD and WT mice. To determine channel expression levels, total cell lysate $Ca_v2.2$ expression was normalized to actin expression to account for the neuronal cell loss that takes place in 12-month old BACHD mice (Doria et al., 2015). Although $Ca_v2.2$ expression was not different when comparing 3-month old BACHD and WT mice (Fig. 5A and B), channel expression was reduced in both the cortex ($t_{10} = 2.32$, $p = 0.0425$) and striatum ($t_{10} = 3.82$, $p = 0.0032$) of 12-month old BACHD mice, as compared to age matched control mice (Fig. 5C and D). This decrease in $Ca_v2.2$ total cellular expression ($t_4 = 22.88$, $p < 0.0001$; Fig. 6A and D) led to decreased levels of the channel at the plasma membrane ($t_4 = 2.86$, $p = 0.0458$; Fig. 6A and B) and in intracellular compartments ($t_4 = 4.872$, $p = 0.0082$; Fig. 6A and C) of BACHD striatal slices, as compared to those of WT slices. In addition, the amount of $Ca_v2.2$ protein that was

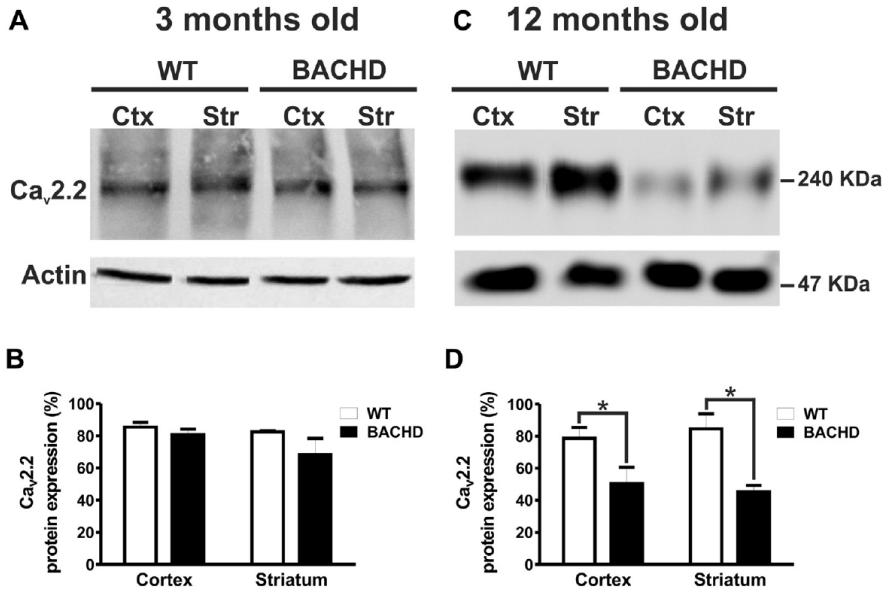

Fig. 5. $Ca_v2.2$ expression is decreased in 12-month old BACHD mice. Shown are representative immunoblots for $Ca_v2.2$ (upper panel) and actin (lower panel) expression in the cortex and striatum of either wild-type (WT) or BACHD mice at 3 months of age (A) and 12 months of age (C). Hundred micrograms of total cell lysate was used for each sample. Graphs show the densitometric analysis of $Ca_v2.2$ expression normalized to actin expression in the cortex and striatum of either WT or BACHD mice at 3 months of age (B) and 12 months of age (D). Data represent the means ± SEM of 4–6 independent experiments, expressed as percentage of actin expression. *Indicates significant difference as compared to WT $Ca_v2.2$ expression ($p < 0.05$). Abbreviations: $Ca_v2.2$, N-type voltage-gated Ca^{2+} channels; SEM, standard error of the mean.

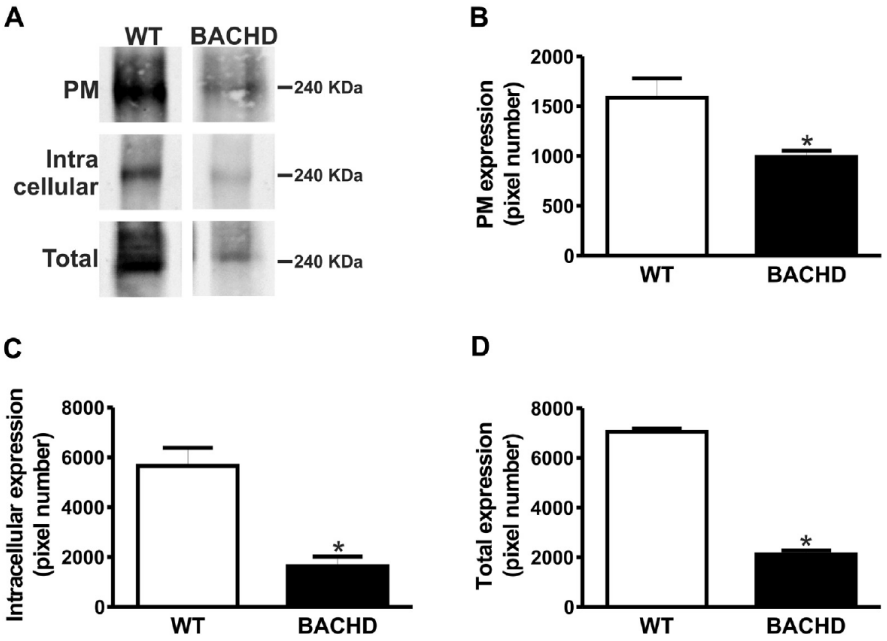

Fig. 6. $Ca_v2.2$ plasma membrane expression is decreased in the striatum of 12-month old BACHD mice. (A) Shown are representative immunoblots for $Ca_v2.2$ cell surface (upper panel), intracellular (middle panel), and total cell lysate expression (lower panel) in the striatum of either wild-type (WT) or BACHD mice at 12 months of age. Graphs show the densitometric analysis of cell surface (B) intracellular (C) and total cell lysate (D) expression of $Ca_v2.2$ in the striatum of either WT or BACHD mice at 12 months of age. Data represent the mean ± SEM of 3 independent experiments. *Indicate significant differences as compared to WT ($p < 0.05$). Abbreviations: $Ca_v2.2$, N-type voltage-gated Ca^{2+} channels; SEM, standard error of the mean.

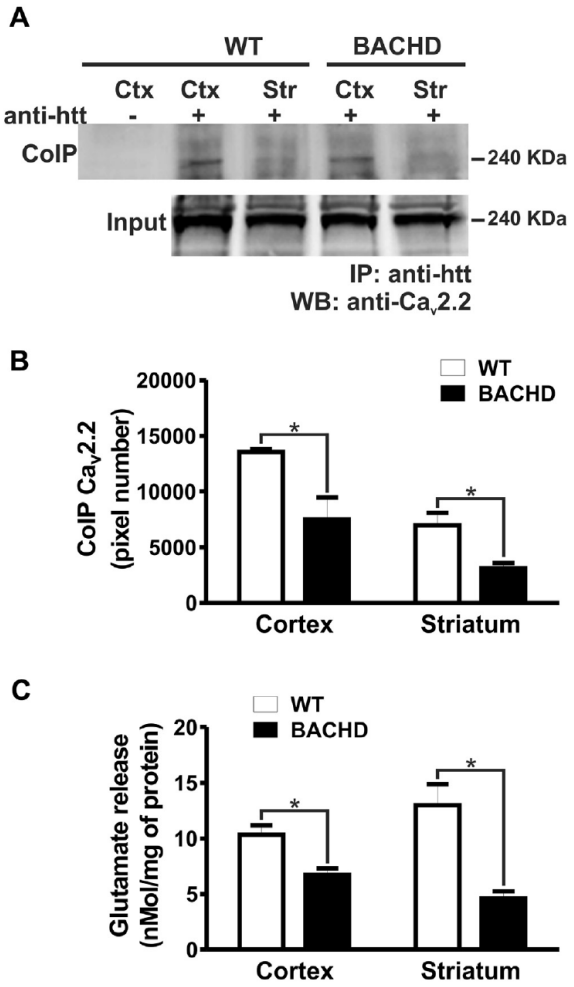

Fig. 7. Interaction between $Ca_v2.2$ and htt is reduced and glutamate release is decreased in 12-month old BACHD striatum and cortex. (A) Upper panel shows representative immunoblots for coimmunoprecipitation (CoIP) of $Ca_v2.2$ with htt in the cortex and striatum of either wild-type (WT) or BACHD mice at 12 months of age. Lower panel shows $Ca_v2.2$ expression in corresponding 100 μg total cell lysate (Input). (B) Graph shows the densitometric analysis of coimmunoprecipitated htt with $Ca_v2.2$ in the cortex and striatum of either WT or BACHD mice at 12 months of age. Data represent the means ± SEM of 3 independent experiments. *Indicate significant differences as compared to WT coimmunoprecipitated $Ca_v2.2$ ($p < 0.05$). (C) Graph shows total glutamate released by high K^+-depolarized synaptosomes from the striatum and cortex of either wild-type (WT) or BACHD mice at 12 months of age. Data represent the means ± SEM of 3 independent experiments. *Indicates significant difference as compared to WT glutamate release ($p < 0.05$). Abbreviations: $Ca_v2.2$, N-type voltage-gated Ca^{2+} channels; htt, huntingtin; SEM, standard error of the mean.

coimmunoprecipitated with htt was reduced in both cortex ($t_4 = 3.04$, $p = 0.0385$) and striatum ($t_4 = 3.15$, $p = 0.0345$) of 12-month old BACHD mice, as compared to that of WT mice (Fig. 7A and B). As htt can compete with syntaxin 1A for $Ca_v2.2$ binding, we tested whether $Ca_v2.2$ could be interacting more with syntaxin in 12-month old BACHD mice. However, because $Ca_v2.2$ protein levels in 12-month old BACHD mice were much reduced (Fig. 5C and D), the amount of syntaxin 1A that was coimmunoprecipitated with the channel was also decreased in both the cortex ($t_4 = 5.84$, $p = 0.0043$) and striatum ($t_4 = 3.39$, $p = 0.0276$) of 12-month old BACHD mice, as compared to age matched WT mice (Supplementary Fig. 2A and B). Interestingly, despite of $Ca_v2.2$ low expression levels, the interaction between $Ca_v2.2$ and Gβγ subunits was augmented in the striatum of 12-month old BACHD mice, as compared to WT animals ($t_4 = 4.15$, $p = 0.0143$; Supplementary Fig. 2C and D). There was no difference in $Ca_v2.2$–Gβγ interaction levels in the cortex of BACHD and WT mice (Supplementary Fig. 2C and D). As $Ca_v2.2$ expression levels at the plasma membrane were decreased and its interaction with Gβγ was increased, we tested whether glutamate release would be altered in 12-month old BACHD mice. Corroborating this hypothesis, levels of glutamate released by synaptosomes from the cortex ($t_4 = 3.58$, $p = 0.0034$) and striatum ($t_4 = 4.07$, $p = 0.0005$) of 12-month old BACHD mice were decreased when compared to that of age matched WT mice (Fig. 7C). Thus, $Ca_v2.2$-related changes are sensitive to mouse age and, thus, disease stage.

4. Discussion

Following on previous reports (Lee et al., 2013; Valencia et al., 2013), the results presented here demonstrate that glutamate release is altered in synaptosomes obtained from BACHD mice. It has been proposed that increased glutamate release could occur as a result of htt-dependent impairment in glutamate uptake (Behrens et al., 2002; Lievens et al., 2001). Moreover, an increase in pyruvate carboxylase protein levels in BACHD astrocytes, which could facilitate glutamate synthesis, has also been proposed as the cause of increased glutamate release (Lee et al., 2013). Nevertheless, our data indicate that $Ca_v2.2$ is likely to be implicated in the alterations of glutamate release observed in HD mouse models, as the increase in glutamate release observed in 3-month old BACHD mice was abolished by blocking N-type Ca^{2+} channels. Moreover, the decrease in $Ca_v2.2$ expression observed in 12-month old BACHD mice was paralleled by decreased glutamate release. Thus, it is possible that different events triggered by mutated htt could contribute to these alterations in glutamate release, including $Ca_v2.2$ dysfunction. HD-related symptoms appear even before detectable levels of neuronal cell loss and altered neurotransmission is pointed as the main feature underlying these early stage symptoms (Diamond et al., 1992; Hahn-Barma et al., 1998). Notably, $Ca_v2.2$ cellular distribution and current-density, as well as its interaction with protein partners, are altered in 3-month old BACHD mice, indicating that this Ca^{2+} channel may have an important role in early stage HD progression.

It has been shown previously that htt interacts with $Ca_v2.2$ through the synaptic protein interaction (synprint) region present in the II–III linker domain of the N-type Ca^{2+} channel (Swayne et al., 2005). Moreover, Swayne et al. (2005) also demonstrated that both wild type and polyglutamine expanded htt amino-terminal fragments bind to $Ca_v2.2$ to the same extent (Swayne et al., 2005). However, our data demonstrate that the amount of htt that binds to $Ca_v2.2$ is diminished in BACHD mice. It is important to note that we examined the interaction between $Ca_v2.2$ and full-length htt in a native system, whereas Swayne et al. (2005) performed an in vitro binding assay using only the amino-terminal fragment of the htt protein and the synprint $Ca_v2.2$ region, which may account for the contradictory results. Importantly, htt has been shown to bind to the same $Ca_v2.2$ synprint region that is involved in syntaxin 1A binding and that the 2 proteins compete for $Ca_v2.2$ interaction (Swayne et al., 2005). Our data corroborate these results, as the decrease in $Ca_v2.2$–htt interaction observed in 3-month old BACHD mice was accompanied by increased $Ca_v2.2$–syntaxin 1A interaction. As syntaxin 1A can facilitate channel inactivation and decrease Cav2.2 currents (Stanley and Mirotznik, 1997), it is possible that htt may control $Ca_v2.2$ activity

by competing with syntaxin 1A for $Ca_v2.2$ binding. As these interactions are dependent on the polyglutamine expansion of htt (Swayne et al., 2005), this may provide a mechanism by which htt dysfunction alters N-type Ca^{2+} channel function. However, although htt interacts less with $Ca_v2.2$ in 12-month old BACHD mice, the interaction between $Ca_v2.2$ and syntaxin 1A is decreased. We hypothesize that although a decrease in the interaction between htt and $Ca_v2.2$ could facilitate $Ca_v2.2$-syntaxin 1A binding, the much reduced expression levels of $Ca_v2.2$ in 12-month old BACHD mice nullifies the augment in $Ca_v2.2$–syntaxin 1A interaction. In fact, because $Ca_v2.2$ levels were so reduced, $Ca_v2.2$–syntaxin 1A interaction was decreased in aged BACHD mice. Thus, mutant htt can regulate $Ca_v2.2$ in many levels by altering channel expression, modifying $Ca_v2.2$ interaction with its protein partners and changing channels activity. Importantly, htt-dependent $Ca_v2.2$ alterations are age dependent.

The increase in $Ca_v2.2$–syntaxin 1A interaction can facilitate the inactivation of N-type Ca^{2+} channels (Bezprozvanny et al., 1995), which would lead to decreased Ca^{2+} current and glutamate release. A direct effect of syntaxin 1A on N-type Ca^{2+} channels has been demonstrated, although channel inhibition by syntaxin 1A also depends on G-protein βγ subunits (Bezprozvanny et al., 1995, 2000; Degtiar et al., 2000; Jarvis and Zamponi, 2001; Jarvis et al., 2000; Stanley and Mirotznik, 1997). Gβγ subunits can directly interact with N-type Ca^{2+} channels through the $Ca_v2.2$ I–II linker domain, amino-terminal and carboxyl-terminal regions, triggering voltage-dependent inhibition of the channel (Agler et al., 2003; Herlitze et al., 1997; Qin et al., 1997; Zamponi et al., 1997; Zhang et al., 1996). Syntaxin 1A can simultaneously bind to $Ca_v2.2$ and Gβγ subunits (Jarvis et al., 2000). Based on that, it has been proposed that syntaxin 1A may facilitate Gβγ-dependent inhibition of N-type Ca^{2+} channels by enabling the colocalization and consequent interaction between Gβγ subunits and $Ca_v2.2$. However, our data indicate that although $Ca_v2.2$ interacts more strongly with syntaxin 1A, the interaction between $Ca_v2.2$ and Gβγ subunits is diminished in 3-month old BACHD mice. Moreover, decreased interaction between $Ca_v2.2$ and syntaxin 1A in 12-month old BACHD mice is accompanied by an increase in $Ca_v2.2$-Gβγ binding. We hypothesize that htt might influence $Ca_v2.2$–Gβγ interaction independently of syntaxin 1A and/or syntaxin 1A might actually disturb $Ca_v2.2$–Gβγ interaction. We also show in this study that htt alters $Ca_v2.2$ cellular trafficking, increasing channel cell surface expression in young BACHD striatum, which could affect the interaction between N-type Ca^{2+} channel and its partners, including syntaxin 1A and Gβγ. Further experiments will be needed to clarify this point. Importantly, the decrease in $Ca_v2.2$–Gβγ interaction in 3-month old BACHD mice could lessen N-type Ca^{2+} channel inhibition, contributing to the observed increase in Ca^{2+} currents and glutamate release. Similarly, increased interaction between $Ca_v2.2$ and Gβγ subunits in 12-month old BACHD mice could contribute to the decrease in glutamate release observed in this HD mouse model. Moreover, the increase in $Ca_v2.2$ plasma membrane expression detected in 3 months and the decrease in cell surface $Ca_v2.2$ observed in 12-month old BACHD striatum may also be key events contributing for the regulation of Ca^{2+} currents and glutamate release.

The increase in glutamate release that takes place in the striatum of 3-month old BACHD mice can lead to activation of glutamate receptors, including N-methyl-D-aspartate receptors (NMDARs), which could modulate neuronal excitability and synaptic transmission, as well as trigger excitotoxicity (Banerjee et al., 2016; Parsons and Raymond, 2014). The striatum is the first and primarily affected brain region in HD, and NMDARs play an important role in HD-related neuronal cell loss (Vonsattel and DiFiglia, 1998; Zeron et al., 2002). Thus, the increase in striatal glutamate release observed in this study could contribute to NMDAR-mediated excitotoxicity. However, neuronal cell loss in the striatum and cortex is only detected in 12-month old BACHD mice (Doria et al., 2015; Gray et al., 2008). Moreover, we show here that glutamate release was decreased in the striatum and cortex of 12-month old BACHD mice. Therefore, it is possible that this increase in glutamate release observed at 3 months of age may not effectively contribute to neuronal cell loss, but modulate other glutamate-related processes, including neurotransmission. Glutamate is the main excitatory neurotransmitter in the brain of mammals and has a key role in neuronal excitability (Banerjee et al., 2016). Thus, one possibility is that this increase in glutamate may influence neurotransmission in young BACHD mice, which could impact HD-related symptoms that appear before overt neuronal cell loss. Future experiments will be important to determine whether increased glutamate release in young adult BACHD mice could either contribute to disease progression or constitute a compensating mechanism present in the early phase of the disease and that is lost in older mice.

In conclusion, the results presented in this study strongly indicate that N-type Ca^{2+} channels are involved in the dysregulation of glutamate release observed in BACHD mice. Moreover, mutated htt protein can influence $Ca_v2.2$ interaction with its protein partners and alter plasma membrane expression of the channel, leading to channel current alterations. Notably, $Ca_v2.2$ blockade by ω-CTx-GVIA does not completely abolish the increase in Ca^{2+} current observed in BACHD neurons, indicating that other Ca^{2+} channels might also play a role in Ca^{2+} current dysregulation in HD. Preliminary data from our group indicate that $Ca_v1.2$ also plays a role in HD-mediated Ca^{2+} current dysregulation (unpublished data). Young adult BACHD mice exhibit increased striatal glutamate release, which can be reduced to WT levels following N-type Ca^{2+} channels block. However, 12-month old BACHD mice exhibit decreased $Ca_v2.2$ expression and decreased glutamate release in the cortex and striatum. Thus, $Ca_v2.2$ alterations are brain region-specific and vary according to mouse age and, consequently, disease stage.

Disclosure statement

The authors have no actual or potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neurobiolaging.2017.03.015.

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Apêndice 2
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Alterations on calcium channels in a mouse model of Huntington´s disease and neuroprotection by blockage of $Ca_V1.2$ channel

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Abstract

Huntington's disease (HD) is a neurodegenerative autosomal dominant disorder, characterized by symptoms of involuntary movement of the body, loss of cognitive function, psychiatric disorder, leading inevitably to death. Disturbances in Ca^{2+} homeostasis contribute to the neurodegenerative process observed in many neurodegenerative diseases. It has been previously described that higher levels of brain expression of $Ca_v1.2$ channel are involved in some neurodegenerative disorders, such as Alzheimer´s and Parkinson´s disease. Our results demonstrate that BACHD mice at the ages of 3 and 12 months exhibit decreased $Ca_v1.2$ total cellular expression in the cortical area. Furthermore, young BACHD mice present higher levels of plasma membrane expression of $Ca_v1.2$ in the cortex and striatum. Electrophysiological analyses confirm a significantly increase in L-type Ca^{2+} currents and also in total Ca^{2+} current density in cortical neurons. By using selective $Ca_v1.2$ antagonists, we were able to promote neuronal survival against insult of glutamate in BACHD neurons without promoting alterations on WT mice. According to our data, $Ca_v1.2$ blockers may be an interesting tool for the treatment of HD. Altogether, our results show that full-length mutant Htt expression may cause a dysregulation of L-type Ca^{2+} channels and we hypothesize that this contributes to neurodegeneration during HD.

Introduction

Huntington's Disease (HD) is a progressive neurodegenerative disorder characterized by symptoms that include involuntary movement of the body, loss of cognitive function, psychiatric disorder and inevitable death [1]. HD is an autosomal dominant disease caused by poly-glutamine expansion in a protein named huntingtin (Htt), leading to aggregate formation, as in a typical case of protein misfolding [2]. However, the molecular mechanisms linking Htt mutation and neuronal cell death have not yet been fully elucidated [3]. The development of HD is determined by the progressive neuronal cell death occurring in the neocortex and striatum of affected patients [1]. It has been demonstrated that the neuronal cell loss that takes place in HD is due to glutamatergic excitotoxicity, which is mediated by excessive influx of intracellular Ca^{2+} [4, 5]. Several studies indicate that mutated Htt promotes Ca^{2+} signaling alterations, which might be closely associated with the death of striatal neurons [6-9]. It has been described that high levels of intracellular Ca^{2+} produce abnormalities in the CNS that may be associated with voltage-gated Ca^{2+} channels [10-15]. In disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), epilepsy and ischemia, alterations on voltage-gated Ca^{2+} channels channels may play an important part [12, 14, 16-18]. Furthermore, studies demonstrated that high levels of $Ca_v1.2$ channel expression in the brain are involved in AD [19] and additional data showed that isradipine, a selective L-type Ca^{2+} channel blocker, offers neuroprotection in a mouse model of PD [20].

Our results show that $Ca_v1.2$ total cellular expression is increased in the cortex of BACHD at 3 and 12 months of age. Although $Ca_v1.2$ total expression in the striatum is not different when comparing BACHD and WT neurons, plasma membrane expression of $Ca_v1.2$ is increased in both cortex and striatum of 3 month old BACHD mice.

Furthermore, whole cell electrophysiology recordings from cortical BACHD neurons show an increase in L-type Ca^{2+} currents and in Ca^{2+} current density, as compared to WT. In addition to that, we performed a concentration-response assay in WT and BACHD cultured neurons using L-type Ca^{2+} channel blockers: nifedipine, isradipine and measured neuronal cell death following glutamate insult. Interestingly, our data indicate that after applying the tested $Ca_v1.2$ channel blockers, there was a decrease in glutamate-induced neuronal cell death in both WT and BACHD neurons. Altogether, these data constitute a previously unrecognized mechanism that may contribute to the pathogenesis of HD. Therefore, we focus on the role of selective L-type Ca^{2+} channel blockers as therapeutic tools for promoting neuroprotection in a mouse model of HD, BACHD.

Results

BACHD mice exhibit increased cortical expression of $Ca_v1.2$

It has been demonstrated that $Ca_v1.2$ expression is increased in some neurodegenerative disorders, including AD and PD [21-23]. Thus, we investigated whether $Ca_v1.2$ expression is altered in BACHD mice, a transgenic mouse model expressing full length human Htt [24]. For that, we performed western blotting experiments to measure $Ca_v1.2$ protein expression in total cell lysates. Our results showed a significantly increase in $Ca_v1.2$ expression in the cortex, but not in the hippocampus or striatum, of 3 month old BACHD, as compared to littermates WT mice (t_6=3.393 p=0.0146, Fig. 1C). Moreover, $Ca_v1.2$ expression in 12 month old BACHD mice is significantly increased in the cortex, but not in the hippocampus or striatum, as compared to WT mice (t_6=6.297, p= 0.0007, Fig. 1D).

$Ca_v1.2$ plasma membrane expression is augmented in the cortex and striatum of young BACHD mice

To verify if the increase in $Ca_v1.2$ total expression was followed by an alteration in the plasma membrane expression of this channel, we decided to use cell surface biotinylation labeling technique to evaluate plasma membrane expression of $Ca_v1.2$ protein in cortical and striatal slices from 3 month old BACHD and WT mice. Plasma membrane expression of $Ca_v1.2$ was significantly increased in both cortical (t_6=5.793, p=0.0012) and striatal slices (t_6=2.495, p=0.0468) from BACHD, as compared to WT slices (Fig. 2B). Levels of $Ca_v1.2$ present in intracellular compartments were also determined and no difference between BACHD and WT was observed (Fig. 2C). Total cell lysate expression of $Ca_v1.2$ was higher in BACHD when compared to WT in cortical slices (t_6=3.011, p= 0.0237, Fig. 2D). This increment in $Ca_v1.2$ plasma membrane expression may contribute to augmented intracellular Ca^{2+} levels, which is observed in most neurodegenerative diseases, as well as in HD.

BACHD neurons display increased L-type Ca^{2+} currents

Whole-cell patch clamp experiments were conducted in primary cultured cortical neurons to evaluate whether increased plasma membrane expression of $Ca_v1.2$ is reflected in an augment in the L-type Ca^{2+} current density. L-type Ca^{2+} current was determined by digital subtraction of the total Ca^{2+} current before and after exposure to nifedipine (nifedipine-sensitive current). Fig. 3A display representative records of the current-voltage relationship of WT (left panel) and BACHD (right panel) cortical neurons before and after the exposure to 5 μM nifedipine. It is evident that both total Ca^{2+} peak current density (-31.74 ± 5.4 pA/pF vs. -54.44 ± 8.3 pA/pF; WT vs. BACHD, Fig. 3B) and L-type Ca^{2+} peak current density (-10.05 ± 1.7 pA/pF vs -20.8 ± 2.9

pA/pF; WT vs BACHD, Fig. 3C) are augmented in BACHD cortical neurons, as compared to WT cortical neurons. Nifedipine was efficient to decrease $Ca_v1.2$-mediated currents from both WT and BACHD neurons (Fig. 3D). Altogether, these data strongly indicate that there is more $Ca_v1.2$ activity in BACHD cortical neurons, which per se could underlie increased intracellular Ca^{2+} levels and, consequently, cell death. Notably, as $Ca_v1.2$ blockage did not completely abolish the increase in total Ca^{2+} current observed in BACHD neurons, these data also suggest that other voltage-gated Ca^{2+} channels might play a part in the Ca^{2+} current alterations observed in HD.

Selective L-type Ca^{2+} channel blockers can protect against glutamate-induced neuronal cell death in BACHD cultured neurons

To test whether the antagonism of $Ca_v1.2$ channel could be efficient to promote neuroprotection in HD, we performed an *in vitro* assay to measure neuronal cell death. Primary cultured corticostriatal neurons from C57/BL6, FVB/NJ (wild type) or BACHD mouse embryos exposed to 50 μM glutamate for 20 h exhibited high level of neuronal cell death (Fig. 4). In order to establish a concentration-response curve, C57/BL6 neurons were incubated with glutamate in the presence of increasing concentrations (0.1, 1 and 10 nM) of nifedipine (Fig. 4A) or israpidine (Fig. 4B), and, as observed, neuronal cell death was significantly decreased. Interestingly, nifedipine at 10 nM was not efficient to promote neuroprotection. In fact, 10 nM nifedipine triggered neuronal cell death even in the absence of glutamate (Fig. 4A). Thus, we settled to perform more experiments using isradipine. To test whether this $Ca_v1.2$ channel blocker could also be efficient to protect neurons expressing mutant Htt, BACHD corticostriatal neurons were also tested and compared to WT neurons (Fig. 4C and 4D). BACHD neurons exhibited increased basal neuronal cell death levels, as compared to WT neurons (Fig. 4C and 4D). Moreover, BACHD neurons were also more susceptible to glutamate insult than

WT neurons (Fig. 4C and 4D). Notably, this excitotoxic effect of glutamate was blocked by 1 nM isradipine in both WT and BACHD neurons (Fig. 4C, 4D). Moreover, 1 nM isradipine diminished cell death induced by glutamate to the same levels as those of untreated neurons (Fig. 4C and 4D). Overall, these data pinpoint that L-type Ca^{2+} channel blockers are capable of preventing the death of WT and BAHCD primary cultured neurons in response to glutamate insult.

Discussion

Ca^{2+} fluxes across the plasma membrane and between intracellular compartments play important roles in neuronal function, including cell survival, synaptic transmission, plasticity and gene transcription [25]. In most neurodegenerative disorders, Ca^{2+} regulation processes are compromised leading the neuronal cells to suffer from synaptic dysfunction, impairment in plasticity, oxidative stress, apoptosis and death [26, 27]. In HD, it is observed neuronal cell death by glutamatergic excitotoxicity, which is mediated by excessive influx, increasing intracellular Ca^{2+} levels [4, 28]. Several studies indicate that mutated Htt promotes Ca^{2+} signaling alterations, which are closely associated with death of striatal neurons [29, 30]. It has been described that mutated Htt is able to sensitize NMDA receptors, increasing NMDA channel permeability to Ca^{2+} in striatal neurons [28, 31]. Also, mutated Htt protein promotes destabilization of mitochondria, decreasing the ability of this organelle to regulate Ca^{2+} levels [32, 33]. In addition, mutated Htt is skillful at sensitizing the inositol trisphosphate receptor ($InsP_3R$), increasing the release of Ca^{2+} from intracellular stores [34]. Thus, Htt-mediated increased intracellular Ca^{2+} levels play an important role in the neuronal cell death that takes place in HD.

Alterations in voltage-gated Ca^{2+} channels are also implicated in age-related neuronal dysfunctions [21, 23, 35, 36]. Interestingly, a relationship between voltage-gated L-type Ca^{2+} channels and AD has already been demonstrated, as published data indicate that β-amyloid peptide is capable of interacting with voltage-gated L-type Ca^{2+} channels, altering channel activity and promoting an increase in the expression of this Ca^{2+}channel at the plasma membrane [19, 37]. As $Ca_v1.2$ proteins are mostly located postsynaptically at somatodendritic locations, this channel regulates neuronal excitability and are known to be involved in translating synaptic activity into alterations in gene expression and neuronal cell death [38-43]. Our investigation started with the observation that $Ca_v1.2$ total expression is increased in the cortex of 3 and 12 month old BACHD mice. Indeed, plasma membrane expression of $Ca_v1.2$ channel is also increased in 3 month old BACHD mice in both cortical and striatal slices. Moreover, the whole-cell patch clamp data confirm that this augment in plasma membrane expression of $Ca_v1.2$ is reflected in an increase in L-type Ca^{2+} current density. Altogether, these data suggest that the increase in intracellular Ca^{2+} observed in HD may also be related to alterations on $Ca_v1.2$ channel expression and activity. Thus, therapeutic agents aiming to diminish this augment in cytosolic Ca^{2+} levels, by blocking L-type Ca^{2+} currents may play an important role in protecting corticostriatal neurons against Htt-mediated neuronal cell death. Importantly, isradipine and nifedipine present neuroprotective potential by inhibiting voltage-gated L-type Ca^{2+} channels, thereby preventing the exaggerated entrance of Ca^{2+} into the cell, which could promote neuronal cell death by excitotoxicity [44]. Notably, L-type Ca^{2+} channel blockers were also found to have neuroprotective effects against Aβ-induced neuronal apoptosis in cultured rat cortical neurons [45] and from amyloid precursor protein (APP)-induced neurotoxicity in neuroblastoma cells [44]. Epidemiological studies also support the hypothesis that L-

type Ca^{2+} channel blockers are capable of preventing or delaying the progression of AD [46-48]. Isradipine is also a voltage-gated L-type Ca^{2+} channel blocker that is well tolerated by patients and is approved for use in humans for the treatment of hypertension. It has been shown that subcutaneous injections of isradipine (2.5 mg/kg for 7 days) antagonize hippocampal damage and memory deficit caused by hypoxia in rats, indicating that this drug has a neuroprotective effect *in vivo* [49]. A dose of 3 mg/kg of isradipine was also able to protect dopaminergic neurons *in vivo* in a mouse model of PD [20, 50]. Currently, isradipine is being evaluated in clinical trials for the treatment of PD patients [51, 52]. Despite these various studies relating voltage-gated L-type Ca^{2+} channel and neurodegenerative diseases, there are no evidences whether these channels could play a role in the Ca^{2+} alteration that occurs in HD patients. According to our results, the excitotoxic effect caused by high concentrations of glutamate in BACHD cultured neurons was diminished by adding 1 nM isradipine to the culture. Importantly, 1 nM isradipine was able to reduce glutamate-induced neuronal cell death to the same levels as control. As observed in our whole cell patch clamp data, $Ca_v1.2$ blockage did not completely abolish the increase in total Ca^{2+} current observed in BACHD neurons, suggesting that other voltage-gated Ca^{2+} channels might play a role in HD dysfunction. Interestingly, even though more subtypes of Ca^{2+}channels seem to be affected by mutated Htt, the blockage of L-type Ca^{2+} currents by isradipine (0.1, 1 and 10 nM) was sufficient for fulling rescuing glutamate-induced neuronal cell death. Nifedipine was also efficient to rescue neuronal cell death at the concentrations of 0.1 and 1 nM. However, 10 nM nifedipine did not prevent glutamate-induced neuronal cell death and was also neurotoxic even in the absence of glutamate. This difference is very intriguing, as both drugs are 1,4-dihydropyridine (DHP) able to bind to $Ca_v1.2$ and $Ca_v1.3$ binding pockets [53, 54]. $Ca_v1.2$ and $Ca_v1.3$ channels display very similar

pharmacological properties, and they exhibit an overlapping expression pattern in the cardiovascular system, endocrine cells and neurons [40, 55, 56]. Voltage-gated L-type Ca^{2+} channels in mammalian brain neurons have either a $Ca_V1.2$ or a $Ca_V1.3$ pore-forming, but $Ca_v1.3$ pore-forming subunits are much less abundant, albeit $Ca_v1.3$ channel dysfunction has been associated to PD [20, 57, 58]. DHP are commonly used to establish the contribution of L-type Ca^{2+} channels in several neuronal processes, but the efficiency of these blockers depends on membrane potential, channel state, and channel subtype [59, 60]. Interesting data using radioreceptor assay demonstrated that nifedipine shows 3- to 4-fold higher affinity for the $Ca_v1.2$ DHP binding pocket in the native membrane environment, as compared to isradipine [54]. On the other hand, isradipine presented 40 times higher affinity for $Ca_v1.3$ channel than nifedipine [20, 54]. Thus, we hypothesise that selectively antagonizing $Ca_v1.3$ L-type Ca^{2+} channels could provide a better option for preventing neuronal cell loss, causing fewer side effects. Importantly, the role of $Ca_v1.3$ channel in HD needs to be addressed in the future.

In conclusion, these results show that L-type Ca^{2+} channels are affected by mutated full length Htt. Indeed, mutated Htt protein may influence $Ca_v1.2$ plasma membrane expression, leading to increased currents, which may contribute to increased intracellular Ca^{2+} levels and consequent cell death. Our data also suggest that other voltage-gated Ca^{2+} channels may be part of this Ca^{2+} dysregulation, since $Ca_v1.2$ blockade by nifedipine does not completely abrogate the increase in Ca^{2+} current observed in BACHD neurons. Finally, *in vitro* concentration-response assay with selective L-type Ca^{2+} channel blockers were able to rescue glutamate-induced neuronal cell death of BACHD cultured neurons. A better comprehension of the mechanisms involved in HD is important to establish the therapeutic window and prospective treatment procedures. Altogether, this data constitutes a previously unrecognized

mechanism that may contribute for the understanding of HD pathogenesis. Besides, L-type Ca^{2+} channel blockers may be potential therapeutic tools to treat HD.

Methods

Animals: All procedures used in this study were approved and strictly followed the ethical principles of animal experimentation adopted by the Ethic Committee on Animal Use of Federal University of Minas Gerais, and institutionally approved under protocol number 139/2013. Mice were housed in an animal care facility at 23ºC on a 12 h light/12 h dark cycle with food and water provided ad libitum. C57/BL6 mice (25–30 g) were purchased from the animal facility (CEBIO) located at the Universidade Federal de Minas Gerais (UFMG). FVB/NJ wild type (WT) and FVB/N-Tg (Htt*97Q) IXwy/J (BACHD) (Gray et al 2008) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA).

Materials: Neurobasal medium, N2 and B27 supplements and GlutaMAX (50 µg/ml penicillin and 50 µg/ml streptomycin) were purchased from Thermo Fisher Scientific. ECL Luminol Prime, G-Sepharose and Neutravidin beads were purchased from GE Healthcare. Anti-β-actin primary antibody, nifedipine, isradipine, protease inhibitors and all the other reagents were purchased from Sigma-Aldrich.

Neuronal primary culture preparation: Neuronal cultures were prepared from the cortical region of either WT or BACHD of E15 mouse embryo brains, as described previously [61]. After dissection, cortical tissue was submitted to trypsin digestion followed by cell dissociation using a fire-polished Pasteur pipette. Cells were plated on poly-L-ornithine coated dishes in Neurobasal medium supplemented with N2 and B27 supplements, 2 mM GlutaMAX, 50 µg/ml penicillin, and 50 µg/ml streptomycin. Cells

were incubated at 37ºC and 5% CO_2 in a humidified incubator and cultured for 8 to 12 days *in vitro* (DIV) with medium replenishment every 4 days.

Cell death assay: Neurons were pre-incubated for 20 h in the presence or absence of L-type Ca^{2+} channel blockers and/or, 50 µM glutamate, as indicated in the Figure Legend, and cell death was determined by Live/Dead viability assay, as described previously [61]. Briefly, neurons were stained with 2 mM calcein acetoxymethyl ester (AM) and 2 mM ethidium homodimer-1 for 15 min and the fractions of live (calcein AM positive) and dead (ethidium homodimer-1 positive) cells were determined. Neurons were visualized by fluorescence microscopy using Floid Microscope (Life Technologies). Cells were analyzed per well in triplicate using ImageJ™ software. Dead cells were expressed as a percentage of the total number of cells.

Cell surface biotinylation: The cortex and striatum of 3 months old WT and BACHD mice were sliced (300 µm) using a McIlwain tissue chopper. Slices were recovered in ACSF (127 mM NaCl, 2 mM KCl, 10 mM glucose, 1.2 mM KH_2PO_4, 26 mM NaH_2CO_3, 1 mM $MgSO_4$, 1 mM $CaCl_2$, pH 7.4) gassed with 95% O_2/5% CO_2 and incubated in a shaking bath at 37°C for 30 min. Plasma membrane proteins of cortical and striatal slices were biotinylated with 1 mg/mL sulfo-NHS-SS-biotin for 1 h on ice, as described previously [30]. To quench the biotinylation reaction, slices were washed and incubated for 30 min with cold 100 mM glycine in ACSF, followed by 3 washes with cold ACSF. Slices were then lysed in RIPA buffer (0.15 M NaCl, 0.05 M tris-HCl, pH 7.2, 0.05 M EDTA, 1% Nonidet P40, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitors (1 mM AEBSF and 10 g/ml of both leupeptin and aprotinin). Biotinylated proteins were separated from nonbiotinylated proteins by NeutrAvidin bead pull-down from equivalent amounts of total cellular protein from

each sample. 20 µL supernatant was saved to determine intracellular $Ca_v1.2$ levels. Total $Ca_v1.2$ expression (input) was determined using 100 µg of protein obtained from whole cell lysate. Proteins were subjected to SDS-PAGE, followed by electroblotting onto nitrocellulose membranes and immunoblot to determine plasma membrane, intracellular and total $Ca_v1.2$ protein levels.

Immunoblotting: The cortex, hippocampus and striatum of BACHD and WT mice were dissected and lysed in RIPA buffer containing protease inhibitors. 100 µg of total cellular protein for each sample was subjected to SDS-PAGE, followed by electroblotting onto nitrocellulose membranes. Membranes were blocked with 10% skim milk in wash buffer (150 mM NaCl, 10 mM Tris-HCl, and 0.075% Triton X 100, pH 7.4) for 1h and then incubated with rabbit anti-$Ca_v1.2$ (1:200) or mouse anti-βactin (1:1.000) antibodies in wash buffer containing 3% skim milk overnight at 4^0C. Membranes were rinsed three times for five minutes with wash buffer and then incubated with either secondary horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5.000) or secondary horseradish peroxidase-conjugated goat anti-mouse IgG (1:5.000) in wash buffer containing 3% skim milk for 1 h at room temperature. Membranes were rinsed three times for 10 minutes with wash buffer and incubated with ECL luminol Prime. Non-saturated, immunoreactive bands were quantified by scanning densitometry using Image Quant LAS software (GE Healthcare). Immuno-band intensity was obtained by ImageJ™ software. In the case of total cell lysate expression, $Ca_v1.2$ expression was normalized to actin expression levels.

Electrophysiology: Whole-cell voltage clamp recordings were obtained using an EPC-10 patch clamp amplifier (HEKA, Holliston, Massachusetts) at room temperature (22-25°C). Current recordings were filtered at 2.9 kHz and digitally

sampled at 10 kHz. Patch pipette resistance was 2.5 to 3.0 MΩ and were filled with an internal solution composed of (120 mM CsCl, 1 mM $MgCl_2.6H_2O$, 10 mM HEPES, 10 mM EGTA, 2 mM Mg-ATP, pH 7.2). Primary cultured cortical neurons with series resistance over 8.0 MΩ were excluded from the analysis. During whole cell experiments neurons were bathed in Tyrode solution (140 mM NaCl, 5.4 mM KCl, 0.5 mM $MgCl_2$, 0.33 mM NaH_2PO_4, 1.8 mM $CaCl_2$, 5 mM HEPES, 11 mM glucose, pH 7.4). After the establishment of the whole-cell configuration, the plate-containing cells were perfused with control solution (130 mM TEA-Cl, 2 mM $MgCl_2.6H_2O$, 10 mM $BaCl_2$, 10 mM HEPES, 10 mM Glucose, pH 7.2) for 5 min followed by 5 min exposure to nifedipine 5 μM added to the same solution. Cortical neurons were hyperpolarized to –100 mV for 50 ms from a holding potential of –80 mV, followed by a ramp protocol from -100 mV to +50 mV at a rate of 1.5 V/s, with a frequency of 0.1 Hz. L-type Ca^{2+} currents were determined by digital subtraction between Ca^{2+} currents before and after the effects of nifedipine. Only cells with stable Ca^{2+} current densities (i.e. without noticeable rundown of the current) were used in the analysis.

Data analysis: Means ± SEM are shown for the number of independent experiments indicated in Figure Legends. GraphPad Prism™ software was used to analyze data for statistical significance determined by either unpaired t-test (for comparing two groups) or two-way analysis of variance (ANOVA) testing followed by Bonferroni post-hoc multiple comparison testing.

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Author contributions statement

L.B.V and F.M.R designed the study. F.R.S conducted Western Blotting, Biotinylation and cell cultures experiments. Electrophysiology recordings were performed by A.S.M and J.S.C. I.G.O carried out the mouse colony as well genotyping. M.V.G made substantial contributions to conception and design of the study and revised the manuscript critically for important intellectual content. Results were analyzed by L.B.V and F.M.R and the article was written by L.B.V. All other authors revised the data and discussed the manuscript.

Additional Information

Competing Financial Interests

The authors declare that they have no conflicts of interest with respect to this report.

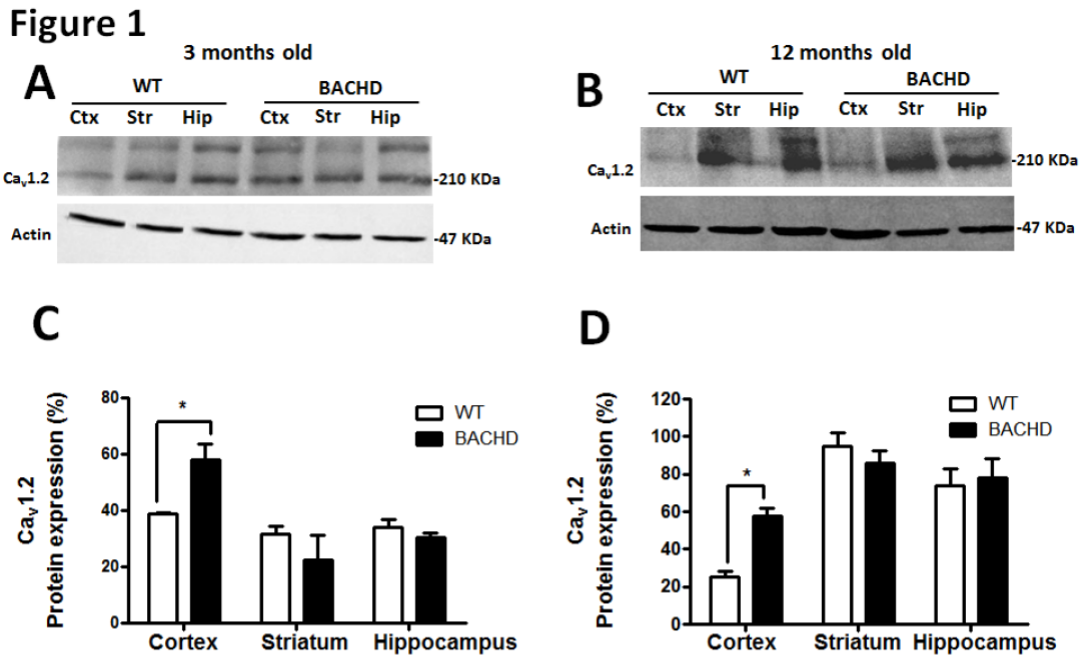

Figure 1: ***$Ca_v1.2$ expression is increased in the cortex of 3 and 12 month old BACHD mice.*** Shown are representative immunoblots for $Ca_v1.2$ (upper panel) and actin (lower panel) expression in the cortex, striatum and hippocampus of either WT or BACHD mice at 3 **(A)** and 12 **(C)** months of age. Graphs show the densitometric analysis of total expression of $Ca_v1.2$ in the cortex, striatum and hippocampus of either WT or BACHD mice at 3 **(B)** and 12 **(D)** months of age. Full-length blots are included in the supplementary information. 100 µg of cell lysate was used for each sample. Data represent the means ± SEM of four independent experiments, expressed as percentage of actin expression. *indicates significant difference as compared to WT $Ca_v1.2$ expression ($p<0.05$).

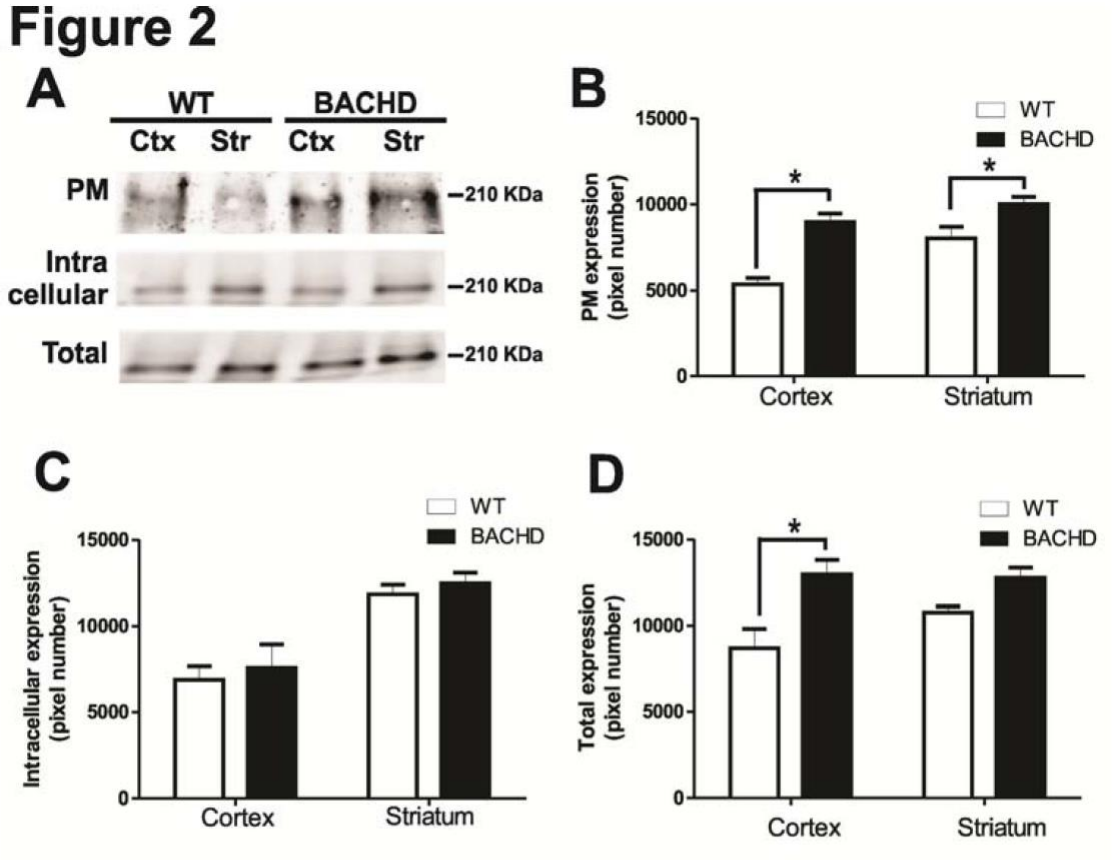

Figure 2: ***$Ca_v1.2$ plasma membrane expression is increased in the cortex and striatum of BACHD mice at 3 months of age.*** **(A)** Shown are representative immunoblots for $Ca_v1.2$ cell surface (upper panel), intracellular (middle panel) and total expression (lower panel) in the cortex and striatum of either WT or BACHD mice at 3 months of age. Graphs show the densitometric analysis of cell surface (**B**) intracellular (**C**) total cell lysate and **(D)** expression of $Ca_v1.2$ protein in the cortex and striatum from either WT or BACHD mice at 3 months of age. Full-length blots are included in the supplementary information. Data represent the mean ± SEM of 4 independent experiments. * indicate significant differences as compared to matched WT ($p<0.05$).

Figure 3

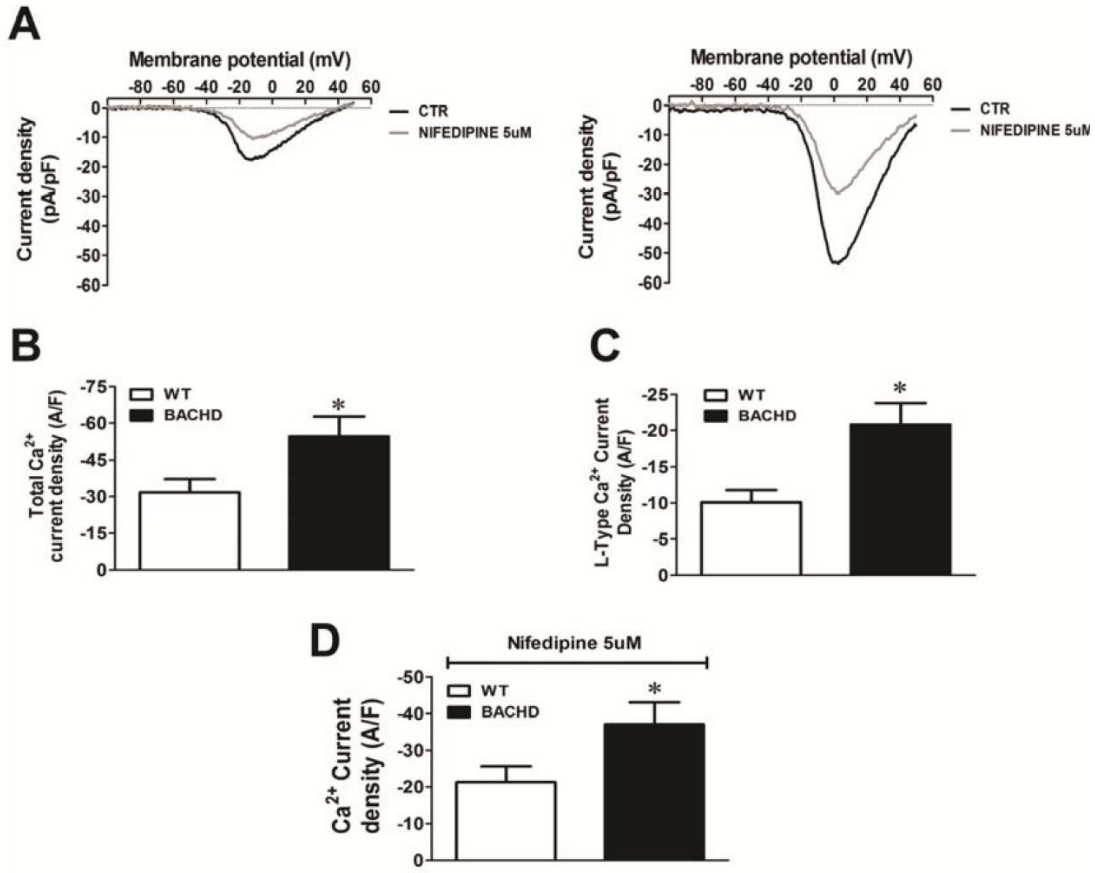

Figure 3: L-type Ca^{2+} currents are increased in BACHD cortical neurons. (A) Shown are representative records of the Ca^{2+} current-voltage relationship from WT (left panel) and BACHD (right panel) in the absence (black) or after exposure to nifedipine 5 µM (gray). **(B)** Graphs show peak total Ca^{2+} current-density and **(C)** L-type Ca^{2+} current-density from WT and BACHD mice. **D)** Graphs show peak total Ca^{2+} current-density after exposure to nifedipine 5 µM from WT (n=13) and BACHD (n=14) mice. *indicate significant differences as compared to matched WT ($p<0.05$).

FIGURE 4

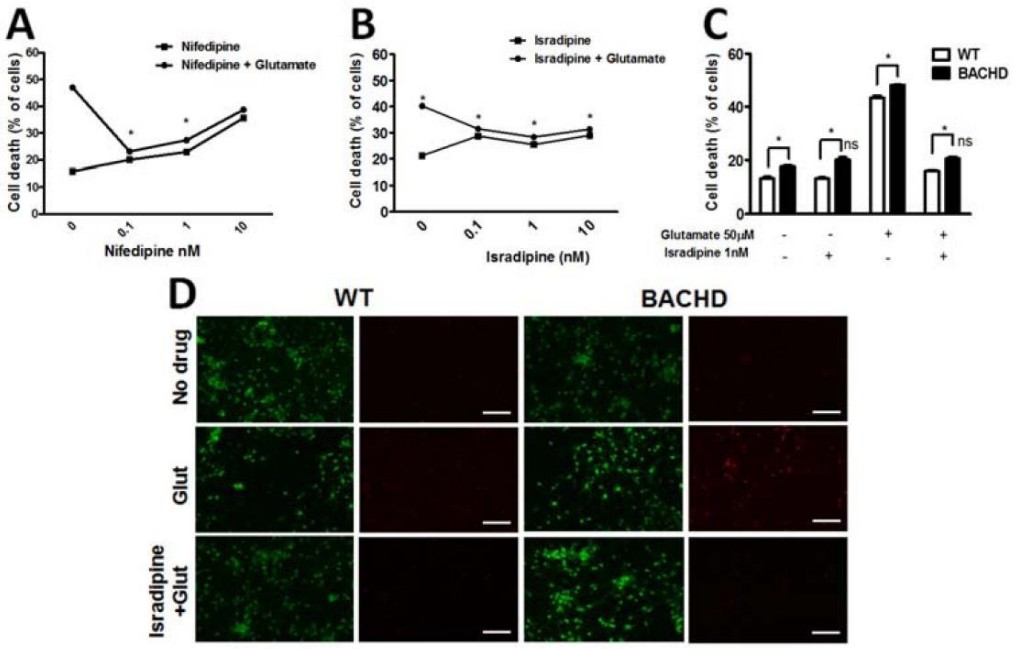

Figure 4: Selective L-type Ca^{2+} blockers protect against glutamate-induced neuronal cell death. (A-B) Graph shows percentage of neuronal cell death of primary cultured corticostriatal of C57/BL6 mice neurons that were either untreated or treated with 0.1 nM, 1 nM or 10 nM of **(A)** Nifedipine or Isradipine **(B)** in the presence or absence of 50 μM glutamate for 20 h. (C) Graph shows percentage of neuronal cell death of primary cultured corticostriatal of WT or BACHD mice neurons that were either untreated or treated with 1 nM of isradipine in the presence or absence of 50 μM glutamate for 20 h. **(D)** Shown are representative images for primary cultured corticostriatal neurons obtained from either WT (FVB) or BACHD embryos that were either untreated or treated with 50 μM glutamate or 50 μM glutamate plus 1 nM isradipine for 20 h and labeled with calcein AM (green, live cells) and ethidium homodimer-1 (red, dead cells). Scale bar = 100 μM. Data represent the means ± SEM of four independent experiments. * indicates significant difference as compared to glutamate-treated neurons and ns indicates no significant difference as compared to untreated neurons (basal cell death) ($p < 0.05$).

Apêndice 3

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The *Phoneutria nigriventer* spider toxin, PnTx4-5-5, promotes neuronal survival by blocking NMDA receptors

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ABSTRACT

Spider toxins are recognized as useful sources of bioactive substances, showing a wide range of pharmacological effects on neurotransmission. Several spider toxins have been identified biochemically and some of them are specific glutamate receptors antagonists. Previous data indicate that PnTx4-5-5, a toxin isolated from the spider *Phoneutria nigriventer*, inhibits the N-methyl-D-aspartate receptor (NMDAR), with little or no effect on AMPA, kainate or GABA receptors. In agreement with these results, our findings in this study show that PnTx4-5-5 reduces the amplitude of NMDAR-mediated EPSCs in hippocampal slices. It is well established that glutamate-mediated excitotoxic neuronal cell death occurs mainly via NMDAR activation. Thus, we decided to investigate whether PnTx4-5-5 would protect against various cell death insults. For that, we used primary-cultured corticostriatal neurons from wild type (WT) mice, as well as from a mouse model of Huntington's disease, BACHD. Our results showed that PnTx4-5-5 promotes neuroprotection of WT and BACHD neurons under the insult of high levels of glutamate. Moreover, the toxin is also able to protect WT neurons against amyloid β (Aβ) peptide toxicity. These results indicate that the toxin PnTx4-5-5 is a potential neuroprotective drug.

1. Introduction

N-methyl-D-aspartate receptors (NMDARs) play a critical role in neurotransmission, acting as an important component of many forms of synaptic plasticity involved in cognition and memory (Bard and Groc, 2011; Paoletti et al., 2013). However, NMDARs are also involved in neurodegeneration and it is well established that overstimulation of NMDARs can lead to excitotoxicity (Parsons and Raymond, 2014; Waxman and Lynch, 2005). Consequently, abnormal expression levels and/or altered NMDAR function have been implicated in numerous neurological disorders and pathological conditions, including schizophrenia, ischemia, Alzheimer's disease (AD), Huntington's disease (HD) and Parkinson's diseases (PD) (Lipton and Rosenberg, 1994; Milnerwood et al., 2010; Parsons and Raymond, 2014; Wang et al., 2013). AD pathology is characterized by the accumulation of neurofibrillary tangles and amyloid plaques, which are mainly comprised of amyloid beta peptide (Aβ) formed by cleavage of the amyloid precursor protein (APP) (Alzheimer, 1907). Recent evidence demonstrates that Aβ oligomers can dysregulate glutamate receptors and disrupt glutamatergic synaptic transmission in hippocampal slices, which parallels early cognitive deficits (Varga et al., 2015). Neurodegeneration and synaptic dysfunction induced by Aβ involves overactivation of the NMDARs resulting in elevated intracellular calcium ion concentration $[Ca^{2+}]_i$ levels and, consequently, cell death (Bordji et al., 2011; Ferreira et al., 2012). HD is characterized by a massive loss of medium sized striatum neurons and is caused by a polyglutamine expansion in the amino-terminal region of the huntingtin protein (Group, 1993). As in the case of AD, overactivation of NMDARs plays a crucial role in HD pathology by

Abbreviations: NMDAR, N-methyl-D-aspartate receptor; DIV, days *in vitro*; ANOVA, analysis of variance; HD, Huntington's disease; AD, Alzheimer's disease; PD, Parkinson's disease; DNQX, 6,7-dinitroquinoxaline-2,3-dione.

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increasing $[Ca^{2+}]_i$ and promoting excitotoxicity (Fan and Raymond, 2007; Zeron et al., 2001). Moreover, NMDARs have been pointed as the key player leading to the selective loss of medium sized spiny neurons as these neurons mainly express NMDARs containing the subunits NR1/NR2B, which are particularly sensitive to mutated huntingtin protein (Chen et al., 1999; Zeron et al., 2002).

The venom of the South American spider *Phoneutria nigriventer* contains potent neurotoxins that act on ion channels (Gomez et al., 2002; Vieira et al., 2005). PnTx4-5-5 is a single chain polypeptide isolated from *P. nigriventer* venom composed of 47 amino acid residues, including 10 cysteines, with a calculated molecular mass of 5175 Da. Previous electrophysiological studies carried out on whole-cell voltage-clamped rat hippocampal neurons indicated that PnTx4-5-5, at a concentration of 1 μM, reversibly inhibits NMDAR evoked currents (de Figueiredo et al., 2001). Our findings in this study corroborate this data, as we show that NMDA receptor-mediated EPSCs (evoked glutamatergic excitatory postsynaptic currents) in CA1 hippocampal slices of adult rats were inhibited by 100 nM Tx4-5-5. Since antagonism of NMDARs has been proposed as a therapeutic intervention to prevent the death of vulnerable neurons and also to curb the progression of neurological diseases, we tested the potential of Tx4-5-5 to promote neuroprotection. Using corticostriatal neuronal cultures from wild type (WT) mice, we discovered that low concentrations of PnTx4-5-5 (0.001 nM–4 nM) are neuroprotective against both glutamate and Aβ-induced neuronal death. Moreover, PnTx4-5-5 also prevented glutamate-induced death of neurons obtained from a mouse model of HD, the BACHD mice.

2. Material and methods

2.1. Material

DNQX (6,7-dinitroquinoxaline-2,3-dione) was purchased from Tocris Bioscience and glutamate was from Sigma Aldrich. Amyloid beta peptide (Aβ) 1-42, Neurobasal medium, N2 and B27 supplements, GlutaMAX (50.0 μg/ml penicillin and 50.0 μg/ml streptomycin), and Live/Dead viability assay were purchased from Thermo Fisher Scientific. Aβ was activated in deionized water at 37 °C for 24 h before incubation with neurons.

2.2. PnTx4-5-5 toxin

PnTx4-5-5 toxin was isolated from the spider *P. nigriventer* venom by reverse phase high performance liquid chromatography (HPLC) and anion exchange HPLC, according to (de Figueiredo et al., 2001). The lyophilized toxin was diluted in deionized water.

2.3. Mouse model

C57/BL6 mice (25–30 g) were purchased from the animal facility (CEBIO) located at the Universidade Federal de Minas Gerais (UFMG). FVB/NJ and FVB/N-Tg(HTT*97Q)IXwy/J (BACHD) transgenic mice (Gray et al., 2008) were purchased from Jackson Laboratory. Mice were housed in an animal care facility at 23 °C on a 12 h light/12 h dark cycle with food and water provided *ad libitum*. Animal care was in accordance with the UFMG Ethics Committee on Animal Experimentation, 139/2013 CETEA/UFMG.

2.4. Neuronal primary culture preparation

Neuronal cultures were prepared from the striatal region of E15 mouse embryo brains, as described previously (Doria et al., 2013). After dissection, striatal tissue was submitted to trypsin digestion followed by cell dissociation using a fire-polished Pasteur pipette. Cells were plated on poly-L-ornithine coated dishes in Neurobasal medium supplemented with N2 and B27 supplements, 2 mM GlutaMAX, 50 μg/ml penicillin, and 50 μg/ml streptomycin. Cells were incubated at 37 °C and 5% CO_2 in a humidified incubator and cultured for 8 days *in vitro* (DIV) with medium replenishment every 4 days.

2.5. Cell death assay

Neurons were incubated for 20 h in the presence or absence of PnTx4-5-5, 50 μM glutamate or 6.64 μM Aβ, as indicated in the *Figure Legend*, and cell death was determined by Live/Dead viability assay, as described previously (Doria et al., 2013). Briefly, neurons were stained with 2 μM calcein acetoxymethyl ester (AM) and 2 μM ethidium homodimer-1 for 15 min and the fractions of live (calcein AM positive) and dead (ethidium homodimer-1 positive) cells were determined. Neurons were visualized by fluorescence microscopy using Floid Microscope (Life Technologies). Cells were analyzed per well in triplicate using ImageJ™ software. Dead cells were expressed as a percentage of the total number of cells.

2.6. Electrophysiology

Whole-cell patch clamp recordings were obtained using a Multiclamp 700B amplifier and pClamp software (Molecular Devices). Glass micropipette electrodes had open-tip resistances of 2–4 MΩ. 7 V, 10 stimuli at 100 Hz were employed to increase the frequency of spontaneous events (Li et al., 2006). 240 μM thick hippocampal slices from 12 to 15 days old Wistar rats were used for whole-cell recordings voltage-clamp of CA1 pyramidal neurons. Hippocampal slices were incubated in ACSF (125 mM NaCl, 3.25 mM KCl, 1.5 mM $CaCl_2$, 1.5 mM $MgCl_2$, 25 mM $NaHCO_3$, 25 mM D-glucose) at 35 °C. The internal pipette solution consisted of 130 CsCl, 7 mM NaCl, 0.1 mM EGTA, 0.3 mM $MgCl_2$ and 10 mM Hepes, pH adjusted to 7.3 with CsOH. The internal solution was supplemented with 2 mM ATP and 0.5 mM GTP, which were added immediately before use. AMPA and Kainate receptors were blocked in all recordings with bath-applied 10 μM DNQX. Under these conditions, spontaneous excitatory post-synaptic currents (sEPSC) were presumed to be mediated exclusively by NMDARs. The introduction of the toxin was controlled by a manual microperfusion system. The tip of perfusion pipette was positioned near the cell body and kept as constant as possible in all experiments.

2.7. Data analysis

Means ± SEM are shown for the number of independent experiments indicated in *Figure Legends*. In the case of cell death assays, GraphPad Prism™ software was used to analyze data for statistical significance and for curve fitting. Statistical significance was determined by analysis of variance (ANOVA) testing followed by Bonferroni post-hoc Multiple Comparison testing. For electrophysiology, sEPSC amplitudes were collected using the program Mini Analysis (Synaptosoft) and compared using the Kolmogrov–Smirnov Two Sample test. For all statistical tests, $p < 0.05$ was considered statistically different.

3. Results

3.1. PnTx4-5-5 reduces NMDAR currents in hippocampal slices

It was shown previously that the toxin PnTx4-5-5 can inhibit NMDAR currents in hippocampal cultures (de Figueiredo et al., 2001). To verify whether the purified PnTx4-5-5 toxin used in this study was active on NMDARs, we applied 100 nM PnTx4-5-5 to

hippocampal slices and measured NMDAR-mediated EPSC after pharmacologically blocking AMPA/kainate receptors with 20 μM DNQX. At the start of each trace, slices were stimulated (7 V, 10 stimuli at 100 Hz) with an electrode positioned to stimulate Schaffer Collateral CA1 afferents to increase the frequency of spontaneous events (Fig. 1A). Cumulative probability histogram of EPSC amplitudes recorded in control conditions or following treatment with 100 nM PnTx-4-5-5 showed that this toxin was able to reduce NMDAR post-synaptic currents (Fig. 1B). Therefore, these data certify the activity of the toxin used in our study and extend previous published results by showing that PnTx4-5-5 blocks NMDARs in hippocampal slices.

3.2. PnTx4-5-5 protects against glutamate-induced neuronal cell death

Increased levels of glutamate promote excitotoxic neuronal cell death mainly due to the activation of NMDARs and augmentation of cytosolic Ca^{2+} concentration (Choi et al., 1988). Since PnTx4-5-5 reduces the amplitude of NMDAR-mediated EPSCs, we hypothesize that this toxin can be neuroprotective. To experimentally test this hypothesis, we performed an *in vitro* assay to measure neuronal cell death. Primary cultured corticostriatal neurons from C57/BL6 mouse embryos exposed to 50 μM glutamate for 20 h exhibited high level of neuronal cell death (Fig. 2). However, when neurons were incubated with glutamate in the presence of PnTx4-5-5, neuronal cell death was significantly decreased (Fig. 2). Even very low concentrations of the toxin, for example 0.001 nM, were sufficient to decrease neuronal cell death (Fig. 2). Moreover, cell death levels observed in neurons co-incubated with glutamate and PnTx4-5-5 were not different than those of untreated neurons (basal neuronal cell death), except for the dose of 0.001 nM PnTx4-5-5, which was not enough to completely rescue glutamate-induced neuronal cell death (Fig. 2). In addition, PnTx4-5-5 in the absence of glutamate was not neurotoxic, as neuronal cell death was not different when comparing neurons treated with the toxin in the absence of glutamate and untreated neurons (Fig. 2).

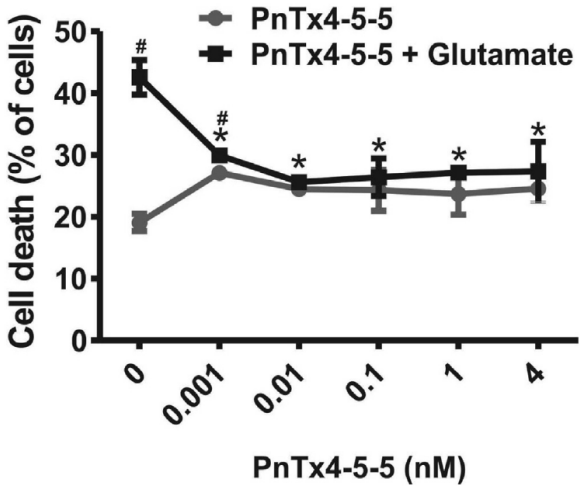

Fig. 2. PnTx4-5-5 protects against glutamate-induced neuronal cell death. Graph shows percentage of neuronal cell death of primary cultured corticostriatal neurons that were either untreated or treated with 0.001 nM, 0.01 nM, 0.1 nM, 1 nM or 4 nM of PnTx4-5-5 in the presence or absence of 50 μM glutamate for 20 h. Data represent the means ± SEM of four independent experiments. * indicates significant difference as compared to glutamate-treated neurons and # indicates significant difference as compared to untreated neurons (basal cell death) ($p < 0.05$).

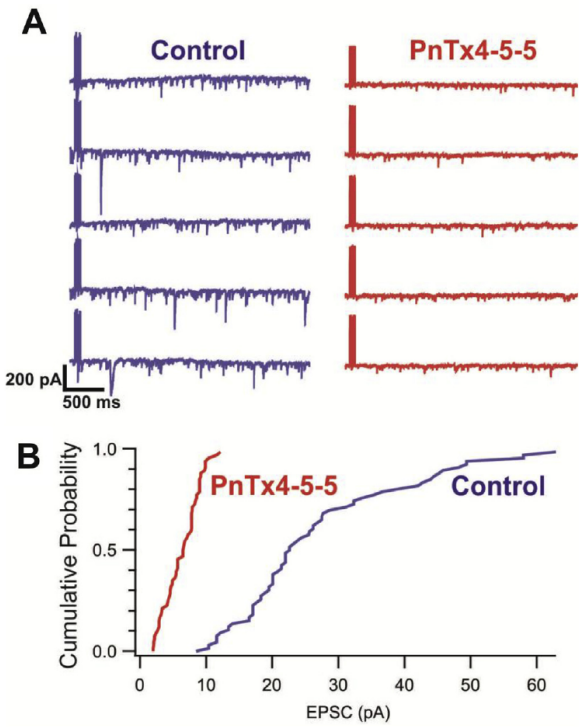

Fig. 1. PnTx-4-5-5 (100 nM) decreases the amplitude of NMDAR-mediated EPSCs. (A) Spontaneous EPSCs recorded in the presence of AMPA/Kainate antagonist (DNQX, 10 μM) in either vehicle- or 100 nM PnTx4-5-5-treated hippocampal slices. At the start of each trace, the slice was stimulated (7 V, 10 stimuli at 100 Hz) to increase the frequency of spontaneous events. (B) Graph shows cumulative probability histogram of EPSC amplitudes recorded in vehicle- or 100 nM PnTx4-5-5-treated hippocampal slices. Data is representative of 3 independent experiments.

3.3. PnTx4-5-5 protects striatal neurons from a mouse model of HD against glutamate-induced neuronal cell death

To test whether PnTx4-5-5 could be efficient to promote neuroprotection in neurodegenerative diseases, we tested this peptide in primary-cultured corticostriatal neurons from a mouse model of HD, BACHD. Corticostriatal neurons obtained from BACHD mice exhibited higher levels of neuronal cell death than those of WT neurons, either in the absence or in the presence of toxin and drugs (Fig. 3). Incubation of neurons with 50 μM glutamate promoted high levels of neuronal cell death in both WT and BACHD cultures, as compared to their respective controls (Fig. 3A, B, D, E, and G). Importantly, this excitotoxic effect of glutamate was blocked by 1 nM PnTx-4-5-5 in both WT and BACHD neurons (Fig. 3C, F, and G). Moreover 1 nM PnTx4-5-5 diminished cell death induced by glutamate to the same levels as that of untreated neurons (Fig. 3G). These data indicate that PnTx4-5-5 present the potential to be neuroprotective in HD.

3.4. PnTx4-5-5 protects against Aβ-induced neuronal cell death

In order to determine whether PnTx4-5-5 could also be efficient to prevent neurodegeneration promoted by other cytotoxic insults, we tested whether the toxin could inhibit Aβ-induced neuronal cell death. Aβ is the main component of AD amyloid plaques and,

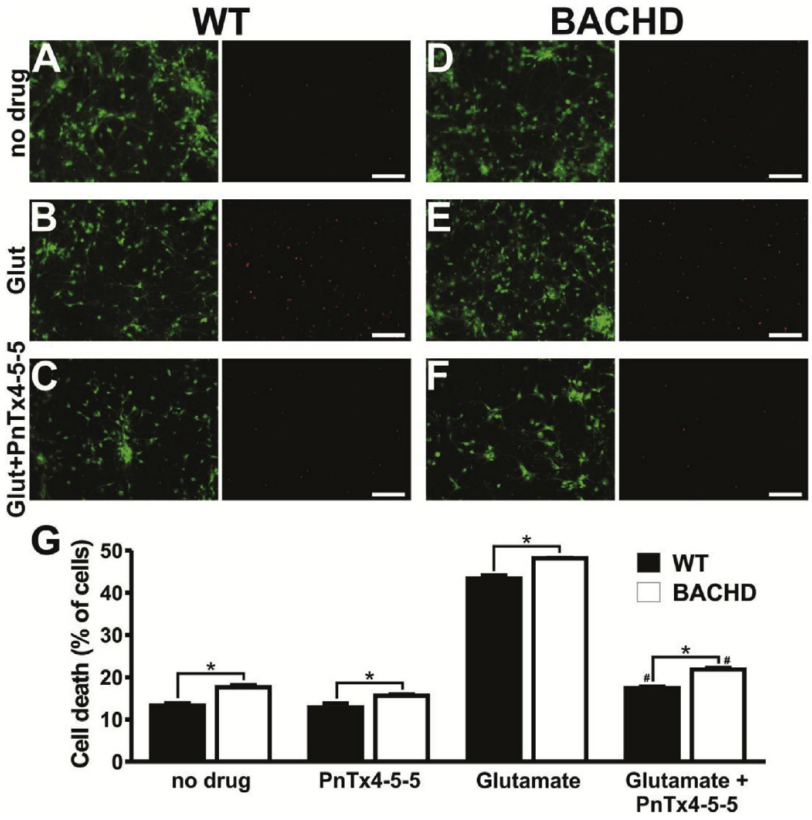

Fig. 3. PnTx4-5-5 protects against glutamate-induced neuronal cell death of neurons from a HD mouse model. Shown are representative images for primary cultured corticostriatal neurons obtained from either WT (FVB) or BACHD embryos that were either untreated (A and D) or treated with 50 μM glutamate (B and E) or 50 μM glutamate plus 1 nM PnTx4-5-5 (C and F) for 20 h and labeled with calcein AM (green, live cells) and ethidium homodimer-1 (red, dead cells). Scale bar = 100 μM. (G) Graph shows percentage of neuronal cell death of primary cultured corticostriatal neurons obtained from either WT (FVB) or BACHD embryos that were either untreated (no drug) or treated with 1 nM PnTx4-5-5 in the presence or absence of 50 μM glutamate for 20 h. Data represent the means ± SEM of four independent experiments. * indicates significant difference between match treated BACHD and WT neurons and # indicates significant difference as compared to glutamate-treated neurons ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

importantly, it has been proposed that soluble oligomers of Aβ peptides can be even more toxic to neurons than amyloid plaques (Lambert et al., 1998). In agreement with these data, corticostriatal neurons incubated with 6.64 μM Aβ oligomers exhibited high levels of neuronal cell death (Fig. 4). To test whether PnTx4-5-5 could be efficient to promote neuronal survival against Aβ-induced cell death, we co-incubated corticostriatal neurons with 6.64 μM Aβ and 1 nM PnTx4-5-5 for 20 h and measured neuronal cell death. 1 nM PnTx4-5-5 was efficient to protect neurons from Aβ-induced insult (Fig. 4), which indicates that this toxin is neuroprotective against a variety of cell death insults.

4. Discussion

Neuronal cell death is the main sequela of several neurodegenerative disorders, such as AD, PD and HD. Thus, the development of new therapies capable of preventing neuronal cell death is a crucial step towards the development of efficacious therapies to treat neurodegenerative diseases. As NMDARs are key players in the excitotoxic neuronal cell death that takes place in various neurodegenerative diseases (Lipton and Rosenberg, 1994; Milnerwood et al., 2010; Parsons and Raymond, 2014; Wang et al., 2013), these receptors are potential targets for the development of neuroprotective drugs. However, blocking NMDARs can lead to serious deleterious effects, as NMDARs are the main excitatory receptors in the brain. Until recently, all the drugs that showed potential as inhibitors of excitotoxicity also blocked normal neuronal function and, consequently, had severe and unacceptable side effects (Lipton, 2004). However, further studies have provided evidences that NMDAR activation could have distinct consequences depending on the synaptic/extrasynaptic location of the receptor. For example, activation of NMDARs directly opposed to the pre-synaptic releasing site, can promote neuroprotection by activating cell signaling pathways and driving the transcription of genes that foster neuronal survival (Papadia and Hardingham, 2007; Papadia et al., 2008). On the other hand, stimulation of extrasynaptic NMDARs has been associated with neuronal death (Hardingham and Bading, 2002; Leveille et al., 2008; Papadia and Hardingham, 2007). Moreover, recent data suggest that chronic activation of extrasynaptic NMDARs leads to sustained neuronal Aβ release and could be involved in the pathogenesis of Alzheimer's disease (Bordji et al., 2011). In the case of HD, it has been shown that neurons from a mouse model of HD, YAC128, exhibit increased extrasynaptic NMDAR-induced currents and signaling, leading to increased sensitivity to neuronal cell death (Milnerwood et al., 2010; Okamoto et al., 2009). Moreover,

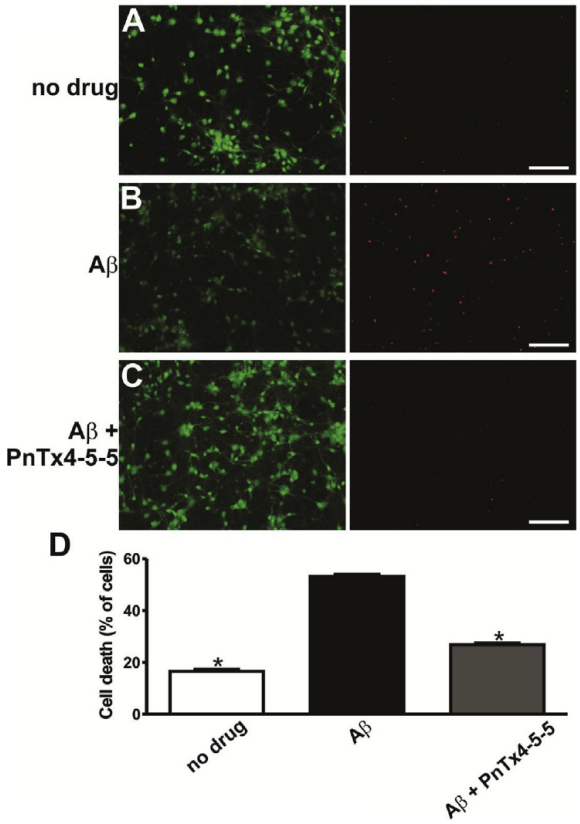

Fig. 4. PnTx4-5-5 protects against amyloid β-induced neuronal cell death. Shown are representative images for primary cultured corticostriatal neurons that were either untreated (A) or treated with 6.64 μM amyloid β (Aβ) (B) or 6.64 μM Aβ plus 1 nM PnTx4-5-5 (C) for 20 h and labeled with calcein AM (green, live cells) and ethidium homodimer-1 (red, dead cells). Scale bar = 100 μM. (D) Graph shows percentage of neuronal cell death in primary cultured striatal neurons that were either untreated (no drug) or treated with 1 nM PnTx4-5-5 in the presence or absence of 6.64 μM Aβ for 20 h. Data represent the means ± SEM of four independent experiments. * indicates significant difference as compared to Aβ-treated neurons ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

activation of synaptic NMDARs promotes resistance to neuronal cell death insults (Okamoto et al., 2009). Thus, as for other neurological diseases, drugs targeting extrasynaptic NMDARs could be a promising strategy to prevent neurodegeneration in AD and HD.

Memantine is a non-competitive NMDAR antagonist with relatively rapid off-rate from the channel and also with strong voltage dependence (Chen and Lipton, 1997; Johnson and Kotermanski, 2006; Lipton, 2006). Therefore, memantine blocks preferentially excessive NMDAR activity without disrupting physiological synaptic transmission, producing less adverse effects than other NMDARs antagonists and being the only drug targeting NMDAR used in clinics for the treatment of neurodegeneration and AD (Chen et al., 1992, 1998bib_Chen_et_al_1998; Leveille et al., 2008; Okamoto et al., 2009). Moreover, memantine appears to preferentially block extrasynaptic over synaptic NMDARs (Xia et al., 2010). However, memantine does not stop the progression of AD and also there is no reliable evidence that this drug can produce long-term positive effects (Ehret and Chamberlin, 2015; Geldenhuys and Darvesh, 2015). Thus, newer and more effective drugs acting on NMDARs are needed for treating neurodegenerative diseases. Although PnTx4-5-5 also blocks NMDARs, this toxin has a chemical structure completely unrelated to memantine and, therefore, might trigger neuroprotection by blocking the receptor through a different mechanism. Thus, PnTx4-5-5 could lead to the development of a new group of NMDAR blockers that might be more effective as disease modifying drugs.

It has been shown previously that PnTx4-5-5, at the dose of 1 μM, attenuates NDMAR evoked currents in rat hippocampal neurons (de Figueiredo et al., 2001). Our results demonstrate that a lower concentration of PnTx4-5-5, 100 nM, is also capable of decreasing NMDAR EPSCs. The concentration of toxin employed in these electrophysiology experiments were higher than that used in the cell death assays performed in this study because it is more difficult for drugs to permeate brain slices (electrophysiology experiments) than dissociated neurons (cell death assays). However, as PnTx4-5-5 was capable of promoting neuroprotection at the concentration of 1 pmol, it is possible that even lower concentrations of the toxin would be effective to block NMDAR currents. Future experiments will be important to determine the lowest effective concentration of the toxin capable of blocking NMDARs and whether NMDAR is the only PnTx4-5-5 target. Indeed, at the micromolar concentration, other targets for PnTx4-5-5 have been proposed, as it has been demonstrated that 2 μM of PnTx4-5-5 can decrease glutamate uptake by rat synaptosomes (Oliveira et al., 2003). These data indicate that the toxin could become toxic if used at high concentrations. Importantly, our results demonstrating that PnTx4-5-5 is a very potent neuroprotective drug, being effective at concentrations as low as 1 pmol, indicate that PnTx4-5-5 can be used at doses that are probably too low to activate toxic mechanisms, including decreased glutamate uptake.

Our data confirmed that PnTx4-5-5 can block NMDARs and showed that PnTx4-5-5 had significant neuroprotective effects, reducing glutamate- and Aβ-induced neuronal cell death. Moreover, PnTx4-5-5 was able to prevent cell death of BACHD neurons stimulated with excitotoxic concentrations of glutamate. However, as mentioned previously, a clinically suitable NMDAR antagonist should spare normal neurotransmission while blocking the ravages of excessive NMDA receptor activation. Therefore, future studies will be important to determine whether PnTx4-5-5 is able to block preferentially extrasynaptic NMDAR or if the toxin is more selective for certain NMDAR subtypes. Furthermore, it will be important to investigate the kinetic properties of NMDAR blockage by the toxin and verify whether the rate of recovery from PnTx4-5-5 blockade is dependent on the open probability of the channel. As targeting NMDARs remains as an area of active research, we believe that our findings may contribute to the field by characterizing the neuroprotective actions of a new class of NMDAR blocker. Because PnTx4-5-5 is a peptide, it cannot be easily delivered to the central nervous system. Nevertheless, this toxin exhibit very potent neuroprotective properties and can be used as a prototype to develop new compounds that could easily cross the blood brain barrier, facilitating the development of new pharmacological tools to treat neurodegenerative diseases.

Conflict of interest statement

The authors declare that they have no conflicts of interest with respect to this report.

Acknowledgments

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.toxicon.2016.01.056.

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Apêndice 4

Demais publicações durante o processo de doutoramento:

1. Publicado (2016) Cellular and Molecular Neurobiology
Thiamine Deficiency Increases Ca^{2+} Current and $Ca_V1.2$ L-type Ca^{2+} Channel Levels in Cerebellum Granular Neurons.
Daniel C. Moreira-Lobo, Jader S. Cruz, **Flavia R. Silva**, Fabíola M. Ribeiro, Christopher Kushmerick, Fernando A. Oliveira

2. Publicado (2016) Current Neuropharmacology
Alzheimer's disease: Targeting the Cholinergic System.
Talita H.Ferreira e Vieira, Isabella M. Guimarães, **Flavia R. Silva** and Fabíola M. Ribeiro

3. Publicado (2016) The Journal of bacteriology
N-Methyl-D-aspartate (NMDA) receptor blockade prevents neuronal death induced by Zika virus infection.
Vivian V. Costa[#], Juliana L. Del Sarto[#], Rebeca F. Rocha, **Flavia R. Silva**, Juliana G. Doria, Isabella G. Olmo, Rafael E. Marques, Celso M. Queiroz-Junior, Gisele Foureaux, Julia Maria S. Araújo, Allysson Cramer, Ana Luíza C. V., Real, Lucas S. Ribeiro, Silvia I. Sardi, Anderson J. Ferreira, Fabiana S. Machado, Antônio C. de Oliveira, Antônio L. Teixeira, Helder I. Nakaya Danielle G. Souza, Fabiola M. Ribeiro and Mauro M. Teixeira.

4. Publicado (2015) Toxicology and Applied Pharmacology
Enhancement of endocannabinoid signaling protects against cocain neurotoxicity.
Luciano R. Vilela, Pedro H. Gobira, Thercia G. Viana, Daniel C.Medeiros, Talita H. Ferreira-Vieira, Juliana G. Doria, **Flávia Rodrigues**, Daniele C. Aguiar, Grace S. Pereira, André R. Massessini, Fabíola M. Ribeiro, Antonio Carlos P. de Oliveira, Marcio F.D. Moraes, Fabricio A. Moreira

5. Publicado (2013) British Journal of Pharmacology
Metabotropic glutamate receptor 5 positive allosteric modulators are neuroprotective in a mouse model of Huntington's disease.
JG Doria*, **FR Silva***, JM de Souza, LB Vieira, TG Carvalho, HJ Reis, GS Pereira, T Dobransky and FM Ribeiro