Dissertação de mestrado

Isabella Monteiro Guimarães

Participação do receptor metabotrópico de glutamato 5 na fisiologia de comportamentos motores e nas alterações motoras que ocorrem em camundongos modelo da doença de Huntington

Universidade Federal de Minas Gerais (UFMG)
Programa de Pós-Graduação em Neurociências
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LISTA DE SIGLAS E ABREVIATURAS

µg: microgramas

µL: microlitros

µM: micromolar

AMPA: ácido aminopropiônico

AMPc: adenosina monofosfato cíclico

DA: doença de Alzheimer

DAG: diacilglicerol

DH: doença de Huntington

DP: doença de Parkinson

ERK: quinase regulada por sinais extracelulares

GPCR: proteína G acoplada ao receptor metabotrópico

GTP: guanosina trifosfato

Gα: subunidade alfa da proteína G

Htt: proteína huntingtina

i.p.: intraperitoneal

IP_3: inositoltri-fosfato

M: molar

mGluR: receptor metabotrópico glutamatérgico

mM: milimolar

MPEP: 2-metil-6(feniletinil) piridina

MTEP: 3-((2-Methyl-4-thiazolyl)ethynyl)pyridine

nm: nanômetro

NMDA: N-metil d-aspartato

NMDAR: receptor N-metil d-aspartado

pb: pares de base

PI: fosfatidilinositol

PI3K: fosfatidilinositol-3-kinase

PIP2: fosfatidilinositol 4,5-bifosfato

PKC: proteína quinase C

PLC: fosfolipase C

RE: retículo endoplasmático

SNC: sistema nervoso central

v/v: volume por volume

SUMÁRIO

RESUMO

Introdução: A doença de Huntington (DH) é uma desordem neurodegenerativa, progressiva e hereditária, que evolui inevitavelmente à morte. Uma vez que vários estudos indicam que o receptor metabotrópico de glutamato 5 (mGluR5) pode ter um papel importante na DH e na modulação de movimentos hipercinéticos, decidimos investigar se o mGluR5 tem um papel na hipercinesia envolvida na DH.

Resultados: Os resultados obtidos em nosso estudo demonstraram que o mGluR5 interfere na atividade locomotora através de substratos neurais específicos. Para determinar o papel mGluR5 em DH, foi realizado cruzamentos entre camundongos modelo para a DH ($Hdh^{Q111/Q111}$) e seu controle ($Hdh^{Q20/Q20}$) e camundongos *knockout* para mGluR5 ($mGluR5^{-/-}$). Foi observado que a deleção do mGluR5 altera atividade locomotora dos camundongos modelo da DH, indicando que existe uma interação funcional entre a Htt mutante e o mGluR5. Ademais, foi caracterizada uma interação funcional da Htt mutante com o mGluR5 capaz de alterar a atividade locomotora de camundongos. Para investigar o mecanismo subjacente a estas alterações de locomoção, foi realizado um ensaio de microarranjo. A expressão de um número de genes envolvidos na regulação do movimento, tal como a dineína cadeia leve, cadeia pesada e dinactina, foram alteradas em camundongos modelo para DH desprovidos da expressão de mGluR5. Tais alterações de expressão foram confirmadas por qPCR.

Conclusão: Os resultados indicam que o mGluR5 pode desempenhar um papel no controle do movimento envolvendo vários substratos neurais, podendo estar envolvido na hipercinesia observada na DH. Além disso, resultados do ensaio de microarranjo sugerem que as alterações motoras promovidas pela deleção gênica do mGluR5 no camundongo modelo da DH podem ocorrer devido à alteração da expressão de genes que codificam proteínas relacionadas a motricidade.

Palavras chave: Doença de Huntington, mGluR5, locomoção, modelo *Knock-in*

ABSTRACT

Introduction: Huntington's disease (HD) is a neurodegenerative disorder, progressive and hereditary, that evolves inevitably to death. As we and others have demonstrated that the metabotropic glutamate receptor 5 (mGluR5) may have a role in HD pathology and that mGluR5 is involved in hyperkinetic movements, we decided to investigate whether mGluR5 has a role in HD-related hyperkinesia.

Results: The results obtained in our study demonstrate that mGluR5 stimulation interferes in locomotor activity by acting on specific neural substrates. To determine mGluR5 role in HD, we have crossed $Hdh^{Q20/Q20}$and $Hdh^{Q111/Q111}$ mice with mGluR5 knockout mice ($mGluR5^{-/-}$). mGluR5 knockout alters HD mice locomotor activity, indicating that there is a functional interaction between mutant Htt and mGluR5. To investigate the underlying changes in this locomotion regulation, a microarray assay was performed. The expression of a number of genes involved in movement control, such as dynein light chain and heavy chain and dynactin, were modified in a mouse model of HD lacking the expression of mGluR5 ($Hdh^{Q111/Q111}/mGluR5^{-/-}$), as compared to its control $Hdh^{Q20/Q20}/mGluR5^{-/-}$. These expression changes were confirmed by qPCR.

Conclusion: The results indicate that mGluR5 plays a role in movement control by acting on specific neural substrates and that the receptor is likely involved in HD hyperkinesia. Moreover, our microarray data suggest that the mGluR5-related movement alterations observed in the HD mouse model might be due to modifications in the expression of genes that codify for movement control related proteins.

Keywords: Huntington's Disease, mGluR5, locomotion, *Knock-in* model

1. INTRODUÇÃO

1.1 – Receptor Metabotrópico de Glutamato 5 (mGluR5)

O glutamato, principal neurotransmissor excitatório do cérebro, desempenha papel importante na plasticidade sináptica, aprendizado, memória e outras funções cognitivas (Mehta, Prabhakar et al. 2013). Os receptores de glutamato podem se dividir em dois tipos: Os ionotrópicos e os metabotrópicos (Nakanishi, Nakajima et al. 1998). Os receptores N-metil-D-aspartato (NMDA), α-amino-3-hidroxi-5-metil-4-isoxazolepropionato (AMPA) e cainato atuam como canais catiônicos após sua ativação pelo glutamato e estão envolvidos na transmissão sináptica excitatória rápida. Já os receptores metabotrópicos (mGluRs), acoplados à proteína G, modulam a transdução de sinal intracelular regulando tanto a abertura dos canais iônicos, como a produção de segundos mensageiros (Pin and Duvoisin 1995, Nakanishi, Nakajima et al. 1998).

São conhecidos oito subtipos diferentes dos receptores mGluR - mGluR1 ao mGluR8 (Pin and Duvoisin 1995). Estes são classificados em três grupos (grupo I, II e III) de acordo com suas semelhanças em sequências de aminoácidos, mecanismos de transdução de sinal e seletividade aos agonistas (Pin and Duvoisin 1995). Os receptores compreendidos pelo grupo I dos mGluRs, mGluR1 e mGluR5, são acoplados à proteína $G\alpha_q$, que estimula a ativação da fosfolipase Cβ1, resultando na formação de diacilglicerol (DAG) e inositol 1,4,5-trifosfato (IP_3), bem como na liberação de Ca^{2+} dos estoques intracelulares e na consequente ativação da proteína quinase C (PKC) (Piers, Kim et al. 2012).

Estudos moleculares demonstram que os receptores do grupo I são amplamente expressos no tecido cerebral (Shigemoto, Nomura et al. 1993, Kerner, Standaert et al. 1997, Yu, Tueckmantel et al. 2005). Através da técnica de hibridização *in-situ* foi

possível localizar as regiões cerebrais com maior expressão do mRNA de mGluR1 e mGluR5, sendo o mGluR1 mais expresso no hipocampo e cerebelo, ao passo que o mGluR5 possui maior expressão no bulbo olfatório, corpo estriado, córtex cerebral e hipocampo (Kerner, Standaert et al. 1997). Resultados semelhantes foram encontrados em estudos de imunohistoquímica realizados por Shigemoto (1993), que observou intensa marcação para o mGluR5 no bulbo olfatório e seus núcleos correspondentes, bem como no córtex cerebral, hipocampo, corpo estriado, núcleo *accumbens*, colículo inferior e núcleos do trigêmeo. Neste mesmo estudo, o padrão de distribuição do receptor foi confirmado através da análise dos níveis de expressão do mRNA de mGluR5 (Shigemoto, Nomura et al. 1993).

1.2 – Receptor metabotrópico de glutamato 5 e atividade locomotora

Estudos funcionais indicam que a sinalização via mGluR5 desempenha papel importante na modulação de diversos aspectos do comportamento, incluindo atividade locomotora espontânea e reação a ambiente novo, bem como de funções cognitivas como memória espacial e ansiedade (Kinney, Burno et al. 2003, McGeehan, Janak et al. 2004, Balschun, Zuschratter et al. 2006, Gray, van den Buuse et al. 2009, Jew, Wu et al. 2013). Antagonistas do mGluR5, tais como 6-methyl-2-[phenylethynyl]-pyridina (MPEP) e 3-((2-methyl-4-thiazolyl)ethynyl)pyridina (MTEP), administrados perifericamente, alteram a atividade locomotora espontânea e a coordenação motora em roedores (McGeehan, Janak et al. 2004, Chan, Lee et al. 2012, Ribeiro, Devries et al. 2014). Além disso, camundongos portadores da deleção global do mGluR5 (*mGluR5*$^{-/-}$) apresentam hiperlocomoção (Kinney, Burno et al. 2003, Gray, van den Buuse et al. 2009, Ribeiro, Devries et al. 2014). Em publicação recente, Jew *et al* (2013) mostrou que a deleção do mGluR5, especificamente no córtex cerebral, modula padrões

locomotores e reação a ambiente novo, mas não afeta a atividade locomotora espontânea. Entretanto, injeções intraperitoneais de antagonista do mGluR5 neste modelo experimental induz pronunciada hiperlocomoção (Jew, Wu et al. 2013). Assim, a depleção do mGluR5 em regiões específicas do cérebro induz respostas divergentes, o que adiciona uma maior complexidade na busca pela função destes receptores na fisiopatologia de comportamentos motores. Além disso, a habilidade do grupo I dos mGluRs em aumentar níveis intracelulares de Ca^{2+} está intimamente relacionada à excitoxicidade e ao mecanismo patológico de doenças neurodegenerativas (Shigemoto, Nomura et al. 1993, Conn, Battaglia et al. 2005), como por exemplo, a doença de Huntington.

1.3 – Doença de Huntington

A doença de Huntington (DH) é uma desordem neurodegenerativa autossômica dominante causada pela perda progressiva de células neuronais do caudado-putâmen e córtex cerebral. A progressão da DH conduz o paciente a um conjunto de sintomas motores, cognitivos e psiquiátricos, dos quais atualmente não há tratamento eficaz, levando o paciente à morte (Ross and Tabrizi 2011). Ainda que sejam encontradas diferenças na prevalência geográfica da DH, o transtorno é observado em todo o mundo, afetando 5 a 8 em cada 100.000 pessoas (Warby, Visscher et al. 2011). A manifestação clínica altera-se ao longo da progressão da doença. Movimentos involuntários, chamados coréia, intensificam durante o curso da doença, causando prejuízos nas atividades de vida diária (Piira, van Walsem et al. 2013). A disfunção da marcha, outro sinal clínico comum, é caracterizada pela instabilidade postural, falta de equilíbrio e incoordenação motora, levando o paciente a quedas constantes (Piira, van Walsem et al. 2013, Williams, Heron et al. 2014). Tais problemas são acompanhados por disfunções

dos movimentos dos olhos (Anderson and MacAskill 2013), disartria (Skodda, Schlegel et al. 2014) e disfagia (Heemskerk and Roos 2011) progressivas. Distúrbios psiquiátricos e cognitivos tipicamente aparecem durante o curso da doença e incluem alteração da personalidade, obsessão, compulsão, depressão maior e demência (Ross and Tabrizi 2011, Papoutsi, Labuschagne et al. 2014, Zarowitz, O'Shea et al. 2014).

A forma mutante de proteína huntingtina (Htt), contendo um número anormal de repetições do aminoácido glutamina na região amino-terminal é a causa da DH (1993). Um estudo realizado com 83 famílias venezuelanas, portadoras desta mutação, demonstrou que a idade de início da doença pode variar devido à interação de componentes genéticos e ambientais (Wexler, Lorimer et al. 2004). Indivíduos portadores de 34 repetições de CAG ou menos não produzem sintomas, enquanto indivíduos que apresentam entre 36 a 39 repetições mostram penetrância incompleta dos sintomas. Por outro lado, pacientes portadores de 40 ou mais repetições de CAG inevitavelmente desenvolvem os sintomas da DH (Wexler, Lorimer et al. 2004).

A principal característica patológica da DH é a perda de células neuronais presentes no caudado-putâmen (corpo estriado em roedores) e em regiões neocorticais de pacientes (figura1) (Schaller 1928, Mitchell, Cooper et al. 1999, Ross and Tabrizi 2011, Dominguez, Egan et al. 2013, Dodds, Chen et al. 2014). Estudos também relatam perda neuronal em outras regiões cerebrais tais como a na região CA1 do hipocampo de pacientes com doença de Huntington (Spargo, Everall et al. 1993), indicando o amplo comprometimento de regiões neurais durante a evolução da doença. Os neurônios do corpo estriado não são igualmente suscetíveis à neurodegeneração na DH. O corpo estriado é composto principalmente (95%) de neurônios espinhosos médios (MSNs), os quais são GABAérgicos, mas também de interneurônios contendo acetilcolina e somatostatina, além dos tipos *fast-spiking* parvalbumina e neuropeptídeo-Y positivo;

tipos celulares importantes em modular os impulsos GABAérgicos dos MSNs (Vincent, Staines et al. 1983, Reiner, Medina et al. 1998, Do, Kim et al. 2012). Os MSNs são os primeiros neurônios a serem atingidos durante a progressão da DH; os interneurônios, por outro lado, são poupados (Ferrante, Kowall et al. 1986, Ferrante, Beal et al. 1987).

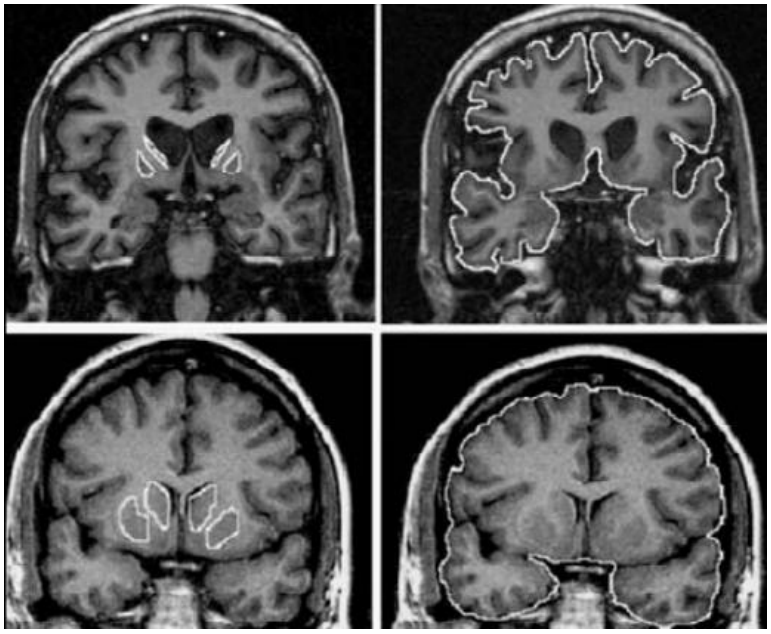

Figura 1:*Imagem de Ressonância Magnética (modificada) do cérebro de indivíduos com DH (acima) e indivíduos que não apresentam a doença (abaixo).* Contornos à esquerda identificam o caudado e putamen, contornos à direita indicam o volume cerebral global. (Ruocco, Lopes-Cendes et al. 2006).

Neurônios do corpo estriado recebem aferência de diferentes núcleos do mesencéfalo, tais como projeções dopaminérgicas da substância *nigra* e serotoninérgicas de núcleos da rafe dorsais, como também projeções glutamatérgicas do tálamo e córtex cerebral (Parent 1990, Joel and Weiner 1994, Reiner, Medina et al. 1998). Assim, o glutamato desempenha um papel importante no circuito núcleo basal-tálamo-cortical.

1.4 – Modelos Experimentais para a Doença de Huntington

A partir da identificação da mutação ocorrida na doença de Huntington, em 1993

(Group 1993), muitos avanços foram conquistados na construção de modelos animais capazes de reproduzir o fenótipo característico da doença. Estes esforços são benéficos no sentido de entender mecanismos patológicos da doença e, desta forma, auxiliar no desenvolvimento de novas possíveis estratégias terapêuticas. Além disso, o cruzamento das diversas linhagens geneticamente modificadas para DH certamente ajudarão a dissecar os mecanismos comportamentais e moleculares da doença.

Grupos independentes desenvolveram linhagens de camundongos transgênicos obtidos através da inserção do gene codificante para a proteína Htt humana inteira (BACHD e YAC128) ou apenas a sua região amino-terminal (R6/2); bem como linhagens de camundongos *knock-in* ($Hdh^{Q111/Q111}$), que são camundongos com uma expansão de 111 glutaminas na região amino-terminal. Estes últimos foram desenvolvidos através da substituição do primeiro exon do gene *htt* murino pelo primeiro exon do gene *htt* humano(Menalled and Chesselet 2002, Menalled, El-Khodor et al. 2009).

1.5 – Doença de Huntington e o receptor metabotrópico de glutamato 5

Em um estudo publicado por Anborgh *et al.* (2005) observou-se em células HEK-293 que os mGluRs do grupo I interagem com a Htt, e que,na presença da Htt mutante, a sinalização do mGluR1 torna-se alterada. Este mesmo estudo também mostrou que a formação de IP_3 pelo mGluR5 em células estriatais do modelo *Knock-in* da DH ($Hdh^{Q111/Q111}$) é comprometida quando comparada ao controle contendo 7 repetições de CAG ($Hdh^{Q7/Q7}$) (Anborgh, Godin et al. 2005). Em camundongos $Hdh^{Q111/Q111}$, a Htt mutante leva à dessensibilização dos receptores mGluR5, resultando em menor formação de IP_3 e aumento da liberação de Ca^{2+} dos estoques intracelulares (Ribeiro, Paquet et al. 2010). Por outro lado, a ativação das vias protetoras da morte

neuronal, envolvendo AKT e ERK1/2, pelo mGluR5, ocorre de maneira mais pronunciada em camundongos *Hdh*$^{Q111/Q111}$ jovens, quando comparados a camundongos controle *Hdh*$^{Q20/Q20}$, indicando uma possível resposta adaptativa para assegurar a sobrevivência dos neurônios estriatais do modelo da DH(Ribeiro, Paquet et al. 2010).

Evidências científicas revelam que a sinalização via mGluR5 modula comportamentos motores, entretanto ainda não está claro quais substratos neurais estão envolvidos nesta modulação. Por outro lado, sabe-se que a sinalização via mGluR5 é sensível à presença da Htt mutante. Como os principais sintomas da DH são as alterações motoras, é possível que a sinalização via mGluR5 participe das alterações motoras observadas em pacientes comDH. Desta forma, o presente estudo testou as seguintes hipóteses: (1) a modulação exercida pelo receptor metabotrópico de glutamato 5 sobre o comportamento motor é dependente de substratos neurais específicos e (2) o receptor metabotrópico de glutamato 5 modula o comportamento motor em modelo animal de DH.

2. OBJETIVOS

2.1 – Objetivo geral

Identificar quais substratos neurais são alvos para a modulação do comportamento motor via mGluR5, bem como investigar o papel do mGluR5 nas alterações motoras observadas em um modelo animal da DH.

2.2 – Objetivos específicos

- Verificar o efeito da supressão do mGluR5, através de antagonistas seletivos ou através do *knockouteamento* do receptor, sobre a atividade locomotora espontânea e coordenação motora;

- Investigar o efeito da inibição do mGluR5 no bulbo olfatório, córtex motor, córtex parietal, hipocampo e corpo estriado sobre a atividade locomotora espontânea e a coordenação motora;

- Determinar se a supressão completa do mGluR5 em um modelo murino da DH, o $Hdh^{Q111/Q111}$, é capaz de alterar os sintomas motores relacionados à DH;

- Determinar se a proteína huntingtina mutante leva a alterações da expressão de proteínas importantes para a regulação da atividade locomotora mediada pelo mGluR5.

3. MATERIAIS E MÉTODOS

3.1 – Materiais

2 – propanol, solução Betaína, clorofórmio, dimetilsulfóxido (DMSO), etanol, cloreto de sódio (NaCl), paraformaldeído, proteinase K, a solução Tris (hidroximetil) aminometano–hidroclorídrico (Tris HCl) e a solução tris (hidroximetil) aminometano - ácido etilenodiamino tetra-acético (Tris-EDTA) foram adquiridos pela Sigma Aldrich®. Tubos tipo eppendorff 0,2; 0,6 e 1mL e ponteiras 10 µL, 200 µL, 1000 µL foram adquiridos pela Axygen Brasil. Agarose, sacarose e sulfato dodecil de sódio (SDS) foram adquiridos pela tanto pela empresa HEXAPUR quanto pela Sigma Aldrich®. Solução contendo sais de desoxirribonucleotídeos fosfatados, ou dNTPs, foi adquirido pela Invitrogen.H_2O – Nucleasefree foi adquirido pela Ambion®. Os iniciadores utilizados pelas reações de PCR e qPCR estão devidamente caracterizados nas tabelas 1 e 2. O kit Power SYBR® Green PCR Master Mix, bem como o SYBR® Safe foram adquiridos pela Life Technologies®. Lamínulas 12mm e laminas foram adquiridas pela Pró cito Laboratórios. As drogas n 2-metil-6-(feniletinil) piridina (MPEP)e 3-((2-Metil-4-tiazolil) etinil) piridina (MTEP) foram obtidas pela Tocris Cookson Inc. Ellisville, MO, USA.

3.2 – Métodos

3.2.1 - Animais

Este trabalho foi realizado utilizando-se camundongos machos C57/BL6 (8-9 semanas de idade) que foram adquiridos do Centro de Bioterismo (CEBIO) do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais - UFMG. Além disso, foram utilizados camundongos machos das linhagens *knock-in* para doença de Huntington – STOCK-*Htt*^{tm2Mem}/J (*Hdh*$^{Q20/Q20}$) e STOCK-*Htt*^{tm5Mem}/J (*Hdh*$^{Q111/Q111}$)

(Wheeler, Auerbach et al. 1999), bem como *knockout* para o mGluR5 – B6;129-Grm5^{tm1Rod}/J (*mGluR5*$^{-/-}$) (Lu, Jia et al. 1997), os quais foram obtidos do *Jackson Laboratory* (Bar Habor, USA). Camundongos das linhagens *Hdh*$^{Q111/Q111}$*/mGluR5*$^{-/-}$ e *Hdh*$^{Q111/Q111}$*/mGluR5*$^{+/+}$ foram obtidos através do cruzamento de *Hdh*$^{Q111/Q111}$, *mGluR5*$^{+/+}$ e *mGluR5*$^{-/-}$. Camundongos das linhagens *Hdh*$^{Q20/Q20}$*/mGluR5*$^{-/-}$ e *Hdh*$^{Q20/Q20}$*/mGluR5*$^{+/+}$ foram obtidas através do cruzamento de *Hdh*$^{Q20/Q20}$, *mGluR5*$^{+/+}$ e *mGluR5*$^{-/-}$. Os animais foram criados e mantidos no biotério do Departamento de Bioquímica e Imunologia do Instituto de Ciências Biológicas da UFMG em ciclo de 12 horas claro/escuro, com temperatura constante de 23°C e recebendo água e ração *ad libitum*. Os procedimentos experimentais utilizados neste trabalho receberam aprovação do CETEA-UFMG (Comitê de Ética em Experimentação Animal – UFMG), protocolo nº 274/2011 (Anexo 1).

3.2.2 – Genotipagem

Extração do DNA

A extração do DNA foi realizada a partir do recorte da cauda dos camundongos mutantes para *mGluR5*. Adicionou-se às amostras 500 µL de solução de extração (SDS 10%, Tris-EDTA, NaCl, Proteinase K), seguido de incubação *overnight* em banho-maria a 55 ºC. Após centrifugação a 15000 x *g* por 10 minutos (min) à temperatura ambiente o sobrenadante foi transferido para novos tubos tipo Eppendorf contendo 500µL de isopropanol. Em seguida foi realizada a homogeneização por inversão (10x) e uma nova etapa de centrifugação a 15000 x *g* por 10 min à temperatura ambiente. Após esta etapa, o sobrenadante foi descartado e os tubos foram deixados invertidos e abertos para secar por 1 hora à temperatura ambiente. Foi adicionado 200 µL de solução TE

0,5X (Tris HCl 1M, EDTA 0,5M) e deixado no banho-maria a 37ºC por 1 hora. As amostras de DNA foram armazenadas a 4ºC

Amplificação do DNA

Os iniciadores utilizados nas reações em cadeia da polimerase (PCR) para identificar as linhagens homozigotas mutantes (*mGluR5*$^{-/-}$), heterozigotas (*mGluR5*$^{+/-}$) e homozigotas selvagens (*mGluR5*$^{+/+}$) foram obtidos a partir do *Jackson Laboratory*, desenvolvidos a partir da sequência depositada no GenBank sob o identificador NM_001081414.2. A reação é realizada através da técnica de Multiplex PCR, no qual um mix de iniciadores é utilizado em cada reação, permitindo a identificação das linhagens numa mesma reação de PCR. As sequencias dos iniciadores bem como o tamanho dos amplicons gerados são mostrados na tabela 1, e um exemplo da identificação é mostrado na figura 2.

As reações foram realizadas utilizando 0,25 U da enzimaTaq DNA polimerase (Invitrogen), 2,00 mM de $MgCl_2$; 0,2 mM de dNTPs; 1,00 µM de cada iniciador (tabela 1),500 ng de DNA genômico, juntamente com o tampão fornecido pelo fabricante diluído para 1X, num volume final de 12 µL de reação. O programa de amplificação consistiu de uma etapa de desnaturação inicial a 94ºC por 3 min, seguida de 35 ciclos formados por uma etapa de desnaturação a 94ºC por 45 segundos, uma etapa de anelamento a 60ºC por 45 segundos e uma etapa de extensão a 72ºC por 45 segundos. A ciclagem termina com uma etapa de extensão final a 72ºC por 2min.

Tabela 1: *Iniciadores utilizados no PCR convencional*

Nome no estoque	Sequência	Amplicon (pb)	%GC
Primer comum	CACATGCCAGGTGACATCAT		50
Selvagem – reverso	CCATGCTGGTTGCAGAGTAA	442	50
Mutante – reverso	CACGAGACTAGTGAGACGTG	650	55

Após a amplificação, 12 µL do produto de PCR juntamente com 3µL de tampão de amostra 5x foram aplicados em gel não desnaturante contendo 2% de agarose, SYBR® Safe (Life Technologies) e solução tampão Tris-Acetato-EDTA (TAE) 0,5X. O gel foi acondicionado em uma cuba de eletroforese horizontal preenchida com TAE 0,5X e submetido a uma corrente de 100 volts por 30 min. A digitalização da imagem foi realizada utilizando o equipamento *ImageQuant LAS 4000* (GE Healthcare Life Sciences).

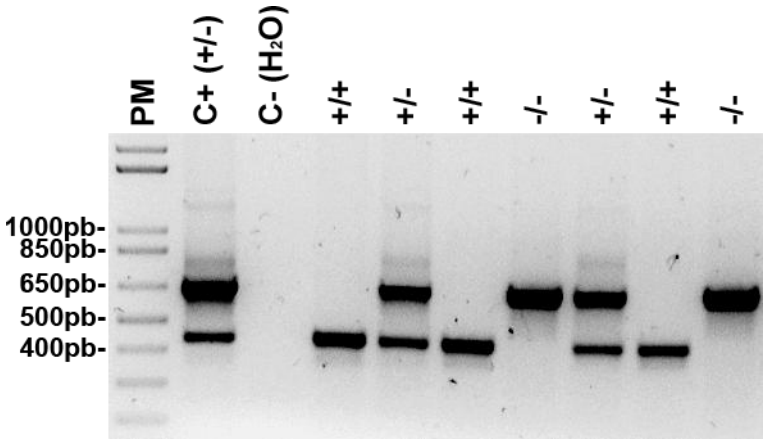

Figura 2:*Genotipagem de camundongos mutantes para o gene mGluR5*. O amplicon gerado pelos iniciadores da linhagem *mGluR5*$^{-/-}$possui 650 pb, da linhagem *mGluR5*$^{+/-}$ possui 650 e 442 pb, e da linhagem *mGluR5*$^{+/+}$possui 442pb.

Apenas os camundongos homozigotos *mGluR5*$^{+/+}$ e *mGluR5*$^{-/-}$ foram utilizados neste estudo.

3.2.3 – Drogas

O antagonista seletivo do mGluR5, 2-metil-6-(feniletinil)-piridina (MPEP), obtido da TocrisBioscience (Bristol, Reino Unido), foi diluído numa solução veículo contendo 50% de dimetilsulfóxido (DMSO) e 50% de solução salina (NaCl 0,9%), sendo administrado em cada estrutura neural na quantidade de 25 nmol, num volume final de 0,5 µL por lado(Phillips, Lam et al. 2006, Martinez-Rivera, Rodriguez-Borrero et al. 2013). Para referências futuras no texto usaremos a descrição 25 nmol/0,5

µL/lado. Já o antagonista seletivo do mGluR5 3-((2-Methyl-4-thiazolyl)ethynyl)pyridine (MTEP), obtido pela Tocris Bioscience (Bristol, Reino Unido), diluído em uma solução salina, foi administrado intraperitonealmente (i.p.) a 5,0 mg/kg.

3.2.4 – Cirurgia

Os camundongos não foram manipulados antes da cirurgia. No momento do procedimento cirúrgico, os animais foram anestesiados com cetamina (80mg/kg) e xilazina (10mg/kg) i.p. e, em seguida, posicionados no aparelho estereotáxico. O bregma e lambda foram alinhados nos planos horizontal e vertical. Pequenos orifícios (0.7 mm) foram feitos no crânio a partir das coordenadas estereotáxicas (tabela 2). Cânulas-guia bilaterais com hastes de metal oclusoras foram fixadas no crânio com cimento de zinco seguido de acrílico dental (Lazaroni, Raslan et al. 2012). Os camundongos receberam uma única dose intramuscular de flunixinameglumina (Banamine, 0,3 mg/kg) e duas doses de cefalexina (72 mg/kg) via oral. O tempo de recuperação pós-cirúrgico foi de 4 a 5 dias antes do início dos testes comportamentais.

Tabela 2: *Descrição das coordenadas estereotáxicas a partir do bregma e características das cânulas guia e injetora*.

Região cerebral alvo	Cânula guia(mm)	Cânula injetora(mm)	Antero Posterior (mm)	Medio lateral (mm)	Dorso ventral (mm)
Bulbo olfatório principal	8	9	+4,28	±1,0	-2,31
Corpo estriado	10	11	+1	±1,5	-3,6
Hipocampo	7	8	-1,9	±1,6	-1,0
Área motora primária (M1)	7	8	+1,94	±2,5	-1,15
Área associativa parietal (V2MM)	6	7	-2,54	±1,6	-0,40

3.2.5 – Micro-infusões

No momento da injeção das drogas, os camundongos foram gentilmente imobilizados e posicionados para retirada das oclusoras. A cânula injetora (30G) foi acoplada à cânula guia. Todos os fármacos foram infundidos em um volume de 0,5µL/lado. Uma bomba de micro-infusão, ligada a uma seringa de 10 µL (Hamilton), foi utilizada para controlar a injeção. As drogas foram injetadas bilateralmente nas regiões cerebrais (tabela 2) a uma taxa de 0,5 µL/min. A cânula injetora permaneceu posicionada por 1 min após a infusão para evitar a difusão da droga ao longo da cânula guia (Lazaroni, Raslan et al. 2012). Os animais foram submetidos aos testes comportamentais, 10 min após a remoção das cânulas injetoras.

3.2.6 – Verificação histológica

Ao final dos experimentos, os animais foram eutanaziados e os cérebros foram imediatamente removidos e estocados em paraformaldeído 4% por um dia, seguido por dois dias em sacarose 30%. Secções coronais (100 µm de espessura a partir dos sinais da cânula) foram obtidas no criostato (-20ºC). As lâminas foram coradas com vermelho neutro e os sítios de injeção foram verificados por microscopia óptica. Apenas os camundongos com cânulas posicionadas corretamente no alvo desejado foram incluídos na análise estatística (Rinaldi, Mandillo et al. 2007, Pereira, Bastos et al. 2014).

3.2.7 – Campo aberto

Para avaliar a atividade locomotora espontânea foi utilizado um sistema automático de campo aberto (LE 8811 IR Monitores de Atividade MotoraPANLAB / HARVARD APPARATUS), com dimensões da caixa acrílicade 450x450x200mm (largura x profundidade x altura) (Pereira, Bastos et al. 2014), como mostrado na figura 3A.

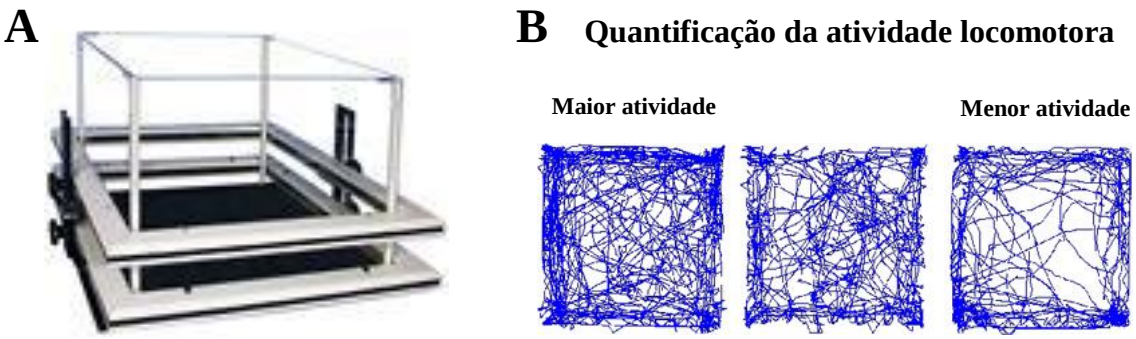

Figura 3: *Representação do LE 8811 - IR Actímetro, aparato de campo aberto.*Em (**A**) o equipamento PANLAB/HARVARD- Actímetro infravermelho (IR) permite o estudo da atividade locomotora espontânea em roedores. Em (**B**) a representação gráfica da atividade locomotora gerada pelo equipamento.

Os camundongos foram habituados na sala de teste comportamental por, no mínimo, 60 min. Durante os testes com infusão da droga, os animais foram inseridos no aparato de campo aberto 10 min após a injeção da droga ou veículo. As atividades detectadas no plano horizontal (distância percorrida) (figura3B) foram medidas durante 60 min. A medida da atividade total foi calculada usando o programa ACTITRACK e as análises estatísticas foram realizadas utilizando o software GraphPad Prism versão 5 (GraphPad Software Inc., San Diego, USA), que serão descritas em detalhes posteriormente.

3.2.8 – Rota Rod teste

O treinamento e o teste comportamental dos camundongos, no sistema Rota Rod (Insight®) (figura4A), ocorreu durante o ciclo de luz entre 08:00 e 12:00 h. Os camundongos foram habituados na sala de testes comportamentais por, no mínimo, 60 min.

A **B**

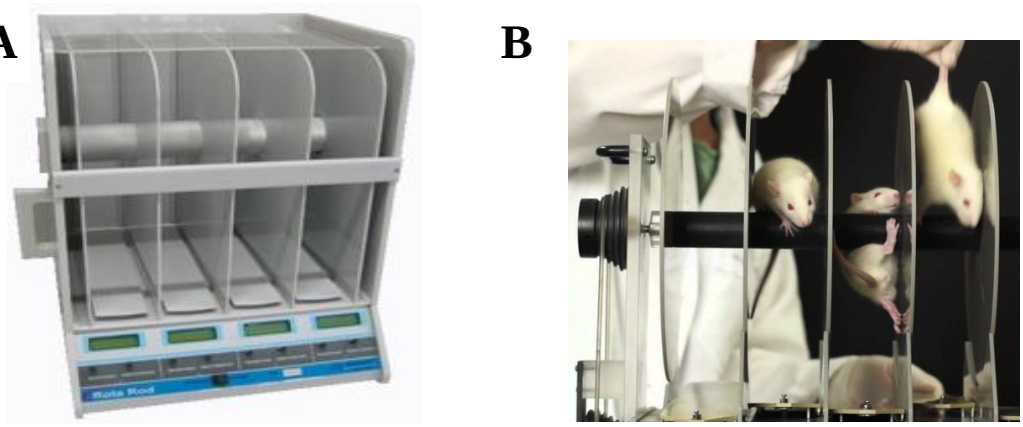

Figura 4: *Representação do aparato de Rota Rod Insight*®. Em (**A**) o aparato de Rota Rod (*Insight*®) permite o estudo da coordenação motora e equilíbrio em roedores. A representação dos camundongos alocados no cilindro giratório do aparato é mostrada em (**B**).

Inicialmente os animais foram submetidos a um protocolo de treinamento no equipamento Rota Rod, exemplificado na figura 4B. No primeiro e segundo dias de treino, os camundongos foram alocados sobre o cilindro giratório durante 2 min, nas cinco primeiras velocidades, que variaram de 5 a 19 rotações por minuto. No dia do teste de infusão das drogas, os camundongos foram inseridos no Rota Rod 10 min após a injeção de veículo ou droga. O protocolo de aceleração foi realizado em três ensaios independentes com um intervalo entre ensaios de 10 min. Caso os camundongos caíssem nos primeiros 10 segundos, eles eram realocados imediatamente no aparelho e sua contagem era reiniciada. A latência de queda do cilindro giratório foi registrada, e a média obtida a partir dos três ensaios foi utilizada para a análise. O tempo limite para os camundongos permanecerem no Rota Rod foi de até 300 segundos. Após este período os camundongos foram removidos do aparelho.

3.2.9 – RT-PCR quantitativo(qPCR)

Amostras de corpo estriado das linhagens $Hdh^{Q20/Q20}/mGluR5^{-/-}$ e $Hdh^{Q111/Q111}/mGluR5^{-/-}$ foram retiradas por microdissecção em solução salina. O RNA

total das amostras foi isolado pelo método TRIzol® (Invitrogen, Burlington, EUA), segundo instruções do fabricante. O RNA foi ressuspendido em 20µL de água livre de nucleases (Ambion®), tendo sua concentração e qualidade analisadas por espectrofotômetro (NanoDrop – ThermoScientific, Wilmington, USA) e eletroforese em gel de agarose, respectivamente. Os cDNAs foram preparados a partir de 40 ng de RNA total em 20 µL final da reação de transcrição reversa.

O PCR quantitativo (qPCR) foi realizado utilizando o kit Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, USA) segundo protocolo do fabricante. Para isso foram utilizadas as plataformas de PCR em tempo real StepOnePlus™ Real-Time PCR Systems e ABI PRISM 7900HT Sequence Detection System (AppliedBiosystems, Foster City, USA). A reação de qPCR foi realizada a fim de quantificar os níveis de mRNA dos genes seguintes: Dineína cadeia leve LC8-tipo 1 – *Dynll1* (NM_019682); dinactina 6 – *Dctn6* (NM_ 011722); dineína cadeia pesada axonemal 6 – *Dnahc6* (NM_ 001164669); dinactina3 – *Dctn3* (NM_016890); dineína cadeia leve Tctex-tipo 1B – *Dynlt1b* (NM_009342). Os iniciadores, dos respectivos genes, foram desenhados com o auxílio do software Primer3plus (Untergasser, Nijveen et al. 2007) e são mostrados na tabela 3. Os iniciadores foram checados utilizando a ferramenta Primer-BLAST (Ye, Coulouris et al. 2012) para avaliação *in silico* da sua especificidade para com seus alvos.

As amostras foram preparadas em triplicatas e as variações da expressão gênica foram determinadas pelo método do ΔCt utilizando o gene da actina (NM_007393.3) como gene constitutivo. Todas as reações de qPCR mostraram boa qualidade de amplificação e a eficiência dos iniciadores foi testada pelo método de diluição seriada.

Tabela 03:*Características dos iniciadores utilizados nas reações deqPCR.*

Código	Sequência	Amplicon (pb)	Tm (ºC)	%GC	Nº identificador
Dynlt1b - F	TCATGCAGAAGAACGGTGCT	64	57,0	50,0	NM_009342.2
Dynlt1b - R	TCTGTGGAGCTGTCCCAGAA		58,1	55,0	
Dnahc6 - F	CGCAAGGAAGATGACACAGA	116	54,9	50,0	NM_001164669.1
Dnahc6 - R	TTAGAGACCCAGCCATGACC		56,4	55,0	
Dctn3 - F	CAGATCCACATCCAGCAGCA	70	57,4	55,0	NM_016890
Dctn3 - R	ACCCTTCCAGGAGAGCCTTA		57,5	55,0	
Dctn6 - R	ATAGGTTTGGGCTCTGTATCTTC	145	54,2	43,4	NM_011722.3
Dctn6 - F	TGATCCACCCTAAAGCACG		54,7	52,6	
Dynll1 - R	CTTAACTGCCCTATCTGTGGTC	143	54,9	50,0	NM_019682.4
Dynll1 - F	TTTGTCCCTGCCAAGTACTG		55,0	50,0	
Actina - R	AATGCCTGGGTACATGGTGGTA	122	58,4	50,0	NM_007393.3
Actina - F	TGGAATCCTGTGGCATCCATGA		58,6	50,0	

3.2.10 – Análise estatística

As análises da média e do erro padrão da média (SEM), nos testes de comportamento, foramrealizadas a partir dos testes *t de Student* e ANOVA. Para os resultados do qPCR, a média e o erro padrão foram calculados a partir de triplicatas técnicas e analisados pelo teste *t* de *Student*, como indicados nas legendas das figuras. Em ambos os métodos, as análises estatísticas foram realizadas com o auxílio do software GraphPadPrism® versão 5 (GraphPad Software Inc., San Diego, USA), sendo considerados significativos os testes que obtiveram valores de $p < 0,05$.

4. RESULTADOS

4.1 –O mGluR5 é capaz de modular a atividade motora em camundongos:

Estudos recentes mostram que o mGluR5 possui um papel importante no controle da atividade locomotora (Jew, Wu et al. 2013, Ribeiro, Devries et al. 2014). Para confirmar a influênciado mGluR5 na atividade locomotora, foi utilizado o modelo experimental mGluR5 *knockout (mGluR5$^{-/-}$)*, o qual apresenta supressão completa deste receptorem camundongo C57/BL6(Lu, Jia et al. 1997). Duas ferramentas de avaliação comportamental foram utilizadas, sendo que ambas permitem avaliar o desempenho motor: campo aberto, que quantifica a atividade locomotora espontânea (figura 3 A-B) e o Rota Rod, que avalia a coordenação motora (figura 4 A-B) (Brooks and Dunnett 2009).

Para avaliar a atividade locomotora espontânea do camundongo, animais *mGluR5$^{-/-}$* foram alocados em um aparato de campo aberto, sendo a distância percorrida pelo animal registrada a cada 5 min. Camundongos *mGluR5$^{-/-}$* apresentaram maior atividade locomotora por intervalo de tempo em comparação ao seu controle *mGluR5$^{+/+}$* ao longo dos 60min avaliados (figura 5A), o que reflete em maior distância total percorrida ao longo deste tempo (figura5B).

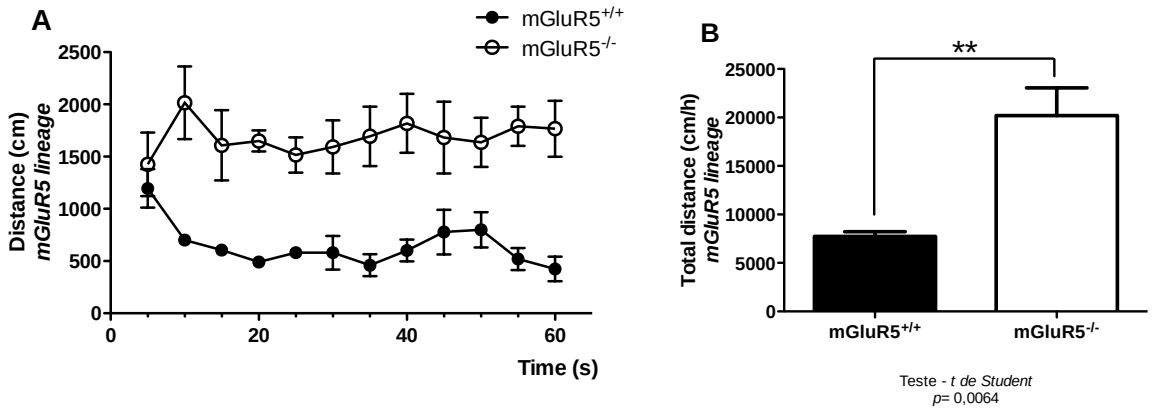

Figura 5: *Camundondos mGluR5*$^{-/-}$ *apresentam maior atividade locomotora.*(**A**) Camundongos *mGluR5*$^{+/+}$(n=3) e *mGluR5*$^{-/-}$(n=3) foram inseridos no aparato de campo aberto e a atividade locomotora foi medida como a distância percorrida (cm) a cada intervalo de 5 min, por um total de 60min. (**B**) Distancia total percorrida em 60min. Os dados representam a média ± SEM. Os ** representa $p < 0,005$ no teste *t* de *Student*.

Com o objetivo de avaliar o equilíbrio e a coordenação motora, os camundongos *mGluR5*$^{-/-}$e *mGluR5*$^{+/+}$ foram submetidos ao teste de Rota Rod. Camundongos *mGluR5*$^{-/-}$ apresentaram latência maior para cair do Rota Rod quando comparados aos camundongos *mGluR5*$^{+/+}$, indicando que camundongos *mGluR5*$^{-/-}$ apresentam melhor coordenação motora (Figura 6).

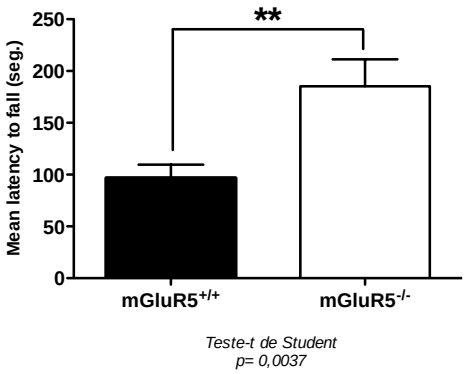

Figura 6: *Camundondos mGluR5*$^{-/-}$ *apresentam melhor desempenho motor no teste de RotaRod.* Os camundongos mGluR5$^{+/+}$(n=8) e mGluR5$^{-/-}$(n=7) foram submetidos ao aparato de Rota Rod e o desempenho motor foi medido como a latência de queda medida em segundos. Os dados representam a média ± SEM. Os ** representa $p < 0,005$ no teste *t* de *Student.*

Além da hiperlocomoção resultante da deleção gênica do mGluR5, estudos indicam que o bloqueio farmacológico deste receptor (via i.p.) também promove aumento da locomoção (Jew, Wu et al. 2013, Ribeiro, Devries et al. 2014). Entretanto, ainda não está claro quais substratos neurais estão envolvidos no controle motor via mGluR5. Está bem estabelecido na literatura que algumas regiões específicas do cérebro, tais como córtex motor primário e os núcleos da base estão envolvidos no controle motor (Reiner, Medina et al. 1998, Herrero, Barcia et al. 2002, DeLong and Wichmann 2007, Stephenson-Jones, Ericsson et al. 2012). Com o objetivo de determinar quais regiões cerebrais são importantes para o controle motor via mGluR5, foram realizadas microinfusões do antagonista do mGluR5, MPEP, em regiões cerebrais primariamente motoras e de ampla expressão do receptor(Shigemoto, Nomura et al. 1993, Yu, Tueckmantel et al. 2005). Para tal, camundongos C57/BL6 foram microinfundidos com MPEP25 nmol/0,5µL/lado e submetidos aos testes comportamentais motores.

Para verificar se o mGluR5 expresso no córtex motor primário (M1) é importante para modular a atividade locomotora espontânea, foi realizada a microinfusão de MPEP na coordenada estereotáxica específica (figura7D) e, após 10 min, o camundongo foi alocado no aparato de campo aberto.

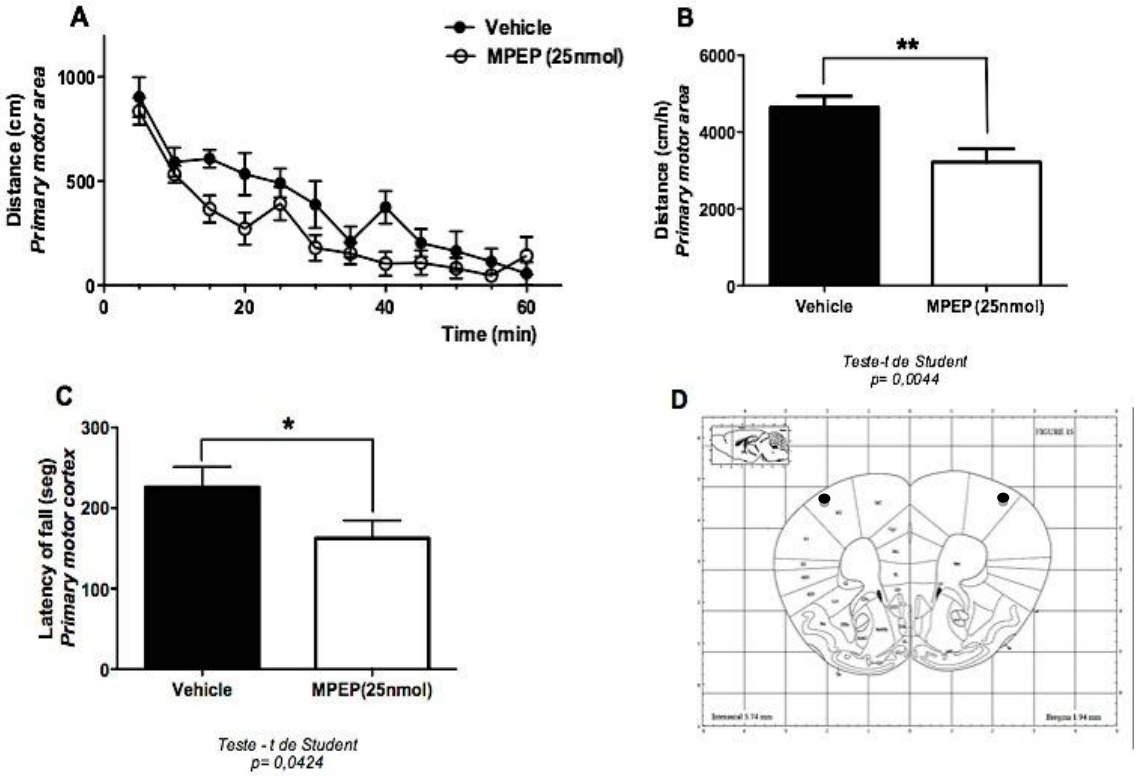

Figura 7: *MPEP reduz aatividade motora quando injetado no córtex motor primário.*Os gráficos **A** e **B** mostram a distância percorrida pelos camundongos selvagens injetados com veículo (n = 7) ou MPEP 25nmol/0,5µL/lado (n = 8). Cada animal foi monitorizado durante 60 min e a distância total foi medida em intervalos de 5 min (**A**) ou cumulativamente durante 60 min (**B**). Os animais foram alocados no aparato de campo aberto após 10 min de microinfusão do veículo ou MPEP. (**C**) O gráfico mostra a latência de queda de camundongos selvagens submetidos ao aparato de Rota Rod que foram injetados com veículo (n= 7) ou MPEP 25 nmol/0,5µL/lado (n = 7). (**D**) Representação gráfica da região de injeção da droga, segundo coordenadas de M1. Esferas pretas indicam a região alvo. Os dados representam as médias ± SEM. Os** representa $p < 0,005$ no teste *t* de *Student* na comparação da droga com o veículo.

A microinfusão de MPEP em M1 promoveu a redução da atividade locomotora comparado à infusão do veículo (figura 7A-B), confirmando o efeito estimulatório do mGluR5 na área motora primária. Para determinar se os receptores mGluR5 expressos em M1 são capazes de alterar o equilíbrio e a coordenação motora dos camundongos, foram realizadas microinfusões de MPEP nesta região e, posteriormente, os camundongos foram submetidos ao Rota Rod. Sustentando a importância da região de M1 na resposta locomotora, os camundongos injetados com a droga apresentaram

latência de queda significativamente reduzida em relação aos animais injetados com o veículo (figura 7C).

Entretanto, dados da literatura indicam que camundongos apresentando deleção gênica específico no córtex cerebral possuem aumento da atividade locomotora quando comparado ao controle (Jew, Wu et al. 2013). Nesse sentido, para melhor entender o papel do mGluR5 expresso no córtex cerebral, investigamos se outra região cortical é capaz de modular a atividade locomotora.

O córtex parietal posterior é uma região cortical associativa envolvida no controle de movimentos visualmente guiados, orientação espacial e de ampla expressão do mGluR5(Calton and Taube 2009). Considerando que tal área poderia alterar o desempenho motor dos camundongos, foi realizada a microinfusão de MPEP em coordenada estereotáxica específica(área cortical visual secundária- V2MM) (figura8 D) e os camundongos foram submetidos aos testes de comportamento. Os camundongos injetados com MPEP não apresentaram diferença significativa em termos de atividade locomotora no campo aberto (figura8 A-B) ou coordenação motora no Rota Rod (figura 8 C), quando comparados aos camundongos injetados com veículo.

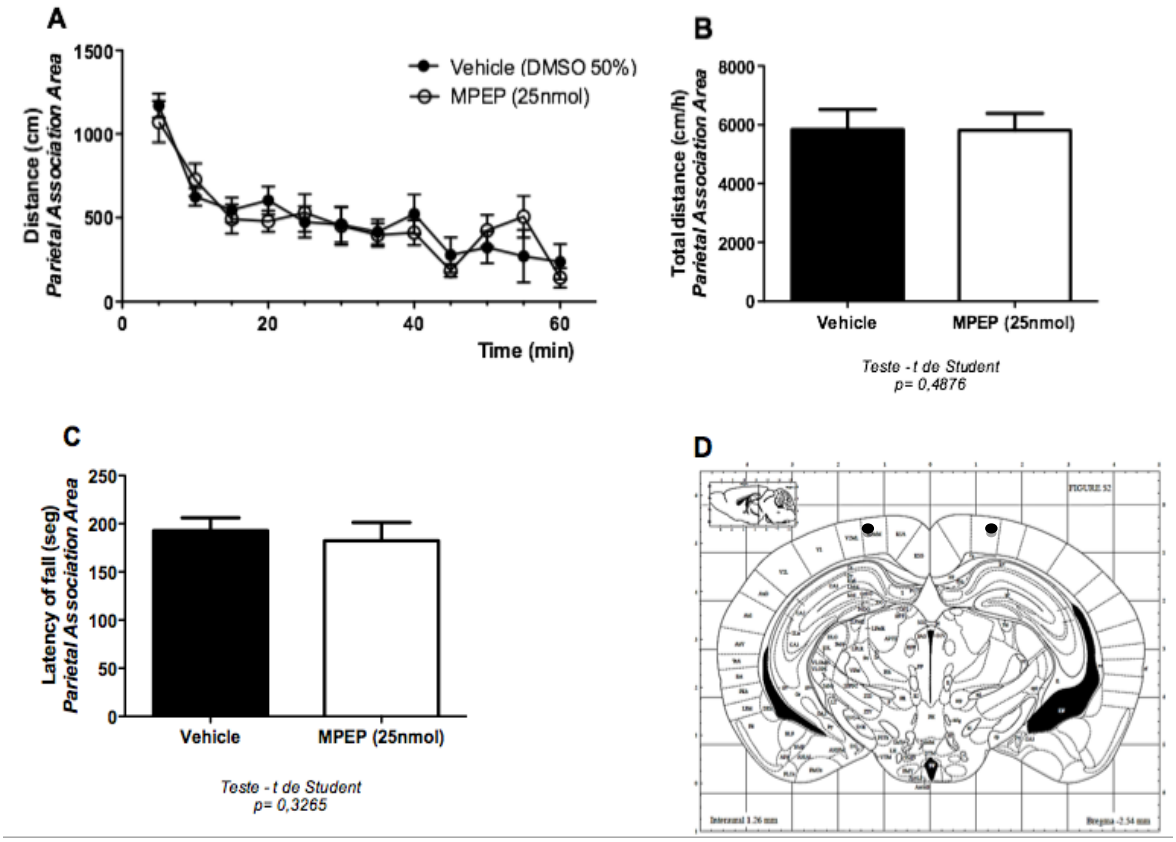

Figura 8:*O antagonismo do mGluR5 no córtex parietal não altera a atividade locomotora.* Os gráficos **A** e **B** mostram a distância percorrida pelos camundongos selvagens injetados com veículo (n = 6) ou MPEP (n = 6). Cada animal foi monitorado durante 60 min e a distância total foi medida em intervalos de 5 min (**A**) ou cumulativamente em 60 min (**B**). Os animais foram alocados no aparato de campo aberto após 10 min de microinfusão do veículo ou MPEP (25 nmol/0,5µL/lado). (**C**) O gráfico mostra a latência de queda de camundongos selvagens submetidos ao aparato de Rota Rod que foram injetados com veículo (n = 6) ou MPEP 25 nmol/0,5µL/lado (n = 6). (**D**) Representação gráfica da região de injeção da droga, segundo coordenadas de V2MM. Esferas pretas indicam a região alvo. Os dados representam a média ± SEM. O * representa $p < 0,05$ no teste *t* de *Student* na comparação da droga com o veículo.

O corpo estriado, onde o mGluR5 é altamente expresso, é uma região importante para o controle motor (Reiner, Medina et al. 1998). Com o objetivo de determinar se o mGluR5 expresso no corpo estriado é importante para o controle motor, foi realizada a microinfusão de MPEP em coordenada específica do corpo estriado de camundongos (figura 9 D) e os animais foram alocados em um aparato de campo aberto. A distância total percorrida pelos animais injetados com MPEP foi significativamente menor quando comparadoaos animais injetados com veículo (figura9 B). No entanto, não

houve diferença significativa no teste de Rota Rod entre os camundongos injetados com MPEP ou com veículo (figura 9C).

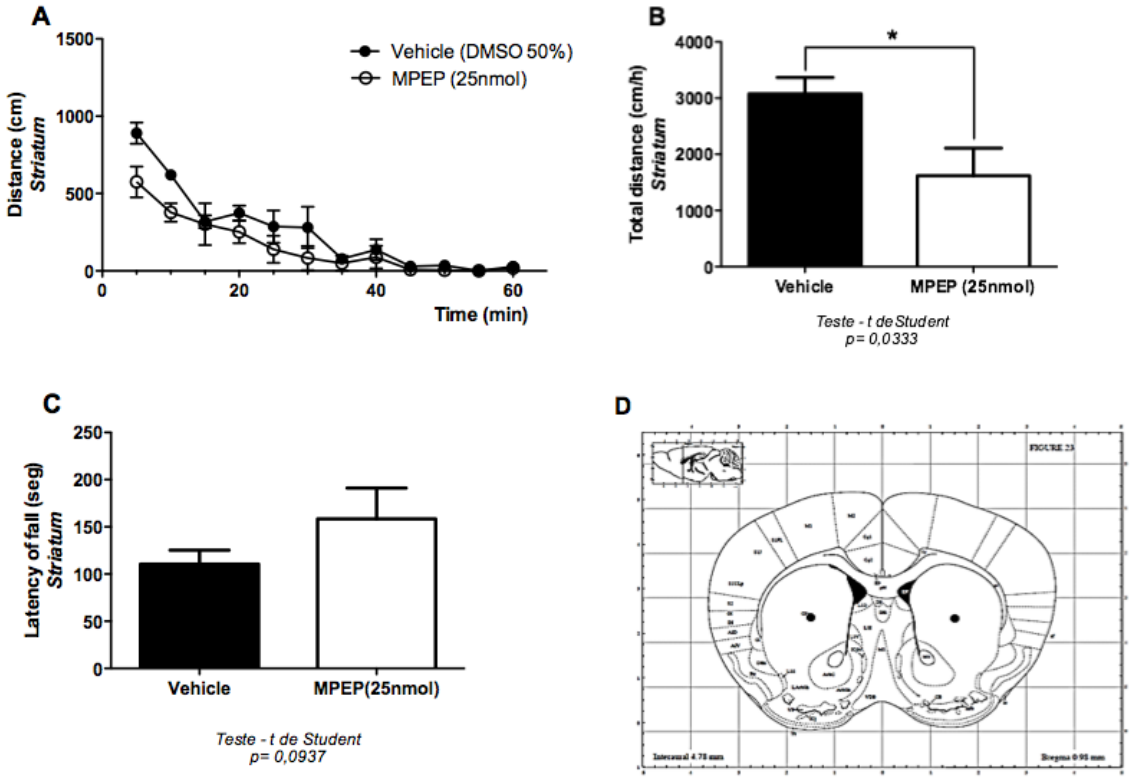

Figura 9: *A microinfusão de MPEP no corpo estriado reduz a atividade locomotora espontânea.* Os gráficos **A** e **B** mostram a distância percorrida pelos camundongos selvagens injetados com veículo (n = 5) ou com MPEP (n = 5). Cada animal foi monitorado durante 60 min e a distância total foi medida em intervalos de 5 min (**A**) ou cumulativamente em 60 min (**B**). Os animais foram alocados no aparato de campo aberto após 10 min de microinfusão do veículo ou MPEP (25nmol/0,5µL/lado). (**C**) O gráfico mostra a latência de queda de camundongos selvagens submetidos ao aparato de rotarod que foram injetados com veículo (n = 7) ou MPEP 25nmol/0,5µL/lado (n = 6). (**D**) Representação gráfica da região de injeção da droga, segundo coordenadas do corpo estriado. Esferas pretas indicam a região alvo. Os dados representam as médias ± SEM. O * representa $p < 0,05$ no teste *t* de *Student* na comparação da droga com o veículo.

A atividade motora dos camundongos injetados com MPEP no M1 e no corpo estriado nos sugere, portanto, que o mGluR5 possui um efeito excitatório sobre estruturas motoras cerebrais. Sendo assim, investigamos o papel do mGluR5 expresso em outras estruturas do cérebro que não estão envolvidas primariamente em movimento, mas que podem interferir no controle locomotor de camundongos.

O hipocampo, outra área cerebral com vasta expressão de mGluR5, está envolvido nos processos de memóriae de emoção (Fanselow and Dong 2010). Embora se trate de uma região que não está diretamente envolvida no controle dos movimentos, foi avaliado o papel do hipocampo na resposta motora aguda. Para isso, foi realizada a microinfusão de MPEP a 25 nmol/0,5µL/lado em coordenada estereotáxica específica (figura 10 D) e, posteriormente, os animais foram submetidos aos testes de comportamento. Camundongos injetados com MPEP percorreram uma distância maior na arena quando comparados ao controle(figura 10 A-B). Por outro lado, não foram observadas diferenças no desempenho motor no teste Rota Rod realizados pelos camundongos injetados com MPEP ou veículo (figura 10 C).

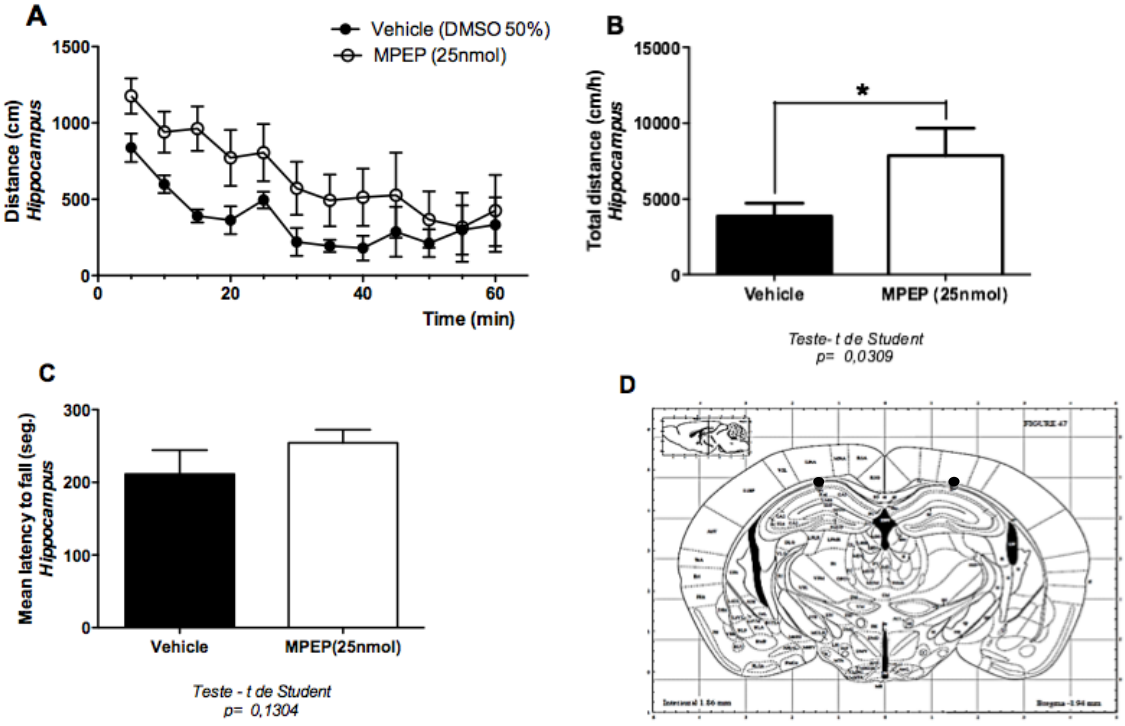

Figura 10:*A injeção de MPEP no hipocampo aumenta a atividade locomotora espontânea.*Os gráficos**A** e **B** mostram a distância percorrida pelos camundongos selvagens injetados com veículo (n = 6) ou com MPEP (n = 5). Cada animal foi monitorado durante 60 min e a distância total foi medida em intervalos de 5 min (**A**) ou cumulativamente em 60 min (**B**). Os animais foram alocados no aparato de campo aberto após 10 min de microinfusão do veículo ou MPEP (25nmol/0,5µL/lado). (**C**) O gráfico mostra a latência de queda de camundongos selvagens submetidos ao aparato de Rota Rod que foram injetados com veículo (n = 7) ou MPEP 25nmol/0,5µL/lado (n = 6). (**D**) Representação gráfica da região de injeção da droga, segundo coordenadas do

hipocampo. Esferas pretas indicam a região alvo. Os dados representam as médias ± SEM. O * representa $p < 0,05$ no teste *t* de *Student* na comparação da droga com o veículo.

Por fim decidimos por bloquear o mGluR5 no bulbo olfatório, que é uma região cerebral que expressa altos níveis de mGluR5 mas que não está envolvida no controle motor. Trata-se de uma região primariamente sensorial, relacionada às impressões olfativas(Auffarth 2013). Dessa forma, foi injetado MPEP na coordenada específica desta região (figura 11 D) e posteriormente os animais foram submetidos a testes comportamentais. Como observado na figura 11 (A-B), a atividade locomotora espontânea dos camundongos injetados com a droga não foi diferente do controle. Por outro lado, surpreendentemente, os camundongos injetados com MPEP e submetidos ao Rota Rod apresentaram um desempenho motor inferior, quando comparados aos camundongos injetados com veículo(figura 11C).

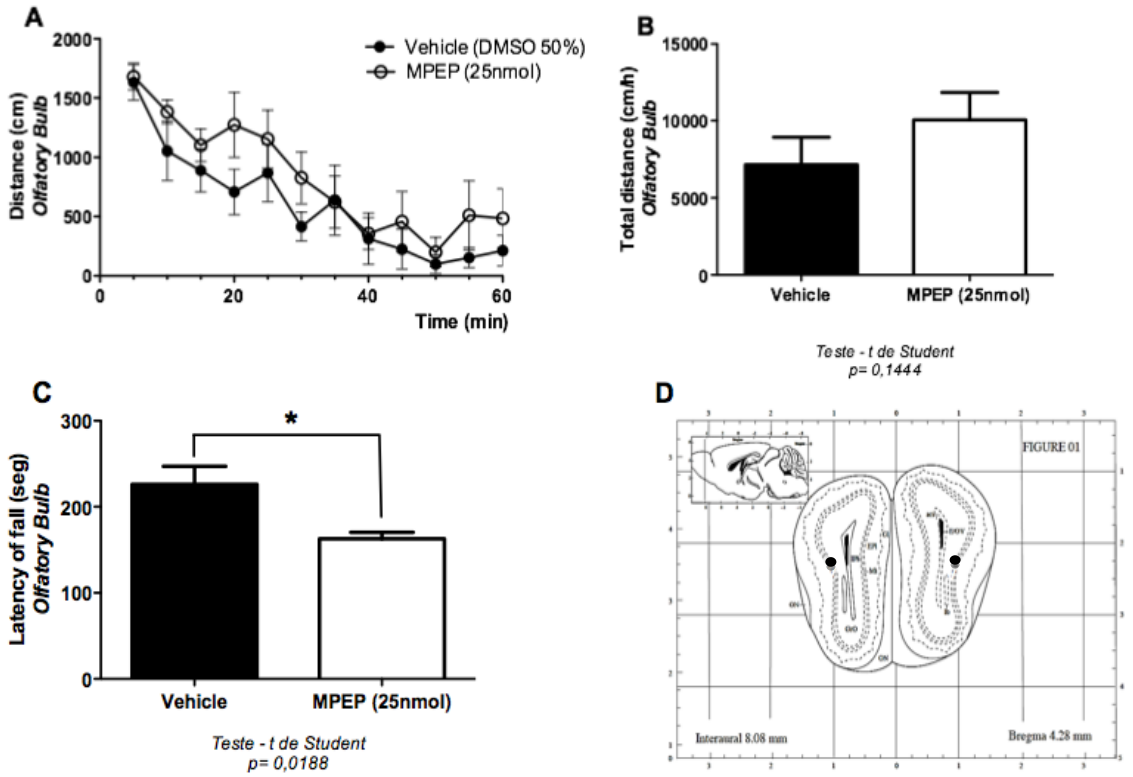

Figura 11:*A injeção de MPEP no bulbo olfatório diminui o desempenho motor no Rota Rod.* Os gráficos **A** e **B** mostram a distância percorrida por camundongos selvagens injetados com veículo (n = 7) ou com MPEP (n = 5). Cada animal foi monitorado durante 60 min e a distância total foi medida em intervalos de 5 min (**A**) ou

cumulativamente por 60 min (**B**). Os animais foram alocados no aparato de campo aberto após 10 min de microinfusão do veículo ou MPEP (25nmol/0,5µL/lado). (**C**) O gráfico mostra a latência de queda de camundongos selvagens submetidos ao aparato de rotarod que foram injetados com veículo (n = 5) ou MPEP 25nmol/0,5µL/lado (n = 4). (**D**) Representação gráfica da região de injeção da droga, segundo coordenadas do *bulbo olfatório*. Esferas pretas indicam a região alvo. Os dados representam as médias ± SEM. O * representa $p < 0,05$ no teste *t* de *Student* na comparação da droga com o veículo.

Nossos dados confirmam que o mGluR5 desempenha um papel importante para o controle do movimento, sugerindo um efeito excitatório sobre o estruturas cerebrais primariamente motoras. Por outro lado, a atividade do mGluR5 na modulação da resposta locomotora parece estar relacionada também a outras estruturas não exclusivamente motoras, como hipocampo e áreas primariamente sensoriais.

4.2 –O modulação da atividade locomotora, via mGluR5, é alterada em camundongos modelo da DH

Como demonstramos que o mGluR5 está intimamente associado à regulação motora, foi questionado se o mGluR5 teria alguma influencia nossinais motores observados na doença de Huntington. Para tal, utilizamos o modelo experimental *knock-in* da DH ($Hdh^{Q111/Q111}$) e seu respectivo controle $Hdh^{Q20/Q20}$ (Wheeler, Auerbach et al. 1999).

Para testar se a alteração na atividade locomotora causada pela supressão do mGluR5 poderia ser reproduzida farmacologicamente nos camundongos modelo para a DH, foi avaliado o efeito do MTEP, antagonista mGluR5, sobre a atividade locomotora em ambas as linhagens $Hdh^{Q20/Q20}$ e$Hdh^{Q111/Q111}$. Injeções i.p. de MTEP, a 5,0mg/kg, foram realizadas e 10 min após os camundongos foram alocados no campo aberto. Tanto a linhagem $Hdh^{Q111/Q111}$quanto $Hdh^{Q20/Q20}$ apresentaram aumento da locomoção quando comparados com animais injetados com solução salina (figura 12A). A

quantificação da atividade locomotora total (figura 12B) confirma a hiperlocomoção nas linhagens $Hdh^{Q111/Q111}$ e $Hdh^{Q20/Q20}$.

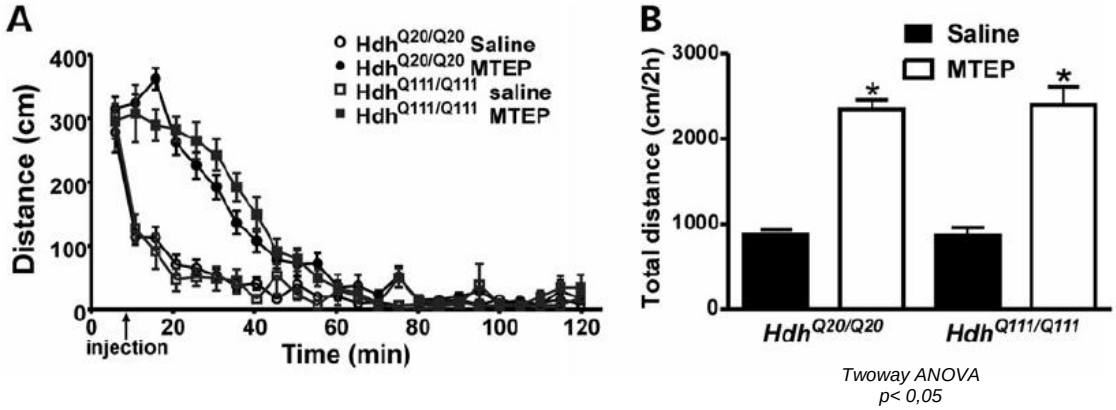

Figura 12: *O tratamento intraperitonial com o antagonista do mGluR5 (MTEP) aumenta a atividade locomotora tanto em camundongos modelo para DH quanto em camundongos controle.*(**A**) Camundongos $Hdh^{Q20/Q20}$ e $Hdh^{Q111/Q111}$ (n=12) foram inseridos no aparato de campo aberto e a atividade locomotora foi medida como distância percorrida (cm) a cada 5 min. 10 min após os camundongos serem introduzidos ao aparato de campo aberto, os mesmos foram injetados com salina ou MTEP (5 mg/kg). O tempo total de medida da atividade locomotora foi de 120 min.**(B)** A distância total percorrida em 120 min foi quantificada. Os dados representam a média ± SEM. $Hdh^{Q20/Q20}$ + salina (n=7), $Hdh^{Q20/Q20}$ + MTEP (n=8), $Hdh^{Q111/Q111}$+ salina (n=6) e $Hdh^{Q111/Q111}$+ MTEP (n=8).O * representa $p < 0,05$ no teste ANOVA de duas vias seguido do pós teste de *Bonferroni* para comparação entre o tratamento com a droga e com o veículo.

Para melhor entender o papel do mGluR5 ao longo do desenvolvimento da DH, foram realizados cruzamentos entre a linhagem $mGluR5^{-/-}$ e o camundongo modelo da doença de Huntington ($Hdh^{Q111/Q111}$) e seu controle ($Hdh^{Q20/Q20}$). A seguir, as linhagens geradas foram submetidas ao campo aberto (figura13 A-B). A atividade motora total da linhagem $Hdh^{Q20/Q20}/mGluR5^{-/-}$ evidencia hipercinesia quando comparado à linhagem $Hdh^{Q20/Q20}/mGluR5^{+/+}$ (figura 13B). Este dado confirma as alterações motoras provenientes da deleção gênica do receptor mGluR5 (figura 5), bem como do antagonismo agudo do MTEP, visto anteriormente (figura12).

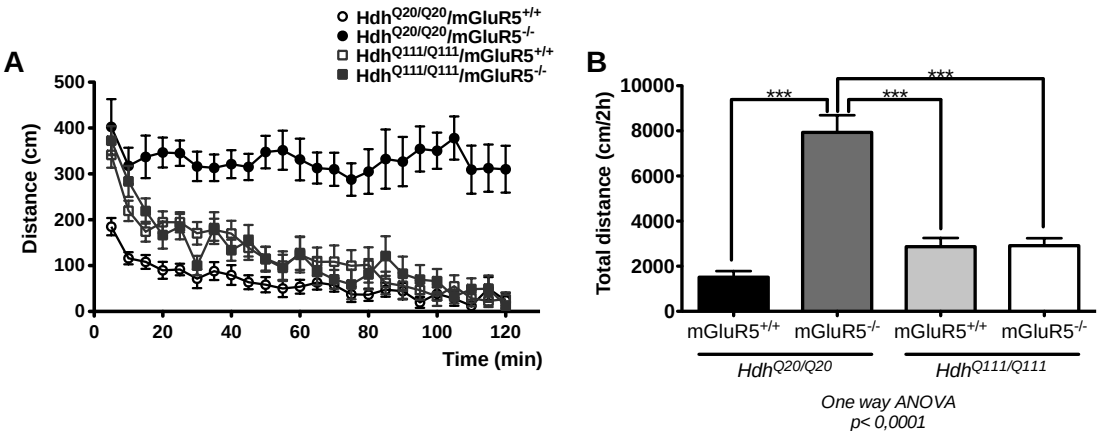

Figura 13: *A Htt mutante anula a hiperlocomoção induzida pelo mGluR5$^{-/-}$.*(**A**) Camundongos *Hdh$^{Q20/Q20}$/mGluR5$^{+/+}$* (n=11), *Hdh$^{Q20/Q20}$/mGluR5$^{-/-}$* (n=11), *Hdh$^{Q111/Q111}$/mGluR5$^{+/+}$* (n=12) e *Hdh$^{Q111/Q111}$/mGluR5$^{-/-}$* (n=11),com 3 meses de idade, foram inseridos no aparato de campo aberto e a atividade locomotora foi medida como distância percorrida (cm) a cada 5 min. **(B)** A distância total percorrida em 120 min foi quantificada. Os dados representam a média ± SEM. Os*** representam $p < 0{,}0001$ no teste ANOVA de uma via seguido do pós-teste de *Bonferroni* para comparação entre as linhagens dos camundongos.

Todavia, os camundongos *Hdh$^{Q111/Q111}$/mGluR5$^{-/-}$* não apresentaram aumento da atividade locomotora como observado com o antagonismo agudo utilizando MTEP(figura 13 B). Esses resultados sustentam a hipótese de que o mGluR5 está intrinsecamente envolvido nas alterações motoras promovidas pela proteína mutante Htt. Além disso, esses dados indicam que a deleção gênica do receptor e a presença da Htt mutante conferem adaptações de longo prazo às quais não são observadas com o antagonismo agudo do mGluR5.

Não se sabe o mecanismo biológico subjacente à modulação da atividade locomotora promovida pelo bloqueio do mGluR5. Desta forma, decidimos analisar se a atividade da Htt mutante no camundongo *mGluR5$^{-/-}$* poderia alterar a expressão de genes que estão supostamente envolvidos nas alterações motoras encontradas na DH. Neste sentido, foi realizado o ensaio de microarranjo com o objetivo de avaliar, em larga escala, as diferenças de expressão gênica entre as linhagens *Hdh$^{Q20/Q20}$/mGluR5$^{+/+}$*, *Hdh$^{Q20/Q20}$/mGluR5$^{-/-}$*, *Hdh$^{Q111/Q111}$/mGluR5$^{+/+}$* e *Hdh$^{Q111/Q111}$/mGluR5$^{-/-}$*.

Os níveis de expressão de mGluR5 (tabela 4) mostraram-se reduzidos nas linhagens *mGluR5*$^{-/-}$ comparadas ao seu controle *mGluR5*$^{+/+}$, ou seja *Hdh*$^{Q111/Q111}$*/mGluR5*$^{-/-}$ vs. *Hdh*$^{Q111/Q111}$*/mGluR5*$^{+/+}$ e *Hdh*$^{Q20/Q20}$*/mGluR5*$^{-/-}$ vs. *Hdh*$^{Q20/Q20}$*/mGluR5*$^{+/+}$. Estes dados pressupõem que o ensaio de microarranjo foi realizado adequadamente, sendo capaz de detectar variações na expressão gênica.

Tabela 4: *Grupo de genes com regulação alterada em Hdh*$^{Q111/Q111}$*/mGluR5*$^{-/-}$*vs. Hdh*$^{Q20/Q20}$*/ mGluR5*$^{-/-}$ *que não estão alterados em Hdh*$^{Q111/Q111}$*/mGluR5*$^{+/+}$*vs. Hdh*$^{Q20/Q20}$*/mGluR5*$^{+/+}$.Dynein heavy chain axonemal 6 - *Dnahc6* (NM_ 001164669); dynactin 3 - *Dctn3* (NM_016890); dynein light Tctex chain-type 1B - *Dynlt1b* (NM_009342); glutamate receptor, metabotropic 5 - *Grm5* (NM_001081414.2). Os efeitos de genótipos foram considerados significativos com base nos seguintes critérios: ANOVA *P* value< 0,05 e aumento ou diminuição de 1,5 vezes (*Fold-Change*). Considerando-se que *Hdh*$^{Q111/Q111}$ é um modelo *knock-in*, foi utilizada a expressão mGluR5 como um controle interno para o ensaio de microarranjo (Ribeiro, Devries et al. 2014).

Linhagem	***Maior expressão (upregulated)***	***Menor expressão (downregulated)***
Hdh$^{Q111/Q111}$*/mGluR5*$^{+/+}$vs. *Hdh*$^{Q20/Q20}$*/mGluR5*$^{+/+}$		*Dnahc6*
Hdh$^{Q111/Q111}$*/mGluR5*$^{-/-}$vs. *Hdh*$^{Q20/Q20}$*/ mGluR5*$^{-/-}$	*Dnahc6* *Dynlt1b*	*Dctn3*
Hdh$^{Q111/Q111}$*/mGluR5*$^{-/-}$vs. *Hdh*$^{Q111/Q111}$*/mGluR5*$^{+/+}$	*Dnahc6*	*Grm5*
Hdh$^{Q20/Q20}$*/ mGluR5*$^{-/-}$vs. *Hdh*$^{Q20/Q20}$*/mGluR5*$^{+/+}$		*Dnahc6* *Grm5*

A análise dos transcritos indicou que a expressão de diversos grupos de genes que codificam proteínas envolvidas em motilidade e transporte celular, tráfico vesicular, desenvolvimento cerebral e agregação de proteínas foram alteradas nos camundongos *Hdh*$^{Q111/Q111}$*/mGluR5*$^{-/-}$ vs. *Hdh*$^{Q20/Q20}$*/mGluR5*$^{-/-}$. Não foi observada alteração na expressão destes genes quando comparados os camundongos *Hdh*$^{Q111/Q111}$*/mGluR5*$^{+/+}$ *vs. Hdh*$^{Q20/Q20}$*/mGluR5*$^{+/+}$.

A fim de validar os resultados do microarranjo, foi realizado PCR quantitativo (qPCR) para determinar a expressão dosgenes de interesse (tabela 4), incluindo aqueles de maior expressão em $Hdh^{Q111/Q111}/mGluR5^{-/-}$: Dineína de cadeia pesada tipo 6 - *Dnahc6* (figura 14 A-B) e dineína cadeia leve Tctex tipo 1B - *Dynlt1b*(figura 15 A-B); bem como os de menor expressão: Dinactina tipo 3 - *Dctn3* (16 A-B). Nossos resultados mostraram que os genes *Dnahc6* (figura 14C) e *Dynlt1* (figura 15C) apresentam maior expressão na linhagem $Hdh^{Q111/Q111}/mGluR5^{-/-}$ quando comparados à linhagem $Hdh^{Q20/Q20}/mGluR5^{-/-}$, confirmando o resultado observado no ensaio de microarranjo (tabela 4).

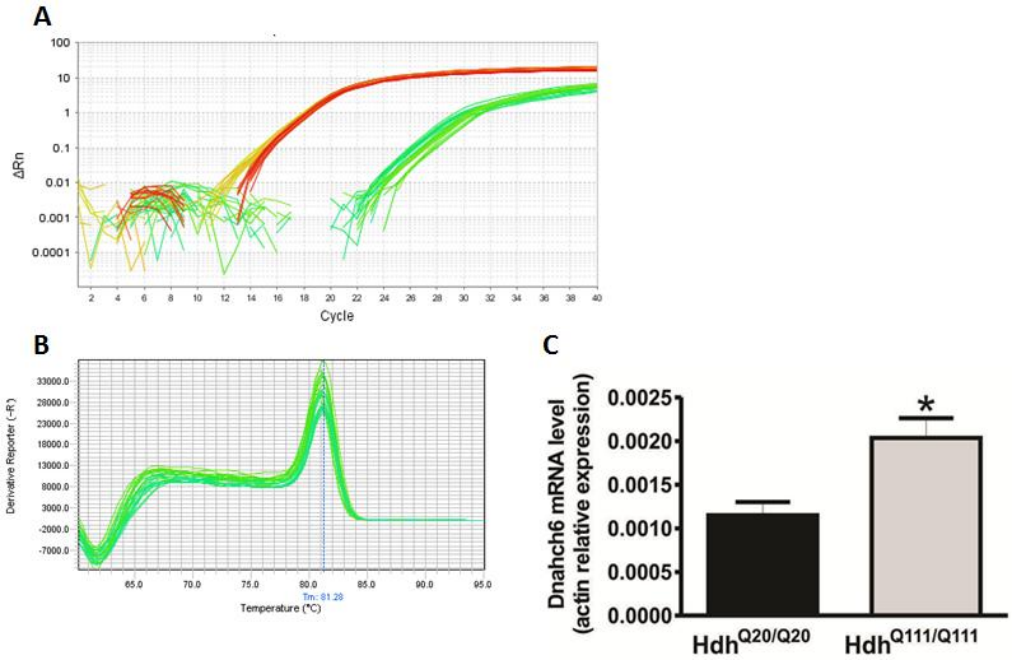

Figura 14: *Há **maior** expressão Dnahc6 em camundongos* $Hdh^{Q111/Q111}/mGluR5^{-/-}$ *em comparação ao* $Hdh^{Q20/Q20}/mGluR5^{-/-}$.Os gráficos mostram a curva de amplificação para a actina (em vermelho) e para a Dineína de cadeia pesada tipo 6 (*Dnahc6)* (em verde) (**A**),a curva de dissociação da amplificação de *Dnahc6*(**B**) e os níveis de mRNA de *Dnahc6* (NM_001164669.1) determinados por qPCR (**C**). As amostras de RNA foram extraídas do corpo estriado de camundongos das linhagens $Hdh^{Q111/Q111}/mGluR5^{-/-}$ e $Hdh^{Q20/Q20}/mGluR5^{-/-}$. A reação de qPCR foi realizada em triplicata e normalizada pelos níveis de RNA de actina. Dados representam a média ± SEM.O * representa $p < 0,05$ no teste *t* de *Student* quando comparado à linhagem $Hdh^{Q20/Q20}/mGluR5^{-/-}$.

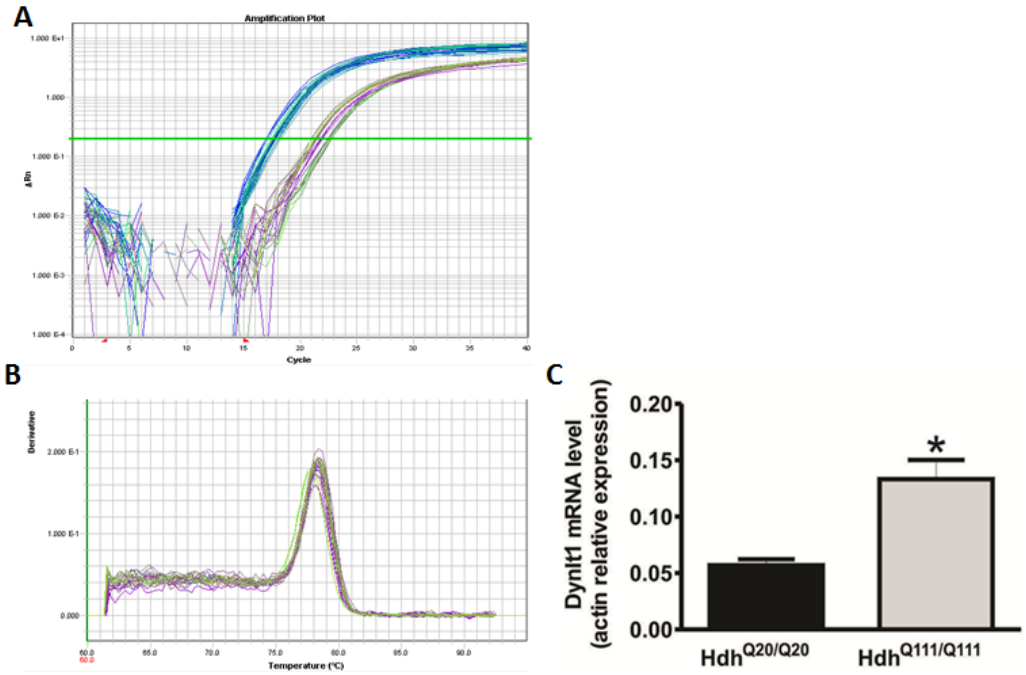

Figura 15: *Há **maior** expressão Dynlt1b em Hdh$^{Q111/Q111}$/mGluR5$^{-/-}$ em comparação ao Hdh$^{Q20/Q20}$/mGluR5$^{-/-}$*.Os gráficos mostram a curva de amplificação para a actina (em azul) e para a dineína cadeia leve Tctex tipo 1B - *Dynlt1b* (em roxo) (**A**), a curva de dissociação da amplificação de *Dynlt1b*(**B**) e os níveis de mRNA de *Dynlt1b* (NM_009342) determinados por qPCR (**C**). Amostras de RNA foram extraídas do corpo estriado de camundongos das linhagens *Hdh$^{Q111/Q111}$/mGluR5$^{-/-}$* e *Hdh$^{Q20/Q20}$/mGluR5$^{-/-}$*. A reação de qPCR foi realizada em triplicata e normalizada pelos níveis de RNA de actina. Dados representam a média ± SEM. O * representa $p < 0,05$ no teste *t* de *Student* quando comparado à linhagem *Hdh$^{Q20/Q20}$/mGluR5$^{-/-}$* .

Entretanto, a expressão do gene *Dctn3* foi reduzida na linhagem *Hdh$^{Q111/Q111}$/mGluR5$^{-/-}$* comparado à *Hdh$^{Q20/Q20}$/mGluR5$^{-/-}$* (figura 16 C), o que está de acordo com o observado no ensaio de microarranjo (tabela 4).

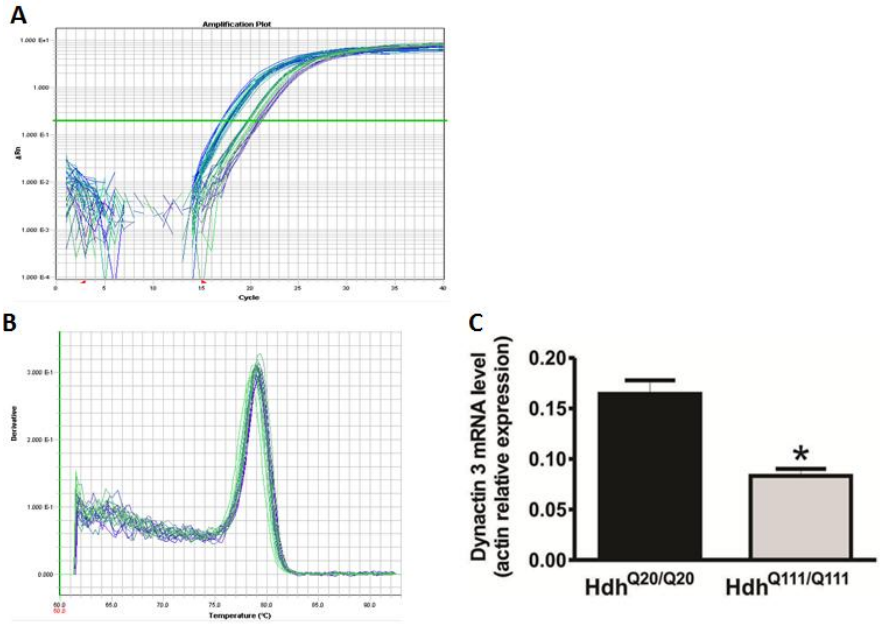

Figura 16: *Há **menor** expressão Dctn3 em Hdh$^{Q111/Q111}$/mGluR5$^{-/-}$ em comparação ao Hdh$^{Q20/Q20}$/mGluR5$^{-/-}$*.Os gráficos mostram a curva de amplificação para a actina (em azul) e para a Dinactina tipo 3 - *Dctn3* (NM_016890) (em verde e roxo) (**A**), a curva de dissociação da amplificação de *Dctn3*(**B**) e os níveis de mRNA de *Dctn3* (NM_016890) determinados por qPCR (**C**). Amostras de RNA foram extraídas do corpo estriado de camundongos das linhagens *Hdh$^{Q111/Q111}$/mGluR5$^{-/-}$* e *Hdh$^{Q20/Q20}$/mGluR5$^{-/-}$*. A reação de qPCR foi realizada em triplicata e normalizada pelos níveis de RNA de actina. Dados representam a média ± SEM. O * representa $p < 0,05$ no teste *t* de *Student* quando comparado à linhagem *Hdh$^{Q20/Q20}$/mGluR5$^{-/-}$* .

Como controle negativo do ensaio de microarranjo, foi avaliada a expressão dos genes dineína cadeia leve LC8 tipo 1 - (*Dynll1*) (figura 16 A-B) e dinactina 6 (*Dctn6*) (figura 17 A-B), os quais estão relacionados ao complexo Dineína-Dinactina mas não se mostraram alterados no ensaio de microarranjo. Os resultados do qPCR não indicaram alteração na expressão relativa destes genes na linhagem *Hdh$^{Q111/Q111}$/mGluR5$^{-/-}$* comparado à *Hdh$^{Q20/Q20}$/mGluR5$^{-/-}$*(figuras 16 C e 17 C), confirmando o resultado obtido previamente no ensaio de microarranjo.

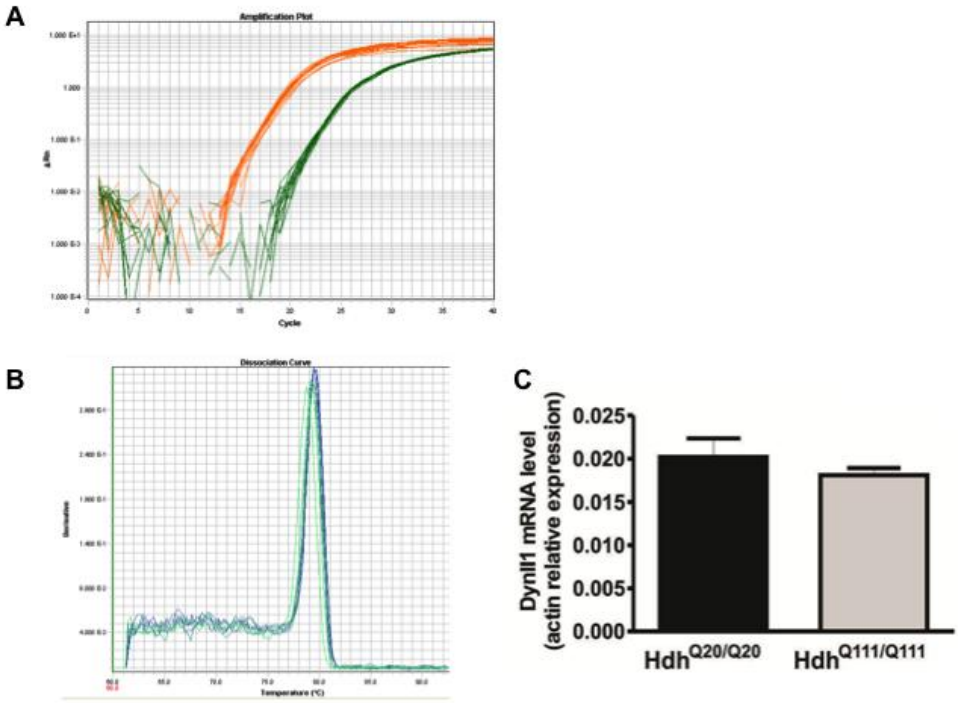

Figura 17: *A expressão de Dynll1 não apresentou diferença entre as linhagens* $Hdh^{Q20/Q20}/mGluR5^{-/-}$ *e* $Hdh^{Q111/Q111}/mGluR5^{-/-}$. Os gráficos mostram a curva de amplificação para a actina (em laranja) e para a dineína cadeia leve LC8-tipo 1 - *Dynll1* (NM_019682) (em verde) (**A**), a curva de dissociação da amplificação de *Dynll1*(**B**) e os níveis de mRNA de *Dynll1*(NM_019682) determinados por qPCR (**C**). Amostras de RNA foram extraídas do corpo estriado de camundongos das linhagens $Hdh^{Q111/Q111}/mGluR5^{-/-}$ e $Hdh^{Q20/Q20}/mGluR5^{-/-}$. A reação de qPCR foi realizada em triplicata e normalizada pelos níveis de RNA de actina. Dados representam a média ± SEM. O * representa $p < 0,05$ no teste *t* de *Student* quando comparado à linhagem $Hdh^{Q20/Q20}/mGluR5^{-/-}$.

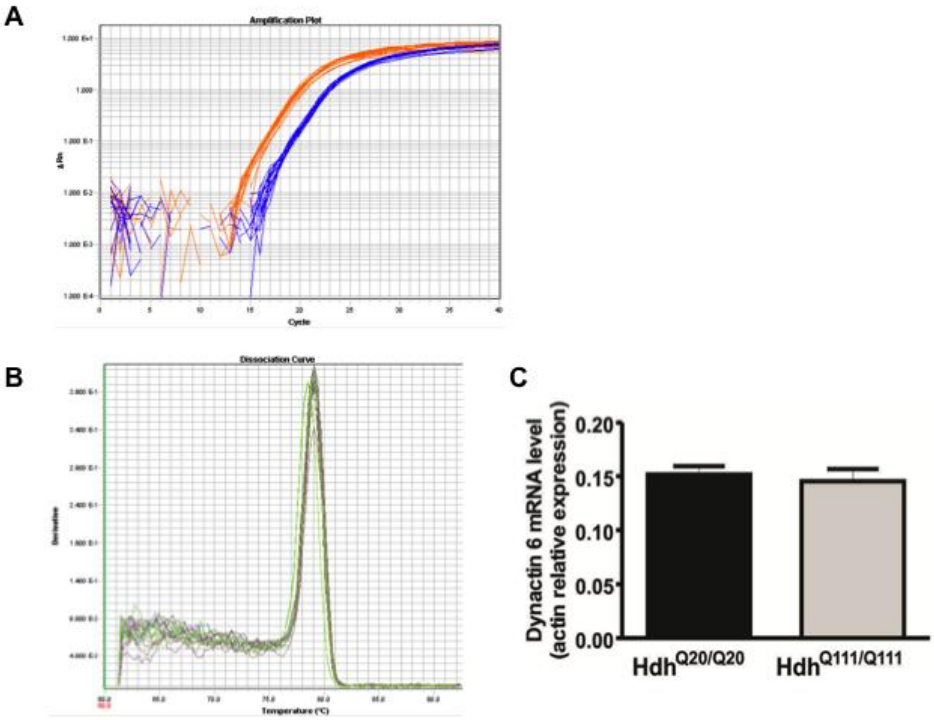

Figura 18: *A expressão de Dctn6 não apresentou diferença entre as linhagens* $Hdh^{Q20/Q20}/mGluR5^{-/-}$ *e* $Hdh^{Q111/Q111}/mGluR5^{-/-}$. Os gráficos mostram a curva de amplificação para a actina (em laranja) e para a dinactina 6 – *Dctn6* (NM_011722), (em azul) (**A**), a curva de dissociação da amplificação de *Dctn6* (**B**) e os níveis de mRNA de *Dctn6* (NM_011722) determinados por qPCR (**C**). Amostras de RNA foram extraídas do corpo estriado de camundongos das linhagens $Hdh^{Q111/Q111}/mGluR5^{-/-}$ e $Hdh^{Q20/Q20}/mGluR5^{-/-}$. A reação de qPCR foi realizada em triplicata e normalizada pelos níveis de RNA de actina. Dados representam a média ± SEM. O * representa $p < 0,05$ no teste *t* de *Student* quando comparado à linhagem $Hdh^{Q20/Q20}/mGluR5^{-/-}$.

5. DISCUSSÃO

Neste trabalho demonstramos que o bloqueio do mGluR5 em diferentes regiões encefálicas é capaz de alterar tanto a locomoção espontânea quanto a coordenação motora de camundongos C57/BL6. Certificamos que não somente substratos neurais primariamente motores como também estruturas límbicas e sensoriais são sensíveis ao bloqueio do mGluR5. Além disso, a supressão global do mGluR5, tanto através do antagonismo agudo quanto pela deleção gênica, promovem hiperlocomoção, confirmando a importância da ativação do mGluR5 na modulação da locomoção. No entanto, o bloqueio agudo do mGluR5 gerou hiperlocomoção no camundongo modelo da DH (*Hdh*$^{Q111/Q111}$), ao passo que o camundongo *Hdh*$^{Q111/Q111}$*/mGluR5*$^{-/-}$ não apresentou aumento da atividade locomotora. Diante disso, para investigarmos as possíveis adaptações biológicas de longo prazo foi realizado o ensaio de microarranjo. Assim, os resultados gerados pelo microarranjo confirmaram que um número de genes importantes para aadaptação locomotora estavam alterados na linhagem *Hdh*$^{Q111/Q111}$*/mGluR5*$^{-/-}$ em comparação à linhagem *Hdh*$^{Q20/Q20}$*/mGluR5*$^{-/-}$. É importante ressaltar que os resultados de qPCR confirmaram as alterações na expressão dos genes *Dnahc6*, *Dynlt1* e *Dctn3* observadas no microarranjo.

Sabe-se que o mGluR5 é amplamente expresso no cérebro de roedores e possui importante papel na excitabilidade neuronal, visto que aumenta níveis de Ca^{2+} intracelulares(Shigemoto, Nomura et al. 1993, Testa, Standaert et al. 1994). Nossos resultados mostram que a depleção deste receptor aumenta a atividade locomotora espontânea observada no campo aberto. Corroborando nossos resultados, Jew*et al.* (2013) observaram hiperlocomoção em camundongos C57/BL6, cujo *knockout* para mGluR5 é específico para a região do córtex cerebral. Por outro lado, não foi observado comprometimento da coordenação motora nesta linhagem (Jew, Wu et al. 2013). Além

disso, em um estudo realizado por Busse *et al.* (2004), foi mostrado que injeções i.p. do antagonista seletivo do mGluR5 não alteram a coordenação motora em roedores (Busse, Brodkin et al. 2004). Em nosso estudo, além da hiperlocomoção dos camundongos *mGluR5*$^{-/-}$, observamos também que estes animais apresentam uma melhor performance no teste Rota Rod. A partir disto, testamos a hipótese de que a modulação de respostas motoras exercida pelo bloqueio de mGluR5 depende do substrato neural envolvido.

Interessantemente, nossos dados mostram que o bloqueio do mGluR5 em uma área primariamente sensorial, como o bulbo olfatório, parece influenciar aspectos importantes da motricidade como equilíbrio e coordenação motora. Em estudo recente foi observado que a bulbectomia olfatória em período neonatal é capaz de alterar comportamento exploratório, atividade locomotora e interação social (Flores, Ibanez-Sandoval et al. 2014). Estas alterações comportamentais, por sua vez, parecem estar relacionadas ao comprometimento de receptores glutamatérgicos(Flores, Ibanez-Sandoval et al. 2014). Em nosso estudo, observamos que o bloqueio específico do mGluR5 no bulbo olfatório gera um pior desempenho locomotor dos camundongos alocados no Rota Rod. Estes dados, portanto, corroboram dados anteriores e indicam que uma estrutura primariamente sensorial como o bulbo olfatório também pode ser capaz de modular o desempenho motor através do sistema glutamatérgico.

Nossos dados indicam ainda que o hipocampo, estrutura conhecida pelo seu papel na memória e aprendizado espacial (Bast and Feldon 2003, Fanselow and Dong 2010), parece modular a atividade locomotora espontânea. Em estudo prévio foi relatado que um surto isquêmico agudo capaz de reduzir em 80% as células do hipocampo dorsal (região de CA1), gera hiperlocomoção em roedores (Kuroiwa, Bonnekoh et al. 1991). Aliado a esse fato, foi observado por Coutureau *et al.* (2000) que os efeitos de lesões no hipocampo parecem exercer algum tipo de inibição na regulação

do comportamento motor, mas a natureza do mecanismo inibitório ainda não está clara (Coutureau, Galani et al. 2000). Em nosso estudo, por sua vez, identificamos hiperlocomoção após microinjeções de MPEP no hipocampo dorsal, o que pode indicar a influencia do sistema glutamatérgico neste processo de modulação da locomoção. Outro estudo realizado em roedores mostrou que microinfusões do antagonista do receptor de NMDA, MK-801, no hipocampo dorsal também gera aumento da atividade locomotora no campo aberto (Zhang, Bast et al. 2000). Embora o hipocampo esteja intimamente relacionado aos processos de memória (Eichenbaum, Schoenbaum et al. 1996, Eichenbaum 2000),suas conexões com áreas associativas, bem como com áreas sensoriais polimodais, fundamentam seu recrutamento em funções sensoriomotoras(Bast and Feldon 2003). A hiperlocomoção gerada pelo antagonismo do mGluR5 no hipocampo, bem como a queda do desempenho motor também gerados pela ação do MPEP no bulbo olfatório deixam clara a influência destas estruturas na regulação do comportamento motor. Tendo em vista estes resultados e análises prévias, ainda não se sabe ao certo como o antagonismo ou a deleção gênica do mGluR5 levam a um aumento da atividade locomotora. Acreditamos que possa existir uma possível interação entre as regiões neurais que levaria ao aumento da atividade locomotora observada no camundongo *mGluR5*$^{-/-}$. Entretanto, mais estudos são necessários no sentido de investigar como os diferentes circuitos – motor, sensorial e límbico – interagem no sentido de modular a resposta motora global. Além destes aspectos, não está claro quais circuitos neuronais estão envolvidos nesta regulação.

Foi recentemente demonstrado por nosso grupo que a hiperlocomoção dos camundongos *mGluR5*$^{-/-}$ é abolida por antagonistas dos receptores da dopamina D1 e D2 (Ribeiro, Devries et al. 2014), sugerindo que vias dopaminérgicas provenientes da *substancia nigra* possam estar hiperativadas nos camundongos *mGluR5*$^{-/-}$. Além disso,

circuitos glutamatérgicos e dopaminérgicos podem interagir em certas regiões do cérebro, incluindo o *nucleus accumbens*, corpo estriado e córtex pré-frontal, e esta interação é importante para o controle do movimento e cognição (Carlsson and Carlsson 1990, Sesack and Pickel 1990, Carr and Sesack 1996, Scott and Aperia 2009). Entretanto, experimentos futuros são necessários para esclarecer quais os mecanismos regulam a alteração da resposta motora gerada pela deleção gênica do mGluR5 e o envolvimento do sistema dopaminérgico neste processo.

O mGluR5 é altamente expresso no corpo estriado, que é a principal região afetada na DH (Testa, Standaert et al. 1994, Ross and Tabrizi 2011). Nosso grupo estabeleceu uma ligação entre o mGluR5 e a DH, mostrando que o grupo I dos mGluRs é capaz de interagir com a proteína Htt(Anborgh, Godin et al. 2005). Além disso, a proteína Htt mutante pode alterar a sinalização do mGluR5, diminuindo a formação de IP_3 e aumentando a ativação de ERK1/2 e AKT (Ribeiro, Paquet et al. 2010). Ademais, demonstramos que o bloqueio do mGluR5 no corpo estriado altera a atividade locomotora de camundongos C57/BL6, sugerindo que a ativação deste receptor pode contribuir na modulação do movimento. Entretanto, a diminuição da atividade locomotora promovida pelo bloqueio do mGluR5 no corpo estriado foi adversa ao que era esperado por nosso grupo. Curiosamente, as microinjeções de MPEP no corpo estriado atingiram a porção ventral desta estrutura, como observado em análise histológica (dados não mostrados). O corpo estriado pode ser funcionalmente dividido em duas sub-regiões: corpo estriado dorsal, baseado nas projeções glutamatérgicas de origem cortical, e corpo estriado ventral, baseado nas projeções dopaminérgicas de origem mesencefálica(Robbins and Everitt 1996, Kakei, Hoffman et al. 1999, Groenewegen 2003). O estriado dorsal está mais envolvido em comportamentos motores, enquanto que o estriado ventral é crucial para processos motivacionais

(Robbins and Everitt 1996, Groenewegen 2003). O estriado dorsal é frequentemente dividido em uma porção externa (dorsolateral), correspondente ao putâmen de primatas, que recebe projeções do córtex sensoriomotor; e uma parte interna (dorsomedial) homóloga ao núcleo caudado em primatas, que recebe projeções do córtex pré-frontal e de associação (Graybiel 1991, Voorn, Vanderschuren et al. 2004). Desta forma, a realização de microinfusões no estriado dorsal-lateral enriqueceria o entendimento do papel do mGluR5 na modulação do comportamento motor, visto que a porção dorsolateral está intimamente envolvida em processos sensório motores. Além disso, esses dados seriam importantes para melhor entender o papel do mGluR5 na coreia associada à DH.

Alterações do sistema locomotor são tipicamente observadas em pacientes com DH e, em nosso estudo, procuramos encontrar a participação do mGluR5 nos sinais locomotores da DH. Injeções agudas i.p. de MTEP geraram hiperlocomoção nos camundongos $Hdh^{Q20/Q20}$ e $Hdh^{Q111/Q111}$, entretanto no caso da linhagem $Hdh^{Q111/Q111}/mGluR5^{-/-}$ não observamos variação na atividade locomotora em resposta às injeções agudas de MTEP. O conflito encontrado nestes resultados nos leva a crer que a ausência do mGluR5, ao longo do desenvolvimento do camundongo modelo da DH, pode ter ativado mecanismos de compensação que anularam a hiperlocomoção induzida pelo mGluR5.Estudos indicam que tanto a presença da proteína Htt mutante quanto a ativação do mGluR5 podem desencadear alterações na expressão gênica (Cha 2000, Huber, Kayser et al. 2000, Vanderklish and Edelman 2002, Sugars and Rubinsztein 2003). Portanto, investigamos se uma alteração na expressão gênica global poderia explicar a falta de hiperlocomoção mostrada pelos camundongos da linhagem $Hdh^{Q111/Q111}/mGluR5^{-/-}$. Os resultados encontrados indicaram que existe uma interação no nível transcricional entre a atividade do mGluR5 e a Htt mutante.

A alteração da expressão de genes envolvidos no tráfico axonal tais como ocorre com as proteínas do complexo dineína-dinactina, contribui no processo patológico de um número variado de doenças neurodegenerativas (Chevalier-Larsen and Holzbaur 2006). Dineínas são estruturas carreadoras de vesículas ao longo do axônio de células neurais. É descrito que a disfunção do complexo dineína compromete o *clearance* autofágico de agregados proteicos, como os agregados Htt, levando à toxicidade proveniente da mutação e agravando o fenótipo em modelos da DH (Ravikumar, Acevedo-Arozena et al. 2005). O modelo de camundongo da DH que expressa o gene *Dnhc1*mutado exibe altos níveis de tremor, redução da força muscular, pior desempenho no teste Rota Rod, disfunção de marcha e diminuição do período de vida, quando comparado ao modelo da DH expressando o gene *Dnhc1* selvagem (Ravikumar, Acevedo-Arozena et al. 2005). Além disso, mutantes de *Caenorhabditis elegans* expressando proteínas alteradas do complexo dineína-dinactina, incluindo dineína cadeia pesada, dineína cadeia leve e dinactina, exibiram disfunção em processos sinápticos, bem como diminuição progressiva da locomoção e diminuição do tempo de vida (Koushika, Schaefer et al. 2004). Outro estudo demonstrou que um modelo de camundongo apresentando mutação do gene dineína cadeia leve – 1 (*Dync1li1*) possui desenvolvimento neural anormal, aumento da ansiedade e menores níveis de atividade locomotora espontânea quando comparado ao controle (Banks, Haas et al. 2011). Concluímos que as alterações genéticas mediadas pela ausência do mGluR5 em camundongos portadores da Htt mutante podem contribuir para a adaptação fenotípica observada, ou seja, diminuição da atividade locomotora.

6. CONSIDERAÇÕES FINAIS

Nossos resultados indicam que o bloqueio do mGluR5, gene amplamente expresso no sistema nervoso central, é capaz de modular a atividade locomotora através de diversos substratos neurais. Observamos ainda que o mGluR5 expresso em regiões não primariamente motoras parecem influenciar a modulação da locomoção. Por outro lado, os circuitos envolvidos nesta regulação merecem ser melhor estudados.

Ademais, foi observado que a proteína huntigtina mutante e o mGluR5 exibem uma interação funcional que pode estar envolvida com os sintomas motores relacionados à DH. Ambos Htt mutante e mGluR5 podem desencadear alterações na expressão de diversos genes, o que poderia explicar as mudanças no comportamento motor observado na linhagem $Hdh^{Q111/Q111}/mGluR5^{-/-}$. Futuros experimentos serão necessários para elucidar os mecanismos subjacentes às alterações observadas nos camundongos $Hdh^{Q111/Q111}/mGluR5^{-/-}$.

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UNIVERSIDADE FEDERAL DE MINAS GERAIS
COMITÊ DE ÉTICA EM EXPERIMENTAÇÃO ANIMAL
- C E T E A -

CERTIFICADO

Certificamos que o **Protocolo nº 274/2011,** relativo ao projeto intitulado "***Investigação da função da Huntingtina mutante sobre os sistemas neurotransmissores dos gânglios da base***", que tem como responsável(is) **Fabiola Mara Ribeiro** , está(ão) de acordo com os Princípios Éticos da Experimentação Animal, adotados pelo ***Comitê de Ética em Experimentação Animal*** **(CETEA/UFMG),** tendo sido aprovado na reunião de **9/ 05/2012.**

Este certificado expira-se em **9/ 05/ 2017.**

CERTIFICATE

We hereby certify that the **Protocol nº 274/2011,** related to the project entitled "***Investigation of the huntingtin function on basal ganglia neurotransmission***", under the supervisiors of **Fabiola Mara Ribeiro,** is in agreement with the Ethical Principles in Animal Experimentation, adopted by the ***Ethics Committee in Animal Experimentation*** **(CETEA/UFMG),** and was approved in **May 9, 2012.**

This certificate expires in **May 9, 2017.**

Belo Horizonte, 11 de Maio de 2012.

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(Mod.Cert. v1.0)

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Metabotropic glutamate receptor 5 knockout promotes motor and biochemical alterations in a mouse model of Huntington's disease

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Huntington's disease (HD) is an autosomal-dominant neurodegenerative disorder caused by a polyglutamine expansion in the amino-terminal region of the huntingtin protein, which promotes progressive neuronal cell loss, neurological symptoms and death. In the present study, we show that blockade of mGluR5 with MTEP promotes increased locomotor activity in both control ($Hdh^{Q20/Q20}$) and mutant HD ($Hdh^{Q111/Q111}$) mice. Although acute injection of MTEP increases locomotor activity in both control and mutant HD mice, locomotor activity is increased in only control mice, not mutant HD mice, following the genetic deletion of mGluR5. Interestingly, treatment of mGluR5 knockout mice with either D1 or D2 dopamine antagonists eliminates the increased locomotor activity of mGluR5 knockout mice. Amphetamine treatment increases locomotor activity in control mice, but not mGluR5 null mutant HD mice. However, the loss of mGluR5 expression improves rotarod performance and decreases the number of huntingtin intranuclear inclusions in mutant HD mice. These adaptations may be due to mutant huntingtin-dependent alterations in gene expression, as microarray studies have identified several genes that are altered in mutant, but not wild-type HD mice lacking mGluR5 expression. qPCR experiments confirm that the mRNA transcript levels of dynein heavy chain, dynactin 3 and dynein light chain-6 are altered following the genetic deletion of mGluR5 in mutant HD mice, as compared with wild-type mutant HD mice. Thus, our data suggest that mutant huntingtin protein and mGluR5 exhibit a functional interaction that may be important for HD-mediated alterations in locomotor behavior and the development of intranuclear inclusions.

INTRODUCTION

Huntington's disease (HD) is an autosomal-dominant neurodegenerative disorder caused by a progressive neuronal cell loss in the caudate-putamen, which leads to involuntary body movement, loss of cognitive function, psychiatric disturbance and death (1,2). HD patients typically exhibit hyperkinesia such as chorea, which is characterized by involuntary, abrupt and irregular movements (1–3). The mutated form of the huntingtin (Htt) protein, exhibiting >37 polyglutamines in the amino-terminal region, is proposed as the cause of the neuronal cell loss observed in the caudate-putamen (striatum in rodents) and neocortical regions of HD patients (4). The striatum is composed mainly (85%) of medium-sized spiny neurons (MSNs), which are GABAergic neurons, but also of interneurons, including cholinergic neurons (5). Although MSNs are the first neurons to be affected during HD progression, cholinergic neurons are spared (6,7).

Striatal neurons receive input from different areas of the basal ganglia and also glutamatergic input from thalamus and cortex (8,9). Moreover, dopaminergic neurons from the substantia

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nigra pars compacta (SNc) receive both GABAergic input from MSNs and glutamatergic input from the cortex (10,11). Thus, glutamate plays a major role in basal ganglia–thalamus–cortical circuits. A number of reports have demonstrated that the metabotropic glutamate receptor 5 (mGluR5) is involved in locomotor activity (12,13). mGluR5 is coupled to the activation of $G\alpha_{q/11}$ proteins, which stimulate the activation of phospholipase Cβ1 resulting in diacylglycerol and inositol-1,4,5-triphosphate formation, release of Ca^{2+} from intracellular stores and the activation of protein kinase C. mGluR5 also activates cell signaling pathways important for cell survival/proliferation (14–16). Moreover, it has been demonstrated that mGluR5 binds to Htt and that mGluR5 G protein signaling is selectively reduced, whereas Akt and extracellular signal-regulated kinase 1/2 (ERK1/2) signaling are increased, in a mouse model of HD, $Hdh^{Q111/Q111}$ knock-in mice, as compared with $Hdh^{Q20/Q20}$ control knock-in mice (17). Treatment of a HD transgenic mouse model with MPEP, which is a mGluR5 antagonist, slightly increases survival of the mutant mice (18). In addition, mGluR5-positive allosteric modulators prevent neuronal cell death of striatal neurons from a BACHD mouse model of HD and increase Akt signalling in the absence of increases in intracellular Ca^{2+} concentrations (19). However, despite of all this evidence, it is still not clear whether mGluR5 plays a role in HD. To shed some light on this issue, we have crossed $mGluR5^{-/-}$ mice with $Hdh^{Q111/Q111}$ and $Hdh^{Q20/Q20}$ mice and performed a series of molecular and *in vivo* studies.

In the present study, we demonstrate that both control and HD mutant mice treated with MTEP, as well as control mice lacking mGluR5 expression, exhibit increased locomotor activity. However, in mice expressing mutant huntingtin protein ($Hdh^{Q111/Q111}$), genetic deletion of mGluR5 does not result in a hyperlocomotor phenotype. Interestingly, D1 and D2 dopamine antagonists are capable of eliminating the increase in locomotor activity promoted by the genetic deletion of mGluR5. Moreover, mGluR5 knockout also improves rotarod performance and decreases huntingtin intranuclear inclusions in mutant HD mice. In addition, microarray studies indicate that mutated huntingtin protein can alter the expression of a number of genes that are involved in the modulation of locomotor activity in mice lacking mGluR5. Thus, our data clearly support the notion that mGluR5 plays an important role in HD pathology and motor symptoms.

RESULTS

To test whether mGluR5 had a role in locomotor activity, we submitted mGluR5 knockout ($mGluR5^{-/-}$) mice to an open-field arena. $mGluR5^{-/-}$ mice were more active in the arena than $mGluR5^{+/+}$ mice as evidenced by the total distance traveled (Fig. 1A and B), confirming that mGluR5 was important for movement control. Moreover, the locomotor activity lines for wild-type and mGluR5 knockout mice have the same slope (Fig. 1A), which indicates that both mouse lines habituate at the same rate.

Abnormal movements including hyperkinesia and chorea are the most typical symptoms observed in HD patients, and we previously demonstrated that mGluR5 signaling was altered in mutant HD ($Hdh^{Q111/Q111}$) mice (1–3,20). Therefore, to further study the role of mGluR5 in HD, we investigated the locomotor activity of control ($Hdh^{Q20/Q20}$) and mutant HD mice that were crossed with mGluR5 knockout mice. At 3 months of age, mGluR5 null control mice ($Hdh^{Q20/Q20}$/$mGluR5^{-/-}$) traveled much further in the open-field arena than wild-type control mice, wild-type mutant HD mice or mGluR5 null mutant HD mice ($Hdh^{Q111/Q111}$/$mGluR5^{-/-}$) (Fig. 2A). To determine whether the hyperlocomotor phenotype was influenced by age, we also submitted the same groups of mice at the ages of 6, 9, 12, 15, 18, 21 and 24 months to the open-field arena (Fig. 3B). Statistical analysis [two-way analysis of variance (ANOVA)] indicated that there is an interaction between age and genotype [$\pm(21.3) = 2.02$, $P = 0.005$]. Moreover, both genotype [$\pm(3.3) = 114.1$, $P < 0.0001$] and age [$\pm(7.3) = 2.65$, $P = 0.0115$] affect locomotor activity, although age did not seem to play a major role. Locomotor activity of mGluR5 null control mice remained higher than that of mGluR5 null mutant HD mice, and Bonferroni posttests indicated that this difference was significant at the ages of 3, 6, 15 and 24 months (Fig. 2B). Moreover, the locomotor activity of the mGluR5 null mutant HD mice was significantly different from that of wild-type control mice at the ages of 9, 12 and 15 months (Fig. 2B). Interestingly, although the distance traveled by mGluR5 null control mice was higher than that of mGluR5 null mutant HD mice, time spent in the center of the arena was not different between the two lines of mice (Fig. 2C and D), indicating that mutant huntingtin affects locomotor activity specifically, but did not appear to affect anxiety behavior. Moreover, the four tested mouse lines did not display differences in

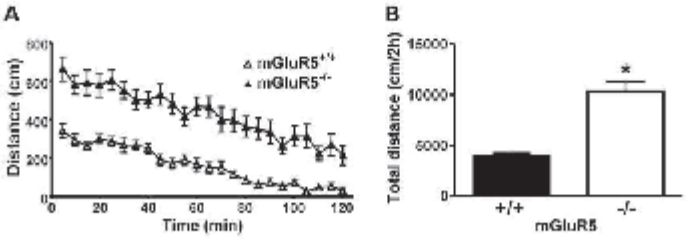

Figure 1. $mGluR5^{-/-}$ mice exhibit increased locomotor activity. Graph shows total distance traveled by wild-type ($mGluR5^{+/+}$) ($n = 11$) and mGluR5 knockout ($mGluR5^{-/-}$) ($n = 12$) mice measured at 5-min intervals (**A**) or cumulatively >120 min (**B**). Each animal was monitored for 120 min in open-field apparatus. Data represent the means ± SEM. * indicates significant difference as compared with $mGluR5^{+/+}$ mice ($P < 0.05$).

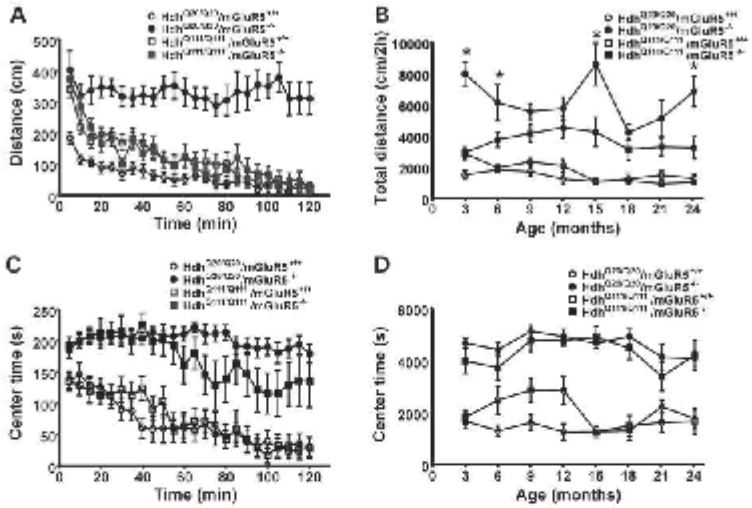

Figure 2. Mutant huntingtin abrogates *mGluR5*$^{-/-}$-induced increased locomotor activity. Graphs show total distance traveled (**A**) and time spent in the center of the apparatus (**C**) by control (*Hdh*$^{Q20/Q20}$/*mGluR5*$^{+/+}$) ($n = 11$), mGluR5 null control (*Hdh*$^{Q20/Q20}$/*mGluR5*$^{-/-}$) ($n = 11$), mutant HD (*Hdh*$^{Q111/Q111}$/*mGluR5*$^{+/+}$) ($n = 12$) and mGluR5 null mutant HD (*Hdh*$^{Q111/Q111}$/*mGluR5*$^{-/-}$) mice ($n = 11$) at 3 months of age measured at 5-min intervals. Each animal was monitored for 120 min. Data represent the means ± SEM. Graphs show total distance traveled (**B**) and time spent in the center (**D**) by *Hdh*$^{Q20/Q20}$/*mGluR5*$^{+/+}$ ($n = 9$–11), *Hdh*$^{Q20/Q20}$/*mGluR5*$^{-/-}$ ($n = 6$–11), *Hdh*$^{Q111/Q111}$/*mGluR5*$^{+/+}$ ($n = 9$–12) and *Hdh*$^{Q111/Q111}$/*mGluR5*$^{-/-}$ mice ($n = 6$–11) tested at 3, 6, 9, 12, 15, 18, 21 and 24 months of age. Each animal was monitored for 120 min in the open-field apparatus. Data represent means ± SEM. * indicates significant differences as compared with *Hdh*$^{Q111/Q111}$/*mGluR5*$^{-/-}$ mice ($P < 0.05$).

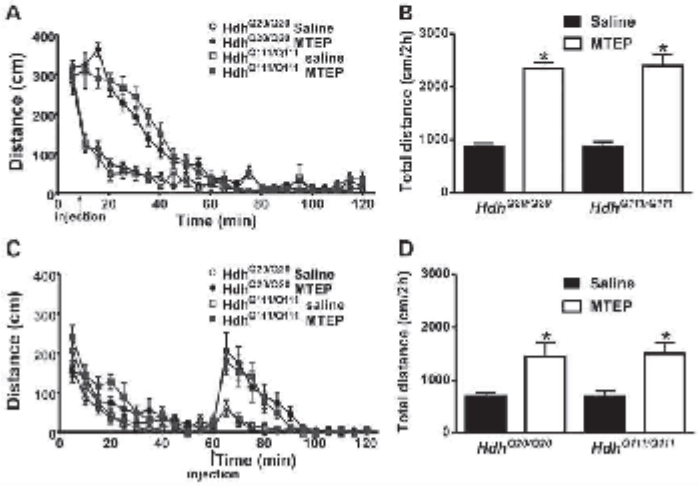

Figure 3. Acute MTEP injection induces increased locomotor activity in both *Hdh*$^{Q20/Q20}$ and *Hdh*$^{Q111/Q111}$ mice. (**A** and **B**) Graphs show total distance traveled by control (*Hdh*$^{Q20/Q20}$) (MTEP $n = 8$ and saline $n = 7$) and mutant HD (*Hdh*$^{Q111/Q111}$) (MTEP $n = 8$ and saline $n = 7$) mice. Animals were placed in the open-field box and injected at an injection point of 5 min with either saline or MTEP (i.p. 5 mg/Kg). Each animal was monitored for 120 min, and total distance was measured at 5-min intervals (**A**) or cumulatively >120 min (**B**). (**C** and **D**) Graphs show total distance traveled by *Hdh*$^{Q20/Q20}$ (MTEP $n = 8$ and saline $n = 7$) and *Hdh*$^{Q111/Q111}$ (MTEP $n = 8$ and saline $n = 7$) mice. Animals were placed in the open-field box and injected at an injection point of 60 min with either saline or MTEP (i.p. 5 mg/Kg). Each animal was monitored for 120 min, and total distance was measured at 5-min intervals (**C**) or cumulatively >120 min (**D**). Data represent the means ± SEM. * indicates significant differences as compared with matched genotype injected with saline ($P < 0.05$).

weight gain (data not shown) or in huntingtin mRNA and protein expression levels (Supplementary Material, Fig. S1). In addition, the tested mouse lines did not exhibit any kind of stereotypical behavior when allocated either in their home cage or in the open-field arena (Supplementary Material, Fig. S2). Together, these data suggest that the expression of mutated huntingtin protein in mGluR5 null mutant mice might be activating compensatory mechanisms that prevented the increase in locomotor activity.

To test whether the observed increase in locomotor activity caused by mGluR5 knockout could be recapitulated pharmacologically in control and mutant HD mice, we assessed the effects of mGluR5 antagonist MTEP on the locomotor activity of both mouse lines. To do this, we injected mice with MTEP (i.p. 5 mg/kg) 10 min after introducing the mice to the open-field arena. Both control and mutant HD mice injected with MTEP traveled much further in the arena than saline-injected mice, and no differences in the locomotor activity of both mouse lines was observed following acute pharmacological blockade of mGluR5 (Fig. 3A and B). However, it was not clear whether mGluR5 blockage caused hyperactivity or a lack of habituation. In order to investigate this, we introduced control and mutant HD mice to the open-field arena, waited 60 min for habituation to the new environment and then injected the mice with MTEP (i.p. 5 mg/kg). Both mouse lines showed an increase in locomotor activity, measured by distance traveled, following MTEP injection, indicating that mGluR5 blockage promotes hyperactivity as opposed to prevention of habituation (Fig. 3C and D).

The mechanism underlying the increase in locomotor activity promoted by mGluR5 blockage is unknown. Therefore, we investigated whether this increase in locomotor activity was dopamine dependent. Thus, mGluR5 null control and mutant HD mice were injected with either saline or the D2 dopamine receptor antagonist haloperidol (i.p. 0.5 mg/kg). Haloperidol caused the levels of locomotor activity, as measured by total distance, in the mGluR5 null control mice to go down to levels observed for mGluR5 null mutant HD mice (Fig. 4A and B). Similar results were observed when mGluR5 null control mice were injected with the D1 dopamine receptor antagonist SCH23390 (i.p. 0.5 mg/kg), as the levels of distance traveled by mGluR5 null control mice treated with SCH23390 were not different than those of mGluR5 null mutant HD mice (Fig. 4C and D). Interestingly, the distance traveled by mutant HD following either haloperidol or SCH23390 treatment was not different than that of saline-treated mutant HD mice that express mGluR5 (Fig. 4A–D). In addition, amphetamine (i.p. 2 mg/kg) treatment promoted a further increase in locomotor activity for mGluR5 null control mice, but had no effect on the locomotor activity of mGluR5 null mutant HD mice (Fig. 4E and F). These data suggested that the increase in locomotor activity observed following genetic deletion of mGluR5 in control mice was dopamine dependent. The lack of effect of amphetamine treatment on mGluR5 null mutant HD mice suggested that polyglutamine-expanded mutant huntingtin protein might activate a developmentally compensatory mechanism that appears to block the hyperactivity induced by both mGluR5 deletion and dopamine stimulation.

Previously, rotarod performance of mutant HD (Q111) mice was shown to not to be impaired when compared with that of control mice (21). Moreover, it was shown that MTEP had no effect on rotarod performance (22). To determine whether mGluR5 knockout could alter control and mutant HD mouse motor coordination, mice were trained and submitted to testing sections on the rotarod. Rotarod learning curves were not different among tested genotypes (Fig. 5A and B). In agreement to previously published data, the latency of control and mutant HD mice that express mGluR5 to fall from rotarod was not different from one another (Fig. 5C). The knockout of mGluR5 did not change the performance of control mice on the rotarod (Fig. 5C). However, genetic deletion of mGluR5 significantly improved the performance of mutant HD mice on the rotarod, when compared with all other genotypes (Fig. 5C). Statistical analysis (two-way ANOVA) indicates that both genotype [$\pm(3.3) = 27.6$, $P < 0.0001$] and age [$\pm(7.3) = 20.1$, $P < 0.0001$] affected rotarod performance. Moreover, Bonferroni posttests indicate that the rotarod performance of mGluR5 null mutant HD mice was better than that of wild-type control mice at the ages of 3, 6 and 21 months (Fig. 5C). These data further support the notion that mGluR5 is intrinsically implicated in the motor alterations promoted by mutated Htt protein.

To further characterize the consequences of polyglutamine-expanded huntingtin expression in an mGluR5 null genetic background, we performed immunohistochemistry experiments using EM48 antibody to determine the pattern of mutated huntingtin aggregation in the striatum of 12-month-old mice. Control mice that either express or do not express mGluR5 did not display any huntingtin aggregates (Fig. 6A and B). However, mutant HD mice that express mGluR5 exhibited high levels of diffuse EM48 labeling in the striatum, as well as darkly stained puncta (Fig. 6C and E), which has been characterized as ubiquitin-positive intranuclear inclusions (23). Interestingly, although diffuse EM48 labeling could be observed in the striatum of mGluR5 null mutant HD mice, intranuclear inclusions were mostly absent in the striata of these mice (Fig. 6D and F). Quantification of these data and statistical analysis demonstrate that both diffuse EM48 labeling (Fig. 6G) and intranuclear inclusions (Fig. 6H) were robustly reduced in mGluR5 null mutant HD mouse striatum as compared with that of wild-type HD mutant mice (Fig. 6E), strongly indicating that mGluR5 is involved in huntingtin aggregation. Statistical analysis (unpaired *t*-test) indicates a significant effect of genotype ($P < 0.0001$).

To further investigate the mechanism behind the observed adaptive phenotype of mGluR5 null mutant HD mice, we decided to analyze whether mutated huntingtin expression in an mGluR5 null background was altering the expression of genes that might be involved in the pattern of huntingtin aggregation and HD-related locomotor alterations. Thus, we performed a microarray assay to compare mRNA expression levels between wild-type control mice, mGluR5 null control mice, wild-type mutant HD mice and mGluR5 null mutant HD mice. As expected, mGluR5 expression was down-regulated in both mouse lines lacking mGluR5 expression when compared with wild-type control and mutant HD mice (data not shown). These data support the assumption that the microarray assay was performed properly and was capable of detecting gene expression variations. To identify pathways and networks of genes significantly altered in mGluR5 null mutant HD mice versus wild-type mutant HD mice, we analyzed the microarray data using ingenuity pathway analysis (IPA). The microarray

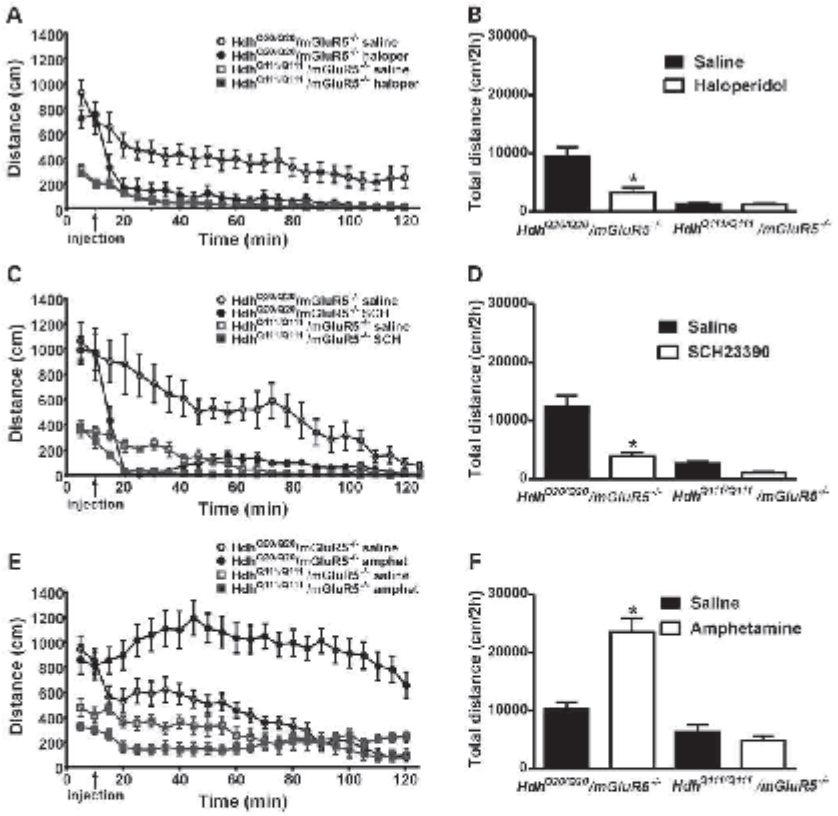

Figure 4. Role of mutated huntingtin protein in locomotor activity involves both mGluR5 and dopamine neurotransmission. (**A** and **B**) Graphs show total distance traveled by mGluR5 null control ($Hdh^{Q20/Q20}$/$mGluR5^{-/-}$)(haloperidol (haloper) $n = 11$ and saline $n = 10$) and mGluR5 null mutant HD ($Hdh^{Q111/Q111}$/$mGluR5^{-/-}$) (haloperidol $n = 7$ and saline $n = 7$) mice. Animals were placed in the open-field box and injected at an injection point of 10 min with either saline or haloperidol (i.p. 0.5 mg/kg). Each animal was monitored for 120 min, and total distance was measured at 5-min intervals (A) or cumulatively >120 min (B). (**C** and **D**) Graphs show total distance traveled by $Hdh^{Q20/Q20}$/$mGluR5^{-/-}$ (SCH23390 (SCH) $n = 9$ and saline $n = 10$) and $Hdh^{Q111/Q111}$/$mGluR5^{-/-}$ (SCH23390 $n = 11$ and saline $n = 10$) mice. Animals were placed in the open-field box and injected at an injection point of 10 min with either saline or SCH23390 (i.p. 0.5 mg/kg). Each animal was monitored for 120 min, and total distance was measured at 5-min intervals (C) or cumulatively >120 min (D). (**E** and **F**) Graphs show total distance traveled by $Hdh^{Q20/Q20}$/$mGluR5^{-/-}$ (amphetamine (amphet) $n = 21$ saline $n = 15$) and $Hdh^{Q111/Q111}$/$mGluR5^{-/-}$ (amphetamine $n = 21$ and saline $n = 15$) mice. Animals were placed in the open-field box and injected at an injection point of 10 min with either saline or amphetamine (i.p. 2 mg/kg). Each animal was monitored for 120 min, and total distance was measured at 5-min intervals (E) or cumulatively >120 min (F). Data represent means ± SEM. * indicates significant differences as compared with matched genotype injected with saline ($P < 0.05$).

analysis indicated that the expression of many genes encoding for animal motility, cell transport, vesicular trafficking proteins, brain development and protein aggregation was altered in mGluR5 null mutant HD when compared with wild-type mutant HD mice (Tables 1 and 2 and Supplementary Material, Fig. S3). The expression of these genes was neither different nor altered when we compared wild-type control and mutant HD mice (data not shown).

In order to confirm the microarray results, we performed quantitative real-time PCR (qPCR) to determine the expression of genes of interest, including the mGluR5 null mutant HD mouse up-regulated genes, dynein heavy chain 6 (*Dnahc6*) and dynein light Tctex chain-type 1B (*Dynlt1*), and the mGluR5 null mutant HD mouse down-regulated gene dynactin 3 (*Dctn3*). The qPCR results confirmed that *Dnahc6* and *Dynlt1* were up-regulated in mGluR5 null mutant HD mouse versus mGluR5 null control mice (Fig. 7A and B). Moreover, in agreement with the microarray results, *Dctn3* was down-regulated in mGluR5 null mutant HD mouse versus mGluR5 null control mice (Fig. 7C). To determine whether the observed expression

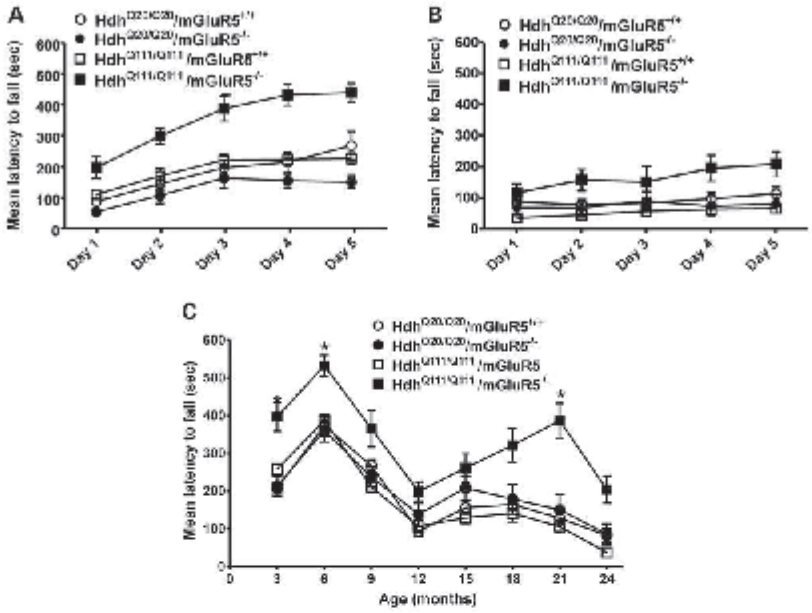

Figure 5. mGluR5 knockout improves $Hdh^{Q111/Q111}$ rotarod performance. (**A** and **B**) Graphs show rotarod learning curve represented as latency to fall from accelerating rotarod by control ($Hdh^{Q20/Q20}/mGluR5^{+/+}$) ($n = 9–11$), mGluR5 null control ($Hdh^{Q20/Q20}/mGluR5^{-/-}$) ($n = 6–11$), mutant HD ($Hdh^{Q111/Q111}/mGluR5^{+/+}$) ($n = 9–12$) and mGluR5 null mutant HD ($Hdh^{Q111/Q111}/mGluR5^{-/-}$) ($n = 6–11$) mice that were 3 (A) and 24 (B) months old. Each animal was trained for 10 min in six trials performed every day for five consecutive days. (**C**) Graph shows latency to fall from accelerating rotarod by $Hdh^{Q20/Q20}/mGluR5^{+/+}$ ($n = 9–11$), $Hdh^{Q20/Q20}/mGluR5^{-/-}$ ($n = 6–11$), $Hdh^{Q111/Q111}/mGluR5^{+/+}$ ($n = 9–12$) and $Hdh^{Q111/Q111}/mGluR5^{-/-}$ ($n = 6–11$) mice tested at 3, 6, 9, 12, 15, 18, 21 and 24 months of age. Each animal was tested in three trials, and the average latency to fall was determined. Data represent means ± SEM. * indicates significant differences as compared with $Hdh^{Q20/Q20}/mGluR5^{+/+}$ mice ($P < 0.05$).

alterations were gene specific, we performed qPCR to measure expression levels of related genes that did not appear altered in the microarray assay. The microarray results did not indicate any changes in dynein light chain LC8-type 1 (*Dynll1*) and dynactin 6 (*Dctn6*) expression levels in mGluR5 null mutant HD mouse versus mGluR5 null control mice. Supporting these results, qPCR data also indicated that the expression of *Dynll1* and *Dctn6* was not different between mGluR5 null mutant HD mouse versus mGluR5 null control mice (Fig. 7D and E).

DISCUSSION

Chorea-like movements are the most characteristic symptoms of HD patients. Currently, these abnormal hyperkinetic movements are treated with antidopaminergic neuroleptic drugs, which are not very efficacious in the case of HD and may promote extrapyramidal side effects (24,25). A better understanding of the neural circuits and alterations promoted by mutated huntingtin will help to develop new therapeutic strategies to treat chorea-like HD symptoms. In the present study, we demonstrate that mGluR5 blockage promotes increased locomotor activity and that this increase is abrogated by D1 and D2 dopamine antagonists. mGluR5 null control (Q20/Q20) mice, as well as wild-type control mice treated with MTEP, exhibit increased locomotor activity as compared with control. However, although MTEP acute injection increases mutant HD mouse locomotor activity, knockout of mGluR5 in mutant HD mice does not promote augmented locomotor activity. Moreover, mGluR5 null mutant HD mice exhibit improved rotarod performance as compared with that of wild-type control, mGluR5 null control and mutant HD mice. In addition to these behavioral alterations, mutated huntingtin aggregation appears to be influenced by mGluR5 as huntingtin intranuclear inclusions observed in mutant HD mouse striatum are significantly reduced in mGluR5 null mutant HD mice. It is possible that mutated huntingtin protein could alter gene expression differently in the absence of mGluR5, which could account for these adaptations. Supporting this hypothesis, our microarray study indicates that mutated huntingtin protein can alter the expression of a number of genes that could be important for the locomotor adaptations and decrease in huntingtin intranuclear inclusions observed in mGluR5 null mutant HD mice. Importantly, qPCR experiments confirmed that the mRNA transcript levels of *Dnahc6* and *Dynlt1*, and dynactin 3 are altered in mGluR5 null mutant HD mice, as compared with mGluR5 null control mice,

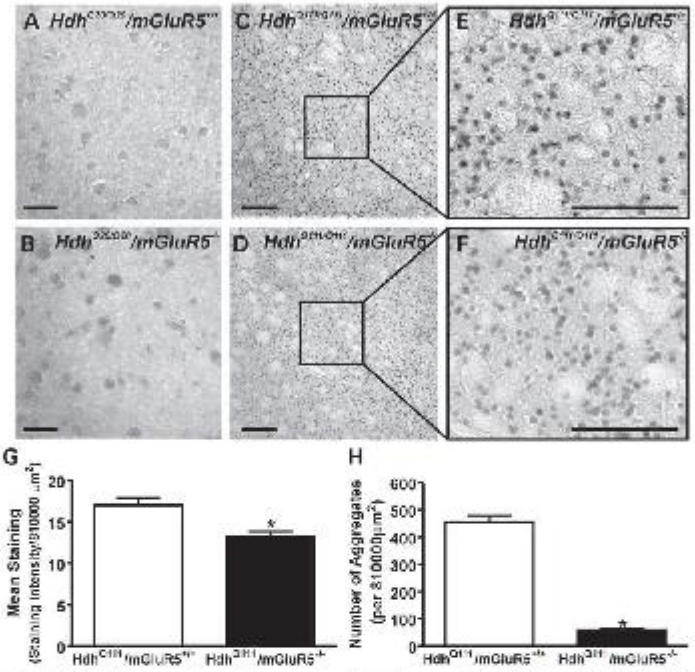

Figure 6. The number of huntingtin (EM48) intranuclear inclusions is reduced by the knockout of mGluR5 in Hdh$^{Q111/Q111}$/mGluR5$^{-/-}$ mice. Shown are representative images for EM48 immunostaining from (A) control (Hdh$^{Q20/Q20}$/mGluR5$^{+/+}$), (B) mGluR5 null control (Hdh$^{Q20/Q20}$/mGluR5$^{-/-}$), (C) mutant HD (Hdh$^{Q111/Q111}$/mGluR5$^{+/+}$) and (D) mGluR5 null mutant HD (Hdh$^{Q111/Q111}$/mGluR5$^{-/-}$) striatal slices. Previous panels were enlarged to show intranuclear inclusions present in Hdh$^{Q111/Q111}$/mGluR5$^{+/+}$ (E) and Hdh$^{Q111/Q111}$/mGluR5$^{-/-}$ (F) striatal slices. Graphs show quantification of diffuse EM48 staining (G) and number of Huntingtin aggregates (intranuclear inclusions) (G) found per 900 × 900 µm^2. Data represent the means ± SEM for three independent experiments. Asterisks indicate significant differences (unpaired *t*-test) as compared with Hdh$^{Q111/Q111}$/mGluR5$^{+/+}$ mice ($P < 0.05$). Scale bar = 150 µm.

although these alteration are not observed when we compare wild-type control and mutant HD mice.

mGluR5 is highly expressed in the striatum, which is the main region affected in HD (26,27). Our group has established a link between mGluR5 and HD by showing that group I mGluRs interact with the huntingtin protein (17). Moreover, mutant huntingtin protein can alter mGluR5 signaling, decreasing inositol-1,4,5-triphosphate (IP3) formation and increasing ERK1/2 and AKT activation (20). A number of reports have demonstrated that mGluR5 blockage can induce hyperkinetic movements (12,13). In agreement with these findings, our data demonstrate that mGluR5 knockout or mGluR5 blockage induce hyperkinesia in mice, suggesting that mGluR5 could contribute to HD chorea.

It is still unknown how mGluR5 blockage induces hyperkinesia and which neuronal circuits are involved in this regulation. We show that the hyperkinesia exhibited by the mGluR5 knockout mice is abolished by D1 and D2 dopamine receptor antagonists, haloperidol and SCH23390, respectively. It is well known that the glutamatergic and dopaminergic systems physically interact in certain brain regions, including the striatum, nucleus accumbens and prefrontal cortex, and that this interaction is important for the control of both cognition and movement (28–31). Interestingly, striatum and cortex are the primary affected areas in HD (6,7). Importantly, our data demonstrate that mutated Huntingtin expression abolishes increased locomotor activity promoted by both mGluR5 knockout and amphetamine, further supporting the idea that mGluR5 blockage in the striatum and cortex could be involved in hyperkinesia. The striatum is mainly composed of GABAergic inhibitory MSNs, which express high levels of mGluR5 and project to different areas of the brain, including the SNc, which is mainly composed of dopaminergic projection neurons that up-regulate locomotor activity via cortical and nigrostriatal stimulation (10,32). We hypothesize that the increase in locomotor activity observed in mGluR5 knockout mice is because of the decreased stimulation of MSNs by glutamatergic cortical projection neurons, which in wild-type mice can occur via mGluR5 activation (8,9). Decreased stimulation of MSNs lessens the inhibition of SNc neurons, leading to dopaminergic disinhibition and increased thalamocortical stimulation, which can increase locomotor activity. Further experiments, including brain region-specific injections of MTEP, will be important to confirm this hypothesis.

Table 1. Genes up-regulated in $Hdh^{Q111/Q111}$/mGluR5$^{-/-}$ versus $Hdh^{Q20/Q20}$/mGluR5$^{-/-}$ mice that are not up-regulated in $Hdh^{Q111/Q111}$/mGluR5$^{+/+}$ versus $Hdh^{Q20/Q20}$/mGluR5$^{+/+}$ mice

Gene name	Gene symbol	RefSeq	Transcript ID	Fold change	*P*-value
Dynein, axonemal, heavy chain 6	*Dnahc6*	ENSMUS00000T114088	10545502	6.00	1.33E-07
5′ nucleotidase domain containing 3	*Nt5dc3*	NM_175331	10365518	3.38	3.04E-06
Serine (or cysteine) peptidase inhibitor	*Serpina3n*	NM_009252	10398075	3.03	1.12E-07
Vomeronasal 1 receptor	*V1rc15*	NM_134170	10545111	2.77	0.000827765
Secreted phosphoprotein 1	*Spp1*	NM_009263	10523717	2.38	0.00137286
Phospholipase A2	*PLA2g4e*	NM_177845	10486403	2.11	1.78E-05
Protocadherin beta 8	*Pcdhb8*	NM_053133	10455078	1.79	0.00190443
Trafficking protein particle complex 2-like	*Trappc2l*	NM_021502	10576152	1.75	0.000105982
Dynein light chain Tctex, type 1	*Dynlt1*	NM_009342	10548785	1.75	0.000488174
Iduronidase, alpha-L-	*Idua*	NM_008325	10524034	1.67	5.08E-05
Double homeobox B-like	*Duxbl*	NM_183389	10413255	1.66	9.54E-05
WD repeat and FYVE domain containing 1	*Wdfy1*	NM_001111279	10355974	1.65	7.18E-05
Aryl hydrocarbon receptor nuclear translocator-like 2	*Arnl2*	NM_172309	10542764	1.63	6.59E-06
Serine/threonine kinase 10	*Stk10*	NM_009288	10375083	1.59	4.62E-05
Filamin, beta	*Flnb*	NM_134080	10412562	1.58	3.26E-05
Hippocalcin-like 1	*Hpcal1*	NM_016677	10399671	1.55	0.000205577
Solute carrier family 35, member F3	*Slc35f3*	NM_175434	10576586	1.52	4.60E-05
Integrator complex subunit 7	*Ints7*	NM_178632	10352735	1.51	0.000138663
Matrilin 2	*Matn2*	NM_016762	10423599	1.47	0.000223666
Natriuretic peptide precursor type A	*Nppa*	NM_008725	10510265	1.37	5.04E-05
Exonuclease 3′–5′ domain containing 1	*Exd1*	NM_172857	10486241	1.35	0.000212041
Ectonucleoside triphosphate diphosphohydrolase 2	*Entpd2*	NM_009849	10470014	1.33	2.82E-05
Rho GTPase activating protein 4	*Arhgap4*	NM_138630	10605143	1.30	0.000340499
Tubby like protein 4	*Tulp4*	NM_054040	10441497	1.25	0.000128779

Motor coordination deficit can be typically observed in HD patients, and this feature is also present in most HD mouse models, such as R6/2, YAC128 and BACHD, which can be assessed by submitting the animals to the rotarod task (21). However, knock-in mouse models of HD with <150 polyglutamines, such as $Hdh^{Q111/Q111}$ mice, do not exhibit any impairment in rotarod performance, as compared with control (21,33). It has also been shown that mGluR5 blockage with MTEP does not improve rotarod performance (22). However, we show here that the knockout of mGluR5 in a mouse expressing mutated huntingtin, but not wild-type huntingtin, improves rotarod performance. One potential hypothesis to explain these finding is that mGluR5 blockage facilitates motor coordination but that improvement in motor coordination in mGluR5 null control mice is abrogated by hyperactivity. Thus, because the mGluR5 null mutant HD mice do not exhibit increased locomotor activity, improvement in rotarod performance can be detected in these mice. Further experiments, including crossing a HD mouse model that exhibit rotarod deficiency to mGluR5 knockout mice, will be necessary to test this hypothesis and determine whether mGluR5 blockage can improve motor coordination in HD. Moreover, the knockout of mGluR5 in a HD mouse model that has a short life span will be important to determine whether the lack of mGluR5 could increase mouse longevity.

MTEP acute injection was sufficient to augment locomotor activity in both control and mutant HD mice. However, lack of mGluR5 expression during the whole life span as in mGluR5 knockout mice fails to increase locomotor activity in mutant huntingtin expressing mice, although mGluR5 null control mice exhibit increased locomotor activity. These data indicate that following the developmental loss of mGluR5 expression, the mutated huntingtin protein might activate compensatory mechanisms that abrogate mGluR5-induced hyperkinesia. Understanding these mechanisms could contribute to a better comprehension of mutant Huntingtin-induced chorea. A number of reports indicate that the mutated Huntingtin protein can alter gene expression (34,35). Moreover, mGluR5 stimulation can also modify gene expression (36,37). Thus, we investigated whether mutated Huntingtin-induced gene expression alterations in an mGluR5 null background could help to explain the lack of hyperkinesia, improved rotarod performance and decreased intranuclear inclusions observed in mGluR5 null mutant HD, as compared with mGluR5 null control mice. In agreement with this hypothesis, we found that the mRNA transcript levels correspondent to a number of genes were altered in mGluR5 null mutant HD, as compared with mGluR5 null control mice, but not in mGluR5 null control versus wild-type mice. These data indicate that there is a functional interaction between the glutamatergic system and mutated huntingtin in terms of controlling gene expression. In agreement with this assumption, it has been shown that the knockout of GluN3A, which is an NMDA receptor subunit, leads to normalization of NMDAR currents, prevention of synapse degeneration and striatal cell death, as well as reversion of motor and cognitive decline exhibited by YAC128 mice (38).

qPCR experiments confirmed that *Dnahc6* and *Dynlt1* genes were up-regulated and that dynactin 3 gene was down-regulated in mGluR5 null mutant HD, as compared with mGluR5 null control mice. Mutation and/or altered expression of proteins involved in axonal transport, such as dynein heavy chain, dynein light chain and dynactin, contribute to pathogenesis in multiple neurodegenerative diseases (39). Dyneins are microtubule motors that move cargo from the distal ends of axons toward neuronal cell bodies. It has been shown that decreased dynein function impairs autophagic clearance of aggregate-prone proteins, such as mutated Huntingtin, leading to increased huntingtin toxicity and enhanced phenotype in mouse and fly models of HD (40). A mouse model of HD expressing mutated

Table 2. Genes down-regulated in $Hdh^{Q20/Q20}/mGluR5^{-/-}$ versus $Hdh^{Q111/Q111}/mGluR5^{-/-}$ mice that are not down-regulated in $Hdh^{Q20/Q20}/mGluR5^{+/+}$ versus $Hdh^{Q111/Q111}/mGluR5^{+/+}$ mice

Gene name	Gene symbol	RefSeq	Transcript ID	Fold change	*P*-value
Protein phosphatase 1, inhibitor subunit 2	*Gm5972*	NR_003650	10467003	−4.21	5.98E-07
Pituitary tumor-transforming gene 1	*Pttg1*	NM_013917	10385325	−3.53	1.02E-06
Retinoic acid early transcript beta	*Raet1b*	BC132022	10362097	−3.07	0.000186535
Ubiquitin-like domain containing CTD phosphatase 1	*Ublcp1*	NM_024475	10385361	−2.34	1.67E-05
Activin A receptor, type II-like 1	*Acvrl1*	NM_009612	10426999	−2.24	3.32E-07
Heat shock protein 8	*Hspa8*	NM_031165	10584572	−2.22	2.14E-06
Hematological and neurological expressed 1-like	*Hn1l*	EF651808	10417359	−2.12	1.68E-05
Interphotoreceptor matrix proteoglycan 1	*Impg1*	NM_022016	10595306	−2.11	0.000146534
Chemokine (C-C motif) ligand 28	*Ccl28*	NM_020279	10598203	−2.05	0.000876406
Progressive myoclonic epilepsy, type 2 gene	*Epm2a*	NM_010146	10361754	−1.95	0.000169655
Dynactin 3	*Dynactin 3*	NM_016890	10512291	−1.87	0.000101524
Retinol binding protein 1	*Rbp1*	NM_011254	10588037	−1.83	0.00083148
Chromobox homolog 7	*Cbx7*	NM_144811	10430649	−1.79	0.000629588
Nucleoporin 35	*Nup35*	NM_027091	10567131	−1.78	0.00012699
Carbonic anhydrase 12	*Car12*	NM_178396	10586591	−1.78	0.000179906
Holliday junction recognition protein	*Hjurp*	NM_198652	10356461	−1.78	4.66E-06
Solute carrier family 7 (cationic amino acid transporter)	*Slc7a11*	NM_011990	10498024	−1.76	0.000298727
Hydroxysteroid (17-beta) dehydrogenase 7	*Hsd17b7*	NM_010476	10359917	−1.73	0.000364903
Abhydrolase domain containing 10	*Abhd10*	NM_172511	10439701	−1.70	0.000383236
Transmembrane protein 44	*Tmem44*	NM_172614	10438942	−1.65	0.000222186
Midline 1	*Mid1*	NM_010797	10603208	−1.64	0.000202454
N-acetylneuraminate pyruvate lyase	*Npl*	NM_028749	10858879	−1.63	0.000374456
Toll-like receptor 1	*Tlr1*	NM_030682	10530145	−1.62	0.00061089
Tensin 1	*Tns1*	NM_027884	10855536	−1.24	7.99E-05
EPM2A (laforin) interacting protein 1	*Epm2aip*	NM_175266	10589756	−1.60	0.000285304
Hedgehog interacting protein-like 1	*Hhlp1*	NM_001044380	10898211	−1.58	1.17E-06
Disintegrin-like and metallopeptidase (reprolysin)	*Adamts3*	NM_001081401	10531193	−1.55	0.000446511
PTPRF interacting protein, binding protein 1 (liprin)	*Ppfibp1*	NM_026221	10542791	−1.55	1.99E-05
Formin 2	*Fmn2*	NM_019445	10851971	−1.54	0.000206781
Serine peptidase inhibitor, Kazal, type 8	*Spink8*	NM_183136	10589407	−1.54	4.04E-05
Cysteine-rich secretory protein LCCL domain containing	*Crispld1*	NM_031402	10344990	−1.50	9.61E-06
Leucine carboxyl methyltransferase 2	*Lcmt2*	NM_177846	10486710	−1.25	0.000273002
FAT tumor suppressor homolog 4	*Fat4*	NM_183221	10491732	−1.26	0.000220214
Purkinje cell protein 4-like 1	*Pcp4l1*	NM_025557	10360053	−1.27	2.36E-05
Solute carrier family 6 (neurotransmitter transporter)	*Slc6a20a*	NM_139142	10597960	−1.29	0.000147968
Glutamate receptor, metabotropic 7	*Grm7*	NM_177328	10540509	−1.37	0.000221335
Deoxyguanosine kinase	*Dguok*	NM_013764	10545697	−1.38	0.000331795
Protein tyrosine phosphatase, receptor, type B	*Ptprb*	NM_029928	10366476	−1.39	0.000361865
Xanthine dehydrogenase	*Xdh*	NM_011723	10452815	−1.41	0.000301857
Thrombospondin 2	*Thbs2*	NM_011581	10447951	−1.42	8.54E-06

Dnhc1 exhibited higher levels of tremor, worse performance in the grip strength and accelerating rotarod, compromised gait and a shorter lifespan, as compared with HD mouse that express wild-type *Dnhc1* (40). Moreover, *Caenorhabditis elegans* expressing mutant proteins that are part of the dynein–dynactin complex, including dynein heavy chain, dynein light chain and dynactin, misaccumulate synaptic proteins at the ends of neuronal processes and exhibit progressive motor neuron disease symptoms (41). As these mutant *C. elegans* age, neuronal misaccumulations increase in size and frequency, locomotion becomes progressively slower and life span is shortened (41). Another study demonstrates that a mouse model that presents a point mutation in the dynein light intermediate chain 1 (*Dync1li1*) displays abnormal neuronal development, increased anxiety and lower levels of spontaneous locomotor activity, as compared with wild-type littermates (42). Thus, mutated huntingtin-mediated alterations could contribute to the phenotypic adaptations, including the diminished levels of huntingtin intranuclear inclusions and the motor alterations, observed in mGluR5 null mutant HD mice.

In conclusion, our data indicate that mutated huntingtin protein and mGluR5 exhibit a functional interaction that might be implicated in HD-related symptoms. Both mutated huntingtin and mGluR5 can regulate gene expression levels, which could account for the changes in motor behavior and pattern of huntingtin aggregation observed in mGluR5 null mutant HD mice. However, although mGluR5 knockout in mutant HD mice decreased huntingtin aggregation, it is still not clear whether mGluR5 antagonists could ameliorate HD-related symptoms. Future experiments will be important to further investigate the mechanisms underlying the alterations observed in mGluR5 null mutant HD mice.

MATERIALS AND METHODS

Materials

TRIzol, Nuclease-Free Water and Power SYBR® Green PCR Master Mix were purchased from Life Technologies (Foster City, CA, USA). Mouse anti-Huntingtin EM48 antibody was

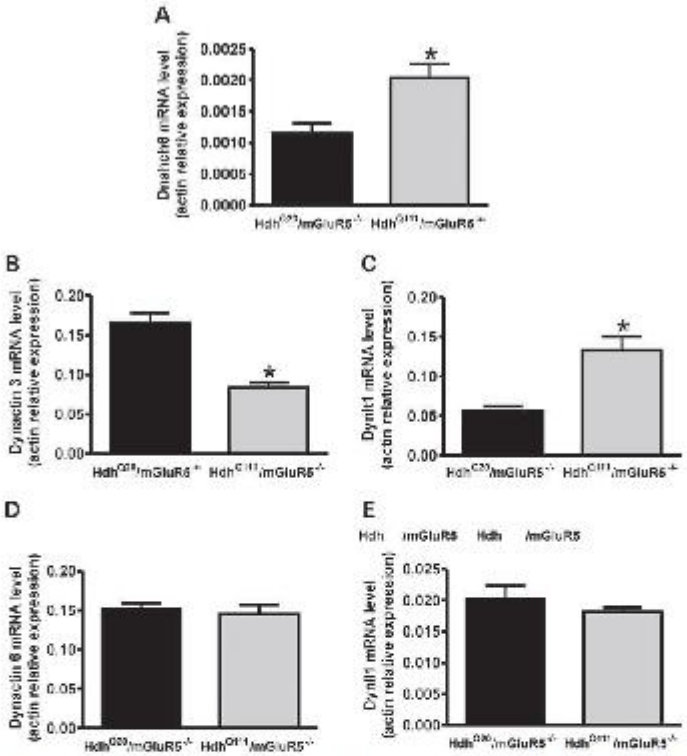

Figure 7. Gene expression changes between $Hdh^{Q20/Q20}/mGluR5^{-/-}$ versus $Hdh^{Q111/Q111}/mGluR5^{-/-}$ mice. Graph shows mRNA levels of dynein heavy chain axonemal 6 (*Dnahc6*), NM_001164669.1 (A), dynactin 3, NM_016890 (B), dynein light Tctex chain-type 1B (*Dynlt1b*), NM_009342 (C), dynactin 6, NM_011722 (D), and dynein light chain LC8-type 1 (*Dynll1*), NM_019682 (D) determined by quantitative RT-PCR. mRNA was extracted from the striatum of age-matched mGluR5 null mutant HD ($Hdh^{Q111/Q111}/mGluR5^{-/-}$) and mGluR5 null control ($Hdh^{Q20/Q20}/mGluR5^{-/-}$) mice. qPCR reaction was performed in triplicate and normalized to actin mRNA levels. Data represent the means ± SEM of four independent experiments. * indicates significant differences as compared with $Hdh^{Q20/Q20}/mGluR5^{-/-}$ mice ($P < 0.05$).

purchased from Millipore (Billerica, MA, USA). MTEP was kindly provided by Merck & Co., Inc. (Rahway, NJ, USA), haloperidol (Cat. H1512) was purchased from Sigma–Aldrich (St. Louis, MO, USA), SCH23390 (cat. 0925) and D-amphetamine (cat. 2813) were purchased from Tocris Bioscience (Bristol, UK). All other biochemical reagents were purchased from Sigma–Aldrich.

Mouse model

STOCK-*Htt*^{tm2Mem}/J ($Hdh^{Q20/Q20}$) and STOCK-*Htt*^{tm5Mem}/J ($Hdh^{Q111/Q111}$) knock-in mice (43) and mGluR5 knockout mice B6;129-Grm5^{tm1Rod}/J ($mGluR5^{-/-}$) (44) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). $Hdh^{Q111/Q111}/mGluR5^{-/-}$ and $Hdh^{Q111/Q111}/mGluR5^{+/+}$ mice were obtained by crossing $Hdh^{Q111/Q111}$ and $mGluR5^{-/-}$ mice. $Hdh^{Q20/Q20}/mGluR5^{-/-}$ and $Hdh^{Q20/Q20}/mGluR5^{+/+}$ mice were obtained by crossing $Hdh^{Q20/Q20}$ and $mGluR5^{-/-}$ mice. 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 University of Western Ontario Animal Care Committee.

Open field

Eight VersaMax Animal Activity Monitors (AccuScan Instruments, Inc., Columbus, OH, USA) were used to measure locomotor activity. Experiments were performed during the light cycle of the mice and between the hours of 08:0 and 14:00. Mice were allowed to explore the open-field boxes (20 × 20 cm) for 120 min during which time movement was measured at 5-min intervals using beam breaks converted to cm. During

drug studies, mice were allowed to explore the open-field boxes for 10 min after which an injection of either saline or drug was administered and the activity was monitored for additional 110 min. Measurement of total activity and time spent in the center of the arena were calculated,, and statistical analyses were performed using GraphPad Prism software.

Rotarod test

The training and testing of the mice on the rotarod treadmill system (Diego Instruments, Sand Diego, CA, USA) occurred during the light cycle between the hours of 08:00 and 14:00. Mice were habituated to the testing room for 15–20 min. To introduce the mice to the rotarod apparatus, mice were placed on the rotarod and left at rest for 5 min on the first day of training before beginning the accelerating protocol. Mice were then trained for a maximum of 10 min in six trials at an accelerating speed (from 4 to 40 RPM in 600 s) for five consecutive days with 10-min breaks between each trial. If mice fell in the first 20 s of training, they were placed back on the apparatus immediately, up to three times. Mice were returned to their home cages at the end of training day 5 and rested for 2 days. On day 8, mice were tested in three trials with accelerating speed separated by a 30-min inter-trial interval. The latency to fall from the rod was recorded, and the average obtained from the three trials was used for analysis. Mice remaining on the rod for >600 s were removed and their time scored as 600 s.

EM48 immunohistochemistry

Mice were transcardially perfused with 4% paraformaldehyde (PFA) in PBS. Brains were then dissected out and stored in 4% PFA in PBS. Prior to sectioning, brains were put into 30% sucrose in PBS overnight at 4°C. Brains were dissected into left and right hemisphere, with the right hemisphere used for histology. Brains were coronally sectioned through the striatum, from +1.18 to −0.4 mm bregma. Immunohistochemistry was performed on 40-µm free-floating sections using a peroxidase-based immunostaining protocol. In brief, endogenous peroxidase activity was quenched using 0.1% hydrogen peroxide, after which the membranes were permeabilized using 1% Triton X-100. Non-specific binding was blocked using 1.5% normal horse serum, followed by incubation in primary antibody (1:100, anti-Huntingtin protein, mouse monoclonal EM48 antibody) overnight at 4°C. Sections were washed in PBS and then incubated in secondary antibody (biotinylated horse anti-mouse, 1:400, Vector Elite ABC kit mouse) for 90 min at 4°C. Finally, sections were incubated in an avidin–biotin enzyme reagent (Vector Elite ABC kit mouse, PK-6102, Vector Laboratories). Immunostaining was visualized using a chromogen (Vector SG substrate). Sections were mounted on slides and visualized using a Zeiss LSM-510 META multiphoton laser scanning microscope with a Zeiss 10× lens, representative 900 × 900-µm areas of striatum were imaged for analysis. The number of EM48-positive cells was analyzed using the multi-threshold plugin on Image J, whereas the number of EM48-positive puncta per image was counted using the cell counter tool in Image J (NIH, USA). The difference in numbers between genotypes was analyzed using unpaired *t*-test.

Microarray hybridization and analysis

Microarray labeling and hybridization were performed at the London Regional Genomics Centre (Robarts Research Institute, London, ON, CA). To prepare 5.5 µg of cDNA using GeneChip® WT Terminal Labeling Kit (Affymetrix, Santa Clara, CA, USA), 20 µg of isolated RNA from striatum extracts was used. cDNAs were then labeled and hybridized in Mouse Gene 1.0 ST chips using GeneChip® WT Terminal Labeling Kit and GeneChip® Hybridization Wash and Stain kit (Affymetrix). The arrays were incubated for 17 ± 1 h, scanned with the GeneChip Scanner 3000 7G (Affymetrix) using Command Console v1.1, and probe set signals were calculated with the multi-array average algorithm. We used Partek Genomics Suite v6.5 (Partek, St. Louis, MO, USA) to determine differences in gene expression levels. Networks were generated through the use of IPA software (Ingenuity Pathway Analysis, Ingenuity Systems). Genotype effects were considered significant based of the following criteria: (i) ANOVA *P*-values < 0.05 and (ii) 1.5-fold increase or decrease. Considering that $Hdh^{Q111/Q111}$ is a knock-in mice, we used mGluR5 expression as an internal control for the microarray assay.

Quantitative RT-PCR

RNA was isolated using Trizol reagent as per manufacture's instructions (Invitrogen, Burlington, ON, USA). RNA was re-suspended in 20 µl of RNase-free water, and its concentration and quality were analyzed by NanoDrop™ (Thermo Scientific, Wilmington, USA) and gel electrophoresis, respectively. cDNAs were prepared from 40 ng of total RNA extracted in a 20 µl final reverse transcription reaction. Quantitative PCR was performed using the Power SYBR® Green PCR Master Mix and the ABI PRISM 7900HT Sequence Detection System platform (Applied Biosystems, Foster City, CA, USA). Quantitative RT-PCR (qPCR) was performed to quantify mRNA levels of the following genes: dynein light chain LC8-type 1—*Dynll1* (NM_019682); dynactin 6—*Dctn6* (NM_011722); dynein heavy chain axonemal 6—*Dnahc6* (NM_001164669); dynactin 3—*Dctn3* (NM_016890); dynein light Tctex chain-type 1B—*Dynlt1b* (NM_009342). Primers were designed using Primer3Plus Program: *Dynll1* (forward: 5′ TTTGTCCCTGCCAAGTACTG 3′; reverse: 5′ CTTAACTGC CCTATCTGTGGTC 3′); *Dctn6* (forward: 5′ TGATCCACCC TAAAGCACG 3′; reverse: 5′ ATAGGTTTGGGCTCTGTA TCTTC 3′); *Dnahc6* (forward: 5′ CGCAAGGAAGATGACA CAGA 3′; reverse: 5′ TTAGAGACCCAGCCATGACC 3′); *Dctn3* (forward: 5′ CAGATCCACATCCAGCAGCA 3′; reverse: 5′ ACCCTTCCAGGAGAGCCTTA 3′); *Dynlt1b* (forward: 5′ TCATGCAGAAGAACGGTGCT 3′; reverse: 5′ TCTGTGGA GCTGTCCCAGAA 3′). Samples were prepared in triplicate, and changes in gene expression were determined with the $2^{-\Delta CT}$ method using actin for normalization. All RT-qPCRs showed good quality of amplification, and the specificity and efficiency of primers were tested and confirmed by the serial dilution method.

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 and for curve fitting. Statistical significance was determined by ANOVA testing followed by *post hoc* Multiple Comparison testing.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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Metabotropic glutamate receptor 5 as a potential therapeutic target in Huntington's disease

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Keywords: Huntington's disease, mGluR5, huntingtin, neurodegeneration, cell signaling, glutamate.

Abstract:

Introduction: Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder caused by a polyglutamine expansion in the amino-terminal region of the huntingtin protein (htt), which underlies the loss of striatal and cortical neurons. Glutamate has been implicated in a number of neurodegenerative diseases and several studies suggest that the metabotropic glutamate receptor 5 (mGluR5) may represent a target for the treatment of HD.

Areas covered: The main goal of this review is to discuss the current data in the literature regarding the role of mGluR5 in HD and evaluate the potential of mGluR5 as a therapeutic target for the treatment of HD. mGluR5 is highly expressed in the brain regions affected in HD and is involved in movement control. Moreover, mGluR5 interacts with htt and mutated htt profoundly affects mGluR5 signaling. However, mGluR5 stimulation can activate both neuroprotective and neurotoxic signaling pathways, depending on the context of activation.

Expert opinion: Although the data published so far strongly indicate that mGluR5 plays a major role in HD-associated neurodegeneration, htt aggregation and motor symptoms, it is not clear whether mGluR5 stimulation can diminish or intensify neuronal cell loss and HD progression. Thus, future experiments will be necessary to further investigate the outcome of drugs acting on mGluR5 for the treatment of neurodegenerative diseases.

1. Huntington's Disease:

Huntington's disease (HD), as first described by George Huntington in 1872, is an autosomal dominant neurodegenerative disorder, involving the loss of striatal and cortical neurons[1]. The most prominent HD symptoms are motor deterioration, cognitive decay and psychiatric alterations that inevitably lead to the death of HD patients within 15-20 years of disease onset[2]. HD clinical manifestations are progressive following disease onset and include involuntary movements (chorea) that intensify during the course of the disease, causing the impairment of daily life activities[3]. HD patients also suffer gait dysfunctions that are characterized by postural instability, lack of balance and motor incoordination, leading to common falls. These problems are accompanied by eye movement abnormalities and progressive dysarthria and dysphagia. Moreover, psychiatric and cognitive disturbances typically appear during disease progression and include personality alterations, obsession, compulsion, major depression and dementia.

In 1983, the chromosomal localization of the HD gene was determined[4]. Ten years later, the HD gene, IT15, encoding the huntingtin protein (htt), was identified and its characterization indicated the presence of a polymorphic CAG repeat that was expanded and unstable in HD patients[5]. Wild type htt contains 15–35 CAG repeats, while in HD the repeat length is increased (40-180 repeats) resulting in a mutant disease-inducing htt protein. Early age of onset and severity of symptoms are directly attributed to CAG trinucleotide expansion length: as the number of CAG increases, the earlier and more severe clinical signs appear. Moreover, CAG repeats tend to increase from one generation to the next, especially when mutant htt is inherited from the father[6]. However, individuals with identical repeat lengths may exhibit significant variability in terms of age of onset, suggesting that other factors, such as environmental influences and genetic modifiers also play a role in the clinical course of the disease[7].

Nevertheless, despite the known cause of HD, there is no disease modifying drug to treat HD patients.

Since the identification of the htt gene, several studies have investigated htt physiological function and its involvement in HD. Wild type htt can interact with a number of different proteins, playing an important role in preventing apoptosis, facilitating vesicular trafficking and regulating gene transcription[8, 9]. Moreover, it has been demonstrated that wild-type htt is important for development as htt knockout mice do not survive embryonic phase[10]. Additionally, htt heterozygous mice present motor activity alterations and cognitive deficits[10]. Mutant htt exhibits a toxic gain-of-function, including formation of protein aggregates, altered gene expression, compromised autophagy and proteasome function, mitochondrial dysfunction leading to accumulation of reactive oxygen species, increased glutamate excitotoxicity and augmented apoptosis (Figure 1)[11-13]. Thus, it is possible that both loss of normal wild type htt physiological function and mutant htt toxic gain-of-function contribute to HD development.

1.1 Animal Models of HD:

Since HD is caused by a single gene mutation, it is feasible to develop animal models that closely recapitulate HD pathology and that can be used to better understand HD mechanisms and to develop treatments. Thus, a number of mouse models of HD have been developed, including transgenic mice expressing only the amino-terminal region of human polyglutamine expanded htt protein, such as R6/2 and N171-82Q[14, 15]; transgenic mice expressing full-length human polyglutamine expanded htt protein, such as yeast artificial chromosome mice (YAC128) and bacterial artificial chromosome mice (BACHD)[16, 17]; and knock-in mice, such as $Hdh^{Q97/Q97}$, $Hdh^{Q111/Q111}$ and

$Hdh^{Q150/Q150}$, generated by switching the first exon of the murine htt gene by the first exon of the human mutated htt protein[18, 19,29].

R6/2 mice exhibit severe neurological signs and premature death at 3-6 months of age[15]. However, despite having a robust phenotype, the R6/2 mice are not regarded as an accurate model of HD, as these mice only express the amino-terminal region of the htt protein, which is mostly comprised of polyglutamines. Thus, R6/2 may be considered a model of polyglutamine diseases rather than a specific HD mouse model. $Hdh^{Q111/Q111}$ mice display a very mild phenotype, exhibiting less behavioral abnormalities than R6/2, BACHD and YAC128 mice[20]. However, $Hdh^{Q150/Q150}$ mice exhibit HD related phenotypic alterations, including motor deficit and gait abnormalities[21, 22]. Thus, as observed in HD patients, HD mouse models with more CAG repeats ($Hdh^{Q111/Q111}$ versus $Hdh^{Q150/Q150}$) exhibit stronger phenotypes.

Although HD rodent models replicate the HD related phenotypic and pathological alterations, most of the mouse models do not display the robust neurodegeneration that takes place in HD patients. Interestingly, mutant htt protein appears to be more toxic to larger animals, such as pig and monkey, than to rodent HD models[23]. However, despite of this benefit, maintaining bigger animals is more expensive and demands more elaborate infrastructure required for the housing of animals that have a much longer life-span.

1.2 Mutant htt and Neuronal Cell Death:

The neuronal cell loss that takes place in HD is the main cause of HD-related symptoms. The striatum begins to degenerate before any other brain areas; however, not all striatal neurons are affected. The first neurons to be lost during the course of HD are the projection striatal GABAergic medium-sized spiny neurons (MSNs), which

account for 95% of the striatal neurons[24]. Interestingly, striatal interneurons are spared in HD[25]. Following HD progression, cortical neurons are also lost[24]. Unfortunately, there is no clear understanding as to why mutant htt, which is expressed throughout the body, leads to the selective death of MSNs and cortical neurons.

Protein aggregation is a common phenomenon in many neurodegenerative diseases[26]. It has been shown that the polyglutamine expanded htt amino-terminal region can be cleaved, forming intranuclear inclusions and cytoplasmic aggregates, which are observed in the striatum and cortex of HD patients and in animal models of the disease[16, 27]. Importantly, the formation of htt aggregates is highly correlated with both striatal neurodegeneration and HD progression[28]. However, it is not clear whether htt aggregate accumulation results in cell death or promotes neuroprotection by buffering the potentially toxic form of the soluble protein[28, 29].

Defects of intracellular Ca^{2+} homeostasis have been reported in the majority of the animal models of HD, as it has been demonstrated that mutant htt leads to increased intracellular Ca^{2+} concentration by employing a number of different mechanisms[30]. Polyglutamine expanded htt protein causes sensitization of both the ionotropic N-methyl-D-aspartate receptor (NMDAR), and the inositol-1,4,5-triphosphate (InsP3) receptor, increasing entrance of extracellular Ca^{2+} and the release of Ca^{2+} from intracellular stores, respectively[31-33] (Figure 1A). Moreover, polyglutamine expanded htt destabilizes mitochondrial Ca^{2+} regulation[34,35]. Mutated htt protein directly binds to mitochondria and, in conjunction with increased intracellular Ca^{2+}, discharges mitochondrial membrane potential, modifies mitochondrial ATP production by uncoupling oxidative phosphorylation, induces opening of the mitochondrial permeability transition pore, releases cytochrome c and activates cell death pathways[35]. Direct binding of mutant htt to mitochondria alone is insufficient to mediated Ca^{2+}

overload and the dysregulation of mitochondrial function and appears to also require an N-methyl-D-aspartate (NMDA) component, as NMDAR blockers can normalize mitochondrial ATP levels and ATP/ADP ratio in striatal cells expressing mutant htt[36]. Although mitochondrial alterations could easily explain the higher susceptibility of HD cells to apoptosis, opposing results have shown that striatal mitochondria from knock-in and transgenic mouse models of HD were equally susceptible to Ca^{2+} loads than their wild type counterparts[37]. Nevertheless, mutant htt-mediated increases in intracellular Ca^{2+} represents the most compelling hypothesis to explain how mutant htt leads to neuronal cell death.

Mutant htt can cause transcriptional deregulation by altering microRNA expression and by interacting with and modifying the function of transcriptional factors, which can contribute to the molecular pathogenesis of HD[9,38]. For example, mutant htt can bind to and sequester into aggregates the cAMP-responsive element binding protein (CREB)-binding protein, which is a coactivator of CREB[39]. Interestingly, by interfering with the CREB/CBP transcriptional pathway, mutant htt represses the peroxisome proliferator-activated receptor γ coactivator-1a (PGC-1α) gene transcription, which leads to decreased mitochondrial biogenesis[40]. Highlighting PGC-1α role in HD, PGC-1α knockout mice exhibit striatum lesions resembling HD and its overexpression has protective effects on striatal neurons expressing mutant htt[40-42]. Brain-derived neurotrophic factor (BDNF) mRNA levels are also decreased in HD, as mutant htt lacks the capacity of the wild type protein to inhibit the neuron restrictive silencer element, which functions to decrease BDNF expression[43]. BDNF is an essential neurotrophin, having a role in modulating dendritic branching, spine morphology, synaptic plasticity, and influencing learning and memory[44, 45]. Importantly, infusion of BDNF protein into the striatum of the R6/1 HD mouse model increases survival of striatal neurons and

improves motor function[46]. In addition, mutant htt also alters expression of a number of other proteins, including protein kinases, cytosketetal proteins, Ca^{2+} binding proteins (for example parvalbumin, calbindin or hippocalcin), and proteins important for neurodevelopment[9, 27, 47, 48]. Thus, HD-related transcription deregulation may, at least partially, help to explain how mutant htt can promote neurodegeneration.

2. Metabotropic Glutamate Receptors:

Glutamate is the major excitatory neurotransmitter in the brain and is responsible for regulating a variety of physiological functions including integrative brain function and neuronal cell development[49, 50]. Conversely, glutamate is also known to influence neuronal cell death[49, 50]. Glutamate acts via both ionotropic glutamate receptors, such as the NMDA and alpha-amino-3-hydroxy-5methyl-4isoxazolepropionic acid receptor, which are ligand gated ion channels; and metabotropic glutamate receptors (mGluRs). There are 8 mGluR subtypes divided into 3 groups that are all members of the G protein coupled receptor (GPCR) superfamily[51]. The dysregulation of glutamatergic signaling is widely associated with the pathophysiology of multiple neurodegenerative diseases[50]. However, this dysregulation has typically been attributed to NMDA-mediated excitotoxic cell death. Increasingly, Group 1 mGluRs (mGluR1 and mGluR5), in particular mGluR5, are being linked to the pathological progression of neurodegenerative diseases including HD[52-54].

2.1 Localization and Signaling:

mGluR5 is widely expressed in the central nervous system in both neuronal and non-neuronal cells including astrocytes, oligodendrocytes and microglia, as well as stem cells[55]. While mGluR5 is found both pre- and post-synaptically, it is predominantly located in the peri-synaptic region of the postsynaptic membrane of the glutamatergic

synapse[56]. mGluR5 is found in high levels in the striatum on MSNs and interneurons, as well as in the nucleus accumbens of the basal ganglia. These receptors are also expressed at high levels in the cerebral cortex, CA1 and CA3 pyramidal cells in the hippocampus, and in the granule cells of the olfactory bulb[56-58].

Group 1 mGluRs are coupled to the heterotrimeric G protein, $G\alpha_{q/11}$, which activates phospholipase C (PLC)[50, 59]. PLC cleaves phosphatidylinositol 4,5, bisphosphate into diacylglycerol (DAG) and InsP3. InsP3 opens ligand-gated inositol phosphate receptors on the endoplasmic reticulum allowing the release of Ca^{2+} from intracellular stores. Ca^{2+} and DAG both contribute to the activation of protein kinase C (PKC). In addition to PKC, the stimulation of Group 1 mGluRs can also mediate the activation of a number of other downstream effector enzymes such as, extracellular regulated kinase (ERK), mitogen activated protein kinase, proline rich tyrosine kinase 2, Akt and mammalian target of rapamycin[60-62]. Additionally, Group 1 mGluRs modulate the activity of ligand gated ion channels. For example, mGluR5 has been shown to enhance NMDA receptor function and there by plays a role in regulating neuronal excitability and synaptic plasticity[63-65]. There are 2 splice variants for mGluR5; mGluR5a and mGluR5b[56]. The mGluR5a splice variant, includes a long C terminal tail which encodes binding motifs for both protein phosphatase 1γ1 and Homer[66,67]. Homer has specifically been identified as an important regulator of mGluR5-mediated ERK activation in spinal cord and striatum[66]. Attenuation of GPCR signaling, including that of Group 1 mGluRs, is mediated by G protein-coupled receptor kinases (GRKs). GRKs typically function by phosphorylating the receptor in order to attract β-arrestin proteins, which function to sterically uncouple the receptor from their heterotrimeric G proteins and promote GPCR endocytosis[50,59,67]. However GRK2-mediated mGluR5 desensitization is phosphorylation-independent and involves the displacement of

mGluR5 interactions with $G\alpha_{q/11}$ via the amino-terminal GRK2 RGS homology domain[68]. This is facilitated by the binding of GRK2 binding to the second intracellular loop, promoting desensitization independently of phosphorylation[59, 62, 68, 69]. Furthermore the desensitization of mGluR5 signaling, can be influenced by proteins such as Rab8 and the htt binding protein optineurin[70, 71].

2.2 Pharmacology:

A variety of selective agonists, antagonists, positive and negative allosteric modulators are available for Group 1 mGluRs including mGluR5. Amongst the agonists and antagonists available a number are phenylglycine derivatives. 3-dihydroxyphenylglycine (DHPG) is one of the earliest developed orthosteric agonists selective for Group 1 mGluRs. However, it is does not selectively activate Group I mGluRs and is equally potent at both mGluR1 and mGluR5[72]. The orthosteric agonist 2-chlorohydroxyphenylglycine (CHPG) is selective for mGluR5, but shows weaker potency and efficacy than DHPG[73]. With respect to phenylglycine derivative antagonists, a number are available which are selective for mGluR5. However, these are typically of low potency making them of limited use. In the case of alpha-methyl-4-carboxyphenylglycine (MCPG), this antagonist also affects Group II mGluRs[74].

Negative allosteric modulators are allosteric ligands, which reduce the affinity of agonists at the receptor, by antagonizing activity in a non-competitive manner. A large number of the negative allosteric modulators for mGluR5 are made up of phenylpyridine derivatives. Early negative allosteric modulators selective for mGluR5 include (E)-2-methyl-6-stryrylpyridine (SIB-1893) and 6-methyl-2(phenylazo)-3-pyrimidol (SIB-1757), which show good selectivity and potency[75]. Over time, a number of negative allosteric modulators for mGluR5 have been developed that show

increasing selectivity and potency. These include 2-methyl-6-(phenylethynyl)pyridine (MPEP)[76], 3-((methyl-4-thiazolyl)ehtynyl)pyridine (MTEP)[77], and most recently 2-chloro-4-((dimethyl-1-(4-(trifluoromethoxy)phenyl)-1H-imidazol-4yl)ethynyl)pyridine (CTEP), which shows the highest potency and selectivity, as well as having an extended half-life of 18 hours[78]. However, SIB-1893 has also shown the ability to act as a positive allosteric modulator for the Group III mGluR; mGluR4[79]. Similarly, MPEP acts as a positive allosteric modulator for mGluR4 and as weak antagonist of the NMDAR[76].

The use of positive allosteric modulators is proving increasingly useful in the investigation of a functional role for mGluR5. Positive allosteric modulators work by binding to a topographically distinct site from the agonist, potentiating agonist response. Of the positive allosteric modulators available and selective for mGluR5, many are N-(1,3-diphenyl-1H-pyrazol-5yl)benzamides. These positive allosteric modulators include 3-cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide (CDPPB), N-(1,3-diphenyl-1H-pyrazol-5yl)nitrobenzamide (VU29) and (4-fluorophenyl)(3-(3-(4-fluorophenyl)-1,2,4-oxodiazol-5yl-piperidin-1yl)methanone (ADX47273)[80].

2.3 Neuroprotection versus Neurotoxicity:

Group 1 mGluRs and their interacting proteins play a key role in the regulation of central nervous system function and these receptors, in particular mGluR5, have been implicated in a number of neurological and neurodegenerative diseases including: fragile X syndrome, schizophrenia, Alzheimer's disease (AD), Parkinson's disease (PD) and HD[51, 52, 81]. However, the literature provides conflicting information concerning whether the activation of endogenous mGluR5 is either neuroprotective or neurotoxic.

The elevation of intracellular Ca^{2+} concentrations, as the consequence of the activation of NMDAR, is associated with excitotoxic cell death and mGluR5 activation

potentiates NMDAR function via calcium-dependent mechanisms (Figure 1A and B)[63, 82]. Moreover, activation of the NMDAR is believed to reduce mGluR5 desensitization and this effect is thought to be mediated by protein phosphate 2B/calcineurin (Figure 1C)[83]. Protein phosphate 2B/calcineurin is activated as the consequence of the entry of Ca^{2+} into the cell via NMDA-gated ion channels. Although it is unclear as to whether the relationship between mGluR5 and NMDA receptors is the cause of neurodegeneration or the result of other processes, it is clear that the excessive activation of glutamate receptors results in neurotoxic effects mediated by both receptors.

mGluR5 is also associated with a number of signaling pathways that are considered to be neuroprotective[50, 84, 85]. These pathways include: ERK1/2, Akt and mTOR. Activation of these intracellular signaling pathways is important for cell survival, growth and proliferation, as well as the regulation of synaptic plasticity[66, 86]. Additionally, the association of Homer proteins with mGluR1a allows the receptor to engage the activation PI3K via the interaction of Homer with the PI3K enhancer PIKE resulting in the antagonism of neuronal apoptosis[66, 86]. In addition to influencing neuroprotective signaling pathways, mGluR5 activation regulates the activity of fragile X mental retardation protein (FMRP), an RNA binding protein known to repress protein synthesis at the synapse[87, 88]. One of the proteins regulated by FMRP is amyloid precursor protein (APP)[88]. Although APP is typically associated with AD, the normal functions of APP include cell proliferation, cell-cell adhesion and synaptogenesis[89]. Predominantly located at the synapse, APP is released from neurons in an activity dependent manner; the activation of mGluR5 results in FMRP mediated translation of APP and under normal conditions this effect may be neuroprotective[81].

3. mGluR5 as a Therapeutic Target in Huntington´s Disease:

Alterations in glutamate neurotransmission and NMDA-mediated excitotoxicity are associated with the development of a number of neurodegenerative diseases[49, 52]. In addition to the NMDAR, Group 1 mGluRs are increasingly being associated with neurodegenerative diseases, especially in AD and HD[52, 53, 90]. mGluR5 is highly expressed in brain regions that are affected in both AD and HD, including the striatum, hippocampus and cortex[56]. Moreover, mGluR5 can physically and directly interact with both wild type and mutant htt[70]. The interaction between mGluR5 and mutant htt appears to be of a functional consequence, as studies from our group indicate that mGluR5 signaling is altered in a knock-in mouse model of HD. Specifically, mGluR5 signaling is uncoupled from $G\alpha_{q/11}$-mediated inositol phosphate formation in $Hdh^{Q111/Q111}$ mice, as compared to control $Hdh^{Q20/Q20}$ mice (Figure 1B)[53]. This occurs as the consequence of increased PKC-mediated desensitization of mGluR5 and PKC antagonist results in the mGluR5-dependent death of neuronal cultures derived from $Hdh^{Q111/Q111}$ mice in response to DHPG treatment. This effect of PKC inhibition is lost in neuronal cultures derived from $Hdh^{Q111/Q111}$ mice that lacked mGluR5 expression. In addition, it has been demonstrated that mGluR5 has a role in movement control, as mGluR5 knockout mice, as well as wild-type mice treated with mGluR5 antagonists, exhibit increased locomotor activity[27, 91].

However, it is not clear whether mGluR5 activation either slows the development of HD pathology or exacerbates HD pathology, as mGluR5 signaling may lead to either activation of neuroprotective pathways or neuronal toxicity, depending on the context of receptor activation[56, 85]. mGluR5 agonists and antagonists were employed to shed some light on the receptor´s role in neuronal cell death. However, pharmacological intervention of mGluR5 has yielded conflicting results with respect to

neuroprotection and neurotoxicity. For example, both mGluR5 agonists and antagonists efficiently promote neuroprotection in a rat model of focal cerebral ischemia[92]. *In vitro* studies using neuronal cultures show that agonists, such as DHPG, are neuroprotective in a number of striatal, hippocampal and cerebellar cultures, as well as in brain slices, while other studies using cortical cultures found that agonists exacerbate excitotoxicity, while the use of antagonists ameliorate neurotoxic insults[85, 93, 94]. In addition, primary mouse cortical astrocytes derived from mGluR5 knockout mice are resistant to oxygen glucose deprivation-mediated cell death[54]. Therefore, it is possible that the effect of stimulating mGluR5 is dependent upon the condition of cells and environment being targeted.

The activation of mGluR5 may exacerbate neuronal toxicity by increasing Ca^{2+} release from intracellular stores[85]. Mutant htt has been found to sensitize InsP3 receptor-mediated release of Ca^{2+} from intracellular stores via its direct interaction with the receptor, potentially leading to cell death in HD[32, 33] (Figure 1B and C). Interestingly, the treatment of neuronal cultures derived $Hdh^{Q111/Q111}$ mice with MPEP protects against PKC inhibitor-mediated death of the cell cultures indicating that a loss of mGluR5-selective desensitization may be contributing HD neuropathology with age[53]. *In vivo* inhibition of mGluR5, using antagonists such as MPEP, has no effect on long-term potentiation, while ameliorating parkinsonian akinesia in PD and improving spatial memory and protecting synapses in AD mice[95, 96]. In agreement with the hypothesis that mGluR5 plays a deleterious role in HD, treatment of R6/2 mice with mGluR5 antagonist MPEP increases survival, offers neuroprotection and improves motor function in these HD mice[97]. Moreover, mGluR5 deletion in $Hdh^{Q111/Q111}$ mice decreases htt aggregate formation (Figure 1B), increases locomotor activity and improves performance on the rotarod[27].

Agonist activation of mGluR5 may also promote receptor signaling via neuroprotective signaling pathways[53]. For example, when cortical neuronal cultures are incubated twice with the mGluR1/5 agonist DHPG, there is an activity-dependent switch from mGluR5-mediated potentiation to mGluR5-mediated inhibition of NMDAR-mediated excitotoxicity[84, 85]. This switch in mGluR5 activity is dependent upon PKC activation[85]. This may be mechanistically similar to the observed neuroprotection in young asymptomatic $Hdh^{Q111/Q111}$ mice, where PKC-mediated mGluR5 desensitization uncouples mGluR5 signal transduction via G proteins. This PKC-mediated desensitization of G protein signaling is accompanied by increased mGluR5 signaling via ERK1/2 and Akt neuroprotective pathways (Figure 1B). The treatment of neurons derived from $Hdh^{Q111/Q111}$ mice with PKC-inhibitors may result in cell death by both preventing mGluR5-stimulated activation of ERK1/2 and Akt signaling and concomitantly increasing G protein-mediated release of intracellular Ca^{2+}.

It is reported that acute blockage of mGluR5 is neuroprotective, whereas chronic mGluR5 blockage increases neuronal death[33, 98]. This observed loss of neuroprotection may involve a loss of mGluR5 signaling via ERK1/2 and Akt pathways following prolonged mGluR5 blockage resulting in reduced cell survival. Consequently, positive allosteric modulators (PAMs) for mGluR5 may favor the activation of pro-survival signaling pathways over the activation of signaling pathways that contribute to excitotoxicity[98]. Interestingly the use of mGluR5 PAMs *in vitro* is neuroprotective in striatal neurons and offers better protection against excitotoxicity than either agonists or antagonists by promoting mGluR5-mediated activation of neuroprotective cell signaling pathways without excessive Ca^{2+} release[98]. This neuroprotection is dependent on AKT activation, as blockage of this kinase leads to loss of PAM-mediated neuroprotection[98].

In addition, treatment of BACHD mice with the mGluR5 PAM, CDPPB, improves recognition memory, further emphasizing mGluR5 PAMs potential as drugs to treat neurodegenerative diseases, especially HD[98].

4. Conclusion:

In conclusion, there is evidence that mGluR5 may serve as a molecular target for the treatment of HD. However, a clear mechanistic understanding of the circumstances by which mGluR5 activation and antagonism are either neuroprotective or neurotoxic remain to be determined. Studies using mGluR5 knockout mice crossed with mutant htt knock-in mice, as well as with either inhibitors or positive allosteric modulators of mGluR5 signaling, suggest that there is potential for the development of drugs targeting mGluR5 for HD treatment. However, understanding the mechanistic "switch" between mGluR5-mediated protection and neuropathology is essential for the successful utilization of these drugs to treat a neurodegenerative disease such as HD.

5. Expert Opinion Section: The future of mGluR5 as a target for the treatment of HD

A significant pitfall facing HD research, as with other neurodegenerative disease research, is the availability of effective animal models that accurately recapitulate the HD disease process. Although many HD mouse models are available that reproduce the HD phenotype more effectively than mouse models available for AD and PD, no individual HD mouse model fully recapitulates the HD disease process. While knock-in models are well accepted models of HD pathogenesis (see section 1.1), they are slow to develop the motor deficits which characterize HD, and often, as with the $Hdh^{Q111/Q111}$ model, the deficits observed are mild despite the use of very long tracks of CAG repeats

in these mice. Conversely, models such as the R6/2 are much more aggressive in the development of disease phenotype. However they do not completely model the disease, as other 'weaker' models do. Moreover, as stated in section 1.1, neuronal cell loss is only marginally observed in mouse models of HD. Until more effective and affordable models for HD are available, such as miniature pigs, there will always be limitations for understanding the mechanisms underlying HD pathogenesis and for the identification of effective targets and strategies to optimally use such targets. Thus, conflicting information, such as whether to inhibit or activate mGluR5, will continue to confound the understanding and treatment of the disease. At present, with respect to mGluR5 there are two exciting, yet apparently opposite strategies to treat HD that have shown considerable promise *in vivo*. These strategies, involve the use of high-affinity negative allosteric modulators (NAMs) or positive allosteric modulators (PAMs).

The genetic deletion of mGluR5 in a mouse model of HD has yielded exciting results with respect to behaviour and pathology (as described in section 4.1)[27]. This presents the possibility that mGluR5 antagonism through the use of NAMs may be a viable strategy for treating HD. For example, MTEP has been used effectively to reverse spatial learning deficits in AD mouse models, and the treatment of fragile X knockout mice with CTEP, an orally available mGluR5 NAM effectively reverses impairments in these mice that are associated with fragile X syndrome[78, 95, 99]. CTEP is currently in phase III in phase III clinical trials for fragile X syndrome (***http://clinicaltrials.gov/ct2/show/NCT01750957?term=RO4917523&rank=2***). Thus, given that mGluR5 knockout reverses neuropathology associated with mutant htt expression in mice and improves the motor function of mutant HD mice, NAMs provide a potentially exciting avenue for HD treatment.

Given the observation that chronic mGluR5 activation may be deleterious *in vitro*, the use of mGluR5 agonists, such as PAMs, that are biased towards the selective activation of neuroprotective signal transduction pathways activated by mGluR5 represent an enticing avenue for HD treatment. This strategy allows for the modulation of cyto-protective mGluR5 signaling in response to activation by endogenous glutamate without necessarily promoting increases in intracellular Ca^{2+} release associated with cell death *in vitro*[98] and *in vivo* (Doria and Ribeiro unpublished observations). PAMs can cross the blood brain barrier and are being tested to treat schizophrenia and migraine and pre-clinical animal tests indicate that these drugs exhibit low toxicity and are well tolerated (http://clinicaltrials.gov/ct2/show/NCT00820105?term=mGluR5&rank=9)[100]. Thus, both mGluR5 NAMs and PAMs hold potential as drugs to treat HD.

6. Article highlights box:

- Glutamate and mGluR5 have been implicated in a number of neurodegenerative diseases, including Huntington's disease
- mGluR5 interacts with htt and mutant htt profoundly affects mGluR5 signaling
- mGluR5 is involved in htt aggregation and in motor function regulation
- mGluR5 stimulation can activate both neuroprotective and neurotoxic signaling pathways, depending on the context of activation.
- Both mGluR5 positive and negative allosteric modulators hold potential as drugs to treat HD.

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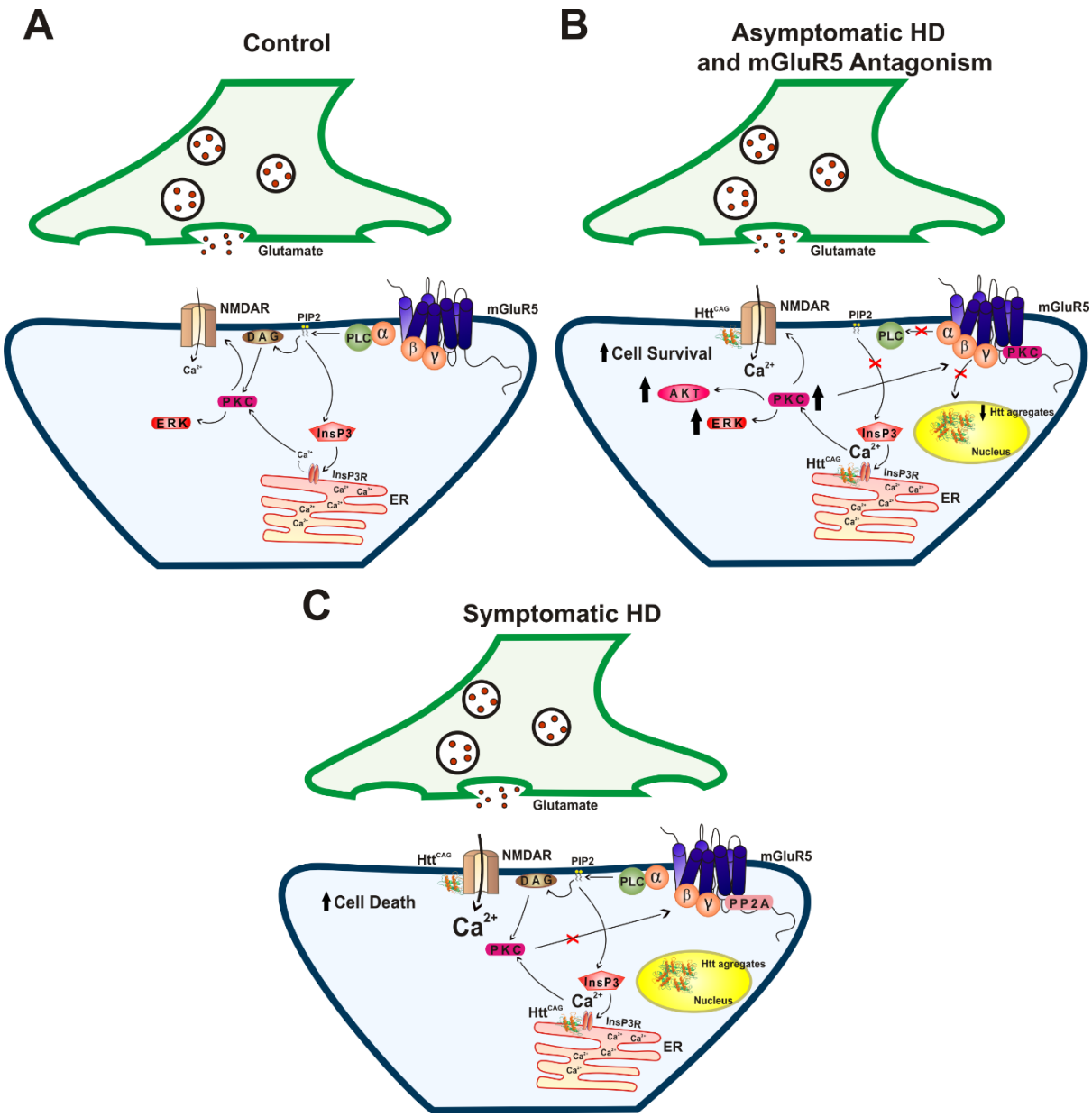
Conflict of Interest Statement: none

Abbreviations: AD, Alzheimer's disease; ADX47273, 4-fluorophenyl)(3-(3-(4-fluorophenyl)-1,2,4-oxodiazol-5yl-piperidin-1yl)methanone; Akt, protein kinase B; APP, amyloid precursor protein; BDNF, brain-derived neurotrophic factor; CREB, cAMP-responsive element binding protein; CTEP, 2-chloro-4-((dimethyl-1-(4-(trifluoromethoxy)phenyl)-1H-imidazol-4yl)ethynyl)pyridine; VU29, N-(1,3-diphenyl-1H-pyrazol-5yl) nitrobenzamide; DAG, diacylglycerol; DHPG, dihydroxyphenylglycine; CHPG, 2-chlorohydroxyphenylglycine; ERK, extracellular regulated kinase; FMRP, fragile X mental retardation protein; GPCR, G protein coupled receptor; GRKs, G protein-coupled receptor kinases; HD, Huntington's disease; htt, huntingtin; InsP3, inositol-1,4,5-triphosphate; mGluR, metabotropic glutamate receptor; MSNs, medium-sized spiny neurons; NAM, negative allosteric modulator; NMDAR, N-methyl-D-aspartate receptor; PAM, positive allosteric modulator; PD, Parkinson's disease; PGC-1α, peroxisome proliferator-activated receptor γ co-activator 1α; PLC, phospholipase C and PKC, activation of protein kinase C.

Figure 1: **Implications of altered mGluR5 signaling in HD.**

A) In normal MSNs, activation of mGluR5 leads to $G\alpha_q$-mediated activation of phospholipase C (PLC). Activation of PLC results in the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2), releasing the second messenger diacylglycerol (DAG) and inositol 14,5 trisphosphate (InsP3), which binds to intracellular InsP3 receptors (InsP3R) present in the endoplasmic reticulum (ER), leading to Ca^{2+} release. The activation PKC activation can lead to the activation of ERK1/2 phosphorylation and the phosphorylation of NMDAR, facilitating NMDAR activation. mGluR5 can also physically couple to NMDAR through scaffolding proteins such as HOMER, SHANK and PSD95. **B)** In assymptomatic HD MSNs, mutant htt (htt^{CAG}) affects Ca^{2+} signaling by sensitizing the InsP3R resulting in increased Ca^{2+} release from intracellular stores and increased PKC activation and sensitizing NMDAR-mediated influx of extracellular Ca^{2+}. In young animals, the increased PKC activity increases mGluR5 desensization prevent G protein coupling, but selective leads stimulation leads to higher levels of ERK and Akt activation, which may be neuroprotective. The knockout of mGluR5 results in diminished intranuclear mutant htt aggregates in the nuclei of MSN. **C)** In older HD MSNs, mutant htt (htt^{CAG}) affects Ca^{2+} signaling by sensitizing the InsP3R resulting in increased Ca^{2+} release from intracellular stores and sensitizing NMDAR-mediated influx of extracellular Ca^{2+}, resulting in MSN degeneration. Protective effects of mGluR5 desensitization are lost potentially as the consequence of either increased protein phosphatase 2B (PP2A)-mediated dephosphorylation of mGluR5 or the reduction of PKC-dependent mGluR5 phosphorylation. The loss of Ca^{2+} homeostatis leads to MSN cell death which is correlated with the formation of htt agreggates.


A
Control
Glutamate
NMDAR
PIP2
mGluR5
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β
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PKC
ERK
InsP3
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Asymptomatic HD
and mGluR5 Antagonism
Glutamate
HttCAG
NMDAR
PIP2
mGluR5
PLC
PKC
Cell Survival
Ca2+
AKT
ERK
InsP3
Htt agregates
Nucleus
InsP3R
ER
C
Symptomatic HD
Glutamate
HttCAG
NMDAR
PIP2
mGluR5
DAG
PLC
PP2A
Cell Death
Ca2+
PKC
InsP3
Htt agregates
Nucleus
InsP3R
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