

## RESEARCH PAPER

# Metabotropic glutamate receptor 5 positive allosteric modulators are neuroprotective in a mouse model of Huntington's disease

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## BACKGROUND AND PURPOSE

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder caused by a polyglutamine expansion in the huntingtin protein. We have previously demonstrated that the cell signalling of the metabotropic glutamate receptor 5 (mGluR5) is altered in a mouse model of HD. Although mGluR5-dependent protective pathways are more activated in HD neurons, intracellular Ca<sup>2+</sup> release is also more pronounced, which could contribute to excitotoxicity. In the present study, we aim to investigate whether mGluR5 positive allosteric modulators (PAMs) could activate protective pathways without triggering high levels of Ca<sup>2+</sup> release and be neuroprotective in HD.

## EXPERIMENTAL APPROACH

We performed a neuronal cell death assay to determine which drugs are neuroprotective, Western blot and Ca<sup>2+</sup> release experiments to investigate the molecular mechanisms involved in this neuroprotection, and object recognition task to determine whether the tested drugs could ameliorate HD memory deficit.

## KEY RESULTS

We find that mGluR5 PAMs can protect striatal neurons from the excitotoxic neuronal cell death promoted by elevated concentrations of glutamate and NMDA. mGluR5 PAMs are capable of activating Akt without triggering increased intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>); and Akt blockage leads to loss of PAM-mediated neuroprotection. Importantly, PAMs' potential as drugs that may be used to treat neurodegenerative diseases is highlighted by the neuroprotection exerted by mGluR5 PAMs on striatal neurons from a mouse model of HD, BACHD. Moreover, mGluR5 PAMs can activate neuroprotective pathways more robustly in BACHD mice and ameliorate HD memory deficit.

## CONCLUSIONS AND IMPLICATIONS

mGluR5 PAMs are potential drugs that may be used to treat neurodegenerative diseases, especially HD.

## Abbreviations

[Ca<sup>2+</sup>]<sub>i</sub>, intracellular Ca<sup>2+</sup> concentration; CDPPB, 3-Cyano-*N*-(1,3-diphenyl-1*H*-pyrazol-5-yl)benzamide; CPPHA, *N*-[4-Chloro-2-[(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)methyl]phenyl]-2-hydroxybenzamide; DFB, [(3-Fluorophenyl)methylene]hydrazone-3-fluorobenzaldehyde; DHPG, (S)-3,5-dihydroxyphenylglycine; HD, Huntington's disease; Htt, huntingtin; InsP3, inositol-1,4,5-triphosphate; LY294002, 2-(4-Morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride; mGluR, metabotropic glutamate receptor; MPEP, 2-methyl-6-(phenylethynyl)-pyridine; PAM, positive allosteric modulator; PIKE, PI3K enhancer; VU1545, *N*-[1-(2-Fluorophenyl)-3-phenyl-1*H*-pyrazol-5-yl]-4-nitrobenzamide; WT, wild type

## Introduction

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder caused by a polyglutamine expansion in the amino-terminal region of the huntingtin (Htt) protein (Group THsDCR, 1993). HD patients exhibit a progressive neuronal cell loss in the caudate-putamen and neocortical regions of the brain, which leads to motor deterioration, loss of cognitive function, psychiatric disturbance and death (Young, 2003; Li and Li, 2004).

Glutamate is postulated to play an important role in excitotoxic neuronal cell loss (DiFiglia, 1990; Nicoletti *et al.*, 1996; Calabresi *et al.*, 1999; Ribeiro *et al.*, 2010a). Although it is well established that overstimulation of ionotropic glutamate receptors, such as NMDAR, leads to excitotoxicity, the role of metabotropic glutamate receptors (mGluRs) in neuronal cell death processes is not completely understood (Zeron *et al.*, 2002; Schiefer *et al.*, 2004). Group I mGluRs (mGluR1 and mGluR5) are coupled to the activation of G<sub>αq/11</sub> proteins, which stimulate the activation of phospholipase C $\beta$ 1 resulting in diacylglycerol and inositol-1,4,5-triphosphate (InsP3) formation, release of Ca<sup>2+</sup> from intracellular stores and activation of protein kinase C. Activation of mGluR1/5 expressed in neurons from a mouse model of HD leads to high levels of Ca<sup>2+</sup> release from intracellular compartments, which can contribute to excitotoxic processes (Tang *et al.*, 2005; Ribeiro *et al.*, 2010b). However, mGluR1/5 stimulation also leads to activation of other signalling pathways important for cell survival/proliferation, such as ERK and Akt (Rong *et al.*, 2003; Hou and Klann, 2004; Mao *et al.*, 2005). Interestingly, mGluR5 activation leads to higher levels of ERK and Akt activation in HD than in control neurons (Ribeiro *et al.*, 2010b). Thus, mGluR5 can produce either excitotoxicity or neuroprotection depending on which cell signalling pathway is activated.

To characterize drugs that could be neuroprotective in HD, we have investigated a number of compounds acting on mGluR5. We find that mGluR5 blockage by 2-methyl-6-(phenylethynyl)-pyridine (MPEP) is not neuroprotective, although mGluR1/5 activation by (S)-3,5-dihydroxyphenylglycine (DHPG) promotes neuroprotection against glutamate-induced neuronal death. However, both MPEP and DHPG treatments increase basal neuronal death. The neurotoxic effects of mGluR5 agonists and antagonists might be due to overstimulation of Ca<sup>2+</sup> release or blockage of protective pathways respectively. mGluR5 positive allosteric modulators (PAMs) can activate one signalling pathway without triggering others (Zhang *et al.*, 2005; Bradley *et al.*, 2009; Chen *et al.*, 2012). Thus, we decided to investigate whether the mGluR5 PAMs, [(3-Fluorophenyl)

methylene]hydrazone-3-fluorobenzaldehyde (DFB), *N*-[1-(2-Fluorophenyl)-3-phenyl-1*H*-pyrazol-5-yl]-4-nitrobenzamide (VU1545) and 3-Cyano-*N*-(1,3-diphenyl-1*H*-pyrazol-5-yl)benzamide (CDPPB) could have a neuroprotective role. We find that mGluR5 PAMs are capable of protecting neurons from glutamate- and NMDA-induced neuronal cell death. The tested PAMs can activate Akt without promoting intracellular Ca<sup>2+</sup> release, and blockage of Akt stimulation abrogates PAM-mediated neuroprotection. PAMs are also capable of promoting neuroprotection in striatal neurons from a mouse model of HD, BACHD, and one of the tested PAMs, VU1545, more efficiently activates Akt and promotes neuroprotection in BACHD neurons than in wild-type (WT) neurons. Moreover, the memory deficit exhibited by BACHD mice was ameliorated by CDPPB treatment. Thus, our results indicate that mGluR5 PAMs exhibit the potential to treat HD as they prevent neuronal death and improve memory deficit in an HD mouse model.

## Methods

### Material

Neurobasal medium, N2 and B27 supplements, GlutaMAX (50.0  $\mu\text{g}\cdot\text{mL}^{-1}$  penicillin and 50.0  $\mu\text{g}\cdot\text{mL}^{-1}$  streptomycin), Fura-2 AM, DAPI, mouse anti-neuronal nuclei (NeuN) monoclonal antibody, Alexa Fluor 633 goat anti-mouse and Alexa Fluor 488 goat anti-rabbit secondary antibodies, and live/dead viability assay were purchased from Life Technologies (Foster City, CA, USA). Rabbit anti-vesicular GABA transporter (VGAT) was purchased from Merck Millipore (Billerica, MA, USA). DHPG, MPEP, DFB, VU1545 and CDPPB were purchased from Tocris Cookson Inc. (Ellisville, MO, USA). HRP-conjugated anti-rabbit IgG secondary antibody was from Bio-Rad (Hercules, CA, USA). ECL Western blotting detection reagents were from GE Healthcare (Little Chalfont, UK). Rabbit anti-phospho Akt and anti-Akt monospecific clonal antibodies were from DB Biotech (Kosice, Slovakia). 2-(4-Morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride (LY294002) and all other biochemical reagents were purchased from Sigma-Aldrich (St Louis, MO, USA). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

### Mouse model

FVB/NJ (wild type) and FVB/N-Tg (HTT\*97Q)IXwy/J (BACHD) transgenic mice (Gray *et al.*, 2008) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). 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 Universidade Federal de Minas Gerais Ethics Committee on Animal Experimentation, CETEA.

### Neuronal primary culture preparation

Neuronal cultures were prepared from the striatal region of E15 mouse embryo brains. Animal procedures were approved by the Ethics Committee on Animal Experimentation, CETEA/UFGM. After dissection, striatal tissue was submitted to trypsin digestion followed by cell dissociation using a fire-polished Pasteur pipette. Cells were plated on poly-L-ornithine-coated dishes in neurobasal medium supplemented with N2 and B27 supplements, 2.0 mM GlutaMAX, 50.0 µg·mL<sup>-1</sup> penicillin and 50.0 µg·mL<sup>-1</sup> streptomycin. Cells were incubated at 37°C and 5% CO<sub>2</sub> in a humidified incubator and cultured for 10–12 days *in vitro* with medium replenishment every 4 days.

### Immunofluorescence and imaging

Striatal neurons were washed twice in PBS and fixed with 4% formaldehyde in PBS for 20 min. After fixation, cells were washed with PBS and pre-incubated with a permeabilization solution (PBS, 0.2% Triton and 3% BSA) for 60 min. Subsequently, rabbit anti-GAT1 (1:200) or mouse anti-NeuN (1:500) antibodies were added to cells and incubated at 4°C in permeabilization solution for 16 h. Cells were washed and incubated with goat anti-rabbit and anti-mouse antibodies conjugated to Alexa Fluor 633 and Alexa Fluor 488, respectively, for 60 min in permeabilization solution at 1:1000 dilution. Following that, cells were washed and stained with DAPI 1:1000 for 10 min. Detection of immunolabelled proteins was performed using filter sets for excitation of 390/40, 482/18 and 586/15 nm and emission of 446/33 nm for DAPI, 533/59 nm for Alexa Fluor 488-labelled anti-NeuN antibody and 646/68 nm for Alexa Fluor 633-labelled anti-GAT1 antibody. Images were obtained by fluorescence microscopy using an Olympus IX70 Multi-Fluorescence Microscope (Olympus, Tokyo, Japan) and an Olympus Evolt E-300 camera (Olympus).

### Akt activation experiments

Neuronal primary cultures from WT and BACHD embryos were incubated in HBSS for 20 min at 37°C. Cultures were then stimulated with DHPG (from a 10 mM stock solution in HBSS), DFB (from a 10 mM stock solution in DMSO), VU1545 (from a 10 mM stock solution in DMSO) and CDPPB (from a 10 mM stock solution in DMSO) at concentrations indicated in the *figure legends* for 5 min at 37°C. When LY294002 (from a 10 mM stock solution in DMSO) was used, it was added 5 min prior to and kept during mGluR5 stimulation. Neurons were lysed in RIPA buffer (0.15 M NaCl, 0.05 M Tris-HCl, pH 7.2, 0.05 M EDTA, 1% nonidet P40, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitors (1.0 mM AEBSF and 10.0 µg·mL<sup>-1</sup> of both leupeptin and aprotinin). About 50.0 µg of total cellular protein for each sample was subjected to SDS-PAGE, followed by electroblotting onto nitrocellulose membranes.

### Immunoblotting

Membranes were blocked with 5% BSA in wash buffer (150.0 mM NaCl, 10.0 mM Tris-HCl, pH 7.0 and 0.05% Tween 20) for 1 h and then incubated with rabbit anti-phospho Akt (S473) (1:1000) antibody in wash buffer containing 3% BSA for 2 h at room temperature. Membranes were rinsed three times with wash buffer and then incubated with secondary HRP-conjugated goat anti-rabbit IgG diluted 1:5000 in wash buffer containing 3% skim milk for 1 h. Membranes were rinsed three times with wash buffer and incubated with ECL Western blotting detection reagents. Antibodies were then stripped and membranes were incubated with anti-Akt (1:1000) for 2 h and probed with secondary antibody to determine total Akt expression. Non-saturated, immunoreactive Akt bands were quantified by scanning densitometry. Immuno-band intensity was calculated using ImageJ™ software and the number of pixels of Akt phospho-bands was divided by the number of pixels of total Akt to normalize phosphorylation levels of kinases to total kinase expression.

### Cell death assay

Neurons were incubated for 20 h with either vehicle or drug, glutamate (from a 10 mM stock solution in H<sub>2</sub>O), DHPG, MPEP, DFB, VU1545, CDPPB and/or LY294002, as indicated in the *figure legend*, and cell death was determined by live/dead viability assay, as described previously (Ribeiro *et al.*, 2010b). Briefly, neurons were stained with 2.0 µM calcein acetoxymethyl ester (AM) and 2.0 µM ethidium homodimer-1 for 15 min and the fractions of live (calcein AM positive) and dead (ethidium homodimer-1 positive) cells were determined. Neurons were visualized by fluorescence microscopy using an Olympus IX70 Multi-Fluorescence Microscope. Images were captured using an Olympus Evolt E-300 camera and scored by a blinded observer. A minimum of 300 cells were analysed per well in triplicate using ImageJ software. Dead cells were expressed as a percentage of the total number of cells.

### Measurement of intracellular Ca<sup>2+</sup> concentration

Neuronal primary cultures from WT and BACHD embryos seeded on 12 mm coverslips were loaded with 0.2 µM Fura-2 AM for 20 min at 37°C. Coverslips were washed with HBSS and inserted into a cuvette filled with 2.0 mL of HBSS. Neurons were stimulated with 10.0 µM DFB, 10.0 µM VU1545, 10.0 µM CDPPB, 10.0 µM DHPG or 30.0 mM KCl and illuminated with alternating 345 and 380 nm light, with the 510 nm emission detected using a PTI spectrofluorimeter. Baseline (60 s) measurements were obtained before addition of agonist. At the end of each experiment, SDS 10% (0.1% final) was added to obtain  $R_{max}$ , followed by 3.0 M Tris + 400.0 mM EGTA (pH 8.6) for  $R_{min}$ , as described by Grynkiewicz *et al.* (1985). The increase in intracellular Ca<sup>2+</sup> concentration ( $[Ca^{2+}]_i$ ) promoted by depolarization induced by 30.0 mM KCl was taken as 100%.

### Novel object recognition task

The object recognition test is based on differential spontaneous exploration of novel and familiar objects (Ennaceur and

Delacour, 1988). The apparatus used was an open box made of PVC (50 × 35 × 25 cm) surmounted by a video camera and a light. Objects were made of glass or plastic and their weight was such that mice could not displace them. As far as we could ascertain, they had no natural significance for mice and they had never been associated with reinforcement. Initial tests showed that mice did not have any preference for the objects used. The general procedure consisted of three different phases: habituation, training and test phase. On the first day, mice were individually submitted to a single habituation session of 10 min, during which they were introduced to the empty arena. On the second day (24 h later), animals were submitted to a single 10 min training session, during which two identical objects were placed in symmetrical positions from the centre of the arena and each object was 15 cm from the side walls. After a 90 min delay, during which mice returned to their home cage, they were reintroduced into the arena for 10 min (test phase) and exposed to two objects, a familiar object and a novel object, placed at the same locations as during the training phase. To control odour cues, the apparatus and objects were cleaned with 70% ethanol between each session and animal. All sessions were performed during the first part of the light cycle, and mice were acclimated to the room for at least 30 min before the beginning of each session. Exploration time was defined as sniffing or touching the object with the nose. Data are expressed as recognition index, calculated according to the following formulae:  $\text{time exploring the new object} \times 100 / (\text{time exploring the familiar object} + \text{time exploring the new object})$  (Lazaroni *et al.*, 2012). Two weeks after submitting untreated (NT) mice to object recognition test, animals were treated with CDPPB (5 mg·kg<sup>-1</sup>) for 7 days and resubmitted to object recognition assay. CDPPB was suspended in 0.5% methylcellulose and injected i.p. at a volume of 2 mL·kg<sup>-1</sup>.

### Data analysis

Means ± SEM are shown for the number of independent experiments indicated in *figure legends*. GraphPad Prism™ software was used to analyse data for statistical significance and for curve fitting. Statistical significance was determined by ANOVA testing followed by Bonferroni *post hoc* multiple comparison testing.

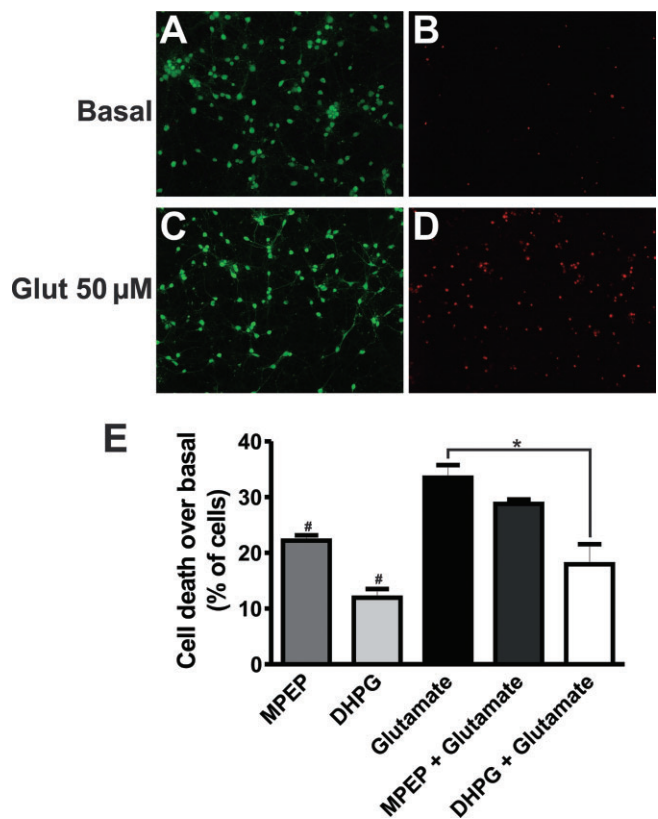
## Results

### *mGluR5* activation protects against glutamate-induced neuronal cell death

In order to characterize the primary cultures employed in this study, we have used various neuronal-specific markers. DAPI, which is a nuclear marker that stains DNA, was used to determine the total number of cells (Supporting Information Fig. S1B and C). Most cells present in these primary cultures were neurons as they were (about 95%) NeuN positive (Supporting Information Fig. S1A, B and C). Moreover, cultured neurons are mainly medium-sized spiny neurons as they are positive for the VGAT (Supporting Information Fig. S1D, E and F). Neurons that were incubated with fluorescent secondary antibodies in the absence of primary antibodies did not exhibit any staining (Supporting Information Fig. S1G, H and

I). In addition, Neurons that were incubated with anti-excitatory amino acid transporter 3 antibody, which is a marker for glutamatergic neurons, exhibited no staining, indicating that the neurons present in the striatal primary cultures were not contaminated with glutamatergic neurons (data not shown).

Increased levels of glutamate promote neuronal cell death by activating excitotoxic mechanisms mainly due to augmentation of cytosolic Ca<sup>2+</sup> concentration (DiFiglia, 1990; Nicoletti *et al.*, 1996; Calabresi *et al.*, 1999). Primary-cultured striatal neurons stimulated with 50.0 μM glutamate for 20 h exhibited 29.46 ± 0.7% neuronal cell death above basal levels (Figure 1). Higher levels of glutamate, such as 100.0 and 250.0 μM glutamate, promoted 36.82 ± 2.1 and 52.23 ± 3.5% neuronal cell death respectively (data not shown).



**Figure 1**

DHPG, but not MPEP, protects against glutamate-induced neuronal cell death. Shown is a representative image for primary-cultured striatal neurons labelled with calcein AM (A, green, live cells) and ethidium homodimer-1 (B, red, dead cells) exhibiting basal neuronal death. Also shown is a representative image for primary-cultured striatal neurons treated with 50.0 μM glutamate for 20 h and labelled with calcein AM (C) and ethidium homodimer-1 (D). (E) Graph shows percentage of neuronal cell death in primary cultured striatal neurons that were treated with 10.0 μM MPEP, 10.0 μM DHPG, 50.0 μM glutamate, 10.0 μM MPEP + 50.0 μM glutamate and 10.0 μM DHPG + 50.0 μM glutamate for 20 h. Data represent the means ± SEM of four independent experiments. \* indicates significant difference as compared with neurons treated with glutamate and # indicates significant differences as compared with basal neuronal death ( $P < 0.05$ ).

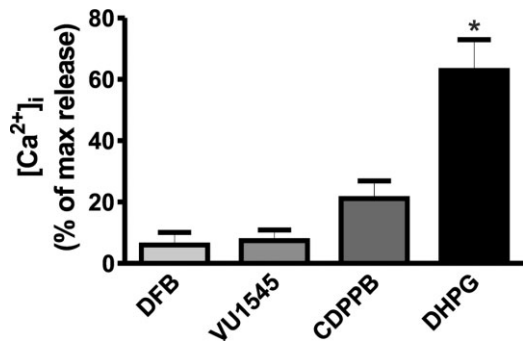


Glutamate-mediated excitotoxic neuronal cell death occurs mainly via NMDAR activation, although group 1 mGluR stimulation may also play a role in this cell-death process (DiFiglia, 1990; Nicoletti *et al.*, 1996; Calabresi *et al.*, 1999). As it is not clear whether mGluR1/5 activation can exacerbate or protect against neuronal cell death, we decided to investigate whether either MPEP, which is an mGluR5 negative allosteric modulator that acts as an inverse agonist, or DHPG, which is an mGluR1/5 agonist, could protect against glutamate-induced neuronal cell death. 10.0  $\mu\text{M}$  MPEP was not efficient in protecting neurons from glutamate-induced neuronal cell death (Figure 1E). Moreover, MPEP in the absence of glutamate was neurotoxic (Figure 1E). About 10.0  $\mu\text{M}$  DHPG treatment decreased glutamate-induced neuronal death; however, DHPG also promoted an increase in neuronal death when applied in the absence of glutamate, which indicates that DHPG can be either neurotoxic or neuroprotective depending on the context of activation (Figure 1E). mGluR5 can both increase cytosolic  $\text{Ca}^{2+}$  concentration and activate protective pathways (Rong *et al.*, 2003; Hou and Klann, 2004; Mao *et al.*, 2005). Thus, the neurotoxic effects of mGluR5 agonists and antagonists might be due to overstimulation of  $\text{Ca}^{2+}$  release and blockage of protective pathways respectively.

### *mGluR5 PAMs activate AKT without increasing intracellular $\text{Ca}^{2+}$ concentration and protect against glutamate-induced neuronal cell death*

Drugs acting on mGluR5 that could activate protective pathways, such as Akt, but without triggering  $\text{Ca}^{2+}$  release might have effective neuroprotective actions. It has been demonstrated that mGluR5 PAMs can activate one signalling pathway without triggering others (Zhang *et al.*, 2005; Bradley *et al.*, 2009; Chen *et al.*, 2012). Thus, we decided to investigate whether PAMs in the absence of agonist were able to promote Akt activation without triggering  $\text{Ca}^{2+}$  release. To test this, striatal neuronal cultures were labelled with Fura-2 AM and stimulated with 10.0  $\mu\text{M}$  DFB, 10.0  $\mu\text{M}$  VU1545, 10.0  $\mu\text{M}$  CDPPB, 10.0  $\mu\text{M}$  DHPG or 30.0 mM KCl. DFB, VU1545 and CDPPB did not promote an increase in  $[\text{Ca}^{2+}]_i$  above basal levels (Figure 2). However, DHPG promoted an increase in  $[\text{Ca}^{2+}]_i$  that was  $62.87 \pm 10.0\%$  of that promoted by 30.0 mM KCl depolarization (Figure 2).

To determine whether PAMs could activate Akt in the absence of an mGluR5 agonist, we stimulated primary-cultured striatal neurons with PAMs and determined Akt phosphorylation by Western blot experiments. About 10.0  $\mu\text{M}$  DFB was not sufficient to activate Akt above basal levels (Figure 3A and B). However, 100.0  $\mu\text{M}$  DFB was efficient in promoting Akt activation above basal levels, and DFB-mediated Akt activation was as robust as that promoted by 10.0  $\mu\text{M}$  DHPG (Figure 3A and B). In addition, both VU1545 at the concentrations of 10.0 and 100.0  $\mu\text{M}$  (Figure 3C and D) and CDPPB also at the concentrations of 10.0 and 100.0  $\mu\text{M}$  (Figure 3E and F) activated Akt above basal levels as robustly as 10.0  $\mu\text{M}$  DHPG. As VU1545 and CDPPB promoted Akt phosphorylation as efficiently at the concentration of 10.0  $\mu\text{M}$  as at the concentration of 100.0  $\mu\text{M}$ , we decided to test whether these drugs could activate Akt when employed



**Figure 2**

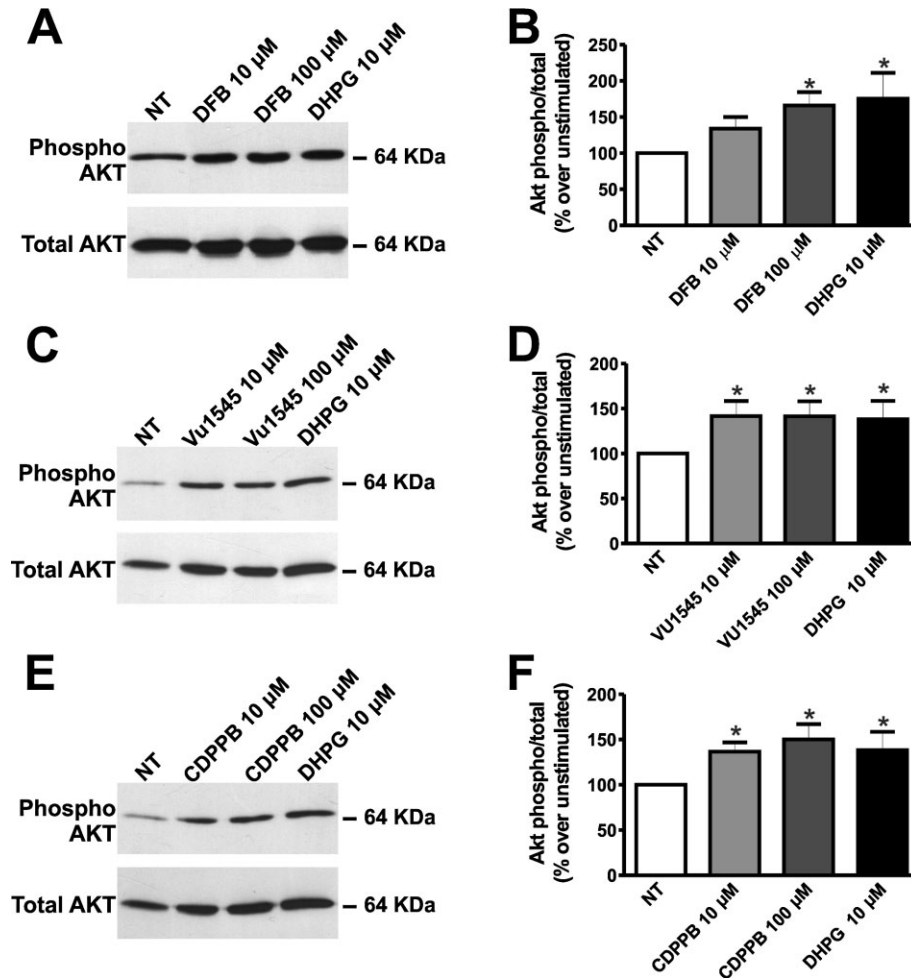
mGluR5 PAMs do not promote increased intracellular  $\text{Ca}^{2+}$  concentration. Graph shows intracellular  $\text{Ca}^{2+}$  concentration in primary-cultured striatal neurons stimulated with 10.0  $\mu\text{M}$  DFB, 10.0  $\mu\text{M}$  VU1545, 10.0  $\mu\text{M}$  CDPPB and 10.0  $\mu\text{M}$  DHPG. Data represent the means  $\pm$  SEM of six independent experiments expressed as percentage of 30.0 mM KCl-induced  $\text{Ca}^{2+}$  release. \* indicates significant difference as compared with basal  $\text{Ca}^{2+}$  release ( $P < 0.05$ ).

at lower concentrations. Both VU1545 (Figure 4A and B) and CDPPB (Figure 4C and D) at the concentrations of 0.1 and 1.0  $\mu\text{M}$  efficiently promoted Akt activation above basal levels. Moreover, Akt activation by VU1545 (Figure 4A and B) and CDPPB (Figure 4C and D) at the concentrations of 0.1 and 1.0  $\mu\text{M}$  were as efficient as 10.0  $\mu\text{M}$  DHPG in promoting Akt activation. These data indicate that mGluR5 PAMs can activate Akt in the absence of an mGluR5 agonist, having the potential to be neuroprotective. Moreover, low concentrations of VU1545 and CDPPB could be sufficient to induce neuroprotection.

As all tested mGluR5 PAMs were efficient in activating Akt without promoting increased  $[\text{Ca}^{2+}]_i$ , we decided to test whether these drugs could be neuroprotective. To investigate this, primary-cultured striatal neurons were treated with glutamate in the presence or absence of PAMs for 20 h. Vehicle, including  $\text{H}_2\text{O}$ , HBSS or DMSO, in the absence of drug was not sufficient to modify neuronal cell death as compared with basal levels (Supporting Information Fig. S2). However, neuronal cell death induced by 50.0 and 100.0  $\mu\text{M}$  glutamate was significantly reduced by co-stimulating the cultures with DFB, VU1545 and CDPPB at the concentrations of 10.0  $\mu\text{M}$  (Figure 5A) and 100 nM (Figure 5B). These data highlight the potential of mGluR5 PAMs as neuroprotective drugs.

### *VU1545 is a very potent neuroprotective drug and this neuroprotection is dependent on Akt activation*

To determine the minimum concentration of mGluR5 PAMs necessary to promote neuroprotection, we performed a dose-response experiment using VU1545 at the concentrations of 1.0, 5.0, 10.0, 50.0, 100.0 and 10 000.0 nM. About 1.0 nM VU1545 sufficed to protect neurons from glutamate-induced cell death, although 5.0 nM VU1545 elicited higher levels of neuroprotection (Figure 6A). Interestingly, 5.0 nM VU1545 was as efficient as 10 000.0 nM VU1545 to promote neuroprotection (Figure 6A). Moreover, when applied in the



**Figure 3**

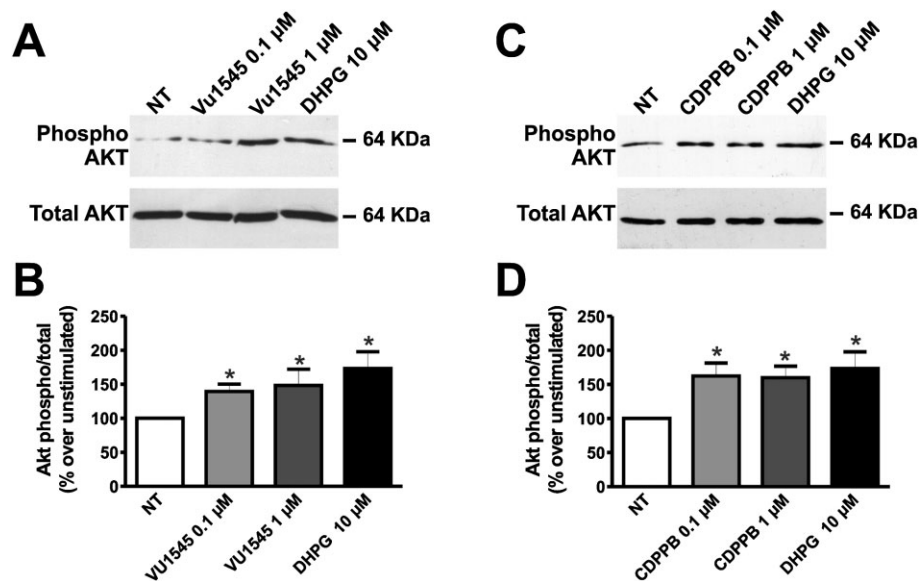
DFB, VU1545 and CDPPB promote AKT activation. Shown are representative immunoblots for phospho- (upper panel) and total-Akt expression (lower panel) in primary-cultured striatal neurons that were either untreated (NT) or treated with 10.0 μM DHPG, 10.0 or 100.0 μM DFB (A), 10.0 or 100.0 μM VU1545 (C) and 10.0 or 100.0 μM CDPPB (E) for 5 min. About 100.0 μg of cell lysate was used for each sample. Graphs show the densitometric analysis of phospho-AKT normalized to total-AKT expression in primary-cultured striatal neurons that were either untreated (NT) or treated with 10.0 μM DHPG, 10.0 or 100.0 μM DFB (B), 10.0 or 100.0 μM VU1545 (D) and 10.0 or 100.0 μM CDPPB (F) for 5 min. Data represent the means ± SEM of six independent experiments, expressed as percentage of basal Akt phosphorylation. \* indicates significant differences as compared with untreated neurons ( $P < 0.05$ ).

absence of glutamate, VU1545 did not increase neuronal death above basal levels at any of the tested concentrations, although we observed a tendency towards augmented neuronal death when VU1545 was applied at high doses such as 10 000.0 nM (Figure 6A). Thus, VU1545 is a very potent neuroprotective drug and, different from DHPG, has no neurotoxic effect when applied in the absence of glutamate.

Most neuronal cell death induced by glutamate is due to NMDAR overstimulation (Zeron *et al.*, 2001). However, it has been shown that stimulation of other glutamate receptors such as group I mGluRs can be neuroprotective (Baskys *et al.*, 2005). To determine whether VU1545-dependent neuroprotection depends on glutamate co-stimulation of mGluRs, we decided to examine whether VU1545 can protect against neurotoxicity induced by NMDA, which does not activate glutamate receptors other than NMDAR. NMDAR stimulation led to high levels of neuronal death, especially when NMDA

was applied at high concentrations such as 10.0 μM (Figure 6B). About 100.0 nM VU1545 led to neuroprotection against NMDA-induced neuronal cell death and this neuroprotection reached statistical significance in the case of neurons treated with 10.0 μM NMDA (Figure 6B). These data indicate that VU1545 neuroprotection is independent of glutamate co-stimulation and that this PAM can protect against both glutamate- and NMDA-induced neurotoxicity.

To confirm that the neuroprotective effect exerted by VU1545 was through mGluR5, we introduced MPEP to primary-cultured striatal neurons that were treated with 50.0 μM glutamate + 100.0 nM VU1545 for 20 h. MPEP completely blocked VU1545-induced neuroprotection (Figure 6C), strongly implicating mGluR5 in this neuroprotective mechanism. To determine whether Akt activation by PAMs is important for mGluR5 PAMs' neuroprotective effects, we blocked Akt stimulation by using a PI3K inhibitor,



**Figure 4**

VU1545 and CDPPB promote AKT activation even at lower concentrations. Shown are representative immunoblots for phospho- (*upper panel*) and total-AKT expression (*lower panel*) in primary-cultured striatal neurons that were either untreated (NT) or treated with 10 μM DHPG, 0.1 or 1.0 μM VU1545 (A) and 0.1 or 1.0 μM CDPPB (C) for 5 min. About 100.0 μg of cell lysate was used for each sample. Graphs show the densitometric analysis of phospho-AKT normalized to total-AKT expression in primary-cultured striatal neurons that were either untreated (NT) or treated with 10.0 μM DHPG, 0.1 or 1.0 μM VU1545 (B) and 0.1 or 1.0 μM CDPPB (D) for 5 min. Data represent the means ± SEM of six independent experiments, expressed as percentage of basal AKT phosphorylation. \* indicates significant differences as compared with untreated neurons ( $P < 0.05$ ).

LY294002. PI3K is responsible for phosphorylating Akt on serine-473, promoting Akt activation (Rong *et al.*, 2003). Thus, blocking PI3K with LY293002 leads to Akt inhibition. Western blot experiments indicated that primary-cultured striatal neurons that were pre-incubated with 25.0 μM LY294002 for 5 min prior to 10.0 μM VU1545 stimulation for 5 min exhibited no Akt phosphorylation above basal levels (data not shown). To test whether Akt activation by VU1545 was responsible for VU1545-induced neuroprotection, we added 25.0 μM LY294002 to primary-cultured striatal neurons that were treated with either 50.0 μM glutamate or 50.0 μM glutamate + 1.0 μM VU1545 for 20 h. VU1545 neuroprotection against glutamate-induced neuronal cell death was eliminated by the addition of LY294002 (Figure 6D). These data indicate that VU1545-mediated neuroprotection is dependent on Akt activation.

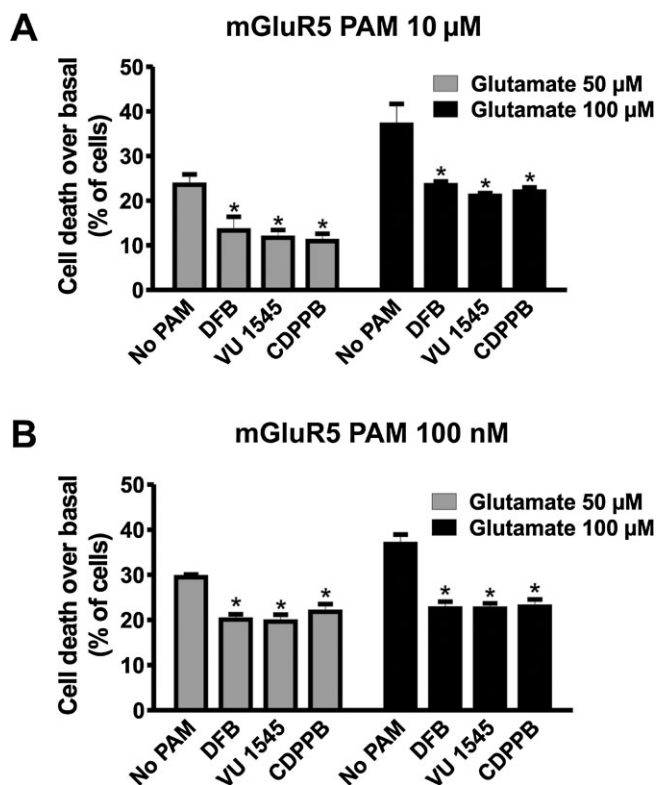
### *mGluR5 PAMs can protect striatal neurons from a mouse model of HD against glutamate-induced neuronal cell death*

To test whether mGluR5 PAMs could be efficient neuroprotective drugs in neurodegenerative diseases, we tested these compounds in primary-cultured striatal neurons from a mouse model of HD, BACHD. When both WT and BACHD neurons were co-stimulated with 1.0 μM DFB (Figure 7A), 1.0 μM VU1545 (Figure 7B) and 1.0 μM CDPPB (Figure 7C), glutamate-induced neuronal cell death was significantly reduced. Interestingly, VU1545 neuroprotective effect appeared higher in BACHD neurons than in WT neurons (Figure 7C). Supporting these observations, statistical analysis

(two-way ANOVA) indicated that there was a significant interaction between genotype and VU1545 treatment [ $\pm(2.030) = 3.446$ ;  $P = 0.045$ ].

We have previously demonstrated that AKT activation by DHPG appeared more pronounced in neurons from a knock-in mouse model of HD than in control mice, although these difference did not reach statistical significance (Ribeiro *et al.*, 2010b). Thus, we decided to investigate whether the increased neuroprotective effect of VU1545 in BACHD striatal neurons could be due to increased Akt activation in BACHD striatal neurons. To test this hypothesis, primary-cultured striatal neurons from WT and BACHD embryos were stimulated with either 10.0 μM DHPG or 10.0 μM VU1545. DHPG promoted Akt activation in BACHD neurons to the same levels as those observed in WT neurons (Figure 8A and B). However, VU1545-mediated Akt phosphorylation was more pronounced in BACHD neurons than in WT neurons (Figure 8A and B).

Stimulation of striatal neurons from a knock-in and from a transgenic mouse model of HD with either glutamate or DHPG causes increased  $[Ca^{2+}]_i$ , as compared with that observed in neurons from control mice (Tang *et al.*, 2003; 2005; Ribeiro *et al.*, 2010b). VU1545 did not increase  $[Ca^{2+}]_i$  in WT striatal neurons above basal levels (Figure 2). To test the effect of VU1545 on  $[Ca^{2+}]_i$  in BACHD neurons, primary-cultured striatal neurons from BACHD mice were labelled with Fura-2 AM and stimulated with 10.0 μM VU1545. As observed in the case of WT neurons, VU1545 did not promote an increase in BACHD neurons  $[Ca^{2+}]_i$  above basal levels (data not shown). Moreover, the levels of  $[Ca^{2+}]_i$  in BACHD neurons were not different from those observed in



**Figure 5**

mGluR5 PAMs protect against glutamate-induced neuronal cell death. Graph shows percentage of neuronal cell death induced by either 50.0 or 100.0  $\mu\text{M}$  glutamate in primary-cultured striatal neurons that were either untreated (no PAM) or treated with DFB, VU1545 and CDPPB at the concentrations of 10.0  $\mu\text{M}$  (A) and 100.0 nM (B) for 20 h. Data represent the means  $\pm$  SEM of four independent experiments. \* indicates significant differences as compared with neurons treated with glutamate in the absence of PAMs ( $P < 0.05$ ).

WT neurons (data not shown). Taken together, these data indicate that VU1545 does not trigger increased  $[\text{Ca}^{2+}]_i$  when stimulating either BACHD or WT striatal neurons. Moreover, VU1545 can more efficiently promote Akt phosphorylation and neuroprotection in BACHD than in WT neurons.

### CDPPB ameliorates BACHD deficit in novel object recognition memory

It has been previously shown that BACHD mice exhibit learning impairment (Southwell *et al.*, 2009). In order to determine whether mGluR5 PAMs exhibit *in vivo* activity, we investigated whether CDPPB could improve BACHD mice performance in the object recognition task, which measures mice preference for a novel object. First, mice are allowed to explore two identical objects for 10 min. Following a 90 min delay, mice are reintroduced into the arena for 10 min and exposed to two objects: a familiar object and a novel object. The time mice explore the new object is calculated as a percentage of the total time exploring both familiar and novel objects. NT WT mice remembered the familiar object as the percentage of time exploring the new object was signifi-

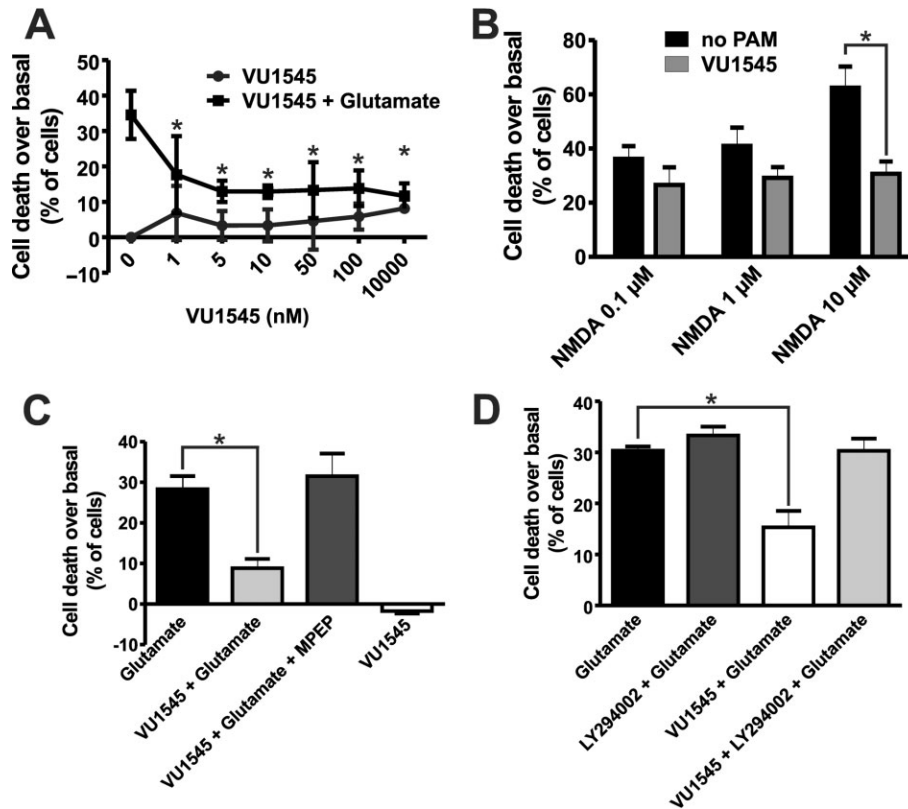
cantly higher than 50% (Figure 9). However, NT BACHD mice displayed no preference for novel object (Figure 9). To determine whether CDPPB could ameliorate the memory deficit exhibited by BACHD mice, we injected WT and BACHD mice with CDPPB 5 mg $\cdot\text{kg}^{-1}$  i.p. for 7 days and measured whether treated mice improved memory performance. Although CDPPB did not improve WT mice performance in the object recognition task, CDPPB-treated BACHD mice exhibited a significant preference for novel object, indicating that CDPPB can reverse the memory deficit exhibited by the HD mouse model (Figure 9). These data highlight the clinical potential of mGluR5 PAMs for treating neurodegenerative diseases.

## Discussion

The loss of neuronal tissue is the main factor leading to the development of neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease and HD (Vonsattel and DiFiglia, 1998; Donev *et al.*, 2009; Bagetta *et al.*, 2010; Ribeiro *et al.*, 2011). Thus, a drug that could protect neurons from cell-death processes would be an important therapeutic tool to treat such diseases. In the present study, we show that mGluR5 PAMs, such as DFB, VU1545 and CDPPB, have significant neuroprotective effects, reducing glutamate- and NMDA-induced neuronal cell death. mGluR5 PAMs are capable of activating Akt without promoting increased  $[\text{Ca}^{2+}]_i$ . Moreover, mGluR5 PAMs' neuroprotective effects are dependent on Akt activation, as blockage of this kinase leads to loss of PAM-mediated neuroprotection. Importantly, PAMs' potential as drugs that may be used to treat neurodegenerative diseases is highlighted by the high levels of neuroprotection exerted by PAMs on BACHD neurons stimulated with excitotoxic concentrations of glutamate and by the higher efficiency of mGluR5 PAMs in activating Akt in primary-cultured BACHD striatal neurons. Moreover, CDPPB treatment reversed the memory deficit exhibited by BACHD mice.

mGluR5 stimulation can lead to activation of pathways that can either protect neurons from cell death or exacerbate neurotoxicity (Bruno *et al.*, 2001; Zeron *et al.*, 2002; Schiefer *et al.*, 2004; Baskys *et al.*, 2005; Tang *et al.*, 2005). Activation of intracellular  $\text{Ca}^{2+}$  release by mGluR5 occurs via  $\text{G}\alpha_{q/11}$  proteins, leading to activation of phospholipase  $\text{C}\beta_1$  and  $\text{InsP}_3$  production. High levels of  $[\text{Ca}^{2+}]_i$  can contribute to excitotoxic processes, exacerbating neuronal death (Orrenius *et al.*, 2003). Moreover, mGluR5 contributes to NMDAR activation, which may also add to neurotoxicity as NMDAR stimulation is intimately associated with excitotoxic neuronal cell death (Awad *et al.*, 2000). In agreement with this rationale, mGluR5 blockage with MPEP decreases glutamate-induced apoptosis of striatal neurons from a transgenic HD mouse model (Tang *et al.*, 2005). However, our data show opposite results, as we have demonstrated that MPEP is not capable of reducing glutamate-induced neuronal cell death (Figure 1E). In addition, MPEP is neurotoxic when applied in the absence of glutamate (Figure 1E) and reverses VU1545-mediated neuroprotective effect (Figure 6C). We have stimulated neurons with glutamate in the presence or absence of MPEP for 20 h instead of 8 h as performed by Tang *et al.* (2005). Therefore, it is possible that MPEP could be neuroprotective initially as it blocks  $\text{Ca}^{2+}$  release, but as it also blocks activation of protec-





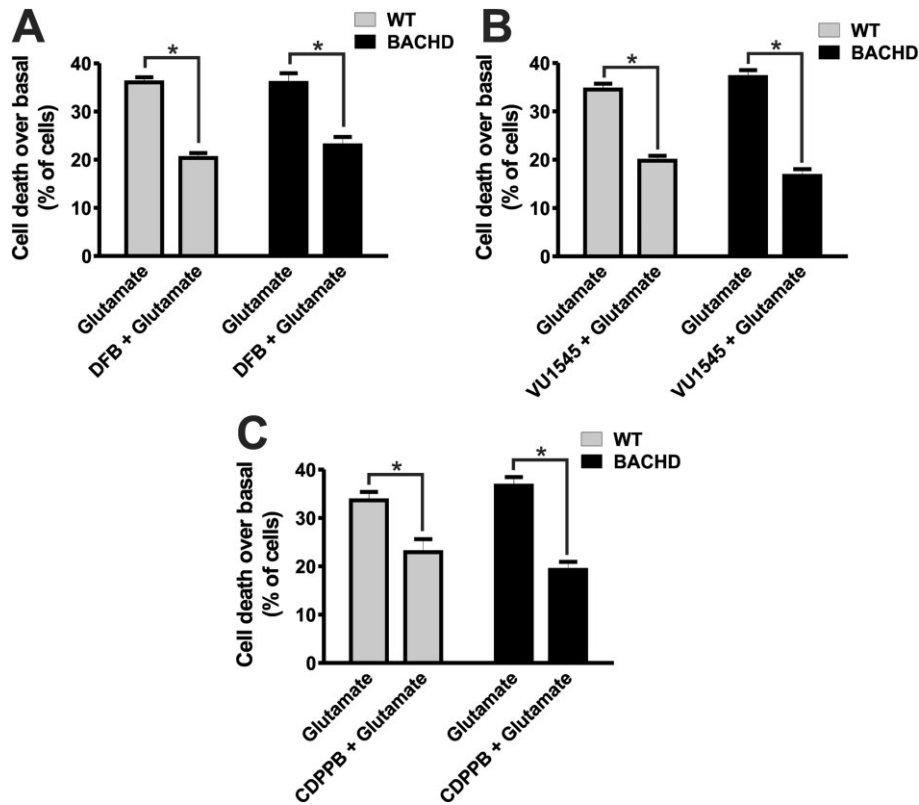
## Figure 6

VU1545 is a very potent neuroprotective drug and this neuroprotection is dependent on Akt activation. (A) Graph shows the effect of VU1545 at the concentrations of 1.0, 5.0, 10.0, 50.0, 100.0 and 10 000.0 nM on the percentage of neuronal cell death in primary-cultured striatal neurons that were either untreated (NT) or treated with 50.0  $\mu$ M glutamate for 20 h. Data represent the means  $\pm$  SEM of four independent experiments. \* indicates significant differences as compared with neurons treated with glutamate in the absence of VU1545 ( $P < 0.05$ ). (B) Graph shows percentage of neuronal cell death induced by NMDA at the concentrations of 0.1, 1.0 or 10.0  $\mu$ M in primary-cultured striatal neurons that were either NT (no VU1545) or treated with VU1545 100.0 nM for 20 h. Data represent the means  $\pm$  SEM of three independent experiments. \* indicates significant differences as compared with neurons treated with 10.0  $\mu$ M NMDA in the absence of VU1545 ( $P < 0.05$ ). (C) Graph shows percentage of neuronal cell death in primary-cultured striatal neurons that were treated with 50.0  $\mu$ M glutamate, 100.0 nM VU1545 + 50.0  $\mu$ M glutamate, 100.0 nM VU1545 + 50.0  $\mu$ M glutamate + 10.0  $\mu$ M MPEP and 100.0 nM VU1545 for 20 h. Data represent the means  $\pm$  SEM of three independent experiments. \* indicates significant difference as compared with neurons treated with glutamate ( $P < 0.05$ ). (D) Graph shows percentage of neuronal cell death in primary-cultured striatal neurons that were treated with 50.0  $\mu$ M glutamate, 25.0  $\mu$ M LY294002 + 50.0  $\mu$ M glutamate, 1.0  $\mu$ M VU1545 + 50.0  $\mu$ M glutamate and 1.0  $\mu$ M VU1545 + 25.0  $\mu$ M LY294002 + 50.0  $\mu$ M glutamate for 20 h. Data represent the means  $\pm$  SEM of four independent experiments. \* indicates significant difference as compared with neurons treated with glutamate ( $P < 0.05$ ).

tive pathways, it could become toxic when applied for longer periods. In addition to activating  $\text{Ca}^{2+}$  release via  $\text{InsP}_3$  receptors, mGluR5 can also activate neuroprotective pathways, such as ERK1/2 and Akt (Rong *et al.*, 2003; Mao *et al.*, 2005). Consistent with an mGluR5 neuroprotective role, most recently published data indicate that mGluR1/5 activation leads to neuroprotection (Bruno *et al.*, 2001; Baskys *et al.*, 2005; Ribeiro *et al.*, 2010b; Chen *et al.*, 2012). We have shown here that DHPG is neuroprotective against glutamate-induced neuronal cell death (Figure 1E), which indicates that mGluR1/5 activation is a better strategy to promote neuroprotection than mGluR1/5 blockage. However, this DHPG-mediated neuroprotection is not as robust as that offered by mGluR5 PAMs (compare Figure 1E and Figure 6). Moreover, different from mGluR5 PAMs, DHPG increases basal neuronal death (Figure 1E). In addition to activating neuroprotective pathways, DHPG also triggers  $\text{Ca}^{2+}$  release from intracellular

stores, which might contribute to excitotoxicity. We hypothesize that neither blocking mGluR5 with MPEP nor activating it with DHPG is the best strategy to promote neuroprotection. Conversely, interaction of PAMs with mGluR5 could stabilize a conformation of the receptor that would facilitate activation of neuroprotective pathways, including Akt and ERK1/2, without triggering  $\text{G}\alpha_{q/11}/\text{InsP}_3/\text{Ca}^{2+}$  activation.

The activation of Akt by group I mGluRs involves PI3K and phosphoinositide-dependent kinase (Rong *et al.*, 2003; Hou and Klann, 2004). A PI3K enhancer (PIKE) couples group I mGluRs to PI3K via Homer proteins (Rong *et al.*, 2003). Importantly, formation of the functional complex mGluR1-Homer-PIKE allows PI3K activation by PIKE, which results in reduced apoptosis (Rong *et al.*, 2003). Thus, activation of Akt by mGluR5 appears to be independent of the  $\text{InsP}_3/\text{Ca}^{2+}$  pathway. It has been demonstrated that mGluR5 PAMs have the potential to activate one signaling pathway without trig-



**Figure 7**

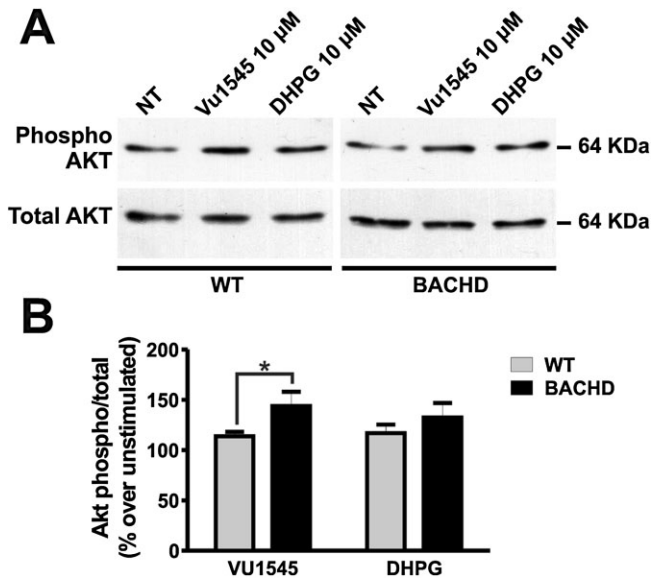
mGluR5 PAMs protect BACHD neurons from glutamate-induced neuronal cell death. Graphs show percentage of neuronal cell death induced by 50.0 μM glutamate in either BACHD or wild-type (WT) primary cultured striatal neurons in the presence or absence of 1.0 μM DFB (A), 1.0 μM VU1545 (B) and 1.0 μM CDPPB (C) for 20 h. Data represent the means ± SEM of four to six independent experiments. \* indicates significant differences as compared with neurons treated with glutamate in the absence of PAMs ( $P < 0.05$ ).

gering others (Zhang *et al.*, 2005; Bradley *et al.*, 2009; Chen *et al.*, 2012). Stimulation of cortical astrocytes with DFB, N-[4-Chloro-2-[(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)methyl]phenyl]-2-hydroxybenzamide (CPPHA) and CDPPB in the absence of mGluR5 agonist fails to increase  $Ca^{2+}$  release above basal levels (Zhang *et al.*, 2005; Bradley *et al.*, 2009). However, DFB and CPPHA increase ERK1/2 activation even in the absence of mGluR5 agonist in cortical astrocytes (Zhang *et al.*, 2005). Moreover, primary-cultured cortical neurons treated with CDPPB exhibit high levels of ERK1/2 activation (Chen *et al.*, 2012). As mGluR5 activates ERK1/2 and Akt in a similar manner, which occurs via Homer, we decided to investigate whether mGluR5 PAMs could activate Akt in the absence of agonist. Our data show that mGluR5 PAMs efficiently activate Akt without promoting increased  $[Ca^{2+}]_i$ . Akt activation by mGluR5 PAMs appears to be essential for preventing glutamate-induced neuronal death, as blockage of Akt activation abrogates PAM-induced neuroprotection.

Akt activation can protect against neuronal death (Datta *et al.*, 1999; Kandel and Hay, 1999). Moreover, Akt can also promote phosphorylation of mutated Htt protein, which functions to reduce Htt aggregate formation and neuronal cell death, providing a protective pathway in HD (Humbert *et al.*, 2002; Warby *et al.*, 2009). We and others have shown that basal Akt phosphorylation is increased in HD (Gines

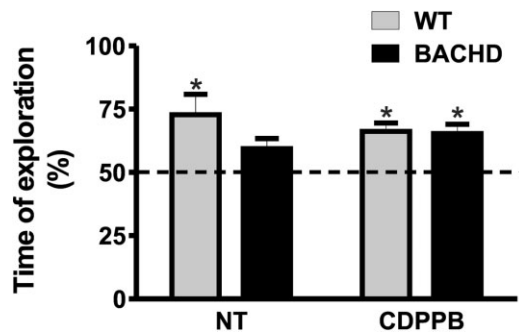
*et al.*, 2003; Ribeiro *et al.*, 2010b). Furthermore, basal Akt activation appears to be dependent on glutamate receptors as both NMDAR antagonist MK801 and mGluR5 antagonist MPEP decrease Akt activation in HD neurons to WT levels (Gines *et al.*, 2003; Ribeiro *et al.*, 2010b). DHPG-mediated Akt activation in striatal neurons from a knock-in (Ribeiro *et al.*, 2010b) and a transgenic (Figure 8) mouse model of HD is not different from that observed in control neurons. Interestingly, VU1545 promotes higher levels of Akt activation in BACHD striatal neurons than in WT neurons (Figure 8). Moreover, VU1545 appears to be more efficient in preventing cell death of BACHD neurons than of WT neurons (Figure 7). These data highlight the potential of mGluR5 PAMs, especially VU1545, as therapeutic tools to prevent the neuronal cell death that takes place in HD.

mGluR5 PAMs are very selective for mGluR5 and are currently being tested to treat other disorders of the CNS (Ritzen *et al.*, 2005; Conn *et al.*, 2009; Rodriguez *et al.*, 2010). *In vivo* animal tests and human clinical trials indicate that mGluR5 PAMs might be good therapeutic options to treat schizophrenia, exhibiting low toxicity and being well tolerated by patients (Rodriguez *et al.*, 2010; Spear *et al.*, 2011; Gray *et al.*, 2012). The data presented here demonstrate that mGluR5 PAMs can activate Akt without promoting increased  $[Ca^{2+}]_i$ , offering neuroprotection against glutamate- and NMDA-



**Figure 8**

VU1545 activates Akt more robustly in BACHD than in wild-type (WT) neurons. (A) Shown is a representative immunoblot for phospho- (upper panel) and total-Akt expression (lower panel) in primary cultured striatal neurons from either WT or BACHD mice that were either untreated (NT) or treated with 10.0  $\mu$ M DHPG or 10.0  $\mu$ M VU1545. About 100.0  $\mu$ g of cell lysate was used for each sample. (B) Graph shows the densitometric analysis of phospho-Akt normalized to total-Akt expression in primary-cultured striatal neurons from either WT or BACHD mice that were either NT or treated with 10.0  $\mu$ M DHPG or 10.0  $\mu$ M VU1545. Data represent the means  $\pm$  SEM of six independent experiments, expressed as percentage of basal Akt phosphorylation. \* indicates significant difference as compared with matched treated WT neurons ( $P < 0.05$ ).



**Figure 9**

CDPPB ameliorates BACHD deficit in novel object recognition memory. Graph shows percentage of time of novel object exploration calculated as an index between time spent exploring a novel object  $\times$  100/time with both objects. A score of 50% indicates no preference. Two weeks after untreated (NT) wild-type (WT,  $n = 6$ ) and BACHD ( $n = 6$ ) mice were submitted to object recognition test, both WT and BACHD were treated with CDPPB i.p. 5 mg $\cdot$ kg $^{-1}$  for 7 days and reassessed for recognition memory. Data represent the means  $\pm$  SEM, expressed as percentage of time exploring both objects. \* indicates significant differences from chance exploration (50%) ( $P < 0.05$ ).

induced neurotoxicity. Moreover, VU1545 activates Akt and protects neurons from a mouse model of HD in a more robust manner than that observed in the case of WT neurons. Importantly, CDPPB sub-chronic treatment ameliorated HD-related memory deficit observed in BACHD mice, further emphasizing mGluR5 PAMs' potential as drugs to treat neurodegenerative diseases, especially HD. As HD diagnosis can be established early in life by genotypic tests, mGluR5 PAMs could be used to slow neuronal loss and, consequently, disease progression.

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## Conflict of interest

None.

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## Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Figure S1** Neuronal primary cultures are mainly composed of medium-sized spiny neurons. Shown are representative fluorescence micrographs demonstrating the presence of both Alexa Fluor 488-conjugated anti-NeuN antibody (green)

(A) and DAPI staining (blue) (B) in the same striatal neurons (C). Shown are representative fluorescence micrographs demonstrating the presence of both Alexa Fluor 633-conjugated anti-VGAT antibody (red) (D) and DAPI staining (blue) (E) in the same striatal neurons (F). Shown are representative fluorescence micrographs demonstrating the absence of either Alexa Fluor 488 or Alexa Fluor 633 (G) and the presence of DAPI staining (blue) (H) in the same striatal neurons (I). Data are representative images of multiple cells from three independent experiments.

**Figure S2** Drug dilution vehicles (water, HBSS and DMSO) do not modify neuronal cell death. Graph shows percentage of neuronal cell death in primary-cultured striatal neurons that were treated with H<sub>2</sub>O (1:1000 v/v), HBSS (1:1000 v/v), DMSO (1:1000 v/v), DMSO (1:10.000 v/v) and 50.0 μM glutamate (1:1000 v/v) for 20 h. All vehicles were diluted as indicated (v/v) in complete neurobasal medium. Data represent the means ± SEM of three independent experiments.