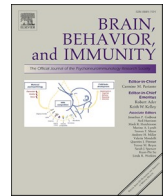


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Full-length Article

Peripheral administration of the Class-IIa HDAC inhibitor MC1568 partially protects against nigrostriatal neurodegeneration in the striatal 6-OHDA rat model of Parkinson's disease

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ABSTRACT

Parkinson's disease (PD) is a neurodegenerative disorder characterised by nigrostriatal dopaminergic (DA) neurodegeneration. There is a critical need for neuroprotective therapies, particularly those that do not require direct intracranial administration. Small molecule inhibitors of histone deacetylases (HDIs) are neuroprotective in *in vitro* and *in vivo* models of PD, however it is unknown whether Class IIa-specific HDIs are neuroprotective when administered peripherally. Here we show that 6-hydroxydopamine (6-OHDA) treatment induces protein kinase C (PKC)-dependent nuclear accumulation of the Class IIa histone deacetylase (HDAC)5 in SH-SY5Y cells and cultured DA neurons *in vitro*. Treatment of these cultures with the Class IIa-specific HDI, MC1568, partially protected against 6-OHDA-induced cell death. In the intrastriatal 6-OHDA lesion *in vivo* rat model of PD, MC1568 treatment (0.5 mg/kg i.p.) for 7 days reduced forelimb akinesia and partially protected DA neurons in the substantia nigra and their striatal terminals from 6-OHDA-induced neurodegeneration. MC1568 treatment prevented 6-OHDA-induced increases in microglial activation in the striatum and substantia nigra. Furthermore, MC1568 treatment decreased 6-OHDA-induced increases in nuclear HDAC5 in nigral DA neurons. These data suggest that peripheral administration of Class IIa-specific HDIs may be a potential therapy for neuroprotective in PD.

1. Introduction

Parkinson's disease (PD) is characterised by the degeneration of nigrostriatal dopaminergic (DA) neurons of the substantia nigra (SN) and of their axonal projections to the striatum (ST), and intracellular aggregation of α -synuclein (Braak et al., 2003; Poewe et al., 2017; Spillantini et al., 1997), which lead to reduced striatal dopamine levels and consequent motor and non-motor symptoms (Fearnley and Lees, 1991; Greffard et al., 2006; Herrera et al., 2005). PD treatment is focused on dopamine replacement pharmacotherapy to relieve motor symptoms (Bloem et al., 2021; Fahn et al., 2004), but no current therapy slows the ongoing neurodegeneration. Development of disease-modifying therapies which can be administered through a peripheral route is a major goal (Bloem et al., 2021; Kalia et al., 2015). Histone deacetylases (HDACs) are key epigenetic regulators and are under

investigation as potential therapeutic targets for PD (Gupta et al., 2020; Mazzocchi et al., 2020; Sharma et al., 2019). HDACs are enzymes that are crucial for chromatin remodeling and can be grouped into four classes: Class I (HDAC1, HDAC2, HDAC3 and HDAC8), Class IIa (HDAC4, HDAC5, HDAC7, HDAC9), Class IIb (HDAC6 and HDAC10), Class III (also called SIRT1-7) and Class IV (HDAC11) (Mazzocchi et al., 2020). HDACs can be inhibited by pharmacological agents known as HDAC inhibitors (HDIs), which can be either pan-HDIs, or class-specific HDIs (Balasubramanian et al., 2009; Bondarev et al., 2021; Porter and Christianson, 2019). Several studies have investigated the therapeutic potential of pan-HDIs such as sodium butyrate (NaB) and valproic acid (VPA), but few have focused on class-specific inhibition (Rane et al., 2012; Ximenes et al., 2015). Some Class I (Choong et al., 2016; Johnston et al., 2013) and Class IIb (Francelle et al., 2020; Jian et al., 2017; Li et al., 2021; Pinho et al., 2016) HDIs have shown neuroprotective

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potential in PD models. However, the Class III HDI nicotinamide exacerbates neurodegeneration in the lactacystin rat PD model (Harrison et al., 2019), and some HDAC1/2 HDIs can exacerbate cell death induced by the DA neurotoxin, 1-methyl-4-phenylpyridinium (MPP⁺) *in vitro* (Park et al., 2016). This underscores the importance of exploring the therapeutic potential of selective HDAC inhibition, including testing class-specific HDIs in *in vivo* PD models.

Class IIa HDACs undergo nucleocytoplasmic shuttling to exert effects on neuronal survival and axonal growth (Cho and Cavalli, 2012). Class IIa HDACs, including HDAC4 and 5, are expressed in DA neurons in mouse SN, located mainly in the cytoplasm (Mazzocchi et al., 2019; Wu et al., 2017). Gene co-expression analysis has shown that HDAC5 and HDAC9 are co-expressed with DA markers in the human SN (Mazzocchi et al., 2021; Mazzocchi et al., 2019). Treatment with the class IIa HDIs, MC1568 and LMK235, protected against MPP⁺- and α -synuclein-induced degeneration of DA neurons *in vitro* (Collins et al., 2015; Mazzocchi et al., 2021). These effects appear to be mediated through HDAC5 inhibition, as siRNAs against HDAC5, but not HDAC4 and HDAC7, protected against α -synuclein-induced neurite degeneration in SH-SY5Y cells (Mazzocchi et al., 2019). However, it is unclear whether Class IIa HDIs, and in particular HDAC5 inhibition, is neuroprotective against DA neurodegeneration *in vivo*.

There is no known selective inhibitor of HDAC5, but the small molecule MC1568 is the most well-characterised Class IIa-specific HDI (Mai et al., 2005). Intraperitoneal (i.p.) administration of MC1568 in rats reduces HDAC5 levels in nucleus accumbens (Taniguchi et al., 2017), highlighting the feasibility of a peripheral administration route. MC1568 (i.p.) has also been shown to reduce thimerosal-induced apoptosis in rat prefrontal cortex, by preventing upregulation of Class IIa HDACs (Guida et al., 2016). In the current study, we examined the neuroprotective potential of MC1568 in *in vitro* models of PD, then investigated the potential of i.p. administration of MC1568 to exert neuroprotective effects in the intrastriatal 6-OHDA lesion rat model of PD.

2. Materials and Methods

2.1. Cell culture

SH-SY5Y cells (ATCC; CRL-2266), used as a model of human DA neurons (Xicoy et al., 2017), were grown in Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (DMEM/F-12), with 10% foetal bovine serum (FBS), 100 nM L-Glutamine, 100U/ml Penicillin, 10 μ g/ml Streptomycin (all Sigma), in a humidified atmosphere containing 5% CO₂ at 37°C. Primary cultures of embryonic day (E)14 rat VM (Biological Service Unit, University College Cork) were dissected as previously described (Hegarty et al., 2016) under license with full ethical approval. Tissue was dissociated following enzymatic treatment and cells were plated at 5×10^4 cells per well on poly-D-lysine-coated 24-well plates (Sigma) in DMEM/F-12 media with 1% penicillin/streptomycin (Sigma), 1% L-glutamine (Sigma), 2% B27 (Invitrogen), 1% FBS. Cells were treated either concurrently or after 24 h delay with MC1568 (Cayman) and 6-OHDA (Sigma) for 72 h, at concentrations indicated in figures. Where indicated, cells were treated for 24 h with 6-OHDA and the protein kinase C (PKC) activator phorbol 12-myristate 13-acetate (PMA) for 1 h before fixation.

2.2. MTT assay

Culture media was removed and 300 μ L of 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) in HBSS was added to each well for 4 h at 37 °C in 5% CO₂. MTT-HBSS solution was carefully aspirated and 100 μ L of DMSO was added to each well to permeabilise any formazan produced by the cells. 75 μ L of DMSO/formazan solution from each well was pipetted into a 96-well plate and absorbance of each sample measured at 620 nm using an

A600 plate reader (Thermo Fisher).

2.3. Immunocytochemistry

For neurite length analysis, SH-SY5Y cells were labelled with the fluorescent vital dye calcein AM (1:1000 Invitrogen) for 1 h at the end of culture period. In primary cultures, DA neurons were identified by immunocytochemical staining for tyrosine hydroxylase (TH). Cell culture plates were fixed for 15 min using 4% paraformaldehyde. Following 3×5 min washes in PBS-T, cultures were incubated in 5% bovine serum albumin (BSA) in 10 mM PBS-T for 1 h at room temperature. They were incubated in primary antibodies: TH (Millipore MAB318; 1:200), HDAC5 (Santa-Cruz; sc-133106 1:200) or beta-actin (Santa-Cruz sc-47778; 1:200), diluted in 1% BSA in 10 mM PBS at 4°C for 16 h. Following 3×5 min washes in 10 mM PBS-T, cells were incubated in 594-conjugated Alexa-Fluor® secondary antibodies (Invitrogen; 1:500 A11005 or A11012) in 1% BSA in 10 mM PBS, prior to 3×5 min washes. Immunostained SH-SY5Y cells and E14 VM neurons were imaged using Olympus IX71 inverted microscope. For analysis of neurite growth, five non-overlapping images were captured from each well in each experimental group and 135–350 cells were analysed per group, as indicated in the figure legends. Fluorescence intensity of individual cells was measured by densitometry using Image J analysis software.

2.4. In vivo study design

32 adult female Sprague-Dawley rats were purchased from Envigo, UK, and maintained on a 12 h:12 h light:dark cycle with access to food and water *ad libitum*. Animals were randomly assigned to one of four experimental groups: Vehicle/Saline (n = 8), Vehicle/MC1568 (n = 8), 6-OHDA/Saline (n = 8) and 6-OHDA/MC1568 (n = 8). Vehicle/Saline group received stereotactic injection of saline with 0.01% ascorbate, followed by 7 daily i.p. injections of saline with 0.5% DMSO. Vehicle/MC1568 group received stereotactic injection of saline with 0.01% ascorbate, followed by 7 daily i.p. injections of 0.5 mg/kg MC1568 in saline with 0.5% DMSO. 6-OHDA/Saline group received stereotactic injection of 7 μ g of 6-OHDA in saline with 0.01% ascorbate, followed by 7 daily i.p. injections of saline with 0.5% DMSO. 6-OHDA/MC1568 group received stereotactic injection of 7 μ g of 6-OHDA dissolved saline with 0.01% ascorbate followed by 7 daily i.p. injections of 0.5 mg/kg MC1568 in saline with 0.5% DMSO. The dose of 0.5 mg/kg MC1568 i.p. was based on a study (Griffin et al., 2017) showing that this dose was safe and well tolerated; this was supported by the lack of an effect of this treatment on the body weight of the animals throughout our study (Supplementary Fig. 1). Rats were housed in groups of four in standard housing cages with environmental enrichment. Behavioural testing was performed on the week before stereotactic surgery, and at day 8 and 12 post-surgery. All experiments were conducted in accordance with the European Directive 2010/63/EU and under authorisation granted by the Health Products Regulatory Authority Ireland (AE19130/P057).

2.5. 6-OHDA lesion surgery

Stereotaxic surgery was performed under general anaesthesia induced by isoflurane inhalation. The mortality rate in this study was 3.1%, as one animal did not recover from stereotaxic surgery. Each animal was then placed into the stereotactic frame, an incision was made on the skull and four small holes were made into the skull. Four-site unilateral intrastriatal lesion was made by infusion of 6-OHDA hydrobromide (7 μ g as free base (Sigma) in 3 μ L saline with 0.01% ascorbate, into each of four sites) at a rate of 1 μ L/min with 2 min for diffusion, at the following co-ordinates: AP + 1.3, ML $\pm$ 2.7; AP + 0.4, ML $\pm$ 3.1; AP – 0.4, ML $\pm$ 4.3; AP – 1.3, ML $\pm$ 4.7 from bregma and DV – 5.0 below dura, with incision bar at – 2.3 mm). After diffusion, the needle was withdrawn, and sutures were performed. As post-surgery care, animals received Carprofen (5 mg/kg s.c.) as analgesic and 5% glucose solution

(i.p.), then were allowed to recover fully on a heated mat before being returned to their home cages.

2.6. Behavioural analysis

The Stepping test for forelimb akinesia was performed as described previously (Olsson et al., 1995). In brief, animals were restrained to retain one forelimb, then hindlimbs and the free forelimb were placed on the countertop. The animal was then moved sideways at steady pace on the countertop across 90 cm in approximately 15 s. Numbers of adjusting steps made by the unrestrained forelimb on ipsilateral and contralateral sides were counted.

2.7. Tissue collection and processing

At 14 days after surgery, rats were sacrificed under terminal anaesthesia with sodium pentobarbital (50 mg/kg) and transcardially perfused with 4% paraformaldehyde. Brains were extracted and placed in 4% paraformaldehyde in PBS overnight, then placed in 30% sucrose for cryoprotection and subsequently snap-frozen in isopentane with liquid nitrogen. 30 μ m cryosections were cut, then placed in cryoprotectant (10 mM PBS with 30% ethylene glycol, 25% glycerol, 20% water) until used for immunohistochemistry.

2.8. Immunohistochemistry

Immunohistochemistry was carried out as previously described (Goulding et al., 2021). Sections were washed three times in 1.2% tris-buffered saline (TBS) solution for 5 min. Where appropriate, sections were quenched using 3% hydrogen peroxide/10% methanol in distilled water for 5 min to block endogenous peroxidase activity, then washed three times in TBS solution. For fluorescent immunostaining, non-specific binding was blocked using 10% goat, horse or rabbit serum as appropriate, diluted in tris-buffered saline containing 0.02% Triton-X100 (TxTBS). Sections were incubated overnight shaking at 4 °C in primary antibody diluted in 1% TxTBS serum. The following primary antibodies were used: TH (Merck Millipore; MAB318 1:1000), IBA1 (Fujifilm; 019-19741 1:1000) or HDAC5 (Abcam; ab1439 1:500). Following three washes in TBS, sections were incubated for 2 h in secondary antibody diluted in TxTBS containing 1% serum. For fluorescence staining, Alexa Fluor 488- or 594-conjugated secondary antibodies were used (1:500; Invitrogen). Sections were washed three times in TBS and, where indicated, nuclei were stained by incubation in DAPI (Sigma; 1:3000) solution for 5 h in the dark, followed by three TBS washes, then cover-slipped using fluorescent mounting media (Dako Diagnostics). For chromogen detection, secondary antibodies were biotinylated goat-anti-rabbit IgG (1:200, Jackson ImmunoResearch Lab), horse-anti-mouse IgG (1:200, Vector Labs) or rabbit-anti-goat IgG (1:200, Vector Labs). Following three TBS washes, sections were incubated in streptavidin-biotin-horseradish peroxidase solution (Vector Labs) for 2 h. Sections were washed three times in TBS before developing with DAB (Vector Labs). Sections were dehydrated using increasing concentrations of ethanol, cleared in Xylene and cover-slipped using DPX mounting media (BDH Chemicals). Images were taken using the Olympus BX53 Upright Microscope, Olympus FV1000 Confocal Laser Scanning Biological Microscope and Olympus FV10i Microscope.

2.9. Image analysis

For the analysis of *in vitro* experiments, three non-overlapping images of each well were acquired and $n = 135$ cells for each group were analysed using ImageJ software analysis. These values were averaged to give one n per group per experiment. Specifically, for neurite length analysis, Image J was calibrated using the appropriate scale bar and the freehand tool was used to manually trace neurites from three cells in each image, which were then measured using the 'analyse' function. To

quantify nuclear accumulation of HDAC5, cells were immunostained for HDAC5 (red) and DAPI (blue). The nuclear outline was traced using the DAPI image of three individual cells in each image, and the mean intensity value was measured. A similar strategy was used to measure HDAC5 nuclear accumulation *in vivo*, in TH-positive neurons in the SN. A similar tracing approach was also used to measure the cell body area of IBA1 + microglia, by tracing the cell body, which was then quantified using the 'analyse' function. For immunohistochemical analysis of nigrostriatal pathway integrity, 3 individual brain slices of striatum or SN were imaged for each of the 8 animals per experimental group. To quantify TH immunoreactivity in striatum, images were converted to 8-bit images using Image J, the segmented line tool was used to trace the outline of the striatum, and mean intensity values were measured to quantify TH-immunopositive staining on the ipsilateral and contralateral sides. To evaluate the numbers of TH neurons in the SN, and numbers of IBA1 + cells in both striatum and SN, images were converted to 8-bit images, 'adjust threshold function' was used to obtain a black and white image with a threshold to allow visualisation of each individual cell, this was maintained throughout the image analysis. Finally, the 'analyse particles' function was used to quantify the numbers of cells in each image.

2.10. Statistical analysis

Statistical analysis was performed using GraphPad Prism 8 (©2020 GraphPad Software, CA USA). Data are presented as mean (expressed as a percentage of control) $\pm$ SEM of the number of experimental replicates, rather than of the number of cells. *In vivo* data are presented as mean $\pm$ SEM as percentage of the contralateral side. Statistical differences were analysed using two-way ANOVA as appropriate, with *post-hoc* test as indicated in the figure legends.

3. Results

3.1. 6-OHDA reduces neurite length and induces HDAC5 nuclear accumulation, which is partially prevented by PKC activation, in SH-SY5Y cells and cultured DA neurons.

We first sought to determine whether 6-OHDA treatment increased nuclear HDAC5 levels in SH-SY5Y cells and whether this could be prevented by PKC activation, which is known to promote nuclear-cytoplasmic shuttling of HDAC5 (Wu et al., 2017). We found that treatment of SH-SY5Y cells with 15 μ M 6-OHDA for 24 h had a significant effect on nuclear HDAC5 levels ($F_{(1-8)} = 183.9$, $P < 0.0001$) (Fig. 1A, B). Pre-treatment for 1 h with 10 μ M PMA, a PKC activator, also had a significant effect on nuclear HDAC5 levels ($F_{(1-8)} = 18.05$, $P = 0.0028$), and there was found a significant 6-OHDA $\times$ PMA interaction ($F_{(1-8)} = 20.52$, $P = 0.0019$). *Post-hoc* testing confirmed that PMA pre-treatment partially prevented 6-OHDA-induced nuclear accumulation (Fig. 1A, B).

We confirmed these findings in primary cultures of E14 rat VM, which were treated with 5 μ M 6-OHDA for 24 h. Densitometry analysis of nuclear HDAC5 in TH-positive neurons revealed significant effects of both 6-OHDA ($F_{(1-8)} = 261.2$, $P < 0.0001$) and PMA treatment ($F_{(1-8)} = 99.17$, $P < 0.0001$), with a significant 6-OHDA $\times$ PMA interaction ($F_{(1-8)} = 124.8$, $P < 0.0001$). Finally, treatment with 6-OHDA ($F_{(1-8)} = 197.2$, $P < 0.0001$) and stimulation with PMA ($F_{(1-8)} = 13.9$, $P = 0.0058$) for 1 h had a significant effect on neurite length, although the 6-OHDA $\times$ PMA interaction was not significant ($F_{(1-8)} = 0.1617$, $P = 0.6982$) (Fig. 1D, E). These data show that 6-OHDA increases nuclear HDAC5 levels in both SH-SY5Y cells and DA neurons *in vitro*.

3.2. Concurrent treatment with MC1568 partially protects against 6-OHDA-induced decreases in neurite length in cultured DA neurons.

We next sought to investigate the effects of MC1568, a specific Class

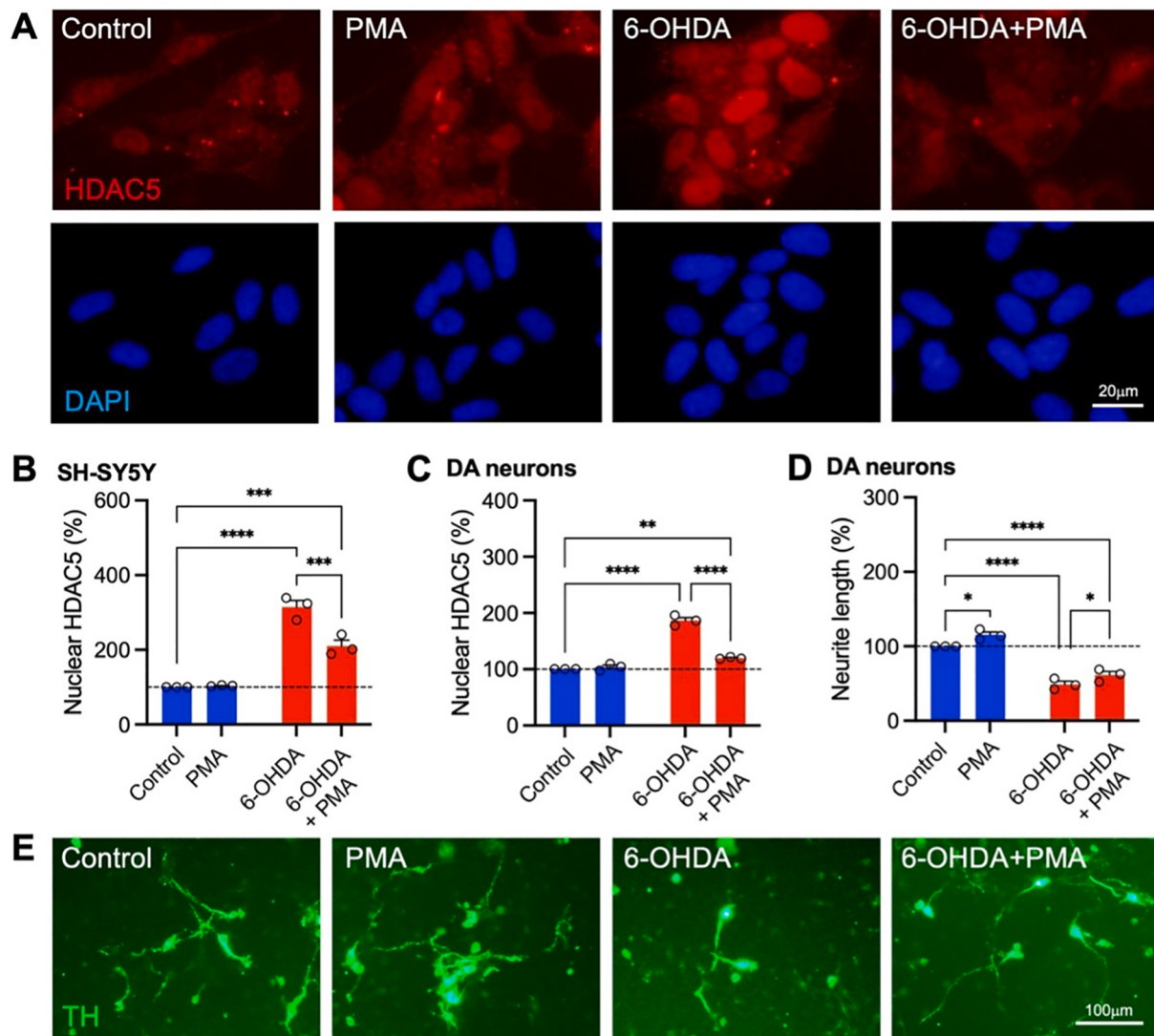


Fig. 1. 6-OHDA reduces neurite length and induces HDAC5 nuclear accumulation, which is partially prevented by PKC activation, in SH-SY5Y cells and cultured DA neurons. (A) Representative photomicrographs of immunocytochemical staining for HDAC5 and of DAPI staining, and (B) graph showing nuclear HDAC5 expression, in SH-SY5Y cells after treatment with or without 10 μ M of the PKC activator, PMA, for 1 h prior to treatment with or without 15 μ M 6-OHDA for 24 h. Graphs showing (C) nuclear HDAC5 expression and (D) neurite length, and (E) representative photomicrographs of TH-positive DA neurons, in primary cultures of E14 rat VM after treatment with or without 10 μ M of the PKC activator, PMA, for 1 h prior to treatment with or without 5 μ M 6-OHDA for 24 h. All data are presented as the mean $\pm$ SEM as a percentage of the control of $n = 3$ experiments. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ vs. control or as indicated; Two-way ANOVA with *post-hoc* Tukey's test.

Ila HDI, on 6-OHDA-induced neurodegeneration of SH-SY5Y cells, using a concurrent treatment paradigm. To do this, SH-SY5Y cells were treated with 15 μ M 6-OHDA or 0.1 μ M MC1568 for 3 DIV and cell survival was analysed using MTT assay (Fig. 2A). We found significant main effects of 6-OHDA ($F_{(1-8)} = 84.65$, $P < 0.0001$) and of MC1568 ($F_{(1-8)} = 11.89$, $P = 0.0087$), with no significant interaction ($F_{(1-8)} = 1.161$, $P = 0.3127$) (Fig. 2B). *Post-hoc* analysis showed that concurrent treatment of MC1568 with 6-OHDA had no significant effect on cell survival.

We next examined neurite length in the same experimental setting and found significant main effects of 6-OHDA ($F_{(1-8)} = 113.3$, $P < 0.0001$) and MC1568 ($F_{(1-8)} = 20.02$, $P = 0.0002$) on neurite growth, with no 6-OHDA $\times$ MC1568 significant interaction ($F_{(1-8)} = 0.0986$, $P = 0.7615$). *Post-hoc* comparisons showed that 6-OHDA had a detrimental effect on neurite growth, that was partially prevented by co-treatment with MC1568 (Fig. 2C).

We also examined the neurite growth-promoting effects of these treatments in DA neurons, using primary cultures of E14 rat VM. When cultures were concurrently treatment with 0.02 μ M MC1568 with or

without 5 μ M 6-OHDA, there were significant main effects of 6-OHDA ($F_{(1-8)} = 113.3$, $P < 0.0001$) and MC1568 ($F_{(1-8)} = 20.16$, $P = 0.0021$) on axon growth, with no significant interaction ($F_{(1-8)} = 0.004$, $P = 0.9855$) (Fig. 2D). *Post-hoc* analysis showed a detrimental effect of 6-OHDA, that was partially prevented by MC1568 (Fig. 2D). These data show that concurrent treatment with MC1568 can partially protect against 6-OHDA-induced DA neurodegeneration *in vitro*.

3.3. Delayed treatment with MC1568 partially protects against 6-OHDA-induced decreases in neurite length in cultured DA neurons.

Although concurrent treatment is useful in an experimental setting, in PD some degree of neurodegeneration would have already occurred before any therapeutic would be applied. To model this *in vitro*, we used a delayed treatment paradigm in which SH-SY5Y cells were firstly treated with 15 μ M 6-OHDA for 24 h, prior to treatment with 0.1 μ M MC1568 for 3DIV (Fig. 2E). Two-way ANOVA analysis of the MTT assay revealed significant effects of 6-OHDA ($F_{(1-8)} = 1272$, $P < 0.0001$) and

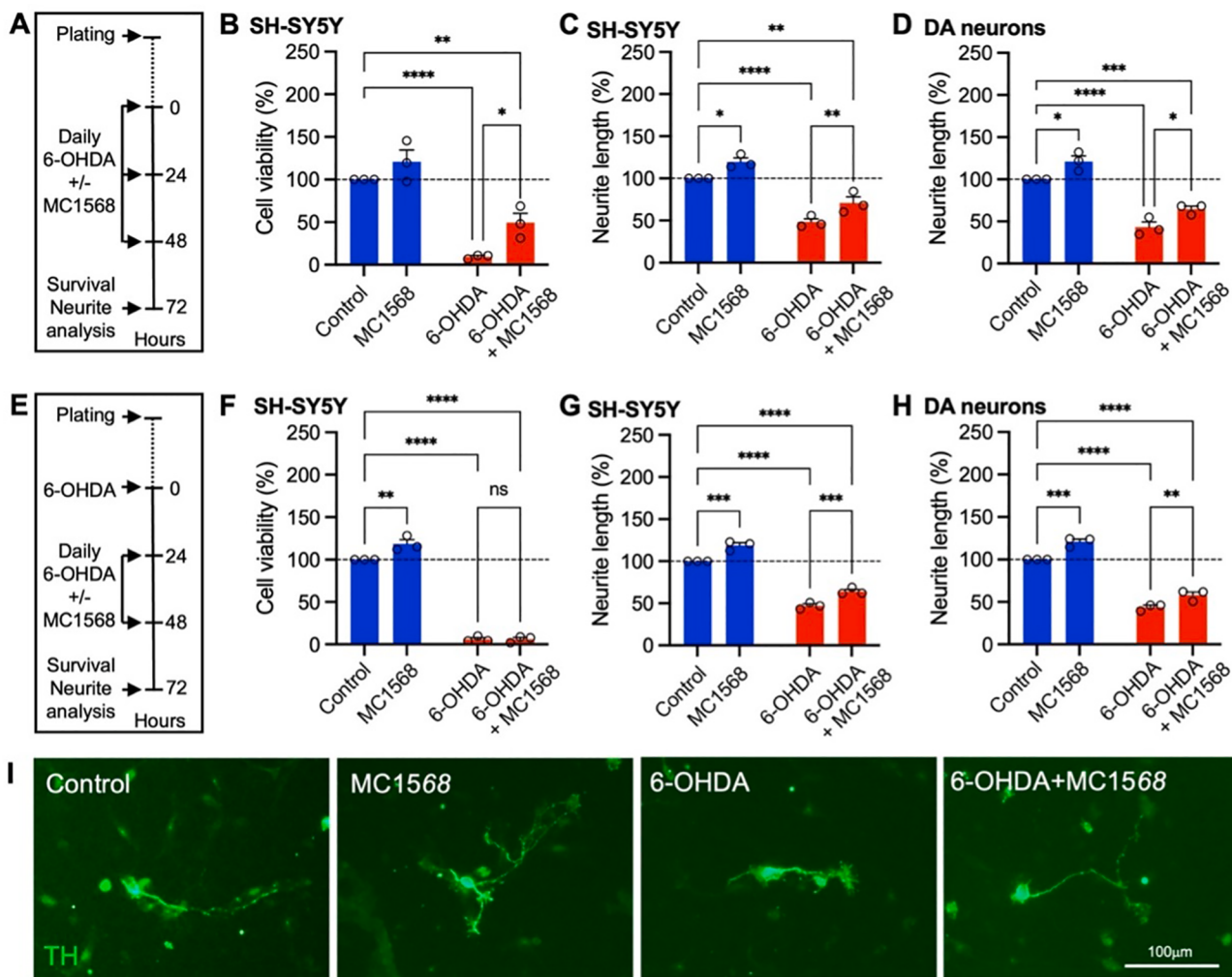


Fig. 2. Concurrent and delayed treatment with MC1568 partially protects against 6-OHDA-induced decreases in neurite length in cultured DA neurons. (A) Schema of the treatment regime used in experiments B-D. Graphs of (B) MTT assay and (C) neurite length of SH-SY5Y cells treated concurrently with or without 15 μ M of 6-OHDA and with or without 0.1 μ M of MC1568 for 3 DIV. (D) Graph of neurite length of DA neurons in primary cultures of E14 rat VM treated concurrently with or without 5 μ M of 6-OHDA and 0.02 μ M of MC1568 for 3 DIV. (E) Schema of the treatment regime used in experiments F-H. Graphs of (F) MTT assay and (G) neurite length of SH-SY5Y cells treated with or without 15 μ M of 6-OHDA for 24 h prior to treatment with or without 0.1 μ M of MC1568 for the remaining 48 h for a total experimental duration of 3 DIV. (H) Graph of neurite length of DA neurons in primary cultures of E14 rat VM treated with or without 5 μ M of 6-OHDA for 24 h prior to treatment with or without 0.02 μ M of MC1568 for the remaining 48 h for a total experimental duration of 3 DIV. (I) Representative photomicrographs of TH-positive neurons from the experiment shown in (H). All data are presented as the mean $\pm$ SEM as a percentage of the control of $n = 3$ experiments. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ vs. control or as indicated; Two-way ANOVA with *post-hoc* Tukey's test.

MC1568 on cell viability ($F_{(1-8)} = 10.25$, $P = 0.0126$), with a significant 6-OHDA $\times$ MC1568 interaction ($F_{(1-8)} = 10.17$, $P = 0.0128$) (Fig. 2F). *Post-hoc* comparisons showed that MC1568 alone had a significant effect on cell viability, but did not significantly affect the 6-OHDA-induced decreases in cell viability (Fig. 2F).

Neurite length analysis of SH-SY5Y cells in the same experimental design showed significant effects of 6-OHDA ($F_{(1-8)} = 550.5$, $P < 0.0001$) and MC1568 ($F_{(1-8)} = 61.53$, $P < 0.0001$) on neurite growth, but no significant interaction ($F_{(1-8)} = 0.1069$, $P = 0.7521$) (Fig. 2G). *Post-hoc* testing showed that 6-OHDA-induced decreases in neurite growth were partially prevented by MC1568 treatment (Fig. 2G).

Finally, experiments using delayed treatment of primary cultures of E14 rat VM, in which MC1568 was administered 24 h after treatment with 5 μ M 6-OHDA, confirmed the significant effect of both treatments individually (6-OHDA; ($F_{(1-8)} = 481.8$, $P < 0.0001$) and (MC1568) ($F_{(1-8)} = 42.12$, $P = 0.0002$), with no interaction between them ($F_{(1-8)} = 1.469$, $P = 0.26010$) (Fig. 2H). *Post-hoc* testing revealed a partial protective effect of MC1568 on 6-OHDA-induced axonal degeneration (Fig. 2H, I). Collectively, these data show that MC1568 can partially

protect against 6-OHDA-induced neurodegeneration in cellular models.

3.4. Peripheral administration of MC1568 exerts partial protective effects on motor behaviour and nigrostriatal integrity in 6-OHDA-lesioned rats.

We next performed an *in vivo* experiment to determine whether i.p. administration of MC1568 could prevent against 6-OHDA-induced nigrostriatal neurodegeneration. For detailed description of the experimental design see the Methods section. In brief, adult female rats received unilateral intrastratial stereotactic injection 6-OHDA or saline, followed by 7 daily i.p. injections of saline or 0.5 mg/kg MC1568. Motor behavior was assessed using the stepping test (Olsson et al., 1995) on days 8 and 12 post-surgery (Fig. 3A). As expected, there was no significant effect of time ($F_{(2-14)} = 1.759$, $P = 0.2083$) or treatment ($F_{(3-21)} = 0.2925$, $P = 0.8303$) on the number of steps taken with the ipsilateral paw. However, there was a significant effect of time ($F_{(2-14)} = 62.61$, $P < 0.0001$) and treatment ($F_{(3-21)} = 5.389$, $P = 0.0066$), and a time $\times$ treatment interaction ($F_{(6-42)} = 5.102$, $P = 0.0005$), on the number of steps taken with the contralateral paw. *Post-hoc* testing revealed a

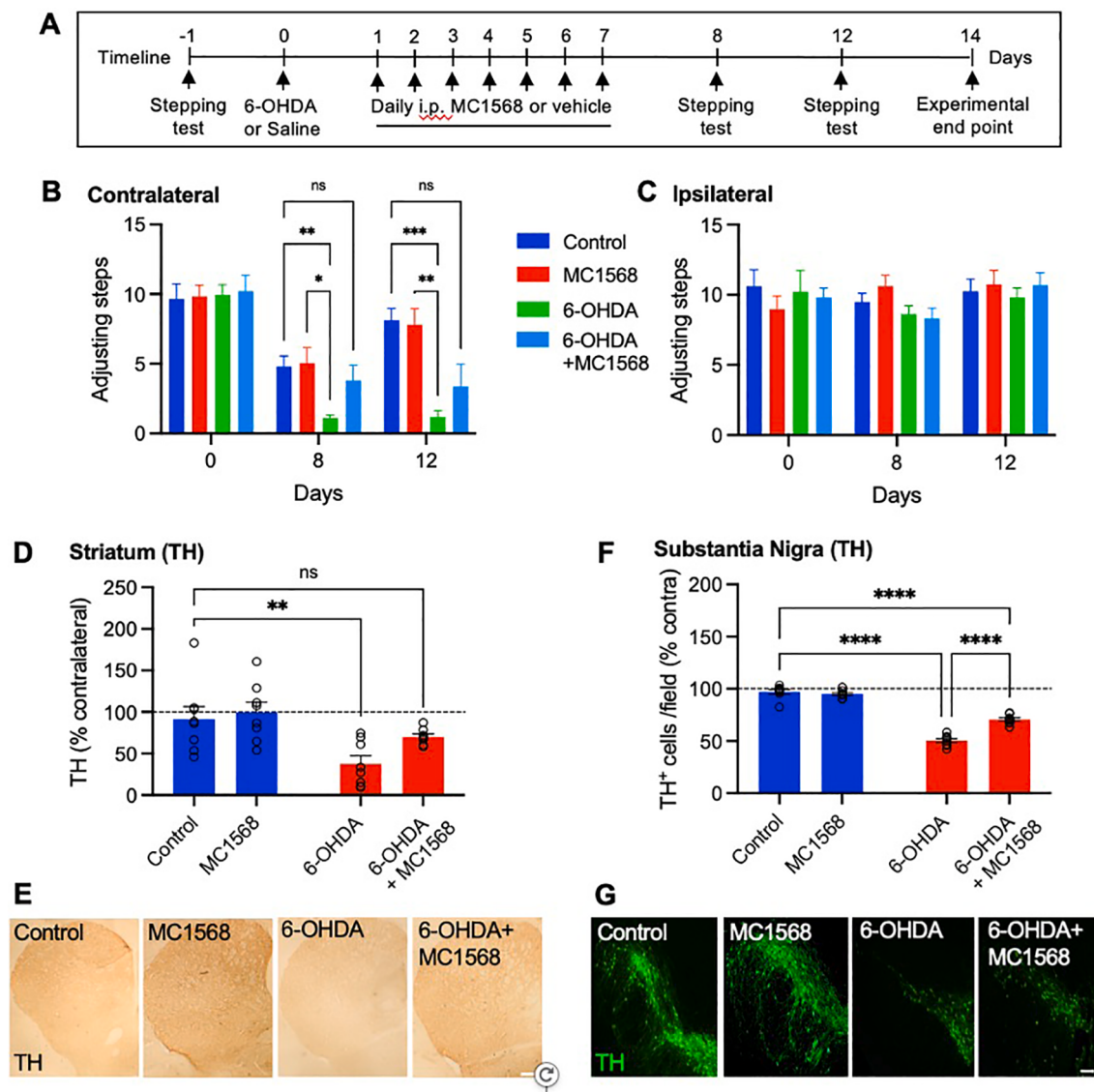


Fig. 3. Peripheral administration of MC1568 exerts partial protective effects on motor symptoms and nigrostriatal integrity in 6-OHDA-lesioned rats. (A) Schema showing the experimental design of the experiment used to test for neuroprotective efficacy of daily i.p. injections of 0.5 mg/kg MC1568 for one week, starting at 24 h after intrastriatal 6-OHDA lesion. (B, C) Graphs showing the numbers of adjusting steps on the Stepping test made by the (B) contralateral (affected) paw and (C) ipsilateral (unaffected) paw, at baseline (one day before surgery) and at 8 and 12 days post-surgery. (D) Quantification of TH immunoreactivity in the ipsilateral ST, expressed as percentage of the contralateral side. (E) Representative photomicrographs of TH-immunostained sections through the ipsilateral ST. (F) Quantification of TH immunoreactivity in the ipsilateral SN, expressed as percentage of the contralateral side. (G) Representative photomicrographs of TH-immunostained sections through the ipsilateral SN. All data are presented as the mean $\pm$ SEM of $n = 8$ per group. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ vs. control or as indicated; Two-way ANOVA with *post-hoc* Tukey's test. Scale bar = 100 μ m.

significant decrease in the number of steps taken with the contralateral paw at day 8 and day 12 post surgery in the 6-OHDA + vehicle group when compared to controls, which was not seen in the 6-OHDA + MC1568 group (Fig. 3B, C).

We examined nigrostriatal integrity by quantifying striatal dopaminergic innervation as measured by TH immunohistochemistry and densitometry (Fig. 3D, E). We found that 6-OHDA lesion had a significant effect on striatal dopaminergic innervation ($F_{(1-28)} = 14.20$, $P = 0.0008$), while MC1568 treatment did not ($F_{(1-28)} = 3.347$, $P = 0.0780$), and there was no significant 6-OHDA $\times$ MC1568 interaction ($F_{(1-28)} = 1.248$, $P = 0.2735$). *Post-hoc* comparisons showed a significant reduction in the 6-OHDA + vehicle group when compared to the control group, which was not seen in the 6-OHDA + MC1568 group, (Fig. 3D, E). In agreement, we found significant main effects of 6-OHDA ($F_{(1-28)} = 404.3$, $P < 0.0001$) and MC1568 ($F_{(1-28)} = 26.41$, $P < 0.0001$) on the numbers of TH-immunopositive neurons in the SN (Fig. 3F, G), with a

significant 6-OHDA $\times$ MC1568 interaction ($F_{(1-28)} = 40.08$, $P < 0.0001$). *Post-hoc* comparison revealed a significant reduction in the number of TH-positive neurons in the 6-OHDA + vehicle group when compared to the control group, that was not seen in the 6-OHDA + MC1568 group (Fig. 3F, G). Collectively, these data show that MC1568 partially protects against 6-OHDA-induced neurodegeneration.

3.5. Peripheral administration of MC1568 prevents microglial activation and decreases HDAC5 nuclear accumulation in DA neurons in 6-OHDA-lesioned rats.

We investigated whether MC1568 treatment modulated 6-OHDA-induced neuroinflammation by counting the numbers of IBA1 + microglia in the striatum and SN, as a measure on neuroinflammation (Thakur et al., 2017). Two-way ANOVA analysis revealed significant effects of 6-OHDA ($F_{(1-28)} = 15.19$, $P = 0.0006$) and MC1568 ($F_{(1-28)} =$

4.982, $P = 0.0338$) on the numbers of IBA1 + cells in the striatum, as well as a significant 6-OHDA $\times$ MC1568 interaction ($F_{(1-28)} = 4.912$, $P = 0.0350$) (Fig. 4A, B). *Post-hoc* comparison revealed a significant increase in the numbers of IBA1 + cells in the striatum in the 6-OHDA + vehicle group, which was prevented by MC1568 (Fig. 4A, B). Similar findings were seen in the SN, where there was a significant main effects of 6-OHDA ($F_{(1-28)} = 34.33$, $P < 0.0001$) and MC1568 ($F_{(1-28)} = 41.98$, $P < 0.0001$) on the number of IBA1 + cells in the SN, with a significant 6-OHDA $\times$ MC1568 interaction ($F_{(1-28)} = 18.71$, $P = 0.0002$) (Fig. 4C, D). Again *post-hoc* testing revealed a significant increase in the numbers of IBA1 + cells in the SN in the 6-OHDA + vehicle group, which was

prevented by MC1568. (Fig. 4C, D).

To investigate whether the increased number of IBA1 + cells may also reflect an increase in microglia activation, we examined the somal area of IBA1 + cells, which has been used as a proxy measurement of microglial activation (Davis et al., 2017). We found that compared to the control group, 6-OHDA led to a significant increase in IBA1 + somal area (47.0 ± 2.3 vs 77.13 ± 3.6 mm²; $p > 0.0001$), which was partially prevented by MC1568 treatment (77.13 ± 3.6 mm² vs 66.0 ± 3.0 mm²; $p > 0.05$).

Finally, we evaluated whether 6-OHDA resulted in an increase in nuclear accumulation of HDAC5 in DA neurons in the SN, and whether

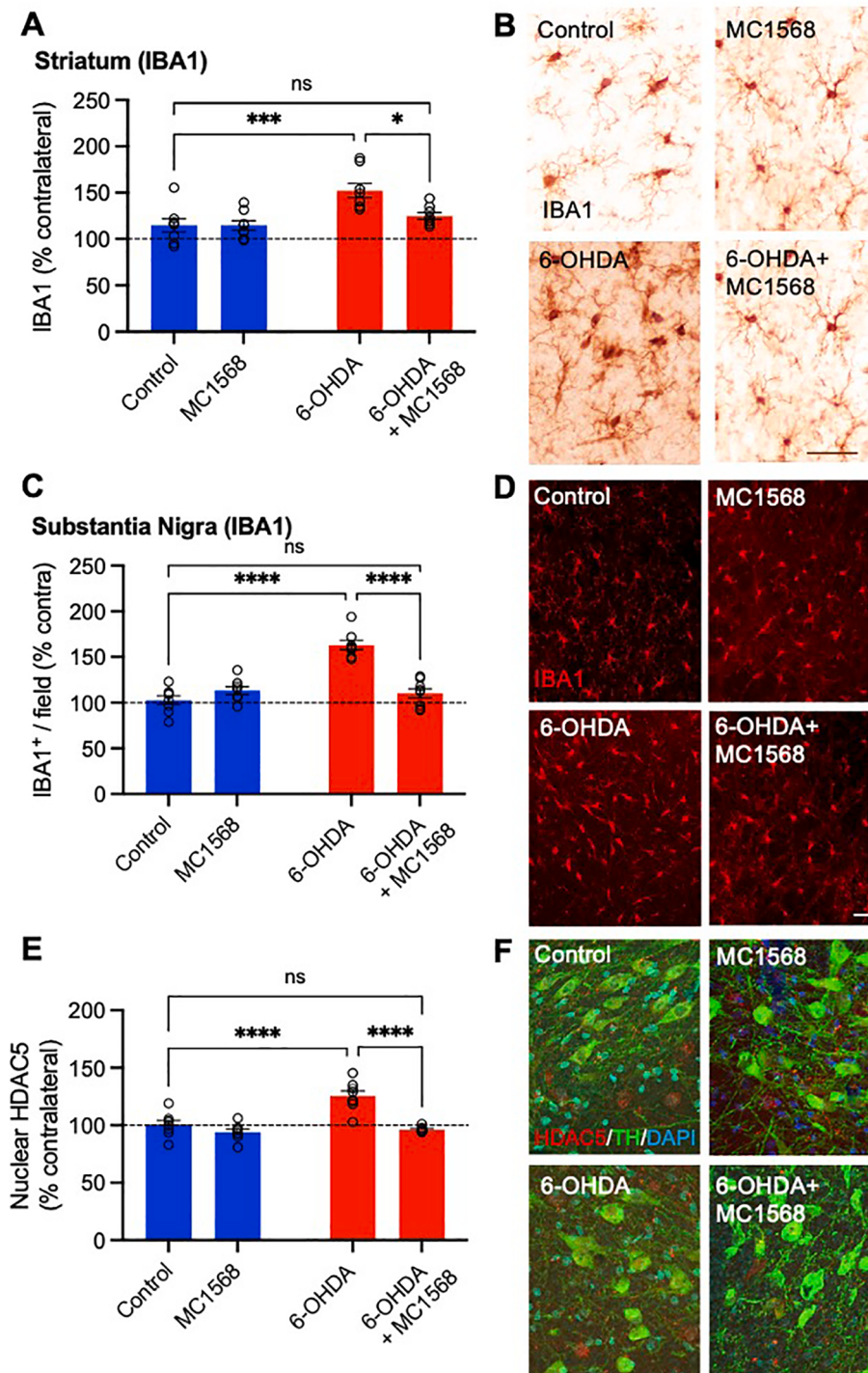


Fig. 4. Peripheral administration of MC1568 prevents microglial activation and HDAC5 nuclear accumulation in DA neurons in 6-OHDA-lesioned rats. (A) Graph and (B) representative photomicrographs showing the numbers of IBA1-immunopositive cells per field in the ST expressed as percentage of the contralateral side, in all treatment groups. (C) Graph and (D) representative photomicrographs showing the numbers of IBA1-immunopositive cells per field in the ipsilateral SN, expressed as percentage of the contralateral side in all treatment groups. (E) Graph and (F) representative photomicrographs showing the density of HDAC5 nuclear staining (red) in TH-immunopositive neurons (green) in the ipsilateral SN, expressed as percentage of the contralateral side, in all treatment groups. Sections were counterstained with DAPI. All data are presented as the mean $\pm$ SEM expression as a percentage of the control of $n = 8$ per group. **** $p < 0.0001$, *** $p < 0.001$, * $p < 0.05$ vs. control or as indicated. ns = not significant. Two-way ANOVA with *post-hoc* Tukey's test. Scale bar = 20 μ m.

this could be prevented by MC1568. Both 6-OHDA and MC1568 treatments resulted in significant changes in HDAC5 nuclear levels ($F_{(1-28)} = 18.27$, $P = 0.0002$ and $F_{(1-28)} = 31.70$, $P < 0.0001$, respectively), and there was a significant 6-OHDA $\times$ MC1568 interaction ($F_{(1-28)} = 12.94$, $P = 0.0012$). Post-hoc testing revealed a significant increase in nuclear HDAC5 levels in TH-positive neurons in the SN in the 6-OHDA + vehicle group, which was prevented by MC1568 treatment (Fig. 4E, F). These data show that 6-OHDA lesion induced nuclear accumulation of HDAC5 in DA neurons, and that this could be prevented by peripheral administration of MC1568.

4. Discussion

Although the role of HDACs in PD is being increasingly studied, the ways in which these proteins specifically contribute to PD pathogenesis are still largely unknown (Didonna and Opal, 2015). Despite this, HDIs are considered to have promise as disease-modifying therapies, since some *in vivo* studies have shown that pan-HDIs can exert neuroprotective effects in animal models of PD (Hou et al., 2021; Lai et al., 2019). However, since distinct HDACs subtypes are differentially expressed within specific cells and tissues, there is a need to investigate which class-specific HDIs have neuroprotective effects, particularly when administered peripherally.

In this study, we examined the effects of 6-OHDA treatment on neurite length, as a single cell readout of neurodegeneration, in cultured DA neurons. We also investigated the effects of 6-OHDA on HDAC5 shuttling between the cytoplasm and nucleus in these cells. We found that neurotoxic insult induced by 6-OHDA administration has a detrimental effect on neurite outgrowth in DA neurons, which is accompanied by nuclear accumulation of HDAC5. Since Class IIa HDACs nucleocytoplasmic shuttling is known to be controlled by the canonical Ca^{2+} /CaM/PKC pathway (McKinsey et al., 2001), we investigated whether HDAC5 nuclear accumulation could be altered by the activation of canonical Ca^{2+} /CaM/PKC pathway. Pre-treatment for the PKC activator, PMA, partially prevented 6-OHDA-induced nuclear accumulation increased neurite outgrowth in both cell types. A previous study reported HDAC4 nuclear accumulation following MPP⁺ treatment of cultured DA neurons overexpressing A53T α -synuclein (Wu et al., 2017). However, in contrast to our findings, that study also found that HDAC5 localisation *in vitro* in A53T cells was unchanged after MPP⁺ administration (Wu et al., 2017). In agreement with our results, another study demonstrated that following neuronal injury, HDAC5 is exported from the nucleus in a PKC-dependent manner in dorsal root ganglia (DRG) neurons, and that this is a fundamental step in triggering pro-regenerative gene expression in these cells (Cho et al., 2013). While there are some differences between these studies, collectively these data support the hypothesis that nuclear accumulation of Class IIa HDACs is associated with neurodegeneration.

Next we assessed the neuroprotective effect of the Class IIa HDI, MC1568, which we had previously showed to have beneficial effects in other cellular models of PD (Mazzocchi et al., 2019), in two *in vitro* PD models. We found that concurrent, but not delayed, treatment protected both SH-SY5Y cells and cultured DA neurons against 6-OHDA-induced decreases in cell viability. Furthermore, both concurrent and delayed MC1568 treatment partially protected against 6-OHDA-induced neurite degeneration in both cell types. The rationale for testing delayed treatment with MC1568 is that in clinical settings, pharmacological interventions are always applied after disease onset, in a context of ongoing neurodegeneration (Löhle et al., 2014). In a previous study, we demonstrated that when administered before the toxin, MC1568 can protect cultured DA neurons from axonal degeneration (Mazzocchi et al., 2019). We also previously reported neuroprotective effects of MC1568 on cultured sympathetic neurons, including preservation of neuronal branching (Collins et al., 2015), showing that MC1568 has effects on other neuronal populations which are affected in PD.

Following positive results in *in vitro* studies, we examined the effects

of peripheral administration of MC1568 in adult rats with intrastriatal 6-OHDA lesions. This is the first study, to our knowledge, to use MC1568 in an *in vivo* model of PD. Class IIa HDAC-specific inhibition has demonstrated neuroprotective effects in animal models of other brain disorders, but its therapeutic potential in PD models has not been previously examined. In a rat model of reperfusion ischemia, i.p. administration of TMP-269, a specific class-IIa HDI, at 30 min prior to the insult, exerted a neuroprotective effect by reducing infarct volume (Su et al., 2020). This indicates the potential neuroprotective benefits of Class IIa HDAC inhibition *in vivo*. MC1568 partially prevented behavioural impairments induced by 6-OHDA, as assessed using the stepping test. In support of our data, oral treatment with either 25 mg/kg or 50 mg/kg VPA for 15 days has been reported to significantly reduce apomorphine-induced rotational behaviour in rats with intrastriatal 6-OHDA lesions (Ximenes et al., 2015). Another study found that treatment with NaB (150 and 300 mg/kg i.p. for 14 days) significantly improved motor function in rats with 6-OHDA lesions of the medial forebrain bundle (MFB) (Sharma, Taliyan, & Singh, 2015). In that study, motor performance was assessed using several behavioural tests, including apomorphine-induced rotations, spontaneous locomotor activity, narrow beam test and rotarod test. In addition to motor effects, we found that MC1568 administration prevented 6-OHDA-induced loss of striatal DA innervation, and partially preserved DA neuronal cell bodies in the SN. These data demonstrate that MC1568 partially protects the nigrostriatal DA system from 6-OHDA-induced anatomical and functional degeneration. These findings are in agreement with several studies showing the neuroprotective potential of the pan-HDIs, VPA and NaB, in *in vivo* PD models (Lai et al., 2019; Sharma et al., 2015; Ximenes et al., 2015). For example, i.p. administration of 300 mg/kg NaB for 14 days protected against decreases in striatal dopamine induced by MFB 6-OHDA lesions (Yuan et al., 2005). Another study found that 6-OHDA-induced increases in pyknotic nuclei in the ST of adult rats were prevented by NaB treatment (Sharma et al., 2015). Another pan-HDI, VPA, prevented loss of striatal DA neurotransmission and protected against nigral DA neuronal cell, in rats with MFB 6-OHDA lesions (Lai et al., 2019). Collectively, these studies show that inhibition of Class IIa HDACs may, at least partially, underlie the neuroprotective effects of pan-HDI *in vivo* in models of PD.

Since HDAC inhibition has previously been demonstrated to have an anti-inflammatory effect in an *in vitro* model of PD (Harrison et al., 2018), we also investigated potential anti-inflammatory effects of MC1568 *in vivo*. We found that MC1568 treatment prevented 6-OHDA-induced increases in microglia, in the ST and SN, as well as preventing 6-OHDA-induced increases in nuclear levels of HDAC5 in DA neurons in the SN. A previous study showed that oral administration of VPA for 15 days reduced microglia inflammation in rats with 6-OHDA striatal lesions (Ximenes et al., 2015).

In conclusion, we have shown that both concurrent and delayed treatment with MC1568 can partially protect DA neurons from 6-OHDA-induced neurodegeneration *in vitro*. Further, we found that peripheral administration of the Class IIa HDI MC1568 exerts neuroprotection and behavioural improvements in an *in vivo* rat model of PD. Our data suggest that MC1568 may exert its effect by acting on DA neurons to limit nuclear accumulation of HDAC5, and also by preventing microglial activation. Elucidating the contributions of each of these mechanisms to the neuroprotective effects of MC1568 will be important for future research. The data rationalise the further study of Class IIa HDACs, and of pharmacological agents that target them, in developing new therapeutic approaches for neuroprotection in PD.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbi.2022.02.025>.

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