Differences in statin associated neuroprotection corresponds with either decreased production of IL-1 β or TNF- α in an *in vitro* model of neuroinflammation-induced neurodegeneration

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Abbreviations: BDNF (brain-derived neurotrophic factor); CNS (central nervous system); DAF-2 (4,5-diaminofluorescein); DAF-2 DA (4,5-diaminofluorescein diacetate); DCF (2',7'-dichlorofluorescein); DCF-DA (2',7'-dichlorofluorescein diacetate); DMEM (Dulbecco's Modified Eagle Medium); DMSO (dimethyl sulfoxide); ELISA (enzyme-linked immunosorbent assay); FBS (foetal bovine serum); HAPI (highly aggressively proliferating immortalized); HAPI-CM (conditioned media from HAPI cells); HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A); IL (interleukin); LPS (lipopolysaccharide); MDC

(monodansylcadaverine); PBS (phosphate buffered saline); PGE₂ (prostaglandin E₂); RA (retinoic acid); ROS (reactive oxygen species); TNF (tumour necrosis factor)

Abstract

3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins) have been associated with conflicting effects within the central nervous system (CNS), with underlying mechanisms remaining unclear. Although differences between individual statins' CNS effects have been reported clinically, few studies to date have compared multiple statins' neuroprotective effects. This study aimed to compare six statins (atorvastatin, fluvastatin, pitavastatin, pravastatin, rosuvastatin, simvastatin; 0-100 µM) using an *in vitro* model of lipopolysaccharide (LPS)-induced neuroinflammation and subsequent neurodegeneration. To achieve this, HAPI microglia were treated with LPS (0.1 µg/mL; 24 h), resulting in increased reactive oxygen species (ROS), nitric oxide, and IL-1β, TNF-α and PGE₂ release. Conditioned media ("HAPI-CM") was then transferred to SH-SY5Y neuroblastoma cells, and effects on cellular viability, mitochondrial membrane permeability, apoptosis, autophagy and ROS production assessed. Of the statins investigated, only atorvastatin, pravastatin and rosuvastatin protected SH-SY5Y cells from LPS-induced decreases in cellular viability; this appeared mediated through reduced caspase 3/7 activation and was associated with decreased IL-1β (atorvastatin, pravastatin) and/or TNF-α (atorvastatin, pravastatin, rosuvastatin). Only pravastatin conferred protection at all tested concentrations. ROS production and autophagic vacuole formation was decreased by all statins, suggesting these two mechanisms are unlikely to be sole mediators of neuroprotection seen with selected statins. Ultimately, our model suggests that despite all statins reducing microglial inflammation, subsequent effects on neuronal viability and cell death signalling pathways varies between statins. Our findings

50 highlight the need to consider individual statins as inducing discrete pharmacological effects
51 within the CNS in future *in vitro* / *in vivo* studies and in clinical practice.

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53 **Keywords:** HAPI, SH-SY5Y, statin, neuroinflammation, neuroprotection, neurotoxicity

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Introduction

HMG-CoA reductase inhibitors (“statins”) are indicated in the treatment of dyslipidaemia and reduction of cardiovascular risk, and are amongst the most highly prescribed drug classes worldwide [1, 2]. Generally, statins are well tolerated with few serious side effects with muscle pain, weakness, and cramps being the most commonly reported [3, 4]. However, in recent years, there has been increasing evidence from clinical studies which suggests that statins may also enter and have pharmacological effects within the central nervous system (CNS). For example, statins have been associated with effects on cognitive function, neurodegenerative disease, and neurological disorders such as stroke, depression and epilepsy [5]. Interestingly, however, the available evidence is conflicting: despite a number of observational studies suggesting a beneficial effect [6-9], there are also several case reports of statin-associated neurological detriment, including memory loss, and cognitive impairment [10-12]. Mechanistic studies which investigate the possible reasons underlying these discrepancies are lacking. Furthermore, our understanding of statins’ pharmacological mechanism(s) within the CNS is hindered by the common assumption that all statins will exert the same neurological effects, despite individual statins being pharmacologically diverse [5]. Interestingly, clinical studies which have stratified statin-associated neurological outcomes by individual compound have reported differences between agents, perhaps indicating a lack of homogeneity with respect to statins’ CNS effects [13-15].

To date, there are several elegantly designed *in vitro* and a limited number of *in vivo* studies which have proposed potential mechanisms through which statins may exert their neurological action [16-20]. Several groups, including ours, have shown that statins exert anti-inflammatory effects *in vitro* across primary, tumour-derived, immortalised and differentiated microglial models [16, 20-22]. To date, the majority of reported protective effects are associated with

reduced neuroinflammatory mediator expression and/or release, including known mediators of neuronal injury such as TNF- α , IL-1 β , and reactive oxidative and nitrosative species [5, 16, 20-22]. These findings are supported by *in vivo* studies which have similarly observed neuroprotective effects associated with statin-induced reduced neuroinflammation [23, 24]. Whilst the anti-neuroinflammatory effects of statins appear well-established, there are limited studies which investigate whether the statin-associated effects in glial models *in vitro* directly translate into neuronal protection [18].

Of the mechanistic studies available to date, a common methodological limitation is the use of only one or two statins to represent the class. Although the statin class has the same ultimate effect with respect to lipid-lowering, they are structurally diverse; this results in a number of pharmacological differences between statin compounds with regards to metabolism, drug interactions, and lipophilicity, amongst others [25, 26]. In clinical studies which have compared statin compounds, differences between statin-associated clinical effects have been observed, which may suggest that they possess differing pharmacological mechanisms within the CNS also [13-15]. Despite these clinical observations, only two studies to our knowledge have compared the effects of five or more clinically relevant statin compounds using neurological or neurological-like models *in vitro* [22, 27]. In a previous study by our group, atorvastatin, fluvastatin, pitavastatin, pravastatin, rosuvastatin and simvastatin decreased LPS-induced increases in inflammatory mediators (PGE₂, TNF- α and IL-1 β), oxidative stress, and nitrosative stress THP-1 monocytes which had been chemically differentiated to a microglial-like phenotype [22]. While this study provided some insight to effects of these compounds on a microglial-like cell line, there have been no studies to date which compare how the different statin compounds affect true microglial inflammation *in vitro*, or how this subsequently affects neuronal health.

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106 Hence, the aim of this study was to compare six clinically relevant, structurally and
107 pharmacologically diverse statin compounds, using an *in vitro* model of neuroinflammation-
108 induced neurodegeneration. To achieve this, we developed an *in vitro* model of
109 neuroinflammation-induced neuronal cell death using lipopolysaccharide (LPS)-conditioned
110 media from highly aggressively proliferating immortalized (HAPI) microglial cells, and
111 transferring this to both differentiated and undifferentiated SH-SY5Y neuroblastoma cells. The
112 present study compares the effects of multiple statin compounds on both a true microglial
113 model of neuroinflammation, and is the first reported use of statins in an *in vitro* model of
114 neuroinflammation-induced neurodegeneration. Unlike previous studies, we employed six
115 statins (namely atorvastatin, fluvastatin, pitavastatin, pravastatin, rosuvastatin and simvastatin)
116 to compare how each compound then influenced LPS-induced neuroinflammation in HAPI
117 microglial cells, as well as subsequent cell death signalling in SH-SY5Y neuroblastoma cells.
118 Furthermore, we investigate possible mechanisms which underlie the observed effects to
119 determine whether these were a class-wide phenomenon, or differ between the various statins.

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Materials and Methods

Cell culture

HAPI rat microglial cells were obtained from EMD Millipore Corporation (Temecula, CA, USA). Cells were cultured to 80% confluence and maintained at 37°C in 5% CO₂ in complete Dulbecco's Modified Eagle Medium (DMEM; Gibco, Victoria, Australia) containing 1 mg/mL glucose, sodium pyruvate, L-glutamine, and Phenol Red. Medium was additionally supplemented with 10% foetal bovine serum (FBS; Bovogen, Australia) and 0.1 mg/mL gentamicin (Gibco, Victoria, Australia). SH-SY5Y human neuroblastoma cells were obtained from Sigma-Aldrich (St Louis, MO, USA). Cells were cultured to 80% confluence and maintained at 37°C with 5% CO₂ in complete DMEM (Gibco, Victoria, Australia) containing 5 mg/mL glucose, sodium pyruvate, L-glutamine, and Phenol Red. Medium was additionally supplemented with 10% FBS (Bovogen, Australia) and 0.1 mg/mL gentamicin (Gibco, Victoria, Australia). To induce differentiation in SH-SY5Y cells, cells were seed at 1.0×10^4 cells/mL. Starting at 24 h post-seeding, cells received sequential treatment with 5 days 10 μ M retinoic acid (RA; abcam, Victoria, Australia) followed by 3 days of 50 μ g/mL brain-derived neurotrophic factor (BDNF; abcam, Victoria, Australia) was used as previously described [28-30]. RA-containing media was replaced every 48 h, BDNF-containing media was replaced every 24 h, and supplementation continued for the duration of each end-point analysis.

RNA extraction

Following either the differentiation process (differentiated samples), or 72 h post-seeding (undifferentiated samples), cells were harvested with TRIzol™ (Gibco Invitrogen, Victoria, Australia) and RNA was extracted according to the manufacturer's instructions. Concentration and purity of RNA samples were determined using a NanoDrop Spectrophotometer (Thermo

Fisher Scientific, Victoria, Australia) and showed minimal degradation. All samples were stored at -80°C until used for reverse transcription.

Reverse transcription

Total RNA was reverse transcribed using iScript™ Reverse Transcription Supermix for real-time polymerase chain reaction (Bio-Rad Laboratories Pty Ltd, NSW, Australia). Each reaction consisted of 1 µL of template RNA, 0.5X of 5x iScript™ Reverse Transcription Supermix, in a final volume of 20 µL. Samples were incubated at 25 °C for 5 min, 42 °C for 60 min and then deactivated at 85 °C for 5 min. The cDNA was stored at -20 °C until required.

Real time polymerase chain reaction

Real-time polymerase chain reaction (rt-PCR) was used to measure the expression of three known neuronal genes, synaptophysin (*SYP*), neuron specific enolase 2 (*ENO2*) and dynamin-1 (*DYNI*). Primers were obtained from Sigma Genosys; primer sequences are listed in Table 1. Master mixes were made using iTaq™ Universal SYBR® Green (Bio-Rad Laboratories, NSW, Australia) as the detection system. Concentrations of each primer were used according to previous optimisation [31]. All PCRs consisted of 1 µL cDNA, 1 µL forward primer (3 µM), 1 µL reverse primer (3 µM), 5 µL SYBR® Green (Bio-Rad Laboratories, NSW, Australia), and 12 µL diethyl pyrocarbonate-treated water for a final volume of 20 µL. The Qiagen Rotor-Gene Q System was used to perform real-time PCR with cycling parameters of denaturation at 95°C for 30 s, then 40 cycles of 95 °C for 5 s, 60 °C for 30 s. Tubes containing mastermix but no template were included for each primer set as negative controls. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal reference for determining ΔC_T values. All samples were amplified in duplicate. To determine the cycle threshold (C_T), the threshold value was set at 0.03 in each reaction. The cycle threshold was

calculated as the cycle number of an amplifying PCR product where it crosses the fixed threshold line. C_T values for each sample were normalized to *GAPDH* and expressed as a ΔC_T value. To generate a fold-change comparison, ΔC_T values for undifferentiated SH-SY5Y cell samples were subtracted from ΔC_T values for differentiated cell samples, generating a $\Delta\Delta C_T$ value. For graphing purposes, fold change values were calculated by converting $\Delta\Delta C_T$ values to $2^{-\Delta\Delta C_T}$ values [32].

In vitro model of neuroinflammation using LPS-conditioned HAPI media

To establish an *in vitro* model of neuroinflammation, LPS-conditioned media was generated from HAPI microglial cells, and used to induce a pro-inflammatory response in differentiated and undifferentiated SH-SY5Y cells. LPS (*Escherichia coli* serotype 055:B5) is a potent immune stimulant, and has been widely used in models of microglial activation and neuroinflammation *in vitro* and *in vivo* [22, 33-36].

To generate LPS-conditioned HAPI media (HAPI-CM), HAPI cells were seeded at 3.0×10^5 cells/mL in 24 microwell plates. At 23 h post-seeding, HAPI cells were pre-treated with 0-100 μ M statin (atorvastatin, fluvastatin, pitavastatin, pravastatin, rosuvastatin or simvastatin; Cayman Chemical Company, MI, USA) or control (culture medium, dimethyl sulfoxide, prednisone; Cayman Chemical Company, MI, USA) and incubated for 1 h at 37°C in 5% CO₂. Each compound (statins and prednisone) was prepared from a DMSO stock through addition of phosphate buffered saline (PBS), with a maximum final concentration of 0.5% (v/v) DMSO in wells. Appropriate DMSO control wells were included for all experiments.

Following incubation, LPS (final concentration 0.1 μ g/mL; Sigma, St. Louis MI, USA) or vehicle were added to statin-containing medium and incubated at 37°C in 5% CO₂ for 24 h.

After this time, cell-free supernatant was collected and either immediately used or stored at -80°C. Maximum storage of conditioned media did not exceed 14 days.

To generate the *in vitro* model of neuroinflammation-induced neurodegeneration, SH-SY5Y cells were seeded at 1.0×10^5 cells /mL in a 96 microwell plate in undifferentiated model, and seeded and treated as previously described for the differentiated model. At 24 h post-seeding or differentiation period, the medium above cells was replaced with HAPI-CM (previously described). SH-SY5Y cells were then incubated for 24-48 h. After incubation, end-point analysis was conducted.

Resazurin (Alamar Blue) cell viability assay

Resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt) is a non-fluorescent compound which is reduced to resorufin, a fluorescent dye, in the presence of metabolically active cells, and thus can be used as a measure of cellular proliferation / viability [37]. Briefly, SH-SY5Y cells and HAPI cells were seeded and treated as described above. At 24 h post-seeding or completion of differentiation/treatment process (previously described), media above cells was replaced with complete media containing 44 µM resazurin (Sigma, St. Louis MI, USA) and plates incubated at 37°C, 5% CO₂ protected from light for 3 h. After this incubation, reduction of resazurin to resorufin was quantified using a Tecan Infinite 200 Pro microplate reader (excitation: 530 nm, emission: 590 nm) (Tecan, Victoria, Australia). Appropriate cell-free controls were included. Results from each tested condition were validated using a Countess® automated cell counter (Invitrogen, Victoria, Australia).

4,5-diaminofluorescein diacetate fluorimetry

4,5-diaminofluorescein diacetate (DAF-2 DA) is a cell permeable nitric oxide probe which is hydrolysed to fluorescent 4,5-diaminofluorescein (DAF-2) in the presence of nitric oxide [38]. HAPI cells were seeded and treated as described above. At 24 h post-treatment, media was replaced with serum-free media containing 10 μ M DAF-2 DA (Cayman Chemical Company, Ann Arbor MI, USA) and incubated in the dark for 45 min at 37°C in 5% CO₂. Following incubation, cells were washed twice with PBS and fluorescence (excitation: 485 nm, emission: 538 nm) measured using a Tecan Infinite 200 Pro microplate reader (Tecan, Victoria, Australia).

2',7'-dichlorofluorescein diacetate fluorimetry

Global reactive oxygen species (ROS) production was determined through the quantification of 2',7'-dichlorofluorescein (DCF) fluorescence as previously described [39]. In the presence of ROS, the non-fluorescent 2',7'-dichlorofluorescein diacetate (DCF-DA) is converted to highly fluorescent DCF. SH-SY5Y cells and HAPI cells were seeded and treated as described above. At 24 h post-differentiation or treatment process, media above cells was replaced with serum-free media containing 10 μ mol/L DCF-DA (Cayman Chemical Company, Ann Arbor MI, USA) for 30 min. Cells were then washed twice using PBS and fluorescence (excitation: 485 nm, emission: 535 nm) measured using a Tecan Infinite 200 Pro microplate reader (Tecan, Victoria, Australia).

IL-1 β , TNF- α and PGE₂ release

To evaluate the release of pro-inflammatory mediators following LPS stimulation of HAPI cells, TNF- α , IL-1 β , and prostaglandin E₂ (PGE₂) were quantified using enzyme-linked immunosorbent assay (ELISA). HAPI or SH-SY5Y cells were seeded and treated as described above. For both cell lines, PGE₂ was quantified using a PGE₂ Express ELISA kit (Cayman

Chemical Company, MI, USA). In HAPI cells, TNF- α was quantified using a TNF- α (rat) ELISA Kit (Aviva Systems Biology, CA, USA), and IL-1 β was quantified using an IL-1 β (rat) ELISA Kit (Aviva Systems Biology, CA, USA) as previously described [40, 41]. In SH-SY5Y cells, TNF- α was quantified using a TNF- α (human) ELISA Kit (Cayman Chemical Company, MI, USA), and IL-1 β quantified using an IL-1 β (human) ELISA Kit (Cayman Chemical Company, MI, USA) as previously described [42]. All steps were performed as per the respective manufacturer's instructions. Absorbance for each assay was read at using a Tecan Infinite 200 Pro microplate reader (Tecan, Victoria, Australia).

JC-1 mitochondrial membrane potential detection assay

Mitochondrial membrane potential, an early apoptotic event, was monitored using the cationic probe JC-1 as previously described [43]. At 24 h post-seeding, the medium above SH-SY5Y cells was replaced with HAPI-CM. Following incubation for a further 24 h, medium above the cells was removed and cells were washed once with PBS (37 °C), then fresh medium containing JC-1 dye (1 μ g/ml) added to cells. Cells were then incubated for 30 min, and washed twice using PBS under limited lighting. Fluorescence intensity was read at red (excitation: 530 nm, emission: 590 nm) and green (excitation 485 nm, emission 535 nm) spectra using a Tecan Infinite 200 Pro microplate reader (Tecan, Victoria, Australia).

Caspase 3/7 fluorescence

Caspase-3/7 activation was used as an index of apoptosis. SH-SY5Y cells were seeded and treated with HAPI-CM as described above. Following incubation for a further 24 h, caspase-3/7 activity was determined using a caspase-3 fluorescence assay kit (Cayman Chemical Company, MI, USA) as previously described [40]. All steps were performed as per the

267 manufacturer's instructions. Fluorescence (excitation: 485 nm, emission: 535 nm) was read at
268 using a Tecan Infinite 200 Pro microplate reader (Tecan, Victoria, Australia).

270 *Monodansylcadaverine fluorescence*

271 Monodansylcadaverine (MDC) is an autofluorescent compound which concentrates in acidic
272 cellular compartments, and was used as previously described to as an indicator of autophagic
273 vacuole formation [44-46]. SH-SY5Y cells were seeded and treated with HAPI-CM as
274 described above. Following 24 h incubation, plates were centrifuged at 400 x g for 5 min.
275 Supernate was removed, and 50 µM MDC solution added to each well except unstained control.
276 Plates were incubated for 15 min at 37°C, and then centrifuged at 400 x g for 5 min. Supernate
277 was removed, cells were washed twice with PBS, and fluorescence intensity measured
278 (excitation: 335 nm, emission: 515 nm) using a Tecan Infinite 200 Pro microplate reader
279 (Tecan, Victoria, Australia).

281 *Statistical analyses*

282 All data were analyzed using one-way analysis of variance (ANOVA) with the Tukey-Kramer
283 multiple comparisons test, using Graphpad InStat software v3.06 (San Diego, California).
284 Significance levels were defined as p<0.05 (*,#), p<0.01 (**,##), and p<0.001 (***,###). All
285 graphs were drawn using Graphpad Prism v6.01 (San Diego, California).

Results

Statin pre-treatment decreases LPS-induced inflammatory response and oxidative stress in HAPI cells

The use of LPS as a model of induced neuroinflammation *in vitro* is well-established [22, 47-49]. To determine the effect of LPS on HAPI cells, a panel of pro-inflammatory mediators associated with the neuroinflammatory response were quantified following LPS stimulation. The treatment of HAPI cells with 0.1 µg/mL LPS did not have any significant effect on cellular viability, as determined by the reduction of resazurin to resorufin (Supplement 1). Similarly, pre-treatment of HAPI cells with atorvastatin, fluvastatin, pitavastatin, pravastatin and rosuvastatin (1-100 µM; Supplement 1A-1E) prior to LPS treatment did not have any significant effect on cellular viability, nor did dimethyl sulfoxide (DMSO) control (data not shown). In contrast, pre-treatment with 100 µM simvastatin induced a significant decrease in cellular viability when compared to both untreated HAPI cell control ($p<0.001$) and compared to LPS only treatment ($p<0.001$; Supplement 1F).

To determine the effect of LPS on global ROS production, DCF fluorescence was used (Figure 1). Treatment of HAPI cells with LPS significantly increased DCF fluorescence ($p<0.01$). Pre-treatment with 10 µM prednisone prior to LPS resulted in a significant decrease in DCF fluorescence compared to LPS alone ($p<0.001$); this was also significantly below baseline DCF readings ($p<0.01$). Pre-treatment of HAPI cells with atorvastatin, fluvastatin, pitavastatin, pravastatin and rosuvastatin (1-100 µM; Figures 1A-1E) reduced DCF fluorescence compared to LPS treatment alone ($p<0.001$). Interestingly, atorvastatin 100 µM (Figure 1A), fluvastatin 10 and 100 µM (Figure 1B), pitavastatin 10 and 100 µM (Figure 1C), and simvastatin 10 µM (Figure 1F) decreased DCF fluorescence to a significantly greater extent than was achieved through using prednisone 10 µM ($p<0.05$).

DAF fluorescence was used as an indicator of nitric oxide production (Figure 2). Similar to DCF fluorescence, treatment of HAPI cells with LPS resulted in a significant increase in DAF fluorescence ($p<0.001$), which was significantly decreased by prednisone ($p<0.01$). All tested concentrations of atorvastatin, pitavastatin, and pravastatin significantly decreased LPS-induced DAF fluorescence ($p<0.001$; Figures 2A, 2C and 2D respectively). Fluvastatin (Figure 2B) only decreased DAF fluorescence at 100 μ M ($p<0.05$), whilst rosuvastatin only decreased DAF fluorescence at 1 and 10 μ M ($p<0.001$). In contrast to all other statins, all three tested concentrations of simvastatin resulted in significant augmentation of LPS-induced DAF fluorescence ($p<0.05$; Figure 2F).

To determine the effect of LPS on the release of pro-inflammatory mediators in the HAPI-CM model, PGE₂, TNF- α and IL-1 β was quantified in the absence and presence of statin pre-treatment. Following LPS treatment, PGE₂ levels significantly increased compared to untreated control (Figure 3; $p<0.001$). This was significantly decreased in the presence of 10 μ M prednisone (Figure 3; $p<0.001$). All tested concentrations of pitavastatin (Figure 3C; $p<0.0001$), and rosuvastatin (Figure 3E; $p<0.05$) significantly decreased PGE₂ release compared to LPS; while fluvastatin (Figure 3B; $p<0.001$) and pravastatin (Figure 3D; $p<0.001$) both decreased PGE₂ at 10-100 μ M. In contrast, atorvastatin pre-treatment did not significantly affect PGE₂ release at any tested concentration compared to LPS only (Figure 3A), and simvastatin (1 μ M and 100 μ M) significantly increased PGE₂ levels (Figure 3F).

LPS treatment significantly increased TNF- α release compared to untreated control ($p<0.001$; Figure 4). Prednisone 10 μ M significantly decreased LPS-induced TNF- α release ($p<0.001$; Figure 4). Pre-treatment with 1-100 μ M atorvastatin (Figure 4A), pitavastatin (Figure 4C), pravastatin (Figure 4D), and rosuvastatin (Figure 4E) significantly decreased LPS-induced

TNF- α release ($p < 0.05$; Figure 4). Although 1 μ M fluvastatin significantly decreased LPS-induced TNF- α release ($p < 0.001$; Figure 4B), 10-100 μ M fluvastatin had no significant effect. In contrast, while 10 μ M and 100 μ M simvastatin significantly decreased TNF- α release ($p < 0.001$), 1 μ M simvastatin had no significant effect (Figure 4F).

IL-1 β was significantly elevated in HAPI-CM following LPS treatment compared to untreated control ($p < 0.05$; Figure 5). This increase was significantly decreased by 10 μ M prednisone pre-treatment ($p < 0.05$). Of all six statins tested, only 100 μ M atorvastatin ($p < 0.05$; Figure 5A) and 1-100 μ M pravastatin ($p < 0.05$; Figure 5D) significantly reduced LPS-induced IL-1 β release. Similarly to results found for PGE₂ and TNF- α , 100 μ M simvastatin significantly attenuated LPS-induced IL-1 β release (Figure 5F). Pre-treatment with DMSO control showed no significant effects on LPS-induced IL-1 β release (data not shown).

Conditioned media from LPS-treated HAPI cells induces cell death in undifferentiated SH-SY5Y cells after 24 h which is protected against by atorvastatin, pravastatin, and rosuvastatin, but attenuated by simvastatin.

To determine the optimal conditions for the present *in vitro* model of neuroinflammation-induced neurodegeneration, HAPI-CM was transferred onto both undifferentiated SH-SY5Y cells, as well as SH-SY5Y cells which had been sequentially differentiated using RA and BDNF. Following the differentiation process, SH-SY5Y cells were found to exhibit neurite projections and had increased genetic expression of *DNM1*, *ENO2* and *SYP* (Supplementary Figure 2).

As shown in Figure 6A, the presence of LPS-only HAPI-CM on undifferentiated SH-SY5Y cells was found to significantly decrease cellular viability to $77.3 \pm 4.1\%$ of control after 24 h

($p < 0.001$), as determined through resazurin reduction. In contrast, HAPI-CM did not affect viability in differentiated SH-SY5Y cells after 24 h (Figure 6B). Increased exposure (48 h) further decreased cellular viability in undifferentiated SH-SY5Y cells to $60.0 \pm 8.8\%$ of control ($p < 0.001$) but consistent with 24 h exposure had no effect on differentiated SH-SY5Y cells (Supplementary Figure 3). The undifferentiated model of neuroinflammation-induced neurodegeneration was chosen for use in all subsequent experiments.

To determine whether statin pre-treated HAPI-CM could increase cellular viability in the undifferentiated SH-SY5Y model, resazurin reduction was again used. HAPI-CM pre-treated with 100 μM atorvastatin was found to significantly increase cellular viability in undifferentiated SH-SY5Y cells compared to LPS-only HAPI-CM ($p < 0.01$; Figure 6C). Similarly, pre-treatment with pravastatin 1-100 μM ($p < 0.05$; Figure 6F), and rosuvastatin 100 μM ($p < 0.01$; Figure 6G) attenuated LPS-induced reductions in cellular viability. In contrast, attenuation of LPS-induced decreases in cellular viability was observed following treatment with HAPI-CM pre-treated with 10 and 100 μM simvastatin ($p < 0.001$; Figure 6H). Neither fluvastatin nor pitavastatin showed any significant change in cellular viability compared to LPS alone in undifferentiated SH-SY5Y cells (Figure 6D and 6E respectively). A summary of each compounds effects on the HAPI-CM, and subsequent SH-SY5Y cellular viability can be found in Table 2.

Atorvastatin, pravastatin and rosuvastatin improve mitochondrial membrane potential and decreases caspase 3/7 activation in undifferentiated SH-SY5Y cells treated with HAPI-CM

JC-1 fluorescence was measured as an index of mitochondrial membrane potential (Figure 7). LPS only HAPI-CM was observed to decrease JC-1 fluorescence ($p < 0.001$; Figure 7). HAPI-CM pre-treated with atorvastatin 10 μM and 100 μM was found to significantly increase JC-1

fluorescence ($p<0.05$; Figure 7A), however HAPI-CM pre-treated with fluvastatin and pitavastatin had no significant effects on JC-1 fluorescence compared to LPS only HAPI-CM (Figure 7B-C). HAPI-CM pre-treated with 10 μ M and 100 μ M pravastatin was associated with significantly increased JC-1 fluorescence compared to LPS only HAPI-CM ($p<0.001$; Figure 7D). Similar to atorvastatin and pravastatin, HAPI-CM pre-treated with 10 and 100 μ M rosuvastatin significantly improved LPS-induced decreases in JC-1 fluorescence ($p<0.001$; Figure 7E), however simvastatin pre-treated HAPI-CM was found to have no significant effect on JC-1 activity (Figure 7F).

To determine the involvement of early apoptosis in HAPI-CM associated changes in SH-SY5Y cellular viability, caspase 3/7 activity (Figure 8) was quantified. LPS only HAPI-CM was found to significantly increase caspase 3/7 fluorescence in SH-SY5Y cells ($p<0.001$; Figure 8). HAPI-CM pre-treated with 100 μ M atorvastatin was associated with a significant decrease in caspase 3/7 activity compared to both LPS-only HAPI-CM, and HAPI-CM pre-treated with prednisone ($p<0.001$; Figure 8A). In contrast, HAPI-CM pre-treated with 1 μ M and 10 μ M atorvastatin was associated with a significant increase in caspase 3/7 activity ($p<0.001$; Figure 8A). Similarly, HAPI-CM pre-treated with fluvastatin (1 μ M, 10 μ M) was associated with a significant increase in caspase 3/7 activity ($p<0.001$; Figure 8B), although pitavastatin had no significant effects on caspase 3/7 activity (Figure 8C). In contrast, all concentrations of pravastatin were found to significantly reduce LPS-induced increases in caspase 3/7 activity ($p<0.001$; Figure 8D). These decreases were also significantly lower than those induced by the anti-inflammatory agent prednisone ($p<0.01$; Figure 8D). Rosuvastatin pre-treatment was also associated with a significant decrease in caspase 3/7 activity compared to LPS-only HAPI-CM ($p<0.001$; Figure 8E), with 100 μ M rosuvastatin decreasing caspase 3/7 activity to a significantly lower extent than was induced by HAPI-CM pre-treated with prednisone

($p < 0.001$; Figure 8E). In contrast, however, 1 μM rosuvastatin was found to significantly attenuate LPS-induced increases in caspase 3/7 activity ($p < 0.001$; Figure 8E). Furthermore, HAPI-CM pre-treated with 10 μM and 100 μM simvastatin was found significantly increase caspase 3/7 activity compared to LPS-only supernatant ($p < 0.001$; Figure 8F).

The attenuation of ROS production by all statin-treated HAPI supernatant does not correlate with protection against SH-SY5Y cell death

DCF fluorescence was again used to evaluate the effect of HAPI-CM $\pm$ statin pre-treatment on undifferentiated SH-SY5Y cells (Figure 9). Treatment of SH-SY5Y cells with HAPI media conditioned with LPS-only induced a significant increase in DCF fluorescence ($p < 0.001$; Figure 9). This increase was significantly attenuated by HAPI-CM pre-treated with prednisone ($p < 0.001$). All statin conditions which were previously associated with protection against LPS-induced decreases in cellular viability in undifferentiated SH-SY5Y cells, namely atorvastatin 100 μM , pravastatin 1-100 μM , and rosuvastatin 100 μM (Figure 8), were similarly observed to protect against LPS-induced decreases in DCF fluorescence (Figure 9). All statins were associated with a significant decrease in DCF fluorescence for at least one tested concentration (Figure 9A-F). Interestingly, some statin pre-treatment conditions which were not associated with changes to HAPI-CM cellular viability were also noted to significantly decrease DCF fluorescence (atorvastatin 10 μM , fluvastatin 1 μM and 100 μM , pitavastatin 1-100 μM , rosuvastatin 10 μM , simvastatin 1 μM ; Figure 9). Simvastatin (100 μM) was observed to significantly augment LPS-induced increases in DCF fluorescence ($p < 0.001$; Figure 9F), which correlated with decreased cellular viability as previously described (Figure 6F).

The reduction of MDC fluorescence is not associated with protection against SH-SY5Y cell death

The use of MDC as a probe for the detection of autophagic vacuoles *in vitro* is well established [50, 51], hence MDC fluorescence was used to evaluate the potential role of autophagy in observed changes in SH-SY5Y cellular viability following HAPI-CM treatment. Following treatment with LPS-only HAPI-CM, a significant increase in MDC fluorescence in SH-SY5Y cells was observed ($p < 0.001$; Figure 10). Prednisone pre-treatment significantly decreased LPS-induced MDC fluorescence in SH-SY5Y cells ($p < 0.05$). HAPI-CM pre-treated with atorvastatin (1-100 μM ; Figure 10A), fluvastatin (1-100 μM ; Figure 10B), pitavastatin (1-100 μM ; Figure 10C), and rosuvastatin (1-100 μM ; Figure 10E) significantly decreased MDC fluorescence compared to LPS-only HAPI-CM ($p < 0.001$). Despite pravastatin pre-treatment significantly protecting against LPS-induced decreases in cellular viability at all tested concentrations (Figure 6D), only 100 μM pravastatin was found to significantly decrease MDC fluorescence ($p < 0.001$; Figure 10D). Simvastatin did not have any significant effect on MDC fluorescence at any tested concentration (Figure 10F).

Discussion

A number of clinical and epidemiological studies have suggested that the statin class may have neuroprotective properties, however conflicting evidence has also been reported which reports either a null or detrimental effect. Consequently, the true neurological effects of these compounds to date has remained unclear. The assumption of many clinical studies and *in vitro* / *in vivo* experiments to date that statins' CNS effects are a class mechanism, despite clinical reports of differences in effect between compounds, has also meant that few studies have compared the various compounds within the statin class [6, 13-15, 52]. Given that individual statins are both structurally and pharmacologically diverse, it is possible that the discrepancies between clinical studies may be due to individual compounds having different clinical effects in the CNS. To our knowledge, however, there are no studies to date which have compared multiple statins in a single *in vitro* model of neurodegeneration.

Here, we report for the first time that despite all statins significantly reducing LPS-induced release of inflammatory mediators in HAPI microglia, this did not translate to neuroprotection for all compounds when HAPI-CM was transferred to undifferentiated SH-SY5Y cells. Statins' abilities to reduce LPS-induced inflammation have been well-established in microglial and microglial-like models [20, 22, 53]. Indeed, in a previous study by our group, we identified that all six statins used in the present study were also able to decrease LPS-induced inflammation in THP-1 cells which had been chemically differentiated to a microglial-like phenotype [22]. Interestingly, while our previous study found all tested statins capable of decreasing pro-inflammatory mediators to a similar extent, the use of rat microglia in the present study demonstrated subtle differences in the ability of different statins to reduce LPS-induced inflammation and oxidative / nitrosative stress [22]. For instance, while LPS-induced TNF- α release was previously consistently suppressed by treatment with all six statins in

differentiated THP-1 cells [22], only atorvastatin, pitavastatin, pravastatin and rosuvastatin suppressed TNF- α release from HAPI cells at all tested concentrations in the present study. Furthermore, although all statins previously reduced LPS-induced IL-1 β production to some extent in differentiated THP-1 cells [22], only high-dose atorvastatin and all tested concentrations of pravastatin were able to attenuate LPS-induced IL-1 β production in HAPI cells in the present study. These findings may suggest that the use of HAPI microglia, as opposed to the 'microglial-like' THP-1 cells, enable the study of statins' differing microglial effects to be modelled more accurately.

As the ability of statins to reduce microglial inflammation in the present study did not always translate into the protection of SH-SY5Y cells against toxic insult, this highlights the importance of multicellular models when considering neuroinflammatory effects *in vitro*. Microglia are considered central orchestrators of the neuroinflammatory response, therefore interactions between neurons and microglia are central to the proper development and homeostatic maintenance of the CNS [54-56]. Therefore, the assessment of both microglial effects and subsequent neuronal effects induced by statin pre-treatment in our model is an advantage compared to previous studies which have looked at either cell type in isolation [16, 19-22, 57].

When examining the inflammatory profile changes induced by each statin individually, it can be seen that atorvastatin and pravastatin were the only compounds to significantly decrease LPS-induced IL-1 β release. This was associated with protection against LPS-induced reductions in cellular viability and significant reductions in caspase 3/7 activity. This could suggest that the ability of both atorvastatin and pravastatin to reduce IL-1 β and subsequent apoptotic activity could be a mechanism through which they confer protection to SH-SY5Y

cells. IL-1 β is well established as a key mediator of neuronal injury in both acute and chronic neurodegenerative conditions, including stroke, brain trauma, Alzheimer's disease and Parkinson's disease [58]. Furthermore, IL-1 β has been found to mediate increases in neuronal cell death induced by LPS in a rodent model of Parkinson's disease [59], as well as induce neuronal death and apoptosis in primary rat and human neuronal cultures [60].

It is interesting to note, however that while the highest dose of rosuvastatin pre-treatment in the present model was associated with no significant decrease in IL-1 β , it was still found to be protective against SH-SY5Y cell death. This may suggest that rosuvastatin acts through mechanisms not relating to IL-1 to protect against SH-SY5Y toxicity. All three protective statins, including rosuvastatin, were also associated with significant reductions in LPS-induced TNF- α production, which may also suggest a role for TNF- α in mediating SH-SY5Y toxicity in this model. Unlike systemic pro-inflammatory responses whereby TNF- α production induces IL-1 β release (and vice versa), it has been found that this mutual activation does not necessarily occur within the neuroparenchyma [61-63]. Consequently, differing levels of each respective cytokine in the present model may represent alternate mechanisms of anti-inflammatory activity by rosuvastatin compared to pravastatin and/or atorvastatin. Further investigation into potential anti-inflammatory mechanisms of rosuvastatin is warranted to better understand this compound's actions.

Acute elevations in TNF- α has been previously shown to mediate neurotoxic effects through mitochondrial dysfunction [64]. This is consistent with our study, where LPS-induced increases in TNF- α were associated with a reduction in mitochondrial membrane potential. TNF- α has previously been implicated as a central mediator of neurodegenerative disease, and cognitive decline associated with chronic neuroinflammation [65, 66]. Notably, while

atorvastatin, pravastatin and rosuvastatin pre-treatments were associated with reductions in TNF- α in HAPI-CM, so too were pitavastatin at all concentrations, as well as fluvastatin and simvastatin at selected concentrations. This suggests that the reduction of LPS-induced TNF- α release is not the sole mediator of SH-SY5Y protection. The reduction of TNF- α by multiple statins is consistent with the aforementioned THP-1 study by our group [22]. Interestingly, however, both fluvastatin and simvastatin were found to decrease TNF- α release in the microglial-like THP-1 model at concentrations which did not have any significant effect on TNF- α in the HAPI microglial model, reinforcing the difference between these two models.

The reduction of caspase 3/7 activity occurs only in statins which were protective against SH-SY5Y toxicity, suggesting that atorvastatin, pravastatin and rosuvastatin afford protection through reducing apoptosis. Furthermore, these three compounds also improved mitochondrial membrane potential in a dose-dependent manner. Mitochondrial dysfunction has been previously associated with being both a cause and consequent of pro-apoptotic activity [67]. Our findings relating to both caspase activity and mitochondrial membrane potential are consistent with previous *in vitro* and *in vivo* studies whereby the reduction of apoptosis has been previously implicated as a mechanism of statin-induced protection [17, 68-72].

Consistent with previously reported literature, all three protective statins were also found to decrease ROS production in our study in a dose-dependent manner, suggestive of decreased oxidative stress [21-23]. Interestingly, reduced ROS production was also observed in statins which did not confer protection against toxicity (fluvastatin, pitavastatin and simvastatin). Similarly, all tested statins with the exception of simvastatin demonstrated the capacity to decrease nitric oxide release, which was consistent with previous studies [21, 77]. The reduction of nitric oxide release, however, also did not correspond with neuroprotection in our

model. Furthermore, PGE₂ release appeared unrelated to statins' ability to decrease CM-induced toxic insult in SH-SY5Y cells, with selected concentrations of atorvastatin and pravastatin found to improve cellular viability exhibiting no significant reduction in PGE₂. This suggests that statins' neuroprotective effects may be independent of their class' ability to reduce oxidative stress, nitrosative stress and PGE₂ in HAPI cells.

Interestingly, while our model suggests that high-dose simvastatin appeared to augment LPS-induced toxicity in the present mode, a number of studies have identified simvastatin as conferring neuroprotective effects in various clinical populations [14, 15, 78]. Simvastatin has been previously associated with neurotoxic effects in spiral ganglion neurons *in vitro* at the same concentrations used in the present study, whereas protective effects cited in other literature have been at lower concentrations perhaps suggesting a dose-dependent effect [17, 57]. This clinical significance of the simvastatin toxicity seen in our study and in other reports is unclear; although the measurement of plasma statin concentrations facilitates our knowledge of clinically relevant systemic concentrations of these agents, the clinical relevant doses for CNS effect remains unknown [79]. Similarly, while it is well established that statins are capable of entering the neuroparenchyma, the extent of compound penetration, retention and efflux remains unclear [5].

Atorvastatin, fluvastatin, pitavastatin and rosuvastatin were found to decrease MDC fluorescence at all tested concentrations in the present study, indicative of decreases in autophagic vacuole formation and potentially reduced autophagy. This, however, did not confer protection against SH-SY5Y cell death. Our findings are in contrast to previous *in vivo* studies where both simvastatin and atorvastatin have been associated with neuroprotection through increased autophagy in models of spinal cord injury [80, 81]. It is important to note

that autophagy is a complex, evolutionarily preserved process, and the effects of statins on this pathway are poorly understood [82, 83]. Furthermore, while the reduction of autophagic vacuole formation by statins in our model provides insight as to the possible effects on the autophagy response, further investigation into this pathway would be required to better understand the comparative effects of statins on autophagy.

To our knowledge, the present study is the first *in vitro* investigation to compare six statin compounds in a model of neuroinflammation-induced neurodegeneration. Our findings here suggest that, as a class, statins appear heterogeneous in their ability to protect against LPS-induced neuronal toxicity. When considering the discrepancies and inconsistencies which exist across current clinical literature with regards to the neurological effects of the statin class, the findings of the present study have considerable implications, as they suggest that the mechanism of statins within the CNS are compound-dependent, and not a class effect. Notably, there are some similarities between our findings and those which have been reported in clinical studies. For example, in a review of clinical trials and prospective observational studies, Geifman and colleagues (2017) found that atorvastatin users were significantly less cognitively impaired than non-users, whereas simvastatin, lovastatin, fluvastatin, pravastatin and rosuvastatin were found to have no effect [13]. Furthermore, while fluvastatin, pravastatin and rosuvastatin were found to have no significant effect on female dementia diagnoses, simvastatin and atorvastatin were found to marginally decrease this risk [14].

When discussing the present findings and their clinical implications, there are a number of key factors which should be considered. Firstly, it is important to acknowledge that concentrations used in the present study are likely to be supra-therapeutic. While it is well accepted that statins (including compounds used in the present study) are able to influence the neuroparenchyma,

the concentration of each statin required to do so is currently unclear, and hence the translational implication of our findings at the highest doses is equally unclear [5]. Interestingly, although the protective effects seen with atorvastatin and rosuvastatin in our study occurred at the higher tested concentrations, pravastatin exhibited protective effects from the lowest tested concentration, which may be clinically relevant. Consequently, while our findings do support the notion that statins possess heterogeneous actions within a neurological model, further studies are recommended *in vivo* and/or clinical populations to clarify compounds and, in particular, concentrations and doses which would be of interest in the neurological setting. A further factor for consideration in future studies is the model or disease state which is used. Our study employed a neuroinflammation model of neurodegeneration, however this approach would not be appropriate for all neurological disorders. Given the highly varied pathogenesis of neurological conditions, future studies both *in vitro*, *in vivo* and in clinical settings should look to compare multiple statin compounds to gain greater insight as to which compounds (if any) demonstrate protective or detrimental activity within the CNS, and how this occurs.

Ultimately, the mechanistic findings from our study support the clinical observation that individual statins exhibit differing pharmacological characteristics in an *in vitro* model of neuroinflammation-induced neurodegeneration, with only pravastatin (all concentrations), atorvastatin (high-dose) and rosuvastatin (high-dose) associated with SH-SY5Y protection. Given the varied pathogenesis of differing neurological and neurodegenerative disorders, it is also quite plausible that individual statins will have differing effects in different clinical populations. As such, future clinical investigations into statins' effects within the CNS should avoid generalising findings to the statin class and indeed across neurological conditions with

628 differing pathogenic mechanisms. We strongly suggest that future studies should consider the
629 comparison of multiple statin compounds when further evaluating statins' CNS effects.

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Conclusion

Here we show that while all members of the statin class significantly reduced LPS-induced release of inflammatory mediators in HAPI cells, subsequent effects on cell death signalling pathways in SH-SY5Y neuronal cells are varied between statin compounds. When considering each compounds' effect on the inflammatory profile, the protective effects of both pravastatin and atorvastatin appear to be mediated through reductions in IL-1 β release, with pravastatin affording protection against SH-SY5Y toxic insult at all tested concentrations. These findings may be important when considering the reported discrepancies between clinical studies which use single statin compounds, or indeed why meta-analyses which fail to stratify by statin may conclude negligible or conflicting effects. Findings from the present study highlight the need to consider individual statins as having discrete effects within the CNS, with potentially varied mechanism(s) of action. Given the limitations surrounding *in vitro* modelling, further investigation into how the neuroprotective effect of atorvastatin, pravastatin, and rosuvastatin translate into an *in vivo* setting are warranted.

Conflict of interest

The authors have no competing interests to declare.

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866 **Table 1:** *Primer sequences used for rt-PCR*

Primer	Sequence
<i>SYP</i>	Forward: 5'-TGAAGGTGCTGCAATGGGT-3' Reverse: 5'-TGTTGGCACAATCCACGC-3'
<i>ENO2</i>	Forward: 5'-TGGAGACAAACAGCGTTACTTAGG-3' Reverse: 5'-GAGAGACCTGAGCTGATGAGGG-3'
<i>DYN1</i>	Forward: 5'-ACACGCTGCCGGGACTTC-3' Reverse: 5'-CAGCTGCCGGTAATCCTT-3
<i>GAPDH</i>	Forward: 5'-TGCACCACCAACTGCTTAGC-3' Reverse: 5'-GGCATGGACTGTGGTCATGAG-3'

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868

869 **Table 2:** Summary of statin-associated effects on pro-inflammatory mediator release in LPS-
870 treated HAPI cells, and subsequent effect on cellular viability in HAPI-CM treated SH-SY5Y
871 cells. Symbol definition: $\uparrow$ Significant increase ($p<0.05$); $\downarrow$ significant decrease ($p<0.05$);
872 – no significant effect

	ROS	NO	PGE ₂	TNF- α	IL-1 β	SH-SY5Y viability
Atorvastatin	1 μ M: $\downarrow$	1 μ M: $\downarrow$	1 μ M: –	1 μ M: $\downarrow$	1 μ M: –	1 μ M: –
	10 μ M: $\downarrow$	10 μ M: $\downarrow$	10 μ M: –	10 μ M: $\downarrow$	10 μ M: –	10 μ M: –
	100 μ M: $\downarrow$	100 μ M: $\downarrow$	100 μ M: –	100 μ M: $\downarrow$	100 μ M: $\downarrow$	100 μ M: $\uparrow$
Fluvastatin	1 μ M: $\downarrow$	1 μ M: –	1 μ M: –	1 μ M: $\downarrow$	1 μ M: –	1 μ M: –
	10 μ M: $\downarrow$	10 μ M: –	10 μ M: $\downarrow$	10 μ M: –	10 μ M: –	10 μ M: –
	100 μ M: $\downarrow$	100 μ M: $\downarrow$	100 μ M: $\downarrow$	100 μ M: –	100 μ M: –	100 μ M: –
Pitavastatin	1 μ M: $\downarrow$	1 μ M: $\downarrow$	1 μ M: $\downarrow$	1 μ M: $\downarrow$	1 μ M: –	1 μ M: –
	10 μ M: $\downarrow$	10 μ M: $\downarrow$	10 μ M: $\downarrow$	10 μ M: $\downarrow$	10 μ M: –	10 μ M: –
	100 μ M: $\downarrow$	100 μ M: $\downarrow$	100 μ M: $\downarrow$	100 μ M: $\downarrow$	100 μ M: –	100 μ M: –
Pravastatin	1 μ M: $\downarrow$	1 μ M: $\downarrow$	1 μ M: –	1 μ M: $\downarrow$	1 μ M: $\downarrow$	1 μ M: $\uparrow$
	10 μ M: $\downarrow$	10 μ M: $\downarrow$	10 μ M: $\downarrow$	10 μ M: $\downarrow$	10 μ M: $\downarrow$	10 μ M: $\uparrow$
	100 μ M: $\downarrow$	100 μ M: $\downarrow$	100 μ M: $\downarrow$	100 μ M: $\downarrow$	100 μ M: $\downarrow$	100 μ M: $\uparrow$
Rosuvastatin	1 μ M: $\downarrow$	1 μ M: $\downarrow$	1 μ M: $\downarrow$	1 μ M: $\downarrow$	1 μ M: –	1 μ M: –
	10 μ M: $\downarrow$	10 μ M: $\downarrow$	10 μ M: $\downarrow$	10 μ M: $\downarrow$	10 μ M: –	10 μ M: –
	100 μ M: $\downarrow$	100 μ M: –	100 μ M: $\downarrow$	100 μ M: $\downarrow$	100 μ M: –	100 μ M: $\uparrow$
Simvastatin	1 μ M: $\downarrow$	1 μ M: $\uparrow$	1 μ M: $\uparrow$	1 μ M: –	1 μ M: –	1 μ M: –
	10 μ M: $\downarrow$	10 μ M: $\uparrow$	10 μ M: –	10 μ M: $\downarrow$	10 μ M: –	10 μ M: $\downarrow$
	100 μ M: $\uparrow$	100 μ M: $\uparrow$	100 μ M: $\downarrow$	100 μ M: $\downarrow$	100 μ M: –	100 μ M: $\downarrow$

Figure 1: DCF fluorescence as an index of global ROS production in LPS-treated HAPI cells in the absence and presence of statin pre-treatment (percentage change relative to untreated control). HAPI cells were seeded at 3.0×10^5 cells/mL and treated 23 h post-seeding with prednisone ('pred'; 10 μ M) or 0-100 μ M (A) atorvastatin, (B) fluvastatin, (C) pitavastatin, (D) pravastatin, (E) rosuvastatin or (F) simvastatin. After 1 h, cells were treated with 0.1 μ g/mL LPS for 24 h, following which DCF fluorescence was measured. Results are expressed relative to cellular viability for each respective condition. Data shows mean + SD of three independent experiments. *vs untreated vehicle control, #vs LPS only

Figure 2: DAF fluorescence in LPS-treated HAPI cells in the absence and presence of statin pre-treatment (percentage change relative to untreated control). HAPI cells were seeded at 3.0×10^5 cells/mL and treated 23 h post-seeding with prednisone ('pred'; 10 μ M) or 0-100 μ M (A) atorvastatin, (B) fluvastatin, (C) pitavastatin, (D) pravastatin, (E) rosuvastatin or (F) simvastatin. After 1 h, cells were treated with 0.1 μ g/mL LPS for 24 h, following which DAF fluorescence was measured. Results are expressed relative to cellular viability for each respective condition. Data shows mean + SD of three independent experiments. *vs untreated vehicle control, #vs LPS only

893

894 **Figure 3:** PGE₂ release in LPS-treated HAPI cell supernatant in the absence and presence of
 895 statin pre-treatment. HAPI cells were seeded at 3.0×10^5 cells/mL and treated 23 h post-
 896 seeding with prednisone ('pred'; 10 μM) or 0-100 μM (A) atorvastatin, (B) fluvastatin, (C)
 897 pitavastatin, (D) pravastatin, (E) rosuvastatin or (F) simvastatin. After 1 h, cells were treated
 898 with 0.1 μg/mL LPS for 24 h, following which PGE₂ was quantified using ELISA. Note different
 899 scale used for Image (F). Data shows mean + SD of three independent experiments. *vs
 900 untreated vehicle control, #vs LPS only

901

902 **Figure 4:** TNF- α release in LPS-treated HAPI cell supernatant in the absence and presence of
 903 statin pre-treatment. HAPI cells were seeded at 3.0×10^5 cells/mL and treated 23 h post-
 904 seeding with prednisone ('pred'; 10 μ M) or 0-100 μ M (A) atorvastatin, (B) fluvastatin, (C)
 905 pitavastatin, (D) pravastatin, (E) rosuvastatin or (F) simvastatin. After 1 h, cells were treated
 906 with 0.1 μ g/mL LPS for 24 h, following which TNF- α was quantified using ELISA. Data shows
 907 mean + SD of three independent experiments. *vs untreated vehicle control, #vs LPS only

Figure 5: IL-1 β release in LPS-treated HAPI cell supernatant in the absence and presence of statin pre-treatment. HAPI cells were seeded at 3.0×10^5 cells/mL and treated 23 h post-seeding with prednisone ('pred'; 10 μ M) or 0-100 μ M (A) atorvastatin, (B) fluvastatin, (C) pitavastatin, (D) pravastatin, (E) rosuvastatin or (F) simvastatin. After 1 h, cells were treated with 0.1 μ g/mL LPS for 24 h, following which IL-1 β was quantified using ELISA. Data shows mean + SD of three independent experiments. *vs untreated vehicle control, #vs LPS only

915

916 **Figure 6:** (A-B) Resazurin reduction of (A) undifferentiated SH-SY5Y cells and (B)
 917 differentiated SH-SY5Y cells with HAPI-CM for 24 h. (C-H) Resazurin reduction of
 918 undifferentiated SH-SY5Y cells treated with conditioned media generated from HAPI cells
 919 treated with LPS ± statins. Briefly, SH-SY5Y cells were seeded at 1.0×10^5 cells/mL and at 24

920 *h treated with HAPI-CM which had been pre-treated with prednisone ('pred'; 10 μ M) or 0-*
921 *100 μ M (C) atorvastatin, (D) fluvastatin, (E) pitavastatin, (F) pravastatin, (G) rosuvastatin or*
922 *(H) simvastatin. After 24 h, the reduction of resazurin to resorufin was measured as an index*
923 *of cellular viability. Data shows mean + SD of three independent experiments. *vs untreated*
924 *vehicle control, #vs LPS only.*

925

926

927

928 **Figure 7:** JC-1 fluorescence of undifferentiated SH-SY5Y cells treated with conditioned media
 929 generated from HAPI cells treated with LPS ± statins. At 24 h post-seeding, SH-SY5Y
 930 supernate was replaced with HAPI-CM which had been pre-treated with prednisone ('pred';
 931 10 μM) or 0-100 μM (A) atorvastatin, (B) fluvastatin, (C) pitavastatin, (D) pravastatin, (E)
 932 rosuvastatin or (F) simvastatin. After 24 h, the JC-1 fluorescence was measured as an index
 933 mitochondrial membrane potential. Results are expressed relative to cellular viability for each
 934 respective condition. Data shows mean + SD of three independent experiments. *vs untreated
 935 vehicle control, #vs LPS only

936

937 **Figure 8:** Caspase 3/7 fluorescence of undifferentiated SH-SY5Y cells treated with conditioned
 938 media generated from HAPI cells treated with LPS ± statins. At 24 h post-seeding, SH-SY5Y
 939 supernate was replaced with HAPI-CM which had been pre-treated with prednisone ('pred';
 940 10 μM) or 0-100 μM (A) atorvastatin, (B) fluvastatin, (C) pitavastatin, (D) pravastatin, (E)
 941 rosuvastatin or (F) simvastatin. After 24 h, caspase 3/7 activity was quantified using ELISA.
 942 Results are expressed relative to cellular viability for each respective condition. Data shows
 943 mean + SD of three independent experiments. Note different scale for Image (F) *vs untreated
 944 vehicle control, #vs LPS only

Figure 9: DCF fluorescence (% change relative to untreated control) as an index of global ROS production in undifferentiated SH-SY5Y cells treated with conditioned media generated from HAPI cells treated with LPS $\pm$ statins. At 24 h post-seeding, SH-SY5Y supernate was replaced with HAPI-CM which had been pre-treated with prednisone ('pred'; 10 μ M) or 0-100 μ M (A) atorvastatin, (B) fluvastatin, (C) pitavastatin, (D) pravastatin, (E) rosuvastatin or (F) simvastatin. After 24 h, DCF fluorescence was measured. Results are expressed relative to cellular viability for each respective condition. Data shows mean $\pm$ SD of three independent experiments. *vs untreated vehicle control, #vs LPS only, †vs prednisone pre-treatment

954

955 **Figure 10:** MDC fluorescence was used to assess autophagy vacuole formation in
 956 undifferentiated SH-SY5Y cells treated with conditioned media generated from HAPI cells
 957 treated with LPS $\pm$ statins. At 24 h post-seeding, SH-SY5Y supernate was replaced with HAPI-
 958 CM which had been pre-treated with prednisone ('pred'; 10 μ M) 0-100 μ M (A) atorvastatin,
 959 (B) fluvastatin, (C) pitavastatin, (D) pravastatin, (E) rosuvastatin or (F) simvastatin. After 24
 960 h, MDC fluorescence was measured. Results are expressed relative to cellular viability for
 961 each respective condition. Data shows mean + SD of three independent experiments. *vs
 962 untreated vehicle control, #vs LPS only, †vs prednisone pre-treatment

964

965 **Supplement 1: Resazurin reduction of LPS-treated HAPI cells in the absence and presence of**
966 *statin pre-treatment. HAPI cells were seeded at 3.0×10^5 cells/mL and treated 23 h post-*
967 *seeding with 0-100 µM (A) atorvastatin, (B) fluvastatin, (C) pitavastatin, (D) pravastatin, (E)*
968 *rosuvastatin or (F) simvastatin. After 1 h, cells were treated with 0.1 µg/mL LPS for 24 h,*
969 *following which reduction of resazurin to resorufin was measured as an index of cellular*
970 *viability. Data shows mean + SD of three independent experiments. *vs untreated vehicle*
971 *control, #vs LPS only*

Supplement 2: Relative gene expression, as determined through $2^{-\Delta\Delta CT}$, of (A) *DYN1*, (B) *SYP*, and (C) *ENO2* in undifferentiated SH-SY5Y (uSH-SY5Y) cells compared to SH-SY5Y cells which have been differentiated to neuronal-like cells through sequential treatment with 5 days RA and 3 days BDNF (dSH-SY5Y).

Supplement 3: Resazurin reduction of (A) undifferentiated SH-SY5Y cells and (B) differentiated SH-SY5Y cells incubated with HAPI-CM for 48 h. Data shows mean + SD of three independent experiments. *vs untreated cell control

985

986 **Supplement 4:** Resazurin reduction of differentiated SH-SY5Y cells treated with conditioned
 987 media generated from HAPI cells treated with LPS $\pm$ statins. At 24 h post-differentiation, cells
 988 were treated with HAPI-CM which had been pre-treated with 0-100 μ M (A) atorvastatin, (B)
 989 fluvastatin, (C) pitavastatin, (D) pravastatin, (E) rosuvastatin or (F) simvastatin. After 24 h,
 990 the reduction of resazurin to resorufin was measured as an index of cellular viability. Data
 991 shows mean + SD of three independent experiments. *vs untreated cell-only control; #vs
 992 untreated vehicle control

993

994 **Supplement 5:** Resazurin reduction of undifferentiated SH-SY5Y cells treated with 0-100 μM
 995 of (A) atorvastatin, (B) fluvastatin, (C) pitavastatin, (D) pravastatin, (E) rosuvastatin or (F)
 996 simvastatin for 24 h. Data shows mean + SD of three independent experiments. *vs untreated
 997 cell control, #vs LPS only