Mol Neurobiol (2016) 53:7284–7297
DOI 10.1007/s12035-015-9611-6

Nociceptin/Orphanin FQ Inhibits the Survival and Axon Growth of Midbrain Dopaminergic Neurons Through a p38-MAPK Dependent Mechanism

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Received: 21 September 2015 / Accepted: 3 December 2015 / Published online: 21 December 2015

Abstract Nociceptin/orphanin FQ (N/OFQ) is an opioid-like neuropeptide that binds and signals through a G-protein-coupled receptor called the N/OFQ peptide (NOP) receptor. N/OFQ and the NOP receptor are expressed in the midbrain and have been implicated in the pathogenesis of Parkinson's disease (PD). Genetic removal of the N/OFQ precursor partially protects midbrain dopaminergic neurons from 1-methyl-4-phenylpyridine-induced toxicity, suggesting that endogenous N/OFQ may be detrimental to dopaminergic neurons. However, whether N/OFQ directly affects the survival and growth of dopaminergic neurons is unknown. Here, we show that N/OFQ has a detrimental effect on the survival of dopaminergic neurons and the growth of their axons in primary cultures of the E14 rat ventral mesencephalon. N/OFQ potentiates the effects of the neurotoxins 6-hydroxydopamine and 1-methyl-4-phenylpyridinium through p38-MAPK signalling. We also show that like α-synuclein, there is a significant reduction in N/OFQ messenger RNA (mRNA) expression in the midbrain of patients with Parkinson's disease. These results demonstrate for the first time that N/OFQ is detrimental to the survival and growth of dopaminergic neurons and that its expression is altered in the midbrain of patients with Parkinson's disease.

Keywords Nociceptin/orphanin FQ · Parkinson's disease · Dopaminergic · Neuron · Survival · Axon · Growth · p38-MAPK · Human

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Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder, which affects 0.5 to 1 % of those 65–69 years of age and 1 to 3 % of people over 80 years of age [1, 2]. PD is characterized by motor deficits, including akinesia, bradykinesia and resting tremor, and non-motor deficits, including cognitive and autonomic disturbances [1, 3]. The progressive degeneration of midbrain dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) and the loss of their axonal terminals in the striatum is the classical neuropathological feature of this disease [4–6]. A number of post-mortem studies have shown that the motor symptoms of PD appear when there is a ~30 % loss of total SN neurons [7–9]. In a PET study of striatal dopaminergic terminal loss, no motor symptoms were apparent with a 51 and 56 % reduction in vesicular monoamine transporter ligand and a dopamine transporter ligand, respectively, however, the motor symptoms of PD became apparent when these markers were reduced by 62 and 71 %, respectively [10]. This pre-symptomatic lag phase has been proposed to be due to sprouting of remaining DA neurons [11, 12] or to adaptive changes in non-DA structures [13]. Although 5–10 % of PD cases are of a genetic aetiology, the remaining 90–95 % of cases are of idiopathic origin [14,

15]. Thus, understanding the molecular and cellular mechanisms that regulate DA neuron survival, and the growth and maintenance of their axons is crucial to develop new understanding of the causative basis of their degeneration in PD and new therapies for the disorder. Various pre-clinical and clinical studies suggest an involvement of Nociceptin/orphanin FQ (N/OFQ) in the pathogenesis of PD; however, whether N/OFQ directly affects DA neuronal survival or axonal growth is unknown.

N/OFQ [16, 17] is a neuropeptide belonging to the opioid family [18, 19] and is the endogenous ligand of the N/OFQ peptide (NOP) receptor [20]. The N/OFQ-NOP receptor system is expressed throughout the central nervous system [21, 22] and is involved in the modulation of a variety of central functions such as pain perception, mood, cognition, food intake, reward and locomotion [18, 19, 23]. We have previously demonstrated that N/OFQ also contributes to motor impairment in PD [24]. Indeed, an increase in N/OFQ levels is observed in the brain of Parkinsonian rodents [24, 25] and the CSF of PD patients [25]. Furthermore, NOP receptor antagonists reverse motor impairment in rodent and non-human primate [26, 27] models of the disease. A recent NOP-eGFP reporter mouse has shown that NOP is expressed throughout the neuronal cell body and in the neuronal processes [28], and NOP messenger RNA (mRNA) was found in DA neurons in the SN [29, 30]. The source of N/OFQ in the midbrain is less clear, but N/OFQ is expressed by both neurons and astrocytes [31, 32]. Specifically in SNc/VTA, about half of N/OFQ-positive neurons also expressed GAD, suggesting that they are GABAergic [30]. The role of N/OFQ in PD, however, may go beyond its involvement in sustaining motor symptoms. Indeed, *N/OFQ* knockout mice were partially protected from the neurotoxic action of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP), with a higher number of DA neurons and striatal tyrosine-hydroxylase (TH)-positive terminals spared after acute neurotoxin injection with respect to controls [24, 33], suggesting that N/OFQ also contributes to the neurodegeneration associated with PD. Interestingly, no neuroprotection was observed in *N/OFQ* knockout mice after injection of methamphetamine [33], which is known to destroy striatal DA terminals and DA neurons in a retrograde manner, possibly indicating that N/OFQ neurotoxic action is accomplished in SNpc, where DA neurons are located. In fact, high levels of N/OFQ are found in both SNpc and SN pars reticulata (SNpr) [34]. It could be speculated that N/OFQ is neurotoxic through indirect mechanisms, e.g. via releasing

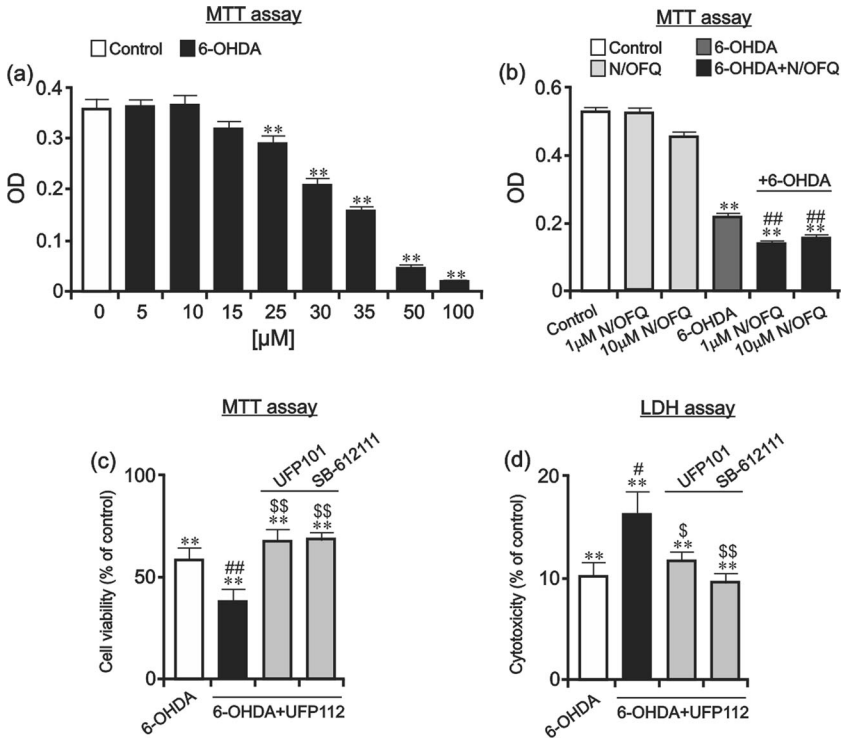

Fig. 1 N/OFQ potentiates the effects of 6-OHDA on SH-SY5Y cells. **a** SH-SY5Y cells were incubated for 24 h with increasing concentrations (5–100 μM) of 6-OHDA after which cell viability was quantified using an MTT assay. **b** MTT assay showing the effect of N/OFQ (1–10 μM), with or without 30 μM 6-OHDA on SH-SY5Y cells viability for 24 h. **c** MTT assay and **d** LDH assay examining the effect of the NOP agonist UFP112 (3 μM) on the neurotoxic effect of 30 μM 6-OHDA after 24 h and whether this was prevented by the NOP antagonists UFP101 (10 μM) and SB612111 (3 μM). Data are means ± SEM of eight determinations per group and are expressed as percent of control. $^{*}p < 0.05$; $^{**}p < 0.01$ different from control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ different from 6-OHDA; $^{\S}p < 0.05$, $^{\S\S}p < 0.01$ different from 6-OHDA + UFP112 (ANOVA followed by Newman-Keuls)

glutamate [35] or pro-inflammatory mediators (for a review see [36]). However, SNpc DA neurons express the NOP receptor [21, 22], and although N/OFQ exerts acute inhibitory effects on the activity of SNpc DA neurons [37], the possibility that prolonged exposure to N/OFQ can exert direct, slower neurotoxic actions cannot be ruled out. In fact, N/OFQ [38], and more generally opioids [39], not only act through the canonical Gi pathway but also through the MAP-kinase pathway, most likely recruited by the βγ subunits [38]. The involvement of MAP-kinase signalling in the degeneration of DA neurons is well established, and administration of the DA neurotoxin 6-hydroxydopamine (6-OHDA) leads to the activation of p38-MAPK signalling in DA neurons [40].

Therefore, the aim of this study was to determine if N/OFQ exerts a direct detrimental effect on DA neuronal survival and axonal growth, and whether there were alterations in N/OFQ and/or its receptor in the human Parkinsonian midbrain. To do this, the SH-SY5Y neuroblastoma cell line, a widely used and well-validated model of human midbrain DA neurons [41], and primary cultures of embryonic rat ventral mesencephalon (VM) [42, 43] were used. Both the SH-SY5Y cell line [44, 45] and midbrain DA neurons [21, 22] express the NOP receptor. Analysis of N/OFQ and NOP expression in the Parkinsonian midbrain was performed using datasets derived from the gene expression omnibus of previously published microarray data of the SN of PD patients.

Methods

Preparation and Treatment of SH-SY5Y Cell Cultures

SH-SY5Y cells were maintained in Dulbecco's Modified Eagle Medium:F12 (DMEM:F12; Sigma), supplemented with 10 % foetal calf serum (FCS; Sigma), 100 nM L-Glutamine (Sigma), 100 U/ml Penicillin (Sigma), and 10 μg/ml Streptomycin (Sigma). The cells were incubated under a humidified atmosphere containing 5 % CO_2 at 37 °C and were treated

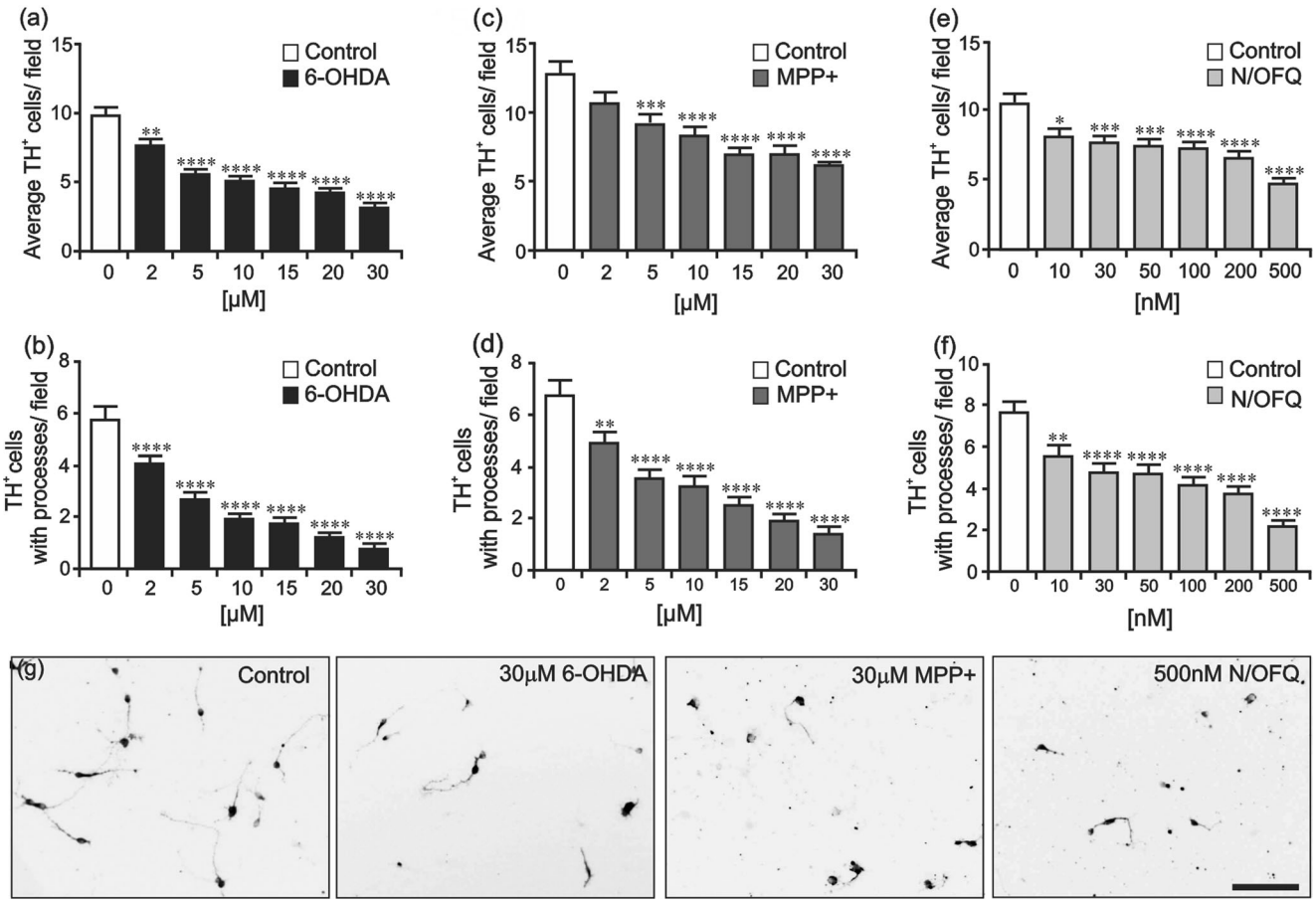

Fig. 2 N/OFQ reduces the numbers of DA neurons in a dose-dependent manner. Primary cultures of E14 rat VM were incubated for 24 h with increasing concentrations of 6-OHDA (2–30 μM), MPP^+ (2–30 μM) or N/OFQ (10–500nM) after which the numbers of DA neurons, and those with a visible neurite were assessed. **a**–**f** Graphical representations of the average number of TH-immunopositive DA neurons and those with intact neurites in **a**, **b** 6-OHDA, **c**, **d** MPP^+ and **e**, **f** N/OFQ-treated E14 VM DA neurons at 24 h post-treatment. **g** Representative photomicrographs of E14 VM DA neurons immunocytochemically stained for TH. Scale bar = 50 μm. Data are expressed as mean ± SEM from three separate experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ vs. control; one-way ANOVA with post hoc Dunnett's test

following 1 day in vitro (DIV) with 0, 5, 10, 15, 25, 30, 35, 50 or 100 μM 6-OHDA (pre-stabilized in 0.01 % ascorbic acid; Sigma) with/without 0.1, 1, 3 or 10 μM N/OFQ, 3 μM UFP112 (NOP receptor agonist [46]), 10 μM UFP101 (NOP receptor antagonist [47]) or 3 μM SB-612111 (NOP receptor antagonist; [48, 49]) for 24 h before analysis of cell viability using an MTT and lactate dehydrogenase (LDH) assay.

MTT Assay

The MTT assay was used to determine the number of viable cells in SH-SY5Y cultures. The assay is based on the cleavage of the yellow tetrazolium salt [3-(4,5-dimethylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide] (MTT) to purple formazan crystals by metabolically active cells. Following the treatments, 0.5 mg/ml of MTT labelling reagent was added to each well, and plates were incubated at 37 °C for 4 h. Cultures were then solubilized in dimethyl sulfoxide (DMSO), and the spectrophotometric absorbance of the samples was detected by a microtiter plate reader. The wavelength to measure absorbance of formazan product is 570 nm, with a reference wavelength of 750 nm.

LDH Assay

The LDH cytotoxicity assay was used to quantitatively measure LDH released into the media from damaged cells as a biomarker for cellular cytotoxicity and cytolysis. All the reactions were performed at room temperature, and samples in each experimental group were prepared in triplicate. Briefly, after seeding cells in a 96-well flat bottom microtiter plate, lysis solution was added to the latter, and after a 45-min incubation in a humidified 37 °C, 5 % CO_2 atmosphere, 50 μl of culture supernatant from each well was collected and transferred to a new microtiter plate. Fifty microliters of LDH assay buffer was added to the supernatants, and the plate was gently agitated for 30 s to allow reagents' mix. After a 10–30-min incubation at room temperature, 50 μl of the stop solution was added to all the wells and, after a gentle agitation, absorbance was measured between 490 and 520 nm.

Preparation of E14 Rat Ventral Mesencephalon (VM) Cultures

VM cultures were prepared from embryonic (E) day 14 embryos of time-mated Sprague-Dawley rats (Biological

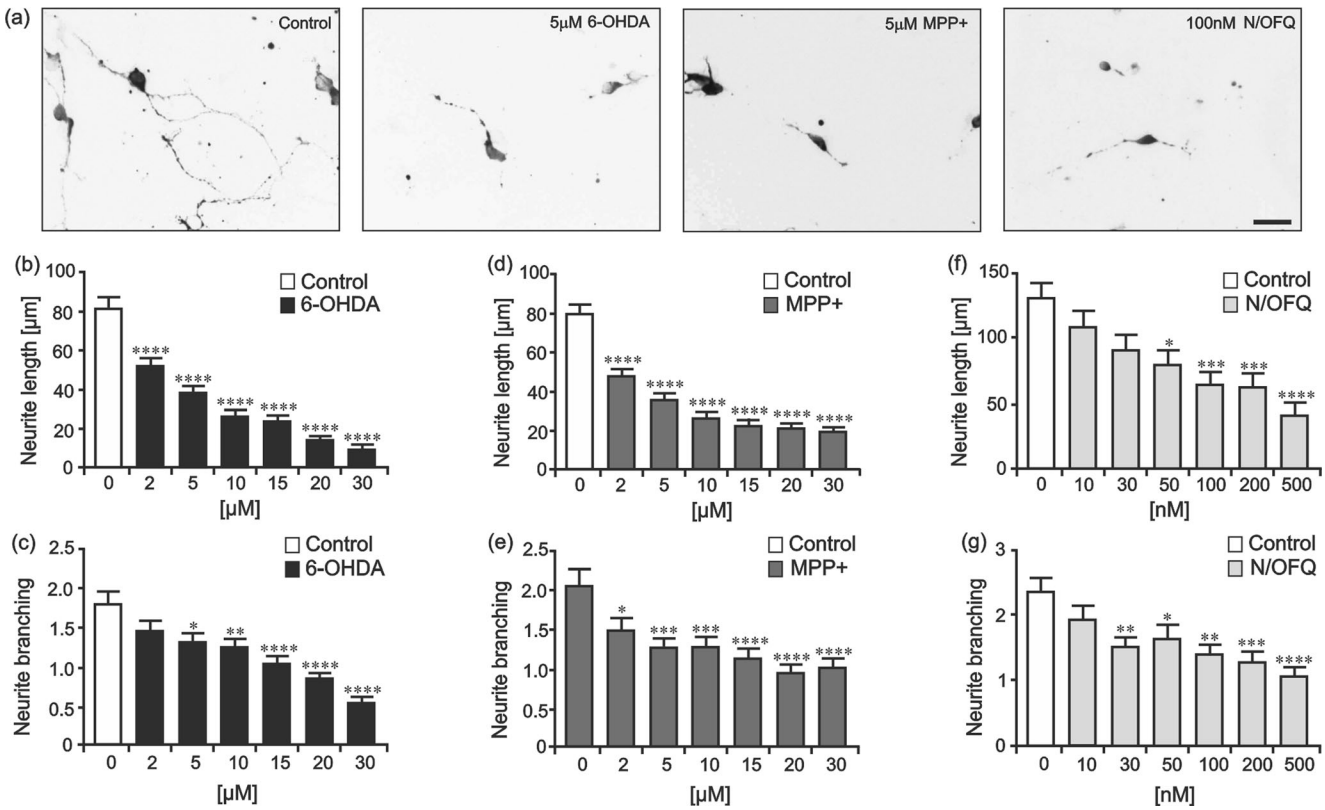

Figure 3 6-OHDA, MPP^+ and N/OFQ reduce the length and branching of VM dopaminergic neurons in a dose-dependent manner. **a** Representative photomicrographs of 6-OHDA, MPP^+- and N/OFQ-treated E14 VM cultures immunocytochemically stained for TH. Scale bar = 50 μm. Graphical representations of the average neurite length and branching of **b**, **c** 6-OHDA, **d**, **e** MPP^+ and **f**, **g** N/OFQ-treated DA neurons in E14 VM cultures. Data are expressed as mean ± SEM from three separate experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ vs. control; one-way ANOVA with post hoc Dunnett's test

Services Unit, University College Cork) as previously described [42]. Briefly, dissociated VM neurons were seeded at a density of 1×10^5 cells per poly-D-lysine-coated 24-well tissue culture dish in DMEM:F12 (Sigma) supplemented with 1 % penicillin/streptomycin (Sigma), 1 % L-glutamine (Sigma), 1 % FCS (Sigma) and 2 % B27 (Invitrogen). Cultures were treated for 24 h with either 6-OHDA (0-30 μM) or MPP^+ (0-30 μM) or N/OFQ (0-500nM) 2 h after plating, and cultures were incubated under a humidified atmosphere containing 5 % CO_2 at 37 °C before being processed for immunocytochemistry. All experiments were repeated at least three times with three individual replicates per group, per experiment.

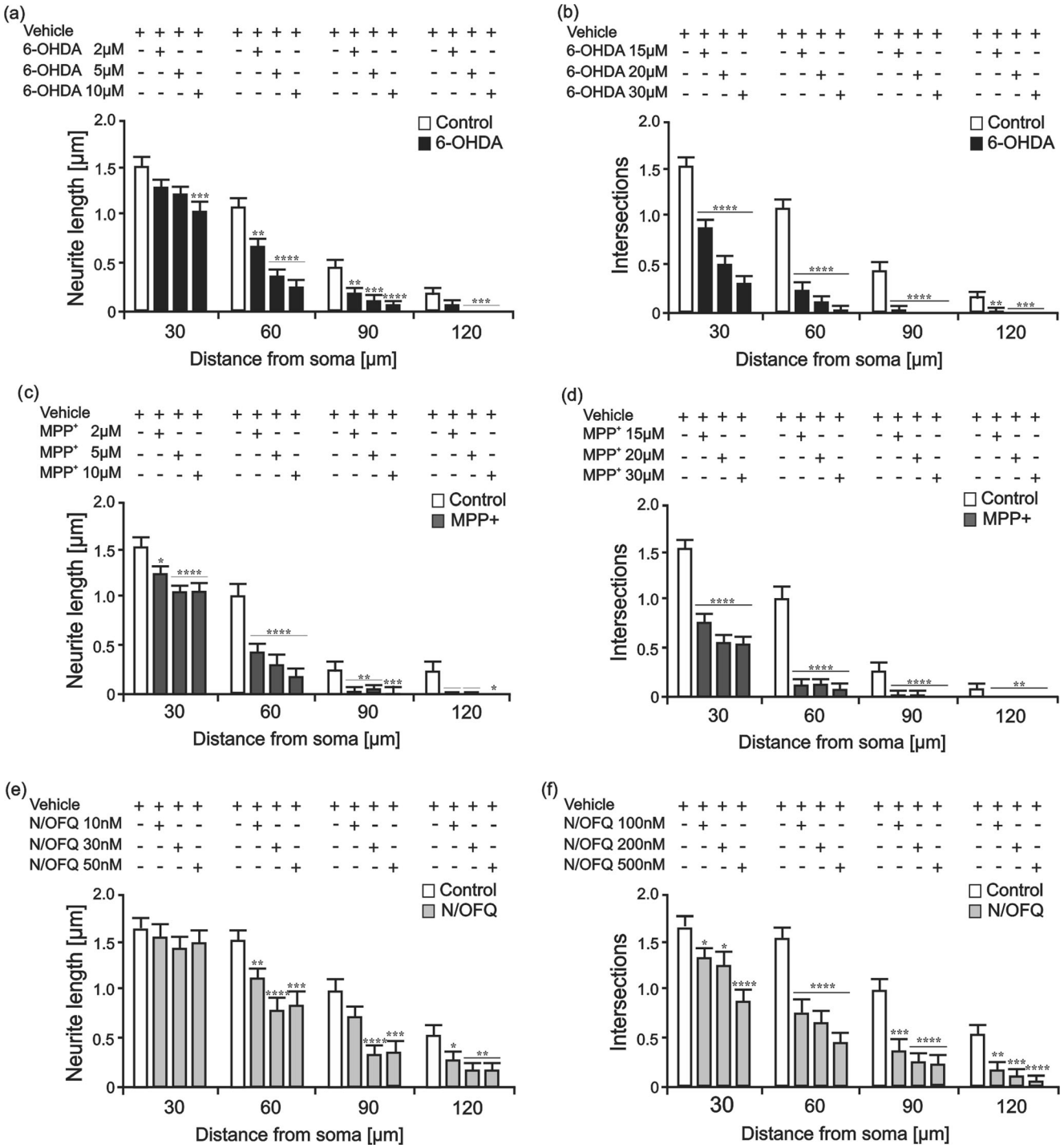

Fig. 4 6-OHDA, MPP^+ and N/OFQ reduce the complexity of VM DA neurons. Modified Sholl plots indicating the complexity of the neurite field of **a**, **b** 6-OHDA, **c**, **d** MPP^+ and **e**, **f** N/OFQ-treated E14 VM DA neurons, as indicated. Data are expressed as mean ± SEM from three separate experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ vs. control; one-way ANOVA with post hoc Dunnett's test

Immunocytochemistry

At the end of the experiment, cells were washed in pre-warmed Hank's balanced salt solution (HBSS) and fixed with ice-cold methanol for 10 min at −20 °C followed by 4 % paraformaldehyde for 15 min at room temperature and washed in 10 mM PBS with 0.02 % Triton (Sigma) (PBST) three times for 5 min. To block non-specific antibody binding, cells were incubated in 5 % bovine serum albumin (BSA) in 10 mM PBS for 1 h at room temperature. Cells were then incubated in an antibody against tyrosine hydroxylase (TH; 1:200; mouse monoclonal; Millipore) in 1 % BSA in 10 mM PBS for 16 h at 4 °C. The cells were washed three times in PBST and incubated with Alexa Fluor 594-conjugated donkey anti-mouse IgG (1:2000; Invitrogen) in 1 % BSA in PBS for 1 h at 20 °C. Following three washes in PBST, cultures were imaged under an Olympus IX70 Provis Inverted microscope fitted with an Olympus DP70 camera and AnalysisD^{TM} software. For preparation of figures, representative photomicrographs were converted to grayscale and inverted.

Analysis of Neuronal Complexity

Images of the neurite arbours of individual DA neurons were used to analyse the Sholl profiles of TH-positive cells using Sholl analysis as previously described [50]. Morphological analysis of total neurite length and number of branch points were carried out as previously described [51]. Briefly, neurite length was calculated using the formula $n \times T \times \pi/2$, where n = the number of times the neurites intersect the grid lines and T =

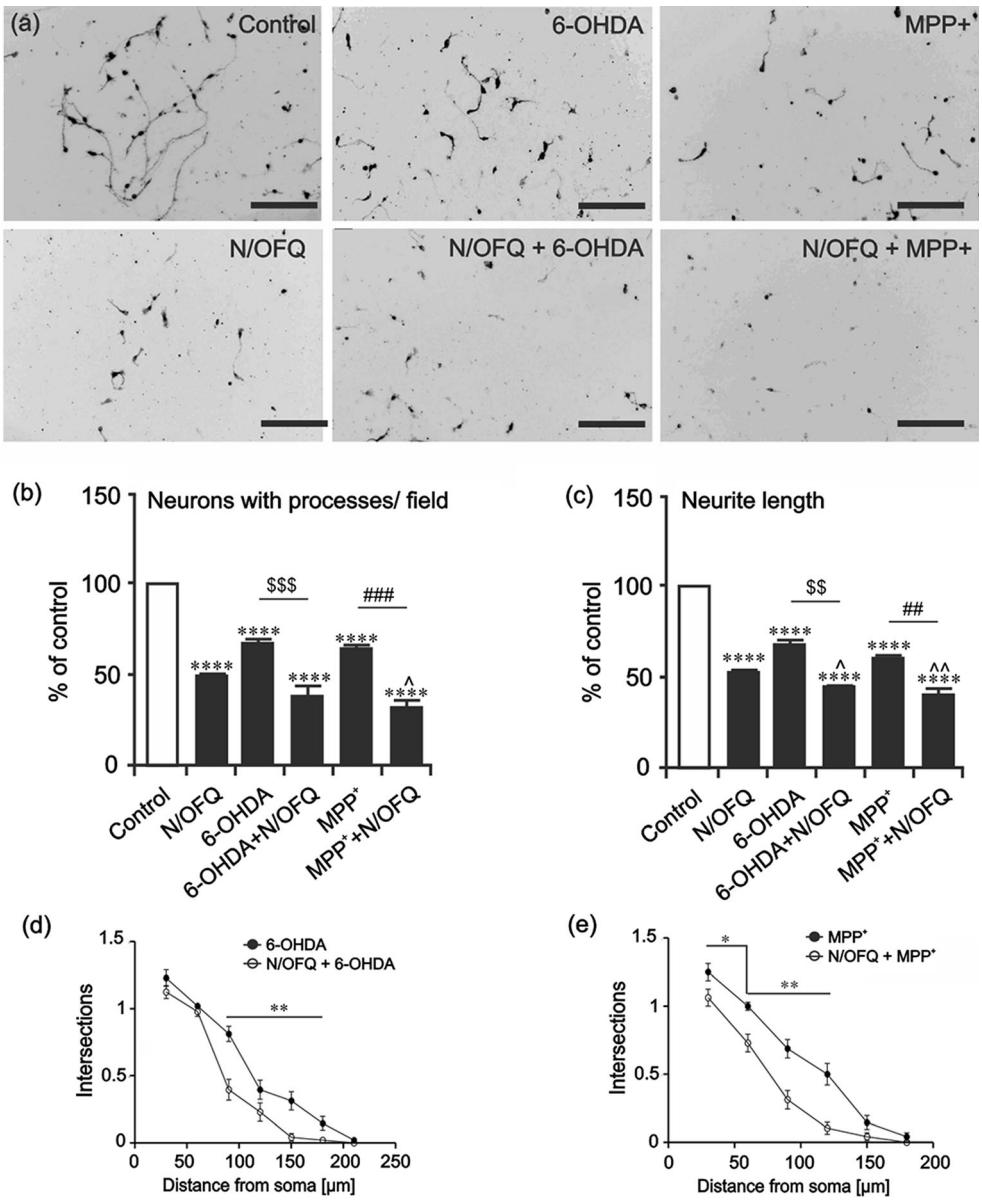

Fig. 5 N/OFQ potentiates the neurotoxic effects of 6-OHDA and MPP+ in DA neurons. **a** Representative photomicrographs and **b** the numbers of DA neurons with processes and **c** DA neurite length as a percentage of control following treatment with 6-OHDA, MPP$^+$, N/OFQ or a combination of N/OFQ and either toxin for 24 h. **d**, **e** Modified Sholl plots indicating the complexity of the neurite field of DA neurons comparing 6-OHDA to N/OFQ + 6-OHDA and MPP$^+$ to MPP$^+$+N/OFQ. Data are expressed as mean ± SEM from three **separate experiments.** ***$p < 0.05$, ****$p < 0.01$, *****$p < 0.0001$ vs. control or as indicated.** $^{\$\$}p < 0.01$ **and** $^{\$\$\$}p < 0.001$ **vs. 6-OHDA alone.** $^{\#\#}p < 0.01$ **and** $^{\#\#\#}p < 0.001$ **vs. MPP$^+$ alone.** $\wedge p < 0.05$, **and** $\wedge\wedge p < 0.01$ **vs. N/OFQ alone. ANOVA followed by Newman-Keuls**

the distance between the gridlines taking the magnification into account.

Statistical Analyses

Statistical analyses were performed using unpaired Student's *t* test or one-way analysis of variance (ANOVA) with a post hoc Tukey's, Newman-Keuls' or Dunnett's test as appropriate. Data were expressed as mean plus and minus standard error of the mean (mean ± SEM) and deemed significant when $p < 0.05$.

Microarray Gene Expression

Microarray gene expression data comparing SN from normal and age-matched PD patients derived from three independent studies was downloaded from Gene Expression Omnibus (GEO, series GSE20295 [52], GSE8397 [53] and GSE7621 [54]). RMA or TMM-normalized probe expression values were summarized to Ensembl Gene IDs by averaging individual probe values annotated to the same Ensembl ID (probes that matched more than one gene were removed from the analysis). The resulting pool of 12,210 genes with expression data in all three datasets was subsequently quantile-normalized before extracting expression values for PNOC, OPRL, SNCA and MAPT. The expression values of these genes were normalized against total RNA to allow comparison across samples. Statistical comparisons between control and disease-derived samples were carried using three separate approaches: RANK products [55], *t* test (parametric) and Wilcoxon test (non-parametric). Charts show the mean

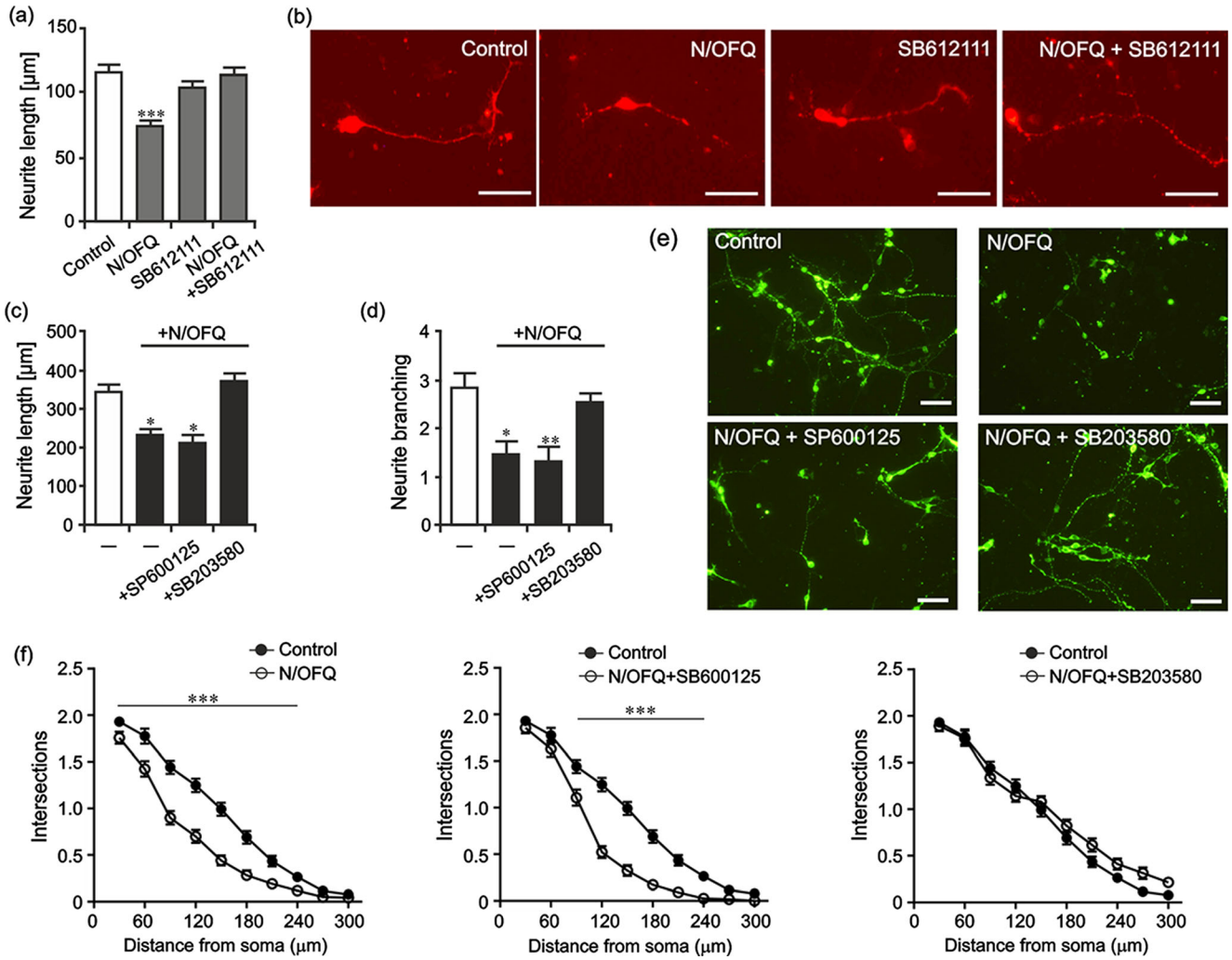

Fig. 6 N/OFQ exerts its effects on DA neurons through a NOP-p38-MAPK pathway. **a** Graphical representation of average DA neuron length and **b** representative photomicrographs of E14 VM DA neurons following treatment with 100 nM N/OFQ in the presence or absence of 10 μM SB612111 (NOP receptor antagonist) for 24 h. **c** Graphical representations of **c** average neurite length and **d** neurite branching, **e** representative photomicrographs and **f** Sholl plots of E14 VM DA neurons treated with 100 nM N/OFQ in the presence or absence of 30 μM SB600125 (JNK inhibitor) or 30 μM SB203580 (p38 inhibitor) for 24 h. Data are expressed as mean ± SEM from three separate experiments. (***$p < 0.001$ vs. control; one-way ANOVA with Tukey's test)

±SEM for the indicated genes comparing all resulting control ($n=38$) and PD samples ($n=51$).

Results

N/OFQ Potentiates the Neurotoxic Effect of 6-OHDA in SH-SY5Y Cells

To examine if N/OFQ affected cell viability, we first used the SH-SY5Y human neuroblastoma cell line, which has been used as a model of human midbrain dopaminergic neurons [56]. A dose-response experiment was initially performed to determine a concentration of 6-OHDA which was submaximal in its neurotoxic effect. SH-SY5Y cells were cultured in increasing concentrations of 6-OHDA (0-100 μM) for 24 h after which time viability was assessed by MTT assay. Increasing concentrations of 6-OHDA resulted in a dose-dependent reduction in cell viability (Fig. 1a). A 30-μM concentration of 6-OHDA was selected for subsequent experiments as this resulted in a 50 % reduction in cell viability, thus facilitating examination of any additive effects of N/OFQ.

SH-SY5Y cells were cultured in the presence of 30 μM 6-OHDA together with either 1 μM or 10 μM of N/OFQ for 24 h at which time an MTT assay was performed. While N/OFQ alone did not significantly reduce cell viability, there was a trend towards a reduction at the higher dose (Fig. 1b). Interestingly, we found that 1 μM or 10 μM N/OFQ significantly exacerbated the effects of 6-OHDA on cell viability (Fig. 1b). To confirm that this was mediated through the NOP receptor, a similar experiment was performed using the NOP agonist, UFP112 (3 μM) and cell viability was assessed using an MTT and LDH assay (Fig. 1c, d). We found using an MTT assay that UFP112 also exacerbated the effects of 6-OHDA on cell viability (Fig. 1c). This effect was prevented by the NOP antagonists UFP101 (10 μM) and SB-612111 (3 μM) (Fig. 1c). These findings were confirmed using a LDH assay (Fig. 1d). In agreement with the MTT assay (Fig. 1c), the NOP agonist, UFP112 combined with 6-OHDA resulted in significantly higher levels of LDH compared to 6-OHDA alone, an effect that was prevented by the NOP receptor antagonists (Fig. 1d). These data suggested that N/OFQ-NOP may affect DA neuronal survival. To investigate this further and to assess whether N/OFQ affected DA axons, we used primary cultures of the E14 rat VM.

N/OFQ Inhibits the Survival and Growth of Midbrain DA Neurons

To confirm our hypothesis that N/OFQ is detrimental to midbrain DA neurons, the effect of N/OFQ only on the survival and axonal growth of primary cultures of DA neurons was examined and compared to the effect of the neurotoxins, 6-OHDA and MPP^+. To do this, a dose

Table 1 GEO accession numbers and GSEs of individual data analysed

GEO series	Samples	Control samples	Parkinson's disease samples
GSE20295 (ref# [52])	GSM508717, GSM508723, GSM508725, GSM508726, GSM508730, GSM508708, GSM508720, GSM508721, GSM508722, GSM508724, GSM508729, GSM508733, GSM508734, GSM508735, GSM521253, GSM606624, GSM606625, GSM606626, GSM508711, GSM508712, GSM508713, GSM508714, GSM508731, GSM508710, GSM508715, GSM508716, GSM508718, GSM508728, GSM508732	18	11
GSE8397 (ref# [53])	GSM208630, GSM208631, GSM208632, GSM208633, GSM208634, GSM208646, GSM208647, GSM208648, GSM208649, GSM208650, GSM208651, GSM208636, GSM208637, GSM208638, GSM208639, GSM208640, GSM208641, GSM208642, GSM208643, GSM208644, GSM208653, GSM208654, GSM208655, GSM208656, GSM208657, GSM208658, GSM208659, GSM208660, GSM208661, GSM208662, GSM208663, GSM208664, GSM208665, GSM208666, GSM208667	11	24
GSE7621 (ref# [54])	GSM184355, GSM184356, GSM184357, GSM184358, GSM184359, GSM184360, GSM184361, GSM184362, GSM184363, GSM184364, GSM184365, GSM184366, GSM184367, GSM184368, GSM184369, GSM184370, GSM184371, GSM184372, GSM184373, GSM184374, GSM184375, GSM184376, GSM184377, GSM184378	9	16

response was initially performed. E14 rat VM cultures were prepared and cultured for 24 h in the presence of increasing concentrations of 6-OHDA (0–30 μM), MPP^+ (0–30 μM) and N/OFQ (0–500nM) and immunocytochemically stained for TH to visualize DA neurons and their axons. Treatment with 6-OHDA (Fig. 2a, b, g) and MPP^+ (Fig. 2c, d, g) significantly reduced the numbers of DA neurons and those with processes when compared to vehicle-treated controls. Intriguingly, N/OFQ was found to be as effective as both DA neurotoxins in reducing the numbers of DA neurons and those with axons (Fig. 2e–g).

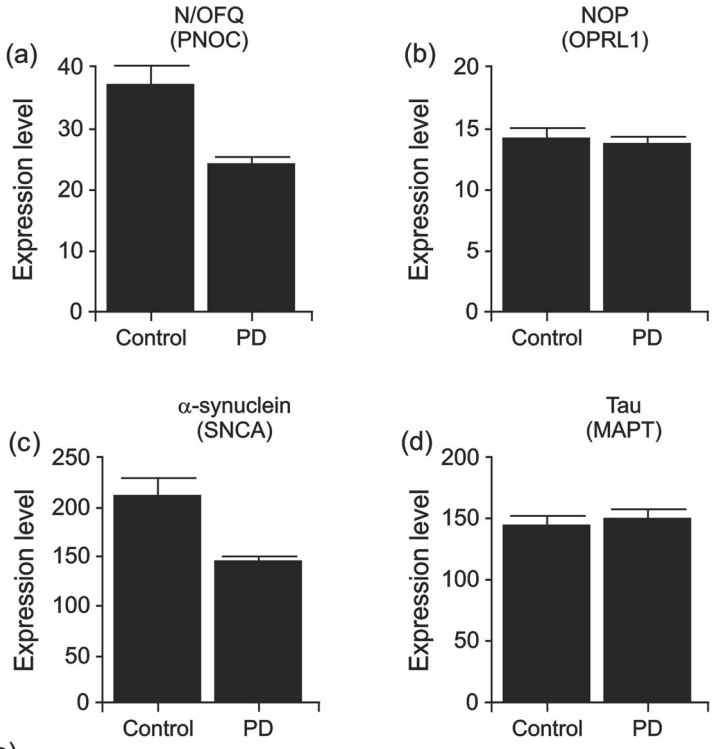

Fig. 7 Reduced expression of N/OFQ and a-synuclein in the human PD midbrain. **a**–**d** Gene expression comparisons for N/OFQ (PNOC), NOP (OPRL1), α-synuclein (SNCA) and Tau (MAPT) using microarray expression data derived from normal and Parkinson's disease SN (see Table 1). **e** Statistical comparisons between control and disease-derived samples were carried using three separate approaches: RANK products, *t* test (parametric) and Wilcoxon test (non-parametric). Charts show the mean ± SEM for the indicated genes comparing all resulting control ($n = 38$) and PD samples ($n = 51$)

(e)

T-Test

Gene	T	df	p value	Mean expression Ctrl	Mean expression PD	Standard error Ctrl	Standard error
PNOC	3.0912	43.144	**0.003487**	36.58820	23.90103	3.94473	1.133435
SNCA	3.5778	44.364	**0.0008524**	211.4323	144.2344	17.91606	5.636193
OPRL1	0.9431	75.159	0.3486	14.38566	13.71466	0.56089	0.4376973
MAPT	-0.6328	84.774	0.5285	142.5390	150.0206	8.40461	8.314332

Wilcoxon test

	W	p value
PNOC	1368	**0.0009485**
SNCA	1331.5	**0.002677**
OPRL1	1118.5	0.2165
MAPT	918.5	0.6783

RANK products

Probability of being **down** regulated in PD						
Symbol	**Ensembl ID**	**gene.index**	**RP/Rsum**	**FC:(Ctrl/PD)**	**Pfp**	**P.value**
PNOC	ENSG00000168081	8706	2720.7477	2.4872	0.025	**0.0022**
OPRL1	ENSG00000125510	4614	5076.6213	1.9794	1.4424	0.7984
SNCA	ENSG00000145335	6734	1212.0731	3.1872	0	0
MAPT	ENSG00000186868	10460	3910.5413	2.037	0.7738	0.1892

Probability of being **up** regulated in PD						
Symbol	**Ensembl ID**	**gene.index**	**RP/Rsum**	**FC:(Ctrl/PD)**	**Pfp**	**P.value**
PNOC	ENSG00000168081	8706	4712.1354	2.4872	1.2692	0.6272
OPRL1	ENSG00000125510	4614	5374.0251	1.9794	1.2566	0.899
SNCA	ENSG00000145335	6734	8697.7132	3.1872	1.0041	1
MAPT	ENSG00000186868	10460	4938.1543	2.037	1.301	0.7431

The effect of N/OFQ on the axonal growth of DA neurons was determined by analysing total neurite length and branching and by performing Sholl analysis, which provides a graphic illustration of neuronal complexity at defined distances from the cell body [50]. These effects of N/OFQ were next compared to those of 6-OHDA and MPP^+. Treatment with increasing concentrations of 6-OHDA (Fig. 3a–c) and MPP^+ (Fig. 3a, d, e) significantly reduced neurite length and neurite branching of DA neurons compared to vehicle-treated controls at the same time point. Similarly, it was found that increasing concentrations of N/OFQ also reduced neurite length and branching in a dose-dependent manner (Fig. 3a, f, g). These data were confirmed by the Sholl profiles which demonstrated that increasing concentrations of 6-OHDA (Fig. 4a, b), MPP^+ (Fig. 4c, d) and N/OFQ (Fig. 4e, f) resulted in a significant decrease in the Sholl profiles of DA neurons, indicating a decrease in the complexity of the neurite field. These data support the hypothesis that N/OFQ exerts a direct neurotoxic and neuritotoxic effect on midbrain DA neurons.

N/OFQ Potentiates the Effects of 6-OHDA and MPP^+ on DA Neurons

It was next examined if N/OFQ could potentiate the effects of 6-OHDA and MPP^+ on DA neurons. To do this, E14 rat VM cultures were cultured in sub-maximal concentrations of 6-OHDA (5 μM), MPP^+ (5 μM), N/OFQ (100 nM) or a combination of N/OFQ and 6-OHDA or MPP^+ for 24 h. Treatment with 6-OHDA, MPP^+ and N/OFQ significantly reduced the numbers of DA neurons with processes when compared to vehicle-treated controls at the same time point (Fig. 5a, b). However, the effects of combined N/OFQ and MPP^+ were significantly greater than either neurotoxin alone (Fig. 5a, b). Similarly, N/OFQ also exacerbated the effects of 6-OHDA and MPP^+ on neurite length (Fig. 5c) and in the Sholl profiles which indicated that the effects of combined N/OFQ and either 6-OHDA or MPP^+ on DA neuronal complexity were significantly greater than either 6-OHDA or MPP^+ alone (Fig. 5d, e).

N/OFQ Affects Midbrain DA Neurons Through a NOP-p38-MAPK Mechanism

The molecular mechanisms by which N/OFQ may exert its effects on midbrain DA neurons were subsequently examined. To determine if the effects of N/OFQ are mediated by the NOP receptor, a pharmacological inhibitor of the NOP receptor, SB612111 was utilized. A dose-response experiment was performed initially which established that treatment of primary cultures of the E14 rat VM with concentrations up to 10 μM SB612111 did not have any adverse effects on DA neurons (data not shown). To determine if the detrimental effects of N/OFQ on DA neurons were mediated by the NOP receptor, primary cultures of the E14 rat VM were pre-treated with 10 μM SB612111 for 1 h prior to treatment with 100 nM N/OFQ for 24 h. Pre-treatment with the NOP receptor antagonist protected DA neurons against the detrimental effects of N/OFQ on DA neurite length (Fig. 6a, b). This demonstrates that N/OFQ exerts its effects on DA neurons through the NOP receptor.

The intracellular mechanisms mediating the effects of N/OFQ on DA neurons may be through the modulation of a number of intracellular pathways. We hypothesized that p38 and/or JNK signalling may be involved as it has previously been demonstrated that these pathways affect DA neuron survival and furthermore that they are activated by N/OFQ, depending on the cellular context. Primary cultures of the E14 rat VM were pre-treated with the p-38 MAPK inhibitor, SB203580 or the JNK inhibitor, SP600125 (both at 30 μM; which does not affect DA neurons at this concentration) for 1 h prior to treatment with 100 nM N/OFQ for 24 h. Pre-treatment with SB203580, but not SP600125, protected DA neurons against the N/OFQ-mediated reductions in total neurite length (Fig. 6c), neurite branching (Fig. 6d, e) and in the Sholl profiles (Fig. 6f). Collectively, these data indicate that N/OFQ exerts its effects on DA neurons through a p38-MAPK-dependent mechanism.

Reduced N/OFQ and α-Synuclein mRNA Expression in the Human Midbrain in PD

We next examined N/OFQ and NOP receptor mRNA expression in the SN of PD patients and age-matched controls using microarray gene expression data. These data were obtained from three independent studies downloaded from Gene Expression Omnibus (GEO, series GSE20295 [52], GSE8397 [53] and GSE7621 [54]) (Table 1). Interestingly, we found a statistically significant down regulation of N/OFQ (Fig. 7a) but no change in the NOP receptor (Fig. 7b) in the PD SN compared to controls using three separate statistical tests RANK products [55], *t* test (parametric) and Wilcoxon test (non-parametric) (Fig. 7e). Interestingly, α-synuclein (SCNA) (Fig. 1c, e) also showed a strong down regulation in the PD SN (Fig. 7c, e), whereas in contrast, Tau (MAPT) mRNA levels were not different between controls and PD samples (Fig. 7d, e). These data provide the first evidence of alterations in N/OFQ expression in the human SN in PD that parallel those of α-synuclein.

Discussion

In this study, the direct effects of the neuropeptide N/OFQ on the survival and growth of midbrain DA neurons were examined. While we have previously reported that N/OFQ contributes to motor impairment in PD [24], and that elevated levels

of N/OFQ are found in the pre-clinical rodent models of PD as well as in patients with PD [24, 25], it was not known whether N/OFQ may contribute to the on-going degeneration of DA neurons, the pathological hallmark of the disorder. Since SNpc DA neurons express the NOP receptor [21, 22], we examined the effects of N/OFQ on survival and neurite growth in the SH-SY5Y neuroblastoma cell line, which has been used extensively as a model of human VM DA neurons [56, 57] and in primary cultures of the E14 rat VM, which is a widely used, experimentally tractable model for studying the physiological and pathological molecular mechanisms regulating these processes [42, 43, 58]. Here, we show that N/OFQ is as potent as the DA neurotoxins 6-OHDA and MPP^+ in reducing DA neuron survival and growth. These findings suggest that elevations of N/OFQ observed in the Parkinsonian brain may play a causative role in degeneration of DA neurons and their processes. In agreement with this hypothesis, we found that N/OFQ exacerbated the detrimental effects of MPP^+ and 6-OHDA on DA neuron survival and growth. This is supported by studies showing that deletion of the N/OFQ gene in mice conferred partial protection against MPTP-induced loss of SN dopamine neurons and against the loss of TH-positive DA nerve terminals in the caudate-putamen [24, 33]. These findings suggest that a perturbation in endogenous N/OFQ-NOP signalling may be involved in DA neuronal degeneration observed in PD. This suggestion is supported by the fact that NOP receptor antagonists reverse motor impairment in rodent [24, 26, 37, 59, 60] and non-human primate [26, 27] models of PD.

Interestingly, we also found that N/OFQ exerted a dose-dependent, detrimental effect on DA neuron survival and growth. Given that N/OFQ levels were found to be approximately 3.5-fold elevated in the CSF of Parkinsonian patients compared with non-parkinsonian neurologic controls [25], CSF profiling of endogenous N/OFQ levels may be a useful indicator of disease progression. This is supported by the finding that DA depletion was associated with increased mRNA N/OFQ levels in the SNpc, whereas no change was observed in globus pallidus, nucleus accumbens, thalamus and motor cortex [25], possibility indicating a specific relationship between N/OFQ and DA neurons.

In terms of molecular mechanisms, N/OFQ [38, 61] and opioids in general [39], not only act through the canonical Gi pathway but also through the MAP-kinase pathway most likely recruited by the $\beta\gamma$ subunits [38]. We hypothesized that p38-MAPK-kinase signalling specifically may be involved in mediating the effects of N/OFQ on DA neurons given that it has been shown to be activated by N/OFQ [38, 61] and multiple lines of evidence implicate p38-MAPK signalling in the DA degeneration observed in PD. Increased staining of phospho-p38 has been shown in the SNpc (but not VTA) DA neurons in human brain sections from PD patients and in MPTP-treated mice [62]. Furthermore, the DA neurotoxins 6-OHDA and MPTP activate p38-MAPK in SNpc DA neurons [40, 62, 63], and the vulnerability of DA neurons to neurotoxic insult is dependent, at least in part, upon p38-MAPK signalling [62, 64–67]. Moreover, neurotrophic factors and kinases which promote DA neuronal survival and neurite growth dampen p38-MAPK signalling, suggesting that activation of p38-MAPK signalling may contribute to DA degeneration. When we examined the molecular mechanisms by which N/OFQ affected DA neurons we found that the neurotoxic and neuritoxic effects of N/OFQ are dependent on p38-MAPK signalling. Indeed, we found that the detrimental effect of N/OFQ on DA neurons is prevented by a p38 but not a JNK inhibitor, implying that the neurotoxic and neuritoxic effects of N/OFQ are dependent on p38-MAPK signalling. Given that SNpc DA neurons express the NOP receptor [21, 22] and that high levels of N/OFQ are found in the SNpc and are increased in the CSF of PD patients [24, 34] along with elevated phospho-p38 [62], these data suggest that elevations in N/OFQ in the parkinsonian brain may act directly on DA neurons through the NOP receptor thereby activating p38-MAPK signalling which leads to DA neuron degeneration.

Under this perspective, the reduction of N/OFQ mRNA observed in the human SNpc might be viewed as a compensatory mechanism to prevent excessive NOP receptor stimulation and protect residual DA neurons. These data do not easily reconcile with previous findings showing an increase in N/OFQ expression in 6-OHDA hemilesioned rats [24, 25, 68] or MPTP/MPP^+-treated mice [68, 69], which correlate with the increase of N/OFQ levels monitored in the rat SNpr [24] and in the CSF of PD patients [25]. However, it is possible that the impact of acute (and selective) loss of SNpc DA neurons on N/OFQ transmission, as that produced by toxins in rodents, is different from that caused by chronic and progressive degeneration of DA and non-DA neurons in humans. Moreover, CSF samples processed in the Marti 2010 study [25] were taken from patients much earlier (mean age 60.6± 1.8), when compared to samples used in the Zhang et al. study (GSE20295 [52]; mean age 76.7 ± 6.2) and in the Moran et al. study (GSE8397 [53]; mean age: 80.0 ± 5.7). This suggests that PD progression might cause biphasic changes of N/OFQ expression in SN, reflecting plastic adaptations inside and outside the SN.

In summary, these results demonstrate for the first time that N/OFQ is detrimental to the survival and growth of DA neurons and support the theory that NOP receptor antagonists may represent a novel class of neuroprotective therapy for the protection of DA neurons and their neurite processes in PD.

Acknowledgements The authors acknowledge Dr. Sean Crampton for the technical assistance. This work was supported by grant support from Science Foundation Ireland (Grant No. :10/RFP/NES2786) (GO'K).

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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