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A substance P mediated pathway contributes to 6-hydroxydopamine induced cell death

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ABSTRACT

The neurotoxin 6-hydroxydopamine (6-OHDA) is used to induce dopaminergic cell death, resulting in insufficient striatal dopamine content in the basal ganglia and motor dysfunction typical of Parkinson's disease. Dopamine induces release of the neuropeptide substance P (SP) within the substantia nigra, whereas SP is able to potentiate striatal dopamine release, thus creating a positive feedback mechanism. Previous studies, however, have shown that elevated SP is detrimental to neuronal survival and motor function in acute brain injury. In the current study, we demonstrate that 6-OHDA increases SP production in meso-striatal organotypic co-culture. Moreover, there was a significant correlation between SP content and lactate dehydrogenase release, a marker of cell death, suggesting elevated SP production may contribute to 6-OHDA induced cell death *in vitro*.

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Parkinson's disease (PD) is the most common motor neurodegenerative disorder affecting 1–2% of the population over the age of 65 [1]. In PD, dysfunction of the basal ganglia (BG) occurs due to the degeneration of dopaminergic neurones from the substantia nigra (SN). This loss of neurones produces a depletion of striatal dopamine (DA), which is integral for proper functioning of the BG. Since the BG is integrally involved in the smooth execution of movement, motor function is therefore disrupted in PD.

The neuropeptide substance P (SP) is found in both the striatum and in particularly high levels within the SN. Here SP is involved in the release of DA by preferentially binding to the tachykinin NK1 receptor located on dopaminergic neurones. Furthermore, DA can potentiate the release of SP within the SN by binding to its receptor (D1) located on striatonigral projection neurones [12]. Thus regulation of DA and SP in the BG is by a positive feedback mechanism. There is accordingly a decrease in nigral SP expression due to the loss of striatal DA stimulation [4,16]. This loss of SP has been postulated to be involved in symptom presentation [4,23]. Notably, all of these observations have been made in post-mortem PD tissue that invariably presented with late PD. Little is known about SP production during early dopaminergic degeneration. This is significant given that elevated SP production has been shown to directly result in cell death [3], and more recently has been shown to be deleterious to overall motor function following both traumatic brain injury and stroke [5,21].

Organotypic cell culture models are often used to determine biochemical and physiological pathways at a cellular level. Importantly they retain the cytoarchitecture of adult tissue and therefore largely replicate *in vivo* environments [8,10,20]. Organotypic culture of the ventral mesencephalon (VM) and striatal tissue has been particularly useful for mechanistic and dopaminergic cell survival studies as interconnections between the striatum and VM are retained. Due to the controlled nature of the culture environment, determination of factors that result in dopaminergic cell death can be easily studied with use of various receptor agonists and antagonists.

The aim of the present study was to use *in vitro* meso-striatal organotypic co-culture to characterize the effects of 6-OHDA treatment on SP expression, and to investigate whether elevated SP is associated with cell death.

All experimental protocols were conducted according to the guidelines established by the National Health and Medical Research Council of Australia and were approved by the animal ethics committee of the Institute of Medical and Veterinary Services, Adelaide, Australia. At all times, adequate measures were taken to minimize pain or discomfort in all animals.

Organotypic slices were prepared from brains of Sprague–Dawley pups of postnatal days 4–6 according to the procedures previously described by Gahwiler and Stoppini et al. [6,7,20] with minor modifications. Briefly, pups were decapitated and the brain gently removed from the skull onto a sterile chilled

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Petri dish, ventral surface upwards. The VM and the striatum were dissected out and coronal sections of 200 μm and 300 μm were cut, respectively. Sections were gently teased apart then carefully transferred to sterile Petri dishes containing chilled Gey's balanced salt solution with 0.1% D-glucose and 0.1% potassium chloride. The tissue was placed at 4 °C for 90 min before mounting slices on a plasma clot of chick plasma and thrombin ensuring that the VM and striatal tissue were no more than 1 mm apart. The coverslips were then placed into a sterile 24-well plate and left for 30 min before 0.5 ml of culture medium (50% DMEM; 25% Hanks balanced salt solution with 10 nM HEPES; 25% heat inactivated horse serum; supplemented with glucose, glutamine and 0.5% gentamycin and penicillin) was added. Plates were placed into a 37 °C incubator with 5% CO_2. After 4 *days in vitro*, cultures were treated with antimitotic agents cytosine-β-D-arabinofuranoside and uridine for 24 h to retard glial and fibroblast growth. Media was changed every 3–4 days ensuring cultures remained sterile.

At day 19 *in vitro* cultures were treated with 0.6 ml of medium containing 6-hydroxydopamine (6-OHDA; 200 μM, Sigma) with NMDA (200 μm, Sigma) and glycine (10 μM, Sigma) for 1 h. A 1-h exposure time for 6-OHDA was used based on the previous study by Kress and Reynolds (2005) who demonstrated that it produces a substantial loss of DA neurones [11]. In a pilot study, 6-OHDA treatment at either 0.5, 1 or 2 h produced an increase in SP content compared to non-treated controls (data not shown), indicating that 6-OHDA treatment *in vitro* generates an initial increase in SP production. Like Kress and colleagues, however, our pilot study confirmed that NMDA and glycine needed to be included in the treatment regime as 6-OHDA treatment alone did not induce cell death (data not shown). Additional cultures were treated with SP (10 μM; Sigma), the NK1 antagonist, N-acetyl-L-tryptophan (NAT; 10 μM; Sigma) or a combination of 6-OHDA with SP or NAT for 1 h. All treatment solutions were sterile filtered before being added to cultures. Media was changed following treatment and at the same time for the consecutive 3 days. All removed media was frozen at −80 °C.

The presence of DA neurones and their neurite outgrowth was determined with tyrosine hydroxylase (TH) immunohistochemistry. Cultures were fixed by adding 1 ml of 10% neutral buffered formalin solution to each well for 10 min following removal of media. Cultures were washed with phosphate buffered saline (PBS; 2×) before 1 ml of PBS with 0.1% Triton X-100 was added for 15 min. Following washes in PBS (2×), 1 ml of PBS with 0.5% hydrogen peroxide was added to each culture for 15 min before being washed in PBS (2×). Normal horse serum (0.5 ml of 3% NHS) was added for 15 min to each well before NHS was removed and TH primary antibody (1:4000, Chemicon, ab151) added to each culture and left to incubate at room temperature overnight. Following washes with PBS (2×), secondary anti-rabbit biotinylated antibody (Vector BA-1000, 1:250) was added for 30 min at room temperature before cultures were washed (2×) and incubated with tertiary streptavidin peroxidase conjugate (SPC; Pierce, 1:1000) for 1 h at room temperature. Finally following washes (2×), the immunocomplex was visualised with 3,3′-diaminobenzidine (DAB, Sigma D-8001) before cultures were counterstained with half strength haematoxylin and mounted with DPX. Cultures were visualised under light microscopy for TH immunoreactive neuronal growth.

The level of SP contained in the media, and the production of lactate dehydrogenase (LDH) as a measure of cell death, was determined using ELISAs. Samples (100 μL; 20 μL of media to 80 μL of Tris Buffered Saline; TBS) were loaded in triplicate into a Maxisorp plate. Blank wells with no loaded protein were included as SP controls while 400 ng/100 μL of bovine serum albumin (BSA) was loaded as LDH controls. Samples were left overnight at 4 °C before they were tipped off and blocking agent (0.2% gelatine solution for SP or 3% BSA for LDH) added to each well. Plates were then gently agitated at room temperature for 1 h. Wells were washed in TBS (3×) then incubated with 100 μL of primary antibody (SP Chemicon; LDH Abcam; 1:1000) at 37 °C for at least 1 h in a humid container. Following washes in TBS (3×), wells were incubated with 100 μL of secondary antibody (SP = anti-rabbit horseradish peroxidase conjugate; HRP; Rockford, 1:1000; LDH = anti-goat HRP, Sigma, 1:2000) for 1 h at 37 °C oven in a humid container. Wells were washed in TBS (4×) and the liquid substrate system 3,3′,5,5′-tetramethylbenzidine (Sigma) used to reveal protein expression by adding 100 μL to each well. The reaction was stopped with 50 μL of 0.5 M sulphuric acid. SP and LDH content was determined by reading the absorbance at 450 nm on an Ascent Multiskan plate reader.

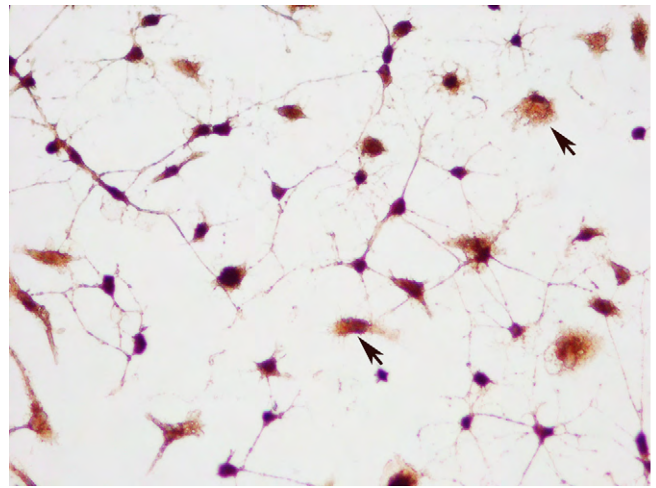

Fig. 1. Tyrosine hydroxylase (TH) immunoreactive neurones in meso-striatal co-cultures following 6-OHDA treatment. Neurite outgrowth and interconnections are apparent between neurones. Black arrows denote dead TH immunoreactive neurones.

SP and LDH ELISA data were examined using analysis of variance (ANOVA) followed by Bonferroni post hoc tests and displayed as mean ± SEM. A Pearson's test was performed to determine correlation between SP and LDH content. Significance was set at $p < 0.05$.

At day 19 meso-striatal co-cultures were well established with good tissue growth. TH immunohistochemistry confirmed the presence of dopaminergic neurones with neurite outgrowth and obvious interconnections. Neuritic outgrowth of TH immunoreactive neurons did not occur when only organotypic cultures of VM were grown (data not shown). Following 6-OHDA treatment, TH immunoreactive cells that appeared dead were also present in cultures (Fig. 1).

Dose response studies for SP and the NK1 antagonist, NAT, were undertaken to determine the optimal dose for use in organotypic culture (results not shown). The optimal dose of 10 μM for both treatments was used to examine the effects of SP and NAT treatment alone and a combination of 6-OHDA with SP or NAT. A non-treated control was also included. The non-treated control had significantly greater SP content than blank control ($p < 0.001$; data not shown), suggesting that meso-striatal organotypic culture express SP under normal conditions. At day 1 following treatment, 6-OHDA treatment resulted in a small but non significant increase in SP production compared to controls (Fig. 2A). Similarly, despite the change in media, SP treatment alone also caused a slight rise in SP content compared to control at day 1 post-treatment. However when SP treatment was combined with 6-OHDA, a highly significant increase in SP content compared to controls was produced ($p < 0.01$). Furthermore, this increase in SP was greater than the SP release generated by SP or 6-OHDA treatment alone, suggesting 6-OHDA with SP generates a cumulative effect on SP production. Treatment with the NK1 antagonist, NAT, both alone and in

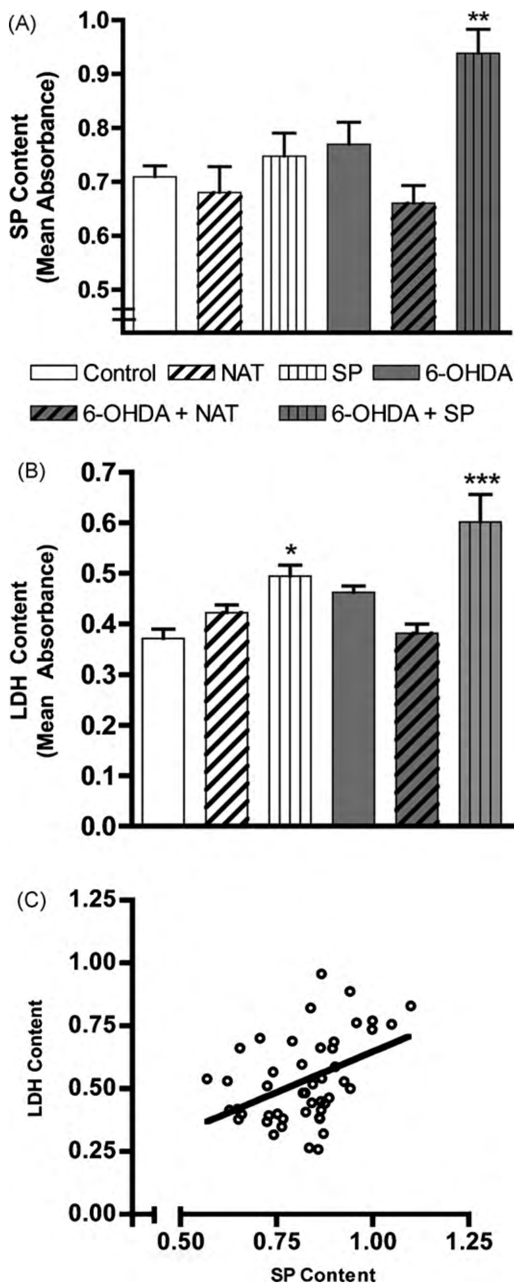

Fig. 2. (A) Effects of combined treatment of 6-OHDA with SP or the NK1 receptor antagonist, N-acetyl-L-tryptophan (NAT) on substance P (SP) content at day 1 post-treatment. Bars denote effects of the following treatments: control (non-treated), NAT (10 μM), SP (10 μM), 6-OHDA (200 μM + NMDA 10 μM + glycine 10 μM), 6-OHDA + NAT, 6-OHDA + SP. Post hoc tests established differences in SP production between 6-OHDA + SP (**$p < 0.01$) to control (ANOVA; $n = 8$/group). (B) Effects of combined treatment of 6-OHDA with SP or NAT on lactate dehydrogenase (LDH) content at day 3 post-treatment. Bars denote effects of the following treatments: control (non-treated), NAT (10 μM), SP (10 μM), 6-OHDA (200 μM + NMDA 10 μM + glycine 10 μM), 6-OHDA + NAT, 6-OHDA + SP. Post hoc tests established differences in LDH production between SP (*$p < 0.05$) and 6-OHDA + SP (***$p < 0.001$) to control (ANOVA; $n = 8$/group). (C) Correlation between SP and LDH content at 1 and 2 days following treatment: control (non-treated), NAT (10 μM), SP (10 μM), 6-OHDA (200 μM + NMDA 10 μM + glycine 10 μM), 6-OHDA + NAT, 6-OHDA + SP ($n = 8$/group). Linear regression (Pearson) established a correlation between SP and LDH content ($p = 0.002$; $r = 0.44$).

combination with 6-OHDA, resulted in a reduction in SP content compared to both 6-OHDA treatment alone and non-treated controls. Thus, treatment with an NK1 antagonist can attenuate the 6-OHDA induced rise in SP seen at day 1 post-treatment in meso-striatal organotypic culture.

LDH content of non-treated controls was significantly greater than the BSA control ($p < 0.001$, data not shown), indicating that organotypic cultures produce LDH. NAT treatment alone did not produce a significant rise in LDH compared to controls, whereas the SP alone treated group had a significant increase in LDH production compared to control ($p < 0.05$; Fig. 2B). 6-OHDA treatment resulted in an increase in LDH content, which returned to control levels when combined with NAT. Thus, 6-OHDA induced cell death could be attenuated through inhibition of SP. In contrast, when SP treatment was combined with 6-OHDA, a highly significant increase in LDH production was observed compared to controls ($p < 0.001$). This group also showed significant increases in LDH content compared to 6-OHDA treatment alone ($p < 0.05$). Accordingly, combination treatment of SP with 6-OHDA exacerbated cell death as measured by LDH production, whereas inhibition of 6-OHDA toxicity using the NK1 antagonist attenuated cell death.

SP content had returned to control levels in all treatment groups by day 2 post-treatment, therefore SP content was not determined past this time point. Combining absorbance readings of LDH and SP content from days 1 and 2 post-treatment demonstrated significant correlation ($r = 0.44$; $p = 0.002$) between elevated SP content and increased LDH production (Fig. 2C).

6-OHDA, a hydroxylated analogue of dopamine, has been used to study PD since 1968 when Ungerstedt [22] demonstrated that an injection of 6-OHDA into the SN resulted in anterograde degeneration of dopaminergic neurones. Like DA, the compound is taken up into neurones by dopamine transporters where it accumulates in the cytosol. It is then either rapidly deaminated by monoamine oxidase or undergoes autooxidation, both of which result in the formation of hydrogen peroxide and reactive oxygen species (ROS) [19]. Glutamate excitotoxicity, which results in high intracellular calcium, also plays a major role in 6-OHDA induced cell death in both cell culture systems and *in vivo*. Indeed in previous studies, glutamate and intracellular calcium levels are elevated in deteriorating cultures exposed to 6-OHDA [2,9].

The neuropeptide SP is located in DA modulated medium spiny projection neurones of the striatum and within the SN, where SP-containing striatal projection neurones make synaptic connections with nigral dopaminergic neurones. Therefore, as 6-OHDA is mechanistically similar to DA, the initial increase in SP by 6-OHDA observed in this study may be due to the ability of 6-OHDA to bind to DA receptors (D1) on SP-containing striatal projection neurons initiating SP release. In addition, DA neurones express NK1. Thus the endogenous SP released from these projection neurones can bind to NK1 receptors to potentiate the release of DA. As the interconnections in meso-striatal organotypic co-cultures resemble those *in vivo*, SP and DA production is controlled through a positive feedback mechanism in this *in vitro* system. Although DA production was not directly measured in this study, the increased SP production caused by 6-OHDA is likely through this feedback mechanism. Furthermore, the combined treatment of SP and 6-OHDA caused a significant increase in SP, which was greater than that produced by either treatment alone. This suggests a cumulative effect in SP production and further confirms that the SP and DA interactions in meso-striatal organotypic co-culture are indeed intact and resemble those *in vivo*.

Activation of the SP NK1 receptor, a G-protein coupled receptor, initiates a biochemical cascade that results in increased turnover of intracellular inositol 1,4,5-triphosphate and elevation in intracellular Ca^{2+}. Located on the NK1 receptor is a cAMP binding protein that responds to elevated cAMP or Ca^{2+} by increasing gene transcription, thereby creating a positive feedback for SP [18]. Furthermore, activation of NK1 facilitates the protein kinase C phosphorylation of NMDA receptors and glutamate release [14]. Indeed, activation of NK1 by SP, along with DA receptor activation is required for striatal glutamate release *in vivo* [15]. Thus SP can potentiate both

DA and glutamate release and therefore oxidative stress and glutamate excitotoxicity, both of which contribute to dopaminergic cell death.

LDH production is a widely accepted marker of *in vitro* cell death. In the current study there was a significant correlation between SP and LDH content in culture media during the subsequent 2 days following treatment, with greater SP content resulting in high LDH levels. This suggests that higher than basal levels of SP may cause cell death in meso-striatal organotypic co-cultures. Further confirmation that SP contributes to cell death was the reduction in LDH production when cultures were co-treated with the NK1 antagonist, NAT, which is a highly potent specific antagonist for the NK1 receptor [13]. The NK1 receptor, which is located on dopamine neurones, is the preferential receptor for SP, although SP can also bind to NK2 and NK3 [18]. The NK3 receptor is also expressed by dopaminergic neurones and thus may also play a role in dopaminergic cell death. However, as NAT is specific for the NK1 receptor, these results confirm a role for SP and activation of NK1 receptor in dopaminergic cell death *in vitro*.

Unfortunately, it cannot be definitively stated that elevated levels of SP is specifically associated with dopaminergic cell death, as increased LDH is a non-specific marker of tissue injury. However as meso-striatal organotypic co-culture resemble *in vivo* conditions, dopaminergic neurones are likely to be the first to undergo cell death due to their pre-existing state of oxidative stress caused by normal DA metabolism [17]. Indeed, cultures that had been treated with 6-OHDA displayed TH immunoreactive neurons that were dead in appearance.

In conclusion, treatment with 6-OHDA in meso-striatal organotypic co-culture increased SP production and exacerbated cell death, as demonstrated by increased LDH content. LDH production was further increased when SP was combined with 6-OHDA treatment, yet was reduced when 6-OHDA was combined with the NK1 antagonist, NAT. These results indicate that 6-OHDA induced cell death is mediated, in part, by SP through a mechanism that can be blocked using an NK1 receptor antagonist. NK1 antagonists may therefore be a useful avenue of investigation for the attenuation of dopaminergic cell death associated with Parkinson's disease.

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