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Investigation of the therapeutic potential of *N*-acetyl cysteine and the tools used to define nigrostriatal degeneration *in vivo*

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Abstract

The glutathione precursor *N*-acetyl-L-cysteine (NAC) is currently being tested on Parkinson's patients for its neuroprotective properties. Our studies have shown that NAC can elicit protection in glutathione-independent manners *in vitro*. Thus, the goal of the present study was to establish an animal model of NAC-mediated protection in which to dissect the underlying mechanism. Mice were infused intrastrially with the oxidative neurotoxicant 6-hydroxydopamine (6-OHDA; 4 µg) and administered NAC intraperitoneally (100 mg/kg). NAC-treated animals exhibited higher levels of the dopaminergic terminal marker tyrosine hydroxylase (TH) in the striatum 10d after 6-OHDA. As TH expression is subject to stress-induced modulation, we infused the tracer FluoroGold into the striatum to retrogradely label nigrostriatal projection neurons. As expected, nigral FluoroGold staining and cell counts of FluoroGold⁺ profiles were both more sensitive measures of nigrostriatal degeneration than measurements relying on TH alone. However, NAC failed to protect dopaminergic neurons 3 weeks following 6-OHDA, an effect verified by four measures: striatal TH levels, nigral TH levels, nigral TH⁺ cell counts, and nigral FluoroGold levels. Some degree of mild toxicity of FluoroGold and NAC was evident, suggesting that caution must be exercised when relying on FluoroGold as a neuron-counting tool and when designing experiments with long-term delivery of NAC—such as clinical trials on patients with chronic disorders. Finally, the strengths and limitations of the tools used to define nigrostriatal degeneration are discussed.

Keywords

Parkinson's disease; *N*-acetylcysteine; Dopamine; Neurodegeneration; Retrograde; FluoroGold; The reference trap

1. Introduction

The glutathione precursor *N*-acetyl-L-cysteine (NAC) has been tested on humans in multiple clinical trials, many of which have shown positive results. For example, NAC improved some aspects of cognition in patients with Alzheimer's disease (Adair et al., 2001). NAC

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doubled the chances of symptom resolution from 42% to 86% in soldiers experiencing traumatic brain injury (Hoffer et al., 2013). NAC has been shown to ameliorate the depressive symptoms associated with bipolar disorder (Berk et al., 2008b) and to mitigate the negative symptoms and akathisia associated with schizophrenia (Berk et al., 2008a). Recent studies have shown that NAC increases glutathione levels in the brain of patients with Parkinson's disease, supporting the view that NAC can indeed cross the blood-brain barrier (Holmay et al., 2013; Katz et al., 2015). NAC is also currently being tested for its neuroprotective properties in Parkinson's patients ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT01470027) identifier: NCT01470027).

Because of its well-established antioxidant properties, NAC is one of the most frequently used positive controls in cellular and animal studies of neuroprotection. For example, we recently showed that NAC prevents neurodegeneration in multiple cellular models of neurodegenerative disorders (Jiang et al., 2013; Posimo et al., 2013; Unnithan et al., 2012; Unnithan et al., 2014). Consistent with these observations, Clark and colleagues have shown that NAC can prevent dopaminergic terminal loss and α -synucleinopathy in a transgenic mouse model of Parkinson's disease (Clark et al., 2010). Second, Berman and colleagues have shown that NAC can reduce the loss of dopaminergic neurons in EAAC1^{-/-} mice, which suffer impairments in cysteine uptake (Berman et al., 2011). Third, NAC has been shown to protect against dopaminergic loss in the MPTP model of Parkinson's disease (Pan et al., 2009; Perry et al., 1985; Sharma et al., 2007). Fourth, Munoz and colleagues reported that subcutaneous NAC robustly protects against the loss of dopaminergic neurons in the 6-hydroxydopamine (6-OHDA) model of Parkinson's disease (Munoz et al., 2004), consistent with studies showing that NAC prevents 6-OHDA toxicity in dopaminergic cells *in vitro* (Choi et al., 1999) and that NAC reduces oxidative stress in 6-OHDA-treated animals *in vivo* (Aluf et al., 2010). However, striatal 6-OHDA infusions are known to lead to progressive dopaminergic cell death over the course of several weeks (Sauer and Oertel, 1994) and it is not yet clear if systemically delivered NAC will prevent nigral cell loss at longer timepoints after 6-OHDA. The Munoz study also did not report dopaminergic neuron measurements in animals treated with NAC by itself (in the absence of 6-OHDA). If NAC raises TH expression in both the absence and presence of 6-OHDA, one cannot then conclude that it changes the impact of 6-OHDA. Furthermore, the impact of systemic NAC on dopaminergic cells in the contralateral substantia nigra was also not reported. Most notably, the mechanism underlying NAC-mediated protection of the nigrostriatal pathway has not been definitively established with, for example, the use of glutathione synthesis inhibitors. This gap is an important one to fill because NAC can engage in glutathione-dependent and glutathione-independent effects. For example, the aforementioned study by Clark and colleagues reported that NAC protected striatal dopaminergic terminals without a long-term increase in glutathione synthesis (Clark et al., 2010). NAC can activate NF κ B and increase Mn superoxide dismutase levels (Das et al., 1995). NAC may also protect cells by increasing phospho-ERK (Sun et al., 2012; Yan and Greene, 1998; Zhang et al., 2011). NAC can induce AP-1 activity by the sequential activation of ERK and Elk-1, which, in turn, binds to the *c-Fos* promoter and induces *c-Fos* expression (Meyer et al., 1993; Muller et al., 1997). We have also shown that NAC can protect cells in a heat shock protein 70-dependent manner (Jiang et al., 2013), perhaps through thiol exchanges with transcription factors that

regulate synthesis of this protein, such as heat shock factor 1. Thus, the goal of the present study was to develop an animal model in which the molecular mechanisms underlying the protective effects of NAC could be dissected.

To improve the rigor of our study and identify potentially transient effects of NAC, we examined TH levels in animals at two separate timepoints: 10 days and 3 weeks after the 6-OHDA insult. This served to determine if the protective effects of NAC were transient or long lasting in the 6-OHDA model, which is relevant for the treatment of chronic, progressive neurodegenerative disorders such as Parkinson's disease. Furthermore, the academia/industry roundtable preclinical study recommendations known as STAIR guidelines for stroke studies recommends sacrificing animals between 2 and 3 weeks after injury (Fisher et al., 2009), as accomplished here. Third, we examined dopaminergic cell loss both at the level of the axon terminal in the striatum and the soma in the substantia nigra in order to ensure the neuroprotection spanned the entire nigrostriatal pathway, in contrast to the aforementioned Clark study (Clark et al., 2010). Fourth, we estimated protection of nigral cell bodies by three methods: 1) overall expression of the dopaminergic marker tyrosine hydroxylase (TH) in the ventral mesencephalon with a low-resolution, high-sensitivity infrared Odyssey imager, 2) counts of TH⁺ cell bodies in the substantia nigra by higher-resolution microscopy, and 3) retrograde labeling of nigrostriatal projection neurons with the fluorescent tracer FluoroGold (Schmued and Fallon, 1986; Wessendorf, 1991). The fourth of these methods involved measurements of FluoroGold⁺ immunoreactivity on the Odyssey Imager as well as FluoroGold⁺ cell counts with a higher resolution microscope. FluoroGold has been widely used as a retrograde tracing tool in dozens of studies employing the 6-OHDA model (for some examples, see Anastasia et al., 2009; Aymerich et al., 2006; Choi-Lundberg et al., 1998; Cohen et al., 2011; Ebert et al., 2007; Kozłowski et al., 2000; Mandel et al., 1997; Sauer and Oertel, 1994; Yamada et al., 1999). We specifically employed FluoroGold as a marker of dopaminergic neurons because TH is subject to modulation by stress (Baruchin et al., 1990; Chang et al., 2000; Kumer and Vrana, 1996; Tank et al., 2008; Tekin et al., 2014). If TH levels rise and fall in individual cells through stress-induced changes in gene expression, measurements of overall TH expression and TH⁺ cell counts may not be in proportion to changes in nigrostriatal cell numbers, which could confound cell count data. In contrast to TH, FluoroGold is not an endogenous protein. Thus, loss of FluoroGold immunoreactivity in this system is expected to reflect true loss of dopaminergic cell numbers (Sauer and Oertel, 1994). Furthermore, the use of FluoroGold as a retrograde tract-tracer permits us to selectively label those dopamine neurons that send efferent projections along the nigrostriatal pathway to the site of infusion, which is important because it is the degeneration of this pathway that is thought to underlie the motor deficits in Parkinson's disease.

2. Methods

2.1. Antibodies and chemicals

Primary and secondary antibodies and their dilutions are listed in Table 1. 6-OHDA was purchased from Sigma-Aldrich (H116-5MG, Sigma-Aldrich, St. Louis, MO) and stored at -80 °C at a concentration of 2.5 µg/µL in saline with 0.02% ascorbic acid to reduce auto-

oxidation. NAC was purchased from Acros Organics (160280250, Acros Organics, Morris, NJ), dissolved in phosphate-buffered saline (PBS), sterile-filtered and stored at a concentration of 22.5 mg/mL at -20°C until use. FluoroGold was purchased from FluoroChrome (Fluorochrome LLC, Denver, CO) and stored at a concentration of 1.5% in PBS at 4°C until use.

2.2. Animals and surgeries

All animal housing and treatments were in accordance with the *NIH Guide* and approved by the Duquesne University Institutional Animal Care and Use Committee. Male CD1 mice from Charles River (Horsham, PA) were bred in the Duquesne vivarium and housed in a 12:12 photoperiod with *ad libitum* access to food and UV-disinfected water. On the day of surgery, animals were anesthetized with 2–3% isoflurane vapors in 3% oxygen and stabilized in a stereotaxic frame. FluoroGold (6 μg in 0.4 μL), 6-OHDA (4 μg in 1.6 μL) or an equal volume of vehicle was infused into the caudoputamen (ML -2.0 mm, AP $+0.5$ mm, and DV -3.2 mm from bregma) with a Hamilton syringe (80085, Hamilton, Reno, NV) over the course of 4 min. For the studies employing FluoroGold, the tracer was delivered one week before 6-OHDA infusions in the same striatal location so that retrograde uptake by healthy dopaminergic neurons could proceed in an unimpeded fashion. The doses chosen for 6-OHDA and FluoroGold are consistent with or lower than doses reported in the recent literature (Bagga et al., 2015; Cohen et al., 2011; Lazzarini et al., 2013; Liang et al., 2008), and were also based on our own pilot studies to ensure lack of striatal necrosis. The needle was withdrawn 4 min after the infusion was completed to minimize diffusion into dorsal structures during withdrawal. Animals were placed on a warm surface until resumption of spontaneous locomotor activity and then returned to their cages until sacrifice. All animals received 0.015 mg/kg buprenorphine subcutaneously during recovery from surgery and antibiotic treatment of their scalp wound. In the NAC experiments, animals received 100 mg/kg NAC in PBS or an equal volume of vehicle (4.44 mL/kg body weight or 0.176 mL per 40 g mouse) as a daily intraperitoneal injection using an insulin syringe with a small diameter (309311, Becton Dickinson, Franklin Lakes, NJ). These daily injections were initiated on the day of the 6-OHDA surgery, immediately after the surgery was completed. At the end of the experiment, animals were reanesthetized with isoflurane and perfused through the left ventricle with 50 mL of saline followed by 100 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (9990244, Thermo Scientific, Kalamazoo, MI).

2.3. Histology

Brains were immersed in 30% sucrose in 10 mM PBS for 48 h and cut on a freezing microtome in the coronal plane. Free-floating sections cut 50 μm in thickness were stored in cryoprotectant at -20°C until immunostaining was performed. Cryoprotectant was rinsed off with three exchanges of PBS and sections were then incubated in a 1:1 solution of Odyssey Block (LI-COR Bioscience, Lincoln, NE) in PBS for 1 h at room temperature on a shaker. Sections were subsequently incubated in primary antibodies at the concentrations listed in Table 1 for 24–48 h on a shaker at 4°C . Unbound primary antibodies were rinsed off with three exchanges of PBS and sections were then incubated in the appropriate fluorescent secondary antibodies as indicated in Table 1, for 1 h on a shaker at room temperature. The Hoechst 33258 reagent (bisBenzimide) was added during the secondary

incubation at a concentration of 0.005 μ M to label nuclei in animals that did not receive FluoroGold (which also fluoresces under UV illumination). Following six washes in PBS, sections were mounted onto glass slides (Superfrost Plus, Fisher Scientific, Pittsburgh, PA) and coverslipped with FluoroMount G (Southern Biotech, Birmingham, AL). Sections were scanned on an infrared Odyssey Imager for lower resolution analyses (21 μ m resolution) or under epifluorescent microscopy (Olympus IX73, B&B Microscopes, Pittsburgh, PA) for higher resolution microscopy in the visible wavelengths, using 4 $\times$ and 10 $\times$ objectives.

2.4. Image analyses

For the infrared measurements of TH levels, regions of interest (ROIs) were traced in three sections by a blinded observer along the boundaries of the striatum, using the anterior commissure, lateral ventricle, and corpus callosum as anatomical landmarks. For the measurements of nigral TH or FluoroGold levels, the nigra was defined by the dorsal boundaries of the TH immunostaining, the lateral boundaries of the ventral tegmental area, and the ventrolateral outer surface of the brain. The ventral tegmental area was distinguished from the substantia nigra by the location of the medial terminal nucleus and the medial lemniscus. For the bilateral cell counts in the substantia nigra, images of the ipsilateral and contralateral nigrae were stitched on the Olympus microscope and a blinded observer counted all nigral TH⁺ cells in three ventral midbrain sections per animal using the manual count tool in ImageJ software (NIH Image, Bethesda, MD). Photographs from all animal groups were captured at the same exposures and with the same intensity scaling. Two independent raters counted nigral cells on select sections, and the results were found to be in agreement. Finally, sections from FluoroGold-infused animals were viewed under confocal microscopy to assess colocalization of FluoroGold with TH (Olympus Fluoview 1200 confocal system on an IX83 inverted frame). All confocal images were captured using a 40 $\times$ silicone oil objective (NA 1.25) with a gain of 1.0. In the confocal studies, the UV-illuminated FluoroGold staining was pseudocolored red to contrast with the green TH.

2.5. Statistical analyses

Data are presented as the mean and SEM in all figures. Depending on the number of independent variables, data were analyzed by one, two, or three-way ANOVA followed by the Bonferroni *post hoc* correction (SPSS Version 20, Armonk, NY). When there were only two groups, the two-tailed, unpaired Student's *t*-test was performed. Differences were deemed significant only when $p < 0.05$. The Grubb's outlier test was performed, but no statistical outliers were found. In Figs. 1 and 2, all experiments had an *n* of 8 per group, with the exception of the 6-OHDA + NAC group, in which one animal was sacrificed due to low bodyweight. In Figs. 3 and 4, all experiments had an *n* of 6 per group, with the exception of the 6-OHDA + PBS group, in which one brain was excluded due to inadequate saline/formaldehyde perfusion and the 6-OHDA + NAC group, in which one animal died. In Figs. 5 and 6, all experiments had an *n* of 8 per group, with the exception of 1) the saline + PBS group, in which two animals died, 2) the 6-OHDA + PBS group, in which one brain was excluded due to poor perfusion, and 3) the saline + NAC group, in which 3 animals died.

3. Results

In the present study we tested the hypothesis that NAC would protect dopaminergic terminals of the nigrostriatal pathway against the oxidative toxicity of 6-OHDA. In the first set of studies, NAC was injected intraperitoneally on a daily basis beginning immediately after 6-OHDA surgeries until sacrifice 10 days later (Fig. 1). Brain sections were then immunohistochemically stained for TH protein and imaged on a high-sensitivity infrared imager. As expected, 6-OHDA elicited significant toxicity in dopaminergic terminals of the ipsilateral striatum. This was evident when ipsilateral TH levels were divided by contralateral TH levels and expressed as a ratio, a common method of presenting striatal TH in the literature that takes into account inter-animal variability in the quality of immunohistochemical staining (Fig. 1A). NAC did not change the impact of 6-OHDA on loss of ipsilateral/contralateral TH. However, when we examined the right and left hemispheres individually, we found that NAC raised TH expression in the contralateral hemisphere in 6-OHDA-treated animals (Fig. 1B). In addition, there was a trend towards a NAC-mediated increase in TH levels in the ipsilateral hemisphere in 6-OHDA-treated animals ($p = 0.058$, two-way ANOVA followed by Bonferroni *post hoc* correction). Striatal area was not significantly changed in any group (Fig. 1C). Although not typically acknowledged in the literature, TH signal in the striatum is sensitive to changes in striatal area, as tracing a bigger region of interest leads to greater TH signal in proportion to the larger size of the trace. Thus, we also expressed TH levels per unit striatal area, in order to approximate the level of TH expression per dopaminergic axon terminal, *i.e.*, the “density” of striatal TH. When we expressed striatal TH signal as a function of striatal area (Fig. 1D), we discovered that NAC raised TH expression in both hemispheres in 6-OHDA-infused mice. These data support the view that NAC can cross the blood-brain barrier and protect against experimental Parkinson's disease 10 days after a severe oxidative insult and are consistent with previous work by Munoz et al. (2004).

In order to determine whether there was concomitant neuroprotection of cell bodies, we first used the infrared imager to screen for changes in the density of TH label in the substantia nigra (Fig. 2). In contrast to the striatum, overall TH expression in the nigra was not significantly affected by NAC treatment (Fig. 2A). 6-OHDA caused significant loss of ipsilateral nigral TH compared to the contralateral side in both PBS and NAC-infused animals (Fig. 2A). The areas of the ipsilateral and contralateral substantia nigrae were significantly reduced by 6-OHDA in both the saline and NAC-treated mice, suggesting commissural effects of the oxidative toxicant (Fig. 2B). When we expressed TH signal as a function of nigral area, 6-OHDA elicited an increase in TH density in the contralateral nigra—a potentially compensatory effect that would not be apparent had we only performed higher resolution dopaminergic cell counts (Fig. 2C). When we expressed the ipsilateral TH signal density as a function of TH levels in the contralateral hemisphere, 6-OHDA was not significantly toxic in the NAC-treated animals (Fig. 2D). On the other hand, NAC did not significantly raise this measure relative to the animals infused intraperitoneally with PBS. We therefore interpret these data as lack of significant protection of the somata. When we performed TH⁺ cell counts in the nigra, we also did not observe a significant neuroprotective effect of NAC (Fig. 2F).

As stated in the Introduction, it is well established that TH expression is modulated by stress. As a result, loss of TH⁺ immunolabeling may not always reflect a true loss of dopaminergic terminals or cell bodies. Thus, we infused the retrograde tracer FluoroGold into the striatum to label dopaminergic cell bodies of the nigrostriatal pathway (Fig. 3A). FluoroGold infusions in the striatum labeled cells in the ipsilateral substantia nigra, pars compacta, as expected. We expected FluoroGold loss in the nigra to be a more sensitive measure of dopaminergic cell loss because only the neurons projecting to the site of infusion would be measured. In contrast, measurements of TH⁺ structures in the nigra do not distinguish between those neurons projecting to the center of the 6-OHDA infusion and those projecting more peripherally that are not affected by 6-OHDA. As retrograde tracing techniques may influence reported death rates in studies of the nigrostriatal pathway (Emsley et al., 2001; Naumann et al., 2000), we began by validating the use of FluoroGold. To this end, we delivered FluoroGold or vehicle into the striatum one week before 6-OHDA, at the same stereotaxic coordinates, so that retrograde tracer uptake could proceed unimpeded by oxidative injury. As an initial screen for nigrostriatal pathway degeneration, we measured TH or FluoroGold staining intensity in the ipsilateral and contralateral striata and nigrae (Figs. 3 and 4). In these experiments, there was a slight but significant loss in ipsilateral TH staining in the vehicle-treated striatum in the absence of FluoroGold (see first two bars in Fig. 3B). FluoroGold did not alter the toxic impact of 6-OHDA on striatal TH loss, suggesting that this tracer could be used in conjunction with 6-OHDA without altering the effects of the toxicant. However, FluoroGold infusions slightly decreased striatal area in the contralateral hemisphere (Fig. 3C). This effect was not apparent in the 6-OHDA group, suggesting that there may have been astrogliosis or some form of hypertrophy in response to dopaminergic terminal loss. As we had hoped, when we expressed striatal TH as a function of striatal area—the most important measure—there was no impact of FluoroGold in any group (Fig. 3D).

After demonstrating that FluoroGold did not alter loss of striatal TH in response to 6-OHDA, we next turned our attention to the ventral mesencephalon. FluoroGold infusions did not change TH expression in the nigra of vehicle or 6-OHDA-infused animals (Fig. 4A). We also examined nigral area in FluoroGold-infused animals and discovered that FluoroGold decreased nigral area in the animals that had been infused with 6-OHDA (Fig. 4B). However, TH expression as a function of nigral area was not affected by FluoroGold (Fig. 4C). As expected, overall FluoroGold loss in the nigra in response to 6-OHDA toxicity was much more dramatic than the parallel loss of TH (Fig. 4D vs A, G). Similarly, cell counts revealed that loss of TH⁺ cells in the nigra was not as dramatic as loss of FluoroGold⁺ cells in this structure (Fig. 4E vs F, H vs I). Thus, the FluoroGold tracer was a more sensitive means of assessing the toxicity of 6-OHDA infusions. In the higher-resolution stitched microscopic images of the ventral mesencephalon, the topography of dopaminergic cell loss in this model is evident; ventrolateral nigral cells are the most vulnerable to cell death after striatal 6-OHDA infusions (see white arrows in Fig. 4H, I), the same pattern described in postmortem nigral tissue from Parkinson's patients (Damier et al., 1999).

A confocal analysis of FluoroGold and TH dual-labeling suggests that some, but not all FluoroGold⁺ cells expressed the dopaminergic marker TH, consistent with previous studies

by Sauer and Oertel (1994) (Fig. 4J). Furthermore, the colocalization studies revealed that the cells that project to the dorsal neostriatum (the epicenter of the infusion) were clustered in the ventral tier of the substantia nigra, pars compacta (see merged images in Fig. 4G, J), as described previously in classic tract-tracing studies by Fallon and Moore (1978).

Aside from ensuring that the marker used to label neurons is sensitive, it is important to ensure that the protection afforded by therapeutic agents is not fleeting, especially in studies of chronic neurodegenerative disorders such as Parkinson's disease. As mentioned earlier, the toxicity associated with striatal 6-OHDA infusions in rodents is known to be progressive (Sauer and Oertel, 1994). In the Munoz study, the examination of TH⁺ structures in NAC-treated animals was completed 1 week after 6-OHDA infusions (see Fig. 8 in Munoz et al., 2004), leaving unanswered the question of whether there was long-term protection of the entire nigrostriatal pathway in this model of oxidative stress. Thus, we repeated the intraperitoneal NAC experiments in 6-OHDA infused mice and sacrificed them 3 weeks following surgeries. In these animals, no striatal protection with NAC was evident (Fig. 5A). Instead, NAC reduced TH expression in the contralateral hemisphere of the animals receiving the ascorbic acid vehicle (0 µg 6-OHDA group or third set of bars in Fig. 5A). Striatal area was not affected by 6-OHDA or NAC at this timepoint (Fig. 5B). Thus, TH staining density or expression per unit area followed the same pattern as in Fig. 5A (Fig. 5C). The ipsilateral/contralateral ratio of striatal TH expression was also not altered by NAC (Fig. 5D). These results demonstrate that intraperitoneal NAC does not protect dopaminergic terminals in the striatum for the long term.

In order to screen the NAC-treated animals for nigral cell loss, we measured TH and FluoroGold signal in the ventral midbrain. Nigral TH levels were reduced with 6-OHDA to the same extent in NAC and PBS-injected animals (Fig. 6A), suggesting again that NAC did not protect nigral dopamine neurons against 6-OHDA toxicity at this timepoint. Indeed, NAC reduced overall TH levels in this structure in all but one group, suggesting some degree of toxicity of NAC. The area of the nigra was not affected by NAC, although it was slightly increased by infusions of the vehicle ascorbic acid in both the PBS- and NAC-injected animals (Fig. 6B). This increase in the area of TH expression may be a hormetic response to the mild stress of vehicle infusion and was blunted by the additional stress of 6-OHDA. Because of these changes in nigral area, TH density or TH expression per unit area was slightly reduced in the ipsilateral hemisphere of animals injected with the ascorbic acid vehicle, an effect that was significant in the presence of NAC (Fig. 6C). Finally, NAC did not significantly change the ratio of ipsilateral over contralateral TH expression in the substantia nigra (Fig. 6D). Similarly, NAC did not increase FluoroGold signal in the 6-OHDA infused animals (Fig. 6E). Instead, NAC significantly reduced FluoroGold immunoreactivity in the ascorbic acid-treated group, suggesting it was somewhat toxic and caused some cell death in the nigrostriatal pathway, consistent with the Odyssey imaging results in Fig. 6A. Given the lack of protective effect of NAC in this set of animals in the initial screen with the Odyssey imager, we decided not to count dopamine neurons in this final study or to measure levels of glutathione or heat shock protein 70.

4. Discussion

Although NAC has been used in many experimental models of injury and administered to humans in clinical trials, the mechanism of action has often not been definitively established. Our recent *in vitro* studies demonstrate that NAC can robustly protect cortical neurons and neuroblastoma cells against proteasome inhibitors and oxidative stress (Jiang et al., 2013; Posimo et al., 2013; Unnithan et al., 2012; Unnithan et al., 2014). Indeed, NAC was so protective that it prevented cell death in response to severe, dual hits of the proteasome inhibitor MG132 and hydrogen peroxide (Unnithan et al., 2012; Unnithan et al., 2014). We also discovered that NAC can prevent loss of heat shock protein defenses in neuroblastoma cells treated with high concentrations of MG132 and that inhibitors of heat shock protein 70 (Hsp70) abolished NAC-mediated protection (Jiang et al., 2013). In the present study we established that intraperitoneal NAC only transiently protects nigrostriatal neurons from oxidative stress in the 6-OHDA animal model and that it exerts some degree of mild toxicity over longer periods. These discrepancies between our *in vitro* and *in vivo* observations warrant further investigation and may reflect the longer timeframes employed for the animal studies. As mentioned in the Introduction, some investigators have already reported the protective impact of NAC in animal models of Parkinson's disease. We have added to this body of work by verifying a NAC-mediated increase in TH in the ipsilateral striatum approximately one week after 6-OHDA delivery (Munoz et al., 2004). However, we also show that this ipsilateral effect is mirrored by a similar increase on the contralateral side. Thus, when the data are expressed as a function of the contralateral side, there is no apparent protection. On the other hand, as TH is the rate-limiting enzyme for dopamine biosynthesis, higher levels of TH in both hemispheres would be expected to translate to bilaterally higher dopamine levels, which could mitigate the symptoms of Parkinson's disease. For this reason, we repeated the NAC studies but sacrificed the animals at a later timepoint to ensure the protection was not fleeting. However, there was no protection in this group of animals. In future studies, we intend to deliver NAC by the oral route, either in food or water, because a daily, chronic injection protocol is much more stressful than food intake even if NAC has an unpleasant thiol odor that may affect palatability.

The negative findings in the three-week study have important implications for the long-term clinical use of NAC, which would be necessary in patients with slowly evolving neurodegenerative disorders such as Parkinson's disease. Our findings are timely in light of the ongoing clinical trials of NAC in Parkinson's patients (NCT01470027). If those trials fail, one explanation might be that NAC does not offer long-lasting protection of dopaminergic neurons against oxidative stress, a major pathological hallmark of Parkinson's disease (Blesa et al., 2015; Kim et al., 2015). Although long-term protection of striatal TH was reported with NAC in the Clark study (Clark et al., 2010), their injury model involved transgenic overexpression of α -synuclein and is therefore different from the oxidative injury model employed here. Clark and colleagues further demonstrated that the NAC-mediated increase in nigral glutathione was transient and no longer evident at the time of the striatal neuroprotection assay. Other authors have argued that NAC may have toxic properties, which would obviously be more likely to emerge after chronic treatments (Munoz et al., 2004; Zaeri and Emamghoreishi, 2015). As NAC exhibits low (~9.1%) oral bioavailability

(Olsson et al., 1988), it was delivered intraperitoneally in the present study at a dose of 100 mg/kg, a dose commonly used in the recent NAC literature and also employed in the Munoz study (Bachle et al., 2011; Chakraborti et al., 2008; Comparsi et al., 2014; Gunay et al., 2014; Jaccob, 2015; Munoz et al., 2004; Prakash et al., 2015; Smaga et al., 2012; Soleimani Asl et al., 2015; Truini et al., 2015). In this context, the World Health Organization guidelines for NAC administration in acetaminophen overdose are 150 mg/kg intravenously over 1 h, followed by 50 mg/kg intravenously over 4 h, and 100 mg/kg intravenously over 16 h (Algren, 2008). In clinical trials on Parkinson's patients, NAC has been delivered intravenously at doses as high as 150 mg/kg (Holmay et al., 2013). In patients with myocardial infarction, NAC was delivered into the heart at 100 mg/kg (Eshraghi et al., 2016). In patients undergoing stem cell transplantation, NAC was delivered intravenously at 100 mg/kg daily for 21 days (Ataei et al., 2015). In patients with oral mucositis, NAC was delivered intravenously at 100 mg/kg per day for at least 2 weeks (Moslehi et al., 2014). In humans, oral NAC administration is associated with nausea and vomiting whereas intravenous administration may result in anaphylactic reactions, which can lead to death in rare instances (Algren, 2008). Based on these observations and the present findings, it is worth bearing the potential toxicity of NAC in mind when using this drug to treat chronic conditions such as Parkinson's disease. Furthermore, future attempts to establish a model of its beneficial properties may wish to employ lower NAC doses or deliver it in a less stressful way, as accomplished by Clark, Swanson, and their colleagues (Berman et al., 2011; Clark et al., 2010).

We used several rigorous methods to quantify nigrostriatal degeneration and validated our tools. First, we used a 16-bit, high sensitivity imager to measure loss of TH protein in the striatum, expressing the data in multiple ways to ensure that changes in striatal area did not confound our interpretations, in contrast to the majority of 6-OHDA studies in the literature. The 16-bit depth (2^{16}) of the grayscale Odyssey imager translates to an unusually wide dynamic range, because 2^{16} (65,536) potential shades of gray afford high resolution, especially compared to 8-bit color imagers, which perceive only 2^8 (256) shades of color. Second, changes in dopaminergic neurons in the nigra were measured by four techniques: 1) measurements in overall TH expression levels and nigral area using the Odyssey Imager and 2) dopaminergic cell counts by higher resolution microscopy, 3) measurements of FluoroGold levels using the Odyssey imager, and 4) counts of individual FluoroGold-labeled nigral neurons. Although Sauer and Oertel report a partial loss of TH immunoreactivity in FluoroGold-labeled nigrostriatal neurons following 6-OHDA lesions (Sauer and Oertel, 1994), FluoroGold was employed in the present study to avoid the potential confound of stress-induced changes in TH expression in the absence of parallel changes in cell numbers (Baruchin et al., 1990; Chang et al., 2000; Kumer and Vrana, 1996; Tank et al., 2008; Tekin et al., 2014). Taken together, the results showed convincingly that NAC does not afford any protection of dopaminergic neurons against oxidative stress when delivered intraperitoneally for several weeks.

As we expected, it was important to examine the therapeutic potential of NAC at multiple timepoints, to report contralateral values, and to examine the cell bodies in addition to the terminals. Many studies in the 6-OHDA model report TH or dopamine values only as an ipsilateral/contralateral ratio without revealing the individual hemispheric values. However,

expressing data as a ratio is subject to the “reference trap,” an important issue discussed in the classic stereology literature (Braendgaard and Gundersen, 1986; Hyde et al., 2007). When data are only expressed as a ratio, it is not possible to know whether changes are attributable to the numerator or denominator or both. Indeed, the reader may be naturally inclined to assume changes in the numerator and not in the denominator, *i.e.*, the reference value. However, our study clearly shows that contralateral TH levels can change with treatments to the same degree as ipsilateral TH levels (Fig. 1), leading to a misleading lack of effect on the ratio of the two. Thus, the most appropriate control for this type of study of the basal ganglia is the ipsilateral hemisphere of the vehicle-treated group, which addresses the possibility of treatment-related effects on the contralateral side. Because we were careful to measure protection with multiple tools and expressed the data in several different ways, we have included a list of the strengths and limitations of the techniques used here to assess nigrostriatal degeneration (Table 2). In order to avoid repetition, we will only expand on some of the major points in the table here. Although numerous studies have employed FluoroGold to assess degeneration of the nigrostriatal pathway, we have shown that FluoroGold can reduce nigral area (see Fig. 4B). The use of FluoroGold to quantify nigrostriatal degeneration is ubiquitous (Anastasia et al., 2009; Aymerich et al., 2006; Choi-Lundberg et al., 1998; Cohen et al., 2011; Ebert et al., 2007; Kozłowski et al., 2000; Mandel et al., 1997; Sauer and Oertel, 1994; Yamada et al., 1999) and offers multiple advantages, such as higher sensitivity, but the potential toxicity is nevertheless a limitation (Naumann et al., 2000).

A limitation of using the Odyssey imager to quantify overall TH levels in the striatum and nigra is that loss of protein expression may not necessarily reflect loss of axon terminals or soma numbers. One can imagine that stressed neurons retract their dendrites or lose TH expression without actually dying. On the other hand, the degree of overall loss of nigral TH expression according to the infrared imager was consistent with the degree of loss of TH⁺ cell numbers, as verified by higher resolution microscopy, supporting its value as an initial screening tool. These two types of measurements should be viewed as complementary, as one may observe increases in TH expression per unit area by the Odyssey imager without any changes in cell numbers per se, suggesting that individual nigral cells are producing more TH, perhaps in a compensatory fashion to preserve homeostatic equilibrium. The second advantage of using the Odyssey imager to complement the cell counts is that nigral and striatal areas are easily measured, loss of which can also reveal some form of toxicity.

Although we were not able to employ stereological techniques for cell counts in the present study, we counted all TH⁺ neurons in three enormous stitched microscopic images of the nigra. Had we chosen to count smaller subregions of the substantia nigra by using a grid and expressed the data as cell numbers per unit area, our interpretations would have been subject to the possible confound of treatment-induced changes in cell density. One can imagine that a toxic treatment may not impact dopamine neuron numbers but reduces the size of the nigra due to glial cell loss, resulting in more tightly packed dopamine neurons. In such cases, if one reports cell counts per square millimeter or per microscopic field of view, one would report high cell numbers simply because of the tighter packing density. Similarly, one can imagine an increase in neuron numbers in a given structure without parallel changes in cell

density per unit area because the size of structure expands slightly to accommodate additional glial cells. On the other hand, cell counting techniques that are not based on stereology and do not only count cells within a counting brick in the center of the section are subject to over-counting or under-counting cell numbers. However, such errors, if indeed present, should have occurred at the same rates for the experimental and control groups in the present investigation.

As with stereological studies of dopamine neurons, TH cell counts in the present report are highly dependent on TH expression levels. For example, dopamine cells of the nigrostriatal pathway may express TH at levels too low to be detected by immunohistochemistry. To circumvent this limitation, previous studies have reported cell counts of Cresyl Violet-stained nigral cells. However, Cresyl Violet-stained Nissl substance is also expressed in glial cells and glial cells can undergo hyperplasia in response to stress, a response that would result in higher nigral cell counts. To circumvent this confound, we employed FluoroGold to specifically label dopamine neurons of the nigrostriatal pathway. Yet another alternative would be to use a neuronal marker such as NeuN, although NeuN staining in the nigra is variable and not even present in some TH-expressing dopamine neurons (Cannon and Greenamyre, 2009). Furthermore, neuronal markers would not exclusively label the efferent projection neurons of the nigrostriatal pathway, the loss of which is an important variable for modeling Parkinson's disease. For all the abovementioned reasons, a combined approach employing multiple types of measurements, as achieved in the present study, seems the best approach. To combat the confound of FluoroGold toxicity, it might be worth examining other more inert tract-tracers, such as biotinylated dextran amines, although these are transported in both retrograde and anterograde directions, and would label the projection from the striatum to the substantia nigra, pars reticulata. This would preclude using the Odyssey imager to measure overall tracer levels in the nigra as a specific measure of the nigrostriatal efferent pathway. Other retrograde tracers such as True Blue fade quickly but would be another option worth testing, as one study reported less toxicity of True Blue compared to FluoroGold over long survival timeframes (Garrett et al., 1991).

In conclusion, we have performed an extensive study of the therapeutic potential of NAC, using multiple independent measurements of nigrostriatal degeneration. NAC may offer transient protection in this model, but the effect wanes within the following few weeks. We have described in detail the strengths and limitations of using an established retrograde tracer to quantify nigrostriatal degeneration and presented alternatives for future studies, such as delivering NAC by oral intake of food or water and switching to more inert tracers. As NAC has already been shown to benefit patients with multiple neurological conditions, it continues to be important to establish animal models of its therapeutic effects to accelerate the discovery of novel drug targets and to shed greater light on the mechanisms underlying drug-induced neuroprotection. However, based on the present findings, it is also important to note that chronic treatment with NAC may not offer long lasting protection against oxidative injury and may exert toxic effects of its own. This has potentially important implications for clinical trials of NAC in patients of Parkinson's disease, an inexorably progressive condition that would necessitate long-term treatment strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

6-OHDA	6-hydroxydopamine
FG	FluoroGold
NAC	<i>N</i> -acetyl cysteine
PBS	phosphate-buffered saline

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Fig. 1.

NAC raises TH levels in the striatum 10 days after 6-OHDA infusions. Mice were stereotactically infused with 4 μg 6-OHDA or an equivalent volume of vehicle (0.02% ascorbic acid in saline) into the right striatum. For the next 10 days, mice received daily intraperitoneal injections of 100 mg/kg NAC or an equivalent volume of phosphate-buffered saline (PBS). Forebrain sections were immunostained for the dopaminergic terminal marker tyrosine hydroxylase (TH) and scanned on a high-sensitivity Odyssey Imager. (A) 6-OHDA significantly reduced ipsilateral/contralateral striatal TH levels. NAC did not affect this ratio. (B) Overall TH levels in the contralateral and ipsilateral striata. 6-OHDA reduced TH levels on the side ipsilateral to the infusion. NAC raised TH levels in the contralateral striatum of 6-OHDA-infused animals. There was a trend towards a NAC-mediated increase in TH levels in the ipsilateral hemisphere in 6-OHDA-treated animals ($p = 0.058$). (C) Striatal area was not affected by 6-OHDA or NAC. (D) TH levels were expressed as a function of striatal area, a measurement that reflects average dopaminergic terminal density (TH per unit area). NAC significantly raised TH density in both hemispheres after 6-OHDA infusions. (E) Representative coronal sections through the forebrain, immunolabeled for TH⁺ dopaminergic terminals and scanned on the Odyssey imager. Shown are the mean and SEM of 7–8 mice per group. * p 0.05, ** p 0.01, *** p 0.001 versus 0 μg 6-OHDA; + p 0.05, ++ p 0.01, +++ p 0.001 ipsilateral versus contralateral; ~ p 0.05, ~~ p 0.01, ~~~ p 0.001 versus 0 mg/kg NAC; two or three-way ANOVA followed by Bonferroni *post hoc* correction.

Fig. 2. NAC fails to protect dopaminergic neurons in the substantia nigra from 6-OHDA toxicity. Mice were stereotactically infused with 4 μ g 6-OHDA or an equivalent volume of vehicle (0.02% ascorbic acid in saline) into the right striatum. For the next 10 days, mice received daily intraperitoneal injections of 100 mg/kg NAC or an equivalent volume of phosphate-buffered saline (PBS). Midbrain sections were stained for the dopaminergic terminal marker tyrosine hydroxylase (TH). (A) Overall TH levels in the contralateral and ipsilateral nigrae. The ipsilateral nigra had lower TH levels than the nigra contralateral to the 6-OHDA infusion site. (B) Nigral area was significantly reduced by 6-OHDA in both hemispheres in the absence or presence of NAC. (C) TH levels were expressed as a function of nigral area, a measurement that reflects average TH density (TH per unit area). 6-OHDA raised TH density in the contralateral nigra and NAC did not significantly affect this measure. (D) 6-OHDA significantly reduced ipsilateral/contralateral nigral TH levels. Although 6-OHDA did not cause a significant loss of this measure in NAC-treated animals, NAC did not significantly increase this ratio. (E) Representative coronal sections through the

mesencephalon, immunolabeled for TH⁺ cell bodies and scanned on the Odyssey Imager. (F) TH cell counts in the nigra reveal that NAC has no impact on the loss of dopaminergic cells in this structure. Shown are the mean and SEM of 7–8 mice per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus 0 μ g 6-OHDA; + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$ ipsilateral versus contralateral; ~ $p < 0.05$, ~~ $p < 0.01$, ~~~ $p < 0.001$ versus 0 mg/kg NAC; two or three-way ANOVA followed by Bonferroni *post hoc* correction.

Fig. 3.

Impact of the retrograde tracer FluoroGold on 6-OHDA toxicity in the striatum. (A) Mice were stereotactically infused with 6 μ g FluoroGold (FG). Seven days later, mice were sacrificed and brain sections viewed under UV illumination on an epifluorescent microscope. The ipsilateral nigrostriatal pathway was retrogradely labeled. cc, Corpus callosum; CPu, caudatoputamen; SNpc, substantia nigra, pars compacta; SNpr, substantia nigra, pars reticulata. (B) In a separate series of experiments, mice were infused with 6 μ g FluoroGold or an equivalent volume of phosphate-buffered saline (PBS) into the right striatum. Seven days after FluoroGold infusions, mice were infused with 4 μ g 6-OHDA or an equivalent volume of vehicle (0.02% ascorbic acid in saline) in the same striatal location. One week following 6-OHDA infusions, forebrain sections were immunostained for the dopaminergic terminal marker tyrosine hydroxylase (TH) and FluoroGold and scanned on

the Odyssey Imager. (b) The impact of 6-OHDA toxicity on overall striatal TH levels was not affected by FluoroGold. (C) FluoroGold slightly reduced striatal area in the hemisphere contralateral to the infusion. 6-OHDA prevented this effect. (D) FluoroGold did not change TH levels per unit area in the striatum of vehicle or 6-OHDA-infused animals. (E) Representative coronal sections through the forebrain, immunolabeled for TH⁺ dopaminergic terminals (green) and FluoroGold (red). Shown are the mean and SEM of 5–6 mice per group. **p* 0.05, ***p* 0.01, ****p* 0.001 versus 0 µg 6-OHDA; +*p* 0.05, ++*p* 0.01, +++*p* 0.001 ipsilateral versus contralateral; ~*p* 0.05, ~~*p* 0.01, ~~~*p* 0.001 versus 0 µg FG; three-way ANOVA followed by Bonferroni *post hoc* correction. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 4. Impact of the retrograde tracer FluoroGold on 6-OHDA toxicity in the substantia nigra. Mice were stereotactically infused with 6 µg FluoroGold (FG) or an equivalent volume of phosphate-buffered saline (PBS) into the right striatum. Seven days later, mice were infused in the same location with 4 µg 6-OHDA or an equivalent volume of vehicle (0.02% ascorbic acid in saline). One week later, brain sections were stained for the dopaminergic terminal marker tyrosine hydroxylase (TH) and FluoroGold. (A) The impact of 6-OHDA on overall TH levels in the nigra was not affected by FluoroGold. (B) FluoroGold slightly reduced

nigral area in both hemispheres of 6-OHDA-infused animals. (C) FluoroGold did not change TH levels per unit area in the nigra of vehicle or 6-OHDA-infused animals. (D) Overall FluoroGold levels in the nigra were significantly reduced by 6-OHDA. (E) FluoroGold did not significantly change TH⁺ cell counts in the nigra in vehicle or 6-OHDA-infused animals. (F) The number of FluoroGold⁺ cells of the nigrostriatal pathway was significantly reduced by 6-OHDA. (G) Representative coronal sections through the midbrain, immunolabeled for TH⁺ dopaminergic neurons (green) and FluoroGold⁺ cells (red) and scanned on the Odyssey Imager. (H–I) Microscopic images of FluoroGold⁺ (h) and TH⁺ (i) cells in the ventral midbrain in 6-OHDA/saline and FluoroGold/PBS-infused animals. The arrow points to the lateral nigra, the area most vulnerable to loss in Parkinson's disease. (J) Confocal analysis of FluoroGold and TH dual labeling. Arrows point to some of the cells that contain both FluoroGold and TH. Asterisks overlie FluoroGold-labeled nigrostriatal cells that do not express TH. FluoroGold⁺ cells were viewed under UV illumination and pseudocolored red for presentation. Shown are the mean and SEM of 5–6 mice per group. **p* 0.05, ***p* 0.01, ****p* 0.001 versus 0 µg 6-OHDA; +*p* 0.05, ++*p* 0.01, +++*p* 0.001 ipsilateral versus contralateral; ~*p* 0.05, ~~*p* 0.01, ~~~*p* 0.001 versus 0 µg FG; three-way ANOVA followed by Bonferroni *post hoc* correction or two-tailed Student's *t*-test for panels d and f. IPN, interpeduncular nucleus; ml, medial lemniscus; SNpc, substantia nigra, pars compacta; SNpr, substantia nigra, pars reticulata; VTA, ventral tegmental area. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 5.

NAC fails to protect dopaminergic terminals in the striatum three weeks following 6-OHDA infusions. Mice were stereotactically infused with 6 μg FluoroGold (FG) into the right striatum. Seven days later, mice were infused in the same location with 4 μg 6-OHDA or an equivalent volume of vehicle (0.02% ascorbic acid in saline). For the next three weeks, mice received daily intraperitoneal injections of 100 mg/kg NAC or an equivalent volume of PBS. Forebrain sections were stained for the dopaminergic terminal marker tyrosine hydroxylase (TH) and FluoroGold (FG). (A) NAC reduced overall TH levels in the striatum contralateral to vehicle infusion and did not protect against 6-OHDA-induced loss of TH. (B) Striatal area was not significantly affected by NAC or 6-OHDA. (C) TH levels were expressed as a function of striatal area, reflecting TH density (TH per unit area). NAC reduced this measure in the contralateral hemisphere of vehicle-infused animals. (D) 6-OHDA significantly reduced ipsilateral/contralateral striatal TH levels and NAC did not impact this measure. (E) Representative coronal sections through the forebrain, immunolabeled for TH⁺ terminals (green) and FluoroGold (red). Shown are the mean and SEM of 5–8 mice per group. **p* 0.05, ***p* 0.01, ****p* 0.001 versus 0 μg 6-OHDA; +*p* 0.05, ++*p* 0.01, +++*p* 0.001 ipsilateral versus contralateral; ~*p* 0.05, ~~*p* 0.01, ~~~*p* 0.001 versus 0 mg/kg NAC; two or three-way ANOVA followed by Bonferroni *post hoc* correction. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 6. NAC fails to protect dopaminergic neurons in the substantia nigra three weeks following 6-OHDA infusions. Mice were stereotactically infused with 6 μg FluoroGold (FG) into the right striatum. Seven days later, mice were infused in the same location with 4 μg 6-OHDA or an equivalent volume of vehicle (0.02% ascorbic acid in saline). For the next three weeks, mice received daily intraperitoneal injections of 100 mg/kg NAC or an equivalent volume of PBS. Midbrain sections were stained for the dopaminergic terminal marker tyrosine hydroxylase (TH) and FluoroGold (FG). (A) NAC reduced overall TH levels in the nigra in all groups except for the vehicle-infused ipsilateral striatum. (B) Nigral area was increased

by infusion of vehicle into the ipsilateral hemisphere and 6-OHDA prevented this effect. NAC had no impact on this measure. (C) TH levels were expressed as a function of nigral area, reflecting TH density (TH per unit area). NAC did not raise TH density in the ipsilateral nigra. (D) 6-OHDA significantly reduced ipsilateral/contralateral nigral TH levels and NAC did not impact this ratio. (E) Overall FluoroGold levels were reduced by 6-OHDA in the ipsilateral nigra. NAC decreased FluoroGold levels in vehicle-infused animals. (F) Representative coronal sections through the mesencephalon, immunolabeled for TH⁺ (green) and FluoroGold⁺ (red) cells. Shown are the mean and SEM of 5–8 mice per group. **p* 0.05, ***p* 0.01, ****p* 0.001 versus 0 µg 6-OHDA; +*p* 0.05, ++*p* 0.01, +++*p* 0.001 ipsilateral versus contralateral; ~*p* 0.05, ~~*p* 0.01, ~~~*p* 0.001 versus 0 mg/kg NAC; two or three-way ANOVA followed by Bonferroni *post hoc* correction. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1

Antibodies.

	Source	Company	Catalog #	Lot #	Dilution
<i>Primary antibody</i>					
Anti-tyrosine hydroxylase	Sheep	Millipore	AB1542	2554850	1:1000
Anti-fluorescent gold	Rabbit	Millipore	AB153-1	2533847	1:3000
<i>Secondary antibody</i>					
Anti-sheep @800	Donkey	Jackson Laboratories	713-655-147	106089	1:500
Anti-sheep @488	Donkey	Life Technologies	A11015	1567206	1:500
Anti-rabbit @ 700	Donkey	Jackson Laboratories	711-625-152	111285	1:500

Table 2

Strengths and limitations of the techniques used here to quantify nigrostriatal degeneration.

Strengths	Limitations
<ul style="list-style-type: none"> • Striatal and nigral TH measurements were completed on a 16-bit imager with high sensitivity 	<ul style="list-style-type: none"> • The Odyssey imager has low resolution (21 μm) and individual cells cannot be resolved
<ul style="list-style-type: none"> • Blinded TH⁺ cell counts were performed by higher resolution microscopy to overcome the spatial limits of the Odyssey Imager and to complete the measurements of striatal TH 	<ul style="list-style-type: none"> • The higher resolution counts were not performed with stereological techniques
<ul style="list-style-type: none"> • Measurements of the area of the striatum and nigra were included 	<ul style="list-style-type: none"> • None
<ul style="list-style-type: none"> • TH levels in the striatum and nigra were expressed per unit area to approximate TH density and to avoid the confounding effect of different-sized tracings of the region of interest 	<ul style="list-style-type: none"> • TH levels may be modulated by stress such that a drop in TH density may not reflect a true loss in dopaminergic terminals or neurons
<ul style="list-style-type: none"> • FluoroGold was used to overcome the limitation of stress-induced modulation of TH expression 	<ul style="list-style-type: none"> • FluoroGold exhibited some toxicity
<ul style="list-style-type: none"> • FluoroGold can be labeled immunohistochemically so that it can be viewed in more channels than under UV illumination and can also be labeled more permanently with diaminobenzidine 	<ul style="list-style-type: none"> • None
<ul style="list-style-type: none"> • Measurements of nigral TH levels on the Odyssey Imager were consistent with nigral TH⁺ cell counts, suggesting that high-throughput Odyssey imaging can be a useful and efficient screening tool in models of Parkinson's disease 	<ul style="list-style-type: none"> • Loss of protein does not necessarily indicate loss of cell numbers
<ul style="list-style-type: none"> • Measurements of loss of FluoroGold⁺ structures in the nigra were more sensitive than loss of TH⁺ structures 	<ul style="list-style-type: none"> • FluoroGold exhibited some toxicity
<ul style="list-style-type: none"> • FluoroGold was infused 7d before 6-OHDA and so specifically labeled healthy projection neurons that form the nigrostriatal pathway and project to the site of infusion 	<ul style="list-style-type: none"> • None
<ul style="list-style-type: none"> • Neuroprotection was assessed at two different survival periods 	<ul style="list-style-type: none"> • None
<ul style="list-style-type: none"> • All labeled cells in the nigra were counted on large stitched images instead of only counting a fraction of the image, in order to limit the confounding impact of potential differences in cell density across groups 	<ul style="list-style-type: none"> • None
<ul style="list-style-type: none"> • We included an ascorbic acid/saline control for every 6-OHDA group and a PBS control for every NAC group 	<ul style="list-style-type: none"> • None
<ul style="list-style-type: none"> • We included measurements of the contralateral hemisphere as an additional control 	<ul style="list-style-type: none"> • None
<ul style="list-style-type: none"> • NAC was given intraperitoneally instead of orally to overcome low oral bioavailability and potential variability from different degrees of food intake due to the strong thiol odor of NAC 	<ul style="list-style-type: none"> • Daily intraperitoneal injections are much more stressful than daily food intake