Free 3-Nitrotyrosine Causes Striatal Neurodegeneration *In Vivo*

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Peroxynitrite formation has been demonstrated in several neurodegenerative disorders; thus far, protein nitration and consequent alterations in protein function are implicated as mechanistic events. Free 3-nitrotyrosine (free-3NT) is also elevated in these settings; a neurotoxic role for this modified amino acid has not been investigated. We tested the hypothesis that free-3NT is neurotoxic *in vivo*, using a mouse model of striatal degeneration. The neurodegenerative effects of the dopaminergic neurotoxin 6-hydroxydopamine (6-OHDA) (unilateral intrastriatal injection, 64 nmol) were compared with free-3NT (32 nmol) or free-tyrosine (free-TYR) (32 nmol). 6-OHDA-treated mice exhibited significant ipsilateral turning behavior after D-amphetamine challenge, indicative of unilateral striatal injury (ipsilateral–contralateral turning differential, 21.1 ± 6.8). Significant turning behavior was also observed in free-3NT-treated mice but not in free-tyrosine-treated mice (free-3NT, 16.0 ± 3.9; free-TYR, 1 ± 2.7; $p < 0.01$). Immunohistochemistry was used to evaluate striatal tyrosine hydroxylase (TH) content. 6-OHDA or free-3NT treatment caused severe reductions in TH immunoreactivity in injected striata compared with the contralateral hemisphere (injected/contralateral immunoreactivity ratio: 6-OHDA, 0.23 ± 0.07; free-3NT, 0.49 ± 0.02). Free-tyrosine treatment had no effect (1.03 ± 0.09). Turning behavior was correlated with striatal TH ratio ($p < 0.01$). Furthermore, we observed a striking unilateral reduction in TH-positive cell body counts in the substantia nigra pars compacta of 6-OHDA- and free-3NT-treated mice (injected/contralateral cell count ratio: 6-OHDA, 0.40 ± 0.04; free-3NT, 0.59 ± 0.02). Free-tyrosine treatment had no effect (1.05 ± 0.04). No evidence for increased striatal protein incorporation of 3NT was observed in any treatment group. These data represent the first evidence that free-3NT can elicit neurodegenerative effects *in vivo*; free-3NT may have a causal role in neurodegenerative conditions.

Key words: peroxynitrite; 3-nitrotyrosine; neurodegeneration; Parkinson's disease; nitration; nitric oxide

Although Parkinson's disease represents an important health care problem, the molecular mechanisms of striatal neurodegeneration remain incompletely defined; as a result, currently optimized therapeutic approaches remain inadequate (Zhang et al., 2000). Nitric oxide-mediated excitotoxicity has long been implicated in several settings of both acute and chronic neurodegeneration, including Parkinson's disease (Hunot et al., 1996). Recognizing the important contribution of oxidative events to chronic neurodegenerative conditions, more recent evidence has suggested that peroxynitrite formation may participate in and/or mediate many of these effects (Squadrito and Pryor, 1998). Peroxynitrite ($ONOO^-$) is a potent oxidant formed during the nearly instantaneous reaction of nitric oxide with superoxide anion and has been shown to selectively nitrate protein tyrosine residues, causing cellular dysfunction, DNA damage, and cell death (Beckman and Koppenol, 1996). $ONOO^-$-mediated protein nitration has been demonstrated in Parkinson's disease, as well as in other neurodegenerative disorders (e.g., Alzheimer's disease and amylotrophic lateral sclerosis; Beckman et al., 1993; Good et al., 1996, 1998). In these settings, neuronal protein nitration and consequent alterations in protein function have been implicated as important contributors to cell dysfunction and apoptosis (Ischiropoulos, 1998). The free nitrated amino acid is also elevated in these settings, but a neurotoxic role for free 3-nitrotyrosine (free-3NT) has not been proposed or investigated previously (Hensley et al., 1998; Pennathur et al., 1999; Tohgi et al., 1999). Here we tested the hypothesis that free-3NT is neurotoxic *in vivo*, using a relevant animal model of Parkinson-like striatal neurodegeneration, and describe evidence that free-3NT itself, in the absence of direct oxidative events, causes neuronal cell loss *in vivo* and may contribute to $ONOO^-$-related neurodegenerative events.

MATERIALS AND METHODS

Animal model of striatal injury. Striatal neurodegeneration was induced in mice (CF-1 strain; 30–35 gm; $n = 8$) via a single unilateral intranigrostriatal injection of the classical dopaminergic neurotoxin 6-hydroxydopamine (6-OHDA) (64 nmol/4 μl saline) with the aid of a stereotaxic instrument, as described previously (Fung and Uretsky, 1980). In parallel investigations, free-3NT or free L-tyrosine (free-TYR) were injected using identical methods (each 32 nmol/4 μl saline). Before injection, free-3NT was recrystallized and shown to be >99.8% pure (by

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capillary electrophoresis analytical assay). After intrastriatal injections, the surgical incision was closed with surgical staples and animals were allowed to recover for 2 weeks. During this 2 week recovery period, mice were housed under 12 hr light/dark cycles with access to food and water *ad libitum*; all ate and drank normally, and no gross neurological deficits were observed. All chemicals were obtained from Sigma (St. Louis, MO), except where noted.

Two weeks after intrastriatal injection, mice were challenged with D-amphetamine (4 mg/kg, i.p.; a stimulant of presynaptic striatal dopamine release) or apomorphine (1.5 mg/kg, s.c.; a postsynaptic dopamine receptor agonist); they were then observed for turning behavior after being placed in a circular jar (13.5 cm diameter). After amphetamine challenge, net ipsilateral (toward the injected hemisphere) turning behavior is indicative of unilateral degeneration of presynaptic striatal dopamine neurons. Net contralateral (away from the injected hemisphere) turning behavior after apomorphine challenge occurs as a result of a unilateral postsynaptic dopamine receptor supersensitivity response. The postsynaptic dopamine receptor supersensitivity response is apparently related to extent of dopamine depletion and is reflective of more severe degenerative events relative to the amphetamine-induced net turning response (Marshall and Ungerstedt, 1977).

Histology and immunohistochemistry. After behavioral analyses, mice were killed by CO_2 inhalation, and brains were formalin fixed and processed for histological analysis as described previously (Mihm et al., 2000; Wattanapitayakul et al., 2000). Five micrometer coronal sections were prepared from two regions along the nigrostriatal axis, the striatum, and the substantia nigra pars compacta. Striatal histology was assessed in coronal sections prepared at 150 μm intervals encompassing the entire striatal region (from the most anterior corpus callosum to the anterior thalamic region). Multiple coronal sections of the substantia nigra pars compacta were prepared at 300 μm intervals, encompassing the entire substantia nigra. Tissues were evaluated for general histology using cresyl violet stain and immunostained using polyclonal primary antibody for tyrosine hydroxylase (TH) (1:750; Novus Biologicals Inc., Littleton, CO) or 3NT (1:2000; Upstate Biotechnology, Lake Placid, NY). Staining controls illustrated the specificity of immunostaining and included nonimmune rabbit IgG (1:750; Vector Laboratories, Burlingame, CA) in place of anti-TH primary antibody, and preincubation of primary antibody with free-3NT (5 mM, preadsorbed control) for anti-3NT immunohistochemistry. Diaminobenzidine (0.06% w/v) followed by methyl green counterstaining provided visualization of immunoreactivity.

Image capture and digital image analysis. Coronal sections of whole striata and the substantia nigra pars compacta were visualized with an Olympus Optical (New York, NY) BX-40 microscope (12.5× or 200× magnification) and captured under identical lighting conditions and optical settings using a Polaroid (Cambridge, MA) digital camera (1290 × 960 resolution). Images were then analyzed using research-based digital image analysis software (Image Pro Plus 4.0; Media Cybernetics, Silver Spring, MD). In striatal studies, integrated optical density analysis of TH staining was performed in each striata for each section (30–35 sections analyzed per treatment group). Striatal areas in each coronal section were delineated from serial sections stained with cresyl violet. Images were then segmented to eliminate background and nuclear counterstain from analysis. Optical densities were determined for each striatum and multiplied by the total positively stained striatal area to give an integrated measure of staining intensity. Integrated optical densities were expressed as the ratio of immunoreactivity in the injected (left) versus the contralateral (right) striatum. In the absence of a treatment effect, a ratio of 1.0 (equal staining intensity for TH content in each hemisphere) would be expected. Over 180 striatal areas were analyzed, and intra-observer and inter-observer variability were each <2%.

For studies in the substantia nigra pars compacta, quantitative cell counts of TH-positive cell bodies were performed in both hemispheres for each coronal section studied. The areas of interest in each coronal section were delineated from the combination of TH specificity in this area (only cell bodies of the pars compacta and ventral tegmental areas stained positive for TH in this region) and serial sections stained with cresyl violet. After segmentation and gating of cell bodies based on size, digital image analysis-assisted cell body counts were obtained in both injected and control hemispheres. Cell counts were expressed as the ratio of TH-positive cell bodies in the injected (left) versus the contralateral (right) striatum. In the absence of a treatment effect, a ratio of 1.0 (equal TH-positive cell body counts in each hemisphere) would again be expected. Average cell counts conducted in the control hemispheres (right side) were not statistically different between treatment groups (free-TYR, free-3NT, and 6-OHDA; $p = 0.41$; NS). Over 4300 cell bodies were counted by this method, and intra-observer and inter-observer variability were each <2%.

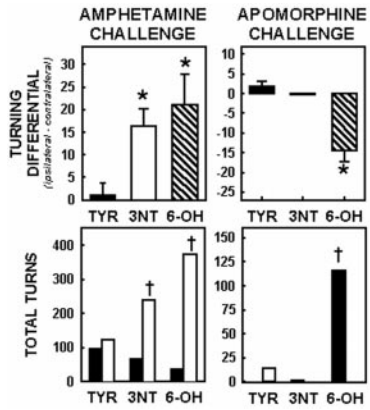

Figure 1. Free-3NT elicited turning behavior consistent with degeneration of striatal dopaminergic nerve terminals. Unilateral nigrostriatal injections of free-TYR, free-3NT, or 6-OHDA were assessed for neurodegenerative effects in mice using the turning model. Net turning behavior after amphetamine or apomorphine challenge is indicative of dopaminergic injury in the nigrostriatal axis. *Left panels*, Free-TYR treatment did not elicit amphetamine-induced turning behavior. Free-3NT and 6-OHDA treatment resulted in significantly increased ipsilateral versus contralateral turning behavior, as well as increased net ipsilateral turning behavior compared with free-TYR controls, consistent with degeneration of striatal dopamine nerve terminals. *Right panels*, Apomorphine challenge did not result in significant net turning behavior in free-TYR- or free-3NT-treated mice but did stimulate significant contralateral turning in 6-OHDA-treated mice, consistent with severe degenerative events. $n = 8$ mice per treatment group. * indicates a significant difference from the free-TYR control, as assessed by ANOVA ($p < 0.05$); † indicates that ipsilateral turning was significantly different from contralateral turning within the treatment group (paired t test; $p < 0.05$).

Data handling. Behavioral circling data were expressed as both net turning differential (ipsilateral minus contralateral) and total turning behavior (ipsilateral vs contralateral). Integrated optical density data for TH immunoreactivity were expressed as a ratio of integrated optical density of injected divided by control striatal values. Quantitative cell counts were expressed as a ratio of pars compacta TH-positive cell body counts in the injected hemisphere divided by the control hemisphere. Statistical analyses were performed using one-way ANOVAs for differential turning behavior, TH immunoreactivity ratios, and cell count ratios between treatment groups. Paired Student's t tests were used to test statistically significant differences for total ipsilateral versus contralateral turning behavior, injected versus control striatal TH immunoreactivity, and injected versus control pars compacta TH-positive cell counts within treatment groups. Spearman's nonparametric correlation analyses were performed to assess significant correlations. In all cases, $p < 0.05$ described statistical significance.

RESULTS

The behavioral results after free-3NT, free-TYR (equimolar negative control), and 6-OHDA (classical dopaminergic neurotoxin, positive control) treatment are shown in Figure 1. Free-TYR-treated mice did not demonstrate significant net turning behavior after either amphetamine or apomorphine challenge (i.e., no apparent neuronal injury) (Fig. 1, *left* and *right panels*, respectively). In contrast, increased net ipsilateral turning behavior was observed in free-3NT-treated mice after amphetamine challenge (Fig. 1, *left panel*); apomorphine-induced contralateral turning was not observed (Fig. 1, *right panel*). 6-OHDA treatment caused net turning behavior after both amphetamine and apomorphine challenges. Locomotive capacity was not impaired in any of the treatment groups, as assessed by rotarod testing (data not shown).

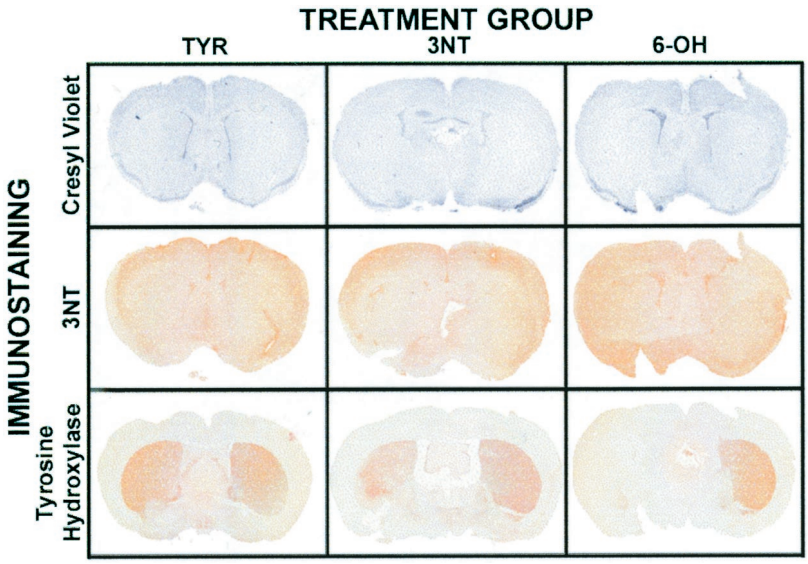

Figure 2. Representative coronal sections from immunohistochemical studies. Immunohistochemical studies were conducted after behavioral analyses. Five micrometer coronal sections were prepared on slides at 150 μm intervals, encompassing the entire striatal region (from the most anterior corpus callosum to the anterior thalamic region). Representative images from general histological (cresyl violet) and immunohistochemical (protein nitration, tyrosine hydroxylase) analyses are shown. Free-3NT treatment did not result in gross morphological changes or significant decreases in striatal cell density (cresyl violet stain), and basal 3NT immunoprevalence was equivalent across treatment groups. Free-3NT and 6-OHDA treatment resulted in unilateral decreases in TH content in injected striata.

After behavioral analysis, brains were processed for histological and immunocytochemical analyses. Striatal cell density was apparently not grossly perturbed by free-TYR, free-3NT, or 6-OHDA treatment, because no relative changes in striatal cresyl violet staining density were apparent in any of the treatment groups (Fig. 2). In addition, no significant evidence of increased striatal protein nitration was observed in any treatment group, as assessed by 3NT immunohistochemistry. In contrast, evidence of unilateral decreases in striatal TH content (an index of dopamine-specific neuronal degeneration in this region) was observed in the free-3NT and 6-OHDA treatment groups. Integrated optical density analysis of TH content was assessed in each striata and expressed as the ratio of immunoreactivity in the injected (left) versus the contralateral (right) striatum (Fig. 3, *left panel*). In the absence of a treatment effect, a ratio of 1.0 (equal staining intensity for TH content in each hemisphere) would be expected. Statistically significant reductions in this ratio were observed in the free-3NT and 6-OHDA treatment groups (51 and 77% reductions, respectively). The relationship of the behavioral turning response to D-amphetamine and striatal TH immunoreactivity is also illustrated in Figure 3 (*right panel*). A significant negative correlation was observed between amphetamine-induced rotational orientation and the TH immunoreactivity ratio (Spearman's correlation; $p < 0.01$), illustrating that behavioral deficits parallel striatal dopaminergic nerve terminal loss in this model.

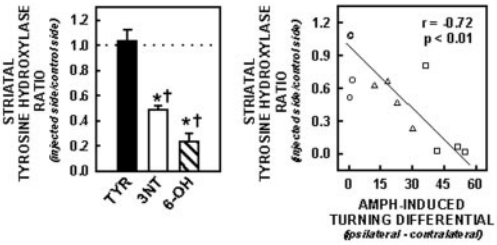

Figure 3. Free-3NT caused significant decreases in striatal tyrosine hydroxylase content. *Left*, Average integrated optical densities for TH content in injected versus contralateral striata in free-TYR-, free-3NT-, and 6-OHDA-treated mice. Relative TH immunoreactivity is expressed as the ratio of integrated optical density for injected divided by contralateral (control) striatum (7 sections per brain at 150 μm intervals, 30–35 sections analyzed per treatment group). In the absence of a treatment effect, a ratio of 1.0 (equal staining intensity for TH content in each hemisphere) would be expected. * indicates a significant difference from the free-TYR control (one-way ANOVA; $p < 0.05$); † indicates that the integrated optical density of the injected hemisphere was significantly different from the integrated optical density of the control hemisphere within the treatment group (paired t test; $p < 0.05$). *Right*, The amphetamine-induced behavioral deficit was inversely correlated with TH content. Spearman's nonparametric correlation analysis was used to assess the relationship between decreased TH content and treatment-induced behavior. A significant negative correlation was observed between amphetamine (*AMPH*)-induced rotational behavior and TH immunoreactivity content ($r = -0.72$; $p < 0.01$). ○, Free-TYR; △, free-3NT; □, 6-OHDA.

Representative photomicrographs of TH staining in the substantia nigra pars compacta of mice treated with free-TYR, free-3NT, or 6-OHDA are shown in Figure 4 (*left panels*). Quantitative cell counts of TH-positive cell bodies were conducted in the same mice studied for behavioral effects and for striatal TH content. TH-positive cell body density was equivalent in the injected versus control pars compacta in free-TYR-treated mice (Fig. 4, *top right panel*). In contrast, striking and statistically significant reductions in TH-positive cell body counts were observed in the injected hemispheres of mice treated with free-3NT and 6-OHDA (41 and 60% reductions in cell body counts compared with contralateral control, respectively).

A correlation analysis was performed to probe the potential relationship between nerve terminal injury and cell body degeneration in this model (Fig. 4, *bottom right panel*). A statistically significant positive association was observed between striatal TH content ratio and the substantia nigra TH-positive cell body count ratio (Spearman's correlation; $p < 0.01$).

DISCUSSION

Reactive oxygen species have been implicated in a variety of progressive neurodegenerative disorders (Parkinson's disease, Alzheimer's disease, amylotrophic lateral sclerosis, Huntington's disease, and others), but the putative species, cellular targets, and molecular mechanisms involved are unclear (Gotz et al., 1994). In several recent reports, peroxynitrite formation and attendant protein nitration have also been detected in these settings, but the putative mechanism or mechanisms by which $ONOO^-$ might induce neuronal injury *in vivo* have not been not defined (Tor-

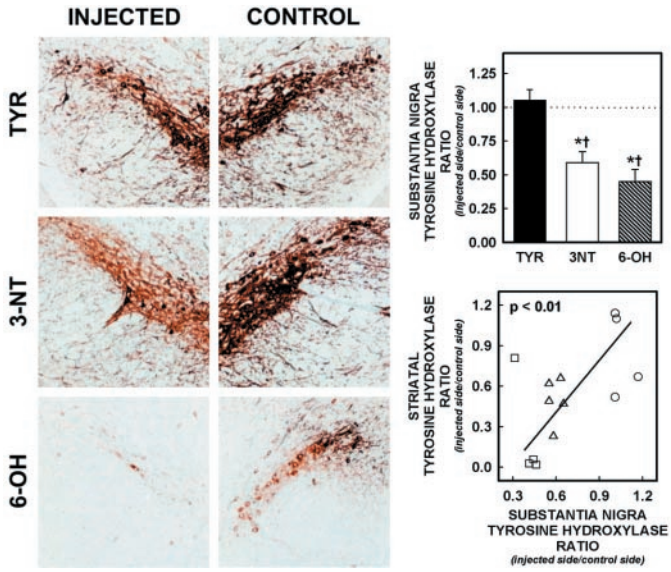

Figure 4. Nigrostriatal cell body loss in free-3NT-treated mice. *Left panels*, Representative images from TH staining in the substantia nigra pars compacta from free-TYR-, free-3NT-, and 6-OHDA-treated mice are shown (200× original magnification). Free-TYR treatment did not alter relative TH-positive cell body counts; treatment with free-3NT and 6-OHDA resulted in a loss of TH-positive cell body density in the injected hemisphere. *Top right panel*, Quantitative cell counts from injected and contralateral hemispheres. Injected side versus control side TH-positive cell counts were not different in free-TYR-treated mice; free-3NT and 6-OHDA treatment caused a statistically significant decrease in TH-positive cell body count ratios compared with the free-TYR control. * indicates a significant difference from the free-TYR control (one-way ANOVA; $p < 0.05$); † indicates that injected hemisphere TH-positive cell counts were significantly different from control hemisphere TH-positive cell counts within the treatment group (paired *t* test; $p < 0.05$). *Bottom right panel*, Spearman's nonparametric correlation analysis was used to assess the relationship between decreased striatal TH content and TH-positive cell counts. A significant relationship ($p < 0.01$) was observed between TH content parameters in the nerve terminals (striatum) and cell bodies (substantia nigra). ○, Free-TYR; △, free-3NT; □, 6-OHDA.

reilles et al., 1999). Thus far, only nitration and consequent alterations in protein function have been proposed (Torreilles et al., 1999). Elevations of the free nitrated amino acid (free-3NT) have also been observed in these settings but have been considered only as a biomarker of $ONOO^-$ formation or increased catabolism of nitrated proteins (Ischiropoulos, 1998; Tohgi et al., 1999). A neurotoxic role for the free modified amino acid has not been investigated.

The symptomology of Parkinson's disease is known to originate primarily from severe degeneration of the dopaminergic nigrostriatal pathway; however, the mechanism or mechanisms by which this degeneration is initiated are unknown (Zhang et al., 2000). The 6-hydroxydopamine turning model has been used for decades to model the pathology of Parkinson's disease and has been predictive of both clinical symptomology and therapeutic outcomes in Parkinson's patients (Uretsky and Schoenfeld, 1971; Fung and Urestky, 1980; Linder et al., 1996; Mandel et al., 1997). As expected, striatal 6-OHDA injection caused a striking ipsilateral turning response after amphetamine dosing, whereas apomorphine dosing caused significant and contralateral turning. We and others have demonstrated previously that these challenge-induced behaviors are consistent with selective destruction of dopaminergic neurons within the striatal region. Striatal injection of free-3NT (at doses half that of 6-OHDA) also elicited similar amphetamine-induced responses, indicative of dopamine neuron injury *in vivo*. In contrast, free-3NT-treated mice did not demonstrate similar evidence of postsynaptic dopamine receptor supersensitivity (apomorphine response). These data could suggest that the striatal injury induced by free-3NT may be less severe for dopaminergic neurons than 6-OHDA at the doses used. In contrast to the effects of free-3NT, equimolar free-TYR was completely devoid of any behavioral effects, also suggesting a unique action of the modified amino acid.

After the behavioral investigations, brains were analyzed for histological evidence of neuronal injury. Although general striatal morphology was apparently preserved in each treatment group (cresyl violet staining), we observed striking evidence of unilateral decreases in striatal TH content (an index of dopamine-specific neuronal degeneration in this region) in the free-3NT and 6-OHDA treatment groups, whereas free-TYR injection produced no effects. Furthermore, we observed significant evidence of cell body degeneration in the origin of these dopaminergic nerve terminals, the substantia nigra pars compacta, in the free-3NT and 6-OHDA treatment groups. Again, free-TYR did not elicit a neurodegenerative effect. These histological results are highly consistent with the behavioral data, in that both striatal nerve terminal injury and pars compacta cell body loss are significant in both the free-3NT and 6-OHDA treatment groups but are more severe in the 6-OHDA-treated mice. This histological data might be predicted from the behavioral data demonstrating an amphetamine-induced turning response in both treatment groups but an apomorphine-induced turning response in only the 6-OHDA treatment group. Together, these data represent the first experimental evidence that free-3NT can elicit potent neurodegenerative effects *in vivo*.

The free modified amino acid free-3NT has been evaluated previously for biologic activity and has been implicated as a potentially cytotoxic species in a variety of cell types *in vitro* (Eiserich et al., 1999; Kalisz et al., 2000; Mihm et al., 2000). Because free-3NT protein incorporation has been suggested as a mechanism of the biologic activity of free-3NT (Eiserich et al., 1999; Kalisz et al., 2000) and because striatal protein nitration has been implicated as a mechanistic event in multiple settings of neurodegenerative injury [most notably the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's (Ara et al., 1998)], the protein incorporation of free-3NT was investigated as a mechanism for the observed behavioral and histological evidence of neurodegeneration in this setting. Interestingly, no significant evidence of protein nitration was observed in any treatment group, suggesting that the protein incorporation of 3NT is apparently not obligatory in the observed behavioral deficits or degenerative events. Continued study into the mechanisms of free-3NT-induced cell death in this setting appears warranted and may include both oxidative (Krainev et al., 1998) and nonoxidative (Mihm et al., 2000) pathways.

Nonparametric correlation analyses yielded a highly significant negative correlation between amphetamine-induced rotational orientation and the TH immunoreactivity ratio (Fig. 3, *right panel*). These findings are the first experimental evidence statistically linking behavioral deficits to decreases in striatal TH content in this model, and they suggest that nigrostriatal neurodegeneration is a potential mechanism of free-3NT-induced behavioral deficits in this model. In addition, we observed a statistically significant positive correlation between the changes in TH content in the striatum (site of injection) and cell body counts in the substantia nigra pars compacta (site of cell body origin).

These results are consistent with the interpretation that the mechanism of behavioral impairments elicited by free-3NT was the result of neurodegenerative effects and that the neuronal injury elicited by free-3NT resulted in cell death in the nigrostriatal axis. Because these associations strongly suggest a neurodegenerative role for free-3NT *in vivo*, additional mechanistic investigations certainly appear warranted and are ongoing in our laboratory.

An intense interest in oxidative biology has developed in several research communities, including the neuroscience, cardiovascular, aging, diabetes, cancer, and human immunodeficiency virus–acquired immunodeficiency syndrome fields (Fridovich, 1998). In all of these settings, $ONOO^-$ has emerged as a participant and potential initiator of progressive disease (Ischiropoulos, 1998; Torreilles et al., 1999). Thus far, $ONOO^-$-related cytotoxicity has been attributed exclusively to local cellular DNA damage or to impaired protein structure and function via tyrosine nitration or other oxidative events (Beckman and Koppenol, 1996). Our findings provide a new and previously undescribed insight into the mechanisms of $ONOO^-$ cytotoxicity *in vivo*. Although $ONOO^-$ formation and attendant protein nitration have been implicated in the pathogenesis of several progressive neurodegenerative disorders, we have described the first experimental evidence that the free modified amino acid free-3NT can elicit potent neurodegenerative effects *in vivo*, independent of $ONOO^-$-mediated oxidative and/or protein nitration events. Rather than serving only as a benign biomarker of oxidative events, as suggested previously, free-3NT may have a causal role in neurodegenerative conditions.

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