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GDNF Reduces Oxidative Stress in a 6-Hydroxydopamine Model of Parkinson's Disease

Michael P. Smith¹ and Wayne A. Cass^{*}

Department of Anatomy and Neurobiology, University of Kentucky College of Medicine, Lexington, Kentucky 40536, USA

Abstract

Many current theories of Parkinson's disease (PD) suggest that oxidative stress is involved in the neurodegenerative process. Potential neuroprotective agents could protect neurons through inherent antioxidant properties or through the upregulation of the brain's antioxidant defenses. Glial cell line-derived neurotrophic factor (GDNF) has been shown to protect and restore dopamine neurons in experimental models of PD and to improve motor function in human patients. This study was designed to investigate GDNF's effect on oxidative stress in a model of PD. GDNF or vehicle was injected into the right striatum of male Fischer-344 rats. Three days later 6-OHDA or saline was injected into the same striatum. The striatum and substantia nigra from both sides of the brain were removed 24 hours after 6-OHDA or saline injection and analyzed for the oxidative stress markers protein carbonyls and 4-hydroxynonenal. Both markers were significantly reduced in GDNF + 6-OHDA treated animals compared to vehicle + 6-OHDA treated animals. In addition, in animals allowed to recover for 3.5 to 4 weeks after the 6-OHDA administration, the GDNF led to significant protection against loss of striatal and nigral tissue levels of dopamine. These results suggest that the protective effects of GDNF against 6-OHDA involve a reduction in oxidative stress.

Keywords

GDNF; reactive oxygen species; protein carbonyls; 4-hydroxynonenal; striatum; substantia nigra; dopamine

Oxidative stress is a common characteristic of many of the current theories of the etiology of Parkinson's disease (PD). It is implicated either as a cause or result of the neurodegeneration associated with PD [4,29,36,38] and is also considered an important component in neurodegeneration in other models and diseases such as traumatic brain injury [3], HIV-related dementia [1], cerebral ischemia [5], Alzheimer's disease [32], and amyotrophic lateral sclerosis [34].

Glial cell line-derived neurotrophic factor (GDNF) has been shown to have both protective and restorative properties *in vivo*, particularly regarding the dopaminergic neurons of the substantia nigra [12,16,22]. In addition to its effects in models of PD, GDNF has demonstrated similar protective and restorative effects in other models of neurodegeneration [6,17,26,48].

¹Present address: Department of Neuroscience, Medical University of South Carolina, 173 Ashley Ave., Suite 403 BSB, Charleston, SC 29425.

^{*}Corresponding Author: Wayne A. Cass, Ph.D. Department of Anatomy and Neurobiology MN-225 Chandler Medical Center University of Kentucky Lexington, Kentucky 40536-0298 Telephone (859) 323-1142 FAX (859) 323-5946 E-mail: wacass1@uky.edu

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Although the protective and restorative properties of GDNF have been well documented, the mechanisms behind these properties are still relatively unknown.

Increases in oxidative stress can be detected before signs of neuronal degeneration [15,47], suggesting that oxidative stress may be an early component of neuronal loss. This study was designed to investigate GDNF's ability to reduce the generation of oxidative stress in a rodent model of PD. To carry out these studies, young adult rats received GDNF prior to 6-OHDA. Dopamine (DA) was measured in the striatum and substantia nigra to confirm GDNF's ability to reduce 6-OHDA-induced depletion of DA. Protein carbonyls and 4-hydroxynonenal (HNE) were assayed to measure GDNF's ability to reduce or inhibit oxidative stress. The measurement of protein carbonyls is one of the most widely accepted techniques to measure oxidative damage [41], and HNE is the most abundant cytotoxic molecule generated *in vivo* under oxidative conditions [45].

Young adult (12-21 weeks, 230g-326g) male Fischer-344 rats (Harlan Sprague Dawley, Indianapolis, IN) were used for all experiments. Animals were housed in groups of two under a 12-hour light/dark cycle with food and water freely available. All animal use procedures were in strict accordance with the NIH *Guide for the Care and Use of Laboratory Animals* and were approved by the Animal Care and Use Committee at the University of Kentucky.

The rats were anesthetized with isoflurane (2.0-2.5% as needed) and placed into a stereotaxic frame. The skull was exposed and a small burr hole was drilled in the skull above the right striatum (0.0 mm posterior to bregma, 3.4 mm right of midline). The site of GDNF administration was selected to be located between the two subsequent 6-OHDA injection sites. The dura was cut and a SGE syringe with a 26 gauge blunt-tipped needle was slowly lowered to a depth of 5.0 mm below the surface of the brain. 5 μ l of either vehicle (10 mM citrate buffer with 150 mM NaCl, pH 5.0) or 5 μ g of GDNF (PeproTech, Rocky Hill, NJ) dissolved in vehicle was injected at a rate of 0.5 μ l/minute for 10 minutes into the striatum. The needle was left in place for an additional 5 minutes following the injection and then slowly withdrawn. The burr hole was filled with Gelfoam and the incision closed with wound clips. Three days after the first surgery, the rats were again anesthetized with isoflurane and placed into a stereotaxic frame. 6-OHDA sites of administration were based upon previous work in our lab [40] and adapted from Kirik et al. [24]. The skull was exposed and 2 small burr holes were drilled in the skull above the right striatum (0.5 mm posterior to bregma, 4.2 mm right of midline; 0.5 mm anterior to bregma, 2.5 mm right of midline). The dura was cut and a SGE syringe with a 26 gauge blunt-tipped needle was slowly lowered to a depth of 5.0 mm below the surface of the brain. 2 μ l of either vehicle (0.9% saline with 0.1% ascorbic acid, pH 5.5) or 10 μ g of 6-OHDA (Sigma-Aldrich, St. Louis, MO) dissolved in vehicle was injected at a rate of 0.4 μ l/minute for 5 minutes into each of the two sites. The needle was left in place for an additional 5 minutes following each injection and then slowly withdrawn. The burr holes were filled with Gelfoam and the incision closed with wound clips.

Striatal and nigral tissue was collected for the measurement of oxidative stress markers 24 hours after the 6-OHDA or saline injections. Tissue was collected for the measurement of DA levels from a separate group of animals 3½ to 4 weeks after 6-OHDA or saline injections. All animals were rendered unconscious with CO₂, decapitated and the brains quickly removed and chilled in ice-cold saline. A coronal slice 2 mm thick was removed at the level of the striatum using a chilled brain mold (Rodent Brain Matrix; ASI Instruments, Warren, MI). The left and right striata were then dissected from the slice. A similar 2 mm coronal slice was made through the midbrain and the substantia nigra removed from both sides of the brain. All tissue samples were placed in pre-weighed vials, weighed and frozen on dry ice. The samples were stored at -80°C until analysis.

Tissue levels of DA were analyzed using high pressure liquid chromatography (HPLC) with electrochemical detection as described previously [7]. The retention times of standards were used to identify peaks, and peak heights were used to determine amount of recovery of internal standard (dihydroxybenzylamine) and amounts of DA.

The primary focus of these studies was on the effects of GDNF on nigrostriatal DA neurons. Thus, for the measurement of oxidative stress markers in the striatum we prepared crude synaptosomes in order to remove some of the non-dopaminergic elements (neuronal and glial cell bodies). Striatal tissue was homogenized with a Teflon pestle in cold 0.32 M sucrose buffer containing protease inhibitors (Complete, Mini; Roche Diagnostics, Indianapolis, IN) and centrifuged for 15 minutes at $1000 \times g$. The supernatant was transferred to a clean vial and centrifuged a second time for 15 minutes at $12,500 \times g$. The supernatant was discarded and the resulting crude synaptosomes were resuspended in 50 μ L of buffer. For the substantia nigra, as we were interested in dopaminergic cell bodies, we used whole tissue rather than synaptosomes to ensure inclusion of the cell bodies. Nigral tissue was homogenized with a sonicator in cold 0.32 M sucrose buffer containing protease inhibitors. Protein concentrations were determined using the Pierce BCA method (Pierce Biotechnology, Rockford, IL).

The methods for determining protein carbonyls and HNE by slot blots have been described previously as rapid and effective methods of measuring markers of oxidative stress [35,42]. For protein carbonyls, equal volumes of protein (15 μ g), 12% SDS, and the derivatizing agent 2,4-dinitrophenylhydrazine (DNP) (OxyBlot Protein Oxidation Detection Kit; Chemicon International, Temecula, CA) were incubated at room temperature for 20 minutes and the reaction was stopped with a neutralizing solution. 250 ng of derivatized proteins were slot-blotted to a nitrocellulose membrane via vacuum filtration through a 48 well template (Bio-Dot SF; BioRad, Hercules, CA). For HNE analysis, 15 μ g of protein per sample was slot-blotted onto the membrane by the same method. The membrane was blocked with 3% BSA for 1 hour, followed by incubations with rabbit anti-DNP (1:150, Chemicon) for 1 hour for protein carbonyls or rabbit anti-HNE (1:10,000, Alpha Diagnostic, San Antonio, TX) for 2 hours for HNE. This was followed by goat anti-rabbit conjugated to alkaline phosphatase (1:15,000, Sigma-Aldrich, St. Louis, MO) for 1 hour, and an alkaline phosphatase substrate (SigmaFast; Sigma-Aldrich, St. Louis, MO). After drying, the developed membrane was scanned and the optical density of the samples quantified densitometrically using Scion Image.

Data from the slot blots were expressed as a ratio of the side ipsilateral to the injections to the side contralateral to the injections (I:C ratio). Tissue levels of DA were expressed as ng/g wet weight of tissue. Results are expressed as mean $\pm$ SEM. All data were analyzed by ANOVA followed by Newman-Keuls post hoc comparisons. P-values ≤ 0.05 were considered statistically significant.

Figure 1 shows striatal and nigral tissue levels of DA measured 3.5 to 4 weeks after 6-OHDA or saline injection. In the animals that received vehicle + 6-OHDA, there was a significant decrease in DA of 93% in the ipsilateral striatum compared to the contralateral striatum, and a significant decrease in DA of 70% in the ipsilateral nigra compared to the contralateral nigra. The GDNF treatments significantly reduced the DA-depleting effects of the 6-OHDA. In the animals treated with GDNF + 6-OHDA, the decrease in striatal DA was 66% in the ipsilateral striatum compared to the contralateral striatum, and the decrease in nigral DA was 19% in the ipsilateral side compared to the contralateral side. In the GDNF + saline treated animals, the GDNF had no effect on striatal DA levels, but did increase nigral DA levels by 36%.

Protein carbonyls and HNE were measured 24 hours after intrastriatal administration of 6-OHDA or saline. This time period was chosen based on previous experiments where increases in striatal levels of protein carbonyls and HNE occurred 1 day, but not 3, 7 or 14 days after

intrastratial injections of 6-OHDA [40]. In animals treated with vehicle + 6-OHDA there was a significant increase in the I:C ratio for both protein carbonyls (+62%) and HNE (+127%) in the striatum (Fig. 2). Administration of GDNF 3 days prior to the 6-OHDA completely prevented the 6-OHDA-induced increases in protein carbonyls and HNE in the striatum. Neither the GDNF nor the 6-OHDA injections had any effect on the I:C ratios for protein carbonyls or HNE in the substantia nigra (Fig. 2).

The present results suggest that GDNF, when administered prior to 6-OHDA, decreases the amount of oxidative stress produced by 6-OHDA and reduces the loss of DA in the striatum and substantia nigra. These results concerning DA levels are analogous to previous findings that a single injection of GDNF into the striatum attenuates the loss of DA and neurons in the nigrostriatal pathway of rodents lesioned with 6-OHDA [2,8,25]. Our studies suggest that the observed reduction in oxidative stress markers is likely due to downstream effects of GDNF. Neuroprotective effects of GDNF are abolished when protein synthesis is inhibited [21], and following a single injection of GDNF into the striatum, GDNF is retrogradely transported to the substantia nigra and is present in the striatum only in very low levels 3 days after administration [43]. The 6-OHDA-induced loss of DA was attenuated in our study when GDNF was administered 3 days prior to 6-OHDA, which suggests that the protective effects we observed are likely due to downstream effects of GDNF. A similar finding in regards to GDNF's downstream effects was found in a primate model of PD in which GDNF was continuously infused into the brain for two months and then stopped [14]. The improved behavioral effects due to the GDNF were still observable for at least two months after the administration of GDNF was halted.

Previous studies in our lab have looked at the generation of oxidative stress following a striatal injection of 6-OHDA [40]. An increase in markers of oxidative stress in the striatum occurred 24 hours after 6-OHDA. This increase was absent at 3, 7, and 14 after 6-OHDA. We did not see an increase in oxidative stress markers at any of these time points in the substantia nigra. DA measurements taken at the same time points showed a significant decrease in the striatum at all time points, while DA in the substantia nigra was not decreased until day 7. It has been suggested that ROS initiate signal transduction pathways, such as that of apoptosis, which commence in neuronal terminals and terminate in the cell bodies [30,31]. Thus, apoptotic events initiated in striatal DA terminals may be leading to retrograde degeneration or damage to DA cell bodies in the absence of observable oxidative stress in the substantia nigra.

We are unaware of any other studies that have demonstrated in an *in vivo* model that the increase in oxidative stress observed after striatal administration of 6-OHDA is attenuated by prior administration of GDNF. *In vitro* studies have shown that GDNF can reduce oxidative stress-induced cell death [13,44,46], and there is evidence that a single injection of GDNF causes an increase in the activity of superoxide dismutase, catalase, and glutathione peroxidase *in vivo* in non-lesioned rodents [9]. Oxidative stress is increased in patients with PD [18,23,37], and GDNF has been demonstrated to improve motor function in patients with PD [33,39]. Thus, our findings that GDNF attenuates neurotoxin-induced oxidative damage *in vivo* may relate to the positive behavioral effects that have been reported in some patients, and adds to the understanding of the protective properties of GDNF. To more fully appreciate these properties it will be important to determine in future studies the effects that GDNF has on endogenous antioxidant systems in *in vivo* models of neurodegeneration.

The I:C ratios for the protein carbonyls and HNE in the GDNF + 6-OHDA treated animals were not statistically different than the non-lesioned controls. However, there was still a significant decrease in striatal DA levels in the GDNF + 6-OHDA animals when comparing these same groups. One possibility for this discrepancy is that neurons lesioned by 6-OHDA may be destroyed by more than one mechanism. Conflicting reports exist in reference to the

mechanisms involved with 6-OHDA-induced cell death. Most research shows that 6-OHDA induces apoptosis [10,28,49]. However, there are reports of increased apoptotic factors without corresponding changes in cellular morphology [19,20,27]. *In vitro* studies have demonstrated that while GDNF enhances the viability of DA neurons against 6-OHDA, it does not completely inhibit cell death [11,46]. Oxidative stress is likely not involved in all aspects of the degeneration associated with 6-OHDA, which could account for the results that we obtained. In addition, indicators of oxidative stress that were not quantified in the present study (such as levels of glutathione, hydrogen peroxide or nitric oxide) may be involved. Thus, further studies are needed to more fully define the protective effects of GDNF.

The results from the present study suggest that GDNF is involved in the attenuation of free radical damage, at least in terms of the 6-OHDA lesioned rodent model. Though the effects of GDNF are well documented, the mechanisms involved in how GDNF induces neuroprotection have not been completely elucidated. Uncovering these mechanisms may give insight into sources of neuronal loss in PD and other neurodegenerative disorders. GDNF's ability to attenuate oxidative stress is only one of its overall effects, and studying these protective effects advances understanding of how GDNF may prevent the further loss of neurons in PD. Because PD is a progressive disease, the prevention of further loss of neurons is a major factor in treatment. At present, GDNF must be administered directly into the brain. Thus, its use as a conventional therapeutic agent may be limited. However, the results that have been demonstrated in both models of PD and in humans have been substantial and promising; and therefore, continued study of GDNF may lead to the development of other compounds or therapeutic strategies that will be beneficial for the treatment of PD and other neurodegenerative disorders.

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Figure 1.

Tissue levels of DA in rats treated with intrastriatal vehicle or GDNF 3 days before intrastriatal saline or 6-OHDA. DA was measured in the striatum and substantia nigra 3.5 to 4 weeks later. Results are means $\pm$ SEM from 6 - 8 animals per group. * $p < 0.05$ vs. contralateral side of same group, # $p < 0.05$ vs. ipsilateral side of vehicle + 6-OHDA group (two-way ANOVA with side as a within factor followed by Newman-Keuls post hoc comparisons).

Figure 2.

Effects of GDNF on protein carbonyl and HNE levels. GDNF or vehicle was injected into the right striatum 3 days before 6-OHDA or saline was injected into the same striatum. Tissue samples were collected 24 hours after the 6-OHDA or saline injections. Protein carbonyls and HNE content were measured in the striatum and substantia nigra and a ratio of the ipsilateral to contralateral side (I:C Ratio) was calculated for each animal for each region. Results are mean $\pm$ SEM for 6 animals per group. * $p < 0.05$ vs. all other groups (one-way ANOVA followed by Newman-Keuls post hoc comparisons).