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IGF-1 Protects Dopamine Neurons Against Oxidative Stress: Association with Changes in Phosphokinases

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Abstract

Insulin-like growth factor-1 (IGF-1) is an endogenous peptide transported across the blood brain barrier that is protective in several brain injury models, including an acute animal model of Parkinson's disease (PD). Motor deficits in PD are due largely to the progressive loss of nigrostriatal dopaminergic neurons. Thus, we examined the neuroprotective potential of IGF-1 in a progressive model of dopamine deficiency in which 6-hydroxydopamine (6-OHDA) is infused into the striatum. Rats received intrastriatal IGF-1 (5 or 50 μ g) 6 hrs prior to infusion of 4 μ g 6-OHDA into the same site and were sacrificed 1 or 4 wks later. Both concentrations of IGF-1 protected tyrosine hydroxylase (TH) immunoreactive terminals in striatum at 4 wks but not at 1 wk, indicating that IGF-induced restoration of the dopaminergic phenotype occurred over several weeks. TH-immunoreactive cell loss was only attenuated with 50 μ g IGF-1. We then examined the effect of striatal IGF-1 on the Ras/ERK1/2 and PI3K/Akt pathways to ascertain if their activation correlated with IGF-1-induced protection. Striatal and nigral levels of phospho-ERK1/2 (pERK1/2) were maximal 6 hrs after IGF-1 infusion and, with the exception of an increase in nigral pERK2 at 48 hrs, returned to basal levels by 7 days. Phospho-Akt (Ser473) was elevated 6–24 hrs post-IGF-1 infusion in both striatum and substantia nigra concomitant with inhibition of pro-death GSK-3 β , a downstream target of Akt. These results suggest that IGF-1 can protect the nigrostriatal pathway in a progressive PD model and that this protection is preceded by activation of key pro-survival signaling cascades

Keywords

6-OHDA; Akt; CREB; Parkinson's disease; 6-OHDA; Striatum; ERK1/2; GSK-3 β ; CREB

1. Introduction

Parkinson's disease (PD) is characterized in part by motor dysfunctions due largely to the loss of dopaminergic cells in the substantia nigra (SN) and a reduction of dopaminergic control over striatal output neurons (Agid and Blin, 1987; Hornykiewicz, 1998). Although

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the connection between dopaminergic cell loss in the SN and motor dysfunctions has been known for decades, the underlying cause of this cell death remains unknown. However, several neurotrophic factors, including glial cell line-derived neurotrophic factors (GDNF), have been shown to reduce the vulnerability of dopaminergic neurons in animal models when administered exogenously (see review by Airavaara et al., 2012), and GDNF has also been reported to be effective in the treatment of PD (Rosenblad et al., 1999; Gill et al., 2003; Nutt et al., 2003; Slevin et al., 2006; Patel et al., 2013), although this is somewhat controversial (see Sherer et al., 2006). Moreover, age is the major known risk factor for PD, and the endogenous level of some neurotrophic factors decrease with age, as will be discussed below. This has lead several investigators to suggest that trophic factor loss may contribute to the etiology of PD (Parain et al., 1999; Sonntag et al., 1999; Howells et al., 2000; Chauhan et al., 2001; Boger et al., 2006; Maggio et al., 2006).

Insulin-like growth factor 1 (IGF-1) is synthesized mainly in the liver and transported across the blood-brain barrier into the brain, although it is also produced locally within the brain (for review see Aberg, et al., 2006). IGF-1 levels in plasma and brain are highest during prenatal development and decrease with age (Sonntag, et al., 1999; Maggio, et al., 2006), as does its receptor IGF-R1 (Sonntag, et al., 1999; Yaghmaie, et al., 2006). Other neurotrophic factors also decline with age (Yurek and Fletcher-Turner 2001; Abe et al., 2010; Calabrese et al., 2013). IGF-1 appears to be of particular importance to dopaminergic neurons. For example, the concentration of IGF-1 mRNA in the SN is 3 times higher than its overall concentration in the brain (Rotwein et al., 1988) and tyrosine hydroxylase (TH) immunoreactive cells in the SN contain the IGF-1 receptor (IGF-1R) (De Keyser, et al., 1994; Quesada, et al., 2007). IGF-1 mRNA is also detected in the striatum (Rotwein et al., 1988), as is the IGF-1R receptor (De Keyser, et al., 1994).

Collectively, these observations raise the possibility that decreased signaling through the IGF-1 pathway is causally related to dopaminergic neuronal degeneration in PD and that increased signaling through this pathway may protect against the loss of dopaminergic neurons in this condition. Indeed, IGF-1 has been reported to protect and promote the survival of dopaminergic neurons in vitro (Beck, et al., 1993; Zawada, et al., 1996). Moreover, chronic administration of IGF-1 or administration of Gly-Pro-Glu, an N-terminal peptide of IGF-1, attenuated loss of TH-immunoreactive cells, terminals, and behavioral deficits in response to 6-OHDA infusion into the dopaminergic axons within the nigrostriatal pathway (Guan, et al., 2000; Krishnamurthi, et al., 2004; Quesada and Micevych, 2004). However, infusion of 6-OHDA into the striatum, the terminal field of dopaminergic neurons in SN may better reflect the progression of the human condition. This is because, after an intrastriatal infusion, the loss of dopaminergic neurons is protracted, occurring over the course of several months instead of a few days, as is the case with 6-OHDA infusion into the dopaminergic axons (Sauer and Oertel, 1994), and also because it is generally believed that degeneration begins at the terminals in PD (Burke and O'Malley 2012).

To determine if IGF-1 could protect the dopaminergic nigrostriatal pathway from oxidative damage in this progressive model, we provided a single bolus injection of IGF-1 into the striatum 6 h prior to intrastriatal infusion of 6-OHDA, and sacrificed animals 1 or 4 wk post

6-OHDA infusion to assess the integrity of the dopaminergic nigrostriatal pathway. The mechanism underlying IGF-1 induced protection was also examined.

2. Materials and Methods

Materials

IGF-1 was purchased from Peprotech (Rocky Hill, NJ, USA) and was prepared in 1 mM filtered phosphate buffer. 6-OHDA was purchased from Regis Technologies (Morton Grove, IL, USA). All other reagents were of the highest available purity and were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless indicated otherwise.

Animals

Eighty-five male *Sprague Dawley rats* (Harlan, Indianapolis, IN, USA) weighing 250 g were used in these experiments. All animals were housed 2 per cage and maintained on a 12 h light/dark cycle with food (Harlan Laboratories, Madison WI) and water available ad libitum. All procedures were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh.

Surgical Procedures

Animals were placed in an induction chamber containing 4–5% isoflurane (Halocarbon, River Edge, NJ, USA) with 100% O₂ as a carrier gas. Animals were then placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA) where the anesthetic plane was maintained with 1–2% isoflurane in 100% O₂. A 10 µl Hamilton syringe and a microinfusion pump (Stoelting, Wood Dale, IL, USA) was used to infuse IGF-1 (5 or 50 µg/3 µl phosphate buffer or vehicle (3 µl phosphate buffer) unilaterally into the striatum at the coordinates +0.7 mm anterior, –3.1 mm lateral to bregma, and 6.0 mm ventral to dura (Paxinos and Watson, 1982). The infusion flow rate was 0.5 µl/min. The infusion syringe was left in place for 5 min after delivery of IGF-1 to allow for diffusion of the neurotrophic factor. To assess the diffusional characteristics of IGF-1 throughout the striatum and the SN, a group of rats was perfused at 6 h, 24 h, 3 d and 7 d post-IGF-1 with ice cold 0.1 M phosphate buffer followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were removed, sectioned, and striatal and nigral sections stained for IGF-1. The remaining animals were again anesthetized with isoflurane 6 h post-IGF-1 infusion and received 0.75 µl of 4 µg 6-OHDA or 0.02% ascorbic acid in sterile 0.9% saline (0.5 µl/min) at the same striatal coordinates as above. The animals were sacrificed 1 wk and 4 wks later by transcardial perfusion, as described above, and the brains harvested for subsequent analysis of TH using immunohistochemistry.

Immunohistochemical Analysis

One and 4 weeks post-6-OHDA infusion, animals were deeply anesthetized with Equithesin and sacrificed via intracardial perfusion with chilled 0.1 M phosphate buffer followed immediately by chilled 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were removed and post fixed for 2 h in the same fixation solution followed by 48 h in 30% sucrose in 0.1 M phosphate buffer (pH 7.4) at 4°C. Brains were then sectioned at 60 µm

thickness on a cryostat. Coronal slices were collected in a one-in-six series. Every sixth section from the SN and the striatum was labeled with antisera against TH or IGF-1.

All sections were rinsed 3 times in 10 mM phosphate buffered saline (PBS, pH 7.6) prior to and between incubations. Sections were pretreated for 15 min with 0.1% H₂O₂ in PBS followed by blocking with 10% normal donkey serum (Jackson ImmunoResearch Labs, Inc., West Grove, PA, USA) and 0.3% Triton-X 100 for 1 h at room temperature. Sections were then incubated with a 1:1000 dilution of the monoclonal mouse anti-TH antibody (catalog # MAB318; Chemicon, Inc., Temecula, CA, USA) or polyclonal IGF-1 (catalog # AF291-NA; R&D Systems, Minneapolis, MN, USA) diluted in 10% normal donkey serum and 0.3% Triton-X 100 overnight at 4°C. This was followed by incubation for 1 h at room temperature in a 1:2000 dilution of the appropriate biotinylated secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA). Tissue was then treated with an avidin biotin peroxidase complex (ABC-Elite, Vector Laboratories, Burlingame, CA) and subsequently incubated for 10 min in diaminobenzidine (20 mg in 100 ml Tris buffer, pH 7.2). The peroxidase reaction was induced by adding 0.01% H₂O₂. After several washes, sections were mounted on gelatin coated slides and allowed to dry overnight at room temperature. To stabilize the diaminobenzidine signal against fading or photobleaching, sections were then exposed to a 0.04% solution of osmium tetroxide in 0.1 M phosphate buffer for 10–20 sec, washed and dehydrated in ascending concentrations of ethanol, and cover slipped with Permount mounting medium (Fisher Scientific, Pittsburgh, PA, USA) after submersion in xylenes. To insure the staining specificity, negative control sections were always included by omitting the primary antibody.

Cell counts in SN

To count dopaminergic neurons in the SN, we selected every sixth section from each animal for staining using a random number generated from a computer program (GraphPad, La Jolla, CA) to select the first section. We then counted TH-immunoreactive cells in the 3 sections of the SN that appeared to show the greatest loss of TH immunoreactive cells for each animal. Cell number was the average $\pm$ SEM of cell numbers in these 3 sections.

Image Analysis

For semiquantitative assessment of dopaminergic nerve terminals within the striatum, we analyzed the lesion area using MetaMorph software (Molecular Devices Corp., Sunnyvale, CA, USA). For quantification of area devoid of TH-immunoreactivity, coronal sections were visualized using a Nikon Supercool scanner (Nikon Inc., Melville, NY, USA). The bright field images were imported into MetaMorph and the region of low staining intensity was circumscribed in blind fashion and the area within this region was calculated as number of pixels by MetaMorph and expressed as a ratio to the contralateral hemisphere. The average optical density of three sections centered on the infusion site that showed the largest area devoid of TH was used as a representation of lesion area for each animal. Vehicle treated animals were used as control.

Temporal analysis of IGF-1 effects on kinases

Rats receiving IGF-1 were sacrificed 6 h to 7 d later. To maintain the phosphorylation integrity of phospho-kinases in the brain post-mortem (O'Callaghan and Sriram, 2004), we sacrificed a separate group of animals (n = 6 per group) using a TMW-6402C microwave fixation system (Muromachi Kikai, Tokyo, Japan) composed of a microwave power generator and applicator unit designed to focus the microwave beam to the head. The animals were exposed to 4.5 kW of microwave radiation for 1.90 seconds. The striatum and SN were then dissected on a dry ice cooled platform and stored at -80°C until assay by Western blot analysis. Frozen samples were sonicated in 1% sodium dodecyl sulfate and boiled for 5 min at 95°C . After determination of protein content (BCA assay, Pierce, Rockford, IL), equal amounts of protein from each sample were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). The blots were then blocked in 5% nonfat milk and incubated overnight at 4°C with polyclonal antibodies targeting one of the following phospho-proteins: pERK1/2 (1:1000, catalog # 9101), pAkt (Ser473) (1:500, catalog # 9271), pCREB (1:500, catalog # 9191), pGSK-3 β ser 9 (1:1000, catalog # 9336), and p β -catenin (1:1000, catalog # 9561) (Cell Signaling Technology, Danvers, MA, USA). After washing, membranes were incubated at room temperature for 60 min with diluted horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:5000; Calbiochem, San Diego, CA, USA). Bound antibody was visualized by chemiluminescence (NEN, Boston, MA) and exposed to radiographic film. The blots were then stripped and re-probed for antibodies recognizing total protein of the phospho-proteins mentioned above (all at 1:1000; ERK1/2 catalog # 9102; Akt catalog # 9272, GSK-3 β catalog # 9315; CREB catalog # 9197; catenin catalog # 9562). Equal protein loading was confirmed by probing for α -tubulin (Sigma Chemical, St. Louis, MO; catalog # T6074). We quantified the bands corresponding to immunoreactivity against the protein of interest by densitometry using UnScan-It software (Silk Scientific, Orem, UT, USA) and all levels were normalized relative to total protein. In many occasions, the same blots were stripped and re-probed for various proteins.

Statistical Analysis

Statistical analyses were performed using one-way ANOVA followed by Newman-Keuls post hoc analysis (GraphPad Software, La Jolla, CA, USA). Results were considered significant when $p < 0.05$ and data represented as mean $\pm$ SEM.

3. Results

Distribution of exogenous IGF-1 after intrastriatal infusion

To determine the diffusional pattern of IGF-1 at the time 6-OHDA would be infused into the striatum, we infused IGF-1 (5 or 50 $\mu\text{g}/3 \mu\text{l}$) into the striatum (0.5 $\mu\text{l}/\text{min}$), and striatal and nigral sections were subjected to immunohistochemical analysis for IGF-1. Rats were perfused at 6 h, 1 d, 3 d, or 7 d post-infusion. At the 6-hr time point, IGF-1 immunoreactivity was observed throughout the ipsilateral dorsal and ventral striatum (Fig 1), with staining also seen in the olfactory tubercle and overlying cortex. IGF-1 immunoreactivity was still present at 24 h post-injection, but decreased to basal levels at 3

and 7 d post-injection (data not shown). No staining was seen in the SN at the time points examined and no staining was seen on the contralateral side of the striatum, which could thus be used as an internal negative control. In addition, no staining was observed in vehicle treated animals in the SN or striatum.

Effect of pre-IGF-1 treatment on 6-OHDA induced loss of DA terminals in the striatum

6-OHDA (0.75 μ l of 4 μ g) resulted in a significant loss of TH-immunoreactivity in the striatum. Image analysis of striatum in a 360 μ m region centered on the 6-OHDA injection showed an approximate 30–35% area devoid of TH, both at 1 (Fig 2, $p < 0.001$ vs. vehicle) and 4 wk post-lesion (Fig 3A and 3B, $\# p < 0.05$ vs. vehicle) in comparison with vehicle treated animals.

IGF-1 infused 6 h before 6-OHDA had no appreciable effect on the response to the toxin within the striatum at 1 wk (Fig 2, $n = 6$). In contrast, by 4 wks the animals pretreated with either concentration of IGF-1 showed a 62% decrease in the striatal area devoid of detectable TH immunoreactivity compared to animals that received 6-OHDA alone (30% vs. 11.5% lesion area, $n = 12$, $p < 0.05$, Fig 3A and 3B), suggesting that restoration of the phenotype occurs gradually over several weeks.

Effect of IGF-1 on the 6-OHDA-induced loss of TH positive cell bodies

To assess the damage incurred to the SN, we counted three randomly selected sections in the SN of each treatment groups at 4 wks post 6-OHDA infusion. The average number of TH-immunoreactive cells in the sections from vehicle injected animals was 698 ± 49 cells ($n = 12$) (Fig 4), which is approximately 5% of the total number of TH immunoreactive cells reported to be in the SN (German and Manaye 1993; Nair-Roberts et al., 2008). Infusion of 6-OHDA reduced this to 485 ± 22 cells ($n = 12$), a reduction of 31% ($p < 0.05$). Animals that received 5 μ g IGF-1 6 hr prior to 6-OHDA had 540 ± 37 TH immunoreactive cells, which was not significantly different than animals that received 6-OHDA. In contrast, pretreatment with 50 μ g IGF-1 6 h prior to 6-OHDA resulted in a significant 38% sparing of TH-immunoreactive cells (652 ± 50 , $p < 0.05$) when compared to 6-OHDA treated animals, suggesting that IGF-1 can protect against loss of TH-immunoreactive cells in the SN in a progressive 6-OHDA model of PD (Fig 4).

Temporal effect of IGF-1 on ERK1/2 signaling

Neurotrophic factors are known to exert their effects through activation of the ERK1/2 and Akt signaling kinases. Thus, to begin to understand the possible involvement of these kinases in IGF-1 induced protection against 6-OHDA, we examined the temporal profile of these kinases and some downstream effectors in response to intrastriatal IGF-1. Intrastriatal infusion of 5 μ g IGF-1 transiently increased ERK1/2 phosphorylation in the ipsilateral striatum and SN. The effect of IGF-1 on pERK1/2 in the striatum was maximal at 6 h, the time point in which 6-OHDA was to be infused, with approximately a 2-fold increase in phospho-ERK1/2 (pERK1/2) ($p < 0.05$) compared to vehicle injected animals (Fig 5A). At 1 d, these kinases had returned to basal levels and remained there for 7 d post-lesion, the last time point assessed.

Intrastriatal IGF-1 also caused an increase in pERK1/2 in the SN at 6 h post-injection (87%, $p < 0.05$ and 34%, $p < 0.05$ for pERK1 and pERK2, respectively; Fig 5B). There was a trend toward slightly higher pERK1 throughout the entire 7 d observation period, but the increase from 1 d to 7 d was not significant, whereas pERK2 appeared to return to basal levels 1 d post-IGF-1, then increased again at 48 hr post-IGF-1 before returning to basal levels at 7 d post-infusion (Fig 5B). Intra-striatal IGF-1 had no significant effect on total ERK1/2 protein levels at any time point examined.

Temporal profile of IGF-1 on Akt signaling

IGF-1 also induced a significant increase in phospho-Akt (pAkt) (Ser473) in the striatum. Similar to its effects on pERK1/2, the increase was maximal at 6 h (184%, $p < 0.005$, Fig 6A), remained elevated at 1 d (87%, $p < 0.05$), but decreased to basal levels by 2 d post IGF-1 infusion. Intra-striatal IGF-1 induced a robust increase in pAkt (Ser473) in the SN at 6 h (186%, $p < 0.001$, Fig 6B) that was sustained until 1 d post-injection (191%, $p < 0.001$). pAkt (Ser473) had returned to vehicle control levels by 2 d post-infusion. No alteration in total protein levels of Akt was seen in response to IGF-1.

Effect of IGF-1 on GSK-3

Akt negatively regulates GSK-3 α and GSK3 β via phosphorylation at Ser 21 and Ser 9 respectively (Markuns et al., 1999; Leininger et al., 2004), so an increase in p-GSK levels at these sites would lead to an inhibition of its activity. Activated GSK-3 β hyperphosphorylates β -catenin, a member of the canonical WNT signaling pathway involved in cell growth and/or maintenance. This destabilizes β -catenin and targets β -catenin for degradation by the proteasome (Aberle et al., 1997). Thus, we performed a Western blot analysis with specific antibodies that recognize phosphorylated GSK-3 α and β at serine 21 and 9, respectively and total GSK-3 α/β , as well as total β -catenin.

Intrastriatal IGF-1 increased pGSK-3 α and β levels by approximately 40% ($p < 0.05$ and $p < 0.001$, respectively) in the striatum at 6 h to 1d compared to vehicle treated animals (Fig 7A), before returning to basal levels at 3 d. In contrast, intrastriatal IGF-1 did not affect the levels of GSK3 α or β phosphorylation in the SN (Fig 7B) or total GSK3 protein levels in the striatum or SN. There were no significant changes in phosphorylated or total β -catenin in the striatum (Fig 8) or SN (data not shown) when compared to vehicle treated animals. However, β -catenin was significantly lower at the 6 hr and 7 d time point than at 1d and 3d post IGF-1 infusion.

ERK1/2-dependent neuronal survival has been shown to activate the transcription factor cAMP response element binding protein (CREB) (Alonso et al., 2002; Ortega et al., 2011). Thus, we also examined the effect of IGF-1 on the phosphorylation state of this protein. Intra-striatal injection of IGF-1 increased phosphorylated CREB ($p < 0.05$, Fig 8B and C) at 6 hrs in the striatum but had no appreciable effects on pCREB in SN (data not shown) or total CREB in the striatum or SN.

4. Discussion

6-OHDA is widely used to model the loss of dopaminergic neurons responsible for motor deficits associated with PD. The striatal 6-OHDA model employed in this study provides a more protracted lesion model than most toxin-based models (Sauer and Oertel, 1994) and a wider time window for possible therapeutic interventions. This model has been used to study the neuroprotective effects of several neurotrophic factors but not IGF-1. However, unlike most neurotrophic factors, IGF-1 is able to cross the blood-brain barrier, and gain access to the brain from the periphery by way of a saturable transport system (Pan and Kastin 2000). Indeed, blood borne IGF-1 has been shown to be pivotal to exercise-induced protection against brain injury (Carro et al., 2001), spinal cord injury (Koopmans et al., 2006), and hippocampal neurogenesis (Trejo et al., 2001). This characteristic presents obvious advantages as a therapeutic intervention for neurodegenerative diseases. Thus, we examined the impact of IGF-1 in a progressive model of DA degeneration. Specifically, we asked whether a single bolus injection of IGF-1 could protect the dopaminergic nigrostriatal pathway from oxidative stress induced by 6-OHDA and if so, whether activation of the Ras/ERK1/2 and PI3K/Akt pathways correlated with this protection.

We observed that both 5 and 50 µg IGF-1 administered 6 hrs prior to 6-OHDA attenuated damage to TH-immunoreactive terminals in the striatum when assessed 4 weeks after 6-OHDA infusion. Our study using the progressive 6-OHDA striatal infusion model of PD agrees with previous observations using an acute PD model (axonal infusion of 6-OHDA) indicating that IGF-1 can mitigate toxicity to dopaminergic terminals induced by 6-OHDA (Quesada et al., 2008; Quesada and Micevych 2004). However, unlike these previous studies where protection was observed as early as 1-week post-6-OHDA infusion, we did not observe protection of TH-immunoreactive terminals in the striatum at the 1 wk time period, which suggests that in the progressive model of dopaminergic neuron loss protection may have resulted from gradual restoration of the TH phenotype or sprouting of residual striatal terminals (Love et al., 2005; Georgievska et al., 2002).

We and others have previously reported that dopaminergic neurotoxins, including 6-OHDA and MPTP, can downregulate the TH phenotype prior to actual cell death and have suggested that this represents an initial focus of the neurons on survival at the expense of normal cellular function (Bowenkamp, et al., 1996; Lu and Hagg 1997; Georgievska et al., 2002; Cohen et al., 2011). In the previous studies of IGF-1, the trophic factor was delivered chronically up to 7 days post-6-OHDA infusion either intracerebroventrically or peripherally, which may have resulted in more widespread distribution of IGF-1 in the striatum and even into SN, which in turn may have lessened the toxic impact of 6-OHDA and either prevented downregulation of TH or restored it more quickly. Indeed, we did not detect IGF-1 protein in the SN at any time point in the current study.

We observed that TH was reduced in 6-OHDA-treated animals despite pretreatment with IGF-1, as previously observed with GDNF (Cohen et al., 2011). Nonetheless, IGF-1 administration led to the gradual restoration of the dopaminergic phenotype that we and others have observed with pre-treatment with another neurotrophic factor, GDNF, using both the progressive and acute 6-OHDA PD models (Bowenkamp, et al., 1996; Lu and Hagg,

1997; Cohen et al., 2011). This further suggested that differences in the experimental paradigm (chronic infusion vs. single bolus injection) underlie the differing outcomes with IGF-1 in our study versus previous observations using this neurotrophic factor. In our own studies we observed that restoration of the TH phenotype in the striatum with GDNF occurred gradually over 8 wks (Cohen et al., 2011). Thus, it is possible that the 62% protection of TH in the striatum in the current study would have been greater had we assessed damage at later time points.

IGF-1 at the lowest dose administered did not protect against the loss of TH-immunoreactive cells in the SN as was observed previously with the more acute 6-OHDA model of PD. However, 50 µg IGF-1 did result in a statistically significant increase in the number of TH-immunoreactive cells in the SN compared to animals that only received 6-OHDA, and was no different than the number of TH-immunoreactive cells in saline treated animals. We and others have observed protection of TH-immunoreactive cells in the SN with other neurotrophic factors using the 6-OHDA striatal infusion model (Bowenkamp, et al., 1996; Lu and Hagg, 1997; Cohen et al., 2011). As previously stated, the loss of the TH phenotype does not necessarily correlate with actual cell loss. Indeed, with GDNF pretreatment we observed that the number of TH-immunoreactive cells decreased in the SN in response to 6-OHDA, but the number of FluoroGold positive cells did not (Cohen et al., 2011). We interpreted this as evidence that the cells did not die, but the phenotype was lost. Thus, the failure to observe a protection of the TH phenotype in the SN with the lower concentration of IGF-1 does not mean that this concentration in our hands did not protect against cell loss. With GDNF, we observed that the rate of recovery of TH in the terminal region was faster than that in the SN (Cohen et al., 2011). Indeed, at the 2 wk time point, GDNF had partially restored TH in the striatum, whereas at this same time point, loss of TH-immunoreactive cells in SN was no different than animals that had received 6-OHDA in the absence of GDNF (Cohen et al., 2011). Thus, it is possible that had we assessed TH-immunoreactivity in the SN at later time points we would have observed protection that reached statistical significance with the lower concentration of IGF-1.

Signaling through the IGF-1R receptor upon binding of IGF-1 activates two classical pathways known to be involved in cell survival, the Ras/ERK1/2 and PI3K/Akt signaling cascades (Aberg, et al., 2006; Cardona-Gomez, et al., 2002; Foncea, et al., 1997; LeRoith, et al., 1993; Willaime-Morawek, et al., 2005). Therefore, to assess the effect of IGF-1 on these signaling cascades at the time 6-OHDA, we examined the effect of IGF-1 on total and phosphorylated ERK1/2 and Akt (Ser473) in the striatum and SN from 6 hr – 7 days post-infusion. Our objective was to determine if protection correlated with activation of these signaling cascades. These signaling pathways have been shown to be upstream of nuclear factor erythroid 2-related factor 2 (Nrf2) (Hwang et al., 2008; Kim et al., 2010). Nrf2 is a stress responsive transcription factor that orchestrates the induction of a myriad of enzymatic and non-enzymatic anti-oxidant defense compounds (e.g. glutathione), and an increase in Nrf2 might act to counterbalance 6-OHDA-induced oxidative stress. Intra-striatal infusion of IGF-1 increased phosphorylation of ERK1/2 and pAkt (Ser473) in the striatum and SN in a time dependent manner. Interestingly, at the 1 d time point pERK1/2 levels returned to basal levels after an initial increase at 6 hrs concomitant with an increase in pAkt (Ser473) that rebounded when the levels of pAkt (Ser473) returned to basal levels. A similar activation

and deactivation of Akt and ERK1/2, respectively, was observed in the SN after chronic intraventricular infusion of IGF-1 and the protective effects of IGF-1 at 7 d post-lesion on TH-immunoreactive neurons and terminals was shown to be dependent on the Akt pathway and not the ERK1/2 pathway (Quesada, et al., 2008). Although ERK1/2 is generally considered to be pro-survival, sustained activation of pERK1/2 has been observed to mediate toxin induced cell death (Kulich and Chu, 2001, Stanciu, et al., 2000, Subramaniam, et al., 2004). Thus, in the context of ERK1/2-mediated toxicity, Akt-dependent negative regulation of ERK1/2 would have an obvious benefit. However, the physiological role of trophic factor induced inhibition of ERK1/2 by Akt is less clear.

We further assessed the effects of IGF-1 on the ERK1/2 and Akt signaling pathways by examining the effect of IGF-1 on the downstream effectors, GSK-3 β , β -catenin, and CREB in the striatum and SN. No effect of IGF-1 was observed on these phosphorylated proteins in the SN. However, GSK-3 α and β , pro-apoptotic kinases that are negatively regulated by Akt via phosphorylation, were inhibited in the striatum from 6 hrs –1 d post-IGF-1 infusion. Inhibition of GSK-3 α and β would allow for the stabilization of β -catenin, its translocation into the nucleus, and transcriptional regulation that could underlie IGF-1 protective effects. Likewise, pCREB was increased in the striatum at 6 hrs and thus could mediate IGF-1 induced protection against 6-OHDA toxicity.

In conclusion, like other neurotrophic factors, including GDNF and BDNF, IGF-1 is protective in a progressive 6-OHDA striatal infusion model and that protection correlated with early activation of two pivotal survival pathways, the Ras/ERK1/2 and PI3K/Akt signaling cascades. However, IGF-1 has the advantage that it can be given systemically. This unique quality of IGF-1 compared to other neurotrophic factors that have thus far been examined, may make it of particular value as a therapeutic intervention in the treatment of PD.

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Fig. 1.

Diffusion pattern of different concentrations of IGF-1 in the striatum. Five and 50 µg IGF-1 was infused into the striatum and animals were perfused at 6 hr post infusion. Striatal sections were labeled with an antibody against IGF-1 as described in Materials and Methods. Six hrs post infusion of IGF-1, staining was observed all over the striatum with some spillover into the olfactory tubercle and the overlying cortex. No staining was observed on the ipsilateral side, in the vehicle treated animals or in sections in which the primary antibody step was omitted.

Fig. 2. Effect of IGF-1 on the loss of striatal TH-immunoreactive terminals induced by 6-OHDA at 1 wk post-6-OHDA infusion. Animals received vehicle, 5 or 50 μg IGF-1 into the striatum 6 hrs prior to 4 μg/0.75 μl 6-OHDA or its vehicle at the same site.***p < 0.001 vs. vehicle.

Fig. 3.

Effect of IGF-1 on the loss of striatal TH-immunoreactive terminals induced by 6-OHDA. Animals received vehicle, 5 or 50 μg IGF-1 into the striatum 6 hrs prior to 4 μg/0.75 μl 6-OHDA or its vehicle at the same site. Animals were sacrificed 4 wks (**A** and **B**) post-6-OHDA infusion. The measured immunoreactivity in the ipsilateral striatum is expressed as a percent of the non-injected contralateral striatum (**A**). (**B**) Representative photomicrographs of animals who received vehicle, 6-OHDA or IGF-1 + 6-OHDA. Data represent the mean ± SEM of three independent experiments. #p < 0.05 vs. vehicle; ***p < 0.05 vs 0 μg IGF-1

Fig. 4.

Effect of IGF-1 on the loss of nigral TH-immunoreactive cells induced by 6-OHDA.

Animals received vehicle, 5 or 50 µg IGF-1 into the striatum 6 hrs prior to 4 µg/0.75 µl 6-OHDA or its vehicle at the same site and were then sacrificed 4 wks later **(A)** Cell counts of TH-immunoreactive neurons in the SN. **(B)** Photomicrographs representative of animals that received vehicle for 6-OHDA and IGF-1 and animals that received 0, 5 or 50 µg IGF-1 prior to 6-OHDA injections. Data represent the mean ± SEM of three independent experiments. #p < 0.05 vs. vehicle; *p < 0.05 vs 0 µg IGF-1

Fig. 5.

Effect of intrastriatal IGF-1 on the temporal activation of ERK1/2 in the striatum and SN. Animals received vehicle or 5 μ g IGF-1 into the striatum prior to being sacrificed by microwave irradiation 6 hr – 7 days post-infusion. The figure shows pERK1/2 as a function of total ERK1/2 in the striatum (**A**) and the SN as well as representative blots for ERK1/2 in the SN (**B**). α -Tubulin was used as a loading control. The data are expressed as the mean $\pm$ SEM of the ratio of phosphorylated to total protein levels. * $p < 0.05$ vs. vehicle

Fig. 6.

Effect of intrastriatal IGF-1 on striatal and nigral pAkt (Ser473) as a function of time. Animals received vehicle or 5 μ g IGF-1 into the striatum prior to being sacrificed by microwave irradiation 6 hr – 7 days post-infusion. Representative blots pAkt (Ser473) and total Akt levels are shown together with densitometry analysis of western blots from the striatum (A) and the SN (B). α -Tubulin was used as a loading control. The data are expressed as the mean $\pm$ SEM of the ratio of phosphorylated to total protein levels. * $p < 0.05$, ** $p < 0.005$ vs. vehicle

Fig. 7.

Effects of IGF-1 infusion in the striatum on the temporal levels of striatal (**A**) and nigral (**B**) GSK-3α and β. The figure shows representative blots of pGSK-3α/β and total GSK-3α/β as well as densitometry analysis of western blots from the striatum (**A**) and the SN (**B**). α-Tubulin was used as a loading control. Data are mean ± SEM of the ratio of phosphorylated to total protein levels. * $p < 0.05$, *** $p < 0.001$ vs. vehicle

Fig. 8.

Effect of intrastriatal IGF-1 on the temporal activation of β -catenin and CREB in the striatum. The figure shows representative blots as well as densitometry analysis of phosphorylated β -catenin and CREB as a function of total β -catenin and CREB in animals that received vehicle or 5 μ g IGF-1 into the striatum. α -Tubulin was used as a loading control. The data are expressed as the mean $\pm$ SEM of the ratio of phosphorylated to total protein. * $p < 0.05$ vs. vehicle; ** $p < 0.01$ vs. 1d and 2 d