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Intranasal insulin protects against substantia nigra dopaminergic neuronal loss and alleviates motor deficits induced by 6-OHDA in rats

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Abstract

Protection of substantia nigra (SN) dopaminergic (DA) neurons by neurotrophic factors (NTF) is one of the promising strategies in Parkinson's disease (PD) therapy. A major clinical challenge for NTF-based therapy is that NTFs need to be delivered into the brain via invasive means, which often shows limited delivery efficiency. The nose to brain pathway is a non-invasive brain drug delivery approach developed in recent years. Of particular interest is the finding that intranasal insulin improves cognitive functions in Alzheimer's patients. In vitro, insulin has been shown to protect neurons against various insults. Therefore, the current study was designed to test whether intranasal insulin could afford neuroprotection in the 6-hydroxylase dopamine (6-OHDA)-based rat PD model. 6-OHDA was injected into the right side of striatum to induce a progressive DA neuronal lesion in the ipsilateral SN pars compact (SNc). Recombinant human insulin was applied intranasally to rats starting from 24 h post lesion, once per day, for 2 weeks. A battery of motor behavioral tests was conducted on day 8 and 15. The number of DA neurons in the SNc was estimated by stereological counting. Our results showed that 6-OHDA injection led to significant motor deficits and a 53% of DA neuron loss in the ipsilateral side of injection. Treatment with insulin significantly ameliorated 6-OHDA-induced motor impairments, as shown in improved locomotor activity, tapered/ledged beam walking performance, Vibrissa-elicited forelimb-placing, initial steps, as well as methamphetamine-induced rotational behavior. Consistent with behavioral improvements, insulin treatment provided a potent protection of DA neurons in the SNc against 6-OHDA neurotoxicity, as shown by a 74.8 % of increase in tyrosine hydrolase (TH) positive neurons compared to the vehicle group. Intranasal insulin treatment did not affect body weight and blood glucose levels. In conclusion, our study showed that intranasal insulin provided strong neuroprotection in the 6-OHDA rat PD model, suggesting that insulin signaling may be a novel therapeutic target in a broad neurodegenerative disorders.

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Keywords

insulin; dopaminergic neurons; striatum; substantia nigra; neurotrophin; Parkinson's Disease

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder in aged population. Despite significant progresses towards better management of symptoms, there is a lack of effective preventive and therapeutic approaches. Neurotrophic factor (NTF) therapy has shown great promise in preclinical PD models (Hegarty et al., 2014; Rodrigues et al., 2014; d'Anglemon de Tassigny et al., 2015). However, recent clinical trials showed limited efficacy (Kalia et al., 2015). One of the factors contributing to the discrepancy between animal studies and clinical trials might be related to limited bioavailability of NTFs in the substantia nigra (SN) using invasive drug delivery approaches, rather than lack of true biological effectiveness. Hence, there is a great research need to overcome current technical hurdles of the brain delivery, in order to achieve therapeutic and sustainable levels of NTFs in the SN. Meanwhile, it is also important to explore new neuroprotective reagents.

Insulin is a hormone synthesized by the beta cells of the pancreas, and its major biological action in the periphery is to regulate blood glucose levels via stimulating glucose uptake in the liver, muscle, and adipose tissue. Since the energy metabolism of the brain is independent of insulin whereas insulin receptors are expressed by neural cells, it has been proposed that insulin might play physiological roles in the CNS (Banks et al., 2012). In fact, emerging evidence suggests that insulin plays important physiological roles in the brain by modulating high brain functions including cognition and appetite. For example, it has been demonstrated that intranasal insulin could enhance cognition in healthy human volunteers (Benedict et al., 2004; Reger et al., 2008) as well as Alzheimer's patients (Reger et al., 2008). Insulin could be detected in the cerebrospinal fluid (CSF) within minutes after nasal application, suggesting that intranasal application is a practical means to deliver insulin into the brain parenchyma. Currently, several clinical trials are being conducted for intranasal insulin therapy in AD.

Insulin is closely related to insulin-like growth factor-1 (IGF-1), which is a well-known growth and trophic factor. In the brain, IGF-1 plays an important role in neurodevelopment as well as in recovery during CNS disorders (Fernandez and Torres-Aleman, 2012). Since both insulin receptor (IR) and IGF-1 receptors (IGF-1R) are widely expressed in the brain, and their intracellular signaling pathways largely overlap, insulin could function as a neuroprotective agent once it reaches brain parenchyma at sufficient levels. Currently, direct evidence supporting this notion is limited to in vitro studies. For instance, it has been shown that insulin could protect neurons against a variety of insults, including glucose-oxygen deprivation (Sun et al., 2010), excitotoxicity (Kim and Han, 2005), and oxidative stress (Ribeiro et al., 2014). The neuroprotective action of insulin in vivo, however, remains unexplored. Therefore, the current study was aimed to test whether intranasal insulin administration affords neuroprotection in a 6-hydroxydopamine (6-OHDA)-lesioned rat PD model.

Experimental procedures

1. Animals

Male Sprague-Dawley rats (250 g) were obtained from Harlan Laboratories (Indianapolis, IN). Animals arrived in the laboratory a week before experiments. All procedures for animal care were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center. A total of 64 rats were used in this study.

2. Intrastriatal injection of 6-OHDA

One hour prior to surgery, rats received an intraperitoneal (i.p.) injection of desipramine HCl (25 mg/kg) to block the uptake of 6-OHDA into noradrenergic terminals (Ling et al., 2004). Rats were anesthetized with isoflurane and were placed on a stereotaxic frame with a rat adaptor. A small hole was made on the skull by a dental drill, and a 10 µl Hamilton syringe was descended into the right striatum. The coordinates used were as following: 0.7 mm anterior to the bregma, 3 mm lateral to the sagittal suture, and 5 mm ventral to the dura (Paxinos et al., 1985). A total of 20 µg of 6-OHDA (in 4 µl saline with 0.2 mg/ml of ascorbic acid) was infused into the right striatum, over a period of 4 min. The needle was kept for additional 5 min before being slowly retrieved. The wound was closed with wound clips and animals were returned to their cages. The control rats received the same amount of vehicle (saline with ascorbic acid).

3. Intranasal insulin treatment

Starting 24 h post-surgery, rats were treated with recombinant human insulin (Cell Sciences, CRI505, Canton, MA) via intranasal application, as previously described (Marks et al., 2009; Cai et al., 2011). Under lightly anesthesia with isoflurane, the animal was held in an upright position and 200 µg of insulin (in 20 µl PBS) was applied to each naris using a 10 µl fine pipette tip. Hence, each rat received a total of 400 µg insulin per day. The daily treatment continued until day 14 post-surgery. Rats in the control group received the same amount of sterile PBS under light anesthesia. Thus, there were 4 experimental groups in this study: saline (intrastriatal injection) + vehicle (intranasal), saline + insulin, 6-OHDA + vehicle, and 6-OHDA + insulin. The Methamphetamine (Meth)-induced rotation test requires that half of the animals in each treatment group to be injected with saline (used as the vehicle for Meth) for calculating the baseline rotations. Therefore, 16 animals were included in each treatment group. All these animals were used in blood glucose and body weight measurements, and all behavioral tests except rotation test. Ten animals per treatment group were used for stereological cell counting study (see below).

4. Blood glucose level and body weight measurement

Blood glucose levels were measured using a Contour Blood Glucose Monitoring System (Bayer, Germany). A small drop of blood obtained from rat tail veins was applied to a test strip and the glucose level was read out immediately. A baseline level was determined 24 h

before surgery. Blood glucose was measured 30 min after insulin administration, once a day until day 15. The body weight of animals was recorded daily.

5. Behavioral tests

A battery of motor behavioral tests were conducted to assess nigrostriatal DA system integrity (Tillerson et al., 2002; Fleming et al., 2004; Schallert T., 2005; Woodlee et al., 2005). Except METH-induced rotational behavior, all other behavioral tests were conducted at 1 day before (day 0), 8 and 15 days after 6-OHDA injection.

5.1. Locomotion—Locomotor activity was measured using the ANY-maze Video Tracking System (Stoelting Co., Wood Dale, IL, USA). Rats were placed in activity chambers ($42 \times 25 \times 40 \text{ cm}^3$) in a quiet room with dimmed light. The total distance traveled by the testing animal was recorded during a 60-min testing period.

5.2. Exposure rearing test—The exposure rearing test was conducted by placing rats in an upright transparent Plexiglas cylinder (20 cm in diameter and 30 cm in height). The cylinder is high enough to prevent rats from reaching the top, and wide enough to allow 2 cm gap between the base of the tail and the cylinder wall when the rat is touching the wall using its forelimbs. Once inside the cylinder, rats typically rear and engage in exploratory behavior by placing their forelimbs along the cylinder wall. The rearing activities were recorded and the number of rearing events (against the wall) was counted in a 5 minutes period.

5.3. Tapered/ledged beam walking test—Sensorimotor function of hindlimbs was evaluated using a tapered/ledged beam test (Schallert T., 2005; Zhao et al., 2005). Foot-faults (slips) made with the hindlimbs can be measured as an index of hindlimb function. The tapered/ledged beam consists of the staging area ($6 \times 15 \text{ cm}$), trapezoid walking beam (6 and 1.5 cm wide and 135 cm long) and off-loading area ($1.5 \times 15 \text{ cm}$). The entire apparatus were elevated to a height of 50 cm above the floor. The beam walking apparatus consist of a tapered beam with underhanging ledges (2 cm wide, dropped 2 cm below the upper beam surface) on each side to permit foot faults without falling. Bedding from the animal's home cage was placed at the end of beam to encourage movement. A bright light was placed above the start point to motivate the rats to traverse the beam. The rats' performance was analyzed by calculating the slip ratio of the hindlimb (number of slips/number of total steps). The time each animal spent to traverse the beam and join their littermates was also recorded. Steps onto the ledge were scored as a full slip, while a half slip was given if the limbs touched the side of the beam. The slip ratio for each hindlimb was recorded. The mean value from three trials was used for statistical analysis.

5.4. Vibrissa-elicited forelimb-placing test—The vibrissa-elicited forelimb-placing is a useful tool to assess sensorimotor function of the forelimbs in rats. Rats use their vibrissae to gain bilateral information from the proximal environment and this information is integrated between the hemispheres. Disruption of the nigrostriatal system integrity leads to deficits in forelimb placing upon stimulation of the rat's vibrissae (De Ryck et al., 1992; Woodlee et al., 2005). In this cross-midline test of forelimb placing, the animal was gently

held by its torso, but was turned sideways so that the vibrissae were perpendicular to the surface of the table. The now downwardly oriented limb was gently restrained by the experimenter as the downwardly oriented vibrissae were brushed against a table edge once per trial for 10 trials. The percentage of trials in which the rat successfully placed its other forepaw onto the tabletop was recorded for each side. Intact animals place the forelimbs of both sides quickly onto the counter top with 100% success in this test, while damages to the nigrostriatal system lead to a decrease in the successful rate of placing response of the contralateral side. If an animal struggled during testing, the data were not included in the overall analysis. The percentage of successful placing response for each forelimb was calculated.

5.5. Movement initiation test—Movement initiation for each forelimb was assessed to test the forelimb akinesia (Tillerson et al., 2002; Fleming et al., 2004; Woodlee et al., 2005). The testing rat was held by its torso and its hindlimbs, and one forelimb was lifted above the surface of the table so that the weight of the animal's body was supported with the other forelimb alone. The animal was allowed to initiate stepping movements in a 60 sec period for one forelimb and then the other in a balanced order. The time to initiate one step was recorded for each forelimb.

5.6. METH-induced locomotion—The test was performed on day 15 after 6-OHDA injection. Rats were first placed in the testing chambers to allow animals acclimatize their surroundings. One hour later, rats received saline or METH (2.5 mg/kg, s.c.) injection, and their activities were recorded for 90 min. Full 360° turns in the direction both ipsilateral (rotation towards the lesion) and contralateral to the lesion were counted. The results were expressed as net rotations towards the lesion side (ipsilateral turns – contralateral turns).

6. Stereological assessment of DA neurons in the SNc

After completion of the behavioral tests on day 15, rats were deeply anesthetized with isoflurane and perfused intracardially with 0.9% saline followed by 4% paraformaldehyde (PFA). Brains were post-fixed with 4% PFA for additional 24 h at 4 °C, and were immersed in sucrose solutions (sequential in 10%, 20% and 30% of sucrose solution in PBS, each for 24 h) for cytoprotection. Coronary free-floating sections at 40 µm of thickness were then prepared using a freezing slide microtome (SM2000R, Leica Biosystems, IL., USA). The number of DA neurons in the SNc was assessed using an unbiased stereological approach.

DA neurons in the SN were identified by tyrosine hydrolase (TH) immunostaining. A pilot immunostaining using a conventional protocol was performed to assess antibody penetration, which is a critical step for accurate stereological cell counting. For this purpose, image stacks with a step size of 1 µm were acquired to cover the full thickness of the section under a motorized microscope (Nikon Nie, NY., USA), and TH+ cells were examined by StereoInvestigator software (MBF Bioscience, Williston, VT, USA). Following this protocol, it appeared that the TH antibody was only able to penetrate ~10 µm on each side of the section, thus leaving about 10 µm in the center unstained. This problem persisted after increasing triton concentration (up to 0.8%) in the buffer and/or extending incubation time (up to 1 wk). We later overcame this technical issue by pre-treating sections with protease

K. Our modified immunostaining protocol yielded excellent antibody penetration into the full thickness of the sections. Briefly, brain sections were washed in PBS and then blocked in 10% normal goat serum in PBS for 30 min. Sections were treated with protease K (EMD Millipore Billerica, MA, USA) diluted 1:50 in PBS for 1 hr at RT. Following washing in large volume of PBS, sections were incubated with anti-TH antibody (EMD Millipore) at 1:1000 dilution in PBS containing 7.5% goat serum and 0.2% triton, overnight at RT. The next day, sections were washed 3 times in PBS and then incubated with Alex fluo-488-conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) in PBS containing 7.5% goat serum. After washing, sections were mounted on slides and air dried. Fluorescence mounting medium containing DAPI was then applied on sections, and sections were covered with coverglass.

A pilot study was conducted to determine optimal parameters for stereological estimation of TH+ cells in the SNc. Various configurations in the size of systemic random grid, the counting frame, the guard zone, and the optical dissection probe depth, were tested to satisfy the following requirement: 1) 0–5 cells identified within the counting frame; 2) a minimum of 500 TH+ neurons counted in the entire SNc; and 3) the coefficient of error (CE), which is used to estimate the precision of stereological cell counting, is less than 0.1. Based on these requirements, the following parameters were found to be appropriate: systematic random grid size at $160\ \mu\text{m} \times 160\ \mu\text{m}$, a counting frame at $140\ \mu\text{m} \times 110\ \mu\text{m}$, the guard zone at $2\ \mu\text{m}$, and the probe depth at $16\ \mu\text{m}$. These parameters were used across all samples. Under SRS image acquisition workflow, the anatomical boundaries of SNc were outlined for each coronary plane under $4\times$ objective lens. Typically 10 sections (in a series of every 6th section encompassing the entire SN region) were used for stereological cell counting. Using the pre-determined parameters as described above, image stacks across the X, Y, and Z axis were acquired for both the FITC channel (TH+ cells) and DAPI channel (nuclei). An optical fractionator probe was then employed to count TH+ neurons in the SNc. As a general rule, TH+ cells that were either inside the counting frame or touching the top and right green borders were counted, while those touching the left and bottom red borders were not counted. Upon completion of cell counting in all sections, the total number of TH+ cells in the entire SNc was calculated by the software, based on the section thickness, the serial section intervals, as well as the total cell counting. A total of 10 rats per treatment group were initially used to prepare brain sections for stereological study. However, due to technical issues, sections from one rat brain in the 6-OHDA+insulin group were found to be unusable for stereology. Therefore, nine animals were used in this group while 10 in other three treatment groups.

7. Statistics

Data were analyzed using SigmaPlot software (version 12.0), and were presented as the mean $\pm$ standard error of mean (SEM). One way analysis of variance (ANOVA) followed by post-hoc Student-Newman-Keuls test was performed to compare differences among multiple treatments for stereological cell counting and METH-induced rotational behavior data. Other behavioral data were analyzed by two-way repeated measures ANOVA followed by the Student-Newman-Keuls test. Results with a $p < 0.05$ were considered statistically significant.

Results

1. Intranasal insulin significantly ameliorated 6-OHDA-induced motor behavioral impairments

Using a comprehensive set of motor behavioral tests, our results showed that insulin treatment resulted in significant functional recovery in the 6-OHDA-lesioned rats.

1.1. Locomotion—A significant decline of locomotor activity (the crossing distances in Fig. 1A) was observed in rats on day 15 post 6-OHDA lesion ($p < 0.05$). There was no significant impairment on day 8, suggesting that locomotor deficits occurred in the second week after 6-OHDA lesion. Intranasal insulin treatment significantly attenuated 6-OHDA-induced locomotor activity deficit ($p < 0.05$).

1.2. Rearing events—The rearing events were significantly reduced in the 6-OHDA exposed rats on day 15 ($p < 0.05$) (Fig. 1B). Similar to locomotion, it was not altered on day 8. Insulin treatment significantly attenuated 6-OHDA-induced exposure rearing deficit ($p < 0.05$).

1.3. Tapered/ledged beam walking test—All control animals successfully performed this test without making any errors during walking. However, a significantly increased incidence of slip was observed in the 6-OHDA-exposed rats on day 8 ($p < 0.05$), and it was further increased on day 15 ($p < 0.05$) (Fig. 2A). In addition to increased incidence of faulty steps, the response latency was also increased significantly in the 6-OHDA-treated rats ($p < 0.05$) (Fig. 2B). Intranasal insulin treatment led to a near complete recovery on both steps and response latency in beam walking performance test ($p < 0.05$) (Fig. 2A&2B).

1.4. Vibrissae-initiated forelimb placement test—As shown in Fig. 3A, the contralateral forelimb placing was markedly impaired on both day 8 and 15 ($p < 0.05$), in the 6-OHDA-injected rats upon stimulating the animal's ipsilateral vibrissae. Insulin treatment abolished the 6-OHDA-induced deficits in this test ($p < 0.05$).

1.5. Movement initiation test—The movement initiation ability was significantly impaired on day 8 post 6-OHDA lesion ($p < 0.05$), and further deteriorated on day 15 ($p < 0.05$) (Fig. 3B). Intranasal insulin treatment completely restored the ability to initiate forelimb movement ($p < 0.05$).

1.6. METH-induced rotational behavior—The rotation test is one of the most reliable tests used in rodent neurotoxin PD models with ipsilateral lesion (Bove et al., 2005). Upon METH challenge, the 6-OHDA-lesioned rats showed significantly increased rotations towards lesion side ($p < 0.05$) (Fig. 4). Intranasal insulin treatment significantly reduced rotations as compared to the 6-OHDA+vehicle rats ($p < 0.05$).

2. Intranasal insulin significantly protected DA neurons against 6-OHDA neurotoxicity in the SN

Following motor behavioral tests, we then assessed DA neuron survival in the SNc, using an unbiased stereological cell counting approach. Intrastriatal 6-OHDA injection led to a

retrograde lesion to DA neurons in the SNc. In agreement with early studies suggesting a rather modest damage to the DA neurons in this model, our data showed that there was a 53% loss of DA neurons in the ipsilateral (right) SNc of 6-OHDA-injected rats (5110 ± 464 cells vs 10302 ± 157 cells in the right side of control rats). We did not find DA neuron loss in the contralateral SNc (10714 ± 246 cells compared to 10473 ± 178 cells of the left side of saline+vehicle rats). Treatment with insulin significantly ameliorated 6-OHDA-induced DA neuron loss in the ipsilateral SNc (8931 ± 626 cells) (Fig. 5B). There was no difference between saline+vehicle and saline+insulin groups (10669 ± 239 cells left side; 10388 ± 217 cells right side) ($P > 0.05$), on either side of the SNc. The CE values for all groups were less than 0.1 (range from 0.075 to 0.096).

3. Intranasal insulin treatment did not alter body weight and blood glucose levels

As shown in Fig. 6A, animals in all experimental groups lost weight during the first few days after surgery. However, animals were able to regain their body weight 4 days after the surgery ($p > 0.05$ compared to day 0). Similarly, blood glucose levels in all experimental animals, including the controls, dropped at 24 h post-surgery, but were recovered to pre-surgery levels afterwards (Fig. 6B). There were no significant differences in blood glucose levels among any experimental groups at any time-points assessed ($p > 0.05$) (Fig. 6B).

Discussion

This is the first study to demonstrate that intranasal insulin could afford a strong neuroprotection in a preclinical PD model. The potent pro-survival effect on DA neurons suggest that intranasal insulin may be a novel therapy for not only PD, but also other neurodegenerative disorders.

The idea that insulin could act as a trophic factor is not new, because insulin shares many biological similarities with IGF-1, which is a potent and universal trophic factor for cell survival. In the CNS, IGF-1 is critically involved in both normal neurodevelopment and regeneration during brain injury (Torres-Aleman, 2010). Because insulin shares 50% homology with IGF-1, whereas IR shares 66% homology with IGF-1R (Banks et al., 2012), it is not surprising that the downstream signaling pathways of IR and IGF-1R largely overlap. The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) and the Ras-MAPK extracellular signal-regulated kinases (Erk1/2) are two major transduction systems (Fernandez and Torres-Aleman, 2012) mediating survival and growth effects by insulin/IGF-1. Early studies have demonstrated that IR and insulin receptor substrates (IRS) are widely expressed in the brain in a regional and cell type-specific manner (Duarte et al., 2012; Fernandez and Torres-Aleman, 2012). Insulin can bind to both IR and IGF-1R; however, the binding affinity for IR is approximately ~ 100 – 1000 higher than IGF-1R. Interestingly, the human brain also expresses a hybrid receptor comprising each chain of IR and IGF-1R, this enables the hybrid receptor to bind insulin and IGF-1 with similar affinity (Fernandez and Torres-Aleman, 2012). Theoretically, once reaching brain parenchyma, insulin could potentially activate IR, IGF-1R, and the hybrid receptor, leading to cell survival and growth. Currently, the expression of IR and the hybrid receptor at cellular level is not clear, although it has been reported that neurons express higher levels of IR than glial

cells (Unger et al., 1991). It is worth mentioning that an early study by Figlewicz and colleagues demonstrated a large overlap between IR and TH immunoreactivity in the VTA and SN regions (Figlewicz et al., 2003). All these evidence suggests that intranasal insulin could be a potential therapeutic agent to treat neurodegenerative disorders. Despite a lack of in vivo evidence, a number of in vitro studies have demonstrated that insulin could protect neuronal injury against various insults (Kim and Han, 2005; Sun et al., 2010; Ribeiro et al., 2014). To the best of our knowledge, this is the first study to demonstrate that intranasal insulin not only protects DA neurons from injury but also significantly ameliorates motor behavioral deficits in an animal model of PD.

Besides its trophic effect on neurons, insulin appears to have much diverse biological actions in the brain. For example, insulin could increase the expression of brain-derived neurotrophic factor (BDNF) in the hypothalamus (Negron et al., 2015). BDNF is a potent NTFs that has been found to be neuroprotective in preclinical PD models (Hegarty et al., 2014). Insulin has also been demonstrated to regulate cytokine release from cultured astrocytes and microglia, and reduce toxicity of activated microglia towards SH-SY5Y neuronal cells (Spielman et al., 2015). Interestingly, insulin is also anti-inflammatory in both the periphery (Jeschke et al., 2004) and the brain (Adzovic et al., 2015). Thus, a marked improvement of functional outcomes in rats observed in this study might also be attributed to indirect effects, at least partially.

The blood brain barrier (BBB) is notoriously known for its impermeability to larger molecules, which creates enormous challenge for brain drug delivery. While there are some success in enhancing central bioavailability of certain small molecules by modifying their chemical properties, nearly all large molecules such as peptides and proteins failed in this attempt (Begley, 2004). Therefore, seeking alternative brain delivery approaches is urgently needed to develop novel drug-based therapies for neurodegenerative disorders. One of the strategies is to enhance drug transport crossing the BBB by modifying their chemical configuration or formulation, such as using nanoparticles or polyethylene glycol (PEG). Alternatively, it is also possible to transiently enhance BBB permeability by novel techniques such as the ultrasound (Beccaria et al., 2015). All those approaches that are aimed at enhancing brain delivery by targeting BBB, however, are currently limited in animal studies. In contrast, the nose-to-brain pathway is a novel noninvasive brain drug delivery approach which bypass the BBB, making it especially useful for delivery of larger molecules such as peptides and/or proteins (Djupesland et al., 2013; Pardeshi and Belgamwar, 2013). Importantly, this approach has been tested in numerous clinical trials. The exact pathways and mechanisms by which intranasally delivered drugs can gain access to the brain parenchyma are not fully elucidated. It is generally acknowledged that the olfactory and trigeminal pathways are two major routes (Thorne et al., 2004; Thorne et al., 2008). One of the possible transport mechanisms is that peptides diffuse into the olfactory and trigeminal nerve terminals, which are in direct contact with the nasal mucus, are transported retrogradely into their respective cell bodies and then into olfactory bulb or brainstem. Alternatively, peptides are transported along the olfactory and trigeminal nerves through extracellular channels (created by ensheathing cells), rather than a simply slow axonal transport (Lochhead and Thorne, 2012), since intranasally administrated peptides could be detected in the CSF rather rapidly (as early as 15 minutes) while it seems that

simple diffusion across brain parenchyma is unlikely to reach such a rapid distribution. Some researchers also suggested additional mechanisms, such as a “perivascular pump” mechanism powered by arterial pulsation and blood pressure to drive peptides rapidly distributed across the brain parenchyma via interstitial fluid (Hadaczek et al., 2006; Liu et al., 2012).

Although the brain delivery efficiency of peptides is likely related to their molecular mass, large proteins such as NTFs have been successfully delivered into brain via intranasal route (Bender et al., 2015). The molecular weight of the current FDA approved drugs for nasal application is relatively small, between 1,000–3,400 Da (Ozsoy et al., 2009). IGF-1, which is 7.5 kDa, was detected at significant levels in the neonatal rat brain after intranasal application (Lin et al., 2009), whereas intranasal IGF-1 has been demonstrated to be neuroprotective in a number of animal models of brain disorders, including stroke (Fletcher et al., 2009), neonatal brain injury (Cai et al., 2011), and Huntington’s disease (Lopes et al., 2014). Recently, it was reported that glial cell line-derived growth factor (GDNF), which is a much large protein with a molecular weight of 15 kDa, could be detected in the rat brain after nasal application (Bender et al., 2015). The same group had previously demonstrated that intranasal GDNF provided a significant neuroprotection in the 6-OHDA rat PD model (Migliore et al., 2014). Compared to IGF-1 and GDNF, insulin is smaller with a molecular weight of 5.8 kDa. A recent study reported that recombinant human insulin could be detected in many brain regions in rats within 30 minutes following intranasal application (Kamei and Takeda-Morishita, 2015).

A major concern regarding potential side effects of intranasal insulin therapy is the risk of hypoglycemia. In the current model, we did not observed significant drop of blood glucose levels at 30 min and 24 h following intranasal insulin application, suggesting a minimal effect on systemic glucose levels. This is consistent with report from human studies (Freiherr et al., 2013).

In conclusion, the current study demonstrated that intranasal insulin provides a strong neuroprotection in the 6-OHDA rat PD model. Given that intranasal insulin therapy has been consistently demonstrated to improve cognitive decline in AD patients, whereas a number of studies showed that insulin could protect neuronal injury in vitro, intranasal insulin may hold therapeutic potential for not only AD and PD, but also other neurodegenerative disorders.

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Abbreviations

CNS	central nervous system
IGF-1	insulin-like growth factor-1
IR	insulin receptor

NTF	neurotrophic factors
6-OHDA	6-hydroxylase dopamine
PD	Parkinson's disease
SN	substantia nigra
SNc	substantia nigra pars compacta
TH	tyrosine hydrolase
MTA	ventral tegmental area

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Highlights

- Intranasal insulin treatment significantly ameliorates 6-OHDA-induced sensorimotor dysfunction in rats
- Intranasal insulin treatment significantly reduced methamphetamine-induced rotation in 6-OHDA-lesion rats
- Intranasal insulin treatment significantly increased DA neuron survival in the SN in 6-OHDA-lesion rats
- Intranasal insulin treatment did not cause hypoglycemia in rats

Fig. 1.

Intranasal insulin significantly ameliorates locomotor and exposure rearing impairment in rats subjected to unilaterally 6-OHDA lesion. 6-OHDA treatment significantly reduced both crossing distance (A: Open field test) and rearing events (B: Exposure rearing response) on day 15. Treatment with intranasal insulin significantly ameliorated deficits in both locomotion and rearing events in 6-OHDA-lesion rats. Data were analyzed by two-way repeated measures ANOVA followed by Student-Newman-Keuls test. N=16 in each group. * $P < 0.05$ represents significant difference between the 6-OHDA+vehicle and the saline+vehicle group, or between the 6-OHDA+vehicle and the saline+insulin group, on the same post-treatment day. # $P < 0.05$ represents significant difference between the 6-OHDA+insulin group and the 6-OHDA+vehicle group on the same post-treatment day.

Fig. 2.

Intranasal insulin significantly improved motor performance in tapered/ledged beam walking tests in 6-OHDA lesion rats. At both day 8 and 15, 6-OHDA lesioned rats showed significantly increased incidence of slip steps and response latency as compared to the control. Treatment with intranasal insulin completely reversed 6-OHDA-induced motor impairment. Data were analyzed by two-way repeated measures ANOVA followed by Student-Newman-Keuls test. N=16 in each group. * $P < 0.05$ represents significant difference between the 6-OHDA+vehicle and the saline+vehicle group, or between the 6-OHDA +vehicle and the saline+insulin group, on the same post-treatment day. # $P < 0.05$ represents significant difference between the 6-OHDA+insulin group and the 6-OHDA+vehicle group on the same post-treatment day.

Fig 3.

Intranasal insulin significantly improved forelimb sensorimotor deficits. A marked decrease in the successful rate of forelimb placing in response to vibrissa stimulation was observed in the 6-OHDA-lesioned rats on day 8 and 15 (A). A significant, progressive increase of the response latency to initiate steps was also observed in 6-OHDA-lesioned rats (B). Daily treatment with intranasal insulin significantly increased the success rate of forelimb-placing (A), and reduced the response latency to initiate steps (B). N=16 in each group. * $P < 0.05$ represents significant difference between the 6-OHDA+vehicle and the saline+vehicle group, or between the 6-OHDA+vehicle and the saline+insulin group, on the same post-treatment day. # $P < 0.05$ represents significant difference between the 6-OHDA+insulin group and the 6-OHDA+vehicle group on the same post-treatment day.

Fig. 4.

Intranasal insulin significantly reduced METH-induced rotational behaviors in 6-OHDA-lesioned rats. The METH-induced rotation was calculated as the net value by subtracting the contralateral rotation from the ipsilateral rotation assessed on day 15 post-lesion. 6-OHDA-lesioned rats showed a markedly increased rotation towards the lesion side, which was significantly reduced by treatment with intranasal insulin. N=8 in each group. * $P < 0.05$ represents significant difference between the 6-OHDA+vehicle and the saline+vehicle group, or between the 6-OHDA+vehicle and the saline+insulin group. # $P < 0.05$ represents significant difference between the 6-OHDA+insulin group and the 6-OHDA+vehicle group.

Fig. 5.

Intranasal insulin treatment protected nigral DA neurons against 6-OHDA neurotoxicity. **A:** representative micrographs illustrate TH immunostaining in the SN regions. 6-OHDA lesion rats showed a marked loss of TH+ DA neurons in the ipsilateral side, primarily located in the SNc but also noticeable in the SN reticulate and VTA regions. In contrast, TH immunostaining in the contralateral side was not affected. Treatment with insulin protected TH+ neurons in the ipsilateral SNc against 6-OHDA lesion. **B:** Stereological cell counting to estimate numbers of DA neurons in both side of the SNc. 6-OHDA exposure led to a 53% loss of DA neurons in the ipsilateral SNc, while insulin treatment significantly increased DA neuron survival in the 6-OHDA-exposed animals ($P < 0.05$). $N = 10$ for the saline+vehicle, saline+insulin, and 6-OHDA+vehicle groups; $n = 9$ for the 6-OHDA+insulin group. * $P < 0.05$ vs the right side of the saline+vehicle or saline+insulin group; # $p < 0.05$ vs the right side of the 6-OHDA+vehicle group.

Fig. 6.

Intranasal insulin did not affect body weight and blood glucose levels. A: The body weight of all experimental animals decreased in the first few days after the surgery; however, all animals were able to regain body weight to the pre-surgery levels afterward. There were no significant differences among groups. Similarly, blood glucose levels dropped sharply in all animals at 24 h post-surgery, but returned to pre-surgery levels afterwards. There were no significant differences of blood glucose levels among treatment groups. * $P < 0.05$ vs before surgery. $N = 16$ in each group.