Published in final edited form as:

J Neurosci Methods. 2018 June 01; 303: 30–40. doi:10.1016/j.jneumeth.2018.03.006.

Methodology and effects of repeated intranasal delivery of DNSP-11 in awake Rhesus macaques

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Abstract

Background—To determine if the intranasal delivery of neuroactive compounds is a viable, long-term treatment strategy for progressive, chronic neurodegenerative disorders, such as Parkinson's disease (PD), intranasal methodologies in preclinical models comparable to humans are needed.

New Method—We developed a methodology to evaluate the repeated intranasal delivery of neuroactive compounds on the non-human primate (NHP) brain, without the need for sedation. We evaluated the effects of the neuroactive peptide, DNSP-11 following repeated intranasal delivery and dose-escalation over the course of 10-weeks in Rhesus macaques. This approach allowed us to examine striatal target engagement, safety and tolerability, and brain distribution following a single ¹²⁵I-labeled DNSP-11 dose.

Results—Our initial data support that repeated intranasal delivery and dose-escalation of DNSP-11 resulted in bilateral, striatal target engagement based on neurochemical changes in DA metabolites-without observable, adverse behavioral effects or weight loss in NHPs. Furthermore, a ¹²⁵I-labeled DNSP-11 study illustrates diffuse rostral to caudal distribution in the brain including the striatum-our target region of interest.

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Disclosure

These results have not been published in any peer-reviewed journal. L.H.B., D.M.G., and G.A.G. declare two awarded patents and two pending patent applications regarding DNSP-11.

Comparison with Existing Methods—The results of this study are compared to our experiments in normal and 6-OHDA lesioned rats, where DNSP-11 was repeatedly delivered intranasally using a micropipette with animals under light sedation.

Conclusions—The results from this proof-of-concept study support the utility of our repeated intranasal dosing methodology in awake Rhesus macaques, to evaluate the effects of neuroactive compounds on the NHP brain. Additionally, results indicate that DNSP-11 can be safely and effectively delivered intranasally in MPTP-treated NHPs, while engaging the DA system.

Keywords

intranasal; Parkinson's disease; peptide; drug delivery

1.0. Introduction

One of the major challenges in delivering large molecular weight (MW) compounds, such as peptides and proteins to the Central Nervous System (CNS), has been their targeted delivery to the brain [1–4]. The presence of the blood-brain barrier (BBB) and the blood-cerebrospinal fluid barriers (BCSFBs) greatly restricts the passive entry of large MW compounds into the CNS following oral and parenteral routes of administration [3–6]. Therefore, invasive surgical techniques are generally used to deliver compounds directly to the brain—often with varying degrees of success [7–15]. For example, based on their ability to promote survival and growth in neuronal populations [16], neurotrophic factors have been extensively pursued as a possible disease-modifying treatment for Parkinson's disease (PD) [2, 7–10, 17]. Although preclinical studies have supported the efficacy of intraparenchymally infused neurotrophic factors into the nigrostriatal system of parkinsonian animal models [18–26]; clinical trials examining the direct, surgical infusion of neurotrophic factors into targeted regions of the basal ganglia system have failed to meet primary end points [9, 12–15, 27, 28]. This lack of clinical efficacy has been strongly attributed to insufficient biodistribution and/or bioavailability following direct infusion into the brain [2, 7–9, 17, 29]. While additional efforts have been explored to improve distribution and/or bioavailability to key pathophysiological regions of the CNS following direct, surgical infusion [9, 30–34], optimal delivery methodologies for neurotrophic factors have yet to be identified [2, 7–9, 17, 33]. Therefore, new avenues need to be examined that overcome challenges associated with delivering large MW compounds to the brain for the treatment of chronic, progressive neurodegenerative diseases and disorders [1, 2, 9]. One possible alternative strategy currently under investigation is the discovery and development of new neuroactive compounds [35–38] that can potentially be delivered to the CNS using non-invasive routes of administration, such as intranasal delivery [1, 39].

An emerging body of preclinical [39–48] and clinical [49–52] evidence supports the intranasal delivery of compounds as a viable, non-invasive alternative route of administration that can potentially bypass the BBB and BCSFBs—directly targeting compounds to the CNS and cerebrospinal fluid (CSF) [50, 52–54]. Several preclinical studies have attributed the rapid nose-to-brain transport of intranasally-administered compounds to a combination of extracellular pathways including: perineural transport associated with the olfactory and trigeminal nerves, perivascular delivery by way of the cerebral vasculature, and

perilymphatic system [1, 53, 55–57]. However, the majority of intranasal studies investigating nose-to-brain transport mechanisms and/or the effects of neuroactive compounds on the CNS, have been conducted in rodents [58, 59]. While the importance of these studies should not be underestimated, differences in rodent nasal anatomy [53, 56, 57, 59–61] and the typical use of sedation to optimize intranasal dosing to the olfactory region [59, 62], may limit the translation of rodent studies into the clinic [58, 59, 63]. For example, our team has reported on the neuroactive effects of the synthetic, amidated 11-amino acid neuroactive peptide, DNSP-11 (dopamine neuron stimulating peptide-11) following repeated intranasal delivery in rats [39]. However, the repeated use of light isoflurane sedation as chemical restraint appeared to enhance the toxicity of the 6-OHDA nigrostriatal lesion [39, 64]. Therefore, the development of intranasal dosing methodologies, amenable to repeated dosing paradigms in awake (unanesthetized) non-human primates (NHP), which are anatomically comparable to the human brain and olfactory system [58–61], remains a critical step in the preclinical evaluation of neuroactive compounds intended for the treatment of chronic, progressive neurodegenerative diseases and disorders such as PD [58, 63].

Here we report an intranasal dosing methodology, using an atomizer in awake NHPs that can be implemented to evaluate the effects of neuroactive compounds, such as DNSP-11, on the brain following prolonged, repeated intranasal dosing. In this proof-of-concept study, Rhesus macaques were administered DNSP-11 (or vehicle) intranasally 4 consecutive days-per-week, with escalation of the DNSP-11 dose (0, 0.3, 1.0, 3.0, 10.0 mg/day) occurring biweekly over the course of 10-weeks. This dosing strategy allowed us to examine striatal target engagement, safety and tolerability of repeated dosing and dose-escalation, and brain distribution following a single ^{125}I -labeled DNSP-11 dose. Our data support that the repeated intranasal delivery and dose-escalation of DNSP-11 resulted in bilateral, target engagement based on changes in striatal tissue levels of DA and DA metabolites: 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). In addition, there were no observable behavioral effects or weight loss following repeated intranasal delivery. Finally, we observed that DNSP-11 is rapidly transported to the CNS following a single, bilateral intranasal dose-as evident from a ^{125}I -DNSP-11 distribution study. Collectively, these results demonstrate that DNSP-11 can be safely delivered intranasally at various concentrations over an extended period of time in awake NHPs, while maintaining its neuroactive properties in the striatum [39].

2.0 Methods

2.1. Ethics statement & animals

The animal facility at the University of Kentucky strictly follows the guidelines set by the National Institutes of Health (NIH), and are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC), with veterinarians experienced in the healthcare of NHPs. All animal use protocols were approved by the University of Kentucky Institutional Animal Care and Use Committee (IACUC), and strictly followed by research staff. For this proof-of-concept study, six, non-research naïve female Rhesus macaques (10–18 yr, 4–8 kg; Covance, Alice, TX) were used.

NHPs were individually housed at the University of Kentucky animal facility and maintained on a 12 hr light/dark cycle in temperature and humidity-controlled rooms, with food provided twice daily and water available *ad libitum*. It should be noted that the NHPs used in the present studies were acquired from another study, which excluded them based on a lower level of hemiparkinsonian symptoms following intracarotid artery (right side) infusion with MPTP (0.12–0.17 mg/kg, Sigma-Aldrich, St. Louis, MO) (data not shown) [65]. NHPs recovered for a minimum of 3 months following surgical MPTP infusion as previously reported, ensuring lesion stability prior to vertical-chair training [65, 66].

2.2. Materials

Unless stated otherwise, all chemicals were of reagent grade and purchased from Sigma-Aldrich (St. Louis, MO) or Thermo Fisher Scientific (Pittsburg, PA). ^{125}I -DNSP-11 was synthesized using a modified, active (R9K) sequence for iodination by the Bolton-Hunter method, and purified > 95 % pure by RP-HPLC (Perkin Elmer, North Billerica, MA), as described previously [39]. For dose-escalation studies, DNSP-11 was dissolved in vehicle (0.9 % saline, pH 7.2–7.4, Hospira, Inc. Lake Forest, IL), and shown to be stable *in vitro* [37]. All sample preparations were blinded from those performing animal dosing.

2.3. Portable atomizer system

To repeatedly deliver DNSP-11 intranasally in awake NHPs over an extended period of time, we developed and calibrated a portable system comprised of a commercially available glass atomizer (DeVilbiss, DV-151; Summit Medical, St. Paul, MN). The atomizer was driven by compressed, medical grade N₂ and controlled by a timed pressure ejection system (Picospritzer III; Parker; Pine Brook, NJ), paralleling previously reported intranasal studies in NHPs [47]. The Picospritzer III allowed for precise regulation of both pressure (PSI) and duration (sec) of a single intranasal spray burst (PSI/sec). Based on similar studies in NHPs [62, 67], a volume of $207\text{ }\mu\text{l} \pm 10\%$ ($1 \times$ per naris) was used. To accurately deliver the designated volume, the Picospritzer III was set at 16 PSI for 1 sec. However, the deposition of the atomizer has not been characterized and will need to be determined in future studies.

2.4. Vertical chair-training and acclimation to intranasal dosing

Prior to the start of training exercises, NHPs were fitted with a hard nylon collar (Primate Products, Inc., Immokalee, FL) used to secure a NHP to a modified, custom-fabricated vertical chair for intranasal dosing (Figure 1A & B) [68, 69]. Chair-training and atomizer acclimation in NHPs occurred over the course of 2–4 weeks, with individual training sessions lasting approximately 15–30 min. To facilitate training, NHPs received veterinary approved positive reinforcement in the form of dietary enrichment and audio-visual entertainment. Training sessions began through the introduction of a primate pole (Primate Products, Inc., Immokalee, FL) into an NHP's home cage, later advancing to attachment of the pole to the NHP's hard nylon collar used for transport [69]. NHPs were then introduced to the vertical chair and familiarized to an attachable head restraint apparatus comprised of a foam wedge placed under the NHP's mandible (Figure 1A & B). Acclimation to the head restraint apparatus and foam wedge was a critical stage in the training period as it allowed for the gentle restraint of the NHP's head at an approximate 45° angle during intranasal administration (Figure 1B) [53, 62]. Atomizer training sessions began by dosing each NHP

with vehicle only, in strict accordance with our intranasal dosing procedure (see section 2.5.).

2.5. Procedure of intranasal administration in awake Rhesus macaques

Intranasal dosing occurred between 09:00 – 11:00 in a designated humidity and temperature-controlled procedure room. Prior to intranasal dosing, NHPs received veterinary-approved positive reinforcement (see section 2.4.), while acclimating to their surroundings for approximately 5 min. Following acclimation, the NHP's head was placed in a head restraint apparatus with mandibular foam stabilizer (Figure 1A & B) [53, 62]. For intranasal dosing, the atomizer was primed by 4 consecutive test sprays. Immediately following atomizer priming, the NHP was administered the first intranasal dose (Figure 1B). A 5 min absorption period was then observed while the NHP's head remained in the angled position, the atomizer was then re-primed and the second intranasal dose administered to the opposite naris (Figure 1B). It should be noted that the atomizer was equipped with a rubber stopper which aided in positioning the nozzle directly in front of the NHP's naris for dosing, while helping to align the metal applicator tube with the NHP's nasal bridge (Figure 1B).

2.6. Experimental timeline

As parameters for a biologically relevant DNSP-11 dose and intranasal dosing paradigm had yet to be established in NHPs, we examined half-log step above the biologically active dose (0.3 mg) found in rats [39], adjusted for changes in brain mass between species [53, 62]. DNSP-11-treated NHPs (n=3) were administered test article 4 consecutive days-per-week, with escalation in DNSP-11 dose (0, 0.3, 1.0, 3.0, and 10.0 mg) occurring biweekly over 10-weeks (Figure 2). The control group (n=3) received only vehicle using the same dosing paradigm (Figure 2). To document general health over the course of the study, NHPs were monitored in their home cages approximately 60 min following the start of the first intranasal dose (t=0), as previous studies have indicated rapid systemic absorption and CNS uptake of various compounds in NHPs [45, 53] and DNSP-11 rats [39]. Monitoring sessions were recorded using a SONY HandyCam Vision Camera (SONY P6-120 MPL; data not shown). In addition, body weight measurements were collected at the end of each dosing week (Figure 2). At the end of the 10-week dosing period, NHPs were administered the final intranasal dose under anesthesia prior to tissue collection.

2.7. Tissue processing

NHPs were anesthetized with an initial dose of ketamine hydrochloride (10–20 mg/kg, Fort Dodge), laid supine with the head tilted back 45°, and administered a terminal intranasal dose of either DNSP-11 (10 mg) or vehicle, while under light isoflurane anesthesia (2% with 1% O₂). To determine brain and systemic distribution, one DNSP-11-treated NHP received iodinated (¹²⁵I) DNSP-11 (5 mCi/10 mg DNSP-11; 2.5 mCi/5 mg DNSP-11 per naris). To administer the ¹²⁵I-labeled DNSP-11 dose, proper personal protective equipment was worn, and dosing was completed in a fume hood, in accordance with radiation safety protocols. Following the start of the first ¹²⁵I-labeled intranasal dose (t=0), 1.0 mL of blood was collected from the saphenous vein in 15 min intervals to determine rate of systemic absorption into the bloodstream. Approximately 60 min following the start of the first intranasal dose (t=0), 0.5 mL cerebrospinal fluid (CSF) was collected by lumbar puncture

and NHPs were then deeply anesthetized (5 % isoflurane with 1 % O₂) and administered a fatal dose of sodium pentobarbital (250–300 mg, intravenously, Vortech, Dearborn, Michigan, MI) as previously described [66]. Prior to transcatheter perfusion, 5.0 mL of urine aspirated from the bladder. NHPs were then transcatheterially perfused with ice-cold heparinized saline (4 ml heparin/4 L of 0.9 % saline). Brain tissue was then harvested and weighed (wet-weight) for neurochemical, gamma counting and autoradiography analyses. Whole brains were immediately processed into 2 mm-thick serial, coronal sections using a brain mold kept on wet-ice that had been stored at 4°C in 0.9 % saline (Baxter, Deerfield, IL).

2.8. Neurochemical analyses of the Rhesus macaque striatum

For neurochemical analysis, brain sections that contained the striatum were laid flat on a piece of foam padding placed over dry ice [66]. Multiple 2 mm-diameter biopsy punches were collected from the right and left striatum: putamen (n = 9 per hemisphere), caudate nucleus (n = 4 – 5 per hemisphere), nucleus accumbens (n = 1 per hemisphere) and globus pallidus (n = 2 – 3 per hemisphere) for analysis by HPLC-EC (Figure 4) as previously described [66]. Biopsy punches were placed in pre-weighed 1.5 ml protein LoBind eppendorf tube (Sigma-Aldrich, St. Louis, MO), weighed (wet-weight), flash frozen with dry ice and stored at –70 °C. Biopsy punches were processed for dopamine (DA), 3,4 – dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA). Samples were separated using an isocratic HPLC system (ESA-Dionex: Thermo Fisher Scientific, Pittsburg, PA) coupled to a dual-channel electrochemical array detector (Coulchem III; 5100A, E1 +0.35 mV and E2 –0.25 mV with a 5011 dual analytical cell). Separation of DA and DA metabolites was achieved using a C18 column (4.6 mm × 75 mm, 3 µm particle size, ODS Hypersil; Keystone Scientific) with mobile phase (5 % MeOH, citrate-acetate, pH 4.0) [70]. Average tissue levels of DA, DOPAC and HVA were determined by combining tissue punches for specific striatal regions.

2.9. Gamma counting and autoradiographic analysis

The olfactory bulbs (right & left), trigeminal nerves (right & left) and pituitary gland were harvested for gamma counting analysis following transcatheter perfusion. Additionally, multiple, 2 mm-diameter biopsy punches for gamma counting analysis were collected from the frontal, motor and occipital cortices (6 punches per cortical area/hemisphere), caudate nucleus (4 punches per hemisphere), putamen (6 punches per hemisphere), globus pallidus (2 punches per hemisphere), nucleus accumbens (1 punch per hemisphere), amygdala (1 punch per hemisphere) and cerebellum (6 punches per hemisphere) (Figure 4). All samples were individually placed in pre-weighed 12 × 75 mm polypropylene disposable culture tubes (Fisher Scientific, Pittsburgh, PA), weighed (wet-weight) and placed on wet-ice for immediate gamma counting analysis. Samples (fluid, whole tissue, biopsy punch) were analyzed using a Perkin-Elmer Cobra II Gamma Counter (Packard; preset energy range 15 to 75 KeV, 1 min runtime) to detect total ¹²⁵I signal (counts-per-min, CPM). To determine the average CPM signal for specific regions of the CNS, the olfactory bulbs, trigeminal nerves and tissue punches were combined. CPM values were then normalized (CPM/mg or CPM/100 µl) to determine average ¹²⁵I signal. For autoradiography analysis, 2-mm thick serial, coronal brain slices were placed in a GE autoradiography cassette and exposed to film for 24 hr, and then visualized using a Typhoon 9400 phosphorimager (GE Health Care).

2.10. Statistical Analyses

Statistical analyses were performed using GraphPad Prism 6 Software (La Jolla, CA). Prior to statistical analysis, the average tissue levels of DA and DA metabolites in the putamen, caudate nucleus, nucleus accumbens and globus pallidus were determined for both the contralateral and MPTP-lesioned striatum of DNSP-11 and vehicle-treated NHPs by HPLC-EC. Neurochemicals (analyte and/or DA turnover) of the contralateral striatum were analyzed using an unpaired two-tailed *t test* * $p < 0.05$ and ** $p < 0.01$. However, variations in lesion severity were apparent in both DNSP-11 and vehicle-treated NHPs following neurochemical analysis of the MPTP-lesioned striatum. Therefore, NHPs were categorized based on the percent depletion of DA in the MPTP-lesioned putamen compared to the contralateral putamen (Table 2). Based on the small sample size, statistical analysis of the MPTP-lesioned striatum was withheld, and qualitative assessments made to compare neurochemical content and turnover ratios (Table 2).

3.0. Results

3.1. Effects of DNSP-11 on striatal tissue levels of DA and DA metabolites

MPTP-treatment was seen to result in robust decreases in tissue levels of DA and DA metabolites ipsilateral to the treatment side in most of the MPTP-treated animals that were treated with vehicle or DNSP-11 (Table 1). The unilateral lesion approach in this study, directly targeting the nigrostriatal system in the right hemisphere of the brain, provided an important opportunity to observe the effects of DNSP-11 on injured dopaminergic circuitry and the more normal contralateral hemisphere (Table 2). Dopamine neurons in the substantia nigra that innervate the putamen and caudate nucleus are especially vulnerable to MPTP neurotoxicity, while dopamine neurons in the ventral tegmental area innervating the nucleus accumbens are more resilient [20]. This pattern is evident in the two DNSP-11 recipients with moderately severe putamenal and caudate DA depletions of ~80–90% (Table 1). Effects on the nucleus accumbens and globus pallidus were less severe, ranging from ~56–68% (Table 1). When compared to the two vehicle recipients with DA depletions in the right putamen and caudate nucleus, the DNSP-11 recipients showed [(DOPAC + HVA)/DA] ratios 3.8 to 2.6 times higher indicating DNSP-11 was increasing dopamine turnover in these striatal areas (Table 1). Dopamine depletion in the nucleus accumbens and globus pallidus was markedly less severe with [(DOPAC + HVA)/DA] ratios 0.8 to 1.5 times higher than in the two comparable vehicle recipients (Table 1). By contrast, one vehicle treated animal had a very extensive depletion of DA of >99% and one MPTP-treated animal had greater levels of DA following DNSP-11 treatment. The present study used animals that were known to have variable DA depletions as this study was a proof of concept design to develop the nasal delivery approach. Thus, the one animal that showed much higher levels of DA and DA metabolites post DNSP-11 intranasal treatment may be attributed to a target activation effect and/or a poorer MPTP-induced lesion. Further studies with larger numbers of animals with more uniform depletion by DA and metabolites by MPTP are needed to better understand the *in vivo* effects of nasal delivery of DNSP-11.

By contrast, there was clear evidence of target activation of the DA neuronal system on the contralateral hemisphere (Table 2). Changes in DA, DA metabolites and DA turnover ratios

were present in the contralateral striatum between DNSP-11 (n=3) and vehicle-treated (n=3) groups (Table 2). Tissue levels of DA in the putamen, caudate nucleus, and globus pallidus were not significantly affected in the DNSP-11-treated group compared to vehicle (Table 2). By contrast, reductions in DOPAC were seen in the putamen, caudate nucleus and nucleus accumbens by 59% (p=0.0317*), 54% (p=0.0078**), and 45% (p=0.0017**), respectively (Table 2). In addition, a reduction in tissue-levels of HVA were found in the putamen, caudate nucleus and globus pallidus by 25% (p=0.0097**), 24% (p=0.0363*), and 21% (p=0.0061**), respectively, in the DNSP-11-treated group compared to vehicle (Table 2).

Due to the low numbers of animals studied, DA turnover ratios were also examined to investigate an index of changes in DA function [71]. In the contralateral striatum, DA turnover [(DOPAC + HVA)/DA] was unchanged in the DNSP-11-treated NHPs compared to vehicle (Table 2). However, DOPAC/DA turnover ratios in nucleus accumbens and globus pallidus were significantly reduced by 12% (p=0.0494*) and 56% (p=0.0223*), respectively, of the DNSP-11-treated group compared to vehicle controls (Table 2). Thus, additional studies are needed but our current studies support that nasal administration of DNSP-11 produced effects on the DA neuronal system.

3.2. Cage-side observations following repeated intranasal delivery and dose-escalation of DNSP-11

To determine safety and tolerability of prolonged, repeated intranasal delivery of DNSP-11 in awake NHPs, DNSP-11 was administered in a dose-escalating manner (Figure 2). Over the course of 10-weeks there were no observable indications of acute epistaxis, gastrointestinal associated irritation, adverse neurological symptoms, such as seizure or dyskinesia, or other general behavioral side-effects documented during daily cage-side observations (data not shown). Additionally, DNSP-11 and vehicle-treated NHPs did not show significant changes in average body weight ($\leq 5\%$) over the course of the 10-week study (Supplemental Figure 1). These results parallel our previous rodent studies in that there were no reported cases of adverse side-effects or significant changes in average body weight associated with repeated intranasal administration or dose-escalation of DNSP-11 [39].

3.3. Radiolabel distribution following a single, terminal intranasal ^{125}I -labeled DNSP-11 dose

To determine brain distribution, CSF and systemic absorption following intranasal delivery of DNSP-11 using our intranasal dosing method, we treated one NHP with a single intranasal dose of ^{125}I -labeled DNSP-11 in vehicle (5 mCi/10 mg DNSP-11; 2.5 mCi/5 mg/0.5 mL per naris). Quantitative gamma counting analysis of CSF (Table 3) and biopsied brain tissue samples revealed the presence of ^{125}I signal 60 min following the start of the intranasal dosing (t=0) (Figure 3), with the greatest normalized (cpm/mg) ^{125}I signal present in the olfactory bulbs and trigeminal nerves (Figure 3). Qualitative autoradiography analysis of serial, coronal brain slices corroborated these findings, as there was visibly greater ^{125}I signal present in the lateral olfactory tracts (Figure 4A–G) when compared to all other brain regions (Figure 4A–T). In addition, the pituitary gland, a circumventricular organ inherently exposed to the systemic circulation, showed a 2-fold increase in normalized ^{125}I signal

(cpm/mg) compared to the olfactory bulbs (Figure 3) [72]. These data provide supporting evidence for a direct nose-to-brain route of transport, paralleling similar intranasal distribution studies in NHPs (Figure 3) [45].

Analogous to our prior ^{125}I -labeled DNSP-11 distribution studies in rats, gamma counting and autoradiography analysis revealed diffuse rostral to caudal distribution in all brain tissue samples examined (Figure 3 & Figure 4A–T), including the striatum (putamen, caudate nucleus, nucleus accumbens and globus pallidus), which is our target region of interest (Figure 3 & 4F–L). Heavy deposits of radiolabeled signal were also found surrounding the ventricles (Figure 4) and major white matter tracks of the brain—most notably in the corpus callosum (Figure 4D–J), and striatal afferent and efferent motor fibers (Figure 4G–I). These data further support the rapid distribution of DNSP-11 throughout the NHP brain following intranasal administration [55].

To determine the rate of ^{125}I -labeled DNSP-11 systemic absorption into the bloodstream as a function of time, blood samples were collected from the saphenous vein at 15, 30, 45 and 60 min following the start of the first intranasal dosing ($t=0$). As expected, ^{125}I signal was present at all examined time points, with the greatest ^{125}I signal observed at 60 min (Table 3). Additionally, ^{125}I signal was found in both urine and thyroid gland samples, further supporting systemic absorption and clearance following intranasal delivery while also denoting the presence of free/unbound ^{125}I in the periphery [73].

4.0. Discussion

One of the major hurdles in the development of long-term treatment strategies for PD and similar CNS neurodegenerative disorders has been the targeted delivery of large MW compounds, such as GDNF, to the brain [2, 5, 9, 17, 52]. While several strategies have emerged to overcome challenges associated with delivering compounds across the BBB [30, 32, 69, 74, 75]; the potential use of GDNF and other neurotrophic factors as a treatment for PD has yet to be realized, in part because of suboptimal delivery to the basal ganglia system [2, 9, 29]. One strategy to circumvent this challenge is to discover and develop smaller, functional neuroactive compounds [35–38] that are amenable to non-invasive delivery methods [39]. Our team has previously described the *in vitro* and *in vivo* neuroprotective and restorative properties of DNSP-11 [35–37], that can engage the nigrostriatal system following repeated intranasal dosing in normal F344 rats and in a unilateral 6-OHDA rat model of PD [39]. However, findings from our rat study indicated the repeated use of light isoflurane sedation for intranasal dosing may have enhanced the toxicity of the nigrostriatal lesion [39, 64]. Therefore, we developed an methodology to examine the effects of intranasally delivered neuroactive compounds, such as DNSP-11, without the need for sedation in Rhesus macaques—a preclinical model anatomically comparable to the human olfactory system and brain [58, 60]. To the best of our knowledge, this is the first demonstration to indicate that the prolonged, repeated intranasal delivery and dose-escalation of DNSP-11 results in bilateral, striatal target engagement based on neurochemical changes in DA and DA metabolites, and a lack of observable, adverse behavioral effects or weight loss in MPTP-treated NHPs. Furthermore, a single ^{125}I -labeled DNSP-11 study provides evidence supporting direct intranasal transport.

To determine neurochemical target engagement, DA, DA metabolites (DOPAC & HVA) and DA turnover ratios were examined in the contralateral and MPTP-lesioned striatum. Here we report a general pattern indicating a reduction in DA metabolites and DA turnover ratios in the contralateral striatum of DNSP-11-treated NHPs compared to vehicle controls.

Compensatory effects from the MPTP-lesions and crossover of MPTP are known to occur using with intra-carotid artery administration of MPTP in nonhuman primates [18, 20, 23]. We found decreases in tissue levels of DOPAC & HVA and DA turnover ratios that we currently interpret as a normalization of the compensatory effects of the MPTP treatment, which can augment DA turnover [18, 20, 23]. Suppression of DA and its metabolites has also been observed at higher doses of GDNF in NHPs [76] and rats [24] following direct brain injection. Interestingly, the present study indicates wide-spread reductions in DA metabolites and DA turnover ratios found in all examined regions of the contralateral striatum in DNSP-11-treated NHPs when compared to vehicle and possible effects on DA systems ipsilateral to the lesion. These results are in contrast to previous studies in NHPs in which GDNF was infused into the putamen by CED, indicating a lack of neurochemical effects to areas of the striatum outside of the distribution volume area [29, 66, 77]. Collectively, neurochemical data support bilateral effects on the striatal system following repeated intranasal delivery and dose-escalation of DNSP-11 compared to vehicle in MPTP-treated Rhesus macaques. These results parallel our previous striatal neurochemical findings in normal and 6-OHDA treated rats following repeated intranasal delivery of DNSP-11 [39]. However, additional studies with greater statistical power are needed to better understand the effects of nasal administration of DNSP-11 in both normal and unilateral MPTP-treated nonhuman primates.

Prior studies examining the transport of select compounds to the brain of NHPs and humans following intranasal delivery have demonstrated rapid uptake into the CSF and/or distribution into the brain in under 30 min [1, 45, 50, 52, 53]. Rapid intranasal transport has been attributed to a combination of paracellular mechanism including: 1) perineural transport associated with the trigeminal and/or olfactory nerves found mainly in the olfactory region of the nasal cavity, 2) perivascular delivery-powered by bulk flow through the propulsion of arterial blood and diffusion, and/or 3) perilymphatic transport to the cervical lymph nodes [1, 53, 56]. In the present study, ^{125}I analysis of CSF and brain samples indicated the presences of radioactive signal 60 minutes post-intranasal dosing. ^{125}I signal was found in the olfactory bulbs and trigeminal nerves, and diffusely though out the brain, including the major white matter tracks (Figure 3 & 4)-supporting anisotropic movement of DNSP-11 [78]. In addition, ^{125}I signal was present at lower levels in grey matter regions (Figure 4), supporting rapid cellular uptake, paralleling previously published studies in rats examining direct intraparenchymal infusion and uptake of DNSP-11 into the nigrostriatal system [35]. When taken together, our study provides evidence supporting the direct nose-to-brain transport of DNSP-11 following intranasal delivery, consistent with our ^{125}I -DNSP-11 studies in normal rats [39] and similar studies in NHPs [45].

^{125}I analysis also indicated rapid systemic absorption of the radio-tracer within 15 min after intranasal administration, and showed a steady increase in ^{125}I signal over time (Table 3). This data is similar to our previous intranasal ^{125}I -DNSP-11 studies in normal rats [39] and similar studies in NHPs [45]. While the possibility of DNSP-11 crossing the BBB following

absorption into the bloodstream after intranasal delivery exists, it is unlikely as the half-life data of DNSP-11 in NHP plasma has been shown to be < 10 min as analyzed by LC-MS/MS (data not shown). Furthermore, the pituitary gland, a circumventricular organ where the BBB is more permeable, had a higher normalized ^{125}I signal compared to all other biopsied brain tissue samples examined, including the olfactory bulbs and trigeminal nerves-providing evidence supporting a direct nose-to-brain route [45, 72]. Although our intranasal ^{125}I -labeled DNSP-11 study supports rapid absorption into the systemic circulation, peripheral tissues and the brain, cage-side monitoring and body weight measurements support that the prolonged and repeated intranasal administration of DNSP-11 or vehicle did not lead to observable behavioral adverse side effects. In addition, we cannot estimate the concentration of DNSP-11 that is achieved from these data. Additional studies are needed to determine the dosage and to optimize the deposition [45] for achieving the *in vivo* brain concentrations necessary for the desired pharmacological effects by the nasal delivery method.

As this was a proof-of-concept study to implement our intranasal dosing methodology to examine the effects of DNSP-11 in awake NHPs, an optimal DNSP-11 dose and intranasal dosing regimen had yet to be determined. Therefore, future studies with larger numbers of animals will be needed to determine an optimal DNSP-11 dose and intranasal dosing regimen, and to investigate the neurochemical contribution of other anatomical relevant neuronal populations, such as the substantia nigra, as ^{125}I signal was found diffusely throughout the brain. In addition, studies in other lesion and/or aged NHP models may help to evaluate the impact of intranasally delivered DNSP-11 on damaged DA neuronal populations.

In summary, this proof-of-concept study supports the utility of our intranasal dosing methodology to evaluate the effects of neuroactive compounds in awake Rhesus macaques, a preclinical model comparable to the human brain and olfactory system [50, 60]. Furthermore, results from this study provide evidence supporting bilateral, striatal target engagement based on neurochemical changes in DA and DA metabolites-without observable, behavioral adverse side effects or weight loss following repeated intranasal delivery or dose-escalation of DNSP-11 in MPTP-treated Rhesus macaques. Additionally, a ^{125}I -labeled DNSP-11 study indicated rapid tracer uptake into CSF and diffuse distribution of ^{125}I signal in the brain-providing supporting evidence of nose-to-brain transport. Collectively, this study supports the further investigation of intranasally delivered DNSP-11 in preclinical NHP models of PD, and demonstrates the importance of evaluating the long-term outcome of intranasally delivered neuroactive compounds intended for the treatment of chronic, progressive CNS disorders such as PD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Dr. Samuel Deadwyler of Wake Forest School of Medicine for his helpful conversations, and Matt Hazzard and Tom Dolan of the University of Kentucky Academic Technology Group for helping with the medical illustrations used in Figures 1 & 4.

Funding

This project was supported by funds from NINDS: (NS039787: all; NS060924: L.H.B.), NCATS (UL1TR000117: L.H.B., G.A.G.), NIA (AG013494: D.M.G., G.A.G.; T32-AG000242: J.T-C., M.J.S.), Kentucky INBRE Pilot (NCRR 5P20RR016481-12, NIGMS 8P20GM103436-12: L.H.B), NIH COBRE pilot (NCRR P20RR20171: L.H.B), Endowed Chair Funds (D.M.G.), Dupree Trust (G.A.G), Estate of Laura C. Miller (L.H.B), PhRMA Foundation (L.H.B.), Columbus Foundation (L.H.B.). The content is solely the responsibility of the authors and does not represent the official views of the NIH. The sponsors had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abbreviations

BBB	blood-brain barrier
BCSFC	blood-cerebrospinal fluid barrier
CED	convection-enhanced delivery
CNS	central nervous system
CSF	cerebrospinal fluid
CPM	counts per min
DA	dopamine
DOPAC	3,4-dihydroxyphenylacetic acid
DNSP-11	dopamine-neuron stimulating peptide-11
GDNF	glial cell line-derived neurotrophic factor
GFR	GDNF-family receptor
HPLC-EC	high performance liquid chromatography with electrical chemical detection
HVA	homovanillic acid
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride
MW	molecular weight
NHP	non-human primate
OB	olfactory bulb
OSN	olfactory sensory neuron
PD	Parkinson's disease
RP-HPLC	reverse phase-high performance liquid chromatography

SN substantia nigra
6-OHDA 6-hydroxydopamine

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Highlights

- DNSP-11 was repeatedly delivered intranasally in awake Rhesus over 10-weeks.
- Neurochemical analysis of the striatum provided evidence for target engagement.
- No observed behavioral side-effects following repeated delivery or dose-escalation.
- Evidence supports direct nose-to-brain transport after a single dose.

Figure 1. Intranasal dosing of awake, chair-trained NHPs using an atomizer

For daily intranasal dosing, (A) NHPs were placed in a custom-fabricated vertical chair and secured. Prior to intranasal dosing, a head restraint with mandibular stabilizer (foam wedge) was used to secure and lift the NHP's head approximately 45°. (B) Each NHP received an intranasal dosing using an atomizer. The atomizer was equipped with a rubber stopper to aid in positioning the tip of the medical applicator directly in front of the NHP's nostril, while helping to align the metal applicator with the bridge of the NHP's nose. The atomizer was connected to a Picospritzer III (not pictured), allowing for the regulation of both duration (sec) and pressure (PSI) of the atomized spray.

Experimental Timeline

Figure 2. Experimental timeline: illustration representing a typical biweekly dosing sequence Over the course of 10-weeks, NHPs received either vehicle or DNSP-11 (0, 0.3, 1.0, 3.0, 10.0 mg/day), 4 consecutive days-per-week with escalation in DNSP-11 dose occurring biweekly-day 15 marks the start of the next dosing sequence. Additionally, body weight measurements were taken on days 5 and 12 (non-dosing day).

Figure 3. Brain tissue levels of ^{125}I 60 min following a single, one-time ^{125}I -labeled DNSP-11 dose

Multiple 2 mm-diameter tissue punches were taken of the frontal cortex ($n = 12$), motor cortex ($n = 12$), occipital cortex ($n = 12$), caudate nucleus ($n = 8$), putamen ($n = 12$), accumbens ($n = 2$), globus pallidus (GP, $n = 4$), amygdala ($n = 2$) and cerebellum ($n = 12$), see Figure 4 for tissue punch mapping. Additionally, the olfactory bulbs and trigeminal nerves were harvested for gamma counting analysis (Perkin-Elmer Cobra II Gamma Counter: Packard; preset energy range 15 to 74 KeV, 1 min runtime). Data are presented as the mean (left and right hemispheres) normalized ^{125}I signal (CPM/mg-wet tissue weight) $\pm$ SEM. NHPs treated with vehicle only were found to have CPM values lower than background levels ~ 50 cpm (data not pictured).

Figure 4. Distribution of ^{125}I signal in the brain 60 min following a single, one-time ^{125}I -labeled DNSP-11 dose

^{125}I signal was determined by autoradiography analysis in 2-mm thick serial, coronal brain slabs. 2-mm thick coronal brain sections were exposed to film for 24 hours on a GE cassette and processed using a Typhoon 9400 (GE Health Care). Based on visual, qualitative assessment the highest ^{125}I was present in the olfactory tracts (A–G). Additionally, an increase in ^{125}I signal was present in the white matter regions of the brain (A–T) including the corpus callosum (A–J) and the internal capsule (G–I). Black box indicates brain slabs used for neurochemical analysis (G, I & K). Circles represent tissue punches taken for gamma counting or neurochemical analysis.

Table 1
MPTP lesion categories of vehicle and DNSP-11-treated NHPs based on DA depletion of the lesioned putamen

NHPs were categorized with groups designated as mild (< 80%; N=1 DNSP-11-treated), moderate (80–90%; N=2 DNSP=11; N=2 vehicle-treated), or severe (> 99%; N=1 vehicle-treated), with respect to total DA depletion when comparing the MPTP-lesioned (right) putamen to the contralateral (left) putamen. Data are presented as the mean.

Neurochemical Analysis of the Right or Ipsilateral striatum (MPTP-treated; N=3 per group)

	Vehicle N = 2 ~80% DA loss	DNSP-11 N = 2 ~90% DA loss	Vehicle N = 1 > 99% DA loss	DNSP-11 N = 1 < 20% DA loss
Putamen				
DA	3280	1679	203	17862
DOPAC	570	257	63	1688
HVA	12573	8567	3102	21794
[(DOPAC + HVA)/DA]	4.50	17.18	60.22	1.44
Caudate Nucleus				
DA	4614	1546	79	25570
DOPAC	526	236	37	1912
HVA	8266	5283	1353	17684
[(DOPAC + HVA)/DA]	2.16	5.69	42.95	0.80
Nucleus Accumbens				
DA	3740	3025	1490	10592
DOPAC	915	759	657	2601
HVA	15960	11721	8636	25540
[(DOPAC + HVA)/DA]	4.98	4.14	10.73	2.66
Globus Pallidus				
DA	133	138	260	303
DOPAC	64	17	39	22
HVA	5313	8893	26	9103
[(DOPAC + HVA)/DA]	25.11	37.21	10.73	30.39

Table 2
Tissue levels of DA, DOPAC, HVA and corresponding DA turnover [(DOPAC+HVA)/DA], DOPAC/DA, HVA/DA in the contralateral striatum following repeated intranasal delivery and DNSP-11 dose-escalation

Average tissue levels of DA and DA metabolites of the contralateral and MPTP-lesioned striatum of vehicle and DNSP-11-treated NHPs. For neurochemical analysis, tissue punches were harvested and weighted (wet weight) from the putamen (n = 9 per hemisphere), caudate nucleus (n = 4 – 5 per hemisphere), nucleus accumbens (n = 1 per hemisphere) and globus pallidus (n = 2 – 3 per hemisphere) over three 2 mm-thick coronal tissue sections, see Figure 4 for tissue punch mapping. Average tissue levels of DA, DOPAC and HVA were determined by combining tissue punches for a specific striatal region (ng/g wet tissue weight). All data were analyzed using an unpaired two-tailed *t* test

Neurochemical Analysis of the Left or Contralateral Striatum (N=3 per group)						
	DA	DOPAC	HVA	[(DOPAC HVA)/DA]	+ DOPAC/DA	HVA/DA
Putamen						
Vehicle	23801 ± 3998	3282 ± 590	27377 ± 698	1.40 ± 0.20	0.15 ± 0.05	1.30 ± 0.19
DNSP-11	21359 ± 2343	1339 ± 110 *	20429 ± 1323 **	1.42 ± 0.10	0.09 ± 0.01	1.34 ± 0.10
	p=0.6261	p=0.0317	p=0.0097	p=0.9234	p=0.2402	p=0.8384
	t ₍₄₎ =0.5270	t ₍₄₎ =0.3.240	t ₍₄₎ =4.696	t ₍₄₎ =0.1023	t ₍₄₎ =1.378	t ₍₄₎ =0.2176
Caudate Nucleus						
Vehicle	27601 ± 3785	3183 ± 299	19906 ± 1043	0.98 ± 0.21	0.13 ± 0.03	0.85 ± 0.19
DNSP-11	22304 ± 1569	1449 ± 183 **	15040 ± 1174 *	0.78 ± 0.04	0.07 ± 0.01	0.72 ± 0.03
	p=0.2657	p=0.0078	p=0.0363	p=0.4113	p=0.1045	p=0.5398
	t ₍₄₎ =0.1293	t ₍₄₎ =4.947	t ₍₄₎ =3.097	t ₍₄₎ =0.9165	t ₍₄₎ =2.092	t ₍₄₎ =0.6696
Nucleus Accumbens						
Vehicle	12572 ± 2681	3705 ± 165	25436 ± 1094	2.48 ± 0.40	0.31 ± 0.05	2.17 ± 0.36
DNSP-11	11790 ± 837	2024 ± 151 **	22344 ± 1460	2.07 ± 0.09	0.17 ± 0.02 *	1.90 ± 0.07
	p=0.7946	p=0.0017	p=0.1654	p=0.3744	p=0.0494	p=0.5021
	t ₍₄₎ =0.2782	t ₍₄₎ =7.510	t ₍₄₎ =1.695	t ₍₄₎ =0.9988	t ₍₄₎ =2.788	t ₍₄₎ =0.7368
Globus Pallidus						
Vehicle	373 ± 17	79 ± 8	10744 ± 276	30.00 ± 1.75	0.22 ± 0.03	29.80 ± 1.72
DNSP-11	411 ± 73	45 ± 17	8445 ± 335 **	22.30 ± 2.70	0.10 ± 0.02 *	22.20 ± 2.72
	p=0.6404	p=0.1473	p=0.0061	p=0.0745	p=0.0223	p=0.0774
	t ₍₄₎ =0.5043	t ₍₄₎ =1.794	t ₍₄₎ =5.298	t ₍₄₎ =2.399	t ₍₄₎ =3.623	t ₍₄₎ =2.364

Data are presented as the mean $\pm$ SEM.

$p < 0.01$.

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$p < 0.05$

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Table 3
CSF, peripheral tissue and blood ^{125}I levels 60 min following a single, one-time ^{125}I -labeled DNSP-11 dose

^{125}I signal was present in CSF, the thyroid gland, urine and blood samples as analyzed by gamma counter (Perkin-Elmer Cobra II Gamma Counter: Packard; preset energy range 15 to 74 KeV, 1 min runtime). Blood samples were taken from the saphenous vein in 15 min intervals following the start of the first intranasal dose (t=0). Data are presented as total ^{125}I signal (CPM) and normalized ^{125}I signal (CPM/mg). NHPs treated with vehicle only were found to have CPM values lower than background levels 50 cpm (data not shown).

Samples	CPM (Counts Per Min)	Normalized CPM/mg
CNS		
CSF	2530	25
Peripheral Samples		
Thyroid Gland	8181000	15480
Urine	1984500	19845
Blood Samples		
15 min	77850	779
30 min	89160	892
45 min	96970	970
60 min	99700	997