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Research paper

Central neuropeptide-S treatment improves neurofunctions of 6-OHDA-induced Parkinsonian rats

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ABSTRACT

Parkinson's disease (PD) is characterized by degeneration of the dopaminergic neurons in substantia nigra (SN). The motor symptoms of PD include tremor, rigidity, bradykinesia and postural impairment. In rodents, central administration of neuropeptide-S (NPS) has been shown to induce locomotor activity, dopamine release and neuronal survival by decreasing lipid peroxidation, additionally, the NPS receptor (NPSR) was detected in SN. Accumulating findings suggest that central NPS may ameliorate the parkinsonian symptoms, however, this has been explored incompletely due to the scarcity of experimental studies. Therefore, the present study was designed to test whether central NPS treatment exerts protective and/or alleviative effects on 6-OHDA-induced rat experimental PD model. Adult male Wistar rats received acute (alleviate; 10 nmol, icv) or chronic (protective; 1 nmol, icv for 7 days) NPS treatment following the central injection of 6-OHDA in medial forebrain bundle. Motor performance tests and in vivo nigral microdialysis were performed before and 7 days after the central 6-OHDA injection. The immunoreactivities for tyrosine hydroxylase (TH), NPSR, 4-hydroxynonenal (4-HNE) and c-Fos were detected by immunohistochemistry in frozen SN sections. Our double immunofluorescence labeling studies demonstrated that NPSR is present in the nigral TH-positive neurons. Central NPS injection caused a remarkable c-Fos expression in SN; whereas, no change was observed following vehicle injection. In both chronic and acute treatment groups, the 6-OHDA-induced motor dysfunction and impaired nigral dopamine release were improved significantly. However, only chronic, but not acute treatment restored the loss of nigral TH-positive cells, while decreasing the 4-HNE immunoreactivity in SN. Our findings demonstrate that central NPS treatment not only exerts a neuroprotective action on nigral dopaminergic neurons, it also improves the striatal dopaminergic signaling. Therefore, the present study candidates the NPSR agonism as a novel therapeutic approach for PD treatment.

1. Introduction

Neuropeptide S (NPS), a 20-amino-acid neuropeptide was identified initially as the endogenous ligand of the orphan G-protein coupled receptor GPR154 which has been lately named as NPS receptor (NPSR) (Xu et al., 2004). In brain, the mRNA of the NPS precursor has been detected mainly in a cluster of neuronal cells that are located strictly between locus coeruleus and the Barrington's nucleus (Clark et al., 2011; Xu et al., 2004, 2007). Activation of NPSR increases cellular excitability by inducing transient increases in intracellular calcium, while causing cAMP accumulation suggesting coupling of Gq and Gs for NPSR (Okamura et al., 2008; Ruzza et al., 2010). Unlike NPS, the NPSR expression has been shown in numerous brain regions including thalamus, hypothalamus, amygdala, cortical areas and the brainstem structures (Clark et al., 2011; Leonard and Ring, 2011; Xu et al., 2007). In brain, the expression of NPSR in dopaminergic neurocircuitry indicates the regulatory role of NPS in dopamine neurotransmission. Recently, the modulatory action of central NPS in dopaminergic signaling in medial prefrontal cortex and the nucleus accumbens has been shown in rodents (Mochizuki et al., 2010; Si et al., 2010). Moreover, the expression of NPSR has been demonstrated in rat substantia nigra (SN) (Xu et al., 2007) suggesting that central exogenous NPS may exhibit beneficial effects on Parkinson's Disease (PD)-induced impaired motor functions. In fact, it has been demonstrated recently that central administration of NPS attenuated the 6-OHDA-induced motor deficits in mice (Didonet et al., 2014).

Oxidative stress is thought to play a pivotal role in degeneration of the dopaminergic neurons in PD (Youdim et al., 2001). The

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overproduction of reactive oxygen species is occurred due to mitochondrial dysfunction and oxidative stress, which in turn causes the accumulation of misfolded proteins, in addition to producing other deleterious events in dopamine-producing neurons (Dauer and Przedborski, 2003). The level of malondialdehyde which is a reactive aldehyde generated as an end product of lipid peroxidation was found to be increased in several brain regions from patients who died with PD when compared to the age-matched control human postmortem brains (Dexter et al., 1989). Interestingly, recent findings indicate the alleviative effect of NPS in oxidative stress and brain injury by attenuation of the oxidative damage to lipids and proteins. Applying NPS centrally to mice, Castro and colleagues have conducted a series of experiments and demonstrated that NPS reduces the carbonylated protein in the cerebellum and striatum, while decreasing the lipid peroxidation in the cortex (Castro et al., 2009b). Furthermore, NPS was shown to attenuate the formation of thiobarbituric reactive species in hippocampus (Castro et al., 2009a).

Therefore, applying chronic-protective (1 nmol) and acute-alleviative (10 nmol) central NPS treatments in 6-OHDA-induced parkinsonian rats, the aim of the present study was to investigate whether NPS improves the impaired motor functions by (i) favoring neuronal protection, (ii) inducing the nigral dopamine release. Additionally, using double immunofluorescence labeling, we tested whether nigral dopaminergic neurons (iii) express NPSR and (iv) are activated upon central administration of NPS.

2. Materials and methods

2.1. Animals

Adult male Wistar rats weighing 250–300 g were housed in stainless steel cages in an air-conditioned room (22 ± 2 °C with a 12:12 h light:dark cycle) with unrestricted access to food and water. All experimental protocols conducted on rats were performed in accordance with the standards established by the Institutional Animal Care and Use Committee at Akdeniz University Faculty of Medicine (with unique authorization number B.30.2.AKD.0.05.07.00/103). All efforts were made to minimize animal suffering and to reduce the number of animal in experiments. During the experimental protocols, to assess the health status, food consumption and body weight were monitored weekly.

2.2. Experimental design

To generate the experimental PD model, the rats underwent unilateral lesion surgery by injection of 6-OHDA into medial forebrain bundle (mfb). Central NPS treatments were applied through an intracerebroventricular (icv) cannula implanted chronically. The microdialysis and motor tests were performed in different group of rats. In each group, the rats were divided as (i) control (received intra-mfb and icv injections of vehicle), (ii) PD (received intra-mfb injection of 6-OHDA and icv injection of vehicle), (iii) PD-chronic/protective NPS treatment (received intra-mfb injection of 6-OHDA and icv injection of NPS, 1 nmol for 7 days), (iv) PD-acute/alleviate NPS treatment (received intra-mfb injection of 6-OHDA and single icv injection of NPS, 10 nmol on the 7th day). The experimental procedures are summarized in Fig. 1.

2.3. Unilateral 6-OHDA lesion

For lesion surgery, following an anesthesia with an intraperitoneal injection of ketamine (60 mg/kg) and xylazine (12 mg/kg), the rats were placed on a stereotaxic frame and 6-ODHA was injected into the right medial forebrain bundle (mfb). For injection, a burr hole was drilled above the injection site and the tip of 33G injection cannula was lowered into the right mfb according to the coordinates estimated in reference to the Paxinos and Watson Rat Brain Atlas (Paxinos, 1997) 1.88 mm anteroposterior (AP), 1.5 mm mediolateral (ML) to the bregma and 8.0 mm dorsoventral (DV) from dura. The 6-OHDA hydrochloride (Sigma-Aldrich, St. Louis, MO USA) was prepared in 0.1% ascorbic acid solution and the lesion was created by a single injection of 6-OHDA-HCl (3 × 4 μg/μl at flow rate of 1 μl/min). To prevent backflow of the solution, the injection needle was kept in place for 3 min following the injection. The control group animals underwent the same injection procedures which received the same volume of 0.1% ascorbic acid solution as vehicle.

2.4. Microdialysis surgery and Icv cannulation

After lesion surgery, a permanent stainless steel guide cannula (ID: 0.4; OD: 0.5 mm) was implanted into SN unilaterally according to the coordinates (5.2 mm RC, 2.6 mm ML from the Bregma; 7 mm DV from the skull surface). A 26G injection cannula was implanted into the right lateral ventricle according to coordinates (−0.8 mm RC, 1.4 mm ML from the Bregma; 4 mm DV from the skull surface) for the icv injections. The cannulas were then secured by dental cement and an anchor screw. A dummy cannula was placed into the guide cannula to prevent contamination and blockage. In order to maintain post-operative pain relief, rats were administered with meloxicam (1 mg/kg, ip) for 2 days. Microdialysis sampling was performed 7 days after lesion surgery. To verify the icv cannula placement in the lateral ventricle, rats were injected centrally with 150 ng human angiotensin-II on the post-operative day-3. Immediately after the icv injection, rats were returned to the home cages with access to a water bottle and the latency to drink was recorded, as previously reported (Sunter et al., 2003). One of the rats was excluded from the experiments as it failed to drink within 120 s.

2.5. Brain microdialysis

A microdialysis probe (CX-I-8-02, Eicom, Kyoto, Japan) with polyimide fused silica membrane (2 mm long, Eicom, Kyoto, Japan) was inserted through the guide cannula, 4 days prior to the sampling. On the experiment day, after rats were placed in microdialysis cages individually, the probe was then connected to a microsyringe pump using FEP tubing and perfused with artificial cerebrospinal fluid (aCSF; 148 mM NaCl, 4 mM KCl, 1.2 mM CaCl2, 0.85 mM MgCl2). After, a 120 min of perfusion for stabilization, the collected perfusates were discarded and the samples (100 μl) were obtained at a flow rate of 1 μl/min for 100 min. In rats received PD-chronic/protective NPS treatment, the microdialysis samples from SN were collected immediately following stabilization (Fig. 1A, upper part); whereas, in the rats administered with 10 nmol of NPS, the dialysates were obtained 100 min before and after the icv injection (Fig. 1A, lower part). At the end of the experiments, the brains were removed and placed in 10% formalin and they were blocked around the anatomic site of the cannulas and coronal sections (50 μm) were made by a vibratome for the histological verification. Two rats were excluded from the study due to the implantation of the microdialysis probe outside of the SN. A representative view of lesion surgery and SN cannulation sites are presented in Fig. 2.

2.6. Quantitative mass spectrometric measurement of dopamine

Standard for dopamine was purchased from Sigma-Aldrich. Dopamine was prepared by weighing 0.01 g of compound into a 10 ml glass tube. Then, 1 ml of 98–100% formic acid (Sigma-Aldrich) and 9 ml LC-grade water was added. An optimized multiple reaction monitoring (MRM) method was developed using ultra-fast liquid chromatography (UFLC) coupled with tandem mass spectrometry (MS/MS) as previously described (Santos-Fandila et al., 2013). A UFLC system (LC-20 AD UFLC XR, Shimadzu Corporation, Japan) was coupled to a LCMS-8040 triple quadrupole mass spectrometer (Shimadzu Corporation, Japan). Chromatographic separations were carried out using an HPLC column (Inertsil ODS-4, 3 × 100 mm, 2 μm, GL Sciences Inc. Tokyo, Japan)

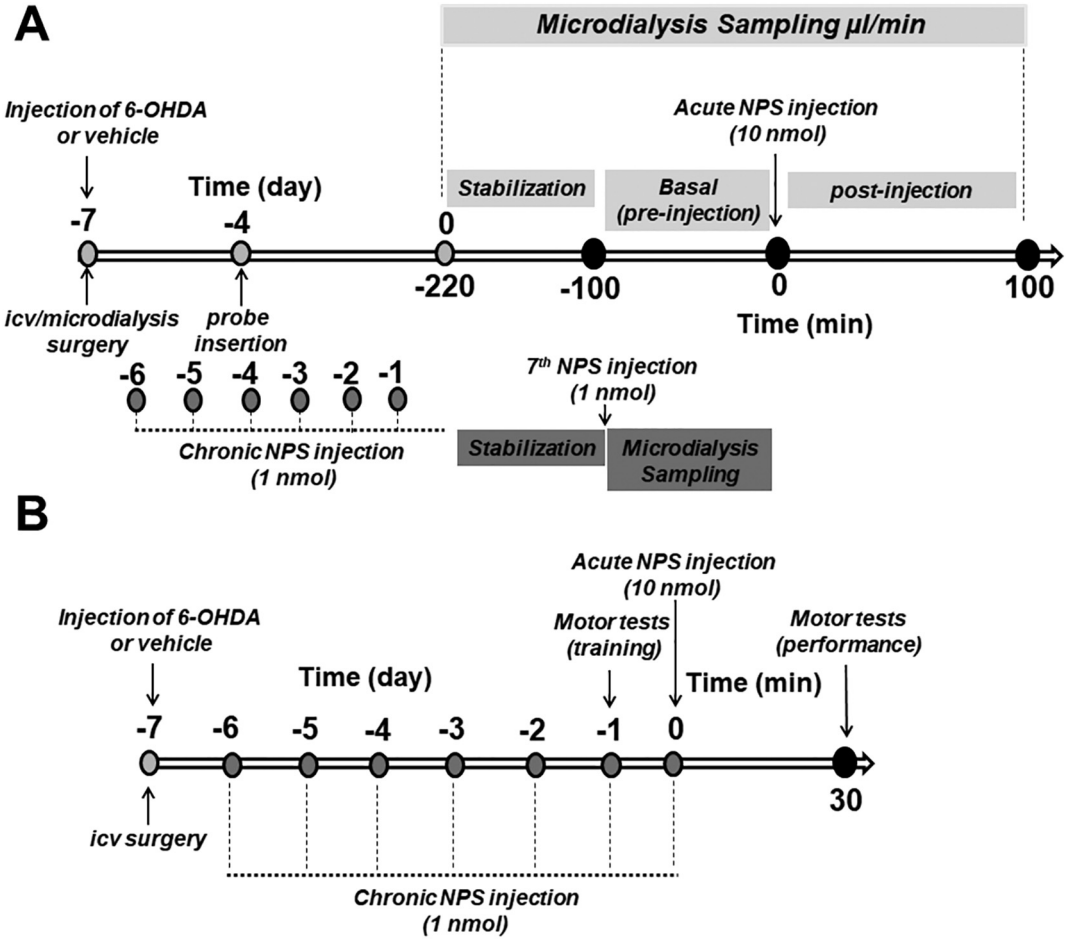

Fig. 1. Representative flow chart of the experimental design (A: microdialysis, B: motor tests). The experiments were performed 7 days after the 6-OHDA surgery. The microdialysis probe was inserted into SN 4 days prior to the sampling.

maintained at 25 °C. Dopamine was detected using a gradient elution with a flow rate of 0.4 ml/min. Mobile phase solvent A was water with 0.1% formic acid and 1% acetonitrile and solvent B was acetonitrile with 0.1% formic acid. Gradient program was solvent B, 5–50% (0–1 min), 50–95% (1–2 min), 95% (2–3 min) and 5% (3–4 min). Injection volume was 5 µl. MRM transitions and responses were automatically optimized for dopamine in positive electrospray ionization (ESI). In the positive ESI-MS mode the precursor and product *m/z* values were as follows: dopamine precursor ion 155 and product ion 136.9. Retention time of dopamine was 2.51 min. Response to dopamine was optimized to a linear calibration range from 50 to 1000 ng/ml and a sample analysis time of 5 min. Samples (typically 100 µl) of microdialysates obtained from in vivo studies were transferred to autosampler vials, 5 µl of formic acid were added and vortexed.

2.7. Motor tests

To assess locomotor activity, the animals were tested in an open field arena with a digital monitoring system for 5 min, as previously described (Parlak et al., 2018). The state of catalepsy was analyzed using a vertical wire netting (size 56.5 × 23.5 cm; mesh 1 × 1 cm; wire diameter 2 mm). Briefly, the rats were placed with all paws on the wire net and the time taken for at least one paw to be actively displaced from the bar (descent latency) was determined as described elsewhere (Hacioglu et al., 2012; Lopez et al., 2007). Finally, the rotarod test was used to assess motor coordination and balance, as described previously (Monville et al., 2006). Briefly, the rats were pre-trained on an automated 4-lane rotarod unit at the rate of 5, 10, 20, 30 and 40 rpm for a 300 s. The assessments were performed 2 times at each speed with a rest of 5 min between each trial. For each animal, the time spent on the rod

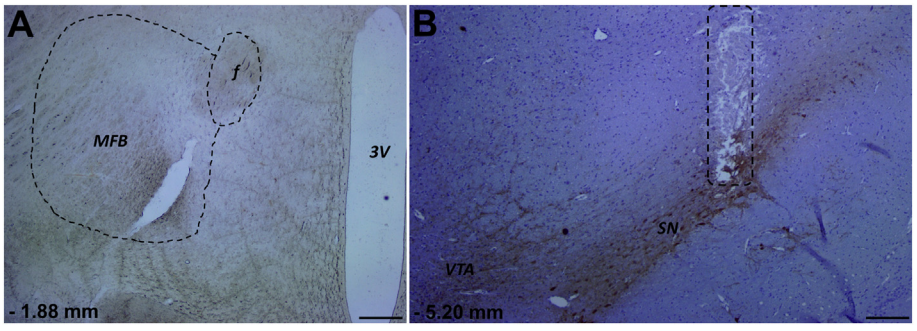

Fig. 2. Representative view of coronal mfb (A) and SN (B) sections depicting the sites of lesion surgery and microdialysis, respectively. The dashed line in the right designates microdialysis probe. The coordinates indicate the distance of the sections from bregma towards lambda. *MFB*: medial forebrain bundle, *f:* fornix, *3V*: third ventricle, *VTA*: ventral tegmental area, *SN*: substantia nigra. Scale bars represent 100 µm.

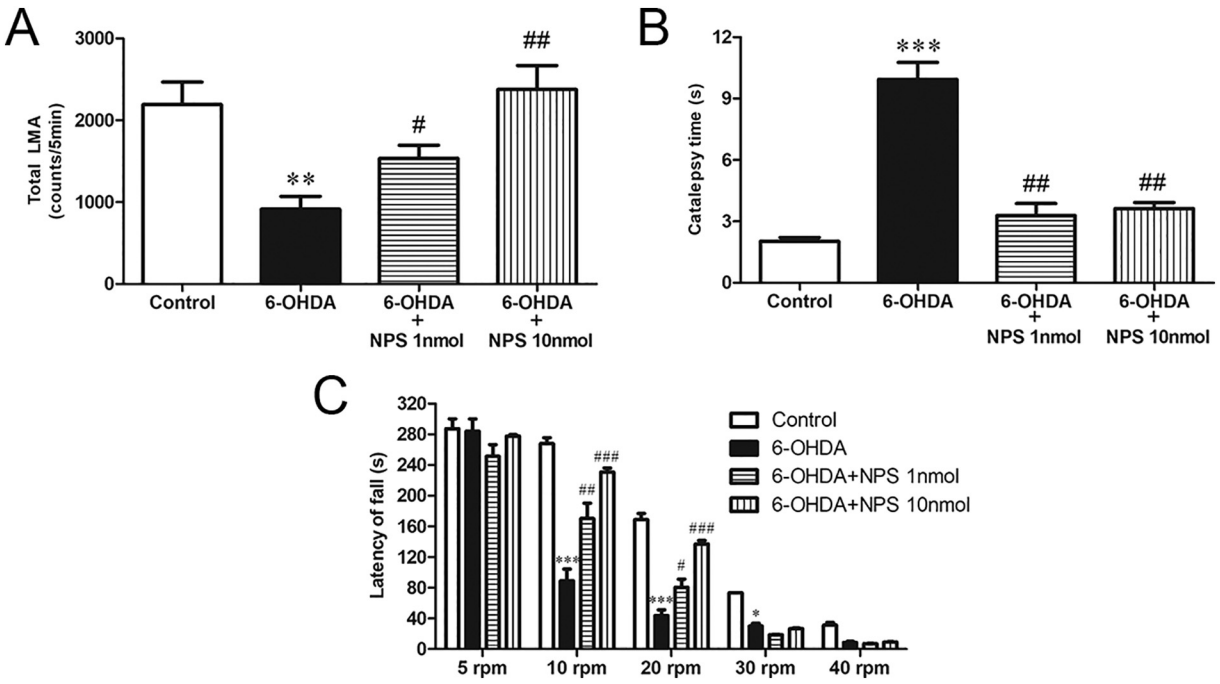

Fig. 3. The effect of central NPS treatment on motor functions assessed by measurement of total locomotor activity (A), catalepsy time (B) and rotarod tests (C). $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$ vs control; $^{\#}p < .05$, $^{\#\#}p < .01$, $^{\#\#\#}p < .001$ vs 6-OHDA. One-way ANOVA followed by Tukey post hoc were carried out to statistical comparisons. In each group, $n = 10$.

was measured for all speeds as the latency to fall.

2.8. Histology

2.8.1. Immunofluorescence labeling

From a separate group of rats ($n = 4$) underwent the same experimental procedures, the brains were obtained following cardiac perfusion (with 50 ml of saline followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffered saline (PBS)). Brains were removed and post-fixed in 4% PFA + 20% sucrose for 24–48 h. Subsequently, 50 μm-thick brain sections were cut using a freezing microtome and kept in long term storage buffer solution (0.1 M phosphate buffer, 30% sucrose and 25% ethylene glycol) at −20 °C. On the day of experiment, the floating sections were processed for double-labeling immunofluorescence using sheep anti-tyrosine hydroxylase (TH) (1:1000 dilution; AB113, Abcam, Cambridge, MA, USA), anti-NPSR (1:200 dilution; ABN12, Merck Millipore, Darmstadt, Germany) or rabbit anti-c-Fos (1:500 dilution, ABE457, Merck Millipore). The antibodies were diluted in 0.1 M PBS (pH 7.4) containing 10% normal horse serum, 0.3% Triton X-100 and 0.01 M TRIS. The sections were incubated with primary antibodies for 24 h at 4 °C and washed in PBS containing 0.3% Triton X and 0.01 M TRIS and incubated with secondary antibodies (donkey anti-rabbit conjugated with Alexa Fluor 488 for NPSR and c-Fos; donkey anti-sheep conjugated with Alexa Fluor 546 for TH; 1:400 for both staining; Invitrogen, Carlsbad, CA, USA) at room temperature for 1 h. To confirm the selectivity of anti-NPSR antibody, negative staining protocol was performed by replacing the primary antibody with the antibody diluent. After several rinses with PBS and mounted with fluoromount-G (Southern Biotechnology, Birmingham, AL, USA), the images were captured using a fluorescent microscope (Olympus BX61, Tokyo, Japan) equipped with appropriate filters for secondary antibodies. The number of TH-positive cells was counted by two examiners who were blind to the experimental protocols.

2.8.2. Immunoperoxidase staining

For immunoperoxidase staining, the slides were incubated with mouse anti- hydroxynonenal (HNE) antibody (AB48506, Abcam) at 1:200 dilution overnight in a humidified chamber at 4 °C. After several rinses with PBS, the sections were incubated with peroxidase-conjugated anti-mouse secondary antibody (Vector Lab. Inc., Burlingame, CA, USA) at 1:400 dilution for 45 min at room temperature. Then the slides were washed with PBS and peroxidase activity was visualized with 3,3′-Diaminobenzidine (DAB) (Sigma-Aldrich Co. LLC, Steinheim, Germany) for 1–2 min. The slides were counterstained with hematoxylin, dehydrated, mounted in entellan (Merck, NJ, USA) and examined by light microscopy. The 4-HNE immunoreactivity was assessed quantitatively by two observers blind to the treatment groups.

2.9. Statistics

Data were presented as the mean ± SEM and statistical analyses were performed using the Graphpad Prism software. The significance among the experimental groups in motor tests and microdialysis was assessed by one-way or repeated measures ANOVA followed by Tukey's post-hoc or paired *t*-test, as appropriate. In histological sections, the immunoreactivies in different groups were evaluated using Kruskal Wallis and Mann Whitney-*U* test. A p value $< .05$ was considered to be significant.

3. Results

3.1. Central NPS treatment attenuates the 6-OHDA-induced motor deficits

Compared to the controls (2192.2 ± 273.9 counts/5 min), total locomotor activity (LMA) was reduced significantly (914.33 ± 157.5, $F = 3.024$, $R^2 = 0.605$, $p < .01$) in rats injected with 6-OHDA. The 6-OHDA-induced reduction in LMA was restored partially by chronic NPS treatment (1533.3 ± 162.8, $F = 1.068$, $R^2 = 0.4182$, $p < .05$); whereas, it was recovered completely in rats received 10 nmol of acute NPS treatment (2375.8 ± 293.1, $F = 3.847$, $R^2 = 0.6153$, $p < .01$), (Fig. 3A). In control rats, the catalepsy time was 2.0 ± 0.1 s which was increased significantly in 6-OHDA-injected rats (9.9 ± 0.8, $F = 1.582$, $R^2 = 0.7601$, $p < .01$). The 6-OHDA-induced increases in cataplexy time were reduced both by chronic (3.2 ± 0.5, $F = 1.750$,

$R^2 = 0.7482$, $p < .05$) and acute (3.6 ± 0.3, F = 8.611, $R^2 = 0.7800$, $p < .05$) treatments of central NPS (Fig. 3B). Compared to the vehicle-injected control rats (267.8 ± 9.2 s at 10 rpm; 168.8 ± 9.3 s at 20 rpm), 6-OHDA decreased the latency time to fall significantly at the rate of 10 rpm (89.0 ± 17.3, F = 3.076, $R^2 = 0.9663$, $p < .001$) and 20 rpm (43.7 ± 8.1, F = 4.241, $R^2 = 0.8743$, $p < .001$). The decreased latency in 6-OHDA-injected rats was improved partially by chronic NPS treatment (170.3 ± 21.6, F = 7.2301, $R^2 = 0.6213$, $p < .01$ at 10 rpm; 80.5 ± 13.8, F = 9.1945, $R^2 = 5.3725$, $p < .05$ at 20 rpm); whereas, it was restored completely by acute NPS treatment (230.8 ± 4.1, F = 1.5780, $R^2 = 0.9678$, $p < .001$ at 10 rpm; 137.3 ± 5.2 at 20 rpm, F = 1.0245, $R^2 = 0.9831$, $p < .001$). In contrast, neither chronic nor acute NPS treatment changed the 6-OHDA-induced reduction at 30 rpm (Fig. 3C).

3.2. Central NPS treatment restores the 6-OHDA-induced reduction in nigral dopamine release

In-vivo brain microdialysis was performed to quantify the amount of extracellular dopamine levels in SN, while neuroprotective action of NPS was tested in parkinsonian rats received the chronic NPS treatment (1 nmol/day) for 7 consecutive days following the 6-OHDA-induced lesion surgery. Compared with the control animals (50.46 ± 6.7 ng/ml, $n = 6$), 6-OHDA decreased the dopamine levels in microdialysates significantly (4.26 ± 1.8 ng/ml, F = 59.39, $R^2 = 0.9480$, $p < .001$, $n = 6$). The 6-OHDA-induced decreases in dopamine levels were prevented by chronic NPS treatment (23.78 ± 3.8 ng/ml, F = 48.36, $R^2 = 0.7506$, $p < .01$, $n = 10$), (Fig. 4A). To test the dopaminergic effect of NPS on surviving SN cells, nigral microdialysis was performed before and after 10 nmol of central NPS injection. Compared with the pre-injection period (10.8 ± 8.1 ng/ml), NPS injection caused a remarkable increase (28.92 ± 9.2 ng/ml, F = 1.694, $R^2 = 0.5552$, $p < .01$, $n = 10$) in dopamine concentration (Fig. 4B).

3.3. entral NPS treatment attenuates the 6-OHDA-induced degeneration of dopaminergic neurons in SN

In control rats, extensive immunoreactivity for TH (88.1 ± 10.3 cells, Fig. 5A) was detected in SN which was abolished remarkably (4.3 ± 2.8, Fig. 5B, F = 11.68, $R^2 = 0.9711$, $p < .001$) by the injection of 6-OHDA. Chronic NPS treatment reversed the loss of nigral TH-positive cells (51.2 ± 9.6, Fig. 5C, F = 6.568, $R^2 = 0.9459$, $p < .01$); whereas, acute NPS treatment did not affect the loss of TH-positive neurons in SN (13.5 ± 6.3, Fig. 5D).

3.4. Central NPS administration induces c-Fos expression in TH-positive cells in SN

In control animals, an intense immunoreactivity was detected for TH-positive cells and fibers in SN in which a subgroup of the TH-IR cell bodies was found to express NPSR protein (Fig. 6A–C). In nigral sections obtained from the rats centrally injected with 10 nmol of NPS, a noticeable c-Fos expression was detected in TH-positive cells which was absent in vehicle-injected rats (Fig. 6D–F).

3.5. Central NPS treatment ameliorates the 6-OHDA-induced lipid peroxidation in SN

Compared with control (6.2 ± 5.3 cells), 6-OHDA caused a remarkable increase in the number of 4-HNE-immunoreactive cells in SN (42.8 ± 11.0, $n = 4$, F = 3.063, $R^2 = 0.9373$, $p < .01$) which was attenuated significantly (16.2 ± 11.8, $n = 4$, F = 1.306, $R^2 = 0.8177$, $p < .05$) by chronic administration of central NPS. In contrast, acute NPS treatment did not affect (40.4 ± 10.2) on the 6-OHDA-induced nigral 4-HNE immunoreactivity (Fig. 7).

4. Discussion

Using an experimental rat PD model, our present study demonstrated the beneficial effects of central NPS treatment on 6-OHDA-induced parkinsonian motor dysfunction through exerting a neuroprotective effect on nigral dopamine-producing cells. NPS reduced the nigral lipid peroxidation significantly in parkinsonian rats. Additionally, the NPS-induced improvements appear not to be restricted with the neuroprotective action, indeed, it also induced the dopamine release from the surviving cells in SN. Moreover, our study provided a morphological evidence that NPSR is present in the TH-positive nigral cells and these cells are activated upon central administration of NPS. In this study, to test whether NPS exerts a neuroprotective effect on nigral cells, we administered low dose of NPS for 7 consecutive days. On the other hand, we tested the effect of single high dose NPS on motor performance tests. In previous rodent studies, centrally administered NPS has been shown to increase locomotion (Castro et al., 2009b; Ensho et al., 2017; Li et al., 2015; Paneda et al., 2009). Unsurprisingly, single acute administration of NPS significantly improved the motor performance in 6-OHDA-induced parkinsonian rats. However, unlike the acute treatment group, chronic NPS treatment increased the survival of TH-positive nigral cells, while reducing the lipid peroxidation in SNpc in parkinsonian rats. Based on these findings, chronic NPS treatment appears to protect nigral cells, whereas, acute high dose NPS injection seems to cause a burst activation of nigral dopaminergic neurons remaining after 6-OHDA treatment.

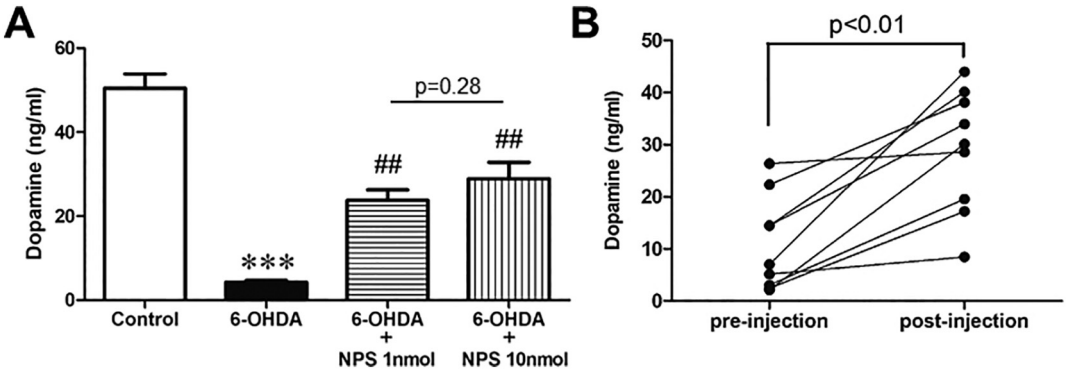

Fig. 4. The effect of chronic and acute central NPS treatments on the dopamine concentrations in nigral microdialysates collected from 6-OHDA-induced parkinsonian rats (A). B: the effect of single high dose (10 nmol) NPS on nigral dopamine release. Each black dot represents an individual rat. One-way or repeated measures ANOVA followed by Tukey post hoc or paired *t*-test were used to test the effect of the chronic and acute NPS treatments, respectively. $^{***}p < .001$ vs control; $^{\#\#}p < .01$ vs 6-OHDA. In control and 6-OHDA, $n = 6$; in acute and chronic NPS treatment groups, $n = 10$.

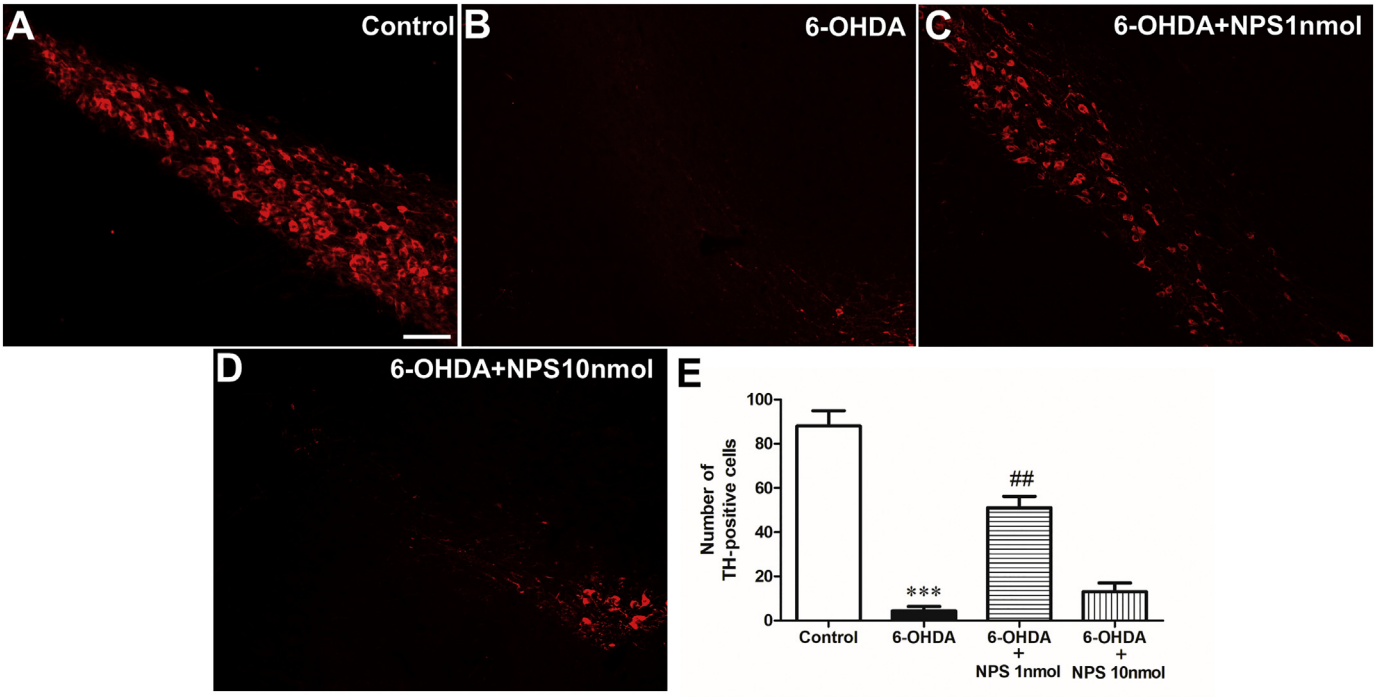

Fig. 5. Effect of central NPS treatment on nigral TH immunoreactivity in 6-OHDA-induced parkinsonian rats (A-D). The scale bar represents 100 μm. The quantitative analysis of TH-positive neurons in SN (E). $^{***}p < .001$ vs control; $^{\#\#}p < .01$ vs 6-OHDA. Kruskal Wallis test followed by Mann Whitney-*U* test was used to carried out to statistical comparisons, $n = 4$ rats per group.

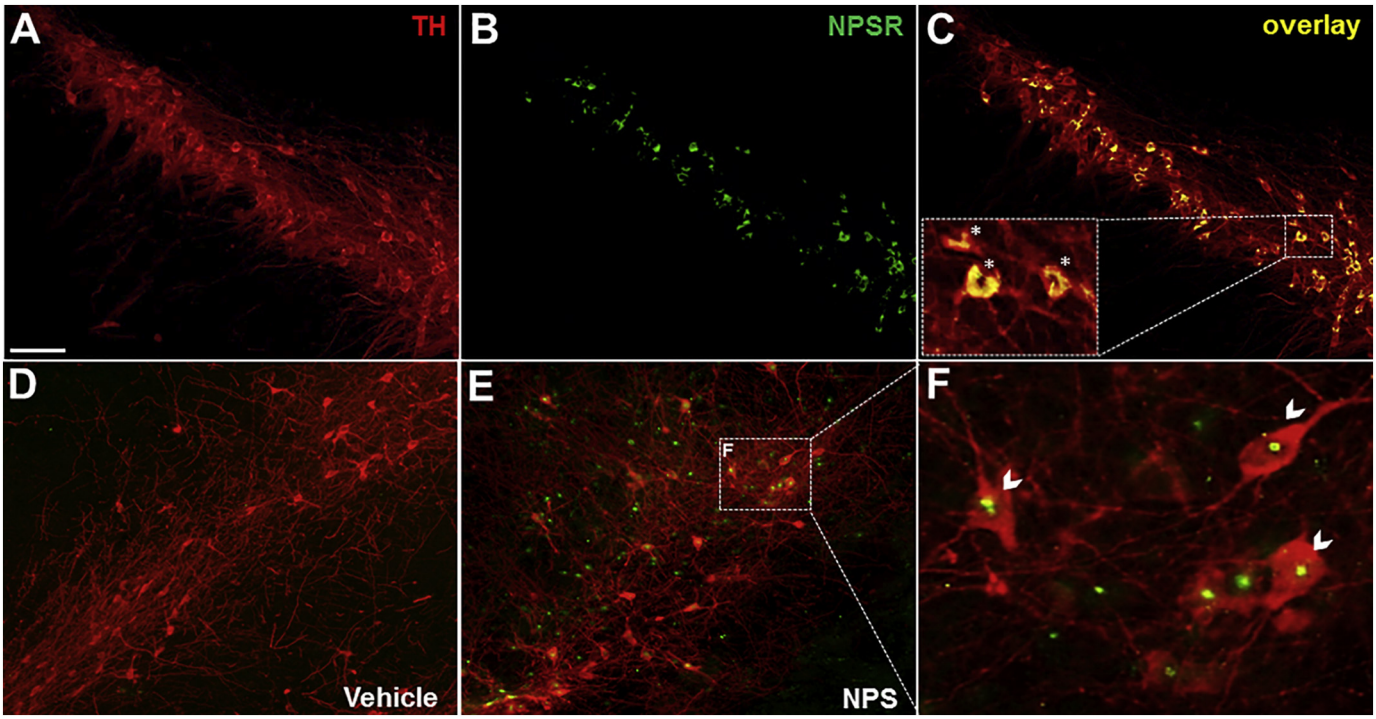

Fig. 6. Representative micrographs showing the immunoreactivity for TH (red, A) and NPSR (green, B) in the SN. NPSR protein was detected in a subgroup of nigral TH-positive cells (yellow, C) which are depicted with asterisks. Central exogenous NPS-induced c-Fos expression in SN is presented in lower panel. Compared with vehicle (D), centrally administered 10 nmol of NPS induced a remarkable c-Fos expression in TH-positive nigral cells (E). In a high magnification of boxed region depicting the double-immunoreactive cells is presented in F which are indicated with arrowheads. The scale bars represent 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

NPS is a novel 20 amino-acid brain peptide identified in the central nervous system (CNS) and peripheral tissues (Xu et al., 2004). Initially, NPS was isolated as the endogenous ligand of the orphan G protein-coupled receptor, GPR-154 (subsequently named NPSR) receptor (Xu et al., 2004) which has been detected later in brainstem and forebrain structures including hypothalamus, amgydala and basal ganglia (Leonard and Ring, 2011; Xu et al., 2007). Despite the production of NPS is restricted to the brainstem, the wide distribution NPSR in brain

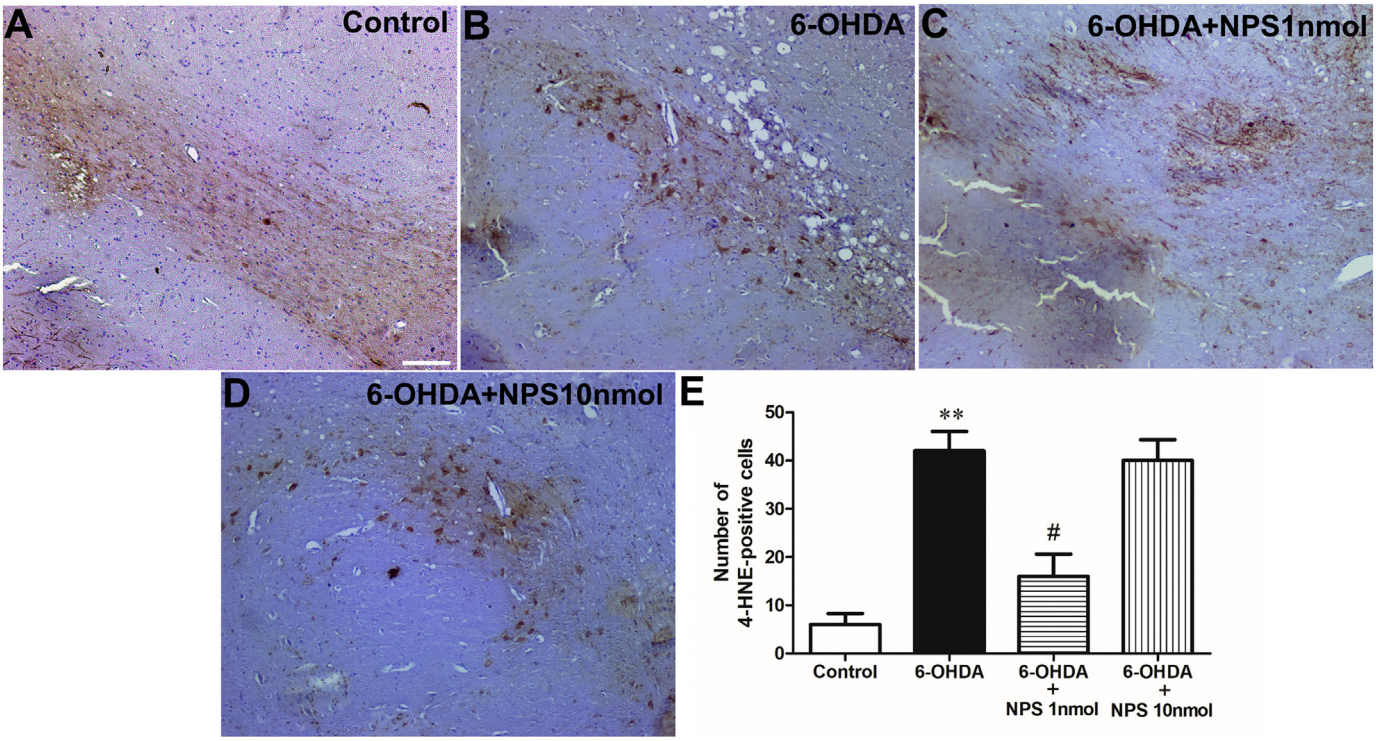

Fig. 7. Effects of acute and chronic central NPS treatment on 4-HNE immunoreactivity in the SN of 6-OHDA-induced parkinsonian rats. Compared to vehicle-treated control rats (A) 6-OHDA increased the immunoreactivity for 4-HNE in SN (B) which was attenuated significantly by chronic (C) but not acute (D) administration of central NPS. Quantitative analysis of 4-HNE-positive neurons in SN (E). Scale bar represents 100 µm. The Kruskal Wallis test followed by Mann Whitney-*U* test was used to carried out to statistical comparisons. $^{**}p < .01$ vs control; $^{\#}p < .05$ vs 6-OHDA. $n = 4$ per group.

suggests the regulatory roles of NPS in numerous physiological functions including stress response (Okamura and Reinscheid, 2007) and anxiety behaviors (Leonard et al., 2008; Vitale et al., 2008), arousal (Rizzi et al., 2008; Xu et al., 2004), locomotion (Leonard et al., 2008; Mochizuki et al., 2010; Pacheco et al., 2011; Roth et al., 2006), memory consolidation (Okamura et al., 2011) and motivational behaviors (Cannella et al., 2009; Cao et al., 2011). Interestingly, these functions are impaired in PD suggesting the modulatory role of NPS/NPSR system in motor and non-motor parkinsonian dysfunctions.

Impaired motor coordination, balance and decreased locomotor activity have been reported in the experimental rodent models of PD. Commonly, motor coordination and balance are evaluated by the rotarod test (Rozas et al., 1998). In a recent mice study, Didonet and colleaugues have reported that single icv administration of NPS attenuated the 6-OHDA-induced motor impairment, while restoring the loss of TH-positive neurons in SN and ventral tegmental area. Previous studies in rodents demonstrated that centrally administrated NPS favors the locomotion. A recent rat study has provided an evidence that central NPS increases motor activity through activation of sympathetic tonus (Ensho et al., 2017). More importantly, NPS infusion into the SN or globus pallidus dose-dependently increased the locomotor activity in mice, however, this effect was blocked by preadministration of SHA68 suggesting that NPS exerts a stimulatory effect on motor neurocircuits of basal ganglia through selective activation of NPSR (Li et al., 2015). On the other hand, Boeck and colleagues prevented the NPS-induced hyperlocomotion by blocking the adenosine A2 receptors in mice (Boeck et al., 2010). In line with the latter findings, our motor performance assessments demonstrated that both acute and chronic treatments of central NPS improved the motor functions in parkinsonian rats. Therefore, further studies seem to be necessary to elucidate the participation of other pathways in stimulatory action of NPS on locomotion.

Previous reports in rodents have shown that NPSR is present in SN. The present study demonstrates the beneficial effects of central NPS treatment in Parkinsonian rats. Our present findings confirm that chronic NPS treatment (1 nmol/day for 7 days) promotes the survival of TH-positive cells by decreasing the 6-OHDA-induced production of 4-HNE, while increasing the release of dopamine from SN which yields improved motor performance. Moreover, we demonstrated that single administration of NPS (10 nmol) caused a restoration of impaired motor functions by inducing the dopamine release from the surviving nigral cells. In fact, NPSR protein was identified previously in the medial amygdala, SN, subiculum, dorsal raphe and several hypothalamic and thalamic regions (Leonard and Ring, 2011). Despite the expression of NPSR in the SN has been detected previously, we reported here a body of evidence for the first time that NPS binds to NPSR expressed in the TH-positive cell population in SN which results in increased c-Fos expression.

PD is characterized by degeneration of the dopamine-producing cells in SN which yields significant motor symptoms including tremor, rigidity, bradykinesia and postural instability (Goetz, 2011). Despite the etiopathogenesis of PD is not elucidated completely, it has been shown in recent years that oxidative stress plays and increased production of reactive oxygen species (ROS) plays an important role in neuronal apoptosis (Youdim et al., 2000; Youdim et al., 2001). Along with the decreased unsaturated fatty acids in the cell membrane, the increased production of free radicals has been demonstrated in PD patients which occurs as a result of impaired protection of anti-oxidant systems (Dexter et al., 1989; Yoritaka et al., 1996). The findings of the recent rodent studies suggest the neuroprotective aspect of NPS. Particularly, it was shown in mice that central administration of NPS attenuated the lipid peroxidation (Castro et al., 2009b). Similarly, it has been demonstrated that centrally administered NPS attenuated the pentylenetetrazole-induced oxidative damage to proteins and lipids in the hippocampus and cerebral cortex in mice (Ramos et al., 2012). Moreover, it was shown in rat retrosplenial cortex that central NPS treatment dose-dependently attenuated the vacuolization and the increases of extracellular acetylcholine levels which were induced by

NMDA receptor antagonist MK-801 (Okamura et al., 2010).

In our study, we have detected a remarkable survival of TH-positive nigral cells in rats underwent chronic NPS treatment for 7 days, moreover, our immunohistochemical analyses demonstrated that NPS prevented the formation of 4-HNE. Although it is unclear how centrally administered NPS exerts neuroprotection, this action might be explained partly by the effect of NPS on cellular excitability by affecting the cytoplasmic calcium concentration. It has been shown previously that NPS-induced antioxidant effects were abolished by lithium (Castro et al., 2009a) which is known to alter cytoplasmic calcium concentration directly (Palty et al., 2004). Moreover, it has been demonstrated in brain slices that NPS increased cytoplasmic levels of calcium in dorsal raphe and laterodorsal tegmentum cells through mediation of NPSR which were found to be dependent on store-mediated calcium as depletion of inositol trisphosphate and ryanodine receptor stores eliminated NPS-induced currents (Roncace et al., 2017). Despite the accumulating evidence suggests the NPSR-mediated direct action, we cannot exclude the possibility that other neurotransmitter systems could contribute or modulate the NPS-induced neuroprotection.

Impaired dopamine neurotransmission plays a pivotal role in the etiopathogenesis of PD (Barone, 2010). For several decades, the dopamine precursor levodopa has been the primary therapy for PD. However, not all of the motor and non-motor features of PD can be attributed solely to dopaminergic dysfunction. To improve the management of PD, recent clinical and preclinical advances provide a basis for the identification of additional innovative therapeutic options which are involved with characterizing alterations in the levels of neurotransmitter receptors and transporters that are associated with the various manifestations of the disease (Brichta et al., 2013). On the other hand, the accumulating evidence indicates the putative regulatory role of NPS dopaminergic neurotransmission in the brain. Cao and colleagues demonstrated that intracerebroventricular administration of NPS exhibited a reward-like effects in rats (Cao et al., 2011). Furthermore, it has been shown in rats that microinjection of NPS into the rat ventral tegmental area induced hyperactivity and increased the extracellular levels of dopamine metabolites in the nucleus accumbens shell (Mochizuki et al., 2010). Last but not least, central administration of NPS dose-dependently enhanced extracellular levels of dopamine in rat medial prefrontal cortex (Si et al., 2010). Our microdialysis experiments have revealed that both acute and chronic treatments of central NPS improved the 6-OHDA-induced impaired motor functions by increasing the dopamine release from SN.

In particular, treatment of central nervous system (CNS) diseases is a challenge due to the blood-brain barrier's (BBB) severely restrict entry of small, non-polar compounds. However, the application of the drugs through intra-nasal route is considered as a non-invasive method of drug delivery which bypass the BBB to allow therapeutic substances direct access to the CNS. Therefore, it appears to be a useful alternative strategy for treatment of CNS diseases/disorders including PD (Lochhead and Thorne, 2012). Despite, the recent rodent studies indicated that nasal application of NPS reduces anxiety and pain-related behaviors, fast in-vivo degradation and short circulating half-life of proteins and peptides appears to be a major concern (Dine et al., 2015; Lukas and Neumann, 2012; Medina et al., 2014). Recently, Ruzza and colleagues synthesized the tetrabranched derivative of NPS (PWT1-NPS) using the peptide welding technology which is a recently developed chemical strategy that allows the synthesis of peptides with extraordinary high yield, purity and reproducibility. In mice, centrally administered PWT1-NPS mimicked the effects of NPS with higher potency and long-lasting action, whereas it yielded a three-fold bigger response in assay of calcium mobilization assay performed in HEK293 cells expressing the mouse NPSR (Ruzza et al., 2015). Therefore, our findings candidate the intra-nasal administration of NPS as a novel therapeutic approach for the treatment of PD patients.

5. Conclusions

Taken together, the data of the present study have revealed that centrally applied NPS exerts beneficial effects on 6-OHDA-induced parkinsonian rats through the mediation of NPSR in SN. The NPS-induced improvements appear to be maintained independently by alleviate and protective mechanisms which appear to be involved with dopaminergic and antioxidant actions on the SN neurons. Therefore, NPS appears to be a potential therapeutic target for treatment of PD.

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Conflicts of interest

No conflicts of interest exist.

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