Pharmacology, Biochemistry and Behavior 91 (2009) 636–642

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

Neuropeptide S produces hyperlocomotion and prevents oxidative stress damage in the mouse brain: A comparative study with amphetamine and diazepam

A.A. Castro [a], M. Moretti [a], T.S. Casagrande [a], C. Martinello [a], F. Petronilho [b], A.V. Steckert [b], R. Guerrini [d], G. Calo' [c], F. Dal Pizzol [b], J. Quevedo [a], E.C. Gavioli [a,*]

[a] Laboratório de Neurociências, Programa de Pós-Graduação em Ciências da Saúde, Universidade do Extremo Sul Catarinense, Criciúma, SC, Brazil
[b] Laboratório de Fisiopatologia Experimental, Programa de Pós-Graduação em Ciências da Saúde, Universidade do Extremo Sul Catarinense, Criciúma, SC, Brazil
[c] Department of Experimental and Clinical Medicine, Section of Pharmacology, and Neuroscience Center, University of Ferrara, and National Institute of Neuroscience, Italy
[d] Department of Pharmaceutical Science and Biotechnology Center, University of Ferrara, Ferrara, Italy

ARTICLE INFO

Article history:
Received 22 May 2008
Received in revised form 24 September 2008
Accepted 10 October 2008
Available online 31 October 2008

Keywords:
Neuropeptide S
Locomotor activity
Oxidative stress
Lipid peroxidation
Protein carbonyls
Catalase
Superoxide dismutase
Amphetamine
Diazepam
Brain structures

ABSTRACT

Neuropeptide S (NPS) is a recently discovered peptide which induces hyperlocomotion, anxiolysis and wakefulness. This study aimed to compare behavioral and biochemical effects of NPS with amphetamine (AMPH), and diazepam (DZP). To this aim, the effects of NPS (0.01, 0.1 and 1 nmol, ICV), AMPH (2 mg/kg, IP) and DZP (1 mg/kg, IP) on locomotion and oxidative stress parameters were assessed in mouse brain structures. The administration of NPS and AMPH, but not DZP, increased locomotion compared to control. Biochemical analyses revealed that AMPH increased carbonylated proteins in striatum, but did not alter lipid peroxidation. DZP increased lipid peroxidation in the cortex and cerebellum, and increased protein carbonyl formation in the striatum. In contrast, NPS reduced carbonylated protein in the cerebellum and striatum, and also lipid peroxidation in the cortex. Additionally, the treatment with AMPH increased superoxide dismutase (SOD) activity in the striatum, while it did not affect catalase (CAT) activity. DZP did not alter SOD and CAT activity. NPS inhibited the increase of SOD activity in the cortex and cerebellum, but little influenced CAT activity. Altogether, this is the first evidence of a putative role of NPS in oxidative stress and brain injury.
© 2008 Elsevier Inc. All rights reserved.

1. Introduction

Neuropeptide S (NPS) is a 20 amino-acid peptide recently identified in the brain and peripheral tissues of distinct species of vertebrates. NPS is the endogenous ligand of a G protein-coupled receptor named NPS receptor (NPSR) (Xu et al., 2004). Cells stably expressing NPSR were used to characterize *in vitro* the pharmacological actions of NPS. Nanomolar concentrations of NPS produce a transient increase in intracellular free Ca^{2+}, increase intracellular levels of cAMP and stimulate phosphorylation of mitogen-activated protein kinase (ERK1/2), thus suggesting that NPSR receptor may be coupled with both Gq and Gs proteins (Xu et al., 2004; Reinscheid et al., 2005b).

The NPSR is widely expressed in the mammalian brain, and higher levels were found in the hypothalamus, amygdala, endopiriform nucleus, cortex, subiculum, and nuclei of the thalamic midline (Xu et al., 2007). In contrast, NPS precursor mRNA is found expressed in a cluster of neurons located between the locus coeruleus and Barrington's nucleus (Xu et al., 2004).

In 2004, Xu and colleagues reported, for the first time, that the intracerebroventricular (ICV) injection of NPS, at nmol doses, produces hyperlocomotion, reduces anxiety-like behaviors and increases wakeful states in rodents. Subsequently, distinct laboratories have also demonstrated that the acute administration of NPS increases locomotion in rats and mice both naïve or habituated to the test chamber (Smith et al., 2006; Roth et al., 2006; Rizzi et al., 2008; Leonard et al., 2008). Recently, several *in vivo* studies have demonstrated the role played by NPS–NPSR system in the control of distinct central functions including wakefulness (Xu et al., 2004), anxiety (Xu et al., 2004; Leonard et al., 2008; Rizzi et al., 2008), drug abuse (Ciccocioppo et al., 2007; Paneda et al., 2007), and also food intake (Beck et al., 2005; Smith et al., 2006; Ciccocioppo et al., 2006; Niimi, 2006; Cline et al., 2007; Cline et al., 2008).

Interestingly, as commented before, the ICV injection of NPS induces a range of effects similar to those described for psychostimulants. Furthermore, at the same range of doses, NPS promotes anxiolytic-like effects as reported for distinct animal models of anxiety and mice strains (Xu et al., 2004; Leonard et al., 2008; Rizzi et al., 2008). However, it should be emphasized that psychostimulant drugs, such as amphetamine, caffeine, and modafinil, increase both locomotion and

* Corresponding author. Programa de Pós-Graduação em Ciências da Saúde, Universidade do Extremo Sul Catarinense (UNESC), 88806-000 Criciúma, SC, Brazil. Tel.: +55 48 3431 2759.
E-mail address: egavioli@hotmail.com (E.C. Gavioli).

0091-3057/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.pbb.2008.10.015

anxiety-related behaviors (for a review see: Reinscheid et al., 2005a). By contrast, standard anxiolytic drugs, i.e. diazepam, reduce anxiety without altering, at least at low doses, locomotor activity in rodents (for a review see: Reinscheid et al., 2005a). In this context, NPS signaling promotes a rather unique neuropeptidergic signal which combines activation and anxiolysis.

Previous studies performed in our labs showed that acute and repeated administration of amphetamine increased damage to lipids and proteins in the rat brain (Frey et al., 2006a,b). Protein carbonyl formation was also increased in primary mesencephalic cell culture exposed to amphetamine for 24 h (Lotharius and O'Malley, 2001). Literature findings point to free radical species, particularly reactive oxygen species and reactive nitrogen species, as a causative factor of amphetamine neuronal toxicity (for a review see: Brown and Yamamoto, 2003). In fact, changes in activity of the free radical-scavenging enzymes superoxide dismutase (SOD) and catalase (CAT) were described for amphetamine in the rat brain (Frey et al., 2006a,b). Additionally, oxidative stress has been demonstrated to occur also in response to high doses of substituted amphetamines such as methamphetamine and 3,4-methlyene-dioxymethamphetamine (MDMA; for a review see: Quinton and Yamamoto, 2006).

Free radical species are also mediating prooxidative processes, and even molecular damage, in different brain regions following acute and repeated administration of diazepam (Musavi and Kakkar, 1998; Musavi and Kakkar, 2000). An enhancement in the TBARS formation was found in the mitochondrial fractions from cerebral cortex and brain stem after single doses of diazepam (3 mg/kg). Additionally, isozymes of SOD and glutathione reductase displayed reduced activity, a region-dependent effect, after the administration of diazepam in rats (Musavi and Kakkar, 1998). Taken together, these data suggest that diazepam caused mitochondrial lipid peroxidation due to an increase in free-radical species production by reducing antioxidant enzyme activity.

Thus, considering that NPS alters central functions and promotes a rather unique behavioral effect, i.e. anxiolysis associated to stimulation, the present study aimed to compare behavioral and biochemical effects of NPS with amphetamine and diazepam, as standard psychostimulant and anxiolytic drug, respectively. The locomotor activity assay was employed in order to evaluate the behavioral effects of NPS, diazepam and amphetamine in mice. Biochemical techniques were used to assess damage to proteins and lipids in homogenate tissues from different brain areas (such as striatum, cortex and cerebellum). Additionally, free radical-scavenging enzyme activity, i.e. SOD and CAT, was measured in homogenate tissues of mice brain, in order to propose a putative relationship between this novel neuropeptidergic system and oxidative stress.

2. Materials and methods

2.1. Animals

Male CF-1 mice (2–3 months, 30–35 g) were obtained from our breeding colony (UNESC). The animals were housed six per cage with food and water available *ad libitum* and were maintained on a 12-h light/dark cycle (lights on at 7:00 am). Each animal was used only once. All experimental procedures involving animals were performed in accordance with the National Institute of Heath's Guide for the Care and Use of Laboratory Animals and the Brazilian Society for Neuroscience and Behavior (SBNeC) recommendations for animal care. This study was approved by the local ethics committee (Comitê de Ética em Pesquisa da Universidade do Extremo Sul Catarinense).

2.2. Drugs

The NPS was synthesized by Dr. R. Guerrini, Department of Pharmaceutical Science and Biotechnology Center, University of Ferrara, according to published methods (Camarda et al., 2008). The level of purity achieved with the NPS synthesis was higher than 95% which was checked by analytical HPLC and mass spectrometry. D-Amphetamine (AMPH) was purchased from Sigma-Aldrich, St. Louis, USA, while diazepam was purchased from Novartis Biociências AS, São Paulo, Brazil. NPS was dissolved in saline, and stock solutions were stored at −20 °C and diluted to the desired concentrations in saline just prior to use. D-Amphetamine was dissolved in saline, and diazepam was solubilized in Tween 80 (0.5%).

2.3. Treatments

Mice were treated with diazepam (1 mg/kg) and D-amphetamine (2 mg/kg) intraperitoneally (IP) 15 min before behavioral tests. NPS (1, 0.1 and 0.01 nmol) was ICV injected 5 min prior locomotor activity assessment. ICV injections (2 µl per mouse) were given as described by Laursen and Belhnap (1986) and previously adopted in our studies (Gavioli et al., 2002, 2003). Briefly, under light (just sufficient for loosing the righting reflex) ether anesthesia, a 27-gauge needle attached to a 10 µl Hamilton syringe was inserted perpendicularly 3 mm deep through the skull, into the left ventricle, at a position 2 mm lateral from the midline on the line drawn through the anterior base of the ears. Each animal received only one ICV or IP injection. Saline solution (NaCl 0.9%) was ICV or IP injected in control groups.

2.4. Locomotor activity assay

An infrared beam array cage (Insight Equipments, Ribeirao Preto, Brazil) connected to a PC was used for assessing locomotor activity in mice. The infrared beam array cage consists of a cubicle made of clear Perspex (48×50 cm) surrounded by 50 cm-high walls. Two facing blocks containing an infrared array record the horizontal activity, and a similar system assesses the vertical activity. The animals were gently placed on the center of the arena and they were allowed to explore the apparatus individually during a period of 30 min. All behavioral experiments were conducted in an illuminated (300 lx in the apparatus center) and quiet room. Locomotor activity was recorded in the light cycle between 13.00 and 16.00 h. After the behavioral evaluation of each mouse, the arena was cleaned with 10% ethanol solution. Locomotor activity was assessed for each mouse individually, during a 30-min period. The total distance traveled (cm) by each animal was accumulated over consecutive 5 min time.

Immediately after the behavioral procedure mice were sacrificed by decapitation, and brain structures were dissected (striatum, cerebellum and cortex), rapidly frozen, and stored at −80 °C until assayed. During brain dissection, when any signs of cannula misplacement or cerebral hemorrhage were observed, that brain was discarded from biochemical assays and also statistical analysis; these brains represent less than 5% of overall injected animals.

2.5. Biochemical assays

2.5.1. Lipid peroxidation

Lipid peroxidation was measured by formation of thiobarbituric acid (TBA) reactive substances (TBARS) after the method of Esterbauer and Cheeseman (1990). After brain dissection, brain structures were washed with PBS, harvested and lysed. Thiobarbituric reactive species, obtained by acid hydrolysis of 1,1,3,3-tetra-ethoxy-propane (TEP), was used as the standard for the quantification of TBARS. TBA 0.67% was added to each tube and vortexed. The reaction mixture was incubated at 90 °C for 20 min and the reaction was stopped by placing samples on ice. The optical density of each solution was measured in a spectrophotometer at 535 nm. Data were expressed as nmol of TBARS equivalents per mg of protein.

2.5.2. Protein carbonyl formation

Protein carbonyl content was measured in brain homogenates using 2,4-dinitrophenylhydrazine (DNPH) in a spectrophotometric

assay (Levine et al., 1994). Briefly, sample tissues were sonicated in ice-cold homogenization buffer containing phosphatase and protease inhibitors (200 nM calyculin, 10 µg/ml leupeptin, 2 µg/ml aprotinin, 1 mM sodium orthovanadate, and 1 µM microcystin-LR) and centrifuged at 1000 ×*g* for 15 min to sediment insoluble material. Three hundred microliter aliquots of the supernatant containing 0.7–1.5 mg of protein were treated with 300 µl of 10 mM DNPH, dissolved in 2 M HCl, and compared with 2 M HCl alone (reagent blank). Samples then were incubated for 1 h at room temperature in the dark and stirred every 10 min. Samples were precipitated with trichloroacetic acid (final concentration of 20%) and centrifuged at 16,000 ×*g* at 4 °C for 15 min. The pellet was washed three times with 1 ml of ethanol/ethyl acetate (1:1 v/v). Each time, the pellet was lightly vortexed and left exposed to the washing solution for 10 min before centrifugation (16,000 ×*g* for 5 min). The final pellet was dissolved in 1 ml of 6 M guanidine in 10 mM phosphate buffer–trifluoroacetic acid, pH 2.3, and the insoluble material was removed by centrifugation at 16,000 ×*g* for 5 min. Absorbance was recorded in a spectrophotometer at 370 nm for both DNPH-treated and HCl-treated samples. Protein carbonyl levels were expressed as nmol of carbonyl per mg of protein.

2.5.3. Superoxide dismutase (SOD) activity assay

SOD estimation was performed based on its ability to spontaneously inhibit oxidation of adrenaline to adrenochrome (Bannister and Calabrese, 1987). 2.78 ml of sodium carbonate buffer (0.05 mM; pH 10.2), 100 µl of EDTA (1.0 mM) and 20 µl of the supernatant or sucrose (blank) were incubated at 30 °C for 45 min. Thereafter, the reaction was initiated by adding 100 µl of adrenaline solution (9.0 mM). The change in the absorbance was recorded at 480 nm for 8 min. Temperature was maintained at 30 °C throughout the assay procedure. One unit of SOD produced approximately 50% of auto-oxidation of adrenaline. Results were expressed as units/mg of protein.

2.5.4. Catalase (CAT) activity assay

The CAT activity was measured by the method that employs hydrogen peroxide (H_2O_2) to generate H_2O and O_2 (Aebi, 1984). Brain tissue was sonicated in 50 mmol/l phosphate buffer (pH 7.0), and the resulting suspension was centrifuged at 3000 ×*g* for 10 min. The sample aliquot (20 µl) was added to 980 µl of the substrate mixture. The substrate mixture contained 0.3 ml of hydrogen peroxide in 50 ml of 0.05 M phosphate buffer (pH 7.0). Initial and final absorbances were recorded at 240 nm after 1 and 6 min, respectively. A standard curve was established using purified catalase (Sigma, MO, USA) under identical conditions.

2.5.5. Protein estimation

Protein was estimated in all the fractions according to the method of Lowry et al. (1951). Bovine serum albumin was used as standard.

2.6. Statistical analyses

Results were analyzed by INSTAT version 3.06 software. Behavioral data are presented as the mean±S.E.M., and each value reflects the mean of 12–15 animals per group. Biochemical data are expressed as mean±S.D., and each value reflects the mean of 4–5 animals per group. Differences among experimental groups were determined by one-way analysis of variance (ANOVA), followed by Dunnett's post-hoc test, except for locomotor activity data which were analyzed by one-way ANOVA with repeated measures, followed by Dunnett's post-hoc test. In all comparisons, statistical significance was set at $P<0.05$.

3. Results

3.1. Locomotor activity assay

Mice ICV or IP injected with saline displayed a cumulative distance traveled in 30 min of 5191±701 and 3928±481 cm, respectively.

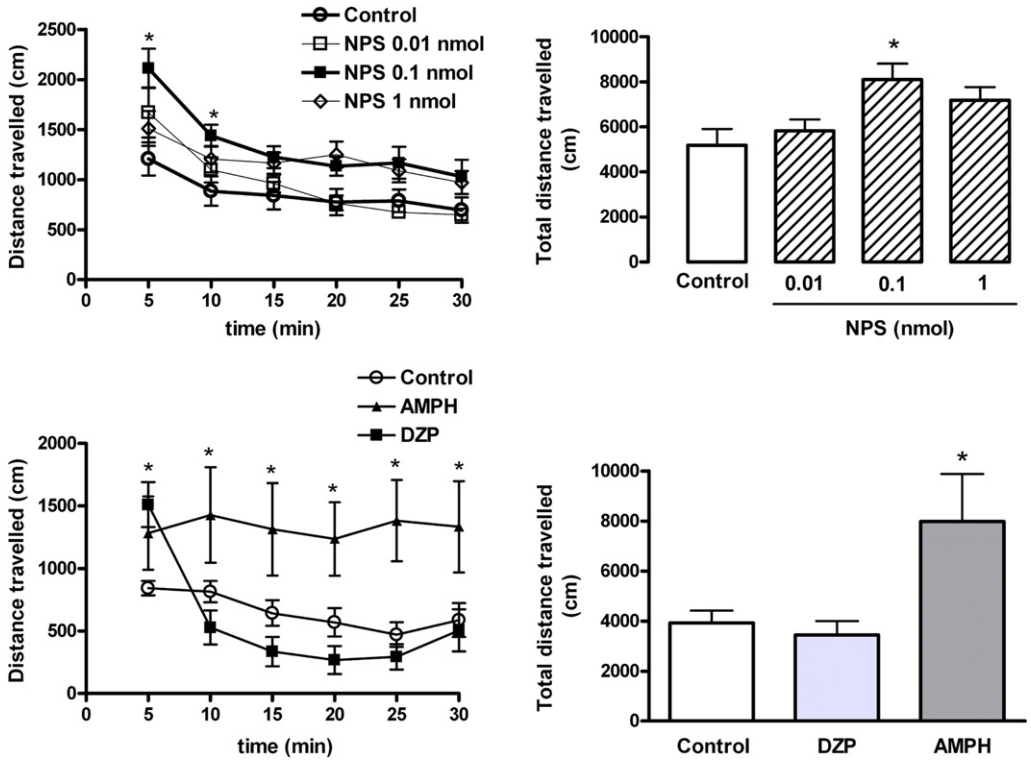

Fig. 1. Effects of the IP administration of amphetamine (2 mg/kg; AMPH), and diazepam (1 mg/kg; DZP; top panels), and the ICV injection of neuropeptide S (0.01, 0.1 and 1 nmol; NPS; bottom panels) on the spontaneous locomotor activity assessed in infrared beam array cages in mice for 30 min. Data are shown as mean±S.E.M. (11–13 mice/group). *$P<0.05$ vs. control group according to one-way ANOVA with repeated measures followed by Dunnett's test (left panels) and one-way ANOVA followed by Dunnett's test (right panels).

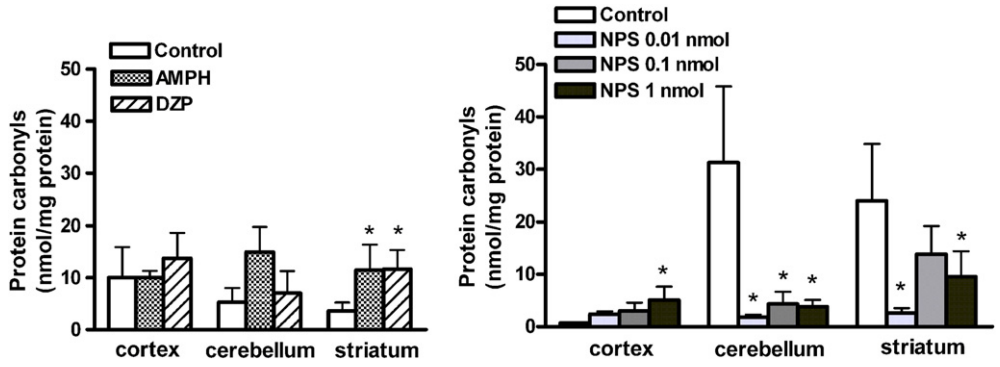

Fig. 2. Effects of the IP administration of amphetamine (2 mg/kg; AMPH), and diazepam (1 mg/kg; DZP; left panel), and the ICV injection of neuropeptide S (0.01, 0.1 and 1 nmol; NPS; right panel) on protein carbonyl content in homogenate tissues of cortex, cerebellum, and striatum of mice assessed by a spectrophotometric assay. Data are shown as mean ± S.D. (4–5 mice/group). *$P<0.05$ vs. control group according to one-way ANOVA followed by Dunnett's test.

Importantly, a statistical comparison of these groups was not provided since these series of experiments were not performed in parallel.

D-Amphetamine (2 mg/kg) administration promoted a robust increase in the distance moved compared to control group (Fig. 1, top panels; $F_{(2,32)}=4.98$; $P=0.013$). The maximum stimulatory effect of D-amphetamine was equal to 203% of control mice. By contrast, mice injected with diazepam (1 mg/kg) did not display any change in the cumulative distance traveled compared to saline, however an increase in locomotion during the first 5 min of observation was registered for diazepam (Fig. 1, top panels). Similar to D-amphetamine, the ICV injection of NPS 0.01–1 nmol/mouse induced an increase in spontaneous locomotion compared to control (Fig. 1; bottom panels). A statistical significant increase of cumulative distance traveled in 30 min was detected in mice treated with NPS 0.1 nmol compared to control group ($F_{(3,43)}=4.24$; $P=0.010$). The stimulatory effect of NPS was not dose-dependent, since the ICV injection of NPS at 0.1 nmol produced a higher stimulatory effect compared to the 1 nmol dose (156% vs. 139%, respectively). Additionally, the ICV injection of NPS 0.1 nmol caused a statistical significant increase of animal locomotion only at the first 10 min of observation (Fig. 1, bottom panels).

3.2. Biochemical assays

As illustrated in Fig. 2 (left panel), the administration of D-amphetamine and diazepam increased carbonylated proteins in the mouse striatum compared to control ($F_{(2,11)}=7.74$; $P=0.008$). In the cerebellum and cortex, the injection of D-amphetamine and diazepam did not modify the amount of carbonylated proteins compared to control ($P>0.05$). By contrast, mice ICV treated with NPS, at distinct doses, displayed a statistical significant reduction of protein carbonyl formation in the cerebellum and striatum compared to control (Fig. 2, right panel). In fact, in the cerebellum, NPS at all doses tested reduced significantly the content of carbonylated proteins (Fig. 2, right panel; $F_{(3,15)}=17.90$; $P<0.0001$). In the striatum, the treatment with NPS also reduced protein carbonyl formation, however the statistical significance was observed only at higher (1 nmol) and lower (0.01 nmol) doses (Fig. 2, right panel; $F_{(3,15)}=9.29$; $P=0.001$). In the cortex, distinctly from other brain areas, the administration of NPS only at the higher dose increased protein carbonyl content (Fig. 2, right panel; $F_{(3,15)}=5.96$; $P=0.007$).

As shown in Fig. 3, a single dose of diazepam (1 mg/kg), but not D-amphetamine (2 mg/kg), caused an increase in peroxidation of polyunsaturated fatty acids in distinct mouse brain structures. An increase in TBARS levels was detected in the cortex ($F_{(2,11)}=4.41$; $P=0.039$) and cerebellum ($F_{(2,11)}=5.81$; $P=0.017$) of diazepam-treated mice compared to control. On the other hand, the acute injection of D-amphetamine did not modify the TBARS formation into those brain structures analyzed. The treatment with NPS at 0.1 and 1 nmol reduced TBARS levels in cerebral cortex of mice in comparison with control (Fig. 3, right panel, $F_{(3,15)}=11.06$; $P=0.0004$). However, no changes in TBARS formation were observed in cerebellum and striatum of NPS-treated mice (Fig. 3, right panel; $P>0.05$).

The changes in the activity of the free radical-scavenging enzymes SOD and CAT were also evaluated in this study (Fig. 4). The single injection of D-amphetamine strongly increased SOD activity only in the striatum (Fig. 4, top panels; $F_{(2,11)}=130.05$; $P<0.0001$). However, no changes in CAT activity were observed after the treatment with D-amphetamine ($P>0.05$). Diazepam did not change the activity of both enzymes SOD and CAT in all the three brain areas analyzed in this study ($P>0.05$).

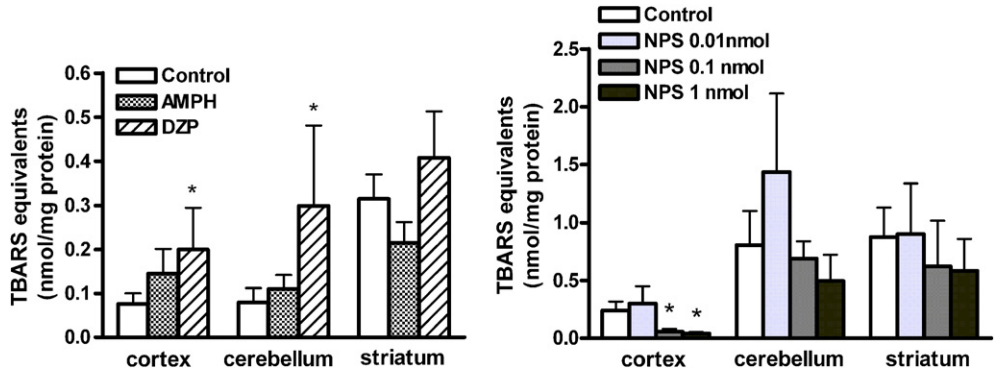

Fig. 3. Effects of the IP administration of amphetamine (2 mg/kg; AMPH), and diazepam (1 mg/kg; DZP; left panel), and the ICV injection of neuropeptide S (0.01, 0.1 and 1 nmol; NPS; right panel) on thiobarbituric acid reactive species (TBARS) formation in homogenate tissues of cortex, cerebellum, and striatum of mice assessed by a spectrophotometric assay. Data are shown as mean ± S.D. (4–5 mice/group). *$P<0.05$ vs. control group according to one-way ANOVA followed by Dunnett's test.

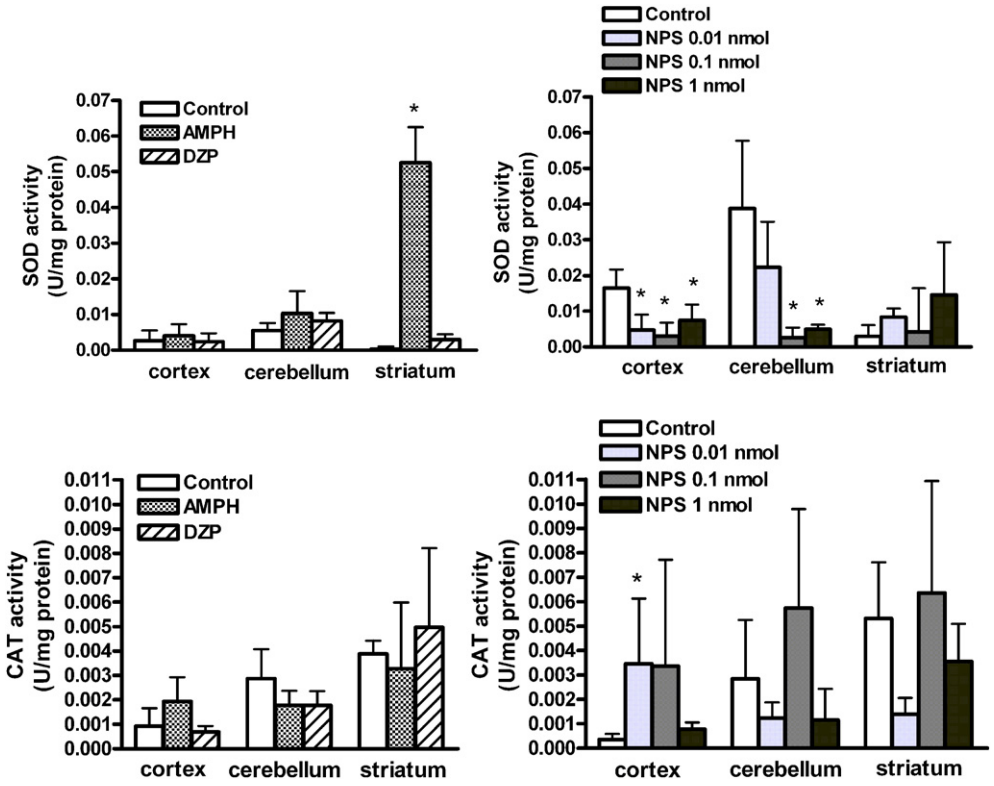

Fig. 4. Effects of the IP administration of amphetamine (2 mg/kg; AMPH), and diazepam (1 mg/kg; DZP; left panels), and the ICV injection of neuropeptide S (0.01, 0.1 and 1 nmol; NPS; right panels) on superoxide dismutase (SOD) and catalase (CAT) activity in homogenate tissues of cortex, cerebellum, and striatum of mice assessed by a spectrophotometric assay. Data are shown as mean±S.D. (4–5 mice/group). *$P<0.05$ vs. control group according to one-way ANOVA followed by Dunnett's test.

Quite distinct from D-amphetamine and diazepam, the ICV injection of NPS significantly changed the activity of free radical-scavenging enzymes SOD and CAT assessed in different brain structures in mice (Fig. 4; right panels). In fact, the treatment with NPS inhibited in a statistical significant manner SOD activity in the cortex and cerebellum compared to control group. SOD activity in the cortex was 68.8%, 81.3% and 56.3% less active in mice treated with NPS at the doses of 0.01, 0.1 and 1 nmol, respectively, than that of saline-treated mice (Fig. 4, top panels; $F_{(3,15)}=9.67$; $P=0.008$). In the cerebellum, the treatment with NPS 0.01, 0.1 and 1 nmol inhibited SOD activity in 50%, 92.5% and 87.5% of control, respectively (Fig. 4, top panels; $F_{(3,15)}=9.73$, $P=0.0008$). No alterations in the striatum were detected in those mice treated with NPS at any dose tested as compared to control ($P>0.05$). Considering CAT activity, in the mouse cortex the treatment with NPS, only at the lower dose (0.01 nmol), increased CAT activity in 1000% of control group (Fig. 4, bottom panels; $F_{(3,15)}=5.84$; $P=0.0075$). No alterations in the activity of this enzyme were observed in striatum and cerebellum of mice injected with NPS ($P>0.05$).

4. Discussion

The present findings demonstrate that both NPS and D-amphetamine, under our experimental conditions, were able to stimulate locomotion in CF-1 mice in an unfamiliar environment. It should be mentioned that similar studies have already described an increase in locomotion induced by NPS in mice (Xu et al., 2004; Roth et al., 2006; Rizzi et al., 2008; Leonard et al., 2008) and in rats (Smith et al., 2006), thus, suggesting that the effects of NPS are highly consistent among experimental conditions and animal species. Interestingly, current data demonstrated that the injection of NPS in mice did not produce a dose-dependent effect, since the dose of 1 nmol displayed lower stimulatory effects compared to 0.1 nmol. This phenomenon has already been described before for NPS-treated mice (Roth et al., 2006; Rizzi et al., 2008), and it could be explained by the fact that the onset of stimulatory action of NPS 1 nmol is slightly delayed. Longer periods of observation, for example 1 h, could possibly detect this postponed hyperlocomotor effect of NPS 1 nmol (Roth et al., 2006). By contrast, Xu et al. (2004) found a potent hyperlocomotor effect for NPS 1 nmol observable at the first minutes of observation. Based on this divergent information, the underlying reasons of this effect are still unknown. Further studies on NPSR knockout mice and with NPSR antagonists could give additional information on this regard.

In addition, under our experimental conditions, D-amphetamine induced a robust and long-lasting stimulatory effect, whereas diazepam did not alter spontaneous activity in CF-1 mice compared to control. Our behavioral findings are in line with literature and suggest that NPS mimicked the stimulatory effects of D-amphetamine in the spontaneous locomotion of CF-1 mice (Wenger, 1989).

Psychostimulant drugs, such as D-amphetamine, cocaine, and MDMA, and also anxiolytic compounds, i.e. diazepam, increase carbonylated proteins and lipid peroxidation in the rodent brain (Quinton and Yamamoto, 2006; Brown and Yamamoto, 2003; Musavi and Kakkar, 2000; Musavi and Kakkar, 1998; Yamamoto and Zhu, 1998). Considering this, the present study aimed to evaluate the effects of distinct doses of NPS on protein carbonylation and lipid peroxidation levels in the striatum, cortex and cerebellum homogenates. The present findings demonstrated that a single administration of D-amphetamine increased protein carbonylation content in the striatum of mice. However, no alterations in TBARS levels were detected in brain structures of D-amphetamine-treated mice. The acute injection of diazepam increased protein carbonyl formation in the striatum, and it caused a significant increase in TBARS formation in the cortex and cerebellum of mice. Opposite to D-amphetamine and diazepam, the ICV injection of NPS reduced protein carbonylation levels in cerebellum and striatum and decreased lipid peroxidation in the mouse cortex, which suggests that NPS could attenuate oxidative damage to proteins and lipids in the mouse brain.

Protein carbonylation is the most widely used biomarker for oxidative damage to proteins (Dalle-Donne et al., 2006). Carbonyl groups are introduced into proteins by a variety of oxidative pathways. Reactive oxygen species can react directly with the protein or they can react with molecules such as sugars and lipids, generating products (reactive carbonyl species) that react with proteins. A large number of neurodegenerative diseases are directly associated with the accumulation of carbonylated proteins in neuronal tissues (Dalle-Donne et al., 2006).

Lipid peroxidation is one of the major consequences of free radical-mediated injury to the brain. Lipid peroxidation changes fluidity and permeability of the cell membranes and, consequently, impairs the activity of membrane-bound enzymes, the binding of molecules to receptors, cellular interactions, nutrient transport, and the function of second messenger systems (Meagher and FitzGerald, 2000). Lipid peroxidation leads to the production of conjugated dienic hydroperoxides. These unstable substances decompose into various aldehydes, which react with thiobarbituric acid, and then produce TBARS that can be measured by a spectrophotometric assay. This approach has some methodological limitations, such as overestimation of TBARS levels due to unspecific reactions of thiobarbituric acid with damaged lipids (Dotan et al., 2004). However, the results presented herein suggested that NPS reduced lipid peroxidation. Thus, it seems unlikely that those methodological limitations could produce a false protective effect for NPS on lipid peroxidation.

Another point to be considered is that a substantial increase in carbonylated proteins and TBARS contents was observed in ICV compared to IP saline-treated mice. These biochemical alterations could be probably due to little, but significant, brain injury caused by saline administration directly into the ventricle. Previously, Kim et al. (1998) demonstrated that the free-hand ICV injection method was able to increase substantially plasma corticosterone levels in mice, which suggests that this method could be employed as an animal model of stress. Taken together, the ICV injection method possibly altered the baseline of those parameters assessed in the present study, which provided an ideal background to assess the protective effects of NPS into the brain. On these bases, further studies aiming to evaluate the role of NPS treatment in distinct stimulus-induced neuronal injury, such as chemical (i.e. chronic administration of psychostimulants or convulsive drugs) and physical (i.e. stressful situations) stimuli are worthy of being performed.

Considering that carbonylated proteins and lipid peroxidation may reflect the effects induced by multiple forms of free radical species, the current study aimed to measure the activity of two free radical-scavenging enzymes: SOD and CAT, in diazepam-, D-amphetamine-, and NPS-treated mice. Diazepam, under our experimental conditions, did not modify SOD and CAT activity. Our data also demonstrated that D-amphetamine increased SOD activity only in the striatum, while no alterations in CAT activity were observed. Only one study is available in the literature about the effects of a single administration of D-amphetamine on SOD and CAT activity. Frey et al. (2006b) reported that D-amphetamine administration increased SOD activity in the prefrontal cortex, while no changes have been described for CAT activity in the rat brain. During physiological states, SOD metabolizes superoxide anion (O_2^-), producing hydrogen peroxide (H_2O_2), which can react with iron to generate highly reactant hydroxyl radicals via the Fenton reaction. CAT is the most important peroxidase in detoxifying excess hydrogen peroxide to prevent hydroxyl production. Alterations on the redox state can lead to an imbalance between SOD and CAT activities and to oxidative stress (Matés and Sánchez-Jiménez, 1999). Thus, in situations in which SOD levels are increased without a concomitant CAT increase, the intermediate product H_2O_2 may accumulate and generate hydroxyl radicals, which may lead to lipid and protein oxidation, and consequently neuronal damage. This is possibly the mechanisms by which D-amphetamine causes damage to lipid and proteins, and it might be the explanation of those biomarker differences in control ICV and IP injected mice.

It should be noted that similar to other biomarkers, such as TBARS and protein carbonyl, SOD activity, but not CAT, appears to be sensitive to free-hand ICV injection method. In fact, a clear increase in SOD activity was observed in control animals treated via ICV compared to IP injections. As commented above, these differences could be due to stress and little brain injury produced by this procedure. In this context, current findings revealed that NPS inhibited SOD activity in the cortex and cerebellum, and slightly increased CAT activity in the mouse cortex, which could suggest that NPS treatment protected against the effects of free-hand ICV injection by inhibiting the increase of SOD activity. Further studies aiming to investigate the participation of antioxidant enzymes, such as glutathione peroxidase, and other non-enzymic antioxidant scavengers should be performed in order to investigate the mechanisms of these protective effects of NPS.

Altogether, the present findings suggest, for the first time, a role played by NPS in attenuating oxidative damage to lipids and proteins in the mouse brain. Our data also revealed that the treatment with NPS attenuated the increase of SOD activity, but not CAT, which was possibly induced by the free-hand ICV injection. Altogether, these observations contribute to provide the first evidence of a putative role of NPS signaling in attenuating oxidative stress and brain injury in mice.

Acknowledgments

This work was supported by funds from the IBRO (Return Home Fellowship to ECG) and the Brazilian National Council Research (grant no. 479760/2007-1 to ECG).

References

Aebi H. Catalase in vitro. Methods Enzymol 1984;105:121–6.
Bannister JV, Calabrese L. Assays for superoxide dismutase. Methods Biochem Anal 1987;32:279–312.
Beck B, Fernette B, Stricker-Krongrad A. Peptide S is a novel potent inhibitor of voluntary and fast-induced food intake in rats. Biochem Biophys Res Commun 2005;332(3):859–65.
Brown JM, Yamamoto BK. Effects of amphetamines on mitochondrial function: role of free radicals and oxidative stress. Pharmacol Ther 2003;99:45–53.
Camarda V, Trapella C, Calo G, Guerrini R, Rizzi A, Ruzza C, et al. Synthesis and biological activity of human neuropeptide S analogues modified in position 2. J Med Chem 2008;51(3):655–8.
Ciccocioppo R, Economidou D, Cannella N, Fedeli A, Cippitelli A, Calo G, et al. Inhibition of palatable food intake by neuropeptide S is reversed by its analogue [Ala3]hNPS. Socienty for Neuroscience; 2006. 809.807/O815.
Ciccocioppo R, Kallupi M, Cannella N, Braconi S, Stopponi S, Economidou D. Neuropeptide S system activation facilitates conditioned reinstatement of cocaine-seeking in the rat. Society for Neuroscience; 2007. 271.218/Z271.
Cline MA, Godlove DC, Nandar W, Bowden CN, Prall BC. Anorexigenic effects of central neuropeptide S involve the hypothalamus in chicks (*Gallus gallus*). Comp Biochem Physiol A Mol Integr Physiol 2007;148(3):657–63.
Cline MA, Prall BC, Smith ML, Calchary WA, Siegel PB. Differential appetite-related responses to central neuropeptide S in lines of chickens divergently selected for low or high body weight. J Neuroendocrinol 2008;20(7):904–8.
Dalle-Donne I, Aldini G, Carini M, Colombo R, Rossi R, Milzani A. Protein carbonylation, cellular dysfunction, and disease progression. J Cell Mol Med 2006;10(2):389–406.
Dotan Y, Lichtenberg D, Pinchuk I. Lipid peroxidation cannot be used as a universal criterion of oxidative stress. Prog Lipid Res 2004;43(3):200–27.
Esterbauer H, Cheeseman KH. Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal. Methods Enzymol 1990;186:407–21.
Frey BN, Martins MR, Petronilho FC, Dal-Pizzol F, Quevedo J, Kapczinski F. Increased oxidative stress after repeated amphetamine exposure: possible relevance as an animal model of mania. Bipolar Disorders 2006a;8:275–80.
Frey BN, Valvassori SS, Réus GZ, Martins MR, Petronilho FC, Bardini K, et al. Changes in antioxidant defense enzymes after D-amphetamine exposure: implications as an animal model of mania. Neurochem Res 2006b;31:699–703.
Gavioli EC, Rae GA, Calo G, Guerrini R, De Lima TCM. Central injections of nocistatin or its C-terminal hexapeptide exert anxiogenic-like effect on behaviour of mice in the plus-maze test. Br J Pharmacol 2002;136:764–72.
Gavioli EC, Marzola G, Guerrini R, Bertorelli R, Zucchini S, De Lima TCM, et al. Blockade of nociceptin/orphanin FQ-NOP receptor signalling produces antidepressant-like effects: pharmacological and genetic evidences from the mouse forced swimming test. Eur J Neurosci 2003;17:1987–90.
Kim DH, Jung JS, Song DK, Suh HW, Huh SO, Kim YH. Intracerebroventricular injection-induced increase in plasma corticosterone levels in the mouse: a stress model. J Pharmacol Toxicol Methods 1998;39(2):71–3.

Laursen SE, Belhnap JK. Intracerebroventricular injections in mice. Some methodological refinements. J Pharmacol Methods 1986;16(4):355–7.
Leonard SK, Dwyer JM, Sukoff Rizzo SJ, Platt B, Logue SF, Neal SJ, et al. Pharmacology of neuropeptide S in mice: therapeutic relevance to anxiety disorders. Psychopharmacology (Berl) 2008;197(4):601–11.
Levine RL, Williams JA, Stadtman ER, Shacter E. Carbonyl assays for determination of oxidatively modified proteins. Methods Enzymol 1994;233:346–57.
Lotharius J, O'Malley KL. Role of mitochondrial dysfunction and dopamine-dependent oxidative stress in amphetamine-induced toxicity. Ann Neurol 2001;49(1):79–89.
Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–75.
Quinton MS, Yamamoto BK. Causes and consequences of methamphetamine and MDMA toxicity. AAPS J 2006;8(2):E337-347.
Matés JM, Sánchez-Jiménez F. Antioxidant enzymes and their implications in pathophysiologic processes. Front Biosci 1999;4:D339-345.
Meagher EA, FitzGerald GA. Indices of lipid peroxidation in vivo: strengths and limitations. Free Radic Biol Med 2000;28(12):1745–50.
Musavi S, Kakkar P. Diazepam induced early oxidative changes at the subcellular level in rat brain. Mol Cell Biochem 1998;178:41–6.
Musavi S, Kakkar P. Pro and antioxidant responses to repeated administration of diazepam in rat brain. Mol Cell Biochem 2000;206(1–2):97-103.
Niimi M. Centrally administered neuropeptide S activates orexin-containing neurons in the hypothalamus and stimulates feeding in rats. Endocrine 2006;30(1):75–9.
Paneda C, de Lecea L, Roberts AJ. Neuropeptide S increases cocaine seeking behaviour. Society for Neuroscience; 2007. 611.611/KK613, San Diego.
Reinscheid RK, Xu YL, Civelli O. Neuropeptide S: a new player in the modulation of arousal and anxiety. Mol Interv 2005a;5(1):42–6.
Reinscheid RK, Xu YL, Okamura N, Zeng J, Chung S, Pai R, et al. Pharmacological characterization of human and murine neuropeptide S receptor variants. J Pharmacol Exp Ther 2005b;315:1338–45.
Rizzi A, Vergura R, Marzola G, Ruzza C, Guerrini R, Salvadori S, et al. Neuropeptide S is a stimulatory anxiolytic agent: a behavioural study in mice. Br J Pharmacol 2008;154:471–9.
Roth AL, Marzola E, Rizzi A, Arduin M, Trapella C, Corti C, et al. Structure–activity studies on neuropeptide S — identification of the amino acid residues crucial for receptor activation. J Biol Chem 2006;281 30,20820809-30,20816.
Smith KL, Patterson M, Dhillo WS, Patel SR, Semjonous NM, Gardiner JV, et al. Neuropeptide S stimulates the hypothalamo-pituitary-adrenal axis and inhibits food intake. Endocrinology 2006;147(7):3510–8.
Xu YL, Reinscheid RK, Huitron-Resendiz S, Clark SD, Wang Z, Lin SH, et al. Neuropeptide S: a neuropeptide promoting arousal and anxiolytic-like effects. Neuron 2004;43:487–97.
Xu YL, Gall CM, Jackson VR, Civelli O, Reinscheid RK. Distribution of neuropeptide S receptor mRNA and neurochemical characteristics of neuropeptide S expressing neurons in the rat brain. J Comp Neurol 2007;500:84-102.
Wenger GR. The role of control activity levels in the reported strain differences to the behavioral effects of drugs in mice. Pharmacol Biochem Behav 1989;32(1):241–7.
Yamamoto BK, Zhu W. The effects of methamphetamine on the production of free radicals and oxidative stress. J Pharmacol Exp Ther 1998;287(1):107–14.