NEUROSCIENCE
RESEARCH ARTICLE
L. de Santana Souza et al. / Neuroscience 448 (2020) 140–148

Role of Neuropeptide S on Behavioural and Neurochemical Changes of an Animal Model of Attention-Deficit/Hyperactivity Disorder

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Abstract—Neuropeptide S (NPS) is a recently discovered peptide signalling through its receptor NPSR, which is expressed throughout the brain. Since NPSR activation increases dopaminergic transmission, we now tested if NPSR modulates behavioural and neurochemical alterations displayed by an animal model of attention-deficit/hyperactivity disorder (ADHD), Spontaneous Hypertensive Rats (SHR), compared to its control strain, Wistar Kyoto rats (WKY). NPS (0.1 and 1 nmol, intracerebroventricularly (icv)) did not modify the performance in the open field test in both strains; however, NPSR antagonism with [^{t}Bu-D-Gly^{5}]NPS (3 nmol, icv) increased, *per se*, the total distance travelled by WKY. In the elevated plus-maze, NPS (1 nmol, icv) increased the percentage of entries in the open arms (%EO) only in WKY, an effect prevented by pretreatment with [^{t}Bu-D-Gly^{5}]NPS (3 nmol, icv), which decreased *per se* the %EO in WKY and increased their number of entries in the closed arms. Immunoblotting of frontal cortical extracts showed no differences of NPSR density, although SHR had a lower NPS content than WKY. SHR showed higher activity of dopamine uptake than WKY, and NPS (1 nmol, icv) did not change this profile. Overall, the present work shows that the pattern of functioning of the NPS system is distinct in WKY and SHR, suggesting that this system may contribute to the pathophysiology of ADHD.

Key words: ADHD, neuropeptide S, animal model, behaviour, dopamine.

INTRODUCTION

Neuropeptide S (NPS) is a 20-amino acid peptide which binds to its cognate receptor NPSR, a G-protein coupled receptor that can trigger intracellular Ca^{2+} mobilisation, cyclic AMP formation and MAPK phosphorylation (Guerrini et al., 2010). Intracerebroventricular (icv) injection of NPS evokes behavioural effects like arousal, hyperlocomotion, anxiolysis and memory improvement (Guerrini et al., 2010).

Robust evidence associates NPS signalling with the modulation of the dopaminergic system, as heralded by the ability of NPS to increase brain dopamine metabolites (Mochizuki et al., 2010; Si et al., 2010) and to improve motor behaviour in a model of Parkinson's disease (Didonet et al., 2014). In addition, NPSR is present in areas with high dopaminergic innervation (Xu et al., 2007).

Attention-deficit/hyperactivity disorder (ADHD) is a developmental disorder characterised by a pattern of persistent inattentiveness and/or hyperactivity/impulsivity which have consequences on social and scholar aspects (American Psychiatric Association, 2013). The most accepted hypothesis about ADHD pathophysiology claims for a monoaminergic hypofunction, specially involving dopamine and noradrenaline, in brain areas involved with planning actions, motivated behaviours and reward, and motor behaviour. In fact, ADHD treatment is based on the improvement of monoaminergic transmission, and methylphenidate (MPH), the gold standard therapy, acts by inhibiting dopamine and noradrenaline transporters thus dampening dopamine uptake (Bush, 2010; Faraone, 2018).

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Abbreviations: ADHD, attention-deficit/hyperactivity disorder; icv, intracerebroventricularly; NPS, Neuropeptide S; SHR, Spontaneous Hypertensive Rats; WKY, Wistar Kyoto rats.

https://doi.org/10.1016/j.neuroscience.2020.09.030
0306-4522/

The most used animal model of ADHD is Spontaneous Hypertensive Rats (SHR), and beyond their behavioural pattern of impulsivity, sustained attention deficit and hyperactivity (Sagvolden et al., 2005b), SHR show several neurochemical impairments such as corticostriatal dopaminergic hypofunction (Russell et al., 1995), increase of dopamine metabolism (Boix et al., 1998) and decrease of dopamine vesicular storage (Russell et al., 1998). Regarding intracellular signalling pathways, SHR have impaired cAMP formation (Marcil et al., 1997) and neuronal Ca^{2+} influx (Lehohla et al., 2004).

Thus, since NPS system impacts on these signalling pathways and affects dopaminergic transmission, we hypothesised that the NPS system might be involved in ADHD pathophysiology. Remarkably, recent studies showed a correlation between ADHD-like symptoms and a polymorphism A/T (Asn-Ile^{107}) in the gene encoding NPSR, that lead to increasing 10-times the potency of this NPSR, without changes of its affinity Laas et al., 2014, 2015).

Hence, the objective of this study was to investigate the effects of NPSR agonism and antagonism on motor, emotional and neurochemical changes present in an animal model of ADHD.

EXPERIMENTAL PROCEDURES

Animals

Adult male inbred Wistar Kyoto rats (WKY)/NIcoCrl (300–400 g) and SHR/NCrl (250–350 g) rats (60–90 days/old) maintained at the Institute of Biology of Fluminense Federal University (Niterói, RJ, Brazil) were used. The animals were housed on plastic cages of 40 × 33 × 17 cm (3–4 per cage) with controlled temperature (22–25 °C), light–dark cycle of 12 h (7:00 am–7:00 pm), and water and food *ad libitum*. A total of 247 rats was used in the present study. All experimental procedures were made according to Brazilian Law 11.794/2008 and were only carried out after approval of Use of Animals Ethics Committee (CEUA) of Federal Fluminense University (protocol n° 782), which follows the principles of Guide for the Care and Use of Laboratory Animals (2011).

Stereotaxic surgery

Animals used in behavioural experiments were anaesthetised intraperitoneally with ketamine (100 mg/kg; Cetamin, Syntec, Brazil) and xylazine (10 mg/kg; Calmiun, União Química, Brazil) to undergo a stereotaxic surgery in order to insert a guide cannula (length of 12 mm and 0.7 mm of diameter) in their lateral ventricle, using the following coordinates: AP = −0.9 mm, ML = +1.5 mm, DV = +2.6 mm (Paxinos and Watson, 2007).

Treatments

The treatments used in this study were either the activation of NPSR with neuropeptide S (Bachem, Switzerland) at doses of 0.1 and 1 nmol (5 μL) or the antagonism of NPSR with its peptidergic antagonist [^{t}Bu-D-Gly^{5}]NPS (CPC Scientific, USA), at doses of 0.3 and 3 nmol (5 μL). These doses and time points of analysis were identical to these selected in previous studies by other groups studying behavioural responses induced by NPS and [tBu-D-Gly5]NPS (Xu et al., 2004; Castro et al., 2009; Rizzi et al., 2009; Boeck et al., 2010; Ruzza et al., 2012). Both treatments were delivered intracerebroventricularly (icv) and the control group received saline solution. Treatments were administered through a needle attached to a polyethylene tube connected to a Hamilton microsyringe (Hamilton Company, USA), and injected with the help of an infusion pump using an infusion rate of 2.5 μL per min.

Behavioural tests

All the animals were acclimated to the laboratory for at least 1 h to minimise the effects of stress due to exposure to a new environment. To evaluate behavioural effects of NPS, animals were treated 5 min before the tests, and to probe the prevention of its effects by a NPSR antagonist, [^{t}Bu-D-Gly^{5}]NPS was injected 15 min prior to NPS. The control groups were similarly injected with saline solution instead of the drugs. The animals were submitted to the open field and then, 30 min later, to the elevated plus-maze.

Open field

The apparatus consists of a square wooden box (60 × 60 × 60 cm). The animals were individually allowed to explore the apparatus for 10 min while being tracked by the software Anymaze (Stoelting, USA) to quantify parameters such as the total distance travelled, central locomotion (in %) and time and latency to enter the central area of the apparatus.

Elevated plus maze

The apparatus is a wooden cross-shaped maze, with two closed arms and two open arms all measuring 50 cm and suspended 50 cm above the floor. The animals were tested for 5 min to quantify the number of entries in the closed arms and the percentage of entries and time spent in the open arms, using the Anymaze software.

Immunobloting

Naive WKY and SHR were anaesthetised with isoflurane (Isoforine, Cristália, Brazil) and had their cerebral cortices removed, rapidly dissected and homogenised in sample buffer (10% glycerol, 5% β-mercaptoethanol, 20% sodium dodecyl sulphate, 0.5 M Tris-HCl, pH 6.8). The homogenates were then heated to 85–95 °C for 5 min to denature proteins and the protein levels were measured by the Bradford method (Bradford, 1976). Samples containing 40 μg of protein were separated by polyacrylamide gel electrophoresis (SDS–PAGE) and proteins were then transferred to a PVDF membrane. Membranes were blocked with skimmed milk (5%) dissolved in TBS-T (200 mM NaCl, 20 mM Tris-HCl, pH 7.6, containing 0.1% Tween-20) and incubated overnight with rabbit

primary antibodies either anti-NPS (1:1000, Cloud-Clone Corp., USA) or anti-NPSR (1:1000, Biorbyt, UK). On the following day, membranes were washed with TBS-T and incubated with a secondary anti-rabbit HRP-antibody (1:3000, Biorad, USA) for 1 h. After washing, the labelled bands were visualised with ECL. Membranes were stripped with glycine 0.2 M, pH 2.2 and re-incubated overnight with a mouse primary antibody anti-alpha-tubulin (1:100,000, Sigma-Aldrich, USA). On the next day, after washing, the membranes were incubated with the secondary anti-mouse HRP-antibody (1:10,000, GE Healthcare, USA) and visualised again with ECL.

[^{3}H]-Dopamine uptake

WKY and SHR received NPS (1 nmol, icv) and, 5 min later, they were anaesthetised to dissect their frontal cerebral cortices, which were placed in wells containing 1 mL Hank's 4 buffered (in mM: 128 NaCl, 4 KCl, 1 $MgCl_2$, 3 $CaCl_2$, 20 HEPES, 4 glucose) at pH 7.4 at 37 °C. Samples from both right and left hemispheres were pooled, cut into approximately 2 mm^3 segments and incubated for 1 h with [^{3}H]-dopamine (0.5 mCi; from PerkinElmer – Massachusetts, USA, specific activity: 71 Ci/mmol) in the presence of ascorbic acid (100 μM, from Sigma-Aldrich, USA) and pargyline (100 μM, from Sigma-Aldrich, USA). The medium was then removed and the tissue washed three times with 3 mL of cold Hank's 4 to wash out the free radioactivity (not taken up by the tissue). Then, 1 mL of water was added and the mixture was subjected to successive freeze–thaw cycles to disrupt the cells before counting radioactivity using a scintillation counter (B281001, PerkinElmer – Massachusetts, USA). The protein content was assayed by the Lowry method (Lowry et al., 1951).

Cyclic AMP assay

Brains from WKY and SHR were removed and the frontal cortices were collected. Samples from the pooled right and left hemispheres were cut into 2 mm^3 segments and incubated for 10 min at 37 °C in Minimum Essential Medium buffered with 20 mM HEPES at pH 7.3 containing 100 mM ascorbic acid, 100 mM pargyline and 0.5 mM IBMX (from Sigma-Aldrich, USA). NPS 5 μM was added to the medium and the tissue suspension was incubated for 30 min at 37 °C. The reaction was stopped by adding 10% trichloroacetic acid (final concentration). cAMP was purified and dosed as previously described (de Mello et al., 1982; Gilman, 1970; Matsuzawa and Nirenberg, 1975) and its levels were normalised to the protein content, evaluated by the Lowry method (Lowry et al., 1951).

Statistical analysis

All the data were submitted to the Shapiro–Wilk normality test, followed by a Levene test to identify variance homogeneity. Behavioural data displayed a normal distribution. Data from immunoblotting, dopamine uptake and cAMP accumulation displayed a non-normal distribution and were analysed using non-parametric tests. Analysis of open field and elevated plus-maze data were made according to the experiment's design, using mixed one-way ANOVA followed by Sidak's multiple comparisons test, and mixed two-way ANOVA with post-hoc analysis using the Duncan's test. Non-parametric data were analysed according to the experiment's design, using Mann-Whitney and Kruskal–Wallis tests. Specific statistical analyses employed are also mentioned in the Figure's captions. The significance threshold assumed in this study was $p < 0.05$. GraphPad Prism 8 (GraphPad, USA) and Statistica 7.0 (Statsoft, USA) were used to perform analysis. In the open field test, outlier rats identified in either of the parameters tested (total distance travelled, central locomotor activity, time in the central area or latency to enter central area) were excluded from analyses. Criteria for animal exclusion in the elevated plus-maze test were the following: rats that did not explore any of the arms, rats that fell from the maze and outliers. Outliers were identified by ROUT test ($Q = 5\%$).

RESULTS

Open field and elevated plus maze

Table 1 shows the effects of NPS and [^{t}Bu-D-Gly^5]NPS administered icv in the performance of WKY and SHR in the open field. The total locomotor activity in the open field test was similar between WKY and SHR and was not modified by both doses of NPS (0.1 and 1 nmol) in any of the strains. Significant differences between WKY and SHR were found on the percentage of central locomotor activity (Two-way ANOVA, strain factor: $F_{1,58} = 41.65$, $p < 0.05$), on the time spent in the central area (Two-way ANOVA, strain factor: $F_{1,58} = 26.71$, $p < 0.05$) and on the latency to first enter the central area (Two-way ANOVA, strain factor: $F_{1,58} = 3.99$, $p < 0.05$). However, both doses of NPS did not further modify any of these anxiety-related parameters in either strains. Similar findings were observed upon treatment with [^{t}Bu-D-Gly^5]NPS: this group of rats also displayed significant differences between WKY and SHR on the percentage of central locomotor activity (Two-way ANOVA, strain factor: $F_{1,48} = 34.12$, $p < 0.05$), time on central area (Two-way ANOVA, strain factor: $F_{1,48} = 20.69$, $p < 0.05$) and latency to first enter on central area (Two-way ANOVA, strain factor: $F_{1,48} = 18.48$, $p < 0.05$), and both doses of the NPSR antagonist did not further modify any of these anxiety-related parameters in either strains. However, [^{t}Bu-D-Gly^5]NPS (3 nmol) increased total locomotion in WKY (Two-way ANOVA, interaction between factors: $F_{2,48} = 3.21$, $p < 0.05$), without significant effects on SHR ($p > 0.05$).

Fig. 1 shows the behavioural parameters of WKY and SHR evaluated on the elevated plus-maze. SHR spent significantly more time in the open arms than control WKY (Two-way ANOVA, interaction between factors: $F_{2,48} = 4.30$, $p < 0.05$), and also did more entries in the closed arms (Two-way ANOVA: $F_{1,48} = 12.69$, $p < 0.05$). NPS had no effects on these parameters in both strains. [^{t}Bu-D-Gly^5]NPS administration was also

Table 1.

NPS

	WKY			SHR		
Parameter	Control (n = 8)	NPS 0.1 nmol (n = 12)	NPS 1 nmol (n = 9)	Control (n = 9)	NPS 0.1 nmol (n = 13)	NPS 1 nmol (n = 13)
Total distance travelled (m)	22.28 ± 2.06	23.64 ± 1.95	17.40 ± 3.29	23.65 ± 1.64	32.40 ± 3.97	18.30 ± 1.66
Central locomotor activity (%)	5.18 ± 2.26	6.41 ± 1.46	2.78 ± 0.94	14.52 ± 2.03#	14.45 ± 1.64#	12.85 ± 1.68#
Time in central area (s)	10.58 ± 5.15	10.16 ± 2.17	4.03 ± 1.80	30.83 ± 6.56#	32.29 ± 5.56#	22.77 ± 4.81#
Latency to enter central area (s)	107.70 ± 53.47	65.18 ± 14.51	58.76 ± 22.97	31.76 ± 9.75#	36.82 ± 15.61#	49.62 ± 17.17#

[^{t}Bu-D-Gly^{5}]NPS (TBU)

	WKY			SHR		
Parameter	Control (n = 10)	TBU 0.3 nmol (n = 7)	TBU 3 nmol (n = 7)	Control (n = 13)	TBU 0.3 nmol (n = 6)	TBU 3 nmol (n = 11)
Total distance travelled (m)	24.74 ± 3.94	24.06 ± 2.28	39.35 ± 5.56	21.65 ± 2.20	32.62 ± 4.45	26.84 ± 4.09
Central locomotor activity (%)	9.29 ± 2.74	4.13 ± 1.53	4.20 ± 1.23	19.87 ± 3.04#	18.98 ± 2.29#	17.08 ± 1.99#
Time in central area (s)	23.39 ± 8.77	8.70 ± 3.02	8.64 ± 2.88	56.55 ± 10.28#	50.03 ± 10.66#	31.82 ± 5.35#
Latency to enter central area (s)	67.48 ± 17.45	136.53 ± 37.26	94.14 ± 29.81	24.58 ± 4.74#	36.28 ± 8.38#	38.60 ± 11.15#

Effect of treatment with NPS (0.1 and 1 nmol) or [^{t}Bu-D-Gly^{5}]NPS (TBU – 0.3 and 3 nmol) in WKY and SHR rats submitted to the open field test. Data are shown as mean ± S.E.M.

# $p < 0.05$ compared to WKY strain, using a Two-way ANOVA followed by Duncan's post hoc test.

devoid of effects on the percentage of time spent in the open arms. Notably, the icv administration of either NPS or [^{t}Bu-D-Gly^{5}]NPS elicited changes in the percentage of entries in the open arms, as shown on Fig. 1. In WKY, NPS (1 nmol) increased the percentage of entries in the open arms (Two-way ANOVA, interaction between factors: $F_{2,48} = 10.16$, $p < 0.05$) without changing entries in the closed arms (Two-way ANOVA, interaction between factors: $F_{2,48} = 0.612$, $p > 0.05$), suggesting an anxiolytic-like effect. Interestingly, the effect was the opposite in SHR rats, where NPS, at both doses, decreased the percentage of entries on these arms when compared with control SHR, and no statistical differences were found between control WKY and NPS-treated SHR rats concerning this parameter ($p > 0.05$). Conversely, the NPSR antagonist, [^{t}Bu-D-Gly^{5}]NPS (3 nmol) significantly reduced the percentage of entries in the open arms in WKY and SHR animals (Two-way ANOVA, treatment factor: $F_{2,55} = 4.41$, $p < 0.05$).

Concerning entries in the closed arms, SHR entered there significantly more times than WKY (Two-way ANOVA, strain factor: $F_{1,55} = 5.95$, $p < 0.05$) and [^{t}Bu-D-Gly^{5}]NPS (3 nmol) increased this parameter in WKY (Two-way ANOVA, treatment effect: $F_{2,55} = 5.31$, $p < 0.05$), but not in SHR ($p > 0.05$).

To evaluate if NPSR antagonism with [^{t}Bu-D-Gly^{5}]NPS (at doses of 0.3 and 3 nmol) was able to prevent the NPS-induced anxiolytic-like effect in WKY, we submitted a new group of WKY to a pretreatment with [^{t}Bu-D-Gly^{5}]NPS 15 min before administration of NPS (1 nmol) to carry out their behavioural analysis in the elevated plus maze. Fig. 2 shows that NPS increased entries in the open arms, and this effect was prevented by pretreatment with 3 nmol [^{t}Bu-D-Gly^{5}]NPS (One-way ANOVA: $F_{3,28} = 6.66$, $p < 0.05$), providing a pharmacological demonstration that this NPS effect is selectively mediated by NPSR.

As treatment with 3 nmol [^{t}Bu-D-Gly^{5}]NPS induced effects *per se* in WKY submitted to the open field (increase of total distance travelled) and elevated plus maze (increase of number of entries on closed arms), we evaluated if a treatment with NPS after pretreatment with [^{t}Bu-D-Gly^{5}]NPS could reverse its effects. Fig. 3 shows that NPS was capable of reversing the effects induced by NPSR antagonism for both the total distance travelled (One-way ANOVA: $F_{3,32} = 5.52$, $p < 0.05$), and entries in the closed arms (One-way ANOVA: $F_{3,28} = 4.54$, $p < 0.05$); this means that [^{t}Bu-D-Gly^{5}]NPS likely acts by blocking endogenous NPS signalling, supporting again the selective involvement of NPSR. Furthermore, this shows that the antagonism of NPSR leads to a hyperlocomotor effect in WKY, which is reversed by NPS administration.

Immunoblotting

Fig. 4 shows the levels of NPS and the density of NPSR, evaluated by Western blot, on frontal cerebrocortical tissue of *naïve* WKY and SHR. The quantification of NPSR density encompassed both bands since they

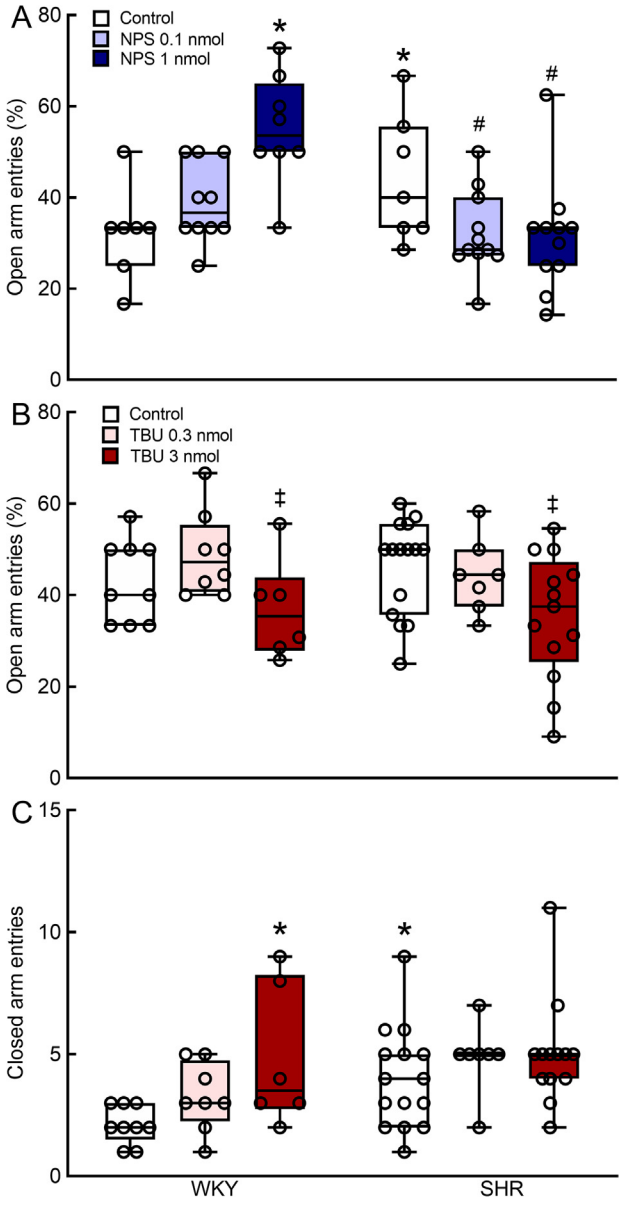

Fig. 1. Effect of the treatment with NPS (0.1 and 1 nmol) or [^{t}Bu-D-Gly^{5}]NPS (TBU – 0.3 and 3 nmol) in WKY (n = 6–10) and SHR (n = 7–11) rats submitted to the elevated plus maze test. The parameters evaluated were the percentage of open arm entries **(A, B)** and the number of closed arm entries **(C)**. Data are shown as median (horizontal line) and the whiskers indicate the 5th and 95th percentile. *p < 0.05 compared to control WKY, $^{\#}p$ < 0.05 compared to control SHR, $^{\ddagger}p$ < 0.05 compared to other treatments, regardless of strain; using a two-way ANOVA followed by Duncan's post hoc test.

reflect different levels of glycosylation of the receptor, as previously described (Clark et al., 2010). In this brain structure, there are no significant differences of NPSR density between the strains (Mann–Whitney, $U_{6,8}$ = 23.0, p > 0.05); however, SHR had a significantly lower content of NPS than WKY (Mann–Whitney, $U_{3,3}$ = 0.0, p < 0.05).

[^{3}H]-Dopamine uptake

The evaluation of dopamine uptake in frontal cerebrocortical tissue of WKY and SHR is shown in Fig. 5. The results obtained corroborate previous studies (Russell et al., 2005; Pandolfo et al., 2013), showing that SHR display higher levels of dopamine uptake than WKY (Mann–Whitney, strain factor, regardless of

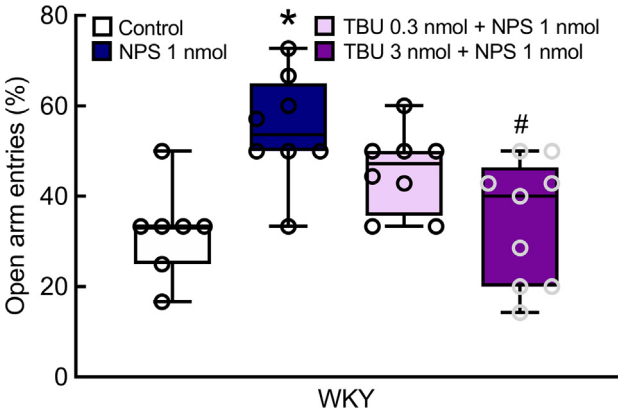

Fig. 2. Effect of pretreatment with [tBu-D-Gly5]NPS (TBU – 0.3 and 3 nmol) in WKY (n = 7–9) before treatment with NPS (1 nmol) in the percentage of open arm entries of an elevated plus maze. Data are shown as median (horizontal line) and the whiskers indicate the 5th and 95th percentile. *p < 0.05 compared to control WKY, $^{\#}p$ < 0.05 compared to NPS (1 nmol); using a one-way ANOVA followed by Sidak's multiple comparisons test.

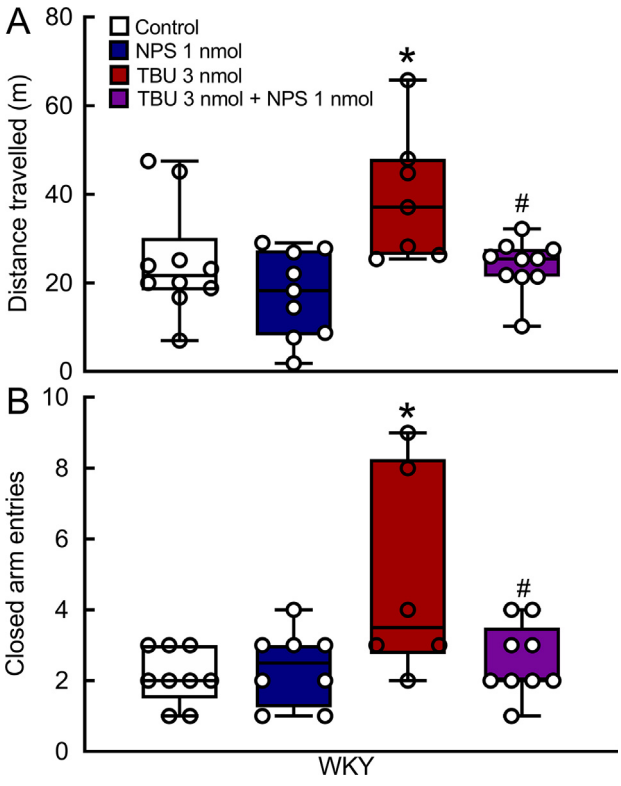

Fig. 3. Effect of pretreatment with [^{t}Bu-D-Gly^{5}]NPS (TBU – 3 nmol) in WKY (n = 6–10) before treatment with NPS (1 nmol) in two estimates of locomotor activity, namely the total distance travelled in the open field **(A)** and the number of closed arm entries in the elevated plus maze **(B)**. Data are shown as median (horizontal line) and the whiskers indicate the 5th and 95th percentile. *p < 0.05 compared to control WKY, $^{\#}p$ < 0.05 compared to TBU; using a one-way ANOVA followed by Sidak's multiple comparisons test.

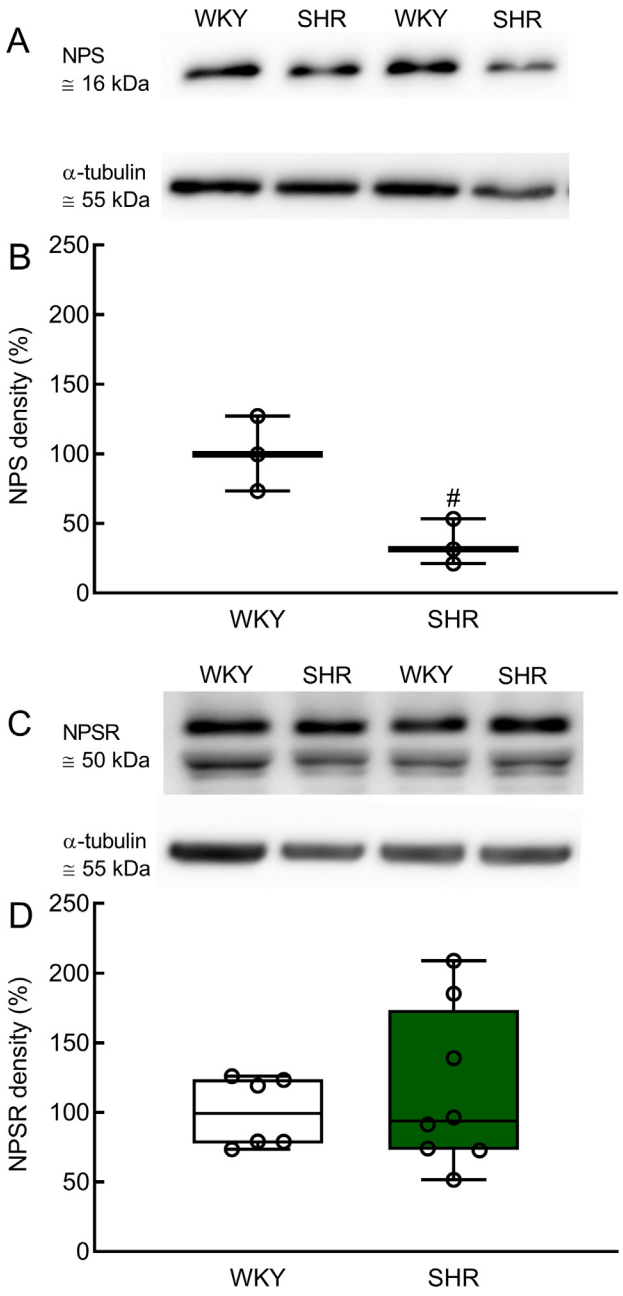

Fig. 4. Representative blots **(A, C)** and average density of NPS (**(B)** $n = 3$) and NPSR (**(D)** $n = 6$ and 8) in samples of the frontal cerebral cortex of naive WKY and SHR. Results were normalised by α-tubulin density and are shown as median (horizontal line) and the whiskers indicate the 5th and 95th percentile. $^{\#}p < 0.05$ compared to WKY; using a Mann–Whitney *U* test.

treatment: $U_{10,12} = 23.5$, $p < 0.05$). However, treatment with NPS (1 nmol) had no effect on [^{3}H]-dopamine uptake in either strains (Kruskal Wallis, $H_3 = 6.27$, $p > 0.05$).

Cyclic AMP assay

Fig. 6 shows the NPS-induced accumulation of cyclic AMP in frontal cerebrocortical tissue of WKY and SHR. The results show that SHR presented a lower cAMP accumulation in the frontal cerebral cortex compared to

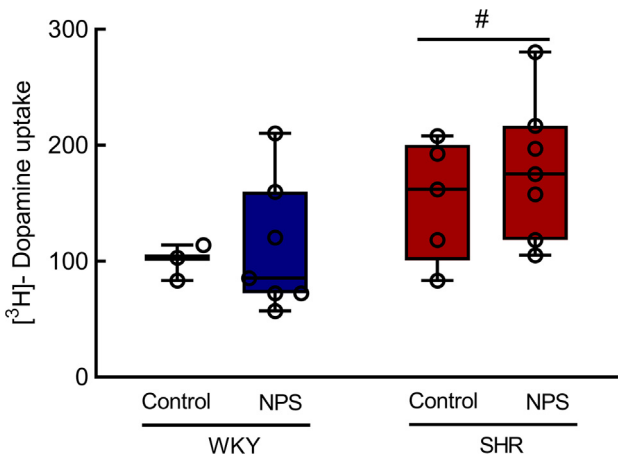

Fig. 5. [^{3}H]-Dopamine uptake in the frontal cerebral cortex after icv administration of saline (control) or NPS (1 nmol) in WKY ($n = 3$–7) and SHR ($n = 5$–7). Results are shown in percentage of the activity determined in WKY, which was 45.66 fmol/mg protein/h on average. Data are shown as median (horizontal line) and the whiskers indicate the 5th and 95th percentile. $^{\#}p < 0.05$ compared to WKY, regardless of treatment; using a Mann–Whitney *U* test.

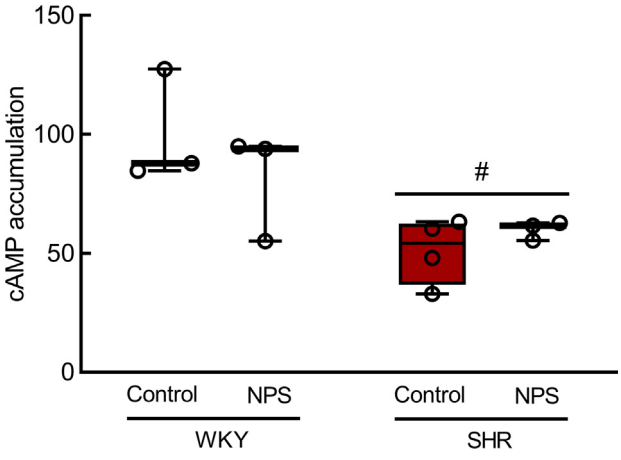

Fig. 6. Ability of NPS (5 μM) to recruit intracellular cAMP accumulation in the frontal cerebral cortex of WKY ($n = 3$) and SHR ($n = 3$–4). Results are shown in percentage of the values quantified in WKY-control, which were 15.67 pmol/mg protein/h on average. Data are shown as median (horizontal line) and the whiskers indicate the 5th and 95th percentile. $^{\#}p < 0.05$ compared to WKY, regardless of treatment; using a Mann-Whitney U test.

WKY (Mann–Whitney, strain factor, regardless of treatment: $U_{6,7} = 5.0$, $p < 0.05$). The treatment with NPS (5 μM) had no effects on cyclic AMP accumulation in WKY (Kruskal–Wallis, $H_3 = 5.93$, $p > 0.05$).

DISCUSSION

This is the first study evaluating the impact of the agonism and antagonism of NPSR in the WKY and SHR, an animal model largely used on ADHD research due to its face, construct and predictive validity (Sagvolden, 2000; Russell et al., 2005; Sagvolden et al., 2005b). However, it is important to point that this model also displays cardiovascular and adrenergic phenotypes that can be act as

confounding factors. Concerning behavioural comparison in the open field, SHR display an increased time spent and distance travelled in the centre of the apparatus, as well as a lower latency to enter this more aversive part of the arena, when compared to WKY. However, no differences were found between the strains with respect to the total distance travelled. Previous work showed that SHR present hyperactivity, although this develops throughout time (Sagvolden et al., 2005b), which can explain the lack of differences during the analysed period of 10 minutes. On the elevated plus maze, SHR spent more time in the open arms and entered more in the closed arms. Taken together, these results corroborate the information that SHR present a different emotional pattern compared to WKY, showing less aversion to potentially dangerous areas, a characteristic already described in previous studies (Howells et al., 2009).

Regarding the effects of NPS on behaviour, our results indicate that NPSR activation triggers different effects in WKY and SHR, that are different from these previously reported to be caused by NPS in mice and Wistar rats (for a review, see: Guerrini et al. 2010). In these different species and strains, the doses tested in our work (0.1 and 1 nmol) simultaneously trigger both hyperlocomotion and anxiolysis. In contrast, our results in WKY and SHR show no significant effects of NPSR activation on locomotion assessed in the open field test. This lack of effects might be due to the length of the tests, and an analysis comprising a larger period of time could eventually reveal some effects. In fact, the 5 minutes time window between the administration of NPS and behavioural analysis allowed to detect behavioural modifications in WKY rats, but it cannot be excluded that different behavioural effects could be present at different time windows after NPS administration. Thus, we observed on the elevated plus maze that NPS (1 nmol) increased the number of entries in the open arms in WKY whereas it reduced these entries in SHR to a level similar of control WKY rats; this suggests that NPS administration, despite its already known anxiolytic-like effect, can modulate exacerbated states to normal levels. It is important to point out that this work was conducted with inbred strains, and, during their development, some specific characteristics concerning the function of the NPS system could be selected, leading to the observed differences on evoked effects compared to other strains already tested.

Previous work has shown that effects evoked by NPS are due to the engagement of different types of G-proteins linked to the NPSR: activation of G_s is associated with the hyperlocomotor effect of NPS, whereas activation of G_q is involved with anxiolysis (Clark et al., 2017; Grund and Neumann, 2018). Thus, it is possible that the effects observed in WKY and SHR may be derived from a different pattern and/or activation efficiency of G protein subunits upon exposure to NPS. The quantification of cAMP levels could help answering this question, since the amount of second messengers formed influences neuronal responses and, consequently, animal behaviour (Sassone-Corsi, 2012). It was already demonstrated that SHR have a lower content of adenylate cyclase and cAMP in the brain when compared to WKY (Zhou et al., 2017), which was confirmed by our results. Moreover, a study done with renal arterioles of WKY and SHR showed that SHR have an increased density and activity of phospholipase C when compared to WKY (Peng et al., 2007), which might also occur in other body tissues. However, measures of brain activity of phospholipase C and association of NPS administration with blockers of adenylyl cyclase and phospholipase C pathways prior to behavioural tests should be attempted in order to define the signalling pathway responsible for the behavioural effects resulting from exposure to NPS. In this context, it was already shown that the anxiolytic-like effect of NPS is dependent of phospholipase C pathway activation in the medial amygdala (Grund and Neumann, 2018).

We also tested [^{t}Bu-D-Gly^5]NPS, a peptidergic antagonist of NPSR, to evaluate if it could trigger effects *per se*, and if it could block the NPS-induced effects. Previous studies in CD-1 mice and Wistar rats showed that [^{t}Bu-D-Gly^5]NPS was unable to alter locomotion and anxiety of these animals (Ruzza et al., 2012). However, our work showed that [^{t}Bu-D-Gly^5]NPS (3 nmol) increased the total distance travelled in the open field test by WKY, whilst it reduced entries in the open arms and increased entries in the closed arms of the elevated plus maze. These results are interesting and suggests that, unlike other strains, the antagonism of NPSR causes hyperlocomotion in WKY. Also, the decrease of entries in the open arms in WKY (which is the opposite of the effect of NPS in these animals) reveals a anxiogenic-like effect of [^{t}Bu-D-Gly^5]NPS, whereas, in SHR, no significant effects were found in this test using this dose (3 nmol), as previously reported (Ruzza et al., 2012).

We also report no differences between the strains of NPSR density in the frontal cerebral cortex, an area intimately involved in ADHD symptoms. However, we show that SHR have lower NPS levels compared to WKY in this brain area. Previous reports described that NPS expression could be regulated by nicotinic receptors (Lage et al., 2007) and purinergic receptors (Lage et al., 2006), so perhaps the expression of NPS is also regulated by dopamine, whose levels are decreased in SHR strain. Indeed, a study showed that rats that received acute or chronic treatment with olanzapine also had changes in NPS mRNA levels, but not in NPSR mRNA levels in the hypothalamus (Pałasz and Rojczyk, 2015) and they also suggested that dopamine could be blocking gene expression of NPS. It is known that NPSR activation increases dopamine levels (Mochizuki et al., 2010; Si et al., 2010) and, considering that SHR have a monoaminergic hypofunction, the lower NPS levels could also be related to lower levels of dopamine. In parallel, the lack of modification of NPSR density between strains should not rule out the involvement of this receptor in ADHD symptoms, since we have not assessed if the cellular membrane expression and/or the NPSR function are modified in SHR compared to WKY.

Studies in humans demonstrated that a NPSR polymorphism could be involved in ADHD symptoms (Laas et al., 2014, Laas et al., 2015). Therefore, it is important to understand how the activation of NPSR could

interfere with different neurochemical features of animals modelling ADHD, in particular concerning dopaminergic activity. Thus, we quantified dopamine uptake, and showed that SHR have a greater uptake capacity than WKY, an effect that was already described (Russell et al., 2005; Pandolfo et al., 2013). However, NPS (1 nmol) had no effects thereupon. This means that, in spite of increased brain dopamine levels triggered by NPSR activation (Mochizuki et al., 2010; Si et al., 2010), this effect does not involve a control of dopamine transporter (DAT) activity, at least in the frontal cerebral cortex. It is already known that SHR have an increased surface density of DAT, and faster dopamine uptake than WKY in the striatum and nucleus *accumbens* (Miller et al., 2012). So, NPS might mediate dopamine release affecting DAT on other brain areas related to ADHD apart from the frontal cortex.

Overall, the present results show that different behavioural effects seen in these two strains might only be related to dopamine or NPS or might also involve other signalling systems. Previous work showed that pre-synaptic activation of NPSR could inhibit serotonin and norepinephrine release in purified synaptic terminals of the pre-frontal cortex of mice, and no effects were found concerning dopamine (Raiteri et al., 2009). Also, it was already published that the NPS system is correlated with other neuromodulators (Niimi, 2006; Massi et al., 2007; Grund et al., 2017), including the adenosinergic system, which is known to be an important target on modulation of SHR behavioural deficits (Boeck et al., 2010; Pacheco et al., 2011; Pandolfo et al., 2013; França et al., 2018).

In conclusion, our results suggest that, in WKY and SHR strains, the functioning of NPS system is different to that previously reported in other animal models, both concerning the effects of NPS on locomotor as well as on emotional aspects. It is important to intensify research on this topic because this NPS system seems to be intimately linked to ADHD pathophysiology, as already suggested by previous work in patients (Laas et al., 2014, Laas et al., 2015). Our work was the first to attempt a behavioural and neurochemical comparison of the NPS system in an ADHD animal model, a disorder for which treatments currently available are focused only on classic neurotransmission. Peptidergic systems are emerging as promising targets to develop new drugs to several diseases, promoting treatments with less side effects and improving the quality of life of patients.

ACKNOWLEDGMENTS

We acknowledge to Elaine C. Gavioli for the great insights to this study through scientific discussions, which provided a better understanding of NPS system.

ROLE OF FUNDING SOURCE

LSS was funded by a fellowship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) during all the time of her PhD and received a PDSE fellowship from the same agency (88881.187855/2018-01). RAC was supported by Fundacion LaCaixa (LCF/PR/HP17/52190001), Centro 2020 (CENTRO-01-0145-FEDER-000008: BrainHealth 2020 and CENTRO-01-0246-FEDER-000010) and FCT-Fundação para a Ciência e Tecnologia (POCI-01-0145-FEDER-03127). The authors gratefully acknowledge the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq - 430746/2016-4), CAPES and Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ - 226440). These agencies had no role in study design, in the collection, analysis and interpretation of data, in the writing of the report, and in the decision to submit the paper for publication.

CONTRIBUTORS

LSS, PP, ASR and RCCK designed the study and wrote the protocol. LSS and PP managed the literature searches and analysis. LSS, PAS, AF and RSM performed the experiments. LSS, RSM and PP undertook the statistical analysis. LSS wrote the first draft of the manuscript and RPC, RAC and PP contributed reviewing data and giving insights to the manuscript. All authors contributed to and have approved the final manuscript.

CONFLICT OF INTEREST

All the authors declare that they have no conflicts of interest.

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(Received 25 April 2020, Accepted 10 September 2020)
(Available online 22 September 2020)