Hormones and Behavior 83 (2016) 23–38

Contents lists available at ScienceDirect

Hormones and Behavior

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

Involvement of nigral oxytocin in locomotor activity: A behavioral, immunohistochemical and lesion study in male rats

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ARTICLE INFO

Article history:
Received 2 October 2015
Revised 21 April 2016
Accepted 13 May 2016
Available online 14 May 2016

Keywords:
Oxytocin
Dopamine
Glutamic acid
Substantia nigra
Oxytocin-saporin
Locomotor activity

ABSTRACT

Oxytocin is involved in the control of different behaviors, from sexual behavior and food consumption to empathy, social and affective behaviors. An imbalance of central oxytocinergic neurotransmission has been also associated with different mental pathologies, from depression, anxiety and anorexia/bulimia to schizophrenia, autism and drug dependence. This study shows that oxytocin may also play a role in the control of locomotor activity. Accordingly, intraperitoneal oxytocin (0.5–2000 μg/kg) reduced locomotor activity of adult male rats. This effect was abolished by d$(CH_2)_5$Tyr$(Me)^2$-Orn^8-vasotocin, an oxytocin receptor antagonist, given into the lateral ventricles at the dose of 2 μg/rat, which was ineffective on locomotor activity. Oxytocin (50–200 ng/site) also reduced and d$(CH_2)_5$Tyr$(Me)^2$-Orn^8-vasotocin (2 μg/site) increased locomotor activity when injected bilaterally into the substantia nigra, a key area in the control of locomotor activity. Conversely, the destruction of nigral neurons bearing oxytocin receptors by the recently characterized neurotoxin oxytocin-saporin injected into the substantia nigra, increased basal locomotor activity. Since oxytocin-saporin injected into the substantia nigra caused a marked reduction of neurons immunoreactive for tyrosine hydroxylase (e.g., nigrostriatal dopaminergic neurons) and for vesicular glutamate transporters VGluT1, VGluT2 and VGluT3 (e.g., glutamatergic neurons), but not for glutamic acid decarboxylase (e.g., GABAergic neurons), together these findings suggest that oxytocin influences locomotor activity by acting on receptors localized presynaptically in nigral glutamatergic nerve terminals (which control the activity of nigral GABAergic efferent neurons projecting to brain stem nuclei controlling locomotor activity), rather than on receptors localized in the cell bodies/dendrites of nigrostriatal dopaminergic neurons.

Introduction

Oxytocin, the neurohypophyseal hormone well known for its hormonal role in lactation and parturition, also exerts widespread actions in central nervous system. Accordingly, a physiological role of this neuropeptide is thought to occur in the control of different behaviors, from sexual behavior and food consumption to empathy, social and affective behaviors (Argiolas and Melis, 2013, Crespi, 2015; Feeser et al., 2012; Liu and Wang, 2003; Lin, 2012; Parker and Bloom, 2012; Gil et al., 2013; Štefánik et al., 2015). An imbalance of central oxytocinergic neurotransmission is also thought to be associated with different mental pathologies, from depression, anxiety and anorexia/bulimia to schizophrenia, autism and drug dependence (Baskerville and Douglas, 2010; Love, 2014; Sarnyai and Kovács, 2014; Guastella et al., 2015). The involvement of oxytocin in the above central functions and mental pathologies is supported by the existence of hypothalamic oxytocinergic neurons that project not only to the neurohypophysis but also to multiple extra-hypothalamic brain areas, such as the nucleus accumbens, the medial frontal cortex, the ventral tegmental area, the substantia nigra, the hippocampus, the amygdala, the medulla oblongata and the spinal cord (Buijs, 1978; Dogterom et al., 1978; Mai et al., 1993; Sofroniew, 1980; Zimmerman et al., 1984). For instance, at the level of the ventral tegmental area oxytocin influences sexual motivation and sexual behavior by acting directly on mesolimbic and mesocortical dopaminergic neurons, which play a key role in motivational and rewarding processes (see Melis et al., 2007; Melis and Argiolas, 2011; Argiolas and Melis, 2013).

Interestingly, evidence for a role of oxytocin in the modulation of locomotor activity has been also suggested by several studies (Crine et al., 1983; Uvnäs-Moberg et al., 1994; Klenerova et al., 2009). Accordingly, low doses of oxytocin given intraperitoneally induced hyperactivity apparently mediated by an anxiolytic effect, while higher doses exerted a sedative effect. However, in these studies the experimental conditions were chosen in order to investigate the anxiolytic effects of oxytocin rather than a possible role of the neuropeptide in the control of locomotor activity or a possible interaction with the nigrostriatal dopaminergic

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http://dx.doi.org/10.1016/j.yhbeh.2016.05.012

system, which plays a key role in the control of locomotor activity. In this regard, the substantia nigra is a brain area of special interest since: 1) oxytocinergic fibres and terminals project here from the paraventricular nucleus of the hypothalamus (Adan et al., 1995; Mai et al., 1993; Sofroniew, 1983; Zimmerman et al., 1984); 2) oxytocinergic receptors and oxytocin receptor messenger RNA are found in the substantia nigra of the human (Loup et al., 1989, 1991) and rat brain (Vaccari et al., 1998), respectively; and 3) the pars compacta of the substantia nigra contains the cell bodies of nigrostriatal dopaminergic neurons which project to the basal ganglia, hence in circuits playing a pivotal role in the control of motor activity (see von Bohlen Und Halbach et al., 2004). Accordingly, the degeneration of dopaminergic nigrostriatal neurons causes motor disturbances such as those found in Parkinson's disease (see Hodaie et al., 2007).

During the studies aimed at investigating the effect of oxytocin injected into the ventral tegmental area on penile erection and sexual activity (Melis et al., 2007, Succu et al., 2008), oxytocin injected into the substantia nigra was found unable to induce penile erection; however it was found able to reduce locomotor activity. This prompted us to study the effect of oxytocin on locomotor activity and its mechanism of action when given systemically and into the substantia nigra. In particular, the effect of low and high doses of oxytocin given intraperitoneally (IP) or into the substantia nigra and the effect of $d(CH_2)_5Tyr(Me)^2$-Orn^8-vasotocin, a selective oxytocin receptor antagonist, given into the lateral ventricles (ICV) or the substantia nigra, on locomotor activity were studied in male rats habituated to the experimental conditions (in order to avoid the anxiety status induced by novelty). Moreover, the presence of nigral oxytocinergic fibres and their localization with respect to nigral neurons immunoreactive for tyrosine hydroxylase (TH) (a marker of dopaminergic neurons) was investigated by immunohistochemistry. Finally, the effect of oxytocin-saporin (OXY-SAP), a recently discovered neurotoxin that specifically destroys neurons presenting oxytocinergic receptors on their surface (Baskin et al., 2010), injected bilaterally into the substantia nigra, on spontaneous locomotor activity was studied. Together, the results of the above experiments with oxytocin, $d(CH_2)_5Tyr(Me)^2$-Orn^8-vasotocin and with OXY-SAP, which revealed the existence of a correlation between the changes in locomotor activity found in OXY-SAP-treated rats and the extent of the changes in nigral TH and vesicular glutamate transporters (VGluT1, VGluT2 and VGluT3) immunoreactivity (IR) measured at 28 days after OXY-SAP, provide support for a modulatory role of oxytocin on locomotor activity at the level of the substantia nigra.

Materials and methods

Animals

Male Sprague Dawley rats (250–300 g at the beginning of the experiments) were obtained from Harlan Nossan (Correzzana, Italy). Animals were kept 4 per cage (38 cm × 60 cm × 20 cm) and were acclimated to the housing facilities of the Department of Biomedical Sciences of the University of Cagliari for at least 10 days before the beginning of the experiments under controlled environmental conditions (24 °C, 60% humidity, 12 h light/dark cycle, with lights on from 08:00 to 20:00 h) and with water and standard laboratory food ad libitum. The experiments were performed between 10:00–14:00 h accordingly to the guidelines of the European Communities Directive of September 22, 2010 (2010/63/EU) and the Italian Legislation (D.L. March 4, 2014, n. 26), and approved by the Ethical Committee for Animal Experimentation of the University of Cagliari.

Drugs and peptides

Oxytocin was purchased from Sigma-Aldrich (S. Louis, MO, USA); $d(CH_2)_5Tyr(Me)^2$-Orn^8-vasotocin from Bachem AG (Bubendorf, CH). Oxytocin-saporin coniugated (OXY-SAP) and Blank-saporin coniugated (BLANK-SAP) from Advanced Targeting Systems (Bemmel, The Netherlands). All other reagents were from available commercial sources.

Systemic treatments

Oxytocin (0.5, 50 and 2000 μg) or saline alone was given to rats IP in a volume of 3 mL/kg of rat body weight.

Microinjections into the substantia nigra and into the lateral ventricles

For microinjections into the substantia nigra or ICV, stainless-steel chronic guide cannulas (22 gauge) aimed bilaterally at the substantia nigra (coordinates: 5.3 mm posterior, 2.0 mm lateral and 2.0 mm ventral to dura), or unilaterally at the lateral ventricle (coordinates: 1.0 mm posterior, 1.5 mm lateral and 2.0 mm ventral to dura) (Paxinos and Watson, 2007) were stereotaxically implanted (Stoelting Co., Wood Dale, IL, USA) in the skull of male rats under isoflurane anaesthesia (1.5–2%). Rats were given one week to recover from surgery.

Oxytocin dissolved in saline was injected bilaterally (50–200 ng/site) into the substantia nigra in a volume of 0.3 μL/site. When $d(CH_2)_5Tyr(Me)^2$-Orn^8-vasotocin was given in combination with oxytocin, it was dissolved in saline and given ICV (2 μg/rat) in a volume of 10 μL 10 min before IP oxytocin (2000 μg/kg) (see above). When $d(CH_2)_5Tyr(Me)^2$-Orn^8-vasotocin was given alone, ICV or bilaterally into the substantia nigra, the compound was dissolved in saline and given ICV at the dose of 10 μg/rat in a volume of 10 μL, or into the substantia nigra at the dose of 2 μg/site in a volume of 0.3 μL/site. Microinjections were performed via an internal cannula (28 gauge), which extended 6.0 mm and 1.5 mm below the tip of the guide cannula for substantia nigra and ICV, respectively (Paxinos and Watson, 2007), and connected by polyethylene tubing to a 10-μL Hamilton syringe driven by a CMA/100 microinfusion pump (Harvard Apparatus, Holliston, MA, U.S.A.). Injections were performed in two min at constant flow rate and after injection the tip of the cannula was left in the injection site for 30 s to allow the spreading of the injected solution.

Lesioning procedure of the substantia nigra with Oxytocin-saporin (OXY-SAP)

The day after a prior assessment of spontaneous locomotor activity (see below), rats were anesthetized with isoflurane (1.5–2%), positioned in a stereotaxic frame (Stoelting Co., Wood Dale, IL, USA) and randomly injected bilaterally with 0.3 μL of OXY-SAP (60 ng/μL/site), or with the same amount of BLANK-SAP (60 ng/μL/site) or with vehicle (0.3 μL/site of phosphate-buffered saline - PBS, pH 7.4). Compounds were infused bilaterally in the substantia nigra (coordinates: 5.3 mm posterior, 2.0 mm lateral and 8.0 mm ventral to dura) (Paxinos and Watson, 2007) with a 28-gauge Hamilton syringe over a period of 3 min (100 nL/min) per site. The needle was left in place for an additional 2 min per site to allow optimal spreading of the neurotoxin. After treatment, rats were weighed and monitored daily for general health conditions. Spontaneous locomotor activity was assessed in each rat other two times, at 14 and 28 days after the injections into the substantia nigra (see below).

Locomotor activity

Before the beginning of the experiments, rats were daily handled for at least one week to avoid stress due to manipulation during the experimental sessions. At the end of this period, each rat underwent one habituation session that lasted for 2 h in order to prevent the influence of novelty factors linked to the experimental procedure and motility apparatus during the experimental sessions. Rats were individually tested for motor activity under standardized environmental conditions (in a soundproof room with a light level of 30 lx) with a Digiscan Animal Activity Analyzer (Omnitech Electronics, Columbus, Ohio). Each cage

(42 cm × 42 cm × 63 cm) had two sets of 16 photocells located at right angles to each other, projecting horizontal infrared beams 2.5 cm apart and 2 cm above the cage floor and a further set of 16 horizontal beams whose height was adapted to the size of the animals (20 cm). Horizontal and vertical activities were measured as total number of sequential infrared beam breaks (counts) in the horizontal or vertical sensors, recorded every 5 min, beginning immediately after placing the animals into the cage, over a period of 30–60 min depending on the experiment. In those experiments in which oxytocin, oxytocin antagonist or saline was injected directly into the substantia nigra rats were put into the motility apparatus 10 min after treatment and recording of locomotor activity lasted for 60 min; in those experiments in which oxytocin or saline was given IP (or oxytocin antagonist was given ICV in combination with oxytocin or saline given IP) rats were put into the motility apparatus 30 min after IP treatment, and recording of locomotor activity lasted for 30 min. In those experiments in which locomotor activity was assessed in substantia nigra OXY-SAP, BLANK-SAP or PBS-injected rats, the animals were moved from their home cage to the motility apparatus and recording lasted for 30 min.

Histology

In those experiments in which microinjections into the substantia nigra were performed, rats were sacrificed by decapitation immediately after the experiments, the brains immediately removed and stored in 4% aqueous formaldehyde for 12–15 days. Forty μm coronal brain sections were then prepared by means of a freezing microtome, stained with Neutral Red and inspected on a phase contrast microscope. The position of the tip of the microinjection cannulas in the substantia nigra was localized by following the track of both microinjection cannulas through a series of brain sections (see Fig. 1). In those experiments in which ICV microinjections were performed, rats were ICV microinjected with 10 μL of methylene blue immediately after the experiments, and then sacrificed by decapitation, the brains immediately removed and visually inspected in order to ascertain the position of the tip of the injection cannula into the lateral ventricle. Only those animals found to have the tip of the injection cannula located correctly into the two substantiae nigrae or ICV were considered for the statistical evaluation of the results.

In those experiments in which rats injected with OXY-SAP, BLANK-SAP or PBS were used, the animals underwent transcardial perfusion immediately after the experiments, the brains were immediately removed, washed in PBS and stored as described below in the Immunohistochemistry section. The position of the tip of the needle of the Hamilton syringe into the two substantiae nigrae was verified as described above for rats receiving substantia nigra microinjections by following the track of both microinjections through a few midbrain sections collected for immunoistochemistry (see above). Only those animals found to have the tip of the injection needle and the lesions located correctly into the two substantiae nigrae were considered for the statistical evaluation of the results.

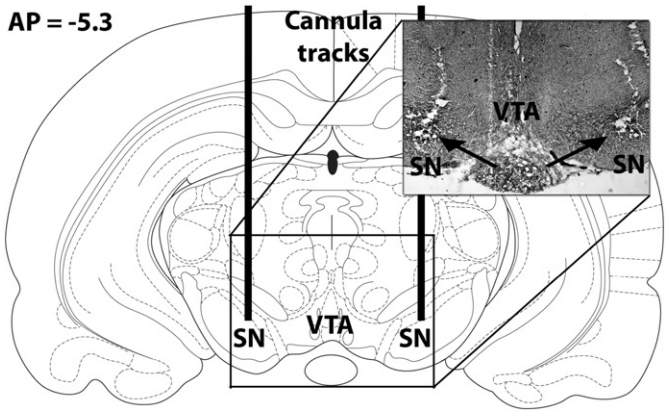

Fig. 1. Schematic representation of a coronal section of the rat brain showing the track of the microinjection cannulas directed to the substantia nigra. The portion of the neutral red-stained section showing the tips of the two microinjection cannulas in the substantia nigra (marked by the black arrows) of a rat bilaterally injected with oxytocin is magnified in the insert. Abbreviations: SN = substantia nigra, VTA = ventral tegmental area.

Immunohistochemistry

Animals were deeply anesthetized with chloral hydrate (400 mg/kg i.p.) and transcardially perfused-fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2–7.4. Brains were rapidly removed; washed overnight in PBS containing 7% sucrose and 0.01% NaN_3, and orientated in aluminium foil moulds in cryoembedding medium (in g/L: polyvinyl alcohol, 80; polyethylene glycol, 42.6; Tween-20, 10; and NaN_3, 0.5) (Cocco et al., 2003), and frozen in melting freon (cooled with liquid nitrogen). Coronal cryosections (10 μm) comprehensive of the whole substantia nigra obtained from the midbrain (starting from section with AP ≈ −6.5 up to section with AP ≈ −4.5) (Paxinos and Watson, 2007), were collected onto poly-L-lysine-coated slides and stored in the vapour phase of a liquid nitrogen tank until used.

For immunohistochemistry aimed at investigating the distribution of oxytocinergic fibres in the substantia nigra, sections prepared as reported above obtained from 6 intact rats, were brought to room temperature and washed in Triton X-100 (1 mL/L, in distilled water). Adjacent serial sections (10 μm) of the substantia nigra were then double-labeled with polyclonal guinea-pig anti-oxytocin (Abcam, Cambridge, UK, ab51637; 1:400), and polyclonal chicken tyrosine hydroxylase (TH) (Millipore, Darmstadt, Germany, ab9702; 1:1500), followed by the incubation with the corresponding species-specific secondary antibodies conjugated with Cy3 or Alexa-488 (Jackson Immunoresearch Laboratories, West Grove, PA).

Similarly, adjacent serial sections (10 μm) prepared as above, within ±300 μm of the injection site, obtained from rats that received bilateral injections into the substantia nigra (e.g., PBS, BLANK-SAP or OXY-SAP, 6 rats per group), were selected and alternatively used for single immunofluorescence labeling with polyclonal chicken TH (Millipore, Darmstadt, Germany) or monoclonal mouse anti-glutamic acid decarboxylase (GAD) (DSHB, Iowa City, Iowa, USA, GAD-6-c; 1:1500) or for double immunofluorescence with polyclonal chicken anti-TH (Millipore, Darmstadt, Germany) and a monoclonal mouse anti-vesicular glutamate transporter (VGluT) directed against one of the three isoforms of the vesicular glutamate transporter (VGluT1, VGluT2 or VGluT3, respectively) (NeuroMAB, Davis, CA, USA, 75-066, 75-067, 75-073; 1:300), followed by the incubation with the corresponding Cy3- or Alexa-488-coniugated species-specific secondary antibodies. Primary and secondary antibodies were routinely diluted in PBS containing 30 mL/L of normal donkey serum, 30 mL/L of normal rat serum and 0.02 g/L NaN_3, in order to prevent the non specific binding. Sections were finally washed with PBS, coverslipped with PBS-glycerol and visualized using an Olympus BX41/BX51 fluorescence microscope (Milan, Italy), equipped with a Fuji FinePix S2 and S3 Pro digital camera (Fujifilm, Milan, Italy). Controls included negative controls (replacement of the primary antibodies by antibody diluent alone) and cross-reactivity controls (each primary antibody used alone, followed by each of the appropriate and inappropriate secondary antibody conjugates, in turn). In order to obtain a semi-quantitative determination of the immunofluorescent signal, the FIJI image processing package, based on ImageJ (NIH) was used. Briefly, 4 animals per group (PBS-, BLANK-SAP- or OXY-SAP-treated) were randomly selected and 3 sections per animal within ±300 μm of the injection site were immunostained with each primary antibody (anti-TH, GAD, VGluT1, VGluT2 or VGluT3; minimum distance of at least 50 μm between sections immunostained with the same primary antibody). For each section, images

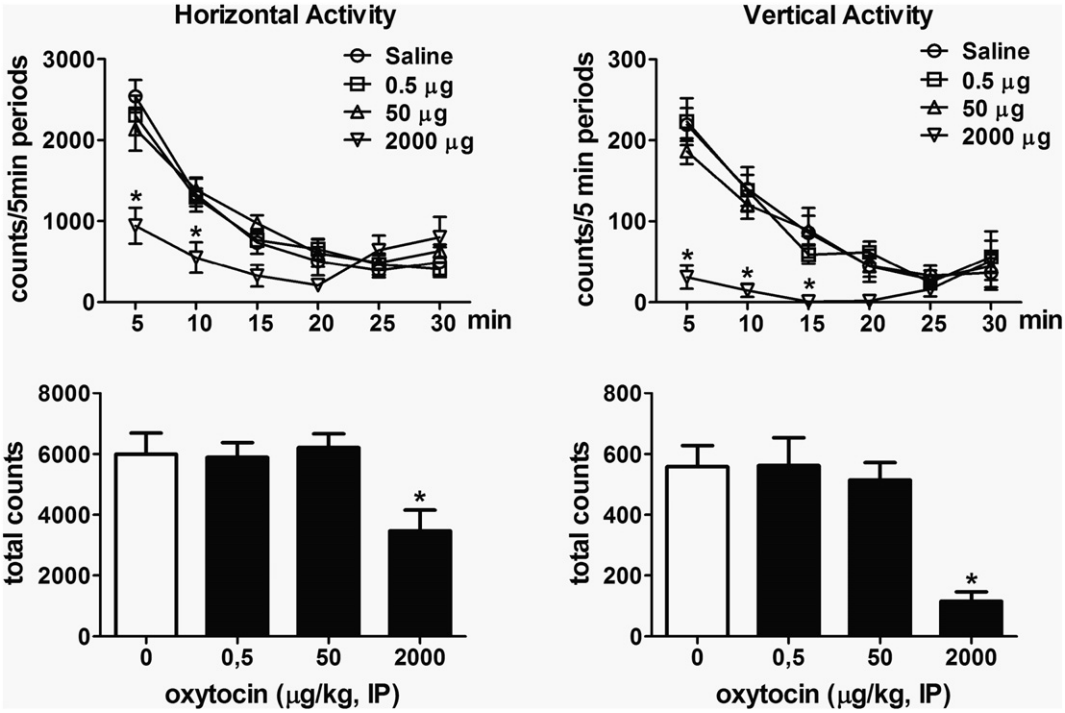

Fig. 2. Horizontal and vertical locomotor activity of male rats treated with IP oxytocin: dose-response curves. Rats treated with oxytocin (0.5, 50 and 2000 μg/kg) were put individually inside the apparatus 30 min after treatment and locomotor activity recorded for 30 min (6 consecutive periods of 5 min) as described in the Materials and methods section. Values are means ± SEM of the counts of 6 consecutive 5 min periods of 6 rats per group or of the total counts of the entire experiment. *P < 0.05, with respect to saline-treated rats (one- or two-way ANOVAs followed by Bonferroni's post hoc tests).

from one side only (left or right) were acquired with a S3 Pro digital camera (magnification: 10× for TH and GAD immunostaining; 20× for VGluTs immunostaining) in order to cover two rectangular areas, one located medially and the other laterally to a line which allows the division of the substantia nigra in two equivalent parts, each one comprehensive of the whole pars compacta and reticulata. For each rectangular area, the RGB TIFF images were first converted to 8-bit grayscale, then the boundaries of both pars compacta and reticulata where manually traced by the user and, finally, background fluorescent signal was removed by a manual thresholding process. For each section the mean density was calculated as the sum of the absolute intensities divided by the sum of the areas of the medial and lateral pars compacta or reticulata. The values of the three sections where then summed and the means calculated to obtain pars compacta and reticulata density values for each animal. The means ± SEM of density values for TH, GAD, VGluT1, VGluT2 and VGluT3 were then calculated for each group of experimental animals (PBS-, BLANK-SAP- and OXY-SAP-treated rats), and changes among groups reported as percent of PBS-treated rats (PBS IR = 100%).

Statistics

Locomotor activity data are presented either as the horizontal and vertical counts measured in recordings of consecutive 5 min periods along the experiment and as the cumulative counts of the same data in the 30 min (or 60 min) recordings of the entire experiment. These data were analyzed by one-way ANOVAs along the general factor "dose" or "treatment" (depending on the experiment) as between subjects factor, or by two-way ANOVAs with the "dose" or "treatment" (depending on the experiment) as between subjects factor and the general factor "time" (i.e., "5 min periods" or "treatment time") as within subjects factor.

Statistical analyses of mean density IR values for pars compacta and reticulata nigral TH, GAD, VGluT1, VGluT2 and VGluT3 IR among groups (PBS, BLANK-SAP and OXY-SAP) were carried out by two-way ANOVAs performed separately for each of the targets investigated (TH, GAD, VGluT1, VGluT2 and VGluT3 IR) with the treatment (PBS, BLANK-SAP or OXY-SAP) as between subjects factor and the sub-portion of the substantia nigra (compacta or reticulata) as within subjects factor.

F values and associated P, degrees of freedom and eta squared (η^2) values were reported for each of the ANOVA analyses done. To facilitate reading, η^2 were transformed in percentages of the total variance, considered as 100%. When ANOVAs revealed statistically significant main effects and/or interactions, post hoc pair wise comparisons were performed by using Bonferroni's test. In addition, size effect estimates (Cohen's *d* for any significant pair wise comparisons) were also reported in the Results section where appropriate.

ANOVAs and the associated Bonferroni's post-hoc comparisons were carried out by using PRISM, Graph Pad 5 Software (San Diego, CA, USA), with the significance level set at P < 0.05. Cohen's *d* were calculated with a free online effect size calculator (http://www.campbellcollaboration.org/escalc/htl/EffectSizeCalculator-SMD1.php).

Results

Effect of IP oxytocin on locomotor activity: dose-response curves and reversal by $d(CH_2)_5Tyr(Me)^2$-Orn^8-vasotocin

As shown in Fig. 2, IP oxytocin (0.5, 50 and 2000 μg/kg) reduced dose-dependently both horizontal and vertical locomotor activity in male Sprague Dawley rats. The effect was observed at the dose of 2000 μg/kg but not at the dose of 0.5 μg/kg or 50 μg/kg, was more pronounced in the first 15 min of the test and tended to disappear in the second 15 min, with motility scores becoming similar to those of vehicle-treated rats. Accordingly, one-way ANOVAs analyses of horizontal and vertical total counts revealed a significant effect of treatment confirmed by repeated measures two-way ANOVAs analyses of 5 min periods counts [$F(3,100) = 4.79$, $P < 0.05$, $\eta^2 = 6.70$, and 10.65,

$P < 0.001$, $\eta^2 = 16.88$], which revealed also a significant effect of periods [$F(5,100) = 66.21$, $P < 0.001$, $\eta^2 = 54.52$, and 42.57, $P < 0.001$, $\eta^2 = 39.74$] and a significant treatment x periods interaction [$F(15,100) = 5.25$, $P < 0.001$, $\eta^2 = 12.98$, and 5.04, $P < 0.001$, $\eta^2 = 14.13$] for both horizontal and vertical activity, respectively. Moreover, post hoc comparisons revealed highly significant differences in both horizontal and vertical locomotor activity between rats treated with 2000 μg/kg oxytocin and saline-treated rats in the first three 5 min periods [horizontal activity: 0–5 min, $P < 0.001$, $d = -3.08$, 5–10 min, $P < 0.01$, $d = -1.58$; vertical activity: 0–5 min, $P < 0.001$, $d = -4.43$, 5–10 min, $P < 0.001$, $d = -2.53$, 10–15 min, $P < 0.01$, $d = -1.62$]. This difference was still significant when considering the total counts of the entire experiment [totals, $P < 0.05$, $d = -1.49$, and $P < 0.001$, $d = -3.38$, for horizontal and vertical activity, respectively] (see Fig. 2 for single points of statistical significance).

As shown in Fig. 3, the inhibitory effect on both horizontal and vertical locomotor activity induced by oxytocin given IP at the dose of 2000 μg/kg was completely abolished by the prior treatment of the rats with the oxytocin receptor antagonist d$(CH_2)_5$Tyr(Me)^2-Orn^8-vasotocin (2 μg/rat ICV 10 min before IP oxytocin). This dose of the oxytocin receptor antagonist was ineffective on basal motor activity levels. Accordingly, one-way ANOVAs analyses of horizontal and vertical total counts revealed a significant effect of treatment confirmed by repeated measures two-way ANOVAs analyses of the 5 min periods counts ($F(3,100) = 5.19$, $P < 0.01$, $\eta^2 = 5.71$, and 7.71, $P < 0.01$, $\eta^2 = 12.33$], which revealed also a significant effect of periods ($F(5,100) = 70.41$, $P < 0.001$, $\eta^2 = 54.50$, and 19.49, $P < 0.001$, $\eta^2 = 31.22$] and a significant treatment x periods interaction ($F(15,100) = 7.32$, $P < 0.001$, $\eta^2 = 17.00$, and 2.86, $P < 0.001$, $\eta^2 = 13.77$] for both horizontal and vertical activity. Finally, post hoc comparisons revealed significant differences in both horizontal and vertical locomotor activity between ICV oxytocin antagonist + IP oxytocin- and ICV saline + IP oxytocin-treated rats (periods, horizontal activity: 0–5 min, $P < 0.001$, $d = 2.64$, 5–10 min, $P < 0.05$, $d = 1.39$, 10–15 min, $P < 0.05$, $d = 1.83$; vertical activity: 0–5 min, $P < 0.001$, $d = 3.94$, 5–10 min, $P < 0.01$, $d = 1.96$, 10–15 min, $P < 0.05$, $d = 1.22$; totals, $P < 0.05$, $d = 1.76$, and $P < 0.01$, $d = 3.52$, for horizontal and vertical locomotor activity, respectively) while no significant differences were found between ICV oxytocin antagonist + IP oxytocin or IP saline-treated rats and ICV saline + IP saline-treated rats (periods, totals, all $P > 0.05$) (see Fig. 3 for single points of statistical significance).

Effect of oxytocin given bilaterally into the substantia nigra on locomotor activity: dose-response curves

As shown in Fig. 4 oxytocin dose-dependently reduced both horizontal and vertical locomotor activity when injected bilaterally (50, 100 and 200 ng/injection site) into the substantia nigra of male Sprague Dawley rats. The effect was immediately evident and lasted for the first 25–30 min. After this period, the reduction of activity in vehicle-treated rats led the different groups of rats to show very similar motility scores, thus making impossible to estimate differences between them. Accordingly, two-way ANOVAs analyses of horizontal and vertical cumulative counts and of 5 min periods counts revealed a significant effect of treatment [$F(3,220) = 3.93$, $P < 0.05$, $\eta^2 = 4.17$, and 9.82, $P < 0.001$, $\eta^2 = 12.92$] and time [periods, $F(11,220) = 101.2$, $P < 0.001$, $\eta^2 = 65.77$, and 25.82, $P < 0.001$, $\eta^2 = 31.65$; totals: $F(2,40) = 188.6$, $\eta^2 = 48.50$, and 57.7, $P < 0.001$, $\eta^2 = 18.12$] and a significant treatment × time interaction [periods, $F(33,220) = 5.11$, $P < 0.001$, $\eta^2 = 9.98$, and 6.02, $P < 0.001$, $\eta^2 = 22.16$; totals: $F(6,40) = 9.94$, $P < 0.001$, $\eta^2 = 7.67$, and 15.69, $P < 0.001$, $\eta^2 = 14.77$] for both horizontal and vertical activity. Post hoc tests revealed that rats treated with oxytocin at the doses of 100 and 200 ng had highly significant different values in horizontal and

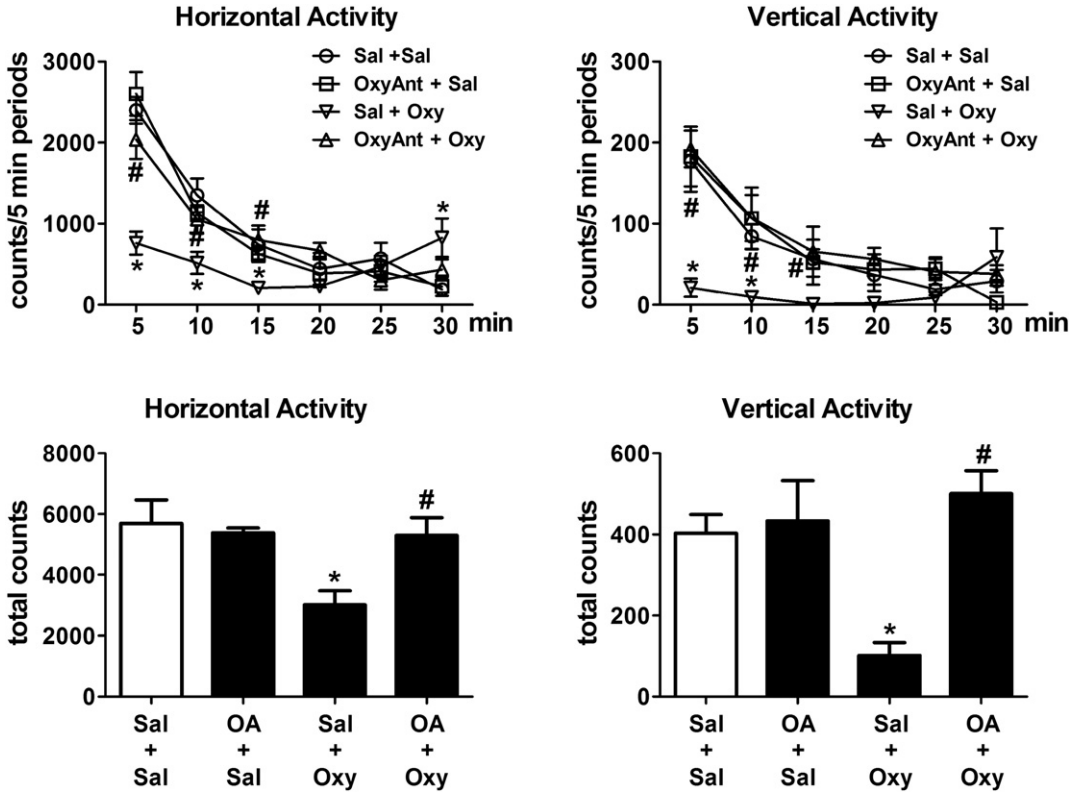

Fig. 3. Reversal by d$(CH_2)_5$Tyr(Me)^2-Orn^8-vasotocin (OA) of the inhibition of horizontal and vertical locomotor activity induced by IP oxytocin. Rats were treated first with ICV OA (2 μg) or saline (10 μL) and 10 min later with IP oxytocin (2000 μg/kg) or saline. After 30 min rats were put individually in the apparatus and locomotor activity recorded for 30 min as described in the legend of Fig. 2. Values are means ± SEM of the counts of 6 consecutive 5 min periods of 6 rats per group or of the total counts of the entire experiment. *$P < 0.05$, with respect to saline-treated rats; #$P < 0.05$, ICV OA + IP Oxy with respect to ICV saline + IP Oxy (one- or two-way ANOVAs followed by Bonferroni's post hoc tests).

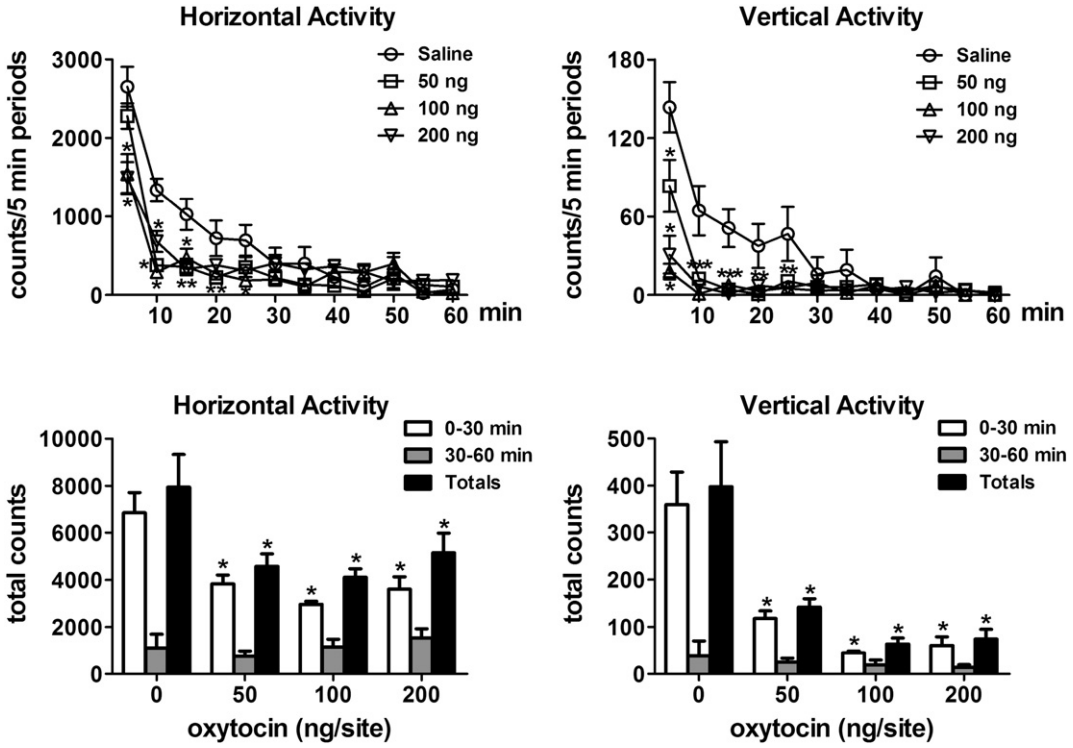

Fig. 4. Horizontal and vertical locomotor activity of rats after bilateral injection of oxytocin into the substantia nigra: dose-response curves. Rats were bilaterally injected into the substantia nigra with oxytocin (50–200 ng/site) or saline (0.3 μL/site) and after 10 min put individually in the apparatus and locomotor activity recorded for 60 min (12 consecutive periods of 5 min) as described in the legend of Fig. 2. Values are means ± SEM of the counts of 12 consecutive 5 min periods of 6 rats per group or of the counts of the first and second 30 min periods and of the entire experiment. *P < 0.05, with respect to saline-treated rats (two-way ANOVAs followed by Bonferroni's post hoc test).

vertical locomotor activity when compared to saline-treated rats along all 5 min periods in the first 20–25 min of the test [periods, saline vs 100 ng, horizontal activity: 0–5 min, $P < 0.001$, $d = -1.79$; 5–10 min, $P < 0.001$, $d = -3.80$; 10–15 min, $P < 0.05$, $d = -1.38$; 15–20 min, $P < 0.05$, $d = -1.12$; 20–25 min, $P < 0.05$, $d = -1.43$; vertical activity: 0–5 min, $P < 0.001$, $d = -3.67$; 5–10 min, $P < 0.001$, $d = -1.93$; 10–15 min, $P < 0.01$, $d = -1.76$; 15–20 min, $P < 0.05$, $d = -1.16$; 20–25 min, $P < 0.01$, $d = -1.15$; saline vs 200 ng, horizontal activity: 0–5 min, $P < 0.001$, $d = -2.09$; 5–10 min, $P < 0.01$, $d = -1.91$; 10–15 min, $P < 0.001$, $d = -1.89$; vertical activity: 0–5 min, $P < 0.001$, $d = -2.76$; 5–10 min, $P < 0.001$, $d = -1.76$; 10–15 min, $P < 0.001$, $d = -2.01$; 20–25 min, $P < 0.01$, $d = -1.15$]. However, oxytocin inhibited both horizontal and vertical locomotor activity already at the dose of 50 ng/injection site/rat (totals, 50 ng vs saline: $P < 0.01$, $d = -1.32$, and $P < 0.001$, $d = -1.53$, for horizontal and vertical activity, respectively], although the inhibitory effect of 50 ng of oxytocin on horizontal activity, at variance from the doses of 100 and 200 ng, was detectable only 10 min after the beginning of the test [periods, 5–10 min, $P < 0.001$, $d = -3.45$]. Nonetheless, this dose showed an efficacy similar to that of the higher ones in inhibiting total horizontal activity in the first 30 min (totals, all $P > 0.05$ when comparing the three doses tested). Finally, no significant differences were observed among the 4 treatment groups in the values of both horizontal and vertical locomotor activity of the second 30 min (totals, all $P > 0.05$) (see Fig. 4 for single points of statistical significance).

Effect of $d(CH_2)_5Tyr(Me)^2$-Orn^8-vasotocin given bilaterally into the substantia nigra on locomotor activity

As shown in Fig. 5, $d(CH_2)_5Tyr(Me)^2$-Orn^8-vasotocin injected bilaterally into the substantia nigra at the dose of 2 μg/injection site increased both horizontal and vertical locomotor activity by about 50% and 75% respectively, when compared to saline-treated rats. Similar results were found when the compound was injected ICV at the dose of 10 μg. In both experimental conditions, the effect was immediately evident and significant in the first 5–10 min, thereafter disappearing along the test; however, this difference was still significant when comparing the total counts values of the entire experiment. Accordingly, one-way ANOVAs analyses of horizontal and vertical total counts revealed a significant effect of treatment confirmed by repeated measures two-way ANOVAs analyses of the 5 min periods counts [$F(3,100) = 3.80$, $P < 0.05$, $\eta^2 = 6.28$ and 5.00, $P < 0.01$, $\eta^2 = 7.67$] which revealed also a significant effect of periods [$F(5,100) = 59.78$, $P < 0.001$, $\eta^2 = 60.91$, and 52.36, $P < 0.001$, $\eta^2 = 57.50$] for both horizontal and vertical activity. Moreover, post hoc comparisons revealed that rats treated ICV with $d(CH_2)_5Tyr(Me)^2$-Orn^8-vasotocin did not differ from those injected with the antagonist directly into the substantia nigra (all $P > 0.05$) (see Fig. 5 for single points of statistical significance).

Effect of Oxytocin-saporin (OXY-SAP) injected bilaterally into the substantia nigra on locomotor activity: comparison with BLANK-SAP and PBS

As shown in Fig. 6, Sprague Dawley male rats bilaterally injected into the substantia nigra with OXY-SAP (0.3 μL/site, 60 ng/μL) showed a higher horizontal (by about 50%) and vertical locomotor activity compared to BLANK-SAP- (0.3 μL/site, 60 ng/μL) and PBS- (0.3 μL/site) injected animals when tested for locomotor activity 28 days after the injection [two-way ANOVAs of 5 min periods data, horizontal activity: treatment, $F(2,75) = 16.30$, $P < 0.001$, $\eta^2 = 16.73$, periods, $F(5,75) = 72.94$, $P < 0.001$, $\eta^2 = 60.25$, treatment x periods, $F(10,75) = 1.77$, $P > 0.05$, $\eta^2 = 2.93$; vertical activity, treatment, $F(2,75) = 2.84$, $P > 0.05$, $\eta^2 = 6.79$, periods, $F(5,75) = 32.21$, $P < 0.001$, $\eta^2 = 47.07$, treatment x periods, $F(10,75) = 2.16$, $P < 0.05$, $\eta^2 = 6.33$]. Augmented horizontal, but not vertical, locomotor activity values were also found in OXY-SAP-injected rats 28 days after treatment when compared to the

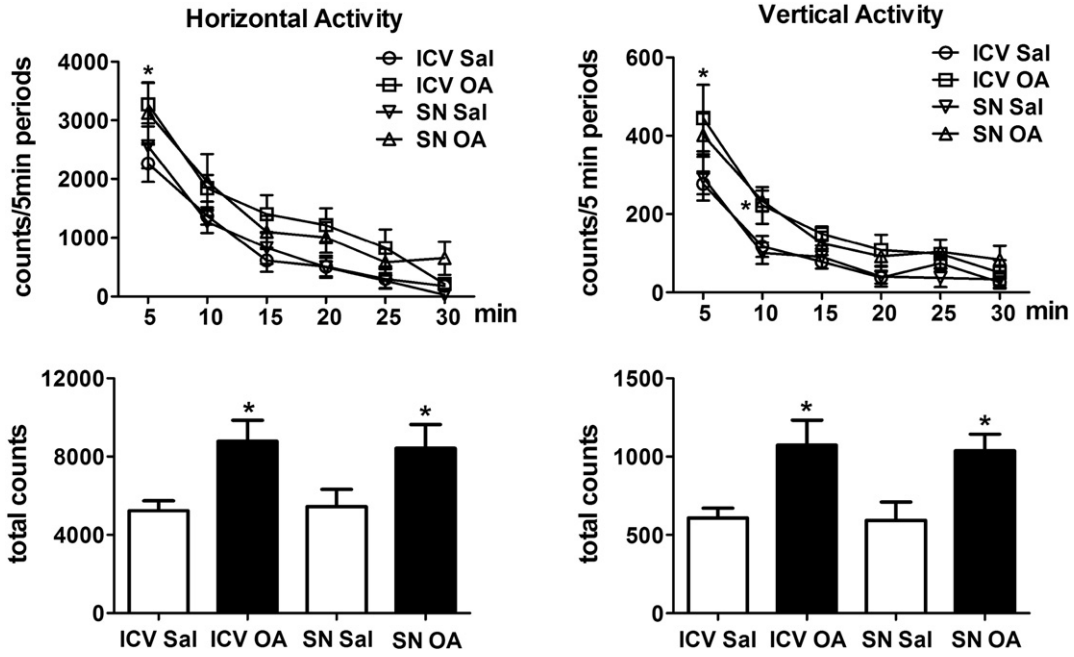

Fig. 5. Horizontal and vertical locomotor activity of rats treated with $d(CH_2)_5Tyr(Me)^2$-Orn^8-vasotocin (OA) injected ICV or bilaterally into the substantia nigra. Rats were injected with OA (10 μg) or saline (10 μL) ICV or bilaterally into the substantia nigra (2 μg/site) or saline (0.3 μL/site). After 10 min rats were put individually in the apparatus and locomotor activity recorded for 30 min (6 consecutive fractions of 5 min) as described in the legend of Fig. 2. Values are means ± SEM of the counts of 6 consecutive 5 min periods of 6 rats per group or of the counts of the entire experiment. *P < 0.05, with respect to saline-treated rats (one- or two-way ANOVAs followed by Bonferroni's post hoc tests).

values obtained in the tests done with these rats before (or 14 days after) the OXY-SAP injection [two-way ANOVAs of 5 min periods data, horizontal activity, treatment time, $F(2,75) = 7.67$, $P < 0.01$, $\eta^2 = 9.71$, periods $F(5,75) = 51.48$, $P < 0.001$, $\eta^2 = 59.98$, treatment time x periods, $F(10,75) = 1.43$, $P > 0.05$, $\eta^2 = 3.34$; vertical activity, treatment time, $F(2,75) = 1.59$, $P > 0.05$, $\eta^2 = 2.45$; periods, $F(5,75) = 21.51$, $P < 0.001$, $\eta^2 = 47.49$, treatment time x periods, $F(10,75) = 1.21$, $P > 0.05$, $\eta^2 = 5.36$].

The above results were further confirmed by two-way ANOVAs of the total motor activity data [horizontal activity, treatment, $F(2,30) = 2.21$, $P > 0.05$, $\eta^2 = 13.16$, treatment time, $F(2,30) = 1.14$, $P > 0.05$, $\eta^2 = 1.80$, treatment × treatment time, $F(4,30) = 5.40$, $P < 0.01$, $\eta^2 = 16.96$; vertical activity, treatment, $F(2,30) = 0.71$, $P > 0.05$, $\eta^2 = 4.52$, treatment time, $F(2,30) = 6.20$, $P < 0.01$, $\eta^2 = 12.27$, treatment x treatment time, $F(4,30) = 1.60$, $P > 0.05$, $\eta^2 = 6.36$]. Accordingly, a higher horizontal locomotor activity was observed in OXY-SAP-injected rats compared to BLANK-SAP- and PBS-injected animals 28 days [OXY-SAP vs PBS, $P < 0.001$, $d = 2.99$; OXY-SAP vs BLANK-SAP, $P < 0.05$, $d = 3.54$], but not before or 14 days after the treatment [all $P > 0.05$], while no significant differences were observed in horizontal activity between BLANK-SAP- and PBS-injected animals before, 14 and 28 days after the treatment [all $P > 0.05$]. Post-hoc tests revealed also that BLANK-SAP and PBS-injected rats showed also a marked tendency of the values of vertical activity to decrease along the tests. Accordingly, in PBS-injected rats these values were found significantly lower at 28 days after treatment when compared with those obtained from the same group before treatment [$P < 0.05$, $d = -1.27$] and with those obtained at the same time from the OXY-SAP- but not BLANK-SAP-injected rats [$P < 0.05$, $d = 1.42$ and $P > 0.05$, respectively].

Immunohistochemistry

As expected, TH IR was found mainly in neuronal perykaria of the substantia nigra pars compacta and, to a lesser extent, in the pars reticulata (Fig. 7a, d). In the pars compacta, a diffuse network of oxytocin-immunoreactive fibres (Fig. 7b, e) approaching to and impinging onto TH immunoreactive perykaria and running close to TH immunoreactive dendrites as well, was also found (Fig. 7c, f). Twenty eight days after treatment, bilateral injections of OXY-SAP in the substantia nigra caused a marked decrease in the number of TH immunoreactive perykaria in the substantia nigra and in their dendrites coursing into the substantia nigra pars reticulata when compared to bilateral BLANK-SAP, which also caused in a few cases a very modest reduction (≈10%) in TH IR when compared to bilateral PBS injections, considered as controls (TH IR valu = 100%) (Fig. 8e, c, a, respectively). The reduction of TH IR in OXY-SAP-treated rats vs BLANK-SAP- and PBS-treated rats was found in the substantia nigra for about 300 μm around the injection sites in all the sections analyzed and was more evident in the pars reticulata (≈90%) than in the pars compacta (≈50%) of the substantia nigra [two-way ANOVA, treatment, $F(2,9) = 9.78$, $P < 0.01$, $\eta^2 = 20.89$, compacta/reticulata, $F(1,9) = 194.0$, $P < 0.001$, $\eta^2 = 65.43$, treatment x compacta/reticulata, $F(2,9) = 1.52$, $P > 0.05$, $\eta^2 = 1.03$; post hoc tests: compacta, OXY-SAP vs PBS, $P < 0.01$, $d = -2.12$; OXY-SAP vs BLANK-SAP, $P < 0.01$, $d = -2.11$; reticulata, OXY-SAP vs PBS, $P < 0.05$, $d = -3.78$; OXY-SAP vs BLANK-SAP, $P < 0.05$, $d = -4.13$].

In contrast, no change was found to occur in GAD IR in the entire susbtantia nigra (pars compacta and reticulata) of OXY-SAP treated rats when compared to BLANK-SAP- or PBS-treated control rats (Fig. 8f, d, b, respectively) [two-way ANOVA, treatment, $F(2,9) = 0.69$, $P > 0.05$, $\eta^2 = 1.75$, compacta/reticulata, $F(1,9) = 245.5$, $P < 0.001$, $\eta^2 = 82.62$, treatment x compacta/reticulata, $F(2,9) = 1.84$, $P > 0.05$, $\eta^2 = 1.24$; post hoc tests: all $P > 0.05$].

The decrease in TH IR found in OXY-SAP treated rats was parallel with a concomitant marked decrease in VGluT1 IR in the substantia nigra pars reticulata (≈75%) and in the substantia nigra pars compacta (≈73%) (Fig. 9h), and of both VGluT2 (Fig. 10h) and VGluT3 (Fig. 11h) (>90% for both VGluT2 and VGluT3 in pars compacta and ≈90% for VGluT2 and 70% for VGluT3 in pars reticulata) in either the substantia nigra pars compacta and reticulata, with minimal residual VGluT3 IR and almost no residual VGluT2 IR in both areas. The decrease in the

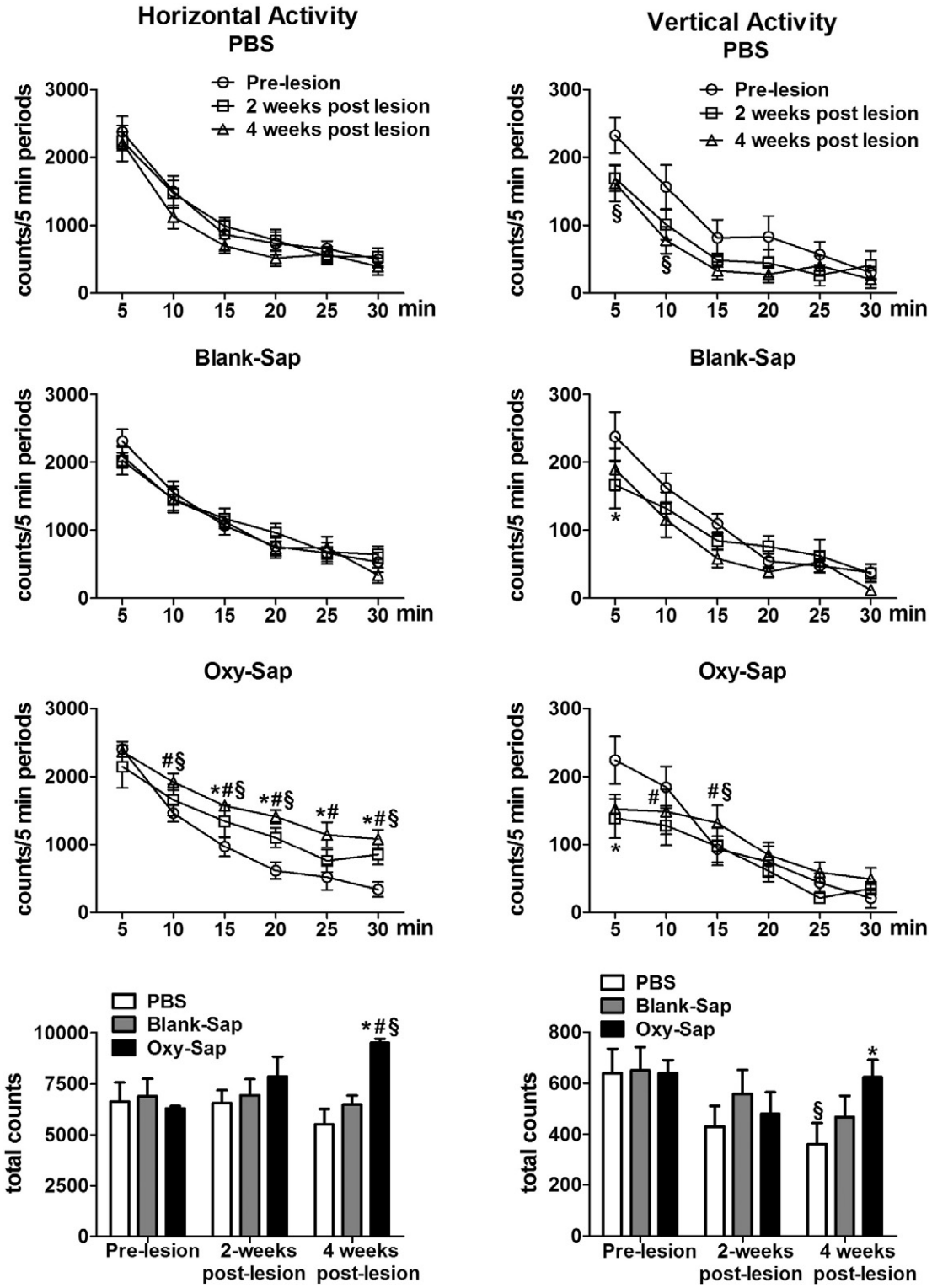

Fig. 6. Horizontal and vertical locomotor activity of rats bilaterally injected with Oxytocin-saporin (OXY-SAP) into the substantia nigra. Rats received OXY-SAP (0.3 μL/site, 60 ng/μL) or BLANK-SAP (0.3 μL/site, 60 ng/μL) or PBS (0.3 μL/site). Locomotor activity was assessed before (pre-lesion), 14 (two weeks) and 28 days after the treatment. Rats were put individually in the apparatus and locomotor activity recorded for 30 min (6 consecutive periods of 5 min). Values are means ± SEM of the counts of 6 consecutive 5 min periods of 6 rats per group or of the total counts of the entire experiment. *P < 0.05, with respect to the corresponding group of PBS-treated rats; #P < 0.05, with respect to the corresponding group of BLANK–SAP-injected rats; §P < 0.05, with respect the corresponding pre-lesion values (two-way ANOVAs followed by Bonferroni's post hoc tests).

VGluT1 IR found in OXY-SAP-treated rats was particularly evident when compared to BLANK-SAP–treated rats (Fig. 9e), in which only a very small decrease (≈15%) in this transporter was found in either substantia nigra pars compacta and reticulata when compared to PBS-treated rats (Fig. 9b), as found for TH IR [two-way ANOVA, VGluT1 IR, treatment, $F(2,9) = 6.77$, $P < 0.05$, $\eta^2 = 17.81$, compacta/reticulata, $F(1,9) = 55.89$, $P < 0.001$, $\eta^2 = 51.77$, treatment x compacta/reticulata, $F(2,9) = 5.52$, $P < 0.05$, $\eta^2 = 10.24$; post hoc tests: compacta, OXY-SAP vs PBS, $P < 0.001$, $d = -2.82$; OXY-SAP vs BLANK-SAP, $P < 0.01$, $d = -1.92$; reticulata, OXY-SAP vs PBS, $P < 0.05$, $d = -5.57$].

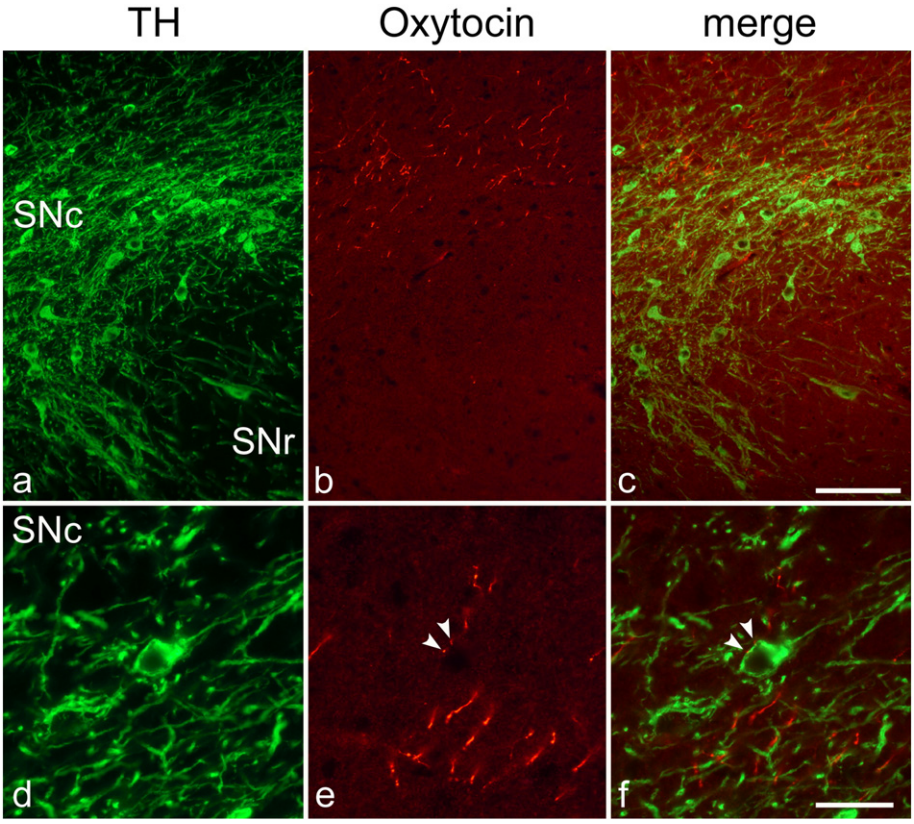

Fig. 7. Substantia Nigra (AP = −5.40 approximately, sections adjacent to the site used for microinjections) immunostained for Tyrosine Hydroxylase (TH, Alexa-488: green labeling) and oxytocin (Cy3: red labeling). Oxytocin fibres were observed almost exclusively in the Substantia Nigra pars compacta and were more abundant in its central portion (a, b and c). Fibres were mainly distributed dorsally and within the pars compacta, where they run close to dopaminergic dendrites and perikarya (d, e and f). e and f: magnification showing an oxytocinergic fibre impinging (arrowheads in e and f) on the perikaryon of a dopaminergic neuron of the pars compacta. SNc: substantia nigra pars compacta; SNr: substantia nigra pars reticulata. a, b and c, scale bar = 100 μm; d, e and f, scale bar = 25 μm.

However, at variance from VGluT1 IR, in BLANK-SAP-treated rats VGluT2 IR (Fig. 10e) and VGluT3 IR (Fig. 11e) were significantly reduced in both the substantia nigra pars compacta (both by ≈46%) and reticulata (VGluT2 by ≈39% and VGluT3 by ≈31%) when compared to PBS-treated rats (Fig. 10b and 11b, respectively) [two-way ANOVA, VGluT2 IR, treatment, $F(2,9) = 25.90$, $P < 0.001$, $\eta^2 = 50.12$, compacta/reticulata, $F(1,9) = 64.34$, $P < 0.001$, $\eta^2 = 24.0$, treatment x compacta/reticulata, $F(2,9) = 18.52$, $P < 0.001$, $\eta^2 = 13.82$; post hoc tests: compacta, OXY-SAP vs PBS, $P < 0.001$, $d = -6.49$, BLANK-SAP vs PBS, $P < 0.001$, $d = -2.02$, OXY-SAP vs BLANK-SAP, $P < 0.001$, $d = -2.64$; reticulata, OXY-SAP vs PBS, $P < 0.05$, $d = -5.59$; VGluT3 IR, treatment, $F(2,9) = 16.69$, $P < 0.001$, $\eta^2 = 46.82$, compacta/reticulata, $F(1,9) = 54.50$, $P < 0.001$, $\eta^2 = 20.72$, treatment x compacta/reticulata, $F(2,9) = 21.57$, $P < 0.001$, $\eta^2 = 16.41$; post hoc tests: compacta, OXY-SAP vs PBS, $P < 0.001$, $d = -4.86$, BLANK-SAP vs PBS, $P < 0.01$, $d = -1.78$, OXY-SAP vs BLANK-SAP, $P < 0.01$, $d = -2.57$; reticulata, OXY-SAP vs PBS, $P < 0.05$, $d = -4.09$].

Discussion

The present study confirms and extends the results of previous studies suggesting that oxytocin is involved in the control of locomotor activity (Klenerova et al., 2009; Uvnäs-Moberg et al.,1994, Maejima et al., 2015). In particular our results confirm that oxytocin decreases locomotor activity of male rats in a non-novelty paradigm when given IP at high doses (2 mg/kg), but not at doses up to 0.05 mg/kg, which were found ineffective when compared to vehicle-treated control rats. The failure of low doses of oxytocin given IP to influence motility is in contrast with the results of above studies, which reported that low doses of oxytocin similar to those used in this study given IP increased motility (Klenerova et al., 2009; Uvnäs-Moberg et al., 1994). This discrepancy may be due to the reduction of anxiety levels secondary to the use in the present study of a non-novelty experimental paradigm, which would reduce anxiety and thus the anxiolytic effect of oxytocin, apparently responsible for the increased locomotor activity induced by low doses of the neuropeptide in the above studies (see Introduction). Nonetheless, the inhibitory effect of high doses of oxytocin on locomotor activity is apparently mediated by the stimulation of central oxytocinergic receptors, being completely antagonized by the selective oxytocin receptor antagonist $d(CH_2)_5Tyr(Me)^2$-Orn^8-vasotocin given ICV (Bankowski et al., 1980) at a dose which was ineffective on locomotor activity. These results are also similar to those of a previous study showing that rats treated with oxytocin given IP at the dose of 0.1 and 1.0 mg/kg or ICV at the dose of 2 and 20 ng showed a reduced exploratory activity in the periphery of and shift their activity to the centre of the arena of the open field apparatus, and that the reduction in the exploratory behavior was antagonized by an oxytocin receptor antagonist injected either IP or ICV, at doses which per se did not induce any behavioral effect (Uvnäs-Moberg et al., 1992).

In agreement with a central effect of oxytocin in reducing locomotor activity, the present study also shows for the first time that oxytocin injected bilaterally into the substantia nigra reduced locomotor activity in a dose-dependent manner. The finding suggests that the substantia nigra may be one of the brain areas where systemic oxytocin acts to inhibit locomotor activity. In line with this hypothesis, the bilateral injection of $d(CH_2)_5Tyr(Me)^2$-Orn^8-vasotocin in the substantia nigra at the dose of 2 μg/site caused an increase of locomotor activity. Together the above findings suggest the existence of a tonic active inhibitory role of

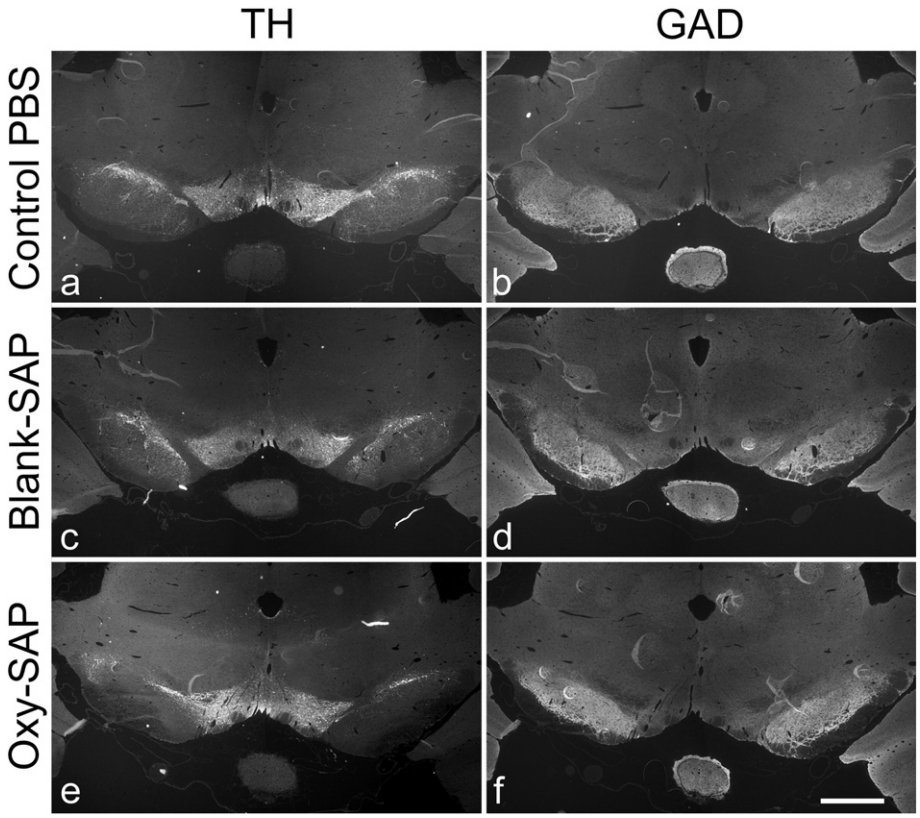

Fig. 8. Coronal sections of the Substantia Nigra (AP = −5.30 approximately, adjacent to the site used for microinjections) immunostained for Tyrosine Hydroxylase (TH) and Glutamic Acid Decarboxylase (GAD) of rats injected with PBS (a and b, respectively), BLANK-SAP (c and d, respectively) and OXY-SAP (e and f, respectively) at 28 days after treatment. OXY-SAP induced a reduction in TH IR both in the perikarya of the pars compacta and in their dendrites coursing into the pars reticulata whereas BLANK-SAP induced no TH IR reduction or only a modest reduction, mainly in the pars reticulata, when compared to PBS. No evident reduction in GAD IR was observed when comparing BLANK-SAP- and OXY-SAP- to PBS-injected animals (d, f and b respectively). Scale bar = 1 mm.

endogenous oxytocin on locomotor activity at the level of the substantia nigra.

As to the mechanism by means of which oxytocin decreases locomotor activity by acting at the level of the substantia nigra, one possibility is that oxytocin reduces the activity of nigrostriatal dopaminergic system. Such a reduction may be mediated by the stimulation of oxytocinergic receptors localized 1) in the cell bodies and dendrites of nigrostriatal dopaminergic neurons, or 2) in other neurons present in the substantia nigra that may interfere directly or indirectly with the activity of the nigrostriatal dopaminergic system. Accordingly, dopaminergic neurons originating in the substantia nigra pars compacta and projecting to striatal neurons, play a pivotal role in the initiation of movement and in its expression (see Björklund and Dunnett, 2007, and references therein)(Fig. 12). Indeed, 1) striatal neurons send inhibitory projections not only to the (internal) globus pallidus and the entopeduncular nucleus but also to the pars reticulata and compacta of the substantia nigra (Costall et al., 1972; Marshall and Ungerstedt, 1977; Hauber, 1998; Radnikow and Misgeld, 1998); and 2) the substantia nigra pars reticulata contains also cell bodies of neurons which project to different brain areas important for postural muscle tone and locomotion initiation and execution, i.e., the superior colliculus, the pedunculopontine nucleus and the thalamus. The striatal inhibitory projections to the globus pallidus and the substantia nigra pars reticulata are part of the so-called direct pathway, while those projecting to the entopeduncular nucleus (external globus pallidus in humans) are part of the so-called indirect pathway (for a review see Takakusaki et al., 2003 and Hauber, 1998) (see also Fig. 12).

In line with studies showing that oxytocin receptors and oxytocin receptor RNA messenger are present in the substantia nigra pars compacta (Loup et al., 1989, 1991; Vaccari et al., 1998) and with the possibility that oxytocin decreases locomotor activity by stimulating oxytocin receptors localized in the cell bodies and dendrites of nigrostriatal dopaminergic neurons, this study also shows that oxytocin immunoreactive fibres are diffuse in the substantia nigra pars compacta running close to and impinging often onto the cell bodies of TH immunoreactive (e.g., dopaminergic) neurons present in this brain area. However, the possibility that the decreased motility induced by oxytocin is secondary to a reduction of the activity of nigrostriatal dopaminergic neurons caused by the stimulation of oxytocinergic receptors localized in the cell bodies of these neurons, is unlikely. Indeed several lines of evidence show that oxytocin facilitates rather than inhibit the activity of dopaminergic neurons, for instance when injected into the ventral tegmental area, where the neuropeptide was found able to induce penile erection, an effect that occurred concomitantly to an increased dopamine release in the nucleus accumbens and in the medial prefrontal cortex (see Melis et al., 2007; Sanna et al., 2012). Thus, it appears more likely that the decrease in locomotor activity induced by intranigral oxytocin is mediated by other mechanisms rather than by the direct inhibition of nigrostriatal dopaminergic neurons. For instance, oxytocin may act on oxytocin receptors localized in other neurons present in the substantia nigra that may interfere directly or indirectly with the activity of the nigrostriatal dopaminergic system, i.e., by modulating the activity of excitatory amino acid (glutamatergic) synapses that activate nigrostriatal dopaminergic neurons, or of GABAergic synapses that inhibit glutamatergic synapses impinging on nigrostriatal dopaminergic neurons, in both cases through the stimulation of presynaptic oxytocinergic receptors localized in these synapses. Alternatively, oxytocin may directly facilitate the activity of glutamatergic synapses that impinge on GABAergic neurons projecting from the substantia nigra pars reticulata to the thalamus (and other nuclei of the brainstem,

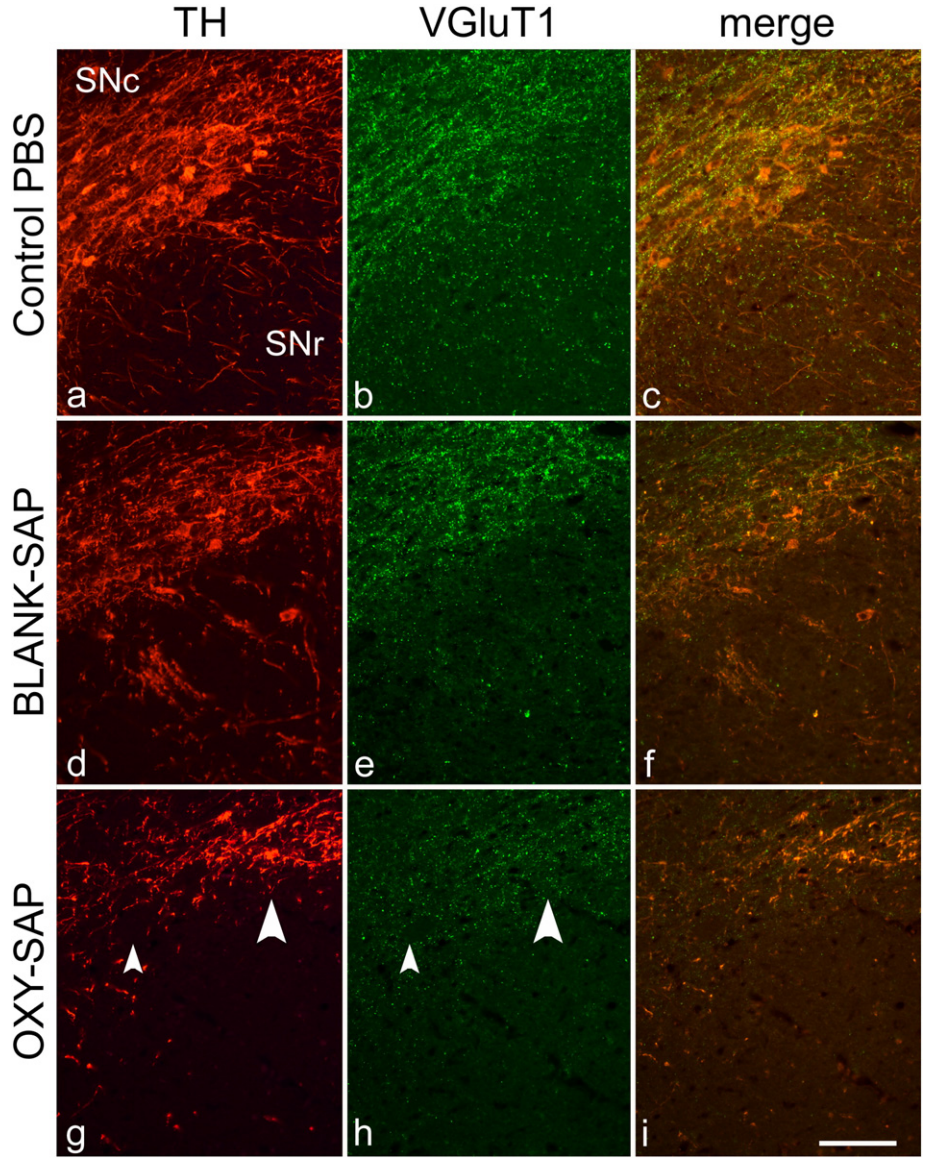

Fig. 9. Substantia Nigra (AP = −5.30 approximately, sections adjacent to the site used for microinjections) immunostained for Tyrosine Hydroxylase (TH, Cy3: red labeling), Vesicular Glutamate Transporter 1 (VGluT1, Alexa-488: green labeling) and merged IR of rats injected bilaterally into the substantia nigra with PBS (a, b and c, respectively), BLANK-SAP (d, e and f, respectively) and OXY-SAP (g, h and i, respectively) at 28 days after treatment. In PBS-injected animals, VGluT1 (b) was expressed in both pars compacta (SNc) and reticulata (SNr), with a higher density in SNc. After 28 days, BLANK-SAP induced no VGluT1 IR reduction (e), except for a slight decrease observable mainly in SNr in the presence of a parallel TH IR reduction. OXY-SAP induced a reduction in VGluT1 IR (h), which was very similar in the SNr and in the SNc, with a parallelism between the extent of TH IR reduction and VGluT1 IR decrease [higher levels of VGluT1 IR corresponded to higher levels of TH IR (big arrowheads in g and h) and vice versa (small arrowheads in g and h)]. Scale bar = 100 μm.

including the superior colliculus and the pedunculopontine nucleus), which play a main role in the control of locomotor activity. The presence of such stimulatory and inhibitory synapses in the substantia nigra, which modulate the activity of nigrostriatal dopaminergic neurons and of GABAergic neurons projecting to the thalamus (and other nuclei of the basal ganglia) is well documented (Hauber, 1998; Kaneda et al., 2005; Windels and Kiyatkin, 2006; Lee et al., 2013; Morales and Root, 2014). The mechanisms recalled above may be not mutually exclusive, contributing all to determine the final activity of nigrostriatal dopaminergic neurons and nigrothalamic GABAergic neurons (Fig. 12).

Support for a role of oxytocin receptors located in neurons different from the cell bodies of nigrostriatal dopaminergic neurons, possibly presynaptically in glutamatergic nerve endings, in the control of locomotor activity is provided by the increase found in the spontaneous locomotor activity of male rats bilaterally injected with OXY-SAP, a neurotoxin characterized for its ability to destroy neurons presenting oxytocin receptors on their surface (Baskin et al., 2010).This increase was very evident 28 days after OXY-SAP treatment, when compared with the locomotor activity of control rats injected bilaterally with an equimolar amount of a control mock peptide-saporin conjugate (BLANK-SAP) or with an equal volume of PBS (motility scores, OXY-SAP > BLANK-SAP ≈ PBS). Indeed, the increase in spontaneous locomotor activity in OXY-SAP-treated rats occurred concomitantly to a marked reduction not only in TH IR in the substantia nigra pars compacta, but also of VGluT1 IR, VGluT2 IR and VGluT3 IR in either the substantia nigra pars compacta and reticulata, although with some difference among them, and no change in GAD IR in both the substantia nigra pars compacta and reticulata. In fact, the marked destruction of nigral dopaminergic neurons (revealed by the reduction in TH IR) and of nigral (local, efferent or afferent) glutamatergic neurons (revealed by the reduction of the IR of all three VGlu transporters)(see Fremeau et al., 2004; Martín-Ibañez et al., 2006; Antal et al., 2014); with no reduction in GABAergic

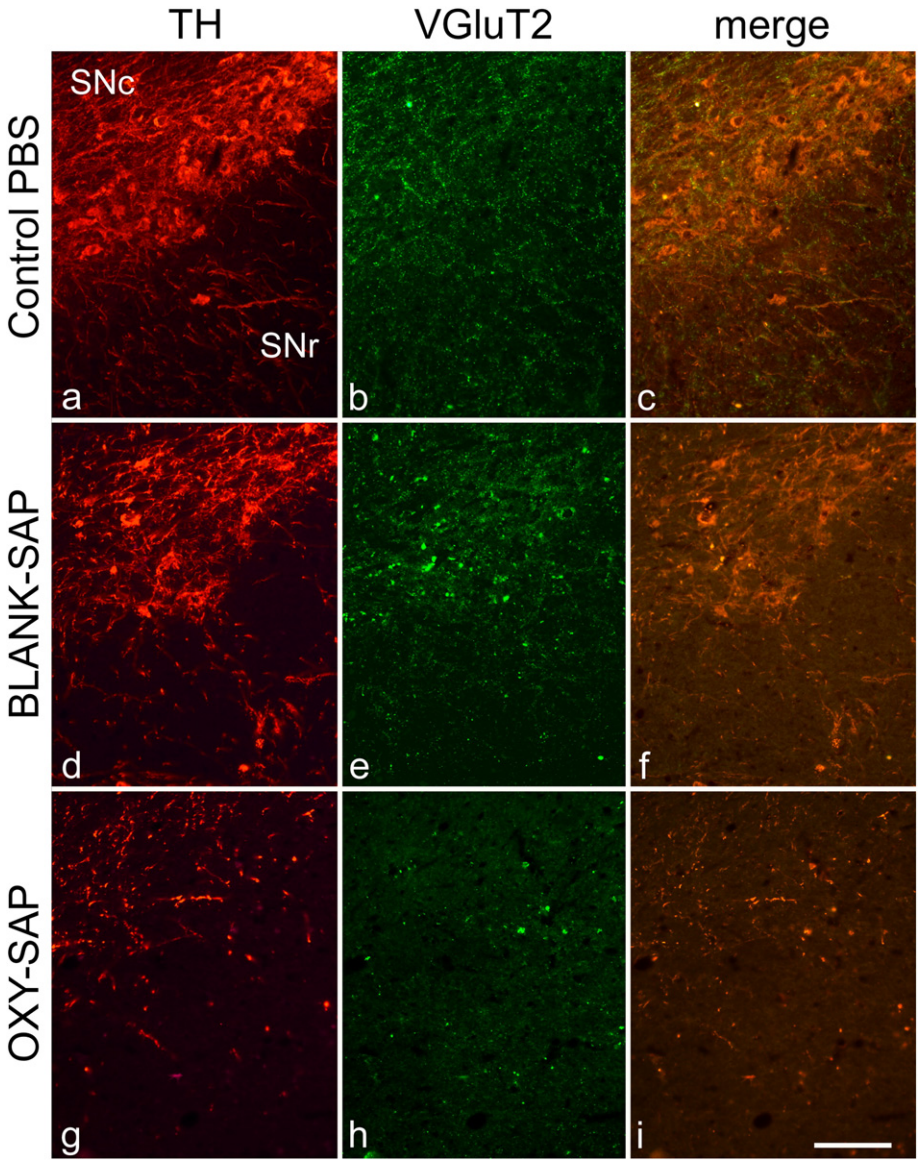

Fig. 10. Substantia Nigra (AP = −5.30 approximately, sections adjacent to the site used for microinjections) immunostained for Tyrosine Hydroxylase (TH, Cy3: red labeling), Vesicular Glutamate Transporter 2 (VGluT2, Alexa-488: green labeling) and merged IR of rats injected bilaterally into the substantia nigra with PBS (a, b and c, respectively), BLANK-SAP (d, e and f, respectively) and OXY-SAP (g, h and i, respectively) at 28 days after treatment. In PBS-injected animals, VGluT2 (b) was expressed in both pars compacta (SNc) and reticulata (SNr), with a slightly higher density in SNc. After 28 days OXY-SAP induced an almost complete reduction in VGluT2 IR in SNr and SNc (h) with a strong parallelism between the extent of VGluT2 decrease and TH IR reduction (g). BLANK-SAP also reduced VGluT2 IR in both SNr and SNc (e) but much less than OXY-SAP and with only minor effects on TH IR (d). Scale bar = 100 μm.

neurons (revealed by the absence of changes in GAD IR) in OXY-SAP-treated rats, suggests that oxytocinergic receptors are present in the substantia nigra in dopaminergic and in glutamatergic neurons as well, while they apparently are not present in nigral GABAergic neurons. Since the destruction of the nigral neurons bearing oxytocin receptors causes an increase in spontaneous locomotor activity, and the destruction of nigrostriatal dopaminergic neurons alone would be expected to cause a decrease rather than an increase in locomotor activity [as found for instance after the bilateral injection of the selective catecholaminergic neurotoxin 6-hydroxy-dopamine (6-OH-DA) into the substantia nigra, i.e., see Grieb et al., 2013], together these findings support the hypothesis that the increased locomotor activity found in OXY-SAP-treated rats is mediated by changes in the activity of nigral glutamatergic rather than nigrostriatal dopaminergic neurons. Accordingly, it is well known that the destruction of nigrostriatal dopaminergic neurons (for instance by 6-OHDA) reduces the activity of striatal GABAergic neuronal efferents and increases the activity of glutamatergic neurons originating mainly in the subthalamic nucleus (but also in other brain areas such as the superior colliculus, the pedunculopontine nucleus, the cortex and so on), and impinging on GABAergic neuronal efferences in the substantia nigra pars reticulata to other nuclei of the basal ganglia motor system (i.e., the thalamus and other nuclei of the brainstem, including the superior colliculus and the pedunculopontine nucleus) (see Hauber, 1998, Melis and Gale, 1983, 1984a, 1984b; Martín-Ibañez et al., 2006) (Fig. 12). In these experimental conditions, in which a nigrostriatal dopamine loss is present, a reduction of glutamatergic tone in the substantia nigra pars reticulata, obtained for instance with a selective glutamic acid receptor antagonist injected in the substantia nigra pars reticulata, facilitates locomotor activity, apparently mediated by the reduction of the burst firing of pars reticulata nigral GABAergic efferences, as expected (see Hauber, 1998; Takakusaki et al., 2003; Kaneda et al., 2005; Windels and Kiyatkin, 2006; Lee et al., 2013; Morales and Root, 2014 and references therein). A similar experimental condition may occur in the OXY-SAP-treated rats of this study.

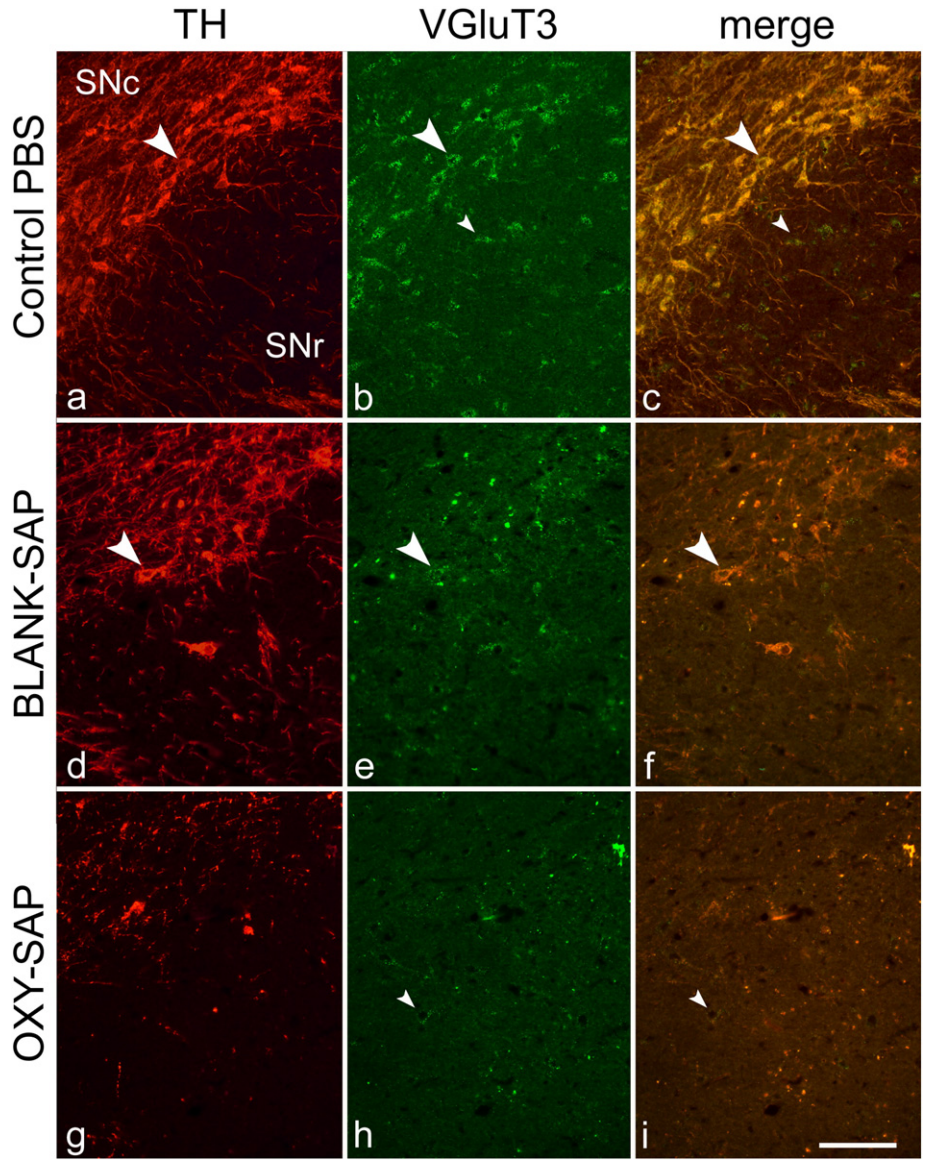

Fig. 11. Substantia Nigra (AP = −5.30 approximately, sections adjacent to the site used for microinjections) immunostained for Tyrosine Hydroxylase (TH, Cy3: red labeling), Vesicular Glutamate Transporter 3 (VGluT3, Alexa-488: green labeling) and merged IR of rats injected bilaterally into the substantia nigra with PBS (a, b and c, respectively), BLANK-SAP (d, e and f, respectively) and OXY-SAP (g, h and i, respectively) at 28 days after treatment. In PBS-injected animals VGluT3 had a perisomatic localization mainly on dopaminergic neurons of the pars compacta (SNc) (big arrowheads), whereas in the pars reticulata (SNr) a perisomatic distribution on a few non-dopaminergic neurons was observed (small arrowheads). After 28 days, OXY-SAP induced an almost complete reduction in VGluT3 IR in both SNr and SNc (h) with a strong parallelism between the extent of VGluT3 decrease and TH IR reduction (g). BLANK-SAP also reduced VGluT3 IR in both SNr and SNc (e), but much less than OXY-SAP and with only minor effects on TH IR (d). Scale bar = 100 μm.

Accordingly, these animals have their nigrostriatal dopaminergic neurons originating in the substantia nigra pars compacta destroyed by OXY-SAP, which has also destroyed glutamatergic nerve terminals of neurons projecting to the substantia nigra pars reticulata (probably originating from the subthalamic nucleus, the superior colliculus, the pedunculopontine nucleus, the frontal cortex and so on) (see Hauber, 1998; Takakusaki et al., 2003; Kaneda et al., 2005; Windels and Kiyatkin, 2006; Martín-Ibañez et al., 2006; Lee et al., 2013; Morales and Root, 2014 and references therein). In fact, the destruction of these glutamatergic neurons should be expected to cause by itself an increase in locomotor activity in intact rats as well as a reduction in the glutamatergic overtone that decreases the impairment of locomotor activity caused by the destruction of nigrostriatal dopaminergic neurons (as found in 6-hydroxy-dopamine-lesioned rats), thereby leading to an increase in spontaneous locomotor activity, as found in OXY-SAP-treated rats.

The interpretation given above is complicated in part by the unexpected decrease in nigral VGluT2 IR and VGluT3 IR found in BLANK-SAP-treated rats, in which only a small decrease in VGluT1 IR was detected, as if BLANK-SAP was able to affect selectively neuronal populations containing VGluT2 IR and VGluT3 IR, but not VGluT1 IR or TH IR, in spite of the fact that BLANK-SAP is supposed to be devoid of any biological activity. Although such decreases were not as dramatic as those found in OXY-SAP-treated rats, they would have been expected to increase basal motor activity in BLANK-SAP-treated rats, at least to some degree, as found in OXY-SAP-treated rats. In fact, in line with the above interpretation, BLANK-SAP-induced VGluT2 IR and VGluT3 IR decreases indicate some lesioning of glutamatergic afferences to the substantia nigra, as discussed above for OXY-SAP-treated rats. One reason why basal motor activity was found to be not changed in BLANK-SAP-treated rats in spite of the reduction in nigral VGluT2 IR and VGluT3 IR, may be the fact that BLANK-SAP-treated rats present only a

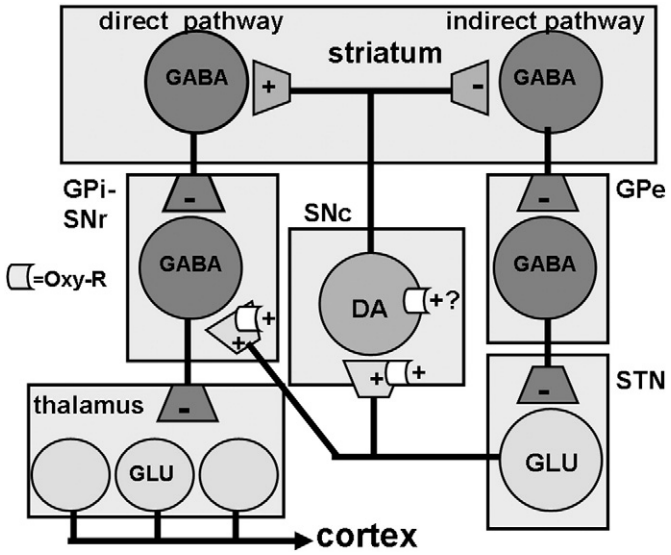

Fig. 12. A hypothetical model that may explain the inhibitory effect of nigral oxytocin on locomotor activity, in line with the results of this study and the canonical view of basal ganglia control of motor activity. Briefly, oxytocin acts mainly on receptors localized presynaptically in the nerve endings of glutamatergic (GLU) neurons originating mainly in the subthalamic nucleus (STN), and impinging on cell bodies and dendrites of nigral dopamine (DA) neurons in the substantia nigra pars compacta (SNc) and reticulata (SNr). Oxytocin receptors (OXY-R) are also present in the cell bodies and dendrites of DA neurons projecting to the striatum. The stimulation of OXY receptors in GLU nerve endings causes an increase in nigral GLU tone, which leads to an increase in the tone of nigral/pallidal (SNr/GPi) GABAergic efferents to the thalamus (and other nuclei of the basal ganglia). This causes a decrease of the activity of excitatory thalamocortical projections, leading to a decrease in locomotor activity. The oxytocin-induced inhibition of locomotor activity does not occur when OXY receptors are blocked by an OXY receptor antagonist or after the destruction not only of GLU neurons, but also of nigrostriatal DA neurons bearing OXY receptors by OXY-SAP. Indeed, in these experimental conditions (e.g. when a marked decrease in nigrostriatal DA activity is present), a reduction in nigrothalamic GABA activity occurs, leading in turn to an increase of the activity of thalamocortical projections and of locomotor activity (see Discussion section for details).

very modest reduction of TH IR when compared to OXY-SAP- treated rats. In fact, this suggests that nigrostriatal dopaminergic neurons are still active in BLANK-SAP-treated rats. Since the absence of nigrostriatal dopaminergic neurons is necessary in order to activate the changes in nigral GABAergic and glutamatergic tone for influencing motor activity as discussed above, this may explain why the lesion of nigral glutamatergic efferences containing VGluT2 and VGluT3 only, does not modify basal motor activity in BLANK-SAP-treated rats. Hence, a concomitant lesion of both dopaminergic and glutamatergic neurons in the substantia nigra, as it occurs in OXY-SAP-treated rats, seems to be necessary to increase basal motor activity.

Whatever mechanism is responsible for the reduction of nigral VGluT2 IR and VGluT3 IR by BLANK-SAP and of the failure of such decreases to increase basal motor activity, together the results of this study show that endogenous oxytocin in the substantia nigra may modulate locomotor activity by acting on oxytocin receptors localized in glutamatergic nerve terminals, leading to a facilitation of the activity of nigral GABAergic efferences and to the concomitant impairment of motor activity usually associated with such increase (see Hauber, 1998), rather than by acting directly on oxytocin receptors localized in nigrostriatal dopaminergic neurons. In fact, in intact rats the net effect of an increase in nigral oxytocinergic tone (as that induced by exogenous oxytocin given into the substantia nigra) results in a decreased locomotor activity, while the net effect of the elimination of the endogenous oxytocinergic tone (as it occurs when the oxytocin receptor antagonist d$(CH_2)_5$Tyr(Me)^2-Orn^8-vasotocin is injected bilaterally into the subtantia nigra, and even more markedly when nigral neurons bearing oxytocinergic receptors, e.g., dopaminergic and glutamatergic, are destroyed by OXY-SAP) is an increased basal level of locomotor activity (Fig. 12).

The interpretation given above of the ability of oxytocin to influence locomotor activity is in line with the canonical view that the main output from the substantia nigra to the thalamic nucleus is represented by GABAergic neurons, which control thalamo-cortical neurons and whose activity is controlled among others by glutamatergic neurons originating in the subthalamic nucleus (Fig. 12). However, the possibility that oxytocin may act on, or that OXY-SAP may also destroy other neurotransmitter/neuropeptide containing neurons bearing oxytocin receptors that have not been investigated in this study or considered in the interpretation given above, which can influence locomotor activity at the level of the substantia nigra, cannot be completely ruled out. Among these are dopaminergic and glutamatergic neurons that project to the thalamus and its nuclei, both of which can modulate the activity of thalamocortical neurons and influence motor activity and other thalamic functions (see Yagüe et al., 2013; Yamaguchi et al., 2013, Morales and Root, 2014 and references therein). If oxytocinergic receptors are also present in the cell bodies of these nigrothalamic neurons, their stimulation by oxytocin or their blockade by d$(CH_2)_5$Tyr(Me)^2-Orn^8-vasotocin or their destruction by OXY-SAP may contribute to modulate in opposite manner the activity of the thalamic efferences to the substantia nigra, hence contributing to the final level of motor activity together with the mechanisms considered above. Interestingly, in adult rats a few of these nigrothalamic neurons have been found to contain both TH and VGLU T2, as if they were also able to release both dopamine and glutamic acid (Yamaguchi et al., 2013). Similar neurons (e.g., positive for TH and VGluT2) have been found in the ventral tegmental area with higher frequency than in the substantia nigra pars compacta (see Morales and Root, 2014, Antal et al., 2014). Unfortunately, whether oxytocin influences locomotor activity by acting on oxytocinergic receptors localized in the surface of nigrothalamic neurons positive for TH, VGlutT2 or both, cannot be determined from our immunohistochemical results, since in our experimental conditions VGluTs immunolabeling is usually observed in synapses only and not in cell perykaria. Another point that needs further investigation is the quantification of the real effect of OXY-SAP on the number of oxytocin receptors in the substantia nigra in our experimental conditions. This may be accomplished by using receptor autoradiography and analyzing oxytocin receptor binding density in the substantia nigra or by measuring the expression of the oxytocin receptor protein in OXY-SAP-, BLANK-SAP- and PBS-treated rats with classic Western Blot analytical techniques.

Despite the uncertainties discussed above, the ability of oxytocin to influence locomotor activity by acting on different neural systems at the level of the substantia nigra, suggests that the neuropeptide may play a modulatory role in the control of locomotion. In this regard, it is noteworthy that the degeneration of dopaminergic nigrostriatal neurons plays a major role in the motor disabilities of Parkinson's disease, such as bradykinesia, rigidity, resting tremor and postural instability (see Lees et al., 2009), and that a significant decrease in the number of oxytocin-immunoreactive neurons was also reported in the hypothalamus of patients affected by Parkinson's disease (Purba et al., 1994). Such a decrease was tentatively related to the increased appetite or to the sexual impotence usually observed in these patients (Purba et al., 1994), in line with the well known inhibitory role of oxytocin in feeding (Maguire et al., 2013) and the facilitatory one on penile erection and sexual behavior (see Argiolas and Melis, 2004, 2013; Melis and Argiolas, 2011, Gil et al., 2013). Oxytocin was also found able to produce an amelioration of the rotenone-induced dopaminergic cell death in the striatum and a suppression of exaggerated striatal neuronal oscillations in a rat model of Parkinson's disease (Erbaş et al., 2012; Erbas et al., 2013). As the majority of oxytocinergic neurons present in the brain originate from the paraventricular nucleus of the hypothalamus and surrounding periventricular regions, it is likely that the decrease in the number of oxytocinergic neurons found in patients with Parkinson's

disease also reflects a decrease of oxytocin levels in the substantia nigra and other brain areas receiving oxytocinergic projections. Together with the ability of oxytocin to influence locomotor activity, this raises the possibility that such a decrease may play a role in the motor disabilities found in these patients. Whether oxytocin may be considered as a target for controlling motor disturbances, as those occurring in Parkinson's disease and/or in other motor disturbances related to basal ganglia dysfunctions, remain to be evaluated.

Conflict of interest

The authors have nothing to declare.

Acknowledgements

This work was partially supported by grants from the University of Cagliari (CAR 2013-2014) to AA, MRM, GLF and CC, from ARS (Autonomous Region of Sardinia) (L.R. 7/2007, "Promotion of Scientific Research and technological innovation in Sardinia", European Social Fund, 2007-2013, Projects Code No. CRP-26206 and CRP-60834 to GLC and CC, and by a MIUR (Ministero dell'Istruzione, dell'Università e della Ricerca Scientifica) grant (SIR 2014, Code no. RBSI14IUX7) to FS. All the authors contributed equally to the work.

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