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Chronic exposure to Δ^9-tetrahydrocannabinol downregulates oxytocin and oxytocin-associated neurophysin in specific brain areas

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Cannabinoids are widely abused drugs. Our goal was to identify genes modulated by Δ^9-tetrahydrocannabinol (Δ^9-THC) treatment. We found that chronic administration of Δ^9-THC (1.5 mg/kg/day, i.p.; 7 days) to rats, downregulates the expression of oxytocin-neurophysin (OT-NP) mRNA and of OT and oxytocin-associated NP (NP_{OT}) immunoreactivity in nucleus accumbens (NAc) and ventral tegmental area (VTA), brain areas involved in reward and addiction. Real-time PCR revealed a 60% and 53% reduction of OT-NP mRNA in NAc and VTA, respectively, under chronic treatment, while no changes were observed in NAc after 24 h. Immunohistochemistry showed a large decrease in number of OT and NP_{OT}-stained fibers in NAc (by 59% and 52%, respectively) and VTA (by 50% and 56%, respectively). No changes in cell staining were observed in the paraventricular nucleus and supraoptic nucleus. As OT is known to inhibit development of drug tolerance and attenuate withdrawal symptoms, we suggest that OT downregulation could play a role during the establishment of the chronic effects of Δ^9-THC.

Keywords: Cannabinoids; Δ^9-THC; Oxytocin; Neurophysin; Nucleus accumbens; Ventral tegmental area; Reward system

Introduction

Marijuana and hashish have been used for centuries for therapeutical and recreational purposes. The major psychoactive compound in these cannabis preparations is Δ^9-tetrahydrocannabinol (Δ^9-THC), which has been shown to affect a variety of physiological functions, including nociception, cognition, memory, locomotion, reward and appetite (Iversen, 2003; Pertwee, 1997). Many of the biological effects of Δ^9-THC in the nervous system are regulated by signaling through the $G_{i/o}$-protein-coupled CB_1 cannabinoid receptor (CB_1-R) (Howlett, 1995, 2004; Mukhopadhyay et al., 2002). After a long debate, it is now accepted that cannabinoids including Δ^9-THC could be addictive, leading to the development of tolerance and dependence (Gardner and Vorel, 1998; Lichtman and Martin, 2005; Martin et al., 2004; Parolaro and Rubino, 2005; Tanda and Goldberg, 2003; and references therein). Moreover, in the last decade, a large body of evidence has been accumulated on the effects of cannabinoids on brain reward processes (Fattore et al., 2001; Gardner, 2002; Gardner and Vorel, 1998; Lichtman and Martin, 2005; Maldonado, 2002; Parolaro and Rubino, 2005; Tanda and Goldberg, 2003), in which several brain regions, including the nucleus accumbens (NAc) and the ventral tegmental area (VTA) have been implicated (Gardner, 2002; Lupica et al., 2004; Pistis et al., 2001; Wise, 2000).

Exposure to drugs of abuse leads to changes in gene expression. Alterations in voltage-gated K^+ channels, synapsin, tyrosine hydroxylase, adenylyl cyclase types VIII and I, and the transcription factors ΔFosB and cAMP response element binding protein (CREB), were observed after chronic opiate administration (Chao and Nestler, 2004; Lane-Ladd et al., 1997; Matsuoka et al., 1994; Matus-Leibovitch et al., 1995, 1996, 1997; Widnell et al., 1996). Chronic exposure to Δ^9-THC was shown to affect the expression of CB_1-R mRNA in cerebellum, hippocampus and corpus striatum (Zhuang et al., 1998). Using gene-array technology, Kittler et al. (2000) reported that chronic Δ^9-THC treatment affected the expression of several genes in rat hippocampus. Recently, we demonstrated that chronic administration of Δ^9-THC increases the expression of brain-derived neurotrophic factor (BDNF), at both mRNA and protein levels, in brain areas associated with reward and addiction (Butovsky et al., 2005).

The peptide oxytocin (OT) is an important neurohormone in the hypothalamo-neurohypophysial system. OT is produced by processing of a precursor molecule (prepro-oxytocin-neurophysin)

Abbreviatiions: BLA, basolateral amygdala; Δ^9-THC, Δ^9-tetrahydrocannabinol; mPFC, medial prefrontal cortex; NAc, nucleus accumbens; OT, oxytocin; NP_{OT}, oxytocin-associated neurophysin; PVN, paraventricular nucleus; SON, supraoptic nucleus; VTA, ventral tegmental area.

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that yields OT and OT-associated neurophysin I (NP_{OT}) (Brownstein et al., 1980). The prepro-OT-NP mRNA as well as OT and NP_{OT} peptides are found in high amounts in neuronal cell bodies located in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus. These areas project to the neurohypophysis, where OT is stored in nerve ending and is released into the blood stream (Gainer and Wray, 1994). In addition, the prepro-OT-NP mRNA and the OT peptide are found in nerve fibers that project from the hypothalamus to extrahypothalamic brain areas, including amygdala, septum, substantia nigra, medulla oblongata and spinal cord (Castel and Morris, 1988; Hallbeck et al., 2001; Onaka, 2004). Moreover, both OT and OT-receptors were found in the NAc of the rat and of the male and female voles, and in the rat VTA (Kremarik et al., 1993; Lim et al., 2004; Yoshimura et al., 1996; You et al., 2000).

OT was found to play a major role in birth contractions and lactation (reviewed by Burbach et al., 2001). In addition, OT was shown to have neurotransmitter functions (Carter, 2003; Shojo and Kaneko, 2000) and it acts in various regions of the nervous system where it controls many complex neuroadaptive processes, such as learning, memory and social interactions (Elliott et al., 2001; Insel and Young, 2001; Reijmers et al., 1998; Sarnyai et al., 1992).

OT was reported to have a role in the regulation of various processes induced by several drugs of abuse. For example, OT was shown to inhibit acute cocaine-induced locomotor hyperactivity, as well as heroin self-administration (Sarnyai and Kovacs, 1994). Downregulation of OT was observed following exposure to several drugs of abuse, such as cocaine, ethanol and morphine (Light et al., 2004; Silva et al., 2002; You et al., 2000). In the latter case, You et al. (2000) showed that chronic morphine treatment leads to a reduction in OT-NP mRNA and OT peptide levels in the NAc and SON, but not in the PVN. As for the effects of cannabinoids, oxytocinergic neuronal activation was shown to prevent the cannabinoid withdrawal syndrome induced by lithium (Cui et al., 2001). However, there are no reports about the effects of cannabinoids on OT expression. In the present study, we show that chronic Δ^9-THC treatment leads to downregulation of OT-NP mRNA, as well as to a reduction in OT and NP_{OT} peptide immunoreactivities in the NAc and VTA, brain areas known to be part of the mesolimbic dopamine rewarding pathway and to be involved in drug addiction.

Results

Chronic exposure to Δ^9-THC downregulates OT-NP mRNA in specific brain areas

Total RNA was extracted from several brain areas of rats chronically treated with Δ^9-THC (1.5 mg per kg per day, i.p. for 7 days) or injected with vehicle. This concentration of Δ^9-THC was chosen as it was found to be rewarding in rats, while higher doses were shown to be aversive (A. Zangen, unpublished data; Sanudo-Pena et al., 1997; Valjent and Maldonado, 2000). RNA was reverse-transcribed and the cDNA subjected to semiquantitative PCR amplifications and agarose gel analysis. The results show that chronic Δ^9-THC treatment led to a large decrease in OT-NP mRNA in the NAc and in the VTA (data not shown).

For more accurate determinations of the changes in the amounts of OT-NP mRNA in various brain areas, we employed the real-time quantitative PCR (Q-PCR) method. The mRNAs for ribosomal protein L19, hypoxanthine-guanine phosphoribosyltransferase (HPRT) and cyclophilin were used as normalizing genes, as they were found not to be affected by the chronic Δ^9-THC treatment. Fig. 1 shows representative examples of Q-PCR for OT-NP mRNA and cyclophilin mRNA in NAc, VTA and PVN. The results show that chronic Δ^9-THC treatment led to a large decrease in OT-NP mRNA in the NAc and the VTA, but did not induce any significant change in the PVN.

The Q-PCR experiments were repeated 3 times with each of 4 different batches of mRNA preparations (each obtained from brain areas of 6 controls and 6 Δ^9-THC-treated rats) and the results were averaged. Fig. 2a shows that the amount of OT-NP mRNA in NAc after Δ^9-THC treatment was markedly downregulated, while no changes were observed in the amounts of the normalizing genes HPRT, cyclophilin and L19. Each of these control genes is therefore suitable for serving as a standard for calculating the changes in OT-NP mRNA expression. The relative amounts of OT-NP mRNA (in comparison with cyclophilin mRNA) are shown in Fig. 2b. It can be seen that high levels of OT-NP are expressed in PVN ($18.2 \pm 4.4 \times 10^{-3}$ relative to the amount of cyclophilin mRNA), whereas much lower levels are found in the NAc and VTA ($0.40 \pm 0.07 \times 10^{-3}$ and $0.30 \pm 0.06 \times 10^{-3}$, respectively). No significant OT-NP mRNA expression was found in the dorsal striatum, a brain region which is close to the NAc anatomically, but which is not considered a part of the limbic system. The Q-PCR data reveal that the 1-week exposure to Δ^9-THC led to a 60% decrease in OT-NP mRNA in the NAc and to a 53% in the VTA. No significant change in OT-NP mRNA expression was detected in the PVN.

While chronic Δ^9-THC led to a significant decrease in OT-NP mRNA in the NAc, no significant change was observed in this area when OT-NP mRNA expression was analyzed 24 h after a single Δ^9-THC injection (Fig. 3). It thus seems that chronic exposure to Δ^9-THC is needed to induce the decrease in OT-NP mRNA expression.

Chronic exposure to Δ^9-THC downregulates the amount of OT and NP_{OT} peptides

Immunohistochemistry was used to examine the effect of chronic Δ^9-THC treatment on OT and NP_{OT} expression in several brain areas. Specificity of immunostaining was confirmed by omitting the primary antibody. Using a rabbit polyclonal antibody immunoreactive against OT peptide (Altstein et al., 1988), we observed staining in the NAc and VTA. Most of the OT immunostaining in these areas was present on neural processes, with no stained cell bodies. In contrast, in PVN and SON, both cell bodies and neural processes were intensively stained. The levels of OT immunostaining, after chronic Δ^9-THC treatment (1.5 mg/kg/day; 7 days), were markedly downregulated in both NAc and VTA, while no changes in OT expression were observed in either PVN or SON (Fig. 4). Similar results were also observed using a higher dose of Δ^9-THC (6 mg/kg/day for 7 days).

To quantify the results, we counted the number of cell bodies (in PVN and SON) and fibers (in NAc and VTA) stained for OT, using the Image ProPlus program. For each tested area, counting was performed using 24–36 sections taken from 12 animals (4 groups, each consisting of 3 vehicle- or 3 Δ^9-THC-treated rats). The results show (Fig. 5) a reduction of 59% in the number of stained fibers in the NAc (being reduced from 35.0 ± 5.3 to 14.4 ± 4.2 stained fibers/field). A reduction of 50% was obtained in the VTA (from 36.3 ± 4.0 to 18.0 ± 3.6 stained fibers/field). The PVN and SON were heavily stained with the anti-OT antibody (showing

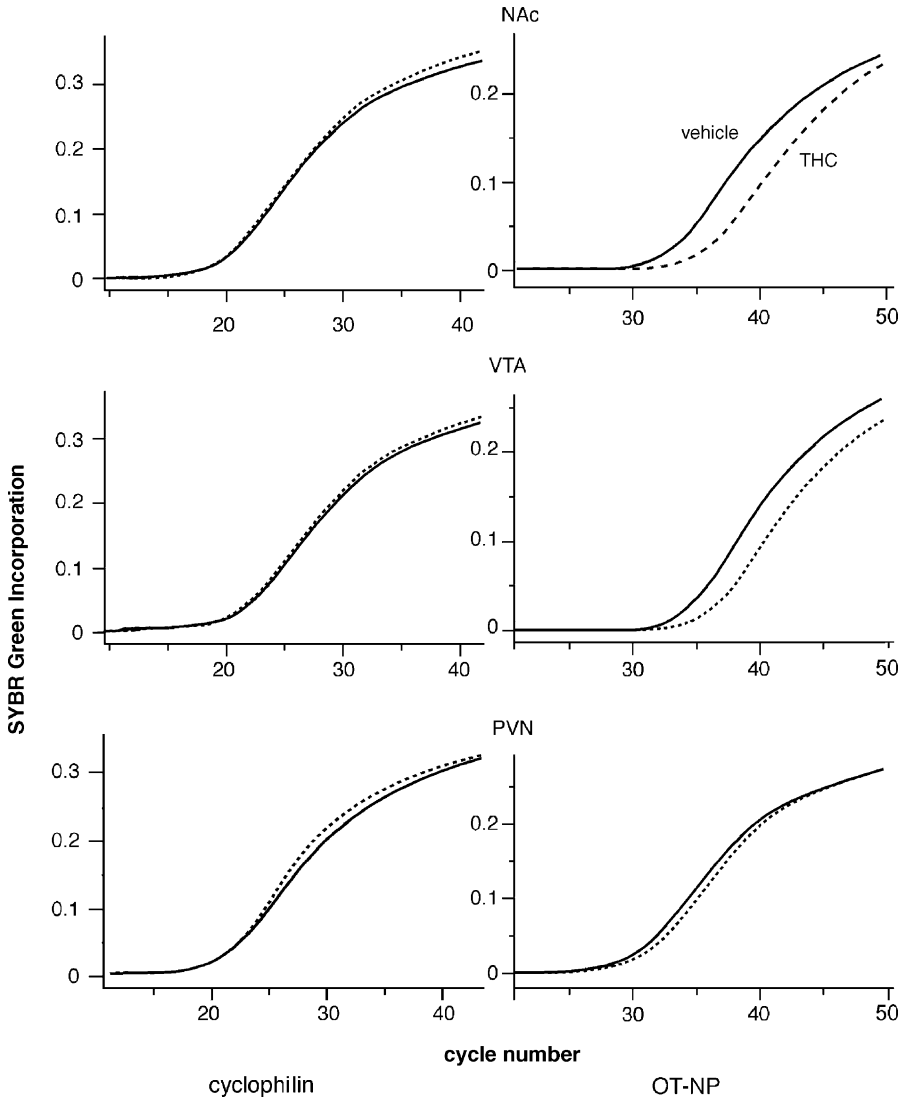

Fig. 1. Analysis of OT-NP mRNA by Q-PCR. Representative curves showing the amounts of OT-NP mRNA in the NAc, VTA and PVN of rats chronically treated with Δ^9-THC (dotted lines) and of control animals treated with vehicle (solid lines), in comparison with the amounts of cyclophilin mRNA in these areas.

211 ± 18 and 96 ± 25 stained cells/field, respectively). No significant change in the number of stained cells was observed in either of these areas after chronic Δ^9-THC treatment (201 ± 44 and 113 ± 23 cells/field, respectively). In addition, the Δ^9-THC treatment did not lead to any significant change in the intensity of staining of cell bodies or neural fibers in these areas.

Since the OT gene encodes both OT and NP_{OT}, we examined the staining of NP_{OT} in similar brain sections from control and chronic Δ^9-THC-treated animals. As expected, NP_{OT} immunostaining showed a very similar pattern to the one observed for OT. A marked reduction in staining was observed in the NAc and VTA (Fig. 6). Again, most of the staining in these areas was observed on neural fibers, with no stained cell bodies. On the other hand, in agreement with the results for OT, a strong NP_{OT} staining of cell bodies and neural fibers was observed in the PVN and SON, and this staining was not affected by the Δ^9-THC treatment. To quantify the NP_{OT} immunostaining, we counted the number of stained cells and fibers as described above. The results (Fig. 7) show a reduction of 52% in the NAc (from 33.3 ± 4.5 to 16.3 ± 2.1 stained fibers/field), and 56% in the VTA (from 38.0 ± 3.6 to 16.7 ± 5.0 stained fibers/field). As described above, the PVN and SON were strongly stained (299 ± 25 and 101 ± 22 cells/field, respectively). No significant change in staining intensity or in the number of stained cells was observed after chronic Δ^9-THC treatment (307 ± 26 and 95 ± 17 stained cells/field, respectively). As a control experiment, we compared the profile of staining of OT to that of NP_{OT} in the same PVN sections. We found that in both vehicle- and Δ^9-THC-treated rats all the cells containing OT also contain NP_{OT} and vice versa. A representative example showing the staining of OT and of NP_{OT} in the PVN of Δ^9-THC-treated rat is presented in Fig. 8. The colocalization of OT and NP_{OT} is in agreement with the production of these peptides from the same precursor at a 1:1 ratio (Gainer and Wray, 1994).

Discussion

The repeated use of drugs of abuse induces neuroadaptive mechanisms that cause remodeling of brain circuits that are normally

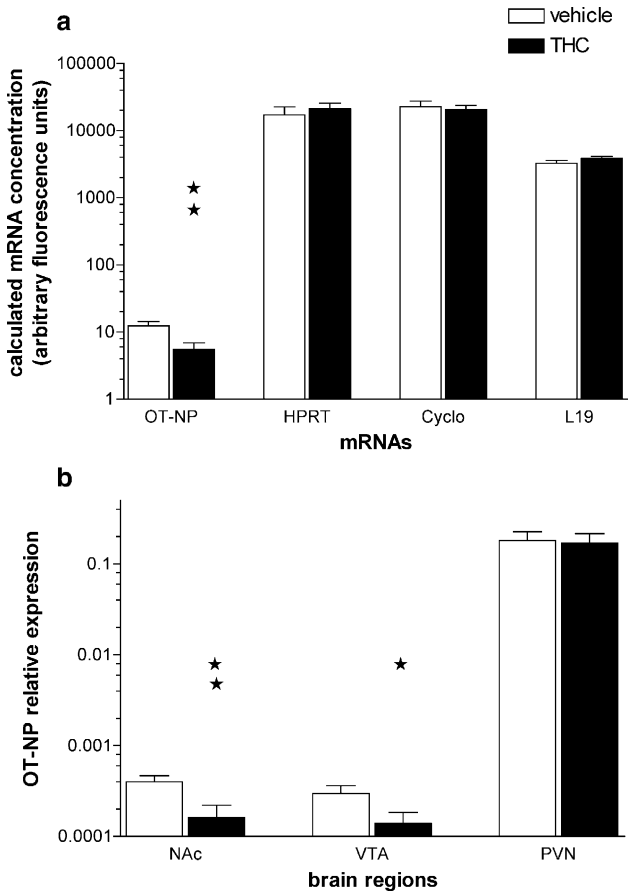

Fig. 2. Q-PCR analysis of OT-NP mRNA in several brain areas of control and chronic Δ^9-THC-treated rats. (a) Calculated concentrations of OT-NP and HPRT, cyclophilin and L19 mRNAs (in arbitrary units of SYBR Green fluorescence) in the NAc of vehicle- and Δ^9-THC-chronic-treated rats. The mRNA concentrations were calculated employing the experimental C_t, using the Rotor-Gene 6 software provided by the thermal cycler Rotor-Gene 3000. (b) Relative expression levels of OT-NP amplicon in comparison with the levels of cyclophilin amplicon in the NAc, VTA and PVN. *$P < 0.05$, **$P < 0.01$.

involved in naturally rewarding effects (i.e. food and water intake, sex and social interaction) and learning behaviors (Chao and Nestler, 2004; Nestler, 2001a,b; Robbe et al., 2003; Volkow et al., 2003). The central neuronal circuits known to be involved in mediating the rewarding processes are located in the VTA, NAc and prefrontal cortex (Gardner, 2002; Lupica et al., 2004; Pistis et al., 2001; Wise, 2000). Drugs of abuse are known to affect the VTA–NAc pathway in a way that leads to marked alterations in reinforcement mechanisms and motivational states (Nestler, 2001a,b, 2004, 2005). High concentration of cannabinoid receptors is present in the NAc and VTA where cannabinoids were shown to affect the reward system (Lupica et al., 2004; Robbe et al., 2003). Cannabinoid receptors were also found in the hypothalamic nuclei, although at a relatively low concentration (Howlett, 1995). The function of these receptors in the hypothalamus is not completely clear.

Changes in gene expression upon exposure to drugs of abuse and after their withdrawal have been reported by us and other groups (Ammon-Treiber and Höllt, 2005; Berhow et al., 1996; Erdtmann-Vourliotis et al., 1999; Matus-Leibovitch et al., 1995, 1996, 1997; McClung and Nestler, 2003; Meredith et al., 2002). However, relatively little is known about the effects of cannabinoids on gene expression in various areas of the brain. In this regard, it was reported that chronic exposure to Δ^9-THC (10 mg/kg/day; i.p.; 21 days) affects the expression of rat CB_1-R mRNA in cerebellum, hippocampus and corpus striatum (Zhuang et al., 1998). A different profile of genes was found to be differentially expressed after 24 h, 7 and 21 days of Δ^9-THC treatment (with 10 mg/kg/day) in rat hippocampus (Kittler et al., 2000). However, oxytocin was not reported to be one of the altered genes affected under both acute and/or chronic exposure to Δ^9-THC in this area. This result is in agreement with our finding that the hippocampus has a very low, if any, detectable amounts of OT-NP mRNA and OT peptide (data not shown), and with Dogterom et al. (1978) who reported undetectable amounts of OT peptide in rat hippocampus.

We recently reported that chronic exposure to a rewarding concentration of Δ^9-THC (1.5 mg/kg/day; i.p.; 7 days) upregulates the expression of BDNF, a neurotrophic factor known to modulate synaptic plasticity and adaptive processes underlying learning and memory. Both mRNA and protein levels were increased in several brain areas involved in reward and addiction including NAc and posterior VTA (Butovsky et al., 2005).

Here we show that chronic exposure to Δ^9-THC leads to a decrease in the amount of OT-NP mRNA, and of OT and NP_{OT} immunostaining in the NAc and VTA. No significant changes were observed in the quantity of OT-NP mRNA in the PVN and of OT and NP_{OT} immunostaining in the PVN and SON areas of the hypothalamus. In addition, the results show that the amounts of OT-NP mRNA and of OT and NP_{OT} peptides in NAc and VTA are very low in comparison with their amounts in PVN and SON. Moreover, in the NAc and VTA, these peptides are located in nerve fibers and are not present in cell bodies. This pattern of staining suggests that the NAc and VTA are innervated by hypothalamic neurons and that the presence of OT-NP mRNA and its peptides in these areas is due to transport of OT-NP mRNA from hypothalamic cell bodies and its local translation in the nerve fibers and nerve endings. Indeed, mRNA transport and translation in neuronal fibers have been observed in many neuronal systems (Bassell et al., 1999). Moreover, transport of OT-NP mRNA itself has been observed in axons (Jirikowski et al., 1990; Mohr and Richter, 1992), and in

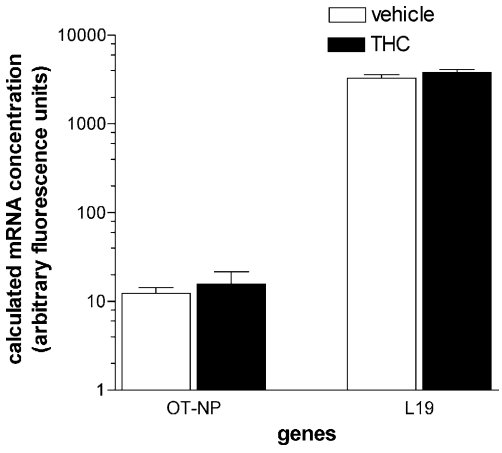

Fig. 3. Calculated concentrations of OT-NP and L19 mRNAs in the NAc after acute Δ^9-THC treatment, obtained by Q-PCR analysis (in arbitrary units of SYBR Green fluorescence). Rats were injected once with a single dose of Δ^9-THC (1.5 mg/kg, i.p.) or vehicle, and decapitated 24 h later.

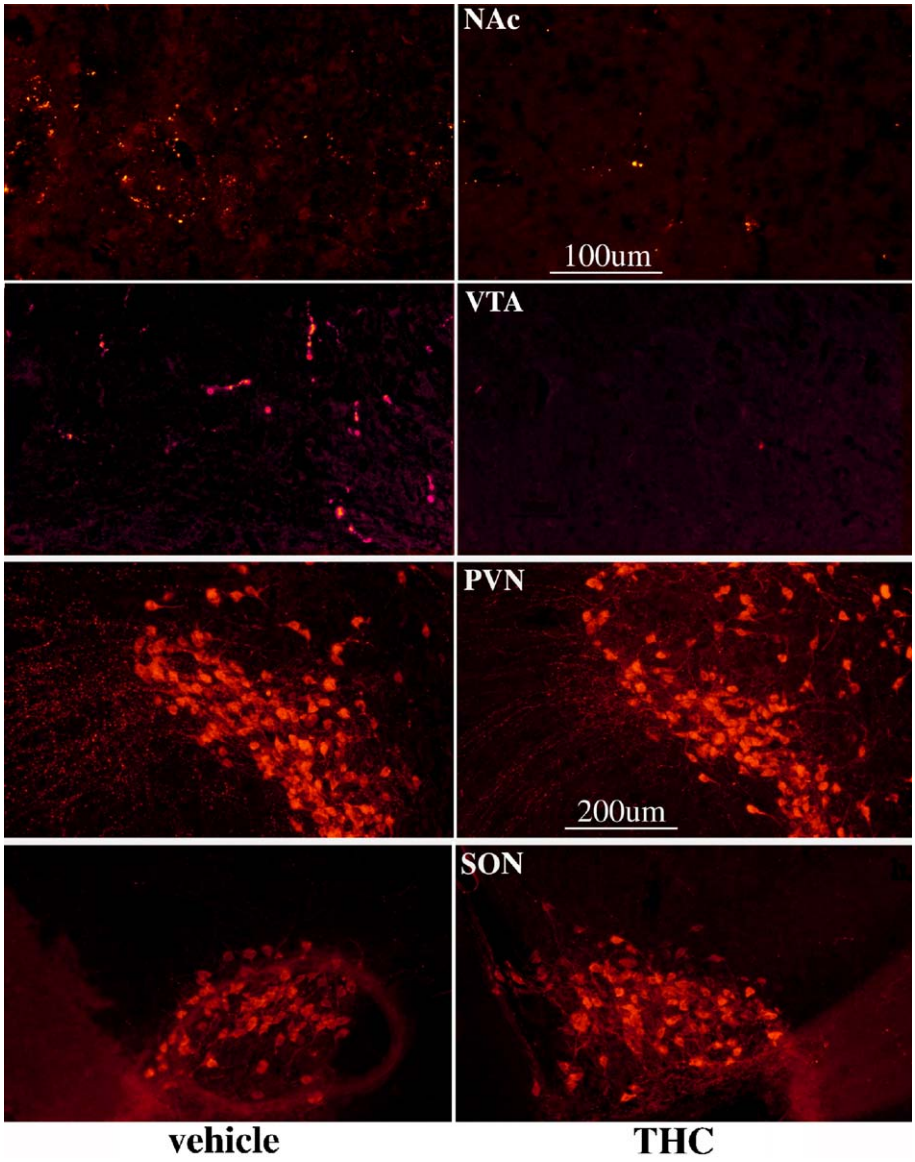

Fig. 4. Immunostaining of OT peptide in the NAc, VTA, PVN and SON following vehicle or chronic Δ^9-THC treatment. Scale bars: 100 μm for NAc and VTA, 200 μm for PVN and SON.

magnocellular dendrites connecting the hypothalamus to several brain regions (Mohr et al., 2002).

The effects of OT are exerted primarily through oxytocin G-protein-coupled receptors (for review see Shojo and Kaneko, 2000). There is evidence for the presence of OT receptors in the VTA, where they are highly expressed during the early postnatal period (Yoshimura et al., 1996). OT receptors were also found in the NAc (Insel and Young, 2001; Young et al., 2001), where OT has been implicated, together with dopamine release, in pair bonding formation (Liu and Wang, 2003). Changes in the expression of OT receptor mRNA were reported to regulate OT action (Caffe et al., 1989; Shojo and Kaneko, 2000). However, we would like to note that we did not find any changes in OT receptor mRNA in NAc following either acute or chronic Δ^9-THC treatment (data not shown).

OT has been implicated in various functions of drugs of abuse. In this regard, it was shown that external application of OT inhibits the development of tolerance to morphine and attenuates various symptoms of morphine withdrawal in mice (Kovacs et al., 1998). In rats, intravenous self-administration of heroin was decreased by OT treatment and OT dose-dependently decreased cocaine-induced hyperlocomotion and grooming behavior (Sarnyai and Kovacs, 1994). Cui et al. (2001) suggested that the prevention by lithium of the cannabinoid withdrawal syndrome is mediated by oxytocinergic neuronal activation. Similarly, lithium potentiated the morphine-induced antinociception and attenuated the withdrawal signs in morphine-dependent rats via a mechanism suggested to be associated with OT release in the CNS (You et al., 2001). In addition, there are indications that several drugs of abuse affect the expression of OT. For example, chronic administration of morphine decreased the levels of OT-NP mRNA and inhibited OT synthesis in NAc and SON (You et al., 2000). Chronic exposure to ethanol led to downregulation of OT mRNA and peptide in the PVN (Silva et al., 2002). Our current results show that chronic exposure to Δ^9-THC downregulates the expression of OT-NP mRNA in NAc and VTA.

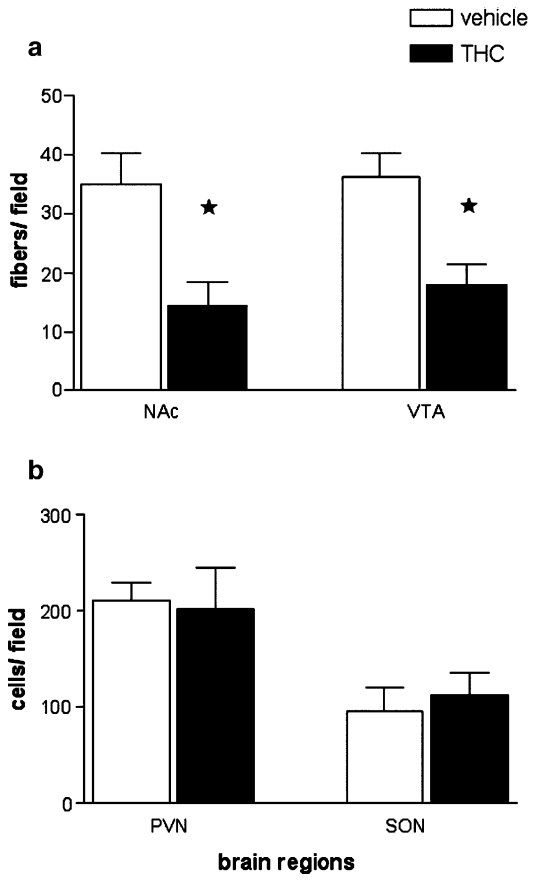

Fig. 5. Quantification of the number of neural fibers (in NAc and VTA) and the number of cells (in PVN and SON) expressing OT, following vehicle or chronic Δ^9-THC treatment. *$P < 0.05$.

The NAc and VTA are key components of the mesolimbic dopaminergic pathway and are involved in the reinforcing and rewarding effects of natural stimuli and of drugs of abuse (Chao and Nestler, 2004; Nestler, 2001a,b, 2005; Robbe et al., 2003; Volkow et al., 2003). It is now well accepted that cannabinoids can increase dopamine levels in the shell of the NAc. This could occur through an opioid receptor-mediated mechanism or via a direct activation of dopaminergic neurotransmission in this area (see Tanda and Goldberg, 2003 for review). In this regard, it is interesting to note that a dopamine D1 agonist (SKF-38393) was shown to affect the expression of vasopressin-associated neurophysin in rat hypothalamic cell cultures (Mathiasen et al., 1996). It is currently unknown whether Δ^9-THC-induced dopamine release could be responsible for the modulation observed in the OT gene expression following Δ^9-THC treatment.

The mesolimbic dopaminergic pathway was also shown to be critical for social signals such as maternal behavior and pair bonding (Insel, 2003; Insel and Young, 2001). Dopamine and OT neurotransmissions in the NAc are involved in partner preference, as mating in the female prairie vole is associated with an increase in dopamine release in the NAc (Gingrich et al., 2000), and activation of both D2 dopamine and OT receptors in the NAc and prelimbic cortex, is necessary for partner preference formation (Gingrich et al., 2000; Liu and Wang, 2003; Young and Wang, 2004; Young et al., 2001).

It is, therefore, tempting to suggest, as indicated above, the existence of an oxytocinergic circuit linking the hypothalamus, where OT-NP mRNA is highly expressed, to the VTA and the NAc and that this circuit is fundamental for the rewarding properties of social interaction and of drugs of abuse.

At this stage, it is not clear how the chronic Δ^9-THC administration affects OT-NP expression and why the effect is observed in the NAc and VTA and is not observed in the PVN and SON. In this regard, it is possible that only few neurons from the hypothalamus send fibers to the NAc and/or VTA and that only these cells are affected by THC. Thus, the chronic Δ^9-THC treatment will affect OT-NP transcription in only few hypothalamic oxytocinergic neurons leading to a very small change in the hypothalamus, while, at the same time, leading to a large reduction of OT-NP mRNA (and its peptide products), in the NAc and VTA. Another possibility is that the reduction of OT-NP mRNA in these neuronal processes is due to a selective cannabinoid-induced inhibition of mRNA trafficking into these areas. In this regard, it was shown that Δ^9-THC and other cannabinoids disrupt the cytoskeleton, including microtubules and microfilaments, and reduce the gene expression of tubulin and actin (Wilson et al., 1996), cytoskeletal proteins that serve as integral part of the mRNA transport system. The effect of Δ^9-THC on OT-NP expression is probably mediated via the CB_1-R and not via the CB_2 cannabinoid receptor (CB_2-R). Our Q-PCR studies showed the presence of CB_1-R but not CB_2-R mRNA in NAc and VTA. Moreover, in an earlier work, we showed that Δ^9-THC is a very weak agonist of the CB_2-R (Bayewith et al., 1966).

In summary, our finding that chronic Δ^9-THC treatment modulates OT expression in the NAc and VTA strengthens the notion that OT has a role in the rewarding effects of drugs of abuse, including cannabinoids. We would like to propose that oxytocinergic neurons and their nerve endings in the VTA and NAc, are integral elements of the adaptive responses to addictive drugs.

Experimental methods

Materials

Δ^9-THC was obtained from the National Institute on Drug Abuse (NIDA; Baltimore, MD, USA) and was solubilized (0.5 mg/ml) in phosphate-buffered saline (PBS) containing 30% (w/v) 2-hydroxypropyl-1-β-cyclodextrin (Cavasol® W7 HP, Wacker-Chemie GmbH, Munich, Germany) (Jarho et al., 1998). The primers for the PCR reactions were as follows: HPRT-sense GGCCAGACTTGTTGGATTTG and antisense CCGCTGTCTTTTAGGCTTTG; cyclophilin-GTCTCTTTTCGCCGCTTGCT and TCTGCTGTCTTTGGAACTTTGTCTG; L19 ribosomal protein-CTGAAGGTCAAAGGGAATGTG and GGACAGAGTCTTGATGATCTC; OT-NP-ATGCAGTCACCCTCTTAGACTGGGC and TATGCCTTGTCCACTGCCATGGT.

Mouse anti-NP_{OT} monoclonal antibody (PS-38) was kindly provided by Dr. H. Gainer (NIH, Bethesda, MD, USA). It recognizes only NP_{OT} and does not interact with OT peptide (Ben-Barak et al., 1985). The preparation of rabbit anti-OT antibody (VA-10), which does not cross-react with NP_{OT}, was detailed previously (Altstein et al., 1988).

Animals and treatment

Adult male Sprague–Dawley rats (12–15 weeks old, 250–300 g) were supplied by the Animal Breeding Center of the Weizmann Institute of Science and handled according to the guidelines of the National Institutes of Health and the Weizmann Institute of Science for management of laboratory animals. Rats were housed in a light- and temperature-controlled room, with food and water provided ad libitum, and were matched for age and weight for each experiment. After 1 week of cage

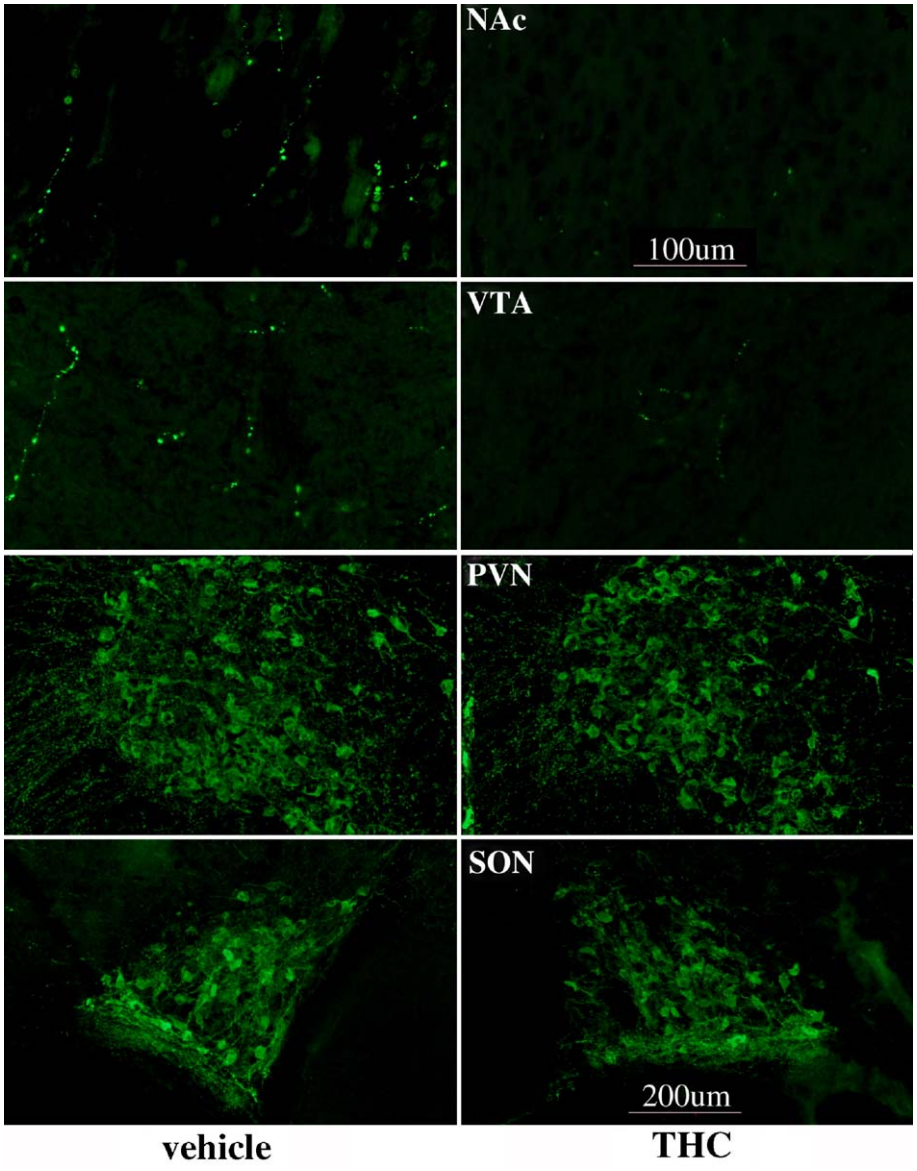

Fig. 6. Immunostaining of NP_{OT} in the NAc, VTA, PVN and SON following vehicle or chronic Δ^9-THC treatment. Scale bars: 100 μm for NAc and VTA, 200 μm for PVN and SON.

adaptation, rats were injected intraperitoneally with either a single dose of Δ^9-THC (1.5 mg/kg) or with daily injections of 1.5 mg per kg per day for 7 days. Control groups of rats were injected with vehicle. Each experiment was performed in groups of six vehicle- and six Δ^9-THC-treated rats and was repeated four times. Δ^9-THC injections and rat killing were performed at constant times (10:00 AM and 1:00 PM, respectively), as expression of genes, including several transcription factors is under circadian regulation (Humphies and Carter, 2004).

RNA extraction and cDNA preparation

RNA extraction and cDNA preparation were carried out as described previously (Butovsky et al., 2005). In brief, rats were decapitated 3 h after the last injection of Δ^9-THC or vehicle, unless otherwise indicated, and specific brain regions including NAc, VTA and PVN were punched out and frozen at −70°C. Frozen brain tissue samples were homogenized and total RNA prepared using the RNeasy purification kit (Qiagen, Hilden, Germany) according to the manufacturer's guidelines, including DNase treatment which was performed on the same RNeasy columns. Quality and quantity of total RNA extracted from each brain area were assessed by measuring the optical density ratio at 260/280 nm and by analysis on ethidium bromide-stained 1% agarose gel electrophoresis. RNA samples (1 μg) were reverse-transcribed using recombinant reverse transcriptase (Omniscript RT Kit; Qiagen, Hilden, Germany) and oligo-dT primer, in a final volume of 20 μl, as indicated in the manufacturer's protocol. To verify complete removal of genomic DNA, RNA samples were subjected to mock reverse transcription (in the absence of reverse transcriptase), and both samples were then analyzed by PCR.

Semiquantitative PCR

The synthesized cDNA was diluted 10 times and amplified by PCR as detailed by Butovsky et al. (2005). Briefly, PCR was carried out in 10 μl reaction volumes containing 4 μl of the diluted cDNA sample, 5 pmol of each primer and 5 μl of ReddyMix PCR Master Mix (ABgenehouse, Epsom, Surrey, UK), yielding a final concentration of 1.5 mM $MgCl_2$. PCR cycling conditions included a 95°C heating step for 1 min at the beginning of every run. Samples were amplified for a specific number of cycles (see example in Fig. 1), with the following cycle profile: 40 s at 95°C, 30 s at 60°C and 40 s at 72°C. This was followed by a 5-min final incubation at

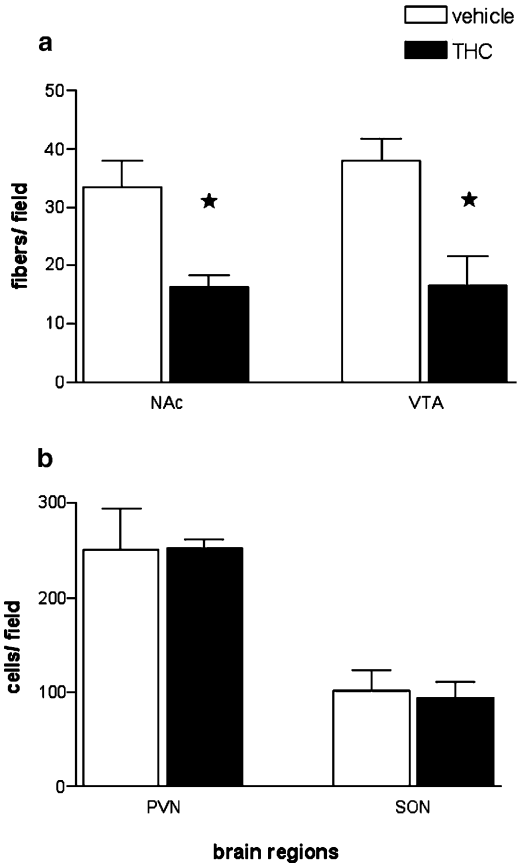

Fig. 7. Quantification of the number of neural fibers (in NAc and VTA) and the number of cells (in PVN and SON) expressing NP_{OT} peptide, in vehicle and chronic Δ^9-THC-treated rats. *$P < 0.05$.

72°C. PCR products were analyzed by electrophoresis on ethidium bromide-stained 2% agarose gels.

Quantitative real-time PCR (Q-PCR)

Q-PCR was carried out in 0.1 ml tubes in the Rotor-Gene 3000 (Corbett Research, Sydney, Australia). PCR reaction mixtures (20 μl) contained cDNA samples (in 3 μl), 5 pmol of each of the relevant two primers (see above) and 10 μl SYBR Green PCR Master kit (Applied Biosystems, Foster City, CA, USA). For each of the examined mRNAs, we ran normal and mock reverse transcribed samples, as well as no template control (total mix without cDNA). In order to obtain the dilution curves (and calculate PCR amplification efficiencies), each set of duplicate PCR reactions was performed with 5 different concentrations of cDNA. The PCR reactions were subjected to the following conditions: 10 min at 95°C to activate the polymerase present in the PCR Master kit, followed by cycles consisting of: 15 s at 95°C, 20 s at 60°C and 20 s at 72°C. Fluorescence was measured at the end of each elongation step. A melting curve was generated at the end of each run to ensure product uniformity and to rule out primer–dimers and splice variants. In addition, the Q-PCR product was analyzed by gel electrophoresis and shown to have the expected amplicon size. For each sample, an amplification plot was generated, showing the increase in the SYBR Green reporter dye fluorescence with each cycle of PCR. A threshold cycle value C_t was calculated from the exponential phase of each PCR sample and a standard curve for each gene was plotted (C_t versus log DNA concentration). The amounts of the various mRNAs in the examined samples were calculated employing the Rotor-Gene 6 software and their calculated concentrations were expressed in relative units of SYBR Green fluorescence.

Immunohistochemistry

Three hours after the last injection of Δ^9-THC, rats were anesthetized with pentobarbital (Pental Veterinary; CTS Chemical Industries, Tel Aviv, Israel; 50 mg/rat; i.p.) and perfused intracardially with 250 ml of phosphate-buffered saline, pH 7.4 (PBS), followed by 250 ml of 2.5% fresh paraformaldehyde in PBS. Brains were removed and kept in 1% paraformaldehyde in PBS until use. One day before sectioning, the brains were kept overnight in 30% sucrose at 4°C and then cut coronally (20 μm) using a freezing microtome (Leica SM 2000R, Oberkochen, Germany). Immunostaining was performed on free-floating sections. In brief, sections were washed twice with Dulbecco's PBS (D-PBS) containing 0.1% Triton (D-PBS-T). After 1 h incubation with blocking solution (20% normal goat serum in D-PBS-T), the sections were incubated overnight with rabbit anti-OT antibody (1:3000 in D-PBS-T containing 2% normal goat serum) or mouse monoclonal anti-NP_{OT} antibody (1:1000 in D-PBS-T containing 2% normal goat serum). Sections were washed three times with D-PBS-T and incubated for 40 min with cy3-conjugated goat anti-rabbit antibody or with

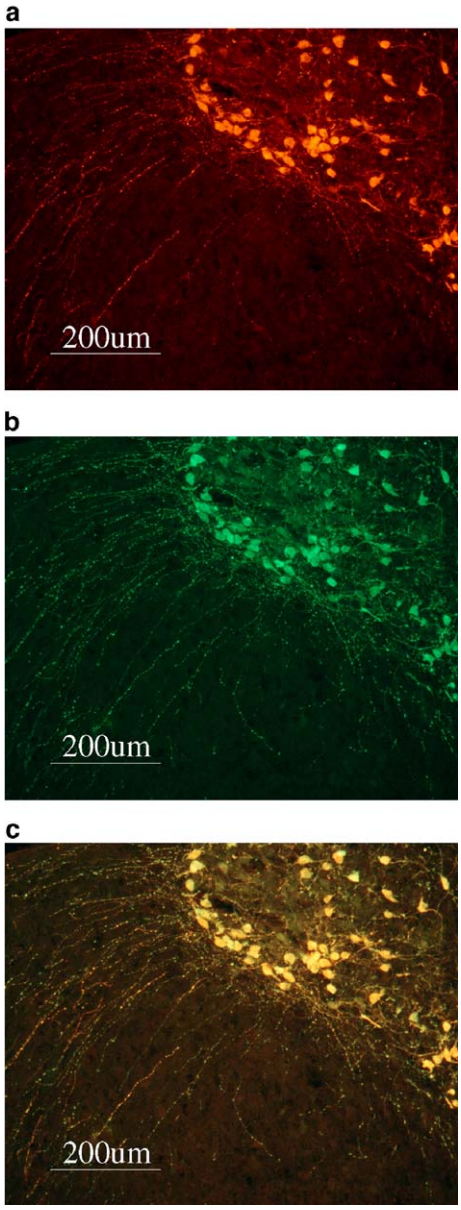

Fig. 8. Immunostaining of (a) OT and (b) NP_{OT} in the PVN of chronic Δ^9-THC-treated rat. Panel c shows the overlay of the two stainings.

cy2-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, Baltimore, MD, USA; 1:200 in D-PBS-T), respectively. After three rinses in D-PBS, the sections were mounted on slides and viewed by fluorescence microscopy (Nikon E600). The number of cells or neural fibers stained for OT or NP_{OT} was counted using the Image ProPlus program (Media Cybernetics, Silver Springs, MD, USA) using 2–3 sections from each of the animals studied. As a control for non-specific staining, parallel sections were incubated as described above but omitting the primary antibody.

Data analysis

Data are expressed as means ± SD of four independent experiments, each performed in duplicate. Results were analyzed using the Student's *t* test.

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