

Vardenafil Enhances Oxytocin Expression in the Paraventricular Nucleus without Sexual Stimulation

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Purpose: Oxytocin is associated with the ability to form normal social attachments. c-Fos is an immediate early gene whose expression is used as a marker for stimulus-induced changes in neurons. The effect of phosphodiesterase-5 (PDE-5) inhibitors on oxytocin activation in the brain without sexual stimuli has not yet been reported. In the present study, we investigated the effects of vardenafil on oxytocin and c-Fos expression in the paraventricular nucleus (PVN) of conscious rats.

Methods: Male Sprague-Dawley rats weighing 300 ± 10 g were divided into 6 groups (n=5 in each group): the control group, the 1-day-0.5 mg/kg, the 1-day-1 mg/kg, the 1-day-2 mg/kg, the 3-day-1 mg/kg, and the 7-day-1 mg/kg vardenafil administration group. The experiment was conducted without sexual stimulation. Vardenafil was orally administered. The animals in the control group received an equivalent amount of distilled water orally. The expression of oxytocin and c-Fos in the PVN was detected by immunohistochemistry.

Results: Oxytocin expression in the PVN was increased by 1 day administration of 2 mg/kg vardenafil, and this effect of vardenafil appeared in a duration-dependent manner. c-Fos in the oxytocin neurons of the PVN was increased by 1 day administration of 2 mg/kg vardenafil, and this effect of vardenafil also appeared in a duration-dependent manner. These results showed that vardenafil augments the expression of oxytocin with activation of oxytocin neurons in the PVN.

Conclusions: In this study, we showed that the PDE-5 inhibitor, vardenafil directly enhances oxytocin expression and also activates oxytocin neurons in the PVN, which indicates that vardenafil may exert positive effects on affiliation behavior and social interaction.

Keywords: Vardenafil; Oxytocin; c-Fos; Paraventricular nucleus; Rats

INTRODUCTION

Oxytocin is synthesized in the supraoptic nucleus and paraventricular nucleus (PVN) of the hypothalamus. Oxytocin neurons in the PVN send projections to the hippocampus, amygdala, and hypothalamus as well as the dorsal horn of the spinal cord. Oxytocin actions are associated with pair bonding, maternal care, sexual behavior, affiliation, attachment, and social memory [1-

5]. Oxytocin is relevant to physiological and behavioral effects induced by social interactions [6]. Moreover, oxytocin treatment was shown to increase social contacts in several species [7]. Oxytocin is known as the key factor in affiliation behavior [8].

c-Fos is an immediate early gene whose expression is used as a marker for stimulus-induced changes in the metabolic activity of neurons. It is induced in the central nervous system under various conditions [9,10]. Expression of c-Fos is used as a meth-

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od of identifying recently activated neuronal population [11,12].

Vardenafil (Levitra) is a selective phosphodiesterase-5 (PDE-5) inhibitor approved for the treatment of erectile dysfunction [13]. PDE-5 is an enzyme found in the corpus cavernosum, and inhibition of PDE-5 allows accumulation of cyclic guanosine monophosphate (cGMP) elaborated by sexual arousal. cGMP causes relaxation of the smooth muscle with concomitant increased blood flow to the penis, leading to an erection [14,15]. In addition, PDE-5 inhibitors have been proved to be effective in various disorders, such as chronic obstructive pulmonary disease, prostatic hyperplasia, hypertension, and coronary heart disease [16].

In animal studies, centrally administered oxytocin induces erection, and oxytocin receptor antagonists prevent non contact erection, which is considered as an index of sexual arousal [17]. Various effects of the PDE-5 inhibitors, besides their use in erectile dysfunction, have been reported [16], however, the effects of PDE-5 inhibitors on oxytocin expression and activation in the brain without sexual stimuli have not yet been reported. Thus, we investigated the possibility of sexual stimuli-independent effects of a PDE-5 inhibitor on oxytocin expression and activation in conscious rats. Specifically, we investigated the effects of vardenafil on the expression of oxytocin and c-Fos in the PVN by use of immunohistochemistry.

MATERIALS AND METHODS

Animals and Treatments

Male Sprague-Dawley rats, weighing 300 ± 10 g, were used for the experiment. The animals were housed at a controlled temperature ($20 \pm 2^\circ\text{C}$) with light-dark cycles, consisting of 12 hours of light and 12 hours of darkness (lights on from 07:00 to 19:00). The experimental procedures were performed in accordance with the animal care guidelines of the National Institutes of Health (NIH) and the Korean Academy of Medical Sciences.

To investigate the dose-dependent effect of vardenafil, we divided the rats into 4 groups ($n=5$ in each group): the control group, the 1-day-0.5 mg/kg vardenafil administration group, the 1-day-1 mg/kg vardenafil administration group, the 1-day-2 mg/kg vardenafil administration group. In order to investigate the duration-dependent effect of vardenafil, the rats were divided into 4 groups ($n=5$ in each group), the control group, the 1-day-1 mg/kg vardenafil administration group, the 3-day-1 mg/kg vardenafil administration group, and the 7-day-1 mg/kg vardenafil administration group.

Vardenafil was obtained from Bayer Healthcare Pharmaceuticals Inc. (Wayne, NJ, USA). Vardenafil was dissolved in distilled water as previously described [18]. It was orally administered (p.o.) once daily. The animals in the control group received an equivalent amount of distilled water orally.

Tissue Preparation

Two hours after the last administration of each drug, the animals were weighed and overdosed with Zoletil 50 (10 mg/kg, i.p.; Virbac, Carros, France). After a complete lack of response was observed, the rats were transcardially perfused with 50 mM phosphate-buffered saline (PBS) and then with 4% paraformaldehyde in 100 mM phosphate buffer at pH 7.4. The brains were dissected, postfixed in the same fixative overnight, and transferred into a 30% sucrose solution for cryoprotection. Serial coronal sections of 40 μm thickness were made with a freezing microtome (CM1510-3, Leica Microsystems GmbH, Wetzlar, Germany).

Oxytocin Immunohistochemistry

For oxytocin immunostaining, free-floating tissue sections were washed twice for 15 minutes in 50 mM PBS and were then permeabilized in 0.2% Triton X-100 for 30 minutes. After being washed twice with PBS, the sections were sequentially incubated in rabbit anti-oxytocin antibody (1:4,000; Oncogene Research Products, Cambridge, MA, USA). The sections were next incubated for 1 hour with biotinylated secondary antibody anti-rabbit (Vector Laboratories Inc., Burlingame, CA, USA). The sections were subsequently incubated with an avidin-biotin-peroxidase complex (Vector Laboratories Inc.) for 1 hour at room temperature. For visualization, the sections were incubated for 5 minutes in 50 mM Tris-HCl (pH 7.6) that contained 0.02% 3,3'-diaminobenzidine (DAB) and 0.03% hydrogen peroxide. The sections were mounted on gelatin-coated glass slides. The slides were allowed to air-dry overnight at room temperature, and the coverslips were mounted by using Permount (Thermo Fisher Scientific Inc., Waltham, MA, USA).

c-Fos Immunohistochemistry

For the detection of c-Fos-positive cells in the PVN, immunohistochemistry was performed as previously described [19]. Free-floating tissue sections were washed twice for 15 minutes in 50 mM PBS and were then permeabilized in 0.2% Triton X-100 for 30 minutes. After being washed twice with PBS, the sections were incubated overnight with rabbit anti-c-Fos anti-

body (1:500; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The sections were next incubated for 1 hour with biotinylated secondary antibody anti-rabbit (1:200; Vector Laboratories Inc.). The sections were subsequently incubated with an avidin-biotin-peroxidase complex (Vector Laboratories Inc.) for 1 hour at room temperature. For visualization, the sections were incubated for 5 minutes in 50 mM Tris-HCl (pH 7.6) that contained 0.02% 3,3'-diaminobenzidine nickel chloride (nickel-DAB) and 0.03% hydrogen peroxide. The sections were mounted on gelatin-coated glass slides. The slides were allowed to air-dry overnight at room temperature, and the coverslips were mounted by using Permount (Thermo Fisher Scientific Inc.). The c-Fos immunoreactivity was localized to the cell nuclei and appeared as a dark gray-black stain.

Data Analysis

To assess oxytocin and c-Fos expressions in the PVN, cell counting was performed by use of the Image-Pro Plus computer-assisted image analysis system (Media Cybernetics Inc., Bethesda, MD, USA) attached to a light microscope (Olympus Co., Tokyo, Japan). The numbers of oxytocin-positive neurons and c-Fos-positive oxytocin neurons were counted hemilaterally at $\times 10$ magnification. The presence of a dark-brown label with round structure was judged as indicative of oxytocin-positive neurons or c-Fos-positive oxytocin neurons. The statistical significance of differences was determined by one-way analysis of variance (ANOVA) followed by Duncan's post-hoc analysis, and the results were expressed as the mean \pm standard error of the mean. Differences were considered significant at $P < 0.05$.

RESULTS

Dose Dependence of the Effect of Vardenafil on Oxytocin Expression

The dose-dependent effect of 1-day vardenafil treatment on the number of oxytocin-positive neurons in the PVN was investigated. Photomicrographs of oxytocin-positive neurons in the PVN in each group are presented in Fig. 1. The number of oxytocin neurons in the PVN was 47.80 ± 3.58 /section in the control group, 49.28 ± 3.37 /section in the 0.5 mg/kg vardenafil-treated group, 52.10 ± 2.39 /section in the 1 mg/kg vardenafil-treated group, and 61.40 ± 1.93 /section in the 2 mg/kg vardenafil-treated group. These results showed that oxytocin expression in the PVN was increased by 1-day treatment with 2 mg/kg vardenafil ($P < 0.05$).

Duration Dependence of the Effect of Vardenafil on Oxytocin Expression

The duration-dependent effect of 1 mg/kg vardenafil treatment on the number of oxytocin-positive neurons in the PVN was investigated. Photomicrographs of oxytocin-positive neurons in the PVN in each group are presented in Fig. 2. The number of oxytocin neurons in the PVN was 47.80 ± 3.58 /section in the control group, 52.10 ± 2.39 /section in the 1-day vardenafil-treated group, 58.62 ± 1.79 /section in the 3-day vardenafil-treated group, and 62.25 ± 1.87 /section in the 7-day vardenafil-treated group. These results showed that oxytocin expression in the PVN

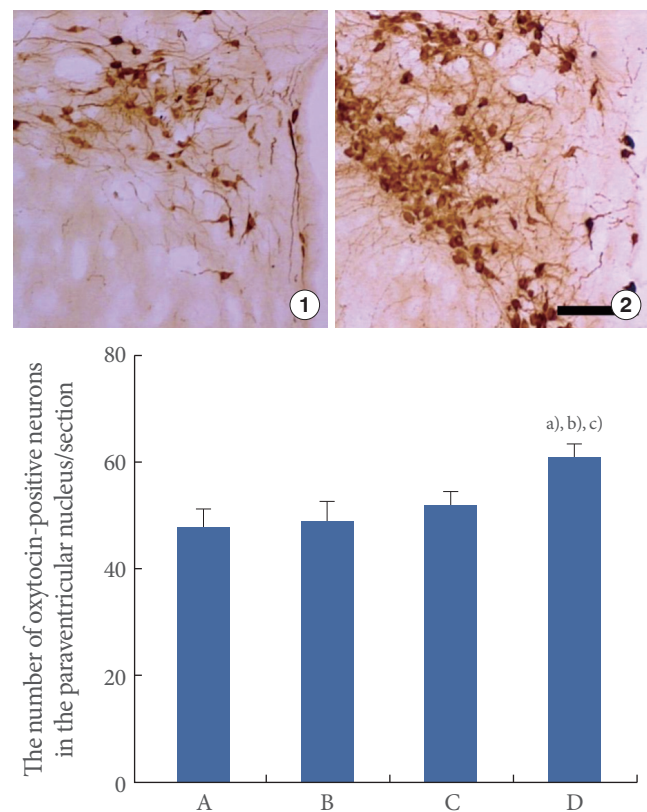


Fig. 1. The dose dependence of the effect of 1-day vardenafil administration on oxytocin expression in the paraventricular nucleus. Above: Photomicrographs of oxytocin-positive neurons. (1) Control group, (2) 2 mg/kg vardenafil administration group. The scale bar represents 100 μ m. Below: Mean number of oxytocin neurons in each group. (A) Control group, (B) 0.5 mg/kg vardenafil administration group, (C) 1 mg/kg vardenafil administration group, (D) 2 mg/kg vardenafil administration group. Values are mean \pm standard error of the mean. ^{a)}Represents $P < 0.05$ compared to the control group. ^{b)}Represents $P < 0.05$ compared to the 0.5 mg/kg vardenafil administration group. ^{c)}Represents $P < 0.05$ compared to the 1 mg/kg vardenafil administration group.

was increased by 1 mg/kg vardenafil in a duration-dependent manner ($P < 0.05$).

Dose Dependence of the Effect of Vardenafil on c-Fos Expression in Oxytocin Neurons

The dose-dependent effect of 1-day vardenafil treatment on the percentage of c-Fos expression in oxytocin neurons was investigated. Photomicrographs of the percentage of c-Fos-positive oxytocin neurons in the PVN in each group are presented in Fig. 3. The percentage of c-Fos-positive oxytocin neurons in the PVN was $15.04 \pm 1.22\%$ /section in the control group, $17.42 \pm 1.96\%$ /section in the 0.5 mg/kg vardenafil-treated group, $19.53 \pm$

1.86% /section in the 1 mg/kg vardenafil-treated group, and $28.80 \pm 2.85\%$ /section in the 2 mg/kg vardenafil-treated group. These results showed that expression of c-Fos in oxytocin neurons of the PVN was increased by 1-day treatment with 2 mg/kg vardenafil ($P < 0.05$).

Duration Dependence of the Effect of Vardenafil on c-Fos Expression in Oxytocin Neurons

The duration-dependent effect of treatment with 1 mg/kg vardenafil on the percentage of c-Fos expression in oxytocin neu-

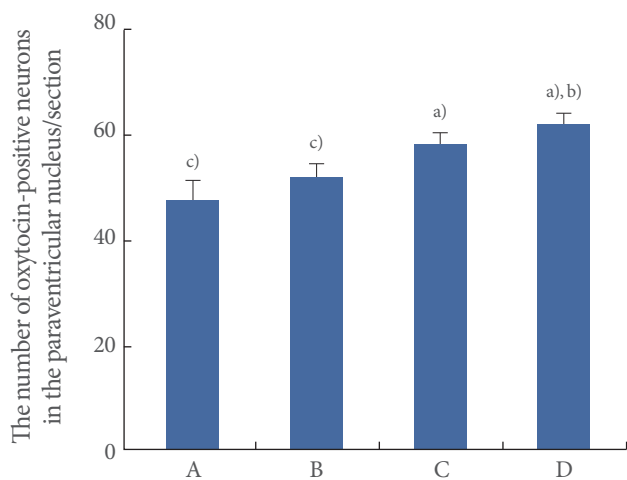
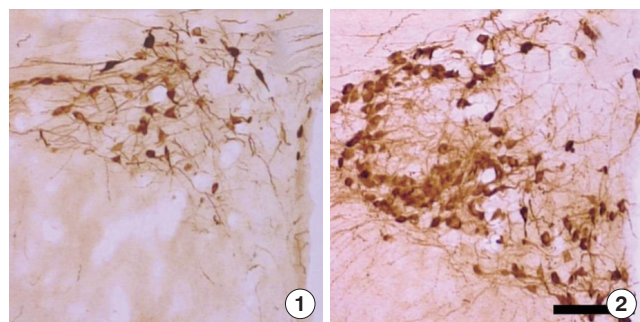


Fig. 2. The duration dependence of the effect of 1 mg/kg vardenafil administration on oxytocin expression in the paraventricular nucleus. Above: Photomicrographs of oxytocin-positive neurons. (1) Control group, (2) 7-day vardenafil administration group. The scale bar represents 100 μ m. Below: Mean number of oxytocin neurons in each group. (A) Control group, (B) 1-day vardenafil administration group, (C) 3-day vardenafil administration group, (D) 7-day vardenafil administration group. Values are mean \pm standard error of the mean. ^{a)}Represents $P < 0.05$ compared to the control group. ^{b)}Represents $P < 0.05$ compared to the 1-day vardenafil administration group. ^{c)}Represents $P < 0.05$ compared to the 3-day vardenafil administration group.

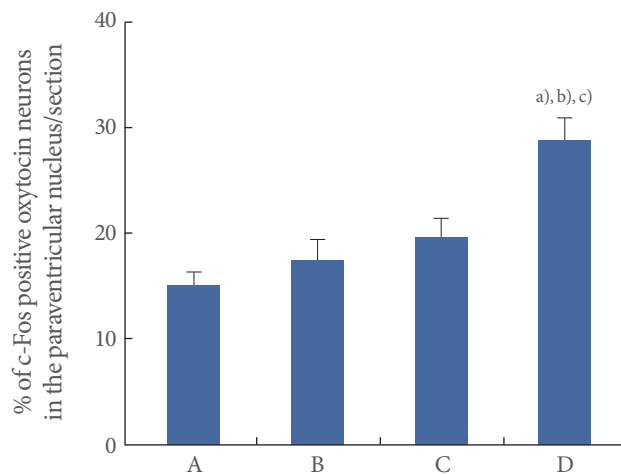
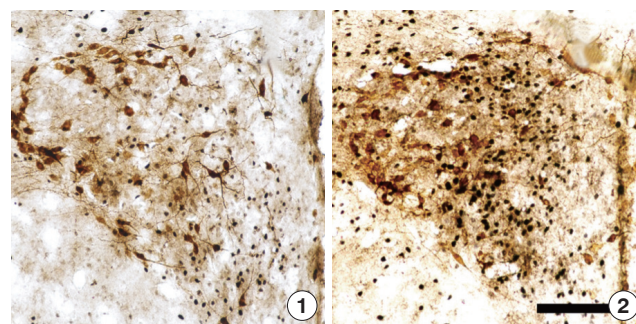


Fig. 3. The dose dependence of the effect of 1-day vardenafil administration on the percentage of c-Fos-positive oxytocin neurons in the paraventricular nucleus. Above: Photomicrographs of c-Fos-positive oxytocin neurons in the paraventricular nucleus. (1) Control group, (2) 2 mg/kg vardenafil administration group. The scale bar represents 100 μ m. Below: Mean percentage of c-Fos-positive oxytocin neurons in each group. (A) Control group, (B) 0.5 mg/kg vardenafil administration group, (C) 1 mg/kg vardenafil administration group, (D) 2 mg/kg vardenafil administration group. Values are mean \pm standard error of the mean. ^{a)}Represents $P < 0.05$ compared to the control group. ^{b)}Represents $P < 0.05$ compared to the 0.5 mg/kg vardenafil administration group. ^{c)}Represents $P < 0.05$ compared to the 1 mg/kg vardenafil administration group.

rons was investigated. Photomicrographs of the percentage of c-Fos-positive oxytocin neurons in the PVN in each group are presented in Fig. 4. The percentage of c-Fos-positive oxytocin neurons in the PVN was $15.04 \pm 1.22\%$ /section in the control group, $19.53 \pm 1.86\%$ /section in the 1-day vardenafil-treated group, $23.75 \pm 1.13\%$ /section in the 3-day vardenafil-treated group, and $28.20 \pm 0.80\%$ /section in the 7-day vardenafil-treated group. These results showed that the expression of c-Fos in oxytocin neurons of the PVN was increased by 1 mg/kg vardenafil in a duration-dependent manner ($P < 0.05$).

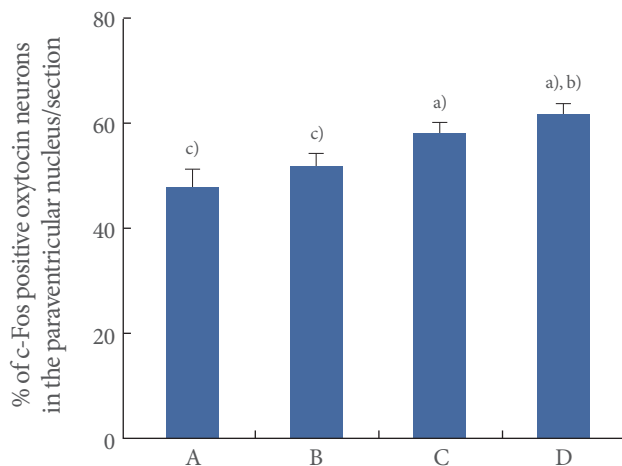
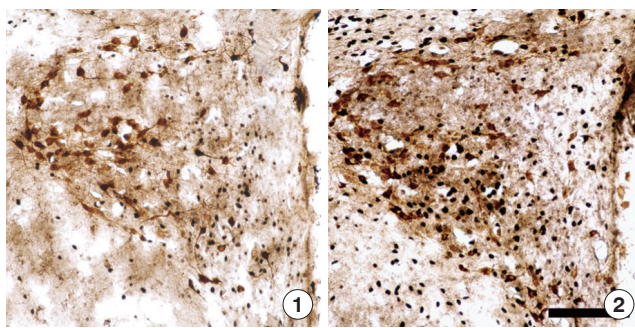


Fig. 4. The duration dependence of the effect of 1 mg/kg vardenafil administration on the percentage of c-Fos-positive oxytocin neurons in the paraventricular nucleus. Above: Photomicrographs of c-Fos-positive oxytocin neurons. (1) Control group, (2) 7-day vardenafil administration group. The scale bar represents 100 μ m. Below: Mean percentage of c-Fos-positive oxytocin neurons in each group. (A) Control group, (B) 1-day vardenafil administration group, (C) 3-day vardenafil administration group, (D) 7-day vardenafil administration group. Values are mean \pm standard error of the mean. ^{a)}Represents $P < 0.05$ compared to the control group. ^{b)}Represents $P < 0.05$ compared to the 1-day vardenafil administration group. ^{c)}Represents $P < 0.05$ compared to the 3-day vardenafil administration group.

DISCUSSION

Vardenafil is a PDE-5 inhibitor developed as an oral therapy for erectile dysfunction [20]. Chronic erectile dysfunction often leads to feelings of humiliation, shame, and negative social interaction [21]. In the present study, we determined the expression of oxytocin in the PVN of the hypothalamus in male rats in response to administration of vardenafil according to dose and duration in the absence of sexual stimulation. The daily tadalafil dose given to the rats (5 mg/kg) was about equivalent to a single 50 mg daily dose in men [22]. Royle et al. [23] treated 10 mg/kg vardenafil twice per day for the middle cerebral artery occlusion in mice. In the current study, Gul et al. [24] treated 0.5 mg/kg of vardenafil as low dosage and 5 mg/kg of vardenafil as high dosage for the determination of the effect of vardenafil on cerebral vasospasm using Wistar rats. Our dosages used in this study are relevant to the human dosage and to the doses of animal studies.

Various neuropeptides and hormones play a pivotal role in social interaction [2]. Of these, oxytocin plays an important role in the brain as a mediator of social behavior [6-8,25]. Oxytocin binds to G-protein coupled receptors, which are localized in numerous brain regions, including the amygdala, hippocampus, and PVN [26]. Moreover, oxytocin activates neurons in the central nucleus of the amygdala where the peptide is anxiolytic and promotes affiliation behaviors [27,28]. Several studies have identified oxytocin as an important mediator for social behavior [29-31]. It was shown that oxytocin treatment increases social contact in several animal species [6,7]. For example, oxytocin was found to suppress anxiety to psychosocial stress [32,33] and to increase trust [30]. Oxytocin may also promote bonding between individuals; between mother and offspring, as has been shown for example in sheep; and also between female and male of some species [34]. Witt et al. [35] reported that oxytocin treatment increases social contact in female prairie voles. These results indicate that, in prairie voles, exogenously administered oxytocin can increase positive social behavior.

The present results showed that 1-day application of vardenafil significantly increased the number of oxytocin-positive neurons in the PVN at a dose of 2 mg/kg. Vardenafil at 1 mg/kg significantly increased the number of oxytocin-positive neurons in the PVN with 3 days of treatment, showing that the enhancing effect of vardenafil on oxytocin expression appeared in a duration-dependent fashion. These data showed that vardenafil, a PDE-5 inhibitor, directly enhances oxytocin expression in

the PVN without sexual stimulation.

Next, we hypothesized that vardenafil could modulate positive social interaction via activation of oxytocin neurons in the PVN. The expression of c-Fos is rapidly induced and has been implicated in the neuronal changes in response to various external stimuli [10]. To confirm whether central oxytocin neurons are activated, determination of c-Fos expression in the oxytocin-positive neurons is required [12]. c-Fos is the product of the immediate early gene c-Fos, and its expression is induced when a neuron is activated. c-Fos immunoreactivity has been extensively used as a marker of neuronal activation.

The present results showed that 1-day application of vardenafil significantly increased the number of c-Fos-positive oxytocin neurons in the PVN at a dose of 2 mg/kg. Vardenafil at 1 mg/kg significantly increased the number of c-Fos-positive oxytocin neurons in the PVN with 3 days of treatment, showing that the enhancing effect of vardenafil on c-Fos expression appeared as the duration-dependent fashion. These data showed that vardenafil augments the activation of oxytocin neurons in the PVN.

We showed here that the PDE-5 inhibitor vardenafil directly enhances oxytocin expression and also activates oxytocin neurons in the brain without sexual stimulation. These results indicate that vardenafil may exert positive effects on affiliation behavior and social interaction.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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