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Orexin-A acts on the paraventricular nucleus of the midline thalamus to inhibit locomotor activity in rats

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ABSTRACT

Orexins (hypocretins) are novel peptides that have been shown to play a role in control of behavioral arousal. The paraventricular nucleus of the midline thalamus (PVT) is one area of the brain that is the most densely innervated by orexin fibers. In addition, the PVT sends a dense projection to the nucleus accumbens, an area of the striatum involved in the regulation of locomotion. This study was done to determine the effect of microinjections of orexin-A (OXA) or the orexin receptor antagonist SB334867 in the PVT on locomotor activity (LA) in morphine-naïve and morphine-sensitized rats. Microinjections of OXA (3 μg/500 nl) in or near the PVT inhibited LA in rats tested in a novel and familiar environment as well as in rats expressing behavioral sensitization to repeated injections of morphine. In contrast, microinjections of SB334867 had no effect on LA in any of the test situations. Using an approach involving experimenter based analysis of ethological behaviors; we found that microinjections of OXA in the midline thalamus decreased LA while at the same time increasing the expression of grooming and freezing. These results suggest that OXA can act on the PVT and the midline thalamus to produce arousal independent of LA.

1. Introduction

Orexin-A (OXA) and orexin-B (OXB) are novel peptides that are exclusively found in the lateral and perifornical areas of the posterior hypothalamus (de Lecea et al., 1998; Sakurai et al., 1998). While having a restricted distribution within the hypothalamus, orexin neurons provide extensive and widespread projections to many regions of the brain where orexins have been shown to regulate a number of physiological, endocrine, and homeostatic functions (Ferguson and Samson, 2003; Harris and Aston-Jones, 2006; Kukkonen et al., 2002; Sakurai, 2006). Orexins act at two different G-protein coupled receptors: the orexin-1 receptor (OX1R) which is selective for OXA and the orexin-2 receptor (OX2R) which is a nonselective for both OXA and OXB (Sakurai et al., 1998). Although different functions have been attributed to orexins, a large body of evidence suggests that orexins play a key role in brain arousal (Carter et al., 2009; Sakurai, 2007). In fact, narcolepsy, a sleep disorder associated with a difficulty in maintaining wakefulness in humans, appears to be due to a deficiency in orexin transmission in the brain (Sakurai, 2007; Taheri et al., 2002). An arousal role for orexins is supported by studies showing that administrations of OXA in the lateral ventricles increased locomotor activity (LA), food intake, and grooming in rodents (Espana et al., 2002; Jones et al., 2001; Nakamura et al., 2000; Rodgers et al., 2000). More recent research evidence also indicates that the orexins may produce arousal of the brain reward system and may be involved in the mechanisms of drug addiction (Boutrel and de Lecea, 2008; de Lecea et al., 2006; Harris and Aston-Jones, 2006; Harris et al., 2005).

Although most nuclei of the thalamus are not innervated by orexin fibers, a group of midline and intralaminar thalamic nuclei that are associated with mechanisms of arousal and attention (Groenewegen and Berendse, 1994; Smith et al., 2004; Van der Werf et al., 2002) receive prominent innervation from orexin neurons (Kirouac et al., 2005; Peyron et al., 1998). Of the midline and intralaminar nuclei, the paraventricular nucleus of the thalamus (PVT) receives an especially dense input from orexin neurons (Kirouac et al., 2005). The PVT also contains a high density of the OX1R and OX2R mRNA (Marcus et al., 2001) and electrophysiological studies show that most PVT neurons are depolarized in the presence of orexins through both OX1R and OX2R mediated mechanisms (Huang et al., 2006; Ishibashi et al., 2005; Kolaj et al., 2007). The PVT is also notable for providing a very dense projection to the nucleus accumbens (Berendse and Groenewegen, 1990; Li and Kirouac, 2008; Moga et al., 1995; Vertes and Hoover, 2008), a part of the ventral striatum strongly associated with the regulation of LA and complex behaviors (Mogenson et al., 1980; Nicola, 2007; Pennartz et al., 1994). In addition to the direct

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projections to the nucleus accumbens, the PVT moderately innervates the medial prefrontal cortex and basolateral nucleus of the amygdala which in turn innervate the nucleus accumbens (Li and Kirouac, 2008; Moga et al., 1995; Vertes and Hoover, 2008). Consequently, the PVT with its direct and indirect projections to the nucleus accumbens forms an impressive neural network that could exert control over LA.

It is well known that most addictive drugs have a stimulant effect on LA and this arousal effect may represent an important mechanism for addiction (Wise and Bozarth, 1987; Wolf, 1998). In fact, repeated administrations of addictive drugs lead to a sensitized locomotor response when an animal is re-exposed to the drug or drug related cues (Vanderschuren and Kalivas, 2000; Wolf, 1998). Studies using c-Fos as a marker of neuronal activity show that the PVT is stimulated following acute administrations of a variety of addictive drugs including cocaine, morphine and ethanol (Deutch et al., 1998; Garcia et al., 1995; Ryabinin and Wang, 1998) as well as exposure to environmental cues that previously paired with addictive substances (Brown et al., 1992; Dayas et al., 2008; Wedzony et al., 2003). Consequently, it is plausible that the PVT is activated by the arousal features of addictive drugs and that PVT may be important for mediating the sensitized locomotor response in drug treated animals. This hypothesis is supported by a study showing that lesions of the PVT attenuated the cue-induced psychomotor response to injections of cocaine (Young and Deutch, 1998). The fact that orexin neurons are active during aroused states and that central administrations of orexins stimulate LA suggests that this neuropeptide may be an important chemical signal mediating the sensitized psychomotor response to addictive drugs.

Consequently, experiments were designed to determine the effects of stimulating or blocking orexin receptors in the PVT on LA in rats that were tested in a variety of conditions that produced enhanced LA including tests of LA in morphine-sensitized rats. The results indicate that activation of orexin receptors in the PVT region inhibited LA in a variety of conditions in which LA was enhanced. The attenuation of LA was accompanied by an increase in grooming and freezing behaviors. We discuss the possibility that an inhibitory effect of orexins in the PVT on LA is consistent with the role of orexins in arousal.

2. Methods

2.1. Animals and housing

Male Sprague–Dawley rats (220–240 g at the beginning of the experiment; Charles River laboratories of Beijing, China) were housed individually in translucent plastic cages (37 cm × 24 cm × 19 cm) in a controlled temperature (20–24 °C) and humidity (50%–70%) colony room on a 12 h/12 h light/dark cycle (lights on at 07:00 h). Food and water were available in the home cage ad libitum. On arrival, rats were handled gently 3 times for 5 min over a 1 week period to acclimatize them to handling. All experiments were conducted in the light phase (8:00–18:00). The experimental protocol and procedures were in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication no. 85-23, revised 1985); the Regulations for the Administration of Affairs Concerning Experimental Animals (China, 1988); and approved by the local committee at Institute of Psychology in Beijing.

2.2. Surgery

The surgery was performed a week after the acclimatization period as described above. Rats were anesthetized with equithesin (0.3 ml/100 g, i.p.) and placed in a Stoelting stereotaxic frame. Stainless steel guide cannula (23 gauge, Plastics One, Roanoke, VA, USA) were unilaterally implanted into the posterior aspect of the PVT (3.1 mm posterior to bregma, 1.3 mm lateral to the midline, and 4.0 mm ventral to the skull, at 10° angle, with the incisor bar set at 3.3 mm below intraaural line). The guide cannulae were secured with 3 stainless steel screws and dental cement. Stylets with caps (Plastics One, Roanoke, VA, USA) were inserted into the length of the guide cannula to prevent occlusion and the rats were treated with penicillin (50,000 Units) to prevent infection. The animals then were returned to their individual home cage to recover over 10–14 days during which the rats were gently handled every other day to adapt them to the microinjection procedure and to reduce the stress associated with handling.

2.3. Drugs and microinjection

Morphine hydrochloride (Qinghai pharmaceutical, China), OXA (Tocris, U.K), SB334867, an OX1 receptor antagonist (Tocris, U.K), DMSO (Sigma, U.S) and physiological saline (NaCl 0.9%) were used in the experiments. Morphine (5.0 mg/ml) and OXA (6.0 μg/μl) were dissolved in saline, SB334867 was dissolved in DMSO to the concentration of 12.0 μg/μl immediately before use. Three days before the drug injection procedure, all the rats were transferred to the injection and test room and stayed there for 2 h and received mock microinjection daily to minimize stress. During the microinjection period, the rats were gently hand-held while the stylet was removed and placed in 75% ethanol; the drugs or vehicle (in a volume of 0.5 μl) were infused through the injector cannula (30 gauge, Plastics One, Roanoke, VA, U.S) which protruded 2.0 mm below the tip of the guide cannula into the PVT region. Infusions were delivered with a Hamilton microsyringe mounted on a motorized pump (Stoelting Company, IL, U.S) at a rate of 0.25 μl/min over 2 min. The injector was placed in the guide cannula for another 2 min to prevent the injectate from flowing back through the guide cannula. The stylet was then placed back into the guide cannula and the rats were returned to their home cage until the start of the test sessions.

2.4. Apparatus

Eight plastic chambers with black walls, 40 cm × 40 cm × 50 cm (L × W × H) were used to measure LA. The testing chambers were placed in a room with dim light provided by three incandescent bulbs (15 W). A video camera suspended on the ceiling was used to track the movement of the rats in the chambers. The video was captured on a computer and subsequently analyzed for distance traveled at different time points by software (Taiji Software Company, Beijing, China).

2.5. Experiment 1: Spontaneous LA in familiar and novel environments

In order to reduce the number of animals used, groups of rats were tested in several test situations in Experiment 1 and 2 (the sequence of treatments and tests are summarized in Fig. 1). In each case, there was a minimum period of 4 days of rest between tests and rats received no more than four injections in total with treatments counterbalanced in each experiment. Each experimental group contained 12–14 animals whereas the group tested in the novel environment contained 8–12 animals.

Two paradigms were used to measure spontaneous LA in naïve rats. In the novel environment paradigm, rats were placed in the novel chamber and LA was measured for 90 min 5 min after receiving OXA (3.0 μg/500 nl), SB334867 (6.0 μg/500 nl) or vehicle (500 nl) infusion in the midline thalamus. After this test was completed, rats were exposed to the familiar environment paradigm by placing the rats in the same chamber for 1 h daily for 4 days to habituate them to the environment. Afterwards, rats were divided into three groups and infused with OXA, SB334867, or vehicle as described before and placed into the chamber for 90 min to evaluate LA in a familiar environment.

2.6. Experiment 2: LA in morphine-sensitized rats

After completion of the experiment 1, we modified the visual and tactile cues of the chamber by adding white horizontal and vertical

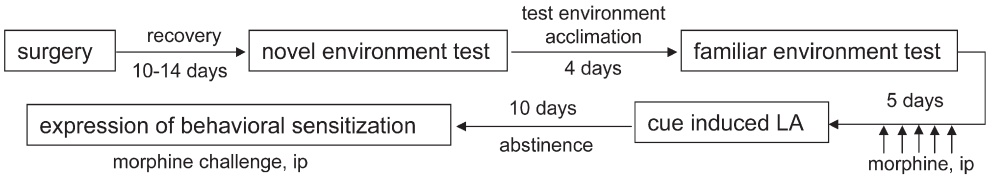


Fig. 1. Diagram showing the sequence of treatments and tests used for Experiments 1 and 2.

stripes (50×3 cm) on the walls of the chamber and converting the smooth surface of the floor to a rough surface. Rats received daily injection of morphine (5.0 mg/kg, i.p.) and placed in the chamber for 2 h per day for total of 5 consecutive days to develop morphine-induced behavioral sensitization. On the sixth day, rats were infused with either OXA (3.0 μg/500 nl), SB334867 (6.0 μg/500 nl) or vehicle (500 nl) and returned to their home cages for 5 min before being placed into the test chamber. Conditioned LA was then measured for 90 min (cue-induced LA in morphine sensitized rats). Following the conditioned LA test, rats were kept in their home cages without morphine treatment for 10 days. After the 10 day morphine abstinence period, rats received microinjections of OXA, SB334867 or vehicle as described above and returned to their home cages. Five minutes later, all rats that received a morphine challenge (5.0 mg/kg, i.p.) were immediately placed into the test chamber to measure LA for 150 min (expression of behavioral sensitization).

2.7. Experiment 3: Co-assessment of LA and ethological behaviors

We also examined the effect of OXA in the midline thalamus on the expression of ethological behaviors because central administrations of OXA have been shown to increase face grooming in rats in addition to increasing LA (Espana et al., 2002; Rodgers et al., 2000). Two groups of naïve rats were test in a circle open field (1 m diameter, 50 cm high) for 10 min 5 min after receiving OXA (3.0 μg/500 nl) or vehicle (500 nl). The behavioral activity of the rats were captured by camera suspended 1.5 m above the open field for the following analysis for LA and behaviors such as grooming, freezing and rearing were rated and analyzed.

2.8. Cannulae placement verification

At the end of the experiments, all rats were deeply anesthetized with chloral hydrate (40 mg/kg), perfused transcardially with heparinized saline followed by 500 ml ice-cold 4.0% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). Brains were removed and postfixed in the same fixative for 1 h, and cryoprotected in 20% sucrose in 0.1 M phosphate-buffered saline (PBS) for 24 h at 4 °C. Coronal sections from the thalamus were made at 100 μm using a vibratome. We used OXA immunoreactivity to verify the locations of the cannula tips in relation to the PVT since work in our laboratory shows that orexin fibers define the boundaries of the PVT (Kirouac et al., 2005; Li and Kirouac, 2008; Parsons et al., 2006). Immunohistochemical staining of OXA was carried out on free-floating sections. Primary and secondary antibodies were diluted in blocking solution containing 0.1% sodium azide, 5% normal donkey serum, and 0.3% Triton-X 100 in PBS. Brain sections were preincubated in the blocking solution for 1 h at room temperature. Sections were then incubated in rabbit anti-OXA (1: 3000; Chemicon, Temecula, CA; catalogue # AB3704, lot 24110715) for 2 days at room temperature. The specificity of the OXA antiserum has been previously established in a cell expression system and preabsorption experiments (Nambu et al., 1999). After rinsing in PBS, sections were transferred to a secondary antibody of biotinylated donkey anti-rabbit (1:500; Jackson Immunoresearch, West Grove, PA) for 2 h at room temperature. Sections were rinsed again and then exposed to an avidin-biotin complex (1:500; Elite ABC Kit; Vector Laboratories, Burlingame, CA) for 60 min at room temperature. Tissue sections were then rinsed and reacted for 5 min with diaminobenzidine (DAB) with nickel intensification (Vector DAB Kit) to produce black orexin fiber labeling. The DAB reaction was terminated by rinsing in PBS before sections were mounted onto gelatin-coated slides and coverslipped. The sections were then examined under a light microscope and mapped on drawings of the thalamus.

2.9. Data analysis

The total distance travelled by rats in all experiments, number of rearing, freezing and grooming duration in experiment 3 were analyzed using one-way ANOVA (all data are shown as mean ± SEM). In order to examine the time-dependent effect of OXA on LA in experiment 1 and 2, the distance travelled for 10 min periods was further analyzed using two-way ANOVA with "treatment" (OXA, SB334867 and vehicle) as between-subject factors and "time" (9×10 min periods) as within factors. Distance traveled during the development of behavioral sensitization to morphine was analyzed using two-way ANOVA with "treatment" (saline and morphine) as between factors and "day" (5 days) as within factors. Post-hoc tests were conducted with the LSD test whenever indicated by the ANOVA test results.

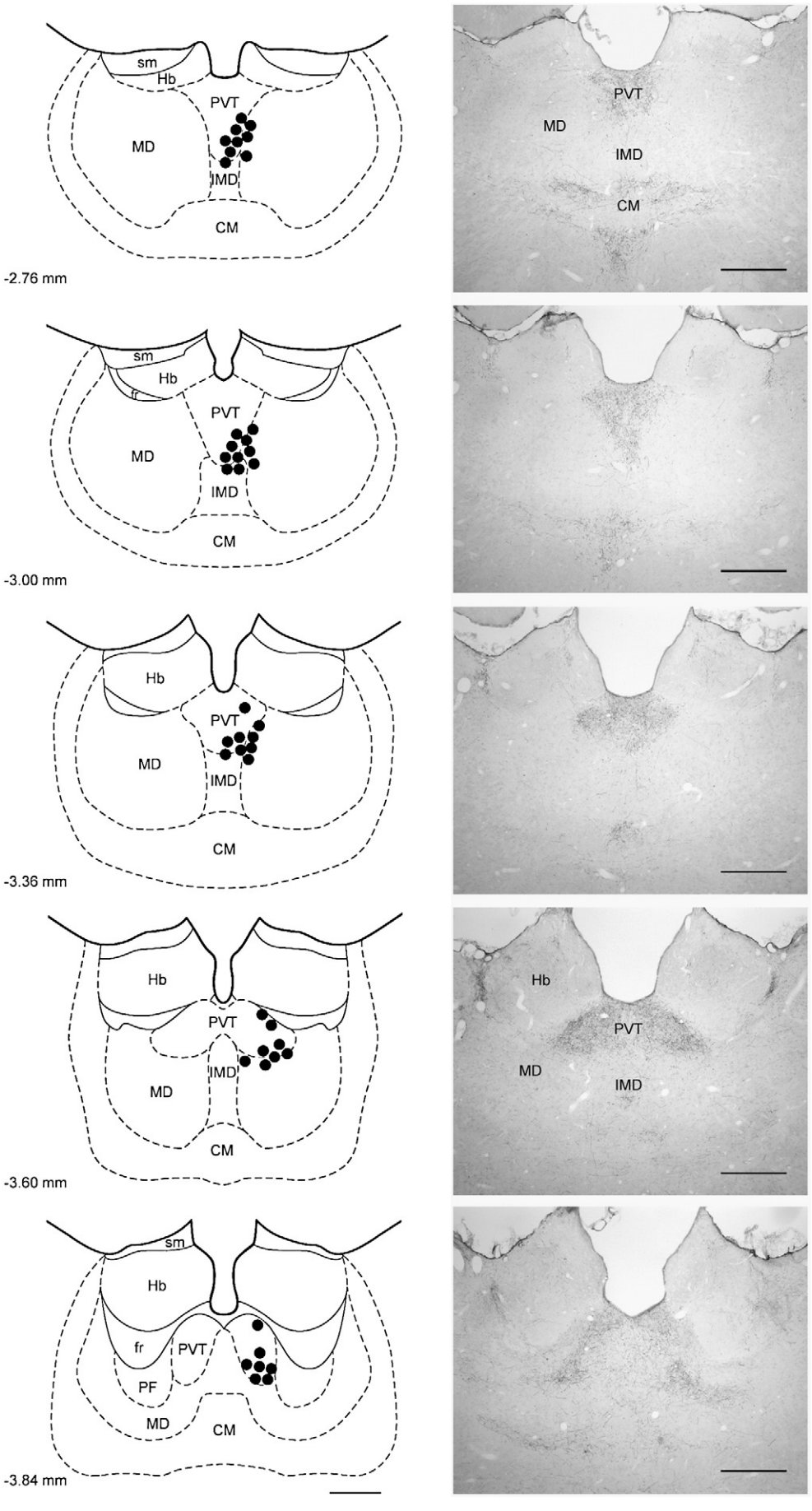
3. Results

The location of the injector cannula placements is shown in Fig. 2. Most of the cannula placements were located in the PVT or near the boundary of the PVT and the mediodorsal nucleus or the intermediodorsal nucleus of the thalamus. This type of placement prevented the damage to the PVT that would likely result from placements in the middle of the PVT.

3.1. Microinjections of OXA in the PVT inhibits LA in novel environment

Figs. 3 and 4 depicted the effect of microinjecting OXA and the OX1 antagonist SB334867 in or near the PVT on LA in rats tested in a familiar or novel environment. In experiments involving rats tested in a novel environment, one-way ANOVA demonstrated that there was a significant difference in the total amount of LA for the full 90 min (Fig. 3A; F (2, 30) = 3.704, $p<0.05$). Post-hoc analysis showed that OXA significantly decreased total LA ($p<0.05$) whereas SB334867 had no effect. In order to investigate the time-dependent effect of OXA on LA, the distance travelled for 10 min periods was calculated and analyzed by two-way ANOVA. The ANOVA showed that there was a significant main effect for "treatment" (Fig. 3B; F (2, 30) = 3.704, $p<0.05$) and an interaction effect between "treatment" and "time

Fig. 2. The figure shows all cannula placements (left) and OXA fibers (right) in corresponding levels of the midline thalamus. Numbers on left indicate the approximate rostrocaudal plane posterior to bregma. Staining for OXA was used to define the boundaries of the PVT for the mapping of injector cannulae. Black dots show the location of the tip of the injector cannula for individual rats. Note that all the injection sites were in or immediately adjacent to the paraventricular nucleus of the thalamus (PVT). The figure is adapted from diagrams of a stereotaxic atlas of the rat brain (Paxinos and Watson, 2005). CM, centromedial nucleus; fr, fasciculus retroflexus; Hb, habenula; IMD, intermediodorsal nucleus; MD, mediodorsal nucleus; PF, parafascicular nucleus; sm, stria medullaris. Scale bar = 500 μm.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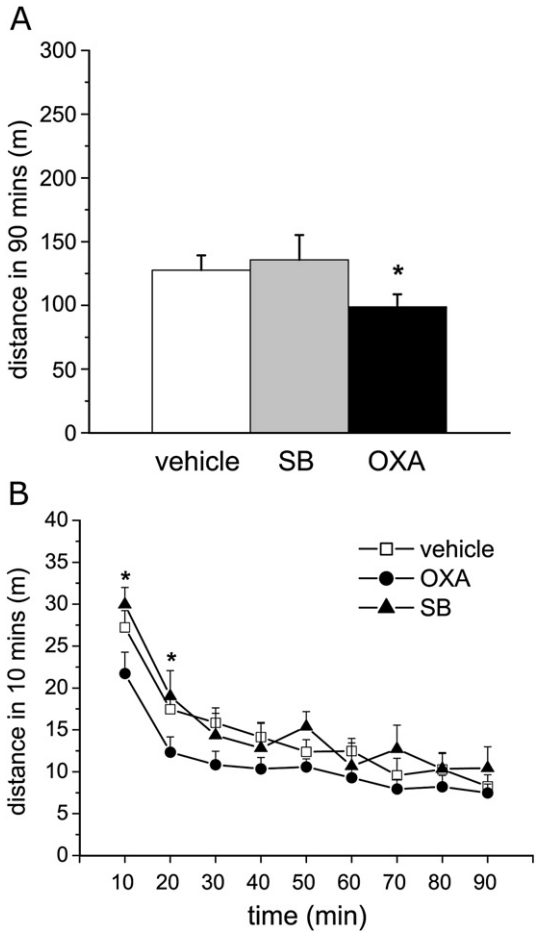

Fig. 3. Effects of microinjections of OXA or SB334867 in the midline thalamus on LA of rats placed in a novel environment. (A) Orexin-A significantly inhibited LA as measured by the total distance travelled (meters) for a 90 min period. (B) The inhibitory effect of OXA on LA was observed in the first and second 10 min periods. * indicates $p<0.05$, compared to vehicle.

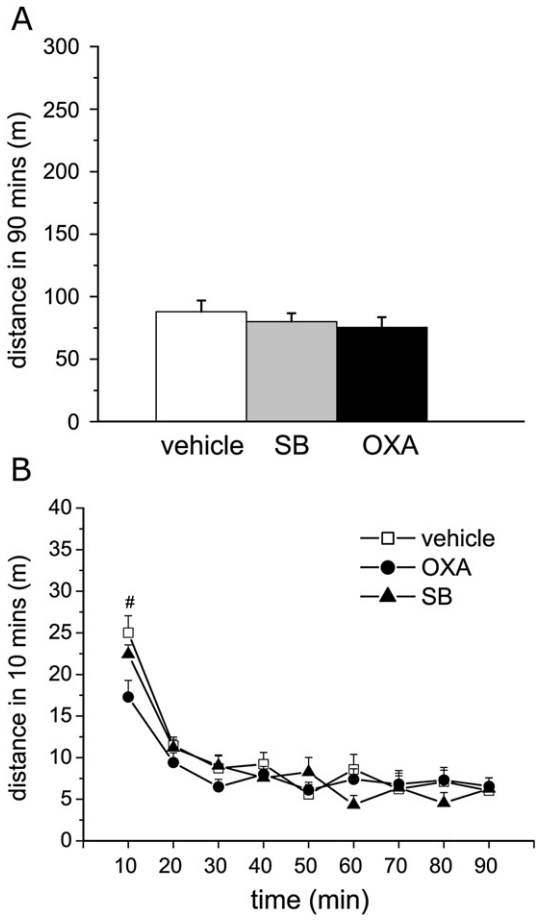

Fig. 4. Effects of microinjections of OXA or SB334867 in the midline thalamus on LA of rats placed in a familiar environment. (A) No differences were found between groups for the total distance travelled for a 90 min period. (B) An inhibitory effect of OXA on LA was observed in the 10 min period. * indicates $p<0.05$, compared to vehicle.

period" (Fig. 3B; F (16, 240) = 2.02, $p<0.05$). Significant differences were observed in the first and second 10 min periods (F (2, 30) = 3.45, $p<0.05$; F (2, 30) = 3.37, $p<0.05$) and the post hoc tests revealed that OXA inhibited LA in the first ($p<0.05$) and second ($p<0.05$) 10 min periods when LA was the highest (Fig. 3B).

In the familiar environment experiments, no difference in total LA between the different groups was observed (Fig. 4A; F (2, 40) = 0.649, $p>0.05$). The two-way ANOVA indicated that there was a significant interaction effect between "treatment" and "time period" (Fig. 4B; F (16, 320) = 2.29, $p<0.01$). Simple effect analysis showed that the difference between groups was significant only in the first 10 min (F (2, 40) = 4.93, $p<0.01$) where OXA inhibited LA ($p<0.005$) at a time when the animals were most active.

These data indicate that microinjection of OXA in the dorsal midline thalamus inhibited LA when rats were tested in a novel and familiar environment and that this inhibitory effect was observed during the first 20 min of the test when the animals were most active. In contrast, blocking of orexin receptors with microinjections of SB334867 in the PVT region had no effect on LA.

3.2. Microinjection of OXA into PVT inhibits LA in morphine sensitized rats

Fig. 5 illustrates the development of morphine induced behavioral sensitization. Two-way ANOVA showed a significant main effect of "treatment" (F (1, 49) = 7.92, $p<0.01$) and a significant interaction effect between "treatment" and "days" (F (4, 196) = 3.66, $p<0.01$). Simple effect analysis revealed that there was a significant difference in LA between different days in morphine group (F (4, 196) = 10.51, $p<0.005$). In addition, LA was higher on the fifth day compared to the first day in morphine treated rats ($p<0.01$) whereas the LA in morphine treated rats was significantly greater than that of saline group on the fifth day ($p<0.01$). The fact that there was no difference between the fourth and fifth day in morphine treated rats ($p>0.05$) indicates that behavioral sensitization had completely developed by the fourth day.

The LA of morphine-sensitized rats was measured following an exposure to the morphine-paired chamber (cue-induced LA). For experiments involving cue-induced LA, one-way ANOVA revealed a significant difference among groups (Fig. 6A; F (2, 40) = 3.867, $p<0.05$) with OXA significantly inhibited LA compared to vehicle group ($p<0.01$). Distance traveled at different 10 min time points was analyzed by two-way ANOVA. A significant interaction effect between "treatment" and "time period" was observed (F (16, 320) = 1.86, $p<0.05$) and simple effect analysis revealed that there was a significant difference between groups in the first (Fig. 6B; F (2, 40) = 9.80, $p<0.01$), third (Fig. 6B; F (2, 40) = 3.39, $p<0.05$) and fourth 10 min periods (Fig. 6B; F (2, 40) = 4.76, $p<0.05$). Post-hoc analysis indicated that OXA inhibited LA during these periods ($p<0.005$; $p<0.05$; $p<0.005$, respectively).

The LA of morphine-sensitized rats was measured following an exposure to the same test chamber immediately after an injection of morphine after a 10 day period of abstinence (morphine-induced LA). In this test situation, LA was measured for 150 min to capture the full

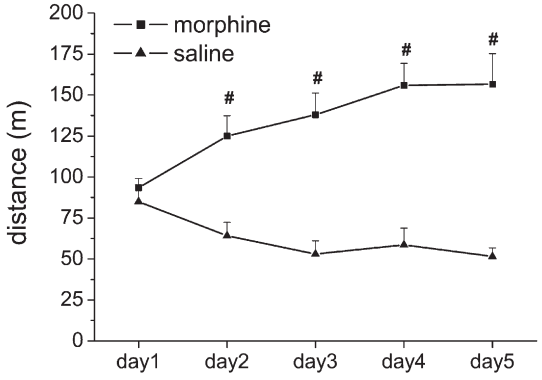

Fig. 5. Development of morphine induced behavioral sensitization over 5 days. Each point represents the total distance traveled in meters for a 120 min test period. # indicates $p<0.01$ compared to vehicle.

expression of the behavioral sensitization. One-way ANOVA revealed that there was no significant difference in the total LA between the different groups (Fig. 7A; F (2, 37) = 1.402, $p>0.05$). However, there was a significant difference in the distance traveled at different time points as indicated by two-way ANOVA (Fig. 7B; F (16, 296) = 2.59, $p<0.005$). The simple effect analysis showed that there was a significant difference among groups during the first and second 10 min (F (2, 37) = 4.16, $p<0.05$; F (2, 37) = 5.15, $p<0.01$) with OXA inhibiting LA during the first two 10 min periods of the test when behavioral activity is high ($p<0.05$; $p<0.005$, respectively).

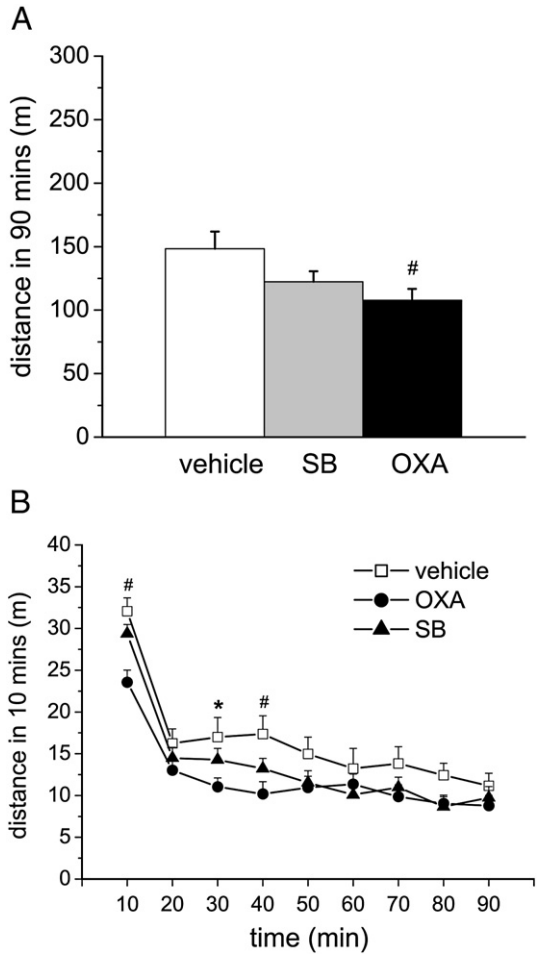

Fig. 6. Effects of microinjections of OXA or SB334867 in the midline thalamus on LA induced by cues paired with morphine in morphine-sensitized rats. (A) Orexin-A significantly inhibited LA as measured by the total distance travelled. (B) The inhibitory effect of OXA on LA was observed in the first, third and fourth 10 min periods. * indicates $p<0.05$ and # indicates $p<0.01$, compared to vehicle.

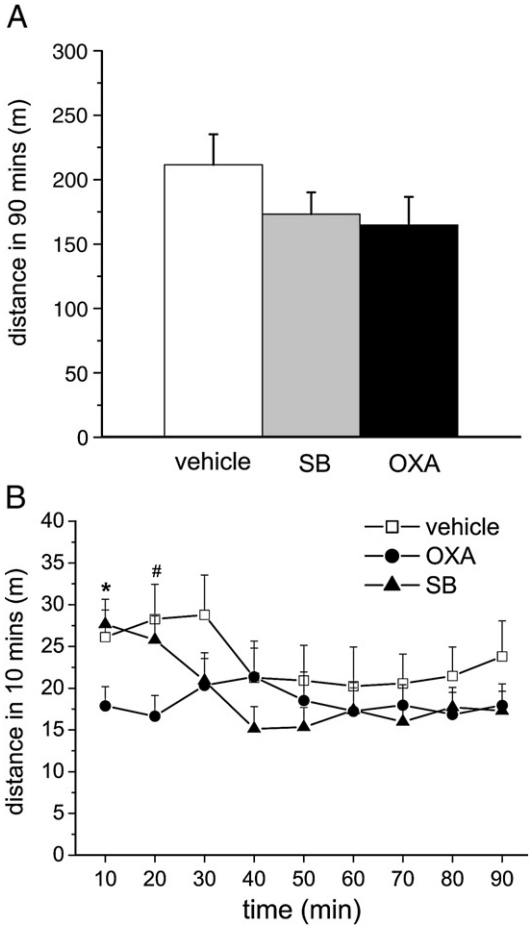

Fig. 7. Effects of microinjections of OXA or SB334867 in the midline thalamus on LA in morphine-sensitized rats with morphine challenge. (A) No differences were found between groups for the total distance travelled for a 90 min period. (B) The inhibitory effect of OXA on LA was observed in the first and second 10 min periods. * indicates $p<0.05$ and # indicates $p<0.01$, compared to vehicle.

These data provide evidence that microinjection of OXA in the midline thalamus inhibited LA induced by cues paired with morphine and in a morphine challenge situation (Figs. 6B and 7B). The inhibitory effect of OXA on LA was only observed in the first 10 to 40 min periods when LA was high while blocking of orexin receptors had no obvious effect on LA in morphine sensitized rats.

3.3. Microinjection of OXA into PVT increases grooming and freezing

One-way ANOVA showed OXA significantly inhibited LA (Fig. 8A; F (1, 19) = 4.10, $p<0.05$) and a tendency to decrease rearing behaviors (Fig. 8B; F (1, 19) = 3.02, $p=0.09$). However, OXA significantly increased grooming duration (Fig. 8C; F (1, 19) = 5.14, $p<0.05$) and freezing duration (Fig. 8D; F (1, 19) = 5.72, $p<0.05$) compared to vehicle group.

4. Discussions

The major purpose of the present investigation was to determine if orexins in the PVT modulate LA in rats tested in states of enhanced arousal. We show using an automated video tracking system that OXA microinjected in or near the PVT of the midline thalamus decreased the LA of rats tested in morphine-naïve and morphine-sensitized

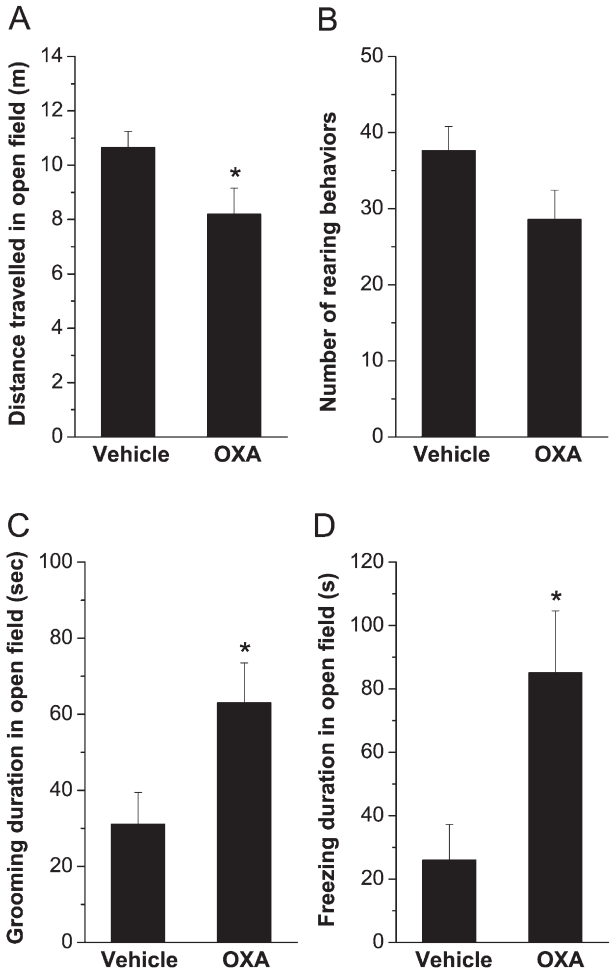

Fig. 8. Effects of microinjections of OXA in the midline thalamus on LA (A), rearing (B), grooming (C), and freezing (D) behaviors in rats tested in the open field. The data show that OXA decreased LA whereas expression of grooming and freezing was enhanced. * indicates $p<0.05$, compared to vehicle.

animals. A consistent observation from all the experiments was that the inhibitory effect of OXA on LA occurred at time points during which LA was high with effects sometimes lasting as long as 40 min post OXA microinjections. Using an approach involving experimenter based analysis of LA and ethological behaviors, we show that microinjections of OXA in the midline thalamus decreased LA while at the same time increasing the expression of grooming and freezing. This suggests that orexins can act on the PVT and midline thalamus to produce behaviors incompatible with high levels of LA.

We propose that the effects on LA of microinjections of OXA in the midline thalamus were mediated mostly by actions of this peptide on orexin receptors in the PVT. This proposal is based on the location of orexin receptors in the midline thalamus, the anatomical relationship between different midline thalamic areas containing orexin fibers and receptors, and the placement of our microinjections. First, the PVT contains the most robust expression of mRNA for both the OX1R and OX2R while the mediodorsal nucleus of the thalamus located immediately lateral to the PVT does not appear to express mRNA for these receptors (Marcus et al., 2001). While other midline and intralaminar nuclei were reported to express a weak mRNA signal for OX1R and a moderate signal for OX2R (Marcus et al., 2001), these thalamic nuclei are located at a least 1 mm or more ventrally or laterally from the injection sites. In fact, the density of orexin receptor mRNA in the midline thalamus is consistent with the density of orexin fibers in the thalamus (Kirouac et al., 2005; Nixon and Smale, 2007) with the PVT appearing to be the most significant target for these peptides. Accordingly, we purposefully targeted our microinjections at the ventral and lateral border of the posterior PVT to avoid damaging neurons of the PVT and to maximize the exposure of the PVT to OXA and SB334867. Substances delivered by pressure through cannulae or micropipettes have a strong tendency to produce more diffusion dorsally in the path of the injector cannula (our personal observations). The placements near the ventral and lateral border of the PVT were seen as advantageous to stimulate orexin receptors in the PVT while minimizing stimulation of receptors in the centromedial and paracentral nuclei. Consistent with this approach, work in our laboratory showed that similar microinjections near the PVT strongly enhance c-Fos expression in the PVT while expression in other midline thalamic area was only slightly enhanced (Kirouac and Li, 2008). It should be emphasized that the view that OXA actions on the PVT attenuate LA does not exclude the role of orexins in other midline thalamic nuclei in the control of LA. The fact that the centromedial and intermediodorsal nuclei also project to similar but different subregions of the striatum and prefrontal cortex (Groenewegen and Berendse, 1994; Van der Werf et al., 2002) would suggest that orexins may have similar functions in all the midline thalamic nuclei.

Previous investigations show that administrations of OXA in the lateral ventricles increased LA, food intake, and grooming in rodents (Espana et al., 2002; Jones et al., 2001; Nakamura et al., 2000; Rodgers et al., 2000) which suggest that orexins are important for controlling behavioral arousal. The present study shows a consistent inhibitory effect on LA following microinjections of OXA in the midline thalamus, an observation that may appear contradictory to the proposed role of orexins in arousal. A previous study reported a decrease in LA immediately after a central administration of a high dose of OXA (Rodgers et al., 2000). This effect lasted approximately 15–20 min from which time normal behavioral activity resumed and, for this reason, these authors suggested that OXA had a short lasting sedative effect (Rodgers et al., 2000). Two observations argue against a sedative effect of OXA in the present study. First, the inhibitory effect of OXA on LA occurred when LA was high and the attenuation lasted longer in conditions where LA was enhanced for a longer period (novel environment and morphine sensitized rats). For example, rats tested in a familiar environment were less active, and in this situation, OXA inhibited LA for only 10 min (Fig. 4B). In contrast, in morphine sensitized rats, LA was enhanced for longer period of time and OXA attenuated LA for up to 40 min (Figs. 6B and 7B). Second, microinjections of the same dose of OXA in the midline thalamus of rats tested in the open field showed a decrease in LA but an enhanced expression of grooming and freezing. Other laboratories have reported that central injections of OXA produced grooming, rearing, and eating in rats (Espana et al., 2002; Jones et al., 2001; Nakamura et al., 2000; Rodgers et al., 2000) which represent behaviors that are likely to compete with ongoing LA. These observations suggest that orexins may act at receptors in the PVT and the midline thalamus to produce arousal of specific behaviors that are independent of LA.

We were also interested in determining if orexins are involved in the expression of behavioral sensitization to repeated systemic injections of morphine. Previous studies have shown that neurons in the PVT are activated by acute administrations of addictive drugs including morphine (Deutch et al., 1998; Garcia et al., 1995; Ryabinin and Wang, 1998) and exposure to environmental cues previously paired with addictive substances (Brown et al., 1992; Dayas et al., 2008; Wedzony et al., 2003). Since the PVT is normally active during periods of arousal (Novak et al., 2000a,b; Novak and Nunez, 1998; Peng et al., 1995), it is reasonable to suggest that the PVT could be activated by the arousal features of addictive drugs. In turn, the PVT could modulate locomotor activity by influencing neural system in the nucleus accumbens, an area of the ventral striatum known to regulate locomotion and drug-seeking behaviors (Mogenson et al., 1980; Nicola, 2007; Pennartz et al., 1994). Accordingly, we predicted that orexin release in the midline thalamus in response to exposure of rats

to cues or to morphine challenges would mediate some of the behavioral sensitization associated with chronic morphine treatment. As shown by the present investigation, this does not appear to be the case since blocking of orexin receptors by microinjections of the OX1R antagonist in the midline thalamus did not attenuate LA to both cue and morphine-induced expression of behavioral sensitization. The inability of microinjections of SB334867 in the midline thalamus to produce an effect on LA in the present study does not appear to be due to an ineffective concentration of the antagonist since the same concentration applied to the ventral tegmental area was shown to attenuate the expression of locomotor sensitization to cocaine (Borgland et al., 2006). It should be pointed out that SB334867 has a stronger affinity to OX1R and that it is possible that blocking of OX2R receptors with a specific antagonist would produce a different result.

Many studies provide evidence that orexins can modulate arousal level (Kukkonen et al., 2002; Sakurai, 2006, 2007; Taheri et al., 2002) and some studies have reported that central injections of OXA increased LA (Espana et al., 2002; Jones et al., 2001; Nakamura et al., 2000; Rodgers et al., 2000). In our own experiments, OXA when injected in the midline thalamus attenuated LA in morphine-naïve and morphine sensitized rats. These results would appear contradictory to what could be expected from central injections of OXA. However, it is possible that orexins released in the midline thalamus during periods of high arousal serve to limit LA to allow the expression of other behaviors. This could represent a mechanism by which ongoing behavioral activity is interrupted to enhance vigilance or attention to situations in the environment. The observation that orexin neurons are activated by stressful or challenging situations is consistent with this hypothesis (Espana et al., 2003; Sakamoto et al., 2004; Watanabe et al., 2005; Zhu et al., 2002). Also consistent with this hypothesis, injections of OXA in the midline thalamus attenuated LA while increased freezing and grooming, two behaviors associated with stressful or fearful situations (Dunn et al., 1979, 1987; Endres et al., 2005; Klemenhagen et al., 2006; Pivina et al., 2007; Roseboom et al., 2007; Spruijt et al., 1992).

The present study suggests that stimulation of orexin receptors in the PVT can decrease ongoing LA possibly by directly modulating neuronal systems that control locomotion (Mogenson et al., 1980; Nicola, 2007; Pennartz et al., 1994) or activating systems that produce behaviors incompatible with locomotion. In addition to modulating locomotion (Mogenson et al., 1980; Nicola, 2007; Pennartz et al., 1994), recent studies indicate that eating behavior can be elicited from the rostral shell of the nucleus accumbens while defensive behavior (defense burying, paw-treading, biting, and emitting distress calls) can be produced from the caudal shell (Reynolds and Berridge, 2001, 2002, 2003, 2008). This suggests that the nucleus accumbens can mediate both appetitive and aversive behaviors depending on the subregions of the nucleus accumbens being modulated. Indeed, the identification of subcompartments with different input/output pattern within the nucleus accumbens provides an anatomical substrate for functionally distinct channels within this area of the striatum (Pennartz et al., 1994). The PVT preferentially projects to the middle to caudal shell of the nucleus accumbens (Berendse and Groenewegen, 1990; Li and Kirouac, 2008) and OXA may act on the PVT to modulate neuronal channels in the caudal shell that mediate defensive or aversive behaviors including the expression of freezing and grooming. From this perspective, a decrease in LA following injections of OXA in the PVT of the midline thalamus is consistent with the role of orexins in arousal. Finally, our findings are likely to translate to other species including humans since a dense orexin innervation has been demonstrated in the PVT of a number of species including non-human primates and humans (Hsu and Price, 2009; Moore et al., 2001). The fact that the orexin system appears to be upregulated in females (Johren et al., 2001, 2002; Taheri et al., 1999) suggests that studying sex-differences produced by the action of orexins in the PVT may also be important in future area of inquiry.

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