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Changes in emotional behavior produced by orexin microinjections in the paraventricular nucleus of the thalamus

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ABSTRACT

The paraventricular nucleus of the midline thalamus (PVT) innervates areas of the extended amygdala known to play a key role in the expression of emotional behaviors. In this study, microinjections of orexins (hypocretins), which have excitatory actions on neurons in the PVT, in the midline thalamus were used to investigate if the PVT modulates the expression of emotional behavior in the open field. First, the approach–avoidance tendency (number and duration of visit to the center area) associated with novelty was examined in orexin treated rats before and after placing a novel object in the center of the open field. Second, the expression of ethological behaviors (rearing, locomotion, freezing, and grooming) in the open field was used to determine the effects of orexins on emotionality. Microinjections of orexin-A (OXA) or orexin-B (OXB) in the PVT decreased exploration of the center area and the novel object indicating that the center area and the object had more aversive properties in orexin treated rats. Both OXA and OXB microinjections in the PVT increased the expression of freezing and grooming behaviors which are indicative of a negative emotional state. The results indicate that microinjections of orexins in the PVT made the test situation more aversive and produced avoidance behaviors. This suggests that orexins may act at the PVT to modulate behaviors associated with a negative emotional state.

1. Introduction

The role of the thalamus in the control of behavioral states has not received as much attention as other regions of the forebrain and midbrain. For example, the midline and intralaminar nuclei of the thalamus are often understood to be important for cortical arousal (Groenewegen and Berendse, 1994) while the contribution of this group of thalamic nuclei to motivational or emotional states remains largely unexplored. Nonetheless, there is growing interest in the midline and intralaminar nuclei because tracing studies have shown that individual members of this group of nuclei project to functionally distinct regions of the cortex and subcortical regions (Groenewegen and Berendse, 1994; Van der Werf et al., 2002). The paraventricular nucleus of the thalamus (PVT) is of special interest because this nucleus provides a unique and very dense projection to the shell of the nucleus accumbens, the bed nucleus of the stria terminalis and the central nucleus of the amygdala (Berendse et al., 1992; Berendse and Groenewegen, 1991; Hsu and Price, 2009; Li and Kirouac, 2008; Vertes and Hoover, 2008), which collectively form an anatomical macrostructure called the extended amygdala (Alheid, 2003; Alheid et al., 1995; Alheid and Heimer, 1988; de Olmos et al., 2004). Recent reviews have presented a compelling case for the extended amygdala as being the key part of the forebrain involved in the modulation of the behavioral and physiological responses associated with motivation and emotions (Davis and Shi, 1999; Heimer, 2003; Koob, 2003). The PVT also innervates areas of the medial prefrontal cortex and basolateral nucleus of the amygdala which in turn exert influence on complex behaviors by way of projections to the extended amygdala (Cardinal et al., 2002).

Review papers on the PVT and other midline and intralaminar nuclei propose that this group of thalamic nuclei have functions related to arousal (Groenewegen and Berendse, 1994; Van der Werf et al., 2002). The fact that several studies using the expression of the immediate early gene c-fos or its protein product have consistently shown that neurons in the PVT are more active during periods of arousal (Novak et al., 2000a; Novak and Nunez, 1998; Novak et al., 2000b; Peng et al., 1995) and strongly activated during aversive conditions (Beck and Fibiger, 1995; Bhatnagar and Dallman, 1998; Bubser and Deutch, 1999; Chastrette et al., 1991; Cullinan et al., 1996; Timofeeva and Richard, 2001) is consistent with an arousal function for the PVT. However, it is unknown if emotional or motivational

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behavior is influenced by an increase in neuronal activity in the PVT. A small number of studies have reported mixed effects on behavior following lesions of the PVT. For example, one study reported that lesions of the PVT did not change baseline locomotor activity but enhanced the locomotor response associated with cocaine (Young and Deutch, 1998). In the same study, lesions of the PVT were found to attenuate the context-induced locomotor responses after the animals were sensitized to cocaine (Young and Deutch, 1998). Another paper reported that lesions of the posterior portion of the PVT resulted in a more pronounced defensive burying in chronically stressed rats suggesting that the PVT may play an important role in dampening anxiety-like behaviors following stress (Bhatnagar et al., 2003). Finally, lesions of the PVT in rats were recently reported to prevent context-induced reinstatement of ethanol intake (Hamlin et al., 2009). While these lesion studies show that the PVT can influence some facet of behavior, it is difficult to make a conclusion about the precise role of the PVT in the modulation of motivated/emotional behavior.

Another approach to examining the role of the PVT in behavior would be to apply excitatory substances to the PVT while observing ongoing behavior. However, one major problem with using this approach is that the PVT is a small midline thalamic structure that extends the full length of the thalamus. This largely makes the PVT inappropriate for pharmacological studies because most drugs or neurotransmitters are likely to bind to receptors located in other midline thalamic nuclei as well as those in the PVT. However, the identification of a discrete distribution of fibers and receptors for orexin (hypocretin) peptides within the midline thalamus represents a means of specifically activating neurons in this area of the thalamus (Date et al., 1999; Kirouac et al., 2005; Marcus et al., 2001; Parsons et al., 2006). Orexins are receiving a large amount of attention for their importance in maintaining states of behavioral arousal (Carter et al., 2009; Sakurai, 2007). The orexin-A (OXA) and orexin-B (OXB) peptide fragments have been shown to produce pronounced excitatory effects on neurons in the PVT (Bayer et al., 2002; Huang et al., 2006; Kolaj et al., 2007), which is consistent with the strong mRNA signal for the orexin-1 (OX1R) and orexin-2 receptor (OX2R) in the PVT (Marcus et al., 2001). In contrast, orexin fibers and receptors are largely absent in nuclei immediately adjacent to the PVT (Date et al., 1999; Kirouac et al., 2005; Marcus et al., 2001). Therefore microinjections of orexins in the midline thalamus represent a viable approach to examining the behavioral effects produced by activation of neurons in the PVT. Using this approach, our laboratory recently reported that microinjections of OXA in the region of the PVT inhibited locomotor activity in morphine naïve and morphine sensitized rats (Li et al., 2009). We also showed that microinjections of OXA caused an increase in the expression of freezing and grooming behavior which may indicate an aversive or fearful response (Li et al., 2009).

In this study, we examined the behavioral effects of microinjections of OXA and OXB in the PVT region of rats tested in the open field. The open field test takes advantage of the fact that there are natural approach and avoidance tendencies that reflect the emotional state of rats placed in a novel environment (Ramos and Mormede, 1998). For instance, rats have an innate motivation to explore a novel environment like the open field (a form of goal-seeking behavior that reflects a positive emotional state) while the center of the open field represents an area that produces some level of avoidance especially in animals expressing a negative emotional state like fear or anxiety (Ramos and Mormede, 1998). We also use the methodology of placing a novel object in the center of the open field after the rat has had some experience with the test environment (Carey et al., 2008; Dai and Carey, 1994) to further examine the effect of activating the PVT region on exploratory/aversive behaviors. In addition, ethological analysis of ongoing behaviors (locomotion, grooming, rearing, and freezing) was combined with traditional spatiotemporal analysis to further examine effects of orexins in the PVT.

2. Methods

2.1. Animals

A total of 68 male Sprague–Dawley rats (Charles River, Beijing, China) weighing 220–240 g were used for this study. The rats were housed individually on a 12 h/12 h light/dark cycle (lights on at 07:00) in a temperature and humidity controlled colony room with food and water available ad libitum. On arrival, rats were handled gently for 5 min every other day to acclimatize them to handling. All tests were performed during the light phrase (08:00–18:00). The experimental procedures were in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985) and the experimental protocol was approved by Research Ethics Review Board of Institute of Psychology, Chinese Academy of Sciences.

2.2. Surgery

Rats were anesthetized with equithesin (0.3 ml/100 g, i.p) to implant a stainless steel guide cannula (23 gauge, Plastics One, Roanoke, VA, USA) unilaterally into the posterior aspect of the PVT region (3.1 mm posterior to bregma, 1.3 mm lateral to the midline, and 4.0 mm ventral to the skull, angle at 10°, with the incisor bar at 3.3 mm below intraaural line). Another group of rats had a guide cannula implanted in the lateral thalamus for control microinjections of OXA (3.1 mm posterior to bregma, 3.0 mm lateral to the midline, and 4.3 mm ventral to the skull, angle at 0°). Three stainless steel screws were attached on the skull to anchor the guide cannula, and dental cement was used to secure the guide cannulae in place. The capped stylets (Plastics One, Roanoke, VA, USA) were inserted to prevent occlusion. The animals were then returned to their individual home cages to recover for 10–14 days during which rats were gently handled every other day to reduce the stress associated with handling. Three days before the behavioral tests, all rats were transferred on a daily basis to the test room where they received mock microinjection to habituate the animals to the microinjection procedure.

2.3. Microinjections

Orexin-A (OXA) and orexin-B (OXB; Tocris, UK), were dissolved in saline and stored in aliquots at −20 °C until the day of the experiment. During the microinjection period, the rat was gently held while the stylet was removed. The drug or vehicle (0.5 μl) was injected through an injector cannula (30 gauge, Plastics One, Roanoke, VA, USA) which protruded 2.0 mm below the guide cannula. An infusion pump mounted with a glass microsyringe was used to deliver the drug at the rate of 0.25 μl/min over 2 min.

2.4. Behavioral tests

With a few modification, an open field (with novel object) procedure was conducted according to previous studies (Carey et al., 2008; Dai and Carey, 1994). Briefly, 5 min after receiving microinjections of vehicle, OXA (3.0 or 10.0 μg) or OXB (3.0 μg) into the PVT region, the rats were placed in a circular open field (1 m diameter, 50 cm high, black floor and grey wall) containing no object in the center area (40 cm in diameter) in the first 10 min (no object test session). Immediately after this period, a metal cylinder object (6 cm in diameter, 10 cm in height) was put into the center area and the behavioral activity of rats was measured for another 10 min (novel object test session). After each test, the open field was cleaned with 2.0% ethanol. As a control, a subgroup of rats was tested using the same procedure following microinjections of OXA (3.0 μg) in the lateral thalamus. The behavioral activity of the rats was recorded by camera suspended 1.5 meters above the open field for subsequent

analysis of locomotor activity, time spent in the center area, and visits to the center area using a commercially available software (Taiji Software Company, Beijing, China). The ethological measures of grooming duration, freezing duration, number of defecation, and number of rearing episodes were also rated and analyzed by two experimenters blind to the treatment. The Pearson coefficients of two observers for the ethological behavioral measures ranged from 0.91 to 0.99.

2.5. Cannula verification

At the end of the experiment, all rats were deeply anesthetized with chloral hydrate (40 mg/kg), perfused transcardially with heparinized saline followed by 4.0% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). Coronal sections from the injection site were obtained at 100 μm using a vibratome. We mapped the locations of the cannula tips in relation to the PVT on brain sections stained immunohistochemically for OXA since previous work in our laboratory showed that OXA fibers define the anatomical boundaries of the PVT (Kirouac et al., 2005). An immunohistochemical staining for OXA fibers was done as previously described (Kirouac et al., 2005).

2.6. Statistical analysis

All data were analyzed using one-way ANOVA (data are shown as mean ± SEM). Post-hoc analysis with Dunnett tests were employed to determine if differences between groups were significant.

3. Results

3.1. Cannula placement

The injector cannulae were targeted towards the posterior half of the PVT or near the boundary of the PVT and the mediodorsal or the intermediodorsal nuclei (Fig. 1). These coordinates were chosen to limit the damage to the PVT caused by the insertion and removal of the injector cannula. In addition, these placements were preferred to prevent the backflow of the injectate into the third ventricle located immediately above the PVT. Work in our laboratory showed that similar microinjections of OXA enhanced c-Fos expression in the PVT (Kirouac and Li, 2008). Microinjections of OXA were also done in the laterodorsal and the posterior thalamic nuclei of the thalamus to evaluate the effect of stimulation orexin receptors lateral to the PVT (placements not shown).

3.2. Effects of microinjection of OXA and OXB in the PVT region on the number and duration of visits to the center area

The effect of microinjections of orexins in the PVT was examined in the same rats tested in the open field without (first 10 min) and with the presence of a novel object in the center area (second 10 min). The one-way ANOVA indicated that there was a significant difference between the groups in the number of visits in both the open field without (Fig. 2A, $F(4,66)=10.166$, $p<0.001$) and with (Fig. 2A, $F(4,66)=4.983$, $p<0.001$) the presence of the novel object. Post-hoc analysis of the data involving rats placed in the open field without the novel object indicated that OXA (3.0 μg, $p<0.05$, $n=12$; and 10.0 μg, $p<0.01$, $n=11$) and OXB (3.0 μg, $p<0.01$, $n=14$) significantly decreased the number of visits. In the presence of the novel object, only rats receiving OXB (3.0 μg, $p<0.01$) showed a significant decrease in the number of visits to the center area. The data also showed that microinjections of 3.0 μg OXB produced more robust effects on the number of visits to the center area than did microinjections of the same ($p<0.01$) or higher concentrations of OXA ($p<0.05$). Similarly, as depicted in Fig. 2B, the one-way ANOVA also revealed a significant difference between groups in the duration of visits without ($F(4,66)=14.356$, $p<0.001$) and with ($F(4,66)=3.252$,

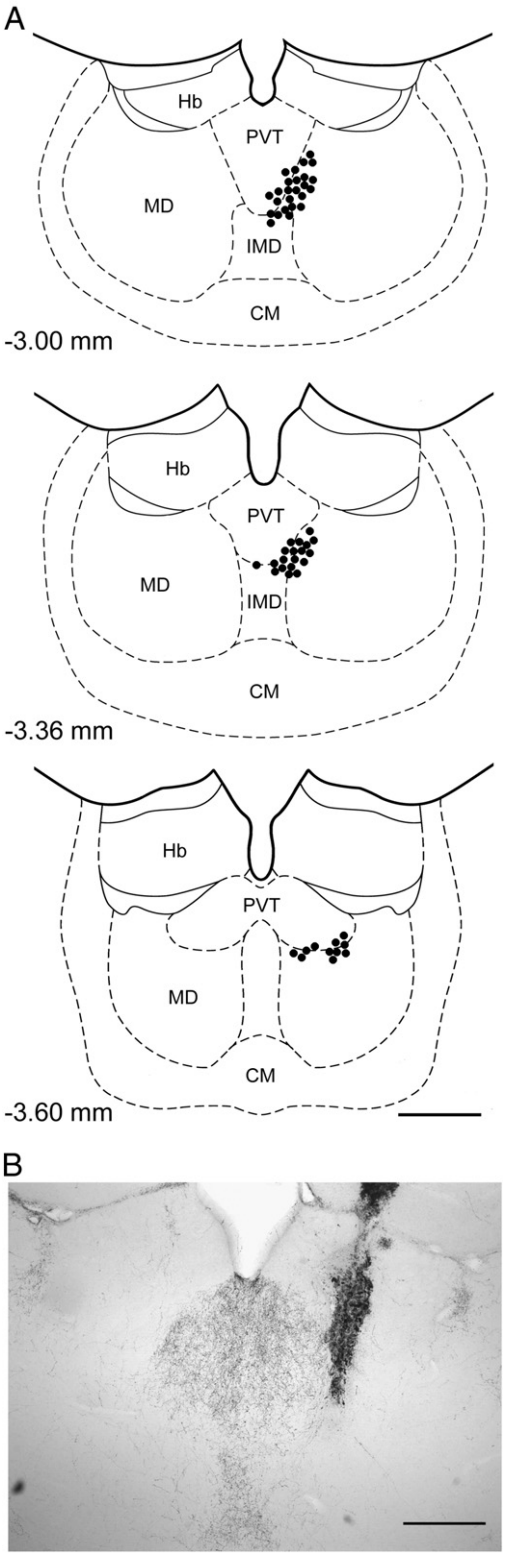

Fig. 1. The figure shows all the microinjection sites in the midline thalamus. (A) The diagram shows the location of the tip of the injector cannula (black dot) in plates of the midline thalamus as adapted from a stereotaxic atlas (Paxinos and Watson, 2005). Note that all the injection sites were in or immediately adjacent to the paraventricular nucleus of the thalamus (PVT). (B) Digital image of the tract produced by the injector cannula located at the outer edge of the PVT as demarcated by orexin-A fiber staining. Numbers on left indicate the rostrocaudal plane posterior to bregma. CM, centromedial nucleus; Hb, habenula; IMD, intermediodorsal nucleus; MD, mediodorsal nucleus. Scale bar, 500 μm.

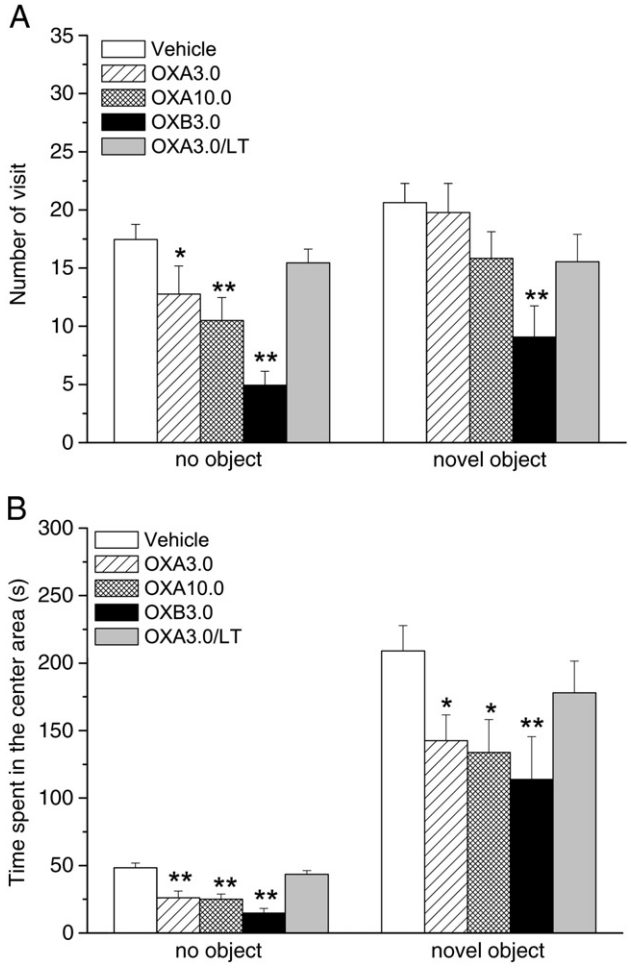

Fig. 2. Effect of microinjections of orexin-A (OXA) and orexin-B (OXB) in the paraventricular nucleus of the thalamus (PVT) region on visits in the center area of the open field. (A) Microinjections of OXA (3.0 and 10.0 μg) and OXB (3.0 μg) in the region of the paraventricular nucleus of the thalamus (PVT) decreased the number of visits to the center area. Only OXB (3.0 μg) decreased the number of visits to the center area when the novel object was present. (B) OXA (3.0 and 10.0 μg) and OXB (3.0 μg) in the PVT region decreased the visit duration to the center without and with the presence of the novel object. Microinjections of OXA (3.0 μg) in the lateral thalamus (LT) had no effect. The values represent mean ± SEM for this and all subsequent figures, * indicates $p<0.05$, ** $p<0.01$, compared to vehicle.

$p<0.05$) the presence of the novel object. Post-hoc analysis demonstrated that rats receiving OXA (3.0 μg, $p<0.05$; and 10.0 μg, $p<0.05$) and OXB (3.0 μg, $p<0.01$) were found to spend less time in the center area without the object. In the presence of the novel object, OXA (3.0 μg, $p<0.01$, and 10.0 μg, $p<0.01$) and OXB (3.0 μg, $p<0.01$) also significantly decreased duration of visits to the center area. Finally, microinjections of OXA approximately 2.5 mm lateral to the PVT were found to have no effect on the number of visits (Fig. 2A, $p>0.05$) and duration (Fig. 2B, $p>0.05$) of the visits to the center area indicating that stimulation of orexin receptors outside the midline thalamus did not produce any apparent behavioral effects in the open field. In summary, the data indicate that stimulation of orexin receptors in the PVT region produces a shift from exploration to avoidance of the central area of the open field.

3.3. Effects of microinjection of OXA and OXB in the PVT region on the expression of ethological behaviors

The expression of ethological behaviors can be quantified to assess the motivational and emotional state of an animal (Ramos and Mormede, 1998). Accordingly, we evaluated the effect of OXA and OXB on a number of ethological measures associated with explorative (locomotion and rearing) and aversive (defecation, freezing and grooming) behaviors.

Rearing and locomotion represent measures of vertical and horizontal investigatory behaviors in the open field (Montgomery, 1955). As shown in Fig. 3A, the one-way ANOVA indicated that there was a significant difference between the treatment groups in the number of rearing episodes without ($F(4,66)=3.842$, $p<0.01$) and with ($F(4,66)=4.105$, $p<0.01$) the presence of the object. Post-hoc analysis of the data before placing the novel object in the center indicated that OXA (10.0 μg, $p<0.05$) and OXB (3.0 μg, $p<0.01$) significantly decreased the number of rearing episodes. In the same way, the results show that after introducing the novel object, OXA (10.0 μg, $p<0.05$) and OXB (3.0 μg, $p<0.01$) significantly decreased the number of rearing episodes. As shown in Fig. 3B, the one-way ANOVA also indicated that there was a significant difference between the treatment groups in locomotor activity in the open field without ($F(4,66)=7.317$, $p<0.001$) and with ($F(4,66)=4.063$, $p<0.01$) the presence of the novel object. Post-hoc analysis of the data without the novel object in the center indicated that OXA (10.0 μg, $p<0.01$) and OXB (3.0 μg, $p<0.01$) significantly decreased locomotor activity. Similarly, with the novel object in the center, OXA (10.0 μg, $p<0.05$) and OXB (3.0 μg, $p<0.01$) significantly decreased locomotor activity. Microinjections of OXA approximately 2.5 mm lateral to the PVT had no significant effect on the rearing behavior (Fig. 3A, $p>0.05$) or locomotor activity (Fig. 3B, $p>0.05$).

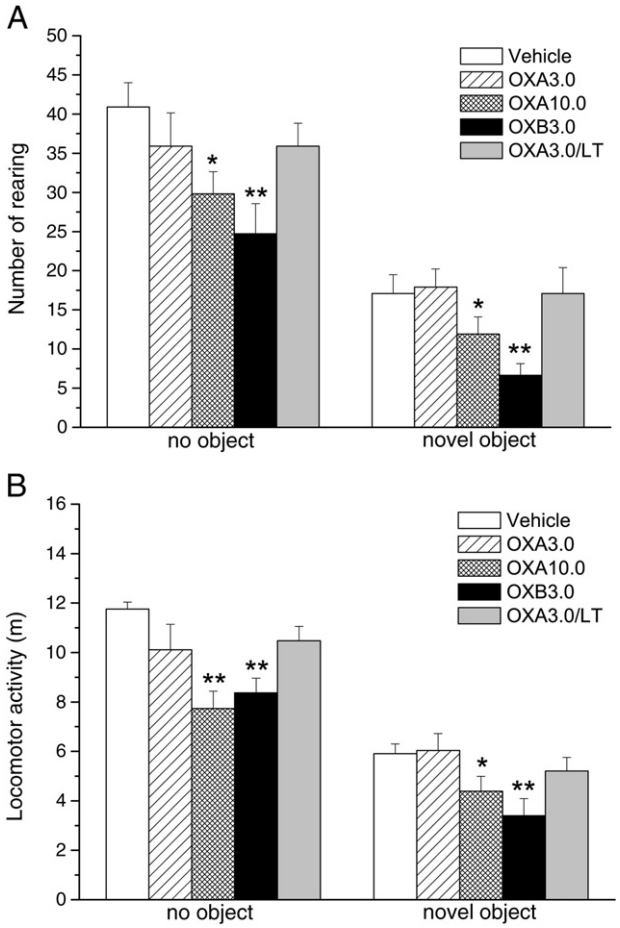

Fig. 3. Effect of OXA or OXB microinjections in the region of the paraventricular nucleus of the thalamus (PVT) on exploratory behaviors in the open field test. (A) Microinjections of OXA (3.0 and 10.0 μg) and OXB (3.0 μg) in the PVT decreased the number of rearing episodes. (B) Microinjections of OXA (3.0 and 10.0 μg) and OXB (3.0 μg) in the PVT decreased locomotor activity. * indicates $p<0.05$, ** indicates $p<0.01$, compared to vehicle.

Defecation, freezing and grooming are usually associated with stressful or conflict situation and represent behavioral correlates of an aversive state (Berridge et al., 1999; Espejo, 1997). As shown in Fig. 4A, the one-way ANOVA indicated that there was a significant difference between the treatment groups in grooming duration without ($F(4,66)=5.866$, $p<0.001$) and with ($F(4,66)=10.525$, $p<0.001$) the presence of the novel object. The post-hoc analysis indicated that OXA (3.0 μg, $p<0.01$; and 10.0 μg, $p<0.05$) significantly increased grooming duration in the open field without a object, and with the novel object in the center, OXA (3.0 μg, $p<0.01$, and 10.0 μg, $p<0.01$) also significantly increased grooming duration. As shown in Fig. 4B, one-way ANOVA also revealed a significant difference between the treatment groups in freezing duration in the open field without ($F(4,66)=6.546$, $p<0.01$) and with ($F(4,66)=6.795$, $p<0.001$) the presence of the novel object. The post-hoc analysis indicated that microinjections of OXA (10.0 μg, $p<0.05$) and OXB (3.0 μg, $p<0.01$) significantly increased freezing duration in the open field without the novel object, whereas only OXB (3.0 μg, $p<0.01$) increased freezing duration in the presence of the novel object. Microinjections of OXA approximately 2.5 mm lateral to the PVT were found to have no significant effect on the grooming duration (Fig. 4A, $p>0.05$) or freezing duration (Fig. 4B, $p>0.05$). Finally, no significant differences in the defecation number were found between the different treatment groups ($F(4,66)=0.758$, $p>0.05$; data not shown).

In summary, the ethological data are consistent with the spatiotemporal results. Rats receiving OXA and OXB showed a decrease in locomotor activity and rearing, which is consistent with the decrease in exploration of the open field and novel object. In contrast, OXA and OXB increased grooming and freezing, which is consistent with the avoidance of the center of the open field and novel object.

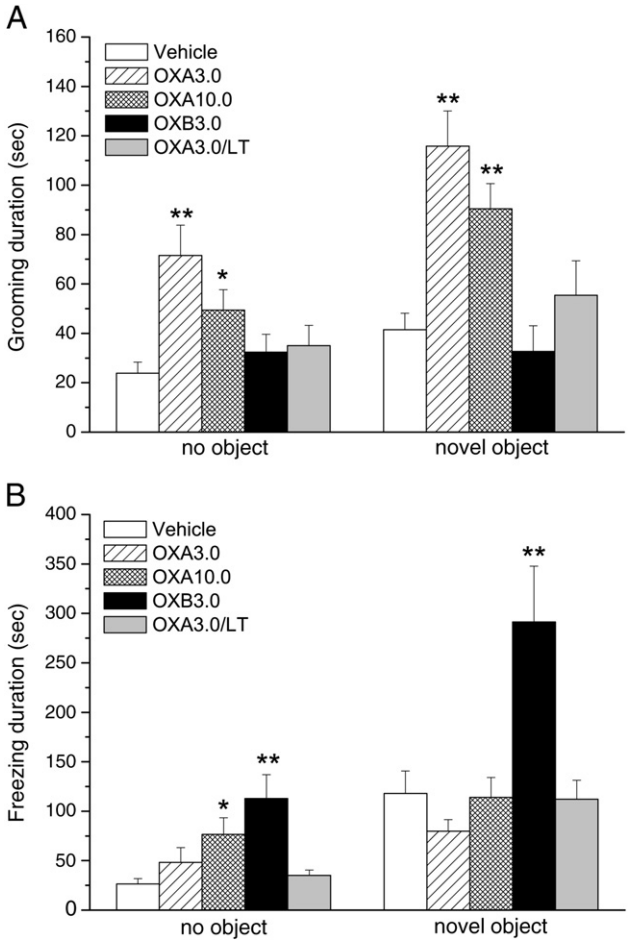

Fig. 4. Effect of OXA or OXB microinjections in the region of the paraventricular nucleus of the thalamus (PVT) on grooming and freezing behaviors in the open field test. (A) Microinjections of OXA (3.0 and 10.0 μg) in the PVT increased grooming time for both the "no object" and the "novel object" test periods whereas OXB (3.0 μg) had no effect. (B) Microinjections of OXB (3.0 μg) in the PVT increased freezing time for both the "no object" and the "novel object" test periods whereas the higher concentration of OXA (10.0 μg) increased freezing time only during the "no object" test session. * indicates $p<0.05$, ** indicates $p<0.01$, compared to vehicle.

3.4. Correlation between explorative and aversive behaviors

In the open field, the center area represents a novel environment that can elicit both approach and avoidance behaviors. At first, rats move around the wall area and largely avoid the center area. Over time, the animal will approach the center area briefly only to retreat back towards the wall with this pattern repeated over test period (approach–avoidance conflict). Grooming and freezing behaviors normally occur when the rat retreats from exploring the central area of the open field. Consequently, a Pearson correlation analysis was used to examine the effect of microinjections of OXA and OXB in PVT region on the relationship between the exploration (measured as the time spent in the center) and aversive behaviors (grooming or freezing time). As depicted in Fig. 5, there is no significant correlation between the time spent in the center and grooming (upper panel of Fig. 5A, $r^2=0.018$, $p=0.469$) or freezing time (lower panel of Fig. 5B, $r^2=0.107$, $p=0.322$) in animals receiving vehicle injection. However, a significant negative correlation between time spent in the center and grooming was observed after the rats treated with OXA (10.0 μg, $r^2=-0.559$, $p<0.05$) and OXB (3.0 μg, $r^2=-0.554$, $p<0.05$), and a trend but not significant correlation in rats injected with OXA (3.0 μg, $r^2=-0.458$, $p=0.06$). As shown in lower panel of Fig. 5B, a significant negative correlation between time spent in the center and freezing was also observed after the rats were treated with OXA (3.0 μg, $r^2=-0.583$, $p<0.05$; 10 μg, $r^2=-0.560$, $p<0.05$) and OXB (3.0 μg, $r^2=-0.499$, $p<0.05$). The correlation analysis indicates that microinjections of orexins in the PVT region shifted the emotional state of the rats to a more negative valence.

4. Discussion

It is well known that the medial prefrontal cortex, basolateral amygdala, subiculum of the hippocampus and dopamine neurons of the ventral tegmental area, all of which represent areas of the brain that provide significant input to the extended amygdala, are important for regulating various facets of motivational and emotional behaviors (Cardinal et al., 2002). In contrast, little information is available concerning the influence of the PVT on complex behaviors despite the fact that the PVT provides one of the most impressive inputs to the extended amygdala (Berendse et al., 1992; Berendse and Groenewegen, 1991; Hsu and Price, 2009; Li and Kirouac, 2008; Vertes and Hoover, 2008). The PVT is commonly believed to play a role in arousal (Groenewegen and Berendse, 1994) and the dense orexin innervation of PVT is consistent with that view (Kirouac et al., 2005). The present study was done to determine the effect of microinjections of orexins in the PVT on innate motivational and emotional behaviors expressed in an open field. The experiments show that microinjections of both OXA and OXB in the PVT decreased exploration in the open field while increasing behavioral indicators of an aversive state (grooming and freezing). These behavioral effects were also observed after placing a novel object in the center of the open field. We interpret the pattern of behavioral responses produced by microinjections of orexins in the PVT as evidence for enhanced emotionality. We propose that the PVT may function as an emotional arousal system which predominantly enhances negative emotions.

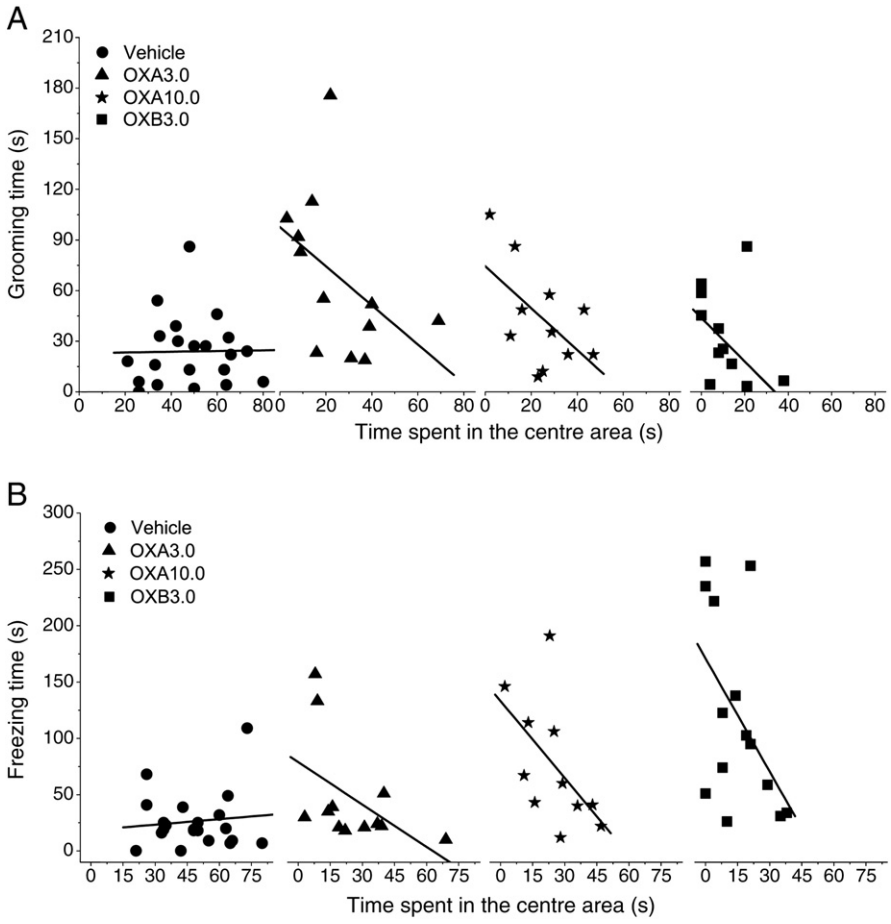

Fig. 5. Correlations between the time spent in the center area in open field and the grooming or freezing time after microinjections of OXA and OXB in the region of the paraventricular nucleus of the thalamus (PVT). (A) There is no correlation between the visit time and grooming time in vehicle group, but microinjection of OXA (3.0 and 10.0 μg) and OXB (3.0 μg) in the PVT produced a significant negative correlation between visit time and grooming time. (B) While there is no correlation between the visit time and freezing time in vehicle group, microinjection of OXA (3.0 and 10.0 μg) and OXB (3.0 μg) in the PVT produced a significant negative correlation between visit time and freezing time.

The dorsal and midline region of the posterior thalamus is composed of several nuclei which could have mediated the effects of orexins observed in the present study. While not completely excluding a role for other midline nuclei, the PVT appears to be the most likely candidate for mediating the behaviors observed in the present study. First, orexin fibers uniquely target the PVT and avoid other thalamic nuclei adjacent to the PVT (Date et al., 1999; Kirouac et al., 2005). Second, the PVT contains a moderate expression of mRNA for the OX1R and strong expression of mRNA for the OX2R whereas the intermediodorsal nucleus, mediodorsal and habenular complex, which are immediately in the vicinity of the microinjection zone, contain at best a weak signal for orexin receptors (Marcus et al., 2001). The comparatively high density of orexin receptors in the PVT in addition to the fact that it is unlikely that the orexin peptides would have diffused more than 1 mm away from the injection site (Thorpe et al., 2003) suggests that the PVT mediated most of the behavioral effects of orexins in our experiments. Electrophysiological studies showing that OXB has a greater postsynaptic excitatory effect on neurons in the PVT than does OXA (Bayer et al., 2002; Huang et al., 2006; Ishibashi et al., 2005) are consistent with the strong expression of OX2R mRNA in the PVT (Marcus et al., 2001) and the robust behavioral effects produced by OXB.

Our experiments show that microinjections of OXA and OXB in the PVT decreased exploration of the center area of the open field. Another way of stating these effects is to say that orexins in the PVT increase avoidance of the central area (Ramos and Mormede, 1998). Likewise, rats receiving OXA and OXB in the PVT region showed less exploration and more avoidance of the novel object placed in the open field. We interpret the results of the spatiotemporal data produced by microinjections of orexins in the PVT as evidence for a shift in the balance of the innate exploration-avoidance behavioral tendency towards favoring avoidance. Furthermore, analysis of the effects of orexins in the PVT on ongoing ethological behaviors showed that locomotion and rearing, which represent behaviors normally associated with exploration (Carey et al., 2008; Dai and Carey, 1994), were decreased in the orexin treated animals. We previously reported that administrations of OXA in the PVT region produced a decrease in locomotion in morphine-naïve and -sensitized rats (Li et al., 2009). As such, the argument could be made that the orexin treated rats spent less time exploring the center area of the open field because of an inhibition in locomotor activity or a motor impairment. However, this does not appear to be the case in the present study because the 3 μg dose of OXA significantly decreased the number of visits and time spent in the center area but did not produce significant changes in locomotor activity. Furthermore, microinjections of orexins in the PVT region increased freezing and grooming, behaviors that are associated with a fear or aversive 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 increase in grooming produced by orexins in the PVT observed in this paper and our previous study (Li et al., 2009) also argues against the possibility that orexins produced sedative effects. As a result, we interpret the pattern of behaviors expressed in rats receiving OXA and OXB in the PVT as indicative of a heightened anxiety state (Ramos and Mormede, 1998). The behavioral effects produced by orexins in the present study is consistent with a previous study showing that administration of OXA in the

cerebral ventricles produce anxiety-like responses in mice (Suzuki et al., 2005).

Placing a novel object in the open field enhanced the amount of grooming and freezing in vehicle treated rats, which supports the view that the novel object had the ability to induce a mild aversive state (referred to as neophobia). It is interesting to note that the increases in grooming and freezing behaviors produced by the novel object were found to be enhanced by microinjections of orexins in the PVT. However, discrepancies were observed with respect to the effects of OXA and OXB on spatiotemporal and ethological behaviors expressed in the presence of the novel object. First, the OXA peptide (3.0 and 10.0 μg) increased grooming when a novel object was placed in the open field whereas OXB (3.0 μg) had no effect. In contrast, OXB (3.0 μg) increased freezing while OXA (3.0 and 10.0 μg) did not have any influence on the expression of this behavior. However, the observation that OXB was more potent in producing freezing is consistent with the decreases in the number and duration of visits to the center area produced by this peptide fragment. It is likely that the increase expression of freezing behavior interfered with the expression of grooming behavior. Following the same logic, the increase in grooming produced by OXA peptide is seen as being consistent with the observation that this peptide produced less avoidance of the center area (hence less freezing). The observations that OXA or OXB produced either grooming or freezing suggest that orexin peptides when administered in the PVT region produced some level of fear or anxiety. As a result, we interpret the pattern of behaviors produced in the open field following microinjections of orexins in the PVT as indicative of an enhanced level of emotionality.

Neurons in the PVT are more active during periods of wakefulness (Novak and Nunez, 1998) and strongly activated following exposure of animals to stressful/aversive situations (Bhatnagar and Dallman, 1998; Bubser and Deutch, 1999) including conditioned fear (Beck and Fibiger, 1995). This, in addition to the fact that the PVT is well positioned anatomically to influence the activity of the key regions of the forebrain involved in emotions (Hsu and Price, 2009; Li and Kirouac, 2008; Vertes and Hoover, 2008), suggests that the PVT may function in the modulation of emotional behaviors. As recently discussed by Pfaff, increases in emotional and behavioral reactivity normally accompany states of heightened arousal (Pfaff, 2006). Endogenously released orexins could act on the PVT and the midline thalamus to enhance the emotional state associated with different levels of arousal. For example, posttraumatic stress disorder (PTSD) is an affective disorder where cues or memories of a traumatic event produce a hyperaroused state that elicits negative emotional and behavioral responses such as avoidance (Frewen and Lanius, 2006; Yehuda and LeDoux, 2007). Bhatnagar et al. have also clearly established that the PVT inhibits the activity of the hypothalamic-pituitary-adrenal (HPA) axis to the same stressor in chronically stressed rat (Bhatnagar and Dallman, 1999; Bhatnagar et al., 2002; Bhatnagar et al., 2000; Jaferi and Bhatnagar, 2006; Jaferi et al., 2003). Since activity of the HPA is an important modulator of behavior (de Kloet, 2003), the influence of the PVT on the HPA represents another mechanism by which the PVT can influence emotional behaviors. While more research will be required to provide an unequivocal support for a role for the PVT in mediating behaviors expressed during a negative emotional state, anatomical and behavioral evidence suggests a possible role for the PVT.

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