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ORIGINAL INVESTIGATION

Orexins in the paraventricular nucleus of the thalamus mediate anxiety-like responses in rats

Yonghui Li · Sa Li · Chuguang Wei · Huiying Wang · Nan Sui · Gilbert J. Kirouac

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Abstract
Rationale Anatomical studies have shown that the paraventricular nucleus of the thalamus (PVT) innervates areas of the forebrain involved in the expression and regulation of emotional behaviors including fear and anxiety. In addition, the PVT is densely innervated by fibers containing orexin-A (OXA) and orexin-B (OXB), peptides that are well-known for their arousal effects on behavior.
Objectives In this study, we investigate whether microinjections of orexin receptor agonists and antagonists in the PVT region alter expression of anxiety-like behaviors in the rat as measured in the elevated plus maze.
Results We report that microinjections of OXA and OXB in the PVT region elicited anxiety-like response as indicated by a reduction in open arm time and entries. In addition, OXA and OXB produced changes in ethological measures indicative of an anxiety state. Central administrations of antagonists for corticotropin releasing factor (CRF) or the opioid kappa receptors attenuated the anxiogenic effects produced by microinjections of OXA in the PVT region. We also provide evidence that endogenously released orexins act at the PVT to produce anxiety by showing that microinjections of TCSOX229, an orexin-2 receptor antagonist, in the PVT region attenuated the anxiogenic effects produced by a previous exposure to footshock stress.
Conclusions This study indicates that endogenously released orexins act on the PVT to regulate anxiety levels through mechanisms involving the brain kappa and CRF receptors.

Keywords Orexins · Hypocretins · Arousal · Midline thalamus · Anxiety · Kappa · CRF

Y. Li · C. Wei · H. Wang · N. Sui
Key Laboratory of Mental Health, Institute of Psychology, Chinese Academy of Sciences, Beijing 100101, China

Y. Li · S. Li · G. J. Kirouac (✉)
Department of Oral Biology, University of Manitoba, 780 Bannatyne Avenue, Winnipeg, MB R3E 0W2, Canada
e-mail: kirouac@cc.umanitoba.ca

Introduction

The midline and intralaminar nuclei of the thalamus consist of a group of nuclei that are proposed to mediate arousal of the cerebral cortex (Groenewegen and Berendse 1994). There is growing interest in this group of thalamic nuclei because specific nuclei within this group can also selectively interact with functionally distinct cortical–subcortical circuits (Van der Werf et al. 2002). For instance, the paraventricular nucleus of the midline thalamus (PVT) provides a dense input to parts of the so-called extended amygdala, which includes the shell of the nucleus accumbens, dorsolateral region of the bed nucleus of the stria terminalis, and the central nucleus of the amygdala (Hsu and Price 2009; Li and Kirouac 2008; Parsons et al. 2007; Vertes and Hoover 2008). The PVT also provides a moderate input to the prefrontal cortex and basolateral amygdala, which in turn project to the same regions of the extended amygdala innervated by the PVT (Hsu and Price 2009; Li and Kirouac 2008; Vertes and Hoover 2008). The medial prefrontal cortex and the basolateral amygdala, along with the parts of the extended amygdala innervated by these cortical regions, represent an important cortical–subcortical circuit involved in emotional behaviors (Cardinal et al. 2002; Walker et al. 2003), all of which can be influenced by the PVT. In addition, afferents from the PVT overlap with

subregions of the extended amygdala that contain neurons densely stained for dynorphin and corticotropin releasing factor (CRF) (Kirouac and Li 2008; Li and Kirouac 2008), two peptides strongly implicated in the expression of negative emotional states (Davis 1998; Davis et al. 2010; Heinrichs and Koob 2004; Shirayama and Chaki 2006).

Orexins (hypocretins), peptides produced exclusively in neurons of the posterior hypothalamus, have received a tremendous amount of attention for their importance in maintaining states of behavioral arousal (Carter et al. 2009; Sakurai 2007), including arousal associated with appetitive behaviors (Boutrel and de Lecea 2008; Harris and Aston-Jones 2006; Harris et al. 2005; Rodgers et al. 2002). Reports showing that orexin neurons are more active during the wake period, active exploration and drug seeking (Dayas et al. 2008; Espana et al. 2003; Estabrooke et al. 2001; Harris et al. 2005; Mileykovskiy et al. 2005) are consistent with a behavioral arousal role for this peptide. In addition, orexin neurons have been reported to be more active following exposure of animals to stress (Espana et al. 2003; Furlong et al. 2009), which suggests that orexins may also serve as a neurochemical signal that controls the arousal levels involved in the expression of behavioral and physiological responses to stressful situations.

Of all the thalamic nuclei, the PVT is especially notable for its very dense orexin innervations and receptor expression (Kirouac et al. 2005; Marcus et al. 2001). The orexin-A (OXA) and orexin-B (OXB) peptide fragments have been shown to produce potent excitatory effects on most neurons in the PVT by actions involving the orexin-2 receptors (OX2R) (Bayer et al. 2002; Huang et al. 2006; Kolaj et al. 2007). Studies showing that neurons in the PVT are activated during states of arousal and following stress (Bhatnagar and Dallman 1998; Chastrette et al. 1991; Hamlin et al. 2009; Novak and Nunez 1998; Peng et al. 1995; Timofeeva and Richard 2001) are supportive of a functional relationship between the orexin system and the PVT. In fact, we recently reported that microinjections of OXA in the PVT region of rats increased freezing and grooming (Li et al. 2009), behaviors associated with fearful and aversive situations. We also reported that microinjections of both OXA and OXB in the PVT region produced an anxiety-like response in animals tested in an open field (Li et al. 2010). As such, these pharmacological studies suggest that orexins may act on the PVT to regulate negative emotional behavior.

In this paper, we show that microinjections of orexins in the PVT region of rats produced anxiety in the elevated plus maze (EPM), a well-established and validated test for measuring anxiety level in rodents (Walf and Frye 2007). We also show that the anxiogenic effects produced by orexins in the PVT region are prevented by the central administrations of kappa opioid and CRF receptor antagonists. Finally, we demonstrate that blocking of the OX2R in the PVT region attenuates the anxiety state produced in rats previously exposed to a shock stress.

Materials and methods

Animals

Male Sprague–Dawley rats (210–230 g, Charles River, Beijing, China) were housed on a 12 h/12 h light/dark cycle (lights on at 07:00 A.M.) with controlled temperature (20–24°C) and humidity (40–70%). 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 Regulations for the Administration of Affairs Concerning Experimental Animals (China, 1988). The experimental protocol was approved by the Research Ethics Review Board of Institute of Psychology, Chinese Academy of Sciences.

Surgery

Rats were anesthetized with equithesin (0.3 ml/100 g, i.p.) and placed in a Stoelting stereotaxic frame. A stainless steel guide cannula (23 gauge, Plastics One, VA, USA) was 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, angle at 10°, with the incisor bar at 3.3 mm below intraaural line). Some rats had a second cannula implanted in the lateral ventricle (LV; 0.8 mm posterior to bregma, 1.4 mm lateral to midline, and 2.5 mm ventral to the skull). Another subgroup of rats had a single guide cannula implanted in the lateral thalamus (3.1 mm posterior to bregma, 3.0 mm lateral to the midline, and 4.3 mm ventral to the skull). Stainless steel screws and dental cement were used to secure the guide cannula in place. A capped stylet (Plastics One, VA, USA) was inserted to prevent occlusion and all rats were treated with penicillin (80,000 units) after surgery. Rats were allowed to recover for 10–14 days, during which they were handled every other day to reduce stress associated with handling at the time of testing.

Drugs and microinjections

The OXA and OXB peptides (Tocris, UK), the non-selective CRF antagonist α-helical CRF (hCRF, Tocris), the kappa receptor antagonist norbinaltorphimine (norBNI; Tocris) and the OX2R antagonist TCSOX229 (TCS, Tocris) were dissolved in saline. The OX1R antagonist SB334867 (SB, Tocris) was dissolved in DMSO (Sigma, USA).

Previous studies using an avoidance memory task have shown that DMSO does not alter the antagonist properties of SB (Akbari et al. 2006, 2007, 2008) nor that it produces behavioral effect when compared to saline (Naghdi and Asadollahi 2004). Drug solutions were stored in aliquots at −20°C and thawed immediately before the microinjections. A mock microinjection was given to the rats each day in the test room for 3 days before the actual experiment to habituate the animals to the injection procedure. The drug or vehicle (0.5 μl for the PVT and 2.0 μl for the LV) was injected through an injector cannula (30 gauge, Plastics One, USA) which protruded 2.0 mm below the guide cannula. Infusions were delivered with a Hamilton microsyringe mounted on a motorized pump (Stoelting Co, IL, USA). The injection was done at the rate of 0.25 μl/min over 2 min. The stylet was inserted back into the guide cannula and the rat was returned to its home cage for 5 min before being placed in the EPM.

Behavioral testing

Subjects from the same group were tested in the EPM at different times of the light phase (8:00–18:00). The EPM (Med Associates, VT, USA) was composed of black Plexiglass and consisted of two open arms (50 cm×10 cm×0.5 cm) and two closed arms (50 cm×10 cm×40 cm) with all the arms extended from a center area (10 cm×10 cm). The maze was elevated 50 cm above the floor and placed in a dimly lit room with two 15-W bulbs. The illumination in open and closed arms was between 2.0–5.0 lx and less than 0.5 lx, respectively. This level of illumination is less anxiogenic and results in rats spending approximately an equal amount of time in the open and closed arms (Zorrilla et al. 2002).

With a few modifications, the EPM test was conducted as described previously (Walf and Frye 2007). Briefly, a rat was placed in the center area of the EPM facing one of the closed arms and the movement of the rat was tracked by infrared sensors and video camera for 5 min. After each test, the maze was cleaned with 2.0% ethanol and dried to prevent interference of subsequent tests by olfactory cues. The time spent in open and closed arms, as well as the number of entries to open and closed arms, were recorded using software (Med Associates, VT, USA). The data were converted into the percent open arm time (time in open arms/time in open arms + time in closed arms) and percent open arm entry (numbers of entries to open arm/numbers of entries to open arm + numbers of entries to closed arms), both of which represent indices of anxiety (Pellow et al. 1985; Rodgers and Johnson 1995). The number of entries into the closed arms was used as a measure of locomotor activity as previously done (Cruz et al. 1994; Espejo 1997; File 2001).

Ethological behavior analysis

The expression of ethological behaviors in the EPM is also useful for assessing anxiety levels (Carobrez and Bertoglio 2005; Hogg 1996; Rodgers et al. 1997). The following behavioral variables were quantified from video records of the test: (1) latency to enter the open arms (open arm latency); (2) duration of head scanning outside of a closed arm with part of the body remaining in the closed arms (scanning time); (3) number of episodes where body and head are stretched forward (stretch-attend postures, SAP); (4) time spent exploring the end of the open arm (end arm time); (5) time spent dipping the head down from the open arms and center area (head dipping time); (6) grooming duration in closed arms (grooming time); and (7) number of defecations (defecation number). The behavioral analysis was done by two experimenters blind to the group identity of the subjects (the correlation coefficient for two observers ranged from 0.91–0.97).

Experiment 1: effect of microinjections of OXA and OXB in the midline thalamus on anxiety

Three different concentrations of OXA (0.3 μg/0.08 nmol, 3.0 μg/0.8 nmol and 10.0 μg/2.8 nmol), OXB (3.0 μg/1.0 nmol) or the vehicle were microinjected in the midline thalamus 5 min prior to the behavioral test. A subgroup of rats was tested in the EPM following microinjections of OXA (3.0 μg) in the lateral thalamus.

Experiment 2: effect of CRF and kappa opioid receptor blockade on anxiety produced by OXA microinjections in the midline thalamus

The CRF antagonist hCRF (1.0 μg/0.26 nmol) was infused in the lateral ventricles 30 min before receiving a microinjection of OXA (3.0 μg) in the midline thalamus. Since there is a debate as to when norBNI should be given to completely block the kappa receptors (Knoll et al. 2007; Land et al. 2008), we chose to infuse norBNI (10.0 μg/13.6 nmol) in the lateral ventricles at both 24 h and 30 min before the OXA microinjections. Another experiment in which hCRF, norBNI, or saline was given in the lateral ventricles and saline in the PVT region was done to determine if the CRF and kappa antagonists produce nonspecific effects on EPM behaviors.

Experiment 3: effect of blocking orexin receptors in the PVT region on anxiety

Sixty rats were placed in a shock chamber (Med Associates) for 180 s before receiving a single intense foot shock (2.0 mA, 10 s, n=30) or no shock (n=30). Previous studies

showed that rats exposed to similar shock subsequently expressed anxiety for up to 2 weeks (Louvart et al. 2005, 2006). Subsequently, we examined the effect of microinjections of OX receptor antagonists in the PVT region on anxiety-like behaviors in the EPM 2 days after shock exposure. The OX1R antagonist SB334867 (10.0 μg/31.3 nmol), OX2R antagonist TCSOX229 (10.0 μg/23.0 nmol), or vehicle were injected into the PVT region 15 min prior to testing the animals in the EPM. The antagonist SB334867 has a 50-fold selectivity for OX1R compared to OX2R whereas TCSOX229 has a 250-fold selectivity for the OX2R over the OX1R (Duxon et al. 2001; Hirose et al. 2003). Similar doses of orexin antagonists were effective at blocking some of the behavioral effects of orexins (Chang et al. 2007; Hollander et al. 2008).

Cannula verification

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 verified the locations of the cannula tips in relation to the PVT on brain sections stained immunohistochemically for OXA as done previously and described by our laboratory (Kirouac et al. 2005).

Statistical analysis

All data were analyzed using one- or two-way ANOVA when appropriate (data are shown as mean ± SEM) and with post-hoc Dunnett analysis to determine if differences between groups were significant. Rats with cannula placements that were more than 0.5 mm away from the PVT were excluded from the analysis.

Results

Cannula placement

The injector cannulae were targeted towards the posterior half of the PVT at the boundary of the PVT and the mediodorsal nucleus or the intermediodorsal nucleus of the thalamus (Fig. 1). Placements that were within the PVT or immediately adjacent to the PVT (<0.5 mm from the PVT) were considered as successful placements. The data from a few subjects were discarded because the cannula placements did not meet this criterion (n=3). These coordinates were chosen to limit the damage to the PVT caused by the insertion and removal of the injector cannula. Similar microinjections of OXA near the PVT strongly enhanced c-Fos expression in the PVT, while the expression in other midline thalamic areas was only slightly enhanced (Kirouac and Li 2008). These placements were also done to prevent the backflow of injectate into the third ventricle located immediately above the PVT. We did some OXA microinjections in the laterodorsal and the posterior thalamic nuclei of the lateral thalamus (placements not shown) to evaluate the effect of stimulation of the orexin receptors outside the PVT and the midline thalamus.

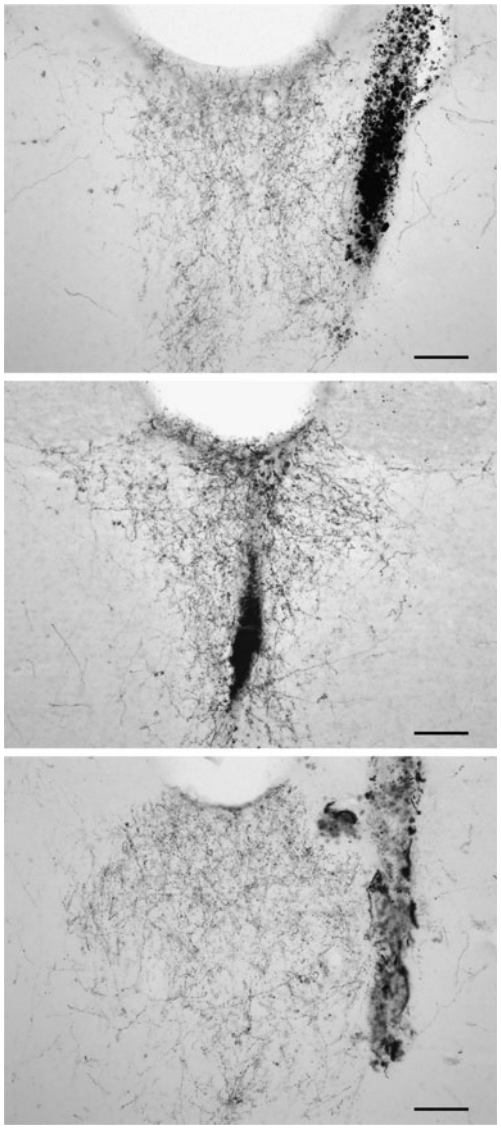

Fig. 1 Digital images of three examples of injector cannula placements in or immediately adjacent to the paraventricular nucleus of the thalamus (PVT) in sections stained for orexin-A. As we previously reported, orexin-A fibers clearly show the boundaries of the PVT within the midline thalamus (Kirouac et al. 2005; Li and Kirouac 2008). *Scale bar* 500 μm

Microinjections of OXA and OXB into midline thalamus produce anxiety-like effects in the EPM

The effect of OXA microinjections in the midline thalamus (0, 0.3, 3.0, and 10.0 μg, n=10 to 11) or lateral thalamus (3.0 μg, n=13) on the behavioral activity of rats placed in the EPM was assessed. The ANOVA revealed a significant main effect for treatment on the percent open arm time (Fig. 2a, $F_{(5,64)}$=7.177; p<0.001) and the percent open arm entry (Fig. 2b, $F_{(5,64)}$=4.682, p<0.01). Post-hoc analysis showed that the percent open arm time (p<0.001, p<0.001) and the percent open arm entry (p<0.05, p<0.01) were significantly decreased in rats treated with the higher concentrations of OXA (3.0 and 10.0 μg) when compared to the vehicle treated group (Fig. 2a and b). There were no significant differences between rats receiving the low concentration of OXA (0.3 μg) and saline (p>0.05). Since OXA binds with equal strength to both the OX1R and the OX2R, whereas OXB binds preferentially to the OX2R, we examined the effect of stimulating OX2R by microinjecting OXB (3.0 μg, n=14) in the midline thalamus. Similar to the higher concentrations of OXA, administrations of OXB (3.0 μg) in the midline thalamus resulted in a reduction in the percent open arm time (p<0.01) and the percent open arm entry (p<0.01). These results indicate that OXA and OXB microinjections in the PVT region produce anxiogenic effects in the EPM.

The behavioral effect of microinjections of OXA (3.0 μg) into the lateral thalamus was measured and compared with microinjections of OXA or vehicle in the midline thalamus. The post-hoc comparison revealed that OXA in the lateral thalamus had no effect on the percent open arm time and percent open arm entry compared to the vehicle group (Fig. 2a and b). The post-hoc analysis also indicated that OXA in the midline thalamus decreased the percent open arm time and percent open arm entry compared to microinjections of OXA in the lateral thalamus (p<0.01, p<0.05). As such, the results indicate that the behavioral effects of microinjections of OXA and OXB in the midline thalamus were due to stimulation of orexin receptors in the midline thalamus.

We have previously reported that administrations of OXA in the PVT region can decrease locomotor activity in some open field conditions (Li et al. 2009). Even so, there were no significant differences between any of the treatments on the number of entries in the closed arm (Fig. 2c, $F_{(5,64)}$=1.714, p>0.05), a measure of locomotor activity in the EPM (Cruz et al. 1994; Espejo 1997; File 2001). This indicates that the anxiety-like effects produced by microinjections of OXA and OXB into midline thalamus were not caused by impairment of locomotion.

Besides the traditional spatiotemporal measures, such as the time spent in the open arm and the number of entries in the open arm, rodents can exhibit a number of other behaviors in the EPM that can be related to the emotional state of the test subject. These behaviors can also be quantified to improve the reliability and validate the traditional measurement of anxiety on the EPM (Carobrez and Bertoglio 2005; Cruz et al. 1994; Moser 1989; Rodgers et al. 1997; Rodgers and Johnson 1995). Accordingly, we evaluated the effect of OXA and OXB on a number of ethological measures associated with emotionality, such as hesitation, risk assessment, exploratory behaviors and displacement behaviors. In all of our experiments, we evaluated the open arm latency, scanning time, SAP number, end arm time, head dipping time, grooming time, and defecation number. These behavioral measures are believed to represent different aspects of the anxiety state of

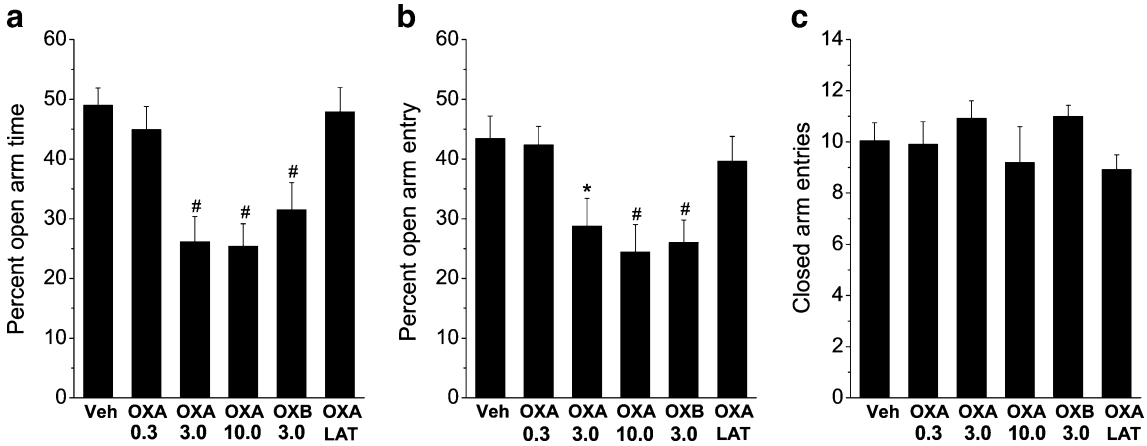

Fig. 2 Effects of orexin-A (*OXA*) or orexin-B (*OXB*) microinjections in the paraventricular nucleus of the thalamus (*PVT*) region and lateral thalamus (*LAT*) on anxiety assessed by spatiotemporal measurement in the elevated plus maze. Effects of OXA (0.3, 3.0 and 10.0 μg), OXB (3.0 μg), or vehicle (*Veh*) in the PVT region and LAT on percent open arm time (**a**), percent open arm entry (**b**), and closed arm entries (**c**). The values represent mean ± SEM for this and all subsequent figures. *indicates p<0.05 and $^{\#}p$<0.01 compared to vehicle, n=10 to14 for each group

the test animal as measured on the EPM (Ohl et al. 2001; Ramos and Mormede 1998).

Increases in open arm latency and scanning time are hypothesized to be measures of hesitation in an approach-avoidance conflict, as well as the avoidance of unprotected areas in the EPM (Espejo 1997; Ohl et al. 2001; Wall and Messier 2001). The ANOVA showed significant main treatment effects for open arm latency (Fig. 3a, $F_{(5,64)}$= 4.197, p<0.01) and scanning time (Fig. 3b, $F_{(5,64)}$=4.751, p<0.01). Post-hoc analysis indicated that OXA (10.0 μg, p <0.01) and OXB (3.0 μg, p<0.05) in the midline thalamus significantly increased the open arm latency compared to the vehicle group. In addition, OXA (3.0 and 10.0 μg, p< 0.05, p<0.05) and OXB (3.0 μg, p<0.01) increased the scanning duration compared to the vehicle group.

An increase in the number of SAP is hypothesized to be a measure of risk assessment in rodents tested in the EPM (Augustsson and Meyerson 2004; Rodgers and Johnson

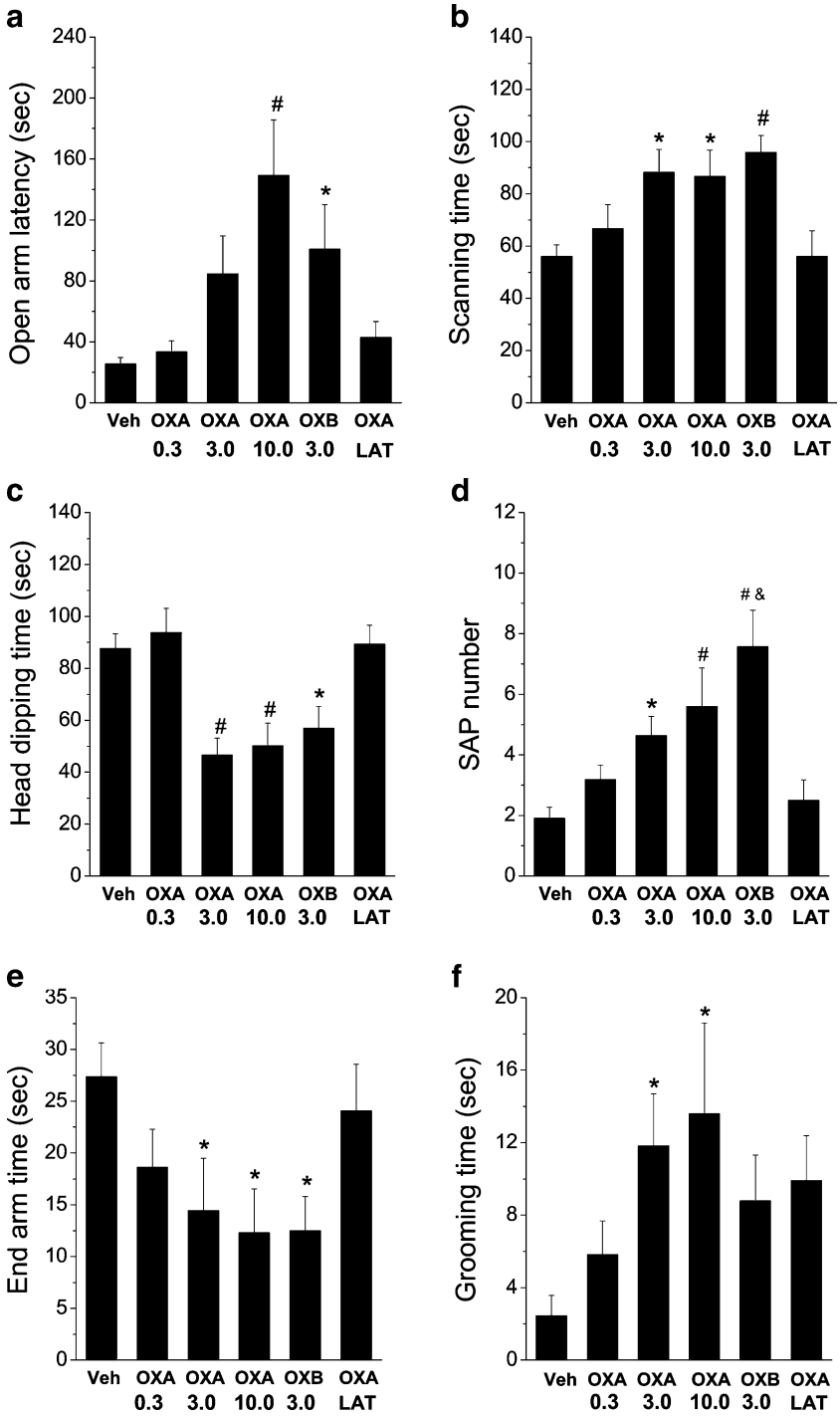

Fig. 3 Effects of orexin-A (OXA) or orexin-B (OXB) microinjections in the midline thalamus on anxiety evaluated by the ethological measurement in the elevated plus maze. Effects of OXA (0.3, 3.0 and 10.0 μg), OXB (3.0 μg), or vehicle (Veh) in the region of the paraventricular nucleus of the thalamus on open arm latency (**a**), scanning time (**b**), head dipping time (**c**), number of stretch-attend postures (**d**), end arm time (**e**), and grooming time (**f**). *indicates p<0.05 and #indicates p<0.01 compared to vehicle, &indicates p<0.05 compared to OXA (3.0 μg), n=10 to 14 for each group

1995). There was a significant difference among the groups for the number of SAP (Fig. 3d, $F_{(5,64)}=6.47$, $p<0.001$) and post-hoc comparisons revealed that rats receiving the OXA (3.0 μg, $p<0.05$; 10.0 μg, $p<0.01$) or OXB (3.0 μg, $p<0.001$) displayed more SAP compared to vehicle treated rats. In addition, the rats treated with OXB (3.0 μg, $p<0.05$) showed more SAP compared to the ones treated with the same concentration of OXA.

A decrease in the end arm time and head dipping time indicates a decrease in active exploration of uncertain areas in the EPM (Borelli and Brandao 2008; Silva and Frussa-Filho 2000; Wall and Messier 2001). In addition, an increase in displacement behaviors such as grooming is usually associated with a stressful or conflict situation and represents behavioral correlates of a negative emotional state (Berridge et al. 1999; Espejo 1997). The ANOVA showed significant differences for the head dipping time (Fig. 3c, $F_{(5,64)}=7.613$, $p<0.001$), end arm time (Fig. 3e, $F_{(5,64)}=2.482$, $p<0.05$), and grooming time (Fig. 3f, $F_{(5,64)}=2.21$, $p<0.05$) among treatment groups. Post-hoc comparison showed that rats injected with OXA (3.0 and 10.0 μg) and OXB (3.0 μg) showed significant decreases in head dipping time ($p<0.01$, $p<0.01$, $p<0.05$) and end arm time ($p<0.05$, $p<0.05$, $p<0.05$). Only OXA-treated rats (3.0 and 10.0 μg, $p<0.05$, $p<0.05$) showed increased grooming time compared to the vehicle group, whereas OXB (3.0 μg) treated rats did not show that increase ($p=0.15$). No differences were found for the number of defecation episodes between the different treatment groups (data not shown, $F_{(5,64)}=0.495$, $p>0.05$). Finally, there were no differences in the expression of ethological behaviors in rats receiving OXA in the lateral thalamus and rats receiving the vehicle in the PVT region (Fig. 3a–f).

Administrations of antagonists for CRF and kappa receptors attenuated the behavioral effect induced by microinjections of OXA in the midline thalamus

CRF antagonist hCRF (1.0 μg, $n=9$) or the kappa antagonist norBNI (10.0 μg, $n=9$) was infused in the lateral ventricles before injecting OXA (3.0 μg) in the midline thalamus. The ANOVA showed significant main effects for treatment on the percent open arm time (Fig. 4a, $F_{(3,34)}=4.69$, $p<0.01$) and the percent open arm entry (Fig. 4b, $F_{(3,34)}=4.231$, $p<0.01$) but not the number of closed arm entries ($F_{(3,34)}=1.352$, $p>0.05$, data not shown). Post-hoc analysis showed that both hCRF and norBNI significantly reversed the decrease in the percent open arm time ($p<0.01$) and the percent open arm entry ($p<0.01$) by OXA. In contrast, hCRF ($n=12$) and norBNI ($n=11$) alone when compared with saline ($n=12$) did not have any effect on the percent open arm time (Fig. 4c, $F_{(2,31)}=0.05$, $p>0.05$) or percent open arm entry (Fig. 4d, $F_{(2,31)}=0.44$, $p>0.05$) in rats that had received only saline in the PVT region. The ethological analysis of EPM behaviors was used to further examine the behavioral effect of both antagonists on the anxiety induced by OXA in the PVT. The ANOVA revealed significant main effects of treatment for the open arm latency (Fig. 5a, $F_{(3,34)}=4.164$, $p<0.05$), scanning time (Fig. 5b, $F_{(3,34)}=7.022$, $p<0.01$), SAP number (Fig. 5d, $F_{(3,34)}=7.256$, $p<0.01$), head dipping time (Fig. 5c, $F_{(3,34)}=7.421$, $p<0.01$), and grooming time (Fig. 5f, $F_{(3,34)}=4.312$, $p<0.01$), but no difference was observed on the end arm time (Fig. 5e, $F_{(3,34)}=1.422$, $p>0.05$). Post-hoc comparison demonstrated that norBNI significantly reversed the changes in open arm latency ($p<0.05$), scanning time ($p<0.01$), head dipping time ($p<0.01$), and SAP number ($p<0.05$) produced by OXA. Similarly, hCRF had similar effects on the open arm latency ($p<0.05$), scanning time ($p<0.01$), head dipping time ($p<0.01$), SAP number ($p<0.01$), and grooming time ($p<0.05$). No significant difference

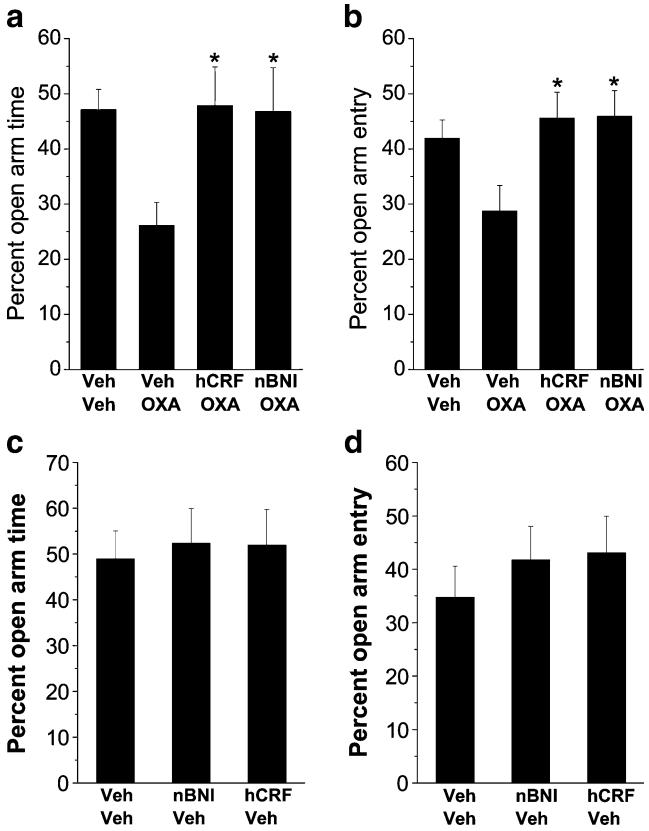

Fig. 4 Effects of intracerebroventricular injections of norbinaltorphimine (*nBNI*) and α-helical corticotropin releasing factor (*hCRF*) on the percent open arm time and percent open arm entry in the elevated plus maze following microinjections of orexin-A (*OXA*) (**a,b**) or vehicle (*veh*) (**d,c**) in the region of the paraventricular nucleus of the thalamus. *indicates $p<0.05$ and #indicates $p<0.01$ compared to OXA, $n=9$ for each group

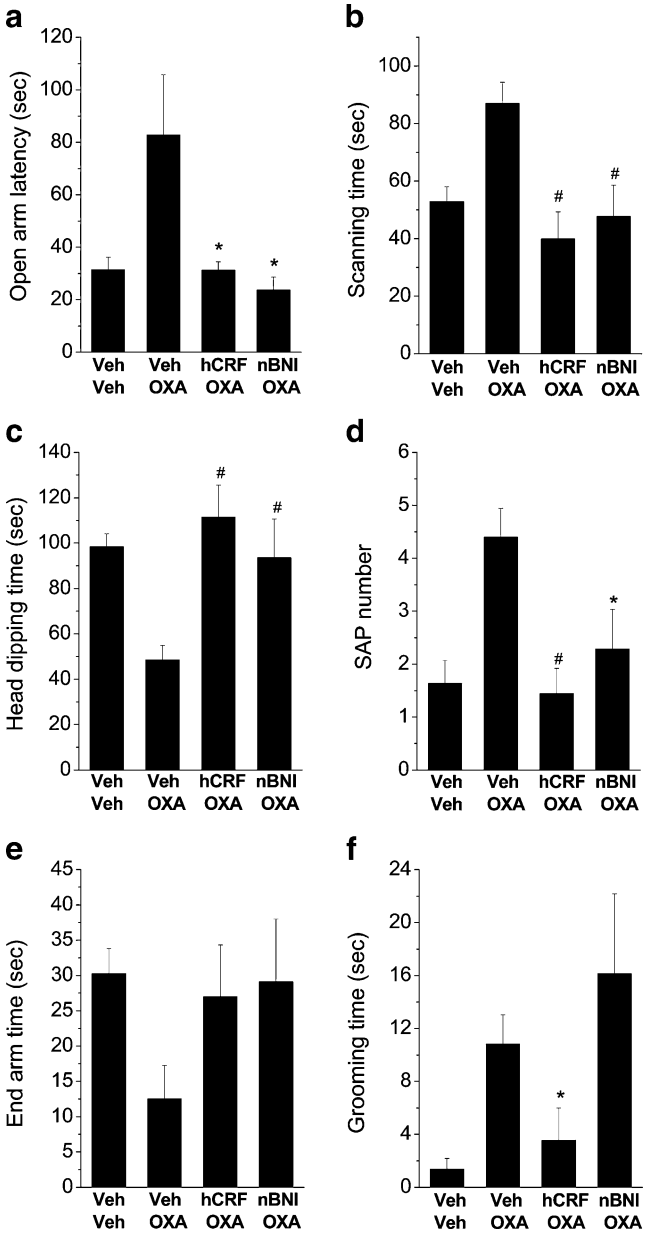

Fig. 5 Effects of intracerebroventricular injections of norbinaltorphimine (*nBNI*) and α-helical corticotropin-releasing factor CRF (*hCRF*) on changes in open arm latency (**a**), scanning time (**b**), head dipping time (**c**), SAP number (**d**), end arm time (**e**), and grooming time (**f**) produced by microinjections of 3.0 μg of orexin-A (*OXA*) in the region of the paraventricular nucleus of the thalamus. *indicates $p<0.05$ and #indicates $p<0.01$ compared to OXA, $n=9$ for each group

was observed on open arm latency ($F_{(2,31)}=0.789$, $p>0.05$), scanning time ($F_{(2,31)}=0.331$, $p>0.05$), head dipping time ($F_{(2,31)}=0.532$, $p>0.05$), SAP numbers ($F_{(2,31)}=0.399$, $p>0.05$), end arm time ($F_{(2,31)}=0.875$, $p>0.05$) and grooming time ($F_{(2,31)}=0.178$, $p>0.05$) when infusing hCRF or norBNI into lateral ventricle before injecting saline into the midline thalamus.

Microinjections of an OX2 receptor antagonist into PVT blocked footshock-induced anxiety effects

We further investigated the effect of endogenously released orexins in the PVT on stress-potentiated anxiety by microinjecting OX receptor antagonist into PVT. It is well established that previous exposure to footshock can potentiate the anxiety state as measured in EPM (Korte and De Boer 2003; Korte et al. 1999; Louvart et al. 2005; Pynoos et al. 1996). As such, we injected OX1 and OX2 receptor antagonists into the PVT region to examine their effect on footshock-induced anxiety. The two-way ANOVA revealed a significant main effect of stress on percent open arm time (Fig. 6a, $F_{(1,54)}=9.50$, $p<0.01$) and the percent open arm entry (Fig. 6b, $F_{(1,54)}=9.96$, $p<0.01$) indicating that the shocked rats expressed an anxiety state. In addition, the ANOVA revealed a significant interaction effect of "group" and "stress" on the percent open arm time (Fig. 6a, $F_{(2,54)}=3.31$, $p<0.05$) and the percent open arm entry (Fig. 6b, $F_{(2,54)}=2.507$, $p<0.05$). The orexin antagonists

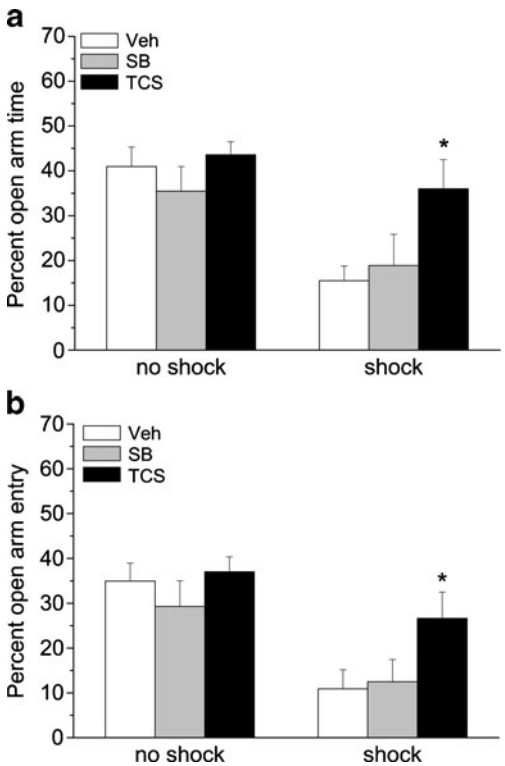

Fig. 6 Effects of microinjections of the orexin-1 receptor antagonist SB334867 (*SB*), orexin-2 receptor antagonist TCSOX229 (TCS) or vehicle (*Veh*) in the region of the paraventricular nucleus of the thalamus on percent open arm time (**a**) and percent open arm entry (**b**) in the elevated plus maze (*EPM*). For these experiments, rats received a single episode of footshock (2 mA for 10 s) or no footshock 2 days prior to the EPM test. *indicates $p<0.05$ compared to Veh group, $n=10$ for each group

had no effect on the percent open arm time (Fig. 6a, $F_{(2,55)}$=0.6, p>0.05) and percent open arm entry (Fig. 6b, $F_{(2,55)}$=0.28, p>0.05) in rats that were not previously exposed to footshock stress. In contrast, there is a significant difference between groups that received the orexin antagonists and the vehicle on the percent open arm entry (Fig. 6a, $F_{(2,55)}$=3.37, p<0.05) and percent open arm entry (Fig. 6b, $F_{(2,55)}$=2.79, p<0.05) in the rats that were exposed to footshock stress. Post-hoc analysis indicated that OX2 receptor antagonist TCSOX229 (n=10) significantly attenuated the decrease of percent open arm time (p< 0.05) and percent open arm entry (p<0.05), whereas the OX1 receptor antagonist SB334867 (n=10) had no effect compared to the vehicle group (n=10). The ethological analysis of EPM behaviors was used to further examine the behavioral effect of both antagonists on anxiety-like behaviors induced by stress. In this case, footshock stress significantly increased open arm latency (Fig. 7a, $F_{(1,54)}$= 24.78, p<0.001), scanning time (Fig. 7b, $F_{(1,54)}$=2.98, p<0.05), SAP number (Fig. 7d, $F_{(1,54)}$=11.49, p<0.01), and grooming time (Fig. 7f, $F_{(1,54)}$=5.05, p<0.05). In contrast, exposure to footshock stress significantly decreased head dipping time (Fig. 7c, $F_{(1,54)}$=7.29, p<0.01) and the end arm time (Fig. 7e, $F_{(1,54)}$=7.02, p<0.01). Consequently, the pattern of ethological behaviors expressed in rats that had been exposed to footshock is indicative of an enhanced anxiety state. The ANOVA also revealed a significant interaction effect on SAP number ($F_{(2,\ 54)}$= 2.78, p<0.05), head dipping time ($F_{(2,\ 54)}$=3.27, p<0.05), and end arm time ($F_{(2,\ 54)}$=2.61, p<0.05). The orexin antagonists had no effect on any of the ethological measures in the rats that were not previously exposed to footshock stress (Fig. 7). For the rats receiving footshock, there is a significant difference between groups receiving the orexin antagonists and vehicle on SAP numbers (Fig. 7d, $F_{(2,\ 55)}$=2.86, p<0.05), head dipping time (Fig. 7c, $F_{(2,\ 54)}$=2.2, p<0.05), and end arm time (Fig. 7e, $F_{(2,\ 55)}$=2.14, p<0.05). Post-hoc analysis indicated that OX2 antagonist significantly attenuated the increase in SAP number (p<0.05) and significantly reversed the decrease in head dipping time (p<0.05) and the end arm time (p<0.05). In contrast, the OX1 antagonist had no effect on ethological behaviors expressed in stressed rats. In summary, the data indicate that OX2 antagonist administered in the midline thalamus can attenuate some of the anxiety-like behaviors induced by footshock stress but had no anxiolytic effect in non-stressed animals.

Discussion

Since its discovery in 1998, the evidence in support of a role for orexins in brain arousal has accumulated to the point where the modulation of arousal states is often seen as the most important function ascribed for these peptides (Sakurai 2007; Siegel 2004; Taheri et al. 2002). In this

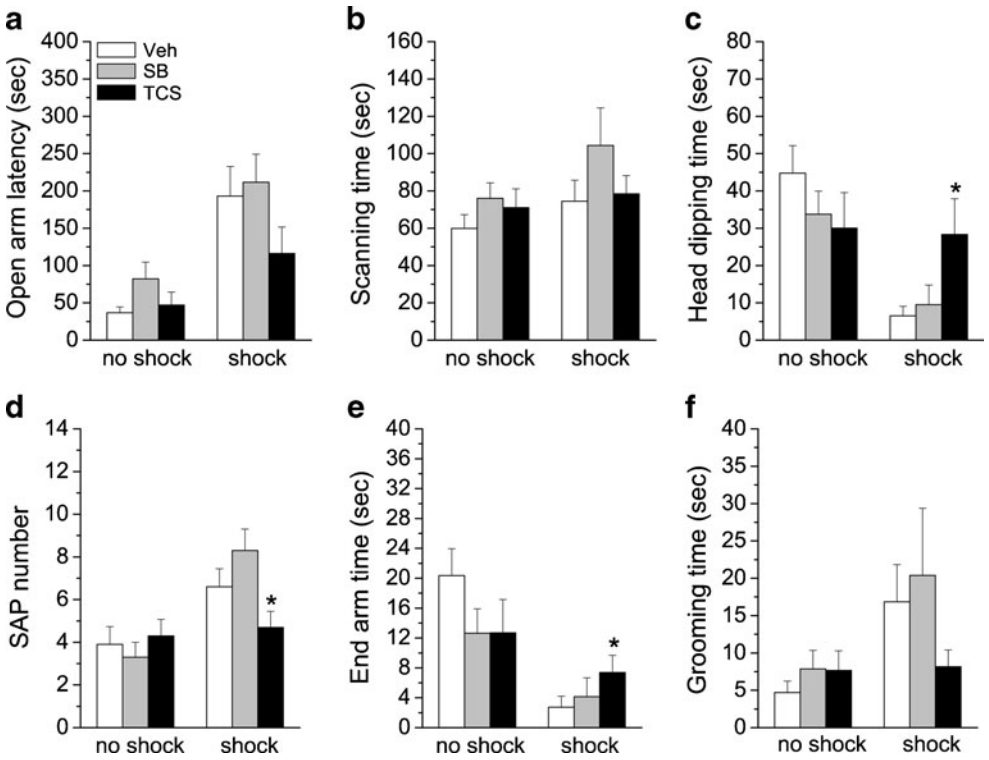

Fig. 7 Effects of microinjections of the orexin-1 receptor antagonist SB334867 (SB), orexin-2 receptor antagonist TCSOX229 (TCS) or vehicle (Veh) in the region of the paraventricular nucleus of the thalamus on open arm latency (**a**), scanning time (**b**), head dipping time (**c**), SAP number (**d**), end arm time (**e**), and grooming time (**f**). For these experiments, rats received a single episode of footshock (2 mA for 10 s) or no footshock 2 days prior to the EPM test. * indicates p<0.05 compared to Veh group, n=10 for each group

paper, we extend these findings by showing that microinjections of OXA and OXB in the midline thalamus produced a form of emotional arousal in rats tested in an anxiety-provoking environment. This was shown by a decrease in open arm time and open arm entries in the EPM following microinjections in the PVT region. Microinjections of orexins in the PVT region were also found to increase ethological behaviors expressed during an approach-avoidance conflict (face grooming, SAP, and scanning time) and in anxiety-provoking situations (Albrechet-Souza et al. 2007; Cruz et al. 1994; Garcia et al. 2005; Rodgers and Johnson 1995). We also provide evidence that these behavioral effects appear to be mediated by the brain opioid and CRF systems by showing that icv injections of antagonists to kappa and CRF receptors attenuated the anxiogenic effects induced by OXA. Finally, we provide evidence that endogenously released orexins in the PVT may be involved in regulating anxiety levels by showing that microinjections of an OX2R antagonist in the PVT attenuated the anxiogenic effects produced by a previous exposure to footshock stress. In summary, the results presented in this study indicate that endogenously released orexins may act on the PVT to regulate anxiety levels through mechanisms involving kappa opioid and CRF receptors.

The dorsal and midline region of the posterior thalamus is composed of several small nuclei which could potentially be involved in the effects of orexins observed in the present study. The PVT, mediodorsal, intermediodorsal, centromedial, paracentral nuclei, as well as the habenula, are all within the general region where the orexin agonists or antagonists were injected in the midline thalamus. However, several facts suggest that the behavioral effects of orexins were largely mediated by actions on the PVT. First, compared to other thalamic nuclei, the PVT contains a high density of orexin fibers, which implies that this nucleus is one of the most important targets for these peptides (Kirouac et al. 2005). Second, the PVT contains a moderate to high expression of mRNA for the OX1R and OX2R, respectively (Marcus et al. 2001). The centromedial nucleus, which is located approximately 1 mm ventral to the PVT, also contains a moderate signal for orexin receptor mRNA (Marcus et al. 2001). On the other hand, it is unlikely that a sufficient amount of the orexin peptide would have diffused to this nucleus to produce behavioral effects (Thorpe et al. 2003). The fact that injections of OXA in the lateral thalamus produced no effect in the EPM is consistent with this idea of limited diffusion and supports our conclusion that the orexin agents acted primarily at the PVT. The intermediodorsal nucleus, which is adjacent to the PVT, contains a very weak signal for orexin receptor mRNA, whereas the mediodorsal nucleus and the habenula do not express mRNA for the orexin receptors (Marcus et al. 2001). Third, the PVT is the only member of the midline and intralaminar group to provide a direct and significant innervation to dynorphin and CRF rich regions of the extended amygdala (Kirouac and Li 2008; Li and Kirouac 2008). Dynorphin and CRF peptides, which are located in subpopulations of neurons in the extended amygdala, have been shown to play a key role in regulation of emotional behaviors (Bruchas et al. 2008; Davis 1998; Heinrichs and Koob 2004; Lang and Davis 2006). Results from our experiments show that blocking CRF and the kappa receptors attenuated the behavioral effects of administrations of orexins in the midline thalamus. While it is not possible to completely exclude a contribution by other midline nuclei in the present study, the PVT appears to be the most likely candidate for mediating the behavioral effects of orexin receptor agonists and antagonist observed here.

The strategy of microinjecting a peptide directly into the brain to examine its behavioral effects represents a means of understanding the function of an endogenously released peptide. This experimental approach has been used to provide evidence for orexins in the regulation of arousal and food intake (Espana et al. 2002; Jones et al. 2001; Nakamura et al. 2000; Rodgers et al. 2000; Thorpe and Kotz 2005; Thorpe et al. 2003). In this study, we found that microinjections of OXA and OXB in the PVT region at concentrations of 3.0 and 10.0 μg led to a decrease in the time spent in the open arm of the EPM, which is considered a behavioral response indicative of an anxiety state (Rodgers and Dalvi 1997; Walf and Frye 2007). While we previously reported that microinjections of orexins in the PVT inhibited locomotion in some situations (Li et al. 2009), it is unlikely that a decrease in the exploration of the open arm in orexin-treated rats was due to an impairment of movement because the number of closed arm entries was not affected. Furthermore, administrations of orexins in the PVT increased the amount of grooming, SAP and scanning observed in the EPM. These ethological behaviors are expressed when an animal experiences anxiety in the presence of an approach-avoidance conflict (Albrechet-Souza et al. 2007; Garcia et al. 2005; Rodgers and Johnson 1995; Spruijt et al. 1992). The fact that behaviors like grooming and SAP were increased in orexin-treated rats also indicates that these rats did not have motor deficits that could account for their lack of exploration in the open arm.

We also assessed if endogenously released orexins in the PVT modulate anxiety levels. First, microinjections of OX1R and OX2R antagonists in the PVT region of normal rats did not affect any of the behavioral measures assessed in the EPM. This indicates that the release of orexins in the PVT does not influence anxiety levels when non-anxious rats are tested in the EPM. Consistent with previous studies showing that an episode of shock stress produces hypervigilant and anxious rodents (Korte and De Boer 2003;

Louvart et al. 2005; Mikics et al. 2008a, b; Pynoos et al. 1996; Siegmund and Wotjak 2007a, b; van Dijken et al. 1992), exposure of rats to footshock 2 days prior to the EPM test in the present study resulted in a decrease in the percent open arm time and entry. More importantly, the anxiogenic effect of footshock stress was attenuated by giving the OX2R antagonist TCSOX229 in the PVT region. We interpret these results as evidence that activation of the OX2R in the PVT is important for modulating the anxiety levels of rats previously exposed to a stress or fear inducing condition. The anxiogenic effects of orexins in the PVT may become functionally more important in situations involving uncertainty and requiring vigilance.

A plausible interpretation of our experiments is that the level of anxiety expressed in a novel situation can be modulated by orexins released in the PVT. This is consistent with anatomical evidence showing that the PVT preferentially and heavily innervates key regions of the extended amygdala involved in regulating emotions and anxiety (Li and Kirouac 2008). As such, activation of orexin receptors in the PVT could enhance the excitability of neurons in the extended amygdala that control anxiety levels while blocking of orexin receptors in the PVT would have the opposite effect. An alternative interpretation is that the anxiety-like response produced by orexins at the midline thalamus may have been due to an increase in generalized brain arousal. This does not appear to be the case because orexin administrations in the thalamus did not have any effect on locomotor activity, which is normally elevated in animals in heightened states of arousal (Pfaff et al. 2008). In fact, we previously reported that microinjections of orexins in the PVT produced a weak inhibition of locomotion while increasing grooming and freezing (Li et al. 2009, 2010). A more likely explanation is that administrations of orexins in the PVT may have produced arousal effects that are specific to neurons that regulate emotional behaviors like anxiety. This interpretation is consistent with anatomical studies showing that the PVT sends an impressive projection to regions of the extended amygdala that densely stain for CRF and dynorphin (Kirouac and Li 2008; Li and Kirouac 2008), two peptides linked to the regulation of emotions and anxiety (Bals-Kubik et al. 1993; Bruchas et al. 2007, 2008; Knoll et al. 2007; Land et al. 2008; Lee and Davis 1997; Newton et al. 2002; Sahuque et al. 2006; Walker et al. 2009a, b; Wittmann et al. 2009). In the present study, we show that icv administrations of CRF or kappa receptor antagonists decreased the anxiety-like behaviors produced by microinjections of OXA in the PVT region. While these results suggest that a possible link between the PVT and the CRF/dynorphin systems, more experiments will be required to show this unequivocally. Furthermore, we cannot exclude the possibility that the CRF and kappa antagonists could have decreased the brain arousal in a nonspecific manner, which in turn could have reduced the effectiveness of OXA to elicit anxiety. As shown in this study, this does not appear to be the case since the CRF and kappa antagonists given alone had no apparent effects on behaviors tested in the EPM. Clearly, more experiments will be required to determine the mechanisms associated with anxiety produced by orexin administrations in the PVT.

Functional consideration of an orexin-PVT emotional arousal system

It is well documented that orexins can produce a stress-like response by enhancing the activity of the hypothalamic–pituitary–adrenal axis and the secretion of stress hormones in the circulation (Chang et al. 2007; Ida et al. 2000; Jaszberenyi et al. 2000; Kuru 2000; Sakamoto et al. 2004; Samson et al. 2002, 2007). In turn, orexin neurons may be activated by novelty-stress, pain, and contextual cues associated with shock (Espana et al. 2003; Furlong et al. 2009; Watanabe et al. 2005; Winsky-Sommerer et al. 2004; Zhu et al. 2002), but not other stressors like exposure to cold and restraint (Furlong et al. 2009; Kiss 2007). Consequently, a relationship between the orexin peptides, arousal and stress is likely to exist, but the nature of this relationship remains poorly understood.

In the present study, we found that exposure to single episode of relatively intense shock stress produced an orexin-mediated anxiogenic effect 2 days later when the rats where tested in a different context. Exposure of rats or mice to shocks of similar intensity and duration, as done in the present study, has been shown to produce long-lasting states of hyperarousal, hypervigilance, and anxiety (Korte and De Boer 2003; Louvart et al. 2005; Mikics et al. 2008a, b; Pynoos et al. 1996; Siegmund and Wotjak 2007a, b; van Dijken et al. 1992). While it is not known if exposure to a fear inducing stimuli like shock leads to a prolonged activation or sensitization of orexin neurons, the present study provides some evidence for the involvement of orexins in the PVT in the anxiogenic effects of shock stress. These results are consistent with reports showing that neurons in the PVT are more active during periods of arousal or aversive situations (Bhatnagar and Dallman 1998; Brown et al. 1992; Bubser and Deutch 1999), and that the PVT projects to the key regions of the forebrain involved in emotional behaviors (Hsu and Price 2009; Li and Kirouac 2008; Parsons et al. 2007; Vertes and Hoover 2008). The PVT could possibly excite CRF neurons and the release of CRF in the extended amygdala where this peptide plays a key role in regulating anxiety states (Lee and Davis 1997; Sahuque et al. 2006; Walker et al. 2009a, b). The PVT could also excite dynorphin neurons that are located in the extended amygdala where the release of this

opioid peptide acting through the kappa receptor has been shown to have dysphoric effects (Bals-Kubik et al. 1993; Bruchas et al. 2007, 2008; Land et al. 2008; Newton et al. 2002) and which may be involved in the anxiolytic effects of systemic administrations of norBNI (Knoll et al. 2007; Wittmann et al. 2009). It is also interesting to consider that some CRF neurons in the central nucleus of the amygdala also contain dynorphin (Marchant et al. 2007) and that PVT strongly innervate the same region of the amygdala (Li and Kirouac 2008). While more research will be required to provide more support for the proposed model, a functional orexins-PVT emotional arousal system may be of importance for understanding how the brain modulates anxiety levels.

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Conflict of interest The authors declare that except for income received from their primary employer, no financial support or compensation has been received from any individual or corporate entity over the time when this research has been conducted and manuscript prepared.

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