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A KEY ROLE FOR OREXIN IN PANIC ANXIETY

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Introductory paragraph

Panic disorder is a severe anxiety disorder with recurrent, debilitating panic attacks. In subjects with panic disorder there is evidence of decreased central GABAergic activity as well as marked increases in autonomic and respiratory responses following intravenous infusions of 0.5M sodium lactate[1–3]. In an animal model of panic disorder, chronic inhibition of GABA synthesis in the dorsomedial/perifornical hypothalamus of rats produces anxiety-like states and a similar vulnerability to sodium lactate-induced cardioexcitatory responses[4–9]. The dorsomedial/perifornical hypothalamus is enriched in orexin (ORX, also known as hypocretin)-containing neurons[10] that play a critical role in arousal[10,11], vigilance[10] and central autonomic mobilization[12], all of which are key components of panic. Here, we demonstrate that activation of the ORX neurons is necessary for developing a panic-prone state in the animal model, and either silencing the hypothalamic ORX gene (*Hcrt*) product with RNA interference or systemic ORX1 antagonists blocks the panic responses. Moreover, we show that subjects with panic anxiety have elevated levels of ORX in the cerebrospinal fluid compared to subjects without panic anxiety. Taken together our results suggest that the ORX system may be involved in the pathophysiology

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AUTHOR CONTRIBUTIONS AS, PLJ, and WT formulated the hypotheses and designed the studies. SDF and PLJ performed telemetrical probe surgeries. SDF and PLJ scored all behavior and SDF performed all stereotaxic surgeries. PLJ performed the immunohistochemistry. PEM and AD performed all RT-PCR assays with technical expertise from WT and SS. PLJ and WT analyzed all animal data. LT-B and LB were responsible for the human subject study, the ORX assays of the CSF samples and the analysis of the human data. PLJ, WT and AS interpreted the data and collectively wrote the main draft of the article. PJ, WT, SDF, AD, PK, LB, LT-B, AG and AS contributed to the writing of the manuscript and have approved of the final version.

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See further details in comprehensive supplemental methods

of panic anxiety, and that ORX antagonists constitute a potential novel treatment strategy for panic disorder.

Body

Panic disorder is characterized by recurrent episodes of severe anxiety accompanied by multiple physical symptoms such as increased cardiorespiratory responses[13], and constitutes a risk factor for suicidal behavior[3]. Panic attacks characterized by sudden onset of fear along with rapid increases in respiration and heart rates[16] can reliably be induced in panic disorder subjects by specific and normally innocuous interoceptive stimuli (e.g., intravenous 0.5M sodium lactate or yohimbine[14–16], or 7% CO_2 inhalations[17]). This suggests that global neural pathways which modulate arousal are perturbed in these subjects. Consistent with this, reduced central GABAergic activity has been reported in subjects with panic disorder[1] and drugs that restore GABAergic inhibition (e.g. benzodiazepines) are clinically effective treatments[2]. In rats, acute disruption of GABAergic inhibition in panic-generating CNS sites such as the dorsomedial/perifornical hypothalamus, amygdala or the dorsal periaqueductal grey leads to panic-like behavior and increased cardiorespiratory responses[18]. Furthermore, after chronically inhibiting GABA synthesis in the dorsomedial/perifornical hypothalamus of rats [with 5 days of local l-allylglycine (l-AG): a GABA synthesis inhibitor infusions using osmotic minipumps connected to a cannula], sodium lactate challenges produce anxiety [measured by social interaction, elevated plus maze, open field test and freezing in defensive probe burying test] as well as panic [defined as increased “flight”-like locomotion and increased heart rate (HR), mean arterial pressure responses (MAP)][4–9]. This is also pharmacologically validated with anti-panic drugs such as alprazolam[8], and provides a robust rat model of human sodium lactate-induced panic attacks.

Coincidentally, ORX producing neurons are exclusive to the dorsomedial/perifornical and lateral hypothalamus[10,19] and are known to regulate feeding, wakefulness[10,11] and vigilance[10]. The ORX neurons are also involved in mobilizing sympathetic responses and desensitizing the parasympathetically mediated baroreflex[12] to permit simultaneous increases of blood pressure and heart rate[5], which are all key components of panic. Autonomic nervous system targets of ORX neurons are also activated by sodium lactate infusions in the above described `sodium lactate panic prone rats' but not in controls[5]. Finally, mice lacking the *Hcrt* gene have attenuated defence responses to panic cues and cardioexcitatory responses following disinhibition of the dorsomedial/perifornical hypothalamus[20]. Based on these data we hypothesised that the ORX system plays a critical role in producing panic attacks.

Utilizing our established panic model[4–9] (also see supplemental materials), we first observed that ORX-positive cells (specifically those in the dorsomedial/perifornical hypothalamus) are selectively (Suppl. Fig. 1) activated (i.e., increased c-Fos) following sodium lactate administration in panic-prone rats ($p=0.001$, Fig. 1a), and this activation correlated with increase in anxiety-related behavior (Fig. 1a).

We then demonstrated that sodium lactate-induced panic responses are dependent on translation of the *Hcrt* gene that produces *preproOrexin mRNA*, by injecting small

interfering (si) RNA targeting the *preproORX* mRNA (siORX) (OnTarget*Plus* SmartPool® Dharmacon) into the dorsomedial/perifornical hypothalamus of panic-prone rats 48 h prior to sodium lactate or saline challenges. We used quantitative RT-PCR to assess mRNA levels in the combined dorsomedial and lateral hypothalamus. Importantly, injecting panic-prone rats with siORX attenuated multiple components of the sodium lactate-induced panic-like responses [anxiety-like behavior (*siRNA x time effect*, p=0.035, Fig. 1b), and cardioexcitatory effects (*siRNA x time effects* for HR, p=0.002; and MAP, p=0.003, Fig. 1d–e)], whereas si control (siCON) rats displayed the predicted panic-like responses (Fig. 1b–e). As expected, treatment with siORX dramatically reduced local *preproORX* mRNA in control (p=0.047, Fig. 1g) and panic-prone rats compared to treatment with siCON, (p=0.025, Fig. 1h, also Fig. 1k, Suppl. Table 1). The effect was selective, as neither pro-dynorphin mRNA (a gene co-expressed in ORX neurons[21]; p=0.184, Fig. 1i) nor local pro-opiomelanocortin mRNA (p=0.207, Fig 1j) was reduced by siORX injection. Interestingly, once a panic-like response occurred, both *preproORX* (p=0.007, Fig. 1g) and pro-dynorphin (p=0.001, Fig. 1i) mRNA levels were rapidly suppressed, suggesting panic-induced negative feedback.

In the next step, we show that sodium lactate-induced panic in panic-prone rats is attenuated by systemic pre-treatment with ORX1 receptor antagonists. The selective ORX1 receptor antagonist (SB334867, 30 mg/kg, Tocris[22]) attenuated the anxiety-like behavior [measured with social interaction (p=0.001, Fig. 2a) and open field tests (0.025, Fig. 2b)]. This ORX1 receptor antagonist also blocked the increases in locomotion (p=0.017, Fig. 2a), heart rate (p=0.001, Fig. 2a) and blood pressure (p=0.001, Fig. 2a; p=0.001, Fig. 2b) responses induced by the sodium lactate challenge. These effects mimicked the effects of pre-treating panic-prone rats with alprazolam [3 mg/kg, Sigma (Fig. 2a)], a clinically effective benzodiazepine that blocks both spontaneous and sodium lactate-induced panic attacks in subjects with panic disorder[14,15]. Similarly, a second ORX1 receptor antagonist (SB408124, 30 mg/kg, Tocris) also attenuated the sodium lactate-induced increases in locomotor activity (p=0.004, Fig. 2c) and tachycardia responses (p=0.001, Fig. 2c) in another group of panic-prone rats (See Suppl. Fig. 2a–c for localization of infusion sites). The SB334867 ORX1 antagonist did not alter anxiety or cardiovascular responses in control rats (n=7/group, Suppl. Fig 3), or baseline measures (see Suppl. Results) in panic-prone rats.

One potential concern is that blocking ORX function might induce general somnolence or narcoleptic behavior[10], thus reducing sodium lactate-induced panic responses. We do not believe this to be the case for the following reasons: in a previous study, acute blockade of ORX receptors did not result in narcoleptic states[11]; and reducing ORX activity for short periods with either ORX 1 receptor antagonists (Fig. 2a–c) or gene silencing (Fig. 1c) did not result in somnolence during testing or alter baseline locomotor activity. In fact, the ORX gene silencing or ORX1 receptor antagonist increased social interaction (Fig. 1b, 2a) and exploration in the open field (Fig. 2b), clearly arguing against induction of sedation. In addition to its attenuation of panic-like responses, the ORX1 receptor antagonist (SB334867, 30 mg/kg i.p.) also blocks sodium lactate-induced freezing (indicative of panic-like fear) observed in the defensive burying test in panic-prone rats (p=0.021, Fig. 3a). Again this is

not due to sedative effects of the ORX1 antagonist, since the number of mid-line crossings was not reduced in the treated panic-prone rats (Fig. 3b).

The sodium lactate-induced anxiety, but not the cardiorespiratory components of the panic response appears to be pivotally linked to the bed nucleus of the stria terminalis[23] (Suppl. Fig. 5 for hypothetical mapping of implicated neural pathways). Therefore, to confirm an end target effect of activating ORX neurons, we focused on the bed nucleus of stria terminalis which receives ORX projections from the dorsomedial/perifornical hypothalamus [see Sakurai et al., 2007 review]. We injected an ORX1 receptor antagonist (SB334867) ipsilaterally into the bed nucleus of the stria terminalis of panic-prone rats, prior to the sodium lactate challenge, which reduced anxiety-like behavior compared to the vehicle-injected rats ($p=0.0002$, Fig. 3c, Suppl. Fig. 2d).

The animal model of panic disorder utilized here was established over the last 10 years and has robust face, predictive and construct validity[4–9]. The model's predictive validity is demonstrated by responses, similar to those observed in subjects with panic disorder, to both panic-inducing agents (e.g. sodium lactate, yohimbine, and inhalations of CO_2) and anti-panic effects of therapeutic agents such as alprazolam and group II metabotropic glutamate agonists[8]. Also, this animal model was recently used in a series of preclinical studies to identify a novel class of translocator protein agonist (that enhances the central inhibitory effects of GABA), which subsequently showed anti-panic properties in clinical trials, further strengthening the model's predictive validity[24]. The construct validity of this model is supported by the fact that neural circuits of the dorsomedial/perifornical hypothalamus regulate behavioral and autonomic components of the “fight or flight” response in rats[25], and are implicated in eliciting panic-like responses in humans[26] and animals[23]. Furthermore, panic disorder subjects have reported deficits in central GABA activity[1] and pharmacological restoration of central GABA activity prevents panic attacks[2], in accordance with our animal model. Also, the panic- and anxiety-like responses noted in this model are not likely due to a general increase in arousal, as there are no changes in baseline acoustic startle responses (Suppl. Fig. 4). Similarly, there is no increase in baseline startle response in human subjects with panic disorder[27,28].

In order to clinically validate the role of ORX in panic disorder, cerebrospinal fluid (CSF) samples were collected from 53 medication-free subjects who presented with suicidal behavior. The subjects were assessed for present symptoms of panic anxiety [as measured by the comprehensive neuropsychopathological rating scale (CPRS)] and CSF samples were assayed for ORX levels. Increased CSF ORX was observed in subjects with panic anxiety compared to subjects without panic anxiety. Furthermore, subjects with only panic anxiety had significantly higher CSF ORX than subjects with panic anxiety and co-morbid major depressive disorder ($p=0.004$, Fig. 4, Suppl. Table 2). Our findings of increased ORX in subjects with panic anxiety are consistent with a previous report that chronic treatment with sertraline, a well known anti-panic and antidepressant drug, reduces ORX levels in the CSF whereas bupropion, an antidepressant with a lower efficacy in treating panic disorder, does not[29].

Taken together, our translational experiments in animal models and subjects suggest that aberrant functioning of the ORX system may underlie panic-attacks. We suggest that ORX1 receptor antagonists may provide a novel therapeutic approach for the treatment of panic disorder.

Methods and Materials

Animals and housing conditions

All experiments used adult male Sprague-Dawley rats (300–350 g, Harlan Laboratories), which were individually housed (22 °C; 12/12 light/dark cycle; lights on at 7:00 A.M.) for 7–10 days prior to surgery. Food and water were provided *ad libitum*. Animal care procedures were conducted in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals (NIH Publication no. 80–23) revised 1996 and the guidelines of the IUPUI Institutional Animal Care and Use Committee.

Inducing panic-prone state in rats and panic response following sodium lactate (NaLac)

Cannulae (Plastics One Inc.) were directed at the dorsomedial/perifornical hypothalamus (DMH/PeF[30]) and connected to an osmotic minipump (DURECT Corporation) filled with l-AG solution (a glutamic acid decarboxylase inhibitor) or when applicable d-allylglycine (d-AG: the inactive isomer of l-AG). Radio-telemetry probes (Data Science International) were surgically implanted into the abdomen of rats[31] to measure cardiovascular and locomotor responses. Rats received intravenous (i.v.) infusions [similar to clinical studies[16]; 10ml over 15 min] of either 0.5M NaLac or 0.9% isotonic saline at least five days following the initiation of l-AG or d-AG infusions.

c-Fos induction in ORX neurons following infusions of NaLac or saline

l-AG or d-AG treated rats received NaLac or saline challenge (n=6/group for 4 groups) and were immediately tested in the social interaction (SI) test. 90 min following the SI test rats were perfused and brains were immunoprocessed into 6 parallel sets of coronal section (30 μm).

Injections of siORX or siCON into the DMH/LH of panic-prone rats

Control rats received stereotaxic injections of OnTarget*Plus* SmartPool® siRNA against rat *ppORX* mRNA (siORX, 100nMol, Dharmacon) into one side of the DMH/PeF, and negative control siRNA (siCON, 100nMol, Dharmacon) into the other side to confirm gene silencing. Panic-prone rats were given bilateral siORX or siCON treatment, and 48 hrs later infused with saline or NaLac (siCon/Sal n=4, siORX/Sal n=6, siCON/Lac n=5, siORX/Lac n=6). SI and cardiovascular responses were recorded and tissue processed for RT-PCR.

Attenuating panic-like responses with systemic ORX1 receptor antagonists

Panic-prone rats received intraperitoneal (i.p.) injections of drug or vehicle 30 min prior to i.v. infusions and panic-like responses were assessed. In 1st experiment, rats were injected with the ORX1 receptor antagonist SB334867 [30mg/kg, Tocris, in 0.2ml, n=12], alprazolam (3mg/kg, Sigma, n=6) or vehicle (0.2ml, n=11) 30 min prior to a NaLac

challenge and the SI test was used to assess anxiety behavior. In 2nd experiment, an open field (OF) test was used to assess anxiety [i.p. vehicle+i.v. saline (n=5); i.p. vehicle+i.v. NaLac (n=5); or i.p. SB334867 (30mg/kg)+i.v. NaLac (n=6)]. In a final experiment, panic-prone rats received i.p. injection of an alternative ORX1 receptor antagonist [30mg/kg SB408124, Tocris, n=4] or vehicle (0.2ml, n=6) 30 min prior to NaLac.

NaLac effects on unconditioned defensive shock-associated behaviors

Rats were acclimated to the testing apparatus for 10 min with a deactivated shock probe. On testing day, panic-prone rats either received an i.p. injection of the ORX1 receptor antagonist, SB334867 [30mg/kg, Tocris, n=3], or vehicle (0.2ml, n=6) 30 min prior to the i.v. challenge. The SB334867+l-AG group of rats (n=3) and the vehicle+l-AG rats (n=3) were given an i.v. infusion of NaLac, whereas the remaining vehicle+l-AG group were infused with saline (n=3). Rats were then immediately placed in the test cage with activated shock probe (0.7 mA, Lafayette Instruments Co.) for 10 min. Time spent burying; in proximity of probe; grooming; freezing; and number of center crossings were assessed[32].

ORX1 receptor antagonist into the bed nucleus of the stria terminalis (BNST) of rats

Panic-prone rats (n=5/group in a crossover design) received unilateral injections of SB334867 (300pmoles/100nl vehicle) or vehicle directed at the BNST [using a 33 gauge injector (Plastics One)] 30 min prior to receiving i.v. infusions of 0.5 M NaLac. An SI test was conducted immediately following the lactate challenge.

Measuring ORX-A in the cerebrospinal fluid of subjects with and without panic anxiety

Subjects (n=53) who presented in acute suicidal crisis were systematically assessed for psychiatric symptoms utilizing the CPRS. A threshold cut off at 1.5 on item 3 (panic, inner tension) on that scale was used to define a subject as having significant panic symptoms. Lumbar punctures were performed to collect CSF[33], and samples stored at −80°C. CSF-ORX-A levels were measured in duplicate using an ^{125}I radioimmunoassay (Phoenix Pharmaceuticals). All subjects with substance abuse and traces of medication in the blood were excluded from the analysis. This study was approved by the Lund University Medical Ethics Committee.

Statistical analyses

All values are expressed as means ± s.e.m. or means ± s.d. as noted. Where indicated, we assessed between groups effects using paired or unpaired t-tests for 2 groups and ANOVA's with Tukey's HSD post-hoc test for >2 groups. A non-parametric Kruskal-Wallis ANOVA test was used for the open field test due to unequal variance (see results) and for clinical data. Within groups effects were assessed using a one-tailed Dunnet's test. The alpha level was set at < 0.05 in all cases. (Excel 2007, SPSS 16.0 and Systat 5.0 were used to analyze data and SigmaPlot 8.0 and CorelDraw 12.0 was used to plot and illustrate data).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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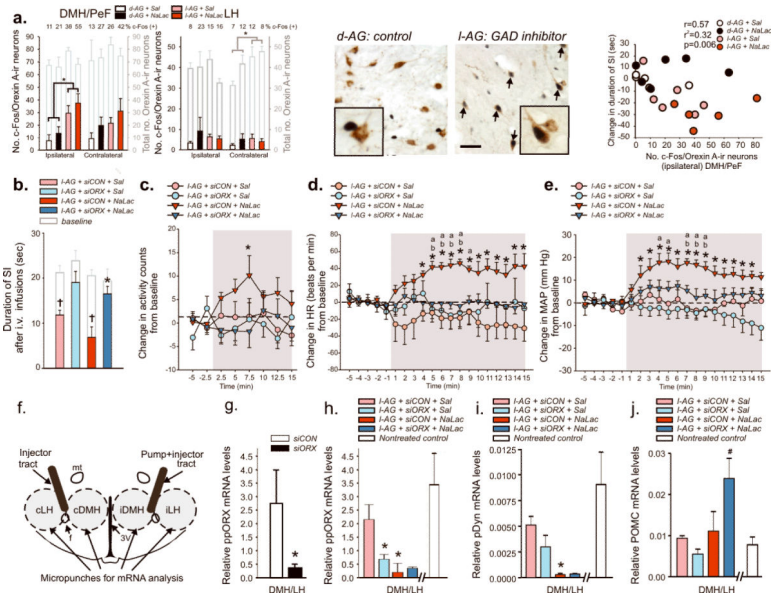

Figure 1.

a) Left: Mean no. of c-Fos/ORX-A-ir neurons in the DMH/PeF and LH of rats challenged with either sodium lactate (Lac) or saline (Sal). Bars with black lines = no. of c-Fos/ORX-A-ir neurons; grey, open bars = total no. of ORX-A-ir neurons. Middle: photomicrographs representing c-Fos (blue nuclei) and ORX-A-ir neurons (brown cytoplasmic) in the DMH with arrows indicating c-Fos/ORX-A-ir neurons. Scale bars = 50 μm, 125 μm for inset. Right: Mean no. of c-Fos/ORX-A-ir neurons in the DMH/PeF correlated with changes in social interaction (SI). Effects of prior (48h) injections of small interfering (si) RNA targeting prepro-orexin mRNA (siORX), but not control siRNA (siCON), into the DMH/PeF of panic-prone rats (l-AG treated) on: b) anxiety-like responses (SI duration, * and † indicate $p<0.05$); c) general locomotor activity; d) heart rate (HR); and e) mean arterial blood pressure (MAP). For locomotor and cardiovascular data, *, a, and b indicate $p<0.05$. f) Coronal illustration of unilateral l-AG infusions, bilateral siORX or siCON injections, and micropunches taken for mRNA assays; g) Effects of siORX into the DMH/PeF of control rats on concentrations of local prepro-ORX (ppORX) mRNA in the combined DMH/LH. Effects of bilateral injections of siORX or siCON into the DMH/PeF of panic-prone rats challenged with Lac or saline on local h) ppORX, i) pro-dynorphin (pDyn) and j) proopiomelanocortin (POMC) mRNA. The last bar in figures 1h-j represents the concentration of mRNA in the DMH/LH of untreated homecage control rats. For Fig. 1h-j, * and # respectively indicate $p<0.05$ compared to siCON/Sal or siORX+Sal groups. All mRNA levels are expressed relative to beta-actin mRNA levels. Bars and lines represent means; error bars represent SEM. Abbreviations: contra, contralateral; DA, dorsal hypothalamic area; DMH, dorsomedial hypothalamus; ipsi, ipsilateral; LH, lateral hypothalamus.

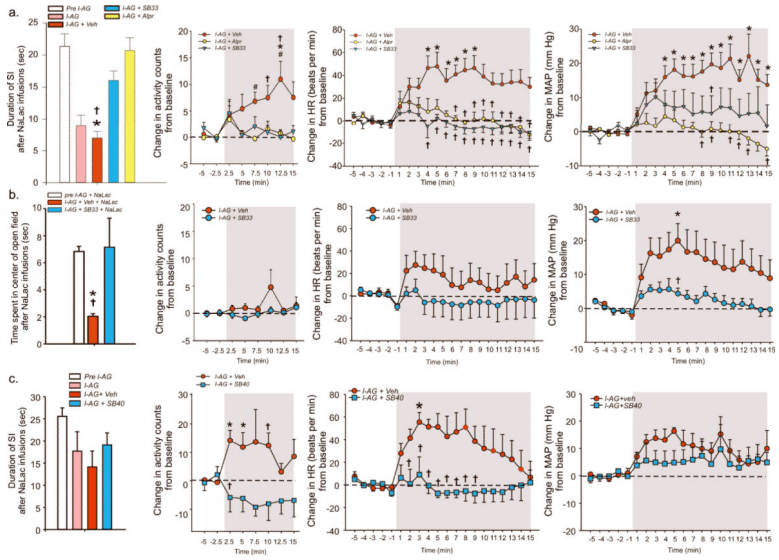

Figure 2.

a) Effect of systemically injecting the ORX1 receptor antagonist (SB334867) or benzodiazepine (alprazolam) into panic-prone rats prior to NaLac challenges on a) anxiety-like responses [social interaction (SI)], general locomotor activity, heart rate (HR), and mean arterial blood pressure (MAP). For anxiety tests in Fig. 2a, and 2b, * and + indicate significant differences between groups using a Tukey's HSD tests with $p<0.05$. For locomotor and cardiovascular data in Fig 2a–c * indicates significantly different from baseline using a Dunnet's test and † indicate significant differences between groups using a Tukey's HSD or paired t-test with $p<0.05$. b) Effects of systemically injecting SB334867 into panic-prone rats prior to NaLac challenges on anxiety-like responses (decreased time spent in center of open field), general locomotor activity, HR, and MAP. c) Effects of systemically injecting a 2nd ORX1 receptor antagonist (SB408124) into panic-prone rats prior to NaLac on SI duration, general locomotor activity, HR and MAP. See Suppl. Fig. 2a–2c for respective probe-placement verification. Bars and lines in graphs represent the mean, and error bars represent the standard error of the mean.

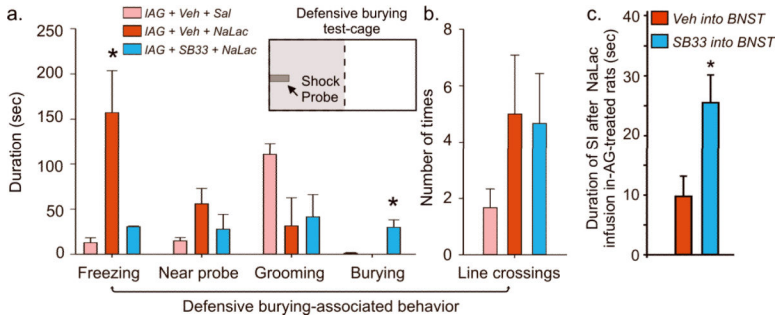

Figure 3.

Assessment of defensive shock associated behaviors in panic-prone rats (l-AG treated) challenged with isotonic saline (Sal) or hypertonic sodium lactate with a prior intraperitoneal injection of either a vehicle (Veh) or an orexin 1 receptor antagonist (30mg/kg SB334868: SB33). Duration of: a) freezing, near probe (see gray shaded area in defensive shock cage illustration in inset in figure 3a) grooming, burying; and b) number of line crossings (see dashed line in illustration inset in figure 3a). * indicates significant differences between all other groups using a Tukey's HSD tests with $p<0.05$. c) Effects of unilateral injections of the ORX1 receptor antagonist SB334867 (300pmoles/100nl) into the bed nucleus of the stria terminalis (BNST) of l-AG treated rats, prior to a sodium lactate challenge, on the duration of SI (compared to SI duration following vehicle injection). Bars in graphs represent the mean, and error bars represent the standard error of the mean. * indicates significant differences between groups using a paired t-test with $p<0.05$. See Supplemental Figure 1d for l-AG infusion placement verification in DMH/PeF and injections sites into the BNST.

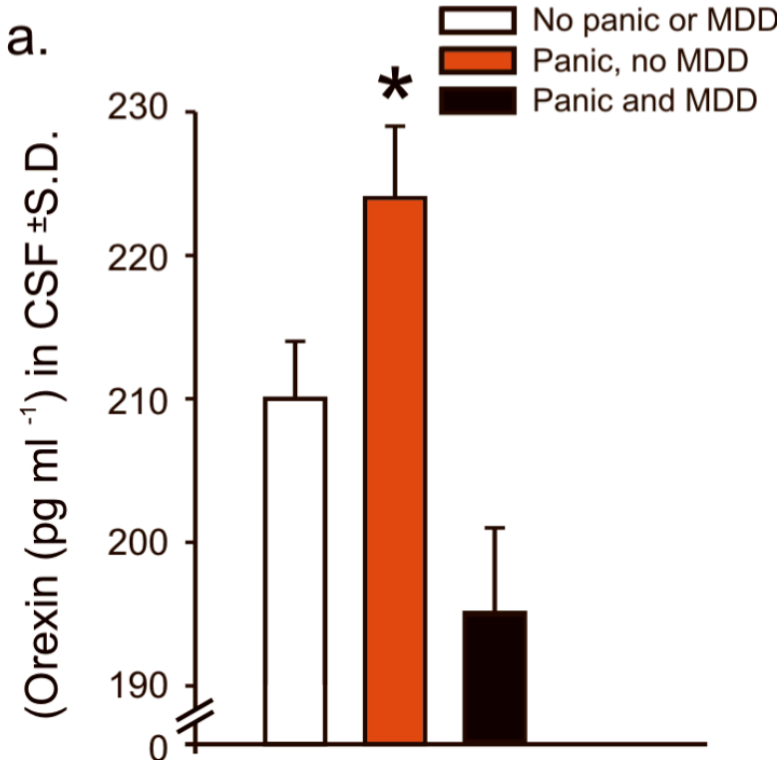

Figure 4.

Results of the human translational study: bar graph illustrates ORX concentrations in cerebrospinal fluid (CSF: obtained by lumbar puncture) samples from all subjects, which were assayed using a radioimmunoassay[33]. A cohort of subjects who presented with acute suicidal behavior was systematically assessed for psychiatric symptoms utilizing the comprehensive psychopathological rating scale (CPRS), where item 3 (inner tension) assesses panic anxiety. A threshold cut off at 1.5 on this item was used to define a subject as having significant panic symptoms. All subjects with substance abuse and traces of antidepressive, neuroleptic or anxiolytic medication in the blood were excluded from the analysis. A total of 53 medication-free subjects were included comprising three groups: subjects with panic anxiety without major depressive disorder (MDD) (n=12); subjects with both panic and co-morbid MDD (n=13); and subjects without panic, without MDD (n=28). * indicates significant differences from other groups, using Kruskall Wallis ANOVA (P=0.004); and two-tailed Mann-Whitney U-test (subjects with panic and MDD, p= 0.002; subjects without panic, p=0.01). Bars in graph represent the mean, and error bars represent standard deviation of the mean. Supplemental Table 2 describes details of subject characteristics. Age and gender did not have any impact on CSF ORX levels (Pearsons R and Mann-Whitney U-tests, P>0.1).