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Putative genes mediating the effects of orexins in the posterior paraventricular thalamus on neuroendocrine and behavioral adaptations to repeated stress

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Abstract

Exposure to repeated stress is often associated with psychopathology. However, our understanding of the underlying neural circuitry that regulates responses to repeated stress is limited. The posterior paraventricular thalamus (pPVT) is a brain region responsible for transmission of multimodal sensory information to limbic structures that regulate responses to both acute and repeated stress. Orexin-containing cells originating in the hypothalamus heavily innervate the pPVT. Our previous work has shown that activation of orexin1 receptors in the pPVT during repeated swim stress is important for facilitation of the hypothalamic-pituitary-adrenal (HPA) axis response to subsequent novel restraint. However, the genes responsible for these orexin-mediated adaptations to repeated stress are not known. Using a custom PCR array we examined the expression of 186 specific mRNAs in the pPVT of animals exposed to repeated swim stress (4 days of 15 min swim/day) with or without direct pPVT microinfusion of the orexin1 receptor antagonist SB334867 prior to each daily swim stress. Tissue was collected the next morning under basal non stressed conditions. Repeated stress and/or orexin receptor blockade significantly altered expression of only 9 specific genes including growth factors (Vegfa, Bax and Mt3), G-protein coupled receptors (Adora2a, Grm2 and Crhr1), immune-related genes (Ptgs2 and Cx3cr1) and an epigenetic-related gene (Hdac5). These genes represent potential targets for further characterization of orexin-mediated adaptations to repeated stress in the pPVT.

Keywords

Hypocretin; Hypothalamic pituitary adrenal; PCR array; Crhr1; Vegfa; Bax; Mt3; Ptgs2; Ador2a; Grm2; Cx3cr1; Hdac5

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Disclosure statement

The authors have nothing to disclose.

1. Introduction

The paraventricular nucleus of the thalamus (PVT) is an interface between sensory inputs and limbic and cortical structures of the extended amygdala (Su and Bentivoglio, 1990; Berendse and Groenewegen, 1991; Turner and Herkenham, 1991; Moga et al., 1995; Kirouac et al., 2005; Dong and Swanson, 2006). The PVT contains moderate to high densities of orexin receptors (Trivedi et al., 1998; Lu et al., 2000; Marcus et al., 2001; Cluderay et al., 2002) and receives amongst the densest orexin innervation in the brain in rodents and primates (Peyron et al., 1998; Nambu et al., 1999; Kirouac et al., 2005; Kerman et al., 2007; Hsu and Prince, 2009). Within the PVT, the posterior division (pPVT) receives the densest inputs compared to the anterior and medial subdivisions (Kirouac et al., 2005). Our previous work indicates the posterior division of the PVT is important for neuroendocrine and behavioral adaptations to repeated stress (Bhatnagar et al., 2002; Jaferi and Bhatnagar, 2006). Adaptations to repeated stress fall into at least two categories: habituation to familiar, homotypic stressors and facilitation to novel, heterotypic stressors (Dallman et al., 1992; Bhatnagar and Dallman, 1998). Two phases to habituation and facilitation have been identified. During the initial development phase, the effects of repeated stress become established, which allows the habituation to the homotypic stressor or facilitation to the novel stressor to be expressed, the second phase (Jaferi and Bhatnagar, 2006; Grissom and Bhatnagar, 2011). Our previous work suggests that different neural substrates mediate development vs. expression phases (Jaferi and Bhatnagar, 2006; Grissom et al., 2008; Grissom and Bhatnagar, 2011; Heydendael et al., 2011). Recently, we examined whether orexin inputs to the pPVT regulate the development (during repeated swim) and/or expression (during novel restraint) of facilitation of HPA activity. We observed that blockade of orexin1 receptors in the pPVT with the orexin1 receptor antagonist SB334867 prior to each of 4 daily swim exposures prevented the expression of the facilitated ACTH and facilitated hypothalamic c-Fos response to subsequent novel challenge restraint. Furthermore, this blockade of pPVT orexin receptors during development prevented the repeated swim stress-induced increase in basal CRH mRNA in the paraventricular hypothalamus (Heydendael et al., 2011). However, blockade of orexin receptor1 prior to restraint (i.e. during expression) had no effect. These results suggest that activation of orexin inputs to the pPVT play a key role in the development of the facilitated response. However the genes that mediate orexin actions in the pPVT during development are not known. Here, we used a custom PCR array to examine the expression of 186 specific mRNAs in the pPVT of animals exposed to repeated swim stress (4 days of 15 min swim/day) with or without direct pPVT microinfusion of the orexin1 receptor antagonist SB334867 prior to the daily swim stress. The genes were chosen from 7 prospective groups of genes selected based on their potential roles in the neurological adaptations to repeated stress as well as genes that have not been studied in this regard. This selection of genes represented a broad, relatively unbiased approach to identify the genes or groups of genes that may be involved in orexin regulation of stress adaptation in the PVT. These groups are: growth factors and their related genes (47 genes), G-protein coupled receptors and related genes (46 genes), immune-related genes (20 genes), epigenetic-related genes (31 genes), intracellular signaling-related genes (26 genes), genes for common ion channels (11 genes) and genes for neuropeptides (6 genes). The complete list of genes analyzed is in supplementary Fig. 1. The results indicate

that of the 186 genes examined, the expression of only 9 genes was significantly altered in the pPVT by either stress and/or blockade of orexin receptors. These results identify a subset of genes that may mediate the actions of orexins in the pPVT in the regulation of neuroendocrine and behavioral adaptations to repeated stress.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (Charles River, Kingston, NY) weighing between 225 and 250 g at arrival were used for these studies. Rats were individually housed in plastic tub cages on a 12:12 h light:dark cycle with lights on at 0600 h with ad libitum access to food and water. Rats were given a 5–7 day acclimation period prior to surgery. All stress and experimentation took place before 1200 h. All procedures were approved by the IACUC at the CHOP Research Institute.

2.2. Procedure

Unilateral guide cannulae (22ga) were implanted in the pPVT to target the posterior division: AP: –2.8, ML: 0.0 mm, DV: –6.4 mm. Half the rats were exposed to swim stress and the other half remained in their home cages and served as controls. Swim stress was performed in large glass chromatography containers filled to 35 cm with 25 °C water with rats placed in the water for 15 min on each of 4 consecutive days. Rats were injected with vehicle or 0.1 µg of SB334867 in 0.25 µl of DMSO directly into the pPVT at 30 min prior to daily swim stress for 4 days. Drug was administered by inserting a 7 mm injector cannula connected to PE tubing. The dose of SB334867 and the stress/injection protocol was chosen based on our previously published data showing that daily pre-swim administration of 0.1 µg SB334867 prevents facilitation of the HPA axis response to restraint (Heydendael et al., 2011). Control rats were injected with vehicle or SB334867 at the same time of day as swim stressed rats. In all experiments cannulae placement was confirmed upon sacrifice using previously described criteria (Bhatnagar et al., 2000, 2006), the success rate was 85%. The pPVT was defined as extending from –2.8 to –3.3 mm from bregma (Bhatnagar et al., 2000). Following exclusion based on histology, the group sizes, which represent the number of biological replicates in the gene array, for the 4 groups were: non-stressed/vehicle: $n = 10$; non-stressed/SB: $n = 9$; swim stressed/vehicle: $n = 7$; 0.1 µg swim stressed/SB: $n = 9$. In order to determine the genes that putatively mediate the effects of orexins in the pPVT, rats were sacrificed under basal conditions on day 5 without any restraint exposure.

2.3. Tissue collection, RNA extraction

24 h after final stress/control exposure, rats were decapitated and circular 1 mm PVT punches were collected and flash frozen on dry ice. Samples were subsequently stored at –80 °C until RNA extraction. Samples were homogenized in QIAzol Lysis Buffer and total RNA was isolated using miRNEasy kit (Qiagen). RNA concentration was assessed using nanodrop spectrophotometer (Thermo Fisher scientific), where 260/230 ratios were in the range of 0.16–1.85 and the 260/280 ratios were all above 1.8. Quality of RNA was assessed using the Agilent technologies bioanalyzer 2100, with a minimum RNA integrity number of 7 being considered acceptable for inclusion into the experiment. Using these criteria, all

samples were included in the experiment. All PCR array related work was conducted at the Nucleic Acid/Protein Research Core Facility at the Children's Hospital of Philadelphia.

2.4. Gene expression assays using custom PCR array

Custom-made PCR arrays (CAPR-10089E, SABiosciences, Supporting information Fig. 1) containing 192 pre-optimized SYBR Green RT PCR assays for 186 genes of interest, 3 housekeeping genes (Beta-actin, Ribosomal protein large P1 and hypoxanthine phosphoribosyltransferase 1) and 3 synthetic control genes (reverse transcription control, positive PCR control and rat genomic DNA contamination control). Samples were run according to manufacturer's instructions. 320 ng RNA per rat was used to synthesize cDNA using the RT² First Strand Kit (SABiosciences). The comparative Ct method (Schmittgen and Livak, 2008) was used to plot mRNA expression differences for genes of interest. Ct values were normalized to the average Ct values of the three housekeeping genes for each rat. Heat maps were constructed using GENE-E software by The Broad Institute (<http://www.broadinstitute.org/cancer/software/GENE-E/>).

2.5. Analysis

First pass analysis used to identify genes differentially expressed between experimental groups was done using the analysis tool provided by SABiosciences (www.sabiosciences.com/pcrarraydataanalysis.php). Further detailed statistical analyses of the differentially expressed genes products was done using Prism (Graphpad software, LaJolla, CA) for 2-way ANOVA for the factors of Drug (vehicle or SB334867) $\times$ Stress (control or repeated swim stress). In the event of significant main or interaction effects, post hoc analyses were conducted. A p value of 0.05 was used for all analyses.

3. Results

3.1. Growth factor-related genes

Of the 47 growth factor-related mRNAs examined in the custom PCR array, three growth factors showed significant effects following two-way ANOVA. There was a significant interaction effect ($F_{1,31} = 6.602$, $p = 0.02$; Fig. 1A) on the expression of vascular endothelial growth factor A (Vegfa). Post hoc tests indicated that four days of repeated swim stress significantly increased Vegfa expression compared to the no swim-vehicle group ($p = 0.03$). This effect was blocked by treatment with the orexin1R antagonist SB334867 ($p = 0.01$) since repeatedly swim stressed rats injected with SB334867 exhibited Vegfa expression significantly lower than vehicle treated repeated swim group. There was also a significant interaction effect ($F_{1,31} = 4.723$, $p = 0.04$; Fig. 1B) on the expression Bcl-2-associated $\times$ protein (Bax). Post hoc tests indicated that the vehicle-injected repeated swim stress group exhibited significantly increased Bax expression when compared with the no swim-vehicle group ($p = 0.01$). However, Bax expression in the SB334867-treated repeated stress group was similar to that in controls. There was a significant interaction effect on the expression of metallothionein-3 (Mt3, $F_{1,31} = 11.19$, $p = 0.00$; Fig. 1C). Post hoc tests revealed that four days of treatment with SB334867 significantly increased Mt3 expression in the no swim control group when compared to the no swim vehicle group ($p = 0.02$). This effect was the opposite in the repeated swim group where 4 days of SB334867 significantly decreased Mt3

expression compared to the vehicle treated repeated swim group ($p = 0.05$). To summarize, expression of *Vegfa* and *Bax* was increased by repeated swim, and this increase was prevented by treatment with SB334867. In contrast, *Mt3* was increased by SB334867 treatment in the non-stressed control group but decreased by SB334867 treatment in the repeated swim stress group.

3.2. GPCR-related genes

Of the 46 G-protein coupled receptor-related mRNAs examined, 3 showed significance following two-way ANOVA. There was a significant main effect of treatment with SB33867 to decrease the expression of adenosine A_{2A} receptor (*Adora2a*; $F_{1,31} = 9.795$, $p = 0.00$; Fig. 2A) regardless of stress condition. There was a significant interaction effect on the expression of group II metabotropic glutamate receptor (*Grm2*; $F_{1,31} = 5.064$, $p = 0.03$; Fig. 2B). Post hoc analysis revealed an effect of SB334867 treatment in the no stress control group to increase *Grm2* mRNA when compared with the no swim-vehicle group ($p = 0.03$). This effect was not present in the repeated swim group. There was a significant interaction effect on the expression of corticotropin-releasing hormone receptor 1 (*Crhr1*; $F_{1,31} = 4.763$, $p = 0.04$; Fig. 2C). Post hoc analysis revealed that vehicle treated swim stressed rats exhibited increased *Crhr1* mRNA expression when compared to the no swim-vehicle group ($p = 0.03$). However, expression of *Crhr1* mRNA in the SB334867 treated repeated swim group was not different from the vehicle treated repeated swim group.

3.3. Immune-related genes

Of the 20 immune-related mRNAs examined, two genes showed significance following two-way ANOVA. There was a significant main effect of drug treatment on the expression of prostaglandinendoperoxide synthase 2 (*Ptgs2*; $F_{1,31} = 9.637$, $p = 0.00$; Fig. 3A) such that repeated treatment with SB334867 decreased *Ptgs2* expression regardless of stress condition. There was a significant main effect of drug treatment on the expression of the CXC3 chemokine receptor 1, also known as the fractalkine receptor or GPCR13 (*Cx3cr1*; $F_{1,31} = 8.817$, $p = 0.01$; Fig. 3B) with repeated treatment with SB334867 increasing *Cx3cr1* expression regardless of stress condition. Thus, two immune-related genes were differentially affected by SB334867 treatment with *Ptgs2* being decreased and *Cx3cr1* being increased by treatment regardless of stress condition.

3.4. Epigenetic-related genes

Of the 31 epigenetic-related mRNAs examined, only histone deacetylase 5 (*Hdac5*) showed significance following two-way ANOVA. There was a significant interaction effect on the expression of *Hdac5* ($F_{1,31} = 5.671$, $p = 0.02$; Fig. 4). Post hoc analysis revealed a significant effect of treatment with SB334867 to increase *Hdac5* mRNA only in the no swim control group ($p = 0.01$). *Hdac5* expression in the repeated swim group was not affected by orexin receptor blockade.

3.5. Other gene groups

Of the remaining 3 gene groups of genes that were chosen for examination there was no effect on the expression of the 26 intracellular signaling-related genes, the 11 genes for

common ion channels or any effect on the 6 genes for neuropeptides examined (Fig. 5). Thus, the expression of only 9 genes was significantly altered in the pPVT by either stress and/or blockade of orexin receptors thereby identifying genes that mediate orexin actions in the pPVT to regulate neuroendocrine adaptations to repeated stress.

3.6. Discussion

Only 9 of the 186 genes examined in the pPVT were significantly affected by repeated stress and/or by orexin receptor blockade. Three of the gene groups selected, intracellular signaling, ion channels or neuropeptides, did not contain a single gene that showed significance in the 2-way ANOVAs. However, this lack of effect does not rule out the possibility that other intracellular messengers, ion channels or neuropeptides that were not examined in the array may still play a role in the orexin-mediated effects on adaptations to repeated stress. The 9 identified genes represent specific gene targets that could mediate orexin-effects in the pPVT in repeatedly stressed rats (Heydendael et al., 2011). Below we discuss the findings by grouping genes by the impact of stress and/or orexin receptor blockade on their expression.

Three of the genes examined were significantly affected by blockade of orexin receptors in the pPVT in both non-stressed control and stressed groups. *Adora2a* and *Ptgs2* were decreased by 4 days of treatment with SB334867 whereas *Cx3cr1* was increased by blockade of orexin receptor in both stress groups. The lack of differential expression of these genes between control and repeated stress groups indicates that these genes do not play a role in the orexin-mediated adaptations that occur specifically in response to repeated stress. However, these genes are clearly regulated by orexin receptor activation suggesting that they do play a role in other orexin-mediated actions in the pPVT that are not related to repeated stress. Furthermore, because of the effects of SB334867 on the expression of these three genes in the nonstressed groups, these results also imply that these three genes are under constitutive regulation by orexin during basal conditions. *Adora2a* mRNA encodes the adenosine A2A receptor which plays an important role in the regulation of glutamate and dopamine release (Ferré et al., 2007; Cunha et al., 2008). Adenosine and its respective receptors, including the 2a receptor, have also been implicated in sleep induction (Huang et al., 2011), which has been directly associated with the orexin system (Sakurai and Mieda, 2011). These findings, in combination with the current results showing orexin regulation of pPVT *Adora2a*, suggest a role for orexin actions in pPVT in the regulation of sleep/wakefulness. *Ptgs2* mRNA encodes for the protein cyclooxygenase-2 (cox-2), which converts arachidonic acid (AA) to prostaglandin endoperoxide-H₂ and is considered pro-inflammatory (Hewett et al., 2006). While little is known about the regulation of cox-2 by orexin in the brain, previous literature reports a similar regulation of cox-2 by orexinA in the gut, with orexinA treatment causing over-expression of cox-2 protein (Brzozowski et al., 2008). Considering that in the current study orexin receptor blockade led to a reduction in *Ptgs2* mRNA even in the non-stressed group, the results suggest that orexin activity in the pPVT positively regulates the inflammatory cascade associated with *Ptgs2* expression. *Cx3cr1* mRNA encodes for the CX3C chemokine receptor 1, which plays a developmental role in the migration of microglia in the central nervous system (Nishiyori et al., 1998). Previous work has reported that deficiencies in *Cx3cr1* signaling impairs cognitive function

and synaptic plasticity with Cx3cr1 knock-out mice exhibiting deficits in the morris water maze, decreased motor learning and impaired long-term potentiation in the hippocampus (Rogers et al., 2011). Thus, these data suggest that orexin inputs into the pPVT may play a role in the synaptic plasticity, and potentially immune function, within the pPVT under basal conditions and that this potential role is independent of the stress state of the animal.

Mt3, Grm2 and Hdac5 were increased by 4 days of orexin receptor blockade in non swim stressed animals. However, in the case of Grm2 and Hdac5 the effect of the SB334867 treatment was not apparent following repeated swim stress. In the case of Mt3, the effect of SB334867 to increase expression was actually reversed following repeated swim stress. These data suggest that orexin regulation of these 3 genes occurs during basal non-stress conditions and that additional inputs to the pPVT during stress may override the effects of orexins and in the case of MT3 reverse the affect orexins. Mt3 mRNA encodes the protein metallothionein-3, which is a metal-binding protein that is associated with resistance to neuronal injury (Uchida et al., 2002; Kim et al., 2008). Grm2 mRNA encodes the G-protein coupled group 2 metabotropic glutamate receptor (mGluR2), which is responsible for modulating glutamate neurotransmission (Feyissa et al., 2010). Work in rats has shown a role for mGluR2 to reduce neuronal excitability in rat PVT that, because of the connections of the PVT with limbic structures, is speculated to contribute to the effectiveness of mGluR2-activating drugs in rodent models of anxiety and psychosis (Hermes and Renaud, 2011). These two studies taken together with our work showing orexin receptor blockade increasing mGluR2 mRNA suggests a possible link with orexin regulation of mGluR2 in stress-related behaviors relevant to psychiatric disorders. Hdac5 mRNA encodes histone deacetylase 5, which represses transcription when tethered to a promoter and is suggested to play a role in the epigenetic mechanisms that contribute to stress-related psychiatric illnesses (Tsankova et al., 2007). Increased Hdac5 function ultimately leads to decreased histone acetylation and decreased transcription of Hdac5 target genes. Knocking out the gene for Hdac5 created a hypersensitive response to chronic social defeat stress that led to increased social avoidance in the social interaction test and decreased sucrose preference when compared with wild-type control (Renthal et al., 2007). When taken in conjunction with our findings, these results suggest that normal suppression of Hdac5 in the pPVT by orexin is a critical factor in how sensitive an animal is to a given stressor, and may determine how an animal responds to subsequent stressors.

Three genes exhibited increased expression following repeated stress that was blocked by pre-treatment with SB334867, but no effect in non-stressed control animals was observed. This response pattern parallels the blockade of the facilitated HPA response to a novel stressor by SB334867 that we previously showed (Heydendael et al., 2011). Two of these genes, Vegfa and Bax, are growth factor-related genes whose specific activity is related to cell death and survival (Kim et al., 2006). Vegfa mRNA encodes vascular endothelial growth factor, which is constitutively expressed in the brain (Marti and Risau, 1998), and exerts neuroprotective effects (Sondell et al., 2000). Vegfa stimulates axonal outgrowth, enhances cell survival and has been shown to protect neurons against ischemic injury (Sondell et al., 2000; Shin et al., 2008). Bax, on the other hand is a pro-apoptotic member of the Bcl-2 protein family (Oltvai et al., 1993), which has been shown to be regulated by Vegfa (Kim et al., 2006). Because Vegfa regulates Bax, it is not surprising that the

expression patterns of the two genes are similar. Therefore, it is difficult to interpret whether Bax is directly regulated by orexin receptor activation or indirectly through effects of orexins on Vegfa. Regardless, the significant interaction effect for both genes suggests that this particular growth factor system involving Bax and Vegfa is involved in the orexin actions in the pPVT during repeated stress. Crhr1 was the third gene exhibiting increased expression following repeated stress that was blocked by treatment with SB334867. Crhr1 mRNA encodes for the Corticotropin releasing hormone receptor 1, a G-protein coupled receptor that mediates endocrine, autonomic, behavioral, and immune responses to stress (Dautzenberg et al., 2001; Aguilera et al., 2004). In addition to regulating neuroendocrine responses to stress, the CRH/Crhr1 system also regulates a variety of stress-related behaviors (Dunn and Berridge, 1990; Valentino and Van Bockstaele, 2002; Hammack et al., 2003) such as locomotor activity, sleep (Romanowski et al., 2010), addictive behavior (Sillaber et al., 2002) and in particular anxiety-related behavior (Dunn and Berridge, 1990; Liebsch et al., 1999). Work using Crhr1 knock-out mice has shown a reduction in anxiety-related behavior in the knock-out mice when compared to wild-type controls (Smith et al., 1998; Timpl et al., 1998). We found that orexin receptor blockade prevented the repeated stress-induced rise in Crhr1 mRNA. It has also been previously reported that CRH receptors are expressed on orexin cell bodies in the hypothalamus, and activation of these receptors can increase the firing frequency of orexin cells, suggesting a close interaction between CRH and the orexin system (Winsky-Sommerer et al., 2004, 2005). Although it is not known whether Crhr1 are expressed in the same neurons that express orexin receptors in the PVT, the results suggest an important interaction between orexin and Crhr1 in the regulation of adaptations to stress. Further work will be necessary to elucidate this interaction between the Crhr1 and orexin systems in the PVT.

4. Conclusion

Of the 9 genes found to be significant, only 3 had expression profiles that mimicked the facilitated HPA response to novel stress that was blocked by daily pre-swim stress treatment with SB334867 (Heydendael et al., 2011). For this reason, the results implicate these genes, Vegfa, Bax and Crhr1, as mediating orexin effects in the pPVT on the facilitated HPA response to novel stress and suggest that appropriate regulation of these genes by orexin is necessary for mounting a proper adaptive stress response. Disruption of this regulation may be involved in the transition from an adaptive stress response toward a detrimental stress response and possible psychiatric illness. Further study of these particular genes is necessary with a focus on their role in other measures of stress adaptation such as habituation of the HPA response to a repeated homotypic stressor. Additionally, research focused on the specific mechanisms through which these genes and gene products operate during exposure to repeated or chronic stress is necessary.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.brainresbull.2012.09.002>.

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Fig. 1.

(A) Relative expression of Vegfa mRNA in the pPVT of non-stressed rats (no swim) or rats exposed to daily 15 min swim for 4 days (repeated swim) that were injected with vehicle or 0.1 μg of orexin receptor antagonist SB334867 directly into the pPVT prior to each of the 4 days of no stress or swim and then sacrificed basally on day 5. Group sizes were: non-stressed/vehicle: $n = 10$; non-stressed/SB: $n = 9$; swim stressed/vehicle: $n = 7$; 0.1 μg swim stressed/SB: $n = 9$. (B) Relative expression of Bax mRNA in rats as described in (A). (C) Relative expression of Mt3 mRNA in rats as described in (A). (D) Heat map showing

relative fold change expression levels of the growth factor-related genes examined in relation to house keeping gene expression. * = $p < 0.05$ compared to vehicle-no swim; # = $p < 0.05$ compared to vehicle-repeated swim.

Fig. 2.

(A) Relative expression of Adora2a mRNA in the pPVT of non-stressed rats or rats exposed to daily 15 min swim for 4 days that were injected with vehicle or 0.1 μ g of orexin receptor antagonist SB334867 (SB) directly into the pPVT prior to each of the 4 days of no stress or swim and then sacrificed basally on day 5. Group sizes were: non-stressed/vehicle: $n = 10$; non-stressed/SB: $n = 9$; swim stressed/vehicle: $n = 7$; 0.1 μ g swim stressed/SB: $n = 9$. (B) Relative expression of Grm2 mRNA in rats as described in (A). (C) Relative expression of Crhr1 mRNA in rats as described in (A). (D) Heat map showing relative fold change

expression levels of the GPCR-related genes examined in relation to house keeping gene expression. * = $p < 0.05$ compared to vehicle-no swim; ** = $p < 0.05$ main effect of drug treatment.

Fig. 3.

(A) Relative expression of Ptgs2 mRNA in the pPVT of non-stressed rats or rats exposed to daily 15 min swim for 4 days that were injected with vehicle or 0.1 μ g of orexin receptor antagonist SB334867 (SB) directly into the pPVT prior to each of the 4 days of no stress or swim and then sacrificed basally on day 5. Group sizes were: non-stressed/vehicle: $n = 10$; non-stressed/SB: $n = 9$; swim stressed/vehicle: $n = 7$; 0.1 μ g swim stressed/SB: $n = 9$. (B) Relative expression of Cx3cr1 mRNA in rats as described in (A). (C) Heat map showing relative fold change expression levels of the immune-related genes examined in relation to housekeeping gene expression. * = $p > 0.05$ compared to vehicle-no swim; # = $p < 0.05$ compared to vehicle-repeated swim; ** = $p < 0.05$ main effect of drug treatment.

Fig. 4. (A) Relative expression of Hdac5 mRNA in the pPVT of non-stressed rats or rats exposed to daily 15 min swim for 4 days that were injected with vehicle or 0.1 μ g of orexin receptor antagonist SB334867 (SB) directly into the pPVT prior to each of the 4 days of no stress or swim and then sacrificed basally on day 5. Group sizes were: non-stressed/vehicle: $n = 10$; non-stressed/SB: $n = 9$; swim stressed/vehicle: $n = 7$; 0.1 μ g swim stressed/SB: $n = 9$. (B) Heat map showing relative expression levels of the epigenetic-related genes examined. * = $p < 0.05$ compared to vehicle-no swim.

Fig. 5.
Heat map showing relative fold change expression levels of the intracellular signaling genes, common ion channel genes and neuropeptide genes examined in relation to housekeeping gene expression.