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Research report

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.

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 innnervation 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 subdivisons (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

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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 RT^2 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.sabiosceneces.com/pcrarraydataanlysis.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) × 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 × 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