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Orexinergic modulation of serotonin neurons in the dorsal raphe of a diurnal rodent, *Arvicanthis niloticus*

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ABSTRACT

The hypothalamic neuropeptide, orexin (or hypocretin), is implicated in numerous physiology and behavioral functions, including affective states such as depression and anxiety. The underlying mechanisms and neural circuits through which orexin modulates affective responses remain unclear. The objective of the present study was to test the hypothesis that the serotonin (5-HT) system of the dorsal raphe nucleus (DRN) is a downstream target through which orexin potentially manifests its role in affective states. Using a diurnal rodent, the Nile grass rat (*Arvicanthis niloticus*), we first characterized the expression of the orexin receptors OX1R and OX2R in the DRN using *in situ* hybridization. The results revealed distinct distributions of *OX1R* and *OX2R* mRNAs, with *OX1R* predominantly expressed in the dorsal and lateral wings of the DRN that are involved in affective processes, while *OX2R* was mostly found in the ventral DRN that is more involved in sensory-motor function. We next examined how the orexin-OX1R pathway regulates 5-HT in the DRN and some of its projection sites using a selective OX1R antagonist SB-334867 (10 mg/kg, i.p.). A single injection of SB-334867 decreased 5-HT-ir fibers within the anterior cingulate cortex (aCgC); five once-daily administrations of SB-334867 decreased 5-HT-ir not only in the aCgC but also in the DRN, oval bed nucleus of the stria terminalis (ovBNST), nucleus accumbens shell (NAcSh), and periaqueductal gray (PAG). HPLC analysis revealed that five once-daily administrations of SB-334867 did not affect 5-HT turnover to any of the five sites, although it increased the levels of both 5-HT and 5-HIAA in the NAcSh. These results together suggest that orexinergic modulation of DRN 5-HT neurons *via* OX1Rs may be one pathway through which orexin regulates mood and anxiety, as well as perhaps other neurobiological processes.

1. Introduction

Orexin, also known as hypocretin, is a hypothalamic neuropeptide with a well-established role in regulating many important physiological functions including the sleep-wake cycle and energy homeostasis (Siegel, 2004; Tsujino and Sakurai, 2009). The orexin system has also been implicated in regulating mood and anxiety (Nollet and Leman, 2013; Pizza et al., 2014). For example, narcoleptic patients have diminished central orexin levels, and have a higher likelihood of mood and anxiety disorders when compared to the general population or to individuals with other neurological disorders (Fortuyn et al., 2010; Ohayon, 2013; Vourdas et al., 2002). Similarly, lower orexin levels have been reported in patients suffering from major depressive disorders or comorbid depression and anxiety (Brundin et al., 2007a; Brundin et al., 2009; Brundin et al., 2007b; Johnson et al., 2010; Rotter et al., 2011). On the other hand, a positive correlation between orexin and positive emotions has been observed in both dogs and humans (Blouin et al., 2013; Wu et al., 2011). Although there is a clear association between orexin and affective state, the underlying neural pathways through which orexin regulates mood and emotion are not well understood.

One of the potential downstream targets of the orexinergic system in regulating affective state is the dorsal raphe nucleus (DRN), which contains the greatest number of midbrain 5-HTergic neurons and is implicated in depression and anxiety among other functions (Graeff, 1993; Michelsen et al., 2007). Orexin neurons project heavily to the DRN (Nixon and Smale, 2007; Peyron et al., 1998), where orexin peptides induce excitatory responses *in vitro* (Soffin et al., 2004) and

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stimulate 5-HT release *in vivo* (Tao et al., 2006). Manipulating the central 5-HT system is the basis of many popular pharmacotherapies for treating affective disorders, albeit controversial because the underlying mechanisms and effectiveness of such drugs are unclear (Cipriani et al., 2018; Harmer et al., 2017). Regardless, our previous findings in a diurnal rodent model of seasonal affective disorder (SAD) support for a role of the orexin-DRN pathway in regulating mood and anxiety (Deats et al., 2014; Ikeno et al., 2016; Leach et al., 2013).

SAD is a major depressive disorder with a seasonal pattern, in which patients experience recurring depression episodes in fall and winter followed by spontaneous remission in spring and summer (Rosenthal et al., 1984). The symptoms of SAD can be alleviated by bright light therapy, suggesting a causal link between reduced light exposure and depression in winter months. Bright light exposure promotes arousal and wakefulness in diurnal mammals including humans; but induces sleep in nocturnal ones (Smale et al., 2003). Therefore, a diurnal model is advantageous for understanding the neuropathology of SAD (Yan et al., 2019). Previous work from this laboratory utilized diurnal *Nile* grass rats (*Arvicanthis niloticus*) that were housed in a winter-like lighting condition with reduced daytime light intensity (Leach et al., 2013). After four weeks, the animals showed higher depression- and anxiety-like behaviors compared to the controls housed in a summer-like condition with bright light during the day. Increased depression- and anxiety-like behaviors were accompanied by attenuated orexin-ir fibers and fewer 5-HT-ir neurons in the DRN, along with a lower density of 5-HT-ir fibers/terminals in the anterior cingulate cortex (aCgC) (Deats et al., 2014; Leach et al., 2013). We also found a functional connection between light, hypothalamic orexin cells and the DRN using the neural activity marker Fos (Adidharma et al., 2012). Bright light exposure increased Fos-ir in both orexin cells and in the DRN, and pretreating grass rats with a selective orexin receptor 1 (OX1R) antagonist, SB-334867, prevented light-induced Fos in the DRN. Furthermore, treating grass rats with SB-334867 led to increased depressive-like behaviors even when the animals were housed in a summer-like bright light condition (Deats et al., 2014). These results collectively suggest that in diurnal grass rats, orexinergic inputs to the DRN underlie light-dependent changes in behavioral paradigms modeling aspects of depression and anxiety. It should be noted that although the distribution of orexin receptors is generally similar between diurnal and nocturnal rodents, there are species-specific expression in brain regions implicated in regulating sleep, emotion and cognition (Ikeno and Yan, 2018). For example, OX1R mRNA has been detected in the caudate putamen and ventral tuberomammillary nucleus only in diurnal grass rats, but not in nocturnal mice (Ikeno and Yan, 2018). Thus, elucidating orexinergic regulation in 5-HT system in diurnal grass rats will contribute to a better understanding on how the two systems interact in humans.

In the present study we first characterized the expression of orexin receptors in the DRN of grass rats. The results revealed a distinct pattern in the distribution of OX1R and OX2R, one that suggests OX1R plays a dominant role in the DRN for modulating affective behaviors. We then assessed 5-HT-ir in the DRN and in several 5-HT neuron projecting sites involved in mood and anxiety, including the aCgC, oval bed nucleus of the stria terminalis (ovBNST), nucleus accumbens shell (NAcSh), and periaqueductal gray (PAG) following either acute (single administration) or subchronic (five daily administrations) orexin receptor antagonism with SB-334867. As discussed above, we previously found that a single administration of SB-334867 increased depression-like behaviors in grass rats housed in a summer-like condition (Deats et al., 2014), and demonstrating changes in the 5-HT system following the same treatment would support it as a downstream target through which orexin regulates affective state. The subchronic paradigm was intended to induce a more sustained attenuation of OX1R-mediated signaling, as in our grass rat SAD model that displays depression-like behaviors when animals are housed under low-intensity daylight (Deats et al., 2014; Leach et al., 2013). Following OX1R antagonism, we measured 5-HT-ir as well as levels of 5-HT and its metabolite 5-HIAA in the DRN and in projecting sites. The results demonstrate that OX1R-mediated signaling regulates the 5-HT system of the DRN, as well as some DRN projection sites, and provide insights into the pathways through which orexin neurons modulate affective functioning in a diurnal species.

2. Methods

2.1. Animals and housing conditions

Adult male grass rats (*Arvicanthis niloticus)* were produced from a breeding colony originally established with animals imported from sub-Saharan Africa in 1993 and since maintained at Michigan State University (for details see McElhinny et al., 1997). The animals in the colony were housed in a 12 h light:12 h dark (LD) cycle with food (Prolab 2000 #5P06, PMI Nutrition LLC, MO, USA) and water available *ad libitum*. The time of lights-on was defined as Zeitgeber time (ZT) 0. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80–23) and were approved by the Institutional Animal Care and Use Committee of Michigan State University.

2.1.1. Experiment 1: distribution of orexin receptors in the DRN

To determine the distribution of orexin receptors in the DRN, male grass rats (n = 5) were transcardially perfused with saline followed by 4% paraformaldehyde around midday between ZT5-7. The brains were post-fixed with 4% paraformaldehyde and cryoprotected in 20% sucrose before being processed for *in situ* hybridization using cRNA probes for *OX1R* and *OX2R* mRNA (details below).

2.1.2. Experiment 2: effects of acute OX1R antagonism on 5-HT immunoreactivity (ir)

Male grass rats (4–6 month old) received a single intraperitoneal (i.p.) injection of either the selective OX1R antagonist, SB-334867 (10 mg/kg, Tocris Biosciences, Bristol, UK), or vehicle (60:40 DMSO/saline, 0.4 ml) at ZT2. This dose was based on our previous study of grass rats (Adidharma et al., 2012; Deats et al., 2014), with effects on their brain and behaviors observed at 3 to 4 h post-injection. Animals were overdosed with pentobarbital, then transcardially perfused with saline followed by 4% paraformaldehyde at ZT5 (*i.e.*, 3 h post-injection, n = 6/group). Brains were post-fixed and cryoprotected, and sectioned at 40 μm for immunostaining of 5-HT.

2.1.3. Experiment 3: effects of subchronic OX1R antagonism on 5-HT-ir, 5-HT content and turnover

Similar to above, male grass rats received an i.p. injection of either the selective OX1R antagonist, SB-334867 (10 mg/kg), or vehicle (60:40 DMSO/saline, 0.4 ml), but once a day for five consecutive days. 24 h after the last injection, one cohort of animals (n = 6/group) was overdosed with pentobarbital and perfused. Their brains were prepared for immunostaining of 5-HT as in Experiment 2. Another cohort of animals (n = 8–9/group) was injected once a day for five days with SB-334867 or vehicle, overdosed and rapidly decapitated the next day, and the brains used for HPLC analysis of 5-HT system measures in the DRN, aCgC and NAc. Fresh-frozen brains were sliced coronally at 200 μm. Micropunches (0.5-mm diameter) through the sites of interest were made bilaterally and stored at −80 °C until being processed for HPLC.

2.2. In situ hybridization

Antisense and sense cRNA probes for *OX1R* and *OX2R* were produced and *in situ* hybridization was performed as described previously (Ikeno and Yan, 2018). In brief, coronal sections (40 μm) containing the DRN were treated with proteinase K and acetic anhydride prior to incubation with DIG-labelled *OX1R* or *OX2R* antisense (0.5 μg/ml) or

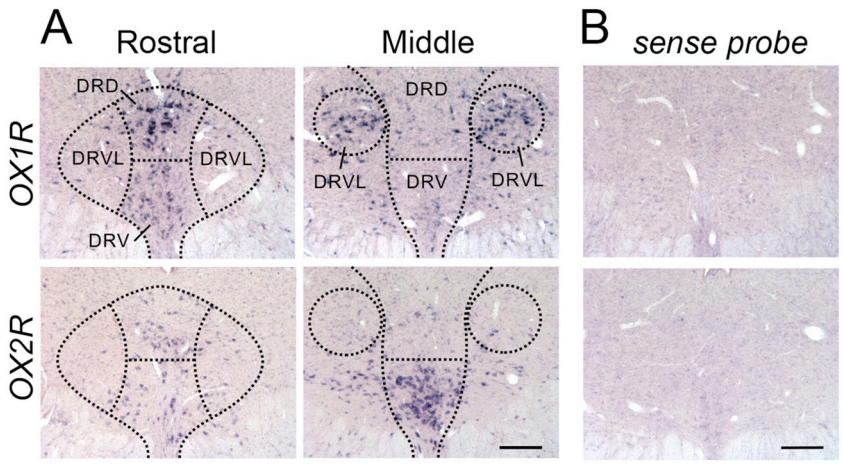

Fig. 1. Distribution of OX1R and OX2R mRNA in the rostral and middle DRN. DRD, DR dorsal; DRV, DR ventral; DRVL, DR ventrolateral wings. Scale bar, 100 μm.

sense probes (0.5 μg/ml) overnight at 60 °C. After a series of washes, sections were treated with RNase A, then incubated in an alkaline phosphatase-conjugated DIG antibody (1:5000, Sigma-Aldrich) for 3 d. Sections were then incubated in NBT/BCIP solution (Roche) overnight at room temperature.

2.3. Immunohistochemistry (IHC)

Single-label IHC for 5-HT was conducted using methods similar to those described in our previous study (Leach et al., 2013). Briefly, coronal sections containing the DRN and four of its target regions of interest were rinsed in 0.1 M phosphate buffer before incubation in a primary rabbit antiserum against 5-HT (1:10,000, NT-102 5HTrab, Protos Biotech, NY) at 4 °C for 2 days. This antiserum was raised against a formaldehyde 5-HT-hemocyanin conjugate, and specificity was established by abolishment of immunostaining following preabsorption with the immunogen (Chalazonitis et al., 2008). Sections were rinsed in phosphate buffer before incubation in a secondary antibody (biotinylated goat anti-rabbit, 1:1000, Vector lab, CA) at 4 °C overnight. Processing was completed with the avidin-biotin-immunoperoxidase technique (VECTASTAIN Elite ABC System, Vector lab, CA) per the manufacturer's protocol. Finally, the 5-HT containing cell bodies and fibers were stained brown using 3′3-diaminobenzidine (Sigma-Aldrich, St. Louis, MO) as chromogen.

2.4. Quantitative analysis of IHC results

Images of the brain sections were captured using a CCD video camera (CX9000, MBF Bioscience, VM, USA) attached to a light microscope (Nikon Instruments Inc., NY, USA). The camera and microscope settings were kept constant for all images. 5-HT-ir was analyzed in the DRN, NAcSh, ovBNST, aCgC and PAG. Sections containing the brain regions of interest (4–8 sections per region) correspond to the following plates in the rat brain atlas (Paxinos and Watson, 2004): planes 92 to 97 (Bregma −7.08 to −7.68 mm) for DRN, planes 20 to 24 (Bregma 1.56 to 1.08 mm) for NAcSh, planes 30 to 33 (Bregma 0.36 to 0 mm) for ovBNST, planes 14 to 33 (Bregma 2.28 to 0 mm) for aCgC and planes 86 to 93 (Bregma −6.36 to −7.20 mm) for PAG. In the DRN, sections from the rostral two-thirds of the DRN were analyzed, with sections containing 5-HT-ir cell bodies clustered at the center defining the rostral subregions (dorsal, ventrolateral, ventral), while the cell bodies spreading laterally defined the middle DRN (dorsal, ventral, ventrolateral “wings”) (Coomans et al., 2013). Observers blind to the animals' experimental conditions analyzed the cell number and optical density measurements using NIH Image as described in previous studies (Adidharma et al., 2012; Deats et al., 2014). Student's *t*-tests were used to determine differences between animals injected with the OX1R antagonist or vehicle on both the number of 5-HT cell bodies and 5-HT fiber density, with *ps* < 0.05 indicating statistical significance and effect size estimated using Cohen's d.

2.5. HPLC-EC analysis of monoamines

Biogenic amines were quantified as described elsewhere (Perez et al., 2005; Spieles-Engemann et al., 2010; Kanaan et al., 2015; Chen et al., 2019). Brain punches were sonicated in 200 μl of antioxidant solution (0.4 N perchlorate, 1.34 mM EDTA, and 0.53 mM sodium metabisulfite), and total protein concentration was determined using BCA assays (Pierce). Samples were centrifuged at 10,000*g* for 10 min at 4 °C. The supernatant was separated on a 150 × 4.6 mm Microsorb MV C18 100–5 column (Agilent Technologies), and simultaneously examined for 5-hydroxytryptamine (5-HT), 5-hydroxy-indoleacetic acid (5-HIAA), dopamine (DA), homovanillic acid (HVA), and 3,4-dihydroxyphenylacetic acid (DOPAC). Analytes were quantified on a 12-channel coulometric array detector (CoulArray 5200, ESA) attached to an autosampler/solvent delivery system (Waters Alliance 2695) under the following conditions: flow rate of 1 ml/min; detection potentials of 25, 85, 120, 180, 220, 340, 420, 480 mV; and scrubbing potential of 750 mV. The mobile phase consisted of 100 mM citric acid, 75 mM Na2HPO4, 80 μM heptanesulfonate monohydrate, pH 4.25, in 11% methanol. Sample values were calculated based on a minimum six-level standard curve of the analytes with quality control samples interspersed within the sample run. Data were expressed in ng/mg protein. Student's *t*-tests were used to determine differences between groups of animals injected with the OX1R antagonist or vehicle for each measure, with *ps* < 0.05 indicating statistical significance and effect size estimated using Cohen's d.

3. Results

3.1. Distribution of OX1R and OX2R mRNA in the DRN of grass rats

In the DRN, hybridization signals for *OX1R* and *OX2R* mRNA showed distinct distribution patterns. Particularly strong signals for *OX1R* mRNA was detected in the dorsal subregion of the rostral DRN, while there was only moderate expression in the ventral subregion at that level (Fig. 1A). There was also very dense OX1R mRNA in the lateral wings of the middle DRN (Fig. 1A). On the other hand, only weak *OX2R* signal was found in the rostral DRN in either the dorsal and ventral subregions, but very dense *OX2R* expression was found in the ventral part of the middle DRN (Fig. 1A). A sense probe for *OX1R* or *OX2R* mRNA revealed no specific labeling in the DRN (Fig. 1B).

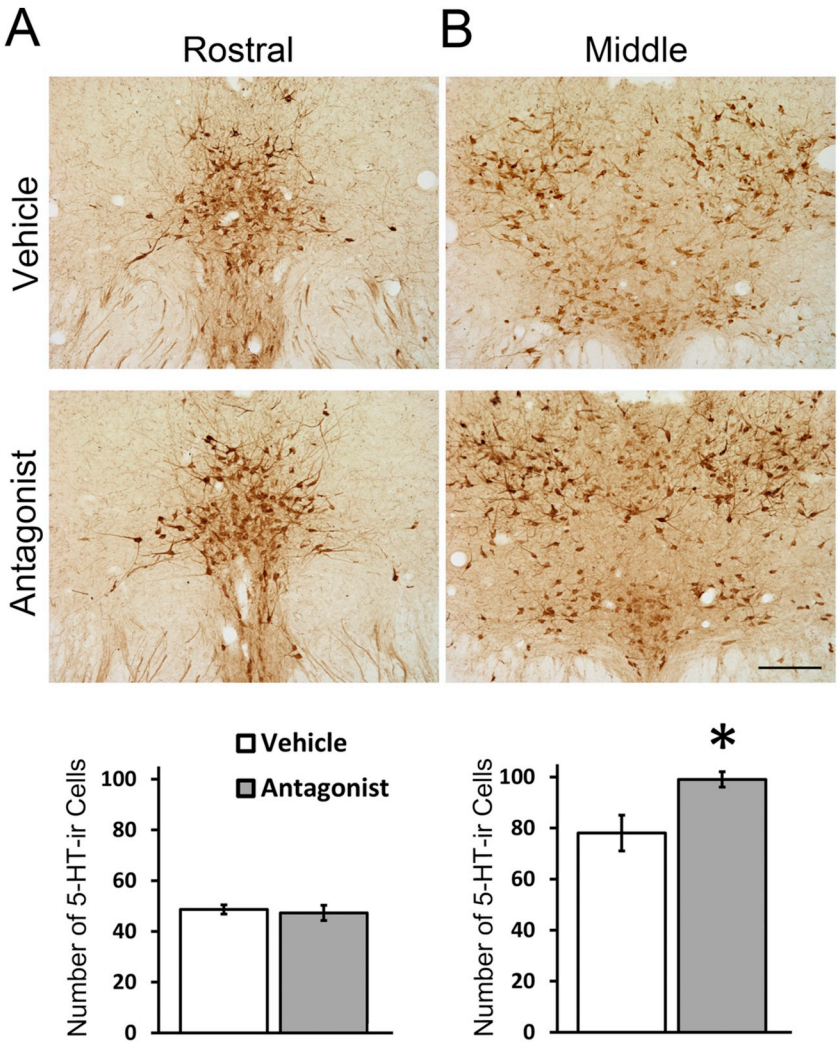

Fig. 2. Representative photomicrographs and quantitative analysis of 5-HT-ir cells in the rostral (A) and middle (B) DRN following a single injection of OX1R antagonist SB-33486 or vehicle. Data are shown as Means ± SEMs (n = 5). *indicates $p < 0.05$. Scale bar, 100 μm.

3.2. Acute OX1R antagonism increased 5-HT-ir in the DRN and reduced 5-HT-ir fiber density in aCgC

Following a single IP injection of the OX1R antagonist, SB-334867, the numbers of 5-HT-ir neurons in the rostral DRN did not differ between the antagonist- and vehicle-treated groups (Fig. 2A, $t_8 = -0.38$, $p = 0.713$, Cohen's $d = 0.24$), but in the middle DRN, the antagonist-treated group had a small but significantly higher number of 5-HT-ir cells compared to controls (Fig. 2B, $t_8 = -2.76$, $p = 0.025$, Cohen's $d = 1.75$). Antagonist-treated animals also showed significantly lower 5-HT-ir fiber density in the aCgC (Fig. 3, $t_8 = -3.01$, $p = 0.017$, Cohen's $d = 1.78$), but not in the BNST, NAcSh or PAG ($ps > 0.05$, Cohen's $d = 0.32$, 1.75 and 0.0 respectively).

Subchronic OX1R antagonism reduced 5-HT-ir in the DRN and its targets.

Five daily treatments of SB-334867 significantly reduced the number of 5-HT-ir neurons in the DRN compared to the number found in the DRN of vehicle-treated animals; this was true for both the rostral (Fig. 4A, $t_9 = 2.85$, $p = 0.046$, Cohen's $d = 1.99$) and middle DRN (Fig. 4B, $t_9 = 8.38$, $p < 0.001$, Cohen's $d = 4.85$). SB-334867-treated animals also showed a marked reduction in 5-HT-ir fiber density in the aCgC ($t_8 = -6.22$, $p < 0.001$, Cohen's $d = 2.26$, Cohen's $d = 1.99$), BNST ($t_9 = 3.58$, $p = 0.006$, Cohen's $d = 1.63$), NAcSh ($t_9 = 8.80$, $p < 0.001$, Cohen's $d = 5.32$) and PAG ($t_9 = 5.75$, $p < 0.001$, Cohen's $d = 3.37$) (Fig. 5).

HPLC analysis revealed no significant difference between the SB-334867- and vehicle-treated groups in 5-HT turnover within the three brain sites analyzed (*i.e.*, DRN, aCgC, NAcSh) (Table 1). However, the levels of 5-HT and its metabolite 5-HIAA (normalized by total protein) were higher in the NAcSh of the OXR1 antagonist treated group compared to controls. The antagonist-treated animals also had higher levels of DOPAC and HVA in the NAc, as well as in the DRN. While these measures were all corrected for total protein per sample, it should be noted that total protein per sample measured in the NAc itself was unexpectedly affected by OXR1 antagonist treatment, with total protein lower in the NAc (but not in the DRN or aCgC) of the antagonist-treated group compared to the controls (Table 1).

4. Discussion

The results of the present studies reveal that in diurnal grass rats, orexin-OX1R signaling has a significant impact on the central 5-HT system and therefore likely plays a role in regulating 5-HT-mediated processes, including affective behaviors. This is probably not exclusive to diurnal grass rats because expression of *OX1R* and *OX2R* mRNA has also been found in the DRN of laboratory mice and rats (Greco and Shiromani, 2001; Marcus et al., 2001; Mieda et al., 2011; Trivedi et al., 1998), although subregional difference in abundance of each receptor has not been previously reported. In the present study, a closer look of the distribution of each receptor subtype revealed a distinct spatial

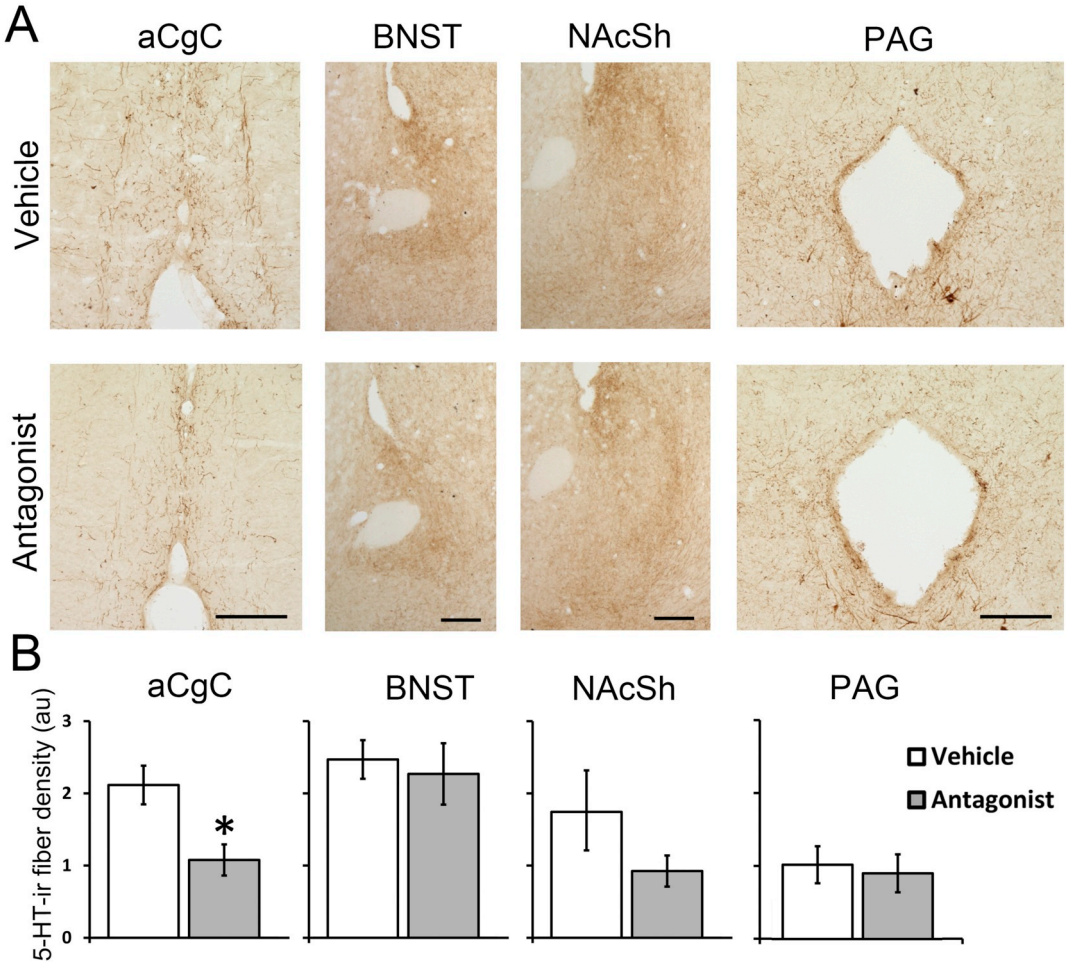

Fig. 3. Representative photomicrographs (A) and quantitative analysis (B) of 5-HT- ir fibers in the anterior cingulate cortex (aCgC), oval bed nucleus of the stria terminalis (ovBNST), nucleus accumbens shell (NAcSh), and periaqueductal gray area (PAG) following a single injection of OX1R antagonist SB-334867 or vehicle. Data are shown as Means ± SEMs (n = 5). *indicates $p < 0.05$. Scale bar, 100 μm.

pattern in the DRN, such that *OX1R* was predominantly expressed in the dorsal and lateral wings while *OX2R* was mainly expressed in the ventral subregion (Fig. 1). The DRN subregions are not only defined by the cytoarchitecture and distribution of 5-HT neurons, but also their afferent and efferent projections together, suggesting functional differences (Hale and Lowry, 2011). For example, the dorsal subnucleus and the lateral wings are considered part of the neural system involved in the behavioral and physiological responses related to stress and anxiety, while the ventral subregion regulates sensory-motor functions (Hale and Lowry, 2011). The distinct distribution pattern of *OX1R* and *OX2R* mRNA in the DRN suggest that OX1R predominantly influences the dorsal and lateral DRN for orexin's effect on mood and emotional behaviors in grass rats. This is consistent with existing literature suggesting distinct functions of OX1R and OX2R, including a genetic study that found a significant association between unipolar depression and a polymorphism of OX1R, but not OX2R (Rainero et al., 2011). OX2R has instead been primarily implicated in narcolepsy and catalepsy (Hasegawa et al., 2014; Lin et al., 1999; Willie et al., 2003).

Orexin peptides have been shown to regulate 5-HT neurons in nocturnal laboratory rats (Liu et al., 2002; Soffin et al., 2004; Tao et al., 2006). Both peptides can excite 5-HT neurons directly, as well as indirectly by inhibiting local GABAergic inputs to 5-HT neurons (Liu et al., 2002). The excitatory effects of orexin A can be blocked by the selective OX1R antagonist, SB-334967 (Soffin et al., 2004). Orexins can also induce the release of 5-HT. Infusion of orexin A (30 μM) into the DRN leads to a 2–3-fold increase in local extracellular 5-HT, while orexin B only leads to 20–30% of increase in 5-HT even at a much higher dose (100 μM), suggesting OX1R plays a more important role in regulating DRN 5-HT neurons (Tao et al., 2006). Three hours following a single intraperitoneal injection of an OX1R antagonist we found a small, but statistically significant, increase in the number of 5-HT-ir neurons in the middle portion of the DRN. We also found a significant decrease in 5-HT-ir fiber density in the aCgC when compared to the fiber density found in vehicle-treated animals. Such a seemingly rapid change in immunoreactivity for 5-HT itself (rather than its precursors or metabolizing enzymes) is consistent with the fact that brain SB-334867 concentrations after an IP injection reach peak levels within 30 min post-injection and remain high for at least 4 h (Ishii et al., 2005). In addition, at least one other study found similarly rapid changes in 5-HT immunoreactivity in neural somata and fibers after a single experimental event (Lorenzi and Grober, 2012).

There was no significant effect of a single OX1R antagonist injection on 5-HT-ir fiber density in the other three brain regions examined (BNST, NAcSH or PAG), though, but did so after five daily injections of the OX1R antagonist. These results may collectively suggest that antagonism of orexin-OX1R signaling impedes 5-HTergic output from the DRN to some of its target regions, resulting in accumulation of 5-HT within the somata and less at the terminals. Conversely, the results could indicate that OX1R antagonism causes rapid neurotransmitter release that depletes 5-HT from the fibers. The former case may be more likely, as previous studies have found that damaging raphe serotonergic cells or their ascending projections, at least initially, reduces rather

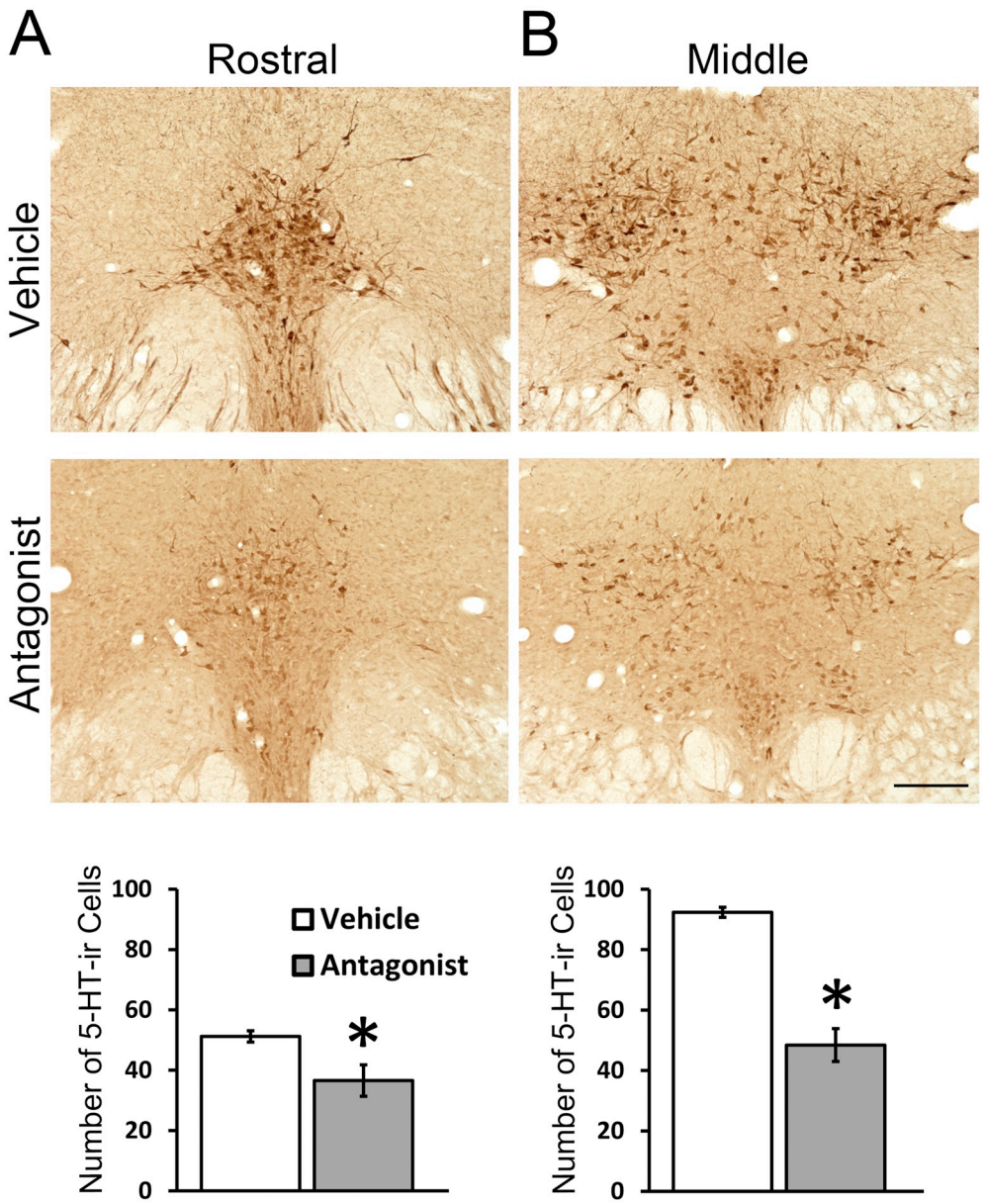

Fig. 4. Representative photomicrographs and quantitative analysis of 5-HT-ir cells in the rostral (A) and middle (B) DRN following five daily injections of the selective OX1R antagonist, SB-334867 or vehicle. Data are shown as Means ± SEMs (n = 6 vehicle/n = 5 antagonist). *indicates $p < 0.05$. Scale bar, 100 μm.

than increases 5-HT fiber density in numerous forebrain targets (*e.g.*, Holschbach et al., 2018; McLean and Shipley, 1987; Meyer-Berstein et al., 1997; Zhou and Azmitia, 1986).

Our HPLC analysis of 5-HT turnover after repeated OX1R antagonist injections aimed to address this issue, but we did not find a significant effect on 5-HT turnover in the DRN or in the aCgC and NAcSh. However, levels of both 5-HT and 5HIAA were higher in the NAcSh of the antagonist-treated group, which was unexpected given their lower 5-HT-ir fiber density compared to controls. We do not have a simple explanation for this finding, especially in light of the fact that the amount of total protein in the NAcSh (but not the DRN or aGgC from the very same animals) used to standardize the neurochemical measurements in each sample was unexpectedly lower in the antagonist-treated group than in the controls. Perhaps the reduction in total protein levels in the NAcSh of SB-334867-treated animals reflects an inhibition of orexin-induced neurotrophic factors involved in mesolimbic function (Harada et al., 2013; Winrow et al., 2010; Yamada et al., 2009). In any case, previous work from our group has shown that acute antagonism of OX1R signaling with SB-334867 provokes a depressive phenotype in the forced-swim test in diurnal grass rats (Deats et al., 2014), which may at least partly due to a change in 5-HTergic inputs to the aCgC, as indicated by a reduction in 5-HT-ir fiber density. The aCgC has long been implicated in regulating emotion and mood (Etkin et al., 2011; Pizzagalli, 2011). Patients with unipolar and bipolar depression show reduced neural activity in the aCgC (Drevets et al., 1997), and lesions of the aCgC in rats significantly increase immobility time in a forced swim test, reflecting greater behavioral despair (Bissiere et al., 2006). 5-HT modulates activity of aCgC, with the antidepressant effects of deep brain stimulation in the aCgC nullified by 5-HT depletion (Hamani et al., 2012), and decreased 5-HT transporter binding in the aCgC found after human subjects are treated with bright light therapy (Harrison et al., 2015). Our results show that a single injection of OX1R antagonist significantly reduces 5-HT-ir fibers in the aCgC within 3 h, highlighting a potential pathway through which the orexinergic activity can acutely and relatively rapidly regulate affective state. Determining some of the cellular mechanisms through which natural or

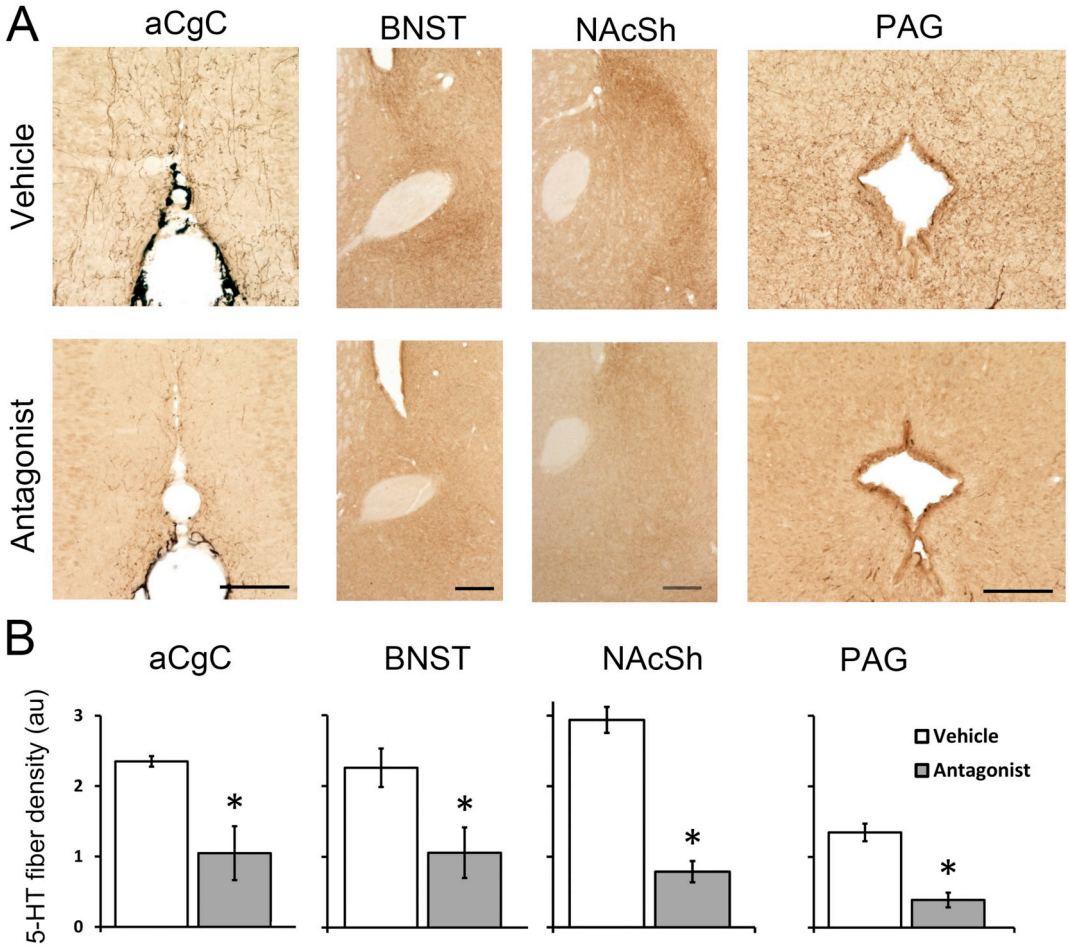

Fig. 5. Representative photomicrographs (A) and quantitative analysis (B) of 5-HT- ir fibers in the anterior cingulate cortex (aCgC), oval bed nucleus of the stria terminalis (ovBNST), nucleus accumbens shell (NAcSh), and periaqueductal gray area (PAG) in animals receiving either 5 daily injection of the OX1R antagonist SB-334867 or vehicle. Data are shown as Means ± SEMs (n = 6 vehicle/n = 5 antagonist). *indicates $p < 0.01$. Scale bar, 100 μm.

Table 1
Intracellular serotonin and dopamine measures indicated by HPLC analysis of the dorsal raphe nucleus (DRN), anterior cingulate cortex (aCgC), and nucleus accumbens (NAc) of male Nile grass rats that received five daily injections of the OXR1 antagonist (SB-334867; Antag) or Vehicle (Veh). Significant differences between groups indicated in bold font.

		5HIAA (pg/μg protein)	5-HT (pg/μg protein)	5HIAA/5-HT	DOPAC (pg/μg protein)	HVA (pg/μg protein)	Total protein (μg/sample)
DRN	Veh (Mean ± SEM)	23.46 ± 1.57	9.92 ± 0.76	2.42 ± 0.15	0.81 ± 0.03	1.09 ± 0.03	11.48 ± 0.90
	Antag (Mean ± SEM)	26.24 ± 1.41	11.15 ± 0.76	2.39 ± 0.11	0.95 ± 0.06	1.26 ± 0.06	11.82 ± 0.86
	T-test (*p; Cohen's d*)	0.27; 0.59	0.33; 0.52	0.91; 0.07	**0.05**; 0.98	**0.04**; 1.14	0.81; 0.12
aCgC	Veh (Mean ± SEM)	1.59 ± 0.12	0.82 ± 0.03	1.93 ± 0.14	0.26 ± 0.03	0.44 ± 0.07	57.13 ± 3.46
	Antag (Mean ± SEM)	1.53 ± 0.07	0.86 ± 0.03	1.79 ± 0.08	0.28 ± 0.03	0.41 ± 0.03	59.91 ± 2.38
	T-test (*p; Cohen's d*)	0.70; 0.29	0.41; 0.54	0.34; 0.74	0.64; 0.13	0.75; 0.21	0.51; 0.37
NAc	Veh (Mean ± SEM)	2.39 ± 0.15	1.43 ± 0.11	1.73 ± 0.17	1.55 ± 0.11	1.58 ± 0.09	35.11 ± 2.28
	Antag (Mean ± SEM)	3.40 ± 0.21	2.20 ± 0.16	1.56 ± 0.06	2.61 ± 0.30	2.43 ± 0.18	22.86 ± 2.17
	T-test (*p; Cohen's d*)	**0.00; 1.98**	**0.00; 1.99**	0.34; 0.49	**0.01; 1.58**	**0.00; 2.07**	**0.00; 1.92**

pharmacological changes in orexin receptor activity can rapidly affect aspects of the 5-HT system that underlie our broader assessments of immunoreactivity and turnover - such as 5-HT cell expression and activity of the TPH2 and AADC enzymes, cell firing rate and pattern, and rates of terminal 5-HT packaging and release - under particular conditions that influence affective state (*e.g.*, different lighting conditions, stress levels, or social environments) would be valuable in future studies.

Following five-days of OX1R antagonism with SB-334867, we found an even more marked change of 5-HT-ir, involving a widespread decrease in the number of 5-HT-ir cells in the DRN and a decrease in 5-HT-ir fiber density not only in the aCgC but also in the NAcSh, BNST and PAG. The latter sites have also been implicated in regulating mood and anxiety (Bennett, 2011; Graeff, 2004; Puig and Gulledge, 2011). For instance, the NAcSh is well known to provide motivational salience to a given stimulus and is tightly linked to the feeling of pleasure, which is a key parameter affected in mood disorders (Ito et al., 2004; Shirayama and Chaki, 2006). 5-HT release in the mesolimbic system is associated with reduced motivated behavior, so one could have expected OX1R antagonism to promote 5-HT signaling (possibly increase 5-HT-ir fiber density in the NAc rather than decrease it). However, optogenetic stimulation of 5-HT release in the NAc does not alone affect responding for saccharine reward in mice (Browne et al., 2019), so perhaps our decrease in 5-HT-ir fiber density is unrelated to orexin's effects on

accumben's control of affective behaviors. The BNST has been implicated in stress and anxiety, with 5-HT 1A receptor activation associated with an anxiolytic response (Garcia-Garcia et al., 2018; Gomes et al., 2011; Levita et al., 2004) and 2C receptor activity associated with an anxiogenic response (Marcinkiewcz et al., 2016). Furthermore, electrical stimulation of the BNST produces behaviors similar to those caused by a stressful stimulus (Casada and Dafny, 1991). Reduced 5-HT input onto 1A receptors in the BNST may therefore impede the ability to cope with stressful stimuli, generating an anxiogenic response as similarly exhibited by diurnal grass rats treated with OXR1 antagonist or housed in dim light during the daytime (Deats et al., 2014; Ikeno et al., 2016). Lastly, OX1R activation disinhibits the PAG (Ho et al., 2011), which depending on the PAG subregion involved is associated with fearful or anxious behaviors (Brandao et al., 2008; Fendt, 1998; Miller et al., 2010; Morgan and Clayton, 2005), and OX1R receptor antagonism in the PAG can reduce anxiety-related behaviors in laboratory rats (Pourrahimi et al., 2019).

Previous research on our grass rat model of SAD found that dim daylight intensity led to decreased 5-HT-ir fiber density in the DRN, aCgC and PAG; these animals also shows more depression- and anxiety-like behaviors compared to animals housed under conditions of bright daylight intensity (Ikeno et al., 2016; Leach et al., 2013). The dim-daylight animals also had fewer orexin-ir neurons in the hypothalamus and lower orexin-ir fiber density in the DRN compared to controls housed in bright light during the day (Deats et al., 2014). The results from the present study support a causal link between the attenuated OXergic activity and 5-HTergic outputs observed in the grass rat model of SAD, and suggest that the 5-HTergic dorsal raphe is one of the downstream targets for orexin's modulation of affective behaviors in diurnal grass rats and other animals.

Declaration of competing interest

None.

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