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A comparison of the orexin receptor distribution in the brain between diurnal Nile grass rats (*Arvicanthis niloticus*) and nocturnal mice (*Mus musculus*)

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Abstract

The neuropeptide orexin/hypocretin regulates a wide range of behaviors and physiology through its receptors OX1R and OX2R, or HCRT-1 and HCRT-2. Although the distributions of these receptors have been established in nocturnal rodents, their distributions in the brain of diurnal species have not been studied. In the present study, we examined spatial patterns of *OX1R* and *OX2R* mRNA expression in diurnal Nile grass rats (*Arvicanthis niloticus*) by in situ hybridization and compared them with those in nocturnal mice (*Mus musculus*). Both receptors showed similar spatial patterns between species in most brain regions. However, species-specific expression was found in several regions that are mainly implicated in regulation of sleep/wakefulness, emotion and cognition. *OX1R* expression was detected in the caudate putamen and ventral tuberomammillary nucleus only in grass rats, while it was detected in the bed nucleus of the stria terminalis, medial division, posteromedial part only in mice. The distribution of *OX2R* mRNA was mostly consistent between the two species, although it was more widely expressed in the ventral tuberomammillary nucleus in grass rats compared to mice. These results suggest that neuronal pathways of the orexin system differ between chronotypes, and these differences could underlie the distinct profiles in behaviors and physiology between diurnal and nocturnal species.

Keywords

orexin; hypocretin; chronotype; OX1R; OX2R

1. Introduction

The orexin system is involved in the regulation of a wide range of physiological and behavioral responses such as sleep and arousal, feeding, motivation, autonomic function,

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emotion, and cognition (Girault et al., 2012; Johnson et al., 2012; Sakurai, 2014; Tsujino and Sakurai, 2013). There are two isoforms of orexin neuropeptides, orexin A and B (also called hypocretin 1 and 2), which are synthesized in a small number of cells located in the lateral hypothalamus (de Lecea et al., 1998; Sakurai et al., 1998). Orexins bind to two G-protein receptors, orexin receptor 1 (OX1R, also called HCRTR-1) and orexin receptor 2 (OX2R, also called HCRTR-2); OX1R has a greater affinity to orexin A than to orexin B, while OX2R has a similar affinity to both orexin A and B (Sakurai et al., 1998).

The neuronal pathways of the orexin system have been well studied in nocturnal model animals, i.e., laboratory rats and mice, which has revealed wide distributions of orexinergic fibers as well as orexin receptors throughout the brain, especially with dense projections in the paraventricular nucleus of the thalamus, arcuate hypothalamic nucleus, tuberomammillary nucleus, dorsal raphe nucleus, medial raphe nucleus, and locus coeruleus, enabling its diverse functions in physiology and behavior (Johnson et al., 2012; Sakurai, 2014; Tsujino and Sakurai, 2013). On the other hand, relatively little is known about the neuronal pathways of the orexin system in diurnal species, and it's unclear whether the neuronal pathways involved in the orexin system are conserved between diurnal and nocturnal species. In fact, different distributions of orexin-immunoreactive cells and fibers have been demonstrated between nocturnal and diurnal rodents, including laboratory rats, golden hamsters, degus, and diurnal Nile grass rats (Nixon and Smale, 2007; Novak and Albers, 2002). For example, nocturnal species, laboratory rats and hamsters, but not diurnal ones, degus and grass rats, have orexin A- and B-immunoreactive fibers in the rhomboid nuclei of the thalamus, which are important for cognitive processes (Hallock et al., 2016). However, the distribution of orexin receptors in diurnal rodents remained unclear.

The objective of the present study is to further elucidate the neuroanatomical pathways of the orexin system in diurnal species and to explore the difference between chronotypes. We utilized a well-established diurnal rodent model, the diurnal Nile grass rats (*Arvicanthis niloticus*), and examined the distribution of orexin receptors (*OX1R* and *OX2R*) mRNA in the brain of grass rats in comparison with those in nocturnal laboratory mice. The diurnal nature of the Nile grass rats has been well documented based on their behaviors in the field and in the laboratory (McElhinny et al., 1999, 1997; Schwartz and Smale, 2005; Shuboni et al., 2012), as well as the anatomy of their retina and visual regions in the brain (Gaillard et al., 2013, 2009, 2008). Grass rats have been widely used for the studies examining chronotype differences in sleep and arousal (Novak et al., 2000, 1999; Schwartz and Smale, 2005; Todd et al., 2012) and in the circadian system (Castillo-Ruiz and Nunez, 2007; Mahoney et al., 2004; Ramanathan et al., 2010, 2008). Moreover, this species has also been used for examining neuronal mechanisms underlying mood and emotional behaviors, in which the orexin system has been suggested to have an important role (Adidharma et al., 2012; Ashkenazy-Frolinger et al., 2009; Deats et al., 2015, 2014; Fonken et al., 2012; Ikeno et al., 2016).

2. Results

2.1 OX1R

2.1.1 General distribution patterns—In both grass rats and mice, specific signals for *OX1R* mRNA were detected throughout the brain. Clear signals were found in both species mainly at the medial septal nucleus, nucleus of the vertical limb of the diagonal band, paraventricular nucleus of the thalamus, anterior hypothalamic area, supraoptic nucleus, medial preoptic nucleus, hippocampus, basolateral amygdaloid nucleus, ventromedial hypothalamic nucleus, lateral mammillary nucleus, dorsal raphe nucleus, median raphe nucleus, and locus coeruleus (Fig. 1).

2.1.2 Comparison between grass rats and mice—The general expression of *OX1R* mRNA was similar between grass rats and mice, e.g., strong *OX1R* signals were observed in the locus coeruleus at comparable levels between these species (Fig. 2A). However, species differences in distribution of *OX1R* signals were clearly found in some regions including the caudate putamen (CPu) of the striatum, ventral tuberomammillary nucleus (VTM) and the bed nucleus of the stria terminalis, medial division, posteromedial part (BSTMPM). In the CPu (Fig. 2B), dense *OX1R* signals were found in some cells in grass rats, but no cells in this region showed *OX1R* signals in mice. Similarly, in the VTM (Fig. 2C), *OX1R* signals were found in grass rats but not in mice, although relatively high *OX1R* signals were detected at the lateral mammillary nucleus in both species. While in the BSTMPM (Fig. 2D), *OX1R* signals were not detected in grass rats, but intense *OX1R* signals were observed in this region in mice.

2.2 OX2R

2.2.1 General distribution patterns—Specific signals for *OX2R* were detected in several regions of both species, i.e., the medial septal nucleus, nucleus of the vertical limb of the diagonal band, paraventricular nucleus of the thalamus, paraventricular nucleus of the hypothalamus, hippocampus, ventromedial hypothalamic nucleus, arcuate hypothalamic nucleus, VTM, subbrachial nucleus, dorsal raphe nucleus, median raphe nucleus, and pontine nuclei (Fig. 3).

2.2.2 Comparison between grass rats and mice—The distribution of *OX2R* signal was very similar between grass rats and mice. For example, both species moderately expressed *OX2R* signals in the pontine nuclei (Fig. 4A) and many other brain regions shown in Figure 3. However, a striking difference was found in the VTM. Grass rats expressed *OX2R* signals in a broader area compared to mice, and some sections from grass rats even showed strong signals along with the ventral edge of the mammillary nucleus, which was not observed in any sections from mice (Fig. 4B).

3. Discussion

In this study, we examined the distributions of *OX1R* and *OX2R* mRNA expression in the brain of diurnal grass rats in comparison with those of nocturnal laboratory mice. The general distributions of these receptors in both species were consistent with previous studies

showing orexin receptors mRNA distributions in nocturnal rats (Marcus et al., 2001; Trivedi et al., 1998), and fiber distributions of orexin neurons in grass rats (Adidharma et al., 2012; Deats et al., 2014; Nixon and Smale, 2007). In addition, the present study revealed distinct spatial patterns of *OX1R* mRNA expression between grass rats and mice in some regions, such that *OX1R* signals were observed in the CPu and VTM only in grass rats, while they were observed in the BSTMPM only in mice. Moreover, *OX2R* signals were distributed in a broader area of VTM in grass rats compared to those in mice. The expression patterns of *OX1R* and *OX2R* in these areas of mice were similar to those of nocturnal rats (Marcus et al., 2001; Trivedi et al., 1998).

Diurnal animals and nocturnal animals show completely different patterns of their sleep and wakefulness in reference to the daily light/dark cycle and in their responses to light (Redlin, 2001). The control of the sleep/wake states is one of the major roles of the orexin system. Orexin-deficient mice (Chemelli et al., 1999) or dogs carrying mutation in the *OX2R* gene (Lin et al., 1999) display a phenotype similar to human narcolepsy. Administration of orexin A promotes wakefulness and suppresses both non-REM and REM sleep in mice and rats (Hagan et al., 1999; Mieda et al., 2011, 2004; Piper et al., 2000), which involves both *OX1R* and *OX2R* receptors (Mieda et al., 2011).

One of the regions that play a role for sleep and wakefulness is the VTM. The VTM is a source of histamine, which is crucial for the maintenance of wakefulness (Lin et al., 1988; Parmentier et al., 2002). In the VTM, histaminergic neurons receive dense orexinergic innervation in mice (Chemelli et al., 1999), and the arousal-promoting effect of orexins requires histamine receptors (Huang et al., 2001; Yamanaka et al., 2002). Most if not all histaminergic neurons in the VTM express *OX2R* mRNA in mice and rats, indicating that orexins stimulate histaminergic neurons through *OX2R* (Mieda et al., 2011; Yamanaka et al., 2002). In the present study, both grass rats and mice highly expressed *OX2R* mRNA in the VTM, consistent with previous reports in mice and rats (Marcus et al., 2001; Mieda et al., 2011). However, *OX2R* expression in grass rats showed a broader distribution compared to mice. Moreover, we found that *OX1R* mRNA was also expressed in the VTM in grass rats, in drastic contrast to nocturnal mice and rats that do not express *OX1R* mRNA at a detectable level in this region (present study; Marcus et al., 2001; Mieda et al., 2011). Although a lack of *OX1R* in the VTM in mice and rats has led to a view that *OX2R* but not *OX1R* mediates the orexins' arousal action via histaminergic neurons in nocturnal animals (Yamanaka et al., 2002), the presence of *OX1R* mRNA in grass rats found in the present study suggests a possible role of *OX1R* in arousal regulation within the VTM in diurnal mammals. Future study will examine the localization of *OX1R* and *OX2R* in VTM histaminergic neurons in grass rats, to better understand how orexin signaling promotes arousal in diurnal animals via histaminergic neurons in the VTM.

The orexin system is also involved in the regulation of mood and emotional behaviors (Flores et al., 2015; James et al., 2017; Johnson et al., 2012). Orexin neurons project to brain regions implicated in depression and anxiety, e.g., the prefrontal cortex, hippocampus, BST and amygdala, both in nocturnal and diurnal rodents (Nixon and Smale, 2007; Peyron et al., 1998). The present study revealed a clear species-difference of *OX1R* expression in the BSTMPM, a region of the extended amygdala, which is highly implicated in emotional

responses (Adhikari, 2014). In the BSTMPM, while *OX1R* mRNA was densely expressed in mice, almost no expression was observed in grass rats. A body of evidence suggests an importance of the BST in anxiety regulation (Duvarci et al., 2009; Sahuque et al., 2006; Sajdyk et al., 2008). The BST is composed of several subdivisions, and subdivision-dependent roles in anxiety regulation have been demonstrated: the oval nucleus of the BST and the anterodorsal BST respectively increases and decreases anxiety-like behaviors (Kim et al., 2013). Although the functional importance of BSTMPM in emotional regulation remains to be determined, it is possible that BSTMPM also participates in modulation of anxiety states. The absence of *OX1R* in grass rats suggests different role of orexin signaling in regulating BSTMPM function as well as in regulating mood and emotional between diurnal and nocturnal species.

The present study revealed clear expression of *OX1R* mRNA in the CPu of grass rats but not in mice. Consistent with our finding, mRNA expression of orexin receptors in the CPu were not detected in nocturnal rats (Marcus et al., 2001; Trivedi et al., 1998). The CPu or dorsal striatum has been implicated in many functions such as learning/memory, reward processing, motivation and decision making in humans and rodents (Macpherson et al., 2014; Packard, 2009; Balleine et al., 2007). The expression of *OX1R* in the CPu of grass rats but not other nocturnal species suggests a unique *OX1R*-mediated orexinergic regulation of striatum-based cognitive process in diurnal mammals. Although it remains unclear the extent to which orexin-*OX1R* pathway regulates the function of the CPu in grass rats, the species-specific expression of *OX1R* in the CPu begs further investigation into the orexinergic modulation of the CPu or dorsal striatum in diurnal mammals.

In conclusion, the present study demonstrated that *OX1R* and *OX2R* mRNA distributions are different between grass rats and mice in several brain regions mainly implicated in sleep/wakefulness, emotion and cognition. mRNA distributions in mice were similar to those in nocturnal rats (Marcus et al., 2001; Trivedi et al., 1998), implicating that the differences between grass rats and mice revealed in the present study are not merely species differences. Thus, our findings indicate that neuronal pathways of the orexin system involved in these functions are distinct between diurnal and nocturnal species. These behavioral and physiological functions are under control of not only orexins, but also other neurotransmitters (Camardese et al., 2014; Eggermann et al., 2001; Lin et al., 1988; Monti, 2010; Neumann and Landgraf, 2012; Parmentier et al., 2002; Suri et al., 2015; Wing et al., 2015). However, neuronal and molecular mechanisms underlying these functions have been established mostly by using nocturnal animals, and therefore, it is necessary to investigate whether interactions between orexin and these neurotransmitters are conserved in diurnal rodents. The results that grass rats express orexin receptors in the brain region where no expression is found in nocturnal laboratory mice or rats, e.g., the CPu and VTM, imply that orexin signaling in grass rats is involved in different functions from that in nocturnal species. Revealing neuronal pathways and action mechanisms of the orexin system in diurnal species will provide a better understanding of orexin-related disorders in humans.

4. Experimental Procedure

4.1 Animals

Adult male Nile grass rats (N = 4) were derived from animals originally imported from sub-Saharan Africa and bred at Michigan State University (McElhinny et al., 1997). Adult male CD1 mice (N = 4) were purchased from Charles River Laboratory (OH, USA). The animals were maintained in 12 h light-12 h dark conditions with *ad libitum* access to food (Prolab 2000 #5P06, PMI Nutrition LLC, MO, USA) and water. All procedures were approved by the Michigan State University Animal Use and Care Committee.

4.2 Tissue collection

The animals were euthanized with sodium pentobarbital (200 mg/kg) and perfused transcardially using saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer during the light phase. Brains were post-fixed for 12–18 h, cryoprotected in a 20% sucrose solution, and frozen using dry ice. Five alternate sets of 30 µm coronal sections were prepared through the rostrocaudal axis of the brain using a cryostat.

4.3 In situ hybridization

To obtain cDNA fragments of grass rat *OX1R* and *OX2R*, total RNAs were extracted from the hypothalamus of grass rats and cDNAs were synthesized as described in Ikeno et al. (2016). cDNA cloning was performed by PCR using PCR Supermix (Life Technologies, Carlsbad, CA, USA) with *OX1R*-F (5'-CTG CCT CCA GAC TAT GAG GAC-3') and *OX2R*-R (5'-GCC TGG GGC ACC ATG ACA G-3') primers, or *OX2R*-F (5'-GCA GGC GGA GAC AAG CTT-3') and *OX2R*-R (5'-AGG GTG GTC TTA TTG GCT AGG-3') primers. PCR products were cloned into plasmids using a TOPO TA Cloning kit for sequencing (Life Technologies) and sequenced. The plasmid containing cDNA fragments of grass rat *OX1R* and *OX2R* was amplified by PCR using M13 Forward and M13 Reverse primers and transcribed with T3 polymerase for antisense probes and with T7 polymerase for sense probes using a DIG RNA labeling kit (Roche, Indianapolis, IN, USA). In situ hybridization was performed as described previously (Shuboni and Yan, 2010; Yan et al., 1999; Yan and Silver, 2002). To directly compare *OX1R* and *OX2R* mRNA distributions between grass rats and mice, the sections collected from individual grass rats and mice were processed together in a single well/tube. Free-floating sections were processed with proteinase K at 37°C and 0.25% acetic anhydride at room temperature for 10 min, and then incubated in hybridization buffer containing DIG-labelled *OX1R* or *OX2R* antisense probes (0.5 µg/1 ml) or sense probes (0.5 µg/1 ml) overnight at 60°C. Sections were washed and treated with RNase A. After incubation in a blocking reagent in buffer 1 (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 1 h at room temperature, sections were incubated at 4°C in an alkaline phosphatase-conjugated DIG antibody diluted 1:5000 in buffer 1 for 3 days. Sections were incubated in a buffer containing NBT/BCIP solution (Roche) with 5% polyvinyl alcohol (Sigma-Aldrich) for 24 h. Sections were mounted and coverslipped.

4.4 Data analysis

All sections were analyzed under a light microscope (Zeiss, Gottingen, Germany) and representative images of each region were captured using a CCD video camera (CX9000, MBF Bioscience, VT, USA). Anatomical localization was determined according to the rat brain atlas (Paxinos and Watson, 2005) and mouse brain atlas (Lein et al., 2007; Paxinos and Franklin, 2001). Specificity of signals detected by antisense probes was confirmed by comparing with sections processed with sense probes.

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- Orexin receptors distribution was compared between diurnal and nocturnal rodents.
- OXRs showed similar distributions between species in most brain regions.
- Species-specific expression was found in regions related to arousal and emotion.
- These differences could underlie the distinct profiles in behaviors and physiology.

Fig. 1.

General distributions of *OX1R* mRNA in the brain of grass rats and mice. Representative photographs with *OX1R* signals are shown. Scale bar: 1 mm. MS: medial septal nucleus; VDB: nucleus of the vertical limb of the diagonal band; PVT: paraventricular nucleus of the thalamus; BSTMPM: bed nucleus of the stria terminalis, medial division, posteromedial part; AHN: anterior hypothalamic area; SO: supraoptic nucleus; Hipp: hippocampus; VMH: ventromedial hypothalamic nucleus; BLA: basolateral amygdaloid nucleus; DR: dorsal raphe nucleus; MnR: median raphe nucleus.

Fig. 2.

Comparison of *OX1R* distribution between grass rats and mice. Representative photographs with *OX1R* mRNA signals in (A) the locus coeruleus, (B) caudate putamen, (C) mammillary nucleus, and (D) bed nucleus of the stria terminalis, medial division, posteromedial part of grass rats and mice. Scale bar: 200 μ m. 4V: fourth ventricle; LC: locus coeruleus; LM: lateral mammillary nucleus; VTM: ventral tuberomammillary nucleus; MRe: mammillary recess of the third ventricle; BSTMPM: bed nucleus of the stria terminalis, medial division, posteromedial part; f: fornix.

Fig. 3.

General distributions of *OX2R* mRNA in the brain of grass rats and mice. Representative photographs with *OX2R* signals are shown. Scale bar: 1 mm. MS: medial septal nucleus; VDB: nucleus of the vertical limb of the diagonal band; PVN: paraventricular hypothalamic nucleus; Hipp: hippocampus; VMH: ventromedial hypothalamic nucleus; ARC: arcuate hypothalamic nucleus; DR: dorsal raphe nucleus; MnR: median raphe nucleus; SubB: subthalamic nucleus; Pn: pontine nuclei.

Fig. 4. Comparison of *OX2R* distribution between grass rats and mice. Representative photographs with *OX2R* mRNA signals in (A) the pontine nuclei and (B) tuberomammillary nucleus (VTM) of grass rats and mice. Scale bar: 200 μ m.