Please cite this article in press as: Palus K et al. Orexins/hypocretins modulate the activity of NPY-positive and -negative neurons in the rat intergeniculate leaflet via OX_1 and OX_2 receptors. Neuroscience (2015), http://dx.doi.org/10.1016/j.neuroscience.2015.05.039

Neuroscience xxx (2015) xxx–xxx

OREXINS/HYPOCRETINS MODULATE THE ACTIVITY OF NPY-POSITIVE AND -NEGATIVE NEURONS IN THE RAT INTERGENICULATE LEAFLET VIA OX_1 AND OX_2 RECEPTORS

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Abstract—Orexins/hypocretins (OXA and OXB) are two hypothalamic peptides involved in the regulation of many physiological processes including the sleep-wake cycle, food intake and arousal. The orexinergic system of the lateral hypothalamus is considered a non-specific peptidergic system, and its nerve fibers innervate numerous brain areas. Among many targets of orexinergic neurons is the intergeniculate leaflet (IGL) of the thalamus – a small but important structure of the mammalian biological clock. In rats, the IGL consists of GABAergic cells which also synthesize different neuropeptides. One group of neurons produces neuropeptide Y (NPY) and sends its axons to the master biological clock known as the suprachiasmatic nuclei. Another neuronal group produces enkephalin and is known to connect contralateral IGLs. This study evaluated the effects of orexins on identified IGL neurons revealing that 58% of the recorded neurons were sensitive to OXA (200 nM) and OXB (200 nM) administration. Both NPY-positive and -negative neurons were depolarized by these neuropeptides. Experiments using selective orexin receptor antagonists (SB-334867, 10 μM and TCS-OX2-29, 10 μM) suggested that both orexin receptors participate in the recorded OXA effects. In addition, IGL neurons were either directly depolarized by OXA or their activity was altered by changes in presynaptic inputs. We observed an increase of GABA release onto the investigated IGL neuron after OXA application, consistent with a presynaptic localization of the orexin receptors. An increase in miniature excitatory postsynaptic current frequency was not observed within the IGL. Our findings reinforce the connection between circadian clock physiology and the orexinergic system.

Key words: orexin, intergeniculate leaflet, neuropeptide Y, biological clock, patch clamp, electrophysiology.

INTRODUCTION

Neuropeptides orexin A (OXA) and orexin B (OXB), also called hypocretin-1 and hypocretin-2, were first identified in the mammalian brain by two independent research groups (Sakurai et al., 1998; de Lecea et al., 1998). Over 15 years of intensive studies have revealed that orexins are the key regulators of many physiological processes. Both orexins are synthesized from a common precursor known as prepro-orexin in neurons located exclusively in the lateral hypothalamic area, dorsomedial hypothalamus, and in a subset of neurons localized around the fornix (de Lecea et al., 1998; Nambu et al., 1999).This relatively small neuronal population innervates numerous areas throughout the brain (Peyron et al., 1998). Orexin actions are mediated by two G-protein-coupled metabotropic receptors named orexin receptor 1 (OX_1 receptor) and orexin receptor 2 (OX_2 receptor) with partially distinct expression patterns among many brain nuclei (Trivedi et al., 1998). The sensitivity of orexin receptors varies between two peptides: OXA has an equal affinity for both receptors, in contrast to OXB which binds predominately to the OX_2 receptor (de Lecea et al., 1998; for review see Kukkonen, 2013). Because of the non-specificity, the orexinergic system plays multiple roles in the physiology and pathology of the central nervous system (for a review see Li et al., 2014). The first proposed orexin function was in appetite control and food intake regulation (Sakurai et al., 1998; for a review see Rodgers et al., 2002). However novel findings highlight its role in the modulation of sleep and wakefulness (Mieda et al., 2011; Tsunematsu et al., 2013; for a review see de Lecea and Huerta, 2014), or even more, a leading role in driving arousal (Boutrel et al., 2010; Kohlmeier et al., 2013; for a review see Alexandre et al., 2013).

One of the brain nuclei innervated by orexinergic neurons is the intergeniculate leaflet (IGL) of the thalamus, which is a small but important structure of the mammalian biological clock (Peyron et al., 1998; Nixon and Smale, 2005). The IGL was first described as a thin

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Abbreviations: Bic, bicuculline methbromide; dLGN, dorsal part of lateral geniculate nucleus; DMSO, dimethyl sulfoxide; ENK, enkephalin; GHT, geniculo-hypothalamic tract; IGL, intergeniculate leaflet; Kyn, kynurenic acid sodium salt; mEPSC, miniature excitatory postsynaptic currents; mIPSC, miniature inhibitory postsynaptic currents; nACSF, normal artificial cerebrospinal fluid; NDS, normal donkey serum; NPY, neuropeptide Y; OX_1 receptor, orexin receptor 1; OX_2 receptor, orexin receptor 2; OXA, orexin A; OXB, orexin B; PBS, phosphate-buffered saline; SB, SB 334867; SCN, suprachiasmatic nuclei; TCS, TCS-OX2-29; TTX, tetrodotoxin citrate; vLGN, ventral part of lateral geniculate nucleus.

http://dx.doi.org/10.1016/j.neuroscience.2015.05.039
0306-4522/

area of neurons intercalated between the dorsal and ventral parts of the lateral geniculate nucleus (Moore and Card, 1994). These three interrelated structures are retinorecipient and form the lateral geniculate complex (for the review see Harrington, 1997). The feature distinguishing IGL from the whole complex is the presence of neuropeptide Y (NPY) immunoreactivity within its borders. The NPY-positive neurons send their axons through the geniculo-hypothalamus tract (GHT) to the suprachiasmatic nuclei of the hypothalamus (SCN) (Harrington, 1997; Glass et al., 2010). However, an even greater number of IGL neurons produce the opioid peptide enkephalin (ENK). These cells are known to project to the contralateral IGL (through the geniculo-geniculate pathway) and are directly innervated by the retina (Harrington, 1997). As shown in our laboratory, ENK-positive neurons express an infra-slow oscillatory pattern of activity *in vivo* (Lewandowski et al., 2000, 2002; Blasiak and Lewandowski, 2013). It has also been suggested that the IGL consists of GABAergic interneurons inhibiting cells within the nucleus (Harrington, 1997; Thankachan and Rusak, 2005; Palus et al., 2013, 2015). Previous *in vitro* experiments demonstrated that most IGL neurons are spontaneously active in the slice, although in these conditions the oscillatory pattern of activity is missing (Blasiak and Lewandowski, 2004; Szkudlarek and Raastad, 2007).

Little is known about the possible role of distinct neuronal IGL subpopulations. It has been recently proposed that NPY-positive neurons convey information related to the metabolic state of the organism to the SCN (Saderi et al., 2013). The well-known function of the whole nucleus is to collect and integrate photic information arising from the retina with non-photic cues delivered by non-specific brain systems (Moore and Card, 1994; Horowitz et al., 2004; Morin and Blanchard, 2005). This integrated information is then sent to the SCN. Hence the IGL is thought to contribute to the modulation of circadian rhythms (for review see Morin, 2012). Electrophysiological studies showed that IGL activity is influenced by many neurotransmitters, including serotonin (Blasiak et al., 2006; Palus et al., 2013), acetylcholine (Pekala et al., 2007; Werhun and Lewandowski, 2009), GABA (Palus et al., 2015) or relaxin-3 (Blasiak et al., 2013). Previous studies found that orexins influence IGL activity in rodents (Pekala et al., 2011). It is proposed that orexinergic neurons in the lateral hypothalamus and IGL neurons cooperate in arousal-induced circadian clock resetting (Webb et al., 2008). Moreover, the relationship between orexins and the biological clock has been established. Orexinergic neurons that are activated by dark-pulses remain under SCN control and show circadian rhythmicity (Yoshida et al., 2001; Martinez et al., 2002; Marston et al., 2008). Orexins themselves can alter the activity of the SCN, exerting a direct hyperpolarizing effect upon the clock cells during the night and potentiating NPY's inhibition during the day (Belle et al., 2014). With regard to electrophysiological studies on IGL cells, only extracellular *in vitro* studies were carried out and demonstrated the excitatory action of these neuropeptides on IGL neuronal activity. It has also been suggested that both OX_1 receptor and OX_2 receptor could play a role in the observed effect (Pekala et al., 2011). However, the target of orexins on the specific subpopulations of IGL neurons and the pre- or postsynaptic character of this modulation remain to be determined. Therefore, the aim of this study was to evaluate the effects of OXA and OXB on immunohistochemically identified NPY-positive and -negative IGL neurons using the patch clamp technique. In addition, we determine how OXA affects the IGL neuron firing rate, membrane properties and synaptic transmission via identified orexin receptors.

EXPERIMENTAL PROCEDURES

Brain tissue preparation

All experiments were performed in accordance with the European Community Council Directive of 24 November 1986 (86/0609/EEC) and Polish Animal Welfare Act of 23 May 2012 (82/2012). They were approved by the Local (Krakow) Ethics Commission. Every effort was made to minimize the number of animals used and their suffering. Animals were held in 12-h/12-h light/dark conditions (light on 8.00 am, light off 8.00 pm) with water and food *ad libitum* in the Jagiellonian University Animal Facility. Male Wistar rats (13–18 days old) were anesthetized with isoflurane (2 ml/kg body weight, Baxter) and decapitated between 1 and 2 h Zeitgeber Time (ZT, 9:00–10:00 am). Brain tissue was quickly removed from the skull and immersed in oxygenated (95% O_2, 5% CO_2) normal ice-cold artificial cerebrospinal fluid (nACSF), composed of (in mM): NaCl 118, $NaHCO_3$ 25, KCl 3, NaH_2PO_4 1.2, $CaCl_2$ 2, $MgCl_2$ 2, and glucose 10 (pH = 7.4; osmolality ~ 295 mOsmol/kg). Then, the block of brain tissue containing the thalamus was placed on the cold plate of a vibroslicer (Leica VT1000S, Heidelberg, Germany) and 250-μm coronal slices were cut. Slices with the IGL were transferred to the pre-incubation chamber for at least 60 min, and then the slices were placed in the recording chamber. During the experimental procedure recorded neurons were filled with biocytin for further immunohistochemical characterization.

Electrophysiology

Patch clamp experiments were performed with borosilicate glass pipettes (Sutter Instruments, Novato, USA; resistance 6–9 MΩ). The recording electrode was placed in the IGL under visual control (through a 100× microscope objective). The IGL neuron was identified under 400× magnification with a Zeiss Examiner microscope fitted with infrared differential interference contrast (Göttingen, Germany). Whole-cell configuration was obtained by using an Ez-gSEAL100B Pressure Controller (Neo Biosystem, San Jose, USA) and applying negative pressure. For recording, Spike2 and Signal (CED, Cambridge, UK) software were used. The recorded signal was amplified by a SC 05LX (NPI, Tamm, Germany) amplifier. The signal was low-pass filtered at 3 kHz and digitized at 20 kHz. A liquid junction

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