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Serotonin differentially modulates excitatory and inhibitory synaptic inputs to putative sleep-promoting neurons of the ventrolateral preoptic nucleus

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ABSTRACT

The role of serotonin (5-HT) in sleep-wake regulation has been a subject of intense debate and remains incompletely understood. In the ventrolateral preoptic nucleus (VLPO), the main structure that triggers non-rapid eye movement (NREM) sleep, putative sleep-promoting (PSP) neurons were shown *ex vivo* to be either inhibited (Type-1) or excited (Type-2) by 5-HT application. To determine the complex action of this neurotransmitter on PSP neurons, we recorded spontaneous and miniature excitatory and inhibitory postsynaptic currents (sEPSCs, sIPSCs, mEPSCs and mIPSCs) in response to bath application of 5-HT. We established in mouse acute VLPO slices that 5-HT reduces spontaneous and miniature EPSC and IPSC frequencies to Type-1 neurons, whereas 5-HT selectively increases sIPSC and mIPSC frequencies to Type-2 VLPO neurons. We further determined that Type-1 neurons display a lower action potential threshold and a smaller soma size than Type-2 neurons. Finally, single-cell RT-PCR designed to identify the 13 serotonergic receptor subtypes revealed the specific mRNA expression of the 5-HT1A,B,D,F receptors by Type-1 neurons. Furthermore, the 5-HT2A-C,4,7 receptors were found to be equivalently expressed by both neuronal types. Altogether, our results establish that the excitatory and inhibitory inputs to Type-1 and Type-2 VLPO PSP neurons are differentially regulated by 5-HT. Electrophysiological, morphological and molecular differences were also identified between these two neuronal types. Our results provide new insights regarding the orchestration of sleep regulation by 5-HT release, and strongly suggest that Type-2 neurons could play a permissive role, whereas Type-1 neurons could have an executive role in sleep induction and maintenance.

1. Introduction

The role of serotonin in sleep-wake regulation has been a topic of great scientific debate over the past 60 years. Indeed, deciphering the effects of serotonin (5-hydroxytryptamine, or 5-HT) on sleep is a complex matter, especially since this monoamine neurotransmitter plays major roles in several physiological functions including appetite, gastrointestinal motility, thermoregulation, nociception, emotion and cognition (Artigas, 2015; Berger et al., 2009; Stiedl et al., 2015). Moreover, 5-HT effects are mediated by a wide family of receptors that are classified into seven main receptor subtypes, designated 5-HT_{1-7}. The 5-HT_4, 5-HT_6 and 5-HT_7 classes presently have one subtype each, whereas the remaining receptor classes are composed of five (5-$HT_{1A\text{-}B\text{-}D\text{-}E\text{-}F}$), three (5-$HT_{2A\text{-}B\text{-}C}$) or two (5-$HT_{3A\text{-}B}$ and 5-$HT_{5A\text{-}B}$) receptor subtypes (Hannon and Hoyer, 2008). All 5-HT receptors (5-HTR) are G protein-couple receptors with the exception of the ligand ion

Abbreviations: 5-HT, serotonin; 5-HTR, serotonergic receptor; Gal, galanin; mEPSC, miniature excitatory post-synaptic current; mIPSC, miniature inhibitory post-synaptic current; NA, noradrenaline; PSP, putative sleep-promoting neuron; scRT-PCR, single-cell reverse transcription polymerase chain reaction; sEPSC, spontaneous excitatory post-synaptic current; sIPSC, spontaneous inhibitory post-synaptic current; VLPO, ventrolateral preoptic nucleus.

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channel 5-HT_3R, and all receptors produce excitatory responses except for types 1 and 5 (Monti, 2011).

The relationship between 5-HT and sleep was first reported in the 1950s, when the decrease in cerebral 5-HT following the administration of reserpine was observed to induce sedation (Brodie et al., 1955). In the following decade, the destruction of serotonergic nuclei, as well as the inhibition of 5-HT synthesis with *p*-chlorophenylalanine, were both shown to induce persistent insomnia, reinforcing the link between 5-HT and sleep induction (Delorme et al., 1966; Jouvet and Renault, 1966). However, *in vivo* recordings from raphe neurons during sleep-wake cycles have refuted the role of serotonergic neurons in the regulation of non-rapid eye movement (NREM) sleep, since the electrical activity of 5-HT neurons in raphe nuclei decreases in frequency during NREM as compared to wakefulness (Lydic et al., 1987; McGinty and Harper, 1976; Trulson and Jacobs, 1979). In addition, *in vivo* microdialysis in cats indicates that the level of 5-HT release in serotonergic dorsal raphe nuclei (DRN) parallels the time course of presumptive serotonergic neuronal activity, which decreases from waking until NREM and continues through rapid eye movement (REM) sleep (Portas and McCarley, 1994).

Hypotheses concerning the serotonergic involvement in sleep-wake regulation are continually evolving, owing to the improvement of technical approaches. *In fine*, the observation that raphe neurons diminish their firing rates during sleep has been revised, since recordings of atypical neurons in the dorsal raphe nucleus were shown to display the highest discharge frequency during NREM sleep, including a suppression of firing during both wakefulness and REM sleep (Sakai and Crochet, 2001). Moreover, the preoptic area, containing the ventrolateral preoptic area (VLPO), was the only brain region in which small doses of 5-HT precursor microinjections could restore long periods of physiological sleep, when a 5-HT synthesis inhibitor was injected to completely induce insomnia in cats (Denoyer et al., 1989). A better understanding of the mode of action and the effects of 5-HT is therefore fundamental to appreciating the physiological role of 5-HT on the behavioral state, in particular within the VLPO. Indeed, this hypothalamic structure is involved in NREM sleep promotion and maintenance by forming reciprocal inhibitory connections with wake-promoting structures (Gallopin et al., 2000; Saper et al., 2001), and it mostly receives serotonergic inputs from innervation by the DRN (Chou et al., 2002).

The VLPO is composed of a small cluster of GABAergic and galaninergic neurons (Chou et al., 2002; Lu et al., 2000; Sherin et al., 1996; Steininger et al., 2001; Szymusiak et al., 1998). The inhibition of these VLPO neurons by wake-promoting neurotransmitters is in agreement with their inactivity during wakefulness, and suggests that they should correspond to the sleep-active cells recorded *in vivo* and that are involved in the inhibition of arousal systems (Saper et al., 2001; Szymusiak et al., 1998). Henceforth, sleep-promoting neurons have been identified according to their inhibitory response to bath application of noradrenaline (NA) (Gallopin et al., 2005, 2000; Liu et al., 2013; Moore et al., 2012; Saint-Mleux et al., 2004; Varin et al., 2015). In contrast to other neurotransmitters that promote wakefulness, 5-HT was shown to induce opposite responses, as revealed by *ex vivo* electrophysiological recordings of presumed sleep-promoting (PSP) neurons in the rat VLPO. Indeed, neurons inhibited by NA were also inhibited (44%; Type-1) or excited (56%; Type-2) by 5-HT, revealing two different neuronal subtypes of PSP neurons (Fort et al., 2009; Gallopin et al., 2005).

In order to better understand the complex regulation of VLPO PSP neuronal excitability by 5-HT, we performed whole-cell patch-clamp recordings of these neurons in slice preparations of juvenile mice. Neuronal excitability and effects on spontaneous/miniature excitatory and inhibitory postsynaptic currents (EPSCs and IPSCs, respectively) were examined in response to 5-HT application. We also identified electrophysiological, molecular and morphological features of Type-1 *vs*. Type-2 PSP neurons. These results characterize two different types of neurons within the VLPO that could play distinct roles in sleep regulation.

2. Methods

2.1. Animals

Male-only C57BL/6J mice (Charles River, France) and FVB Gal-GFP mice (Mutant Mouse Regional Resource Center (MMRRC), USA), 14–18 days old, were used to record the serotonergic modulation of afferent inputs and to perform scRT-PCR, respectively. The Gal-GFP mouse strain STOCK Tg (Gal-EGFP)HX109Gsat/Mmucd (identification number 016342-UCD) was obtained from the MMRRC, a NIH-funded strain repository, and was donated to the MMRRC by the NINDS-funded GENSAT BAC transgenic project. Dr. N. Heintz, the donating investigator of this Gal-GFP mouse strain, was the first to publish it (Gong et al., 2003). All animals were housed in a temperature-controlled room (20–22 °C) under a 12-h light-dark cycle (lights on at 09:00 a.m.) with *ad libitum* access to food and water. All animal procedures were conducted in strict compliance with our institutional protocols and were approved by the European Community Council Directive of 22 September 2010 (010/63/UE) and the local ethics committee (Comité d'éthique en matière d'expérimentation animale number 59, C2EA—59, 'Paris Centre et Sud'). The number of animals in our study was accordingly kept to the necessary minimum.

2.2. Slice preparation

Animals were decapitated at the beginning of the light phase, between 09:00 and 10:00 a.m. Brains were quickly extracted without removing the meninges and submerged in cold slicing artificial cerebrospinal fluid (aCSF, 4 °C) containing (in mM): 130 NaCl; 5 KCl; 2.4 $CaCl_2$; 20 $NaHCO_3$; 1.25 KH_2PO_4; 1.3 $MgSO_4$; 10 D-glucose; 15 sucrose; and 1 kynurenic acid (pH = 7.35). Brains were constantly oxygenated with 95% O2 − 5% CO_2. During slicing, 1 mM kynurenate was added to the aCSF. Coronal brain slices (300 μm thick) containing the VLPO were cut with a vibratome (VT2000S; Leica) and transferred to a constantly oxygenated (95% O2 − 5% CO2) holding chamber containing aCSF. Subsequently, individual slices were placed in a submerged recording chamber maintained at 32 °C and perfused (1.5 mL/min) with oxygenated kynurenate-free aCSF, and placed under a microscope (Axioscop2FS; Zeiss) for observation.

2.3. Morphological analysis

The targeted neurons were located in the VLPO cluster and were photographed prior recording with a CoolSnapHQ2 CCD camera (CoolSNAP HQ^2; Roper Scientific) controlled by the Image-Pro 7 software (Media Cybernetics Inc., San Diego, CA). Morphological features were measured from these images after calibration with a standard 24-μm grid. In order to describe morphological features of VLPO neurons, 8 parameters related to features of their soma were extracted from infrared pictures taken prior to whole-cell recording in the cell-attached configuration. Their area, perimeter, form factor and maximal (Feret max) and minimal (Feret min) diameters passing through the centroid were computed. The degree of flatness of a contour shape, taken as the ratio of its minimum diameter to its maximum diameter, was indicated as the aspect ratio. The measurement of how closely this shape approached that of a circle