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GABAergic REGULATION OF THE PERIFORNICAL–LATERAL HYPOTHALAMIC NEURONS DURING NON-RAPID EYE MOVEMENT SLEEP IN RATS

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Abstract—The perifornical–lateral hypothalamic area (PF–LHA) has been implicated in the regulation of behavioral arousal. The PF–LHA predominantly contains neurons that are active during behavioral and cortical activation and quiescent during non-rapid eye movement (nonREM) sleep, that is, are nonREM-off neurons. Some *in vitro* and *in vivo* studies indicate that PF–LHA neurons, including hypocretin-expressing neurons, are under GABAergic control. However, a role of GABA in suppressing the discharge of PF–LHA neurons during spontaneous nonREM sleep has not been confirmed. We recorded the sleep–wake discharge profiles of PF–LHA neurons and simultaneously assessed the contributions of local $GABA_A$ receptor activation and blockade on their wake- and nonREM sleep-related discharge activities by delivering $GABA_A$ receptor agonist, muscimol (500 nm, 5 μM, and 10 μM) and its antagonist, bicuculline (5 μM, 10 μM, and 20 μM), adjacent to the recorded neurons via reverse microdialysis. Muscimol dose-dependently decreased the discharge of PF–LHA neurons including nonREM-off neurons. Muscimol-induced suppression of discharge during nonREM sleep was significantly weaker than the suppression produced during waking. In the presence of bicuculline, PF–LHA neurons, including nonREM-off neurons, exhibited elevated discharge, which was dose-dependent and was significantly higher during nonREM sleep, compared to waking. These results suggest that $GABA_A$ receptor mediated increased GABAergic tone contributes to the suppression of PF–LHA neurons, including nonREM-off neurons, during spontaneous nonREM sleep. Published by Elsevier Ltd on behalf of IBRO.

Key words: perifornical–lateral hypothalamus, hypocretin, GABA, posterior–lateral hypothalamus, sleep.

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Abbreviations: aCSF, artificial cerebrospinal fluid; Fos-IR, c-Fos protein immunoreactivity; HCRT, hypocretin; MCH, melanin-concentrating hormone; NonREM, non-rapid eye movement sleep; PF–LHA, perifornical–lateral hypothalamic area; TBS, tris buffered saline.

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The perifornical–lateral hypothalamic area (PF–LHA) has been implicated in the regulation of behavioral arousal (Gerashchenko and Shiromani, 2004; Datta and Maclean, 2007; McCarley, 2007; Szymusiak and McGinty, 2008). The PF–LHA predominantly contains neurons that are active during behavioral and cortical activation and quiescent during non rapid eye movement (nonREM) sleep, that is, are nonREM-off neurons (Alam et al., 2002; Koyama et al., 2003; Suntsova et al., 2007). Stimulation of the PF–LHA evokes locomotor activity, EEG activation, and arousal, whereas its lesions increase sleep (Stock et al., 1981; Sinnamon et al., 1999; Gerashchenko et al., 2003; Alam and Mallick, 2008). Neurochemically, the PF–LHA is a heterogeneous structure and includes populations of neurons expressing hypocretin (HCRT), melanin-concentrating hormone (MCH), GABA, and glutamate (Bittencourt et al., 1992; Peyron et al., 1998; Abrahamson and Moore, 2001; Gerashchenko and Shiromani, 2004; Ohno and Sakurai, 2008). Amongst these neuronal groups, HCRT neurons have been implicated in the facilitation and/or maintenance of arousal (Ohno and Sakurai, 2008). These neurons exhibit wake-associated discharge and c-Fos expression (Fos-IR) and are quiescent during both nonREM and REM sleep (Estabrooke et al., 2001; Espana et al., 2003; Lee et al., 2005; Mileykovskiy et al., 2005; Takahashi et al., 2008). A loss of HCRT signaling is linked with symptoms of narcolepsy in human and animals (Lin et al., 1999; Peyron et al., 2000; Thannickal et al., 2000). While glutamateric neurons have been implicated in the regulation of HCRT neuronal excitability and are wake-active, GABAergic and MCH neurons in the PF–LHA have been implicated in the regulation of sleep, especially REM sleep (Li et al., 2002; Kumar et al., 2005; Hassani et al., 2009). Although, *in vitro* studies have identified several neurotransmitters and neuromodulators that influence the activity of PF–LHA neurons including HCRT neurons (Kukkonen et al., 2002; Ohno and Sakurai, 2008), the neurotransmitter(s) that regulate the suppression of PF–LHA neurons during nonREM sleep remains poorly understood.

GABAergic neurons are major contributors to inhibitory tone throughout the mammalian brain and acts via two different types of receptors, viz., $GABA_A$ and $GABA_B$ (Cooper et al., 1996). A role of GABAergic system in sleep regulation is evident from the fact that most commonly used hypnotics are $GABA_A$ receptor analogues, although $GABA_B$ receptor has also been implicated (Gottesmann, 2002; Mohler, 2006; Matsuki et al., 2009; Winsky-Sommerer, 2009). Administration of selective $GABA_A$ receptor

agonists, for example, muscimol and THIP, increase the duration of nonREM and REM sleep and electroencephalogram (EEG) delta frequency in rats and humans (Lancel and Faulhaber, 1996; Faulhaber et al., 1997; Lancel et al., 1997). Majority of sleep-active neurons in the preoptic (POA) region are GABAergic (Gong et al., 2004; Gvilia et al., 2006). It has been hypothesized that GABAergic system is a predominant contributor of the inhibitory tone to multiple arousal systems during sleep (Saper et al., 2005; Szymusiak and McGinty, 2008).

Evidence suggests that PF–LHA neurons, including HCRT neurons are subject to GABAergic inhibitory tone. For example, (a) PF–LHA contains local GABAergic neurons and receives projections from sleep-active GABAergic neurons in the POA region (Abrahamson and Moore, 2001; Kumar et al., 2005; Uschakov et al., 2007); (b) both $GABA_A$ and $GABA_B$ receptors are localized on HCRT neurons and modulate HCRT neuronal activity *in vitro* (Li et al., 2002; Eggermann et al., 2003; Backberg et al., 2004; Xie et al., 2006); (c) GABA levels in the posterior hypothalamus are higher during nonREM and REM sleep (Nitz and Siegel, 1996); (d) THIP administration into the PF–LHA increases sleep (Thakkar et al., 2008); and (e) in presence of bicuculline into the PF–LHA, Fos-IR is increased in HCRT and non-HCRT neurons and animals spent more time in waking and less time in nonREM/REM sleep (Alam et al., 2005; Goutagny et al., 2005). However, the contributions of GABAergic inhibitory tone in suppressing the discharge of PF–LHA neurons during spontaneous nonREM sleep has not been directly confirmed. In this study, we recorded sleep–wake discharge profiles of the PF–LHA neurons and simultaneously assessed the contributions of local $GABA_A$ receptor activation and blockade on their wake- and nonREM sleep-related discharges by delivering $GABA_A$ receptor agonist, muscimol, and its antagonist, bicuculline, adjacent to the recorded neurons via reverse microdialysis.

EXPERIMENTAL PROCEDURES

Procedure

Experiments were conducted on six Sprague–Dawley, unanesthetized, unrestrained male rats weighing between 300–350 g and in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals. These rats were maintained on 12:12 h light–dark cycle (lights on from 8:00 AM) and with food and water available *ad libitum*. The experiments were conducted during light-on phase so that the extracellular discharge activity of PF–LHA neurons could be recorded through multiple sleep–wake cycles during baseline and drug treatment.

The experimental procedure has been described in detail earlier (Alam et al., 1999; Kumar et al., 2007). In brief, under surgical anesthesia (Ketamine+Xylazine: 80:10 mg/kg; i.p.) and aseptic conditions, rats were stereotaxically implanted with EEG and electromyogram (EMG) electrodes for polygraphic determination of sleep–waking states. A microdrive-microdialysis guide cannula assembly, consisting of a single barrel mechanical microdrive and an adjacent guide cannula for microdialysis probe insertion, was implanted such that their tips rested 3 mm above the dorsal aspect of the PF–LHA (A, −3.0 to −3.20; L, 1.40 to 1.60; H, 4.5 to 5.0 from bregma) (Paxinos and Watson, 1998). Five pairs of microwires, each consisting of two 20 μm insulated stainless steel wires glued together except for 2.0 mm at the tip, were passed through the microdrive barrel such that their tips projected into the PF–LHA.

Data acquisition

Experiments were started at least 10 days after surgery and acclimatization of rats with the recording chamber. At least 12 h before the experiment, the stylet of the microdialysis guide cannula was replaced by a microdialysis probe (semi-permeable membrane tip length 1 mm; outer diameter, 0.22 mm; molecular cut off, 50 kDa; Eicom, Japan), which was fixed in place with dental cement. After implantation, the probe was flushed with artificial cerebrospinal fluid (aCSF; composition in mM, 145 Na^+, 2.7 K^+, 1.0 Mg^{2+}, 1.2 Ca^{2+}, 1.5 Cl^- and 2 Na_2HPO_4, pH, 7.2) at a flow rate of ~2 μl/min for 3–4 h. In this study, the microdialysis probe was fixed and microwires were advanced adjacent to the microdialysis probe to minimize the tissue trauma and ensure maximum stability of the unit recording. The length of the probe was set such that the microwires were within 200–500 μm from the semi-permeable membrane.

The experimental paradigm included recording of EEG, EMG, and unit activity during baseline, during drug delivery and during washout. The microdrive was advanced in 20–30 μm steps until an isolated single unit was found. First, the discharge rate of an isolated PF–LHA neuron was recorded through two to three stable sleep–wake cycles with aCSF perfusion as a baseline. After baseline recording, known concentrations of muscimol (500 nM, 5 μM, and 10 μM), a $GABA_A$ receptor agonist, or bicuculline (5 μM, 10 μM, and 20 μM), a $GABA_A$ receptor antagonist, was dialyzed adjacent to the recorded neurons for 10 min so that the transient effects of each drug on the discharge activity of neuron(s) could be studied without triggering a strong behavioral response. After delivery of drugs, the perfusion medium was switched back to aCSF and the recording continued for another 45–90 min as wash out or recovery. During the entire recording session, the animal was undisturbed except, if necessary, a stable episode of waking was achieved by mild auditory stimuli or gentle touch. EEG, EMG, and raw microwire signals were digitized (Cambridge Electronic Design 1401, London; supporting software, Spike 2) and stored on a disc for subsequent analyses. Multiple spikes, if present, were sorted on the basis of spike shape parameters from the amplified unprocessed microwire signals.

At the end of the recording session, under deep anesthesia (100 mg/kg i.p., pentobarbital), rats were perfused and the brain tissue was processed for histology. The location of the microdialysis probe and the microwire tracts were histologically confirmed and the locations of the various neuronal types encountered were plotted (Fig. 1).

Data analysis

The sleep–wake discharge profiles of neurons were determined by the criteria adopted earlier (Alam et al., 2002). Neurons were classified as “wake-active” if their nonREM/active-wake as well as REM/active-wake discharge ratios were <0.5. Neurons were classified as “wake/REM-active” if REM/active–wake ratio was >0.5 and <1.5. Neurons were classified as “REM-active” if the REM/active-wake and REM/nonREM ratios were >1.5. Neurons exhibiting a change of less than 50% in all states were classified as “state-indifferent” neurons.

This study was aimed at determining GABAergic contribution on the suppression of PF–LHA neurons during nonREM sleep. Since wake- and wake/REM-active neurons are quiescent during nonREM sleep, we specifically focused on these two neuronal types, which were grouped together as “nonREM-off” neurons. Effects of muscimol or bicuculline on the discharge activity of