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THE CHOLINERGIC AGONIST CARBACHOL INCREASES THE FREQUENCY OF SPONTANEOUS GABAERGIC SYNAPTIC CURRENTS IN DORSAL RAPHE SEROTONERGIC NEURONS IN THE MOUSE

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Abstract—Dorsal raphe nucleus (DRN) serotonin (5-HT) neurons play an important role in feeding, mood control and stress responses. One important feature of their activity across the sleep–wake cycle is their reduced firing during rapid-eye-movement (REM) sleep which stands in stark contrast to the wake/REM-on discharge pattern of brainstem cholinergic neurons. A prominent model of REM sleep control posits a reciprocal interaction between these cell groups. 5-HT inhibits cholinergic neurons, and activation of nicotinic receptors can excite DRN 5-HT neurons but the cholinergic effect on inhibitory inputs is incompletely understood. Here, *in vitro*, in DRN brain slices prepared from GAD67-GFP knock-in mice, a brief (3 min) bath application of carbachol (50 μM) increased the frequency of spontaneous inhibitory postsynaptic currents (sIPSCs) in GFP-negative, putative 5-HT neurons but did not affect miniature (tetrodotoxin-insensitive) IPSCs. Carbachol had no direct postsynaptic effect. Thus, carbachol likely increases the activity of local GABAergic neurons which synapse on 5-HT neurons. Removal of dorsal regions of the slice including the ventrolateral periaqueductal gray (vlPAG) region where GABAergic neurons projecting to the DRN have been identified, abolished the effect of carbachol on sIPSCs whereas the removal of ventral regions containing the oral region of the pontine reticular nucleus (PnO) did not. In addition, carbachol directly excited GFP-positive, GABAergic vlPAG neurons. Antagonism of both muscarinic and nicotinic receptors completely abolished the effects of carbachol. We suggest cholinergic neurons inhibit DRN 5-HT neurons when acetylcholine levels are lower i.e. during quiet wakefulness and the beginning of REM sleep periods, in part via excitation of muscarinic and nicotinic receptors located on local vlPAG and DRN GABAergic neurons. Higher firing rates or burst firing of cholinergic neurons associated with attentive wakefulness or phasic REM sleep periods leads to excitation of 5-HT neurons via the activation of nicotinic receptors located postsynaptically and presynaptically on excitatory afferents.

Key words: patch-clamp, presynaptic modulation, sleep, GAD67-GFP knock-in mice, ventrolateral periaqueductal gray.

INTRODUCTION

Wakefulness and rapid-eye-movement (REM) sleep are conscious brain states which appear superficially similar, based on examination of the cortical electroencephalogram (EEG). However, brain function is dramatically different in these two states. One major neuromodulatory difference which may help explain the difference between these states is the reduced firing during REM sleep of dorsal raphe nucleus (DRN) serotonergic, and other aminergic neurons (Brown et al., 2012). The DRN contains the largest number of forebrain projecting 5-HT neurons (Jacobs and Azmitia, 1992; Monti, 2010). Extracellular recordings showed that these neurons discharge fastest during wakefulness and discharge at a much lower rate during REM sleep (McGinty and Harper, 1976; Trulson and Jacobs, 1979), although firing may occur following phasic REM events (Sakai and Crochet, 2001).

One neurotransmitter system which may be involved in the silencing of DRN neurons during REM sleep is the brainstem cholinergic system, located in the laterodorsal and pedunculopontine tegmental nuclei (LDT/PPT), since, in contrast to aminergic neurons, these cholinergic neurons are highly active during REM sleep (Steriade et al., 1990; Williams et al., 1994; Thakkar et al., 1998; Sakai, 2012) and project to the DRN and surrounding regions (Rye et al., 1987; Woolf and Butcher, 1989). In fact, an influential theory of REM sleep control, the reciprocal interaction theory of Hobson and McCarley (Hobson et al., 1975; McCarley and Hobson, 1975), posits state-dependent interactions between cholinergic and brainstem aminergic neurons (see McCarley, 2007; Brown et al., 2012 for a critical discussion of this and other REM sleep control models).

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Abbreviations: 5-HT, serotonin; ACSF, artificial cerebrospinal fluid; AHP, afterhyperpolarization; ANOVA, analysis of variance; AP, action potential; AP5, (2R)-amino-5-phosphonovaleric acid; DNQX, 6,7-dinitroquinoxaline-2,3-dione; DRN, dorsal raphe nucleus; EGTA, ethylene glycol tetraacetic acid; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; LDT, laterodorsal tegmentum; mIPSCs, miniature inhibitory postsynaptic currents; NMDA, *N*-methyl-D-aspartate; PGO, Ponto-geniculo-occipital; PnO, pontine nucleus, pars oralis; PPT, pedunculopontine tegmentum; REM, rapid-eye-movement; RMP, resting membrane potential; sIPSCs, spontaneous inhibitory postsynaptic currents; SubC, subcoeruleus; TTX, tetrodotoxin; vlPAG, ventrolateral periaqueductal gray; xscp, decussation of the superior cerebellar peduncle.

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Several *in vitro* (Luebke et al., 1992; Leonard and Llinás, 1994) and *in vivo* (Thakkar et al., 1998) studies have confirmed inhibition of cholinergic LDT/PPT neurons by 5-HT. In addition, direct and indirect *excitatory* effects of nicotine on DRN serotonin neurons have been shown (Li et al., 1998; Mihailescu et al., 1998, 2001, 2002; Galindo-Charles et al., 2008; Chang et al., 2011; Garduño et al., 2012). In some of these studies, inhibitory effects were also seen prior to excitatory effects and/or in particular subsets of neurons. Thus, inhibitory effects of cholinergic stimulation may also occur, likely in a state-dependent manner.

Recent experiments and theories of REM sleep control have emphasized a role for GABAergic neurons (Xi et al., 1999; Luppi et al., 2006; Mallick et al., 2012; Brown et al., 2012). A microdialysis study showed that GABA release in DRN is increased during REMS (Nitz and Siegel, 1997), suggesting that increased GABAergic input is important for inhibiting DRN serotonergic neurons during REM sleep. Furthermore, unit recording studies show that the discharge rate of DRN neurons loses its relationship to the vigilance state with iontophoretic application of a $GABA_A$ receptor antagonist into DRN (Gervasoni et al., 2000), supporting the hypothesis that GABAergic inputs are involved.

The DRN 5-HT neurons are surrounded by a large number of GABAergic neurons in the DRN and neighboring ventrolateral periaqueductal gray (vlPAG) (Maloney et al., 2000; Brown et al., 2008). Using retrograde-tracing, Gervasoni et al. (2000) revealed that DRN receives GABAergic inputs from multiple regions, including a strong input from the vlPAG. Furthermore, retrograde tracing combined with Fos immunohistochemistry to identify recently active neurons, suggested that the vlPAG contains REM-active GABAergic neurons (Sapin et al., 2009). A unifying model to combine cholinergic and GABAergic influence of REM sleep control would suggest cholinergic excitation of GABAergic neurons which inhibit DRN neurons (McCarley, 2007; Brown et al., 2012). Thus, here, we tested the hypothesis that cholinergic neurons inhibit DRN REM-off 5-HT neurons by increasing GABAergic inputs using whole-cell recordings in coronal brain slices prepared from GAD67-GFP knock-in mice (Tamamaki et al., 2003; Brown et al., 2008). Application of the mixed cholinergic agonist, carbachol was used to stimulate both muscarinic and nicotinic receptors. Excitatory glutamatergic synaptic events were blocked so as to focus on inhibitory inputs. We then removed particular parts of the brainstem slices to determine which parts of the brainstem slice are required for carbachol effects on inhibitory inputs to DRN neurons. Lastly, we tested the effect of carbachol on one possible source of GABAergic inputs to DRN, vlPAG GABAergic neurons.

EXPERIMENTAL PROCEDURES

Animals

All experiments conformed to U.S. Veterans Administration, Harvard University, and U.S. National Institutes of Health guidelines and were reviewed by the institutional animal care and use committee (IACUC) of the VA Boston Healthcare System. Experimental male and female GAD67-GFP knock-in animals were obtained by crossing male heterozygous GAD67-GFP knock-in mice (Swiss-Webster background) with wild-type female Swiss-Webster mice (Charles River, Wilmington, MA, USA). GFP-positive animals were phenotyped under a fluorescent microscope within 3 days after birth. GAD67-GFP knock-in animals have similar sleep–wake behavior and cortical rhythms as wild-type animals (Chen et al., 2010; McNally et al., 2011). The selective expression of green fluorescent protein in brainstem GABAergic neurons was validated in our previous study (Brown et al., 2008). Mice were housed under constant temperature and a 12:12 light:dark cycle (7AM:7PM), with food and water available *ad libitum*.

Slice preparation

Slices from young mice (9–20 d) were used for all experiments since visualization of neurons in brain slices is easier at this age and young animals have large amounts of REM sleep (Jouvet-Mounier and Astic, 1968). Mice were deeply anesthetized with isofluorane and then decapitated. Coronal brainstem slices (250-μm thickness) were cut between −4.48 and −4.80 mm with respect to Bregma rostrocaudally in ice-cold sucrose solution (in mM: 208.6 sucrose, 1.8 KCl, 25.6 $NaHCO_3$, 1.2 KH_2PO_4, 0.6 $CaCl_2$, 3.3 $MgSO_4$, 10 glucose, saturated with 95% O_2/5% CO_2). After slicing they were placed into artificial cerebrospinal fluid (ACSF) (in mM: 124 NaCl, 1.8 KCl, 25.6 $NaHCO_3$, 1.2 KH_2PO_4, 2 $CaCl_2$, 1.3 $MgSO_4$ and 10 glucose, 300 mOsm, saturated with 95% O_2/5% CO_2) for > 1 h at room temperature before being transferred to the recording chamber and superfused with warmed ACSF (32 °C) at 2–3 ml/min.

Whole-cell patch-clamp recordings

For putative 5-HT neurons, electrophysiological recordings were made from somata of GFP-negative neurons in the ventral, interfascicular parts of DRN where 5-HT neurons are particularly highly concentrated (Monti, 2010). For GABAergic neurons, electrophysiological recordings were made from somata of GFP-positive neurons in lateral vlPAG (Brown et al., 2008). Neurons were photographed prior to recording using a Hamamatsu ORCA-ER CCD camera (Hamamatsu Corporation, Middlesex, NJ, USA). Fluorescent neurons were observed with Zeiss filters (GFP: filter set 38, excitation filter 470/40 and emission filter 525/50). Long-axis cell diameter was measured from these images and calibrated using a standard 25-μm grid. Intrinsic membrane properties of the neurons were tested with 1-s long current steps as previously reported (McKenna et al., 2013). The amplitude of these steps was titrated according to the input resistance of the neurons so that the maximal hyperpolarization with the largest current step was the same for each cell (McKenna et al., 2013).