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ALPHA-2 ADRENERGIC REGULATION OF PEDUNCULOPONTINE NUCLEUS NEURONS DURING DEVELOPMENT

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Abstract—Rapid eye movement sleep decreases between 10 and 30 days postnatally in the rat. The pedunculopontine nucleus is known to modulate waking and rapid eye movement sleep, and pedunculopontine nucleus neurons are thought to be hyperpolarized by noradrenergic input from the locus coeruleus. The goal of the study was to investigate the possibility that a change in α-2 adrenergic inhibition of pedunculopontine nucleus cells during this period could explain at least part of the developmental decrease in rapid eye movement sleep. We, therefore, recorded intracellularly in 12–21 day rat brainstem slices maintained in oxygenated artificial cerebrospinal fluid. Putative cholinergic vs. non-cholinergic pedunculopontine nucleus neurons were identified using nicotinamide adenine dinucleotide phosphate diaphorase histochemistry and intracellular injection of neurobiotin (Texas Red immunocytochemistry). Pedunculopontine nucleus neurons also were identified by intrinsic membrane properties, type I (low threshold spike), type II (A) and type III (A+low threshold spike), as previously described. Clonidine (20 μM) hyperpolarized most cholinergic and non-cholinergic pedunculopontine nucleus cells. This hyperpolarization decreased significantly in amplitude (mean±S.E.) from −6.8± 1.0 mV at 12–13 days, to −3.0±0.7 mV at 20–21 days. However, much of these early effects (12–15 days) were indirect such that direct effects (tested following sodium channel blockade with tetrodotoxin (0.3 μM)) resulted in hyperpolarization averaging −3.4±0.5 mV, similar to that evident at 16–21 days. Non-cholinergic cells were less hyperpolarized than cholinergic cells at 12–13 days (−1.6±0.3 mV), but equally hyperpolarized at 20–21 days (−3.3±1.3 mV). In those cells tested, hyperpolarization was blocked by yohimbine, an α-2 adrenergic receptor antagonist (1.5 μM). These results suggest that the α-2 adrenergic receptor on cholinergic pedunculopontine nucleus neurons activated by clonidine may play only a modest role, if any, in the developmental decrease in rapid eye movement sleep. Clonidine blocked or reduced the hyperpolarization-activated inward cation conductance, so that its effects on the firing rate of a specific population of pedunculopontine nucleus neurons could be significant. In conclusion, the α-2 adrenergic input to pedunculopontine nucleus neurons appears to consistently modulate the firing rate of cholinergic and non-cholinergic pedunculopontine nucleus neurons, with important effects on the regulation of sleep–wake states.

Key words: arousal, cholinergic, clonidine, reticular activating system, yohimbine.

Rapid eye movement (REM) sleep in man decreases from about 8 h (50% of total sleep time) in the newborn to about 1 h (15% of total sleep time) in the adult, and this decrease occurs mostly from birth until the end of puberty (Roffwarg et al., 1966). In the rat, the decrease in REM sleep occurs between 10 and 30 days of age, declining from over 70% of total sleep time in the newborn to the adult level of about 15% of sleep time (Jouvet-Mounier et al., 1970). It has been suggested that the direction of these changes indicates that there is a REM sleep inhibitory process that develops during the first two weeks of life in the rat (Vogel et al., 2000). We have been investigating the decrease in REM sleep across this critical period in development because we hypothesize that, if this developmental decrease in REM sleep drive does *not* occur, lifelong increases in REM sleep drive may ensue. We speculate that the greater the degree of excessive REM sleep drive (i.e. the greater the lack of decrease due to developmental dysregulation), the more pronounced the severity of subsequent REM sleep drive symptomatology, which may include hallucinations (REM sleep intrusion into waking), frequent nocturnal arousals and hypervigilance. A number of disorders exhibit increases in REM sleep drive, including such post-pubertal onset diseases as schizophrenia, panic attacks, bipolar disorder, and obsessive–compulsive disorder (Garcia-Rill 1997, 2001). Moreover, changes in REM sleep regulation later in life are evident in depression, insomnia, and such degenerative conditions as Alzheimer's, Huntington's and Parkinson's diseases (Garcia-Rill 1997, 2001). Dysregulation of mesopontine cholinergic systems has also been proposed in narcolepsy (Boop et al., 1993; Sinton and McCarley, 2000).

The pedunculopontine nucleus (PPN), as the cholinergic arm of the reticular activating system (RAS), is known to modulate waking and REM sleep (Steriade and McCarley, 1990). PPN neurons increase their firing rates during synchronization of fast rhythms in waking and REM sleep (have tonic activity in waking, increased bursting activity during REM sleep and reduced activity during slow wave

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Abbreviations: ACh+, cholinergic; ACh−, non-cholinergic; aCSF, artificial cerebrospinal fluid; AHP, afterhyperpolarization; ANOVA, analysis of variance; AP, action potential; CLO, clonidine; Ih, hyperpolarization activated cation current; LC, locus coeruleus; LDT, laterodorsal tegmental; LTS, low threshold spike; NA, noradrenaline; NADPH, nicotinamide adenine dinucleotide phosphate; PPN, pedunculopontine nucleus; REM, rapid eye movement; Rin, input resistance; RMP, resting membrane potential; TTX, tetrodotoxin; YOH, yohimbine; ZD-7288, 4-(N-ethyl-N-phenylamino)-1,2-dimethyl-6(methylamino) pyrimidinium chloride.

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sleep) (Steriade et al., 1990a,b). We have been studying the changes in PPN neuronal properties, synaptic inputs and neurochemical control during the most drastic decrease in REM sleep in the rat, 12–21 days postnatally. The present studies were undertaken to determine if the responsiveness of PPN neurons to the α-2 adrenergic agonist clonidine (CLO) changes during this period. Inhibition of PPN neurons by noradrenergic locus coeruleus (LC) neurons has long been proposed as a mechanism for behavioral state control. Specifically, a subset of PPN neurons increases firing in advance of, and as, REM sleep ensues, whereas they slow their firing during waking ("Wake-Off/REM-On" cells) and non-REM sleep (El-Mansari et al., 1989; Steriade et al., 1990a,b). Other cells show "Wake-On-REM-On" firing characteristics. It has been hypothesized that these "REM-on" type neurons promote REM sleep via their projections to ascending and descending sites (Steriade and McCarley, 1990; McCarley and Massaquoi, 1992). It would be these cells that would most likely be inhibited by noradrenaline (NA) to prevent the occurrence of REM sleep. NA has been reported to hyperpolarize 7–15 day (i.e. during the first half of the developmental decrease in REM sleep) cholinergic mesopontine neurons in the laterodorsal tegmental nucleus (LDT) (Williams and Reiner, 1993), although similar studies have never been carried out in the PPN, or at later stages (15–30 days) of the developmental decrease in REM sleep. Moreover, α-2 adrenergic receptor development is known to undergo marked changes in the LC and other mesopontine regions *after* 15 days of age in the rat (Happe et al., 2004). Interestingly, in adult cholinergic mesopontine neurons, only about one half showed α-2 adrenergic receptor immunocytochemical labeling, while one third of cholinergic cells showed α-1 adrenergic receptor labeling (Hou et al., 2002). These authors suggested that differential activation of different populations of cholinergic cells may underlie aspects of behavioral state control. However, they did not perform triple labeling studies in order to determine if different populations of cholinergic cells are differentially modulated by the two receptor subtypes. This would determine if there is indeed differential adrenergic regulation of Wake-Off/REM-On vs. Wake-On/REM-On cells. It is, therefore, important to determine the nature of the responses to adrenergic agents on identified and electrophysiologically classified PPN neurons throughout the developmental decrease in REM sleep, but especially after 15 days of age. Intracellularly recorded 12–21 day PPN neurons were first classified according to type based on intrinsic membrane properties and then were injected with biocytin or neurobiotin at the end of recording in order to label studied neurons using Texas Red–avidin immunofluorescence. Nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase histochemistry was used to determine which of the recorded cells were cholinergic PPN neurons using light microscopy. Preliminary findings were described in abstract form (Mamiya et al., 2004).

EXPERIMENTAL PROCEDURES

Subjects

The procedures were approved by the Institutional Care and Use committee and abided by all U.S. National Institutes of Health guidelines for the ethical treatment of animals. The number of animals used was minimized, and the procedures ensured their lack of suffering. The methods employed in the present study were identical to those used in the study of other transmitter types, e.g. glutamatergic inputs (Kobayashi et al., 2004). Timed-pregnant Sprague–Dawley rats were used and the litters culled to 10. At 12–21 days of age, pups were anesthetized using ketamine (70 mg/kg, i.m.) until tail pinch and corneal reflexes were absent, then were rapidly decapitated. The brains were dissected free under cooled (4 °C), oxygenated (95% O_2, 5% CO_2) artificial cerebrospinal fluid (aCSF) and sagittal brainstem slices were cut along the axis of the PPN. The block of tissue was glued onto a stage and 400 μM slices cut with a Vibroslicer (World Precision Instruments, Sarasota, FL, USA) under cooled, oxygenated aCSF, and then allowed to equilibrate for 1 h in oxygenated aCSF at room temperature before recording. The composition of the aCSF was (in mM): NaCl 122.8; KCl 5; $MgSO_4$ 1.2; $CaCl_2$ 2.5; NaH_2PO_4 1.2; $NaHCO_3$ 25; and dextrose 10. Only one to two of the 400 μm slices from each side of the brain contained the PPN. In this study, we used a total of 150 pups (15 litters). Typically, we recorded from one cell in the PPN from each slice. Once a well-studied cell was injected intracellularly, we recorded from the other ipsilateral slice or from the contralateral PPN.

Recording procedures

Brain slices were suspended in a recording chamber on a nylon mesh, allowing gravity-fed aCSF to flow entirely around the slice. The aCSF flowed through a sleeve of circulating warmed water so that the temperature of the aCSF in the chamber was 30 ± 1 °C. Outflow was removed by suction, and flow adjusted to 2–3 ml/min. Microelectrodes were pulled in a Sutter Instruments puller using Omega-Dot^{TM}, thin-wall borosilicate glass and filled with 3 M K^+ acetate and 1% biocytin or neurobiotin. Ideal electrodes had a resistance of 85–105 MΩ. Signals were amplified with an Axoclamp 2b amplifier (Axon Instruments, Foster City, CA, USA) in the current clamp mode. Neurons were impaled and allowed to stabilize for about 5 min before testing. Neurons that showed a consistent resting membrane potential (RMP) ≤ -50 mV and action potentials (AP) $\geq$40 mV, and which provided stable, long-term recordings were accepted for data analysis. The RMPs were verified and adjusted when the electrode was withdrawn at the end of recordings (usually only 1–2 mV difference, but sometimes $\geq$5 mV after biocytin or neurobiotin injection). In bridge mode, a series of hyperpolarizing and depolarizing current steps of 0.1–1.0 nA at RMP was applied to allow the calculation of a preliminary *I–V* curve during the linear range of voltage deflections using SuperScope software (GW Instruments, Somerville, MA, USA).

The basic properties of PPN neurons between 12 and 21 days postnatally, including RMP, membrane input resistance (Rin), AP amplitude, duration and threshold, afterhyperpolarization (AHP) amplitude, AHP duration (Kobayashi et al., 2002), and cell type (based on the presence of a low-threshold spike (LTS) (type I), an A current (type II) or both A+LTS (type III)) (Kamondi et al., 1992; Kobayashi et al., 2002; Leonard and Llinas, 1990; Takakusaki et al., 1997), were recorded and analyzed as previously reported. Briefly, type I cells are all non-cholinergic, type II cells are 2/3 cholinergic, and type III cells are 1/3 cholinergic (Kang and Kitai, 1990; Leonard and Llinas, 1990; Takakusaki and Kitai, 1997). We should note that some of these studies were performed on mesopontine neurons in the guinea-pig (Leonard and Llinas, 1990), an animal which exhibits an adult sleep–wake pattern at birth compared with that in the rat (Jouvet-Mounier et al., 1970).