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Serotonin hyperpolarizes cholinergic low-threshold burst neurons in the rat laterodorsal tegmental nucleus *in vitro*

(pontogeniculooccipital wave/REM sleep)

J. I. LUEBKE*, R. W. GREENE*, K. SEMBA†, A. KAMONDI‡, R. W. MCCARLEY*, AND P. B. REINER‡§

*Neuroscience Laboratory, 151C, Department of Psychiatry, Harvard Medical School/Veteran's Administration Medical Center, Brockton, MA 02401; †Department of Anatomy, Dalhousie University, Halifax, NS, Canada B3H 4H7; and ‡Kinsmen Laboratory of Neurological Research, Department of Psychiatry, University of British Columbia, Vancouver, BC, Canada V6T 1Z3

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ABSTRACT **Serotonergic suppression of cholinergic neuronal activity implicated in the regulation of rapid eye movement sleep and its associated phenomenon, pontogeniculooccipital waves, has long been postulated, but no direct proof has been available. In this study, intracellular and whole-cell patch-clamp recording techniques were combined with enzyme histochemistry to examine the intrinsic electrophysiological properties and response to serotonin (5-HT) of identified cholinergic rat laterodorsal tegmental nucleus neurons *in vitro*. Sixty-five percent of the recorded neurons demonstrated a prominent low-threshold burst, and of these, 83% were cholinergic. In current-clamp recordings 64% of the bursting cholinergic neurons tested responded to the application of 5-HT with a membrane hyperpolarization and decrease in input resistance. This effect was mimicked by application of the selective 5-HT type 1 receptor agonist carboxamidotryptamine maleate. Whole-cell patch-clamp recordings revealed that the hyperpolarizing response was mediated by an inwardly rectifying K^+ current. Application of 5-HT decreased excitability and markedly modulated the discharge pattern of cholinergic bursting neurons: during a 5-HT-induced hyperpolarization these neurons exhibited no rebound burst after hyperpolarizing current input and a burst in response to depolarizing current input. In the absence of 5-HT, the relatively depolarized cholinergic bursting neurons responded to an identical hyperpolarizing current input with a burst and did not produce a burst after depolarizing current input. These data provide a cellular and molecular basis for the hypothesis that 5-HT modulates rapid eye movement sleep phenomenology by altering the firing pattern of bursting cholinergic neurons.**

The neurobiological basis of behavioral state regulation has intrigued neurobiologists since the classic studies of Moruzzi and Magoun (1). In recent years attention has focused upon a subpopulation of neurons in the laterodorsal (LDT) and the pedunculopontine (PPT) tegmental nuclei, which are immunoreactive to choline acetyltransferase and are, therefore, defined as cholinergic (2, 3). These nuclei have been implicated in regulation of the ascending reticular activating system, arousal, locomotion, relay of central autonomic pathways, and rapid eye movement (REM) sleep (for review, see ref. 4). There is evidence for a role of bursting cholinergic LDT and PPT neurons in the production of pontogeniculooccipital (PGO) waves, a hallmark sign of REM sleep, including the following: (*i*) these neurons project heavily to the thalamus, including the lateral geniculate nucleus (5, 6); (*ii*) a population of "PGO-burst" neurons in the LDT/PPT area fires bursts consisting of two to six spikes that are tightly linked with the occurrence of PGO waves (7–10); (*iii*) stimulation of the LDT/PPT area elicits field potentials identical to PGO waves in the lateral geniculate nucleus (11); (*iv*) excitotoxic lesion of the LDT/PPT area reduces PGO waves during REM sleep by 75% (12); and (*v*) injection of nicotinic antagonists into the lateral geniculate nucleus abolishes geniculate PGO waves (13).

A considerable body of evidence also implicates serotonergic mechanisms in the control of PGO wave generation (for review, see ref. 4). Depletion of serotonin (5-HT) by systemic administration of *p*-chlorophenylalanine leads to a condition in which spontaneous PGO waves are recorded not only during REM sleep but also during wakefulness (14). In a similar vein, parasaggital lesions that isolate the serotonergic raphé nuclei also result in the appearance of PGO waves during wakefulness and slow-wave sleep (15). Consistent with these data, serotonergic neurons exhibit marked state-related changes in spontaneous activity, firing most during waking, firing less during slow-wave sleep, and falling silent during REM sleep when spontaneous PGO waves are seen (16). There is anatomical evidence that serotonergic neurons in the dorsal and median raphé nuclei project to the LDT (17, 18). From these observations, 5-HT has been hypothesized to exert an inhibitory effect on the generation of PGO waves.

Although the intrinsic membrane properties that mediate the activity and response patterns of LDT and PPT neurons have recently begun to be characterized (19–21), there are currently few or no data on how other neurotransmitters, such as 5-HT, might modulate this activity. In the present study, we have examined the effects of 5-HT and the specific 5-HT type 1 receptor agonist, carboxamidotryptamine maleate (5-CT) on identified cholinergic neurons in the LDT. The results indicate that 5-HT can alter the response of LDT neurons to hyperpolarizing and depolarizing inputs in a manner consistent with the proposed role of these neurons in the generation of PGO waves.

METHODS

***In Vitro* Brainstem Slice Preparation.** Long–Evans rats 8–14 days old were anesthetized with halothane and decapitated; their brains were then rapidly removed. The brain was trimmed to form a block that contained the pontomesencephalic tegmentum and was cut with a Vibratome into transverse slices ≈500 μm thick. The slice containing the LDT was submerged in a recording chamber and constantly superfused with a solution of oxygenated modified Ringer's solution containing 124 mM NaCl, 26 mM $NaHCO_3$, 3 mM KH_2PO_4, 2.5 mM KCl, 2.5 mM $CaCl_2$, 1.3 mM $MgCl_2$, and 10 mM glucose, pH 7.2. The slice was maintained at 32°C, and the

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Abbreviations: LDT, laterodorsal tegmental nucleus; 5-HT, serotonin (5-hydroxytryptamine); 5-CT, carboxamidotryptamine maleate; REM, rapid eye movement; PGO, pontogeniculooccipital; PPT, pedunculopontine tegmental nucleus; *I–V*, current–voltage; V_m, membrane potential.

§To whom reprint requests should be addressed.

flow rate of the perfusate was 1.5–2 ml/min. Intracellular recordings were obtained with electrodes filled with 2% biocytin/2 M KCl. Electrode resistances ranged from 60 to 130 MΩ. Current-clamp and single microelectrode voltage-clamp experiments were done using an Axoclamp-2A amplifier (Axon Instruments, Burlingame, CA) and the software program BASIC-FASTLAB (Indec Systems, Sunnyvale, CA). Drugs were bath-applied in the following concentrations: 1–50 μM 5-HT (Research Biochemicals, Natick, MA), 100–500 nM 5-CT (Research Biochemicals), 0.3 μM tetrodotoxin (Sigma), and 2–4 mM cadmium (Sigma). All cells from which intracellular recordings were obtained were simultaneously injected with biocytin (Sigma). After the recordings, slices were placed in 4% paraformaldehyde at 4°C overnight and treated with Texas red avidin (Vector Laboratories) the next day, as described (22). After visualization of the electrophysiologically characterized, biocytin-labeled neuron, the slice was processed for NADPH-diaphorase histochemistry, as described (23).

Whole-Cell Patch-Clamp Preparation. For whole-cell patch-clamp recordings from LDT neurons in slices, we used the protocol of Blanton *et al.* (24). Electrodes were fabricated from 1.5-mm thin-wall borosilicate glass and were filled with a solution of the following composition: 110 mM potassium methanesulphonic acid, 15 mM NaCl, 1 mM $CaCl_2$, 2 mM MgATP, 0.3 mM GTP, 11 mM EGTA, and 10 mM Hepes (pH 7.2); to dissolve the EGTA, KOH was added, bringing the final potassium concentration to 140 mM. In some experiments the extracellular potassium concentration was increased from 2.5 to 5 mM. Electrodes had resistances ranging from 3 to 5 MΩ. Recordings were obtained by using either a List EPC-7 patch-clamp amplifier or an Axoclamp 2A amplifier in the continuous voltage-clamp mode at a gain of 50–75 nA/mV. Series-resistance compensation was not used. Experiments were done at room temperature.

RESULTS

Intrinsic Membrane Properties of LDT Neurons. Intracellular recordings were obtained from 34 LDT neurons, all of which had a resting membrane potential (V_m) more negative than −55 mV and action potential overshoots greater than +10 mV. The mean input resistance of these cells was 178 ± 69 (SD) MΩ. Sixty-five percent of the neurons were of the bursting type, exhibiting a low-threshold spike upon the crest of which rode a burst of action potentials that had a mean interspike interval of 15.6 ± 5.4 (SD) msec, and the other 35% did not display a low-threshold burst. Representative voltage traces of a bursting and a non-bursting neuron are shown in Fig. 1. Fig. 1*A* shows the rebound burst response to a hyperpolarizing current step of a bursting neuron held at −65 mV (left trace) and the burst response to a depolarizing step of the same neuron held at −75 mV (right trace). In the presence of tetrodotoxin, the fast sodium spikes were eliminated, revealing a typical low-threshold spike (Fig. 1*B*, left trace); this spike was abolished by the application of the calcium channel blocker cadmium (Fig. 1*B*, right trace), revealing the calcium dependence of the low-threshold spike. The characteristics of the low-threshold burst in these cells (i.e., voltage and calcium dependence) were similar to those described in thalamic (25), basal forebrain (26), and medial pontine reticular formation neurons (27), except that the interspike interval was considerably longer in the cells examined in the present study. The nonbursting neuron shown in the last two traces did not display a burst in response to a hyperpolarizing step from a depolarized membrane potential (Fig. 1*C*, left trace) or in response to a depolarizing step from a hyperpolarized membrane potential (Fig. 1*C*, right trace).

Identification of Cholinergic and Non-cholinergic Neurons. Because all choline acetyltransferase-immunoreactive neurons of the mesopontine tegmentum stain positively for the histochemical marker NADPH-diaphorase (23), we used this histochemical technique to determine the cholinergic nature of physiologically characterized LDT neurons. Eighty-three percent of the bursting neurons examined (15 of 18) were cholinergic, whereas 33% of the nonbursting neurons examined (3 of 9) were cholinergic. False negative results were unlikely, as darkly stained NADPH-diaphorase-positive cells were always seen in the same focal plane as the biocytin-labeled neurons. Representative examples of a NADPH-diaphorase-positive bursting neuron and a NADPH-diaphorase-negative non-bursting neuron are shown in Fig. 2.

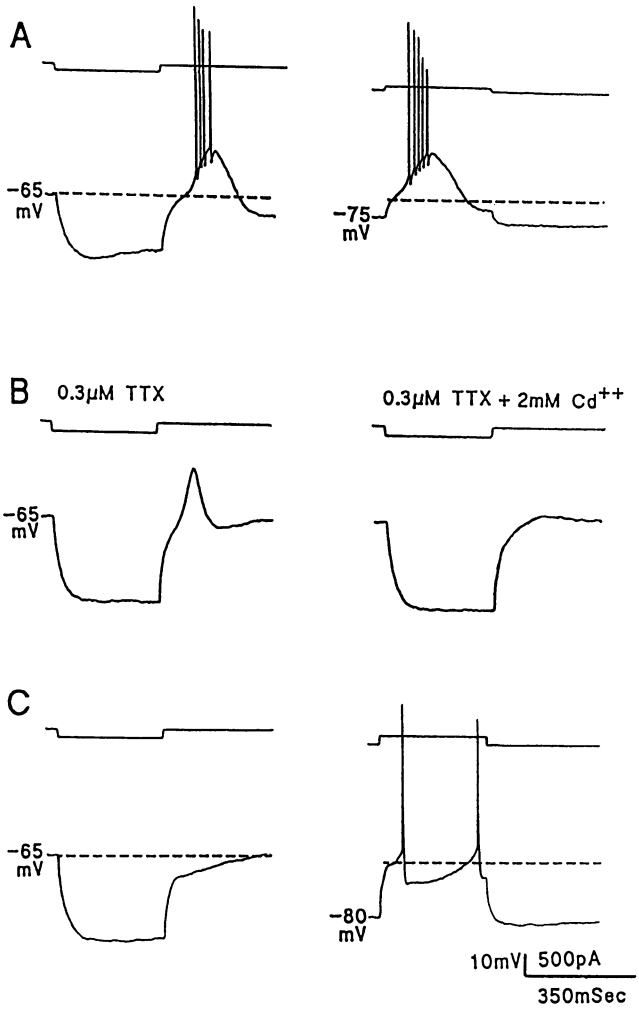

FIG. 1. Characteristics of bursting and non-bursting neurons. (*A*) Left trace: the resting V_m was held at −65 mV, and a hyperpolarizing current step (upper trace) resulted in a rebound low-threshold burst. Right trace: when resting V_m was held at −75 mV, a depolarizing current step (upper trace) caused a prominent low-threshold burst. (*B*) Left trace: with 0.3 μM tetrodotoxin (TTX), a low-threshold spike is revealed. Right trace: application of 2 mM cadmium abolished the spike. (*C*) Left trace: response of a non-bursting neuron held at −65 mV and given a hyperpolarizing step (upper trace). Right trace: response of the non-bursting neuron held at −80 mV and given a depolarizing step (upper trace).

Response of LDT Neurons to 5-HT and 5-CT: Current-Clamp Recordings. Seventy-two percent of the bursting neurons tested (13 of 18) responded to 5-HT with a membrane hyperpolarization and decrease in membrane resistance. Of the cholinergic bursting neurons tested, 64% (7 of 11) responded in this manner. Thirty-three percent (3 of 9, one cholinergic) of the nonbursting neurons tested responded to 5-HT with a membrane hyperpolarization and decrease in input resistance. Neurons that were not hyperpolarized by

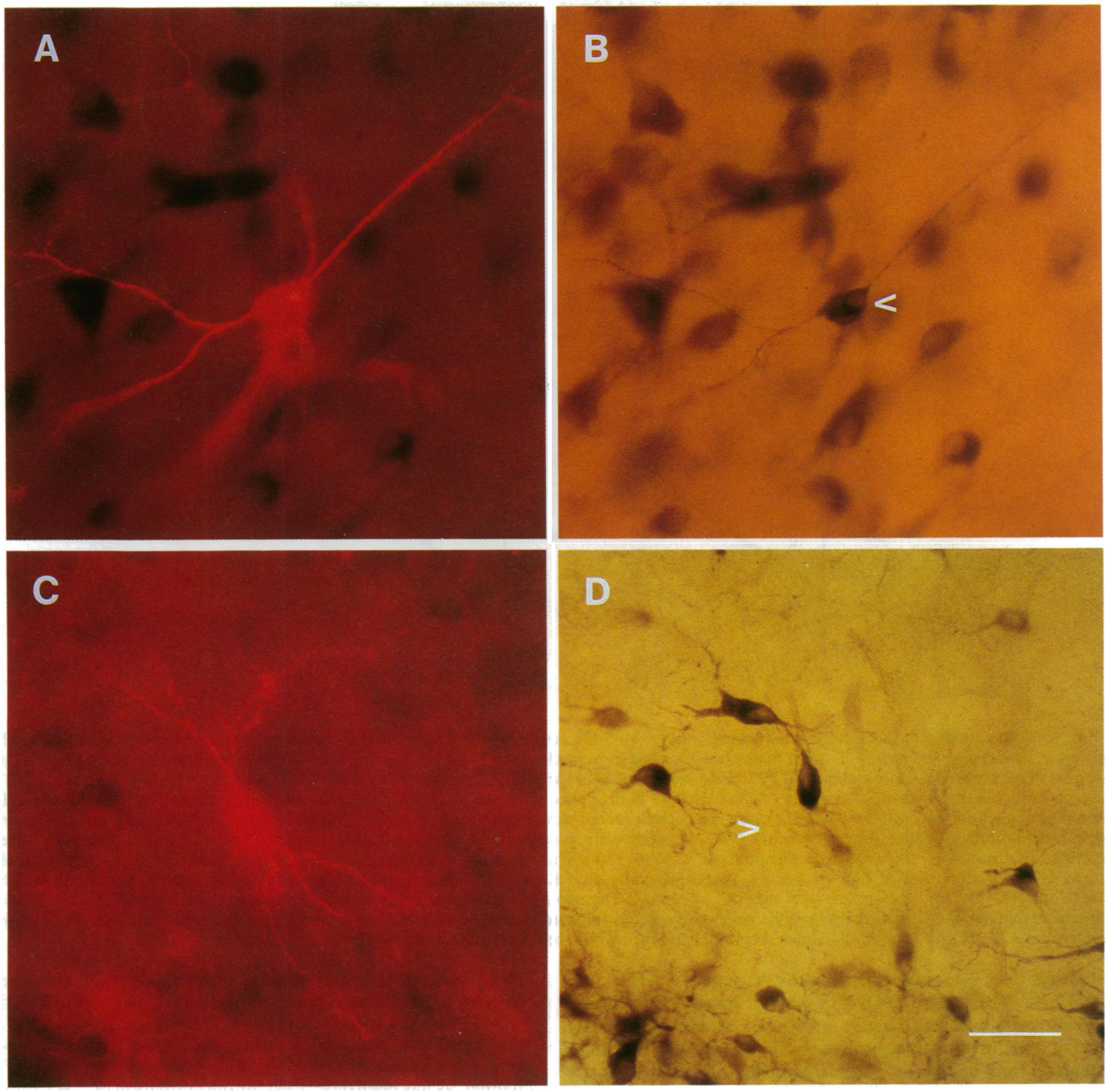

FIG. 2. Identified cholinergic and non-cholinergic LDT neurons. (*A*) Fluorescent photomicrograph of a low-threshold burst neuron labeled with Texas red avidin. (*B*) Bright-field photomicrograph of the identical field as in *A* but after NADPH histochemistry. Arrow indicates the bursting neuron, which is NADPH-diaphorase positive. (*C*) Fluorescent photomicrograph of a non-bursting neuron labeled with Texas red avidin. (*D*) Bright-field photomicrograph of identical field as in *C*. Arrow indicates site of the non-bursting neuron that was negative for NADPH-diaphorase activity. (Bar = 50 μm.)

5-HT exhibited no change in membrane potential or input resistance in response to 5-HT (data not shown). Each cell that responded to 5-HT responded in a like manner to 5-CT. The response of a bursting neuron to 5-HT and 5-CT during current-clamp recording is shown in Fig. 3*A*. 5-HT (10 μM, left trace) and 5-CT (500 nM, right trace) evoked a membrane hyperpolarization with a decrease in input resistance.

Response of LDT Neurons to 5-HT: Whole-Cell Patch-Clamp Recordings. 5-HT (10–50 μM) produced a hyperpolarization, or outward current, in 78% (7 of 9) of the bursting neurons recorded with whole-cell patch-clamp techniques (Fig. 3*B*). In three neurons, current–voltage (*I–V*) ramps were carried out before and during 5-HT application to characterize further the induced outward current (Fig. 3*C*). In each case, the *I–V* curves crossed very near to the predicted K^+ equilibrium potential, suggesting that the hyperpolarization was due to opening of K^+ channels. When control *I–V* curves were subtracted from those obtained during 5-HT application, the resulting traces revealed that the K^+ current induced by 5-HT exhibited marked inward rectification (Fig. 3*D*).

Effect of 5-HT and 5-CT on the Low-Threshold Burst. Hyperpolarization of the cell membrane by 5-HT and 5-CT altered the response of bursting neurons to hyperpolarizing and depolarizing current steps (Fig. 4). Under control conditions, (V_m = −65) a hyperpolarizing step resulted in a rebound burst (Fig. 4*A*, left trace), whereas a depolarizing step resulted in a nonburst firing pattern of four action

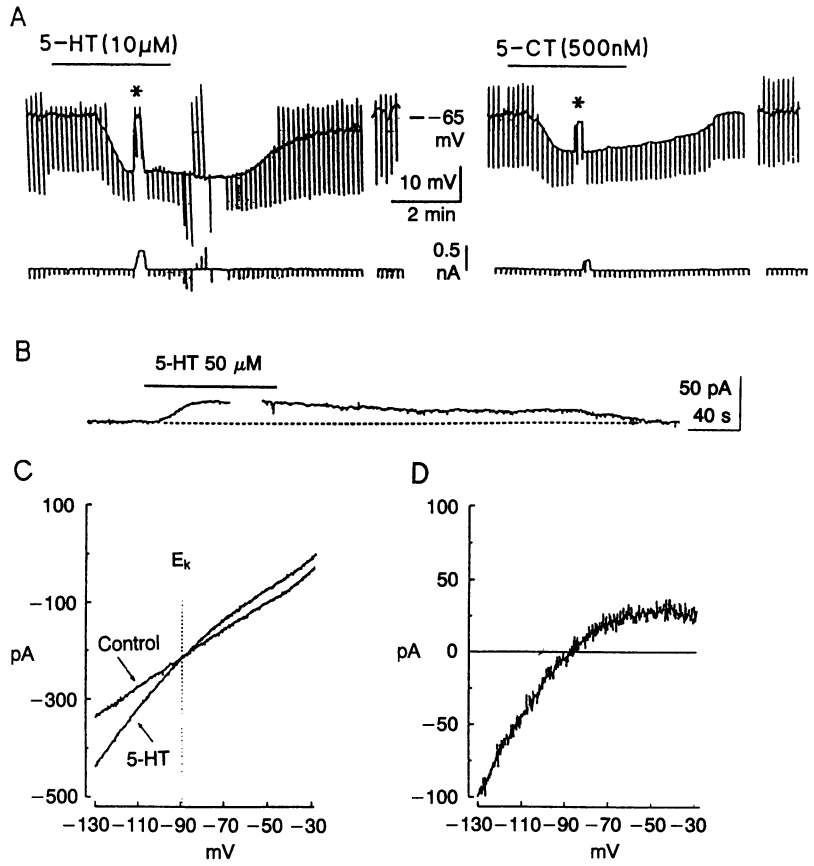

FIG. 3. Intracellular and whole-cell patch-clamp analysis of 5-HT response. (*A*) Response of a cholinergic bursting neuron to 10 μM 5-HT (left upper trace) and 500 nM 5-CT (right upper trace) during current-clamp recordings. Repolarization of the membrane to resting levels (at asterisk) demonstrated a decrease in input resistance independent of the change in membrane potential and thus due to direct drug action. (*B*) Response of a bursting neuron clamped at −60 mV to 5-HT bath applied at 50 μM. A slowly developing outward current was induced by 5-HT (period of application indicated by overline), and the current record slowly returned to baseline after termination of 5-HT application. Gap in the current record indicates the time when 5-HT *I–V* ramps were generated, which have been omitted. (*C*) Steady-state *I–V* curves were generated by "ramping" the voltage command from −130 to −30 mV at a rate of 1 mV/sec under control conditions and after application of 50 μM 5-HT. Vertical dotted line marked E_K represents calculated K^+ equilibrium potential under the experimental conditions in which intracellular $[K^+]$ = 139 mM and extracellular $[K^+]$ = 5 mM. The two *I–V* curves cross very near E_K. (*D*) Digital subtraction of control *I–V* curve from that obtained during 5-HT application reveals a steady outward current depolarized to E_K, which exhibits marked inward rectification negative to −70 mV. (*A* is an intracellular recording; *B–D* are whole-cell patch-clamp recordings.)

potentials (Fig. 4*A*, right trace). In the presence of 5-HT (10 μM, V_m = −80), a hyperpolarizing current step failed to elicit a low-threshold burst (Fig. 4*B*, left trace), whereas a depolarizing step did elicit this mode of firing but with fewer action potentials than in Fig. 4*A*, right trace (Fig. 4*B*, right trace). Thus the hyperpolarizing effect of 5-HT strongly altered both the burst response pattern and the excitability of this bursting neuron to identical hyperpolarizing and depolarizing inputs.

DISCUSSION

The first principal finding of this study is that the majority of low-threshold burst neurons in the LDT are cholinergic. Earlier studies of the LDT/PPT suggested that the low-threshold burst neurons were not cholinergic and the nonbursting neurons were cholinergic (20, 21). These discrepant results might be accounted for by differences in age, species, location of the recorded neurons, and/or differences in experimental techniques. We recorded exclusively in the LDT of neonatal rats, whereas Leonard and Llinas recorded primarily in the PPT of the adult guinea pig (20) and Kang and Kitai recorded neurons only in the adult rat PPT (21). We note that our identification of the bursting neurons in the LDT as cholinergic is consistent with experiments showing blockade of geniculate PGO activity by application of cholinergic antagonists (13).

The second principal finding of this paper is that the majority of cholinergic bursting neurons are hyperpolarized by 5-HT and the specific 5-HT type 1 receptor agonist 5-CT. Whole-cell patch-clamp recordings revealed that this phenomenon was due to activation of an inwardly rectifying K^+ channel, as has been described for the activation of 5-HT type 1A receptors on several other types of neurons in the central nervous system (28). The finding that 5-HT hyperpolarizes bursting cholinergic LDT neurons *in vitro* places the hypothesis that 5-HT exerts an inhibitory effect on PGO wave generation on firm cellular and molecular grounds.

We hypothesize that (*i*) during waking, when serotonergic activity is high (16), bursting LDT cholinergic neurons are hyperpolarized via the opening of inwardly rectifying K^+ channels positively coupled to 5-HT type 1 receptors and (*ii*) during REM sleep, when serotonergic activity is absent, serotonergic inhibition of bursting cholinergic LDT neurons is removed. On the basis of our data we would predict that the generation of PGO waves during REM sleep is due to transient hyperpolarizing synaptic events, the offset of which would induce a rebound burst in a depolarized cholinergic LDT neuron. In contrast, during waking, when the serotonergic influence is high and bursting cholinergic LDT neurons are hyperpolarized, excitatory but not inhibitory synaptic inputs would lead to burst generation.

LDT neuronal function and activity are known to be heterogeneous (10). This paper has focused on cholinergic

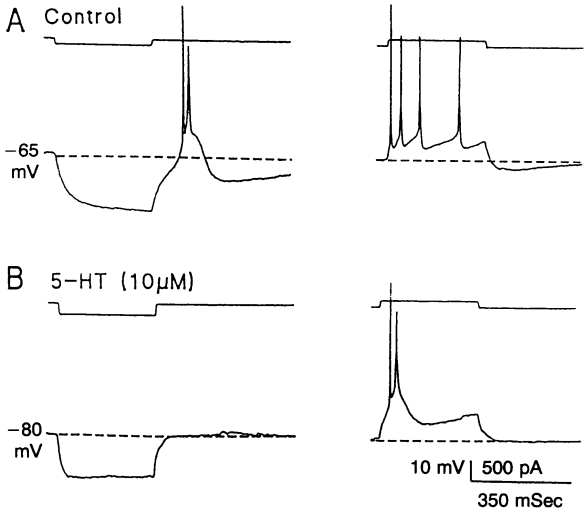

FIG. 4. Effect of 5-HT on the low-threshold burst. (*A*) Left trace: under control conditions ($V_m = -65$) a hyperpolarizing step results in a rebound low-threshold burst. Right trace: a depolarizing step results in nonburst-like firing. (*B*) Left trace: with 5-HT ($V_m = -80$) the cell does not respond to a hyperpolarizing step with a burst. Right trace: a depolarizing step causes a rebound burst but with fewer action potentials than in *A*, right trace.

low-threshold burst neurons that are likely related to PGO waves. Based on *in vivo* studies, PGO-burst neurons represent only a small minority of all LDT neurons (9, 10). It should be emphasized that PGO-related but non-bursting and non-PGO-related neurons are also likely to be modulated by 5-HT. Non-PGO-related neurons with various afferent and efferent projections may influence muscle atonia, saccadic eye movements, and/or electroencephalogram desynchronization (for review, see ref. 4). We suggest that the presence or absence of a PGO-related burst-discharge pattern likely depends not only on the intrinsic electrophysiological properties and serotonergic response but also on the particular anatomical connectivity (i.e., excitatory or inhibitory synaptic inputs) of an LDT neuron.

This work is not only relevant to the understanding of the monoaminergic–cholinergic interactions in the regulation of REM sleep but is also of potential relevance to the understanding of mood disorders, such as depression in which monoaminergic–cholinergic interactions appear important (for review, see ref. 29). This demonstration of a powerful modulation of excitability of cholinergic LDT neurons by serotonin echoes invertebrate work on behavioral state-related effects of serotonin (30–32) and points to an emerging area of work on the cellular foundations of mammalian behavioral state control.

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