

NORADRENALINE EXCITES NON-CHOLINERGIC LATERODORSAL TEGMENTAL NEURONS VIA TWO DISTINCT MECHANISMS

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Abstract—Cholinergic neurons of the laterodorsal tegmental nucleus have been hypothesized to play a critical role in the generation and maintenance of rapid eye movement sleep. Less is known about the function of non-cholinergic laterodorsal tegmental nucleus neurons. As part of our ongoing studies of the brainstem circuitry controlling behavioral state, we have begun to investigate the functional properties of these neurons. In the course of these experiments, we have observed a novel response to the neurotransmitter noradrenaline. Whole-cell patch-clamp recordings of laterodorsal tegmental nucleus neurons were carried out in 21- to 35-day-old rat brain slices. A subpopulation of laterodorsal tegmental nucleus cells responded to a 30-s application of 50 μ M noradrenaline with depolarization and a decrease in input resistance which lasted several minutes. Following return to resting membrane potential, these cells invariably exhibited barrages of excitatory postsynaptic potentials which lasted at least 12 min. These excitatory postsynaptic potentials were reversibly abolished by bath application of tetrodotoxin, as well as by the non-*N*-methyl-*D*-aspartate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione, but were insensitive to application of the *N*-methyl-*D*-aspartate receptor antagonist 2-amino-5-phosphonopentanoic acid. To examine whether these neurons were cholinergic, the recorded cells were labeled with biocytin and tested for co-localization with reduced nicotinamide adenine dinucleotide phosphate-diaphorase, a marker for laterodorsal tegmental nucleus cholinergic neurons. In every instance, neurons with these properties were non-cholinergic. However, they were always located in close proximity to reduced nicotinamide adenine dinucleotide phosphate-diaphorase-positive laterodorsal tegmental nucleus cells.

The present data indicate that noradrenaline, in addition to directly inhibiting cholinergic cells of the laterodorsal tegmental nucleus, also results in the direct and indirect excitation of non-cholinergic cells of the laterodorsal tegmental nucleus. The indirect excitation is long lasting and mediated by glutamatergic mechanisms. Our working hypothesis is that these non-cholinergic cells are local circuit inhibitory interneurons and that prolonged excitation of these neurons by noradrenaline may serve as a mechanism for inhibition of cholinergic laterodorsal tegmental nucleus cells during wakefulness, when noradrenaline tone is high. © 1999 IBRO. Published by Elsevier Science Ltd.

Key words: REM sleep, rat, pons, wakefulness, GABAergic neurons.

Human beings cycle between a succession of behavioral states: alert wakefulness, quiet wakefulness, slow-wave sleep and rapid eye movement (REM) sleep. The mechanisms which govern the cycles of sleep and wakefulness are incompletely understood. Nevertheless, there exists a consensus that brainstem cholinergic mechanisms play a pivotal role in the generation and maintenance of REM sleep. Specifically, it is hypothesized that cholinergic projections from the laterodorsal tegmental nucleus (LDT) to the medial pontine reticular formation (mPRF) are responsible for state-dependent release of acetylcholine (ACh), which triggers REM sleep.

Considerable information is available regarding the cellular mechanisms which regulate LDT ACh release. Given its apparent state dependence, primary modulators of this activity include neurons whose firing patterns co-vary with behavioral state. Noradrenergic neurons of the locus ceruleus (LC), serotonergic neurons of the raphe nuclei and histaminergic neurons of the tuberomammillary nuclei show state-

dependent firing, being most active during wakefulness, slower during slow-wave sleep and becoming nearly silent during REM sleep.^{1,39,53} These neurons have all been shown to project to the LDT.^{13,14,17,47} Based on these physiological and anatomical properties, it has been hypothesized that monoaminergic inputs to the LDT play a critical role in regulating cholinergic activity. Supporting this conclusion are the findings that noradrenaline (NA) and serotonin inhibit cholinergic neurons of the LDT.^{28,29,55} Further inhibition of cholinergic LDT cells may be provided by cholinergic input which has been shown to arise from the contralateral LDT.⁴⁷ Moreover, cholinergic agonists inhibit cholinergic LDT cells.^{28,30} Based on these and other data, it has been hypothesized that inhibition of cholinergic LDT cells contributes to wakefulness and release from this inhibition contributes to generation of REM sleep.

Compelling as this set of events may be, it is clear that this represents an incomplete account of the cellular basis of behavioral state control. A key issue is that the LDT is heterogeneous. Indeed, the majority of LDT neurons are non-cholinergic and many may be GABAergic.⁷ Presumably, these cells also receive monoaminergic and cholinergic input; however, the potential role of non-cholinergic neurons in the LDT on the generation or maintenance of behavioral state has not been specifically addressed previously. Given the paucity of information regarding the function of non-cholinergic LDT cells, we have begun to study the anatomy, physiology and pharmacology of these cells. Specifically, we hypothesized that if these cells play a role in the mediation of

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Abbreviations: ACh, acetylcholine; AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate; AP5, 2-amino-5-phosphonopentanoic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; EGTA, ethyleneglycolbis(aminoethyl ether)tetra-acetate; EPSP, excitatory postsynaptic potential; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; LC, locus ceruleus; LDT, laterodorsal tegmental nucleus; LGN, lateral geniculate nucleus; mPRF, medial pontine reticular formation; NA, noradrenaline; NADPH, reduced nicotinamide adenine dinucleotide phosphate; PBS, phosphate-buffered saline; PGO, ponto-geniculo-occipital; REM, rapid eye movement; TTX, tetrodotoxin.

behavioral state, they may exhibit responses to neurotransmitters implicated in behavioral state control.

EXPERIMENTAL PROCEDURES

Wistar rats (62 rats; male and female, three to five weeks old, 55–120 g; Animal Care, UBC) were anesthetized with halothane and decapitated. The brain was rapidly removed and trimmed to form a block that contained the LDT, which was then cut into coronal slices (400 μm thick) on an Oxford Vibratome. The slice containing the LDT was placed in a recording chamber and constantly superfused with a solution of standard artificial cerebrospinal fluid containing (in mM): 126 NaCl, 25 NaHCO_3 , 1.2 NaH_2PO_4 , 2.5 KCl, 2.5 CaCl_2 , 1.2 MgCl_2 and 11 glucose (pH 7.3 when saturated with 95% O_2 /5% CO_2). Slices were allowed to equilibrate to room temperature and all electrophysiological experiments were carried out at 21°C.

The whole-cell configuration of the patch-clamp technique as applied to brain slices was used to record from neurons in the LDT using bridge mode (for details, see Ref. 19). Patch pipettes were constructed from thin-wall borosilicate glass capillary tubes (1.5 mm o.d., 1.17 mm i.d.; Warner Instrument Co.). The electrode solution contained (in mM): 120 potassium gluconate, 10 HEPES, 24 NaCl, 15 KCl, 11 EGTA, 1 CaCl_2 , 2 MgATP . Baseline and post-drug recordings of synaptic activity, input resistance and resting membrane potentials of LDT neurons were collected and compared. Input resistance was determined by maximum voltage deflection of the membrane potential upon the injection of 0.03 nA hyperpolarizing current at the resting potential. Data are reported as mean \pm S.E.M. Statistical significance was assessed using Student's paired *t*-test or a between-group repeated measure ANOVA. Because there was not a statistical difference between baseline membrane potential or input resistance values of cells in any of the treatment groups, data are reported as either the actual change or a percentage change from baseline. All drugs were applied at known concentrations within the artificial cerebrospinal fluid. Tetrodotoxin (TTX), NA, carbachol, 5-hydroxytryptamine hydrochloride (serotonin) and propranolol were obtained from Sigma Biochemicals. 2-Amino-5-phosphonopentanoic acid (AP5), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), histamine dihydrochloride, isoproterenol bitartrate, atenolol, dobutamine hydrochloride, prazosin, phenylephrine and UK-14304 were obtained from RBI Biochemicals.

Electrical stimulation was applied to the tissue at the lateral boundary of the ipsilateral LDT with a bipolar electrode connected to an isolated voltage source (300–700 μA , 200 μs duration, 0.1 Hz).

Data were collected on a PC using the P-Clamp 6 suite of programs (Axon Instruments). Off-line analyses of spontaneous and drug-elicited synaptic activity were conducted by manual scoring of 10 episodes, each of duration 1.84 s during each experimental condition. Traces were visually inspected for depolarizing or hyperpolarizing synaptic events, and the amplitude of each event was marked manually using the mouse-measure command. This method ensured that these analyses were not corrupted by any slight change in the noise level or by membrane fluctuations. In a minority of cells, excitatory postsynaptic potential (EPSP) activity was present prior to drug application, and the largest EPSP amplitude was used as a cut-off for both pre- and post-drug EPSP analyses. In most cells, no EPSPs were seen under basal conditions; in these cases, only the largest post-drug EPSPs were included.

Histochemical identification of recorded neurons was achieved by inclusion of 0.2% biocytin in the normal patch pipette solution, which was allowed to passively diffuse into the cell during recording. Only one LDT cell was recorded per side in each slice.

After the experiment, all slices were fixed in 2% paraformaldehyde and 15% picric acid in 0.1 M phosphate-buffered saline (PBS). The following day, slices were removed from the fixative and rinsed three times for 15 min in PBS. Slices were then incubated in PBS containing 20 $\mu\text{g}/\text{ml}$ Texas Red®-conjugated avidin and 0.3% Triton-PBS solution. Following washout, slices were placed in 15% sucrose until they were completely saturated. Following this cryoprotective protocol, slices were cut into 50- μm sections with a cryostat and mounted onto coated slides.

After these procedures, the slice was then processed for NADPH-diaphorase histochemistry to determine if the neurons labeled with biocytin were cholinergic or, alternatively, if they were located in a region rich in cholinergic cells.⁵⁴ Briefly, slides were immersed in a solution containing 1 mg/ml NADPH and 0.1 mg/ml nitroblue tetrazolium in PBS and incubated at 37°C for 30–60 min. Subsequently,

slices were rinsed in PBS and stored in the dark to dry. Slides were coverslipped and examined under a microscope equipped with both bright-field optics and epifluorescence for identification of NADPH- and Texas Red-positive neurons, respectively.

RESULTS

The data are derived from recordings of 160 LDT neurons. Histological examination of the recorded cells which exhibited membrane depolarization and the appearance of, or an increase in ongoing, EPSP activity following the application of NA (see below) revealed that these biocytin-filled cells were NADPH-diaphorase negative, but were found within the borders of the LDT as defined by NADPH-diaphorase histochemistry (Fig. 1). We thus conclude that these cells were not cholinergic. These cells appeared to be much smaller (8.7–18 μm) than cholinergic cells. However, the majority of biocytin-labeled cells were found in close proximity to cholinergic cells (Fig. 1). In many cases, the processes of the labeled cells could be visualized and it appeared, at the light microscope level, that these processes contacted cholinergic processes, although this cannot be determined without a more detailed level of analysis, such as at the electron microscopic level and with confocal analysis techniques.

Noradrenaline

NA (50 μM) was applied to 47 biocytin-labeled cells. Of these, 45 cells were not cholinergic (Fig. 1), whereas two were NADPH-diaphorase positive and thus cholinergic. Forty of 45 non-cholinergic cells responded with depolarization of the membrane potential and a decrease in input resistance within 30 s of application of NA (Fig. 2). The membrane potential of these cells prior to drug application was -65.13 ± 0.73 mV; that following bath-applied NA was -58.68 ± 0.96 mV. The mean input resistance prior to drug application was 611.14 ± 39.8 M Ω and that following NA was 527.25 ± 36.16 M Ω ($n=20$), as measured at the resting membrane potential via injection of direct current. These changes in membrane potential and input resistance induced by NA were significant ($P < 0.05$).

In five of 45 non-cholinergic LDT cells, application of NA caused a hyperpolarization of the membrane potential; similar responses were seen in both of two cholinergic LDT neurons, as has been reported previously.⁵⁵

To examine the hypothesis that the changes in membrane potential and input resistance induced by NA were directly mediated, we applied TTX (300 nM) prior to the application of NA in an additional five cells. The efficacy of the effect of TTX in blocking synaptic transmission was determined by abolishment of action potentials elicited in response to depolarizing current pulses. Application of TTX did not prevent NA-induced depolarization of the membrane potential ($n=5$) nor the decrease in input resistance in these cells ($n=4$), and the amplitudes of the depolarization and changes in input resistance were similar to those seen in non-TTX-treated cells (data not shown).

Synaptic potentials were elicited upon electrical stimulation of the ventral lateral aspect of the LDT in 26 of 40 cells that exhibited membrane depolarization in response to NA. The amplitude of the resultant compound EPSP prior to NA was 4.42 ± 0.63 mV, during NA application it was 3.35 ± 0.63 mV and, following recovery of the resting

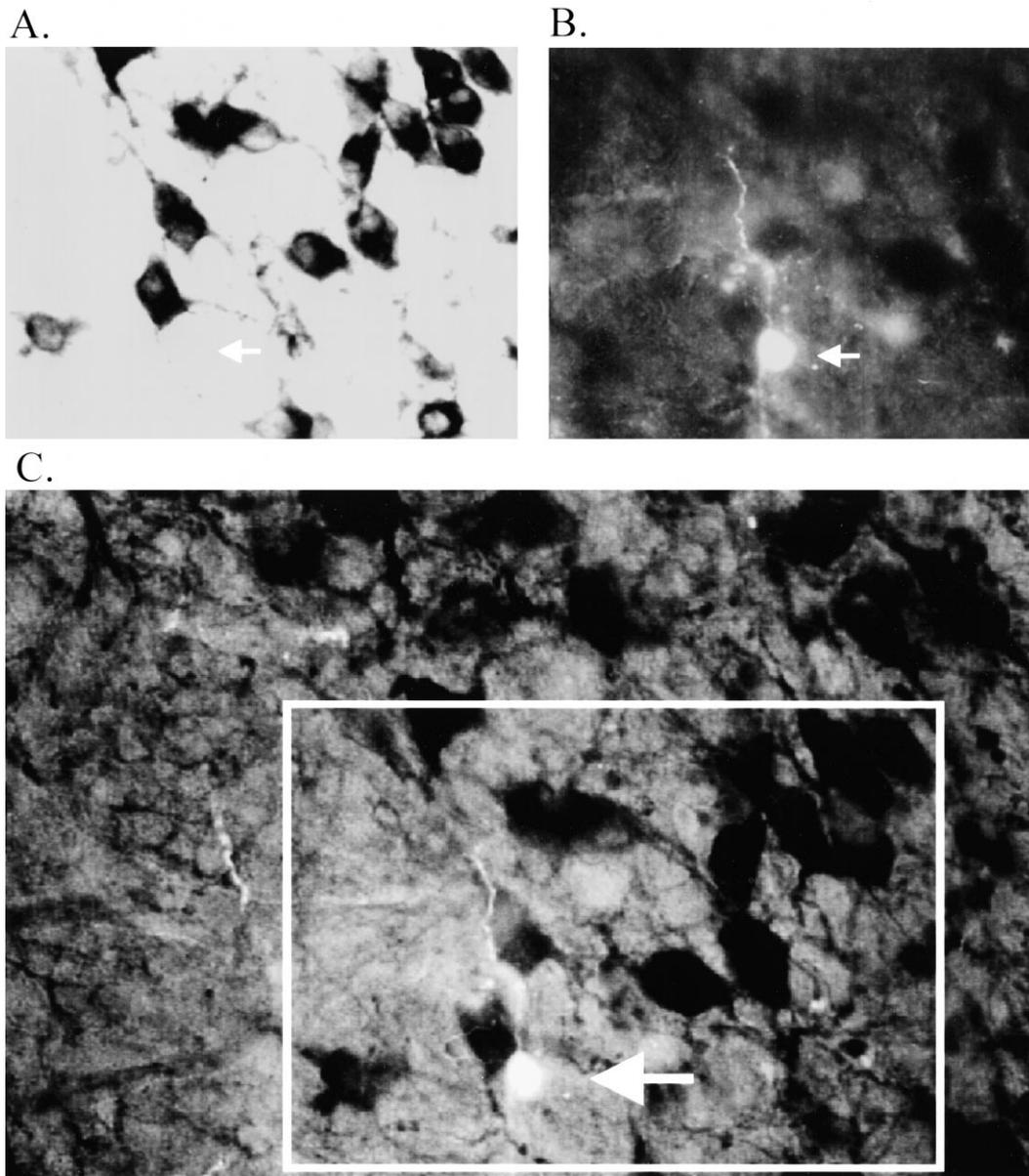


Fig. 1. Histochemical identification of the LDT. Neurons from which electrophysiological data were recorded were biocytin filled and subsequently visualized with fluorescence. The top right photo (B) is a fluorescent photomicrograph of one of the recorded cells in this report. NADPH-diaphorase histochemistry shows that the biocytin-labeled cell from which electrophysiological recordings were obtained was NADPH-diaphorase negative and thus non-cholinergic. The bright-field photomicrograph at the top left (A) is the identical field as that in the top right to demonstrate that the biocytin-filled cell was NADPH-diaphorase negative and thus non-cholinergic. Arrows in the top photos indicate the location of the biocytin-filled, NADPH-diaphorase-negative cell. The photo at the bottom (C) is observed under both bright-field and fluorescent illumination, and shows the identical biocytin-filled cell as shown in B (see boxed region), as well as a wider field than the field represented in panels A and B. As is apparent in this photo, the LDT NADPH-diaphorase-negative, biocytin-filled cells were found in a region rich in cholinergic cells and, commonly, their processes surrounded the neurites and somas of NADPH-diaphorase-positive and thus cholinergic cells.

membrane potential, it was 2.30 ± 0.48 mV. Of these, only the latter was significantly different from control.

All of the cells which depolarized with the application of NA repolarized to their pre-drug membrane potential, and in some cases to a more hyperpolarized potential within 3–6 min (4.17 ± 0.44 min). Remarkably, following this repolarization, barrages of EPSPs were observed; similar EPSPs were not seen prior to application of NA ($n=34$; Fig. 2). Prior to the administration of NA, 4.0 ± 0.9 ($13.04 \pm 3.06/60$ s) excitatory potentials were present ($n=34$). During the depolarization of the membrane induced by administration of NA, 17.0 ± 2.61 ($55.42 \pm 8.51/60$ s) depolarizing potentials were recorded in the same group of cells ($n=34$). Following

repolarization, 30.64 ± 4.47 ($99.87 \pm 14.60/60$ s) potentials were present. Quantification of these data revealed that there was a significant ($P < 0.05$) 13-fold increase in the incidence of EPSPs following repolarization of the cell from NA-induced depolarization. In many cases, EPSPs were seen during the late portion of the NA-induced depolarization, but in many cases EPSPs were not apparent until the cell had repolarized to the resting potential; in a few neurons, these EPSPs were of sufficient magnitude to evoke action potentials. This increased EPSP activity lasted a minimum of 12 min following application of NA (mean: 18.44 ± 1.17 min).

To test the hypothesis that this effect was presynaptically

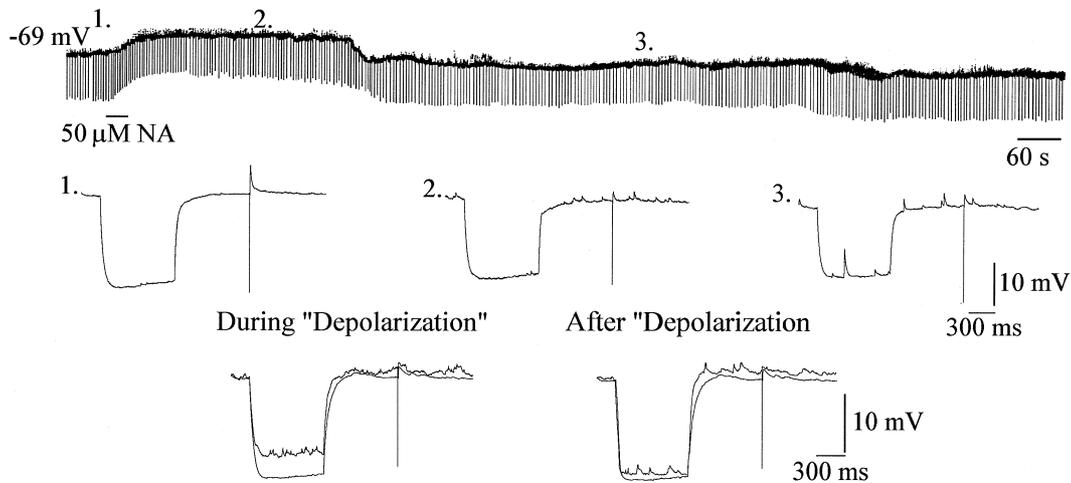


Fig. 2. Application of NA resulted in a short-lived membrane depolarization. Following recovery of the membrane potential, excitatory synaptic activity arose in recorded non-cholinergic LDT cells. Bridge mode experiments on a neuron in the LDT. Top trace is the chart recording of the membrane potential of one cell. NA-induced membrane depolarization lasted approximately 4.5 min. NA-induced excitatory synaptic activity was much longer lived, with a duration of at least 12 min post-NA. Middle traces are individual traces of a single voltage deflection in response to a hyperpolarizing current pulse prior to (1), immediately following (2) and several more minutes following (3) NA application. Bottom traces are overlaid averaged traces taken from another neuron during different points in the drug treatments taken at the same membrane potential (-70 mV). Averages of 10 episodes were utilized to calculate input resistance in order to remove possible contamination of these data by EPSP activity. Note that, even in the averages held at the resting membrane potential, EPSP activity is evident and may contribute to the calculated input resistance. In 1 through the bottom traces, stimulation of the ventral aspect of the LDT is being applied to monitor changes in amplitude of the resultant EPSP; the amplitude of this elicited potential was significantly reduced following repolarization of the membrane potential (3 and "After Depolarization").

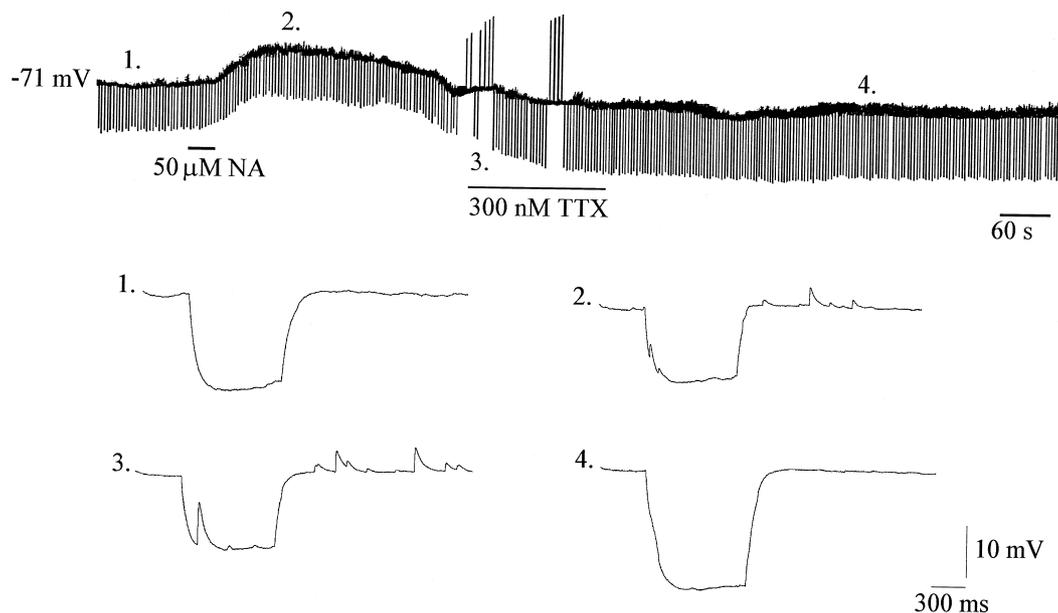


Fig. 3. TTX effectively blocked ongoing EPSP activity elicited by NA. Bridge mode experiments on a neuron in the LDT. Top trace is the chart recording of the membrane potential of a cell during application of TTX and NA. These data show that excitation of a presynaptic neuron(s) is responsible for the appearance of EPSPs in the recorded cells. Bottom traces are individual traces of a single voltage deflection in response to a hyperpolarizing current pulse prior to (1) and immediately following the effect of NA (2). (3, 4) Traces collected prior to and after the effect of TTX, respectively. When applied prior to the application of NA, TTX did not block NA-induced membrane depolarization (data not shown).

mediated, we applied 300 nM TTX to four cells in which excitatory activity had arisen following the application of NA. In every case, TTX abolished this EPSP activity (Fig. 3), suggesting that these EPSPs arose secondary to action potentials generated within the slice. The application of TTX also abolished the synaptic potential elicited by electrical stimulation of the lateral aspects of the LDT ($n=4$). To test the hypothesis that the EPSP activity induced by NA application was mediated by excitatory amino acids, we

applied the kainate/quisqualate/ α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor antagonist CNQX (10 μ M) or the *N*-methyl-D-aspartate receptor antagonist AP5 (50 μ M) to the slice following confirmation that NA application induced EPSPs in a given cell. Both NA- and stimulation-evoked EPSPs were significantly reduced by CNQX ($n=5$; Fig. 4). In one cell, it was possible to monitor the recovery of the EPSP activity following washout of CNQX. By contrast, AP5 did not have any observable effect

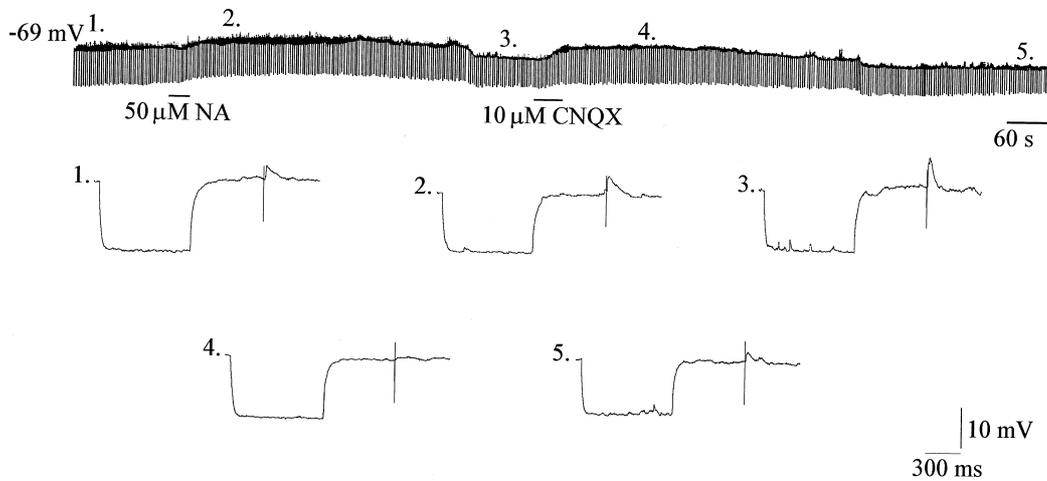


Fig. 4. CNQX was found to block ongoing NA-induced excitatory synaptic activity. Bridge mode experiments on a NADPH-negative neuron in the LDT. Top trace is the voltage of the cell. Bottom traces are individual traces of a single voltage deflection in response to a hyperpolarizing current pulse prior to (1) and immediately following (2) application of NA. Traces 2 and 3 show that NA elicited direct and indirect effects in this cell. Application of CNQX completely abolished the excitatory synaptic activity that arose following the administration of NA. CNQX also significantly reduced the amplitude of the EPSP elicited in these cells by electrical stimulation of the ventral aspect of the LDT. This reduction is apparent by comparing traces 3 and 5. It should be noted that, in this particular cell, the amplitude of the elicited EPSP was greater following repolarization of the cell (3); however, typically, this potential was significantly reduced in amplitude from that recorded pre-drug (see Results). Because the membrane potential was altered by the application of drugs and the amplitude of the elicited EPSP was not examined at one fixed potential, interpretation of these data is difficult. AP5 did not have any effect on NA-induced EPSPs and only slightly reduced the amplitude of the elicited potential (data not shown).

upon the suppression of excitatory activity that arose in these neurons following the application of NA ($n=4$). AP5 did significantly reduce the amplitude of the potential evoked by electrical stimulation of the ventral LDT (8.0%).

The pharmacological identity of the receptors mediating the excitatory responses of non-cholinergic LDT cells to NA was investigated. In all of seven cells which depolarized in response to NA, application of the α_1 -adrenergic agonist phenylephrine (10 μM) significantly depolarized the cell by 3.15 ± 0.14 mV, accompanied by a decrease in input resistance of 13.8% (Fig. 5), but did not induce EPSPs. Following washout of phenylephrine, NA was applied to the cells and induced a depolarization and EPSPs ($n=7$). Application of the α_1 -adrenergic antagonist prazosin (50 μM) blocked the NA depolarization, but did not prevent the ability of NA to evoke EPSPs (Fig. 5). The α_2 -adrenergic agonist UK-14304 (10–50 μM) had no consistent effect on the membrane potential or EPSP activity ($n=4$; data not shown).

The β -adrenergic agonist isoproterenol (10 μM) had no significant effects upon membrane potential or input resistance of all of 14 non-cholinergic LDT cells which depolarized in response to NA. Isoproterenol did evoke EPSP activity in these cells, lasting 16.56 ± 0.88 min (Fig. 6). The β -adrenergic agonist dobutamine (10 μM) also had no significant effect on membrane potential, but evoked EPSP activity in all of three cells tested (Fig. 6). The β_1 -adrenergic antagonist atenolol (50 μM) hyperpolarized non-cholinergic LDT neurons with a concurrent decrease in input resistance ($P < 0.05$). Atenolol was ineffective in blocking the depolarization evoked by NA ($n=5$), but did block the appearance of EPSPs (Fig. 6). The non-specific β -adrenergic antagonist propranolol (10 μM) did not significantly alter membrane potential or the input resistance of non-cholinergic LDT neurons. Propranolol did not block the NA-induced depolarization (3.4 ± 0.19 mV) or change in input resistance (15.7 ± 4.0 M Ω), but did block the NA-evoked EPSPs ($n=6$; data not shown).

Acetylcholine, histamine and serotonin

Because there is a rich innervation of the LDT by cholinergic neurons of the contralateral LDT,⁴⁷ we tested the effects of the cholinergic agonist carbachol (10 μM) upon non-cholinergic LDT neurons. In all of 11 non-cholinergic LDT cells which subsequently depolarized to NA, application of carbachol resulted in depolarization of the membrane potential (12.79 ± 0.61 mV) with a concurrent decrease in input resistance (118.8 ± 24.6 M Ω ; $P < 0.05$) and did not increase excitatory synaptic activity (Fig. 7). These effects were not blocked by TTX ($n=3$). Two of 13 cells hyperpolarized following carbachol and, following washout, subsequent application of NA. One cell was subsequently determined to be NADPH-diaphorase positive and the other negative. The finding that the NADPH-diaphorase-positive cell hyperpolarized in response to both carbachol and NA was not surprising in that it has been demonstrated previously that cholinergic LDT cells respond with hyperpolarization to muscarinic cholinergic agonists^{28,30} and to NA.⁵⁵

Histaminergic cells of the tuberomammillary nucleus of the hypothalamus also exhibit state-dependent firing and send projections to the LDT; therefore, we examined the effects of histamine (10 μM) on non-cholinergic LDT cells which depolarized to NA. Histamine significantly depolarized 13 of 14 cells (4.7 ± 0.67 mV) with a concurrent decrease in input resistance (46.7 ± 29.52 M Ω) (Fig. 7). These changes persisted in the presence of TTX ($n=4$). Depolarization of the membrane was long lasting compared to the depolarization induced by NA (8.3 ± 0.6 min, $P < 0.05$). We were able to confirm, in the rat LDT, a previous report²² that histamine excites cholinergic pedunculopontine neurons in the guinea-pig. We found that two NADPH-diaphorase-positive LDT cells which hyperpolarized in the presence of NA responded to histamine with a rapid depolarization (4.7 and 8.4 mV), a slight increase in input resistance (13 and 43 M Ω), and a large increase in excitatory synaptic activity and/or action

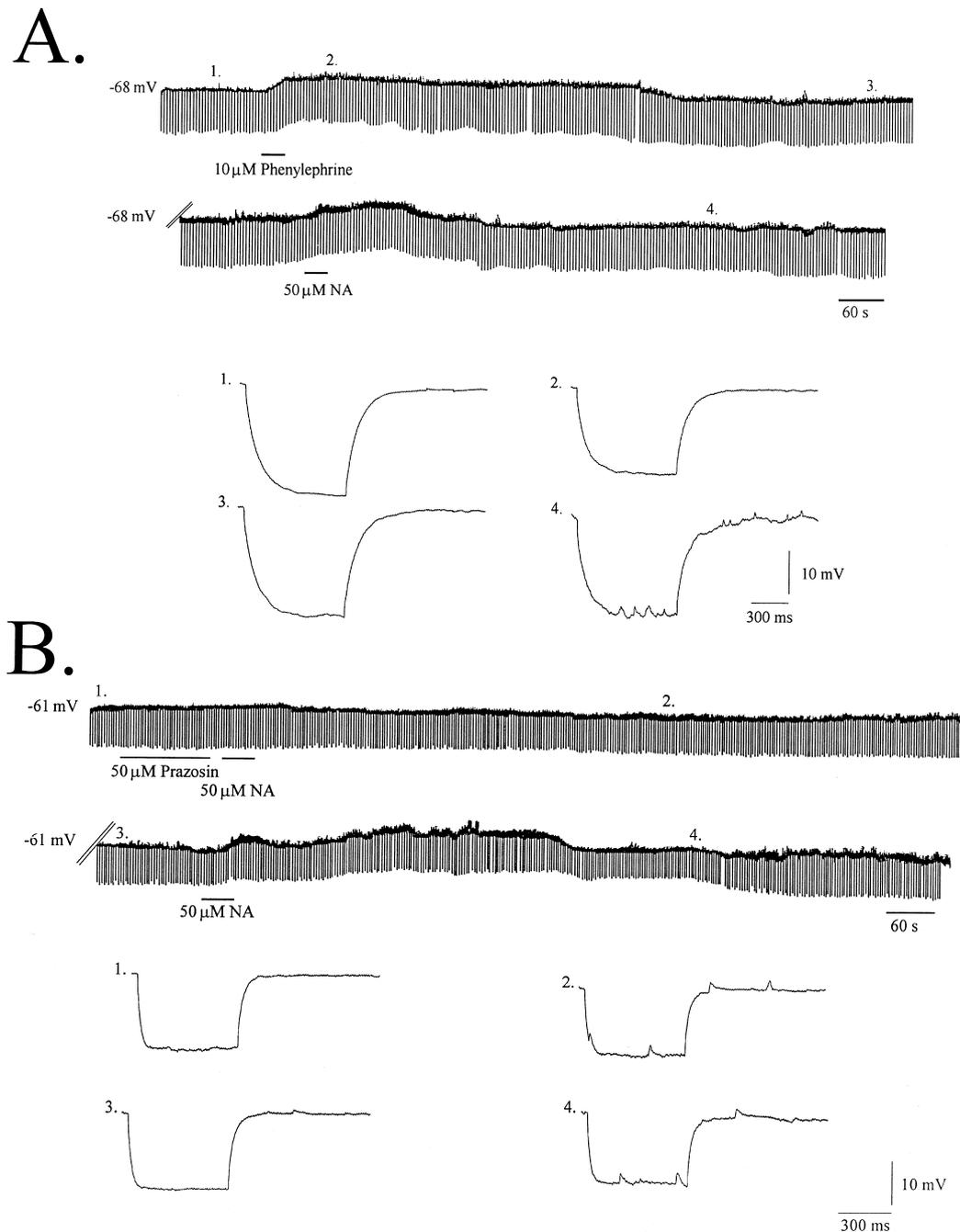


Fig. 5. Application of α -adrenergic agonists elicited membrane depolarization without an increase in excitatory synaptic activity. α -Adrenergic antagonists blocked the elicitation of depolarization of the membrane by NA. Bridge mode experiments on a neuron in the LDT examining the adrenergic receptor responsible for the depolarization of the membrane potential and the increase in excitatory synaptic activity. Top traces are the chart recordings of the membrane potential of the cells. Bottom traces are individual traces of a single voltage deflection in response to a hyperpolarizing current pulse prior to (1), immediately following (2) and, in the case of agonists, several minutes following (3) application of α -adrenergic agonists and antagonists.

potentials, which persisted for 20–30 min following drug application; these effects persisted in the presence of TTX.

The LDT receives a dense serotonergic input, and serotonin has been shown to have profound effects on behavioral state (see Discussion). Therefore, we tested whether this neurotransmitter modifies the activity of non-cholinergic LDT cells which depolarized in response to NA. In 12 of 13 non-cholinergic LDT cells, serotonin induced depolarization (4.3 ± 0.1 mV) and a decrease in input resistance (144.5 ± 6.4 M Ω) (Fig. 7); these effects persisted in the presence of TTX ($n=4$). EPSPs were not induced by

serotonin in these cells. Congruent with the results of Leubke *et al.*²⁹ and Leonard and Llinás,²⁸ in five of 15 NADPH-diaphorase-positive cells, serotonin and NA induced hyperpolarization of the membrane potential.

DISCUSSION

In the present study, non-cholinergic cells of the LDT exhibited changes in electrophysiological activity upon application of neurotransmitters hypothesized to play a major role in the control of behavioral state. Additionally, this study

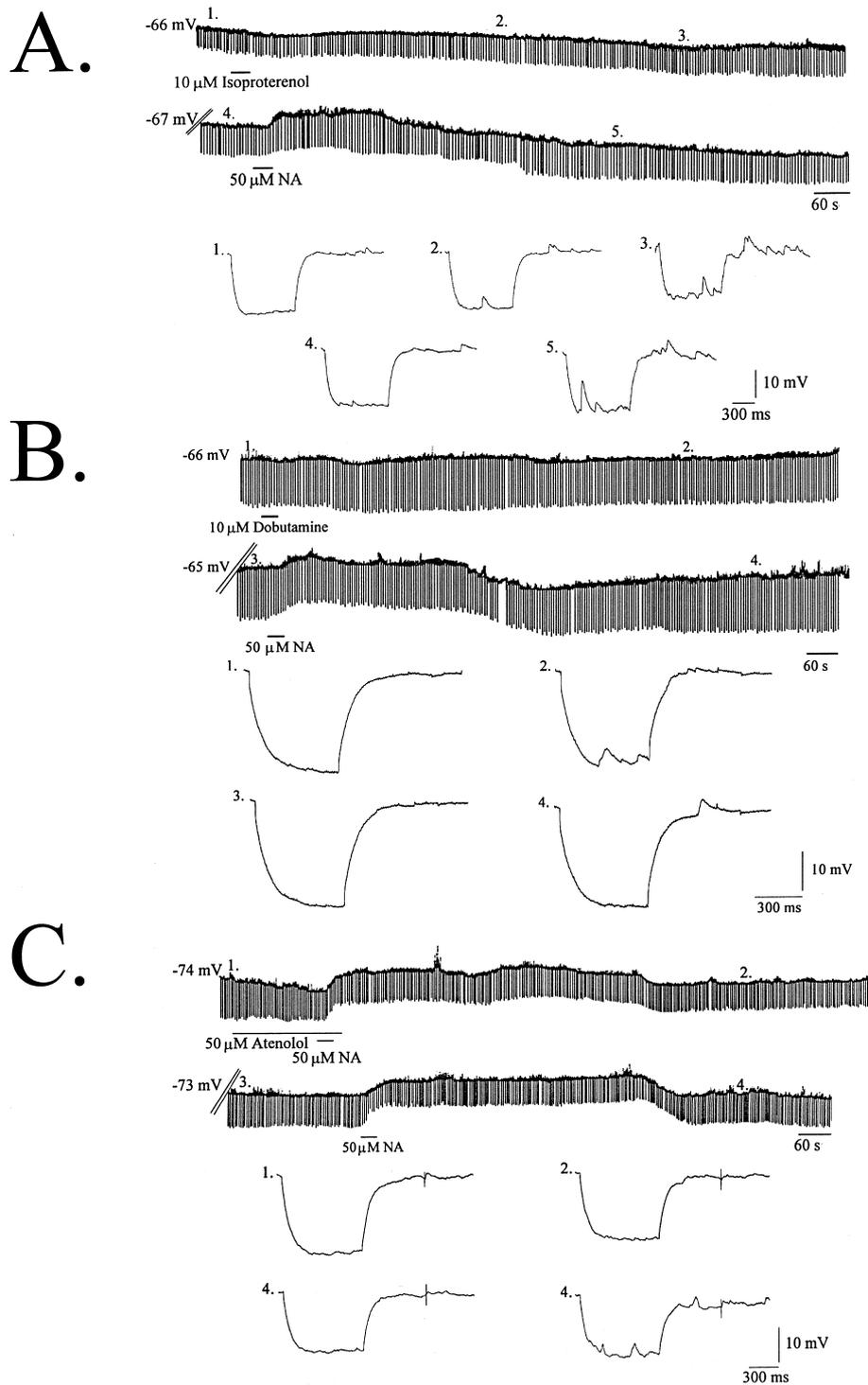


Fig. 6. β -Adrenergic agonists elicited an increase in EPSPs without a depolarization of the membrane potential and β -adrenergic antagonists prevented the elicitation of EPSPs by NA. Bridge mode experiments on a neuron in the LDT. Top traces are the chart recordings of the membrane potential of the cells. Bottom traces are individual traces of a single voltage deflection in response to a hyperpolarizing current pulse prior to (1), immediately following (2) and, in the case of agonists, several minutes following (3) application of drugs.

documents a novel response to NA that is long lasting and, to the best of our knowledge, has not been reported previously. Our working hypothesis is that these non-cholinergic LDT neurons may provide state-dependent inhibition of cholinergic LDT cells.

In recent years, significant advances have been made in the elucidation of the neuronal mechanisms which generate and

maintain the state of REM sleep. Numerous lines of evidence suggest that cholinergic excitation of neurons in the dorsal mPRF triggers REM sleep: (i) a REM-like state can be generated by the injection of cholinergic agents into the mPRF;^{2,3,57} (ii) microdialysis studies have demonstrated that ACh is naturally released in the mPRF during REM sleep²³ and during cholinergically induced REM sleep;³¹ (iii) some neurons in

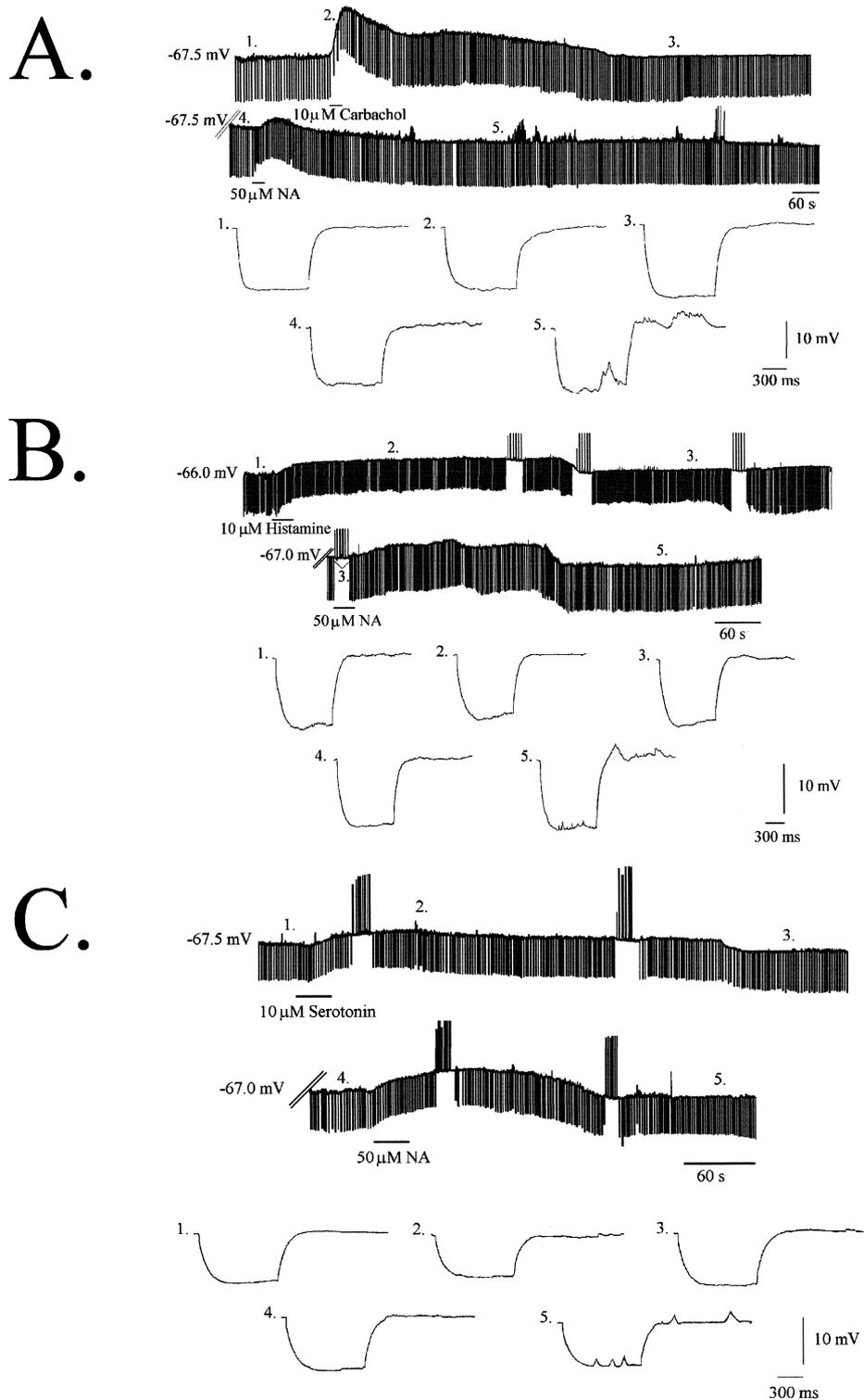


Fig. 7. Cholinergic, histamine and serotonin agonists induced a membrane depolarization in non-cholinergic LDT cells. Although all these neurotransmitter agonists induced membrane depolarization, excitatory synaptic activity was not elicited. Bridge mode experiments examining the effects of carbachol, histamine and serotonin on NADPH-negative neurons in the LDT which were found to subsequently depolarize and demonstrate EPSP activity upon NA administration. Top traces (in A–C) are the chart recordings of the membrane potential. Note that the response to carbachol was composed of a rapid depolarization followed by a longer-lived depolarization and that recovery from histamine was long lasting. Bottom traces (in A–C) are individual traces of a single voltage deflection in response to a hyperpolarizing current pulse prior to (1) and immediately following (2) application of carbachol, histamine and serotonin, and before (3) and after (4) NA.

the mPRF show state-dependent increases in firing rates during REM;⁴⁹ (iv) application of cholinergic agonists depolarizes some mPRF neurons.⁸

Neurons of the LDT, a region of the pons which is just rostral to the mPRF, are suspected to play a role in the control of behavioral state. (1) LDT neurons are the major site of

cholinergic neurons in the brainstem.⁴⁰ (2) Cholinergic LDT neurons send projections caudally to the mPRF and rostrally to the lateral geniculate nucleus (LGN).^{14,33} (3) Phenotypically unidentified neurons in this region demonstrate state-dependent firing increases during REM sleep.⁶ (4) Some neurons in this region exhibit burst firing profiles just prior to recordings of ponto-geniculo-occipital (PGO) waves in the lateral LGN.^{50,51} PGO waves, which are biphasic electrical events which appear spontaneously just prior to, and continue during, REM sleep, are generated in the LGN upon REM sleep-related activity of cholinergic LDT cells, and are one of the hallmarks of REM sleep utilized to polygraphically define the state.

Based on these data, one hypothesis regarding the mechanisms involved in the generation of REM sleep is that cholinergic neurons of the LDT are excited (or released from inhibition) and this excitation (or disinhibition) results in release of ACh into the mPRF, activating circuitry involved in the generation of the physiological signs of REM. However, the mechanisms which regulate the cholinergic neurons of the LDT still remain to be fully elucidated. For example, while state-dependent activity of LDT cells has been documented, it is still unclear if the activity of cholinergic LDT cells varies between different behavioral states.^{56,58} One logical focus regarding the control of cholinergic LDT cells is on the activity of other neuronal groups that exhibit state-dependent firing and send projections to the LDT.

State-dependent firing of noradrenergic neurons of the LC, serotonergic neurons of the dorsal raphe nucleus and histaminergic neurons of the tuberomammillary hypothalamic nucleus has been demonstrated, and neurons from these monoaminergic nuclei send projections to the LDT (see below for details). Noradrenergic, serotonergic and histaminergic neurons show their highest frequency of firing during wakefulness, decreased firing during slow-wave sleep and their lowest firing during REM sleep.^{1,32,53} It has been demonstrated that serotonin and NA inhibit identified cholinergic neurons of the LDT,^{28,29,55} and NA presynaptically inhibits ACh release in the mPRF by actions on cholinergic terminals.⁴⁸ Monoaminergic inhibition of cholinergic LDT cells has been suggested to play a principle role in the suppression of REM sleep in waking, in which noradrenergic and serotonergic neuronal firing is highest. Another source of inhibition of cholinergic LDT cells may arise from the cholinergic neurons themselves. There is evidence that the majority of cholinergic LDT cells are projection neurons,²⁵ and it has been demonstrated that many reciprocal cholinergic connections between contra- and ipsilateral LDT nuclei exist,⁴⁷ and interestingly, it has been reported that muscarinic agonists inhibit identified cholinergic LDT cells.^{28,30} Taken together, these data suggest that inhibition of cholinergic LDT cells may arise from cholinergic activity in the contralateral LDT; however, at variance with this conclusion are the findings that cholinergic LDT neurons do not appear to be the primary target of cholinergic synaptic input.¹⁰

We have shown in the present report that the application of NA results in a directly mediated membrane depolarization and an indirectly mediated increase in EPSP activity in a subpopulation of non-cholinergic LDT neurons. We found that membrane depolarization was mediated by α_1 -adrenergic receptors and increases in EPSP activity by β -adrenergic receptors. Autoradiography studies of adrenergic receptors in the rat brain have demonstrated that β -adrenergic receptors

exist within the pontine reticular formation; however, the precise position within the pons is difficult to determine from these reports.^{34,37} α_1 -Adrenoceptor binding site studies have demonstrated high binding levels in the pontine reticular formation, particularly in the peribrachial region of the rat, a region which encompasses the LDT.¹⁸ The increase in EPSPs was abolished not only by the application of TTX, but also by the presence of CNQX. These data suggest that the increase in EPSP activity is mediated by glutamatergic mechanisms, and that the cell bodies and axon projections of these glutamatergic neurons are intact and within the slice. Glutamatergic neurons have been localized in the rat and squirrel monkey LDT, although the efferent projections of these cells are not known, nor if these cells have local collaterals onto non-cholinergic LDT cells;^{4,27} however, a variety of glutamatergic neurons from many different regions have been shown to project to the LDT.^{27,47} Neurons in the mPRF, a region demonstrated to be critically involved in the mediation of behavioral state (see above), send projections to the LDT and this region contains numerous cells that were found to be immunoreactive for glutamate.²⁶ The brainstem slices from which we recorded the present data contained the contralateral LDT and rostral regions of the mPRF, and glutamatergic input to the LDT cells from which we recorded could have originated from any of these regions.

The increase in EPSP activity induced by NA in the present study was abolished by CNQX but not by AP5, suggesting that these EPSPs are mediated by AMPA and/or kainate receptors. AMPA receptors are composed of glutamate subunits 1, 3 and 4, which have been shown by immunohistochemistry to exist on NADPH-diaphorase-negative neurons of the LDT.¹² Additionally, we found that electrical stimulation of the ventral aspect of the LDT resulted in CNQX- and, to a lesser extent, AP5-sensitive synaptic potentials. These data further support the hypothesis that glutamatergic receptors exist on the neurons in the present study and that glutamatergic neurons project to these cells. Whether or not the NA- and stimulation-induced EPSPs arise from activity in the same glutamatergic population of neurons remains to be determined.

It has been demonstrated, in the rat, that extensive projections to cholinergic and non-cholinergic cells of the LDT arise from noradrenergic neurons in the LC.^{11,16,25,46} Given the state-dependent firing of noradrenergic neurons of the LC, it seems reasonable to assume that if the population of cells in the present report receive NA innervation then they might exhibit firing patterns in which they are most active during wakefulness and less so during slow-wave sleep, when LC firing is highest and slightly reduced, respectively. This pattern of activity would have a tremendous impact on the activity of cells upon which the present cells send inputs. However, it has not been demonstrated unequivocally that a subpopulation of cells exist within the LDT that exhibit such a firing profile (see below).

The effects on non-cholinergic LDT cells of other neurotransmitters believed to play a role in behavioral state were also examined in this study. Cholinergic input to neurons of the LDT has been demonstrated from the contralateral LDT;⁴⁷ it has been reported that cholinergic LDT cells make synaptic contacts mostly with non-cholinergic LDT neurons.¹⁰ Serotonergic fibers exist in the LDT,⁴⁷ and the primary targets of serotonergic terminals in the LDT appear to be non-cholinergic cells.⁹ Histaminergic fibers have also been demonstrated

in the LDT,³⁶ as well as histamine receptors.³⁵ In the present study, we found that carbachol, a cholinergic agonist, and serotonin directly depolarized the membrane potential and decreased the input resistance of non-cholinergic LDT cells, without altering the synaptic activity of these cells. Histamine depolarized both non-cholinergic and cholinergic LDT cells, and our findings thus collaborate those of Khateb *et al.*,²² who reported previously that histamine depolarized cholinergic pedunculopontine tegmentum neurons of the guinea-pig.

The non-cholinergic LDT cells which responded with depolarization to application of NA were smaller in size than cholinergic cells identified using NADPH-diaphorase histochemistry. Moreover, the processes of the recorded cells were intermingled with the somata and processes of cholinergic cells. GABA-containing cells, smaller and more numerous than cholinergic LDT neurons, have been reported in the rat LDT.^{7,15,24,52} Moreover, Ford *et al.*⁷ found glutamate decarboxylase-positive boutons surrounding the cell bodies and proximal dendrites of choline acetyltransferase-positive LDT cells. Data from a study by Honda and Semba¹⁰ suggested that the non-cholinergic LDT cells which received synaptic contact from cholinergic fibers could be LDT GABAergic cells, based on their small size and ultrastructure. Evidence for a GABAergic inhibitory influence on cholinergic LDT cells is provided by the findings of Sakai and Koyama,⁴⁵ who found that application of a GABA_A antagonist in the cat LDT caused excitation of putative cholinergic LDT cells. Taken together, these data suggest that GABAergic LDT cells could be local circuit inhibitory interneurons, projecting to and inhibiting cholinergic neurons, and we believe that these cells might represent those from which the present data were collected. Unfortunately, we were unable to directly test this hypothesis, as we found no GABA immunoreactivity in the LDT slice, despite extensive efforts (Kohlmeier and Reiner, unpublished observations), probably because colchicine pretreatment is impractical in the slice preparation, and available glutamate decarboxylase and GABA antibodies that do not require such pretreatment do not stain well in the LDT. Additionally, in a collaborative project, we were unable to demonstrate reliable co-localization of other proteins in glutamate decarboxylase- or GABA-positive LDT cells which could then be used as markers of GABA cells (Semba, Kohlmeier and Reiner, unpublished observations), as has been reported in other GABA neurons in the brain. These cells are unlikely to be glutamatergic or glycinergic, as Clements and Grant⁴ reported that glutamate-immunoreactive LDT cells were similar in size and morphology to cholinergic neurons, and Rampon *et al.*³⁸ verified previous reports in which glycine immunoreactivity was not found in the rat LDT.

The simplest prediction of these data would be that the firing of these cells would parallel that of aminergic neurons: firing highest during wakefulness and reduced during slow-wave sleep, and slowest during REM sleep. However, there is little or no experimental evidence for cells with such firing patterns in the LDT,^{5,6,20,21,41,43,44,50,51} and there are only two reports of cells (unidentified histochemically) within the LDT that are specifically wake-on/REM-off.^{21,42} Presumably, the multiplicity of inputs which neurons receive in the intact brain accounts for these differences between the predictions of *in vitro* experiments and the reality of the situation *in vivo*.

An alternative hypothesis is that these non-cholinergic LDT neurons represent the so-called PGO-off neurons reported by Steriade *et al.*⁵⁰ These cells increased firing in REM as compared to wakefulness and slow-wave sleep, and ceased firing 50–180 ms prior to recordings of PGO waves in the LGN. If these PGO-off neurons are GABAergic (as suggested by these authors), the arrest of their firing prior to and during PGO waves could disinhibit adjacent PGO-on, presumably cholinergic neurons, allowing burst firing of the cholinergic LDT cells.

Irrespective of the *in vivo* behavior of non-cholinergic LDT cells, our data suggest that NA may be acting to modulate firing of cholinergic LDT cells by two mechanisms. It has been shown that NA directly inhibits cholinergic neurons of the LDT.⁵⁵ The present study shows that NA directly excites non-cholinergic neurons of the LDT, and we hypothesize that this results in an indirect inhibition of cholinergic LDT cells. Such concerted inhibition of cholinergic neurons is likely to be of physiological significance.

CONCLUSION

To summarize, we report data on a population of non-cholinergic LDT cells. These cells exhibited a novel response to the application of NA. Additionally, these cells depolarized with the application of a cholinergic agonist, histamine and serotonin. The response of these cells to neurotransmitters implicated in behavioral state control and their localization in a region of the pontine reticular formation known to have a pivotal role in the control of behavioral state suggest that these cells might also be involved in the mediation of behavioral state or perhaps on activity of neuronal groups implicated in generating the physiological signs of different behavioral states.

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