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Cholinergic Regulation of the Posterior Medial Thalamic Nucleus

Radi Masri*, Jason C. Trageser*, Tatiana Bezdudnaya, Ying Li, and Asaf Keller

Program in Neuroscience and Department of Anatomy and Neurobiology, University of Maryland School of Medicine, Baltimore, Maryland

Abstract

We previously showed that the GABAergic nucleus zona incerta (ZI) suppresses vibrissae-evoked responses in the posterior medial (POm) thalamus of the rodent somatosensory system. We proposed that this inhibitory incertothalamic pathway regulates POm responses during different behavioral states. Here we tested the hypothesis that this pathway is modulated by the ascending brain stem cholinergic system, which regulates sleep–wake cycles and states of vigilance. We demonstrate that cholinergic inputs facilitate POm responses to vibrissae stimulation. Activation of the cholinergic system by stimulation of brain stem cholinergic nuclei (laterodorsal tegmental and the pedunculopontine tegmental) or by tail pinch significantly increased the magnitude of POm responses to vibrissae stimulation. Microiontophoresis of the muscarinic receptor agonist carbachol enhanced POm responses to vibrissae stimulation. Application of carbachol to an in vitro slice preparation reduced the frequency but not the amplitude of miniature inhibitory postsynaptic currents, indicating a presynaptic site of action for carbachol. We conclude that the cholinergic system facilitates POm responses by suppressing GABAergic inputs from ZI. We propose the state-dependent gating hypothesis, which asserts that differing behavioral states, regulated by the brain stem cholinergic system, modulate the flow of information through POm.

INTRODUCTION

Thalamic nuclei can be characterized by the type of information they relay to the neocortex (reviewed in Sherman and Guillery 2005). Nuclei concerned with transmitting information from the periphery are referred to as “first-order” nuclei, which include the ventral posterior medial (VPM) nucleus in the somatosensory system and the lateral geniculate nucleus (LGN) in the visual system. These nuclei, whose receptive fields are determined by ascending inputs from the periphery, respond robustly and reliably to peripheral stimulation. In contrast are “higher-order” nuclei, such as the posterior medial (POm) nucleus in the somatosensory thalamus and the pulvinar nucleus in the visual system. These nuclei are hypothesized to transmit information from one cortical area to another, as their receptive fields are determined by descending cortical inputs (Sherman and Guillery 2001). In anesthetized rats POm neurons respond poorly to vibrissae stimulation, displaying labile responses that are abolished after cortical inactivation (Diamond et al. 1992a; Lavallée et al. 2005; Sosnik et al. 2001; Trageser and Keller 2004). These data support the classification of POm as a higher-order nucleus.

We previously demonstrated that POm neurons are regulated by feedforward inhibition from the GABAergic nucleus zona incerta (ZI): When ZI activity is suppressed, POm neurons respond robustly and at short latencies (Trageser and Keller 2004). The responses revealed by

Address for reprint requests and other correspondence: A. Keller, Department of Anatomy and Neurobiology, University of Maryland School of Medicine, 20 Penn Street, Baltimore, MD 21201 (E-mail: akeller@umaryland.edu)..

*R. Masri and J. C. Trageser contributed equally to this work.

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suppressing ZI are determined by direct inputs from the trigeminal nuclei because they are resistant to cortical inactivation (Lavallée et al. 2005). Thus ZI regulates POM responses, determining whether POM functions as a first-order or a higher-order nucleus.

We recently demonstrated that both spontaneous and vibrissae-evoked activity of ZI neurons are suppressed by cholinergic inputs from the brain stem reticular activating system (Trageser et al. 2006). Because these cholinergic inputs are responsible for transitions between behavioral states, such as sleep and alertness (Steriade 2003), we suggested a state-dependent gating hypothesis, where differing behavioral states—regulated by the brain stem cholinergic system—modulate ZI activity, thereby modulating the response properties of POM neurons.

A prediction of this hypothesis is that the cholinergic reticular activating system regulates the responses of POM neurons by suppressing the incertothalamic inhibitory inputs. Our aim in this study was to test this prediction. Some of the findings reported here were previously presented in abstract form (Masri et al. 2005).

METHODS

We used 62 female Sprague–Dawley rats weighing 250 to 350 g for in vivo recordings. Anesthetized rats were placed in a stereotaxic device for the duration of the experiments. All incision sites were infused with local anesthetics. We maintained body temperature at 37°C with a servo-controlled heating blanket. All procedures adhered strictly to institutional and federal guidelines.

Halothane anesthesia (15 rats)

We anesthetized rats with halothane (3%) administered through a nosepiece. The trachea was then cannulated and halothane was administered through a tracheal tube. We monitored electroencephalographs (ECoGs) to assess the stage of anesthesia and maintained the rats at stage III/3–4 (Friedberg et al. 1999).

Fentanyl analgesia (5 rats)

We anesthetized the animals initially with halothane (3%) and inserted a venous catheter in the jugular vein for drug delivery and a second catheter in the femoral artery for monitoring blood pressure and heart rate. After the insertion of catheters, we discontinued the administration of halothane and infused the rats intravenously with fentanyl ($10 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$) for the rest of the experiment. We immobilized the rats with pancuronium bromide ($1.5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$) and they were then artificially respired with a positive-pressure respirator at 90 breaths/min. We monitored blood pressure, heart rate, and ECoGs throughout the experiment to ensure that the animal was in no pain or distress.

Urethan anesthesia (42 rats)

Rats received an intraperitoneal injection of urethan (1.5 g/kg). We monitored ECoGs to assess the stage of anesthesia and maintained the rats at stage III/3–4 (Friedberg et al. 1999). We administered supplementary injections (150 mg/kg) as needed.

In vivo POM extracellular recording

We obtained extracellular unit recordings with quartz-insulated platinum electrodes (2 to 4 M Ω) from POM neurons. We advanced electrodes in the right hemisphere based on stereotaxic coordinates (AP 3.2, ML 2.6, relative to Bregma; Paxinos and Watson et al. 1998). We digitized waveforms (40 kHz) recorded from well-isolated units through a Plexon (Dallas, TX) data acquisition system and sorted units off-line with Plexon's Off-line Sorter, using dual thresholds

and principal component analyses. We generated autocorrelograms with Neuroexplorer software (Littleton, MA) to confirm that we obtained recordings from single units.

We marked recording sites with electrolytic lesions (5 μ A for 10 s) at the end of the experiment and then deeply anesthetized the rats with sodium pentobarbital (60 mg/kg) and perfused them transcardially with buffered saline followed by 4% buffered paraformaldehyde. We obtained coronal brain sections (70 μ m thick) and Nissl-stained the sections to identify recording and stimulation sites.

LDT–PPT stimulation

We targeted a concentric bipolar stimulating electrode (250 μ m diameter; FHC, Bowdoin, ME) to the laterodorsal tegmentum (LDT) and the pedunculopontine tegmentum (PPT) nuclei, based on stereotaxic coordinates (AP 8 –9.0, ML 0.5–1.5, 5– 6.0 mm deep). Electrical stimulation (200 μ A) consisted of 200- μ s pulses delivered at 100 Hz for 1 s.

Tail pinch

We pinched the midpoint region of the tail using a plastic clamp (Samco Silicone Products, Warwickshire, UK). We recorded individual POM neuronal responses to vibrissae stimulation and changes in ECoGs before, during, and after applying the tail pinch.

Vibrissae stimulation

After isolation of vibrissae-sensitive neurons in POM, we stimulated vibrissae with air puffs delivered through a tube (0.5-mm diameter) by a computer-controlled Picospritzer (General Valve, Fair-field, NJ). We delivered air puffs at 0.5, 2, 5, 8, or 11 Hz with a pressure of 60 psi, resulting in vibrissae deflections of about 30°. We deflected vibrissae in their preferred direction, i.e., the direction that elicited the shortest-latency, highest-magnitude response. We simultaneously deflected at least four vibrissae to evoke responses in POM neurons.

Carbachol iontophoresis

We microiontophoretically applied carbachol to individual POM neurons through a multibarrel pipette attached to a carbon fiber used for single-unit recordings (1 to 3 M Ω , Carbostar, Kation Scientific, Minneapolis, MN). Barrels were filled with carbachol (100 μ M in saline) and 4% pontamine sky blue, and a retaining current (–10 to –12 nA) was applied through a current generator (Model 6400A, Dagan, Minneapolis, MN). After isolating a vibrissae-sensitive neuron, we stimulated the vibrissae with air puffs (50-ms duration) delivered through a tube (0.5-mm diameter) and a computer-controlled Picospritzer. We recorded neuronal responses to 0.5-Hz vibrissae stimulation for 3 min and then applied +20 to +50 nA of current for 3 min to eject carbachol, while applying a balancing current in another barrel filled with saline.

At the end of the experiment we marked the recording sites by ejecting pontamine sky blue from the pipette by applying current (–20 μ A) for 20 min. We then deeply anesthetized the animals with sodium pentobarbital (60 mg/kg) and perfused them transcardially with buffered saline followed by 4% buffered paraformaldehyde. We obtained coronal brain sections (80 μ m thick) and stained them with neutral red to identify recording sites.

Data analysis

We isolated single units off-line with Off-line Sorter, as described above. We exported time stamps of well-isolated units and of stimulus triggers to Matlab (The MathWorks, Natick, MA) for analyses using custom-written algorithms. We constructed peristimulus time histograms (PSTHs, 1-ms bins) and defined significant stimulus-evoked responses as PSTH bins whose

response magnitude significantly exceeded (99% confidence interval) spontaneous activity levels, computed from a 200-ms period preceding the stimuli.

We defined response onset as the first two consecutive bins (post-stimulus) displaying significant responses (defined as above) and defined response offset as three consecutive bins in which response magnitude fell below the 99% confidence interval. We defined response magnitude as the total number of spikes per stimulus occurring between response onset and offset. We performed statistical analyses in SPSS (SPSS, Chicago, IL) and assessed, in individual neurons, changes occurring in response magnitude and spontaneous activity using the Student's *t*-test or the nonparametric Wilcoxon signed-ranks test; $P < 0.05$ was considered significant.

In vitro POM recordings

We anesthetized 23 Sprague–Dawley rats, 12 to 27 days old, with ketamine (30 mg/kg); removed the brains; and prepared 400- μ m-thick coronal slices. Next, we obtained whole cell patch-clamp recordings with an EPC10 amplifier (HEKA Elektronik, Lambrecht, Germany), digitized at 20 kHz using Pulse software (HEKA), and stored on a personal computer. The impedances of the patch electrodes were 3 to 5 M Ω . We recorded action-potential insensitive, miniature inhibitory postsynaptic currents (mIPSCs), by using a high-chloride pipette solution containing (in mM) 100 K-gluconate, 60 CsCl, 10 HEPES, 1 MgCl₂, 2 MgATP, 0.3 Tris-GTP, 1 BAPTA, and 5 biocytin (pH adjusted to 7.3). The extracellular solution contained, in mM, 124 NaCl, 3 KCl, 25 NaHCO₃, 2 CaCl₂, 1.3 MgSO₄, 5 BES, and 15 glucose. We obtained the following agents from RBI-Sigma (Natick, MA) and bath-applied them to the perfusate: carbachol (2 μ M), D-2-amino-5-phosphopentanoic acid (AP5, 50 μ M), tetrodotoxin (TTX, 1 μ M), and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20 μ M).

We analyzed recorded waveforms off-line with MiniAnalysis software (Synaptosoft, Decatur, GA). To calculate resting membrane potential (V_m) and input resistance (R_{in}) we applied a series of voltage commands and plotted the current–voltage relationships (*I*–*V* curve). We estimated R_{in} from the slope of the *I*–*V* curve and defined the V_m as the *I*–*V* curve's intersection with the abscissa. We analyzed the kinetics of mIPSCs by averaging the waveforms and fitting the decay (10–90%) with a single exponential.

Statistical comparisons were performed with unpaired or paired (for the same cell) Student's *t*-test or the nonparametric Kolmogorov–Smirnov statistic (K-S test); $P < 0.05$ was considered significant. We present data as means $\pm$ SE.

We filled cells with biocytin through the recording pipette and fixed slices overnight in a buffered solution containing 4% paraformaldehyde. To visualize cells and to confirm that all recorded cells were in POM we reacted sections with the ABC *Elite* kit (1:1,000; Vector Labs, Burlingame, CA) and 3,3'-diaminobenzidine (DAB; 0.5 mg/ml), urea H₂O₂ (0.3 mg/ml), and CoCl₂ (0.2 mg/ml) in 0.05 M Tris buffer containing 0.5 M NaCl.

RESULTS

POM responses to vibrissae stimuli are labile

To allow comparisons with previous studies that used a variety of anesthetics, we characterized the responses of POM neurons under the following conditions: fentanyl analgesia ($n = 21$ neurons), urethane anesthesia ($n = 53$), or halothane anesthesia ($n = 22$). In total, we recorded responses from 96 well-isolated POM neurons to vibrissae stimuli delivered at 0.5, 2, 5, 8, or 11 Hz (see METHODS). POM neurons displayed labile, long-latency responses to repetitive vibrissae deflections, with increasing failure rates as stimulation frequency increased. Figure 1A depicts a representative example of a neuron recorded under fentanyl analgesia. This neuron

displayed weak, long-latency responses (33 ms) to repetitive vibrissae deflections. Response magnitude decreased as stimulation frequency was increased. Figure 1B depicts similar responses recorded from a POM neuron under urethan anesthesia. In this cell response magnitude was larger and response latency was shorter, compared with the neuron depicted in Fig. 1A; however, we found no significant differences ($P > 0.05$) in the response magnitude, spontaneous activity rates, or response latency between the population of neurons recorded under the different anesthetics.

POM neurons responded with low probability and 75% of the neurons produced no significant responses to vibrissae stimulation at 11 Hz. Further, the responses were highly variable, with onset latencies ranging from 5 to 69 ms; coefficient of variation (CV): 0.5 Hz = 0.58; 2 Hz = 0.59; 5 Hz = 0.49; 8 Hz = 0.82; 11 Hz = 0.82. Thus consistent with previous reports (Diamond et al. 1992b; Lavallée et al. 2005; Sosnik et al. 2001; Trageser and Keller 2004), POM responses recorded under a variety of anesthetics are highly labile.

LDT–PPT stimulation gates POM

Brain stem cholinergic neurons in the laterodorsal tegmental nucleus (LDT) and the pedunculo pontine tegmental nucleus (PPT) are causally responsible for transitions in sleep–wake cycles (Steriade 2003). During sleep or anesthetized states, cortical EEGs show high-amplitude, slow oscillations, whereas during arousal and activated states, high-frequency oscillations are evident (Moruzzi and Magoun 1949; Steriade and Timofeev 2003). These transitions in EEG activity can be mimicked by stimulating the LDT–PPT complex in anesthetized animals (Castro-Alamancos and Oldford 2002; Steriade 2003).

To test the hypothesis that the cholinergic reticular activating system gates POM responses, we stimulated LDT–PPT (Fig. 2F) while recording from POM neurons in response to repetitive vibrissae stimulation at 2, 5, 8, or 11 Hz. We performed these experiments under urethan because urethan induces distinct, high-amplitude, low-frequency cortical oscillations similar to those observed during slow-wave sleep. These oscillations are evident in the cortical ECoG (Fig. 2, “Control”). After LDT–PPT stimulation these high-amplitude oscillations are replaced with low-amplitude, high-frequency oscillations lasting about 7 s (Fig. 2A, “Test”). Thus consistent with previous reports, LDT–PPT stimulation mimics the transitions in ECoG recordings observed during sleep–wake cycles (Castro-Alamancos and Oldford 2002; Steriade 2003).

We tested the effects of LDT–PPT stimulation on 19 POM neurons. Four of these neurons failed to generate statistically significant responses to vibrissae deflections delivered at 2 Hz, either before, during, or after LDT–PPT stimulation. These four neurons, recorded from three different animals, were located in close proximity to other POM neurons that did produce significant responses. Figure 2B depicts PSTHs constructed from a representative neuron tested at each frequency. Under control conditions, this neuron responded poorly to vibrissae stimulation at all frequencies. In contrast, after LDT–PPT stimulation, the neuron responded robustly and with high probability to vibrissae stimulation, with a fourfold increase in response probability across frequencies (Fig. 2B, “Test”).

Figure 2C depicts population PSTHs, constructed from data recorded from 15 POM neurons. Under control conditions, POM responses to vibrissae stimuli were variable and labile; two of these neurons failed to produce significant responses to 2-Hz stimulation and two others failed to produce significant responses to 11-Hz stimuli. After LDT–PPT stimulation the population response probability increased (Fig. 2C) and, as a result, the variability in response latency decreased, especially in response to stimulation at higher frequencies (Fig. 2D) [e.g., CVs: 8 Hz = 0.45 (54% decrease); 11 Hz = 0.46 (46% decrease)]. Furthermore, the neurons that failed

to respond to vibrissae stimulation at 2 and 11 Hz under control conditions responded robustly to these stimuli after LDT–PPT stimulation.

After LDT–PPT stimulation response magnitudes for all frequencies also significantly increased ($P \leq 0.001$; Wilcoxon test; spikes per stimulus pre/post values: 2 Hz, $0.24 \pm 0.39/0.42 \pm 0.53$; 5 Hz, $0.09 \pm 0.09/0.19 \pm 0.16$; 8 Hz, $0.01 \pm 0.01/0.13 \pm 0.090$; 11 Hz, $0.001 \pm 0.001/0.014 \pm 0.005$). Figure 2E depicts the effects of LDT–PPT stimulation on response magnitudes of individual neurons. LDT–PPT stimulation did not significantly affect response duration ($P > 0.05$) or spontaneous activity rates ($P > 0.05$).

An additional method for activating the brain stem cholinergic system is the tail pinch (Kayama et al. 1991). Similar to electrical stimulation, tail pinch resulted in a transition in the cortical ECoG from high-amplitude, low-frequency oscillations, to low-amplitude, high-frequency oscillations (Fig. 3A). Figure 3B depicts PSTHs constructed from responses of a representative POM neuron. Before the tail pinch, the neuron displayed labile, low-probability responses to vibrissae stimulation. However, after the tail pinch, the neuron responded to vibrissae stimulation (at 2 Hz) with increased response probability and increased response magnitude (pre/post values: $0.40/0.85$ spikes per stimulus). We obtained similar responses from 19 additional neurons (pre/post: $0.19 \pm 0.25/0.48 \pm 0.39$ spikes per stimulus; $P < 10^{-4}$). Figure 3C depicts the effects of tail pinch on response magnitudes of individual neurons.

These findings suggest that activating the cholinergic brain stem system, either by LDT–PPT stimulation or by tail pinch, enhances the flow of information through POM, allowing neurons with weak responses to respond more robustly and reliably to vibrissae stimulation.

Carbachol increases the response magnitude of POM cells

Stimulating the reticular activating system evokes acetylcholine (ACh) release in widespread brain structures, including various dorsal and ventral thalamic nuclei and the cerebral cortex (reviewed in Steriade 2003). To test whether cholinergic activity directly affects neuronal activity in POM we used microiontophoresis of the muscarinic receptor agonist carbachol onto individual, well-isolated POM neurons. We recorded from 21 neurons that responded to stimulation of the vibrissae with air puffs delivered at 0.5 Hz. Fifteen of the neurons were recorded under urethan (nine rats) and the remaining six under halothane (three rats).

Figure 4A depicts a representative plot of PSTH versus time constructed from a POM neuron recorded before, during, and after carbachol application. Carbachol application (3 min) produced a significant ($P = 0.003$) increase in response magnitude (from 0.36 to 0.65 spikes/stimulus, a 79% increase). This increase was completely reversible, with responses returning to predrug magnitudes 8 min after carbachol application was terminated. Similar significant increases in the magnitude of vibrissae-evoked responses occurred in 12 of 21 neurons (57%), with increases averaging 83% ($\pm 60\%$). The magnitude of the evoked responses was reduced in one neuron and was not affected significantly in the remaining eight neurons (Fig. 4B).

In some POM neurons carbachol also significantly increased spontaneous firing rates. In the example depicted in Fig. 4A the neuron had an average spontaneous firing rate of 0.3 Hz, a value that was significantly ($P < 10^{-4}$) increased (to 1.5 Hz) during carbachol application. Similar significant increases in spontaneous firing occurred in 10 of 21 neurons (48%), with facilitation averaging 255% ($\pm 223\%$). Spontaneous firing was reduced in two other neurons and was not significantly affected in the remaining cells. Increases in spontaneous firing rates were always accompanied by increases in the magnitude of evoked responses. We found no correlation ($P > 0.48$; $r = 0.16$, Pearson product) between carbachol's effects and any of the following metrics: precarbachol spontaneous firing rate, precarbachol response magnitude, or

response onset latency. Carbachol-sensitive and -insensitive neurons were intermingled throughout POM.

These findings demonstrate that local application of a cholinergic agonist significantly enhances both the spontaneous and sensory-evoked activity in a significant population of POM neurons.

Presynaptic regulation of GABA release in POM

GABAergic terminals from ZI that target POM neurons express a high density of the presynaptic muscarinic m2 receptors (Bartho et al. 2002), receptors that presynaptically regulate γ -aminobutyric acid (GABA) release in a variety of CNS pathways (Baba et al. 1998; Barnabi and Cechetto 2001; Lupica et al. 1992). We therefore hypothesized that ACh acts presynaptically to suppress GABA release onto POM neurons.

To test this hypothesis we recorded, in vitro, mIPSCs in voltage-clamp mode (-65 mV) in the presence of TTX (1 μ M), AP5 (50 μ M), and CNQX (20 μ M). Gabazine (GABA_A receptor antagonist; 10 μ M) completely suppressed all mIPSCs, indicating that they were mediated by GABA_A receptors. Processes that affect the frequency but not the amplitude of mIPSCs involve presynaptic but not postsynaptic mechanisms (Scanziani et al. 1995). Therefore if carbachol reduces mIPSC frequencies, but not their amplitude, this would support a presynaptic mechanism of action.

Figure 5A depicts a representative sample of mIPSCs recorded in control conditions (*left traces*) and during application of carbachol (2 μ M; *right traces*). Because of the high-chloride content of the recording pipettes, mIPSCs are evoking inward currents. The cumulative probability plots in Fig. 5B depict the effects of carbachol on the amplitudes and frequencies of mIPSCs recorded from this neuron. There was no significant change in mIPSC amplitudes after carbachol application (K-S test, $P = 0.68$). By contrast, carbachol resulted in a rightward shift in the cumulative distribution of interevent intervals, indicating a significant decrease in mIPSC frequencies (Fig. 5B, $P < 10^{-4}$, K-S test).

The frequency of mIPSCs was significantly ($P = 10^{-4}$, K-S test) suppressed in 25 of 33 neurons (76%). On average, mIPSC frequencies were reduced from 0.26 Hz (± 0.23) to 0.09 Hz (± 0.07), a decrease of $236 \pm 385\%$ ($P = 0.007$). In most of these neurons (14/25; 56%), there were no significant changes in the amplitudes of mIPSCs, suggesting that carbachol acted only presynaptically to suppress GABA release (Fig. 5C). In the remaining 11 cells (44%), carbachol also significantly ($P = 10^{-4}$, K-S test) suppressed the amplitudes of mIPSCs. On average, amplitudes decreased from 17.9 ± 4.8 to 16.1 ± 4.8 pA, a decrease of $10.0 \pm 8.4\%$ ($P = 0.01$, t -test). This suggests that, in these 11 neurons, carbachol may have had both presynaptic and postsynaptic effects.

To further test for carbachol's site of action, we compared, in the same group of neurons, the kinetics of mIPSCs. We reasoned that a purely presynaptic effect would have no effect on their decay time constants. Figure 5D depicts averaged, scaled, and superimposed mIPSCs recorded from a single neuron before and during carbachol application. In this example, and in all cells tested, carbachol had no significant effects on mIPSC kinetics ($P = 0.4$). On average, decay time constants were $\tau_{\text{control}} = 14.2 \pm 6.7$ and $\tau_{\text{carbachol}} = 15.7 \pm 7.1$, values that were indistinguishable ($P = 0.12$). Furthermore, carbachol application did not significantly affect the estimated resting membrane potentials ($P = 0.21$; $\Delta V_m = 0.7 \pm 2.4$ mV) or estimated input resistances ($P = 0.08$; $\Delta R_{\text{in}} = 13.8 \pm 28.5$ M Ω). These findings suggest that carbachol had no discernible effect on postsynaptic properties.

DISCUSSION

Our goal was to test the hypothesis that the cholinergic reticular activating system regulates the responses of POM neurons by acting on incertothalamic inhibitory inputs. We attempted to falsify this hypothesis by testing the validity of several predictions; all results were consistent with our underlying hypothesis.

Direct stimulation of the reticular activating system resulted in significant increases in the magnitude of POM responses to vibrissae inputs. We obtained similar results by activating this cholinergic system with a tail pinch. We recognize that LDT–PPT stimulation may inadvertently activate fibers of passage and nearby nuclei. Further, both tail pinch and LDT–PPT stimulation may directly or indirectly affect brain regions that could influence POM activity (Steriade and Llinás 1988). For example, cholinergic inputs from LDT–PPT may directly affect neurons in the spinal trigeminal subnucleus interpolaris, which provides a major afferent input to POM (Timofeeva et al. 2005). For these reasons we tested, through use of both in vivo and in vitro experiments, the direct effects of cholinergic agents on POM neurons. In vivo, carbachol application enhanced the responses of most POM neurons, mimicking the effects of LDT–PPT stimulation and the tail pinch. In vitro, carbachol presynaptically suppressed GABAergic inputs to POM neurons. We recently demonstrated that the cholinceptive, GABAergic inputs to POM arise from the zona incerta (see following text; Trageser et al. 2006).

Thus notwithstanding the caveats stated above, converging data from this and previous studies are consistent with the hypothesis that a critical component in the regulation of higher-order thalamic nuclei, such as POM, involves cholinergic modulation of the incertothalamic pathway.

Cholinergic effects on POM neurons

Activation of the brain stem cholinergic system facilitated POM responses to vibrissae stimulation, an effect that was mimicked by the direct iontophoresis of carbachol onto individual POM neurons. Roughly half of the neurons were thus facilitated, indicating that POM may contain several neuronal populations that have different sensitivities to cholinergic agonists. This conjecture is consistent with previous reports on POM heterogeneity (e.g., Lavallée et al. 2005; Varela and Sherman 2004). For example, we previously showed that POM contains a population of neurons responding to both cortical and peripheral inputs and a second population that responds only to cortical inputs (Trageser and Keller 2004).

Alternatively, the lack of cholinergic effects may simply reflect methodological limitations. For example, LDT–PPT stimulation may have failed to activate cholinergic inputs to the recorded cells or insufficient concentrations of carbachol may have been realized through iontophoresis.

In addition to facilitating POM responses to vibrissae stimulation, carbachol increased the spontaneous firing rate of POM neurons. However, spontaneous firing rates were not significantly affected by LDT–PPT stimulation. Whether this discrepancy reflects different mechanisms for regulating evoked versus spontaneous activity in POM remains to be determined.

Mechanisms of cholinergic regulation

Like its action in POM, ACh enhances spontaneous and evoked activity of VPM neurons (Castro-Alamancos 2004). In VPM, as in other first-order thalamic nuclei, ACh is thought to act by hyperpolarizing inhibitory cells and depolarizing principal neurons (Steriade 2003; Varela and Sherman 2004). Because LDT–PPT projects to both POM and VPM (Hallanger et al. 1987), ACh may facilitate POM neurons through a similar, depolarizing mechanism. Indeed,

the muscarinic agonist methylcholine (250 μ M), applied in vitro, depolarizes most POM neurons (Varela and Sherman 2004). In contrast, we show that the cholinergic agonist carbachol (2 μ M) has no effect on V_m , R_{in} , or mIPSCs kinetics of POM neurons, arguing against a postsynaptic action for carbachol. We cannot exclude the possibility that at higher concentrations carbachol may have postsynaptic effects or that space-clamp limitations obscured these effects. We also note that our in vitro data were obtained from animals that are younger than those used in the in vivo studies. To our knowledge, there are no reported differences in the anatomical or electrophysiological properties of the relevant circuit elements. Nevertheless, we cannot exclude the possibility of such differences.

These caveats notwithstanding, converging evidence supports the hypothesis that cholinergic regulation of POM occurs in large part through suppression of inhibitory, incertothalamic inputs. We recently demonstrated that activation of the brain stem cholinergic system in vivo and carbachol application in vitro and in vivo suppresses spontaneous and vibrissae-evoked activity of ZI neurons that project to POM (Trageser et al. 2006). Our current in vitro findings support the hypothesis that ACh also acts by suppressing GABA release from inhibitory terminals in POM.

These terminals may arise from any of the three sources of GABAergic inputs to POM, the ZI, the thalamic reticular nucleus (TRN), or the anterior pretectal nucleus (APT) (Bokor et al. 2005). At least one line of evidence strongly implicates ZI terminals; their axon terminals express a high density of the presynaptic muscarinic m2 receptors (Bartho et al. 2002), receptors that presynaptically regulate GABA release in a variety of CNS pathways (Baba et al. 1998; Barnabi and Cechetto 2001; Lupica et al. 1992). In contrast, TRN axon terminals are reported not to express m2 receptors (Cox and Sherman 2000). Whether APT terminals in POM express m2 receptors remains to be determined.

Thus ACh regulates sensory transmission in POM through at least three mechanisms: regulation of ZI firing (Trageser et al. 2006), presynaptic regulation of GABA release (present finding), and regulation of TRN firing (Fuentelba and Steriade 2005). This suppression will lead to the disinhibition of POM neurons, promoting enhanced responses to peripheral sensory stimulation.

The state-dependent gating hypothesis

POM neurons receive potent tonic and feedforward GABAergic inputs from ZI (Bartho et al. 2002; Lavallée et al. 2005). Inactivating ZI disinhibits POM neurons and allows them to respond robustly to sensory stimuli (Trageser and Keller 2004; see also Lavallée et al. 2005). Cholinergic inputs from the reticular activating system control this incertothalamic regulatory mechanism (Trageser et al. 2006; present findings).

LDT–PPT forms part of the brain stem–activating system that regulates sleep–wake cycles and states of vigilance (Steriade 2003). The fact that cholinergic inputs from these nuclei regulate activity in the incertothalamic pathway suggests that behavioral states regulate this system. We propose a state-dependent gating hypothesis, in which behavioral states modulate ZI's inhibitory regulation of POM. This regulation may allow POM to function in both relay modes proposed by Sherman and Guillery (2001): 1) a first-order mode, in which POM relays sensory information from the periphery to the cortex; and 2) a higher-order mode, in which POM relays information between cortical areas (see also Trageser and Keller 2004). During slow-wave sleep (and anesthetic states), when cholinergic activity is diminished, POM neurons fail to respond to ascending sensory inputs, and may function primarily as a higher-order relay. By contrast, increased cholinergic activity during wakefulness and enhanced vigilance suppresses ZI-mediated inhibition, thereby ungating POM responses to ascending inputs. We therefore

predict that during this state POM functions as a first-order relay. Findings presented here are fully consistent with this hypothesis.

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FIG. 1.

Response probability of posterior medial (POM) neurons decreases as stimulus frequency increases. *A*: rasters (*top*) with peristimulus time histograms (PSTHs, *bottom*) constructed from recordings of a single POM neuron in response to a 50-ms air puff delivered to the vibrissae at time $t = 0$. Air puffs were delivered at 2, 5, 8, or 11 Hz. Recordings obtained under fentanyl analgesia. *B*: stimulation and recording as in *A*, except that in this POM neuron, the recordings were obtained under urethan anesthesia. POM neurons produced weak and labile responses to vibrissae stimulation >2 Hz.

FIG. 2.

Laterodorsal tegmentum–pedunclopontine tegmentum (LDT–PPT) stimulation increases POM response probability. **A**: electrocorticogram (ECoG), spikes, and stimulus time stamps (Stimuli) recorded before and after LDT–PPT stimulation. Box delineates period after LDT–PPT stimulation (vertical black bar). Note, in the ECoG, transition from low-frequency, high-amplitude oscillations to high-frequency, low-amplitude oscillation after LDT–PPT stimulation, and the increase in single-unit POM responses to stimulus presentations during this period. **B**: PSTHs constructed from a single unit recorded in POM before (Control) and after LDT–PPT stimulation (Test). **C**: group PSTHs constructed from POM neurons recorded before (Control) and after LDT–PPT stimulation (Test). **D**: coefficient of variation of response

latencies plotted as a function of vibrissae stimulation frequency before (open symbols) and after (filled symbols) LDT–PPT stimulation. Coefficient of variation was significantly reduced at 8- and 11-Hz stimulation frequencies. *E*: plots depicting magnitudes of responses to 2-Hz vibrissae deflections recorded from individual neurons, before (Control) and during (Test) LDT–PPT stimulation. Response magnitude significantly increased for all frequencies ($P \leq 0.001$; Wilcoxon signed-ranks test). *F*: location of LDT–PPT stimulation sites (open circles), plotted on maps modified from Paxinos and Watson (1998).

FIG. 3.

Tail pinch stimulation increases POM response probability. *A*: ECoG transitions from high-amplitude, low-frequency oscillation before tail pinch to low-amplitude, high-frequency oscillation after tail pinch. *B*: PSTHs constructed from a POM neuron in response to vibrissae stimuli delivered at 2 Hz before (Control) and after tail pinch. After tail pinch the neuron responded at high probability with a greater than 2-fold increase in response magnitude (pre/post values: 0.40/0.85 spikes per stimulus). *C*: plots depicting magnitudes of responses to 2-Hz vibrissae deflections recorded from individual neurons, before (Control) and during tail pinch. Response magnitude significantly increased for all frequencies ($P \leq 10^{-4}$; Wilcoxon).

FIG. 4.

Carbachol increases POM response probability. *A*: cumulative PSTH vs. time plot constructed from responses of a POM neuron to vibrissae stimulation, before and after the iontophoresis of carbachol (100 μ M). This analysis depicts the dynamics of the PSTH over time and is generated by calculating multiple PSTHs using a sliding window in time. Each histogram is shown as a vertical stripe with colors representing response magnitude. Horizontal axis represents the position of the sliding window in time. Carbachol increased the response magnitude by 79% ($P = 0.003$). Apparent shift in latency during carbachol application is not significant and is exaggerated by the sliding window and by filtering. *B*: plots depicting magnitudes of responses to 0.5-Hz vibrissae deflections recorded from individual POM neurons, before (Control) and during carbachol iontophoresis.

FIG. 5.

Effects of carbachol on miniature inhibitory postsynaptic currents (mIPSCs) of POM neurons. **A:** whole cell voltage-clamp recording of a POM neuron before and after bath application of carbachol. Carbachol suppressed the frequency of mIPSCs recorded. **B:** cumulative probability plots of mIPSCs amplitude (*left*) and frequency (*right*) before (control) and after carbachol application. **C:** summary of the effect of carbachol on the amplitude and frequency of mIPSCs in individual POM neurons. **D:** superimposed and scaled averages of mIPSC traces before and after carbachol application.