Published in final edited form as:

J Neurophysiol. 2006 September ; 96(3): 1456–1463.

State-Dependent Gating of Sensory Inputs by Zona Incerta

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Abstract

We have previously shown 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 incerto-thalamic pathway regulates POm responses during different behavioral states. Here we tested the hypothesis that the cholinergic reticular activating system, implicated in regulating states of arousal, modulates ZI activity. We show that stimulation of brain stem cholinergic nuclei (laterodorsal tegmental and pedunculopontine tegmental) results in suppression of spontaneous firing of ZI neurons. Iontophoretic application of the cholinergic agonist carbachol to ZI neurons suppresses both their spontaneous firing and their vibrissae-evoked responses. We also found that carbachol application to an in vitro slice preparation suppresses spontaneous firing of neurons in the ventral sector of ZI (ZIV). Finally, we demonstrate that the majority of ZIV neurons contain parvalbumin and project to POm. Based on these results, we present the state-dependent gating hypothesis, which states that differing behavioral states—regulated by the brain stem cholinergic system—modulate ZI activity, thereby regulating the response properties of higher-order nuclei such as POm.

INTRODUCTION

The brain stem activating system regulates the transmission of sensory information through the thalamus during different behavioral states. For example, during sleep, cholinergic inputs from the brain stem are causally related to the suppression of transmission of sensory inputs to the neocortex, a hallmark of sleep states (Steriade 2003). This cholinergic modulation affects dorsal thalamic nuclei and inhibitory neurons in the thalamic reticular nucleus (TRN). We recently showed that the ability of the thalamic posterior medial (POm) nucleus—a nucleus responsible for transmitting vibrissae derived information in the rodent—to reliably relay sensory information depends on the state of the thalamic GABAergic nucleus zona incerta (ZI) (Trageser and Keller 2004). Inactivating ZI—the neurons of which respond to vibrissae deflections (Nicolelis et al. 1992) and densely innervate POm (Bartho et al. 2002)—disinhibits POm neurons, allowing them to respond robustly to vibrissae stimulation (Lavallée et al. 2005; Trageser and Keller 2004). These results suggest that both TRN and ZI modulate the flow of information through POm.

In contrast to the TRN that targets all thalamic nuclei, ZI neurons preferentially target a subpopulation of thalamic nuclei termed higher-order nuclei, of which POm is a member (Bartho et al. 2002; Diamond et al. 1992; Sherman 2005). This suggests a novel gating

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GRANTS

This work was supported by National Institute of Neurological Disorders and Stroke Grant NS-051799 to A. Keller and Fellowship F31-NS-046123 to J. C. Trageser.

mechanism whereby ZI controls the flow of information through select thalamic nuclei. For this to occur, a mechanism must exist for regulating ZI output.

The brain stem cholinergic system responsible for modulating TRN also densely innervates ZI (Kolmac and Mitrofanis 1998; Mesulam et al. 1983), the neurons of which express high levels of muscarinic receptors (Bartho et al. 2002). We therefore reasoned that ZI neurons could be regulated by these cholinergic inputs. We show that cholinergic agonists suppress spontaneously active ZI neurons in vivo and in vitro and that this effect is preferentially restricted to the ventral portion of ZI, which targets POM. Further, we show that excitation of the brain stem activating system inhibits ZI neurons. Based on these observations we suggest that the transmission of information through higher-order relays may depend on the state of ZI the activity of which is modulated by behavioral states.

METHODS

In vivo surgical procedures

We used 11 female Sprague-Dawley rats weighing 250–350 g for in vivo recordings. The rats were anesthetized with urethan (1.5 g/kg body wt), and we monitored electrocorticograms (ECoGs) to assess the stage of anesthesia, which was maintained at stage III/3–4 (Friedberg et al. 1999). We maintained body temperature at 37°C with a servo-controlled heating blanket. All procedures strictly adhered to institutional and federal guidelines.

In vivo ZI extracellular recording

We obtained extracellular unit recordings with quartz-insulated platinum electrodes (2–4 M Ω) from spontaneously active ZI neurons. We advanced electrodes in the right hemisphere based on stereotaxic coordinates (AP 3.5, ML 2.8), maintaining the rats in the stereotaxic frame throughout the recordings. We digitized (40 kHz) waveforms recorded from well-isolated units through a Plexon (Dallas, TX) data-acquisition system, and isolated units off-line with Plexon's off-line sorter, using dual thresholds and principal component analyses. We generated auto-correlograms with Neuroexplorer software (Littleton, MA) to confirm that we obtained recordings from single units.

Recording sites were marked with electrolytic lesions (5 μ A for 10 s) at the end of the experiment. Rats were then deeply anesthetized with sodium pentobarbital (60 mg/kg) and perfused transcardially with buffered saline followed by 4% buffered paraformaldehyde. We obtained coronal brain sections (80 μ m thick) and Nissl-stained them to identify recording sites.

Carbachol iontophoresis

We micro-iontophoretically applied carbachol to individual ZI neurons through a multi-barrel pipette attached to a carbon fiber used for single-unit recordings (1–3 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 Corporation, Minneapolis, MN). Once we isolated a vibrissae-sensitive neuron in ZI, we stimulated the vibrissae with air puffs (50-ms duration) delivered through a tube (0.5-mm diam) 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 1 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 μm thick) and stained them with neutral red to identify recording sites.

We isolated single units off-line with off-line sorter as described in the preceding text. We exported time stamps of well-isolated units and of stimulus triggers to Matlab (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 the response magnitude of which 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 in the preceding text), and defined response offset as two 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 after carbachol iontophoresis using Student's *t*-test.

LDT-PPT stimulation

We targeted a concentric bipolar stimulating electrode (250- μm diam; Frederick Haer, Bowdoinham, ME) to the laterodorsal tegmentum (LDT) and the pedunculopontine tegmentum (PPT) nuclei, based on stereotaxic coordinates (AP 9.0, ML 0.7, 6.0 mm deep). Electrical stimulation (200 μA) consisted of 200 μs pulses delivered at 100 Hz for 1 s.

In vitro ZI recordings

We anesthetized 22 Sprague-Dawley rats, 12–27 days old, with ketamine (30 mg/kg), removed the brains, and prepared 400- μm -thick slices. Slices were submerged in a recording chamber mounted on a fixed-stage microscope, and continuously perfused (at 2 ml/min) with artificial cerebral spinal fluid containing (in mM) 125 NaCl, 3 KCl, 2 CaCl_2 , 2 MgSO_4 , BES, and 15 D-glucose, aerated with 95% O_2 -0.5% CO_2 , pH 7.4. We obtained visually guided whole cell patch-clamp recordings with an Axon 1D amplifier (Axon Instruments, Union City, CA), digitized at 20 kHz with an A/D board (ITC-18; Instrutech, Great Neck, NY) using Pulse software (Heka Elektronik), and stored on a personal computer. The impedances of the patch electrodes were 3–5 M Ω . The intracellular recording solution contained, in mM, 120 K-gluconate, 10 KCL, 10 HEPES, 1 MgCl_2 , 2.5 MgATP , 0.2 Tris-GTP, 0.1 BAPTA, and 5 biocytin (pH adjusted to 7.3). We obtained the following agents from RBI-Sigma (Natick, MA) and bath applied them to the perfusate: carbachol (30 μM), atropine (30 μM), D-2-amino-5-phosphopentanoic acid (AP5; 50 μM), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20 μM), and gabazine (10 μM).

We filled cells with biocytin through the recording pipette and fixed slices overnight in a buffered solution containing 4% paraformaldehyde. To visualize cells, we reacted sections with the ABC *Elite* kit (1:1000; Vector Labs, Burlingame, CA) and 3–3' diaminobenzidine (DAB; 0.5 mg/ml), urea H_2O_2 (0.3 mg/ml), and CoCl_2 (0.2 mg/ml) in 0.05 M Tris buffer containing 0.5 M NaCl. Using the NeuroLucida (MicroBrightField, Williston, VT) morphometry system, we reconstructed labeled cells.

In vivo neuroanatomy

To retrogradely label incerto-thalamic neurons, in four female Sprague-Dawley rats (250–350 g), we injected the thalamus with the retrograde tracer FluoroGold (Fluorochrome, Denver). We performed surgery using sterile techniques on rats anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg), maintaining body temperature at 37°C using a thermostatically

regulated heating pad. We placed the rats in a stereotaxic device and created a craniotomy over the POM nucleus. We targeted a glass pipette (30- to 50- μ m tip diameter) containing 3% FluoroGold (in saline) to the physiologically identified vibrissae representation in POM and ejected the tracer by applying air pulses to the back of the pipette with a Picospritzer.

Five to 8 day after recovery from surgery, we killed the rats with sodium pentobarbital (60 mg/kg ip) and perfused them transcardially with buffered saline followed by a phosphate buffered 4% paraformaldehyde solution. We cut coronal sections (50 μ m thick) with a Vibratome, and counterstained every other section with the fluorescent stain SYTOX Green (Molecular Probes, Eugene, OR). We reacted the remaining sections for parvalbumin immunocytochemistry, using an antibody to parvalbumin raised in mice (Swant, Bellinzona, Switzerland). We incubated the sections in this antibody (1:120,000 in phosphate-buffered saline containing 0.4% Triton-X) for 48 h at 4°C, and after several rinses incubated the sections in a secondary antibody, goat anti-mouse conjugated to FITC (1:600; Jackson ImmunoResearch, West Grove, PA). We mounted the sections on glass slides and examined them with a confocal microscope (Olympus FV-500).

RESULTS

To test the hypothesis that the activity of ZI neurons is regulated by cholinergic inputs from the reticular activating system, we proceeded in the following steps. To guide our electrophysiological recordings, we first used neuroanatomical approaches to locate the relevant neuronal population: the inhibitory neurons that project to POM. We then used whole cell recordings from and intracellular labeling of ZI neurons in vitro to test whether the activity of the relevant neuronal population is modulated by cholinergic agents. We then stimulated the cholinergic reticular activating system in vivo to determine whether it affects the activity of these ZI neurons. Finally, we tested whether individual ZI neurons are modulated by direct application of cholinergic agents.

Inhibitory ventral ZI neurons preferentially target POM

ZI is a heterogeneous structure, commonly divided into four sectors (dorsal, ventral, rostral, and caudal), containing both excitatory and inhibitory neurons that project to a number of subcortical and cortical structures (reviewed by Mitrofanis 2005). To identify the neuronal population that forms the substrate for GABAergic suppression of POM responses by ZI, we labeled incerto-thalamic neurons by injecting a retrograde tracer into POM and stained the retrogradely labeled cells in ZI with an antibody against parvalbumin (see METHODS).

We identified 701 retrogradely labeled cells in 19 50- μ m coronal sections obtained from three rats. The majority (680; 97%) of these cells were in the lateral aspect of ventral ZI (ZIV), whereas we only rarely observed labeled cells in the dorsal region of ZI (ZID; Fig. 1A).

To determine whether these incerto-thalamic neurons were inhibitory, we processed the same sections with an antibody against parvalbumin. We chose parvalbumin as a marker because it is expressed by GABAergic neurons of the ZI (Kolmac and Mitrofanis 1999; Nicolelis et al. 1995) and because of the superior sensitivity and specificity of antibodies available for its detection. Almost all (679 of 680; 99.8%) retrogradely labeled cells in ZIV stained positively for parvalbumin (Fig. 1B). In a control experiment, we deposited FluoroGold into the ventral posterior medial nucleus of the thalamus (VPM). We did not observe any retrogradely labeled cells in ZI. These findings confirm previous reports that ZI projects to POM but not to VPM (Bartho et al. 2002; Lavallée et al. 2005; Power et al. 1999). Further, these findings demonstrate that all ZIV neurons that project to POM are parvalbumin-positive and thus likely to be inhibitory.

Heterogeneity of ZI neurons in vitro

In light of our anatomical results confirming the differential projection pattern of dorsal and ventral ZI neurons to POM (see preceding text), we sought to characterize the neurons in these sectors electrophysiologically and morphologically. We obtained whole cell current-clamp recordings from ZI neurons ($n = 107$) in an in vitro slice preparation. The intracellular recording solution contained biocytin, which allowed us to subsequently identify the recorded cells in histological sections, and to determine whether their somata were in ZIv ($n = 70$ cells), ZId ($n = 18$), or along the border between these sectors (ZIv/d; $n = 19$; see Fig. 2).

Nearly all of the neurons recorded in ZIv (64/70; 91%) elicited spontaneous and rhythmic action potentials (median firing rate = 9.3 Hz; 7.4 ± 1.6 Hz; Fig. 3, A and B). By contrast, only 7 of the 19 ZId neurons (37%), and 11/18 ZIv/d neurons (61%) fired spontaneously (Fig. 3, C and D). Spontaneous firing rates of these ZId (6.8 ± 1.7 Hz) and ZIv/d (7.8 ± 3.4 Hz) were statistically indistinguishable ($P = 0.1$) from those of ZIv neurons. Spontaneous firing of ZI neurons recorded in all sectors persisted in the presence of antagonists of *N*-methyl-D-aspartate receptors (2-amino-5-phosphonopentanoic acid, 50 μ M), AMPA/kainate receptors [6-cyano-7-nitroquinoxaline-2,3-dione (CNQX); 20 μ M] and GABA_A receptors (gabazine, 10 μ M), suggesting that the spontaneous firing reflects membrane properties intrinsic to the cells.

This conclusion is also supported by the finding that spontaneous firing in ZIv neurons was suppressed by hyperpolarizing ($\Delta V_m = 7 \pm 4$ mV) current injections. Further, injecting depolarizing currents into spontaneously silent ZId neurons ($n = 9$) evoked accommodating spike trains that rarely entrained to the duration of the current pulses (Fig. 3C). Thus the higher incidence of spontaneous firing in ZIv neurons, compared with ZId neurons, is unlikely the result of differences in resting V_m among these populations.

Pronounced afterhyperpolarizations (AHPs) of relatively long duration followed the spontaneous action potentials of ZIv neurons (Fig. 3A, Table 1). Action potentials in ZId neurons, evoked by depolarizing current injections, had significantly ($P < 0.05$) smaller and shorter AHPs (Fig. 3C; Table 1). In addition, all spontaneously active ZIv neurons responded to hyperpolarizing current injections with a burst of rebound spikes (Fig. 3A, *), whereas none of the non-spontaneously firing neurons did. The properties of ZIv/d neurons fell in between those of ZIv and ZId neurons (Table 1). The three classes of ZI neurons did not differ in their estimated input resistance (Table 1); we did not compare resting membrane potentials, because the spontaneous firing of ZIv neurons rendered these comparisons unreliable.

We reconstructed the morphologies of labeled neurons (15 in ZIv, 12 in ZId, and seven in ZIv/d). Neurons in both ZIv and ZId had a bipolar morphology with relatively long dendrites emanating from each of the fusiform somatic poles, and arborizing exclusively within their parent sector, without entering the adjacent sector (Fig. 2). ZIv/d neurons had either a multipolar or a bipolar morphology with dendrites spanning both the ZIv and ZId sectors. We recovered only relatively short axonal segments and therefore cannot comment on their arborization patterns. The size of the somata and the number of dendrites were similar for the three groups of ZI neurons; the only significant difference was the longer average dendritic length of ZIv/d neurons (Table 1). The morphologies we encountered resemble those described for ZI neurons in the cat and monkey (Ficalora and Mize 1989; Ma et al. 1992).

Carbachol inhibits ZIv neurons in vitro

ZI receives direct cholinergic inputs from the laterodorsal tegmental (LDT) and the pedunclopontine tegmental (PPT) brain stem nuclei (Kolmac et al. 1998), suggesting that acetylcholine modulates ZI neurons. To test this prediction, we obtained in vitro whole cell recordings from spontaneously firing ZIv ($n = 22$) and ZId ($n = 6$) neurons recorded before,

during, and after bath application of the cholinergic agonist carbachol (30 μ M). We performed these recordings in the presence of 2-amino-5-phosphonovaleric acid, CNQX, and gabazine as described in the preceding text. Infusion of carbachol to the bath completely suppressed the spontaneous firing in most ZIv neurons (18 of 22; 82%; Fig. 3E). By contrast, carbachol suppressed firing in only two of the six (33%) spontaneously firing ZId neurons. Application of the cholinergic antagonist atropine reversed the effects of carbachol (7 of 8 neurons; Fig. 3E). Carbachol application did not significantly affect the estimated resting membrane potentials or the estimated input resistances ($\Delta V_m = -1.2 \pm 6.4$ mV; $P > 0.1$; $\Delta R_{in} = 10.6 \pm 27.1\%$; $P > 0.1$). These findings demonstrate that cholinergic inputs suppress spontaneously active ZI neurons, and that the primary targets of these cholinergic inputs are neurons in ZIv.

LDT-PPT stimulation inhibits ZI

Having demonstrated the suppressive effects of carbachol on ZI activity in vitro, we next asked whether activation of cholinergic pathways in vivo has a similar effect on ZI neurons. Brain stem cholinergic neurons in the LDT-PPT densely innervate ZI, and electrical stimulation of the brain stem reticular activating system (LDT-PPT) results in the widespread efflux of acetylcholine (ACh) throughout the brain, including the thalamus (Castro-Alamancos 2002; Paré et al. 1990). ACh release leads to the suppression of high-amplitude, slow cortical oscillations indicative of sleep and anesthetized states, and the induction of low-amplitude, high-frequency oscillations reflecting states of arousal and alertness that are evident in cortical EEG recordings (Moruzzi and Magoun 1949; Steriade 2003). For this reason, brain stem stimulation is a physiologically relevant tool because it mimics, in anesthetized preparations, transitions in behavioral states.

To test the effects of brain stem stimulation, we recorded, in urethan-anesthetized rats, from well-isolated single units ($n = 35$) in ZI before and after LDT-PPT stimulation. As previously reported (Castro-Alamancos 2002; Paré et al. 1990), LDT-PPT stimulation results in transitions in electrocorticogram (ECoG) signals from high-amplitude, low-frequency oscillations to low-amplitude high-frequency oscillations. Recordings from a representative ZI neuron during these transitions are depicted in Fig. 4A. Consistent with previous reports (Lavallée et al. 2005; Nicolelis et al. 1992), ZI neurons fire spontaneously in vivo (median firing rate = 3.3 Hz; 4.0 ± 0.6 Hz). Immediately after LDT-PPT stimulation, the spontaneous firing rate of this ZI neuron decreased and remained suppressed for 74 s. The decrease in spontaneous firing outlasted the transition in the ECoG (6 s), continuing even after reinstatement of the high-amplitude, low-frequency oscillations. The changes in firing rate are evident in the instantaneous firing frequency plot (Fig. 4B), where we plot firing frequency as a function of time. Of the ZI neurons tested ($n = 35$), 37% were significantly suppressed, 14% showed an increase in spontaneous firing rates, and 49% were unaffected by LDT-PPT stimulation. Suppression times were variable (29.4 ± 9.9 s) with half of the neurons displaying relatively short periods of suppression (4.7 ± 0.9 s) lasting for the duration of cortical activation. The remaining neurons responded to LDT-PPT stimulation with prolonged periods of suppression (54.1 ± 13.5 s) outlasting the duration of cortical activation.

Histological analyses confirmed that our recordings were from ZI neurons. However, the size of the lesions used to mark the recording sites, and the fact that almost all lesions were adjacent to the border between the dorsal and ventral sectors of ZI, rendered it impossible to localize the recordings to one of these sectors.

These findings indicate that LDT-PPT stimulation suppresses the activity of a large population of ZI neurons. These findings are consistent with the hypothesis that excitation of brain stem arousal centers modulates ZI, gating the transmission of information through higher-order thalamic nuclei.

Carbachol inhibits ZI neurons in vivo

Although LDT-PPT stimulation evokes acetylcholine release and mimics the physiological activation of the brain stem reticular system (see preceding text), we cannot exclude the possibility that the stimulation inadvertently activated other classes of neurons or fibers of passage. To confirm that the effects of LDT-PPT stimulation reflect cholinergic activity, we tested whether in vivo micro-iontophoresis of carbachol onto individual ZI neurons mimics the effects of LDT-PPT stimulation.

We recorded from 18 ZI neurons that responded to air puff stimulation of the vibrissae (see METHODS). Histological analyses confirmed that all recording sites were in the lateral sector of ZI, a region previously shown to contain vibrissae-responsive neurons (Nicolelis et al. 1992). However, due to the size of the dye deposit used to mark recording sites and due to the relatively small dorsoventral size of ZI, we were unable to ascertain whether the recordings were from ZIv, ZId, or ZIv/d.

All vibrissa-responsive neurons fired spontaneous action potentials (median firing rate = 4.9 Hz; 9.2 ± 1.8 Hz), and responded to vibrissa stimulation at relatively short latencies (median = 7 ms; 7.4 ± 0.9 ms). Figure 4C depicts representative PSTHs recorded from one of these neurons before, during and after carbachol application. Carbachol application (60-s duration) produced a significant ($P = 0.001$) suppression in the magnitude of this neuron's response to vibrissae stimulation (from 1.14 to 0.80 spikes/stimulus, 29.8% decrease). This suppression was completely reversible with responses returning to predrug magnitudes 50 s after we terminated carbachol application. Similar significant reductions in the magnitude of vibrissae evoked responses occurred in 10 of 18 neurons (56%) with reductions averaging $35.9 \pm 11.7\%$. The magnitudes of evoked responses increased in three other neurons and were not affected significantly in the remaining five neurons.

Carbachol application also suppressed the spontaneous firing of ZI neurons. A representative example is depicted in Fig. 4D where we plot the firing rate of a ZI neuron as a function of time. Prior to carbachol application, this neuron fired, on average, 4.8 spikes/s, a value that was significantly ($P < 10^{-4}$) decreased (to 1.9 Hz; 60.3% reduction) during carbachol application. Similar significant reductions in spontaneous firing occurred in 10 of 18 neurons (56%), with reductions averaging $68.5 \pm 17.9\%$. Spontaneous firing increased in two other neurons and was not affected significantly in the six remaining neurons.

DISCUSSION

We present findings from both in vitro and in vivo experiments, demonstrating cholinergic suppression of ZI activity. Furthermore, our in vivo findings demonstrate that LDT-PPT stimulation can suppress ZI activity. Our anatomical findings are consistent with previous descriptions of inhibitory inputs from ZI to the POm nucleus of the somatosensory thalamus (Bartho et al. 2002; Lavallée et al. 2005; Power et al. 1999). We extend these findings by showing that these inhibitory inputs arise almost exclusively from the lateral sector of ventral ZI.

We previously demonstrated that inactivating ZI significantly enhances POm responses to vibrissae stimulation (Trageser and Keller 2004; see also Lavallée et al. 2005). Based on the anatomical and physiological data presented here, we propose that ACh—released after excitation of the brain stem activating system—suppresses both the spontaneous and the vibrissae-evoked activity of ZI neurons. This leads to disinhibition of POm neurons, promoting enhanced responses to sensory inputs. In addition to regulating the activity of a population of ZI neurons, ACh may regulate POm responses by presynaptically suppressing GABA release from ZI terminals in POm (Bartho et al. 2002). In a companion paper (Masri et al. 2006), we

present evidence supporting both of these predictions: that cholinergic activity disinhibits POM responses and that this occurs through presynaptic regulation of GABA release.

LDT-PPT stimulation

We recognize that LDT-PPT stimulation may inadvertently activate fibers of passage and nearby nuclei (Steriade and Llinás 1988). Although we made every attempt to limit these possibilities, it is important to consider that brain stem nuclei other than cholinergic nuclei may play a role in regulating ZI responses. Furthermore, the response properties of ZI neurons after LDT-PPT stimulation most likely reflect the actions of neuromodulators on the entire thalamocortical/corticothalamic network. These caveats notwithstanding, the anatomical and physiological 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 incerto-thalamic pathway. Further support for this supposition comes from our finding that carbachol suppresses both the spontaneous and stimulus-evoked activity of some ZI neurons, suggesting that this modulation occurs by affecting both tonic and feed-forward inhibition from ZI to POM.

Functional consequence of the heterogeneity of ZI

In contrast to our in vitro results, where cholinergic agonists suppressed essentially all ZIv neurons, both LDT-PPT stimulation and cholinergic agonist micro-iontophoresis suppressed approximately half of the ZI neurons recorded in vivo. These differences likely reflect the heterogeneity of ZI with neurons in different sectors expressing different chemical markers and having different afferent and efferent relationships (see Mitrofanis 2005). Our findings confirm previous reports demonstrating that ZI inputs to POM arise almost exclusively from neurons in ZIv (Lavallée et al. 2005; Power et al. 1999). We further demonstrate that these incerto-thalamic ZIv neurons are spontaneously active in vitro (see also Eaton and Moss 1989), that they contain parvalbumin (and are therefore likely inhibitory), and that their spontaneous activity is suppressed by cholinergic agonists. It is possible that our in vivo recordings sampled a larger proportion of ZId rather than ZIv neurons (see RESULTS), thus accounting for the lower percentage of ZI neurons suppressed after LDT-PPT stimulation or iontophoresis of carbachol.

State-dependent modulation of ZI

Higher-order thalamic nuclei, such as POM, receive extrinsic inhibitory inputs from ZI, the anterior pretectal nucleus, and the TRN (Bokor et al. 2005; Mitrofanis 2005). By contrast, first-order nuclei such as the ventral posterior medial (somatosensory) and the lateral geniculate (visual) nuclei, receive extrinsic GABAergic inputs exclusively from TRN (see Fuentealba and Steriade 2005). The reticular and extrareticular systems differ significantly in their postsynaptic influences on thalamic neurons, in the intrinsic properties of their constituent neurons and in the input-output relationships of their parent nuclei (see Bokor et al. 2005). These differences suggest that the selective extra-reticular GABAergic control of higher-order nuclei plays a distinct role in thalamic regulation, a role that remains to be determined.

Our findings are consistent with the hypothesis that behavioral states determine the function of ZI. This hypothesis predicts that ZI-mediated inhibition of POM is most potent during slow-wave sleep (and anesthetic states)—when cholinergic activity is diminished. This prediction is supported by anecdotal evidence (Koyama et al. 2003; Parmeggiani and Franzini 1973). As a result, POM neurons fail to respond to ascending sensory inputs, and function primarily in “higher-order” mode, concerned with relaying *trans*-cortical information (Sherman 2005). 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 nucleus that directly relays peripheral inputs to the cortex.

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FIG. 1.

Inhibitory ventral sector of zona incerta (ZIv) neurons project to posterior medial thalamus (POM). *A*: pseudocolor confocal image of a SYTOX green nuclear counterstained (blue) section through ZI, depicting neurons (white) retrogradely labeled following FluoroGold injection into POM. Note that most incerto-thalamic neurons are in the lateral part of ZIv. *B*: pseudocolor confocal image of a section through ZI stained with an antibody against parvalbumin (blue). Note that most parvalbumin-positive cells are in ZIv. Retrogradely labeled incerto-thalamic neurons are double-labeled for parvalbumin (white).

FIG. 2.

Morphology of ZI neurons. The morphology and location of representative ZI neurons are depicted in the reconstructions of biocytin-filled cells. Note that neurons with somata in ZIv or ZId have their dendrites confined to their parent sector, and that neurons along the ZIv/ZId border have dendrites (and axons; thin lines) that cross this border.

FIG. 3.

Heterogeneity of ZI neurons in vitro. Whole cell current-clamp recordings from neurons in ZIv (A) and ZId (C and D). The ZIv neuron fires spontaneously (*top*) and responds to hyperpolarizing current injections with rebound spikes (*bottom*, *). A histogram of interspike intervals recorded from a ZIv neuron (B) illustrates the rhythmicity of their spontaneous firing. Most ZId neurons (e.g., C) do not fire spontaneously (*top*) nor do they evoke rebound spikes (*bottom*). An example of a spontaneously firing ZId neuron is depicted in D. All scale bars (A–D) = 20 mV/200 ms. E: whole cell current-clamp recording from a ZIv neuron in response to application of the cholinergic agonist carbachol (30 μ M), which inhibits spontaneous activity; this effect is blocked by atropine (30 μ M; *right*).

FIG. 4.

Cholinergic suppression of spontaneous and evoked activity in ZI. *A*: electrocorticograms (ECoGs) and time stamps of spikes recorded prior to, during and after laterodorsal tegmentum (LDT)-pedunculopontine tegmentum (PPT) stimulation. Note, in the ECoG, the transition from low-frequency, high-amplitude oscillations to high-frequency, low-amplitude oscillations after LDT-PPT stimulation, and the decrease in spontaneous firing in the ZI neuron. *B*: instantaneous firing frequency computed for the neuron in *A*, prior to and after LDT-PPT stimulation ($\rightarrow$). Note that, in this example, the suppression in neuronal firing outlasts the duration of the LDT-PPT stimulation. *C*: peristimulus time histograms depicting responses of a ZI neuron to vibrissae stimuli delivered at 0.5 Hz before, during, and after carbachol iontophoresis. This neuron displayed significant ($P = 0.001$), reversible suppression of vibrissae-evoked responses during carbachol administration. *D*: instantaneous firing frequency plot computed from a spontaneously active ZI neuron showing a significant ($P < 10^{-4}$), reversible reduction in spontaneous activity during carbachol application.

TABLE 1

Biophysical and morphological properties of ZI neurons in vitro

	Rin, MΩ	Spike width	AHP amplitude, mV	AHP duration, ms	Soma area, μm ²	Primary dendrites (number)	Dendritic length, μm
ZIv	154.1 ± 57.5	1.3 ± 0.3	19.7 ± 3.9*	124.2 ± 51.6*	190.04 ± 75.82	3.13	890.37 ± 398.1
ZId	128.2 ± 30.6	1.2 ± 0.5	10.4 ± 2.9	35.4 ± 27.3	226.11 ± 54.53	3.33	895.99 ± 535.72
ZIv/d	134.1 ± 48.9	1.5 ± 0.5	12.1 ± 7.3	103.2 ± 65.3	201.03 ± 68.16	3.29	1364.68 ± 126.34*

Values are ± SE.

* $P < 0.05$. AHP, after hyperpolarization; ZI, zona incerta; ZIv, ventral sector of ZI.