Abnormal Anterior Pretectal Nucleus Activity

Contributes to Central Pain Syndrome

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ABSTRACT

Central pain syndrome (CPS) is a debilitating condition that affects a large number of patients with a primary lesion or dysfunction in the central nervous system, most commonly due to spinal cord injury, stroke, and multiple sclerosis lesions. The pathophysiological processes underlying the development and maintenance of CPS are poorly understood. We have recently shown, in an animal model of CPS, that neurons in the posterior thalamic nucleus (PO) have increased spontaneous and evoked activity. We also demonstrated that these changes are due to suppressed inhibitory inputs from the zona incerta (ZI). The anterior pretectal nucleus (APT) is a diencephalic nucleus that projects upon both the PO and ZI, suggesting that it might be involved in the pathophysiology of CPS. Here we test the hypothesis that CPS is associated with abnormal APT activity by recording single units from APT in anesthetized rats with CPS resulting from spinal cord lesions. The firing rate of APT neurons was increased in spinal-lesioned animals, compared to sham-operated controls. This increase was due to a selective increase in firing of Tonic neurons that project to and inhibit ZI, and an increase in bursts in Fast Bursting and Slow Rhythmic neurons. We also show that, in normal animals, suppressing APT results in increased PO spontaneous activity and evoked responses in a sub-population of PO neurons. Taken together, these findings suggest that APT regulates ZI inputs to PO, and that enhanced APT activity during CPS contributes to the abnormally high activity of PO neurons in CPS.

KEYWORDS: maladaptive plasticity, posterior thalamic nucleus, pretectum, spinal cord injury, zona incerta
INTRODUCTION

Many patients with insults to the spinal cord or brain suffer from excruciating, unrelenting chronic pain, a condition called central pain syndrome (CPS). CPS affects over half of spinal cord injury patients, almost 30% of multiple sclerosis patients and 8% of stroke patients, and therefore millions of people worldwide (Bonica 1991; Andersen et al. 1995; Siddall et al. 2003; Osterberg et al. 2005).

There is no effective treatment for CPS. An understanding of the pathophysiological mechanisms of CPS is needed for the development of effective long-term treatments. Although the underlying insults and their location vary among CPS patients there is general agreement that CPS involves insults to the spinothalamocortical tract and abnormal inhibition in the thalamus (Head and Holmes 1911; Bowsher 1995; Boivie 2005; Canavero and Bonicalzi 2007). We have recently reported that CPS resulting from spinal cord injury is associated with suppressed inputs from the inhibitory nucleus zona incerta (ZI) to the posterior thalamus (PO), resulting in higher spontaneous firing rates and evoked responses in PO (Masri et al. 2009). ZI projects exclusively to higher-order thalamic nuclei (Bartho et al. 2002; for review see Mitrofanis 2005), many of which are involved in the pathophysiology of pain disorders (Dostrovsky and Craig 2009).

PO activity is regulated by inhibitory inputs from two additional nuclei: the reticular thalamic nucleus and the anterior pretectal nucleus (APT). The reticular nucleus innervates both first-order and higher-order thalamic nuclei (Jones 2007). We have recently shown that this nucleus is unlikely to be directly involved in CPS resulting from spinal cord injury (Masri et al. 2009). APT, like ZI, exclusively innervates higher-
order thalamic nuclei (Bokor et al. 2005; Wanaverbecq et al. 2008), and several lines of evidence indicate that APT is involved in somatosensory functions, including nociception (for review see Berkeley and Mash 1978 and Rees and Roberts 1993; Prado and Roberts 1985; Roberts and Rees 1986; Rees and Roberts 1987; Prado 1989; Brandao et al. 1991; Terenzi et al. 1995; Rosa and Prado 1997; Rosa et al. 1998; Neto et al. 1999; Porro et al. 1999; Prado and Faganello 2000; Villarreal et al. 2003, 2004; Villarreal and Prado 2007). For example, APT is part of the descending pathways that modulate responses to noxious input in the spinal cord (for review see Rees and Roberts 1993). Peripheral noxious stimulation activates APT neurons (Rees and Roberts 1989; Neto et al. 1999; Porro et al. 1999; Villarreal et al. 2003), electrical and chemical stimulation of APT produces long-lasting antinociceptive effects in both acute and persistent pain models (Rhodes and Liebeskind 1978; Prado and Roberts 1985; Roberts and Rees 1986; Rees and Roberts 1987; Chiang et al. 1989; Prado 1989; Wilson et al. 1991; Wang et al. 1992; Villarreal et al. 2004), and APT inactivation increases responses to noxious stimuli (Villarreal et al. 2003, 2004). In a chronic pain model, APT inactivation resulted in increased autotomy, suggesting an increase in spontaneous pain (Rees et al. 1995). Along with its projections upon PO, APT also innervates the ventral subregion of ZI (Terenzi et al. 1995; May et al. 1997; Giber et al. 2008), the ZI subregion that projects to PO (Trageser et al. 2006; Giber et al. 2008). Given the potential for APT to regulate both PO and ZI, and the dramatic changes recorded in PO and ZI of animals with CPS (Masri et al. 2009), we hypothesized that APT activity would be abnormal in CPS. Specifically, the changes in PO and ZI could be caused by decreased inhibitory drive from APT to PO and/or increased inhibitory drive from APT to ZI. The experiments described here test these hypotheses.
MATERIALS AND METHODS

All procedures were approved by the University of Maryland School of Medicine Animal Care and Use Committee. Experiments were conducted according to institutional guidelines, federal regulations, and the guidelines of the International Association for the Study of Pain.

**Spinal lesions**

Seven adult female Sprague-Dawley rats weighing 250-300 g were used in the spinal lesions portion of this study. Three of these rats underwent spinal lesion and four rats underwent sham lesion surgery (Wang and Thompson 2008; Masri et al. 2009). Surgeries were conducted under strict aseptic conditions. Rats were anesthetized with ketamine/xylazine (100/8 mg/kg, i.p.) and placed on a thermoregulated heating pad to maintain body temperature. A laminectomy was performed to expose the spinal cord between C6 and T2. A quartz-insulated platinum electrode (5 μm tip) was targeted to the anterior spinothalamic tract on one side of the spinal cord. Current (10 μA for 10 sec, repeated 4 times) was passed through the electrode to produce an electrolytic lesion (approximately 0.6 mm³). Sham surgery was performed without laminectomy. The analgesic buprenorphine (0.05 mg/kg) was administered every 12 hours for 24 hours postoperatively.

**Behavioral testing**
Rats were habituated to the testing room for 30 min before behavioral testing, which consisted of measuring mechanical hindpaw withdrawal thresholds bilaterally using calibrated von Frey filaments (Stoelting, IL). Filaments were applied to the dorsal surface of the hindpaw based on studies demonstrating that threshold changes are more reliably and consistently detected at this site (Ren 1999). Mechanical withdrawal threshold was defined as the force at which the animal withdrew to 3 of the 5 stimuli delivered. Rats underwent behavioral testing for 3 days in the week before surgery, to establish baseline thresholds, and on day 28 post-surgery.

**Extracellular recordings**

Following confirmation of decreased mechanical withdrawal thresholds 28 days after spinal lesion/sham surgery, rats were anesthetized with urethane (1.5 g/kg, i.p.) and infused with local anesthetics at surgical sites, head-fixed in a stereotaxic apparatus, and placed on a thermo-regulated heating pad to maintain body temperature at 37°C. Depth of anesthesia was monitored every 15 minutes by testing reflexes to pinching of the skin and cornea stimulation. Supplemental doses of urethane (0.15 g/kg) were given when necessary. Urethane was selected because it is the only anesthetic that has no, or negligible, effects on glutamatergic and GABAergic transmission, and therefore produces only minimal disruption of signal transmission in the neocortex (Sceniak and Maciver 2006).

A craniotomy was performed over the APT contralateral to the spinal lesion site or over PO in naive animals. Extracellular recordings of single units were
obtained with quartz-insulated platinum electrodes (1 μm diameter tip; 2-4 MΩ).

Spike waveforms were digitized through a Plexon (Dallas, TX) data acquisition system and sampled at 40 kHz. Electrodes were advanced to APT or PO based on stereotaxic coordinates. APT: anteroposterior (AP), -4.8; mediolateral (ML), 1.9; PO: AP, -3.6; ML, 2.6 (Paxinos and Watson 1998). Animals were maintained within the stereotaxic frame throughout the recordings. Once a well-isolated unit was identified in APT spontaneous activity was recorded for 3 minutes.

In PO thalamus, responses were recorded to 50 deflections of the contralateral vibrissae by air puffs delivered through a tube (0.5 mm diameter) by a computer-controlled Picospritzer. Spontaneous activity was computed from a 100 msec period preceding stimulus onset. Air puffs were delivered at 1 Hz with a pressure of 60 psi, resulting in deflections of multiple whiskers of <30°. An orientation of the air tube was chosen that deflected the maximal number of whiskers and was not changed for the duration of each recording session.

At the end of each experiment, electrolytic lesions (20 μA of negative current for 20 seconds, performed 2 times) were made to confirm the recording sites. Animals were then deeply anesthetized with sodium pentobarbital (60 mg/kg) and perfused transcardially with buffered saline followed by buffered 4% paraformaldehyde. Coronal brain and spinal sections (80 μm thick) were obtained and Nissl-stained to identify recording and lesion sites.

**APT inactivation**
Seven adult female Sprague-Dawley rats weighing 250-300 g were used in the APT inactivation portion of this study. APT lesions were performed in 4 of these rats; the remaining 3 rats served as naive controls and did not receive lesions. Lesions were made by advancing a quartz-insulated platinum electrode into the APT following stereotaxic coordinates (AP, -4.8; ML, 1.9) and passing negative current through the electrode at 40 μA for 10 sec, repeated 4 times at 5.9 mm, 5.5 mm and 5.1 mm from the pial surface, resulting in lesions 0.07 to 0.5 mm³ in volume. Verification of lesion sites was performed as described above. Only animals with clear lesions in the APT were included in the analyses. PO recordings were obtained within 15 min of creating lesions in the APT, and the recording session lasted up to 12 hr. No significant differences were observed in any of the analyzed parameters over the course of an experiment.

Data analysis

Statistical analyses were performed with Intercooled Stata (Stata Corporation, College Station, TX) and GraphPad Prism (GraphPad Software Inc., San Diego, CA). Between-group statistical comparisons were assessed with the nonparametric Mann Whitney U test. Proportional data were analyzed using the Chi-squared test. The significance level was set at $p < 0.05$ for all tests.

Behavioral data: To test whether hindpaw withdrawal thresholds changed over time after surgery, data from spinal-lesioned rats and sham-operated rats were analyzed separately with the Wilcoxon matched pairs test.
Vibrissa evoked responses: Time stamps of well-isolated units and of stimulus triggers were exported to Matlab (MathWorks, Natick, MA) for analyses using custom-written algorithms. Peristimulus time histograms (PSTHs; 1 msec bins) were constructed from these time stamps. Significant stimulus-evoked responses were defined as PSTH bins with a response magnitude that significantly exceeded (99% confidence interval) spontaneous activity levels, computed from a 100 msec period preceding the stimuli.

RESULTS

Behavioral confirmation of CPS in spinal-lesioned animals

We use an established rodent model of CPS in which electrolytic lesions of the spinal cord result in mechanical hyperalgesia below the lesion site (Endo et al. 2008; Wang and Thompson 2008; Masri et al. 2009). The time course and clinical features of this hyperalgesia are consistent with CPS symptoms in human patients (reviewed in Canavero and Bonicalzi 2007). Animals with such spinal lesions develop mechanical and thermal hyperalgesia within 14 days of spinal lesion surgery (Masri et al. 2009). Consistent with previous reports, all animals in the present study that underwent spinal lesion surgery developed a significant reduction in hindpaw withdrawal thresholds to mechanical stimuli by 28 days following surgery. Mechanical thresholds decreased from 93.3 g (SD = 24.2 g, median = 80, range 80 to 140) to 66.7 g at 28 days post-surgery (SD = 16.3 g, median = 60, range 60 to 100; p = 0.03, the Wilcoxon matched-pairs test; n = 3 rats). Sham surgery had no effect on mechanical withdrawal thresholds of either the ipsilateral or contralateral hindpaw (pre-surgery mean = 90.0 ± 21.4 g, median = 100,
range 60 to 120; 28 days post-surgery mean = 95.0 ± 14.1 g, median = 100, range 60 to 100; p = 0.6; n = 4).

Neuronal activity in APT following CPS

Within one week after hyperalgesia was confirmed (at 28 days post-lesion), we obtained recordings from 164 well-isolated single units in APT of urethane-anesthetized rats (57 from sham-operated control and 107 from spinal-lesioned animals). Post hoc histological analysis of electrode tracts and lesion sites (see Methods) confirmed that all neurons were located within APT, throughout both dorsal and ventral APT. Recorded cells were located between 4.8 mm and 5.2 mm posterior to bregma. There was no difference in location of cells recorded from sham-operated and spinal-lesioned animals. Consistent with a previous report (Bokor et al. 2005), APT neurons exhibited a heterogeneous pattern of spontaneous firing, with a high degree of variability, ranging from 0.2 to 17.5 Hz in sham-operated controls and 0.06 to 25.3 Hz in spinal-lesioned rats. We describe the heterogeneity of responses in detail below.

To test the hypothesis that CPS is associated with abnormal APT activity, we compared the spontaneous firing of APT neurons recorded from animals with CPS to those recorded from sham-operated rats. Neurons from CPS rats had a significantly higher mean spontaneous firing rate (CPS: mean = 6.2 ± 6.0 Hz, median = 3.6, range 0.06 to 25.3; Sham: mean = 3.3 ± 4.3 Hz, median = 1.3, range 0.2 to 17.5; p < 0.001, two-tailed Mann-Whitney U test; Figure 1A). This finding is consistent with the hypothesis that CPS is associated with increased inhibitory drive from APT to ZI, and inconsistent with the hypothesis of decreased inhibitory drive from APT to the posterior thalamus.
APT is a heterogeneous nucleus, forming at least two regions with distinct anatomical, physiological and behavioral characteristics. Projections from the brainstem trigeminal and dorsal column nuclei preferentially terminate in ventral APT, a region that projects to areas involved in motor functions (Berman et al. 1977; Yoshida et al. 1992; Terenzi et al. 1995). Villarreal et al. (2004) found that stimulating dorsal APT was more effective than stimulating the ventral APT in inducing antinociception of acute pain, while stimulation of ventral APT was more effective for antinociception of persistent pain. We sought to determine if the increase in APT activity in CPS occurred preferentially in one of these APT subregions. Figure 1B shows spontaneous firing rates plotted against brain depth. Linear regression fits show a significant deviation from zero for neurons from spinal-lesioned animals ($r^2 = 0.05; p = 0.02$) such that a greater increase in activity is seen in ventral APT compared to dorsal APT. The same analysis for sham animals is not significant ($r^2 = 0.04; p = 0.2$). The directionality of the increase in APT activity is consistent with prior evidence that APT is heterogeneous and supports the notion that the ventral APT is more involved in somatosensory processes than the dorsal APT. A cumulative probability plot of spontaneous firing rate shows a significant rightward shift in lesioned animals compared to sham-operated ($p = 0.003$, Kolmogorov-Smirnov test; Figure 1D), indicating that not only is the firing in lesioned animals increased but that a greater proportion of neurons show increased firing rate following spinal lesions.

Besides its involvement in pain processing, APT is also involved in the processing of non-noxious somatosensation (for review see Rees and Roberts 1993; Terenzi 1995). We tested the effects of spinal lesions on innocuous, sensory-evoked responses in APT
by deflecting the contralateral vibrissae with air puffs or by tactile stimulation of various body parts. None of the neurons recorded responded to these stimuli. The failure to find neurons in APT that respond to low-threshold cutaneous and whisker stimuli agrees with the report by Rees and Roberts (1989) that APT neurons do not respond to hair movements, light pressure or limb flexion.

Bursting activity in APT also showed a high degree of variability, with sham-operated rats displaying 0 to 206 bursts per minute and spinal-lesioned rats 0 to 214 bursts per minute (Figure 1E). The percentage of APT neurons exhibiting at least one burst was significantly higher in CPS rats compared to sham-operated controls (44% vs. 23%; $p = 0.01$, $\chi^2$ test). This increase in the population of bursting neurons was also reflected in a significant increase in burst rate (CPS: mean $= 5.6 \pm 14$ bursts per minute, median $= 0$, range 0 to 71; $n = 107$; Sham: mean $= 2.4 \pm 11$, median $= 0$, range 0 to 68; $p = 0.004$, two-tailed Mann-Whitney U test; $n = 57$; Figure 1E).

To test if the increase in spontaneous firing affects both bursting and non-bursting APT neurons, we computed the mean spontaneous firing rate separately for each group. Spontaneous activity was significantly higher in the non-bursting neurons from spinal-lesioned animals: CPS: mean $= 5.3 \pm 5.5$ Hz, median $= 2.4$, range 0.06 to 25; $n = 60$; Sham: mean $= 3.0 \pm 4.1$ Hz, median $= 1.3$, range 0.2 to 15; $p = 0.02$; $n = 44$; Figure 1F).

By contrast, in bursting neurons spinal lesions had no effect on either spontaneous firing rate or on any of the burst properties (sham-operated, $n = 13$; spinal-lesioned, $n = 47$; Table 1).

**Effects on specific neuronal classes**
Bokor et al. (2005) have shown that APT neurons can be classified into three populations based on their firing properties. These are: (1) Fast Bursting cells that periodically fire high frequency bursts of action potentials (> 350 Hz); (2) Tonic cells that fire monotonous or irregularly spaced action potentials; (3) Slow Rhythmic cells that fire short bursts of action potentials (at < 150 Hz) at a rhythm locked to the slow cortical oscillations of anesthetized animals (0.7 to 8 Hz).

Using these criteria, and analyzing interspike interval (ISI) plots and autocorrelograms (see Materials and Methods), we categorized APT neurons into these 3 classes. Example ISIs and autocorrelograms of neurons in each category from sham-operated rats are shown in Figure 2. Firing parameters for the three example neurons are as follows: Fast Bursting – spontaneous firing rate 20 Hz; burst frequency 71 bursts per minute; mean frequency within bursts 256 ± 53 Hz; Tonic – spontaneous firing rate 25 Hz; burst frequency 1 burst per minute; mean frequency within bursts 147 ± 32; Slow Rhythmic – spontaneous firing rate 9.4 Hz; burst frequency 5.1 bursts per minute; mean frequency within bursts 147 ± 21. These measurements are consistent with those observed previously (Bokor et al. 2005), thus validating our categorization method.

We first asked if spinal lesions affected the prevalence of neurons in each class. They did not: the percentage of neurons in each class was indistinguishable in CPS and sham-operated animals (p = 0.3; χ² test; Figure 2G).

We next asked if the increase in firing rates following spinal lesions was reflected differentially in one or more of the APT neuronal subpopulations. Only Tonic cells showed a significant, nearly 2-fold increase in spontaneous activity following spinal lesion (CPS: mean = 8.2 ± 5.5 Hz, median = 7.6, range 0.06 to 25; Sham: mean = 4.6 ±
4.5 Hz, median = 4.0, range 0.3 to 15; \( p = 0.03 \); two-tailed Mann-Whitney U test; Figure 3B). Conversely, Fast Bursting and Slow Rhythmic neurons showed no change in spontaneous firing rate (Fast Bursting, CPS: mean = 3.7 ± 5.5 Hz, median = 1.2, range 0.2 to 20; Sham: mean = 2.0 ± 3.4 Hz, median = 1.1, range 0.2 to 18, \( p = 0.1 \); Figure 3A; Slow Rhythmic, CPS: mean = 9.3 ± 5.7 Hz, median = 9.4, range 0.5 to 25; Sham: mean = 6.1 ± 5.4 Hz, median = 5.2, range 0.3 to 15; \( p = 0.1 \); Figure 3C).

Because bursting has been implicated in mechanisms of CPS (Lenz et al. 1989; Vierck et al. 1990; Weng et al. 2003; Lee et al. 2005; but see Dostrovsky 2007), we asked if spinal lesions affected the propensity of neurons in each class to fire bursts of action potentials. We defined bursts in extracellularly recorded spike trains as clusters of at least four spikes with interspike intervals of \( \bullet 10 \) msec. Consistent with the qualitative classification criteria (see above), this quantitative analysis revealed that, in sham-operated rats, the Fast Bursting class exhibited the highest incidence of bursting neurons (35%) while bursting neurons were rare among both Tonic (7%) and Slow Rhythmic (11%) neurons.

Following spinal lesions, Fast Bursting and Slow Rhythmic neurons showed an increase in bursting activity (Figure 3). Fast Bursting neurons showed an increase in burst rate (Sham: mean = 4.0 ± 14 bursts per minute, median = 0, range 0 to 69; CPS: mean = 9.1 ± 18, median = 0.3, range 0 to 71; \( p = 0.02 \); Figure 3A) but no change in the proportion of neurons exhibiting bursts (35% in sham-operated, 57% in spinal-lesioned, \( p = 0.08 \), \( \chi^2 \) test). Slow Rhythmic neurons showed a dramatic increase in the proportion of neurons exhibiting bursts (11% in sham-operated vs. 56% in spinal-lesioned, \( p = 0.04 \)). This increase in bursting population was accompanied by an over 100-fold
increase in overall bursting rate (0.04 ± 0.11 bursts per minute in sham-operated vs. 6.1 ± 8.3 in spinal-lesioned animals; Sham: median = 0, range 0 to 0.33; CPS: median = 0.33, range 0 to 27; p = 0.008; two-tailed Mann-Whitney U test; Figure 3C).

Meanwhile, Tonic neurons showed no significant change in either incidence of bursting neurons (Sham: 7%; CPS: 19%, p = 0.4) or burst rate (Sham: mean = 0.05 ± 0.2 bursts per minute, median = 0.2, range 0 to 0.7; CPS: mean = 0.2 ± 0.5, median = 0, range 0 to 3; p = 0.3; Figure 3B).

We analyzed firing properties of bursting cells within each group to determine if they were altered by spinal lesions. Compared to sham-operated rats, no group showed a significant change in spontaneous firing rate, burst frequency, burst duration, number of spikes per burst, ISIs within bursts, or average of frequencies or peak frequencies within bursts (Table 2).

**PO activity is increased following APT lesion**

The hypothesis—which we disproved—was that CPS would be associated with decreased activity of APT neurons. This hypothesis was based on our finding that CPS is associated with a dramatic increase in spontaneous and evoked activity in PO (Masri et al. 2009), and on the assumption that APT inhibits PO neurons. The latter assumption was based on anatomical data showing that APT sends inhibitory, GABAergic projections to PO (Bokor et al. 2005), and on *in vitro* findings showing that stimulation of APT evokes inhibitory potentials in PO neurons (Bokor et al. 2005; Wanaverbecq et al. 2008). However, to our knowledge there is no direct evidence for the prediction that APT inhibits spontaneous or evoked activity of PO neurons *in vivo.*
We tested this prediction by inactivating large parts of APT with electrolytic lesions (see Materials and Methods). The resulting lesions were 0.07 to 0.5 mm$^3$ and affected both the dorsal and ventral subregions of APT (Figure 4A, C). One lesion was located medial and dorsal; the second medial and ventral; the third was located slightly posterior compared to the first two and covered large areas of both dorsal and ventral APT.

We tested the effects of APT lesions on the spontaneous activity of PO neurons, and on their responses to sensory stimulation (see Materials and Methods). To allow for meaningful comparisons between data from control and APT-lesioned animals, we focused on neurons in the dorsolateral portion of the medial posterior nucleus (POm), a region containing vibrissae-responsive neurons, and included only neurons that responded to vibrissae stimulation. We compared data from neurons recorded from rats with intact APT ($n = 17$ neurons) with data from neurons recorded from rats in which APT was lesioned 15 min before the recordings ($n = 28$ neurons).

As we and others reported previously, PO neurons fire spontaneously at low rates and respond to vibrissae stimulation weakly and at long latencies (Diamond et al. 1992; Trageser and Keller 2004; Lavallee et al. 2005; Masri et al. 2008). As seen in Figure 4B, the spontaneous firing rate in PO increased following APT lesion (Naive: median = 0.0 Hz, range 0 to 0; Lesion: median = 0.2 Hz, range 0 to 3; $p < 0.001$; two-tailed Mann-Whitney U test).

Previous work in our lab (Trageser and Keller 2004) has shown that PO contains two neuronal populations: cells that respond to vibrissae deflections at relatively short latencies ($\leq 30$ msec) and cells that respond at longer latencies ($> 30$ msec). We also
showed that these two cell populations are regulated differently by the inhibitory nucleus zona incerta (Trageser and Keller 2004). We therefore sought to determine how APT regulates short- \((n = 8\) control; \(n = 7\) post-lesion) and long- \((n = 9\) control; \(n = 21\) post-lesion) latency cells in PO. Inactivating APT resulted in a selective increase in response magnitude of short-latency cells (median = 0.94 spikes per stimulus, range 0.08 to 2.8; \(p = 0.04\); Mann-Whitney U; Figure 4D and E) compared with control (median = 0.24 spikes per stimulus, range 0.04 to 0.74) and had no effect on long-latency cells \((p = 0.1\); Figure 4F and G). The disinhibition of short-latency cell responses following APT lesion suggests that, like the inhibitory subththalamic nucleus ZI (Trageser and Keller 2004), APT functions to selectively suppress sensory-evoked responses in this subset of PO neurons.

**DISCUSSION**

Our goal was to elucidate the role of APT in the pathogenesis of central pain syndrome (CPS). We considered two hypotheses: (1) decreased inhibitory input from APT to PO contributes to the increased spontaneous and evoked activity of neurons in PO during CPS; (2) increased inhibitory input from APT to ZI decreases ZI activity and, consequently, increases PO activity (Masri et al. 2009). The first hypothesis arises from the observation that APT neurons can powerfully inhibit PO neurons (Bokor et al. 2005). The prediction follows that the activity of APT neurons would be abnormally low in CPS. Contrary to this prediction, APT activity was elevated in rats with CPS, with increases in both spontaneous activity and incidence of bursting.
Given this unexpected finding we asked whether APT did indeed inhibit PO under normal conditions. Suppression of APT with electrolytic lesions resulted in increased spontaneous and sensory-evoked activity in PO neurons. These findings indicate that APT, like its counterpart ZI (Trageser and Keller 2004; Lavallee et al. 2005), exerts potent inhibition onto PO neurons.

How, then, can we reconcile the apparently contradictory findings that in CPS APT activity increases are associated, not with enhanced inhibition of PO, but rather with enhanced activity of PO neurons? We consider several possibilities.

**Efficacy of APT inputs to PO**

One possibility is that the increased spontaneous activity of APT neurons is insufficient to significantly affect PO neurons. We have previously shown that, during CPS, spontaneous activity of PO neurons increases 30-fold, and that this increase is associated with dramatically reduced inhibition from ZI (Masri et al. 2009). It is possible, therefore, that the < 2 fold increase in the spontaneous activity of APT neurons cannot significantly counteract the dramatically enhanced activity of PO neurons in animals with CPS.

**Differential Effect on Neuronal Subtypes**

APT neurons can be classified into three groups based on firing pattern, morphology and parvalbumin expression (Bokor et al. 2005). Two key findings in the current study were that CPS was associated with a selective increase in spontaneous firing rate of Tonic neurons, and a selective increased burst firing of Fast Bursting and Slow Rhythmic neurons (Fig. 3).
All three neuronal APT subtypes project to both ZI and PO (Giber et al. 2008). Their target distribution, however, is not homogeneous (Fig. 5). The GABAergic APT-ZI projections are thought to be dominated by Tonic neurons while Fast Bursting neurons, the other GABAergic group in APT, project mainly to PO and account for only a small percentage of GABAergic projections from APT to ZI (Bokor et al. 2005; Giber et al. 2008). Therefore, the increase in spontaneous firing rate of Tonic neurons during CPS likely leads to increased GABAergic input to ZI and provides a possible explanation for the decrease in ZI activity seen during CPS (Masri et al. 2009). This preferential increase in inhibitory output to ZI, and the subsequent increase in PO activity, explains how changes in APT activity could affect PO through an indirect pathway.

Tonic neurons project not only upon ZI, as they make up approximately 20% of APT projections upon PO (Giber et al. 2008). It is possible that the relatively modest increase in inhibitory Tonic neuron activity is not sufficient to counteract the 30-fold increase in PO activity, driven by disinhibition from ZI (Masri et al. 2009).

Most (60%) of the synapses formed by APT axon terminals in ZI are excitatory, and available evidence suggests that these originate from Slow Rhythmic APT neurons (May et al. 1997; Bokor et al. 2005; Giber et al. 2008). During CPS the incidence of burst firing is increased in these neurons, but their overall firing rate remains unchanged (Fig. 3C). Because ZI activity is dramatically suppressed during CPS (see above), it is possible that the increased bursting of these excitatory APT-ZI inputs has little or no effect on the firing rate of ZI neurons. This would be consistent with the notion that presynaptic bursts can affect the temporal fidelity of postsynaptic neurons (Sherman and Guillery 1998, 2006), without affecting their overall excitability.
Alternatively, it is possible that the excitatory Slow Rhythmic neurons do not
project upon PO-projecting ZI neurons, but rather that they drive inhibitory
interneurons within ZI to produce feed-forward inhibition of PO-projecting neurons.
There is precedence for such feed-forward regulation of the incerto-thalamic pathway:
Urbain and Deschenès (2007) argued that excitatory inputs from motor cortex
preferentially target local interneurons in ZI, which, through feed-forward inhibition,
inhibit other ZI neurons that project to PO. It is therefore possible that, during CPS, the
increase in bursting of excitatory Slow Rhythmic neurons activates this intra-incertal
circuit and consequently inhibits ZI.

While Tonic and Slow Rhythmic cells make up the majority of inputs to ZI, Fast
Bursting cells preferentially target the thalamus (Bokor et al. 2005; Giber et al. 2008).
The firing rate of Fast Bursting neurons remains unchanged during CPS, but the
incidence of burst firing is increased (Figure 3B). The fact that there is no change in the
firing rate of these neurons, which are inhibitory, is consistent with the increased PO
activity seen during CPS (Masri et al, 2009) and suggests that changes in APT activity
could affect PO indirectly via ZI rather than through changes in PO-projecting neurons.
The increase in PO activity following APT lesion confirms that APT does inhibit PO
neurons under normal conditions and further supports the notion that changes in APT
during CPS are not global but target specific neuron groups. As noted above regarding
Slow Rhythmic neurons that project to ZI, the increased burst firing in Fast Bursting
neurons without a change in firing rate may not significantly affect the excitability of PO
neurons.

*Increased Activity Preferentially in Ventral APT*
Besides the heterogeneity in neurotransmitter expression, cellular morphology, electrophysiological properties, APT projection targets differ along its rostrodorsal and caudoventral axes (Terenzi et al. 1995; Scalia and Arango 1979; Giber et al. 2008; for review see Rees and Roberts 1993 and Mitrofanis 2005). A loose topography exists such that the dorsal APT, the APT subregion associated with visual systems, projects to the lateral ventral ZI (vZI), dominated by neurons involved in communication with visual areas, whereas the ventral APT, associated with somatosensory systems, projects preferentially to the somatosensory and motor subregions of vZI (Berman 1977; Terenzi et al. 1995). Interestingly, the greatest increase in spontaneous activity occurred in the ventral/somatosensory APT (Fig. 1B). Thus, the effects of the APT activity increase would be expected to have a greater affect on vZI neurons involved in somatosensory and motor areas, including PO (Mitrofanis 2005).

**Other targets of APT**

Besides PO and ZI, APT is part of the descending pain modulation system and projects to a number of areas involved with the processing of nociceptive information. These areas include the posterior hypothalamus, intralaminar thalamus, somatosensory cortex, rostral ventrolateral medulla, and possibly periaqueductal gray (Berman 1977; Beitz 1982; Foster et al. 1989; Terenzi et al. 1995; Zagon et al. 1995). It is likely that the changes we observe in APT following spinal lesions also affect these targets, along with its effects on ZI and PO, demonstrated here.

Support for a role for APT in pain comes also from studies demonstrating that stimulation of APT induces anti-nociception (Prado and Roberts 1985; Roberts and Rees 1986; Prado 1989), and that inhibiting APT results in exaggerated responses to painful
stimuli (Villarreal et al. 2003, 2004). These effects of APT are thought to occur through descending modulation of processing in the spinal cord (Price and Mayer 1975). At first glance, these findings appear to contradict our finding that APT activity is increased in CPS. However, the studies cited above examined the role of APT in regulating responses to acute pain, whereas our study focused on the maladaptive plasticity that occurs in APT weeks after spinal cord injury. To our knowledge, only one other study examined the role of APT in a chronic pain condition: Rees et al. (1995) showed that, following dorsal horn rhizotomy, inhibiting APT increases autotomous behavior, suggesting an increase in spontaneous pain. However, the activity of APT was not directly tested in that study.

It is also likely that the effects of stimulating or inactivating large regions of APT are confounded by the heterogeneity of neurons in this structure (Bokor et al. 2005; and see present findings). Immediately relevant to pain processing, Villarreal et al. (2004) showed that stimulating dorsal APT is more effective at producing antinociceptive effects on brief noxious stimuli, whereas stimulating ventral APT is more effective at producing anti-nociception to persistent noxious stimuli. Likewise, inhibiting ventral APT caused a greater increase in incisional pain than did inhibiting dorsal APT.

A Role for APT in CPS

APT innervates exclusively higher order thalamic nuclei and, as such, is poised to regulate sensory information that involves communication between one cortical area to another. The findings in this study suggest that changes in APT activity are part of the maladaptive plasticity underlying the development of CPS; specifically, that the increase in spontaneous and bursting activity in APT contributes to the changes seen in ZI and
PO function, resulting in aberrant pain processing that contributes to the development of CPS.
**FIGURE LEGENDS**

**Figure 1**
APT activity is increased during CPS. (A) Spontaneous firing rate of APT neurons in sham-operated controls ($n = 57$) and in spinal-lesioned rats with behaviorally confirmed CPS ($n = 107$). (A, E, F) Boxes represent the 25th and 75th percentile of distribution; dashed lines represent mean values; whiskers show the 10th and 90th percentiles. (B) Spontaneous firing rate plotted against recording depth and fitted with a linear regression line for sham-operated (dashed; $r^2 = 0.04$) and lesioned (solid; $r^2 = 0.05$) rats. Spontaneous firing rate increases with recording depth for lesioned, but not sham, rats. (C) A micrograph of a coronal section depicting a representative lesion (red asterisk) marking the location of a recorded neuron indicated in B (red). The outline demarcates the borders of APT, and the numerals indicate depth from the pial surface. (D) Cumulative probability plot showing the distribution of spontaneous firing rates of APT neurons in sham-operated and lesioned rats. Spinal lesion (black circles) results in a significant rightward shift in the distribution of spontaneous firing rate. (E) Incidence of bursts of action potentials is increased in APT of CPS animals, compared to sham-operated controls. (F) Spontaneous firing rate of non-bursting cells in APT in control ($n = 44$) and CPS animals ($n = 60$). Non-bursting cells in APT show increased spontaneous firing rate during CPS, compared to controls.

**Figure 2**
Firing patterns of representative APT neurons from each of the 3 distinct neuronal populations. (A, C, E) Interspike intervals (ISI; left column) and autocorrelograms (right) are shown for each representative neuron. Fast Bursting neurons (A) are characterized by sharp autocorrelogram peaks and a narrow ISI distributions at very
short interval (< 0.01 sec). Tonic neurons (C) are characterized by autocorrelograms that lack a central peak and a wide ISI distribution due to the paucity of very short ISIs (see also Table 2). Slow Rhythmic neurons (E) are characterized by a broad central peak in the autocorrelogram and wider ISI distribution compared to Fast Bursting cells. Bin size in all histograms is 1 msec. (B, D, F) Example raster plots of spike patterns (top) and waveforms (bottom) for each unit. Distinct firing patterns characteristic of the three neuron populations are evident. (G) Relative incidence of Fast Bursting, Tonic and Slow Rhythmic neurons is not changed in CPS.

**Figure 3**

Effect of spinal lesions on APT neuron subtypes. Spontaneous firing rate (left) and bursts per minute (right) of Fast Bursting (A), Tonic (B) and Slow Rhythmic (C) neurons in sham-operated and spinal-lesioned rats. (A, B, C) Boxes represent the 25th and 75th percentile of distribution; dashed lines represent mean values; whiskers show the 10th and 90th percentiles. Significant increases were observed in spontaneous firing rate of Tonic neurons (B, left) and in bursts per minute of Fast Bursting (A, right) and Slow Rhythmic (C, right) neurons.

**Figure 4**

Effects of APT inactivation on PO response properties. (A) Line drawings superimposed on serial brain atlas sections (Paxinos and Watson 1998) summarizing the location and size of APT lesions (n = 3). Red trace corresponds to lesion shown in 4C. APT, anterior pretectal nucleus; DG, dentate gyrus; MGd, dorsal region of medial geniculate nucleus; MGv, ventral region of medial geniculate nucleus; MPT, medial pretectal nucleus; OPT, olivary pretectal nucleus; OT, nucleus of the optic tract; PO, posterior thalamic nucleus; PoT, posterior triangular thalamic nucleus; PPT, posterior pretectal nucleus; SG,
suprageniculate nucleus; VPM, ventral posteromedial thalamic nucleus; ZId, dorsal zona incerta; ZIv, ventral zona incerta. (B) Spontaneous activity of PO neurons is increased following APT lesions. (C) Photomicrograph or a coronal section from a rat that received APT lesion (5.30 mm posterior to bregma). Note that the extent of the lesion, delineated by red trace in 4A, covers nearly the entire APT (demarcated with a broken line). (D, F) PSTHs constructed from responses of PO neurons to a 50 msec air puff delivered to the vibrissae at time $t = 0$ in naive and in APT-lesioned animals. Horizontal dashed lines depict response magnitude levels exceeding (99% confidence interval) spontaneous activity levels (computed from a 100 msec period preceding stimulus onset). (D) PSTHs from two representative short-latency (onset $\leq 30$ msec) neurons from a control animal and an animal following APT lesion. (E) Grouped data of response magnitude of short latency neurons from naive ($n = 8$) and APT-lesioned ($n = 7$) animals. Lesioning APT caused a significant increase in response magnitude of PO cells. (F) PSTHs from two representative long latency (onset $> 30$ msec) neurons from a naive animal and APT-lesioned animal. (G) Grouped data of response magnitude of long latency neurons from naive ($n = 9$) and APT-lesioned animals ($n = 21$). APT lesion did not affect response magnitude in long latency PO neurons.

**Figure 5**

Schematic representation of activity changes in APT neuronal subtypes during CPS. Increased activity of GABAergic Tonic neurons (orange) projecting to ZI during CPS leads to a decrease in ZI activity and, consequently, to decreased inhibitory drive to PO (bright red). The increased activity of PO-projecting Tonic neurons is insufficient to counter the abnormally high PO activity that results. Excitatory Slow Rhythmic neurons (green), which project to both ZI and PO, show increased bursting activity (represented
by dashed lines) without changing their overall firing rate. Fast Bursting neurons (dark red), which project almost exclusively to PO, show increased bursting during CPS while their overall firing rate remains unchanged. Abnormally high PO activity leads to increased activation of cortical areas responsive to somatosensory input.
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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.
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Table 1
Firing properties of bursting APT neurons from spinal-lesioned and control rats.

<table>
<thead>
<tr>
<th></th>
<th>Spont. firing rate (Hz)</th>
<th>Bursts per min.</th>
<th>% Spikes in bursts</th>
<th>Mean burst duration (msec)</th>
<th>Mean spikes in burst</th>
<th>Mean ISI in burst (msec)</th>
<th>Mean freq. in burst (Hz)</th>
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<tbody>
<tr>
<td>Sham (n = 13)</td>
<td>4.1 ± 5.2</td>
<td>4.1 ± 5.2</td>
<td>12.3 ± 13.9</td>
<td>17 ± 7</td>
<td>4.8 ± 1.3</td>
<td>4.4 ± 1.3</td>
<td>248 ± 62</td>
</tr>
<tr>
<td>Lesion (n = 47)</td>
<td>7.3 ± 6.4</td>
<td>7.3 ± 6.4</td>
<td>12.7 ± 18.3</td>
<td>20 ± 10</td>
<td>5.2 ± 1.7</td>
<td>4.8 ± 1.3</td>
<td>232 ± 71</td>
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<tr>
<td>p-value</td>
<td>0.08</td>
<td>0.2</td>
<td>0.5</td>
<td>0.3</td>
<td>0.4</td>
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Values are means ± SD, n = 13 sham, 47 lesion. See METHODS for burst criteria.
Table 2
Firing properties of bursting cells within specific classes of APT neurons from spinal-lesioned and control rats

<table>
<thead>
<tr>
<th>Class</th>
<th>Sham (n = 12)</th>
<th>Lesion (n = 28)</th>
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<tr>
<td></td>
<td>Spont. firing rate (Hz)</td>
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<td>Bursts per min.</td>
<td>12 ± 24</td>
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<td>Mean burst duration (msec)</td>
<td>15 ± 6.1</td>
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<td></td>
<td>Mean spikes in burst</td>
<td>4.6 ± 1.1</td>
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<td></td>
<td>Mean ISI in burst (msec)</td>
<td>4.1 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>Mean freq. in burst (Hz)</td>
<td>261 ± 54</td>
</tr>
<tr>
<td></td>
<td>Mean peak freq. in burst (Hz)</td>
<td>340 ± 80</td>
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**Fast Bursting**

<table>
<thead>
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<th>Class</th>
<th>Sham (n = 1)</th>
<th>Lesion (n = 7)</th>
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<tr>
<td></td>
<td>Spont. firing rate (Hz)</td>
<td>4.7</td>
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<td></td>
<td>Bursts per min.</td>
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<td></td>
<td>Mean burst duration (msec)</td>
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<td></td>
<td>Mean spikes in burst</td>
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<tr>
<td></td>
<td>Mean ISI in burst (msec)</td>
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<tr>
<td></td>
<td>Mean freq. in burst (Hz)</td>
<td>220</td>
</tr>
<tr>
<td></td>
<td>Mean peak freq. in burst (Hz)</td>
<td>343</td>
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</table>

**Tonic**

<table>
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<th>Lesion (n = 10)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Spont. firing rate (Hz)</td>
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<tr>
<td></td>
<td>Bursts per min.</td>
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<td>Mean burst duration (msec)</td>
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<td></td>
<td>Mean spikes in burst</td>
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<td></td>
<td>Mean ISI in burst (msec)</td>
<td>7.7</td>
</tr>
<tr>
<td></td>
<td>Mean freq. in burst (Hz)</td>
<td>130</td>
</tr>
<tr>
<td></td>
<td>Mean peak freq. in burst (Hz)</td>
<td>156</td>
</tr>
</tbody>
</table>

Values are means ± SD. Only means are given for sham-operated Tonic and Slow Rhythmic neurons where n = 1. See METHODS for burst criteria. No parameter showed a significant difference between sham and lesion groups.