Network Actions of Pentobarbital in the Rat Mesopontine Tegmentum on Sensory Inflow Through the Spinothalamic Tract

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INTRODUCTION

Despite the advances made in our understanding of the molecular mechanistic actions of general anesthetics, very little is known about the in vivo neural circuits involved in expressing the behavioral state of general anesthesia (Belelli et al. 1999; Campagna et al. 2003; Collins et al. 1995). How ever, few electrophysiological studies have actually identified which sensory projection neurons are responsible for this anesthetic-induced cortical deafferentation.

On the other hand, Devor and colleagues recently discovered a discrete brain stem area in the rat, termed the mesopontine tegmental anesthesia area (MPTA), that was claimed to be the quintessential “hot spot” for pentobarbital (PB) anesthesia (Devor and Zalkind 2001). These investigators reported that microinjections of PB into the MPTA of conscious behaving rats resulted in a state that resembled general anesthesia that was characterized by antinociception, atonia, and the loss of consciousness, along with a shift in the electroencephalog raphic (EEG) waveform signature from a low-voltage, desynchronized, “fast-wave” pattern to a high-voltage, synchronized, “slow-wave” pattern (Devor and Zalkind 2001). Further detailed anatomical studies by Devor and colleagues also indicated that MPTA neurons project to sensory (Sukhotinsky et al. 2005) and motor (Sukhotinsky et al. 2005) control systems in the brain and the spinal cord that could mediate the antinociceptive and atonic components of PB anesthesia, respectively. However, a dearth of electrophysiological evidence exists for understanding the mechanisms underlying the antinociceptive and atonic components of the anesthesia-like state following PB microinjections into the MPTA.

The antinociception, reported by Devor and Zalkind (2001) as “analgesia,” was based on standard rodent behavioral tests and may arise as a consequence of any number of network processes, including a brain stem-mediated reduction of spinal sensory inflow via classical ascending spinal sensory pathways and/or spinal motor outflow. Antinociception scores in the studies of Devor and Zalkind (2001) may also be the consequence of atonia alone.

Accordingly, in the present study we tested the principal hypothesis that the bilateral microinjections of PB into the MPTA of the isoflurane-anesthetized rat suppress sensory inflow through the spinothalamic tract (STT). Specifically, the spontaneous and both the antidromically and the orthodromically evoked spike activities of STT neurons in an acute, in vivo rat preparation were quantified before and after the bilateral microinjections of PB into the MPTA.

METHODS

All experimental procedures were approved by the University of British Columbia Committee on Animal Care and complied with the...
Surgical procedures

INDUCTION OF ANESTHESIA. Anesthesia was induced by placing the rat in an anesthetizing box (# 500108, Harvard Apparatus, Holliston, MA) that was supplied with a continuous stream of isoflurane (4%) and nitrous oxide (0.6 L/min) in oxygen (3 L/min). Once the animal lost its righting reflex, it was removed from the chamber and the anesthetic mixture thereafter was delivered through a custom-made nose cone until tracheal intubation. Once the trachea was intubated, the isoflurane and oxygen levels were then lowered to 2.5% and 1 L/min, respectively. The isoflurane levels were controlled using a calibrated isoflurane solution vaporizer (Model 100F; Ohio Medical Products, Airco, Madison, WI). A surgical plane of anesthesia was confirmed by the absence of blink and toe-pinch reflexes. The rectal body temperature was constantly monitored and maintained at 37°C using a feedback-controlled heating blanket. Enrofloxacin (5 mg/kg, administered intramuscularly) was given and lubricating ophthalmic ointment (Lacrilube) was applied to the eyes to prevent corneal drying. Warm, lactated Ringer solution was administered as a continuous drip (10 mL/h) using the following stereotaxic coordinates: anteroposterior (AP): -1.2, medial-lateral (ML): 0, dorsoventral (DV): 0.5. A surgical plane of anesthesia was confirmed by the absence of blink and toe-pinch reflexes. The lungs were inflated and deflated with a mechanical respirator (Inspira; Harvard Apparatus) for the entire length of the experiment. The animal was artificially ventilated using a mechanical respirator (Inspira; Harvard Apparatus) for the entire length of the experiment.

PERIPHERAL NERVE SURGERY, SPINAL LAMINECTOMY, AND CRANIO- OTOMY PROCEDURES. The left sciatic (Sc) and sural (Su) nerves were exposed and bathed in a pool of warm mineral oil. These nerves were carefully draped on bipolar hook electrodes to synaptically activate the recorded STT neurons (Fig. 1).

A laminectomy was then performed to expose the T3 and L1 spinal cord segments. The exposed dura mater was carefully cut, reflected away from the spinal cord, and the spinal cord was covered with a small pool of warm mineral oil. The T3 and L1 vertebrae were tightly secured by two spinal clamps (Model 986B, David Kopf Instruments, Tujunga, CA) that, in turn, were anchored to a stereotaxic frame (Model 1430, David Kopf Instruments).

The rat’s head was secured in the frame at stereotaxic zero using ear bars and a palate bar. A midline incision was made in the scalp to expose the calvarium. A trephination was made in the parietal bone, contralateral to the site of the peripheral nerve surgery for introducing a microelectrode into the ventral posterior lateral (VPL) nucleus of the thalamus. In addition, two trephinations were made bilaterally along the central suture in the left and right parietal bones for insertion of microinjection cannulae into the MPTA. Finally, bilateral trephinations were made in the frontal bones to fix screws for recording the cortical EEG, which was used to confirm the surgical plane of anesthesia during neuromuscular blockade. The filters on the recording amplifier were set between 0.5 (high-pass) and 35 (low-pass) Hz (half-amplitude attenuation) (Bjorvatn et al. 1998). A 60-Hz notch filter was also used.

Identification of spinothalamic tract (STT) neurons

On completion of all surgical procedures, isoflurane levels were then adjusted to the lowest levels possible (1.5–1.75%) while still maintaining a steady plane of surgical anesthesia, as confirmed by the EEG and the absence of corneal and pinch reflexes. A tungsten microelectrode (“S” in Fig. 1) was positioned into the VPL nucleus using the following stereotaxic coordinates: anteroposterior (AP): -1.2, medio-lateral (ML): 0, dorsoventral (DV): 0.5. A monopolar tungsten electrode (R) was directed toward the ventral posterior lateral (VPL) nucleus of the thalamus, contralateral to the L1 spinal cord segment; the L1 spinal cord was recorded through another monopolar tungsten electrode (R). The identified STT neuron was synaptically activated by stimulating sciatic (Sc) and sural (Su) nerves via 2 bipolar hook electrodes. Conventional techniques were used to amplify and quantify STT neuron activity. PB and/or Vh solutions were microinjected into the MPTA bilaterally using microinjection cannulae (MIC). A, amplifier; CED, spike data acquisition hardware; DRG, dorsal root ganglion cell; PC/Spike2: spike date acquisition, storage, and analysis software system; WD, window discriminator.
procedures are provided in the following text.

and test (postmicroinjections) conditions. Brief descriptions of these neurons—were quantified under control (baseline, premicroinjection) parameters—spontaneous firing rate (SFR), antidromic firing index (AFI).

Indeed, Devor and Zalkind (2001) were adamant on the importance of “on-target” PB microinjections in the MPTA, per se, for inducing all of the classical signs of anesthesia. Accordingly, we focused only on intra-MPTA microinjections in the present study.

The extracellular spike activity of an individual L1 STT neuron was recorded using a tungsten microelectrode ("R" in Fig. 1) that was gradually lowered into the spinal gray matter using a Kopf hydraulic microdrive (Model 650, David Kopf Instruments). Spike activity was amplified (×10,000) and filtered (band-pass: 100–10,000 Hz) using an AC-coupled differential amplifier (Model 1800, A-M Systems, Sequim, WA; "A" in Fig. 1). The amplified signal was then routed to an oscilloscope (Model D11, Tektronix, Beaverton, OR) and a spike processor (Model D130; Digitimer, Hertfordshire, UK; "WD" in Fig. 1), which was manually set to detect only the STT action potential waveforms. The amplified and filtered STT spike activity as well as the EEG signals were recorded and digitized “on-line” into separate waveform channels using a Pentium 4 computer equipped with spike acquisition and analysis software (Spiketrack, version 5; Cambridge Electronics Design (CED), Cambridge, UK) and hardware (Power1401 plus; CED). The sampling rates for the STT spike wave data and EEG were 20 kHz and 100 Hz, respectively. Data files of each STT neuron recording were stored on a data server for subsequent off-line analysis using specialized subroutine scripts.

A spike-template algorithm was used routinely to ensure that action potentials of the STT neuron were stable throughout each experiment. Once a stable spike waveform was obtained, four electrophysiological parameters—spontaneous firing rate (SFR), antidromic firing index (AFI), and both Sc- and Su-evoked responses (ERs) of the STT neurons—were quantified under control (baseline, preinjection) and test (postmicroinjections) conditions. Brief descriptions of these procedures are provided in the following text.

Spike data acquisition and amplification procedures

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SPONTANEOUS SPIKE ACTIVITY. The ongoing spontaneous spike activity for each STT neuron was assessed by quantifying firing rate (Soja et al. 1996; Taeppavarapruk et al. 2002). The average SFR (spikes/s) for each STT neuron was calculated from 2-min epochs of the spike waveform under control and test conditions.

PERIPHERAL-NERVE-EVOKED RESPONSES. Synaptic responses of STT neurons were evoked by electrical stimulation of the sciatic (Sc, mixed) and sural (Su, cutaneous) nerves. Briefly, bipolar hook electrodes were used to deliver single-pulse stimuli (0.1 ms, 0.67 Hz) via a stimulator (Model 2100, A-M Systems, Carlsborg, WA) to the Sc and Su nerves. For each nerve, the threshold intensity (T) was first determined as the minimum intensity (μA) required to produce a single or short train of orthodromic action potentials recorded from the STT neuron (Soja et al. 1993, 1995). Then, peripheral-nerve–evoked responses to 50 consecutive stimuli (at 2T) were quantified using specialized Spike2 scripts. The scripts generated poststimulus time histograms (PSTHs; bin width: 0.5 ms) of spike activity, calculated the average response magnitude (mean number of spikes/stimulus trial), and response latency (ms) from user-defined PSTH epochs. Stimulation of the peripheral nerves at the intensities used during the control and test paradigms produced a hindlimb twitch. Thus before assessing peripheral-nerve–evoked responses, the rat was paralyzed with a single dose of pancuronium bromide (0.03–0.3 mg/kg, iv). The dose of pancuronium was repeated as required throughout the experiment. During the pancuronium-induced paralysis, the surgical plane of anesthesia was confirmed by characteristic large-amplitude, slow EEG wave activity. The amplitude, waveform, and spike rate of recorded STT neurons were not altered by pancuronium.

ANTIDROMIC FIRING INDEX (AFI). To determine the FI, threshold, antidromic stimuli, consisting of 100 triple-pulse trains (pulse width: 0.1 ms, interpulse interval: 3 ms, 0.67 Hz), were delivered to the VPL nucleus using a stimulator (Model 2100, A-M Systems). The FI was expressed as a ratio of antidromic spikes/75 stimulus trials, which were corrected for any spontaneous collisions using the following equation (Lloyd and McIntyre 1955): Firing Index (FI) = (Number of antidromic spikes/number of stimulus trials) × 100.

Drug and control solutions

Pentobarbital sodium (Dumex Medical Surgical Products, Pickering, ON, Canada), generously provided by Dr. Ernest Puil (Professor Emeritus, Department of Anesthesiology, Pharmacology and Therapeutics, University of British Columbia), was dissolved in a vehicle composed of 10% ethanol and 20% propylene glycol (Sigma–Aldrich, St. Louis, MO) in water to make a final concentration of 200 μg/mL (800 nM) (Devor and Zalkind 2001). This was the highest effective dose reported by Devor and Zalkind (2001) and was thus appropriately chosen for the present study. PB-free vehicle was used as control. Both PB and vehicle control solution (Vh) were filtered through a 0.2-μm Millipore filter prior to use.

Intra-MPTA microinjections

Devor and Zalkind (2001) emphasized clearly in their study that “off-site” microinjections of PB either induced no anesthesia or only induced partial sedation or insignificant atonia and/or antinociception. Indeed, Devor and Zalkind (2001) were adamant on the importance of “on-target” PB microinjections in the MPTA, per se, for inducing all of the classical signs of anesthesia. Accordingly, we focused only on intra-MPTA microinjections in the present study.

Two custom-length (~5.5 cm) hypodermic stainless steel tubes (Catalog No. 832400, regular wall, 29-gauge; A-M Systems) were used as cannulae for the microinjections of PB or Vh into the MPTA (“MIC” in Fig. 1). Each cannula was firmly fastened to a micromanipulator (Kopf 1460-61, David Kopf Instruments). The cannulae were prefilled with either Vh or PB and then slowly lowered bilaterally into the brain toward the MPTA (Fig. 1), targeting the following stereotaxic coordinates: −7.2 to −8.0 mm posterior to bregma, ±1.1 to 1.2 mm lateral to the midline, and 7.0 to 7.5 mm ventral to the surface of the brain (Devor and Zalkind 2001; Paxinos and Watson 2007; Voss et al. 2005). A total volume of 1 μL/side of PB or Vh was slowly injected simultaneously into the both sides of MPTA using a calibrated syringe infusion pump (Model 22, Harvard Apparatus, South Natick, MA). The microinjection cannulae were left in place for 15 min after completion of the injections to avoid backtracking of the injection fluid into the cannula trajectory (Devor and Zalkind 2001). The bilateral microinjections in all the experiments were completed over a time period of 4 min.

Baseline and postmicroinjection data collection

In each experiment, before the microinjections of Vh/PB, the four STT parameters (SFR, FI, Su-ER, and Sc-ER) were sequentially measured over a collection cycle of 4.5 min to establish a baseline. The epoch times for SFR, FI, Su-ER, and Sc-ER in the collection cycle were 2, 0.5, 1, and 1 min, respectively. Following the baseline measurements, the microinjections were carried out and the time when bilateral microinjections of PB or Vh were completed was defined as time 0. The collection cycles were then repeated at four time points, each starting at 2, 15, 30, and 60 min following time 0. Bar histograms of mean data are plotted in figures corresponding to the 2-, 15-, and 60-min time points.
Histology of the microinjection sites

In six rats, at the end of the experiment, pontamine blue dye (2% in 4 M NaCl, 1 μL/side) was bilaterally microinjected at the same stereotaxic coordinates used for Vh/PB microinjections in that experiment. One hour after the dye microinjections the animal was perfused; the brain was removed, blocked, placed in cold 4% paraformaldehyde, and postfixed for 24 h. Coronal sections (40–50 μm) of the brain were then made with a microtome (Vibratome 1000 Classic, St. Louis, MO), slide mounted, counterstained with hematoxylin QS (Vector Laboratories, Burlingame, CA), and permanently fixed for analysis. Selected brain sections were imaged using a digital scanner (Vector Laboratories, Burlingame, CA), and permanently fixed for the brain were then made with a microtome (Vibratome 1000 Classic, St. Louis, MO), slide mounted, counterstained with hematoxylin QS (Vector Laboratories, Burlingame, CA), and permanently fixed for analysis. Selected brain sections were imaged using a digital scanner system (ScanScopeCS, Aperio Technologies, Vista, CA) at ×40 magnification. The images were digitally processed with ImageScope (Aperio Technologies) and Adobe Photoshop version 7.0 (Adobe Systems, Seattle, WA). Brains of six additional rats were perfused without pontamine blue dye microinjections and counterstained as described earlier. In these six brains, the microinjection sites were determined by locating the tips of the track marks made by the microinjection cannulae. The anatomical sites of microinjections obtained from all of the 12 rats were plotted on standard coronal section of the brain from the rat brain atlas of Paxinos and Watson (2007).

Statistical analyses

Due to the inherent technical difficulties associated with this type of experiment (Fig. 1), it was not possible to test every STT neuron with both Vh and PB microinjections. Thus STT neurons examined in this study were divided post hoc into three groups according to the type of data collected. Accordingly, Group I consisted of six STT neurons whose spike parameters were measured before and after microinjections of Vh only. Group II consisted of six STT neurons whose spike parameters were measured before and after microinjections of PB only. Finally, Group III consisted of six STT neurons where propitious recording conditions allowed data collection before and after Vh as well as before and after PB microinjections. In the case of STT neurons in Group III, Vh was bilaterally microinjected first and the four parameters were assessed at the standard four time points as described earlier. After the last data collection cycle (i.e., 60-min time point), a 90-min time gap was allowed before baseline parameters were measured again. Then, PB was microinjected into the MPTA and subsequent postmicroinjection collection cycles were repeated.

The statistical analysis was performed using SigmaStat (version 3.5; Systat Software, Chicago, IL). A one-way ANOVA was used to compare the respective baseline values of each parameter of STT neurons among the three groups. Data between two groups were combined for further statistical analyses only if initial ANOVA analyses of baseline values indicated no differences existed. In this study, the principal objective was to detect significant differences in each STT neuron parameter postmicroinjection at each time point against the respective premicroinjection baseline values rather than among the time points. Values of each electrophysiological parameter measured before microinjections served as the baseline (control). Values at each time point (i.e., 2, 15, 30, and 60 min) following microinjections (test) were compared against the respective control values using a one-way repeated-measures (RM) ANOVA. The significant differences were further analyzed with a Bonferroni test. Nonnormally distributed data were analyzed using Friedman RM-ANOVA on ranks. In all cases, P < 0.05 was considered statistically significant. All the values in RESULTS are expressed as means ± SE unless otherwise noted, such as mean ± SD.

RESULTS

General characteristics of STT neurons

In all, 18 spinothalamic tract (STT) neurons were recorded extracellularly from the L1 spinal segment. Each STT neuron satisfied three standard antidromic criteria: constant latency, high-frequency following, and collision between antidromically and orthodromically propagated action potentials (Fig. 2, A–C). All STT neurons were recorded in vertical electrode tracks located about 0.5 mm lateral from the midline. The mean (±SD) recording depth of all 18 STT neurons was 772 ± 397 μm (range: 107–1,528 μm) below the surface of the spinal cord. The estimated depths of spinal recording and thalamic stimulating sites of STT neurons are depicted in Fig. 2, D and E, respectively. The mean (±SD) antidromic latency of all 18 STT neurons, as measured from the onset of the stimulus artifact to the onset of the antidromic action potential, measured 4.4 ± 0.6 ms (range: 3.7–5.8 ms). The mean (±SD) antidromic conduction distance measured 81.4 ± 7.6 mm (range: 60–90 mm). This corresponds to an average axonal conduction velocity of 18.6 ± 2.5 m/s (range: 14–23 m/s).

Effect of pentobarbital microinjections into the MPTA on STT neuron spike activity

In the present study, the most significant observation following bilateral microinjections of PB into the MPTA was suppression of all STT neuron parameters, i.e., SFR, FI, Sc-, and Su-ER. The action of PB was observed within 2 min of microinjections and lasted for 15–30 min following the microinjections. Detailed results of each of the parameters are reported in the following text.

Spontaneous firing rate

BASELINE VALUES. All of the STT neurons examined in this study under isoflurane anesthesia (see METHODS) displayed spontaneous spike activity. The mean (±SE) baseline SFR for the sample population of 18 STT neurons measured 13.2 ± 3 spikes/s (range: 4–39). The mean (±SE) baseline SFR of STT neurons in Groups I, II, and III measured 8.6 ± 2.1 spikes/s (range: 4–18), 11.8 ± 2.9 spikes/s (range: 5–22), and 17.7 ± 5.1 spikes/s (range: 3–39), respectively (Fig. 3, C and D). The baseline firing rates did not differ significantly among STT neurons in all three groups [F(2,15) = 2.5, P = 0.12, one-way ANOVA].

VEHICLE CONTROL SOLUTION (Vh) MICROINJECTIONS. Bilateral microinjections of Vh into the MPTA did not alter the mean SFR of STT neurons in Group I [Fig. 3C, n = 6, checkered bars; F(4,18) = 0.3, P = 0.87, one-way RM-ANOVA] or Group III [Fig. 3C, n = 6, gray bars; F(4,20) = 0.72, P = 0.59, one-way RM-ANOVA]. Similarly, SFR values were not altered by Vh microinjections when the corresponding SFR values for Groups I and III were combined [Fig. 3C, n = 12, black bars; F(4,42) = 0.47, P = 0.76, one-way RM-ANOVA]. A typical continuous rate-meter histogram depicting the SFR of a STT neuron around bilateral Vh microinjections is presented in Fig. 3A.

PENTOBARBITAL (PB) MICROINJECTIONS. In contrast to Vh, the mean (±SE) SFR of Group II STT neurons (Fig. 3D, diagonal lines-filled bars) was significantly reduced by about 53% from a baseline SFR of 11.8 ± 2.9 to 5.3 ± 1.4 spikes/s 2 min following the completion of the PB microinjections [F(4,20) = 4.05, P = 0.04, one-way RM-ANOVA]. The suppressed SFR...
recovered toward the baseline within 15 min following PB microinjections. Similarly, the mean (±SE) SFR of Group III STT neurons was significantly reduced by 36% from the baseline SFR of 20.1 ± 4.82 to 12.8 ± 6.1 spikes/s following PB microinjections [Fig. 3D, gray bars; \( F(4,18) = 3.37, P = 0.04 \), one-way RM-ANOVA]. The SFR returned toward baseline within 15 min.

Interestingly, in four of six STT neurons in Group III, following recovery, there was a significant decrease in the mean SFR at the 60-min time point. As a result, when compared with the baseline SFR, the mean SFR at the 60-min time point for all six STT neurons in Group III was decreased by about 56% to 8.7 spikes/s [Fig. 3D, gray bars; \( F(4,18) = 3.37, P = 0.04 \), one-way RM-ANOVA]. The SFR returned toward baseline within 15 min.

Peripherally evoked STT responses

In the present study, low-intensity stimuli not exceeding 2T were applied to the Su (cutaneous) and Sc (mixed) nerves to synaptically activate STT neurons. Stimuli at 2T intensity do not activate C fibers. Therefore nonnoxious synchronous input to the STT neurons provided by 2T stimuli would not be expected to induce long-term potentiation (LTP) or long-term depression (LTD) mechanisms impinging on STT neurons (Ikeda et al. 2000; Liu and Sandkuhler 1995, 1997) and primary afferents (Randic et al. 1993). The mean (±SE) threshold intensity for Su and Sc nerve stimulation measured 1.8 ± 0.4 mA (range: 0.2–5 mA) and 1.4 ± 0.7 mA (range:...
0.08–10 mA), respectively. In the present series of experiments, the synaptic activation of most STT neurons by Su and Sc nerves under isoflurane anesthesia resulted in a complex response. The evoked response consisted of a short-duration presynaptic afferent volley, followed by a field potential and, in most cases, a burst of STT neuron spikes (Figs. 4A and 5A). Interestingly, in two (#7 and #8) of 18 STT neurons, no action potentials occurred following stimulation of either Su or Sc nerve at 2T intensity. In two other STT neurons, the responses occurred with stimulation of either only Su (#9) or Sc (#11) nerve. Thus Su- and Sc-ER were recorded and measured in 15 of 18 STT neurons. The synaptic responses of all STT neurons were obtained by stimulating Su or Sc nerve at 2T.

For three STT neurons in particular, stimulation of only Sc nerve evoked short-latency “early” responses and long-latency “late” responses (see an example in Fig. 5A, Baseline). Beall et al. (1977) observed similar early and late burst discharges of STT neurons, evoked by Su nerve stimulation in pentobarbital-anesthetized primates, which were related to volleys in Aβ and A6 fibers, respectively. It is unlikely that the “late” component of the evoked responses for STT neurons in the present study was due to activation of small-diameter fibers.

Prior to pharmacological studies with Vh or PB, additional control experiments were performed (data not shown) to verify that our peripheral nerve stimulation protocol did not induce habituation phenomenon (Macdonald and Pearson 1979). Thus the observed effects of microinjected PB were due to its action in the MPTA (see following text).

**SURAL-NERVE–EVOCKED STT RESPONSES.** Baseline values. The mean (±SE) Su-ER magnitude of STT neurons in Groups I (n = 5), II (n = 6), and III (n = 4) at baseline measured 6 ± 2 spikes/trial (range: 2–14), 8.8 ± 2.3 spikes/trial (range: 3–18), and 7.1 ± 1.1 spikes/trial (range: 4–10), respectively. The corresponding baseline latencies measured 14.8 ± 5.9 ms (range: 13–17), 16.1 ± 5.2 ms (range: 15–18), and 14 ± 2.9 ms (range: 13–15), respectively. The baseline values of either the response magnitude [F(2,12) = 0.53, P = 0.6, one-way ANOVA] or response latency [F(2,12) = 0.054, P = 0.96, one-way ANOVA] of the three groups did not differ significantly from each other. The results of Su-ER of STT neurons are illustrated in Fig. 4.

**Vehicle control solution (Vh) microinjections.** Microinjections of Vh did not alter the magnitude or latency of Su-ER of STT neurons, irrespective of whether it was Group I [Fig. 4C, n = 6, checked bars; F(4,14) = 1.53, P = 0.24, one-way RM-ANOVA], Group III [Fig. 4C, n = 4, gray bars; F(4,12) = 0.35, P = 0.84, one-way RM-ANOVA], or a combined data set of Groups I and III [Fig. 4C, n = 12, black bars; F(4,30) = 1.17, P = 0.34, one-way RM-ANOVA].

**Pentobarbital (PB) microinjections.** The effects of PB microinjections on the magnitude Su-ER of STT neurons are summarized in Fig. 4D. Within 2 min following PB microinjections, the mean (±SE) response magnitude of STT neurons in Group II was significantly decreased by 45% from the baseline magnitude of 8.8 ± 2.3 spikes/trial (Fig. 4D, n = 6, diagonal lines-filled bars; P = 0.007, Friedman one-way repeated-measures [RM] ANOVA).
In contrast to Vh, the mean (± SE) response magnitude of Sc-ER of STT neurons in Group II was significantly reduced within 2 min following PB microinjections by about 35% to 6.3 ± 2.7 spikes/trial compared with the baseline magnitude of 9.7 ± 2.8 spikes/trial (Fig. 5D, n = 6, diagonal lines-filled bars; P = 0.03, Friedman one-way RM-ANOVA on ranks). Similar to Su-ER, the mean response magnitude of Sc-ER of STT neurons in Group III was not altered following PB microinjections [Fig. 5D, n = 4, gray bars; F(4,12) = 0.88, P = 0.51, one-way RM-ANOVA]. However, when STT neuron data of Groups II and III were combined, the mean response magnitude was significantly reduced within 2 min following PB microinjections by 26% to 7.2 ± 1.6 spikes/trial (Fig. 5D, n = 10, black bars; P = 0.006, Friedman one-way RM-ANOVA on ranks). Recovery following PB microinjections toward the preinjection baseline occurred within 15 min. The mean latency of Sc-ER of STT neurons in all three groups did not change following PB microinjections (for all groups, P > 0.05, one-way RM-ANOVA).

**Vehicle control solution (Vh) microinjections.** Microinjections of Vh did not alter the response magnitude of Sc-ER of STT neurons (Fig. 5C, n = 5, checkered bars; F(4,15) = 0.1, P = 0.44, one-way RM-ANOVA) and III [Fig. 5C, n = 9, black bars; F(4,31) = 0.51, P = 0.73, one-way RM-ANOVA].

**Sciatic-nerve-evoked responses.** Baseline values. Results of Sc-ER of STT neurons are illustrated in Fig. 5. The mean (± SE) baseline response magnitudes of STT neurons in Groups I (n = 5), II (n = 6), and III (n = 4) measured 6.3 ± 2.3 spikes/trial (range: 3–15), 9.7 ± 2.8 spikes/trial (range: 1–17), and 8.1 ± 1.2 spikes/trial (range: 5–11), respectively. The corresponding mean (± SE) baseline response latencies measured 47.2 ± 2.4 ms (range: 44–54), 42.3 ± 3.6 ms (range: 31–54), and 44.5 ± 0.5 ms (range: 43–46), respectively. The baseline values of either response magnitude [F(2,12) = 0.51, P = 0.61, one-way ANOVA] or response latency [F(2,12) = 0.78, P = 0.58, one-way ANOVA] did not differ significantly among the three groups.
Finally, the latency and magnitude of the afferent volley recorded in response to stimulation of either Su or Sc nerve were not altered following microinjections of either Vh or PB (data not shown).

Antidromic firing index

The antidromic firing index (FI) was used to indirectly measure the postsynaptic excitability of the STT neurons. The FI method has been used to assess changes in the postsynaptic excitability of motor neurons (Hunt 1955; Lloyd and McIntyre 1955; Wilson and Burgess 1962), group Ia and Ib afferents (Wall 1958; Willis et al. 1976), tooth pulp afferents (Cairns et al. 1996; Lisney 1979), and some descending fiber systems (Rudomin and Jankowska 1981). A decrease in the antidromic FI can be attributed to hyperpolarization due to supraspinal-mediated shunting of the soma (Lipski 1981) and/or disfacilitation (Wilson and Burgess 1962; Zhang et al. 1991a,b).

BASELINE VALUES. Figure 6 illustrates the results of effects of Vh and PB microinjections on FI. The mean (±SE) baseline FI of all 18 STT neurons was 87.9 ± 3.3 (range: 53–100). The mean (±SE) baseline FI of STT neurons in Groups I, II, and III were 82.3 ± 6.1 (range: 63–99), 88.9 ± 7.4 (range: 53–100) and 93.8 ± 2.1 (range: 86–100), respectively (Fig. 6, C and D). The baseline FI of the three groups did not differ significantly from each other [F(2,15) = 1.04, P = 0.58, one-way ANOVA].

VEHICLE CONTROL SOLUTION (VH) MICROINJECTIONS. Microinjections of Vh into the MPTA did not change the mean FI of the STT neurons in either of Groups I [Fig. 6C, n = 6, checkered bars; F(3,13) = 1.11, P = 0.38, one-way RM-ANOVA] or III [Fig. 6C, n = 6, gray bars; P = 0.18, Friedman’s one-way RM-ANOVA on ranks] as well as their combined data set [Fig. 6C, n = 12, black bars; F(4,42) = 1.33, P = 0.27, one-way RM-ANOVA].

PENTOBARBITAL (PB) MICROINJECTIONS. In the case of the STT neurons in Group II the mean (±SE) FI significantly reduced by 45% to 49.2 ± 13 within 2 min of the PB microinjections when compared with the baseline FI of 88.9 ± 7.4 [Fig. 6D, diagonal lines-filled bars; F(3,15) = 4.51, P = 0.009, one-way RM-ANOVA]. Similarly, following PB microinjections, the mean (±SE) FI of the STT neurons in Group III was significantly reduced to 49.9 ± 20.2 within 15 min, corresponding to a 46% decrease compared with the baseline FI of 92.7 ± 2.1 [Fig. 6D, gray bars; F(4,18) = 4.6, P = 0.04, one-way RM-ANOVA]. The combined FI data of STT neurons from Groups II and III also showed a significant reduction in the mean FI by about 41% within 2 min of PB microinjections following PB microinjections [Fig. 6D, black bars; F(4,42) =
In all cases, FI suppression by PB was significantly sustained for 15–30 min and returned toward the baseline value within 60 min of the microinjections.

Locations of the microinjection cannulae in the MPTA

In six rats, pontamine blue dye solution was microinjected bilaterally into the MPTA using the same stereotaxic coordinates that were used for PB/Vh microinjections. Figure 7, B and C illustrates the anatomical location of the MPTA sites in the brain stem marked by microinjections of pontamine blue dye. In six additional rats, the anatomical locations of the microinjections were identified from the tips of the track marks made by the microinjection cannulae (Fig. 7A). Figure 7D depicts anatomical locations of the microinjection sites within MPTA obtained from 12 rats by locating either the dye marks (filled circles) or the tip of the track marks (filled squares) plotted on standard coronal brain section (−7.92 mm from bregma) from the rat brain atlas of Paxinos and Watson (2007).

The dye microinjections were localized in brain areas corresponding to the following stereotaxic coordinates (Paxinos and Watson 2007): AP: −7.2 to −8 mm from bregma; ML: 1.1 to 1.2 from the midline; and DV: −7.0 to −7.7 mm from the dorsal surface of the brain. The dye spread was found to be limited to a distance of 1.5–2 mm in the mediolateral and dorsoventral directions from the microinjection sites (Fig. 7C, boxed area). This corresponds precisely to the location of MPTA as reported in the earlier studies (Devor and Zalkind 2001; Sukhotinsky et al. 2005, 2006, 2007; Voss et al. 2005).

**DISCUSSION**

The present study provides experimental evidence supporting our hypothesis that the bilateral microinjections of PB into the MPTA of the isoflurane-anesthetized rat suppress sensory inflow through the STT. Accordingly, certain electrophysiological properties of the identified STT neurons in the lumbar spinal cord of the isoflurane-anesthetized rat preparation were studied before and after bilateral microinjections of PB into the
MPTA using extracellular recording techniques. The following discussion focuses on technical considerations, general properties of STT neurons, and possible neural mechanism(s) of suppression of STT neuron excitability following PB microinjections into the MPTA.

**Technical considerations**

Given the previously documented location of the MPTA, the behavioral consequences of barbiturates focally applied into the rat MPTA (Devor and Zalkind 2001; Voss et al. 2005), and the established electrophysiological characteristics of lumbar STT neurons in the acute rat preparation (Giesler et al. 1976; Menetrey et al. 1984; Palecek et al. 1992), it was appropriate to examine pentobarbital’s actions in the MPTA on STT neuron excitability in the same species. Even though Devor and Zalkind (2001) reported on the anatomical structures included in the “MPTA,” such as the subpeduncular tegmental nucleus and dorsomedial part of pontine reticular nucleus pars oralis, exactly which neuronal population in the MPTA mediates the anesthetic effects of PB is still unknown. It is thus appropriate to use the term “MPTA” as originally used by Devor and Zalkind (2001).

Probably the most critical experimental caveat of this study was the presence of background anesthetic (isoflurane) that was continuously present throughout the experimental protocol. Ethical constraints necessitated having the animal anesthetized at a surgical plane of anesthesia at all times. Although recent evidence suggests that isoflurane minimally affects dorsal horn neurons (Jinks et al. 2008), and isoflurane levels in the present study were minimized while still maintaining a surgical plane of anesthesia, it cannot be unequivocally ruled out that complex interactions of isoflurane and PB may have occurred on the neural circuitry involved in modulating STT neuron excitability via the MPTA. It is inevitable that the isoflurane used to anesthetize the animal may have already suppressed the excitability of STT neurons before PB microinjection (Cuellar et al. 2005; Jinks et al. 1999; cf. Jinks et al. 2008) and therefore may have contributed to an “occlusion” phenomenon. It is thus the most striking part of the present study that we observed a reproducible suppression of STT neurons by PB microinjections, whose activity may already have been suppressed by isoflurane. Had the STT neurons been tested with PB in a drug-free preparation, the suppression would aptly have been greater than that observed in the present study. However, in the absence of “true baseline” (i.e., under the ideal conditions that represent a surgery- and anesthesia-free control state) further assessment of such interactions is impossible in conventional “acute” animal preparations (Soja 2001, 2007). Despite this caveat, the actions of PB microinjections into the MPTA on STT neuron activity recorded under isoflurane anesthesia were nevertheless reproducible and interpretable and occurred with nearly the same time course as that of the behavioral antinociception reported by Devor and Zalkind (2001).

**General properties of STT neurons**

STT neurons recorded in this study were located at depths ranging from 107 to 1,528 μm below the surface of the spinal cord, which correspond to laminae I–V of the rat spinal gray matter (Grant and Koerber 2004; Molander et al. 1984), as indicated by Palecek et al. (1992). The mean antidromic latency and the estimated mean axonal conduction velocity of 18 STT neurons measured 4.4 ± 0.6 and 18.6 ± 2.5 m/s,
respectively. These values corroborate those reported previously for the lumbar STT neurons in the rat (Giesler et al. 1976; Ménetre et al. 1984; Palecek et al. 1992). Finally, L₁ STT neurons in the isoflurane-anesthetized rat preparation in medially placed spinal electrode tracts display spontaneous spike activity and are readily activated polysynaptically by large-diameter afferents of the lower limb driven by low-intensity electrical stimuli applied to cutaneous (Su) and mixed (Sc) nerve trunks.

Interestingly, the baseline SFRs (mean of 13 spikes/s) of the 18 STT neurons recorded in the T₁₃–L₁ segment as in this study are higher than those of the STT neurons recorded in the lower lumbar (L₄–L₅) spinal cord segments of PB-anesthetized rats reported in previous studies (Chen and Pan 2002; Palecek et al. 1992). In the latter studies, the mean firing rates were <5 spikes/s. The differences between the background activities of the STT neurons reported herein and those by Chen and Pan (2002) and Palecek and colleagues (1992) may be due to a number of factors, including—but not limited to—the type of anesthetic used (isoflurane vs. pentobarbital) and/or segmental differences in the properties of L₁ versus L₄–L₅ STT neurons. Recently Barter et al. (2008) reported paradoxically lower firing rates (mean of 5–6 spikes/s) of several ascending dorsal horn neurons recorded in the L₁ segment using comparable minimum alveolar concentrations of isoflurane anesthesia. Additionally, these neurons were located at more superficial layers (mean depth of 580 μm) within the dorsal gray than those reported herein. However, Barter et al. (2008) also reported that the majority of their “superficial” dorsal horn ascending projection neurons displayed firing rates (mean of 16 spikes/s) that were very similar to those of our population of STT neurons. It should also be noted that in the study by Barter et al. (2008), their recorded neurons were antidromically activated from the upper cervical segments only (as opposed to from the VPL nucleus in the present study) and, as discussed by these authors, may have comprised several (supra)segmental cell populations, each with different membrane properties that could account, in part, for different baseline firing rates (Jiang et al. 1995; Ruscheweyh and Sandkühler 2002).

**Suppression of STT neuron excitability following PB microinjections into the MPTA**

Results of the present study clearly show that the excitability of STT neurons was significantly suppressed following PB microinjections into the MPTA. Given that the Vh, containing ethanol, a CNS depressant itself, did not alter these parameters indicates that the effects were very likely due to PB itself.

Microinjections of PB into the MPTA resulted in a significant decrease in the mean SFR, peripheral-nerve–evoked response magnitude, and FI of the STT neurons. This suppression was rapid in onset, occurring within 1.5 min following the microinjections, and suggests a local action site of action of PB in the MPTA, which, in turn, alters descending modulation. Microinjections of Vh did not affect any of these parameters.

The decrease in the spontaneous and synaptic responses of STT neurons may be due to several mechanisms, including but not limited to: 1) supraspinal-mediated shunting of the soma (Lipski 1981), 2) disfacilitation (Wilson and Burgess 1962; Zhang et al. 1991a,b), and/or 3) decrease in the transmitter release from central terminal arbors of primary afferent fibers in the spinal cord as a consequence of enhanced impulse traffic through axoaxonic synapses (Rudomin 1999; Rudomin and Schmidt 1999).

In this study, we addressed the possibility of somatic shunting by quantifying the antidromic firing index (FI) around PB microinjections. The FI was substantially suppressed following PB microinjections over the same time period when SFR and peripheral-nerve–evoked responses were suppressed. This finding indicates a neural network-induced reduction in STT neuron excitability, which may involve somatic membrane hyperpolarization that is further accompanied by an increase in STT neuron conductance (Lipski 1981; Whitehorn and Burgess 1973; Willis et al. 1976).

**Time course of suppression of STT neuron excitability following PB microinjections into the MPTA**

As indicated in results, the SFR was significantly suppressed within 90 s after PB microinjections commenced. Although it was not possible to confirm PB’s onset here for the other parameters (i.e., Su-ER, Sc-ER, and FI), it may seem reasonable that PB may have exerted a shorter onset of action on these parameters as well, given the consistent suppression observed for these parameters with SFR at later time points (2 and 15 min). Thus the reduction in sensory inflow through the STT by PB microinjections may begin before the behavioral antinociception as reported by Devor and Zalkind (2001).

The results show that not only the spontaneous spike activity but also the orthodromic and antidromic evoked responses of the STT neurons were suppressed 2 min following PB microinjections. This suppression returned to baseline within 30–60 min of microinjections. In the behavioral studies reported by Devor and Zalkind (2001), the anesthesia-like state usually occurred within 1 min of the completion of PB microinjections into the MPTA, peaking in 2–5 min. The animals reawakened 10–30 min following PB microinjections with full recovery occurring from 23 to 45 min depending on the anesthesia score of each of the animals. Voss et al. (2005) replicated the results of Devor and Zalkind (2001) with thiopental and reported that atonia and antinociception began during the thiopental microinjections, lasting between 15 and 120 min.

Thus the overall time course of the suppression and subsequent recovery of spontaneous firing rate, antidromic firing index, and peripheral-nerve–evoked responses of STT neurons following microinjections of PB into the MPTA reported herein remarkably overlaps with the time course of behavioral antinociception (Devor and Zalkind 2001; Voss et al. 2005).

**Possible neural pathways underlying pentobarbital-mediated suppression of STT neurons arising from the MPTA**

Several network scenarios may underlie PB-mediated reduction in STT neuron excitability (Fig. 8). Recent anatomical studies have shown that MPTA neurons project to endogenous pain-modulating pathways, including the periaqueductal gray (PAG), rostral ventromedial medulla (RVM), locus ceruleus (LC), and dorsal raphe nuclei (Reiner et al. 2007; Sukhotinsky et al. 2003, 2006). Under baseline conditions, MPTA neurons may inhibit neurons in
neurons in the MPTA also send direct projections in the spinal transmission through the STT (Fig. 8). In addition, because excite the serotonergic and noradrenergic neurons in the 2005). The disinhibited PAG neurons, in turn, may further disinhibition of the PAG as well as relay centers in the RVM, probably through GABAergic mechanisms (Sukhotinsky et al. 2003, 2005). The PAG exerts robust descending inhibition on the spinal pain transmission neurons, including STT neurons through the relay centers in the RVM (Bajic and Proudfit 1999; Gerhart et al. 1984; Mason 1999; Millan 2002). Microinjections of PB and other γ-amino butyric acid type A (GABA\(_A\)) receptor agonists into the MPTA would likely suppress the ongoing activity of the MPTA, thereby causing disinhibition of the PAG as well as relay centers in the RVM and LC (Devor and Zalkind 2001; Sukhotinsky et al. 2003, 2005). The disinhibited PAG neurons, in turn, may further excite the serotonergic and noradrenergic neurons in the RVM and LC, respectively, which, in turn, may reduce transmission through the STT (Fig. 8). In addition, because neurons in the MPTA also send direct projections in the spinal cord dorsal horn (Reiner et al. 2007; Sukhotinsky et al. 2006) it is possible that these projections provide direct (or indirect through spinal interneurons) synaptic inputs to the STT neurons and directly modulate their activities. Thus the “antinociception” observed following microinjections of barbiturate anesthetics into the MPTA in the behavioral studies (Devor and Zalkind 2001; Voss et al. 2005) can be attributed, in part, to a reduction in sensory inflow through the STT.

An unaddressed question is whether MPTA, via its bulbospinal projections (Sukhotinsky et al. 2006), serves as a source of classical tonic descending inhibition (TDI) akin to that reported for unidentified spinal (inter)neurons by Wall (1967) and others in the cat (Hall et al. 1982; Rudomin et al. 2004; Soja and Sinclair 1983) and in the rat (Necker and Hellon 1978). If so, PB-mediated suppression of MPTA neurons would result in a reduction of TDI, which of course may be offset by other network actions described earlier. Alternatively, if the MPTA constitutes a source of tonic descending facilitation (TDF) to STT neurons, our results could also be explained by the depressant effects of PB on those MPTA bulbospinal projection neurons mediating TDF. It should be pointed out that the aforementioned scenarios are speculative and warrant supporting evidence from future investigations.

Conclusions

Results of the present study indicate that the behavioral antinociception following microinjections of barbiturate “GABA\(_A\) mimetic” anesthetics into the MPTA (Devor and Zalkind 2001; Voss et al. 2005) may be due, in part, to attenuation of sensory inflow through a classical ascending sensory pathway—i.e., the STT. This provenance warrants further studies on spinal sensory neurons and motoneurons to disclose the neurotransmitters and switching mechanisms that arise from the MPTA that eventuate in dampening of sensorimotor processing during anesthesia. Such efforts are not only important for understanding the network-induced sensorimotor dampening caused by barbiturates (Devor and Zalkind 2001; Voss et al. 2005), but also by other current agents used clinically, that affect GABA receptor mechanisms (e.g., propofol, etomidate, etc.), as well as that which occurs during natural sleep states and sleep states induced by “GABA\(_A\)ergic” drugs (Soja 2007).

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REFERENCES


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