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Research Report

GABA-mediated oxytocinergic inhibition in dorsal horn neurons by hypothalamic paraventricular nucleus stimulation

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In anaesthetized rats, we tested whether the unit activity of dorsal horn neurons that receive nociceptive input is modulated by electrical stimulation of the hypothalamic paraventricular nucleus (PVN). An electrophysiological mapping of dorsal horn neurons at L3–L4 let us choose cells responding to a receptive field located in the toes region of the left hindpaw. Dorsal horn neurons were classified according to their response properties to peripheral stimulation. Wide Dynamic Range (WDR) cells responding to electrical stimulation of the peripheral receptive field and presenting synaptic input of Aδ, Aβ, and C-fibers were studied. Suspected interneurons that are typically silent and lack peripheral receptive field responses were also analyzed. PVN electrical stimulation inhibits Aδ (−55.0± 10.2%), C-fiber (−73.1±6.7%), and post-discharge (−75.0±8.9%) peripheral activation in WDR cells, and silent interneurons were activated. So, this last type of interneuron was called a PVN-ON cell. In WDR cells, the inhibition of peripheral responses caused by PVN stimulation was blocked by intrathecal administration of a specific oxytocin antagonist or bicuculline. However, PVN-ON cell activation was blocked by the same specific oxytocin antagonist, but not by bicuculline. Our results suggest that PVN stimulation inhibits nociceptive peripheral-evoked responses in WDR neurons by a descending oxytocinergic pathway mediated by GABAergic PVN-ON cells. We discuss our observation that the PVN electrical stimulation selectively inhibits Aδ and C-fiber activity without affecting Aβ fibers. We conclude that Aδ and C-fibers receive a presynaptic inhibition mediated by GABA.

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1. Introduction

The most studied structures involved in the phenomenon of stimulation-induced analgesia are the ventral periaqueductal gray matter and the nucleus raphe magnus (see Villanueva and Fields, 2004). However, more recently it has been shown that hypothalamic paraventricular nucleus (PVN) stimulation also produces analgesia in rats (Yirmiya et al., 1990; Shiraishi et al., 1995). Recent work in our laboratory (Miranda-Cardenas et al., 2006; Condés-Lara et al., 2006) shows that PVN electrical stimulation or intrathecal oxytocin (OT) administration produces analgesia in freely moving rats. Moreover, PVN stimula-

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tion or OT reduced the incoming peripheral Aδ and C-fiber activation in dorsal horn spinal cord neurons. However, the mechanism involved in the PVN descending pathway related to the analgesic effects is not well defined.

PVN neurons project directly to different segments of the spinal cord (Sawchenko and Swanson, 1982). In addition, PVN neurons send oxytocinergic fibers mainly to the superficial laminae of the dorsal horn (Swanson and McKellar, 1979), and a close correlation between the sites of PVN oxytocinergic projections and oxytocin-binding sites in the dorsal horn has been shown elsewhere (Reiter et al., 1994). Several studies have demonstrated that oxytocin (OT) has analgesic effects (Arletti et al., 1993; Yang, 1994; Uvnäs-Moberg et al., 1992; Yirmiya et al., 1990); however, the mechanisms by which OT exerts antinociceptive effects are not clear. In the spinal cord, OT inhibits sensory glutamatergic transmission between afferent fibers and dorsal horn neurons (Robinson et al., 2002; Condés-Lara et al., 2003). On the other hand, in cultured dorsal horn neurons, OT increases the frequency of AMPA receptor-mediated spontaneous post-synaptic currents (Jo et al., 1998). These results suggest that OT indirectly inhibits sensory transmission in dorsal horn neurons by exciting spinal inhibitory interneurons.

In this work, we propose that the antinociceptive effect of the PVN oxytocinergic projection to the spinal cord is mediated by GABAergic interneurons. The mechanism involves a selective reduction of presynaptic Aδ and C-fiber activity.

2. Results

2.1. *Modulation of dorsal horn neurons activity by PVN stimulation*

Sixty dorsal horn neurons were characterized according to their response properties to peripheral receptive field (RF) stimulation and to PVN electrical stimulation. Recorded dorsal horn neurons were classified according to their characteristics as Wide Dynamic Range (WDR) and PVN-ON cells (see below). Forty-four cells classified as WDR cells neurons showed clear peripheral (RF) responses and could present wind-up or slight wind-up increased response according to the characteristics of Seagrove et al. (2004). This type of cell presented Aβ, Aδ, C-fibers, and post-discharge responses (see Fig. 1). The PVN-ON cells were silent, without apparent sensory input, and they were not activated by peripheral stimulation. In the present study, we report on 16 PVN-ON neurons. During 9 recordings, we were able to record simultaneously WDR and PVN-ON cells. In

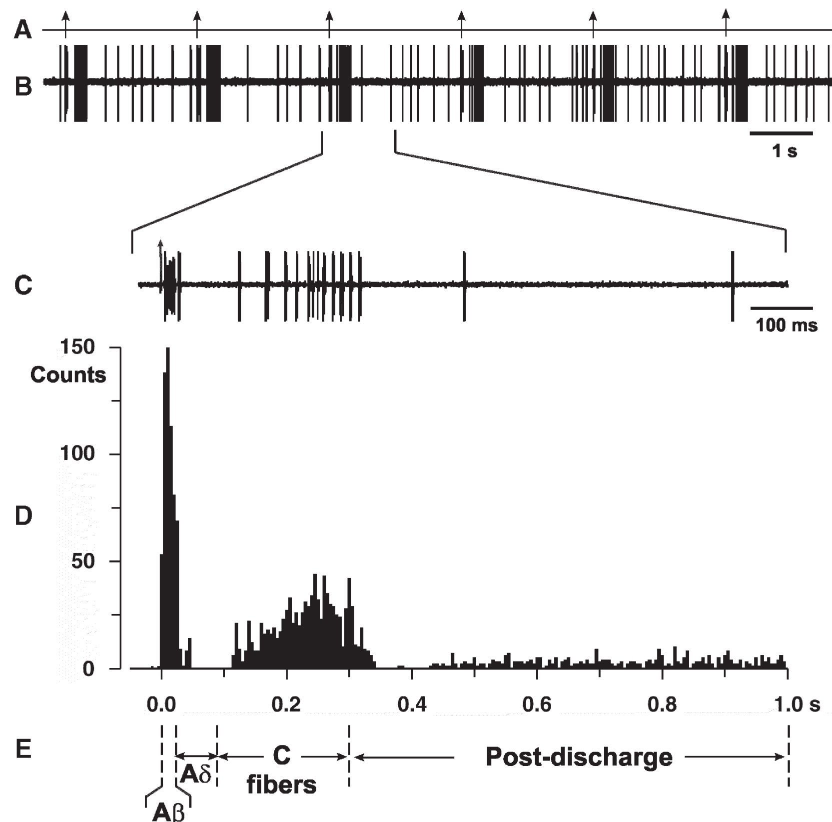

Fig. 1 – Neuronal responses to receptive field stimulation (RFs). Panel A shows the stimulus artifact. Panel B shows six consecutive responses to RFs in an identified dorsal horn WDR neuron. Panel C is single response to the same neuron. Panel D shows a peri-stimulus time histogram for the same neuron, 50 ms prior and 1000 ms before the RFs. Panel E shows the time intervals used to measure the different fiber components of the response: Aβ, Aδ, C-fibers, and the post discharge. We evaluated the amount of each of the different fiber components.

Fig. 2 – Effects of PVN stimulation on dorsal horn cell responses. Panel A is an example of extracellular recording of a WDR dorsal horn neuron showing its responses (2) to RF electric stimulation (1 shows the artifacts of the RF stimulation) before and after PVN stimulation (PVN stim). The horizontal bar below the record indicates the time of PVN stimulation (black square over the records and histograms). Panel B shows a firing rate histogram of the same (WDR) neuron. Panel C is an example of a PVN-ON neuron recording. Panel D is the firing rate histogram of the same PVN-ON cell. Notice that while the WDR cell responded to RFs and reduced these responses before PVN stimulation, the PVN-ON cell did not respond to peripheral stimulation, and it was activated only after a PVN electric stimulus. Also, notice that during PVN electrical stimulation the increases observed in the firing rate histograms are due to electrical artifacts. Panel E shows the averaged activity histograms of WDR (filled triangles) and PVN-ON (open triangles) neurons. The data represent the mean ± SE of 22 WDR and 14 PVN-ON neurons. The continuous lines are running averages of the cell activity. Panel F is a graph showing a significant negative correlation ($r = -0.75$) between WDR and PVN-ON neuronal activity.

addition, WDR neurons reduced their evoked responses to electric stimulation of the receptive field after PVN electrical stimulation. In contrast, PVN-ON neurons were silent before PVN stimulation and became activated by the PVN stimulus, which is why we called these neurons PVN-ON cells. The electrophysiological activity of these neurons was analyzed in

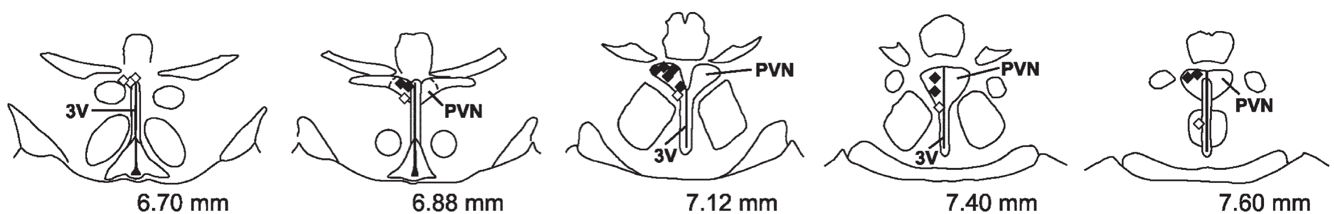

Fig. 3 – Schematic coronal sections indicating the location of electric PVN stimulation. Filled symbols represent the zones in which stimulation causes a significant change in dorsal horn cell activity. The stimulation sites that do not change neuronal activity are indicated with open symbols. The coordinates (mm from the interaural line) are indicated below each drawing. PVN, hypothalamic paraventricular nucleus; 3V, third ventricle.

Table 1 – The action potentials were measured according to their latencies to reach and activate WDR cells

	% of change after BIC administration (mean ± SE)
Aβ-fibers	+12.6 ± 6.6
Aδ-fibers	+143.9 ± 61.9 *
C-fibers	+15 ± 11.6
Post-discharge	+64.2 ± 42.19

* $p < 0.05$, Student's t test. $n = 8$.

unitary and also in multiunit recordings (no more than two cells). In multiunit recordings, the activity of both classes of neurons was usually recorded simultaneously. Fig. 2 shows examples of a WDR cell and a PVN-ON dorsal horn cell recordings. The histograms in Figs. 2B and D show the activity before and after PVN stimulation of WDR and PVN-ON cells, respectively. The average activity histogram of 22 WDR and 14 PVN-ON dorsal horn neurons is shown in Fig. 2E before and after PVN stimulation. Notice that while PVN-ON neurons became active after PVN stimulus, WDR neurons were inhibited. There was an inverse correlation ($r = -0.75$) between the activity of WDR and PVN-ON cells (Fig. 2F).

The effect of central stimulation on dorsal horn cells depends of the electrode location. Changes in the activity of spinal neurons were evoked by the stimulation of the medial part of parvicellular PVN (at 7.12 mm from interaural line). Fig. 3 shows the location of the stimulating electrodes in 18

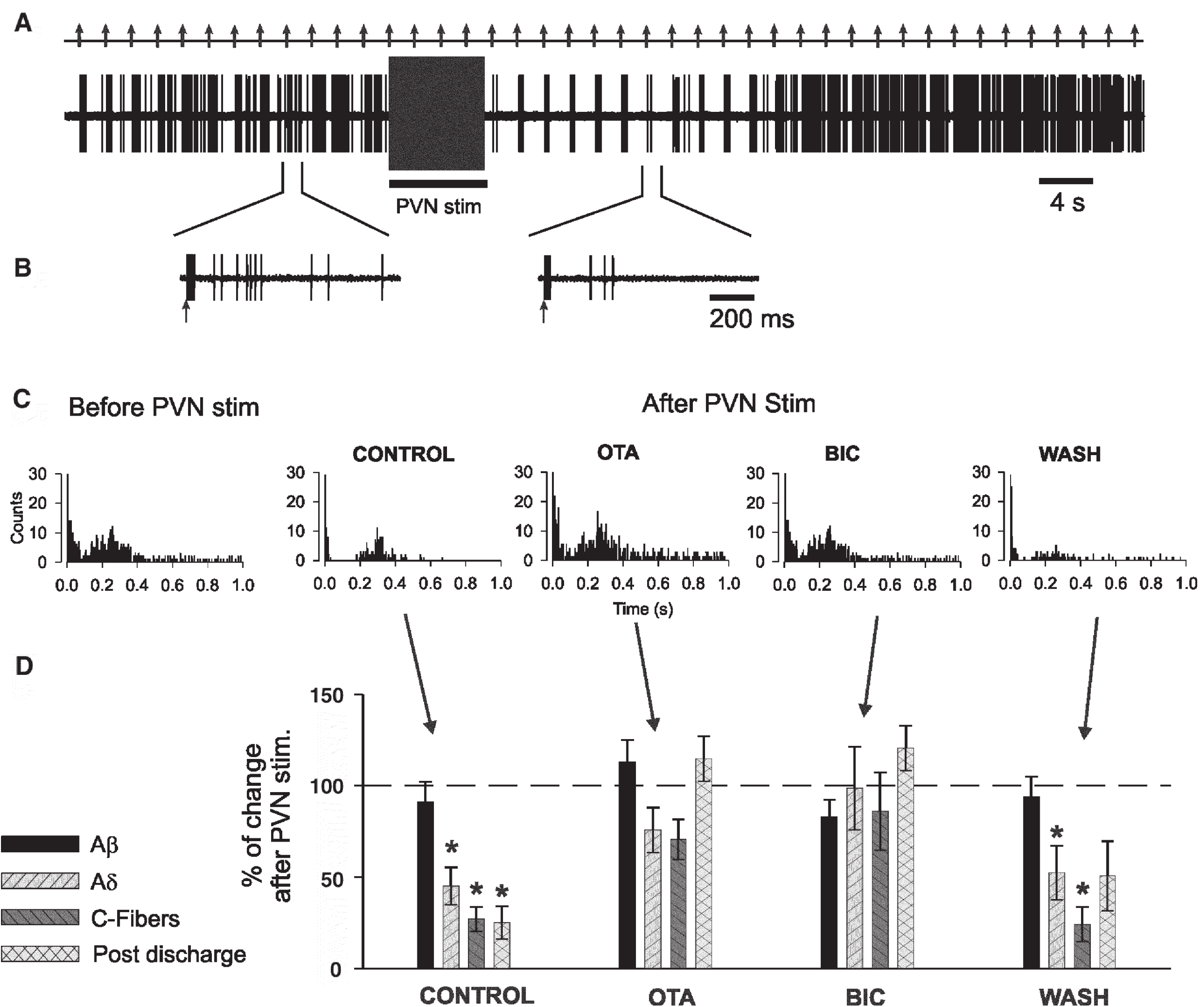

Fig. 4 – Intrathecal OTA and BIC effects on the PVN inhibition of nociceptive spinal cord cell responses. Panel A is an electrophysiological recording of dorsal horn WDR cells showing their responses to peripheral receptive electric field stimulation before and after PVN stimulation. The horizontal line at the top of the record shows artifacts of the RFs (arrows). The black square above the recorded activity indicates the PVN stimulation (PVN stim). In panel B, two records of RFs responses before and after PVN stimulation in a WDR cell are illustrated. Notice the reduction in the response. In panel C, the effects of the PVN stimulation in the control situation and the effects of PVN stimulation 20 min after OTA or BIC administration are shown in the form of post-stimulus-time histograms. Also shown are the effects on the PVN 30 min after wash. Panel D illustrates the percent change for Aβ, Aδ, C-fiber, and post-discharge responses (see Fig. 1 for methods description). Notice that the significant decrease (*$p < 0.05$, paired t test; $n = 8$) in the neuronal responses to Aδ, C-fiber, and post-discharge observed after PVN stimulation (CONTROL) was reversed in the presence of OTA and BIC. Also notice that 30 min after wash, the Aδ and C-fiber reduction again became significant.

experiments. Each symbol represents the location of the electrolytic lesion produced by the stimulating electrode.

2.2. OTA and BIC prevent the effects of PVN stimulation on dorsal horn neurons

Since dorsal horn GABAergic interneurons are important in the processing of nociceptive information (Schmidt and Schaible, 1998), GABAergic interneurons might be involved in the inhibition produced by PVN stimulation. To test this hypothesis, in eight experiments we examined the effect of BIC and OTA, applied intrathecally, on the inhibition of nociceptive neuronal responses of WDR dorsal horn cells produced by PVN stimulation.

The neuronal responses of RF stimulation on WDR dorsal horn neurons did not change after PVN stimulation in the presence of OTA. Intrathecal BIC administration caused a generalized increase, but this was only significant ($p<0.05$ paired t test) for the Aδ responses in the 8 cells analyzed (Table 1). However, it was observed that BIC (in 7 of 8 neurons) and OTA (8 cells) block the effects of PVN stimulation (Fig. 4), and the maximum effect of BIC and OTA occurred 20 min after administration. These effects were reversed 30 min after washing the spinal cord with saline solution (Fig. 4C). Using post-stimulus time histograms constructed with 10 stimuli before and 10 stimuli after PVN stimulation, we compared in WDR cells the number of spikes corresponding to Aβ, Aδ, C-fibers, and post discharge latencies (Fig. 4D). PVN stimulation reduced significantly ($p<0.05$; paired t test; $n=8$) the responses of Aδ (−55±10.2%), C-fibers (−73.1±6.7%), and the post discharge (−75±8.9%) compared to the control values. No significant change was observed in Aβ responses (−8.8±10.9%). The decrease in Aδ, C-fiber, and post discharge responses caused by PVN stimulation was not observed in the presence of OTA or BIC, and it was restored after washing (Figs. 4C and D).

The intrathecal application of OTA during recordings of PVN-ON cells allowed us to observe a dramatic suppression of the activation presented by these cells. The suppression of the activation could be reversed by the spinal cord wash. Both effects, the suppression of the activation produced by OTA and the wash latencies, were maximal around 20 min, i.e. we observed a progressive effect reaching a maximum at 20 min as shown in Fig. 5. These effects were observed in 3 expe-

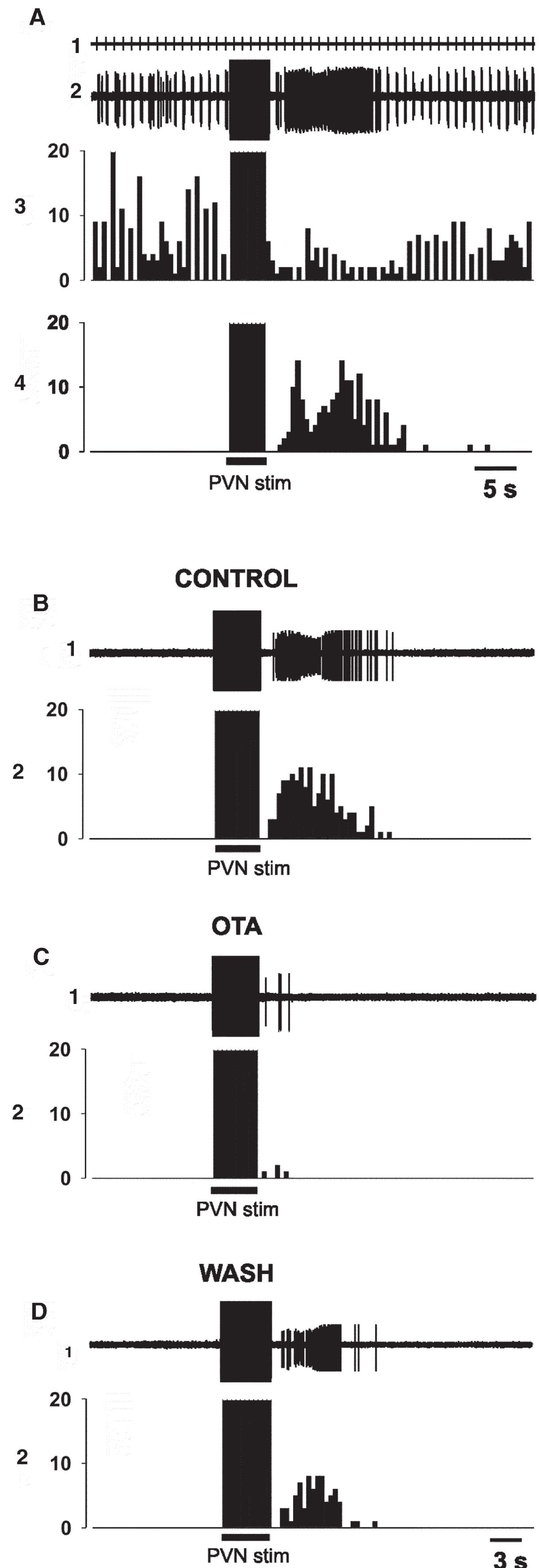

Fig. 5 – OTA effects on the PVN-ON cells. Panel A illustrates a simultaneous recording of WDR and PVN-ON cells during nociceptive RFs. The artifacts of RFs that generated Aδ and C-fibers responses in a WDR cell are shown in 1. The black square represents the PVN stimulation that generates the activation of a PVN-ON cell. A3 and 4 show the firing rate histograms of the two cells: WDR and PVN-ON cells, respectively. Notice that after the PVN stimulation, the WDR cell showed diminished responses to peripheral stimulation (3), and the PVN-ON cell increased its firing rate (4). Panels B–D show the effects of PVN stimulation on a recorded PVN-ON cell (1) and its firing rate histogram (2). Notice that PVN stimulation activated this cell in the control situation (B), but this activation was blocked by the intrathecal OTA action (C), and it was recovered after wash (D).

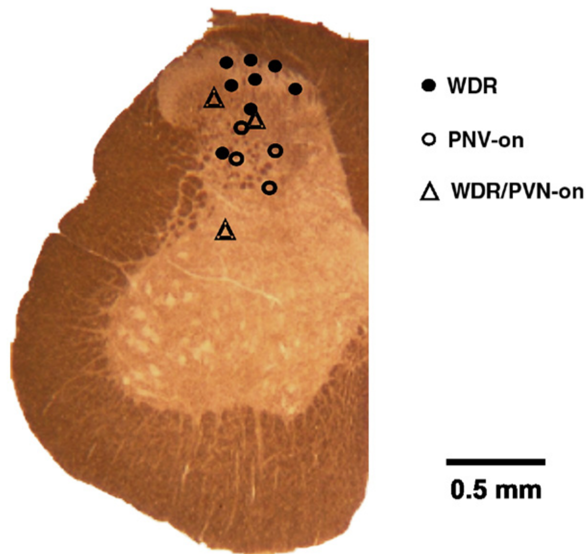

Fig. 6 – Histological location of recorded cells on the left side of a spinal cord section. WDR or PVN-ON cells and simultaneously recorded WDR and PVN-ON cells are illustrated. Due to overlap, only some cell positions are illustrated.

riments. Topical application of BIC on cells neighboring the PVN-ON recorded cells caused only a small, non-significant increase of the firing rate discharge produced by the PVN stimulation (data not shown).

At the end of the recordings, we made iontophoretic injections through the recording pipettes and, using the microdrive references and the blue point of pontamine, we reconstructed and positioned the location of cells recorded. These locations are plotted in Fig. 6.

3. Discussion

The present study indicates that the oxytocinergic projection originating in the PVN modulates the activity of ipsilateral dorsal horn neurons that receive nociceptive input and that GABA mediates this modulation.

Several lines of evidence obtained in humans (Yang, 1994; Madrazo et al., 1987) and in animal models (Arletti et al., 1993; Uvnäs-Moberg et al., 1992; Brown and Perkowski, 1998; Ge et al., 2002) have suggested that oxytocin plays an important role in pain modulation and analgesia. In the spinal cord, Reiter et al. (1994) reported that OT receptors are distributed primarily in the marginal zone and substancia gelatinosa of the dorsal horn, supporting a role for OT in pain modulation at the spinal cord level.

It has previously been shown that PVN electrical stimulation induces antinociception in rats (Yirmiya et al., 1990; Shiraishi et al., 1995). In addition, our results indicate that the presence of an OT antagonist blocks the inhibition of neuronal responses caused by PVN stimulation. The finding that stimulation of most locations outside the PVN did not produce significant changes in the activity of dorsal horn neurons suggests that the inhibition observed after stimulation is caused by the activation of hypothalamic PVN neurons (Fig. 3). These results support the idea that the PVN is part of the endogenous pain inhibitory system. In fact, an ipsilateral descending oxytocinergic hypothalamic projection has been described (Sawchenko and Swanson, 1982), suggesting that "endogenous" OT modulates the transmission of nociceptive information in the dorsal horn.

Recent data from our laboratory (Miranda-Cardenas et al., 2006; Condés-Lara et al., 2006) show by behavioral tests and unit activity recordings of dorsal horn cells responding to noxious stimulations that the electrical activation of the PVN as well as the local application of OT blocks the incoming Aδ and C-fiber responses. A selective OT antagonist reverses these effects. Nevertheless, the synaptic mechanism involved was unclear until our present data.

Accumulated results have implicated GABA in the inhibition of transmission of nociceptive information in the dorsal horn (see Malcangio and Bowery, 1996 for review). Spinal cord GABAergic neurons have been described predominantly between lamina I–II (Mogoul et al., 1987; Todd and McKenzie, 1989; Alvarez et al., 1996), where they inhibit nociceptive information acting at a presynaptic (Bernardi et al., 1995; Todd, 1996) or postsynaptic level (Mogoul et al., 1987).

Here we report that BIC prevents the inhibition of nociceptive responses of dorsal horn cells that are caused by PVN hypothalamic stimulation, suggesting that $GABA_A$ receptors participate in this inhibition. The facts that GABAergic interneurons (Heinke et al., 2004) and oxytocin receptors (Reiter et al., 1994) are located in laminae I and II are consistent with the idea that descending oxytocinergic fibers inhibit nociceptive responses of dorsal horn cells through GABAergic action. However, we cannot exclude the participation of other neurotransmitters in this modulation, since endogenous opioids have been implicated in the modulation of spinal nociceptive responses mediated by OT (Yu et al., 2003).

In experiments with spinal cord slices, Robinson et al. (2002) showed that OT inhibits glutamatergic transmission between afferent fibers and dorsal horn neurons. On the other hand, using dorsal horn neuron cultures, Jo et al. (1998) reported that OT receptor activation facilitates AMPA-type receptor-mediated glutamatergic synaptic transmission. These results suggest that OT indirectly inhibits sensory transmission between afferent fibers and dorsal horn neurons by exciting inhibitory interneurons. In this context, our results show that PVN stimulation diminished the responses of dorsal horn neurons (WDR cells) that receive nociceptive input and, at the same time, it also activates other cells that showed no relationship with receptive field stimuli (PVN-ON neurons). The lack of sensorial input in PVN-ON cells and the activation by a descending OT pathway is consistent with the proposition that these cells are interneurons. In addition, the reduction of WDR cell responses correlates with the increase of PVN-ON cell activity (Fig. 2). Our results further indicate that BIC blocks the inhibitory effect of PVN stimulation by PVN-ON cells, which could be GABAergic inhibitory interneurons. With this background, we propose that PVN oxytocinergic neurons could activate PVN-ON cells, which, in turn, inhibit the transmission of nociceptive signals in the spinal cord.

Our results also show that PVN stimulation significantly and specifically inhibits Aδ and C-fiber responses in WDR dorsal horn neurons and does not affect the activation of Aβ fibers. These observations suggest that the spinal inhibition caused by the descending oxytocinergic pathway occurs at the presynaptic level. GABAergic inhibition in the spinal cord has been widely studied. Several lines of evidence have shown that GABA is the neurotransmitter of spinal presynaptic inhibition, causing a hyperpolarization of spinal neurons and a depolarization of primary afferent terminals by activating bicuculline-sensitive $GABA_A$ receptors, thus increasing chloride conductance. In addition, the stimulation of a number of brain structures can result in primary afferent depolarization and presynaptic inhibition at the spinal cord level (for review see Rudomin and Schmidt, 1999). In accord with the gate control theory (Melzack and Wall, 1965), it has been shown that cutaneous C-fibers could be depolarized by A-fiber input (Fitzgerald and Woolf, 1981; Calvillo et al., 1982), and this depolarization is mediated by GABAergic mechanisms (Randic et al., 1980). Under our experimental conditions, electrical stimulation of the receptive field activates Aβ, Aδ, and C-fibers. This could produce a primary afferent depolarization, and concomitantly, C-fiber inhibition that is mediated by $GABA_A$ receptors (Schmidt and Schaible, 1998; Rudomin and Schmidt, 1999). Here it is important to note that BIC increased the Aδ fiber input (similar results have been shown by Seagrove et al., 2004; Rojas-Piloni et al., 2006) leading to a possible increase of C-fiber inhibition. However, during BIC administration we observe no effect on C-fiber activity (see Table 1). This lack of effect was observed also during PVN or RF stimulation. In brief, BIC suppressed the OT descending inhibition produced by PVN stimulation (see Fig. 4). These results indicate that the BIC effects on PVN-evoked inhibition and on Aδ and C-fiber responses in WDR neurons are not related to primary afferent depolarization mediated by Aβ fibers. Nevertheless, further work is necessary to establish whether PVN stimulation increases the excitability of fine afferents and causes a presynaptic inhibition mediated by primary afferent depolarization. An interesting puzzle remains about the latency of the OTA and BIC effects, which were maximal around 20 min. The rate of diffusion may contribute to the latency, but in reality we do not yet have a good explanation for this latency. Nevertheless, this latency is consistent with other experiments (Seagrove et al., 2004; Condés-Lara et al., 2006).

4. Experimental procedures

4.1. General procedures

Guidelines contained in the *NIH Guide for the Care and Use of Laboratory Animals* (85-23, revised in 1985) and IASP recommendations (Zimmermann, 1983) were followed throughout. Wistar rats (280–310 g) were initially anesthetized in a hermetic box with halothane (1.5%) in a mixture of two-thirds N_2O and one-third O_2. A cannula was inserted into the trachea to provide artificial ventilation and to maintain the anesthesia throughout the experiment. End tidal CO_2 and electrocardiogram were monitored during the experiment, and the temperature was maintained at 38 °C by means of a circulating water pad. The rats were fixed in a stereotaxic apparatus, and two small holes were drilled in the skull to place silver ball electrodes for electrocorticogram recording to assure a continuous and stable anesthesia state. The animals were secured in a spinal cord unit frame, and lumbar vertebrae were fixed to improve stability at the recording site. A laminectomy was performed to expose spinal cord segments L4–L6 (see Condés-Lara et al., 2003).

4.2. Recording, stimulation, and drug administration

As a first step a parylene-coated tungsten electrode was inserted into the spinal cord, and recordings were made from neurons of the exposed segments, which receive afferent input from the hindpaw. Then, we moved the electrode and mapped the region in a rostral–caudal direction until we found the region receiving input from the toe area. At this time we changed to glass microelectrodes filled with a 4% solution of pontamine sky blue in 1 M KCl with a resistances between 7 and 10 MΩ. Neuronal activity was recorded with pre-amplifier filters set to 300 Hz in the low range and 3 kHz in the high range. The electrodes were coupled to a hydraulic microdrive, and the recorded activity was digitized and analyzed off-line using the spike 2 system (CED, Cambridge, UK). The microdrive records and the marked points (see Histology procedures) help us to reconstruct the locations of the recorded cells. In 9 recordings, we were able to distinguish 2 cells that were differentiated using the spike 2 software. For each recorded cell, the specific somatic receptive field was located by tapping on the entire hindpaw and toes. On some occasions we were able to record multiunit activity (two cells): one responded to receptive field stimulation (called a WDR cell), and the second was a silent cell, non-responsive to receptive field stimulation, but activated after PVN stimulation (called a PVN-ON cell).

Two electrodes connected to a stimulus-isolator unit were inserted into the somatic receptive field of the recorded neurons. The peripheral stimulation consists of single, 1-ms pulses at 0.5 Hz. The stimulus–intensity threshold was adjusted by giving progressive (0.1–1.5 mA) electrical pulses until a C-fiber response was evoked (90–300 ms post-stimulus). Then, the stimulation intensity was set at 3 times the threshold to assure C-fiber response, and under these conditions we studied WDR and PVN-ON neurons of the dorsal horn spinal cord that responded to C-fiber activation. A train of 50 stimulations was repeated each 5 min in order to test the temporal excitability changes produced by OTA and BIC.

A concentric bipolar stainless steel electrode with 0.5 mm separation between the tips was placed in the PVN using the stereotaxic coordinates (AP 7.12; L 0.45; H 2.00) of Paxinos and Watson (1998). PVN electrical stimulation consisted of a train of 1-ms pulses at 60 Hz with an intensity of 300 μA over a period of 6 seconds. Using similar parameters it has been reported that PVN stimulation completely inhibits the tail flick response (Yirmiya et al., 1990), the paw pressure pain test in rats (Shiraishi et al., 1995), and thermal and mechanical withdrawal responses in neuropathic rats (Miranda-Cardenas et al., 2006) without apparent side effects.

The specific OT antagonist $d(CH_2)^5[Tyr(Me)^2,Thr^4,Tyr\text{-}NH_2^9]$ OVT (OTA) and the $GABA_A$ receptor antagonist bicuculline

(BIC) were applied directly onto the spinal cord at concentrations of 10^{-6} M and 10^{-4} M, respectively, in a volume of 20 μl. The effect of each drug was analyzed by comparing the neuronal responses before and after PVN stimulation at 5 min intervals. Maximal effects were typically observed between 20 and 30 min after drug administration.

4.3. *Data analysis*

Single unit activity responses were plotted as frequency rate histograms and receptive field post-stimulus time histograms. The RFs-evoked activities were quantified by classifying the action potentials according to their latency (see Fig. 1) of response (0–20 ms Aβ, 20–90 ms Aδ, 90–300 ms C-fiber, and 300–1000 ms post-discharge). A paired *t* test was used to compare the responses corresponding to Aβ, Aδ, C-fiber, and post discharge of 10 neuronal responses before PVN stimulation with 10 neuronal responses after PVN stimulation. The effects of OTA or BIC were tested each 5 min over a 30-min period and compared with the pretreatment activity. During these tests, we also compared the neuronal responses before and after PVN stimulation.

4.4. *Histology*

The recording sites were marked iontophoretically with pontamine blue (cathodic current 10–15 μA, 25 min). In addition, the tip location of the stimulating electrode was labeled with an electrolytic lesion (100 μA, 10 s). At the end of the experiments, the rats were perfused with 10% formalin, and the spinal cord and brains were removed. Spinal cord and brain sections were frozen to locate the recording and stimulating regions.

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