Monoaminergic descending pathways contribute to modulation of neuropathic pain by increasing-intensity treadmill exercise after peripheral nerve injury

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Abstract

This study characterizes the impact of increasing-intensity treadmill exercise (iTR) on noradrenergic (NE) and serotonergic (5HT) modulation of neuropathic pain. Following sciatic nerve transection and repair (SNTR) rats developed significant mechanical and thermal hyperalgesia that was partially prevented by iTR performed during the first 2 weeks after injury.

Marked decrease in the expression of $5HT_{2A}$ and α_{1A} and β-, but not α_{2A} adrenergic receptors in the spinal cord dorsal horn was associated to SNTR and recovered by iTR, particularly in lamina II. iTR significantly increased $5HT_{2A}$ in periaqueductal grey (PAG), raphe magnus (RM) and dorsal raphe nucleus (DRN), with a pattern suggesting reorganization of serotonergic excitatory interconnections between PAG and DRN. iTR also increased the expression of α_{1A} in locus coeruleus (LC) and DRN, and β_2 in LC, indicating that exercise enhanced activity of NE neurons, likely by activating autologous projections from DRN and PAG.

iTR hypoalgesia was antagonized by blockade of β_2 and $5HT_{2A}$ receptors with administration of butoxamine and ketanserin. The neurotoxin DSP4 was injected to induce depletion of NE projections from LC before starting iTR. DSP4 treatment worsened mechanical hyperalgesia, but iTR hypoalgesia was similarly produced. Moreover, $5HT_{2A}$ expression in LC further increased after DSP4 injection, all these results suggesting an intrinsic regulation of 5HT and NE activity between PAG, DRN and LC neurons activated by iTR.

Finally, iTR significantly reduced microglial reactivity in LC and increased non-microglial BDNF expression, an effect that was reverted by butoxamine, implicating BDNF regulation in central 5HT/NE actions on neuropathic pain.

Keywords

Abbreviations

iTR, increasing-intensity treadmill exercise; 5HT, serotonergic; NE, noradrenergic; SNTR, sciatic nerve transection and repair; i.p., intraperitoneal; RM, raphe magnus; DRN, dorsal raphe nucleus; PAG, periacqueductal grey; LC, locus coeruleus; Bu, butoxamine; Ke, ketanserin; DSP4, N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine; BDNF, brain-derived neurotrophic factor.

1. Introduction

Increasing-intensity treadmill exercise (iTR), performed during the first days after injury, has been shown to significantly reduce hyperalgesia in neuropathic pain models, such as sciatic nerve chronic constriction injury (CCI) (Cobianchi et al., 2010) and section and suture repair (SNTR) (Cobianchi et al., 2013). iTR decreased the expression of BDNF and other neurotrophins although without impairing nerve regeneration (Cobianchi et al., 2013, Lopez-Alvarez et al., 2015). iTR-induced decrease of BDNF expression was associated to reduction of microgliosis and restoration of the expression of chloride transporters in the primary sensory neurons and along the central pain pathways (Lopez-Alvarez et al., 2015; Modol et al., 2014). Little is known, however, on the central mechanisms by which iTR induces hypoalgesia, and we hypothesize that it may act by activating descending pathways for inhibition of pain transmission at the spinal level.

Brain areas can modulate the ascending pain signals by serotonergic and noradrenergic projections to spinal cord neurons, which can facilitate or inhibit the afferent sensory neurons. Periaqueductal grey (PAG) areas receive pain and temperature fibers and activate defensive and stress responses by sending axons to both locus coeruleus (LC) and raphe magnus nucleus (RM), whose antinociceptive output trigger descending inhibition (Millan, 2002). Serotonergic RM and noradrenergic LC projections normally activate spinal enkephalinergic and GABA/glycinergic interneurons. Enkephalin released from terminals of enkephalinergic dorsal horn interneurons acts on the opioid receptors located on the central processes of nociceptive primary afferents, reducing Ca^{2+} entry into their terminals and decreasing the release of nociceptive neurotransmitters such as glutamate and substance P (Ossipov et al., 2010). Similarly, activation of dorsal horn interneurons containing GABA or glycine also inhibit the spinal transmission of noxious sensory signals, and previous studies indicate that spinal GABAergic inhibition is reduced after experimental nerve injury (Moore et al., 2002). The loss of tonic inhibition by spinal interneurons is also associated with dysregulation of NKCC1/KCC2 chloride cotransporters expression, inducing an inversion of GABAergic depolarizing currents in neuropathic conditions (Modol et al., 2014), that is prevented by iTR (Lopez-Alvarez et al., 2015).

Besides the demonstrated peripheral effects (Cobianchi et al., 2010, 2013; Lopez-Alvarez et al., 2015; Udina et al., 2011), we hypothesized that iTR may activate pain central inhibition normally gating the nociceptive input to supraspinal, medullary and cortical areas, which are decreased after peripheral nerve injury. By stimulating the descending noradrenergic and serotonergic projections to the dorsal horn, specific exercise training may result in the activation of inhibitory circuits in the dorsal horn and in the consequent inhibition of second-order spinothalamic neurons by presynaptic and postsynaptic mechanisms.

We studied the expression of noradrenaline (NE) and serotonin (5HT) receptors in sensory neurons of the spinal cord dorsal horn, that participate in pain modulation. Among NE receptors, α_{1A} receptors are expressed in GABAergic and glycinergic neurons

of dorsal horn lamina II, where they may participate in endogenous inhibition of afferent pain by exciting inhibitory interneurons (Baba et al., 2000). α_{2A} receptors are expressed in the spinal cord predominantly on the terminals of primary C-fibers afferents, where they inhibit nociception (Stone et al., 1998). β_2 receptor is an excitatory adrenoreceptor expressed in dorsal horn neurons (Nicholson et al., 2005), which activation induces antinociceptive effects (Yalcin et al., 2009a,b, 2010; Zhang et al., 2016). We also assessed changes in serotonergic $5HT_{2A}$ receptor since it is involved in spinal chloride homeostasis, which dysregulation is associated with spinal disinhibition and neuropathic pain (Gackiere and Vinay, 2014; Jacobs et al., 2002). Under hyperalgesic states the $5HT_{2A}$ receptor was found to be expressed in laminae I-III NK1R-positive projection neurons (Mantyh et al., 1997) and in lamina II galanin-containing neurons expressing GABAergic boutons (Tiong et al., 2011), receiving emerging interest as a potential target for treating nerve injury-induced pain and spasticity (Bos et al., 2013).

In this study, we investigated the changes induced by iTR on noradrenergic and serotonergic circuitry that may be related to antinociception. For this purpose, we analyzed the expression of adrenergic α_{1A}, α_{2A} and β_2 receptors, and serotonergic $5HT_{2A}$ receptor after injury to the sciatic nerve in rats, and their changes under increasing-intensity exercise when neuropathic pain is prevented. We also relate the exercise-induced hypoalgesia to the expression of β_2 receptor and the reduction of microgliosis in noradrenergic neurons.

2. Materials and methods

2.1. *Animals and surgery*

Adult female Sprague-Dawley rats (240 ± 30 g) were housed in standard cages with access to food and water *ad libitum* under a light–dark cycle of 12 hours. All the experimental procedures were approved by the Ethics Committee of the Universitat Autonoma de Barcelona and followed the guidelines of the European Commission on Animal Care (EU Directive 2010/63/EU). Rats were anesthetized by intraperitoneal (i.p.) injection of ketamine (10 mg/kg, Imalgene 500; Rhone-Merieux, Lyon, France) and xylazine (1 mg/kg, Rompun; Bayer, Leverkusen, Germany).

Rats were submitted to a sciatic nerve transection and repair (SNTR), a well characterized model that allows the evaluation of neuropathic pain and nerve regeneration (Cobianchi et al., 2014). The right sciatic nerve was exposed at the mid thigh, transected at 92 mm from the tip of the third toe, and repaired by epineural sutures (10-0). The wound was closed in two layers and disinfected with povidone iodine. Rats were kept in a warm environment until their recovery from anesthesia.

2.2. *Experimental design*

Seven days before surgery, all the animals were habituated to the experimental device for treadmill locomotion (Treadmill LE 8706; LETICA, Barcelona, Spain) and

pretrained to the task, by leaving them to explore the stopped treadmill for 5 minutes and then trained in a single iTR session. Each iTR session consisted of 1 hour running, starting at a locomotion speed of 10 cm/s that was increased 2 cm/s every 5 minutes, until a maximal speed of 32 cm/s (Cobianchi et al., 2013; Lopez-Alvarez et al., 2015). All rats were evaluated during follow-up with sensory tests performed during the morning, whereas treadmill running sessions were performed during the afternoon.

At day 3 after surgery, animals were randomly selected to follow or not the iTR training. Training sessions were performed daily over 12 consecutive days from day 3 to 14 after injury.

SNTR rats were divided in several groups: a group of rats performed iTR (SNTR-iTR group, n=10), and a second group remained sedentary (SNTR-sed group, n=8). Other groups performed or not iTR with pharmacological blockade of β_2-receptors with butoxamine (Sigma-Aldrich, 8 mg/Kg in saline, i.p.; SNTR-iTR+Bu group, n=6, and SNTR-sed+Bu group, n=6), or blockade of $5HT_{2A}$-receptors with ketanserin (Sigma-Aldrich, 8 mg/Kg in saline, i.p.; SNTR-sed+Ke group, n=6, and SNTR-iTR+Ke group, n=6). These drugs were administered each day of training, 30 minutes before starting the exercise. Doses were chosen over the basis of previous studies using rats. Control groups for both drugs were injected with saline vehicle only. A naive group of rats was added for comparison with injured rats (n=6).Finally, other two groups of animals were injected with N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine (DSP4, Sigma-Aldrich, 50 mg/kg in saline, i.p.), a neurotoxin that selectively induces degeneration of NE neurons in the LC, thus depleting NE projections originating from the LC (Jonsson et al., 1981; Prieto and Giralt, 2001). DSP-4 was injected within 10 min of preparation (Grzanna et al., 1989), and its administration was performed 4 days before the injury to ensure effect from the beginning of the training. One group of DSP4 injected rats followed iTR (SNTR-iTR+DSP4 group, n=8), and another remained untrained (SNTR+DSP4 group, n=10).

2.3. *Nociceptive tests for pain threshold measurement*

Three days before surgery, all the injured animals were habituated to the experimental devices, and then tested for baseline nociceptive thresholds recording. The nociceptive behavior tests for mechanical and thermal stimuli were performed on both hind paws before and at different days after injury (dpi), the experimenter being blind to assignment of rats to the different groups. Lateral and medial sites of the paw were tested to differentiate changes in sensory thresholds produced respectively by sciatic nerve injury from those due to saphenous nerve sprouting (Cobianchi et al., 2014).

Sensitivity to mechanical stimuli was measured by means of an electronic Von Frey algesimeter (Bioseb, Chaville, France). Rats were placed on a wire net platform in plastic chambers. Then, a non-noxious pointed probe was gently applied to each test site, slowly increasing the pressure. The threshold was expressed as the force (in grams) at which rats withdrew the paw in response to the stimulus. A cutoff force was set at 40 g,

when the stimulus lifted the paw without response. The mechanical nociceptive threshold was calculated as the mean of 3 measurements per test site, with a 3 minute interval between each measurement.

Thermal sensitivity was assessed by means of a Plantar test algesimeter (Ugo Basile, Comerio, Italy). Rats were placed into a plastic box with an elevated plexiglass floor. The beam of a lamp was pointed at the same test sites as above in the hind paw plantar surface. Intensity was set to low power (40 mW/cm2) with a slow heating rate. A cutoff time for the stimuli was set at 20 seconds to prevent tissue damage. Heat pain threshold was calculated as the mean of 3 trials per test site, with a 5 minute interval between trials, and expressed as the latency (in seconds) of paw withdrawal response. For both mechanical and thermal thresholds, values are reported as the percentage ratio between the ipsilateral injured and the contralateral normal paw at each test day. This allows a representation of neuropathic pain induced by the injury since no significant variations of contralateral thresholds were found in previous studies (Cobianchi et al., 2013; Lopez-Alvarez et al., 2015).

2.4. *Immunohistochemistry of spinal cord and brain*

At the end of follow-up at 14 dpi, all rats were euthanized and spinal cord and brain samples collected for immunohistochemical assays. Lumbar (L4-L5) spinal cord segment and brain were removed from perfused animals and kept in fixative for 1 hour and 4 hours respectively, and then cryoprotected with 30% sucrose in PBS. Transverse sections, 25 μm thick, were cut on a cryostat and mounted on slides. Sections were blocked with specific serum and incubated overnight with primary antibodies in 0.3% Triton X-100 in PBS, used for staining 5HT and NE receptors in spinal cord and brain sections (anti-β_2 receptors, rabbit, 1:500, Santa Cruz; anti-α_{1A} receptors, goat, 1:500, Santa Cruz; anti-α_{2A} receptors, goat, 1:500, Santa Cruz; anti-5HT$_{2A}$ receptors, goat, 1:500, Santa Cruz), along with antibodies to identify 5HT and NE neurons (anti-tyrosin hydroxylase (TH), mouse, 1:500, Santa Cruz; anti-tryptophan hydroxylase (TPH), mouse, 1:500, Santa Cruz), microglial cells (anti-Iba1, goat, 1:200, Abcam) and BDNF (anti-BDNF, sheep, 1:200, Millipore).

The following day sections were incubated for 2 hours with Alexa Fluor 488 and/or Alexa Fluor 594 conjugated secondary antibodies (1:200, Life Technologies). After washing in PBS, sections were coverslipped with a solution of DAPI (1 mL/mL) in Mowiol mounting medium. Positive and negative controls of antibodies were previously checked for non-specific labeling. For each marker, three sections from 4 samples per group were used for quantification of immunolabeling. The experimenter was blinded to the sample group. Anatomical structures of interest were previously identified under microscope in spinal cord and brain atlas coordinates using specific sections and Nissl or Hematoxylin staining. 10X and 20X images were captured with a Zeiss LSM 700 confocal microscope, and analyzed using ImageJ software (NIH, USA). Thresholding of fluorescent signal was adjusted over the background level of a negative control (samples stained

without primary antibody). The integrated density of immunoreactivity was calculated within regions of interest drawn with ImageJ software tool. For spinal cord immunoreactivity, data are shown for both ipsilateral and contralateral sides. For brain immunoreactivity, data are shown only for projection areas of the injured side.

2.5. *Data analysis*

Data are presented as mean ± SEM. Statistical analysis of nociceptive thresholds was made by two-way analysis of variance (ANOVA) with group and time after injury as factors, followed by Bonferroni's post hoc comparisons. Statistical comparisons for immunofluorescence data were made by one-way and two-way ANOVAs followed by Tukey's post hoc test when necessary. The level of statistical significance was 5% ($p<0.05$) in all the analyses.

3. Results

3.1. *iTR reduced hyperalgesia after SNTR*

Changes in sensory thresholds were recorded at both medial and lateral sites of the hindpaw to monitor the contribution to hyperalgesia of collateral sprouting of saphenous nerve, and of denervation by the injured sciatic nerve (Cobianchi et al., 2013; Lopez-Alvarez et al., 2015). As showed in Fig. 1A-B, mechanical and thermal thresholds in uninjured contralateral paws were not significantly affected by injury with an ipsi/contra% threshold ratio of about 100% at 3 dpi. However SNTR produced a marked decrease of the mean mechanical threshold at the medial side from 3 to 14 dpi (Fig. 1A; 48 to 32% of contralateral in SNTR-sed group). The decrease of thermal threshold at medial side was comparatively lower (Fig. 1B; 92 to 69% in SNTR-sed group). Statistical analyses for both group and time factors, and also their interaction, showed significant effects for the changes observed in mean medial mechanical (Group factor: $F=26.39$, $p<0.0001$; Time factor: $F=71.45$, $p<0.0001$; Interaction: $F=9.35$, $p<0.0001$) and thermal thresholds (Group factor: $F=17$, $p=0.0001$; Time factor: $F=4$, $p<0.0114$; Interaction: $F=8.83$, $p<0.0001$). iTR significantly prevented the reduction of both mechanical and thermal thresholds at 7 and 14 dpi in the SNTR-iTR group (Fig. 1; $p<0.05$ and $p<0.001$, SNRT-iTR vs. SNRT-sed Bonferroni posttest).

On the other side, the lateral side of the injured hindpaw was unresponsive during the 14 days following SNTR, since this territory remains denervated until at least 4 weeks after injury (Cobianchi et al., 2014). Since the development of thermal hyperalgesia was slower than the mechanical, we did not analyze changes of thermal thresholds in further experiments, and focused on the changes observed at the medial side of the paw.

3.2. *iTR counteracted the decrease of α_{1A} and β_2 adrenergic and $5HT_{2A}$ serotonergic receptors expression in the dorsal horn after SNTR*

We studied the expression of adrenergic and serotonergic receptors in laminae I, II and III of the spinal cord dorsal horn. We found α_{1A} receptor mainly expressed in lamina II and at lower intensity in laminae I and III (Fig. 2A). After SNTR, α_{1A} expression strongly decreased in all laminae of both dorsal horn ipsilateral and contralateral to injury (Fig. 2A,B). iTR produced an increase of α_{1A} immunoreactivity in laminae II-III of both dorsal horns, although only significantly in the ipsilateral side (Fig. 2B; $p<0.001$, SNTR-iTR vs. SNTR-sed). On the other hand, the expression of α_{2A} receptor was almost unchanged in the dorsal horn of injured sedentary and exercised rats (data not shown).

The β_2 receptor was expressed in all laminae I-III of the dorsal horn in the naïve rats (Fig. 3A). SNTR reduced the expression of β_2 receptor in both the ipsilateral and contralateral sides of lamina I, II and III ($p<0.001$ naive vs. SNTR-sed; Fig. 3A,B). The expression of β_2 was significantly recovered by iTR in the lamina II bilaterally ($p<0.001$ SNTR-sed vs. SNTR-iTR) and in the contralateral lamina I ($p<0.01$).

In naïve animals, the $5HT_{2A}$ receptor was more densely expressed in lamina II, even if spot clusters of immunofluorescent labeling were present in all dorsal horn laminae (Fig. 4A). After SNTR, $5HT_{2A}$ immunoreactivity decreased significantly in lamina II bilaterally ($p<0.001$ naive vs. SNTR-sed; Fig. 4B) and in the ipsilateral lamina I ($p<0.05$ naive vs. SNTR-sed). Interestingly, iTR significantly recovered $5HT_{2A}$ receptor expression in lamina II ($p<0.01$ SNTR-sed vs. SNTR-iTR), and in the ipsilateral lamina I ($p<0.05$, SNTR-sed vs. SNTR-iTR; Fig. 4B).

These results indicate that nerve injury determines a decrease of NE and 5HT tone in the dorsal horn neurons that may reflect the drive from normal to neuropathic conditions, and highlight the iTR potential to reverse these changes.

3.3. Changes in $5HT_{2A}$, α_{1A} and β_2 receptors expression in midbrain areas induced by SNTR and after iTR

Since iTR showed to significantly impact the expression of $5HT_{2A}$ serotonergic and α_{1A} and β_2 adrenergic receptors in the lumbar dorsal horn of spinal cord, we investigated the changes that peripheral nerve injury and iTR treatment determined on the expression of these receptors at higher integrating brain centers that participate in pain descending modulation, such the periaqueductal grey matter (PAG), the locus coeruleus (LC), the dorsal raphe (DRN) and the raphe magnus nucleus (RM) (Fig. 5A).

The normal expression of α_{1A} receptor was found in medium to large-size neurons of LC and DRN and on terminals of NE projections to RM (Fig. 5B, naïve group). Slightly stronger staining was observed in the DRN after injury (Fig. 5B, SNTR-sed group), in contrast with the reduced expression in the dorsal horn. iTR significantly increased α_{1A} immunoreactivity in LC and DRN, and in adjacent areas, such as the subcoeruleus nucleus, and in more neurons of dorsomedial DRN areas (Fig. 5B, SNTR-iTR group). Compared with LC and DRN, where the increase of α_{1A} immunoreactivity was significant (Fig. 5C; SNTR-iTR vs. naïve, $p<0.01$, and SNTR-iTR vs. SNTR-sed, $p<0.05$), only slightly increased amount of immunopositive clusters was observed surrounding the soma of

RM neurons. The changes in the pattern of α_{1A} receptor expression across LC and raphe nuclei suggest that autologous α_{1A} receptors in LC may have been activated in a positive loop to increase LC activity under exercise training.

Autologous β_2 receptors were also found in LC and sparse immunoreactivity in DRN and RM of naïve rats (Fig. 6A, naïve group). Nerve injury did not induce significant changes in β_2 receptors (Fig. 6A, SNTR-sed group). iTR rats showed immunoreactivity in a larger area of LC (Fig. 6A-B, SNTR-iTR vs. SNTR-sed p<0.01; SNTR-iTR vs. naïve p<0.05), and also a slight not significant higher density in DRN and RM, as terminals of noradrenergic projections from LC (Fig. 6B). These results confirmed that iTR enhanced the activity of NE LC neurons.

The immunolabeling of $5HT_{2A}$ receptor in naïve rats showed positive clusters in PAG and DRN regions and rarely in the RM nucleus extending laterally into the adjacent reticular formation, where are large-size serotonergic neurons (Fig. 7A, naïve group). Peripheral nerve injury decreased the expression of $5HT_{2A}$ in PAG and DRN (Fig. 7A-B, SNTR-sed group). The pattern of expression appeared different in injured animals, with higher presence of scattered $5HT_{2A}$ clusters through dorsomedial areas of RM than in the PAG compared with naïve samples. iTR strongly increased $5HT_{2A}$ receptor expression in PAG and DRN (Fig. 7A-B, SNTR-iTR vs. SNTR-sed p<0.01, and SNTR-iTR vs. naïve p<0.05). There was strong immunolabeling in the soma of many large-size neurons in these areas and even in dendrites of PAG and of DRN neurons projecting to other nuclei. Spot clusters indicating terminal $5HT_{2A}$ contacts were significantly increased in the RM (Fig. 7A-B, SNTR-iTR vs. SNTR-sed p<0.05, and SNTR-iTR vs. naïve p<0.01). This result suggests a reorganization of serotonergic connections between PAG and raphe nuclei, with the possible function to increase their activity to descending output.

3.4. Blockade of β_2 or $5HT_{2A}$ receptors antagonized the iTR induced hypoalgesia after SNTR

We wanted to test if the activation of β_2 and $5HT_{2A}$ receptors was directly involved in the prevention of mechanical hyperalgesia observed in iTR rats after SNTR. Inhibition of β_2 receptors with butoxamine, a selective β_2 blocker, before starting iTR sessions antagonized its hypoalgesic effect at both 7 and 14 dpi (Fig. 8A, SNTR-iTR vs. SNTR-iTR+Bu p<0.05). However, butoxamine treatment alone had no effects on mechanical hyperalgesia, indicating a specific β_2 activity contribution on the iTR effects.

Similarly, treatment with ketanserin, an inhibitor of $5HT_2$ receptors (also with weak α_1 adrenergic blocking properties), significantly antagonized the iTR hypoalgesic effect, but only at 14 dpi (Fig. 8B). iTR hypoalgesia was only partially blunted at 7 dpi (Fig. 8B, SNTR-iTR vs. SNTR-iTR+Ke, p<0.001 at 14 dpi). Ketanserin treatment alone partially reduced SNTR induced mechanical hyperalgesia, indicating that $5HT_2$ receptors (and maybe α_1 receptors blocked by ketanserin) may mediate mechanisms of sensitization after nerve injury.

3.5. Depletion of NE output from locus coeruleus with DSP4 did not modify the hypoalgesic effect of iTR

The NE specific neurotoxin DSP4 was injected to induce depletion of NE projections from the LC (Grzanna et al., 1989), thus preventing the activation of NE descending projections during iTR (Fig. 9). In rats receiving DSP4 injection the hyperalgesia was even slightly increased compared with untreated rats (Fig. 9A). However, iTR treatment similarly produced hypoalgesia in injured rats ($p < 0.01$ SNTR-iTR+DSP4 vs. SNTR-sed+DSP4), despite the depletion of NE output significantly reduced the expression of β_2 and α_{1A} receptors in the LC, which was not compensated by iTR (Fig. 9B-C). This result suggests that the activation of NE descending projections may be not the only mechanism inducing pain relief by iTR, and points out the possibility that mesencephalic 5HT system play a supportive or modulatory role for NE.

To confirm this hypothesis, we looked at the $5HT_{2A}$ expression in LC. Sparse labeling of $5HT_{2A}$ receptors was found in the LC of naïve rats (Fig. 10A), probably corresponding to terminals of PAG excitatory and DRN inhibitory projections that regulate the LC activity (Haddjeri et al., 1997; Kim et al., 2004). SNTR decreased the expression of $5HT_{2A}$ receptors, which was prevented in the groups performing iTR (Fig. 10A-B). The inhibition of β_2 receptors by butoxamine did not have any effect on the expression of $5HT_{2A}$ in the LC of injured rats. In contrast, injection of DSP4 induced a significant increase of $5HT_{2A}$ immunoreactivity ($p < 0.01$ and 0.05 SNTR-sed vs. SNTR-sed+DSP4 and SNTR-iTR+DSP4 respectively), even higher than by iTR alone. The strong effect induced by DSP4 on the expression of $5HT_{2A}$ receptors may suggest the activation of DRN 5HT to increase the inhibition of depleting LC neurons, while iTR exercise seems to restore the normal tonic PAG and DRN 5HT control on NE activity. All together these results demonstrate that iTR hypoalgesia is conveyed by both β_2 and $5HT_{2A}$ receptors likely through parallel activation of $5HT_{2A}$ in PAG and DRN along to adrenergic LC neurons to balance the impairment of their activity due to injury.

3.6. iTR reduced microgliosis in locus coeruleus dependent on activation of β_2 receptor after SNTR

Since activation of microglia has been described to be regulated by β_2 adrenergic receptor activation (Fujita et al., 1998; O'Donnell et al., 2012), and plays an active role in the pathway that leads to BDNF regulation and KCC2 dephosphorylation in neuropathic pain states (Modol et al., 2014), we evaluated the microglial activation after SNTR (Fig. 11). We focused in LC in order to analyze the reaction of microglial cells adjacent to noradrenergic neurons projecting to the spinal cord. Iba1 immunoreactivity in the LC showed significant microglia activation after nerve injury compared to naïve rats (Fig. 11A-B, $p < 0.01$, naïve vs. SNTR-sed). Interestingly, iTR significantly reduced Iba1 immunoreactivity ($p < 0.05$, SNTR-sed vs. SNTR-iTR). However, the effect of iTR was reverted by butoxamine injection ($p < 0.001$, naïve vs. SNTR-sed+Bu and SNTR-iTR+Bu). Butoxamine treated groups showed highly ramified microglial cells suggesting that

inhibition of β_2 receptors may favor proliferation of microglia (Fig. 11A). These results show that β_2 receptors are involved in the iTR reduction of microglial reaction after nerve injury in the LC, and this mechanism could represent a potential shift from hyperalgesic to hypoalgesic state.

Since the expression of injury-induced microglial BDNF reactivity in the spinal cord dorsal horn neurons is associated to neuropathic pain and is reduced by iTR (Cobianchi et al., 2013; Lopez-Alvarez et al., 2015; Udina et al., 2011), we also analyzed BDNF expression in the LC. BDNF immunoreactivity was scant in LC of naïve rats, and not colocalized with Iba1 (Fig. 11A, naïve group). The level of BDNF expression was not influenced by nerve injury, but it was present in reactive microglia (Fig. 11A, SNTR-sed group), and increased above normal levels by iTR (Fig. 11A, SNTR-iTR group). In contrast, BDNF immunoreactivity was lowered by butoxamine treatment (Fig. 11A, SNTR-sed+Bu and SNTR-iTR+Bu), although changes were not significant between the different groups (Fig. 11B). This result shows that iTR increases BDNF expression in midbrain areas but not dependent on β_2 receptors differently from the activation of microglia.

4. Discussion

In this study we aimed to analyze the contribution of 5HT and NE descending pathways to the hypoalgesic effect of iTR on peripheral neuropathic pain. We showed that iTR reduced hyperalgesia was associated with parallel recovery in the expression of serotonergic $5HT_{2A}$ and noradrenergic α_{1A} and β_2 receptors in sensory neurons of lumbar spinal cord and in brainstem areas known to integrate sensory and motor inputs in order to modulate the descending responses.

The immunohistochemical characterization of NE receptors expression in spinal cord dorsal horn revealed that they are mainly expressed in laminae II-III, where they were reduced after SNTR. The recovery of α_{1A} and β_2 but not of α_{2A} receptors observed after iTR may be related to the inhibition of the afferent pain. Baba et al. (2000) demonstrated the contribution of α_1 receptors to antinociception by activation of GABAergic and glycinergic inhibitory interneurons. Our results suggest that the downregulation of α_{1A} receptor in dorsal horn interneurons contributes to hyperexcitability and spinal disinhibition after peripheral nerve injury. iTR training upregulated the α_{1A} receptor in the lamina II, a region rich of GABAergic and glycinergic interneurons, thus promoting their inhibitory action.

iTR also reverted the reduction of β_2 receptor, particularly in lamina II of the dorsal horn. The β_2 receptor is present in the central terminals of nociceptive afferents and on dendrites of superficial dorsal horn neurons (Nicholson et al., 2005; Patterson and Hanley, 1987; Mizukami, 2004). Interestingly capsaicin treatment in rats decreased the β adrenergic binding sites in the spinal cord (Patterson and Hanley, 1987), as we have found after sciatic nerve injury. The facts that iTR increased β_2 expression, and that inhibition of β_2 with butoxamine antagonized the hypoalgesic effect of exercise, support the view that β_2 receptor is contributing to mediate the hypoalgesia induced by iTR. This

suggestion is in line with the antiallodynic effect demonstrated by Yalcin et al. (2009a,b, 2010) by pharmacological stimulation of β_2 after sciatic lesion.

Activation of $5HT_{2A}$ receptor is known to enhance glycine and/or GABA responses in spinal neurons (Xu et al., 1996; Liu et al., 2000), and to shift the chloride equilibrium potential in the hyperpolarizing direction (Bos et al., 2013), restoring endogenous inhibition at the dorsal horn. We have found that iTR increased $5HT_{2A}$ expression, suggesting that activation of $5HT_{2A}$ mediated tonic inhibition may contribute to the iTR effect on reducing hyperalgesia after nerve injury. However, an increase in dorsal horn $5HT_{2A}$ density was reported in a model of persistent pain (Van Steenwinckel et al., 2009), and upregulation and activation of $5HT_{2A}$ was observed after spinal nerve ligation, associated to spinal hyperexcitability (Aira et al., 2010). Furthermore, administration of a selective $5HT_{2A}$ agonist in the spinal nerve ligation model significantly increased C-fiber evoked potentials (Aira et al., 2010).

On the other hand, we showed that immunoreactivity to $5HT_{2A}$ after SNTR significantly decreased in laminae I-III, similarly to β_2 receptor, and the prevention of $5HT_{2A}$ receptor activation during exercise by pretreatment with ketanserin reduced the iTR hypoalgesic effect. Then if restoration of both spinal $5HT_{2A}$ and β_2 receptors is a direct consequence of iTR, $5HT_{2A}$ may be not directly involved as β_2 in the mechanism of early pain suppression. This may be explained by the multiple roles that $5HT_{2A}$ receptor may play on neuropathic pain conditions, by differently acting on neuronal subpopulations of dorsal horn to inhibit or facilitate pain signals. After injury, a shift of $5HT_{2A}$ receptors to inhibitory interneurons was shown (Aira et al., 2010). Since iTR counteracted the spinal loss of $5HT_{2A}$, we suggest that our treadmill protocol may potentiate the activity of inhibitory interneurons that intervene in nociception.

Besides changes in the spinal cord, antinociception may be conveyed by iTR by activation of $5HT_{2A}$ receptor as well as adrenoreceptors at higher brain centers. It is known that projections from PAG-RM and LC to the spinal cord play a pivotal role in pain control. Pain and temperature fibers project to PAG through the spinomesencephalic tract, and PAG can control afferent pain by means of parallel actions on RM and LC (Basbaum and Fields, 1978). In these nuclei, antinociceptive mechanisms can be activated through μ_1 opioid, $5HT_{2A}$ and $5HT_{2C}$ serotonergic, and α_{1A} adrenergic receptors of the LC (de Freitas et al., 2016). β_2 receptor is also expressed in areas directly participating in pain (Nicholson et al., 2005), and human genetic studies confirmed the contribution of β_2 to chronic pain disorders (Diatchenko et al., 2006). Moreover, β_2 receptor is essential for the antiallodynic action of antidepressant drugs (Yalcin et al., 2009a,b), since the absence or blockade of β_2 suppressed the hypoalgesic effect of antidepressant drugs on mechanical allodynia (Yalcin et al., 2009b).

The actions played by iTR on mesencephalic nuclei to activate central pain control may be multiple, and conveyed through synaptic regulation of NE and 5HT interconnections. Indeed, PAG is known also to be excited or inhibited by $5HT_{2A}$ and $5HT_{1A}$ receptors expressing fibers from DRN, which form a regulatory circuitry negatively

modulated by GABA and opioids (Jolas and Aghajanian, 1997; Liu et al., 2000). The DRN is the largest serotonergic nucleus providing most supply of 5HT to forebrain, and its dorsal subnuclei are located adjacent to the PAG, where dense clusters of 5HT neurons project to the RM (Kwiat and Basbaum, 1990; Cho and Basbaum, 1991) and to the LC (Kim et al., 2004). Activation of the brainstem 5HT system modulates both nociceptive and motor spinal cord circuits (Jordan and Slawinska, 2011; Pearlstein et al., 2005), and exercise increases the release of 5HT in supraspinal areas associated to reducing mechanical hyperalgesia (Gerin et al., 2008; Korb et al., 2010; Jacobs et al., 2002). In a recent work, Bobinski et al. (2015) found increased brainstem levels of 5HT, its metabolites and $5HT_{1B/2A/2C}$ receptors after a 2-weeks low-intensity treadmill training following sciatic nerve injury. Interestingly, the inhibition of 5HT but not of catecholamines reversed the hypoalgesic effect of such low-intensity exercise. At difference, our study suggests that NE antinociception can be further activated by increasing the intensity of exercise. Indeed, the iTR training induced an increase of α_{1A}, β_2 and $5HT_{2A}$ receptors expression in the PAG-DRN and PAG-LC pathways. 5HT and NE systems may interact in these areas during exercise. Earlier studies revealed that NE causes an increase in 5HT neuronal firing in DRN, mediated via activation of α_1 adrenoceptor located on 5HT neurons (Day et al., 1997; Vandermaelen and Aghajanian, 1983). We hypothesize that the activation of α_{1A} receptor in the DRN may form part of a regulatory loop that is activated by iTR between DRN and LC to enhance LC and RM descending pain-suppressing neurons. NE axons could also activate α_1 receptors on GABAergic and glycinergic inhibitory interneurons leading to inhibition of pain-relay neurons (Pertovaara, 2006).

On the other hand, pharmacological stimulation of β_2 receptor suppressed neuropathic pain after sciatic nerve insult (Yalcin et al., 2010). We found that iTR strongly increased the expression of β_2 receptor in the dorsal horn lamina II interneurons and in the LC, and that activation of β_2 receptor was necessary to induce iTR hypoalgesia. To understand which part of this circuitry is involved in the hypoalgesic effect, we depleted the NE output from LC by using the neurotoxin DSP4 (Jonsson et al., 1981; Prieto and Giralt, 2001). Administration of DSP4 has been shown to revert antinociception in different pain models (Zhong et al., 1985; Kudo et al., 2010) and to regulate 5HT agonists induced analgesia (Garcia et al., 2003). In our model, pretreatment with DSP4 increased the hyperalgesia but did not block the effect of iTR. Since the activity of LC neurons is subordinated to the activation of 5HT projections from PAG and DRN, we suggest that iTR training can trigger NE-induced descending inhibition by reestablishing also $5HT_{2A}$ receptor activity in the brainstem. Our results highlight the 5HT and NE reciprocal actions under neuropathic pain and blockade of NE neurons.

Brain and spinal expression of neurotrophins, particularly BDNF, can be modulated by activation of NE and 5HT pathways, with relevant effects on motor and sensory recovery after nerve injuries. NE activation via β adrenergic receptors seems to be essential for exercise-induced BDNF regulation (Garcia et al., 2003), and β adrenergic

blockade significantly attenuates the increase of BDNF mRNA due to exercise in the cortex (Ivy et al., 2003). On the other hand, BDNF released by activated microglia triggers neuropathic mechanisms, such as downregulation of chloride cotransporter KCC2 (Coull et al., 2005; Ferrini and De Koninck, 2013), associated to disinhibition at spinal interneurons (Modol et al., 2014). iTR increased the BDNF expression in LC parallel to decreasing microgliosis and BDNF-expressing microglia, as we previously showed in association to the recovery of KCC2 levels (Lopez-Alvarez et al., 2015). The blockade of β_2 receptor also increased microgliosis, suggesting that activation of β_2 receptor during iTR plays a role in modulation of the neuroinflammatory response to nerve injury. $5HT_{2A}$ receptor activity has been described to downregulate the BDNF expression in the rat brain (Vaidya et al., 1997). The reduction of neurotrophins NGF and BDNF observed in DRG sensory neurons after iTR (Cobianchi et al., 2013; Lopez-Alvarez et al., 2015) could be associated to the sustained increase of β_2 and $5HT_{2A}$ receptors expression in brain and spinal cord, which are reduced by sciatic nerve injury. These results indicate that the increased neurotrophic factor production in sensory neurons after peripheral nerve injury can be reversely modulated by increased intensity exercise in order to prevent maladaptive plastic changes associated to neuropathic pain.

5. Conclusions

The results of this study bring new knowledge on the contribution of descending inhibition, by increasing activity of serotonergic and noradrenergic projections from brainstem centers, to the beneficial effects of specific exercise training programs in reducing neuropathic pain. Future studies aimed at understanding which neuronal populations and which molecular mechanisms underlying pain inhibition and central brainstem circuits are still needed to foster the therapeutic possibilities for treating sensorimotor disorders with specific exercise programs.

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Figures
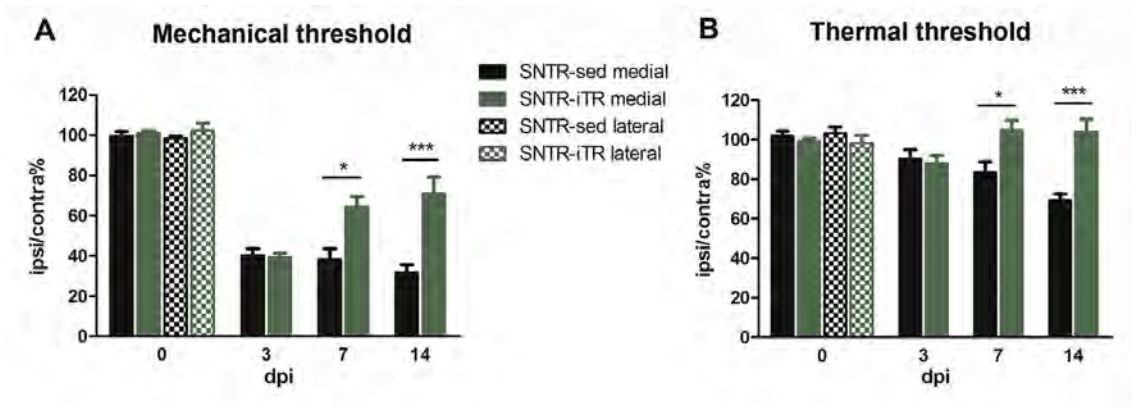
Figure 1. *Hyperalgesia is reduced by iTR after SNTR*. Changes in mechanical (A) and thermal (B) sensory thresholds recorded at the medial (filled colour bars) and lateral (dotted colour bars) test sites in rats after SNTR that were untrained (-sed) or followed daily iTR from 3 days (-iTR). Values are represented as the percent ratio between the mean ipsilateral and the contralateral paw threshold at 3, 7 and 14 days post-injury (dpi). * $p < 0.05$, *** $p < 0.001$.
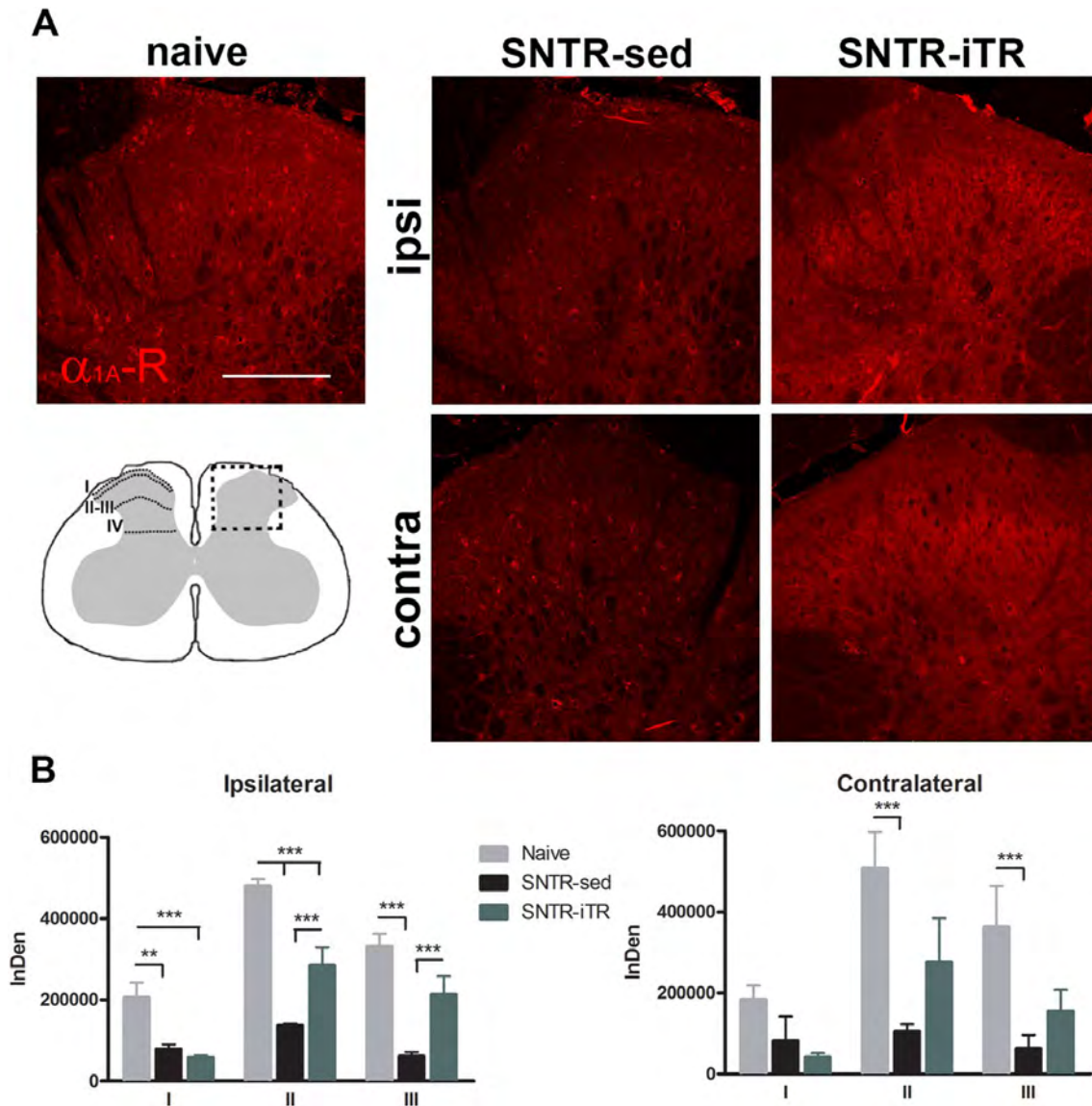
Figure 2. *iTR counteracted the decrease of α$_{1A}$ receptor expression in dorsal horn after SNTR*. (A) Representative confocal images of α$_{1A}$ adrenergic receptor immunoreactivity at 14 dpi in the spinal dorsal horn of naïve rats, and in the ipsilateral (ipsi) and contralateral (contra) dorsal horns of SNTR-sed and SNTR-iTR rats, with graphic representation of laminae I-IV as regions of interest included in captured images. Scale bar 100μm. (B) Quantification of α$_{1A}$ immunoreactivity in the ipsilateral and contralateral dorsal horn laminae (I, II, III) of SNTR-sed and SNTR-iTR rats compared with to naïve rats. ** $p \leq 0.01$, *** $p < 0.001$.
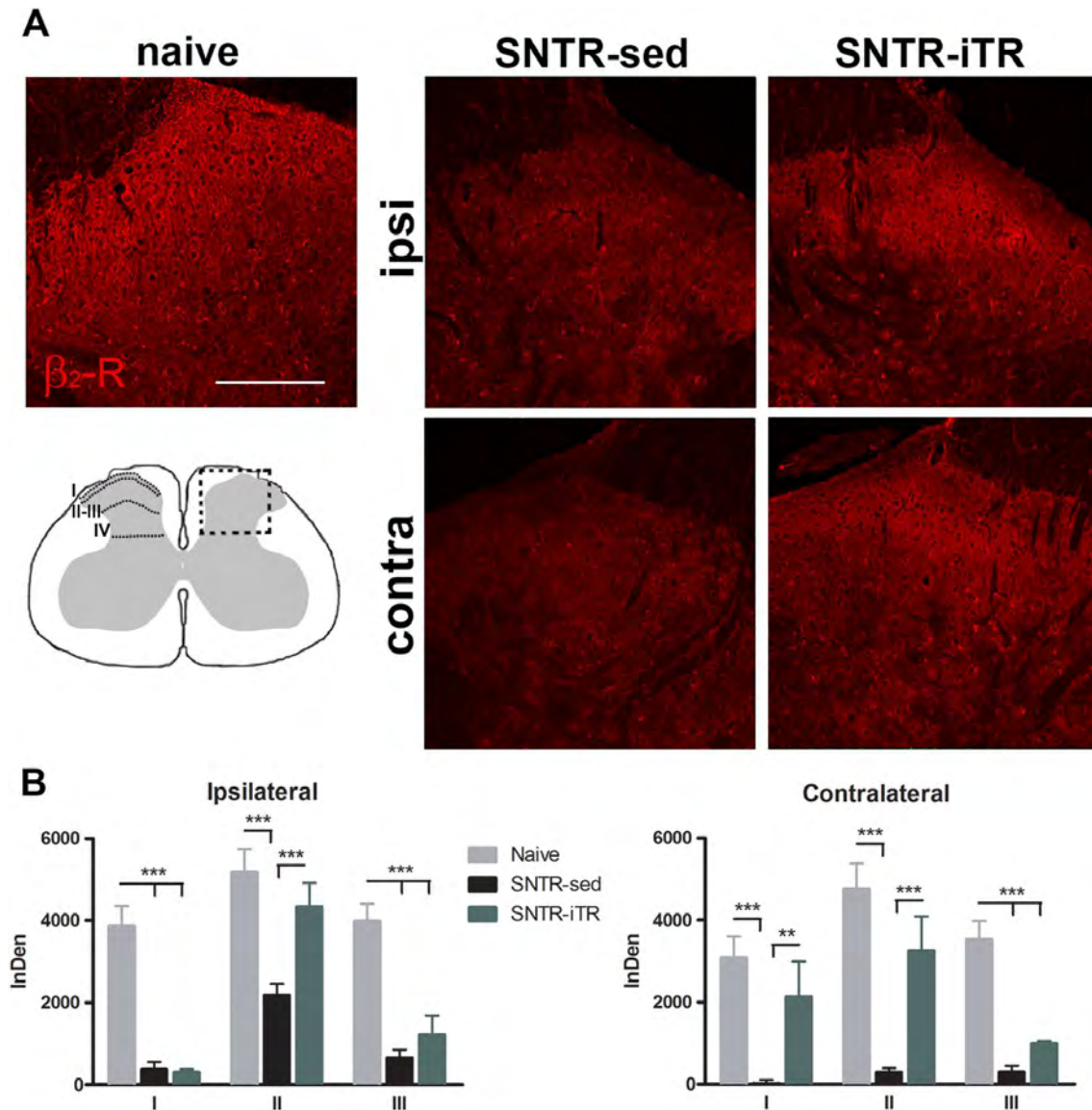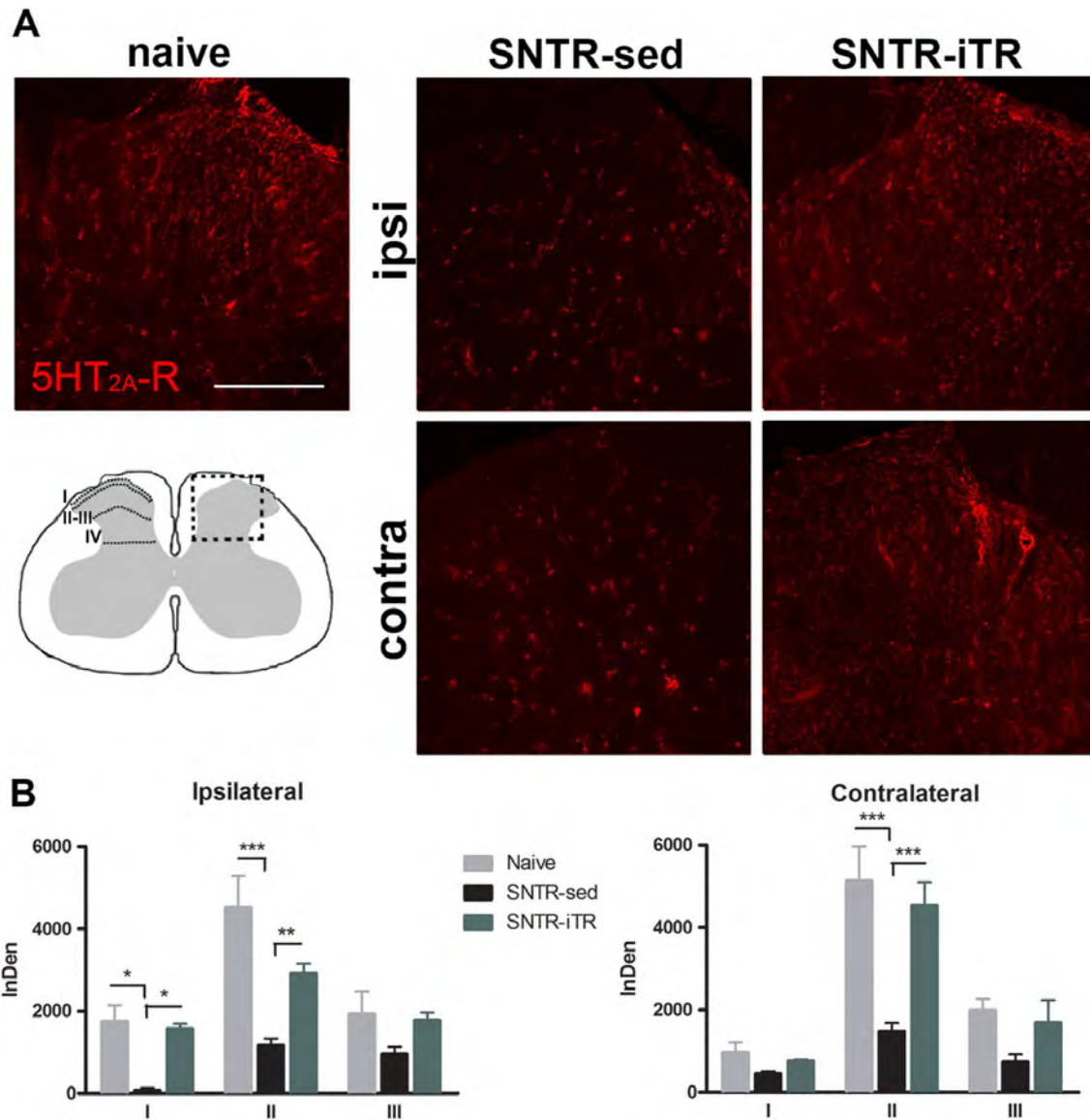
Figure 3. *iTR counteracted the decrease of β$_2$ receptor expression in dorsal horn after SNTR.* (A) Representative confocal images of the β$_2$ adrenergic receptor immunoreactivity at 14 dpi in the spinal dorsal horn of naïve rats, and in the ipsilateral (ipsi) and contralateral (contra) dorsal horns of SNTR-sed and SNTR-iTR rats, with graphic representation of laminae I-IV as regions of interestincluded in captured images. Scale bar 100µm. (B) Quantification of β$_2$ immunoreactivity in the ipsilateral and contralateral dorsal horn laminae (I, II, III) of SNTR-sed and SNTR-iTR rats compared to naïve rats. ** p<=0.01, *** p<0.001.
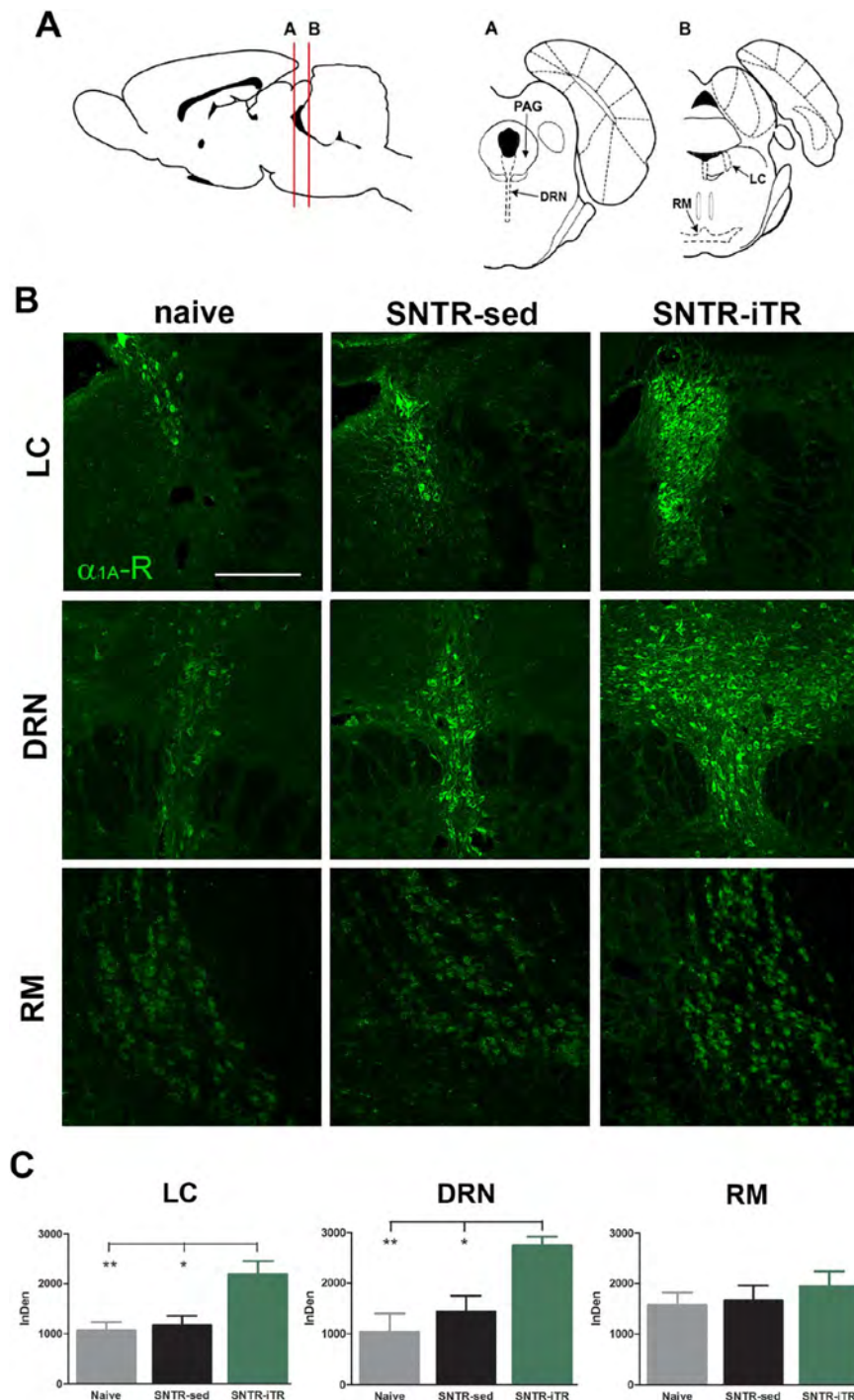
Figure 4. *iTR counteracted the decrease of 5HT$_{2A}$ receptor expression in dorsal horn after SNTR.* (A) Representative confocal images of the 5HT$_{2A}$ serotonergic receptor immunoreactivity at 14 dpi in the spinal dorsal horn of naïve rats, and in the ipsilateral (ipsi) and contralateral (contra) dorsal horns of SNTR-sed and SNTR-iTR rats, with graphic representation of laminae I-IV as regions of interestincluded in captured images. Scale bar 100μm. (B) Quantification of 5HT$_{2A}$ immunoreactivity in the ipsilateral and contralateral dorsal horn laminae (I, II, III) of SNTR-sed and SNTR-iTR rats compared to naïve rats. * $p<0.001$, ** $p<=0.01$, *** $p<0.001$.

Figure 5. *iTR increased the expression of 5HT$_{2A}$ receptor in PAG, DRN and RM after SNTR*. (A) Graphic representation of PAG, RM, DRN and LC nuclei considered as regions of interest for quantification in coronal sections. (B) Representative confocal images of the 5HT$_{2A}$ serotonergic receptor immunoreactivity at 14 dpi in PAG, DRN and RM of naïve, SNTR-sed and SNTR-iTR rats. Scale bar 100µm. (C) Quantification of 5HT$_{2A}$ immunoreactivity in PAG, DRN and RM of SNTR-sed and SNTR-iTR rats compared to naïve rats. * p<0.05; ** p<0.01.
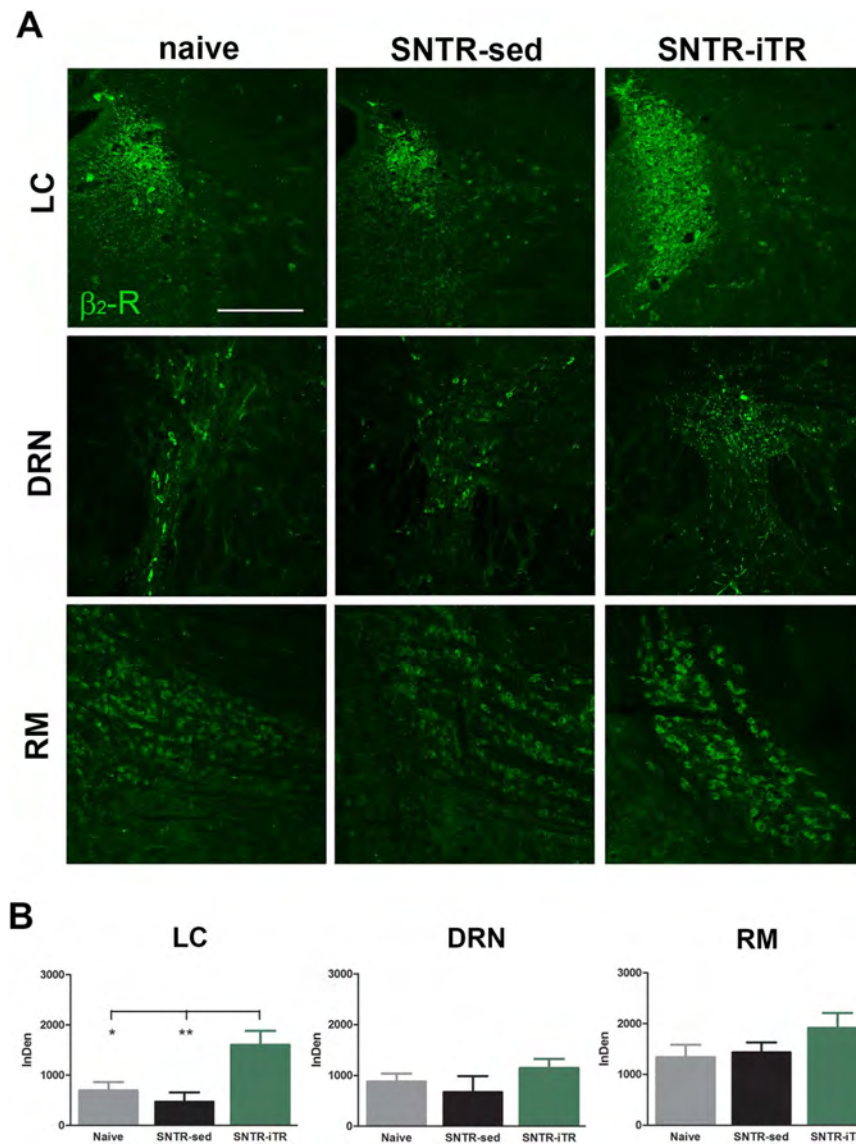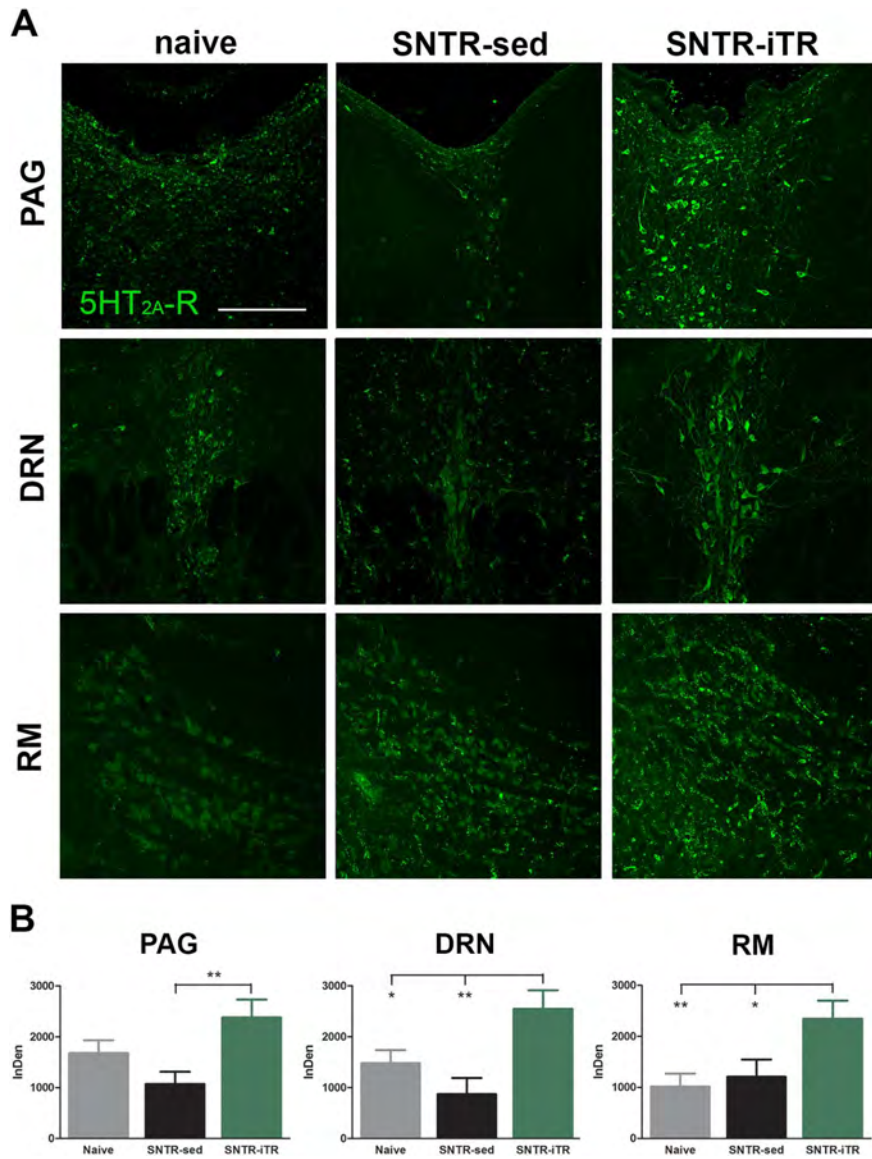
Figure 6. *iTR increased the expression of α_{1A} receptor in LC and DRN after SNTR*. (A) Representative confocal images of the α_{1A} receptor immunoreactivity at 14 dpi in LC, DRN and RM of naïve, SNTR-sed and SNTR-iTR rats. Scale bar 100μm. (B) Quantification of α_{1A} immunoreactivity in LC, DRN and RM of SNTR-sed and SNTR-iTR rats compared to naïve rats. * $p<0.05$; ** $p<0.01$.
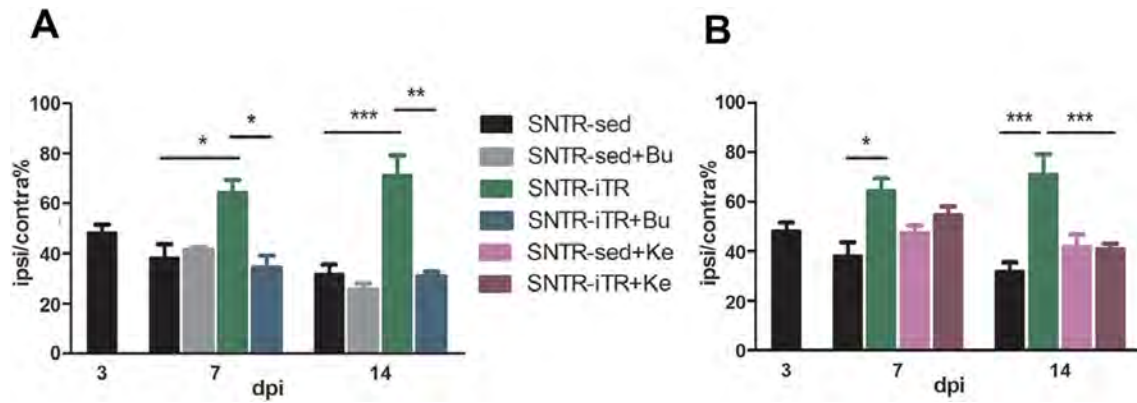
Figure 7. *iTR increased the expression of β₂ receptor in LC after SNTR*. (A) Representative confocal images of the β_2 adrenergic receptor immunoreactivity at 14 dpi in LC, DRN and RM of naïve, SNTR-sed and SNTR-iTR rats. Scale bar 100µm. (B) Quantification of β_2 immunoreactivity in LC, DRN and RM of SNTR-sed and SNTR-iTR rats compared to naïve rats. * p<0.05; ** p<0.01.

Figure 8. *Blockade of β₂ or 5HT₂ₐ receptors antagonized the iTR induced hypoalgesia after SNTR*. (A-B) Changes in mechanical threshold of SNTR-sed and SNTR-iTR groups compared with rats treated with Butoxamine (A, SNTR-sed+Bu and SNTR-iTR+Bu groups) or Ketanserin (B, SNTR-sed+Ke and SNTR-iTR+Ke groups), recorded at medial test sites at 3, 7 and 14 days post-injury (dpi) and represented as the percent ratio between the ipsilateral and the contralateral paw. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.
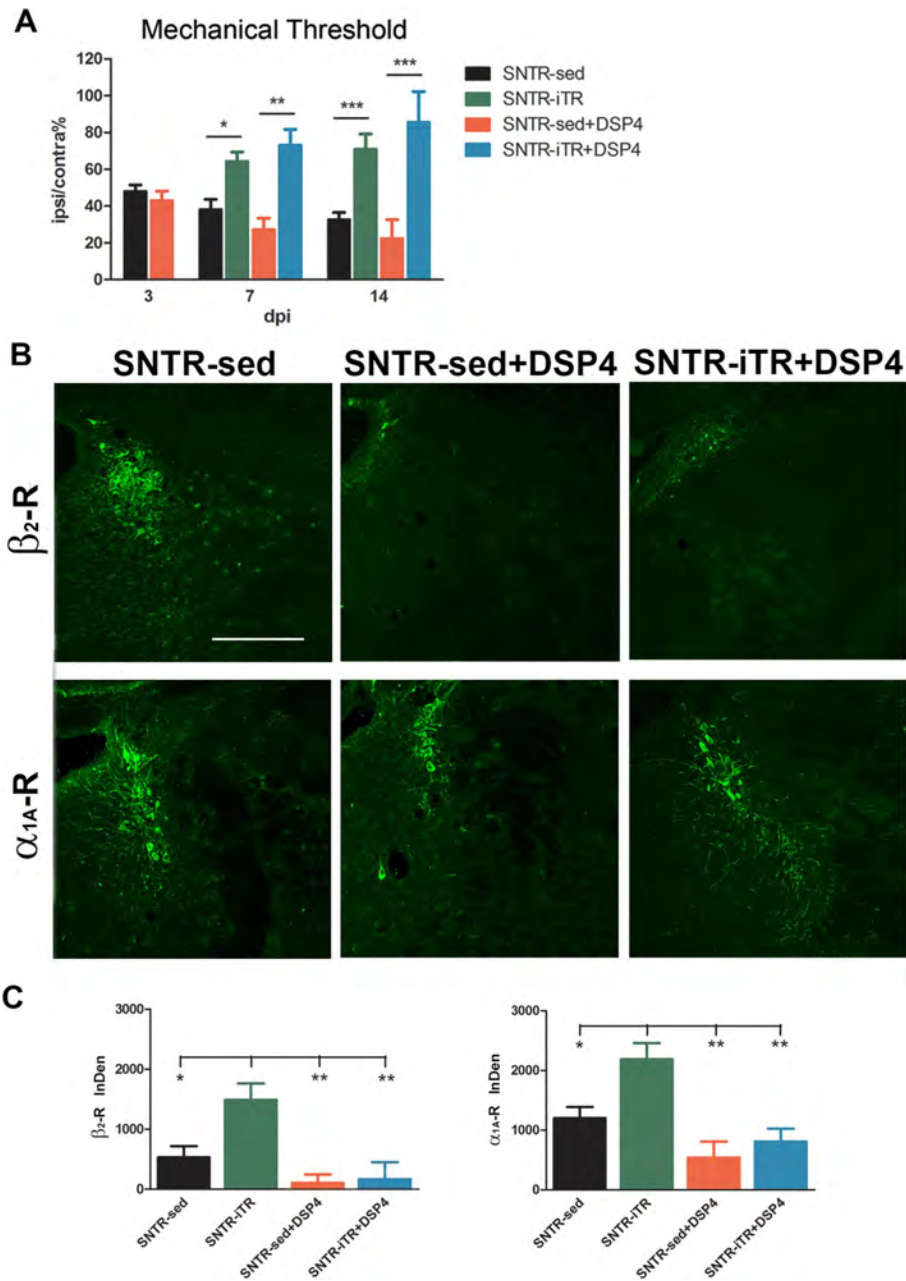
Figure 9. *Depletion of NE output from locus coeruleus with DSP4 did not affect the hypoalgesic effect of iTR*. (A) Changes in mechanical threshold of SNTR-sed and SNTR-iTR groups compared with rats treated also with DSP4, recorded at medial test sites at 3, 7 and 14 days post-injury (dpi) and represented as the percent ratio between the ipsilateral and the contralateral paw. (B) Representative confocal images of α_{1A} and β_2 adrenergic receptors immunoreactivity at 14 dpi in LC of SNTR-sed, SNTR-sed+DSP4 and SNTR-iTR+DSP4 rats. Scale bar 100μm. (C) Quantification of α_{1A} and β_2 immunoreactivity in LC of SNTR-sed and SNTR-iTR groups compared with rats treated with DSP4 (SNTR-sed+DSP4 and SNTR-iTR+DSP4 groups). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.
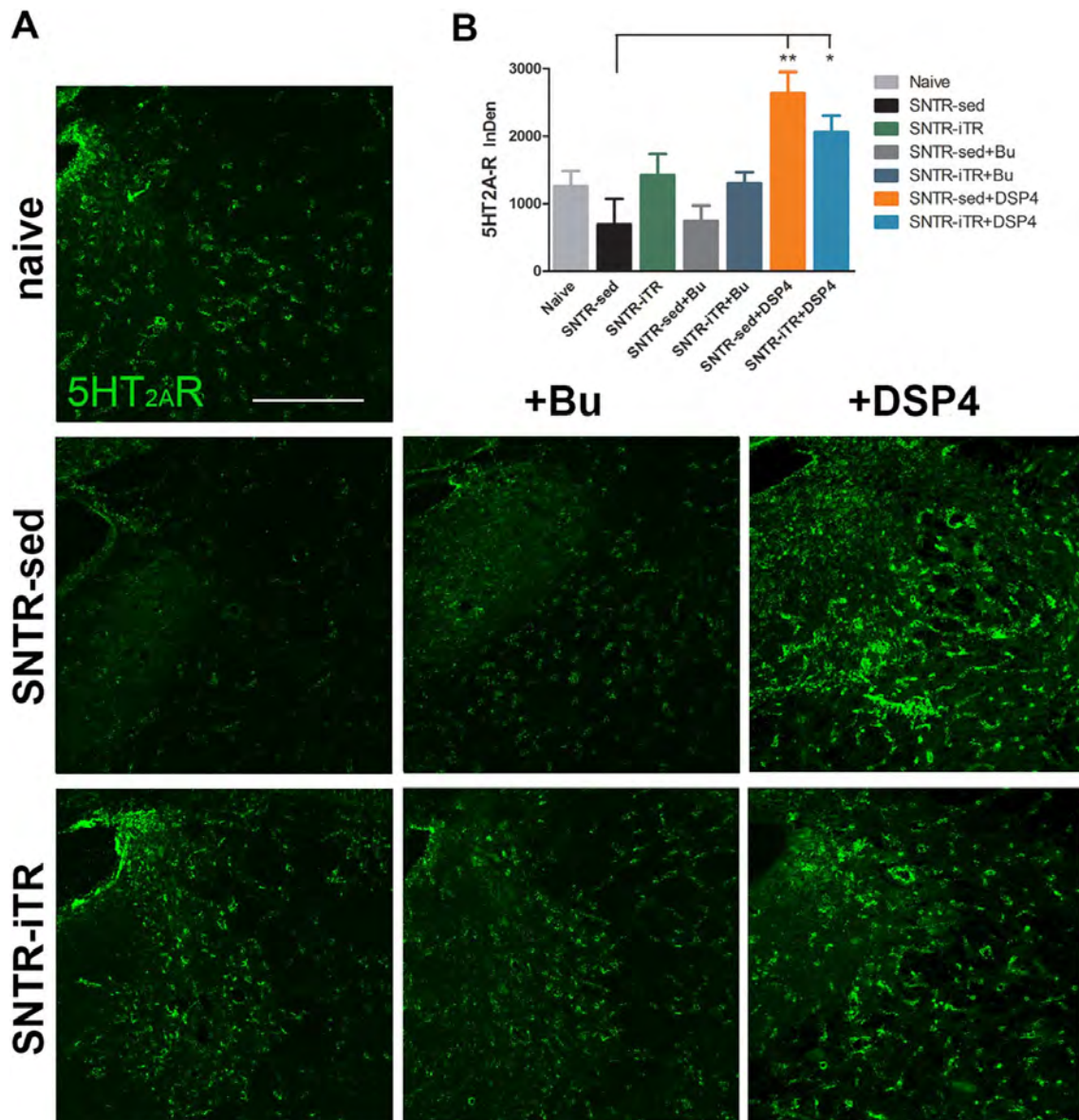
Figure 10. *The expression of 5HT$_{2A}$ receptor in locus coeruleus was normalized by iTR and further increased by DSP4 injection after SNTR.* (A) Representative confocal images of the 5HT$_{2A}$ serotonergic receptor immunoreactivity at 14 dpi in LC of naïve, SNTR-sed and SNTR-iTR rats, and those treated with Butoxamine or with DSP4. Scale bar 100μm. (B) Quantification of 5HT$_{2A}$ immunoreactivity in LC of naïve, SNTR-sed and SNTR-iTR groups compared with the same groups treated also with Butoxamine (SNTR-sed+Bu and SNTR-iTR+Bu groups) or DSP4 (SNTR-sed+DSP4 and SNTR-iTR+DSP4 groups). * $p<0.05$; ** $p<0.01$.
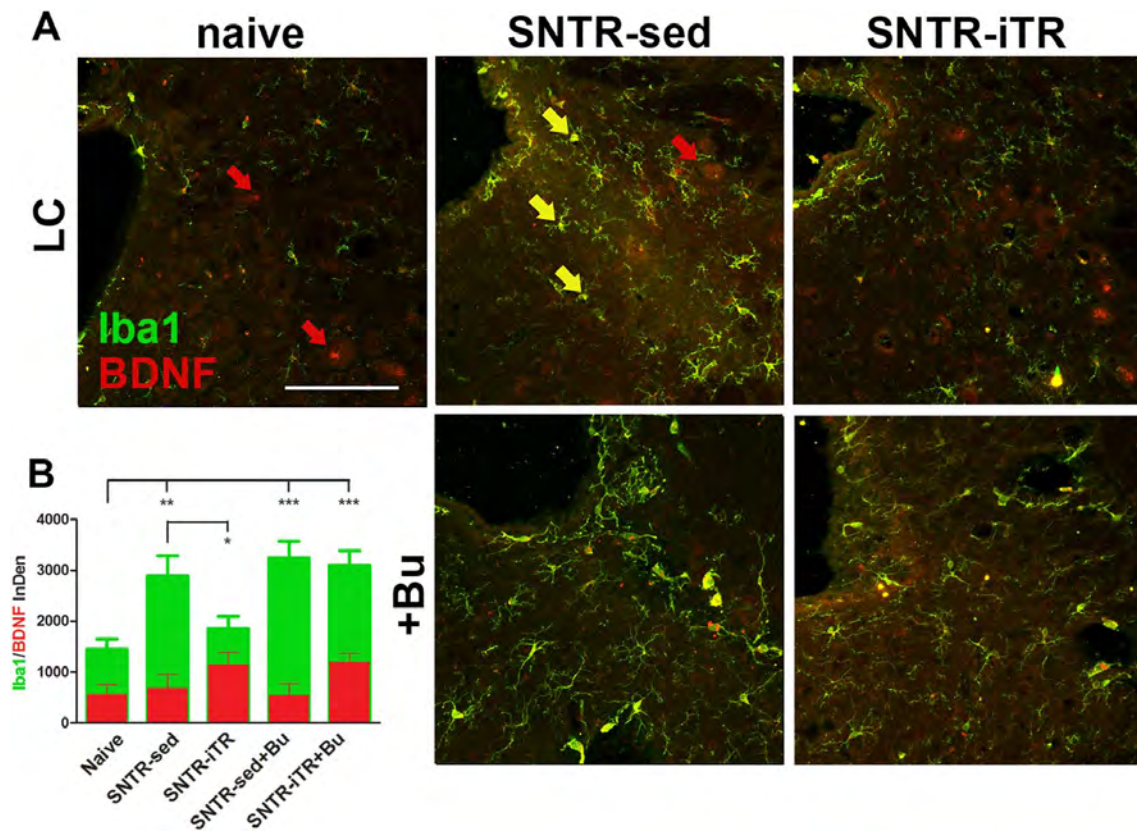
Figure 11. *iTR reduced microgliosis in locus coeruleus dependent on activation of β₂ receptor after SNTR*. (A) Representative confocal images of Iba1 and BDNF immunoreactivity at 14 dpi in LC of naïve, SNTR-sed and SNTR-iTR groups compared with groups treated also with Butoxamine (SNTR-sed+Bu and SNTR-iTR+Bu groups). Yellow arrows point to microglial cells expressing BDNF; red arrows point to BDNF labeling not colocalized with Iba1. Scale bar 100µm. (B) Relative quantification of Iba1 and BDNF immunoreactivity (Integrated Density, InDen) in double-labeled LC samples of naïve, SNTR-sed and SNTR-iTR rats compared with rats treated also with Butoxamine (SNTR-sed+Bu and SNTR-iTR+Bu groups). * $p<0.05$; ** $p<0.01$; *** $p<0.001$.