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Differential adaptive changes on serotonin and noradrenaline transporters in a rat model of peripheral neuropathic pain

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ABSTRACT

Serotonin and noradrenaline reuptake inhibitors have shown to produce antinociceptive effects in several animal models of neuropathic pain. In the present work, we have analyzed the density of brain and spinal serotonin and noradrenaline transporters (5-HTT and NAT) in a rat model of neuropathic pain, the spinal nerve ligation (SNL). Quantitative autoradiography revealed a significant decrease in the density of 5-HTT ([^{3}H]citalopram binding) at the level of the lumbar spinal cord following 2 weeks of neuropathic surgery (lamina V, −40%: 6.01 ± 0.64 nCi/mg tissue in sham-animals *vs* 3.59 ± 1.56 in SNL-animals; lamina X, −30%: 9.10 ± 2.00 *vs* 6.40 ± 1.93 and lamina IX, −22%: 12.01 ± 2.41 *vs* 9.42 ± 1.58). By contrast, NAT density ([^{3}H]nisoxetine binding) was significantly increased (lamina I–II, +34%: 2.20 ± 0.45 *vs* 2.96 ± 0.65; lamina V, +57%: 1.34 ± 0.28 *vs* 2.11 ± 0.66; and lamina IX, +58%: 2.39 ± 0.71 *vs* 3.78 ± 1.10). At supraspinal structures, SNL induced adaptive changes only in the density of 5-HTT (septal nuclei, +33%: 10.18 ± 2.03 *vs* 13.53 ± 1.14; CA3 field of hippocampus, +18%: 6.94 ± 1.01 *vs* 8.21 ± 0.81; paraventricular thalamic nucleus, +21%: 15.18 ± 1.88 *vs* 18.35 ± 2.08; lateral hypothalamic area, +40%: 12.68 ± 1.90 *vs* 17.8 ± 2.55; ventromedial hypothalamic nuclei, +19%: 7.16 ± 0.92 *vs* 8.55 ± 0.40; and dorsal raphe nucleus, +15%: 35.22 ± 3.88 *vs* 40.68 ± 3.11). Thus, we demonstrate, in the SNL model of neuropathic pain, the existence of opposite changes in the spinal expression of 5-HTT (down-regulation) and NAT (up-regulation), and the presence of supraspinal adaptive changes (up-regulation) only on 5-HTT density. These findings may help understanding the pathogeny of neuropathic pain and the differential analgesic action of antidepressants targeting 5-HTT and/or NAT transporters.

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1. Introduction

Peripheral nerve injury results in hypersensitivity to mechanical and thermal stimuli associated with a plethora of changes in both injured primary sensory afferents and those uninjured. Neurochemical and molecular adaptive changes in the spinal cord also contribute to central sensitization and the persistence of protracted pain [40]. L5–L6 spinal nerve ligation (SNL) model is one of the most commonly neuropathic paradigms used in rats. It exhibits robust mechanical hypersensitivity, thus provides a useful tool to explore the adaptive anatomical and neurochemical changes underlying neuropathic pain [17]. Antidepressants are widely used in chronic pain states. Among them serotonin and noradrenaline reuptake inhibitors have been demonstrated to produce analgesic actions in neuropathic pain in clinical and basic studies [16]. These drugs modulate monoamines neurotransmission and they induce analgesic effects not only through the activation of the descending pain modulatory systems [30], but also by acting on spinal cord targets [22]. Serotonin and noradrenaline reuptake inhibitors exert analgesic or proalgesic effects depending on the site of action and on the receptor subtype involved [8]. The serotonin transporter is highly expressed in the rat spinal cord and its distribution parallels the innervation by serotoninergic fibers originating from the medullary raphe complex, central gray and reticular formation of the midbrain [7]. Noradrenaline-containing fibers expressing noradrenaline transporter are descending from the A5, A6 and A7 cell loci in the pons to the spinal cord [10], and this inhibitory pathway appears to be activated by persistent noxious input [38]. As 5-HTT and NAT are the primary targets of antidepressants, it is deemed to be of interest to analyze whether neuropathic pain could induce

Abbreviations: 5-HTT, serotonin transporter; NAT, noradrenaline transporter; SNL, spinal nerve ligation; PWT, paw withdrawal threshold; PPWT, paw-pressure withdrawal threshold; HPA, hypothalamic–pituitary–adrenal.

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adaptive changes in their expression either at spinal or supraspinal sites. In the present study, we have used autoradiographic techniques to quantify 5-HTT and NAT density in the brain and lumbar spinal cord of rats subjected to L5–L6 spinal nerve ligation.

2. Materials and methods

2.1. Animals

Adult (200–250 g) male *Sprague-Dawley* rats were used. All the experiments were carried out in accordance to the guidelines of The European Communities Council Directive 86/609/EEC and were approved by Universidad de Cantabria Animal Research Ethical Committee. The animals (6 animals *per* group) had *ad libitum* access to food and water. Temperature ($22 \pm 2\,^{\circ}C$) and humidity ($55 \pm 10\%$) were maintained within a narrow range and the animals were on a 12-h light/dark cycle.

2.2. L5/L6 spinal nerve ligation (SNL)

Neuropathy was induced following the method originally described by Kim and Chung [17]. Rats were anesthetized with chloral hydrate (400 mg/kg, i.p.). A dorsal midline incision was made from L3–S2. Once exposed, L5 and L6 spinal nerves were ligated with 3–0 silk sutures ($n = 6$). Sham-operated rats were used as control group ($n = 6$).

2.3. Assessment of mechanical allodynia and hyperalgesia

Pain responses were evaluated 2 weeks postsurgery. Mechanical allodynia was tested by stimulation of the plantar surface of the hindpaw with *von Frey* monofilaments (Stoelting Europe, Dublin, Ireland). The paw withdrawal threshold (PWT) was taken as the lowest force that evoked 1 hindpaw withdrawal response out of 5 stimuli [35]. Mechanical hyperalgesia was assessed by measuring the paw-pressure withdrawal threshold (PPWT) when exposed to increasing noxius mechanical stimulation with an analgesiometer (*Randall-Sellito test*, Ugo Basile, Italy) [2]. The two mechanical tests were performed in both hindpaws, ipsi- and contralateral on both sham- and SNL-animals.

2.4. Tissue preparation for autoradiographic studies

Twenty-four hours after the completion of nociceptive assays, animals (12 animals in total) were sacrificed and the brain and spinal cord were rapidly removed, frozen and stored at $-80\,^{\circ}C$ until sectioning. Coronal sections (20 μm) were cut at $-20\,^{\circ}C$ using a cryostat and gelatinized slides. Several brain regions were taken according to Paxinos and Watson atlas [25] and corresponding approximately to cingulate cortex, anterior hippocampus, central gray, locus coeruleus and raphe magnus. Spinal cord transverse sections were obtained from the lumbar level, roughly corresponding to L4–L5–L6 segments.

2.5. 5-HTT and NAT autoradiography

Both 5-HTT and NAT autoradiographies were performed as previously described [12] using [^{3}H]citalopram (3 nM; specific activity 81.2 Ci/mmol; PerkinElmer Life Sciences, Madrid, Spain) and [^{3}H]nisoxetine (3 nM; specific activity 85 Ci/mmol, PerkinElmer Life Sciences, Madrid, Spain) for 5-HTT and NAT studies respectively.

Three consecutive sections of each animal (two for total binding and one for non-specific binding) were thawed and dried at room temperature for 30 min, then preincubated at $25\,^{\circ}C$ for 15 min in Tris–HCl 50 mM buffer (pH 7.7) containing NaCl 120 mM (5-HTT experiments) or NaCl 300 mM (NAT experiments) and KCl 5 mM. Sections were then incubated in the same buffer at $25\,^{\circ}C$ for 2 h for the 5-HTT and at $4\,^{\circ}C$ for 4 h for the NAT. Fluoxetine 10 μM and mazindol 1 μM (Sigma Aldrich Inc., Spain) were used to determine 5-HTT and NAT non-specific binding, respectively. Following incubation, sections were washed four times for 2 min (5-HTT autoradiography) or three times for 5 min (NAT autoradiography) in ice-cold buffer, briefly dipped in deionized water at $4\,^{\circ}C$, and then cold air-dried over night.

Autoradiograms were generated by apposing the incubated slides to Biomax MR films (GE Healthcare, Madrid, Spain) along with tritium labeled standards (GE Healthcare, Madrid, Spain) and developed after 3 months of exposition at $4\,^{\circ}C$.

2.6. Data analysis and statistics

Autoradiograms digitalized and then quantified using an image analysis system (Scion Image, Scion Corporation, Maryland, USA). Unilateral readings (by duplicate and in two consecutive sections per animal) were carried out to detect differences between ipsi- and contralateral structures. Statistical analysis demonstrated no differences between ipsi- and contralateral values in any of the areas measured. Therefore, data were pooled and given as bilateral readings (the value for each animal was obtained from 4 readings).

Results represent the autoradiographic density of 5-HTT and NAT (nCi/mg tissue) and are expressed as mean ± S.D. The statistical comparison (PRISM, version 4.03, GraphPad Software, San Diego, CA, USA) was done using an unpaired two-tail Student's *t*-test with a level of significance set at $p < 0.05$.

3. Results

3.1. Mechanical allodynia and hyperalgesia induced by SNL

Two weeks after SNL surgery, the mechanical paw withdrawal threshold (PWT) in the ipsilateral hind paw of SNL rats was decreased in comparison to sham-animals (3.07 ± 1.13 g *vs* 17.28 ± 3.12 g; $p < 0.001$; $n = 6$ animals/group). A certain degree of contralateral mechanical allodynia was also found in SNL rats (PWT: 11.09 ± 5.40 g; $p = 0.061$ *vs* sham-animals). Mechanical hyperalgesia was also detected, as evidenced by a decreased paw-pressure withdrawal threshold (PPWT) in the ipsilateral paw hind of SNL rats in comparison to sham-animals (183.56 ± 7.25 g *vs* 113.20 ± 4.15 g, respectively; $p < 0.001$).

3.2. Autoradiographic density of 5-HTT

In sham-operated animals, the highest density of brain 5-HTT was found in brainstem nuclei, amygdala, thalamus and the lateral hypothalamic area. The autoradiographic density of 5-HTT was significantly increased in SNL animals in the septal lateroventral nuclei (+33%), hypothalamic areas (+40%, +19%), paraventricular thalamic nucleus (+21%), CA3 field of hippocampus (+18%), and the dorsal raphe nucleus (+15%) (Table 1; Fig. 1). At the level of the lumbar spinal cord, an intense labeling of [^{3}H]citalopram was observed in ventral horn, central area and dorsal horn in sham-operated animals. After SNL, 5-HTT density was significantly reduced in almost all the regions analyzed (lamina V: −40%; lamina X: −30%; lamina IX: −22%), and a tendency toward reduction was observed in lamina I–II (−18%) (Table 1, Fig. 3).

3.3. Autoradiographic density of NAT

The greatest densities of NAT in the brain from sham-operated animals were found in brainstem nuclei (locus coeruleus and

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