Differential galanin upregulation in dorsal root ganglia and spinal cord after graded single ligature nerve constriction of the rat sciatic nerve

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Received 11 May 2007; received in revised form 11 July 2007; accepted 14 July 2007

Abstract

Single ligature nerve constriction (SLNC) is a newly developed animal model for the study of neuropathic pain. SLNC of the rat sciatic nerve induces pain-related behaviors, as well as changes in the expression of neuropeptide tyrosine and the Y1 receptor in lumbar dorsal root ganglia (DRGs) and spinal cord. In the present study, we have analyzed the expression of another neuropeptide, galanin, in lumbar DRGs and spinal cord after different degrees of constriction of the rat sciatic nerve. The nerve was ligated and reduced to 10–30, 40–80 or 90% of its original diameter (light, medium or strong SLNCs). At different times after injury (7, 14, 30, 60 days), lumbar 4 and 5 DRGs and the corresponding levels of the spinal cord were dissected out and processed for galanin-immunohistochemistry. In DRGs, SLNC induced a gradual increase in the number of galanin-immunoreactive (IR) neurons, in direct correlation with the degree of constriction. Thus, after light SLNC, a modest upregulation of galanin was observed, mainly in small-sized neurons. However, following medium or strong SLNCs, there was a more drastic increase in the number of galanin-IR neurons, involving also medium and large-sized cells. The highest numbers of galanin-IR neurons were detected 14 days after injury. In the dorsal horn of the spinal cord, medium and strong SLNCs induced a marked ipsilateral increase in galanin-like immunoreactivity in laminae I–II. These results show that galanin expression in DRGs and spinal cord is differentially regulated by different degrees of nerve constriction and further support its modulatory role on neuropathic pain.

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Keywords: Neuropeptides; Neuropathic pain; Peripheral nervous system; Primary afferent neurons; Immunohistochemistry

1. Introduction

Single ligature nerve constriction (SLNC) is a newly developed animal model for the study of neuropathic pain (Brumovsky et al., 2004) based on the widely used chronic constriction injury (CCI) model (Bennett and Xie, 1988). However, whereas CCI is achieved with four loose ligatures around the sciatic nerve, SLNC only requires a single ligature. It also has the advantage of permitting different degrees of controlled constriction of the nerve, resulting in three main levels of injury: light, medium or strong, with a reduction of 10–30, 40–80 or 90% of the original diameter of the nerve, respectively. Using this model, it has already been shown that the development of neuropathic pain-related behaviors is dependent on the degree of constriction (Brumovsky et al., 2004). Moreover, intensity-graded SLNCs of the sciatic nerve also induce changes of different magnitude in the expression of neuropeptide tyrosine (NPY) and one of its associated receptors, the Y1 receptor, in primary afferent neurons (Brumovsky et al., 2004).

Galanin, another neuropeptide expressed at low levels by dorsal root ganglion (DRG) neurons (Skofitsch and Jacobowitz, 1985), has also been reported to undergo a strong upregulation after different types of sciatic nerve lesions, such as axotomy, partial nerve transaction (PNT), crush and CCI (Hökfelt et al., 1987; Villar et al., 1989; Nahin et al., 1994; Ma and Bisby, 1985).
1997; Zhang et al., 1998; Shi et al., 1999). However, there is no information on galanin expression in primary afferent neurons after graded constrictions of the rat sciatic nerve. Both pro- and anti-nociceptive effects have been attributed to galanin, probably related to the activation of different receptors (Liu et al., 2001). In fact, three galanin receptors have been cloned so far: GalR1, -R2 and -R3 (Branchek et al., 2000). In normal conditions, both GalR1 and -R2 have been detected in DRGs (see Kerekes et al., 2003) and spinal cord (see Waters and Krause, 2000; Brumovsky et al., 2006).

Since the exact role of galanin in pain processing is not yet fully understood, it seemed relevant to study its expression after sciatic nerve injury, with a different experimental model to the ones used before. For this purpose, we induced different degrees of controlled constriction of the rat sciatic nerve using SLNC, and analyzed galanin-like immunoreactivity (LI) in the lumbar DRGs and the dorsal horn of the spinal cord.

2. Materials and methods

2.1. Nerve injury model

Young adult Sprague-Dawley male rats (200–300 g, Fucal, Buenos Aires, Argentina) were anesthetized with chloral hydrate (350 mg/kg, i.p.) and their sciatic nerve was exposed at the mid-thigh level and dissected free from the surrounding tissue. The nerve was then wrapped with a thin square strip (5 mm) of polyethylene and constricted to varying degrees with a tie around the strip using 3.0 silk suture (Barbour Threads, Lisburn, Ireland). In this way, the diameter of the nerve was reduced 10–30% (light SLNC), 40–80% (medium SLNC) or 90% (strong SLNC) (Brumovsky et al., 2004). In sham animals used as controls, the strip of polyethylene and the silk suture were placed around the sciatic nerve without constriction. Seven animals were included in each experimental group. The degree of constriction of each nerve was confirmed after perfusion of the animals and dissection under a surgery microscope using a 10 mm ruler (Brumovsky et al., 2004), and also by microscopic observation of 16 μm sections stained with neutral red, using a grid attached to the ocular of a photomicroscope (see below).

2.2. Immunohistochemistry

2.2.1. Tissue preparation

After 7, 14, 30 or 60 days, the animals were deeply anesthetized with an overdose of chloral hydrate (1.5 g/kg i.p.), and perfused through the heart with 60 ml of warm (37 °C) Tyrode’s buffer (pH 7.4), followed by 60 ml of fixative (4% paraformaldehyde and 0.2% picric acid in 0.16 M phosphate buffer, pH 7) (Zamboni and De Martino, 1967) at 37 °C and 300 ml of the same fixative at 4 °C. The ipsi and contralateral lumbar 4 and 5 (L4–5) DRGs, as well as the corresponding levels of the spinal cord, were removed and post-fixed in the same fixative for 90 min at 4 °C. The tissues were then rinsed in 20% sucrose in phosphate buffer (pH 7.2) containing 0.1% sodium azide and stored in the same solution at 4 °C.

2.2.2. Immunoperoxidase procedure

Tissues were embedded in OCT compound (Tissue Tek, Miles Laboratories, Elkhart, USA) and cut longitudinally (DRGs) or transversely (spinal cord) at 14 μm thickness in a cryostat (Microm, Heidelberg, Germany). The sections were mounted onto chrome alum-gelatin-coated slides, allowed to dry for at least 1 h, rinsed twice in phosphate-buffered saline (PBS) and dehydrated. Endogenous peroxidase was inactivated with 0.5% hydrogen peroxide (H2O2), followed by rehydration and rinses in PBS. Sections were incubated overnight in a humid chamber at 4 °C with anti-galanin antibody (1:3000, rabbit, Peninsula Laboratories, Belmont, USA), diluted in PBS containing 0.2% bovine serum albumin, 0.03% Triton X-100 and 0.1% sodium azide. The sections were then rinsed twice in PBS and incubated at room temperature for 1 h with biotinylated goat anti-rabbit secondary antibody (1:200, Vector Laboratories, Burlingame, California, USA), rinsed twice in PBS, and incubated according to the avidin–biotin complex (ABC) protocol (Vectorstain Elite kit, Vector Laboratories) for 30 min at room temperature. Peroxidase activity was demonstrated by reaction with 3,3-diaminobenzidine using H2O2 and nickel salts for enhancement of the reaction product. After dehydration, the sections were coverslipped using synthetic Canada balsam as mounting media. Control sections were incubated with a mixture of galanin antibody and the antigenic peptide (10−5, 10−6 M, Peninsula Laboratories) or by omitting the primary or secondary antibodies.

2.3. Microscopy

All sections were examined under bright-field illumination using a Nikon Eclipse E-800 photomicroscope (Nikon, Tokyo, Japan). Photographs were taken using a Nikon D700 digital camera. Resolution, brightness and contrast of the digital images were optimized using the Adobe Photoshop software (Adobe Systems Inc., San Jose, California, USA).

2.4. Quantification

The number of neurons exhibiting galanin-LI was determined in L4–5 DRGs by counting immunostained neuronal profiles under bright-field illumination using a 20 × objective, in randomly, systematically sampled sections throughout the DRGs (every 6th section, 10 sections per ganglion). In a second step, the sections were counterstained with cresyl violet, and the total number of neuronal profiles per section was determined. In all cases, only neuronal profiles with a visible nucleus were counted. Finally, the percentage of galanin-immunoreactive (IR) neuronal profiles was obtained by correlation with the total number of neurons present in each section. For the assessment of neuronal size, a grid (30 μm × 30 μm) attached to the ocular of the microscope was used. Galanin-IR neuronal profiles similar in size to those observed in control ganglia (<30 μm in diameter) were grouped as small-sized, whereas neuronal profiles exhibiting diameters >30 μm were defined as medium to large-sized (Brumovsky et al., 2006).

2.5. Statistical analysis

Statistical analysis was carried out by applying One-way analysis of variance (ANOVA) and Newman–Keuls multiple comparison post-test. Results were expressed as mean ± S.E.M. p values are presented as following: ns p > 0.05; *0.05 > p > 0.01; **0.01 > p > 0.001 and ***p < 0.001.

3. Results

3.1. DRGs

In control ganglia, galanin-LI was restricted to small-sized neurons and could be observed only in 2.3 ± 0.3% of total DRG neurons (Figs. 1a and 2a–d). In contralateral ganglia, no changes in galanin expression could be detected, independently of the degree of constriction and the evaluated survival time (Figs. 1b and 2a–d). SLNC of the sciatic nerve induced a gradual increase in the number of galanin-IR primary afferent neurons, paralleling the degree of constriction, in all the evaluated survival times (Figs. 1 and 2). Seven days after all three degrees of SLNC, the total count of galanin-IR neurons was significantly increased in the ipsilateral DRGs (light SLNC: 12.8 ± 1.4%, medium SLNC: 46.5 ± 2.4%, strong SLNC: 55.0 ± 2.2%), when compared to the contralateral ganglia (p < 0.001 in all cases) (Fig. 2a and b). In animals with medium or strong SLNCs, this increase was even higher 14
days after surgery (medium SLNC: 56.3 ± 2.4%, strong SLNC: 64.2 ± 4.6%, p < 0.001 in both cases) (Fig. 1d and e). In contrast, the number of galanin-IR neurons induced by light SLNC 7 and 14 days after the lesion, was virtually identical (12.8 ± 1.4% versus 13.1 ± 1.7%, p > 0.05).

Light SLNC induced galanin-upregulation mostly in small-sized primary afferent neurons, in all the evaluated survival times (Figs. 1c and 2a-d). However, following medium or strong SLNCs, galanin-LI was also detected in medium and large-sized DRG neurons, 7 (Fig. 2c) and 14 (Figs. 1d and e and 2d) days post-injury. One month after all three degrees of SLNC, the number of galanin-IR neuronal profiles decreased significantly (light SLNC: 5.1 ± 1.1%, medium SLNC: 19.3 ± 2.0%, strong SLNC: 27.8 ± 2.2%, p < 0.01 in all cases). After 2 months, the longest survival time studied, galanin-LI returned to normal in animals with light (2.4 ± 0.8%) or medium (3.1 ± 1.0%) SLNCs. However, rats with strong SLNC, still exhibited high numbers of small-sized galanin-IR neurons (11.0 ± 1.1%), when compared to controls (p < 0.001).

3.2. Dorsal horn of the spinal cord

In control animals, as well as contralateral to the lesion in all SLNC-treated animals, galanin-LI could be detected in the neuropil of laminae I–II (Fig. 3b, d and f). Light SLNC only induced a slight increase in galanin-IR neuropil in the ipsilateral laminae I–II. In contrast, after medium (Fig. 3a) and strong (Fig. 3e) SLNCs, there was a noticeable ipsilateral increase in galanin-LI in laminae I–II.

4. Discussion

The present results show that the extent of galanin upregulation observed in lumbar DRGs and spinal cord of...
rats subjected to a sciatic nerve SLNC, is dependent on the degree of the constriction. Thus, a small but still significant increase in the number of galanin-IR neuronal profiles, mostly of small size, was observed after light SLNC. However, following medium or strong SLNCs, there was a more drastic increase in the number of neurons expressing galanin, involving also medium and large-sized cells.

In a previous study, we demonstrated that the development of pain-related behaviors also depends on the degree of constriction (Brumovsky et al., 2004). Using the von Frey filaments Test, it was shown that medium SLNC is the most effective in inducing mechanical allodynia, a pain-like behavior produced by normally non-noxious mechanical stimuli (Chaplan et al., 1994). Moreover, animals with medium SLNC also develop intense cold allodynia (Musolino et al., 2007), when assessed using the Choi Test (Choi et al., 1994). On the contrary, light lesions induce a modest, but still significant increase in mechanical sensitivity, while animals subjected to a strong SLNC do not show signs of mechanical allodynia, probably due to the lack of conduction of pain information through the site of severe constriction (Brumovsky et al., 2004).

Graded SLNCs also induce gradual changes in the expression of NPY and the Y1 receptor, two markers of mainly large and small-sized DRG neurons, respectively (Brumovsky et al., 2004). The increase observed in NPY expression is directly related to the intensity of the nerve constriction, with progressively larger numbers of NPY-positive neurons induced by higher degrees of constriction. In contrast, a decrease in the number and intensity of Y1-IR DRG neurons, inversely correlated to the intensity of constriction, is observed (Brumovsky et al., 2004). Both NPY and Y1 receptor have been shown to undergo robust changes in expression after other types of sciatic nerve lesions and they both participate in the modulation of neuropathic pain (Brumovsky et al., 2007; Gibbs et al., 2007; Hökfelt et al., 2007; Smith et al., 2007).

Galanin has also been involved in pain modulation (Wiesenfeld-Hallin et al., 1992, 2005; Xu et al., 2000; Liu et al., 2001; Wynick et al., 2001; Brumovsky et al., 2006, 2007) and linked to survival and regeneration of lesioned neurons (Kerr et al., 2000; Mahoney et al., 2003; Holmes et al., 2005; Shi et al., 2006). This peptide is normally expressed at low levels in DRGs and local dorsal horn neurons, and is strongly upregulated after different types of peripheral nerve lesions (Hökfelt et al., 1987; Villar et al., 1989; Nahin et al., 1994; Ma and Bisby, 1997; Zhang et al., 1998; Shi et al., 1999). In the present work, we show that galanin expression in primary afferent neurons is also regulated by sciatic nerve ligation, being the magnitude of galanin upregulation directly related to the degree of constriction of the sciatic nerve.

Ma and Bisby (1997) have previously shown that different degrees of nerve injury induce a differential upregulation of galanin in primary afferent neurons. Thus, PNT and CCI upregulate galanin in a higher number of ipsilateral DRG neurons, when compared to axotomy. Independently of the type of lesion, this upregulation mostly involves small-sized DRG neurons. However, more medium and large-sized galanin-IR

neuronal profiles are present after PNT and CCI of the sciatic nerve, than after axotomy (Ma and Bisby, 1997). These data suggest a differential reaction of primary afferent neurons to different levels of nerve injury, as supported by our present results. The observation that a light constriction of the sciatic nerve induces a small but noticeable upregulation of galanin in small DRG neurons suggests that even under very discrete adverse conditions, unmyelinated primary afferents could be more sensitive to injury than large myelinated fibers. In fact, Mosconi and Kruger (1996) have shown that increasing the degree of constriction of the sciatic nerve using polyethylene cuffs of decreasing inner diameters results in correspondingly higher numbers of myelinated fibers being affected, in addition to the ongoing alteration of unmyelinated fibers (Mosconi and Kruger, 1996).

This progressive increase in the number and type of fibers severed by increasing degrees of constriction could be associated to the gradual upregulation of galanin, as well as to the progressive development of pain-related behaviors. Thus it could be speculated that light SLNC only affects a small population of unmyelinated fibers, therefore inducing galanin upregulation in a few small-sized neurons and resulting in a slight increase in mechanical sensitivity. In contrast, medium and strong SLNCs most likely lesion all types of nerve fibers, inducing galanin upregulation in small, medium and large-sized cells. Moreover, it is possible that the presumably larger number of small-caliber fibers affected and the incorporation of myelinated axons contribute to the development of the intense mechanical allodynia observed in animals with medium SLNC. Finally, strong lesions, similarly to axotomy, are more difficult to evaluate in their generation of allodynia (Brumovsky et al., 2004), since the intensity of the lesion probably alters the conduction of pain information, making it impossible to determine the state of pain perception.

With regard to the temporal pattern of galanin expression, the highest numbers of galanin-IR neuronal profiles were...
observed 14 days after the lesion in animals with medium or strong constrictions. In contrast, in animals with light SLNC, there were no significant differences in galanin expression 7 or 14 days after the lesion. These results are in agreement with previous studies showing that the upregulation of galanin mRNA and protein reach a maximum 10–14 days after axotomy (Villar et al., 1989; Ma and Bisby, 1997) or CCI (Nahin et al., 1994; Ma and Bisby, 1997). Two months after surgery, galanin expression returned to control levels in animals with light and medium SLNCs. However, animals with strong constrictions still exhibited higher numbers of galanin-IR neurons. This observation may reflect the persistent efforts of DRG neurons to remain healthy and attempt regeneration of their severed axons. In fact, galanin has been associated to regeneration and survival mechanisms (see Holmes et al., 2005; Shi et al., 2006).

In the superficial laminae of the lumbar dorsal horn, we observed a marked ipsilateral increase in galanin-LI following medium and strong SLNCs. This observation supports previous data showing that the upregulated synthesis of galanin after peripheral nerve injury leads to its increased transport and release in the dorsal horn of the spinal cord (Colvin et al., 1997; Colvin and Duggan, 1998).

In summary, we show that galanin expression in primary afferent neurons is differentially regulated after sciatic nerve constriction, being the magnitude of galanin upregulation directly related to the degree of constriction of the sciatic nerve. Furthermore, the progressive increase in galanin-LI suggests that the number and type of sciatic nerve fibers affected by the lesion depend on the degree of constriction. Thus, light SLNC predominantly alters a small population of unmyelinated fibers and results in a modest increase in the number of galanin-IR small-sized neurons, whereas medium and strong SLNCs probably affect all types of axons, inducing galanin upregulation in a larger number of small, medium and large-sized DRG neurons.

Acknowledgements

This work was supported by Austral University, PICTO-CRUP 30930 and Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina (CONICET). We are grateful to Silvina Ruffolo, Germán Ruffolo and Guillermo Gastón for their skilled technical assistance.

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