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TACTILE ALLODYNIA CAN OCCUR IN THE SPARED NERVE INJURY MODEL IN THE RAT WITHOUT SELECTIVE LOSS OF GABA OR $GABA_A$ RECEPTORS FROM SYNAPSES IN LAMINAE I–II OF THE IPSILATERAL SPINAL DORSAL HORN

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Abstract—Although there is evidence that reduced inhibition in the spinal dorsal horn contributes to neuropathic pain, the mechanisms that underlie this are poorly understood. We have previously demonstrated that there is no loss of neurons from laminae I–III in the spared nerve injury (SNI) model [Polgár E, Hughes DI, Arham AZ, Todd AJ (2005) Loss of neurons from laminas I-III of the spinal dorsal horn is not required for development of tactile allodynia in the SNI model of neuropathic pain. J Neurosci 25:6658–6666]. In this study we investigated whether there was a difference between ipsilateral and contralateral sides in the levels of GABA, the vesicular GABA transporter (VGAT), or the β_3 subunit of the $GABA_A$ receptor at synapses in the medial part of the superficial dorsal horn in this model. Tissue from rats that had undergone SNI 4 weeks previously was examined with an electron microscopic immunogold method to reveal GABA, following pre-embedding detection of $GABA_A$ β_3 to allow identification of GABAergic terminals. Assessment of labeling for the $GABA_A$ β_3 subunit and VGAT was performed by using immunofluorescence and confocal microscopy. We found no difference in the intensity of immunolabeling for any of these markers on the two sides of the superficial dorsal horn. These results suggest that there is no significant loss of GABAergic boutons from the denervated area after SNI (which is consistent with the finding that neuronal death does not occur in this model) and that there is no depletion of GABA or $GABA_A$ receptors at GABAergic synapses within this region. An alternative explanation for disinhibition after nerve injury is that it results from reduced excitatory drive to GABAergic dorsal horn neurons following loss of primary afferent input to these cells.

Key words: neuropathic pain, dorsal horn, $GABA_A$ receptor β_3 subunit, postembedding immunocytochemistry, vesicular GABA transporter, antigen retrieval.

Peripheral nerve injury can result in allodynia, hyperalgesia and spontaneous pain, which are characteristic features of neuropathic states. The mechanisms underlying these phenomena are complex and remain controversial. Altered properties of primary afferent fibers in damaged nerves and changes in sensory processing within the CNS are both thought to play a role in the development of neuropathic pain.

One proposed mechanism is loss of inhibition in the superficial laminae of the spinal dorsal horn. GABA, the major inhibitory neurotransmitter in this region, acts on postsynaptic $GABA_A$ receptors to produce inhibitory postsynaptic currents (IPSCs) in spinal neurons. Several lines of evidence suggest that changes affecting GABAergic transmission in the dorsal horn contribute to neuropathic pain. Yaksh (1989) showed that intrathecal administration of a $GABA_A$ antagonist produced tactile allodynia in naïve rats. Loss of GABA and its synthesizing enzyme glutamate decarboxylase (GAD) from the dorsal horn has been reported following nerve injury (Castro-Lopes et al., 1993; Ibuki et al., 1997; Eaton et al., 1998; Moore et al., 2002). Moore et al. (2002) and Scholz et al. (2005) reported that some neurons in this region underwent apoptosis following nerve injury, and suggested that this contributed to loss of GAD. They also found substantial reduction in the GABAergic component of primary-afferent evoked IPSCs in lamina II neurons, which they attributed, at least in part, to apoptosis of GABAergic neurons. However, there is also evidence to suggest that death of GABAergic neurons does not occur after nerve injury. We showed that there was no loss of neurons, and no change in the proportion that were GABA-immunoreactive, in laminae I–III of the ipsilateral dorsal horn in the chronic constriction injury (CCI) model (Polgár et al., 2003, 2004). We also found that 4 weeks after spared nerve injury (SNI) the number of neurons in laminae I–III was not altered, and that the apoptotic cells seen in the spinal cord at earlier stages were microglia, rather than neurons (Polgár et al., 2005).

However, even if GABAergic neurons do not undergo apoptosis, loss of inhibition after nerve injury could still result from depletion of GABA from their axon terminals, leading to reduction of transmitter release, and thus the size of IPSCs. Castro-Lopes et al. (1993) reported that transection of the sciatic nerve led to loss of GABA from the denervated region in the dorsal horn that started 2 weeks after injury and progressively increased up to 4 weeks. Since SNI involves transection of two branches of the sciatic nerve, this procedure might be expected to lead to significant depletion of GABA from GABAergic axon terminals in the denervated territory, and in this study we

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Abbreviations: CCI, chronic constriction injury; DAB, 3,3′-diaminobenzidine; EPSC, excitatory postsynaptic current; GAD, glutamate decarboxylase; IB4, isolectin B4; IPSC, inhibitory postsynaptic current; SNI, spared nerve injury; TSA, tyramide signal amplification; TUNEL, terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling; VGAT, vesicular GABA transporter; VIP, vasoactive intestinal peptide.

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have tested this hypothesis. We used antibody against the $GABA_A$ receptor β_3 subunit (Todd et al., 1996) to identify GABAergic synapses in laminae I–II on each side with electron microscopy in tissue from rats that had survived 4 weeks after SNI. We then compared immunogold labeling for GABA over their presynaptic boutons on ipsilateral and contralateral sides within the territory of the tibial and common peroneal nerves. We also used confocal microscopy to look for evidence that levels of the vesicular GABA transporter (VGAT, a marker for GABAergic terminals) or synaptic $GABA_A$ receptors differed between the two sides.

EXPERIMENTAL PROCEDURES

Animals and operative procedures

All experiments were approved by the Ethical Review Process Applications Panel of the University of Glasgow and were performed in accordance with the European Community directive 86/609/EC and the UK Animals (Scientific Procedures) Act 1986. All efforts were made to minimize the number of animals used and their suffering.

Fourteen adult male Sprague–Dawley rats (250–330 g) were used in this study, and 11 of these underwent unilateral SNI (Decosterd and Woolf, 2000). The SNI rats were anesthetized with halothane and the left sciatic nerve was exposed through an incision of the skin and muscle at the level of its trifurcation. The common peroneal and tibial branches were tightly ligated with 4.0 silk and transected just distal to the ligation, while the sural nerve was left intact. The wound was then closed, and the animals made an uneventful recovery from surgery.

Behavioral testing

Testing to detect signs of tactile allodynia was carried out on all of the rats that had undergone SNI on nine occasions: 6, 3 and 1 days before and 1, 4, 7, 14, 21 and 28 days after the operation. Responses to mechanical stimuli were tested by using von Frey filaments with logarithmically incremental stiffness (Chaplan et al., 1994). The animals were placed in a Perspex cage with a wire-mesh floor and left to acclimatize for 15 min prior to testing. The filaments were applied only to the sural nerve territory of the plantar surface of the hind paw (lateral side) (Decosterd and Woolf, 2000). Lifting or flinching of the stimulated foot was recorded as a positive response. The 50% paw withdrawal threshold was calculated by Dixon's nonparametric test (Dixon, 1980; Chaplan et al., 1994). A one-tailed unpaired *t*-test was used to determine whether the threshold was lower on the ipsilateral side at each post-operative time-point.

Tissue processing

On the 28th post-operative day, the rats that had undergone SNI were deeply anesthetized with pentobarbitone and perfused with a fixative that contained either 4% freshly depolymerized formaldehyde ($n=5$, for confocal microscopy), or a mixture of 1% glutaraldehyde and 1% formaldehyde ($n=6$, for electron microscopic immunogold labeling of GABA). The three unoperated rats were also anesthetized and perfused with the glutaraldehyde/formaldehyde fixative. Since suboptimal fixation can result in variable retention of GABA, great care was taken to ensure that perfusion with the glutaraldehyde-containing fixative was rapid and efficient (Somogyi et al., 1985). This was achieved by minimizing the time between opening the thoracic cavity and commencing the perfusion, by using a brief (~5 s) rinse with Ringer's solution, and by warming both rinsing solution and fixative to 37 °C to minimize vasoconstriction (Polgár et al., 2003). The L4 and L5 segments were removed from the rats fixed with formaldehyde and stored in the same fixative at 4 °C for 8 h, while the L4 segments of the rats fixed with glutaraldehyde/formaldehyde were stored in this fixative overnight at 4 °C. Tissue blocks were marked by cutting a tapering notch in the ventral white matter on the right hand side and were then cut into 60 μm transverse sections with a Vibratome. The notch was needed to allow the two sides to be distinguished, and the approximate rostrocaudal location of the sections within the segment to be identified, since the sections were processed free-floating.

Immunocytochemistry and lectin binding

Sections from the L4 segment of the six SNI rats that were fixed with glutaraldehyde/formaldehyde were cut sequentially into three series, which were treated for 30 min in 50% ethanol to enhance antibody penetration, followed by 30 min in 1% sodium borohydride to reduce free aldehyde groups. Sections in each series were then processed according to one of the following protocols: (1) a pre-embedding immunoperoxidase reaction with antibody against $GABA_A$ receptor β_3 subunit; (2) a peroxidase reaction to reveal binding of *Bandeiraea simplicifolia* isolectin B4 (IB4; which labels a population of intact unmyelinated afferents); or (3) a fluorescence reaction to reveal vasoactive intestinal peptide (VIP). Sections reacted according to the first protocol were then processed for electron microscopy and used for subsequent post-embedding immunogold detection of GABA, while the second and third reactions were used to delineate the region in the superficial dorsal horn that contained axotomized unmyelinated afferents (identified by depletion of IB4 and up-regulation of VIP; Shehab et al., 2004), and the boundary between laminae II and III (seen with dark-field illumination). For the first protocol, sections were incubated for 72 h in antibody against the $GABA_A$ receptor β_3 subunit (gift from Prof. W. Sieghart, Medical University of Vienna, Austria; 0.96 μg/ml; Todd et al., 1996), overnight in biotinylated donkey anti-rabbit IgG (1:500; Jackson ImmunoResearch, West Grove, PA, USA) and for 4 h in ExtrAvidin peroxidase (1:1000; Sigma-Aldrich, Gillingham, UK; catalogue number E2886). They were then reacted with 3,3′-diaminobenzidine (DAB), osmicated (1% OsO_4 for 20 min), dehydrated in acetone, block stained with uranyl acetate and flat-embedded in Durcupan. Sections reacted to reveal IB4 were incubated for 72 h in biotinylated IB4 (1 μg/ml; Sigma-Aldrich) and overnight in ExtrAvidin peroxidase (1:1000; Sigma-Aldrich). Following the DAB reaction, the sections were dehydrated, cleared and coverslipped on glass slides. Sections reacted to reveal VIP were incubated for 72 h in rabbit antibody against VIP (1:5000; gift from Prof. J. Allen, University College Dublin, Ireland) and overnight in donkey-anti-rabbit cyanine-5.18 (1:100; Jackson ImmunoResearch). Sections were mounted on glass slides in antifade mounting medium (Vector Laboratories, Peterborough, UK). Antibodies and lectins used in protocols 2 and 3 were diluted in PBS that contained 0.3% Triton X-100, while for protocol 1 the diluents did not contain detergent. All incubations were carried out at 4 °C.

L4 sections from the three unoperated rats were treated with 50% ethanol and sodium borohydride, and then processed for pre-embedding electron microscopic immunoperoxidase detection of the $GABA_A$ β_3 subunit as described above (protocol 1).

Sections from L4 and from the rostral half of the L5 segment of each of the five SNI rats that were perfused with 4% formaldehyde were cut, treated for 30 min in 50% ethanol, and then reacted according to one of the following immunofluorescence protocols: (1) antigen retrieval with pepsin (Watanabe et al., 1998; Nagy et al., 2004) followed by detection of $GABA_A$ receptor β_3 subunit; (2) immunostaining for VGAT. For the first of these protocols, sections were incubated for 10 min at 37 °C in pepsin (0.5 mg/ml; DAKO, Glostrup, Denmark; Watanabe et al., 1998) and then for 72 h in $GABA_A$ β_3 antibody (1.6 μg/ml) and overnight in donkey anti-rabbit IgG conjugated to Alexa 488 (1:500; Invitrogen, Paisley, UK). Sections reacted to reveal VGAT were incubated for 72 h