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Upregulation of the GABA-transporter GAT-1 in the spinal cord contributes to pain behaviour in experimental neuropathy

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ABSTRACT

Sciatic nerve ligation in rats (chronic constriction injury (CCI)) induces signs and symptoms that mimic human conditions of neuropathy. The central mechanisms that have been implicated in the pathogenesis of neuropathic pain include increased neuronal excitability, possibly a consequence of decreased availability of spinal GABA. GABA availability is regulated by the presence of the GABA-transporters (GATs). This study investigates the dorsal horn expression of the transporter GAT-1 and its functional involvement towards pain behaviour in the CCI model. Male Lewis rats (total $n = 37$) were subjected to CCI or to a sham procedure. A sub-group of animals was treated with the GAT-1 antagonist NO-711. Behavioural testing was performed pre-surgery and at 7 days post-surgery. Testing included evaluation of mechanical allodynia using Von Frey filaments, thermal allodynia with a hot-plate test and observational testing of spontaneous pain behaviour. Subsequently, spinal protein expression of GAT-1 was assessed by Western blotting. Animals were sacrificed 7 days following surgery. CCI markedly increased mechanical and thermal allodynia and spontaneous pain behaviour after 7 days, while the sham procedure did not. GAT-1 was increased in spinal cord homogenates compared contralateral to the ligation side after 7 days. NO-711 treatment significantly reduced all tested pain behaviour. These data provide evidence for possible functional involvement of GAT-1 in the development of experimental neuropathic pain. The latter can be derived from observed analgesic effects of early treatment with NO-711, a selective GAT-1 inhibitor. The obtained insights support the clinical employment of GAT-1 inhibitors to treat neuropathic pain.

Altered synaptic transmission within the spinal cord dorsal horn plays a key role in the pathogenesis of neuropathic pain. Gamma aminobutyric acid (GABA) is a principal inhibitory neurotransmitter of the central nervous system. The inhibitory effects of GABA are mediated by ionotropic GABAa and c receptors and metabotropic GABAb receptors [9]. Extracellular GABA levels are regulated by specific high-affinity plasma membrane transporters termed GABA-transporters (GATs). GATs are functionally involved in modulation of magnitude and duration of GABA-ergic inhibition. Four GABA-transporters (GAT-1–GAT-4) have been cloned. GAT-1 is the most predominant GAT and is expressed on presynaptic as well as postsynaptic GABA-ergic and non-GABA-ergic neurons [5,20]. Moreover, GAT-1 is expressed in glial cells [15].

Sciatic nerve ligation in rats (chronic constriction injury (CCI)) induces signs and symptoms that mimic human conditions of neuropathic pain [3]. Reduced levels of GABA [7,8,11] and GABA receptors [4,6] have been recorded after axotomy and CCI, implicating a functional loss of GABA-ergic transmission in the superficial dorsal horn. More recently, knockdown of GAT-1 was shown to induce hypoalgesia after hind paw formalin injection [22], whereas transgenic GAT-1 overexpression was associated with hyperalgesia [17,10]. This prompted us to investigate dorsal horn expression of GAT-1 and its correlation with pain behaviour in the CCI model. Furthermore, we investigated the functional role of GAT-1 by treating CCI animals with the GAT-1 antagonist NO-711 which readily penetrates the blood–brain barrier [12].

Male Lewis rats weighing between 200 and 250 g were subjected to CCI or to a sham procedure [3]. Animals were anesthetized with ketamine 80 mg/kg and xylazine 10 mg/kg intraperitoneally. In CCI animals (total $n = 25$), four chromic catgut ligatures were tied loosely around the right sciatic nerve just proximal to its trifurcation. Sham animals were subjected to the same procedure without ligation of the sciatic nerve ($n = 8$). A sub-group of CCI rats ($n = 7$) was treated twice with a subcutaneous injection in the neck (60 mg/kg) of the GAT-1 antagonist 1-[2-[[(diphenylmethylene)imino]oxy] ethyl]-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid hydrochloride

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(NO-711) (Sigma, St. Louis, MO) dissolved in 0.5 ml 0.9% NaCl. The latter was employed at the time of surgery and 5 days following surgery. This treatment regime was based on observed therapeutic effects with respect to mechanical allodynia in pilot studies. Separate groups of control CCI rats were treated with vehicle only ($n = 12$) or not treated ($n = 6$). Since no differences were observed between the latter two groups, we decided to pool CCI control animals in one group ($n = 18$). All animals were sacrificed 7 days following surgery and performed experiments were approved by the Maastricht University animal ethical board. Behavioural testing was performed pre-surgery and 7 days later. Testing included evaluation of mechanical allodynia using Von Frey filaments (4.31 and 5.07 mN applied to the right footpad ipsilateral to the side of the CCI/sham procedure), thermal allodynia with a hot-plate test at 40 °C (2 min acclimatisation, measuring time of foot elevation during 3 min) and observational testing of spontaneous pain behaviour (according to nominal scale as introduced by Attal et al. [1]). Using a standard Western blotting technique, commercially available polyclonal primary antibodies (Millipore, Billerica, MA) were applied to homogenates of ipsilateral and contralateral L4 and L5 lumbar spinal cord hemisections in a sub-group of rats ($n = 4$) subjected to CCI that received vehicle only. After stripping membranes were reprobed with anti-β-actin antibodies to confirm equivalent loading. Parametric data are presented as mean ± S.E.M., and Student's *t*-tests were used for statistical comparison between groups of animals.

First, with respect to the operated right side of animals that were not subjected to pharmacological intervention: our behavioural data reveal increased mechanical allodynia in rats subjected to CCI as compared to sham-operated animals when 5.07 mN filaments were employed (33% vs. 7.9% of maximal response, $p < 0.01$) (Fig. 1). When the 4.31 mN filaments were employed, animals subjected to CCI showed a non-significant increase in mechanical allodynia as compared to sham-operated animals (11% vs. 6.3% of maximal response, NS) (Fig. 1). Thermal allodynia was increased in rats subjected to CCI as compared to sham-operated controls (19 s vs. 0 s of paw elevation, $p < 0.001$) (Fig. 2). Observational testing of spontaneous pain behaviour showed increased pain in rats subjected to CCI as compared to sham-operated controls (mean of 2.4 vs. 0 on the Attal scale, $p < 0.05$) (Fig. 3). No significant pain behaviour in rats was observed pre-surgery. Western blotting revealed increased GAT-1 protein expression in spinal cord hemisections ipsilateral to the ligated side compared to constitutive expression in contralateral hemisections (Fig. 4) or specimens obtained from sham-operated controls (data not shown). Equivalent loading was confirmed by reprobing stripped membranes with anti-β-actin antibodies (Fig. 4).

Second, with respect to the operated right side of animals subjected to pharmacological intervention with NO-711: rats subjected to CCI and treated with NO-711 showed significantly decreased mechanical allodynia (9.4% vs. 33% of maximal response in 5.07 mN setting, $p < 0.05$ and 4.9% vs. 11% in 4.31 mN setting, $p < 0.05$), thermal allodynia (4.9 s vs. 19 s of hindpaw elevation, $p < 0.01$) and spontaneous pain behaviour (0.3 vs. 2.4 on the Attal scale, $p < 0.05$) as compared to vehicle only treated CCI animals. No behavioural effects of NO-711 treatment were seen in sham-operated rats (data not shown).

These data show that CCI leads to mechanical as well as thermal allodynia and also spontaneous pain. Moreover, we show upregulation of spinal GAT-1 protein after CCI. Functional involvement of this upregulated spinal GAT-1 in pain-related behaviour following CCI is suggested by the analgetic effects of the GAT-1 antagonist NO-711 in all the performed behavioural tests. These data are in line with other reports that reveal increased expression of GAT-1 and GAT-3 in the spinal trigeminal nucleus and increased synaptosomal GABA uptake in brain stem after painful facial carrageenan injections [17]. Moreover, mice overexpressing GAT-1 showed enhanced susceptibility to develop hyperalgesia [10]. More recently, mice lacking GAT-1 were shown to exhibit decreased nocicepetive responses to various painful stimuli [22]. Our study is the first to show a functional increase in spinal GAT-1 after CCI.

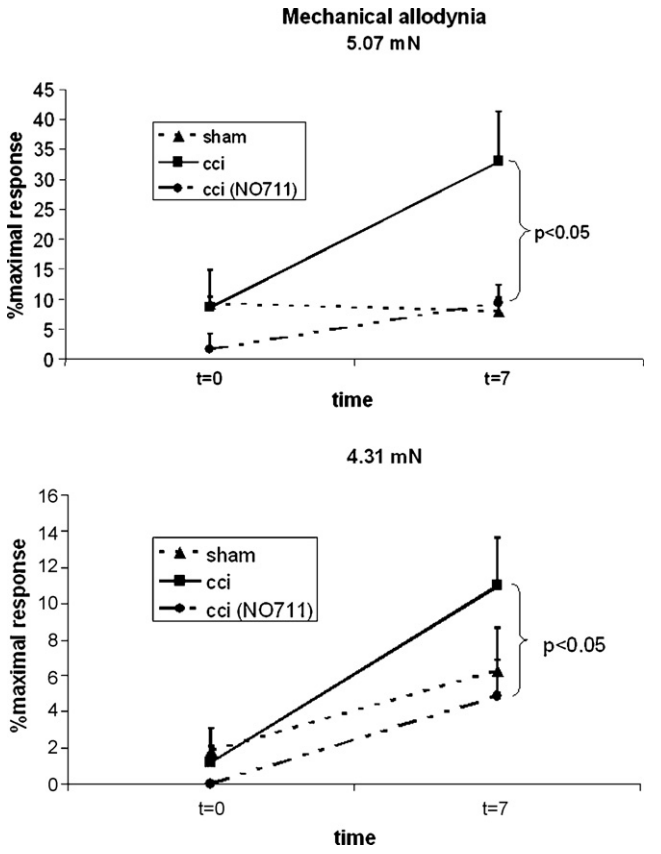

Fig. 1. Mechanical allodynia in the footpad ipsilateral to the side of ligation. Mechanical allodynia as measured by application of 4.31 mN (lower panel) as well as 5.07 mN (upper panel) Von Frey filaments, before ($t = 0$) and 7 days following CCI. Allodynia is expressed as % of maximal response. Maximal response (100%) is defined as a positive footpad withdrawal in response to each individual application of the filament. A total of nine individual applications per measurement with an interval of >1 min were employed. Shown are means and bars represent S.E.M.

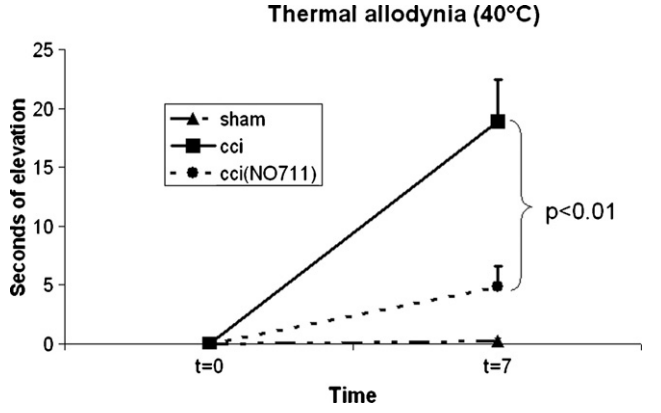

Fig. 2. Thermal allodynia in the footpad ipsilateral to the side of ligation. Thermal allodynia as measured by the hot-plate test, before ($t = 0$) and 7 days following CCI. Allodynia is expressed as total time of hindpaw elevation in seconds. Shown are means and bars represent S.E.M.