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Superficial NK1 expressing spinal dorsal horn neurones modulate inhibitory neurotransmission mediated by spinal $GABA_A$ receptors

Wahida Rahman [a,*], Shafaq Sikander [a], Rie Suzuki [a], Stephen P. Hunt [b], Anthony H. Dickenson [a]

[a] *Department of Pharmacology, University College London, Gower Street, London WC1E 6BT, UK*
[b] *Department of Anatomy and Developmental Biology, University College London, Gower Street, London WC1E 6BT, UK*

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Abstract

Lamina 1 projection neurones which express the NK1 receptor (NK1R+) drive a descending serotonergic pathway from the brainstem that enhances spinal dorsal horn neuronal activity via the facilitatory spinal 5-HT3 receptor. Selective destruction of these cells via lumbar injection of substance P-saporin (SP–SAP) attenuates pain behaviours, including mechanical and thermal hypersensitivity, which are mirrored by deficits in the evoked responses of lamina V–VI wide dynamic range (WDR) neurones to noxious stimuli. To assess whether removing the origin of this facilitatory spino-bulbo-spinal loop results in alterations in GABAergic spinal inhibitory systems, the effects of spinal bicuculline, a selective $GABA_A$ receptor antagonist, on the evoked neuronal responses to electrical (Aβ-, Aδ-, C-fibre, post-discharge and Input) and mechanical (brush, prod and von Frey (vF) 8 and 26 g) stimuli were measured in SAP and SP–SAP groups. In the SAP control group, bicuculline produced a significant dose related facilitation of the electrically evoked Aδ-, C-fibre, post-discharge and input neuronal responses. The evoked mechanical (prod, vF8 g and 26 g) responses were also significantly increased. Brush evoked neuronal responses in these animals were enhanced but did not reach significance. This facilitatory effect of bicuculline, however, was lost in the SP–SAP treated group. The generation of intrinsic GABAergic transmission in the spinal cord appears dependent on NK1 bearing neurons, yet despite the loss of GABAergic inhibitory controls after SP–SAP treatment, the net effect is a decrease in spinal cord excitability. Thus activation of these cells predominantly drives facilitation.
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NK1 receptor expressing (NK1R+) superficial dorsal horn cells play an integral part in the transmission and enhancement of nociception. In particular these cells are essential for the full expression of hypersensitive "pain-like" behaviours, such as pain in response to low threshold normally innocuous stimuli (allodynia) and enhanced pain to noxious stimuli (hyperalgesia), which often manifest in chronic pain states since either destruction of NK1R+ neurones, pharmacological blockade or genetic knock-out of NK1Rs results in attenuation of these hypersensitive responses [10,24,30]. Several lines of evidence show that these neurons drive descending controls projecting from the rostroventromedial medulla (RVM) via the dorsolateral funiculus (DLF) back onto spinal neurones to enhance nociceptive transmission [9,24]. Disruption of this spino-bulbo-spinal loop at various points in the anatomical pathway attenuates allodynia and hyperalgesia in animal models of inflammation, nerve and spinal cord injury [10,15,18,24,28,30]. At the level of the spinal cord, selective ablation of these cells with the neurotoxin saporin (SAP) conjugated to substance P (SP) – the NK1R preferring endogenous agonist – results in marked deficits in the coding of polymodal peripheral inputs under normal conditions, as well as for the more persistent chemical evoked responses by deep wide dynamic range (WDR) dorsal horn neurones [24]. Pharmacological block of spinal 5-HT3 receptors mimicked almost all the effects of SP–SAP treatment [24] on WDR neurones suggesting these neuronal changes arise through disruption of a facilitatory serotonergic influence from the brainstem.

Under normal conditions it is well known that spinal activity is a balance between incoming excitations and spinal excitatory and inhibitory mechanisms (and descending controls). Thus noxious peripheral inputs can drive supraspinal pathways to further enhance subsequent peripheral inputs. To balance

* Corresponding author. Tel.: +44 207 679 3737; fax: +44 207 679 3742.
E-mail address: w.rahman@ucl.ac.uk (W. Rahman).

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facilitations, noxious peripheral inputs are also subject to inhibitory influences, via the activation of descending inhibitory controls and also, in the first instance, by intrinsic spinal inhibitory systems. One of the principal mediators of local endogenous inhibition is γ-aminobutyric acid (GABA).

Under normal conditions superficial NK1R+ cells are activated only by noxious stimuli, however a recent in vitro study demonstrated that NK1R+ neurones within the superficial dorsal horn are under strong GABAergic control [27], by removing this inhibitory control a long-lasting polysynaptic activity generated by A-fibre stimulation of NK1R+ neurones was revealed [27]. Furthermore, loss of GABA inhibition within the cord via intrathecal injection of the selective $GABA_A$ receptor antagonist, bicuculline, is sufficient to produce tactile allodynia and thermal hyperalgesia, [13,29] which correlates well with enhanced responses of lamina I and deep dorsal horn (lamina V–VI) neurones seen after spinal application of bicuculline *in vivo* [11,19,21]. Thus it might be predicted that superficial NK1R+ cells could play a significant role in modulating spinal GABA receptor systems. Therefore, to assess whether loss of NK1R+ cells produces any potential alterations in GABAergic spinal inhibitory systems we compared the effects of topical spinal bicuculline, on the evoked activity of deep dorsal horn WDR neurones in SP–SAP with those in SAP control animals. These WDR cells, with both innocuous and noxious inputs, allow us to gauge the effects of GABAergic controls on tactile and noxious processing.

Male Sprague-Dawley rats weighing approximately 130–150 g at time of intrathecal injection and 300–350 g at time of immunohistochemistry/electrophysiology were employed for this study (Central Biological Services, University College London, UK). All experimental procedures were approved by the UK Home Office and follow the guidelines under the International Association for the Study of Pain [32].

Selective ablation of lumbar NK1R+ neurones was achieved via intrathecal injection of the toxin saporin conjugated to substance P (Advanced Targeting Systems, San Diego, California) as previously described [15,24]. Rats were anaesthetised with ketamine (1.5 mg/kg i.p.). A cannula attached to a Hamilton syringe, was inserted into the subarachnoid space and 10 μl of 1 μM SP–SAP or SAP (control group) was injected into the L4–5 regions. The cannula was withdrawn, wound closed and animals were allowed to recover and not used for further study until 28 days after intrathecal injection.

To confirm selective ablation of superficial NK1R+ cells in SP–SAP treated animals, NK1R immunohistochemistry reactions were carried out as previously described [20,24]. Briefly, deeply anaesthetised animals were transcardially perfused with 4% paraformaldehyde in PBS (0.15 M, pH 7.4), at 10 °C. The spinal cord was removed and cryoprotected overnight in 30% sucrose solution. Transverse sections (40 μm thick) were cut and serially collected. Antibody reactions were carried out against NK1 (1:10,000; Eurogentec), NeuN (1:1000, Chemicon) and GFAP (1:1000, Dako). Immunoreactive sites were revealed using biotinylated secondary antibodies followed by avidin conjugated fluorescent antibodies Cy3 or FITC. Sections were mounted and coverslipped in Gel Mount (Sigma).

In vivo electrophysiology was conducted 4 weeks after intrathecal injection. Animals were anaesthetised with halothane (1.0–1.2 %) delivered in a gaseous mix of N_2O (66%) and O_2 (33%) and a laminectomy performed to expose the L4–5 segments of the spinal cord. Extracellular recordings were made from ipsilateral deep dorsal horn neurones using parylene coated tungsten electrodes (A-M Systems, USA). A train of 16 transcutaneous electrical stimuli (2 ms wide pulses, 0.5 Hz) was applied at three times the threshold current for C-fibres, following which a post-stimulus histogram was constructed. Responses evoked by Aβ- (0–20 ms), Aδ- (20–90 ms) and C-fibres (90–350 ms) were separated and quantified on the basis of latency. Responses occurring after the C-fibre latency band were taken to be the post-discharge of the cell (350–800 ms). The number of action potentials evoked by the first stimulus in the train of electrical stimuli response × 16 was referred to as 'Input' and is recorded from 90 to 350 ms post-stimulus; i.e. the baseline C-fibre-evoked response. Under normal conditions, there are clear peaks of activity attributable to the evoked activity produced by the different fibre types, however after bicuculline this distinction is less clear. It is impossible to determine whether the increased activity in the latency band usually attributed to e.g. C-fibre evoked activity, is partly due to a prolongation of firing due to A-delta fibre inputs, but at least some of the increased activity will be due to disinhibition or excitation of C-fibre inputs. Thus, to allow for comparison between groups we have used the same latency bands. The peripheral receptive field was also stimulated using a range of natural mechanical stimuli—dynamic brush, prod (5 mm-diameter; 4 N/cm^2) and mechanical punctate stimuli applied using von Frey filaments (vF) 8 and 26 g over a 10 s period. Data were captured and analysed by a CED 1401 interface coupled to a Pentium computer with Spike 2 software (Cambridge Electronic Design; PSTH and rate functions).

Prior to drug administration, stable control responses to electrical and natural stimuli were established at 10 min intervals. (−)-Bicuculline methoiodide (Sigma–Aldrich) was administered spinally (5, 50 and 250 μg/50 μl). Each drug dose was followed for 40 min with tests made at 10 min intervals.

All data are expressed as mean ± standard error of mean (S.E.M.). Pre-drug baseline neuronal responses between groups were compared using unpaired Student's *t*-test. Drug effects are expressed as mean maximal evoked neuronal response per dose. Drug effects within a group were analysed using analysis of variance with repeated measures (RM-ANOVA) followed by Dunnet's post hoc tests as appropriate. Comparison of drug effects between groups was analysed using two-way RM-ANOVA. Level of significance was set at $^*P < 0.05$.

Animals were monitored during the 1 month period following intrathecal injection and none of the animals employed showed any motor deficits and displayed normal grooming behaviour and weight gain.

Histological examination of lumbar cord sections confirmed a selective and marked depletion of NK1R immunoreactivity in the superficial lamina of SP–SAP treated animals compared with SAP controls. Immunoreactivity for neurone-specific nuclear protein (NeuN, a marker for neurones) and glial fibrillary acidic protein (GFAP, a marker for astrocytes) showed no abnormal

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