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Functional differences between neurochemically defined populations of inhibitory interneurons in the rat spinal dorsal horn

Erika Polgár [a,*], Thomas C.P. Sardella [a], Sheena Y.X. Tiong [a], Samantha Locke [a], Masahiko Watanabe [b], Andrew J. Todd [a,*]

[a] Spinal Cord Group, Institute of Neuroscience and Psychology, University of Glasgow, Glasgow G12 8QQ, United Kingdom
[b] Department of Anatomy, Hokkaido University School of Medicine, Sapporo 060-8638, Japan

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In order to understand how nociceptive information is processed in the spinal dorsal horn we need to unravel the complex synaptic circuits involving interneurons, which constitute the vast majority of the neurons in laminae I–III. The main limitation has been the difficulty in defining functional populations among these cells. We have recently identified 4 non-overlapping classes of inhibitory interneuron, defined by expression of galanin, neuropeptide Y (NPY), neuronal nitric oxide synthase (nNOS) and parvalbumin, in the rat spinal cord. In this study we demonstrate that these form distinct functional populations that differ in terms of sst_{2A} receptor expression and in their responses to painful stimulation. The sst_{2A} receptor was expressed by nearly all of the nNOS- and galanin-containing inhibitory interneurons but by few of those with NPY and none of the parvalbumin cells. Many galanin- and NPY-containing cells exhibited phosphorylated extracellular signal-regulated kinases (pERK) after mechanical, thermal or chemical noxious stimuli, but very few nNOS-containing cells expressed pERK after any of these stimuli. However, many nNOS-positive inhibitory interneurons up-regulated Fos after noxious thermal stimulation or injection of formalin, but not after capsaicin injection. Parvalbumin cells did not express either activity-dependent marker following any of these stimuli. These results suggest that interneurons belonging to the NPY, nNOS and galanin populations are involved in attenuating pain, and for NPY and nNOS cells this is likely to result from direct inhibition of nociceptive projection neurons. They also suggest that the nociceptive inputs to the nNOS cells differ from those to the galanin and NPY populations.

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1. Introduction

The great majority of neurons in laminae I–III of the dorsal horn are interneurons with axons that arborize locally, and these play a major part in the neuronal circuits that process sensory inputs, including those perceived as pain [2,11,37,52,60,66,77,80]. Our understanding of the organisation of these circuits remains limited, mainly as a result of the difficulty of defining functional populations among the interneurons [11,66,80]. Inhibitory interneurons that use GABA and/or glycine constitute 25–40% of the neurons in laminae I–III in the rat [45]. Several roles have been suggested for these cells, including prevention of different types of pain [52,60,77] and suppression of itch [50]. In addition, loss of function of the inhibitory interneurons (eg, as a result of the decreased synthesis of GABA or reduction of its postsynaptic action) may contribute to neuropathic pain [6,7,39,44,56]. Previous attempts to classify dorsal horn interneurons on the basis of morphological and electrophysiological criteria have met with limited success. Although some inhibitory interneurons in lamina II have been identified as islet or central cells [12,15,16,32,36,67,79,81], many do not belong to these classes and are morphologically diverse [16,36,79]. Even less is known about inhibitory interneurons in laminae I and III.

Neurochemistry provides an alternative approach for classifying these cells, and we have identified 4 non-overlapping populations of inhibitory interneurons in laminae I–III of the rat, based on expression of neuropeptide Y (NPY), galanin, neuronal nitric oxide synthase (nNOS) or parvalbumin [28,46,54,64]. Between them, these account for at least half of the inhibitory interneurons in laminae I–II [54], and it has been demonstrated that there are

* Corresponding authors. Address: Spinal Cord Group, West Medical Building, University of Glasgow, University Avenue, Glasgow G12 8QQ, UK.
E-mail addresses: erika.beresford-polgar@glasgow.ac.uk (E. Polgár), andrew.todd@glasgow.ac.uk (A.J. Todd).

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differences in the postsynaptic targets of their axons [21,46,47,49,54]. Developmental studies also indicate a different lineage for NPY- and galanin-containing cells in the mouse [4].

The somatostatin receptor sst_{2A}, which is present at high levels in the superficial dorsal horn [55,57,70], is restricted to inhibitory interneurons in this region and contributes to disinhibition in the spinal cord [70,79,80]. Because it is found on 13–15% of neurons in laminae I–II [70], we estimate that around half of the inhibitory interneurons in these laminae possess the receptor. However, we do not yet know whether it is associated with particular neurochemical types of interneuron. Some inhibitory interneurons are activated by painful stimuli [15,19,69,83,84], but little is known about the responses of cells belonging to these 4 classes. There is controversy over the extent to which nNOS-containing neurons are activated by noxious stimuli [5,18,29,31,41], and there have apparently been no studies of the responses of cells belonging to the other 3 populations.

In this study, we examined sst_{2A} expression among the different neurochemical classes and used 2 different activity-dependent markers, phosphorylation of extracellular signal–regulated kinases (ERKs) [23] and expression of Fos [22], to test their responses to noxious mechanical, thermal and chemical stimuli. The aim was to determine whether inhibitory interneurons belonging to these classes differ in their expression of sst_{2A} receptor and their responses to noxious stimuli, as this would support the idea that they represent functionally distinct populations and help to elucidate their roles in somatosensory processing.

2. Methods

2.1. Animals and tissue processing

Experiments were approved by the Ethical Review Process Applications Panel of the University of Glasgow and were performed in accordance with the UK Animals (Scientific Procedures) Act 1986.

Thirty-seven male Wistar rats (220–350 g; Harlan) were used in the study. Seven of these were deeply anaesthetized with pentobarbitone (300 mg i.p.) and perfused through the left ventricle with fixative. For 4 of the rats this contained 4% freshly depolymerized formaldehyde, while for the other 3 it contained 4% formaldehyde/0.2% glutaraldehyde. The other 30 rats were used to investigate phosphorylation of ERK or expression of Fos after noxious stimulation. Four different types of noxious stimulus (heat, pinch, or injection of capsaicin or formalin) were applied to one hind paw of these animals. For most phospho-ERK (pERK) experiments (n = 4–6 rats per stimulus type) the stimuli were applied while the animals were under general anaesthesia with urethane (0.4–0.8 g, i.p.), and this was maintained for 5 min after the end of the stimulus, at which point the animals were perfused with fixative (containing 4% formaldehyde). For Fos experiments (n = 3 rats per stimulus type), the animals were briefly anaesthetized with isoflurane (2.5–3%) while the stimulus was applied and were then allowed to recover from general anaesthesia. They were reanaesthetized with pentobarbitone and perfused with fixative (4% formaldehyde) 2 h after the stimulus. The heat stimulus involved immersion of the hind paw in water at 52°C for 20 s, while the pinch stimulus consisted of repeated pinching of folds of skin (6 each on the dorsal and ventral surface of the hind paw, applied with forceps for 5 s at each point over the course of 1 min) [42]. Chemical stimulation involved injection of 25 μL of 1% capsaicin (dissolved in 7% Tween-80, 20% ethanol, saline) [63] or 100 μL of formalin (2% formaldehyde) [8] into the plantar surface of the paw. In preliminary experiments we found that relatively few cells in the superficial dorsal horn were positive for Fos after the pinch stimulus, and this stimulus was therefore not used to investigate Fos expression. The noxious stimuli were applied while animals were anaesthetized in order to minimize discomfort. Continuous general anaesthesia with urethane was used in the pERK experiments because ERK phosphorylation peaks within 5 min after noxious stimulation, and it was therefore necessary to carry out the perfusion fixation promptly at this time [23].

In order to test for pERK expression during the second phase of the formalin response [9], 3 rats received a formalin injection in the foot while under brief isoflurane anaesthesia. They were reanaesthetized with pentobarbitone and perfused at 30 min after formalin injection. This survival time was chosen as it is near the peak of the second phase, which starts around 15 min after injection [65,78].

After perfusion fixation, midlumbar (L4–5) segments were removed from all animals and cut into 60-μm-thick sections with a Vibratome. Transverse sections were used for all parts of the study.

Sections were immersed in 50% ethanol for 30 min, and those from glutaraldehyde-fixed animals were treated with 1% sodium borohydride for 30 min (to reduce free aldehyde groups), followed by extensive rinsing. Sections were then processed for multiple-labelling immunofluorescent detection, as described below. Details of the sources and concentrations of primary antibodies are listed in Table 1. All secondary antibodies were raised in donkey and were species specific. Fluorescent secondary antibodies were conjugated to Rhodamine Red, DyLight 649 (1:100, 1:500, respectively; both from Jackson Immunoresearch) or Alexa 488 (1:500; Invitrogen). In some cases, secondary antibodies conjugated to biotin (1:500) or horseradish peroxidase (HRP; 1:1,000, both from Jackson Immunoresearch) were used. The biotinylated antibodies were revealed with avidin conjugated to Pacific Blue (1:1,000; Invitrogen) or with avidin–HRP (Sigma; 1:1,000) followed by tyramide signal amplification (TSA; tetramethylrhodamine kit; PerkinElmer Life Sciences). The HRP-labelled secondary antibodies were revealed with TSA. TSA reactions were used when 2 of the primary antibodies in an immunoreaction were raised in the same species (Fos combined with either NPY or galanin). In these cases, the initial incubation included one of these antibodies at low concentration (Table 1), and this was revealed with TSA. The sections were subsequently reacted with the other primary antibody, which was revealed with secondary antibody conjugated to a different fluorochrome [3]. For all other reactions sections were initially incubated in a cocktail containing all primary antibodies and then in a corresponding mixture of secondary antibodies. Primary antibody incubations were for 3 days and those in secondary antibodies were overnight (both at 4°C). Antibodies were diluted in PBS that contained 0.3% Triton-X100, except for reactions involving anti-sst_{2A}, in which 5% normal donkey serum was included in both

Table 1
Antibodies used.

Antibody	Species	Dilution	Source
Galanin	Rabbit	1:1,000 1:20,000[a]	Bachem
NPY	Rabbit	1:1,000 1:100,000[a]	Bachem
nNOS	Sheep	1:2,000	P.C. Emson
Parvalbumin	Rabbit	1:500	M. Watanabe
Parvalbumin	Guinea pig	1:2,500	M. Watanabe
sst_{2A}	Guinea pig	1:2,000	Gramsch Laboratories
GABA	Rabbit	1:5,000	D.V. Pow
NeuN	Mouse	1:500	Millipore
pERK	Mouse	1:500	Santa Cruz Biotechnology
Fos	Rabbit	1:5,000 1:40,000[a]	Santa Cruz Biotechnology

[a] Used in combination with the TSA (tyramide signal amplification) method.

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