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A QUANTITATIVE STUDY OF NEUROCHEMICALLY DEFINED POPULATIONS OF INHIBITORY INTERNEURONS IN THE SUPERFICIAL DORSAL HORN OF THE MOUSE SPINAL CORD

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Abstract—Around a quarter of neurons in laminae I–II of the dorsal horn are inhibitory interneurons. These play an important role in modulating somatosensory information, including that perceived as pain or itch. Previous studies in rat identified four largely non-overlapping neurochemical populations among these cells, defined by expression of galanin, neuropeptide Y (NPY), neuronal nitric oxide synthase (nNOS) or parvalbumin. The galanin cells were subsequently shown to coexpress dynorphin. Several recent studies have used genetically modified mice to investigate the function of different interneuron populations, and it is therefore important to determine whether the same pattern applies in mouse, and to estimate the relative sizes of these populations. We show that the neurochemical organization of inhibitory interneurons in mouse superficial dorsal horn is similar to that in the rat, although a larger proportion of these neurons (33%) express NPY. Between them, these four populations account for ~75% of inhibitory cells in laminae I–II. Since ~25% of inhibitory interneurons in this region belong to a novel calretinin-expressing type, our results suggest that virtually all inhibitory interneurons in superficial dorsal horn can be assigned to one of these five neurochemical populations. Although our main focus was inhibitory neurons, we also identified a population of excitatory dynorphin-expressing cells in laminae I–II that are largely restricted to the medial part of the mid-lumbar dorsal horn, corresponding to glabrous skin territory. These findings are important for interpretation of studies using molecular-genetic techniques to manipulate the functions of interneuron populations to investigate their roles in somatosensory processing.

Key words: neuropeptide Y, neuronal nitric oxide synthase, dynorphin, galanin, inhibitory interneuron.

INTRODUCTION

The spinal dorsal horn receives sensory input from a wide variety of primary afferents, including nociceptors, pruritoceptors, thermoreceptors and low-threshold mechanoreceptors, and these terminate in a highly organized pattern within specific laminae (Todd, 2010, 2017; Abraira and Ginty, 2013; Braz et al., 2014). The incoming sensory information is processed through complex synaptic circuits before being transmitted via projection neurons to the brain (for conscious perception), as well as to neurons involved in spinal reflex pathways. The main components involved in these modulatory circuits are local interneurons, which are extremely numerous, and are thought to constitute around 99% of the neurons within the dorsal horn (Abraira and Ginty, 2013). Dorsal horn interneurons can be divided into two broad functional classes: inhibitory neurons, which use GABA and/or glycine as their principal fast transmitter, and excitatory (glutamatergic) neurons (Todd et al., 2003; Yasaka et al., 2010; Zeilhofer et al., 2012). Quantitative studies in the mouse have shown that the inhibitory interneurons account for around one quarter of the neurons in lamina I–II and ~40% of those in lamina III. These cells are known to have an important role in suppressing pain and itch, and loss of this function is thought to contribute to pathological pain states (Yaksh, 1989; Coull et al., 2003; Sandkuhler, 2009; Kardon et al., 2014; Foster et al., 2015; Petitjean et al., 2015).

The inhibitory interneurons can be divided into specific classes, based on the expression of certain neuropeptides and proteins (Todd, 2010, 2017; Braz et al., 2014), and there is increasing evidence that these neurochemical classes correspond to functional populations. For example, they differ in laminar location, which is likely to reflect specific patterns of primary afferent input to each class, and in their responses to noxious stimuli (Polgar et al., 2013b). In 2011, we identified four largely

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Abbreviations: AAV, adeno-associated virus; DAPI, 4′,6-diamidino-2-phenylindole; eGFP, enhanced green fluorescent protein; nNOS, neuronal nitric oxide synthase; NPY, neuropeptide Y; PKCγ, protein kinase Cγ; PPD, preprodynorphin; tdTom, tdTomato; TSA, tyramide signal amplification.

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non-overlapping populations among the inhibitory interneurons in laminae I–III of the rat spinal cord, defined by expression of galanin, neuropeptide Y (NPY), neuronal nitric oxide synthase (nNOS) and parvalbumin (Tiong et al., 2011). We subsequently reported that most of the galanin-containing cells also express the opioid peptide dynorphin, although dynorphin was also found in some excitatory interneurons (Sardella et al., 2011a). Between them, these four populations (galanin/dynorphin, NPY, nNOS and parvalbumin) were thought to account for just over half of the inhibitory interneurons in laminae I–II (Todd, 2017).

Since then, several studies have made use of these neurochemical features to manipulate the activity of neuronal populations in genetically modified mouse lines and test the effects on pain and itch behavior (Duan et al., 2014; Bourane et al., 2015; Foster et al., 2015; Petitjean et al., 2015). These studies have provided important insights into the functions of inhibitory interneuron populations. However, interpretation of findings from studies of this type is complicated, firstly because proteins of interest may be transiently expressed by certain neurons in the dorsal horn (Duan et al., 2014; Bourane et al., 2015; Gutierrez-Mecinas et al., 2017), meaning that behavioral changes cannot necessarily be attributed to the intended neuronal populations. In addition, single neurochemical/genetic markers are often expressed by more than one functional population. For example, nNOS, parvalbumin and dynorphin are also found in significant numbers of excitatory interneurons. Finally, although similar neurochemical populations to those that we defined in the rat can be identified in the mouse dorsal horn, less is known about the sizes of these populations, or the extent to which they overlap. Indeed, there appear to be some species differences, because unlike the situation in the rat, nNOS and galanin show significant overlap in the mouse (Iwagaki et al., 2013; Kardon et al., 2014).

The main aim of this study was to define and quantify neurochemical populations among the inhibitory interneurons in the mouse superficial dorsal horn, and to determine what proportion of the inhibitory neurons they account for. This information will be of importance for interpreting studies in which specific interneuron populations are targeted in genetically modified mice.

EXPERIMENTAL PROCEDURES

Animals

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.

Nine adult C57Bl/6 mice of either sex (20–28 g) were deeply anesthetized with pentobarbitone (30 mg i.p.) and perfused through the left cardiac ventricle with fixative consisting of 4% freshly depolymerized formaldehyde in phosphate buffer. Lumbar spinal cord segments were removed and stored at 4 °C for 2 h in the same fixative. Tissue from these mice was processed for immunocytochemistry (see below) to reveal interneurons belonging to different neurochemical populations.

As an additional way of identifying neurons that express dynorphin, we also analyzed tissue from mice in which Cre recombinase had been knocked into the prodynorphin gene ($Pdyn^{Cre}$) (Krashes et al., 2014). $Pdyn^{Cre}$ mice were crossed with the Ai9 reporter line (Jackson Laboratory; Stock number 007909), in which Cre-mediated excision of a STOP cassette drives expression of the red fluorescent protein tdTomato (tdTom). The resulting mice ($Pdyn^{Cre}$;Ai9) should have tdTom in all neurons that have expressed dynorphin at any stage during development. Four male $Pdyn^{Cre}$;Ai9 mice (20–25 g) were anesthetized and perfused with fixative, and spinal cord tissue was removed and processed as described above.

To distinguish neurons that continue to express dynorphin past the early postnatal period, we performed intraspinal injections of adeno-associated (AAV) virus (serotype 1) carrying a conditional (Cre-dependent) enhanced green fluorescent protein (eGFP) expression cassette (AAV.flex.eGFP; Penn Vector Core, Philadelphia, PA USA) (Gutierrez-Mecinas et al., 2017). The virus encodes the inverted sequence for eGFP between pairs of heterotypic LoxP sites with antiparallel orientation (Atasoy et al., 2008). The rationale for this approach is that in infected dorsal horn neurons that express Cre at the time of injection, there will be permanent reversal of the coding sequence, resulting in expression of eGFP. Three $Pdyn^{Cre}$;Ai9 mice (either sex, 15–17 g, aged P30–44) were anesthetized with isoflurane and received two injections of AAV.flex.eGFP (each 1.7×10^9 GC in 300 nl) into the dorsal horn on the right side, as described previously (Gutierrez-Mecinas et al., 2017). The injections were made on either side of the T13 vertebra, at ~300 μm from the midline and at a depth of 300 μm below the pial surface. The wound was closed and the animals allowed to recover with appropriate postoperative analgesia. After a survival period of 13 or 14 days, they were re-anesthetized and fixed by perfusion, and tissue was processed as described above.

General features of immunostaining and confocal microscopy

Lumbar spinal cord segments were cut into 60-μm-thick transverse sections with a vibrating blade microtome. These were immersed for 30 min in 50% ethanol to enhance antibody penetration and reacted for multiple-labeling immunofluorescence staining as described previously (Gutierrez-Mecinas et al., 2014, 2016; Cameron et al., 2015; Ganley et al., 2015). Details of the antibodies used in this study, including the sources and concentrations, are provided in Table 1. Unless otherwise stated, the sections were incubated for 3 days at 4 °C in primary antibodies diluted in PBS that contained 0.3 M NaCl, 0.3% Triton X-100 and 5% normal donkey serum, and then overnight in species-specific secondary antibodies (Jackson Immunoresearch, West Grove, PA, USA) that were raised in donkey and conjugated to Alexa 488, Alexa 647, Rhodamine Red, Pacific Blue or biotin. All secondary antibodies were diluted 1:500 (in the same diluent), apart from those conjugated to Rhodamine Red