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Research article

Pax2 is persistently expressed by GABAergic neurons throughout the adult rat dorsal horn

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HIGHLIGHTS

- Pax2 was expressed in neurons in all laminae of the dorsal horn in the adult rat.
- GABA immunoreactivity was detected in essentially all Pax2$^+$ neurons.
- Pax2 immunoreactivity was detected in essentially all GABA$^+$ neurons.
- Pax2 is a robust somatic marker of inhibitory neurons in the adult rat dorsal horn.
- Persistent Pax2 expression may be required to maintain a GABAergic phenotype.

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ABSTRACT

The transcription factor Pax2 is required for the differentiation of GABAergic neurons in the mouse dorsal horn. Pax2 continues to be expressed in the adult murine spinal cord and has been used as a presumed marker of GABAergic neurons in the superficial dorsal horn of the adult mouse, although a strict association between adult Pax2 expression and presence of GABA throughout the dorsal horn has not been firmly established. Moreover, whether Pax2 is selectively expressed in GABAergic dorsal horn neurons also in the rat is unknown. Here, immunofluorescent labeling of Pax2 and GABA in the lumbar spinal cord of adult rats was used to investigate this issue. Indeed, essentially all GABA immunoreactive neurons in laminae I–V were immunolabeled for Pax2. Conversely, essentially all Pax2 immunopositive neurons in these laminae exhibited somatic GABA immunolabeling. These results indicate persistent Pax2 expression in GABAergic neurons in the adult rat dorsal horn, supporting the hypothesis that Pax2 may be required for the maintenance of a GABAergic phenotype in mature inhibitory dorsal horn neurons in the rat. Furthermore, Pax2 may be used as a selective and specific general somatic marker of such neurons.

1. Introduction

The generation of inhibitory neurons in the dorsal spinal cord relies on a network of transcription factors that ultimately leads to expression of effector genes involved in the γ-aminobutyric acid (GABA-)ergic and glycinergic machinery, including glutamate decarboxylase (GAD) isoforms, the vesicular inhibitory amino acid transporter and glycine transporter 2 [1,3,5,6,11,17]. Near the down-stream end of this network is the expression of paired box 2 (Pax2), which is required for the maturation of a GABAergic and glycinergic phenotype in these neurons [6,11,17]. Indeed, Pax2 is continually expressed, at least partially in GABAergic neurons, in the murine dorsal horn perinatally and into adulthood in the mouse [e.g.,6,9], suggesting that this transcription factor may be necessary for the persistent expression of inhibitory transmitter effector genes in the mature dorsal horn.

The cell bodies of GABAergic neurons in the brain and spinal cord have in principle been easily identifiable immunohistochemically by direct detection of GABA ever since the first GABA-specific antisera were generated [23,24]. Other selective markers for GABAergic neurons are the GAD isoforms GAD65 and GAD67 [7]. However, these markers suffer from limitations that preclude or complicate their use in many circumstances. For instance, because amino acids exhibit poor retention in tissue fixed without glutaraldehyde [25,26], and because most amino acid antisera are raised against glutaraldehyde conjugates, amino acids including GABA can not be

Abbrevations: GABA, γ-aminobutyric acid; GAD, glutamate decarboxylase; GAD67, glutamate decarboxylase 67; Pax2, paired box 2; PBS, phosphate-buffered saline.

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reliably detected in tissue fixed with paraformaldehyde alone [but see Ref. [19]]. In addition, GABA immunolabeling is for unknown reasons restricted to the surface of thick tissue sections [21,22], further complicating its use in quantitative immunofluorescence microscopy and colocalization studies. Moreover, GABA may not be retained in neuronal cell bodies in slice preparations [24]. Similarly, GAD isoforms, although universal markers of GABAergic axons and nerve endings, are often not detectable in cell bodies without colchicine treatment, including in the spinal cord [8,12,14]. Thus, a general somatic marker of GABAergic neurons in the dorsal horn that is usable in paraformaldehyde-fixed tissue and compatible with conventional immunohistochemical techniques would be of considerable value. As mentioned, Pax2 has in recent years been used by several groups as such a marker in the murine superficial dorsal horn at various postnatal ages [e.g.,4,9,16,20]. However, a direct relationship between Pax2 expression and presence of GABA has not been established in adult dorsal horn neurons. In particular, many $Pax2^+$ neurons in the deep dorsal horn were found to not express *Gad1* at birth, suggesting that in this region, not all mature $Pax2^+$ neurons are GABAergic [6]. Moreover, whether Pax2 is expressed by inhibitory dorsal horn neurons also in the rat has not been examined. The present study aimed to investigate these issues by assessing the colocalization of Pax2 and GABA immunofluorescence in the rat dorsal horn.

2. Experimental procedures

2.1. Tissue preparation

Four adult (~250–300 g) male Sprague-Dawley rats were anaesthetized with sodium pentobarbital (60 mg, i.p.) and rapidly perfused transcardially with phosphate-buffered saline (PBS, 300 mOsm, pH 7.4) followed by PBS containing 4% paraformaldehyde and 1% glutaraldehyde or 4% paraformaldehyde alone (0.5–1 L, 20 min). After perfusion, the lumbar spinal cord was removed. The L1-L4 segments were either cut transversely on a Vibratome at 50 μm thickness or placed in 30% sucrose in PBS and cut on a freezing microtome to yield 40 μm thick transverse sections. All animal experiments were approved by the local Animal Care and Use Committee.

2.2. Immunofluorescence

Spinal cord sections were treated with 1% $NaBH_4$ in PBS for 30 min, washed in PBS and incubated in PBS containing 3% normal goat serum, 0.5% bovine serum albumin and 0.5% Triton X-100 (blocking solution), before being incubated in primary antibody solution at room temperature overnight or for three days. The primary antibody solution contained rabbit anti-Pax2 (1:100; Atlas Antibodies, HPA047704, lot R44792), mouse anti-GABA (1:1000; Swant, clone 3D5, lot ps1) and guinea pig anti-NeuN (1:250; Synaptic Systems, 266 004, lot 266004/5) in blocking solution. In an additional experiment, sections from tissue fixed with paraformaldehyde and glutaraldehyde or only paraformaldehyde were incubated with three NeuN antibodies: the guinea pig antibody, as well as a rabbit antibody (1:1000; Abcam, ab177487; 1:1000) and mouse anti-NeuN (1:1000; Millipore, clone A60). For secondary detection, sections were incubated in goat anti-rabbit Alexa Fluor 488, goat anti-mouse IgG_1 Alexa Fluor 568 and goat anti-guinea pig Alexa Fluor 647 for 2–4 h (all 1:500; Life Technologies). Sections were mounted on slides using Prolong Gold or Prolong Diamond with DAPI (Life Technologies).

2.3. Microscopy

The immunolabeled sections were examined in a Zeiss LSM700 confocal microscope. For quantitative analysis of Pax2/GABA colocalization, z-stacks of optical sections at 1 μm separation were acquired of the entire dorsal horn throughout the extent of GABA immunolabeling at the surfaces of the tissue section using the automatic tile scan function with a 40x/1.3 oil immersion objective. For assessment of the specificity of the guinea pig NeuN antibody, four dorsal horns immunolabeled for the three NeuN antibodies were imaged using tile scan and the 40x/1.3 objective.

2.4. Image analysis

For quantitative analysis, sections from the L1 segment of three animals were used. For each animal, two or three dorsal horns from two sections were analyzed. The z-stack of each tissue section was opened in ImageJ and laminar borders outlined based on NeuN immunoreactivity. As GABA immunolabeling was confined to the surface of the section, only optical sections at the surface that exhibited immunofluorescence in the GABA channel were analyzed. In those optical sections, NeuN immunolabeled cells containing a nucleolus or DAPI staining were marked in each lamina, and such cells were subsequently checked for immunopositivity for GABA and Pax2 in their respective fluorescence channel. In order to estimate the specificity of the guinea pig NeuN antibody, the dorsal horns were examined in the channel showing mouse anti-NeuN immunoreactivity, and 50 immunolabeled cells exhibiting a prominent nucleolus were semirandomly marked in each dorsal horn. Marked cells were subsequently examined in the other channels for immunoreactivity produced by the guinea pig and rabbit NeuN antibodies.

3. Results

The distribution of Pax2 immunolabeling in the spinal cord was similar to that in the mouse [6]; numerous immunolabeled nuclei were found in the dorsal horn, whereas fewer labeled nuclei were found in intermediate and ventral gray matter (Fig. 1). GABA staining was confined to the surfaces of the section, and closely mimicked the expected distribution [13,18,28]. Neuropil staining was extensive in all laminae, although particularly concentrated in lamina I–III (Fig. 1). Cell bodies throughout the gray matter were immunolabeled with varying intensity. Immunolabeled axons were also found in the white matter, in particular in the ventral and lateral funiculi and the pyramidal tract.

NeuN immunolabeling using the guinea pig antibody from Synaptic Systems showed the expected distribution of labeled cells in paraformaldehyde fixed spinal cord [27], although weak diffuse neuropil staining was also observed (Fig. 2A). In tissue fixed with paraformaldehyde and glutaraldehyde, the staining of neuropil was increased, sometimes making delineation of NeuN immunolabeled cell bodies difficult (Figs. 1, 2B). Notably, many DAPI-stained nuclei were devoid of labeling, suggesting that also the diffuse staining was selective for certain cell types (presumably neurons) (Fig. 2B). Quantitative analysis of spinal dorsal horn co-immunolabeled with the guinea pig antibody and two monoclonal NeuN antibodies, including the original clone A60 [15], showed that all cells labeled with the guinea pig antibody were also labeled with the other antibodies (Fig. 2B). Conversely, 97.5% (195/200) of dorsal horn cells labeled with clone A60 were also clearly discernible with the guinea pig antibody (whereas 100% were distinctly labeled by rabbit anti-NeuN) in paraformaldehyde/glutaraldehyde fixed tissue (data not shown).