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# DISTRIBUTION OF CALCIUM CHANNEL $Ca_V1.3$ IMMUNOREACTIVITY IN THE RAT SPINAL CORD AND BRAIN STEM

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**Abstract—The function of local networks in the CNS depends upon both the connectivity between neurons and their intrinsic properties. An intrinsic property of spinal motoneurons is the presence of persistent inward currents (PICs), which are mediated by non-inactivating calcium (mainly $Ca_V1.3$) and/or sodium channels and serve to amplify neuronal input signals. It is of fundamental importance for the prediction of network function to determine the distribution of neurons possessing the ion channels that produce PICs. Although the distribution pattern of $Ca_V1.3$ immunoreactivity ($Ca_V1.3$-IR) has been studied in some specific central nervous regions in some species, so far no systematic investigations have been performed in both the rat spinal cord and brain stem. In the present study this issue was investigated by immunohistochemistry. The results indicated that the $Ca_V1.3$-IR neurons were widely distributed across different parts of the spinal cord and the brain stem although with variable labeling intensities. In the spinal gray matter large neurons in the ventral horn (presumably motoneurons) tended to display higher levels of immunoreactivity than smaller neurons in the dorsal horn. In the white matter, a subset of glial cells labeled by an oligodendrocyte marker was also $Ca_V1.3$-positive. In the brain stem, neurons in the motor nuclei appeared to have higher levels of immunoreactivity than those in the sensory nuclei. Moreover, a number of nuclei containing monoaminergic cells, for example the locus coeruleus, were also strongly immunoreactive. $Ca_V1.3$-IR was consistently detected in the neuronal perikarya regardless of the neuronal type. However, in the large neurons in the spinal ventral horn and the cranial motor nuclei the $Ca_V1.3$-IR was clearly detectable in first and second order dendrites. These results indicate that in the rat spinal cord and brain stem $Ca_V1.3$ is probably a common calcium channel used by many kinds of neurons to facilitate the neuronal information processing via certain intracellular mechanisms, for instance, PICs.** 

**Key words: L-type calcium channel, alpha 1D subunit, immunohistochemistry, persistent inward current, plateau potential.**

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*Abbreviations:* APC, adenomatous polyposis coli; BSA, bovine serum albumin; Ca-PIC, calcium channel mediated persistent inward current; GFAP, glial fibrillary acidic protein; Iba1, ionized calcium-binding adaptor molecule 1; IR, immunoreactivity; LC, locus coeruleus; NeuN, neuronal nuclei; NGS, normal goat serum; PAP, peroxidase antiperoxidase; PBS, phosphate-buffered saline; PIC, persistent inward current; TBS-T, Tris-buffered saline with 0.3% Triton X-100; 5N, motor trigeminal nucleus; 7N, facial nucleus; 10N, dorsal motor nucleus of vagus; 12N, hypoglossal nucleus.

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The operation of neuronal networks in the CNS depends upon both the specific patterns of connectivity of groups of neurons and the information processing performed by its constituent neurons. The latter is dependent upon the intrinsic properties of the neurons, which is determined in part by their specific profile of ion channels and receptors. Recently, there has been an increased interest in the role of ion channels producing voltage-dependent non-inactivating persistent inward currents (PICs) that permit short-lasting inputs to produce long-lasting increases in firing of motoneurons in addition to providing amplification of dendritic synaptic inputs. Most of the voltage-gated ion channels mediating PICs are neurotransmitter-controlled, creating a high degree of state-dependent flexibility (for a review on motoneurons, see Rekling et al., 2000). In the late 1970s Schwindt and Crill first demonstrated that PICs could trigger all-or-none plateau potentials and self-sustained firing in spinal cord motoneurons (Schwindt and Crill, 1977, 1980; see reviews by Powers and Binder, 2001 and Hultborn et al., 2004). These PICs were shown to be mediated mainly by nifedipine-sensitive voltage-gated L-type calcium channels (Hounsgaard and Kiehn, 1989). PICs could be triggered at a relatively hyperpolarized levels, close to firing threshold (Hounsgaard et al., 1988; Hounsgaard and Mintz, 1988) and thus were attributed to a particular subgroup of L-type calcium channels, the $Ca_V1.3$ (previously $\alpha_{1D}$ subunit, see Ertel et al., 2000). These calcium channels are transmitter-controlled (reviewed by Alaburda et al., 2002), are facilitated by a number of metabotropic receptors for noradrenalin (Conway et al., 1988), 5-HT (Hounsgaard et al., 1988; Hounsgaard and Kiehn, 1989) as well as glutamate and acetylcholine (Svirskis and Hounsgaard, 1998) and are inhibited by GABA (Guertin and Hounsgaard, 1999). Given that the threshold for these calcium channels is close to the firing threshold they have been hypothesized to amplify the classical synaptic excitation of motoneurons, the degree of facilitation being set by the facilitation/inhibition of the $Ca_V1.3$ channels (reviewed by Heckman et al., 2003 and Hultborn et al., 2004).

Electrophysiological investigations of PICs in spinal motoneurons were initially performed in cats (Schwindt and Crill, 1980; Hounsgaard et al., 1984) and turtles (Hounsgaard and Kiehn, 1985, 1989), and more recently in rats (Bennett et al., 2001; Button et al., 2006) and mice (Carlin et al., 2000). The ability to generate PICs is not limited to spinal ventral horn motoneurons but also appears in other spinal neuronal groups including dorsal horn projection neurons and interneurons (Hounsgaard and

Kjaerulff, 1992; Russo and Hounsgaard, 1996; Morisset and Nagy, 1999; Smith and Perrier, 2006) as well as parasympathetic preganglionic and intermediolateral neurons (Derjean et al., 2005). The ability to generate PICs (or bistability) is not restricted to the spinal cord and has also been demonstrated in different neuronal groups from many brain regions in several species. For example, PICs have been found in motoneurons in the motor trigeminal nucleus (5N; Hsiao et al., 1998), the hypoglossal nucleus (12N; Powers and Binder, 2003) and the facial nucleus (7N; Cramer et al., 2007) and the sensory neurons in the principal trigeminal nucleus (Sandler et al., 1998).

The distribution pattern of $Ca_V1.3$-immunoreactivity (IR) in the spinal cord has been partially described in rats (Westenbroek et al., 1998; Dobremez et al., 2005), mice (Jiang et al., 1999; Carlin et al., 2000), turtles (Simon et al., 2003) and cats (Zhang et al., 2006, 2008). However, the results regarding the specific localization of this channel are inconsistent across studies and from the anatomical perspective a systematic description is still lacking at different spinal levels with the exception of one study that was recently performed by us in the cat (Zhang et al., 2008). Due to the increasing popularity of the rat as an experimental model, it is now necessary to obtain a systematic profile on the distribution of $Ca_V1.3$-IR in the rat spinal cord. Only a few studies exist concerning the distribution of $Ca_V1.3$-IR in the brain stem (e.g. Takada et al., 2001; Grunnet and Kaufmann, 2004; Westenbroek et al., 2005), however these studies have usually focused on only a small region or a particular neuronal group. Given that the neurons in many brain stem nuclei, especially the motor nuclei, can generate PICs it is important to perform a systematic investigation of the distribution of this channel also in the brain stem.

## EXPERIMENTAL PROCEDURES

### Animals and tissue preparations

All experimental procedures were conducted in accordance with the guidelines of EU Directive 86/609/EEC and were approved by the Danish Council for Animal Experiments. All efforts were made to minimize the number of animals used and their suffering. Thirteen adult male Wistar rats (Charles River Laboratories, Sulzfeld, Germany) with body weights 250–300 g were used in this study. Under deep anesthesia induced by isoflurane (Baxter, Allerød, Denmark) and Nembutal (50 mg/kg body weight, i.p.; Sygehus Apotekerne, Copenhagen, Denmark) eight rats were transcardially perfused. Following a rinse with saline containing 7500 IU/L heparin the animal was perfused with 500 ml 0.1 M cold phosphate buffer containing 4% paraformaldehyde with ($n$=6) or without ($n$=2) 0.36% L-lysine and 0.05% sodium $m$-periodate. The whole spinal cord and the brain stem were removed immediately and postfixed in the same perfusion solution overnight. The spinal cord and the brain stem were blocked and cryo-protected in phosphate-buffered saline (PBS) with 30% sucrose at 4 °C until cut. Selected segments from different (cervical, thoracic, lumbar, sacral and caudal) parts of the spinal cord and the brain stem (medulla oblongata, pons and midbrain) were cut transversely into 40-$\mu$m-thick sections using a sliding microtome.

### Immunohistochemistry

To investigate the general $Ca_V1.3$ distribution pattern in the spinal cord and the brain stem conventional peroxidase anti-peroxidase (PAP) immunohistochemistry was used. Every fifth section from the spinal cord and every fourth section from the brain stem were processed. Because this staining method has been described in detail elsewhere (e.g. Zhang et al., 2002), we only describe the major steps in this study. 1) The sections were quenched in 0.6% $H_2O_2$ in PBS (pH 7.4) for 40 min. 2) After rinsing in 0.05 M Tris-buffered saline (pH 7.5) containing 0.3% Triton X-100 (TBS-T) the sections were preincubated in TBS-T containing 1% bovine serum albumin (BSA), 1% human serum albumin and 5% normal goat serum (NGS) overnight. 3) The sections were incubated in a rabbit anti-$Ca_V1.3$ primary antibody (Millipore-Chemicon, Temecula, CA, USA) diluted 1:100 in the same solution 20–24 h at 4 °C. 4) After a thorough rinse in TBS-T the sections were incubated in goat anti-rabbit immunoglobulin G (1:50; Dako, Glostrup, Denmark) in TBS-T with 1% BSA, 1% human serum albumin and 2% NGS for 1 h at room temperature. 5) After rinsing sections were incubated in rabbit polyclonal PAP (1:100; Dako) diluted in the same solution for 2 h. 6) The binding peroxidase was visualized by incubating the section in 0.05 M Tris buffer (pH 7.5) containing 0.04% diaminobenzidine tetrahydrochloride and 0.005% $H_2O_2$ for 10–20 min. Finally, the sections were mounted, dried, cleared in toluene and coverslipped with Pertex (HistoLab, Gothenburg, Sweden). To compare the $Ca_V1.3$ immunostaining pattern with the cytoarchitecture of the spinal cord and the brain stem, one set of adjacent sections was stained with Thionin (Nissl staining).

To examine if all the neurons in the spinal cord were labeled by the $Ca_V1.3$ antibody and if glial cells contain $Ca_V1.3$ channels, double-immunostaining was carried out using a $Ca_V1.3$ antibody (1:100) and (1) a mouse anti–neuronal nuclei (NeuN, a neuronal marker) monoclonal antibody (1:2000; Millipore-Chemicon), (2) a mouse anti–glial fibrillary acidic protein (GFAP, an astrocyte marker) monoclonal antibody (1:500; clone GA5, Millipore-Chemicon), (3) a mouse anti–adenomatous polyposis coli (APC, an oligodendrocyte marker) monoclonal antibody (1:100; clone CC-1, Merck, Nottingham, UK) or (4) a goat anti–ionized calcium-binding adaptor molecule 1 (Iba1, a microglial marker) polyclonal antibody (1:100; Abcab, Cambridge, UK). The lumbar spinal sections from two rats that were perfused with only 4% paraformaldehyde were used for double staining. The sections were incubated in $Ca_V1.3$ antibody as above and visualized with a donkey anti-rabbit secondary antibody conjugated with Alexa Fluor 594 (1:100, Invitrogen, Eugene, OR, USA). Subsequently the sections were incubated in a second mouse/goat primary antibody overnight at 4 °C and visualized with a donkey anti-mouse/goat secondary antibody conjugated with Alexa Fluor 488 (1:200, Invitrogen). The sections were washed in distilled water and mounted on SuperFrost slides and then coverslipped with Fluorescent Mounting Medium (Dako).

### Antibody specificity and controls

The rabbit anti-$Ca_V1.3$ antibody used in this study was purchased from Millipore-Chemicon, and was an affinity-purified, polyclonal antibody raised against amino acid residues 859–875 of $Ca_V1.3$ subunit of rat brain voltage-gated calcium channel (accession No.: P27732). This antibody recognizes all forms of $Ca_V1.3$ subunits from the voltage-gated calcium channel and does not cross-react with any other calcium channel antigens tested so far (manufacturer's data sheet). To compare if the staining pattern is the same by using the present antibody and the rabbit $Ca_V1.3$ antibody used in our previous study (raised against amino acids 2169–2203 of the $Ca_V1.3$ subunit, accession No.: P27732; Zhang et al., 2008) immunohistochemistry was also performed with that antibody (generously provided by Dr. Ilya Bezprozvanny from University of Texas Southwestern Medical Centre, will be referred as Texas below). In addition, to verify if these two antibodies label the same protein components we performed a Western blot analysis (see below). Control staining was performed with antibodies that were preadsorbed with a sufficient amount of the corresponding