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DEVELOPMENTAL CHANGES IN THE EXPRESSION OF CALBINDIN AND POTASSIUM-CHANNEL SUBUNITS Kv3.1b AND Kv3.2 IN MOUSE RENSHAW CELLS

Z.-M. SONG,[a,b]* J. HU,[b,c] B. RUDY[d] AND S. J. REDMAN[b]

[a]*Medical School, Australian National University, Canberra, ACT, Australia*

[b]*Division of Neuroscience, John Curtin School of Medical Research, Australian National University, Building 54 Mills Road, Canberra, ACT 0200, Australia*

[c]*Zhongshan Ophthalmic Centre, Zhongshan University, Guangzhou, China*

[d]*Department of Physiology and Neuroscience, New York University School of Medicine, New York, NY, USA*

Abstract—One class of spinal interneurons, the Renshaw cells, is able to discharge at very high frequencies in adult mammals. Neuronal firing at such high frequencies requires voltage-gated potassium channels to rapidly repolarize the membrane potential after each action potential. We sought to establish the pattern of expression of calbindin and potassium channels with Kv3.1b and Kv3.2 subunits in Renshaw cells at different developmental stages of postnatal mice. The pattern of expression of calbindin changed dramatically during early postnatal development. An adult pattern of calbindin reactive neurons started to emerge from postnatal day 10 to postnatal day 14, with cells in laminae I and II of superficial dorsal horn and the ventral lamina VII. Renshaw cells were identified immunohistochemically by their expression of calbindin and their location in the ventral horn of the spinal cord. Western blot results of the lumbar spinal cord showed that Kv3.1b expression became faintly evident from postnatal day 10, reached a maximum at postnatal day 21 and was maintained through postnatal day 49. Double labeling results showed that all Renshaw cells expressed Kv3.1b weakly from postnatal day 14, and strongly at postnatal day 21. Western blot results showed that Kv3.2 expression became detectable in the lumbar cord from postnatal day 12, and increased steadily until reaching an adult level at postnatal day 28. In contrast to the Kv3.1b results, Kv3.2 was not expressed in Renshaw cells, although some neurons located at laminae VIII and VI expressed Kv3.2. We conclude that Renshaw cells express Kv3.1b but not Kv3.2 from postnatal day 14. © 2006 Published by Elsevier Ltd on behalf of IBRO.

Key words: spinal cord, voltage-gated potassium channel, development, ventral horn, mouse.

The excitation and inhibition of motoneurons in the spinal cord are largely controlled by spinal interneurons, which are located mainly in the ventral half of the spinal cord. One class of spinal interneurons, the Renshaw cells, mediates recurrent inhibition of spinal motoneurons. Renshaw cells are strongly immunoreactive for calbindin D28K and gephyrin clustering (Carr et al., 1998). They are located at the border between the ventral horn and the overlying white matter. Morphologically they can be identified by their distinct location and calbindin immunoreactivity. Recent evidence indicates that Renshaw cells are differentiated from an embryonic class of interneurons that transiently express the En1 transcription factor (Sapir et al., 2004).

Mature Renshaw cells can discharge more rapidly than any other type of neurons in the CNS. Interspike intervals shorter than 1 ms have been recorded in response to ventral root stimulation (Eccles et al., 1954). The membrane conductances underlying the fast spiking of Renshaw cells are unknown. The main known candidates permitting rapid repolarization of the action potential and recovery of the sodium conductance from inactivation are the voltage-gated potassium channels expressing Kv3.1 and Kv3.2 subunits. These channels have been shown to enable sustained high frequency bursts of impulses in fast spiking neocortical inhibitory interneurons (Erisir et al., 1999). These channels are rapidly activating delayed rectifiers that require large membrane depolarization (>-10mV) to produce significant activation and they deactivate 7–10$\times$ faster on repolarization than other known voltage-gated K^+ channels (reviewed in Rudy and McBain, 2001). Several studies demonstrated dramatic changes in the expression of channels during postnatal development, including β subunits of Kv channels (Downen et al., 1999) and Kv2.1 channel (Wilson et al., 2004). Although mRNA for Kv3.1, but not for Kv3.2, has been found in both the dorsal and ventral horns of the spinal cord (Weiser et al., 1994), their cellular localization and developmental profile are unknown.

The present experiments aimed to establish whether potassium channels with Kv3.1b and Kv3.2 subunits are present in Renshaw cells, and if so, the age at which mature pattern of expression occurs. The answer to these questions helps to put the development changes of various channel types in the spinal cord into functional perspective.

EXPERIMENTAL PROCEDURES

Membrane preparation

The expression of Kv3.1b and Kv3.2 was investigated in the lumbar spinal cord of C57/B6 mice at ages from newborn to adult (postnatal days (P) 1, 7, 10, 12, 14, 21 and 49). Crude membrane fractions were

*Correspondence to: Z.-M. Song, Division of Neuroscience, John Curtin School of Medical Research, Australian National University, Building 54 Mills Road, Canberra, ACT 0200, Australia. Tel: +61-2-6125-4963; fax: +61-2-6125-2687.
E-mail address: zan-min.song@anu.edu.au (Z.-M. Song).

Abbreviations: P, postnatal day; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; TBS, Tris-buffered saline.

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prepared as described previously (Tansey et al., 2002) with modification. Mice were deeply anesthetized with sodium pentobarbitone (100 mg/kg, i.p.). Segments of lumbar spinal cords (L1–L5) were removed and homogenized in 1 ml of ice-cold "homogenization buffer" (20 mM Tris–HCl, pH 7.4, 0.3 M sucrose, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 20 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, all from Sigma-Aldrich, Sydney, Australia). Homogenates were centrifuged at 10,000×*g* for 10 min (4 °C). The supernatant was then centrifuged at 100,000×*g* for 60 min (4 °C). Pellets were then washed in "suspension buffer" (20 mM Tris–HCl, 1 mM EDTA, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 20 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, pH 7.4) and recentrifuged at 40,000×*g* for 20 min at 4 °C. The pellets were resuspended with suspension buffer, and were either used immediately or stored at −80 °C for later use (up to 2 weeks). Protein concentrations were determined using a Bradford protein assay kit (Sigma-Aldrich), using bovine serum albumin as standards.

SDS–polyacrylamide gel electrophoresis (PAGE) and Western blot

Twelve micrograms of proteins from each spinal cord were resuspended in equal volumes of the 2× Laemmli sample buffer (Sigma-Aldrich) and denatured for 5 min at 80 °C. The proteins were resolved by SDS-PAGE for 2 h at 100 V using precast 4–12% gradient NuPAGE Gel in Tris/glycine/SDS running buffer (Invitrogen, Mount Waverley, VIC, Australia). The resolved proteins were transferred onto Hybond ECL nitrocellulose membranes (Amersham Biosciences, Castle Hill, NSW, Australia) for overnight at 30 V using a Bio-Rad Gel Cell containing Bio-Rad Tris/glycine buffer with 20% methanol (v/v). The membranes were blocked in phosphate-buffered saline (PBS) containing 0.1 (v/v) Tween 20 and 5% non-fat milk for 1 h, and incubated for 2 h at room temperature with anti-Kv3.1b antibody (1:1000, Alomone Laboratories, Jerusalem, Israel) or rabbit anti-Kv3.2 (1:50, raised against the mouse Kv3.2 proteins in the laboratory of Bernard Rudy, fully characterized in Chow et al., 1999 and Tansey et al., 2002). Membranes were rinsed three times in Tris-buffered saline (TBS) containing 0.1% (v/v) Tween 20 (Sigma-Aldrich) and then incubated for 1 h with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:5000, Jackson Immunoresearch Laboratories, West Grove, PA, USA). After three washes in TBS, bands were visualized using SuperSignal Chemiluminescence kit (Pierce, Rockford, IL, USA) and exposed to Kodak X-Omat AR Film (Sigma-Aldrich). The films were digitized using a Luminescent Image Analyzer LAS-1000

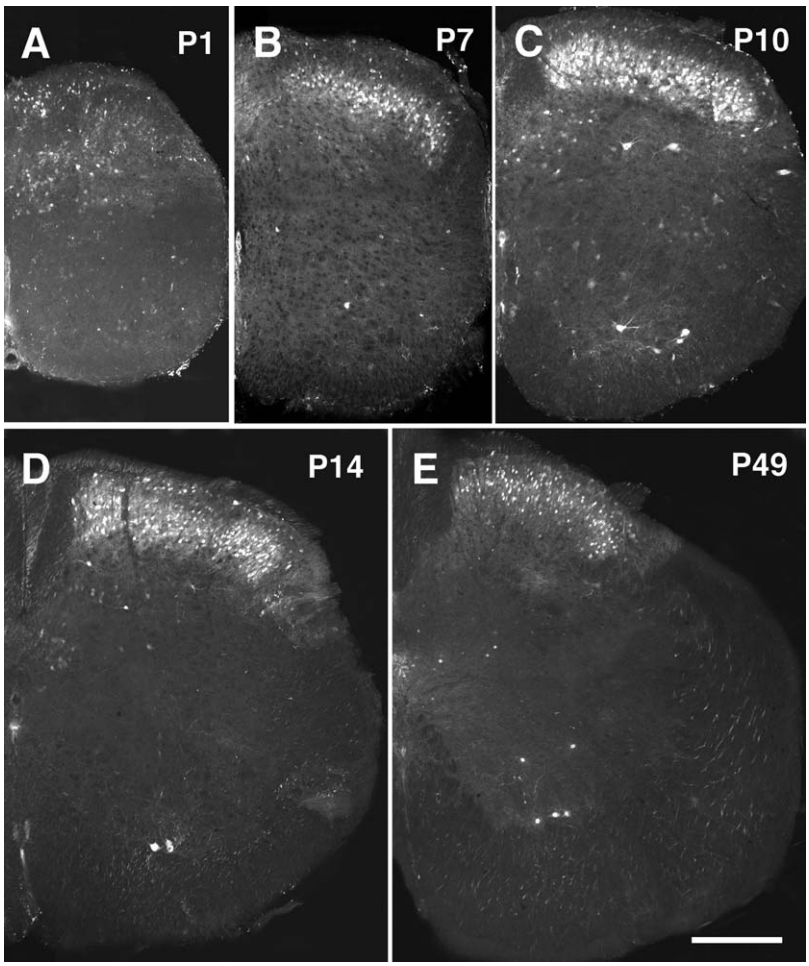

Fig. 1. Representative confocal images showing the postnatal development of calbindin-immunoreactive neurons in the spinal cord. (A) At P1, calbindin-immunoreactive (IR) neurons were scattered in the dorsal half of the spinal cord, but not in the ventral horn. (B) At P 7, calbindin-IR neurons were densely expressed in dorsal spinal cord especially in the lamina II, but rarely at ventral horn. (C) At P10, more calbindin-IR neurons were found in the lamina II and several neurons appeared in the ventral border of the gray matter with white matter, an area that contains Renshaw cell. (D and E). From P14 (D) through P49 (E), dorsal horn calbindin-IR neurons occupied both laminae I and II and a number of reactive cells were present at Renshaw cell area. Scale bar=200 μm.