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Heterogeneous expression of the voltage-gated calcium channel α2 subunit and the voltage-gated sodium channel α subunit in chicken spinal motoneurons

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ABSTRACT

The localization of the voltage-gated calcium channel (VGCC) α2 and the voltage-gated sodium channel (VGSC) α subunits was immunohistochemically investigated in chicken spinal motoneurons. Approximately 83% and 46% of spinal motoneurons were positive for VGCCα2 and VGSCα subunits, respectively. Almost all VGSCα subunit-positive motoneurons exhibited the VGCCα2 subunit immunoreactivity. There were different patterns in occurrence, intensity or nuclear/cytoplasmic stainability of the VGCCα2 and VGSCα subunits among the motoneurons. This study presents the first cellular morphological evidence for the VGCCα2 and VGSCα subunits in spinal motoneurons, postulating that the heterogeneous expression of VGCCα2 and VGSCα subunits in the motoneurons may reflect various motor activities.

The dynamic regulation of the excitability of motoneurons is largely determined by voltage-gated ion channels, which are also the targets of several neuromodulators that affect excitability (for review, see 17). Spinal motoneurons contain almost all of the types of voltage-gated calcium channels (VGCCs): L-, N-, P-, Q-, and R-type. However, the proportion of currents mediated by the different subtypes varies between cells (3). As the electrical excitability is strikingly different between different types of motoneurons (9), spinal motoneurons appear to be heterogeneous with regard to discharge properties. VGCCs are complexes that include a pore-forming α1 subunit, an intracellular auxiliary β subunit, a disulfide-linked complex of α2 and δ subunits, and in some cases, a transmembrane γ subunit (for review, see 7). Recently, high levels of α2δ2 and α2δ3 mRNAs were detected by *in situ* hybridization in motoneurons of the ventral horn of the rat spinal cord (5). In contrast, α2δ1 mRNA was detected in only a few motoneurons (5). Because the VGCCα2 subunit is reserved as a common component in all types of VGCCs (19), we performed an immunohistochemistry using an antibody to the α2 subunit, which may allow broad identification of any type of VGCCs, and revealed the expression of the VGCCα2 subunit in chicken spinal neurons (12).

Four types ($Na_v1.1$, $Na_v1.2$, $Na_v1.3$, and $Na_v1.6$) of voltage-gated sodium channel (VGSC) α subunits are expressed in the central nervous system (22). Of these, 3 types ($Na_v1.1$, $Na_v1.3$, and $Na_v1.6$) are present in spinal motoneurons (10, 13, 21), and motoneurons display different patterns of the VGSCα subunit expression (21). An electrophysiological study reported that VGSCs are critical for both rhythmic firing and propagating action potentials of motoneu-

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rons. The depolarizing sodium currents further activate VGCCs, which subsequently open to contribute to inward calcium currents to the upstroke and plateau of the action potential (11). This may suggest that the VGCCs and VGSCs expressed in the motoneurons are largely involved in various motor activities of skeletal muscles. However, there is no evidence of a morphological correlation of VGCCs and VGSCs in motoneurons. To verify this relationship, we used an antibody that potentially recognizes VGSCs in motoneurons (see below), together with the antibody to the VGCCα2 subunit we employed previously.

Six adult female Leghorn chickens (*Gallus domesticus*, 0.8–1.75 kg) were used for the study. The experiment was performed in accordance with the guidelines of the National Institute of Health Guide for the Care and Use of Laboratory Animals. The chickens under deep anesthesia (60 mg/kg sodium pentobarbital) were perfused with 100 mL physiological saline through their hearts, followed by the perfusion of 400–500 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The detailed perfusion procedure has been described previously (2). The spinal cord of each chicken was removed at the C15 segmental level and was fixed in the same fixative for 3 h at 4°C. The spinal cords were then washed in cold sucrose solutions with increasing concentrations. They were embedded in Tissue-Tek and frozen in cold acetone. The frozen samples were cut into 20-μm-thick transverse sections by use of a cryostat. After pretreatment with methanol for 30 min at room temperature and with 3% goat serum containing 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 30 min, the sections were double-immunolabeled by overnight incubation at 4°C with a cocktail of the following primary antibodies: mouse anti-VGCCα2 subunit monoclonal antibody (1 : 200; A6, Swant, Bellinzona, Switzerland) and rabbit anti-choline acetyltransferase (ChAT) antibody (1 : 500; AB143, Chemicon International, Temecula, USA); rabbit anti-pan VGSCα (SP19) (1 : 1000; S6936, Sigma, St. Louis, USA), which was raised by rabbit using a synthetic peptide corresponding to amino acids 1491–1508 of the α subunit of rat type I ($Na_V1.1$) VGSC (SP19), and mouse monoclonal anti-acetylcholinesterase (AChE) antibody (1 : 500; MAB5422, Chemicon International); or mouse anti-VGCCα2 subunit monoclonal antibody (1 : 200) and rabbit anti-VGSCα subunit antibody (1 : 1000). After overnight incubation, the sections were rinsed in PBS and incubated with biotinylated horse anti-mouse IgG (BA-2000 from VECTASTAIN Elite ABC Kit: PK-6102), Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. The sections were washed in PBS and incubated with a cocktail of Cy2-conjugated goat anti-rabbit IgG (1 : 200; 286769, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and Cy3-conjugated streptavidin (1 : 1000; PA43001, Amersham Life Science, Buckinghamshire, UK) for 1 h at room temperature. The sections were mounted in Fluoroguard (Bio-Rad, Hercules, CA, USA). Fluorescence signals were detected by use of a confocal laser microscope (TCS4D; Leica, Heidelberg, Germany) and the images were captured by use of a CCD camera (VB7010; KEYENCE, Osaka, Japan). For control, the sections were incubated with the normal rabbit or mouse serum instead of a primary antibody or without the addition of a primary antibody. There was no immunostaining detected in the control sections. For quantitative analysis, more than 3 fluorescence sections were randomly selected from each of the six animals, and neurons with a visible nucleus were selected for counting. Staining of the VGCCα2 subunit in the cytoplasm was scored only by Li (Y.N.) as weak or moderate (+), or strong (++) according to the intensity of the staining. Because VGSCα subunits can be localized to the nucleus and cytoplasm, the cells were divided into 3 subgroups based on their immunoreactivity (IR). The neurons were classified as follows: neurons with only nuclear staining were scored as +, those that had a weak or moderate signal in both the nucleus and cytoplasm as ++, and those that had intense staining in both nucleus and cytoplasm as +++. The percentage of neurons positive and negative for VGCCα2 and VGSCα was determined.

Immunoreactivity of the VGCCα2 subunit was mainly seen in large neurons in Lamina IX of the ventral horn (Fig. 1A). These subunits were occasionally seen in small neurons in Lamina VIII, consistent with our previous report (12). Approximately 83% of the total ChAT-positive neurons (n = 520) from 37 fluorescence sections (Fig. 1B) also showed the VGCCα2 subunit-IR (Table 1). All of the neurons that exhibited VGCCα2 subunit-IR were also positive for ChAT (Fig. 1A–C). Based on colocalization of VGCCα2 with ChAT and the distribution and morphology of the VGCCα2-positive neurons, these neurons were confirmed to be motoneurons. VGCCα2 staining was localized in the somata and proximal dendrites, but not in the nuclei of the motoneurons (Fig. 1A). The intensity of the immunostaining in the somata varied between cells. In total,

64.7% of the motoneurons were scored as having weak or moderate intensity (+) and 18.3% as having strong intensity (++) (Table 1). Immunostaining for the VGSCα subunit was observed in some large neurons in the ventral horn and in numerous myelinated axons throughout the spinal cord. Some neurons showed intense IR only in the nucleus, while some had intense staining in the nuclei and weak to intense staining in the cytoplasm (Fig. 1D, H). In addition, intense IR was seen in the proximal

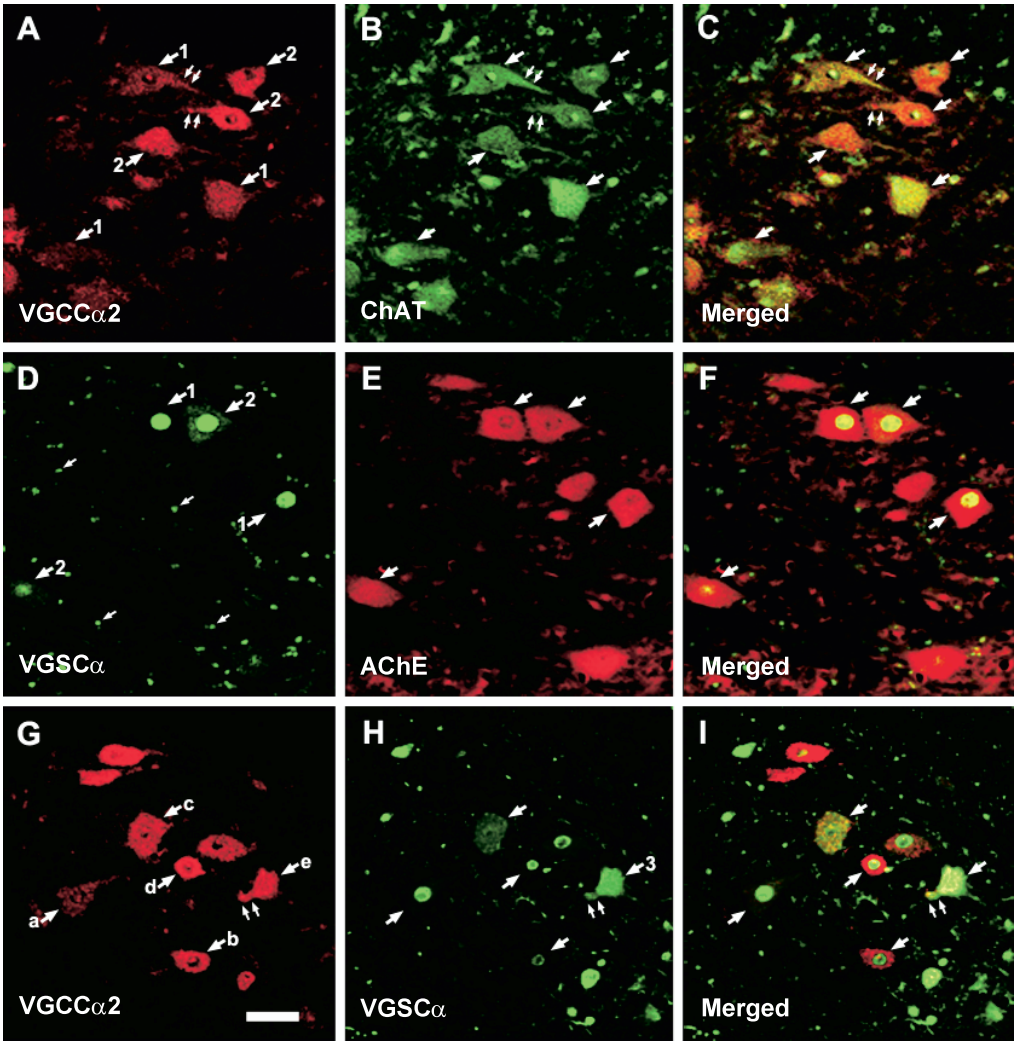

Fig. 1 Fluorescence micrographs showing the expression of the VGCC*α*2 subunit (**A**, **G**), choline acetyltransferase (ChAT) (**B**), the VGSC*α* subunit (**D**, **H**) and acetylcholinesterase (AChE) (**E**) in motoneurons (large arrows) located at lamina IX of the chicken spinal cord. Panels **C**, **F**, and **I** show merged immunnoreactivity (IR) images. **A**–**C**: VGCC*α*2 subunit-IR was localized in neuronal somata and proximal dendrites (small arrows) but not in the nuclei. Motoneurons positive for the VGCC*α*2 subunit were divided into two subgroups based on the intensity of the IR in the cytoplasm; the numbers (1, 2) indicate motoneurons showing weak or moderate (+) and strong (++) IR, respectively (**A**). All neurons positive for VGCC*α*2 were also immunoreactive for ChAT (**A**: VGCC*α*2 subunit; **B**: ChAT; **C**: merged). **D**–**F**: Motoneurons positive for VGSC*α* subunit were divided into three subgroups. Positive neurons that had only nuclear IR were scored as + and those that had a positive nucleus and weak or moderate staining in the cytoplasm were scored as ++ and are shown as 1 and 2, respectively (**D**). Those that had intense nuclear and cytoplasmic staining were scored as +++ (3) (**H**). Myelinated axons (small arrows) showed intense IR (**D**). Almost all motoneurons positive for VGSC*α* subunit were also positive for AChE (**D**: VGSC*α* subunit; **E**: AChE; **F**: merged). **G**–**I**: The VGCC*α*2 subunit-IR was co-localized with the VGSC*α* subunit-IR in some motoneurons (**G**: VGCC*α*2 subunit; **H**: VGSC*α* subunit; **I**: merged). The VGSC*α* subunit-IR was observed in the proximal dendrite (small arrows) of a motoneuron indicated as by 3 in **H**. In **G** **a**–**e** indicate motoneurons with different immunolabeling patterns of the VGCC*α*2 and VGSC*α* subunits; **a**, **b**: VGCC*α*2 subunit (+)/VGSC*α* subunit (+), **c**: VGCC*α*2 subunit (+)/VGSC*α* subunit (++), **d**: VGCC*α*2 subunit (++)/VGSC*α* subunit (+), **e**: VGCC*α*2 subunit (++)/VGSC*α* subunit (+++). Bar = 50 μm.

dendrites of some neurons (Fig. 1H). Approximately 46% of the neurons positive for AChE (n = 442) from 30 fluorescence sections (Fig. 1E) were also positive for the VGSCα (Table 2). All of the neurons positive for VGSCα were also AChE positive (Fig. 1D–F). This confirmed that the neurons positive for VGSCα were also motoneurons. The proportions of the 3 types of the motoneurons scored for the VGSCα subunit described above were 15.2%, 18.6% and 11.8%, respectively (Table 2). Approximately 47% of the VGCCα2 subunit-positive motoneurons (n = 310) from 23 sections were also positive for the VGSCα subunit. Almost all of the motoneurons positive for VGSCα subunit were VGCCα2 subunit positive (Fig. 1G–I). The spinal motoneurons were divided into subgroups based on the intensity of the immunostaining for the VGCCα2 and VGSCa subunits (Table 3). The proportion of motoneurons positive for VGCCα2 with strong staining intensity for VGSCα appeared to be lower than those of other populations.

The present study revealed that most (approximately 83%) of motoneurons in the chicken spinal ventral horn contain the VGCCα2 subunit. This finding is similar to that of an *in situ* hybridization study, in which the VGCCα2δ2 and VGCCα2δ3 subunits were highly expressed in rat spinal motoneurons (5). It is suggested that amyotrophic lateral sclerosis (ALS) is mediated by antibodies directed against the VGCCs (8, 16). Pharmacological experiments have showed that sera from patients with ALS contain factors that can modify the spontaneous release of neurotransmitters by altering the calcium currents through the L-type and N-type VGCC, and can induce changes in sensitivity to the L-type VGCC blocker (15). Therefore, the high proportion of the motoneurons expressing VGCCα2 subunits shown in this study may suggest that the α2δ subunit is essential for somatic movements (5) and that deficiency or changes in its expression may be involved in abnormalities of VGCCs that result in the onset of ALS. The localization of the VGCCα2 subunit in the neuronal somata and proximal dendrites is similar to that of the α1 subunits of L-type VGCCs (25). An electron cryomicroscopic study of the L-type VGCCs showed that the extracellular α2 subunit protrudes from the membrane in close proximity to the channel-forming α1 subunit (23). In addition, the VGCCα2δ subunit enhances the activity of the α1 subunit (6). These findings suggest an important role of the α2 subunit in the function of the α1 subunit in some VGCCs. In the present study, the signal intensity of the VGCCα2 subunit varied among the motoneurons, which suggests that different VGCCs associated with different α2δ subunits may play roles in motor control at multiple levels (14). The expression of the VGCCα2 subunit in proximal dendrites may be important, because L-type VGCCs mediate long-lasting increases in intracellular calcium in the cell body in response to excitatory input to the dendrites (1). They also mediate the entry of calcium in response to summed excitatory input to initiate intracellular regulatory events (21). In contrast, the variation in the expres-

Table 1 *Percentages of motoneurons with different intensities of labeling of the VGCCα2 subunit*

VGCCα2	−	+	++
	17.0	64.7	18.3

Motoneurons were divided as follows: those negative for VGCCα2 were scored as −. Those with staining in the cytoplasm were scored as + or ++ based on the intensity of the staining. Approximately 83.0% of the total number of motoneurons (n = 520) in 37 sections of spinal cord were positive for the VGCCα2 subunit.

Table 2 *Percentages of motoneurons with different intensities of labeling of the VGSCα subunit*

VGSCα	−	+	++	+++
	54.4	15.2	18.6	11.8

Motoneurons were divided into those negative for VGSCα (−), those with only nuclear VGSCα staining (+), those with positive nuclear staining and weak or moderate cytoplasmic staining (++), and those with strongly positive nuclear and cytoplasmic staining (+++). Approximately 45.6% of the total motoneurons (n = 442) in 30 sections of spinal cord were positive for the VGSCα subunit.

Table 3 *Percentages of motoneurons with different labeling of the VGCCα2 subunit and VGSCα subunit*

	VGCCα2 (+)	VGCCα2 (++)	Total
VGSCα (+)	14.8	1.6	16.4
VGSCα (++)	13.9	3.9	17.8
VGSCα (+++)	10.0	2.6	12.6
Total	38.7	8.1	–

Approximately 46.8% of the total VGCCα2 subunit-positve motoneurons (n = 310) from 23 sections of the spinal cord were also positive for VGSCα subunit. Almost all the motoneurons positive for VGSCα subunit were also positive for VGCCα2 subunit.

sion of the VGCCα2 subunit in motoneurons may be partially responsible for the difference in calcium channel inactivity kinetics among spinal motoneurons, as described by Carlin *et al.* (3).

In the spinal cord, cell types within a given region display different patterns of VGSC expression, and the VGSC levels vary between motoneurons. For example, type I VGSC is the most frequently expressed type of VGSCs in neuronal somata (21), while type III VGSC is present at very high densities in myelinated axons (13). Similarly, the present study showed a high density of VGSCα subunits in myelinated axons and in the cytoplasm, as well as in the nucleus of some motoneurons. Such a situation of cellular immunolabeling for VGSCs ($Na_v1.1$, $Na_v1.2$ or $Na_v1.6$) has been observed also in Purkinje cells or other neurons in the cerebellum (4, 20). Thus, the heterogeneous cellular localization of the VGSCα subunit subtypes in the motoneurons may represent different excitabilities of the motoneurons; however, the functional significance of VGSCα in the nucleus is unclear. It is known that the depolarizing upstroke of the action potential is associated with the opening of sodium channels and the entry of positively charged sodium ions into the cell. In most neurons, inward sodium currents further activate calcium channels, which are responsible for both the upstroke and the plateau of the action potential (11). Sodium channels in the motoneuronal somata (18) are assumed to be able to initiate local depolarization to amplify the excitatory postsynaptic potentials on their way to the axon initial segment (24). In the present study, the different intensities of labeling of the VGCCα2 and VGSCα subunits in spinal motoneurons allowed the motoneurons to be divided into subgroups. Therefore, it is postulated that the subgroups of the motoneurons may reflect various motor activities.

In conclusion, this study presents the first cellular morphological evidence for the presence of the VGCCα2 and VGSCα subunits in chicken spinal motoneurons. The finding that spinal motoneurons possess a heterogeneous pattern of expression of VGCCα2 and VGSCα subunits may provide the molecular basis for the different properties of calcium and sodium currents and also provide intriguing cues for further experimental analysis of the motoneuronal specializations. Further study is needed on the mechanism of motoneuron diseases such as an ALS.

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REFERENCES

1. Ahlijanian MK, Westenbroek RE and Catterall WA (1990) Subunit structure and localization of dihydropyridine-sensitive calcium channels in mammalian brain, spinal cord, and retina. *Neuron* **4**, 819–832.
2. Atsumi S, Sakamoto H, Yokota S and Fujiwara T (1985) Substance P and 5-hydroxytryptamine immunoreactive presynaptic boutons on presumed alpha-motoneurons in the chicken ventral horn. *Arch Histol Jpn* **48**, 159–172.
3. Carlin KP, Jiang Z and Brownstone RM (2000) Characterization of calcium currents in functionally mature mouse spinal motoneurons. *Eur J Neurosci* **12**, 1624–1634.
4. Chung YH, Joo KM, Kim MJ and Cha CI (2003) Age-related changes in the distribution of $Na_v1.1$ and $Na_v1.2$ in rat cerebellum. *NeuroReport* **14**, 841–845.
5. Cole RL, Lechner SM, Williams ME, Prodanovich P, Bleicher L, Varney MA and Gu G (2005) Differential distribution of voltage-gated calcium channel alpha-2 delta (α2δ) subunit mRNA-containing cells in the rat central nervous system and the dorsal root ganglia. *J Comp Neurol* **491**, 246–269.
6. Davies A, Douglas L, Hendrich J, Wratten J, Tran Van Minh A, Foucault I, Koch D, Pratt WS, Saibil HR and Dolphin AC and (2006) The calcium channel alpha2delta-2 subunit partitions with CaV2.1 into lipid rafts in cerebellum: implications for localization and function. *J Neurosci* **26**, 8748–8757.
7. Davies A, Hendrich J, Tran Van Minh A, Wratten J, Douglas L and Dolphin AC (2007) Functional biology of the alpha 2delta-2 subunits of voltage-gated calcium channels. *Trends Pharmacol Sci* **28**, 220–228.
8. Drachman DB (2000) Dose autoimmunity play a role in

amyotrophic lateral sclerosis? In: *Amyotrophic Lateral Sclerosis* (Brown RH, Meininger V, Swash M, eds.), pp 327–339, Dunits, Lpndon.
9. Kernell D, Bakels R and Copray JC (1999) Discharge properties of motoneurones: how are they matched to the properties and use of their muscle units? *J Physiol Paris* **93**, 87–96.
10. Krzemien DM, Schaller KL, Levinson SR and Caldwell JH (2000) Immunolocalization of sodium channel isoform NaCh6 in the nervous system. *J Comp Neurol* **420**, 70–83.
11. Levitan IB and Kaczmarek LK (2002) The neuron. In: *Cell and Molecular Biology* (Levitan IB, ed), pp 45–161, Oxford University Press, New York.
12. Li YN, Li YC, Kuramoto H, Liu ZR, Kawate T, Atsumi S, Deng YC and Huang YG (2007) Immunohistochemical demonstration of calcium channel α2 subunit in chicken dorsal root ganglion and spinal cord: with special reference to colocalization with calbindin-D28k in the DRG neurons. *Neurosci Res* **59**, 304–308.
13. Lindia JA and Abbadie C (2003) Distribution of the voltage gated sodium channel Na(v)1.3-like immunoreactivity in the adult rat central nervous system. *Brain Res* **960**, 132–141.
14. Manto M-U and Pandolfo M (2002) The cerebellum and its disorders. In: *The Cerebellum and its Disorders* (Manto M-U, Pandolfo M, eds.), Cambridge University Press, Cambridge.
15. Muchnik S, Losavio A and De Lorenzo S (2002) Effect of amyotrophic lateral sclerosis serum on calcium channels related to spontaneous acetylcholine release. *Clin Neurophysiol* **113**, 1066–1071.
16. Pagani MR, Reisin RC and Uchitel OD (2006) Calcium signaling pathways mediating synaptic potentiation triggered by amyotrophic lateral sclerosis IgG in motor nerve terminals. *J Neurosci* **26**, 2661–2672.
17. Rekling JC, Funk GD, Bayliss DA, Dong XW and Feldman JL (2000) Synaptic control of motoneuronal excitability. *Physiol Rev* **80**, 767–852.
18. Safronov BV and Vogel W (1995) Single voltage-activated Na^+ and K^+ channels in the somata of rat motoneurones. *J Physiol* **487**, 91–106.
19. Vacher H, Mohapatra DP and Trimmer JS (2008) Localization and targeting of voltage-gated ion channels in mammalian central neurons. *Physiol Rev* **88**, 1407–1447.
20. Van Wart A and Matthews G (2006) Impaired firing and cell-specific compensation in neurons lacking Nav1.6 sodium channels. *J Neurosci* **26**, 7172–7180.
21. Westenbroek RE, Merrick DK and Catterall WA (1989) Differential subcellular localization of the RI and RII Na^+ channel subtypes in central neurons. *Neuron* **3**, 695–704.
22. Whitaker WR, Faull RL, Waldvogel HJ, Plumpton CJ, Emson PC and Clare JJ (2001) Comparative distribution of voltage-gated sodium channel proteins in human brain. *Brain Res Mol Brain Res* **88**, 37–53.
23. Wolf M, Eberhart A, Glossmann H, Striessnig J and Grigorieff N (2003) Visualization of the domain structure of an L-type Ca^{2+} channel using electron cryo-microscopy. *J Mol Biol* **332**, 171–182.
24. Wolff M, Vogel W and Safronov BV (1998) Uneven distribution of K^+ channels in soma, axon and dendrites of rat spinal neurones: functional role of the soma in generation of action potentials. *J Physiol* **509**, 767–776.
25. Zhang M, Sukiasyan N, Møller M, Bezprozvanny I, Zhang H, Wienecke J and Hultborn H (2006) Localization of L type calcium channel Cav1.3 in cat lumbar spinal cord with emphasis on motoneurons. *Neurosci Lett* **407**, 42–47.