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Contribution of the delayed-rectifier potassium channel Kv2.1 to acute spinal cord injury in rats

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Recent studies have reported that delayed-rectifier Kv channels regulate apoptosis in the nervous system. Herein, we investigated changes in the expression of the delayed-rectifier Kv channels Kv1.2, Kv2.1, and Kv3.1 after acute spinal cord injury (SCI) in rats. We performed RT-PCR analysis and found an increase in the level of Kv2.1 mRNA after SCI but no significant changes in the levels of Kv1.2 and Kv3.1 mRNA. Western blot analysis revealed that Kv2.1 protein levels rapidly decreased and then dramatically increased from 1 day, whereas Kv3.1b protein levels gradually and sharply decreased at 5 days. Kv1.2 protein levels did not change significantly. In addition, Kv2.1 clusters were disrupted in the plasma membranes of motor neurons after SCI. Interestingly, the expressional changes and translocation of Kv2.1 were consistent with the apoptotic changes on day 1. Therefore, these results suggest that Kv2.1 channels probably contribute to neuronal cell responses to SCI. [BMB reports 2010; 43(11): 756-760]

INTRODUCTION

Trauma to the spinal cord causes devastating damage, resulting in widespread cell death. In addition to the primary mechanical insults to the spinal cord, secondary injuries cause neuronal and glial apoptosis, axonal degeneration, demyelination, and gliatic scars over a period of hours to weeks after the initial spinal cord injury (SCI) (1-3). This secondary damage can lead to a permanent loss of sensory and voluntary motor functions (3, 4). Although spinal cord research is focused on preventing cell death and repairing motor function after SCI, the mechanisms of secondary injury after SCI remain poorly understood.

Delayed-rectifier K^+ currents are important in regulating membrane excitability in the central nervous system (CNS). Numerous voltage-gated potassium (Kv) channels with distinct localization and properties are expressed in the CNS, where they govern diverse functions of membrane excitability (5). For instance, Kv1.3 and Kv1.5 are components of the delayed-rectifier K^+ currents in glial cells, whereas Kv1.1, Kv1.2, Kv2.1, and Kv3.1b that are involved in delayed-rectifier K^+ currents are prominently expressed in neurons (5). Recent studies have reported that delayed-rectifier Kv channels play a critical role in neuronal apoptosis and glial cell proliferation (6-11). However, it is still not known which delayed-rectifier Kv channel is involved in the response to acute SCI.

In this study, we investigated the mRNA and protein levels of the primary delayed-rectifier Kv channels after acute SCI. We found that the expression levels of Kv2.1 channels changed after acute SCI and that Kv2.1 clusters in spinal motor neurons were disrupted in injured spinal cords. Therefore, this study provides evidence for the contribution of delayed-rectifier Kv2.1 channels to SCI.

RESULTS

To investigate the role of delayed-rectifier Kv channels after acute SCI, we performed reverse transcriptase-polymerase chain reaction (RT-PCR) to examine the expression levels of 3 of these channels-Kv1.2, Kv2.1, and Kv3.1-at 1, 3, 5, and 7 days after SCI. We used β3-tubulin mRNA as the normalization control (12). The mRNA levels of Kv1.2 and Kv3.1 in rats with SCI were not significantly different from those in sham-injured rats at any of these time points. However, the mRNA levels of Kv2.1 were significantly elevated after SCI (Fig. 1A). The Kv2.1 mRNA level increased 2.6-fold from day 1 to day 7 post SCI ($P < 0.01$; Fig. 1B). These results suggest that the delayed-rectifier Kv2.1 channel may play a more important role in injured spinal cords than the other Kv channels investigated in this study.

Further, to examine SCI-induced changes in delayed-rectifier channels at the protein level, we performed western blot analysis on the spinal cords of the SCI and sham-injury groups at 1, 3, 5, and 7 days post SCI. The protein expression levels of Kv1.2, Kv2.1, and Kv3.1b were investigated. As shown in Fig. 2A, we observed that Kv2.1 protein levels decreased rapidly and then increased dramatically after SCI, whereas Kv3.1b pro-

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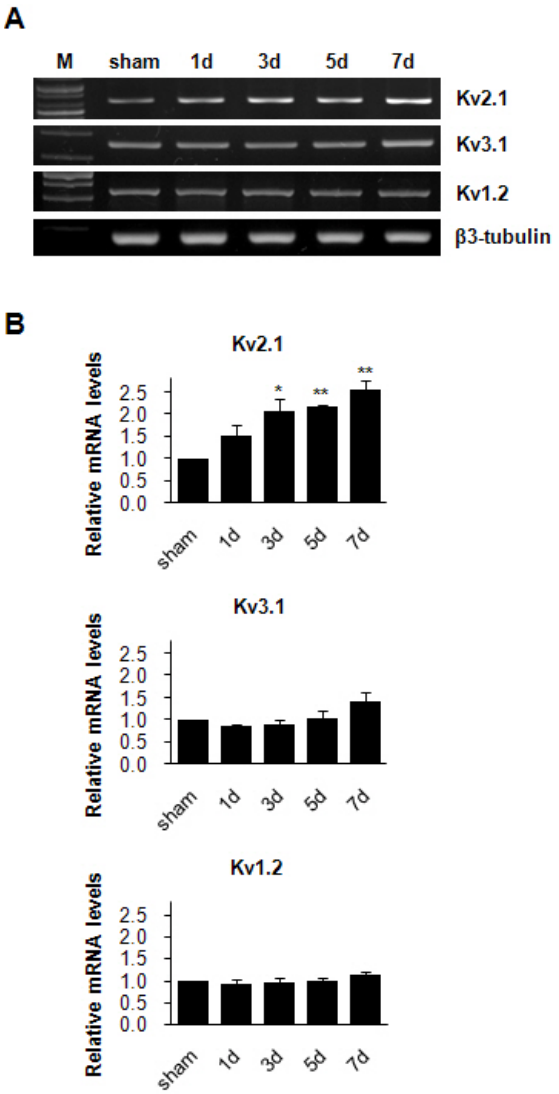

Fig. 1. Relative mRNA levels of delayed-rectifier Kv channels at different time points after acute spinal cord injury (SCI). (A) RT-PCR image showing the relative expression of delayed-rectifier Kv channels at different time points after SCI or sham injury. (B) Quantification of the delayed-rectifier Kv channel expression levels normalized to β3-tubulin mRNA. Expression levels of Kv2.1 were significantly increased at all time points post-SCI compared with those of the sham-injured rats. Data are expressed as the mean (SEM) (*P < 0.05, **P < 0.01, Student's *t* test).

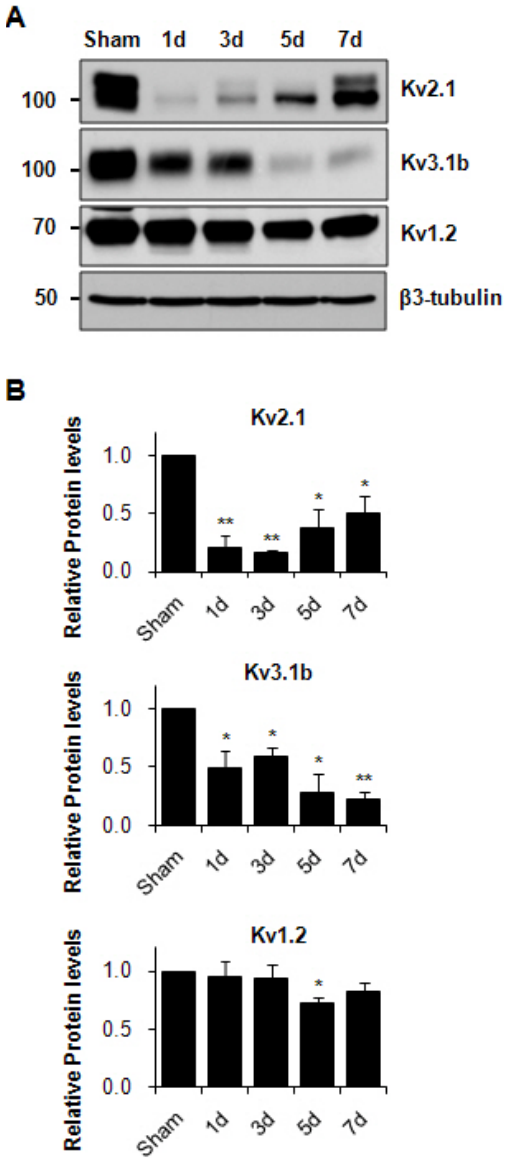

Fig. 2. Relative protein levels of delayed-rectifier Kv channels at different time points post-SCI. (A) Western blot showing the relative expression of delayed-rectifier Kv channels from 1 to 7 days after SCI or sham injury. (B) Quantification of the levels of delayed-rectifier Kv channels normalized to that of β3-tubulin. Levels of Kv2.1 were significantly increased at 1 day post-SCI. Data are presented as the mean (SEM) (*P < 0.05, **P < 0.01; Student's *t* test).

tein levels, unlike Kv2.1, decreased gradually and then sharply on day 5. Quantitative analysis showed that the Kv2.1 protein levels on day 7 were 2.5-fold higher than those on day 1 ($P < 0.05$) (Fig. 2B). Kv1.2 protein levels did not significantly change after SCI.

Kv2.1 channels form large clusters in the somatodendritic membrane of neurons, and pathogenic conditions such as brain ischemia result in the surface dispersion of Kv2.1 clusters in neurons (13). Kv2.1 clusters have also been detected in spinal motor neurons and other neurons (14). To investigate the extent of the dispersion of Kv2.1 clusters in spinal motor neurons after SCI, we performed immunostaining of tissue sections of spinal cord sections. We stained for Kv2.1 in the ventral

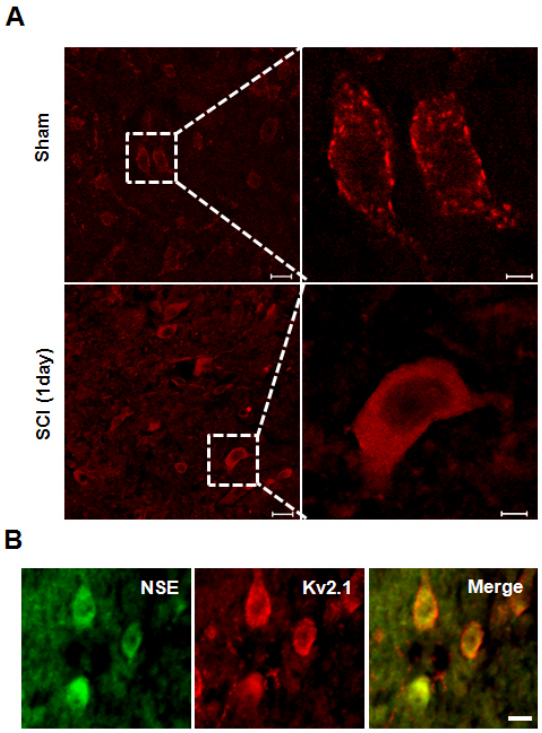

Fig. 3. Translocation of Kv2.1 clusters after SCI. Rat spinal cord sections from sham-injured and SCI rats were stained for Kv2.1 at 1 day post-injury. (A) Clusters of Kv2.1 in sham-injured spinal cord (top). Scale bar, 20 μm. Dispersion of Kv2.1 clusters 1 day after SCI (bottom). Scale bar, 20 μm. Right column images are higher-magnification views of Kv2.1 staining corresponding to the boxed areas. Scale bar, 5 μm. (B) Kv2.1 channel protein is expressed in spinal cord neurons. The sections are stained for Kv2.1 (red) and neuronal specific enolase (NSE) (green).

horn of the spinal cord to detect spinal motor neurons. At day 1 in the control rats, Kv2.1 exhibited somatic membrane clusters in spinal motor neurons, as previously reported (14). However, in the injured spinal cords, the Kv2.1 clusters were completely dispersed over the somatic plasma membranes of the motor neurons (Fig. 3).

We performed terminal transferase dUTP nick end labeling (TUNEL) staining to assess apoptosis in injured spinal cords. In the gray matter, TUNEL-positive cells, reflecting neuronal apoptosis, were detected at 8 h; the number of cells peaked at 1 day after SCI, and declined at 3 days after SCI (Fig. 4). These changes were similar to those reported in a study (2). Interestingly, the maximum number of TUNEL-positive neurons at 1 day after SCI is in accordance with our observations that Kv2.1 protein levels rapidly decrease and then dramatically increase at 1 day after SCI, whereas Kv3.1b protein levels gradually and then sharply decrease at 5 days after SCI. Moreover, the Kv2.1 clusters were completely dispersed at 1 day after SCI (Fig. 2, 3).

DISCUSSION

Traumatic insults to the spinal cord initially lead to mass necrosis of the neurons and glial cells and hours or days later result in neuronal and glial apoptosis (1, 2). Numerous studies have shown that K^+ efflux and a low intracellular K^+ concentration induce apoptosis. Delayed-rectifier Kv channels have been shown to play a key role in this neuronal apoptosis (10, 15-17). Kv-channel blockers have been examined as potential therapeutic agents for SCI (18).

Here, we investigated changes in the expression of several delayed-rectifier Kv channels from 1 to 7 days after acute SCI. Interestingly, we found that only the Kv2.1 mRNA level was

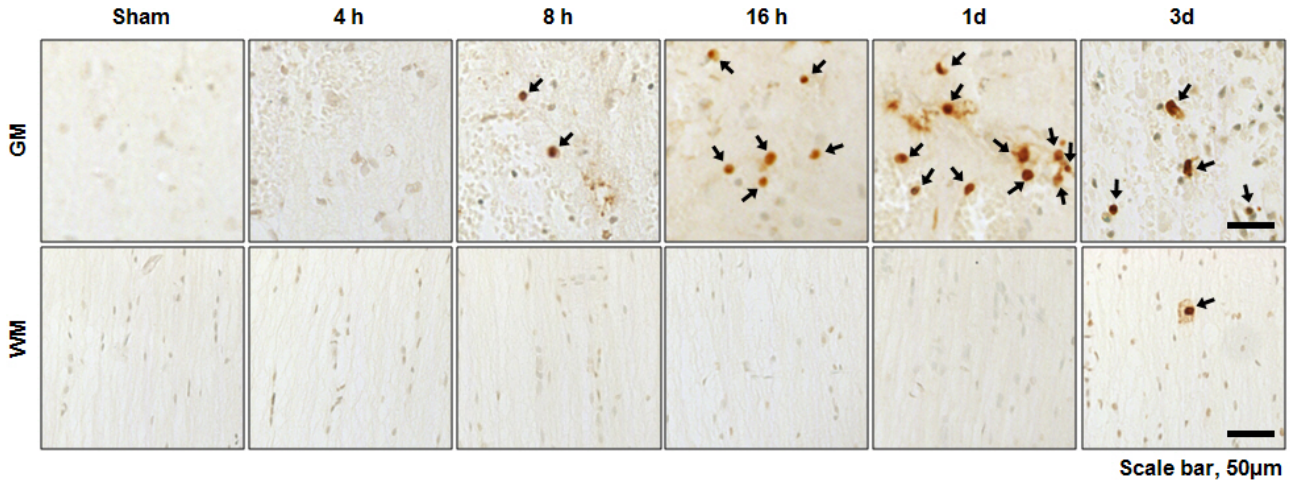

Fig. 4. TUNEL-labeled cells in injured spinal cord at different time points post-injury. TUNEL-positive cells were detected at 8 h post-SCI in the gray matter (GM), whereas a few TUNEL-positive cells (arrow) were detected in presumptive glial cells in the white matter (WM) at 3 days post-SCI. Scale bar, 50 μm.

consistently increased after SCI (Fig. 1). Additionally, the level of the Kv2.1 protein quickly decreased after SCI but then gradually increased (Fig. 2). In addition, Kv2.1 clusters were completely dispersed over the somatic plasma membranes of motor neurons at 1 day post-SCI (Fig. 3). The maximum number of TUNEL-positive neurons at 1 day after SCI is consistent with the expression changes and the translocation of the Kv2.1 protein at 1 day after SCI (Fig. 4).

Neuronal apoptosis is induced between 4 and 24 h after traumatic SCI in rats, whereas glial apoptosis occurs between 4 h and 14 days after SCI (2). Kv2.1 is highly expressed in mammalian central neurons but not in glial cells (5). It has been suggested that Kv2.1 is involved in the regulation of neuronal apoptosis (8, 10, 17, 19). Kv2.1 also contributes to glutamate-induced apoptosis in cultured rat hippocampal neurons (7). It has been reported that increased excitatory synaptic activity in mammalian neurons leads to the disruption of Kv2.1 clusters (20). Hypoxia/ischemia in neurons results in enhanced neuronal delayed-rectifier K^+ currents and in the translocation of Kv2.1 from clusters to a uniform distribution (13). Together, our results suggest that Kv2.1 might be involved in acute SCI-induced neuronal apoptosis and might contribute to the neuronal response to SCI.

MATERIALS AND METHODS

Spinal cord injury

Adult rats (Sprague-Dawley, male, 230-250 g, Sam:TacN [SD] BR; Samtako, Osan, Korea) were anesthetized with 4% chloral hydrate, and a laminectomy was performed at the T9-T10 level, exposing the cord beneath without disrupting the dura. The exposed dorsal surface of the cord was subjected to contusion injury (10 g, 25 mm) using a New York University impactor (21). For the sham-injury controls, the animals underwent a T9-T10 laminectomy without weight-drop injury. All surgical interventions and postoperative animal care were performed in accordance with the Guidelines and Policies for Rodent Survival Surgery provided by the Animal Care Committee of Kyung Hee University.

Total RNA isolation and RT-PCR analysis

Total RNA from the spinal cords was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. First-strand cDNA was prepared from total RNA (1 μg) with oligo$(dT)_{15}$ primers using Moloney murine leukemia virus (MMLV) RNA transcriptase (Invitrogen). After first-strand cDNA synthesis, PCR amplification was conducted using gene-specific primers (Supplemental Table 1). The reaction included an initial denaturation at 94°C for 4 min, followed by 30 cycles of 94°C for 45 s, each primer-specific annealing temperature for 45 s, and extension at 72°C for 1 min. The PCR products were detected in a 1.5% agarose gel. Band densities were measured using ImageJ software (National Institutes of Health, Bethesda, MD).

Membrane preparation and western blot analysis

Spinal cords from different injury time points were homogenized in cold membrane preparation buffer containing 5 mM phosphate (pH 7.4), 320 mM sucrose, 0.1 M NaF, 2 μg/ml aprotinin, 1 μg/ml leupeptin, 2 μg/ml antipain, and 10 μg/ml benzamidine. The homogenate was centrifuged at 700 × g at 4°C for 10 min to remove unbroken cell debris. The supernatant was centrifuged at 100,000 × g at 4°C for 60 min to prepare membrane fractions. For western blot analysis, proteins were separated by 7.5% SDS polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and immunoblotted with monoclonal anti-Kv2.1 (K89/34; Neuromab, Davis, CA), anti-Kv1.2 (K14/16; Neuromab), anti-Kv3.1b (N 16B/8; Neuromab), or β3-tubulin (2G10; Santa Cruz Biotechnology, CA). The membranes were incubated with goat anti-mouse IgG HRP-conjugated secondary antibody (Assay Designs, Ann Arbor, MI) in 4% nonfat milk/TBS. The protein bands of the Kv channels were detected by enhanced chemiluminescence reagent (Pierce, Rockford, IL). The density of the protein bands was measured with ImageJ software.

Immunohistochemistry

Rats were anesthetized with 4% chloral hydrate (500 mg/kg) and briefly perfused with a saline solution followed by 4% paraformaldehyde in phosphate buffer. The spinal cord was cut into 10-μm thick transverse sections on a cryostat (CM1850; Leica, Germany). The sections were blocked with 5% BSA and 0.1% Triton X-100 in PBS for 1 h at room temperature, and then incubated overnight at 4°C with anti-Kv2.1 (K89/34). Sections were then incubated with fluorescein isothiocyanate-conjugated or cyanine 3-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA). Images were made using a laser confocal microscope (LSM 510; Zeiss, Germany).

TUNEL staining

To access apoptotic status, we performed a TUNEL assay using the ApopTag peroxidase in situ apoptosis detection kit (Chemicon, Temecula, CA) according to the manufacturer's instructions. Briefly, the endogenous peroxidase activity in spinal cord sections was quenched using 3% hydrogen peroxide in PBS, and the sections were then washed in PBS. The sections were placed in equilibration buffer and incubated with a working strength of TdT enzyme at 37°C for 1 h. The reaction was terminated with stop/wash buffer and incubated for 30 min with anti-digoxigenin antibody conjugated to peroxidase. Slides were incubated with peroxidase substrate, counterstained with methyl green, and mounted.

Statistical analysis

Data are expressed as the mean (SEM) of 3 independent experiments or as specified for each figure. The significance of the differences among the means was evaluated using the Student's *t* test. A value of $P < 0.05$ was considered statistically significant.

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REFERENCES

1. Keane, R. W., Davis, A. R. and Dietrich, W. D. (2006) Inflammatory and apoptotic signaling after spinal cord injury. *J. Neurotrauma.* **23**, 335-344.
2. Liu, X. Z., Xu, X. M., Hu, R., Du, C., Zhang, S. X., McDonald, J. W., Dong, H. X., Wu, Y. J., Fan, G. S., Jacquin, M. F., Hsu, C. Y. and Choi, D. W. (1997) Neuronal and glial apoptosis after traumatic spinal cord injury. *J. Neurosci.* **17**, 5395-5406.
3. Thuret, S., Moon, L. D. and Gage, F. H. (2006) Therapeutic interventions after spinal cord injury. *Nat. Rev. Neurosci.* **7**, 628-643.
4. Lapointe, N. P., Ung, R. V. and Guertin, P. A. (2007) Plasticity in sublesionally located neurons following spinal cord injury. *J. Neurophysiol.* **98**, 2497-2500.
5. Vacher, H., Mohapatra, D. P. and Trimmer, J. S. (2008) Localization and targeting of voltage-dependent ion channels in mammalian central neurons. *Physiol. Rev.* **88**, 1407-1447.
6. Yao, H., Zhou, K., Yan, D., Li, M. and Wang, Y. (2009) The Kv2.1 channels mediate neuronal apoptosis induced by excitotoxicity. *J. Neurochem.* **108**, 909-919.
7. Shen, Q. J., Zhao, Y. M., Cao, D. X. and Wang, X. L. (2009) Contribution of Kv channel subunits to glutamate-induced apoptosis in cultured rat hippocampal neurons. *J. Neurosci. Res.* **87**, 3153-3160.
8. Redman, P. T., He, K., Hartnett, K. A., Jefferson, B. S., Hu, L., Rosenberg, P. A., Levitan, E. S. and Aizenman, E. (2007) Apoptotic surge of potassium currents is mediated by p38 phosphorylation of Kv2.1. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 3568-3573.
9. Jiao, S., Liu, Z., Ren, W. H., Ding, Y., Zhang, Y. Q., Zhang, Z. H. and Mei, Y. A. (2007) cAMP/protein kinase A signalling pathway protects against neuronal apoptosis and is associated with modulation of Kv2.1 in cerebellar granule cells. *J. Neurochem.* **100**, 979-991.
10. Pal, S. K., Takimoto, K., Aizenman, E. and Levitan, E. S. (2006) Apoptotic surface delivery of K+ channels. *Cell Death Differ.* **13**, 661-667.
11. Preussat, K., Beetz, C., Schrey, M., Kraft, R., Wolfl, S., Kalff, R. and Patt, S. (2003) Expression of voltage-gated potassium channels Kv1.3 and Kv1.5 in human gliomas. *Neurosci. Lett.* **346**, 33-36.
12. Liu, N. K. and Xu, X. M. (2006) beta-tubulin is a more suitable internal control than beta-actin in western blot analysis of spinal cord tissues after traumatic injury. *J. Neurotrauma.* **23**, 1794-1801.
13. Misonou, H., Mohapatra, D. P., Menegola, M. and Trimmer, J. S. (2005) Calcium- and metabolic state-dependent modulation of the voltage-dependent Kv2.1 channel regulates neuronal excitability in response to ischemia. *J. Neurosci.* **25**, 11184-11193.
14. Muennich, E. A. and Fyffe, R. E. (2004) Focal aggregation of voltage-gated, Kv2.1 subunit-containing, potassium channels at synaptic sites in rat spinal motoneurones. *J. Physiol.* **554**, 673-685.
15. Yu, S. P. (2003) Regulation and critical role of potassium homeostasis in apoptosis. *Prog. Neurobiol.* **70**, 363-386.
16. Yu, S. P., Yeh, C. H., Sensi, S. L., Gwag, B. J., Canzoniero, L. M., Farhangrazi, Z. S., Ying, H. S., Tian, M., Dugan, L. L. and Choi, D. W. (1997) Mediation of neuronal apoptosis by enhancement of outward potassium current. *Science* **278**, 114-117.
17. Pal, S., Hartnett, K. A., Nerbonne, J. M., Levitan, E. S. and Aizenman, E. (2003) Mediation of neuronal apoptosis by Kv2.1-encoded potassium channels. *J. Neurosci.* **23**, 4798-4802.
18. McBride, J. M., Smith, D. T., Byrn, S. R., Borgens, R. B. and Shi, R. (2007) 4-Aminopyridine derivatives enhance impulse conduction in guinea-pig spinal cord following traumatic injury. *Neuroscience* **148**, 44-52.
19. Burg, E. D., Remillard, C. V. and Yuan, J. X. (2006) K+ channels in apoptosis. *J. Membr. Biol.* **209**, 3-20.
20. Misonou, H., Mohapatra, D. P., Park, E. W., Leung, V., Zhen, D., Misonou, K., Anderson, A. E. and Trimmer, J. S. (2004) Regulation of ion channel localization and phosphorylation by neuronal activity. *Nat. Neurosci.* **7**, 711-718.
21. Gruner, J. A. (1992) A monitored contusion model of spinal cord injury in the rat. *J. Neurotrauma.* **9**, 123-126.