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# CHRONIC LENTIVIRAL EXPRESSION OF INWARDLY RECTIFYING $K^+$ CHANNELS (Kir2.1) REDUCES NEURONAL ACTIVITY AND DOWNREGULATES VOLTAGE-GATED POTASSIUM CURRENTS IN HIPPOCAMPUS

**M. OKADA* AND H. MATSUDA**

*Department of Physiology, Kansai Medical University, Moriguchi, Osaka 570-8506, Japan*

**Abstract—Strongly inwardly rectifying $K^+$ (Kir2) channels are endogenously expressed in rat brains and have recently been used as a tool to reduce the neuronal activity. But little is known about the role of Kir2 channels and the chronic effect of the reduced activity on the intrinsic excitability of neurons. Here we constructed a lentiviral vector that coexpressed Kir2.1 and GFP (LvKir2.1) and infected the vector to the hippocampal slice cultures. The LvKir2.1-infected CA1 neurons showed clear inwardly rectifying $K^+$ currents for more than 15 days. The resting membrane potential was more negative by approximately 10 mV than those uninfected or infected with the lentiviral vector expressing GFP alone. The infection of LvKir2.1 reduced the voltage change in response to current injections and the amplitude of mEPSPs with a shunting effect. The LvKir2.1 infection significantly reduced the firings evoked by depolarizing currents in the CA1 neurons. The reduction of the firing was attributed to the hyperpolarized potential rather than to the shunting effect. These reductions were limited to modest current injections, suggesting that the *overexpressed* Kir2.1 plays the role of a noise-filter. Moreover, the chronic overexpression of Kir2.1 downregulated the expression of the delayed rectifier potassium current in a homeostatic manner, indicating a usefulness of this viral vector to study the activity-dependent neuronal development.** 

**Key words: Kir channel, lentiviruses, firing, shunting, $I_K$, homeostatic regulation.**

Each neuron has its intrinsic firing property, and neuronal intrinsic excitability is controlled by the delicate balance between inward and outward currents. Potassium channels play a pivotal role in regulation of neural excitability. Strongly inwardly rectifying $K^+$ channels (Kir2) are homo- or hetero-tetramers (Tinker et al., 1996). Inward rectification, the ability of an ion channel to allow smaller efflux than influx of ions, is due to a voltage-dependent block by intracellular $Mg^{2+}$ ions (Matsuda et al., 1987) and polyamines (Ficker et al., 1994; Lopatin et al., 1994). mRNAs for the Kir2 channel subfamily are expressed throughout the brain (Horio et al., 1996; Karschin et al., 1996). The Kir2 channel proteins have a PDZ domain in their C-terminus, which binds PSD-95 at the postsynaptic density (Nehring et al., 2000; Leonoudakis et al., 2004). These data suggest that Kir2 channels regulate intrinsic excitability and synaptic transmission of the central neurons.

Some aspects of neural development are dependent on neuronal activity (Katz and Shatz, 1996). However little is known about the neuronal activity-dependent regulation of the expression of the potassium channels in developing central neurons. A $Na^+$ channel blockade with TTX inhibited the developmental upregulation of the expression of the transient potassium current ($I_A$) channel (Grosse et al., 2000; Casavant et al., 2004). On the other hand, recent studies have shown the significance of inter-neuron competition rather than the overall level of activity in synapse formation. Indeed, the reduction of activity, only in the Kir2.1 expressing plasmid transfected neurons, inhibited the synapse formation and axon arborization, but the overall blockade with TTX did not (Burrone et al., 2002; Hua et al., 2005). Therefore, a novel method that reduces neuronal activity in chronic and selective (cell by cell) ways is needed to elucidate the role of neuronal activity in the development of neuronal excitability.

Lentiviral vectors derived from human immunodeficiency virus 1 have several useful features for experimental neuroscience. They can infect both dividing and non-dividing cells and be integrated into the host genome to enable long lasting expression. Pseudotyping into vesicular stomatitis virus (VSV) G glycoprotein envelope improved the infectious ability and stability of the virus vector particles. Generation of self-inactivating vectors achieved biosafety of the vectors, and the addition of the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) improved expression efficacy. Therefore, these vectors are considered as the most promising for chronic expression experiments of the gene of interest (for review, Follenzi and Naldini, 2002).

In this report, we attempted to: 1) construct a lentiviral vector that stably expresses wild-type Kir2.1 channels, 2) clarify how the overexpressed Kir2.1 reduces the excitability of the CA1 neurons using the whole-cell patch-clamp technique, and 3) examine the effect of the neuronal activity reduction on the expression of other potassium channels. We successfully expressed the channel with the vector and showed that the channel reduces neuronal activities in a limited voltage range because of the strong

*Corresponding author. Tel: +81-6-6993-9422; fax: +81-6-6993-5319.
E-mail address: mokada-tky@umin.ac.jp (M. Okada).

*Abbreviations:* aCSF, artificial CSF; $I_A$, transient potassium current; $I_K$, delayed rectifier potassium current; IRES, internal ribosomal entry site; I–V, current–voltage; Kir, inwardly rectifying $K^+$ channel; TTX, tetrodotoxin; VSV, vesicular stomatitis virus; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element.

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inward rectification. Furthermore, the overexpression downregulated other potassium currents, suggesting a homeostatic regulation of voltage-gated potassium channels.

## EXPERIMENTAL PROCEDURES

### Construction of lentivirus vectors

The self-inactivating expression lentiviral vector plasmid (CS-CDF-CG-PRE) was generously donated by Dr. Hiroyuki Miyoshi (Riken Tsukuba Institute, Ibaraki, Japan), and we replaced the CMV promoter with that of chicken $\beta$-actin. Packaging and VSV-G plasmids were also donated by Dr. Miyoshi. The cDNA of hrGFP (Stratagene) was subcloned between $\beta$-actin promoter and WPRE (referred to as LvGFP). Internal ribosomal entry site (IRES) and cDNA of mouse Kir2.1 (Kubo et al., 1993) was inserted in the downstream of hrGFP (referred to as LvKir2.1).

We prepared lentiviral vectors with calcium-phosphate transfection of the plasmid to HEK293 cells, and 48 h after transfection filtered the cell supernatant with a 0.45 $\mu$m filter (Miyoshi et al., 1998; Lois, 2006). After concentrating virus particles with a two-round centrifugation (50,000$\times g$ 2 h and 23,000$\times g$ 5 h at 4 °C), the pellets were suspended in a small volume of PBS. We then determined the titers of lentiviral vectors with 293 cells by counting GFP-positive cells.

### Infection of recombinant lentivirus vectors to hippocampal slice cultures

We prepared hippocampal slices (350 $\mu$m thickness) from postnatal day 7 rats and kept them in culture in a $CO_2$ incubator at 33 °C as described previously (Stoppini et al., 1991; Okada and Corfas, 2004). We injected the virus solution 3 or 5 days after the start of cultivation. The virus solution (0.1–0.2 $\mu$l) containing the lentivirus vector expected to express GFP alone (LvGFP; $3\times10^8-5\times10^8$ pfu/ml) or LvKir2.1 ($5\times10^7-3\times10^8$ pfu/ml) was injected into the extracellular space of the pyramidal cell layer of the slice culture using Femtojet (Eppendorf, Hamburg, Germany) as described by Okada et al. (2000). All animal experiments were performed in accordance with the guidelines of the Physiological Society of Japan, and approved by the committee of animal experiments of Kansai Medical University. All experiments conformed to international guidelines on the ethical use of animals. All efforts were made to minimize the number of animals used and their suffering.

### Electrophysiology

We recorded whole-cell currents from the HEK293 cells grown on the coverslips and the neurons in the CA1 pyramidal cell layer. After transferring those cells onto a superfusing chamber on a stage of upright microscope (Olympus BX51WI; Tokyo, Japan), we viewed under Nomarski optics with a 60× water immersion objective. The superfusing artificial CSF (aCSF) contained (in mM): NaCl 131, KCl 2.75, $NaH_2PO_4$ 1.1, $NaHCO_3$ 26, glucose 12, $CaCl_2$ 2.5, and $MgCl_2$ 1.3 and was equilibrated with 95% $O_2$–5% $CO_2$ (pH 7.4). We recorded whole-cell currents from GFP-positive cells using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA) at 25.5±1 °C. Tetrodotoxin (TTX) (1 $\mu$M, Nakarai tesque, Kyoto, Japan) was added to inhibit $Na^+$ channel except the firing experiments. Patch pipettes pulled from borosilicate glass (Narishige, Tokyo, Japan) were filled with an internal solution containing (in mM): K-aspartate 66, KCl 71.5, $KH_2PO_4$ 1, EGTA 5, Hepes 5, and $K_2ATP$ 3 (pH 7.4 adjusted with KOH) for the voltage-clamp experiments and K-methanesulfonate 115, KCl 10, $KH_2PO_4$ 1, EGTA 5, Hepes 5, $MgCl_2$ 5, and $K_2ATP$ 3 (pH 7.4) for the current-clamp experiments. The electrode resistance was 3–5 MΩ. When the access resistance (typically 10–20 MΩ) exceeded 25 MΩ, or changed more than 20% of initial value, data were discarded. In addition, when the cellular whole-cell membrane resistance at −70 mV was smaller than 100 MΩ, or the resting membrane potentials of the CA1 neurons were more positive than −40 mV, data were discarded. For recording of spontaneous miniature EPSPs, we added bicuculline (10 $\mu$M, Tocris, Avonmouth, UK) and D-AP5 (25 $\mu$M, Tocris) to the aCSF to block the $GABA_A$ and NMDA receptor-mediated currents, respectively, in addition to TTX. Membrane potentials were corrected for the liquid junction potential according to the calculation of pClamp (Axon). Records were digitized at 10 kHz, and low-pass filtered at 2 kHz. Step pulses of 400 ms from −150 to −20 mV in 10 mV increments were applied from a holding potential of −70 mV. Whole-cell conductances were calculated as the slope of the current-voltage (I–V) relation from −150 mV to −110 mV. Recordings were done 8–15 days after the virus injection except for the time-course experiments.

For the measurement of extracellular $K^+$-sensitive conductance, KCl in the sASCF was replaced by NaCl. The whole cell currents were recorded in the presence and absence of $K^+$ ions, and the currents in the absence of external $K^+$ were subtracted from those in the presence of $K^+$. The conductance was calculated within the linear portion of I–V relation after subtraction (−150 mV to −110 mV).

All values are expressed as means and SEMs. Statistical significances were evaluated by Student's unpaired *t*-test.

## RESULTS

### Lentiviral construction

We constructed two lentiviral vectors: LvKir2.1 that coexpressed GFP and Kir2.1, and LvGFP that expressed only GFP as a control. Chick $\beta$-actin promoter and IRES were used for the neuron-preferential coexpression of GFP and Kir2.1 channels, and WPRE increased the efficacy of the expression.

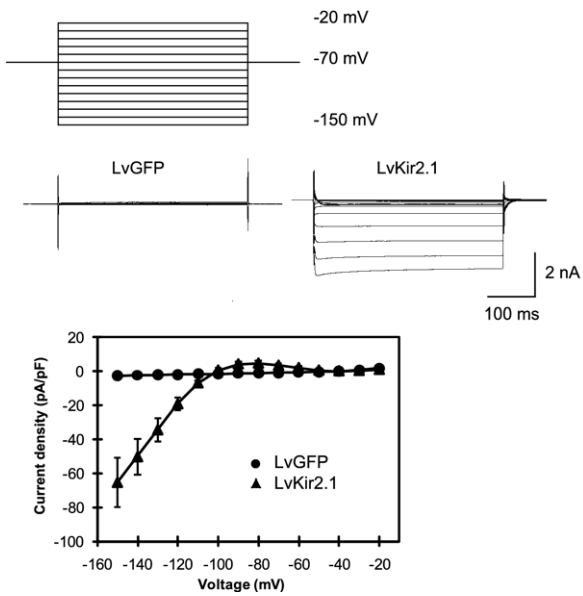


**Fig. 1.** Expression of Kir2.1 in HEK293 cells 48 h after infection of LvKir2.1. Whole-cell voltage-clamp recordings were made from the relatively isolated LvGFP- and LvKir2.1-infected cells ($n$=4) because HEK293 cells express the gap junctions endogenously. Step pulses between −150 and −110 mV elicited large inward currents in LvKir2.1-infected cells, but not in LvGFP-infected cells. I–V relation of LvKir2.1-infected cells showed the inward rectification.