Heterologous Expression of a Glial Kir Channel (KCNJ10) in a Neuroblastoma Spinal Cord (NSC-34) Cell Line

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Summary
Heterologous expression of Kir channels offers a tool to modulate excitability of neurons which provide insight into Kir channel functions in general. Inwardly-rectifying K⁺ channels (Kir channels) are potential candidate proteins to hyperpolarize neuronal cell membranes. However, heterologous expression of inwardly-rectifying K⁺ channels has previously proven to be difficult. This was mainly due to a high toxicity of the respective Kir channel expression. We investigated the putative role of a predominantly glial-expressed, weakly rectifying Kir channel (Kir4.1 channel subunit; KCNJ10) in modulating electrophysiological properties of a motoneuron-like cell culture (NSC-34). Transfection procedures using an EGFP-tagged Kir4.1 protein in this study proved to have no toxic effects on NSC-34 cells. Using whole cell-voltage clamp, a substantial increase of inward rectifying K⁺ currents as well as hyperpolarization of the cell membrane was observed in Kir4.1-transfected cells. Na⁺ inward currents, observed in NSC-34 controls, were absent in Kir4.1/EGFP motoneuronal cells. The Kir4.1-transfection did not influence the NaV1.6 sodium channel expression. This study demonstrates the general feasibility of a heterologous expression of a weakly inward-rectifying K⁺ channel (Kir4.1 subunit) and shows that in vitro overexpression of Kir4.1 shifts electrophysiological properties of neuronal cells to a more glial-like phenotype and may therefore be a candidate tool to dampen excitability of neurons in experimental paradigms.

Key words
Kir channels • Motoneuron • Patch clamp • Neuronal excitability

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Introduction
In general, inwardly rectifying potassium (Kir) channels are transmembrane proteins with the unique property to generate a large potassium conductance at potentials negative to E[K⁺] and vice versa to reduce the conductance upon depolarizing membrane potentials (Nichols et al. 1997). Seven subfamilies (Kir1.x to Kir7.x) have been described with distributions throughout the body, e.g., in skeletal and cardiac muscle (Hibino et al. 2010, Kurachi et al. 1985), CNS (Neusch et al. 2003, Seifert et al. 2009) stomach, kidney (Bockenhauer et al. 2009, Scholl et al. 2009), eye (Kofuji et al. 2000, Reichenbach et al. 2007) and ear (Marcus et al. 2002, Hibino et al. 2010, Rozengurt et al. 2003). Among these channels, Kir 4.1 subunit can function as a heteromer (with the Kir5.1 subunit) or as a homomer (Hibino et al. 2004, Ishii et al. 2003, Tanemoto et al. 2000). The two main functions (Reimann et al. 1999) of these Ba²⁺ blockable channels are the regulation of the resting membrane potential (RMP) (Neusch et al. 2006, Neusch et al. 2001) and the contribution to potassium homoeostasis (Doupink et al. 1995, Haj-Yasein 2011).

In the CNS, Kir4.1 channels are predominantly expressed in glial cells (Li et al. 2001) and contribute substantially to the RMP (Kofuji et al. 2000, Neusch et al. 2001, Neusch et al. 2006). Furthermore, a role in
extracellular K⁺ regulation and cell swelling has been demonstrated in the retina (Newman et al. 1984, 1986, Reichenbach et al. 2007) and in the spinal cord (Dibaj et al. 2007). Neuronal expression has been proposed by some authors in a subset of neurons in the brainstem and in the cortex (Bredt et al. 1995, Li et al. 2001), however the bulk of expression appears in glial cells. In the spinal cord, high expression levels of Kir4.1 have been observed in grey matter surrounding motoneurons and to a lesser extend in white matter structures (Neusch et al. 2001), indicating a role in controlling motoneuronal excitability.

In recent years, several approaches have been made to express Kir and chloride channels in mammalian cell lines, especially in neuronal cell lines (Nadeau et al. 2000, Slimko et al. 2002, Ehrengruber et al. 1997). The goal of these studies was to unravel specific functions of these channels by expressing them in systems lacking respective channel expression. The other reason was to develop a tool that dampens neuronal activity, e.g., in order to develop experimental models for diseases with hyperexcitable neurons such as epilepsy (Loscher et al. 1998) or to reduce the cellular damage after cerebral ischemia. Among the Kir channel family, the Kir4.1 channel subunit has not been tested before in neuronal cell lines.

Here, we report the successful heterologous expression of the Kir4.1 subunit in an embryonic motoneuron-like cell line (NSC-34 cells) that expresses no functional Kir channels. Transient transfection of Kir4.1 channels leads to hyperpolarization of NSC-34 cells, increased K⁺ conductance and reduction of Na⁺ influx upon depolarization, however, without significantly affecting cell survival.

Our study shows that weakly inward-rectifying Kir channels can be successfully introduced in cell lines with a neuronal phenotype with sufficient efficiency. On an electrophysiological basis, Kir4.1 channels modulate NSC-34 cells to a more glial phenotype and offer a tool to study silencing in neuronal cells.

Materials and Methods

Gene construction

N-terminal fusion constructs of Kir4.1 channel subunit with enhanced green fluorescent protein (EGFP) were generated by insertion of the respective cDNA in-frame into the EGFP-C1 eukaryotic expression plasmid (Clontech) using the EcoRI and BamHI restriction sites (Hartel et al. 2007).

Cell culture and transfection

NSC-34, a mouse embryonic spinal cord-neuroblastoma cell line with a motoneuronal phenotype was used as an in vitro model system to investigate the effect of heterologous transfection with a Kir4.1/EGFP construct (Hartel et al. 2007). Cells were kindly provided by Dr. J. Weishaupt, Dept. Neurology, University of Ulm, Germany. Cells were cultured at 37 °C under 5 % CO₂/humidified air in Dulbecco’s modified Eagle’s medium (DMEM) (PAA, Coelbe, Germany) supplemented with 10 % heat-inactivated fetal calf serum (FCS) (PAA), 100 µg/ml streptomycin sulphate and 100 U/ml penicillin 8 (Olsen et al. 2006, Reichenbach et al. 2007). The medium was replaced every second day and cells were passaged once a week. To differentiate cells, the medium was replaced by DMEM/Ham’s F12 supplemented with 1 % FCS, 100 µg/ml streptomycin sulphate, 100 U/ml penicillin and 1 % non-essential amino acids (NEM). Eighty per cent confluent differentiated NSC-34 cells were cultured in dishes on glass coverslips (15 mm diameter) at a density of 10 000 cells/cm². Cells were transiently transfected with the Kir4.1/EGFP fusion protein and for control purposes with EGFP alone using Lipofectamine™ 2000 (Invitrogen, Germany) according to the manufacturer’s instructions. Two earlier described NSC-34 populations were found in cell culture: one population that is characterized by a round morphology and the inability to generate action potentials. These cells are depolarized and have a RMP not more negative than −20 mV, retain inward sodium channel activation and are considered to be more ‘immature’ (Ryan et al. 2009, Cashman et al. 1992, Durham et al. 1993). The other population is able to generate action potentials and has a more negative RMP. Transfection efficacy was 30 % for the more immature cell type. Confocal microscopy and patch clamp experiments were performed 7 days after transfection.

Immunocytochemistry

For immunocytochemistry, transiently transfected NSC-34 cells were washed with PBS once for 5 min at room temperature and fixed in 4 % PFA/PBS. After blocking in 0.1 % Triton X-100 and 10 % normal goat serum (NGS) in PBS, samples were washed and exposed to the primary Kir4.1 antibody (1:200; Alomone Laboratories, Jerusalem, Israel) or the primary NaV1.6 (Scn8a, Type VI Sodium Channel, NaCh6, PN4, CerIII) antibody (1:400; Alomone Laboratories, Jerusalem, Israel) in 1 % NGS at 4 °C overnight. Samples were
incubated with a fluorescent Cy-3 (1:500) conjugated secondary antibody (Jackson Immunoresearch, West Grove, PA, USA) for 1 h. Negative controls were performed by omission of respective primary antibody in every staining. Neuronal death was assessed quantitatively by staining the nuclei with 4',6-diamidino-2-phenylindole (DAPI). Cells were stained with DAPI using a ready-to-use medium containing DAPI (Vectashield; Vector). The nuclei of dying cells were highly condensed compared to those of healthy cells. Cell death was scored by counting in three randomly chosen subfields of three coverslips with a 40x objective for each sample. Results are expressed as mean ± SEM of at least three different experiments. Analysis was verified by a second observer who was unaware of the culture conditions. Confocal images for the Kir4.1 staining were obtained on a Zeiss Axiovert 200/LSM 510 Meta confocal laser-scanning microscope (Zeiss, Jena, Germany) equipped with argon (488 nm) and HeNe (543 nm) laser.

For quantitative assessment of NaV1.6 immunolabeling, photomicrographs were taken by a confocal laser-scanning microscope (Leica TSC SP2; Leica Microsystems Heidelberg GmbH) equipped with an Acousto Optical Beam Splitter (AOBS) and a 40× NA 1.25 oil objective. For the detection of EGFP fluorescence, slices were excited using the 488-nm Argon laser line. For Cy3 fluorescence, excitation was performed with the 543-nm HeNe laser line. Whole cell currents were recorded using Heka amplifier EPC9 (Heka Elektronik GmbH, Lambrecht, Germany) and stored on a computer using Pulse software (Heka Elektronik GmbH, Lambrecht, Germany). Resting membrane potentials were measured at zero holding current directly after gaining whole cell access. Series resistance ranged from 5 to 25 MΩ. The percentage of series resistance compensation was between 70-80%. Potential values were corrected for liquid junction potentials using the auto-function of the used Heka Software. Hyper- or depolarizing steps (10 mV increments) were applied from a holding potential of −60 mV and measured over a voltage range from −150 mV to 50 mV with a pulse duration of 20 ms. Capacitive artefacts were minimized prior to recording. Sodium currents were isolated using an offline leak subtraction protocol implemented in Igor pro. The current measured during negative 10 mV subtraction pulses was multiplied and digitally subtracted from the test pulse current.

Raw data were analyzed with Igor Pro 3.1 (WaveMetrics, Inc., USA). Unpaired t-test and ANOVA with Tukey’s post-hoc test were used to determine the significance of changes when comparing values obtained from non-transfected controls, EGFP transfected controls and Kir4.1 transfected cells. For statistical analysis GraphPad Prism V4.0 (GraphPad Software, San Diego, CA, USA) was used with * P<0.05, ** P<0.01, and *** P<0.001 as significant values. Results were expressed as mean ± SEM.

Results

Transient overexpression of the Kir4.1/EGFP fusion protein in NSC-34 cells

First, we examined if the transient overexpression of the Kir4.1/EGFP fusion protein in a neuroblastoma spinal cord cell line results in apoptosis as it was observed in hippocampal neurons for the inward rectifier ROMK1 (Kir1.1, KCNJ1) (Nadeau et al. 2000). We identified NSC-34 cells transfected with EGFP only
or EGFP-tagged Kir4.1 by their fluorescence after 48-72 h. Transfected cells showed an increase in fluorescence intensity over the following 3 days. Transfected cells could be maintained for at least one week without changes in cellular morphology or electrophysiological properties. DAPI staining of nuclei revealed no significant differences between Kir4.1/EGFP cells and controls (Fig. 1). The number of apoptotic nuclei in Kir4.1/EGFP transfected cells (12.06±1.3 %) was comparable to EGFP transfected cells (11.77±1.6 %) and non-transfected cells (13.91±2.2 %, mean ± SEM).

A) B) C)

Fig. 1. Transient Kir4.1 overexpression in motoneurons is not cell toxic. Transfected and non-transfected NSC-34 cells were stained with DAPI to analyze nuclei morphology. (A) Control NSC-34 cells were analyzed with bright field microscopy. (B, C) Exemplary images of EGFP – (B) and Kir4.1/EGFP – (C) transfected NSC-34 cells stained with DAPI. (D) Nuclei were counted by using general criteria for apoptosis (fragmented and highly condensed nuclei). No difference between control and Kir4.1-transfected cells was observed (p>0.5), mean ± SEM. Scale bar: 20 µm.

In order to verify Kir4.1 expression in transfected Kir4.1/EGFP cells, counterstaining with a Kir4.1 polyclonal antibody was performed. This staining showed an overlap of the transfected fusion protein (green) with Kir4.1 (red, Fig. 2). We observed a punctuate expression pattern for Kir4.1. EGFP- or non-transfected cells displayed no Kir4.1 immunoreactivity (data not shown). To verify a functional expression of the construct, we performed whole-cell voltage-clamp recordings from non-transfected, EGFP-controls and Kir4.1/EGFP transfected cells.

Fig. 2. Confocal images of Kir4.1/EGFP transfected and Kir4.1 counterstained cells. Kir4.1/EGFP transfection of NSC-34 cells led to a transient overexpression of Kir4.1 channels (left). Cytoplasmic and membrane expression was confirmed by immunolabeling with a Kir4.1 antibody (middle) showing a marked overlap with the EGFP fluorescence signal of the Kir4.1/EGFP fusion protein (right). Scale bar: 20 µm.

Patch-clamp recording of Kir4.1/EGFP transfected NSC-34 cells

NSC-34 cells were voltage clamped at a holding potential of ~60 mV and the current-voltage (I-V) relationship was measured over a voltage range from −150 mV to +50 mV in 10 mV increments (Fig. 3). Kir currents were isolated by subtracting currents taken in 50 mM K+ from corresponding currents that were obtained in high K+ + 500 µM BaCl2 (Fig. 3D). We compared Kir currents of WT, EGFP and Kir4.1/EGFP cells by quantifying the inward current evoked by a voltage step from −60 mV (holding potential) to −150 mV (L−150). In normal saline solution inward currents were nearly absent in WT and EGFP controls while in Kir4.1-transfected cells prominent inward currents were measured (Table 1). Furthermore, K+ current in Kir4.1/EGFP transfected cells increased upon application of 50 mM extracellular K+ from −2.4±0.5 to −5.72±0.9 nA (n=8) compared to WT control (from −0.12±0.09 to −0.12±0.1 nA, n=7) and EGFP transfected cells (from −0.18±0.07 to −0.15±0.09 nA, n=7). The results were statistically significant (p<0.001) for WT vs. Kir4.1 and EGFP vs. Kir4.1, but not for EGFP vs. WT (p>0.05). The BaCl2 sensitive current in Kir4.1 transfected cells was −4.33±0.8 nA, but virtually no BaCl2 blockable current was measured in non-transfected cells (−0.03±0.02 nA) and EGFP controls (−0.02±0.02). BaCl2 blockable current was significantly different between WT and Kir4.1 (p<0.001) and between EGFP and Kir4.1-transfected cells (p<0.001).
Fig. 3. Functional expression of Kir4.1 channels in NSC-34 cells. NSC-34 cells were voltage clamped at −60 mV and jumped to test potentials between −150 mV and 50 mV in 10 mV increments. Extracellular K⁺ was increased from 3 mM K⁺ to 50 mM K⁺ followed by additional application of 500 µM BaCl₂. (A) Exemplary whole-cell voltage clamp recordings of EGFP controls (left) and Kir4.1 transfected cells (right) in saline recording solution, 50 mM K⁺ and in 50 mM K⁺ + 500 µM BaCl₂. EGFP controls show weak K⁺ currents, outward directed currents and inward sodium currents. Transfection with Kir4.1 led to an increase in K⁺ currents under negative potentials as well as to an increase in outward K⁺ current. (B, C) illustrates the corresponding I-V curves. Note that in (B) EGFP transfected cells have Na⁺ inward currents which are not detected in Kir4.1-transfected cells (C). Note also in (C) that Kir4.1/EGFP cells show an increase of Kir current in 50 mM K⁺ solution which was completely blocked by BaCl₂. (D) The averaged I-V curves were determined from EGFP and Kir4.1 transfected cells by subtracting currents in 50 mM K⁺ from corresponding currents taken during BaCl₂ blockade (mean ± SEM; EGFP: n=6; Kir4.1: n=8).
Table 1. Cell culture recordings of NSC-34 cells. Values are mean ± SEM. Number in parentheses indicates the number of whole cell voltage-clamp experiments.

|                           | Control        | EGFP           | Kir4.1          |
|---------------------------|----------------|----------------|-----------------|
| $I_{K} (-150)$ nA         | $-0.12 \pm 0.09$ (7) | $-0.18 \pm 0.07$ (7) | $-2.4 \pm 0.50$ (8) ** |
| Change of $I_{K} (-150)$ in 50 mM $K^+$, nA | $-0.12 \pm 0.10$ (7) | $-0.15 \pm 0.09$ (7) | $-5.72 \pm 0.90$ (8) ** |
| Ba$^{2+}$-sensitive $I_{K} (-150)$ current, nA | $-0.09 \pm 0.09$ (7) | $-0.14 \pm 0.09$ (7) | $-1.39 \pm 0.45$ (8) ** |
| in 50 mM $K^+$ RMP         | $-2.36 \pm 0.73$ (11) | $-1.92 \pm 0.48$ (12) | $-30.83 \pm 1.44$ (12) *** |

Significant values: ** (p<0.01), *** (p<0.001)

Kir4.1 cells showed an almost linear I-V curve with weak inward rectifying properties ($I_K$) as shown earlier (Neusch 2001, 2006), whereas controls had prominent outward rectifying currents (Fig. 3A-C) as well as Na$^+$ inward currents but almost no inward rectifying currents. In addition, Kir4.1 transfected cells showed an increase in K$^+$ conductance compared to non-transfected controls ($g=2.09\pm0.36$ pS vs. Kir4.1 transfected $g=56.32\pm8.24$ pS under 50 mM K$^+$ solution, mean ± SEM, Fig. 4A).

In Kir4.1 transfected NSC-34 cells (n=12), RMP was hyperpolarized when compared to non-transfected (n=11) and EGFP-transfected controls (n=12) (Fig. 4C). The RMP of Kir4.1 transfected cells was hyperpolarized ($-30.83\pm1.44$ mV, n=12) when compared to EGFP transfected cells ($-1.92\pm0.48$ mV, n=12, p<0.001) and non-transfected cells ($-2.36\pm0.73$ mV, n=11, p<0.001, mean ± SEM).

Furthermore, at more depolarizing voltage steps, we detected a typical transient inward current which we suggest is mainly evoked by sodium currents. These Na$^+$ inward currents were observed in ~80% of non-transfected NSC-34 cells and in ~70% of EGFP-cells upon depolarizing pulses. Sodium currents showed a broad range (160-1355 pA, Fig. 4B) most likely reflecting different maturity states of the NSC-34 cells. Na$^+$ inward currents induced by depolarizing pulses were not observed in Kir4.1-transfected cells. One-way-ANOVA analysis revealed a significant difference in Na$^+$ current for non-transfected cells when compared with Kir4.1-transfected cells (p<0.01).

NaV1.6 staining is present in non-transfected and Kir4.1/EGFP transfected cells

The influence of heterologous Kir4.1 expression on the expression of a voltage-gated sodium channels was addressed by immunolabeling for NaV1.6. This channel is known to be expressed in NSC-34 cells (Usaka et al. 2008). NaV1.6 immunoreactivity was strong within the soma of NSC-34 cells sparing the nuclear region. NaV1.6...
immunoreactivity was also detected in Kir4.1/EGFP transfected cells (Fig. 5A-C). NaV1.6 fluorescent intensity analysis was performed and photomicrographs processed by ImageJ using the subtracting background function and threshold adjustment. This approach did not reveal a significant difference of the NaV1.6 immunosignal in control versus Kir4.1 expressing cells (unpaired t-test) and suggest that NaV1.6 expression is not downregulated by a transient Kir4.1 transfection (Fig. 5).

**Fig. 5.** Sodium channel (NaV1.6) expression is not changed in Kir4.1/EGFP transfected cells. Fluorescence micrographs display **A**) control cells, **B**) Kir4.1/EGFP transfected cells. Confocal images showing **C**) Kir4.1/EGFP transfection (green), **D**) NaV1.6 (red) and **E**) overlay. Immunoreactivity of NaV1.6 is observed in NSC-34 cells with and without Kir4.1/EGFP construct. Histogram (**F**) is demonstrating the integrated density of NaV1.6 immunoreactivity which reveals no significant reduction of NaV1.6 immunosignal for Kir4.1/EGFP transfected cells compared to controls. Values are mean ± SEM.

**Discussion**

The development of molecular genetic approaches to control neuronal activity started more than a decade ago spanning from the invertebrate model of the fruit fly Drosophila (White et al. 2001a,b, Zhao et al. 1995) to various vertebrate models (McClelland et al. 2011, Falk et al. 2001, Li et al. 2001, Falk et al. 2008, Sutherland et al. 1999). Several candidates, e.g. G protein-gated inward rectifier K⁺ channel subunits 1-4 (GIRK1-4), the renal outer medullar K⁺ channel (ROMK1 or Kir1.1), transcriptional expression of the Na-channel type II (NaCh II) promoter by NRSF, or the Kir2.1 channel subunit, have been tested with different results (Ehrengruber et al. 1997, Johns et al. 1999a,b, Nadeau et al. 2000, Nadeau and Lester 2002, Slimko et al. 2002, Okada et al. 2008). Some earlier candidate channels needed the application of agonist or inducer factors to activate the respective channel just shortly before the electrophysiological experiment (Slimko et al. 2002). Long-term effects of the genetic alteration, which might be of relevance for neuronal compensatory mechanisms or toxicity, could not be properly analyzed or detected. Other channels, such as ROMK1 led to chronic silencing but in addition evoked apoptosis under physiological extracellular K⁺ levels (Nadeau et al. 2000).

The Kir channel subunit we chose for our study has different to, e.g. ROMK1, only weak inwardly-rectifying properties at rest and is ubiquitously expressed in the central nervous system (Bredt et al. 1995, Li et al. 1998, Neusch et al. 2001, Olsen et al. 2006).

So far, the Kir4.1 channel subunit has only been tested in a few non-neuronal cell cultures, e.g. to elucidate Kir4.1 function on ammonia and hypotonicity in HEK-293 cells (Obara-Michlewska et al. 2010) or
oocytes (Pessia et al. 2001). In COS-1 cells a transfection of Kir4.1 channels was sufficient to evoke K⁺-induced calcium transients (Härtel et al. 2007). Up to now, a study that investigates Kir4.1 channel function in neuronal or neuronal-like cell cultures is not published.

Here, we expressed the Kir4.1 potassium channel subunit in an immortalized neuroblastoma spinal cord cell line (NSC-34), which is widely used as an in vitro model for motoneuronal diseases such as amyotrophic lateral sclerosis (Colombrita et al. 2012, Moreno-Martet et al. 2012, Chen et al. 2011). NSC-34 cells display characteristics of a multipolar neuron-like phenotype (Cashman et al. 1992) and combines the advantage of a reproducible and efficiently transfectable system while having a defined expression of neurofilament and membrane proteins. Specifically, NSC-34 cells do not express Kir channels as it has been described, e.g., for the NG108-15 neuroblastoma-glioma hybrid cell line (Ma et al. 1999).

The well-known limitation is the existence of two described NSC-34 populations in cell culture: one population that is predominantly observed retains fewer motoneuronal properties and is characterized by a round morphology and the inability to generate action potentials. These cells have a more depolarized RMP around 0 to −20 mV (Ryan et al. 2009, Cashman et al. 1992, Durham et al. 1993), but retain inward sodium channel activation and are considered to be more ‘immature’. This phenomenon is in line with the fact that developing and proliferating cells in vitro tend to have more depolarized RMP values (Sundelacruz et al. 2009). The other population is able to generate action potentials. For our study, we chose the immature cell population since the overall number of the latter cell type was too low.

Heterologous expression of Kir4.1 channels resulted in a hyperpolarization of the cell membrane by ~30 mV, increased K⁺ conductance and abolished virtually all Na⁺ currents. Kir4.1 transfection proved to be efficient and non-toxic to NSC-34 cells. Furthermore, transfection of Kir4.1 causes a hyperpolarizing shift in the voltage dependence of inactivation without decreasing NaV1.6 channel distribution. Therefore, hyperpolarization of the Kir4.1/EGFP transfected NSC-34 cell appears independent from the NaV1.6 expression, although we cannot rule out that NaV1.6 sodium channels activity is functionally dampened.

In conclusion, introducing the Kir4.1 channel subunit into mammalian/neuronal cell lines may serve as a tool to modulate and reduce neuronal excitability by direct hyperpolarization of the RMP in experimental designs.

Conflict of Interest

There is no conflict of interest.

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