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RNA interference-based functional knockdown of the voltage-gated potassium channel Kv7.2 in dorsal root ganglion neurons after in vitro and in vivo gene transfer by adeno-associated virus vectors

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Abstract

Activation of the neuronal potassium channel Kv7.2 encoded by the *KCNQ2* gene has recently been shown to be an attractive mechanism to inhibit nociceptive transmission. However, potent, selective, and clinically proven activators of Kv7.2/Kv7.3 currents with analgesic properties are still lacking. An important prerequisite for the development of new drugs is a model to test the selectivity of novel agonists by abrogating Kv7.2/Kv7.3 function. Since constitutive knockout mice are not viable, we developed a model based on RNA interference-mediated silencing of *KCNQ2*. By delivery of a *KCNQ2*-specific short hairpin RNA with adeno-associated virus vectors, we completely abolished the activity of the specific Kv7.2/Kv7.3-opener ICA-27243 in rat sensory neurons. Results obtained in the silencing experiments were consistent between freshly prepared and cryopreserved dorsal root ganglion neurons, as well as in dorsal root ganglion neurons dissociated and cultured after in vivo administration of the silencing vector by intrathecal injections into rats. Interestingly, the tested associated virus serotypes substantially differed with respect to their transduction capability in cultured neuronal cell lines and primary dorsal root ganglion neurons and the in vivo transfer of transgenes by intrathecal injection of associated virus vectors. However, our study provides the proof-of-concept that RNA interference-mediated silencing of *KCNQ2* is a suitable approach to create an ex vivo model for testing the specificity of novel Kv7.2/Kv7.3 agonists.

Keywords

Dorsal root ganglion, electrophysiology, adeno-associated virus, Kv7.2, KCNQ2

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Introduction

The potassium channel protein Kv7.2 is widely distributed throughout the peripheral and central nervous system (CNS) and plays a key role in regulating the membrane potential and the excitability of neurons including those involved in nociceptive transmission.[1] It is encoded by the *KCNQ2* gene and exerts its full function as heterodimer with Kv7.3. Activation of Kv7.2/Kv7.3 channels leads to a slowly activating and noninactivating potassium outward current.[2] Kv7.2/Kv7.3 ion channels are involved in pathophysiological mechanisms contributing to epilepsy as well as inflammatory, chronic nociceptive, and neuropathic pain.[3–5] Loss of function of these channels results in the resting membrane potential (RMP) to depolarize

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thereby increasing the excitability of neuronal cells. This is believed to contribute to different diseases, including overactive bladder syndrome[6] and diabetes-induced neuropathic pain, where the hyperexcitability and spontaneous firing of the primary afferent neurons leads to hyperalgesia and allodynia.[3] The Kv7.2/Kv7.3 channel is thus an attractive target for the discovery of new analgesic drugs, which are urgently required for the treatment of chronic pain, since currently available drugs still lack long-term effectiveness.[4]

The selective ICA-27243[7,8] is a Kv7.2/Kv7.3 channel opener with the potential to be used as a tool for the development of new Kv7-specific analgesics with high potency and efficacy. However, comprehensive characterization of candidate molecules as Kv7.2/Kv7.3 openers is hampered by the fact that the engagement of Kv7.2/Kv7.3 to their effects could not be proven due to the lack of viable constitutive Kv7.2 knockout mice[9] and suitable Kv7.2 small molecule antagonists. The antagonists XE991 and linopirdine are not selective for Kv7.2 and also address Kv7.1, excluding their use to demonstrate selective target engagement for Kv7.2.

In our present study, we resolved this dilemma by using RNA interference (RNAi), which is an alternative approach for target validation and in addition provides new treatment options.[10,11] We and others have shown that RNAi-mediated silencing has potential to study pain biology.[12–16] In fact, a small interfering RNA (siRNA) targeting the Transient Receptor Potential Vanilloid 1 receptor has already reached the status of clinical testing for the treatment of ocular pain.[17] Delivery of siRNAs has been the limiting factor in their therapeutic use for a long time, but this has meanwhile been overcome by promising approaches. The use of viral vectors for the expression of short hairpin RNAs (shRNAs) facilitates delivery of the double-stranded RNA molecules to the target cells and permits the long-term silencing of a target gene.[18,19] Vectors based on adeno-associated viruses (AAVs) are used as particularly promising vehicles for the delivery of transgenes[20,21] and have become the most commonly used gene therapy vectors for the CNS.[22] Ten different serotypes of AAV vectors, with different tissue tropisms, are in routine research use. AAV vectors of various serotypes have been used for the silencing of pain targets.[23] Serotypes 5, 6, 8, and 9 are among the most widely used AAV vector types for the transduction of dorsal root ganglia (DRGs).[16,24–36] Frequent routes of in vivo AAV application for gene transfer to DRGs include intrathecal,[16,25–30,32] intravascular,[35] intraperitoneal,[36] and direct intraganglionic injections.[29,31,33,34,37,38]

The first challenge we had to overcome was finding a suitable AAV serotype which would allow the efficient transduction of DRGs in cell culture and the SH-SY5Y neuronal cell line. Once having found a suitable serotype, *KCNQ2* expression was silenced by intrathecal administration of an shRNA-expressing AAV vector in rats. We found that the Kv7.2/Kv7.3-specific ICA-27243 no longer hyperpolarized dissociated DRG neurons in those cells in which the expression of Kv7.2 had been silenced. Our experiments demonstrate that an AAV-mediated shRNA-based *KCNQ2* silencing strategy can be used to investigate new drug candidates which target specificity and mode of action has not fully been demonstrated before.

Materials and methods

Animals

For in vitro experiments, DRGs from male Sprague–Dawley (SD) rats (n = 16, Janvier, Le Genest-Saint-Isle, France) and Wistar rats (n = 8, Charles River Laboratories, Sulzfeld, Germany) were used (with an average animal weight of 175–199 g). In vivo experiments were conducted using male SD rats (n = 64, Janvier; weight 75–99 g at the time of intrathecal administration). After shipment, the animals were allowed at least four days to recover before the experiments. At the time point of DRG preparation, animal weight was between 250 and 350 g. All animals were housed under a 12:12-h light–dark cycle (lights on at 06:00 h), room temperature of 20°C to 24°C, relative air humidity of 35%–70%; 15 air changes per hour, air movement of less than 0.2 m/s. Laboratory food and tap water were available ad libitum.

Ethics statement

All procedures described here were licensed and approved by the appropriate governmental bodies (LANUV AZ84–02.05.20.12.231, AZ84–02.05.40.14.011) and were designed to reduce the numbers and undue suffering in accordance with International Association for the Study of Pain ethics guidelines,[39] the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the German Animal Welfare Law.

siRNAs and generation of the shRNA-expressing AAV vector

The siRNA sequence targeting rat and mouse *KCNQ2* was as follows (purchased from GE Healthcare Dharmacon Inc., Lafayette, CO; USA cat. no. D-047386–01):

Sense strand: 5′-GGUAUUCGGUGUUGAGUA CUU-3′

Antisense strand: 5′-GUACUCAACACCGAAUA CCUU-3′

The siRNA was used for initial tests and then converted into an shRNA. Synthetic DNA oligonucleotides encoding the respective shRNA were cloned into the pSilencer 2.1 U6 neo vector (Thermo Fisher Scientific, Braunschweig, Germany).

For this, the following sense-strand oligonucleotide was used (the duplex-forming sequence of the shRNA is underlined, and the antisense oligonucleotide was designed to generate overhangs for BamHI/HindIII-cloning into the pSilencer vector):

5′-GATCCCGGTATTCGGTGTTGAGTACTTCA AGAGAGTACTCAACACCGAATACCTTTTTTGG AAA-3′

A control shRNA was used that does not share substantial homology to the human genome or the genomes of mice and rats. The control sense-strand oligonucleotide was as follows:

5′-GATCCACTACCGTTGTTATAGGTGTTCAA GAGACACCTATAACAACGGTAGTTTTTTGG AAA-3′

The shRNA expression cassette consisting of the U6 promoter and the sequence encoding the shRNA was subcloned into an AAV shuttle plasmid containing an AAV backbone that leads to the generation of self-complementary AAV (scAAV) vectors.[40] Production of the scAAV vectors was carried out as described previously.[41] In brief, AAV vectors of serotypes 1–6 and 8 were produced with a double transfection system. Packaging plasmids were obtained from PlasmidFactory (Bielefeld, Germany). Serotype 7 was omitted, since for legal reasons, the packaging plasmids were not readily commercially available, and this serotype has not been used for DRG transduction in any of the published studies described above. For AAV vector production, HEK293T cells were cotransfected with the AAV shuttle plasmid and a helper plasmid that provided essential adenoviral helper functions, the Rep proteins of AAV2 and the capsid proteins of the specific AAV serotype using polyethyleneimine as transfection reagent. AAV vectors containing the capsid of AAV9 were generated by triple transfection as described.[40] Cells were grown in roller bottles (850 cm^2; Corning, Tewksbury, MA) and harvested after 72 h. AAV vectors were purified by iodixanol gradient centrifugation[42] followed by filtration with PD10 gel columns (GE Healthcare, Freiburg, Germany). Concentration of the AAV vector preparation was determined by quantitative polymerase chain reaction (qPCR).[41]

Cell culture

Cell lines

HEK293 and HEK293T cells (human embryonic kidney cell line) were cultured in Dulbecco's modified Eagle's medium (BioWest, Nuaillé, France) supplemented with 4 g/l D-glucose (Sigma, St Louis, MO), 10% fetal calf serum (c-c-pro, Oberdorla, Germany), 2 mM L-glutamine (BioWest), and 1× minimal essential medium non-essential amino acids (BioWest). SH-SY5Y cells (human neuroblastoma cell line) were cultured in RPMI1640 (BioWest) supplemented with 2 mM L-glutamine (BioWest) and 10% fetal calf serum (c-c-pro).

Transfection and Western blotting for reporter constructs

For the reporter assays, a fusion mRNA composed of green fluorescent protein (*GFP*) and *KCNQ2* was expressed from the plasmid pcDNA3.1/NT-GFP. As both cDNAs were separated by a stop codon, only GFP was translated. Plasmids were transfected into HEK293T cells with Lipofectamine, 2000 according to the manufacturer's instructions. At 48 h after transfection, cells were lysed in 24-well plates. After boiling the lysate at 95°C for 5 min, proteins were separated on a 16% (w/v) denaturating sodium dodecyl sulfate polyacrylamide gel. Transfer of proteins to polyvinylidene fluoride membranes (GE Healthcare/Amersham, Freiburg, Germany) was performed with a semidry blotter (Peqlab, Erlangen, Germany). Subsequently, blocked membranes were incubated with rabbit GFP antiserum (1:2000; abcam, ab6556, Cambridge, UK) overnight in TBS + 0.1% Tween 20 buffer at 4°C. Secondary goat anti-rabbit antibodies was conjugated with peroxidase (Pierce, #31460, Rockford, IL). Chemiluminescence was determined by the treatment with enhanced chemiluminescence substrate (Pierce) and subsequent detection in the ChemiDoc MP (BioRad, Munich, Germany). To confirm equal loading of the samples, membranes were reprobed with a monoclonal mouse antibody (1:5000) specific for actin (Merck Millipore, MAB1501, Darmstadt, Germany). In this case, a goat anti-mouse antibody was used as secondary antibody (Pierce, #31430). The Marker Seeblue Plus2 (Invitrogen, Karlsruhe, Germany) served as size standard.

Culture of cryopreserved SD rat DRG neurons from Lonza

Cryopreserved rat DRG neurons (R-DRG-505, Lonza, Walkersville, MD) were cultured on Biocoat Poly-D-Lysine/Laminin 12 mm coverslips (Becton Dickinson, Franklin Lakes, NJ) in a 24-multiwell plate according to the recommended instructions provided by Lonza.

Primary culture of SD and Wistar rat DRG neurons

Animals were decapitated and the spinal column was quickly removed and placed in cold Hank's balanced salt solution with 1% antibiotic/antifungal agent

(AA; PAA Laboratories, Cölbe, Germany). Isolated DRGs were enzymatically digested with collagenase 2 (PAA Laboratories) for 45 min at 37°C followed by a 15-min treatment with 2.5% Trypsin (PAA Laboratories) in Ham's F21 culture medium supplemented with 10% FCS; 2 mM L-glutamine; and 1% AA solution at 37°C. Afterwards the enzyme solution was deactivated by fresh culture medium. DRG tissue was triturated through different sizes of pipette tips and then filtered over a 70-µm Falcon mesh (Becton Dickinson). Cells were washed (Eppendorf Centrifuge, 5810 R, at 225 g, RT) and plated in 24-multiwell plates on Biocoat Poly-D-Lysine/Laminin 12 mm coverslips (Becton Dickinson) at a density of 3×10^5 cells/well. Cells were maintained in culture for five to seven days at 37°C, 5% CO_2, 95% relative humidity. Every third day, half of the culture medium was replaced by fresh medium. Culture conditions and media were according to the Lonza instructions to ensure comparability with cryopreserved DRG neurons from Lonza.

AAV vector-mediated transduction of cells

In vitro. HEK293 and SH-SY5Y cells were seeded at a density of 5×10^4 cells/well in a 96-multiwell plate. After 24 h, transduction with the library of pseudotyped AAV vectors was performed using 100,000 virus genomes (VG)/cell. Microscopic analysis 48 h post transduction was carried out with an Axio Observer Z1 microscope (Carl Zeiss, Jena, Germany, 10× magnification, bright-field phase contrast 60 ms, GFP, 2500 ms).

Primary cultured DRG neurons from SD rats were transduced with the library of emerald green fluorescent protein (EmGFP)-expressing AAV vectors with 100,000 VG/cell. Fluorescence images were taken 48 h after transduction in a Biozero BZ8000 microscope (Keyence Corp., Osaka, Japan).

AAV2.6-Ctrl-DsRed and AAV2.6-shKCNQ2-DsRed at a final concentration of 100,000 VG/cell were used with cryopreserved DRG neurons from Lonza and primary cultured DRG neurons from SD rats. The transduction of the neurons was performed twice, initially on day 1 and a second time on day 2. The electrophysiological experiments were conducted 48, 72, and 96 h after the second AAV treatment.

In vivo. AAV vectors were administered intrathecally in isoflurane-anesthetized animals. The injection was placed in the lumbal region between L4 and L5 of the spinal cord. Success in reaching intrathecal space was confirmed by tail flick reaction.[43] A volume of 5 µL was injected with a Hamilton syringe (Hamilton Laboratory Products, Reno, NV) and a $27 \times 1/2$ gauge needle. The injections were carried out at the indicated time points. After the last injection, the animals were housed for the indicated period before further analysis.

Electrophysiological patch-clamp recording

Whole-cell patch-clamp experiments were carried out with a HEKA EPC 9 patch-clamp amplifier and using Patchmaster software 2.4 (HEKA Elektronik Dr. Schulze GmbH, Ludwigshafen, Germany). The sampling rate was 50 kHz, and a Bessel filter with frequency borders of 3–1000 Hz was used. Borosilicate patch electrodes (Harvard Apparatus, Holliston, MA) manufactured with a DMZ-universal puller (Zeitz-Instrumente GmbH, Planegg, Germany) and resistances of 3–5 MΩ were used. The electrodes were filled with an internal solution containing (in mM): 130 KCI, 2 $MgCI_2$, 0.5 $CaCl_2$, 5 BAPTA, 10 HEPES, 3 Na_2ATP, and pH 7.2 adjusted with KOH. The extracellular solution contained (in mM): 150 NaCl, 3 KCl, 2 $CaCl_2$, 2 $MgCI_2$, 10 HEPES, and 10 glucose. The pH was adjusted to 7.4 with NaOH. A continuous gravity-driven whole bath perfusion was performed in all recordings. Small diameter DRG neurons, which are putative nociceptive neurons, with a mean diameter of 27.10 µm (± 0.72, n = 55) were first voltage-clamped at a holding membrane potential of −60 mV to record an I–V protocol from −80 to +60 mV in 20 mV steps. Afterwards DRG neurons were current-clamped, and the RMP was recorded. After a stable potential was observed, the Kv7.2/Kv7.3 opener ICA-27243 (10 µM) was directly applied to the cell for 10 s by a multivalve perfusion system (ALA Scientific Instruments, New York, NY), and the hyperpolarization effect on each neuron was monitored. Patch-clamp data were analyzed using the Patchmaster online measuring tool.

qPCR quantification of *KCNQ2* expression

After RNA isolation with the RNeasy mini Kit (Qiagen, Hilden, Germany) and DNAse I digestion (RQ1, Promega, Mannheim, Germany), the mRNA was reverse transcribed with random hexamer primers using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA) according to the manufacturer's recommendations.

For *KCNQ2* expression analysis, the *KCNQ2* TaqMan gene expression assay (Rn00591249_m1, 20×; Applied Biosystems/Thermo Scientific) in combination with the TaqMan gene expression master mix (2×; Thermo Scientific) was used. As a reference, the level of the 18S rRNA was analyzed with the SYBR green-based qPCR SsoFast EvaGreen supermix (BioRad) using the following primer set:

forward primer: 5′-CGCGGTTCTATTTT GTTGGT-3′

reverse primer: 5′-AGTCGGCATCGTTTATG GTC-3′

The relative *KCNQ2* expression was determined by the ΔΔc(t) method.[44]

Statistics

Statistical evaluation of the effect of RNAi-mediated *KCNQ2* knockdown on RMP and on ICA-27243-mediated hyperpolarization was performed using Student's *t* test (GraphPad Prism 5, GraphPad Software, Inc., La Jolla, CA). Data are presented as mean ± SEM, and p values are considered as significant by *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

Results

Choice of AAV serotype and rat strain

We began by attempting to identify the best AAV serotype for the transduction of neuronal cells. For this purpose, we generated a library of pseudotyped AAV vectors consisting of the backbone of serotype 2 and capsids of the most commonly used serotypes. Each of these vectors contains a sequence for the expression of *EmGFP* to enable measurement of the transduction efficiency into HEK293 cells and the human neuroblastoma cell line SH-SY5Y. As can be seen in the Supplementary Figure S1, AAV2.1 and AAV2.2 showed high transduction efficiency in both cell lines, whereas AAV2.3 and AAV2.6 had high transduction efficiency in SH-SY5Y cells, but transduced HEK293 cells with much lower efficiency.

In a recent publication, the biological function of Kv7.2 was only investigated in Wistar rats.[45] We extended the findings from the Wistar strain to SD rats, as the latter are the strain of choice for behavioral models in pain research. For this purpose, DRGs from both strains were prepared and showed marked differences with respect to their response to the selective Kv7.2 opener ICA-27243. The RMP and the hyperpolarization effect of ICA-27243 were determined by patch-clamp recordings in the current clamp mode (Figure 1(a)). DRG neurons from SD rats had a significantly lower RMP than those from Wistar rats (-60.50 ± 1.58. vs. -54.68 ± 1.65 mV, $p = 0.014$, Figure 1(a)). The hyperpolarizing effect by ICA-27243 in DRG neurons from SD rats was significantly stronger than in neurons from Wistar rats (-10.90 ± 0.88 mV vs. -7.8 ± 0.63 mV, $p = 0.005$). In addition, the response rate, indicating the fraction of DRG neurons that respond to ICA-27243 was 100% for neurons derived from SD rats, while it was only 70% for Wistar rat neurons. Due to the higher response rate and the more pronounced hyperpolarization, SD rats were clearly the better model animal and were used for all subsequent in vitro and in vivo experiments.

At this point, the serotypes with the best transduction efficiency had been determined in standard cell lines. To prepare for in vivo experiments, we next identified the AAV serotype with the highest transduction efficiency in primary small diameter DRG neurons. Cultured DRG neurons prepared from SD rats were transduced with vectors from the library of pseudotyped AAV vectors. The transduction efficiency was examined 48 h after application of the AAV vectors by fluorescence microscopy. Serotypes AAV2.1 and AAV2.9 showed some transduced neurons (2.5% and 1.3% of all cells), but the highest number of *EmGFP* expressing neurons was found in the AAV2.6 culture with 10.3% of all cells being transduced (Figure 1(b), Supplementary Table 1), so that we chose this serotype for subsequent electrophysiological experiments.

Construction of AAV vectors for silencing of KCNQ2

For initial experiments to silence *KCNQ2*, an siRNA sequence targeting the mouse and the rat mRNA was tested for its ability to silence the expression of a fusion gene of *GFP* and *KCNQ2* in cotransfection experiments. The siRNA demonstrated high silencing capacity (data not shown). As next step, oligonucleotides encoding the respective shRNA were cloned into the pSilencer 2.1 neo vector to receive the construct pSil-shKCNQ2. The silencing capacity of the vector was as well confirmed in cotransfection experiments with a reporter vector expressing the *GFP-KCNQ2* fusion construct. We found that only the *KCNQ2*-specific shRNA silenced the target with high efficiency, whereas the control shRNA did not influence gene expression, as demonstrated in Figure 2(a). Quantitative evaluation revealed a knockdown of approximately 70% (Supplementary Figure S2).

As off-target effects have been observed in RNAi applications, we tested for the specificity of our approach. Kv7.2 and Kv7.3 function as dimers. Thus, unintended knockdown of *KCNQ1*, *KCNQ4*, or *KCNQ5* could be side effects that might reduce the usability of our approach. We therefore carried out cotransfection reporter assays. Quantitative evaluation of these experiments by qRT-PCR revealed that only *KCNQ2* is substantially silenced by more than 90%, whereas the other *KCNQ* genes under investigation were only modulated in the typical range of variation of this type of experiments by ± 20% (Supplementary Figure S3).

As our experiments show that the chosen shRNA is efficient and specific, it was justified to transfer the shRNA expression cassette into the backbone of the AAV vector. In addition to the shRNA expression cassettes, the resulting vector encoded *Discosoma*

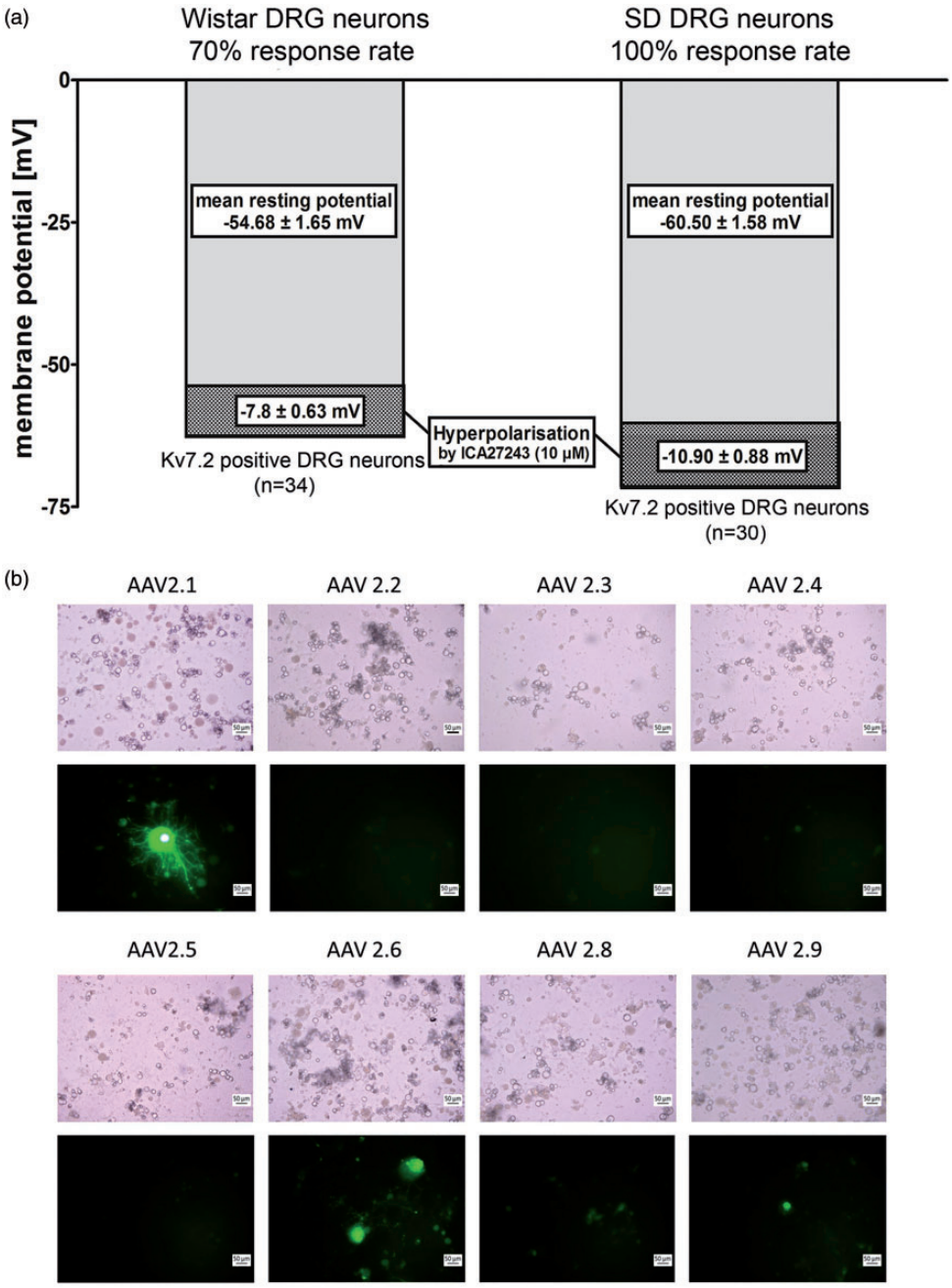

Figure 1. Characterization and AAV transduction of primary DRG neurons. (a) Comparison of resting membrane potential (RMP) and hyperpolarization effect of the Kv7.2/Kv7.3 opener ICA-27243 (10 μM) on DRG neurons derived from Wistar and SD rats. The response rate reflects the fraction of successfully patched DRG neurons that were hyperpolarized upon application of ICA-27243. (b) EmGFP expression in DRG neurons from SD rats transduced with pseudotyped AAV vectors. DRG neurons were transduced with 100,000 VG/cell. Fluorescence images were taken 48 h after transduction. The scale bar represents a length of 50 μm. AAV: adeno-associated virus; DRG: dorsal root ganglion; SD: Sprague–Dawley.

red (DsRed) as a marker to identify successfully transduced cells for use in patch-clamp experiments. We selected the DsRed fluorophore as initial experiments revealed DsRed fluorescence to be better discriminated from the background than fluorescence by EmGFP. In addition, EmGFP tended to induce slightly more cytotoxic effects (not shown). Based on the results described above, pseudotyped AAV2.6 vectors were produced.

The AAV vectors were initially tested in cultured primary DRG neurons from SD rats. Patch-clamp measurements were carried out to determine the

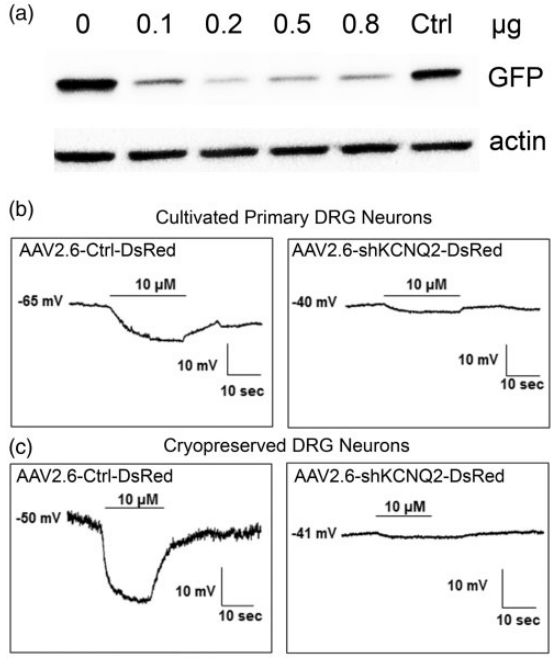

Figure 2. shRNA-mediated silencing of KCNQ2. (a) Western blot of a cotransfection experiment of HEK293T cells with pSil-shKCNQ2 and GFP-KCNQ2. HEK293T cells were cotransfected with 0.8 µg of the plasmid encoding the GFP-KCNQ2 fusion construct and increasing concentrations of pSil-shKCNQ2, as indicated. In the control lane, 0.8 µg of pSil-Ctrl was cotransfected with the plasmid encoding GFP-KCNQ2. Cells were harvested 48 h after transfection. Actin is shown as a loading control. The experiment was carried out four times to confirm robust and efficient knockdown. Bands appeared at the expected apparent masses of 27 kDa for GFP and 42 kDa for actin. (b) Original electrophysiological recordings of hyperpolarization in cultured primary DRG neurons activated by the Kv7.2/Kv7.3 opener ICA-27243 (10 µM). The DRG neurons were freshly prepared from SD rats. Neurons were transduced with AAV2.6-Ctrl-DsRed and AAV2.6-shKCNQ2-DsRed, respectively. Patch-clamp measurements were performed after an incubation period of 96 h. The left trace represents a recording from a DRG neuron transduced with AAV2.6-Ctrl-DsRed, and the right trace shows a DRG neuron transduced in vitro with AAV2.6-shKCNQ2-DsRed. Baseline represents the resting membrane potential of the cell. (c) Experiments as in (b) but with thawed cryopreserved DRG neurons. For all experiments, DRG neurons were transduced with the respective AAV vector twice on two consecutive days. AAV: adeno-associated virus; DRG: dorsal root ganglion; GFP: green fluorescent protein.

hyperpolarization induced by treatment of the neurons with the Kv7.2/Kv7.3 opener ICA-27243 (Figure 2(b)). For neurons that were treated with the control AAV vector, hyperpolarization of approximately 10 mV was observed (Figure 2(b), left trace) which is in line with the results for the untreated neurons shown in Figure 1(a). In contrast, ICA-27243-induced hyperpolarization was substantially diminished after having treated the neurons with the AAV vector that expresses a shRNA targeting *KCNQ2* (Figure 2(b), right trace).

To test whether cryopreservation is detrimental to the functional analysis of Kv7.2/Kv7.3, commercially available DRG neurons intermittently cryopreserved were tested in parallel to freshly prepared primary neurons. Figure 2(c) shows the Kv7.2-mediated hyperpolarization and reduction of this effect after RNAi treatment were similar for both cell types, demonstrating that the freeze/thaw procedure does not change neuron functionality and therefore, the cryopreserved DRG neurons can be used to study hyperpolarization by Kv7.2/Kv7.3 openers.

The mean diameter of measured DRG neurons of the AAV-Ctrl group was 25.9 ± 0.89 µm and 27.0 ± 1.17 µm for the AAV-shKCNQ2 group confirming that recordings were made from similar cell populations.

For both, directly cultivated primary and cryopreserved DRG neurons, the RMP was less negative following treatment with the shRNA silencing *KCNQ2* (-66.0 ± 2.25 mV in the control group vs. -54.0 ± 5.64 mV in the shRNA-treated group for primary cultivated DRG neurons and -48.0 ± 1.98 mV vs. -43.0 ± 2.40 mV for the cryopreserved DRG neurons). This effect can be expected as Kv7.2 contributes to the resting potential and further corroborates successful knockdown of the target.

Time course of RNAi-mediated knockdown of KCNQ2 *in DRG neurons*

Next, we investigated the time course of *KCNQ2* silencing and reduction of the ICA-27243-induced hyperpolarization. For efficient transduction, thawed DRG neurons were transduced with AAV vectors twice on two consecutive days. The hyperpolarization was monitored at different time points after the second transduction. As initial experiments showed no significant changes of the hyperpolarization at 48 h after the second transduction (hyperpolarization of -10.6 ± 1.61 mV and -7.65 ± 1.62 mV for the control and *KCNQ2*-specific shRNAs, respectively; $N = 5$; $p = 0.23$), all further analyses were carried out at 72 and 96 h. Quantitative PCR confirmed stable knockdown of approximately 80% at both time points (Figure 3(a)).

In line with these data, hyperpolarization induced by the Kv7.2 opener ICA-27243 in DRG neurons freshly prepared from SD rats was significantly reduced after silencing of *KCNQ2* (Figure 3(b)). Hyperpolarization was reduced from -8.4 ± 2.1 mV to -1.85 ± 1.56 mV ($p = 0.03$) measured 72 h after the second transduction. ICA-27243-induced hyperpolarization in cells treated with the control vector, 96 h after the second transduction, was slightly stronger at -11.76 ± 1.85 mV. As observed after 72 h, hyperpolarization induced by the Kv7.2/Kv7.3 opener was significantly ($p = 0.005$)

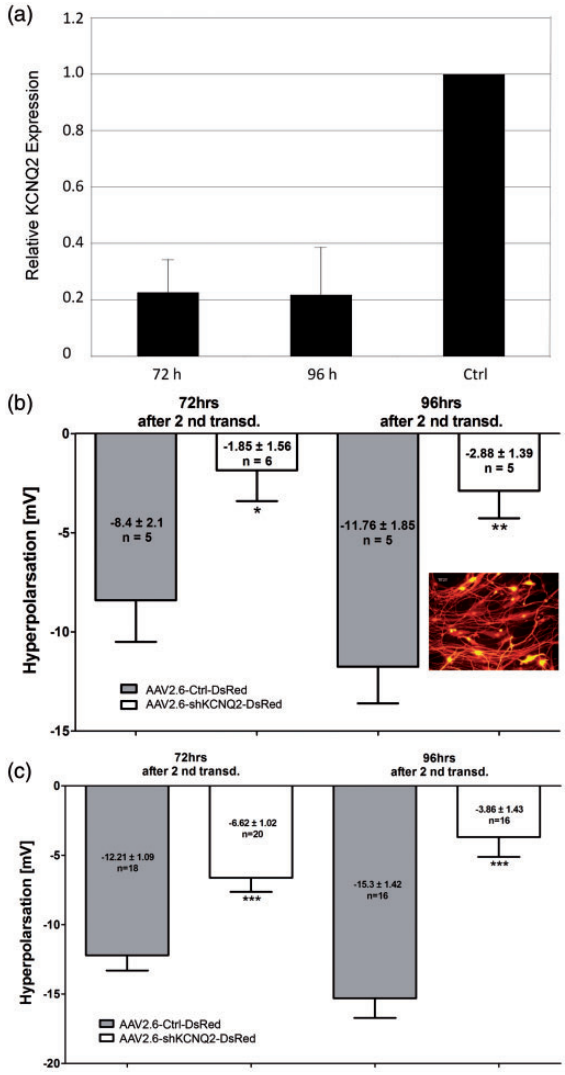

Figure 3. Time course of silencing and hyperpolarization 72 and 96 h after the second transduction. (a) Thawed cryopreserved DRG neurons were transduced with 100,000 VG/cell of AAV 2.6 vectors twice on two consecutive days. RNA was isolated from the cells 72 and 96 h after transduction, and the level of KCNQ2 mRNA was determined by qRT-PCR. KCNQ2 expression level was normalized to the 18S rRNA and determined by the ΔΔCT method. Mean and standard deviation of three independent experiments, each carried out in triplicate, are shown. (b) Time course of hyperpolarization by ICA-27243 (10 μM) in primary cultured DRG neurons after in vitro transduction with AAV2.6-Ctrl-DsRed and AAV2.6-shKCNQ2-DsRed. Hyperpolarization was measured by patch-clamp experiments 72 and 96 h after the second transduction. Inset: Fluorescence image of an in vitro transduced DRG neuron culture from SD rats 96 h after the second transduction with AAV2.6-Ctrl-DsRed. The DRG neurons showed an intensive red fluorescence signal, and the DsRed signal is also visible in the neuronal network. (c) Experiments as in (b) but with thawed cryopreserved DRG neurons. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ (Student's t test). AAV: adeno-associated virus.

reduced after silencing the channel with AAV2.6-shKCNQ2-DsRed (-2.88 ± 1.39 mV).

Thawed cryopreserved DRG neurons produced the same results (Figure 3(c)). Hyperpolarization induced by ICA-27243 in control-treated cryopreserved DRG neurons was slightly more pronounced 96 h after the second transduction in comparison to the first measurement after 72 h (-15.3 ± 1.42 mV after 96 h compared to -12.21 ± 1.09 mV after 72 h). Silencing of *KCNQ2* expression significantly reduced hyperpolarization to -6.62 ± 1.02 mV ($p = 0.0006$) and -3.86 ± 1.43 mV ($p < 0.0001$) after 72 and 96 h, respectively. These results confirmed that the cryopreservation did not have detrimental effects on the silencing and hyperpolarization experiments.

Transduction of whole DRGs

While all transduction experiments described so far were carried out with dissociated DRG neurons, we also tested whether the AAV vectors are capable of transducing neurons in a freshly prepared whole DRG with an intact cell structure and composition. The neurons of the whole DRG were transduced by AAV2.6-Ctrl-DsRed and the dissociation procedure did not destruct the DsRed signal, as can be seen in Figure 4. After an incubation period of 12 days, the DRG was dissociated, and patch-clamp recordings were carried out three days later. Treatment of the cells with the Kv7.2/Kv7.3opener ICA-27243 resulted in a hyperpolarization of 10 mV, in line with the previous experiments with dissociated cells, i.e., transduction of the whole DRG and its subsequent dissociation to perform patch-clamp experiments did not have any influence on the recording of Kv7.2/Kv7.3 activity in comparison to first dissociating the cells and then transfecting them.

In vivo transduction of DRG neurons by AAV vectors

Guided by the results from the in vitro experiments described above, an AAV2.6 control vector encoding DsRed at a dose of 1.9×10^{11} vector genomes (VG) per animal per injection was used for initial in vivo experiments. DRG neurons were prepared from SD rats 14 to 35 days after intrathecal administration of the vector. Surprisingly, we could not identify any fluorescent DRG neurons. In a second series of experiments, the animals were intrathecally administered with AAV2.6 vectors twice, on day 0 and day 5, and the DRG neurons were prepared on days 14, 21, 27, and 34. Again, no transduced neurons were found. Thus, despite the high transduction efficiency of AAV2.6 in vitro, our results indicate that the transduction efficiency in vivo is very low.

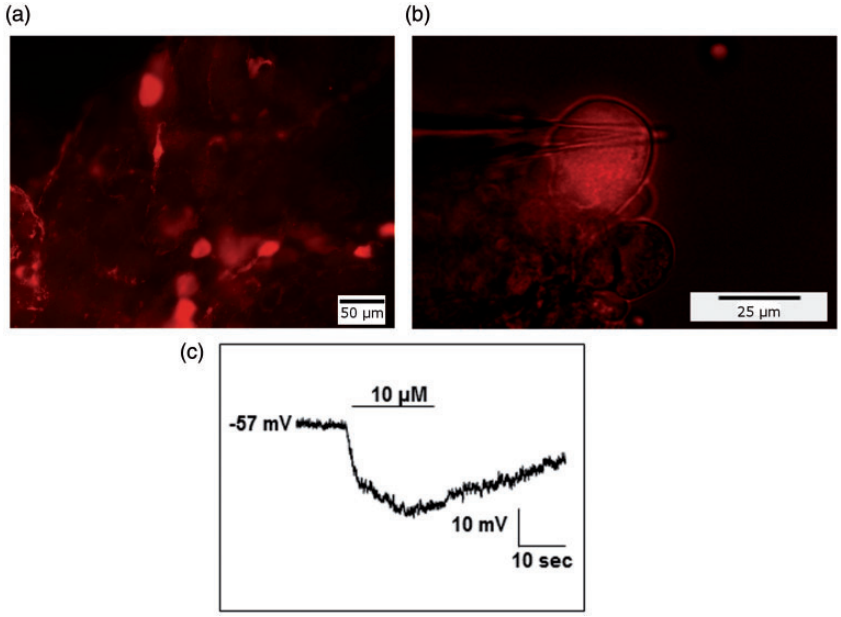

Figure 4. (a) Fluorescence image of a retrieved whole DRG transduced in vitro with AAV2.6-Ctrl-DsRed over a period of 12 days. Transduced neurons showed an intensive red fluorescence signal. The scale bar represents a length of 50 µm. (b) DRG transduced in vitro with AAV2.6-Ctrl-DsRed and dissociated on day 12. Neurons were cultured for another three days to perform patch-clamp recordings. The scale bar represents a length of 25 µm. (c) Original recording of a cultured DRG neuron hyperpolarized by the Kv7.2/Kv7.3 opener ICA-27243 (10 µM). Baseline represents the RMP of the cell.

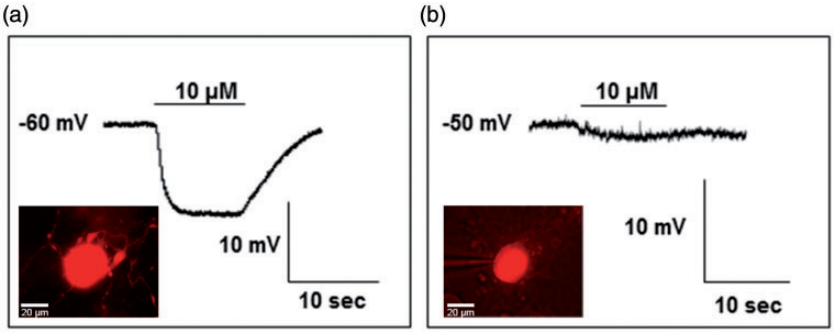

Figure 5. ICA-27243-mediated hyperpolarization in DRG cells. Animals received three intrathecal injections of the AAV vectors and after an incubation period of 21 days. DRGs were retrieved and DRG neurons were cultured on coverslips up to seven days. Trace A represents a recording from a DRG cell transduced with AAV2.5-Ctrl-DsRed and trace B shows a DRG neuron transduced with AAV2.5-shKCNQ2-DsRed. Baseline represents the RMP of the cell. Inset: Fluorescence image demonstrating the successful transduction of AAV2.5-shKCNQ2-DsRed into a DRG neuron after intrathecal application. The scale bar represents a length of 20 µm.

Based on previous reports,[16,28,33] we carried out the next set of experiments with pseudotyped AAV2.5 vectors. In addition, the number of intrathecal vector injections was increased to three during the first five days of the experiment (days 1, 3, and 5). Regarding the unsuccessful transduction experiment with AAV2.6, the highest available concentrations of Ctrl-AAV2.5 and KCNQ2-AAV2.5 were used. The dose for the control vector was 1.5×10^{11} VG per animal. For technical reasons, the vector encoding the KCNQ2-specific shRNA was 8×10^{10} VG per animal per injection. Importantly, despite the slightly higher dose, the control AAV vector did not exert observable unspecific effects in any of the experiments, and the KCNQ2-specific shRNA led to a complete knockdown of the target.

Animals were then kept for 14 to 42 days before primary cultures of DRG neurons were prepared.

Contrary to the unsuccessful experiment with AAV2.6 vectors, we observed fluorescent cells in dissociated DRG neurons after intrathecal application of AAV2.5 vectors indicating successful transduction. Red fluorescing cells were used for patch-clamp experiments. Exemplary recordings of neurons prepared after 21 days are shown in Figure 5. In line with all previous

experiments, application of ICA-27243 to neurons prepared from animals that received the control AAV vector resulted in hyperpolarization of approximately 10 mV (Figure 5(a)). In contrast to the previous two experiments with the AAV2.6 vectors, the AAV2.5 vectors managed to silence ICA-27243-mediated hyperpolarization to a marginal response (Figure 5(b)).

Analysis of a large number of DRG neurons revealed that the RMP was significantly changed after in vivo treatment with AAV2.5-shKCNQ2-DsRed. The RMP was depolarized from -57.15 ± 2.16 mV in DRG neurons from control-treated animals to -47.64 ± 3.17 mV in DRG neurons from animals treated with the active vector. Importantly, the hyperpolarizing effect induced by ICA-27243 was significantly reduced ($p \leq 0.001$) from -10.15 ± 1.22 mV in the AAV2.5-Ctrl-DsRed neurons to -2.05 ± 0.62 mV in AAV2.5-shKCNQ2-DsRed neurons (Figure 6(a)). To assess the hyperpolarization effect induced by ICA-27243 in relation to the RMP, we calculated the relative hyperpolarization of each group (AAV2.5-Crtl-DsRed and AAV2.5-shKCNQ2-DsRed). Similar to the absolute values, we found a strong and statistically significant reduction ($p \leq 0.0001$) of the hyperpolarization from $18.69 \pm 2.68\%$ in the control group down to $5.55 \pm 1.67\%$ in the group with the active silencing vector.

As a control experiment, we compared the RMP and hyperpolarization by ICA-27243 of DRG neurons that were not transduced with AAV2.5-shKCNQ2-DsRed (no fluorescence, Figure 6(b)) with those that were transduced. As expected, in DRGs from animals treated with the control vector, both the transduced and untransduced neurons showed the same degree of hyperpolarization (-10.53 ± 2.03 mV and -14.04 ± 5.09 mV, respectively). In contrast, neurons successfully transduced with the active vector AAV2.5-shKCNQ2-

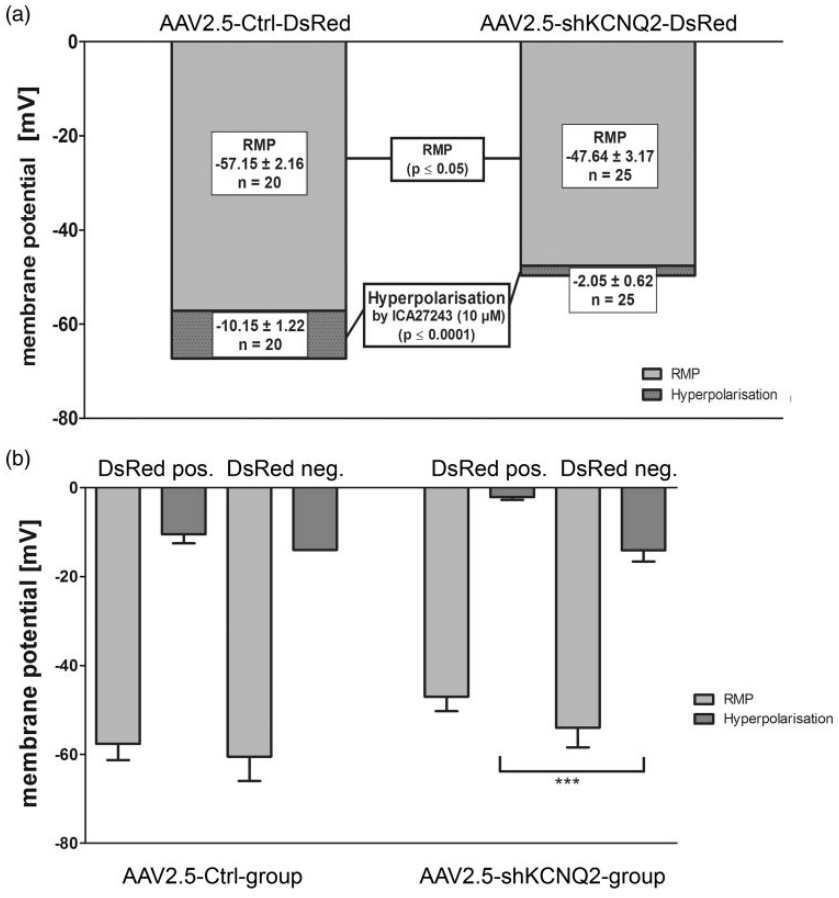

Figure 6. Resting membrane potential (RMP) and hyperpolarization by ICA-27243 (10 μM) of primary cultured DRG neurons from SD rats after in vivo transduction with AAV2.5-Ctrl-DsRed and AAV2.5-shKCNQ2-DsRed. (a) DRGs were retrieved and neurons were cultured for 14 to 42 days after intrathecal administration of the viral vector. Successfully transduced neurons were selected for the measurements by fluorescence microscopy on the basis of the expression of the reporter DsRed. (b) Comparison of transduced vs. nontransduced neurons (n = 2–24). Animals were treated with AAV2.5-Ctrl-DsRed and AAV2.5-shKCNQ2-DsRed, respectively. RMP and hyperpolarization of cultured neurons were measured in red fluorescing, DsRed positive (i.e., transduced) and nonfluorescing, DsRed negative (i.e., nontransduced) cells. ***$p \leq 0.001$ (Student's t test).

DsRed (i.e., red fluorescing cells) had a significantly ($p \leq 0.0001$) diminished hyperpolarization compared to nontransduced neurons from the same culture (-2.14 ± 0.64 mV vs. -14.09 ± 2.55 mV). As expected, treatment of the animals with the active vector AAV2.5-shKCNQ2-DsRed did not change the extent of hyperpolarization compared to the control vector in nonfluorescent (i.e., nontransduced) cells (-14.04 ± 5.09 mV and -14.09 ± 2.55 mV, respectively). These findings indicate that the reduction of hyperpolarization after AAV treatment is due to the RNAi-induced selective knockdown of *KCNQ2*.

Discussion

The neuronal potassium channel Kv7.2 encoded by the *KCNQ2* gene is an attractive target for the development of innovative analgesics. However, specific small molecule compounds that activate Kv7.2 are still lacking, and the mode of action of Kv7.2 is complex.[1] Retigabine is an enhancer of Kv7 currents that showed analgesic activity in animal models of chronic inflammatory and neuropathic pain.[46] However, retigabine is not selective among neuronal Kv7 channels and not even specific to this class of channels, as it also enhances $GABA_A$ currents.[47] ICA-27243 is a selective opener of Kv7.2/Kv7.3[7,8] and thus a valuable tool compound. It shows anticonvulsant and analgesic efficacy in several animal models of epilepsy and pain but is poorly tolerated upon repeated dosing, which led to the cessation of its clinical development.[5] Potent, selective and clinically effective enhancers of Kv7-mediated currents thus remain to be developed.

Our study therefore aimed at providing a model to test the specificity of new drug candidate substances that enhance Kv7.2/Kv7.3 channel activity. The specificity of a channel opener can be tested by eliminating the presumed target by gene silencing. While unspecific substances will retain functional activity, specific agonists will lose their activity in the absence of the target channel. Since constitutive *KCNQ2* knockout mice are not viable,[9] we chose the RNAi approach to abrogate Kv7.2 function.

As striking differences in physiological functions have been described for different rat strains,[48] we performed a comparative analysis of cell viability and determined the fraction of ICA-27243 responsive small diameter DRG neurons derived from SD and Wistar rats. DRG neurons derived from SD rats showed a more hyperpolarized RMP and a more pronounced hyperpolarization after application of ICA-27243 than DRG neurons from Wistar rats. Interestingly, responses of neuronal tissue to ischemia were reported to be much more pronounced in Wistar rats than in SD rats.[48] The enzymatic treatment required to dissociate the prepared DRGs might cause more damage to neurons prepared from Wistar rats, resulting in a higher fraction of dead neurons and a more depolarized RMP of the remaining vital neuron population. Due to the more pronounced hyperpolarization effect in DRG neurons prepared from SD rats and the fact that SD rats are widely used in behavioral models of pain, we chose to carry out all further experiments with SD rats.

For efficient transfer of genetic material to the CNS, AAV vectors have become the tool of choice because of their safety and efficiency.[22] In the present study, we used the latest generation of self-complementary, pseudotyped AAV vectors consisting of the AAV2 genome packaged in the capsid proteins of various AAV serotypes. The shRNA for silencing of the target gene *KCNQ2* was expressed under the control of the RNA polymerase III U6 promoter. In addition, the AAV vectors encoded a fluorescent reporter to allow the identification of transduced cells.

Two factors determine the success of gene transfer by AAV vectors, the use of the optimal serotype and the choice of a suitable route of application. The different AAV serotypes are known to have specific tissue tropisms.[20] For AAV-mediated gene transfer to the DRG, direct administration into the DRG and intrathecal injections are the most widely used approaches.[29] In addition, intravascular and intraperitoneal injection of specific AAV serotypes have been used, although these modes of application require the blood–brain barrier to be crossed.[35,36]

To identify the best suited serotype for our intended application, we carried out initial experiments with cell lines. We observed pronounced differences between the transduction efficiency in the nonneuronal HEK293 and the neuronal SH-SY5Y cell lines. While serotypes 1 and 2 efficiently transduced both cell lines, serotypes 3 and 6 had high transduction efficiency in the neuronal cell line, but not in HEK293 cells. A screening experiment with cultured DRG neurons also revealed high transduction efficiency and low toxicity with serotype 6. As AAV2.6 was previously shown to be suitable for in vivo applications to the DRG,[24,27] we continued our experiments with AAV vectors of serotype 6. Experiments comparing freshly prepared with thawed cryopreserved DRG neurons yielded very similar results, i.e., cryopreservation does not negatively impact on the efficiency of subsequent transduction

In additional experiments, we observed that AAV2.6 vectors not only transduced dissociated DRG neurons but also delivered the transgene to neurons in whole retrieved DRGs. This finding is in line with published results that AAV2.6 can efficiently deliver transgenes to the peripheral sensory nervous system in SD rats after intraganglionic administration.[38] The same group also reported successful gene transfer with AAV2.8 using

this route of application.[31,37] Using AAV2.5 for direct DRG injection, Samad et al.[34] reported successful transduction of approximately 45% of total neuronal profiles. This approach was sufficient to knockdown the voltage-gated sodium channel type III ($Na_v1.3$) and thereby reduced nerve injury-induced neuropathic pain.

Although direct injection of AAV vectors into the DRG is appealing, as the transduction rates are comparatively high, in the in vivo setting, this method requires very invasive procedures, and gene transfer is usually locally restricted. For our in vivo experiments, we therefore chose to apply the AAV vectors by intrathecal injection, which is rapid, less invasive, and even clinically viable.[16,24,26,32] A disadvantage of this procedure, however, is that large amounts of the viral vector are required, and the transduction efficiency was reported to be as low as 1.5%.[29] When we administered AAV2.6 vectors by intrathecal injection in vivo, we did not observe any transduced neurons at all. The reasons for this discrepancy between in vitro and in vivo transduction efficiency are currently unclear. It may indicate differences in the cell surface structure and composition of membrane proteins. Further details need to be analyzed in future experiments.

In addition, our finding points at species-specific differences in the axonal (retrograde) transport of AAV2.6 vectors into sensory neuron cell bodies after intrathecal administration. Towne et al.[24] found high levels of transduction after intrathecal injection of AAV2.6 vectors in mice, while the approach was inefficient in our experiments with rats. Another detrimental factor might be the induction of an immune response by AAV2.6 vectors. While AAV vectors normally do not induce an immune response in neuronal cells, AAV2.6 vectors (but not any other of the tested serotypes) were reported to transduce DRG satellite cells and thereby might provoke an immune response.[33]

In their study, Mason et al.[33] compared various AAV serotypes and found AAV2.5 vectors to be superior to all tested serotypes for the delivery of transgenes by direct injection into the DRG. As other groups also used serotype 5 for intrathecal application of vectors aiming at the transduction of DRG neurons,[16,28] we carried out additional silencing experiments with AAV2.5 vectors. Following three intrathecal applications, we did indeed find transduced DRG neurons expressing the reporter DsRed. In these neurons, silencing of *KCNQ2* with AAV2.5-shKCNQ2-DsRed significantly depolarized the RMP and also diminished hyperpolarization induced by ICA-27243 almost completely from 10.15 mV to 2.05 mV ($p \leq 0.001$). As a control experiment, we determined ICA-27243-induced hyperpolarization in nontransduced neurons from animals that were treated with AAV2.5-shKCNQ2-DsRed. Transduction of neurons was classified by fluorescence microscopy with a red fluorescent signal indicating a successful transduction, while the lack of fluorescence indicated that a cell was not transduced. Hyperpolarization remained unaffected in neurons that were not transduced, despite treatment with the active vector, suggesting that the reduction of hyperpolarization of successfully transduced (i.e., red fluorescing) neurons was due to silencing of *KCNQ2* by AAV2.5-shKCNQ2-DsRed.

Intrathecal administration of AAV2.5 and a subsequent in vivo incubation time period from 21 to 42 days transduced a sufficient number of DRG neurons to carry out patch-clamp measurements with no deterioration in the expression of the fluorescence marker DsRed. The long-lasting effect is a typical finding for AAV vector-mediated transgene expression in nondividing cells. However, the percentage of neurons was too low to reasonably expect behavioral consequences of the treatment, which presumably would require engagement of a larger fraction of DRG neurons. In recent publications, AAV2.9 vectors were suggested as a promising alternative to deliver genes to the DRG, as the vectors cross the blood–brain barrier allowing intraperitoneal or intravascular administration of the vector to cause transgene expression in the CNS.[35,36] However, the transduction efficiency of these routes of application is not known yet.

Throughout our experiments with freshly prepared DRG neurons, cryopreserved DRG neurons and DRG neurons prepared after in vivo application of the AAV vectors, we observed the RMP to depolarize (i.e., to become less negative) after RNAi-mediated knockdown of *KCNQ2*. This finding can easily be explained as Kv7.2/Kv7.3 contributes to M-like currents and thereby influences the RMP.[1] Knockdown of *KCNQ2* results in reduced Kv7.2/Kv7.3-mediated outward current and therefore a more depolarized RMP.

More important in the context of our study is the finding that we no longer observed Kv7.2/Kv7.3-mediated hyperpolarization after silencing of *KCNQ2*. ICA-27243 was used as a selective Kv7.2/Kv7.3 channel opener.[7,8] In untreated or control-treated DRG neurons, stimulation of Kv7.2/Kv7.3 with ICA-27243 resulted in hyperpolarization of approximately 10 mV. In contrast, no hyperpolarization was observed after administration of ICA-27243 to transduced DRG neurons after treatment with AAV vectors that express a shRNA targeting *KCNQ2*.

In summary, we established a model to investigate the selectivity of novel Kv7.2/Kv7.3 openers. RNAi-mediated silencing of *KCNQ2* almost completely abolished hyperpolarization induced by a selective Kv7.2/Kv7.3 opener. This system was stable in freshly prepared, as well as in cryopreserved DRG neurons, i.e., the freeze/thaw procedure is not detrimental to the study of Kv7.2/Kv7.3 function. In addition, DRG

neurons taken into culture after in vivo application of the silencing vector also yielded comparable results. An unexpected finding was that the suitability of AAV serotypes for efficient gene transfer may vary substantially between in vitro experiments in neuronal cell lines as well as cultured primary DRG neurons and in vivo applications by intrathecal injections. This needs to be taken into account when establishing appropriate test systems. Our in vivo experiments demonstrate that intrathecal administration of AAV2.5 vectors can be used for silencing of a target gene in DRG neurons. DRG neurons prepared from these animals are suitable for patch-clamp experiments to analyze changes in functional properties induced by specific channel openers.

Author Contributions

MV contributed to the experimental design, performed all in vivo and patch-clamp experiments, and wrote parts of the manuscript. AW and VR produced the AAV vectors. JB carried out in vitro silencing experiments. HF commented on the experimental design and revised the manuscript. TMT and TC initiated the studies, contributed to the experimental design, and revised the manuscript. GB and WS conceptualized the experimental design and revised the manuscript. JK designed the experiments and wrote the manuscript. All authors have read and approved the final manuscript.

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Supplemental Material

Supplementary material is available for this article.

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