Increased Expression of Hyperpolarization-Activated Cyclic Nucleotide-Gated (HCN) Channels in Reactive Astrocytes Following Ischemia

Pavel Honsa,1 Helena Pivonkova,1 Lenka Harantova,1 Olena Butenko,1 Jan Kriska,1 David Dzamba,1 Vendula Rusnakova,2 Lukas Valihrach,2 Mikael Kubista,2 and Miroslava Anderova1

Astrocytes respond to ischemic brain injury by proliferation, the increased expression of intermediate filaments and hypertrophy, which results in glial scar formation. In addition, they alter the expression of ion channels, receptors and transporters that maintain ionic/neurotransmitter homeostasis. Here, we aimed to demonstrate the expression of Hcn1–4 genes encoding hyperpolarization-activated cyclic nucleotide-gated (HCN) channels in reactive astrocytes following focal cerebral ischemia (FCI) or global cerebral ischemia (GCI) and to characterize their functional properties. A permanent occlusion of the middle cerebral artery (MCAo) was employed to induce FCI in adult GFAP/EGFP mice, while GCI was induced by transient bilateral common carotid artery occlusion combined with hypoxia in adult rats. Using FACS, we isolated astrocytes from non-injured or ischemic brains and performed gene expression profiling using single-cell RT-qPCR. We showed that 2 weeks after ischemia reactive astrocytes express high levels of Hcn1–4 transcripts, while immunohistochemical analyses confirmed the presence of HCN1–3 channels in reactive astrocytes 5 weeks after ischemia. Electrophysiological recordings revealed that post-ischemic astrocytes are significantly depolarized, and compared with astrocytes from non-injured brains, they display large hyperpolarization-activated inward currents, the density of which increased 2–3-fold in response to ischemia. Their activation was facilitated by cAMP and their amplitudes were decreased by ZD7288 or low extracellular Na+ concentration, suggesting that they may belong to the family of HCN channels. Collectively, our results demonstrate that regardless of the type of ischemic injury, reactive astrocytes express HCN channels, which could therefore be an important therapeutic target in post-stroke therapy.

Key words: astrocytes, focal and global cerebral ischemia, HCN channels, ZD7288, cortex, hippocampus

Introduction

Following central nervous system (CNS) injury astrocytes undergo changes in their morphology as well as functional properties. Within several days, they transform into reactive astrocytes, which are characterized by hypertrophied morphology and the increased expression of glial fibrillary acidic protein (GFAP), vimentin and nestin; moreover, a subset of astrocytes starts to proliferate. The functions of reactive astrocytes are numerous and diverse. They modulate the inflammatory response, produce molecules of extracellular matrix (Ridet et al., 1997), reduce the impact of ionic imbalance and separate healthy tissue from the core of the injury, thus preventing the propagation of seizure activity from the injured to the healthy tissue (Faulkner, 2004; Sofroniew and Vinters, 2009). Some of these functions directly depend on the composition of the ion channels in the astrocytic cytoplasmic membrane, which substantially change in response to CNS injury. A large number of studies has described marked...
changes in the expression of different K⁺ channels (Perillán et al., 2000; Kang et al., 2008; Lichter-Konecki et al., 2008) in response to CNS pathology, with respect to the type of CNS disorders, the proliferative activity of the astrocytes or even their distance from the site of injury (for review see, Chvatal et al., 2008). In addition, non-selective cationic channels, which are functionally coupled to sulfonylurea receptor type 1, were identified in reactive astrocytes (Chen and Simard, 2001; Chen et al., 2003). One of the potential candidates for nonspecific cationic influx into astrocytes is the family of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, which were considered to be expressed only in excitable cells; nevertheless, their transcripts have been recently identified also in primary astrocytic cultures (Li et al., 2011) and adult post-ischemic astrocytes (Rusnakova et al., 2013). This family of channels is coded by four genes, Hcn1–4, which were cloned a decade ago (Marx et al., 1999), and their protein products can form homotetramers as well as heterotetramers in the cell membrane. They are primarily permeable for Na⁺ and K⁺ in the ratio 1:4 (Biel et al., 2009; Wahl-Schott and Biel, 2008). Their expression and function has been described in neurons, where they are activated at hyperpolarized membrane potentials, and HCN2 and HCN4 are modulated by the direct binding of cAMP as a response to hormones or neurotransmitters. In neurons, they stabilize the membrane potential and are involved in the process of dendritic integration, constraining long-term potentiation and playing an important role in synaptic transmission (Lewis and Chetkovich, 2011). One of the most important features of HCN channels is their contribution to membrane potential oscillations in neuronal or cardiac cells (Biel et al., 2009; Wahl-Schott and Biel, 2008).

In this study we aimed to detect the expression of HCN channels in cortical or hippocampal astrocytes under physiological as well as ischemic conditions employing permanent focal cerebral ischemia in adult GFAP/EGFP mice or transient global cerebral ischemia in adult rats. We performed single-cell RT-qPCR, immunohistochemistry and the patch-clamp recordings to characterize the HCN channels in post-ischemic astrocytes in both types of ischemic injury on mRNA, protein and functional levels.

Materials and Methods

Induction of Focal Cerebral Ischemia (FCI) in Adult GFAP/EGFP Mice

All procedures involving the use of laboratory animals were performed in accordance with the European Communities Council Directive 24 November 1986 (86/609/EEC) and animal care guidelines approved by the Institute of Experimental Medicine, Academy of Sciences of the Czech Republic (Animal Care Committee on April 7, 2011; approval number 018/2011). All efforts were made to minimize both the suffering and the number of animals used. Experiments were performed on Wistar rats, GFAP/EGFP transgenic mice [line designation TgN(GFAP-EGFP)], in which the expression of enhanced green fluorescent protein (EGFP) is controlled by the human GFAP promoter (Nolte et al., 2001) and on Tg(Slc1a3-cre/ERT)1Nat/J/B6;129S6-Gt(ROSA)26Sor<tm14(CAG-tdTomato)Het/J (Slc1a3/Tomato) transgenic mice (Jackson Laboratory, Bar Harbor, Maine, USA).

Prior to the induction of focal ischemia, 50-day-old mice were anaesthetized with 1.5% isoflurane (Abbott, Illinois, USA) and maintained in 1% isoflurane using a vaporizer (Tec-3, Cyprane, Keighley, UK). A skin incision between the orbit and the external auditory meatus was made, and a 1–2 mm hole was drilled through the frontal bone 1 mm rostral to the fusion of the zygoma and the squamosal bone and about 3.5 mm ventrally to the dorsal surface of the brain. The middle cerebral artery (MCA) was exposed after the dura was opened and removed. The MCA was occluded by short coagulation with bipolar tweezers (SMT, Czech Republic) at a proximal location, followed by transection of the vessel to ensure permanent occlusion. During the surgery, body temperature was maintained at 37 ± 1°C using a heating pad. Sham-operated animals (controls) were subjected to the same surgery procedure, without dura opening and vessel occlusion. The numbers of mice used in the experiments were 19 in the control group (RT-qPCR, immunohistochemistry, electrophysiology), four animals at 7 days (D7) after FCI (RT-qPCR), five animals at 2 weeks (2W) after FCI (RT-qPCR, immunohistochemistry, electrophysiology) and 22 animals at 5 weeks (5W) after FCI (immunohistochemistry, electrophysiology). This MCA occlusion (MCAo) model yields small infarct lesions in the cortical region (Honsa et al., 2012, 2013).

Induction of Global Cerebral Ischemia (GCI) in Adult Rats

Wistar rats (males, 7-9-week-old, 220–260 g) were premedicated with atropin (100 μg/kg, s.c.; Biotika, Slovak Republic) and anaesthetized with sodium pentobarbital (PTB; 65 mg/kg, i.p.). The rats were intubated using a cannula tube (Abbocath-T 16G, Abbott, Sligo, Ireland) and connected to a mechanical ventilator [33.3% O₂ and 66.6% N₂; rate 60 cycles/minutes (min); animal ventilator CIV-101, Columbus Instruments, Columbus, OH, USA] with an appropriate stroke volume to maintain normocapnia. Both common carotid arteries were exposed and occluded for 15 min using aneurysm clips. During occlusion, rats were concurrently ventilated with 6% O₂ and 94% N₂ (Linde Gas, Prague, Czech Republic) as described previously (Anderova et al., 2010; Pivonkova et al., 2010). The core temperature was maintained at 37°C with a heating pad throughout the procedure and recovery from anesthesia. After the operation, the rats were injected with 2 mL saline s.c. in the hip area to prevent dehydration. They were left to survive (reperfusion period) for 2 or 5W prior to sacrifice. The animals were housed individually and allowed food and water ad libitum. Controls received exactly the same surgical procedure without artery occlusion. The numbers of rats used in the experiments were 23 in the control group (RT-qPCR, immunohistochemistry, electrophysiology), 2 animals at 2W after GCI (RT-qPCR) and 17 animals at 5W after GCI (RT-qPCR, immunohistochemistry,
The mouse cells were collected based on EGFP or Tomato fluorescence and viability. We tested the rate of contamination with non-
expressed microglial or neuronal markers. The plates were placed on a pre-cooled rack. The collected tissues were incubated with continuous shaking at 37°C for 60 min in 5 mL of papain solution (20 U/mL) and 0.2 mL DNase (both from Worthington, NJ) prepared in isolation buffer. After papain treatment, the tissue was mechanically dissociated by gentle triturating using a 1 mL pipette. Dissociated cells were layered on the top of 5 mL of Ovomucoid inhibitor solution (Worthington) and harvested by centrifugation (140 × g for 6 min). This method routinely yielded ~2 × 10^6 cells per mouse or rat brain. Cell aggregates were removed by filtering with 30 μm cell strainers (Becton Dickinson, NJ), and the cells were kept on ice until sorting.

**Collection of Single Cells**

Single cells were sorted using flow cytometry (FACS; BD Influx, San Jose, CA). The flow cytometer was manually calibrated to deposit a single cell in the centre of each collection tube. Hoechst 33258 (Life Technologies, Carlsbad, CA) was added to the suspension of cells to facilitate cell identification after the patch–clamp experiments, the slices were fixed post-fixing with 4% PFA in 0.1 M phosphate buffer (PB). Brains were dissected out, post-fixed for 3 h (for anti-HCN4 antibody) or overnight (for anti-HCN1–3) with PFA and treated with a sucrose gradient (10–20–30%) for cryoprotection. Coronal 20 or 40 μm thick slices were prepared using a microtome (HM400, Thermo Scientific Microm, Germany) or a cryostat (Leica CM1850, Leica Microsystems, Wetzlar, Germany), respectively. To facilitate cell identification after the patch–clamp experiments, the recorded cells were dialyzed with the patch pipette solution containing Alexa Fluor 488 or 594 hydrazide. The slices were fixed post-recording with 4% PFA in 0.1 M PB for 1 h and then kept at 4°C in phosphate-buffered saline (PBS). For immunohistochemical staining, the slices were washed in PBS followed by blocking of the non-specific binding sites with 5% Chemiblocker (Millipore, Billerica, MA) and 0.2% Triton in PBS. The blocking solution was also used as the diluent for the antisera. The slices were incubated with the primary antibodies at 4–8°C overnight, and the secondary antibodies were applied for 2 h. The following primary antibodies were used: rabbit anti-HCN1 (1:200; Abcam, Cambridge, UK), rabbit anti-HCN2 (1:200; Alomone Labs, Jerusalem, Israel), rabbit anti-HCN3 (1:200; Pierce, Rockford, IL, USA), rabbit anti-HCN4 (1:200, from single cells was transcribed into cDNA directly in the wells of 96-well plates. Samples were divided into two aliquots. The first aliquots were used for pre-testing, where the expression of two astrocytic markers was determined (Slc1a3 and Glut). Only cells expressing at least one marker were selected for further analysis. To increase the sensitivity of RT-qPCR, the second aliquots of cDNA from preselected cells were first preamplified. Subsequently, such pre-processed samples were analyzed for the expression of 14 genes of interest (Table 1) using the BioMark qPCR platform (Fluidigm, San Francisco, CA). All qPCR reactions were run in technical duplicates. Data were analysed in GenEx software (MultiD, Sweden).
TABLE 1: Sequences of Primers used in Single-Cell qPCR (GFAP/EGFP Mice)

<table>
<thead>
<tr>
<th>Gene</th>
<th>PubMed ID</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>Aif1</td>
<td>NM_019467.2</td>
<td>GATGAGGATCTGCGGTCCA</td>
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<td>Aqp4</td>
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<tr>
<td>Slc1a3</td>
<td>NM_148938.3</td>
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<tr>
<td>Gfap</td>
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<td>Gfapδ</td>
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<td>Hcn1</td>
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<td>Hcn2</td>
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<td>Hcn3</td>
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<td>TGCCAACCTTTTCTTTCCTG</td>
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TABLE 2: Sequences of Primers used in Single-Cell qPCR (Rats)

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<th>Gene</th>
<th>PubMed ID</th>
<th>Forward primer</th>
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<td>Cpg4</td>
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<td>TCCATAACCTCATAGGATAGC</td>
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<td>Slc1a3</td>
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<td>CTGCAACATCCACGCGTATATT</td>
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<td>Pdgfra</td>
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<td>NM_023970.1</td>
<td>TTTGCTCTTATTCTACTCTCTC</td>
<td>GCTGGCTTAGGTGACTCC</td>
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</table>

Alomone Labs), mouse anti-GFAP (1:800, Cy3-conjugate). The secondary antibodies were goat anti-rabbit IgG conjugated with Alexa Fluor 488 or 594 (Life Technologies). To visualize the cell nuclei, the slices were incubated with 300 nM 4,6-diamidino-2-phenyldiade (DAPI) in PBS for 5 min at room temperature and mounted using Aqua Poly/Mount (Polysciences, Eppelheim, Germany). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise stated. A Zeiss 510DUO LSM confocal microscope.
equipped with Ar/HeNe lasers and 40× or 63× oil objectives was used for immunohistochemical analysis. Stacks of consecutive confocal images taken at intervals of 1 μm were acquired sequentially with the two lasers to avoid crosstalk between fluorescent labels. The background noise of each confocal image was reduced by averaging four image inputs. Colocalization images and maximum z projection images were made using Zeiss LSM Image Browser.

**Preparation of Acute Brain Slices**

The rats or mice were deeply anesthetized with PTB (100 mg/kg, i.p.) and perfused transcardially with cold (4°C) isolation solution containing (in mM): 110 NMDG-Cl, 3 KCl, 23 NaHCO₃, 1.25 Na₂HPO₄, 0.5 CaCl₂, 7 MgCl₂, 20 glucose, osmolality 290 mOsm/kg. The animals were decapitated, the brains were quickly dissected out and transversal 200 μm thick slices were cut using a vibrating microtome. The slices were incubated for 30 min at 34°C in the isolation solution and then transferred to aCSF containing (in mM): 122 NaCl, 3 KCl, 28 NaHCO₃, 1.25 Na₂HPO₄, 1.5 CaCl₂, 1.3 MgCl₂, 10 glucose, osmolality ~305 mOsm/kg, where they were kept at room temperature for the duration of the experiments. Solutions were equilibrated with 95% O₂/5% CO₂ to a final pH of 7.4. Osmolality was measured using a vapor pressure osmometer (Vapro 5520, Wescor, Logan, UT).

**Patch–Clamp Recordings**

Acute brain slices were gently transferred to a recording chamber mounted on the stage of an upright microscope (Axioskop, Carl Zeiss, Oberkochen, Germany) equipped with a high-resolution digital camera (AxioCam HRC, Carl Zeiss) and electronic micromanipulators (Luigs & Neumann, Ratingen, Germany). The chamber was constantly perfused with oxygenated aCSF at a rate of 5 mL/min. All electrophysiological measurements were done at room temperature. Patch–clamp recordings were performed in the whole-cell configuration in the voltage–clamp mode using an EPC-9 patch–clamp amplifier in combination with TIDA software or an EPC-10 amplifier in combination with PATCHMASTER software (HEKA Elektronik, Lambrecht/Pfalz, Germany), as described previously (Pivonkova et al., 2010). Patch pipettes were pulled (P-97 Flaming/Brown Micropipette Puller, Sutter Instruments, Novato, CA) from borosilicate capillaries (Sutter Instruments) and filled with an intracellular solution containing (in mM): 130 KCl, 0.5 CaCl₂, 2 MgCl₂, 5 EGTA, 10 HEPES, and Alexa Fluor 488 or 594 hydrazide (0.1 mM; Molecular Probes, Invitrogen, NY); the final pH was kept at room temperature for the duration of the experiments. Solutions were equilibrated with 95% O₂/5% CO₂ to a final pH of 7.4. Osmolality was measured using a vapor pressure osmometer (Vapro 5520, Wescor, Logan, UT).

**Results**

**Reactive Astrocytes Express mRNA Coding HCN Channels**

The expression of transcripts for HCN channels is well documented in neurons throughout the entire mammalian brain (Noebels et al., 2012); however, based on in vitro studies, also glial cells, namely astrocytes, may express these channels (Li et al., 2011). To detect the expression of Hcn genes in astrocytes, we used the transgenic GFAP/EGFP mouse strain, in which only astrocytes and rarely polydendrocytes express EGFP. Adult sham-operated males of this mouse strain were used as controls and animals at two different time points after FCI as ischemic animals. We prepared single cell suspensions from the cerebral cortex (Fig. 1A), and using FACS, we collected only EGFP-positive (EGFP⁺) cells, which were analyzed for the expression of 12 genes using the BioMark qPCR platform. Cells expressing only markers of polydendrocytes (Cspg4, Pdgfra) were excluded, so we analyzed EGFP⁺ cells with astrocytic markers, such as Aqp4, Slc1a3, Gfap, Glul. The total numbers of collected and analyzed cells were 235 EGFP⁺ cells from control brains (5 animals), 183 EGFP⁺ cells from 7 days after FCI (4 animals) and 120 EGFP⁺ cells from 14 days after FCI (3 animals).

In the adult mouse cerebral cortex approximately 5% of these cells contained detectable level of mRNA for Hcn1 and Hcn3, 12% expressed Hcn2 and in 17% of EGFP⁺ astrocytes, we detected mRNA for the Hcn4 channel (Fig. 1B); nevertheless, their relative expression levels were very low in astrocytes from control mice (Fig. 1C). To avoid the possibility that only a small fraction of all cortical astrocytes from the GFAP/EGFP mouse strain was purified by FACS in the control group, we used the Slc1a3/Tomato mouse strain, in...
which almost 90% of Slc1a3 expressing astrocytes express Tomato red fluorescent protein. Slc1a3 is a gene coding excitatory amino acid transporter 1 (EAAT1), which is specifically expressed in astrocytes and thereby labels a different and even larger astrocytic population than that labeled in GFAP/EGFP mice. We isolated and analyzed 47 cells from control uninjured mouse brain of which 89.4% expressed the astrocytic markers Slc1a3 and Glul while only 10.6% expressed HCN channel mRNA. Therefore, using another transgenic mouse strain for the isolation of astrocytes proved that the low expression of HCN channels in the control mouse brain was not due to selecting a subpopulation of EGFP-positive cells from GFAP/EGFP mice.

To determine whether the number of cells expressing Hcn genes is influenced by FCI, we isolated EGFP+ cells from the injured hemisphere 7 and 14 days after FCI. As shown in Fig. 1B, the number of EGFP+ astrocytes expressing Hcn1, 2 genes markedly increased following ischemia and reached approximately 30% of analyzed cells 2W after FCI (Fig. 1B). Moreover, we analyzed the relative expression levels of Hcn genes after ischemia and discovered a strong upregulation of Hcn1–4 expression 2W after FCI. The number of analyzed cells was 235 EGFP+ cells from control brains (5 animals), 183 EGFP+ cells from 7 days after FCI (4 animals) and 120 EGFP+ cells from 14 days after FCI (3 animals). The values are presented as mean ± SEM. Statistical significance was calculated using one-way ANOVA. *P<0.05, significant; **P<0.01, very significant; ***P<0.001, extremely significant.

Immunohistochemical Identification of HCN Channels in the Mouse Cortex After FCI
To confirm the expression of HCN channels in astrocytes in the control brains and those after ischemia, we performed immunohistochemical analyses of the cortex of non-injured...
mice and those 2W and 5W after ischemia. Although we could identify astrocytes in the cortex of GFAP/EGFP mice based on EGFP expression, we stained the sections with an antibody against GFAP to distinguish weakly EGFP$^+$ polydendrocytes from astrocytes. Since it is known that in the GFAP/EGFP mouse strain a small subpopulation of GFAP$^+$/EGFP$^+$ cells are predominantly polydendrocytes (Matthias et al., 2003). HCN1 channel expression was generally very weak in the control mouse cortex (Fig. 2A). In addition to HCN1$^+$ neurons, several EGFP$^+$/GFAP$^+$ cells also expressed this protein, and based on their morphology and the absence of GFAP expression we considered these cells as polydendrocytes. Almost no EGFP$^+$ astrocytes in the control mouse cortex expressed HCN1. After 2 weeks of FCI, HCN1 protein expression in GFAP$^+$ astrocytes was comparably low as in controls (data not shown), so we further analyzed later time points after FCI. A significantly different expression pattern was found in the astrogliotic tissue surrounding the ischemic core 5W after FCI. At the site of the ischemic lesion, there were numerous hypertrophic GFAP$^+$ astrocytic processes around the lesion site (Fig. 2D). The expression pattern of HCN3 channels in the control mouse cortex was very similar to that of HCN2, i.e. predominant localization in neuronal bodies and absence in astrocytes (Fig. 2E). However, numerous reactive astrocytes in the post-ischemic tissue strongly expressed HCN3 channels (Fig. 2F). We also analyzed the expression of HCN channels in the EGFP$^+$ cells in the contralateral post-ischemic hemispheres, but we never detected the expression of any HCN channels in EGFP$^+$ cells in these regions. We were unable to detect HCN4 channels in the cortical cells at any of the analyzed time points, and these channels were expressed only in the subcortical regions (data not shown).

**Immunohistochemical Identification of HCN Channels in the Rat Hippocampus after GCI**

To visualize and detect astrocytes in the rat hippocampus we stained the sections with an antibody against GFAP. The expression of HCN1 channels in the control rat hippocampus was predominantly localized in neuronal cells and virtually absent in astrocytes (Fig. 3A). In contrast, 5W after GCI the expression of GFAP was significantly increased, and HCN1 channels were also found on the cell membrane of many reactive astrocytes (Fig. 3B). A very similar expression pattern was found for HCN2 channels, which were expressed in neuronal bodies in the stratum pyramidale in the hippocampus of control rats (Fig. 3C); however, GCI induced only a slightly increased expression of these channels in reactive astrocytes (Fig. 3D). HCN3 channels showed strong expression in the control hippocampus predominantly in the neurons (Fig. 3E), and 5W after GCI reactive astrocytes in the vicinity of the stratum pyramidale were HCN3-positive as well (Fig. 3F). HCN4 channel expression was not found in either the control or the ischemic rat hippocampus (data not shown).

Taken together, our results from immunohistochemical analyses confirmed the increased expression of HCN channels in reactive astrocytes, predominantly at later time points after both types of ischemic injury. Interestingly, although the single-cell RT-qPCR analyses showed the increased expression of Hcn1–3 mRNA in the mouse cortex after FCI, markedly increased protein expression was confirmed only for the HCN1 and HCN3 channels.

**Membrane Properties of Astrocytes After FCI in GFAP/EGFP Mice**

Since we demonstrated the presence of HCN channels on the mRNA as well as the protein level, we further focused on their functional identification/characterization using the patch–clamp technique in the whole-cell configuration. Initially, we recorded cortical astrocytes in mouse brain slices that were identified based on their EGFP expression 2W after FCI, but we did not find any significant evidence of HCN channel presence or function at this time point. Therefore, we performed further electrophysiological analyses on astrocytes at later time point, 5W after FCI, when strong protein expression was detected. In controls, 100% of EGFP$^+$ cells displayed the membrane properties of mature astrocytes, i.e. time- and voltage-independent symmetrical non-decaying passive currents (Fig. 4A top). In agreement with our previous findings 5W after FCI, 70% of recorded EGFP$^+$ cells were passive astrocytes while the rest had membrane properties attributed to polydendrocytes (Honsa et al., 2012). Cells displaying the membrane properties of polydendrocytes were excluded from further data processing. The average resting membrane potential (RMP) of astrocytes in controls was $-68.7 \pm 1.8$ mV ($n = 32$), and it shifted to more positive values of $-53.7 \pm 1.6$ mV; ($n = 16; P < 0.001$) 5W after FCI (Table 3). The astrocyte input resistance reached $115.4 \pm 9.5$ M$\Omega$ ($n = 32$) in controls and decreased to $74.4 \pm 6.1$ M$\Omega$ ($n = 16; P < 0.01$) after FCI. Their membrane capacitance increased from $15.1 \pm 3.2$ pF ($n = 23$) in controls to $44.1 \pm 10.2$ pF ($n = 12; P < 0.01$) after FCI (Table 3). Moreover, astrocytes 5W after FCI displayed hyperpolarization-activated, time- and voltage-dependent, non-inactivating inward currents (Fig. 4A, B). The astrocytic inward current increased from $73.3 \pm 29.6$ pA ($n = 20$) in controls to $251.3 \pm 38.9$ pA ($n = 16; P < 0.001$) 5W after FCI (Fig. 4C).
FIGURE 2: HCN1–3 staining in the cortex of GFAP/EGFP mice in controls and 5 weeks after focal cerebral ischemia. Brain slices were stained with anti-HCN1–3 antibodies and an antibody directed against glial fibrillary acidic protein (GFAP) in controls and 5 weeks (5W) after focal cerebral ischemia (MCAo). HCN1 channels are rarely expressed by EGFP astrocytes in the control cortex, but their expression is increased in reactive astrocytes 5W after MCAo. A similar expression pattern was found in the case of HCN2 and HCN3 channels. Arrows indicate representative cells. Scale bars: 50 μm.
FIGURE 3: HCN1–3 staining in the CA1 region of the rat hippocampus in controls and 5 weeks after global cerebral ischemia. Brain slices were stained with anti-HCN1–3 antibodies and an antibody directed against glial fibrillary acidic protein (GFAP) in controls and 5 weeks (5W) after global cerebral ischemia (GCI). Arrow indicate the HCN-positive astrocytes after ischemia (s.p., stratum pyramidale; s.r., stratum radiatum). Scale bars: 50 μm.
FIGURE 4: Inward currents triggered by focal cerebral ischemia in mouse EGFP+ cortical astrocytes. A: The inward currents in control astrocytes ($n = 20$) and 5 weeks (5W) after focal cerebral ischemia (FCI, $n = 16$) activated by 1 s hyperpolarizing pulses from a holding potential of $-60$ to $-160$ mV. Astrocytes predominantly displayed passive conductance with a linear current/voltage relationship and, 5W after FCI, additional time- and voltage-dependent non-inactivating inward currents in response to hyperpolarization.

B: Current/voltage relationship generated from the currents activated by 1 s hyperpolarizing pulses in astrocytes in controls and 5W after FCI. Note that time- and voltage-dependent, non-inactivating inward currents were present in astrocytes 5W after FCI, but not in control astrocytes.

C: Inset represents the hyperpolarization-activated inward current obtained by passive current subtraction (left), and the bar graphs indicate the average amplitudes of inward currents after passive subtraction (right) evoked by hyperpolarization to $-150$ mV for 1 s in astrocytes of controls and those 2 weeks (2W) or 5W after FCI. Statistical significance was calculated using ANOVA; ***P < 0.001, extremely significant.

D: The hyperpolarization-activated, time- and voltage-dependent, non-inactivating inward currents were reduced by 50 μM ZD7288 ($n = 5$), a blocker of neuronal $I_h$ currents.

E: Current/voltage relationship generated from the currents activated by 1 s hyperpolarizing pulses in astrocytes 5W after FCI and after the application of 50 μM ZD7288.

F: Inset represents the hyperpolarization-activated inward current obtained by passive current subtraction (left), and the bar graphs indicate the average amplitudes of inward currents after passive subtraction (right) evoked by hyperpolarization to $-150$ mV for 1 s in astrocytes after ischemia, prior to and after the application of 50 μM ZD7288 (expressed in percentages). Statistical significance was calculated using a paired t test; **P < 0.01.
This current was sensitive to 50 μM ZD7288 (Fig. 4D,E), a blocker of neuronal hyperpolarization-activated (i_h) currents (Gasparini and DiFrancesco, 1997), which reduced the current by 63.9 ± 13.9% (n = 5; P < 0.01) (Fig. 4F). Thus, the hyperpolarization-activated, time- and voltage-dependent, non-inactivating inward currents expressed in cortical astrocytes after ischemia might strongly resemble the i_h currents permeable for both K^+ and Na^+ ions observed in cortical astrocyte cultures by (Guatteo et al., 1996). Since HCN channels are known to be modulated by cAMP, the binding of which increases their conductance, we used the extracellular application of 100 μM 8-Br-cAMP. The exposure of post-ischemic astrocytes to cAMP increased the inward current evoked by hyperpolarization by 28.8 ± 5.4% in 25 cells out of 28 (P < 0.05, Fig. 5A–C) and shifted the voltage dependence of HCN channel opening towards more positive potentials (by 4.0 ± 1.6 mV, n = 25). The depolarization of astrocytes 5W after ischemia might be underlain by the downregulation of Kir4.1 protein expression (Pivonkova et al., 2010); however, an additional ion conductance, presumably for Na^+ ions, might contribute to such depolarization as well. To test this hypothesis and to reveal the possible contribution of Na^+ ions to the inward currents in astrocytes after ischemia, we partially replaced Na^+ ions in the aCSF by NMDG^+ (the Na^+ ion concentration was decreased from 150 to 75 mM). After reducing extracellular Na^+ concentration, astrocytes 5W after FCI displayed a marked decrease in the inward current amplitude by 34.9 ± 3.4% (n = 17; P < 0.001, Fig. 5D–F).

**Membrane Properties of Astrocytes After GCI in Rats**

Membrane currents were recorded from astrocytes in the CA1 region (stratum radiatum) of adult rat hippocampi in controls and rats 5W after GCI. Cells were selected for patch–clamp recordings based on their morphology, and post-recording identification using immunohistochemical staining for GFAP was carried out. In hippocampal astrocytes of controls and ischemic rats, clamping the cell membrane from the holding potential of −70 mV to values ranging from −160 to +20 or +40 mV elicited predominantly time- and voltage-independent currents with a linear current/voltage relationship (Pivonkova et al., 2010). Similarly to reactive astrocytes after FCI, the hippocampal astrocytes 5W after GCI displayed a considerable depolarization (Table 3). In controls, the average RMP of astrocytes was −75.7 ± 0.7 mV (n = 61), and it shifted to −65.9 ± 0.7 mV (n = 84; P < 0.001) 5W after GCI. In addition, hippocampal astrocytes 5W after GCI also displayed hyperpolarization-activated, time- and voltage-dependent, non-inactivating inward currents (Fig. 6A–C). The astrocytic inward current increased from 103.3 ± 32.7 pA in controls (n = 18) to 278.7 ± 21.4 (n = 49; P < 0.001) 5W after GCI (Table 3 and Fig. 6C). To confirm that the inward currents could be carried by HCN channels, we used 50 μM ZD7288 and the current was inhibited by 27.4 ± 8.4% (n = 10; P < 0.01, Fig. 6D–F). Although the application of ZD7288 caused a decrease in inward currents, no shifts in the membrane potentials were detected. Despite the fact that ZD7288 is known/used as a specific HCN channel blocker, it also inhibits/activates the functioning of several ion channels, such as Nav1.4 channels (Wu et al., 2012), NMDA-evoked currents (Klar et al., 2003) or low-threshold Ca^{2+} channels (Felix et al., 2003). Therefore, it is possible that the application of ZD7288 simultaneously activates or inhibits other channels, which could compensate for the shift towards more negative potentials caused by blocking the HCN channels.

Moreover, astrocytes display large passive membrane conductance carried by numerous types of two-pore domain potassium channels that are expressed in the astrocytic membrane. These large currents, mostly carried by K^+, could mask the activation of channels with considerably smaller...
FIGURE 5: Effect of 8-bromoadenosine 3', 5'-cyclic monophosphate (cAMP) and decreased extracellular Na⁺ concentration (dNa) on hyperpolarization–activated inward currents in post-ischemic mouse cortical astrocytes. A: The inward currents in astrocytes 5 weeks (5W) after focal cerebral ischemia (FCI, n = 16) activated by 1 s hyperpolarizing pulses from −50 to −160 mV (see the inset at the right) are potentiated by 100 μM cAMP. B: Representative current/voltage curve generated from the currents activated by 1 s hyperpolarizing pulses in a reactive astrocyte 5W after FCI during the application of normal aCSF (+) and aCSF containing 100 μM cAMP (△). C: Inset represents the hyperpolarization-activated inward current obtained by passive current subtraction (left), and the bar graphs indicate the average amplitudes (in percentages) of inward currents after passive subtraction (right) evoked by hyperpolarization to −150 mV for 1 s in astrocytes after ischemia in normal aCSF (5W) and after the application of 100 μM cAMP (cAMP). D: The inward currents in astrocytes 5 weeks (5W) after focal cerebral ischemia (FCI, n = 16) activated by 1 s hyperpolarizing pulses from −50 to −160 mV (see the inset at the right) are decreased after application of aCSF with dNa (△). E: Representative current/voltage curve generated from the currents activated by 1 s hyperpolarizing pulses in a reactive astrocyte 5W after FCI during the application of normal aCSF (+) and during the application of aCSF with dNa from 150 to 75 mM (△). F: Inset represents the hyperpolarization-activated inward current obtained by passive current subtraction (left), and the bar graphs indicate the average amplitudes (in percentages) of inward currents after passive subtraction (right) evoked by hyperpolarization to −150 mV for 1 s in astrocytes after ischemia in normal aCSF (5W) and in aCSF with dNa. Statistical significance was calculated using paired t test; *P < 0.05, ***P < 0.001.
FIGURE 6: Hyperpolarization-activated inward currents triggered by global cerebral ischemia in rat hippocampal astrocytes. A: The inward currents in astrocytes in controls (n = 18) and 5 weeks (5W) after global cerebral ischemia (GCI, n = 49) activated by 1 s hyperpolarizing pulses from a holding potential of −60 or −70 to −160 mV (see the inset at the bottom of section D). Astrocytes predominantly display passive conductance with a linear current-voltage relationship and, 5W after GCI, additional time- and voltage-dependent non-inactivating inward currents activated by hyperpolarization. B: Current/voltage relationship generated from the currents activated by 1 s hyperpolarizing pulses in astrocytes in controls and 5W after GCI. Note that time- and voltage-dependent, non-inactivating inward currents are present in astrocytes 5W after GCI, but not in control astrocytes. C: Inset represents the hyperpolarization-activated inward current obtained by passive current subtraction (left), and the bar graphs indicate the average amplitudes of inward currents after passive subtraction (right) evoked by hyperpolarization to −150 mV for 1 s in astrocytes of controls and those 5W after GCI. D: The hyperpolarization-activated, time- and voltage-dependent, non-inactivating inward currents were reduced by 50 μM ZD7288 (n = 10), a blocker of neuronal Ih currents. E: Current/voltage relationship generated from the currents activated by 1 s hyperpolarizing pulses in astrocytes 5W after GCI and after the application of 50 μM ZD7288. F: Inset represents the hyperpolarization-activated inward current obtained by passive current subtraction (left), and the bar graphs indicate the average amplitudes of inward currents after passive subtraction (right) evoked by hyperpolarization to −150 mV for 1 s in astrocytes 5W after GCI, prior to (ctrl) and after the application of 50 μM ZD7288 (expressed in percentages). Statistical significance was calculated using a paired t test; **P < 0.01; ***P < 0.001.
conductance. This was observed in our previous study in which we described the increased expression of TRPV4 channels in reactive astrocytes. These currents were masked by high potassium conductance, and the application of a TRPV4 antagonist did not result in a shift of the membrane potential. Nevertheless, blocking potassium conductance with cesium revealed TRPV4-mediated currents together with a shift of the membrane potential after blocking these currents (Butenko et al., 2012). However, we could not use cesium to block potassium conductance in our experiments with ZD7288, because cesium simultaneously blocks HCN conductance and removing potassium ions from the solutions is not possible due to the potassium conductance of HCN channels (Lau et al., 2011).

The hyperpolarization-activated current was further potentiated by the extracellular application of 100 μM cAMP by 116.3 ± 33.3% in nine cells out of 21 (P < 0.01, Fig. 7A–C); however, no shift in HCN channel opening to more positive potentials was observed. Similarly to FCI, the hyperpolarization-activated, time- and voltage-dependent, non-inactivating inward currents detected in rat astrocytes after GCI strongly resemble Ih currents, permeable for both K⁺ and Na⁺ cations, so we also decreased the extracellular Na⁺ ion ([Na⁺]ₑ) concentration from 150 to 75 mM, upon which astrocytes 5W after GCI displayed a marked decrease in their inward current amplitude by 34.7 ± 11.0% (n = 10, P < 0.05, Fig. 7D–F). In addition, the decreased Na⁺ concentration in the aCSF induced an outward current in reactive astrocytes 5W after GCI when the cells were voltage clamped at −70 mV, accompanied by membrane hyperpolarization from −65.5 ± 2.2 to -74.3 ± 1.6 mV (n = 8, P < 0.001) (Fig. 7E). These observations indicate that the inward currents in reactive astrocytes 5W after GCI are partially carried by Na⁺ ions and that reactive astrocytes display resting Na⁺ conductance.

In summary, electrophysiological analyses confirmed the presence of hyperpolarization-activated, time- and voltage-dependent, non-inactivating inward currents in reactive astrocytes after both types of ischemic injury. Their sensitivity to the HCN channel blocker ZD7288, their potentiation by cAMP and their permeability for Na⁺ ions suggest that these currents are carried by HCN channels as was anticipated based on mRNA and protein expression.

**Discussion**

In the present study we demonstrated the strong upregulation of mRNA coding the HCN channels in reactive astrocytes in the mouse cortex 2W after focal cerebral ischemia as well as in those in the rat hippocampus following global cerebral ischemia. The presence of these channels, until now described primarily in excitable cells, was also confirmed on the protein level using immunohistochemistry 5W after ischemia. Final confirmation that HCN channels contribute to the changed membrane properties of reactive astrocytes after ischemia was obtained from patch-clamp recordings. We have demonstrated that hyperpolarization-activated inward currents in reactive astrocytes are sensitive to ZD7288, cAMP and a decreased [Na⁺]ₑ concentration, thus we provide here, for the first time, evidence that HCN channels are strongly expressed by reactive astrocytes and significantly alter their electrophysiological properties at later time points following ischemic brain injury.

The single-cell RT-qPCR analyses revealed an increased number of cells expressing Hcn genes and their increased relative expression 2W after FCI or GCI. Interestingly, these changes on the mRNA level occurred with a relatively long delay after injury and lasted for a long time, since at later time points we were also able to detect Hcn transcripts. Based on these data, it seems that the onset of HCN channel expression correlates with glial scar formation (Sofroniew, 2009). Focal cerebral ischemia, the permanent and more severe type of ischemia, led to a marked increase in the number of Hcn expressing astrocytes when compared with GCI, so the levels of Hcn transcripts may correlate with the severity of ischemic damage. Moreover, the Hcn1,2 genes exhibited strong and frequent expression among all of the measured genes in reactive astrocytes 2W after ischemia, which makes them a potentially suitable marker of reactive astrocytes. Nevertheless, it is very difficult to distinguish reactive astrocytes from neural stem cells, which can be found in the proximity of the ischemic regions. Reactive astrocytes share almost all markers with neural stem cells (Buffo et al., 2008; Robel et al., 2011) and moreover, when exposed to the proper factors reactive astrocytes can adopt neural stem cells phenotype (Sirko et al., 2013). Therefore, we cannot exclude the possibility that some of the reactive astrocytes in our analyses adopted neural stem cells phenotype and also expressed increased levels of HCN channels transcripts.

Our immunohistochemical analysis in control brains confirmed that HCN channels are predominantly present in neuronal cells in the mouse cortex or the rat hippocampus (Wahl-Schott and Biel, 2008) and that they are virtually absent in glial cells with the exception of HCN1 channels, which were detected in EGFP⁺ cells with a morphology corresponding to that of polydendrocytes. Although we detected the strong expression of Hcn genes in reactive astrocytes 2W after ischemia, we were unable to confirm the presence of HCN channels at this time point in the cytoplasmic membrane of reactive astrocytes. Nevertheless, 5W after ischemia we found a number of HCN⁺ reactive astrocytes after both types of ischemia. These results are quite unique since HCN channels were known to be expressed only in excitable cells, and only the
HCN1 channel was reported to be expressed in the cortex (Biel et al., 2009). Here we detected also HCN2 and HCN3 channels after ischemia in the mouse cortex as well as in the rat hippocampus. The relatively long delay between the strong transcription of *Hcn* genes and detectable protein levels could be explained by delayed translation into protein, a
phenomenon that was previously shown, for example, in freshly isolated human circulating monocytes (Guo et al., 2008). Although the frequency and expression rates of Hcn1, 2, and 3 were increased 2W after FCI, immunohistochemistry showed the predominant presence of only HCN1 and HCN3 5W after FCI. This discrepancy could be caused by a different half-time of Hcn2 mRNA or its protein products. Alternatively, mRNA coding Hcn2 may not be translated into a protein product at all. Different types of miRNAs, which have recently been shown to regulate many downstream targets for example in the inflammatory response of astrocytes, could play a crucial role in this process (Iyer et al., 2012).

All functional studies we performed point towards delayed protein expression, since in the earlier stages after ischemia we were unable to detect currents corresponding to HCN channels. However, in brains 5W after ischemia we found many reactive astrocytes with significantly depolarized RMP and decreased RM. These altered properties suggest changes in the composition of cytoplasmic membrane channels, such as Kir4.1 or two-pore domain K+ channels as shown previously (Kucheryavkh et al., 2009; Pivonkova et al., 2010; Wu et al., 2013). The recorded inward currents, sensitive to HCN channel blocker and co-agonist, together with the demonstrated Na+ conductance, imply that the HCN channels are one of the main players in the altered membrane properties of reactive astrocytes. Thus, these channels with permeability for Na+ ions could be partially responsible for the significant depolarization of reactive astrocytes that we observed after both types of ischemia. Although the passive membrane properties as well as the hyperpolarization-activated inward currents in reactive astrocytes were very similar after both types of ischemia, we also found some differences in the sensitivity of the currents to their blockers and co-agonists. ZD7288 decreased the amplitude of the hyperpolarization-activated currents by 64% in mice, but only by 28% in rats. Since different current reductions caused by ZD7288 were not described among different HCN homotetramers (Stieber, 2005), we suppose that different sensitivities to ZD7288 might be caused by an unusual composition of HCN channels in rodent reactive astrocytes. Another difference between reactive astrocytes after both types of ischemia was observed in the potentiation of hyperpolarization-activated currents by cAMP. In reactive astrocytes after FCI, cAMP increased the amplitudes of the HCN currents only by 29% in almost all astrocytes (in 25 cells out of 28), while in rat reactive astrocytes following GCI, cAMP application markedly increased the current amplitude by 116%, but only in 9 astrocytes out of 21. Since it is well known that cAMP increases the conductance of channels composed of HCN2 or 4 (Altomare et al., 2003), we hypothesize that these isoforms might be enriched predominantly in reactive astrocytes in the rat. However, this presumption was not confirmed by immunohistochemistry, which could be explained by the different properties of HCN channels in astrocytes; nevertheless, similar cell type-specific differences in ion channel properties were already shown, for example, in NMDA receptors, which have strikingly different properties between neurons and astrocytes (Dzamba et al., 2013; Palgyin et al., 2011). Interestingly, the activation of HCN channels by cAMP resulted in the shift of the voltage dependence of HCN channel opening towards more positive potentials only in reactive astrocytes after FCI (by ~4.0 mV, n = 25). This phenomenon could be caused by the concomitant activation of inwardly rectifying K+ channels, which could entirely counteract this effect (Roy and Sontheimer, 1995). Additionally, the HCN channel brain-specific auxiliary subunit TRIP8b, which is in part responsible for the correct membrane targeting of these channels, was shown to inhibit the cAMP-induced shift of voltage gating of HCN channel (Hu et al., 2013).

The reduction of HCN current amplitudes in reactive astrocytes after a decrease of the [Na+]i concentration was comparable after both types of ischemia, which suggests their similar Na+ conductance. The conductance of HCN channels is carried by K+/Na+ at a ratio of 4:1 (Wahl-Schott and Biel, 2008), which explains why the reduction of the [Na+]i concentration had a relatively small impact on the amplitude of inward currents. Moreover, the intracellular concentration of Na+ in astrocytes is higher than in neurons (Black et al., 2010), which could result in a lower influx of Na+ through HCN channels into astrocytes when compared with neurons.

Taken together, it seems that the strong expression of HCN channels is a common feature of reactive astrocytes at later stages after ischemic brain injury and that therefore these channels may play an important role in the regeneration of ischemic tissue. Hyperpolarization-activated cyclic nucleotide-gated non-selective cation channels are important regulators of neuronal physiology, contributing to passive membrane properties, such as resting membrane potential and input resistance, and to intrinsic oscillatory activity and synaptic integration. But what is the real purpose of this massive HCN channel upregulation in reactive astrocytes? As mentioned before, reactive astrocytes protect the CNS tissue, especially in the initial phase after ischemia, by taking up excitotoxic glutamate (Sidoryk-Wegrzynowicz et al., 2011) and protecting nervous tissue from oxidative stress (Liddell et al., 2006). At later stages, astrocytes facilitate blood brain barrier repair (Tian et al., 2011), stabilize extracellular homeostasis, reduce the seizure threshold (Stanimirovic et al., 1997) and limit the spread of inflammatory cells (Karimi-Abdolrezaee and Billakanti, 2012). It is obvious that the HCN channels are involved in the later processes, possibly contributing to ionic homeostasis maintenance by enabling Na+ and K+ influx into astrocytes or by...
Reducing the [Na\(^+\)] concentration and therefore preventing seizures, since total tissue sodium levels are elevated in stroke (Thulborn et al., 2005) or in brain tumor tissues (Ouwerkerk et al., 2003). Moreover, Na\(^+\)/K\(^+\) ATPase activity within astrocytes appears to depend on a constant Na\(^+\) influx through Na\(^+\) channels, and their role may be a prerequisite for the viability of these cells (Sontheimer et al., 1994). We hypothesize that the upregulated expression of Na\(^+\) permeable channels within astrocytes may provide a compensatory mechanism that supports Na\(^+\)/K\(^+\) ATPase-dependent ionic homeostasis in areas of CNS injury (Black et al., 2010). Membrane depolarization due to HCN channel activity might also activate a part of the low/high threshold voltage-gated Ca\(^{2+}\) channels, which are expressed in post-ischemic astrocytes (Chung et al., 2001; Westenbroek et al., 1998), and thus enhance Ca\(^{2+}\) signaling within astrocytic networks in response to injury. Additionally, HCN channels were also shown to play an essential role in cell proliferation as in the case of embryonic stem cells (Lau et al., 2011), and since we showed that astrocytes proliferate at later time points after ischemia (Anderova et al., 2010), these channels could play a role in controlling the proliferation of reactive astrocytes.

In view of the fact that HCN channel blockers have been recently introduced into human medicine as analogs of beta blockers or calcium channel blockers in the treatment of, e.g., angina pectoris—Ivabradine (Borer, 2003), our study shows for the first time another possible target for these drugs during ongoing stroke, when reactive astrocytes are impaired. We will discuss these ideas more extensively in the following references and suggest that including HCN channel blockers in the treatment of stroke could be an interesting approach to improving the outcome of ischemia lesions upregulate sodium channel Nav1.5. Brain 133:835–846.

References


cloning of a putative voltage- and cyclic nucleotide-gated ion channel progressive.


