Role of hyperpolarization-activated cyclic nucleotide-gated channel HCN2 in embryonic neural stem cell proliferation and differentiation

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A B S T R A C T
Hyperpolarization-activated cyclic nucleotide-gated channels (HCN channels) are involved in spontaneous activity in many electrically active cell types such as cardiomyocytes and neurons. In this study, the role of HCN channels in proliferation and migration of Nestin and Sox2 expressing embryonic neural progenitor cells (NPC) originating from the subventricular zone (SVZ) was examined. Immunostaining and PCR data showed that the HCN2 subtype was highly expressed in these cells. Patch clamp recordings revealed a hyperpolarization-activated current, which was sensitive to inhibitors of HCN channels. Using the fluorescence dye bis-(1,3-dibutylbarbituric acid)-trimethineoxonol (DiBAC(4)(3)) we found that a prompt reduction of the extracellular K+ concentration, or exposing the cells to acute hypoxia, induced an instant hyperpolarization in the whole cell population. Recovery from low K+ induced hyperpolarization after extracellular calcium removal, or by re-oxygenation of hypoxic cells, was sensitive to ZD7288, a HCN channel inhibitor. Treatment of neurosphere cultures from the SVZ with ZD7288 caused a significant and reversible inhibition of neurosphere formation from single cells indicating that proliferation of progenitor cells was reduced. Furthermore, the migration of neuronal cells from neurospheres was considerably retarded in the presence of ZD7288. The results suggest that HCN2 channels are involved in controlling the proliferation of NPC and that HCN2 channel-induced spontaneous electrical activity may trigger the motility response of neurosphere-derived neurons in concert with other ion channels. Furthermore, the response to hypoxia suggests that HCN2 channels may trigger the chemotactic response of NPC to ischemic brain regions seen in many studies.

1. Introduction
HCB channels regulate pacemaker activity in the heart, rhythmic activity in the thalamus and a variety of other brain functions such as dendritic integration and synaptic functions (Biel et al., 2009). In the larger dorsal root ganglion (DRG) neurons, they are involved in both inflammatory and neuropathic pain (Acosta et al., 2012; Dini et al., 2018). HCN channels belong to the superfamily of voltage-gated pore loop channels (Ludwig et al., 1998). These, Na+/K+ permeable channels, are activated by hyperpolarization and deregulated by depolarization. The voltage-dependency of channel opening involves regulation by intracellular cyclic adenosine monophosphate (cAMP) (Chen et al., 2001; Wainger et al., 2001), intracellular pH (Biel et al., 2009; Zong et al., 2001) and chloride concentration (Biel et al., 2009).

The HCN channels are encoded by four genes (HCN1–4) and are widely expressed throughout the heart and the central nervous system (CNS) (Rivolta et al., 2020). The expression pattern of HCN channels, (HCN1–4), has been studied in several species. HCN1 is highly expressed in the neocortex, hippocampus, cerebellar cortex, brainstem and spinal cord (Notomi and Shigemoto, 2004). HCN2 is nearly ubiquitously expressed across the CNS but especially abundantly in hippocampus, thalamic and brainstem nuclei (Benarroch, 2013; Santoro et al., 2000; Vasilyev and Barish, 2002). Interacting proteins, protein kinases and smaller molecules regulate the expression of HCN channels (He et al., 2014). Expression of HCN channel isoforms is upregulated in a variety of pathological states including epileptic seizures and chronic pain (Biel et al., 2009; Sartiani et al., 2017).

Some studies indicate a significant role of HCN channels in neuronal development. HCN channels are abundantly expressed in the mouse brain during embryonic development (Seo et al., 2015) and these channels contribute to spontaneous network activity in embryonic neurons (Klueva et al., 2012). It has also been reported that the cell cycle and proliferation of neural stem cells (NSC) are regulated by HCN
channel-mediated spontaneous calcium oscillations (Johard et al., 2020). Furthermore, differentiation of PC12 neuroblastoma cells is promoted by HCN channel activity (Zhong et al., 2019).

Neonatal brain ischemia causes significant alterations in brain development and may be an etiologic factor for the development of neuropsychiatric disease (Basovich, 2010) on the one hand but the development and may be an etiologic factor for the development of experiments consisted of (in mM): 137 NaCl, 5 KCl, 2 CaCl2, 2.2 solutions containing 500 nM DiBAC4(3) and the fluorescence signal was monitored. The fluorescence intensities of the experiments were converted into the corresponding membrane potential from a linear standard curve where fluorescence values for the standard K+ solutions contained 0.44 KH2PO4, 0.44 KH2PO4, 10 glucose, 10 HEPES, and 0.5 MgCl2. The pH was adjusted to 7.35 with NaOH. Low K+ medium contained 0.44 KH2PO4 (medium from which the KCl had been omitted). All experiments were performed at 37 °C under normal atmospheric conditions (21% O2, 0.038% CO2 in air). Hypoxic HBM was prepared by bubbling 100% N2 gas. 2.3. Cell culture and cell differentiation

Neural progenitor cells (NPCs) were generated as previously described (Clarke et al., 2000). Cells were scraped from the anterior portion of the lateral wall of the lateral ventricles of E14 embryonic mouse brains. The cells were grown as free-floating aggregates termed neurospheres. Dissociated cells were plated in DEME/F-12 “complete” culture medium (Gibco) containing 2 mM L-glutamine, 15 mM HEPES, 100 U/ml penicillin, 100 μg/ml Streptomycin (all from Sigma-Aldrich), B27 supplement (Gibco), 20 ng/ml epidermal growth factor (EGF, PeproTech EC Ltd, London, UK), 10 ng/ml basic fibroblast growth factor (bFGF, PeproTech EC Ltd, London, UK) and maintained in a humidified 5% CO2/95% air incubator at 37 °C. Within 3–5 days, the cells grew as free-floating neurospheres and were passaged after mechanical dissociation. For neuronal differentiation, neurospheres were plated on poly-DL-ornithine (Sigma-Aldrich) coated culture plates in the absence of growth factors. Withdrawal of growth factors induces spontaneous migration of cells from the neurospheres and subsequent differentiation. The differentiated neural progenitor cells selected for measurement of membrane potential were single cells migrating towards the periphery, outside the neurosphere body. The total amount of cells in a typical neurosphere cluster varied between 300 and 1000 cells.

2.4. Measurement of membrane potential

Changes in the resting membrane potential of mice neural progenitor cells differentiated for 3 days were monitored using the potentiometric bisoxonol dye (DiBAC4(3)), an anionic probe that, exhibits enhanced fluorescence upon cell membrane depolarization (increased intracellular fluorescence do to dye influx). Conversely, hyperpolarization of the membrane potential leads to efflux of the probe and a decrease in fluorescence intensity. The advantage of using DiBAC4(3) as compared to patch clamp is that changes in membrane potential can be simultaneously recorded from a large cell population. Cells cultured on 25 mm round coverslips were washed 3 times with HBM pH 7.35, placed in the measuring chamber and transferred to the heat controlled chamber holder on the microscope (Nikon, Diaphot 200 inverted microscope, 20× objective). For the experiment, 500 nM DiBAC4(3) was added to the perfusion solution (HBM pH 7.35) and allowed to equilibrate across the cell membrane for 15 min (1 ml/min) before the data acquisition process was started. The cells were excited with 470 nm wavelength light for 80 ms (rate of data capture 30/min) and the emitted fluorescence captured at 530 nm. The data was collected and further processed with the Origin 6.0 (OriginLab Corp., Northampton, MA) software. At the end of each experiment, the cells were treated with depolarizing (20, 40 or 140 mM) K+ solutions containing 500 nM DiBAC4(3) and the fluorescence signal was monitored. The fluorescence intensities of the experiments were converted into the corresponding membrane potential from a linear standard curve where fluorescence values for the standard K+ solutions were plotted against the calculated membrane potential values. Membrane potential (E) was calculated using the Nernst equation assuming an intracellular potassium concentration of 140 mM and a temperature of 37 °C. E = 2.393 × RT/zF × log10 ([K+]i/[K+]).

2.5. Patch clamp recordings

Neural stem cells were differentiated for 3 days, in the absence of growth factors, in poly-L-ornithine coated 35 mm Petri dishes in a

| Abbreviations |
|----------------|
| HCN channels | Hyperpolarization-activated cyclic nucleotide-mediated channels |
| NPC | neural progenitor cells |
| SVZ | subventricular zone |
| (DiBAC4(3)) | bis-(1,3-dibutylbarbituric acid)-trimethineoxonol |
| ZD7288 | 4-Ethylphenylamino-1,2-dimethyl-6-methylamino pyrimidinium chloride |
| DRG | dorsal root ganglion |
| cAMP | cyclic adenosine monophosphate |
| CNS | central nervous system |
| HBM | HEPES buffered medium |
| PBS | phosphate-buffered saline |
| Tuj 1 | neuronal class III β-tubulin |
| VGCC | voltage-gated calcium channels |
| BSA | bovine serum albumin |

The HEPES buffered medium (HBM) used in the ion imaging experiments consisted of (in mM): 137 NaCl, 5 KCl, 2 CaCl2, 2.2 NaHCO3, 10 glucose, 10 HEPES, and 0.5 MgCl2. The pH was adjusted to 7.35 with NaOH. Low K+ medium contained 0.44 KH2PO4 (medium from which the KCl had been omitted). All experiments were performed at 37 °C under normal atmospheric conditions (21% O2, 0.038% CO2 in air). Hypoxic HBM was prepared by bubbling 100% N2 gas directly into the solution for a minimum of 20 min at 37 °C. When O2 had been removed (dissolved O2 < 0.5 mg/l), the 50 ml tube was capped and the perfusion tube was connected to a valve hole at center of the cap. A Jenway Dissolved Oxygen Meter having a Clark type polarographic sensor was used to measure the oxygen concentration in the solution. A pH of 7.4 was maintained in the buffer solution after bubbling with N2 gas.

2.3. Measurement of membrane potential

Changes in the resting membrane potential of mice neural progenitor cells differentiated for 3 days were monitored using the potentiometric bisoxonol dye (DiBAC4(3)), an anionic probe that, exhibits enhanced fluorescence upon cell membrane depolarization (increased intracellular fluorescence do to dye influx). Conversely, hyperpolarization of the membrane potential leads to efflux of the probe and a decrease in fluorescence intensity. The advantage of using DiBAC4(3) as compared to patch clamp is that changes in membrane potential can be simultaneously recorded from a large cell population. Cells cultured on 25 mm round coverslips were washed 3 times with HBM pH 7.35, placed in the measuring chamber and transferred to the heat controlled chamber holder on the microscope (Nikon, Diaphot 200× inverted microscope, 20× objective). For the experiment, 500 nM DiBAC4(3) was added to the perfusion solution (HBM pH 7.35) and allowed to equilibrate across the cell membrane for 15 min (1 ml/min) before the data acquisition process was started. The cells were excited with 470 nm wavelength light for 80 ms (rate of data capture 30/min) and the emitted fluorescence captured at 530 nm. The data was collected and further processed with the Origin 6.0 (OriginLab Corp., Northampton, MA) software. At the end of each experiment, the cells were treated with depolarizing (20, 40 or 140 mM) K+ solutions containing 500 nM DiBAC4(3) and the fluorescence signal was monitored. The fluorescence intensities of the experiments were converted into the corresponding membrane potential from a linear standard curve where fluorescence values for the standard K+ solutions were plotted against the calculated membrane potential values. Membrane potential (E) was calculated using the Nernst equation assuming an intracellular potassium concentration of 140 mM and a temperature of 37 °C. E = 2.393 × RT/zF × log10 ([K+]i/[K+]).

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humidified 5% CO\textsubscript{2}/95% air incubator at 37 °C. The cell population in the Petri dish was perfused with room temperature recording solution at a flow rate of 0.5 ml/min. Signals were filtered at 3.3 kHz and sampled at 10 kHz using an EPC patch clamp amplifier (HEKA Electronic Lambrrecht, Germany, Pulse softet software). The extracellular solution was composed of (in mmol/l) 137 NaCl, 5 KCl, 2 CaCl\textsubscript{2}, 0.5 MgCl\textsubscript{2}, 10 glucose and 10 HEPES (pH 7.4 adjusted with NaOH). Pipette solution was composed of (in mmol/l) 140 KCl, 5 MgATP, 5 EGTA, 10 HEPES (pH 7.2 adjusted with KOH). Patch electrodes (model PG-150T, Harvard Apparatus, Kent, UK) were prepared with a PC-10 puller and flamed polished with microforge MF-900 (Narishige, London, UK) to a resistance of 3–4 MΩ when filled with standard internal solution. Liquid junction potential was corrected offline. Results were analyzed and graphed using the OriginPro 8.5 software. HCN2 channel recordings was performed in whole-cell configuration. HCN currents were elicited by using a hyperpolarizing pulse step protocol from a holding potential of −60 mV to −140 mV in 20 mV steps for 1.52 s).

2.6. Reverse transcription – Polymerase chain reaction (RT-PCR)

Total RNA was isolated from mouse embryonic neural stem cells using RNAeasy Mini Kit (Qiagen). The RNA (2 μg) was reverse transcribed using Transcriptor High Fidelity cDNA Synthesis Kit (Roche). Primers were adapted from Franz et al. (2000) (Franz et al., 2000).

HCN1: forward: 5′-TCTTGGCAGTTACGCCCTT-3′
reverse: 5′-TTTTCTGGCTACCCCGTCG-3′
HCN2: forward: 5′-TACTTGCTAGCTGTGTCTG-3′
reverse: 5′-GAAATAGGAGCCATCCGACA-3′
HCN3: forward: 5′-GCATCCAGCAGTACTACGA-3′
reverse: 5′-CACCCTCAGCGCCTTACCC-3′
HCN4: forward: 5′-TCTGATCATCATACCCGTG-3′
reverse: 5′-GAAGACCTCAGGACAAT-3′
PCR: Initial denaturation 3 min at 95 °C, after which denaturation was performed for 30 s at 95 °C, annealing for 30 s at 55 °C, and elongation at 72 °C for 1 min (35 repeats). Negative controls included omission of cDNA. PCR products were separated by 110V (40 min) by gel electrophoresis with ethidium bromide and photographed under ultraviolet illumination.

2.7. Immunocytochemistry

Cells differentiated for 3 days were fixed for 20 min at room temperature using 4% paraformaldehyde (Sigma) in phosphate-buffered saline (PBS, pH 7.4). Cells were permeabilized with PBS containing 0.1% Triton X–100 and 1% bovine serum albumin (BSA) (all from Sigma), for 20 min at room temperature. Blocking for unspecific staining was done for 20 min with PBS containing 0.1 M glycine and 1% albumin. Cells were incubated with primary antibodies in PBS containing 1% BSA at room temperature for 1 h. After washing, secondary antibodies were applied for 1 h at room temperature in the dark in 1% BSA-PBS. The primary antibodies used to stain migrating neurons and radial glia progenitor cells were neuronal class III b-tubulin (TUJ1), IgG (MMS-435P, Covance) and anti GLAST, (glial glutamate-aspartate transporter, ab416, Abcam, Cambridge, UK). The secondary antibody used was goat anti-mouse IgG (HL) Supercornbiant Secondary Antibody, Alexa Fluor 488, ThermoFisher Scientific (A28175). Rabbit polyclonal antibody from Life Technologies Europe BV (OSR00120W) was used to detect HCN2. Secondary antibody used was Alexa Fluor 555 goat anti-rabbit IgG (HL) (Life Technologies Europe BV (A21428). To identify neural stem/progenitor cells we used as primary antibodies rabbit monoclonal antibody to Nestin (ab221660) and mouse monoclonal antibody to Anti-Sox2 (ab79351), both from Abcam. For secondary antibody control, primary antibody was omitted, resulting in no fluorescent staining. Cells were mounted using ProLong Diamond Antifade Mountant containing 4′,6-diamidino-2-phenyl-indole (DAPI) for nuclear staining (Invitrogen) and viewed and photographed using an Olympus AX70 Provis microscope, 20 × (numerical aperture 0.50) or 40 x (numerical aperture 0.75) object magnification, equipped with a DP71 high resolution color digital camera (Olympus). A background reading was obtained from areas devoid of cells. This reading was reduced from fluorescence intensity values.

2.8. Neurosphere formation assay

For the assay, a suspension of single cells was seeded in 24-well plates (2 ml/well) and incubated for 3 days in DMEM/F-12 “complete” culture medium (see the section Cell culture and Cell differentiation). Neurospheres formed in the cultures were visualized and photographed by using an Axiovert 135 inverted microscope equipped with a Zeiss AxioCamHRm digital camera (10x objective). The diameter, of the neurospheres formed in the culture (indicative of proliferation potential), was measured by using the ImageJ software. To determine the cell viability of cells treated for 72h with ZD7288 we used a live/dead fluorescent assay (Molecular Probes/Invitrogen) containing Ethidium homodimer-1 and Calcein acetoxymethyl ester. Control and ZD7288-treated cells were incubated with 2 μM of each reagent for 20 min, visualized and photographed with a Fluorescence microscope Ex 475/Em 535 for Calcein-AM (stained live cells green) and Ex 535 nm/Em 620 for EthD-1 (stained dead cells orange).

2.9. Neuronal differentiation assay

For neuronal differentiation neurospheres were plated on poly-dl-ornithine- (Sigma) coated culture dishes in the absence of EGF and FGF. Growth factor withdrawal induced spontaneous neuronal differentiation. After 3 days, images were taken by using an Axiovert 135 inverted microscope equipped with a Zeiss AxioCamHRm digital camera (20x objective). The distance of cell migration was measured by using the ImageJ software.

2.10. Measurement of intracellular Ca\textsuperscript{2+}

Cells differentiated for 3 days on poly-ornithine coated 25 mm round coverslips were loaded with 2 μM Fura-2-AM (Invitrogen, Carlsbad, California, USA) in HBM for 20 min at 37 °C and subsequently transferred to a perfusion chamber. For high potassium induced calcium signaling, NaCl was iso-osmotically replaced with 40 mM KCl. The imaging experiments were performed using an Incyt\textsubscript{6} fluorescence imaging system (Intracellular Imaging, Cincinnati, OH). The cells were perfused in HBM at 37 °C and excited by alternating wavelengths of 340 and 380 nm using narrow band excitation filters. Fluorescence was measured through a 430 nm dichroic mirror and a 510 nm barrier filter with a Cohu CCD camera. One ratiometric image was acquired per second and on-line ratio values were converted to [Ca\textsuperscript{2+}], by using a calibration curve. The data collected was analyzed with the Incyt 4.5 software and further processed with Origin 6.0 software (OriginLabCorp.).

2.11. Statistics

Data are presented as means ± S.E.M (n = number of cells, N = number of independent experiments). In each imaging experiment 60–99 individual cells, located from the edge of the neurosphere body to the outer edge of the culture, were randomly chosen. Each experimental condition was repeated at least three times with independent preparations of neural progenitor cell. In all imaging experiments, membrane potential was allowed to decline for 3 min followed by calcium removal to initiate the recovery phase. Cell migration and cell proliferation (neurosphere diameter) results are presented as Box-and-whiskers plot; “box” depicting the median (mean indicated by a dot), and the 25th and 75th quartiles and the “whisker” showing the 5th and 95th percentile. One-way ANOVA and Tukey's post hoc test were performed to test for statistical significance (p < 0.05).
3. Results

3.1. Neurosphere cultures as a model to study neural stem/progenitor cell proliferation and physiological properties of differentiated cells

To study HCN channels in neurosphere-derived neuronal cells, neurospheres were plated on poly ornithine in the absence of growth factors (EGF and FGF), and allowed to differentiate for 3 days. Two morphologically distinct populations of cells emerged from the neurosphere. A thick inner layer of cells staining with radial glial markers and an outer layer containing neuron-like bipolar cells, which frequently exit from the inner layer and move in an apparent random fashion (Jansson et al., 2012; Louhivuori et al., 2013) (Fig. 1.).

These two cell types are easily distinguished by their different morphology, immunostaining and response to neurotransmitters. The cells have been characterized in previous studies and essentially they consist of cells staining for radial glia markers glutamate aspartate transporter (GLAST), brain lipid-binding protein (BLBP), Nestin, epidermal growth factor receptor (ErbB1) and metabotropic glutamate receptor 5 (mGluR5) and neuronal cells staining for microtubule associated protein 2 (MAP2), neuron specific class III beta-tubulin (Tuj1), NEUronal Nuclei; clone A60 (NeuN) and α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Jansson et al., 2011, 2012; Louhivuori et al., 2013, 2015, 2020). The different glutamate receptor subtype expression allows us to separate the cell types also in functional experiments using calcium imaging or intracellular pH (Nordstrom et al., 2019). The neurosphere-derived cell population used in this study can be considered to be neural stem/progenitor cells since they stain positive for the two most well-described neural progenitor markers Nestin (type IV intermediate filament) and Sox2, a transcriptional factor which is essential for maintaining self-renewal/proliferation/pluripotency of undifferentiated embryonic stem cells and multipotency of neural stem cells (Fig. 2).

3.2. Expression of HCN channels in differentiating neural stem cells

RT-PCR showed that mRNA for HCN2 was present in the cell population after 3 days while expression of the other HCN channel subtypes was not detected (Fig. 3A). Immunostaining with an antibody to HCN2 verified that the channel protein was abundantly expressed in neuron-like unipolar and bipolar cells and proliferating progenitor cells (Fig. 3B and C). Co-staining with TUJ-1, a neuronal marker indicated that the channel protein was mainly expressed in the cell soma.

Fig. 1. Immuno- and DAPI staining showing expression of GLAST (red) and Tuj-1 (green) in neural stem cells differentiated for 3 days.

Fig. 2. Expression of Nestin and Sox2 in differentiated neural stem cells. Immuno- and DAPI staining showing expression of (A) Nestin (red), (B) Sox2 (green) in neural stem cells differentiated for 3 days. (D). Overlay image of the Nestin and Sox2 stained cells. No fluorescence was seen when the primary antibodies were omitted. Images were taken using a 40x objective.
Patch clamp recordings from neuronal cells, outside the dense radial layer, in the whole-cell configuration supported these findings. The inward current generated by applying negative voltages in 20 mV steps for 1.5s triggered an instantaneous current that could be blocked by the HCN channel blocker ZD7288 (50 μM). As shown in Fig. 2 D and E, applying negative voltages, in 20 mV steps from a holding potential of −60 mV, triggered a linear inward current that was sensitive to both Cs⁺ (1 mM) and ZD7288 (50 μM). These results supported the view that HCN2 channels mediate hyperpolarization-activated currents in differentiated neural stem cells. Representative whole-cell currents in a cell patched in the absence (left trace) and presence (right trace) of 50 μM ZD7288. Currents were evoked by hyperpolarizing from −60 to −140 mV for 1.5 s in 20 mV steps from a holding potential of −60 mV. (E) Current-voltage relationship (I/V) in control cells (■) or cells patched in the presence of 1 mM Cs⁺ (▲) or 50 μM ZD7288 (●). Data are presented as means ± SEM from three independent experiments.

3.3. Rapid recovery in membrane potential after low K⁺ induced hyperpolarisation is sensitive to ZD7288, a potent HCN channel inhibitor

We have previously shown that the resting membrane potential of neurosphere-derived neuronal cells is hyperpolarized upon reduction in extracellular K⁺ (Nordstrom et al., 2019, Nordstrom et al., 2020). By using DiBAC4(3)-equilibrated cells, to measure membrane potential, we observed that cells perfused with modified HBM pH 7.35 containing 0.44 mM K⁺ induced hyperpolarization in the neural stem cell population that was sensitive to extracellular calcium. Reduction in extracellular calcium enhances the activity of many nonselective cation channels (Formenti et al., 2001; Hablitz et al., 1986; Smith et al., 2004). Removal of all extracellular Ca²⁺, to uncover potential-activated Na⁺ permeable channels, initiated a prompt depolarization in 50% of the cells (from −98.9 ± 2.2 mV to −51.2 ± 4.0 mV) (Fig. 4A). Likewise, lowering the extracellular K⁺ in the absence of extracellular calcium induced a transient hyperpolarization (Nordstrom et al., 2019). When cells were kept hyperpolarized in the low K⁺-medium, the recovery of membrane potential after calcium removal was blocked by 50 μM ZD7288 (from −100 ± 3.5 mV to −96.5 ± 2.5 mV). ZD7288 did not alter the DiBAC4(3) response under basal conditions. The inhibition was reversible since washout of ZD7288 resulted in rapid recovery in membrane potential (Fig. 4B). To characterize the cell population that showed strong membrane potential recovery after ZD7288 washout, we used kainate, a strong activator of ionotropic glutamate receptors present on migrating neuronal cells (Jansson et al., 2013). Cells responding to kainate, giving a transient depolarization signal, showed much weaker membrane potential recovery after ZD7288 and Ca²⁺ removal (Fig. 4C, blue trace). On the other hand, cells not responding to kainate, showed strong recovery after ZD7288 removal (Fig. 3C, red trace).
3.4. Re-oxygenation induced recovery in membrane potential in cells hyperpolarized by hypoxic conditions is sensitive to ZD7288

We have previously shown that acute hypoxic conditions triggers hyperpolarization in neural stem cells, a hyperpolarization that recovers by re-oxygenation (Nordstrom et al., 2012). As shown in Fig. 5A, exposing the cell population to oxygen-free HBM buffer induced hyperpolarization of the membrane potential. Perfusion of the cells with normoxic HBM buffer induced an immediate recovery in the membrane potential (from $-93.5 \pm 2.87$ mV to $-68.8 \pm 3.80$ mV). On the contrary, when cells were re-oxygenized in the presence of 50 μM ZD7288 only a modest recovery could be seen (from $-95.3 \pm 1.9$ mV to $-90.3 \pm 1.87$ mV) (Fig. 4B). Washout of ZD7288 allowed the cells to recover from the hyperpolarization.
3.5. ZD7288 sensitive HCN2 channel activity is important for neural progenitor cell proliferation

As shown above the HCN2 channel appears to be widely expressed in neural progenitor cells. To study the impact of HCN2 channel activity on neural stem cell proliferation, single cell suspensions of neural stem cells were prepared and incubated for 72h in the presence of various concentrations of ZD7288. As shown in Fig. 6, 10 μM ZD7288 already
affected neural stem cell proliferation (neurosphere formation). The average neurosphere diameter (μm ± S.E.M) in the cultures treated with 100, 50, 25 or 10 μM ZD7288 was 40.81 ± 1.0, 52.97 ± 1.54, and 77.48 ± 2.73, 84.43 μm ± 2.78 μm, respectively, as compared to 148 ± 4.18 and 178.41 μm ± 4.55 μM in control and DMSO treated cultures. In cultures treated with 100 μM ZD7288 only small irregular-shaped spheres could be observed.

Our imaging experiments indicated that the inhibitory effect observed with ZD7288 (50 μM) was reversible. The results obtained from our stem cell proliferation experiments supported this view. Washout of ZD7288 from the cell culture allowed cells to resume proliferation and neurosphere growth (Fig. 7). The average diameter of the neurosphere formed in these experiments (total incubation time of 144 h), were also considerably larger than the one observed after 72h incubation. In control cultures, DMSO, a molecule known to stimulate proliferation in some cell types, slightly increased the neurosphere diameter. By using a Live/Dead cell viability assay, we found that cells treated with 50 μM ZD7288 for 72h were alive (showing esterase activity giving intracellular Calcein staining and no Ethidium bromide homodimer-1 uptake).

3.6. The HCN channel inhibitor ZD7288 affects neural stem cell differentiation

Neural stem cell differentiation was studied by culturing neurospheres for 3 days in the absence of mitogens (see methods section). A typical cell differentiation pattern with apparently randomly moving neuron-like cells is shown in Fig. 8A. As shown in Fig. 8B, the presence of 10 μM ZD7288, induced stellate shaped migration pattern from the neurosphere body. Very few cells moved longer distances and most cells appeared attached to the neurosphere by their processes. In the presence of a higher concentration, 100 μM, their migration distance significantly shortened (Fig. 8C). The average migration distance (μm) in control (DMSO) culture was 179.4 ± 11.9 as compared to 104.2 ± 3.4 and 66.5 ± 2.4 in cells treated with 10 and 100 μM ZD7288.

3.7. Use of calcium imaging to assess ZD7288 specificity

To evaluate whether ZD7288 interfere with T-type calcium channels (low-voltage activated channels) that are expressed in our neural progenitor cell population (Louhivuori et al., 2013), we next performed calcium imaging experiments on cells differentiated for three days. As shown in Fig. 9A, the presence of 50 μM ZD7288 only modestly affected high extracellular K+ induced intracellular calcium increase (Ca2+ increase by 54.8 ± 5.1% as compared to 61.5 ± 7.9% in control). On the
other hand, subsequent perfusion with high K⁺ solution containing 10 μM mibebradil, a specific T-type calcium channel inhibitor, largely blocked K⁺ induced calcium increase (8.45 ± 3.2% increase in Ca²⁺).

4. Discussion

The results of the present study indicate that the hyperpolarization-activated channel, HCN2, is of importance for the proliferation and migration of embryonic neural stem cells. HCN channels have previously been shown to be of importance in the development of hippocampus (Seo et al., 2015), hair cells of the inner ear (Yoshimoto et al., 2015) and differentiation of spiral ganglion cells (Li et al., 2016). HCN2 channels are expressed in neural stem cells of the SVZ in agreement with the data presented here (Johard et al., 2020). Based on the experiments in the present study, using the membrane potential indicator dye DiBAC₄(3), differentiated cells (neurons and glia-like cells) show ZD7288-sensitive HCN channel activity. Although not quantified a large proportion of cells also showed immunoreactivity to HCN2. Interestingly, our electrophysiology recording on cells expressing native HCN2 channels, indicated that a ZD7288 and Cs⁺ sensitive instantaneous inward current was activated within a few milliseconds after applying hyperpolarizing voltages. In most studies the current profile of HCN2 appears to be biphasic consisting of a transient instantaneous current Iᵢ瞬 (HCN2) and a slowly activating current The Iᵢ瞬 (HCN2) current has been shown to be specifically associated with HCN2 expression in recombinant systems (Macri and Accili, 2004; Proenza et al., 2002). However, the HCN2 current and it’s I/V relationship seems to show a significant variation depending on the cells studied (Santoro et al., 2000) with the instantaneous current Iᵢ瞬 (HCN2) being dominant in cells from some brain areas and a monophasic current in others. Furthermore, HCN channels are regulated by a variety of factors including interacting proteins and ionic composition (Biel et al., 2009). For instance co-expression of the Mink-related peptide 1 (MrpP1), which interact with many channel proteins, leads to a monophasic current profile similar to that seen in this study (Proenza et al., 2002).

In agreement with a study performed on rat hippocampal neurons (Hyllienmark and Brismar, 1999), we found that exposing differentiated neuron- and glia-like cells to acute hypoxia, triggers a prompt hyperpolarization of the membrane potential. However, the mechanism for the recovery phase in hippocampal neurons during normoxic conditions remained unclear. In this work, we present evidence that the recovery in membrane potential in cells differentiated from embryonic neural stem cells is to most part due to activation of HCN2 channels. Furthermore, ischemia induces the expression of HCN channels in neurons and glia (Honsh et al., 2014; Park et al., 2019) suggesting that these channels may be involved in neuroprotective responses. It has, indeed been shown in several studies, that neural stem cells migrate towards the ischemic area after an insult (Park et al., 2006; Jin et al., 2005; Kelly et al., 2004; Kim et al., 2004). It seems plausible that this migratory behavior is dictated by HCN channel-mediated spontaneous activity in the progenitor cells.

Spontaneous calcium fluctuations in adult neural stem cells have previously been shown to be sensitive to blockers of HCN channels (Johard et al., 2020). Furthermore, blockade of HCN2 channels with ZD7288 has been reported to provide neuroprotection against oxygen-glucose deprivation induced ischemia/reperfusion injury (Chen et al., 2020). Our results indicate that the reperfusion (re-oxygenation) induced repolarization phase overshoots do to sodium influx via HCN2 channels and this might make the cells more susceptible to glutamate induced intracellular calcium overload and excitotoxicity. Oxygen-glucose deprivation is associated with acute cell swelling do to Ca²⁺ sensitive influx of Na⁺ and increased extracellular glutamate concentration (Goldberg and Choi, 1993). In agreement with this study, we also found a calcium sensitive repolarization phase in cells hyperpolarized by low K⁻-medium. The repolarization phase is most likely do to unhindered Na⁺ influx through HCN2 channels in the absence of Ca²⁺. In the presence of ZD7288, this rapid recovery in membrane potential is inhibited. These findings are in contradiction with the results published by Vay et al. (2020) where they reported that ZD7288 by itself induces cell depolarization and a slow increase in intracellular calcium in primary microglia cells. Generally, in excitable cells, membrane hyperpolarization activates HCN channels and causes a depolarizing, ZD7288-sensitive inward current. Obviously, HCN2 channels present in non-excitable primary cells, respond differently to ZD7288.

Inhibition of HCN channels caused a significant reduction in cell proliferation. Fluctuation in membrane potential and K⁺ channel activity has been implicated in the control of cell-cycle progression (Sachs et al., 1974; Urrego et al., 2014). Depolarization increases proliferation by promoting S/G2/M progression while hyperpolarization correlates with reduced proliferation due to arrest in G1/G0 and differentiation. Here, we present evidence that HCN2 play a critical role in regulation of cell proliferation in neural progenitor cells. Supporting this assumption are our findings that HCN2 is present and functionally active in our cell population and that the inhibitor ZD7288 attenuated progenitor cell proliferation in a concentration-dependent manner. ZD7288-sensitive HCN channels has previously been shown to regulate cell cycle progression in a mouse embryonic stem cell lines derived from blastocysts R1/E (Omelyanenko et al., 2016), mouse embryonic stem cell lineage D3.
et al., 2021). This is in line with a recent study on adult neural progenitor cells (Johard et al., 2020). Adult NSC cells in S/G2/M express HCN channel activity while this activity is lacking in G1/Go (Johard et al., 2020). Interestingly, blocking of voltage-gated calcium channels (VGCC) also causes an arrest in G1/Go (Zeitler et al., 1997) and expression of T-type currents is low in Go and increases in the S phase (Guo et al., 1998; Kuga et al., 1996). A coupling between VGCC and HCN channels in neurosphere-derived cells is thus very likely. Previous reports has shown that high concentrations of ZD7288 might interfere with voltage gated T-type calcium channels 

e.g. in sperm cells and hippocampal pyramidal cells (Felix et al., 2003; Sanchez-Alonso et al., 2008). In our hands, triggering of Ca2+ influx in differentiated cells by addition of depolarizing concentrations of extracellular K+ generated a mibebradil sensitive calcium influx that was only modestly affected by 50 μM ZD7288. Thus, as suggested by Sanchez-Alonso, the potency of ZD7288 in blocking T-type calcium channels might be cell type specific. Additionally, ZD7288 is known to interfere with sodium channels in DRG neurons and Na,1.4 channels expressed in HEK293 cells (Wu et al., 2012). However, proliferating undifferentiated neural stem cells do not express Na+ channels or lack functional Na+ channels (Li et al., 2008). Na+ channel expression increase during the neuron maturation process and the expression of neuronal voltage-gated Na+ channels is a hallmark of neuronal differentiation and necessary for generation of action potentials in functional neurons (Biella et al., 2007; Mirsadeghi et al., 2017). Na channel expression in cultures similar to ours is not present at 3 days of differentiation but appears after 5–6 days and seen only in neurons with differentiated morphology (Louhivuori et al., 2013). Thus, in our cell system, HCN2 channels are the most likely target for ZD7288-mediated inhibition on stem cell proliferation.

The results presented here also indicate that HCN channel activity is of importance for the migratory behavior of neurosphere-derived neuronal cells. While control cells exit from the neurosphere to move randomly, cells with neuronal morphology (bipolar cells) stay close to the neurosphere border when the HCN channel is blocked. Interestingly blocking of T- and L-type VGCC also prevents the exit of neuronal cells from the neurosphere (Louhivuori et al., 2013). Previous studies have shown that HCN2 channels are required for normal patterning of the developing brain (Pai et al., 2018) indicating a role of HCN channels in neuronal migratory activity (or neuronal guidance by glia-like cells). Furthermore, the gliomerular formation of in the olfactory system requires HCN channels (Nakashima et al., 2013). However, in these experiments, an effect of ZD7288 on Na+ channels present in differentiated cells, cannot be completely excluded.

Our results indicate that HCN channels are involved in the regulation of the cell cycle of neural progenitor cells. Furthermore the motility of neuronal cells and thus probably also fundamental properties of developing neurons such as migratory responses seem to depend on functional HCN channels. The resting potential of neurons exiting neurospheres range from −68 to −75 mV, which means that this potential, or potentials slightly more negative to this threshold value, would be sufficient to trigger HCN2 channel activation (Nordstrom et al., 2012). The involvement of HCN channels in the response of NPCs to hypoxia, as shown in this study, is of considerable interest because of the chemotactic response triggered by ischemic areas. This mechanism is likely to be a significant mechanism of repair of small ischemic injuries of the nervous system.

Author contribution statement

Tommy Nordström performed the experiments (except for RT-PCR experiments that was performed by Jarno Hörhà, performed data analysis and wrote the preliminary manuscript. Leif Andersson and Karl Åkerman participated in the design of the study and contributed to the writing of the final version of the manuscript.

Declaration of competing interest

All authors declare no conflicts of interests.

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