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POTASSIUM CHANNEL EXPRESSION IN ADULT MURINE NEURAL PROGENITOR CELLS

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Abstract—Neural progenitor cells (NPCs) are a source of new neurons and glia in the adult brain. Most NPCs reside in the forebrain subventricular zone (SVZ) and in the subgranular zone of the dentate gyrus, where they contribute to plasticity in the adult brain. To use their potential for repair, it is essential to identify the molecules that regulate their growth, migration and differentiation. Potassium (K^+) channels are promising molecule candidates for NPC regulation as they are important components of signal transduction and their diversity is ideal to cover the complex functions required for cell proliferation and differentiation. There is increasing evidence that K^+ channels influence cell growth and neurogenesis, however, very little is known regarding K^+ channel distribution in NPCs. We therefore explored the expression of a variety of voltage-gated (Kv), inwardly rectifying (Kir) and two-pore (K2P) K^+ channels in the SVZ of adult mice and in neurosphere cultures of NPCs during growth and differentiation. Immunocytochemical analysis revealed a differential expression pattern of K^+ channels in nestin$^+$ SVZ precursor cells, early SVZ doublecortin$^+$ neurons and (sub)ependymal cells. These findings were confirmed in neurosphere cultures at the protein and mRNA levels. The expression of some K^+ channel proteins, such as Kir4.1, Kir6.1, TREK1 or TASK1, suggests a role of K^+ channels in the complex regulation of NPC proliferation, maturation and differentiation.

Key words: potassium channel, neural progenitor cells, subventricular zone, rostral migratory stream, doublecortin, neurospheres.

Adult neural progenitor cells (NPCs) are promising tools for the treatment of neurodegenerative diseases, brain and spinal cord injury, multiple sclerosis, or stroke (e.g Pluchino et al., 2003; Hofstetter et al., 2005). Beside self renewal, these cells are endowed with the ability to generate neuronal and glial cells, and most of the NPCs proliferate in the subventricular zone (SVZ) of the adult mammalian forebrain (Doetsch et al., 1999; Garcia et al., 2004). NPCs are commonly cultured in the presence of epidermal and fibroblast growth factor (EGF/FGF2) leading to clonal expansion of neurospheres (Reynolds and Weiss, 1992). In order to increase the yield in neuronal cells for brain repair, it is essential to identify the molecules and signal cascades that regulate NPC growth, migration and differentiation.

Potassium (K^+) channels are a group of promising molecule candidates for NPC regulation as they are important components of the signal transduction machinery in almost all cells of the mammalian body. The channels form highly regulated pores in cell membranes and can be divided into three structural classes: voltage-gated K^+ channels (Kv), inwardly rectifying K^+ channels (Kir) and two-pore 'background' (K2P) channels (Coetzee et al., 1999). At least 75 different K^+ channel genes have been identified to date (Caterall et al., 2002). Distinct temporal and spatial expression, multiple splice variants and the ability to form hetero-oligomers, underscore the impressive diversity of K^+ channels, which may permit the complex regulation needed to control cell proliferation and differentiation. The molecular diversity of K^+ channels allows for the development of highly specific drugs, which target selective cell types or functional systems. Interestingly, spadin was recently identified as the first K2P channel inhibitor and natural anti-depressant peptide, which enhances hippocampal neurogenesis (Mazella et al., 2010).

There is increasing evidence that K^+ channels influence cell growth, maturation and neurogenesis. Changes in K^+ channel activity are required for proliferation at critical cell cycle checkpoints (DeCoursey et al., 1984; Gallo et al., 1996; Wonderlin and Strobl, 1996; MacFarlane and Sontheimer, 2000; Pardo, 2004; Vautier et al., 2004). Pharmacological channel modulation revealed that K^+ current changes may be not only concurrent with, but necessary for progression through the cell cycle (Pappas et al., 1994; MacFarlane and Sontheimer, 2000; Chittajallu et al., 2002; Liebau et al., 2006; Yasuda et al., 2008). It has long been known that K^+ channels are expressed at neural induction (Ribera, 1990). Moreover, lack of certain functional Kv channels results in megencephaly mice with increased proliferation, neurogenesis and enhanced hippocampal cell survival (Almgren et al., 2007). Recently,

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Abbreviations: BMPs, bone morphogenetic proteins; DCX, doublecortin; EGF, epidermal growth factor; FGF2, fibroblast growth factor; GFAP, glial fibrillary acidic protein; Kir, inwardly rectifying K^+ channels; Kv, voltage-gated K^+ channels; K2P, two-pore K^+ channels, K^+, potassium; NPCs, neural progenitor cells; RMS, rostral migratory stream; SVZ, subventricular zone.

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Yasuda et al. (2008) demonstrated that the differentiation of NPCs into neuroblasts in the rodent SVZ is accompanied by changes in the resting membrane potential and down-regulation of Kir channel expression. However, a detailed analysis of K^+ channel expression and function in NPCs does not exist.

We therefore explored the expression of a variety of voltage-gated, inwardly rectifying and two-pore K^+ channels in the adult murine SVZ and in neurosphere cultures using immunocytochemistry, Western blotting, gene expression profiling by microarray and real-time PCR, as well as transient transfection experiments. A differential expression pattern of K^+ channels was observed during growth and differentiation of NPCs, which may contribute to the complex regulation of NPC behavior and lineage determination. Some channels, such as Kir4.1, Kir6.1, TREK1 or TASK1, might selectively participate in SVZ neurogenesis and K^+ channel variability during the cell cycle.

EXPERIMENTAL PROCEDURES

NPC culture—neurospheres

Adult mouse neurospheres were derived from the SVZ using a standard experimental protocol (Galli et al., 2008). All animal procedures were performed according to the local guidelines for animal research. Briefly, SVZ of C57Bl/6 mice (~8 weeks old) were isolated, diced and enzymatically dissociated with 0.05% trypsin–EDTA for 7 min at 37 °C, washed, and triturated into a single cell suspension. Cells were plated in serum-free NBMA medium supplemented with retinoic acid-free B27, L-glutamine, 20 ng/ml EGF and 10 ng/ml basic FGF2 (all substances from Invitrogen, San Diego, CA, USA). Primary SVZ cells were plated at a density of 3.5×10^3 cells/cm^2 in low-attachment culture flasks (Nunc, Roskilde, Denmark), incubated for 7 days at 37 °C and 5% CO_2 to allow neurosphere formation. The secondary neurospheres or subcultures hereof (passage 2) were dissociated into single cells and used for differentiation assays, or immunochemical studies. Differentiation was induced by removal of growth factors (EGF, FGF2).

Immunochemistry

Mice were deeply anesthetized and perfused transcardially with PBS followed by 4% paraformaldehyde in PBS. Brains were dissected, fixed in 4% paraformaldehyde at 4 °C overnight, cryoprotected with 30% sucrose, shock-frozen in hexane at −70 °C, and stored at −80 °C. Frozen tissues were cut into 20 μm sections on a cryostat and washed in PBS. Sections were preincubated in 10% normal goat/donkey serum in PBS containing 0.3% Triton X-100. Sections were washed three times in PBS and incubated at 4 °C for 36 h in primary antibody solutions (10% normal goat serum, 0.3% Triton X-100). K^+ channel proteins were detected using the following primary antibodies: rabbit Kv1.1 [1:1000] and Kv1.4 [1:100] (Veh et al., 1995); Kv1.5 [1:150] (generously provided by Prof. H.G. Knaus; Koschak et al., 1998); mouse Kv1.6 [1:500] (NeuroMab, Davis, CA, USA); rabbit Kv2.1 [1:100] (Sigma, Munich, Germany); Kv4.2 [1:500] (NeuroMab); Kv4.3 [1:500] (NeuroMab); Kir1.1 [1:100] (Alomone, Jerusalem, Israel); Kir2.1 [1:250], Kir2.2 [1:100], Kir2.3 [1:100] and Kir2.4 [1:1000] (Prüss et al., 2005); Kir3.1 [1:500] and Kir3.2 [1:200] (Eulitz et al., 2007); Kir4.1 [1:1000] (Chemicon, Billerica, MA, USA); Kir4.2 [1:50] (Alomone); Kir5.1 [1:100] (provided by Dr. Veh); Kir6.1 [1:700] and Kir6.2 [1:100] (Thomzig et al., 2005); Kir7.1 [1:2000] (Derst et al., 2001); TASK1 [1:100] (Alomone); goat TASK3 [1:100] (Santa Cruz Biotechnology, Santa Cruz, CA, USA); TWIK1 [1:200] (Santa Cruz); rabbit TWIK2 [1:100] (Alomone); goat TREK1 [1:1000] (Santa Cruz); TREK2 [1:500] (Santa Cruz); TRAAK [1:250] (Santa Cruz). Further antibodies: goat/rabbit doublecortin (DCX) [1:500] (Santa Cruz); mouse class III beta tubulin (TuJ-1) [1:500] (Covance, Princeton, NJ, USA); mouse nestin [1:200] (Chemicon); rabbit glial fibrillary acidic protein (GFAP) [1:200] (Dako, Hamburg, Germany); mouse RIP [1:5000] (Chemicon).

After two washes, sections were incubated in PBS-A (2 mg bovine serum albumin in 1 ml PBS) for 1 h and subsequently treated with the secondary antibody (biotinylated IgG, 1:2000 in PBS-A; Vector Laboratories, Burlingame, CA, USA) at room temperature for 16 h. Two further washes were followed by avidin-biotin complex (Elite ABC, 1:1000 in PBS-A; Vector Laboratories) at room temperature for 6 h. After another three washes and preincubation in 3,3′-diaminobenzidine solution (Sigma, Munich, Germany) containing 0.5 mg 3,3′-diaminobenzidine in 1 ml 50 mmol/L Tris–buffer, pH 7.6, 10 mmol/L imidazole and 0.3% ammonium nickel sulfate. Peroxidase activity was visualized for 3 min after addition of H_2O_2 (0.015% final concentration). Omission of primary antibodies served as negative controls.

For immunofluorescence staining, brain sections or coverslips with cultured cells were treated with the primary antibodies as described. After 16 h incubation with secondary antibodies at 4 °C (Alexa 488-/594-IgG 1:500; Invitrogen, Darmstadt, Germany), sections or coverslips were mounted with Mowiol 4-88 (Hoechst, Paris, France) and examined using a fluorescence or confocal microscope (Leica TCS SPE, Leica Microsystems, Wetzlar, Germany).

Western blot analysis

Neurospheres were homogenized on ice in a dounce-homogenizer (Wheaton, Millville, NJ, USA) in homogenization buffer (4 mmol/L HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.4, 250 mmol/L sucrose, 1 mmol/L EDTA, 5 mmol/L sodium azide, 2 mg/ml aprotinin, 1 mg/ml pepstatin A, 1 mg/ml leupeptin and 0.5 mmol/L phenylmethylsulfonylfluoride). The homogenate was centrifuged at 2500 g for 10 min, followed by centrifugation of the supernatant at 100,000 g. The pellet was resuspended in 50 mmol/L Tris–HCl, pH 7.5, and protein concentration was determined with bicinchoninic acid assay (Pierce, Rockford, IL, USA). Homogenates were heated at 90 °C in sodium dodecyl sulfate sample buffer for 3 min. 40 μg of protein per lane was loaded on 8% sodium dodecyl sulfate-polyacrylamide gels followed by electrophoresis. After separation, proteins were electroblotted onto nitrocellulose (Schleicher & Schuell, Dassel, Germany). The membranes were blocked with 5% low-fat milk powder in PBS containing 0.5% Tween-20 for 1 h, incubated over night at 4 °C with anti-K^+ channel antibodies, secondary horseradish peroxidase-linked antibody (1:1000; Sigma, St Louis, MO, USA), and visualized with enhanced chemoluminescence (Pierce) using ImageMaster VDS-CL (Amersham, Freiburg, Germany).

Gene expression profiling

At 0 days (spheres) and 7 days of NPC differentiation of identical subculture, cells were harvested, pelleted and total RNA was prepared from tissue specimens using Trizol (Gibco BRL, Gaithersburg, MD, USA) according to the manufacturer's protocol. RNA obtained from three independent experiments was pooled for each time point. 5 μg of RNA was used for analysis with GeneChip Mouse Genome 430A 2.0 oligonucleotide microarray (Affymetrix, Santa Clara, CA, USA). Labeling of RNA targets, hybridization and calculation of gene expression levels were performed according to the manufacturer's protocol in the Charité Laboratory of Functional Genome Research.

Quantitative real-time polymerase chain reaction

At 0, 1, 2, 3, 4, 7 and 9 days of NPC differentiation, RNA was prepared as described above. Total RNA was reverse-transcribed using random hexamers and moloney murine leukemia virus re-