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THE NOTCH CO-REPRESSOR PROTEIN NKAP IS HIGHLY EXPRESSED IN ADULT MOUSE SUBVENTRICULAR ZONE NEURAL PROGENITOR CELLS

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Abstract—In the adult mammalian brain niches for neural stem cells are maintained, which enable a steady-state neurogenesis. This process is tightly regulated by multiple niche factors, including Notch and NF-κB signaling. The NF-κB-activating-protein (NKAP) has previously been shown to act as Notch co-repressor component by binding CIR and recruiting HDAC3 in T-cell development and furthermore to regulate NF-κB-dependent transcription. Here, we provide first evidence for the expression of NKAP in neurogenic cells of the adult mammalian brain. NKAP is highly expressed in Mash1$^+$ transit amplifying cells and PSA-NCAM$^+$ migrating neuroblasts throughout the subventricular zone (SVZ) and the rostral migratory stream (RMS), as well as in the hippocampus. We further show that NKAP expression levels are downregulated during the course of the RMS. Eventually, most differentiated cells in the olfactory bulb (OB) and the corpus callosum only display low levels of NKAP expression. Finally, large subsets of mature neurons in the OB, the hippocampus and the thalamus express NKAP at high levels, suggesting an additional role of NKAP outside of SVZ progenitor cells.

Key words: adult neurogenesis, NKAP, notch signaling, NF-κB signaling, mature neurons.

INTRODUCTION

Mammalian adult neurogenesis is a tightly regulated process that persists in the hippocampal dentate gyrus (DG) and the forebrain subventricular zone (SVZ) (Zhao et al., 2008; Faigle and Song, 2012). In the DG new neurons are produced by neural stem cells (NSCs) residing in the subgranular zone and designated mostly for local integration in the granule cell layer (GCL) of the DG. Instead, in the SVZ GFAP$^+$/Nestin$^+$ astrocyte-like NSCs (type B cells) generate a mobile PSA-NCAM$^+$ neuroblasts cell type (type A cells) via amplification through local Mash1$^+$ transit amplifying cells (type C cells). Along the rostral migratory stream (RMS) neuroblasts migrate toward the olfactory bulb (OB), where they differentiate into interneurons in the GCL and the glomerular layer (GL). Besides neuronal differentiation, RMS cell derived oligodendrogenesis in the corpus callosum (CC) has also been described (Menn et al., 2006; Walker et al., 2007).

The Notch signaling pathway is of outstanding importance for the regulation of adult SVZ neurogenesis (Pierfelice et al., 2011). Canonical Notch signaling, through the DNA binding protein RBPj (also: CBF1), is involved in the regulation of stem cell maintenance, proliferation, fate decision processes and, in more differentiated cells, in the regulation of cellular and synaptic plasticity. Although there is still some debate in the field, it seems to be the case that most, if not all, SVZ cell types express Notch receptors, indicating a widespread potential for Notch signaling in the SVZ (Stump et al., 2002; Nyfeler et al., 2005; Givogri et al., 2006; Carlen et al., 2009; Wang et al., 2009; Basak et al., 2012). This raises the question, how cell context specific expression of Notch target genes, like glial fibrillary acidic protein (GFAP) expression or the repression of Mash1 by Hes transcription factors, is mediated, if most SVZ cell types have the ability to respond to Notch signaling. However, it is conceivable that the different SVZ cell types could differ in their

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Abbreviations: CC, corpus callosum; CIR, CBF1-interacting co-repressor; DG, dentate gyrus; dpi, days post infection; DMEM, Dulbecco's Modified Eagle's Medium; EDTA, ethylenediaminetetraacetic acid; GAD67, glutamate decarboxylase 1; GAPDH, glyceraldehydes-3 phosphate dehydrogenase; GCL, granule cell layer; GFAP, glial fibrillary acidic protein; GL, glomerular layer; GST-Pi, glutathione S-transferase Pi; HDAC3, histone deacetylase 3; Hes, hairy/enhancer of split; LV, lateral ventricle; Mash1, mammalian achaete-scute homolog 1; MCL, mitral cell layer; NeuN, neuronal nuclei antigen; NF-κB, nuclear factor of kappa light chain gene enhancer in B cells; NKAP, NF-κB activating protein; NSCs, neural stem cells; Olig2, oligodendrocyte lineage transcription factor 2; PSA-NCAM, polysialylated-neural cell adhesion molecule; RBPj, recombination signal-binding protein for immunoglobulin kappa j region; RMS, rostral migratory stream; OB, olfactory bulb; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SVZ, subventricular zone; TH, tyrosine hydroxylase; TuJ1, neuron-specific class III beta-tubulin.

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potential to respond to Notch receptor activation. This can be achieved by modifications downstream of the Notch receptor, where the Notch intracellular domain competes with Notch co-repressor molecules for the binding to RBPj (Borggrefe and Liefke, 2012).

Recently, the NF-κB activating protein (NKAP) has been described to directly interact and co-localize with the known Notch co-repressors CIR and HDAC3 in the regulation of mammalian T-cell development, resulting in repression of Notch target genes (Pajerowski et al., 2009). Other phenotypes in hematopoiesis mediated through loss of NKAP, though, seemed to be independent from Notch signaling, indicating additional, yet unknown, mechanisms (Pajerowski et al., 2010; Hsu et al., 2011). But also the eponymous signaling pathway of the NKAP protein, the NF-κB pathway, was shown to have various pro- and anti-neurogenic functions in the regulation of adult neurogenesis (Denis-Donini et al., 2005; Widera et al., 2006; Worlitzer et al., 2012). In this pathway NKAP was shown to act as activator (Chen et al., 2003).

First evidence of an implication of NKAP in neurogenesis came from an RNAi screen, which showed that the knockdown of the *Drosophila melanogaster* gene CG6066, an NKAP ortholog, leads to overproliferation of *D. melanogaster* neural precursor cells resulting in lethal tumor formation (Neumuller et al., 2011). Therefore, we decided to investigate, if NKAP is expressed in the mouse brain, with focus on the neurogenic region of the SVZ.

EXPERIMENTAL PROCEDURES

Material

The following antibodies were used: anti-GAD67 (Sigma–Aldrich, St. Louis, MO, USA), rabbit anti-GAPDH (Abcam, Cambridge, UK), mouse anti-GFAP (Millipore, Billerica, MA, USA), mouse anti-GFP (Santa-Cruz, Santa Cruz, CA, USA), mouse anti-GST-Pi (BD Bioscience, Franklin Lakes, NJ, USA), mouse anti-Nestin (BD Bioscience), rabbit anti-NKAP (1:50–1:100, Sigma–Aldrich, HPA000916), mouse anti-NeuN (Millipore), mouse anti-Mash1 (BD Bioscience), mouse anti-Olig2 (Millipore), mouse-anti PSA-NCAM (Millipore), mouse-anti-Tuj1 (Covance, Princeton, NJ, USA), rabbit-anti-Tuj1 (Covance), mouse anti-TH (Millipore), mouse anti-α-tubulin (Sigma–Aldrich). Alexa-fluorophore conjugated antibodies (Invitrogen, Carlsbad, CA, USA) were used as secondary antibodies for immunofluorescence staining. DNA was stained with Hoechst 33342 (Invitrogen).

The following plasmids were used: pMX-EGFP (kindly provided by Prof. Hans Schöler, Münster), pcDNA-DEST53-EGFP-NKAP, pcDNA-Dest47-EGFP-NKAP, pMO93-FLAG-IRES-EGFP-NKAP.

Mice

All mice were kept under standard conditions according to governmental rules and regulations and were of 3–6 month of age at the time point of experiment. C57/BL6N mice and transgenic GFAP-EGFP (Nolte et al., 2001) reporter mice were used.

RNA isolation, cDNA generation and NKAP cloning

Total C57/BL6N mouse brain mRNA was isolated (RNeasy Mini Kit, Qiagen N.V., Hilden, NRW, Germany) and NKAP cDNA was produced using SuperScript II Reverse Transcriptase (Invitrogen). Flanking *attB* sites (*italic primer parts*) for Gateway technology (Invitrogen) were added to NKAP cDNA using SYBR Green Jump Start Taq ReadyMix for Quantitative PCR (Sigma–Aldrich) via PCR reaction (Primers: (1) *ggggacaagttt gtacaaaaaagcaggctca*-accatggctcctgtatcgggctcgcgta; (2) *ggggaccactttgtacaagaaagctgggtt*-cttgtcatccttccctttggtcttt). Subsequently, the PCR product was recombined into pMO93 gateway destination vector (kindly provide by Prof. Manuel Grez, Frankfurt), pDEST53-EGFP (Invitrogen) or pDest47-EGFP (Invitrogen) using Gateway technology following the manufacturer's instructions.

Western blotting

HEK293T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum, 2 mM L-Glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin and transfected using Turbofect (Fermentas, Glen Burnie, MD, USA) following the manufacturer's instructions. Cells were lysed 48 h after transfection (in 50 mM Tris, pH 7.5, 0.5 M NaCl, 1% NP-40, 1% DOC, 0.1% sodium dodecyl sulfate (SDS), 2 mM EDTA, 1× Complete Protease Inhibitor (Roche Grenzach-Wyhlen, Germany)), incubated for 30 min (4 °C), then centrifuged for 30 min (4 °C, 13,000 rpm (Centrifuge 5417R, Eppendorf AG, Hamburg, Germany)) and the supernatant was used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blotting.

NSC culture

NSCs were cultured as previously described (Conti et al., 2005). Murine E12.5-E14.5 embryonic brain-derived NSCs were grown on poly-D-Lysine-coated dishes in NSC maintenance medium consisting of NS-A medium (Euroclone, Milan, Italy) supplemented with 10 ng/ml b-FGF2 (Peprotech, Rocky Hill, NJ, USA), 10 ng/ml EGF (Peprotech), 1× N2-Supplement (Invitrogen), Pen/Strep (Invitrogen) and L-Glu (Invitrogen). Neuronal differentiation was induced as described previously (Hillje et al., 2011). Briefly, the medium was changed to Neurobasal medium (Invitrogen), supplemented with 1× N2-Supplement (Invitrogen), 1× B27 Supplement (Invitrogen), Pen/Strep (Invitrogen) and L-Glu (Invitrogen).

Retrovirus production and NSC transduction

For retrovirus production TLA-HEK293T cells (Open Biosystems, Huntsville, AL, USA) were transfected with the packaging plasmid PCL-ECO (kindly provided by Prof. Manuel Grez, Frankfurt) and the transfer vector of