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Temporal and spatial expression pattern of Nnat during mouse eye development

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ABSTRACT

Background: Neuronatin (Nnat) was initially identified as a highly expressed gene in neonatal mammalian brain. In this study, we analyze the spatial and temporal expression pattern of Nnat during mouse eye development as well as in the adult.
Methods: The expression of Nnat was analyzed on mRNA as well as protein level. The presence of Nnat transcripts in the adult retina was examined using reverse transcription-polymerase chain reaction (RT-PCR). Nnat protein expression was evaluated by Western blot and immunohistochemistry during eye development at embryonic day (E) 12, 15, 16 and postnatal day (P) 7, 14, 30 and 175 (adult).
Results: Immunohistochemical studies of the developing mouse eye revealed Nnat expression in embryonic and adult neuroretina as well as in corneal epithelial, stromal, endothelial cells and in lens epithelium. Expression of Nnat was detected from E12 onwards and was also present in adult eyes.
Conclusions: The expression pattern suggests that Nnat may play an important role during eye development and in the maintenance of mature eye.

1. Introduction

The vertebrate neuroretina evolves from the neuroectoderm to finally form a multi-layered organ composed of six neuronal cell types (ganglion cells, amacrine cells, horizontal cells, bipolar cells, cone and rod photoreceptors), and the Müller glia cells (Byerly and Blackshaw, 2009; Nakhai et al., 2007). The characteristically layered structure of the retina is a consequence of the highly ordered organization into three nuclear cell layers separated by two synaptic (plexiform) layers. The cell nuclei of cone and rod photoreceptors reside within the outer nuclear layer (ONL). The inner nuclear layer (INL) accommodates the nuclei of amacrine, horizontal, bipolar, amacrine and Müller glia cells whereas, the ganglion cell layer (GCL) is composed of displaced amacrine cells and ganglion cells. The outer plexiform layer (OPL) and the inner plexifrom layer (IPL) contain the axons and dendrites of the nuclear layers wiring the different cell types (Hoon et al., 2014). Following a relatively ordered chronological sequence, the different retinal cell types are generated from a pool of multipotent retinal progenitor cells (RPCs)(Cayouette et al., 2006; Cepko, 2014). Hereby, retinal ganglion cells are the first cells to be generated, followed in overlapping periods by the generation of horizontal cells, cone photoreceptors, amacrine cells, rod photoreceptor, bipolar and Müller glia cells (Marquardt and Gruss, 2002).

The cornea, about a half millimeter thick transparent tissue, consists of three cellular (corneal epithelium, stroma and endothelium) and two acellular (Bowman's and Descemet's membrane) anatomically different layers. During embryonic development, the corneal epithelium drives from the surface ectoderm. These stratified squamous cells rest on the basement membrane (Bowman's layer) and regenerate lifelong by proliferation of limbal stem cells (Pajoohesh-Ganji and Stepp, 2005). The main component of corneal stroma consists of extracellular matrix proteins and has only a few neural crest-derived keratocytes. Adult corneal endothelial cells are postmitotic and are derived from a monolayer of neural crest cells during embryogenesis. Corneal endothelium rest on Descemet's membrane and regulates the fluid and solute transport between corneal stroma and aqueous humor compartments (Swamynathan,

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2013). The lens is derived from the surface ectoderm. In mice, at around embryonic day 10 (E10), the lens placode is formed and develops by invagination to lens vesicle. From the overlying surface ectoderm the presumptive corneal epithelium is formed. By E15.5 neural crest-derived cells migrate between the surface ectoderm and the lens vesicle and form the presumptive corneal stroma and the monolayered corneal endothelium. (Graw, 2010; Kondoh, 2002; Swamynathan, 2013).

The neuronatin (Nnat) gene consist of 3 coding exons and has two alternative splice forms that are named as Nnat-alpha and Nnat-beta. The alpha isoform of Nnat gene is encoded by exon 1–3, whereas in the beta isoform exon 2 is absent. Former studies identified neuronatin (Nnat) as a selectively expressed gene in the brain, with high abundance in the neonatal rat brain and a subsequent decrease to baseline expression levels in the adult stages (Joseph et al., 1994). Physiologically, Nnat is a cytoplasmatic protein which is primarily localized in the membrane of the endoplasmic reticulum (ER) (Lin et al., 2010). Nnat is highly conserved between the mammalian species rat, human and mouse. Regular mammalian development requires the availability of both parental genomes (Gold and Pedersen, 1994; Latham et al., 1995). A substantial amount of cells within the neocortex are gynogenetic cells, whereas androgenetic cells are mainly contributing to the hypothalamus (Keverne et al., 1996). Interestingly, Nnat belongs to a group of genes, which are imprinted and display only parental contribution (Kagitani et al., 1997). Although, no apparent structural differences were found in mice either displaying no paternal allele or duplicated paternal alleles, a decrease in cerebellar folding could be detected when comparing maternal duplicated with paternal duplicated mice (Kagitani et al., 1997).

In vitro, treatment of PC12 cells with nerve growth factor (NGF) resulted in neuronal differentiation associated with a decrease of Nnat mRNA levels (Joseph et al., 1996). These *in vitro* experiments indicate a role for Nnat during developmental stages and the necessity to downregulate Nnat during differentiation. However, PC12 cell lines deficient of Nnat exhibit an increased sensitivity to toxic effects associated with nigericin, rotenone and valinomycin treatment. This effect is reversible by reconstitution of Nnat by transfection suggesting that Nnat might protect cells from toxic reagents (Zheng et al., 2002). Although *in vitro* data give hints towards the possible role of Nnat, the function of neuronatin *in vivo* remains still unknown. Nevertheless, due to its structural similarity to other proteolipids, such as phospholamban and plasma membrane ATPase associated proteolipid 1 (PMP1), a function in ion, and in particular calcium homeostasis has been suggested (Joseph, 2014; Lin et al., 2010).

Although initially identified as a brain specific gene, two independent research groups could detect *Nnat* expression in non-neuronal tissue as well as in the eye (Kagitani et al., 1997; Wijnholds et al., 1997). In this study, we focused on analyzing in detail the temporal expression and localization of Nnat on both mRNA and protein level in the developing mouse eye.

2. Materials and methods

2.1. Animals

All experiments were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement, and the local animal ethics committee. For the investigation of Nnat during eye development, we used C57BL/6-N mice which were inbreeds of the animal facility of the Martin-Luther University Halle (Germany). The mice were kept in a 12 h light and dark cycle with free access to food and drinking water (Sel et al., 2012, 2013).

2.2. Tissue preparation

Mouse eyes of both sexes aged embryonic day 12 (E12), E14, E16, E18, postnatal day 0 (P0), P1, P4, P7, P14, P30 and P175 (adult) were used as described in our earlier studies (Sel et al., 2012, 2013). Briefly, for immunohistochemical analysis we washed the eyes with 4% formaldehyde fixation buffer (A + E. Fischer, Wiesbaden Germany) and stored in this fixative until further use. For Western blot and RT-PCR we isolated the retina of adult mice (P175). For retina isolation, we induced a retinal detachment and peeled away the neuroretina from the retinal pigment epithelium (RPE). To avoid any contamination with RPE cells we checked the neuroretina by microscopy. For RNA extraction, retina were submerged in the RNAlater RNA stabilization reagent (Qiagen, Hilden, Germany) and stored at −20 °C (Sel et al., 2012, 2013).

2.3. RT-PCR

As descried in our previous studies (Sel et al., 2012, 2013) we isolated total RNA from adult retina by using RNeasy Micro kit (Qiagen). Briefly, after digestion of the genomic DNA 1 U DNase I per μg total RNA (Fermentas, St. Leon-Rot, Germany) at 37 °C for

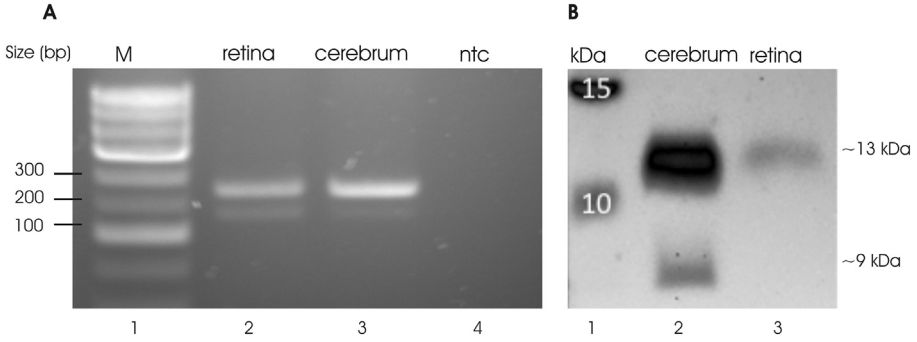

Fig. 1. Nnat is expressed in adult neuroretinal cells.
(A) RT-PCR analysis showing Nnat gene products from adult retina and cerebrum (as control tissue). The DNA marker (M) sizes as base pairs (bp) are shown on the left. The 252 bp and 173 bp products indicate the expression of the alpha and the beta isoform of Nnat gene in retina and cerebrum of adult mice, respectively. To exclude DNA contamination, template control (ntc) was performed by replacing cDNA template with ddH20 in the PCR amplification reaction.
(B) Western blot analysis of Nnat protein expression in cerebrum and retina of adult mice is shown. Equal protein amounts of total protein extracts from adult cerebrum and retina were resolved by 10% SDS–PAGE. The proteins were transferred to a nitrocellulose membrane and detected using anti-Nnat antibody. Protein marker masses (kDa) are indicated on the left. A 13 kDa protein, Nnat, is present in the control tissue (cerebrum) and retina. The 9 kDa protein, which is detectable in cerebrum, is not present in the adult retina.