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NEURONATIN IS A STRESS-RESPONSIVE PROTEIN OF ROD PHOTORECEPTORS

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Abstract—Neuronatin (NNAT) is a small transmembrane proteolipid that is highly expressed in the embryonic developing brain and several other peripheral tissues. This study is the first to provide evidence that NNAT is detected in the adult retina of various adult rod-dominant mammals, including wild-type (WT) rodents, transgenic rodents expressing mutant S334ter, P23H, or T17M rhodopsin, non-human primates, humans, and cone-dominant tree shrews. Immunohistochemical and quantitative real time polymerase chain reaction (qRT-PCR) analyses were applied to detect NNAT. Confocal microscopy analysis revealed that NNAT immunofluorescence is restricted to the outer segments (OSs) of photoreceptors without evidence of staining in other retinal cell types across all mammalian species. Moreover, in tree shrew retinas, we found NNAT to be co-localized with rhodopsin, indicating its predominant expression in rods. The rod-derived expression of NNAT was further confirmed by qRT-PCR in isolated rod photoreceptor cells. We also used these cells to mimic cellular stress in transgenic retinas by treating them with the endoplasmic reticulum stress inducer, tunicamycin. Thus, our data revealed accumulation of NNAT around the nucleus as compared to dispersed localization of NNAT within control cells. This distribution coincided with the partial intracellular mislocalization of NNAT to the outer nuclear layer observed in transgenic retinas. In addition, stressed retinas demonstrated an increase of NNAT mRNA and protein levels. Therefore, our study demonstrated that NNAT is a novel stress-responsive protein with a potential structural and/or functional role in adult mammalian retinas.

Key words: neuronatin, retinal degeneration, rod photoreceptors, autosomal dominant retinitis pigmentosa, ER stress.

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Abbreviations: ADRP, autosomal dominant retinitis pigmentosa; ANOVA, analysis of variance; ER, endoplasmic reticulum; LCA, Leber congenital amaurosis; NNAT, neuronatin; OS, outer segment; P, postnatal day; PBS, phosphate-buffered saline; PNA, peanut agglutinin lectin; qRT-PCR, quantitative real time polymerase chain reaction; SD, Sprague–Dawley; WT, wild-type.

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INTRODUCTION

The retina is a specialized neuronal tissue that plays a vital role in vision. The structural and functional integrity of the retina is supported by the coordinated work of six types of neurons and glial cells. All retinal neurons express a unique protein signature that defines their roles in the functioning of the whole retina. Thus the photoreceptor cells express opsin, a key protein of phototransduction; retinal pigment epithelium cells express pigment epithelium-derived factor, a multifunctional protein promoting photoreceptor survival and supplying the retina with anti-angiogenic action; ON and bipolar cells express glutamate receptor proteins, allowing them to provide a distinct response to the release of glutamate in transmitting a signal from the photoreceptors to the ganglion cells. Despite the recent breakthrough in retinal functional proteomics, many key regulators of the vision process remain to be identified and their function remains to be characterized.

The downstream target of NeuroD1, neuronatin (NNAT), is a proteolipid (Dou and Joseph, 1996) that originally was discovered as a gene involved in the development and differentiation of the central nervous system (Chu and Tsai, 2005). Later, its expression was detected in other non-neuronal adult tissues (Dugu et al., 2010). In humans, the imprinted NNAT gene is expressed only as a paternal allele (Joseph et al., 1994, 1995; Wijnholds et al., 1995) encoding two major isoforms. NNAT-α encodes an 81-aa protein that arises from exons 1, 2, and 3 while NNAT-β encodes a 54-aa protein that originates from the first and the third exons. The proteins encoded by the α and β isoforms have distinct peaks of accumulation during brain development (Joseph et al., 1995; Siu et al., 2008). Thus NNAT-α is expressed earlier than NNAT-β, suggesting different regulatory mechanisms governing their expression. While highly expressed during development, NNAT expression subsequently declines in the adult brain (Joseph et al., 1994; Usui et al., 1996). In the retina, a study demonstrated NNAT mRNA expression at postnatal day (P) 0 and its consequent decline in adult retinas (Dorrell et al., 2004). Despite this significance, the above-mentioned study did not reveal the precise localization of NNAT in different retinal neurons.

In addition to the developing brain, the expression of NNAT has also been reported in the pituitary gland, lungs, adrenal gland, uterus, skeletal muscle, ovaries, pancreas, and skin (Wijnholds et al., 1995; Niwa et al., 1997; Arava et al., 1999; John et al., 2001). These reports have highlighted different patterns of expression of the

NNAT isoform. Thus the reduction of NNAT-α was observed in the β-cells of diabetic rodents (Chu and Tsai, 2005; Joe et al., 2008), while the endothelial cells of blood vessels in obese and diabetic mice showed increased levels of NNAT-α (Mzhavia et al., 2008). All these findings indicate potentially different roles for NNAT isoforms in development and during adulthood.

As of today the precise role of NNAT in healthy and diseased adult human tissues has not been addressed. Thus, Zheng et al. have proposed the protective role for NNAT during neuronal development from the external toxic insults (Zheng et al., 2002), while other studies conducted with non-neuronal cells suggested that NNAT could act as an intracellular Ca^{2+} modulator (Suh et al., 2005; Poon et al., 2006; Joe et al., 2008). In addition to maintaining cellular homeostasis, NNAT can play a role in pathological processes. Thus in patients with glioblastoma, the elevated NNAT level is associated with poor prognosis and serves as a prognostic biomarker (Xu et al., 2012) while in patients with prostate cancer, NNAT suppression is involved in the mechanism of metformin-induced apoptosis in cells (Yang et al., 2015). Recently, Mzhavia et al. have also described the pathophysiological role of NNAT in diabetic vascular disease and have demonstrated NNAT-induced upregulation of NF-kB, increase in inflammatory gene expression, and the promotion of p38 and Jun-N signaling molecules in endothelial cells (Mzhavia et al., 2008). In patients with Lafora disease, NNAT has been reported to contribute to neuro pathogenesis by mediating aberrant Ca^{2+} signaling, the activation of endoplasmic reticulum (ER) stress, and the formation of aggregates called Lafora bodies (Sharma et al., 2013).

All these reports have indicated that NNAT could be one of the key molecules that maintain cellular homeostasis. However, although the potential role of NNAT in several organs and tissues has been highlighted, no comprehensive study has been conducted to examine the role of NNAT in developing and adult retinas. This fact demonstrates the gap in the knowledge of the retinal proteome affecting our comprehensive concepts of retinal physiology. Therefore we initiated a study of NNAT in which we analyzed the expression and localization of the NNAT protein in the mammalian retinas of a wide spectrum, ranging from mice to humans. Using quantitative real time polymerase chain reaction (qRT-PCR), western blot and Immunohistochemical (IHC) analyses, we detected NNAT in adult retinas and additionally revealed that the intercellular localization of NNAT could differ in healthy and diseased adult retinas.

EXPERIMENTAL PROCEDURES

Animal models

The animal protocol was carried out with approval from the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham and in accordance with the guidelines of the Association for Research in Vision and Ophthalmology statement for the use of Animals in Ophthalmic and Vision Research. All efforts were made to minimize the number and the suffering of the animals used.

Homozygous S334ter Rho (line 4), P23H Rho (line 3) transgenic rats, and C57/BL6 rhodopsin knockout and T17M RHO mice were maintained in the UAB housing facility. Heterozygous transgenic rats were bred with wild-type (WT) Sprague–Dawley (SD) rats to generate heterozygous S334ter-4 Rho and P23H-3 Rho rats. The T17M RHO mice were bred with C57/BL6 mice to generate heterozygous T17M RHO mice.

RNA preparation and real-time PCR analysis

Retinas from SD and S334ter-4 Rho rats were isolated at postnatal day (P) 21, P40 and P60. Total RNA was isolated from the individual retinas from each strain using Trizol ($n = 5$). cDNA was prepared using a cDNA reverse transcription kit (Applied Biosystems) from the RNA extracts of SD and S334ter-4 Rho retinas. Each cDNA (20 ng) was subjected to qRT-PCR using Applied Biosystems TaqMan assays (validated for each selected gene) on a One Step Plus instrument (Applied Biosystems, Foster City, CA, USA) to compare the number of cycles (Ct) needed to reach the midpoint of the linear phase. All observations were normalized to the GAPDH housekeeping gene.

Western blot analysis

The retinal protein extract was obtained from SD, S334ter and P23H Rho rats by sonication in a buffer containing 25 mM of sucrose, 100 mM of Tris–HCl, pH = 7.8, and a mixture of protease inhibitors (PMSF, TLCK, aprotinin, leupeptin, and pepstatin). The total protein concentration in the right and left retinas from individual rats were measured using a Biorad protein assay, and 40–60 μg of total protein was used to detect individual proteins. The detection of proteins was performed using an infrared secondary antibody and an Odyssey infrared imager (LiCor Inc., Lincoln, Nebraska, USA). The NNAT antibody from abcam (Cat # ab27266) was used at a dilution of 1:1000.

IHC analysis

Rats and mice were euthanized separately using a CO_2 chamber. The eyeballs were enucleated, affixed in 4% freshly made paraformaldehyde (Cat# S898-09 J.T. Baker, Phillipsburg, NJ, USA), and kept at 4 °C for 8 h. Then, the eyes were hemisected and the eyecups were transferred to fresh phosphate-buffered saline (PBS) to remove formaldehyde, and then immersed in a 30% sucrose solution for cryoprotection. Eyecups were then embedded in a cryostat compound (Tissue TEK OCT, Sakura Finetek USA, Inc., Torrance, CA, USA) and frozen at −80 °C. Twelve-micron sections were obtained using a cryostat. Twelve-micron sections of rat (SD and S334ter-Rho) and mouse (C57 and T17M) retinas were obtained and fixed on polylysine-treated glass slides. Slides were warmed for 30 min at 37 °C and washed in 0.1 M PBS for 10 min three times. The antigen retrieval procedure was performed, using10 mM citrate buffer. First, we placed the slides in the slide holder containing the citrate solution. The assembly was then placed in a