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# Effect of neurturin deficiency on cholinergic and catecholaminergic innervation of the murine eye

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ABSTRACT

Neurturin (NRTN) is a neurotrophic factor required for the development of many parasympathetic neurons and normal cholinergic innervation of the heart, lacrimal glands and numerous other tissues. Previous studies with transgenic mouse models showed that NRTN is also essential for normal development and function of the retina (J. Neurosci. 28:4123–4135, 2008). NRTN knockout (KO) mice exhibit a marked thinning of the outer plexiform layer (OPL) of the retina, with reduced abundance of horizontal cell dendrites and axons, and aberrant projections of horizontal cells and bipolar cells into the outer nuclear layer. The effects of NRTN deletion on specific neurotransmitter systems in the retina and on cholinergic innervation of the iris are unknown. To begin addressing this deficiency, we used immunohistochemical methods to study cholinergic and noradrenergic innervation of the iris and the presence and localization of cholinergic and dopaminergic neurons and nerve fibers in eyes from adult male wild-type (WT) and NRTN KO mice (age 4–6 months). Mice were euthanized, and eyes were removed and fixed in cold neutral buffered formalin or 4% paraformaldehyde. Formalin-fixed eyes were embedded in paraffin, and 5 μm cross-sections were collected. Representative sections were stained with hematoxylin and eosin or processed for fluorescence immunohistochemistry after treatment for antigen retrieval. Whole mount preparations were dissected from paraformaldehyde fixed eyes and used for immunohistochemistry. Cholinergic and catecholaminergic nerve fibers were labeled with primary antibodies to the vesicular acetylcholine transporter (VAChT) and tyrosine hydroxylase (TH), respectively. Cholinergic and dopaminergic cell bodies were labeled with antibodies to choline acetyltransferase (ChAT) and TH, respectively. Cholinergic innervation of the mouse iris was restricted to the sphincter region, and noradrenergic fibers occurred throughout the iris and in the ciliary processes. This pattern was unaffected by deletion of NRTN. Furthermore, functional experiments demonstrated that cholinergic regulation of the pupil diameter was retained in NRTN KO mice. Hematoxylin and eosin stains of the retina confirmed a marked thinning of the OPL in KO mice. VAChT and ChAT staining of the retina revealed two bands of cholinergic processes in the inner plexiform layer, and these were unaffected by NRTN deletion. Likewise, NRTN deletion did not affect the abundance of ChAT-positive ganglion and amacrine cells. In marked contrast, staining for TH showed an increased abundance of dopaminergic processes in the OPL of retina from KO mice. Staining of retinal whole mounts for TH showed no difference in the abundance of dopaminergic amacrine cells between WT and KO mice. These findings demonstrate that the neurotrophic factor NRTN is not required for the development or maintenance of cholinergic innervation of the iris, cholinergic control of pupil diameter, or for development of cholinergic and dopaminergic amacrine cells of the retina. However, NRTN deficiency causes a marked reduction in the size of the OPL and aberrant growth of dopaminergic processes into this region.



## 1. Introduction

Adrenergic and cholinergic neurons play important roles in regulating the function of many organs, including the eye. For example, the sympathetic and parasympathetic limbs of the autonomic nervous system, which release norepinephrine and acetylcholine, respectively, innervate a number of ocular

*Abbreviations:* BSA, bovine serum albumin; ChAT, choline acetyltransferase; GDNF, glial cell-line derived neurotrophic factor; GFRα2, GDNF family receptor α2; H&E, hematoxylin & eosin; KO, knockout; NRTN, neurturin; OPL, outer plexiform layer; PBS, phosphate buffered saline; TH, tyrosine hydroxylase; VAChT, vesicular acetylcholine transporter; WT, wild-type.

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structures, including the iris and ciliary body. At such sites, these neurotransmitters generally exert opposite effects on tissue/organ function (Westfall and Westfall, 2006; Neuhuber and Schrödl, 2011). Within the retina, acetylcholine and dopamine influence a number of different physiological processes including contrast, motion and direction sensitivity, visual acuity, and circadian rhythms (Jackson et al., 2012; Lee et al., 2010; Origlia et al., 2012; Stone et al., 2013). At this location, these neurotransmitters are localized to subpopulations of ganglion cells, amacrine cells, and their processes. While much is known about the function of these transmitter systems within the anterior and posterior segments of the eye, far less is know about the role that neurotrophic factors play in their development and maintenance. Neurturin (NRTN) is one such neurotrophic factor, which has been implicated recently in the development of the retina and cholinergic neurons of many parasympathetic ganglia.

Neurturin (NRTN), is a member of the glial cell line-derived neurotrophic factor (GDNF) family of ligands, which also includes artemin, persephin and GDNF (Paratcha and Ledda, 2008). Transgenic mice with deletion of NRTN or of its specific receptor, GDNF family receptor α2 (GFRα2), show widespread loss of parasympathetic neurons and nerve fibers of the autonomic nervous system (Heuckeroth et al., 1999; Rossi et al., 1999; Mabe and Hoover, 2009). For example, the lacrimal glands of NRTN knockout (KO) mice lack cholinergic nerves and consequently have reduced tear production, which contributes to the ocular phenotype of thick, sagging eyelids with a crusty discharge (Heuckeroth et al., 1999). It is unknown if NRTN deletion also affects cholinergic innervation of the iris, which derives from the ciliary ganglion, but previous studies have shown that developing ciliary neurons express NRTN receptors (Enomoto et al., 2000). Recent studies have also shown that NRTN is required for normal retinal function (Brantley et al., 2008). Electroretinograms from NRTN deficient mice showed marked reduction in scotopic a- and b-waves and photopic b-waves (Brantley et al., 2008). Morphological studies of these mice revealed abnormal bipolar and horizontal cell extensions into the outer nuclear layer of the retina and a reduction in the size of the outer plexiform layer (OPL), providing an anatomical basis for reduced retinal function in NRTN deficient mice (Brantley et al., 2008). However, it is unknown if NRTN deletion affects retinal cholinergic and dopaminergic neurons and nerve fibers.

Evidence for the involvement of NRTN in neuronal survival and function in the retina has also been provided by studies of retinal degeneration (rd) mice. The structure of the retina in rd mice starts to change 10 days after birth with a decreasing number of photoreceptor nuclei. After 20 days a single layer of photoreceptors remains, and by 60 days the layer is gone (Jomary et al., 1999). The inner nuclear layer, inner plexiform layer, and the ganglion cell layer do not vary from WT mice, but the OPL is attenuated (Jomary et al., 1999) just as seen in NRTN deficient mice (Brantley et al., 2008). These changes in rd mice are associated with a deficiency of the NRTN receptor and increased NRTN expression (Jomary et al., 1999). Together, these data and studies of NRTN deficient mice suggest that NRTN is an essential neurotrophic factor for development of the retina and could be required as well for cholinergic innervation of the iris.

The goal of this study was to determine if NRTN deficiency affects cholinergic and catecholaminergic innervation of the mouse ciliary body, iris, and retina. Specifically, we wanted to determine if NRTN was required for development of cholinergic innervation of the iris and if NRTN deletion affected the abundance of cholinergic and dopaminergic neurons in the retina and/or the distribution of their retinal nerve processes. Eyes from NRTN knockout (KO) and WT mice were evaluated by immunohistochemistry using paraffin sections and whole mounts of iris or retina. Cholinergic nerves in the anterior segment of the eye and the retina were identified by immunostaining for the vesicular acetylcholine transporter (VAChT). Cholinergic cell bodies in the retina were labeled using an antibody to choline acetyltransferase (ChAT). Noradrenergic nerves in the anterior segment and dopaminergic amacrine cells and their processes in the retina were labeled by immunostaining for tyrosine hydroxylase (TH). Cholinergic regulation of pupil diameter was evaluated in anesthetized WT and KO mice.

## 2. Methods

### 2.1. Mice

NRTN KO mice, on a C57BL/6 background, were obtained from Jeffrey Milbrandt (Washington University, St. Louis, MO), and a breeding colony was set up using homozygous ($nrtn^{KO}/nrtn^{KO}$) animals. C57BL/6 mice were purchased from Harlan (Indianapolis, IN). Male NRTN KO and WT mice were used at age 4–6 months. Animal protocols were approved by the East Tennessee State University Animal Care and Use Committee and conformed to guidelines of the National Institutes of Health as published in the Guide for the Care and Use of Laboratory Animals (Eighth Edition, National Academy of Sciences, 2011).

### 2.2. Tissue preparation

Mice were euthanized using isoflurane, and the eyes were dissected carefully from the sockets. The intact globes were fixed in either cold 10% neutral buffered formalin for 5–7 days at 4 °C or in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS, pH 7.3) overnight at 4 °C. Formalin-fixed eyes were embedded in paraffin and cut in 5 μm sections using a Microm HM 310 microtome. Cross-sections including anterior and posterior segments of the eye were collected on charged slides, deparaffinized for 1 h at 60 °C and treated for antigen retrieval prior to immunostaining. Paraformaldehyde-fixed eyes were washed in PBS and dissected around the sclera to remove the lens and obtain separate anterior and posterior segments.

### 2.3. Immunostaining

Paraffin sections and the eye segments were immunostained as described previously (Mabe et al., 2006; Mabe and Hoover, 2009) except the segments were processed free-floating in 1.5 ml polypropylene tubes with gentle agitation. Tissues were rinsed 4× 5 min in 0.1 M PBS (pH7.3), 10 min in 0.5% BSA + 0.4% Triton-X100 + 0.1 M PBS, and blocked for 90 min in a solution of PBS containing 5% normal donkey serum, 1% bovine serum albumin (BSA), and 0.4% Triton-X100. After incubation the blocking buffer was replaced with the primary antibody solution of goat anti-VAChT (1:1000; Immunostar, 24286) and rabbit anti-TH (1:500; Pel-Freez, P40101) or goat anti-ChAT (1:50; Millipore, AB144P) in blocking buffer and incubated overnight. Samples were then rinsed 4× 5 min in PBS and 10 min in PBS with 0.5% BSA and 0.4% Triton-X100 before blocking again (60 min) and incubating for 2 h in secondary antibody solution containing 1:200 dilution of Alexa Fluor 555 donkey anti-goat (Invitrogen, A21432) and Alexa Fluor 488 donkey anti-rabbit (Invitrogen, A21206) or Alexa Fluor 555 donkey anti-goat. Samples were then rinsed with PBS, and coverslips were attached to paraffin sections with Citifluor medium and clear nail polish.