INFLUENCE OF THE NEURAL CELL ADHESION MOLECULE ON AGE-RELATED CHANGES IN VISION

by

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DEDICATION

For my family

Weei-Yuarn, Amy and Alyssa Huang

Thank you so much for your patience and encouragement, for always believing in me, and for giving me all the support that I needed to chase after my dream…

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ABSTRACT

The Neural Cell Adhesion Molecule (NCAM) is involved in cell migration, axonal fasciculation, neurite outgrowth, and the formation and stabilization of synapses during development. It also plays an important survival role in the adult nervous system. There has been extensive research focusing on how NCAM affects age-associated cognitive decline; however, little is known concerning the effect of NCAM in the visual system. Using a battery of behavioral, functional, and anatomical assays, I investigated the visual function of young and aged wild type and NCAM deficient (−/−) mice. My results provide evidence that NCAM −/− mice have altered retinal architecture and physiology, impaired pattern discrimination ability, and premature loss of visual acuity during aging.

These observations lead me to further investigate whether NCAM plays a role in protecting retinal neurons following injury in the adult mouse. Using light-induced retinal degeneration, I found that NCAM protects retinas from light-induced injury, and that the protective effect of NCAM is, in part, attributed to its effect on $p75^{NTR}$. To determine whether NCAM is involved in visual system plasticity, I subjected adult mice to long-term monocular deprivation, and demonstrated that PSA-NCAM is required for the reactivation of visual cortical plasticity and recovery of visual function.

Together, I have shown that NCAM plays vital roles in promoting retinal cell survival and in maintaining visual physiology in the nervous system during aging.

LIST OF ABBREVIATIONS USED

α_2M	Alpha$_2$-macrogloblin
ANOVA	Analysis of variance
ARVO	Association for Research in Vision and Ophthalmology
BDNF	Brain derived neurotrophic factor
bp	Base pair
CAM	Cell adhesion molecule
CAMKIV	Calcium/ calmodulin-dependent protein kinase IV
C/DEG	cycles/ degree
ChAT	Choline acetyltransferase
CREB	cAMP responsive element binding protein
CNS	Central nervous system
CSPG	Chondroitin sulfate proteoglycans
E	Embryonic
ECL	Enhanced chemiluminescence system
E/I	Excitatory and inhibitory
Endo N	Endoneuraminidase N
eNOS	Endothelial NOS
ER	Eyelid reopening
ERG	Electroretinogram
ERK	Extracellular signal regulated kinase
FGFR	Fibroblast growth factor receptor

FAK	Focal adhesion kinase
FN	Fibronectin type
FYN	Proto-oncogene tyrosine-protein kinase
GAD65/67	Glutamic acid decarboxylase 65/67
GCL	Ganglion cell layer
GFRα1	Glial cell-derived neurotrophic factor family receptor alpha 1
GPI	Glycosylphosphatidylinositol
HCl	Hydrochloride
HNE	4-hydroxynonenal
H_2O_2	Hydrogen peroxide
ICD	Intracellular domain
IGF-1	Insulin-like growth factor-1
IgSF	Immunoglobulin superfamily
Ig	Immunoglobulin
INL	Inner nuclear layer
iNOS	inducible NOS
IPL	Inner plexiform layer
IOVS	Investigative Ophthalmology & Visual Science
JNK	c-Jun N-terminal kinase
–/–	Knockout
dLGN	dorsal lateral geniculate nucleus
LIRD	Light induced retinal degeneration
LTP	Long-term potentiation

MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase
MCAO	Middle cerebral artery occlusion
MD	Monocular deprivation
MSD1	Muscle specific domain 1
NaCl	Sodium chloride
NCAM	Neural cell adhesion molecule
NeuN	Neuronal Nuclei
NFL	Nerve fiber layer
NFT	Neurofibrillary tangle
NP-40	Nonyl phenoxypolyethoxylethanol
NRAGE	Neurotrophin Receptor Interacting MAGE Homolog
NS	Not significant
NO	Nitric oxide
NOS	Nitric oxide synthase
nNOS	neuronal NOS
OD	Ocular dominance
OLM	Outer limiting membrane
ON	Optic nerve
ONT	Optic nerve transection
ONL	Outer nuclear layer
OPL	Outer plexiform layer
Otx2	Orthodenticle homeobox 2

$p75^{NTR}$	p75 neurotrophin receptor
PAGE	Polyacrylamide gel electrophoresis
PCD	Programmed Cell Death
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PI-PLC	phosphatidylinositol phospholipase C
PI3K–PKB	Phosphatidylinositol-3-kinase protein kinase B
PKA	Protein kinase A
PKC	Protein kinase C
PND	Postnatal day
PNN	Perineuronal net
Pro-NGF	Pro-form of nerve growth factor
PSA	Polysialic acid
PV	Parvalbumin
PVDF	Polyvinylidene fluoride
PST	Polysialyltransferase ST8SiaIV
Rd1	Retinal degeneration 1
REX	$p75^{NTR}$ function blocking antibody
RGC	Retinal ganglion cell
RPE	Retinal pigmented epithelium
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean

STX	Polysialyltransferase ST8SiaII
SVZ	Subventricular zone
T	Thoracic
TACE	Tumor necrosis factor α converting enzyme
TEM	Transmission electron microscopy
TNF-α	Tumor Necrosis Factor α
Trk	Tropomyosin receptor kinase
Tris	(hydroxymethyl) aminomehane
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
V1	Primary visual cortex
VASE	Variable alternatively spliced exon
VC	Visual cortex
VDCC	Voltage dependent calcium channels
VEP	Visual Evoked Potential
WFA	Wisteria floribunda agglutinin
WT	Wild type

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CHAPTER 1: INTRODUCTION

1.1. The Visual System

Development of the Mammalian Visual System: a Brief Review Focusing on the Retina and the Primary Visual Cortex

1.1.1 Development of the Retina

The visual system is a part of the central nervous system (CNS) that allow us to see the surrounding environment. It requires a sophisticated processing system to receive and interpret the visual information that includes the eye, retina, optic nerve, thalamus, superior colliculus, optic radiations and visual cortex (VC). During development, the retina and optic nerve (ON) are derived from the neuroectoderm and are part of the embryonic diencephalon, thus considered as an extension of CNS. The retina develops from the walls of the optic cup. The thinner and outer layer of the optic cup becomes the retinal pigmented epithelium (RPE), whereas the thicker inner layer differentiates into the neural retina (reviewed by Harada et al., 2007). In early fetal development, the two retinal layers are separated by an intraretinal space, but the space between the layers gradually diminishes, fusing together just before birth. Under the influence of the lens development, the inner layer of the optic cup cells (neuroepithelium) is induced and thickens; subsequently, the cells differentiate into the neural retina. There are six types of neurons (retinal ganglion cells (RGCs), horizontal cells, amacrine cells, bipolar cells, rod and cone photoreceptor cells) and one type of glia (Müller glia) in the neural retina. The other glial cells are astrocytes and microglia. Astrocytes enter the retina from the brain along the optic nerve during development, and microglia migrate in from the retinal

margin and the optic disc (Chen et al., 2002). The cells are arranged in three major nuclear layers: the ganglion cell layer (GCL), the inner nuclear layer (INL), and the outer nuclear layer (ONL). All retinal cells are derived from common retinal progenitor cells located on the inner surface of the optic cup. Through the control of various transcription factors (e.g. the basic helix-loop-helix and the homeobox families) and Notch-Delta mediated lateral inhibition, retinal cells are produced in a unidirectional orderly manner (reviewed by Harada et al., 2007; Reese, 2011). The anatomy of a fully developed vertebrate retina is shown in Figure 1-1 and illustrates the 10 distinct layers, from innermost to outermost: (1) inner limiting membrane; (2) nerve fiber layer; (3) GCL; (4) inner plexiform layer (IPL); (5) INL; (6) outer plexiform layer (OPL); (7) ONL; (8) external limiting membrane; (9) photoreceptor layer; and (10) RPE.

1.1.2 Primary Visual Cortex (V1)

Research on V1 has tended to focus on the development of its neural circuitry. The development of mouse V1 takes place as a series of events (Figure 1-2) (reviewed by Espinosa and Stryker, 2012). The dorsal lateral geniculate nucleus (dLGN) of the thalamus acts as a relay centre of the visual pathway, rearranges retinal inputs received from the retinas into distinct regions, and sends information directly to V1 in an organized manner. Retinotopy, which is guided developmentally by a combination of EphA-ephrinA signaling in the cortex and spontaneous waves of neural activity, occurs before eye opening and before RGCs are driven electrochemically by photoreceptor cells, forming a precise topographic map that aligns visual input from the retina with neurons in V1.

Figure 1-1. Schematic cross-section of a mouse eye and the anatomy of the adult retina that converts light signals into neural impulses. The retina is composed of ten distinct layers (from innermost to outermost): 1. Inner limiting membrane; 2. Nerve fiber layer; 3. Ganglion cell layer (GCL); 4. Inner plexiform layer (IPL); 5. Inner nuclear layer (INL); 6. Outer plexiform layer (OPL); 7. Outer nuclear layer (ONL); 8. External limiting membrane; 9. Photoreceptor layer; and 10. Retinal pigmented epithelium (RPE). Adapted and modified from Veleri et al. (2015).

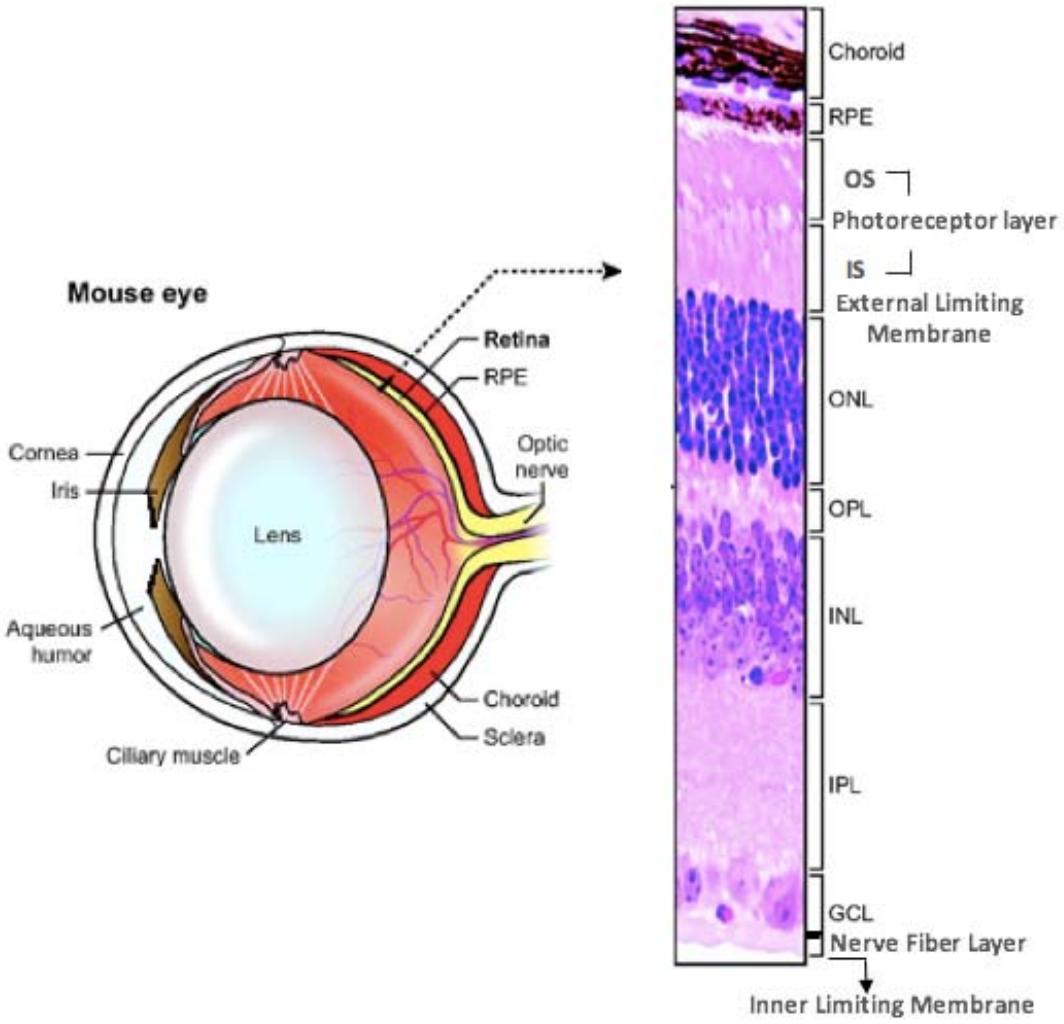
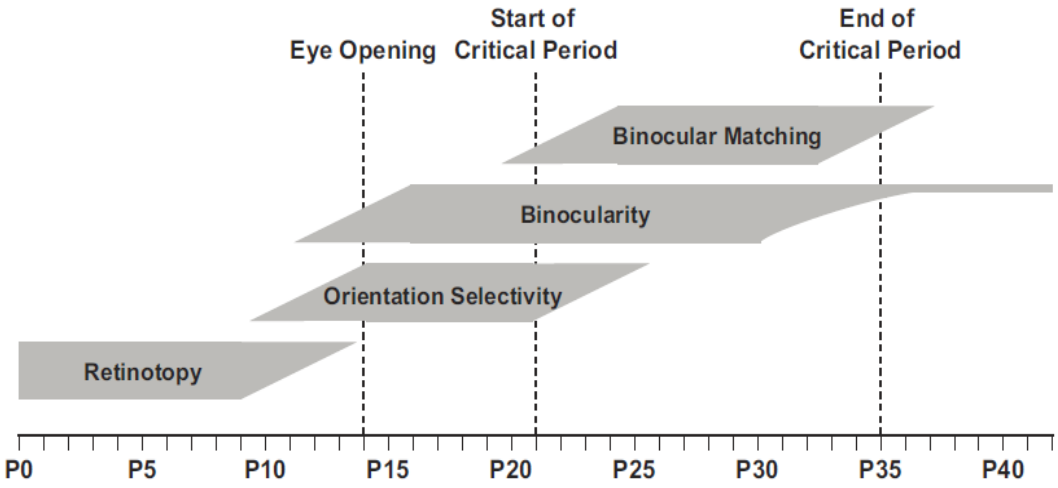


Figure 1-2. Development of the mouse V1. Retinotopy refers to neurons in V1 map representing visual input from the retina. Orientation selectivity occurs when V1 neurons respond to stimuli present at specific orientations. Note that mouse V1, unlike human V1, does not have columnar organization of orientation, so it is not indicated here. Cortical neurons in the critical period are highly sensitive to visual experience, which refines local synaptic connections between neurons and their targets. When neuronal circuits lose plasticity, the critical period is brought to an end. Adapted from Espinosa and Stryker (2012).



After topographic mapping, neurons in the V1 receive input that instructs them to arrange in response to specific features: the orientation column and orientation selectivity. This process begins around the time of eye opening. The initial formation of neuronal circuits in the visual system does not depend on the influence of vision, but the spontaneous activity that contributes to the anatomical segregation of thalamocortical inputs into ocular dominance (OD) columns. Neuronal connections to V1 from each eye are arranged separately and independently. Neurons develop responses to stimuli demonstrated at specific orientations, and cortical responses to the contralateral eye are faster and more organized than those to the ipsilateral eye.

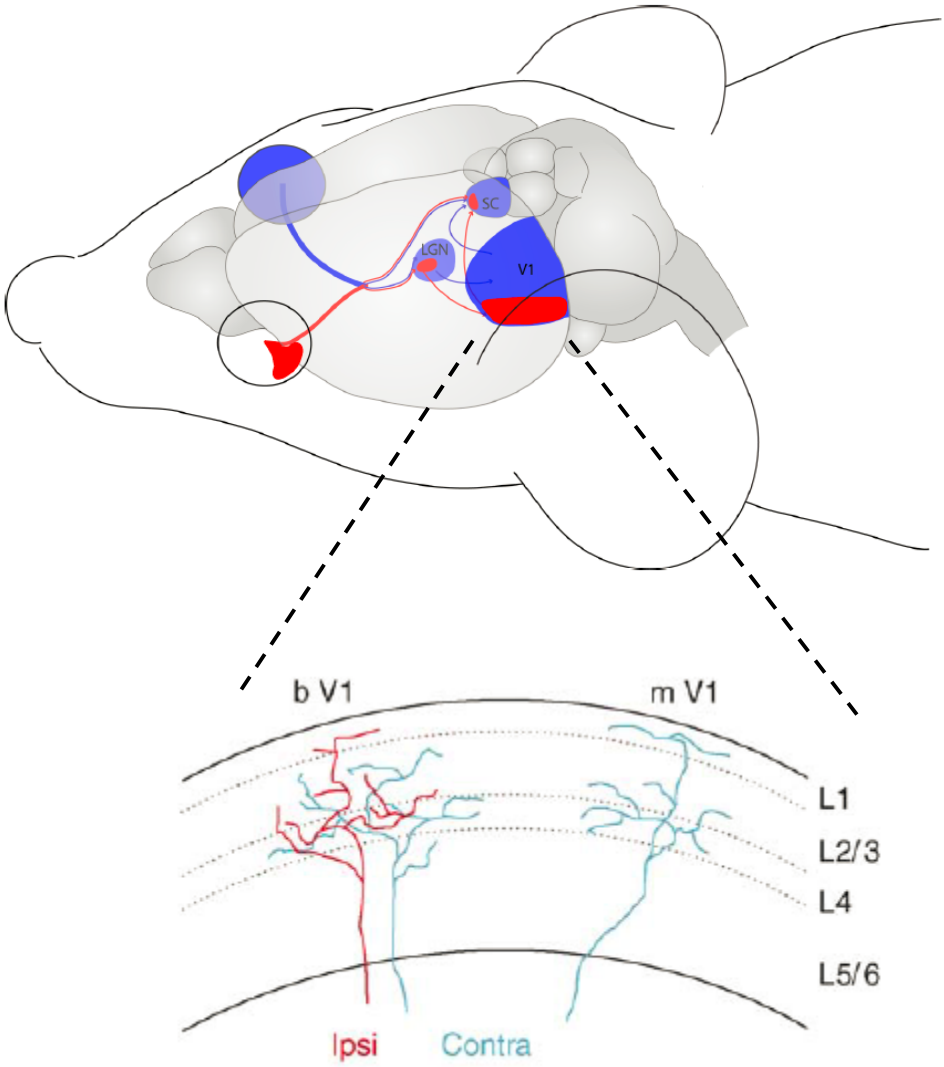
The critical period is the time window during early nervous system development that has profound effects on the brain for subsequent normal function (Hooks and Chen, 2007). Based on the description by Hubel and Wiesel, the timing of OD plasticity, or the period of robust plasticity in response to sensory experience, is a defined period during visual system development (Hubel and Wiesel, 1970; Wiesel and Hubel, 1963). During this critical period, visual experience actively shapes and refines synaptic connections between neurons and their targets. Cortical neurons are highly sensitive to external stimulation. At this stage, information from the two eyes is combined in V1, and simultaneous binocular visual input then drives neurons to alter their selectivity so that their orientation response from both eyes come to match. Any manipulation in vision prevents binocular matching and affects strength and organization of synaptic inputs. Once the critical period ends, directional selectivity becomes mature and fixed. Circuitry of V1 neurons normally remains stable throughout life.

1.2 The Rodent Visual Pathway

The lens and cornea cast an image of the visual world onto the retina, which contains photoreceptor cells that capture photons of light and transduce them into electrochemical signals. The visual information undergoes intraretinal processing via interconnecting neurons, including horizontal, bipolar, amacrine, and RGCs. The visual signals then leave the retina via the eye's sole output path, RGC axons (Figure 1-3). Mammalian RGC axons have more than 20 subcortical targets (Ling et al., 1998). In rodents, almost all (~97%) RGC axons decussate at the optic chiasm, then terminate at the contralateral superior colliculus of the midbrain (Drager and Olsen, 1980; Ellis et al., 2016; Hofbauer and Drager, 1985; Jeffery, 1984) and/or the contralateral dLGN of the thalamus (Grubb and Thompson, 2003; Sefton et al., 2015).

Neurons from the dLGN further relay complex sensory information to layer 4 of the V1. In contrast to humans, the medial 2/3 of mouse V1 receives purely monocular contralateral inputs, whereas the lateral 1/3 contains binocular signals (Montero, 1973). Neurons in the VC are dispersed in a "salt-and-pepper" organization, without columnar groupings of cells (Kaschube, 2014). Nevertheless, visual inputs from contralateral and ipsilateral projections converge onto the complex cells in layer 2/3 of V1, where integration of binocular information occurs; signals are then sent to extrastriate cortex for higher order processing of the visual image (Wilks et al., 2013).

Figure 1-3. A schematic diagram of the mouse visual pathway. Photoreceptors convert light into electrochemical signals that are delivered to other retinal neurons for processing and integration. Information then leaves the eye via RGC axons. In rodents, almost all RGC axons in the optic nerve cross at the midline optic chiasm and transmit information contralaterally to the superior colliculus of the midbrain and the dLGN of the thalamus. Neurons in the dLGN send signals to V1, where information from both eyes is received. The medial 2/3 of mouse V1 receive purely monocular contralateral inputs (mV1), whereas the lateral 1/3 contains binocular signals (bV1). The visual image created in V1 is processed further in extrastriate cortex. (dLGN = dorsal Lateral Geniculate Nucleus, V1= Primary Visual Cortex). Adapted and modified from Wilks et al. (2013) and Hofer et al. (2006).



1.3 Studying Vision in Rodents

Considerable controversy remains as to whether vision research in rodents can be used better understand in the human vision. I acknowledge that there are limits to this line of research (reviewed by Huberman and Niell, 2011). Rodent eyes are positioned laterally on each side of the head, leading to a hemi-panoramic field of view that covers a narrow binocular zone flanked by regions of monocular vision. This contrasts with species with forward facing eyes, such as cats and primates, that allows a wider-range of binocular vision. Rodents see poorly with extremely low visual acuity (0.5 -1 cycle/degree, equivalent to 20/2000 human vision), and thus are unable to perform complicated visual behavior tests e.g. facial recognition or visual attention tasks. As well, the rodent visual system is comparatively primitive: the dLGN does not have distinct cytoarchitectural laminae, and the rodent V1 lacks anatomical columnar organization of orientation (ocular dominance).

Despite differences in anatomical architecture, given the fundamental conservation of visual neural circuitry (including, for example, V1 neurons in the binocular region of rodents that are highly orientation selective in favour of the contralateral eye), it can be beneficial to explore some fundamental neurobiological questions related to vision in rodents (reviewed by Baker, 2013; Priebe and McGee, 2014). The rationale for doing so, first and foremost, includes the application of an ever-increasing diverse palette of genetic tools and consortiums (for example, the International Knockout Mouse Consortium) available in rodents (Sung et al., 2012). Using available tools, there are unique opportunities to decipher the structure of a defined visual cell type, map its

connections, and record its activity in response to stimuli. A greater variety and sophistication of tools, such as neuroanatomical tracing and *in-vivo* live imaging, also allows examination of complex circuits and probing of fundamental mechanisms. Such experiments are long, difficult, and costly to perform in larger animals. Furthermore, rodents have a relatively short lifespan, which greatly facilitates aging studies. In addition, the overall smaller size of the mouse visual system also allows researchers to study with larger sample sizes and across a larger spatial scale. Although still complex, it is reasonable to expect that understanding the simpler rodent visual system can shed light on the more complex biological structure of the human brain.

1.4 Effect of Aging on the Central Nervous System

Aging is characterized as a progressive, generalized impairment of function. It is the disruption of cellular processes arising from failure of tissue maintenance and repair, affected by multiple genes and the accumulation of environmental challenges throughout life. Aging's biological outcome is dictated by this progression of impairment, with or without concomitant disease, and ultimately results in death. During normal aging in the brain, subtle morphological changes occur in neurons, including loss or regression of dendrites and dendritic spines, shrinkage in soma size, alterations in neurotransmitter receptors, and changes in electrophysiological properties (Dickstein et al., 2007; Duan et al., 2003; Nakamura et al., 1985). For certain individuals, changes in the structure of CNS may result in progressive functional decline and/or neuron death, as seen in several age-associated neurodegenerative disorders such as Parkinson's disease and Alzheimer's disease.

Aging also affects brain plasticity, which is thought to support cognition. Deterioration in synaptic plasticity has been shown to result in the impairment of long-term potentiation (LTP) (Fabel and Kempermann, 2008). Besides structural alterations, neuro-chemical changes in the aging brain induce neurofilament protein expression and neurofibrillary tangle (NFT) formation (Yuan et al., 2012). NFTs are commonly found in neuropathological specimens from patients with dementia and/or Alzheimer's disease (Blennow et al., 2006). Aging in the brain is also accelerated by oxidative stress and a reduction of glutamate receptors (Segovia et al., 2001), which disrupt intracellular calcium homeostasis and can result in apoptosis (Mattson, 2007).

1.5 Age-Related Retinal Degeneration

As with other CNS tissue, the retina also degenerates with age. The photoreceptor cells and the RPE are particularly susceptible to cellular senescence. Both a- and b- wave electroretinogram (ERG) amplitudes, the mass electrode responses of the retina to photic stimulation, decline with age in rats and are accompanied by age-related changes in visual behavior, as assessed by a modified acoustic startle reflex (DiLoreto et al., 1995). These age-related changes are thought to be due to impairment of phagocytosis by the RPE of oxidized membranous discs shed from photoreceptor outer segments. This highlights the critical role the RPE plays in providing retinal support, as this layer serves as an avenue for metabolite exchanges between photoreceptors and the choroidal blood supply, and prevents accumulation of damaged proteins, formation of toxic metabolic byproducts, and invasion of inflammatory cells that leads to cell death (reviewed

by Mustafi et al., 2012). The progressive loss of vision due to the death of photoreceptor cells is a shared trait of most inherited human retinal degenerative diseases, including retinitis pigmentosa and age-related macular degeneration. A large number of genes have been identified whose mutations are responsible for retinal degeneration, including rhodopsin (Rao and Oprian, 1996), arrestin (Fuchs et al., 1995), transducin (Dryja et al., 1996), and α and β catalytic subunits of cGMP phosphodiesterase (Huang et al., 1993; McLaughlin et al., 1995), which are key molecules involved in visual transduction pathways. In addition, many genetic mutations have been linked to individual retinal dystrophies, suggesting the existence of multiple pathways involved in effecting photoreceptor degeneration. Consistent with this, both caspase dependent and caspase independent mechanisms have been shown to be simultaneously active in inducing photoreceptor cell death (Lohr et al., 2006).

1.6 Plasticity of the Primary Visual Cortex (V1)

Coordinated, experience-driven changes are known to shape the maturation of cortical circuits for the development of functional brain networks. Hubel and Wiesel, who studied the brain's visual processing system over fifty years ago (Hubel and Wiesel, 1970; Wiesel and Hubel, 1963), showed that neurons in the VC are organized in precise architecture columns (OD columns), and are particularly susceptible to changes when influenced by visual experience during development. Their groundbreaking insights are foundational to modern animal models used for studying cellular and molecular mechanisms underlying plasticity of cortical circuits. Transient eyelid closure (monocular deprivation (MD)) in kittens during the critical period results in a shift of OD towards the

eye that remained open. The perturbed cortical areas can be enlarged, shrunken, or even absent. Such structural and functional anomalies are dependent on the duration and onset of visual deprivation.

Recently, the mouse has become a widely-used model for studying mechanisms involved in critical period plasticity, as only a few days of MD between postnatal days (PND) 28 and 32 are sufficient to induce a shift of OD in the VC. Plasticity is normally attained by the interaction of environmental influences and physiological mechanisms, which induce cytoarchitecture and chemoarchitecture changes through a variety of intracellular signaling pathways that modulate gene expressions, DNA methylation patterns, and histone post-translational modifications (Maya-Vetencourt and Origlia, 2012). Although detailed mechanisms remain unclear, the development of GABAergic circuitry determines onset of the critical period (Hensch et al., 1998). In the absence of GABA release, the critical period's onset is delayed indefinitely (Fagiolini and Hensch, 2000). Many molecules have been identified that trigger GABA neural circuit development; for example, the overexpression of brain derived neurotrophic factor (BDNF) promotes postnatal maturation of cortical circuitry, and induces an earlier critical period in the mouse VC (Hanover et al., 1999). An enriched environment mediates increased expression of insulin-like growth factor 1 (IGF-1) that modulates intracortical inhibitory circuitry and promotes the development of a specialized extracellular matrix structure, the perineuronal net (PNN) (Ciucci et al., 2007). Furthermore, homeobox protein orthodenticle homeobox 2 (Otx2) governs the timing of GABAergic neuron

development, thereby controlling onset of the critical period during VC development (Di Cristo et al., 2007; Sugiyama et al., 2008).

After a period of heightened sensitivity to experience, further modifications become limited or absent in response to visual stimulation, and the complete maturation of GABAergic inhibitory neurons leads to the end of a critical period (He et al., 2006; Morales et al., 2002). This has been studied in MD cats, where dark rearing prolongs the period of sensitivity to visual experience (Cynader, 1983; Timney et al., 1980), an effect that can be reversed by the overexpression of BDNF, which enhances the development of the inhibitory neurons in animals reared in complete darkness (Gianfranceschi et al., 2003). The extended duration of the critical period in dark rearing can also be reversed by environmental enrichment (Bartoletti et al., 2004), which promotes neural plasticity by intensifying the release of BDNF, IGF-1 as well as glutamic acid decarboxylase 65/67 (GAD65/67) proteins in the VC during development (Cancedda et al., 2004; Ciucci et al., 2007; Sale et al., 2004). All of these factors enhance physiological maturation of GABAergic circuitry and consolidation of visual cortical connections.

Non-GABAergic processes also influence the closure of the critical period and include development of PNNs and expression of Nogo-66 (neurite outgrowth inhibitor); they inhibit axonal sprouting and reduces synaptic plasticity of the excitatory neurons (McGee et al., 2005; Pizzorusso et al., 2002) resulting in synaptic stabilization and maturation of the extracellular matrix. In addition, epigenetic mechanisms are highly engaged in the regulation of the critical period. The activity of the cAMP responsive element binding

protein (CREB), controlled by cAMP-dependent protein kinase A (PKA), is upregulated in response to a short period of visual stimulation during the critical period, but this response decreases significantly in adult animals (Fischer et al., 2004; Pham et al., 1999; Putignano et al., 2007; Rao et al., 2004). The transient upregulation of CREB signaling mediates histone post-translational modification and light-induced microRNA (miR-132) expression in the VC, which facilitates epigenetic regulation of dendritic spine activity and OD plasticity (Mellios et al., 2011; Tognini et al., 2011). Thereafter, the down-regulation of CREB, in part, leads to the end of the critical period during VC development.

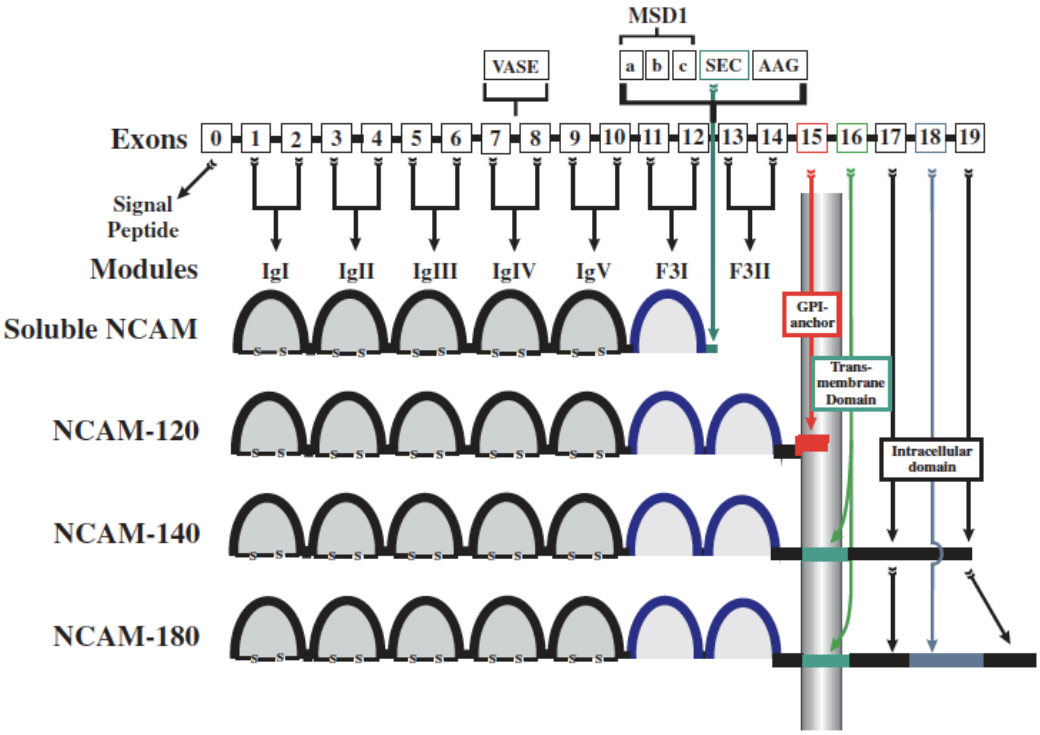
In summary, an orderly sequence of molecular and cellular events occur during the critical period is involved in the modulation of visual cortical plasticity. Thus, understanding the mechanisms that underlie the critical period may lead to strategies for reopening visual plasticity in adulthood to promote the recovery of vision later in life.

1.7 The Neural Cell Adhesion Molecule

1.7.1 Structure, Synthesis and Expression

The cell adhesion molecules (CAMs) are capable of mediating cell-cell interactions, connecting the extracellular matrix, and transmitting biochemical signals across cell membranes. CAMs are categorized into 4 main families: the immunoglobulin superfamily (IgSF), integrins, cadherins and selectins. The IgSF is the largest family of proteins in the human genome that contain a single transmembrane region and a

Figure 1-4. NCAM isoforms. This figure shows schematically the exon structure and alternative splicing patterns of NCAM. (IgI-V = Immunoglobulin I-V (black); F3I-II = Fibronectin I-II (blue)). Adapted from Walmod et al. (2004).



membrane-anchored and transmembrane glycoprotein with variable Ig modules (Lander et al., 2001). The neural cell adhesion molecule (NCAM), also known as CD56, was the first CAM discovered in the IgSF, primarily identified as synaptic membrane protein D2 in the rat brain. It was subsequently characterized as a cell-cell adhesion molecule (Brackenbury et al., 1977; Cunningham et al., 1987; Jorgensen and Bock, 1974). NCAM is predominantly expressed in both neurons and glial cells during neural tube closure; in adulthood, it has also been found in heart, liver, kidney and skeletal muscles (Knittel et al., 1996; Nouwen et al., 1993; Romanska et al., 1996).

There are at least 27 alternatively spliced mRNAs produced from a single gene, NCAM1, (Cunningham et al., 1987), located on chromosome 11 in humans, chromosome 8 in rats, and on chromosome 9 in mice (D'Eustachio et al., 1985; Nguyen et al., 1986; Yasue et al., 1992). In mice, the NCAM1 gene is comprised of 0-19 major exons and an additional 6 smaller exons that give rise to a variety of distinct NCAM isoforms (Figure 1-4) (Walmod et al., 2004). There are three major isoforms of NCAM that are generated from alternative splicing and vary in their cytoplasmic domains. The first 15 exons encode NCAM's extracellular domain, containing 5 N-terminal homologous immunoglobulin (Ig 1-5) modules, followed by 2 fibronectin type (FN) III repeats that are closest to the membrane. The sequence downstream of exon 14 determines the specific NCAM isoform. NCAM 120 (NCAM-C) is a 725 amino acid (120 kDa) membrane protein that contains a STOP codon and two polyadenylation sites in exon 15, which anchors to the cell surface by a glycosylphosphatidylinositol (GPI) linker and is mainly expressed in glial cells. Alternatively, the replacement of exon 15 with 16 gives rise to a

transmembrane segment. NCAM 180 (NCAM-A) is a 950 amino acid (180 kDa) transmembrane protein that is comprised of exons 17-19 intracellular domains and is predominantly expressed in neurons. In contrast, NCAM 140 (NCAM-B) has a shorter cytoplasmic tail (exon 18 absent), and gives rise to an 850 amino acid (140 kDa) transmembrane polypeptides, found in both neurons and glia.

NCAM also exhibits in a truncated or soluble form, that can be found in cerebrospinal fluid, serum, and amniotic fluid in humans (Ibsen et al., 1983; Jørgensen and Bock, 1975), as well as in the cultural media of chick retinal cells, rat neurons and astrocytes (Nybroe et al., 1989; Rutishauser et al., 1976). The truncated form of NCAM can result from proteolytic cleavage of the extracellular segment of any of the 2 major isoforms (Nybroe et al., 1989; Vawter et al., 2001) or by the enzymatic removal of NCAM 120 from the cell surface via phosphatidylinositol phospholipase C (PI-PLC) (He et al., 1986). In addition, the soluble form of NCAM can be produced by alterative splicing through the introduction of a small secreted exon (SEC) with a stop codon between exons 12 and 13 of the NCAM1 transcript (Figure 1-4), resulting in the production of a truncated form of NCAM (115 kDa) with an incomplete extracellular domain (Bock et al., 1987; Secher, 2010).

The extracellular domain of NCAM can be modified by adding any of the 6 mini exons into the main transcript. Besides the SEC codon described above, there are 4 more known exons inserted between exons 12 and 13 of the NCAM1 transcript (Figure 1-4). These are the muscle specific domains 1 (MSD1), which comprises MSD1a (15 base pairs (bp)),

MSD1b (48 bp), MSD1c (42 bp), and a single nucleotide triplet, AAG or MSD1d (Dickson et al., 1987; Hamshere et al., 1991; Santoni et al., 1989). Although the MSD1 peptides of NCAM are isolated from muscle, they are regulated in distinctive manners and may be specialized to perform different functions; as well, some MSD1 are not tissue specific, as they can be found in other cell types, such as cultured neuroblastoma cells (Phimister et al., 1994). Besides MSD1, the inclusion of a 30 bp long sequence, variable alternatively spliced exon (VASE) at the exon 7 and 8 junction may cause a downregulation of neurite outgrowth promoting activity (Arce et al., 1996; Doherty et al., 1992; Liu et al., 1993). The unique NCAM VASE exon is predominantly expressed in the heart and nervous system, and its dysregulation has been linked to schizophrenia and bipolar disorder (Small and Akeson, 1990; Vawter, 2000).

Besides structural variation through alternative splicing of the NCAM1 transcript, NCAM protein can be post-translationally modified by Ser/Thr – phosphorylation, sulphation, palmitoylation, and N – and O – linked glycosylation (Gegelashvili et al., 1993; Krog and Bock, 1992; Linnemann et al., 1985; Ponimaskin et al., 2008; Sorkin et al., 1984). Notably, research studies predominantly focus on the post-translational modulation of NCAM through the attachment of polysialic acid (PSA), the polysialylated form of NCAM (PSA-NCAM), that was first reported by Finne and colleagues over 30 years ago (Finne et al., 1983). The homopolymer chain of PSA is formed by more than 90 sialic acid residues, which are large, negatively charged glycans with high water binding capacity. NCAM has six N- linked glycosylation sites, and the long PSA glycans attach to the 5^{th} and 6^{th} N-glycosylation sites of the Ig5 module of NCAM. The

polysialylation of NCAM increases the range and magnitude of intermembrane repulsion, thereby disrupting the adhesive properties of NCAM (Johnson et al., 2005). PSA-NCAM (PSA in short) is abundantly expressed in the embryonic brain. The first appearance of PSA occurs at embryonic (E) day 8 – 8.5 in mice (Probstmeier et al., 1994), and levels are maximized in the perinatal phase. The biosynthesis and expression of PSA on NCAM is controlled by the coordinated action of two polysialyltransferases, ST8SiaII (STX) and ST8SiaIV (PST). ST8SiaII is predominantly expressed during embryonic development, whereas ST8SiaIV is present at relatively high levels in the adult brain (Galuska et al., 2006; Kurosawa et al., 1997). NCAM is the main carrier of PSA in mammalian brain development, genetic ablation of NCAM removes almost all PSA (Cremer et al., 1994).

The transmembrane isoforms of NCAM, –140 and –180, are the major polysialylated molecules in the mouse brain, and nearly all NCAMs are polysialylated during development until PND 9. Thereafter, with the completion of major morphogenetic processes, PSA levels are reduced by approximately 70% within a week, resulting in the conversion of polysialylated to non–polysialylated NCAM (Oltmann-Norden et al., 2008). NCAM –140 and –180 are the predominant isoforms during development, and are expressed at constant levels from PNDs 1 to 21, but decrease by almost half in adulthood. NCAM –120 expression begins at PND 5, peaks around PND 17, then remain constant and become one of the major isoform in the mouse adult brain (Oltmann-Norden et al., 2008). Although a majority of the polysialylated form of NCAM diminishes within the first 3 weeks of postnatal brain development, some remains in certain brain regions for ongoing neurogenesis and synaptic plasticity during adulthood (Bonfanti, 2006; Seki and

Arai, 1993).

1.7.2 Cell Signaling and Interactions

NCAM plays a major role in signal transduction and the stabilization of synaptic connections. To ensure stable and reliable control of neuronal and network functions, NCAM mediates cell-cell adhesion through cis- and trans- homophilic actions, meaning NCAM on one cell binds to NCAM on the other. In one of the binding models, NCAM – NCAM interactions are involved in all five Ig domains, where they are engaged in binding through a pairwise antiparallel orientation (Ranheim et al., 1996). One of the hallmarks of homophilic interactions is the activation of calcium influx signal transduction pathway to stimulate neurite extension (Knittel et al., 1996; Sheng et al., 2013). NCAM is also capable of binding to a number of other molecules on the cell surface through heterophilic interactions, and this results in a variety of cellular responses; for instance, the Ig4 module of NCAM forms cis-interaction with L1 (the other closely related CAM protein in the IgSF) to induce L1 phosphorylation, that promotes neuronal migration and axon guidance (Kristiansen et al., 1999; Schmid and Maness, 2008). The other known interacting partner of NCAM is fibroblast growth factor receptor (FGFR), the 2nd FNIII module of NCAM that binds to the 3rd Ig domain of FGFR1 in a heterophilic manner and mediates neurite outgrowth through the activation of intracellular signaling events, such as the phosphatidylinositol-3-kinase protein kinase B (PI3K–PKB/Akt) pathway (Ditlevsen et al., 2003; Kiselyov et al., 2003). Furthermore, NCAM can promote axonal growth and cell migration in an FGFR-independent manner through the association with the glial cell-derived neurotrophic factor (GDNF) family

receptor alpha1 (GFRα1) that mediates the downstream of GDNF signaling via tyrosine-protein kinase (Fyn), focal adhesion kinase (FAK) and the extracellular signal regulated kinase (ERK1/2) signaling pathways (Paratcha et al., 2003). Besides homophilic and heterophilic NCAM interactions, the presence of PSA on NCAM also promotes a number of signaling cascades. In BDNF related pathways, PSA facilitates signaling by presenting BDNF to the tropomyosin receptor kinase B (TrkB) receptor (Vutskits et al., 2001). As well, the TrkB receptor directly interact with NCAM via the sequences in their intracellular domains, and this binding mediates tyrosine phosphorylation of NCAM and promotes NCAM-dependent neurite outgrowth (Cassens et al., 2010; Kleene et al., 2010).

1.7.3 Neural Cell Adhesion Molecule in Learning and Synaptic Plasticity

As mentioned earlier, NCAM regulates a number of cellular events through mediating cell adhesion and signal transduction, such as neurite outgrowth, migration, and axon pathfinding during CNS development (Bruses and Rutishauser, 2001). In addition, NCAM is implicated in synaptic plasticity, learning, and emotional processes in adulthood (Gascon et al., 2007b). NCAM deficient mice display pronounced learning and memory impairment in spatial learning tasks and open field tests (Cremer et al., 1994). The intraventricular infusion of NCAM antibodies interferes with NCAM function, inhibits LTP in normal rat hippocampal slices and impairs learning in wild type (WT) rats and chicks (Doyle et al., 1992b; Luthl et al., 1994; Scholey et al., 1993). Similar effects have been observed in organotypic slice cultures prepared from NCAM knockout (−/−) mice, as well as from WT mice treated with endoneuraminidase-N (Endo N) selectively removed PSA from NCAM (Muller et al., 1996). However, how does PSA and NCAM

affect synaptic plasticity? High levels of PSA are expressed on axons and dendrites prior to synapse formation, but decrease rapidly once the contacts are established (Kiss et al., 2001). Hence, the mechanism of action between PSA and NCAM is associated with the dynamic balance between stability and plasticity of synaptic connections. The non-polysialylated NCAM stabilizes cell – cell adhesion, and the polysialylated NCAM hinders/ loosens membrane – membrane apposition that enables structural modification of the cells (Rutishauser and Landmesser, 1996). Thus, the presence of PSA on NCAM reduces intercellular adhesion and promotes synaptic structural remodeling, by which long-term memory formation and learning-dependent synaptic plasticity are supported.

1.8 Goals of the Thesis

The primary goal of this thesis is to investigate, in the adult and aging animal, the influence of NCAM on vision. I have studied the importance of NCAM in the visual system during aging, the mechanism by which NCAM promotes retinal survival, and the role of PSA in the restoration of visual cortical function. Particularly, using anatomical, electrophysiological and behavioral assays, I analyzed age-related changes in visual function of NCAM deficient and WT mice, providing insight into how NCAM supports normal retinal architecture and physiology, and helps to maintain vision from early adulthood to senescence. To my knowledge, this is the first study showing that PSA and NCAM expression are required to maintain normal visual function during aging (Chapter 2). To gain insight into the mechanism by which NCAM protects retinal neurons, I used light-induced injury of the retina as a model to study how NCAM influences $p75^{NTR}$ signaling to promote retinal cell survival (Chapter 3). I then went on to determine

whether the PSA moiety of NCAM is required for neuroplasticity and the recovery of vision in adult mice after long-term MD (Chapter 4). Collectively, results from these experiments provide insight into the possible role of NCAM in human age-dependent visual impairment, offer opportunities for targeted molecular treatment in retinal degeneration diseases, and open up new therapeutic avenues in visual recovery through the manipulation of brain plasticity.

CHAPTER 2: EFFECT OF NEURAL CELL ADHESION MOLECULE ON AGE-RELATED DETERIORATION IN VISION

2.1 Preface and Significance to Thesis

The neural cell adhesion molecule (NCAM) is involved in developmental processes and age-associated cognitive decline; however, little is known concerning the effects of NCAM in the visual system during aging. In this Chapter, using anatomical, electrophysiological, and behavioral assays, I analyzed age-related changes in visual function of NCAM deficient and WT mice. Anatomical analyses show that aging NCAM −/− mice have thinner retinas, fewer RGCs and photoreceptor cell layers than age-matched controls. Electroretinogram testing of retinal function in young adult NCAM −/− mice show a 2-fold increase in a- and b-wave amplitude compared with WT mice, but the retinal activity drops dramatically to control levels by the age of 10 months. In behavioral tasks, NCAM −/− mice have no visual pattern discrimination ability and show premature loss of vision as they age. Together, these findings demonstrate that NCAM plays significant roles in the adult visual system in establishing normal retinal anatomy, physiology and function, and in maintaining vision during aging.

This Chapter has been published in *Neurobiology of Aging* (Luke et al., 2016a). Preliminary results of this study have been presented at the 40th and the 41st Annual Meetings of the *Society for Neuroscience* in San Diego (2010) and Washington D.C. (2011) respectively and published in abstract forms (Luke et al., 2010, 2011).

2.2 Introduction

As we age, our vision deteriorates due to the progressive decline in visual acuity, decreased contrast sensitivity, and an increase in the dark adaptation threshold (Salvi et al., 2006). These progressive changes are associated with changes in lens flexibility, altered retinal electrophysiological properties, a reduction in the number of neurons and regression of dendritic arbors in the retina (Freund et al., 2011; Samuel et al., 2011). Aging may be accompanied by a number of degenerative diseases of the eye, including glaucoma, cataracts, diabetic retinopathy and macular degeneration, all of which can lead to irreversible blindness. Although the underlying causes remain unclear, the decline of visual function in aging is associated with both genetic and environmental factors (Hogg et al., 2009). The development of the visual system involves a temporal sequence of neurochemical signals that regulate synaptic connections and those processes are modulated by visual experience (Belanger and Di Cristo, 2011; Chen et al., 2012). The neural cell adhesion molecule (NCAM) is involved in many stages of the development of the mouse visual system (Bartsch et al., 1990; Clandinin and Feldheim, 2009; Missaire and Hindges, 2015). Little is known, however, concerning the role of NCAM during age-related disorders of visual functions.

NCAM is a glycoprotein of the immunoglobulin superfamily, containing two major transmembrane isoforms (180 and 140 kDa) and a smaller glycophosphatidyl inositol-linked isoform (120 kDa) (Soroka et al., 2008). All major NCAM isoforms can be modified by PSA that is widely expressed during CNS development. NCAM is the most abundant PSA carrier in mammals (Nelson et al., 1995) and the removal of NCAM

abolishes almost all of the PSA in the nervous system (Finne et al., 1983). In the retina, the highly sialylated form of NCAM is expressed throughout all retinal layers during development (Bartsch et al., 1990). However, in adult mice, it is located exclusively on astrocytes and Müller glial cells of the retina and on astrocytes of the ON (Bartsch et al., 1990). Although PSA-NCAM is not expressed in adult RGCs, it has been demonstrated that Müller glia processes ensheath most retinal neurons; therefore, PSA-NCAM resides in close proximity to neurons, including RGCs (Bartsch et al., 1990; Stone et al., 1995). In the mouse visual cortex, PSA and NCAM are highly expressed during development; however, the expression of PSA declines in response to visual system sensory input shortly after eye opening (Di Cristo et al., 2007).

There are a number of age-related changes in neural circuitry underlying vision in the mouse (Samuel et al., 2011). NCAM plays an important role in age-related cognitive impairments (Bisaz et al., 2013), but no research has examined the role of NCAM in age-related visual dysfunction. The purpose of the current work was to investigate the effects of NCAM in the visual system during aging. I used anatomical, functional and behavioral analyses to determine how NCAM deficiency in mice affects age-related changes in visual function. I have found that, in aging WT animals, PSA levels are lower in the retina, but higher in the visual cortex, and both regions exhibit differential expression of NCAM isoforms. Despite the fact that young adult NCAM −/− mice have more RGCs, the total number of optic nerve axons remains the same as in WT mice. In the absence of NCAM, retinal activity increases in young adult mice; however, as the knockout animals age, there is a dramatic drop in retinal activity amplitude and thinning

of the retinas. These NCAM-dependent changes are associated with significant functional consequences: impaired pattern discrimination ability in adulthood and premature loss of visual acuity as the animals age.

2.3 Methods

2.3.1 Subjects

Homozygous NCAM −/− mice and their WT littermates (n = 10 mice of mixed sex for each of the 2, 10, 18 and 24 month groups) on a C57BL/6J background were obtained by crossing heterozygous male and female animals (generously provided by Dr. Victor Rafuse), and genotypes of mice were determined by polymerase chain reaction (PCR) using primers and conditions as indicated previously (Cremer et al., 1994). Animals were bred in-house and were cared for according to the Guide to the Care and Use of Experimental Animals of the Canadian Council on Animal Care. The experimental protocol (number 13-081) was approved by the Dalhousie Committee on Laboratory Animals.

2.3.2 Immunohistochemistry

Animals were anesthetized and transcardially perfused with chilled 4% paraformaldehyde (PFA). The eyes were removed, post-fixed overnight at 4^{o}C, and cryo-protected in 30% sucrose. Retinal sections (16 μm) were prepared by orienting the eyes in OCT (Tissue-Tek, Miles Laboratories), and cutting with a cryostat (Leica CM1850) along the temporal-nasal orientation of the eye. Coronal brain sections through the VC (25μm) were also sliced with a cryostat. The sections were immunoreacted with anti-NCAM (MAB310, Millipore; 1:100) and anti-PSA-NCAM (MAB5324, Millipore; 1:500)

antibodies. Immunohistochemistry was performed as previously described (Murphy et al., 2009).

2.3.3 Immunoblot

Ten retinas and VC per group were used in the 2, 10 and 18-month-old WT samples, but only 5 were collected for the 24-month-old animals. Anti-NCAM (MAB310, Millipore; 1:500), anti-PSA-NCAM (MAB5324, Millipore; 1:1000), and anti-actin (A2066, Sigma; 1:5000) antibodies were used to determine protein expression by standard SDS-PAGE immunoblotting (Murphy et al., 2007a).

2.3.4 Morphometric Analysis

To determine the thickness of 2-month-old and 18-month-old retinas, retinal sections (16 μm) were prepared as indicated above. To ensure consistency in counting cells, only retinal sections that included the ON stump were selected. The sections were stained with nuclear (Hoechst; 1: 50,000, Sigma) and synaptic (Bassoon; 1: 500, Enzo Life Sciences) markers. Five animals were examined per group, and retinal images (n=5 section/ mouse retina) were taken using fluorescence microscopy (Zeiss LSM510 META). Thicknesses of the whole retinas, and each of their relative layers, were measured 300 μm (central), 1100 μm (middle) and 1900 μm (peripheral) from the edge of the ON head using ImageJ. The number of rows of photoreceptor cells in the ONL was counted on the same retinal images using the method of Smith et al. (2008).

2.3.5 Retinal Ganglion Cells: Labeling and Counting

Using the retinal sections (n=5/ group) prepared above, I labeled RGCs using an anti-Neuronal Nuclei (NeuN) (1:500; Millipore) antibody and an anti-Choline

Acetyltransferase (ChAT) (1:400; Millipore) antibody, a method shown to be effective for RGC quantification in young adult and senescent animals (Buckingham et al., 2008). RGCs were counted in the GCL of the whole retinal cross-sections using the method of Smith et al. (2008).

2.3.6 Transmission Electron Microscopy (TEM) Analysis

For TEM analysis, the ONs were prepared as previously described (Murphy et al., 2007a). The images of 100-130nm ON sections were obtained under an electron microscope (EM300; Philips). For RGC axon counting, whole ON cross sections were photographed (~20-30 micrographs) and counted manually using the method of Murphy et al. (2007).

2.3.7 Electroretinogram Recording

Mice were anaesthetized using a mixture of ketamine (100 mg/kg), acepromazine (0.62 mg/kg), and xylazine (15 mg/kg), placed into a stereotaxic frame, and their pupils were dilated with 0.5% cyclopentolate HCl drops (Alcon, Fort-Worth, TX). They were kept at a constant body temperature (37^{o}C) by a heated pad with rectal temperature feedback. An active electrode (Dawson-Trick-Litzkow-plus microconductive fiber, Diagnosys, Littleton, MA) was placed on the mouse's corneal surface and hydrated with 2.5% hydroxypropyl methylcellulose solution. A reference electrode was inserted at the base of the nose and a ground electrode was placed on the tail (Platinum subdermal electrodes, Grass Instruments, Quincy, MA). Signals recorded from the corneal electrode were first collected and amplified 10,000-fold by a differential amplifier with a bandwidth of 3-1000 Hz (P511, Grass Instruments), then digitized into 300 sample points at a rate of

1000 Hz by an A/D instrument converter (GW Instruments, Summerville, SC) (Smith et al., 2013).

After overnight dark adaptation, animals were exposed to a series of strobe flash visual stimuli generated by a PS3 photic-stimulator (Grass Instruments) with increasing intensity from -5.8 to 1 log cd·s/m^2 in scotopic conditions presented against a Ganzfield bowl (LKC Technologies, Gaithersburg MD, USA). Following 10 minutes of light adaptation, a series of flashes ranging from 0.8 to 1.0 log cd·s/m^2 were delivered with a static background illumination at 2.1 log cd·s/m^2. Signals were averaged in each intensity scale following exposure to 16 successive flashes.

2.3.8 Visual Evoked Potentials (VEPs)

Recording procedures for flash VEP have been described elsewhere (Smith et al., 2013). After a craniotomy was performed, an active electrode (Varnish coated platinum subdermal electrode) was placed 2mm lateral to the midline and 3mm anterior to lambda coordinate point over the left occipital cortex without penetrating the dural surface. Visual stimuli ranging from -5.0 to 1.0 log cd·s/m^2 were delivered for the dark adapted animals through a PS3 photic-stimulator in a Ganzfeld diffuser.

2.3.9 Visual Ability Testing

Experimental procedures and the apparatus for the visual behavior test have been previously described in detail (Wong and Brown, 2006, 2007). The visual ability test consists of 4 phases totaling 25 days of testing: 1. Pre-training (1 day); 2. Visual

Detection (8 days); 3. Pattern Discrimination (8 days); and 4. Visual Acuity (8 days). The number of correct trials for each day was converted into a percent correct; 70% of the trials must be successful in order to reach threshold for each task.

2.3.10 Data Analysis

The differences in western blot densitometry from three independent experiments were quantified by one-way ANOVA, followed by Fisher's LSD post hoc analysis. For RGC density, axon counting and retinal thickness analysis, data obtained were compared by independent two-sample *t* tests. A customized program from Matlab (Mathworks, Natick, MA) was used to examine the electrophysiology recordings. Specifically, to analyze amplitudes and implicit times (time to peak) of the ERG responses, the photoreceptor cells' a-wave was measured from the baseline to the trough. The b-wave, which reflects mainly on ON-bipolar activity, was measured from the trough of the a-wave to the peak of the b-wave. To evaluate the effect of NCAM on the age-related changes of the ERG amplitudes in a scotopic environment, parameters V*max* (value of the maximum amplitude), *n* (slope of the intensity-response function), and *k* (response sensitivity) of the Naka-Rushton hyperbolic equation were derived from the measurement values based on the curve fitting procedure (Anastasi et al., 1993). Differences were analysed by all three parameters to compare NCAM −/− with the WT mice. For ERG and VEP analyses, differences obtained between genotypes were evaluated with Student's *t* tests. For the visual ability test, percent correct data were analyzed by a 2x8 (strain by days/ frequencies) between-within analysis of variance (ANOVA) (Wong and Brown, 2006). The age effect was analyzed by a 4x8 (age by days/frequencies) between-within

ANOVA. Post hoc analyses were done using Bonferroni's test. Strain differences in percent correct on day 8 were investigated using two-sample *t* tests. Statistics were expressed as a mean percentage ± standard error of the mean (SEM). Differences were considered statistically significant if $p < 0.05$ and not significant (ns) if $p \geq 0.05$. All analyses were performed using Statview 5.0 (Abacus Concepts) and Minitab 16.

2.4 Results

2.4.1 PSA and NCAM expression in the visual system

To determine whether NCAM plays a role in visual function during aging, I assessed PSA and NCAM expression in the retinas and VC of 2, 10, 18 and 24-month WT mice. In the retina, immunostaining showed no difference in the expression patterns of PSA and NCAM between 2 and 18 months of age (Figure 2-1). Both PSA and NCAM were expressed throughout the retinas, but NCAM immunoreactivity was most prominent in the inner retina. Immunoblot analyses (Figure 2-2a) of PSA levels in the retina at 10 months of age, compared with 2 months, were slightly increased but then dropped significantly when the animals reached 18 and 24 months. Evaluation of NCAM isoforms showed higher levels of 120 kDa and lower levels of 180 kDa were expressed in the retina at 18 months. Furthermore, an additional 150 kDa NCAM isoform appeared in the 2, 10 and 24-month-old retinas.

Figure 2-1. PSA and NCAM staining in normal retinas. Representative PSA and NCAM immunofluorescence staining (red) in WT retina, showing no difference in the expression patterns between retinas of 2- and 18-month-old mice. (a) 2 months, PSA staining; (d) 18 months, PSA staining; (g) 2 months, NCAM staining; (j) 18 months, NCAM staining; (b, e, h, and k) TO-PRO-3 labeled nuclei (green); (c, f, i, and l) merged images. Abbreviations: GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; NCAM, neural cell adhesion molecule; ONL, outer nuclear layer; OPL, outer plexiform layer; PSA, polysialic acid; WT, wild type.

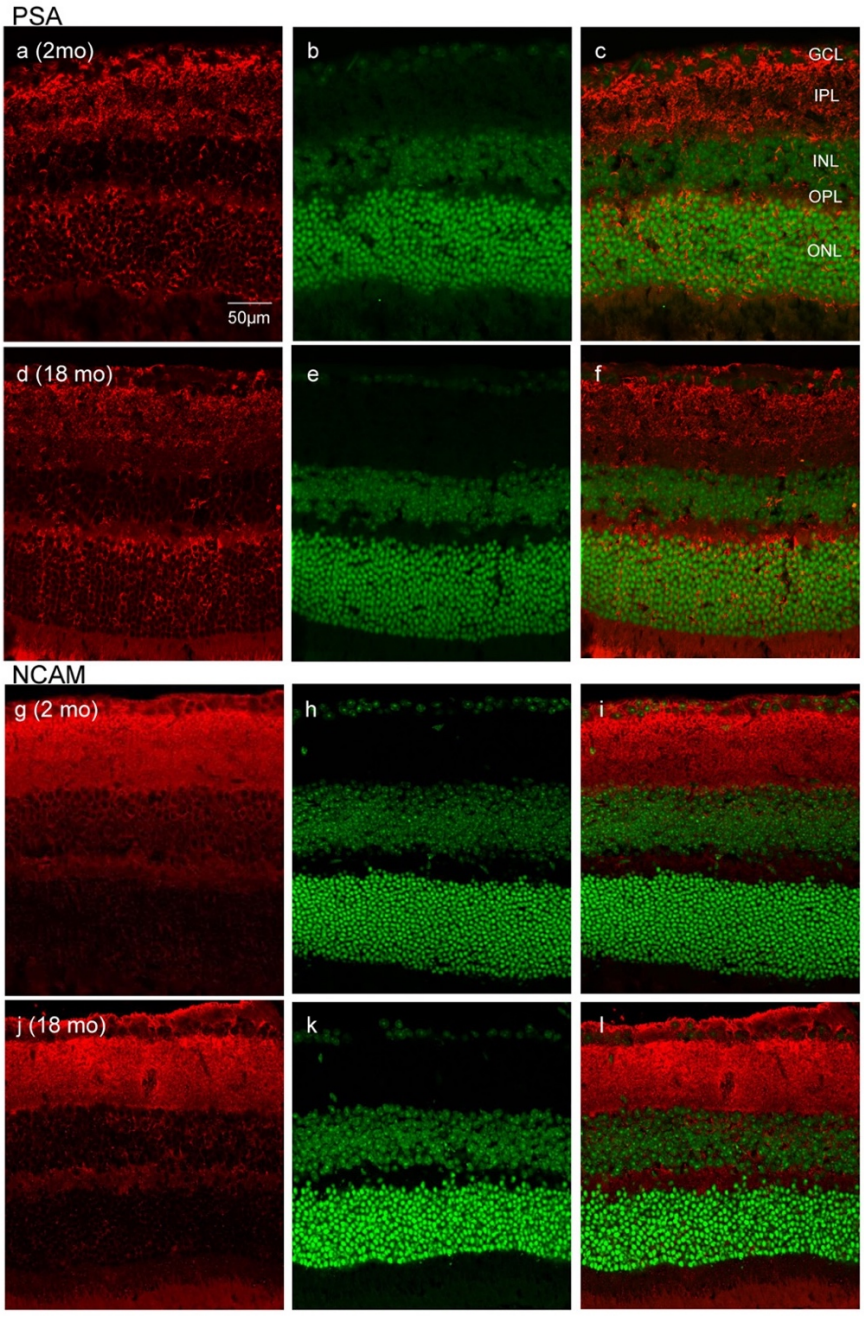
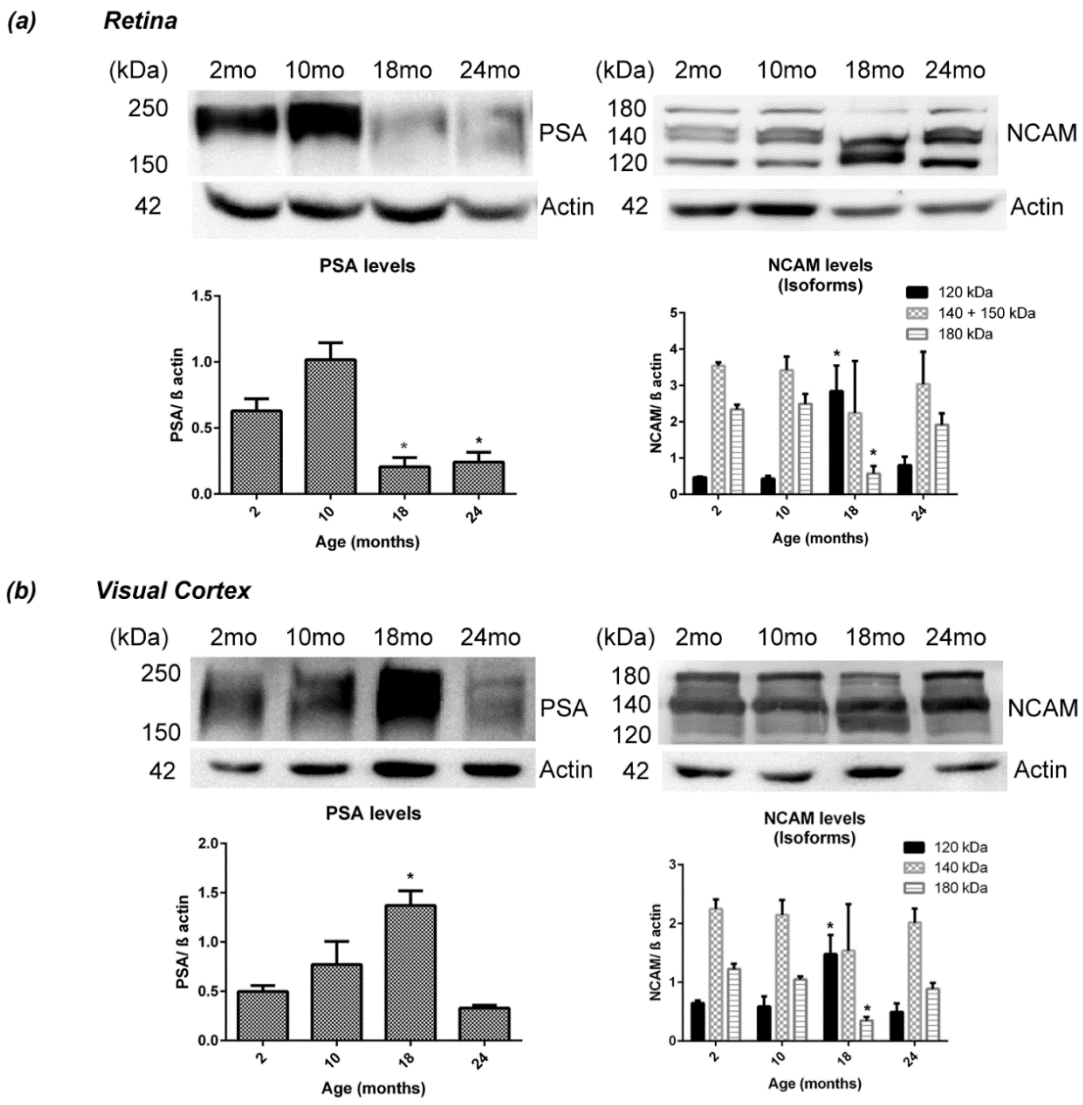


Figure 2-2. PSA and NCAM expression in WT retina and visual cortex. Immunoblot analysis of PSA and NCAM protein expression in (a) retinas and (b) visual cortex of 2-, 10-, 18-, and 24-month-old mice. PSA levels decline with age in the retina but increase in the visual cortex, and both regions show differential expression of NCAM isoforms as the mice age. Asterisk (*) denotes significantly different from 2-month-old protein expression. Abbreviations: NCAM, neural cell adhesion molecule; PSA, polysialic acid; WT, wild type.



There was no detectable PSA immunostaining in the V1 (data not shown). PSA protein levels in the VC were generally low and the highest expression was at 18 months of age (Figure 2-2b). Examination of NCAM isoforms revealed lower levels of 180kDa in the VC at 18 months. NCAM-140 expression was seen predominantly in the VC and did not appear to change over time. NCAM-120 levels were scarcely detectable during adulthood, but increased sharply in the VC when the animals reached 18 months of age.

2.4.2 Anatomical Analysis

2.4.2.1 NCAM −/− mice have altered retinal morphometry

I compared histologic sections of WT and NCAM −/− retinas at 2 and 18 months of age, and found no observable changes in basic retinal layer arrangement between genotypes and ages (Figure 2-3). To examine whether the absence of NCAM altered retinal morphometry, I measured the thickness of the whole retina and five individual retinal layers: the GCL, the IPL, the INL, the OPL, and the ONL (Kolesnikov et al., 2010; Samuel et al., 2011).

At 2 months, the total retinal thickness in NCAM −/− mice was comparable to the controls, with only one small, but statistically significant difference in the peripheral temporal region (Figures 2-4a). However, comparison of the individual layers revealed increased thickness in the inner layers of NCAM −/− retinas, including in the GCL and peripheral IPL (Figures 2-4b and c). This thickening was most marked in the GCL (Figure 2-4b), which showed over 50% increased thickness in the middle and the

Figure 2-3. Representative retinal sections from (a) 2- and (b) 18-month-old mice labeled with Bassoon (red, synaptic staining) and Hoechst (green, nuclear staining) in the central, middle, and peripheral areas show no observable difference in basic retinal layer arrangement between WT and NCAM −/− mice. Abbreviations: GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; NCAM, neural cell adhesion molecule; ONL, outer nuclear layer; OPL, outer plexiform layer; WT, wild type.

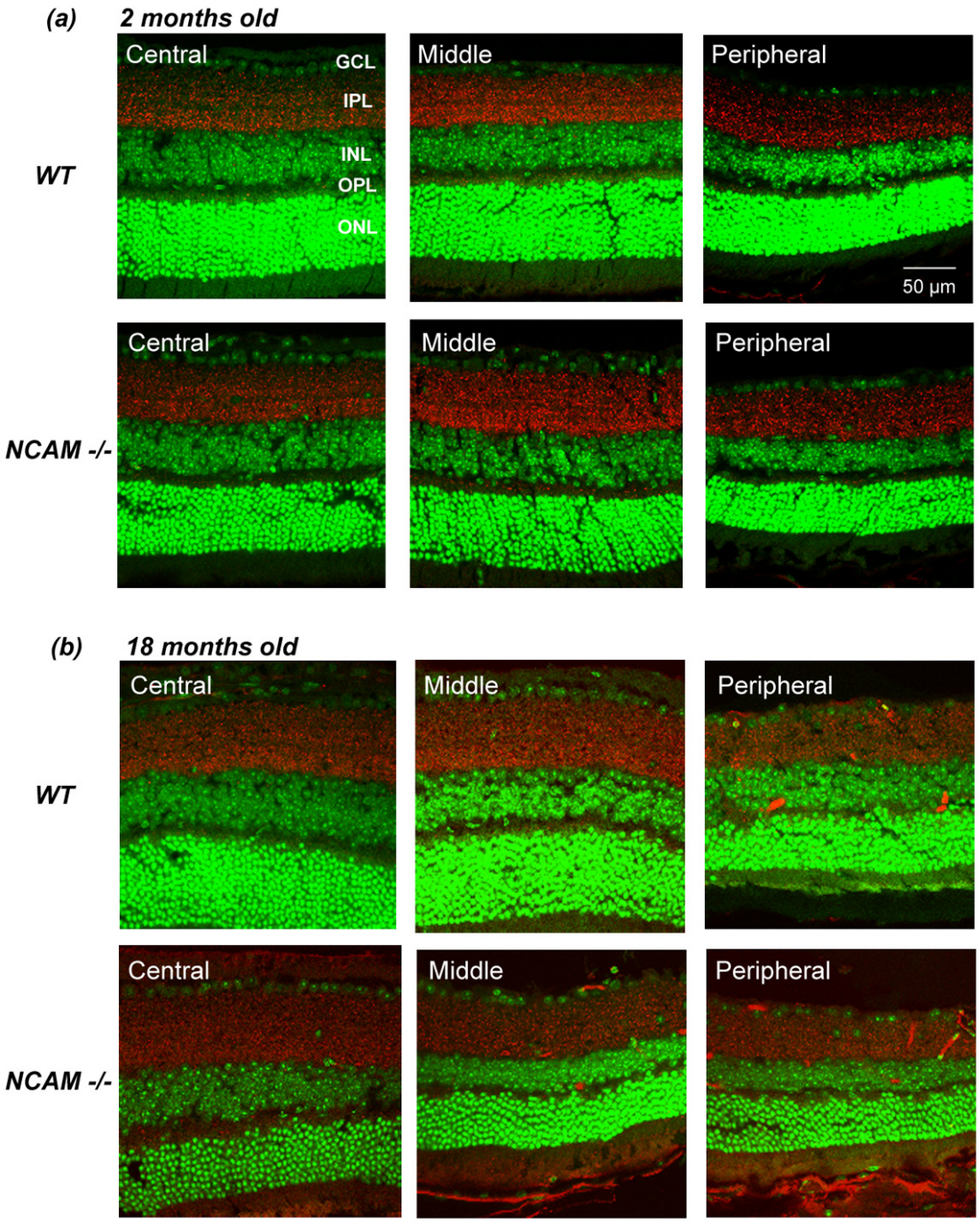
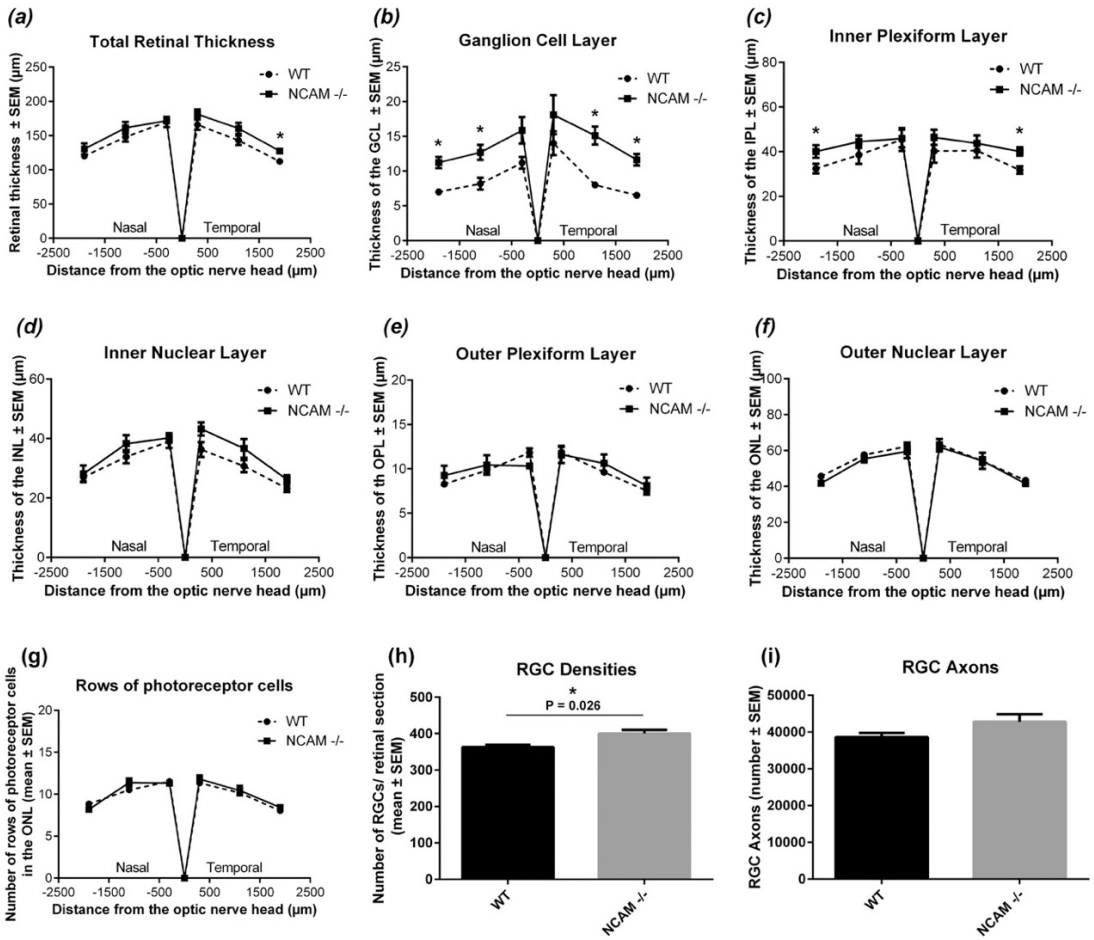


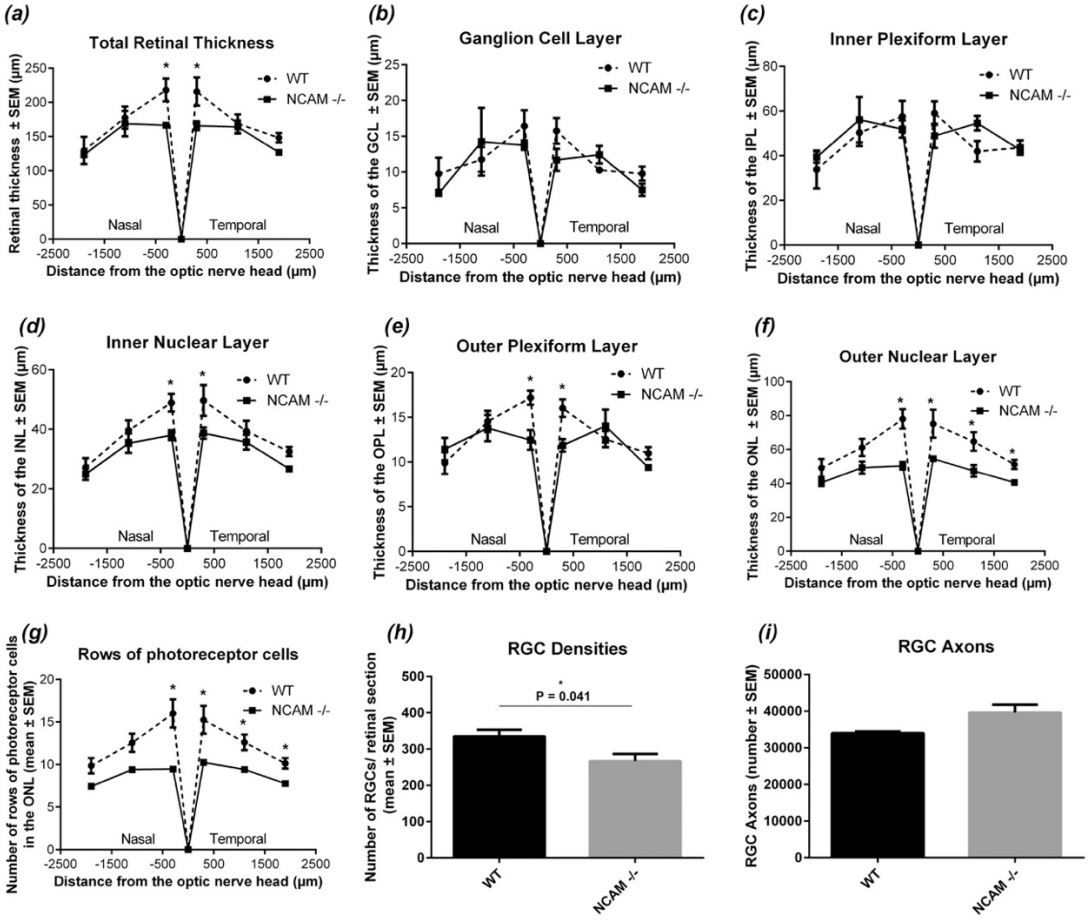
Figure 2-4. Quantitative comparison of WT and NCAM −/− retinas at 2 months. Young adult NCAM −/− mice have thicker ganglion cell layers and more RGCs. (a–f) Morphometric analysis of the retinal sections. (g) The number of rows of photoreceptor cells in the ONL at 2 months. (h and i) RGCs and RGC axon densities showing relative number of RGCs and axons in the NCAM −/− and WT animals. Asterisk (*) denotes significantly different between WT and NCAM −/− retinas. Abbreviations: GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; NCAM, neural cell adhesion molecule; ONL, outer nuclear layer; OPL, outer plexiform layer; RGC, retinal ganglion cell; SEM, standard error of the mean; WT, wild type.



peripheral regions [nasal middle: 12.7 ± 1.1µm (NCAM −/−) vs. 8.2 ± 0.9µm (WT), p = 0.001; temporal middle: 15.1 ± 1.3µm (NCAM −/−) vs. 8.0 ± 0.5µm (WT), p = 0.012; nasal peripheral: 11.2 ± 0.8µm (NCAM −/−) vs. 7.0 ± 0.3µm (WT), p = 0.000; and temporal peripheral: 11.6 ± 0.8µm (NCAM −/−) vs. 6.5 ± 0.2µm (WT), p = 0.001]. I examined the GCL in greater detail and found that the measured thickness in NCAM −/− mice at 2 months of age could be due to a significantly greater number of GCL cells [601 ± 16 cells/section (NCAM −/−) vs. 494 ± 19 cells/ section (WT)]. The NCAM −/− mice showed no differences from the WT mice in the thickness of the INL, OPL or ONL at 2 months of age (Figures 2-4 d-f).

At 18 months, retinal thinning in NCAM −/− mice (Figure 2-5a) relative to WT retinas was restricted to the central areas [nasal: 166.6 ± 4.9µm (NCAM −/−) vs. 218.1 ± 16.7µm (WT), p = 0.018; and temporal: 165.7 ± 6.5µm (NCAM −/−) vs. 215.7 ± 20.6µm (WT), p = 0.043]. Unlike in 2 month NCAM −/− mice, exaggerated thinning occurred more broadly in the 18-month-old NCAM −/− retinas (Figures 2-5 d-f), in the INL, the OPL, and especially in the ONL, which showed approximately one third loss of its thickness in the central areas [nasal central: 50.4 ± 2.3µm (NCAM −/−) vs. 78.0 ± 5.8µm (WT), p = 0.002; temporal central: 54.5 ± 1.5µm (NCAM −/−) vs. 75.2 ± 8.2µm (WT), p = 0.039]. Additional quantitative comparisons revealed a significant reduction in the number of rows of photoreceptor cells in the ONL (Figure 2-5g). Both retinal thickness and photoreceptor cell layer measurements were congruent in showing excessive thinning of the ONL in 18-month-old NCAM −/− mice.

Figure 2-5. Quantitative comparison of WT and NCAM −/− retinas at 18 months. Aging NCAM −/− mice have thinner retinas and contain fewer RGCs and rows of photoreceptor cells. (a–f) Morphometric analysis of the retinal sections. (g) The number of rows of photoreceptor cells in the ONL at 18 months. (h and i) RGCs and RGC axon densities showing relative number of RGCs and axons in the NCAM −/− and WT animals. Asterisk (*) denotes significantly different between WT and NCAM −/− retinas. Abbreviations: GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; NCAM, neural cell adhesion molecule; ONL, outer nuclear layer; OPL, outer plexiform layer; RGC, retinal ganglion cell; SEM, standard error of the mean; WT, wild type.



In summary, compared with WT mice there was thickening in the inner retinal layers and a greater number of cells in the GCL of 2-month-old NCAM −/− mice. As NCAM −/− animals age, exaggerated thinning occurred in all layers of the retina, disproportionately so in the ONL.

2.4.2.2. Fewer RGCs in aging NCAM −/− retina but no change in the number of ON axons

Our morphometric results (section 3.2.1) showed thickening of the GCL with a greater number of cells in the retinal layer of young adult NCAM −/− mice. Consistent with this, previous studies from our laboratory have shown an increase in RGC density in young adult NCAM −/− mice, as well as altered RGC densities in response to injury (Murphy et al., 2007a; Murphy et al., 2009). Therefore, I compared 2 and 18 month old mice to determine whether the rate of RGC loss in NCAM −/− animals is altered during aging using NeuN antibody (Buckingham et al., 2008). At 2 months (Figure 2-4h), I found more RGCs (400 ± 10 cells/retinal section) in the NCAM −/− retinas compared with WT mice (363 ± 6 cells, $p = 0.026$). But, by 18 months of age (Figure 2-5h), the NCAM −/− retinas lost approximately one third of their RGCs and had 20% fewer RGCs (267 ± 18 cells) than WT (335 ± 19 cells, $p = 0.041$).

Since axon degeneration can precede cell body death (Buckingham et al., 2008), I determined whether RGCs in NCAM −/− mouse retinas were anatomically intact by quantifying the number of RGC axons in the ONs. I was surprised to find that there were no differences in the number of ON axons between NCAM −/− (2 mo: 42754 ± 2096; 18

mo: 39613 ± 2152) and WT (2 mo: 38592 ± 1165; 18 mo: 34034 ± 434) mice at either age (Figures 2-4i and 2-5i).

Together, these results show that young adult NCAM −/− mice have more RGCs than WT mice; however, as they age, the NCAM −/− mice lose RGCs more rapidly than WT, but at least at 18 months of age, they do not differ from WT with the loss of ON axons.

2.4.3 Electrophysiological Studies

Whether or not NCAM plays a physiological role in visual function is unknown. To investigate this, I measured ERGs to assess retinal function and VEPs to determine the functional integrity of the visual pathway.

2.4.3.1 ERG is altered in young NCAM −/− mice

Figure 2-6a shows representative ERG intensity-response series to test flashes for groups of WT and NCAM −/− mice at 2, 10, 18 and 24 months. As both groups of animals age, ERG waveform amplitudes decrease as expected and there were no observable differences in the waveform pattern. There were comparable response times to the light stimuli: higher intensity stimuli produced faster responses, and aged animals had relatively longer response times than young adult mice (Figure 2-7). Using the Naka-Rushton equation, intensity-response functions of ERG a- and b- waves in WT and NCAM −/− mice were analyzed in detail (Table 2-1) and results are discussed below.

Figure 2-6. Analysis of ERG responses in 2-, 10-, 18-, and 24-month-old mice. (a) Representative dark-adapted ERGs recorded from WT (blue) and NCAM −/− (purple) mice showing weakening of the ERG waveform as both groups of animals age. Flash intensity, increasing from −3.8 to 1 log cd × s/m^2, is marked on the right on each pair of the waveforms. (b) Mean dark-adapted a- and b-wave amplitudes plotted against log flash intensity for NCAM −/− and WT mice. Two-month-old NCAM −/− mice have significantly higher a- and b-wave amplitudes than WT mice. Points represent means ± SEM ERG amplitude. Abbreviations: ERG, electroretinogram; NCAM, neural cell adhesion molecule; WT, wild type.

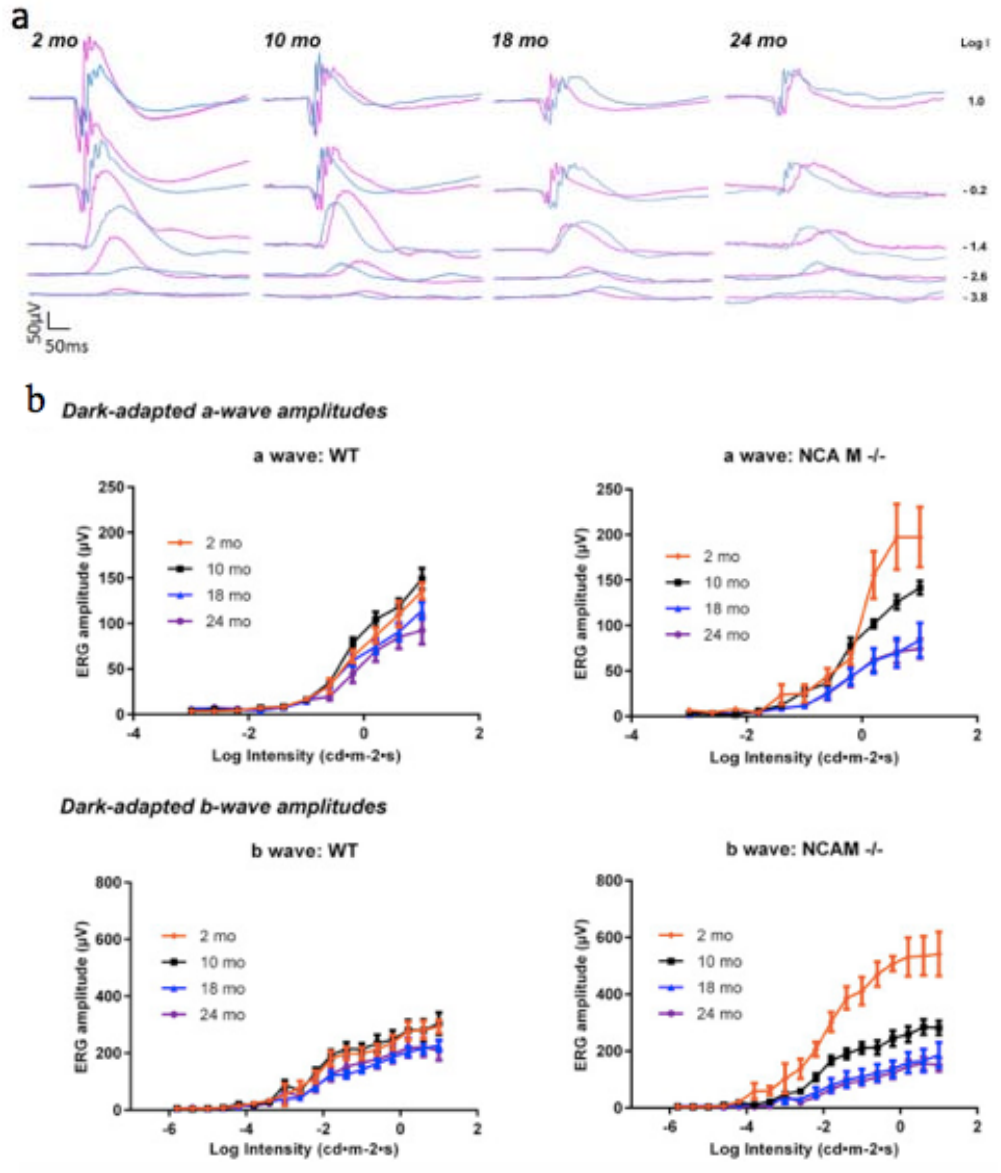


Figure 2-7. Mean dark-adapted a- and b-wave implicit times (time to peak) plotted against flash intensity for WT and NCAM −/− mice at 2, 10, 18 and 24 months old showing consistently longer response times occur in both groups as the animals aged. Points represent means ± SEM.

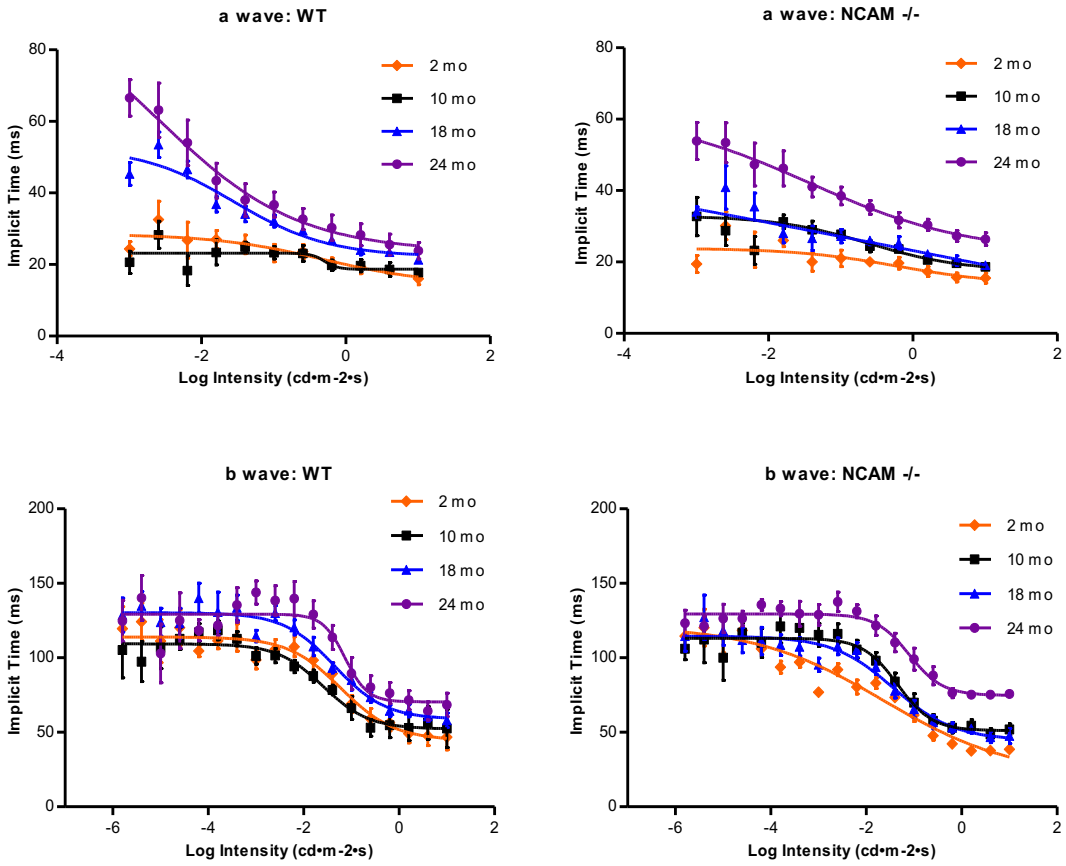


Table 2-1. Vmax, log k and n parameters derived from scotopic ERG a- and b- wave intensity-response function for 2, 10, 18 and 24 months old NCAM −/− and WT mice show that young adult NCAM −/− mice have higher than usual retinal activity levels. Values are means ± SEM.

(a)	*Vmax*	
A wave	***WT***	***NCAM -/-***
2 mo *	110 ± 9	230 ± 37
10 mo	144 ± 9 [a]	164 ± 12 [a]
18 mo	93 ± 10 [a, b]	100 ± 18 [a, b]
24 mo	90 ± 16 [b]	82 ± 11 [a, b]
B wave	***WT***	***NCAM -/-***
2 mo *	306 ± 20	632 ± 111
10 mo	291 ± 32	262 ± 27 [a]
18 mo	213 ± 18 [a]	222 ± 43 [a]
24 mo	201 ± 31 [a]	172 ± 24 [a, b]

(b)	*log k*	
A wave	***WT***	***NCAM -/-***
2 mo	- 0.28 ± 0.14	- 0.15 ± 0.14
10 mo	- 0.069 ± 0.21	- 0.078 ± 0.10
18 mo	- 0.044 ± 0.33	0.27 ± 0.38
24 mo	0.45 ± 0.44	0.039 ± 0.26
B wave	***WT***	***NCAM -/-***
2 mo	-2.28 ± 0.14	-2.06 ± 0.24
10 mo	-2.19 ± 0.23	-1.94 ± 0.10
18 mo *	-1.91 ± 0.11	-0.88 ± 0.31 [a,b]
24 mo	-1.80 ± 0.17	-1.28 ± 0.19 [a,b]

(c)	*n*	
A wave	***WT***	***NCAM -/-***
2 mo	1.08 ± 0.14	1.18 ± 0.11
10 mo	0.98 ± 0.11	0.88 ± 0.07 [a]
18 mo	1.14 ± 0.11	0.79 ± 0.15 [a]
24 mo	0.89 ± 0.24	1.01 ± 0.06 [a]
B wave	***WT***	***NCAM -/-***
2 mo	0.63 ± 0.08	0.58 ± 0.13
10 mo	0.65 ± 0.06	0.71 ± 0.10
18 mo *	0.50 ± 0.01 [b]	0.39 ± 0.02 [b]
24 mo	0.62 ± 0.05	0.51 ± 0.03 [c]

* = WT differs from NCAM −/− mice
a = differs from 2 months old
b = differs from 10 months old
c = differs from 18 months old

A-waves

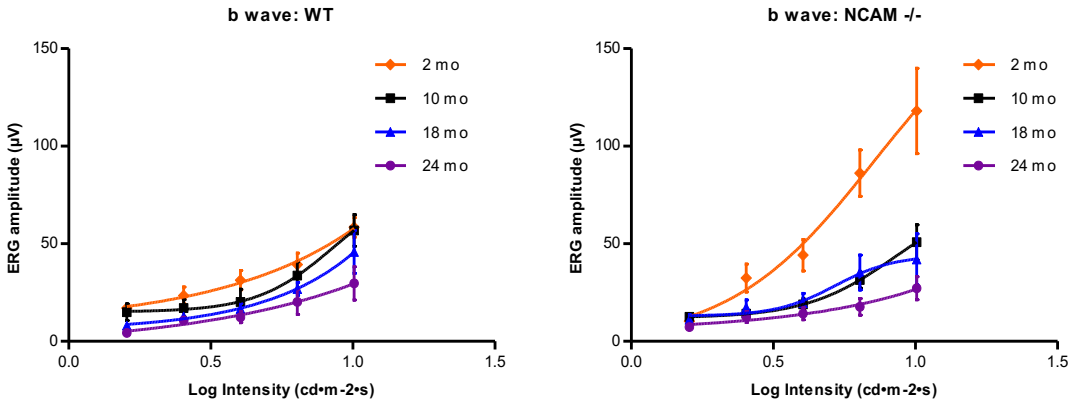
Two-month-old NCAM −/− mice had an a-wave V*max* that was more than double that observed in WT (230 ± 37μV vs. 110 ± 9μV, respectively); these high values subsequently fell to WT levels by 10 months and older (Figure 2-6b, Table 2-1a). In NCAM −/− mice, this represents a decrease of 64% in the mean a-wave V*max* between 2 and 24 months, that is three and one half times the decrease seen in controls (18%). Even so, the retinal sensitivity, parameter *K* (reflects retinal sensitivity to a light stimulus: a higher log *K* value indicates decreased sensitivity to light), did not change over time, suggesting that individual photoreceptors maintain their ability for phototransduction (Table 2-1b). In addition, knockout mice showed a significant decrease in the slope (n) of the intensity-response curve over time, suggesting that NCAM −/− animals' responses to light decline in older animals (Table 2-1c).

B-waves

The age-dependent b-wave V*max* changes in NCAM −/− mice showed a pattern very similar to that seen with the a-wave: two-month-old NCAM −/− mice had a b-wave V*max* that was more than double that observed in WT (632 ± 111μV vs. 306 ± 20μV, respectively) and which subsequently fell to similar levels as WT by 10 months and older (Figure 2-6b, Table 2-1a). This represents a decrease of 73% in the mean b-wave V*max* values between 2 and 24 months old in NCAM −/− mice, which is more than double the decrease seen in WT controls. Similar findings were observed in the light-adapted amplitudes (Figure 2-8). Results show NCAM −/− mice's log *K* values increase significantly with age as expected (Table 2-1b); however, there were significant

Figure 2-8. Mean light-adapted b-wave amplitudes plotted against flash intensity for NCAM −/− and WT mice at 2, 10, 18 and 24 months old showing young adult NCAM −/− mice have significantly higher b-wave amplitudes than normal. Points represent means ± SEM.

Light-adapted b-wave amplitudes



differences between WT and NCAM −/− animals at 18 months, suggesting premature light sensitivity loss in NCAM −/− mice as the animals age.

B/A wave ratio

To evaluate the effect of NCAM in retinal function and visual-signal transmission, the maximum b-wave amplitudes were normalized to their corresponding maximum a-wave amplitudes in each age group (V*max* b-wave / V*max* a-wave = b/a wave ratio). NCAM −/− mice do not differ from WT b/a wave ratios at all ages (Table 2-2), suggesting that the higher b wave responses seen in the 2-month-old knockout mice could be driven by increased photoreceptor activity.

2.4.3.2 VEP responses in aged NCAM −/− mice

To examine the physiological integrity of the visual pathway from retina to visual cortex, flash VEPs were recorded over the occipital cortex in 2 and 18-month-old NCAM −/− and WT animals. No major waveform differences were found between both groups of animals (Figure 2-9). Short flashes of strong light produced a large negative potential, followed by a minor, then a major, peak. In the absence of a recognized way to analyze the VEP data fitted to a standard curve, I compared responses by measuring VEP amplitudes from the baseline to the trough; between WT and NCAM −/− mice, there were no differences in amplitudes at either age (Table 2-3).

Table 2-2. b/a wave ratios for 2-, 10-, 18- and 24- month old show that NCAM −/− and WT mice have similar amplitude ratios. Values are means ± SEM.

			2 mo	***10 mo***	***18 mo***	***24 mo***
b/a wave ratio	***NCAM -/-***		2.45 ± 0.38	1.64 ± 0.19	2.33 ± 0.34	2.14 ± 0.17
	WT		2.75 ± 0.38	1.93 ± 0.21	2.35 ± 0.09	2.34 ± 0.17
t-test	2 months	NCAM -/-		0.014	*NS*	*NS*
		WT		0.032	*NS*	*NS*
	10 months	NCAM -/-			*NS*	*NS*
		WT			*NS*	*NS*
	18 months	NCAM -/-				*NS*
		WT				*NS*
	Between group		*NS*	*NS*	*NS*	*NS*

Figure 2-9. Analysis of VEP responses show no major differences between WT and NCAM −/− waveform patterns at ages 2 and 18 months. Representative scotopic waveforms of flash VEP at 7 different light intensities ranging from −5.0 to 1.0 log cd × s/m^2 for 2 and 18 months, WT (blue) and NCAM −/− (purple) mice. Abbreviations: NCAM, neural cell adhesion molecule; WT, wild type.

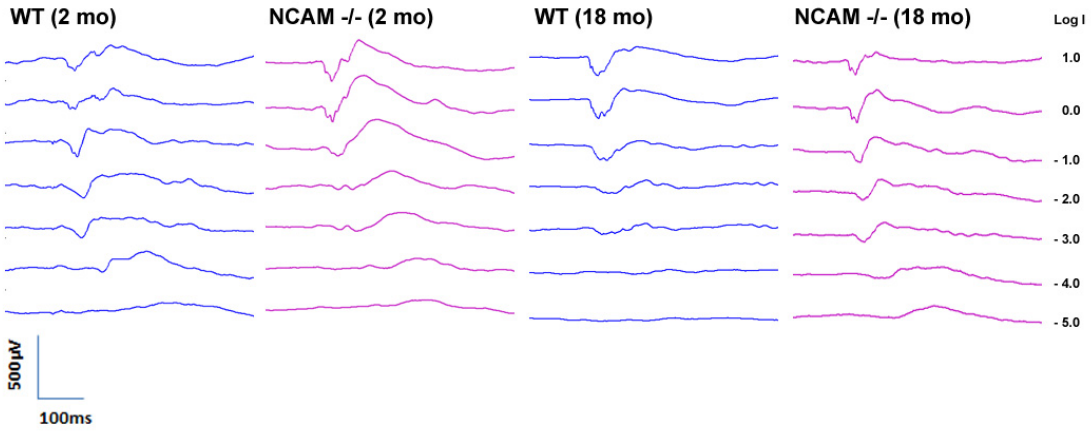


Table 2-3. VEP responses at 2- and 18- month old show no difference in amplitudes between WT and NCAM −/− mice. Values are means ± SEM.

	VEP		***t-test***		
	WT	***NCAM -/-***	2 months		*Between group*
			WT	NCAM -/-	
2 mo	80.06 ± 5.70	111.58 ± 26.29			*NS*
18 mo	86.49 ± 21.25	79.01 ± 10.22	*NS*	*NS*	*NS*

2.4.4 Behavioral Studies

When considering behavioral testing, I first established that the absence of NCAM did not impair mouse motor skills (Figure 2-10). Except for the 24-month-old mice, there was no overall difference in swimming speed between WT and NCAM −/− mice. To investigate the functional integrity of the visual system, I compared visual ability between NCAM −/− and WT mice using computer-based visual behavioral testing. The visual water task consists of visual detection, visual acuity, and pattern discrimination. The results of the visual ability testing are discussed in detail below and summarized in Table 2-4, showing that the knockout mice have premature loss of visual detection, earlier than expected loss of visual acuity, and no pattern recognition ability at any age.

2.4.4.1 Visual Detection Task

Performance at 2, 10, 18 and 24 months: Mice were required to distinguish between a vertical grating (S+) and a gray screen (S-) in the visual detection task (Figure 2-11a). At 2 months, WT and NCAM −/− mice showed similar improvement in accuracy over the 8 days of testing [$F(7, 126) = 35.53$, $P < 0.001$] and reached the 70% criterion on day 2 and day 3 respectively [$F(1, 18) = 4.41$, ns]. However, at 10 months, the WT animals reached the 70% criterion on day 3, whereas it took 5 days of testing for the NCAM −/− mice. Although both groups showed improvement over the 8 days of testing [$F(7, 126) = 20.35$, $P < 0.001$], the NCAM −/− mice did not perform as well as WT [$F(1, 18) = 6.275$, $P < 0.05$]. By 18 months NCAM −/− performance was even worse: WT mice met the 70% criterion after 5 days of testing whereas NCAM −/− mice did not reach this criterion over the full testing period. WT mice outperformed NCAM −/− in accuracy over days

Figure 2-10. Motor skills assessment shows that the latency time for NCAM −/− mice (the time the animal needs to reach the platform in the visual detection task) is not impaired relative to WT at 2, 10 and 18 months. NCAM −/− mice at 24 months old take a comparatively longer time to complete the task. Points represent means ± SEM. Asterisk (*) denotes significantly different between WT and NCAM −/− mice.

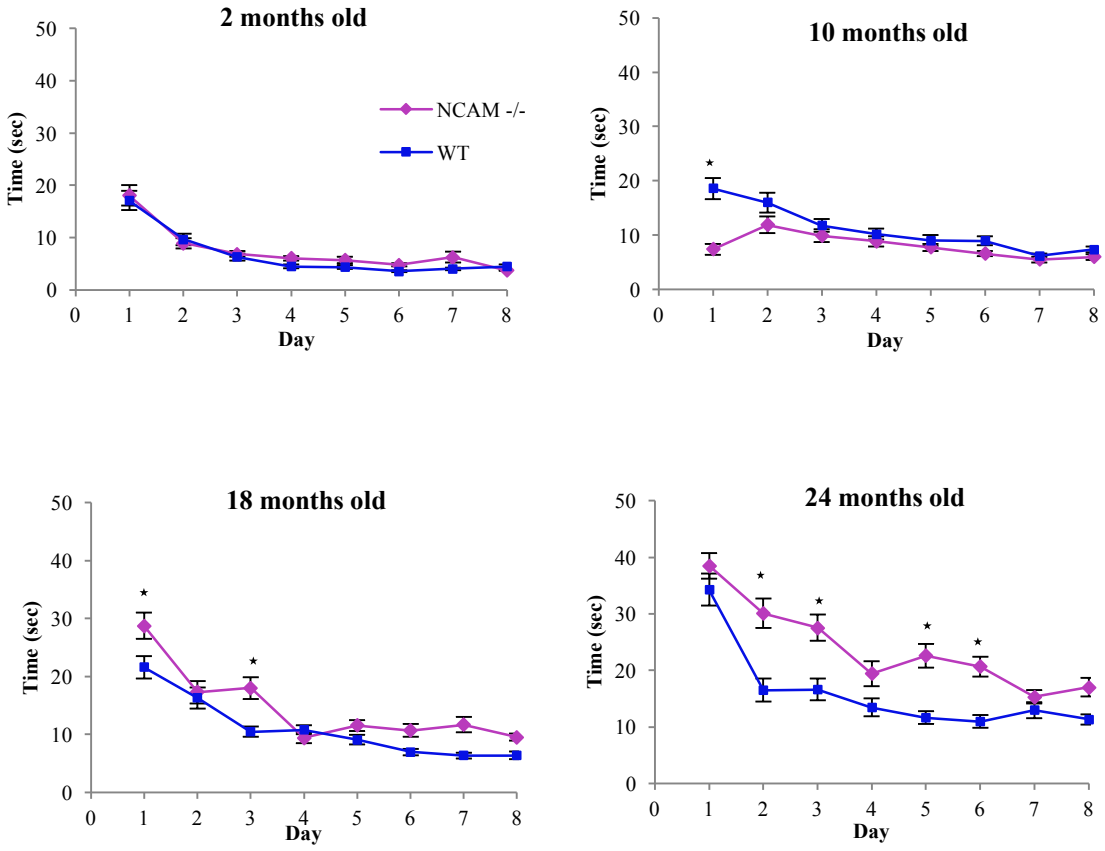
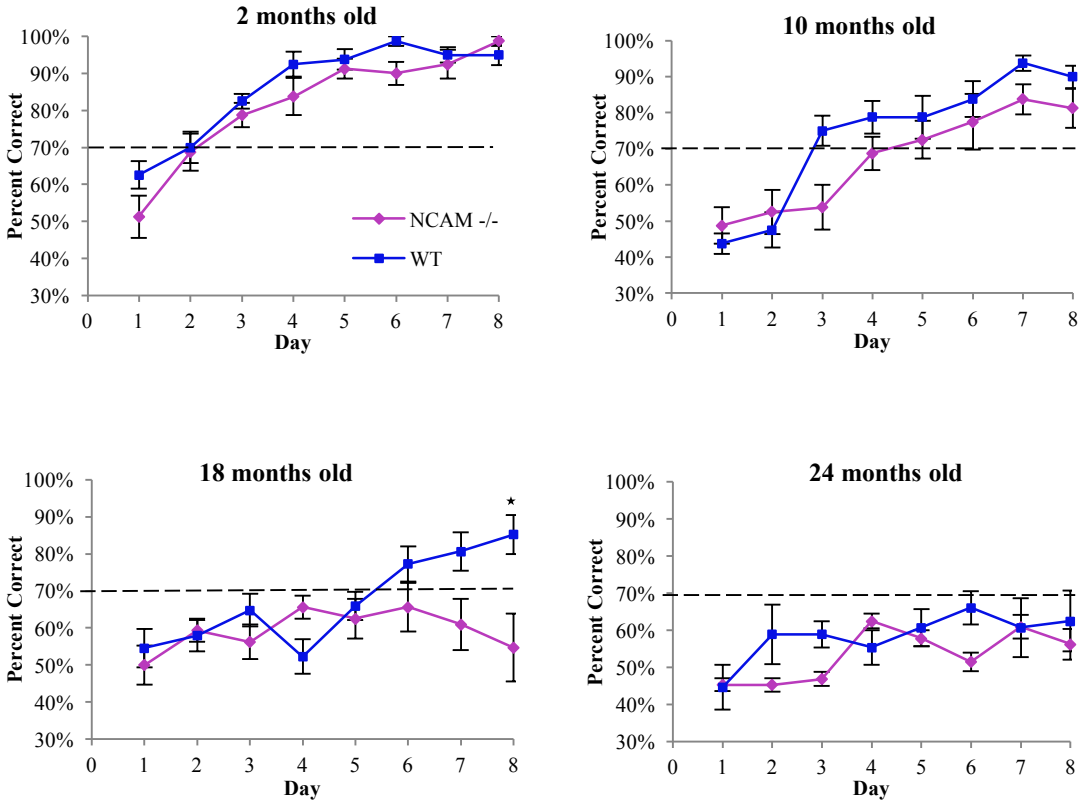


Table 2-4. Summary of performance in the visual detection task, visual acuity task and pattern discrimination task shows that NCAM −/− animals have no pattern recognition ability, and exhibit premature loss of visual acuity as the mice age (see Figure 2-11 for detailed results).

	Visual Detection		Visual Acuity		Pattern Discrimination	
	Day(s) to reach criterion		Threshold (cycles/degree)		Day(s) to reach criterion	
Age (months)	WT	NCAM -/-	WT	NCAM -/-	WT	NCAM -/-
2	2	2	0.64	0.64	4	Did not reach criterion
10	3	5	0.62	0.32	6	Did not reach criterion
18	6	Did not reach criterion	0.32	0	7	Did not reach criterion
24	Did not reach criterion	Did not reach criterion	0	0	Did not reach criterion	Did not reach criterion

Figure 2-11. Visual impairment in aging WT and NCAM −/− mice. NCAM −/− mice had significant differences in all aspects of the visual ability testing: premature loss of visual detection ability *(a)*; earlier and poor performance on the visual acuity task *(b)*; and inability to detect patterns at any age *(c)*. The mean "percent correct" for each age group (2, 10, 18, and 24 months old) is presented for each task. Performances on day 8 were used to compare strains on visual detection and pattern discrimination tasks. Asterisk (*) denotes significantly different between WT and NCAM −/− mice.

(a) Visual Detection Task



tested [F (7, 119) = 4.149, P < 0.01] and performance [F (7, 119) = 3.871, P < 0.01]. At 24 months of age, neither WT nor NCAM −/− mice were able to meet the 70% criterion.

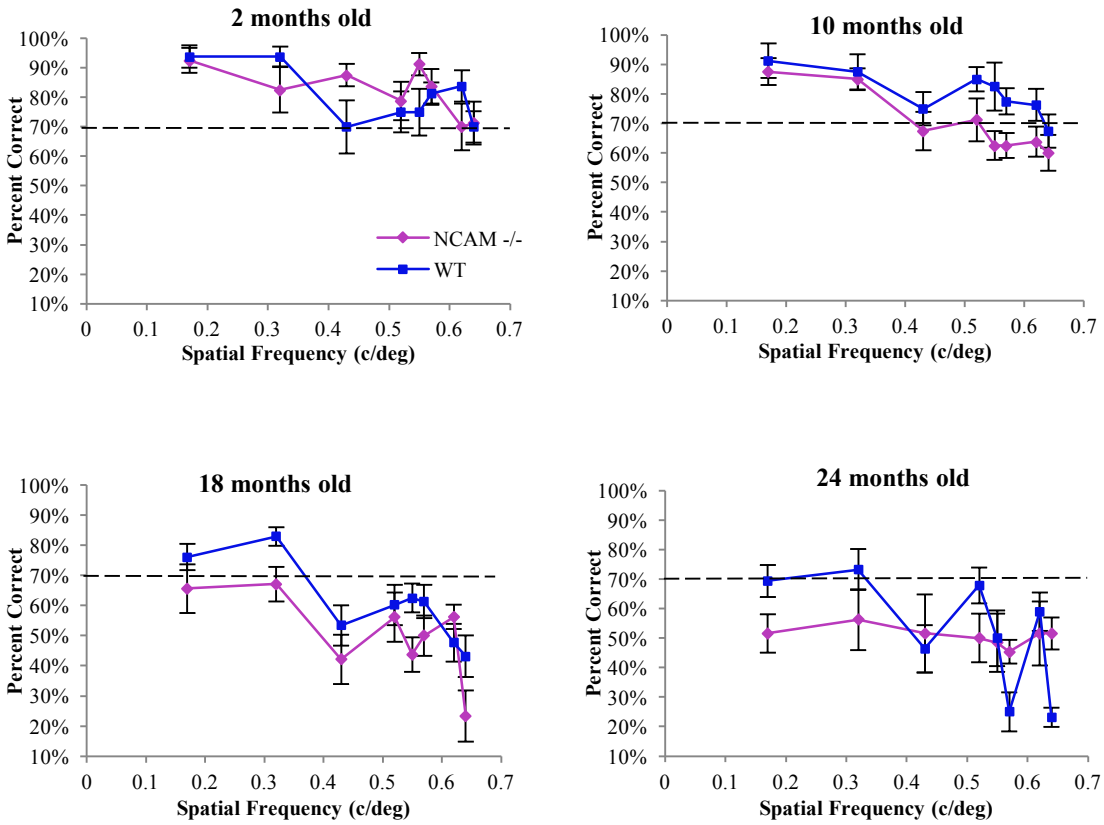
Day 8 performance: To assess visual detection after 7 days of testing, a single performance measure was made on day 8 in the visual detection task. NCAM −/− mice, at 18 months, were significantly outperformed (p = 0.015) by the WT animals. No difference was found when the animals were 2 (p = 0.24), 10 (p = 0.18), or 24 (p = 0.51) months of age.

2.4.4.2 Visual Acuity Task

Animals were required to distinguish between a vertical grating with different spatial frequency (S+) and a gray screen (S-). The visual acuity performance was calculated for each spatial frequency (Figure 2-11b). At 2 months, WT and NCAM −/− mice were equally able to reach the 70% criterion across the range of spatial frequencies [F (1, 18) = 0.119, ns]. However, at 10 months, the WT animals were able to discriminate the spatial frequencies tested up to 0.62 cycles/degree (c/deg), whereas the NCAM −/− mice could only discriminate the spatial frequencies up to 0.32 c/deg. Over the 8 days of testing, there was a significant strain difference favoring the WT group [F (1, 18) = 6.783, P < 0.05]. At 18 months, NCAM −/− mice did not reach the 70% correct criterion at any spatial frequency tested; the threshold of WT mice was 0.32 c/deg [F (1, 17) = 8.445, P < 0.01]. At 24 months, both groups of mice did not reach the criterion and there was no strain difference [F (1, 13) = 0.172, ns].

Figure 2-11

(b) Visual Acuity Task



2.4.4.3 Pattern Discrimination Task

Performance at 2, 10, 18 and 24 months: In the pattern discrimination test, animals were required to distinguish between a vertical grating (S+) and a horizontal grating (S-) (Figure 2-11c). At 2, 10 and 18 months of age, the WT animals reached the 70% correct criterion on day 4, 6 and 7 respectively. In contrast, NCAM −/− mice did not reach this criterion at any age tested. By 24 months, neither NCAM −/− nor WT mice were able to reach the criterion and there was no significant strain difference [$F(1, 13) = 0.295$, ns].

Day 8 performance: NCAM −/− mice did not reach the criterion for discrimination at any age, using the parameters of the present experiment. In contrast, WT mice reached criterion for pattern discrimination test at 2, 10 and 18 months of age.

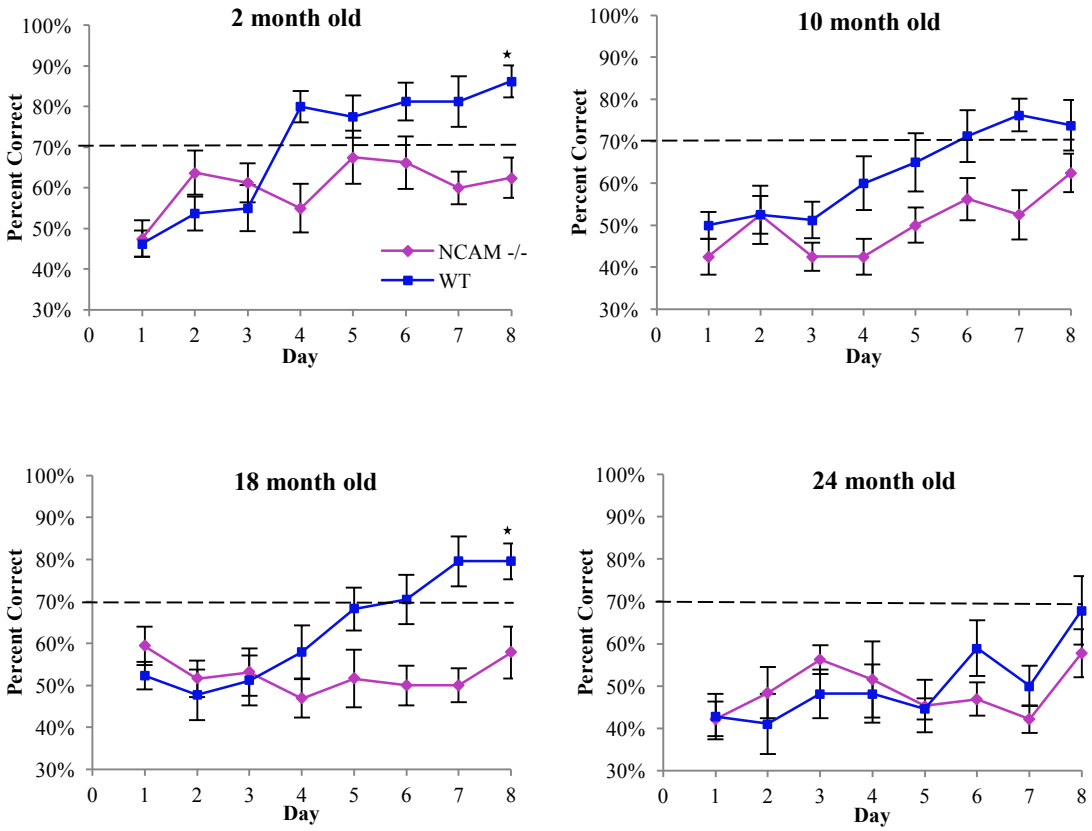
2.5 Discussion

2.5.1 Summary of the Results

Our results show that NCAM has important anatomical, electrophysiological and behavioral effects on the visual system in young adult mice and during aging. Specifically, I have demonstrated that the absence of NCAM has profound effects on vision: 1) NCAM −/− mice have no pattern recognition ability using the parameter tested; 2) at 2 months, despite having more than normal number of RGCs, thicker than usual inner retinal layers and higher than ordinary retinal activity levels, NCAM −/− mice showed normal VEP responses in V1; 3) by 10 months, there is a rapid decline in ERG response amplitudes as well as early onset of deterioration in visual acuity; 4) by 18 months, NCAM −/− mice retinas have particularly thinned photoreceptor cell layers, and

Figure 2-11

(c) Pattern Discrimination Task



fewer RGCs; and 5) premature visual impairment of at least 8 months in these aging animals.

2.5.2 NCAM and the visual system

PSA-NCAM is highly expressed in the developing visual system in all retinal layers, the ONs and the visual cortex (Bartsch et al., 1990; Di Cristo et al., 2007). In the adult retina, PSA is predominantly located in astrocytes and Müller glia, whose cellular processes provide structural scaffolding, regulate synaptic activity and facilitate neuronal plasticity (Reichenbach and Bringmann, 2013). Previous studies have shown that endogenous PSA supports the survival of neonatal as well as injured and normal adult RGCs (Murphy et al., 2009), and is expressed in all cell layers of the VC (Di Cristo et al., 2007); however, no studies to date have examined the effects of NCAM in the visual system during aging.

NCAM expression, including expression of its isoforms, is highly regulated in the CNS during development (Ronn et al., 1998). NCAM dysregulation has been correlated with cancer and tumor progression in humans, suggesting that NCAM may contribute to age-associated changes in cellular architecture and function (Gattenlohner et al., 2009; Huerta et al., 2001; Sasaki et al., 1998). Different isoforms of NCAM play distinct roles in biological processes, such as mediating neurite outgrowth, myoblast fusion and synaptic maturation (Berezin, 2010). A significant change in levels of NCAM isoform 180kDa was found in retinas and in VC of 18 month old animals (Figure 2-2), similar to findings reported in brains and hearts of aging rats, and in central synapses of aged mice telencephalons (Bahr et al., 1993; Gaardsvoll et al., 1993; Linnemann et al., 1993). In

addition to changes in known isoforms, I found an additional NCAM 150 isoform in young and aged WT retinas (Figure 2-2a). Isoforms other than the 3 dominant forms have been described (Andersson et al., 1993; Reyes et al., 1991) and the influences of the 150 isoform on normal retinal architecture and function remain to be elucidated.

2.5.3 Absence of NCAM has major consequences on vision during aging

NCAM −/− mice, at 2 months, have more RGCs than WT, which may be at least in part due to NCAM's effect on RGC migration and survival during development (Canger and Rutishauser, 2004; Murphy et al., 2007a; Ronn et al., 1998). Despite having a surplus amount of RGCs at a young age (Figure 2-4h), I find that NCAM −/− mice have excessive loss of RGCs as they grow older (Figure 2-5h), contrasting the minimal loss of RGCs during aging normally observed in WT controls and across mammalian species (Harman and Moore, 1999; Kim et al., 1996; Samuel et al., 2011). Although RGC axons are particularly vulnerable during aging, our NCAM −/− mice showed an insignificant loss of RGC axons when they reached 18 months old (Calkins, 2013). Why there is a preferential loss of RGC bodies over axons in NCAM −/− mice is not clearly understood but may, in part, be influenced by changes in macrophage responses, BDNF levels, and tau hyperphosphorylation and aggregation during aging (Bull et al., 2012; Luo et al., 2010; Watanabe et al., 2003).

Significant changes were also seen in the photoreceptor layer of the retina. Photoreceptor neurons are particularly susceptible to age-related loss (Cunea and Jeffery, 2007; Cunea et al., 2014; Panda-Jonas et al., 1995). The loss of rods, which comprise 97% of

photoreceptor cells in rodents (Jeon et al., 1998), has physiological and behavioral consequences: a decline of a- and b- wave ERG amplitudes, and a 2-fold reduction in visual acuity and spatial contrast sensitivity (Kolesnikov et al., 2010). Our results show that the absence of NCAM accelerates this age-dependent photoreceptor loss (Figure 2-5g), corresponding temporally with a dramatic drop of ERG amplitudes (Figure 2-6) and a premature loss of visual acuity (Table 3) as the mice age.

2.5.4 How NCAM influences visual function during aging: possible mechanisms

The absence of NCAM may adversely affect visual function at a number of levels: I considered potential effects in the retina, in the projection of visual information to the cortex, and in the cortex itself. In the retina, I observed exaggerated retinal activity in the young adult followed by a rapid decline in ERG levels as the animals age. How might NCAM deficiency affect ERG levels and how might this affect vision? Dysregulation of nitric oxide (NO), which is involved in retinal signal processing, can play a significant role in altering retinal activity: exogenous NO increases ERG a- and b- wave amplitudes, oscillatory potentials, and the scotopic threshold response by 100% in rats (Vielma et al., 2010). Elevated concentrations of NO result in photoreceptor apoptosis and functional damage, thinning of the retina, and marked reduction in ERG response amplitudes (Fawcett and Osborne, 2007; Takahata et al., 2003b). NCAM is known to regulate NO that, via the cGMP pathway, is involved in synaptic plasticity, neurite outgrowth and neuronal survival (Ditlevsen and Kolkova, 2010; Fiscus, 2002; Hindley et al., 1997; Monfort et al., 2004). Consistent with my results, and in light of the above findings, I

speculate that higher amplitudes of ERG found in the young NCAM −/− mice may be a consequence of greater than normal amounts of NO in the retina. Furthermore, prolonged elevation of NO levels would be expected to impair photoreceptor function and significantly reduce ERG response amplitudes as the animals age.

Besides NO effects, I have considered the possibilities of increased survival, and reduced or delayed programmed cell death (PCD), of retinal cells during development. The mechanism by which this occurs is speculative, but two of the major signaling molecules, BDNF and $p75^{NTR}$, involved in regulation of retinal PCD have been shown to interact with PSA-NCAM in modulating neuronal survival in the hippocampus and olfactory bulb (Braunger et al., 2014; Gascon et al., 2007a; Muller et al., 2000). These observations, together with previously reported findings from our laboratory that young adult NCAM −/− mice have greater numbers of RGCs (Murphy et al., 2007a), raise the possibility that PCD and the number of retinal neurons surviving to adulthood may be altered in the absence of NCAM. While alterations in developmental PCD may explain greater numbers of RGCs in young NCAM −/− mice, the preferential survival of axons vs. cell soma during aging raises the possibility that not all of these surviving RGCs are functional. If so, the higher level of retinal activity I observed in early adulthood may be required to maintain normal vision; conversely, as the animals age, a relative decrease in retinal output may no longer support normal vision and could contribute to the premature vision loss in these mice.

Despite a greater number of RGCs in young adult NCAM −/− mice, there was an exaggerated loss of these same neurons as the animals aged. It has been shown that NMDA-induced calcium dynamics are altered in RGCs from NCAM −/− mice, suggesting that calcium-induced excitotoxicity disrupts normal RGC function in the absence of NCAM (Murphy et al., 2012). Consistent with this, PSA has been shown to antagonize competitively the NR2B containing-NMDA receptor to prevent glutamate-induced excitotoxicity (Hammond et al., 2006). Consequently, altered NMDA-induced calcium dynamics in NCAM −/− mice may be important in helping to explain the vulnerability of RGCs to age-related impairment in visual function and the excessive RGC loss I have observed as these mice age.

I have shown that NCAM −/− mice have no pattern discrimination ability, a task that involves a complex integration of visual information between higher-order somatosensory and visual processing cortical areas (Van Essen, 1991). This implies that the visual dysfunction I describe in aging NCAM −/− mice is not solely a consequence of retinal pathology and implicates other CNS regions. Genetic ablation of NCAM alters thalamocortical axon guidance and organization of the somatosensory map, suggesting that NCAM is involved in thalamocortical tract guidance and topographic sorting of thalamic projections (Enriquez-Barreto et al., 2012). Consistent with the idea that NCAM has a significant influence on topographic targeting in the somatosensory system, mutations and dysregulation of NCAM have been implicated in psychiatric disease, including schizophrenia (Arai et al., 2006; Vicente et al., 1997), where visual recognition impairment is observed (Quintana et al., 2011; Spaulding et al., 1980). Thus, our finding

of impaired pattern discrimination ability supports the notion that dysregulation of NCAM affects the integration of visual information in higher cortical areas.

NCAM and PSA-NCAM are required for different forms of neural plasticity in the embryonic and adult nervous system, raising the possibility that the absence of NCAM may have important effects on plasticity in the visual pathway, from the eye to the VC (Bonfanti, 2006; Gascon et al., 2007b). PSA is known to regulate plasticity and learning through modulation of NMDA receptor signaling (Kochlamazashvili et al., 2010) and, with age, NR2A- and NR2B-containing NMDA receptors have different critical roles in cortical visual plasticity (de Marchena et al., 2008). Other molecules under the influence of PSA have been identified in the regulation of developmental plasticity in the visual cortex, including those involved with functional and structural maturation of GABAergic signaling and control of the onset of the critical period during OD plasticity (Di Cristo et al., 2007). Using environmental enrichment as a strategy to induce plasticity in adult amblyopic rats, reduction in intracortical inhibition and recovery of visual acuity have been demonstrated (Sale et al., 2007). Hence, enhancing experience-dependent plasticity improves visual function in adulthood, and possibly throughout life. Furthermore, the loss of synaptic plasticity has been recently shown to result in an orientation response deficit in V1 neurons (Gonzalo Cogno and Mato, 2015). These findings suggest that the loss of pattern discrimination and age-related functional decline in vision I have observed in adult NCAM deficit mice may, at least in part, be due to impaired VC plasticity.

2.5.5 Considerations in interpreting our results

In interpreting our results, there are practical considerations. The absence of NCAM has been associated with depression and impaired learning (Aonurm-Helm et al., 2008a; Kochlamazashvili et al., 2012), CNS effects that could potentially influence the interpretation of our results. I addressed the issue of depression by using a forced swim test, which is widely used as a behavior screen for depression, and found no performance difference between NCAM −/− and WT mice (data not shown), suggesting that depression was not a prominent factor in affecting their performance on visual testing. It is possible, however, that there was impaired learning in the NCAM −/− mice, as they showed no sign of improved performance after 7 days of training in the pattern discrimination task at any age. Similar findings were present in forebrain-restricted BDNF mutant mice, who exhibit difficulty in performing more challenging discrimination tasks (Gorski et al., 2003). Although learning may be impaired, our results do show that NCAM −/− mice can learn. In the visual detection task, for example, they took extra time to learn and reach the 70% criterion, but made over 90% (2 months old) and 80% (10 months old) correct choices by day 8. Finally, the genetic homogeneity of NCAM −/− mice can significantly influence experimental results. For example, although NCAM −/− mice at 2 months of age have approximately 2 times higher than normal ERG responses compared with WT mice, I observed high variability in the ERG/VEP patterns of the knockout animals. These inter-individual variations may have been at least in part due to biological discrepancies; despite extensive backcrossing, the presence of different modifier genes produces differences in expressivity, which can affect phenotype expression (Doetschman, 2009; Eisener-Dorman et al., 2009).

2.6 Conclusion

The absence of NCAM results in altered retinal architecture and visual physiology that have profound effects on vision in the adult rodent. This is the first demonstration of the effects of NCAM on vision during aging and raises the possibility that this molecule may be important in human age-dependent visual impairment.

CHAPTER 3: POLYSIALYLATED NEURAL CELL ADHESION MOLECULE PROTECTS AGAINST LIGHT-INDUCED RETINAL DEGENERATION

3.1 Preface and Significance to Thesis

The low affinity neurotrophin receptor, $p75^{NTR}$, is known to control retinal cell death caused by intense light illumination. Our previous findings suggest that NCAM plays an essential role in supporting the survival of injured retinal ganglion cells. In this Chapter, using light-induced retinal degeneration (LIRD) as a model, I sought to investigate whether NCAM plays a role in LIRD and whether NCAM influences the effect of $p75^{NTR}$ signaling in retinal cell survival. Our results indicated that low levels of caspase 3 activation were detected on the first day in WT mice, followed by an increase of up to 4 days after LIRD. Conversely, in NCAM −/− mice, higher baseline cleaved caspase 3 levels were found earlier at Day 1, followed by reduced levels by Day 4. Removal of PSA prior to LIRD by treatment with Endo N induced an earlier onset of retinal cell death (Day 0). Co-administration of Endo N and $p75^{NTR}$ function-blocking antibody (REX) to WT mice delayed the initiation of apoptosis. Hence, our findings indicate that NCAM protects WT retinas from LIRD; furthermore, the protective effect of NCAM is, at least in part, attributed to its effects on $p75^{NTR}$.

This chapter has been published in *Investigative Ophthalmology & Visual Science* (*IOVS*) (Luke et al., 2016b). Preliminary results of the findings have been presented and published in abstract form at the *Association for Research in Vision and Ophthalmology* (*ARVO*) Conference in Seattle, Washington, USA (Luke et al., 2013).

3.2 Introduction

The retina has the ability to capture photons of light efficiently and enact visual transduction, but excessive or continuous light exposure has been shown to result in cumulative oxidative stress, photo-transduction impairment and photoreceptor cell death, leading to retinal damage, vision impairment and blindness (Grimm and Reme, 2013). Extensive research in LIRD has been conducted in rodents (Duncan and LaVail, 2010), a model that shares many characteristics of human retinal degenerative diseases, including those caused by environmental insult (Organisciak and Vaughan, 2010). The cleavage of NCAM is involved in cortical neuronal death under oxidative stress (Fujita-Hamabe and Tokuyama, 2012), and previous results from our laboratory have demonstrated that NCAM is important in RGC survival and age-related deterioration in vision (Luke et al., 2016a; Murphy et al., 2007a; Murphy et al., 2009). However, the molecular basis of NCAM's role in retinal degeneration remains elusive.

NCAM is a transmembrane protein that is involved in axonal fasciculation, cell migration, neurite outgrowth, synaptic plasticity, and formation and stabilization of synapses during development (Walmod et al., 2004; Zhang et al., 2008). It contains five immunoglobulin-like and two fibronectin type III repeats. NCAM is differentially expressed in two major transmembrane isoforms (180 and 140 kDa) and a glycophosphatidyl inositol-linked isoform (120 kDa) (Soroka et al., 2008). Using immunohistochemical and electron microscopic techniques, NCAM was found to be present on all retinal neurons, and in all layers in the developing and adult mouse retinas

(Bartsch et al., 1989, 1990). All major NCAM isoforms can be modified by PSA, inserted as chains into N-glycosylation sites of the fifth immunoglobulin-like domain (Colley, 2008). NCAM is the most abundant PSA carrier in mammals (Nelson et al., 1995) and the removal of NCAM abolishes almost all of the PSA in the nervous system (Finne et al., 1983). PSA-NCAM is expressed throughout all retinal layers during development (Bartsch et al., 1990). However, in adulthood, it is located exclusively on astrocytes and Műller glial cells of the mouse retina and on astrocytes of the optic nerve (Bartsch et al., 1990).

$p75^{NTR}$ is an important neuronal type I transmembrane signaling protein that interacts with numerous ligands and co-receptors to regulate cellular survival and apoptosis, neurite outgrowth and repulsion, myelination and long-term depression (Barker, 2009). In the retina, $p75^{NTR}$ is found in the Müller cell processes (Hu et al., 1998), which provide an environment for neurons to regulate synaptic activity and facilitate neuronal plasticity (Bringmann et al., 1998). $p75^{NTR}$ and Sortilin forms a cell surface receptor complex for the pro-form of nerve growth factor (ProNGF) to induce death of RGCs in adult rodents (Lebrun-Julien et al., 2010; Nykjaer et al., 2004; Santos et al., 2012). Furthermore, the absence, or blockade, of $p75^{NTR}$ promotes structural and functional photoreceptor cell survival after LIRD (Harada et al., 2000).

Using light-induced injury to the retina as a model, I investigated whether NCAM plays a functional role in neuroprotection and whether NCAM influences $p75^{NTR}$ signaling in modulating retinal cell survival. Our results in WT retinas after LIRD show an

upregulation of PSA-NCAM and enhanced expression of $p75^{NTR}$. NCAM deficient mice had diminished ERG amplitudes and, as also observed in WT mice treated with Endo N, exhibited earlier onset of retinal cell death. Blockade of $p75^{NTR}$ in Endo N-treated WT and NCAM −/− retinas altered retinal apoptosis, suggesting that NCAM plays an important role in the modulation of the death inducing effect of $p75^{NTR}$ after LIRD.

3.3 Materials and Methods

3.3.1 Subjects

2-4 months old WT and homozygous NCAM −/− mice on a C57Bl/6J background were obtained by crossing heterozygous male and female animals (generously provided by Victor Rafuse, PhD) (Cremer et al., 1994). The primer sequences were reported by Cremer et al., and the genotyping PCR conditions were 94°C for 5 min; 30 cycles of 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 1 minute; then 72°C for 10 minutes (Cremer et al., 1994). Animals were subjected to in-house breeding and were cared for according to the Guide to the Care and Use of Experimental Animals of the Canadian Council on Animal Care. All animals were treated in accordance with the *ARVO* Statement for the Use of Animals in Ophthalmic and Vision Research.

3.3.2 Photic injury

Mice (n = 5-10/ group; the exact number is provided in each figure legend) were anesthetized with a mixture of ketamine (100 mg/kg), acepromazine (0.62 mg/kg), and xylazine (15 mg/kg). Their pupils were dilated with 0.5% cyclopentolate hydrochloride

(HCl) drops (Alcon, Fort Worth, TX, USA). Animals were dark adapted overnight, then exposed to 18,000 lux of white fluorescent light in a well ventilated, air conditioned room for 6 hours. During exposure, a drop of normal saline was applied on the cornea every 20 minutes, and an additional 1/5 of the anesthetic mixture would be given if the animals awakened during the photic injury procedure. Mice were sacrificed at specific times from 0 to 4 days after photic injury.

3.3.3 Immunoblot

Protein lysates were prepared by gently pipetting the retinal tissues up and down in a 50-mM (hydroxymethyl)aminomehane(Tris)-HCl (pH 7.5), 5 mM EDTA, 300 mM sodium chloride (NaCl), 0.1% nonyl phenoxypolyethoxylethanol (NP-40), and 1X protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN, USA) several times and incubating for an hour in ice. Protein concentrations were quantified by the Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). A total of 20 μg for each of the WT and NCAM −/− proteins were adjusted with an equivalent volume of distilled water and mixed with 4x SDS-sample buffer (0.08% SDS, 250 mM Tris-HCl [pH 8.0], 40% glycerol, 20% β-mecaptoethanol, and a trace amount of bromophenol blue), boiled for 5 minutes, loaded in a polyacrylamide gel, ran for 2.5 hours at 80 V, then transferred to a polyvinylidene fluoride (PVDF) membrane at 0.25 A for 1.5 hours. Anti-PSA-NCAM (MAB5324, 1:2000; Millipore, Billerica, MA, USA), anti-NCAM (MAB310, 1:500; Millipore), anti-$p75^{NTR}$ (8238, 1:1000; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-Neurotensin Receptor 3 (Sortilin; 612100, 1:500; BD Transduction Laboratories, San Jose, CA, USA), anti-pro-NGF (AB9040, 1:2000; Millipore), and anti-

actin (A2066, 1:5000; Sigma-Aldrich Corp., St. Louis, MO, USA) antibodies were used to determine protein expressions by standard SDS-polyacrylamide gel electrophoresis (PAGE) immunoblotting (Murphy et al., 2007a). To capture active caspase 3 signals, protein lysates were loaded in a 15% polyacrylamide gel, separated via electrophoresis at 55 V for 5 hours and then transferred onto a PVDF membrane for 1 hour at 50 V. Anti-caspase 3 (cleaved) (AB3623, 1:100; Millipore) and anti-actin antibodies were used to determine protein levels. The chemiluminescent signals were detected by the Pierce Enhanced Chemiluminescence System (ECL) 2 Western Blotting Substrate (80196, Thermo Scientific, Rockford, IL, USA) and captured by exposure to X-ray films or by scanning with the Typhoon Variable Mode Imager (Amersham Biosciences, Sunnyvale, CA, USA). The levels of proteins were then quantified using Image J (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) and the data obtained were converted to percentages of the controls.

3.3.4 Immunohistochemistry

Wild-type and NCAM −/− mice (n = 5 per group) were anesthetized and transcardially perfused with chilled 4% paraformaldehyde in phosphate-buffered saline (pH 7.4). The eyes were removed, post-fixed for 30 minutes, and cryo-protected in 30% sucrose overnight at 4°C. Retinal sections (16 μm) were obtained by cutting along superior-inferior orientation of the eye with a cryostat apparatus (Leica CM1850; Leica Biosystems, Wetzlar, Germany). PSA-NCAM (MAB5324, 1:500; Millipore), Bassoon (SAP7F407, 1:500; Enzo Life Sciences LTD., Exeter, UK), $p75^{NTR}$ (REX antibody: A gift from Dr. Louis Reichardt, University of California, San Francisco, CA, USA), and

Hoechst (33258, 1:50,000; Sigma-Aldrich Corp.) immunostaining techniques, previously described (Lebrun-Julien et al., 2009b; Murphy et al., 2009), were performed on sections from both light-induced and noninduced retinas.

3.3.5 Apoptosis Assay

After LIRD, the 16 μm cryostat sections were prepared as described above. The WT retinal samples were fixed in 4% PFA for 15 minutes, post-fixed in precooled (-20 degree celsius) ethanol : acetic acid (2:1) for 5 minutes, permeabilized with proteinase K (20 μg/ml) for 10 minutes, then stained with the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Catalog #S7100, Millipore) according to the manufacturer's instructions.

3.3.6 Electroretinography

WT and NCAM −/− mice were used for visual electrophysiology examinations, which were performed 3 days before and 1 day after LIRD using the method as described previously (Smith et al., 2013). Briefly, mice were dark-adapted overnight and anesthetized. Pupils were dilated with 0.5% cyclopentolate HCl drops (Alcon). ERGs were recorded using a silver-impregnated nylon fiber electrode (Diagnosys, Littleton, MA, USA), which was placed on the mouse's corneal surface and hydrated with 2.5% hydroxypropyl methylcellulose solution. The protocol consisted of recording ERGs from a series of strobe flash visual stimuli with increasing intensity from -5.8 to 1 log $cd \cdot s/m^2$ in scotopic conditions.

3.3.7 Surgery

Animals were anesthetized with isofluorane, and intravitreal injections were performed under an operating microscope using a microliter syringe (7102; Hamilton, Reno, NV, USA) with a glass micropipette tip. The WT mouse eye was injected with 1µl of vehicle (50% PBS/ glycerol solution), or vehicle containing Endo N (6.7 U/µl), Endo N and anti-$p75^{NTR}$ REX antibody (1 mg/ml), or EndoN and IgG antibody (1 mg/ml). For NCAM −/− mice, their eyes were injected with the REX or control IgG antibody. A drop of antibiotic solution (Polysporin, Johnson and Johnson, New Brunswick, NJ, USA) was applied to the cornea of the anesthetized mouse immediately after the intraocular injection. Animals were subjected to LIRD 4 to 5 days after the intravitreal injection.

3.3.8 Data Analysis

The differences in western blot densitometry from the three/four independent experiments were quantified by two-sample *t* tests. Data from electrophysiology experiments were analyzed with a customized program from Matlab (Mathworks, Natick, MA, USA). To analyze amplitudes and implicit times of the ERG responses, the a-wave, which represents the function of photoreceptor cells, was measured from the baseline to the trough. The b-wave, which reflects mainly on ON-bipolar activity, was measured from the trough of the a-wave to the peak of the b-wave. The maximum response amplitude *Vmax*, the sensitivity parameter *log K* (the intensity of the stimulus at half *Vmax*), and the exponent *n* (slope of the intensity-response function) of the Naka-Rushton hyperbolic equation were derived from the measurement values based on the curve fitting procedure (Anastasi et al., 1993). Differences (*Vmax*, *log K* and *n*) were evaluated with

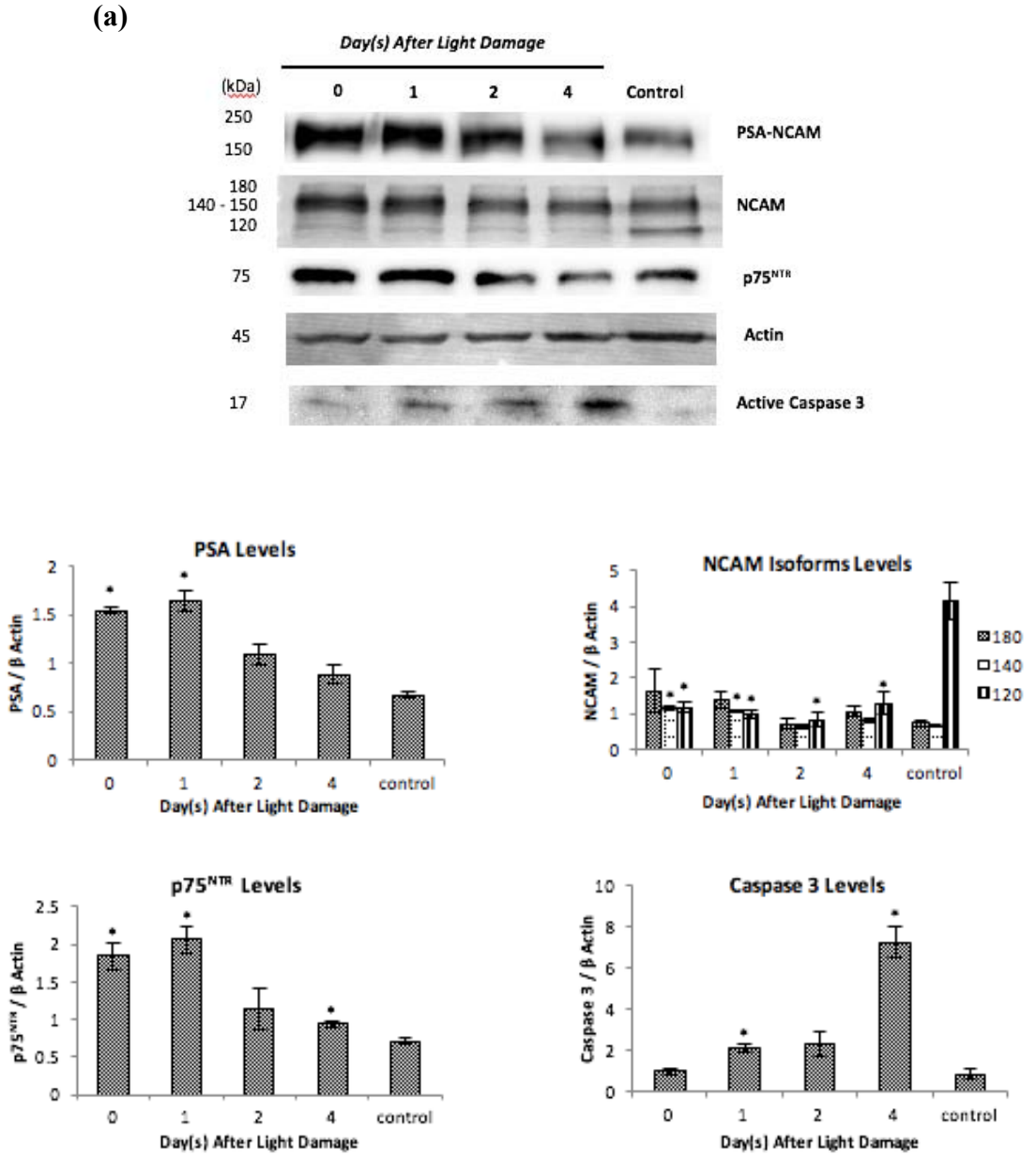
Student's *t* tests to compare NCAM −/− with the WT mice. Statistics were expressed as mean percentages ± SEM. Differences were considered statistically significant if $p < 0.05$. All analyses were performed using Minitab 16 (Minitab, Inc., State College, PA, USA).

3.4 Results

3.4.1 Increase PSA and p75NTR levels after photic injury

To investigate whether NCAM plays a role in LIRD, I first assayed NCAM and PSA expression in WT retinas 0, 1, 2 and 4 days after 6 hours of photic injury (Figure 3-1a). Compared with the non-induced control, I observed notably decreased levels of the 120 kDa, and increased levels of the 140-150 kDa, NCAM isoforms in the LIRD samples. NCAM-180 (180 kDa) expression did not appear to change over time. High levels of PSA (150-250 kDa) were expressed in the retinas immediately after light-induced damage, and their levels remained elevated for at least 2 days. An upregulation of $p75^{NTR}$ (75 kDa) was found in the light damaged retinas; these findings were consistent with previous studies (Harada et al., 2002; Harada et al., 2000). Interestingly, the patterns of $p75^{NTR}$ and PSA expression following LIRD were similar; furthermore, immunostaining showed co-expression of $p75^{NTR}$ and PSA in the retinas after LIRD (Figure 3-1b). Cleaved caspase 3 (17 kDa), an indicator of apoptosis, was detected at low levels on day 0, followed by an increase up to 4 days after LIRD, results that were consistent with a Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Figure 3-2).

Figure 3-1. Western Blot analysis *(a)* showing high levels of PSA-NCAM and p75^{NTR} proteins expressed in the WT retinas (n=5/ group) shortly after light exposure. Active caspase 3 was detected at low levels on day 0, followed by increases up to 4 days after LIRD. Representative immunofluorescence staining *(b)* showing increased p75^{NTR} (green) and PSA-NCAM (red) expression in the WT retinas after light-induced injury: *(i)* non-induced, p75^{NTR} staining; *(ii)* non-induced, PSA-NCAM staining; *(iv) induced,* p75^{NTR} staining; *(v)* induced, PSA-NCAM staining; and *(iii & vi)* merged images of p75^{NTR} and PSA-NCAM staining. Asterisk (*) denotes significantly different from control.



(b)

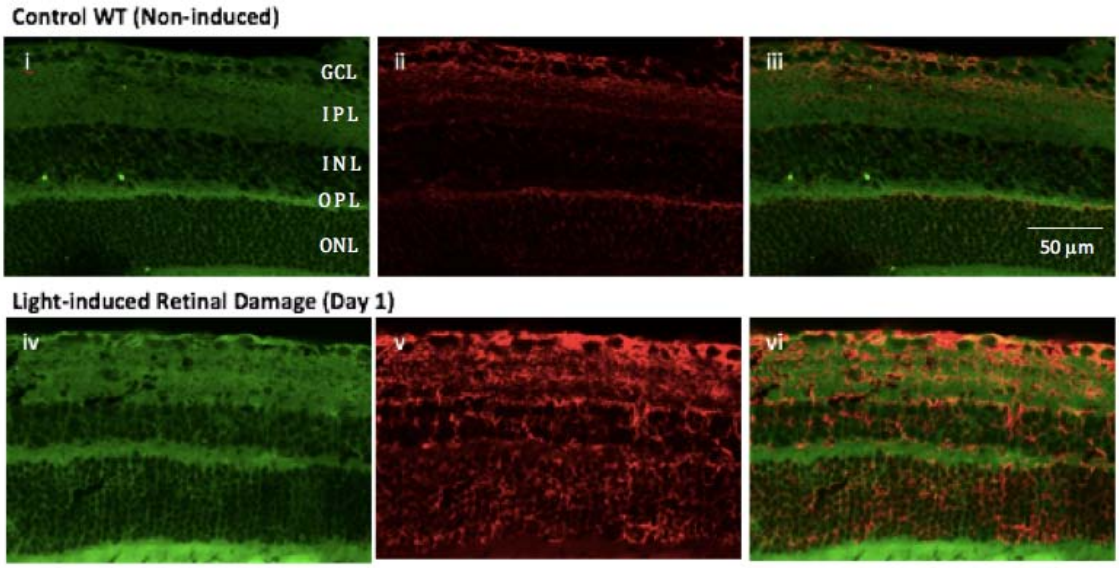
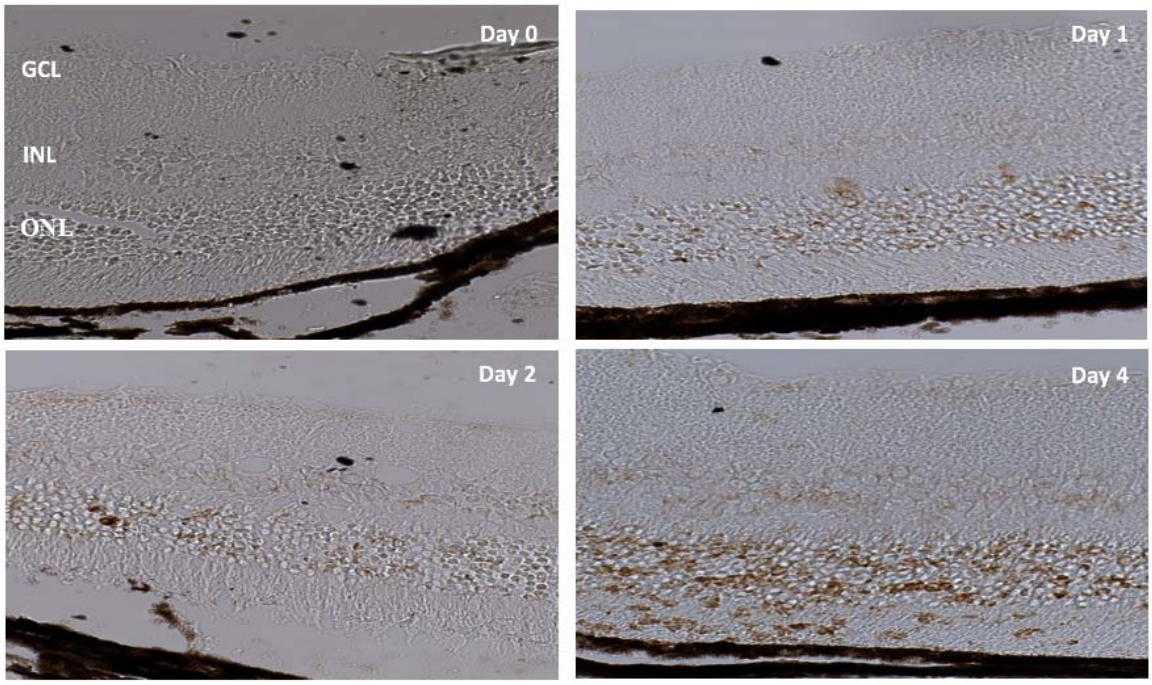

Control WT (Non-induced)
i
GCL
IPL
INL
OPL
ONL
ii
iii
50 µm
Light-induced Retinal Damage (Day 1)
iv
v
vi

Figure 3-2. Extensive cell death on day 4 WT retina. TUNEL assay shows the effect of light induced retinal damage on days 0, 1, 2 and 4. Extensive cell death occurred 4 days after light exposure.



3.4.2 Effect of LIRD on NCAM −/− retina

3.4.2.1 Early apoptosis in NCAM −/− retina

I next examined whether the timeline of retinal cell death I observed in LIRD is altered by the absence of NCAM (Figure 3-3a). Relatively thinned photoreceptor cell layers were observed in WT and NCAM −/− retinas 4 days after LIRD (Figure 3-4). However, in NCAM −/− mice, higher baseline cleaved caspase 3 levels were found several days earlier at Day 0 and Day 1, compared with WT retinas; by day 4, cleaved caspase 3 levels were reduced in NCAM −/− retinas whereas they were high in WT. This earlier onset of cell death in NCAM −/− retinas correlated with higher levels of $p75^{NTR}$ expression on days 0 and 1 after LIRD.

3.4.2.2 Higher p75NTR level in NCAM −/− retina

As higher levels of $p75^{NTR}$ were expressed in the NCAM −/− retinas after LIRD, I next investigated whether $p75^{NTR}$ and its associated proteins were differentially expressed in the knockout's retinas. The pro-NGF/ sortilin/ $p75^{NTR}$ complex has been shown to participate in light-dependent photoreceptor degeneration (Santos et al., 2012), so I compared their expressions in the retinas of WT and NCAM −/− mice using immunoblot analysis (Figure 3-3b). Interestingly, whereas no difference was found in sortilin (95 kDa) and pro-NGF (30 and 32 kDa) levels, $p75^{NTR}$ expression in NCAM −/− retinas was significantly higher than in controls.

Figure 3-3. *(a)* Western Blot analysis of $p75^{NTR}$, actin and active caspase 3 protein expressions in WT and NCAM −/− retinas (n=5/ group) after LIRD. Higher level of active caspase 3 expressed in NCAM −/− at Days 0 and 1 after light exposure suggests photic injury induces earlier cell death in these retinas. The control sample *(C)* was prepared from non-induced NCAM −/− retinas. *(b)* Immunoblot analysis of $p75^{NTR}$, sortilin, pro-NGF and actin protein expressions in uninjured mice shows elevated levels of $p75^{NTR}$ expressed in NCAM −/− retinas. Asterisk (*) denotes the significant difference between WT and NCAM −/− protein expression.

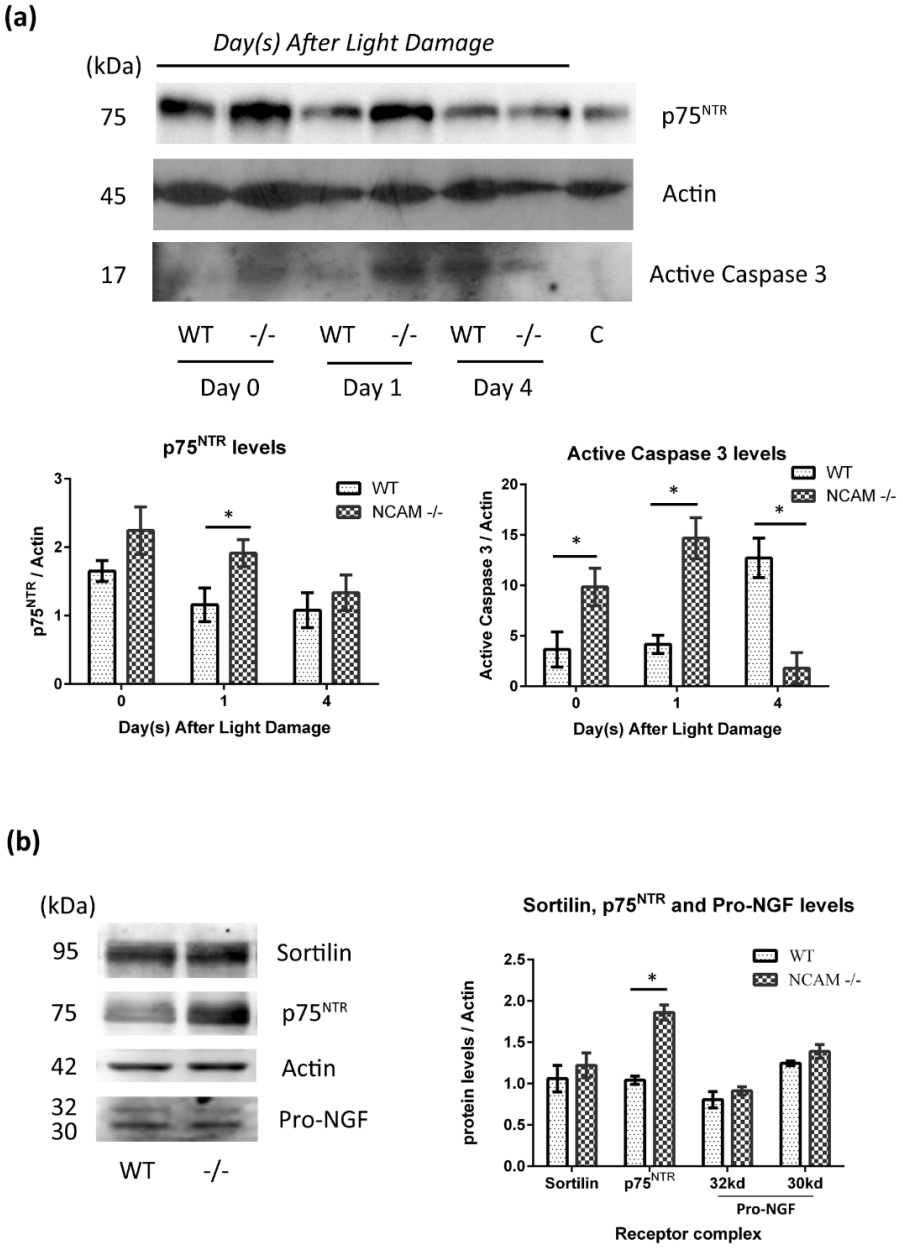
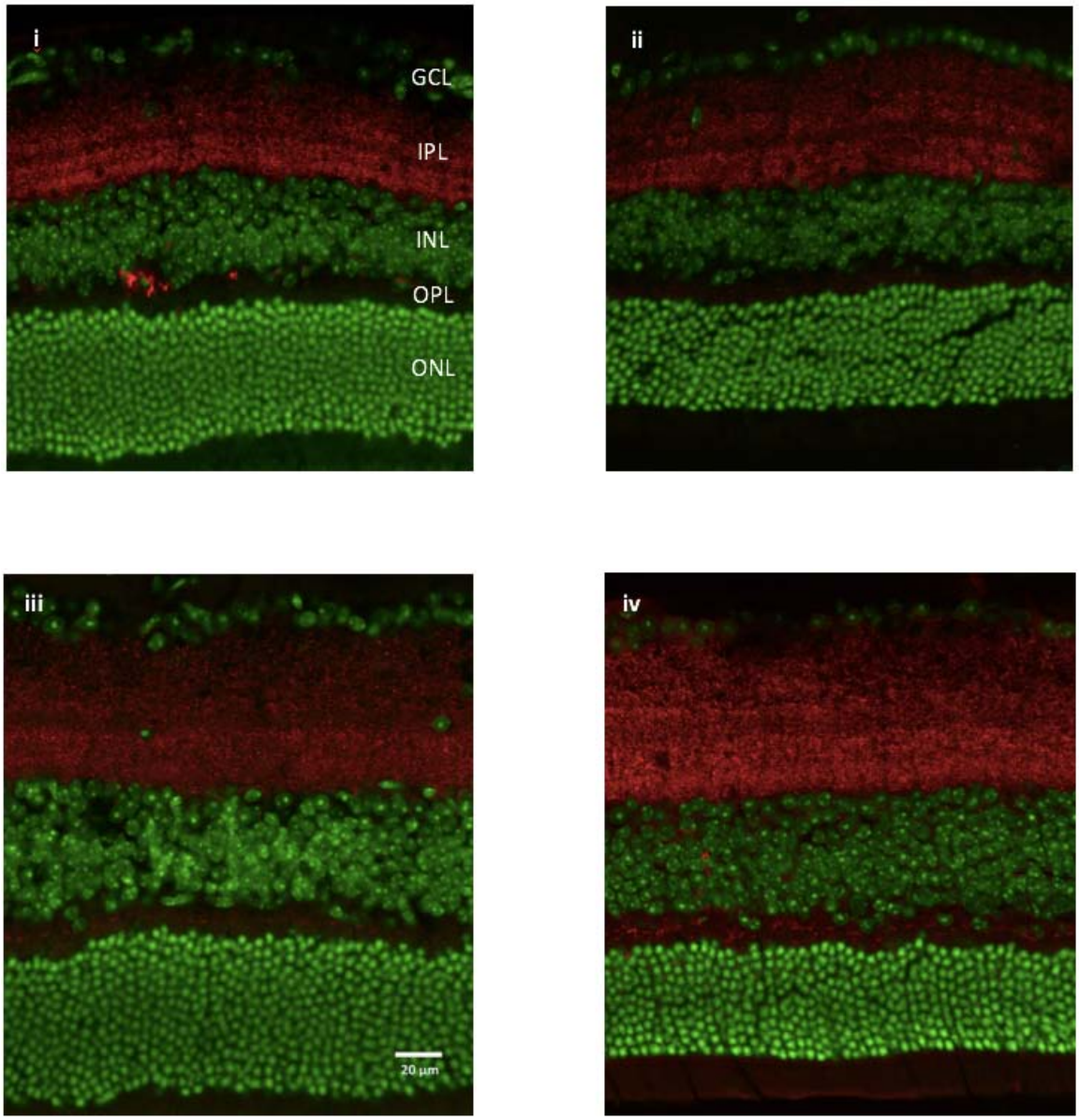


Figure 3-4. Representative Bassoon synaptic (red) and Hoechst nuclear (green) staining of non-induced and light-induced WT and NCAM −/− retinas show reduction of photoreceptor cell layers 4 days after LIRD: *(i)* WT, non-induced; *(ii)* WT, light-induced; *(iii)* NCAM −/−, non-induced; *(iv)* NCAM −/−, light-induced. Images were taken approximately 500µm from the optic disk.



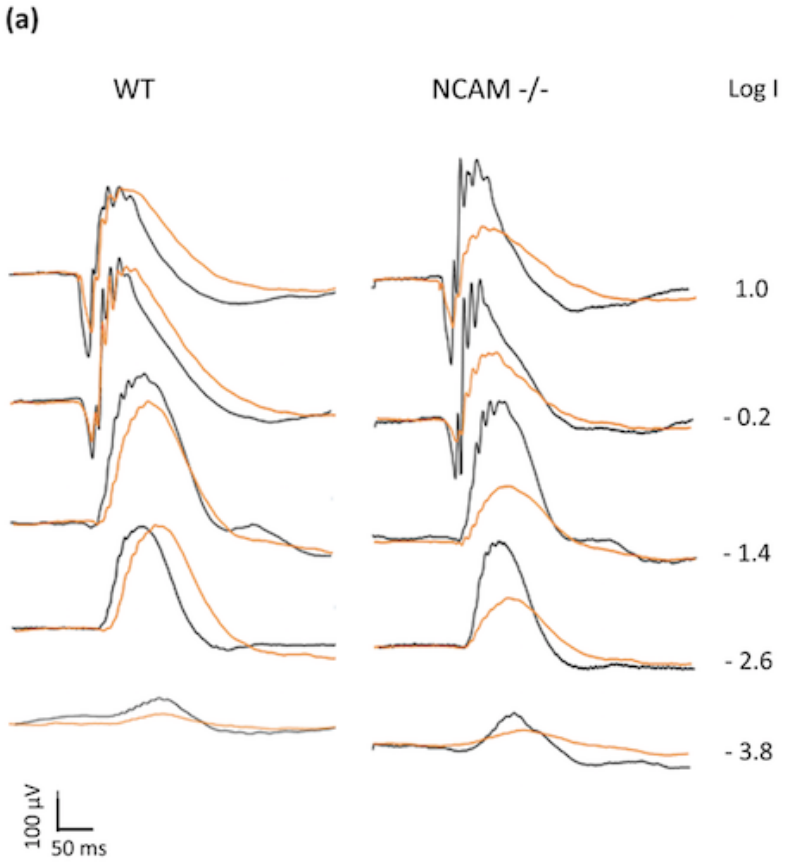
3.4.2.3 Decreased ERG amplitudes in NCAM −/− retina after LIRD

In addition to examining the rate of apoptosis in WT and NCAM −/− mice, I also assessed retinal function before and after photic injury. Figure 3-5a showed representative ERG intensity-response series recorded from WT and NCAM −/− mice. Compared with pre-LIRD values, ERG results showed significant drops of a- and b-wave amplitudes in both groups of mice a day after LIRD (Figure 3-5b). Similar findings were observed in the b-wave light-adapted amplitudes (Figure 3-6). There were no differences in response times to the light stimuli for both groups of animals (Figure 3-7). Furthermore, our baseline results are similar to what has been recently reported: young adult NCAM −/− mice have significantly higher a - and b - wave Vmax than WT mice (Table 3-1a) (Luke et al., 2016a). Using the Naka-Rushton equation, intensity-response functions indicated that the mean a - and b - waves *Vmax* were reduced by 47% and 41% respectively in NCAM −/− mice after LIRD; in contrast, I observed a smaller 26% and 13% reduction in the a- and b- wave *Vmax* for the WT animals. The parameter *K* reflects retinal sensitivity to a light stimulus; pre- and post- LIRD values of the mean a - and b - wave *log K* remained unaffected in WT and NCAM −/− animals (Table 3-1b). There was a significant decrease in the slope *(n)* parameters in the b-wave of the NCAM −/− mice, suggesting that the animals' responses to light decline one day after photic injury (Table 3-1c).

3.4.3 Removal of PSA induced early apoptosis

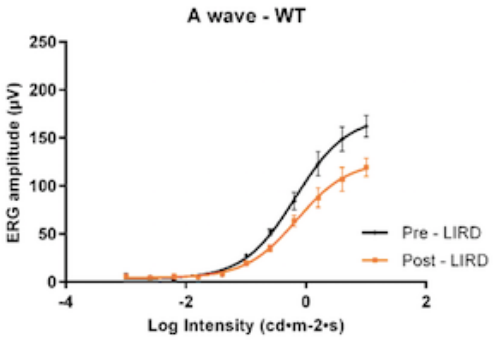
To determine whether the presence of PSA found in WT retinas protects against LIRD, PSA was selectively removed by Endo N 4-5 days before photic injury (Figure 3-8). For eyes that received Endo N, the onset of extensive cell death commenced broadly and

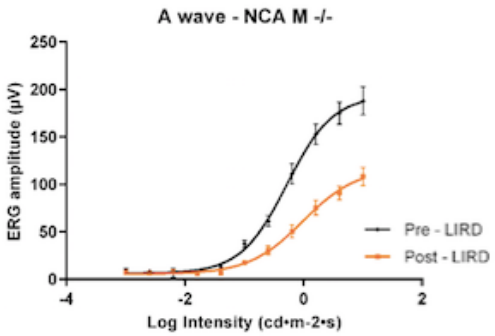
Figure 3-5. Analysis of pre- and post- LIRD ERG responses (n = 6/ group). *(a)* Representative dark-adapted ERGs recorded from WT and NCAM −/− mice showing three days pre- (black) and one day post- (orange) LIRD ERG waveforms. Flash intensity, increasing from -3.8 to 1 log cd*s/m^2, is marked on the right on each pair of the waveforms. *(b)* Mean dark-adapted a- & b- wave amplitudes plotted against log flash intensity for NCAM −/− and WT mice. NCAM −/− mice have significantly lower a- and b-wave amplitudes one day after LIRD.



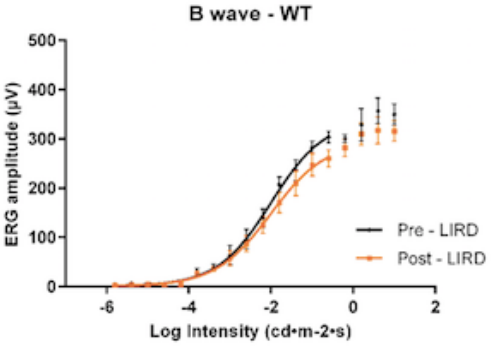
(b)

Dark-adapted a-wave amplitudes





Dark-adapted b-wave amplitudes



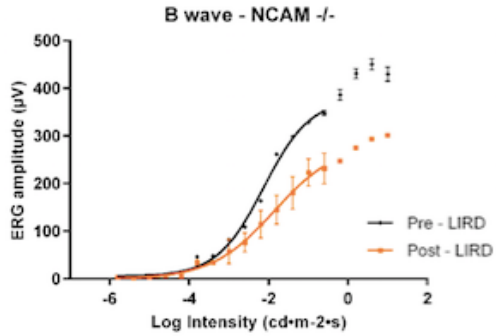


Figure 3-6. Mean light-adapted b-wave amplitudes plotted against flash intensity for NCAM −/− and WT mice show a significant drop of amplitude in WT animals and an even greater drop in NCAM −/− mice one day after LIRD.

Light–adapted b wave amplitudes

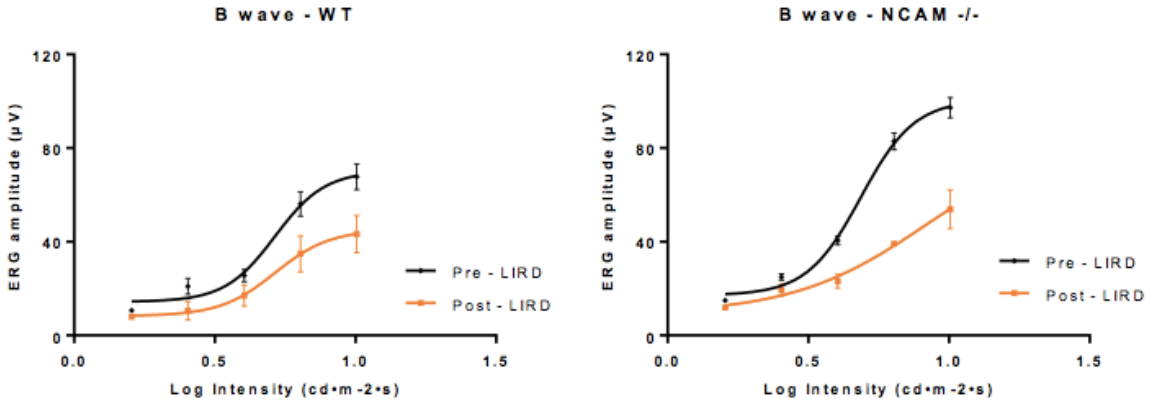


Figure 3-7. Mean dark-adapted a- and b-wave implicit times (time to peak) plotted against flash intensity for NCAM −/− and WT mice showing no differences in response times three days before and one day after LIRD. Points represent means ± SEM.

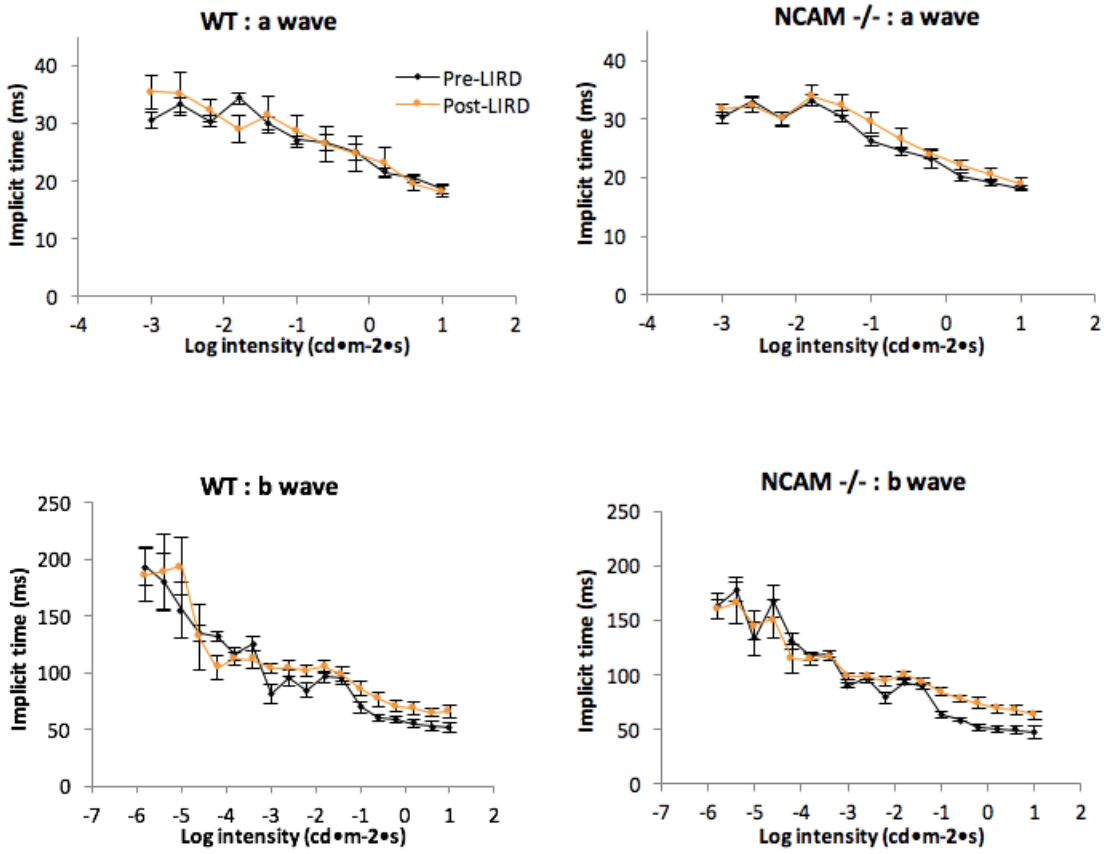
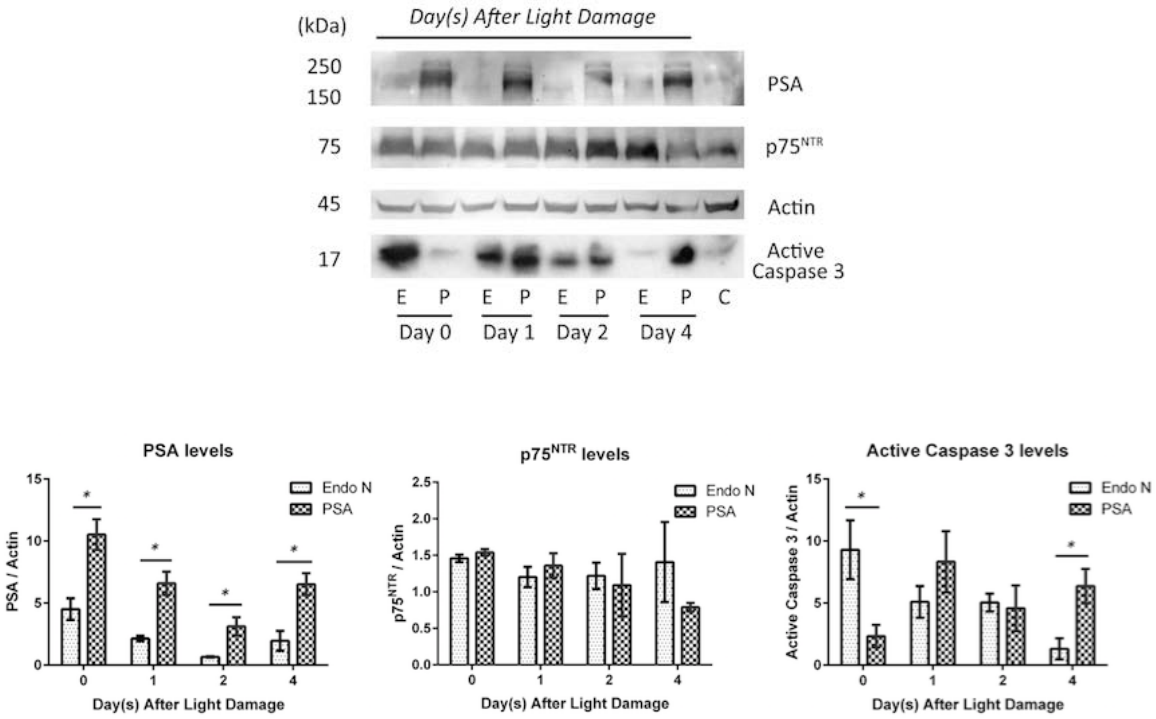


Table 3-1. *Vmax*, *log k* and n parameters derived from scotopic ERG a- and b- wave intensity-response function for NCAM −/− and WT animals show that both groups of mice have lower than normal Vmax levels one day after LIRD.

(a)	***Vmax***		***t-test***		
a wave	***WT***	***NCAM -/-***	***Pre-LIRD*** WT	***Pre-LIRD*** NCAM -/-	***Between group***
Pre - LIRD	173.73 ± 4.98	210.34 ± 6.07			*0.001*
Post - LIRD	129.07 ± 6.18	110.59 ± 4.54	*0.000*	*0.000*	*0.039*
b wave					
Pre - LIRD	343.97 ± 7.22	435.94 ± 13.51			*0.000*
Post - LIRD	300.27 ± 9.97	255.92 ± 23.41	*0.006*	*0.000*	*ns*
(b)	***log k***		***t-test***		
a wave	***WT***	***NCAM -/-***	***Pre-LIRD*** WT	***Pre-LIRD*** NCAM -/-	***Between group***
Pre - LIRD	– 0.22 ± 0.06	– 0.20 ± 0.06			*ns*
Post - LIRD	– 0.12 ± 0.09	– 0.13 ± 0.10	*ns*	*ns*	*ns*
b wave					
Pre - LIRD	– 2.01 ± 0.09	– 1.95 ± 0.05			*ns*
Post - LIRD	– 1.89 ± 0.15	– 1.89 ± 0.15	*ns*	*ns*	*ns*
(c)	***n***		***t-test***		
a wave	***WT***	***NCAM -/-***	***Pre-LIRD*** WT	***Pre-LIRD*** NCAM -/-	***Between group***
Pre - LIRD	1.06 ± 0.09	0.95 ± 0.04			*ns*
Post - LIRD	0.93 ± 0.07	1.03 ± 0.12	*ns*	*ns*	*ns*
b wave					
Pre - LIRD	0.70 ± 0.05	0.87 ± 0.06			*0.043*
Post - LIRD	0.74 ± 0.07	0.57 ± 0.04	*ns*	*0.002*	*ns*

Figure 3-8. Immunoblot analysis shows that removal of PSA induces early onset of retinal cell death; active caspase 3 levels are elevated early after LIRD when PSA is removed by Endo N. 4-5 days prior to light-induced retinal damage treatment, WT animals received an intraocular injection of Endo N *(E)* in the right eye and PBS *(P)* injection in the left eye. Mice (n=10/ group) were sacrificed 0, 1, 2, and 4 days after photic injury. The non-induced, PBS-injected WT retinas served as control *(C)*. Asterisk (*) denotes the significant differences between Endo N and PBS injected retinas.



immediately (Day 0) after LIRD, followed by decreasing active caspase 3 expression by days 2 and 4. However, for eyes that received a PBS intraocular injection, the onset of massive retinal apoptosis was delayed until one day after LIRD, with strong cleaved caspase 3 activity also seen at day 4. Furthermore, although elevated levels of $p75^{NTR}$ appeared immediately after LIRD, there were no major differences in the $p75^{NTR}$ expression between Endo N and PBS – injected retinas on the first 2 days (Days 0 and 1), times when I observed significant differences in the activated caspase 3 levels.

3.4.4 Blockade of $p75^{NTR}$ delays retinal cell death in Endo N-treated WT mice

To determine whether $p75^{NTR}$ has an effect on the premature onset of apoptosis in Endo N – treated WT retinas, I applied *REX* antiserum, which inhibits $p75^{NTR}$ function by binding to its extracellular domain (Figure 3-9). The co-administration of Endo N and *REX* antibody delays the onset of retinal apoptosis by one day, compared with controls (IgG-Endo N). Delaying apoptosis by blocking $p75^{NTR}$ was independent of $p75^{NTR}$ levels, which remained similarly elevated in both groups.

3.4.5 Blockade of $p75^{NTR}$ delays retinal cell death in NCAM −/− mice

The absence of NCAM removes almost all PSA in the null mutant mice. Based on our findings in WT retinas, I also investigated the effect of elevated $p75^{NTR}$ levels in NCAM −/− retinas on the photic injury response (Figure 3-10). For the controls (IgG), as expected, robust active caspase 3 levels (17 and 19 kDa) appeared immediately (Day 0) after light – induced damage, whereas the administration of *REX* antiserum delayed the

Figure 3-9. Immunoblot analysis shows that the blockade of p75^{NTR} binding site, as well as the removal of PSA, delay early onset of retinal cell death. 4-5 days prior to light-induced retinal damage treatment, WT animals received co-administration of Endo N and *REX* antiserum *(R)* in the right eye and Endo N and IgG *(I)* in the left eye. Mice (n=10/ group) were sacrificed 0, 1, 2, and 4 days after photic injury. The non-induced, Endo N and IgG – injected WT retinas served as control *(C)*. Asterisk (*) denotes the significant differences between Endo N – *REX* and Endo N – IgG injected retinas.

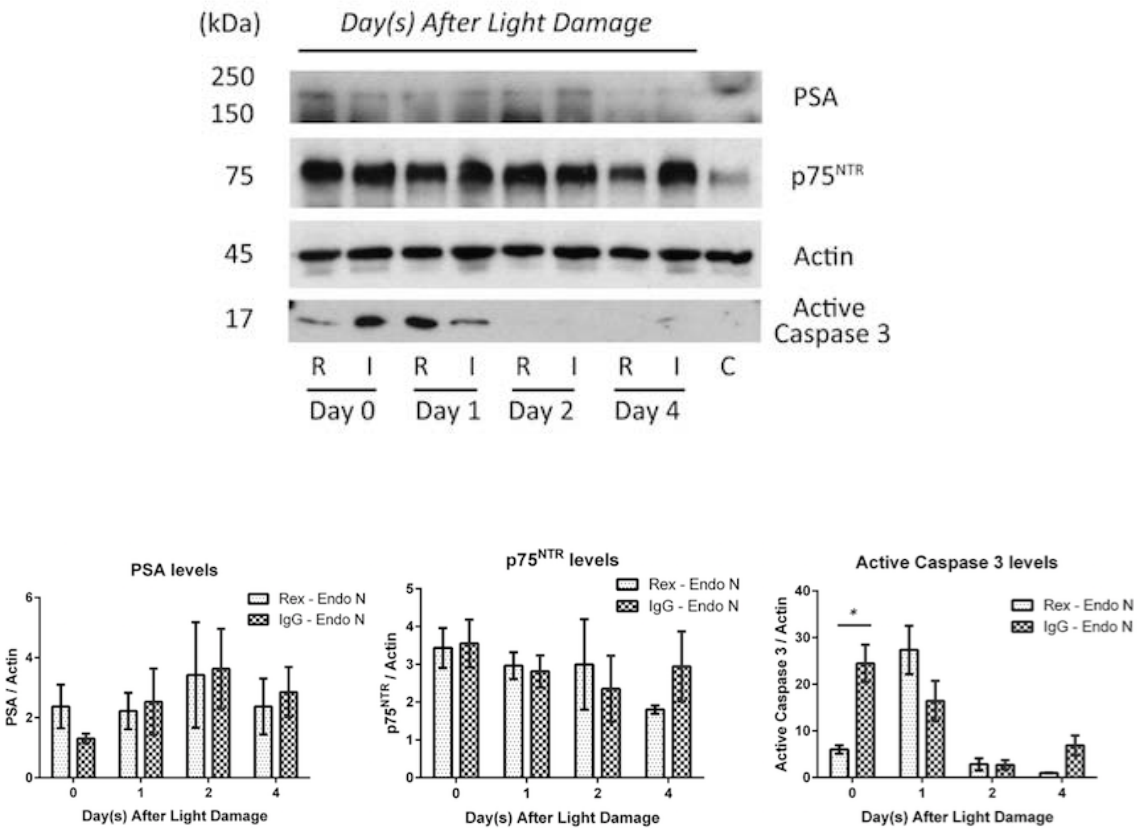
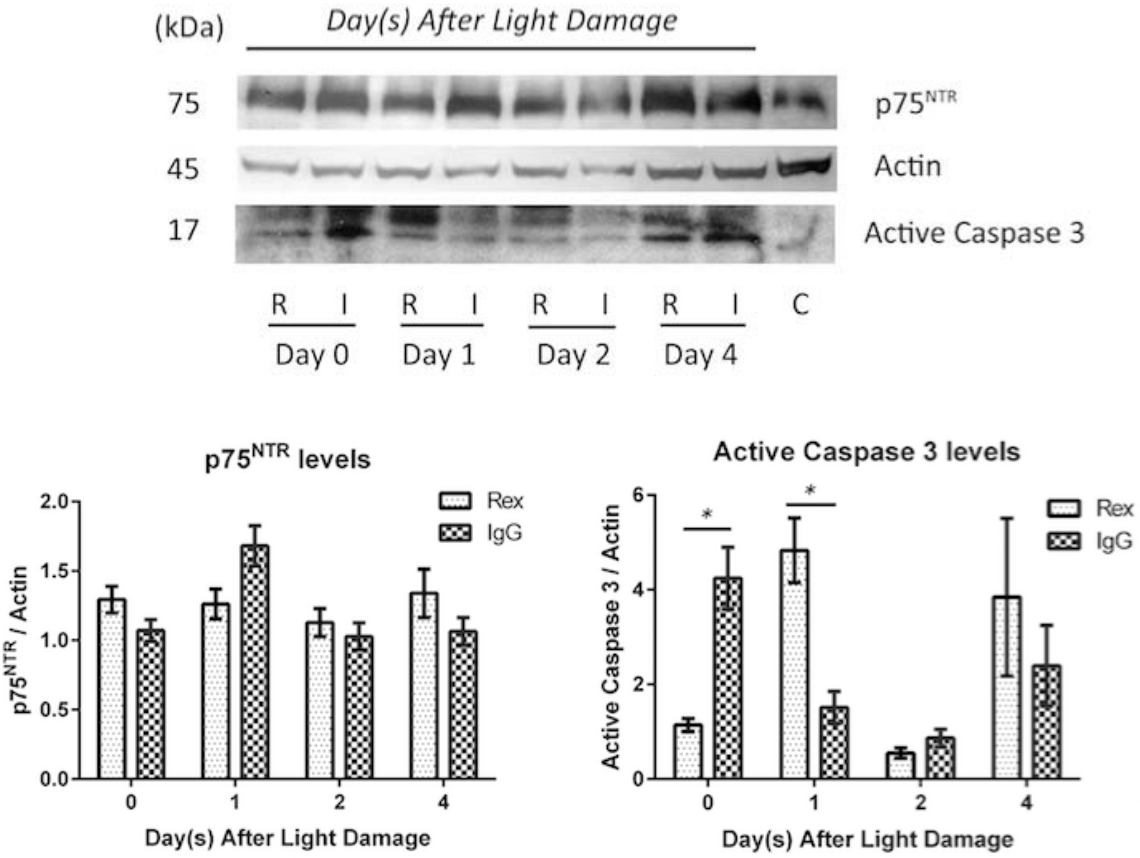


Figure 3-10. Immunoblot analysis shows blockade of $p75^{NTR}$ delayed early onset of retinal cell death in NCAM −/− mice. 4−5 days prior to light-induced retinal damage treatment, NCAM −/− mice received an intraocular injection of *REX (R)* in the right eye and IgG *(I)* injection in the left eye. The animals (n=8/ group) were sacrificed 0, 1, 2, and 4 days after photic injury. The non-induced, IgG-injected NCAM −/− retinas served as control *(C)*. Asterisk (*) denotes the significant differences between *REX* and IgG injected retinas.



onset of apoptosis (Day 1). Again, delaying apoptosis by blockade of $p75^{NTR}$ was independent of $p75^{NTR}$ expression levels.

3.5 Discussion

3.5.1 Summary of the Results

In this study, I have investigated the role of NCAM and its polysialylated derivative, PSA-NCAM, in retinal apoptosis resulting from phototoxicity. My results show that an upregulation of PSA was expressed in the WT retinas immediately after 6 hours of photic injury. Retinal cell death occurred sooner than normal with the removal of PSA, as well as in the absence of NCAM. The magnitude of retinal apoptosis in NCAM −/− mice was correlated with a marked reduction of the mean a- and b- waves *Vmax* in ERG analysis. I specifically investigated expression of the low affinity neurotrophin receptor $p75^{NTR}$, known to promote retinal cell death caused by intense light illumination (Harada et al., 2000). Compared with the WT, I detected higher levels of $p75^{NTR}$ in young adult NCAM −/− retinas. Blockade of $p75^{NTR}$ delayed retinal cells from degeneration in NCAM −/− mice and in WT retinas where PSA was removed, suggesting the presence of NCAM protected retinas from $p75^{NTR}$ induced apoptosis after photic injury. In spite of this, the blockade of $p75^{NTR}$ by a single injection of blocking antibody only postpones the onset of retinal cell death, implicating involvement of alternate pathways independent of $p75^{NTR}$ activation. Taken together, my findings strongly support the notion that NCAM plays an important role in protecting retinal cells from photic injury-induced death, at least in part through the modulation of $p75^{NTR}$.

3.5.2 Light damage as a model for retinal degeneration

Excessive light can cause retinal degeneration experimentally and may be a contributing factor in the progression of human retinal dystrophies and age-related retinal diseases (Grimm and Reme, 2013). Since its first use in rodents by Noell et al., 1966 , LIRD has been used in many studies to examine the effects of white or visible light of different wavelengths and intensities on inducing photoreceptor cell damage (De Vera Mudry et al., 2013; Organisciak and Vaughan, 2010; Ortin-Martinez et al., 2014). Animal models of inherited retinal degeneration such as the rd1 (retinal degeneration 1) mouse, have used LIRD to study cellular, molecular and biochemical events associated with the regulation of photoreceptor cell death (Paquet-Durand et al., 2006; Samardzija et al., 2006; Yang et al., 2007). LIRD can be applied by long term exposure (days) with low illumination or by short term exposure (hours) with high intensity (Grimm and Reme, 2013); the former results in a slow progression of photoreceptor cell death, whereas the latter induces robust cell degeneration, causing at least 90% of photoreceptor cell loss over 10 days (Reme et al., 1998). The induction of photoreceptor cell death by light exposure has also been used to identify new compounds or to evaluate the effectiveness of pharmacological treatments such as the neuroprotective agent, Minocycline (Campbell et al., 2009; Imai et al., 2010; Zhang et al., 2004).

3.5.3 The association between PSA-NCAM and p75^{NTR}

***3.5.3.1** PSA-NCAM and p75^{NTR} in the rodent retina*

The highly sialylated form of NCAM and the p75^{NTR} protein are multifaceted receptors capable of fulfilling a wide number of biological functions (Chen et al., 2009; Durbec and Cremer, 2001). They are expressed widely during development in the nervous system, but decline dramatically by adulthood. During retinal development, PSA-NCAM is expressed in neuroblasts, young post-mitotic neurons, astrocytes and Müller cells of the mouse retina, but the levels progressively diminish in retinal neurons during the third postnatal week (Bartsch et al., 1990). Likewise, high levels of p75^{NTR} are expressed in the inner nuclear layer, inner plexiform layer and ganglion cell layer of the postnatal rat retina (Ding et al., 2001; Hu et al., 1998). In adulthood, the expression of PSA-NCAM and p75^{NTR} are markedly reduced and expressed only in Müller glia, that have structural and functional roles for neurons in the retina (Bartsch et al., 1990; Hu et al., 1998). Injury to the adult nervous system reactivates PSA-NCAM and p75^{NTR} expression, as observed in the visual system after RGC injury, and in models of glaucoma (Hu et al., 1998; Lobanovskaya et al., 2015b; Lonngren et al., 2006; Murphy et al., 2009; Murphy et al., 2007b; Rudzinski et al., 2004). I now know, based on the work of Wada group (Harada et al., 2000), as well as my current study, that intense and prolonged light exposure on the retina results in the activation of p75^{NTR}.

3.5.3.2 The interplay of PSA-NCAM and p75^{NTR} in neuronal survival

The polysialyated form of NCAM has been shown to be a pro-survival molecule in numerous studies (Gago et al., 2003; Gascon et al., 2007a; Lobanovskaya et al., 2015b; Murphy et al., 2009; Murphy et al., 2007b; Vutskits et al., 2001), but the mechanisms by which PSA-NCAM contributes to the survival of neurons remain unclear. There are a few studies that have examined the regulation of neurogenesis, and its association with p75^{NTR}. The absence of NCAM, as well as with the removal of PSA, results in enhanced apoptosis in postnatally generated new neurons of the olfactory bulb (Gascon et al., 2007a). These changes are accompanied by an elevated level of p75^{NTR}, and pharmacological blockade of the p75^{NTR} signaling pathway enhances survival of these neurons (Gascon et al., 2007a). Furthermore, the administration of LM11A-31, that selectively inhibits the binding of NGF and proNGF to p75^{NTR}, also increases PSA-NCAM expression and promotes survival of progenitor neuronal cells in the subgranular zone (Shi et al., 2013). Here, following light-induced retinal injury, I provide further evidence that NCAM, and its polysialyated moiety PSA-NCAM, modulate the activity of p75^{NTR} to promote retinal cell survival.

3.5.4 The role of NCAM in RGC survival

NCAM plays an important role in supporting neuronal survival in the mouse retina. Previous reports from our laboratory have shown that NCAM and its PSA moiety influence RGC survival during development and following injury: (1) NCAM −/− mice have more RGCs in the retinas and have higher levels of BDNF in the superior colliculus than WT mice (Murphy et al., 2007a); (2) following optic nerve transection, the onset of

RGCs loss is earlier in mice lacking NCAM (Murphy et al., 2007a); and (3) and removal of PSA from the surface of neonatal RGCs in vitro, as well as from the adult injured and uninjured retina in vivo, promotes significant RGC death (Murphy et al., 2009). How NCAM influences RGCs survival in these models remain speculative but may involve interaction with BDNF-induced TrkB cell surface receptor (Cassens et al., 2010). PSA presents BDNF to TrkB, thus concentrating the neurotrophin close to its site of action (Vutskits et al., 2001). Several studies have demonstrated that neurotrophins activate the PI3K-Akt signaling cascade through their corresponding receptor tyrosine kinases to promote neuronal survival (Crowder and Freeman, 1998; D'Mello et al., 1997; Dudek et al., 1997; Yao and Cooper, 1995). Therefore, although little is known concerning how NCAM deficiency influences RGCs death, a loss of pro-survival BDNF – TrkB signaling in NCAM deficient mice may be an important mechanism.

3.5.5 $p75^{NTR}$ in response to retinal injury and degeneration: molecular basis

$p75^{NTR}$ does not have intrinsic catalytic activity. Depending on its binding partners or the physiological state of the cell, $p75^{NTR}$ associates selectively with a unique array of proteins, including sortilin, Trk and Nogo receptors, to influence a wide range of cellular functions. The activation of $p75^{NTR}$ results in distinct and even opposing actions, including promoting cell survival, activating apoptotic pathways, or supporting growth cone retraction. Several studies have examined the effect of $p75^{NTR}$ activation in the retina. During light-induced retinal damage, the presence of $p75^{NTR}$ in Müller glial cells suppresses the release of fibroblast growth factor, bFGF, that supports the survival of

retinal neurons, and that the blockade of $p75^{NTR}$ protects photoreceptor cells from apoptosis (Harada et al., 2000). However, the absence of $p75^{NTR}$ does not protect photoreceptor cells against light induced injury, suggesting an alternative cell death pathways exist in $p75^{NTR}$ deficient mice (Rohrer et al., 2003). Using chronic (glaucoma) and acute (optic nerve axotomy) injury models to induce RGC degeneration, activation of $p75^{NTR}$ triggers the release of tumor-necrosis factor α (TNF-α) and α_2-macrogloblin (α_2M) neurotoxic proteins, that act against the protective effect of TrkA receptor and lead to neuronal cell death (Bai et al., 2010). Furthermore, the nerve growth factor precursor, proNGF, activates a non-cell autonomous signaling pathway in response to CNS injury; $p75^{NTR}$ as well as its co-receptors Neurotrophin Receptor Interacting MAGE Homolog (NRAGE) and sortilin, are required to stimulate the release of TNF-α in Müller glial cells to induce RGC death (Lebrun-Julien et al., 2010). Similar findings have been reported using glutamate – induced excitotoxicity in the mouse retina (Lebrun-Julien et al., 2009a). Taken together, in a variety of cells and in response to various stimuli, retinal apoptosis can involve an upregulation of $p75^{NTR}$, that associates selectively with specific receptors to activate a cascade of cell death signaling events.

3.6 Conclusion

I have demonstrated that NCAM protects WT retinas from LIRD through a mechanism that is, at least in part, due to modulation in $p75^{NTR}$ signaling. This greater understanding of the molecular mechanisms involved in LIRD may provide therapeutic opportunities for treatment in this injury model and may also be relevant to other diseases characterized by retinal degeneration.

CHAPTER 4: POLYSIALYLATED NEURAL CELL ADHESION MOLECULE PROMOTES RECOVERY OF VISUAL ACUITY AFTER LONG TERM MONOCULAR DEPRIVATION

4.1 Preface and Significance to Thesis

As shown previously in the aging study (Chapter 2), NCAM −/− mice encounter premature onset of visual acuity impairment as they age; however, the underlying cause of the early deterioration in visual function remains unknown. The polysialylated form of NCAM is known to be intimately involved in different forms of neural plasticity in the adult brain, thus raising the possibility that NCAM may play an important role in visual cortical plasticity. To address this question, I have examined whether PSA is required for visual cortical plasticity in adult mice after long-term MD. My results show that elevated PSA and BDNF levels, associated with increased degradation of perineuronal nets (PNNs), are present in the contralateral visual cortex (VC) of the reopened eye in WT animals. Furthermore, the removal of PSA in the VC of these animals reduces BDNF expression and decreases PNNs degradation, and results in impaired recovery of visual acuity after chronic MD. Collectively, my results demonstrate that PSA is necessary for the reactivation of visual cortical plasticity in adult mice.

This chapter has been submitted for publication. Preliminary results of the findings in this chapter have been presented at the 44th Annual Meeting of the *Society for Neuroscience* in Washington, DC, USA (2014), and published in abstract form (Luke et al., 2014).

4.2 Introduction

Neuronal circuits in the brain exhibit profound plasticity and can be modified in response to environmental changes over a developmental time frame, known as a "critical period". An animal's response to environmental stimuli during a critical period enhances functional adaptation and survival later in life. In the VC, experience drives binocular matching of orientation preference; hence, critical period plasticity is especially important for proper development of the visual system (Levelt and Hubener, 2012). Wiesel and Hubel (1963) identified cortical areas of the cat brain that could be activated by visual experience during the critical period and provided insight into how ocular dominance (OD) columns are established. After the end of the critical period, cortical plasticity diminishes, and neural connections become fixed (Hubel and Wiesel, 1970). Based on this early experimental work, the visual system has become a popular model for studies on the mechanisms of neural plasticity underlying experience-induced cortical changes. In addition, there is an interest in understanding the mechanisms underlying the opening and closing of critical periods in order to develop strategies to promote new neuronal connections and to enhance brain plasticity after the closure of a critical period (Morishita and Hensch, 2008; Toyoizumi et al., 2013).

Using monocular visual deprivation to alter normal binocular vision, the molecular mechanisms underlying OD plasticity have been identified and characterized (Berardi et al., 2003; Maya-Vetencourt and Pizzorusso, 2013). In the past two decades, many molecules in the CNS, such as brain-derived neurotrophic factor (BDNF) and perineuronal nets (PNNs), have been implicated in the shift of balance between excitatory

and inhibitory (E/I) neural circuits, which may remodel experience-dependent plasticity later in life (Maya-Vetencourt and Origlia, 2012). PNNs are lattice-like extracellular matrix structures composed of chondroitin sulfate proteoglycans (CSPGs), and the majority form around the parvalbumin (PV) interneurons, inhibiting axonal growth during development (Karetko and Skangiel-Kramska, 2009). It plays a crucial role in the closure of the critical period (Pizzorusso et al., 2002). PNNs are widely considered as markers of neuronal stabilization and maturation. The degradation of CSPGs promotes PV networking and reactivates cortical plasticity (Lensjo et al., 2017). BDNF is critically important for neuronal plasticity, as increased BDNF expression allows for a functional modification of neural circuitries underlying sensitivity to MD in adult animals (Maya Vetencourt et al., 2008).

In the current study, I examine the role of the polysialylated neural cell adhesion molecule in plasticity of the adult VC. PSA-NCAM is considered to be an essential signaling molecule as it both regulates GABAergic inhibitory maturation and controls the onset of critical period plasticity in the VC during development (Di Cristo et al., 2007); however, to date, its role in plasticity in the VC later in life has not been fully examined.

NCAM is a glycoprotein of the immunoglobulin superfamily that is predominantly expressed in the central and peripheral nervous systems as one of three major isoforms: NCAM-120, a cell surface protein anchored by the glycosylphosphatidylinositol structure, and NCAM-140 and -180, two transmembrane proteins that differ only in their cytoplasmic domains (Soroka et al., 2010). Polysialic acid is a long linear homopolymer

of α-2-8-N acetylneuraminic acid (Finne et al., 1983). NCAM is the primary carrier of PSA in mammals (Nelson et al., 1995). All major NCAM isoforms can be post-translationally modified by polysialylation, giving rise to PSA-NCAM, that is widely expressed in the CNS during development (Bonfanti, 2006). In the mouse VC, PSA expression declines around the time of eye opening (Di Cristo et al., 2007), but is re-expressed in 18-month old mice (Luke et al., 2016a).

Two well-described models have been used to study postnatal plasticity in the visual system, each with distinct molecular mechanisms: the OD shift towards the non-deprived eye after short-term MD, and the recovery of vision in adults following chronic MD. PSA is not required for the shifts of OD in the VC after short-term MD (Guirado et al., 2016); however, the role of PSA in the restoration of visual function in adults after chronic MD is unknown. In light of this, I investigated whether PSA plays a role in the recovery of visual function in adult C57Bl/6J mice following long-term MD. I found an upregulation of PSA and BDNF, as well as a reduced density of GABAergic PV interneurons surrounded by PNNs, in the VC contralateral to the reopened eye. These are typical indications of experience-dependent visual cortical plasticity (Liu et al., 2013). In contrast, removal of PSA in the VC reduces neural plasticity, as indicated by the decreased expression of BDNF and limited degradation of PNNs, resulting in impaired restoration of visual acuity. Taken together, these results indicate that PSA is required for the reactivation of visual cortical plasticity and the recovery of visual function following long-term MD in adult mice.

4.3 Materials and Methods

4.3.1 Generation of Monocularly Deprived Mice

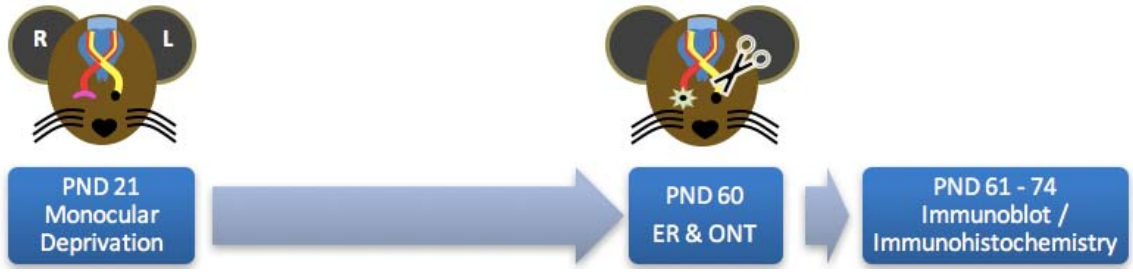
Male and female C57Bl/6J mice were purchased from The Jackson Laboratory (Bar Harbour, ME, USA) bred in-house at Dalhousie University. They were cared for following the Guide to the Care and Use of Experimental Animals of the Canadian Council on Animal Care. The experimental protocol (number 14-137) was approved by the Dalhousie Committee on Laboratory Animals. To generate monocularly deprived mice, their right eyes were occluded by having eyelids trimmed and sewn shut on postnatal day (PND) 21 under 2% of isoflurane anaesthesia (Figure 4-1a). The eyelid closure was inspected regularly under a surgical microscope until complete cicatrization, and animals were excluded from the study if incomplete suturing or spontaneous eye reopening occurred. On PND 60, the eyelids were reopened (ER) and, under the same anesthetic, optic nerve transection (ONT) was performed at approximately 0.5mm behind the globe of the non-deprived left eye, so as to force the animal to employ its reopened eye (Berkelaar et al., 1994; Murphy et al., 2007a). After ER, topical eye drops Polysporin (Johnson & Johnson, New Brunswick, NJ) were administered to the reopened right eye for the first 3 days to prevent inflammation and infection.

4.3.2 Immunoblot

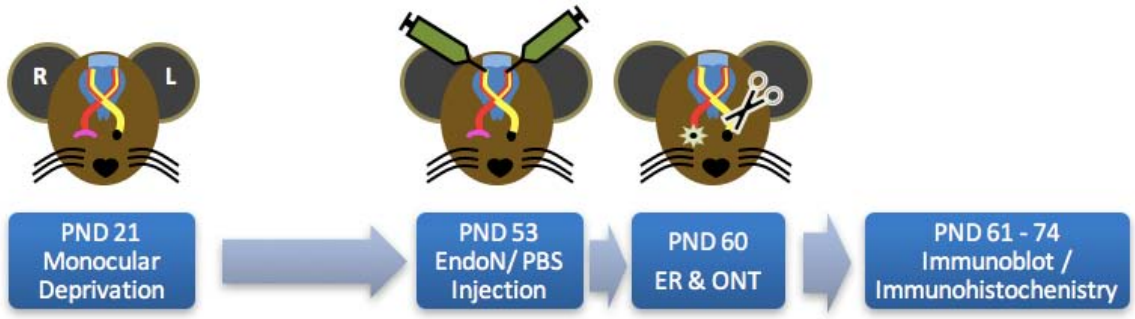
For immunoblot analysis, the monocularly deprived animals (n = 7/ group) were sacrificed on days 1, 2, 3, 4, 7, 10 and 14 after ER and ONT. Their visual cortices were carefully dissected out and pooled together for each group, then incubated in a buffer

Figure 4-1. Generation of monocular amblyopic mice: C57Bl/6 mice underwent a period of monocular deprivation by suturing their right eyelids (PND 21) before the peak of the critical period. Three experiments were conducted: (a) at 2 months old, the sutured eyelids were reopened (ER), and the optic nerves of the previously non-deprived (left) eyes were transected (ONT); (b) PSA was removed through intracranial injection of Endo N (PND 53) before ER and ONT; and (c) visual abilities were examined 24 days before (PND 36) and a week after (PND 67) ER and ONT.

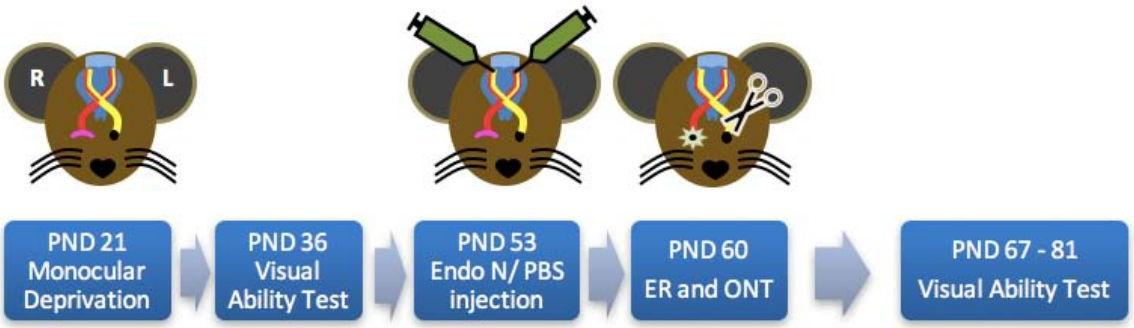
(a) Experimental procedures for the generation of monocular amblyopic mice (re data shown in Figures 4-2 & 4-4).



(b) Experimental procedures for bilateral PBS or Endo N intracranial injection prior to ER and ONT (re data shown in Figures 4-6 & 4-7).



(c) Experimental procedures for the addition of the visual ability test before and after prescheduled treatments (re data shown in Figure 4-9).



containing 50mM Tris, pH 7.5; 5mM EDTA; 0.1% NP-40, 40mM NaCl; and 1X protease inhibitor cocktail (11836170001; Roche Molecular Biochemicals, Indianapolis, IN, USA) for 30 minutes. Anti-NCAM (MAB310, 1:500; Millipore, Billerica, MA, USA), anti-PSA (MAB5324, 1:2000; Millipore), anti-BDNF (N-20) (sc546, 1:500; Santa Cruz Biotechnology, Dallas, TX, USA), and loading control anti-GAPDH (G8795, 1:20,000; Sigma-Aldrich Corp., St. Louis, MO, USA) antibodies were used to determine protein expressions by standard SDS-PAGE immunoblotting.

4.3.3 Immunohistochemistry

Long-term monocularly deprived mice were anesthetized and transcardially perfused with chilled 4% paraformaldehyde 1, 4 and 7 days after ER and ONT. The brains were removed, post-fixed overnight at 4^{o}C, and cryo-protected in 30% sucrose. Coronal brain sections (16 μm) containing primary visual cortex (V1) were obtained by cutting with a cryostat apparatus (Leica CM1850; Leica Biosystems, Wetzlar, Germany), washed 3 times with 1X phosphate buffer, and incubated in Wisteria Floribunda Agglutinin (WFA) (L1516, 1:100; Sigma) overnight to visualize PNN (Hartig et al., 1992). V1 was identified according to the images referenced in the Mouse Brain Atlas (Paxinos and Franklin, 2004). The sections were then immunostained using antibodies against PSA (MAB5324, Millipore; 1:100) and PV (PV-235, 10,000; Swant, Marly, Switzerland) as previously described (Murphy et al., 2009). Two-dimensional immunofluorescent images at 25X magnification acquired in multiple slabs covering the whole brain were stitched together computationally (Zeiss Axio Imager Z2; Thornwood, NY, USA). To quantify the density of PNN and PV neurons in V1, 5 coronal sections from each animal, and 5 animals per group, were analyzed.

4.3.4 Intracranial Injection

At PND 53, the monocularly deprived mice were anesthetized with isoflurane and stabilized in a stereotactic unit. Immediately after a craniotomy procedure, both VC were injected with 1µl of either Endo N (6.7 U/µl) to remove PSA from NCAM or vehicle (PBS) in a 50% glycerol solution using a microliter syringe (7102, Hamilton) fitted with a glass pipette tip (Figure 4-1b). To target the mouse occipital cortex (Paxinos and Franklin, 2004), the needle was placed 0.75 mm below the dura surface, 2.5 mm lateral to the midline and 3.8 mm anterior to the lambda suture (Di Cristo et al., 2007).

4.3.5 Visual Ability Testing

Detailed experimental procedures for the visual ability test have been previously described (Wong and Brown, 2006). Briefly, following the pre-training day, mice (n = 5/ group) at age PND 36 (before intracranial injection) and PND 67 (after ER and ONT) were subjected to eight swim trials per day, for a period of eight days, in the visual detection and visual acuity tasks (Figure 4-1c). The positive and negative visual stimuli were shown randomly on the left and right computer screens, and a hidden platform was positioned under the positive signal. Mice had to swim to the positive signal directly for a successful trial to be scored. The number of correct trials each day was converted into a percent correct, with the passing criterion set at 70% (Wong and Brown, 2007).

4.3.6 Data Analysis

The *t* tests (paired *t* tests for Figures 4-2 and 4-4, independent-*t* tests for Figures 4-6 and 4-7) were used to compare PNN and PV cell densities, as well as immunoblots, that were obtained from 3-4 independent experiments. In the visual behavior test, data were

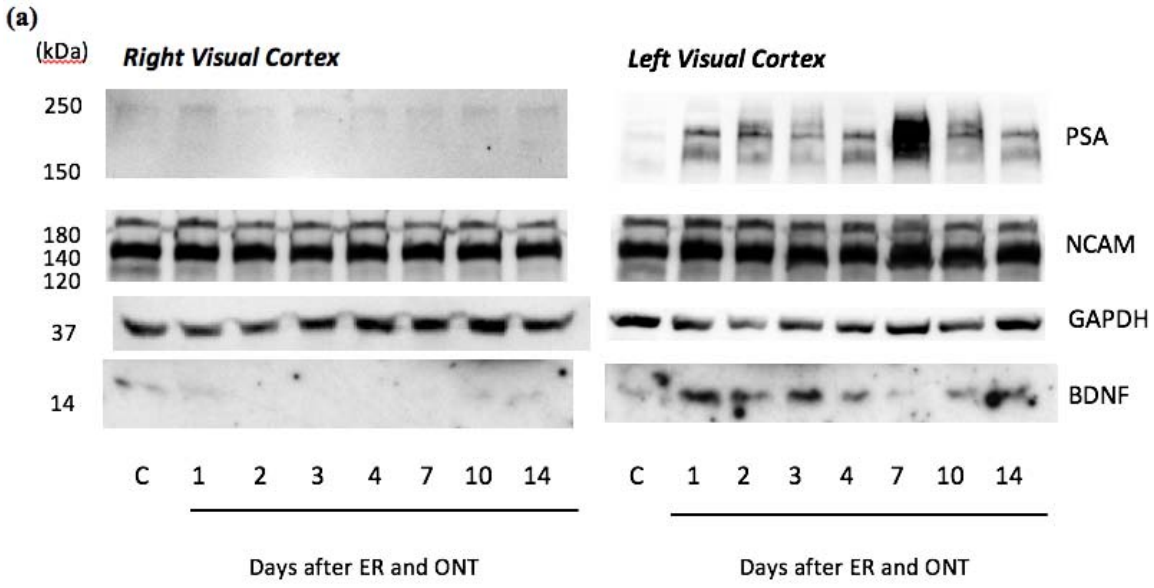
analyzed by 2x8 between-within repeated measures ANOVA, followed by Bonferroni's test (Wong and Brown, 2006). Differences in day 8 performance were examined using independent-*t* tests. Statistics are presented as a mean percentage ± standard error of the mean (SEM). Differences were considered statistically significant if $p < 0.05$, and all statistical analyses were performed using Minitab 17 and Statview 5.0 (Abacus Concepts).

4.4. Results

4.4.1 Increase in levels of PSA, NCAM and BDNF after ER and ONT

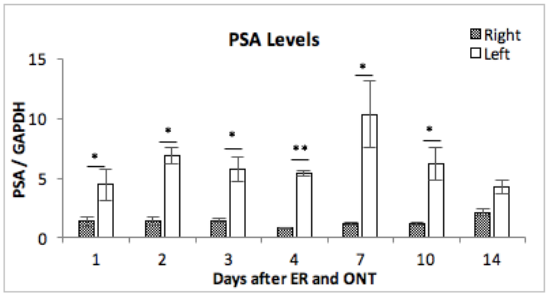
To determine whether PSA plays a role in visual cortical plasticity, I assessed protein expressions in the left and right VC on days 1, 2, 3, 4, 7, 10 and 14 after long-term MD (Figure 4-2a). Changes in protein levels in the VC were expected to correspond to input from the contralateral eye since most the retinal fibres (over 95%) decussate at the optic chiasm. Immunoblot results showed increased expression of PSA (150 – 250 kDa) in the VC (left) contralateral to the reopened eye on almost all days after ER and ONT, with a peak at day 7 (Figure 4-2b-i). Similar results were obtained for NCAM 140 expression in the left VC, that was significantly increased on days 1, 3, 4, 7, 10 and 14 after ER and ONT (Figure 4-2b-ii). For the VC (right) contralateral to the lesioned optic nerve, PSA and NCAM isoform levels remained unchanged compared with the control sample. Furthermore, BDNF levels (14 kDa), which are substantially involved in the recovery of visual function (Kaneko et al., 2008; Sale et al., 2007), were highly expressed only in the left VC on days 1, 2, 3, 10 and 14 after ER and ONT (Figure 4-2b-iii).

Figure 4-2. Molecular changes following MD 1, 2, 3, 4, 7, 10 and 14 days after ER and ONT [as per Figure 4-1(a)]. (a) Western blot analysis shows high levels of PSA, NCAM 140, and BDNF expressed in the mouse's left VC contralateral to the reopened eye. (b) Quantitative analysis of western blot results for PSA (i), NCAM isoforms (ii) and BDNF (iii). Control *(C)* VC samples were prepared from sham operated animals; asterisk (*) denotes significant differences between left and right V1, where *p < 0.05, **p < 0.01, ***p < 0.001.

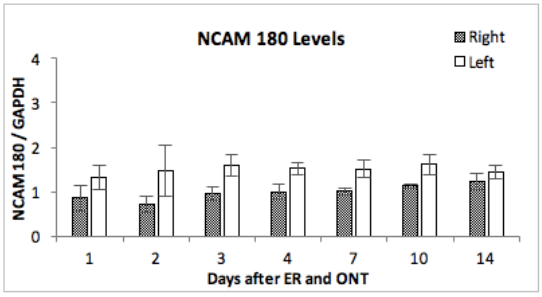


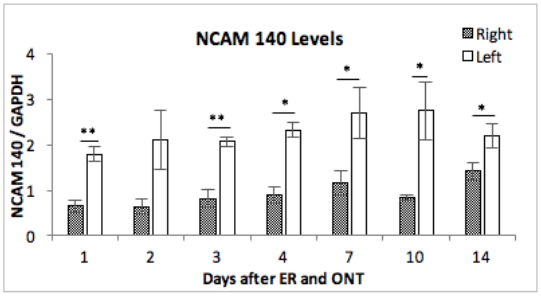
(b)

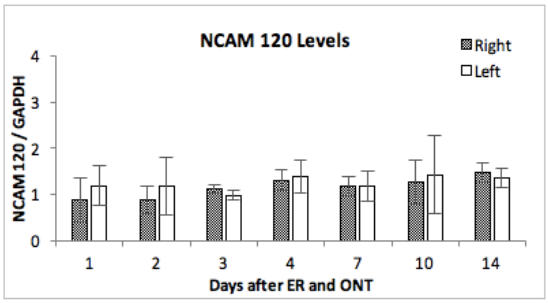
(i)


PSA Levels
Right
Left
PSA / GAPDH
15
10
5
0
1
2
3
4
7
10
14
Days after ER and ONT

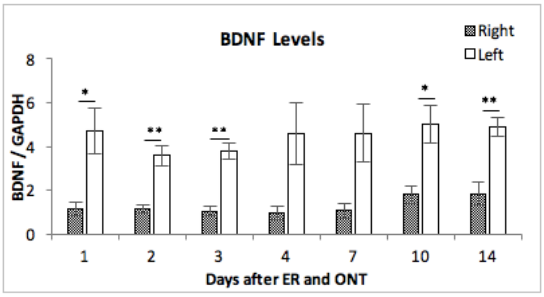
(ii)


NCAM 180 Levels
Right
Left
NCAM180 / GAPDH
4
3
2
1
0
1
2
3
4
7
10
14
Days after ER and ONT


NCAM 140 Levels
Right
Left
NCAM140 / GAPDH
4
3
2
1
0
1
2
3
4
7
10
14
Days after ER and ONT


NCAM 120 Levels
Right
Left
NCAM120 / GAPDH
4
3
2
1
0
1
2
3
4
7
10
14
Days after ER and ONT

(iii)


BDNF Levels
Right
Left
BDNF / GAPDH
8
6
4
2
0
1
2
3
4
7
10
14
Days after ER and ONT

4.4.2 Reactivation of neural plasticity in adult primary visual cortex

The numbers of PNN-labelled, and PV-PNN co-labelled cells, as well as the ratio of PV neurons surrounded by PNNs, decrease during the reactivation of visual cortical plasticity in adulthood (Liu et al., 2013; Sale et al., 2007). Hence, I examined the number of PNN and PV-PNN neurons, and the percentage of PV-PNN within PV-labelled cells in the left and right V1 on days 1, 4 and 7 after ER and ONT. PSA is expressed in a subpopulation of the mature cortical interneurons (Gomez-Climent et al., 2011). It also co-expresses with PV in the puncta surrounding pyramidal neurons somata in the cortex (Nacher et al., 2013). Our immunofluorescence staining using confocal microscopy showed representative labeling of PV, PSA, and WFA labeled PNNs in the V1 (Figure 4-3). Compared to the right V1, immunostaining shows a higher intensity of PSA (Figure 4-4a), and a reduced density of PV-PNN co-labeled cells (Figure 4-4a, insets), in the left V1 4 days after ER and ONT. Quantitative analysis is supportive of reactivation of left visual cortical plasticity by showing significant reductions in the numbers of PNN (Figure 4-4b-i) on days 1 [$t_4 = 4.09$, $p < 0.01$] and 4 [$t_4 = 4.76$, $p < 0.01$], and PV-PNN co-labeled neurons on day 4 [$t_4 = 2.06$, $p < 0.05$] (Figure 4-4b-ii) after ER and ONT. The percentage of PV cells surrounded by PNNs is also reduced in the left V1 on days 1 [$t_4 = 3.60$, $p < 0.05$], 4 [$t_4 = 5.81$, $p < 0.01$] and 7 [$t_4 = 3.59$, $p < 0.05$] after ER and ONT (Figure 4-4b-iii).

Figure 4-3. PSA (a), PV (b), and PNN (c) staining in the left V1 four days after ER and ONT. Representative confocal microscopy images of immunofluorescence staining, showing (d) co-expression (arrows) of PSA (green), PV (red) and PNN (blue) in the mouse V1.

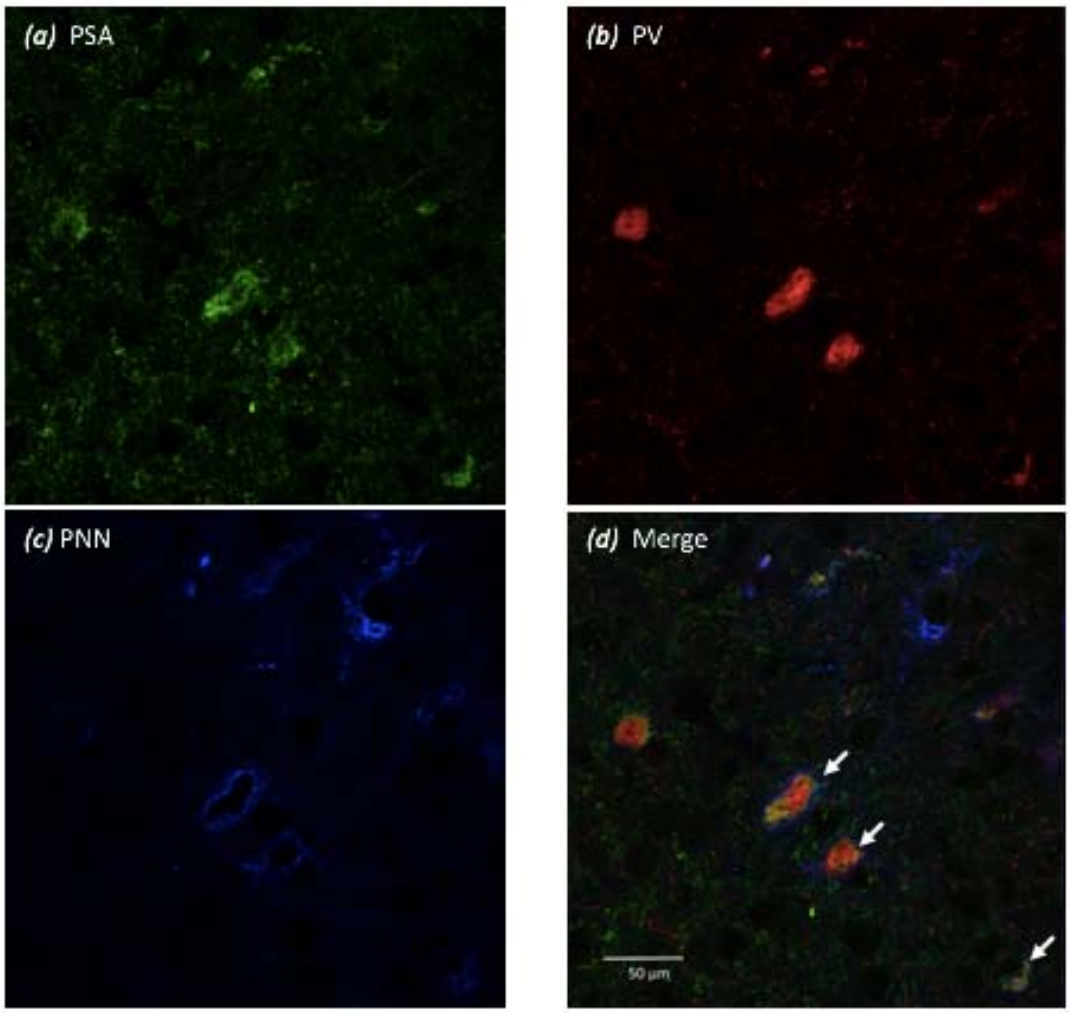
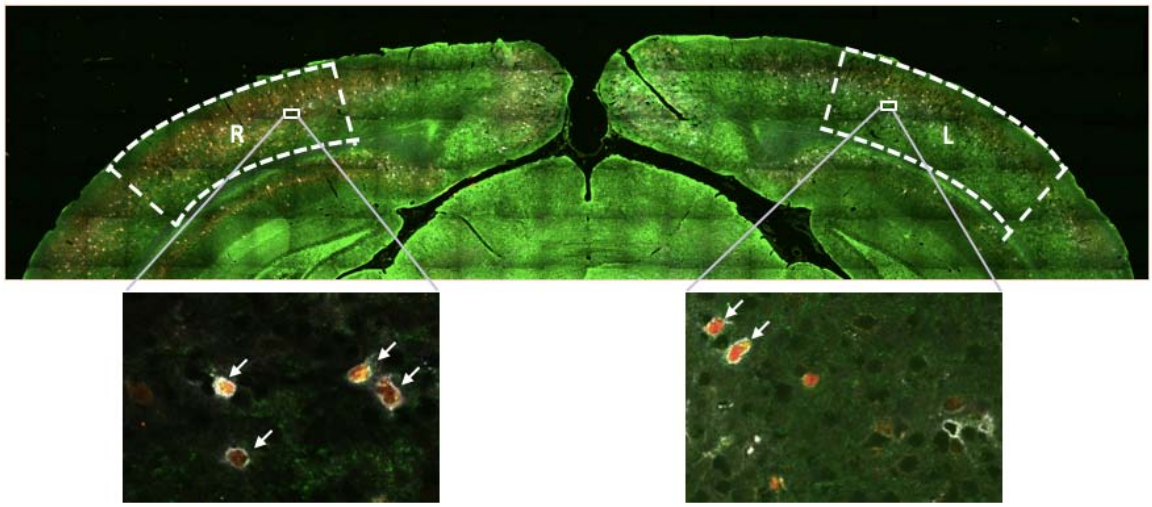
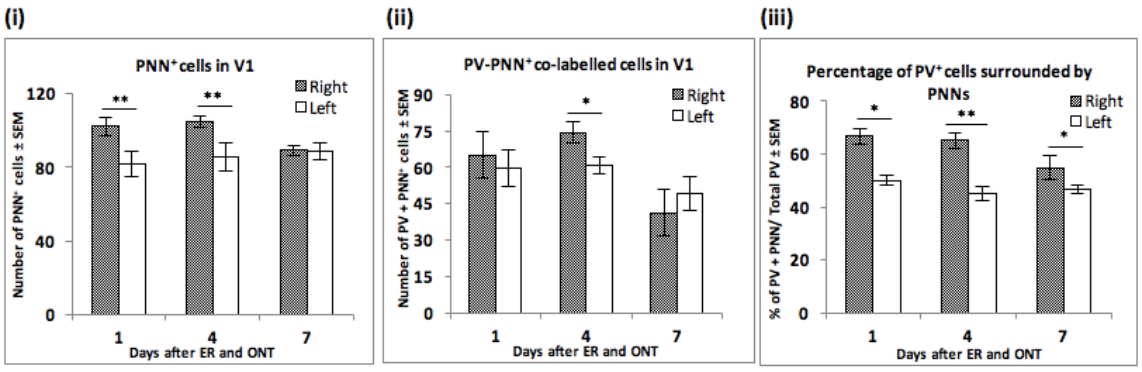


Figure 4-4. Representative immunofluorescence staining (a) shows PSA (green), PV (red), and PNN (white) expressions in V1 four days after ONT and ER [as per Figure 4-1(a)]. Coronal brain section and insets show a higher intensity of PSA staining and fewer PV neurons wrapped by PNNs (arrows) in the left V1. *(b)* Quantitative analysis of WFA-labeled PNN (i), PV-PNN double-labeled neurons (ii), and the percentage of PV^+ cells surrounded by PNN (iii) in the right and left V1 on days 1, 4 and 7 after ER and ONT. Asterisk (*) denotes significant differences between left and right V1, where *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

(a)



(b)

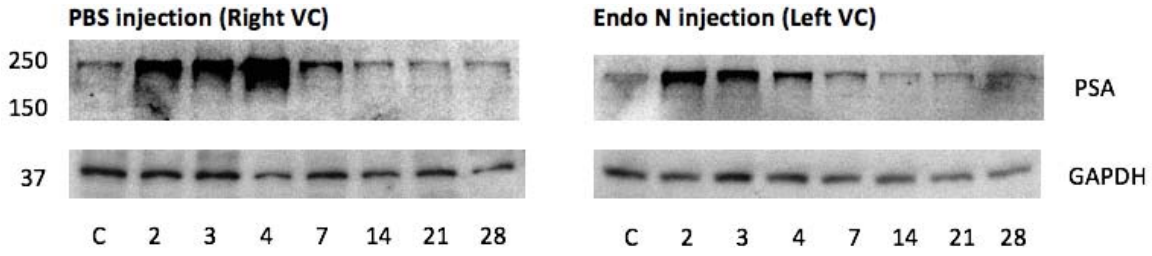


4.4.3 Removal of PSA reduces levels of NCAM and BDNF after ER and ONT

To determine whether high levels of PSA play a role in the reactivation of visual cortical plasticity in adulthood, I selectively removed PSA from NCAM using bilateral Endo N intracranial injections into the VC a week before ER and ONT (day 53) (Figure 4-1b). PSA is barely detectable in the VC after the 4th postnatal week (Di Cristo et al., 2007), but single injections of vehicle (PBS) or Endo N into the young adult VC induced an early injury response, characterized by an increase in PSA expression that appeared in the first 4 days (Figure 4-5); Endo N effectively removes PSA from the VC by 3 days after injection, an effect that has been shown to persist for at least 3 weeks (Ono et al., 1994). Upon removal of PSA from NCAM, I compared the response of BDNF expression between Endo N and PBS treatment on the left VC following ER and ONT; my results focused on the VC contralateral to the reopened eye (Figure 4-6a). Western blotting detected elevated levels of BDNF in the PBS – injected VC that had begun on day 3, and levels were mostly sustained until the end of the observation period (Figure 4-6b-iii). Enhanced expression of PSA was found a day after ER and ONT, and levels were substantially increased on days 10 and 14 (Figure 4-6b-i). Similarly, greater levels of the NCAM140 isoform were detected on day 14 following ER and ONT (Figure 4-6b-ii). Conversely, in the Endo N – injected left VC, I observed neither an increase in BDNF nor a raise in NCAM140 isoform protein levels.

Figure 4-5. Endo N injection reduces PSA levels in V1. Endo N and PBS were injected into the left and right V1, respectively, of previously unoperated adult animals. (a) Immunoblot shows PSA expression in the right and left VC on day(s) 2, 3, 4, 7, 14, 21 and 28 after PBS or Endo N injection. Higher levels of PSA were expressed on days 3 and 4 in the right V1. (b) Quantitative analysis of western blot results; asterisk (*) denotes significantly different between left and right V1, where $p < 0.05$.

(a)



(b)

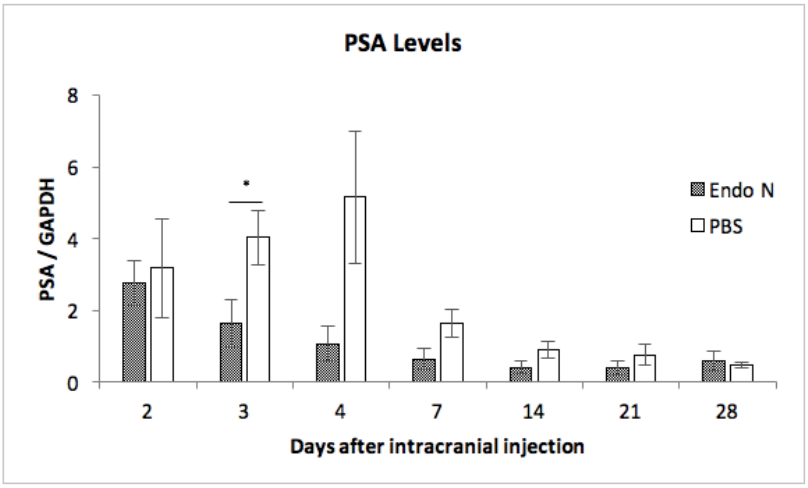
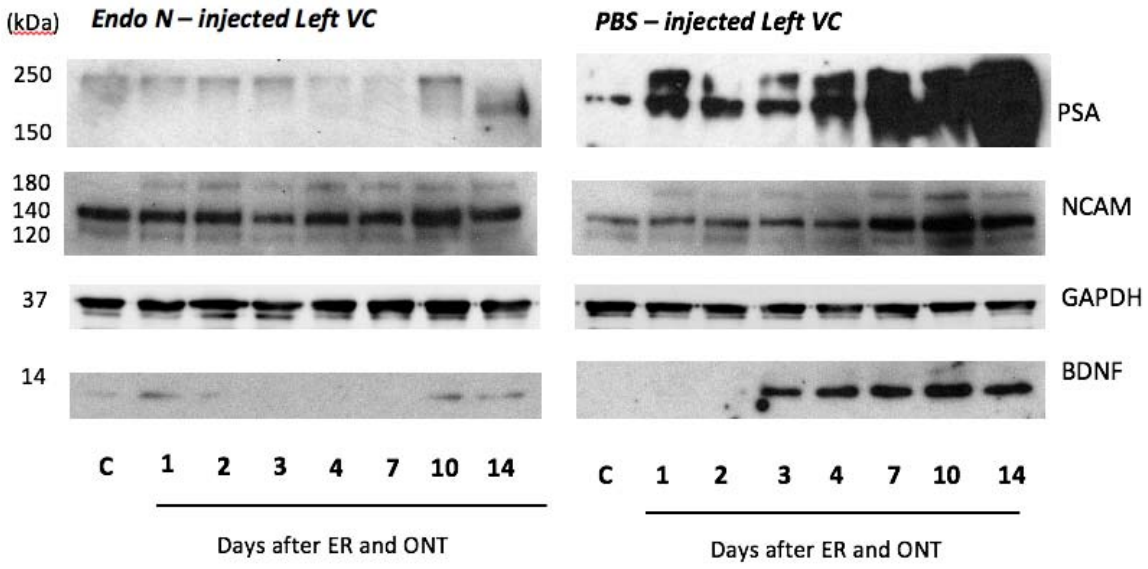


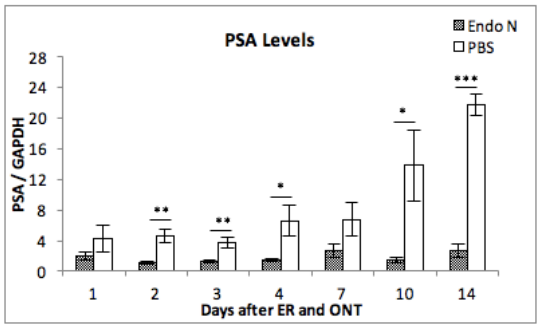
Figure 4-6. (a) Immunoblot analysis of the left VC shows that removal of PSA reduces NCAM and BDNF levels in the visual cortex. Monocularly deprived animals at age PND53 received bilateral VC injection [as per Figure 4-1(b)] of either Endo N or vehicle (PBS). Mice were sacrificed 1, 2, 3, 4, 7, 10 and 14 days after ER and ONT. Sham operated Endo N or PBS-injected animals without ER and ONT served as control *(C)*. (b) Quantitative analysis of western blot results for PSA (i), NCAM isoforms (ii) and BDNF (iii). Asterisk (*) denotes the significant different between Endo N – and PBS – injected V1, where *p < 0.05, **p < 0.01, ***p < 0.001.

(a)

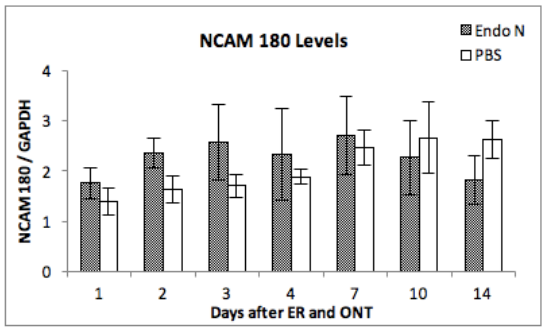


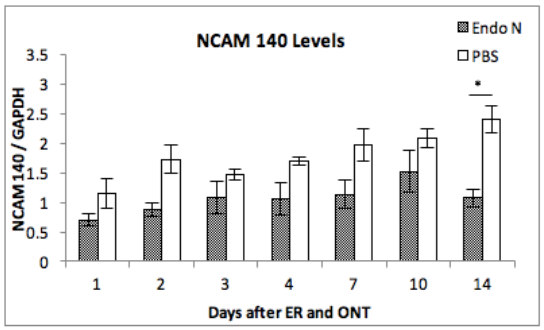
(b)

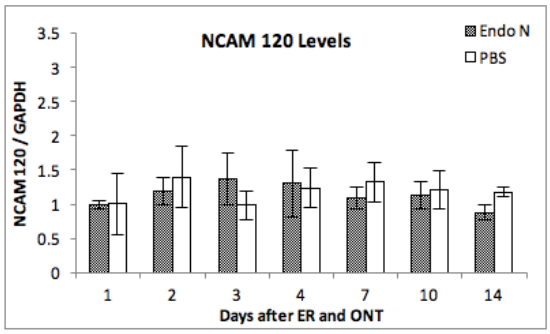
(i)


PSA Levels
Endo N
PBS
PSA / GAPDH
28
24
20
16
12
8
4
0
**
**
*
*
***
1
2
3
4
7
10
14
Days after ER and ONT

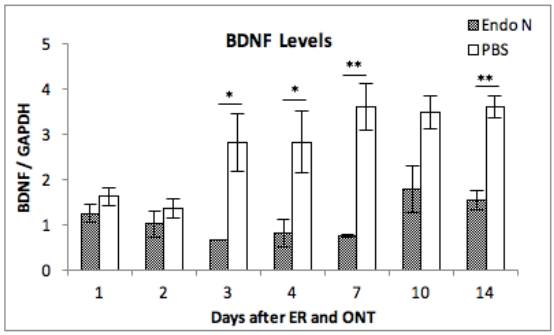
(ii)


NCAM 180 Levels
Endo N
PBS
NCAM180 / GAPDH
4
3
2
1
0
1
2
3
4
7
10
14
Days after ER and ONT


NCAM 140 Levels
Endo N
PBS
NCAM140 / GAPDH
3.5
3
2.5
2
1.5
1
0.5
0
*
1
2
3
4
7
10
14
Days after ER and ONT


NCAM 120 Levels
Endo N
PBS
NCAM120 / GAPDH
3.5
3
2.5
2
1.5
1
0.5
0
1
2
3
4
7
10
14
Days after ER and ONT

(iii)


BDNF Levels
Endo N
PBS
BDNF / GAPDH
5
4
3
2
1
0
*
*
**
**
1
2
3
4
7
10
14
Days after ER and ONT

4.4.4. Removal of PSA inhibits reactivation of visual cortical plasticity

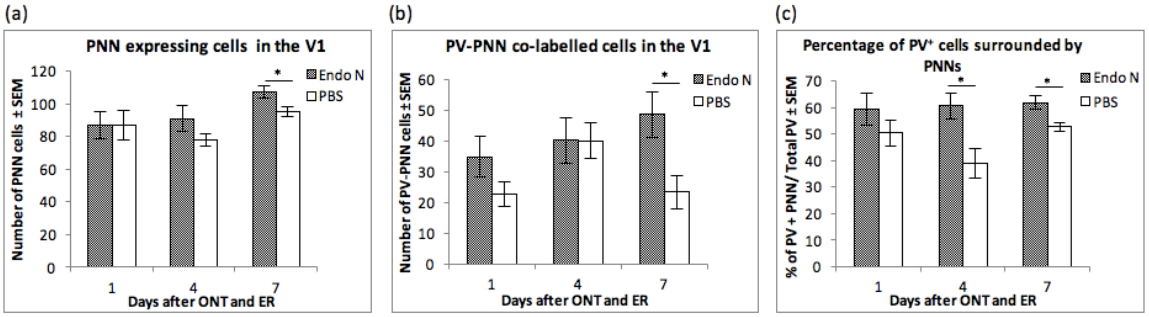
To evaluate whether PSA is required for the reactivation of visual cortical plasticity, I removed PSA from NCAM by Endo N injection into V1 one week prior to ER and ONT, then compared the number of PNNs as well as the number and the percentage of PV-PNN expressing cells in PBS – or in Endo N – injected V1 on days 1, 4 and 7 after ER and ONT. The left V1 of PBS – injected animals had a significantly smaller number of PNNs (Figure 4-7a) on day 7 [$t_8 = 2.81$, $p < 0.05$], fewer PV-PNN co-labelled cells (Figure 4-7b) on day 7 [$t_8 = 2.74$, $p < 0.05$], and a lower percentage of PV neurons surrounded by PNNs (Figure 4-7c) on days 4 [$t_8 = 2.95$, $p < 0.05$] and 7 [$t_8 = 2.97$, $p < 0.05$]. These findings indicate impaired reactivation of visual cortical plasticity when PSA is removed prior to ER and ONT.

4.4.5 Removal of PSA affects recovery of visual ability

To determine the effect of PSA on the recovery of vision, I examined the animals' visual ability 24 days before and one week after ER and ONT. There was no difference in swimming speed between PBS – and Endo N – injected animals [$F(1, 8) = 1.549$, ns] after ER and ONT, indicating that the absence of PSA does not impair the motor skills of mice (Figure 4-8). Recovery of vision was assessed using the computer – based visual behavior test, consisting of visual detection and visual acuity tasks.

In the visual detection task, mice were required to distinguish between a vertical grating and a grey screen. Before intracranial injection, both groups of mice were able to reach

Figure 4-7. The removal of PSA inhibited reactivation of visual cortical plasticity**.** Quantitative analysis of (a) PNN, (b) PNN-PV double-labeled neurons, and (c) percentage of PV^+ cells surrounded by PNNs in Endo N /PBS – injected V1 on days 1, 4 and 7 after ONT and ER [as per Figure 4-1(b)]. Asterisk (*) denotes significantly different between Endo N – and PBS – injected V1, where $p < 0.05$.



the 70% criterion on days 4 and 5 respectively [$F(1, 8) = 1.732$, ns], and improved their accuracy [$F(7, 56) = 14.78$, $P < 0.001$] with over 90% correct on day 8 (Figure 4-9a). After the initial assessment of their visual ability, animals underwent a PBS or Endo N bilateral intracranial injection on day 53, followed by ER and ONT on day 60 (as per Figure 4-1(c)). Their visual capacity was examined again on days 67-75, a week after the last surgery (Figure 4-9b). The PBS-injected mice reached the 70% criterion on day 2 of testing, whereas it took an extra two days of testing for the Endo N injected animals to reach the 70% criterion. Both groups showed improvement of accuracy [$F(7, 56) = 3.115$, $P < 0.008$] over the 8 days of testing, but the PBS – injected mice performed better than the Endo N – injected group [$F(1, 8) = 9.391$, $P < 0.016$]. Performance on day 8 was used to evaluate the mice's visual detection ability after 7 days of testing (Wong and Brown, 2006). Whereas before ER and ONT there was no difference between these two groups of mice, the PBS – injected animals significantly outperformed the Endo N – injected mice on day 8 ($p < 0.014$), showing over 90% correct compared with the Endo N – injected animals who barely achieved the 70% criterion.

Immediately after 8 days of testing in the visual detection task, animals were required to distinguish between a vertical grating with different spatial frequencies and a gray screen in the visual acuity test. Before intracranial injection (Figure 4-9c), there was no significant difference between these 2 groups of mice [$F(1, 8) = 0.714$, ns]. All animals were able to reach the 70% criterion across the range of spatial frequencies tested, and had a similar visual acuity threshold, 0.64 cycle /degree (cyc/deg). Fifteen days after ER and ONT, I examined their visual acuity again: PBS – injected animals maintained their

Figure 4-8. Motor skills evaluation, assessed after 7 days of training, shows no difference in the time to reach the target (latency time) between PBS – or Endo N – injected mice. Animals in the Endo N – injected group took a comparatively longer time to learn the task in the early pre-injection test period. Points represent means ± SEM. Asterisk (*) denotes significantly different between PBS – and Endo N – injected mice, where $p < 0.05$.

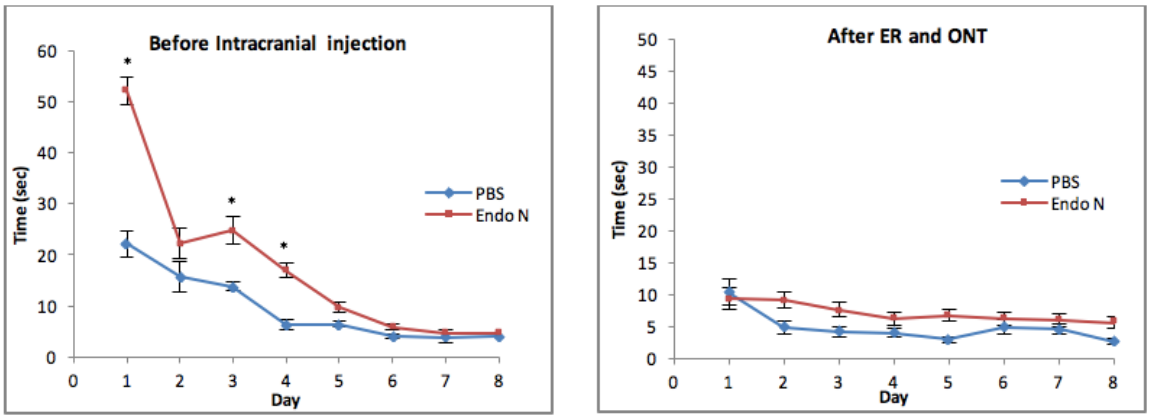
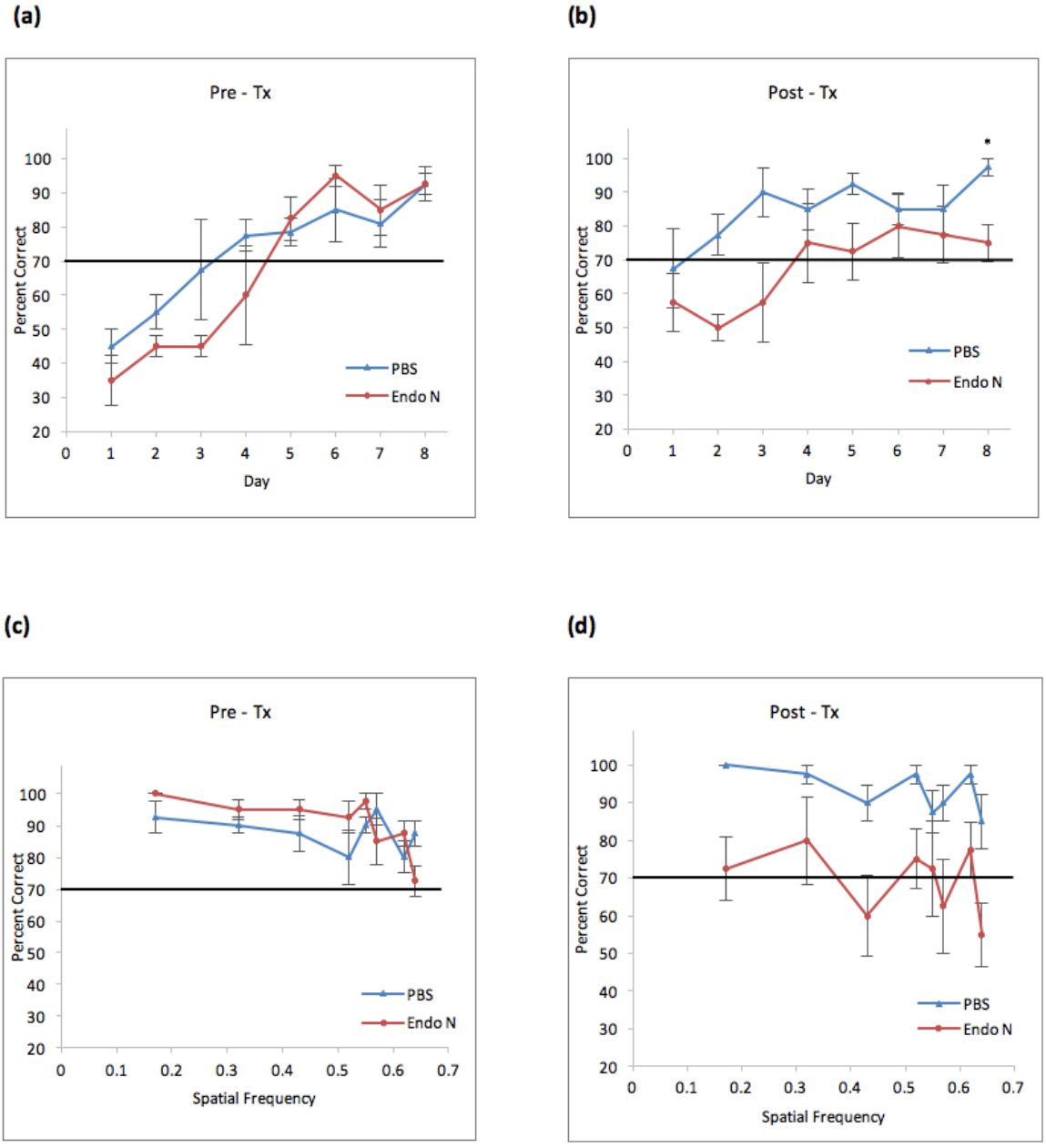


Figure 4-9. The removal of PSA impairs visual acuity recovery after ER and ONT [as per Figure 4-1(c)]. The mean "percent correct" for each group [PBS (blue) – and Endo N (red) – injected animals] is presented for each task. *(a & b)* Performances on day 8 of testing, used to compare treatment groups on the visual detection task, show Endo N – injected mice had a significantly lower percentage of correct responses after ER and ONT. *(c & d)* Mice injected with Endo N also showed poor performance on the visual acuity task after treatment. Pre – Tx = Before intracranial injection; Post – Tx = After ER and ONT.



ability to discriminate spatial frequencies (up to 0.64 cyc/deg), whereas Endo N – injected mice's performance was poor, having their visual acuity threshold significantly reduced to 0.32 cyc/deg [$F(1, 8) = 12.105$, $P < 0.009$] (Figure 4-9d).

4.5 Discussion

4.5.1 Summary of results

In this study, I investigated the role of PSA in the recovery of vision following chronic MD in the adult mouse visual system. My results, after ER and ONT, show high levels of PSA and BDNF in the contralateral VC, accompanied by reduced numbers and percentage of PV-PNN co-labelled cells. Enzymatic removal of PSA in the VC reduces expression of BDNF, and decreases degradation of PNNs, implicating attenuated visual cortical plasticity. In addition, visual ability testing shows that removing PSA from NCAM impairs both visual detection and visual acuity. Taken together, my findings demonstrate that the presence of PSA is required for neural plasticity and functional recovery of vision in adult mice after long-term MD.

4.5.2 PSA-NCAM in neuroplasticity

The PSA moiety of NCAM is expressed abundantly throughout the brain, influencing a wide range of neural cell migration and differentiation during embryonic and early postnatal periods. However, the expression of polysialylated NCAM is restricted to a limited number of regions in the adult brain, including the olfactory bulb and hippocampus, that are associated with ongoing neurogenesis and plasticity in adulthood (Seki and Arai, 1993). In the VC, developmental PSA levels are governed by the

ST8SiaII gene, one of the polysialyltransferase enzymes capable of synthesizing PSA in a sensory experience – dependent manner to control the timing of critical period plasticity and maturation of GABAergic synapses (Belanger and Di Cristo, 2011; Di Cristo et al., 2007).

How does PSA-NCAM regulate neuroplasticity? NCAM and PSA-NCAM may act antagonistically through a dynamic balance between the stability and plasticity of synaptic contacts (Aonurm-Helm et al., 2016). NCAM promotes signal transduction through stabilization of synaptic contacts, whereas the negatively charged PSA chains of NCAM are considered as spacers, reducing adhesion force between cells so as to destabilize contacts and thereby regulate cell interactions and structural modifications (Bruses and Rutishauser, 2001; Rutishauser and Landmesser, 1996). The over-expression of NCAM-140, a finding of note in my VC results after ER and ONT (Figures 4-2 and 4-4), has been implicated in encouraging adhesion and neurite outgrowth of previously deprived VC neurons (Ditlevsen and Kolkova, 2010; Ditlevsen et al., 2008). PSA-NCAM mediates change in cortical reorganization by promoting synaptic plasticity, remodeling dendritic and axonal arbors, and recruiting new neurons to functional networks (Gascon et al., 2007b). For instance, PSA-NCAM is necessary for the structural remodeling of synaptic connections that are associated with long-term potentiation in memory acquisition and consolidation (Senkov et al., 2006), governing appropriate target muscle reinnervation after peripheral axon damage in motor neuron regeneration (Franz et al., 2005), and promoting neurogenesis, migration, and survival of the newly generated neurons in the subventricular zone of the lateral ventricle and the subgranular zone of

dentate gyrus in the hippocampus (Ming and Song, 2005; Ono et al., 1994; Vutskits et al., 2006). My finding that PSA is necessary for visual cortical plasticity and functional recovery after long-term MD is consistent with findings from these previous studies in other area of the nervous system.

4.5.3 The classical models of VC plasticity

My current study shows that PSA-NCAM promotes VC plasticity and functional recovery after long-term MD. These results appear to contrast previous findings where Fluoxetine, an antidepressant that restores plasticity in the VC (Maya Vetencourt et al., 2008), was used by Guirado and his colleagues, who reported that the reactivation of OD plasticity in short-term MD was independent of PSA (Guirado et al., 2016). However, short-term MD induces only an OD shift of visual cortical neurons, whereas long-term MD used in my experiments involves additional steps such as structural remodeling that tends to be more persistent (Krahe et al., 2005). Furthermore, the molecular mechanisms that underlie the OD shift in response to the initial loss of the deprived eye after MD seem to be distinct from the recovery of the deprived eye responses following reverse MD. For example, sleep and CREB activity are necessary for OD plasticity, but not for recovery after MD (Dadvand et al., 2006; Frank et al., 2001; Mower et al., 2002); in contrast, tissue plasminogen activator is not required for MD-induced plasticity, but is essential for the structural remodeling of axons (Muller and Griesinger, 1998).

4.5.4 Animal Models of amblyopia

Visual experience is a potent force that shapes neural circuit connectivity and function, and the experimental manipulation of vision during the critical period of the early postnatal life has led to a growing understanding of the underlying mechanisms of experience-dependent neuronal plasticity. Different experimental approaches have been used to study changes in visual plasticity and the restoration of visual function: the commonly used visual deprivation paradigms where animals are monocularly deprived by enucleation alone without further treatment (reviewed by Nys et al., 2015), or eyelid suturing (Wiesel and Hubel, 1963), some of which can be followed by ER that allows binocular recovery (Wiesel and Hubel, 1965b), or forcing the animal to use the originally deprived eye by reverse lid-suture, enucleation, or ONT (Movshon, 1976; Smith, 1981; Tognini et al., 2012; Wiesel and Hubel, 1965a). Both monocular enucleation and ONT are drastic approaches, resulting in irreversible vision loss that profoundly affects contralateral retinorecipient subcortical and cortical structures. These models are also particularly sensitive to the identification of candidate plasticity genes (Majdan and Shatz, 2006; Nys et al., 2015; Toldi et al., 1996). Depending on the age of surgical intervention and the duration of MD (Lehmann and Lowel, 2008; Nys et al., 2015), these strategies affect different degrees and/or patterns of cortical activity, and result in different rates of visual recovery (Antonini et al., 1999; Maffei and Turrigiano, 2008; Mitchell et al., 2001). Besides experimental paradigms, the selection of testing modalities as well as the choice of animal model may yield diverse results. For example, there are discrepancies between visual acuity assessed by electrophysiology and behavioral techniques following chronic MD, and the data obtained from optical imaging of intrinsic

signals or single-unit recordings reflect functional changes in cortical activity, independent of mRNA translation or protein synthesis (Fischer et al., 2007a; Krahe et al., 2005; Tinelli et al., 2008). The rate and extent of vision restoration also appears more pronounced in rodents than in other *mammalian* species (Blakemore et al., 1978; Blakemore and Van Sluyters, 1974; Fischer et al., 2007b; Hubel and Wiesel, 1970).

4.5.5. Restoration of visual function and plasticity

In this study, I designed an experimental approach to investigate whether a particular molecular candidate, PSA-NCAM, is linked to visual recovery in the adult mouse. I observed a dramatic recovery of visual acuity in PBS – injected control animals after long-term MD: restoration of vision occurred rapidly, within the first 3 weeks following ER and ONT. This observation was in line with previous studies that found that permanent removal of inputs from the non-deprived eye were associated with more rapid recovery and higher visual acuity in cats and mice after long-term MD (Drager, 1978; Smith, 1981). It is possible that once ONT lowers the threshold for synaptic potentiation, subsequent visual experience promotes synaptic strengthening and increases responsiveness in the VC (Abraham and Bear, 1996; Kirkwood et al., 1996; Kuo and Dringenberg, 2009; Philpot et al., 2007). This idea was also supported by a recent study that temporarily blocks all impulse activities in the optic nerve, promoting rapid recovery of function from MD-driven visual impairments (Fong et al., 2016).

Previous animal studies have shown that it is possible to reopen certain levels of plasticity in the VC after the end of a critical period, albeit to a limited extent in humans.

Until now, there has been no intervention that can overcome this barrier in adults (Hensch and Bilimoria, 2012; Sengpiel, 2014). OD plasticity is driven by E/I balance, and any disruption of the balance alters timing of the critical period; for instance, the removal of PSA or genetic disruption of GABA synthesis alters the onset of OD plasticity (Di Cristo et al., 2007; Fagiolini et al., 2004; Hensch, 2005). In light of this, strategies have been developed to influence E/I balance by manipulating molecules and identifying pharmacological and non-invasive interventions underlying reactivation of OD plasticity in adult life. Previous studies using animal models of amblyopia have shown significant recovery of vision in the deprived eye through exercise, caloric restriction, environmental enrichment, dark exposure, and chronic fluoxetine treatment (Duffy and Mitchell, 2013; Kalogeraki et al., 2014; Maya Vetencourt et al., 2008; Sale et al., 2007; Spolidoro et al., 2011). Together, these findings and my own results provide hope that similarly effective therapeutic strategies in humans may some day be implemented (Li et al., 2011; Thompson et al., 2008).

4.6 Conclusions

Using visual deprivation in mice as a model, I have demonstrated that PSA is a crucial factor in visual cortical plasticity and functional recovery after long term monocular deprivation in adults. These findings advance our understanding of PSA-NCAM's involvement in experience-dependent cortical plasticity, and demonstrate that it is possible to reopen OD plasticity and promote functional recovery in the adult brain.

Chapter 5: DISCUSSION

5.1 Summary of Thesis

There are numerous anatomical and physiological changes that occur in our visual system with age, and these modulations usually progress over time. With increasing age, vision deterioration may be accelerated by degenerative diseases of the eye, such as glaucoma, diabetic retinopathy and macular degeneration, all of which can lead to irreversible blindness. Consistent with this line of thought in other areas of the CNS, characteristics of normal aging include decline in memory and learning in the setting of widespread neuronal loss. These age-related changes are associated with the progressive reduction of neuronal networks, as well as a reduction in structural and functional plasticity in the senescent brain (Dorszewska, 2013). NCAM, as well as PSA-NCAM, regulate neuronal and synaptic plasticity in the adult nervous system (Hammond et al., 2006; Kochlamazashvili et al., 2010; Parkash and Kaur, 2007; Vaithianathan et al., 2004). Removal of PSA inhibits spatial and reversal learning ability, while ablation of NCAM impairs spatial learning and memory performance (Becker et al., 1996; Cremer et al., 1994; Markram et al., 2007; Ronn et al., 1998). Furthermore, previous studies from our laboratory have shown a significant effect of the germline deletion of NCAM on the numbers of RGCs in young adult animals (Murphy et al., 2007a). With this background of research demonstrating NCAM's pivotal role in modulating cognitive processes in the adult brain, as well as NCAM's more specific role in influencing RGC survival, I have asked the following question: does NCAM play any functional role (and, if so, how) in the visual system during normal aging?

To begin to answer this question, I used behavioral, functional and anatomical analyses to determine how NCAM deficiency affects vision in mice from young adults to senescent animals (Chapter 2). My results showed that NCAM-deficient mice have impaired pattern discrimination ability. Despite the fact that young adult NCAM −/− animals have higher retinal activity, more RGCs and thicker inner retinal layers, ON axon counts showed that their total numbers remained the same as the WT. As the animals age, PSA levels were higher in the VC, but lower in the retina. Compared with WT, NCAM −/− mice have fewer RGCs and photoreceptor layers, and they also exhibit premature vision loss associated with dramatic drops in retinal activity amplitudes and thinning of the retinas.

These observations lead to further investigations into the potential mechanisms of how NCAM affects vision during aging. To address this question, I focused on two indispensable processing areas in the visual system: the retina (Chapter 3) and V1 (Chapter 4).

In experiments involving the retina (Chapter 3), I examined NCAM's role in LIRD, that has been used as an animal model to mimic the clinical pathology of human age-related retinal degeneration. My results in WT retinas show that the level of PSA increases immediately after light induced retinal alteration. In the absence of NCAM, elevated levels of $p75^{NTR}$ and reduced ERG amplitudes are detected in the retina. NCAM deficient mice, as well as mice treated with Endo-N, exhibit earlier onset of retinal cell death. Furthermore, blockade of $p75^{NTR}$ in Endo-N treated WT and NCAM −/− mice alters

retinal apoptosis, suggesting that NCAM protects WT retinas from LIRD by modulating the detrimental effect of $p75^{NTR}$.

In Chapter 4, I examined the influence of PSA-NCAM on neuroplasticity in the adult animal at the level of the VC. My results show that elevated levels of PSA and BDNF, associated with increased degradation of PNNs, are seen in the VC contralateral to the reopened eye. The removal of PSA in the VC reduces BDNF expression, decreases PNNs degradation, and impairs recovery of visual acuity after ER and ONT. Collectively, I have shown that PSA-NCAM is required for the reactivation of visual cortical plasticity and the recovery of vision in adult mice following chronic MD.

Taken together, these results strongly implicate a pivotal role of NCAM in neuronal survival and synaptic plasticity in the visual system during aging. In this chapter, I will discuss the possible mechanisms of how NCAM protects neurons from light-induced apoptosis, and promotes neuroplasticity from MD-induced vision loss. I will then speculate on possible future treatments by means of synthetic peptide ligands of PSA and NCAM, that could be used to promote structural and functional integrity of neural circuits in aging. To begin, I will address the importance of NCAM on retinal survival and visual cortical plasticity, with particular emphasis on its influence during aging.

5.2 Role of NCAM in Retinal Survival: Highlight concerning RGCs and Photoreceptors

In nervous system development, an overproduction of neurons is generated; consistent with this, a surplus of RGCs is produced during development. Programmed cell death (PCD) is an essential adaptive process that provides cell number homeostasis (Buss et al., 2006). Using a PSA gain-of-function approach, a high number of TUNEL – positive cells were observed in the E8-E12 outer retina of chick whereas PCD is normally undetected at this stage of development (Canger and Rutishauser, 2004). These findings indicate that the over-expression of PSA leads to an increase in apoptosis in the retina and may also result in RGCs being found in ectopic locations. Studies from our laboratory have shown that PSA is essential for supporting RGC survival in the neonatal retina; significant RGC death is observed when PSA is removed from the surface of neonatal RGCs *in vitro* (Murphy et al., 2009).

Besides supporting RGC survival during development, the presence of NCAM also plays a vital role in maintaining normal health and determining the number of RGCs in adulthood. In 2-5 month old NCAM −/− mice, retinas have more than the normal amount of RGCs (Lobanovskaya et al., 2015a; Luke et al., 2016a; Murphy et al., 2007a), an effect that can be understood in the context of altered PCD during development as discussed above. When the animals reached 18 months old, WT retinas show no difference in RGC densities, but exhibit significantly decreased RGC axon numbers. These results are consistent with previous studies, suggesting that RGC axons are highly vulnerable to age-related loss, whereas the number of RGC bodies across mammalian species has been

reported to show minimal decline with age (Buckingham et al., 2008; Harman and Moore, 1999; Kim et al., 1996; Samuel et al., 2011). Conversely, I observed excessive loss of RGCs in the 18-month old NCAM −/− retinas that I think can be explained by the absence of NCAM making the aging RGCs more susceptible to cell death. Such speculation is supported by previous studies from our laboratory showing that the onset of RGC death in NCAM −/− mice is earlier after ON injury, and the RGC densities are reduced by one-fourth 14 days after PSA removal from the retina (Murphy et al., 2007a; Murphy et al., 2009). In addition, the Zharkovsky group showed that the administration of Endo N significantly enhanced the toxicity of kainic acid to WT RGCs (Lobanovskaya et al., 2015a). Furthermore, the absence of ST8SiaII or ST8SiaIV in mature mice reduces the expression of PSA and the number of RGCs in sialyltransferase −/− retinas (Lobanovskaya et al., 2015a). Taken together, my findings coupled with those of other laboratories, indicate that PSA and NCAM promote the survival of RGCs in adult retinas, while RGCs in NCAM −/− mice are more prone to cell death as the mice age.

Unlike RGC bodies, photoreceptor neurons are highly susceptible to age-related loss. In rodents, there is a significant decline in rod and cone cells over 1-2.5 years of age; and in humans, 20-30% of photoreceptors are lost in normal aging (Cunea and Jeffery, 2007; Cunea et al., 2014; Curcio et al., 1993; Panda-Jonas et al., 1995). Retinal shape changes and neuronal density decreases are seen with aging, and such changes are mirrored by thinning of the retinal layers (Samuel et al., 2011). The loss of rods, which comprise 97% of photoreceptor cells, are also accompanied by the decline of a- and b- wave ERG amplitudes, and 2-fold reduction of visual acuity and spatial contrast sensitivity (Jeon et

al., 1998; Kolesnikov et al., 2010). Indeed, these findings are in line with my results obtained from the WT mice, as shown in Figure 2-6 in Chapter 2 and Figure 3-5 in Chapter 3. Furthermore, during aging, NCAM −/− animals display excessive thinning of the ONL that is associated with a dramatic drop of ERG amplitudes and early onset of visual acuity loss. The excessive reduction in ERG amplitudes was also found in adult NCAM −/− mice after light-induced retinal damage. Collectively, these findings support the idea that NCAM also protects the retina against age and light- induced photoreceptor degeneration.

5.3 NCAM and Neurotrophic Factors in Neuronal Survival

BDNF plays important roles in promoting brain development and enhancing the survival of adult neurons, and PSA is involved in many cellular events associated with BDNF. Hence, impaired PSA-BDNF signaling influences the survival, growth, and normal functioning of CNS neurons. An early report observed that the removal of PSA reduces BDNF-mediated cell survival and differentiation in cortical cell cultures, an effect that can be reversed by adding exogenous BDNF into the culture medium and suggesting a direct association between PSA and BDNF (Vutskits et al., 2001). Deficient LTP was found in hippocampal organotypic slice cultures treated with Endo N, and when similar slices were prepared from NCAM −/− mice (Muller et al., 2000). Again, this effect can be reversed by the addition of BDNF. The authors from these 2 studies have proposed that PSA presents BDNF to TrkB in the receptor complex, thus concentrating neurotrophin nearby for more effective BDNF – induced cell signaling. In this way, PSA can promote survival and differentiation of cortical neurons and regulate activity-

dependent synaptic plasticity (Muller et al., 2000; Vutskits et al., 2001). Using native-PAGE and gel filtration chromatography (*in vitro* analysis), BDNF dimers bind directly to the long polymerized chains of PSA to form a large complex that then interacts with TrkB to facilitate the growth and survival of neuroblastoma cells (Kanato et al., 2008). Furthermore, physical interaction occurs between the intracellular domains of TrkB and NCAM, resulting in NCAM phosphorylation and NCAM dependent neurite outgrowth (Cassens et al., 2010). These direct molecular interactions suggest that they work in concert to promote neuronal growth and survival, normal neuronal activity, and proper functioning of the CNS.

My experimental findings described in Chapter 3 demonstrate that the blockade of $p75^{NTR}$ delays apoptosis in mice after LIRD and suggest that PSA influences $p75^{NTR}$ signaling to facilitate the survival of retinal neurons. This concept is supported by previous work where PSA was removed from cultured subventricular zone (SVZ)-derived neurons, that are entirely devoid of TrkA expression (Gascon et al., 2005). Under these conditions, where NGF's effect is mediated only by $p75^{NTR}$-induced signaling, the addition of NGF induces higher levels of cell death (Gascon et al., 2007a). In contrast, following removal of PSA, pharmacological blockade of $p75^{NTR}$ signaling promotes the survival of SVZ-derived neural precursor cells (Gascon et al., 2005). $p75^{NTR}$ induces neuronal cell death through ceramide and c-Jun N-terminal kinase (JNK) pro-apoptotic signaling pathways (Casaccia-Bonnefil et al., 1996; Dobrowsky et al., 1994); blockade of these 2 pathways prevents neuronal cell death in the absence of PSA (Gascon et al.,

2007a), suggesting that PSA modulates the activation of the $p75^{NTR}$-induced apoptotic signaling cascade.

5.4 Role of NCAM in Visual Cortical Plasticity

There is limited research investigating the effect of NCAM in VC development. One study examined the developmental expression pattern of NCAM-180, that is solely seen in neurons and is highly associated with the formation, modification, and stabilization of synaptic contacts in the VC (Delius et al., 1997). In this study, immunoblot experiments revealed age-dependent increases in NCAM-180 levels, and immunohistochemistry showed developmental changes of positive signals in neuropils and neuronal cell bodies, together suggesting that age-dependent modifications of NCAM-180 expression regulates the activity of synaptic contacts during development. Besides protein expression patterns, other reports have concentrated primarily on studying PSA and NCAM effects and mechanisms of action in visual cortical plasticity during development. As described above, the study conducted by Di Cristo and colleagues is perhaps the most intriguing, showing that PSA levels in the mouse VC decline around the time of eye opening, regulate the timing of GABAergic development, and influence the onset of OD plasticity (Di Cristo et al., 2007). The interplay between PSA and GABAergic neurons may be critical to understanding PSA's effects on development. GABAergic interneurons form perisomatic synapses and are necessary for the regulation of neural networks. Furthermore, and as mentioned previously, GABAergic neurons shape functional maturation of the cortex during development, and alterations in GABAergic signaling is associated with delays in synaptic and cellular maturation (Le Magueresse and Monyer,

2013). If the removal of PSA is necessary for the onset of a critical period, what is the role of NCAM in visual cortical plasticity? Using a single cell-gene knockout technique to remove NCAM from individual basket interneurons at different developmental stages, Chattopadhyaya et al. found that NCAM isoforms -120 and -140 promoted basket cell axonal branching and bouton formation during the time perisomatic synapses formed through the Fyn kinase signaling pathway (Chattopadhyaya et al., 2013). PSA and NCAM work in concert to stabilize and promote the maturation of synaptic contacts. It is likely, therefore, that PSA regulates cellular and synaptic plasticity during the early stages of VC development and during the time of eye opening. Decreasing the expression of PSA consolidates NCAM – NCAM interactions; NCAM-180 modifies and stabilizes synaptic connections; and NCAM-140 and -120 promote axonal branching and bouton formation of the GABAergic neurons (basket cells).

The identification of cellular and molecular mechanisms of brain plasticity is critical to understanding the specific determinants of normal development; in addition, it is foundational to developing strategies to enhance both the repair of injured nervous tissue and restoration of function later in life. The maturation of intracortical inhibitory circuitry causes the end of OD plasticity and the closure of the critical period. However, studies in other animal models have shown that it is possible to restore plasticity in adult life by reducing the levels of intracortical inhibition (Harauzov et al., 2010). In rodents, a number of experimental paradigms have been used to modulate GABAergic inhibition in adulthood, such as environmental enrichment (Baroncelli et al., 2010; Sale et al., 2007), dark exposure (He et al., 2006; He et al., 2007), food restriction (Spolidoro et al., 2011),

genetic manipulations (reviewed by Bavelier et al., 2010), IGF-1 administration (Maya-Vetencourt et al., 2012), and long-term Fluoxetine treatment (Maya Vetencourt et al., 2008). Of interest, Prozac is the common trade name of Fluoxetine, that is an antidepressant of the selective serotonin reuptake inhibitor class, and is widely prescribed for the treatment of major depressive diseases and anxiety disorders. Chronic fluoxetine treatment induces juvenile plasticity through the alteration in GABA-mediated neurotransmission and the modification of E/I balance (Guirado et al., 2014). The exact mechanism of how Fluoxetine affects GABAergic synapses and reactivates OD plasticity in adulthood remains elusive, but the administration of the antidepressant increases expression of factors typically seen in plastic changes, including extracellular serotonin (Castren, 2005), PSA, BDNF and TrkB (Saarelainen et al., 2003; Varea et al., 2007), and promotes neurogenesis and synaptogenesis in the adult hippocampus (Hajszan et al., 2005; Malberg et al., 2000). It is well recognized that BDNF signaling and serotonergic systems are important molecular pathways that can act synergistically in the treatment of patients with mood disorders (reviewed by Martinowich and Lu, 2008). In addition, patients with depression have lower levels of BDNF and altered synaptic plasticity (Dowlatshahi et al., 1998).

How are BDNF-TrkB signaling and serotonin related to PSA-NCAM? First and foremost, PSA and NCAM physically interact with BDNF and TrkB receptors respectively (Cassens et al., 2010; Kanato et al., 2008), and the absence of NCAM affects synaptic plasticity and BDNF neurotrophic signaling (Muller et al., 2000). Second, NCAM −/− mice show significant reductions in CaMKIV (Calcium/ calmodulin –

dependent protein kinase IV) and CREB phosphorylation (Aonurm-Helm et al., 2008b), both of which control the transcription of the BDNF gene (Conti et al., 2002; Shieh et al., 1998) to reduce protein expression (Aonurm-Helm et al., 2015). Third, in animal experiments, NCAM deficient mice express lower levels of serotonin in the hippocampus and in frontal cortex (Aonurm-Helm et al., 2015), and show depression-like behavior in tail suspension and sucrose preference tests (Aonurm-Helm et al., 2008a), suggesting that the presence of NCAM facilitates serotonergic transmission in the CNS. In view of all of these findings, together with my experimental results showing reduced levels of BDNF following removal of PSA (Chapter 4), NCAM has a significant influence on BDNF signaling and in the serotonergic system in the CNS. It is also clear, based on my experimental findings, that NCAM is an essential molecule in reinstating neuroplasticity and promoting recovery in the adult visual system.

5.5 The Role of NCAM in Aging

If PSA and NCAM expression are necessary for establishing neuronal circuitry and facilitating plasticity during development and in adulthood, it follows that re-expression of PSA and specific NCAM isoforms may be associated with structural remodeling of neuronal connections and function in the aging brain. Earlier studies have reported that significantly reduced levels of NCAM-180 are observed in the brains (hippocampus) of aged mice and rats (Bahr et al., 1993; Linnemann et al., 1993) and associated with cognitive impairment during early aging (Sandi and Touyarot, 2006). The NCAM -180 isoform is highly enriched in synaptic terminals, where it promotes the stabilization of complex and mature synapses (Polo-Parada et al., 2004). Reduced NCAM-180 levels in

the hippocampus, therefore, might be correlated with the decrease in number and size of perforated synapses in the CA1 stratum radiatum region during normal aging (Nicholson et al., 2004). NCAM is also linked to age-associated neurodegenerative disorders, where altered PSA and NCAM levels have been reported in the hippocampal formation and in cerebrospinal fluid of patients with Alzheimer's disease (Gnanapavan et al., 2010; Mikkonen et al., 1999; Strekalova et al., 2006). Other changes in Alzheimer's disease resulting from altered NCAM expression include NCAM-FGFR interaction and the modulation of synaptic plasticity, thought to influence neuroprotection and cognitive performance in these patients (Enevoldsen et al., 2012). In the peripheral nervous system, and following denervation of muscle fibres, a highly sialylated form of NCAM is re-expressed in regenerating young adult neurites (Franz et al., 2005); in contrast, levels of polysialylated and non-polysialylated NCAM are markedly decreased in elderly mice and rats and are associated with a reduced capacity for neuro-muscular regeneration during aging (Gillon and Sheard, 2015; Olsen et al., 1995). In addition, the age-dependent decline in PSA expression in the dentate gyrus and entorhinal cortex implies that it is linked to reduced structural and functional plasticity of the elderly brain (Fox et al., 1995a; Murray et al., 2016). Along similar lines, visual function declines with age. Consistent with the above findings, I also found the re-expression of PSA in the VC, as well as reduced levels of NCAM-180 in the retinas and in the VC of the 18 months old WT mice (Figure 2-2) (Luke et al., 2016a). Furthermore, in the absence of NCAM, animals have premature loss of vision (Table 2-4), indicating that PSA and NCAM expression are required to maintain normal visual function during aging.

As a biological process, aging in adults is marked by an inevitable decline in physiological and psychological functions that may accompany or exacerbate neurodegenerative diseases, and eventually leads to death. One of the particularly vulnerable characteristics of normal aging is the progressive reduction in cognitive ability, that is associated with an extensive loss of dynamic dendritic spines resulting from the decline in structural and synaptic plasticity in certain brain regions such as the hippocampus and the prefrontal cortex (Burke and Barnes, 2006; Driscoll et al., 2006; Seki and Arai, 1995). LTP and LTD in the hippocampus result from synaptic plasticity, the ability of synapses to change their strength in response to external stimuli, and is mainly regulated by glutamatergic NMDA/AMPA receptors and second messenger pathways (Pozo and Goda, 2010). The CAMs influence synaptic plasticity through the modulation of NMDA receptors, GABAergic system, L-type calcium channels, and mitogen activated protein kinase p38 (Cotman et al., 1998; Dityatev et al., 2008). NCAM, as one of the major CAM molecules, is implicated to play a pivotal role in synaptic plasticity and required to modulate cognitive processes. Ablation of NCAM or removal of PSA impairs LTP formation, which causes cognitive deficits, worsens learning and memory, and is strongly associated with aging (reviewed by Conboy et al., 2010; Kiss and Muller, 2001). This idea is supported by work from the Dityatev group, who observed premature decline in LTP in NCAM −/− hippocampus between 3-12 months, compared with 24 months in WT mice (Kochlamazashvili et al., 2012). This impairment can be restored by increasing the activity of a subtype of NMDA receptors (GluN): blockade of GluN activity in normal aged animals impairs synaptic plasticity and cognitive ability (Clayton et al., 2002), whereas administration of the GluN agonist, D-

cycloserine, rescues the deficits of contextual and cued fear conditioning behavior (behavioral tests that assess the ability of mice to learn and remember) in aging NCAM −/− mice (Kochlamazashvili et al., 2012). Furthermore, altered NCAM expression in glutamatergic neurons of the forebrain during aging is associated with pronounced impairments of working and episodic-like memory, which strongly relies on structural and functional integrity of the prefrontal cortex and the hippocampus (Bisaz et al., 2013). This body of research supports the notion that NCAM expression regulates NMDA receptors and is crucial to maintain healthy cognition in aged animals.

Besides changes in synaptic plasticity, NCAM is also necessary for structural plasticity in the mature CNS. NCAM's role in structural plasticity during development is well-studied and involves cell proliferation, migration, differentiation, axonal/ dendritic fasciculation and growth/ retraction (reviewed by Bonfanti, 2006). PSA is highly expressed in adult neurogenic regions, contributing to the control of adult neurogenesis, a process of generating functional neurons from neural progenitor cells and integrating them into established neuronal circuits. PSA is involved in the migration and maturation of newly generated granule cells in the adult dentate gyrus, a process that depends on structural changes. Consistent with this, several reports have described the reduction of the rate of hippocampal neurogenesis with age and its association with a decrease in PSA expression (Casadesus et al., 2005; Kuhn et al., 1996; Ni Dhuill et al., 1999). Structural plasticity where PSA plays a crucial role is not limited to neurogenic brain regions and is found in non-neurogenic regions where neurite and synaptic remodeling are regulated, and where neuronal connectivity within local cortical circuits is modulated (reviewed by Nacher et

al., 2013). PSA is predominantly expressed in calbindin and somatostatin interneurons, but is also co-expressed with PV in the perisomatic puncta of pyramidal neurons in the adult rat neocortex (Gomez-Climent et al., 2011). Inhibitory interneurons expressing PSA show reduced perisomatic puncta, dendritic arborization and spine density, suggesting that PSA may be involved in the structural remodeling of inhibitory circuits (Gomez-Climent et al., 2011). Interestingly, experimentally reduced levels of PSA are correlated with an increased number of PV expressing perisomatic puncta on pyramidal cells (Castillo-Gomez et al., 2011), implying that PSA depletion activates the maturation of perisomatic inhibitory innervation by basket cells. Similarly, chronic fluoxetine treatment also enhances the degradation of PNNs and increases PSA expression in the adult VC after MD (Maya Vetencourt et al., 2008; Pizzorusso et al., 2002). PNNs are lattice-like extracellular matrix structures, and are widely considered as markers of neuronal stabilization and maturation. Several research studies, looking at various brain regions including the prefrontal cortex, dentate gyrus, visual cortex, and the somatosensory cortex of aged rodents, have reported an increase in the density of PNNs with age (Karetko-Sysa et al., 2014; Tanaka and Mizoguchi, 2009; Yamada and Jinno, 2013). Given that mature PNNs inhibit experience-dependent plasticity, a high number of PNNs may structurally alter the aging brain. Nevertheless, PSA expression is maintained in many regions of the CNS during normal aging, including the substantia nigra, caudate nucleus, and cerebellum of old rats and elderly humans (Murray et al., 2016; Varea et al., 2009) and is, therefore, potentially able to mediate structural plasticity in these brain regions.

5.6 Possible Mechanisms - NCAM in Stress Management

Oxidative stress is the resultant damage that arises due to redox imbalances, that are tightly associated with aging and age-related disorders (Birch-Machin and Bowman, 2016). Aging is highly influenced by genetic and environmental components. Many genetic factors participate in tissue regeneration, maintenance and repair, and modulate the process of aging; however, environmental stimuli that affect cellular macromolecules or interfere with repair processes can accelerate the aging process. Prominent stimuli that increase oxidative stress and induce cell damage include ultraviolet light, environmental pollution, an unhealthy life-style (e.g. smoking), prolonged infection, and chronic inflammation. One of the common theories of aging, the Free Radical Theory, states that the accumulation of free radicals/reactive oxygen species (ROS) over time lead to an increase in mitochondria damage, causing cells to die and organisms to age (Birch-Machin and Bowman, 2016). A free radical is an atom with one or more unpaired electrons, and ROS are chemically reactive radicals containing oxygen (e.g. peroxides, superoxides and hydroxyl radicals). In a biological system, ROS are toxic byproducts generated by normal mitochondrial oxidative metabolism that generates the cell's energy to enable cell signaling and maintenance of homeostasis. Generally, these can be neutralized by antioxidants. However, oxidative stress arises with age due to imbalance between the production of ROS and the ability of the body to counteract the harmful effects. For instance, during times of environmental stress (e.g. excessive light exposure), ROS increases dramatically and becomes a very toxic substance. The brain is especially vulnerable to oxidative stress, as CNS tissue requires a massive amount of oxygen consumption in order to maintain intracellular ion homeostasis. High ROS levels or

oxidative damage induces functional decline and becomes the major contributor to aging and decay in neuroplasticity, as well as to many different neurodegenerative diseases. In light of my findings, I speculate that impaired neural plasticity in NCAM −/− mice prevents them from effectively responding to daily environmental stressors; an aging visual system that is more vulnerable to stress-induced damage has an earlier onset of cell death and functional deterioration.

By virtue of its high metabolic rate, the brain is a target of oxidative stress and a successful outcome of stress is structural remodeling of neural architecture to adapt to new experiences (McEwen et al., 2015). However, the brain also responds to environmental stress (fear, for example). PSA and NCAM are involved in modulating stress exerts in memory function (reviewed by Bisaz et al., 2009). Using different levels of environmental stress (good, tolerable and toxic), the role of NCAM have been investigated in the hippocampus, amygdala, and prefrontal cortex, regions involved with learning and memory. The first experimental evidence in the 1990s, showed that a transient increase in PSA level, together with the formation and remodeling of synapses, were observed in the hippocampus following stressful learning in passive avoidance and water maze tasks (Doyle et al., 1992a; Fox et al., 1995b; Murphy et al., 1996). These findings suggest that stress-induced PSA expression promotes hippocampal synaptic plasticity and memory consolidation (Seki and Arai, 1999). In contrast, if a toxic level of stimuli is given in fear conditioning, hippocampal NCAM polysialyation is significantly reduced, indicating that traumatic fear conditioning suppresses PSA-induced plasticity. These findings support the idea that an optimal level of stress promotes PSA expression

and functionally enhances storage of newly acquired information and that, beyond a certain threshold level of stress, PSA expression is inhibited and retention is impaired (Sandi et al., 2003).

Likewise, NCAM's expression patterns are altered during stressful learning. For example, significant reduction of NCAM-180 is noted in the dentate gyrus a few hours after stressful training (time window for memory consolidation) and is associated with increased expression of ubiquitin C-terminal hydrolase, implying that during the phase of memory consolidation NCAM undergoes controlled proteolysis mediated by the ubiquitin-protein degradation pathway (Foley et al., 2000). In addition, 24 hours after a toxic stress conditioning NCAM expression is induced in the hippocampus, with animals (rats) showing pronounced freezing in memory retention testing (Merino et al., 2000). Furthermore, the effects of changes in NCAM expression on memory formation with different intensities of stress have been examined in pharmacological studies. Administration of NCAM antibodies or synthetic peptides C3d interferes with NCAM binding; both have been shown to impair learning and memory formation (reviewed by Bisaz et al., 2009). Together, these findings support PSA and NCAM involvement in different levels of stress-induced memory consolidation and retention.

Besides studying the influence of stress on NCAM in learning and memory, middle cerebral artery occlusion (MCAO) has been used as a model of ischemic stress in mice. The Tokuyama group showed that MCAO resulted in NCAM-180 degradation, increased NCAM-65 (cleavage product) expression, activated MMP-2/ MMP9 enzymatic activity, and induced cortical neuronal death (Shichi et al., 2011). NCAM is one of the proteolysis

targets of matrix metalloproteinases (MMP-2 and MMP-9), a superfamily of proteolytic enzymes (Hinkle et al., 2006; Hubschmann et al., 2005). The removal of NCAM by siRNA significantly increased neuronal damage following MCAO, whereas the blockade of MMPs decreased infarction size and reduced NCAM-180 fragmentation, suggesting that MMP -2/ MMP-9 amplifies MCAO induced neuronal death by NCAM-180 proteolysis. Similarly, *in vitro* experiments using cultured cortical neurons have demonstrated that exposure to oxidative stress through administration of hydrogen peroxide into the culture medium induces cleavage of NCAM-180 and the production of NCAM-65 via proteolytic activity of MMP-9 (Fujita-Hamabe and Tokuyama, 2012). In fact, the homophilic binding of NCAM-180 is known to activate neuronal survival pathways, including PKA pathway, the protein kinase C (PKC) activating pathway, and the mitogen-activated protein kinase (MAPK) signaling pathways (reviewed by Ditlevsen and Kolkova, 2010; Ditlevsen et al., 2008). Additionally, the heterophilic binding of NCAM-180 and FGF/ GDNF receptors stimulate survival associated signaling cascades (Cassens et al., 2010; Ditlevsen et al., 2008); hence, it is reasonable to expect that the down-regulation of NCAM-180 would have a detrimental effect on neuronal survival. Such thinking is consistent with my data presented in Chapter 2, where I also observed lower levels of NCAM-180 in the retinas, as well as the VC of aged WT mice. In the absence of NCAM, I found that aging retinas have fewer photoreceptor cell layers, and a significant loss of RGCs, suggesting that NCAM-180 may help protect retinas against daily environmental stress during adulthood. In contrast, lowered levels of NCAM-180 seen in normal aging animals may reduce this retino-protective effect and contribute to premature retinal cell death and earlier vision loss.

I used light-induced oxidative damage as a model to identify potential molecular candidates mediating early retinal cell death in NCAM −/− mice. My results showed that NCAM protects retinas from $p75^{NTR}$-induced apoptosis. Oxidative stress triggers $p75^{NTR}$-induced signaling via the activation of 4-hydroxynonenal (HNE), that is a major end product of lipid peroxidation generated during oxidative stress (Dalleau et al., 2013), and disrupts mitochondrial function, deplete cellular antioxidants, modify transmembrane receptor activity, and activate apoptosis of neurons in conditions associated with increased ROS (Kruman et al., 1997). Exposure of sympathetic neurons to HNE causes proteolytic cleavage of $p75^{NTR}$ by metalloprotease and γ-secretase, resulting in axonal fragmentation and neuronal death (Kraemer et al., 2014). In fact, HNE induces $p75^{NTR}$ internalization and proteolytic cleavage, and releases the intracellular domain (ICD) of $p75^{NTR}$. The $p75^{NTR}$-ICD translocates to the nucleus and induces tumor necrosis factor α converting enzyme (TACE) expression (Frade, 2005; Kenchappa et al., 2006), that mediates apoptosis through the activation of PKC/ c-Jun/ AP-1 signaling pathway (Kutuk and Basaga, 2007). Therefore, in view of the results I have obtained, it is reasonable to believe that ROS generated by intense light activates the release of HNE; in NCAM −/− retinas, this causes an excessive amount of $p75^{NTR}$ to undergo proteolytic cleavage, resulting in early onset of apoptosis.

I observed doubling of ERG a- and b- wave amplitudes in 2-month old NCAM −/− animals (Chapter 2). To my knowledge, such an unusual 2-fold increase in ERG responses from light have been reported previously only in retinas having slightly higher

than normal concentrations of NO (Vielma et al., 2010), an important free radical cell signaling molecule with diverse physiological functions including immune response, insulin secretion, vascular tone angiogenesis, and neuronal communication (Bredt, 1999). NO is produced by oxidizing L-arginine to L-citrulline, a reaction that is catalyzed via nitric oxide synthase (NOS) in amacrine cells, RGCs, and photoreceptor inner segments. The synthesis of retinal NO is controlled by light, acetylcholine, melatonin, dopamine, and glutamate (reviewed by Vielma et al., 2012). All three isoforms of NOS (neuronal NOS [nNOS], endothelial NOS [eNOS], and inducible NOS [iNOS]) are expressed to variable degrees in the retina, but nNOS plays the most important part in visual response (reviewed by Vielma et al., 2012). The presence of NO modulates phototransduction and triggers different visual signal processing in the retinas, leading to a significantly intensified retinal light response (Saenz et al., 2007; Saenz et al., 2002). NCAM is known to regulate NO in events associated with synaptic plasticity, neurite outgrowth and neuron survival (Ditlevsen and Kolkova, 2010; Fiscus, 2002; Hindley et al., 1997; Monfort et al., 2004); hence, I speculate that without NCAM, the −/− retinas have higher than normal levels of NO.

NO can give rise to nitrogen reactive species through covalent reactions with target proteins; the exact mechanism remains elusive, but it appears to be involved in protein S-nitrosylation and tyrosine nitration (reviewed by Vielma et al., 2012). Protein S-nitrosylation has been implicated in the interaction with various ion channel proteins to promote retinal cell proliferation, dendritic growth, and neuroprotection (Cheung et al., 2000; Koriyama et al., 2010; Magalhaes et al., 2006). In contrast, tyrosine nitration is associated with pathological conditions caused by highly oxidative environments (Miyagi

et al., 2002) and contributes to light-induced photoreceptor cell death after exposure to intense fluorescent light (Palamalai et al., 2006). Prolonged incubation or excessive application of NO significantly diminishes ERG amplitudes and is toxic to the retinas (Ostwald et al., 1997; Takahata et al., 2003a; Takahata et al., 2003b), pathology that has been implicated in various retinal diseases resulting in photoreceptor damage (Liversidge et al., 2002; Wu et al., 1997). This may explain why I observed a significant decrease in photoreceptor layers and drastic drop-off of a- and b- wave ERG amplitude in aging NCAM −/− mice. These findings may elucidate my ERG results that, at first glance may seem to be paradoxical. In young adult animals with no NCAM, I propose that exposure to slightly higher than normal levels of NO amplifies visual responses to light resulting in elevated ERG signals; however, chronic exposure to NO in aging NCAM −/− animals results in interference with redox homeostasis that ultimately damages retinal cells.

I have shown that NCAM −/− mice have premature loss of visual acuity (Chapter 2). I have also demonstrated, using the animal model of amblyopia via long-term MD, that PSA is required for the reactivation of visual cortical plasticity and the recovery of visual function (Chapter 4). Before discussing how NCAM facilitates visual plasticity and protects neurons from oxidative stress, I will review key molecular events involved in over-production of ROS. Increasing evidence has shown that the presence of ROS is necessary for many physiological cellular activities (reviewed by Schieber and Chandel, 2014). Low levels of ROS participate in synaptic plasticity processes as second messengers/signaling molecules, requiring a certain redox environment to induce structural and functional changes. Anti-oxidative enzymes are used to neutralize the

harmful oxidizing effect. However, ROS production increases with age, and oxidative damage is often related to age-dependent loss of synaptic plasticity, that may have deleterious effects on neurons under pathological conditions (reviewed by Beckhauser et al., 2016). The sequence of events leading to detrimental effects of ROS on synaptic plasticity is as follows: The over-release of glutamate causes NMDA receptor hyperactivation, and drives a massive influx of calcium ions inside cells. Cytoplasmic free calcium and calmodulin controls the activity of nNOS. Excessive amounts of intracellular calcium binds with calmodulin, then activates nNOS to produce NO, inducing superoxide formation via NADPH oxidase to convert into hydrogen peroxide (H_2O_2). Increased levels of H_2O_2 triggers L-type voltage-dependent calcium channels (VDCC) and further induces Ca^{2+} influx. After a series of signaling transduction events, the release of cytochrome C from mitochondria induces apoptosis, and the accumulation of mitochondrial ROS produces excessive amounts of H_2O_2 into the cytosol. While it cannot be completely neutralized by limited antioxidant enzymes, it can enter the nucleus and provoke DNA fragmentation.

How does NCAM protect neurons against oxidative stress? As mentioned above, PSA has been shown to antagonize competitively the NR2B containing-NMDA receptor to prevent glutamate-induced excitotoxicity (Hammond et al., 2006). Likewise, the Dityatev group has demonstrated that NCAM associated with PSA regulates synaptic plasticity through the modulation of GluN2B receptor. The deficiency of NCAM or PSA enhances GluN2B calcium transmission in the CA1 of the hippocampus (Kochlamazashvili et al., 2010). In addition, NMDA-induced calcium dynamics are disrupted in NCAM −/−

RGCs, suggesting that in the absence of NCAM, calcium-induced excitotoxicity affects normal RGC function (Murphy et al., 2012). Furthermore, 50% of neural activities in the L-type VDCC are involved in intracellular signaling triggered by NCAM (Kiryushko et al., 2006). These studies suggest that NCAM modulates calcium dynamics by controlling the activity of NMDA subtype receptors (GluN2B) and L-type VDCC, protecting neurons against oxidative damage. Thus, together with the aforementioned findings, it is reasonable to speculate that neurons, in the absence of NCAM, are unable to adapt to oxidative stress and restore homeostasis. Continuous disparity in calcium signaling affects the balance between healthy signaling and neurotoxicity, leading to reduced synaptic efficacy, and weakening neural connections in an age-dependent decay.

5.7 Future Directions: NCAM Mimetic Peptides and Therapeutic Potential

The response of the brain to stressors is a complicated process, dependent on the stressor's duration, severity, frequency, and timing of onset. Continuous changes in neural architecture, networks, and activities are necessary in order to cope with stress. Yet these necessary neuroplastic adaptation processes significantly decline with age, increasing cellular vulnerability to oxidative stress, and risking neuronal dysfunction and cell death. Since PSA and NCAM are important regulators in different levels of brain plasticity, they are also attractive targets for the development of pharmacological tools in the treatment of age-associated neurodegenerative disorders.

A mimetic peptide is a small protein-like chain (14-20 amino acids) that mimics the active binding site of a molecule. It may antagonize, stimulate, or modulate the physiological activity of its natural ligands. A peptide mimetic is relatively easy to manufacture and modify, and thus can serve as a lead compound for future drug development. Based on the structural and functional properties of NCAM, three types of mimetic peptides have been developed (reviewed by Berezin and Bock, 2010): (1) NCAM binding peptides with artificial (modified) sequences, e.g. C3d (Ronn et al., 1999); (2) synthetic peptides that contain NCAM homophilic binding (NCAM – NCAM interaction) sites, e.g. P2, and plannexin (Kohler et al., 2010; Soroka et al., 2002); and (3) synthetic NCAM-derived peptides targeting heterophilic NCAM interaction partners (NCAM binds with other proteins), e.g. FGL and Encamins (Hansen et al., 2008; Neiiendam et al., 2004) . Aside from NCAM, mimetic peptides and small compounds are also generated from its polysialic acid moiety, e.g. Tegaserod and PR-21(Marino et al., 2009; Pan et al., 2014). I think that these small peptides and compounds will provide an opportunity for the development of new treatments in humans. In the following sections, I will discuss the effects of NCAM (*C3d*, *P2* and *FGL*, one from each category) and PSA (PR-21) mimetic peptides, and focus on their roles in synaptic function and neuroplasticity, as well as their potential therapeutic effects (Dallerac et al., 2013).

C3d is the first artificial NCAM mimetic peptide identified by screening a combinatorial library. It binds with the first Ig domain of NCAM to mediate cell adhesion and signal transduction with high potency (Ronn et al., 1999). The effect of C3d on presynaptic function of hippocampal neurons follows a bell-shaped curve dose-response relationship

(Kiryushko et al., 2003). C3d increases presynaptic releases at a low dose, but reduces its releases when applied at a higher concentration. The dose-dependent effect is possibly a function of calcium signaling: low dose of C3d treatment activates cAMP/ PKA pathway and promotes CREB phosphorylation, which leads to synaptic strengthening, whereas a high concentration of C3d affects NCAM interaction that causes rapid uncoupling of synaptic contacts (Sheppard et al., 1991). Furthermore, the C3d synthetic peptide promotes choline acetyltransferase activity in cultures of rat embryonic septal neurons through the association of FGFR and MAPK pathway, suggesting that C3d may stimulate cholinergic function and improve cognition in neurodegenerative disorders, such as Alzheimer's disease (Burgess et al., 2009). C3d has also been found in rats to relieve neuropathic pain effectively, and to prevent neurodevelopmental defects induced by pyrimethamine (Klementiev et al., 2002; Sakai et al., 2008). Collectively, these studies suggest that C3d may have therapeutic potential for enhancing neuron survival and for improving synaptic function.

The NCAM-derived ***P2*** peptide corresponds to the trans-homophilic binding site in the second Ig domain of NCAM and has several effects. Under certain circumstances, the P2 peptide acts as a competitive antagonist to reduce NCAM cell-cell interactions (Berezin and Bock, 2010). If not participating in trans-homophilic binding, P2 facilitates neuronal plasticity by binding with NCAM to increase cytoplasmic calcium concentrations (Kiryushko et al., 2006; Li et al., 2005). It also stimulates the MAPK/ ERK pathway to induce neurite outgrowth in primary hippocampal rat neurons, as well as in the primary cerebellar and mesencephalic dopaminergic neurons (Pedersen et al., 2004; Soroka et al.,

2002). Through the activation of Akt signaling cascade, the P2 peptide promotes the survival of cerebellar neurons (Pedersen et al., 2004). P2 has also been studied as a therapeutic for the treatment of traumatic brain injury by protecting cells against oxidative stress-induced damage, and promoting neuronal plasticity and regeneration in the sublesional area, to facilitate recovery of sensorimotor and cognitive functions (Klementiev et al., 2008).

The ***FGL*** peptide encompasses the interconnected loop region of the second fibronectin type III module, which has been shown to activate FGFR1 (Kiselyov et al., 2003). The peptide mimics heterophilic interactions with FGFR1 and supports hippocampal synaptic transmission and plasticity by facilitating delivery of AMPA receptors at synapses upon activation of NMDA receptors, resulting in LTP and cognition enhancement (Dallerac et al., 2011; Knafo et al., 2012). The FGL peptide also mediates neuroprotective effects in aged rats, and promote neuronal survival after ischemic brain injury (Ojo et al., 2012; Skibo et al., 2005). Because of its significant effects on cognition, synaptic plasticity, and neuroprotection, there is considerable interest in FGL peptide's potential in treating neurological disorders and age-related neurodegenerative diseases. For instance, it alleviates cognitive impairment induced by amyloid β peptide$_{25\text{-}35}$, a pre-aggregated oligomeric β-amyloid that generates neuropathological findings associated with Alzheimer's disease through the inhibition of intracellular signaling mediated by GSK3β (Klementiev et al., 2007). It also acts as an anti-inflammatory agent by attenuating microglia activation, that is normally seen in aging or age-related cognitive decline (Downer et al., 2009; Downer et al., 2010). Furthermore, the FGL peptide reduces

deleterious effects of chronic stress on cognitive decline during aging (Borcel et al., 2008). These findings suggest that NCAM mimetic peptide, FGL, might be of therapeutic relevance to treat age-related cognitive impairment.

As discussed previously, the polysialyated moiety of NCAM is largely implicated in synaptic transmission and in regulating network connectivity through synaptic reorganization and remodeling. In animal models, several PSA mimetic peptides have been demonstrated to mediate functional recovery from both spinal cord and peripheral nerve injuries (Marino et al., 2009; Mehanna et al., 2010; Pan et al., 2014). For instance, the delivery of PSA mimetic PR-21 to the lesion site after dorsal hemisection of the spinal cord at the thoracic (T) – 9 level enhances motor function, increases serotonergic axon density, and decreases reactive gliosis (Marino et al., 2009). In addition, using lentivirus to induce overexpression of PSA can promote astrocyte infiltration and permit the penetration of regenerating axons into the lesion cavity (Zhang et al., 2007a; Zhang et al., 2007b). These studies support the notion that the expression of PSA, by either lentivirus or mimetic peptide treatments, provides a favorable environment for axonal regeneration and functional recovery after CNS injury.

In the past decade, mimetic peptides have been of growing interest in the biotechnology industry due to high potency and selectivity of their therapeutic targets. Peptides are easy to design and synthesize, are nontoxic and biodegradable, and are less likely to cause an immunogenic reaction. The application of mimetic peptides is currently big business,

estimated to generate more than 10% of the world's pharmacological sales revenues (Agyei et al., 2017).

Despite their therapeutic appeal, there are major drawbacks to peptide therapeutics including their low bioavailability (parenteral administration is generally required) and short duration of action; they are rapidly degraded by proteolytic enzymes of the digestive system or in the blood's plasma, and removed from circulation by means of hepatic clearance (reviewed by McGowan et al., 2015). Furthermore, it is difficult to deliver peptides beyond the blood brain barrier; hence, the option of peptide therapeutics in CNS disorders seems limited in humans. The future success of these peptides will depend on the development of more effective ways of drug delivery, specifically targeted to the brain, regions of the brain, or particular cellular populations in the brain. One possible way to overcome these obstacles is to develop mimetic peptides bio-conjugated with nanoparticles (reviewed by Yadav et al., 2011). The nanocarriers are able to cross the blood brain barrier and have the ability to target specific sites, reducing potential toxicity to the surrounding tissues (reviewed by Lalatsa et al., 2014). Encapsulated nanopeptides retain activity, stability, and structural integrity, with high drug loading and controlled release abilities. Furthermore, the nanoparticles can be delivered by various non-invasive means, including aerosol-based and oral drug formulations (reviewed by Serrano Lopez and Lalatsa, 2013). As such, I think that the incorporation of PSA and NCAM peptides into nanoparticles offers therapeutic potential in the treatment of human CNS disease.

Lastly, besides designing PSA and NCAM mimetic peptides to enhance neuroprotective potency, there are far less costly ways of maintaining the health of our CNS. Antioxidants can be used to attenuate/delay the damaging effects that contribute to cellular aging. Many dietary supplements, present as both natural and synthetic antioxidants, are available to scavenge excessively produced ROS. As well, regular exercise helps increase antioxidant levels and promotes adaptation to oxidative stress. Experimentally, it would be interesting to investigate whether the administration of specific antioxidant supplements could reduce premature vision loss, delay the onset of retinal apoptosis after LIRD, and promote the recovery of vision following long-term MD in NCAM −/− animals.

Collectively, the results of my experimental work outlined in this thesis have demonstrated that NCAM plays vital roles in promoting retinal cell survival and in maintaining visual physiology in the nervous system during aging. This work not only highlights the importance of NCAM in the rodent visual system but also adds to our understanding of the fundamental biology of NCAM. My work provides insights into our basic understanding of cellular interactions within the CNS and strengthens the rationale for exploring new therapeutic tools to promote human CNS plasticity, to treat age-related visual impairment and to alleviate neurodegenerative disease.

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