Université de Montréal

The expression of netrin-1 in the intact and injured adult mice retina

par
Pegah CHEHRAZI
Département de Neuroscience
Faculté de la médecine

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Résumé

La netrine-1 joue un rôle important en tant qu'un élément de guidance pour la croissance axonale au début du développement du système nerveux central. Des études récentes ont démontré une expression de la netrin-1 dans le cerveau antérieur adulte, où elle régule la fonction synaptique et la plasticité dans les neurones corticaux. Cependant, la contribution de la netrine-1 dans la rétine adulte reste encore inconnue. Le but de cette étude est donc de caractériser l'expression de la netrine-1 dans la rétine des souris adultes sauvages (rétine intacte) et malades (rétine blessée).

L'expression rétinéene de la netrine-1 et de son récepteur, supprimée dans le cancer colorectal (DCC), a été déterminée, à partir des immunobuvardages, chez des souris post-natales de jour 0 (P0), P14 et adultes. Le recours au double marquage de la netrine-1 avec un anticorps spécifique contre RBPMS, un marqueur sélectif pour les cellules ganglionnaires de la rétine (RGC), a permis l'identification de sa localisation sur les sections transversales de rétine. De plus, les niveaux de netrin-1 ont également été quantifiés à trois et sept jours après l'axotomie du nerf optique.

Nous avons démontré que la netrine-1 et DCC sont fortement exprimés dans la rétine à P0, toutefois l'expression de netrin-1 est relativement stable pendant le développent alors que l'expression de DCC est remarquablement réduite à l'âge adulte. De plus, ces expériences ont conclu une expression robuste de la netrin-1 dans le soma RGC adulte et, une expression des récepteurs DCC autour du corps cellulaire. Fait important, nous avons aussi pu démontrer que les niveaux d'expressions de la netrine-1 et DCC sont réduits à trois et sept jours suivant l'axotomie du nerf optique. Cependant, la surexpression de la protéine netrin-1 n'a pas eu un effet significatif sur la survie du RGC par rapport aux témoins injectés par un véhicule.

Les résultats obtenus suggèrent que : (i) la netrine-1 est abondamment exprimée dans la rétine néonatale et subit une diminution importante à l'âge adulte, (ii) la netrine-1 et son récepteur DCC sont présents dans les RGC et, (iii) l'expression de la netrine-1 diminue considérablement suite à une lésion axonale. Ensemble, ces résultats suggèrent un rôle pour la netrin-1 dans le système visuel chez les adultes.

Mots-clés : Rétine, Adulte, Netrine-1, DCC, Axotomie

Abstract

Netrin-1 plays a highly-conserved role as a guidance cue directing axonal growth during the early stages of central nervous system development. Recent data has shown that netrin-1 is continued to be expressed in the adult forebrain where it regulates synaptic function and plasticity in cortical neurons. However, the contribution of netrin-1 in the adult retina remains unknown. The purpose of this study was to characterize the expression of netrin-1 in the intact and injured adult mouse retina.

The retinal expression of netrin-1 and its receptor, deleted in colorectal cancer (DCC), was examined at postnatal day 0 (P0), P14, and adult mice using western blots. Double labeling of netrin-1 with an antibody against RBPMS, a selective marker for retinal ganglion cells (RGCs), was used to determine its location on retinal cross-sections. Netrin-1 levels were also quantified at 3 and 7 days after optic nerve axotomy.

We demonstrate that netrin-1 and DCC are abundantly and widely expressed in the retina at P0, but although the expression level of netrin-1 remains relatively stable during development, the expression level of DCC is markedly downregulated in adulthood. Adult RGC soma were shown to be endowed with robust netrin-1 expression. DCC receptors were also found to be expressed around the cell body. Importantly, we show that netrin-1 and DCC levels are further downregulated at 3 and 7 days after optic nerve axotomy. However, the over-expression of netrin-1 protein failed to exert any significant effect on RGC survival in comparison to vehicle-injected controls.

Our data support that: 1) netrin-1 is abundantly expressed in the neonatal retina and undergoes marked downregulation in adulthood, 2) netrin-1 and DCC are present in RGCs, 3) netrin-1 expression decreases rapidly after axonal injury. Together, these results suggest a role for netrin-1 in the mature visual system.

Keywords: Retina, Adult, Netrin-1, DCC, Axotomy

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ALS	Amyotrophic lateral sclerosis
BDNF	Brain derived neurotrophic growth factor
BRB	Blood retinal barrier
cAMP	Cyclic adenosine monophosphate
Cdc42	Cell division cycle 42
CGNs	Cerebellar granule neurons
CNS	Central nervous system
DB	DCC-binding
DCC	Deleted in colorectal cancer
DD	Death domain
DSCAM	Down syndrome cell adhesion molecule
ECM	Extracellular matrix
EGF	Epidermal growth factor
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
GCL	Ganglion cell layer
ICD	Intracellular domain
IHC	Immunohistochemistry
INL	Inner nuclear layer
IPL	Inner plexiform layer
ITRs	Inverted terminal repeats
kDa	KiloDalton
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase;
MMP-9	Matrix metalloprotease 9
mTOR	Mammalian target of rapamycin
N-WASP	Neuronal Wiskott-Aldrich syndrome protein
OB	Olfactory bulb
ONL	Outer nuclear layer
OPL	Outer plexiform layer
P0	Post-natal day 0
P14	Post-natal day 14
Pack1	Serine/threonine kinase p21 activated kinase 1
Pak1	P21-activated kinase
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol (4,5) bisphosphate; IP3, inositol triphosphate;
PKC	Protein kinase C
PLCγ,	Phospholipase Cγ
PNS	Peripheral nervous system
Rac1	Ras-related C3 botulinum toxin substrate 1
RGC	Retinal ganglion cells
RhoA	Ras homologous member A
Rho-GTPases	Rho-guanidine triphosphatase

RBPMS	RNA binding with multiple splicing
RGCs	Retinal ganglion cells
RPE	Retinal pigmented epithelium
RT-PCR	Reverse-transcription polymerase chain reaction
siRNAs	Short interfering RNAs
Src	Tyrosine kinase sarcoma
TLR	Toll like receptors
TSP-1	Thrombospondins type I
UNC-5	Uncoordinated locomotion-5
VDCCs	Voltage-dependent Ca2+ channels in the plasma membrane
VEGF	Vascular endothelial growth factor
ZU5	Zona occludens-5

"If a little kid ever asks you just why the sky is blue, you look him right in the eye and say, "It's because of quantum effects involving Rayleigh scattering combined with a lack of violet photon receptors in our retinae."

Philip Plait

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There are few people without whose involvement this project would never have come to be. So,

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Introduction

Netrin-1 is an extracellular protein belonging to the family of netrin proteins that direct cell and axon migration during neural development. Netrin-1 is a laminin-related protein with dichotomous biological effects. Depending on the receptor it binds, netrin-1 can act as attractant for some cell types and repellent for others. Receptors for netrin-1 include the Deleted in Colorectal Cancer (DCC) family, the Down's Syndrome Cell Adhesion Molecule (DSCAM), the Uncoordinated-5 Homolog Family (UNC5A-D), and the recently described A2b. Canonical signaling through DCC leads to chemoattraction, while signaling through UNC5 mediates chemorepulsion [1].

Although originally recognized for its axon guidance during the development of the nervous system, it was later found that netrin-1 has a rich biology: it is expressed in multiple tissues outside the central nervous system (CNS) such as lung, pancreas, mammary gland, vasculature and muscle where it contributes to organogenesis by mediating cell migration and cell-cell adhesion [2]. Of interest, netrin-1 expression has also been reported in several regions of the adult CNS. For example: it plays an important role in regulating the migration of adult neural stem cells to sites of injury in the mature spinal cord [3]. It is also required for the maintenance of appropriate neuronal and axon–oligodendroglial interactions in the mature nervous system [4]. Most interestingly, netrin-1 and its receptor DCC are present in the adult forebrain and play an important role in regulating synaptic function and plasticity in pyramidal neurons [5].

The role of netrin-1 during retinal development is well established, however, its potential role in the mature retina remains unknown. In the developing mouse retina, netrin-1 is secreted by epithelial cells at the optic disk and guides the DCC expressing embryonic retinal ganglion cell (RGC) axons towards the optic nerve head [6]. It is also shown that the expression of DCC is essential for the survival of RGCs and amacrine cells during postnatal development [7]. However, previous studies provided conflicting results on the expression of netrin-1 in the adult retina. While original data showed that netrin-1 is downregulated to below detection levels in the adult rat retina, subsequent studies contradicted this by showing that netrin-1 is constitutively expressed in adult retinal neurons [8-10]. Therefore, to resolve these issues, we

investigated the expression of netrin-1 and its receptor DCC as well as netrin-3, the closest netrin-1 homolog, in the adult mouse retina using a variety of complementary techniques. Our data revealed that netrin-1 and DCC are present in adult mouse retinas and they are constitutively expressed by adult RGCs. In addition, we showed that netrin-3 is abundantly present in the ganglion cell layer and the inner nuclear layer of the adult mouse retina. We also demonstrated that the expression of netrin-1, netrin-3 and DCC are downregulated after axotomy. Taken together, our results suggest a potential role for netrin-1 in the mature visual system.

I.1. The retina: cellular and functional organization

The retina is the innermost neural layer of the eye where the light energy is transformed into a neural signal. The retina forms a sheet of only ~200 μm thick and is comprised of complex neuronal circuits and glial cells. More than 60 distinct types of neurons are arranged into distinct morphological and functional circuits working in parallel to encode visual information in the retina. In fact, the retina has a stereotypical laminar structure comprised of three nuclear layers, where retinal cell bodies are located, and two intermediate plexiform layers where synaptic interactions between these nuclear layers take place [11, 12]. The retinal neurons are grouped into five major classes of cells: the photoreceptors (rods and cones), three major classes of interneurons (horizontal, amacrine, and bipolar cells) and RGCs (Fig. 1)[13].

Light rays entering the eye cross through the entire retina to reach to the light sensitive outer segments of rods and cones in the outer nuclear layer (ONL) where they are converted into electrical signal. The signal is further relayed to the inner nuclear layer (INL) where bipolar, horizontal, and amacrine cells perform initial information processing. From the bipolar cells the signal is finally transferred to the innermost layer of the retina, the ganglion cell layer (GCL) where the projection neurons the RGCs are located and conveyed the visual image information via their axons bundles in the optic nerve to higher processing centers within the brain (Fig. 1). Dividing these nerve cell layers are two plexiform layers where synaptic connections occur: the outer plexiform layer (OPL), a single lamina where photoreceptors make synaptic connections with bipolar and horizontal cells, and the inner plexiform layer (IPL) where bipolar and amacrine axon terminals synapse onto RGCs [14].

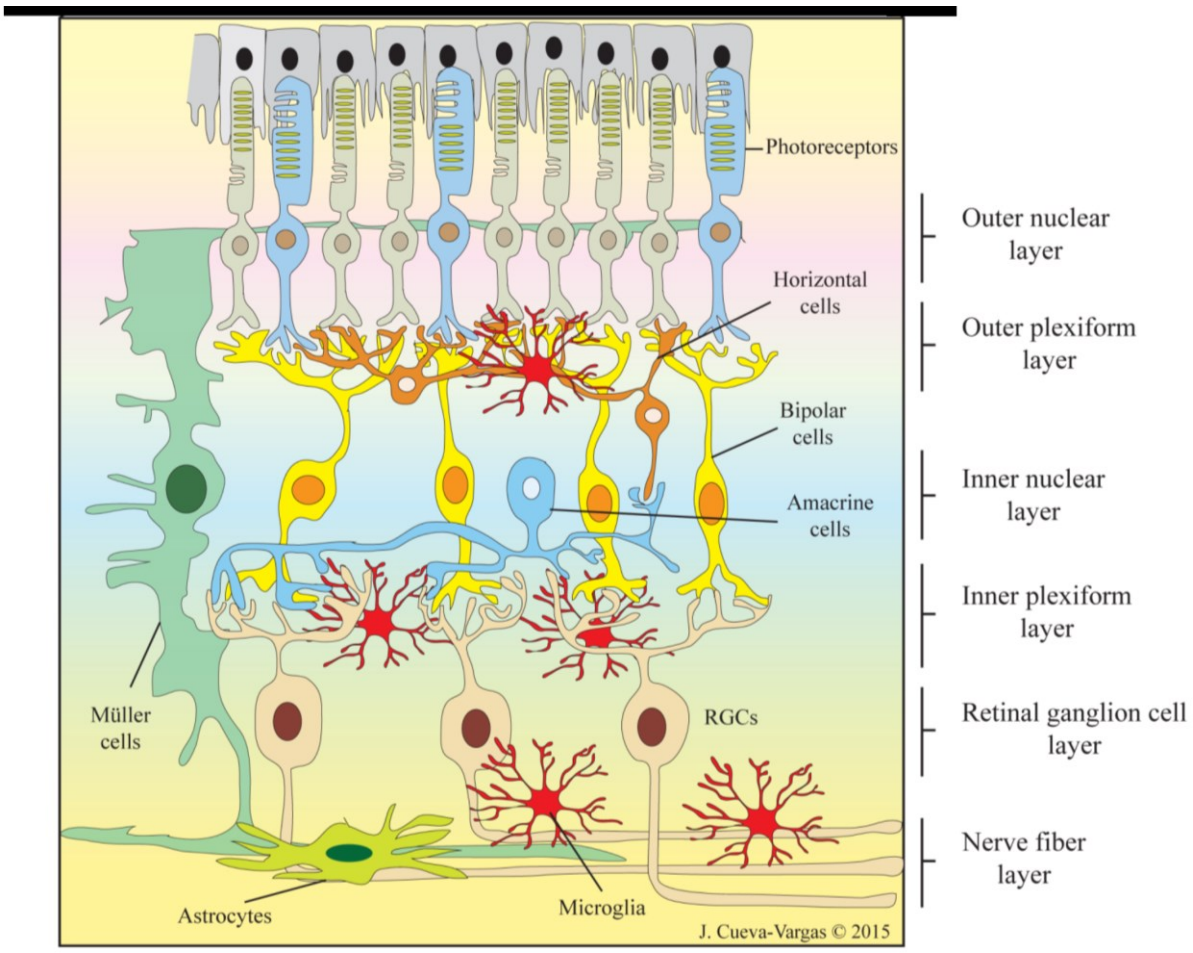


Figure 1. A schematic diagram of the retina demonstrating the principal cell types involved in retinal signaling. The retinal neurons are photoreceptors cells, bipolar cells, horizontal cells, amacrine cells, and retinal ganglion cells (RGCs). These cells are organized into five interconnected layers. Of the five layers, the ganglion cell layer (GCL), inner nuclear layer (INL) and outer nuclear layer (ONL) consist mainly of cell bodies while synaptic connections among the neurons are located in the inner plexiform layer (IPL) and the outer plexiform layer (OPL). The retina also contains glial cells that interact with neurons and blood vessels. The glial cells are categorized into Muller cells, astrocytes and microglia. Muller cells span across the entire thickness of the neural retina and provide an anatomical and functional link between retinal neurons and blood vessels. The astrocytes surround the blood vessels and microglial cells keep the central nervous system under surveillance for infections and injuries. Source of image: Jorge Luis Cueva Vargas.

I.1.1 Photoreceptors

The photoreceptors form a single sheet of regularly spaced light-sensitive cells comprised of rods and cones. Photoreceptors are conical-shaped structures consisting of a membranous outer segment containing photopigments (rhodopsin or cone opsins) and an inner segment containing the cell nucleus. The light absorbing pigments of photoreceptors contains 11-cis retinal which, upon absorption of light, undergoes conformational changes and dissociates itself from opsin. The opsin moiety can now bind and activate a trimeric G-protein called transducin. The activated α subunit of transducin further binds to cGMP phosphodiesterase or PDE, which converts the available supply of cGMP to its non-cyclic form 5'-GMP. The dropping cGMP levels leads to cation channel closure in the plasma membrane blocking sodium and calcium influx. Consequently, the photoreceptor outer segment hyperpolarizes generating a direct flow of electrical current along the membrane to the synaptic terminals promoting the release of the neurotransmitter glutamate [15].

Although both rods and cones respond to light with a slow hyperpolarizing response, they report quite different image properties. The fovea the central region of the retina exclusively contains cones which require high levels of light to generate signals, and therefore work best during the day time illumination. In addition, cones are most sensitive to specific wavelengths of light and are therefore responsible for high visual resolution and color vision. There are three types of cones: long (red or L-), middle (green or M-) and short (blue or S-) wavelength [16]. In the peripheral region of the retina, rod photoreceptors outnumber cone photoreceptors by about 20-fold in almost all mammalian retinas. In fact, after the cerebellar granule cells, ~125 million rod photoreceptors appear to be the second most numerous neurons of the human body. Rods have exquisite sensitivity to light with the resolution of a single photon detection and thus are specialized for vision in dim light [17].

I.1.2 Bipolar cells

Retinal bipolar cells comprise a diverse class of interneurons in the INL, transmitting the photoreceptor signals on amacrine and ganglion cells. There are at least 13 distinct types of bipolar cells which differentially collect and shape photoreceptor signals into parallel

information pathways, each encoding a highly processed feature such as motion, contrast or edges [18, 19]. Bipolar cells fall into two main groups based on their response to glutamate released by photoreceptor: ON (glutamate hyperpolarization) and OFF (glutamate depolarization) bipolar cells. In response to light increments, ON bipolar cells that have inhibitory glutamate receptors, depolarize. On the other hand, OFF bipolar cells that are depolarized in the dark will hyperpolarize. OFF bipolar cells contact OFF RGCs, and ON bipolar cells contact ON RGCs [11, 20].

I.1.3 Horizontal cells

Horizontal cells are the laterally interconnecting neurons in the INL that release inhibitory γ-aminobutyric acid (GABA) neurotransmitters and, as such, help integrate and regulate the input from multiple photoreceptor cells by providing inhibitory feedback. In fact, the horizontal cells measure the average level of illumination across the broad region of photoreceptors and subtract the signal from less illuminated ones, resulting in the reduction of redundancy of the signal transmitted to the RGCs. This selective suppression of certain neuronal signals is called lateral inhibition, and its overall purpose is to improve the contrast and definition and therefore the acuity of the visual stimulus [21].

I.1.4 Amacrine cells

Amacrine cells are important interneurons that establish synaptic contacts with bipolar cells and RGCs in the IPL. Due to the lack of clear polarity, which makes it difficult to differentiate the site of their input from output, they were named amacrine cells meaning axon less neurons [22]. Amacrine cells modulate the synaptic input to ganglion cells in three different ways: either directly by feedforward inhibition on RGC dendrites or by feedback inhibition of axon terminals of bipolar cells that drive them. They also provide lateral inhibition by making connection to other amacrine cells [11, 23]. They mediate their inhibitory functions largely by releasing two neurotransmitters: γ-aminobutyric acid (GABA) and glycine. Amacrine cells exhibit great structural diversity and complexity and are divided into 42 different

morphological subtypes and are thus considered the most diverse cell class in the retina. Amacrine cells are also grouped into four subtypes of narrow-field (30-150 μm), small-field (150-300 μm), medium-field (300-500 μm) and wide-field (>500 μm) based on the measurement of their dendritic fields [22, 24].

I.1.5 Retinal ganglion cells (RGCs)

RGCs are the largest retinal neurons with a density of 2,500 cells per square millimeter. RGCs are the output neurons of the retina by extending axons through the optic nerve to higher processing centers in the brain. The signals from bipolar cells are integrated by RGCs and are further transferred as a diverse set of parallel action potentials travelling down the optic nerve to the brain. RGC action potentials are generated spontaneously and change accordingly when stimulated by the appearance of light in the RGC receptive fields.

RGCs exhibit strikingly varied dendritic morphology and field size. Based on their patterns of dendritic arborization, RGCs were initially categorized in approximately 20 subtypes [25, 26]. However, in addition to their structural heterogeneity, RGCs can be distinguished by their diverse functions and connectivity. In fact, besides being a light detector due to the presence of the pigment melanopsin in an RGC subtype, these neurons can also behave as feature detectors: each neuron tuned to distinct visual features such as contrast, color, or specific motion directions [27, 28]. Therefore, the anatomical classification of RGCs thus far cannot encompass the functional diversity of these neurons and a further categorization of RGCs is necessary to better understand the visual outcome in both physiological and pathophysiological states. Recently, new molecular, genetic, and functional approaches have complemented the RGC classification scheme increasing the estimated number of RGC types to >30 [25, 27, 29].

RGC axons converge at the optic nerve head (ONH) to form the optic nerve which projects to four main targets in the human brain: 1) the lateral geniculate nucleus of the thalamus from where signals are relayed to the visual cortex, 2) the superior colliculus for orienting responses, 3) the pretectal nucleus for the pupillary light reflex, and 4) the suprachiasmatic nucleus for light entrainment of the sleep–wake cycle [30].

I.1.5.1 RGCs are the cellular target in many retinal diseases

RGC death is the final common pathway that leads to loss of vision in several retinal diseases such as glaucoma [31]. RGCs are the sole retinal neurons that generate action potentials transmitted over long distances along the optic nerve trajectory (~50 mm) to the brain visual centers [32]. Therefore, RGCs are the most energetically demanding neurons in the retina. In addition, each RGC subcellular compartments: the dendrites, cell body, non-myelinated axon, and myelinated axon, are located in a different extracellular environments with different energy requirements. Therefore, intracellular energy distribution and consumption is different and specific to each RGC cell relative to other neurons. Accordingly, for the maintenance of optimal neuronal function, mitochondria are unevenly distributed within RGCs to meet the local energy demands of the cell [33]. All these factors render RGCs more vulnerable to minor disruptions of energy homeostasis and predispose them to respiratory chain dysfunction, oxidative stress and ultimately apoptosis [33, 34].

I.1.6 Retinal glial cells

In addition to the neuronal network, the retina contains three basic types of glial cell subdivided into macroglial cells, astrocytes and Muller cells, and microglial cells (Fig. 1). In general, retinal glial cells regulate the microenvironment and ensure optimal neuronal function. Glial cells not only provide the neurons with structural and metabolic support but are also involved in maintaining retinal homeostasis by forming blood-retinal barrier (BRB) and regulating local blood flow [35]. As such, they participate in retinal glucose metabolism, elimination of metabolic waste products, release of certain transmitters, trophic factors and potassium uptake [36].

Under pathophysiological conditions, glial cells also participate in local immune responses and protect retinal neurons through a process called reactive gliosis. Reactive gliosis involves both morphological and functional alteration of the glial cells and is associated with cellular hypertrophy, proliferation, migration and cytokine release with the aim to maintain retinal homeostasis [37]. However, glia cells can also undergo chronic gliosis which constitute

the primary pathogenic element exacerbating disease progression by increasing vascular permeability, infiltration of toxic compounds, and even neovascularization [35, 36, 38]

I.1.6.1 Muller glia

Muller glia are cylindrical, fiber-like cells that constitute the predominant glial cells of the retina. They are arranged in parallel spanning the entire width of the retina with their apical processes in intimate contact with photoreceptor cell bodies and their end feet enveloping RGCs and displaced amacrine cells. Muller cell are endowed with many side branches that are involved in the formation of the blood retinal barrier (BRB) allowing intimate association with both neuronal elements such as synapses as well as the blood vessels. In this way, they play a role in neurotransmission and support the neurons by providing neurotrophic factors and blood-derived nutrients as well as removing metabolic waste [36]. Furthermore, they are involved in maintaining the BRB by providing a permanent anti-proliferative environment for retinal vascular endothelial cells [39].

Müller cells act as living fibers guiding the light through the randomly oriented and irregularly shaped neurons providing a low-scattering light passage from the retinal surface to the photoreceptor cells. As a result, light arrives at individual photoreceptor with high intensity and minimal distortion. Interestingly, it has been shown that Müller cells can generate neural stem cells and as such could be considered as the primary source of new neurons in mammalian retinas [38, 40].

I.1.6.2 Astrocytes

Astrocytes represent the most abundant and heterogeneous retinal glial cell. Astrocytes are mostly located in the nerve fiber layer and support the function of RGC axons. They comprise almost 50% of the cells in the optic nerve head. It is believed that astrocytes migrate from the optic nerve to the retina along the blood vessels and locate themselves exclusively in the inner retina. Accordingly, the presence and distribution of retinal astrocytes is correlated with the presence and distribution of retina blood vessels. They are considered as the main

producer of vascular endothelial growth factor (VEGF) and, as such, are strongly implicated in retinal vascularization under both physiological and pathological states [41]. It is noteworthy that astrocytes communicate by gap junctions and play an important role in the maintenance of the extracellular pH and ion homeostasis as well as glutamate clearance in the retina [36].

I.1.6.3 Microglia

Microglia are distributed ubiquitously in different retinal layers. Microglia can exist in two different states: surveillance and activated states in the retina. In the healthy and homeostatic state, microglia have a small cell body with a rather large nucleus forming a dynamic and highly organized non-overlapping network. This configuration allows the microglia to survey the microenvironment and participate in cell-cell interactions with retinal neurons and other microglia. The presence of surface proteins such as receptors for cytokines, growth factors, purinergic ligands, toll like receptors (TLR) and complement components in addition to their intricate interactions with other neurons allows microglia to systematically sense their environment [42]. Microglia rapidly change their distribution and organization within the retinal layers in pathological states: they exhibit ameboid cell shapes with pseudopodia and contribute to the remodeling of neuronal circuits, as well as the elimination of cell debris and misformed synapses. In this regard, in the experimental model of axotomy, microglia containing RGC debris can be detected months after performing axotomy, indicating both the phagocytotic capacity of microglia and their relative longevity [43-45].

I.2. The retina as a model to study neurodegenerative diseases

The retina is an extension of the CNS and exhibits many structural similarities to the brain and spinal cord [46]. Furthermore, the eye is a very accessible organ providing the opportunity for non-invasive imaging of retinal neuron as well as the potential for drug discovery and delivery using methods such as eye drops or intraocular injections [47]. The intravitreal injection of short interfering RNAs (siRNAs), plasmids and recombinant proteins allow the selective manipulation of RGCs without confounding effects on other neighboring

neurons or surrounding glia. Moreover, different retinal neurons and glial cells can be selectively targeted by the injection of specific viral vectors in the retina.

Diseases of the retina often share similarities and common neurodegenerative mechanisms with other disease of the brain or the spinal cord [48]. For example, accumulating evidence suggest an array of common physiological and pathological changes between glaucoma, the leading cause of blindness worldwide characterized by RGC loss, and Alzheimer disease. In this regard, Chiasseu *et al*. demonstrated that tau accumulation and altered phosphorylation, hallmarks of Alzheimer disease, also plays a neurotoxic effect in glaucoma [49]. These features render the retina a particularly attractive system to investigate pathophysiologic mechanisms underlying neurodegenerative diseases [13]. A better understanding of these mechanisms could have implications for the development of therapeutic approaches for retinal/optic nerve and brain diseases.

I.2.1 Optic nerve axotomy

RGCs are a well-characterized CNS neuronal population with cell bodies located in the inner retina and axonal processes along the optic nerve that reach specific targets in the brain. The optic nerve can be accessed within the orbit of the eye and completely transected or crushed producing a well characterized temporal course of RGC death. Optic nerve axotomy is considered a reproducible model of apoptotic neuronal cell death and has been widely used to study the response of CNS neurons to axonal injuries as well as to test neuroprotective therapies and regenerative strategies [50, 51]. The mechanisms of RGC death in axotomy are still poorly understood. It is believed that following axotomy the physical separation of the cell body from its target results in neurotrophic factor deprivation followed by oxidative stress [52]. Finally, calcium influx destabilizes the cytoskeleton and RGCs die by apoptosis as they do in glaucoma and other CNS diseases [30, 53]. Ultimately, 90% of the injured RGCs die within 14 days post-axotomy and the few remaining neurons are severely impaired and inactive. After axotomy, the pathways involved in the RGCs apoptosis as well as survival can be further investigated by intravitreal injections of modulatory factors. Strategies to promote cell survival in this system may be extrapolated to other neurodegenerative diseases and CNS trauma [54].

I.2.1.1 Optic nerve axotomy and dynamics of RGC death

RGC apoptosis represents a characteristic bi-phasic time-course after axotomy. In fact, RGC apoptosis has a well-defined onset at approximately 3 days post-axotomy providing a time window for experimental manipulations of the apoptotic pathways [55]. After this 3-day interval, the cells degenerate rapidly and only 50% of RGCs survive at 7 days and less than 10% remain at 14 days after injury. Approximately 5% of RGCs remain up to 20 months after transection, but the functional state of these neurons is unknown [56-58]. Interestingly, early morphological changes in dendrites (dendritic retraction) were shown to occur at 3 days post axotomy prior to overt RGC death suggesting that dendrite pathology is an early neurodegenerative event following axonal injury. The identification of pathways that contribute to RGC dendritic arbor maintenance and synaptic integrity will be useful to understand the molecular basis of pathological changes after axotomy [59].

The survival of RGCs after optic nerve axotomy can be tracked over time with ease and accuracy. In fact, because the ganglion cell layer is a monolayer (one cell thick), RGC densities can be quantified in flat-mounted tissue, without the need for stereology. For this, retinal whole-mounts are labelled with RGC specific markers such as brain-specific homeobox/POU domain protein 3A (Brn3a) [60] and RNA binding protein with multiple slicing (RBPMS) [61] and counted at specific time points.

I.2.1.2 Neuroprotective strategies to promote RGC survival after axotomy

Detailed knowledge of the temporal course of RGC death and the mechanisms that lead to neuronal loss is essential to develop efficient neuroprotective strategies after optic nerve injury. In this regard, axotomy-induced RGC loss has been mainly attributed to the withdrawal of trophic factors from target neurons. The focus of many studies has been to activate pathways that are involved in RGC survival, including TrkB signaling, the Erk1/2 pathway following the intravitreal administration of growth factors such as brain derived neurotrophic growth factor (BDNF) [62-64]. However, recent data suggest that axonal injury leads to early changes in RGC dendritic structure prior to cell death, compromise synaptic integrity, and lead to functional deficits [59, 65]. Accordingly, emerging data revealed that dendritic abnormalities and loss of

synapses contributes to the pathology of many psychiatric and neurodegenerative disorders [66, 67]. Therefore, the focus of recent studies is the elucidation of molecular mechanisms leading to RGC dendrite degeneration and synaptic rearrangements after axonal injury. The identification of new pathways that contribute to RGC dendritic arbor maintenance will be necessary for the development of the strategies to protect RGCs connectivity and enhance survival [51, 68, 69].

I.3. Netrin-1: structural organization and evolutionary history

During the development of the nervous system, neurons are produced in specialized regions and migrate through defined pathways until they reach their final location. Each neuron develops a dendritic arbor that is characteristic by its phenotype and an axon that extends to reach its synaptic target. The interaction of neuronal cell surface receptors with attractive and repulsive guidance cues secreted by intermediate and final cellular targets dictates a defined trajectory that the neuron will navigate [70, 71]. Until now, molecular studies have led to the identification of several families of guidance cues over the past 15 years including netrin and its receptors, Robo/Slit, and semaphorins/collapsins [72].

Netrin proteins were originally identified in embryonic chick brain, where they direct commissural axons in a circumferential trajectory to the floor plate at the ventral midline of the spinal cord [73]. Later, netrin was found to be capable of repelling trochlear motor axons, that grow away from the floor plate underlining their role as bi-functional chemotropic guidance cues [74]. Netrins are phylogenetically conserved in all bilaterally symmetrical animals directing a wide range of outgrowing axons and migrating neurons. Subsequent work established that netrins have multivalent ability and are also involved in the development and maintenance of several non-neuronal tissues by regulating processes such as cell adhesion, migration, survival, differentiation and branch morphogenesis [2].

Netrin-1 is a member of the family of secreted netrins, which consists of netrin-1, netrin-3, and netrin-4. Recently, netrin-5 was also identified [75]. In addition to secreted netrins, there are two membrane-tethered netrins, G1 and G2, which are attached to the plasma membrane by a glycophosphatidylinositol (GPI) anchor and are believed to have evolved independently of

secreted netrins. Netrin family proteins consist of an N-terminus laminin-like VI-V domain which is attached to a C-terminal netrin-like domain (NTR) in secreted netrins (Fig. 2A). The N-terminal domains of netrin 1 and netrin 3 share homology with the laminin γ1 short arm, and those of netrins 4, G1 and G2 are homologous to the laminin β1 chain (Fig. 2B). The C-terminal sequence of secreted netrin is rich in basic amino acid residues which interacts with heparin sulfate proteoglycans (HSPGs) which presents secreted netrins on cell surfaces or retains them in the extracellular matrix (ECM) (Fig. 1C). For this reason, the majority of netrin proteins are not freely soluble *in vivo* but are retained on the cell membranes in the ECM [2].

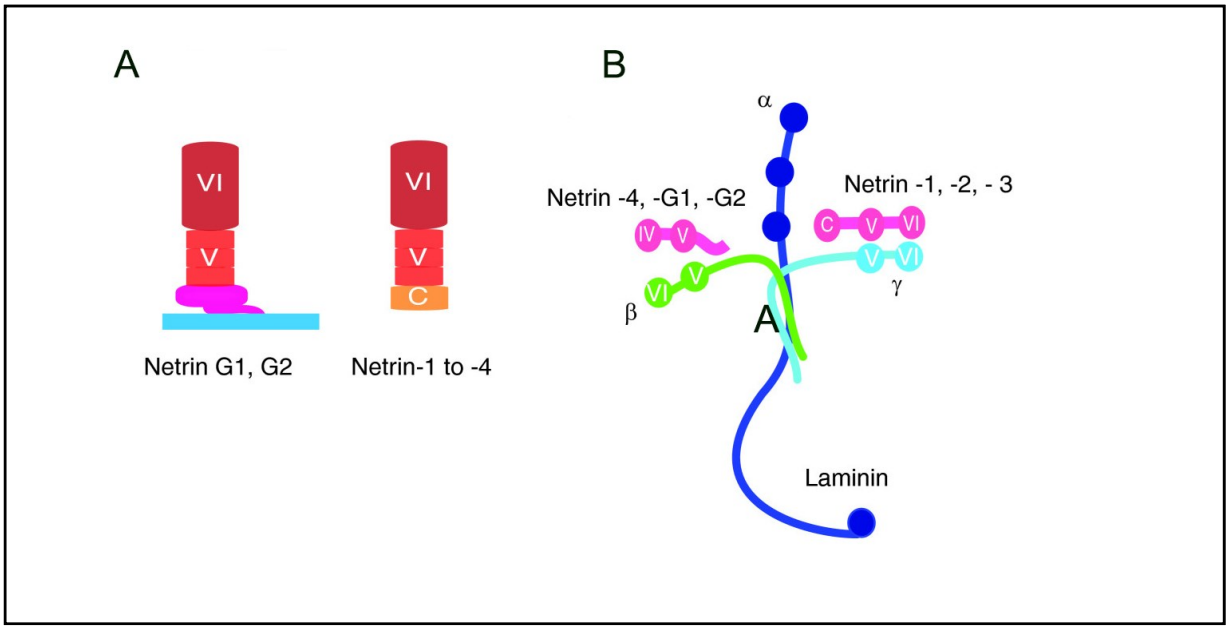


Figure 2. The netrin family of proteins. (A) Netrins 1 to 4 are secreted proteins that are attached to a C-terminal domain whereas netrins G1 and G2 are linked to the plasma membrane by a GPI linker. (B) Laminin 1 is a heterotrimer composed of α (blue), β (green), and γ (turquoise) chains. The amino-terminal VI and V domains of netrins 1 to 3 (red) are homologous with the γ chain of laminin 1. These domains in netrins 4, G1 and G2 resemble β chain of laminin 1. Source of image: Rajasekharan and Kenned, 2009

I.3.1 Netrin-1: the prototypical member of the netrin family

Since their discovery, secreted netrins have been shown to have overlapping functions and receptor repertoires. However, the diverse functional roles of netrins have been best characterized in studies of netrin-1. In fact, netrin-1 has been extensively shown to be implicated in axonal outgrowth and neuronal migration during embryogenesis. The molecule's cDNA encodes a 603 amino acid (aa) protein precursor of approximately 75 (kDa) [76]. Mouse netrin-1 shares 52% aa identity with mouse Netrin-3, and 98% and 87% aa identity with human and chicken. Netrin-1 and netrin-3 are essentially functionally equivalent: both bind to the same receptor proteins and evoke chemoattractant or chemorepellent responses from responsive cells [77]. Loss-of-function mutations in netrin-1 or in certain netrin-1 receptors are lethal in mice, highlighting the importance of netrin-1 signaling during development [2]. In the following chapters, I will summarize the current state of knowledge on netrin-1 and its receptors and discuss netrin-1 functions in the CNS both during development and in adulthood.

I.3.2 Multiple receptors for netrin-1

Netrin-1 exerts most of its regulatory functions through the signaling pathways downstream of its two main receptors: Deleted in Colorectal Cancer (DCC) and the Uncoordinated-5 homologous family (UNC5A, -B, -C, and –D). Other secreted netrins are known to interact with these receptors with different affinity. Recently Down's syndrome cell adhesion molecule (DSCAM) and adenosine receptors (A2b) have also been classified as netrin-1 receptors. All netrin-1 receptors identified thus far are single-pass type I transmembrane proteins and are members of the immunoglobulin (Ig) superfamily (Fig. 3)[1].

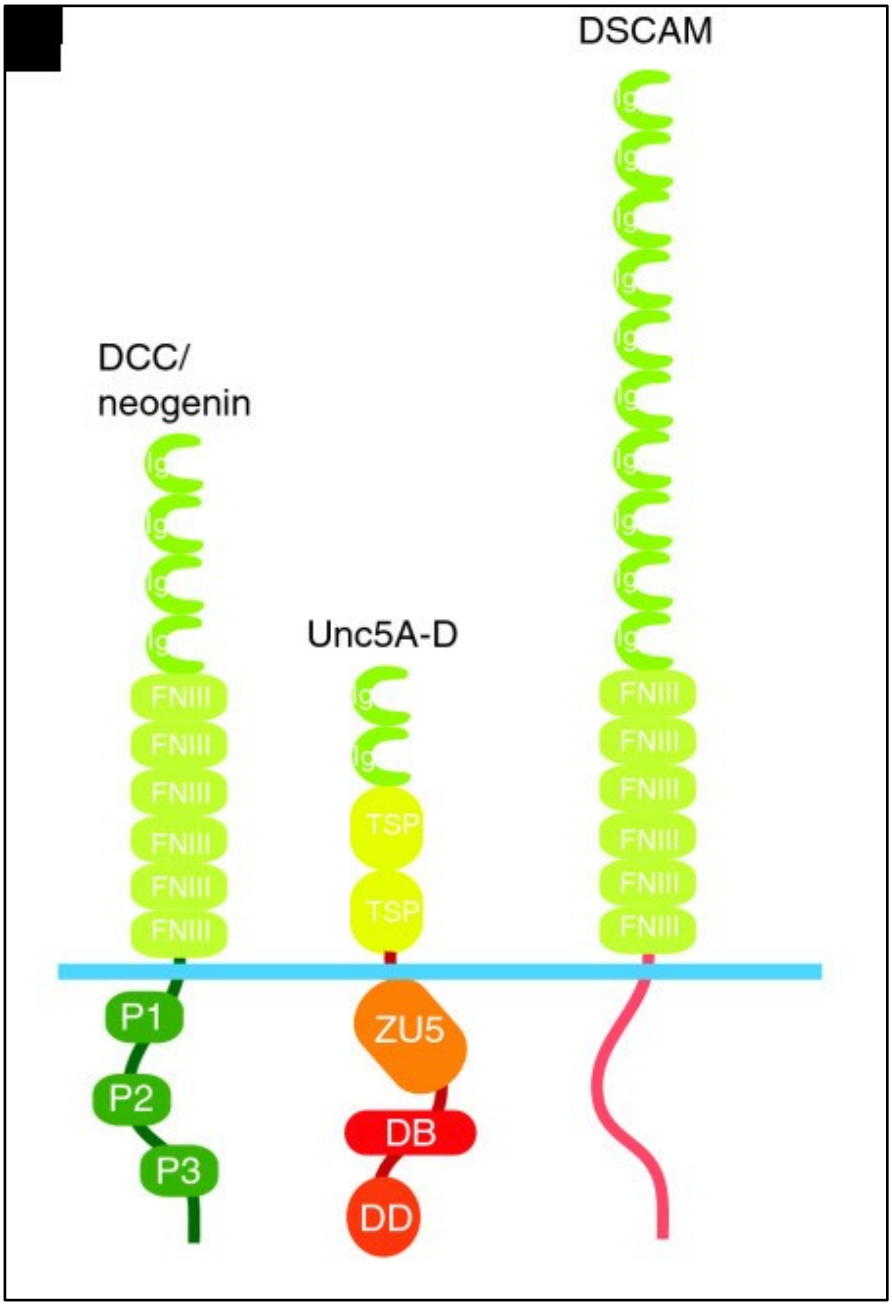


Figure 3. Canonical netrin-1 receptors. The DCC receptor family, the UNC-5 homologs (Unc5A-D), and DSCAM are receptors for netrin-1. DB, DCC-binding domain; DD, death domain; Ig, immunoglobulin domain; FNIII, fibronectin type III repeat; TSP, thrombospondin type-I module; ZU5, domain homologous to part of Zona Occludens-1. Source of the image: Rajasekharan and Kennedy, 2009.

I.3.2.1 The DCC receptors

The DCC receptor family was discovered in 1996 as a cell surface receptor encoded within a 370-kb region on chromosome 18q that is deleted in tumors. Therefore, initially it was proposed as a putative tumor suppressor gene whose absence on colon cells implied a malignant state of colon cancer [78]. Later, it was proposed as a receptor for netrin-1. The DCC receptor family is comprised of both DCC and neogenin with approximately 50% aa identity. Neogenin receptor functions are generally associated to cell adhesion rather than axon guidance. The interaction of neogenin with netrin-1 regulates cell-cell adhesion at paranodal junctions and synapses [2].

Extracellularly, the DCC receptor is composed of four Ig-like domains at its N-terminus, followed by six fibronectin (FN) type III domains, an approximately 50-residue long membrane-proximal stalk and a transmembrane segment. The cytoplasmic tail of DCC is catalytically inactive and comprises three highly conserved domains (P1, P2, and P3) that are involved in conducting netrin-1 downstream signal transduction pathways [79].

Several axon tracts in the spinal cord and the brain express DCC and the loss of DCC expression in mice causes defects in the formation of spinal and cerebral commissures that are comparable to those observed in netrin-1deficient mice [80]. Moreover, mutations in the DCC gene locus are associated with mirror movement disorders in humans and mice, a reported consequence of midline crossing defects [2].

Netrin-1 protein can simultaneously bind to two DCC molecules through a DCC-specific site and a unique generic receptor binding site. This characteristic two-site feature is the key for the interpretation of netrin-1 bi-functionality which will be recapitulated in detail in the chapter 3.3 [81].

I.3.2.2 The UNC5 receptor family

The family of UNC5A-D receptors are transmembrane protein with an extracellular domain consisting of two immunoglobulin repeats followed by two thrombospondins type I groups (TSP-1). The immunoglobin repeats of UNC5 proteins are required for netrin-1 binding.

The UNC5 intracellular domain is about 550 residues and encodes three distinct domains that are required for receptor signaling: (A) ZU-5 domain, a domain of yet undetermined function, named for its homology with a portion of Zona Occludens-1, (B) DCC-binding (DB) motif, and (C) Death Domain (DD), associated with apoptotic signaling [1]. UNC5A-D receptors are widely expressed across the nervous system including neurons that express DCC. In fact, in the presence of UNC5, DCC is replaced by UNC5A on the generic receptor binding site of netrin-1 switching the netrin-1 response from chemoattraction to repulsion [81].

I.3.3 Netrin-1: bi-functionality and reverse signaling

Netrin-1 is a guidance cue that depending on the receptor it binds to, it triggers either attractive or repellent effects on migrating neurons. In general, netrin-1 exerts its chemoattraction activity when it binds to DCC receptors. Upon binding, netrin-1 brings two DCC receptors in close proximity and mediates DCC homo-dimerization by bringing DCC cytoplasmic P3 motifs in close proximity. The dimerized P3 motifs serve as a structural unit for further signaling and recruit intracellular signaling complexes leading to the release of calcium, kinase activation and a rearrangement of the cytoskeleton [2]. On the other hand, netrin-1-mediated chemorepulsion is induced when netrin-1 binds to a DCC and a UNC-5 receptor. In the presence of netrin-1, a ternary complex is formed between netrin-1 and the extracellular portions of UNC-5 and DCC. This heterodimerization through a cytoplasmic interaction between the P1 motif of DCC and the DCC-binding domain (DB) of UNC-5 gives rise to an alternate signaling complex leading to chemorepulsion [81]. Therefore, netrin-1 appears to serve as a scaffold both for symmetric clustering of a single receptor, and asymmetric clustering of a pair of different receptors with remarkable versatility [79, 81, 82]. An important generalization drawn from these data is that the intracellular domain of netrin-1 receptors is determinant in netrin-1 signaling as an attractant or repellent factor. The signaling pathways downstream of DCC and UNC-5 receptors are explained in the next chapter.

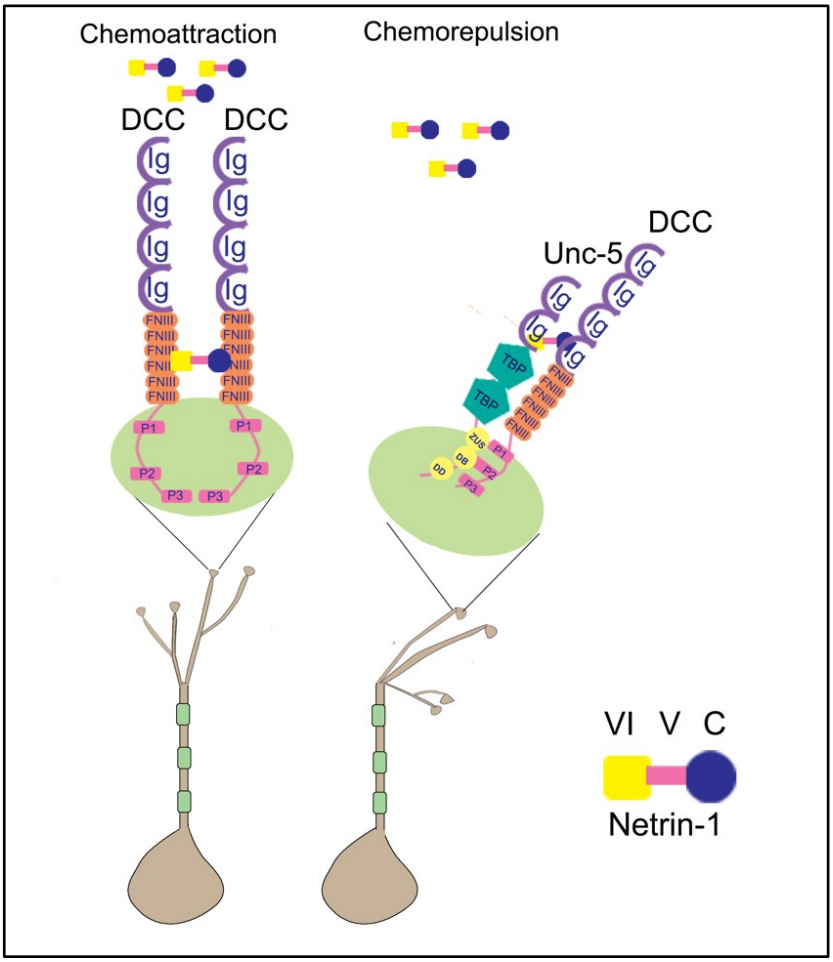


Figure 4. Netrin-1 is a bifunctional axonal guidance cue. Netrin-1 can bind to the ecto-domains of two receptors DCC/DCC or DCC/UNC5, and brings them close enough to initiate interactions between their cytoplasmic domains leading to either chemoattraction or chemorepulsion. Source of image: Pegah Chehrazi

I.3.4 Netrin-1 signaling mechanisms

Netrin-1 signaling has been largely studied in relation to axon guidance where axon motility is directed by orchestrating the dynamic reorganization of actin cytoskeleton in axonal growth cones. The growth cone, a fan-like structure located at the tip of an extending axon, contains the machinery necessary to detect and respond to guidance cues and provides the motor energy necessary for neurite outgrowth [83]. Upon netrin-1 binding, cytoplasmic signal transduction molecules in the growth cone link the activation of axon guidance receptors to the reorganization of the actin cytoskeleton [84]. In the case of chemoattraction, the

membrane extension toward the source of netrin-1 is derived by the insertion of DCC at the leading edge of growth cones, the stabilization of DCC in the plasma membrane and the linking of DCC to actin filaments [85]. However, DCC is a transmembrane protein without an obvious catalytic domain, and thus for a long time it remained unclear how it initiates downstream signaling to regulate axonal outgrowth. Recently, it was shown that DCC functions as a tyrosine kinase-associated receptor and the intracellular domain (ICD) of DCC is constitutively bound to the adaptor protein Nck1 and focal adhesion kinase (FAK). With the binding of netrin-1 to DCC, NCK1 and FAK phosphorylate DCC ICD resulting in the recruitment of a number of intracellular signaling components that activate several parallel cascades: Src family kinases, Rho GTPases, the release of Ca^{2+} stores and protein translation. All these pathways ultimately converge on the rearrangement of the actin cytoskeleton [86, 87]. The signaling mechanisms that underlie netrin-1-induced chemorepulsion are considerably less well understood than those underlying chemoattraction.

Activation of small GTPases

Netrin-1 binding to DCC results in FAK activation which recruits SFKs, Src, and Fyn to the DCC ICD, leading to Nck1-dependent activation of p21 activated kinase1 (PAK1) as well as Rho GTPases [86, 88]. Rho GTPases, in particular Rac1 and Cdc42, are important signaling molecules involved in promoting actin polymerization at the extending edge of the growth cone. They further activate the actin nucleation factor Arp2/3 through the stimulation of the Wiskott-Aldrich syndrome protein (WASP) family members. Another member of Rho GTPases, RhoA, is inhibited during DCC activation leading to DCC endocytosis and thus promoting the trafficking of the DCC receptor on plasma membranes [1, 74, 89].

Regulation of mRNA translation machinery

Extending axons contain the machinery for local protein translation, providing the growth cone with a substantial level of functional autonomy from the cell body during embryogenesis. Netrin-1/ DCC signaling is shown to regulate protein translation via PI-3 kinase and rapamycin-sensitive mTOR protein synthesis. In particular, in response to actin-dependent

changes in growth cone motility, netrin-1 mediated DCC signaling regulates the local translation of β-actin mRNA by relieving its translational inhibition from Zipcode Binding Protein 1(ZBP1) [2, 90, 91].

DCC-dependent signaling also involves the activation of the mitogen-activated protein kinase (MAPK) cascade by extracellular signal-regulated kinase (ERK)-1 and 2 which are phosphorylated following netrin-1 receptor activation. The activation of MAPK pathway further results in the activation of specific transcription factors such as ELK1 underlining a potential mechanism by which netrin-1 may control transcription and protein translation [92].

Calcium in regulating neurite growth and motility

Notably, cytoplasmic Ca^{2+} participates in the concerted regulation of the actin cytoskeleton to promote growth cone extension. The binding of netrin-1 to DCC promotes 1,4,5-trisphospate (IP3)-mediated Ca^{2+} release from internal stores, which is necessary to maintain Ca^{2+} within the optimum range for neurite extension. The release of Ca^{2+} from intracellular stores also activates transient receptor potential (TRP) channels to trigger a Ca^{2+} influx across the plasma membrane [93, 94].

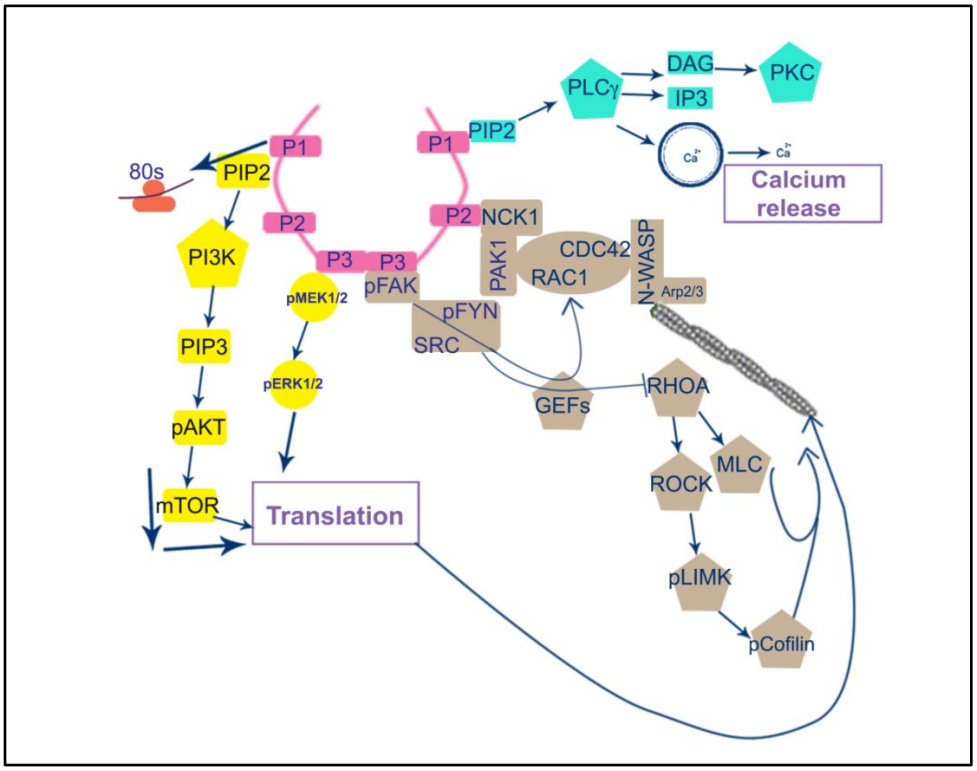


Figure 5. Netrin-1 signal transduction mechanisms. In response to netrin-1, DCC receptors homodimerize via their P3 domains interaction. The DCC P3 domain is bound to FAK while DCC P2 domain is constitutively bound to NCK1. With netrin-1 binding, Nck1 and their downstream effector Pak1 serve as a scaffold for the recruitment of Rho family of small GTPases, in particular Rac1, and Cdc42 and RhoA. Furthermore, FAK recruits and activates the tyrosine kinases Src and Fyn, which also participate in regulating Rac1 and Cdc42 activity. Activated Cdc42 further recruits N-WASP, which promotes the nucleation of F-actin via the Arp2/3 complex modulating the dynamics of actin polymerization. In addition, PI3K and PLCγ are activated in response to netrin-1 binding. PLCγ hydrolyses PIP2 into IP3 and DAG. IP3 stimulates the release of intracellular calcium, and DAG activates PKC, which in turn result in cytoskeleton remodeling. Abbreviations: Pak1, p21-activated kinase; FAK, focal adhesion kinase; Src, tyrosine kinase sarcoma; ERK, extracellular-signal-regulated kinase; MAPK, mitogen-activated protein kinase; PLCγ, phospholipase Cγ; PI3K, phosphatidylinositol 3-kinase; PIP2, phosphatidylinositol (4,5) bisphosphate; IP3, inositol triphosphate; Rac1, Ras-related C3 botulinum toxin substrate 1; Cdc42, cell division cycle 42; N-WASP, neuronal Wiskott–Aldrich syndrome protein; Pack1, serine/threonine kinase p21 activated kinase 1; and Cdc42, cell division cycle 42; RhoA, Ras homologous member A; PKC, protein kinase C. Source of image: Pegah Chehrazi

I.3.5 Netrin-1: a role both as a long-range and a short-range cue

In addition to bi-functionality, netrin-1 has the dual characteristic of functioning at short or long distance. Netrin-1 functions at short-range when it induces signaling in the immediate vicinity of its cellular source. For example, in the mature nervous system, netrin-1 mediates cell-cell contacts between neurons at synapses, as well as between oligodendrocytes and axons at paranodal junctions [1]. By contrast, netrin-1 functions as a long-range cue when acting far from its secreting source. For example, netrin-1 guides the axonal projections across the midline in the embryonic brain and spinal cord [95]. Long-range netrin-1-induced repulsion, which requires higher sensitivity to netrin-1, is mediated by multimerization of UNC5 with DCC as a co-receptor; while the homodimerization of UNC5 homologues in the absence of DCC is enough to mediate short-range repulsion in response to netrin-1 [96].

I.3.6 Calcium modulates netrin-1 signaling

Cyclic adenosine monophosphate (cAMP) is a second messenger that is mainly recognized for its role on axon guidance and axon regeneration. Bi-directional turning responses of growth cones to netrin-1 depend on the relative activities of cAMP dependent pathways, which regulate the cytosolic level of Ca^{2+} signals in two different ways: 1) by regulating Ca^{2+} influx through voltage-dependent Ca^{2+} channels in the plasma membrane (VDCCs) [97], and 2) by controlling the release of Ca^{2+} from intracellular stores. The intracellular level of Ca^{2+} in turn regulates Rho GTPase activation and cytoskeletal dynamics [98, 99]. The netrin-1-induced chemoattractive activity is associated with a high level of cAMP, whereas the netrin-1-mediated chemorepulsive activity is related to a low cAMP level [100].

I.3.7 Other putative netrin-1 receptors

I.3.7.1 DSCAM

The Down's syndrome cell adhesion molecule (DSCAM) receptor was originally identified as a candidate gene duplicated in Down's syndrome. However, for a long time, it was considered as an orphan receptor without an identified ligand. The recent identification of netrin-

1 as a ligand for DSCAM, suggests that some of the deficits associated with this disorder might result from altered netrin-1 signaling. In this regard, Ly *et al*. showed that DSCAM is expressed on spinal commissural axons where it mediates axonal outgrowth and turning in response to netrin-1 both alone or in collaboration with DCC [80]. However, these data were contradicted by the analysis performed in DSCAM-null mice where axons outgrowth toward the floor plate were comparable to the level of axonal extension mediated by DCC alone [101]. However, signaling mechanisms activated by netrin-1 downstream of DSCAM have not yet been identified. Further investigation of the signaling molecules downstream of DSCAM will help clarify the discrepancy between the aforementioned studies.

It is interesting to mention that DSCAM is also shown to function as a repulsive netrin-1 receptor, which in collaboration with UNC5C, mediates netrin-1-induced growth cone collapse. Importantly, main signaling components involved in netrin-1-mediated attraction such as Fyn/FAK/PAK1 signaling were suggested to be required for the coordination of netrin-1/DSCAM and netrin-1/UNC-5-mediated repulsive signaling [102].

I.3.7.2 A2B

A2b is a member of the family of the adenosine-specific receptors that mediates adenylate cyclase activity in response to adenosine binding. A2b receptors are emerging as a regulator of netrin-1 receptor trafficking and thus modulating the response of axons to netrin-1.

As a growth cone extends toward its target, it modulates its direction in response to different guidance cues that are expressed in distinct multiple places along the path. For example, as RGC axons migrating from the retina reach the optic nerve head, a developmental switch from chemoattraction to chemorepulsion is required to convert the growth-promoting signal of netrin-1 into a repulsive one [91]. This allows the RGCs axons to leave the optic nerve head toward the optic chiasm [103]. It is shown that the activity of intracellular signaling intermediates, such as protein kinase C-alpha (PKCa) and adenylyl cyclase activated by A2b receptors, regulates cell surface levels of netrin-1 receptors underlining the versatile nature of the response of axons to netrin-1. The A2b receptor regulates multiple signaling pathways

including PKC and cAMP, with high levels of cAMP favoring chemoattraction while low levels of cAMP encouraging chemorepulsion [104, 105].

I.3.8 The functional role of netrin-1 in the nervous system during development

I.3.8.1 Axon guidance

During development, a gradient of netrin-1 emanating from the floor plate directs the circumferential projection of commissural axons toward and across the ventral midline in the embryonic spinal cord. In contrast, it repels the trochlear motor neuron axons away from the floor plate towards the dorsal midline in the brain stem [106].

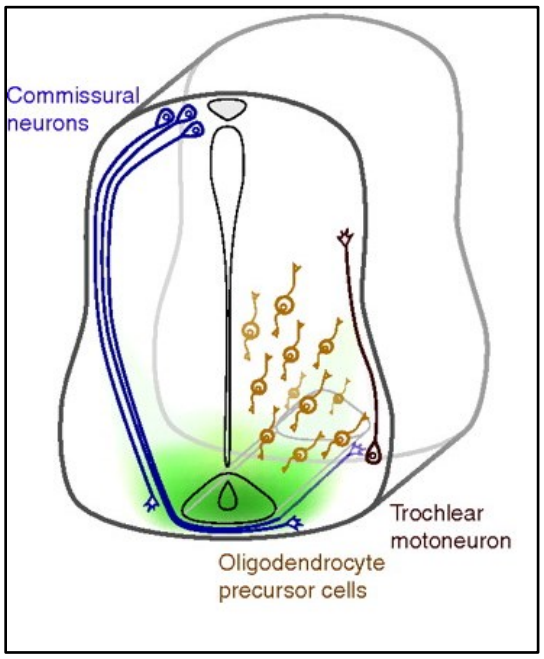


Figure 6. Netrin-1 orients axonal migration during development. In the developing spinal cord, commissural neurons located dorsally extend axons toward a gradient of netrin-1 at the floor plate resulting in the development of the dorsal-ventral axis of the brain and spinal column. The chemorepulsive activity of netrin-1 was shown on the axons of trochlear motor neurons, whose cell bodies are located in the ventral neural tube and their axons innervate the extraocular muscles of the eye. Source of image: Lai Wing Sun *et al,* 2009

I.3.8.2 Neural precursor cell migration

Netrin-1 also acts as a bifunctional regulator of neuron migration. During cerebellar development, netrin-1 acts as an attractant for precerebellar neurons directing them to ultimately populate the pontine nuclei. Subsequently, during postnatal cerebellar development, netrin-1 acts as a repellent for the cerebellar granule neurons (CGN) precursors, which upregulate Unc5 expression as they exit the external granule layer [2]. Netrin-1 is also required for the development of the olfactory bulb at late embryogenesis where it directs the migration of neuronal cells to specific regions of the lateral olfactory tract where they act as guidepost cells for olfactory bulb axons [1].

I.3.8.3 Synaptogenesis

Although primarily recognized for its role as an axon guidance cue in the early stages of CNS development, accumulating data indicate that netrin-1 is a synapse-enriched protein with the capacity to regulate synapse formation and stability in two major ways. First, netrin-1 increases the probability of axon and dendrite contact by increasing the number and complexity of their arbors [107]. Second, at the site of contact, netrin-1 promotes synaptogenesis by increasing the focal accumulation of synaptic proteins through enrichment and reorganization of the actin cytoskeleton [108]. In this regard, it is suggested that netrin-1-related changes in synapse function might influence the development and progression of certain forms of human neurodegenerative diseases. For example, functional DCC heterozygosity and a subsequent reduction in DCC gene dosage were introduced as the main cause of neural circuit disruption in congenital mirror movements disease [2].

I.3.8.4 Netrin-1 in oligodendroglial development and maturation

Developing oligodendrocytes extend multiple branching processes over considerable distances, surveying the local environment to locate unmyelinated axons. Recently, it is shown that netrin-1 plays an important role in oligodendroglial maturation by facilitating the detection of target axons. At early stages of development, netrin-1 promotes the morphological maturation

of oligodendrocytes and thereby increases their chances of encountering with appropriate axonal targets [2]. At later stages of maturation, oligodendrocytes begin to express netrin-1 themselves which participates in the formation of large myelin membrane sheets via process extension and branching. Interestingly, the lamella elaborated by the tip of an extending oligodendrocyte process has been shown to be comparable with the neuronal growth cone. Notably, common intracellular signaling proteins are shown to be implicated in netrin-1-mediated axon guidance and oligodendrocyte development [109].

I.3.8.5 Retinal circuit development

Visual information is relayed from the eye to the brain via the axons of retinal ganglion cells (RGCs). Over 50,000 RGC axons in mice and over a million RGC axons in humans must be guided accurately into the optic nerve during retinal embryonic development. The extension of RGC axons along this precise path as well as synapse formation with appropriate target cells are shown to be highly dependent on netrin-1/DCC signaling [110]. At the time of optic fissure closure, netrin-1 is expressed strongly by the neuroepithelial cells that surround and extend into the optic disc while DCC is expressed by RGC axons. The interaction between netrin-1 and DCC locally guide the growing RGC axons to leave the retina and form the optic nerve. In mice lacking either netrin-1 or DCC, RGC axons navigate normally to the optic disc but fail to exit the eye resulting in smaller optic nerves known as optic nerve hypoplasia [111].

As mentioned above, processes of retinal interneurons (amacrine and bipolar cells) form synapses on dendrites of RGCs in the inner plexiform layer (IPL). The IPL is divided into at least 10 parallel sublaminae. Interestingly, subsets of interneurons and RGCs arborize and form synapses in only one or a few sublaminae. These lamina-specific circuits determine the visual features to which RGC subtypes respond. Interestingly, it has been shown that DSCAM is one of the netrin-1 receptors that plays an important role in this specific laminar targeting by directing correct process arborization and synapse formation between RGCs, amacrine cell and bipolar cells [112, 113].

I.3.9 The role of netrin-1 in the adult nervous system

Although originally identified for its developmental role in guiding axons and neurons to their appropriate target, important information about the expression and function of netrin-1 in adult CNS is beginning to emerge and will be discussed in the following sections.

I.3.9.1 Netrin-1 regulates synaptic function and plasticity in the adult brain

Increasing evidence suggests that many proteins that are essential for normal neural development are also expressed in the adult brain where they influence synapse formation and plasticity [114]. For example, Horn *et al*. have demonstrated that DCC is enriched in dendritic spines of mature forebrain neurons and is implicated in regulating synaptic function and plasticity. Notably, by regulating NMDAR dependent long-term potentiation (LTP), netrin-1-mediated DCC signaling plays a major role in spatial and recognition forms of memory [5].

I.3.9.2 Netrin-1 as an anti-apoptotic agent: the dependence-receptor hypotheses

In recent years, DCC and UNC-5 have been proposed to function as so-called 'dependence receptors' meaning that they are dependent on the availability of netrin-1 to ensure cell survival. Based on this theory, netrin-1-activated receptor pathways were suggested to play an important part in tumorigenesis where the loss of receptor expression or upregulation of netrin-1 expression is predicted to provide a selective advantage to tumor cell growth [115]. These studies were further validated by observations showing that netrin-1/DCC signaling is frequently inactivated in many human malignancies [116]. However, the 'dependence receptor' hypothesis remains controversial. Mice lacking netrin-1 do not exhibit increased apoptosis in the CNS, arguing that netrin-1 is not an essential dependence ligand in the developing CNS. DCC is also shown to be required for the survival of RGCs and amacrine cells in the developing mouse retina [7]. Considering these data, the formerly demonstrated role of netrin-1 as a cell survival factor is proposed to be restricted to specific cells within the nervous system.

I.3.9.3 Netrin-1 expression in the adult retina

While the mature and embryonic functions for netrin-1 have been extensively examined in many organs of rodents, to our knowledge, only few studies have analyzed the expression of netrin-1 in the adult retina. Indeed, while an initial study demonstrated that the expression of netrin-1 completely disappears in adult rat retina [9], later studies asserted that netrin-1 expression is maintained at the level of the ganglion cell layer and the inner nuclear layer in adult mouse and rat retinas [8, 10]. Further work is required to clarify the expression and role of netrin-1 in the adult visual system.

II. Objective of the thesis, hypothesis and experimental approaches

Accumulating data support the presence of netrin-1 in the adult nervous system, suggesting the possibility that netrin-1 is also expressed in the adult retina. Although there is clear evidence of netrin-1 expression in the adult brain, the results on the analysis of netrin-1 expression in the adult retina have been contradictory. While some studies claim that the adult retina is virtually devoid of netrin-1, others show robust netrin-1 expression in adult retina [8-10]. Based on this, I sought to investigate the expression **of netrin-1 and its receptor DCC in the adult mouse retina.** For this purpose, I used three complementary techniques to ensure rigorous results: reverse-transcription polymerase chain reaction (RT-PCR), Western immunoblotting, and retinal immunohistochemistry using C57BL6 wild type mouse retinal tissue. In addition, I used the well-characterized and reproducible model of optic nerve axotomy to investigate the expression of netrin-1 in the injured retina with the goal of gaining insights into the potential role of netrin-1 in the mature retina.

III. Material and methods

III.1 Experimental animals

Animal procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care for the use of experimental animals (www.ccac.ca). Post-natal day 0 (P0), post-natal day 14 (P14), or adult (>2 months) C57BL/6 mice were used in all experiments. All surgeries were carried out in adult, female C57BL6 mouse (19-21g).

III.2 Western-blot analysis

Whole fresh retinas were rapidly dissected and homogenized with an electric pestle (Kontes, Vineland, NJ) in ice-cold lysis buffer (20 mM Tris PH 8.0, 135 mMm NaCl, 1% NP-40, 1% SDS and 10% glycerol supplemented with protease inhibitors). Protein homogenates were centrifuged at 14,000 rpm for 10 min, and the supernatants containing the solubilized proteins were collected. Retinal extracts (100 µg) or (75 µg) were resolved on 7.5% or 10% SDS polyacrymide gels and transferred to nitrocellulose membrane (Bio-Rad life science, Mississauga, ON). To block nonspecific binding, membranes were incubated in 5% dry skim milk dissolved in PBS containing 0.1% Tween-20 (PBST) for 1 hr at room temperature, followed by incubation with primary antibodies overnight at 4°C (Table 1). Membranes were washed and incubated in anti-rabbit or anti-mouse peroxidase-linked secondary antibodies (0.5 µg/ml, GE Healthcare, Baie d'Urfé, QC) for 1 hr at room temperature. Blots were then developed using chemiluminescence reagents (ECL; GE Healthcare) and exposed to autoradiographic film (X-OMAT; Eastman Kodak, Rochester, NY). Densitometric analysis was performed using ImageJ software (NIH, USA) on scanned autoradiographic films obtained from a series of 3 independent western blots each carried out using retinal samples from distinct experimental groups. The densitometric values obtained for netrin-1, netrin-3 and DCC were normalized with respect to their loading controls (β-actin or γ-actin) in the same blot to obtain the final protein ratios.

Table.1: List of primary antibodies used for immunofluorescence and Western Blot experiments.

Antibody	Source	Catalog #	Host	Dilution	Experimental Approach
Netrin-1	Abcam	ab126729	Rabbit	1.5 µg/ml	WB-IHC
DCC	BD Pharmingen™	554223	Mouse	0.5 µg/ml	WB-IHC
β-actin	Sigma-Aldrich	A5316	Mouse	0.5 µg/ml	WB
γ-actin	Sigma-Aldrich	A8481	Mouse	0.5 µg/ml	WB
RBPMS	Phospho Solutions	1832	Guinea pig	0.01 µg/ml	WB-IHC
Myc	Abcam	Ab9106	Rabbit	0.02 µg/ml	IHC

WB: Western blot; IHC: Immunohistochemistry

III.3 Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

Total RNA was purified from adult retinas and cerebellum (used as control). Animals were deeply anesthetized and the eyes were rapidly enucleated. The cornea and the lens were removed and the retina was collected and immediately homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA) with an electric pestle (Kontes, Vineland, NJ) followed by several passages through a 28-gauge needle. Total RNA was extracted and its purity was monitored by A260/A280 ratio. cDNAs were generated from 0.5 µg of total RNA using QuantiTect Reverse Transcription Kit (QIAGEN). The cDNA mixture (2 µl) was subjected to PCR in a 20 µL reaction in a thermal controller minicycler (MJ Research, Inc., Watertown, MA) (Table 2). The primers used are summarized in Table 3. Amplification conditions were: 3 min at 94°C, 31 cycles of 45 sec at 94°C, 30 sec at optimal annealing temperature, 1 min at 72°C and final extension of 10 min at 72°C (Table 3). PCR products (20 µL) were separated on agarose gels,

visualized under UV light and digitalized using Gel Doc TM EZ system (Biorad,Hercules,CA). Densitometric analysis were performed with ImageJ software (NIH, USA).

Table.2: PCR (Invitrogen Platinum Taq)

Master mix components	Amount (µl)
10X Buffer	2.0
50 mM MgCl2	0.6
10 mM dNTP	0.4
Forward primer 10 uM	1.0
Reverse primer 10 uM	1.0
Formamide	1.25
Platinum Taq (5u/µl)	0.5
20% Glycerol	10
dH2O	1.25
Total Volume	20

Table.3: List of primers used for RT-PCR experiments.

Target	Forward primer	Reverse primer	Annealing T°C	Product size
Netrin-1	5'TGCACACGTTCGGCGACGAG 3'	5'CAAGCCTTCCGGTGGGTGAT 3'	58.1	459
γ-actin	5'GCTTACACTGCGCTTCTT 3'	5'TGAGTCTTTCTGGCCCATG 3'	55.2	213

III.4 Retinal immunohistochemistry

Retinal cross-sections

Mice were perfused transcardially with 4 % paraformaldehyde in 0.1 M PBS (pH 7.4). The cornea and the lens were removed and the remaining eyecup was fixed for an additional 2h in 4 % paraformaldehyde at 4°C. Subsequently, the eyecup was equilibrated overnight in 30% sucrose solution and then embedded in optimal cutting temperature (OCT) compound (Tissue-Tek, Miles Laboratories, Elkhart, IN) and frozen in a 2-methylbutane/liquid-nitrogen bath. Retinal radial cryosections (16 µm) were collected onto gelatin-coated slides using a microtome (Leica Biosystems) and stored at -80°C. Next, retinal sections were incubated in 3% Bovin Serum Albumin (BSA) and 0.3% Triton X-100 (Sigma-Aldrich, St.Louis, MO) in PBS for 1h at room temperature to block non-specific binding. Subsequently, the primary antibodies dissolved in blocking solution were added to the retinal sections and incubated overnight at 4°C (Table 1). Sections were then washed and incubated with secondary antibodies: donkey anti-rabbit IgG Alexa 555, donkey anti-mouse IgG Alexa 627, and donkey anti-guinea pig IgG Alexa 488 (1.5 µg/ml, Jackon Immunoresearch Laboratories). Fluorescent staining was examined with an Axioskop 2 Plus Microscope (Carl Zeiss Canada). Images were captured with a CCD video camera (Retiga, Qimaging) and analyzed with ImageJ software (NIH, USA).

Flat-mounted retinas

Mice were perfused transcardially with 4% paraformaldehyde. The eyes were immediately collected and the retinas were carefully dissected out. Retinas were free floated overnight at 4°C in blocking solution (10% normal donkey serum (NGS), 2% Bovine Serum Albumin (BSA), 0.5% Triton X-100 in PBS). Next, sections were incubated for 5 days at 4 °C in blocking solution (2% NGS, 1% BSA, 0.5% Triton X-100) containing an antibody against the RGC-specific marker RNA binding protein with multiple splicing (RBPMS, 0.01 µg/ml, PhosphoSolutions, Aurora, CO). Samples were then incubated in secondary donkey anti-rabbit IgG Alexa Fluor 488 (1µg/ml Molecular Probes, Eugene, OR) and processed for cell density quantification as described below (section II.7).

III.5 Axotomy-induced RGC death assay

Axonal injury was induced by complete transection (axotomy) of the mouse optic nerve leading to rapid onset and predictable apoptotic loss of RGCs [55, 58, 117]. The left optic nerve was transected at 0.5-1 mm from the optic nerve head avoiding damage to the ophthalmic artery. Fundus examination was immediately performed at 3 days after axotomy to check the integrity of the retinal circulation after surgery. Animals showing signs of compromised blood supply were excluded from the study. Retinal whole-mounts were prepared as described above and analyzed histologically at 7 and 14 days after axotomy to determine the density of surviving RGCs.

III.6 Intravitreal injections

Recombinant full length netrin-1 protein (50 ng/µl) or truncated netrin-1 (1000 ng/µl) (kind gift of Dr. Timothy Kennedy, McGill University) (Table 4), as well as an adeno-associated virus (AAV) encoding netrin-1 (AAV.netrin-1, 5.06x1013 vg/ml) or control GFP (AAV.GFP, 8.23x1012 vg/ml) were injected into the vitreous chamber of the left eye using a custom-made glass microneedle (total volume: 2 µl). Control eyes were injected with sterile PBS or control AAV). The sclera was exposed and the tip of the needle was inserted at a 45° angle through the sclera and retina into the vitreous space using a posterior approach. This route of administration avoided injury to the iris or lens [118]. The injection was performed within ~30 sec, after which the needle was gently removed. Intraocular injections were performed immediately after optic nerve axotomy.

III.7 Quantification of RGC survival

At one or two weeks after axotomy, mice were euthanized by transcardial perfusion with 4% paraformaldehyde. The operated eye was dissected and fixed for an additional 2h. The retinas were removed and flat mounted with the vitreal side up on a glass slide for examination of the ganglion cell layer. The flat-mounted retinas were labeled with the RGC-specific marker

RBPMS. The labelled RGCs were counted within 3 square areas at distance of 0.25, 0.625 and 1 mm from the mouse optic disc in each of the retinal quadrants for a total of 12 retinal areas encompassing a total area of 1 mm2. Fluorescent staining was examined with an Axioskop 2 plus Microscope (Carl Zeiss Canada). Images were captured with a CCD video camera (Retiga, Qimaging) and analyzed with ImageJ software (NIH, USA).

II.8 Statistical analysis

Data analysis and statistics were performed using the Instat software (GraphPad Software Inc., San Diego, CA) by a one-way analysis of variance (ANOVA), followed by the Tukey's Multiple Comparison *post-hoc* test as indicated in the figure legend.

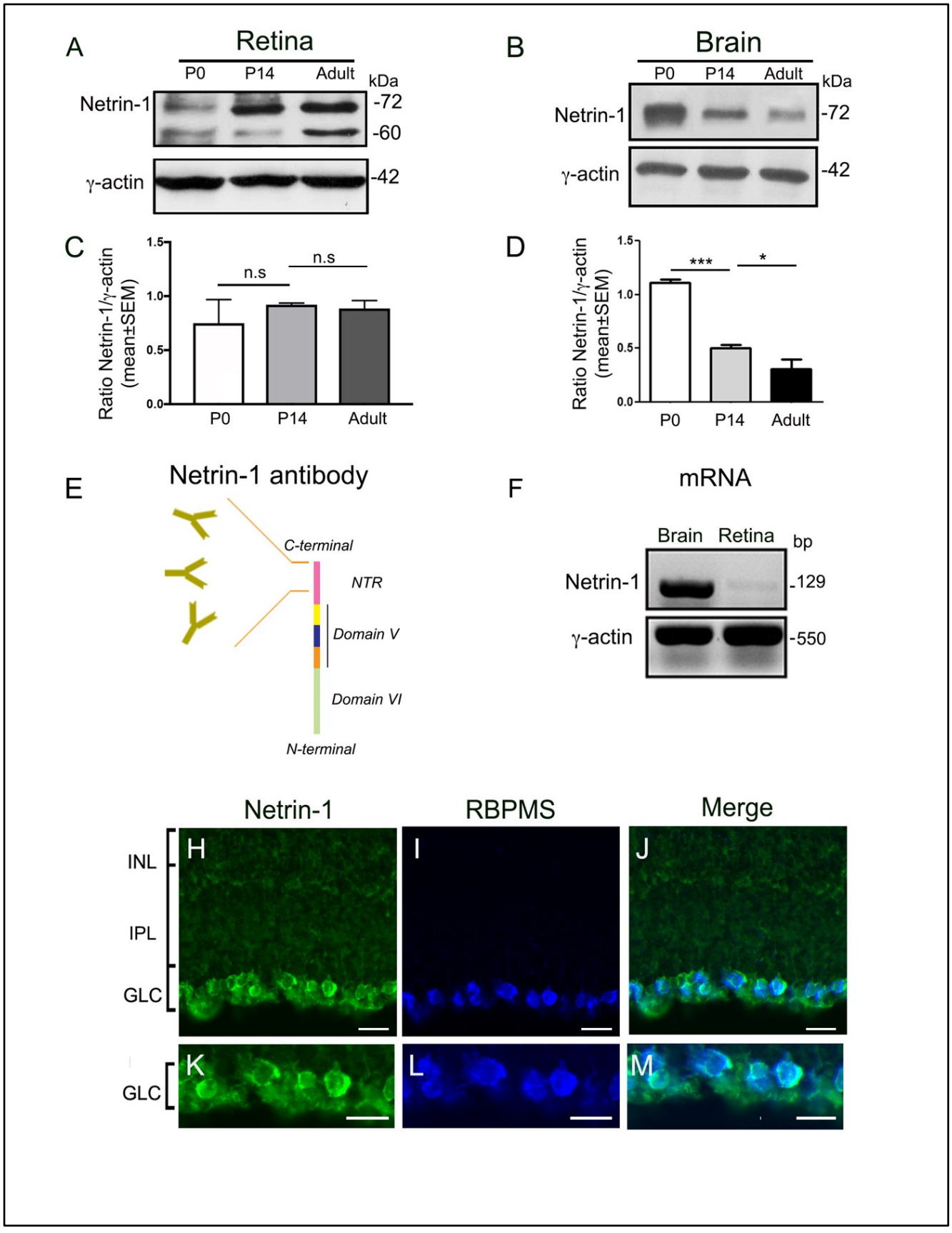
IV. Results

IV.1 Netrin-1 is expressed in the developing and adult mouse retina

During embryonic development of the retina, netrin-1 is expressed by epithelial cells that surround the optic nerve disc playing a crucial role in guiding growing RGC axons toward the optic nerve head [119]. However, the expression and role of netrin-1 in the adult retina is poorly understood. Thus, we first investigated the expression of netrin-1 by western-blot analysis of retinal protein homogenates from C57BL6 wild-type mice during early postnatal developmental (P0), at the time of eye opening (P14), and in adulthood (~2 months) (Fig. 1A). Given that netrin-1 is expressed in the mouse cerebellum postnatally [108], protein homogenates extracted from this tissue was used as positive control (Fig. 1B). As mentioned earlier, full-length netrin-1 contains the N-terminus laminin-like domains of VI and V and a Netrin-Like Domain called NTR module. In this study, we used a well-characterized netrin-1 antibody with specificity to sequences within NTR domain (Fig. 1E) [108]. Our results revealed a major band of 72 kDa corresponding to netrin-1 both during development (P0 and P14) and in the adult retina (Fig. 1A). An additional band of lower molecular weight (~60kDa), possibly a truncated form of netrin-1, was also observed in all retinal samples (Fig. 1A). Densitometric analysis revealed a trend towards the increase of netrin at P14 and adulthood relative to P0, but it was not statistical significant likely due to greater variability found in P0 samples (Fig. 1C). In the cerebellum, netrin-1 was abundantly expressed at P0 followed by a significant decrease in adult mice (Fig. 1D). Together, these data show that the expression of netrin-1 is not restricted to the developing retina during development but that its expression is maintained in the adult retina as well.

We also investigated the expression of netrin-1 by RT-PCR using template RNA extracted from adult mouse retinas (~2 months). RNA extracted from age-matched cerebellum samples were used as positive control. Using specific intron-spanning primers for netrin-1, positive 129-bp bands were observed (Fig. 1F). To establish the cellular localization of netrin-1, we next performed immunohistochemical analysis on retinal cross sections obtained from adult mice eyes (Fig. 1E). The expression of endogenous netrin-1 was detected primarily in the ganglion cell layer (GCL) where RGC bodies are located (Fig.1H-K). In addition to RGCs, displaced amacrine cells and astrocytes populate the ganglion cell layer. Hence, we performed co-localization analysis using

antibodies against netrin-1 and RBPMS, a selective RGC marker [120] (Fig.1I-L). Most RBPMS-positive neurons were immunoreactive for netrin-1 (Fig. 1J-M), indicating that adult RGCs are endowed with high levels of constitutive netrin-1 protein.


A
Retina
P0
P14
Adult
kDa
Netrin-1
-72
-60
γ-actin
-42
B
Brain
P0
P14
Adult
kDa
Netrin-1
-72
γ-actin
-42
C
Ratio Netrin-1/γ-actin (mean±SEM)
1.5
1.0
0.5
0.0
n.s
n.s
P0
P14
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D
Ratio Netrin-1/γ-actin (mean±SEM)
1.5
1.0
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E
Netrin-1 antibody
C-terminal
NTR
Domain V
Domain VI
N-terminal
F
mRNA
Brain
Retina
bp
Netrin-1
-129
γ-actin
-550
Netrin-1
RBPMS
Merge
INL
IPL
GLC
H
I
J
K
L
M
GLC

Figure 1. Netrin-1 expression is expressed in adult mouse retina. (A-B) Representative western blots of retinal and brain samples from P0, P14 and adult C57BL6 mouse probed with a specific netrin-1 antibody. Western blot analysis of equal amounts of retinal homogenates revealed the presence of a 72 kDa band corresponding to netrin-1 in samples from both developing and adult mice. An additional band of ~60 kDa related to a truncated netrin-1 was only observed in retinal samples. The lower panel represents the same blot as in the upper panels but probed with an antibody against β-actin and used to confirm equal protein loading. (C-D) Densitometric analysis revealed that the expression level of netrin-1 remains rather steady throughout retinal development, while it was sharply downregulated in adult brain. Values are expressed as the mean ± S.E.M. (n = 5/group, ANOVA, *p<0.1, **p<0.01, ***p<0,001; Tukey's Multiple Comparison *post-hoc* test). (E) Netrin-1 antibody targets a specific peptide sequence in netrin-1 NTR domain. (F) RT-PCR analyses confirmed the expression of netrin-1 in adult retina and brain samples. (H) Netrin-1 localization in the ganglion cell layer (GCL) in the adult mouse retina. (I-J) Co-labeling of netrin-1 and RBPMS, a RGC-specific marker, revealed that RGC soma are endowed with robust netrin-1 expression. Scale bars: (H-J) =20 μm; (K-M) =22.5 μm. INL: inner nuclear layer, IPL: inner plexiform layer.

IV.2 DCC is abundantly expressed in early developing mouse retina and it is downregulated in adulthood

The growth of embryonic RGC axons out of retina toward the developing optic nerve is mediated by a DCC-dependent response of the RGC axons to netrin-1 [121]. DCC also plays a crucial role in the survival of newly born RGCs and displaced amacrine cells [7]. However, the expression of DCC in adult retina is largely unknown. Therefore, we aimed to investigate the expression of DCC protein in the adult retina by western blot analysis. For this purpose, adult retinal protein samples were analyzed at developmental stages P0, P14, and adult. Samples from age-matched cerebellum were used as a positive control. Visualization of blots probed with a monoclonal antibody that recognizes the intracellular domain of DCC (DCCIN) revealed a ~185 kDa band corresponding to DCC in P0, P14 and adult samples (Fig. 2A-C). Densitometric analysis demonstrated that DCC is abundantly expressed during early postnatal developmental stages and undergoes a marked downregulation in adulthood both in the retina and the brain (Fig. 2B-D).

To determine which retinal cells express DCC, immunolabeling of adult mice retinas was performed using the DCC_{IN} antibody. Our results showed abundant expression of DCC in RGC somata located in the ganglion cell layer (Fig. 2E). Co-labeling of DCC with RBPMS revealed that many RGCs display robust DCC immunoreactivity indicative of DCC protein expression (Fig. 2F, G).

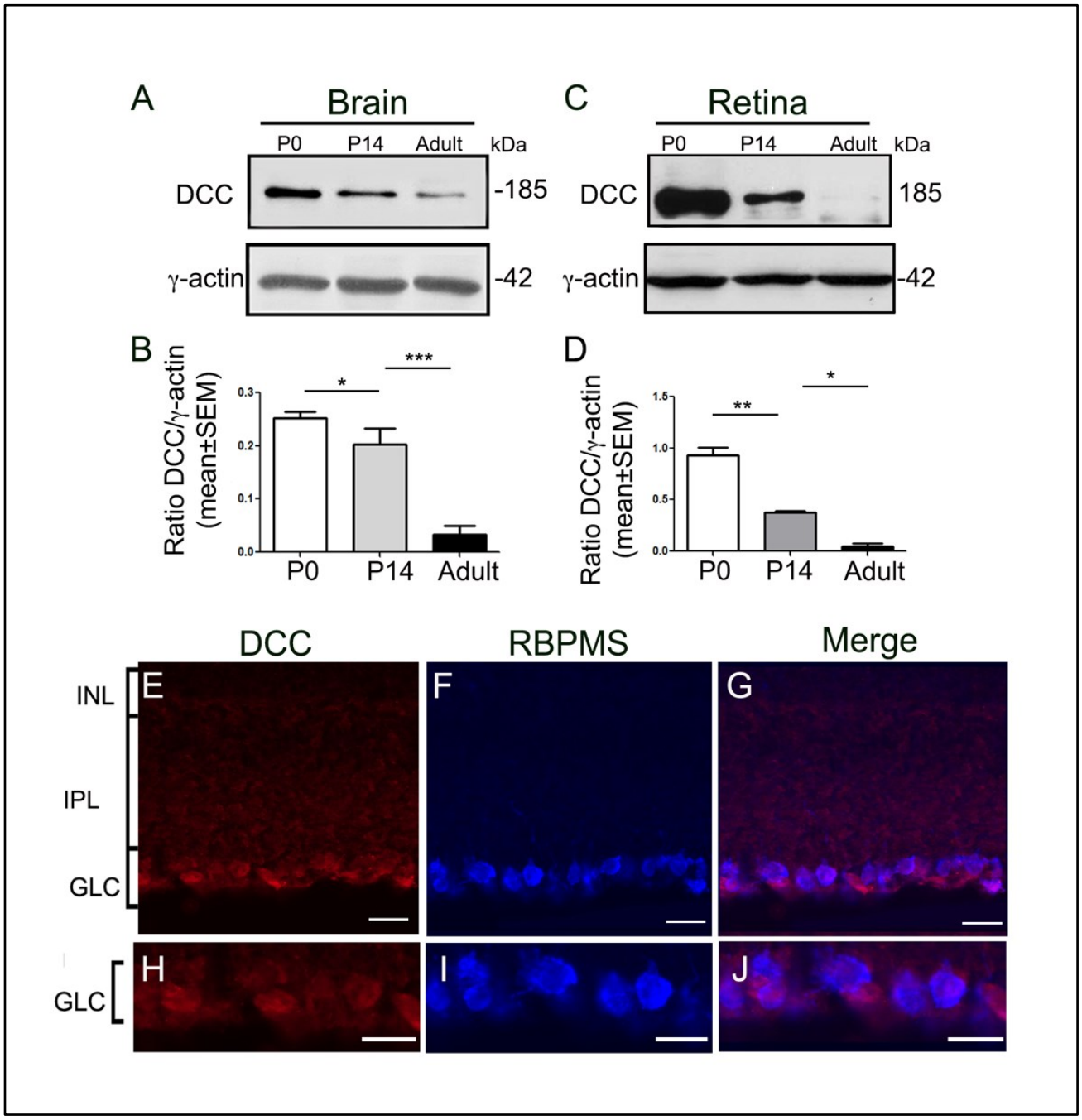

Figure 2. DCC is expressed in the adult mouse retina. (A-C) Representative western blots of soluble retinal and brain extracts from P0, P14 and adult C57BL6 mice probed with a DCC_{IN} antibody demonstrated the presence of a ~185 kDa band corresponding to DCC. (B-D) Densitometric analysis showed a high level of DCC expression at P0 with a marked decrease in P14 and adult samples in both brain and retinal samples. The lower panel represents the same blot as in the upper panels but probed with an antibody that recognizes *β*-actin used to confirm equal protein

loading (n=5/group, ANOVA, *p<0.1, **p<0.01, ***p<0,001; Tukey's Multiple Comparison *post-hoc* test). (E) Immunohistochemistry of retinal cross sections using DCC_{IN} antibody revealed the expression of DCC predominantly in the ganglion cell layer (GCL) of the adult retina. (F-G) Co-labeling of DCC with RBPMS, revealed that RGC soma abundantly express DCC. Scale bars: (E-G)=20 µm and (H-J)=22.5 µm. INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer.

IV.3 Adult RGC soma abundantly express netrin-1 and DCC

Next, we investigated the colocalization of netrin-1 with DCC in adult retinas (Fig.3A, B). Netrin-1 is known to bind and regulate its receptors both in a paracrine and autocrine fashion [77, 122]. Double-labeling of retinal cross-sections with netrin-1 and DCC antibodies displayed a robust colocalization of netrin-1 with DCC in the ganglion cell layer of adult mouse retinas (Fig. 3C).

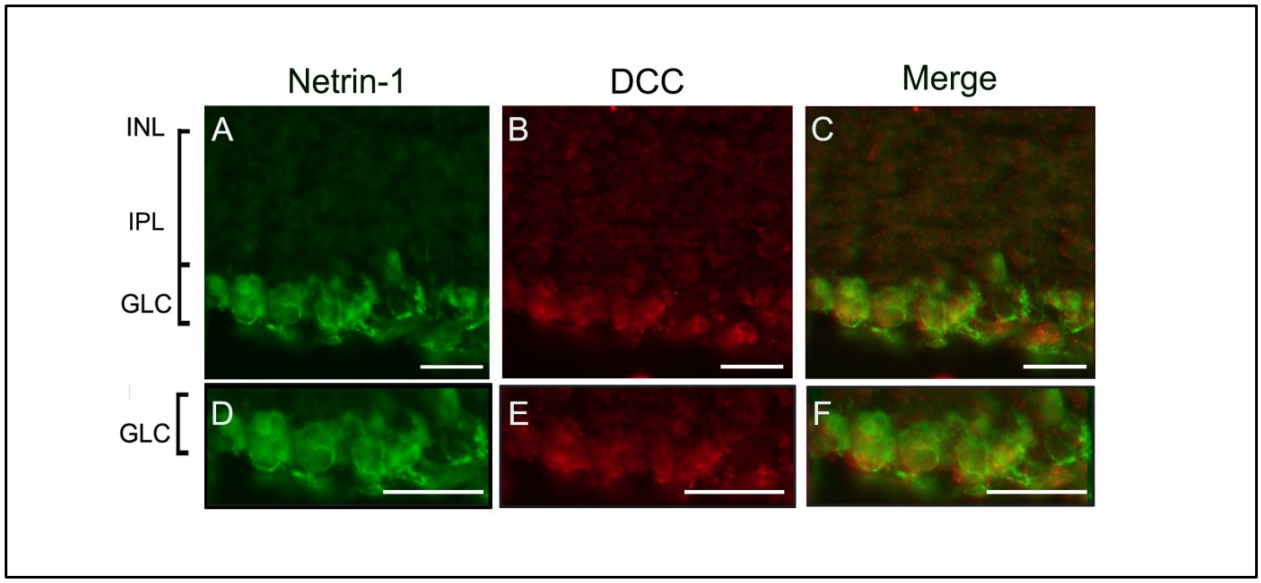

Figure 3. DCC and netrin-1 immunoreactivities co-localize. Adult retina cross-sections colabeled with netrin-1 (A) and DCC (B) demonstrate a strong colocalization of the two markers in the ganglion cell layer (GCL) (C). (Intact, N = 4). Scale bars: (A-C)=20 µm, (D-F)=30 µm. INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer.

IV.4 Netrin-3, the closest homolog of netrin-1 is expressed in the adult retina

Within the netrin protein family, netrin-3 has the highest homology to netrin-1 and is considered as its functional equivalent. Netrin-3 has been shown to bind to DCC, although with different affinity, and mimics the outgrowth-promoting activity of netrin-1 on commissural axons explants, albeit with lower specificity [123]. In vivo studies have shown the presence of netrin-3 in the peripheral nervous system where it is implicated in axon pathfinding and fasciculation [123, 124]. However, very little information is available on the expression and activity of netrin-3 in the adult nervous system.

Here, we aimed to characterize the expression of netrin-3 in the adult retina. Western blot analysis of retinal homogenates from adult mice retinas were carried out using Nora-1 antibody (clone NORA-1 ALX-804-838-C100), an antibody that specifically targets the N-terminal end of netrin-1, a domain that share sequence homology between netrin-1 and netrin-3 both showing globular homology to laminin γ1 short arm (Fig. 4A) [10]. Our results revealed two distinct bands corresponding to netrin-1 (~72 kDa) and netrin-3 (~63 kDa) in all samples suggesting that netrin-3 is expressed in the adult retina and brain (Fig. 4B). A U87 cell lysate, which express netrin-3 but not netrin-1, was loaded as positive control to further confirm the specificity of the observed bands (Fig. 4B).

To identify which retinal cells show Nora-1 immunoreactivity, retinal cross-sections obtained from adult mouse were stained with Nora-1 antibodies. Nora-1 labeling was detected predominantly in two retinal cell layers, the ganglion cell layer (GCL) and the inner nuclear layer (INL) (Fig. 4C). Double labeling of Nora-1 with RBPMS revealed that adult mouse RGCs are endowed with robust Nora-1 immunoreactivity(Fig. 4D, G)

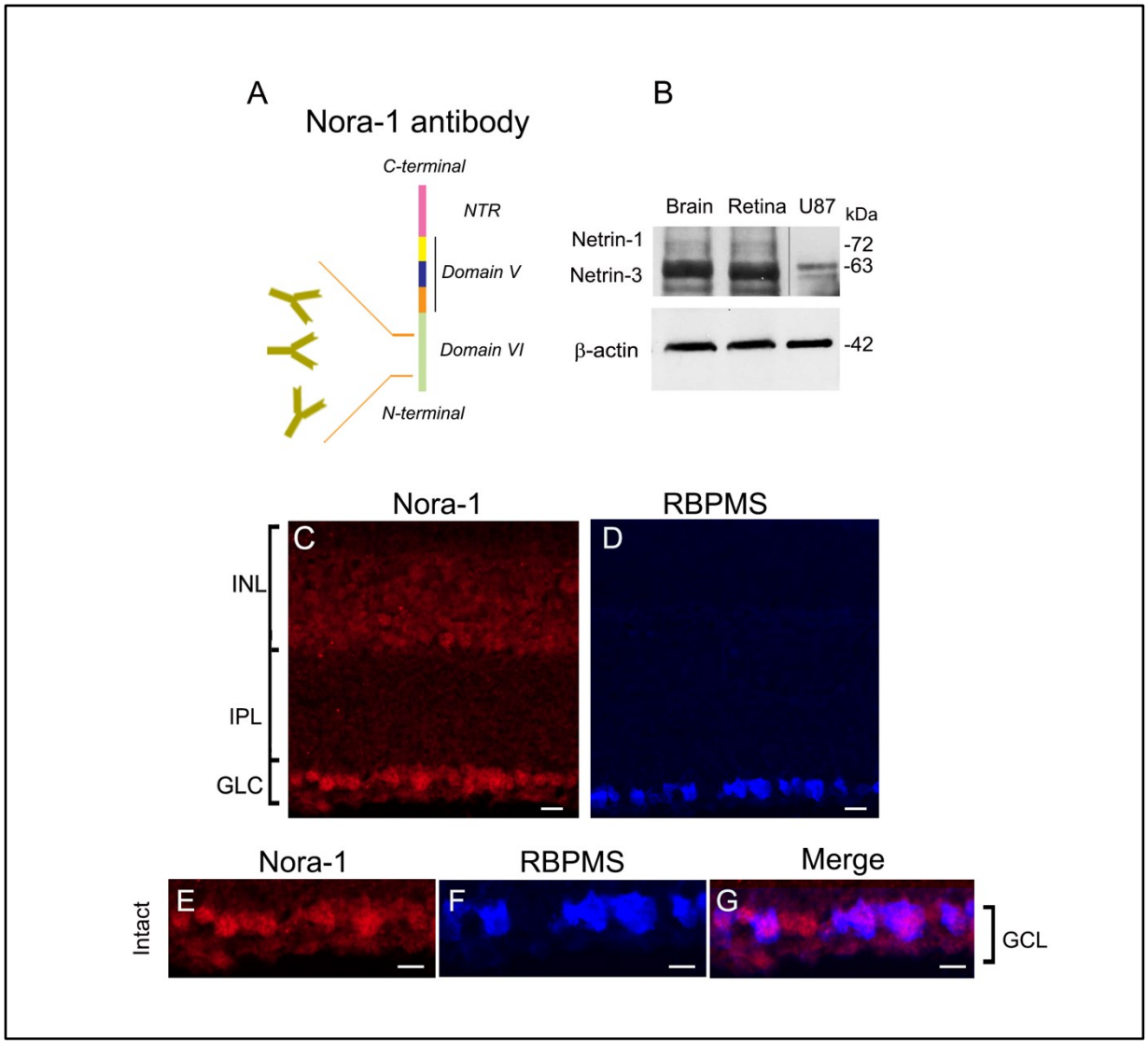

Figure 4. Netrin-1 and Netrin-3 are both expressed in the adult retina. (A-B) Western blot analysis using Nora-1 antibody shows two distinct bands corresponding to netrin-1 (~72 kDa) and netrin-3 (~63 kDa) in retinal and brain samples. The lower panel represents the same blot as in the upper panels but probed with an antibody that recognizes *β*-actin used to confirm equal protein loading. (C) Immunolabeling of retinal cross-sections using Nora-1 antibody revealed strong Nora-1 immunoreactivity in both the ganglion cell layer (GCL) and the inner nuclear layer (INL). (D-G) Co-labeling with RBPMS, a RGC-specific marker, demonstrated robust Nora-1 immunoreactivity in RGC soma (H). (Intact, N = 2). Scale bars: C-D=10 µm, E-G=12 µm. INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer.

IV.5 RGC survival in experimental optic nerve damage

After axotomy, RGCs in the adult mammalian retina degenerate and die by apoptosis in a predictable, time-dependent manner. RGCs begin to disappear from the retina between 5 and 7 day postaxotomy with a cell population of only ~10% remaining after two weeks after injury [64, 125]. To establish whether axonal damage induces changes in netrin-1 levels in RGCs, we first confirmed the time course of injury-induced RGC death by labelling retinal flat-mounts with an antibody against RBPMS (Fig. 5A-C) [126]. We then assessed RGC survival by quantification of RBPMS-labeled neurons in whole-mounted retinas. Consistent with earlier studies, we found that ~50% of RGCs are lost at 7 days after axotomy, and ~90% die at 14 days post-lesion relative to non-injured intact eyes (Fig. 5D) [55, 58, 117].

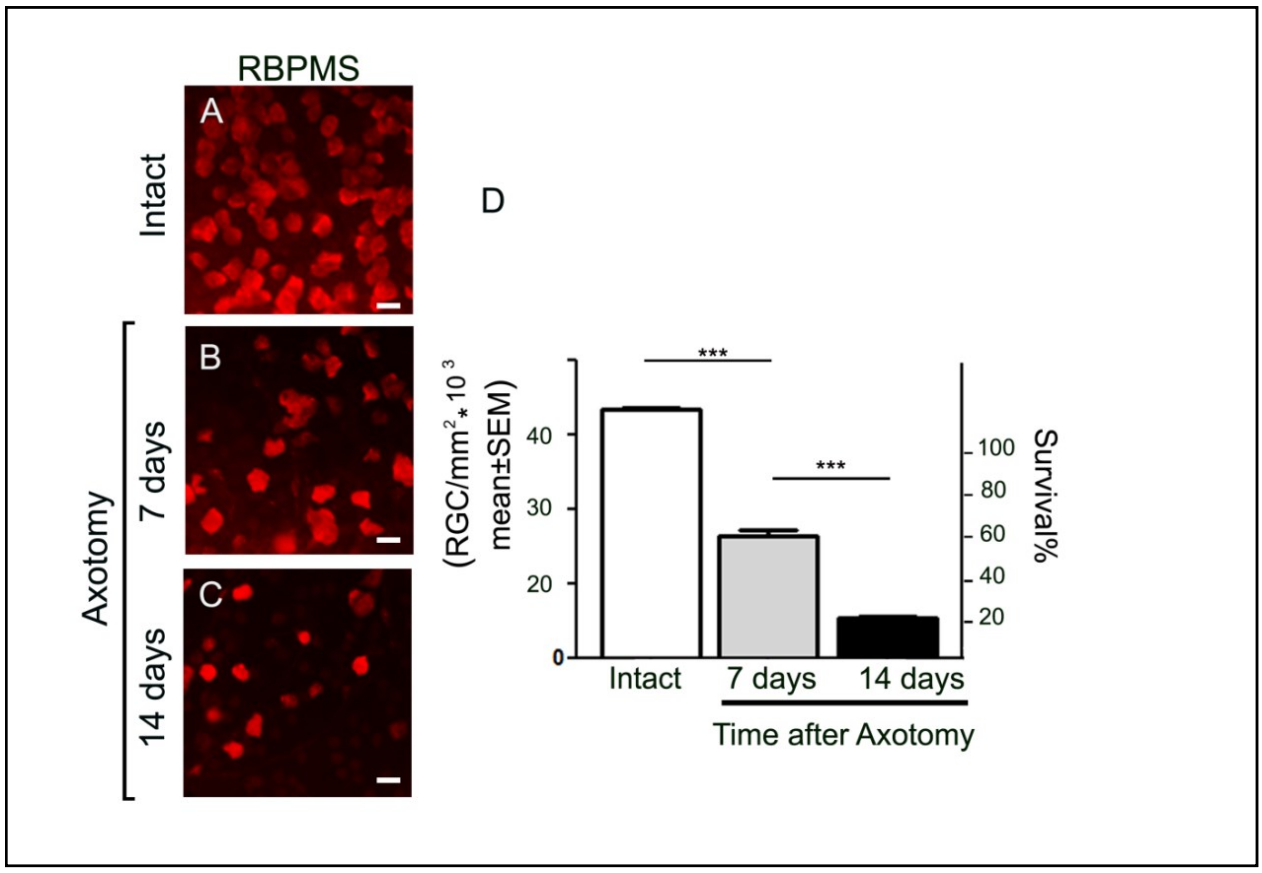

Figure 5. RGCs in the adult mammalian retina die by apoptosis in a time-dependent manner. Representative examples of retinal flat mounts showing RGC somas labeled with RBPMS in intact (A) and axotomized eyes (B-C). (D) Quantitative analysis of RGC densities showed a substantial neuronal loss accounting for 50% and 80% of RGCs at 7 and14 d after axotomy, respectively, compared to the intact retinas (Intact: n=5, axotomy 7 days: n=6, axotomy 14 days: n=7). Data are shown as the mean ± S.E.M (ANOVA, *p<0.1, **p<0.01, ***p<0,001, Tukey's Multiple Comparison *post-hoc* test). Scale bars: A-C=10 μm.

IV.6 Netrin-1 expression is downregulated after optic nerve axotomy

To assess potential alterations in netrin-1 expression after axotomy, we performed western blot analysis of intact and axotomized retinas at 3 days, prior to the onset of RGC degeneration, and at 7 days, a time when ~ 50% of RGCs are lost (Fig. 6A). The time-course of netrin-1 protein expression in retinal protein homogenates from axotomized retinas showed a marked decrease in full length netrin-1 levels which coincided with an increase in truncated netrin-1 (Fig. 6B). Densitometric analysis confirmed a significant decrease in full length netrin-1 protein levels at 3 and 7 days post-injury relative to non-injured retinas, with no further decrease between 3 and 7 days (Fig. 6C). In contrast, truncated netrin-1 increased at these time points (Fig. 6D). Together, our results indicate that the levels of full-length netrin-1 decrease after axotomy, while truncated netrin-1 increases, suggesting the interesting possibility that axonal injury might trigger signals that induce the cleavage of full length netrin-1.

Immunohistochemical analysis of retinal cross sections revealed a visible decrease in netrin-1 in the GCL at 3 and 7 days after axotomy compared to non-injured, intact retinas (Fig. 6E-G). Co-labeling of axotomized retinas with netrin-1 and RBPMS antibodies demonstrated injury-induced downregulation of netrin-1 RGCs (Fig.6H-P). The reduction in netrin-1-positive labeling in the GCL could not be solely attributed to cell death because many RGCs were alive at these time points, with netrin-1 protein expression visibly reduced in surviving RGCs (Fig. 6H-P).

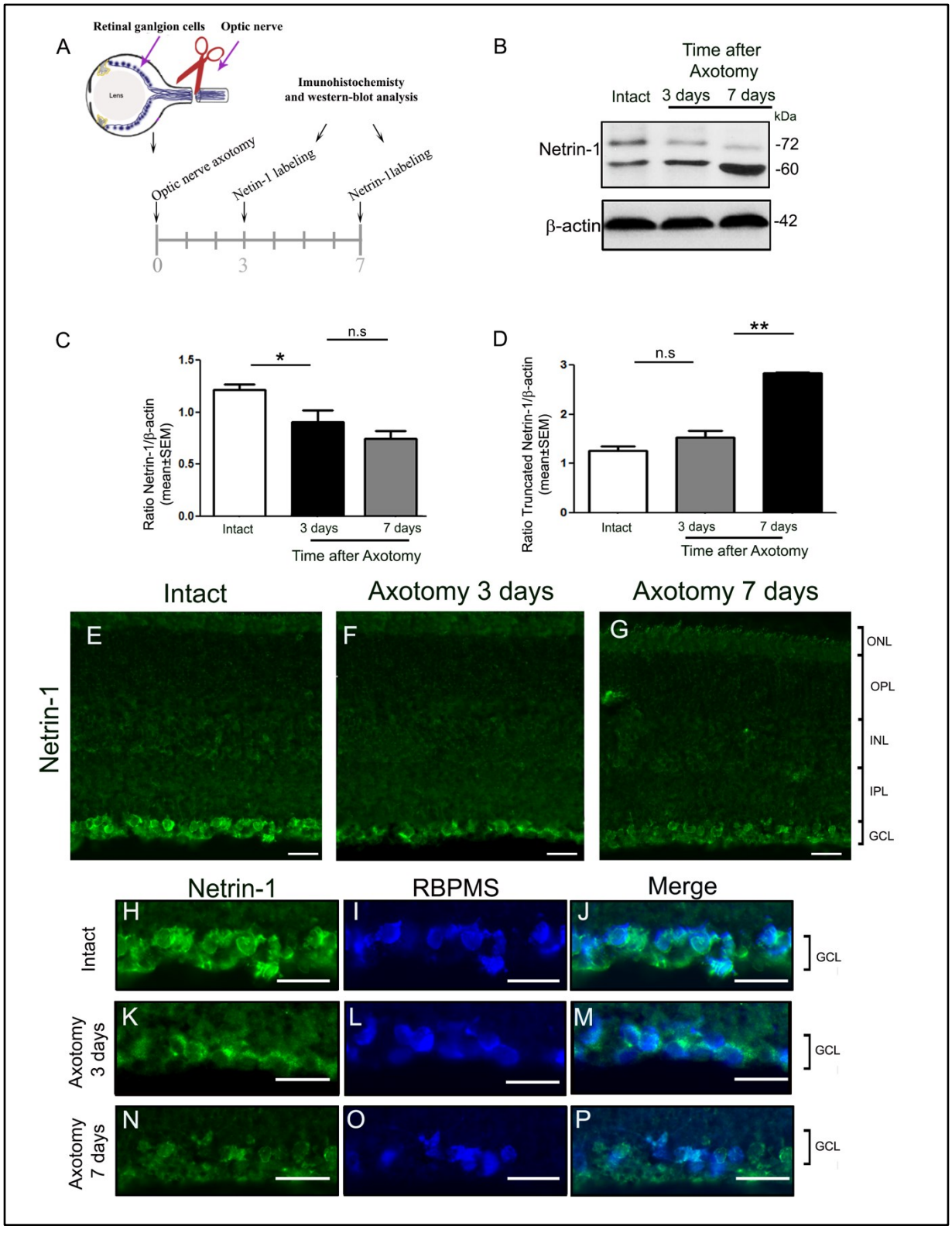

Figure 6. Netrin-1 level decreases after optic nerve axotomy. (A) Outline of the experimental protocol used to investigate netrin-1 expression at 3 and 7 days after axotomy by immunohistochemical and western blot analysis using

the same netrin-1 antibody that is demonstrated in (Fig. 1E). (B-D) Western blot and densitometric analysis revealed that the 72-kDa full-length netrin-1 is downregulated as early as 3 days after injury, coinciding with an increase in truncated netrin-1 level. The lower panel represents the same blot as in the upper panels but probed with an antibody that recognizes β-actin used to confirm equal protein loading. Data are the mean ± S.E.M (n=5/group, ANOVA, *p<0.1, **p<0.01, ***p<0.001, Tukey's Multiple Comparison post-hoc test). (E-G) Immunohistochemical analysis revealed a visible decrease in netrin-1 in the GCL at 3 and 7 days after axotomy compared to non-injured, intact retinas (H-P) RBPMS and netrin-1 co-labeling confirmed that netrin-1 protein decreases in injured RGCs. Scale bars: (E-G)=20 μm, (H-P)=25 μm. ONL: outer nuclear layer, OPL: outer plexiform layer, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer.

IV.7 DCC expression decreases after optic nerve axotomy

Injury-induced downregulation of netrin-1 receptors may limit the response of RGCs to netrin-1. Therefore, we also compared DCC protein expression in axotomized and intact retina. Western blot analysis of soluble retinal extracts from axotomized retinas revealed that DCC protein drastically decreases to below detection levels relative to retinal samples from non-injured eyes (intact) (Fig.7A). Densitometric analysis confirmed a significant decrease of DCC expression at 7 days after axotomy compared to intact controls (Fig.7B). Immunohistochemical confirmed that DCC labeling in the GCL substantially decreased after axotomy (Fig.7 C, D). Colabeling of axotomized retinas with DCC and RBPMS antibodies confirmed DCC protein expression decreases in surviving RGCs (Fig.7E-J). These results indicate that the reduction of DCC signaling in GCL cannot be solely attributed to cell death caused by axotomy because ~ 50% of RGCs remain at 7 days after optic nerve axotomy.

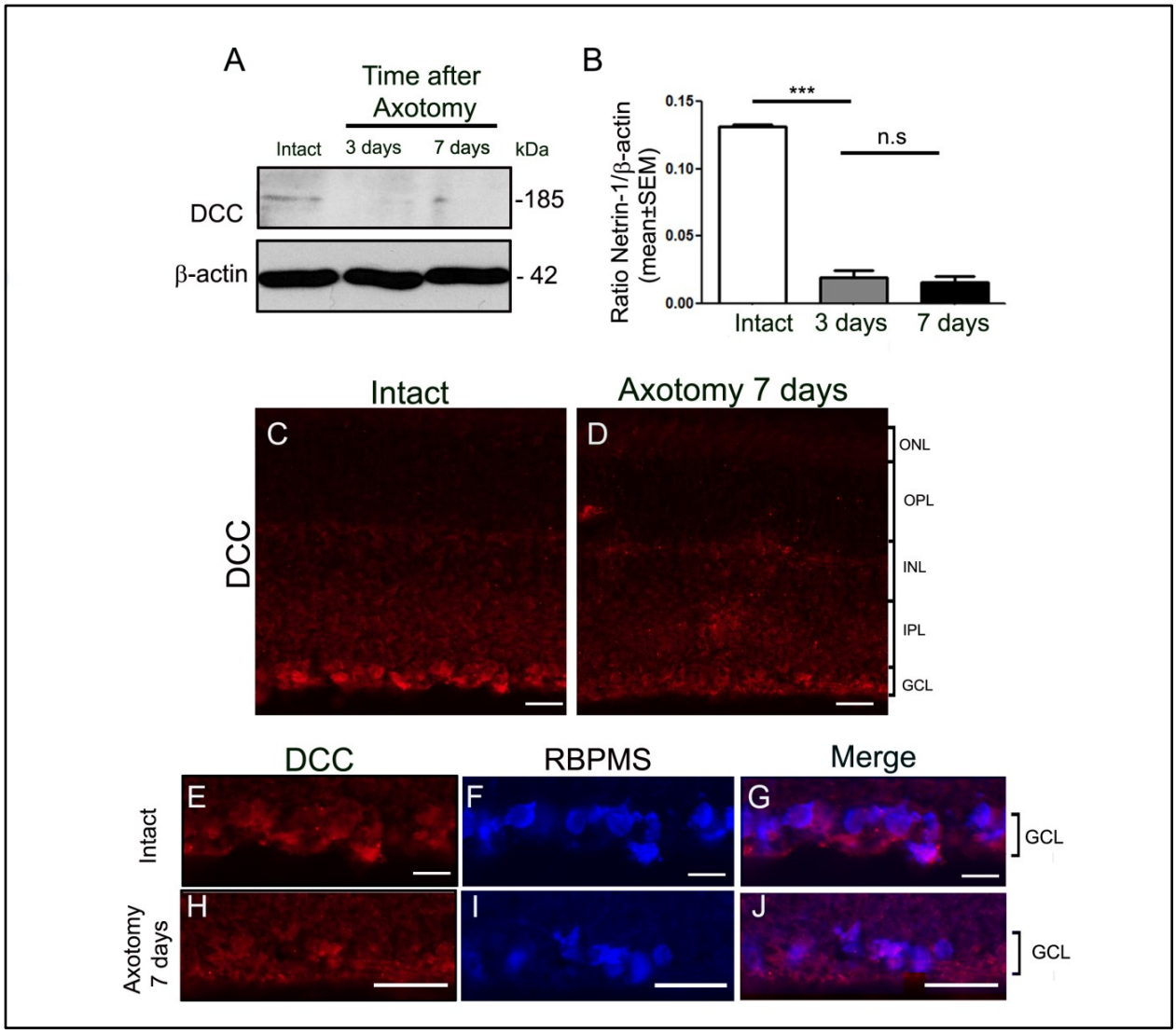

Figure 7. DCC is downregulated after optic nerve axotomy. (A-B) Western blots and densitometric analysis of axotomized retinas revealed a significant decrease in DCC compared to intact retinas. Data are the mean ± S.E.M (n=4/group, ANOVA, ***p<0.001, Tukey's Multiple Comparison *post-hoc* test). (C-D) Immunhistochemical analysis revealed a marked reduction of DCC in the ganglion cell layer (GCL) of axotomized retinas. (E-H) RBPMS labeling confirmed that surviving RGCs express less DCC in axotomized retinas. Scale bars: (A-G) =20 μm, (H-J)=30 μm. ONL: outer nuclear layer, OPL: outer plexiform layer, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer.

IV.8 Netrin-3 expression decreases after optic nerve axotomy

Next, we compared the expression of netrin-3 in intact and axotomized retinas. Western blot analysis of retinal homogenates probed with the Nora-1 antibody revealed that as early as 3 days post-axotomy, netrin-3 expression undergoes a drastic decline followed by a subtle decrease until 7 days (Fig. 8A). Densitometric analysis confirmed a significant decrease of netrin-3 expression at 3 days after lesion with no further decrease at 7 days post-axotomy (Fig. 8B). In retinal cross-sections, intense Nora-1 labeling was observed predominantly in the INL and GCL of non-injured, intact retinas, which decreases substantially at 3 and 7 days after axonal injury (Fig. 8C-E). Co-labeling of retinal sections using Nora-1 and RBPMS demonstrated loss of Nora-1-positive labeling in damaged RGCs at 3 and 7 days post-axotomy (Fig.8F-N). Again, a reduction in Nora-1 labeling could not be solely attributed to RGC death because surviving RGCs displayed low levels of Nora-1 staining.

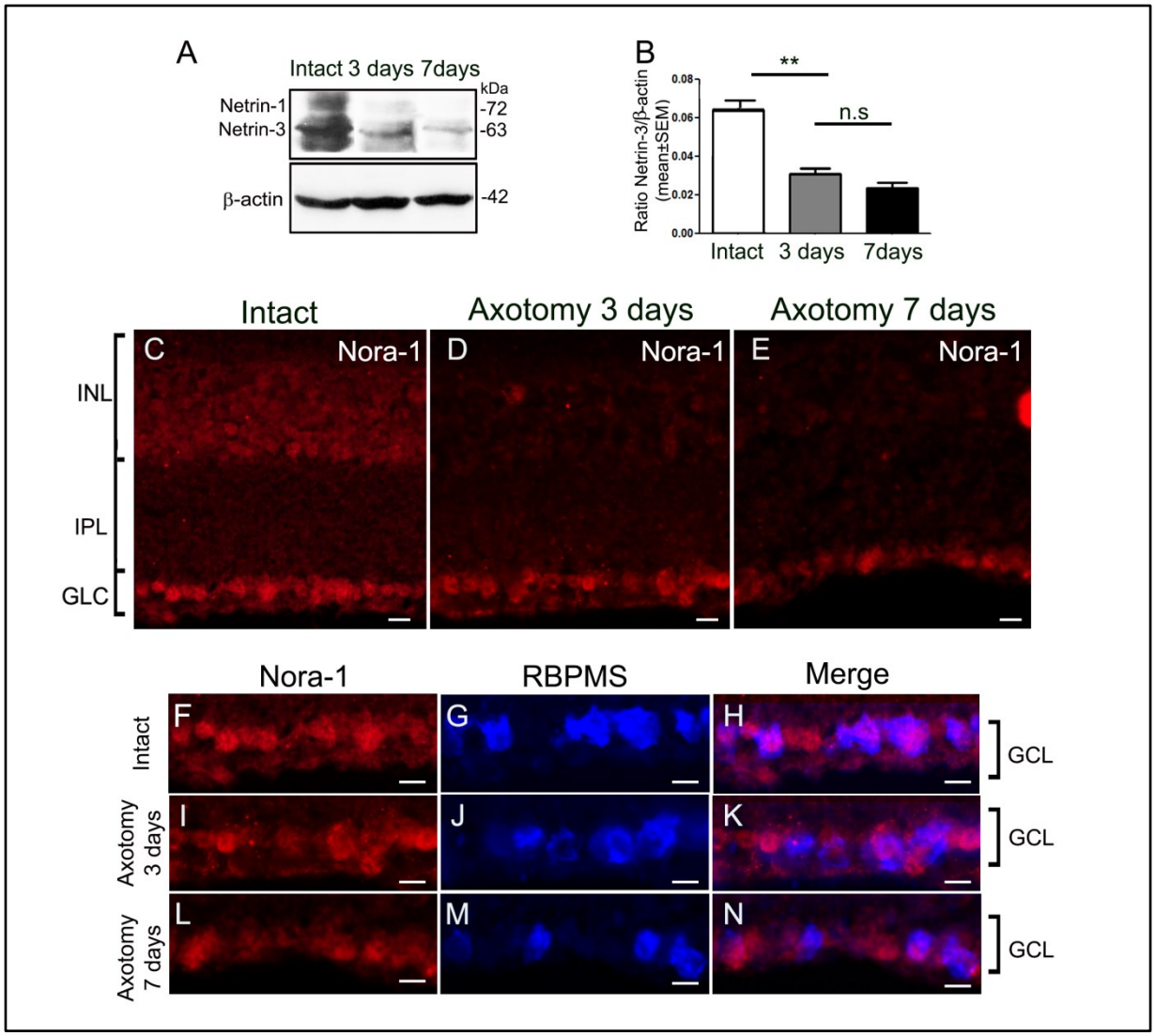

Figure 8. Netrin-3 expression declines after optic nerve axotomy. (A-B) Western-blots and densitometric analysis of axotomized retinas revealed a significant decrease in netrin-3 expression at 3 and 7 days after axotomy. Data are the mean ± S.E.M (n=5/group, ANOVA, **p<0.01, Tukey's Multiple Comparison *post-hoc* test). (C-E) A reduction in Nora-1 immunoreactivity in the ganglion cell layer (GCL) was detected after axotomy. (F-N) RBPMS labeling confirmed that surviving RGCs in axotomized retinas express less Nora-1 protein staining relative to intact RGCs. Scale bars: (C-E)=10 μm, (F-N)=12 μm. INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer.

IV.9 Experimental protocol to study the neuroprotective effect of netrin-1 on axotomized RGCs

The effect of recombinant netrin-1 on RGC survival was tested using the experimental protocol outlined in Figure 9. After intraocular injection of recombinant netrin-1 protein, RGC axons were immediately transected and the density of surviving RGCs, visualized with the cell-specific RBPMS marker, was quantified at 7day post-lesion.

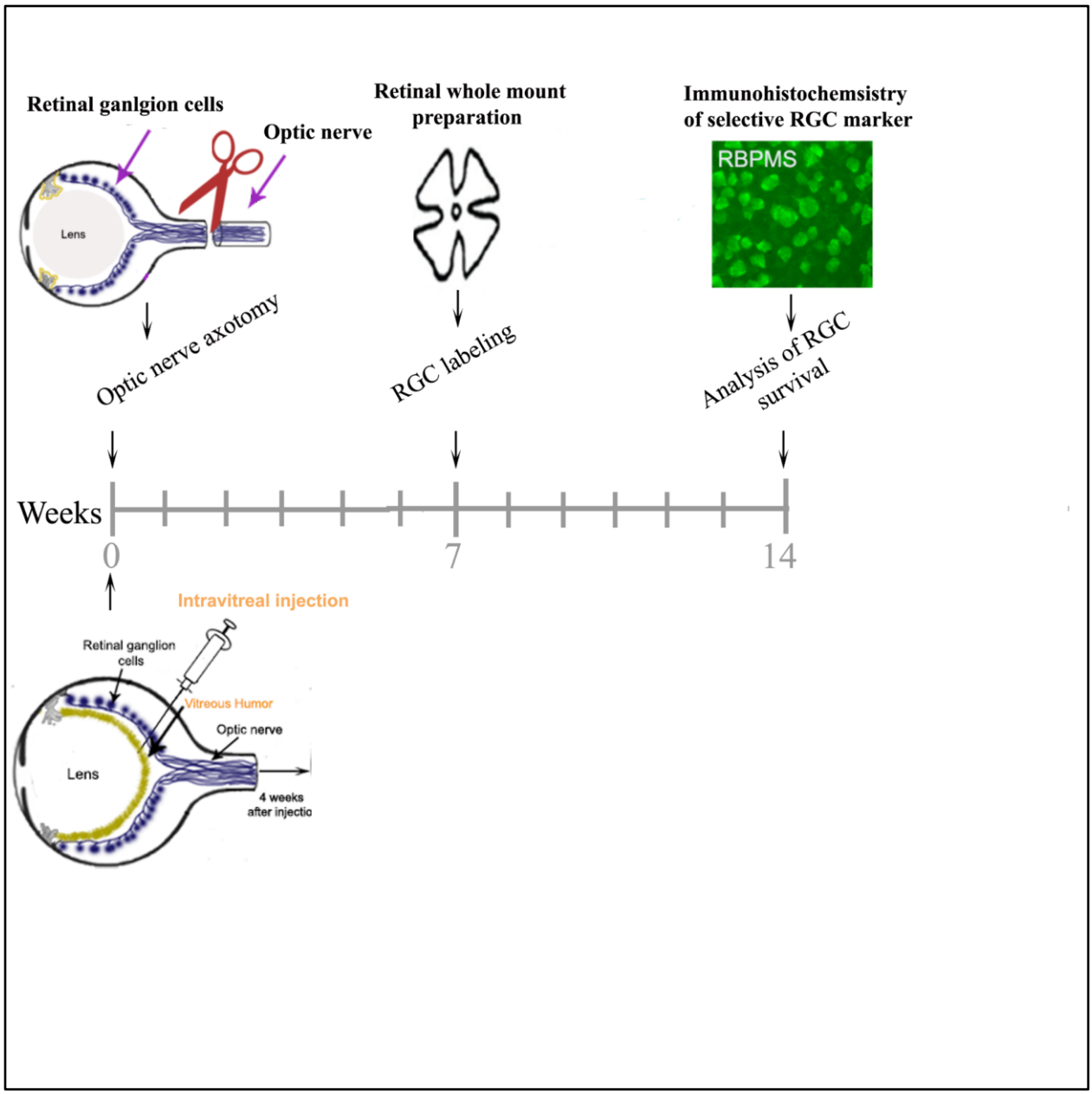

Figure 9. Outline of the experimental protocol used to test the effect of recombinant netrin-1 on RGC survival. Immediately after the intraocular injection of recombinant netrin-1, RGC axons were transected. Seven days after axotomy, mice were perfused and retinal whole mounts were prepared for quantification of surviving RGCs using an RBPMS antibody.

IV.10 Recombinant netrin-1 does not extent RGC survival

Recombinant netrin-1 proteins, either full-length or bioactive VI-V domain (provided by Dr. T. Kennedy, McGill University) were injected intravitreally prior to optic nerve axotomy (Fig. 9). One day after netrin-1 protein administration, retinas were collected and the distribution of the injected protein was examined by immunostaining using an antibody against the c-myc tag present in recombinant netrin-1 (Fig. 10A). Co-labeling of retinal sections using RBPMS and c-myc antibodies demonstrated robust labeling in the GCL, primarily around RGC bodies. (Fig. 10A-C). Control intact contralateral retinas or retinas injected with PBS did not show positive c-myc staining (Fig.10D-F).

The effect of recombinant netrin-1 on RGC survival *in vivo* was tested using the experimental protocol outlined in Figure 9. Quantitative analysis of the number of RGCs in the retinas injected with recombinant full length or truncated netrin-1 did not show neuronal survival relative to control retinas injected with PBS (Fig. 10G). Collectively, our data suggest that, in these experimental conditions, intraocular injection of either full length or the truncated recombinant netrin-1 did not promote RGC survival.

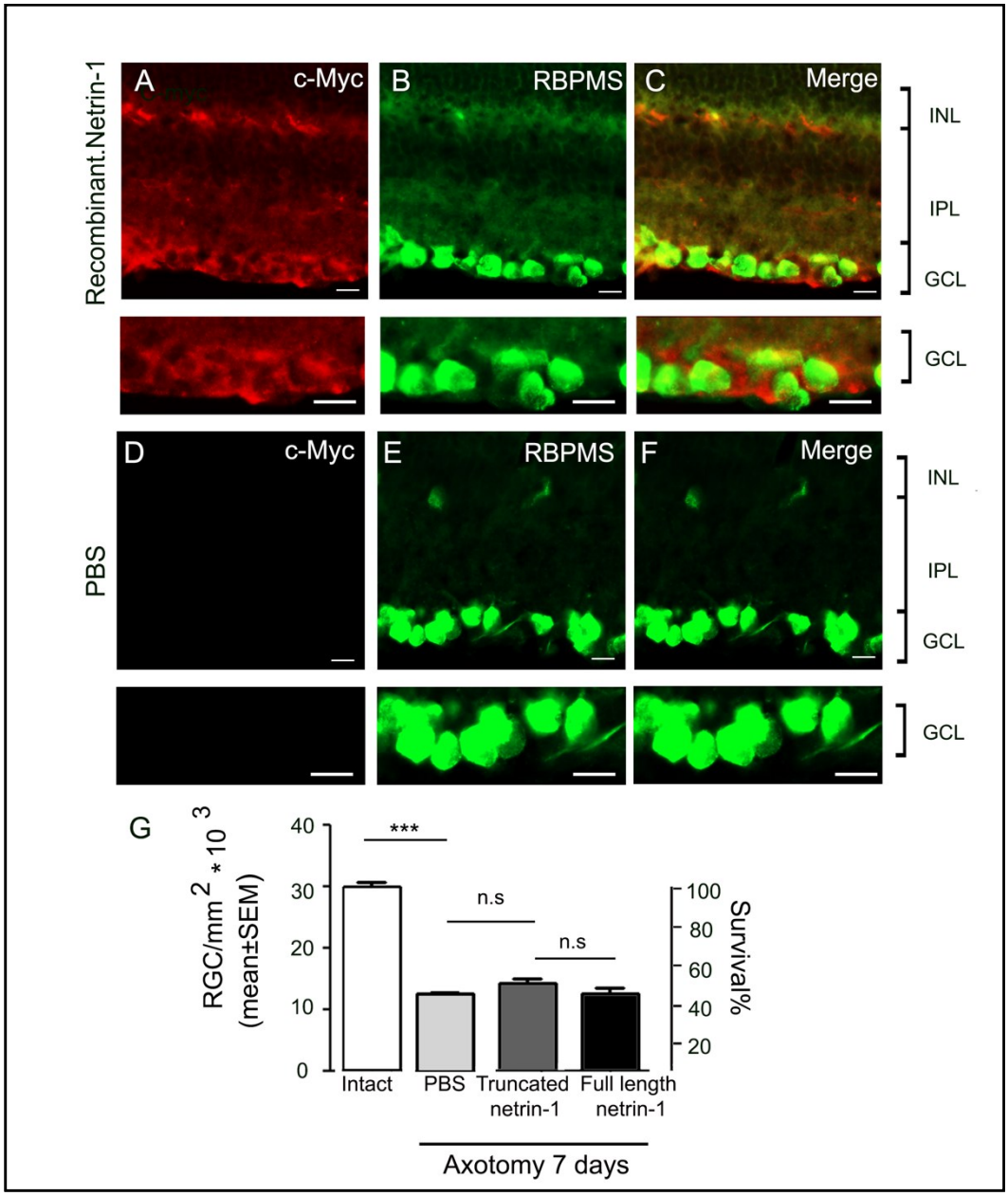

Figure 10. Recombinant netrin-1 does not promote survival of injured RGCs. (A-C) Co-labeling of retinal sections using RBPMS and c-myc antibodies demonstrated robust labeling in the GCL, primarily around RGC bodies. (D-F) Control intact contralateral retinas or retinas injected with PBS did not show positive c-myc staining. Scale bars: (A-C)=10 µm, (D-F)=12 µm. (G) Quantitative analysis of the number of RGCs in the retinas injected with recombinant full length or truncated netrin-1 did not show neuronal survival relative to control retinas injected with PBS (n=6/group ANOVA, ***p<0.001, Tukey's Multiple Comparison *post-hoc* test). The density of RGCs in intact, untreated retinas and axotomized, untreated retinas are shown as reference. INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer.

IV.11 Netrin-1 gene transfer to the retina

An important limitation of applying a bolus intravitreal injection of netrin-1 recombinant protein is that its short *in vivo* half-life might prevent a noticeable neuroprotective effect. To overcome this limitation, we used recombinant adeno-associated virus serotype 2 (AAV2) to deliver netrin-1 to the adult retina. This vector system was selected based on our previous data showing that RGCs are the primary cellular target for AAV2 transduction after intravitreal injection [64].

IV.11.1 AAV.netrin-1 structure

AAV is a member of the parvoviridae family that requires a helper virus for replication. Wild-type AAV houses a single-stranded genome of 4.7 kilo base-pairs (kb) containing two genes, rep and cap, that encode proteins involved in replication and encapsidation, respectively. The AAV genome is flanked by two identical 145-bp inverted terminal repeats (ITRs), which are essential for packaging, replication and integration (Fig. 11A). Several factors such as the site of injection, the AAV serotype and titer, the amount of cargo DNA, and the specific gene promoters and enhancing elements are determinant in the efficiency of AAV transfection in the retina [65, 127]. In this study, a recombinant AAV vector containing a c-myc-tagged full-length mouse netrin-1 gene under control of the Synapsin 1 (SYN1) promoter was produced. SYN1 belongs to a phosphoprotein protein family, also comprising synapsin 2 and 3 isoforms. Members of the synapsin family associate with the surface of synaptic vesicles and have common protein domains that are implicated in synaptogenesis, and maintenance of mature synapses and modulation of neurotransmitter release involved in nerve signal transmission [128].

In AAV control vector netrin-1 sequence was replaced with the green fluorescent protein (GFP) gene which contained no c-myc tag (Fig. 11B).

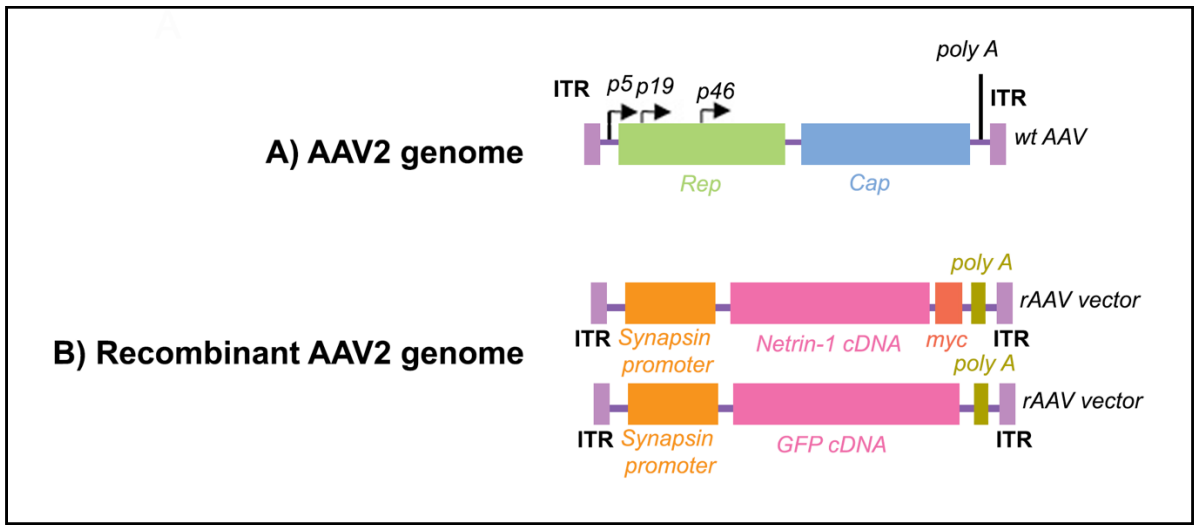

Figure 11.1. Schematic representation of a wild type and recombinant AAV vectors. (A) The wild-type AAV is a single-stranded genome containing two genes, rep and cap, flanked by two identical inverted terminal repeats (ITRs). (B) Our recombinant AAV vector contained a c-myc-tagged full-length mouse netrin-1 gene under control of a SYN1 promoter. The control vector harbors the same construct except that netrin-1 sequence is replaced by GFP. Source of image: Pegah Chehrazi

IV.11.2 AAV-mediated netrin-1 is not expressed by adult RGCs

AAV mediated transgene expression reaches a plateau at 4 weeks after intravitreal injection [58, 64]. Therefore, to determine the efficacy of our AAV.netrin-1 to transduce RGCs and express netrin-1, we analyzed c-myc and RBPMS-labeled cells in retinal sections at 4 weeks after AAV administration. Intravitreal injection of control AAV.GFP showed robust GFP protein expression (shown in red) in the majority of RGCs, visualized by RBPMS staining, validating the specificity of the SYN1 promoter and AAV2 virus at targeting adult RGCs (Fig. 11.2 D-F). In retinas treated with a positive control virus encoding c-myc tagged Inhibitor of Apoptosis-Stimulating Protein of p53 (AAV.iASPP), most RGCs displayed a strong c-myc labeling as previously demonstrated by us [129] (Fig. 11.2 J-L) validating the c-myc antibody used here. Surprisingly, however, retinas treated with AAV.netrin-1, robust c-myc staining was observed in a large number of cells within the inner plexiform layer (IPL). Co-localization of c-myc with RBMPS demonstrated that positive cells were not RGCs (Fig. 11.2 G-I), therefore RGCs did not express AAV-mediated netrin-1. Although the nature of the AAV.netrin-1-transduced cells is currently unknown, their structure

resembles that of astrocytes/microglia. Future work is needed to clarify the identity of the cells targeted by this virus.

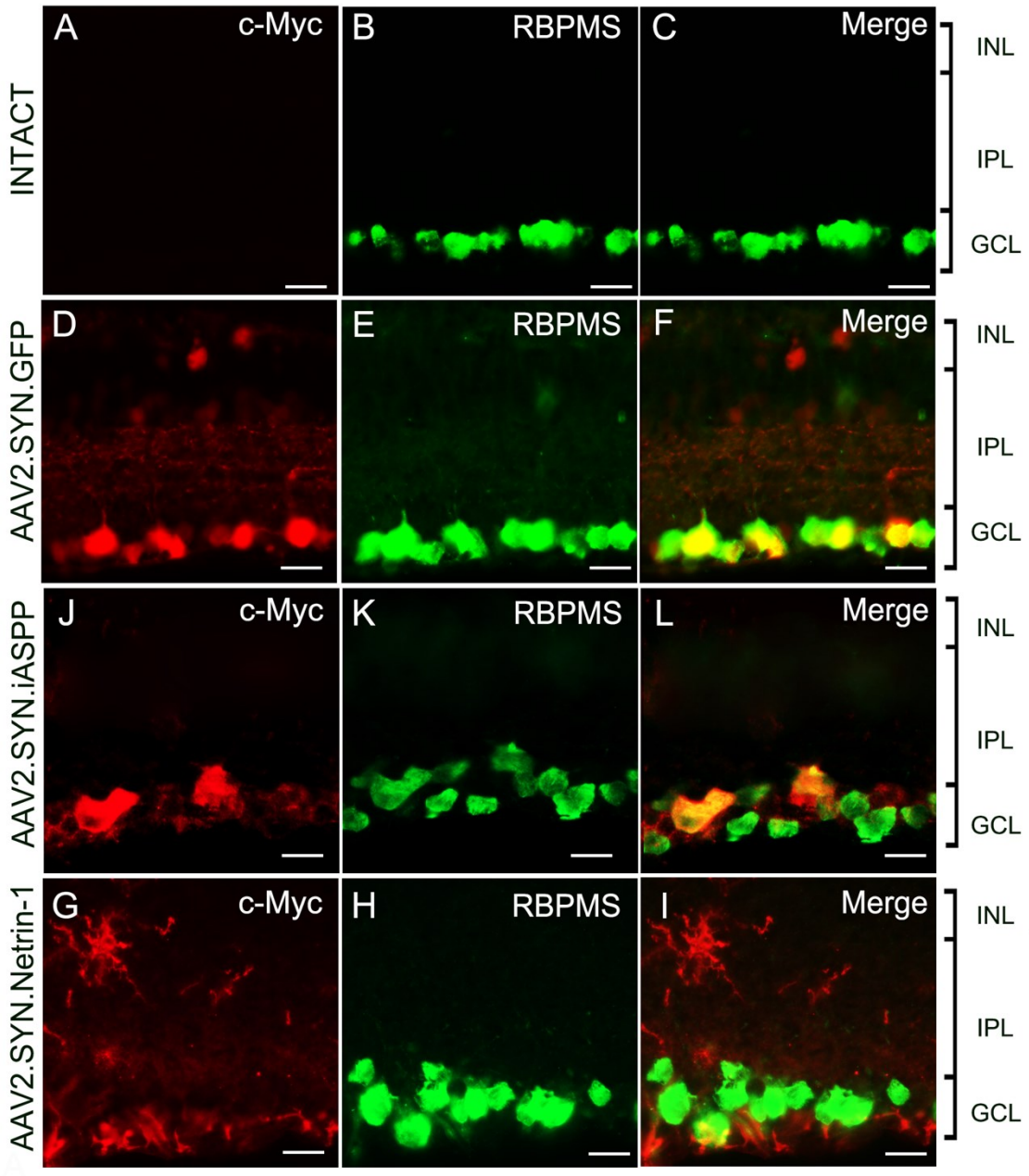

Figure 11.2. The analysis of adult RGCs for AAV-mediated gene product expression at 4 weeks after intraocular expression of the vector. Retinal sections were colabeled with the RGC-specific marker, RBPMS, and anti-c-myc antibody. (A-B) No c-myc staining was detected in control intact retinas. (D-F) Endogenous GFP was detected in RGCs in retinas injected with AAV.GFP. (D) Endogenous GFP signaling is shown in red here. (J-L) Robust c-myc labeling was observed in RGCs in retinas injected with AAV.iASPP. (G-I) RGCs were devoid of c-

myc signaling in retinas treated with AVV. Netrin-1. Scale bars: (A-I) =12 μm INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer.

V. Discussion

This study supports several conclusions: 1) full-length and fragmented forms of netrin-1 are abundantly expressed in the retina of neonatal and young mice, and their expression levels are maintained throughout adult life, 2) DCC is highly expressed during early stages of retinal development but its expression sharply declines in adulthood, 3) netrin-1 and DCC are predominantly expressed by adult RGCs, 4) netrin-3 is expressed in the adult retina primarily in the ganglion cell layer and inner plexiform layer, and 5) netrin-1, netrin-3 and DCC levels in the retina are markedly downregulated after optic nerve axotomy. The observation that netrin-1, netrin-3, and DCC protein levels are altered early after axonal damage and prior to overt retinal neuron death, suggest that these molecules might play a role in the response of the mature visual system to injury.

V.1 Netrin-1 and its receptor DCC are expressed in the adult retina

Netrin-1 is best characterized for its diverse conserved roles in neuronal morphogenesis and stability. However, recent data suggest that netrin-1 is continued to be expressed in adult mouse brain and plays an important role in cortical neuron synaptogenesis during the critical postnatal period of peak synapse formation [108]. DCC and netrin-1 are also shown to be enriched in dendritic spines of pyramidal neurons and they activate proteins that regulate synaptic function and plasticity in the adult brain [5]. These findings incited us to investigate the expression of netrin-1 in the adult retina.

During retinal development, netrin-1/DCC signaling plays a crucial role in guiding growing RGC axons toward the optic nerve head [130]. Netrin-1/DCC signaling is also implicated in the survival of newborn RGCs and displaced amacrine cells in the developing mouse retina [7]. Netrin-1 is also considered an important mediator of RGC axon branching and synaptogenesis in the developing vertebrate visual system [113]. However, contradictory results have confounded our understanding of netrin-1 expression in the adult retina. Indeed, early data demonstrated that the expression of netrin-1 is virtually absent from the retina of adult rats [89]. These findings were later contradicted by studies showing that netrin-1expression is present in the ganglion cell layer of adult mice [8, 10]. Here, we aimed to investigate the expression of netrin-1 in the early postnatal

and adult mouse retina using three complementary techniques: western blot analysis, RT-PCR, and retinal immunohistochemistry using well-characterized antibodies [108]. Our results revealed that netrin-1 and its receptor DCC are constitutively expressed in the neonatal (P0), young (P14) and adult (~2-month-old) mouse retina. We detected constitutive netrin-1 and DCC expression in adult RGCs. Our results show that netrin-1 expression is relatively stable in the developing and adult retina, while DCC protein is abundantly expressed during early postnatal developmental stages of retina and undergoes a progressive decrease in adulthood.

In addition to full-length netrin-1 protein, fragments of netrin-1 (truncated) were also detected in neonatal and adult retinal protein homogenates. So far, two main forms of truncated netrin-1 obtained by proteolytic processing of full-length netrin-1 have been recognized which contain either the domain IV or the netrin like domain (NTR) [10, 131]. It should be mentioned that, alternative splicing of netrin-1 mRNA that would result in the production of shorter netrin-1 isoforms has not been reported in the CNS or any other tissue in normal physiological conditions [131]. Therefore, due to the specificity of the antibody against the netrin-1 C-terminus, we speculate that the smaller netrin-1 peptides detected in this study are likely proteolytic fragments of full-length netrin-1 that are comprised of V and NTR domains. In contrast to the well-defined role of full length netin-1 in the maintenance of homeostasis, fragmented netrin-1 have been suggested to be involved in pathological events [10]. For example, netrin-1 degradation products were shown to be capable of exacerbating vascular permeability in a murine model of diabetic retinal edema [10].

V.2 Netrin-3 is expressed in the adult retina

While the role of netrin-1 has been extensively studies in the CNS, very little information is available on the expression and activity of netrin-3, its closest homologous protein. Mouse netrin-3 share 52% amino acid identity with netrin-1 and also binds to DCC and UNC5 receptors [132]. Netrin-3 mimics the outgrowth-promoting activity of netrin-1 on commissural axons by binding to DCC, although with lower affinity. Netrin-3 is found predominantly in motor neurons and subpopulations of sensory and sympathetic ganglia where it plays a key role in axon pathfinding and fasciculation within the peripheral nervous system [123, 124]. Our study is among the first to show that netrin-3 is present in the adult CNS, both in the retina and cerebellum. Our immune-histochemical analysis demonstrated that netrin-3 is mainly expressed in RGC cell bodies

in the ganglion cell layer as well as in the inner nuclear layer of the retina. These results can be accounted as an additional example of the emerging pattern of ligand–receptor co-expression in RGCs that has been observed for other axonal guidance molecules such as ephrinA-EphA, BDNF-trkB, and slit2-robo [8]. Together, the maintenance of netrin-1 and netrin-3 along with DCC expression in adult retina, as well as their distribution in RGCs, suggest a potential functional role in this system.

V.3 Possible roles of netrin-1 in adult retina

V.3.1 Netrin-1/DCC signaling may regulate synaptic integrity in the adult retina

Recent data show that many molecules that were initially discovered for their function in axon guidance also play important roles in synapse formation and plasticity [114]. Much like axon guidance, the process of forming a new synapse involves coordinated cell morphological and structural changes that are instructed through ligand-receptor interactions followed by intracellular signaling cascades that lead to actin network remodeling [114]. In the same manner, netrin-1 signaling which has been largely studied in relation to axonal guidance, has been shown to be implicated in synapse formation in mammalian cortical neurons [108]. In addition, netrin-1/DCC signaling is shown to be required for the induction of LTP that contributes to spatial and recognition forms of learning and memory in the adult brain [5].

It is now well established that netrin-1 activation leads to the stimulation of multiple signaling pathways downstream of DCC including Src family kinases, Rho GTPases and mTOR-dependent protein translation that all converge on the dynamic reorganization of filamentous actin (F-actin) in axonal growth cones [133]. Similarly, in cortical neurons undergoing synapse formation, netrin-1 locally activates Src family kinase and mTOR-dependent protein synthesis to stimulate rapid reorganization of the underlying actin cytoskeleton that leads to local clustering of presynaptic and postsynaptic proteins [108]. In addition, netrin-1-mediated activation of Rho GTPases increases dendritic arborization and enhances the probability of axon-dendrite contacts [134]. Recently, mTOR signaling has been recognized as a key molecule promoting dendrite stability and synaptic integrity [67, 69]. Based on this, it is plausible to assume that mechanisms

downstream of netrin-1 and netrin-3 signaling play a role in the maintenance of synaptic integrity and function in adult RGCs. In other words, although RGCs lose dendritic spines as they mature [135], the stability of their dendrites and synapses depends on the fine regulation of cytoskeletal dynamics, which could be controlled by netrin-1/3-DCC signaling. This hypothesis is consistent with our immuno-histochemical findings in which the distribution of netrin-3 is observed in the inner plexiform layer, where the synapses between bipolar cells and RGCs are located, providing another line of evidence for the implication of netrin-3 in regulating synaptic complexes or activity. In this regard, with the restricted expression of DCC around the RGC soma, further analysis of other netrin-3 receptors such as neogenin and UNC5 will be helpful to better understand the role of netrin-3 in the adult retina.

V.3.2 DCC mediates an adhesive interaction with substrate-bound netrin-1

The observation of the distribution of netrin-1 and netrin-3 in the adult mouse retina provides some clues about their possible role in the structural maintenance of RGCs. In this regard, the maintenance of the expression of guidance molecules in the adult retina has been proposed to contribute to maintaining the structural integrity of retinal cells [2]. Secreted netrins are known to be tightly bound to extracellular matrix and cell surfaces, thus they are thought to play an important role in cell adhesion via binding to their transmembrane receptors [87]. Consistent with these, and as depicted in our results, the strong co-localization of netrin-1/3 with DCC in individual RGCs as well as the close proximity of the RGCs expressing them suggest that netrin-1/3-DCC interactions might regulate cell-cell or cell-matrix adhesion in the adult retina in a way similar to the maintenance of paranodal junctions between axons and myelinating oligodendrocytes [136].

An additional line of evidence showing that an appropriate cellular adhesion and organization is stabilized by autocrine and paracrine actions of netrin-1/3, was provided in the study of A.Jarjour *et al*, 2011 [77]. In fact, they have shown that in addition to its well-characterized chemotropic activity, netrin-1 as well as other members of netrin family such as netrin-3 play a role as autocrine inhibitors of cell motility by promoting the maturation of focal complexes, structures associated with cell movement, into focal adhesions. In this regard, a reduced expression of netrin-1-DCC has been documented in several brain tumors, including glioblastoma and netrin-1-DCC over-expression was proposed as a potential therapeutic target to

inhibit tumor cell migration and dispersion. Interestingly, other netrin-1 receptors such as neogenin and UNC5 homologues were not sufficient to substitute for DCC function to reduce cell motility in cancer cells [77].

V.4 Netrin-1, netrin-3 and DCC are downregulated after axotomy

In a search for a possible role of netrin-1 and netrin-3 in adult retina, we asked whether their expression changed after optic nerve axotomy. Our analysis of whole retinal lysates revealed a substantial decrease of netrin-1 and netrin-3 as well as DCC at 3 and 7 days after axotomy. This finding is consistent with a prevoius study by Ellezam *et al*. in which the mRNA levels of retinal netrin-1 and DCC were shown to be downregulated following optic nerve axotomy [8]. In addition, the immunohistochemical analysis of individual RGCs labeled with RBPMS shown here demonstrated that surviving RGCs have less netrin-1/3 and DCC proteins relative to non-injured controls.

V.4.1 Truncated netrin-1 gradually increases after axotomy

Despite the selective downregulation of netrin-1, our data showed a gradual increase in shorter netrin-1 fragments after axotomy. Netrin-1 is metabolized into bioactive fragments under pathological conditions. For example, in a murine model of diabetic retinopathy, a 55 kDa-fragmented netrin-1 is shown to be selectively generated through the cleavage of full length netrin-1 by collagenase matrix metalloprotease 9 (MMP-9) and contributes to pathological vascular permeability exacerbating retinal and macular edema [10]. A role for the fragmented netrin-1 in pathology has also been proposed in models of multiple sclerosis (MS) in which fragmented netrin-1 found in plaques lacked the chemoattractant activity of full-length netrin-1 and contributed to disease progression by repelling migrating oligodendrocyte precursor cells and thus preventing remyelination [131].

In a similar manner, the axotomy-mediated gradual increase in truncated netrin-1 we found through our biochemical analysis of retinal samples might result from the abnormal processing of full length netrin-1 by collagenases in the RGC extracellular milieu. As a result, diffusible netrin-1 fragments might be released from the extracellular matrix and compromise the integrity of the

RGC neurovascular unit. Along these lines, Kilic *et al*. demonstrated that protecting the vascular integrity by elevating the endogenous level of vascular endothelial growth factor (VEGF) in a mouse line constitutively expressing human VEGF, promoted the survival of axotomized RGCs [137].

Based on these findings, our data suggest that axotomy-induced stress might lead to netrin-1 cleavage into shorter fragments which can evoke vasomodulatory responses and as such participate in axotomy-induced RGC apoptosis. Therefore, it would be of interest to investigate whether the expression of MMP-9 or other related collagenases increase after axotomy and if so, how short interfering RNA (siRNA)-mediated knockdown of the activated collagenase can rescue RGC death in the context of truncated netrin-1 upregulation. If confirmed, the development of strategies to block netrin-1 cleavage after axotomy might provide a promising strategy to promote the survival of injured RGCs.

V.4.2 Netrin-1-dependent protein synthesis and RGC death

Netrin-1 directs neural and axonal migration by regulating protein synthesis in the growth cones via multiple signaling pathways including the MEK/mitogen-activated protein kinase (MAPK) and the mammalian target of rapamycin (mTOR) kinase [138]. Interestingly, these pathways were shown to also regulate synapse formation and maintenance [59]. In agreement with this, recent studies have identified mTOR as a critical regulator of RGC dendritic arbor stability and synaptic integrity in the adult retina [67]. Notably, mTOR undergoes rapid downregulation in RGCs after axotomy resulting in dendritic retraction and loss of branch complexity soon after axotomy and prior to the onset of RGC death [59]. Axotomy-induced loss of netrin-1, which is an upstream regulator of mTOR, might contribute to mTOR inactivation leading to RGC dendrite pathology, synapse disassembly, and disconnection from pre-synaptic targets. The identification of target proteins synthesized downstream of netrin-1/mTOR pathway activation might lead to a better understanding of how netrin-1 downregulation after axonal injury contributes to RGC disconnection, dysfunction and subsequent death.

V.5 The effect of netrin-1 on RGC survival after axotomy

In this study, we demonstrated that netrin-1 protein levels are reduced in RGCs soon after axotomy. Therefore, we next asked whether increasing the level of netrin-1 could extend or prevent RGC survival. For this purpose, we used recombinant netrin-1 peptides corresponding to either full length or truncated netrin-1 containing domain VI and V of the full length molecule and administered them by intraocular injection. Quantitative analysis of RGC survival demonstrated that recombinant netrin-1 protein administration, either the full length or the truncated form, failed to exert any significant effect on RGC survival relative to vehicle-injected controls. The potential explanations for these unexpected negative results are discussed below.

V.5.1 Netrin-1 supplementation: recombinant protein versus gene delivery

There are several limitations regarding the use of recombinant proteins as a neuroprotective therapy for RGCs. First, a disadvantage of using a bolus intravitreal injection of a recombinant protein is that its short *in vivo* half-life and rapid extrusion from the eye can severely block its biological effect. Second, the diffusion of a recombinant protein can be very limited within the retinal tissue depending on its size and amino acid composition. Third, peptides can induce non-specific responses due to receptors in off-target cells [139]. Fourth, intraocular injection of exogenous proteins can result in pleiotropic, adverse side effects. For example, upon intraocular injection, BDNF can induce nitric oxide upregulation which may in turn limit its neuroprotective action [140]. Collectively, these factors may explain the lack of neuroprotective effect observed in our RGC survival analysis in response to intraocular administration of recombinant netrin-1.

To overcome these limitations, we next investigated the efficacy of a gene transfer approach to supplement injured retinas with a sustained source of netrin-1 by using AAV2 vectors. AAV2 has several advantages over other delivery systems including its ability to target a large number of adult RGCs (>80%), long-term transgene expression that may last for several years, and the lack of pathologies associated with this virus[141, 142]. Previous studies from our laboratory have demonstrated the efficacy of this strategy to upregulate the expression of BDNF, its receptor TrkB, and other anti-apoptotic molecules to promote RGC survival [64, 129, 143, 144]. It is also likely that the biological activity of AAV-mediated transgene expression which is synthesized *in situ*, is higher than that of recombinant protein.

In addition to AAV2 vector specificity, we have also demonstrated synapsin 1 (SYN1) promoter activity and specificity previously in the study of Wilson *et al*, 2014 where a single intravitreal injection of AAV2 vector carrying SYN1-iASPP construct, displayed a very robust level of iASPP expression in RGCs [129]. Similarly, the overexpression of AAV2 vector containing SYN1-iASPP or SYN1-GFP reporter constructs in this study, confirmed the same expression pattern and cell specificity for RGCs as demonstrated in result section (Fig. 11.2 J-L) and (Fig. 11.2 D-F), respectively. However, the AAV vector carrying SYN1-netrin-1 did not behave in the expected manner. Indeed, AAV2.netrin-1 resulted in the transfection of cells that were not RGCs thus questioning the specificity and quality of this viral vector. Future studies using an effective and specific viral vector are required to resolve this issue.

V.5.2 DCC downregulation may compromise the RGC response to recombinant netrin-1

An alternative explanation to the absence of a neuroprotective effect of recombinant netrin-1 on RGCs survival is that due downregulation of DCC after injury, the intrinsic capacity of these neurons to respond to netrin-1 is compromised. A previous study in our laboratory reported that downregulation of the TrkB receptor after axotomy is a key mechanism underlying the short-lived survival effect of BDNF on RGCs [64]. Notably, the neuroprotective effect of BDNF dramatically increased when it was combined with a single intraocular injection of AAV.TrkB. In the same manner, the striking reduction of DCC level in RGCs within 3 days of axotomy may contribute to the desensitization of RGCs to exogenous netrin-1. Therefore, the co-administration of netrin-1 and AAV2 encoding DCC might be necessary to increase the capacity of axotomized RGCs to respond to exogenous netrin-1.

V.5.3 Netrin-1 might promote chemorepulsion after axotomy

Netrin-1 elicits a dichotomous response based on the integration of information from multiple intracellular and extracellular factors [85]. For example, the intracellular level of cAMP switches the netrin-1-mediated DCC response from attraction to repulsion [145]. Experiments in the adult rat retina showed that cAMP levels decrease in RGCs following optic nerve axotomy,

raising the possibility that injured RGCs could interpret netrin-1 as a repulsive cue [146]. Therefore, increasing the level of netrin-1 after axotomy might not provide a supportive environment for maintaining the structural integrity of the injured RGCs and conversely might actually aid degeneration. Therefore, it is also important to investigate the expression pattern of UNC5 after axotomy, which is the primary receptor implicated in the chemorepulsive response to netrin-1.

In parallel, it has been reported that although the bioactive VI-V netrin-1 fragment can bind to both DCC and UNC-5, it is only sufficient to activate a chemorepellent response. In fact, in the absence of the C-terminal domain, netrin-1 loses its ability to induce DCC multimerization, and thus does not retain the chemoattractant capacity of full-length netrin-1. In addition, VI-V fragments block the chemoattractive function of full length netrin-1, suggesting that it competes with full-length netrin-1 for DCC binding sites. Therefore, future studies will be needed to test whether: 1) netrin-1 does have an effect on injured adult RGCs, 2) it promotes or inhibits RGCs survival, and 3) its activity is modulated by different intracellular and extracellular factors after axotomy.

V.5.4 Recombinant netrin-1 concentration

For the sake of protein stability, recombinant proteins are purified from cell cultures in an accompanying amount of salt. Therefore, to decrease the salt concentration to a physiological level, prior to performing intravitreal injection, recombinant netrin-1 proteins were diluted in the appropriate amount of vehicle (PBS). However, due to the concomitant dilution of protein, the concentration of netrin-1 recombinant protein could have been too low to exert a biological effect on RGCs after axotomy and might explain the lack of neuroprotective effect of netrin-1 observed in our results.

In conclusion, our data suggest the presence of an interacting network of netrin-1/DCC or netrin-3 /DCC in RGCs of the adult mammalian retina. Together, these data open the door to a range of assumptions about the functions of netrin-1 and netrin-3 in the mature retina. Based on the functions of netrin-1 previously identified in the mature CNS, netrin-1/3 could act to regulate

dendritic integrity and synapse stability as well as promote structural maintenance in the adult mouse retina. However, functional analysis is needed to evaluate the actual importance of netrin-1 and netrin-3 signaling in this system. In this regard, it would be interesting to investigate the probable modifications in synaptic transmission or retinal structure in the absence of netrin-1/3 proteins. In addition, based on our finding that netrin-3 is strongly expressed in the adult retina, it would be of future interest to elucidate its function in developing and adult retinas.

VI. References

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