

Candidate Genes Involved in Establishment of the Connection between Retina and Superior Colliculus

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Abbreviations

Retina ganglion cells (RGCs)

Direction selective ganglion cells (DSGCs)

ON-OFF direction selective ganglion cells (ooDSGCs)

Intrinsically photosensitive retina ganglion cells (ipRGCs)

Neural retina (NR)

Retinal pigment epithelium (RPE)

Retinal progenitor cells (RPCs)

Superior colliculus (SC)

Stratum zonale (SZ)

Stratum griseum superficial (SGS)

Upper stratum griseum superficial (uSGS)

Lower stratum griseum superficial (lSGS)

Stratum opticum (SO)

Stratum griseum intermediate (SGI)

Lower stratum griseum intermediate (lSGI)

Upper stratum griseum intermediate (uSGI)

Stratum griseum profundum (SGP)

Stratum album profundum (SAP)

Parabigeminal nucleus (PBN)

Lateral geniculate nucleus (LGN)

Ventricular zone (VZ)

Subventricular zone (SVZ)

Intermediate zone (IZ)

Strata superficial (SS)

Strata intermedium (SI)

Strata profundum (SP)

Neuron-glia cell adhesion molecule (NrCAM)

Gene ontology (GO)
Immunohistochemistry (IMC)
N-terminal slit (SlitN)
C-terminal slit (SlitC)
Neuropilin (Nrp)
L1 cell adhesion molecule (L1CAM)
Semaphorin3 (Sema3)
Cadherin (Cdh)
Neurofascin (Nfasc)
Ankyrin (Ank)
Neurologin (NLGN)
Neurexin (Nrxn)
EphrinB (EphB)
Plexin (Plxn)

Abstract

The proper neural connection controls variant neurological functions in the nervous system. The mutation and disruption in neural connections lead to neurological malfunction and diseases. The nervous system is composed of several neural connections in different areas. One of these examples is the connection between the retina and the superior colliculus, namely retino-collicular connection. The retina is a neural part of the eye and has a role in image processing and vision. The superior colliculus is a midbrain structure and plays a role in innate behaviors and head and eyes movement. The correct development of the retino-collicular connection is integral for proper visual processing.

Axon guidance, cell-cell adhesion, and synaptic formation are some of the developmental processes that are involved in the establishment of the retino-collicular connection. Many genes regulate these processes during embryonic and postnatal developmental periods. Here in this research, new candidates which can be involved in the establishment of the retino-collicular connection are investigated by bioinformatics tools and transcriptomics databases such as gene ontology, immunohistochemistry, in situ hybridization, and single cell databases. Finally, five potential families with different developmental functions were chosen and evaluated for the final conclusion. Slit/Robos (Slit1/2, Robo1/2), Neuropilins (Nrp1/2), Cadherins type II (Cdh6/7/8), Neurofascin (Nfasc), Neuroligins (NLGN1/2/3) are our candidates' gene for this research.

Keywords: Retino-collicular connection, Axon guidance, Cell adhesion, Synaptic formation, Bioinformatics tools, Transcriptomics data, Slit/Robo, Neuropilin, Cadherins, Neurofascin, Neuroligins.

Introduction

Retina

The retina is an inner, neural, and light-sensitive layer of the eye that belongs to the central nervous system. The role of the retina is to receive light signals, convert them into electrical signals, and transfer them into the brain for visual processing (Purves *et al.*, 3th edition). Visual ability in both dark and light environments, colour discrimination, and a high degree of visual precision are some functions of the retina (Erskine and Herrera, 2014).

The mouse retina consists of more than 60 different types of neurons that are classified into five major groups with different functions: photoreceptors (rods and cones), bipolar cells, horizontal cells, amacrine cells, and ganglion cells. There is also one major group of glial cells in retina: Müller cells (Jeon, Strettoi and Masland, 1998; Graw, 2010; Masland, 2012). In addition, the retina is made of different cellular layers. These layers transmit immature nervous signals to the optic nerve to reach retinoreceptive areas in the brain. Pigment epithelium, photoreceptor outer segments, outer nuclear layer, outer plexiform layer (synaptic layer), inner nuclear layer, inner plexiform layer (synaptic layer), and ganglion cell layer are the main layers of the retina from the outer part to the inner part, respectively (Grossniklaus, Geisert and Nickerson, 2015).

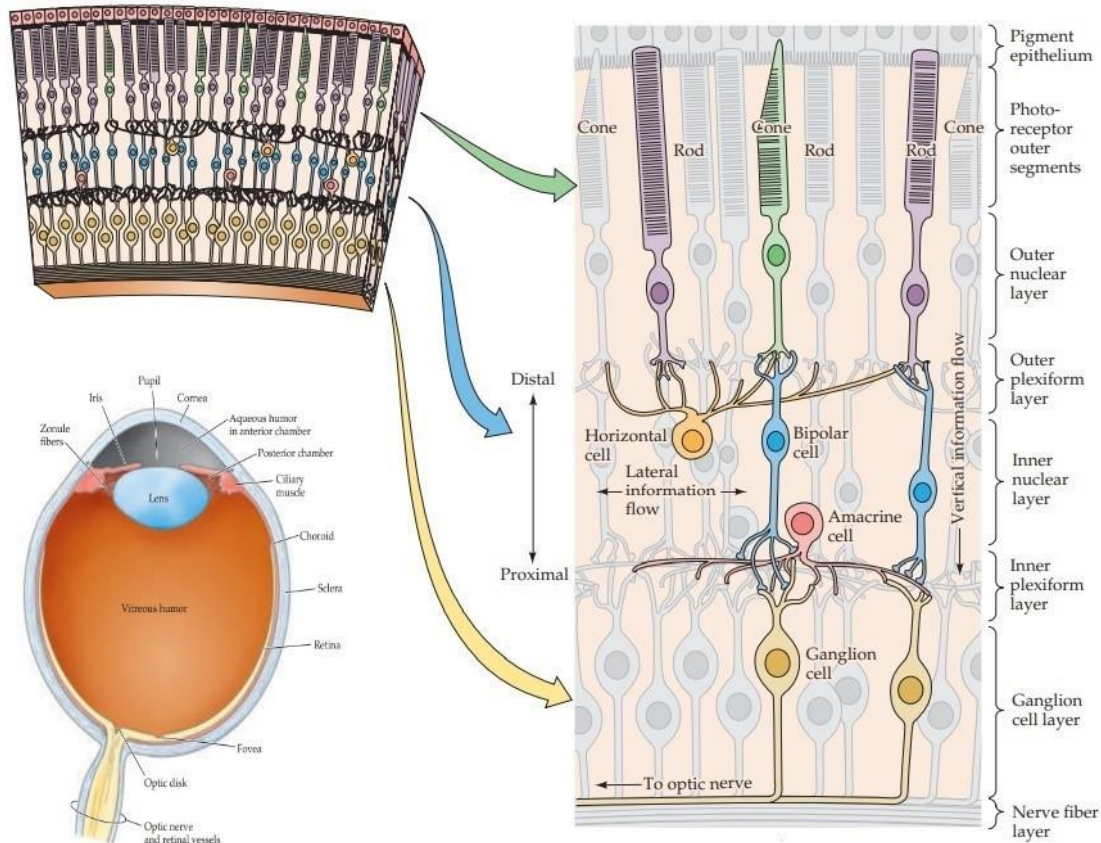


Figure 1: The anatomy of human eye and retina. The figure displays the neural cells and cellular layers from outer (distal) part of the retina to the inner (proximal) part. This figure is taken from (Purves *et al.*, third edition).

The neural activities circuit into two planes in the retina: radial (vertical through the thickness of the retina) and tangential (horizontal, parallel to the retinal surface) (Wang *et al.*, 2017). The Müller glial cells control the organization of neurons among these two planes and maintain the homeostasis of retinal tissues such as regulation of synapse development, neurovascular coupling, electrolyte balance, cellular metabolism, and organization of integration structures (Wang *et al.*, 2017). Their cell bodies place in the inner nuclear layer and they transverse throughout the retina and make a connection with different layers like ganglion cell layers' blood vessels (forms inner limiting membrane), both synaptic layers, and outer nuclear layer (forms outer limiting membrane) (Wang *et al.*, 2017).

Firstly, the image processing begins via the initiation of the visual transduction cascade in the retina photoreceptors (Masland, 2012). The mouse retina has two different types of photoreceptors, namely rod and cone. These receptors have two parts: outer and inner segments. While the outer segment detects a light signal, the inner segment has a role in metabolisms via its nucleus and other cellular organelles (Kawamura and Tachibanaki, 2008).

When rod and cone photoreceptors receive a light signal by their photopigments in the outer photoreceptor layer, they are hyperpolarized and send signals to bipolar and horizontal cells in the inner nuclear layer to establish approximately 12 different parallel streams (Masland, 2001; Masland, 2012; Grossniklaus, Geisert and Nickerson, 2015). In addition, rods are highly sensitive to light and respond to a single photon but they have a delay in response to flash (on-off light stimulus). Thus, they are responsible for the dark vision, but are not suitable for moving object detection. Conversely, cones are less sensitive to light and they respond without delay to the flash. Nevertheless, cones are responsible for daylight vision and moving object detection (Kawamura and Tachibanaki, 2008).

Secondly, the 12 parallel information streams start from the activation of bipolar cells. These neurons transfer signals to amacrine and ganglion cells in the ganglion layer (Masland, 2012). The mammalian retina has several types of bipolar cells. Functionally, all bipolar cells are classified into ON and OFF cells and sub-classified into ON-transient, ON sustained, OFF transient, and OFF sustained regarding their light responses. Moreover, they make synapses with either cone or rod photoreceptors (Wässle *et al.*, 2009; Masland, 2012). Each bipolar cell express unique and different neurotransmitter receptors, ion channels, and intracellular signaling pathways to maintain different visual processions by excitatory or inhibitory functions (Euler *et al.*, 2014). The reason for functional dichotomy is the different expression of glutamate receptors between photoreceptors and bipolar cell's dendrite synapses (Wässle *et al.*, 2009). The OFF bipolar cells are hyperpolarized in response to light, because glutamate expression leads to opening of cation channels; meanwhile, ON bipolar cells are

depolarized due to the closure of their cation channels by glutamate receptors (Masland, 2012).

Finally, neural signals are transmitted from retinal ganglion cells (RGCs) to different areas of the brain through the optic nerve for future processing (Masland, 2001; Masland, 2012; Baden *et al.*, 2016; Grossniklaus, Geisert and Nickerson, 2015). While all RGCs and most amacrine cells receive electrical inputs from cone bipolar neurons, a few of them receive signals from rod receptors by the assistance of cone bipolar cells indirectly. Based on each stream that circulates among cells, the retina responds differently to starlight and daylight (Masland, 2001). Two-thirds of brain areas receive retina's signals from RGCs (Dhande and Huberman, 2014).

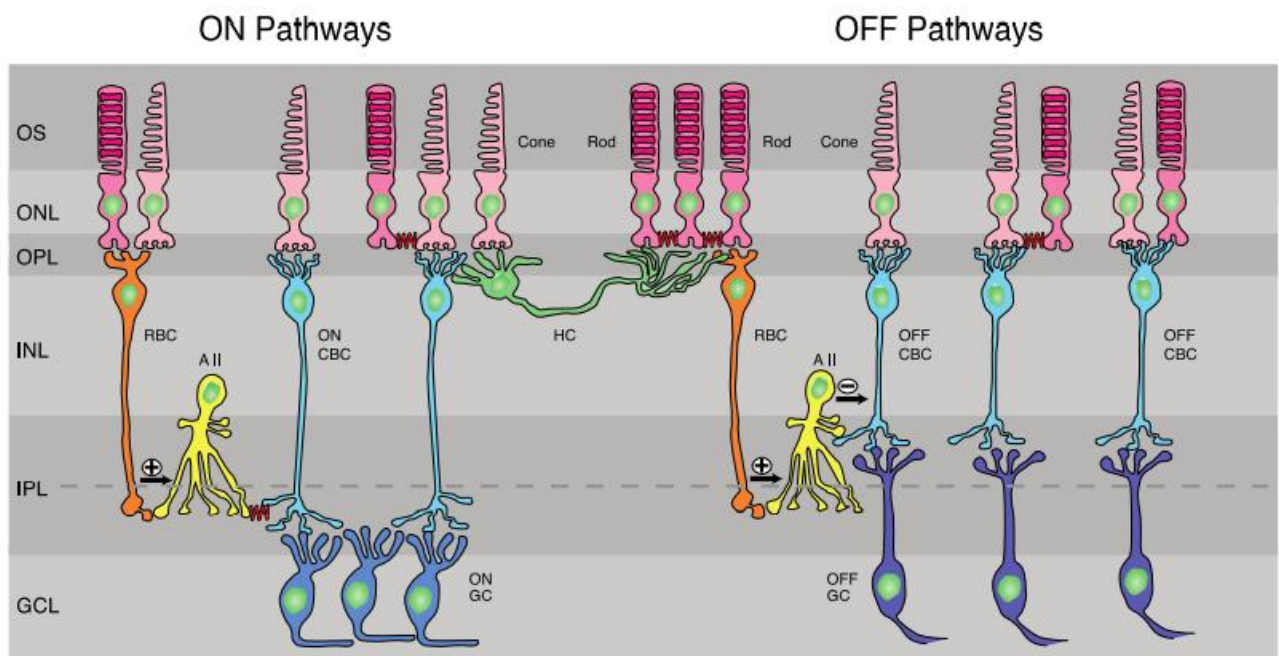


Figure 2: The mouse retina's rod and cone pathways. The rod or cone receptor interacts with ON/OFF bipolar cells. They send signals to ON/OFF ganglion cells in the ganglion layer. Based on which pathways (ON/OFF) is activated, future processing will occur. This figure is taken from (Fain and Sampath, 2018).

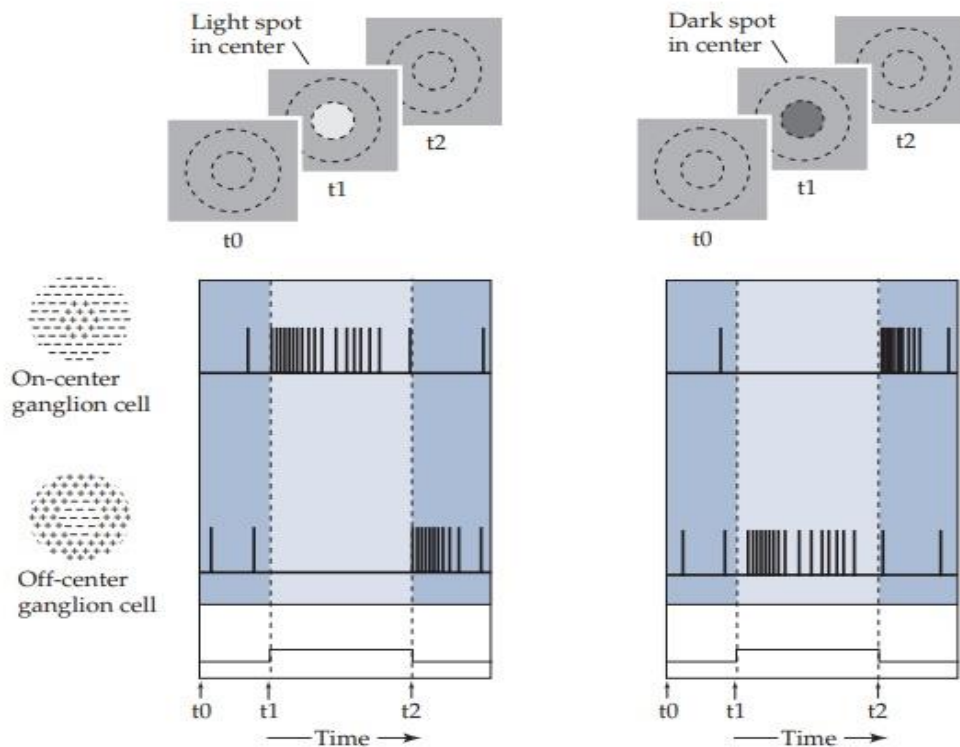


Figure 3: The responses of ON-center and OFF-center ganglion cells. When the light spot appears in the center of the receptive field, the ON ganglion cells will be activated. In contrast, the dark spot in the center of the receptive field leads to the activation of OFF ganglion cells through ON/OFF pathways in the retina. This figure is taken from (Purves *et al.*, third edition).

Amacrine cells are the most diversified retinal neurons. They are inhibitory interneurons. Amacrine cells are subdivided by their dendritic morphology and stratification into three groups: narrow-field, medium-field, and wide-field amacrine cells (Balasubramanian and Gan, 2014). Besides, based on their neurotransmitter expression they are categorized into two major groups: GABAergic and glycinergic types. GABAergic amacrine cells are mostly wide-field cells and stratify into the ganglion cell layer and inner nuclear layer (Balasubramanian and Gan, 2014). Starburst amacrine cell is one subtype of GABAergic cells that they contribute in direction selectivity and motion detection by interacting with RGCs (Tiran-cappello, 2012). Glycinergic cells are narrow-field and they synapse into the inner nuclear layer (Balasubramanian and Gan, 2014).

Retinal ganglion cells interpret inputs based on the interaction within bipolar and amacrine cells, and information about contrast and intensity that present on them to detect lighting patterns in the retina (Grossniklaus, Geisert and Nickerson, 2015). Each RGC has a specific role in visual processing such as respond to specific directions of motions or orientations (Dhande and Huberman, 2014). More than 30 types of RGCs have been identified based on their morphology, localization, function, and susceptibility to degeneration (Rheume *et al.*, 2018). Direction-selective ganglion cells, alpha RGCs, intrinsically photosensitive RGCs, and F-RGCs are the most known types of RGCs in the mouse retina (Rheume *et al.*, 2018; Tran *et al.*, 2019).

Direction selective ganglion cells (DSGCs) are one type of ganglion cells that have a role in motion detection (Tiran-cappello, 2012). DSGCs have been divided into three classes, including ON-OFF DSGCs (ooDSGCs), ON DSGCs, and OFF DSGCs. The ooDSGCs are the main motion detector in the retina (Tiran-cappello, 2012). They detect moving stimuli in four different axes: dorsal, ventral, nasal, and temporal. They also respond to high velocities. Moreover, ooDSGCs are sensitive to both starlight and daylight. It means they are sensitive to dark-light and light-dark transition in their receptive fields and synapse in both OFF and ON inner plexiform layers (Tiran-cappello, 2012; Sanes and Masland, 2015). In contrast, ON DSGCs make synapse into the ON layer of inner plexiform. They are sensitive to dark-light transition implying they can detect the light spot on the center of their receptive fields. They detect moderate and low speeds in dorsal, ventral, and temporal directions (Sun *et al.*, 2006; Tiran-cappello, 2012). Also, OFF DSGCs stratify into the OFF inner plexiform layer of the retina and respond to light-dark transition through upward motion in their receptive fields (Tiran-cappello, 2012). One population of OFF DSGCs that has been identified is J-RGCs (Rousso *et al.*, 2016).

Alpha RGCs are another type of retinal ganglion cells that are classified into four subclasses (Tran *et al.*, 2019). The OFF-sustained Alpha RGCs maintain action potential during the darkness without bursting. Conversely, OFF-transient Alpha RGCs make an action potential in the dark phase by bursting and spiking rapidly. ON-

sustained and ON-transient alpha RGCs have a similar function as OFF types RGCs but they are activated during the daylight (Krieger *et al.*, 2017; Rheaume *et al.*, 2018). OFF types of Alpha ganglion cells stratify into the outer part of the inner plexiform layer, meanwhile, ON types synapse to the inner part of it. All alpha ganglion cells respond to moving objects equally in all directions without direction-selective mechanisms (Krieger *et al.*, 2017).

Intrinsically photosensitive RGCs (ipRGCs) make up below 10% of total retinal ganglion cells that are directly photoreceptive due to melanopsin photopigment expression (Hattar *et al.*, 2008; Sonoda, Okabe and Schmidt, 2011). There are six different ipRGCs in the retina, named M1-M6. Besides, they mediate non-image-forming processes such as pupillary movements (pupillary light reflex), synchronization of circadian rhythm, hormonal and mood regulation, and image-formation (Hattar *et al.*, 2008; Sonoda, Okabe and Schmidt, 2011; Zhao *et al.*, 2014). Previous studies have shown that some subtypes of ipRGCs such as M4 and M5 have a major role in image-formation vision like color opponency (Sonoda, Okabe and Schmidt, 2011). Photoreceptors in the retina convey chromatic signals by different opponent mechanisms to reduce the redundancy in color detection. Red-green and yellow-blue are two opponent mechanisms that are used for color discrimination and vision. During color vision, each mechanism will be dominated and one spectrum will be seen eventually (Lee, Wachtler and Sejnowski, 2002). One example of ipRGCs responsibilities in color opponency is M4 cells reaction to a light stimulus that makes excitatory actions. The M4 cells receive signals from rod receptors and have an overlap and interaction with ON alpha RGCs (Sonoda, Okabe and Schmidt, 2011).

Another kind of RGCs that has been identified is F-RGCs. It expresses mainly the transcription factor *Foxp2* (Tran *et al.*, 2019). Moreover, F-RGCs are divided into four subtypes based on the intersectional expression of *Foxp1* and *Brn3* (Tran *et al.*, 2019). F-minON and F-mini OFF are one pair of them. They are direction-selective. The other pair is F-midiON and F-midiOFF which are direction non-selective ganglions (Rousso *et al.*, 2016). F-RGCs have a role in visual perception, because their axons project to

different retinoreceptive areas of the brain like other non-image forming RGCs such as superior colliculus, lateral geniculate nucleus, suprachiasmatic nucleus, and accessory optic nuclei (for example, medial terminal nucleus and olivary pretectal nucleus) (Rousso *et al.*, 2016).

In mice, eye development begins at embryonic day 8 (E8). At first, during gastrulation, the anterior neuroectoderm which is adjacent to the telencephalic/diencephalic boundary, forms a single eye field that splits in the developing forebrain into the bilateral optic pits by assistance of two morphogenes, sonic hedgehog and Six3 (Heavner and Pevny, 2012; Miesfeld and Brown, 2019). After that, the walls of the diencephalon evaginate and form optic vesicles which have close contact with the ectoderm surface. At E10 the optic cup is formed. The retinoic acid signaling and transcription factor LIM homeobox protein 2 are the important regulatory keys in this process (Graw, 2010; Heavner and Pevny, 2012).

At E12, the development of the retina starts by differentiation of the inner layer of the optic cups to the neural retina (NR) and the outer to retinal pigment epithelium (RPE) (Graw, 2010). The retina development will continue until 2 weeks after birth when the eyes are open (Graw, 2010). In the neural retina, all classes of retinal cells (photoreceptors, bipolar, horizontal, amacrine, RGC, Müller) are derived from retinal progenitor cells (RPCs) in the optic cup. The competence state changes during different developmental stages due to a lengthening of the cell cycle. The RGCs appear firstly in contrast, the Müller glia are raised lastly (Miesfeld and Brown, 2019).

As mentioned before, RGCs are the first neural retinal cell types to arise from the dorsocentral optic cup. Some transcription factors have a role in this developmental pathway such as Sox2, Six3, Six6, and Pax6 (Miesfeld and Brown, 2019). For example, Pax6 expression leads to activation of two other proneural bHLH transcription factors Nurog2 and Atoh7 in early neurogenesis in RGCs (Miesfeld and Brown, 2019). Parallel to RGCs' development, amacrine cells are born from RPCs too. GABAergic amacrine cells are born before glycinergic cells (Balasubramanian and Gan, 2014). Although these cells originate from RPCs which express Pax6, Foxn4, Ptf1a transcription factors,

for subtype's differentiation amacrine cells need other transcription factors too, like Barhl2, Isl1, Bhlhb5, Neurod2, Neurod6, and the Ebf family. Barhl2 is required for glycinergic amacrine cells and Bhlhb5 for GABAergic ones (Graw, 2010; Miesfeld and Brown, 2019).

Cone, rod, and bipolar cells are derived from a subpopulation of RPCs that express Otx2 after generation of RGCs and amacrine cells. Cone photoreceptors appear first, then rods and finally, the bipolar cells are generated. Otx2 activates Vsx2, Prdm1, and Crx for photoreceptors and bipolar cells differentiation (Fei, 2003; Miesfeld and Brown, 2019). The Müller glia is the latest cell that is raised from RPCs. The Müller cells are generated across the retina from outer to the inner limiting membrane and finally enclose all neuronal cells. These cells develop after laminar structures of the retina. In addition, Lhx2 is one of the important regulators in the Müller glial genesis (Wang *et al.*, 2017; Miesfeld and Brown, 2019).

Retinal pigment epithelium (RPE) is a monolayer of cells located between photoreceptors and the choroidal vasculature and has a role in the outer retina's homeostasis. RPE is crucial for the outer retina-blood barrier and support of photoreceptors (Cechmanek and McFarlane, 2017). Mitf and Otx2 are the vital transcription factors for RPE specification and Pax6 is critical for differentiation. Mitf and Vsx2 (for neural retina specification) act antagonistically with each other during development in the optic vesicle to maintain the early boundary between RPE and NR for future developmental processes (Heavner and Pevny, 2012; Miesfeld and Brown, 2019). The Mitf is expressed highly through the dorsal part of the optic vesicle but after the expression of Vsx2, the Mitf expression will be decreased (Heavner and Pevny, 2012; Miesfeld and Brown, 2019).

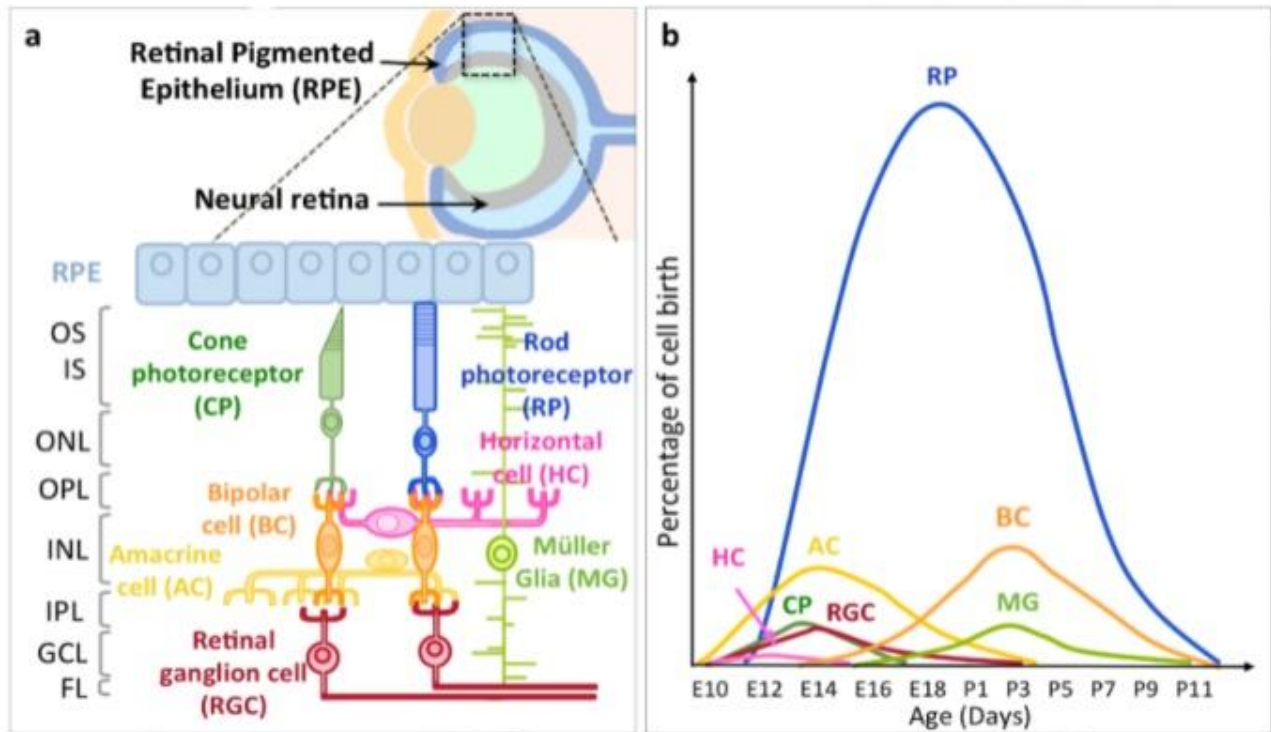


Figure 4: The illustration of RPE and NR and their differentiations to different cells. The time of each retinal cell differentiation is shown in b. The RGCs develop first and the Müller and bipolar cells develop last. This figure is taken from (Nguyen-ba-charvet and Rebsam, 2020).

All in all, retina is a neural part of the eye that first visual processing begins in. The visual signal converts into the neural signal in the retina (Erskine and Herrera, 2014). The retina is composed of five major neurons and six different cellular layers, respectively (Jeon, Strettoi and Masland, 1998; Graw, 2010; Masland, 2012; Grossniklaus, Geisert and Nickerson, 2015). Visual processing begins at the photoreceptors and then transmits to bipolar cells for establishment nearly 12 parallel neural streams (Masland, 2012). After that, the neural signal transmits to the amacrine cells and retinal ganglion cells. Finally, retinal ganglion cells send signals to different part of the brain for future processing (Maland, 2001; Masland, 2012; Grossniklaus, Geisert and Nickerson, 2015; Baden *et al.*, 2016). In addition, the development of the retina initiates at E12 from inner layer of the optic cup. Neural retina and retinal epithelium pigment layer are developed (Graw, 2010). Each neuron and layer develops via

assistance of variant expression factors during different developmental stages to form mature retina (Graw, 2010).

Superior Colliculus

The superior colliculus (SC) is a paired sensory-motor and multimodal integration structure in the roof of the midbrain. It receives more than 85% of retinal ganglion cell's projections and has a connection with the visual cortex (Ito and Feldheim, 2018; Wang *et al.*, 2020). The SC has an integral role in visual processing and control of innate behaviors such as eye, head and body movement, gaze control, escaping and approaching prey, and freezing (Erskine and Herrera, 2014; Ito and Feldheim, 2018; Wang *et al.*, 2020). For instance, medial neurons of the SC mediate escape responses through dorsal periaqueductal gray. They also mediate freezing behavior through the amygdala in the mouse brain (Wang *et al.*, 2020). In addition, the SC's neurons transmit signals to the brainstem and spinal cord motor neurons to establish and guide orientation responses and gaze control (May, 2006; Wang *et al.*, 2020).

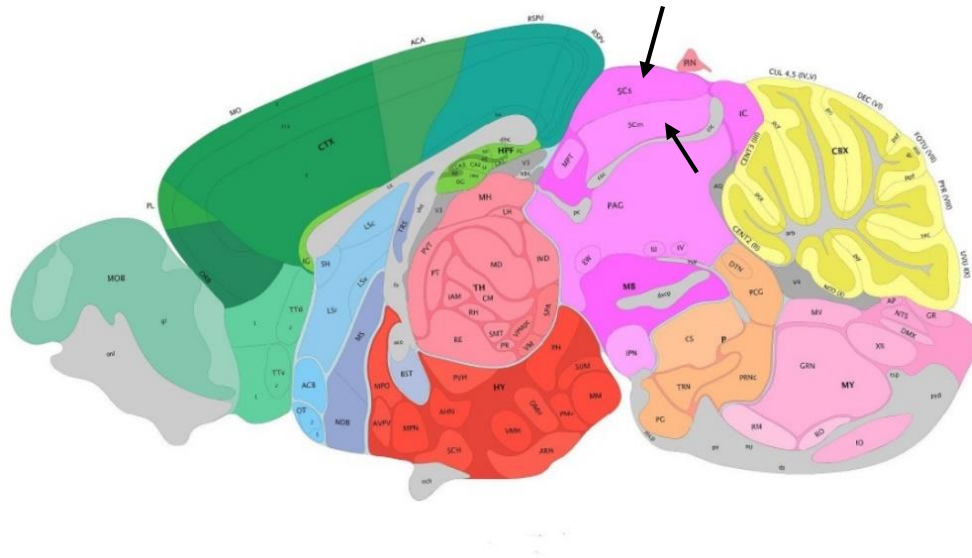


Figure 5: The anatomy of P56 mouse brain (Sagittal). The two black arrows show the sensory and motor part of the superior colliculus. This figure is taken from (Reference mouse atlas map from Allen atlas brain).

The SC is a laminated multilayer structure and its layers consist of fibers and neurons (May, 2006). Superficial and deep (deep and intermediate layers) are two major parts of the SC which each layer has a different function (Wang and Burkhalter, 2013; Ghose *et al.*, 2014). The outermost superficial part is the zonal layer (stratum zonale-SZ) that has

the least amount of cells. The superficial gray layer (stratum griseum superficial-SGS) is beneath the SZ. The SGS is divided into two sublayers: The upper stratum griseum superficial (uSGS) and lower stratum griseum superficial (lSGS) (May, 2006). The stratum opticum (SO) is another part of the superficial layer and exists below the lSGS. The SO is made of fibers and axons of the optic nerve which originate from retinal ganglion cells in the retina (May, 2006; Wang and Burkhalter, 2013).

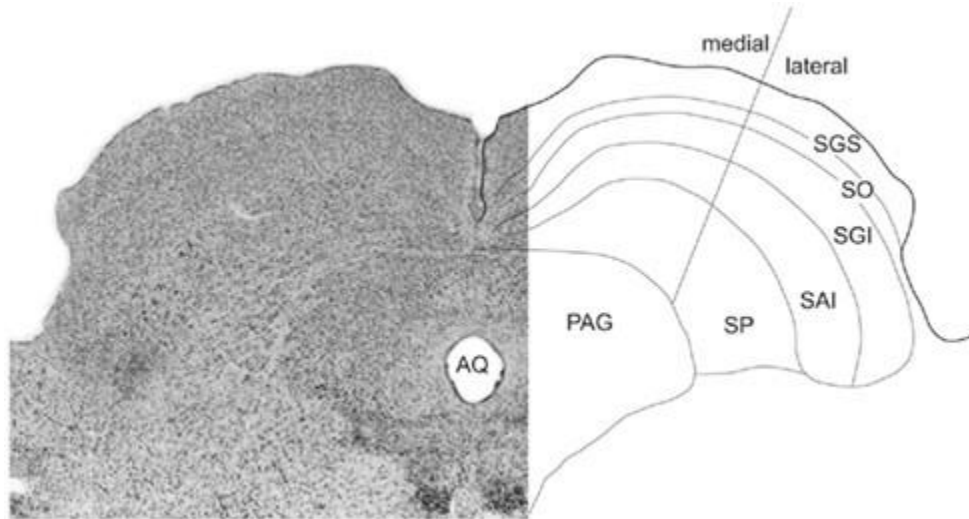


Figure 6: The SC structure. Different layers of the SC from superficial region to deep region is shown. This figure is taken from (Comoli *et al.*, 2012).

The superficial part is for visual sensory processing (May, 2006). Most neurons in the superficial layers, including SZ, SGS, and SO, respond to both ON and OFF flash stimulation (ON-OFF receptive field), whereas few neurons respond to only ON or OFF flashes. Moreover, neurons present direction-selectivity in their activities (Wang *et al.*, 2010). For instance, some of them respond to moving objects strongly while others can detect stimulation within one direction or in the grating orientation (Wang *et al.*, 2010; Solomon, 2018).

The neurons of superficial layers have linear and non-linear responses to the luminance modulation. In non-linear responses, neurons respond to both black and white stimulation in contrast to linear mechanisms. Contrast sensitivity is another function of the superficial layers of neurons in the SC (Solomon, 2018). Visual contrast sensitivity is

the ability to detect differences between patterns and shades in image-forming processing. Contrast sensitivity is important in the discrimination of objects from their backgrounds (Alghwiri and Whitney, 2020).

The intermediate gray layer (stratum griseum intermediate-SGI) in the SC has two sublayers by itself, namely lower sublamina (lSGI) and upper sublamina (uSGI). The intermediate layer also has a white layer too. That is stratum album intermediate (SAI) (May, 2006). Deep layers of the SC are multimodal and they receive inputs from the primary somatosensory cortex, the auditory nuclei of the midbrain, the trigeminal nucleus of the brain stem, and visual inputs from the superficial SC and visual cortex (Cang *et al.*, 2018). Stratum griseum profundum (SGP) is the gray deep layer and stratum album profundum (SAP) is the innermost layer of the SC and is a white deep layer (May, 2006).

Deep layers have a role in the integration of different sensory inputs and premotor signals interpretation (Cang *et al.*, 2018). For example, when deep areas of the SC are damaged, the eye and head movements will be disrupted during exposure to the sensory stimuli (Barth, 1987). Nevertheless, deep and intermediate layers are crucial for saccadic eye and head movements. GABAergic interneurons in the SGI are necessary for this purpose (Sooksawate *et al.*, 2011). In fact, the disinhibition of these interneurons should happen for initiation of the burst activity in the SGI neurons for eye saccadic movement. Thus, GABAergic interneurons based on their input organization will either participate in the recurrent inhibitory processing or feedforward inhibitory circuits when they receive signals from adjacent neurons. If they receive signals from distant neurons they will mediate lateral inhibition or exert feed-forward inhibition in the SGI (Sooksawate *et al.*, 2011).

Selectivity for the looming feature, spatial invariance, and habituation to familiar stimuli are the other three features of the deep SC neurons. In contrast to superficial areas of the SC, deep neurons respond stronger to certain figural stimulation such as expanding dark disks and they do not respond sustainably (Lee *et al.*, 2020). Regarding deep neurons, they have larger receptive fields for the detection of looming objects in any

direction. The deep neurons make a burst spiking at their first exposure to the stimulation but after that, they will repeat the same spiking pattern during the neural activities (habituation) (Lee *et al.*, 2020).

The SC has several types of cells in each layer: Horizontal cells (interneuron), narrow field (vertical), wide-field (vertical), stellate cells (their dendrites do not have any specific orientation extension), and marginal cells are the major cells of superficial layers (May, 2006). Horizontal cells are situated in the upper SGS and they are inhibitory GABAergic neurons (Cang *et al.*, 2018). Narrow and wide-field cells are two excitatory (glutamatergic) cells in the SGS which narrow field cells exist in the lower SGS and their dendritic fields' project in a narrow column in the upward and downward direction. Wide-field cells are found in lower SGS and SO and their dendrites extend toward the surface of the SC (May, 2006; Cang *et al.*, 2018). Marginal cells locate on the SZ and extend downwardly toward SGS and upper SO. They can be both inhibitory and excitatory neurons (Mooney, Lewin and Hess, 1998; Cang *et al.*, 2018).

These neurons project to different areas from superficial areas of the SC. For instance, wide-field cells extend to the lateral posterior nucleus or narrow-field cells have projections to the deeper SC and parabigeminal nucleus (PBN). Stellate and horizontal cells project to the lateral geniculate nucleus (LGN) and parabigeminal nucleus (Cang *et al.*, 2018). PBN is a cluster of satellite that belongs to the SC at the edge of the midbrain. The PBN has a major interaction with superficial layers of the ipsilateral and contralateral layers of the SC (Cui and Malpeli, 2003). Also, deep layers of the SC can provide inputs for this nucleus. The role of PBN is in regulation of saccadic movements by detecting saccades targets and facilitation eye movements (Cui and Malpeli, 2003). The LGN is thalamic nucleus that has a connection with retina and superior colliculus. The LGN projects to the visual cortex for visual processing and neural activity (Wang, 2009).

Although there is a connection between superficial layers and deep SC, most types of superficial layers' cells cannot project to the deep laminations. The deep SC has its own structure (Mooney, Lewin and Hess, 1998). Predorsal bundle cells are one of the unique

cells in the deep SC that contribute to the shifting gaze and gaze control in the organism. Predorsal bundles project to the brainstem gaze centers and cervical spinal cord for gaze function (Bickford and Hall, 1989). The predorsal bundles are large multiple neurons (May, 2006).

The development of the SC begins from the dorsal part of the midbrain. This process peaks around E13 in the mouse and consists of different stages such as neurogenesis, cell migration, and early morphological differentiation from inside to outside, respectively (Cang *et al.*, 2018). At E13, the mesencephalic plate is transformed from neuroepithelium to three cellular layers which are situated between the ventricular wall and the stratum zonale: ventricular zone (VZ), subventricular zone (SVZ), and intermediate zone (IZ) (Edwards, Caviness and Schneider, 1986; Tsunekawa, Yanagawa and Obata, 2005). At E15, the IZ differentiates into other three layers, including strata superficial (SS), strata intermedium (SI), and strata profundum (SP). Then, at E17 cellular and fiber layers are arisen from these layers to form mature six major laminates of the SC (Edwards, Caviness and Schneider, 1986; Tsunekawa, Yanagawa and Obata, 2005).

The majority of neurons are generated from E11 to E14. Next, neurons migrate toward three primary layers and then to the different six layers of the SC. Finally, laminar organization develops from E13-E17 (Edwards, Caviness and Schneider, 1986; Tsunekawa, Yanagawa and Obata, 2005). For example, neurons migrate to IZ at E13 and populate densely in the SS and SP at E15. Conversely, in the SI cells are populated at E13 (Edwards, Caviness and Schneider, 1986). The neural migration completes until birth in the mouse. By the end of the first postnatal week (P7) most parts of the SC will be matured and final maturation will be completed until the first month of the postnatal development (Edwards, Caviness and Schneider, 1986; Cang *et al.*, 2018).

Two forms of cell dispersions are observed during the migration of cells from germinal zones: radial and tangential (Tan *et al.*, 2002). The radial pattern is dominant in the migration of cells in the SC development. Those cells with radial dispersions form a

radial column from VZ to the surface. In contrast, tangential patterns are limited to the superficial layers and upper radial column (Tan *et al.*, 2002; Cang *et al.*, 2018). During postnatal patterning, tangential cells organize into the SGS and SO of the SC and radial columns organize into the intermediate and deep layers (Tan *et al.*, 2002; Cang *et al.*, 2018).

Many transcription factors are involved in the development of the SC. For example, Tal2 a bHLH transcription factor play a role in the formation of the superficial layers in the mice (Cang *et al.*, 2018). Moreover, cytoskeleton proteins are involved in the neural migration and laminar organization in the SC. Tuba1a is an alpha-tubulin gene that controls radial migration in the SC (Cang *et al.*, 2018).

Overall, the superior colliculus is a sensory-motor multimodal structure. It is one of the major retinoreceptents in the brain and has a connection with visual cortex (Ito and Feldheim, 2018; Wang *et al.*, 2020). The SC has a role in visual processing and innate behaviors (Ito and Feldheim, 2018). Anatomically, the SC is a laminated structure and is divided into two major areas (superficial and deep) and some sublayers. The superficial layers is specified for visual processing and the deep layers are for motor processing and integration (May, 2006; Wang and Burkhalter, 2013; Cang *et al.*, 2018). Moreover, the SC has several types of cells which have interaction between each other and other structures like PBN or LGN (May, 2006). The development of the SC initiates at E13. The mesencephalic layer is specified into three primary layers and finally at E17 the main layers appear. Some transcriptional factors are involved in different developmental processes in the SC (Cang *et al.*, 2018).

Retino-collicular Connection

The SC is one of the major retinorecipient areas in the brain. As mentioned earlier, more than 85% of RGCs axons terminate to the superficial layers of the SC directly (Reinhard *et al.*, 2019). Nevertheless, the retina, to be specific RGCs, has a direct neural connection with superior colliculus and this connection controls some processes in visual pathways and behaviors (Ito and Feldheim, 2018; Reinhard *et al.*, 2019).

The Retina is connected to the SC by the retinoptic pathway. The optic axons move from the optic disk of two eyes and form the optic nerve. Then, they reach the optic chiasm and establish either contralateral or ipsilateral projections toward their targets by optic tract (Godement, Salaun and Imber, 1984; Erskine and Herrera, 2014).

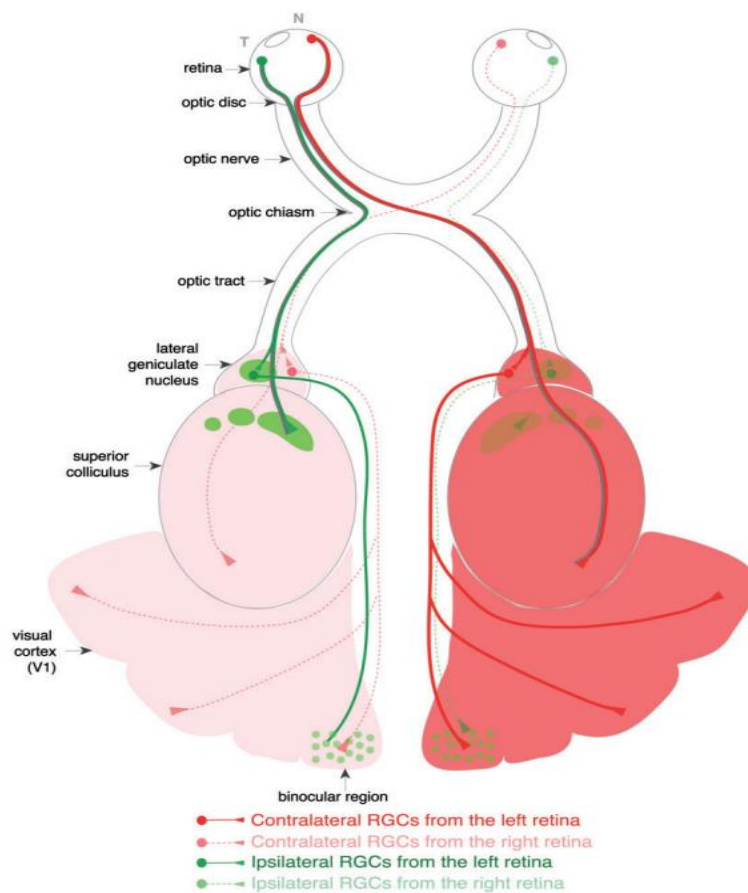


Figure 7: Schematic representation of the visual pathway in the mouse. The ipsilateral and contralateral projections from right and left eyes extend toward optic chiasm and reach the superior colliculus. This figure is taken from (Erskine and Herrera, 2014).

In mice, the development of the retinoptic pathway initiates around E12 and will be mature approximately by the P10 (Guillery, 1992; Demyanenko and Maness, 2002). The optic fiber appears at this time in the optic stalk. At E14 some retinal fibers reach the optic chiasm and by the E16 the optic tract development is almost complete (Guillery, 1992). Regarding the development of the contralateral projections, collicular plates are visible at the surface of the mesencephalon at E16. They migrate medially toward their destinations until E18. Later, the medial edges move toward each other and form contralateral SC (Godement, Salaun and Imber, 1984). Contralateral axons mainly terminate in the superficial layers. At P4, the SZ and uSGS are enriched with a high density of these axons and ISGS have a moderate density of them (Godement, Salaun and Imber, 1984). In ipsilateral development, the first ipsilateral axons appear at E18 at the brachium of the SC. At P1, most ipsilateral fibers migrate to the major part of the SC specifically the SO (Godement, Salaun and Imber, 1984). They will continue to move towards the SC and the density of those axons will be higher (Godement, Salaun and Imber, 1984).

Some studies have tried to investigate a topographic map for the RGCs' efferent axons to the SC. The topographic map is a map that provides information about the ordered projections of sensory surfaces like the retina and is used for the observation of spatial information (Triplett *et al.*, 2010). One type of topographic map is retino-collicular that represents the projections of the retina to the SC (Kaas, 1997). Here are some examples of the topographic alignment between different retinal ganglion cells and different areas of the SC: ON-OFF DSGCs terminate into the uSGS, alpha RGCs into the ISGS, F-RGCs into both uSGS and ISGS. IpRGCs also project to the SC (Rousso *et al.*, 2016; Martersteck *et al.*, 2017; Ito and Feldheim, 2018).

Neural connections are critical for the appropriate function of the brain in maintaining vital processes like breathing, controlling behaviors, vision, movements, and so on (Ito and Feldheim, 2018). The establishment of a neural connection between RGCs and the SC requires different developmental mechanisms such as axon guidance, cell adhesion, synaptic formation, axon-axon competition, and neural activity (Demyanenko

and Maness, 2002; Dai *et al.*, 2013; Duan *et al.*, 2014; Ito and Feldheim, 2018). Each mechanism in the neural connection between the retina and SC is regulated by different sets of genes. Thus, differential gene expression is an integral part of retino-collicular development and function (Ito and Feldheim, 2018).

The RGC axon guidance is the first step in retino-collicular development. Axon guidance is the process that neurons extend their axons toward their targets for the neural connection. Axon guidance occurs in the growth cone of the axon (Russell and Bashaw, 2019). The growth cone is a motile structure that is situated on the tip of the axon and has an interaction by their receptors with the external environment for extending or preventing axonal growth (Russell and Bashaw, 2019). Guidance cues (attractants or repellants) interact with their receptors on axons and mediate axon pathfinding, axon outgrowth, and axon cone collapse. Most axon guidance molecules regulate Rho family (small GTPases) and their different downstream effectors to modify cytoskeletal and membrane dynamics for axon extension (Russell and Bashaw, 2019). In addition, local protein synthesis in axons (growth cones) is necessary for the growth or collapse of axons (Russell and Bashaw, 2019).

As mentioned before, the RGCs axons extend toward the optic nerve to finally reach the SC and make a synaptic connection with their targets. The pattern of axon extension among RGCs is medial, RGCs from the central part of the retina extend to the peripheral part (Inatani, 2005). Some genes are involved in the retino-collicular axon guidance development. Here are some examples of this:

To begin with, the Ephrins family is one of the guidance cues that have a role in the connection between RGCs and the SC (Dai *et al.*, 2014). Ephrins are tyrosine kinase receptors that divide into two major categories with their ligands: EphA-ephrin A and EphB-ephrin B (Ye *et al.*, 2019). EphAs and their ligands express along the temporal-nasal axis of the retina and the anterior-posterior of the SC (Ito and Feldheim, 2018). According to the previous researches, a mutation in the EphA-ephrinA signaling pathways leads to ectopic termination of RGCs' axons in the SC. Thus, it causes incorrect receptive field location in the anterior-posterior part of the SC (Ito and

Feldheim, 2018). EphB-ephrinB is expressed along the dorsal-ventral axis of the retina and lateral-medial part of the SC. EphBs have a role in the extension of the RGCs axon forwardly to the SC and establishment of the connection reversibly from the SC (Triplett and Feldheim, 2013). Nevertheless, they involve in synaptic maturation too. The mutation in EphBs family can cause ectopic termination of axons in the target and malfunctioning of proper connectivity (Triplett and Feldheim, 2013).

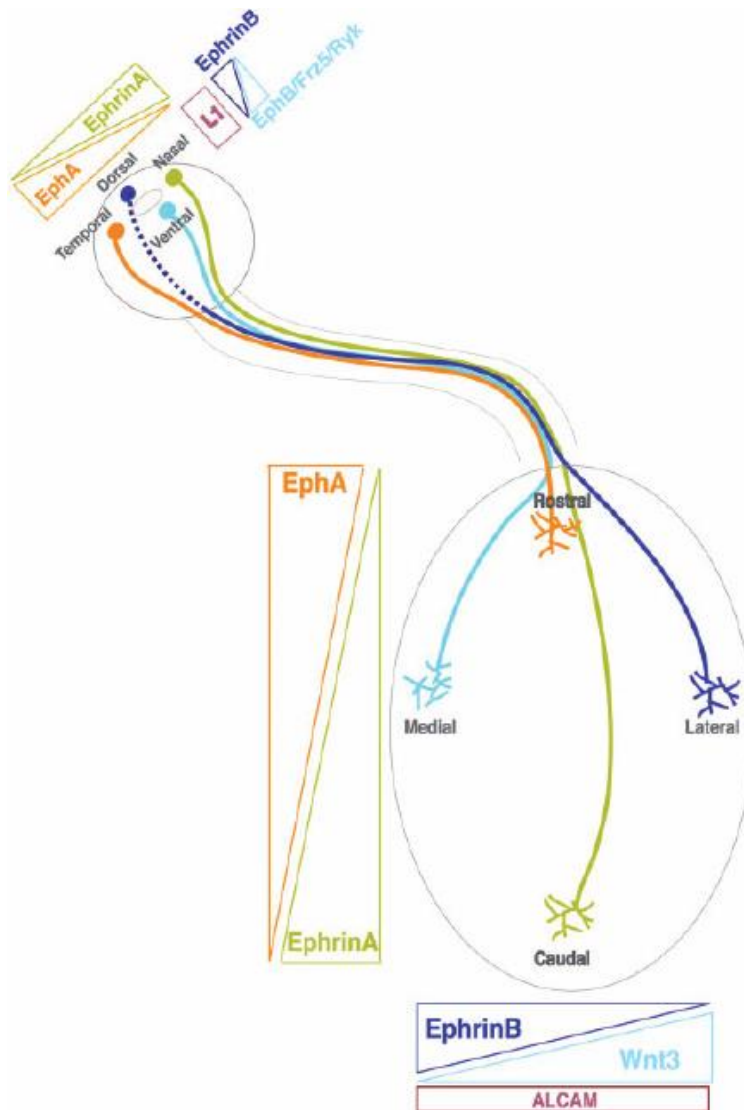


Figure 8: Guidance molecules in development of retino-collicular topographic map. The forward and backward of EphB receptor and ligand gradients initiate axon guidance and interact with other developmental mechanisms in the retino-collicular pathway. This figure is taken from (Erskine and Herrera, 2014).

Another example is Semaphorin 3A (Sema3A) that has a local effect in axon guidance in the retino-collicular map. The Sema3A activates two other proteins, Rhoa and NF-protocadherin, to regulate cytoskeletal dynamic for axon collapse and preventing excessive extension in this process (Russell and Bashaw, 2019).

The synaptic formation is another mechanism that happens in the development of the neural connection between the retina and the SC. A synapse is a small space between two neurons or neurons and muscles/glands and connects cells to each other for neurotransmission (Garner *et al.*, 2002; Sanes, 2019). Each synapse has presynaptic cells, synaptic clefts, and postsynaptic cells. Axon from one neuron (at the presynaptic site) participate in the synapse with dendrite or soma of another neuron (at the postsynaptic site) (Garner *et al.*, 2002; Sanes, 2019).

There are two types of synapses in the nervous system: chemical and electrical synapses. In chemical synapses, the electrical signals in presynaptic neurons convert to the chemical substances, named neurotransmitters (Sanes, 2019). Neurotransmitters can be classified by themselves into different categories such as amino acids, amines, purines, peptides, and nitric oxide (Nagastu, 2000). An electrical synapse transmits electrical signals by gap junctions and they have rapid transfer than chemical synapses (Shin *et al.*, 2020). Moreover, synapses are different from each other based on their morphology. Type I belongs to synapses that have a connection between axonal terminals and dendritic spines and they are asymmetric. Type II belongs to those which have axonal terminal and soma/dendritic shafts in their structures (Ko, Choii and Um, 2015).

Most types of neurotransmitters synthesize in the presynaptic neuroterminals and store in the synaptic vesicles (Nagastu, 2000). When axons are activated, neurotransmitters are released from the active zone of the presynaptic cell into the synaptic cleft (Sanes, 2019; Shin *et al.*, 2020). The action potential causes the influx of calcium ions in the presynaptic neuron and activates vesicular exocytosis to release the neurotransmitter (Shin *et al.*, 2020). Then, neurotransmitters bind to the receptors on the postsynaptic neuron to induce or inhibit action potential in the postsynaptic neuron. Neurotransmitters

will either be re-uptaken by presynaptic terminals again after their activities or degraded by metabolizing enzymes (Nagastu, 2000).

Synaptic formation happens when RGCs axons terminate to functional presynaptic sites at the SC (Sanes, 2019). When axons reach the presynaptic site, some molecular signals induce growth cones to differentiate from migratory structure to presynaptic terminals. Superficial SC cells have to differentiate too for making postsynaptic cells for synaptic development (Sanes, 2019). Cadherins, protocadherins, and neuroligin are the most important molecules that have a role in the induction and maturation of synapse (Garner *et al.*, 2002).

Chemical synapses are classified based on the expression of their neurotransmitters. Some chemical synapses are excitatory such as glutamatergic synapses (Graner *et al.*, 2002). In contrast, GABAergic and glycinergic synapses are inhibitory (Sanes, 2019). Some synapse are modulatory that means they express noradrenaline and dopamine as a neurotransmitter (Garner *et al.*, 2002). The balance of the excitatory and inhibitory ratio is critical for correct neurotransmission and neural activity in the brain. The disruption of this balance leads to several neurologic disorders (Ko, Choi and Um, 2015).

GABAergic synapses are the main inhibitory synapses in the mammalian brain. Inhibitory synapses like GABAergic inhibit the action potential through postsynaptic neurons. They are important in the balance between excitatory and inhibitory synapses during development (Ko, Choi and Um, 2015). Neuroligin-2 has a principal role in the establishment of inhibitory synapses. It can connect the pre and postsynaptic cells to each other and recognize trans synaptic signaling (Ko, Choi and Um, 2015). GABA is synthesized by glutamate precursor via glutamate decarboxylase enzyme in the cytoplasm of presynaptic neurons. GABA binds to two different types of receptors on the postsynaptic neurons. GABA-A receptors are the ionotropic and ligand-gated ion receptors. When GABA attach to these receptors, chloride ions influx inside the postsynaptic cells and prevent the action potential. Another GABA receptor is GABA-B (Jewett and Sharma, 2021). This receptor interacts with G-protein coupled receptors to

inhibit action potential by increasing postsynaptic potassium and decreasing presynaptic calcium (Jewett and Sharma, 2021).

Glycine is another inhibitory neurotransmitter and has a similar action to GABA in inhibiting the action potential. Glycine leads to an increase in the level of chloride in postsynaptic cells (Legendre, 2001). Glycine synapses have a role in locomotor behavior and spinal reflex action mainly. In fact, the presence of glycinergic synapses are not confined to the brainstem and spinal cord, and they can be found in many other areas of the central nervous system (Legendre, 2001).

The glutamatergic synapse is the main excitatory synapse in the brain. A glutamate neurotransmitter is pivotal in the establishment of neural connection, neural growth, neural migration, and plasticity (Moretto *et al.*, 2017). There are two major glutamate receptors in the nervous system: ionotropic and metabotropic. Ionotropic receptors divide into several groups in glutamatergic synapses: N-methyl-D-aspartate (NMDA) receptors, α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors and Kainate (KA) receptors (Moretto *et al.*, 2017). These receptors have a role in synapse maturation and plasticity. They are involved in postsynaptic membrane depolarization via influxes of sodium and calcium ions. Metabotropic receptors participate in synaptic transmission by interacting with second messenger molecules such as G-protein (Moretto *et al.*, 2017). In addition, Glutamate will be synthesized by glutamine deamination in presynaptic cells and store in synaptic vesicles for future neurotransmission (Danbolt, 2014). After its neurotransmission into the astrocyte the sodium ion-dependent excitatory amino acid transporter uptakes glutamate. It is for the conversion of the glutamate to the glutamine by glutamine synthase and localization of it into the presynaptic cell (Danbolt, 2014).

Cell-cell adhesion is another mechanism that occurs during retino-collicular development. Some genes regulate cell-cell adhesion in the retino-collicular pathway. L1 cell adhesion molecule is a transmembrane protein that has a role not only in axon guidance but also in synaptic target by mediating cell-cell adhesion. It is expressed on the growth cone of axons and neurons in the SC (Demyanenko and Maness, 2002). L1

can activate MAPkinase signaling through intermediates Src, phosphoinositide-3 kinase, Rac1, and p21-activated kinase for neuron growth and migration. L1 mutant mice illustrate abnormalities in forming optic nerve and synaptic targeting in the SC (Demyanenko and Maness, 2002). Neuron-glia related cell adhesion molecule (NrCAM) is another protein that has a role in cell adhesion in retino-collicular pathway. NrCAM belongs to the L1 family of immunoglobulin-class cell adhesion molecules (L1, CHL1, Neurofascin) and plays a role in axon guidance (Dai *et al.*, 2013). NrCAM mutation causes mistargeting in the RGCs termination axons in mediolateral areas of the SC (Dai *et al.*, 2013).

Identification of genes that are involved in retino-collicular development is crucial for understanding the function and development of this circuit and its role in visual system and vision. Also, by finding the genes and proteins that have a role in this connection, neurological malfunction and related disorders can be better diagnosed and proper treatment can be found.

Gene mutation and dysfunction in each step or structure of the connection between RGCs and the SC can cause problems in the vision and leads to neurological diseases. Glaucoma is a neurodegenerative disease causing by progressive loss of RGCs (Chen *et al.*, 2015). Glaucoma leads to visual impairment and in severe cases blindness. Recent studies have been identified that visual target of RGCs such as the SC impacts on survival of RGCs and prevention of this disease (Geeraerts *et al.*, 2019). Nevertheless, we think that investigating genes that have a role in the direct connection between RGCs and the SC is important for a new treatment for visual diseases.

We hypothesized that the direct connection between the retina and superior colliculus is controlled by the differential spatial (partial) expression of the genes in different clusters of these two regions. Although some genes have been identified and proved experimentally for the development of retino-collicular connection several genes that might have a role in this connection have not been recognized due to the complexity of establishing neural circuits and connection (Demyanenko and Maness, 2002; Dai *et al.*, 2013). In this research, other potential candidates that might contribute to the

connection between the retina and the SC are investigated via transcriptomic databases and bioinformatics analysis.

To be specific, in order to investigate the main purpose of the research the development, anatomy, and function of the retino-collicular connection were questioned. After that, the purpose was categorizing candidate genes via gene ontology. Whether the candidate genes have a role in the developmental processes of retino-collicular connection was the question for the second part of this project. Moreover, in the third part of the research, searching gene expression at cellular level and certain time in RGCs and SC's neuron were questioned. Finally, for the final conclusion, our question was whether the RGCs that are expressed our candidate genes in certain developmental time have a topographical alignment with SC's neurons or not and whether our candidate genes have a role in the development of other neural connection in the nervous system or not?

Methods and Materials

In order to investigate new candidate genes that play a role in the direct connection between retina and SC, the anatomy, function, and development of these two regions and their connection in mice have been studied. Then, the potential candidates have been chosen and evaluated by transcriptomic databases and bioinformatics tools to answer the purpose and hypothesis of this research.

Gene Ontology (GO)

Gene ontology is a tool for studying and classifying genes and their products based on their cellular and molecular components, biological processes and genes' functions (Ashburner *et al.*, 2011). I used the MGI gene ontology tool for the classification of genes based on their function in the development of the retino-collicular connection. Axon guidance, cell-cell adhesion, synaptic formation and transmission, cell differentiation, and neuron migration are the major categories that candidates are classified into for further evaluation. If a gene does not belong to these developmental processes and functions, it will not count as a potential candidate. In order to search the genes for gene ontology the batch query search in the Genes, Genome features and maps section of the MGI database was used. Each gene was investigated in the gene ontology option in batch query search individually and the result was observed for further evaluation (<http://www.informatics.jax.org/batch>: GO).

Gene Expression

Candidate genes expressions are important in their validation. Spatial and temporal expressions were investigated by single cell databases and other molecular detective techniques like RNA in situ hybridization and immunohistochemistry databases.

Single cell expression: Generally, single cell analysis is used for studying genomics, transcriptomic, proteomics, cell-cell interaction, and metabolomics at a single cell level (Hu *et al.*, 2016). Different single cell isolation methods are used to isolate single cells

from the total sample and then different detective techniques in each field are used to detect expression of genes, proteins, and so on in each cell.

In this research, single cell RNA-seq (transcriptomics) databases are used to observe spatial expression of each candidate gene in the different RGCs and SC neurons. Here are single cell databases resources for this purpose:

1. Single cell transcriptome profiling of retinal ganglion cells identifies cellular subtypes (Rheume *et al.*,2018): In this research, 6225 RGCs from left and right eyes in postnatal day 5 (P5) mice were analyzed by single RNA-seq. Retinal cells were purified and validated by immunostaining and fluorescent in situ hybridization and categorized into 40 clusters. Three subclasses are identified in this experiment, including alpha, ON-OFF DSGC, and ipRGCs (Rheume *et al.*,2018). I investigated the expression of each candidate individually in left and right RGCs eyes. I searched each gene in the specific database for the mentioned study, namely subtypes gene browser. The neonatal RGCs (Atlas- Left & Right Eyes) section was used to search the gene. Each gene was investigated based on the bar graph (Means and SE) in the search box option (select chart type). The bar graphs result for candidate genes were analyzed for final conclusion (<https://health.uconn.edu/neuroregeneration-lab/rgc-subtypes-gene-browser>).

2. Single nuclei dataset, SCS (MD 705) from single cell portal database: The study provides information about the 5105 nuclei from the SC. RNA-seq method is used for these data. The superior colliculus cells are divided into 9 clusters (MD 705 dataset). I analyzed each candidate in the search option in the single cell portal in order to validate the spatial expression among excitatory neuron, inhibitory neuron, and astrocytes in the SC. Each potential candidate was searched in the distribution section of the study database. The options box was changed manually to these parameters: t-SNE in clustering, cell type ontology in annotation, all cells in subsampling, and box plot with all data points in distribution (https://singlecell.broadinstitute.org/single_cell/study/SCP644/single-nuclei-dataset-scs-md705).

Timing of gene expression: The retino-collicular pathway develops around E12 until P10. Nevertheless, candidate genes should be expressed during this period to play a role in the development. For this purpose, MGI and Allen brain atlas gene expression tools were used.

1. Allen brain atlas: Every gene was investigated with the Allen brain atlas gene expression tool at E11.5 to P56. I used the developing mouse brain database, gene search option for this purpose. Either immunohistochemistry (IMC) or RNA in situ hybridization is their method for detecting genes in different tissues of the mouse brain. The result of search bases on the raw expression value and normalized value for transcriptomes per tissue. Allen brain atlas website for this search:

<http://developingmouse.brain-map.org/>.

2. MGI: For more accuracy, the MGI gene expression tool was used too. Similar to Allen brain atlas, IMC or RNA in situ hybridization is used for detection. In contrast to the Allen brain, the retina and some retinoptic structures have data for embryonic and postnatal gene expression. I used the MGI gene expression tool for this project. The genes were investigated in the batch query search in Genes, Genome features and maps, the gene expression option (<http://www.informatics.jax.org/batch>).

Further Validation

After previous steps, the topographic map for the connection between different RGCs subtypes and the SC is evaluated by reviewing the viral tracing experiments and those related information is combined with data from gene expression and gene ontology. In addition, the protein interaction between candidates is observed. Moreover, results are compared with positive controls (genes that are proved earlier experimentally like Ephs). For protein interaction String database assists (<https://string-db.org/cgi/input?sessionId=b8loVk9EruV1>). In this research, experimental data is used. At the final step, I searched some evidence for each high potential candidate to make a stronger conclusion. The found evidence is related to the establishment of neural connections in other parts of the brain instead of our target connection.

Results

By using applications that are introduced in the methods and materials section, 16 genes that belong to different gene families were picked. Three of them are omitted from the final result because they do not have expression in the most of RGCs and SC's neurons during the developmental stages of the target connection. Also, they do not have strong evidence for their participation in different neural connections.

Table 1: Primary genes

Gene	Whether are picked for final result or not	Cause of rejection
Slit 1	Yes	-
Robo 1	Yes	-
Slit2	Yes	-
Robo 2	Yes	-
Slit 3	No	Lack of expression in most of SC's neurons and RGCs. Lack of supportive evidence.
Robo 3	No	No expression in SC's neurons and RGCs. Lack of supportive evidence.
Nrp 1	Yes	-
Nrp 2	Yes	-

Cdh 6	Yes	-
Cdh 7	Yes	-
Cdh 8	Yes	-
Cdh 23	No	Lack of expression in most of SC's neurons and RGCs.
Nfasc	Yes	-
NLGN 1	Yes	-
NLGN 2	Yes	-
NLGN 3	Yes	-

Here are the final candidates that are suggested for the development of the retino-collicular pathway:

Slits/Robos

Slits are one type of glycoproteins that have a role in axon guidance, cell proliferation, and cell migration in the nervous system. They interact with their receptors, Robo, to regulate these processes in the development (Tong *et al.*, 2019). Slit proteins are made of single peptides with approximately 1500 amino acids. They consist of six parts: one N-terminal signal peptide, four leucine-rich domains, six epidermal growth factor-like domains, laminin-G-like domain, three epidermal growth factor domains, and a C-terminal cysteine tail (Tong *et al.*, 2019). Robo1/2 receptors consist of five immunoglobulins (Ig) like domains, three fibronectin type 3 repeats, and transmembrane

domain in their extracellular structures. Their intracellular regions have a proline-rich domain (Hohenester, 2008; Tong *et al.*, 2019).

Slit proteins bind to the Ig domain of the Robos by their second leucine-rich domain. Also, slit proteins can generate homodimers by binding to other fourth leucine-rich domains (Blockus and Chédotal, 2016). In fact, the slit protein is cleaved by proteolytic enzymes at the fifth and sixth of the epidermal growth factor-like domain to make a long N-terminal slit (SlitN) and short C-terminal slit (SlitC) for future signaling transductions (Tong *et al.*, 2019). The SlitN initiates signal transduction pathways by interacting with Robos whereas SlitC binds to the plexin receptor to maintain its function (Tong *et al.*, 2019). Heparan sulfate proteoglycans are required for Slit/Robo functions. Moreover, Slits can bind to other extracellular matrix molecules like Neurexins, Type IV Collagens, and Netrin 1 by heterophilic binding (Blockus and Chédotal, 2016).

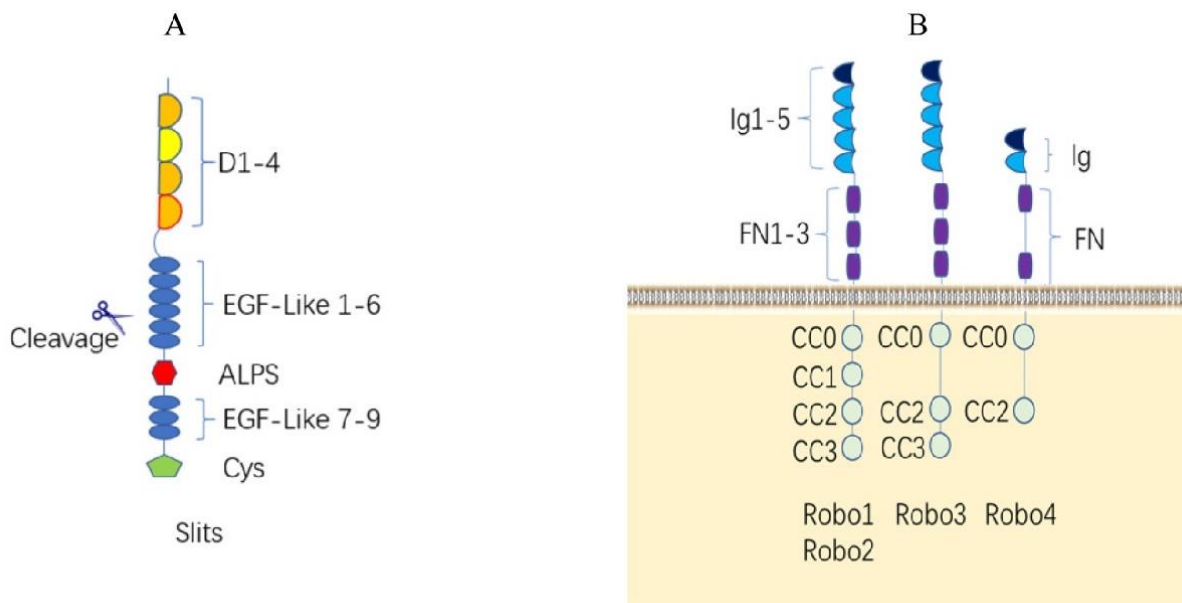


Figure 9: The structure of Slits and Robos proteins. This figure is taken from (Tong *et al.*, 2019).

Slit/Robo signaling pathways participate in different processes such as axon guidance, cell migration, muscle cell formation, stem cell growth, angiogenesis, and organ development (Tong *et al.*, 2019). Slit/Robo signaling downstream pathways depend

mainly on cytoplasmic kinases, actin and microtubule cytoskeleton molecules (Blockus and Chédotal, 2016).

Cytoplasmic kinase Abelson (Abl) is the major molecule in Slit/Robo signaling pathways. Abl promotes axon guidance via binding to the Capulet protein. Interaction with Capulet protein leads to affect the activity of beta-catenin and N-cadherin. Thus, cell adhesion alters and repellent guidance activity occurs (Blockus and Chédotal, 2016; Tong *et al.*, 2019). Moreover, Abl cooperates with actin-binding proteins to control the cytoskeletal elements to control axon guidance and neural migration. Abl can phosphorylate Robo internal domain to regulate the pathway (inhibit axon guidance) (Blockus and Chédotal, 2016; Tong *et al.*, 2019).

GTPases are other molecules that have a role in Slit/Robo signaling. GTPases are regulated by several genes such as Nck, slit/robo GTPase activating proteins, and guanine nucleotide exchange factors (Tong *et al.*, 2019).). For example, Nck attaches to the Robo receptors to maintain axon outgrowth and branching by activating Rho GTPase, Rac (Hohenester, 2008; Blockus and Chédotal, 2016). Another example belongs to the interaction of Slit2/Robo1 with Slit/Robo Gtpase proteins which mediate RhoA and Cdc42 for neural migration. Most Rho GTPases affect cytoskeletal dynamics for axon growth (Hohenester, 2008). The enabled (Ena) protein also have an important role in axonal repulsion of filopodia in axon extension by interacting with slits and regulate cellular actin formation and cell motility (Tong *et al.*, 2019).

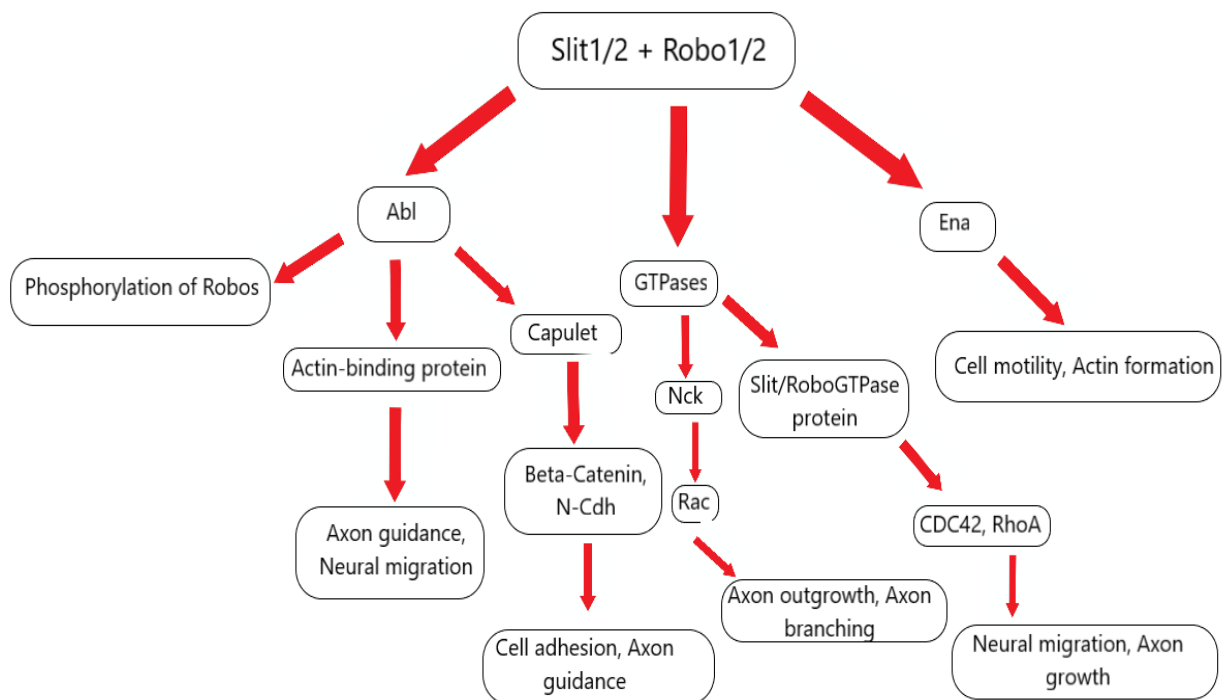


Figure 10: The Slit/Robo cellular pathways.

Slit1, Slit2, Robo1, and Robo2 are potential candidates of this family in this research. The tables and figures below are results of bioinformatics analyses for these genes:

Slit1:

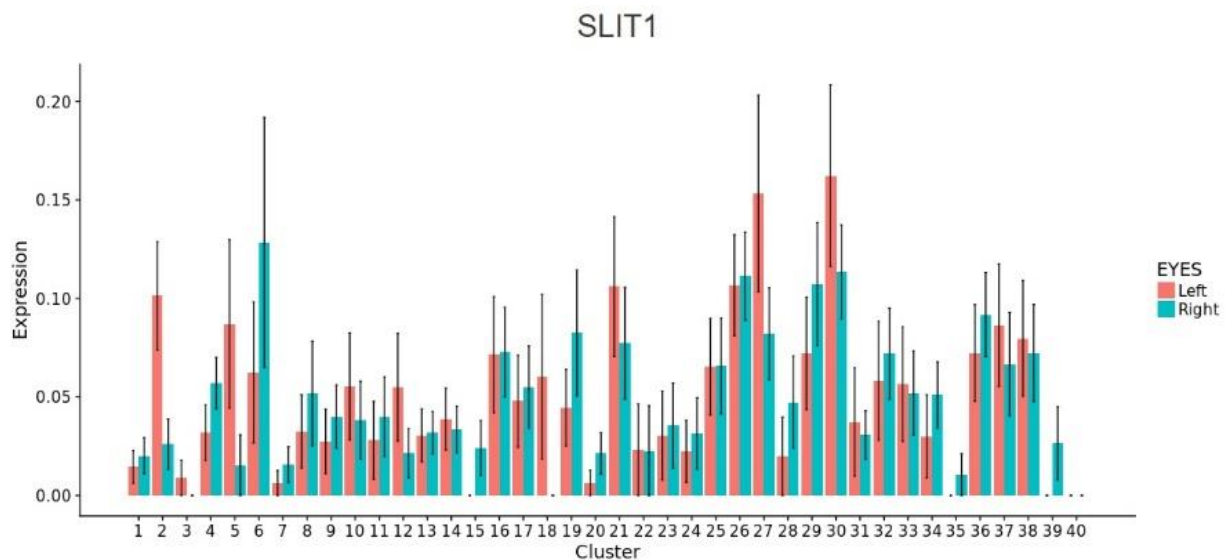
Table 2: Slit1 data analysis results

Gene ontology (MGI):
Axon extension involved in axon guidance
Axon guidance
Axonogenesis
Dorsal-Ventral axon guidance
Negative regulation of axon extension involved in axon guidance (repulsion)
Neuron projection morphogenesis
RGC axon guidance
Cell differentiation
Nervous system development

Nuclear migration
Single cell expression analysis for RGCs at P5 (Rheaume <i>et al.</i>,2018): ON-OFF DSGC (Cluster number 22): Slit1 is detected in both eyes ON-OFF DSGC (Cluster number 23): Slit1 is detected in both eyes lpRGC (Cluster number 6): Slit1 is detected in both eyes lpRGC (Cluster number 25): Slit1 is detected in both eyes lpRGC (Cluster number 26): Slit1 is detected in both eyes lpRGC (Cluster number 37): Slit1 is detected in both eyes Alpha RGC (Cluster number 21): Slit1 is detected in both eyes Alpha RGC (Cluster number 33): Slit1 is detected only in right eye
Single cell expression analysis for the SC (Single nuclei dataset, MD705): Astrocyte: Slit1 is detected in 2.03% of astrocyte population with the mean = 0.0264. Excitatory neuron: Slit1 is detected in 19.05% of excitatory neuron population with the mean = 0.2380. Inhibitory neuron: Slit 1 is detected in 19.54% of inhibitory neuron population with the mean = 0.2442.
Embryonic expression (Allen brain and MGI): Midbrain E10.5: Slit1 is detected Midbrain E11.5: Slit1 is detected Midbrain E13.5: Slit1 is detected Midbrain E15.5: Slit1 is detected Midbrain E17.5: Slit1 is detected Midbrain E18.5: Slit1 is detected Retina E12: Slit1 is not detected Neural retina E17.5: Slit1 is detected
Postnatal expression (Allen brain and MGI) Midbrain P4: Slit1 is detected Midbrain P14: Slit1 is detected Midbrain P28: Slit1 is detected Midbrain P56: Slit1 is detected Retina P0-P1: Slit1 is detected

Interaction (String): Robo1, Robo2

According to the table 1, the Slit1 gene has a role in axon guidance, especially in RGC axon guidance. With one exception in the cluster 33 in retina cells, other identified RGCs (ON-OFFDSGCs, ipRGCs, and Alpha RGC) express slit1. Besides, inhibitory and excitatory neurons of the SC express this gene. These neurons involve in the development of the retino-collicular connection (Reinhard *et al.*, 2019). Regarding embryonic and postnatal expression, the slit1 expresses during the development of retino-collicular due to its expression during E10-P56 in the midbrain and during E17.5-P5 in the retina. Nevertheless, it seems that the slit1 gene involves in the establishment of retino-collicular connection between ON-OFFDSGCs, ipRGCs, and Alpha RGCs and the SC's neurons via axon guidance mechanisms and by interacting with their receptors, Robos and other effectors.



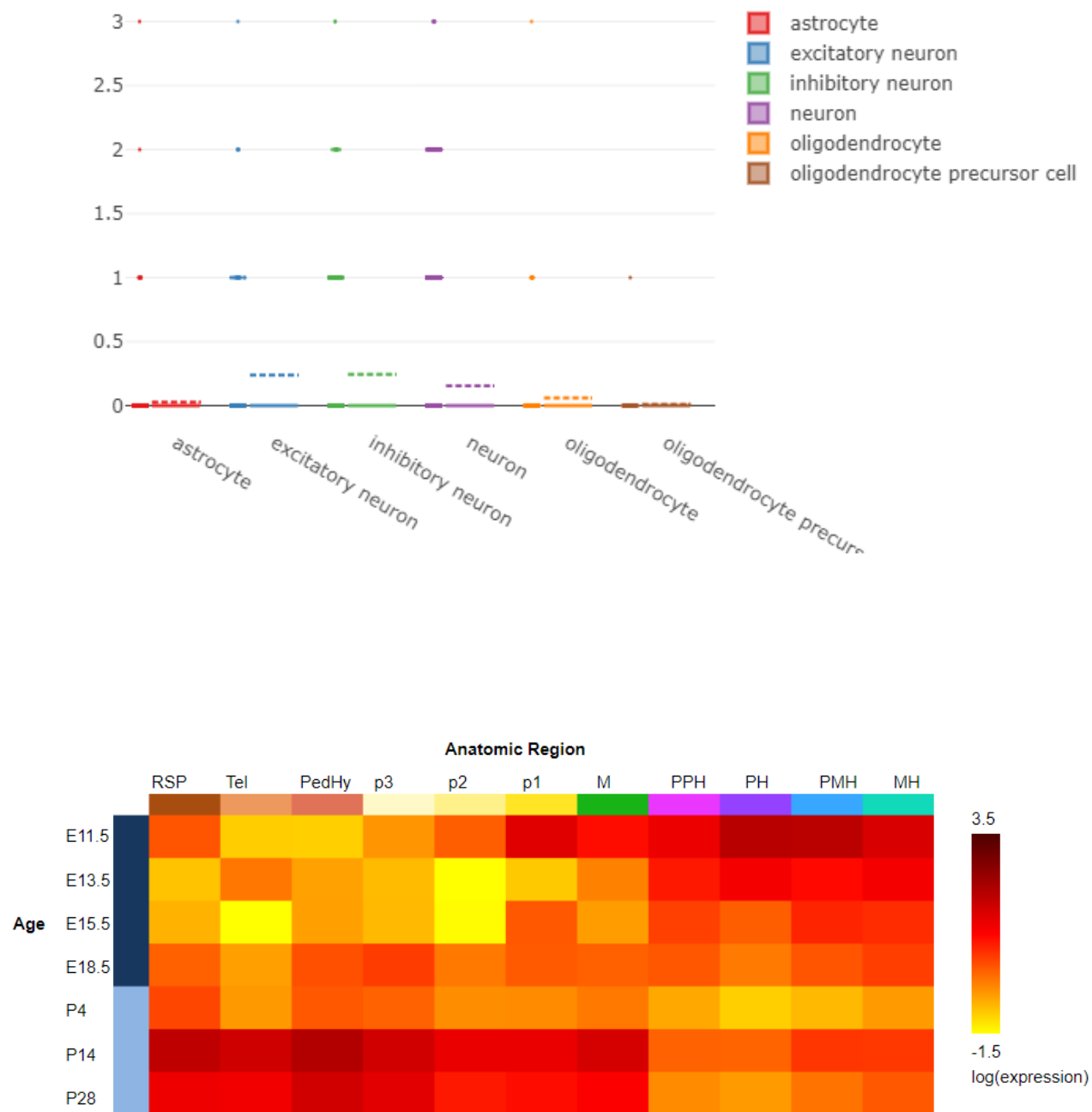


Figure11: A) Mean of left and right eyes Slit1 gene expression in RGCs subtypes and SEM error bars. This figure is taken from (Rheume et al.,2018). B) Distribution of Slit1 gene expression in the SC. This figure is taken from (MD 705). C) Slit1 embryonic and postnatal gene expression. This figure is taken from (developing mouse brain, Allen Brain atlas).

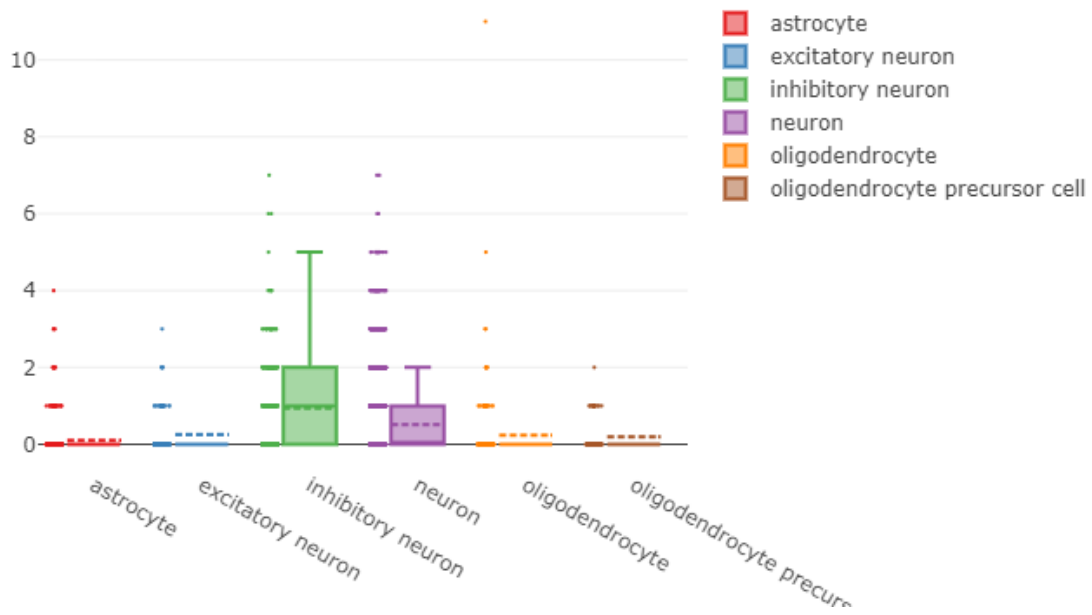
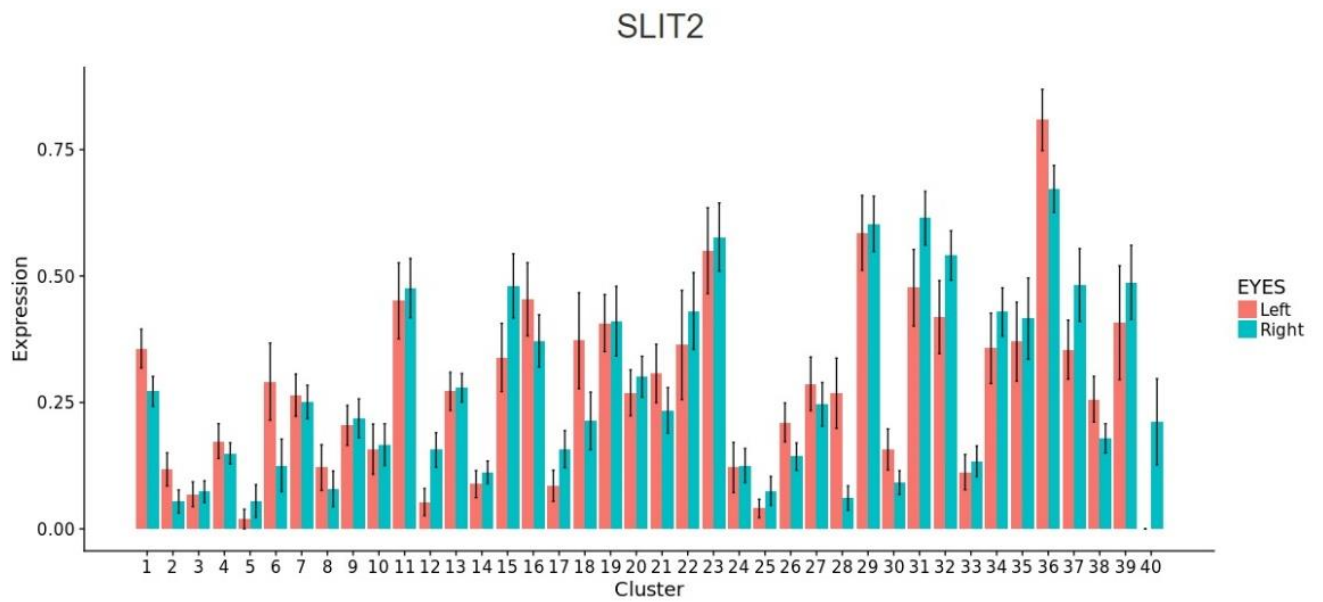
Slit2:

Table 3: Slit2 data analysis results

Gene ontology (MGI): Axon extension involved in axon guidance Axon guidance Cell-cell adhesion Negative regulation of axon extension Negative regulation of retinal ganglion cells axon guidance Retinal ganglion cell axon guidance Cell differentiation Nervous system development Neuron projection morphogenesis
Single cell expression analysis for RGCs at P5 (Rheaume <i>et al.</i>, 2018): ON-OFF DSGC (Cluster number 22): Slit2 is detected in both eyes ON-OFF DSGC (Cluster number 23): Slit2 is detected in both eyes lpRGC (Cluster number 6): Slit2 is detected in both eyes lpRGC (Cluster number 25): Slit2 is detected in both eyes lpRGC (Cluster number 26): Slit2 is detected in both eyes lpRGC (Cluster number 37): Slit2 is detected in both eyes Alpha RGC (Cluster number 21): Slit2 is detected in both eyes Alpha RGC (Cluster number 33): Slit2 is detected in both eyes
Single cell expression analysis for the SC (Single nuclei dataset, MD705): Astrocyte: Slit2 is detected in 6.91% of astrocyte population with the mean = 0.1016. Excitatory neuron: Slit2 is detected in 20.63% of the excitatory neuron population with the mean = 0.2539. Inhibitory neuron: Slit2 is detected in 54.02% of the inhibitory neuron population with the mean = 0.9396.
Embryonic expression (Allen brain and MGI): Midbrain E 10.5-11: Slit2 is detected Midbrain roof plate E11: Slit2 is detected Midbrain E11.5: Slit2 is detected

Midbrain roof plate E 13.5: Slit2 is detected
Midbrain E13.5: Slit 2 is detected
Superior colliculus E 14.5: Slit2 is detected
Midbrain E 15.5: Slit2 is detected
Midbrain E18.5: Slit2 is detected
Retina E 14.5: Slit2 is detected
Neural retina E 15.5: Slit2 is detected
Optic nerve E 15.5: Slit2 is detected
Postnatal expression (Allen brain and MGI)
Midbrain P 4: Slit2 is detected
Midbrain P 14: Slit2 is detected
Midbrain P 28: Slit2 is detected
Midbrain P56: Slit2 is detected
Interaction (String): Robo1, Robo2

According to the data regarding table 2, the Slit2 gene plays a role in axon guidance especially RGC axon guidance, cell-cell adhesion, and neuron projection morphogenesis. These processes are key factors in the development of the connection between the retina and the SC. In addition, similar to the Slit1 gene, the Slit2 express in all identified retinal RGCs at P5 and in the SC's neurons. The Slit2 is expressed more in inhibitory neurons than excitatory. They also are detected during E11-P56 in the midbrain and at E15.5 and P5 in the retina. Overall, this gene probably has the role in RGC axon guidance in the establishment of the retino-collicular connection via cooperating with the Slit1 and interacting with two other receptors, Robo1/2.



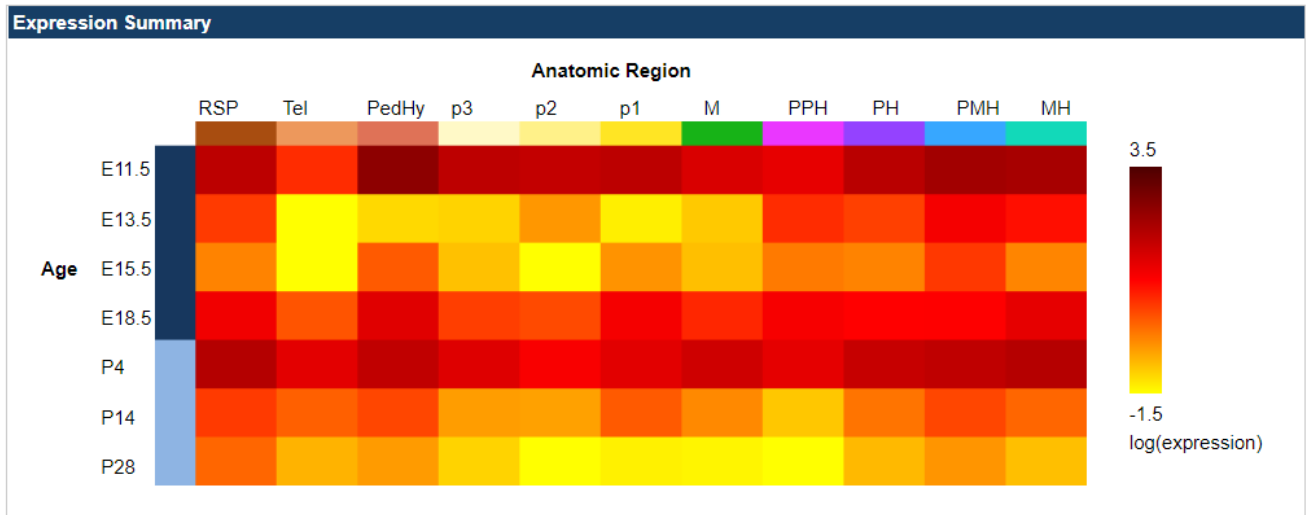


Figure 12: A) Mean of left and right eyes Slit2 gene expression in RGCs subtypes and SEM error bars. This figure is taken from (Rheume et al., 2018). B) Distribution of Slit2 gene expression in the SC. This figure is taken from (MD 705). C) Slit2 embryonic and postnatal gene expression. This figure is taken from (developing mouse brain, Allen Brain atlas).

Robo1:

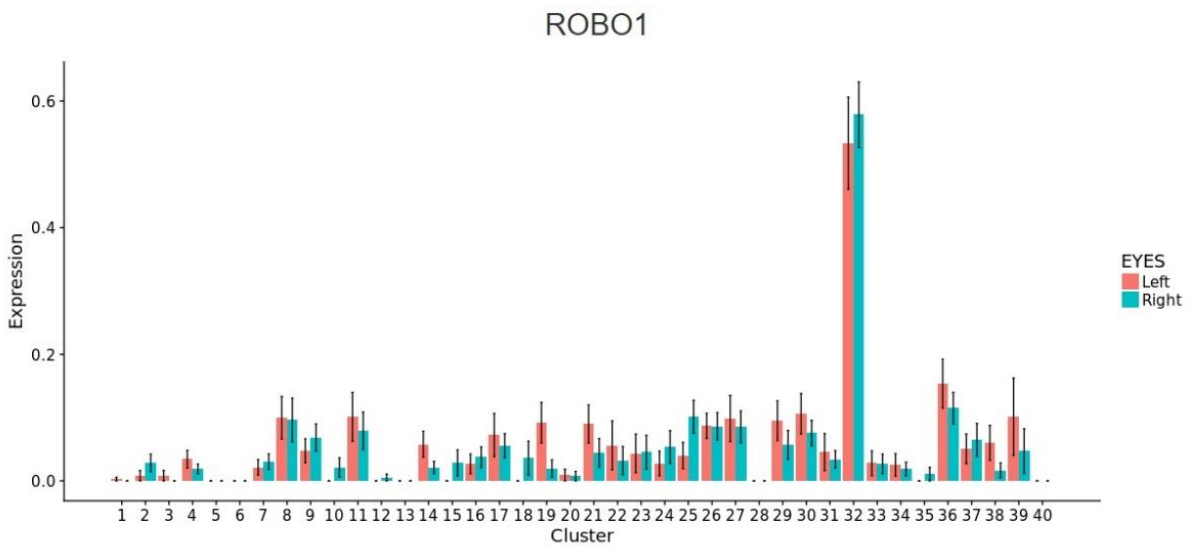
Table 4: Robo1 data analysis results

Gene ontology (MGI): Axon guidance Hemophilic cell adhesion via plasma membrane adhesion molecule Axon guidance receptor activity Cell differentiation Cell projection Nervous system development
Single cell expression analysis for RGCs at P5 (Rheume et al., 2018): ON-OFF DSGC (Cluster number 22): Robo1 is detected in both eyes ON-OFF DSGC (Cluster number 23): Robo1 is detected in both eyes lpRGC (Cluster number 6): Robo1 is not detected in both eyes lpRGC (Cluster number 25): Robo1 is detected in both eyes lpRGC (Cluster number 26): Robo1 is detected in both eyes

lpRGC (Cluster number 37): Robo1 is detected in both eyes Alpha RGC (Cluster number 21): Robo1 is detected in both eyes Alpha RGC (Cluster number 33): Robo1 is detected in both eyes
Single cell expression analysis for the SC (Single nuclei dataset, MD705): Astrocyte: Robo1 is detected in 21.95% of astrocyte population with the mean = 0.2784. Excitatory neuron: Robo1 is detected in 42.06% of the excitatory neuron population with the mean = 0.7301. Inhibitory neuron: Robo1 is detected in 19.83% of the inhibitory neuron population with the mean = 0.2586.
Embryonic expression (Allen brain and MGI): Midbrain E 10.5: Robo1 is detected Midbrain E 11.5: Robo1 is detected Midbrain E 13.5: Robo1 is detected Midbrain E 15.5: Robo1 is detected Midbrain E 18.5: Robo1 is detected Optic stalk E 10.5: Robo1 is detected Optic nerve E 15.5: Robo1 is detected Retina E 16: Robo1 is detected
Postnatal expression (Allen brain and MGI): Midbrain P 4: Robo1 is detected Midbrain P 14: Robo1 is detected Midbrain P 28: Robo1 is detected Midbrain P 56: Robo1 is detected
Interaction (String): Slit1, Slit2

According to the table 3, Robo1 plays a role in axon guidance as a receptor for Slit1 and Slit2. With one exception in cluster 6 in the RGCs, Robo1 expresses in all identified RGCs cells. Robo1 is expressed more in excitatory neurons in the SC. Nevertheless, because the Robo1 is a receptor for the Slit1 and Slit2 and express during E10.5-P56 in

the midbrain and in E10.5, E16, and P5 in the retina, it will be a potential candidate for axon guidance in the target connection.



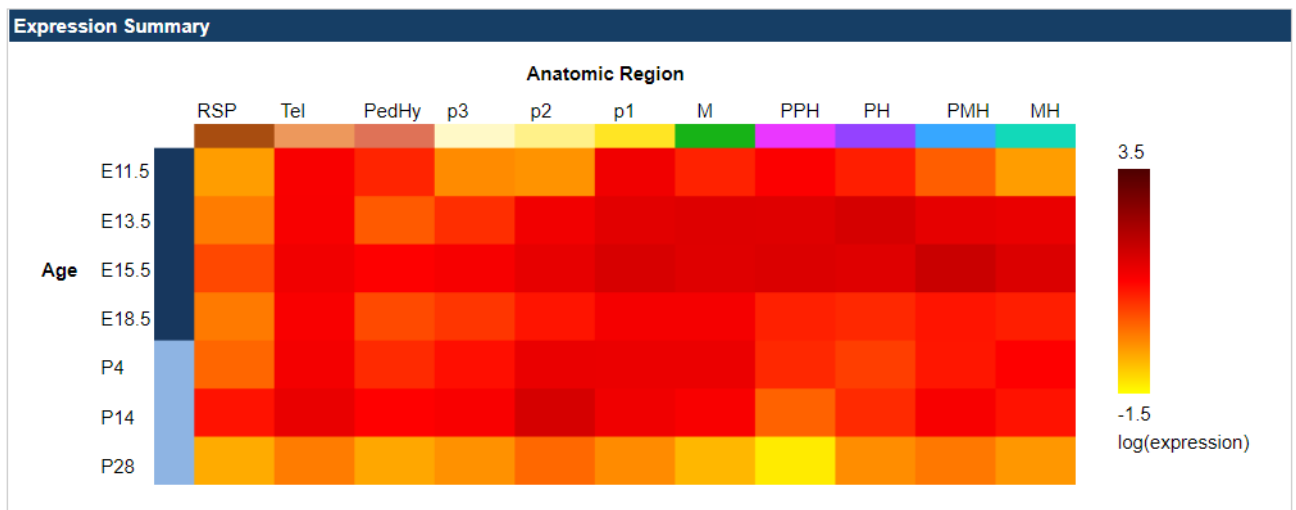
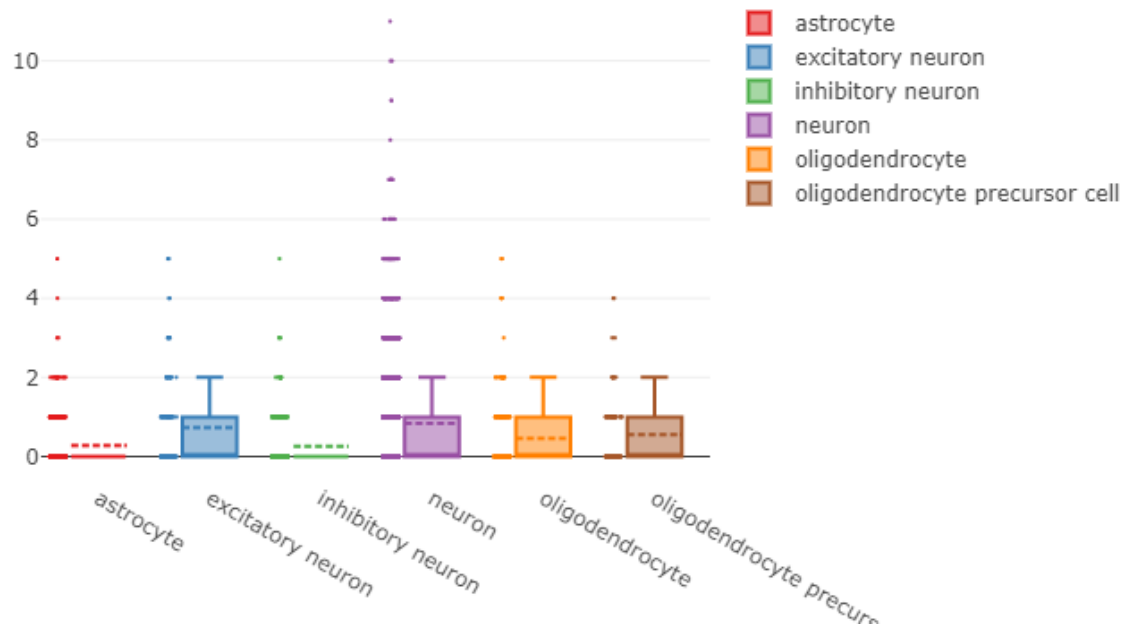


Figure 13: A) Mean of left and right eyes Robo1 gene expression in RGCs subtypes and SEM error bars. This figure is taken from (Rheume et al.,2018). B) Distribution of Robo1 gene expression in the SC. This figure is taken from (MD 705). C) Robo1 embryonic and postnatal gene expression. This figure is taken from (developing mouse brain, Allen Brain atlas).

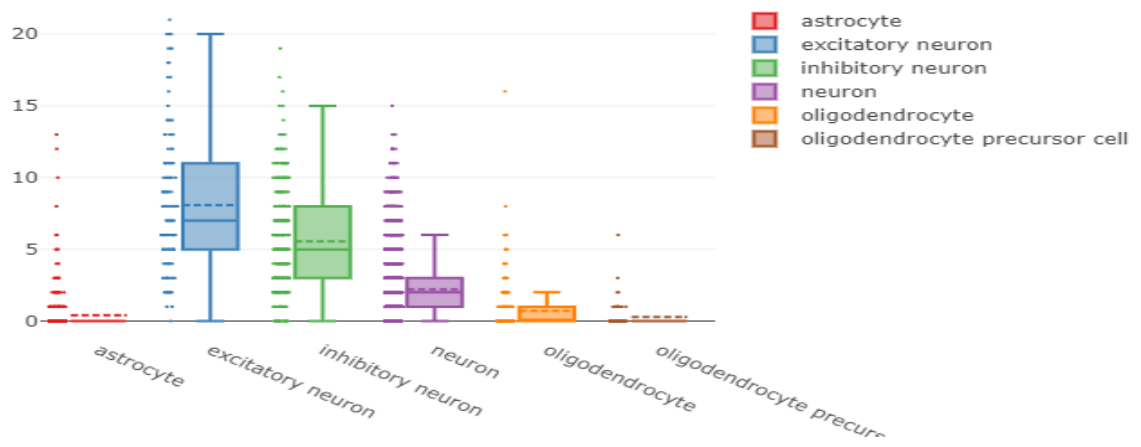
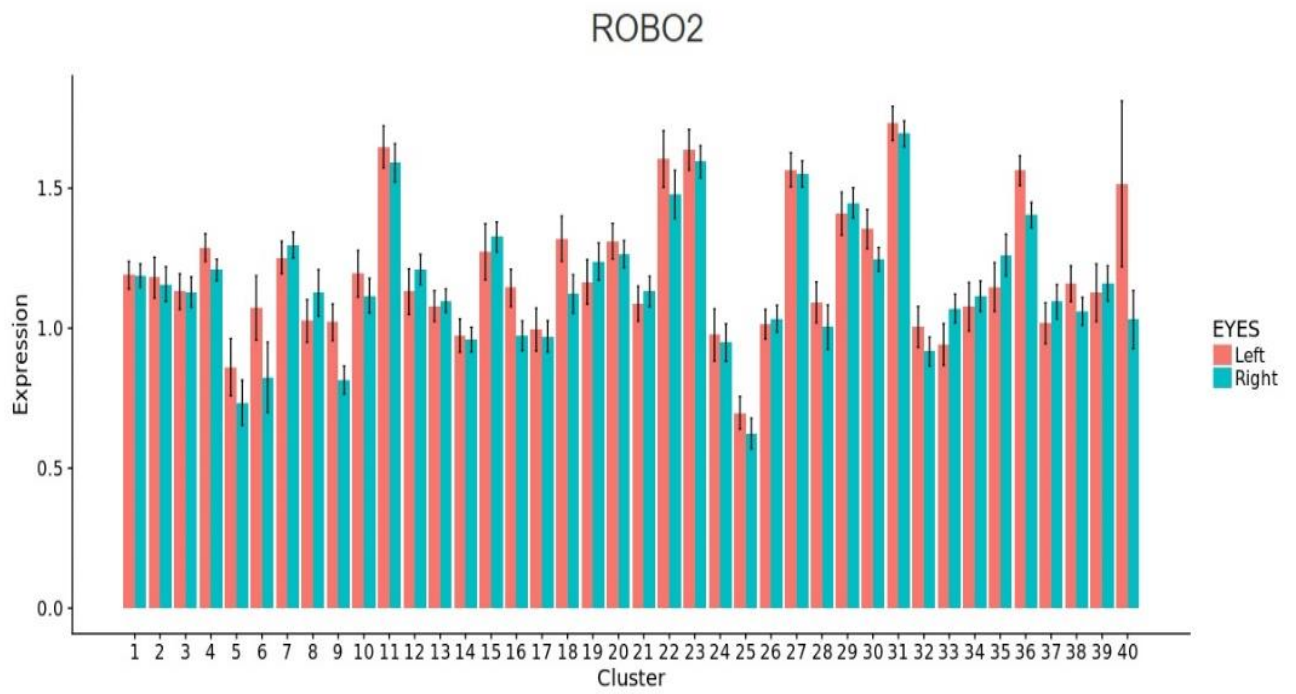
Robo2:

Table 5: Robo2 data analysis results

Gene ontology (MGI): Axon guidance receptor activity Axon guidance Brain development Hemophilic cell adhesion via plasma membrane adhesion molecules Retinal ganglion cell axon guidance Cell differentiation Nervous system development
Single cell expression analysis for RGCs at P5 (Rheaume <i>et al.</i>, 2018): ON-OFF DSGC (Cluster number 22): Robo2 is detected in both eyes ON-OFF DSGC (Cluster number 23): Robo2 is detected in both eyes IpRGC (Cluster number 6): Robo2 is detected in both eyes IpRGC (Cluster number 25): Robo2 is detected in both eyes IpRGC (Cluster number 26): Robo2 is detected in both eyes IpRGC (Cluster number 37): Robo2 is detected in both eyes Alpha RGC (Cluster number 21): Robo2 is detected in both eyes Alpha RGC (Cluster number 33): Robo2 is detected in both eyes
Single cell expression analysis for the SC (Single nuclei dataset, MD705): Astrocyte: Robo2 is detected in 21.14% of astrocyte population with the mean = 0.4065. Excitatory neuron: Robo2 is detected in 99.21% of excitatory neuron population with the mean = 8.0793. Inhibitory neuron: Robo2 is detected in 94.83% of inhibitory neuron population with the mean = 5.5517.
Embryonic expression (Allen brain and MGI): Midbrain E 11.5: Robo2 is detected

Midbrain E 13.5: Robo2 is detected
Midbrain E 15.5: Robo2 is detected
Midbrain E 18.5: Robo2 is detected
Postnatal expression (Allen brain and MGI):
Midbrain P 4: Robo2 is detected
Midbrain P 14: Robo2 is detected
Midbrain P 28: Robo2 is detected
Midbrain P 56: Robo2 is detected
Retina P 7: Robo2 is detected
Interaction (String): Slit1, Slit2

Regarding the Robo2 chart, similar to the Robo1, the Robo2 acts as receptor of the Slit1 and the Slit2 gene in the axon guidance mechanisms. Moreover, this gene is expressed in all identified RGCs at the P5 and has the highest rate of expression among the Slit1, Slit2, and Robo1 in excitatory and inhibitory neurons in the SC and during the embryonic and postnatal developmental stages. This gene due to the reasons mentioned above can be a potential candidate for the target connection.



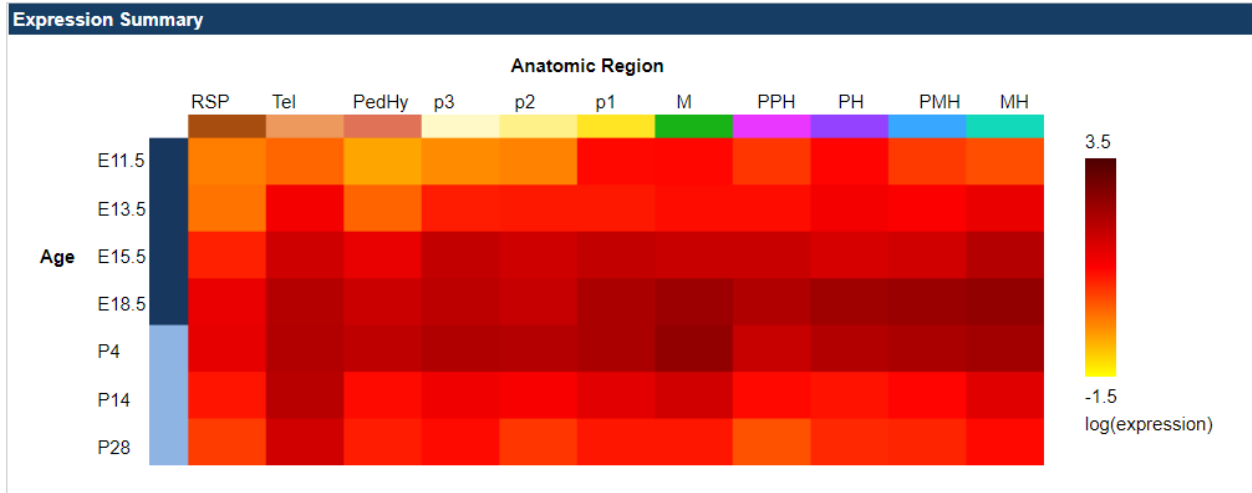


Figure 14: A) Mean of left and right eyes Robo2 gene expression in RGCs subtypes and SEM error bars. This figure is taken from (Rheume et al., 2018). B) Distribution of Robo2 gene expression in the SC. This figure is taken from (MD 705). C) Robo2 embryonic and postnatal gene expression. This figure is taken from (developing mouse brain, Allen Brain atlas).

Overall, the Slit/Robo family is a potential candidate in this research. According to the results, the Robo2 has priority over another receptor in this family for being a potential candidate. Nevertheless, our priority for this family is Robo2, Slit1/2, and Robo1, respectively for involved in the establishment of the connection between retina and SC.

Neuropilins

Neuropilins are non-tyrosine cell surface glycoproteins that have an essential role in the development of the nervous and the cardiovascular system (Roy et al., 2017). They act as a co-receptor for class III semaphorins and some of the vascular endothelial growth factors in axon guidance and angiogenesis (Pellet-Many et al., 2008). The two neuropilin isoforms (Nrp1 and Nrp2) are chosen for this research purpose.

Nrp1 and Nrp2 are transmembrane glycoproteins with 923 and 926 amino acids, respectively. They have an N-terminal extracellular domain that consists of a transmembrane region (Pellet-Many et al., 2008; Roy et al., 2017). In addition, their structures are made of two CUB (complement binding factors C1s/C1r, Uegf, BMP1 (bone morphogenetic protein 1)) (a1/a2) domains, two Factor V/VIII (b1/b2) domains,

and a MAM (meprin, A5 antigen, receptor tyrosine phosphatase μ) (c) domain (Pellet-Many *et al.*, 2008; Roy *et al.*, 2017). They have a cytoplasmic domain with 43 and 44 amino in Nrp1 and Nrp2 with a PDZ binding motif at their intracellular carboxyl-terminal (Niland and Eble, 2019). Semaphorins bind to the CUB domain of neuropilins and b domains are required for interaction of neuropilins with semaphorins and VEGFs for cell adhesion or axon guidance function (Roy *et al.*, 2017).

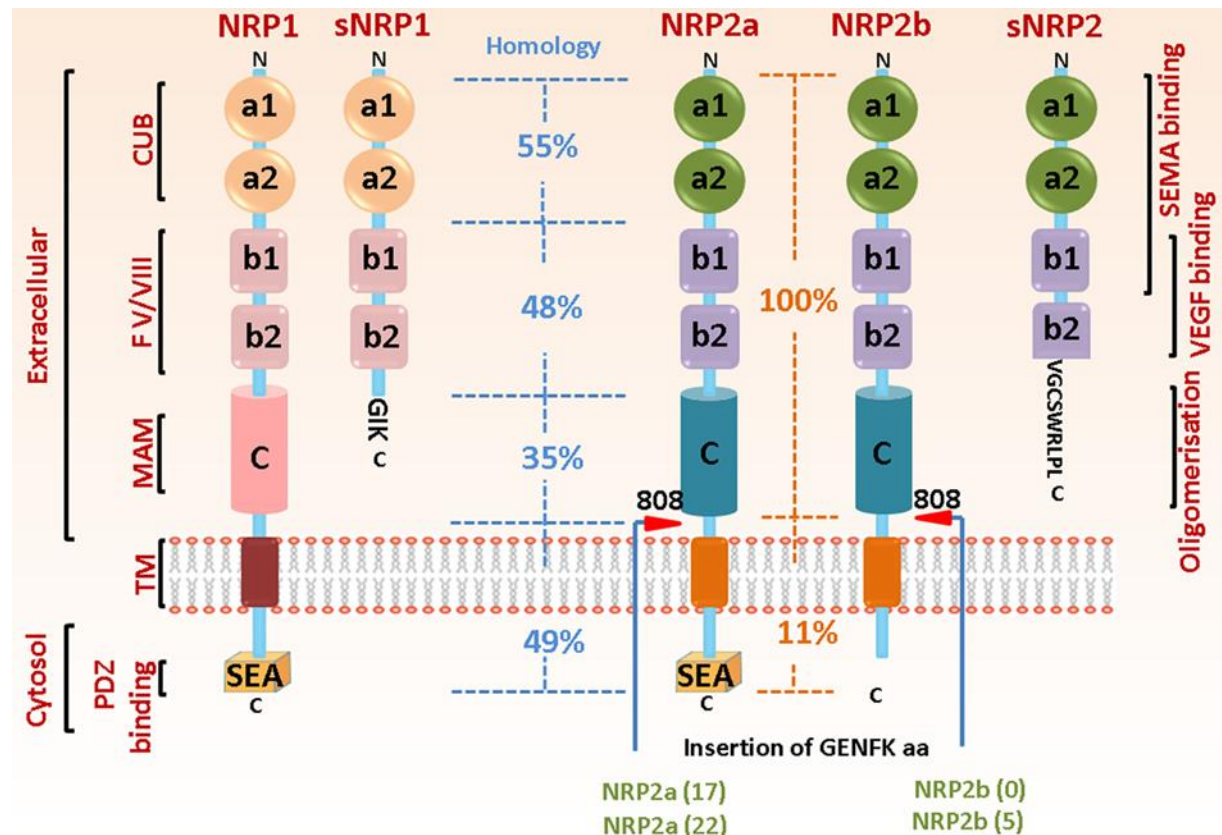


Figure 15: The structure of Nrp isoforms, including Nrp1 and Nrp2 and their soluble isoforms. This figure is taken from (Roy *et al.*, 2017).

Neuropilins have an integral role in the survival of mice during development. Severe abnormal projections and connections are observed in Nrp1/2 mutant mice in both central and peripheral nervous system (Pellet-Many *et al.*, 2008). Nrp1 null mice die between E12-E13.5 due to the cardiovascular and nervous system defections (Pellet-Many *et al.*, 2008). In contrast, Nrp2 mutant mice survive until adulthood but suffer from abnormal guidance and fasciculation of cranial and spinal nerves (Pellet-Many *et al.*,

2008). Since Nrps do not have cytosolic protein kinase domains, they act as a co-receptor in most signal transduction pathways (Niland and Eble, 2019). For example, in axon guidance they cooperate with other receptors, plexins, to maintain axon guidance. They also bind to L1CAM for cell adhesion role (Niland and Eble, 2019).

One example of neuropilin signaling pathways in the nervous system is the interaction of Nrp1 with Sema3f and Plexin A. This complex activates other kinases such as MAPK and CDK5 to regulate protein synthesis, cytoskeletal dynamics, and eventually axon repulsion effect in axon guidance (Szabó *et al.*, 2012). Moreover, plexin activation by Nrps leads to inhibition of actin cytoskeleton proteins, Rac/Rho protein and integrin, so migration and axon guidance will be inhibited in this pattern (Niland and Eble, 2019). In contrast, the inactivation of talin by plexin receptors causes activation of GIPC1 and Paxillin, which eventually affects actin cytoskeletal proteins (Niland and Eble, 2019).

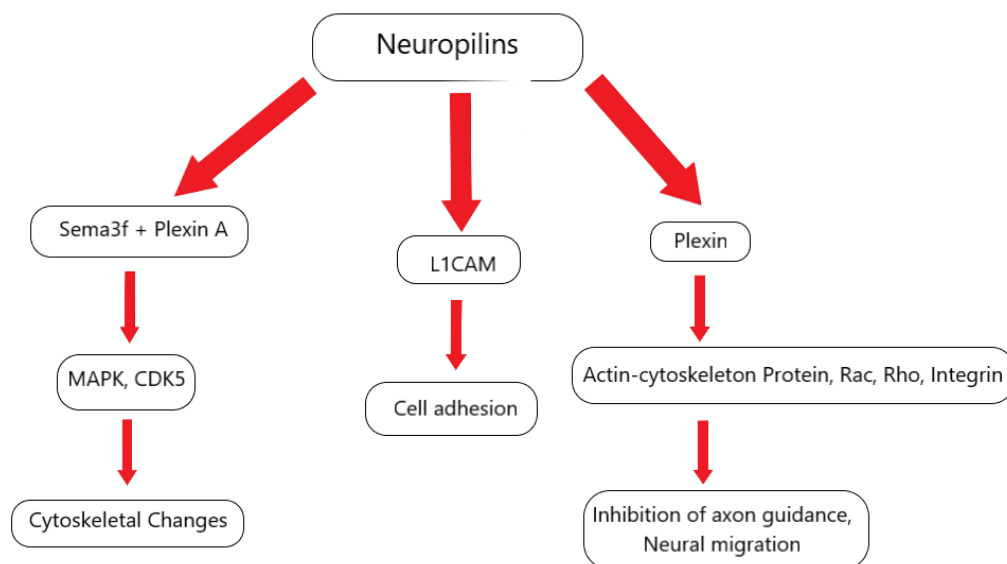


Figure 16: The neuropilin cellular pathways.

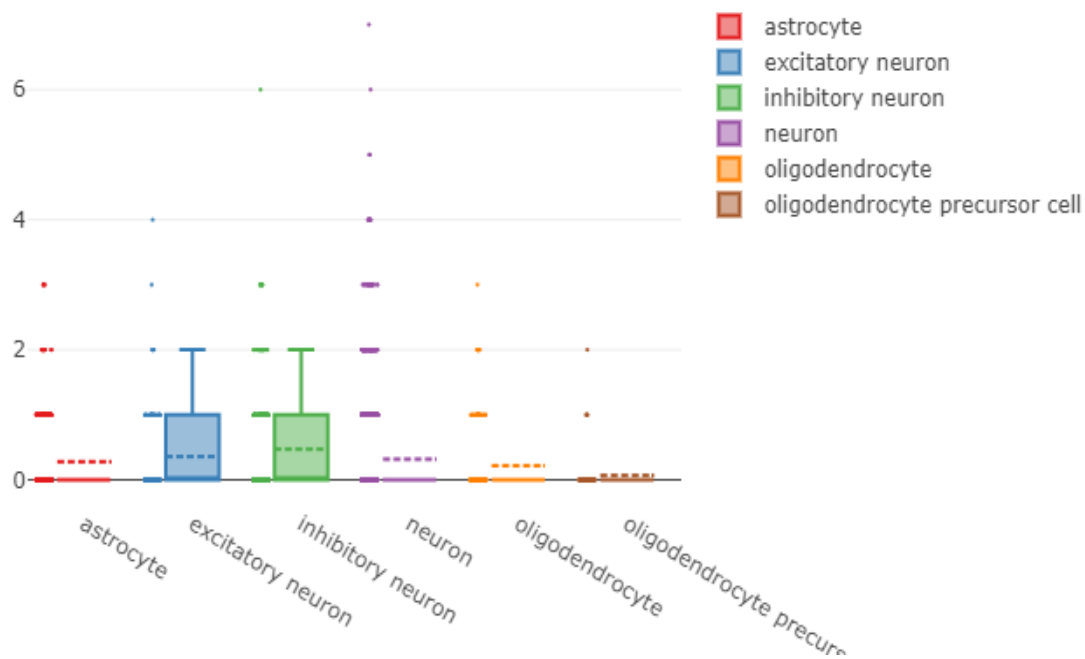
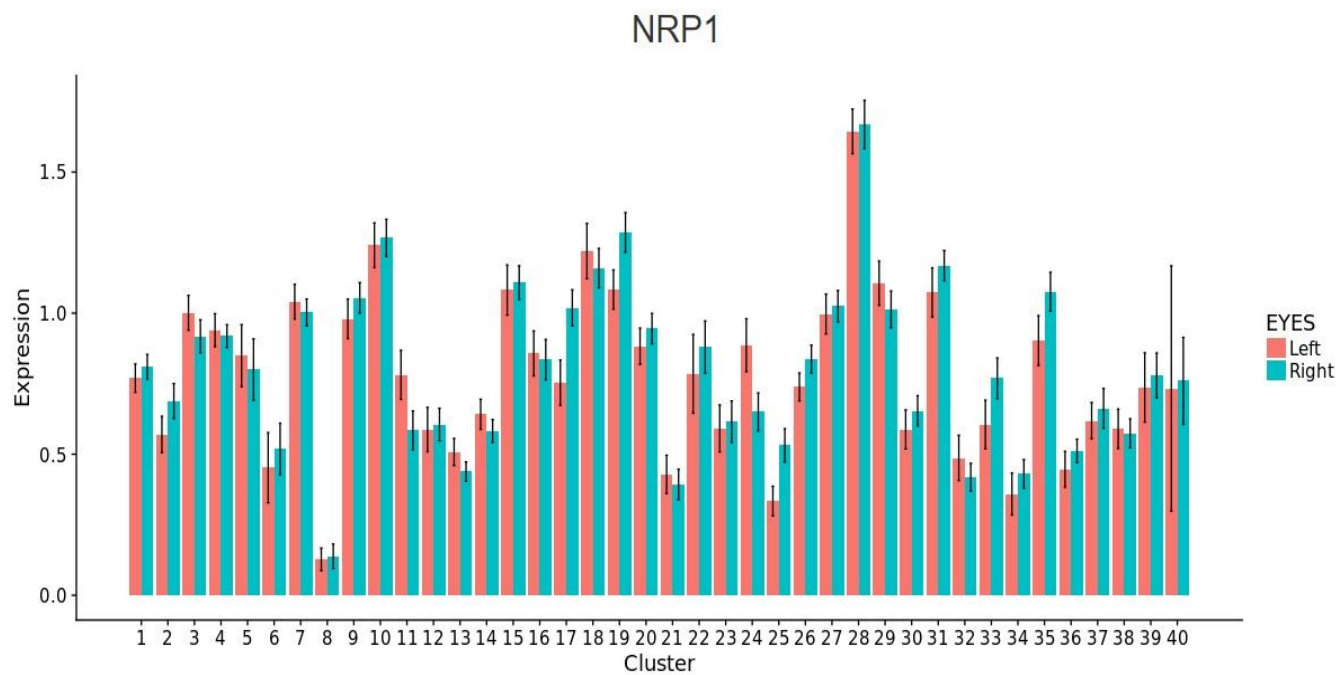
Nrp1:

Table 6: Nrp1 data analysis results

Gene ontology (MGI): Axon guidance Axon extension involved in axon guidance Axonal fasciculation Axonogenesis involved in innervation Cell migration Neuron migration Positive regulation of RGC guidance Cell differentiation Negative regulation of axon extension Nervous system development Neuron projection Sensory neuron axon guidance
Single cell expression analysis for RGCs at P5 (Rheaume <i>et al.</i> , 2018): ON-OFF DSGC (Cluster number 22): Nrp1 is detected in both eyes ON-OFF DSGC (Cluster number 23): Nrp1 is detected in both eyes lpRGC (Cluster number 6): Nrp1 is detected in both eyes lpRGC (Cluster number 25): Nrp1 is detected in both eyes lpRGC (Cluster number 26): Nrp1 is detected in both eyes lpRGC (Cluster number 37): Nrp1 is detected in both eyes Alpha RGC (Cluster number 21): Nrp1 is detected in both eyes Alpha RGC (Cluster number 33): Nrp1 is detected in both eyes
Single cell expression analysis for the SC (Single nuclei dataset, MD705): Astrocyte: Nrp1 is detected in 23.58% of astrocyte population with the mean = 0.2784. Excitatory neuron: Nrp1 is detected in 27.78% of excitatory neuron population with the mean = 0.3571. Inhibitory neuron: Nrp1 is detected in 34.77% of inhibitory neuron population with the mean = 0.4712.

Embryonic expression (Allen brain and MGI): Midbrain E 11.5: Nrp1 is detected Midbrain E 13.5: Nrp1 is detected Midbrain E 15.5: Nrp1 is detected Midbrain E 18.5: Nrp1 is detected Optic nerve E 13.5: Nrp1 is detected Retina E 14.5: Nrp1 is detected Retina E 15.5: Nrp1 is detected Neural retina E 15.5: Nrp1 is detected
Postnatal expression (Allen brain and MGI): Midbrain P 4: Nrp1 is detected Midbrain P 7: Nrp1 is detected Midbrain P 14: Nrp1 is detected Midbrain P 28: Nrp1 is detected Midbrain P 56: Nrp1 is detected Retina P 7: Nrp1 is detected Retina P 21: Nrp1 is detected
Interaction (String): Sema3a, Sema3b, Sema3c, Sema3f, PlexinA2, PlexinA3, PlexinA4.

Based on the Table 5, the Nrp1 receptor has a role in axon guidance and neural migration via interaction with Sema3a/b/c/f and PlexinA1-4. Nevertheless, because this gene is expressed at a single cell level in both excitatory and inhibitory neurons in the SC and in the ON-OFF DSGCs, Alpha RGCs, and ipRGCs in the retina during E11.5-P21, it might be a potential candidate for this research.



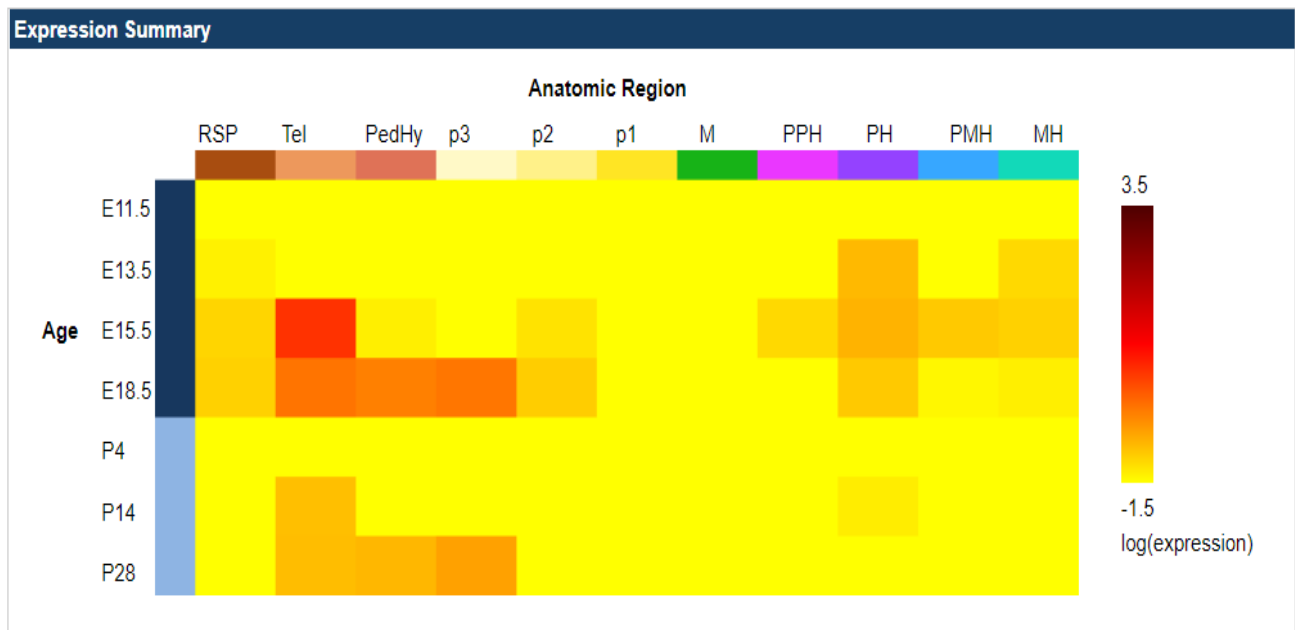


Figure 17: A) Mean of left and right eyes Nrp1 gene expression in RGCs subtypes and SEM error bars. This figure is taken from (Rheume et al., 2018). B) Distribution of Nrp1 gene expression in the SC. This figure is taken from (MD 705). C) Nrp1 embryonic and postnatal gene expression. This figure is taken from (developing mouse brain, Allen Brain atlas).

Nrp2:

Table 7: Nrp2 data analysis results

Gene ontology (MGI): - Axon extension involved in axon guidance - Axon guidance - Neuron migration - Semaphorin-plexin signaling pathway involved in neuron projection guidance - Axonogenesis - Cell differentiation - Nervous system development - Sensory neuron axon guidance
Single cell expression analysis for RGCs at P5 (Rheume et al., 2018): - ON-OFF DSGC (Cluster number 22): Nrp2 is detected in both eyes - ON-OFF DSGC (Cluster number 23): Nrp2 is detected in both eyes

lpRGC (Cluster number 6): Nrp2 is detected in both eyes lpRGC (Cluster number 25): Nrp2 is detected in both eyes lpRGC (Cluster number 26): Nrp2 is detected in both eyes lpRGC (Cluster number 37): Nrp2 is detected in both eyes Alpha RGC (Cluster number 21): Nrp2 is detected in both eyes Alpha RGC (Cluster number 33): Nrp2 is detected in both eyes
Single cell expression analysis for the SC (Single nuclei dataset, MD705): Astrocyte: Nrp2 is detected in 3.46% of astrocyte population with the mean = 0.0406. Excitatory neuron: Nrp2 is detected in 43.65% of excitatory neuron population with the mean = 0.5952. Inhibitory neuron: Nrp2 is detected in 44.25% of inhibitory neuron population with the mean = 0.6752.
Embryonic expression (MGI): Midbrain E 11.5: Nrp2 is detected Midbrain roof plate E 13.5: Nrp2 is detected Superior colliculus E 15.5: Nrp2 is detected Retina E 15.5: Nrp2 is detected
Postnatal expression (MGI): Superior colliculus P 21: Nrp2 is detected Retina P 7: Nrp2 is detected Neural retina P 7: Nrp2 is detected Retina P 21: Nrp2 is detected
Interactions (Strings): Sema3a, Sema3b, Sema3c, Sema3f, PlexinA1

Regarding the table 6, the Nrp2 gene is a potential candidate in this research. Due to the gene ontology, it has a role in axon guidance especially in the semaphorin-plexin pathway and neural migration. This gene also is expressed in the retina during E 15.5-P 21 and in the midbrain during E 11.5-P 21(SC). The Nrp2 has a higher expression in excitatory and inhibitory neurons of the SC but the expression in identified RGCs is lower than the Nrp1.

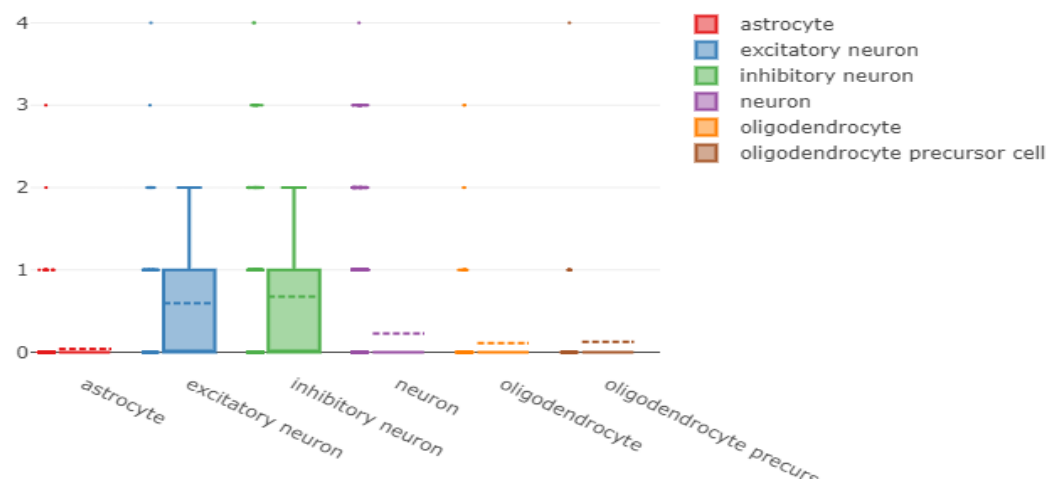
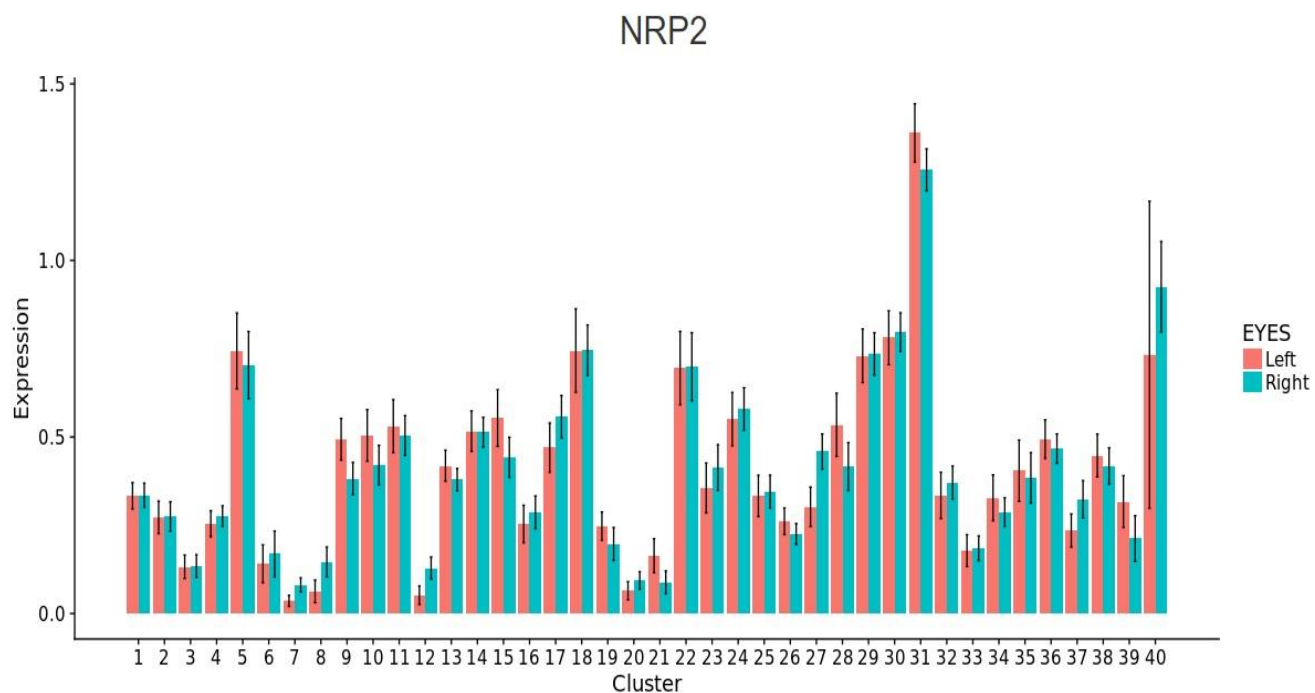


Figure 18: A) Mean of left and right eyes *Nrp2* gene expression in RGCs subtypes and SEM error bars. This figure is taken from (Rheume et al., 2018). B) Distribution of *Nrp2* gene expression in the SC. This figure is taken from (MD 705).

All in all, both Nrp1 and Nrp2 probably have a role in the development of the retino-collicular connection via semaphorin-plexin mechanisms mainly. There is no priority in choosing these genes for the final conclusion.

Cadherins

Cadherins (Cdh) are transmembrane, calcium-dependent proteins that have a role in the development of the nervous system by mediating cell-cell adhesion and synaptic organization (Marître and Heisenberg, 2013; Jontes, 2018). Classic cadherin groups like type II Cadherins have a role in several neural development stages. For example, they participate in neurogenesis, neuronal migration, neuronal polarity, axonal outgrowth, synaptogenesis, synaptic rearrangement, and synaptic plasticity (Jontes, 2018). In this research, three cadherins from the type II family are covered.

Cdh6 is a type II cadherin that is a calcium-dependent molecule. This protein has five extracellular domains, a transmembrane region, and a cytoplasmic domain for interaction with catenin molecules (Casal and Bartolom, 2019). Beta-catenin attaches to the cytoplasmic tail directly and induces the Wnt signaling pathway and cell-cell adhesion. Moreover, Cdh6 consists of RGD and His-Ala-Val (HAV) motif in the extracellular domains, respectively (Casal and Bartolom, 2019).

Cdh7 is another type II cadherin that is investigated in this research. It has different domains in its structure. For instance, Cdh7 consists of HELICc, SNF2-related helicase/ATPase, BRK, and chromodomains (Vuorela *et al.*, 2007). Cdh7 has an important role in embryonic development and gene expression due to the chromatin remodeling function by the chromodomain. In addition, similar to Cdh6, Cdh7 has five extracellular domains but it does not have an HAV motif (Vuorela *et al.*, 2007).

As like the two previous cadherins mentioned earlier, Cdh8 is a calcium-dependent type II Cadherin protein that mediates cell-cell adhesion (Kido *et al.*, 1998). Cdh8 has an extracellular domain and cytoplasmic tail too and a transmembrane region connects

these two domains with each other. Each extracellular domain has a cadherin binding motif for hemophilic interaction (Kido *et al.*, 1998).

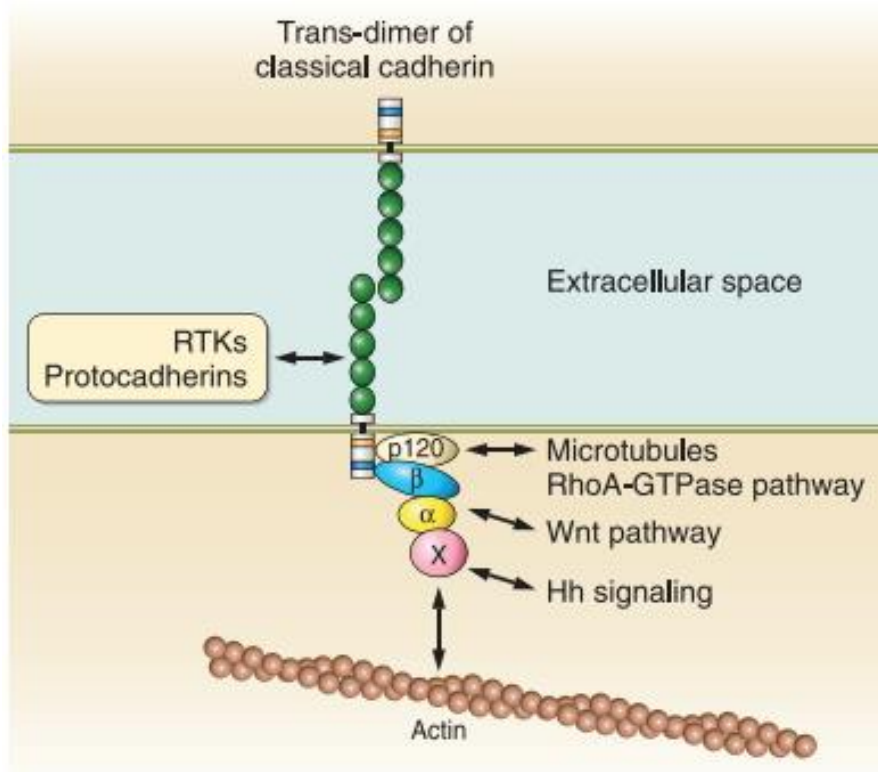


Figure 19: Classic cadherin's cell-cell adhesion. Cadherins attach to each other by their extracellular domains (green) and activate signaling pathways via their internal motifs. This figure is taken from (Hirano and Takeichi, 2021).

Generally, Cadherins act as a major cell adhesion molecule in maintaining and homeostasis of different tissues. Different types of Cadherins present at spaces between contacted cells to regulate cell-cell adhesion (Maître and Heisenberg, 2013). Cell-cell adhesion is regulated by Cadherins in three different ways, including reducing the local interfacial tension via adhesion tension, signaling transduction to affect actomyosin cytoskeleton, and establishment the mechanical coupling between contacted cells (Maître and Heisenberg, 2013). Classic cadherins like type II cadhs are localized in pre and postsynaptic cells. They can interact with catenins to induce some signaling pathways for maintaining synapses (Seong, Yuan and Arikktah, 2015). These types of Cdhs bind to Beta-catenin and induces some pathways to control cell

proliferation, survival, migration, and differentiation at embryonic developmental stages (Seong, Yuan and Arikktah, 2015).

To regulate cell-cell adhesion between two neurons, beta and P120 catenins bind to both cadherin proteins in pre and postsynaptic cells. Also, both Cadherins interact hemophilically or heterophilically with each other in synaptic clefts (Brigidi and Bamji, 2011). Next, at the presynaptic neurons, they activate Scrib for the recruitment of synaptic vesicles to release neurotransmitters. Also, at the postsynaptic neurons, the cadherin-catenin complex activates Rac1 for spine head width. This complex can induce S-SCAM signaling pathway at the post-synaptic site to affect synaptic vesicles by neurexin in the pre-synaptic space (Brigidi and Bamji, 2011).

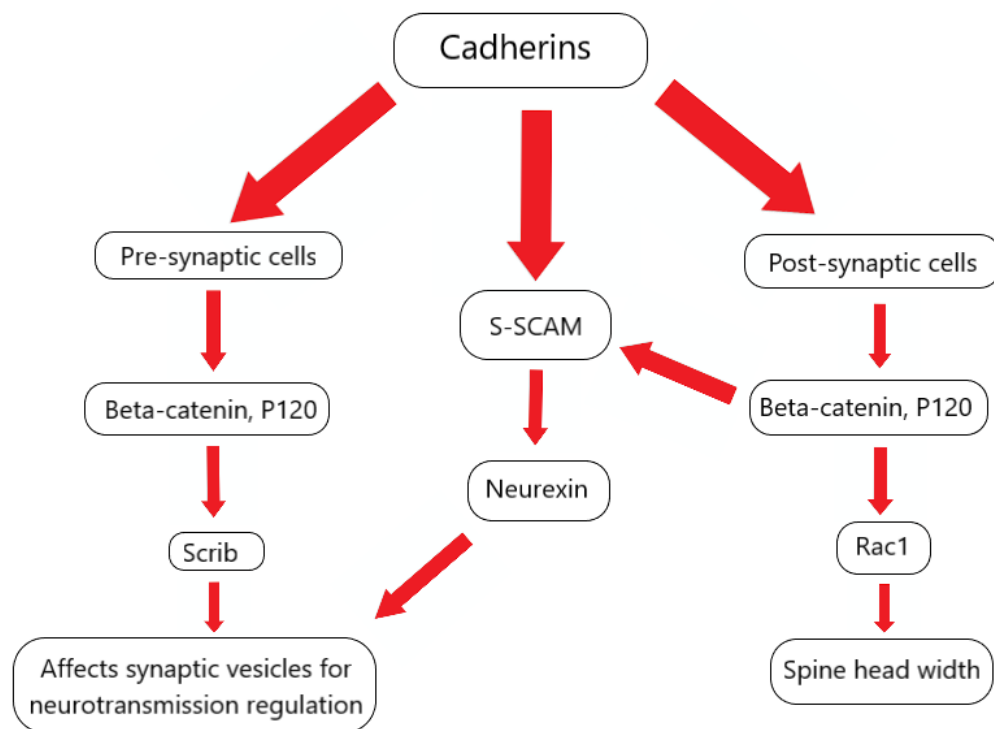


Figure 20: The cadherins cellular pathways.

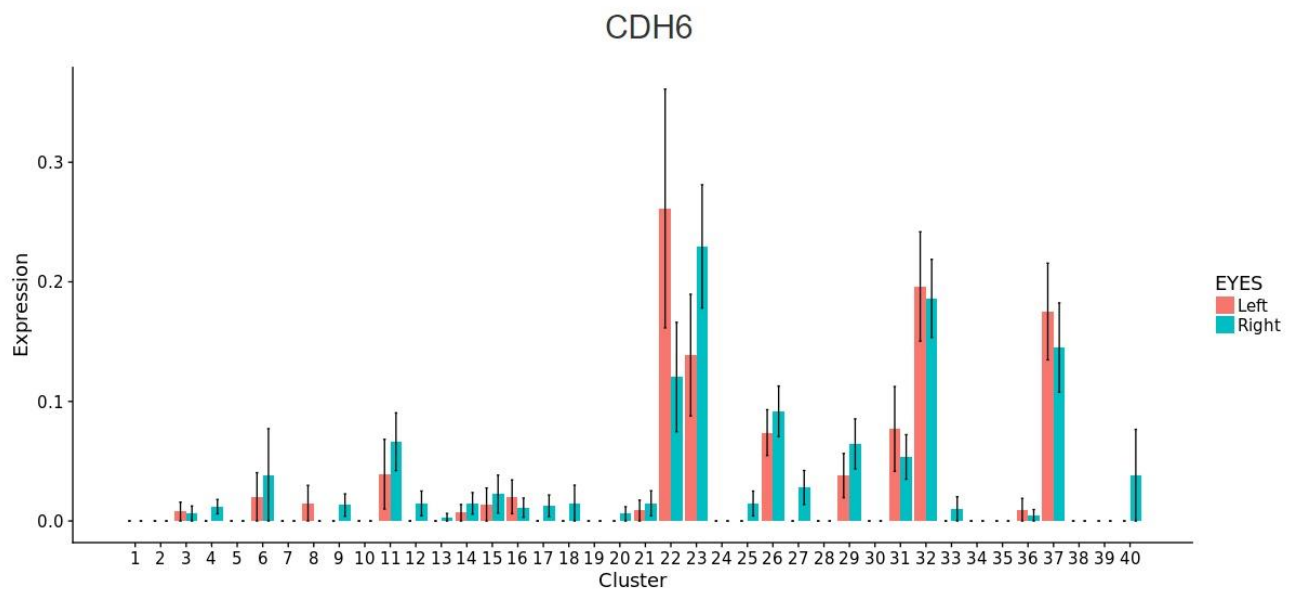
Cdh6:

Table 8: Cdh6 data analysis results

Gene ontology (MGI): Cell-cell adhesion via plasma-membrane adhesion molecules Hemophilic cell adhesion via plasma membrane adhesion molecules Cadherin binding
Single cell expression analysis for RGCs at P5 (Rheaume <i>et al.</i>, 2018): ON-OFF DSGC (Cluster number 22): Cdh6 is detected in both eyes ON-OFF DSGC (Cluster number 23): Cdh6 is detected in both eyes lpRGC (Cluster number 6): Cdh6 is detected in both eyes lpRGC (Cluster number 25): Cdh6 is detected in both eyes lpRGC (Cluster number 26): Cdh6 is detected only in the right eye lpRGC (Cluster number 37): Cdh6 is detected in both eyes Alpha RGC (Cluster number 21): Cdh6 is detected in both eyes Alpha RGC (Cluster number 33): Cdh6 is detected only in the right eye
Single cell expression analysis for the SC (Single nuclei dataset, MD705): Astrocyte: Cdh6 is detected in 4.67% of astrocyte population with the mean = 0.0609. Excitatory neuron: Cdh6 is detected in 18.25% of excitatory neuron population with the mean = 0.2380. Inhibitory neuron: Cdh6 is detected in 32.18% of inhibitory neuron population with the mean = 0.8103.
Embryonic expression (MGI and Allen brain): Midbrain E 11.5: Cdh6 is detected Superior colliculus E 14.5: Cdh6 is detected Midbrain E 13.5: Cdh6 is detected Midbrain E 15.5: Cdh6 is detected Midbrain E 18.5: Cdh6 is detected Retina E 14.5: Cdh6 is detected Optic stalk E 12: Cdh6 is detected
Postnatal expression (MGI and Allen brain):

Midbrain P 4: Cdh6 is detected
Midbrain P 7: Cdh6 is detected
Midbrain P 14: Cdh6 is detected
Midbrain P 28: Cdh6 is detected
Midbrain P 56: Cdh6 is detected
Retina P 7: Cdh6 is detected
Retina P 14: Cdh6 is detected
Retina P 21: Cdh6 is detected
Interactions (String): Cdh7, Cdh9, Cdh10, Cdh15, Cdh18

According to the data regarding table 7, the Cdh6 has some chances to be picked as a potential candidate in this research. The Cdh6 has a role in cell-cell adhesion and cadherin binding mechanisms. Thus, it probably involves synaptic formation between the retina and the SC. Moreover, this gene is expressed in most of the identified RGCs especially in ON-OFF DSGCs (Figure 21) and both excitatory and inhibitory neurons during E11.5-P21. Overall, the Cdh6 might be involved in the establishment of retino-collicular connection via hemophilic interaction with other cadherins in the synaptogenesis.



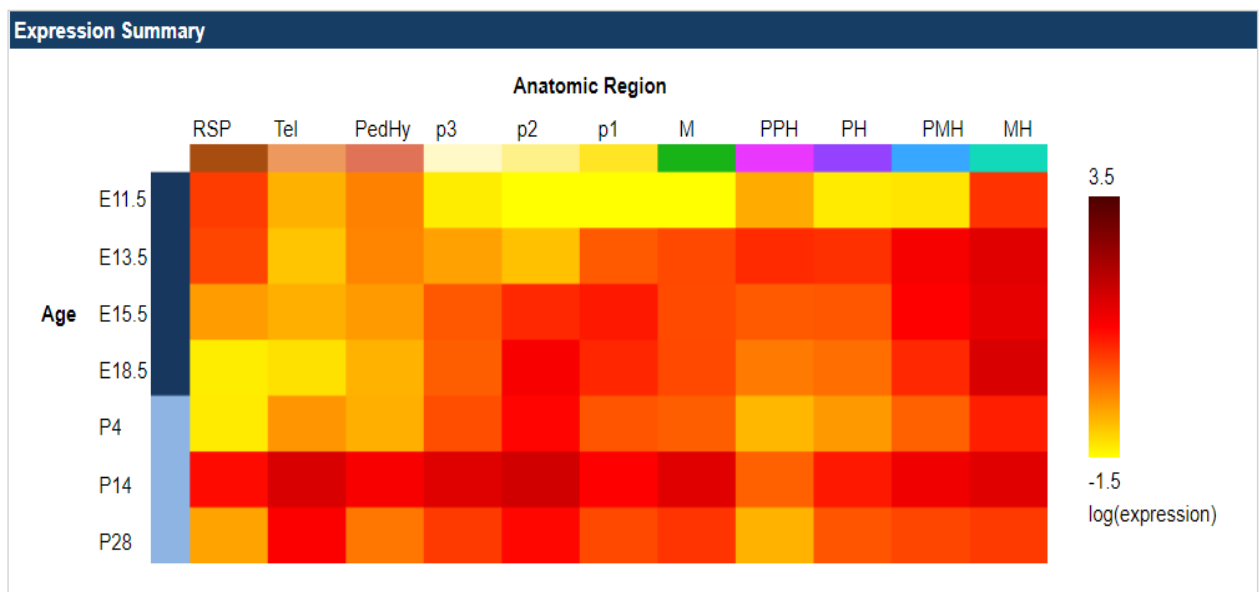
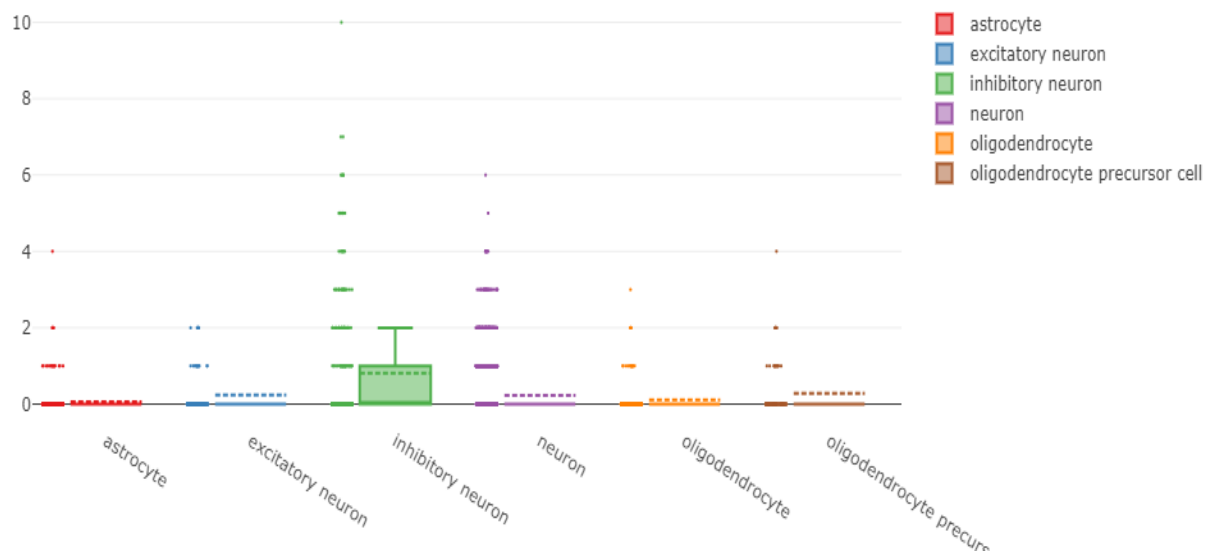


Figure 21: A) Mean of left and right eyes Cdh6 gene expression in RGCs subtypes and SEM error bars. This figure is taken from (Rheume et al., 2018). B) Distribution of Cdh6 gene expression in the SC. This figure is taken from (MD 705). C) Cdh6 embryonic and postnatal gene expression. This figure is taken from (developing mouse brain, Allen Brain atlas).

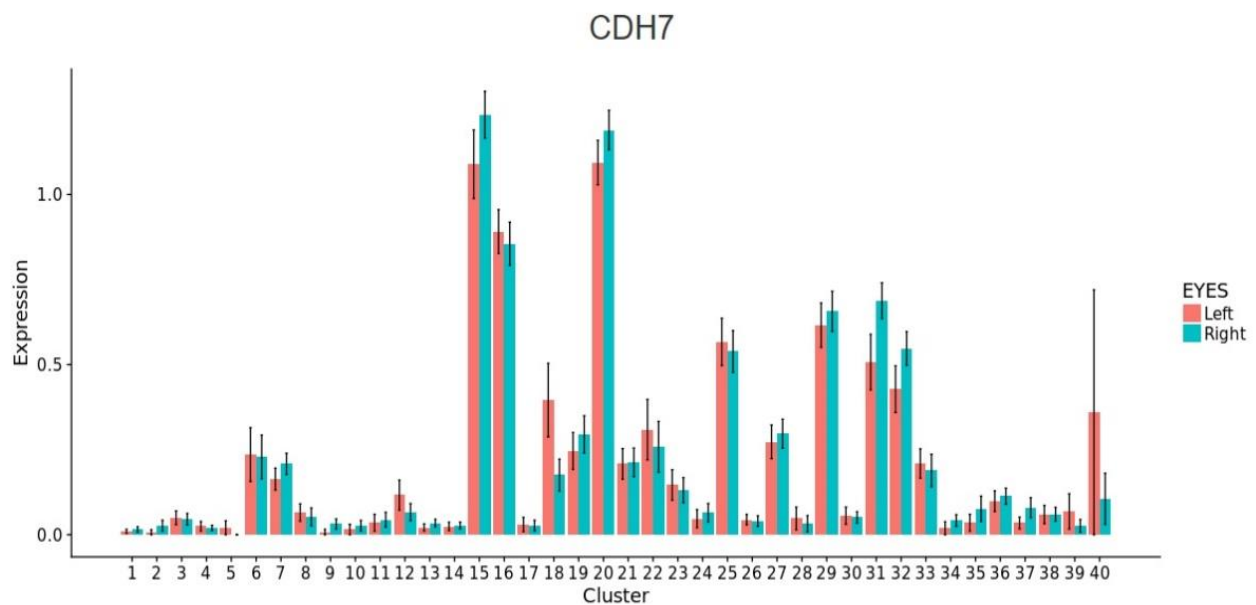
Cdh7:

Table 9: Cdh7 data analysis results

Gene ontology (MGI): Cadherin binding calcium-dependent cell-cell adhesion via plasma membrane cell adhesion molecules cell-cell adhesion cell-cell adhesion via plasma-membrane adhesion molecule hemophilic cell adhesion via plasma membrane adhesion molecules
Single cell expression analysis for RGCs at P5 (Rheaume <i>et al.</i>, 2018): ON-OFF DSGC (Cluster number 22): Cdh7 is detected in both eyes ON-OFF DSGC (Cluster number 23): Cdh7 is detected in both eyes lpRGC (Cluster number 6): Cdh7 is detected in both eyes lpRGC (Cluster number 25): Cdh7 is detected in both eyes lpRGC (Cluster number 26): Cdh7 is detected in both eyes lpRGC (Cluster number 37): Cdh7 is detected in both eyes Alpha RGC (Cluster number 21): Cdh7 is detected in both eyes Alpha RGC (Cluster number 33): Cdh7 is detected in both eyes
Single cell expression analysis for the SC (Single nuclei dataset, MD705): Astrocyte: Cdh7 is detected in 2.64% of astrocyte population with the mean = 0.0447. Excitatory neuron: Cdh7 is detected in 26.19% of excitatory neuron population with the mean = 0.2936. Inhibitory neuron: Cdh7 is detected in 15.80% of inhibitory neuron population with the mean = 0.1925.
Embryonic expression (MGI and Allen brain): Midbrain E11.5: Cdh7 is detected Midbrain E13.5: Cdh7 is detected Midbrain E15.5: Cdh7 is detected Midbrain E18.5: Cdh7 is detected
Postnatal expression (MGI and Allen brain): Midbrain P4: Cdh7 is detected

Midbrain P7: Cdh7 is detected
Midbrain P14: Cdh7 is detected
Midbrain P28: Cdh7 is detected
Midbrain P56: Cdh7 is detected
Retina P7: Cdh7 is detected
Retina P14: Cdh7 is detected
Retina P21: Cdh7 is detected
Interactions (String): Cdh6, Cdh9, Cdh10, Cdh12, Cdh15

According to the table 8, the Cdh7 can be a potential gene for involvement in the connection between the retina and SC. Cdh7 interacts with other types of cadherins like Cdh6 in a cadherin binding manner. In addition, this gene has a role in cell-cell adhesion and is expressed in the identified RGCs and excitatory and inhibitory neurons in the SC. Also, there is sufficient results about the expression time of this gene in the midbrain and retina. Based on the table, the Cdh7 is expressed in the midbrain during E 11.5-P 56 and is expressed in the retina during P5 until P21. Nevertheless, probably the Cdh7 has a role in cell-cell adhesion between neurons in the development of the retino-collicular connection.



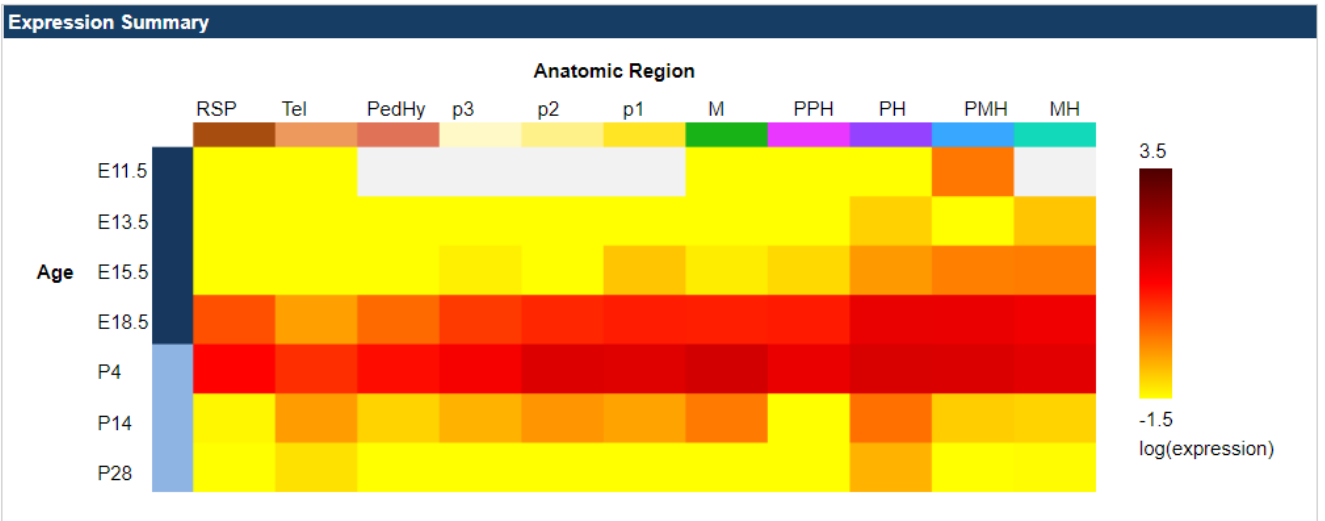
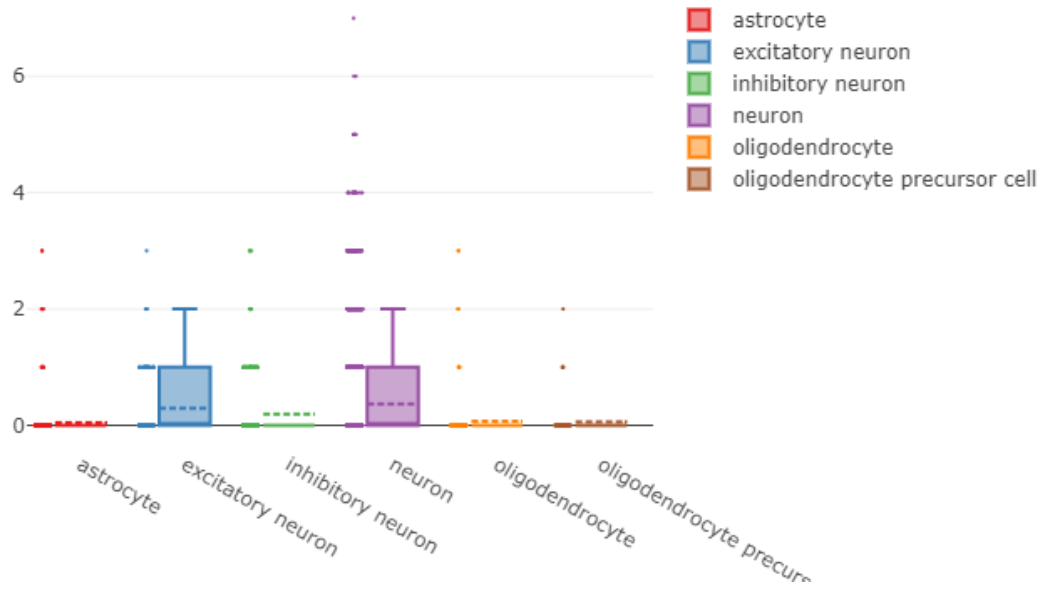


Figure 22: A) Mean of left and right eyes Cdh7 gene expression in RGCs subtypes and SEM error bars. This figure is taken from (Rheaume et al., 2018). B) Distribution of Cdh7 gene expression in the SC. This figure is taken from (MD 705). C) Cdh7 embryonic and postnatal gene expression. This figure is taken from (developing mouse brain, Allen Brain atlas).

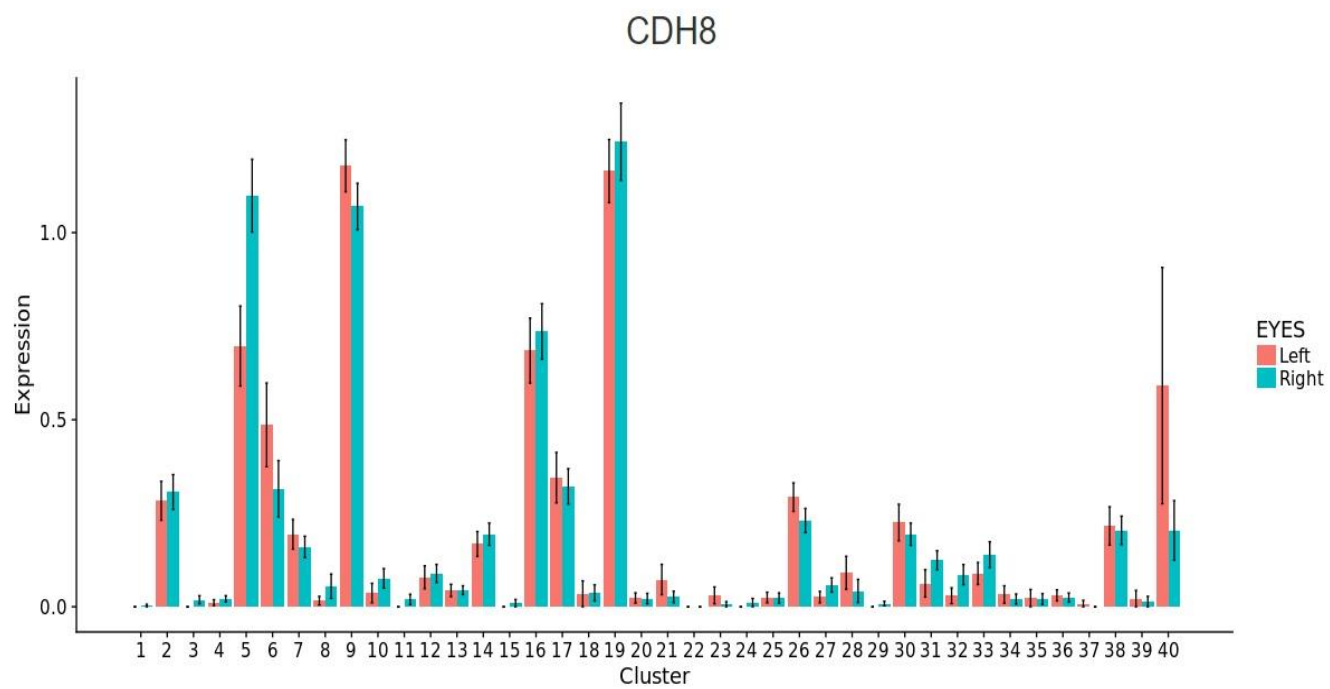
Cdh8:

Table 10: Cdh8 data analysis results

Gene ontology (MGI): Cell-cell adhesion via plasma membrane adhesion molecules. Calcium dependent cell-cell adhesion via plasma membrane adhesion molecules. Chemical synaptic transmission Regulation of synapse organization Axon terminus Cadherin binding Synaptic cleft
Single cell expression analysis for RGCs at P5 (Rheaume <i>et al.</i>, 2018): ON-OFF DSGC (Cluster number 22): Cdh8 is not detected in both eyes ON-OFF DSGC (Cluster number 23): Cdh8 is detected in both eyes lpRGC (Cluster number 6): Cdh8 is detected in both eyes lpRGC (Cluster number 25): Cdh8 is detected in both eyes lpRGC (Cluster number 26): Cdh8 is detected in both eyes lpRGC (Cluster number 37): Cdh8 is detected only in the left eye Alpha RGC (Cluster number 21): Cdh8 is detected in both eyes Alpha RGC (Cluster number 33): Cdh8 is detected in both eyes
Single cell expression analysis for the SC (Single nuclei dataset, MD705): Astrocyte: Cdh8 is detected in 10.98% of astrocyte population with the mean = 0.1768. Excitatory neuron: Cdh8 is detected in 80.95% of excitatory neuron population with the mean = 2.6904. Inhibitory neuron: Cdh8 is detected in 33.62% of inhibitory neuron population with the mean = 0.9712.
Embryonic expression (Allen brain and MGI): E11.5 Midbrain: Cdh8 is detected E12.5 Midbrain roof plate: Cdh8 is detected Midbrain E13.5: Cdh8 is detected

Superior colliculus E14.5: Cdh8 is detected
Midbrain E15.5: Cdh8 is detected
Midbrain E18.5: Cdh8 is detected
Postnatal expression (Allen brain and MGI):
Midbrain P4: Cdh8 is detected
Midbrain P7: Cdh8 is detected
Midbrain P14: Cdh8 is detected
Midbrain P28: Cdh8 is detected
Midbrain P56: Cdh8 is detected
P7 Retina: Cdh8 is detected
P21 Retina: Cdh8 is detected
Interactions (String): Cdh2, Cdh4

Regarding the table 9, the Cdh8 gene plays a role in the synaptic transmission, synaptic organization, and cell-cell adhesion regulation. Also, ON-OFF DSGCs, ipRGCs, and AlphaRGCs express this gene at the single cell level in the retina. A majority of the excitatory neurons' population express the Cdh8 in the SC. Moreover, the related structures, including the midbrain and superior colliculus express the Cdh8 gene during embryonic and postnatal stages. The Cdh8 is expressed at P5, P7, and P21 in the retina. Overall, the Cdh8 is an appropriate candidate in this study because it might be involved in synaptic regulation and cell-cell adhesion in the establishment of the connection between retina and SC.



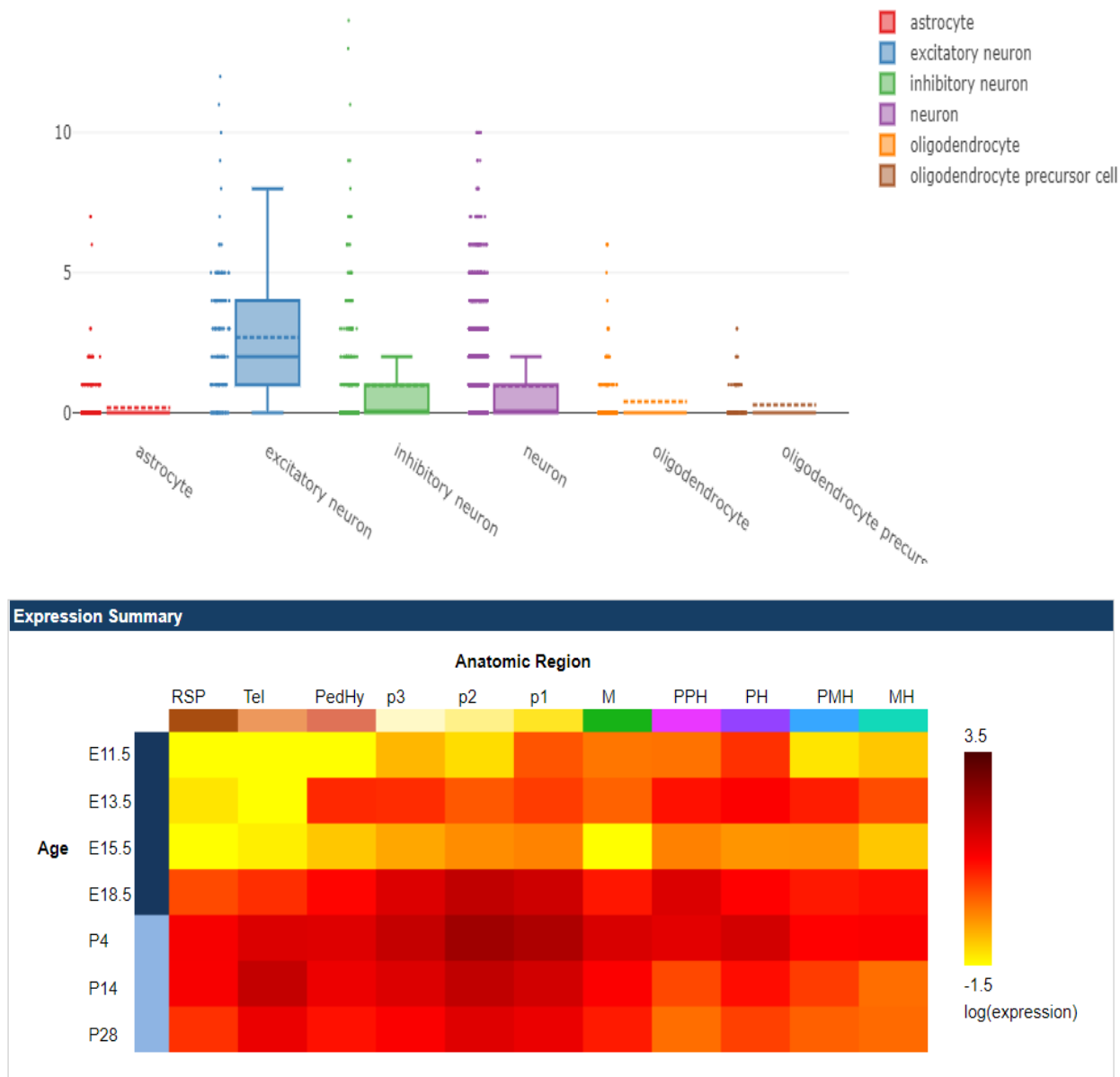


Figure 23: A) Mean of left and right eyes *Cdh8* gene expression in RGCs subtypes and SEM error bars. This figure is taken from (Rheume et al., 2018). B) Distribution of *Cdh8* gene expression in the SC. This figure is taken from (MD 705). C) *Cdh8* embryonic and postnatal gene expression. This figure is taken from (developing mouse brain, Allen Brain atlas).

All in all, Cdh6/7/8 are potential candidates in this research. The Cdh8 has a priority to other genes because it has a role in both synaptic regulation and cell adhesion. Besides, the Cdh8 has stronger expression among other genes during embryonic and early postnatal stages. There is no priority between Cdh6 and Cdh7. Both of them have a chance to be involved in the development of the retino-collicular connection via cell adhesion manner.

Neurofascin

Neurofascin is one member of L1 of family cell adhesion molecules that belongs to the immunoglobulin superfamily proteins (Liu, Focia and He, 2011). Nfasc is a member of L1CAM family with NrCAM, L1, and Chl1 (Ango *et al.*, 2004). This gene involves cell adhesion, axon guidance, neurite extension, synaptic formation, and neuron-glia interaction (NCBI (Refseq), 2009). This protein consists of six immunoglobulin-like (Ig) domains, four to five fibronectin type III like repeats, a transmembrane domain, and a cytoplasmic domain (Ebel *et al.*, 2014). PAT/mucin-like domain is a specific feature of neurofascin. This domain consists of proline, alanine, and threonine amino acids residues mainly (Ebel *et al.*, 2014). The mammalian Nfasc splices to variant isoforms. For example, NF186 and NF155 belong to axonal and glial isoforms, respectively (Liu, Focia and He, 2011). The difference between these two isoforms is the NF155 has an additional small exon in the Ig domain and it is shorter than NF186 due to different splicing (Liu, Focia and He, 2011).

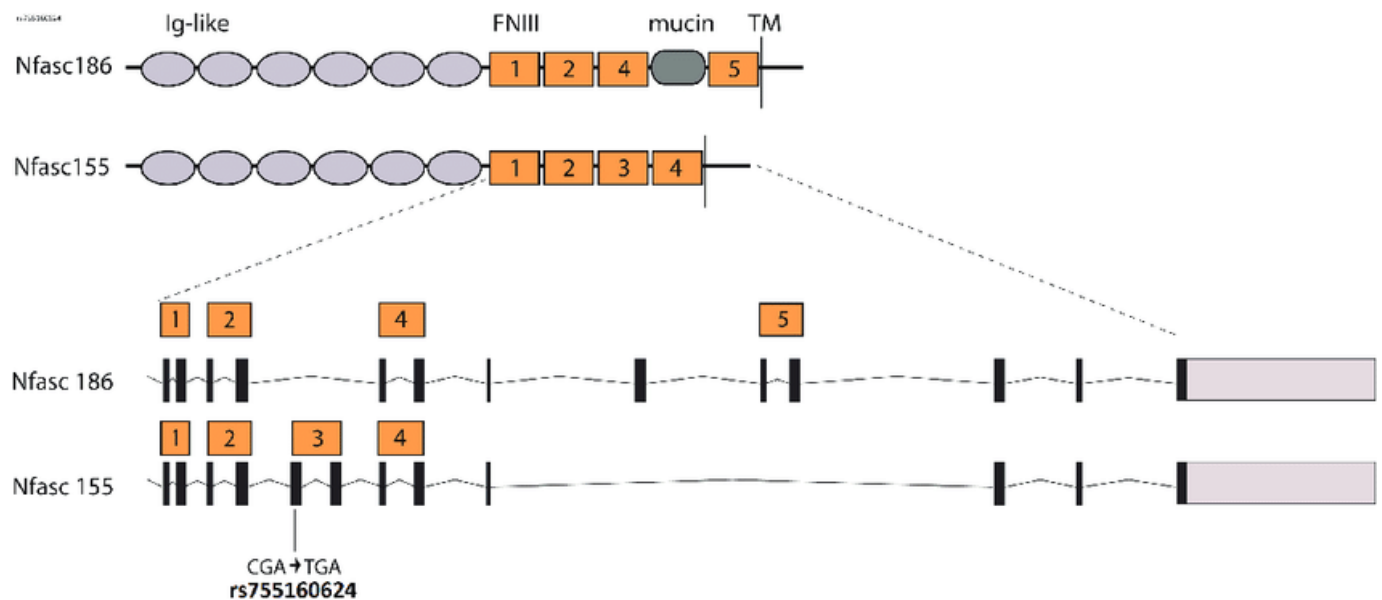


Figure 24: The different isoforms of neurofascin structure. This figure is taken from (Smigiel *et al.*, 2018)

Neurofascin, especially NF186, cooperates with AnkyrinG to induce other signal transductions. AnkyrinG is a scaffold protein that connects membrane molecules to the actin-based cytoskeleton (Ebel *et al.*, 2014). As mentioned earlier, the connection of basket cells and Purkinje cells is regulated by Nfasc through the axon initial segment (Ango *et al.*, 2004). Here is the signaling pathway of the interaction of the Nfasc and AnkG for this purpose. AnkG binds to the NF186, then affect alphaII spectrin and beta IV spectrin on the actin ring. Next, this complex binds to the Ndel1 at the microtubule or actin cytoskeleton (Huang and Rasband, 2018). This pathway can maintain neural polarity at synapses cleft (Huang and Rasband, 2018). Moreover, NrCAM can attach to Nfasc and recruit sodium and potassium membrane channels at the membrane of the axon initial segment. Then similar to NF186-AnkG pathway, spectrins and then Ndel1 or EB1/EB3 are activated at cytoskeletal components (Fujitani, Otani and Miyajima, 2021). Also, recruitment of the sodium and potassium channel leads to maintenance of the synapses function at the axon initial segment (Fujitani, Otani and Miyajima, 2021). In contrast, Nfasc interacts with brevican an extracellular matrix component to stabilize the synapse between the pre and postsynaptic neurons (Dityatev, Seidenbecher and Schachner, 2010).

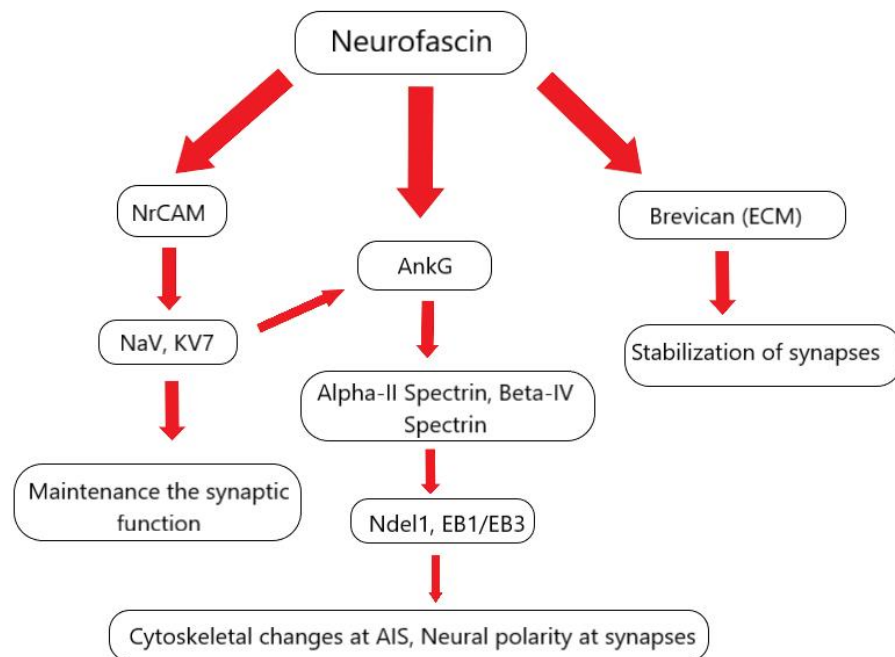


Figure 25: The Neurofascin cellular pathways.

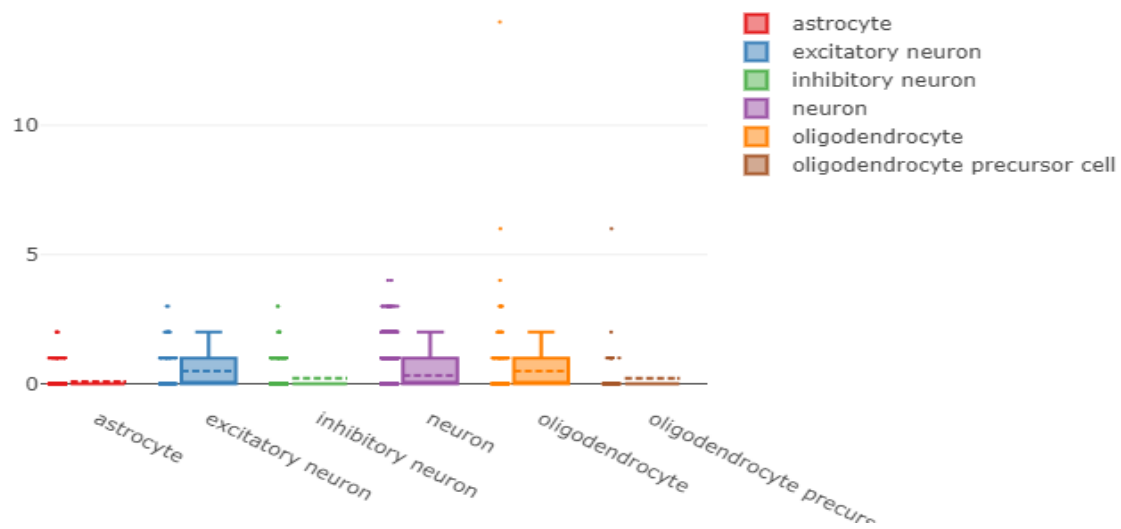
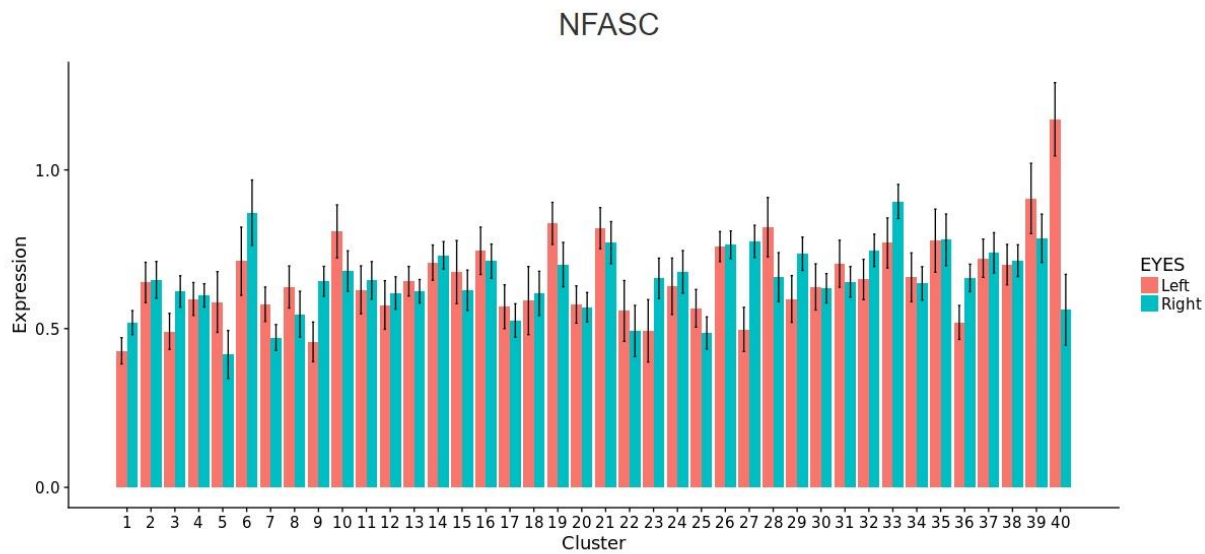
Table 11: Nfasc data analysis results

Gene ontology (MGI):
Axon
Axon guidance
Axon initial segment
Cell adhesion
Synapse organization
Single cell expression analysis for RGCs at P5 (Rheaume <i>et al.</i> , 2018):
ON-OFF DSGC (Cluster number 22): Nfasc is detected in both eyes
ON-OFF DSGC (Cluster number 23): Nfasc is detected in both eyes
IpRGC (Cluster number 6): Nfasc is detected in both eyes
IpRGC (Cluster number 25): Nfasc is detected in both eyes
IpRGC (Cluster number 26): Nfasc is detected in both eyes
IpRGC (Cluster number 37): Nfasc is detected in both eyes
Alpha RGC (Cluster number 21): Nfasc is detected in both eyes

Alpha RGC (Cluster number 33): Nfasc is detected in both eyes
Single cell expression analysis for the SC (Single nuclei dataset, MD705): Astrocyte: Nfasc is detected in 7.72% of astrocyte population with the mean = 0.0853. Excitatory neuron Nfasc is detected in 37.30% of excitatory neuron population with the mean = 0.4920. Inhibitory neuron: Nfasc is detected in 18.68% of inhibitory neuron population with the mean = 0.2155.
Embryonic expression (Allen brain): Midbrain E11.5: Nfasc is detected Midbrain E13.5: Nfasc is detected Midbrain E15.5: Nfasc is detected Midbrain E18.5: Nfasc is detected
Postnatal expression (Allen brain): Midbrain P4: Nfasc is detected Midbrain P7: Nfasc is detected Midbrain P14: Nfasc is detected Midbrain P28: Nfasc is detected Midbrain P56: Nfasc is detected
Interactions (String): AnkR, AnkB, AnkG

According to the Nfasc data analysis results, the function of the Nfasc is axon guidance and synaptic regulation. The Nfasc interacts with Anykrin families for its function. The Anykrins has been proved that have a role in the retino-collicular topographic alignment (Buhusi *et al.*, 2008). Also, the Nfasc is expressed in both neurons of the SC and identified RGCs. About the time of Nfasc gene expression, there are no data for the retina's embryonic and postnatal expression in our database with one exception at P5 at the single cell level. On the other hand, Nfasc is expressed in the midbrain during E11.5-P56. Overall, the Nfasc can be a potential candidate in this research due to its

expression in both structures and suitable gene ontology function. This gene might be playing a role in the synaptic maintenance and regulation in the target connection.



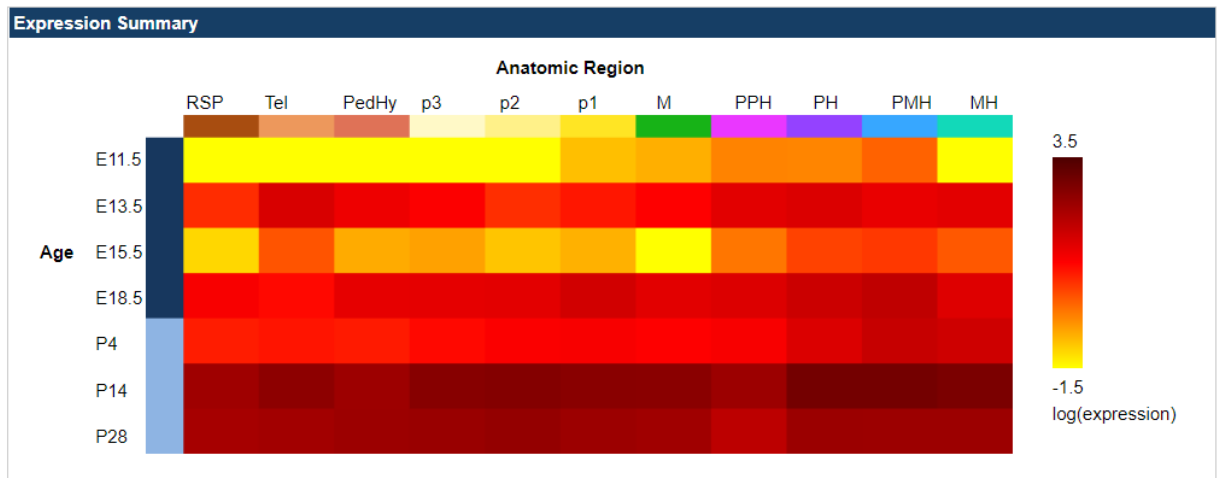


Figure 26: A) Mean of left and right eyes Nfasc gene expression in RGCs subtypes and SEM error bars. This figure is taken from (Rheaume et al., 2018). B) Distribution of Nfasc gene expression in the SC. This figure is taken from (MD 705). C) Nfasc embryonic and postnatal gene expression. This figure is taken from (developing mouse brain, Allen Brain atlas).

Neuroligins

Neuroligins (NLGN) are cell-surface proteins that mediate synaptic signal transmission and regulate synaptic function. Neuroligins are postsynaptic cell adhesion molecules and interact with neurexin in postsynaptic neurons to connect neurons with each other (Südhof, 2008; Marchot and Bourne, 2014). Also, Neuroligins can recruit other synaptic proteins for synaptic functions (Südhof, 2008). Deficient in the NLGN leads to malfunctioning of synapses and several mental and neurological disorders (Chubykin et al., 2013). There are three isoforms of NLGNs in the nervous system that are covered in this research for retino-collicular connection.

Neuroligins are composed of a single N-terminal extracellular domain, a type I transmembrane region, and a cytoplasmic C-terminal tail. The cytoplasmic C-terminal domain composes of several binding/scaffold motifs such as PDZ, WW-binding, and a gephyrin binding site (Marchot and Bourne, 2014). The N-terminal extracellular domain is linked to the transmembrane and C-terminal tail by the highly O-glycosylated region. The neurexin binds to neuroligins only at the extracellular region (Fabrichny et al., 2009). The mammalian NLGN have five variant isoforms, namely NLGN1-4/4y. They

have 70% similarity in their structure (Fabrichny *et al.*, 2009). NLGN1 and NLGN2 are localized more at the glutamatergic (excitatory) in a different part of the nervous system and then are localized at GABAergic/ glycinergic (inhibitory) synapses, respectively. The NLGN3 are found on both excitatory and inhibitory synapses equally (Babaev *et al.*, 2016).

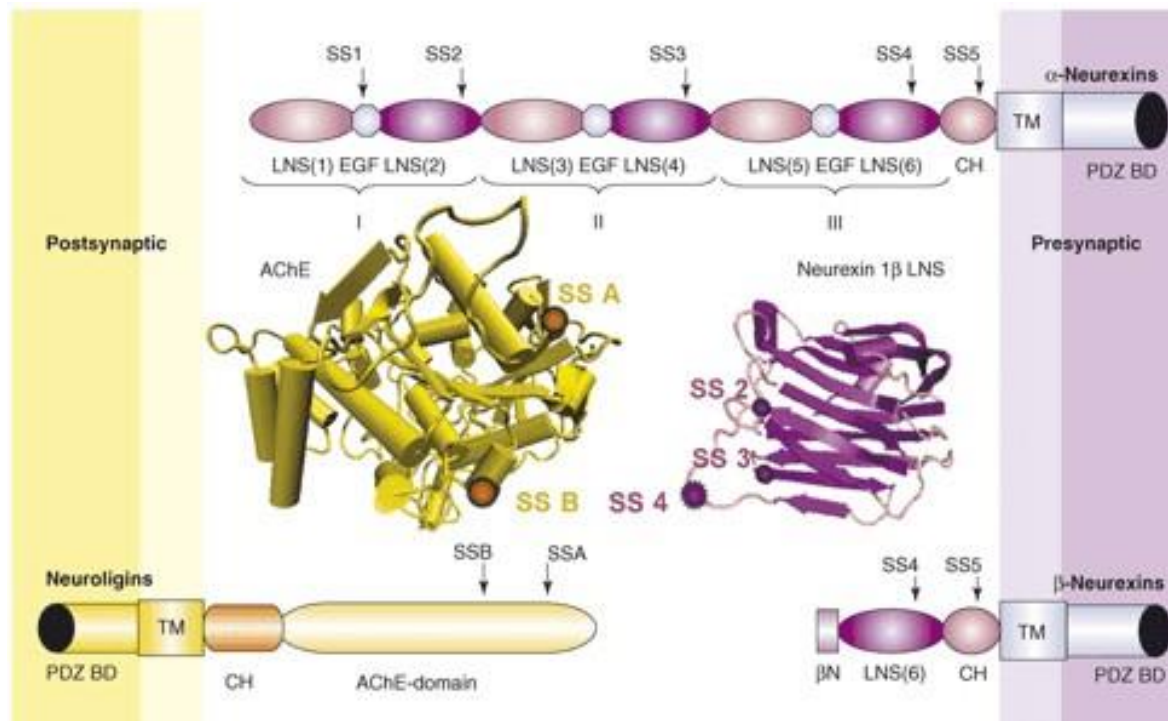


Figure 27: The structure of Neuroligin and Neurexins in post and pre-synaptic neurons. This figure is taken from (Craig and Kang, 2007).

The neuroligin-Neurexin signaling pathway is essential for the recruitment of pre and postsynaptic proteins to regulate synaptic transmission and function (Krueger *et al.*, 2012). At the presynaptic stage, NLGN-Nrxn complex activates Munc18 (interacting protein) and CASK (calcium/calmodulin-dependent serine protein kinase) to affect synaptic vesicles (Krueger *et al.*, 2012). On the other hand, at the postsynaptic site, NLGN-Nrxn complex recruits scaffolding protein PSD 95. This complex induces different signaling transduction at the different types of synapses (excitatory/ inhibitory) (Krueger *et al.*, 2012).

To be more specific, at excitatory synapses, Mun18, Mint, and CASK bind to the alpha-neurexin and this complex interacts with NLGN1/3. Due to this interaction PSD95 is activated (Reichelt and Dachtler, 2015). Then Shank and S-SCAM are recruited. Also, LRRTM and NMDAR are activated (Reichelt and Dachtler, 2015). Another signaling pathway is observed in excitatory synapses. Synaptogamin binds to the beta- neurexin and then they are interacting with NLGN1/3. PSD95, Pick1, and AMPAR are recruited in this pathway (Reichelt and Dachtler, 2015). At inhibitory synapses, Mun18/Mint/Cask complex attaches to the alpha-neurexin and then after interaction with NLGNs, gephyrin and some inhibitory receptors like GABA-AY5 are activated (Reichelt and Dachtler, 2015).

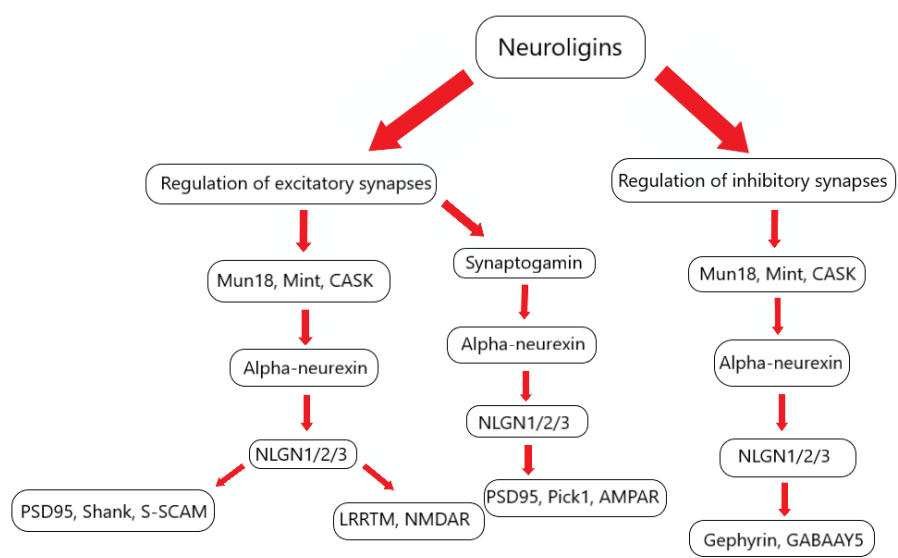


Figure 28: The neuroligins cellular pathways

NLGN1:

Table 12: NLGN1 data analysis results

Gene ontology (MGI):
calcium-dependent cell-cell adhesion via plasma membrane cell adhesion molecules
cell adhesion
cell adhesion molecule binding
chemical synaptic transmission

excitatory synapse assembly
 nervous system development
 neurexin clustering involved in presynaptic membrane assembly
 neuron cell-cell adhesion
 positive regulation of excitatory postsynaptic potential
 positive regulation of synapse assembly
 postsynaptic membrane assembly
 presynaptic membrane assembly
 synapse assembly
 synapse organization
 synaptic membrane adhesion
 integral component of postsynaptic specialization membrane
 positive regulation of synaptic transmission, GABAergic
 positive regulation of synaptic transmission, glutamatergic
 positive regulation of synaptic vesicle clustering
 positive regulation of synaptic vesicle endocytosis
 positive regulation of synaptic vesicle exocytosis
 protein localization to synapse

Single cell expression analysis for RGCs at P5 (Rheume *et al.*, 2018):

ON-OFF DSGC (Cluster number 22): NLGN1 is detected in both eyes

ON-OFF DSGC (Cluster number 23): NLGN1 is detected in both eyes

lpRGC (Cluster number 6): NLGN1 is detected in both eyes

lpRGC (Cluster number 25): NLGN1 is detected in both eyes

lpRGC (Cluster number 26): NLGN1 is detected in both eyes

lpRGC (Cluster number 37): NLGN1 is detected in both eyes

Alpha RGC (Cluster number 21): NLGN1 is detected in both eyes

Alpha RGC (Cluster number 33): NLGN1 is detected in both eyes

Single cell expression analysis for the SC (Single nuclei dataset, MD705):

Astrocyte: NLGN1 is detected in 87.60% of astrocyte population with the mean = 3.0426.

Excitatory neuron NLGN1 is detected in 96.83% of excitatory neuron population with the mean = 7.3174. Inhibitory neuron: NLGN1 is detected in 84.20% of inhibitory neuron population with the mean = 3.5431.
Embryonic expression: No data is available
Postnatal expression: No data is available
Interactions (String): Nrnx1, Nrnx2, Nrnx3

Regarding table 11, although the NLGN1 expression data is not complete during embryonic and postnatal stages, the NLGN1 has a strong gene ontology function in the development of the nervous system. For example, the NLGN1 plays a role in the neuron cell-cell adhesion, chemical synaptic transmission, neurexin clustering involved in presynaptic membrane assembly, positive regulation of postsynaptic potential, an integral component of postsynaptic specialization membrane, positive regulation of synaptic transmission-GABAergic, positive regulation of synaptic transmission-glutamatergic, positive regulation of synaptic vesicle clustering (GO). The mentioned processes are integral for synaptic maintenance and formation in the establishment of a neural connection in the nervous system. Besides, a majority of the excitatory and inhibitory neurons' population in the SC express the NLGN1 gene. Also, at the P5, ON-OFF DSGCs, ipRGCs, and AlphaRGCs express this gene. Nevertheless, the NLGN1 could be a potential candidate for us because the NLGN1 has a crucial role in variant synaptic regulation processes in the nervous system.

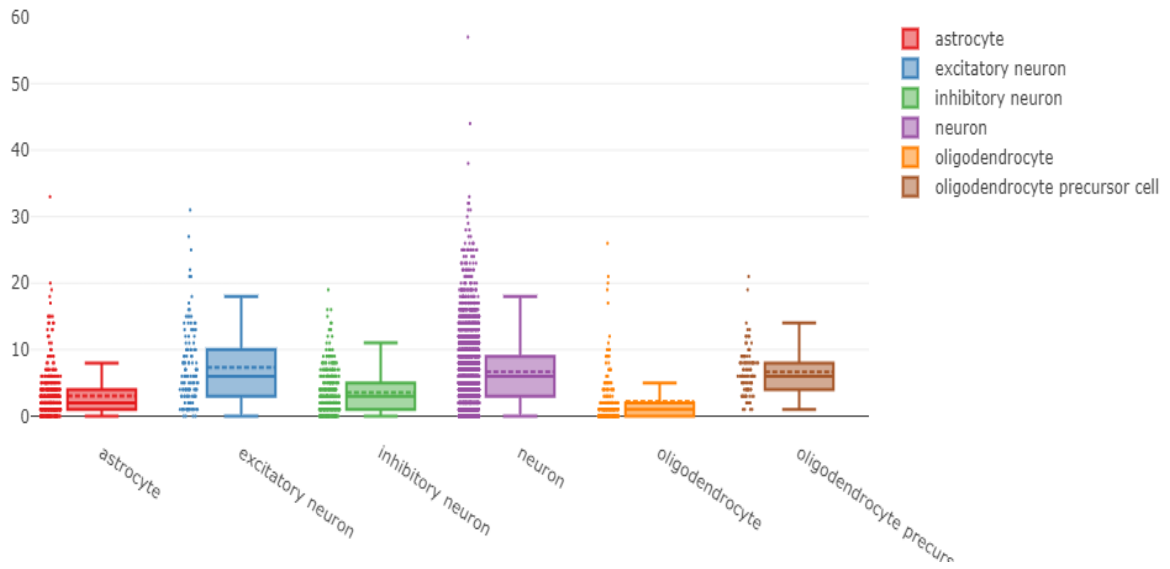
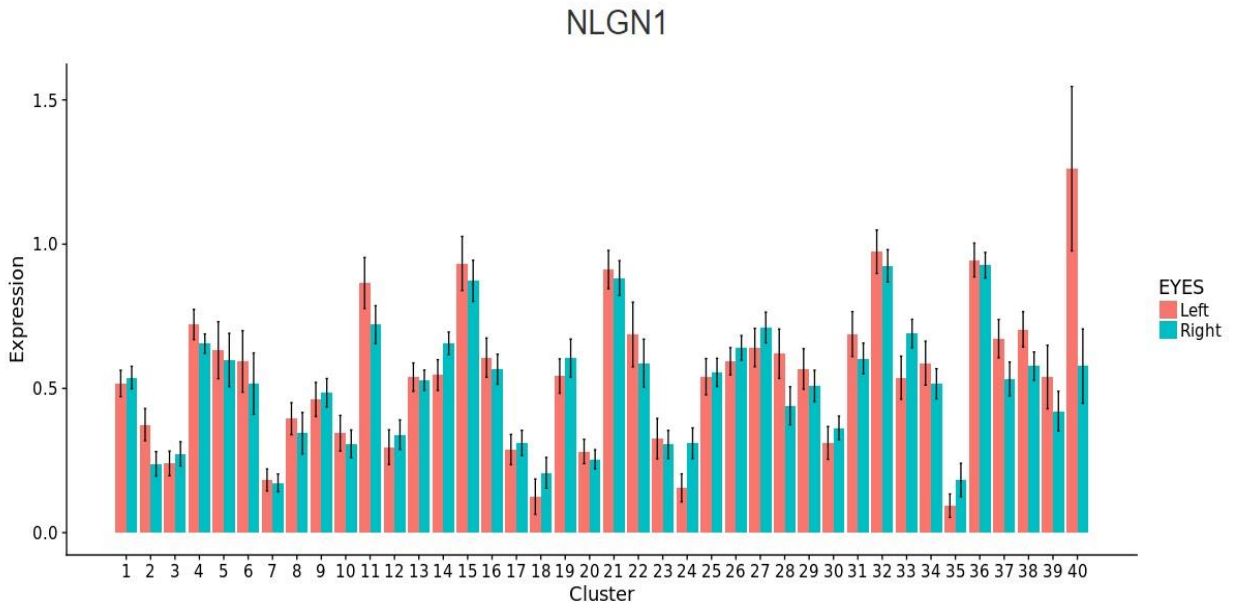


Figure 29: A) Mean of left and right eyes NLGN1 gene expression in RGCs subtypes and SEM error bars. This figure is taken from (Rheume et al.,2018). B) Distribution of NLGN1 gene expression in the SC. This figure is taken from (MD 705).

NLGN2:

Table 13: NLGN2 data analysis results

Gene ontology (MGI): Cell adhesion Cell adhesion molecule binding Chemical synaptic transmission Excitatory synapse GABA-ergic synapse glycinergic synapse inhibitory synapse inhibitory synapse assembly integral component of postsynaptic membrane integral component of postsynaptic specialization membrane modulation of chemical synaptic transmission neurexin family protein binding neuron cell-cell adhesion positive regulation of excitatory postsynaptic potential positive regulation of inhibitory postsynaptic potential positive regulation of protein localization to synapse positive regulation of synapse assembly positive regulation of synaptic transmission, GABAergic positive regulation of synaptic transmission, glutamatergic positive regulation of synaptic vesicle clustering postsynaptic membrane assembly postsynaptic specialization assembly protein localization to synapse synapse assembly synapse organization
Single cell expression analysis for RGCs at P5 (Rheaume <i>et al.</i> , 2018): ON-OFF DSGC (Cluster number 22): NLGN2 is detected in both eyes ON-OFF DSGC (Cluster number 23): NLGN2 is detected in both eyes

lpRGC (Cluster number 6): NLGN2 is detected in both eyes lpRGC (Cluster number 25): NLGN2 is detected in both eyes lpRGC (Cluster number 26): NLGN2 is detected in both eyes lpRGC (Cluster number 37): NLGN2 is detected in both eyes Alpha RGC (Cluster number 21): NLGN2 is detected in both eyes Alpha RGC (Cluster number 33): NLGN2 is detected in both eyes
Single cell expression analysis for the SC (Single nuclei dataset, MD705): Astrocyte: NLGN2 is detected in 7.52% of astrocyte population with the mean = 0.0772. Excitatory neuron NLGN2 is detected in 32.54% of excitatory neuron population with the mean = 0.4206. Inhibitory neuron: NLGN2 is detected in 23.85% of inhibitory neuron population with the mean = 0.2844.
Embryonic expression: No data is available
Postnatal expression: No data is available
Interactions (String): Nrnx1, Nrnx2, Nrnx3

According to the results in the table 12, similar to the NLGN1 gene, the NLGN2 gene has variant roles in the synaptic regulation such as chemical synaptic transmission, excitatory/ inhibitory synapse assembly, an integral component of postsynaptic membrane, an integral component of postsynaptic specialization membrane, neurexin family protein binding, neuron cell-cell adhesion (GO). Less neurons in the SC express the NLGN2 in comparison to NLGN1 and three identified RGCs express this gene at the P5 too. Thus, the NLGN2 has some chances to be involved in the development of the retino-collicular connection.

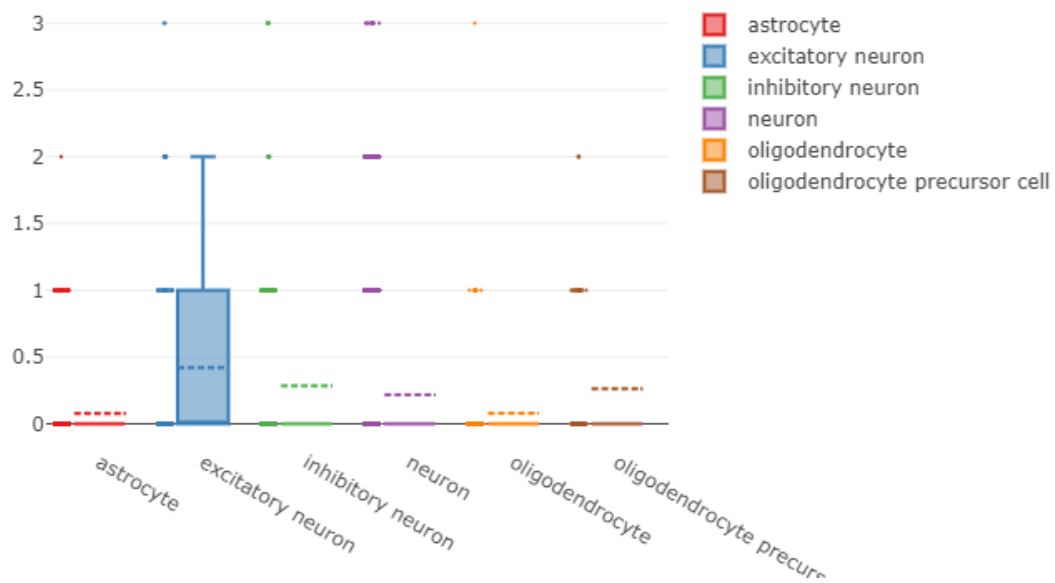
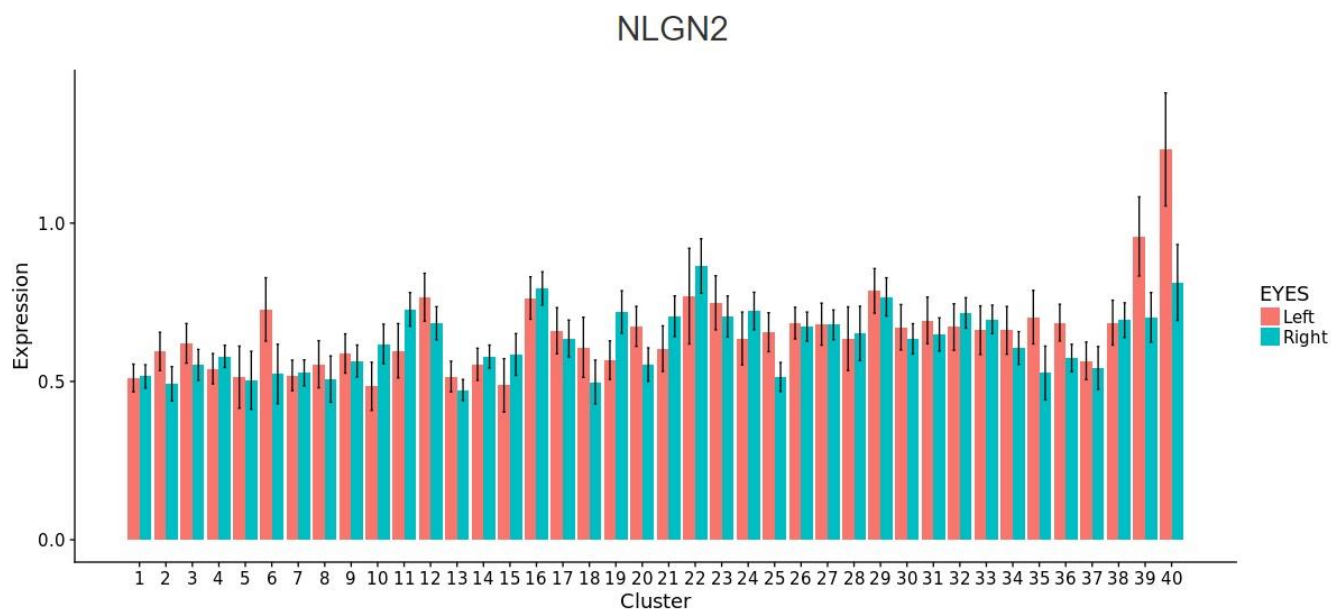


Figure 30: A) Mean of left and right eyes NLGN2 gene expression in RGCs subtypes and SEM error bars. This figure is taken from (Rheaume et al.,2018). B) Distribution of NLGN2 gene expression in the SC. This figure is taken from (MD 705).

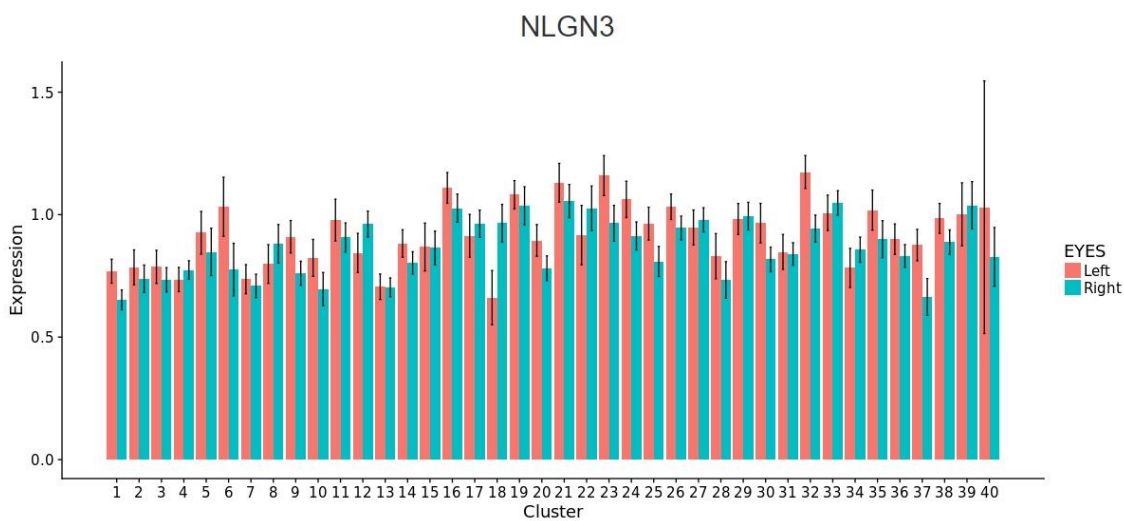
NLGN3:

Table 14: NLGN3 data analysis results

Gene ontology (MGI):
Cell adhesion
Cell adhesion molecule binding
Chemical synaptic transmission
excitatory postsynaptic potential
excitatory synapse
GABA-ergic synapse
glutamatergic synapse
inhibitory postsynaptic potential
inhibitory synapse
integral component of postsynaptic membrane
integral component of postsynaptic specialization membrane
long-term synaptic potentiation
modulation of chemical synaptic transmission
negative regulation of excitatory postsynaptic potential
neurexin family protein binding
neuron cell-cell adhesion
positive regulation of excitatory postsynaptic potential
positive regulation of inhibitory postsynaptic potential
positive regulation of protein localization to synapse
positive regulation of synapse assembly
positive regulation of synaptic transmission, glutamatergic
positive regulation of synaptic vesicle clustering
postsynaptic membrane assembly
postsynaptic specialization assembly

presynaptic membrane assembly regulation of long-term synaptic potentiation regulation of synaptic transmission, glutamatergic synapse assembly synapse organization synaptic vesicle endocytosis
Single cell expression analysis for RGCs at P5 (Rheume <i>et al.</i>, 2018): ON-OFF DSGC (Cluster number 22): NLGN3 is detected in both eyes ON-OFF DSGC (Cluster number 23): NLGN3 is detected in both eyes lpRGC (Cluster number 6): NLGN3 is detected in both eyes lpRGC (Cluster number 25): NLGN3 is detected in both eyes lpRGC (Cluster number 26): NLGN3 is detected in both eyes lpRGC (Cluster number 37): NLGN3 is detected in both eyes Alpha RGC (Cluster number 21): NLGN3 is detected in both eyes Alpha RGC (Cluster number 33): NLGN3 is detected in both eyes
Single cell expression analysis for the SC (Single nuclei dataset, MD705): Astrocyte: NLGN3 is detected in 5.69% of astrocyte population with the mean = 0.0569. Excitatory neuron NLGN3 is detected in 23.02% of excitatory neuron population with the mean = 0.2777. Inhibitory neuron: NLGN3 is detected in 13.79% of inhibitory neuron population with the mean = 0.1494.
Embryonic expression (MGI): Midbrain E11.5: NLGN3 is detected Midbrain E15.5: NLGN3 is detected Retina E15.5: NLGN3 is detected
Postnatal expression (MGI): Midbrain P7: NLGN3 is detected Retina P7: NLGN3 is detected Retina P21: NLGN3 is detected
Interactions (String): Nr1x1, Nr1x2, Nr1x3

According to the table 13, the NLGN3 gene interacts with presynaptic molecules, Nrnx1/2/3 to regulate excitatory/inhibitory postsynaptic potential, chemical transmission in synapses, and synaptic vesicles organization (GO). The NLGN3 is expressed in both RGCs and neurons in the SC. Moreover, the NLGN3 is expressed at E 11.5, 15.5, and P 7 in the midbrain and at E 15.5, P 7, and P 21 in the retina during the development of the retino-collicular connection. All in all, this NLGN isoform is a potential candidate for the mentioned purpose.



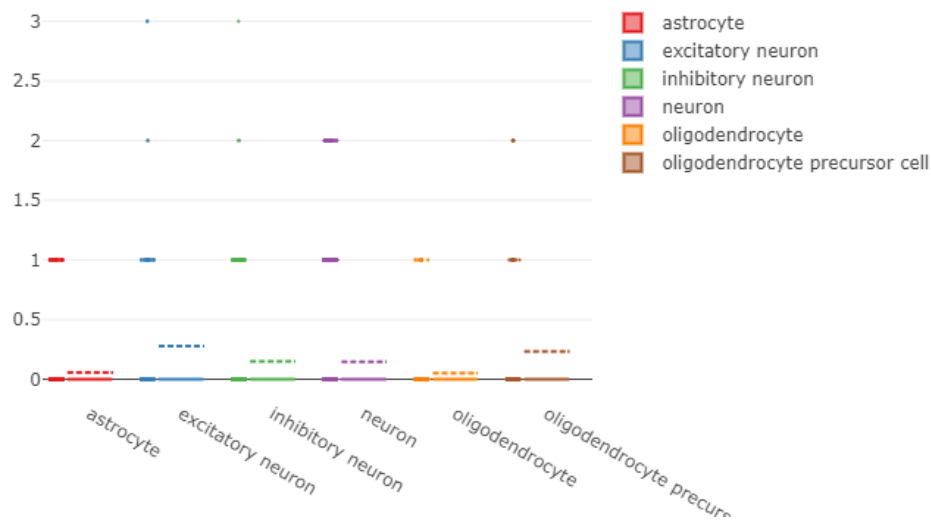


Figure 31: A) Mean of left and right eyes NLGN3 gene expression in RGCs subtypes and SEM error bars. This figure is taken from (Rheaume et al.,2018). B) Distribution of NLGN3 gene expression in the SC. This figure is taken from (MD 705).

Overall, the NLGN family has considerable potential to participate in the development of the connection between the retina and the SC because of its function in the synaptic maintenance and organization of the excitatory and inhibitory synapses. Our priority for this family is NLGN1, NLGN2, and NLGN3 in this research due to their amount of expression in the population of the SC's neurons and RGCs.

Discussion

Slits/Robos

These ligands and receptors act together to regulate some neural connections. **Neuron migration:** Slit/Robo has a role in astrocytic tubes organization during neural migration in the establishment of neural connections (Kaneko *et al.*, 2014). According to Kaneko *et al.*, Slit1^{-/-} mice has a slower rate of neural migration from the rostral migratory stream to the olfactory bulb *in vitro*. In addition, Slit1 interaction with Robo2 is involved in astrocytic organization due to their repulsive effects (Kaneko *et al.*, 2014). Also, Slits are necessary for guiding the midline migration of oculomotor neurons. Homozygous double mutant of Slit1/Slit2 or Robo1/Robo2 causes premature neural migration in oculomotor connection (Bjorke *et al.*, 2016).

Axon guidance: Slit1/Robo2 complex is critical for axon targeting of vomeronasal sensory neuron axons to the posterior accessory olfactory bulb (Sahay *et al.*, 2004). Regarding Skutella and Nitsch, Slit2 mutation leads to abnormal outgrowth of dentate fiber toward the entorhinal cortex. Slit2 has a repulsive effect on dentate gyrus in axon extension via interacting with Robo receptors. Slit1/2 are responsible for the laminar organization of dentate molecular layers including, layer-specific target, innervation and synaptic formation. Overall, Slit2/Robo signaling has a role in hippocampal pathfinding (Skutella and Nitsch, 2001). Slit2/Robo1 participates in axon guidance in habenula-interpendicular connection by preventing excessive habenular axons extension to the thalamic floor plate especially in the midline (Moreno-Bravo *et al.*, 2016). Thus, both Slit1/Slit2 mutation leads to abnormal cross over of left retroflex tract over the midline of the thalamic floor plate in this connection (Moreno-Bravo *et al.*, 2016). In addition, Slit1/2 have a role in preventing abnormal axonal extension in the optic nerve and optic chiasm. Slit1/2 mutations cause ectopic RGCs axon growth from the retina into the optic nerve (Thompson *et al.*, 2006).

Candidate's Validation

Slits (Slit1/2) and their receptors Robos (Robo1/2) are potential candidates for involvement in the retino-collicular connection development due to reasons will be mentioned: The role of Slit1/2 genes in axon guidance especially RGCs axon guidance is a considerable reason. Because the SC is one of the retinoptic targets, the regulation of axonal growth from RGCs toward optic nerve, optic chiasm, and SC via Slit/Robo signaling is important (Thompson *et al.*, 2006). All of these genes with a few exception, in Robo1, are expressed in ON-OFF, Alpha, and Ip RGCs at the P5 in the retina (Table 1/4, Result). Also, these genes are expressed in the excitatory and inhibitory neurons of the SC (Table 1/4, Result). The mutual expression in both retina and SC shows it is possible that Slit/Robo mechanism has a role in retino-collicular connection because proper molecular gradients can establish between retina and the target. Another reason belongs to the anatomical connection of all identified RGCs that express all of these genes (Single cell, Result). All types of identified RGCs (ON-OFF DSGCs, AlphaRGCs, and ipRGCs) have a direct connection with the SC topographically (Plachez *et al.*, 2008; Rheume *et al.*, 2018). Finally, all of these genes are expressed during developmental stages of the target connection (E12-P10). Because gene expression patterns are changed during different developmental phases, it is important that our candidates express between the certain time to impact on the establishment of retino-collicular connection (Rheume *et al.*, 2018).

Neuropilins

Axon guidance: neuropilins interact with class III semaphorins to control axon guidance in several parts of the nervous system development. For example, Nrp2/Sema3f complex has a role in targeting apical vomeronasal axons to the anterior part of the accessory olfactory bulb. Also, the complex participates in fasciculation of the vomeronasal nerve and separation of main and accessory olfactory projections during development (Sahay *et al.*, 2004). Nrp1 and Nrp2 have a function in hippocampus pathfinding too. For instance, Nrp1 has a repulsive impact on axonal pathfinding in the dentate gyrus, entorhinal cortex and cornu ammonis. Nrp2 repulsive guidance cue belongs to the dentate gyrus and cornu ammonis (Skutella and Nitsch, 2001). Another

function of Nrp1 is observed in axonal arborization between basket cells and Purkinje cells in the cerebellum (Telley *et al.*, 2016). Nrp1 mutation leads to aberrant basket cells axon organization in mice. With this mutation, Sema3a cannot act properly in axon guidance and the remarkable reduction of basket cells axon in Purkinje cells is observed (Telley *et al.*, 2016).

Neural migration and synaptic formation: Nrp1 can interact with neurofascin 186 in Purkinje cells and control proper target innervation and synaptic organization in the connection between basket cells and Purkinje cells (Telley *et al.*, 2016). Both Nrps play a role in control neural crest migration. Nrp/null mutant mice show ectopic neural crest migration to hyoid and trigeminal neural crest during development at the dorsal neural tube. Consequently, error in neural crest migration causes ectopic neuron positing in the head. Nrp1 mutations affect this process more than Nrp2 (Schwarz *et al.*, 2009).

Candidate's Validation

Although the role of Nrps is less studied in the nervous system than angiogenesis and vascularization, these genes can be potential candidates for the establishment of the connection between the retina and the SC. Both Nrp1 and Nrp2 interact with semaphorins and plexins that have a role in axon guidance (Table 5/6, Result). This is considerable result as the Class 3 Semaphorins have been suggested to be involved in axon guidance in the retino-collicular pathways (Sharma *et al.*, 2014). At the P5 in RGCs, Nrps have high expression, especially Nrp1. They are also expressed moderately in the excitatory and inhibitory neurons of the SC (Table 5/6, Result). Nrps have low expression during embryonic and postnatal stages (E11.5-P21) but previous studies have shown that lack of Nrps lead to death or severe abnormalities in mice (Pellet-Many *et al.*, 2008). There are acceptable evidence such as the role of Sema3F (one of the ligands of Nrps (Niland and Eble, 2019)) in proper targeting of vomeronasal axons to the anterior part of the accessory olfactory bulb (Sahay *et al.*, 2004) or the importance of Nrp1 in connectivity between the basket cells and the Purkinje in the cerebellum (Telley *et al.*, 2016). All in all, it seems that Nrps can affect RGC axonal growth by the repulsive effect of semaphorins (Sharma *et al.*, 2014) in the retina and

impact on proper targeting from RGCs such ON-OFF DSGC, AlphaRGCs, and ipRGCs to the SC (Table5/6, Result).

Cadherins

Members of cadherin are hypothesized that have a role in central nervous system connectivity by interacting with the same cadherins or make a combination with other sub-families (Osterhout *et al.*, 2012). Because most of the cadherin members work in a combination with each other, the mutation in one member can be compensated by the expression of other cadherins (Dewitz, Duan and Zampieri, 2019). Here are some evidence for cadhre6/7/8 in establishment the neural connectivity and circuits:

Axon targeting: Cdh6 promotes axon-target recognition in non-image-forming visual circuits. Cdh6 works with Cdh3 for this goal. Cdh6^{-/-} mutants causes the aberrant dispersion of Cdh3-RGCs along the margin of the optic tract (Osterhout *et al.*, 2012). (Inoue *et al.*, 1998) Not only Cdh6 defection leads to error in the targeting of RGCs into the non-image retinorecpients, but also it can cause failure in proper termination of axons. For example, RGCs axons' Cdh6 mutant mice fail to terminate properly in their targets (Osterhout *et al.*, 2012). Cdh8 is required for normal initial targeting of bipolar cells in the connection with retinal ganglion cells (Duan *et al.*, 2014).

As mentioned before, cadherins are important in pre and postsynaptic organization. For instance, Cdh6 promotes neurite branching and presynaptic differentiation by enabling Neuroligin-dependent mechanisms in the synaptic organization (Yamagata, Duan and Sanes, 2018). Another example relates to the role of Cdh7 in the inhibition of axonal growth in the pontine nucleus after the arrival of axons at the cerebellum during E17-P4. This leads to upregulation of the synaptogenesis in the connection between the pontine nucleus and cerebellum (Kuwako *et al.*, 2014). Loss of Cdh7 leads to impairment of the synaptic connectivity between the pontine nucleus and the cerebellum (Kuwako *et al.*, 2014). In addition, Cdh7 mediates synaptogenesis between pontine neurons and granule neurons. Cdh7 knockdown mouse shows a significant decrease in the number

of synapses between these two regions (Kuwako *et al.*, 2014). In fact, Cdh7 affect synaptic development through cytoskeletal remodelling between pre and postsynaptic structures (Kuwako *et al.*, 2014).

According to Duan *et al.*, Cdh8 is required for synaptogenesis between bipolar cells and ooDSGCs during postnatal development. The functional connectivity between these two neurons decreases from 53% to 11% in Cdh8 mutant mice (Duan *et al.*, 2014). Besides, Cdh8 has a role in synapse adhesion in the dorsal stratum and participates in the targeting of excitatory synapses on MSNs (Friedman *et al.*, 2016). Cdh8 function is also crucial for stabilizing developing synaptic contacts in stratum lucidum, pyramidal cell layer, and stratum oriens, and so on. For instance, the number of presynaptic terminals in CA3 is reduced in Cdh8 mutation (Bekirov *et al.*, 2009).

Candidate's Validation

It seems Cdh6 is a potential candidate for establishing the connection between ON-OFF DSGCs and the SC because it has the most expression in these RGCs than others at P5 (Table7, Result). In addition, the role of Cdh6 in axon-target recognition in non-image-forming visual centers might be a strong evidence for the purpose (Osterhout *et al.*, 2012). The expression of this gene happens between E12-P10 too (Table7, Result). Cdh7 is another candidate that might have a role in synapse formation in the target connection due to cell-cell adhesion and cadherin binding gene ontology (Table8, Result). This gene has a considerable expression in either embryonic or postnatal period in both retina and the SC (Result). While Cdh7 expression is low during E11.5 to E15.5 and P14 to P56, the expression during E18.5-P4 is significantly high and it can affect the connection due to the maturation of the connection that happens at this time (Table8, Result). Cdh8 has the highest expression in the midbrain during E11.5 to P14, so it has more possibility than other Cdhs to have a role in the retino-collicular connection. Another strong reason is the role of Cdh8 in retinal connectivity, between bipolar cells and ON-OFF DSGCs (Duan *et al.*, 2014). Also, Cdh8 has a variant functions in synaptic regulation in other nervous system connections than other cadherin (6/7) members (Table 9, Result).

Neurofascin

Synaptic formation: One function of Nfasc in the synaptic organization is the role of Nfasc gradient in the initial contact between basket cells' axons and Purkinje cells' soma or dendrite through axon initial segment in the cerebellum. Nfasc cooperates with ankyrinG in this process to regulate synaptogenesis in this connection (Ango *et al.*, 2004). A negative dominant mutation in Nfasc disrupts synapse formation and leads to the death of the mice at P6 (Ango *et al.*, 2004). Retinal connectivity is a novel function of Nfasc (NF186) in the nervous system. Nfasc participates in stabilizing synapses between rod photoreceptors and rod-bipolar cells at the outer plexiform layer of the retina (Pourhoseini *et al.*, 2021). Loss of Nfasc leads to destabilization of synapses between rod photoreceptors and rod-bipolar cells due to a reduction in the number of presynaptic (Basson) and postsynaptic (Glutamate receptor 6) proteins in synapse spaces. On the other hand the loss of Nfasc does not affect synaptic formation (Pourhoseini *et al.*, 2021). The cooperation of Nrp1 with NF186 leads to controlling the recognition and axon contact at synapses between basket cells and Purkinje cells. NF186 can interact with Sema3a for basket cells' target recognition and innervation too (Telley *et al.*, 2016).

Candidate's Validation

The Neurofascin gene is a potential candidate for synaptic regulation in the retino-collicular connection (Table10, Result). First and foremost, Nfasc has an interaction with Anks. Regarding previous studies, Anks are proved to have a role in cell-cell adhesion in the retino-collicular pathway (Buhusi *et al.*, 2009). L1CAM binds to AnkB to regulate this connection. According to the result, Nfasc also interacts with AnkB. The signaling pathways of Nfasc-AnkG at the axon initial segment where that involves in synaptic spaces is proved too (Buhusi *et al.*, 2009). In addition, the rate of Nfasc's expression between E12-P10, and its expression in the ON-OFF DSGCs, alphaRGCs, and ipRGCs is considerable to hypothesize that Nfasc can be a potential candidate. In fact, this gene has a synaptic regulation role in a different parts of the nervous system like the retina and the cerebellum (Table10, Result).

Neuroligins

NLGNs (NLGN1/2/3) have a role in the regulation of synaptic transmission and function. The knockout NLGN1/2/3 mouse shows that the lack of these genes causes abnormal synaptic proteins or vesicles recruitment at postsynaptic neurons (Rawson *et al.*, 2006). Triplet mutation of these genes leads to death at the birth time in the mouse because of disruption neural circuits due to the synaptic transmissions failure (Südhof, 2008). According to Hoon *et al.*, NLGN2 has a role in the maintenance of GABAergic (inhibitory) synapses in the retina. NLGN2 is observed in the GABAergic synapses at IPL or OPL in the retina (Hoon *et al.*, 2009). Although the knockout NLGN2 mouse shows the reduction of GABAergic synapses in the retina, the loss of this gene does not disrupt the whole organization of the retina. NLGN2 deficiency alters the light processing in the retina (Hoon *et al.*, 2009). For example, ON-type ganglion cells have a more increment in their neural firing in the NLGN2 deficient mouse because of GABAergic synapses disruption (Hoon *et al.*, 2009).

In addition, the synaptic transmission is impaired by NLGN2 knockout mice in inhibitory synapses at the basal amygdala (Babaev *et al.*, 2016). NLGN3 is required for synaptic transmission in CA1's hippocampus. The knockdown of this gene causes the decrease of the VGT3+ GABAergic synaptic transmission (Uchigashima *et al.*, 2020).

All in all, the loss of NLGNS do not disrupt synaptic formation or initial contact of neurons but the balance of inhibitory and excitatory neuron is disrupted (Babaev *et al.*, 2016). The NLGNS mutations alter the chemical composition of synapses and the function of neurotransmission. Thus, it can cause modification in the behavior of individuals or leads to some neurological diseases such as autism or other mental retardations (Südhof, 2008; Babaev *et al.*, 2016).

Candidate's Validation

As discussed earlier, Cdhs are important in synaptic formation and neuron contacts (Maître and Heisenberg, 2013). NLGNS can be important in synaptic regulation and making the balance in the connection between retina and SC. NLGNS can act

complementary with Cdh5 in the retino-collicular connection due to Cdh signaling pathways can induce neurexin signaling transduction (Brigidi and Bamji,2011). Because NLGNs are postsynaptic proteins it is important that they express at superior colliculus neurons. According to the result, NLGNs express in the SC's neurons. NLGN1 has a strong expression at the excitatory and inhibitory neuron occupying 96.83% and 84.20%, respectively (Table11/12/13, Result). The NLGN1 has more probability to be involved in the target connection due to gene ontology results and high expression in SC's neurons than other genes. Moreover, neurexin is expressed in presynaptic RGCs in the retino-collicular connection. ON-OFF DSGCs, alpha RGCs, and ipRGCs express neurexin at P5 (Rheume *et al.*,2018).This pattern of expression shows it is possible that neurexin 1/2/3 and NLGN 1/2/3 have a role in the establishment of the connection between the retina and SC.

Comparison with Positive Controls

As mentioned in the methods and materials, some genes are proved experimentally that have a role in the establishment of retino-collicular connection. These genes are chosen as positive controls in this research. Here is a comparison of the potential candidates and these genes to discuss more the potential of our candidates. The expression, interaction, and similarity/complementarity of these two groups (candidates, controls) are evaluated.

Table 15: Comparison gene expression between positive controls and candidates

Genes	Anatomical expression at RGCs and SC (single cell data, (Rheume <i>et al.</i> , 2018), MD 705).	Time of expression during E11.5-P14 (Allen brain and MGI)
EphB2 (Control)	Expressed	Expressed
Sema3a (Control)	Expressed	Expressed
L1CAM (Control)	Expressed	Expressed
NrCAM (Control)	Expressed	Expressed

Slit/Robo (1/2/3, Candidates)	Expressed	Expressed
Neuropilin (1/2, Candidates)	Expressed	Expressed
Cadherin (6/7/8, Candidates)	Expressed	Expressed
Neurofascin (Candidates)	Expressed	Expressed
Neurologin (1/2/3, Candidates)	Expressed	No data is available

The table 14 provides information about the gene expression of controls and candidates. According to the table, all of the mentioned genes with one exception in NLGNs are expressed during the development of retino-collicular connection. We hypothesized that the time of our candidates' gene expression is crucial due to the pattern of gene expression in each developmental stage is different (Rheume *et al.*, 2018). However, our candidates should be expressed during E12-P14 approximately because the development and maturation of the retina and the SC and retino-collicular connection occurs during this time (Guillery, 1992; Demyanenko and Maness, 2002; Graw, 2010; Cang *et al.*, 2018). Nevertheless, our candidates' genes have the possibilities to be involved in the development of the retina and the SC connection.

Table 16: Interactions of controls and candidates genes

Genes	Interactions with controls/candidates/related (String)
EphB2 (Control)	No interaction with candidates
Sema3a (Control)	Nrp1, Nrp2, Plxna1, Plxna2, Plxna3, Plxna4
L1CAM (Control)	No interaction with candidates
NrCAM (Control)	No interaction with candidates

Slit/Robo (1/2/3, Candidates)	No interaction with controls
Neuropilin (1/2, Candidates)	Sema3a, Plxna1, Plxna2, Plxna3, Plxna4
Cadherin (6/7/8, Candidates)	No interaction with controls
Neurofascin (Candidates)	No interaction with controls
Neurologin (1/2/3, Candidates)	No interaction with controls

The table 15 illustrates the comparison between controls and candidates in gene interaction. For instance, the Sema3a (control) has an interaction with Nrp1 and Nrp2 (candidates) experimentally. Although using String or other databases provides some beneficial information, it is not efficient completely for conclusion about the candidates. For example, other candidates and controls do not have interaction with each other basis on the mentioned database. In this case, only the interaction of Nrps and Sema3a is considerable. Thus, because the Nrps interact with one of our controls, they can be potential candidates in addition to other results.

Here are some signaling pathways of control genes and their relation with the candidates. EphBs family impact on the axon guidance, neural migration, and neural connectivity in the retino-collicular pathway by bidirectional signaling transduction (Wan *et al.*, 2017).). At forward signaling, the EphBs receptor activates RasGAP, ERK/MAPK, or PI3, AKT for proliferation. The attachment of Ephexin to the EphBs receptors induces Rho, Rac, and Cdc42 for neurite growth (Wan *et al.*, 2017). Src-EphB complex induces synaptic plasticity. Besides, the interaction of EphBs with FAK, JAK2, and SHP2 leads to cell migration (Wan *et al.*, 2017). At reverse signaling, EphBs bind to the PTP-BL and PDZ-RGS3 for cell migration and proliferation, respectively (Wan *et al.*, 2017).

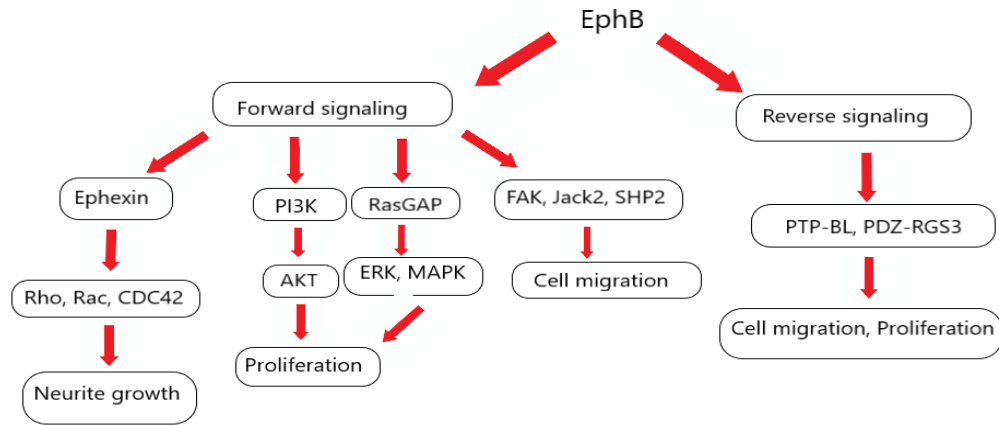


Figure 32: The EphB cellular pathways.

As discussed in the discussion for slit/robo and neuropilins, slit/robo and neuropilins have a role in axon guidance in the nervous system. Their signaling pathways have some similarities with proved axon guidance cues, EphBs. For instance, slit/robo induces axon and neurite growth by Rac, Rho, and CDC42 like EphBs (Hohenester, 2008; Blockus and Chédota, 2016; Wan *et al.*, 2017). The Neuropilin and EphBs signaling pathways are similar in signal transduction of MAPK and Rac Signaling (Szabó *et al.*, 2012; Wan *et al.*, 2017).

L1CAM is another control gene that mediates cell-cell adhesion in the retino-collicular pathway (Buhusi *et al.*, 2008). L1CAM can interact with Ankyrin to induce axon growth, cell-cell adhesion, and axonal fasciculation. L1CAM-Ankyrin activates spectrin on the actin (Schäfer and Frotscher, 2012). Also, L1CAM binds to the Nrp1-Sema3a to activate p-ezrin on the actin for alternation of cytoskeletal organization of growth cone collapse and axonal repulsion (Schäfer and Frotscher, 2012).

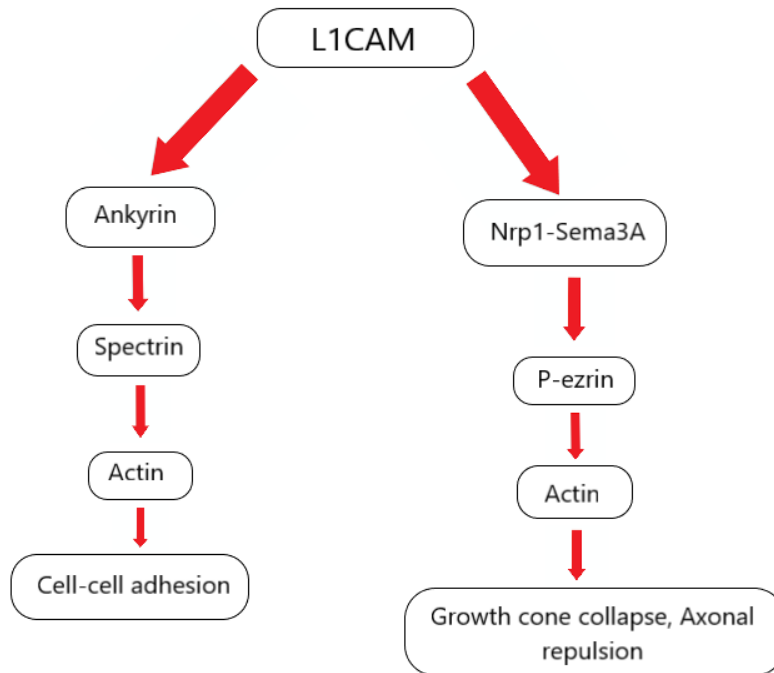


Figure 33: The L1CAM cellular pathways.

Neurofascin is another CAM molecule and its signaling mechanisms is similar to L1CAM (Liu, Focia and He, 2011). Both of them interact with Ankyrin and spectrin to alter the actin cytoskeletal (Schäfer and Frotscher, 2012; Huang and Rasband, 2018). Of course, they have some differences in their role in neural connection such as the role in the synaptic formation in the Nfasc (Ango *et al.*, 2004).

Limitations

In this research, genes are covered that has a high possibility in bioinformatics to involve in the connection between the retina and superior colliculus. Because these genes are not tested experimentally, there is no certainty to conclude that all of candidates' genes are required for the development of retino-collicular connection. In addition, bioinformatics tools and databases are not cover all information about our candidates. For example, the gene expression of all genes is not investigated at the

single-cell level during the whole E11.5-P14. The lack of these data causes inaccuracy in the conclusion because the expression pattern changes during developmental stages. The topographic map for RGCs to the SC are not investigated during the development. Most studies show the map at P56 when the mouse is matured. For this research, the topographic map during E12-P10 is required and one of our research illustrates three types of RGCs at P5 (Rheaume *et al.*, 2018). These are some limitation for this research that mentioned above.

Conclusion

Regarding the result and discussion section, all of the mentioned genes (Slit1/2, Robo1/2, Nrp1/2, Cdh6/7/8, Nfasc, and NLGN1/2/3) can be involved in the development of the retina and superior colliculus connection, but in different contribution. Generally, all of them are expressed in those types of RGCs (ON-OFF DSGCs, ipRGCs, alphaRGCs) that have a direct connection with the superior colliculus at P5. Their gene ontology results and the time of expression are also remarkable for their contribution in this developmental process.

To conclude, our analysis has shown that the neuropilin1 and 2 have more probability to have a role in retino-collicular connection than the other guidance cues (slits/robos), due to their interaction with Sema3a (our positive control, as its role has been shown in this developmental process). Neurofascin is the best candidate among the synaptic formation genes, because it has significant expression during E12-P14 at both structures and has an interaction with NrCAM positive control (Fujitani, Otani and Miyajima, 2021). Cadherins and Neuroligins have an equal chance to be involved in this connection. Their gene ontology and evidence are strong but their expression patterns at some points are not significant as neurofascin.

Future Work

This research introduces potential candidates' genes that have a role in the development of retino-collicular connection. This work can be used for future experimental examinations to confirm the role of these candidates in this process, and to find which other genes are involved in the retino-collicular map. It is important to illustrate more accurate map for neural connections like retino-collicular to understand the nervous system and neurological diseases better.

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