# Defining the expression pattern of Repulsive Guidance Molecules and Netrin-1 in neurons of the mouse cortex and hippocampus

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**Abstract**

The development of the nervous system involves the formation of precise and intricate neural networks which determine how we think, act, and respond to external cues from the environment. Proper formation of the neural system is a complicated process that requires multiple signaling pathways and molecules to work synchronously. Numerous ligand-receptor interactions are involved in the development and maintenance of cortical circuits. The transmembrane receptor Neogenin has been implicated in a wide variety of cellular processes, including cell differentiation and migration. Recent studies suggest that Neogenin also plays an important role in regulating adult hippocampal neurogenesis. The function of Neogenin in these processes is modulated by binding to a variety of ligands that belong to different families of molecules, including Repulsive Guidance Molecules (RGMs) and Netrins. Neogenin is expressed in cells of the developing cortex and hippocampus and has been proposed to influence the development of circuitry in these regions of the nervous system. To begin to assess how these ligands may regulate Neogenin function in the cortex and hippocampus, my project aims at defining the pattern of expression of three Neogenin ligands in these maturing structures by employing *in situ* hybridization approaches. These studies will pave the way to the design of experimental approaches to address the function of these ligands in synapse function.

**Résumé**

Le développement du système nerveux implique la formation de circuits neuronaux précis et complexes, ce qui détermine nos comportements et nos pensées, et qui nous permet d'interagir avec notre environnement. La formation appropriée du système nerveux est un processus compliqué qui nécessite plusieurs molécules et voies de signalisation pour progresser de façon synchronisée. De nombreuses interactions ligand-récepteur sont impliquées dans le développement et le maintien des circuits corticaux. Le récepteur transmembranaire Neogenin est exprimé dans les cellules du cortex et de l'hippocampe et est proposé d'influencer le développement de circuits dans ces régions du cerveau. La Neogenin a été identifiée initialement pour son rôle dans l'orientation des axones au cours du développement du système nerveux, mais depuis cette découverte, d'autres activités importantes ont été définies pour ce récepteur, dans une grande variété de processus cellulaires, y compris la différenciation et la migration cellulaires. Des études récentes suggèrent également un rôle important pour la Neogenin dans la régulation de la neurogenèse dans l'hippocampe adulte. La fonction de la Neogenin dans ces processus est modulée par sa liaison à une variété de ligands appartenant à différentes familles de molécules, notamment les Repulsive Guidance Molecules (RGMs) et les Netrins. Pour commencer à évaluer comment ces ligands peuvent réguler la fonction de la Neogenin dans le cortex et l'hippocampe, mon projet vise à définir le profil d'expression des ligands de la Neogenin dans ces structures pendant leur maturation, en utilisant des approches d'hybridation *in situ* pour examiner l'expression de l'ARNm des ligands dans les neurones excitateurs et les interneurones inhibiteurs. Ces études faciliteront la conception d'approches expérimentales pour mieux comprendre la fonction de ces ligands dans l' activité synaptique.

## Preface and Contribution of Authors

The work in this thesis is original and unpublished work by the author, Aarushi Chaudhry. All work was supervised by Dr. Jean-François Cloutier, and all resources provided by the Cloutier Lab at McGill University. The abstract was translated to French by Sabrina Quilez.

## List of Abbreviations

| Abbreviation | Definition |
|---|---|
| ASD | Autism Spectrum Disorder |
| BMP | Bone Morphogenetic Protein |
| c | cortex |
| cRGM | chick Repulsive Guidance Molecule |
| CA | *Cornu Ammonis* |
| C-terminal/terminus | Carboxy-terminus/terminal |
| CCK | Cholecystokinin |
| DCC | Deleted in Colorectal Cancer |
| Dig | Digoxygenin |
| DG | Dentate Gyrus |
| E | Embryonic day |
| EGF | Epidermal Growth Factor |
| FISH | Fluorescent *in situ* hybridization |
| Flu | fluorescein |
| FN | Fibronectin |
| GABA | Gamma-aminobutyric acid |
| GPI | Glycophosphatidylinositol |
| h | Hippocampus |
| Ig | Immunoglobulin |
| ISH | *in situ* hybridization |

| | |
|---|---|
| mEPSC | miniature excitatory postsynaptic potential |
| mRNA | messenger RNA |
| MS | Multiple Sclerosis |
| μm | Micrometre |
| N-terminal/terminus | amino-terminus/terminal |
| Neo1 | Neogenin |
| OSN | Olfactory Sensory Neuron |
| P | post-natal day |
| PBS | Phosphate Buffered Saline |
| PD | Parkinson's disease |
| PFA | Paraformaldehyde |
| PV | Parvalbumin |
| RGD | Arginine-Asparagine-Aspartic |
| RGMa | Repulsive Guidance Molecule a |
| RGMb | Repulsive Guidance Molecule b |
| RGMc | Repulsive Guidance Molecule c |
| SCZ | Schizophrenia |
| SUS | Sustentacular |
| VIP | Vasoactive Intestinal Peptide |
| vWD | von Willebrand Type D |
| WT | wild-type |

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## 1.Introduction

Neurodevelopmental disorders have a tremendous impact on an individual's behavioural, cognitive, and/or motor functions. Interpreting the underlying molecular underpinnings of these disorders can help uncover the exact mechanisms which might be involved in their onset and progression. Neurodevelopmental disorders are often associated with gene disruptions and mutations (Gilissen et al.,2014; Niemi et al.,2018). The formation of precise neural networks involves several genes and transcription factors which are essential for prenatal neural patterning as well as postnatal synapse development. There is emerging evidence suggesting that dysfunction of these regulators can lead to synaptic dysfunction. As an example, a disruption of the gene *SHANK3* leads to altered glutamatergic synaptic transmission, which is associated with intellectual disability, abnormal social behaviours, repetitive motor movement and delayed speech which are all characteristics of ASD (Wilson et al.,2003; Bonaglia et al.,2006).

Proper functioning of neural circuits relies on the maintenance of a fine balance between excitation and inhibition of these circuits. Indeed, disruption of this balance in several regions of the brain, including in the cortex and the hippocampus, has been associated with several neurodevelopmental disorders (Rubenstein and Merzenich,2003; Siu et al.,2016). The cerebral cortex and hippocampus play important roles in regulating key brain functions. In both structures, excitatory pyramidal neurons and inhibitory interneurons form complex neuronal circuits that are regulated by their levels of activity. Hence proper formation of these neurons during development and their function in mature circuits is essential to maintain a proper excitation/inhibition balance. Given the

involvement of dysfunction of the cerebral cortex and hippocampus in neurodevelopmental disorders, it is important to understand the molecular mechanisms that affect their development and normal function.

In this introduction, I describe the cellular components of the mouse cortex and hippocampus that are essential for their function. I also review the literature related to the function of the transmembrane receptor Neogenin and of some of its ligands in the nervous system to provide rationale for my examination of the pattern of expression of specific Neogenin ligands in these structures.

### 1.1 Cellular organization in the mouse cortex

The cortex is organized into six layers to facilitate a stereotypical flow of information that is essential for decision making, awareness, perception, thought, memory, and emotional states (Blakemore et al.,1995; Fernández et al.,2016). It is mainly composed of two prominent neuronal subtypes: pyramidal cells and interneurons, which constitute about 70-80% and 20-30% of the neuronal population respectively. Together, these cells integrate sensory, motor, and cognitive information (Molyneaux et al.,2007). Cortical evolution reveals that although there has been a substantial increase in the size of the cerebral cortex, its neuronal composition has remained constant throughout (Raybaud and Widjaja,2011). Both these neuronal subpopulations exhibit outstanding cellular diversity that is responsible for the precise synaptic connectivity of cortical cell types. These various classes of neurons can have different shapes and functions and can be identified based on the expression profiles of different biochemical markers.

### 1.1.1 Projection/Principal Cells

Projection neurons, also referred to as principal neurons, represent the most abundant class of neurons spanning about 80% of all cells in the cerebral cortex (Braitenberg and Schüz,1991). These neurons are glutamatergic excitatory neurons, meaning that they transmit signals to downstream targets via the action of the neurotransmitter glutamate (Braitenberg and Schüz,1991). Glutamate depolarizes the postsynaptic neuron and functions through ionotropic and metabotropic receptors. These projection neurons extend their dendritic shaft up to the most superficial layer of the cortex (layer 1) and ramifies into a prominent dendritic arbour and basal dendrites (Jones et al.,1975; Staiger et al.,2004). These neurons also extend axonal arborisations which helps them connect to the cell bodies as well as target the proximal dendrites of the surrounding projection cells and other inhibitory interneurons (Hu et al.,2014). Due to their distinct morphology, they are also termed pyramidal neurons (Quiquempoix et al.,2018). These extensive axonal arborizations allow the projection neurons to form synapses on the targets (local and distal) within the cortex and subcortical areas. Projection neurons are further classified into two types of cells on the basis of their long-range projections i.e., intracortical and corticofugal neurons (Molyneaux et al.,2007). Intracortical neurons are predominantly present in the layers 2/3 and are further subdivided into associative or callosal neurons if they contact the ipsilateral or contralateral hemispheres (Tjia et al.,2017). The corticofugal neurons, on the other hand, are located in the lower layers, and are subdivided into corticothalamic or subcerebral neurons (Baker et al.,2018) The corticothalamic neurons are primarily responsible for regulating the activity of the thalamus as well as modulating the activity of other neurons in cortical layers 5 and 6, whereas the subcerebral projection neurons project away from the cortex (Molyneaux et al.,2007).

### 1.1.2 Interneurons

Interneurons are not as abundantly present in the cerebral cortex as pyramidal neurons and account for about 20-30% of the total neuronal population (Butt et al.,2017). They form synapses onto local targets hence their name. These neurons release GABA neurotransmitter which hyperpolarizes the postsynaptic neurons and thus shunts the firing of action potentials (Butt et al.,2017). Though most interneurons are inhibitory in nature, some of them produce excitatory, depolarizing postsynaptic currents, which are involved in regulating the homeostatic mechanisms of cortical circuits (Kepecs et al.,2014).

Despite representing a smaller population of cells in the cortex, inhibitory interneurons are quite diverse. The diversity of interneurons can be represented by their specific expression of molecular markers. For example, 40% of the total interneuron population express the calcium binding protein parvalbumin (Mi et al.,2018). These parvalbumin (PV) interneurons modulate the firing rate of projection neurons and can be subdivided into three types based on their morphology (Rudy et al.,2011). The first subtype is the PV+ basket cells which primarily innervate the cell body and proximal dendrites of projection neurons and sometimes of other interneurons too (Kepecs et al.,2014). In layer 4 of the cerebral cortex, these basket cells are usually the interneuron targets of thalamocortical projections (Rudy et al.,2011). The second subtype is the chandelier cell which makes synapses on the axon initial segment of projection neurons. These neurons populate layers 1, 2, 5a and 5b of the cerebral cortex (Jiang et al., 2013; Lee et al., 2015). These chandelier neurons innervate projection neurons and are critical for processing information in cortical circuits (Butt et al.,2017). The third subtype of parvalbumin expressing interneurons are the translaminar

interneurons which are specifically located in layer 6 of the cortex (Rudy et al.,2011). These neurons are recruited by layer 6 corticothalamic projection neurons and are required for regulating sensory responses in all the layers (Rudy et al.,2011).

The second major type of interneuron population is identified by the expression of somatostatin (neuropeptide). SST neurons constitute 30% of the total inhibitory interneuron population within the cortex, and these are further classified into Martinotti and non-Martinotti cells (Rudy et al.,2011). The former are mainly located in layer 5 and modulate disynaptic inhibition. Additionally, they are also enriched in layer 2/3 and co-express calretinin (calcium binding protein) (Yavorska and Wehr,2016). The non Martinotti cells are located in layers 4 and 5, and they send their axonal branches to layer 4 to modulate thalamic sensory information (Sultan et al.,2018). These neurons in layer 4 target the fast-spiking interneurons, and disinhibit layer 4 projection neurons, thus increasing their firing rate in the cerebral cortex (Yavorska and Wehr,2016). These somatostatin expressing neurons also have inhibitory effects on amygdala pyramidal cells, and thus might be essential in fear behaviour (Yavorska and Wehr,2016).

Finally, the third subclass is the serotonin receptor 3A-expressing interneurons which also constitute 30% of the total GABAergic population (Rudy et al.,2011). They are mainly located in the superficial layers i.e., Layers 1, 2 and 3 of the cerebral cortex. The most prominent subclass of these neurons has been reported to be Vasoactive Intestinal Peptide expressing neurons (Rudy et al.,2011). These are mainly present in layers 2/3 and target Somatostatin and Parvalbumin expressing neurons (Kepecs et al.,2014). Because of their inhibitory activity, they reduce the firing rate of projection neurons and are implicated in learning and plasticity (Antonoudiou et al.,2020).

Although most functions of these serotonin receptor 3A-expressing interneurons are still under investigation, it has been reported that they may be involved in the processing of sensory information as well as in learning during postnatal development (Rudy et al.,2011).

### 1.2 Cellular organization in the mouse hippocampus

The hippocampus is a seahorse shaped structure located bilaterally within the temporal lobe of the mammalian brain (Squire and Zola-Morgan,1991). It is known to play an integral role in memory formation, and it is precisely crucial for the processing and storage of object and place information (Scoville et al.,1957; O'Keefe and Nadel,1979; Eichenbaum et al.,2007). The hippocampal formation consists of *Cornu Ammonis* (CA1, CA2, CA3), dentate gyrus, entorhinal cortex, parasubiculum, presubiculum and subiculum (Amaral and Witter,1995). The hippocampus proper is a subset of the hippocampal formation and includes the CA regions and dentate gyrus. Like the cortex, the hippocampus is a highly organized brain region. It represents a heterogeneous population of different neuronal subtypes based on their molecular, morphological, and electrophysiological features (Freund and Buzsaki,1996; McBain and Fisahn,2001; Somogyi and Klausberger,2005).

In the hippocampal circuit, the dentate gyrus is involved in receiving input signals and these signals are then further diverted to the CA3, CA1 and the subiculum regions. All of these regions including the CA3, CA1 and the subiculum are primarily embodied by excitatory pyramidal cells whose properties differ along the three axes of the hippocampus: proximal – distal (transverse) axis, the superficial– deep (radial) axis and the dorsal – ventral (long) axis.

### 1.2.1 Projection/Principal Cells

The CA1 region of the hippocampus is considered to be the simplest region to study the neural circuitry because the cell bodies and dendrites of its neurons are organized in distinct layers and their inputs and outputs are well segregated (Szilágyi et al.,2011). In this region, pyramidal neurons are mainly distributed in the CA1 area referred to as the stratum pyramidale. These pyramidal neurons are arranged in a way that their base faces the superficial surface, and their dendritic processes extend both superficially and deep (Benavides-Piccione et al.,2020). The basal dendrites receive glutamatergic input from the CA3 region of the hippocampus, amygdala, and the local axon collaterals, whereas the apical dendrites receive their glutamatergic input from the entorhinal cortex and thalamus (Amaral and Witter,1995). Dendrites of pyramidal neurons receive their inhibitory inputs from interneurons. The cell body and axon initial segment receive GABAergic input only.

The stratum radiatum is located superficially to the stratum pyramidale, and this layer houses CA3-CA1 Schaffer collaterals (Ding, Haber and Hoesen,2010). Cell bodies of pyramidal cells in the CA1 region are much smaller and dense in the stratum pyramidale layer as compared to CA2 and CA3 regions. Moreover, these neurons have varied dendritic lengths depending on their position i.e., pyramidal neurons in CA3 are longer than those in CA1(Scorza et al.,2011). Deep to the stratum pyramidale is the stratum oriens which consists of about 80% of the dendritic processes of pyramidal neurons, with very few of them in the stratum lacunosum-moleculare layer i.e., the most superficial layer where the perforant pathway terminates (Slomianka et al.,2011). Interestingly, most of the pyramidal neurons have long-range axons and are located predominantly in the CA1,

CA3 and Dentate Gyrus (DG). Moreover, it has been suggested that pyramidal neurons of the hippocampus have different dendritic lengths depending on the layer they are localized (Amaral and Witter,1995). The excitatory principal cells are often referred to as place-cells in the hippocampi where they play an important role in encoding spatially relevant information.

### 1.2.2 Interneurons

Inhibitory neurons make about 10-15% of all neurons in the hippocampus (Pelkey et al.,2017). These GABAergic neurons are known to have organized innervation patterns. These interneurons are not only important for inhibition, but they also modulate the firing properties of pyramidal neurons (Udakis et al.,2020). It has been shown that GABAergic interneurons are responsible for organizing the principal cell activity both spatially and temporally for cognitive processing (Roux et al.,2015).

Inhibitory interneurons in the hippocampus are broadly divided into VIP, SST, PV and CCK cells categorized by the expression of specific protein markers (Kawaguchi and Kondo,2002; Xu et al.,2010; Rudy et al.,2011). All these different interneuron subtypes originate from different structures. The SST and PV interneurons originate from the medial ganglionic eminence, whereas the VIP and CCK interneurons originate from the caudal ganglionic eminence (Xu et al.,2004; Fogarty et al.,2007; Miyoshi et al.,2007). The VIP interneurons are involved in hippocampal-dependent cognition and memory formation by impacting synaptic processes such as long-term potentiation and long-term depression (Cunha-Reis et al.,2010; Cunha-Reis et al.,2014; Turi et al.,2019; Luo et al.,2020). The SST interneurons are implicated in diverse processes and functions

including lateral inhibition, synaptic plasticity, and generation of gamma rhythms (Stefanelli et al.,2016; Chiu et al.,2013; Wilson et al.,2012; Veit et al.,2017). The PV interneurons are restricted to the stratum pyramidale in the hippocampus and are primarily involved in regulating the feed-forward inhibition of pyramidal neurons and for generating gamma rhythms which are important for cognitive processes (Klausberger et al.,2003; Cardin et al.,2009). Furthermore, Parvalbumin containing interneurons have been shown to regulate the spike timing of place cells whereas the somatostatin expressing interneurons regulate their firing rate. The axons of CCK interneurons are located in the stratum radiatum and oriens (Vida et al.,1998; Cope et al.,2002; Pawelzik et al.,2002) as well as in the stratum lacunosum-moleculare (Pawelzik et al.,2002; Klausberger et al.,2005) The CCK interneurons play a role in gating the CA3 and entorhinal inputs to CA1 pyramidal neurons (Basu et al.,2013), which in turn is important for long-term associative memory formation (Basu et al.,2016; Kitamura et al.,2014). Studies suggest that the GABAergic interneurons might also be critical in spatial navigation (Hangya et al.,2010).

The diverse hippocampal neuronal subtypes are involved in regulating network rhythms and cognitive processes, such as memory and spatial navigation. In summary, both the pyramidal neurons and the interneurons populating the hippocampus are important in regulating the neuronal network function. Thus, any aberrations in the excitatory or inhibitory neurotransmission might lead to nervous system disorders such as ASD and SCZ among others (Hutsler et al.,2010; Chattopadhyaya et al.,2012; Glausier et al.,2013).

### 1.3 The Multi-Ligand Receptor - Neogenin

During the development of the nervous system, axons extend and innervate their targets to form proper connections in a process referred to as axonal pathfinding. DCC has been previously shown to be an important receptor playing a well-documented role in this process of pathfinding (Shekarabi et al.,2002). Neogenin is a transmembrane protein that can serve as a functional homolog for DCC (Phan et al.,2010). It belongs to the immunoglobulin superfamily (IgSF) and consists of an extracellular region with four immunoglobulin like loops, six fibronectin type III domains (FnIII), followed by a single-pass transmembrane (TM) region, and an intracellular domain that contains three conserved cytoplasmic domains (P subdomains) which contains phosphorylation sites (Vielmetter et al.,1994; Wilson and Key,2007). Although DCC and Neogenin are closely related, they have a very different pattern of expression during development and differ greatly in their functions. At Embryonic Day 8.5, Neogenin can be found to be widely expressed in the ectodermal, mesodermal, and endodermal layers (Gad et al.,1997; Fitzgerald et al.,2006; van den Heuvel et al.,2013). At E11.5, this expression seems to be elevated in the developing cortex of the embryo (Gad et al.,1997; Fitzgerald et al.,2006; van den Heuvel et al., 2013). Throughout development, Neogenin expression could be found to be expressed in the CNS more specifically in the olfactory bulb, cerebral cortex, hippocampus, and cerebellum as well as localized to mesoderm derived tissues including bone and cartilage of the head (van den Heuvel et al.,2013). Owing to its widespread expression in the growing embryo, it is implicated in a range of different roles during neural development including cell adhesion, axonal guidance, neuronal regeneration, apoptosis, cell differentiation, proliferation and cell survival (Kang et al., 2004; Rajagopalan et al.,2004; Matsunaga et al.,2006; Kee et al.,2008). The function of Neogenin in

these different cellular processes is modulated by binding to several families of ligands, including Repulsive Guidance Molecules (RGMs) and Netrin-1 (Rajagopalan et al.,2004; Keino-Masu et al.,1996).

Neogenin and its ligands contribute to the development of the supraoptic tract which links the diencephalon to the telencephalon in the vertebrate forebrain (Wilson and Key,2006). In lower vertebrates, this supraoptic tract is the precursor of the internal capsule in mammals. Knock-down of Neogenin causes axons to follow aberrant trajectories within the supraoptic tract (Fujita et al.,2008). There is evidence indicating that improper neural tube formation is associated with impaired Neogenin expression (Mawdsley et al., 2004; Kee et al.,2008; Kee et al.,2013). Neogenin plays a crucial role in maintaining the integrity of adherens. junction, by recruiting with components of the Wave Regulatory Complex (Lee et al.,2016). Loss of Neogenin has been linked with reduced frequency of mEPSCs in the hippocampal dentate gyrus indicating a potential role in synaptogenesis (Sun et al.,2018) There is increasing evidence suggesting that this transmembrane receptor is also involved in maintaining blood vessel homeostasis (Yao et al.,2020). Interestingly, it plays a role in promoting astrocytic differentiation in the cortex (Huang et al.,2016) and suppressing it in the adult hippocampus (Sun et al.,2018). Further, it has been reported that ablating Neogenin from hippocampal astrocytes increases susceptibility to seizures in mice, and thus, it can be implicated in controlling epileptic response (Sun et al.,2021).

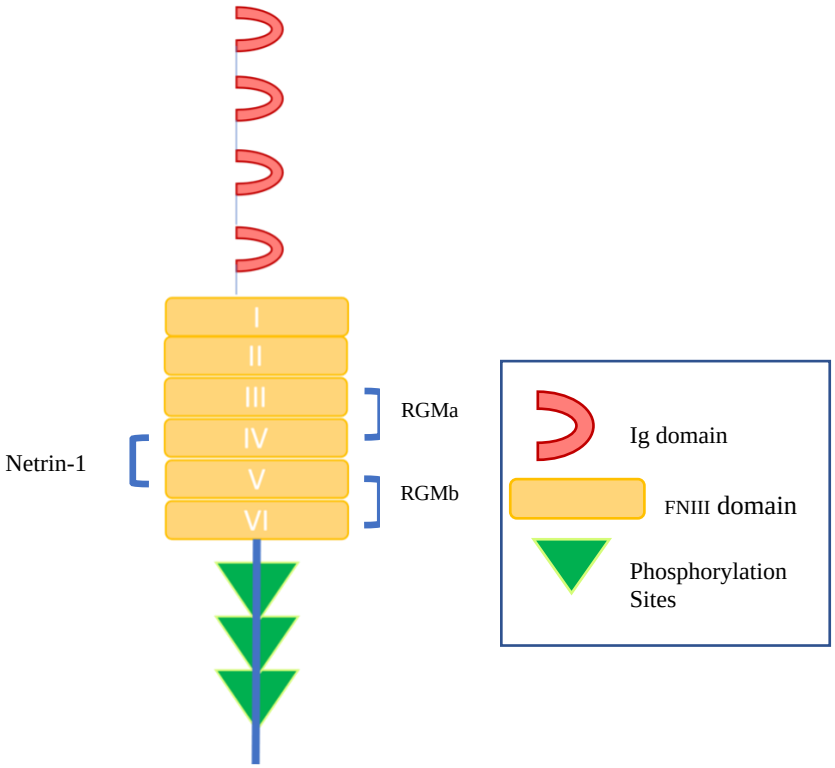



**Figure 1: Structure of Neogenin**

Neogenin is a transmembrane protein consisting of 4 Ig-like domains and 6 FNIII comprising the extracellular region, and three conserved cytoplasmic regions, P1, P2 and P3. Neogenin interacts with Netrin through its FN4 domain and FN5 domains, RGMa through FN3 and FN4, and both RGMb and RGMc through FN5 and FN6.

### 1.4 Repulsive Guidance Molecules – RGMs

The Repulsive Guidance Molecules are a family of GPI-anchored glycoproteins that encode for three identified membrane proteins: RGMa, RGMb, and RGMc (Monnier et al.,2002). This family of molecules was discovered in an effort to isolate genes guiding retinal ganglion cell axons in the chick embryo. c*RGM* is expressed mainly in the developing and adult chick central nervous system

and can act as a repulsive cue for temporal retinal ganglion cell axons projecting into the optic tectum, preventing them from entering the posterior region and promoting their innervation of the anterior region. (Monnier et al.,2002).

Analysis of the chick RGM gene suggests that RGMs do not share any sequence motifs with any other known guidance molecules. Sequence comparison indicated that RGMa is most likely to be the mouse orthologue of chick RGM and that RGMb and RGMc are close homologs (Niederkofler et al.,2004). The structural hallmarks present in all three family members include an N-terminal signal sequence, an RGD motif, a partial von Willebrand type D (vWD) domain, and a C terminal GPI-anchor domain (Severyn et al.,2009). These RGM family members are known to bind to the versatile Neogenin receptor and can modulate BMP signaling by acting as co-receptors for BMP receptors or else by binding directly to BMP ligands (Samad et al.,2005; Babitt et al.,2005; Xia et al.,2005; Halbrookset al.,2007; Xu et al.,2014). *RGMa* and *RGMb*, but not *RGMc*, are expressed in the developing nervous system. *RGMc* expression is observed in striated muscle and in the liver (Papankolaou et al.,2003). Together all the members of the RGM family are known to play critical roles in regulating axonal pathfinding, neural tube closure, neurogenesis, apoptosis, cell differentiation, migration, and iron metabolism (Severyn et al.,2009; Liu et al., 2012; Isaken et al.,2020).


**Figure 2: Structure of RGM**

The Repulsive Guidance Molecule is a GPI anchored glycoprotein that does not share significant homology with any other known protein. It contains an unstable cleavage site; an N-terminal signal peptide; an RGD site; a partial, structurally related, a von Willebrand type D domain; and a hydrophobic domain of unknown function.

### 1.4.1 Repulsive Guidance Molecule a - RGMa

The GPI anchored molecule cRGM is implicated in guiding temporal retinal axons to their correct topographic position within the optic tectum (Monnier et al.,2002). Sequence analysis indicates that RGMa is the mouse orthologue of cRGM, and it is the most widely studied member of the RGM family. Unlike cRGM, the mammalian *RGMa* does not appear to be expressed along the anterior posterior axis of the superior colliculus and is thus not implicated in the targeting of retinal ganglion cell axons (Niederkofler et al.,2004). Soon after RGMa was discovered, there was increasing evidence suggesting that Neogenin mediates RGMa's effects during neural development (Matsunaga et al.,2004; Rajagopalan et al.,2004). *RGMa* functions as a repulsive cue for axons expressing Neogenin. The six FN type III domains, more specifically the FNIII-3 and FNIII-4 sub-regions on the extracellular portion of Neogenin, have been identified to bind with *RGMa* (Rajagopalan et al.,2004). Interactions between RGMa and Neogenin lead to the activation of a range of different molecules, and these signaling pathways have been well elucidated. RGMa-

Neogenin interaction has been reported to be crucial for mediating neural tube closure in mice and *Xenopus* as it regulates the neuroepithelium morphology (Kee et al.,2008). It is also important in the mammalian hippocampus for mediating the laminar patterning of the afferent neuron terminations during development (Brinks et al.,2004). Interestingly, overexpression of RGMa leads to pathfinding defects and it has also been suggested to play a role in neuronal differentiation (Matsunaga et al.,2006). Further, it has been reported to have pro-inflammatory effects in the brain and has been implicated in nervous system disorders such as PD and MS (Fujita et al.,2017; Tanabe et al.,2018). In addition, series of experiments have demonstrated that RGMa expression is increased following brain injury and/or ischemia. As mentioned previously, RGMa is a repulsive guidance cue which is known to inhibit axon growth and prevent nerve regeneration (Hata et al.,2006). Further, application of anti-RGMa antibody has been shown to stimulate growth of neuron fibers as well as enhance synaptogenesis (Hata et al.,2006).

### 1.4.2 Repulsive Guidance Molecule b- RGMb

The *RGMb* gene encodes a Glycosylphosphatidylinositol-anchored protein having an N-terminal peptide sequence as well as a C-terminal hydrophobic region (Samad et al.,2004). *RGMa* and *RGMc* share 50-60% of homology to *RGMb*. Previous studies suggest that *RGMb* is prominently expressed in the dorsal root ganglia of both embryonic and adult mouse, spinal cord, and brain; and this expression can be seen as early as Embryonic Day 7 (Samad et al.,2004 and Liu et al.,2009).

*RGMb* has neuronal adhesive properties which can be implicated in axon guidance and the formation of synaptic connections (Samad et al.,2004). Moreover, it is known that *RGMb* binds to

Bone Morphogenetic Proteins which belong to the superfamily of Transforming Growth Factor – β (Tian et al.,2013). This interaction is crucial for the formation as well as patterning of heart, neural crest cells, limb, muscle, and kidney (Mueller et al.,2006). Subsequent studies suggest that RGMb is involved in the neural system patterning and may as well influence neural differentiation and induce peripheral nerve regeneration (Mueller et al.,2006; Tian et al.,2013).

Further reports suggest that *RGMb* and Neogenin are expressed in the developing cerebral cortex and hippocampus (van den Heuvel et al., 2013). RGMb binds to the FNIII 5 and FNIII 6 domains of Neogenin (Bell et al.,2013). It has been revealed that RGMb interaction with Neogenin seems to play a key role in neuron migration, specifically in the dentate gyrus of the hippocampus (Conrad et al.,2010). Previous work from our lab suggests that Neogenin-RGMb binding is necessary for tuning the balance between OSN and SUS in the developing mouse olfactory epithelium (Kam et al.,2016).

**1.5 Netrin-1**

Netrins are bifunctional secreted proteins belonging to the laminin superfamily that have the capacity to attract some axons and repel others depending on the receptors expressed on neurons (Kang et al.,2004). Six netrin genes have been identified to date, and five of them have been found in mammals – *Netrin-1*, *Netrin-2*, *Netrin-3*, *Netrin-4*, *Netrin-G1* and *Netrin-G2* (Rajasekharan et al.,2009). The Netrin protein is about 600 amino acids in length and is a part of the epidermal growth factor family, and they consist of a C terminal, three EGF domains, and a V globular domain (Ishii et. al.,1992; Sun et al.,2011). They are known to be expressed in both neuronal and

non-neuronal tissues. *Netrin-1* is the most widely studied member of the Netrin family. Initially, DCC and Unc 5 receptors were recognized to mediate responses to netrins (Serafini et al.,1994). While DCC mediates attraction, DCC-Unc5 heterodimeric receptors mediate repulsion in response to Netrin-1 (Hong et al.,1999). The DCC family includes not only DCC but also Neogenin, and they are both members of the Ig superfamily of cell adhesion molecules. *Netrin-1* binds to DCC and Neogenin via their FNIII-4 and FNIII-5 domains (Vielmeter et al.,1994; Xu et al.,2014; Finci et al.,2014). Apart from axon guidance, both DCC and Neogenin can mediate cell adhesion and tissue organization in response to *Netrin-1* (Mann et al.,2004; Meijers et al.,2020). *Netrin-1* is best known for its function in long and short-range chemoattraction and chemorepulsion during axonal pathfinding. It has been reported that Netrin-1 is enriched at cortical synapses *in vivo* during the early postnatal peak of synaptogenesis (Micheva and Beaulieu,1996). Moreover, it has been shown that the majority of *Netrin-1* is associated with excitatory synapses, and overexpressing *Netrin-1* protein in cortical neurons *in vitro* results in its association with synapses (Goldman et al.,2013). Thus, Netrin-1 has been demonstrated to enhance the number and function of excitatory synapses made by cortical pyramidal neurons (Goldman et al.,2013). Furthermore, *Netrin-1* is critical for regulating glutamatergic synaptic plasticity in the adult hippocampus (Glasgow et al.,2018). Although, Neogenin is a Netrin-1 receptor much of its role in response to Netrin-1 binding is yet to be explored. But we do know that Neogenin-Netrin-1 interaction activates focal adhesion as well as extracellular-signal related kinases which are essential for myotube formation (Kang et al.,2004).

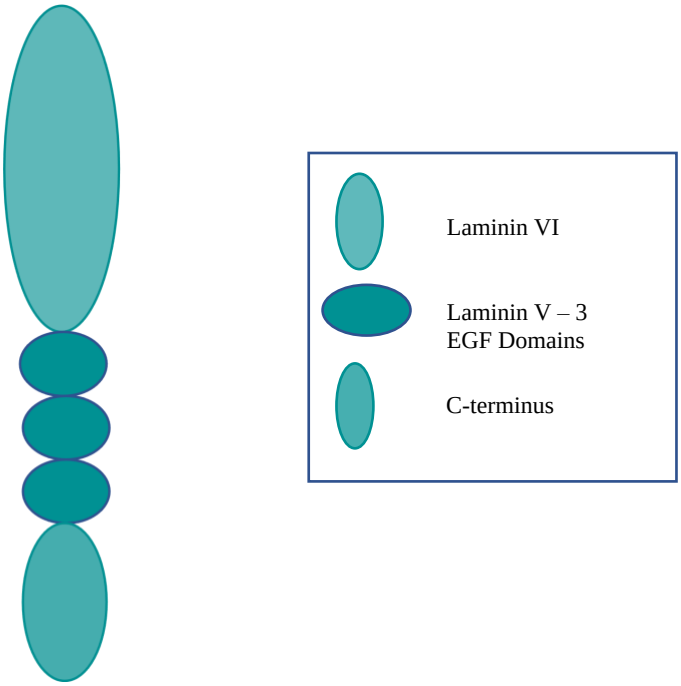



**Figure 3: Structure of Netrin-1**

Netrin1 protein is about 600 amino acids in length. Its N-terminus consists of domain VI and the three EGF repeats of domain V, which are also found in the N-terminus of laminins. Netrin-1's C-terminus does not share any homology with laminins.

## 2. Rationale for the study

During my master's thesis, I've been interested in studying the neural circuit development which involves a diversity of different mechanisms. It is known that there are several families of ligands and receptors that are involved in neural development. All the different molecular players involved in the development of the cortical and hippocampal circuitry, need to be expressed at the right place at the right time. It is important that they interact with the right partners to initiate the right signaling cascades. So, there is a need to understand the expression and various roles of these different molecules.

Neogenin is one of the numerous receptors implicated in modulating the establishment of cell diversity, development of axonal projections, neuronal migration, and synaptic physiology in the nervous system (Monnier et al.,2002; Wilson and Key,2006; O'Leary et al.,2013; Kam et al.,2014; O'Leary et al.,2015; Sun et al.,2018). For example, Neogenin and some of its ligands have been implicated in the development of the cortex and proposed to influence the migration of cortical pyramidal neurons and interneurons through *in vitro* and *in utero* electroporation assays (O'Leary et al.,2013; O'Leary et al.,2015). Unpublished results from our lab have revealed that Neogenin is expressed in the maturing and adult cortex and hippocampus, including in pyramidal and inhibitory interneurons, suggesting it may play a role in modulating neuronal function in the adult cortex. While Neogenin ligands are expressed in the cortex and hippocampus throughout development, the types of cells expressing RGMs and Netrin1 in these structures need to be identified to gather insight into their potential functions in these structures. To begin to gain insight into the potential functions for Neogenin ligands in the maturing cortex and hippocampus, I have examined their spatial patterns of expression in two specific populations of neurons, excitatory

pyramidal and inhibitory neurons, that are present in these structures. My analysis of the pattern of expression of Neogenin ligands in these brain regions will provide key information for the development of loss-of-function approaches to interrogate their function in brain circuitry.

## 3. Materials and Methods

### 3.1 Animals

For *in situ* experiments, the date of birth was designated P0. Experiments were performed on mice at age P25. We used CD1 strain WT mice. All animal procedures have been approved by the Neuro Animal Care Committee, in accordance with the guidelines of the Canadian Council of Animal Care.

### 3.2 Probe design

Dioxygenin or fluorescein-labeled cRNA probes for *RGMa, RGMb, Netrin-1,* and *Gad67* were synthesized by *in vitro* transcription using digoxigenin (DIG) or Fluorescein (Flu) labeling mix (Roche, Mannheim, Germany) according to the manufacturer's recommendations. Probes were synthesized from previously described cDNA clones for *RGMa, RGMb* (Kam et al.,2016), *Netrin-1* (Kind gift of Dr. Timothy E. Kennedy), *Gad67*. *Emx1* probe sequence was PCR amplified using mouse brain cDNA and primer sequence (Forward primer: AAGGGTTCCCACCATATCAACCG and Reverse Primer: ACTAAGAACTACAGCAGGACCTGG) from the Gene Paint Database and cloned into the pBluescript vector. There was almost no overlap of sequences among the probes.

### 3.3 *In situ* Hybridization (ISH) – Digoxygenin

Fresh frozen brains from P25 mice were cryosectioned (20μm), fixed with 4% PFA in 0.1M PBS pH 7.4 and rinsed with 0.1M PBS pH 7.4. Sections were then subjected to a 10 minute acetylation with 0.25% acetic anhydride in 1% triethanolamine followed by washes with PBS and 2x standard saline citrate (SSC). Afterwards sections were prehybridized with hybridization solution (5x

Denhardt's solution, 100mg/mL baker yeast tRNA, 5x SSC and 50% formamide) at 60˚C followed by hybridization of sections with appropriate cRNA probes diluted in hybridization solution at 60˚C overnight. All solutions used up to and including the hybridization step were made using diethylpyrocarbonate (DEPC) treated water. Following hybridization, sections were subjected to a series of stringency washes with 5x SSC, 2x SSC, 50% formamide in 0.2x SSC all at 60˚C and 0.2x SSC at room temperature. Next, sections were washed in a Tris buffered saline solution (100mM Tris and 150mM NaCl, pH 7.5, 43 Tris buffer), blocked for 1 hour at room temperature in a 1% solution of blocking reagent (Roche) in Tris buffer, and hybridized Digoxigenin (DIG) labelled RNA probes were detected with an anti-DIG Fab fragment antibody conjugated to alkaline phosphatase (anti-DIG-AP; 1:3000; Roche) that was diluted in Tris buffer and applied to sections for 3 hours at room temperature. Washes in Tris buffer and a second Tris buffered saline solution (100mM Tris, 100mM NaCl with 5mM MgCl2, pH 9.5) followed immunological detection. Finally, sections were subjected to a colour reaction by incubation with a colour solution made of NBT/BCIP stock solution (Roche) diluted in Tris buffered saline (100mM Tris, 100Mm NaCl and 5mM MgCl2, pH 9.5) overnight at room temperature. The next day sections were rinsed with PBS and mounted with Mowiol (Protocol modified from Giger et al.,1996).

### 3.4 *In situ* Hybridization (ISH) – Double Fluoroescent

Fresh frozen brains from postnatal day (P) 25 mice were cryosectioned (20µm), fixed with 4% PFA in 0.1M PBS pH 7.4 and rinsed with 0.1M PBS pH 7.4. Sections were then subjected to a 10-minute acetylation with 0.25% acetic anhydride in 1% triethanolamine followed by washes with PBS and 2x standard saline citrate (SSC). Afterwards sections were prehybridized with hybridization solution (5x Denhardt's solution, 100mg/mL baker yeast tRNA, 5x SSC and 50%

formamide) at 60˚C followed by hybridization of sections with appropriate cRNA probes diluted in hybridization solution at 60˚C overnight. All solutions used up to and including the hybridization step were made using diethylpyrocarbonate (DEPC) treated water. Following hybridization, sections were subjected to a series of stringency washes with 5x SSC, 2x SSC, 50% formamide in 0.2x SSC all at 60˚C and 0.2x SSC at room temperature. Next, sections were washed in a Tris buffered saline solution (100mM Tris and 150mM NaCl, pH 7.5, 43 Tris buffer), blocked for 1 hour at room temperature in a 1% solution of blocking reagent (Roche) in Tris buffer, and hybridized fluorescent-labelled RNA probes before incubation for 3 hours with anti-DIG fragment antibody conjugated to alkaline phosphatase (anti-DIG-AP; 1:3000; Roche). Next, the slides were placed in 0.2 M HCl for 10 minutes and acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0) for 10 minutes. The sections were again washed in DEPC-PBS for 3x3min. Before prehybridization, the sections are dehydrated in a series of 60%, 80%, 95%, 100% and 100% ethanol. The Sections were prehybridized for 4 h at 37° C in hybridization buffer containing 5x Denhardt's solution, 100mg/mL baker yeast tRNA, 5x SSC and 50% formamide Following prehybridization, they were hybridized by incubating in hybridization buffer containing a combination of either *GAD67* (DIG-labeled) or *Emx1* (Flu-labeled) probes with either *RGMa*/ *RGMb*/*Netrin-1*(Flu or DIG- labelled) at 37°C overnight. Post-hybridization washes were performed sequentially 2x 5 min at 60° C in 5X SSC, 1x1min at 60° C in 2X SSC, 30 min wash 50% formamide/0.2X SSC at 60° C, 1x5min in 0.2X SSC, 1x5min in 1X TN (Tris-HCL + NaCl). 1x15 min in wash buffer containing 0.05% Tween-20 in TBS (0.15 M NaCl in 0.1 M Tris-HCl, pH 7.5), 1x 5 minutes 2x SSC, 1x 30 minutes in 50% formamide/0.2x SSC, and 1x 15 min in wash buffer. They were incubated for 1 hour in blocking buffer (1% blocking reagent from Roche in TN). They were incubated overnight in the dark at 4° C with Anti- Digoxigenin-POD, Fab

fragments (Roche Diagnostics, Mannheim, Germany) and anti-FLU-POD diluted 1:500 and 1:200 respectively in blocking buffer.

After washing 3x 5 minutes in wash buffer (TN+0.05%Tween 20), sections were subsequently incubated for 7-8 min in Biotinyl Tyramide by diluting1:50 in the amplification reagent. They were washed 3x 5 min with wash buffer and incubated for 1hour SA-Alexa 488 in blocking buffer., Next, the sections were incubated for 10min in detection buffer in the dark and on the shaker. Following washing 3x 10 min with wash buffer, they were then mounted onto slides using Fluoromount mounting medium (Southern Biotech, Birmingham, AL) and stored in the dark room at 4° C.

**3.5 Image capture and analysis**

Images were captured using a Carl Zeiss Axio Imager M1 microscope and a QImaging Retiga EXi digital camera. Four different regions of the somatosensory cortex were imaged from each coronal brain section. Both the right and left hippocampi were imaged from each coronal brain section and used for analysis. ImageJ (NIH) software was used to quantify the number of fluorescent positive cells in the regions of interest. Each set of experiment was repeated thrice on three different animals to validate the observed expression pattern.

Colocalization ratio of Cell type/Ligand was calculated by dividing the number of neurons expressing the ligand of interest by the total number of neurons expressing *GAD67* or *Emx1* mRNAs and multiplying by 100. This formula is applied to the calculation of all colocalization ratios in this study.

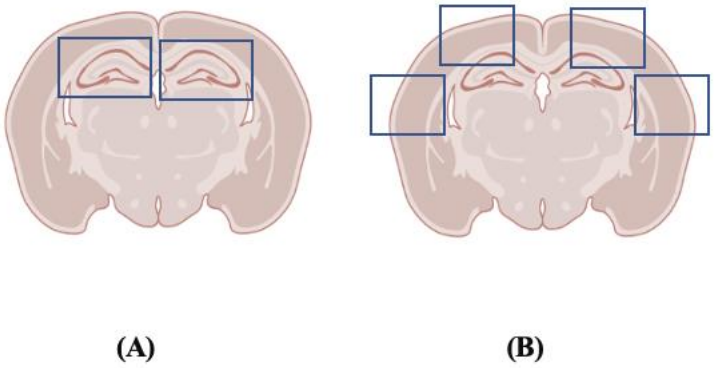



**Figure 4. Schematics illustrating regions of interest per section for analyses**

(A) Both right and left hippocampi were imaged from each coronal brain section and used for analysis. (B) Four different regions of the somatosensory cortex were imaged from each coronal brain section and used for analysis.

## 4. Results

To define the patterns of expression of Neogenin ligands in the somatosensory cortex, I used an *in situ* hybridization approach to detect their mRNA transcripts in sections of brains isolated from P25 mice, a time in development when layers of the cortex and hippocampus are well defined and their circuitry has largely been established (Semple et al.,2013; Chen et al.,2017). Previous studies have shown that these ligands are expressed during development of the cortex and hippocampus but their specific expression in excitatory and inhibitory neurons of these regions has not been characterized, especially at later stages of development (Stanco et. al.,2009; Yamagishi et al.,2011; van den Heuvel et al.,2013; Yamagishi et al.,2021). To identify GABAergic inhibitory neurons and glutamatergic excitatory neurons, I used cRNA probes recognizing *Gad67* (Esclapez et al.,1994; Guo et al.,1997) and *Emx1* (Guo et al.,2000; Chan et al.,2001; Gorski et al.,2002) transcripts, which are expressed in these two respective populations of neurons (Fig 5. A-B). To visualize cells that express *Rgma*, *Rgmb*, and *Netrin-1*, I used cRNA probes that have been extensively characterized by our lab and others in the past (Fig. 5C-E) (Kam et al.,2016; Oldekamp et al.,2004). At P25, all three ligands are expressed throughout the somatosensory cortex and in the different regions of the hippocampus. While *Rgma* and *Netrin-1* are expressed at similar levels in the DG and in the CA1-CA3 region, *Rgmb* showed higher levels of expression in the DG (Fig. 5 C-E). Furthermore, no signal was detected with sense cRNA probes, indicating the specificity of the signals observed with the antisense probes (Fig. 5 C'-E').

To assess whether these ligands are expressed in GABAergic inhibitory neurons and glutamatergic excitatory neurons, I performed double fluorescent *in situ* hybridization experiments with cRNAs

for individual ligands and markers of these two populations of neurons in coronal sections of P25 mouse brains. High magnification views of the cortex revealed that *Rgma*, *Rgmb*, and *Netrin-1* are expressed in all layers of the somatosensory cortex (Fig. 6 A-C). The majority of glutamatergic excitatory neurons in the somatosensory cortex expressing *Emx1* were found to also be positive for *RGMa* (96.1%), RGM*b* (95.2%), and *Netrin1* (94%). Similarly, these three molecules are expressed in most *Emx1*-positive neurons in the hippocampus at this age with 91.4%, 93% and 92.7% of Emx1-positive neurons expressing *RGMa, RGMb*, and *Netrin1*, respectively (Fig. 6 D-F). An examination of the distribution of *RGMa*, *RGMb* and *Netrin-1* probes in *Gad67*-positive inhibitory interneurons revealed that the three molecules are highly expressed in the majority of these neurons. In the somatosensory cortex, I observed that 92% (*RGMa*), 96.1% (*RGMb*), and 97.4% (*Netrin-1*) of Gad*67*-positive neurons (Fig. 7 A-C) were also expressing these individual molecules. In addition, similar proportions of inhibitory neurons of the hippocampus (Fig. 7 D-F) expressed *RGMa* (91.5%), *RGMb* (93.3%), and Netrin-*1* (94%).

In summary, our quantification of double-positive cells showed that more than 90% of *Emx1*-expressing and GAD67-expressing cells in the somatosensory cortex and hippocampus express *Rgma*, *Rgmb*, and *Netrin-1* (Fig 6G; Fig 7G). Hence, these results demonstrate that *Rgma*, *Rgmb*, and *Netrin-1* are expressed in both excitatory and inhibitory neurons of the cerebral cortex and hippocampus following circuitry development, suggesting they may contribute to the maintenance and function of these circuits.

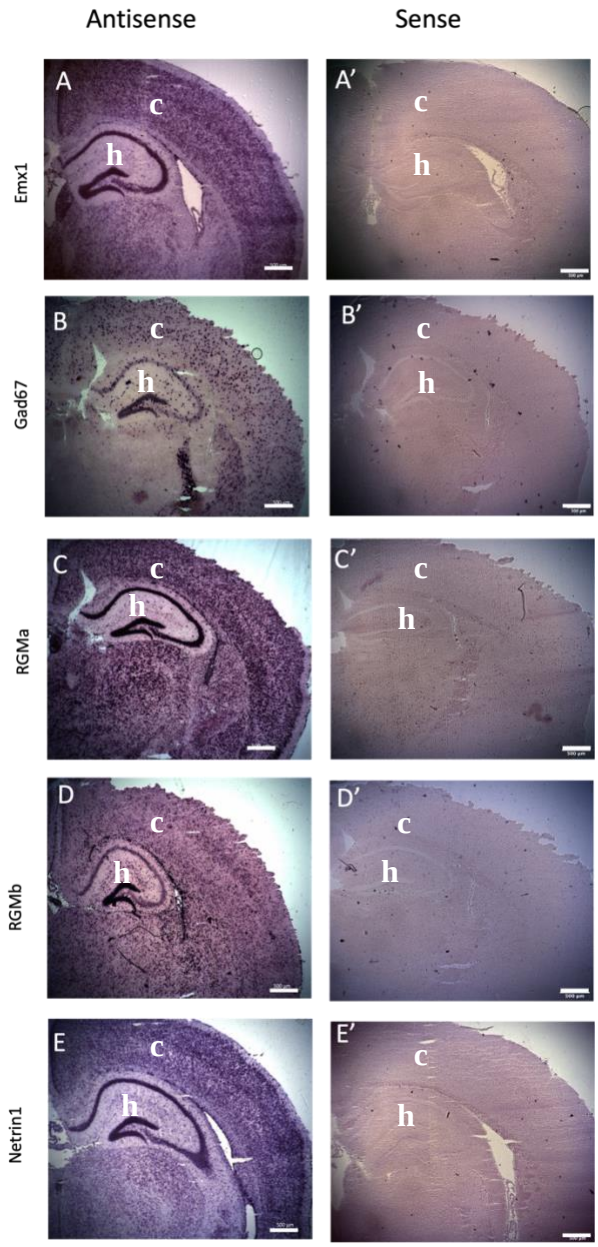
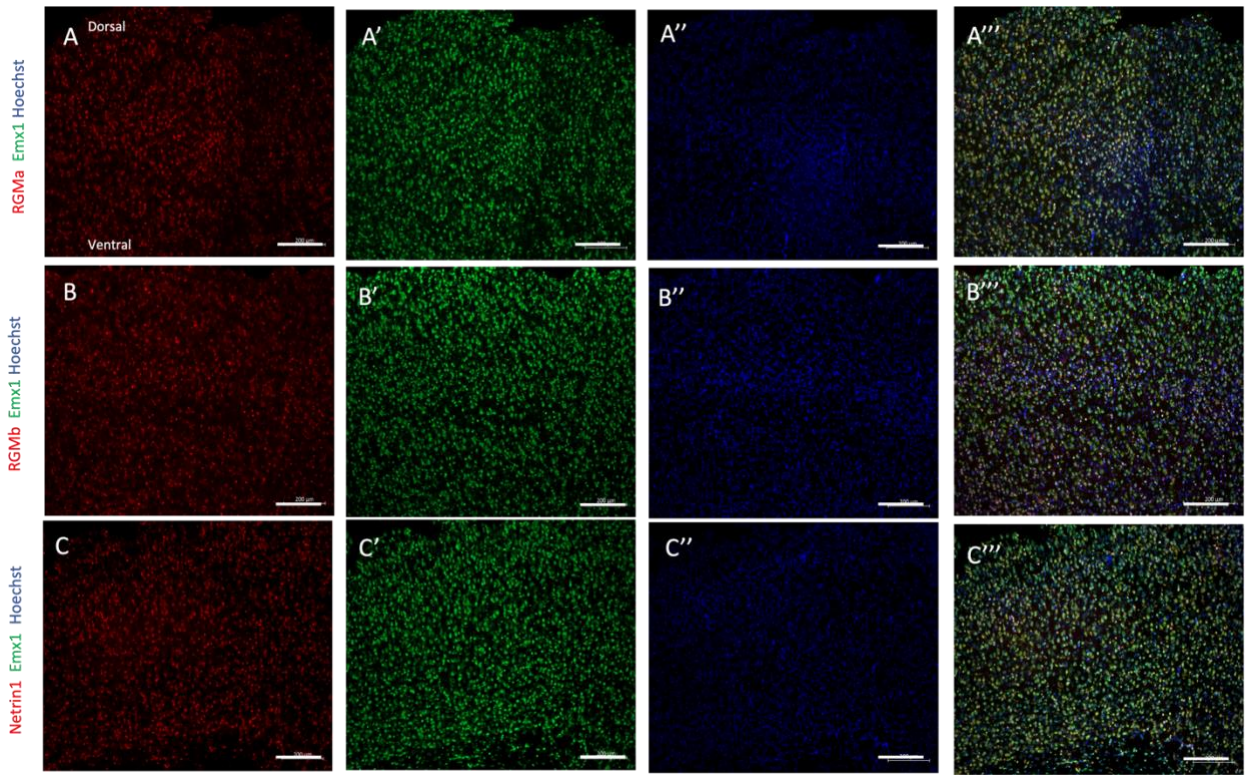



**Figure 5. Expression of *Emx1*, *Gad67*, *RGMa*, and *RGMb* in the cortex and hippocampus**

*Emx1* (A), *Gad67* (B), *RGMa* (C), *RGMb* (D) and *Netrin-1* (E) antisense or sense (A'-E') cRNA probes were hybridized to sections of cortex and hippocampus from P25 mouse brains. Hybridization signal was observed in the cortex (c) and hippocampus (h) for all antisense probes while no signal was observed with the sense probes. Scale bar = 500 μm.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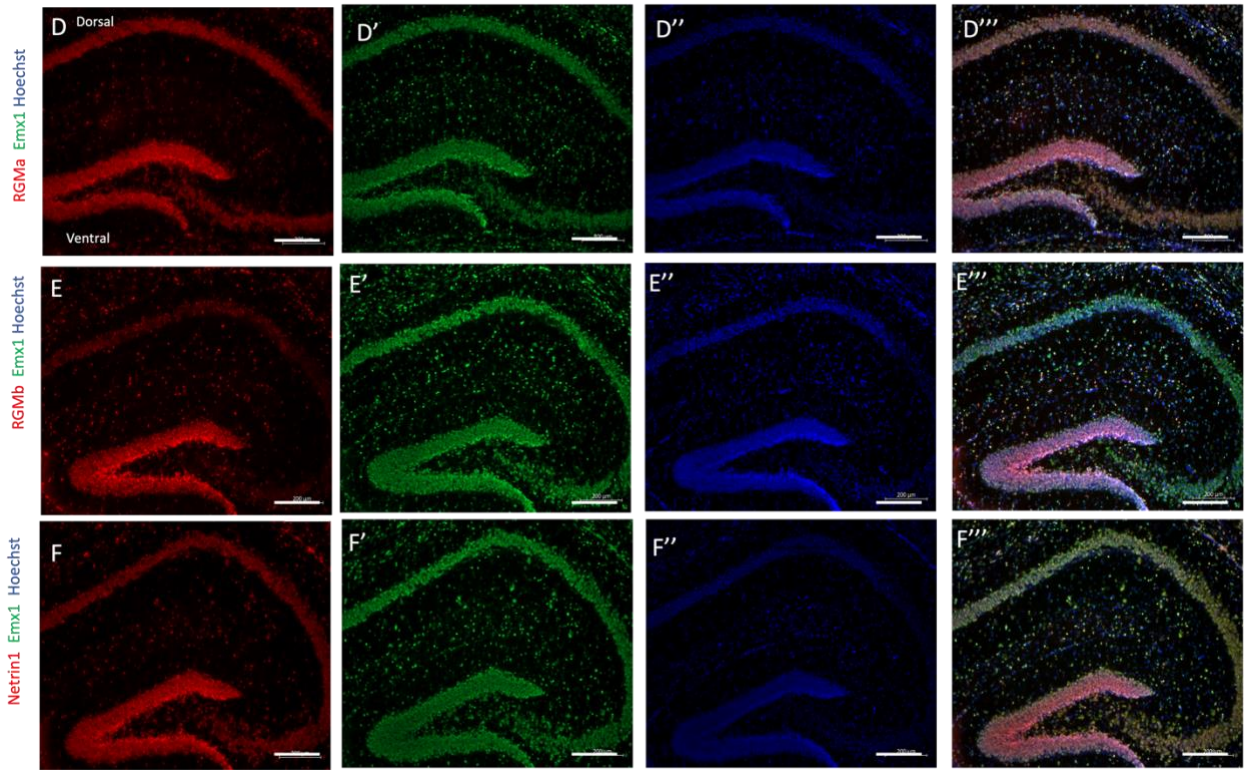
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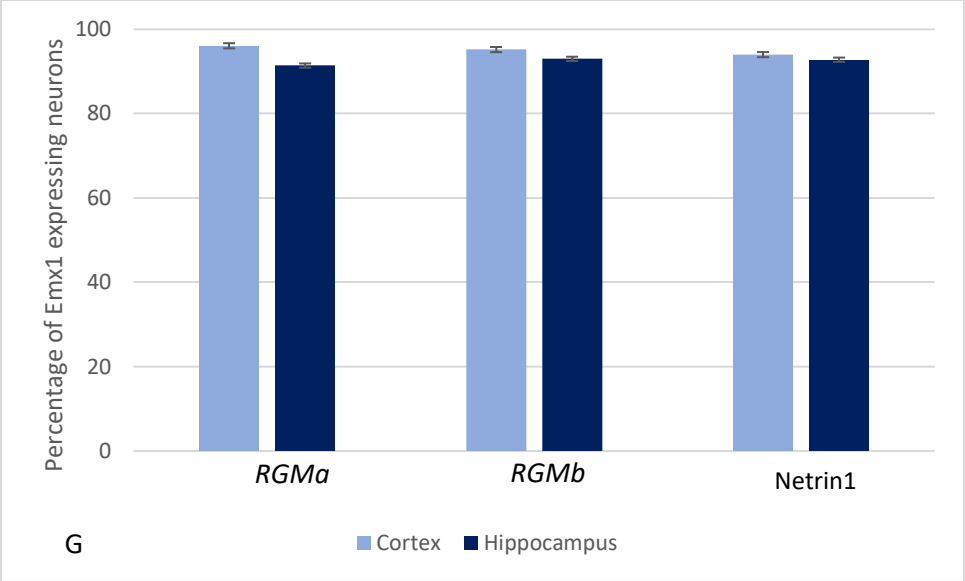
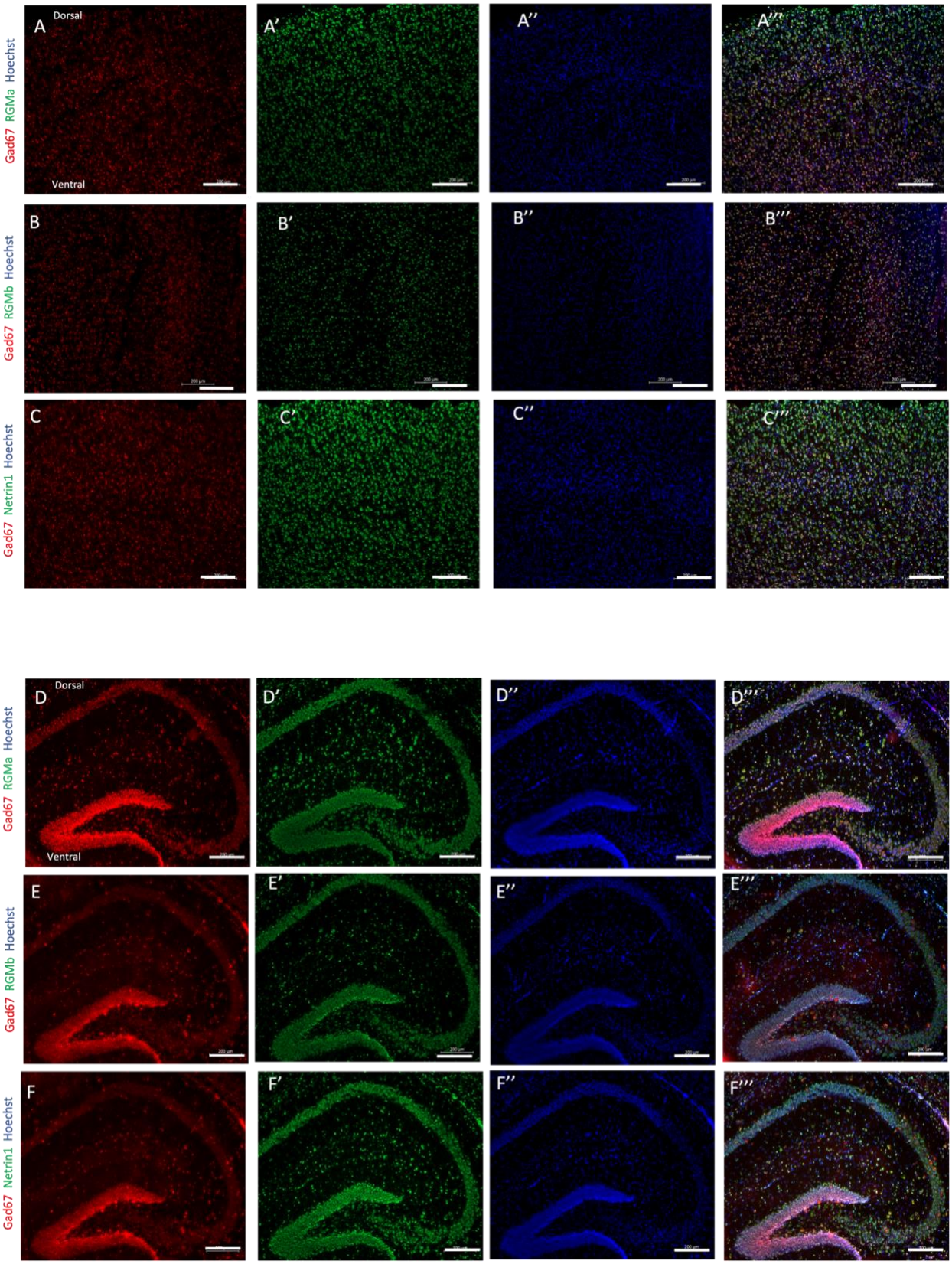



**Figure 6**. **Expression of *RGMa, RGMb, and Netrin-1* in excitatory neurons of the cortex and hippocampus.**

*In Situ* Hybridization of coronal sections of cortex (A-C) and hippocampus (D-F) isolated from P25 mice with DIG-labeled cRNA probes specific for *RGMa, RGMb and Netrin-1* and fluorescein-labeled probes specific for *Emx1* (A'-F'). Nuclei stain with Hoechst (A''-F'') and co-expression of each of the ligand mRNA in *Emx1*-positive neurons (A'''-F'''). Quantification of the percentage of *Emx1*-positive neurons that express *RGMa*, *RGMb*, and *Netrin1* is represented in the bar plots; means± SEM of three experiments are reported (G). Scale bar = 200 μm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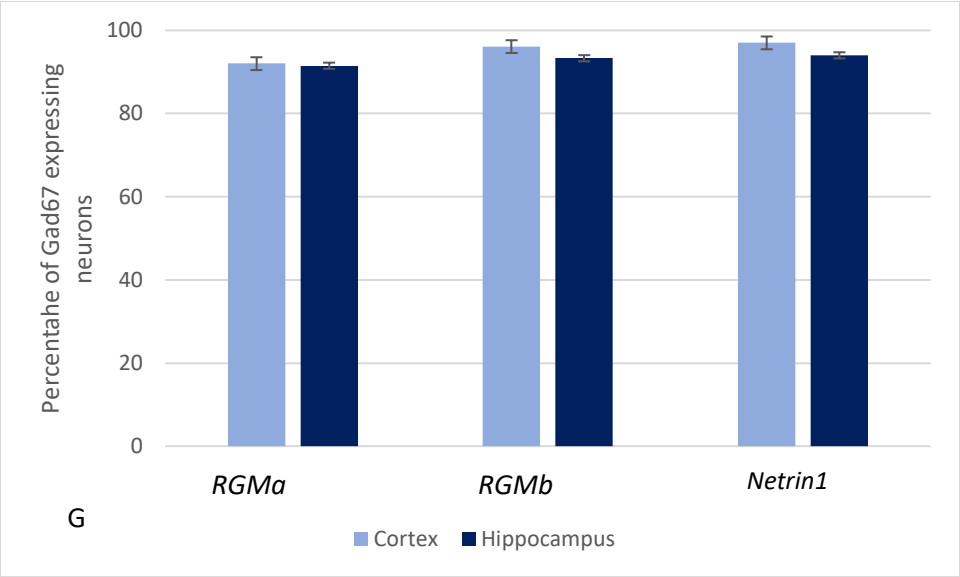


**Figure 7. Expression of *RGMa, RGMb, and Netrin-1* in inhibitory neurons of the cortex and hippocampus.**

*In Situ* Hybridization of coronal sections of cortex (A-C) and hippocampus (D-F) isolated from P25 mice with DIG-labeled cRNA probes specific for *Gad67* (A- F) and fluorescein- labeled probes specific for *RGMa, RGMb and Netrin-1* (A'-F'). Nuclei stain with Hoechst (A''-F'') and co-expression of each of the ligand mRNA in *Gad67* positive neurons (A'''-F'''). Quantification of the percentage of *Gad67*-positive neurons that express *RGMa*, *RGMb*, and *Netrin-1* is represented in the bar plots; means± SEM of three experiments are reported (G).

Scale bar = 200μm

## 4. Discussion

### Expression of RGMs and Netrin-1 in maturing cortical and hippocampal circuitry

My studies were aimed at defining the pattern of expression of the Neogenin ligands *RGMa, RGMb,* and *Netrin-1* in the maturing somatosensory cortex and hippocampal formation to define whether they are restricted to a specific subtype of neurons in these structures. My analyses have revealed that *RGMa, RGMb, Netrin-1* are expressed in both the pyramidal neurons and inhibitory interneurons of the maturing cortex and hippocampus. Through double *in situ* hybridization approaches, I determined that the vast majority (>90%) of pyramidal neurons and inhibitory interneurons express all three molecules during post-natal development, more specifically at P25. Our colocalization data suggest that the vast majority of glutamatergic neurons and GABAergic interneurons express mRNAs for *RGMa, RGMb,* and *Netrin-1*. The expression patterns of these Neogenin ligands in the post-natal brains indicate that they could potentially be involved in late developmental processes including neuronal regeneration or synapse formation, maintenance, and function.

Neogenin has been implicated in the regulation of several processes in the mature nervous system, including the formation and regulation of excitatory synapse function in the amygdala. Loss of Neogenin leads to a reduction in spine density and decreased frequency of miniature excitatory postsynaptic currents. These defects are associated with impaired fear memory, suggesting Neogenin is involved in information processing and synaptic plasticity in the amygdala (Sun et al.,2018). Neogenin has also been shown to influence neuronal activity in the hippocampus. In this case, loss of Neogenin expression in astrocytes leads to impaired GABAergic synaptic

transmission, which is associated with increased epileptiform spikes and seizures in these mice. These defects are likely due to impairments in the GLAST transporter-mediated glutamate-glutamine cycle (Sun et al.,2021). Neogenin also regulates adult neurogenesis in the hippocampal dendate gyrus. Loss of Neogenin in adult neural stem cells reduced neurogenesis and promoted astrogliogenesis in the hippocampus. These defects were associated with impaired glutamatergic neurotransmission and depressive-like behavior in these mice (Sun et al.,2018). Interestingly, activation of Neogenin by RGMa in the dentate gyrus was shown to inhibit adult neurogenesis and affect the migration of newborn neurons in the hippocampus, suggesting that Neogenin may contribute in various ways to adult neurogenesis, possibly by binding to different ligands (Isaken et al.,2020). Based on their expression in the somatosensory cortex and hippocampus at P25, RGMs and Netrin-1 represent good candidates to modulate Neogenin signaling in synaptic function and adult neurogenesis.

Previous research has demonstrated that Netrin-1 is expressed by cortical neurons during the early postnatal phase of synaptogenesis (Micheva and Beaulieu,1996; Goldman et al.,2013). Further, it has also been demonstrated to promote the strength and number of excitatory synapses formed between pyramidal neurons of the cortex (Goldman et al.,2013). In addition, it has also been reported to be involved in facilitating glutamatergic synaptic plasticity in the hippocampus between the CA3 and CA1 pyramidal neurons (Glasgow et al.,2018). While most of these Netrin-1 functions are dependent on the DCC receptor, it remains possible that Netrin-1 binding to Neogenin could contribute to Netrin-1 effects in these biological processes.

Although Neogenin has been implicated in the regulation of synaptic function, it remains to be assessed whether RGM family proteins may modulate this function. Netrin-1has been shown to localize to synapses and to modulate their function but the exact localization of RGM proteins in neuronal circuits remains to be fully examined. In the future, it will be essential to examine the sub-cellular localization of RGMa and RGMb protein in pyramidal neurons and inhibitory interneurons to establish if they are localized at excitatory or inhibitory synapses. A combination of immunohistochemistry and electron microscopy in cultured primary neurons and brain slices should reveal whether these two Neogenin ligands are found at the synapse. Should they be found to be localized at the synapse, their potential role in modulating synapse formation and function could be assessed using primary neuronal cultures in which RGMs expression has been knocked down or enhanced. Furthermore, *in vivo* loss-of-function analyses by ablating expression of RGMa or RGMb specifically in excitatory pyramidal neurons or interneurons using Cre-mediated approaches, combined with histological and electrophysiological studies, should reveal whether they contribute to synapse formation, maintenance, and function in the cortex and hippocampus. Hence, my studies demonstrating that *RGMa*, *RGMb*, and *Netrin-1* are expressed in most pyramidal neurons and inhibitory interneurons in the cortex and hippocampus will pave the way to the development of loss-of-function approaches to address their roles in the maintenance and function of these circuits affected in several neurodevelopmental and psychiatric disorders.

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