Role of Slitrk Family Members in Neurodevelopment

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ABSTRACT

The development of the nervous system is an extremely complex process where gene expression is tightly regulated, both spatially and temporally. Any gene disruption during neurodevelopment, from the complete non-transcription of the gene to a single nucleotide mutation, has the potential to lead to severe consequences in the organism. This situation is particularly well illustrated by the whole spectrum of neurological disorders affecting humans. Thanks to basic research at the gene and protein levels, some genetic causes for certain mental illness have been identified.

This thesis focuses on a novel family of proteins termed the Slitrks. Initial characterization of the Slitrk family genes revealed that their expression is enriched in the central nervous system. Herein, I have performed a detailed analysis of the patterns of expression of the six members of the family in the mouse nervous system. I demonstrate that, despite some overlapping expression, several key brain regions express different combinations of Slitrks suggesting that the different family members may have distinct functions during nervous system development. I further demonstrate that members of the Slitrk family can regulate synapse formation in hippocampal neurons. More precisely, Slitrk1 is required for the formation of both excitatory and inhibitory synapses. Taken together, the results presented in my thesis indicate that Slitrks play an important role in the developing nervous system.

RÉSUMÉ

Le développement du système nerveux est un processus extrêmement complexe pendant lequel l'expression des gènes est contrôlée de façon précise temporellement et localement. Durant le neurodéveloppement, chaque dérégulation génétique, de l'arrêt complet de la transcription d'un gène jusqu'à la mutation d'un seul nucléotide, a le potentiel de mener à de graves conséquences pour l'organisme. Cette situation est particulièrement bien illustrée par l'ensemble des troubles neurologiques qui affectent l'humain.

Cette thèse se concentre sur une nouvelle famille de protéines nommées Slitrks. La description préliminaire de cette famille a révélé que leur expression est enrichie dans le système nerveux central. Par conséquent, j'ai réalisé une analyse détaillée du patron d'expression des six membres de la famille dans le système nerveux de la souris. J'ai ainsi pu démontrer que malgré certains chevauchements d'expression, plusieurs régions du cerveau expriment différentes combinaisons de Slitrks. Cela laisse présager que certains membres de la famille Slitrks peuvent avoir des fonctions distinctes durant la formation du système nerveux. Au cours de mes travaux, j'ai aussi pu démontrer que les Slitrks peuvent réguler la formation des synapses dans les neurones de l'hippocampe. Plus précisément, Slitrk1 est requis à la fois pour la formation des synapses excitatrices et inhibitrices. Dans l'ensemble, les résultats présentés dans cette thèse indiquent que les Slitrks jouent un rôle important dans le développement du système nerveux.

LIST OF FIGURES

LIST OF ABBREVIATIONS

aa: amino acid

Ach: acetylcholine

AChR: ACh receptor

ADAM: a disintegrin and metalloproteinase

ADHD: attention deficit hyperactivity disorder

AM: anteromedial thalamic nuclei

AMPA: α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid

AMPAR: AMPA receptor

ApoE: apolipoprotein E

Aq: aqueduct

AS: antisense

ASD: autism spectrum disorder

Au: arbitrary units

AV: anteroventral thalamic nuclei

BD: bipolar disorder

CAM: cell adhesion molecule

Cbln1: cerebellin 1 precursor protein

CK2: casein kinase II

CM: centromedial thalamic nuclei

CNS: central nervous system

CxP: cortical plate

DAG: diacylglycerol

DEPC: diethylpyrocarbonate

DG: dentate gyrus

DIG: digoxigenin

dLG: dorsal lateral geniculate nucleus

DLS: dorsolateral septum

DN: deep cerebellar nuclei

DRG: dorsal root ganglion

Dvl1: dishevelled-1

E: embryonic day

ECD: extracellular domain

EGF: epidermal growth factor

FGF: fibroblast growth factor

Fig: figure

FLRT: fibronectin leucine-rich repeat transmembrane protein

Fr: fasciculus retroflexus

GCL: granule cell layer of the OB

GL: glomerular layer

GluR: glutamate receptor

GPCR: G protein-coupled receptor

GPI: glycosylphosphatidylinositol

H: hippocampus

Ig: immunoglobulin

IGL: inner granular cerebellar layer

IL-1: interleukin-1

INL: inner nuclear retinal layer

kDa: kilodalton

KO mice: knockout mice

LAR: leukocyte common antigen-related

Lat: lateral cerebellar nucleus

LD: laterodorsal thalamic nucleus

LNS: laminin/neurexin/sex hormone-binding globulin

LP: lateral posterior thalamic nuclei

LTD: long-term depression

LRR: leucine-rich repeat

LRRTM: leucine-rich repeat transmembrane

MCL: mitral cell layer

MD: mediodorsal thalamic nuclei

miRNA: microRNA

mGluR: metabotropic glutamate receptor

Mo: molecular layer of the cerebellum

MuSK: muscle-specific kinase

Narp: neuronal activity–regulated pentraxin

NBL: neuroblastic layer of the retina

Ncad: neural (N-) cadherin

NCAM: neural cell adhesion molecule

NMJ: neuromuscular junction

NP1: neuronal pentraxin 1

NPR: neuronal pentraxin receptor

OB: olfactory bulb

OCD: obsessive-compulsive disorder

ONL: olfactory nerve layer

ORN olfactory receptor neurons

OV: olfactory ventricule

PBS: phosphate-buffered isotonic saline

PF: parafascicular thalamic nuclei

Pk: Purkinje cell layer

PKA: protein kinase A

PKC: protein kinase C

PNET: supratentorial primitive neuroectodermal tumor

PNS: peripheral nervous system

Po: posterior complex thalamic nuclei

PSA: polysialic acid

PSD-95: postsynaptic density protein-95

PTP: protein-tyrosine-phosphatase

PSD: postsynaptic density

PT: paratenial thalamic nuclei

PV: paraventricular thalamic nuclei

RGC: retinal ganglion cell

RGCL: retinal ganglion cell layer

RT: room temperature

S: sense

SALM: synaptic adhesion-like molecule

SC: superior colliculi

siRNA: small interfering RNA

SS: splice site

SynCAM: synaptic cell adhesion molecule

SCZ: schizophrenia

SP: subplate

SSC: standard saline citrate

TG: trigeminal ganglia

Trk: tropomyosin-related kinase

TSP: thrombospondin protein

UTR: untranslated region

VL: ventrolateral thalamic nuclei

VNO: vomeronasal organ

VP: ventroposterior thalamic nuclei

VZ: ventricular zone

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AUTHORS CONTRIBUTIONS

Chapter 1: Literature review

François Beaubien: Wrote all material and prepared all figures.

Chapter 2: Differential expression of Slitrk family members in the mouse nervous system

François Beaubien: Developed rationale, performed all experiments, assembled all figures and co-wrote manuscript.

Jean-François Cloutier: Developed rationale and co-wrote manuscript.

Chapter 3: Slitrk1 and Slitrk2 promote excitatory synapse development

François Beaubien: Developed rationale, performed all experiments, assembled all figures and co-wrote manuscript.

Jean-François Cloutier: Developed rationale and co-wrote manuscript.

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CHAPTER 1

LITERATURE REVIEW

1. General introduction

"The problem of neurology," Wilder Penfield once wrote, "is to understand man himself." The road toward this long-time goal has been filled with amazing discoveries made by scientists of various disciplines, from psychiatry to neurobiology. Recently, developments of techniques in molecular biology and advances in genetics were able to shed some light on the basic principles involved in the development of our human mind. The progress made in our understanding of brain development, from the initial closure of the neural tube to the discovery of adult neurogenesis, is now a central aspect of the brain theory. Importantly, the biology of some neurological diseases is now better understood with easy access to the human genome. In this literature review, I will emphasise on another remarkable feature of the brain: the extreme precision with which synaptic connections are formed during development and maintained throughout the entire lifespan. Synaptic plasticity is perhaps the pillar on which the brain's amazing malleability rests. Thus, the study of synaptogenesis is at the leading edge of research "to understand man himself".

Before describing the details of synapse formation, it is worth placing this process in a bigger picture. Indeed, synaptogenesis only occurs after axon extension, which comes after the birth of neural stem cells and their differentiation into neurons. Initially, the generation of postmitotic neurons from dividing progenitor cells occurs by a process of signaling between adjacent cells in the proneural region of the ectoderm that is regulated by the delta and notch transmembrane proteins. The differentiation of each generated neural precursor cell depends on diverse signals present in their environment. The final

step toward the establishment of synaptic connections is the growth and extension of the neuron's axon to its target site. The tip of the axon, called the growth cone, allows the axon to detect and respond to cues in the environment that guide its growth in the proper direction. Once axons reach their targets, synaptogenesis can begin.

The present literature review will focus on two major topics. First, the question of complex synapse formation will be covered in detail. In addition to explaining the classical model of the formation of neuromuscular junction, synaptogenesis in the more complex peripheral and central nervous systems will be discussed. In the second section of this review, I will present a new family of molecules that I hypothesize plays a critical role in the formation of synapses in the central nervous system (CNS). The section will conclude with the rationale and objectives of this thesis.

2. Synaptogenesis

2.1. Historical perspective

The term *synapse* was adopted by Charles Sherrington in 1897, but the concept of synapses emerged from multiple hypotheses dating back as far as the pre-Socratic era of the fifth century BC. In terms of duration of influence, Aristotle's (384-322 B.C.) theory of "vital pneuma" dominated history for the longest period. Indeed, Aristotle's great contribution to neural science is the introduction of the concept that a substance (the vital pneuma) has to travel to an organ to allow it to function (Everson, 1995). A few hundred years later, Galen (131-200) proposed a refined version of Aristotle's theory in which a delicate substance called "psychic pneuma" is passed from the brain to the spinal cord and then to the nerves to induce movement (Major, 1961). For the next 1300 years, scientists continued to build on Galen's theory. However, with René Descartes (1596-1650) came a new revolution. Based on his new mechanistic philosophy, Descartes proposed that nerve conduction involves the passage of small particles derived from the heart. Here, the novelty resides in the idea that transmission relies on particles passing from the brain to different target locations (Bennett, 1999). It was only with advances in the field of physiology, more specifically, the discovery of the biological applications of electricity, that the formulation and consolidation of the cellular theory emerged, a theory

that recognizes the cell as the fundamental unit of structure, function and organization in all living organisms (Kölliker, 1863). Due to their more complex morphology, it took the work of several people to extend the cellular theory to nerve cells. For a long time, it was believed that neurons must be fused with their targets cells in order to communicate. The "reticular theory" stated that central nerve endings would not end freely, but would have protoplasmic extensions that constitute a fine nerve fibre network (Gerlach, 1871). It was not until the work of Santiago Ramon y Cajal (1852-1934) that the neuron doctrine, namely that each neuron is an independent cell that does not connect with surrounding cells, was adopted. Using a staining method developed by Camillo Golgi (1842-1926), Cajal developed his theory based on the observation that the ends of neurons showed no signs of continuity with other neurons in the cerebellum of birds (Cajal, 1888). Cajal was the first to conclude that discontinuity between neurons represents the true nature of their connectivity. Based on his observations, Cajal also speculated that action potentials flowed only from terminal bulb to dendrite or soma between cells, and then from soma to axon within a cell. However, it was the experimental findings of a physiologist, namely Charles Sherrington (1858-1952), which proved Cajal's doctrine of the polarisation of the neuron. It is while working on spinal reflexes that Sherrington imagined a 'valve-like function' for the end of the nerve:

"*So far as our present knowledge goes, we are led to think that the tip of a twig of the arborescence is not continuous with but merely in contact with the substance of the dendrite or cell-body on which it impinges. Such a special connection of one nerve cell with another might be called a synapse*" (Sherrington and Foster, 1897)

By observing the effect of adrenaline on the peripheral nervous system (PNS), Thomas Renton Elliott (1877-1961) came out with the concept of chemical neurotransmission by postulating that the action potential could cross the synapse through chemical substances, which took the name of "chemical mediators" (Elliott, 1904; Lopez-Munoz and Alamo, 2009). The discovery of multiple neurotransmitters over subsequent years consolidates this theory. Noteworthy, there was a delay of about a decade between the acceptance of the notion of chemical transmission in the PNS and of that in the CNS. Any remaining doubts regarding the synapse as the critical site for neural communication were dispelled with the advent of electron microscopy. By way of conclusion, while it is

here relevant to briefly cover the history behind the current theory of neurotransmission, this story is also a great example of the evolution of scientific progress.

2.2. Example of the neuromuscular junction

In a classical review paper of 1993, Zach Hall and Joshua Sanes wrote that "virtually all of our current understanding of synaptogenesis derives from the study of just one synapse, the vertebrate skeletal neuromuscular junction."(Hall and Sanes, 1993). Our current knowledge about the formation of synapses is indeed much more elaborate than twenty years ago, however some fundamental characteristics of the vertebrate skeletal neuromuscular junction (NMJ) are still highly relevant today for research in synaptogenesis. The synapses between spinal motor neurons and skeletal muscle fibers represent a good research model as they are simple, relatively large, and accessible for dissection compared to the highly complex CNS synapses. Three types of cells are present at the NMJ: a motor neuron, a muscle fiber, and a few Schwann cells. Each motor neuron has its cell body in the spinal cord or brain stem, and it sends an axon to innervate one single muscle, but several muscle fibers. The nerve terminals contain multiple 50 nm diameter synaptic vesicles that are full of the transmitter, acetylcholine (ACh). The synaptic vesicle-rich region facing the muscle fibers is named the active zone. With neuronal depolarization, the action potential travels into the terminal and causes exocytotic release of ACh. However, the active zone is also the site of endocytosis of synaptic vesicles. The region of the muscle fiber opposite the active zone is also a specialised region within the muscle fibre with junctional folds, which are shallow gutters in the membrane filled with ACh receptors (AChRs). A layer of extracellular material called basal lamina traverses the 50 nm-wide synaptic cleft and extends into these junctional folds. The basal lamina continuously encircles the muscle fiber and fuses with the Schwann cell's basal lamina (Hall and Sanes, 1993). Only at the synaptic cleft, this extracellular layer contains the important enzyme, acetylcholinesterase, which terminates transmitter action.

The question of how a mature neuromuscular junction further develops from the initial contact between a motor axon and a myotube is particularly interesting. In rodent, this

entire process occurs over a surprisingly long period of about three weeks and normally begins at embryonic day (E) 13. Synapse formation can start anywhere on the muscle fiber since there is no predetermined synaptic site. The intrinsic development of synaptic machinery in the motor neuron and in the myotube occurs independently. Motor axons can form synaptic vesicles filled with neurotransmitter without any contact with muscle fiber while uninnervated myotubes can synthesize functional ACh receptors when cultured in the absence of neurons (Fischbach and Cohen, 1973; Hartzell and Fambrough, 1973). It is the initial contact between the growth cone and the myotube that alters the nerve morphology and initiates a series of changes in the muscle cell. The growth cone begins its transformation into a nerve terminal by accumulating synaptic vesicles that lead to membrane thickening. The motor neurons also synthesize and release from their nerve terminals a proteoglycan named agrin. This basal lamina-associated protein has a key role in synapse formation by inducing the clustering on the muscle of existing AChRs and several other proteins including muscle agrin, acetylcholinesterase, rapsyn and a heparan sulphate proteoglycan that are distributed throughout the membrane at a low density (McMahan, 1990). In muscles of *agrin*-deficient mutant mice, postsynaptic AChR clusters are markedly reduced in number, size, and density after having initially developed normally (Gautam et al., 1996). Though several molecules that interact with agrin are present in myotube membrane and could have physiological functions, it is now established that agrin most likely acts via a receptor-like tyrosine kinase termed MuSK, for muscle-specific kinase which is part of an agrin receptor complex (Valenzuela et al., 1995; Glass et al., 1996). Mice lacking MuSK die at birth, owing to an inability to breathe resulting from the fact that these mice lack NMJs (DeChiara et al., 1996). However, even if agrin induces prominent and rapid tyrosine phosphorylation of MuSK, isolated MuSK receptor is not sufficient to bind agrin suggesting that MuSK requires additional components for it to bind to agrin (Glass et al., 1996). This long-sought receptor for agrin was simultaneously identified by two groups as Lrp4, a low-density lipoprotein receptor-related protein (Kim et al., 2008b; Zhang et al., 2008a). Lrp4 was already know to play a role in NMJ formation, based on *lrp4* mutant mice that display defects in presynaptic and postsynaptic differentiation that are strikingly similar to those found in MuSK mutant mice, but its exact function was

unclear (Weatherbee et al., 2006). Hence agrin, secreted by motor neurons, binds to the lipoprotein receptor, Lrp4, which induces aggregation and activation of the MuSK receptor complex and its downstream signaling. The cytoplasmic protein rapsyn is recruited and will link the receptors to the cytoskeleton of the muscle. Indeed, agrin signaling triggers activation of Rac, Pak, and Src, which are required to cluster more AChR. (Gautam et al., 1995). Taken together, the actual model suggests a two-steps process of NMJ formation: A MuSK-dependent, agrin-independent mechanism of AChR aggregation is followed by nerve-derived, agrin-dependent elaboration and maintenance of clusters (Song et al., 2006).

Classically, a protein, called neuregulin, that was purified from brain extracts on the basis of its ability to stimulate the synthesis of AChRs in cultured myotubes was believed to be the most likely candidate to mediate the transcription of AChR genes on motor endplates (Falls et al., 1993). Neuregulin is expressed by both motor neurons and muscles, but its receptors, the tyrosine kinases erbB2, erbB3, and erbB4, are expressed by the muscle only. Mice lacking neuregulin, erbB2, or erbB4 die during development which prevent analysis of their function in synapse development (Gassmann et al., 1995; Lee et al., 1995; Meyer and Birchmeier, 1995), but a 50% decrease in the density of AChRs is observed in neuregulin heterozygotes mice (Sandrock et al., 1997). While expecting a decrease in the density of AChRs in mice lacking ErbB2/4 in skeletal muscle or neuregulin in motor neurons and muscle fibers, only modest or no phenotypes were detected in these mice, suggesting that neuregulin might not in fact be essential for synapse-specific transcription of AChR subunit genes (Escher et al., 2005; Jaworski and Burden, 2006). Alternatively, neuregulin is known to act on the phosphorylation of α-dystrobrevin1, a component of the postsynaptic apparatus involved in the anchoring of the AChRs in the synaptic membrane, for the maintenance of the NMJ (Schmidt et al., 2011). Neuregulin also potentiates AChR clustering when injected into muscles of embryonic mice by increasing the tyrosine phosphorylation of MuSK in the ongoing presence of agrin (Ngo et al., 2012). To date, there is no other motor neuron molecule suggested to increase the synthesis of AChRs.

Another surprising property of NMJ maturation is the transition of the organisation of the AChR clusters from having a small oval-like shape with uniform receptor density to having multiperforated elaborate branches with a pretzel-like shape (Balice-Gordon and Lichtman, 1993; Marques et al., 2000). This topological maturation of the postsynaptic apparatus seems to occur in a nerve-independent manner by a still poorly understood mechanism (Kummer et al., 2004). The AChR clusters do not form a mature pretzel-like structure in mice that are mutant for the Rho guanine nucleotide exchange factor, ephexin1 (Shi et al., 2010). It was already known that key regulators of actin dynamics regulate agrin-induced clustering of AChRs in cultured myotubes (Weston et al., 2003). Adult *ephexin1*$^{-/-}$ mice display muscle weakness and impaired neuromuscular transmission associated with NMJ abnormalities, including an imprecise synaptic apposition of the presynaptic and postsynaptic sides (Shi et al., 2010). Overall, this suggests that ephexin1 affects the reorganization of the actin cytoskeleton in muscle cell.

In conclusion, research on how NMJs form and function remains a good paradigm for gaining insight about general synaptogenesis, but it is also critical for our understanding of neurological disorders affecting the NMJ like congenital myasthenic syndromes (Engel and Sine, 2005).

2.2.1. Comparison between nerve terminals of neuromuscular junctions and central synapses

The vertebrate skeletal neuromuscular synapse lies outside of the brain and does not have a neuron as its postsynaptic element, but it is still remarkably quite similar to the central synapse. This resemblance is true, at least for the major protein components of synaptic vesicles, for the mechanism of transmitter release, and for the clustering of neurotransmitter receptors in the postsynaptic membrane. However, certain major differences exist between central and peripheral synapses. First, there is the absence of a basal lamina-like structure ensheathing the central synaptic clefts. Instead, the adhesion between the pre- and postsynaptic membranes relies on the interaction of matched adhesion molecules (Sanes and Jessel, 2000). Second, while the NMJ is restricted to have only one active zone per synapse, CNS synapses can include multiple active zones at

each synapse. Finally, another major difference is the ratio between the number of presynaptic axons and postsynaptic targets at each synapse, which is one-to-one for the NMJ, but 1000 to 1 on average in the CNS.

Despite its clear benefits, research on the NMJ has certain limitations with respect to providing specific details about how central nervous system synapses form. For example, the NMJ model is not helpful when studying differences between excitatory and inhibitory synapse formation or when characterizing postsynaptic machinery. As our knowledge of the formation of individual synapse advances, neuroscientists are becoming increasingly interested in more complex questions related to the dynamics of synapse formation. For this purpose, NMJ model is unlikely to suffice.

2.3. CNS synaptogenesis

2.3.1. Introduction

One of the remarkable features of synaptogenesis in the CNS is the fact that it occurs throughout the entire life of the organism. Synapse formation first occurs in the embryo during the initial development of the nervous system, and it is now known to continue into adulthood where it plays a role in the process of learning and memory. The establishment of mature synapses between neurons is fundamental, and a considerable machinery must be put in place to ensure that axons connect to their correct targets (Fig. 1).

After performing his ground-breaking experiments using the retino-tectal circuit of adult frogs, Roger Sperry proposed that each neuron has its own molecular marker that is recognized by a specific synaptic partner (Sperry, 1963). However, knowing the enormous number of neurons in the brain, and therefore the gigantic number of synapses, it was unlikely that so many different recognition molecules would exist. Instead, target recognition cues may direct growth cones to specific regions or layers of tissue, rather than specific cells. Thus, the current hypothesis proposes that target selection occurs at the level of groups of identical neurons, rather than single cells (Munno and Syed, 2003). Nevertheless, the task of understanding synaptogenesis remains immense.

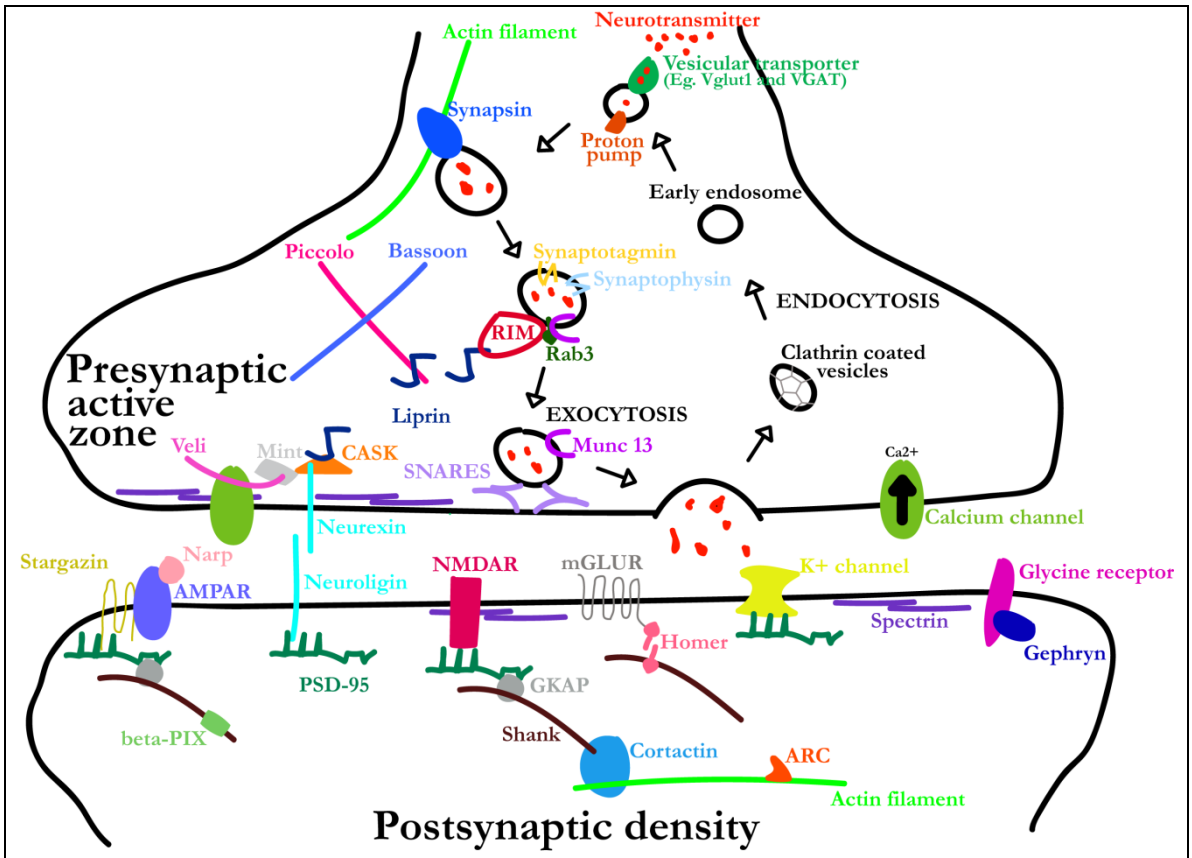


Figure 1: The molecular organization of the synapse.

The various molecules illustrated here regulate synapse function, morphology, trafficking and localization of adhesion molecules and neurotransmitter receptors. Neurexins are anchored to the presynaptic cytomatrix via a trimeric protein complex of CASK, Mint, and Veli, which in turn links to Ca^{2+} channels and liprins. Liprin also directly interacts with RIM active zone proteins that regulate neurotransmitter release, and is indirectly linked to the active zone protein Piccolo which, with Bassoon, is a very large multidomain scaffolding molecule. RIM forms a tripartite complex with Munc 13 and Rab3 that controls priming of synaptic vesicles for the upcoming synaptotagmin/snares-mediated vesicle fusion. Prior to exocytosis, the vesicles are first loaded with a specific neurotransmitter depending on the vesicular transporter and directed to the active zone via the binding of synapsin to the cytoskeleton actin. Following the release of neurotransmitter in the synaptic cleft, synaptic vesicles are recycled via a clathrin-mediated pathway. The postsynaptic compartments of excitatory and inhibitory synapses

differ in their composition and structure. For simplicity's sake, all major players are represented together in this cartoon. PSD-95 is a major scaffolding molecule localized in excitatory glutamatergic synapses. PSD-95 modulates trafficking of AMPA receptors via stargazin. The PDZ domains of PSD-95 also bind to other postsynaptic membrane proteins, including potassium channels, tyrosine kinases ErbB4 (not shown) and the cell adhesion molecule, neuroligin. PSD-95 is linked to actin filaments through GKAP, Shank and cortactin complex. Shank also binds to Homer, which interacts directly with the cytoplasmic tail of the metabotropic glutamate receptor (mGluR) and with the guanine nucleotide exchange factors beta-pix. Gephyrin, a tubulin-binding protein, is at the core of inhibitory postsynaptic scaffolds stabilizing glycine receptors and/or $GABA_A$ receptors (not shown). Finally, the cytoskeletal protein, spectrin, lines the intracellular side of the pre- and post-membranes, playing a role in maintenance of plasma membrane.

2.3.2. The four steps of synaptogenesis

During development, many axons travel long distances to reach their target zones even before forming any synapses. Thus, the transition between axon growth and synaptogenesis must be highly regulated. While synapse formation is generally categorized into four steps, readers need to bear in mind that the entire process is a fluid succession of events rather than discrete steps (Missler et al., 2012). In order to form the correct neuronal connections, axons and dendrites first start by establishing initial, often transient, contacts. This step relies on interactions between sets of cell adhesion molecules that are involved in cell-cell recognition, but also on secreted factors, receptors, and signaling molecules that make neurons receptive to synapse formation. This initial establishment of synaptic contacts is followed by the assembly of pre- and postsynaptic molecular machinery. This second step of synapse formation includes the recruitment of synaptic vesicles, the formation of the active zone, and the development of postsynaptic density structures. However, synapses are still not functional at this point. The third step of the process is the functional specification of the synapses, during which the organization of the molecular components of the synapse leads to the

acquisition of distinct physiological properties. The second and third steps are dependent on synaptogenic proteins, often different from the ones implicated in step one, which specialized in the recruitment of synaptic proteins. In the final step of synaptogenesis, synaptic activity determines whether these synapses will be stabilized or eliminated.

2.3.3. Synaptogenic proteins

Synaptic organizing proteins exist in two main classes: secreted factors and synaptic adhesion complexes (Fig. 2). Significant progress has been made over the last ten years in identifying factors derived from either the target neurons or the surrounding glia that accelerate and guide synaptogenesis. Indeed, prior to the initial contact, certain factors transform axons and dendrites into competent pre- and postsynaptic structures to undergo synaptogenesis. These molecules will be referred to as "priming factors" (Waites et al., 2005). During maturation of the synapse, other secreted factors from the presynaptic and postsynaptic terminal participate in receptor clustering. On the other hand, the synaptogenic adhesion complexes are composed of transmembrane presynaptic and postsynaptic partners that bind *in trans* across the cleft. The knowledge about these synaptic proteins has also increased tremendously over the last two decades. Many more families of molecules are now known to serve the process of synaptogenesis. Here will be reviewed the major players in this continuously growing field.

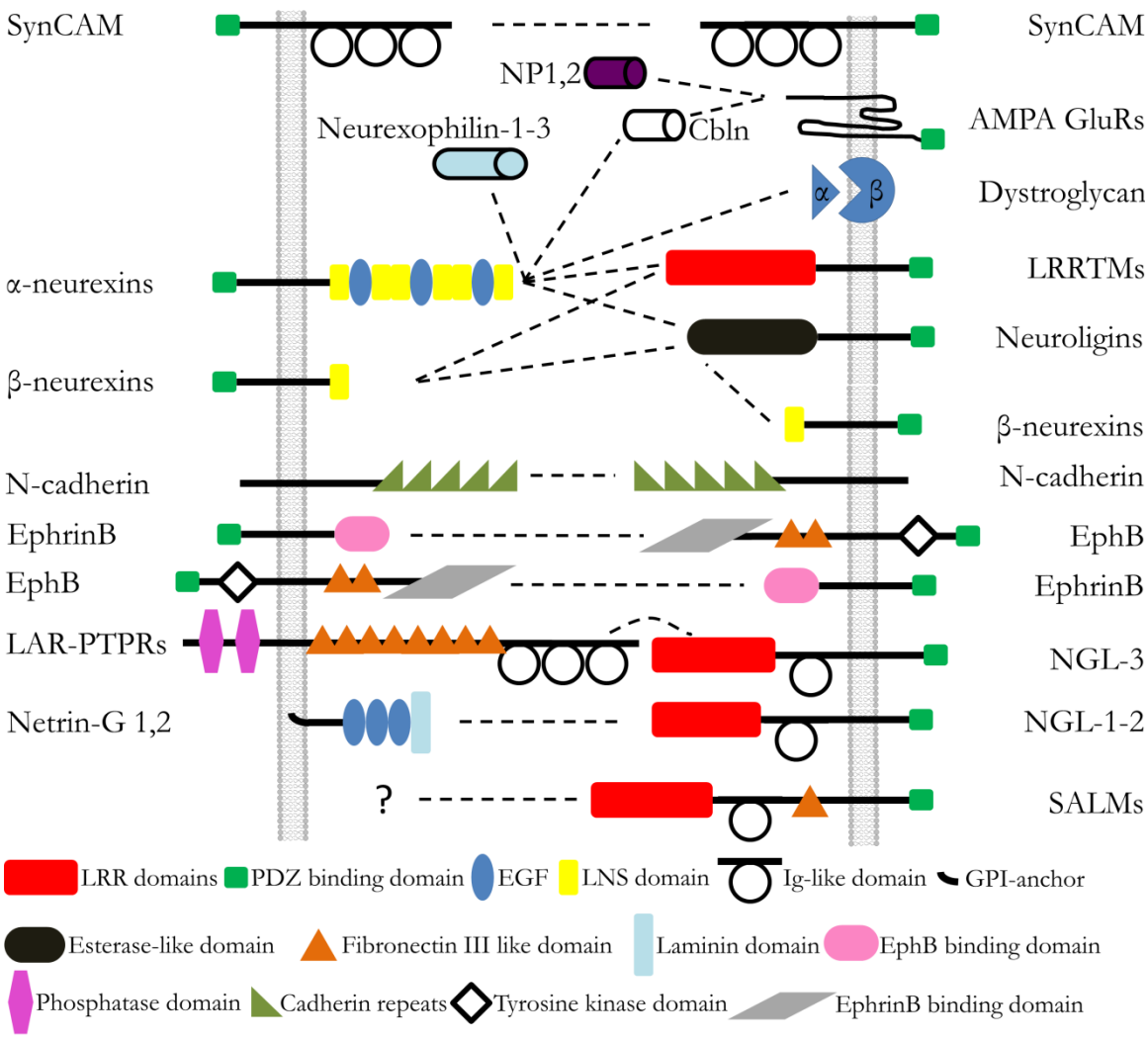


Figure 2: An inventory of synaptogenic molecules.

Schematic diagram of the domain structure of synaptic proteins present on pre- and postsynaptic sites, as discussed in this thesis. Protein-protein interactions between molecules are shown by dotted lines.

2.3.3.1. Secreted factors

2.3.3.1.1. Neuronally derived priming factors

Classically, priming factors derived from the target neurons include members of two families of proteins: the Wnt and the fibroblast growth factors (FGF) (Scheiffele, 2003).

2.3.3.1.1.1. Wnt

Since its discovery almost 20 years ago, the Wnt family of secreted signaling molecules has been implicated in various developmental functions such as embryonic induction, the generation of cell polarity, and the specification of cell fate (Sylvie et al., 2011). Wnts can signal through different receptors including Frizzled, Ryk and Ror2. Thus far, two Wnt family members have been shown to play a role in early steps of the formation of neuronal connections: WNT-7a and WNT-3. WNT-7a induces growth cone enlargement, axonal spreading, and increases the clustering of presynaptic markers in their presynaptic partners potentially through the Dishevelled-1 (Dvl1) pathway (Lucas and Salinas, 1997). More precisely, Wnt7a preferentially stimulates excitatory synapse formation (Ciani et al., 2011). A lack of Wnt7a in mice causes a delay in the morphological maturation of the synapse and accumulation of synaptic proteins while the double Wnt-7a/Dvl1 mutant mice exhibit even more severe defects (Hall et al., 2000; Ahmad-Annuar et al., 2006). Similar to the effect of Wnt7a, inhibition of Gsk3β, a serine/threonine kinase known to be a signaling molecule in Wnt pathways, leads to axonal remodelling and clustering of synapsin I in developing neurons (Hall et al., 2000; Hall et al., 2002). These effects are likely to be due to the binding of Wnt7a to Frizzled-5 (Fz5) which is expressed during the peak of synaptogenesis. Expression of Fz5 during early stages of synaptogenesis increases the number of presynaptic sites in hippocampal neurons. Conversely, Fz5 knockdown blocks the ability of Wnt7a to stimulate synaptogenesis (Sahores et al., 2010). These findings demonstrate that Wnt7a acts as a retrograde signal to regulate presynaptic assembly through the binding of Fz5 and through Dvl- and Gsk3b-mediated signalling pathways. Interestingly, Wnts also play roles in synaptogenesis by inhibiting synapse formation. The balance between the synapse-promoting effects of Wnt7a and Wnt7b and synapse-inhibiting effects of Wnt5a

proteins are related to the activation of canonical and non-canonical Wnt signalling pathways, respectively (Davis et al., 2008). In the formation of specific sensory-motor neuron synapses, WNT-3, secreted by motor neuron dendrites, inhibits axonal extension, increases growth cone size and the clustering of the presynaptic marker, synapsin I, in the innervating sensory axons (Krylova et al., 2002). Because of the embryonic lethally of the *WNT-3*$^{-/-}$ mice, a conditional knockout mouse will be required to better address the implication in synapse formation of WNT-3 *in vivo* (Liu et al., 1999). Nevertheless, existing data suggest that the WNTs could act as a retrograde signal from postsynaptic neurons to regulate axonal remodeling and expression of synaptic proteins on presynaptic terminals.

2.3.3.1.1.2. FGF

The ability to cluster presynaptic vesicles in cultured neurons was used to identify FGF22 as a target-derived molecule that promotes differentiation of growth cones into presynaptic nerve terminals (Umemori et al., 2004). FGFs make up a family of polypeptide growth factors of 22 members with functions in a wide range of processes, including cell proliferation, migration, differentiation, tissue repair, and response to injury (Ornitz and Itoh, 2001). FGF22 and its closest relatives, FGF7 and FGF10, that are expressed by other subpopulations of neurons promote vesicle clustering and neurite branching but have no detectable effect on the length of neurites (Umemori et al., 2004). None of the other FGFs have these effects. Using blocking reagents and mice deficient in their main FGF receptor, FGFR2, these FGFs were confirmed to be involved in presynaptic differentiation in the cerebellum (Umemori et al., 2004). Recently it was shown that FGF22 and FGF7 specifically promote the organization of excitatory and inhibitory presynaptic terminals, respectively, as target-derived presynaptic organizers (Terauchi et al., 2010). FGF22 and FGF7 overexpression in cultured hippocampal neurons results in an increase in the clustering of vGlut1 and VGAT puncta, respectively. Strikingly, *FGF22*-deficient mice are resistant to epileptic seizures while *FGF7*-deficient mice are prone to them (Terauchi et al., 2010). This observation is consistent with the hypothesis that an alteration in the number of excitatory or inhibitory synapses or a change in the balance of these synapses is related to epilepsy-like behavior.

Overall, these results demonstrate a selective differentiation of glutamatergic and GABAergic presynaptic terminals by these target-derived molecules.

2.3.3.1.2. Glial-derived priming factors

Until recently, the function of astrocytes, that constitute nearly half of the cells in the brain, was a neurobiological mystery. The evidence that astrocytes directly enhance synapse formation has been obtained from a number of studies in different neuronal cell lines (van den Pol and Spencer, 2000; Nagler et al., 2001; Song et al., 2002) and supported by the fact that in many regions of the CNS, most synapses are formed after the differentiation of astrocytes and oligodendrocytes (Pfrieger and Barres, 1996; Ullian et al., 2004a). It has been shown that astrocyte membrane-bound factors promote synaptogenesis. Indeed, local contact of neurons with astrocytes promotes global synaptogenesis, with PKC signaling responsible for signal propagation (Hama et al., 2004). However, the first direct demonstration that astrocytes enhance the formation of new synapses or stabilize existing synapses through secreted factors was done by coculturing purified retinal ganglion cells (RGCs) with a feeder layer of astrocytes. The removal of the astrocytes resulted in the disappearance of the majority of synapses potentially due to the lack of release of soluble factors that prime neurons to undergo synaptogenesis (Ullian et al., 2001). Cholesterol complexed to apolipoprotein E (ApoE) was initially proposed to be one of these factors promoting synaptogenesis (Mauch et al., 2001). In the CNS, cholesterol is synthesized *in situ* rather than imported from blood (Kabara, 1973; Dietschy and Turley, 2001). Based on these evidences, it was proposed that the large amount of cholesterol needed for synaptogenesis is dependent on its production by glial cells and its delivery via ApoE-containing lipoproteins. However, another group found that treatment of neurons with either ApoE or cholesterol had no effect on synapse number while confirming that cholesterol enhanced synaptic efficacy (Ullian et al., 2004b; Christopherson et al., 2005). Thrombospondin family members (TSPs) were identified as another glial-derived factor family while comparing the ability of astrocyte-conditioned medium and astrocyte feeding layers to induce synapses in rat RGCs (Christopherson et al., 2005). The five TSPs are large oligomeric extracellular matrix proteins that mediate cell-cell and cell-matrix interactions by binding an array of

membrane receptors, other extracellular matrix proteins, and cytokines (Adams, 2001; Bornstein, 2001). Interestingly, TSP1 and TSP2 double null showed a reduced density of immunohistochemically identified synapses in the cortex (Christopherson et al., 2005). TSP1 and TSP2, as astrocyte-secreted proteins, are sufficient to induce the formation of ultrastructurally normal CNS synapses. The induced synapses are presynaptically active, but lack functional α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors, or so called postsynaptically silent, suggesting the existence of an unidentified astrocyte-derived signal necessary for postsynaptic development (Christopherson et al., 2005). Finally, glypican 4 and glypican 6 were identified as astrocyte-secreted signals sufficient to induce functional synapses between neurons, and it was shown that depletion of these molecules from astrocyte-conditioned medium significantly reduces their ability to induce postsynaptic activity (Allen et al., 2012). Another molecule, estrogen, synthesized and secreted by astrocytes was found to regulate synapse formation and synaptic transmission (Hu et al., 2007). The effect of estrogen in the promotion of synaptogenesis was already known (Brake et al., 2001; Yankova et al., 2001; Kretz et al., 2004), but the origin of estrogen was uncertain. The effect of astrocyte-conditioned medium on synaptic formation and transmission is blocked by an estrogen receptor antagonist, tamoxifen, in culture. Finally, two other astrocyte-derived factors, hevin and SPARC, have been characterized for their role in synapse development. Hevin induces the formation of synapses between cultured rat retinal ganglion cells while SPARC specifically antagonizes the synaptogenic function of hevin (Kucukdereli et al., 2011). Interestingly, Hevin null mice have fewer excitatory synapses; conversely, SPARC null mice have increased excitatory connections (Jones et al., 2011; Kucukdereli et al., 2011). Moreover, the loss of SPARC increases surface AMPA receptors levels at the excitatory synapses through its interaction with β-integrins (Jones et al., 2011). This study suggests that SPARC inhibits glutamate responsiveness of synapses by decreasing surface levels of AMPA glutamate receptors.

2.3.3.1.3. Neuronal pentraxin family

The neuronal pentraxin family consists of two secreted proteins, neuronal pentraxin (NP) 1 and NP2 (also known as neuronal-activity-regulated pentraxin (Narp)), and of the

neuronal pentraxin receptor (NPR), a presynaptic integral membrane protein. NP1 and NP2, which are expressed in both the developing and adult CNS, are homologous to the serum pentraxins, which are molecules involved in acute immunological responses (Schlimgen et al., 1995; Tsui et al., 1996; Dodds et al., 1997). NP2 was shown initially to induce neuronal migration and neurite outgrowth (Tsui et al., 1996) and later, to be secreted from both pre and postsynaptic neurons and selectively enriched at excitatory synapses (O'Brien et al., 1999). In an overexpression paradigm, NP2 can induce the aggregation of AMPA receptor subunits which is a major characteristic of excitatory synapse formation (O'Brien et al., 1999). Accordingly, a dominant-negative NP2 mutant that blocks the secretion of endogenous NP2 has a decreased ability to induce AMPA receptor (AMPAR) subunit clusters on contacted dendrites of spinal neurons (O'Brien et al., 2002). Moreover, NP1 and NP2 interact with AMPAR more precisely, with the N-terminal extracellular domain of the AMPAR subunit, GlurR4 (Xu et al., 2003; Sia et al., 2007). Furthermore, the membrane bound NPR also binds AMPAR and contributes to synapse formation. Mechanistically, NPR is cleaved and consequently released as a soluble form by the extracellular protease TACE (Cho et al., 2008b). Soluble NPR turns out to be essential for the internalization of AMPAR and for mGluR1/5-dependent long-term depression (LTD) in both the hippocampus and the cerebellum (Cho et al., 2008b). Knockdown of NP1 by siRNA or genetic knockout of all three neuronal pentraxins abolishes GluR4 clustering at reconstituted and neuronal synapses (Sia et al., 2007). At the systems level, studies using mice lacking NP1 and NP2 reveal defects in the segregation of eye-specific RGC projections to the dorsal lateral geniculate nucleus (Bjartmar et al., 2006). It was also shown that the presynaptic secretion of Narp from excitatory synapses increases synaptic strength by regulating levels of GluR4-containing AMPARs (Chang et al., 2010). More specifically, Narp prominently accumulated at excitatory synapses on parvalbumin-expressing interneurons. Increasing network activity resulted in a homeostatic increase of excitatory synaptic strength. This synaptic function of Narp is also reflected in the *Narp*$^{-/-}$ mice by an increased sensitivity to kindling-induced seizures suggesting a problem of balance between excitatory and inhibitory synapses (Chang et al., 2010).

2.3.3.2. Synaptic adhesion complexes

2.3.3.2.1. Cadherins

The cadherins constitute a superfamily including more than 100 members in vertebrates. They are grouped into subfamilies that are designated as classic cadherins, desmosomal cadherins, and protocadherins (Nollet et al., 2000). The largest subfamily, the protocadherins, is itself divided into several subgroups: α-, β-, γ-, flamingo (CELSR), fat-protocadherins (Redies et al., 2005). As candidates for target recognition and synapse formation, the protocadherins are interesting because they undergo alternative splicing and have region-specific expression patterns (Wu and Maniatis, 1999; Wang et al., 2002a). While their expression is not restricted to synaptic sites, there are at least some isoforms present at the synapse (Phillips et al., 2003). Protocadherin variants were also proposed to mediate isoform-specific homophilic interactions (Fernandez-Monreal et al., 2009). Unfortunately, the ablation of multiple members of this family did not result in any defects in synaptogenesis or any alterations in synaptic ultrastructure (Wang et al., 2002b). The protocadherins seem to rather contribute to neuron-glia junctions (Garrett and Weiner, 2009). Although the currently available expression profile of the protocadherins at the synapse is clear, the significance of their molecular diversity for synapse formation has remained obscure and requires further study.

Several members of a second subfamily of cadherins, the classical cadherins, have been shown to have a synaptic expression pattern (Obst-Pernberg and Redies, 1999; Junghans et al., 2005), with cadherins and their cytosolic partner, catenin, localized to both sides of the synapse. Classical cadherins are able to dimerize both between molecules presented on the same cell (*cis*) and between molecules from different cells (*trans*) (Shapiro et al., 1995). About 20 members of this subfamily are expressed in the CNS (Yagi and Takeichi, 2000), but neural N-cadherin (Ncad) is the most widely expressed and best-studied subtype. Indeed, Ncad is known to be present at the synapse since biochemical approaches revealed its expression as a major glycoprotein in isolated PSD fractions (Beesley et al., 1995). In regard to its role in synapse formation, Ncad is clustered at nascent synapses in all immature neurons *in vitro*, but is progressively lost from inhibitory synapses during development to become restricted to excitatory synapses in adult cells

(Benson and Tanaka, 1998). In zebrafish, Ncad has been shown to be transported in association with active zone components, to form stable puncta in the wake of the migrating growth cones (Jontes et al., 2004). In cultured hippocampal neurons, the blockade of Ncad leads to a reduction in synapsin puncta and a diffused distribution of synapsin in axons (Togashi et al., 2002). Despite all these interesting features, Ncad on its own is not able to induce synapse formation in an artificial synapse formation assay (Scheiffele et al., 2000; Sara et al., 2005). Moreover, when the Ncad gene was conditionally deleted in the mouse cerebral cortex, although the laminar identity and layer structures were lost, neurons appeared to form normal synapses with normal levels of pre and postsynaptic proteins (Kadowaki et al., 2007). *In vitro* neuronal differentiation of mouse embryonic stem cells from *Ncad*$^{-/-}$ mice, which die at birth, also reveals that neither the initial formation nor the ultrastructural characteristics of synapses are altered (Jungling et al., 2006). The current model for the synaptic role for Ncad is that this CAM first plays an important role in supporting adhesion between apposed membranes prior to synaptogenesis, and at later stages, is involved in synaptic plasticity. Interestingly, two groups recently suggested that cadherin might enhance synapse formation by recruiting and functionally interacting with another molecule at the synapse, neuroligin-1 (Aiga et al., 2010; Stan et al., 2010). These papers open the exciting question of the functional interplay between cadherins and other synaptic cell adhesion systems.

2.3.3.2.2. Nectins

While intercellular junctions in the nervous system mostly consist of an axon and a dendrite, certain cases of synapses between dendrites do exist (Kaba and Nakanishi, 1995; Gulyas et al., 1996; Lohmann and Wong, 2001). For example, dendrodendritic microcircuits in the olfactory bulb mediate feedback to and lateral inhibition of the mitral cells, the neurons that transmit olfactory sensory information to higher brain regions (Shepherd et al., 2007). CAMs are known to link similar or "like" cells, thus it is interesting that certain CAMs only participate in axodendritic synaptic junction formation and not in homotyptic dendrodendritic connections. The nectin family of CAMs provide an example to explain how certain CAMs can regulate the type of synapse that is formed. The four family members of the Nectin family form homodimers in *cis*,

which then heterodimerize in *trans,* forming tetramers to create adhesive contact between cells (Takahashi et al., 1999; Miyahara et al., 2000; Satoh-Horikawa et al., 2000; Reymond et al., 2001). Nectin-1 is located at the synapse predominantly in the presynaptic membrane, and nectin-3 is at the postsynaptic membrane (Mizoguchi et al., 2002). It is by way of recruitment of cadherins that the nectins can suppress dendrodendritic synapse formation. During the formation of an axodendritic synapse, the trans-interaction of nectin promotes the recruitment of cadherin molecules (Tachibana et al., 2000; Honda et al., 2003). In neurons, cadherins are localized to both axons and dendrites, but curiously, are not implicated in the formation of contacts between dendritic spines. The current model suggests that the axon-biased localization of nectin-1 and its trans-interaction with nectin-3 on the dendrite promotes homophilic cadherin–cadherin interactions and strengthens synaptic junctions. In the case of dendrodendritic interactions, the homophilic interactions between nectin-3 would not be stable enough to recruit cadherin (Togashi et al., 2006). Thus, dendrodendritic synapses cannot be maintained by nectin-3 alone, as cadherin homophilic interactions will not be formed to strengthen the cell-cell adhesion.

2.3.3.2.3. NCAM

Several lines of evidence suggest NCAM participation in mechanisms of synaptogenesis. For instance, in cultured hippocampal neurons, NCAM accumulates rapidly, within minutes, at sites of contact formation in nascent synapses (Sytnyk et al., 2002). Another study, using heterogenotypic cultures of neurons from *NCAM*$^{-/-}$ and wild-type mice resulted in a reduced number of synapses compared to a homogenotypic culture of only wild-type neurons, indicating preferential formation of synapses with NCAM expressing cells (Dityatev et al., 2000). Also, the treatment of hippocampal cultures with a synthetic peptide that represents a part of the binding site of NCAM to the FGF receptor-1, known to stimulate neurite outgrowth (Kiselyov et al., 2003), enhanced the number of new synapses (Cambon et al., 2004). In addition, *NCAM*$^{-/-}$ mice show deficits in learning and memory, processes that rely heavily on synaptic function (Cremer et al., 1994). In the brain, NCAM undergoes a post translational modification whereby polysialic acid (PSA) chains are added to NCAM disrupting the adhesive properties of NCAM (Hildebrandt et

al., 2010). Indeed, PSA chains in general have been implicated in neuronal migration (Ono et al., 1994), axonal fasciculation, branching (Yamamoto et al., 2000), guidance (Tang et al., 1992), synaptogenesis (Dityatev et al., 2004) and activity-dependent plasticity (Eckhardt et al., 2000). The current model suggests that PSA-NCAM, by interfering with NCAM homophilic binding, reduces adhesive connections but plays a permissive role in promoting growth and reorganization of postsynaptic structures required for the formation of new contacts (Muller et al., 2010). Accordingly, elimination of PSA from NCAM in visual cortex results in earlier onset of synaptic adhesion leading to premature onset of ocular dominance plasticity and modification of synaptic transmission (Di Cristo et al., 2007).

2.3.3.2.4. Neurexins and Neuroligins

Neurexins were first discovered as the receptor for α-latrotoxin, a toxin found in black widow spider venom, which binds to presynaptic nerve terminals and triggers massive neurotransmitter release (Ushkaryov et al., 1992). In mammals, six main neurexin isoforms are derived from three genes (neurexin1 to -3) having two different promoters each, giving rise to an alpha (α), long isoform and a beta (β), short isoform (Ushkaryov et al., 1994; Tabuchi and Sudhof, 2002). α- and β-neurexins are presynaptic type 1 membrane proteins composed of canonical sets of domains. Whereas neurexins are still thought to function primarily presynaptically, certain publications have speculated that neurexins are postsynaptic regulators as well (Peng et al., 2004; Taniguchi et al., 2007). Indeed, neurexin has been shown to directly bind and specifically inhibit postsynaptic $GABA_A$ receptors in a neuroligin-independent mechanism (Zhang et al., 2010).) The large extracellular domain of α-neurexins contains up to six LNS domains (for laminin A, neurexins, and sex hormone-binding globulin (Sasaki et al., 1988; Joseph and Baker, 1992)), with epidermal growth factor (EGF) domains located after the first, third, and fifth LNS domains, whereas β-neurexins possess only the last LNS and EGF domains. The short cytoplasmic tail of α- and β-neurexins contains a PDZ binding motif that binds to CASK, a hybrid kinase/MAGUK protein (Hata et al., 1996). Alternative splicing occurring at five splice sites in α-neurexin and two in β-neurexins contributes to making neurexins one of the most diverse protein families of the mammalian nervous system,

with over 1000 isoforms (Ushkaryov and Sudhof, 1993; Ullrich et al., 1995). With different isoforms expressed in distinct cell populations, neurexins were rapidly seen as potential candidates for neuronal cell recognition in early steps of synapse formation (Missler and Sudhof, 1998b). This hypothesis was supported by the results of *in vitro* assays where synapse-inducing activity was first demonstrated for β-neurexins (Scheiffele et al., 2000; Dean et al., 2003; Graf et al., 2004) and these observations were extended to α-neurexins (Boucard et al., 2005; Kang et al., 2008). The first identified ligand for the neurexins was neuroligin-1 (Ichtchenko et al., 1995). Neuroligins are postsynaptic type I membrane proteins with an extracellular domain largely composed of a single esterase-like domain. The relatively short intracellular domain of neuroligins includes a PDZ-domain binding sequence that recruits different PDZ-domain-containing proteins, such as postsynaptic density protein-95 (PSD-95), and a tyrosine-based motif that binds to gephyrin (Ichtchenko et al., 1996; Irie et al., 1997; Poulopoulos et al., 2009). In vertebrates, four genes encode the neuroligins, neuroligin-1 to -4 (except in humans and higher primates where a fifth neuroligin also exists (Graves, 2006)). All the neuroligins can bind α- and β-neurexins to either the sixth LNS domain of the α-neurexins or the single LNS domain of the β-neurexins (Boucard et al., 2005). Indeed, the binding is facilitated by the splice variation of β-neurexin that lacks an insert in the splice site 4 (SS4) (Nguyen and Sudhof, 1997). Neuroligin mRNAs are also susceptible to splicing at two positions, referred to as A and B (Ichtchenko et al., 1995). The splice variant with no insert in B was shown to bind to all β-neurexins and presumably to all α-neurexins (Ichtchenko et al., 1996; Boucard et al., 2005). Among neuroligin-1, SynCAM, EphB2, NGL-2 and N-Cadherin, only neuroligin-1ΔB recruits the presynaptic marker Bassoon within 1 hour in a mixed-culture assay (Lee et al., 2010). The importance of splicing for these interactions was confirmed by mutational analysis and crystal structure work (Sheckler et al., 2006; Koehnke et al., 2008; Reissner et al., 2008; Shen et al., 2008). Different neuroligins have specific expression patterns. Neuroligin-1 localizes primarily at glutamatergic synapses (Song et al., 1999) while neuroligin-2 is primarily at GABAergic synapses (Graf et al., 2004; Varoqueaux et al., 2004). The expression level of neuroligin-3, localized at both glutamatergic and GABAergic synapses, coincides with the peak of synaptogenesis during postnatal development.(Budreck and Scheiffele, 2007). Finally,

neuroligin-4 is also present at glutamatergic synapses and was recently shown to also be localized to inhibitory glycinergic postsynaptic membranes (Hoon et al., 2011).

Using the mixed-culture system, in which primary neurons are combined with non-neuronal cells transfected with a cDNA encoding the protein of interest (Biederer and Scheiffele, 2007), neuroligin-1 and -2 were found to trigger the *de novo* formation of presynaptic structures that can be block by the addition of soluble β-neurexin to this coculture (Scheiffele et al., 2000). Likewise, the contact of neurons with neurexin-expressing nonneuronal cells recruits postsynaptic markers (Graf et al., 2004; Nam and Chen, 2005). In order to further study the synaptic role of neurexins and neuroligins, loss-of-function experiments were required. Unfortunately, different RNAi-mediated knockdown experiments yielded mixed results. In fact, the loss of a single neurexin or neuroligin results in some studies in a dramatic decrease in synapses number in rodent neurons (Chih et al., 2005; de Wit et al., 2009; Shipman et al., 2011) while RNAi-mediated knockdown did not lead to any effect in other studies (Zhang et al., 2010; Ko et al., 2011; Soler-Llavina et al., 2011). On the other hand, gain-of-function approaches demonstrated that overexpression of neurexin-1β induced differentiation of postsynaptic receptors (Graf et al., 2004) and that overexpression of neuroligin-1 promotes excitatory synapse formation while neuroligin-2 stimulates the formation of inhibitory terminals (Prange et al., 2004; Chih et al., 2005; Chubykin et al., 2007). Transgenic mice overexpressing neuroligin-1 show an increased ratio of excitation to inhibition accompanied with significant deficits in memory acquisition, while neuroligin-2 expression results in an increase in inhibitory synaptic markers (and to a lesser extent in excitatory presynaptic markers), as well as in the maturation and transmission of inhibitory synapses (Hines et al., 2008; Dahlhaus et al., 2010).

Using the triple-knockout mice of α-neurexins, which die perinatally, presumably of a strong impairment in neurotransmitter release, it was shown that α-neurexins are not required for the initial induction of synapse formation, per se. In fact, these mice display no major decrease in the number of excitatory synapses, with only a moderate decrease in inhibitory synapses with synapses that are ultrastructurally normal (Missler et al., 2003; Dudanova et al., 2007). However, a dramatic reduction in spontaneous and evoked

neurotransmission at both glutamatergic and GABAergic synapses is observed in the triple knockout (Missler et al., 2003). Also a reduction in NMDA-receptor-mediated postsynaptic currents without affecting AMPA-receptor-mediated currents is seen in these mice (Kattenstroth et al., 2004). It was also shown that the deletion of only neurexin-1α generates mice with electrophysiological phenotypes that are associated with pervasive behavioral abnormalities (Etherton et al., 2009). Interestingly, these mice exhibit increased repetitive grooming behaviors. It is suggested that the lack of α-neurexins cause a reduced function of the N-type calcium channels at the synaptic active zones. The fact that α-neurexins perform a non-redundant role in calcium-dependent neurotransmitter release was confirmed by transgenic rescue experiments. Indeed, α-neurexin KO mice overexpressing only neurexin-1α have normal synaptic transmission and Ca^{2+} currents while overexpression of neurexin-1β has no effect (Zhang et al., 2005b). On the other side of the synaptic cleft, the neuroligin-1, -2 and -3 triple knockout mice die shortly after birth, likely because of an impairment of transmission (Varoqueaux et al., 2006). Similar to the triple α-neurexin knockout mice, electrophysiological and morphological findings indicate that the initial formation of synaptic contacts does not depend on neuroligins. It was rather observed that the loss of neuroligins results in a dramatic decrease in spontaneous GABAergic and glycinergic activity and a moderate reduction in spontaneous glutamatergic activity in the respiratory brainstem, causing respiratory failure (Varoqueaux et al., 2006). Reduction in both GABAergic and glycinergic inhibitory transmission was found in the absence of neuroligin-2 while glutamatergic transmission remained unaffected, which is consistent with neuroligin-2 localization (Chubykin et al., 2007; Poulopoulos et al., 2009). Moreover, anxiety-like behavior is increased in these mice (Blundell et al., 2009). Interestingly, the deletion of neuroligin-2 affects only a subtype of inhibitory neurons (Gibson et al., 2009). In regard to the single knockout of neuroligin-1, a reduction of NMDAR-dependent synaptic responses compared with AMPAR-dependent responses is found whereas there is no effect on inhibitory synapses (Chubykin et al., 2007). Again synapse number is not significantly affected in these mice (Varoqueaux et al., 2006).

Altogether, the current model proposes that neurexin-neuroligin interactions are more likely to participate in activity-dependent modulation, in terms of the maturation, remodeling, and specification of synapses, rather than in *de novo* synaptogenesis (reviewed in (Sudhof, 2008; Missler et al., 2012)). This proposed view is very nicely illustrated by recent work done in *Aplysia* (Choi et al., 2011) where depleting neurexin in the presynaptic sensory neuron or neuroligin in the postsynaptic motor neuron abolishes both long-term facilitation and the associated presynaptic growth induced by repeated pulses of serotonin.

2.3.3.2.5. Tripartite complexe neurexin/Cbln1/GluRδ2

Neurexins also bind the secreted protein, cerebellin 1 precursor protein (Cbln1) (Uemura et al., 2010). The Cbln family consists of four members that are expressed in various brain regions and accumulate in synaptic clefts (Hirai et al., 2005; Miura et al., 2006; Miura et al., 2009). Cbln1 binds the N-terminal domain of the glutamate receptor (GluR) δ2 located on the postsynaptic membrane (Matsuda et al., 2010; Uemura et al., 2010). Although its structure is similar to ionotropic glutamate receptors, GluRδ2 has no channel function. Instead, studies have recently suggested that GluRδ2 acts as an adhesion molecule (Uemura and Mishina, 2008; Mandolesi et al., 2009). There is clear *in vivo* evidence that GluRδ2 plays an essential role synapse formation through neurexin and Cbln1 signaling (Kashiwabuchi et al., 1995; Kurihara et al., 1997; Takeuchi et al., 2005; Uemura et al., 2010). For example, in Cbln1 mutant mice, analysis of cerebellar synapses by electron microscopy revealed the appearance of naked spines lacking presynaptic contacts and mismatched synapses with expanded PSDs over active zones (Uemura et al., 2010). Thus, the characterization of a trimeric complex comprising neurexin, Cbln and GluRδ2 shed light on the mechanism of synapse formation in the cerebellum as well as in various other brain regions. It also highlights a third type of synaptic organizer distinct from cell adhesion molecules, which directly links pre- and postsynaptic elements, and soluble factors functioning on either the pre- or postsynaptic sites. Secreted Cbln1 is sandwiched between presynaptic neurexin and postsynaptic GluRδ2 and serves as a bidirectional synaptic organizer. Recent work went even further in the characterization of this trans-synaptic triad. It was found that the synaptogenic triad is

assembled from tetrameric GluRδ2, hexameric Cbln1, and monomeric NRXN in the ratio of 1:2:4 (Lee et al., 2012). This phenomenon is reminiscent of the binding of the secreted leucine-rich glioma inactivated 1 (LGI1) to both pre- and postsynaptic receptors: a disintegrin and metalloproteinase (ADAM) 22 and ADAM23, respectively (Fukata et al., 2010).

2.3.3.2.6. Neurexins and Dystroglycans

Dystroglycan was identified to be another binding partner for neurexin through affinity chromatography of brain lysate using immobilized neurexin. The interaction between α- and β-neurexins and dystroglycan was confirmed and found to be located to a single LNS domain (Sugita et al., 2001). Dystroglycan is composed of two subunits (α- and β-dystroglycan), bound to each other on the cell surface, and they are derived by proteolytic cleavage from a single precursor (Ibraghimov-Beskrovnaya et al., 1992). Dystroglycan links the intracellular actin cytoskeleton to the extracellular matrix by binding intracellularly to dystrophin and utrophin, which in turn are connected to the actin cytoskeleton (Ahn and Kunkel, 1993), and by binding extracellularly to the LNS domains of several extracellular matrix proteins, namely neurexin, laminin, agrin, and perlecan (Gee et al., 1994; Henry et al., 2001). Dystroglycan is selectively associated with a subset of inhibitory GABAergic synapses but is not detectable at excitatory glutamatergic synapses (Levi et al., 2002). Altogether, dystroglycan could represent a good candidate to mediate neurexin-dependent postsynaptic inhibitory differentiation along with neuroligin-2. However, neither α- nor β-dystroglycan was clustered by neurexin in the coculture assay (Graf et al., 2004). Moreover, deletion of brain dystroglycan is not essential for GABAeric synaptogenesis (Moore et al., 2002). These results suggest that dystroglycan is not essential for the neurexin-induced clustering of postsynaptic proteins, but the interaction between neurexin and dystroglycan may have a unique function in synapse development.

2.3.3.2.7. Neurexins and Neurexophilins

α-neurexin is also tightly complexed to a secreted glycoprotein called neurexophilin-1 (Petrenko et al., 1993). Four related genes code for neurexophilins in mammals, referred

to as neurexophilins 1-4 (Petrenko et al., 1996), but only neurexophilin-1 and -3 interact biochemically with α-neurexins. Furthermore, neurexophilin-1 and -3 occupy the same binding site as dystroglycan on neurexin, but bind with a higher affinity, suggesting an antagonistic relationship between neurexophilin and dystroglycan (Missler and Sudhof, 1998a). Neither obvious abnormalities nor changes in synaptic protein patterns were found in *neurexophilin-3*$^{-/-}$ brains (Beglopoulos et al., 2005). However, impairments in sensorimotor gating and motor coordination tasks were observed in these mutant mice. Nonetheless, it remains unclear whether synaptic neurexophilins are involved in synaptic transmission as modulator molecules of neurexin activity.

2.3.3.2.8. Neurexins and LRRTMs

The most recently identified binding partners for the neurexins are the leucine-rich repeat transmembrane (LRRTM) proteins. Using bioinformatics tools, four novel genes encoding LRRTMs were identified for their similarity to the repulsive guidance cue, Slit (Lauren et al., 2003). LRRTMs are typical cell surface proteins with a signal sequence, 10 extracellular LRRs flanked by cysteine-rich domains and a transmembrane region followed by an intracellular tail that bears similarity to a PDZ domain binding motif. LRRTMs localize to excitatory synapses and can instruct excitatory presynaptic differentiation (de Wit et al., 2009; Ko et al., 2009; Linhoff et al., 2009). Overexpression of LRRTM2 in neurons increases excitatory, but not inhibitory, synapse numbers (Ko et al., 2009; Linhoff et al., 2009) and, conversely, LRRTM2 knockdown results in a reduction of excitatory synapses without affecting inhibitory synapse density (de Wit et al., 2009). Moreover, deletion of LRRTM1 in mice leads to a subtle phenotype where the distribution of the vesicular glutamate transporter in the hippocampus is altered (Linhoff et al., 2009). Thus, knockout of multiple family members may be required to reveal a stronger phenotype. Binding experiments showed that all the four LRRTM proteins bind to neurexin-1β (Ko et al., 2009) and that only neurexin-1, but not neurexin-2 or -3 acts as a presynaptic receptor for LRRTM (de Wit et al., 2009). However, a cell surface binding assay partially contradicts these findings by presenting evidence that LRRTM2 binds neurexin-1, -2, and -3 α and β (Siddiqui et al., 2010). Interestingly, LRRTM binding to neurexin is highly competitive with neuroligin since both proteins cannot bind

simultaneously to neurexin (Ko et al., 2009; Siddiqui et al., 2010). In cultured neurons, only the combined loss-of-function of neuroligin-1 and -3 and LRRTM2 and LRRTM3 caused a decrease in excitatory, but not inhibitory, synaptic density (Ko et al., 2011). This observation is hard to reconcile with the data showing that the knockdown of LRRTM2 alone results in a reduction in the number of excitatory synapses (de Wit et al., 2009). An *in vivo* guided injection of a lentivirus capable of expressing three shRNAs simultaneously (neuroligin-3, LRRTM2 and LRRTM3) into the hippocampal CA1 region of neuroligin-1 KO mice caused a substantial decrease in excitatory transmission (Soler-Llavina et al., 2011). Strikingly, the density of synapses was unaffected. It is suggested that neuroligins and LRRTMs can partly functionally compensate for each other during development, but they can also perform distinct functions at mature excitatory synapses (Soler-Llavina et al., 2011). In the future, it would be interesting to explore whether these two non-similar classes of molecules that bind to the same receptor, neurexin, also share postsynaptic signaling pathways.

2.3.3.2.9. SALMs

The synaptic adhesion-like molecule (SALM) family contains five identified members, SALM1 to SALM5 (Ko et al., 2006; Morimura et al., 2006; Wang et al., 2006), which have the ability to interact with each other (Seabold et al., 2008). The domain structure of the SALMs includes six extracellular LRR domains, an immunoglobulin C2-like domain, a fibronectin type III domain, and a transmembrane region. SALM1, SALM2 and SALM3 additionally contain an intracellular PDZ binding domain. The synaptic localization of SALM proteins has been demonstrated by several approaches (Ko et al., 2006; Wang et al., 2006; Seabold et al., 2008; Mah et al., 2010). SALM1 forms a complex with PSD-95 *in vivo* and also interacts directly with the NR1 subunit of NMDA receptors, but not with AMPA receptors. In this way, SALM1 promotes dendritic clustering of NMDA receptors in cultured neurons (Ko et al., 2006; Wang et al., 2006), suggesting a role for SALM1 in the recruitment of NMDA receptors to early synapses. In contrast, SALM2 associates with AMPA receptors and, to a lesser extent, with NMDA receptors (Ko et al., 2006). During excitatory synaptic development, AMPA receptor clustering at the synapse occurs at later stages suggesting that SALM2 might promote maturation,

rather than initial formation, of synapses. Consistently, SALM2 lacks the ability to induce presynaptic differentiation in mixed-culture assays (Ko et al., 2006) while SALM3 and SALM5 have the potential to do so (Mah et al., 2010). Moreover, SALM2 overexpression at early stages (6-12 days *in vitro* (DIV)) in cultured neurons did not have any effect on synapse number, while it had a significant effect at later stages (12-18 DIV), increasing the number of excitatory synapses. However, the number of both excitatory and inhibitory presynaptic contacts is increased when SALM3 and SALM5 are overexpressed (Mah et al., 2010). SALM2 knockdown in neurons reduces the number of excitatory synapses (Ko et al., 2006) while knockdown of SALM5 reduces the number of excitatory and inhibitory synapses (Mah et al., 2010). Antibody-induced clustering of SALM3 and not SALM5, which lacks the PDZ binding domain, on dendrites induces co-clustering of PDS-95. In the near future, the characterization of the *SALM*-deficient mice or transgenic mouse models will shed light on the function of individual SALMs and in their involvement in synapse formation.

2.3.3.2.10. SynCAMs

Containing three extracellular immunoglobulin (Ig)-like domains and an intracellular PDZ consensus motif, the different members of the synaptic cell adhesion molecule (SynCAM) family have been identified by a number of independent approaches in different systems (Pletcher et al., 2001; Urase et al., 2001; Wakayama et al., 2001; Kakunaga et al., 2005). The SynCAM family comprises four genes that are found solely in vertebrates (Biederer, 2006). Early reports state that SynCAM proteins are predominantly expressed by the brain and are located at the pre- and postsynaptic sites of both excitatory and inhibitory synapses (Biederer et al., 2002; Fogel et al., 2007; Thomas et al., 2008). Consistent with its localization at the synapse, SynCAM-1 was shown to induce presynaptic specializations dependent on the Ig domains using the mixed-culture of neurons and heterologous cell lines (Biederer et al., 2002). It was later demonstrated that this induction of synapses was likely to rely on SynCAM self-assembly in *cis* (Fogel et al., 2011). Indeed, SynCAM proteins engage each other in highly specific homophilic and heterophilic interactions (Biederer et al., 2002; Fogel et al., 2007). Overexpression and ablation of SynCAM-1 in mice specifically increases or decreases,

respectively, excitatory synapse numbers without altering their ultrastructure, while the number of inhibitory synapses is unaffected (Robbins et al., 2010). Nevertheless, the increase in excitatory synapses is only a modest 20% from an 8-fold increase of protein compared to around 100% increase from only a 2-fold increase for neuroligin-1 overexpressing mice (Robbins et al., 2010). Surprisingly, SynCAM-1 overexpressors have impairments in spatial learning and memory while SynCAM-1 KO mice exhibit significantly enhanced spatial memory compared to wild-type (Robbins et al., 2010). Recently, it was shown that SynCAM-1 performs successive functions in developing neurons, from shaping growth cones to the assembly of axo-dendritic contacts (Stagi et al., 2010). In summary, contrarily to other synaptogenic molecules, SynCAMs are early players in axo-dendritic contact differentiation. Later during synapse maturation, SynCAM proteins are engaged in specific homo- and heterophilic adhesive interactions, representing a trans-interacting adhesion system.

2.3.3.2.11. NGLs

Netrin-G/laminet is a family of two CAMs, netrin-G1 and netrin-G2. Structurally, they are similar to the classical axon guidance molecules, netrins, but they diverge in that they do not bind to known netrin receptors, and in that they are glycosylphosphatidylinositol (GPI) anchors (Nakashiba et al., 2000). Netrin-G1 ligand (NGL-1) and netrin-G2 ligand (NGL-2) have been found to be the receptors for the two members of the netrin-G/laminet family in an isoform-specific manner (Lin et al., 2003; Zhang et al., 2005a; Kim et al., 2006). Vertebrates have three NGL proteins, NGL1-3, and their expression is mainly restricted to the brain. NGLs are putative type I transmembrane proteins with nine LRRs followed by one Ig domain in the extracellular segment, and with a PDZ domain binding motif in the cytoplasmic region that interacts with PSD-95 (Lin et al., 2003; Kim et al., 2006). This interaction promotes the localization of NGLs to the postsynaptic membrane (Cheng et al., 2006). In addition, NGL proteins are mainly detected at postsynaptic sites of excitatory, but not inhibitory, synapses (Kim et al., 2006). This synaptic localization suggested a role for these proteins in synaptogenesis that was supported by induction of functional presynaptic differentiation in contacting neurites of NGL-2 and NGL-3 expressing heterologous cells (Kim et al., 2006; Woo et

al., 2009). For instance, when overexpressed in cultured neurons, NGL-3 increases excitatory, but not inhibitory, presynaptic contacts while the loss of NGL-3 reduces excitatory synapse number (Woo et al., 2009). Interestingly, NGL-2 and netrin-G2 knockout mice show mild behavioral defects but have an identical phenotype of impaired startle response to acoustic stimuli, supporting the notion that they functionally interact (Zhang et al., 2008b). To date, the analysis of the synapses in NGLs and netrin-Gs knockout mice is still not published in detail except for the observation that NGL-1 and -2 are diffused along the dendrites in netrin-G1 and netrin-G2 deficient mice (Nishimura-Akiyoshi et al., 2007).

While NGL-1 binds to netrin-G1, and NGL-2 to netrin-G2, NGL-3 interacts with neither netrin-G1 nor netrin-G2. NGL-3 instead interacts with the leukocyte common antigen-related (LAR) subfamily of receptor protein tyrosine phosphatases (PTPs). This subfamily is composed of three vertebrate homologs, LAR, PTP-σ, and PTP-δ (Chagnon et al., 2004) and NGL-3 binds all three of these proteins (Woo et al., 2009; Kwon et al., 2010). Application of soluble LAR in a mixed-culture assay inhibits NGL-3-dependent presynaptic induction. In contrast to netrin-G2 that does not induce postsynaptic clustering when expressed in the coculture system (suggesting the requirement of an additional protein), LAR alone can trigger postsynaptic clustering (Woo et al., 2009; Kwon et al., 2010). Also, ablating LAR expression in hippocampal cultures leads to a decreased number and function of excitatory synapses (Dunah et al., 2005). Recently, it was found that presynaptic PTP-σ binds in *trans* to the postsynaptic neurotrophin receptor tropomyosin-related kinase (Trk) C (Takahashi et al., 2011). This bidirectional synaptic interaction between TrkC and PTP-σ is required in excitatory, but not inhibitory, synapse formation. Altogether, the precise mechanism of how NGLs are involved in synaptogenesis is still not fully understood, but the trans-binding interaction to the presynaptic LAR family is a promising field of study as these phosphatases are now found to be part of multiple pathways. Finally, it should be noted that NGL-3 binds to the first two PDZ domains of PDS-95, whereas neuroligins bind to the third PDZ domain (Irie et al., 1997). Hence, NGLs and neuroligins could simultaneously bind to a single molecule of PSD-95 and therefore modulate each other's effects.

2.3.3.2.12. Ephrins and Eph Receptors

The first Eph receptor (EphAl) was identified in 1987, whereas their ephrin ligands were cloned in the mid-90s (Flanagan and Vanderhaeghen, 1998). Since then, Ephs and ephrins have been implicated in numerous developmental processes of the nervous system (Reber et al., 2007). Eph receptors consist of two subclasses, EphA and EphB that are distinguished based on their binding to GPI-anchored ephrinA and to transmembrane ephrinB, respectively, with the exception of EphA4 which binds to both classes of ephrins. One unique feature of the Eph-ephrin signaling is the possibility for the Eph receptor to also act as a ligand in the same manner that an ephrin ligand can act as a receptor. This so called "reverse signaling" becomes very useful during synaptogenesis where signaling cascades are needed on both sides of the synaptic cleft. More specifically, EphA-ephrinA signaling is involved in regulation of spine length and retraction of spines in hippocampal neurons (Murai et al., 2003; Fu et al., 2007). Indeed, activation of EphA4 forward signaling reduces spine length, whereas inhibition of EphA4 signaling increases spine length (Murai et al., 2003). Even though the activation of EphA by ephrinAs does not directly form synapses, the modification of dendritic spines indirectly affects synaptogenesis. In contrast, activation of EphB-ephrinB signaling promotes excitatory synapse formation with EphB forward signaling controlling dendritic filopodia motility, potentially allowing pre- and postsynaptic partners to initiate contact, and after EphB trans-synaptic interactions stabilizing nascent synaptic contacts (Kayser et al., 2008). Neurons cultured from *EphB1*$^{-/-}$, *EphB2*$^{-/-}$, *EphB3*$^{-/-}$ triple-knockout mice have a major reduction of postsynaptic dendritic filopodia motility. The increased filopodia motility initiated by EphBs signaling engage several Rho family GTPases, thereby remodeling the actin cytoskeleton of postsynaptic spines (Kayser et al., 2008; Lai and Ip, 2009). Furthermore, EphrinB binding to EphB induces a direct extracellular interaction between EphB and NMDARs (Dalva et al., 2000), resulting in NMDAR clustering (Takasu et al., 2002). In mature and synaptically active neurons, EphB receptor stimulation leads to an activation of Src family kinases which in turn phosphorylate NMDAR subunits resulting in increased channel gating (Grunwald et al., 2001). In the hippocampus of *EphB1*$^{-/-}$, *EphB2*$^{-/-}$, *EphB3*$^{-/-}$ triple-knockout mice,

dendritic spine development is impaired with less dendritic NMDAR and AMPAR clusters detected (Henkemeyer et al., 2003; Kayser et al., 2006). Overall, activation of ephrins by Eph receptors can induce synapse formation and spine morphogenesis, whereas in the mature nervous system, ephrin signaling modulates synaptic function and long-term changes in synaptic strength (Hruska and Dalva, 2012). However, there is still no explanation for how repulsive guidance by Eph/ephrins is converted into adhesive responses during synapse formation.

2.3.3.2.13. FLRTs

As mentioned above, neurexins were first discovered as the receptor for α-latrotoxin, which binds to presynaptic nerve terminals and triggers massive neurotransmitter release (Ushkaryov et al., 1992). However, another receptor for α-latrotoxin has been reported: latrophilins (Krasnoperov et al., 1997; Lelianova et al., 1997). The latrophilin family consists of three isoforms with a similar domain organization, consisting of a G protein-coupled receptor (GPCR) subunit and an unusually large adhesion-like extracellular N-terminal fragment with lectin, olfactomedin, and hormone receptor domains (Sugita et al., 1998; Ichtchenko et al., 1999). Though much effort has been expended investigating these molecules, it was only recently that the fibronectin leucine-rich repeat transmembrane (FLRT) proteins were identified as the endogenous ligands for latrophilins (O'Sullivan et al., 2012). The FLRTs, a family composed of three isoforms, are single-pass transmembrane proteins with ten extracellular leucine-rich repeat domains and a juxtamembrane fibronectin type 3 domain (Lacy et al., 1999). Roles in axon guidance and cell migration, through Unc5 proteins, were recently reported for the FLRTs (Yamagishi et al., 2011). Interestingly, FLRT3 is located on the postsynaptic membrane where it interacts with presynaptic latrophilin3 (O'Sullivan et al., 2012). Knockdown of FLRT3 results in a highly significant reduction in dendritic protrusion density relative to controls, which is associated with a decrease in the strength of synaptic input onto these cells. These results suggest a dual function for the FLRT in mediating both axon guidance and synapse formation.

2.3.3.2.14. IL1RAPL1 and IL-1RAcP

In addition to the regulation of innate and adaptive immune responses, the interleukin-1 (IL-1) family of cytokines is involved in sleep regulation, learning, memory, stress response, and control of lipid metabolism in the brain (Horai et al., 1998; Rothwell and Luheshi, 2000; Goshen and Yirmiya, 2009). A member of the receptor complex for IL-1, IL1-receptor accessory protein-like 1 (IL1RAPL1), was initially identified as the product of an X-linked gene responsible for a nonsyndromic form of mental retardation. The IL1RAPL1 knock-out mice have impaired hippocampal long-term potentiation and a decrease in number of hippocampal dendritic spines (Pavlowsky et al., 2010). In the zebrafish, IL1RAPL1 regulates synapse formation in vivo in olfactory sensory neurons (Yoshida and Mishina, 2008). It was later demonstrated that IL1RAPL1 also plays a role in synapse formation in mammalian neurons (Yoshida et al., 2011). Notably, this synaptogenic activity of IL1RAPL1 is specific for excitatory synapses in the neuron-fibroblast coculture assay. Even more interesting, postsynaptic IL1RAPL1 binds to presynaptic PTP-δ, and this interaction is shown to be required for the synaptogenic effect of IL1RAPL1 since the activity of IL1RAPL1 was abolished in primary neurons from PTP-δ knock-out mice (Yoshida et al., 2011). When compared to the binding abilities of NGL-3 to PTP-δ, since NGL-3 interacts with PTP-δ (Kwon et al., 2010), it was found that the binding ability to PTP-δ was much stronger for IL1RAPL1 (Yoshida et al., 2011). Finally, another essential component of receptor complexes for IL-1, interleukin-1 receptor accessory protein (IL-1RAcP), also has robust synaptogenic activity (Yoshida et al., 2012). Knockdown of IL-1RAcP isoforms in cultured cortical neurons suppressed synapse formation. Furthermore, the spine densities of cortical and hippocampal pyramidal neurons were reduced in IL-1RAcP knock-out mice. Like IL1RaPL1, IL-1RAcP interacts with PTP-δ. These results tremendously increase the complexity of the synaptic code generated by the PTP LAR family.

2.3.4. Role of Leucine-Rich Repeat proteins in synaptogenesis

Although synaptic cell adhesion proteins are involved in different steps of synapse formation, their functional specificity is based on a limited number of extracellular

domains (Missler et al., 2012). The Ig-domains, cadherin domains, LNS domains, and LRRs are the most common building blocks of synaptic molecules. LRR is one of the most frequently seen protein domain repeats across species (Bjorklund et al., 2006), and the most recent count compiled a list of 375 human LRR-containing proteins (Ng et al., 2011). Of these, 139 are part of the extracellular LRR superfamily (Dolan et al., 2007), of which many are involved in various aspects of nervous system development (Chen et al., 2006; Homma et al., 2009; de Wit et al., 2011). Recently characterized families implicated in synapse formation, SALMs, NGLs and LRRTMs and now Slitrks, all have LRR domains. These proteins are added to other synaptic LRR proteins, which include densin-180, Erbin, TrkC, and LGI1. The LRR is a 20-29 aa motif that contains a conserved 11-residue sequence rich in leucines at the N-terminal (LxxLxLxxN/CxL where x is any aa) (Kobe and Kajava, 2001). When present in proteins, LRRs usually occur in tandem arrays of a few to more than a dozen that together constitute the LRR domain (Matsushima et al., 2005). Early X-ray crystallographic studies revealed that LRRs proteins have a curved, horseshoe-shaped structure, wherein parallel β-sheets line the inner circumference of the horseshoe (Kobe and Deisenhofer, 1993, 1995). The particular structure makes the LRR domain a very effective protein-binding motif. The presence of such a large array of proteins with LRR domains at the synapse might be explained by their potential to ensure reliable synaptic connectivity based on the LRR protein-interaction motif.

3. The Slitrk family

3.1. Identification

The Slitrk family of genes was discovered in nucleotide sequence database searches aimed at identifying genes that are deregulated in mutant mice with neural tube defects (Aruga and Mikoshiba, 2003). This new family of molecules was given the name Slitrk based on sequence similarity with Slits and Trks. Simultaneously, the gene KIAA0918 (now known as the fifth member of the Slitrk family) was reported as a gene expressed in early hematopoietic progenitors but not in mature hematopoietic cells (Shmelkov et al., 2001). The *Slitrk2* gene was also identified independently in a study focused on the transcriptional map of the entire q27 band of the Human X (Zucchi et al., 1999).

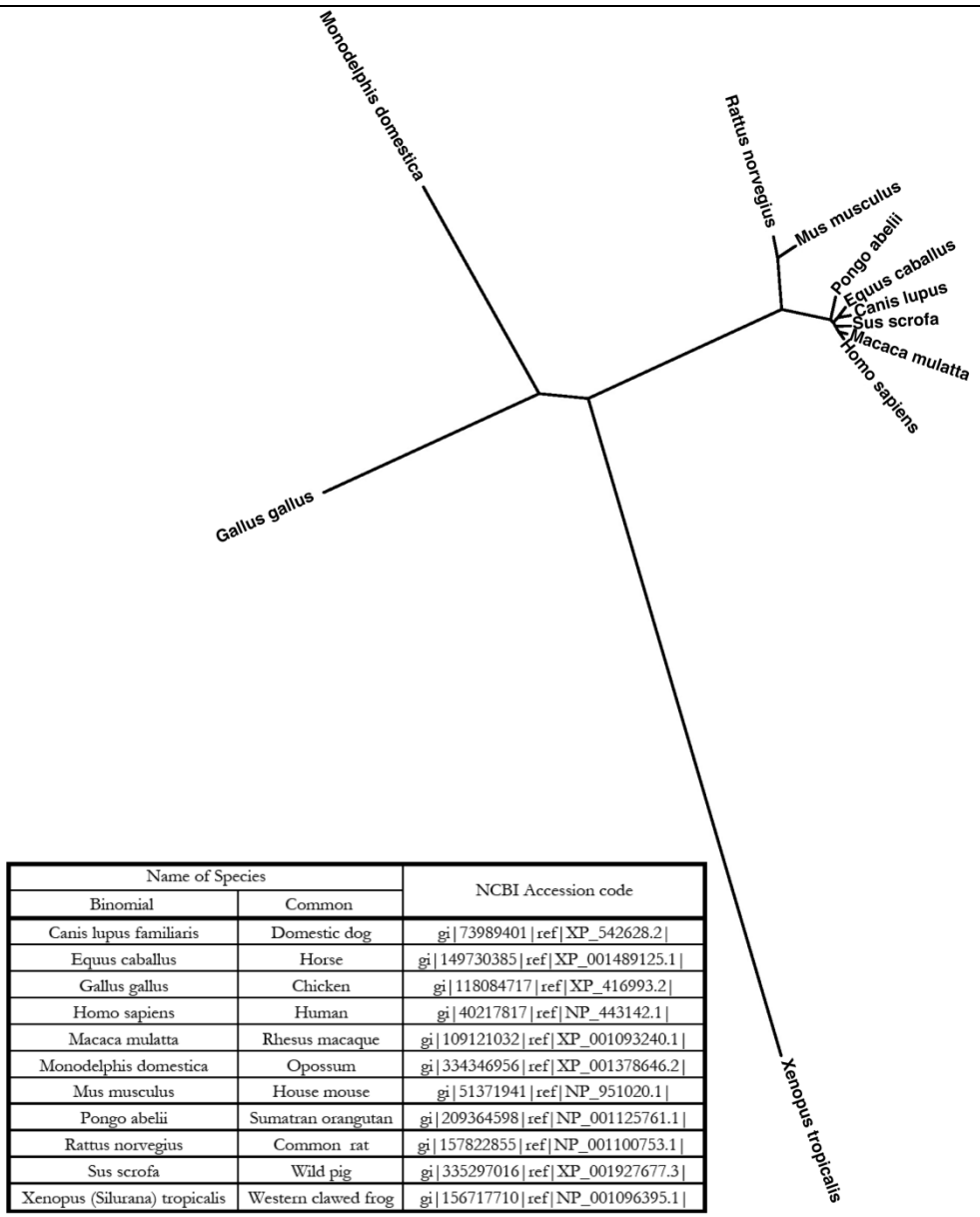
Interestingly, this portion of the chromosome X contains a gene candidate responsible for mental retardation conditions such as Fragile X syndrome (genes FMR1 and FMR2) (Gu et al., 1996) and other similar syndromes (Kondo et al., 1991). Zucchi et al.'s initial characterization of this novel gene predicted a protein with similarity to other members of the LRR protein superfamily such as Slit. The gene was then termed SLIT like protein 1 (SLITL1) (Submitted in September 1999 to the EMBL/GenBank/DDBJ databases).

3.2. Genomic organization of the mouse and human *Slitrks*

The Slitrk family is composed of six genes that are dispersed on three chromosomes: on chromosome 3 (*Slitrk3*), 14 (*Slitrk1*, *5* and *6*) and X (*Slitrk2* and *4*) (Aruga and Mikoshiba, 2003). This chromosomal organization is conserved in human, such that the *Slitrks* are located on homologous chromosomes (Aruga et al., 2003). Closer analysis of the six *Slitrk* genes reveals that *Slitrk1* and *Slitrk5* are composed of a single exon and that *Slitrk2*, *Slitrk3*, *Slitrk4* and *Slitrk6* have two exons. This pattern of gene organisation is conserved in mouse, rat, and human, with the exception of *Slitrk2* in human that has 3 exons. However, the protein-coding region of all the Slitrks is located in a single one of their exons (Aruga et al., 2003).

3.3. Phylogeny analysis for Slitrk1

In terms of evolutionary conservation, it is worth mentioning that unlike some other LRR proteins, the six Slitrk genes are not present in invertebrates. This may be surprising, considering the fact that multiple LRR-containing proteins that have functions in higher organisms are also critical in more ancient species. Nevertheless, the Slitrks have homologues in a broad range of vertebrates, from the chick and the frog to the human. This homology is best illustrated with the example of Slitrk1. As shown by the phylogenetic tree for this protein (Fig. 3), the aa sequence of Slitrk1 varies between different vertebrates.



Name of Species		NCBI Accession code
Binomial	Common	
Canis lupus familiaris	Domestic dog	gi|73989401|ref|XP_542628.2|
Equus caballus	Horse	gi|149730385|ref|XP_001489125.1|
Gallus gallus	Chicken	gi|118084717|ref|XP_416993.2|
Homo sapiens	Human	gi|40217817|ref|NP_443142.1|
Macaca mulatta	Rhesus macaque	gi|109121032|ref|XP_001093240.1|
Monodelphis domestica	Opossum	gi|334346956|ref|XP_001378646.2|
Mus musculus	House mouse	gi|51371941|ref|NP_951020.1|
Pongo abelii	Sumatran orangutan	gi|209364598|ref|NP_001125761.1|
Rattus norvegius	Common rat	gi|157822855|ref|NP_001100753.1|
Sus scrofa	Wild pig	gi|335297016|ref|XP_001927677.3|
Xenopus (Silurana) tropicalis	Western clawed frog	gi|156717710|ref|NP_001096395.1|

Figure 3: Phylogenetic tree for Slitrk1.

The phylogenetic tree for Slitrk1 was constructed with average distance between species using percent of aa identity. The calculation of the identity percentage was obtained via the multiple sequence alignment program Clustal Omega (Larkin et al., 2007) and the graphic was reconstructed with the bioinformatics program Phylogeny.fr (Dereeper et al., 2008). The different species shown above are the ones in which Slitrk1 homologues are found.

3.4. Slitrks protein structure

All the members of the Slitrk family are type I (single pass) transmembrane proteins with the N-terminal outside of the cell and the C-terminal inside (Fig. 4). This orientation was deduced from the hydrophobicity profiles of the six proteins (Hofmann and Stoffel, 1993; Aruga and Mikoshiba, 2003). The sizes of the Slitrks vary between 696 aa and 980 (Aruga et al., 2003). The extracellular portion of the Slitrks contains two LRR domains. Each LRR domain is composed of 13-17 LRR motifs and each domain is flanked by four conserved cysteine residues. The length of the cytoplasmic tail varies between the different members but none contain any known functional domain. Slitrk1 has a shorter tail relative to the five other Slitrks. While it may be useful in understanding the mode of action of these proteins, to date, no crystal structure of the Slitrks has been solved.

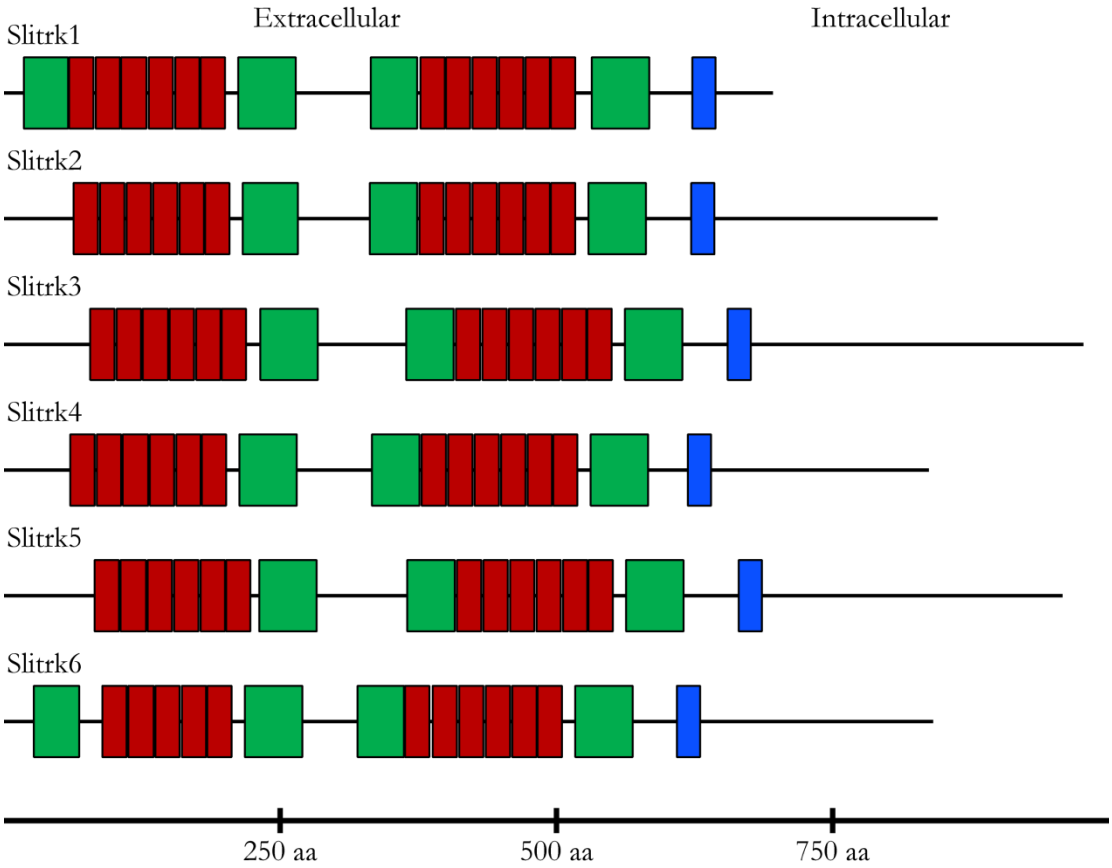


Figure 4: Structure of the Slitrk proteins.

Analysis of the protein domains found in the human sequences of the six Slitrks. The precise delimitation of each domain was obtained on the UniProt Knowledgebase (UniProtKB) database (www.uniprot.org) and confirmed with the Protein database from NCBI (www.ncbi.nlm.nih.gov/protein). Note that these two bioinformatic tools did not always define the region in N-terminal of the first LRR as a domain per se even if the sequence is cysteine-rich in all cases. Red box, leucine-rich repeat domain; green box, leucine-rich repeat terminal domain in both amino- and carboxy- region; blue box, transmembrane domain. The scale bar at the bottom represents the length of the proteins in aa.

3.5. Expression of Slitrks in the nervous system

As a first attempt to examine the tissue distribution of the Slitrks genes, Northern blot analyses were performed in the adult mouse (Aruga and Mikoshiba, 2003) and in human brain (Aruga et al., 2003). With expression beginning around embryonic day (E) 10-12, the Slitrks were mostly found in the brain. Using *in situ* hybridization, all the Slitrk mRNAs were also detected in embryonic brain ((Aruga and Mikoshiba, 2003); chapter 2). Unlike Slitrk1 to 5, which are mainly expressed in the brain, Slitrk6 is also expressed mainly outside the CNS (Aruga and Mikoshiba, 2003). Using a series of *in situ* hybridization experiments, Slitrk6 expression was found in visceral organs and in head structures in the developing mouse. Slitrk6 is highly expressed in taste buds (Hevezi et al., 2009). Interestingly, Slitrk6 mRNA levels were increased the most in the lungs of the glucocorticoid receptor-null mice (Bird et al., 2007).

Anti-Slitrk1 antibody strongly detects proteins in the olfactory bulb, frontal cortex, hippocampus and amygdala (Katayama et al., 2008). This expression during mammalian brain development seems to be evolutionarily conserved. At least, its regional and developmental expression is preserved in mouse, monkey and human brain (Stillman et al., 2009). Using immunoelectron microscopy in the cerebral cortex of adult mouse and rhesus monkey, Slitrk1 was found in neuronal dendrites, in the periphery of the Golgi apparatus and in the early and late endosomes (Stillman et al., 2009). These observations were confirmed by the colocalization of Slitrk1 with the dendritic marker, microtubule-associated protein 2 (MAP2) (Garner et al., 1988), but not with the axonal microtubule-associated protein, tau (Dotti et al., 1987). Slitrk1 also colocalizes with RAB5, an early endosome marker. Furthermore, in the adult striatum, the few neurons that maintain Slitrk1 protein expression are positive for the cholinergic interneuron marker, choline acetyltransferase (CHaT) (Stillman et al., 2009). Interestingly, striatal cholinergic interneurons have recently been implicated in the induction of synaptic plasticity, motor learning and motor dysfunction (Pisani et al., 2007).

3.6. Relationship to Slits and Trks proteins

Since the Slitrks were given their name according to their similarity to the Slit and the Trk proteins, the relation between them will be describe here. The Slits belong to a family of potent secreted chemorepellents that can regulate the growth of growing axons through their receptors, the Robos. Structurally, the Slits are large secreted glycoproteins containing four LRR domains at their N-terminus followed by six EGF-like motifs, three laminin G domains, and a C-terminal cysteine-knot motif (Beaubien et al., In press). The homology between the Slits and the Slitrks spans the entire extracellular domains of Slitrk proteins. However, elevated percentages of identical aa residues are only present in the most amino-terminal LRR domains of the Slits and the Slitrks (Fig. 5) (Aruga and Mikoshiba, 2003). In fact, the LRRs of Slitrks are no closer in terms of homology to Slits than to other LRRs proteins. In regard to function, Slitrks and Slits are likely to be different as they do not bind the same receptor. While Slits bind to Robo, no direct physical interaction between the extracellular domain of Slitrk and the extracellular domain of Robo has been reported ((Aruga and Mikoshiba, 2003); our unpublished observation).

On the intracellular side of the membrane, the Slitrks are homologous to the neurotrophin receptors, Trks. This similarity is located at the carboxyl-terminus of both proteins, more precisely around specific tyrosine residues (Y791 in TrkA and their corresponding tyrosines in TrkB and TrkC and Y833 in Slitrk2 and their corresponding tyrosines in Slitrk3-4-5) (Fig. 5). Y791 is phosphorylated in Trks upon activation and dimerization of these receptors by neurotrophins (Obermeier et al., 1993; Perez et al., 1995; Arevalo and Wu, 2006). At present, there is no information in the literature about the possible phosphorylation of this conserved tyrosine residue in Slitrks 2 to 5. Finally, the same segment of the TrkA sequence (PPXY) is also responsible for the Nedd4-2 E3 ubiquitin ligase binding that affects the internalization of the receptor upon NGF binding (Arevalo et al., 2006). Knowing that Slitrks are also type 1 transmembrane proteins, it would be interesting to assess the role of E3 ubiquitin ligase in the recycling of the Slitrks.

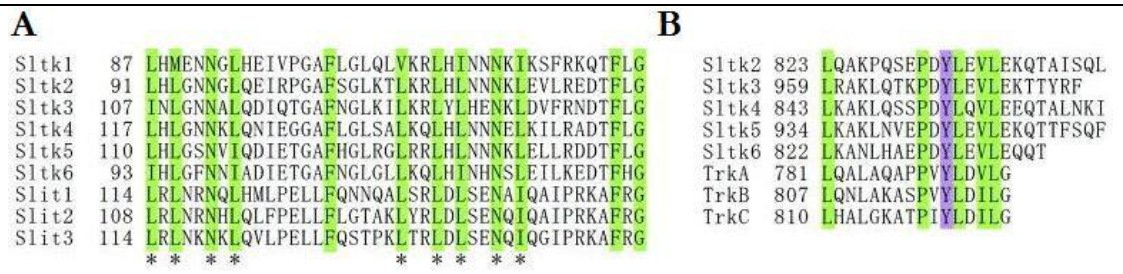


Figure 5: Amino acid sequence alignment of Slitrks/Slits and Slitrks/Trks.

(**A**) Alignment in the leucine-rich repeat domain between mouse Slitrk protein aa sequences and the human slits. The leucine rich repeat L–L–L–N motifs are indicated by asterisks below the sequences. The green shaded letters indicate completely conserved amino acid residues among the Slitrks and Slits. (**B**) aa sequence alignment of the carboxyl-terminus of the Slitrk and human Trk neurotrophin receptor. Purple-colored letters indicate the tyrosine residues conserved between the Trk proteins and Slitrk proteins, and green-colored letters indicate the conserved aa residues between Slitrk proteins and Trk proteins. (Adapted from (Aruga and Mikoshiba, 2003))

3.7. Functions of the Slitrks

3.7.1. Slitrks and neuropsychiatric disorders

3.7.1.1. Tourette's syndrome

The first evidence for a role of the Slitrks in any neurological disease came when mutations in the *Slitrk1* gene were identified in a Tourette's syndrome (TS) patient (Abelson et al., 2005). TS is a complex neuropsychiatric disorder characterized by a large range and variable number of unwanted repetitive simple or complex motor and vocals tics (American_Psychiatric_Association, 2000). Once considered a rare disorder, the newest estimation of the prevalence of TS is approximately 4 to 6/1000 children (Khalifa and von Knorring, 2003; Jin et al., 2005). The onset is during childhood and the tics usually reach their worst point between ages of 9 to 12 (Gadow et al., 2002). In terms of comorbidity, as high as 60% of TS patients are diagnosed with attention deficit

hyperactivity disorder (ADHD) (Coffey et al., 2000; Eapen et al., 2004) and 50% have prominent obsessive-compulsive symptoms (Pauls, 2003). It is now accepted that the etiology of TS involves the interaction of environmental and genetic risk factors. A number of epigenetic factors have also been implicated in the pathogenesis, such as gestational and perinatal insults, exposure to androgens, heat, and fatigue as well as postinfectious autoimmune mechanisms (Swain et al., 2007). In terms of the genetic contribution to TS, comparison between monozygotic and dizygotic twins initially suggested major gene effects (Pauls et al., 1981). However, more recent studies instead support poly- or oligogenic (a few genes) inheritance (Walkup et al., 1996). Slitrk1 was proposed as a TS susceptibility factor when a *de novo* inversion in chromosome 13 was identified in a Caucasian boy presenting with TS and ADHD (Abelson et al., 2005). Fine mapping of the inverted region revealed that the *Slitrk1* gene is located near one of the borders of the inversion. Screening 174 TS patients also led to the identification of one proband with a single-base deletion in the coding region of the *Slitrk1* gene, creating a frameshift that resulted in the formation of a stop codon in the second LRR domain, and consequently, a truncated Slitrk1 protein. In addition to this stop codon mutation, a single-base change that maps to the 3' untranslated region (UTR) of the Slitrk1 transcript was found in two unrelated patients with TS and obsessive-compulsive symptoms and not in 4296 control chromosomes (Abelson et al., 2005). This noncoding sequence variant (var321) is within the predicted binding site of a microRNA (miRNA), hsa-miR-189 (Lagos-Quintana et al., 2003; Miska et al., 2004). The miRNAs are small (18-25 nucleotides) noncoding RNAs that are capable of regulating gene expression. In a later study with 92 TS Austrian patients, no nucleotide change was found in the coding region of *Slitrk1* but, a new 3' UTR variant was identified in one patient and segregated in two additional family members with tic symptoms (Zimprich et al., 2008). Overall, the identification of Slitrk1 as a new candidate gene that might be associated with a subset of TS patients, provided hope that it will help in understanding the fundamental pathways underlying the disease pathogenesis. However, multiple subsequent studies in North American, Costa Rican, Ashkenazi, Dutch and Taiwanese individuals with TS failed to identify Slitrk1 mutations (Deng et al., 2006; Keen-Kim et al., 2006; Verkerk et al., 2006; Chou et al., 2007). Similar negative results were obtained in the largest screen (1048

individuals) performed to date using the samples collected by the Tourette Syndrome Association International Consortium for Genetics (TSAICG, 2007; Scharf et al., 2008). This study concluded that var321 is only rarely present in patients with TS.

3.7.1.2. Other potential implications in neuropsychiatric disorders

Belonging to obsessive-compulsive disorder (OCD) spectrum with TS, trichotillomania (TTM) is a chronic behavioral disorder characterized by hair loss due to repeated urges to pull or twist hair until it breaks off (Hallopeau, 1889; American_Psychiatric_Association, 2000). The prevalence of TTM in adult and child populations is largely unknown as the necessary large-scale epidemiological studies have not been published (Duke et al., 2009). In terms of genetic causes of the syndrome, a recent concordance study examined differences in TTM rates occurring in monozygotic and dizygotic twin pairs and found significant differences supporting that heritability is an important contributor to the manifestation (Novak et al., 2009). Interestingly, the mother of the TS patient initially identified with a stop codon mutation in Slitrk1 gene was diagnosed with TTM (Abelson et al., 2005). Later, the sequencing of the *Slitrk1* gene in 44 TTM families in which one or more individuals are diagnosed uncovered two novel non-synonymous mutations in the extracellular portion of Slitrk1 (Zuchner et al., 2006). The altered genotype was not detected in 2192 non-TTM controls. However, the two Slitrk1 sequence variants represent a frequency of 4.5% which is considered as a rare variation.

Because 40% of TS probands have OCD or at least moderate obsessive-compulsive symptoms (Bloch et al., 2006), in the next study 322 OCD probands and 390 controls were genotyped for the Slitrk1 var321 and the frameshift mutation inducing an premature stop codon (Wendland et al., 2006). Wendland et al. did not observe any of the rare Slitrk1 variants in the OCD probands. However, one var321 heterozygote subject was found in the negative controls, somewhat reducing the probability of an exclusive association between the variant and TS.

Recently, *Slitrk2* was suggested to be bipolar risk gene (Smith et al., 2009). Bipolar disorder (BD) is characterized by episodes of mania and depression

(American_Psychiatric_Association, 2000). The present literature based on family, twin, and adoption studies supports a strong genetic component for this disorder (the heritable component of bipolar disorder ranges between 80 and 90%) (Rice et al., 1987; Pregelj, 2011). In an effort to identify specific genetic variations influencing BD, a genome-wide association study was performed on various populations. Residing on chromosome X, Slitrk2 gene is located near one of the top hits of the study (Smith et al., 2009).

Fragile X syndrome is the most common form of inherited intellectual disability in boys. Most often, it is due to an abnormal expansion of DNA triplet repeats (CGG) in the 5' UTR of the *FMR1* gene that eventually reduces the expression of the protein (Turk, 2011). However, other mutational mechanisms, such as deletions of FMR1, also cause the syndrome. Sometimes these deletions can be up to 13 Mb in size, which may be cytogenetically visible and often include adjacent genes (Coffee et al., 2008). Recently, a mother and her son were described, with a large Xq27.3–q28 deletion encompassing the *FMR1*, *FMR2* genes as well as, interestingly, the *Slitrk2* gene (Cavani et al., 2010). Since the phenotype of Fragile X syndrome patients with large deletions is more severe than patients with the CGG expansion, the loss of the *Slitrk2* gene could be partly responsible for the symptoms.

The *Slitrk2* gene also reported in a screen for rare variants in genes that could predispose individuals to autism spectrum disorder (ASD) and schizophrenia (SCZ) in a fraction of cases (Piton et al., 2010b). Indeed, *Slitrk2* was included in a selection of 111 potential candidates based mainly on its location on chromosome X and its brain expression. The authors focused on X-linked genes, as several pieces of evidence have implicated this chromosome in ASD and SCZ (Marco and Skuse, 2006; Loat et al., 2008). The sequencing of *Slitrk2* in a cohort of 142 ASD and 143 SCZ individuals reveals two novel non-synonymous variants, 265G>A and 1646C>T, in females with SCZ and in their affected siblings. These two mutations were not found in the control group which included 277 X chromosomes.

3.7.2. Slitrks and their CNS functions

3.7.2.1. Early experiments with cell lines and primary neurons

Our understanding of the role of Slitrks in neurodevelopment remains very limited. Early studies in PC12 cells suggested that Slitrks can modulate neurite outgrowth (Aruga and Mikoshiba, 2003). PC12 is a cell line derived from a pheochromocytoma of the rat adrenal medulla that has the ability to stop dividing and to terminally differentiate into a sympathetic-like neuronal phenotype characterized by neurite outgrowth when treated with nerve growth factor (NGF), FGF2 or cAMP analogues (Greene and Tischler, 1976; Gunning et al., 1981; Rydel and Greene, 1987). In a non-NGF induced paradigm, overexpression of Slitrk1 leads to neurite outgrowth whereas overexpression of other Slitrks did not have a significant effect. When the PC12 cells were induced with NGF, overexpression of Slitrk1 had no effect on neurite outgrowth whereas neurite outgrowth was severely suppressed in Slitrk2 and Slitrk3-transfected cells and weakly suppressed in Slitrk4, Slitrk5 and Slitrk6 expressing cells compared to control cells treated with NGF. The intracellular region of Slitrk2 is required for its neurite outgrowth inhibition. This function might depend on the phosphorylation of the tyrosine residues located in this portion of the Slitrks. Overexpression of Slitrk1 can also promote neurite outgrowth in mouse cortical neurons (Abelson et al., 2005; Kajiwara et al., 2009).

3.7.2.2. Interaction between Slitrk1 and the 14-3-3 proteins

Remarkably, the neurite outgrowth effect of Slitrk1 is abolished with the mutation of a single aa, Ser_{695} (Kajiwara et al., 2009). This serine is important for Slitrk1 binding to all the seven members of the 14-3-3 family of proteins (Kajiwara et al., 2009). The 14-3-3 family consists of seven distinct isoforms that are adapter proteins that interact with specific phospho-aa motifs within a number of binding proteins and consequently regulate diverse cellular processes (Garbe and Bashaw, 2004; Jin et al., 2004; Bridges and Moorhead, 2005; van Heusden, 2005; Clandinin and Feldheim, 2009; Kent et al., 2010). The 14-3-3s recruit substrate proteins in specific subcellular localizations and therefore control their spatial and temporal activity (Muslin and Xing, 2000). Slitrk1 colocalizes with 14-3-3s in the soma, dendrites and growth cones of rat cortical neurons (Kajiwara et

al., 2009). Several potential sites of serine/threonine phosphorylation have been identified in Slitrk1 including Ser_{695}, which is specifically phosphorylated by casein kinase II (CK2). Interestingly, Slitrk1 bearing a phosphomimetic S695E mutation enhances neurite outgrowth in neurons to a similar level of wild-type Slitrk1, while a S695A Slitrk1 mutant does not. The mutation S695A also affects the 14-3-3 binding suggesting that 14-3-3/Slitrk1 interaction might be involved in the modulation of neuritogenesis (Kajiwara et al., 2009).

3.7.2.3. Characterization of Slitrk knockout mice

The importance of Slitrks in the development and maintenance of the nervous system has been emphasized by gene ablation studies in mice. *Slitrk1*-deficient mice do not display any external visible abnormalities other than a slightly lower body weight in males (Katayama et al., 2008). Their brains did not reveal any obvious anatomical or histological abnormalities. *Slitrk1*$^{-/-}$ mice do not have any unusual behaviors, including stereotypy, tremor, seizure or abnormal repetitive behaviors during global observation and behave normally in a paradigm used to detect OCD symptoms in animal. However, *Slitrk1*$^{-/-}$ mice demonstrate anxiety-like behaviors while performing the elevated plus-maze test (Pellow et al., 1985; Treit et al., 1993). Interestingly, treatment of *Slitrk1*$^{-/-}$ mice with clonidine, an agonist of the α2-adrenoceptor often prescribed to TS patients (Schlicker and Gothert, 1998), alleviates the anxiety-like behaviors (Katayama et al., 2008). *Slitrk1*$^{-/-}$ mice also present a depression-like behavior measured by the forced swimming test and a decrease in locomotor activity (Katayama et al., 2008), behaviors that are not alleviated by treatment with clonidine. *Slitrk1*$^{-/-}$ mice may therefore represent a valuable model to better understand complicated neuropsychiatric diseases.

In contrast to Slitrk1 mutant mice, Slitrk5 mutant mice exhibit behaviors that are more reminiscent of OCD (Shmelkov et al., 2010). These mice develop severe facial skin lesions and hair loss at three months of age as a result of excessive self-grooming. Administration of fluoxetine, a selective serotonin reuptake inhibitor commonly used to treat OCD (Mataix-Cols et al., 1999), to these mice alleviated these behaviors. Interestingly, *neurexin-1a*-deficient mice exhibit the same repetitive grooming behaviors

(Etherton et al., 2009). *Slitrk5*$^{-/-}$ mice showed an increase activity in the marble-burying behavior test indicating possible OCD. Furthermore, orbitofrontal cortex is over-activated in the *Slitrk5*$^{-/-}$ mice, reminiscent of OCD patients who have elevated activity in this brain region (Whiteside et al., 2004; Menzies et al., 2008). These behaviors have been proposed to result from a decrease in the overall number of glutamate receptor subunits in the striatal neurons in *Slitrk5*$^{-/-}$ mice. Hence, the ablation of Slitrk5 seems to generate some of the hallmarks of OCD. However, it is still unknown why removal Slitrk5, which is broadly expressed in the brain, affects more strongly the orbitofrontal cortex and striatal neurons.

Slitrk3-deficient mice also have normal gross brain morphology (Takahashi et al., 2012). Interestingly, occasional convulsive seizures were observed in *Slitrk3*$^{-/-}$ mice together with increased susceptibility to chemoconvulsant-induced seizures. These mutant mice will be further discussed in chapter 4.

While ablation of Slitrk1, Slitrk3 and Slitrk5 results in behavioral defects associated with TS, SCZ, and OCD, ablation of Slitrk6 expression does not appear to lead to abnormal behaviors in anxiety tests (Matsumoto et al., 2011). In contrast, *Slitrk6*$^{-/-}$ mice show defects in the development of the inner ear that include marked alterations in the spacing of radial fibers projecting to the organ of Corti and cochlea (Katayama et al., 2009). It is reminiscent of previous observations on the expression of Slitrk6 in various developing organs outside the central nervous system including the cochlea (Aruga, 2003). Explants of spinal ganglia show reduced neurite outgrowth when grown in the presence of cochlear sensory epithelia isolated from *Slitrk6*$^{-/-}$ mice when compared to epithelia isolated from wild-type mice, suggesting that Slitrk6 may have a neurotrophic effect (Katayama et al., 2009). Finally, auditory function tests revealed that *Slitrk6*$^{-/-}$ mice have a mid-frequency range hearing loss and a mild vestibular function defect (Matsumoto et al., 2011). Even if Slitrk6 expression in the brain is low, it can be hypothesized that *Slitrk6*$^{-/-}$ mice may bear additional yet unidentified defects in neurodevelopment.

4. Rationale and objectives

At the onset of my graduate studies, very little was known about the function of Slitrks in the nervous system (Aruga, 2003; Aruga and Mikoshiba, 2003; Aruga et al., 2003). Based on the few published observations and the interesting protein structures of the Slitrks, I hypothesized that this novel family may play a role in the development of the nervous system and therefore, decided to answer two specific questions described below:

OBJECTIVE 1:

WHAT IS THE SPATIO-TEMPORAL EXPRESSION PATTERN OF SLITRKS IN THE NERVOUS SYSTEM?

I have performed a detailed analysis of the expression patterns of *Slitrks* during neurodevelopment at embryonic and postnatal ages. Using *in situ* hybridization, I demonstrated that, despite some overlap, each of the six *Slitrks* have a unique expression profile suggesting they may have different roles during neurodevelopment. These results are presented in Chapter 2.

OBJECTIVE 2:

ARE THE SLITRKS IMPLICATED IN SYNAPSE FORMATION?

Based on the expression pattern of Slitrks and the synaptogenic activity reported for proteins with similar structures, I examined whether Slitrk1 and Slitrk2 are involved in the development of new synapses. Working with rat hippocampal neuron cultures, I have shown that Slitrks possess the potential to induce new synapses. These results are presented in Chapter 3.

CHAPTER 2

DIFFENTIAL EXPRESSION OF SLITRK FAMILY MEMBERS IN THE MOUSE NERVOUS SYSTEM

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1. Preface

This chapter was published as a research article in 2009 in Developmental Dynamics (Beaubien and Cloutier, 2009). At the onset of these studies, several members of the Slitrk family had been shown to be expressed in the nervous system. However, a detailed and comprehensive analysis of their patterns of expression in the prenatal and early postnatal brains of mice was not available. I therefore present in this chapter a comprehensive description of the expression of Slitrks in the mouse developing nervous system. These studies allowed us to begin to define what potential roles Slitrks may play in neurodevelopment.

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2. Abstract

The Slitrk family of transmembrane proteins is composed of six members that are highly expressed in the nervous system. To date, the function of Slitrks during development of the nervous system has yet to be defined. The high homology between the extracellular region of Slitrks and the repulsive axon guidance molecules Slits suggest that Slitrks may regulate axon outgrowth during development. To begin to evaluate their role during development, we have examined the expression of the *Slitrk* genes in the developing murine nervous system using *in situ* hybridization. Here, we show that despite some overlap in expression, the Slitrks display distinct patterns of expression in the olfactory system, the eye, forebrain structures, the cerebellum, the spinal cord, and dorsal root ganglia. These diverse patterns of expression suggest that Slitrk family members may have different functions during development of the nervous system.

3. Introduction

The development of the nervous system requires a wide variety of processes to take place including cellular differentiation, cellular migration, axonal guidance, and synapse formation and refinement. A large number of type 1 transmembrane proteins containing LRR domains are highly expressed in the nervous system and have been implicated in regulating various phases of neuronal development (Chen et al., 2006; Dolan et al., 2007; Ko and Kim, 2007). The Slitrks are a large subfamily of LRR-containing transmembrane proteins (Slitrk1 to 6) that were first identified in a screen for differentially expressed genes in mice with neural tube defects (Aruga and Mikoshiba, 2003). They are type 1 transmembrane proteins composed of an extracellular domain containing two LRR highly homologous between members of the family and a cytoplasmic region that varies in size between the six members. The LRR domains of Slitrk family members show a high homology to LRR regions found in members of the Slit family of secreted molecules that have been implicated in the regulation of several biological processes including cell migration, axonal pathfinding, and axonal and dendritic branching (Nguyen-Ba-Charvet and Chedotal, 2002). In addition, the cytoplasmic region of most Slitrks contains tyrosine residues with surrounding aa sequences homologous to Trk

family neurotrophin receptors (Aruga and Mikoshiba, 2003). The differences observed between the intracellular domains of Slitrk family members may allow them to perform different roles during neuronal development.

The function of Slitrk family members during development of the nervous system remains largely unknown. Expression of Slitrk1 in PC12 cells and cortical neurons induces the growth of unipolar neurites and dendritic branching in these respective cells (Aruga and Mikoshiba, 2003; Abelson et al., 2005). In contrast, expression of other Slitrk family members inhibits NGF-induced neurite outgrowth to varying levels in PC12 cells (Aruga and Mikoshiba, 2003). The differential effect of Slitrk1 on neurite outgrowth in PC12 cells compared to other Slitrks may be related to the presence of a very short cytoplasmic tail on Slitrk1 when compared to Slitrk2 to 6. Ablation of *Slitrk1* expression in the mouse leads to increased anxiety-like behaviour despite the absence of any gross anatomical abnormalities in the brain (Katayama et al., 2008). These results suggest that Slitrk1 may regulate the formation or maintenance of proper brain wiring. In keeping with this possibility, *Slitrk1* was proposed as a susceptibility gene for TS (Aruga and Mikoshiba, 2003; Abelson et al., 2005; Miranda et al., 2008) and Slitrk1 is highly expressed in neurons believed to be affected in these patients (Stillman et al., 2009). However, other studies did not report an association between mutations in *Slitrk1* and TS (Deng et al., 2006; Verkerk et al., 2006; Wendland et al., 2006; Scharf et al., 2008).

In an effort to further understand the roles of Slitrks during development of the mouse nervous system, we have performed a comprehensive analysis of their expression in the late prenatal and early postnatal periods using *in situ* hybridization. The partially overlapping yet differential expression of the six *Slitrk* genes in the nervous system that we report here suggests they may play different roles in the development of specific regions of the brain.

4. Experimental Procedures

Animals

E18 mouse embryos were obtained from timed-pregnant CD1 females and brains were isolated from P10 CD1 mice purchased from Charles River (Saint-Constant, Quebec, Canada). Date of vaginal plug was considered as E0 and the day of birth was taken as day P0. The embryos and brains were embedded in Tissue-Tek O.C.T. compound (Miles, Elkhart,IN) and frozen in dry ice-cooled 2-methylbutane. Tissues were stored at -80°C for future sectioning. All animal procedures used were in accordance with the guidelines and approval of the McGill University animal care committee.

Riboprobe synthesis

Nonradioactive, digoxigenin (DIG)-labeled cRNA probes with either sense or antisense orientation were synthesized by *in vitro* transcription using DIG labelling mix (Roche, Mannheim, Germany) according to the recommendations of the manufacturer. *Slitrk* probes were synthesized from mouse cDNA clones of the full coding sequence purchased from Open Biosystems (Huntsville, AL): *Slitrk1* (EST IMAGE number 9007276), *Slitrk2* (EST IMAGE number 40060548), *Slitrk3* (EST IMAGE number 6844461), *Slitrk4* (EST IMAGE number 40096585), *Slitrk5* (EST IMAGE number 30357422), *Slitrk6* (EST IMAGE number 8861685). Transcription of the full length cDNA clones synthesized cRNA probes of 600-1000 base pairs that were partially hydrolyzed for 25 min at 60°C in 10 mM DTT, 200 mM $NaHC0_3/Na_2C0_3$, pH 11, to an average length of 100-250 nucleotides (Schaeren-Wiemers and Gerfin-Moser, 1993). Hydrolysis was stopped by neutralization with 100 mM acetic acid, and the cRNA probes were precipitated with 1/10 volume of 4M LiCl and 2.5 volumes of ethanol at -80°C. Precipitated cRNA fragments were recovered in 20 µl diethylpyrocarbonate (DEPC)-treated water.

In situ hybridization with digoxigenin-labeled riboprobes

Fresh frozen brains were cryosectioned at 20 µm at -17°C and thaw mounted on Fisherbrand Superfrost/Plus microscope slides (Fisher Scientific, Hampton, NH).

Sections were allowed to dry for 1 hour, fixed for 20 minutes in 4% paraformaldehyde in 0.1 M phosphate-buffered isotonic saline (PBS; pH 7.4), rinsed three times for 5 minutes in PBS, and rinsed once briefly in DEPC-treated water. Sections were incubated for 10 minutes with 0.25% acetic anhydride in 1% triethanolamine, washed twice in PBS, rinsed in 1x standard saline citrate (SSC) for 5 minutes, and prehybridized for 3 hours at 60°C in 50% formamide, 5x Denhardt's solution, 5x SSC, 200 μg/ml baker's yeast tRNA. Two hundred microliters of hybridization mixture containing approximately 100 ng/ml DIG-labeled riboprobe were applied per slide, covered with Nescofilm (Karlan research products corporation, Cottonwood, AZ), and hybridized overnight at 60°C. On the following day, sections were washed for 5 minutes at 60°C in 5x SSC, 1 minutes at room temperature (RT) in 2x SSC, 30 minutes at 60°C in 50% formamide containing 0,2x SSC, and 5 minutes in 0,2x SSC at RT. Sections were then washed in Tris-buffered saline (TBS; 100 mM Tris-HC1, pH 7.5, 150 mM NaCl) for 5 minutes and blocked for 1 hour in a 1% solution of blocking reagent (Roche, Mannheim, Germany) in TBS, followed by a three hour incubation with anti-DIG Fab fragments conjugated to alkaline phosphatase (1:3000) and two 15 minute washes in TBS. The color reaction was performed overnight at room temperature. Sections were rinsed extensively in PBS and coverslipped with Mowiol 4-88 (Calbiochem, San Diego, CA). Brightfield *in situ* hybridization signals on sections were photographed using a Zeiss Axio imager upright microscope equipped with a monochrome Qimaging Retiga Exi camera. Each *in situ* hybridization experiment was repeated a minimum of three times on a minimum of three different animals to eliminate any variability in expression between animals.

5. Results

5.1. Specificity of the cRNA probes used in this study

To examine the expression of *Slitrk* genes in the developing nervous system, coronal sections of E18 mouse embryos were incubated with antisense cRNA probes for *Slitrk* mRNAs and representative sections showing defined patterns of expression are presented in Figure 1. To assess potential cross-reactivity of our cRNA probes among different *Slitrk* family members, we carefully compared their general patterns of expression on the premise that regions detected by only one *Slitrk* probe would provide

direct evidence that probes for other *Slitrks* do not also recognize this specific family member. For 4 probes (*Slitrk2, Slitrk3, Slitrk5, and Slitrk6),* there were regions expressing only a single *Slitrk* family member. For example, the neuroepithelium (*Slitrk2)* (Fig. 1B), the granular cell layer of the olfactory bulb (OB) (*Slitrk3*) (Fig. 2C), the molecular layer of the cerebellum (*Slitrk5)* (Fig. 7E), and the outer aspect of the neuroblastic layer of the retina (*Slitrk6*) (Fig. 10F) were each uniquely detected by only one probe.

Slitrk1 and *Slitrk5* were only expressed in regions coincident with other family members. Therefore, we used the deductive reasoning that follows to demonstrate the specificity of these two probes. *Slitrk1* was expressed in the ganglion cell layer of the retina but *Slitrk2*, *Slitrk4*, and *Slitrk6* were not (Fig. 10A, B, D, F). *Slitrk1* was also expressed in the ventricular zone of the cortex, but *Slitrk3* and *Slitrk5* were not (Fig. 3A, E, I). Together, these findings demonstrate that cRNA probes for *Slitrks2-6* do not recognize *Slitrk1* transcripts. In a similar fashion, we deduced the specificity of our probe for *Slitrk4,* which is expressed in the mitral cell layer of the OB (*Slitrk2* and *Slitrk6* are not; Fig. 2B, D, F) and the inner granule layer of the cerebellum at P10 (*Slitrk3* is not; Fig. 7C). *Slitrk1* and *Slitrk5* transcripts were detected in all regions expressing *Slitrk4*, and therefore we cannot completely exclude the possibility that these two cRNA probes may cross-react with *Slitrk4* transcripts. However, the signals observed with the *Slitrk1* and *Slitrk5* cRNA probes are consistently more robust than the signal observed with the *Slitrk4* cRNA probe in all regions examined, suggesting these probes specifically detect *Slitrk1* and *Slitrk5* transcripts rather than *Slitrk4* transcripts. Taken together, we conclude that the each of the cRNA probes used in this study is specific for its target, and does not cross-react with other members of the family. A summary of brain structures where *Slitrk1* to *Slitrk6* expression is observed is presented in Table 1 and a description of their expression in specific regions of the nervous system is provided below.

TABLE 1. Summary of Slitrks Gene Expression in the Developing Murine Nervous System[a]

	E18						P10					
I. Telencephalon												
Eye												
Neuroblastic layer	-	2	-	-	-	6						
Retinal GCL/INL	1	-	3	-	5	-						
Olfactory system												
Vomeronasal organ	-	-	-	-	-	-						
Olfactory epithelium	-	-	-	-	5	6						
OB glomerular layer	1	2	-	-	5	-	-	-	-	-	-	-
OB mitral cell layer	1	-	3	4	5	-	1	-	3	-	5	-
OB granular cell layer	-	-	3	-	-	-	1	-	3	4	5	-
Cerebral cortex												
Ventricular zone	1	2	-	-	-	-						
Subplate	1	-	-	4	5	-						
Cortical plate	1	2	3	4	5	-						
CI							-	-	-	-	-	-
CII – IV							1	-	3	-	5	-
CV							-	-	3	-	5	-
CVI							1	-	3	-	5	-
Piriform cortex	1	-	3	4	5	-						
Hippocampal formation												
CA1	1	2	3	4	5	-	1	2	3	4	5	-
CA3	1	2	3	4	5	-	1	2	3	4	5	-
Dentate gyrus	-	-	3	-	5	-	1	2	3	4	5	-
Striatum	1	2	3	4	5	-						
Amygdala	1	2	3	4	5	-						
Septum	1	-	3	4	5	6						
II. Diencephalon												
Thalamus	1	-	3	4	5	6						
Hypothalamus	1	2	3	4	5	6						
III. Mesencephalon												
Superior colliculus	1	-	3	-	5	-						
Inferior colliculus	1	-	3	-	5	-						
IV. Rhombencephalon												
Cerebellum												
Molecular layer	-	-	3	-	5	-	-	-	-	-	5	-
Purkinje layer	1	2	3	4	5	6	1	-	3	4	5	6
Inner granular layer							1	2	-	4	5	-
Deep cerebellar nuclei	1	-	3	4	5	6	1	-	3	4	5	-
VI. Others												
Spinal cord	1	2	3	4	5	6						
Dorsal root ganglia	1	2	3	-	5	-						
Trigeminal ganglion	1	2	3	4	5	-						

[a]-, not expressed; E, embryonic day; P, postnatal day.

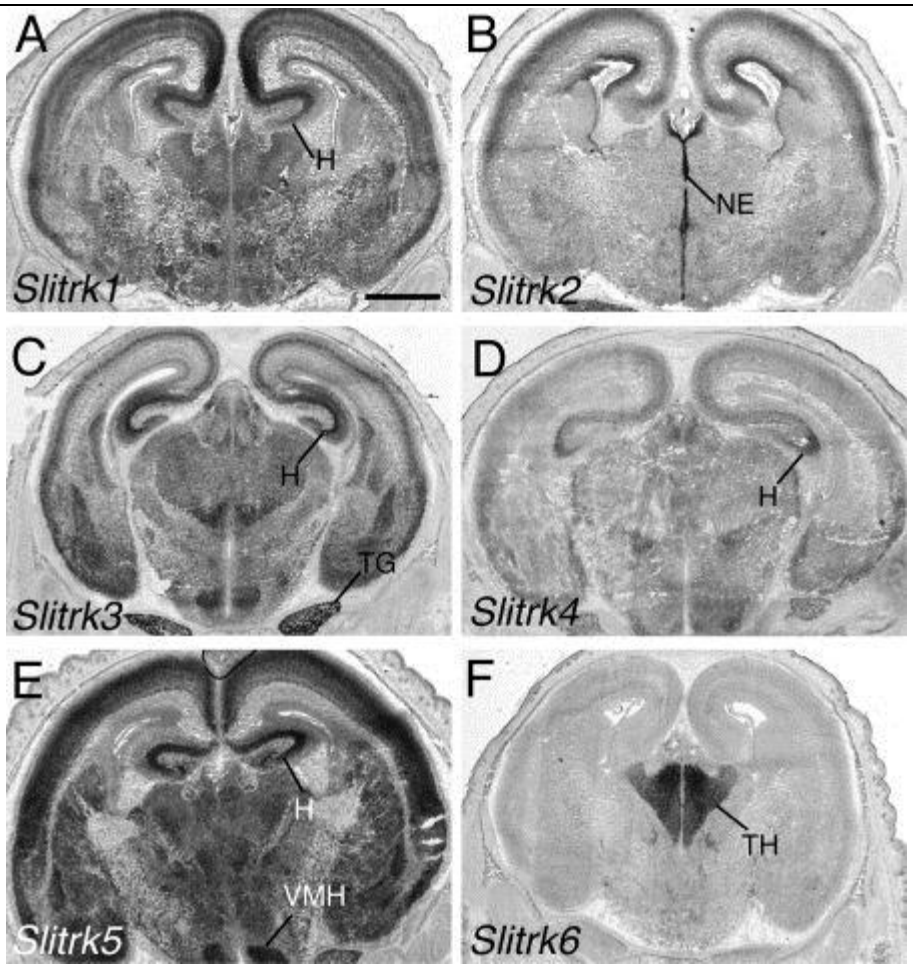

Figure 1: mRNA expression of Slitrk family members in the developing brain.

In situ hybridization of coronal sections of brains at E18 with antisense cRNA probes specific for *Slitrk1* (**A**), *Slitrk2* (**B**), *Slitrk3* (**C**), *Slitrk4* (**D**), *Slitrk5* (**E**), and *Slitrk6* (**F**). *Slitrk1-5* are widely expressed in the brain while *Slitrk6* expression is restricted to the thalamus. *Slitrk1, 3, 4,* and *5* are highly expressed in the hippocampus (H) while *Slitrk2* is expressed in the neuroepithelium (NE) (**A, B, C, D, E**). *Slitrk3* mRNA is detected in the trigeminal ganglia (TG) (**C**). *Slitrk5* is expressed in the ventral medial hypothalamic nucleus (VMH) (**E**). Scale bar = 1 mm.

5.2. Slitrks expression in the olfactory system

The olfactory bulb (OB) receives olfactory information coming from olfactory receptor neurons (ORNs) located in the olfactory epithelium and relays this information to higher brain structures (Cho et al., 2008a). ORNs project their axons to the OB where they form synapses with dendrites of second order neurons in neuropil structures termed glomeruli. *Slitrk1* mRNA is detected in the developing glomerular layer (GL) and in the mitral cell layer (MCL) containing second-order neurons which receive input from ORNs and send projections to pyramidal cells in the cortex (Fig. 2A). In contrast to *Slitrk1*, *Slitrk2* is expressed in cells lining the olfactory ventricule (OV) and in the GL (Fig. 2B). *Slitrk2* is also the only family member to be observed in the olfactory nerve layer (ONL). The *Slitrk3* gene is strongly expressed in the granule cell layer of the OB (GCL), which contains cells implicated in lateral inhibition of the mitral cells, and in the MCL (Fig. 2C). *Slitrk4* expression is restricted to the MCL while *Slitrk5* shows a pattern of expression similar to *Slitrk1* with expression in the GL and MCL (Fig. 2D, E). At E18, *Slitrk6* is the only member of the family that is not expressed in the OB (Fig. 2F). In contrast, expression of both *Slitrk5* and *Slitrk6* is detected in the olfactory epithelium (Table 1; data not shown). None of the Slitrks are expressed in the sensory organ of the accessory olfactory system, the vomeronasal organ (VNO; Table 1; data not shown). The expression of the Slitrk family members in the OB observed at E18 is mostly maintained at P10. At that age, the patterns of expression of *Slitrk1*, *Slitrk3* and *Slitrk5* are similar to E18 with signal detected in the MCL and in the GCL (Fig. 1G, I, K). *Slitrk2* expression in the OB is lost by P10 (Fig. 2H). *Slitrk4* mRNA expression is low and restricted to the GCL while *Slitrk6* mRNA is not detected at P10 in the OB (Fig. 2J, L). Interestingly, the expression of *Slitrk1*, 2, and 5 observed in the glomerular layer at E18 disappears by P10.

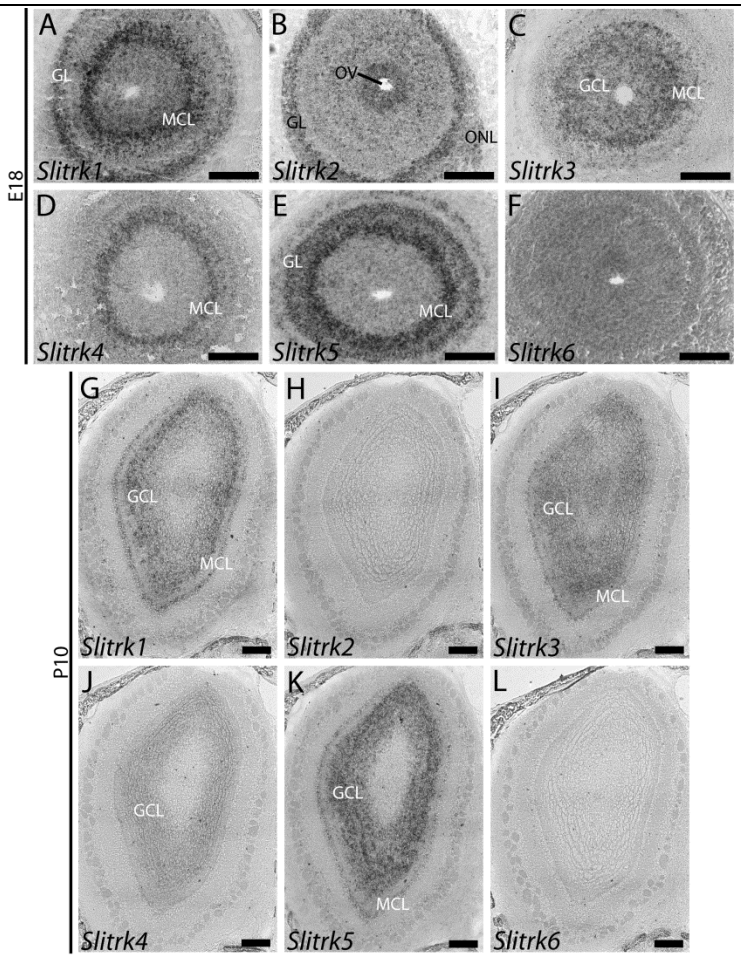

Figure 2: Slitrks mRNA expression in the developing olfactory bulb (OB) at E18 and P10.

In situ hybridization of coronal sections of OB with antisense cRNA probes for *Slitrk* genes. *Slitrk1* mRNA is detected in the glomerular layer (GL) and in the mitral cell layer (MCL) of the OB at E18 (**A**). By P10, *Slitrk1* expression is upregulated in the granular cell layer (GCL) (**G**). *Slitrk2* mRNA expression is observed in cells lining the olfactory ventricule (OV), in the GL, and at low levels in the ONL at E18 (**A**) but its expression in the OB is downregulated by P10 (**H**). *Slitrk3* mRNA expression is observed in the granule cell layer (GCL) and in MCL at E18 (**C**) and maintained at P10 (**I**). *Slitrk4* mRNA expression is restricted to the MCL at E18 (**D**) and downregulated by P10 (**J**). *Slitrk5* mRNA is detected in the MCL, GCL, and ONL at E18 (**E**) and in the MCL and

GCL at P10 (**K**). *Slitrk6* is not expressed in the OB at E18 and P10 (**F**, **L**). Scale bar = 250 μm.

5.3. Slitrks expression in the developing cerebral cortex

The fully formed cerebral cortex is composed of six distinct layers that become distinguishable after birth in the mouse. These layers are established as cortical neurons migrate radially from the proliferative ventricular zone to their final position in the cortex between E11 and E18 in response to local extracellular cues (Gupta et al., 2002). Axons of cortical neurons respond to guidance cues in the environment as they grow toward several targets including the thalamus, the spinal cord, and the contralateral cortex (Whitford et al., 2002). At E18, *Slitrks* are highly expressed in the developing cortex and their expression is maintained postnatally (Fig. 3). *Slitrk1* mRNA is most prominently expressed in the cortical plate (CxP) and in the subplate (SP) (Fig. 3A). In addition, low levels of expression are observed in the basal part of the ventricular zone (VZ; Fig.3A). In contrast to *Slitrk1*, *Slitrk2* is highly expressed in the apical layer of the VZ at E18 where mitotically active cells are located (Leone et al., 2008). *Slitrk2* mRNA is also detected at lower levels in the CxP (Fig. 3C). We detected *Slitrk3* mRNA expression only in a single layer of the CxP that can hardly be identified at E18 (Fig. 3E). *Slitrk4*, is expressed at low levels in the CxP and in the SP while *Slitrk5* mRNA is detected in the CxP and in the SP (Fig. 3G, I). *Slitrk6* is not expressed in the cortex (Fig. 3K, L). As development progresses, distinct populations of projection neurons are formed, are located in different cortical layers and areas, have unique morphological features, express different complements of transcription factors, and ultimately serve different functions (Molyneaux et al., 2007). At P10, *Slitrk1* is restricted to cortical layers II to IV (CII-IV) and to cortical layer VI (CVI) (Fig. 3B). The expression of *Slitrk2* and *Slitrk4* is decreased at P10 when compared to E18 (Fig. 3D, H) whereas *Slitrk3* and *Slitrk5* expression is maintained and restricted to layers 2 to 6 (Fig. 3F, J).

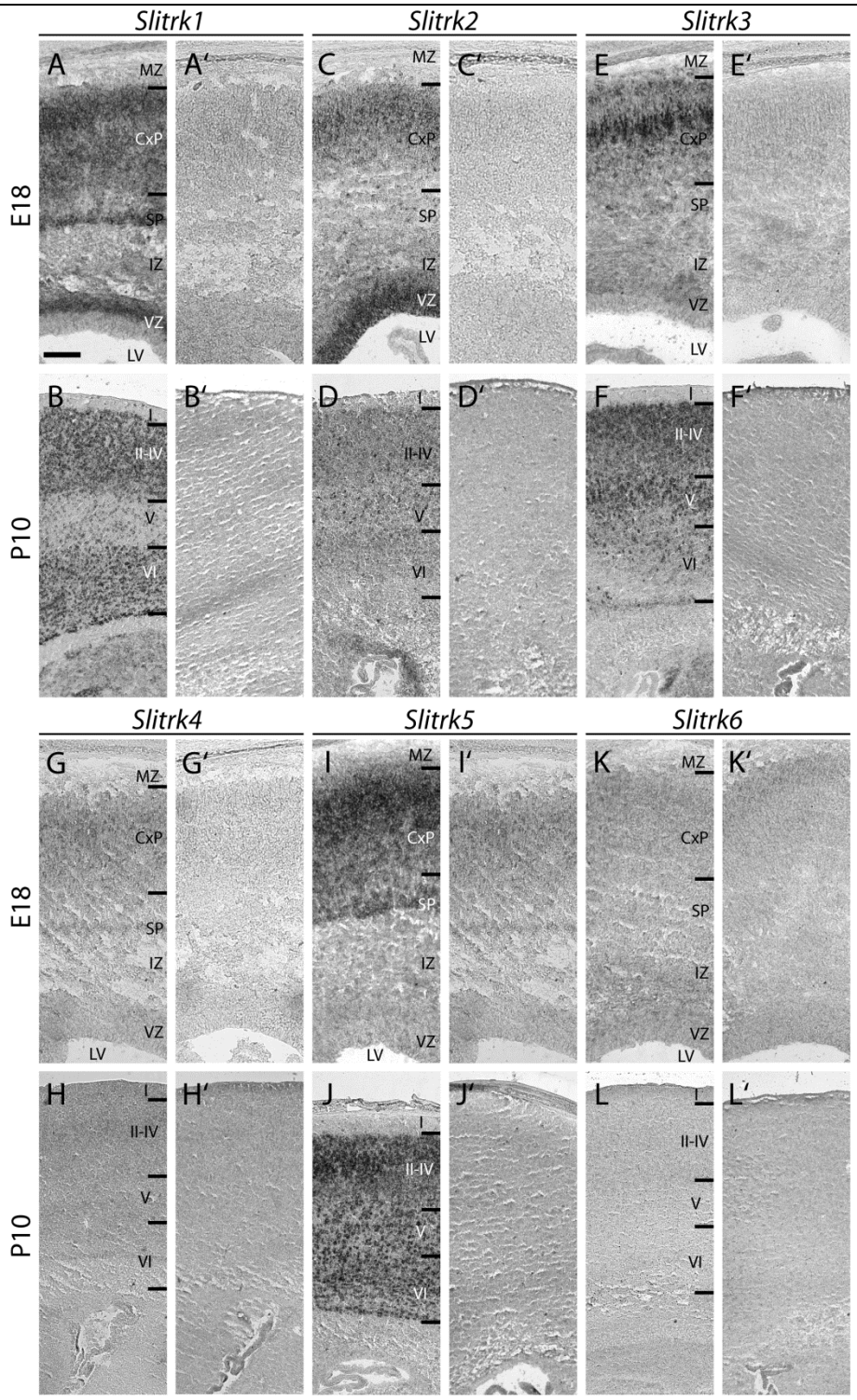

Figure 3: Slitrks mRNA expression during development of the cerebral cortex at E18 and P10.

In situ hybridization of coronal sections of cerebral cortex with sense (S) (**A'**-**L'**) or

antisense (AS) (**A-L**) cRNA probes for *Slitrk* genes. *Slitrk1* is highly expressed in the cortical plate (CxP), the subplate (SP), and in the apical part of the ventricular zone (VZ) at E18 (**A**). At P10, *Slitrk1* mRNA is observed in cortical layers II to IV (CII-IV) and CVI (**B**). *Slitrk2* mRNA is detected in the basal layer of the VZ and at low levels in the CxP at E18 (**C**). At P10, *Slitrk2* expression is significantly lower than at E18 (**H**). *Slitrk3* is expressed in the CxP at E18 (**E**) and is broadly expressed in cortical layers II to VI at P10 (**F**). *Slitrk4* mRNA is detected at low levels in the CxP and SP at E18 (**G**) and is absent in the cortex at P10 (**H**). *Slitrk5* is highly expressed in the CxP and SP at E18 (**I**) and its expression is maintained in all cortical layers at P10 (**J**). In contrast to *Slitrk5*, *Slitrk6* is not expressed in the cerebral cortex (K, L). Scale bar = 125 μm (**A, A', C, C', E, E', G, G', I, I', K, K'**) and 250 μm (**B, B', D, D', F, F', H, H', J, J', L, L'**).

5.4. Slitrks expression in the hippocampal region

The hippocampus is a brain structure that belongs to the limbic system and plays major roles in short term memory and many other memory processes (Riedel and Micheau, 2001). The hippocampal region also includes the dentate gyrus (DG), which is thought to contribute to the formation of new memories and is a site of continuous neurogenesis in the adult (Kempermann, 2002). *Slitrk1* mRNA is expressed abundantly in the CA1 and CA3 fields of the hippocampus at E18 and P10. In contrast, *Slitrk1* mRNA is not detected in the DG at E18 but low levels of expression is observed in the DG at P10 (Fig. 4A, G). Low levels of *Slitrk2* and *Slitrk4* expression are observed in the CA1 and CA3 fields of Ammon's horn at E18 and P10 whereas no expression is detected in the DG at E18 (Fig. 4B, D, H, J). As observed for *Slitrk1*, the expression of *Slitrk2*, and to a lesser extent *Slitrk4*, in the DG appears to be upregulated in the postnatal brain (Fig. 4H, J). The *Slitrk3* and *Slitrk5* patterns of expression are similar with strong signals across the hippocampal formation throughout development (Fig. 4C, E, I, K). *Slitrk6* is not expressed in the hippocampal region (Fig. 4F, L).

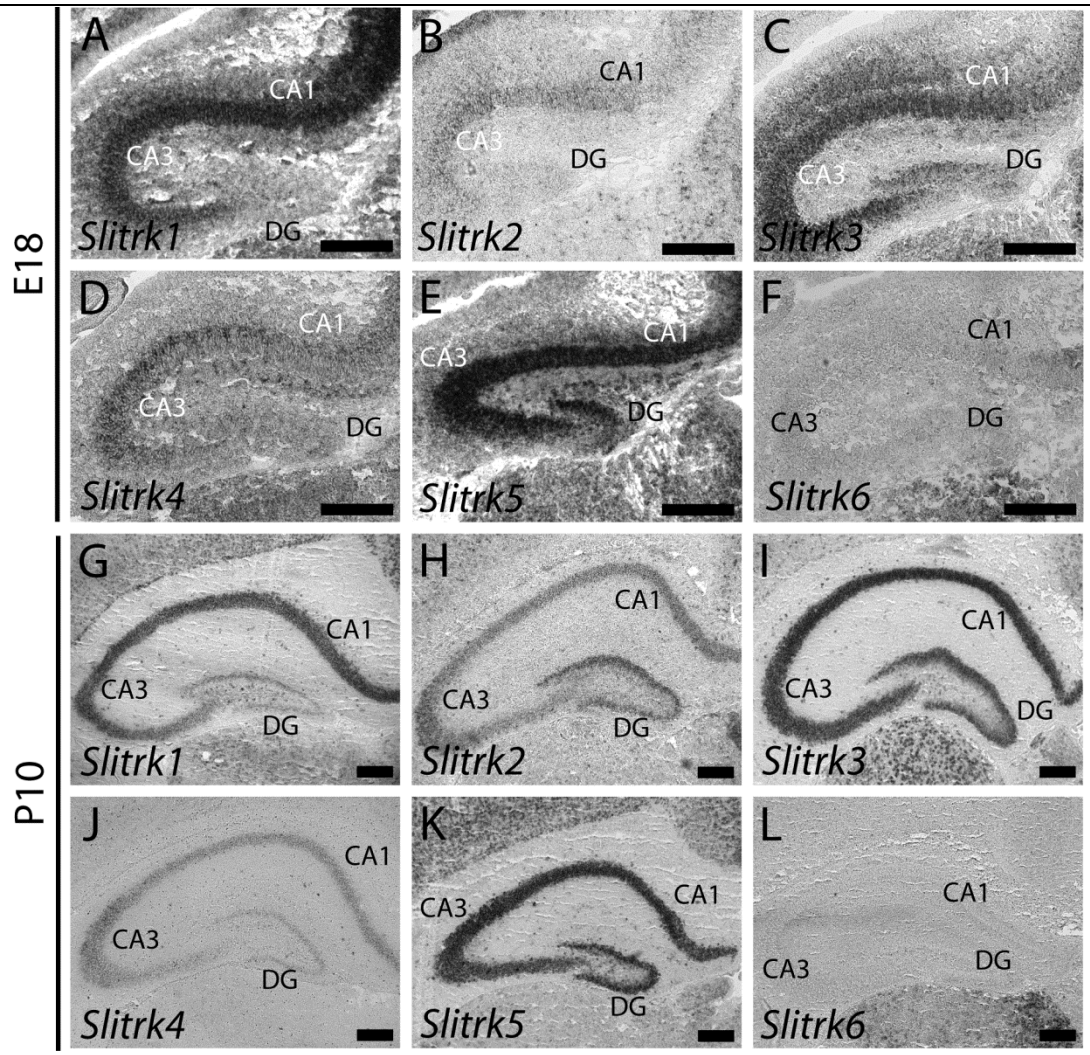

Figure 4: Slitrks mRNA expression in the developing hippocampal region at E18 and P10.

In situ hybridization of coronal sections of the hippocampal region with antisense cRNA probes for *Slitrk* genes. *Slitrk1* mRNA is observed in the CA1 and CA3 region of the hippocampus at E18 and P10 (**A, G**). While *Slitrk1* mRNA is not detected in the dentate gyrus (DG) at E18, low levels of *Slitrk1* mRNA are observed in this cell layer at P10 (**A, G**). *Slitrk2* and *Slitrk4* are expressed at low levels in the CA1 and CA3 regions at E18 and P10 and are also expressed in the DG at P10 (**B, D, H, J**). *Slitrk3* expression is observed in the CA1, CA3, and DG at E18 and P10 (**C, I**). High levels of *Slitrk5* mRNA are observed in all regions of the hippocampus at E18 and P10 (**E, K**). *Slitrk6* is not expressed in the hippocampus (**F, L**). Scale bar = 250 μm.

5.5. Expression of *Slitrk6* in the diencephalon

The highly compartmentalized expression of *Slitrk6* we observed in the developing mouse brain has previously been reported (Aruga, 2003). Since we predominately detected *Slitrk6* mRNA signal in the thalamus, we performed a detailed analysis of its expression within the diencephalon. *In situ* hybridization on consecutive sections starting from the most rostral part of the brain, first revealed *Slitrk6* expression in the dorsolateral septum (DLS), a structure that receives projections from CA1 region pyramidal neurons of the hippocampus and relays information to various brain regions including the medial septum–diagonal band complex (data not shown) (Amaral and Witter, 2004; Risold, 2004). *Slitrk6* signal was detected in several thalamic regions including the paraventricular (PV), paratenial (PT), anteromedial (AM), anteroventral (AV), mediodorsal (MD), and ventrolateral thalamic nuclei (VL) whereas low levels of *Slitrk6* expression were detected in the laterodorsal nucleus (LD) (Fig. 5 A, B). *Slitrk6* expression was also observed in the centromedial (CM), lateral posterior (LP), ventroposterior (VP), posterior complex (Po), and parafascicular thalamic nuclei (PF) (Fig. 5 C, D). Lower levels of *Slitrk6* mRNAs were detected in the dorsal lateral geniculate nucleus (dLG) (Fig. 5 C).

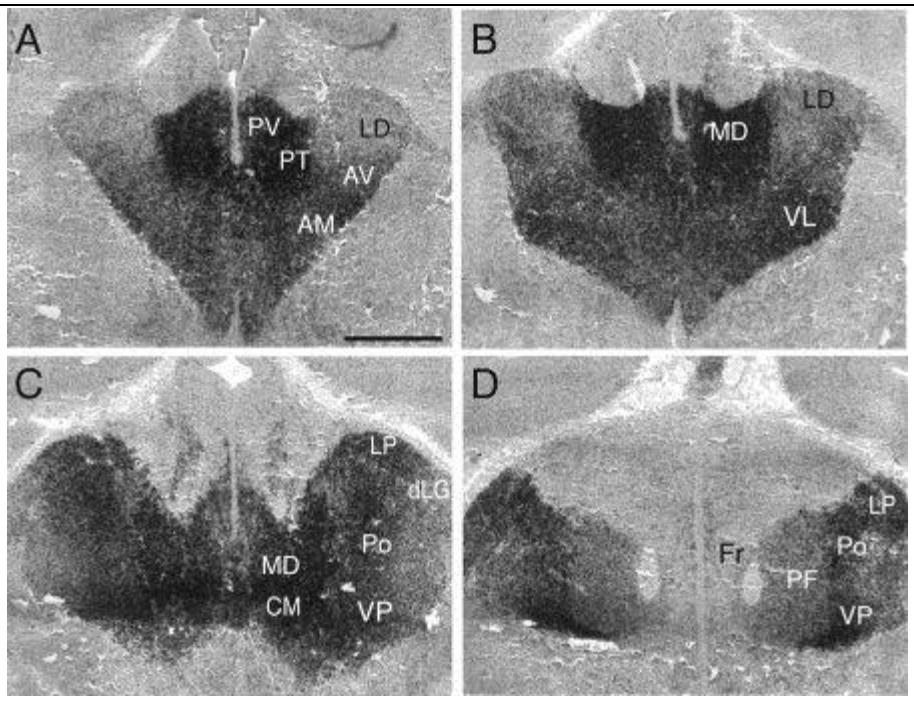

Figure 5: *Slitrk6* mRNA expression in thalamic regions at E18.

In situ hybridization of coronal sections of the diencephalon with an antisense cRNA probe for *Slitrk6*. *Slitrk6* mRNA is detected in the paraventricular (PV), paratenial (PT), anteroventral (AV), anteromedial (AM), mediodorsal (MD), ventrolateral (VL), and laterodorsal (LD) thalamic nuclei (**A, B**). *Slitrk6* is also expressed in the centromedial (CM), lateral posterior (LP), posterior complex (Po), ventroposterior (VP), parafascicular (PF), and dorsal lateral geniculate (dLG) nuclei (**C, D**). Fr: fasciculus retroflexus. Scale bar = 500 μm.

5.6. Slitrks expression in the cerebellum

The cerebellum plays a critical role in the integration of sensory inputs and in the regulation of motor control. The layered structure of the cerebellum is generated through extensive neuronal migration that takes place during late embryonic and early postnatal development (Komuro and Yacubova, 2003). At E18, all *Slitrk* genes were found to be expressed at various levels in the Purkinje cell layer (Pk) of the developing cortex (Fig. 6).

The variable expression of *Slitrk1* in the Pk cell layer may represent parasagittal stripes (Fig. 6A). In addition, *Slitrk3* and *Slitrk5* mRNAs were detected in the molecular layer of the cerebellum (Mo) (Fig. 6C, E). By P10, when a large proportion of granular neurons have migrated, *Slitrk1*, *Slitrk2*, *Slitrk4*, and *Slitrk5* are expressed in the inner granular layer (IGL) (Fig. 7A, B, D, E) while *Slitrk5* is the only member of the family expressed at significant levels in the Mo (Fig. 7E). Interestingly, *Slitrk1* mRNA seems to be located only in the IGL of anterior lobes of the cerebellum (Fig. 7A). The expression of Slitrk family members in the Pk observed at E18 is maintained at P10 except for *Slitrk2* whose expression is decreased in Purkinje cells at that stage of development. *Slitrk1*, *Slitrk3*, *Slitrk4*, and *Slitrk5* expression is also detected in the deep cerebellar nuclei (DN) (Fig. 7A, C, D, E). The *in situ* hybridization experiments performed on coronal sections at E18 also revealed high expression of *Slitrk3* and *Slitrk5* in the inferior (IC) and superior colliculi (SC) (Fig. 6C, E) which develop from the dorsal portion of the mesencephalon. *Slitrk2* is also expressed in the cells lining the aqueduct (Aq) (Fig. 6B).

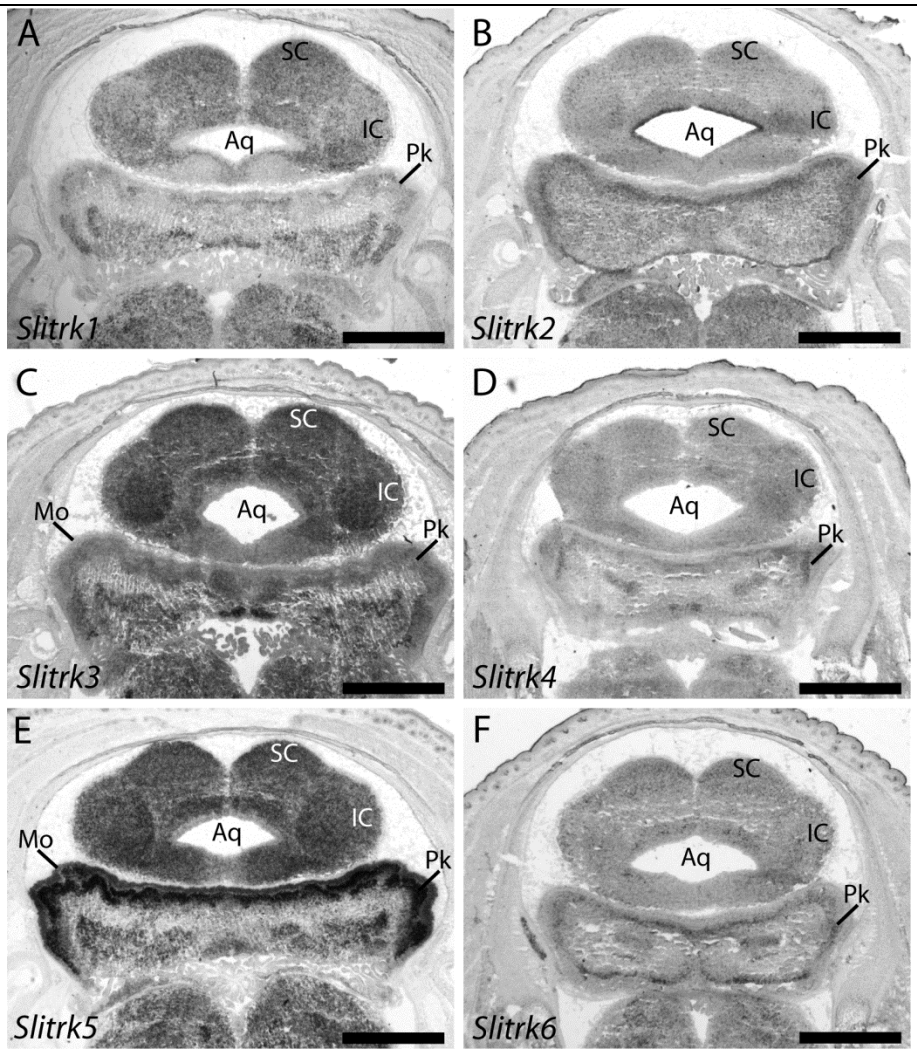

Figure 6: Slitrks mRNA expression in the developing cerebellum at E18.

In situ hybridization of coronal sections with antisense cRNA probes for *Slitrk* genes. *Slitrk1* is expressed in the Purkinje cell layer (Pk), the medial cerebellar nucleus (Med), and the lateral cerebellar nucleus (Lat) of the developing cerebellum. Low levels of *Slitrk1* are also observed in the superior colliculus (SC) and in the inferior colliculus (IC) (**A**). *Slitrk2* mRNA is also detected in the Pk and in cells lining the aqueduct (Aq) (**B**). *Slitrk3* mRNA is observed in the molecular layer (Mo), the Pk, the Med, the Lat, and at high levels in the SC and IC (**C**). *Slitrk4* is expressed at low levels in the Pk and Med, while *Slitrk5* is expressed at high levels in all layers of the developing cerebellum (**D, E**). *Slitrk6* mRNA is restricted to the Pk and Med (**F**). Scale bar = 1 mm.

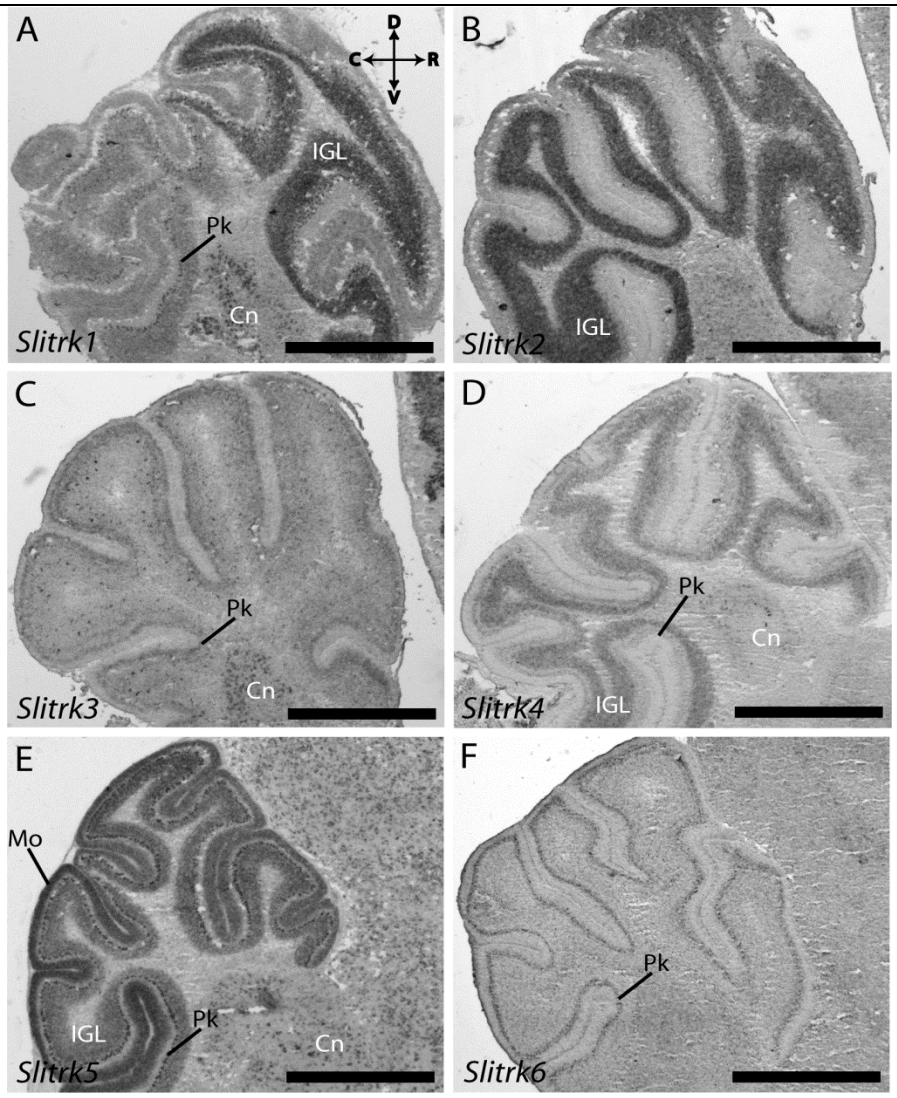

Figure 7: Slitrks mRNA expression in the cerebellum at P10.

In situ hybridization of sagittal sections of the cerebellum with antisense cRNA probes for *Slitrk* genes. *Slitrk1* is expressed in the Purkinje cell layer (Pk), in the inner granular layer of the cerebellum (IGL), and in the deep cerebellar nuclei (CN) (**A**). Interestingly *Slitrk1* expression in the IGL appears restricted to the anterior lobes of the cerebellum. *Slitrk2* is strongly expressed in the IGL while low levels of *Slitrk3* mRNA are detected in the Pk and CN (**B, C**). *Slitrk4* and *Slitrk5* mRNAs are observed in the IGL, the PK, and in the CN (**D, E**). In addition, *Slitrk5* is also expressed in the molecular layer (Mo) of the cerebellum. *Slitrk6* mRNA is restricted to the Pk. Scale bar = 500 μm.

5.7. Slitrks expression in the embryonic spinal cord

In the spinal cord, the Slitrks are expressed at different levels in the gray matter without any clear restriction to circumscribed areas although higher levels of *Slitrk1*, *Slitrk2*, *Slitrk3*, and *Slitrk5* are detected in the ventral motor pools (Fig. 8 A, B, C, E). It is also interesting to note that *Slitrk1*, *Slitrk2*, *Slitrk3* and *Slitrk5* mRNAs are present in a subset of sensory neuron nuclei in the dorsal root ganglia (DRGs). Whether different Slitrks are expressed in subsets of DRG neurons with specific sensory modalities remains to be determined.

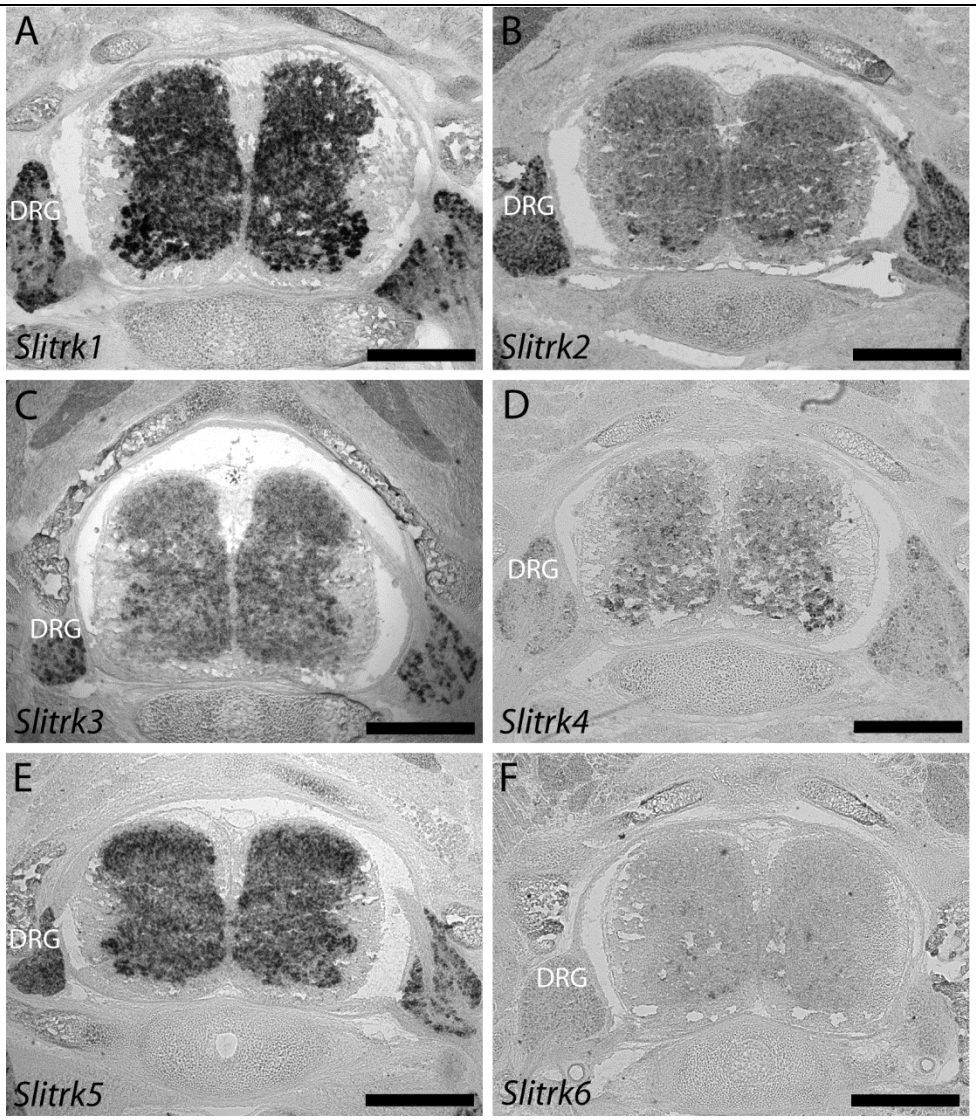

Figure 8: Slitrks mRNA expression during spinal cord development at E18.

In situ hybridization of coronal sections of the spinal cord with antisense cRNA probes for *Slitrk* genes. *Slitrk1*, *Slitrk2*, *Slitrk3*, and *Slitrk5* mRNAs are detected at various levels in the gray matter of the spinal cord (**A, B, C, E**). Higher levels of expression of *Slitrk1*, *Slitrk2*, *Slitrk4*, and *Slitrk5* are observed in the ventral part of the spinal cord where motor neurons are located (**A, B, D, E**). *Slitrk1*, *Slitrk2*, *Slitrk3*, *Slitrk4*, and *Slitrk5* are expressed in sensory neurons within the dorsal root ganglia (DRG) (**A-E**). *Slitrk6* mRNA is not detected in the spinal cord and DRG (**F**). Scale bar = 1 mm.

5.8. Slitrks expression in the trigeminal ganglion

Trigeminal ganglia (TG) are involved in several sensory modalities in the face including touch, pain, and temperature. TG sensory neurons can be classified in different groups based on their sensory modalities and they innervate mainly mechanoreceptors, thermoreceptors and nociceptors in the face, oral cavity and nasal cavity (Lazarov, 2002). Several members of the Slitrk family are detected in the trigeminal ganglia at E18. *Slitrk1*, *Slitrk2*, *Slitrk3*, and *Slitrk5* are highly expressed in the TG (Fig. 9A, B, C, E) while low levels of *Slitrk4* are observed and *Slitrk6* mRNA is not detected (Fig. 9D, F). It is interesting to note that the levels of expression of *Slitrk1*, *Slitrk2*, and *Slitrk4* appear to vary from cell to cell within the ganglia with a subset of cells expressing higher levels of these transcripts. It remains possible that Slitrks may be expressed differentially in TG neurons subserving specific sensory modalities.

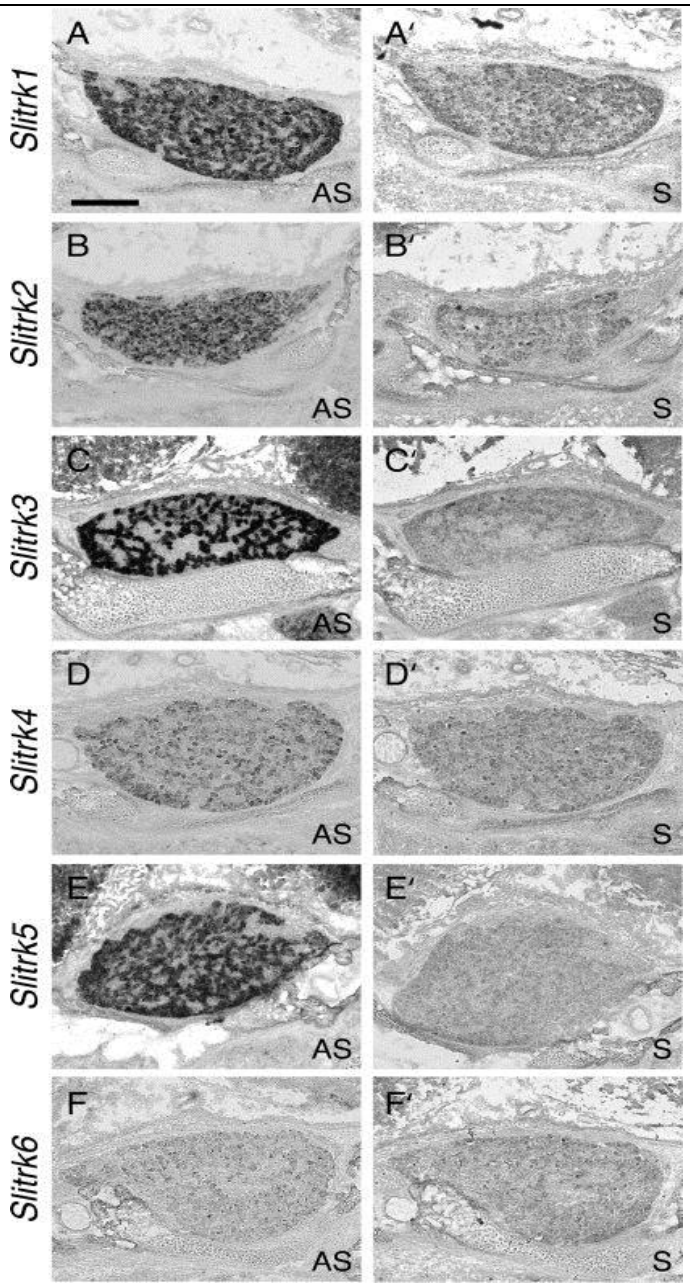

Figure 9: Slitrks mRNA expression in the developing trigeminal ganglion (TG) at E18.

In situ hybridization of coronal sections of the TG with sense (S) (**A'-F'**) or antisense (AS) (**A-F**) cRNA probes for *Slitrk* genes. *Slitrk1*, *Slitrk2*, *Slitrk3*, and *Slitrk5* are expressed at high levels in subsets of cell bodies in the TG (**A, B, C, E**). Low levels of *Slitrk4* mRNA are detected in the TG while no signal above background levels is observed for *Slitrk6* (**D, F**). Scale bar = 250 μm.

5.9. Slitrks expression during eye development

At E18, the neural retina consists of the neuroblastic layer (NBL), which is destined to give rise to horizontal cells and photoreceptor cells, a transient intermediate anuclear layer (of Chievitz), a retinal ganglion cell layer, and a developing inner nuclear layer (INL) (Kaufman, 1992). The expression patterns of *Slitrk* genes at E18 are particularly interesting in the developing retina where they are mostly expressed in a complementary fashion. *Slitrk1* and *Slitrk5* are exclusively detected in the retinal ganglion cell layer (RGCL) and presumptive INL (Fig. 10A, E). Low levels of *Slitrk3* are also detected in the RGCL (Fig. 10C). In contrast, *Slitrk2* expression is restricted to the NBL and *Slitrk6* is observed in the outermost region of the NBL (Fig. 10B, F). *Slitrk4* expression is not observed in the retina (Fig. 10D).

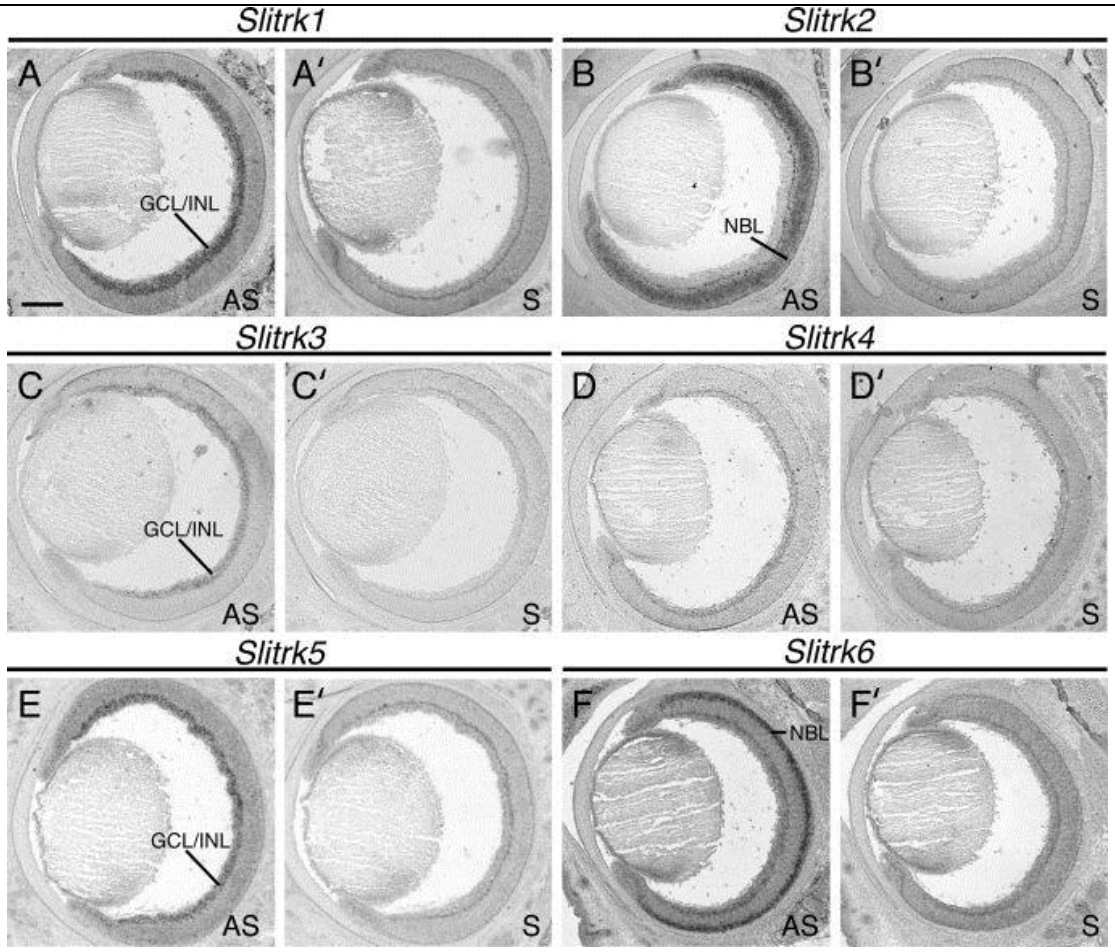

Figure 10: Slitrks mRNA expression in the developing eye at E18.

In situ hybridization of coronal sections of the retina with sense (S) (**A'-F'**) or antisense (AS) (**A-F**) cRNA probes for *Slitrk* genes. *Slitrk1*, *Slitrk3*, and *Slitrk5* expression is restricted to the retinal ganglion cell layer (RGCL) and prospective inner nuclear layer (INL) while *Slitrk2* is specifically expressed in the inner aspect of the neuroblastic layer (NBL) (**A, B, E**). *Slitrk6* expression is restricted to the outer aspect of the neuroblastic layer and *Slitrk4* is not expressed in the retina (**D, F**). Scale bar = 250 μm.

6. Discussion

Our understanding of the role of Slitrk family members during development of the nervous system is quite limited. To gain more insight into the potential functions of these proteins, we have performed a detailed analysis of the expression of all six members of the family in the developing murine nervous system. Our results show that each Slitrk family member is expressed in a unique spatiotemporal manner. Within several structures analyzed, including the olfactory bulb, the cortex, and the cerebellum, Slitrk family members are complementarily expressed in different regions. This observation is perhaps most striking in the developing retina where *Slitrk1*, *Slitrk3*, and *Slitrk5* expression is restricted to the RGCL while *Slitrk2* and *Slitrk6* are expressed in the NBL (Fig. 10). The regional segregated expression of different Slitrks in these structures is suggestive of functional specialization for the different members of the family. In addition, the expression of several family members in post-natal brains suggests that while Slitrks may play a role in regulating developmental processes taking place during embryogenesis, the majority of Slitrks may also be required for processes occurring at later time during development including synapse formation and plasticity.

Our observation that some members of the Slitrk family, such as *Slitrk1* and *Slitrk2*, are expressed in varying levels among populations of neurons located in the trigeminal and dorsal root ganglia raises the possibility that Slitrks can regulate the differential fasciculation of axons through homophilic or heterophilic interactions. Other LRR-containing transmembrane proteins, such as FLRT3 and AMIGO, have been shown to regulate adhesion (Kuja-Panula et al., 2003; Robinson et al., 2004; Karaulanov et al., 2006). Hence, the differential expression of Slitrks on specific populations of axons may promote segregation of subsets of axons within large fascicles.

We have observed high levels of *Slitrk2* expression in several regions containing neuroepithelium, where generation of newborn neurons takes place in the CNS. These include the ventricular zone of the developing cortex, the ventricule of the olfactory bulb, and the neuroblastic layer of the retina. In addition, expression of *Slitrk2* in the ventricular zone of the spinal cord early during development has previously been

reported (Aruga and Mikoshiba, 2003). These results suggest that *Slitrk2* may be involved in controlling the proliferation of progenitor cells or their differentiation into neurons. In addition, the restricted expression of Slitrk family members could control the differentiation of different populations of cells within specific regions. For example, in the retina, *Slitrk1* and *Slitrk5* are expressed in retinal ganglion cells while *Slitrk2* and *Slitrk6* are expressed in the neuroblastic region giving rise to photoreceptors and horizontal cells.

In conclusion, our study shows that members of the Slitrk family are widely expressed in the central nervous system, with partially overlapping yet distinct patterns of expression. These analyses will permit the future development of *in vitro* and *in vivo* assays to define the role of the different Slitrks during development of the nervous system.

CHAPTER 3

SLITRK1 AND SLITRK2 PROMOTE SYNAPSE DEVELOPMENT

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1. Preface

This chapter is a manuscript that will shortly be submitted to The Journal of Biological Chemistry after the deposit of the thesis. In the previous chapter, we studied the expression pattern of the six Slitrk family members in the nervous system. The strong expression of *Slitrks* in the hippocampus combined with the fact that multiple LRR-containing proteins can modulate synaptogenesis in hippocampal neurons lead me to hypothesis that Slitrks may regulate synaptogenesis. In this chapter, I examine the role of Slitrk1 and Slitrk2 in synaptogenesis using a set of biochemical and *in vitro* approaches. I demonstrate that both Slitrk1 and Slitrk2 can promote synaptogenesis in hippocampal neurons.

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2. Abstract

Following the migration of the axonal growth cone to its target area, the initial axo-dendritic contact needs to be transformed into a functional synapse. This multi-step process relies on overlapping but distinct combinations of molecules that confer synaptic identity. Slitrk molecules are members of a family of transmembrane proteins that are highly expressed in the central nervous system. We found that two members of the Slitrk family, Slitrk1 and Slitrk2, can regulate synapse formation between hippocampal neurons. Slitrk1 and Slitrk2 are enriched in postsynaptic fractions and can dimerize when expressed in heterologous cells. Expression in nonneural and neural cells leads to presynaptic differentiation in contacting axons. While overexpression of Slitrk1 and Slitrk2 in hippocampal neurons increased the number of excitatory synaptic contacts on these neurons, it did not modulate the number of inhibitory synaptic contacts. In contrast, treatment of hippocampal neurons with a Slitrk1 function-blocking antibody reduced the number of excitatory and inhibitory synapses formed in hippocampal neurons. Similarly, perturbing Slitrk1 interactions with recombinant protein interfered with excitatory synapse formation. Altogether, our results indicate that Slitrk family proteins regulate synapse formation.

3. Introduction

One of the key steps in the development of the nervous system is the formation of new connections between different neurons. This process, referred to as synaptogenesis, also plays a critical role in the mature brain where the dynamic modification of circuitry has a profound effect on functions such as learning and memory. Multiple families of cell adhesion molecules have been implicated in various aspects of synapse formation, such as the formation of initial contacts and synapse maturation. These include members of the neuroligin (Ichtchenko et al., 1995; Scheiffele et al., 2000), neurexin (Ichtchenko et al., 1995; Graf et al., 2004), LRRTM (de Wit et al., 2009; Ko et al., 2009; Linhoff et al., 2009), SynCAM (Biederer et al., 2002; Robbins et al., 2010), netrin G-ligand (NGL) (Kim et al., 2006), SALM (Mah et al., 2010), and EphB (Kayser et al., 2006) families of cell

surface proteins. Whether these different families of molecules function cooperatively or redundantly remains to be fully addressed.

It has been suggested that differences in neural connectivity or synaptic patterning underlie many neurodevelopmental disorders including autism and SCZ (Wright and Washbourne, 2010). For example, familial forms of autism-spectrum disorders have been linked to mutations in several neuroligin and neurexin (reviewed in (Sudhof, 2008; Bourgeron, 2009)) as well as in SynCAM 1 and cadherin (Zhiling et al., 2008; Wang et al., 2009). Another family of transmembrane proteins that has been implicated in the etiology of brain disorders is the Slitrks. While SLITRK1 was proposed as a susceptibility gene for TS (Abelson et al., 2005; Miranda et al., 2008), variants of the SLITRK2 gene were found in patients with SCZ (Piton et al., 2010a).

Slitrks form a family of six structurally similar proteins that contain two LRR motifs in their extracellular portion and a cytoplasmic region that varies in size between members of the family (Aruga and Mikoshiba, 2003). Leucine-rich repeat motifs are protein-protein interaction domains commonly found in synaptogenic proteins (Ko and Kim, 2007). Despite some overlap in their expression, the Slitrks display mostly distinct patterns of expression in the developing murine nervous system suggesting they may play different roles in specific regions of the brain (Beaubien and Cloutier, 2009). In keeping with this possibility, gene ablation studies in mice for different Slitrk family members have yielded distinct phenotypes. While ablation of *Slitrk1* expression leads to increased anxiety-like behaviour (Katayama et al., 2008), *Slitrk5* mutant mice display obsessive compulsive-like behaviors (Shmelkov et al., 2010) and *Slitrk3* mutant mice exhibit increased susceptibility to seizures (Takahashi et al., 2012). In contrast, *Slitrk6*$^{-/-}$ mice display specific defects in development of the inner ear, including disorganized innervation and neuronal loss (Katayama et al., 2009). Based on the structure of Slitrks and the nature of phenotypes observed in some *Slitrk* mutant mice, Slitrks were proposed to regulate synapse formation in the CNS. Recent evidence has shown that Slitrks can promote presynaptic differentiation in a neuron-fibroblast coculture assay,

and that Slitrk3 is specifically required for the formation of inhibitory synapses both *in vitro* and *in vivo* (Linhoff et al., 2009; Takahashi et al., 2012).

Here we have examined the function of two members of the Slitrk family, Slitrk1 and Slitrk2, in synapse formation. We show that while Slitrk1 and Slitrk2 can promote excitatory synapse formation in cultures of hippocampal neurons, they are not sufficient to induce inhibitory synapse development in these cells. Furthermore, inhibition of Slitrk1 function using an antibody raised against its extracellular region reduces the number of excitatory and inhibitory synapses between hippocampal neurons in culture. Taken together, our results indicate that Slitrks 1 and 2 can regulate synapse formation.

4. Experimental Procedures

cDNA constructs

Full-length human *Slitrk1* (aa 1-696), human *Slitrk2* (aa 1-845) and *Slitrk2⊿ICD* (aa 1-646) lacking the intracellular portion were subcloned into pEGFP-N1 vector (Clontech). For C-terminal MYC-tagged Slitrk construct, full-length human *Slitrk1* (aa 1-696), full-length human *Slitrk2* (aa 1-845), *Slitrk1⊿ICD* (aa 1-660) and *Slitrk2⊿ICD* (aa 1-646) were subcloned into pcDNA3.1 Myc-His A vector or pcDNA3.1 V5-His A vector (Invitrogen). GFP-tagged LAR, mVenus-tagged neuroligin-1 lacking inserts in splice sites A and B, GFP-tagged N-cadherin, and HA-tagged neurexin1β (-S4) were kind gifts from Dr Wiljan Hendricks, Dr Thomas C. Südhof, Dr David R. Colman, and Dr Peter Scheiffele, respectively.

Antibodies

A rabbit polyclonal antibody recognizing the intracellular portion of Slitrk1 (referred to as Slitrk1-C) was generated against the peptide DGSHRVYDCGSHS (aa 680-693 of the mouse sequence) and purified against the same epitope. Another Slitrk1 rabbit polyclonal antibody (namely Slitrk1-N) was obtained from an animal immunized with the complete extracellular portion of the protein (aa 2-600). The other antibodies were purchased commercially: Slitrk2 (R&D Systems), vGlut1 (NeuroMab), PSD-95 (BD Pharmingen,

NeuroMab), MYC tag (clone 9E10), GFP (Novus), synapsin I (Milipore), synaptotagmin I luminal, Gephyrin and VGAT (Synaptic Systems), p42 MAP kinase (Cell signaling), HA tag (Abcam), synaptophysin and α-Tubulin (Sigma-Aldrich), and MAP2 (GeneTex).

Reverse Transcription PCR

For RT-PCR experiments, total RNA was isolated from embryonic day 18-19 (E18-19) rat hippocampal neurons cultured 10 days in vitro (DIV), and total E18-19 rat brain using an RNeasy kit (Qiagen). cDNA was prepared with a ThermoScript reverse transcriptase kit (Invitrogen). Primers for PCR detection were as follows: Slitrk1F, 5' - ccgctggaaacgttacaggggac; Slitrk1R, 5'- cgtttcaccagctgcagccccag; Slitrk2F, 5' - ctgagcggcgtctggttcctc; Slitrk2R, 5'- cccaggtcggatctcctgcaacc; Slitrk3F, 5' - gagcgaacctcggagatcctgagc; Slitrk3R, 5'- gttcccaaggttaatggacacagcattattc; Slitrk4F, 5' - tggctctttctgattgtgtcagccctg; Slitrk4R, 5'- gcactgagcccgagaaatgctccac; Slitrk5F, 5' - cccca gtaactttggaacaggaccttcac; Slitrk5R, 5'- cattgctacccagatgtaaaattgaagcccc; Slitrk6F, 5' - ctgaccgactttggacacctcttccagc; Slitrk6R, 5'- ccaaggccattaaatgcacctgtctcg; GAPDHF, 5' - gcatcctgcaccaccaactg; GAPDHR, 5'-cggccgcctgcttcaccaccttct.

Cell culture

E18-19 rat embryos were obtained from timed pregnant Sprague Dawley females purchased from Charles River (Saint-Constant, Québec, Canada). Cultures of hippocampal neurons were prepared from the embryos according to previously described protocols (Banker and Goslin, 1998; Kaech and Banker, 2006). Mixed-culture assays of HEK293T and neurons were performed essentially as described previously (Biederer and Scheiffele, 2007). Briefly, HEK293T cells were transfected with the constructs of interest for 24 hrs, trypsinized, plated onto cultured hippocampal neurons at 9 DIV, followed by immunostaining at 11 DIV. For the synaptotagmin I antibody uptake assay, neurons were incubated with antibodies to the synaptotagmin I luminal domain (1:10) or antibodies against MYC at the same concentration in isotonic depolarizing solution (KCl) for 5 min at 37°C (Biederer and Scheiffele, 2007).

Transfection of neurons and immunocytochemistry

Cultured neurons were transfected using a mammalian transfection kit (Clontech) at 13 DIV, fixed with 4% paraformaldehyde/4% sucrose (vol/vol) at 15 DIV, and permeabilized with 0.2% Triton X-100 (vol/vol) in phosphate-buffered saline (pH=7.4). Neurons were then incubated with specific primary antibody followed by Alexa-488-, Alexa-546-, Alexa-647-conjugated secondary antibodies (1:500; Invitrogen).

Synaptosomal fractionation

PSD fractionation was performed on adult rat brain (Unstripped brain, 7-8 week old, ID 56004-2, Pel-Freez Biologicals) as described (Fallon et al., 2002) with some minor modifications: a purified P2 pellet was incubated in 0.5% Triton-X 100, 40 mM Tris-HCl (pH=8.0) and then centrifuged at 32,000 x *g* to generate the PSD1 fraction. This fraction was then further extracted with 0.5% Triton-X 100, 40 mM Tris-HCl (pH=8.0), and subsequently centrifuged at 200,000 x *g* to isolate the PSD2 fraction.

Cell surface biotinylation

COS cells were transfected with the different plasmids for 24 hrs, washed 3 times with PBS 1X, incubated with EZ-Link Sulfo-NHS-LC-Biotin 1 mg/ml (Thermo Scientific Pierce) at 4°C for 30 min, and washed 3 times with PBS + 100mM glycine to quench the biotin reagent. The cells were then lysed and surface proteins were isolated using streptavidin agarose beads (Thermo Scientific Pierce).

Image acquisition and quantification

Images were acquired using a confocal microscope with 40x or 63x oil objective (LSM710; Zeiss) and Z-stacks were used in the analysis. The settings were kept constant for all scanning in each experiment. To determine the average fluorescent intensity of synaptic protein clusters in coculture experiments, randomly chosen HEK293T cells were used for quantification with ImageJ software (NIH). Acquired images were thresholded, and the integrated intensity of the clusters on the HEK293T cell was normalized to the cell area. To determine the average fluorescent intensity of synaptic

protein clusters in overexpression experiments, the same approach was used but using the cell area of the transfected neurons excluding the cell body. All values are presented as mean ± s.e.m. and significance of the quantification was determined by Student's *t* test.

Slitrk1 neutralization experiment

The rabbit Slitrk1-N polyclonal antibody was used for all Slitrk1 neutralizing antibody experiments. Non-immunized purified rabbit IgG (Sigma-Aldrich) was used as the negative control. Recombinant human Slitrk1 protein (Ala16 to Ser616 with a C-terminal 6-His tag) was obtained commercially (R&D Systems). Slitrk1-C antibodies (~10 ug/ml) and recombinant Slitrk1 (1.2 ug/ml) were added in parallel into culture media of hippocampal neurons at 9, 10 and 11 DIV. The treated neuronal cultures were immunostained at 12 DIV. For the coculture of HEK293T and neurons experiments, the two reagents were added separately 4 hrs after plating the transfected HEK293T with neurons at 9 DIV followed by another application at 10 DIV and by immunostaining at 11 DIV. The images were then acquired using a confocal microscope.

5. Results

5.1. Slitrk1 and Slitrk2 are enriched in synaptosomal and postsynaptic density fractions

The Slitrks are predominately expressed in neural tissues at embryonic ages and postnatally (Aruga and Mikoshiba, 2003; Aruga et al., 2003; Beaubien and Cloutier, 2009). More specifically, their localization at the synapse has been recently suggested based on the presence of Slitrk3 and Slitrk5 at synaptic sites in cultured neurons (Shmelkov et al., 2010; Takahashi et al., 2012). To determine whether other Slitrk family members are found at the synapse, we examined the distribution of Slitrk1 and Slitrk2 in subcellular fractions generated from adult rat brain using specific antibodies against the intracellular domain of Slitrk1 (Slitrk1-C) or the extracellular portion of Slitrk2. Both Slitrk1 and Slitrk2 were found in synaptic membrane fractions (Fig. 1A). Interestingly, Slitrk2 appeared to be more selectively enriched than Slitrk1 in PSD fractions. For

Slitrk1, two closely migrating bands were observed likely representing differentially glycosylated forms of Slitrk1 that have previously been described (Kajiwara et al., 2009). Since Slitrk1 and Slitrk2 show high sequence similarity, we confirmed the specificity of both antibodies by examining their ability to detect MYC-tagged Slitrk1 or Slitrk2 proteins expressed in HEK293T cells. Both Slitrk1 and Slitrk2 antibodies specifically recognized Slitrk1 or Slitrk2, respectively, and did not cross-react with the other Slitrk (Fig. 1B). Hence, these results indicate that Slitrk1 and Slitrk2 can be localized to synapses and may thus play a role in synaptogenesis. To further explore the properties of Slitrks, we examined their ability to dimerize when expressed in heterologous cells. Immunoprecipitation experiments revealed that Slitrk1 and Slitrk2 can homodimerize when expressed in HEK293T cells (Fig. 1C). Furthermore, co-expression of Slitrk1 and Slitrk2 in these cells led to the formation of heterodimers between the two proteins, indicating that Slitrk family members can form both homo- and heterodimers at the cell surface (Fig. 1C).

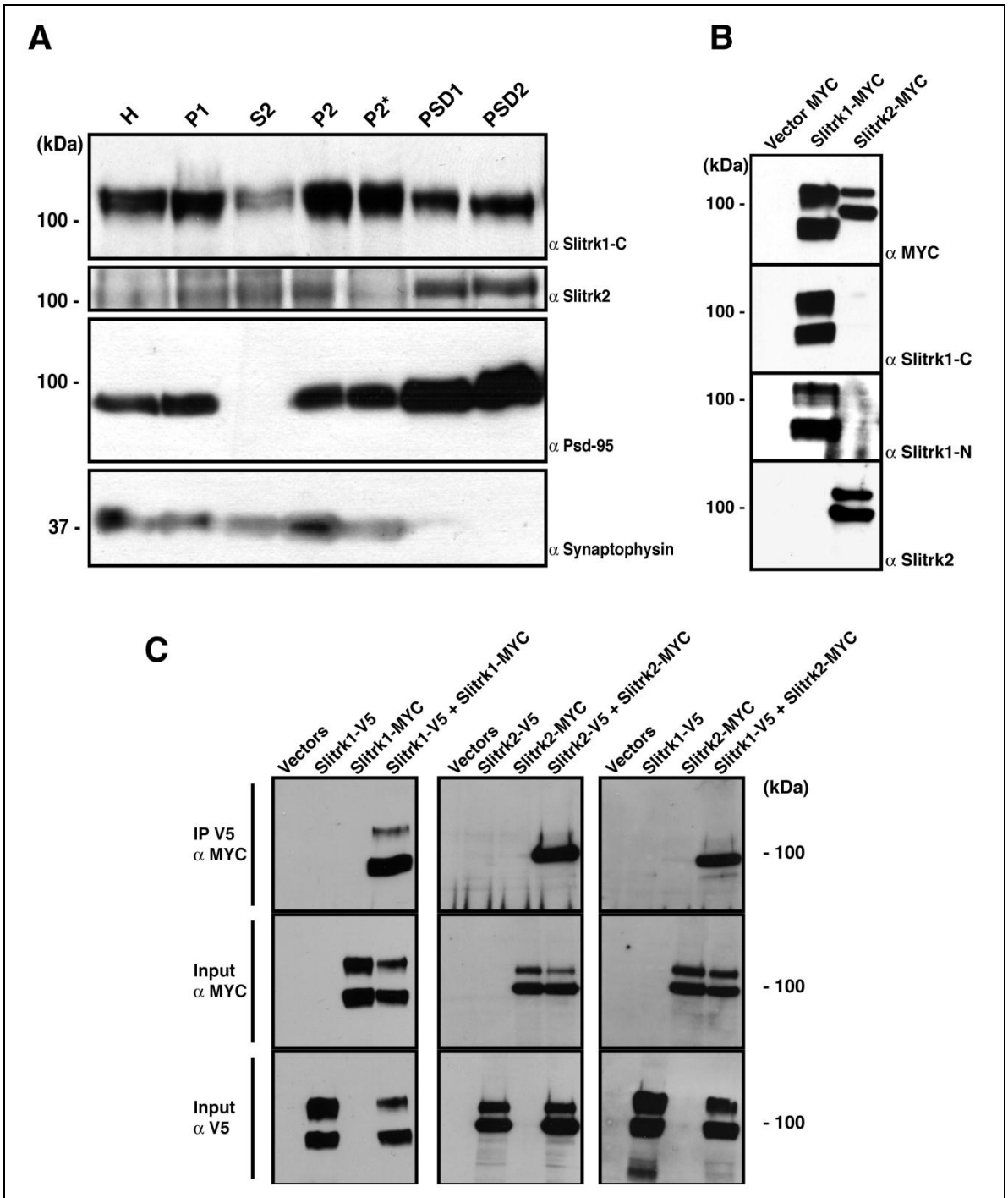

Figure 1: Localization of the Slitrks at the synapse and their capacity to dimerize.

(**A**) Distribution of Slitrk1 and Slitrk2 in subcellular fractions of adult rat brain. Note that Slitrk1 and Slitrk2 are detected in synaptic fractions including P2, P2* and in the PSD. PSD-95 and synaptophysin (SynPhy) were probed for comparison. H, homogenate; P1,

crude nuclear fraction; S2, supernatant after P2 precipitation; P2, crude synaptosomes; P2*, purified synaptosomes; PSD1, pellet after the first Triton X-100 extraction; PSD2, pellet after the second Triton-X 100 extraction. (**B**) Characterization of Slitrk1 and Slitrk2 antibodies. Slitrk1 and Slitrk2 (C-terminally MYC tagged) expressed in HEK293T cells were immunoblotted with Slitrk1-C, Slitrk1-N, Slitrk2, and Myc antibodies. Myc antibodies were used to normalize the data. These antibodies are isoform specific. (**C**) Slitrks form complexes in heterologous cells. HEK293T cells were co-transfected with Slitrk1-V5 + Slitrk1-MYC, Slitrk2-V5 + Slitrk2-MYC, Slitrk1-V5 + Slitrk2-MYC, or Slitrk1ΔICD-V5 + Slitrk2ΔICD-MYC. Proteins were solubilised with RIPA buffer 48hrs post-transfection and immunoprecipitated with anti-V5 antibodies. Blots were then probed with anti-MYC (Input = immunoblots of the lysate). These results indicate that Slitrk1 and Slitrk2 can form homomeric and heteromeric complexes independent of their intracellular portion when expressed together in HEK293T cells.

5.2. Slitrk1 and Slitrk2 induce presynaptic differentiation in a mixed-culture assay

To investigate whether Slitrk1 and Slitrk2 show synaptogenic activity, we used a mixed-culture assay where hippocampal neurons are grown in the presence of nonneuronal cells expressing a transmembrane protein of interest, and the differentiation of presynaptic specializations in contacting axons is assessed (Scheiffele et al., 2000; Biederer and Scheiffele, 2007). In this assay, expression of the well-characterized synaptogenic protein neuroligin-1 in nonneuronal cells promotes clustering of presynaptic vesicle markers in hippocampal neurons, while expression of N-cadherin fails to concentrate synaptic vesicles in contacting axons (Fig. 2A,B) (Scheiffele et al., 2000; Sara et al., 2005). Expression of either Slitrk1 or Slitrk2 induced clustering of synapsin I, a presynaptic vesicle marker, in contacting axons of cultured hippocampal neurons (Fig. 2A,B). Interestingly, the extent of synapsin I clustering observed with either Slitrk1 or Slitrk2 was lower than the clustering observed with neuroligin-1 expression despite the fact that Slitrk1 and Slitrk2 are expressed at similar or higher levels at the surface of the COS cells

(Fig. 2C). Based on these observations, we hypothesized that Slitrk1 and Slitrk2 may require the presence of additional Slitrk family members to achieve their full synaptogenic potential. In keeping with this possibility, Slitrk1 and Slitrk2 can form homomeric and heteromeric complexes when expressed in HEK293T cells (Fig. 1C). However, co-expression of Slitrk1 and Slitrk2 in HEK293T cells did not significantly increase synapsin I clustering when compared to cells expressing either Slitrk1 or Slitrk2 (Fig. 2A,B). Furthermore, the extracellular region of Slitrk2 is sufficient to promote synapsin I clustering (Fig. 2A,B).

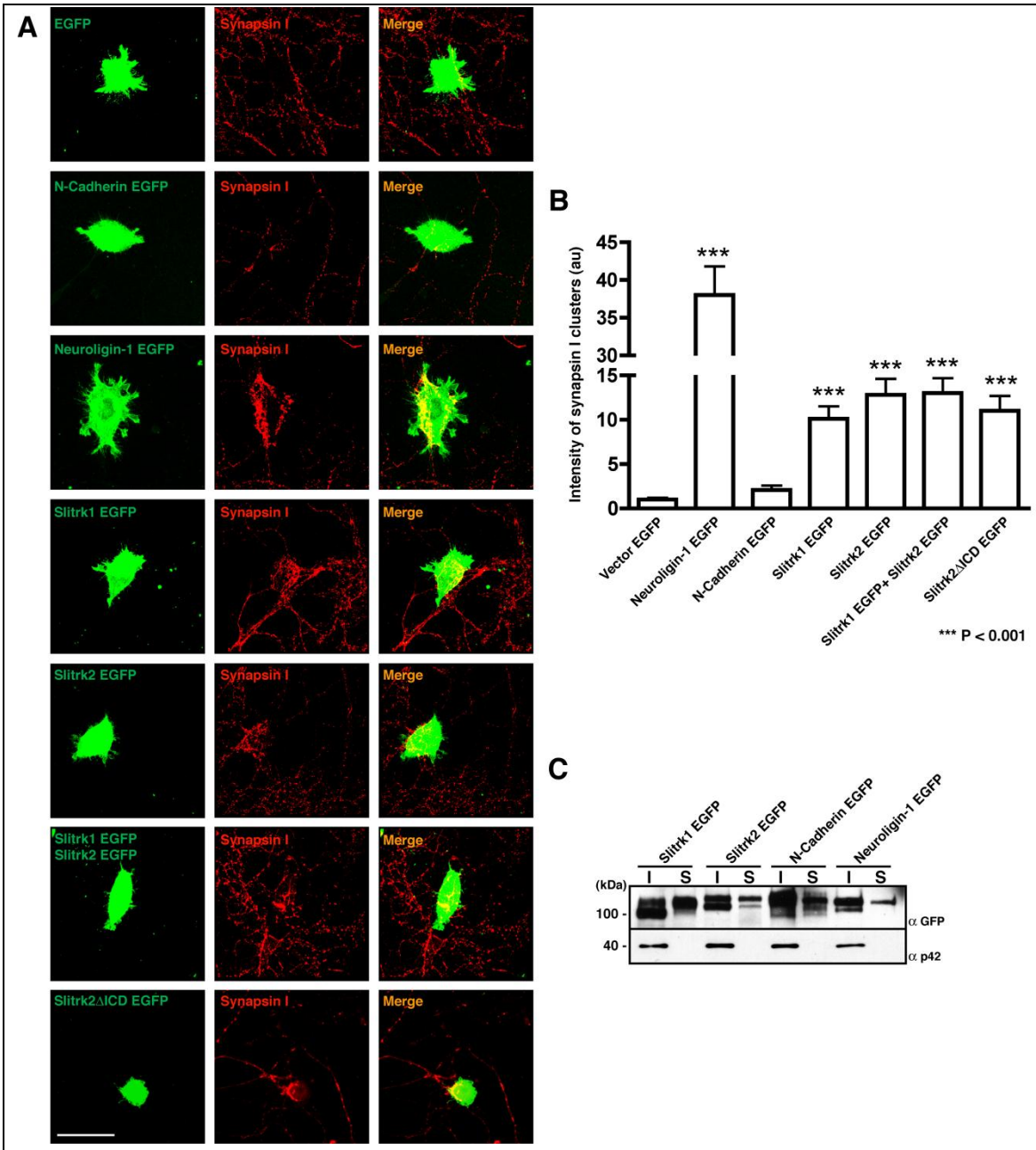

Figure 2: Slitrk1 and Slitrk2 expressed in nonneural cells induce presynaptic differentiation in contacting axons.

(**A**) Induction of clustering of the presynaptic protein synapsin I in contacting axons by Slitrk1 and Slitrk2. HEK293T cells expressing EGFP alone, N-Cadherin EGFP, neuroligin-1 EGFP, Slitrk1 EGFP, Slitrk2 EGFP, Slitrk1 EGFP + Slitrk2 EGFP, or Slitrk2ΔICD EGFP were cocultured with hippocampal neurons (9 DIV) for 48hrs and stained for synapsin I. Scale bar, 50 um. (**B**) Quantification of the results in **A** of the

integrated intensity of synapsin I clusters induced; values were normalized to the integrated intensity associated with HEK293T expressing EGFP alone (defined as 1). Mean ± s.e.m. (Vector EGFP, 1.0 ± 0.2, n = 80; neuroligin-1 EGFP, 38.0 ± 3.8, n = 80; N-Cadherin EGFP, 2.1 ± 0.5, n = 80; Slitrk1 EGFP, 10.1 ± 1.4, n = 80; Slitrk2 EGFP, 12.8 ± 1.8, n = 80; Slitrk1 EGFP + Slitrk2 EGFP, 13.0 ± 1.7, n = 100; Slitrk2ΔICD EGFP, 11.0 ± 1.3, n = 80; Done in 4 separate experiments; ***$p < 0.001$, Student's t test; au, arbitrary units). (**C**) Comparison of surface expression levels of the different EGFP-tagged proteins in COS cells. COS cells transfected with Slitrk1 EGFP, Slitrk2 EGFP, N-Cadherin EGFP, and neuroligin-1 EGFP were treated with biotin to isolate surface proteins, followed by immunoblotting with EGFP antibodies. p42 MAP kinase is used as an intracellular control. Expression of the Slitrk1 and Slitrk2 proteins at the surface was similar or greater than that of neuroligin-1. Input: 2%. I, Input; S, Surface protein.

To assess if the presynaptic structures induced by Slitrk1 and Slitrk2 are functional (i.e. release neurotransmitters), we used an assay designed to follow the uptake of antibodies directed against the luminal domain of the synaptic vesicle protein synaptotagmin I, which is present during the recycling of presynaptic vesicles (Kraszewski et al., 1995). Both Slitrk1 and Slitrk2 induced the uptake of the synaptotagmin I antibodies in contacting axons upon depolarization for 5 minutes (Fig. 3A,B). Depolarisation of the cells in the presence of a control MYC antibody did not lead to any reuptake suggesting that the uptake of the synaptotagmin I antibody we observed is specific (Fig. 3C). These results demonstrate that Slitrk1 and Slitrk2 can induce the differentiation of presynaptic specializations with the capacity for depolarization induced synaptic vesicle recycling.

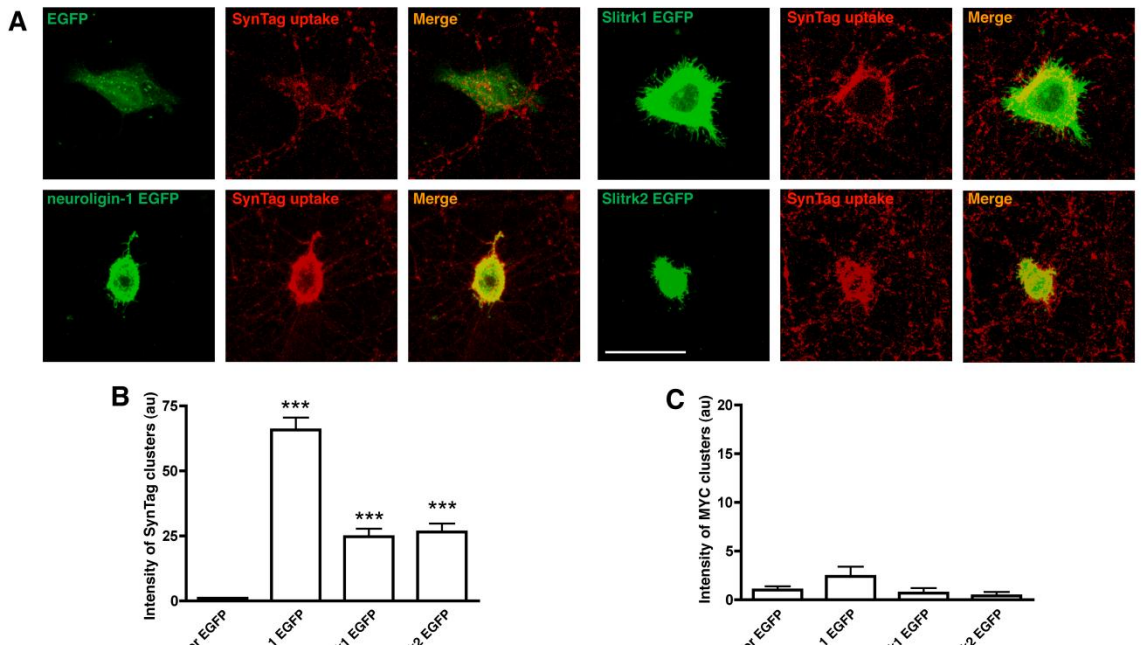

Figure 3: Slitrk1 and Slitrk2 induce the uptake of synaptotagmin I luminal domain antibodies in contacting axons of cocultured neurons.

(**A**) HEK293T cells were transfected with EGFP alone, neuroligin-1 EGFP, Slitrk1 EGFP, or Slitrk2 EGFP were cocultured with rat hippocampal neurons (9 DIV) for 48hrs, followed by incubation of the live neurons with synaptotagmin I (SynTag) luminal domain antibodies to tag functional presynaptic nerve terminals and double staining for SynTag and EGFP. Scale bar, 50 um. (**B**) Quantification of the results in **A** of the integrated intensity of synaptotagmin I clusters induced; values were normalized to the integrated intensity associated with HEK293T expressing EGFP alone (defined as 1). Mean ± s.e.m. (Vector EGFP, 1.0 ± 0.2, n = 80; neuroligin-1 EGFP, 65.8 ± 4.7, n = 80; Slitrk1 EGFP, 24.7 ± 3.1, n = 88; Slitrk2 EGFP, 26.5 ± 3.3, n = 88; Done in 5 separate experiments; ***$p < 0.001$, Student's t test; au, arbitrary units). (**C**) Quantification of the results of the uptake of control antibodies. Antibodies against MYC epitope were used at the same concentration as antibodies against synaptotagmin I in the above protocol. The integrated intensity of the clusters derived from the uptake of MYC antibodies is negligible and not significantly different for the four proteins. Mean ± s.e.m. (Vector

EGFP, 1.0 ± 0.4, *n* = 30; neuroligin-1 EGFP, 2.4 ± 1.0, *n* = 30; Slitrk1 EGFP, 0.7 ± 0.5, *n* = 30; Slitrk2 EGFP, 0.4 ± 0.4, *n* = 30; Done in 3 separate experiments; Student's *t* test).

5.3. Overexpression of Slitrk1 and Slitrk2 in neurons induces excitatory presynaptic differentiation

To determine whether Slitrk1 and Slitrk2 can also promote presynaptic differentiation in neurons, we first examined which members of the family are expressed in hippocampal neurons using RT-PCR. While we did not detect *Slitrk6* in hippocampal neurons, all other members of the family show robust expression (Fig. 4A). We therefore overexpressed Slitrk1 or Slitrk2 in hippocampal neurons and examined their effect on synapse formation. Cultured hippocampal neurons were transfected at 13 DIV with either EGFP-tagged Slitrk1 or Slitrk2 and immunostained two days later with synapsin I antibodies. Overexpression of either Slitrk1 or Slitrk2 induced a robust increase in the amount of presynaptic contacts, as measured by the intensity of synapsin I clusters on dendrites (Fig. 4B,C). Since synapsin I is a presynaptic protein found at both excitatory glutamatergic and inhibitory GABAergic synapses, we then sought to determine if Slitrks may preferentially promote the formation of either type of synapses. Overexpression of either Slitrk1 or Slitrk2 induced excitatory presynaptic differentiation in contacting axons, as measured by the intensity of vGlut1 clusters (Fig. 5A,B), but had no effect on the formation of inhibitory presynaptic contacts as measured by the intensity of VGAT clusters (Fig. 5C,D). Hence, overexpression of either Slitrk specifically promotes the formation of excitatory synapses but is not sufficient to promote the formation of inhibitory synapses.

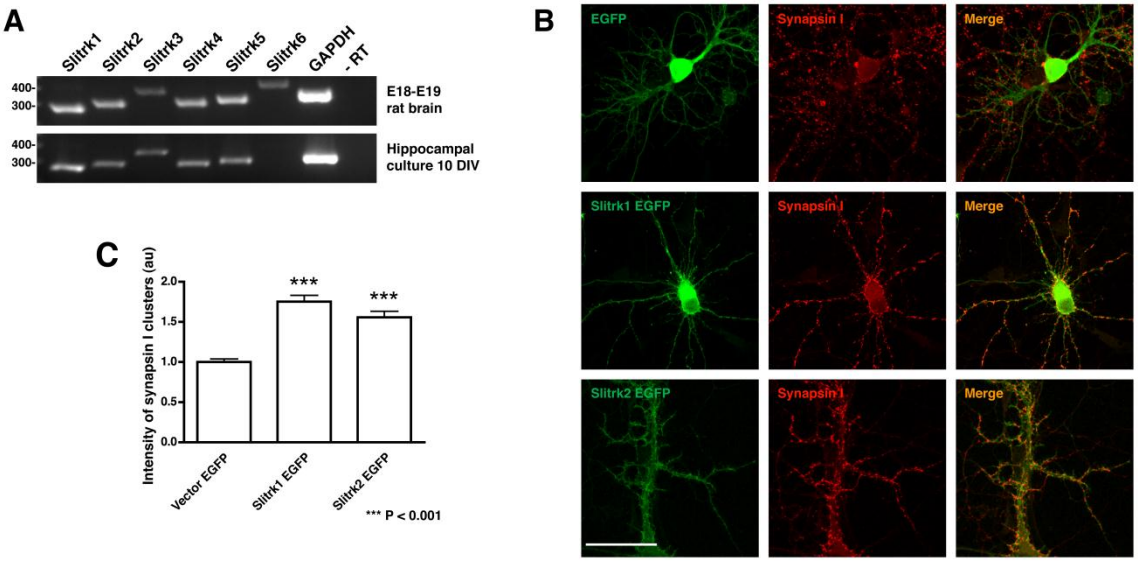

Figure 4: Overexpression of Slitrk1 and Slitrk2 in cultured neurons increases the amount of presynaptic contacts.

(**A**) Expression of Slitrk family members in rat brain and in hippocampal neuron cultures. RT-PCR analysis was performed on cDNA prepared from embryonic day 18-19 rat brain and from hippocampal neurons cultures at 10 DIV. Expression of all the six Slitrks is detected in brain while only Slitrk1, 2, 3, 4 and 5 are detected in cultured hippocampal neuron (**B**) Cultured hippocampal neurons were transfected with EGFP alone, Slitrk1 EGFP, or Slitrk2 EGFP at 13 DIV and immunostained for synapsin I at 15 DIV. Scale bar, 50 um. (**C**)Quantification of the results from **B**; values were normalized to the integrated intensity associated with neurons transfected with EGFP alone (defined as 1). Mean ± s.e.m. (Vector EGFP, 1.00 ± 0.04, n = 70; Slitrk1 EGFP, 1.75 ± 0.08, n = 91; Slitrk2 EGFP, 1.56 ± 0.08, n = 29; Done in 4 separate experiments; ***$p < 0.001$, Student's t test; au, arbitrary units).

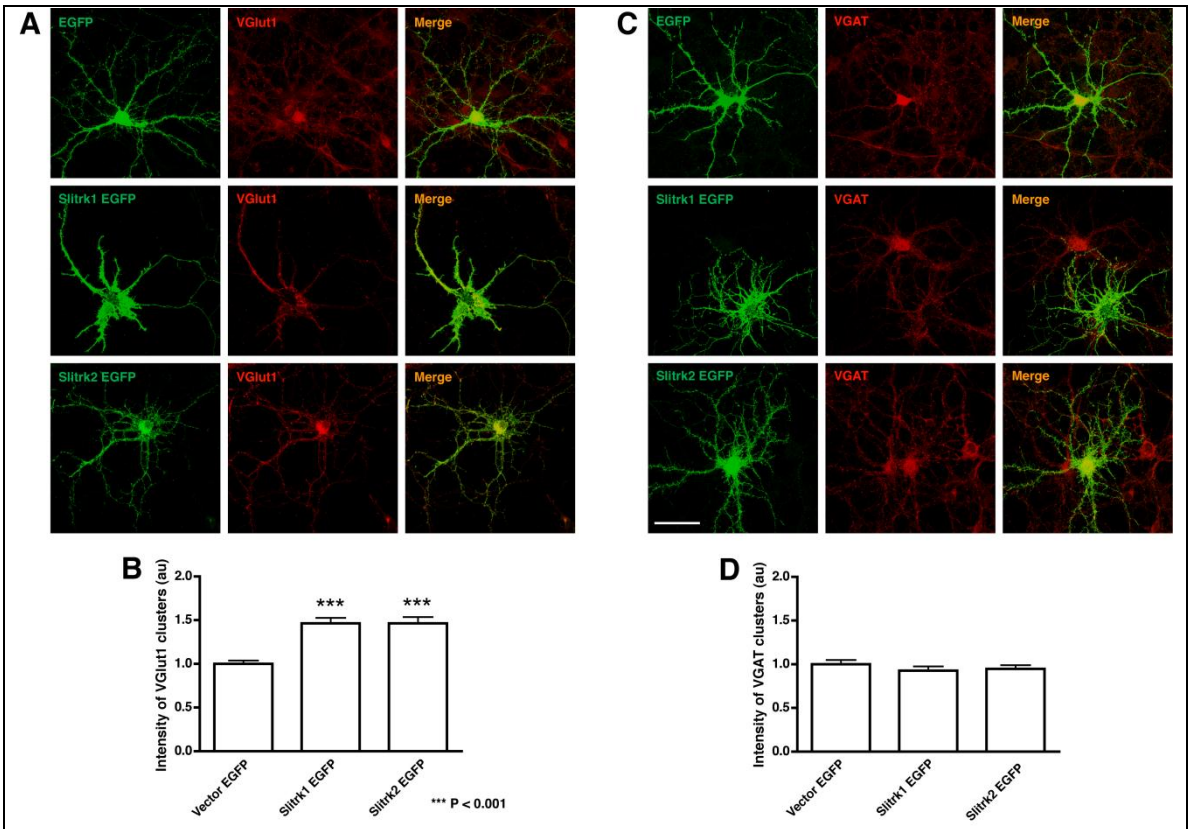

Figure 5: Overexpression of Slitrk1 and Slitrk2 in cultured neurons increases the amount of excitatory presynaptic but not inhibitory contacts.

(**A, C**) Cultured hippocampal neurons were transfected with EGFP alone, Slitrk1 EGFP, or Slitrk2 EGFP at 13 DIV and immunostained for vGlut1 **A** and VGAT **C** at 15 DIV. Scale bar, 50 um. (**B**) Quantification of the results from **A**; values were normalized to the integrated intensity associated with neurons transfected with EGFP alone (defined as 1). Mean ± s.e.m. (Vector EGFP, 1.00 ± 0.04, n = 90; Slitrk1 EGFP, 1.46 ± 0.06, n = 84; Slitrk2 EGFP, 1.46 ± 0.07, n = 73; Done in 5 separate experiments; ***p < 0.001, Student's t test; au, arbitrary units). (**D**) Quantification of the results from **C**; values were normalized to the integrated intensity associated with neurons transfected with EGFP alone (defined as 1). Mean ± s.e.m. (Vector EGFP, 1.00 ± 0.05, n = 55; Slitrk1 EGFP, 0.93 ± 0.05, n = 55; Slitrk2 EGFP, 0.95± 0.04, n = 55; Done in 3 separate experiments; Student's t test).

5.4. Slitrk1 and Slitrk2 do not induce postsynaptic differentiation in a mixed-culture assay

Our observations that Slitrk1 and Slitrk2 are enriched in the PSD fraction, that they promote the formation of excitatory synapses when overexpressed in neurons, and that they can induce presynaptic protein clustering in a mixed-culture assay indicate that they are likely be localized to the postsynaptic side of the synapse and have their effect on presynaptic boutons. Nonetheless, it remains possible that localization of Slitrk1 and Slitrk2 to the presynaptic side of the cleft can promote clustering of postsynaptic proteins.

Since existing biochemical methods are not entirely adequate for isolating presynaptic membrane proteins (Cotman and Taylor, 1972; Crawford et al., 1981; Ratner and Mahler, 1983; Phillips et al., 2001) we used the mixed-culture assay to determine whether expression of either Slitrk1 or Slitrk2 could induce the clustering of postsynaptic proteins. Expression of Slitrk1 or Slitrk2 in HEK293T cells did not induce detectable levels of clustering of the abundant postsynaptic scaffolding protein PSD-95 (Fig. 6A,B). In contrast, expression of neurexin1β, an inducer of glutamatergic and GABAergic postsynaptic structures promoted the formation of PSD-95 clusters on contacting axons (Chih et al., 2006; Kang et al., 2008).

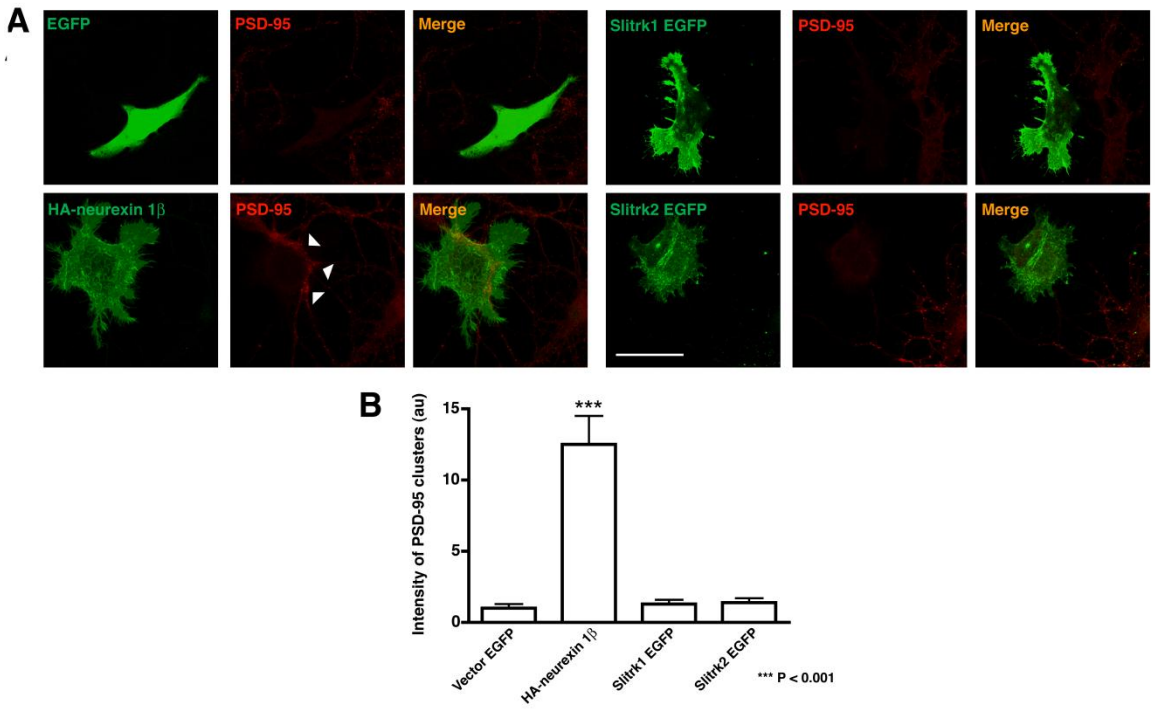

Figure 6: Slitrk1 and Slitrk2 expressed in nonneural cells do not induce clustering of the excitatory postsynaptic protein PSD-95 in contacting axons.

(**A**) HEK293T cells expressing EGFP alone, LAR EGFP, HA-neurexin 1β, Slitrk1 EGFP, or Slitrk2 EGFP were cocultured with hippocampal neurons (9 DIV) and stained for PSD-95 (11 DIV). White arrowheads indicate PSD-95 clustering. Scale bar, 50 um. (**B**) Quantification of the results in **A** of the integrated intensity of PSD-95 clusters induced; values were normalized to the integrated intensity associated with HEK293T expressing EGFP alone (defined as 1). Mean ± s.e.m. (Vector EGFP, 1.0 ± 0.3, n = 45; LAR EGFP, 1.6 ± 0.4, n = 25; HA-neurexin 1β, 12.5 ± 2.0, n = 30; Slitrk1 EGFP, 1.3 ± 0.3, n = 25; Slitrk2 EGFP, 1.4 ± 0.3, n = 30; Done in 3 separate experiments; ***p < 0.001, Student's t test; au, arbitrary units).

5.5. Application of Slitrk1 antibodies reduces synapse number in hippocampal neuron cultures

The ability of Slitrk1 to promote clustering of presynaptic proteins may be dependent on its interaction with an as yet unidentified membrane protein expressed on the presynaptic terminal. The synaptogenic activity of Slitrk1 may therefore be affected by blocking this interaction using an antibody that recognizes the extracellular region of Slitrk1. A previous study has demonstrated the efficacy of applying antibodies directed against the extracellular domain of endogenous TrkC to block its ability to regulate synapse formation (Takahashi et al., 2012). We therefore raised a rabbit polyclonal antibody against the Slitrk1 ectodomain (Slitrk1-N) and examined its effect on the synaptogenic potential of Slitrk1. The antibody recognized Slitrk1, did not cross-react with Slitrk2, and blocked the synaptogenic activity of Slitrk1, but not Slitrk2, in a mixed-culture assay (Fig. 1B; Fig. 7A). To test whether Slitrk1 is necessary for synapse formation, we treated cultured hippocampal neurons with the Slitrk1 function-blocking antibody and examined its effect on the number of excitatory and inhibitory synapses present on these neurons. Treatment with the antibody significantly reduced the densities of both apposed synapsin I/PSD-95 and VGAT/Gephyrin clusters indicating that Slitrk1 is required for formation of both excitatory and inhibitory synapses in this system (Fig. 7B-D).

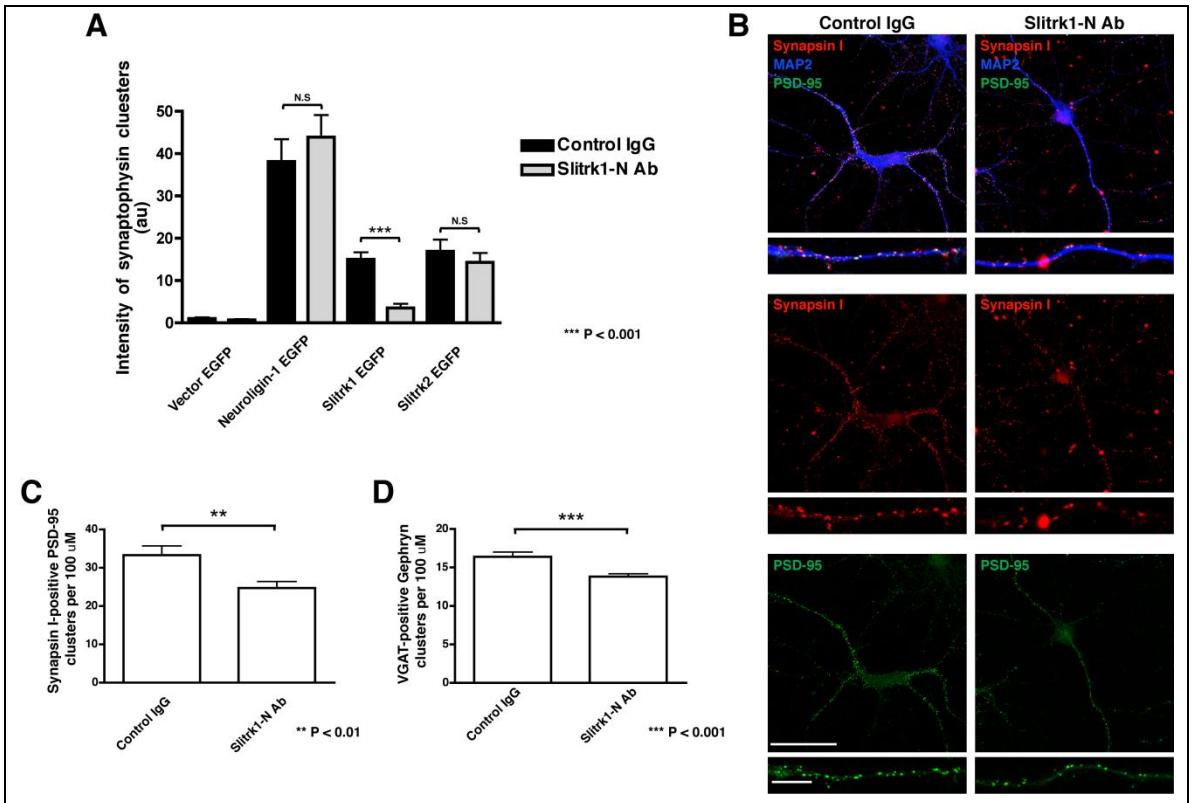

Figure 7: Treatment of hippocampal neurons with Slitrk1 antibodies reduces synapse density.

(**A**) Induction of clustering of the presynaptic protein synaptophysin in contacting axons by Slitrk1 is reduced by Slitrk1 antibody application. HEK293T cells expressing EGFP alone, neuroligin-1 EGFP, Slitrk1 EGFP, Slitrk2 EGFP, were cocultured with hippocampal neurons (9 DIV) for 48hrs and stained for synaptophysin. At 9 and 10 DIV, ~10 ug/ml of Slitrk1-N antibodies were added to the culture media. Values were normalized to the integrated intensity associated with HEK293T expressing EGFP alone treated with nonimmune IgG (defined as 1). Mean ± s.e.m. (EGFP alone treated with IgG, 1.0 ± 0.3, with αSlitrk1-N 0.7 ± 0.2; neuroligin-1 EGFP treated with IgG, 38.1 ± 5.3, with αSlitrk1-N 43.9 ± 5.2; Slitrk1 EGFP treated with IgG, 15.0 ± 1.7, with αSlitrk1-N 3.5 ± 1.0; Slitrk2 EGFP treated with IgG, 16.9 ± 2.8, with αSlitrk1-N 14.3 ± 2.2;, n = 42 for each condition; Done in 5 separate experiments; ***$p < 0.01$, Student's t test; au, arbitrary units). (**B**) Addition of Slitrk1-N antibodies at ~10 ug/ml into culture media on each of 9, 10 and 11 DIV results in a reduction of the density of apposed synapsin

I/PSD-95 clusters at 12 days in culture. Control nonimmune rabbit IgG had no effect. MAP2 staining is used to show the integrity of the neuron. Scale bar, 50 um and 10 um for the zoom boxes. (**C**) Quantification of the results in **B** of the synapsin I-positive PSD-95 clusters per 100 um. Mean ± s.e.m. (Control IgG, 33.3 ± 2.4, n = 48; Slitrk1-N antibodies, 24.7 ± 1.7, n = 48; Done in 5 separate experiments; $^{**}p < 0.01$, Student's t test). (**D**) Quantification of the VGAT-positive gephyrin clusters per 100 um after addition of Slitrk1-N antibodies into culture media following the same protocol as in **B**. Mean ± s.e.m. (Control IgG, 16.5 ± 0.5, n = 53; Slitrk1-N antibodies, 13.8 ± 0.5, n = 53; Done in 5 separate experiments; $^{***}p < 0.01$, Student's t test).

The strong expression of five out of the six Slitrk family members in hippocampal neurons (Fig. 4A), along with the previously described synaptogenic activity of other Slitrks may explain why blocking the function a single Slitrk does not completely abolish synapse formation. Since Slitrk1 and Slitrk2 can interact with each other when expressed in heterologous cells (Fig. 1C), we reasoned that addition of a soluble recombinant Slitrk1 extracellular domain (ECD) to hippocampal cultures may interact with the extracellular region of multiple Slitrks and thereby block their association with potential presynaptic partners. Addition of the recombinant Slitrk1 protein blocked the ability of Slitrk1, but not neuroligin-1, to induce presynaptic synapsin I clusters in a mixed-culture assay (Fig. 8A). The recombinant Slitrk1 extracellular domain slightly reduced the synaptogenic potential of Slitrk2 but did not completely block it (Fig. 8A). We next examined the effect of treating hippocampal neuron cultures with recombinant Slitrk1 extracellular domain on synapse number. Cultures treated with Slitrk1 ECD showed a reduction in the density of apposed synapsin I/PSD-95 clusters that was very similar to the effect observed in cultures treated with the Slitrk1 function-blocking antibody (Fig. 8C-D). Altogether, these results indicate that endogenous Slitrks are required for synapse formation.

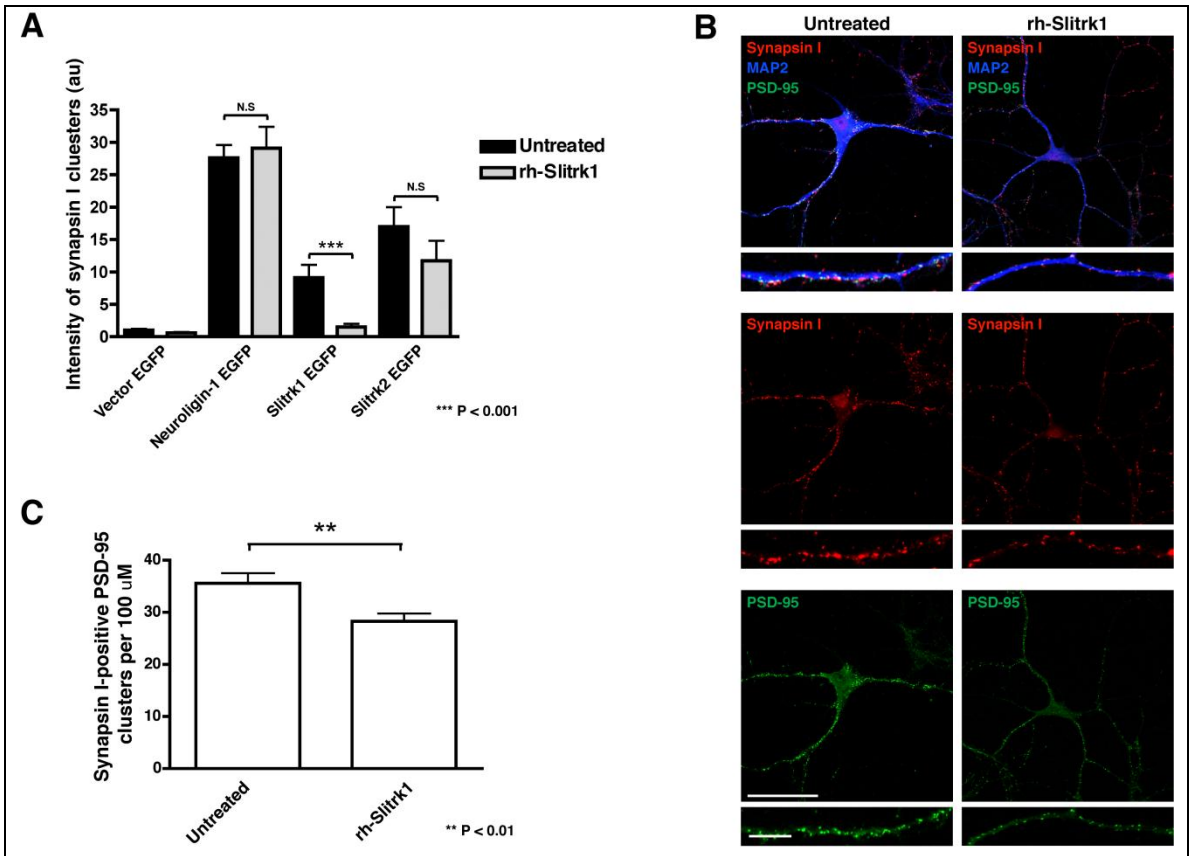

Figure 8: Treatment of hippocampal neurons with Slitrk1 recombinant protein reduces synapse density.

(**A**) Induction of clustering of the presynaptic protein synapsin I in contacting axons by Slitrk1 is reduced by the application of recombinant Slitrk1 protein. HEK293T cells expressing EGFP alone, neuroligin-1 EGFP, Slitrk1 EGFP, Slitrk2 EGFP, were cocultured with hippocampal neurons (9 DIV) for 48hrs and stained for synapsin I. At 9 and 10 DIV, 1.2 ug/ml of recombinant Slitrk1 was added to the culture media. Mean ± s.e.m. Values were normalized to the integrated intensity associated with HEK293T expressing EGFP alone without treatment (defined as 1). (EGFP alone untreated, 1.0 ± 0.2, with rh-Slitrk1 0.6 ± 0.1; neuroligin-1 EGFP untreated, 27.6 ± 2.0, with rh-Slitrk1 29.1 ± 3.3; Slitrk1 EGFP untreated, 9.1 ± 2.0, with rh-Slitrk1 1.5 ± 0.5; Slitrk2 EGFP untreated, 17.0 ± 3.0, with rh-Slitrk1 11.7 ± 3.1; Minimum n = 40 for each condition; Done in a minimum of 3 separate experiments; ***$p < 0.01$, Student's t test; au, arbitrary units). (**B**) Addition of recombinant Slitrk1 proteins at 1.2 ug/ml into culture media on

each of 9, 10 and 11 DIV results in a reduction of the density of apposed synapsin I/PSD-95 clusters at 12 days in culture compared to untreated neurons. MAP2 staining is used to show the integrity of the neuron. Scale bar, 50 um and 10 um for the zoom boxes. (**C**) Quantification of the results in **B** of the synapsin I-positive PSD-95 clusters per 100 um. Mean ± s.e.m. (Control IgG, 33.3 ± 2.4, $n = 48$; Slitrk1-N antibodies, 24.7 ± 1.7, $n = 48$; Done in 5 separate experiments; $^{**}p < 0.01$, Student's t test).

6. Discussion

Members of the Slitrk family of proteins have been implicated in the etiology of multiple neuropsychiatric disorders (Abelson et al., 2005; Zuchner et al., 2006; Piton et al., 2011). Here we show that two members of this family, Slitrk1 and Slitrk2, can promote presynaptic, but not postsynaptic, differentiation of synapses in a mixed-culture assay. Furthermore, overexpression of either Slitrk1 or Slitrk2 in hippocampal neurons induced the formation of excitatory, but not inhibitory, synapses on contacting axons. Blocking Slitrk1 function with an antibody leads to a decrease in the number of both excitatory and inhibitory synapses in hippocampal neuron cultures, indicating that endogenous Slitrk1 contributes to the formation of these synapses.

Our observation that both Slitrk1 and Slitrk2 are present in postsynaptic density fractions isolated from brain supports a role for these proteins in regulating synapse formation. However, the unavailability of specific antibodies that are suitable for immunocytochemistry analyses prevented us from determining the specific localization of Slitrk1 or Slitrk2 at excitatory and inhibitory synapses. Nonetheless, our observation that both excitatory and inhibitory synapse numbers are reduced in hippocampal neurons upon treatment with a Slitrk1 function-blocking antibody indicates that Slitrk1 contributes to the formation of both types of synapses (Fig. 7). This is consistent with the previously reported observation that Slitrk1 can promote presynaptic clustering of both vGlut1 and VGAT in a mixed-culture assay (25). It is therefore somewhat surprising that overexpression of Slitrk1 in hippocampal neurons did not increase the number of inhibitory synapses formed in these cultures (Fig. 5). It remains possible that

the level of overexpression of Slitrk1 achieved is not sufficient to promote inhibitory synapse formation. Alternatively, the effect of Slitrk1 on presynaptic differentiation of inhibitory synapses may be regulated by the levels of expression of a Slitrk1-binding protein on the presynaptic side of the cleft. Low levels of expression of such a protein on the presynaptic side would limit any effect overexpressing Slitrk1 could have on inhibitory synapse formation.

Slitrk3 has recently been identified as a specific inducer of inhibitory presynaptic differentiation by binding to the receptor protein tyrosine phosphatase, PTP-δ, on the presynaptic side of the cleft (Takahashi et al., 2012). Ablation of *Slitrk3* in mice leads to specific reductions in both inhibitory synapse density and synaptic transmission in the hippocampus. In contrast to the specific role that Slitrk3 plays in regulating inhibitory synapse formation, our results indicate that Slitrk1 function contributes to the development of both excitatory and inhibitory synapses in hippocampal neurons (Fig. 7,8). Since multiple Slitrk family members interact with PTP-δ, this interaction has been proposed to mediate their ability to promote the formation of inhibitory synapses (25). While the binding partners for Slitrk1 and Slitrk2 that regulate their effect on presynaptic differentiation of excitatory synapses remain to be identified, our *in vitro* mixed-culture assays suggest that Slitrk1 and Slitrk2 promote presynaptic, but not postsynaptic, differentiation. Although Slitrk family members share substantial amino acid sequence identity, it is unclear whether both Slitrk1 and Slitrk2 function by binding to a single or multiple different membrane proteins on the surface of the presynaptic cleft. Such diversity in binding partners within families of synaptogenic proteins has previously been described for the Netrin-G ligand family. While NGL-1 and NGL-2 interact with netrin-G1 and netrin-G2, respectively, NGL-3 interacts with the LAR family protein tyrosine phosphatases (Lin et al., 2003; Kim et al., 2006; Woo et al., 2009; Kwon et al., 2010).

Our observation that Slitrk1 and Slitrk2 can homo- and heterodimerize when expressed in HEK293T cells suggests that multiple members of the Slitrk family have the ability to interact with each other (Fig. 1). While the structural requirements for dimerization of Slitrks remain to be determined, the intracellular region of Slitrk1 interacts with adaptors of the 14-3-3 family of family (Kajiwara et al., 2009). Dimeric 14-

3-3 proteins may therefore serve as a bridge to promote either homophilic or heterophilic interactions between Slitrks. Future studies should reveal whether dimerization of Slitrks is required for their function at the synapse.

Findings in various Slitrk mutant mice are consistent with a role for these proteins in regulating synapse development or physiology. While *Slitrk1* mutant mice have increased anxiety-like behaviour (Katayama et al., 2008), *Slitrk5* mutant mice display compulsive-like behaviours associated with impaired corticostriatal synaptic transmission (Shmelkov et al., 2010). Moreover, *Slitrk3* mutant mice exhibit increased susceptibility to seizures (Takahashi et al., 2012). In conclusion, our studies demonstrate an important role for Slitrk1 in the regulation of presynaptic differentiation of both excitatory and inhibitory synapses. Our results further indicate that multiple members of the Slitrk family have a function in synaptic development.

CHAPTER 4

GENERAL DISCUSSION

1. Original contributions

The major aim of this thesis was to study the role of the Slitrks, a recently discovered family of molecules present in the brain, during neurodevelopment. To begin to address the involvement of Slitrks in the development of the nervous system, I analysed their patterns of expression in the nervous system. These analyses provided interesting information about potential distinct roles for Slitrks. For example, Slitrk2 is the only Slitrk family member that is highly expressed in brain regions where active generation of neurons takes place, such as in the ventricular zones. In contrast, other regions, such as the hippocampus, express multiple members of the Slitrk family suggesting they may have redundancy functions in this region. This study was the first to compare the patterns of expression of all six Slitrk family members in the nervous system and remains the most comprehensive report to date. Based on the expression analyses, I hypothesized that Slitrks may regulate hippocampal development and examined their roles in hippocampal synapse formation. My examination of the role of Slitrks in synapse formation has shown that both Slitrk1 and Slitrk2 can act as synaptogenic proteins to promote the formation of excitatory synapses. Furthermore, it revealed that Slitrk1 function is needed for the development of both excitatory and inhibitory synapses. My results represent the first demonstration that Slitrk1 functions in the development of synapses in hippocampal neurons and further support a recent report demonstrating an involvement for members of the Slitrk family in synaptogenesis (Takahashi et al., 2012).

2. Slitrks as synaptogenic proteins

During the redaction of this thesis, a recent report was published demonstrating a role for Slitrk3 in the development of inhibitory synapses in the hippocampus (Takahashi et al., 2012). Using a mixed cell culture assay that I have described in Chapter 3, Takahashi et al. demonstrated that all members of the Slitrk family have synaptogenic potential *in vitro* (Linhoff et al., 2009; Takahashi et al., 2012). Most Slitrk family members, including Slitrk1 and Slitrk2, can promote clustering of the inhibitory presynaptic marker VGAT, and of the excitatory presynaptic marker vGlut1. In contrast, Slitrk3 specifically promotes the clustering of VGAT in a mixed-culture assay. Based on the specific effect of Slitrk3 on VGAT clustering that is shared only by neuroligin-2 so far, the authors further examined its function in inhibitory synapse formation. Slitrk3 localizes at inhibitory synapses and knockdown of its expression leads to reduced numbers of inhibitory synapses in hippocampal neuron cultures. Slitrk3 also appears to play a critical role in inhibitory synapse function *in vivo*. Ablation of Slitrk3 expression in mice leads to a reduction in both inhibitory synapse density and inhibitory transmission. *Slitrk3*$^{-/-}$ mice exhibit the interesting phenotype of occasional spontaneous seizures accompanied by increased seizure susceptibility to chemoconvulsants (Takahashi et al., 2012). Since normal brain function requires a finely tuned balance of excitation and inhibition, the reduction of inhibitory connections might explain the increase in seizure susceptibility.

Interestingly, my results support the findings reported by Takahashi et al. and provide additional insight into the function of Slitrks at the synapse. I demonstrate that Slitrk1 and Slitrk2 are localized to the postsynaptic density fraction and that their overexpression in hippocampal neurons can promote the formation of excitatory synapses. Furthermore blocking Slitrk1 function using a function-blocking antibody or a soluble recombinant form of the Sitrk1 extracellular domain leads to a reduction in the number of both excitatory and inhibitory synapses in hippocampal neurons. My results therefore indicate that Slitrk1 and Slitrk2 can modulate synapse formation in addition to the previously reported role for Slitrk3 in this process.

3. Cooperativity or redundancy at the synapse

Over the last decade, several new molecules have been found to have the potential to induce synapse formation. Two important questions that remain to be addressed are 1- whether these cell adhesion molecules are necessary or sufficient for synaptogenesis and 2- whether these cell adhesion molecules function synergistically or redundantly (Wright and Washbourne, 2011). Knowing that neurexins coordinate multiple adhesion events at the synapse, one could have imagined them to be necessary for synaptogenesis. However, the knockout mice studies suggest that even neurexins are dispensable for synapse formation and seem to play a role at later stages of synapse development (Missler et al., 2003). At this point, it is unlikely that a molecule necessary for synaptogenesis in every synapse will be discovered. However, it is possible that specific synaptic cell adhesion molecules will be found to be necessary for the formation of specific subsets of synapses in different brain regions.

The question of whether the cell adhesion molecules function synergistically or redundantly is even more relevant for the case of the Slitrks. In certain regions such as in the hippocampus, Slitrks may have redundant roles in synapse formation. In contrast, in other regions where expression does not overlap, Slitrks may have specific roles. In support for non-redundant functions for the Slitrks, the publications of the single *Slitrk1*$^{-/-}$, *Slitrk3*$^{-/-}$ and *Slitrk5*$^{-/-}$ mice have shown important behavioural phenotypes that are specific to each knockout mice. Importantly, the single *Slitrk* mutant mice phenotypes are much more striking than for those of most other single knockout CAMs at the synapse.

4. Slitrks in the molecular organization of the synapse

Slitrks as trans-synaptic proteins

Our results strongly suggest that Slitrk1 and Slitrk2 are located on the postsynaptic side of the synapse and promote presynaptic differentiations. However, we cannot totally exclude the possibility that Slitrk proteins could also be located on the presynaptic side of the synapse and interact in *trans* with postsynaptic Slitrks to mediate synapse formation. First, it is difficult to biochemically isolate presynaptic proteins, thus it is not possible to

directly address the presence of the Slitrks on this side of the synapse. Secondly, although Slitrk1 and Slitrk2 do not induce clustering of postsynaptic proteins in a mixed-culture assay, it remains possible that they may do so *in vivo*. For example, cadherin function on the presynaptic side is well characterized *in vivo*, but it does not induce clustering of postsynaptic proteins in the coculture assay (Scheiffele et al., 2000; Sara et al., 2005). Thirdly, while we were able to demonstrate that Slitrks have the potential to interact in *cis,* we did not have the right molecular tools to test their binding in *trans*. Thus, the possibility that the Slitrk proteins interact in *trans* at the synapse cannot yet be discarded.

Slitrks as postsynaptic organizers

The presynaptic PTP-δ was found to bind Slitrk3 (and all the other Slitrks) and to be required trans-synaptically for Slitrk3 to induce inhibitory presynaptic differentiation (Takahashi et al., 2012). Knocking down PTP-δ in neurons of a coculture assay selectively blocks the inhibitory synaptogenic activity of Slitrk3, but not of neuroligin-2. Moreover, knocking down PTP-δ blocks the Slitrk2-induced clustering of inhibitory presynaptic markers while having no effect on the excitatory marker aggregation (Takahashi et al., 2012). This finding suggests the existence of a different presynaptic receptor for Slitrk2, other than PTP-δ, to mediate excitatory differentiation. Previously, PTP-σ has been demonstrated to be the functional presynaptic receptor by which TrkC exclusively induces excitatory presynaptic differentiation during synapse formation (Takahashi et al., 2011). Thus, it remains unclear how these phosphatases can contribute selectively at GABAergic (with Slitrk3) and glutamatergic synapses (with TrkC). Alternative splicing of the PTP-δ gene can provide one possible explanation for this issue, whereby only certain splice variants can interact specifically with Slitrk3 at inhibitory synapses (Yoshida et al., 2011). Another interesting question is whether Slitrks could also interact at the synapse with the other two members of the LAR family of receptor PTPs: PTP-σ and LAR. It may be hypothesized that different combinations of interactions between the LAR family members and the Slitrks could generate synapse connection specification. Another level of complexity is added with the consideration of another postsynaptic protein, NGL-3, which binds to LAR, PTP-σ, and PTP-δ (Woo et al., 2009; Kwon et al., 2010). NGL-3 binding to the LAR family was previously shown to

regulate only excitatory synapse formation (Woo et al., 2009). In the near future, it will be critical to first assess if the Slitrks functionally interact with the three LAR family members, and then to determine whether the Slitrks and NGL-3 compete for the binding sites on LAR, PTP-σ, and PTP-δ. This theory could be tested using the coculture system with bath applications of either Slitrk3 or NGL-3 recombinant protein to potentially block the effect of the other protein.

5. Mechanism of action of the Slitrks at the synapse

Based on the current results, a more fundamental question remains: how does the interaction between PTP-δ and the Slitrks promote presynaptic differentiation? One possibility is that the Slitrks-PTP-δ interaction is important for the initial step of synaptogenesis when the axonal growth cone first contacts the dendrite. In this scenario, both proteins act as cell adhesion molecules to begin stabilization of the new synapse. In support of this hypothesis, the structure of the large extracellular domains of LAR family members is highly reminiscent of the structure of cell adhesion molecules. It consists of three Ig-like domains and four to eight fibronectin type III domains, depending on the alternative splicing that take place during transcription (Pulido et al., 1995). On the postsynaptic side, the fact that the Slitrks do not have in their intracellular domains any known signaling protein motifs suggests they may act solely as adhesion molecules. It is also possible that Slitrks-PTP-δ interactions promote presynaptic differentiation by initiation a signaling cascade on either the presynaptic, the postsynaptic or both sides of the synapse. Such a case would suggest that their interaction plays a role at later stages of synaptogenesis, as the synapse matures. This hypothesis is supported by the fact that the second cytoplasmic phosphatase domain of the LAR family, which is catalytically inactive, interacts with liprin-α (Pulido et al., 1995), a cytoplasmic adaptor protein that is important for presynaptic development (Zhen and Jin, 1999; Kaufmann et al., 2002). Also, their first phosphatase domain, which is catalytically active, may be responsible for the dephosphorylation of downstream effectors upon binding of the Slitrks. Finally, while there are no obvious known signaling domains in the intracellular portion of the Slitrks, bioinformatic analyses predict that Slitrk1 (and potentially the other Slitrks) might be phosphorylated by protein kinase A (PKA), protein kinase C (PKC), and CK2

(Kajiwara et al., 2009). If confirmed, this option could reveal the existence of postsynaptic downstream signaling. Hence, it is important to highlight here that it is still unclear whether Slitrks act as cell adhesion molecules during synapse formation or as instructive molecules that participate in synapse assembly.

Our current model favors a role in adhesion for Slitrks at the synapse. However, it remains possible that Slitrks could regulate synapse formation by being proteolytically processed at the membrane surface. It has been suggested that Slitrks might undergo proteolysis to release the extracellular domain (Kajiwara et al., 2009). Indeed, it was found that the activation of PKC, known to promote secretion of transmembrane proteins (Gabuzda et al., 1993; Izumi et al., 1998), led to the release of a Slitrk1 extracellular domain fragment in the growth media of transfected COS cells. This cleavage is abolished by the ADAM-protease inhibitor TAPI-2 (Kajiwara et al., 2009). This family of metalloproteases is frequently responsible for the shedding of transmembrane proteins. This α-secretase cleavage can be followed by an intramembranous cleavage catalysed by the γ-secretase complex, which seems to also cleave Slitrk1 (Kajiwara et al., 2009). These results raise important questions for the involvement of Slitrks in synapse formation. The Slitrk family may join the classical synaptogenic priming factors derived from the target neurons (described in chapter 1), the Wnt and the FGFs. The synaptic effect of the Slitrks could be achieved in two steps. First, Slitrks could be released from the dendrites and travel to prime the growth cone for synaptogenesis. Later, the transmembrane Slitrks could help in stabilizing the synapse. Future experiments will need to be designed to address this specific possibility.

6. Towards a dual function for Slitrks

The Slitrks begin to be expressed before synapse formation occurs. As described in detail in the first chapter, there are multiple evidences that Slitrks are indeed implicated in neurite outgrowth modulation. In terms of the impacts of the Slitrks on neural circuit formation, the work done with the *Slitrk6*$^{-/-}$ mice are insightful. The survival and neurite outgrowth of the sensory neurons in the inner ear is reduced in the absence of Slitrk6. Histological examinations also revealed that vestibular innervation in the inner ear was

markedly decreased and sometimes misguided without Slitrk6 (Katayama et al., 2009). Therefore, we now have indications that Slitrks participate in at least two functions during development: neurite outgrowth and synapse formation. This dual role is not without precedent among synaptogenic proteins. So far, cadherins, L1-CAMs and SALMs have been shown to mediate neurite outgrowth as well as synapse formation both *in vitro* and *in vivo* (Redies, 2000; Nishimura et al., 2003; Godenschwege et al., 2006; Wang et al., 2008; Mah et al., 2010). Even the key postsynaptic protein, neuroligin-1, was recently shown to induce neurite outgrowth through interaction with neurexin-1 and activation of FGF receptor 1 (Gjorlund et al., 2012). Some of the processes involved in synapse formation and neurite outgrowth could be potentially shared. How the switch between the two functions is controlled is still unknown. Interestingly, impairs in both neuritogenesis and synaptogenesis in the establishment of neuromuscular connections were associated to the Slitrk genes (Marteyn et al., 2011). Overall, the characterization of the different *Slitrk* mutant mice in well-established developmental paradigms will help distinguish when and where the Slitrks are implicated in synapse formation or in neurite outgrowth.

7. Synaptic cell adhesion molecules and disorders of the nervous system

Recent advances in research on proteins implicated in synapse formation that cross the synaptic cleft have contributed to our understanding of brain disorders. Multiple human genetic studies have linked mutations in synaptic proteins with various psychiatric and neurological diseases of so-called synaptopathies (reviewed in (Grabrucker et al., 2011; Waites and Garner, 2011)). Even though most of these studies conclude that complex brain disorders are likely to be due to multiple genetic and environmental causes, the correlation between synaptic protein mutations and diseases remains extremely insightful. Among the studies that are related to the proteins discussed in this thesis, LRRTM1 was shown to be linked, via paternal transmission, to both handedness and SCZ (Francks et al., 2007). Genetic linkage studies found a correlation between autism spectrum disorders (ASD) and mutations in cadherins (Wang et al., 2009; Pagnamenta et al., 2011). SALM5 has been associated with severe progressive autism and mental retardation (de Bruijn et al., 2010) and with familial SCZ (Xu et al., 2009). Netrin-G1 is implicated in Rett

syndrome, a neurodevelopmental disorder that predominantly affects females, causing loss of purposeful hand movements, impaired social contact, and stereotypic hand movements (Borg et al., 2005). In addition, single nucleotide polymorphism studies have linked netrin-G1 and netrin-G2 with SCZ (Aoki-Suzuki et al., 2005; Ohtsuki et al., 2008). Mutations within neuroligin genes have been correlated with ASD (Jamain et al., 2003; Talebizadeh et al., 2006; Yan et al., 2008; Glessner et al., 2009). Moreover the mental retardation–associated protein, IL1RAPL1, triggers excitatory, but not inhibitory, presynaptic differentiation via trans-interaction with PTP-δ (Valnegri et al., 2011; Yoshida et al., 2011). Mutations in SHANK3, a scaffolding protein at the postsynaptic density that modulates dendritic spine morphology and synaptic signaling, lead to global developmental delay and autism (Durand et al., 2007; Moessner et al., 2007; Gauthier et al., 2009; Durand et al., 2012). Interestingly, mice with targeted mutations in the different domains of SHANK3 displayed physiological and behavioral consequences reminiscent of mild autism (Bozdagi et al., 2010; Peca et al., 2011; Wang et al., 2011; Yang et al., 2012). However, most likely due to their central role in presynaptic organisation and the fact that they bind multiple proteins, neurexin deletions associated with disorders are the most commonly documented. Indeed, several reports have suggested that deletions of *NRXN1* confer a substantial increase in risk of SCZ (Consortium, 2008; Kirov et al., 2008; Walsh et al., 2008; Kirov et al., 2009; Need et al., 2009; Rujescu et al., 2009) or ASD (Szatmari et al., 2007; Kim et al., 2008a; Morrow et al., 2008; Glessner et al., 2009). Altogether, these observations suggest that many brain disorders result from very subtle differences in neural connectivity caused by mutations in synaptic proteins.

Multiple recent studies, described in the literature review of the thesis, describe certain Slitrk mutations in patients with disorders such as TS, TTM, BD, ASD, and SCZ. These genetic studies represent another proof of principles that the Slitrks are synaptic proteins that play a role the connectivity between synapses. Moreover, determining how the same gene is mutated in patients with different mental illness might eventually shed light on our understanding of the pathophysiology of these disorders. To a certain extent, TTM and TS, both of which are forms of OCD, might represent alternative expressions of a single underlying genetic alteration. In line with this possibility, it is interesting that the

mouse mutant for the transcription factor Hoxb8 shows a behavior manifested by compulsive grooming and hair removal, similar to behavior in humans with OCD (Greer and Capecchi, 2002). In the brain, Hoxb8 cell lineage marking exclusively labels bone marrow-derived microglia. Surprisingly, restriction of Hoxb8 deletion to the hematopoietic system, which gives rise to microglia, results in mice with the excessive grooming and hair removal behavioral defects (Chen et al., 2010). Immunological dysfunctions have been associated with neuropsychiatric disorders like autism, OCD, BD or SCZ (reviewed in (Ashwood et al., 2006; Strous and Shoenfeld, 2006; da Rocha et al., 2008)). Knowing that the Slitrks are expressed by hematopoietic stem cells (Milde et al., 2007) and that *Slitrk5*$^{-/-}$ mice exhibit an excessive grooming behaviour highly similar to the *Hoxb8*$^{-/-}$ mice (Shmelkov et al., 2010), it is possible that the Slitrks are implicated in the etiology of brain disorders by playing role outside the synapse formation, for example in the generation of microglia.

8. Conclusion

I would like to conclude my thesis just as it started; with a citation by Dr Wilder Penfield, founder of the Montreal Neurological Institute:

"The brain is the organ of destiny. It holds within its humming mechanism secrets that will determine the future of the human race."

In my humble opinion, this single quote summarizes why neuroscience is a world apart from all other scientific disciplines: learning about the brain makes us more human.

The results I have presented in my thesis shed light on how the Slitrk family of proteins can regulate the fundamental process of synaptic formation during development of the nervous system. As usual, these few answers have raised multiple exciting questions about the mode of action of Slitrks at the synapse that will be pursued in the future.

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