CHARACTERIZATION OF TWO ASD-ASSOCIATED GENES IN PRIMARY

HIPPOCAMPAL NEURONS: SEMA5A AND PTEN

by

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Abstract

Autism spectrum disorder (ASD) is a pervasive neurodevelopmental disorder primarily characterized by stereotypic behaviors, deficits in social interaction and difficulties with communication. Extensive epidemiological studies suggest a major role for genetics in the etiology of ASD. To date, 600-1,200 human genes have putatively been linked to ASD, including SEMA5A and PTEN. A large number of these ASD-associated genes play a role in the formation, maintenance, elimination or stabilization of synapses, while others are involved in broader elements of neurodevelopment, such as dendrite arborization, dendritogenesis, and soma size. Consistent with neurological dysfunction in ASD are observations that individuals with ASD often have supernumerary synapses, disrupted excitatory/inhibitory balance, and patterns of hypo- and hyper-connectivity compared to the general population. Despite this, many of the neurological functions of ASD-associated genes or gene disruptions remain poorly elucidated.

In this study, we examine the role of Sema5A in activity-mediated synapse elimination, notably hippocampal long-term potentiation (LTP) and long-term depression (LTD). We describe the enhanced trafficking of Sema5A to the surface membrane during LTD and the subsequent Sema5A-dependent elimination of excitatory synapses. Furthermore, we demonstrate that Sema5A selectively mediates excitatory—and not inhibitory—synapse elimination, suggesting a mechanism by which the dysregulation of Sema5A could disrupt excitatory junibitory balance. Secondly, we describe the role of PTEN in negatively regulating excitatory synapse density, total dendritic arbor length, and soma size. Moreover, we characterize alterations to the neurological functions of PTEN in mature hippocampal neurons following the introduction of ASD-associated single nucleotide variants (SNVs). We

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demonstrate that most of the ASD-associated PTEN SNVs tested are broadly loss of function, with two notable exceptions: P38H PTEN exhibits a single altered neurological function, while H123Q PTEN phenocopies wild type human PTEN across all measures, further stressing the importance of biological functionalization. Lastly, we establish a PTEN knockdown assay in which PTEN SNVs could be tested for synaptic, dendrite and somal phenotypes. Combined and integrated, the functionalization of ASD-associated genes and gene variants could permit greater accuracy in ASD diagnoses and prognoses, as well as the improved targeting of therapeutic interventions.

Lay Summary

Individuals with autism spectrum disorder (ASD) have difficulties with socializing, trouble communicating and repetitive behaviors, among other symptoms. One of the leading theories about the cause of ASD is disruption to genes within these individuals. These disruptions include when or how long the gene is turned on or off, as well as changes to the protein produced by that gene. However, ASD individuals do not all have the same gene disruptions and the number of genes potentially involved is large. Little is understood about how these gene disruptions alter brain function or development. In the first half of this thesis, I describe the movement of Sema5A protein, the product of an ASD-associated gene, during brain cell activity and its control of incoming excitatory communication. In the second half of this thesis, I explore the effect of mutations in the PTEN gene, another ASD-associated gene, on brain cell communication and geometry.

Preface

This thesis contains the original, unpublished work of the author, Riki Dingwall. I performed all of the experiments and data analysis contained within, excluding the following exceptions: The experiments and data analysis shown in Figure 3.2a was performed by Wei Xiao in the O'Connor lab, while the experiments and data analysis shown in Figure 3.2b was performed by Rachel Gomm-Kalisko in the Bamji lab; the immunocytochemistry experiments in Chapter 4 were performed with the assistance of a graduate student in the Bamji lab, Matthew Edwards, under my supervision. The animal research contained within this thesis was performed with ethical approval from the UBC Animal Care Committee (certificates #A14-0338).

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List of Abbreviations

- ADHD Attention Defecit Hyperactivity Disorder
- ADI-R Autism Diagnostic Interview-Revised
- ADOS Autism Diagnostic Observation Schedule
- AKT Protein Kinase B
- AMPA α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
- **AMPARs AMPA Receptors**
- ANOVA Analysis of Variance
- AP2 Clathrin Adaptor Protein 2
- ASD Autism Spectrum Disorder
- BDNF Brain-Derived Neurotrophic Factor
- CA Cornus Ammonis
- CAMKII Calmodulin-Dependent Protein Kinase II
- cAMP Cyclic Adenosine Monophosphate
- cGMP Cyclic Guanosine Monophosphate
- cLTD Chemical Long-Term Depression
- CNQX 6-cyano-7-nitroquinoxaline-2,3-dione
- CNS Central Nervous System
- CNV Copy Number Variation
- CRD Cysteine-Rich Domain
- DG Dentate Gyrus
- DIV Days In Vitro

DRGs - Dorsal Root Ganglions

- DSM-5 Diagnostic and Statistical Manual of Mental Disorders
- DTT Dithiothreitol
- E Embryonic Day, e.g. E18.5
- E-LTP Early Long-Term Potentiation
- EC Entorhinal Cortex
- ECS Extracellular Solution
- EDTA Ethylenediaminetetraacetic Acid
- EGFP Enhanced Green Fluorescent Protein
- EPSCs Excitatory Postsynaptic Currents
- EPSPs Excitatory Postsynaptic Potentials
- ER Endoplasmic Reticulum
- fMRI Functional Magnetic Resonance Imaging
- GABA γ-Aminobutyric Acid
- GABA_AR γ-Aminobutyric Acid Receptor Type A
- $GABA_BR$ γ -Aminobutyric acid Receptor Type B
- GAP GTPase-Activating Protein
- GFP Green Fluorescent Protein
- GI Gastrointestinal
- GPCRs G-Protein Coupled Receptors
- GSK Glycogen Synthase Kinase
- GWAS Genome-Wide Association Study
- HA Human Influenza Hemagglutinin

- HFS High-Frequency Stimulation
- HRP Horseradish Peroxidase
- IQ Intelligence Quotient
- KD Knockdown
- KO Knockout
- L-LTP Late Long-Term Potentiation
- LFS Low-Frequency Stimulation
- LOF Loss of Function
- LTD Long-Term Depression
- LTP Long-Term Potentiation
- MAPs Microtubule Associated Proteins
- MEM Minimum Essential Media
- mGluRs Metabotropic Glutamate Receptor
- mTOR Mechanistic Target of Rapamycin
- MWM Morris Water Maze
- NMDA N-Methyl-D-aspartic acid
- NMDAR-LTD N-Methyl-D-aspartic acid Receptor Dependent Long-Term Depression
- NMDAR-LTP N-Methyl-D-aspartic acid Receptor Dependent Long-Term Potentiation
- NMDAR N-Methyl-D-aspartic acid Receptor
- NPCs Neural Progenitor Cells
- NSF N-ethylmaleimide Sensitive Susion Protein
- **OE** Overexpression
- P Postnatal Day, e.g. P1

- PBD Phosphatidylinositol 4,5-bisphosphate Binding Domain
- PBS Phosphate Buffered Saline
- PDZ-BD Postsynaptic Density Binding Domain
- PFA Paraformaldehyde
- PHTS Phosphatase and Tensin Homolog Hamartoma Tumor Syndrome
- PI3K Phosphoinositide 3-kinase
- PIP2 Phosphatidylinositol 4,5-bisphosphate
- PIP3 Phosphatidylinositol (3,4,5)-triphosphate
- PKA Protein Kinase A
- PKC Protein Kinase C
- PP1 Protein Phosphotase 1
- PSD-95 Postsynaptic Density Protein 95
- PSI Plexin-Semaphorin-Integrin
- PTEN Phosphatase and Tensin Homolog
- PTEN* Short Guide RNA Resistant Phosphatase and Tensin Homolog
- qPCR Quantitative Polymerase Chain Reaction
- RFP Red Fluorescent Protein
- ROI Region of Interest
- SEP Super Ecliptic pHluorin
- shRNA Short Guide RNA
- SNPs Single Nucleotide Polymorphisms
- SNVs Single Nucleotide Variants
- STVs Synapse Vesicle Protein Transport Vesicles

synCAM1 - Synaptic Cell Adhesion Molecule 1

- TBST Tris-Buffered Saline with Tween
- TMS Transcranial Magnetic Stimulation
- TSC Tuberous Sclerosis
- TSRs Thrombospondin Type-1 Repeats
- VGAT Vesicular y-Aminobutyric Acid Transporter
- VGluT1 Vesicular Glutamate Transporter
- WES Whole Exome Sequencing

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Chapter 1: Introduction

1.1 Introduction to Neurodevelopmental Disorders

Neurodevelopmental disorders refer conventionally to a range of impairments to the growth and formation of the central nervous system (CNS) during prenatal or early postnatal development. Causes of individual neurodevelopmental disorders range from genetic to immunological to environmental, and various multifactorial combinations therein (for review, see Sharin & Sur, 2015; Parikshak et al., 2015). Any or all of these disruptions to the tightly orchestrated development of the nervous system can lead to distinct phenotypes later in life across an array of functional measures. One of the best-studied subcategories of neurodevelopmental disorders is autism spectrum disorder (ASD). ASD is characterized, according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-5; American Psychiatric Association, 2013), by deficits in social interaction and communication, as well as the presence of stereotypic behaviors. Further, these symptoms must appear early in life and persist throughout development in a manner inconsistent with mere developmental delay. While the clinical phenotypes observed are heterogenous, and thus too are the probable etiologies, all ASD patients converge at the broad level of neurodevelopmental impairment.

1.2 Typical Neurodevelopment: Focus on the Hippocampus

1.2.1 Adult Hippocampal Anatomy

The crude anatomy of the adult hippocampus is complex (for review, see van Strien et al., 2009; Insausati, 1993; Gogtay et al., 2006). There are several substructures, referred to as the

hippocampus proper, dentate gyrus (DG), subiculum, presubiculum, parasubiculum and entorhinal cortex (EC). Viewed from a mid-coronal section, the adult hippocampus reveals a folded, S-shaped internal structure. A distinct region known as the DG lies at the anterior tip of the S, which caps the end of the hippocampus proper. Travelling posteriorly along the S-axis, the subsequent hippocampus proper (or Cornus Ammonis; CA) begins with a small CA4 region, followed by the CA3, another small zone known as the CA2, and finally the CA1 region. The hippocampus proper eventually transitions into the subiculum, presubiculum, parasubiculum then, finally, the EC and the parahippocampal gyrus. Within this anatomical framework, the adult hippocampus is further organized into several horizontal layers. Starting from the lateral ventricle border and working our way down, the alveus is the most superior layer of the hippocampus made up of myelinated fibres en route to the fimbria. The next later, stratum oriens, spans CA1-CA3 and primarily hosts inhibitory basket cells. Importantly, the basal dendrites of pyramidal hippocampal neurons are also located in the stratum oriens, where commissural fibres communicate between hippocampi. Stratum pyramidale, the following thin later, contains the cell bodies of the canonical excitatory pyramidal cells of the hippocampus, as well as interneurons of various morphology and output. The subsequent stratum radiatum makes up the vast majority of the hippocampus proper and contains Schaffer collateral fibres and several types of interneuron. The next stratum-stratum lacunosum-also contains Schaffer collaterals and is often combined with the most inferior superficial stratum, just prior to the hippocampal fissure, the stratum moleculare. Meanwhile, the DG is made up of three major components: the hilus, stratum granulosum and stratum moleculare. Working inferior from the hippocampal fissure, the stratum moleculare contains perforant path fibres that synapse and excite distal apical dendrites of granule cells. Granule cell somas reside in the subsequent stratum granulosum and largely

form excitatory glutamatergic synapses, though some also release neuropeptides. The last layer, known as the hilus, is comprised mostly of DG cell axons en route to CA3.

1.2.2 Formation of Neural Circuits

While alterations to developmental migration and differentiation produce robust neurological phenotypes—such as lissencephaly—, disruption to the formation of neural circuits likely results in more targeted functional alterations, akin to those observed in most ASD and schizophrenia cases (Weiner et al., 2013). These changes could occur (i) prenatally during the establishment of neural circuitry, (ii) postnatally as the brain undergoes temporal and contextual changes, or (iii) both. Once neuronal cell fate has been specified and migration has halted, the cell begins to extend processes that will eventually form the axon and dendrites (Fukata et al., 2002). The axon is a specialized region of the neuron for propagating action potentials and thus must begin to extend itself and initiate appropriate connections with target cells both proximal and distal. Conversely, the dendrites are the primary site at which input from other neurons is received and thus must respond to target axons and begin generating a synapse, both structurally and functionally (Barnes & Polleux, 2011). In concert, the processes of axogenesis and dendritogenesis respectively, produce the primitive neural circuits in the developing brain. At birth, the neuronal population is large and the number of synapses overabundant. However, as postnatal development progresses, neuronal density is reduced and synapses are pruned as the circuits are refined and altered by experience (Penzes et al., 2011).

1.2.3 Axogenesis and Axon Plasticity Throughout Development

At the tip of the process destined to form the axon is a specialized region, known as the growth cone, which senses the local molecular environment and directs axonal outgrowth (Dent et al., 2011). These growth cones rely on cell-surface receptors that respond to either diffusible

(such as netrins and some semaphorins) or surface-bound (such as cell adhesion molecules, ephrins and some semaphorins) guidance cues from intermediate targets. These guidance cues may be either attractive or repellant, and accordingly direct the axon pathfinding of the developing neuron (for review, see Chilton, 2006; Bashaw & Klein, 2010; Dickson, 2002). The action of a guidance cue as either attractive or repellant depends upon the timing of release and concentration, as well as the receptors and second messengers expressed in the growth cone of the responding axon. It is through the concerted efforts of these guidance cues that the axon navigates the developing CNS and initiates connections with appropriate targets. When an axon encounters a filopodia present on a target dendrite, a synapse is formed through the association of adhesion molecules and the recruitment of synaptic machinery (Ziv & Smith, 1996; Fiala et al., 1998). In later development, axons retain their ability to grow and elongate locally, but largely remain unchanged (Hu & Strittmatter, 2004; Lewis et al., 2013).

1.2.4 Dendritogenesis and Dendrite Plasticity Throughout Development

The rest of these aforementioned processes produced by early neurons eventually form dendrites. The exact process by which a single neurite becomes destined to form the axon and the remainder the dendrites is not yet fully elucidated and may indeed differ between common model organisms (Jan & Jan, 2001). Despite their common beginnings, mature dendrites and axons differ greatly. Dendrites contain microtubules of mixed polarity, while axons contain polarized microtubules that extend their plus end to the axon terminal (Baas et al., 1988; Craig and Banker, 1994). The complex microtubule cytoskeleton of the dendrite provides structural stability, anchors cellular compartments and permits the bidirectional trafficking of cargo (for review, see Conde & Cáceres, 2009). Though the cytoskeletal turnover in mature dendrites is rapid and overt, the structures themselves are stable and thus likely require extensive local

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protein generation (Tsaneva-Atanasova et al., 2009). Moreover, certain motor proteins, ion channels and other proteins are exclusively expressed in either the dendrites or the axons, and mature dendrites contain almost all organelles, including ribosomes, Golgi outposts and endoplasmic reticulum (ER; Gao, 1998; Gardiol et al., 1999; Wells et al., 2000; Steward & Schuman, 2001). Once the axon has been specified, early dendrites display highly dynamic behavior and are constantly being extended and retracted (Dotti et al., 1988; Craig & Banker, 1994). During development, the formation of synapses along the dendritic arbor promotes stabilization, while the lack of synapses or the loss of synapses leads to destabilization and sometimes retraction of the dendrite (for review, see Cline & Haas, 2008; Haas et al., 2006). Indeed, early dendrites with fewer synapses typically have fewer spines and branches (Jan & Jan, 2003). Some minor dendritic pruning is thought to occur later in development, independent of synapse loss and formation (Wu et al., 1999; McAllister, 2000). However, as development progresses, the plasticity of dendrites markedly reduces under normal physiological conditions (Luener & Gould, 2010), The uncoupling of dendrite retraction and synapse elimination as development proceeds is believed to maintain the network integration of a given mature neuron, while allowing it to modulate its synaptic input. Canonically, dendrites are stabilized by microtubule-associated proteins (MAPs), which may in part be regulated by the synaptic release of brain-derived neurotrophic factor (BDNF; Vaillant et al., 2002). The location of a synapse within the context of the dendrite arbor determines the electrophysiological influence of that synapse on the firing of that neuron. Further, the dendritic arbor determines the spatial scope of inputs a neuron can receive within the broader CNS context. Thus, disruption to the outgrowth, maintenance and stability of dendrites could have broad functional implications (for review, see Spruston, 2008). Indeed, the retraction of dendrites at mature stages is a known feature of some

neuropsychiatric and neurodegenerative diseases (Leuner & Gould, 2010). In the hippocampus, postnatal dendritic pruning has been observed in the pyramidal neuron population (Liu et al., 2005), while chronic stress in adulthood can induce dendritic regression in the CA1, CA3 and DG (McKittrick et al., 2000; Sousa et al., 2000).

1.2.5 Synaptogenesis: Recognition and Assembly

The generation of chemical synapses in the CNS is an integral part of neural circuit formation and dendrite stability, while their maintenance and plasticity is integral to neuronal computation. In vivo, rat hippocampal neurons begin generating synapses at postnatal day 1 (P1), while in dissociated rat hippocampal cultures, synapses begin forming during the second week in vitro and multiply two-fold by the third week of culture (Boyer et al., 1998). These synapses consist of a presynaptic axonal compartment responsible for the release of neurotransmitters and a postsynaptic dendritic component responsible for transducing incoming neurotransmitter signals. These synaptic compartments must be held in close apposition across a synaptic cleft by various cell adhesion molecules (for review, see Dalva et al., 2007). The conventional paradigm for synapse formation espouses that the initial contact between a growing axon and a dendritic filopodium—a thin extension from the dendritic shaft that displays exploratory, sampling behavior unless stabilized—creates a stable adhesion that recruits the pre- and post-synaptic machinery to the appropriate cellular compartments and stimulates cytoskeletal restructuring (Ziv & Smith, 1996). For example, cadherins are thought to direct synapse morphology and integrity through their transynaptic interactions and by coupling to the cytoskeleton via α - and β catenins (Togashi et al., 2002). Further, in the postsynaptic compartment, ephrinB receptor ligands can induce the clustering of N-methyl-D-aspartate (NMDA) receptors, though this may not have developmental importance (Penzes et al., 2003). Presynaptic specialization, on the other hand, has only a few identified regulators: the neuroligins and synaptic cell adhesion molecule 1 (synCAM1). The neuroligins are tethered to the postsynaptic membrane by associations with postsynaptic density protein 95 (PSD-95) and signal through presynaptically localized neurexins to promote presynaptic specialization (Scheiffele et al., 2000). However, challenging this paradigm is the observation that clusters of prepackaged presynaptic and postsynaptic molecules form along neurites, even in the absence of synaptic contact (Friedman et al., 2000; Umeda et al., 2001). These include functional units of neurotransmitter release machinery, scaffold proteins and neurotransmitter receptors. For example, in cultured hippocampal neurons, clusters of NMDA receptors (NMDARs) and postsynaptic contact (Umeda et al., 2001; Craig & Lichtman, 2001). In the axons, synapse vesicle protein transport vesicles (STVs) form at predefined sites and form stable connections with dendritic filopodia (Sabo et al., 2006).

1.2.6 Synaptogenesis and Synapses Throughout Development

Following initial contact, synaptophysin and bassoon accumulate presynaptically, shortly followed by PSD-95 and NMDARs postsynaptically (Umeda et al., 2001). Within one hour, synaptic transmission may occur, likely in part due to presynaptic assembly from the aforementioned preassembled vesicles (Li & Sheng, 2003; Shapira et al., 2003). In the postsynaptic compartment, however, PSD-95 originates largely from cytoplasmic pools and does not colocalize initially with NMDA or α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Rao et al., 1998; Marrs et al., 2001). Instead, the trafficking of NMDA and AMPA receptors (AMPARs) is independently regulated through their different binding partners (Washbourne et al., 2002; Scannevin & Huganir, 2000). Most early synapses indeed lack AMPARs and thus are silent. However, as development proceeds, the fraction of excitatory

synapses without AMPARs greatly reduces until almost all contain them (Durand et al., 1996). The mechanism by which this switch occurs has not been fully established, though some have hypothesized that γ -Aminobutyric acid (GABA) signaling—which may be excitatory during development—may provide sufficient depolarization to remove the Mg²⁺ block on NMDARs and promote AMPAR insertion (Ben-Ari, 2002). This topic shall be explored in greater detail in Sections 2.7 and 2.8. The developmental switch from predominantly AMPAR-lacking to AMPAR-containing excitatory synapses coincides with a shift in predominant spine morphology from filopodial to mushroom (Matsuzaki et al., 2001). During early synaptogenesis, synapses are largely present on the shaft and filopodia (Ziv & Smith, 1996). However, as these synapses mature, inhibitory synapses are localized largely to the dendritic shaft, while excitatory synapses become localized primarily to large, wide-necked protrusions known as mushroom spines (Villa & Nedivi, 2016), as shown in Figure 1.1. Some have hypothesized that this morphological shift represents a maturation lineage from filopodial to mushroom spines, although this has not been conclusively demonstrated (Ziv & Smith, 1996). Filopodia have an average turnover of minutes in culture, while mushroom spines last anywhere from hours to days (Dailey & Smith, 1996). It is also important to note that some extrinsic factors, such as the Cajal-Retzius cells in the hippocampus (Del Rio et al., 1997) and various Wnt ligands, can instruct regional and appropriate synaptogenesis (Salinas & Zou, 2008). Lastly, although the developmental framework of neural networks appears initially independent of synaptic activity, it is synaptic communication that ultimately regulates the stabilization, enhancement, maintenance and elimination of synapses throughout the rest of development (Flavell & Greenburg, 2008).

1.2.6.1 Excitatory Synapses

There are two major classifications of synapses in the CNS: excitatory and inhibitory. These synapses differ not only in their output, but also in their molecular constituents and dendritic location (Villa & Nedivi, 2016). In the hippocampus, glutamate-releasing or glutamatergic synapses are the major form of excitatory synapse and their activity promotes the depolarization of the neuron (for review, see Scannevin & Huganir, 2000). On the presynaptic side, glutamate is generated in the cytoplasm from glutamine by the enzyme glutaminase, and transported into synaptic vesicles by vesicular glutamate transporter 1 (VGluT1) along a H⁺ gradient (Takamori, 2006). These glutamate-containing vesicles remain tethered to the presynaptic terminal until Ca²⁺ influx from depolarization triggers vesicle fusion to the surface membrane and exocytosis, thus releasing glutamate into the synaptic cleft (Sudhof, 2013). Secreted glutamate must then be sensed by the postsynaptic compartment and transduced into an excitatory signal in the postsynaptic neuron. The major responsive elements are the glutamate receptors, which are maintained at the synapse by a range of scaffolding proteins, such as the canonical PSD-95 (Kim & Sheng, 2004). Glutamate receptors can be further subdivided into two major classes: ionotopic and metabotropic. Ionotropic receptors include NMDA, AMPA, kainite and delta receptors and are ligand-gated ion channels, while metabotropic glutamate receptors (mGluRs) include mGluR1-mGluR8 and are G-protein coupled receptors (GPCRs) that modulate NMDAR activity (Greger & Esteban, 2007). Though ionotropic receptors vary greatly, most are tetramers with two extracellular domains that sense glutamate, a transmembrane domain that permits cation conduction and an intracellular domain that tethers the receptor to the membrane. Most excitatory synaptic transmission in the adult brain occurs through AMPARs, which respond to glutamate and permit the influx of Na⁺ and outflux of K⁺. The calcium permeability

of the AMPAR, as well as a host of other functional differences, is defined by the subunit composition (Greger & Esteban, 2007). Indeed, there are four subunits (GluA1-GluA4), with GluA2 conferring calcium impermeability. In the mature hippocampus, most AMPARs are GluA1/GluA2 heteromers, suggesting that they are not integral sources of Ca²⁺ influx into the postsynaptic compartment during activity (Lu et al., 2009). However, AMPARs are highly responsive to activity and are known to modulate synaptic strength and response through their trafficking to and from the surface membrane (Greger & Esteban, 2007). NMDARs, on the other hand, are thought to underlie synaptic plasticity (for review, see Hunt & Castillo, 2012). Unlike AMPARs, NMDARs are blocked by Mg²⁺ under basal conditions and require the depolarization of the postsynaptic cell to shift this Mg^{2+} block, as well as the binding of glutamate and glycine to the receptor extracellularly, in order to fire. Owing to this phenomenon, NMDARs are often referred to as coincidence detectors. Further, NMDARs are calcium permeable, and thus likely contribute to calcium influx to the postsynaptic cell when active (Huganir & Nicoll, 2013). NMDARs have a range of subunits: GluN1, GluN2A-D, and GluN3A-B. Similar to AMPARs, subunit composition of the NMDARs also confers a range of functional properties. For example, during early development, GluN2B, GluN2D and GluN3A subunits predominate. However, as development proceeds, there is a switch in subunit composition and GluN2A-containing NMDARs eventually predominate. GluN2B-containing NMDARs are known to remain open longer than GluN2A-containing NMDARs and thus this switch alters signaling and excitotoxicity properties of the neuron (Wyllie et al., 2013). In the conventional paradigm of glutamatergic firing, glutamate release at the presynaptic side stimulates AMPARs, which depolarizes the postsynaptic compartment through the movement of Na⁺ and K⁺. This depolarization shifts the Mg²⁺ block present at NMDARs, which also received glutamate and

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glycine from presynaptic vesicle release. The combined depolarization and binding of glutamate and glycine to the NMDAR leads to further depolarization through Na^+ and K^+ ions, as well as the influx of Ca^{2+} to the postsynaptic compartment (Scannevin & Huganir, 2000).

1.2.6.2 Inhibitory Synapses

Inhibitory synapses, on the other hand, promote hyperpolarization or reduce depolarization of the postsynaptic neuron (for review, see Moss & Smart, 2001). The main inhibitory neurotransmitter in the hippocampus is GABA, which is generated in the presynaptic neuron from glutamate by glutamate decarboxylase and its co-factor pyridoxal phosphatase (Tritsch et al., 2016). GABA is then packaged into synaptic vesicles by vesicular GABA transporter (VGAT) for eventual release. Similarly, there are two classes of GABA receptors: the ionotropic, ligand-gated GABA_A receptors (GABA_AR), and the metabotropic, G-protein coupled GABA_B receptor (GABA_BR). Binding of two GABA molecules to the GABA_AR opens an ion pore and permits the influx of Cl⁻ ions into the postsynaptic compartment and thus causes hyperpolarization. GABA_ARs are tethered to the postsynaptic density by a scaffolding protein known as gephyrin, which is akin to the tethering of AMPARs by PSD-95 in excitatory synapses (Moss & Smart, 2001). Though initial studies suggested that GABA_ARs may be excitatory during development due to the lack of Cl pumps creating a negative gradient and thus creating the outflux—as opposed to the influx observed in the mature CNS—of Cl⁻ ions when GABA_ARs are active, recent evidence suggests this may be an artifact of methodology (Zilberter, 2016). Overexcitation of these receptors, however, leads to autoinhibition via conformational remodeling that seals the ion pore (Sigel & Steinmann, 2012). There are a number of GABA_AR subunits that confer different functional properties: α (1-5), β (1-3), γ (1-3), δ , ε , π , θ , and ρ (1-3). The canonical GABA_AR consists of two α subunits, two β subunits and one γ subunit, though the minimum required to form a receptor is two α and two β subunits. The slow response to GABA release is mediated through the GABA_BR, which stimulates the opening of K⁺ channels through a G-protein signaling cascade. K⁺ then leaves the cell and similarly hyperpolarizes the postsynaptic compartment, reducing the likelihood of neuronal firing (Moss & Smart, 2001).



Figure 1.1. General neuronal morphology, excitatory synapses and inhibitory synapses. (**a**) A 20x confocal image of a green fluorescent protein (GFP)-expressing hippocampal neuron is shown. Evident is the long-range axon, as well as the complex dendritic arbor. Site 1 indicates a spiny dendritic protrusion, the morphological correlate of excitatory synapses, while site 2 indicates the dendritic shaft, the primary site of dendritic inhibitory synapses. (**b**) AMPAR-mediated postsynaptic depolarization removes the Mg²⁺ block present at NMDARs.

Coincidental binding of glutamate to NMDARs leads to an influx of Ca²⁺ into the postsynaptic compartment, leading to the activation of kinases, such as CaMKII and PKA. These kinases phosphorylate downstream signaling molecules causing the insertion of AMPARs and alteration of the postsynaptic density and f-actin cytoskeleton through local protein synthesis (Adapted by Rachel Gomm-Kalisko with permission from Kauer & Malenka, 2007). (c) Binding of GABA to GABA_ARs results in the opening of the ion pore and a subsequent influx of Cl⁻ ions into the postsynaptic compartment. Thus, GABA_A receptor activation leads to hyperpolarization of the neuron and contributes to the prevention of firing in the postsynaptic neuron.

1.2.6.3 Synapse Plasticity

However, as aforementioned, synapses are constantly being formed and eliminated throughout development, and this is referred to as synapse plasticity (for review, see Neves et al., 2008). Indeed, synapse formation is exuberant during fetal development and synapses are systematically pruned during childhood and adolescence, culminating at \sim 50% reduced levels in adulthood (Stiles & Jurnigen, 2010; Penzes et al., 2011), as shown in Figure 1.2a. Despite this overarching theme towards synapse elimination, on a microscopic level, synapses are also being extensively formed and enhanced throughout life. It is thought that the plasticity of synapses allows for the modulation of neural networks in response to experience and activity. This is of particular interest in regions of the brain known to be associated with learning and memory, such as the hippocampus, which has well-defined roles in spatial learning and declarative memory (Neves et al., 2008). One of the first studies to suggest the plasticity of neuronal responses in the hippocampus was performed by Bliss and Lomo (1973). In which, high frequency stimulation (HFS) was applied to fibers of the perforant pathway and excitatory postsynaptic potentials (EPSPs) recorded in the DG. When a single-pulse stimuli was then applied to these HFS-exposed fibers, there was a long-term enhancement of EPSPs in the DG, which they coined as long-term

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potentiation (LTP). These experiments provided the first insight into a mechanism by which functional alterations to neural networks might occur.

1.2.6.4 Long-term Potentiation

LTP is thus defined as a persistent strengthening of a synapse in response to activity (for review, see Lynch, 2004), as shown in Figure 1.2b. Though the mechanism of LTP induction can differ by brain region, signaling molecules and neuron type, most induction protocols rely on NMDAR activation. However, some brain regions, such as the mossy fibre pathway, undergo NMDAR-independent LTP. The best-studied form of LTP is NMDAR-dependent LTP (NMDAR-LTP) at excitatory synapses in the CA1 region of the hippocampus, which shall be the focus of this section (Nicoll & Schmitz, 2005). Some of the initial evidence for the mechanism underlying LTP came from experiments wherein the application of NMDAR antagonists to Schaffer collaterals of the CA1 prohibited LTP (Collingridge et al., 1983) and the hyperpolarization of the postsynaptic cell during HFS prevented LTP at both inhibitory and excitatory synapses (Malinow & Miller, 1986). There are now two canonical mechanisms for the induction of LTP: one that mediates the initial, fast response to activity, and another that mediates the maintenance of the potentiated state over longer periods of time (for review, see Lüscher & Malenka, 2012). During early LTP (E-LTP), glutamate-induced depolarization of the postsynaptic cell via AMPARs displaces the NMDAR Mg^{2+} block, creating a Ca^{2+} influx. This influx activates protein kinases (importantly protein kinase A, protein kinase C, and calmodulindependent protein kinase II; PKA, PKC and CAMKII respectively), leading to the phosphorylation of AMPARs, which improves their ion conduction, and the insertion of AMPARs into the postsynaptic membrane (Hayashi et al., 2000). The late LTP (L-LTP) response that maintains the potentiated state requires the phosphorylation of a number of second

messengers, like cyclic adenosine monophosphate (cAMP), that regulate long-range gene transcription and promote the local synthesis of PSD-95 and AMPARs, which then become integrated into the synapse (Sacktor, 2008). Though the precise mechanism is unclear, spines undergoing NMDAR-LTP also increase in size, volume and postsynaptic density through reorganization and enlargement of the F-actin cytoskeleton (Fortin et al., 2010). If LTP induction is blocked, these structural changes do not occur, suggesting they share common biochemical pathways. The trafficking of GluA1 subunits in particular was demonstrated to be required for activity-dependent LTP and this insertion is likely mediated through phosphorylation by CAMKII (Hayashi et al., 2000). Accordingly, following LTP, the majority of nascent AMPARs inserted into the membrane are GluA1/A2 heteromers (Henley & Wilkinson, 2013).

1.2.6.5 Long-term Depression

Long-term depression (LTD), on the other hand, is a persistent and activity-dependent decrease in the strength of a synapse (for review, see Collingridge et al., 2010), as shown in Figure 1.2b. The first evidence for the existence of LTD came from studies that performed low frequency stimulation (LFS) of CA1 Schaffer collaterals and observed a depotentiation of the postsynaptic response (Dudek and Bear, 1992). This protocol is still widely used today to induce LTD, as well as a spike-timing protocol and the bath application of NMDA and glycine to simulate a chemical form of LTD (cLTD). Though most LTD induction paradigms are electrophysiological in nature, cLTD could not further enhance LFS-induced LTD, suggesting they act through common pathways (Lee et al., 1998). Moreover, many of the surrogate markers of LTD, such as AMPAR internalization, are observed in both electrophysiological and chemical LTD induction paradigms (Lin & Huganir, 2007). The best-elucidated forms of LTD occur in the hippocampus and cerebellum, though they differ in mechanism. For example, in the cerebellum,

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strong stimulation of synapses leads to LTD, while weak but persistent stimulation of excitatory synapses induces LTD in the hippocampus (Massey & Bashir, 2007). The canonical form of LTD is NMDAR-dependent LTD (NMDAR-LTD) at excitatory synapses of the hippocampus, which shall thus be the focus of this section. However, it is important to note that LTD can also depend on mGluRs and endocannabinoids. NMDAR-LTD is thought to rely on the minimal activation of NMDARs and the resulting small calcium influxes insufficient to breach a certain threshold at which LTP is initiated. Instead, these low levels of calcium bind to calmodulin and activate the high affinity calcium sensor calcineurin (Mulkey et al., 1993), as shown in Figure 1.2c. Calcineurin is positioned near the mouth of the NMDA channel by its association with PSD-95 and its activation leads to the subsequent activation of a number of phosphatases, including protein phosphatase 1 (PP1). These protein phosphatases work in opposition to the kinases mentioned in the previous section by dephosphorylating GluA1, PKA and PKC, leading to the internalization and weakening of AMPARs (Collingridge et al., 2010). Further, hippocalcin is also activated during LTD, causing clathrin adaptor protein 2 (AP2) to replace Nethylmaleimide sensitive fusion protein (NSF) at a GluA2 binding site, leading to the dynamindependent endocytosis of NMDARs via clathrin-coated vesicles (Palmer et al., 2005). This appears to be an integral process in some forms of LTD, as the inhibition of hippocalcin signaling prevents LTD. Though different NMDAR subtypes may play specific roles in LTD, the evidence thus far has been inconclusive. For example, GluN2B is required for NMDAR-LTD only under specific circumstances (Bartlett et al., 2007; Morishita et al., 2007). Circumstantial evidence, such as the increasing difficulty to initiate NMDAR-LTD as development progresses despite the comparable presence of NMDARs (Yashiro & Philpot, 2008), however, does suggest some subtype involvement, though the genetic replacement of all AMPARs with kainite

receptors or single NMDAR subtype knockout (KO) did not seem to impair or alter CA1 LTD (Meng et al., 2003; Selcher et al., 2012; Granger & Nicoll, 2014). As in LTP, during LTD spines are dramatically restructured. Studies have regularly identified a reduction in spine size and volume that is dependent on NMDAR activation in the CA1 region of the hippocampus. These effects are similarly mediated through restructuring of the actin cytoskeleton and postsynaptic density (Zhou et al., 2004). For example, PSD-95 is dephosphorylated at Ser295 during NMDAR-LTD and this leads to its eventual removal and permits AMPAR endocytosis (Kim et al., 2007). Lastly, long-term alterations to protein synthesis also underlie the maintenance of the depotentiated state of a synapse undergoing LTD, but the mechanisms regulating this process remain poorly elucidated (Manahn-Vaughan et al., 2000).



Time (min)



Figure 1.2. Developmental regulation of spine density, electrophysiological effects of LTP and LTD at excitatory synapses and LTD signaling pathways. (a).During early childhood, spine formation—a morphological correlate of excitatory synapses—is exuberant even in neurotypical humans. A development trend towards the systematic elimination of spines occurs throughout adolescence and adulthood as neural circuits are refined by experience. A leading theory of ASD postulates that individuals with ASD form an even greater number of exuberant spines during childhood and, despite showing a similar trend towards net elimination, spine number remains excessive in ASD patients throughout life. Thus, these differences could represent alterations to the mechanisms underlying spine formation, maintenance and/or elimination. (Used with permission from Penzes et al., 2011). (b) In the CA1 region of the hippocampus, LTP, a persistent strengthening of synapse strength, can be induced by HFS, while LTD, a persistent weakening of synapse strength, can be induced by LFS. LTP and LTD are typically induced and assessed electrophysiologically through the respective increase or decrease in amplitude of excitatory postsynaptic currents (EPSCs). However, LTP and LTD can also be chemically induced using glycine or NMDA. (Adapted with permission from Collingridge et al., 2010). (c) NMDAR activation leads to the influx of Ca^{2+} into the postsynaptic compartment. When the number of NMDARs activated is low, the Ca^{2+} preferentially binds to its high affinity-binding partner, calcineurin, leading to its activation. Activated calcinuerin in turn activates protein phosphatases, including PP1, capable of dephosphorylating AMPARs and glycogen synthase kinase 3s (GSK3s), leading to AMPAR internalization. Further, PSD-95 is dephosphorylated, leading to its removal from the synaptic membrane and shrinkage of the postsynaptic density. A number of other downstream signaling molecules mediate LTD-triggered elimination of synapses and the long-term maintenance of LTD (Used with permission from Rachel Gomm-Kalisko).

1.2.6.6 In Vivo Correlates of Long-term Potentiation and Long-term Depression

Though great attention has been paid to the mechanisms of LTD/LTP in the hippocampus, debate still reigns about the contribution of these phenomena to learning and memory (for review, see Takeuchi et al., 2014). Some early studies demonstrated that an
NMDAR antagonist blocked both LTP induction in the hippocampus and attenuated performance in the hippocampus-dependent Morris Water Maze (MWM) task (Morris et al., 1986). Conditional KO mice lacking the GluN1 subunit of NMDARs in the CA1 of the hippocampus similarly had impaired spatial learning and LTP (Tsien et al., 1996). Conversely, overexpression of NMDAR2B led to enhanced LTP and spatial memory (Wang et al., 2009). However, it was later discovered that these findings only hold true in task-naïve animals and are not recapitulated in pretrained animals. Indeed, in one eye-blink conditioning study, associative learning before, but not after, conditioning could not be diminished by LTP induction (Moser & Moser, 2000). Saturating LTP in the hippocampus in some studies prohibited spatial learning, though this finding has been inconsistent (Moser et al., 1998; Robinson, 1992). Heterozygotic CAMKII KO mice, an important downstream signaling molecule during LTP, displayed fine hippocampal-dependent memory initially after training, but impaired memory 10-50 days posttraining, suggesting a loss of L-LTP or LTP maintenance (Frankland et al., 2001). Hippocampal NMDAR-LTD, on the other hand, in GluN2B and calcineurin KO mice is impaired, as well as their performance in hippocampal-dependent spatial tasks (Brigman et al., 2001; Zeng et al., 2001). Similarly, disruption to forebrain calcineurin or Ser/Thr protein phosphatase 2A leads to reduced NMDAR-LTD and increased latency in the MWM reversal phase, suggesting a role for NMDAR-LTD in the pruning of memories and behavioural flexibility (Zeng et al., 2001; Nicholls et al., 2008). Further, depotentiation in the CA1 and DG has been observed in rats exploring either a novel environment or a familiar environment containing novel objects, suggesting LTD might also play a role in novelty detection (Manahan-Vaughan & Braunewell, 1999; Abraham et al., 2002).

1.2.7 Note on Gliogenesis

Though secondary to the focus of the present thesis, it is important to note that glial proliferation, migration, differentiation and integration largely occurs postnatally and likely continues indefinitely throughout all later stages of development (for review, see Clarke & Barres, 2013). Despite some preliminary studies that highlight the overarching importance of glia to the maturation and function of neural circuits, the dynamics of neuron-glia interactions are poorly elucidated. However, the contribution of oligodendrocytes to action potential propagation and thus circuit firing patterns, as well as astrocytic contribution to tripartite synapses and synaptic cleft maintenance (Hama et al., 2004; Hughes et al., 2010; Pfrieger & Barres, 1997; Verbich et al., 2012), undisputedly contribute to a broad range of CNS functions with wide-reaching implications.

1.3 Clinical and Postmortem Features of ASD

1.3.1 Clinical Phenotypes of ASD

Any number or combination of impairments to the development of the CNS could contribute to the etiology of ASD. ASD is currently diagnosed based on behavioural characteristics observed in the clinic, typically through the use of diagnostic scales such as Autism Diagnostic Observation Schedule (ADOS) and Autism Diagnostic Interview-Revised (ADI-R; for review, see Fakhoury, 2015). Though the clinical phenotype in individuals with ASD is broad, some core phenotypes do exist: stereotypic behaviours, as well as impairments in communication and sociability (American Psychiatric Association, 2013). Further, a number of patients experience secondary symptoms, such as self-injury, hyperactivity, atypical obsessions, difficultly building social relationships, aggression and impaired theory of mind. There are also a number of frequently occurring co-morbid conditions, including anxiety, depression, intellectual disability, Attention Deficit Hyperactivity Disorder (ADHD) and gastrointestinal (GI) disorders. These symptoms typically become noticeable from 1-3 years of age when developmental targets are not being appropriately reached in a timely manner (Fakhoury, 2015). Though most individuals with ASD have a below average intelligence quotient (IQ) and impaired cognitive function, the range is broad such that it encapsulates high-functioning individuals with above-average intelligence, as well as individuals with severe delays in lingual and intellectual development (Constantino, 2011). This broad range, or spectrum, indicates the likely many pathways by which cohorts present with ASD clinically.

1.3.2 Neurodevelopmental Disruptions in ASD

Evidence for the disruption of the CNS in ASD patients comes from neuropathology, functional magnetic resonance imaging (fMRI) and transcranial magnetic stimulation (TMS) studies. In postmortem brain samples, individuals with ASD were found to have alterations to neuron density, neuron size, inflammation, excitability, dendrite morphology, dendrite outgrowth, and dendritic spine density compared to the unaffected general population (Hustler & Zhang, 2010; Courchesne et al., 2011; Raymond et al., 1996; Gupta et al., 2014; Rubenstein & Merzenich, 2003). Neuronal disruptions in ASD brains have been identified in a broad range of brain regions, including the frontal cortex, cerebellum, hippocampus and amygdala (for review, see Amaral et al., 2008). One of the leading theories on the cause of ASD is the lack of longrange and overabundance of short-range neuronal connections. Indeed, fMRI and TMS studies have identified hypo- and hyper-connectivity patterns in the brains of individuals with ASD compared to controls (Kana et al., 2014; Muller et al., 2011). A complementary, but distinct, hypothesis on the etiology of ASD postulates that an imbalance in the excitatory to inhibitory

ratio of neurotransmission underpins the symptoms of ASD. This imbalance could occur at almost any point in neurodevelopment, including the formation, maintenance, elimination or enhancement of excitatory or inhibitory synapses (for review, see Rubenstein & Merzenich, 2003; Nelson & Valakh, 2015). Indeed, many of the genes associated with ASD were demonstrated in animal models to play a role in synapse plasticity, strength or number, which shall be discussed in detail in Section 1.4. Our lack of understanding about the likely varied etiologies of ASD prohibits the ability of researchers to create ASD subcategories. This likely reduces the power of these studies to identify common neurophenotypes by masking subgroup differences within a heterogenous ASD cohort. Despite valiant attempts to identify a common neuropathology, no consensus currently exists on the underlying neurological changes of ASD (Won et al., 2013).

1.4 ASD Epidemiology and Genetics

1.4.1 Broad Genetics of ASD

20 in every 10,000 children, however, are affected by ASD with a sex ratio of 4.3:1, though this sex difference depends on the comorbid factors and is less evident in certain types of ASD, such as ASD with intellectual disability (Newschaffer et al., 2007). Twin studies were among the first to suggest the heritability of ASD, wherein the concordance rate in monozygotic twins was consistently higher (70-90%) than in dizygotic twins (0-10%; Abrahams & Geschwind, 2008). Further, epidemiological studies identified a 10-fold increase in ASD risk for siblings of individuals with ASD and a greater frequency of subthreshold characteristics in unaffected family members of particular individuals with ASD compared to the general population (Sandin et al., 2014). A recent meta-analysis of all ASD twin studies found that

additive genetic effects explained ~65% of the concordance of ASD, while shared environmental effects accounted for the remaining 35% (Tick et al., 2016). Genome-wide association studies (GWAS) looking at mutation rates in whole-exome sequencing (WES) data have identified 600-1,200 potentially ASD-associated genes (for review, see De Rubeis & Buxbaum, 2015). These mutations include large chromosomal alterations, frameshift mutations and various forms of single nucleotide variation (SNV). Chromosomal alterations refer to the amplification, deletion or translocation of a large section of a chromosome, while frameshift mutations involve the deletion or insertion of a single amino acid into the genetic sequence, thereby shifting the reading frame. SNVs, on the other hand, refer to the alteration of a single nucleotide in the genetic sequence. SNVs can be (i) synonymous, and thus not alter the transcribed protein sequence, due to redundancy in the codon sequence; (ii) nonsynonymous, and thus alter the transcribed amino acid at that genetic loci; or (iii) nonsense, and thus introduce a premature stop codon.

1.4.2 Making Sense of Genetic Heterogeneity in ASD

Before it is possible to appreciate how these mutations may collectively contribute to ASD, it is important to consider the background mutation rate in the general population (for review, see Bourgeron, 2015). Every individual carries three million genetic variants from the reference human genome sequence. 18-74 of these variants, 1-4 of which will be present in exons, will not be shared by either parent, and thus are classified as *de novo* (O'Roak et al., 2011). These include SNVs, as well as insertion, deletions and copy number variations (CNVs). 40-110 of the variants in any given individual are classified as disease-causing and appear in the Human Gene Mutation Database (Xue et al., 2012). Thus, it is the norm and not the exception for an individual to carry *de novo* mutations. The difficulty comes in assessing the penetrance and polygenic contribute of any single mutation to the development of disorders. Interestingly, the

proportion of ASD thought to be explained by single nucleotide polymorphisms (SNPs)—which are SNVs that occur in >1% of the population—is 17-60%, suggesting that while these variants may contribute to polygenic forms of ASD, genetic buffering at other loci can sufficiently prohibit the development of ASD (Gaugler et al., 2014). Despite this, GWAS studies have lacked the power to identify a single SNP with genome-wide significance for ASD (Anney et al., 2012). Most cases of ASD are likely polygenic, though some monogenic forms have been described, demonstrating that a single mutation (10-20%) or an accumulation of mutations has the capacity to underlie the disorder in any individual (Huguet et al., 2013). Currently, a single chromosomal alteration, gene insertion, gene deletion or SNV can sufficiently explain most of an individual's ASD symptoms in only 15-25% of cases (Bourgeron, 2015). Indeed, no single gene can account for more than 1% of all ASD cases (Huguet et al., 2013). In ASD individuals with normal IQ measures, no causative de novo SNVs have been found, suggesting that in more common ASD cases with intellectual impairment, rare and penetrant variants might unveil ASD by reducing IQ or genetic buffering at other loci (Skuse, 2007; Bourgeron, 2015). However, 5-15% of ASD individuals carry de novo CNVs, versus only 0-2% in the general population (Glessner et al., 2009). These CNVs can be either insertions or deletions of the same gene or genes, but often occur at sites observed in several individuals with ASD, suggesting hypermutability of these regions (Michaelson et al., 2012). Further, de novo SNVs are not observed more frequently in ASD patients compared to their unaffected siblings or than predicted by chance alone, but there is a two- to three-fold increase in likely gene disruptive de novo mutations in individuals with ASD (O'Roak et al., 2011; Bourgeron, 2015). Despite this 'likely gene disrupting' classification, a lot of the genetic variants associated with ASD have yet to be functionally characterized and rely on *in silico* models as predictors. Attention is now

shifting to the functional annotation of exonic mutations in ASD, as the present thesis attests. While this work has broad diagnostic and therapeutic implications, variants in non-coding regions of the genome remain largely ignored (Yuen et al., 2016). These non-coding regions may also indirectly change gene function by altering gene expression temporally, intrinsically or in response to stimuli. One method of selecting SNVs that may contribute to ASD involves identifying *de novo* non-synonymous SNVs that occur either with greater frequency or exclusively in ASD patients and characterizing those SNVs in animal models using assays with relevance to known functions of that gene, or with relevance to correlates of ASD neuropathology or behavior.

1.5 Environmental Factors and ASD Risk

As aforementioned, some evidence exists for the contribution of environmental factors to ASD, though no single environmental factor has been demonstrated to influence risk. Instead, these environmental factors may act in concert with one another, particularly within a given genetic context, to increase ASD risk (Fakhoury, 2015). Environmental exposure to a range of factors has the capacity to alter cell differentiation patterns, synaptogenesis, axon myelination, and gene expression (Lyall et al., 2014). Some examples of environmental factors that may influence ASD risk include interuterinal environment (James, 2014), viral infection during pregnancy (Knuesel et al., 2014), oxygen deprivation during birth (Gardener et al., 2011), parental age at the time of conception (Durkin et al., 2008), timing between pregnancies (Cheslack-Postava et al., 2011), the use of prenatal vitamins before conception (Schmidt et al., 2011), the use of valproic acid and other mood stabilisers (Roullet et al., 2013), and maternal obesity or diabetes (Li et al., 2016). One of the ways in which environment may alter gene

expression is through the alteration of epigenetic states—such as DNA methylation, chromatin remodeling or histone modification—, which modulate the expression of genes without altering the DNA code. In fact, some studies have described altered DNA methylation in a number of synaptic genes, including SHANK3 (Zhu et al., 2014; Loke et al., 2015). However, it is important again to note that no environmental factor has yet been consistently and conclusively associated with ASD.

1.6 Animal Models of ASD

Animal models are an invaluable source of information on the function of genetic mutations observed in ASD. As previously mentioned, attention has recently shifted to the functional annotation of genetic mutations in the hope that it'll (i) provide greater insight into the etiology of ASD, (ii) allow for greater specificity in diagnoses and accuracy in prognoses, and (iii) permit the generation and recommendation of targeted therapies and interventions. Generally, these animal models focus on the ability of gene disruption or environmental exposure to phenocopy ASD behaviors and neuropathology. In mice and rats, ASD-like behaviors are classified as repetitive self-grooming, the lack of a preference for sociability or social novelty, reduced social interactions, reduced ultrasonic vocalizations and behavioral inflexibility, such as the swim path reorienting required during the reversal phase of the MWM task (for review, see Crawley et al., 2012). ASD-like neuropathology, on the other hand, is considered in rodent models to be neuronal hyperexcitability, synaptic alterations, neuronal migration or differentiation deficits, and alterations to neuronal morphology (Pardo & Eberhart, 2007). As well as assessing the ability of these models to mimic ASD, animal models are also used to elucidate the functional and neuropathological consequences of different ASD-associated genetic alterations or environmental exposures upon neurodevelopment, behavior, biochemical pathways, and cellular physiology. Most ASD models are transgenic mice, though a number of other organisms are often used to model particular aspects of gene function, such as yeast, fruit fly and Caenorhabditis elegans (Halladay et al., 2009; Crawley, 2012). The common ASD model mice include *Cntnap2* KOs, which exhibit seizures, reduced social measures, failed MWM reversal phase, reduced differentiation of GABAergic interneurons and altered neuronal migration (Peñagarikano et al., 2011). Further, mutation of the Fragile X Syndrome-associated gene *Fmr1*, which has high comorbidity with ASD in humans, leads to impairments in LTP, aberrant social behaviors and atypical cognitive and anxiety-like behaviors (Gantois et al. 2001; Crawley, 2012). Interestingly, the mutation of specific regions of the ASD-associated Shank3 gene led to distinct phenotypes in mouse models. For example, a mutation in the ankyrin domain led to impaired excitatory transmission and LTP, but typical social behaviors despite the presence of some stereotypies (Bozdagi et al., 2010). However, a Homer binding site mutation led to reduced LTP and enhanced LTD, despite producing no differences in spine density (Bangash et al., 2011; Crawley, 2012). Lastly, a mutation in the PDZ binding domain (PDZ-BD), which is important for the association of this protein with synaptic scaffolding proteins such as PSD-95, led to severe ASD-like behavioral phenotypes, longer spines, reduced spine density and reduced excitatory neurotransmission (Peca et al., 2011). The perturbations observed in animal models of ASD thus provide important insights into the potential neurobiologies, etiologies and neuropathologies of ASD.

1.7 Semaphorins: Focus on Semaphorin 5A and ASD

1.7.1 SEMA5A and ASD

In the first chapter of my results, I focus on the role of SEMA5A, an ASD-associated gene, at the synapses of mature neurons. The initial evidence for the association of SEMA5A with ASD came from a GWAS study of 1,500 individuals with ASD, which found a significant enrichment of SNVs in or near the SEMA5A gene, compared to unaffected family members (Weiss et al., 2009). Further, the blood and brains of some individuals with ASD exhibit downregulation of SEMA5A expression (Weiss et al., 2009; Melin et al., 2006). Supporting the downregulation of SEMA5A, previous work had already described the enrichment of SEMA5A regulatory genes in rare SNPs and CNVs associated with ASD (Cheng et al., 2013). Sema5A belongs to a family of over twenty different proteins known as the semaphorins, which were initially identified as growth cone-collapsing proteins involved in repulsive axon guidance (for review, see Pasterkamp, 2012). Some of these semaphorins are secreted, while others are membrane-bound or GPI-anchored, and contemporarily semaphorins are thought to play a role in the generation of precise neural networks through a variety of mechanisms. These proteins are further subdivided into 8 classes (SEMA1-7 and SEMAV) based on their extracellular motifs, type of membrane association and phylogenetic relationship. Some of the semaphorins are found exclusively in invertebrates, vertebrates or viruses, as outlined in Figure 1.3a. However, all of the semaphorins contain a 500 amino acid, cysteine-rich extracellular Sema domain, which is highly conserved across phyla (Kolodkin et al., 1993). This Sema domain is integral for the binding of Sema to its canonical receptors, the plexins (Tamagnone et al., 1999). All vertebrate semaphorins also contain a plexin-semaphorin-integrin (PSI) or cysteine-rich domain (CRD), but it can vary in its proximity to the Sema domain (Yazdani & Terman, 2006). In addition, some of the

semaphorins, including class 5 semaphorins, have Ig-like or type-1 thrombospondin repeats (TSRs), which may modulate the effects of these semaphorins by binding to co-receptors distinct from the plexins. Indeed, the bidirectional—repulsive or attractive depending on context—axon guidance cue function of Sema5A is likely mediated through its TSRs in certain regions of the developing brain (Kantor et al., 2004).

1.7.2 Roles of Semaphorins in the Nervous System

The range of functions attributed to the semaphorins is diverse. This thesis shall focus solely on identified neurodevelopmental functions, of which there are several. For example, in cultured hippocampal neurons, Sema3A inhibits axon formation in developing neurites and promotes dendrite identity by reducing cAMP and PKA activation, while increasing cyclic guanosine monophosphate (cGMP) activation (Shelly et al., 2011). Sema3A is also thought to play a role in axon pathfinding, acting as a repulsive guidance cue in dorsal root ganglions (Luo et al., 1993), motoneurons (Moret et al., 2007), sympathetic ganglion cells (Koppel et al., 1997), cerebellar mossy fibres (Rabacchi et al., 1999) and hippocampal neurons (Chedotal et al., 1998). Interestingly, Sema3B and Sema3C may competitively bind to the neuropilin receptor thought to underpin the effects of Sema3A and preclude its repulsive activity, permitting flexibility in the response to Sema3A (Takahashi et al., 1998). In the retina, Sema6A was shown to restrict dendrite arborization to the proper lamina of the inner plexiform layer through the Plexin-A4 receptors. Further, the class 5 semaphorins are important for the correct arborization of retinal ganglion cells (Matsuoka et al., 2011). Lastly, a number of semaphorins have demonstrated roles in synapse specificity, plasticity and signaling. In the spinal cord and striatum, Sema3E is important for the targeted formation of excitatory synapses (Pecho-Vrieseling et al., 2009; Ding et al., 2011), while Sema3F plays this role in the hippocampus and cortex. Indeed, SEMA3F KO

mice exhibit greater numbers of proximal dendritic spines in the DG (Tran et al., 2008). The bath application of Sema3F, which is secreted in vivo, in hippocampal slices leads to the increased frequency and amplitude of AMPAR-dependent miniature EPSCs (mEPSCs) in the DG, but only frequency—and not amplitude—increases in CA1 neurons (Sahay et al., 2005). Bath application of Sema3A, on the other hand, leads to dose-dependent decreases in the amplitude of CA1 EPSPs without altering presynaptic excitability or release probability (Bouzioukh et al., 2006). In the postnatal cerebellum, Sema3A knockdown (KD) leads to increases in synapse elimination and decreased amplitude of excitatory neurotransmission (Uesaka et al., 2014). Importantly for the present thesis, class 5 semaphorins localize to the postsynaptic compartments of postnatal cortical and hippocampal neurons (Duan et al., 2014; Inagaki et al., 2001). In cultured hippocampal neurons, the KD of Sema4B reduced both excitatory and inhibitory synapses, while the KD of Sema4D reduced only inhibitory synapse density, indicating the capacity of semaphorins to influence synapses of both major classifications (Paradis et al., 2007). The overexpression (OE) and KD of Sema5B also led to specific effects on excitatory synapse density (O'Connor et al., 2009), which shall be discussed in further detail in Section 1.7.6.

1.7.3 Signal Diversity in the Semaphorin Family

The various roles performed by the semaphorins likely rely on the composition of the different semaphorins, alternate splicing, location, timing of exposure, receptor expression and signaling pathways (for review, see Pasterkamp, 2012). The semaphorins are largely thought to signal through plexin receptors, though evidence exists for functional semaphorin-semaphorin interactions and the indirect activation of plexins through neuropilin co-receptors. The canonical semaphorin signaling pathway dictates that the binding of a semaphorin activates the GTPase-activating protein (GAP) domain of a plexin receptor, leading to the downstream activation of

protein kinases, GTPases and cytoskeleton-associated proteins (Janssen et al., 2010). Over nine plexins have been identified in the vertebrate genome: PLXNA1-4, PLXNB1-3, PLXNC1 and PLXND1. However, the functional consequences of semaphorin signaling depend upon their temporal and spatial patterns of expression, as well as the various responsive elements and associated signaling pathways (Pasterkamp, 2012). Further complicating the picture, these plexins can undergo heterodimerization with other plexins, likely contributing to signal diversity (Usui et al., 2003). Some semaphorins also exhibit bidirectional function, as aforementioned. For example, Sema5A is known to act as an attractive cue when axons express heparin sulphate proteoglycan alongside an as yet unidentified receptor subunit, while it acts repulsively when axons express condrotin sulphate proteoglycans (Kantor et al., 2004). Transmembrane semaphorins can also act as both ligands that initiate signaling and receptors that transduce semaphorin signals. For example, the receptor activity of Sema1A in Drosophila drives synapse formation and neurite targeting (Sweeney et al., 2011), which complements data demonstrating an association of cytosolic signaling proteins with membrane-associated semaphorins (Wang et al., 1999). Lastly, signaling diversity in the semaphorins may be partially mediated by cis inhibition. In the developing hippocampus, Sema6A is coexpressed alongside PLXNA2 in neurons of the stratum lucidum. This PLXNA2 receptor inhibits the repulsive signaling of Sema6A and thus permits the entry of PLXNA4-expressing mossy fibres into regions from which they'd otherwise be repulsed (Suto et al., 2007).



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Figure 1.3. Semaphorin classes and receptor subtypes. (a) Semaphorins are subdivided into 8 classes (SEMA1-7 and SEMAV) based on their extracellular motifs, type of membrane association and phylogenetic relationship. Class 1 and 2 semaphorins are found exclusively in invertebrates, while classes 3-7 are found in vertebrates and class V found exclusively in viruses. Some of these semaphorins are secreted, others are transmembrane, and others still are GPI-anchored. This protein family is defined by the presence of a Sema domain, while all vertebrate semaphorins contain a CRD. (Used with permission from Gherardi et al., 2004) (b) A number of receptors have been identified for the semaphorin proteins. The canonical signaling pathway involves the plexin receptors, which are further subdivided into four classes: A, B, C, and D. All plexin classes are transmembrane and similarly contain a sema domain capable of binding to all vertebrate semaphorin proteins. Neuropilins, on the other hand, are co-receptors for the class 3 semaphorins, while immune system associated proteins like Tim-2 and CD72 are known to respond to class 4 semaphorins. (Used with permission from Yazdani & Terman, 2006).

1.7.4 Class 5 Semaphorins

There are two class five semaphorins in vertebrates, Sema5A and Sema5B, which share 58% protein homology. The majority of their similarity resides in the sema domain, while their divergence is most obvious in cytoplasmic regions. The class five semaphorins are distinguished from the other semaphorin classes by the presence of seven thrombospondin repeats in the extracellular domain. During murine development, both Sema5A and Sema5B are expressed in a variety of tissues from E10 to birth in temporal and spatial patterns (Adams et al., 1996). Their expression patterns are non-overlapping in the spinal cord, suggesting the existence of distinct expression pathways (Masuda et al., 2014). In the adult, Sema5B expression is restricted to the brain, while Sema5A is present in various organs, including the brain (Adams et al., 1996).

1.7.5 Signaling Pathways of the Class 5 Semaphorins

The signaling pathways of the class five semaphorins have yet to be fully elucidated. However, some evidence from *in vitro* fibroblast studies suggests that Sema5A-PlexB3 interaction is functional and results in a collapsing response (Artigiani et al., 2004). Further, the proliferation and migration of endothelial cells may be regulated through the interaction of Sema5A and PlexB3 (Sadanandam et al., 2010). Nrp3 and PlexB3 are often co-expressed with Sema5A throughout development, putatively suggesting a role for this neuropilin co-receptor in Sema5A signaling also (Atigiani et al., 2004; Sadanandam et al., 2008). PlexA3 and Sema5A also demonstrate overlapping expression during motor neuron development in the Zebrafish (Hilario et al., 2009). However, another study in COS7 cells identified high affinity binding of Sema5A to PlexA1 and PlexA2, with minimal binding of Sema5A to PlexA3. Further, the KO of PlexA2, and not PlexA1, led to reductions in DG excitatory synapse density and sociability, akin to those observed in Sema5A KO mice (Duan et al., 2014). Thus, the repulsive axon guidance cue function of Sema5A and Sema5B may be mediated through PlexA1, PlexA2, PlexA3 or PlexB3, with potential for Nrp3 to act as a co-receptor.

1.7.6 Class 5 Semaphorins in the Nervous System

The class five semaphorins have also been implicated in a number of neurodevelopmental roles, including axon guidance, circuit development and synapse plasticity. For example, Sema5B acts to repel axons in the chick dorsal root ganglions (DRGs) both *in vitro* and *in vivo* (Liu et al., 2014), as well as corticofugal axons *in vitro* (Lett et al., 2009). Sema5A, however, functions bidirectionally. For example, the TSRs of Sema5A have been demonstrated to promote axon outgrowth in the ventral myotome (Hilario et al., 2009). Furthermore, these TSRs regulate the aforementioned proteoglycan-dependent responses in the developing rat midbrain (Kantor et

al., 2004) and can promote the exit of DRG sensory neurons towards their targets in the spinal cord. Conversely, in the notochord, Sema5A acts as a repellent, preventing the entrance of DRG axons (Masuda et al., 2014). The KO of Sema5A or Sema5B is also sufficient to cause aberrant dendrite arborization in retinal ganglion cells, amacrine cells and bipolar cells (Matsuoka et al., 2011). Lastly, the role of the class five semaphorins in synapse plasticity is just now being evaluated. In one study, using cultured hippocampal neurons, the overexpression of Sema5B led to a reduction in excitatory synapse density, while the short hairpin RNA (shRNA)-mediated knockdown of Sema5B led to increased excitatory synapse density. Using time-lapse imaging, O'Connor et al. (2009) further demonstrated that these alterations were due to synapse elimination and not a lack of synapse formation. Another study characterized increased dendritic spines and excitatory synapse density, but no changes to inhibitory synapse density, in the DG of Sema5A KO mice. Interestingly, the ablation of Sema5B expression in the DG did not lead to any spine or synapse density changes in this study (Duan et al., 2014).

1.8 Overall Objective of Chapter 3: SEMA5A

The overall objective of chapter 3 is to identify alteration to the cellular localization of Sema5A during activity-dependent excitatory synapse elimination.

1.9 Hypothesis: SEMA5A

We hypothesize that LTD enhances the trafficking of Sema5A to the cell surface, mediating the elimination of excitatory synapses.

1.10 PTEN and ASD

1.10.1 Association of PTEN with ASD

The third chapter of my thesis focuses on phosphatase and tensin homolog (PTEN). PTEN is an enzyme that exhibits both lipid and protein phosphatase activity and is an integral negative regulator of the PI3K/AKT pathway, as outlined in Figure 1.4 (for review, see Sansal & Sellers, 2004). The association of PTEN with ASD was first suggested when it was noted that germline PTEN mutations in individuals with PTEN-hamartoma tumour syndrome (PHTS), including Cowden's syndrome, often present with macrocephaly and a higher incidence of sporadic ASD (Butler et al., 2005; Blumenthal & Dennis, 2008). Further, 10-20% of ASD children develop macrocephaly and a preliminary study found that $\sim 17\%$ of children with both ASD and macrocephaly carry a PTEN mutation, suggesting a potential causal link between these factors (Lainhart et al., 1997; Butler et al., 2005; Fidler et al., 2000). Contemporarily, over 20 reports have implicated PTEN in ASD with 3 associated clinical CNVs and 57 rare human variants (SFARI Gene, 2017). In order to functionalize these mutations, over 21 transgenic mice have been generated, as well as a variety of other animal models (SFARI Gene, 2017; Halladay et al., 2009). In mice, the deletion of PTEN is embryonically lethal (Backman et al., 2001; Di Cristofano et al., 1998), but the selective KO of PTEN in postmitotic neurons of the cortex leads to macrocephaly, behavioural inflexibility, social behavior deficits, anxiety-like behaviours and seizures, recapitulating many of the neurophenotypes and behaviours observed in ASD patients (Jiang et al., 2005). Germline PTEN mutations are present in 1-5% of the total ASD population (McBride et al., 2010). However, due to their heavy reliance on WES, this data does not factor in mutations present in introns or the promoter region, so this is likely a conservative estimate. Further, blood is often used as the source material in these studies and thus fails to capture brainspecific regulation of PTEN via either genetic or epigenetic modulation (Buxbaum et al., 2007; Zhou & Parada, 2012). Most germline PTEN mutations are point mutations, some of which had been previously identified in PHTS and are known to alter lipid phosphatase activity (Endersby & Baker, 2008; Zhou & Parada, 2012). For example, in UU87MG glioblastoma cells, the H93R point mutation reduced PTEN membrane binding and phosphatase activity (Redfern et al., 2010). Interestingly, a comprehensive study using a humanized yeast bioassay revealed that most purely ASD-associated PTEN mutations do not substantially reduce the lipid phosphatase activity of PTEN and often have less severe biochemical effects than PHTS-associated PTEN mutations (Rodriguez-Escudero et al., 2011). This data was consistent with the absence of a correlation between PTEN-associated ASD and tumour-related PTEN mutations (Zhou & Parada, 2012).

1.10.2 Signaling Pathways of PTEN

PTEN is expressed at high levels in many tissues, including the CNS, skin and thyroid in humans, and ubiquitously transcribed in the mouse embryo (Gimm *et al.*, 2000; Luukko *et al.*, 1999). The lipid phosphatase activity of PTEN counteracts phosphoinositide 3-kinase (PI3K) activity by dephosphorylating phosphatidylinositol (3,4,5)-trisphosphate (PIP₃) to phosphatidylinositol 4,5-bisphosphate (PIP₂), resulting in a decrease in the downstream activation of protein kinase B (AKT). The PI3K/AKT pathway is involved in cell growth, cell survival, cell proliferation, LTP, protein synthesis and microtubule restructuring (Sansal & Sellers, 2004; Manning & Cantley, 2007). It is perhaps unsurprising then that PTEN is classified as a tumor suppressor gene and inactivation of this gene has been observed in a broad range of human cancers (Sansal & Sellers, 2004). Further, various germline mutations of PTEN are known to increase the risk of neurological disorders, such as macrocephaly, ASD and epilepsy (Endersby & Baker, 2008). There are two major pathways thought to underlie many of the

cellular functions of PTEN: the GSK3 and mechanistic target of rapamycin (mTOR) complex pathway. In the former pathway, AKT activation leads to the phosphorylation of GSK-α and GSK-β at serine 21 and serine 29 respectively, thus inactivating their kinases (Gartner et al., 2006; Zhou & Parada, 2012). This GSK inactivation reduces the phosphorylation of microtubulebinding proteins, which is essential for axon formation in the neurite tip and associated with neurogenesis, neuronal migration and neuronal polarity (Jiang et al., 2005; Gartner et al., 2006; Hur & Zhou, 2010). In the latter pathway, activation of AKT inhibits the tuberous sclerosis 1 and 2 (TSC1/TSC2) complex. This complex typically inhibits Rheb, which in turn activates mTOR complex 1, which is comprised of mLST, mTOR and Raptor (Manning & Cantley, 2007). This signaling pathway regulates cell growth by controlling protein synthesis (Zhou & Parada, 2012). In humans, the functional loss of TSC1 or TSC2 leads to tuberous sclerosis complex, a genetic disease that causes benign tumors to develop in the brain and other organs (Kwiatkowski & Manning, 2005). Autistic behaviors are observed in 25-50% of TSC patients (Wizniter, 2004). In mice, TSC1 or TSC2 KO in the developing brain leads to mTOR-dependent neuronal hypertrophy, mTOR-independent ectopic axon formation, macrocephaly and seizures (Meikle et al., 2007; Tavazoie et al., 2005). Further, TSC2^{+/-} mice exhibit elevated mTORC1 activity and deficits in learning and memory (Ehninger et al., 2008). Interestingly, the treatment of brainwide PTEN KO mice with an mTORC1 inhibitor known as rapamycin prevented and reversed the major neuronal changes associated with brain-specific PTEN loss and ameliorated PTENassociated ASD-like behaviors, suggesting that the mTORC1 pathway may underlie a lot of the neurological changes present in ASD patients with PTEN mutations (Zhou et al., 2009).

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PBD	Phosphatase Domain	C2 Domain	C- ⁻	^r ail <mark>P</mark> DZ-BD
1 12 22 38	65 78 92 101 118 130 139 157 167 176	5 188 202 211 241 252 274 285	320 335 349	403

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Figure 1.4. Linear protein structure of PTEN and PTEN signaling pathways. (a) PTEN is an intracellular enzyme comprised of 403 amino acids, which can be divided crudely into five functional domains. Working from the N-terminal to the C-terminal, these domains include the PIP₂-binding domain (PBD), a phosphatase domain, a C2 domain, a carboxy-terminal tail and PDZ-binding domain (PDZ-BD). (b) PTEN signaling promotes the conversion of cytosolic PIP₃ to PIP₂, leading to a reduction in the PIP₃-dependent activation of AKT. AKT, in turn, regulates two important pathways through GSK3 and the mTOR complex. In the former pathway, AKT activation results in the phosphorylation of GSK3s, inactivating their kinase activity and reducing microtubule-binding protein associated axon formation, neurogenesis and neuronal migration. In the second pathway, AKT inhibits the TSC1/TSC2 complex, activating Rheb, which in turn activates the mTOR complex 1, comprised of mLST, mTOR and Raptor, leading to a reduction in protein synthesis and dependent cell growth. (Used with permission from Zhou & Parada, 2012).

1.10.3 PTEN in the Nervous System

The KO of PTEN in the whole murine brain leads to dramatic anatomical disruption, premature death and seizure activity (Backman et al., 2001). As such, these mice can only provide a degree of insight into gene function throughout neurodevelopment. Many of the subsequent models have subverted these problems by using conditional or regional KO, as well as shRNA-mediated knockdowns (Jiang et al., 2005; Zhou et al., 2009). For example, the KO of PTEN exclusively in NPCs leads to increased proliferation (Groszer et al., 2001), while PTEN KO specifically in the cortex and hippocampus of postmitotic neurons leads to decreased sociability, anxiety, macrocephaly, hypertrophy, increased excitatory synapse density, and seizures (Jian et al., 2005; Luikart et al., 2011). Further, the KO of PTEN in the DG of PTENflx/flx mice using Cre lentiviruses for 7-25 days from P1 leads to neuronal hyperexcitability, increased dendrite length, increased mushroom spine and filopodia densities, somal hypertrophy, exaggerated intrinsic electrophysiological properties and supernumerary dendritic protrusions (Williams et al., 2015). The targeted deletion of PTEN in developing neurons led to soma, dendrite and axon outgrowth. These alterations included increases in dendritic arborization, dendrite thickness and dendritic spine density, as well as the presence of ectopic axons and axonal projections (Kwon et al., 2006; Jaworski et al., 2005; Jiang et al., 2005). Neither the proliferation nor the hypertrophic effects, however, were observed in astrocytes devoid of PTEN, suggesting some effect of cell type on PTEN signaling (Chow et al., 2011). Further supporting this finding are studies wherein PTEN KO in neural progenitor cells

(NPCs) of the adult subventricular zone leads to constitutive neurogenesis (Gregorian et al., 2009), while PTEN deletion in the subgranular zone of the DG causes excessive self-renewal of NPCs initially, followed by preferential astrocytic over neuronal differentiation (Bonaguidi et al., 2011). This data further suggests that PTEN may have different effects in different cell populations, depending on developmental stage and specification. Lastly, PTEN^{+/-} mice develop macrocephaly over time and females exhibit impairments in sociability (Page et al., 2009). Interestingly, both of these phenotypes were enhanced in mice heterozygotic for both PTEN and Scl6a4, another ASD-associated gene, suggesting that PTEN may contribute as part of the multi-hit model proposed by modern genetic studies (Page et al., 2009; Zhou & Parada, 2012; Bourgeron, 2015).

1.11 Overall Objective of Chapter 4: PTEN

The overall objective of chapter 4 is to determine whether overexpression of ASDassociated PTEN SNVs in cultured hippocampal neurons disrupts soma size, dendrite morphology and synapse density.

1.12 Overall Objective of Chapter 4: PTEN

We hypothesise that a subset of ASD-associated PTEN SNVs result in the disruption of PTEN function and that expressing these variants in primary hippocampal cultures will perturb soma size, dendrite morphology and synapse density.

Chapter 2: Methods

2.1 DNA Constructs

Enhanced green fluorescent protein (EGFP)-pN1 was obtained from Clontech (6085-1). Human HA-Sema5A and human HA-Sema5B in the pDisplay vector were kind gifts from Dr. Timothy O'Connor (UBC; University of British Colombia, Vancouver, Canada). PSD-95-RFP (red fluroscent protein) in the PGW1 vector was a kind gift from Dr. David Bredt (University of California, San Diego, CA). pCAG-GFP was obtained from Addgene (11150) and was originally deposited by Dr. Connie Cepko (Harvard University, Cambridge, MA). Dr. Kurt Haas (University of British Colombia, Vancouver, Canada) excised the GFP of the pCAG-GFP construct and cloned in human PTEN (isoform 1; P60484-1) containing three N-terminal Human influenza hemagglutinin (HA) tags and five synonymous mutations to prevent shRNA binding. The following synonymous mutations were introduced: 573 GTG>GTT, 579 CTG>CTA, 585 TTT>TTC and 591 AAG>AAA. Henceforth, PTEN* shall refer to the shRNA-resistant form of human PTEN containing these five synonymous mutations. Site-directed mutagenesis was then performed by Dr. Kurt Haas (University of British Colombia, Vancouver, Canada) to generate various SNVs in PTEN*: K6E, K6I, P38H, H123Q, C124S, R130L, T131I, G132D, T167N, and P354Q. These PTEN* plasmids were then kindly gifted to us by Dr. Kurt Haas (University of British Colombia, Vancouver, Canada). The dual-promoter shRNA vector pLL3.7 was obtained from Addgene (11795) and originally synthesized by Dr. Luk Parijs.

2.2 shRNA Cloning

The rat PTEN and scramble shRNA sequences used were obtained from Qiagen (KR47268G) and the following oligos were synthesized as duplexes by Integrated DNA Technologies (IDT):



Figure 2.1. Duplexed oligos used for shRNA cloning. The duplexed oligos used to generate the rat PTEN and scrambled shRNA plasmids are shown as linear DNA sequences with one blunt end and one sticky end. Once inserted and expressed by the pLL3.7 vector, the shRNA will fold at the loop site and bind to itself, forming a hairpin structure. Processing of this shRNA into an siRNA capable of targeting mRNA for degradation subsequently occurs. The thymine nucleotide required to complete the U6 promoter of the pLL3.7 vector after HpaI and XhoI double digestion is shown in red, while the shRNA sequences are shown in green. The underlined region of the shRNA sequence indicates the target sequence. The PTEN shRNA is designed against rat PTEN, while the scramble shRNA corresponds to no cDNA sequences present in the rat genome. The loop sequence is indicated in magenta, the transcription termination sequence is shown in cyan and the XhoI overhang site is depicted in purple. PTEN, phosphatase and tensin homolog; shRNA, short hairpin RNA.

The pLL3.7 vector was then cut with HpaI and XhoI (New England Biolabs) before the shRNA sequences were ligated into the vector using T4 DNA ligase as per the manufacturer's instructions (New England Biolabs). Thus, dual-promoter pLL3.7 plasmids were generated with EGFP under the CMV promoter and either the scramble or the rat PTEN shRNA under the U6 promoter.

2.3 Cell Culture and Transfection

Primary hippocampal dissociated cell cultures were prepared from E18.5 Sprague-Dawley rats, as per previously published protocols (Xie *et al.*, 2000). In short, hippocampi were dissected, incubated with 0.25% trypsin for 20 minutes, incubated with 0.05% DNase for 3 minutes and dissociated by trituration. Cells were plated at a density of either (i) 75,000 cells/coverslip on poly-L-lysine coated glass coverslips for immunocytochemistry, or (ii) 250,000 cells/well in a 6-well plate for biotinylation. Cells were allowed to adhere in plating media consisting of minimum essential media (MEM) supplemented with Pen/Strep, 10% (vol/vol) heat-inactivated fetal bovine serum, GlutaMAX, sodium pyruvate, and 0.5% glucose. After three hours, the plating media was removed by suction and replaced with maintenance media consisting of neurobasal media supplemented with NeuroCult SM1 (replaces B27 in the original protocol), GlutaMAX and Pen/Strep. Cultures were maintained in an incubator at 37°c and 5% CO₂. Neurons were subsequently transfected using Lipofectamine 2000, as per the manufacturer's protocol, at either day in vitro (DIV) 7 or DIV10, and used for experiments on DIV13-14.

2.4 Neuronal Stimultion: cLTP and cLTD

Neuronal activity and synapses were amplified by a previously described chemical LTP (cLTP) protocol (Lu *et al.*, 2001). In which, on DIV13, the maintenance media is replaced by Mg^{2+} -free extracellular solution (Mg^{2+} -free ECS; 140mM NaCl, 5.4mM KCl, 1.3mM CaCl₂, 25mM HEPES, and 33mM D-Glucose, pH 7.35) with 0.5µM tetrodotoxin (Alamone Labs) and 20µm bicuculline methiodide (Fluka BioChemika). The cells were incubated with this solution for 20 minutes before being replaced with Mg^{2+} -free ECS supplemented with 200µM glycine (Roche) for three minutes to chemically simulate LTP. Cells were then washed with Mg^{2+} -free ECS and allowed to incubate at 37°c with 5% CO₂ for 30 minutes before being used for experimentation. Control cells were subject to the same number of washes using the same stock solutions as the experimental group, but were not exposed to glycine during the 3-minute incubation.

Similarly, neuronal activity and synapses were dampened by a previously described chemical LTD (cLTD) protocol (Lee *et al.*, 1998). Briefly, DIV13 neurons are washed twice with Mg^{2+} -free ECS before being incubated with Mg^{2+} -free ECS with 20µM NMDA (Abcam) and 10µM glycine (Roche) for 3 minutes to chemically simulate LTD. Following incubation, the cells were washed for a final time with Mg^{2+} -free ECS and then incubated for 15, 30, 45 or 60 minutes as indicated in ECS containing 2mM MgCl₂ before being used for experimentation. As before, the control cells were exposed to the same number of washes using the same stock solutions as the experimental groups. However, no glycine or NMDA was present during the 3-minute incubation.

2.5 Immunocytochemistry

Neurons were fixed for 10 minutes in 4% paraformaldehyde (PFA) with 4% sucrose in 1x phosphate buffered saline (PBS; 137mM NaCl, 2.7mM KCl, 4.3mM Na₂HPO₄, 1.4mM KH₂PO₄ in H₂O). Then, the membranes were either permeabilized using 0.1% Triton-X/PBS or washed in 1X PBS for 10 further minutes. Blocking was performed to prevent nonspecific antibody binding with 10% goat serum in PBS for 1 hour at room temperature with gentle rocking. Primary antibodies were diluted in 1% goat serum in PBS and were applied, after blocking, overnight in the dark at 4°C with gentle rocking. The primary antibodies used included rabbit anti-HA (Cell Signaling Technology; C29F4), mouse anti-PSD-95 (Abcam; ab2723), rabbit anti-VGAT (Synaptic Systems; 131 003) and mouse anti-gephyrin (Synaptic Systems; 147011). The following day, the coverslips were washed three times for 10 minutes in 1X PBS before being incubated with secondary antibodies in the dark for 1 hour at room temperature with gentle rocking. The secondary antibodies were also diluted in 1% goat serum in PBS and include goat anti-mouse IgG₁-647 (Life Technologies; A21240), goat anti-mouse IgG_{2a}-568 (Life Technologies; A21134), goat anti-rabbit-405 (Life Technologies; A31556) and goat anti-rabbit 633 (Life Technologies; A21070). After secondary antibody incubation, another three 10-minute washes were performed with 1X PBS. The coverslips were then mounted onto microscope slides with ProLong Gold Anti-Fade Reagent (Molecular Probes).

2.6 Image Acquisition

Neurons were imaged on an Olympus Fluoview 1000 inverted confocal microscope using both 20x and 60x oil-immersion objectives. The acquisition parameters were set using the control cells for any given experiment and kept consistent throughout imaging within

experimental replicates. All cells were imaged within 3-4 days of setting the acquisition parameters.

2.7 Biotinylation

For the biotinylation experiments, cLTP and cLTD was performed as previously described. 30 minutes post-induction, the neurons were washed with ice-cold PBS-CM (0.1mM CaCl₂ and 1mM MgCl₂ in 1X PBS, pH 8) before being incubated at 4°c with gentle rocking with 0.5mg/ml NHS-SS-Biotin in ice-cold PBS-CM. After incubation, the cells were washed again with PBS-CM and any unbound biotin quenched with two seven-minute incubations with quenching buffer (20mM glycine in PBS-CM). Lysis was then performed using mechanical scraping in lysis buffer (Roche cOmplete protease inhibitor tablets, 1% IGEPAL-CA630 and 1mM PMSF) before being spun down at 500 x g for 5 minutes at 4°c. The samples were then vortexed, run through a 26 1/2 gauge syringe and left at 4°c to nutate for 30 minutes, before being spun down at 16,000 x g for 30 minutes at 4°c to clear the cell lysate. Protein quantification was then performed on the cell lysates using a BCA assay kit (Thermo Scientific) as per the manufacturer's instructions. 10 µg of each whole cell lysate sample was then combined with SDS-sample buffer (50mM Tris-HCl, 2% SDS, 10% glycerol, 14.5mM EDTA and 0.02% bromophenol blue with 1% β-mercaptoethanol) before being boiled at 95°C for 5 minutes and stored at -20°C until the following day.

100µg of the remaining protein sample would be added to 50ul/condition of a 50% slurry of Neutravidin-conjugated agarose beads (Thermo Scientific) that were pre-washed three times with lysis buffer. Every condition was topped up to 500uL with additional lysis buffer to facilitate movement of the beads and then the mixture was placed on a nutator at 4°C overnight.

The following day, the beads were pelleted and washed seven times by spinning at 500 x g for 3 minutes and discarding the supernatant. Once complete, the beads were dried by pipetting and 40μ L SDS-sample buffer (as before, but with 100mM DTT instead of β -mercaptoethanol) added to elute the beads. The samples were boiled at 80°C for 10 minutes and then incubated at room temperature before being run on a Western blot alongside the whole cell lysates from the day before.

2.8 Western Blots

9% SDS-PAGE Acrylamide-Bis resolving gels (9% Acrylamide/Bis, 0.374M pH 8.8 Tris, 0.1% SDS, 0.05% TEMED and 6% APS) with 4% stacking gels (4% Acrylamide/Bis,) were generated to run the whole cell lysate alongside the biotinylated fractions. The samples were run at 70V for 30 minutes and then, once through the resolving gel, switched to 120V for 1 hour in 1X running buffer (192mM glycine, 25mM Tris and 0.1% SDS). Gels were then wet transferred to 0.2µM pore PVDF membrane—pre-activated in methanol—for 12 hours at 25V in 1X Transfer Buffer (192mM glycine and 25mM Tris). Successful transfer was checked with 0.4% Ponceau-Red staining before blocking in 5% BSA in 1X TBST (20mM, 150mM, 0.1% Tween-20, pH 7.6). Rabbit Sema5A antisera (gifted to us by Dr. Alex Kolodkin; Johns Hopkins University, Baltimore, MD) was added 1:50 to the blocking solution and allowed to incubate at 4°C overnight with gentle rocking. The following day, the antibody was maintained at 4°C for subsequent use and the membranes washed with 1X TBST three times for 10 minutes. Then, the goat anti-rabbit HRP-conjugated secondary antibody was applied in 1X TBST for 1 hour at room temperature with gentle rocking. Another three 10-minute washes were performed with 1X TBST before the membrane was transferred to 1X PBS for imaging. A chemiluminescent

substrate kit (Thermo Fisher Scientific) was used as per the manufacturer's instructions for visualization and all exposures and imaging was performed using the Gel DocTM XR+ Gel Documentation System or the C-DiGit Blot Scanner with Image StudioTM Lite software.

2.9 Synapse Density Analysis

For the HA-Sema5A localisation experiments, neurons were co-transfected at DIV10 with pCMV.GFP, PSD-95-RFP and human HA-Sema5A. Chemical LTD, or a comparable number of washes for the control condition, was used to stimulate these neurons at DIV13 and cells were fixed 15 minutes, 30 minutes, 45 minutes or 60 minutes post-cLTD induction. These non-permeabilized neurons were fluroscently immunolabeled with an antibody raised against HA to identify surface membrane-localised exogenous HA-Sema5A. Whereas in the Sema5A overexpression experiments looking at inhibitory synapses, neurons were co-transfected at DIV10 with pCMV.GFP, pDisplay and human HA-Sema5A as indicated: Control — pCMV.GFP and pDisplay, and Sema5A overexpression — pCMV.GFP and human HA-Sema5A. These neurons were then fixed at DIV13, permeabilized and fluorescently immunostained with antibodies against VGAT and gephyrin to visualize excitatory synapses.

However, in the PTEN overexpression experiments, cells were co-transfected at DIV10 with combinations of pCAG.GFP and pCAG.HA₃-human PTEN* either with or without SNVs as indicated: Control — pCAG.GFP; WT overexpression — pCAG.GFP and pCAG.HA₃-WT PTEN*; and SNV overexpression (x10) — pCAG.GFP and pCAG.HA₃-SNV PTEN*. These neurons were subsequently fixed at DIV14, permeabilized and fluroscently immunostained with antibodies against PSD-95 to visualize excitatory synapses, gephyrin to visualize inhibitory synapses and HA to confirm exogenous PTEN expression.

Lastly, in the PTEN knockdown experiments, cells were co-transfected at DIV7 with combinations of either the scramble or the rat PTEN shRNA in the pLL3.7 vector and pCAG.HA₃-PTEN* either with or without SNVs as indicated: control — scramble shRNA; PTEN knockdown — rat PTEN shRNA; WT rescue — rat PTEN shRNA and pCAG.HA₃-WT PTEN*; and SNV rescue — rat PTEN shRNA and pCAG.HA₃-C124S PTEN*. Morphology of these neurons and confirmation of shRNA expression was visualized using the GFP present under the CMV promoter in the dual-promoter pLL3.7 vector. These neurons were subsequently fixed on DIV14, permeabilized and fluroscently immunostained for PSD-95 to visualize excitatory synapses and HA to confirm exogenous PTEN expression.

In order to focus explicitly on synapses in the sparsely transfected hippocampal neurons, Adobe Photoshop CS6 was used to create masks of the dendrites using the 60x GFP cell fill image. In doing so, the axon and soma of the imaged cell is removed, as well as any neurites of unknown origin or from neighboring transfected cells. Using the magic wand tool noncontiguously at a tolerance of 30, the black background was selected and the dendrite mask then pasted onto the protein marker images (VGluT1, PSD-95, PSD-95-RFP, HA-Sema5A, gephyrin and VGAT) to limit visualized staining only to dendrites in the neuron of interest.

These 60x images were then opened in ImageJ software, converted to 8-bit and thresholded. During the Sema5A experiments, an average threshold for every protein layer was set within each culture using the control neurons and then applied uniformly to every condition within that culture and experiment. However, during the PTEN experiments, noise was reduced in the images using the Subtract Background tool in ImageJ with a 10-pixel rolling ball radius. Images were then individually thresholded for accurate assessment of puncta, regardless of minor fluctuations in staining intensity or focus between images. Puncta for all experiments were

defined as between $0.05\mu m^2$ and $3\mu m^2$. Quantification of individual puncta density, integrated density and the colocalisation of presynaptic and postsynaptic synapse markers were performed using the ImageJ Colocalization plugin (available here:

https://imagej.nih.gov/ij/plugins/colocalization.html). Only puncta that overlapped by more than 4 pixels and shared >50 intensity ratio between the two channels were considered colocalised by this software.

Subsequently, the masked 8-bit GFP images were opened in ImageJ through the NeuronJ plugin (available here: <u>http://imagej.net/NeuronJ</u>). The Add Dendrites tool of this plugin was then used to trace the dendrites of each image and provide a measure of the total length of all the dendrites remaining in the image after masking. The total number of puncta, derived from the aforementioned Colocalisation software, was then divided by the total length of the dendrites for each cell and represented in graphs as a measure of length (density, or number of puncta/µm) and thus average distribution along the dendrites.

2.10 Western Quantification

Western blot exposure images were opened as 300dpi tiffs in ImageJ and converted to 8bit. The Rectangular selection tool was used to highlight our bands of interest within a horizontal region of interest (ROI). Then, Analyze > Gels > Select First Lane was selected before plotting the lanes as an intensity profile. The Straight tool was then used to remove the background noise and separate the intensity peaks produced by our various lanes. The Wand tool was used to individually select the area beneath the peaks. These area measurements were then transferred to Excel. Due to the qualitative nature of Western blots, within each blot, the control intensity or peak area was considered 1 and the resulting conditions normalized accordingly.

2.11 Total Dendrite Length Analysis

Adobe Photoshop CS6 was similarly used to create masks of the neurons using the 20x GFP cell fill image. In doing so, the axon of the imaged cell is removed, as well as any surrounding neurons. This masked image was then opened in ImageJ, converted to 8-bit, saved and then opened in ImageJ again through the NeuronJ plugin. The Add Dendrites tool was then used to trace the dendrites of that neuron. As the entire dendritic arbor can be captured at 20x, this protocol provides the total dendrite length of a given neuron in µm.

2.12 Soma Size Analysis

Adobe Photoshop CS6 was also used to create soma masks using the 20x GFP cell fill image. In doing so, all neurites are removed from the image, as well as any surrounding neurons, leaving only the soma of the neuron of interest. The masked image was then opened in ImageJ, converted to 8-bit and thresholded. Analyze > Measure was then selected and the somal area (in μm^2) of each neuron individually calculated.

2.13 Statistical Analysis

All data values are expressed as means ± SEM. In the immunocytochemistry experiments, the 'n' specified represents the number of cells in each condition, while the 'n' for the biotinylation experiment refers to the number of cultures. Raw data handling and number crunching was performed in Microsoft Excel, and then copied into Graphpad Prism 5 for statistical analysis. Statistical significance was determined using one-way analyses of variance (ANOVAs) with either Bonferroni or Sidak's post-hoc tests, as indicated in the figure legends. Statistical significance was defined as P<0.05 and P-values are indicated in figures as: * = p<0.05, ** = p<0.01, *** = p<0.001, and **** = p<0.0001, or # = p<0.05, ## = p<0.01, ### = p<0.001, and #### = p<0.0001.

Chapter 3: Results - Sema5A

3.1 Class 5 Semapohrins and Inhibitory Synapses

Sema5B, a 58% homologous protein to Sema5A, was previously demonstrated by O'Connor et al. (2009) to cause synapse elimination in cultured hippocampal neurons. Our group—prior to me joining the collaboration—had similarly demonstrated that the overexpression of Sema5A in cultured hippocampal neurons resulted in a significant decrease in excitatory synapse density (data not shown; Rachel Gomm-Kalisko, Bamji Lab, UBC), as assessed by VGluT1/PSD-95 colocalisation. This work was performed as part of a collaborative effort between the Bamji (Riki Dingwall & Rachel Gomm) and O'Connor (Wei Xiao) labs at UBC. Thus, this chapter shall focus on my contribution to the study and highlight the work performed by others only when necessary to justify hypotheses and conclusions. For example, I demonstrated that no significant decreases were observed in inhibitory synapses—assessed by VGAT/gephyrin colocalisation—when overexpressing Sema5A (Figure 3.1a). Further, the shRNA-mediated knockdown of Sema5A led to an increase in excitatory synapse density, which did not demonstrate additive or synergistic effects when Sema5B was concurrently knocked down (data not shown; Wei Xiao, O'Connor Lab, UBC). Though Sema5A is a transmembrane semaphorin and likely not secreted in vivo, bath application of a soluble form of Sema5A (Sema5A-Fc) was sufficient to cause reductions to PSD-95-RFP density and integrated density in a time-dependent manner (data not shown; Wei Xiao, O'Connor Lab, UBC). Taken together, these results indicate that Sema5A can specifically reduce excitatory synapse density, likely through synapse elimination, as opposed to a deficiency in synapse formation.


Figure 3.1. Sema5A or Sema5B overexpression did not alter inhibitory synapse density. (a) Quantification of the colocalised VGAT and gephyrin puncta density, referred to as inhibitory synapse density, in cells overexpressing GFP only, Sema5A, or Sema5B. Control, Sema5A OE, Sema5B OE, n = 34, 37, 34 respectively, in three separate cultures; one way-ANOVA p>0.05. n.s. = non-significant.

3.2 Surface Localisation of Exogenous Sema5A Post-cLTD

The aforementioned bath application of Sema5A-Fc resulted in the rapid elimination of excitatory synapses in a manner consistent with synapse elimination during LFS or chemically induced LTD. Further, the shRNA-mediated KD of Sema5A prohibited the elimination and formation of excitatory synapses typically observed five hours post-cLTD and post-cLTP respectively (Figure 3.2; Rachel Gomm-Kalisko, Bamji Lab, UBC; Wei Xiao, O'Connor Lab, UBC). This led us to hypothesize that cLTD induction may cause the elimination of synapses through Sema5A.



Figure 3.2. Knockdown of Sema5A precludes LTD-mediated synapse elimination and LTP-mediated synapse formation. (a) Quantification of excitatory synapse density (assessed by VGluT1/PSD-95 colocalisation) in DIV13 neurons expressing either empty shRNA vector controls or a Sema5A shRNA from DIV10. These neurons were then stimulated with 200 μ M glycine, fixed five hours later and immunostained for VGlut1 and PSD-95 to quantify excitatory synapses. The GFP present in the shRNA plasmid permitted the masking of dendrites, such that synapses being formed only on the transfected neuron could be assessed. n = 21-29 cells in three separate cultures; one-way ANOVA p<0.001, with post-hoc Bonferroni's test: *** = p<0.001 compared to the control -cLTP condition. n.s. = not significant. Black line denotes statistical comparison between Sema5A KD neurons with and without cLTP

stimulation. (b) Similarly, the second figure shows quantification of excitatory synapse density (assessed by VGluT1/PSD-95 colocalisation) in DIV13 neurons expressing either empty shRNA vector controls or a Sema5A shRNA from DIV10. These neurons were then stimulated with 10 μ M glycine with 20 μ M glycine and then fixed, immunostained and masked as before. n = 23-61 cells in 3-5 separate cultures; one-way ANOVA p<0.0001, with post-hoc Bonferroni's test: ** = p<0.01, *** = p<0.001 compared to the control -cLTD condition. n.s. = not significant. Black line denotes statistical comparison between Sema5A KD neurons with and without cLTD stimulation.

Coupled with the low levels of Sema5A present at the surface membrane under basal conditions (Duan et al., 2014), we further hypothesized that cLTD may alter the localization of Sema5A, specifically by targeting it to the surface membrane. In order to probe these hypotheses, we first validated the previously published cLTD protocol in our system. This induction protocol relies on the low-frequency activation of NMDARs by low concentrations of NMDA and glycine to internalize AMPARs. In order to quantify the surface membrane-localised AMPARs, a GluA1 construct with an extracellular super ecliptic pHluorin tag (SEP; SEP-GluA1) was transfected into neurons and live-imaged post-cLTD every 5 minutes for one hour. Due to the pH sensitivity of the tagged flurophore, the relative intensity of intracellular versus extracellular GluA1 allowed for visualization and quantification of AMPAR internalization. A significant decrease in density and integrated density of SEP-GluA1 puncta was observed 60 minutes post-cLTD induction (data not shown; Rachel Gomm-Kalisko, Bamji Lab, UBC). We thus determined that this chemical method of LTD induction recapitulated some of the molecular changes observed with LTD *in vivo* and was suitable for assessing synapse elimination in our system. We then subsequently performed a quantitative polymerase chain reaction (qPCR) for Sema5A and observed a two-fold increase in mRNA expression 2 and 4 hours post-cLTD (data

not shown; Rachel Gomm-Kalisko, Bamji Lab, UBC). Furthermore, I overexpressed HA-Sema5A in hippocampal neurons, performed cLTD and fixed neurons 15 minutes, 30 minutes, 45 minutes or 60 minutes post-induction. As the HA tag is expressed on the N-terminus of Sema5A and thus is exposed to the extracellular matrix when inserted into the cell surface membrane, a non-permeabilized immunocytochemistry experiment could be performed to quantify surface membrane-localized Sema5A. A significant increase in the surface localization of HA-Sema5A, as assessed by dendritic puncta density, was observed 30 minutes post-cLTD, which was no longer statistically noticeable by 60 minutes post-cLTD (Figure 3.3a-b). Further, the insertion of HA-Sema5A occurred before increases in mRNA and thus likely represents trafficking as opposed to increases in gene transcription. The surface membrane insertion of Sema5A also coincided with a significant decrease in PSD-95-RFP integrated density and a trending but not statistically significant decrease in PSD-95-RFP density (Figure 3.3c-d), suggesting that Sema5A has the temporal salience to elicit excitatory synapse elimination. A live-imaging experiment of Sema5A-SEP also demonstrated a significant increase in surface membrane insertion at 30 minutes post-cLTD that was not significantly different from controls at 60 minutes post-cLTD. Like the fixed data, the insertion was generalizable to the entire surface membrane and not specific to synaptic—PSD-95-RFP associated—or extrasynaptic—non-PSD-95-RFP associated—locations (data not shown; Rachel Gomm-Kalisko, Bamji Lab, UBC).



Figure 3.3. Exogenous Sema5A is inserted into the surface membrane 30 minutes post-cLTD. (a) DIV13 hippocampal neurons overexpressing HA-Sema5A, PSD-95-RFP and GFP were stimulated with either control treatment or 20 μ M NMDA and 10 μ M glycine to chemically induce LTD. 15, 30, 45 and 60 minutes post-cLTD, these neurons were fixed and immunostained for extracellularly exposed HA epitopes, which are indicative of surface membrane inserted exogenous HA-Sema5A. GFP images were used to mask the HA and PSD-95-RFP images to assess density changes associated solely with the transfected neuron of interest. Scale bar = 5 μ m. (b) Quantification of dendritic HA puncta density (surface-bound HA-Sema5A) in cells overexpressing Sema5A at five time points post-cLTD, or with control treatment. n = 20-26 cells in three separate cultures; one-way ANOVA p<0.01, with post-hoc Bonferroni's test: ** = p<0.01 compared to HA-Sema5A overexpressing cells with control treatment. n = 20-26 cells in three separate cultures; one-way ANOVA p>0.05. n.s. = not significant. (d) Quantification of PSD-95-RFP integrated density 15, 30, 45 or 60 minutes post-cLTD, or with control treatment. n = 20-26 cells in three separate cultures; n = 20-26 cells in the separate cultures; n = 20-26 cells in three separate cultures; n = 20-26 cells in three separate cultures; n = 20-26 cells in three separate cultures is n = 20-26 cells in three separate cultures; n = 20-26 cells in three separate c

three separate cultures; one-way ANOVA p<0.01, with post-hoc Bonferroni's test: * = p<0.05 compared to HA-Sema5A overexpressing cells with control treatment.

3.3 Surface Localisation of Endogenous Sema5A Post-cLTD and Post-cLTP

Despite the insight into the localisation of Sema5A provided by these exogenous experiments, our primary interest was in the action of Sema5A endogenously. As a time point for capturing the surface membrane insertion of Sema5A had now been established, I performed a biotinylation experiment 30 minutes post-cLTD or post-CLTP, wherein all surface membrane proteins are tagged with biotin and the cells subsequently lysed. A fraction of the sample was retained for assessing whole cell Sema5A levels, while the rest was applied to Neutravidin beads, which bind to biotin and thus separate the biotinylated surface protein fraction. This surface fraction was then eluted and run alongside the whole cell lysate in a Western blot to assess qualitative differences in whole cell and surface Sema5A expression between control and 30minute post-cLTD or post-cLTP neurons. No significant differences were observed in the whole cell expression levels of Sema5A (Figure 3.4a-b), suggesting that total Sema5A expression in neurons had not altered 30 minutes post-cLTD or post-cLTP. However, significant differences were observed in the surface fraction of Sema5A post-cLTD compared to control (Figure 3.4ab). As previously reported, very little Sema5A was present at the surface membrane under control conditions. However, a four-fold increase in antibody reactivity was observed in the biotinylated fraction 30 minutes post-cLTD, suggesting that the transient surface localization of Sema5A also occurs endogenously and along a similar timescale.



Figure 3.4. Endogenous Sema5A is inserted into the surface membrane 30 minutes post-cLTD. (a) Western blots for the whole cell lysate and surface protein fraction, separated using biotinylation, from DIV13 hippocampal neurons are shown following antibody staining for endogenous Sema5A. Neurons were stimulated with either control, cLTP or cLTD treatment for 30 minutes before being biotinylated and lysed. (b) Quantification of the surface fraction of endogenous Sema5A 30 minutes after control, cLTP or cLTD treatment. n = three separate cultures; one-way ANOVA p<0.05 with post-hoc Bonferroni's test: * p<0.05. n.s. = not significant. Also shown, is the quantification of the whole cell protein levels of endogenous Sema5A 30 minutes after control. cLTP or cLTD treatment. n = three separate cultures; one-way ANOVA p>0.05. n.s. = not significant.

Overall, I demonstrated that the overexpression of Sema5A or Sema5B does not alter the density of inhibitory synapses. Further, both exogenous and endogenous Sema5A is trafficked to the surface membrane 30 minutes post-cLTD. This trafficking coincided with a decrease in the size of postsynaptic densities, which is often attributed to early synapse loss or weakening.

Chapter 4: Results - PTEN

4.1 Overexpression of ASD-associated PTEN* SNVs: Synapses

The overexpression of a GFP-tagged PTEN in neurons had previously been reported to decrease the density of dendritic spines, a morphological correlate of excitatory synapses (Zhang et al., 2012). Zhang et al. (2012) further demonstrated that these spine density decreases were phosphatase-dependent, as they were not observed in neurons overexpressing GFP-C124S PTEN, a phosphotase dead biochemical mutant of PTEN. Due to the greater than 85% colocalisation between VGluT1 and PSD-95, as well as between VGAT and gephyrin (data not shown; Jordan Shimell, Bamji Lab, UBC), single postsynaptic markers were used in our study as surrogates for excitatory and inhibitory synapses respectively. In doing so, the number of excitatory and inhibitory synapses being formed onto a single transfected neuron could be quantified simultaneously. As shown in Figure 4.1, a transfected cell fill was used to remove the axon and soma of the imaged cell, as well as any neurites of unknown origin or neighboring cells. The dendrite mask was then pasted onto the fluorescently immunostained images (for PSD-95, gephyrin and HA) to limit visualized staining only to dendrites in the neuron of interest. The remaining dendrites, after masking, were then traced and the total number of each puncta type divided by the total length of dendrite imaged, thus providing protein measures as densities, or average distribution throughout the dendritic arbor.



Figure 4.1. Immunocytochemistry image processing and analysis. The image processing and analysis of fixed DIV13-14 neurons after 60x (A) and 20x (B) confocal imaging are depicted. (**a**) A dendrite mask is generated using the cell fill layer and applied uniformly to all other layers. The synaptic layers undergo number and integrated density analyses, while the dendrites are traced. (**b**) A somal and dendritic mask is generated using the cell fill layer. The dendrites of this neuron are then traced to assess total dendrite length. Subsequently, the dendrites are removed, leaving just the soma. Somal area is then calculated to examine conditional alterations to soma size.

As shown in Figure 4.2, a number of SNVs in the shRNA-resistant human PTEN (PTEN*) sequence were generated, including eight ASD-associated SNVs, a phosphatase dead mutant, and a population-derived positive control.



Figure 4.2. Structure of human PTEN and location of ASD-associated SNVs. PTEN is made up of a number of functional domains, including the PDB, the phosophotaste domain, the C2 domain, the C-tail and the PDZ binding domain. The ASD-associated PTEN SNVs tested in the present study, indicated by blue lollipops, cluster in the PDB and phosphotase domains. These mutations include K6E, K6I, P38H, H123Q, R130L, T131I, G132D, and T167N. The green lollipop indicates the C124S mutation site within the amino acid code, which is a catalytically dead biochemical variant of PTEN. The location of a positive control SNV (P354Q) derived from a member of the general population through the ExAC Browser database is indicated by a purple lollipop. PIP2, phosphatidylinositol-4,5-bisphosphate; PBD, PIP2 binding domain. C-Tail, carboxyl-terminal tail. PDZ-BD, postsynaptic density protein, *Drosophila* disc large tumor suppressor, and zonula occludens-1 protein-binding domain.

The overexpression of human WT PTEN* or P354Q PTEN*—an ExAC-derived population control—in cultured hippocampal neurons resulted in a significant decrease in PSD-95 density compared to GFP-expressing controls (Figure 4.3a-4b), akin to the previously published dendritic spine decreases. However, no significant changes to gephyrin density were observed (Figure 4.3b-c). Similarly, these PSD-95 changes were phosphatase-dependent and thus not observed in neurons overexpressing the C124S phosphatase dead biochemical mutant of PTEN*. The majority of the ASD-associated PTEN* SNVs similarly overexpressed in neurons had PSD-95 and gephyrin densities statistically indistinguishable from controls (Figure 4.3a-c). suggesting they are loss-of-function (LOF), specifically the phosphatase function of PTEN and its regulatory influence upon excitatory synapses. These putative loss-of-function ASDassociated variants include K6E, K6I, P38H, R130L, T131I, G132D and T167N. However, the overexpression of one variant (H123Q)-perhaps surprisingly given that it occurs in the enzymatic pocket of PTEN, one amino acid away from C124S-produced a PSD-95 density significantly reduced from control neurons and not significantly different from human WT PTEN* overexpression, suggesting that the phosphatase activity of this variant is still intact. While this result does not preclude the variant from having other altered PTEN functions, these functional assessments allow the annotation of ASD-associated variants and may eventually contribute to phenotyping ASD patients and providing accurate prognoses with targeted, individualized therapies. Further, the selective effect of PTEN on excitatory synapses and not inhibitory synapses has the potential to disrupt the excitatory/inhibitory balance or excitability of these neurons, though it is difficult to derive electrophysiological conclusions from molecular staining alone.





dead C124S PTEN*, ASD-associated SNVs of PTEN*, or the population control P354Q PTEN*. n = 20-65 cells in 2-4 separate cultures; one-way ANOVA p>0.05.

4.2 Overexpression of ASD-associated PTEN* SNVs: Dendrite Length and Soma Size

Though PTEN is present in the soma and dendrites of mature neurons, their influence on total dendrite length and soma size had not yet been elucidated. We demonstrate that the overexpression of human WT or P354Q PTEN* from DIV10-DIV14 led to a significant reduction in total dendrite length and soma size compared to GFP controls (Figure 4.4a-c). These effects were again dependent on the phosphatase activity of PTEN and were thus not observed in the phosphatase dead mutant (C124S)-overexpressing condition. Further, the overexpression of K6I, R130L, T131I, G132D or T167N PTEN* in wild-type hippocampal neurons did not significantly alter total dendrite length or soma size, while the overexpression of H123Q PTEN* significantly reduced both, comparable to WT PTEN* overexpression. Interestingly, the overexpression of P38H PTEN* significantly reduced soma size compared to controls, while the overexpression of K6I PTEN* was not significantly different from controls or WT PTEN* overexpression, suggesting a mild though statistically indistinguishable reduction in soma size. Overall, the phosphatase activity of PTEN* seems to regulate dendrite outgrowth or maintenance, as well as soma size, and a number of likely gene disrupting ASD-associated PTEN* variants demonstrate a loss of these protein functions. Alterations to the dendritic arbor and somal volume have capacity to perturb electrophysiological output and neural networking in the brains of patients with ASD, though further testing would be required to establish if these ASD-associated PTEN SNVs produce these effects in vivo, particularly when expressed heterozygotically throughout development.

100µm Control Human WT C124S b С 1.5 1.5 #### ##### Total Dendrite Length ### ##### Normalised Soma Size #### #### #### ##### ##### ### ### Normalised 1.0 1.0 0.5 0.5



= Population Control

= ASD-Associated Variant

Figure 4.4. PTEN* overexpression reduces total dendrite length and soma area, while the overexpression of most ASD-associated SNVs does not. (a) Representative masked 20x confocal images demonstrating the dendritic and somal morphology of DIV14 neurons overexpressing GFP only, human WT PTEN* or phosphatase dead C124S PTEN* from DIV10. Scale bar = 100 μ m. (b) Quantification of total dendrite length (in μ m) for neurons overexpressing GFP only, wild type PTEN*, phosphatase dead C124S PTEN*, ASD-associated SNVs of PTEN*, or the population control P354Q PTEN*. n = 20-80 cells from 2-4 separate cultures; one-way ANOVA p<0.001, with post-hoc Sidak's test: * p<0.05, ** p<0.01, *** p<0.001, **** p<0.001 compared to GFP-expressing controls. # p<0.05, ## p<0.001, #### p<0.0001 compared to human wild type PTEN* overexpressing neurons. (c)

Quantification of soma size (in μ m²) for neurons overexpressing GFP only, wild type PTEN*, phosphatase dead C124S PTEN*, ASD-associated SNVs of PTEN*, or the population control P354Q PTEN*. n = 20-80 cells from 3-5 separate cultures; one-way ANOVA p<0.0001, with post-hoc Sidak's test: * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 compared to GFP-expressing controls. # p<0.05, ## p<0.01, #### p<0.001, #### p<0.0001 compared to human wild type PTEN* overexpressing neurons.

4.3 PTEN KD and Synapses

While any putative effects of PTEN SNVs in patients with ASD are observed despite the presence of one functional genetic copy of PTEN, another imperfect but informative way to elucidate the functional consequences of SNVs upon PTEN is through knockdown studies. In the present study, shRNAs designed against rat PTEN were expressed in wild-type neurons at DIV7. These shRNAs target PTEN mRNA for degradation and thus silence or 'knockdown' the expression of that gene. A scramble shRNA, on the other hand, is designed to target none of the genes present in a particular organism, but provides a suitable control for the stress placed on a cell by DNA loading and the transcription of constitutively active exogenous promoters. The phenotypes of these KD neurons, including cellular morphology and synapses, were then compared to scramble shRNA-expressing neurons at DIV14 and the function of that gene inferred from any observed cellular or molecular deficits. Human PTEN*-either with or without SNVs—can then be cointroduced to neurons during the shRNA-mediated genetic interference, such that the wild-type rat PTEN expression is silenced, while exogeneous human PTEN* is replacing or 'rescuing' this genetic loss. If indeed these neurons fail to develop the deficits associated with PTEN KD, the replacement PTEN* is said to sufficiently 'rescue' the KD, and demonstrates that the shRNA is specifically targeting PTEN and likely not having offtarget effects. If the exogenous expression of a PTEN* SNV within this context fails to rescue

the KD phenotype(s) or produces an enhanced phenotype compared to wild-type, it can be inferred that for that particular function, the PTEN SNV is either loss-of-function or gain-offunction, respectively. In doing so, an assay is generated for the functional annotation of PTEN SNVs.

Prior to our study, evidence for the role of PTEN in a range of neuronal phenotypes came from PTEN KO and conditional KO animals. For example, Williams *et al.* (2015) used the injection of Cre lentiviruses into the DG of PTEN flx/flx mice at P7 to elucidate the role of PTEN in mature neurons. This regional knockout of PTEN in neurons of the DG led to increased excitatory synapses, hyperexcitability, increased dendritic protrusions, a shift in excitatory synapse location and increased density of mushroom spines compared to GFP-expressing withinanimal controls. These phenotypic alterations were significant as early as 7.5 days after PTEN knockout and persisted until their final time point at 24.5 days post-injection.

In our study, the DIV7-DIV14 knockdown of PTEN in hippocampal neurons led to a significant increase in PSD-95 density compared to controls (Figure 4.5a-b). This result is comparable to the reduction in PSD-95 staining observed in PTEN overexpressing neurons and further highlights the role of PTEN at excitatory synapses. The human wild-type PTEN* rescue condition successfully ameliorated the PSD-95 density changes observed in the PTEN KD rat neurons, suggesting that rat and human PTEN have comparable activity profiles and the shRNA used specifically targeted rat PTEN mRNA. Attempting to rescue with the phosphatase dead mutant C124S PTEN*, however, failed to prevent PTEN KD-associated PSD-95 changes, indicating that these effects are once again dependent on the phosphatase activity of PTEN.



Figure 4.5. PTEN knockdown increases excitatory synapse density, which can be rescued by wild type PTEN, but not most ASD-associated PTEN SNVs (a) Representative images for DIV14 hippocampal neurons expressing a scramble shRNA ('Control'), a rat PTEN shRNA ('PTEN KD'), a PTEN shRNA and human wild-type PTEN* ('WT Rescue'), or a PTEN shRNA and phosphatase dead C124S PTEN* ('C124S Rescue') from DIV7 immunostained for endogenous PSD-95 and HA. Shown are 30µm masked segments of dendrite and the associated PSD-95 puncta and HA staining. Scale bar = 5μ m. (b) Quantification of dendritic PSD-95 puncta density (surrogate for excitatory synapses) in neurons expressing a scramble shRNA, a rat PTEN shRNA, a PTEN shRNA and wild-type human PTEN*, or a PTEN shRNA and the phosphatase dead C124S PTEN*. *. n = 8-10 cells in 1 culture; one way-ANOVA p<0.001, with post-hoc Sidak's test: * p<0.05, ** p<0.01, *** p<0.001 compared to scramble shRNA expressing controls. # p<0.05, ## p<0.01, ### p<0.001 compared to human WT Rescue neurons.

4.4 PTEN KD, Dendrite Length and Soma Size

Further, in the aforementioned PTEN KO study, Williams *et al.* (2015) also observed increased somal hypertrophy and increased dendrite outgrowth in PTEN KO neurons compared to controls from 7 days post-injection onwards. The shRNA-mediated knockdown of PTEN from DIV7-DIV14 in our dissociated hippocampal cultures led to a two-fold significant increase both in total dendrite length and soma size compared to scramble shRNA-expressing controls (Figure 4.6a-c), recapitulating the findings from Williams *et al.* (2015). The cotransfection of human wild type PTEN* was able to ameliorate the alterations in both total dendrite length and soma size, while the coexpression of the PTEN shRNA with the phosphatase dead biochemical mutant of PTEN* (C124S) was not able to rescue the knockdown phenotype. Thus, the effects of PTEN on dendritic outgrowth and soma size are dependent upon its phosphatase activity in both the overexpression and knockdown context.





size (in μ m²) for neurons expressing a scramble shRNA, a rat PTEN shRNA, a PTEN shRNA and wildtype human PTEN*, or a PTEN shRNA and the phosphatase dead C124S PTEN*. n = 12-20 cells from 2 separate cultures; one-way ANOVA p<0.001, with post-hoc Sidak's test: * p<0.05, ** p<0.01, *** p<0.001 compared to scramble shRNA controls. # p<0.05, ## p<0.01, ### p<0.001 compared to PTEN KD neurons.

Overall, I demonstrate that the overexpression of human wild type PTEN in rat hippocampal neurons leads to reductions in excitatory synapse density, total dendrite length and soma size. The overexpression of most ASD-associated PTEN SNVs did not cause alterations to any of these neurophenotypes, suggesting they are loss of function. Interestingly, I report that H123Q PTEN* phenocopied wild type PTEN across all measures, while P38H and potentially K6I PTEN* phenocopied wild type only in their ability to reduce soma size. This diversity of consequences among ASD-associated SNVs in the same gene highlights the importance of functionalizing genetic variants in biological systems. Further, I present a rat PTEN knockdown assay with the potential to probe human PTEN SNVs systematically in rat hippocampal neurons in a moderately high-throughput manner. Ongoing, a total of 22 stable ASD-associated SNVs are being tested in the overexpression context, and a number of them characterized also in the KD context.

Chapter 5: Discussion

5.1 Sema5A

This present study produced the novel finding that the localization of Sema5A is altered during NMDAR-LTD. Notably, we demonstrated that Sema5A is inserted into the dendritic surface membrane 30 minutes post-cLTD. Further, the overexpression of Sema5A or Sema5B in hippocampal neurons did not alter inhibitory synapse density, as assessed by VGAT/gephyrin colocalisation, demonstrating that the class 5 semaphorins selectively alter excitatory synapses (data not shown; Rachel Gomm-Kalisko, Bamji Lab, UBC; Wei Xiao, O'Connor Lab, UBC).

5.1.1 Class 5 Semaphorins do not Regulate Inhibitory Synapses

Previous studies had identified a role for Sema5A (Duan et al., 2014) and Sema5B (O'Connor et al., 2009) at mature excitatory synapses of the hippocampus, in addition to their previously elucidated developmental roles at other brain regions in axon guidance (Liu et al., 2014; Hilario et al., 2009; Kantor et al., 2004; Masuda et al., 2014) and dendritic outgrowth (Matsuoka et al., 2011). Duan et al. (2014) used both *in vitro* and *in vivo* methodologies to assess the dendritic and synaptic effects of class 5 semaphorins in DG cells of the hippocampus. They identified that the KO of Sema5A or Sema5B in mice produced no obvious maturation defects, such as patterning or dendrite outgrowth, in the hippocampus. However, the germline or conditional P15-P29 KO of Sema5A increased the spine density of DG, but not CA1, neurons by ~25%. P1-P2 cultures of these Sema5A^{-/-} DG neurons further demonstrated that these supernumerary spines were associated with a ~40% increase in PSD-95 density compared to Sema5A^{+/-} neurons, though no changes were observed in gephyrin density. This is in line with our findings that the shRNA-mediated KD of Sema5A in rat hippocampal neurons increased,

while the overexpression of Sema5A reduced, excitatory synapse density, as assessed by VGlut1/PSD-95 colocalization (data not shown; Wei Xiao, O'Connor Lab, UBC; Rachel Gomm-Kalisko, Bamji Lab, UBC). Similarly, in our study, the overexpression of Sema5A or Sema5B did not alter inhibitory synapses, assessed by VGAT/gephyrin colocalization. The sparse introduction of Sema5A by transfection into either Sema5A^{-/-} or Sema5A^{+/-} DG neurons demonstrated that these effects were cell autonomous and not reliant on *trans* interactions (Duan et al., 2014). Lastly, hippocampal slices from these mice identified an increase in mEPSC frequency and amplitude associated with Sema5A KO, suggesting these additional excitatory synapses are functional. These electrophysiological effects were ameliorated by bath application of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), an AMPAR antagonist, indicating a role for AMPARs at these excess synapses.

Interestingly, no alterations to spine density in the DG or CA1 of Sema5B^{-/-} mice were reported in the Duan et al. (2014) study. This is contrary to an *in vitro* study of hippocampal neurons performed by O'Connor et al. (2009), wherein the sparse DIV7-8 transfection of primary rat hippocampal cultures with GFP-Sema5B and PSD-95-RFP for 2-3 days led to a ~70% decrease in excitatory synapse density, assessed by PSD-95-RFP/synaptophysin colocalization. Transfection with GFP-Sema5B and synaptophysin-RFP, however, identified no deficits in the number of synapses formed by Sema5B overexpressing neurons compared to wild type controls, suggesting this effect is cell autonomous and postsynaptic. Lastly, the cotransfection of a Sema5B shRNA and PSD-95-RFP in hippocampal neurons led to an 18% increase in the number of PSD-95-RFP puncta associated with a synaptophysin puncta compared to wild type neurons. Further, O'Connor et al. (2009) reported a puncta size increase of 60-80% for PSD-95 and 50% for synaptophysin in Sema5B KD neurons compared to wild type controls. The discrepancy in the results could, however, emanate from a number of factors. For example, some alterations *in vivo* may not be observed *in vitro*. Furthermore, Duan et al. (2014) looked only at germline Sema5B KO mice, which may undergo compensatory mechanisms during development, and not specifically at the loss of Sema5B at mature synapses like O'Connor et al. (2009). Another potential source of divergence is the use of molecular synapse density versus morphological spine density to assess excitatory synapses. Finally, O'Connor et al. (2009) used a broad population of rat hippocampal neurons to elucidate the effects of Sema5B, while Duan et al. (2014) focused specifically on CA1 and DG neurons of the hippocampus in mice.

Combined and integrated, our results are consistent with Duan et al. (2009)'s finding that Sema5A acts as a negative modulator of synapse formation during development and postnatally (data not shown; Wei Xiao, O'Connor Lab, UBC; Rachel Gomm-Kalisko, Bamji Lab, UBC). These effects are specific to excitatory synapses and no such alterations were observed either at developmental Sema5A KO inhibitory synapses or at the inhibitory synapses of Sema5A overexpressing neurons. Follow up work using time-lapse imaging demonstrated that the Sema5A-mediated loss of mature excitatory synapses was due to increased synapse elimination and not reduced synapse formation (data not shown; Wei Xiao, O'Connor Lab, UBC).

5.1.2 Class 5 Semaphorins are Regulated by Activity

The LTD mediated surface membrane localisation of Sema5A is a novel finding of our study, though the regulation of other semaphorins by neuronal activity has been previously identified (Carrillo et al., 2010; Cheadle & Biederer, 2014). Initially in our study, we demonstrated that cLTD in hippocampal neurons increased the transcription of Sema5A and Sema5B two hours post-induction, suggesting a role for these genes in the maintenance of LTD (data not shown; Rachel Gomm-Kalisko, Bamji Lab, UBC). However, we also identified an

almost four-fold increase in the insertion of HA-Sema5A into the dendritic surface membrane of hippocampal neurons 30 minutes post-cLTD compared to baseline in Sema5A overexpressing neurons. This insertion occurred throughout the dendritic arbor and was not specifically associated with PSD-95-RFP (data not shown). Despite this, the membrane insertion of HA-Sema5A coincided with a significant ~50% decrease in PSD-95-RFP integrated density and a ~30% (statistically insignificant) decrease in PSD-95-RFP puncta. Furthermore, subsequent work using hippocampal neurons overexpressing SEP-Sema5A and PSD-95-RFP further elucidated the effects of NMDAR-cLTD on Sema5A localization. These aforementioned overexpressing neurons underwent cLTD and were imaged every 5 minutes for 2 hours. The pH sensitivity of the SEP tag allowed for the visual separation of extracellular and intracellular SEP-Sema5A localization by relative signal intensity due to the difference in pH between the intracellular and extracellular compartments. In doing so, we identified the trafficking of SEP-Sema5A to the dendritic membrane post-cLTD, which peaked at 30 minutes and returned to control levels by 80 minutes. Furthermore, this insertion was observed at both synaptic (PSD-95-RFP associated) and extrasynaptic (non-PSD-95-RFP associated) sites, supporting the suggestion that Sema5A is not specifically targeted to excitatory synapses, but to the surface membrane generally following LTD (data not shown; Rachel Gomm-Kalisko, Bamji Lab, UBC). Although the transcription of Sema5A was not increased by 30 minutes post-cLTD, it is not possible to rule out changes in protein synthesis using these methodologies. However, the use of plasmids with constitutively active exogenous promoters unlikely to be regulated by neuronal activity, as well as the exclusive tracking of tagged exogenous Sema5A, lends some credence to the hypothesis that these results represent the surface membrane targeting of existing Sema5A and not an increase in protein expression.

A major caveat of both of these localization experiments, however, is that they rely on the overexpression of Sema5A, which may alter the localization or activity of endogenous Sema5A. Thus, we followed up with a biotinylation experiment intended to look at the localization and protein expression of endogenous Sema5A after cLTD and cLTP. In this experiment, the surface proteins of wild type rat hippocampal neurons were biotinylated 30minutes after the chemical induction of NMDAR-LTD or -LTP. Whole cell lysates from these neurons and a pull down for the biotinylated surface fraction were run together on a Western blot and endogenous Sema5A detected using an antibody. In this way, we identified a four-fold increase in the surface localization of endogenous Sema5A 30 minutes post-cLTD compared to neurons that did not undergo cLTD or cLTP induction. No significant increase in Sema5A surface localization post-cLTP was observed compared to controls, suggesting this effect is specific to LTD and not generalizable to all neuronal activity. Furthermore, no increases in whole cell Sema5A protein levels was observed 30 minutes post-cLTD or -cLTP, indicating that detectable increases in protein synthesis do not occur and thus are not able to sufficiently explain the four-fold increase in surface localization of Sema5A post-cLTD. Interestingly, three bands were detected using polyclonal Sema5A antisera. The larger band of the three falls ~120kDa, the predicted molecular weight of rat Sema5A, suggesting that processing of the Sema5A protein may occur or that the antibody exhibits some non-specific binding.

5.1.3 Possible Mechanism of Sema5A Function

The signaling pathways involved in mediating the neuronal effects of Sema5A have not yet been fully elucidated. A number of plexins, the canonical semaphorin receptors, have been suggested to interact functionally with Sema5A as a ligand (Artigiani et al., 2004; Hilario et al., 2009; Matsuoka et al., 2011; Sadanandam et al., 2008; Sadanandam et al., 2010). Indeed,

Sema5A has a relatively small cytoplasmic domain with no known functional motifs, suggesting that Sema5A acts as a ligand more than as a receptor. Using COS7 cells, Duan et al. (2014) demonstrated the capacity of Sema5A to bind to PlexA1 and PlexA2, but not PlexA3, at low nanomolar concentrations. Moreover, Sema5A binding in the DG was greatly reduced in Plxna1⁻ ^{/-} mice and Plxna2^{-/-} mice, with almost no Sema5A binding observed in Plxna1^{-/-}:Plxna2^{-/-} double KO mice. In situ hybridization of the WT mouse hippocampus demonstrated that PlexA1, PlexA2 and PlexA3 are all expressed in distinct patterns. In the DG, PlexA2, but not PlexA1, is expressed throughout the synaptogenic period, while PlexA3 is only expressed in immature DG neurons and restricted to the SGZ in adolescence. Furthermore, fractionation analysis revealed that PlexA2 and A3 localizes to extrasynaptic sites of hippocampal neurons, while PlexA1 is enriched at the postsynaptic density. Thus, the effect of Sema5A at excitatory synapses of the DG is likely mediated through extrasynaptically localized PlexA2 receptors, which corroborates our hypothesis that the insertion of Sema5A throughout the dendrite 30 minutes post-cLTD mediates excitatory synapse elimination. Duan et al. (2014) went on to demonstrate that this is likely a *cis* interaction that requires the sema domain of Sema5A and the cytoplasmic domain of PlexA2. Importantly, the ablation of PlexA2 was able to ameliorate DG excitatory synapse elimination caused by the overexpression of Sema5A. In our mixed population of hippocampal neurons, a number of plexins, including PlexA2, or co-receptor neuropilins could be involved in Sema5A signaling (Pasterkamp, 2012).

5.1.4 Sema5A and ASD

Prior to our study, Sema5A was genetically associated with ASD when two GWASs identified a significant enrichment of SNVs near or in the SEMA5A gene in the ASD cohort compared to the general population (Cheng et al., 2013; Weiss et al., 2009). Furthermore, two

gene expression analyses identified the downregulation of SEMA5A in the blood and brains of individuals with ASD (Weiss et al., 2009; Melin et al., 2006). We have demonstrated that Sema5A may contribute to the neuropathology of ASD by causing the elimination of excitatory synapses, alongside its established role in axon guidance (Kantor et al., 2004). As aforementioned, one of the leading explanations for the etiology of ASD is disruption to the balance of excitatory and inhibitory synapses (Rubenstein & Merzenich, 2003; Nelson & Valakh, 2015). Our work on Sema5A's select elimination of excitatory synapses indicates a potential for its dysregulation to create a synaptic imbalance in certain brain regions of individuals with ASD. Particularly, the activity-regulated relocalization of Sema5A to the dendritic membrane following cLTD demonstrates potential for aberrant synapse elimination throughout life in ASD patients with Sema5A dysregulation (Penzes et al., 2011). Many of the genes associated with ASD to date have known synaptic functions (Bourgeron, 2015). Our study, along with the work of Duan et al. (2014), suggests that Sema5A similarly belongs to this subcategory.

5.2 PTEN

The second portion of the present thesis functionally annotated SNVs of the PTEN gene in rat hippocampal neurons. Prior to our study, these variants had only been genetically associated with ASD and not functionally characterized in mammals. In particular, the effect of these SNVs on mature excitatory and inhibitory synapse density, as well as dendrite outgrowth and soma size was elucidated in a PTEN overexpression context. All of the ASD-associated PTEN SNVs tested, except H123Q, were loss-of-function in our assays, leading to the alteration of excitatory synapses, dendritic outgrowth and soma size.

5.2.1 PTEN Regulates Excitatory Synapses, Dendrite Outgrowth and Soma Size

The germline ablation of PTEN in mice is embryonically lethal (Di Cristofano et al., 1998). On the other hand, the brain-specific KO of PTEN using Pten^{loxp/loxp}; Gfap-Cre mice leads to broad anatomical disruptions of the cerebellum, DG and CA3 region of the hippocampus, as well as macrocephaly, seizures, ataxia, cell-autonomous hypertrophy of DG neurons, and premature death (Backman et al., 2001). Thus, it has been known for some time that PTEN is an essential component of both embryonic and neural development. Accordingly, the downstream signaling cascade of PTEN is broad and has been associated with a diverse range of cellular functions, including proliferation, axon growth, neuronal polarity and cell survival (for review, see Sansal & Sellers, 2004; Jiang et al., 2005; Zhou & Parada, 2012). Perhaps unsurprisingly, severe disruptions to the function of this gene in humans have long been associated with cancer and PTEN is thusly considered a tumor suppressor gene (Sansal & Sellers, 2004).

However, recent evidence using more targeted methodologies has identified a role for PTEN in the maturation and maintenance of neurons. Zhang et al. (2012) overexpressed GFP-PTEN in mature CA1 pyramidal neurons of rat hippocampal slice cultures. Two days after transfection, GFP-PTEN overexpressing neurons exhibited a ~75% reduction in dendritic spine density compared to GFP expressing controls. This effect was not observed when overexpressing a phosphatase dead biochemical mutant of PTEN (C124S). However, PTEN has both lipid and protein phosphatase activity. Using two further biochemical variants of PTEN, one of which lacks solely protein phosphatase activity (Y138L) and another that lacks solely lipid phosphatase activity (G129E), Zhang et al. (2012) demonstrated that the effects of PTEN on spine morphology were dependent upon its protein, but not lipid, phosphatase activity. Similarly, in our study, the overexpression of human WT PTEN* in rat hippocampal cultures led to a

reduction in PSD-95 density compared to WT controls. PSD-95 is a major component of excitatory synapses, which are present largely at dendritic spines, allowing for the direct comparison of these two metrics (Li & Sheng, 2003). Furthermore, the overexpression of C124S PTEN* in our system did not alter PSD-95 density, akin to the findings by Zhang et al. (2012). In addition to alterations to PSD-95 density, we also report that the overexpression of human WT PTEN* in hippocampal neurons leads to a reduction in total dendrite length and increases in soma size in a phosphatase-dependent manner. As our overexpression began at DIV10, when the dendritic arbor has largely stabilised, these changes likely represent the atypical retraction of dendrites (Dotti et al., 1998). As such, the role of PTEN during dendritic outgrowth was not captured by this experimental design and could represent a potentially fruitful follow-up endeavor. Interestingly, the overexpression of WT PTEN* in our system did not reduce gephyrin density, a surrogate marker of inhibitory synapses.

Williams et al. (2015) looked at the electrophysiological, dendritic, synaptic and somal consequences of PTEN ablation in newborn DG mouse neurons. P7 Pten^{flx/flx} mice were coinjected with two retroviruses into the DG. One of the retroviruses encoded GFP, while the other encoded mCherry-T2A-Cre. The latter of the two retroviruses, after entering DG neurons, results in the expression of Cre and subsequent cell-specific excision of the Pten gene. However, at the multiplicity of infection ratios used, these two retroviruses only labeled a small subset of cells, such that in a single injected mouse, DG neurons mostly expressed nothing exogenous, while a few expressed (i) just GFP, (ii) just mCherry-T2A-Cre, or (iii) GFP and mCherry-T2A-Cre. In doing so, they were able to elucidate the effects of PTEN specifically in newborn DG neurons without inducing broad anatomical or cellular disruption in the mouse. Further, the colabeling allowed for the comparison of neurons lacking PTEN (mCherry positive) with wild

type neurons (only GFP positive) in the same animal. Hippocampal slices from these mice revealed significant neuronal hyperexcitability, increased dendrite length and arborization, increased mushroom spines and filopodia, somal hypertrophy, exaggerated intrinsic electrophysiological properties and increased dendritic protrusions from 7.5-24.5 days postinjection in PTEN KO neurons compared to controls. Similarly, in our in vitro study, we described a significant increase in PSD-95 density after shRNA-mediated knockdown of PTEN in a mixed hippocampal culture. We also identified a significant increase in soma size and dendritic outgrowth, comparable to the results from Williams et al. (2015). Unlike our overexpression study, the PTEN shRNA was expressed from DIV7, when hippocampal dendrites in culture are still being extended and stabilized, and thus this experiment captures dendritogenesis (Dotti et al., 1998). One of the biggest caveats to such studies is the potential for the introduced shRNA to target genes other than those intended, known as off-target effects. However, the coexpression of human PTEN* during the shRNA-mediated knockdown of rat PTEN was able to ameliorate the synaptic, dendritic and somal phenotypes associated with PTEN KD, to a degree consistent with wild type rat neurons. Thus, the reintroduction of PTEN was sufficient to rescue the phenotypic alterations induced by the PTEN shRNA in our system, disputing the presence of off-target effects. Lastly, the coexpression of C124S PTEN* failed to rescue the effects of PTEN KD and these neurons were comparable to KD neurons across all three aforementioned measures, suggesting these effects are dependent upon the phosphatase activity of PTEN. In addition to recapitulating previous PTEN findings in our system, we also report that the KD of PTEN did not significantly alter dendritic gephyrin density. Despite this recapitulation of some of the demonstrations by William et al. (2015), it is not possible for us to conclude, though one might speculate, that the electrophysiological properties of our *in vitro* KD

neurons match those reported in their study. Though a selective increase in PSD-95 density suggests an excess specifically of excitatory synapses and thus potential hyperexcitability, neuronal output cannot be deduced from molecular staining alone. This is especially true when one considers that Williams et al. (2015) specifically characterized mouse neurons of the DG, while our rat neurons are of broad hippocampal identity with varied morphologies and intrinsic properties. Subsequent studies using calcium imaging or patch clamping to characterize neuronal firing patterns would have to be performed. Nonetheless, many of the morphological and molecular phenotypes thought to underpin these physiological changes were likewise observed in our *in vitro* model.

5.2.2 Role of PTEN in ASD

As aforementioned, the function of PTEN as a tumor suppressor gene in humans has been widely characterized (Sansal & Sellers, 2004). PTEN has since also been associated with a number of neurodevelopmental disorders, including ASD (Zhou & Parada, 2012). 1-5% of individuals with ASD are thought to carry a germline PTEN mutation (McBride et al., 2010). These mutations include CNVs, large chromosomal alterations, rare SNPs and SNVs. Our study focused on *de novo* PTEN SNVs found only in individuals affected with ASD and not in either of their unaffected parents, in order to increase the likelihood that the mutation is at least partially causative. This may indeed be an effective approach, as genetic studies have identified that individuals with ASD do not carry more *de novo* mutations than the general population, but that the *de novo* mutations they carry are more likely to be disruptive (O'Roak et al., 2011; Bourgeron, 2015). However, it is not entirely possible to rule out the contribution of other genomic sites, as each individual typically carries 18-74 *de novo* mutations, which might

themselves be causative of ASD or provide genetic buffering (O'Roak et al., 2011). Some germline PTEN mutations result in a form of PHTS, known as Cowden's syndrome, which is a syndromic form of ASD. These individuals often present with macrocephaly, which is itself associated with germline PTEN mutations and ASD (Butler et al., 2005; Blumenthal & Dennis, 2008; Lainhart et al., 1997; Fidler et al., 2000). In these patients, one could hypothesize that aberrant signaling during PTEN's elucidated role in NPC proliferation and neurogenesis in various subpopulations might underpin their macrocephaly (Gregorian et al., 2009; Sansal & Sellers, 2004). The resulting disorganization of typical neurodevelopment might then contribute to their clinical ASD phenotype. However, the vast majority of individuals with ASD and a germline PTEN mutation do not exhibit such robust anatomical phenotypes (McBride et al., 2010). Thus, if PTEN does indeed play a role in the etiology of ASD in these patients, it is likely from secondary effects of PTEN, such as those on neural circuit formation and maintenance (Williams et al., 2015). That is not to say, however, that these secondary effects may not also underlie some of the neuropathology and behavior of patients with macrocephaly, germline PTEN mutations and ASD. Nonetheless, the previously explored effects of PTEN on soma size and PSD-95 density may alter the excitatory/inhibitory balance of particular neural circuits. Such imbalances have been proposed as a common neuropathology in ASD, though the findings are inconsistent (Rubenstein & Merzenich, 2003; Nelson & Valakh, 2015). Further, the aberrant regulation of dendritic arborization by PTEN could alter the connectivity of neural pathways, in line with hypo- and hyper-connectivity findings in individuals with ASD, another leading theory in ASD neuropathology (Kana et al., 2014; Muller et al., 2011).

5.2.3 Most De Novo ASD-associated SNVs of PTEN are Loss of Function in Neurons

Most of the de novo PTEN* SNVs tested in our in vitro hippocampal model were loss-offunction. Indeed, the overexpression of K6E, R130L, T131I, G132D or T167N PTEN* did not significantly reduce PSD-95 density, total dendrite length, or soma size. Statistically speaking, these ASD-associated PTEN SNV overexpressing neurons were indistinguishable from controls and C124S PTEN overexpressing neurons, yet significantly different from human WT PTEN overexpressing neurons. Intriguingly, the only ASD-associated SNV capable of producing overexpression effects akin to WT PTEN was H123Q. This may not be immediately intuitive as histidine and glutamine have different amino acid properties and this amino acid substitution has altered the function of other enzymes (Schröder & Schröder, 1992). Moreover, the H123Q site lies within the binding pocket of PTEN, just one amino acid away from the C124S mutation, which results in a complete loss of phosphatase activity (Zhou & Parada, 2012; Rodríguez-Escudero et al., 2011). This discrepancy between the knowledge used to make predictions and biological assays highlights the importance of functionalization in elucidating the true effects of ASD-associated SNVs. It is especially important to note that in a humanized yeast bioassay of phosphatase function performed by Rodríguez-Escudero et al. (2011), ASD-associated mutations partially reduced phosphatase activity, compared to the severe loss of function observed in tumour-associated PTEN SNVs. In particular, they characterized the phosphatase function of H93R, H118P, H123Q, E157G, Y176C, F241S, D252G, N276S and D326N. Interestingly, in their assay, they found the H123Q PTEN mutation to exhibit minimal loss of phosphatase function. The phenotypic characterizations performed in our study did not suggest a partial loss of function of H123Q, as it was comparable to human WT PTEN in the overexpression context. This could indicate that our assays were not sensitive enough to capture various degrees of

phosphatase function loss, or that in neurons there is a threshold of PTEN phosphatase function required to prevent synaptic, dendritic and somal consequences. Furthermore, our own humanized yeast mini-array found that H123Q, as well as all the other ASD-associated PTEN SNVs in our study, largely lacked phosphatase activity (data not shown; Kathryn Post, Haas Lab, UBC). The discrepancy between these biochemical results makes our data difficult to interpret. However, combined and integrated, they represent the possibility of an as yet non-elucidated function of PTEN, distinct from its phosphatase activity, which regulates these neuronal characteristics.

5.2.4 Functional Genomics of ASD: Future Implications

Historically, studies of ASD etiology have focused on population genetics and clinical case reports (Bourgeron, 2015). A lot of attention has contemporarily shifted to the functionalization of the mutations found in ASD-associated genes in a variety of model organisms. Though this approach often does not consider the polygenic contribution of germline mutations and SNPs to most ASD cases, it shall likely contribute greatly to our understanding of gene function, etiology, neuropathology and behaviour. Interestingly, one of the ASD-associated SNVs tested in this study (P38H PTEN*) appeared as a loss-of-function mutation in two of the three neurophenotypes characterized, yet phenocopied wild type PTEN in its effects upon soma size. This kind of diversity of effects caused by different gene disruptions can only be captured by biological functionalization and may go some way to explaining symptom heterogeneity amongst individuals with ASD. The eventual goal of mutation functionalization research is to allow for (i) the phenotyping of ASD patients, (ii) better prognosis, and (iii) targeted therapies. Indeed, some attempts are already being made to subcategorize ASD patients according to a range of functional measures (Chang et al., 2015). Better understanding of how a patient's

genetic background contributes to their ASD neuropathology and behavior will permit greater accuracy in predictions of clinical measures from genetic sequencing. Lastly, the characterization of the genes and signaling pathways disrupted in individuals with ASD opens up the possibility for targeted treatments. Of importance to the present study, Zhou et al. (2009) demonstrated that the neuronal hypertrophy and ASD-associated behaviors observed in mice deficient of PTEN in postmitotic neurons of the cortex and hippocampus could be ameliorated by rapamycin treatment. Rapamycin specifically inhibits the mTORC1 complex signaling pathway downstream of PTEN. This work preliminarily suggests that some of the neural consequences of gene alteration—such as those observed in ASD—may be sensitive, even at mature stages, to pharmacological intervention.
Bibliography

- Abraham, W. C., Logan, B., Greenwood, J. M., & Dragunow, M. (2002). Induction and experience-dependent consolidation of stable long-term potentiation lasting months in the hippocampus. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 22(21), 9626–9634.
- Abrahams, B. S., & Geschwind, D. H. (2008). Advances in autism genetics: on the threshold of a new neurobiology. *Nature Reviews. Genetics*, *9*(5), 341–355.
- Adams, R. H., Betz, H., & Püschel, A. W. (1996). A novel class of murine semaphorins with homology to thrombospondin is differentially expressed during early embryogenesis. *Mechanisms of Development*, 57(1), 33–45.
- Amaral, D. G., Schumann, C. M., & Nordahl, C. W. (2008). Neuroanatomy of autism. *Trends in Neurosciences*, 31(3), 137–145.
- American Psychiatric Association. (2013). Diagnostic and statistical manual of mental disorders (5th ed.). Arlington, VA: American Psychiatric Publishing.
- Anney, R., Klei, L., Pinto, D., Almeida, J., Bacchelli, E., Baird, G., ... Devlin, B. (2012).
 Individual common variants exert weak effects on the risk for autism spectrum disorders.
 Human Molecular Genetics, 21(21), 4781–4792.
- Artigiani, S., Conrotto, P., Fazzari, P., Gilestro, G. F., Barberis, D., Giordano, S., ... Tamagnone,
 L. (2004). Plexin-B3 is a functional receptor for semaphorin 5A. *EMBO Reports*, 5(7),
 710–714.
- Baas, P. W., Deitch, J. S., Black, M. M., & Banker, G. A. (1988). Polarity orientation of microtubules in hippocampal neurons: uniformity in the axon and nonuniformity in the

dendrite. *Proceedings of the National Academy of Sciences of the United States of America*, 85(21), 8335–8339.

- Backman, S. A., Stambolic, V., Suzuki, A., Haight, J., Elia, A., Pretorius, J., ... Mak, T. W.
 (2001). Deletion of Pten in mouse brain causes seizures, ataxia and defects in soma size resembling Lhermitte-Duclos disease. *Nature Genetics*, 29(4), 396–403.
- Bangash, M. A., Park, J. M., Melnikova, T., Wang, D., Jeon, S. K., Lee, D., ... Worley, P. F. (2011). Enhanced polyubiquitination of Shank3 and NMDA receptor in a mouse model of autism. *Cell*, 145(5), 758–772.
- BARNES, A. P., & POLLEUX, F. (2009). Establishment of Axon-Dendrite Polarity in Developing Neurons. *Annual Review of Neuroscience*, 32, 347–381.
- Bartlett, T. E., Bannister, N. J., Collett, V. J., Dargan, S. L., Massey, P. V., Bortolotto, Z. A., ... Lodge, D. (2007). Differential roles of NR2A and NR2B-containing NMDA receptors in LTP and LTD in the CA1 region of two-week old rat hippocampus. *Neuropharmacology*, 52(1), 60–70.
- Bashaw, G. J., & Klein, R. (2010). Signaling from Axon Guidance Receptors. Cold Spring Harbor Perspectives in Biology, 2(5).
- Ben-Ari, Y. (2002). Excitatory actions of gaba during development: the nature of the nurture. *Nature Reviews. Neuroscience*, *3*(9), 728–739.
- Bliss, T. V., & Lomo, T. (1973). Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. *The Journal of Physiology*, 232(2), 331–356.
- Blumenthal, G. M., & Dennis, P. A. (2008). PTEN hamartoma tumor syndromes. *European Journal of Human Genetics: EJHG*, *16*(11), 1289–1300.

- Bonaguidi, M. A., Wheeler, M. A., Shapiro, J. S., Stadel, R. P., Sun, G. J., Ming, G., & Song, H. (2011). In vivo clonal analysis reveals self-renewing and multipotent adult neural stem cell characteristics. *Cell*, 145(7), 1142–1155.
- Bourgeron, T. (2015). From the genetic architecture to synaptic plasticity in autism spectrum disorder. *Nature Reviews. Neuroscience*, *16*(9), 551–563.
- Bouzioukh, F., Daoudal, G., Falk, J., Debanne, D., Rougon, G., & Castellani, V. (2006).
 Semaphorin3A regulates synaptic function of differentiated hippocampal neurons. *The European Journal of Neuroscience*, 23(9), 2247–2254.
- Boyer, C., Schikorski, T., & Stevens, C. F. (1998). Comparison of hippocampal dendritic spines in culture and in brain. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 18(14), 5294–5300.
- Bozdagi, O., Sakurai, T., Papapetrou, D., Wang, X., Dickstein, D. L., Takahashi, N., ...
 Buxbaum, J. D. (2010). Haploinsufficiency of the autism-associated Shank3 gene leads to deficits in synaptic function, social interaction, and social communication. *Molecular Autism*, 1, 15.
- Brigman, J. L., Wright, T., Talani, G., Prasad-Mulcare, S., Jinde, S., Seabold, G. K., ... Holmes,
 A. (2010). Loss of GluN2B-containing NMDA receptors in CA1 hippocampus and cortex impairs long-term depression, reduces dendritic spine density and disrupts learning. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, 30(13), 4590–4600.
- Butler, M. G., Dasouki, M. J., Zhou, X.-P., Talebizadeh, Z., Brown, M., Takahashi, T. N., ...
 Eng, C. (2005). Subset of individuals with autism spectrum disorders and extreme macrocephaly associated with germline PTEN tumour suppressor gene mutations.

Journal of Medical Genetics, 42(4), 318–321.

- Buxbaum, J. D., Cai, G., Chaste, P., Nygren, G., Goldsmith, J., Reichert, J., ... Betancur, C.
 (2007). Mutation screening of the PTEN gene in patients with autism spectrum disorders and macrocephaly. *American Journal of Medical Genetics. Part B, Neuropsychiatric Genetics: The Official Publication of the International Society of Psychiatric Genetics*, 144B(4), 484–491.
- Carrillo, R. A., Olsen, D. P., Yoon, K. S., & Keshishian, H. (2010). Presynaptic activity and CaMKII modulate retrograde semaphorin signaling and synaptic refinement. *Neuron*, 68(1), 32–44.
- Chang, J., Gilman, S. R., Chiang, A. H., Sanders, S. J., & Vitkup, D. (2015). Genotype to phenotype relationships in autism spectrum disorders. *Nature Neuroscience*, 18(2), 191– 198.
- Cheadle, L., & Biederer, T. (2014). Activity-Dependent Regulation of Dendritic Complexity by Semaphorin 3A through Farp1. *The Journal of Neuroscience*, *34*(23), 7999–8009.
- Chédotal, A., Del Rio, J. A., Ruiz, M., He, Z., Borrell, V., de Castro, F., ... Soriano, E. (1998).
 Semaphorins III and IV repel hippocampal axons via two distinct receptors. *Development* (*Cambridge, England*), 125(21), 4313–4323.
- Cheng, Y., Quinn, J. F., & Weiss, L. A. (2013). An eQTL mapping approach reveals that rare variants in the SEMA5A regulatory network impact autism risk. *Human Molecular Genetics*, 22(14), 2960–2972.
- Cheslack-Postava, K., Liu, K., & Bearman, P. S. (2011). Closely spaced pregnancies are associated with increased odds of autism in California sibling births. *Pediatrics*, 127(2), 246–253.

- Chilton, J. K. (2006). Molecular mechanisms of axon guidance. *Developmental Biology*, 292(1), 13–24.
- Chow, L. M. L., Endersby, R., Zhu, X., Rankin, S., Qu, C., Zhang, J., ... Baker, S. J. (2011). Cooperativity within and among Pten, p53, and Rb pathways induces high-grade astrocytoma in adult brain. *Cancer Cell*, 19(3), 305–316.
- Clarke, L. E., & Barres, B. A. (2013). Emerging roles of astrocytes in neural circuit development. *Nature Reviews. Neuroscience*, 14(5), 311–321.
- Cline, H., & Haas, K. (2008). The regulation of dendritic arbor development and plasticity by glutamatergic synaptic input: a review of the synaptotrophic hypothesis. *The Journal of Physiology*, 586(6), 1509–1517.
- Collingridge, G. L., Kehl, S. J., & McLennan, H. (1983). Excitatory amino acids in synaptic transmission in the Schaffer collateral-commissural pathway of the rat hippocampus. *The Journal of Physiology*, *334*, 33–46.
- Collingridge, G. L., Peineau, S., Howland, J. G., & Wang, Y. T. (2010). Long-term depression in the CNS. *Nature Reviews. Neuroscience*, *11*(7), 459–473.
- Conde, C., & Cáceres, A. (2009). Microtubule assembly, organization and dynamics in axons and dendrites. *Nature Reviews. Neuroscience*, *10*(5), 319–332.
- Constantino, J. N. (2011). The quantitative nature of autistic social impairment. *Pediatric Research*, *69*(5 Pt 2), 55R–62R.
- Courchesne, E., Mouton, P. R., Calhoun, M. E., Semendeferi, K., Ahrens-Barbeau, C., Hallet, M. J., ... Pierce, K. (2011). Neuron number and size in prefrontal cortex of children with autism. *JAMA*, 306(18), 2001–2010.

Craig, A. M., & Banker, G. (1994). Neuronal polarity. Annual Review of Neuroscience, 17, 267-

310.

- Craig, A. M., & Lichtman, J. W. (2001). Getting a bead on receptor movements. *Nature Neuroscience*, *4*(3), 219–220.
- Crawley, J. N. (2012). Translational animal models of autism and neurodevelopmental disorders. *Dialogues in Clinical Neuroscience*, *14*(3), 293–305.
- Dailey, M. E., & Smith, S. J. (1996). The dynamics of dendritic structure in developing hippocampal slices. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 16(9), 2983–2994.
- Dalva, M. B., McClelland, A. C., & Kayser, M. S. (2007). Cell adhesion molecules: signalling functions at the synapse. *Nature Reviews. Neuroscience*, 8(3), 206–220.
- De Rubeis, S., & Buxbaum, J. D. (2015). Genetics and genomics of autism spectrum disorder: embracing complexity. *Human Molecular Genetics*, *24*(R1), R24–R31. 37
- Del Río, J. A., Heimrich, B., Borrell, V., Förster, E., Drakew, A., Alcántara, S., ... Soriano, E. (1997). A role for Cajal-Retzius cells and reelin in the development of hippocampal connections. *Nature*, 385(6611), 70–74.
- Dent, E. W., Gupton, S. L., & Gertler, F. B. (2011). The Growth Cone Cytoskeleton in Axon Outgrowth and Guidance. *Cold Spring Harbor Perspectives in Biology*, *3*(3).
- Di Cristofano, A., Pesce, B., Cordon-Cardo, C., & Pandolfi, P. P. (1998). Pten is essential for embryonic development and tumour suppression. *Nature Genetics*, *19*(4), 348–355.
- Dickson, B. J. (2002). Molecular mechanisms of axon guidance. *Science (New York, N.Y.)*, 298(5600), 1959–1964.
- Ding, J. B., Oh, W.-J., Sabatini, B. L., & Gu, C. (2011). Semaphorin 3E-Plexin-D1 signaling controls pathway-specific synapse formation in the striatum. *Nature Neuroscience*, *15*(2),

215-223.

- Dotti, C. G., Sullivan, C. A., & Banker, G. A. (1988). The establishment of polarity by hippocampal neurons in culture. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 8(4), 1454–1468.
- Duan, Y., Wang, S.-H., Song, J., Mironova, Y., Ming, G., Kolodkin, A. L., & Giger, R. J.
 (2014). Semaphorin 5A inhibits synaptogenesis in early postnatal- and adult-born hippocampal dentate granule cells. *eLife*, *3*.
- Dudek, S. M., & Bear, M. F. (1992). Homosynaptic long-term depression in area CA1 of hippocampus and effects of N-methyl-D-aspartate receptor blockade. *Proceedings of the National Academy of Sciences of the United States of America*, 89(10), 4363–4367.
- Durand, G. M., Kovalchuk, Y., & Konnerth, A. (1996). Long-term potentiation and functional synapse induction in developing hippocampus. *Nature*, *381*(6577), 71–75.
- Durkin, M. S., Maenner, M. J., Newschaffer, C. J., Lee, L.-C., Cunniff, C. M., Daniels, J. L., ... Schieve, L. A. (2008). Advanced parental age and the risk of autism spectrum disorder. *American Journal of Epidemiology*, 168(11), 1268–1276.
- Ehninger, D., Han, S., Shilyansky, C., Zhou, Y., Li, W., Kwiatkowski, D. J., ... Silva, A. J.
 (2008). Reversal of learning deficits in a Tsc2+/- mouse model of tuberous sclerosis. *Nature Medicine*, 14(8), 843–848.
- Endersby, R., & Baker, S. J. (2008). PTEN signaling in brain: neuropathology and tumorigenesis. *Oncogene*, *27*(41), 5416–5430.
- Fakhoury, M. (2015). Autistic spectrum disorders: A review of clinical features, theories and diagnosis. International Journal of Developmental Neuroscience: The Official Journal of the International Society for Developmental Neuroscience, 43, 70–77.

- Fiala, J. C., Feinberg, M., Popov, V., & Harris, K. M. (1998). Synaptogenesis via dendritic filopodia in developing hippocampal area CA1. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 18(21), 8900–8911.
- Fidler, D. J., Bailey, J. N., & Smalley, S. L. (2000). Macrocephaly in autism and other pervasive developmental disorders. *Developmental Medicine and Child Neurology*, 42(11), 737–740.
- Flavell, S. W., & Greenberg, M. E. (2008). Signaling mechanisms linking neuronal activity to gene expression and plasticity of the nervous system. *Annual Review of Neuroscience*, 31, 563–590.
- Frankland, P. W., O'Brien, C., Ohno, M., Kirkwood, A., & Silva, A. J. (2001). Alpha-CaMKIIdependent plasticity in the cortex is required for permanent memory. *Nature*, 411(6835), 309–313.
- Friedman, H. V., Bresler, T., Garner, C. C., & Ziv, N. E. (2000). Assembly of new individual excitatory synapses: time course and temporal order of synaptic molecule recruitment. *Neuron*, 27(1), 57–69.
- Fukata, Y., Kimura, T., & Kaibuchi, K. (2002). Axon specification in hippocampal neurons. *Neuroscience Research*, 43(4), 305–315.
- Gao, F. B. (1998). Messenger RNAs in dendrites: localization, stability, and implications for neuronal function. *BioEssays: News and Reviews in Molecular, Cellular and Developmental Biology*, 20(1), 70–78.
- Gardiol, A., Racca, C., & Triller, A. (1999). Dendritic and postsynaptic protein synthetic machinery. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 19(1), 168–179.

- Gärtner, A., Huang, X., & Hall, A. (2006). Neuronal polarity is regulated by glycogen synthase kinase-3 (GSK-3beta) independently of Akt/PKB serine phosphorylation. *Journal of Cell Science*, 119(Pt 19), 3927–3934.
- Gaugler, T., Klei, L., Sanders, S. J., Bodea, C. A., Goldberg, A. P., Lee, A. B., ... Buxbaum, J.
 D. (2014). Most genetic risk for autism resides with common variation. *Nature Genetics*, 46(8), 881–885.
- Gimm, O., Attié-Bitach, T., Lees, J. A., Vekemans, M., & Eng, C. (2000). Expression of the PTEN tumour suppressor protein during human development. *Human Molecular Genetics*, 9(11), 1633–1639.
- Glessner, J. T., Wang, K., Cai, G., Korvatska, O., Kim, C. E., Wood, S., ... Hakonarson, H. (2009). Autism genome-wide copy number variation reveals ubiquitin and neuronal genes. *Nature*, 459(7246), 569–573.
- Gogtay, N., Nugent, T. F., Herman, D. H., Ordonez, A., Greenstein, D., Hayashi, K. M., ...Thompson, P. M. (2006). Dynamic mapping of normal human hippocampaldevelopment. *Hippocampus*, *16*(8), 664–672.
- Granger, A. J., & Nicoll, R. A. (2014). LTD expression is independent of glutamate receptor subtype. *Frontiers in Synaptic Neuroscience*, 6.
- Gregorian, C., Nakashima, J., Le Belle, J., Ohab, J., Kim, R., Liu, A., ... Wu, H. (2009). Pten deletion in adult neural stem/progenitor cells enhances constitutive neurogenesis. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 29(6), 1874–1886.
- Groszer, M., Erickson, R., Scripture-Adams, D. D., Lesche, R., Trumpp, A., Zack, J. A., ... Wu, H. (2001). Negative regulation of neural stem/progenitor cell proliferation by the Pten

tumor suppressor gene in vivo. Science (New York, N.Y.), 294(5549), 2186-2189.

- Gupta, S., Ellis, S. E., Ashar, F. N., Moes, A., Bader, J. S., Zhan, J., ... Arking, D. E. (2014).
 Transcriptome analysis reveals dysregulation of innate immune response genes and neuronal activity-dependent genes in autism. *Nature Communications*, *5*, 5748.
- Haas, K., Li, J., & Cline, H. T. (2006). AMPA receptors regulate experience-dependent dendritic arbor growth in vivo. *Proceedings of the National Academy of Sciences of the United States of America*, 103(32), 12127–12131.
- Halladay, A. K., Amaral, D., Aschner, M., Bolivar, V. J., Bowman, A., DiCicco-Bloom, E., ... Threadgill, D. W. (2009). Animal models of autism spectrum disorders: information for neurotoxicologists. *Neurotoxicology*, 30(5), 811–821.
- Hama, H., Hara, C., Yamaguchi, K., & Miyawaki, A. (2004). PKC signaling mediates global enhancement of excitatory synaptogenesis in neurons triggered by local contact with astrocytes. *Neuron*, 41(3), 405–415.
- Hayashi, K., Kubo, K., Kitazawa, A., & Nakajima, K. (2015). Cellular dynamics of neuronal migration in the hippocampus. *Frontiers in Neuroscience*, *9*.
- Hayashi, Y., Shi, S. H., Esteban, J. A., Piccini, A., Poncer, J. C., & Malinow, R. (2000). Driving AMPA receptors into synapses by LTP and CaMKII: requirement for GluR1 and PDZ domain interaction. *Science (New York, N.Y.)*, 287(5461), 2262–2267.
- Henley, J. M., & Wilkinson, K. A. (2013). AMPA receptor trafficking and the mechanisms underlying synaptic plasticity and cognitive aging. *Dialogues in Clinical Neuroscience*, 15(1), 11–27.
- Hilario, J. D., Rodino-Klapac, L. R., Wang, C., & Beattie, C. E. (2009). Semaphorin 5A is a bifunctional axon guidance cue for axial motoneurons in vivo. *Developmental Biology*,

326(1), 190–200.

- Hu, F., & Strittmatter, S. M. (2004). Regulating axon growth within the postnatal central nervous system. *Seminars in Perinatology*, *28*(6), 371–378.
- Huganir, R. L., & Nicoll, R. A. (2013). AMPARs and synaptic plasticity: the last 25 years. *Neuron*, *80*(3), 704–717.
- Hughes, E. G., Elmariah, S. B., & Balice-Gordon, R. J. (2010). Astrocyte secreted proteins selectively increase hippocampal GABAergic axon length, branching, and synaptogenesis. *Molecular and Cellular Neurosciences*, 43(1), 136–145.
- Huguet, G., Ey, E., & Bourgeron, T. (2013). The genetic landscapes of autism spectrum disorders. *Annual Review of Genomics and Human Genetics*, *14*, 191–213.
- Hunt, D. L., & Castillo, P. E. (2012). Synaptic plasticity of NMDA receptors: mechanisms and functional implications. *Current Opinion in Neurobiology*, 22(3), 496–508.
- Hur, E.-M., & Zhou, F.-Q. (2010). GSK3 signalling in neural development. *Nature Reviews*. *Neuroscience*, 11(8), 539–551.
- Hutsler, J. J., & Zhang, H. (2010). Increased dendritic spine densities on cortical projection neurons in autism spectrum disorders. *Brain Research*, 1309, 83–94.
- Insausti, R. (1993). Comparative anatomy of the entorhinal cortex and hippocampus in mammals. *Hippocampus*, *3 Spec No*, 19–26.
- James, W. H. (2014). An update on the hypothesis that one cause of autism is high intrauterine levels of testosterone of maternal origin. *Journal of Theoretical Biology*, *355*, 33–39.
- Jan, Y.-N., & Jan, L. Y. (2001). Dendrites. Genes & Development, 15(20), 2627–2641.
- Janssen, B. J. C., Robinson, R. A., Pérez-Brangulí, F., Bell, C. H., Mitchell, K. J., Siebold, C., & Jones, E. Y. (2010). Structural basis of semaphorin-plexin signalling. *Nature*, 467(7319),

1118–1122.

- Jaworski, J., Spangler, S., Seeburg, D. P., Hoogenraad, C. C., & Sheng, M. (2005). Control of dendritic arborization by the phosphoinositide-3'-kinase-Akt-mammalian target of rapamycin pathway. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 25(49), 11300–11312.
- Jiang, H., Guo, W., Liang, X., & Rao, Y. (2005). Both the establishment and the maintenance of neuronal polarity require active mechanisms: critical roles of GSK-3beta and its upstream regulators. *Cell*, 120(1), 123–135.
- Kana, R. K., Uddin, L. Q., Kenet, T., Chugani, D., & Müller, R.-A. (2014). Brain connectivity in autism. *Frontiers in Human Neuroscience*, 8.
- Kantor, D. B., Chivatakarn, O., Peer, K. L., Oster, S. F., Inatani, M., Hansen, M. J., ... Kolodkin,
 A. L. (2004). Semaphorin 5A is a bifunctional axon guidance cue regulated by heparan and chondroitin sulfate proteoglycans. *Neuron*, 44(6), 961–975.
- Kim, E., & Sheng, M. (2004). PDZ domain proteins of synapses. *Nature Reviews. Neuroscience*, 5(10), 771–781.
- Kim, M. J., Futai, K., Jo, J., Hayashi, Y., Cho, K., & Sheng, M. (2007). Synaptic accumulation of PSD-95 and synaptic function regulated by phosphorylation of serine-295 of PSD-95. *Neuron*, 56(3), 488–502.
- Kitazawa, A., Kubo, K., Hayashi, K., Matsunaga, Y., Ishii, K., & Nakajima, K. (2014).
 Hippocampal pyramidal neurons switch from a multipolar migration mode to a novel "climbing" migration mode during development. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 34(4), 1115–1126.

Knuesel, I., Chicha, L., Britschgi, M., Schobel, S. A., Bodmer, M., Hellings, J. A., ... Prinssen,

E. P. (2014). Maternal immune activation and abnormal brain development across CNS disorders. *Nature Reviews. Neurology*, *10*(11), 643–660.

- Kolodkin, A. L., Matthes, D. J., & Goodman, C. S. (1993). The semaphorin genes encode a family of transmembrane and secreted growth cone guidance molecules. *Cell*, 75(7), 1389–1399.
- Koppel, A. M., Feiner, L., Kobayashi, H., & Raper, J. A. (1997). A 70 amino acid region within the semaphorin domain activates specific cellular response of semaphorin family members. *Neuron*, 19(3), 531–537.
- Kwiatkowski, D. J., & Manning, B. D. (2005). Tuberous sclerosis: a GAP at the crossroads of multiple signaling pathways. *Human Molecular Genetics*, 14 Spec No. 2, R251-258.
- Kwon, C.-H., Luikart, B. W., Powell, C. M., Zhou, J., Matheny, S. A., Zhang, W., ... Parada, L.
 F. (2006). Pten regulates neuronal arborization and social interaction in mice. *Neuron*, *50*(3), 377–388.
- Lainhart, J. E., Piven, J., Wzorek, M., Landa, R., Santangelo, S. L., Coon, H., & Folstein, S. E. (1997). Macrocephaly in children and adults with autism. *Journal of the American Academy of Child and Adolescent Psychiatry*, 36(2), 282–290.
- Lee, H. K., Kameyama, K., Huganir, R. L., & Bear, M. F. (1998a). NMDA induces long-term synaptic depression and dephosphorylation of the GluR1 subunit of AMPA receptors in hippocampus. *Neuron*, 21(5), 1151–1162.
- Lee, H. K., Kameyama, K., Huganir, R. L., & Bear, M. F. (1998b). NMDA induces long-term synaptic depression and dephosphorylation of the GluR1 subunit of AMPA receptors in hippocampus. *Neuron*, 21(5), 1151–1162.

Lett, R. L. M., Wang, W., & O'Connor, T. P. (2009). Semaphorin 5B is a novel inhibitory cue

for corticofugal axons. Cerebral Cortex (New York, N.Y.: 1991), 19(6), 1408–1421.

- Lewis, T. L., Courchet, J., & Polleux, F. (2013). Cellular and molecular mechanisms underlying axon formation, growth, and branching. *J Cell Biol*, *202*(6), 837–848.
- Li, M., Fallin, M. D., Riley, A., Landa, R., Walker, S. O., Silverstein, M., ... Wang, X. (2016).
 The Association of Maternal Obesity and Diabetes With Autism and Other
 Developmental Disabilities. *Pediatrics*, *137*(2), e20152206.
- Li, Z., & Sheng, M. (2003). Some assembly required: the development of neuronal synapses. *Nature Reviews. Molecular Cell Biology*, *4*(11), 833–841.
- Liu, R. Q., Wang, W., Legg, A., Abramyan, J., & O'Connor, T. P. (2014). Semaphorin 5B is a repellent cue for sensory afferents projecting into the developing spinal cord. *Development (Cambridge, England)*, 141(9), 1940–1949.
- Loke, Y. J., Hannan, A. J., & Craig, J. M. (2015). The Role of Epigenetic Change in Autism Spectrum Disorders. *Frontiers in Neurology*, 6.
- Lu, W., Man, H., Ju, W., Trimble, W. S., MacDonald, J. F., & Wang, Y. T. (2001). Activation of synaptic NMDA receptors induces membrane insertion of new AMPA receptors and LTP in cultured hippocampal neurons. *Neuron*, 29(1), 243–254.
- Lu, W., Shi, Y., Jackson, A. C., Bjorgan, K., During, M. J., Sprengel, R., ... Nicoll, R. A. (2009). Subunit composition of synaptic AMPA receptors revealed by a single-cell genetic approach. *Neuron*, 62(2), 254–268.
- Luikart, B. W., Schnell, E., Washburn, E. K., Bensen, A. L., Tovar, K. R., & Westbrook, G. L.
 (2011). Pten Knockdown in vivo Increases Excitatory Drive onto Dentate Granule Cells. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, 31(11), 4345–4354.

- Luo, Y., Raible, D., & Raper, J. A. (1993). Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. *Cell*, *75*(2), 217–227.
- Lüscher, C., & Malenka, R. C. (2012). NMDA Receptor-Dependent Long-Term Potentiation and Long-Term Depression (LTP/LTD). *Cold Spring Harbor Perspectives in Biology*, *4*(6).
- Luukko, K., Ylikorkala, A., Tiainen, M., & Mäkelä, T. P. (1999). Expression of LKB1 and PTEN tumor suppressor genes during mouse embryonic development. *Mechanisms of Development*, 83(1–2), 187–190.
- Lyall, K., Schmidt, R. J., & Hertz-Picciotto, I. (2014). Maternal lifestyle and environmental risk factors for autism spectrum disorders. *International Journal of Epidemiology*, 43(2), 443–464.
- Lynch, M. A. (2004). Long-term potentiation and memory. *Physiological Reviews*, 84(1), 87–136.
- Malinow, R., & Miller, J. P. (1986). Postsynaptic hyperpolarization during conditioning reversibly blocks induction of long-term potentiation. *Nature*, *320*(6062), 529–530.
- Manahan-Vaughan, D., & Braunewell, K. H. (1999). Novelty acquisition is associated with induction of hippocampal long-term depression. *Proceedings of the National Academy of Sciences of the United States of America*, 96(15), 8739–8744.
- Manahan-Vaughan, D., Kulla, A., & Frey, J. U. (2000). Requirement of translation but not transcription for the maintenance of long-term depression in the CA1 region of freely moving rats. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 20(22), 8572–8576.
- Manning, B. D., & Cantley, L. C. (2007). AKT/PKB signaling: navigating downstream. *Cell*, *129*(7), 1261–1274.

- Marrs, G. S., Green, S. H., & Dailey, M. E. (2001). Rapid formation and remodeling of postsynaptic densities in developing dendrites. *Nature Neuroscience*, 4(10), 1006–1013.
- Massey, P. V., & Bashir, Z. I. (2007). Long-term depression: multiple forms and implications for brain function. *Trends in Neurosciences*, 30(4), 176–184.
- Masuda, T., Sakuma, C., Yaginuma, H., & Taniguchi, M. (2014). Attractive and permissive activities of semaphorin 5A toward dorsal root ganglion axons in higher vertebrate embryos. *Cell Adhesion & Migration*, 8(6), 603–606.
- Matsuoka, R. L., Chivatakarn, O., Badea, T. C., Samuels, I. S., Cahill, H., Katayama, K.-I., ...
 Kolodkin, A. L. (2011). Class 5 transmembrane semaphorins control selective
 Mammalian retinal lamination and function. *Neuron*, *71*(3), 460–473.
- Matsuoka, R. L., Nguyen-Ba-Charvet, K. T., Parray, A., Badea, T. C., Chédotal, A., & Kolodkin,
 A. L. (2011). Transmembrane semaphorin signalling controls laminar stratification in the mammalian retina. *Nature*, 470(7333), 259–263.
- Matsuzaki, M., Ellis-Davies, G. C., Nemoto, T., Miyashita, Y., Iino, M., & Kasai, H. (2001).
 Dendritic spine geometry is critical for AMPA receptor expression in hippocampal CA1
 pyramidal neurons. *Nature Neuroscience*, 4(11), 1086–1092.
- McAllister, A. K. (2000). Cellular and molecular mechanisms of dendrite growth. *Cerebral Cortex (New York, N.Y.: 1991)*, *10*(10), 963–973.

McBride, K. L., Varga, E. A., Pastore, M. T., Prior, T. W., Manickam, K., Atkin, J. F., & Herman, G. E. (2010). Confirmation study of PTEN mutations among individuals with autism or developmental delays/mental retardation and macrocephaly. *Autism Research: Official Journal of the International Society for Autism Research*, 3(3), 137–141.

McKittrick, C. R., Magariños, A. M., Blanchard, D. C., Blanchard, R. J., McEwen, B. S., &

Sakai, R. R. (2000). Chronic social stress reduces dendritic arbors in CA3 of hippocampus and decreases binding to serotonin transporter sites. *Synapse (New York, N.Y.)*, *36*(2), 85–94.

- Meikle, L., Talos, D. M., Onda, H., Pollizzi, K., Rotenberg, A., Sahin, M., ... Kwiatkowski, D. J. (2007). A mouse model of tuberous sclerosis: neuronal loss of Tsc1 causes dysplastic and ectopic neurons, reduced myelination, seizure activity, and limited survival. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 27(21), 5546–5558.
- Melin, M., Carlsson, B., Anckarsater, H., Rastam, M., Betancur, C., Isaksson, A., ... Dahl, N.
 (2006). Constitutional downregulation of SEMA5A expression in autism.
 Neuropsychobiology, 54(1), 64–69.
- Michaelson, J. J., Shi, Y., Gujral, M., Zheng, H., Malhotra, D., Jin, X., ... Sebat, J. (2012).
 Whole-genome sequencing in autism identifies hot spots for de novo germline mutation. *Cell*, 151(7), 1431–1442.
- Moret, F., Renaudot, C., Bozon, M., & Castellani, V. (2007). Semaphorin and neuropilin coexpression in motoneurons sets axon sensitivity to environmental semaphorin sources during motor axon pathfinding. *Development (Cambridge, England)*, 134(24), 4491– 4501.
- Morishita, W., Connor, J. H., Xia, H., Quinlan, E. M., Shenolikar, S., & Malenka, R. C. (2001). Regulation of synaptic strength by protein phosphatase 1. *Neuron*, *32*(6), 1133–1148.
- Morris, R. G., Anderson, E., Lynch, G. S., & Baudry, M. (1986). Selective impairment of learning and blockade of long-term potentiation by an N-methyl-D-aspartate receptor antagonist, AP5. *Nature*, 319(6056), 774–776.

- Moser, M. B., & Moser, E. I. (2000). Pretraining and the function of hippocampal long-term potentiation. *Neuron*, *26*(3), 559–561.
- Moss, S. J., & Smart, T. G. (2001). Constructing inhibitory synapses. *Nature Reviews*. *Neuroscience*, *2*(4), 240–250.
- Mulkey, R. M., Herron, C. E., & Malenka, R. C. (1993). An essential role for protein phosphatases in hippocampal long-term depression. *Science (New York, N.Y.)*, 261(5124), 1051–1055.
- Müller, R.-A., Shih, P., Keehn, B., Deyoe, J. R., Leyden, K. M., & Shukla, D. K. (2011). Underconnected, but How? A Survey of Functional Connectivity MRI Studies in Autism Spectrum Disorders. *Cerebral Cortex (New York, NY)*, 21(10), 2233–2243.
- Nakahira, E., & Yuasa, S. (2005). Neuronal generation, migration, and differentiation in the mouse hippocampal primoridium as revealed by enhanced green fluorescent protein gene transfer by means of in utero electroporation. *The Journal of Comparative Neurology*, 483(3), 329–340.
- Nelson, S. B., & Valakh, V. (2015). Excitatory/Inhibitory balance and circuit homeostasis in Autism Spectrum Disorders. *Neuron*, 87(4), 684–698.
- Neves, G., Cooke, S. F., & Bliss, T. V. P. (2008). Synaptic plasticity, memory and the hippocampus: a neural network approach to causality. *Nature Reviews. Neuroscience*, 9(1), 65–75.
- Newschaffer, C. J., Croen, L. A., Daniels, J., Giarelli, E., Grether, J. K., Levy, S. E., ... Windham, G. C. (2007). The epidemiology of autism spectrum disorders. *Annual Review* of Public Health, 28, 235–258.

Nicholls, R. E., Alarcon, J. M., Malleret, G., Carroll, R. C., Grody, M., Vronskaya, S., & Kandel,

E. R. (2008). Transgenic mice lacking NMDAR-dependent LTD exhibit deficits in behavioral flexibility. *Neuron*, *58*(1), 104–117.

- Nicoll, R. A., & Schmitz, D. (2005). Synaptic plasticity at hippocampal mossy fibre synapses. *Nature Reviews. Neuroscience*, *6*(11), 863–876.
- O'Connor, T. P., Cockburn, K., Wang, W., Tapia, L., Currie, E., & Bamji, S. X. (2009). Semaphorin 5B mediates synapse elimination in hippocampal neurons. *Neural Development*, *4*, 18.
- O'Roak, B. J., Deriziotis, P., Lee, C., Vives, L., Schwartz, J. J., Girirajan, S., ... Eichler, E. E. (2011). Exome sequencing in sporadic autism spectrum disorders identifies severe de novo mutations. *Nature Genetics*, 43(6), 585–589.
- Page, D. T., Kuti, O. J., Prestia, C., & Sur, M. (2009). Haploinsufficiency for Pten and Serotonin transporter cooperatively influences brain size and social behavior. *Proceedings of the National Academy of Sciences of the United States of America*, 106(6), 1989–1994.
- Palmer, C. L., Lim, W., Hastie, P. G. R., Toward, M., Korolchuk, V. I., Burbidge, S. A., ... Henley, J. M. (2005). Hippocalcin functions as a calcium sensor in hippocampal LTD. *Neuron*, 47(4), 487–494.
- Paradis, S., Harrar, D. B., Lin, Y., Koon, A. C., Hauser, J. L., Griffith, E. C., ... Greenberg, M.
 E. (2007). An RNAi-based approach identifies molecules required for glutamatergic and GABAergic synapse development. *Neuron*, *53*(2), 217–232.
- Pardo, C. A., & Eberhart, C. G. (2007). The neurobiology of autism. *Brain Pathology (Zurich, Switzerland)*, *17*(4), 434–447.
- Parikshak, N. N., Gandal, M. J., & Geschwind, D. H. (2015). Systems biology and gene networks in neurodevelopmental and neurodegenerative disorders. *Nature Reviews*.

Genetics, 16(8), 441–458.

- Pasterkamp, R. J. (2012). Getting neural circuits into shape with semaphorins. *Nature Reviews*. *Neuroscience*, *13*(9), 605–618.
- Peça, J., Feliciano, C., Ting, J. T., Wang, W., Wells, M. F., Venkatraman, T. N., ... Feng, G.
 (2011). Shank3 mutant mice display autistic-like behaviours and striatal dysfunction. *Nature*, 472(7344), 437–442.
- Pecho-Vrieseling, E., Sigrist, M., Yoshida, Y., Jessell, T. M., & Arber, S. (2009). Specificity of sensory-motor connections encoded by Sema3e-Plxnd1 recognition. *Nature*, 459(7248), 842–846.
- Peñagarikano, O., Abrahams, B. S., Herman, E. I., Winden, K. D., Gdalyahu, A., Dong, H., ... Geschwind, D. H. (2011). Absence of CNTNAP2 leads to epilepsy, neuronal migration abnormalities, and core autism-related deficits. *Cell*, 147(1), 235–246.
- Penzes, P., Beeser, A., Chernoff, J., Schiller, M. R., Eipper, B. A., Mains, R. E., & Huganir, R.
 L. (2003). Rapid induction of dendritic spine morphogenesis by trans-synaptic ephrinBEphB receptor activation of the Rho-GEF kalirin. *Neuron*, *37*(2), 263–274.
- Penzes, P., Cahill, M. E., Jones, K. A., VanLeeuwen, J.-E., & Woolfrey, K. M. (2011). Dendritic spine pathology in neuropsychiatric disorders. *Nature Neuroscience*, 14(3), 285–293.
- Pfrieger, F. W., & Barres, B. A. (1997). Synaptic efficacy enhanced by glial cells in vitro. Science (New York, N.Y.), 277(5332), 1684–1687.
- Rabacchi, S. A., Solowska, J. M., Kruk, B., Luo, Y., Raper, J. A., & Baird, D. H. (1999).
 Collapsin-1/semaphorin-III/D is regulated developmentally in Purkinje cells and collapses pontocerebellar mossy fiber neuronal growth cones. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 19(11), 4437–4448.

- Rao, A., Kim, E., Sheng, M., & Craig, A. M. (1998). Heterogeneity in the molecular composition of excitatory postsynaptic sites during development of hippocampal neurons in culture. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience, 18*(4), 1217–1229.
- Raymond, G. V., Bauman, M. L., & Kemper, T. L. (1996). Hippocampus in autism: a Golgi analysis. Acta Neuropathologica, 91(1), 117–119.
- Redfern, R. E., Daou, M.-C., Li, L., Munson, M., Gericke, A., & Ross, A. H. (2010). A mutant form of PTEN linked to autism. *Protein Science: A Publication of the Protein Society*, 19(10), 1948–1956.
- Robinson, G. B. (1992). Maintained saturation of hippocampal long-term potentiation does not disrupt acquisition of the eight-arm radial maze. *Hippocampus*, *2*(4), 389–395.
- Rodríguez-Escudero, I., Oliver, M. D., Andrés-Pons, A., Molina, M., Cid, V. J., & Pulido, R. (2011). A comprehensive functional analysis of PTEN mutations: implications in tumorand autism-related syndromes. *Human Molecular Genetics*, 20(21), 4132–4142.
- Roullet, F. I., Lai, J. K. Y., & Foster, J. A. (2013). In utero exposure to valproic acid and autism--a current review of clinical and animal studies. *Neurotoxicology and Teratology*, *36*, 47–56.
- Rubenstein, J. L. R., & Merzenich, M. M. (2003). Model of autism: increased ratio of excitation/inhibition in key neural systems. *Genes, Brain, and Behavior*, *2*(5), 255–267.
- Sabo, S. L., Gomes, R. A., & McAllister, A. K. (2006). Formation of presynaptic terminals at predefined sites along axons. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 26(42), 10813–10825.

Sacktor, T. C. (2008). PKMzeta, LTP maintenance, and the dynamic molecular biology of

memory storage. Progress in Brain Research, 169, 27-40.

- Sadanandam, A., Rosenbaugh, E. G., Singh, S., Varney, M., & Singh, R. K. (2010). Semaphorin
 5A promotes angiogenesis by increasing endothelial cell proliferation, migration, and
 decreasing apoptosis. *Microvascular Research*, 79(1), 1–9.
- Sadanandam, A., Varney, M. L., & Singh, R. K. (2008). Identification of semaphorin 5A interacting protein by applying apriori knowledge and peptide complementarity related to protein evolution and structure. *Genomics, Proteomics & Bioinformatics*, 6(3–4), 163– 174.
- Sahay, A., Kim, C.-H., Sepkuty, J. P., Cho, E., Huganir, R. L., Ginty, D. D., & Kolodkin, A. L. (2005). Secreted semaphorins modulate synaptic transmission in the adult hippocampus. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 25(14), 3613–3620.
- Sahin, M., & Sur, M. (2015). Genes, circuits, and precision therapies for autism and related neurodevelopmental disorders. *Science (New York, N.Y.)*, *350*(6263).
- Salinas, P. C., & Zou, Y. (2008). Wnt signaling in neural circuit assembly. *Annual Review of Neuroscience*, *31*, 339–358.
- Sandin, S., Lichtenstein, P., Kuja-Halkola, R., Larsson, H., Hultman, C. M., & Reichenberg, A. (2014). The familial risk of autism. *JAMA*, *311*(17), 1770–1777.
- Sansal, I., & Sellers, W. R. (2004). The biology and clinical relevance of the PTEN tumor suppressor pathway. *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology*, 22(14), 2954–2963.
- Scannevin, R. H., & Huganir, R. L. (2000). Postsynaptic organization and regulation of excitatory synapses. *Nature Reviews. Neuroscience*, 1(2), 133–141.

- Scheiffele, P., Fan, J., Choih, J., Fetter, R., & Serafini, T. (2000). Neuroligin expressed in nonneuronal cells triggers presynaptic development in contacting axons. *Cell*, 101(6), 657–669.
- Schmidt, R. J., Hansen, R. L., Hartiala, J., Allayee, H., Schmidt, L. C., Tancredi, D. J., ... Hertz-Picciotto, I. (2011). Prenatal vitamins, one-carbon metabolism gene variants, and risk for autism. *Epidemiology (Cambridge, Mass.)*, 22(4), 476–485.
- Schröder, G., & Schröder, J. (1992). A single change of histidine to glutamine alters the substrate preference of a stilbene synthase. *The Journal of Biological Chemistry*, 267(29), 20558– 20560.
- Selcher, J. C., Xu, W., Hanson, J. E., Malenka, R. C., & Madison, D. V. (2012). Glutamate receptor subunit GluA1 is necessary for long-term potentiation and synapse unsilencing, but not long-term depression in mouse hippocampus. *Brain Research*, 1435, 8–14.
- Shapira, M., Zhai, R. G., Dresbach, T., Bresler, T., Torres, V. I., Gundelfinger, E. D., ... Garner,
 C. C. (2003). Unitary assembly of presynaptic active zones from Piccolo-Bassoon
 transport vesicles. *Neuron*, 38(2), 237–252.
- Shelly, M., Cancedda, L., Lim, B. K., Popescu, A. T., Cheng, P., Gao, H., & Poo, M. (2011). Semaphorin3A regulates neuronal polarization by suppressing axon formation and promoting dendrite growth. *Neuron*, 71(3), 433–446.
- Shi, S., Hayashi, Y., Esteban, J. A., & Malinow, R. (2001). Subunit-specific rules governing AMPA receptor trafficking to synapses in hippocampal pyramidal neurons. *Cell*, 105(3), 331–343.
- Sigel, E., & Steinmann, M. E. (2012). Structure, function, and modulation of GABA(A) receptors. *The Journal of Biological Chemistry*, 287(48), 40224–40231.

- Skuse, D. H. (2007). Rethinking the nature of genetic vulnerability to autistic spectrum disorders. *Trends in Genetics: TIG*, *23*(8), 387–395.
- Sousa, N., Lukoyanov, N. V., Madeira, M. D., Almeida, O. F., & Paula-Barbosa, M. M. (2000).
 Reorganization of the morphology of hippocampal neurites and synapses after stressinduced damage correlates with behavioral improvement. *Neuroscience*, 97(2), 253–266.
- Spruston, N. (2008). Pyramidal neurons: dendritic structure and synaptic integration. *Nature Reviews. Neuroscience*, *9*(3), 206–221.
- Steward, O., & Schuman, E. M. (2001). Protein synthesis at synaptic sites on dendrites. *Annual Review of Neuroscience*, 24, 299–325.
- Stiles, J., & Jernigan, T. L. (2010). The Basics of Brain Development. *Neuropsychology Review*, 20(4), 327–348.
- Südhof, T. C. (2013). Neurotransmitter release: the last millisecond in the life of a synaptic vesicle. *Neuron*, *80*(3), 675–690.
- Suto, F., Tsuboi, M., Kamiya, H., Mizuno, H., Kiyama, Y., Komai, S., ... Fujisawa, H. (2007).
 Interactions between plexin-A2, plexin-A4, and semaphorin 6A control lamina-restricted projection of hippocampal mossy fibers. *Neuron*, *53*(4), 535–547.
- Sweeney, L. B., Chou, Y.-H., Wu, Z., Joo, W., Komiyama, T., Potter, C. J., ... Luo, L. (2011). Secreted semaphorins from degenerating larval ORN axons direct adult projection neuron dendrite targeting. *Neuron*, 72(5), 734–747.
- Takahashi, T., Fournier, A., Nakamura, F., Wang, L. H., Murakami, Y., Kalb, R. G., ... Strittmatter, S. M. (1999). Plexin-neuropilin-1 complexes form functional semaphorin-3A receptors. *Cell*, 99(1), 59–69.

Takahashi, T., Nakamura, F., Jin, Z., Kalb, R. G., & Strittmatter, S. M. (1998). Semaphorins A

and E act as antagonists of neuropilin-1 and agonists of neuropilin-2 receptors. *Nature Neuroscience*, *1*(6), 487–493.

- Takamori, S. (2006). VGLUTs: "exciting" times for glutamatergic research? *Neuroscience Research*, *55*(4), 343–351.
- Takeuchi, T., Duszkiewicz, A. J., & Morris, R. G. M. (2014). The synaptic plasticity and memory hypothesis: encoding, storage and persistence. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 369(1633).
- Tamagnone, L., Artigiani, S., Chen, H., He, Z., Ming, G. I., Song, H., ... Comoglio, P. M. (1999). Plexins are a large family of receptors for transmembrane, secreted, and GPIanchored semaphorins in vertebrates. *Cell*, 99(1), 71–80.
- Tavazoie, S. F., Alvarez, V. A., Ridenour, D. A., Kwiatkowski, D. J., & Sabatini, B. L. (2005). Regulation of neuronal morphology and function by the tumor suppressors Tsc1 and Tsc2. *Nature Neuroscience*, 8(12), 1727–1734.
- Tick, B., Bolton, P., Happé, F., Rutter, M., & Rijsdijk, F. (2016). Heritability of autism spectrum disorders: a meta-analysis of twin studies. *Journal of Child Psychology and Psychiatry,* and Allied Disciplines, 57(5), 585–595.
- Togashi, H., Abe, K., Mizoguchi, A., Takaoka, K., Chisaka, O., & Takeichi, M. (2002). Cadherin regulates dendritic spine morphogenesis. *Neuron*, *35*(1), 77–89.
- Tran, T. S., Rubio, M. E., Clem, R. L., Johnson, D., Case, L., Tessier-Lavigne, M., ... Kolodkin,
 A. L. (2009). Secreted semaphorins control spine distribution and morphogenesis in the postnatal CNS. *Nature*, 462(7276), 1065–1069.
- Tritsch, N. X., Granger, A. J., & Sabatini, B. L. (2016). Mechanisms and functions of GABA corelease. *Nature Reviews. Neuroscience*, 17(3), 139–145.

- Tsaneva-Atanasova, K., Burgo, A., Galli, T., & Holcman, D. (2009). Quantifying neurite growth mediated by interactions among secretory vesicles, microtubules, and actin networks. *Biophysical Journal*, 96(3), 840–857.
- Tsien, J. Z., Huerta, P. T., & Tonegawa, S. (1996). The essential role of hippocampal CA1 NMDA receptor-dependent synaptic plasticity in spatial memory. *Cell*, 87(7), 1327– 1338.
- Uesaka, N., Uchigashima, M., Mikuni, T., Nakazawa, T., Nakao, H., Hirai, H., ... Kano, M. (2014). Retrograde semaphorin signaling regulates synapse elimination in the developing mouse brain. *Science (New York, N.Y.)*, 344(6187), 1020–1023.
- Umeda, T., & Okabe, S. (2001). Visualizing synapse formation and remodeling: recent advances in real-time imaging of CNS synapses. *Neuroscience Research*, *40*(4), 291–300.
- Usui, H., Taniguchi, M., Yokomizo, T., & Shimizu, T. (2003). Plexin-A1 and plexin-B1 specifically interact at their cytoplasmic domains. *Biochemical and Biophysical Research Communications*, 300(4), 927–931.
- Vaillant, A. R., Zanassi, P., Walsh, G. S., Aumont, A., Alonso, A., & Miller, F. D. (2002).
 Signaling mechanisms underlying reversible, activity-dependent dendrite formation.
 Neuron, 34(6), 985–998.
- van Strien, N. M., Cappaert, N. L. M., & Witter, M. P. (2009). The anatomy of memory: an interactive overview of the parahippocampal-hippocampal network. *Nature Reviews*. *Neuroscience*, 10(4), 272–282.
- Verbich, D., Prenosil, G. A., Chang, P. K.-Y., Murai, K. K., & McKinney, R. A. (2012). Glial glutamate transport modulates dendritic spine head protrusions in the hippocampus. *Glia*, 60(7), 1067–1077.

- Villa, K. L., & Nedivi, E. (2016). Excitatory and Inhibitory Synaptic Placement and Functional Implications. In *Dendrites* (pp. 467–487). Springer, Tokyo.
- Wang, D., Cui, Z., Zeng, Q., Kuang, H., Wang, L. P., Tsien, J. Z., & Cao, X. (2009). Genetic enhancement of memory and long-term potentiation but not CA1 long-term depression in NR2B transgenic rats. *PloS One*, 4(10), e7486.
- Washbourne, P., Bennett, J. E., & McAllister, A. K. (2002). Rapid recruitment of NMDA receptor transport packets to nascent synapses. *Nature Neuroscience*, *5*(8), 751–759.
- Weiner, J. A., Jontes, J. D., & Burgess, R. W. (2013). Introduction to mechanisms of neural circuit formation. *Frontiers in Molecular Neuroscience*, 6.
- Weiss, L. A., Arking, D. E., Gene Discovery Project of Johns Hopkins & the Autism Consortium, Daly, M. J., & Chakravarti, A. (2009). A genome-wide linkage and association scan reveals novel loci for autism. *Nature*, 461(7265), 802–808.
- Wells, D. G., Richter, J. D., & Fallon, J. R. (2000). Molecular mechanisms for activity-regulated protein synthesis in the synapto-dendritic compartment. *Current Opinion in Neurobiology*, 10(1), 132–137.
- Williams, M. R., DeSpenza, T., Li, M., Gulledge, A. T., & Luikart, B. W. (2015). Hyperactivity of newborn Pten knock-out neurons results from increased excitatory synaptic drive. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 35(3), 943–959.
- Wiznitzer, M. (2004). Autism and tuberous sclerosis. *Journal of Child Neurology*, *19*(9), 675–679.
- Won, H., Mah, W., & Kim, E. (2013). Autism spectrum disorder causes, mechanisms, and treatments: focus on neuronal synapses. *Frontiers in Molecular Neuroscience*, 6, 19.

- Wu, G. Y., Zou, D. J., Rajan, I., & Cline, H. (1999). Dendritic dynamics in vivo change during neuronal maturation. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 19(11), 4472–4483.
- Wyllie, D. J. A., Livesey, M. R., & Hardingham, G. E. (2013). Influence of GluN2 subunit identity on NMDA receptor function. *Neuropharmacology*, 74, 4–17.
- Xie, C., Markesbery, W. R., & Lovell, M. A. (2000). Survival of hippocampal and cortical neurons in a mixture of MEM+ and B27-supplemented neurobasal medium. *Free Radical Biology & Medicine*, 28(5), 665–672.
- Xue, Y., Chen, Y., Ayub, Q., Huang, N., Ball, E. V., Mort, M., ... 1000 Genomes Project Consortium. (2012). Deleterious- and disease-allele prevalence in healthy individuals: insights from current predictions, mutation databases, and population-scale resequencing. *American Journal of Human Genetics*, *91*(6), 1022–1032.
- Yazdani, U., & Terman, J. R. (2006). The semaphorins. Genome Biology, 7(3), 211.
- Yuen, R. K. C., Merico, D., Cao, H., Pellecchia, G., Alipanahi, B., Thiruvahindrapuram, B., ... Scherer, S. W. (2016). Genome-wide characteristics of de novo mutations in autism. NPJ Genomic Medicine, 1, 160271–1602710.
- Zeng, H., Chattarji, S., Barbarosie, M., Rondi-Reig, L., Philpot, B. D., Miyakawa, T., ...
 Tonegawa, S. (2001). Forebrain-specific calcineurin knockout selectively impairs
 bidirectional synaptic plasticity and working/episodic-like memory. *Cell*, 107(5), 617–629.
- Zhang, X. C., Piccini, A., Myers, M. P., Van Aelst, L., & Tonks, N. K. (2012). Functional analysis of the protein phosphatase activity of PTEN. *The Biochemical Journal*, 444(3), 457–464.

- Zhou, J., Blundell, J., Ogawa, S., Kwon, C.-H., Zhang, W., Sinton, C., ... Parada, L. F. (2009). Pharmacological inhibition of mTORC1 suppresses anatomical, cellular, and behavioral abnormalities in neural-specific Pten knock-out mice. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 29(6), 1773–1783.
- Zhou, Q., Homma, K. J., & Poo, M. (2004). Shrinkage of dendritic spines associated with longterm depression of hippocampal synapses. *Neuron*, 44(5), 749–757.
- Zhu, L., Wang, X., Li, X.-L., Towers, A., Cao, X., Wang, P., ... Jiang, Y.-H. (2014). Epigenetic dysregulation of SHANK3 in brain tissues from individuals with autism spectrum disorders. *Human Molecular Genetics*, 23(6), 1563–1578.
- Zilberter, M. (2016). Reality of Inhibitory GABA in Neonatal Brain: Time to Rewrite the Textbooks? *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, 36(40), 10242–10244.
- Ziv, N. E., & Smith, S. J. (1996). Evidence for a role of dendritic filopodia in synaptogenesis and spine formation. *Neuron*, 17(1), 91–102.