DEFECTIVE TrkB SIGNALING PATHWAYS

IN IDIOPATHIC AUTISM

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In Search of the Molecular Mechnisms Underlying Autistic Behaviour

By

CHIARA NICOLINI, B.Sc., M.Sc.

A Thesis

Submitted to the School of Graduate Studies in Partial Fulfillment

of the Requirements for the Degree Doctor of Philosophy

DOCTOR OF PHILOSOPHY Medical Sciences (2016)

McMaster University, Hamilton, ON

TITLE: Defective TrkB Signaling Pathways in Idiopathic Autism

AUTHOR: Chiara Nicolini, B.Sc. (University of Modena and Reggio Emilia), M.Sc. (University of Trieste)

SUPERVISOR: Dr. Margaret Fahnestock

NUMBER OF PAGES: xviii, 191

ABSTRACT

Autism is a neurodevelopmental disorder characterized by impairments in social communication and interaction and by repetitive patterns of behaviour, interests and activities. It is perhaps the most common and handicapping neurological disorder of childhood and as such represents a significant public health problem and a huge burden for education and social service systems. Currently there is no diagnostic test or cure available for autism and the molecular mechanisms underlying autistic behaviour remain to be elucidated. Mutations in genes linked to autism adversely affect molecules involved in synapse development and plasticity including brain-derived neurotrophic factor receptor (TrkB) and its downstream effector mammalian target of rapamycin (mTOR), which is increased in several forms of syndromic autism.

Here, we investigated whether TrkB, mTOR and their signaling pathways are disrupted in postmortem brain tissue from subjects with idiopathic autism, that is, cases of autism without a known genetic cause and thought to be of environmental/epigenetic origin. We next further examined the contribution of defective TrkB signaling to autistic behaviour in mice exposed to the histone deacetylase inhibitor valproic acid (VPA), a well-established model of environmental/epigenetic origin of autism.

We found that TrkB signaling pathways were reduced in idiopathic autism and that these disruptions were associated with decreased excitatory postsynaptic marker PSD-95, suggesting fewer excitatory synapses. Moreover,

we showed that similar molecular deficits were present in VPA-exposed mice that lacked sociability and displayed increased repetitive, stereotyped behaviour. We also determined that behavioural deficits in these mice were rescued by administration of the partial TrkB agonist LM22A-4 but not by treatment with the active tripeptide fragment of the insulin-like growth factor-1, (1-3)IGF-1. Lastly, reduced TrkB signaling in VPA-exposed mice was normalized by LM22A-4 administration combined with behavioural enrichment.

The present work provides a better understanding of the molecular mechanisms that contribute to autistic behaviour and implicates TrkB signaling in autism pathogenesis. Furthermore, these data demonstrate that molecular changes observed in brains of patients with idiopathic autism differ from syndromic forms and highlight that both too much and too little signaling can be equally disruptive. The present work also shows that maternal challenge with VPA resulted in social deficits, increased repetitive, restrictive behaviour and reduced TrkB signaling in mice, pointing to epigenetic modifications as a potential underlying mechanism of molecular and behavioural disruptions in autism. Lastly, these findings suggest that pharmacological activation of TrkB using compounds such as the partial TrkB agonist LM22A-4 might play a role in treating sociability and repetitive, perseverative behaviour in autism.

ACKNOWLEDGEMENTS

It would have not been possible to write my thesis without the help and support of the many people who accompanied me through this journey. First and foremost, I would like to express my sincerest gratitude to my supervisor, Dr. Margaret Fahnestock, who welcomed me into her lab and gave me the opportunity to carry out this work. She helped me grow both scientifically and personally and will always be a model I aspire to. Her immense love and devotion to science struck me from the very first time we met in Trieste and will always inspire me. I thank her deeply for her guidance, mentorship, and constant encouragement that kept me motivated throughout.

I would next like to greatly thank my other supervisory committee members, Dr. Jane Foster and Dr. Ram Mishra, for their mentorship and valuable guidance. They were extremely supportive and provided me with much advice throughout my work. Their insightful comments helped me focus my ideas and choose the right direction to complete this work.

I am forever grateful to Bernadeta, our lab technician, for everything she taught me, all her help and support. She was of great importance and I would have never made it through without her.

I am also extremely grateful and indebted to Elyse for her help, support and friendship. I couldn't have done it without her. I am very happy we shared this journey.

I owe my eternal gratitude to Vadim for all he taught me and for being always ready to help and support me at any time. He never hesitated. I would have not been able to do this without him.

A very special thanks to my parents for their lifelong support and encouragement.

I would also like to thank my sister, my brother and my friends, especially Ele, Elisa, Denise, Mike and Miriana, Otti, Nadia, Paolo, Samina and Simon, for their extreme patience and for being always there for me.

Last but not least, I would like to acknowledge my partner, Emily. I would have not made it through without her love, constant support and help. She allowed me to keep the strength, the determination and the clarity of mind to reach this goal. I thank her immensely.

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α-CaMKII	α-Ca2+/Calmodulin kinase II
Akt	RAC-alpha serine/threonine-protein kinase (also known as protein kinase B, PKB)
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Arc	Activity-regulated cytoskeletal-associated protein
ASD	Autism Spectrum Disorder
BDNF	Brain-derived neurotrophic factor
CNS	Central nervous system
CpG	Cytosine phosphatidyl guanine
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dTTP	Deoxythymidine triphosphate
4E-BP1	4E-Binding protein 1
EDTA	Ethylenediaminetetraacetic acid
eIF4B	Eukaryotic translation initiation factor 4B
eIF4E	Eukaryotic translation initiation factor 4E
eIF4F	Eukaryotic translation initiation factor 4F
eIF4G	Eukaryotic translation initiation factor 4G
Eps8	Epidermal growth factor receptor pathway substrate 8
ERK	Extracellular signal-regulated kinase (also known as mitogen-activated protein kinase, MAPK)
FDA	Food and Drug Administration
FMR1	Fragile X mental retardation 1 gene

FMRP	Fragile X mental retardation protein
FXS	Fragile X syndrome
GAP	GTPase-activating protein
GFAP	Glial fibrillary acidic protein
GSK3β	Glycogen synthase kinase 3 beta
GTP	Guanosine triphosphate
HDAC	Histone deacetylase
i.p.	Intraperitoneal
ICR(CD-1)	Institute for Cancer Research; Caesarean Derived-1
ID	Intellectual disability
IGF-1	Insulin-like growth factor 1
IRES	Internal ribosomal entry site
LTD	Long-term depression
LTP	Long-term potentiation
MeCP2	Methyl-CpG-binding protein 2
MMP-7	Matrixmetalloprotease-7
mTOR	Mammalian target of rapamycin
n.s.	Non-significant
NF	Neurofibromatosis (type I)
NF1	Neurofibromin 1
NGF	Nerve growth factor
NICHD	National Institute of Child Health and Human Development
NLGN4	Neuroligin-4
NMDA	N-methyl-D-aspartate
NT-3	Neurotrophin-3

NT-4/5	Neurotrophin-4/5
NTRK2	Neurotrophic tyrosine kinase receptor type 2 (also known as tyrosine kinase receptor B, TRKB)
P or PD	Postnatal day
p70S6K	p70 kDa-Ribosomal protein S6 kinase
$p75^{NTR}$	p75 kDa-Neurotrophin receptor
PBS	Phosphate-buffered saline
PI3K	Phosphoinositide-3'-kinase
PIP3	Phosphatidylinositol (3,4,5)-triphosphate
PMI	Postmortem interval
proBDNF	Precursor brain-derived neurotrophic factor
PSD-95	Postsynaptic density protein 95
PTEN	Phosphatase and tensin homolog on chromosome ten
PV	Parvalbumin
PVDF	Polyvinylidene difluoride membrane
qPCR	Quantitative real-time polymerase chain reaction
Rac	Ras-related C3 botulinum toxin substrate
Ras	Rat sarcoma
Rheb	Ras homolog enriched in the brain protein
rhIGF-1	Recombinant human insulin-like growth factor 1
RhoA	Ras homolog gene family member A
RT-PCR	Reverse transcription polymerase chain reaction
RTT	Rett syndrome
S6 or rp S6	Ribosomal protein S6
SD	Standard deviation
SDS	Sodium dodecyl sulfate

SE	Standard error
Shc	Src homology 2 domain containing
SKI-1	Subtilisin-kexin-like isozyme-1
SNP	Single nucleotide polymorphism
SSRI	Selective serotonin re-uptake inhibitor
TrkB	Tropomyosin related kinase B
TSC	Tuberous sclerosis complex
TSC1	Hamartin
TSC2	Tuberin
VPA	Valproic acid

CHAPTER 1: INTRODUCTION

1.1 Autism

Autism is a life-long neurodevelopmental disorder, clinically characterized by social communication and interaction impairments, restricted interests and repetitive behaviours (DSM-5, 2013; Lahey, 2006). It belongs to a heterogenous group of disorders called Autism Spectrum Disorders (ASDs) that show considerable overlap but vary in severity and combination of symptoms. With a prevalence in the US and Canada of 1 in 68 individuals (CDC MMWR, 2014; NEDSAC, 2012), autism is perhaps the most common and handicapping neurological disorder of childhood (Offord, 1982). It represents a significant public health problem and a huge burden for education and social service systems (Järbrink & Knapp, 2001). Currently, there is no diagnostic test or cure available for autism and the etiology of this disease is still unknown.

Autism has a strong genetic component with a complex pattern of transmission (Bailey et al., 1995; Fombonne et al., 1998) thought to be the result of perhaps as many as 1000 interacting genes (De Rubeis et al., 2014; Gaugler et al., 2014). Indeed, recent efforts have identified hundreds of rare genetic events that carry increased risk for ASD, many of them arising *de novo* (Iossifov et al., 2014; Neale et al., 2012; O'Roak et al., 2012; Ronemus et al., 2014; Sanders et al., 2012). However, there are many cases of autism, termed "idiopathic", for which there is no known genetic cause. Idiopathic autism is likely due to environmental factors (Sandin et al., 2014) including *in utero*

exposure to drugs, such as valproic acid, and environmental toxins, such as pesticides, infection, paternal age, or other insults (Berko et al., 2014; Moore et al., 2000; Rasalam et al., 2005; St-Hilaire et al., 2012; Tordjman et al., 2014; Volk et al., 2013; Williams et al., 2001). These environmental influences might confer autism susceptibility by inducing epigenetic changes (Bushnell, 2013; Chaste & Leboyer, 2012; Flashner et al., 2013; LaSalle, 2013; Miyake et al., 2012; Siniscalco et al., 2013; Wong et al., 2014). Studies showing that autistic behaviour arises from epigenetic mutations (Fragile X syndrome) or disruption of key epigenetic regulatory factors (Rett syndrome) corroborates the hypothesis that epigenetic mechanisms are involved in the etiology of idiopathic autism. Further support for this hypothesis comes from recent exome sequencing studies showing that rare sequence variations occur in genes coding for transcriptional and chromatin-remodeling proteins (De Rubeis et al., 2014). Collectively, these findings highlight that epigenetic dysregulation of gene expression might provide a link between gene and environment in autism pathogenesis. Nevertheless, the proteins and pathways that lead from genes/environment to behaviour remain to be elucidated.

1.1.1 Synaptic Dysfunction in ASDs

Functional/structural imaging and human post-mortem brain studies have shown that impairments in cortical connectivity and synaptic dysfunction are a hallmark of autism and likely account for cognitive and behavioural deficits of autistic patients (Amaral et al., 2008; Just et al., 2007; Minshew & Williams, 2007; Rubenstein & Merzenich, 2003; Shukla et al., 2011a,b).

Autism-linked mutations have been found in genes that code for central synaptic organizing molecules including postsynaptic adhesion and scaffolding molecules such as neuroligins and shanks and their presynaptic partners neurexins (Berkel et al., 2010; Bourgeron, 2009; Durand et al., 2007; Lionel et al., 2013; Parikshak et al., 2013; Pinto et al., 2010; Südhof, 2008). Remarkably, animal models carrying these mutations exhibit autistic-like behaviours (Blundell et al., 2010; Jamain et al., 2008; Peça et al. 2011; Tabuchi et al., 2007; Won et al., 2012). Also, single-gene mutations causing disorders with high rates of autism such as tuberous sclerosis (mutations in TSC1/TSC2), neurofibromatosis (mutations in NF1), and macrocephaly (mutations in PTEN) (Ehninger & Silva, 2011; Hoeffer & Klann, 2010; Kelleher & Bear, 2008; Levitt and Campbell, 2009) dysregulate the Akt-mTOR pathway, that controls protein translation at the synapse (Hay & Sonenberg, 2004; Jaworski et al., 2005; Santos et al., 2010). Lastly, specific SNP haplotypes of brain-derived neurotrophic factor (BDNF), a key regulator of cortical development and synaptic function and plasticity (Kaplan & Miller, 2000; Yoshii & Constantine-Paton, 2010), as well as multiple SNPs and haplotypes of its receptor gene *(NTRK2)* have been linked to autism (Correia et al., 2010; Nishimura et al., 2007). Taken together, these findings have focused attention on aberrant connectivity resulting from alterations at synapses as an underlying cause of both genetically-linked ASD and idiopathic autism.

1.2 BDNF & Its Receptors

BDNF is a member of the mammalian protein family of neurotrophins including nerve growth factor (NGF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5), originally identified as neuronal survival factors. Since their discovery, neurotrophins have been implicated in a multitude of processes that are crucial for prenatal and postnatal brain development (Croll et al., 1998) and synaptic plasticity (Bramham et al., 2005; Lu B., 2003). BDNF, especially, plays a key role in neuronal development and differentiation, axonal and dendritic growth, synaptogenesis, dendritic spine homeostasis, excitation/inhibition balance, synaptic transmission and cortical organization in the central nervous system (CNS) (Ann et al., 2008; Chapleau et al., 2009; Horch, 2004; Huang & Reichardt, 2001; Jeanneteau et al., 2010; Leal et al., 2014; Rutherford et al., 1998; Tanaka et al., 2008). It also regulates long-term potentiation (LTP) in the hippocampus, modulating hippocampal plasticity and memory consolidation (Egan et al., 2003; Jankowsky & Patterson, 1999; Tyler et al., 2002).

BDNF is synthesized as a 32kDa precursor, proBDNF, which can be further processed to either a 14kDa mature or a 28kDa truncated form (**Figure 1**). Mature BDNF is generated intracellularly by furin (Mowla et al., 2001) and extracellularly by plasmin or matrixmetalloprotease-7 (MMP-7) (Lee et al, 2001; Pang et al., 2004). Truncated BDNF is generated through cleavage of proBDNF by a specific, membrane-bound, calcium-dependent serine proteinase known as subtilisin-kexin-like isozyme-1 (SKI-1) (Seidah et al., 1999) and cannot be further processed into mature BDNF.

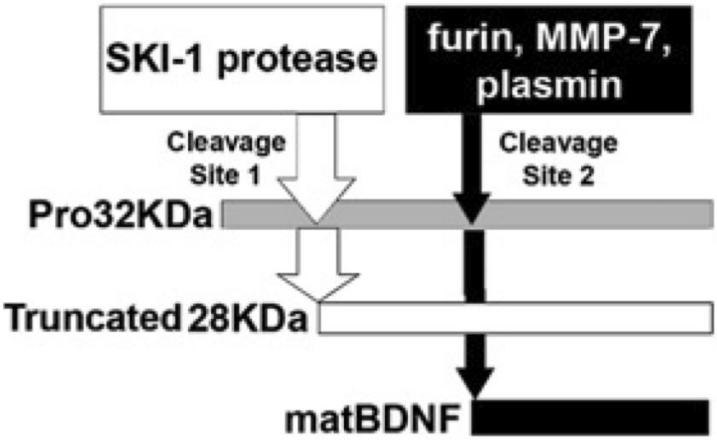

Figure 1. Schematic diagram of precursor BDNF proteolytic processing. Cleavage of proBDNF by SKI-1 protease generates truncated BDNF, while cleavage by furin, MMP-7 or plasmin produces mature BDNF (Adapted from Carlino et al., 2008).

ProBDNF and mature BDNF have distinct and sometimes opposing functions in the brain (Lu et al., 2005). ProBDNF reduces neuronal differentiation and dendritic spines and induces apoptosis and long-term depression (LTD) in cultured neurons, while mature BDNF promotes spine formation, neuronal survival and LTP (Koshimizu et al., 2009; Pang et al., 2004; Teng et al., 2005; Woo et al., 2005). This suggests that the correct balance between proBDNF and mature BDNF is crucial for synaptic development and plasticity, and that an imbalance of these two BDNF isoforms may result in synaptic abnormalities. The biological activities and roles of truncated BDNF are still unknown.

ProBDNF and mature BDNF bind to two different types of receptors. Mature BDNF binds primarily to the tropomyosin related kinase B receptor (TrkB) leading to the activation of several intracellular pathways, including the phosphoinositide-3'-kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR) pathway, the extracellular signal-regulated kinase (ERK) pathway and the Epidermal growth factor receptor pathway substrate 8 (Eps8)-Rac pathway (**Figure 2**) (Fahnestock & Nicolini, 2015). These pathways regulate synapse formation and function in the developing and adult brain by controlling actin cytoskeletal remodeling, protein synthesis and PSD-95 trafficking at synapses (Kaplan & Miller, 2000; Offenhäuser et al., 2004; Santos et al., 2010; Yoshii & Constantine-Paton, 2010). Specifically, the PI3K-Eps8-Rac pathway modulates actin cytoskeletal reorganization (Innocenti et al., 2002), thus influencing synaptic plasticity and dendritic spine stability and density (Menna et al., 2013). The Akt-mTOR pathway controls spine protein synthesis via two downstream cascades, 4E-BP1/eIF4E and S6 kinase/eIF4B/S6, while the ERK signaling cascade regulates both translation via eIF4E (Bramham & Wells, 2007; Takei et al., 2001) and cytoskeletal remodeling via Eps8 (Innocenti et al., 2002). Lastly, PI3K/Akt contributes to TrkB-mediated PSD-95 trafficking to excitatory synapses (Yoshii & Constantine-Paton, 2010).

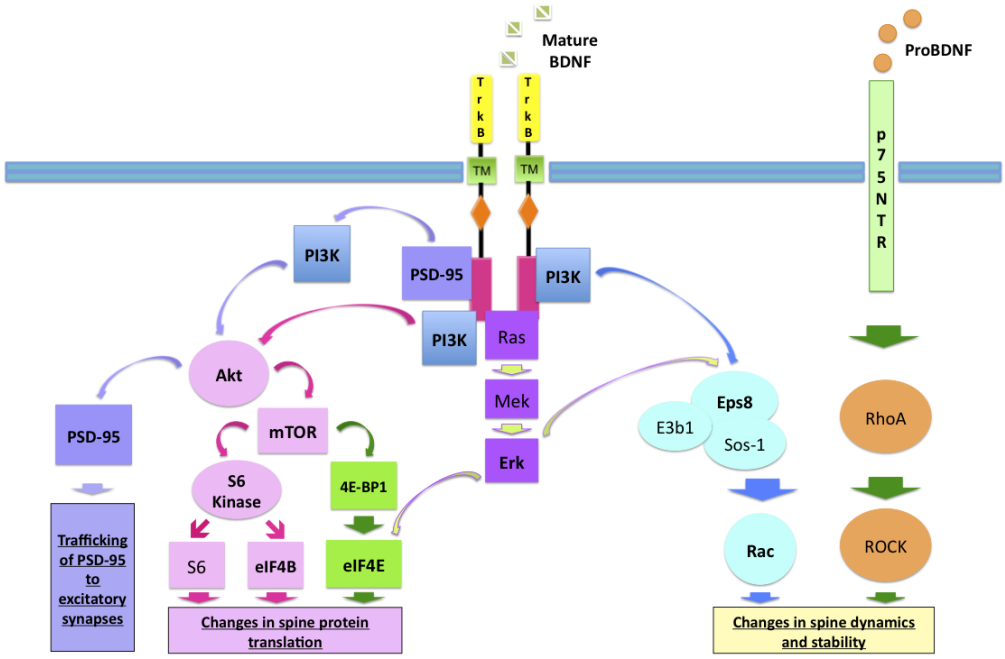

Figure 2. BDNF/TrkB-activated intracellular signaling cascades involved in spine protein synthesis and actin remodeling at synapses. These include the PI3K-Akt-mTOR pathway, which regulates spine protein synthesis via two downstream signaling cascades, 4E-BP1/eIF4E and S6 kinase/S6, the PI3K/Eps8/Rac pathway modulating Rac-dependent actin cytoskeletal reorganization and the ERK cascade controlling both translation and actin remodeling. Additionally, PSD-95 forms a complex with TrkB that signals via PI3K-Akt, promoting recruitment of PSD-95 to excitatory synapses. Lastly, proBDNF/$p75^{NTR}$ via RhoA opposes Eps8/Rac and induces dendritic spine destabilization and neurite retraction. (Fahenstock & Nicolini, 2015)

TrkB is densely expressed on cortical and hippocampal neurons and is involved in formation of both glutamatergic and GABAergic synapses during development (McAllister, 2002; Seil & Drake-Baumann, 2000; Spenger et al., 1995). However, with maturation it becomes enriched at excitatory synapses (Gomes et al., 2006). TrkB modulates synaptogenesis, neural survival and differentiation and activity-dependent synaptic plasticity (Binder & Scharfman, 2004; Kang et al., 1997; Yamada & Nabeshima, 2003). The human TrkB gene is large and contains 24 exons (Stoilov et al., 2002). The first 5 exons have internal ribosomal entry site (IRES) activity that guides ribosomes to the translation start site and enhances translation initiation (Dobson et al., 2005). The TrkB extracellular domain containing the signal sequence for membrane localization is encoded by exons 5-14 (Schneider & Schweiger, 1991; Shelton et al., 1995), while the TrkB intracellular portion with the tyrosine kinase domain is encoded by exons 20-24 (Middlemas et al., 1991). Exon 12 encodes the immunoglobulin-like domain responsible for neurotrophin binding and exon 15 codes for the transmembrane domain (Urfer et al., 1995). TrkB has three major splice variants which generate a full-length receptor (TrkB-FL), a truncated isoform lacking the tyrosine kinase domain (TrkB-T1) and a truncated isoform lacking the tyrosine kinase domain but containing a Shc binding site (TrkB-Shc) (**Figure 3**) (Klein et al., 1990; Stoilov et al., 2002). The TrkB transcripts encoding full-legth receptors and truncated TrkB-T1 are the most abundant in the cerebral cortex (Luberg et al., 2010). The expression levels of TrkB isoform mRNAs change during postnatal brain development (Luberg et al., 2010). Specifically, in the human

prefrontal cortex TrkB-FL mRNA expression peaks in toddlers, decreases slightly during school age and then remains unchanged until early adulthood, decreasing significantly in adults. Conversely, TrkB-T1 mRNA levels decrease in toddlers and during school age, and increase throughout adulthood. Lastly, TrkB-Shc mRNA expression peaks during infancy and then decreases gradually (Luberg et al., 2010). Interestingly, TrkB isoform transcripts have different expression profiles from their corresponding proteins (Luberg et al., 2010). TrkB-FL protein levels peak during infancy and decrease gradually throughout adulthood, while TrkB-T1 protein expression rises steadily into adolescence and decreases only slightly into adulthood. Protein expression levels of TrkB-Shc do not show significant changes (Luberg et al., 2010). While the exact mechanisms underlying this disconnect between TrkB isoform mRNA and protein expression patterns remain to be determined, Luberg et al. (2010) proposed that post-transcriptional mechanisms are likely to be implicated. These may be microRNA-mediated (see Discussion).

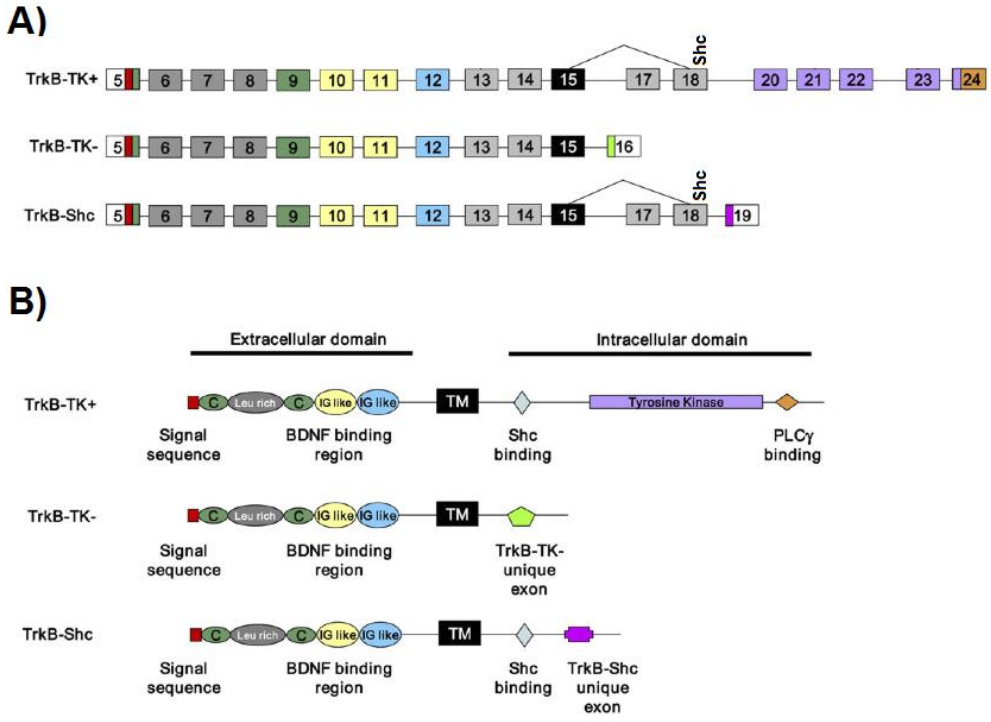

Figure 3. Structure of the three best characterized TrkB transcripts (**A**) and protein isoforms (**B**): Full-length TrkB (TrkB-TK+), truncated TrkB (TrkB-TK-) and src homology binding site-containing TrkB (TrkB-Shc). (**A**) Filled and numbered boxes represent sequences encoding protein. (**B**) Functional domains of TrkB proteins. C, cysteine rich region; Leu rich, leucine rich region; IG like, immunoglobulin-like domain; TM, transmembrane domain; TK, tyrosine kinase domain. (Adapted from Wong et al., 2011).

Full-length receptors are expressed almost exclusively in pyramidal neurons and interneurons, contain an intracellular catalytic tyrosine kinase domain and hence mediate classic neurotrophic BDNF signaling (Klein et al., 1989, 1991; Reichardt, 2006). Conversely, truncated TrkB isoforms, which are located on both neurons and glia (Frisén et al., 1993; Ohira & Hayashi, 2003), are able to bind BDNF but, lacking tyrosine kinase activity, cannot elicit the normal cellular response to BDNF. Hence, truncated TrkB isoforms might inhibit BDNF signaling either by sequestering BDNF and reducing its availability for binding to TrkB-FL or by acting as dominant-negative and forming inactive heterodimers with the full-length receptors (Eide et al., 1996; Fenner, 2012). Neurotrophic effects of BDNF depend on the relative levels of TrkB isoforms (Armanini et al., 1995; Eide et al., 1996).

Mature BDNF and proBDNF also bind to the low-affinity pan-neurotrophin receptor $p75^{NTR}$, a member of the tumor necrosis factor receptor superfamily (Dechant & Barde, 2002; Smith et al., 1994) and the first neurotrophin receptor to be discovered (Johnson et al., 1986; Radeke et al., 1987). $p75^{NTR}$ lacks intrinsic enzymatic activity and associates with cytoplasmic protein such as RhoA to signal (Yamashita et al., 1999). It is highly expressed by many populations of neurons during the outgrowth of axons and dendritic arborization (Buck et al., 1987; Yan & Johnson, 1988). Opposite to TrkB, $p75^{NTR}$ mediates apoptosis, neurite retraction and LTD (Bamji et al., 1998; Kaplan & Miller 2000; Roux & Barker, 2002; Woo et al., 2005). In particular, $p75^{NTR}$ reduces the length of filopodia and neurites via RhoA activation (Yamashita et al., 1999). $p75^{NTR}$ also negatively regulates

dendrite morphology and dendritic spine density in hippocampal pyramidal neurons (Zagrebelsky et al., 2005). Mature BDNF binds more strongly to TrkB receptors (Huang & Reichardt, 2003), wheras proBDNF binds more strongly to $p75^{NTR}$ (Lee at al., 2001) hence providing a balance between synaptic strengthening (BDNF/TrkB) and synaptic weakening (proBDNF/$p75^{NTR}$). It follows that maintaining a balance between the relative levels of BDNF isoforms and their receptors is critical for normal synaptic function and cortical circuitry development. Changes in BDNF and TrkB isoform levels are implicated in neuropsychiatric disorders marked by altered cortical maturation and synaptic dysfunction including schizophrenia (Carlino et al., 2011; Hung & Huang, 2013; Weickert et al., 2005; Wong et al., 2010, 2013), depression (Hung et al., 2010; Karege et al., 2002) and Alzheimer's disease (Holsinger et al., 2000; Michalski & Fahnestock, 2003; Peng et al., 2005).

1.3 BDNF, TrkB, Their Downstream Pathways & Autism

While the molecular underpinnings of autism remain unknown, genetic, anatomical and functional imaging studies suggest that defects in synaptic development and plasticity, which impair establishment and maintenance of functional neuronal networks, result in autism. Because of their pivotal role in guiding synaptic development, plasticity and cortical organization (Minichiello, 2009; Yoshii & Constantine-Paton, 2010), BDNF, TrkB and their signaling pathways, including Akt-mTOR and Eps8/Rac, are essential molecules in brain development and thus prime candidates for involvement in

ASD. Specific BDNF SNP haplotypes are associated with autism (Nishimura et al., 2007), as are multiple SNPs and haplotypes of its receptor TrkB gene (*NTRK2*) (Correia et al., 2010). Multiple studies using ELISAs have reported elevated BDNF-immunoreactive protein in archived samples of neonatal blood, serum, platelet-rich plasma and basal forebrain in autistic patients compared to normal controls (Connolly et al., 2006; Correia et al., 2010; Miyazaki et al., 2004; Nelson et al., 2001; Perry et al., 2001). In agreement with these studies, we found elevated BDNF-immunoreactivity in postmortem fusiform gyrus of subjects with idiopathic autism compared to controls (Garcia et al., 2012). Since ELISAs cannot distinguish between the three BDNF protein isoforms, BDNF isoforms were quantified in autistic and control fusiform gyrus using Western blotting. We found elevated proBDNF and decreased truncated BDNF protein levels in fusiform gyrus of autism subjects *vs.* controls. This evidence confirms a neurochemical abnormality in a brain region, fusiform gyrus, involved in facial recognition and implicated in social interaction deficits in ASD (Allison et al., 1994; Boucher et al., 1992; Gauthier et al., 1997). It also corroborates the hypothesis that imbalances in BDNF isoforms contribute to synaptic dysfunction and altered neuronal connectivity. Potential links between alterations in BDNF/TrkB-mediated signaling pathways and autism are supported by decreased Akt total protein and phosphorylation in the frontal cerebral cortex from autistic patients (Sheikh et al., 2010). Mutations in Akt-mTOR cascade components including hamartin (TSC1), tuberin (TSC2), neurofibromin 1 (NF1), phosphatase and tensin homolog on chromosome ten (PTEN) and methyl-CpG-binding protein 2

(MECP2), cause disorders with a high prevalence of autism (Kelleher & Bear, 2008; Levitt & Campbell, 2009). Additionally, single nucleotide insertions in the promoter of mTOR downstream effector eIF4E have been found in individuals with autism (Neves-Pereira et al., 2009), and autism-like phenotypes have been observed in eIF4E-overexpressing mice (Gkogkas et al., 2013). Lastly, knockout of Eps8 in mice resulted in spine abnormalities, decreased LTP and autism-like symptoms (Menna et al., 2013). Taken together, these findings strengthen the hypothesis that defective BDNF, TrkB and their effector pathways contribute to autism, likely by disrupting mTOR-dependent protein synthesis and actin cytoskeletal remodeling.

1.4 mTOR & Local Protein Synthesis

Mammalian target of rapamycin (mTOR) is a large serine-threonine protein kinase that mediates a plethora of functions in the developing and mature brain. It regulates neuronal survival, differentiation and development, dendrite arborization, synaptogenesis, synaptic plasticity, dendritic spine morphogenesis and protein synthesis, with the latter being its best-characterized function (Jaworski et al., 2005; Kumar et al., 2005; Santini & Klann, 2011; Swiech et al., 2008). mTOR-dependent local protein synthesis in neuronal dendrites is upregulated by binding of BDNF to its receptor TrkB (**Figure 4**) (Takei et al., 2004).

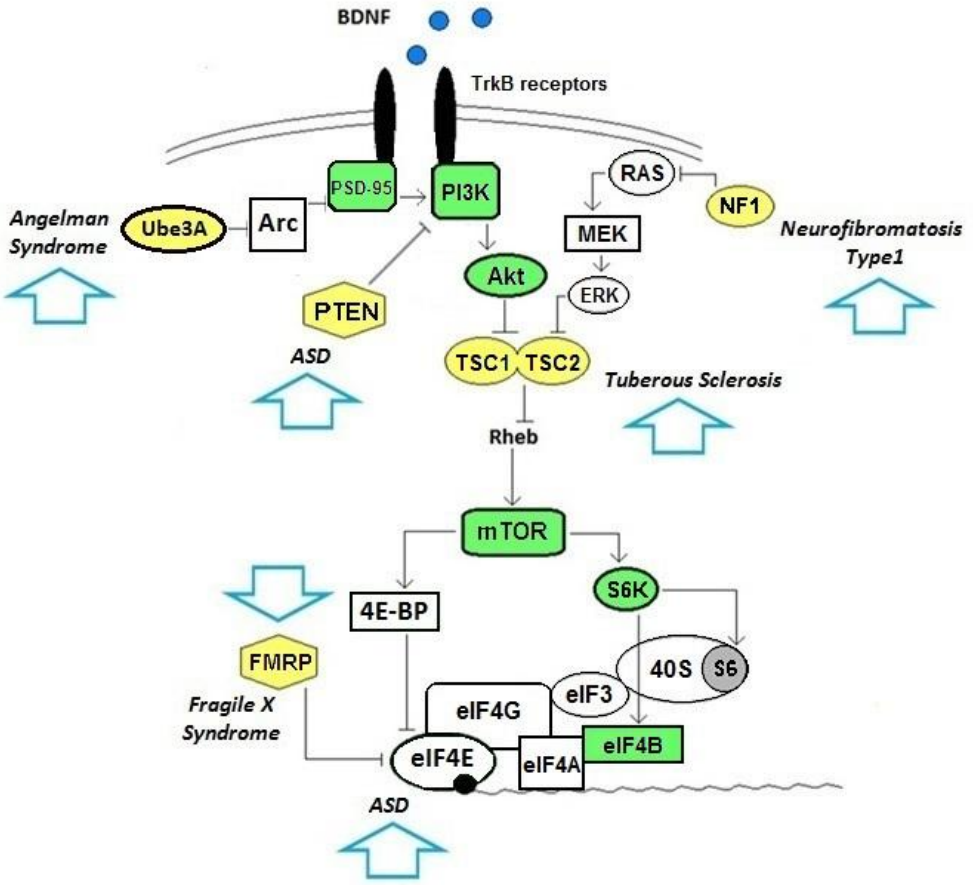

Figure 4. Schematic diagram of mTOR pathways activated upon binding of BDNF to TrkB and regulating protein synthesis. **Yellow** indicates proteins implicated in single-gene disorders associated with high rates of autism. **Green** indicates proteins found to be reduced in idiopathic autism in this study. **White** indicates proteins that do not exhibit differences between idiopathic autism and control (ERK, 4E-BP, eIF4E) or that have not yet been tested (Fahnestock & Nicolini, 2015).

BDNF binding to TrkB activates phophoinositide-3' kinase (PI3K) which catalyzes the production of phosphatidylinositol 3,4,5-triphosphate (PIP3). PIP3 binds to Akt and promotes Akt recruitment to the cell membrane where it is phosphorylated. Activated Akt phosphorylates and thus inhibits tuberin (TSC2), a GTPase-activating protein (GAP) for the Ras homolog enriched in the brain protein (Rheb). Unphosphorylated TSC2 together with hamartin (TSC1) forms the tuberous sclerosis complex (TSC1/2) and negatively regulates mTOR by inhibiting the small GTP-binding protein Rheb. Following Akt-mediated phosphorylation of TSC2, GTP-Rheb can activate mTOR, which acts primarily by phosphorylating p70 S6 kinase (p70S6K) and the inhibitory eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), critical regulators of protein synthesis at the synapse (Burnett et al., 1998; Hay & Sonenberg, 2004). 4E-BP1 prevents the interaction of the eukaryotic initiation factor 4E (eIF4E) with the eukaryotic initiation factor 4G (eIF4G) to form the translation initiation complex eIF4F required for translation to begin (Proud, 2007). mTOR-mediated phosphorylation of 4E-BP1 releases eIF4E and promotes cap-dependent translation (Beretta et al., 1996). Locally translated mRNAs include α-Ca^{2+}/Calmodulin kinase II (α-CaMKII), activity-reguated cytoskeletal-associated protein (Arc), Homer 2 and receptor subunits such as GluA1/GluR1, GluA2/GluR2 and GluN1/NR1 (Ju et al., 2004; Messaoudi et al., 2007; Miller et al., 2002; Schratt et al., 2004; Yin et al., 2002). mTOR also activates p70S6K which phosphorylates the ribosomal protein S6 (rp S6) and the eukaryotic initiation factor 4B (eIF4B) (Holz et al., 2005; Shahbazian et al., 2006). Phosphorylated S6 controls translation of

mRNAs coding for proteins involved in translation regulation such as ribosomal proteins and elongation factors (Jefferies et al., 1997). Hence, translation of these mRNAs promotes the synthesis of new components of the translational machinery which are critical for maintaining protein synthesis (Meyuhas, 2000). mTOR-induced protein synthesis in response to BDNF activation of TrkB receptors plays a crucial role in dendrite and spine morphogenesis and synaptic plasticity (Santos et al., 2010; Waterhouse & Xu, 2009). It is therefore not surprising that de-regulated mTOR is implicated in several syndromic ASDs characterized by synaptic dysfunction and impaired cortical networks (**Figure 4**) (Kelleher & Bear, 2008; Ricciardi et al., 2011; Troca-Marín et al., 2012). In particular, mutations in TSC1/2 and PTEN, which activate mTOR, lead to tuberous sclerosis complex (TSC) and macrocephaly, respectively, while mutations in the transcriptional regulator MeCP2 reduce mTOR and cause Rett syndrome (RTT) (Ehninger & Silva, 2011; Li & Pozzo-Miller, 2014). This suggests that either hyper- or hypo-activation of mTOR, leading to excessive or deficient protein synthesis at synapses, may be equally disruptive and result in autistic behaviour (**Figure 5**) (Zoghbi & Bear, 2012).

1.5 De-regulated mTOR and Dendritic Spines in Syndromic ASDs

Dendritic spines are small and specialized protrusions from dendrites and represent the principle postsynaptic sites of excitatory synapses in the CNS (Harris, 1999). A spine consists of a head connected by a neck to the dendrite. Head shape and neck length/diameter vary. There are three major

types of spines: stubby, mushroom and thin (Peters & Kaiserman-Abramof, 1970). Stubby spines have no obvious neck. Mushroom spines have a large head and a narrow neck. Lastly, thin spines are characterized by a small head and a long neck (Nimchinsky et al., 2002). Changes in spine number, structure and dynamics shape connectivity of cortical networks that subserve high-order cognitive functions and behaviour (Dunaevsky et al., 2001; Grutzendler et al., 2002; Segal, 2005). It follows that alterations in dendritic spines are likely to impair establishment and remodeling of neuronal circuits and thus negatively affect behaviour and cognition. As seen in TSC, fragile X syndrome (FXS) and RTT patients and animals models, both up- and down-regulation of the mTOR pathway are associated with abnormal dendritic spine morphology and density (Troca-Marín et al., 2012). This evidence suggests that hyper- or hypo-activated mTOR, by perturbing dendritic spines during critical periods in brain development, might disrupt neuronal circuitry and result in autism (**Figure 5**).

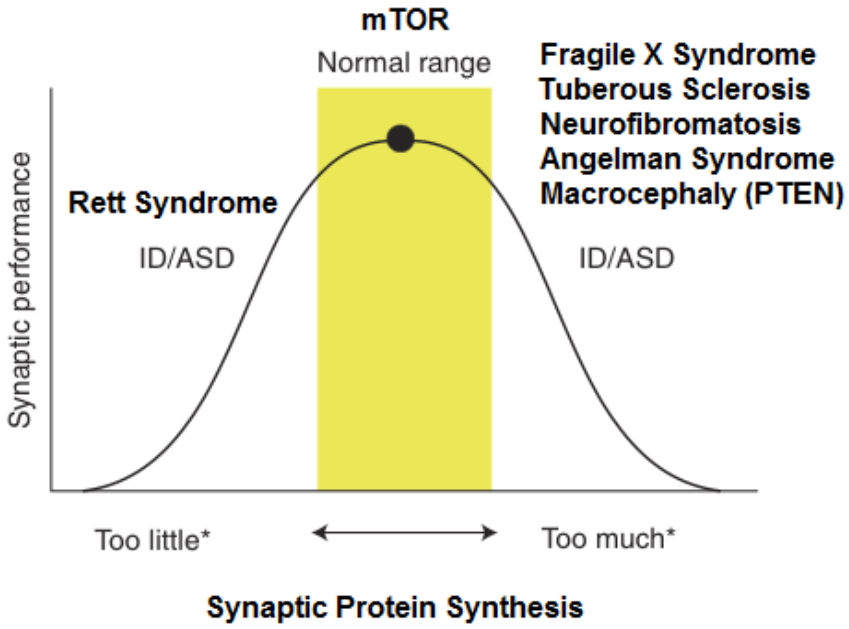

Figure 5. Either too much or too little signaling through the mTOR pathway can result in autistic behaviour. Optimal synaptic function appears to occur within a narrow range. ID, intellectual disability; ASD, Autism Spectrum Disorder; mTOR, mammalian target of rapamycin; PTEN, phosphatase and tensin homolog on chromosome ten. (Adapted from Kelleher & Bear, 2008; Zoghbi & Bear, 2012).

1.5.1 Tuberous Sclerosis

Tuberous sclerosis complex (TSC) is a genetic disorder caused by mutations in hamartin (TSC1) or tuberin (TSC2) (Kwiatkowski & Manning, 2005) and associated with ASDs in 25-60% of cases (Kelleher & Bear, 2008). The high prevalence of ASDs in TSC suggests that mutations in the TSC1 or TSC2 gene contribute to an increased vulnerability to autism (Ehninger & Silva, 2011). TSC1 and TSC2 form a heterodimeric complex which accelerates the inactivation of Rheb, hence inhibiting mTOR. Mutations of either TSC1 or TSC2 result in increased Rheb activity and thus aberrant up-regulation of mTOR signaling and mTOR-mediated protein synthesis (**Figure 6**) (Keheller & Bear, 2008; Troca-Marín et al., 2012). Up-regulated mTOR is associated with structural abnormalities of dendrites and dendritic spines in both TSC patients and mouse models. Specifically, TSC mice and patients have fewer and enlarged dendritic spines (Machado-Salas, 1984; Tavazoie et al., 2005).

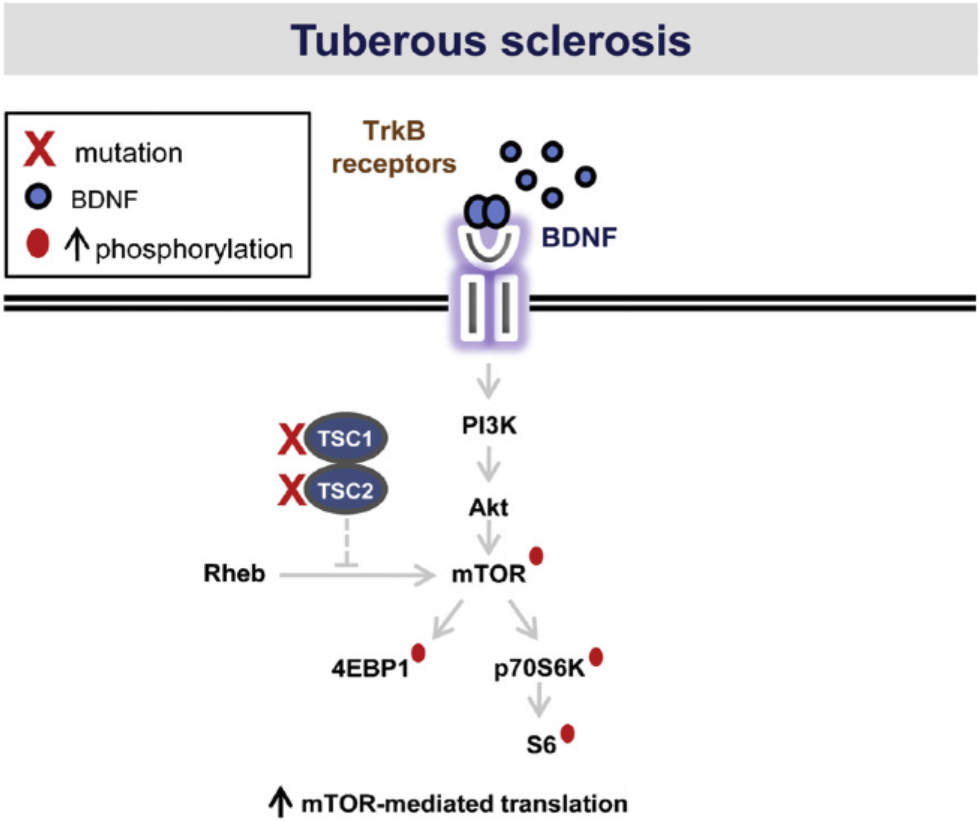

Figure 6. Deregulation of mTOR-dependent protein synthesis in tuberous sclerosis. Mutations of either TSC1 (hamartin) or TSC2 (tuberin) result in increased GTP-Rheb and hyperphosphorylation of mTOR, 4E-BP1, p70S6K and S6, suggesting elevated mTOR-mediated translation (Adapted from Troca-Marín et al., 2012).

1.5.2 Rett Syndrome

Rett syndrome (RTT) is a neurodevelopmental disorder caused by loss-of-function mutations in the X-linked methyl-CpG binding protein 2 (MECP2) gene (Amir et al., 1999; Chahrour & Zoghbi, 2007). It occurs almost exclusively in girls and is associated with autistic features (42%-58%) (Hagberg et al., 1983). The product of the MECP2 gene, MeCP2, is a DNA-binding protein that was believed to function as a transcriptional repressor by binding to methylated CpG dinucleotides and recruiting co-repressors and histone deacetylase complexes (Jones et al., 1998; Nan et al., 1997, 1998). It has now been demonstrated that MeCP2 recruits transcriptional activators, such as the transcription factor CREB, to promoters, and thus also promotes gene activation (Chahrour et al., 2008). This finding is consistent with the data showing decreased BDNF mRNA and protein in Mecp2-null mice (Chang et al., 2006), and suggests that MeCP2 acts as both repressor and activator of transcription.

In addition to decreased BDNF, hypo-phosphorylated Akt, mTOR, p70S6K and S6 have been found in *Mecp2* KO mice, suggesting decreased mTOR signaling and lower protein synthesis (**Figure 7**) (Ricciardi et al., 2011). Down-regulated mTOR is associated with decreased dendritic complexity and spine number (Belichenko et al., 1994). RTT patients exhibit small dendritic arbors and regional loss of dendritic spines, named “naked” areas (Belichenko et al., 1994). Mouse models of RTT also have fewer spines, with the spines present being characterized by elongated necks and reduced heads (Belichenko et al., 2009).

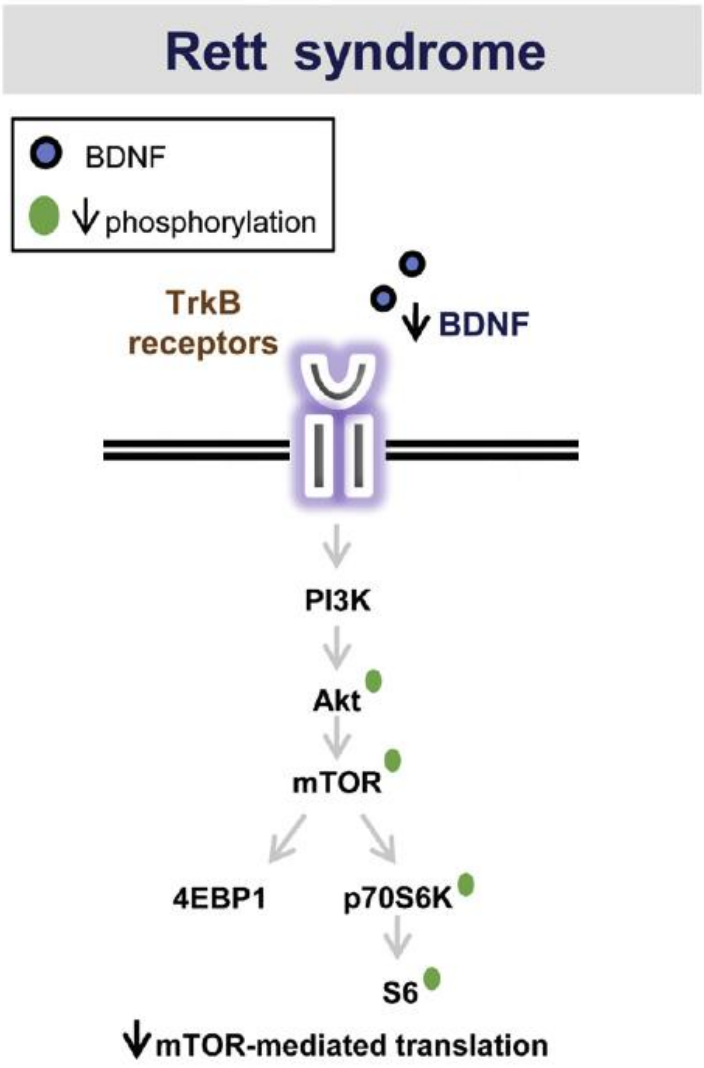

Figure 7. Deregulation of mTOR-dependent protein synthesis in Rett syndrome. Hypophosphorylation of Akt, mTOR, p70S6K and S6 is associated with reduced mTOR-mediated translation (Adapted from Troca-Marín et al., 2012).

1.5.3 Fragile X syndrome

Fragile X syndrome (FXS) is an X-linked form of mental retardation characterized by a high prevalence of ASDs (15%-30%) and caused by mutations in the fragile X mental retardation 1 (FMR1) gene coding for an RNA binding protein, the fragile X mental retardation protein (FMRP) (Bagni & Greenough, 2005). FMRP regulates mRNA transport and translation (De Rubeis & Bagni, 2010). Normally, it binds to specific mRNAs, repressing their translation (Li et al., 2001; Napoli et al., 2008; Zalfa et al., 2003). Silencing of the FMR1 gene results in absence of FMRP and increased translation of FMRP-target mRNAs (Bassel & Warren, 2008; Gross et al., 2010). In particular, translation of FMRP-target mRNA p110β coding for PI3K catalytic subunit is increased in Frm1 KO mice (Gross et al., 2010), pointing to elevated Akt-mTOR signaling. Reports of increased phosphorylated PI3K, Akt, mTOR, 4E-BP1 and p70S6K in Fmr1 KO mice confirm higher mTOR activity (**Figure 8**) (Gross et al., 2010; Sharma et al., 2010). Up-regulated mTOR is associated with increased density of abnormally long and thin dendritic spines (immature) in FXS patients and rodent models (Comery et al., 1997; Irwin et al., 2000, 2001; Nimchinsky et al., 2001).

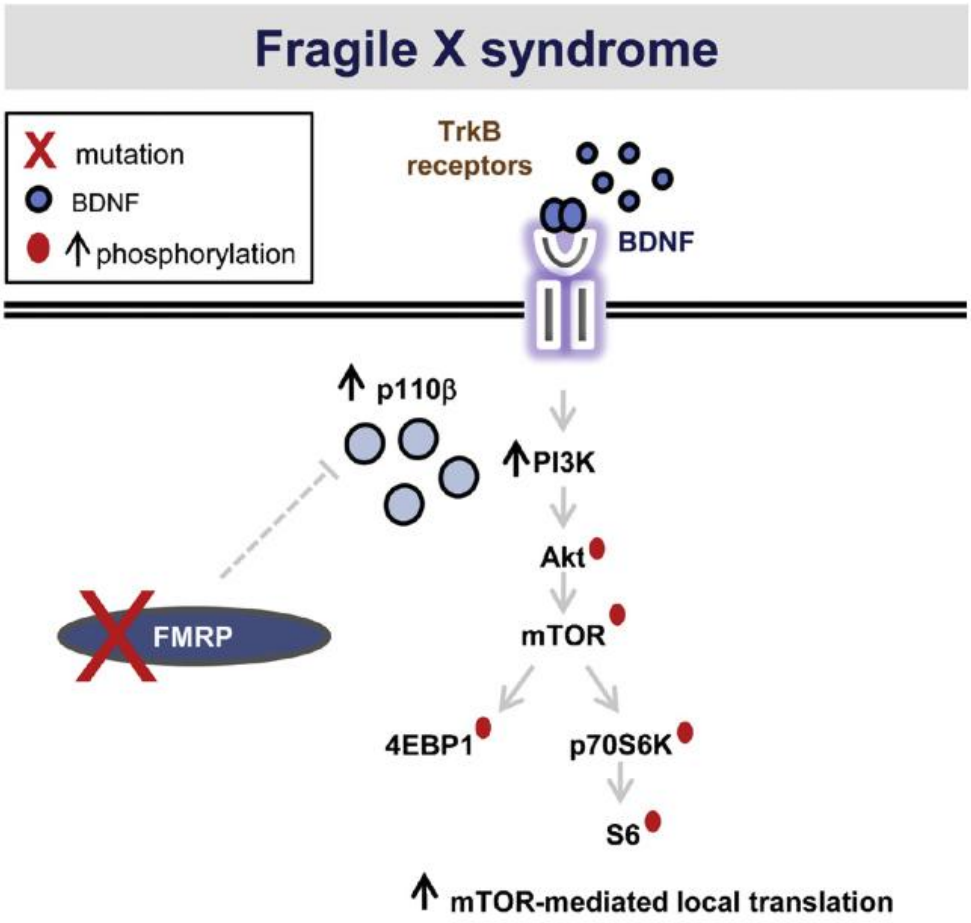

Figure 8. Deregulation of mTOR-dependent protein synthesis in Fragile X syndrome. Absence of the FMRP protein results in increased levels of PI3K catalytic subunit, p110β and hence hyperphosphorylation of Akt, mTOR, 4E-BP1, p70S6K and S6, ultimately leading to elevated mTOR-mediated translation (Adapted from Troca-Marín et al., 2012).

Collectively, these findings support the model that either increased or decreased mTOR disrupts spine density, shape and function and, thus ultimately brain connectivity, causing autistic behaviour in syndromic ASDs. De-regulation of the mTOR pathway may also be implicated in the emergence of autistic traits outside of these genetic disorders and might represent a link between syndromic ASDs and idiopathic autism. Whether this pathway is involved in idiopathic autism was investigated in this study.

1.6 Pharmacological Intervention in Syndromic ASDs

There seems to be a great potential for pharmacological intervention in the TrkB-mTOR pathway. Autistic-like behaviors have been improved in adult rodent models of syndromic ASDs by administration of inhibitors (Ehninger & Silva, 2011; Sato et al., 2012; Zhou et al., 2009) or activators (Tropea et al., 2009; Schmid et al., 2012) of this pathway.

1.6.1 Rapamycin

Rapamycin (Sirolimus) is an antibiotic produced by the bacterium *Streptomyces hygroscopius* that specifically interferes with mTOR activation (Veverka et al., 2008). It has been approved by the Food and Drug Administration (FDA) as an anti-rejection drug and for the treatment of cardiovascular diseases (Tsang et al., 2007). More recently, rapamycin treatment has been used to correct altered mTOR signaling and behavioural deficits in animal models of TSC. *Tsc2+/-* mice have an inactivating mutation

in the *Tsc2* gene that results in increased Akt-mTOR signaling and impaired cognitive and social behavior (Ehninger et al., 2008; Sato et al., 2012). Promisingly, treatment with rapamycin reduced mTOR signaling to wild-type levels and reversed learning and social behavioural deficits in *Tsc2+/-* mice (Ehninger et al., 2008; Sato et al., 2012). Moreover, there are currently several ongoing clinical trials assessing the effects of rapamycin (Sirolimus) and its analogs (rapalogs), such as Everolimus, on autistic behaviour, seizures and tumors in children with TSC (Curatolo & Moavero, 2012: Silverman & Crawley, 2013). Treatment with rapamycin has also been reported to ameliorate autistic-like behaviours, including anxiety and reciprocal social interaction deficits, in conditional *Pten* KO mice (Zhou et al., 2009). Lastly, rapamycin has been tested in animal models of FXS. Applications of this drug reduced protein synthesis in *Fmr1* KO hippocampal neurons (Gross et al., 2010) but failed to correct excess protein synthesis in *Fmr1* KO hippocampal slices (Osterweil et al., 2010). Thus, whether rapamycin might normalize increased mTOR and protein translation in FXS remains unclear.

1.6.2 LM22A-4

LM22A-4 is a non-peptide small molecule that was developed by Longo, Massa and colleagues and functions as a specific partial TrkB agonist (Massa et al., 2010) (**Figure 9**). It mimics the loop II region of BDNF, which is capable of activating TrkB with high specificity (Massa et al., 2010). Indeed, LM22A-4 binds selectively and directly to TrkB, triggering its downstream signaling cascades including PI3K/Akt (Massa et al., 2010). This

small-molecule BDNF loop-2-domain mimetic might represent a better approach than BDNF itself to correct BDNF/TrkB signaling deficits. BDNF does not have favorable drug-like characteristics. Its half-life is limited and its blood brain barrier and brain intraparenchymal penetration is poor (Morse et al., 1993; Poduslo & Curran, 1996). Moreover, since it functions through two receptors ($p75^{NTR}$ and TrkB) (Chao & Hempstead, 1995), BDNF might have multiple undesired effects. Lastly, direct injection of BDNF into the brain has been demonstrated to result in hyperexcitability and seizure activity in rats (Xu et al., 2004).

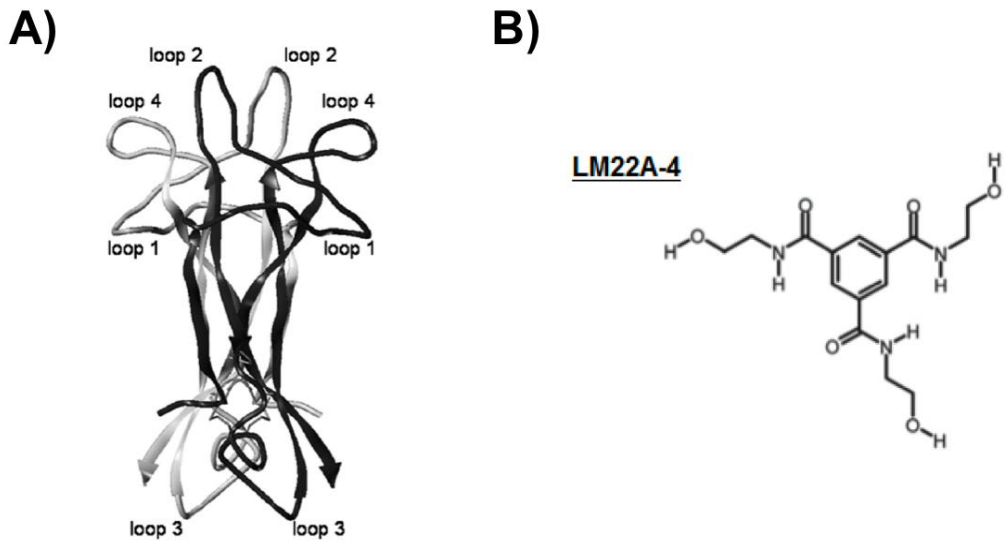

Figure 9. (**A**) Three-dimensional structure of BDNF. (**B**) Structure of LM22A-4. (Adapted from Fletcher & Hughes, 2006; Massa et al., 2010)

LM22A-4 has been used to increase TrkB activation and improve breathing deficits in MeCP2 mutant mice, a well-established model of human RTT. MeCP2 mice have reduced BDNF in cortex, cerebellum, and brainstem (Chang et al., 2006; Wang et al., 2006) as well as decreased Akt-mTOR signaling and protein synthesis in the primary sensory cortex and in the CA1 region of the hippocampus (Ricciardi et al., 2011). Intraperitoneal injections of LM22A-4 ameliorated brainstem TrkB and Akt phosphorylation and respiratory abnormalities in these mice (Kron et al., 2014; Schmid et al., 2012).

1.6.3 (1-3)IGF-1

(1-3)IGF-1 is the active N-terminal tripeptide (glycine-proline-glutamate) of the insulin-like growth factor 1 (IGF-1) (Yamamoto & Murphy, 1994). Similar to BDNF, IGF-1 stimulates neural cell proliferation and survival and promotes growth and development of the CNS (D'Ercole et al., 1996). Its expression is highly regulated during brain development, peaking at times when neuron progenitors proliferate and when neurite outgrowth, dendritic maturation and synaptogenesis occur (Bartlett et al., 1991; Bondy, 1991; O'Kusky et al., 2000). IGF-1 mediates its functions acting through the PI3K/Akt/mTOR and ERK pathways (Bondy & Cheng, 2004; Corvin et al., 2012; Ozdinler & Macklis, 2006; Zheng & Quirion, 2004). Its active fragment (1-3)IGF-1, unlike BDNF, is able to cross the blood-brain barrier (Baker et al., 2005) and to retain neurotrophic activity (Guan et al., 2004; Sizonenko et al., 2001), supporting a potential therapeutic role for this molecule in treatment of

brain disorders. Indeed, systemic treatment with (1-3)IGF-1 has been shown to partially restore dendritic spine density, increase excitatory postsynaptic marker PSD-95 and improve locomotor and breathing functions in MeCP2 mutant mice, ultimately extending their lifespan (Tropea et al., 2009). Similarly, administration of the full-length, recombinant human IGF-1 (rhIGF-1) to these mice has been demonstrated to increase dendritic spine density, PSD-95 and Akt phophorylation and restore sociability, locomotor activity and respiratory patterns (Castro et al., 2014). However, a previous study by Pitcher and colleagues (2013) reported that treatment with full-length IGF-1 did not improve impaired motor ability and breathing or memory deficits in *Mecp2* null mice. Also, contrary to the increase in dendritic spine density observed in visual cortex pyramidal neurons following treatment with (1-3)IGF-1, full-length recombinant human IGF-1 failed to ameliorate dendritic spine density in *Mecp2* null mouse hippocampal slice cultures (Pitcher et al., 2013). Lastly, while low-dose administration of full-length IGF-1 slightly extended lifespan and ameliorated heart rate in *Mecp2* null mice, high-dose treatment negatively impacted survival of these mice by worsening their metabolic syndrome. Although these opposing results warrant caution with IGF-1 compounds in clinical trials, rhIGF-1 (mecasermin), which is already FDA-approved for treatment of growth failure in children with growth hormone insensitivity (Kemp, 2009), appears to improve breathing and behavioural deficits in RTT patients (Khwaja et al., 2014).

Improvement of autistic behaviour in these genetic models of ASD, particularly in Rett syndrome, which arises from a mutation in an epigenetic regulatory gene, supports the exciting possibility that behavioural deficits can also be ameliorated in idiopathic autism.

1.7 Maternal Challenge with VPA as a Model of Idiopathic Autism

To investigate the relationship of TrkB-mTOR signaling deficits to autistic behaviour, and to test whether therapeutic intervention in this pathway reverses behavioural deficits, it is necessary to move to an animal model.

Valproic acid (VPA) or 2-propylpentanoic acid is a short-branched chain fatty acid which has been used worldwide as an antiepileptic drug (Löscher, 2002) and for the treatment of mood disorders (Lambert et al., 1975; Emrich et al., 1980). VPA modulates neurotransmission (Johannesen & Johannesen, 2003; Löscher, 1999; Owens & Nemeroff, 2003; Gobbi & Janiri, 2006) and regulates gene expression through (epigenetic) chromatin remodeling by inhibition of histone deacetylase (HDAC) activity (Phiel et al., 2001). Indeed, Kataoka et al. (2013) demonstrated that exposure to a 500 mg/kg VPA dose on gestational day 12.5 (E12.5) resulted in increased levels of acetylated histones H3 and H4 in mouse embryonic brains up to 6 hours after mothers were injected intraperitoneally. Conversely, prenatal exposure to valpromide, a VPA analog that lacks HDAC inhibitory activity, did not increase the levels of acetylated H3 and H4 histones (Kataoka et al., 2013). This evidence suggests that VPA causes transient changes in histone acetylation levels of embryonic brains through HDAC inhibition. Interestingly, only mice prenatally exposed

to VPA but not to valpromide exhibited autistic-like behavioural deficits. This supports the hypothesis that HDAC inhibition during a critical window of embryonic brain development might contribute to autism pathogenesis. However, it does not preclude that other VPA mechanisms of action could be involved in inducing autistic-like behavioural alterations following exposure to this drug during brain development. In particular, VPA could impact axonal remodeling in developing neurons by inhibiting GSK3β and thus promoting Wnt signaling (Hall et al., 2002). The Wnt signaling pathway regulates the differentiation of cortical intermediate progenitors into neurons (Munji et al., 2011) and the patterning of the cerebral cortex (Chenn, 2008). Given its crucial functions in the differentiation of neural precursor populations and in the regional specification of the cortex, abnormal activation of the Wnt pathway induced by *in utero* VPA exposure could disrupt spatio-temporal windows of embryonic brain development that are essential for the establishment of normal neuronal networks. VPA could also affect differentiation and proliferation of neural cell progenitors via the β-catenin-Ras-ERK-p21 pathway (Jung et al., 2008). Specifically, VPA promotes β-catenin activation by inhibiting GSK3β (Jung et al., 2008). Activated β-catenin regulates Ras, which in turn increases the levels of phosphorylated ERK (Jung et al., 2008). Subsequent ERK-mediated induction of p21 modulates stimulation of neural cell progenitor differentiation and inhibition of their proliferation (Jung et al., 2008). Lastly, VPA exposure could result in elevated GABA levels, as VPA inhibits an enzyme involved in GABA degradation, GABA transaminase, while it promotes GABA synthesizing enzyme glutamic

acid decarboxylase (Löscher, 2002). GABA plays a critical role in maturation of neural networks in the developing brain (Baltz et al., 2010; Ben-Ari et al., 2007; Sernagor et al., 2010). Thus, changes in GABA levels due to exposure to VPA during early development could disrupt the developing neuronal circuitry and result in autistic behavioural phenotypes. However, the involvement of these VPA mechanisms of action in autistic-like behaviours and whether these mechanisms are shared by valpromide remain to be demonstrated.

VPA is a known risk factor for autism. Maternal exposure to VPA at the time of neural tube closure increases the incidence of autism in humans (Arndt et al., 2005; Chomiak & Hu, 2013; Christensen et al., 2013; Christianson et al., 1994; Rodier et al., 1996) and causes autism-like symptoms in rodents (Roullet et al., 2013; Schneider & Przewlocki, 2005; Stanton et al., 2007). In particular, a single exposure to VPA *in utero* leads to impaired social interactions, increased repetitive, restrictive behaviour and sensory/communication deficits in rodents' offspring (Kataoka et al., 2013; Markram et al., 2008; Roullet et al., 2010; Schneider & Przewlocki, 2005). Rodents prenatally exposed to VPA also exhibit anatomical and molecular alterations similar to human autism including reduced cerebellar volume and Purkinje cell number (Ingram et al., 2000; Raber et al., 2000; Rodier et al., 1996, 1997), decreased expression of the postsynaptic adhesion molecule Neuroligin 3 (Kolozsi et al., 2009), which is encoded by an autism-associated gene (Jamain et al., 2003), decreased BDNF mRNA (Roullet et al., 2010), and increased NR2A and NR2B NMDA receptor subunits (Rinaldi et al., 2007).

Furthermore, we showed that VPA rats have hypophosphorylated Akt, mTOR, 4E-BP1 and S6 (Nicolini et al., 2015), suggesting that maternal challenge with VPA results in decreased mTOR and likely negatively affects mTOR-mediated protein translation. Rats exposed to VPA prior to formation of the neocortex also have perturbed cortical connectivity along with abnormal synapse formation and pruning (Rinaldi et al., 2008 a,b). Additionally, in line with the evidence that postulates a disruption of the excitatory/inhibitory circuit balance in human autism (Rubenstein & Merzenich, 2003), VPA-exposed mice exhibit decreased parvalbumin (PV)-positive interneurons in parietal and occipital cortices (Gogolla et al., 2009). Lastly, like humans, VPA-treated rats respond successfully to early environmental enrichment (Schneider et al., 2006). Taken together, these findings support face, construct and predictive validity of VPA-induced rodent models. Thus, VPA-treated rodents appear to be a suitable animal model for investigating the contribution of defective TrkB-mTOR signaling to autistic behaviour and for screening compounds that might ameliorate autistic behavior by intervening in this pathway. Additionally, as supported by the findings that prenatal exposure to VPA leads to transient hyperacetylation of H3 and H4 histones in the embryonic mouse brain (Kataoka et al., 2013), VPA-exposed mice appear to be a model of epigenetic changes. It follows that, although much of today's ASD research uses transgenic mouse models carrying mutations in single genes known to cause autism or related developmental disorders, the VPA-exposed mouse might better represent the many idiopathic autism cases having no known genetic basis.

1.8 Evaluating Autism-Like Behaviours in Mouse Models

Presently, there is not a medical test that can diagnose autism. Clinicians rely on detection of behavioural impairments to make a diagnosis. Two symptom domains define ASD: (1) Social interaction and social communication deficits; (2) restricted, repetitive, patterns of behaviour, interests and activities (DSM-5, 2013). In addition, subsets of patients show associated symptoms which include anxiety, seizures, hyperactivity and sleep disruptions (Lecavalier, 2006; Leyfer et al., 2006; Malow et al., 2006; Volkmar & Nelson, 1990; White et al., 2009). Because of this solely behavioural diagnosis, to evaluate specific behaviours relevant to the core symptoms of autism in animal models of this disease is critical. Standardized tasks for scoring behaviours with face validity to each autism symptom domain are now in place and are routinely used for phenotyping autism animal models.

1.8.1 Sociability

Deficits in reciprocal social interaction are the identifying feature of autism. Autistic individuals lack interest in others and exhibit unusual and inappropriate social approach behaviours (Baron-Cohen, 1988; Kanner, 1943; Mundy et al., 1986; Volkmar & Pauls, 2003; Volkmar et al., 1987). It follows that it is important to quantify social behaviours in animal models of autism. Laboratory mice are a social species. They display parental care of the pups, juvenile play and communal nesting, sleep in group huddles and have a natural tendency to approach unfamiliar conspecifics (Branchi, 2009; Champagne et

al., 2007; Francis & Meaney, 1999; Francis et al., 2003; Grant & MacIntosh, 1963; Laviola & Terranova, 1998; Manning et al., 1992, 1995; Terranova et al., 1993). Social behaviours in mice are crucial for mating, foraging, defending territories and avoiding predators (Laviola & Terranova, 1998; Miczek et al., 2001; Moles & D'Amato, 2000; Panksepp & Lahvis, 2007). Moreover, social experiences in early postnatal life in the form of juvenile play among littermates and maternal care have a major impact on brain and behaviour development and shape adult social behaviour (Dyer & Southwick, 1974; Cirulli et al., 1997; Laviola, 1996; Namikas & Wehmer, 1978). Since mice have a natural tendency to approach unfamiliar conspecifics, low direct social approach in mice has strong face validity to social interaction deficits characteristics of autism (Crawley, 2007; Moy et al, 2007, 2008; Silverman et al., 2010b). Crawley and colleagues developed a social approach task that allows evaluation of direct social approach in mice using a three-chambered apparatus (Crawley, 2004; Moy et al., 2004; Nadler et al., 2004; Yang et al., 2011). A subject mouse is given the choice to spend time in a room with a novel mouse located inside an inverted wire pencil cup or an empty room. Sociability is defined as the tendency to spend time in the room with the novel conspecific *vs.* the empty chamber. This test has been widely used to assess social deficits in mouse models of autism including BTBR T+ tf/J (BTBR) (Chadman, 2011; McFarlane et al., 2008; Moy et al., 2007; Pobbe et al., 2011; Yang et al., 2007, 2009), Eps8 KO (Menna et al., 2013), *Fmr1* KO (Liu et al., 2011; Moy et al., 2009), NLGN4 KO (Jamain et al., 2008), *in utero* VPA-

exposed (Kang & Kim, 2015; Kim et al., 2014) and *Pten* haploinsufficient mice (Page et al., 2009).

1.8.2 Repetitive, Restricted Patterns of Behaviour

Stereotyped, repetitive behaviours characteristic of autism include inflexibile insistence on sameness, resistance to change and motor stereotypies (Bodfish et al., 2000; Matson et al., 2009; Turner, 1999; Szatmari et al., 2006; Zandt et al., 2007). Mice display spontaneous repetitive motor behaviours including hindlimb jumping, backflips, self-grooming and digging (Lewis et al., 2007). Rodent tasks with face validity to this symptom domain of autism measure reversal learning, excessive self-grooming and repetitive digging (Crawley, 2012; Lewis et al., 2007; Silverman et al., 2010a). The latter is evaluated by calculating the marbles that are buried after a 30-minute session (Deacon et al., 2006). The marble-burying test was first described by Broekkamp et al. (1986), who demonstrated that anxiolytics are more potent than antipsychotics in decreasing marble burying. However, subsequent studies showed that antidepressants, including selective serotonine re-uptake inhibitors (SSRIs) citalopram, paroxentine and fluoxetine, and other compounds not used to treat anxiety such as yohimbine, atropine, morphine and D-amphetamine, also reduce marble-burying (Borsini et al., 2002; Nicolas et al., 2006; Njung'e & Handley, 1991). These findings, combined with the evidence of lack of habituation to marbles and lower burying rates when marbles are placed in clusters (Njung'e & Handley, 1991), suggest that marble-burying has limited validity for anxiety and might instead measure

digging behaviour unrelated to anxiety (Thomas et al., 2009). Mice do not deliberately bury marbles but dig heartily in the deep wood chip bedding material, and marbles fall through the displaced bedding (Gyertyan, 1995; Thomas et al., 2009). Glass marbles are not a fear-provoking stimulus and provide a means of measuring the intensity of digging activity, which is a compulsive behaviour (Gyertyan, 1995; Njung'e & Handley, 1991; Thomas et al., 2009; Sherwin et al., 2004). Hence, marble-burying represents a useful tool to evaluate repetitive/perseverative responses in mice. The marble-burying test has been used to examine repetitive behaviour in several mouse models of autism including BTBR (Amodeo et al., 2012, 2014), *Fmr1* KO (Veeraragavan et al., 2012), *Eif4ebp2* KO (Gkogkas et al., 2013) and prenatally VPA-exposed mice (Kang & Kim, 2015; Kim et al., 2014; Mehta et al., 2011)

1.8.3 Anxiety-Related Behaviour

Anxiety is one of the most common co-occurring symptoms in autism (Gillott et al., 2001; Kim et al., 2000; Leyfer et al., 2006; Vasa & Mazurek, 2015; White et al., 2009). The elevated plus maze test is a widely used test to measure anxiety-related behaviours in rodents (Handley & Mithani, 1984; Lister, 1987; Pellow et al., 1985, 1986; Sidor et al., 2010). This test is based on a spontaneous tendency of rats and mice to actively explore new environments and on their natural aversion to threatening areas such as open and elevated spaces (Lister, 1987; Rodgers & Dalvi, 1997). The elevated plus maze consists of two open arms and two enclosed arms, raised from the floor.

Animals are allowed to enter and spend time in both enclosed non-aversive areas (closed arms) and open aversive zones (open arms). The extent to which a subject animal explores the unprotected open arms is used as an index of anxiety, with decreased exploration indicating elevated open space-induced anxiety (Sidor et al., 2010). The elevated plus maze test has been used to measure anxiety-like responses with face validity to autism in mouse models of this disease including *Fmr1* KO (Bilousova et al., 2009; Busquets-Garcia et al., 2013; Liu et al., 2011; Heulens et al., 2012), BTBR (Benno et al., 2009; Chadman et al., 2011; Moy et al., 2007; Pobbe et al., 2011), *MeCP2* KO (Castro et al., 2014) and VPA-exposed mice (Kataoka et al., 2013).

CHAPTER 2: HYPOTHESES & OBJECTIVES

As the molecular mechanisms underlying autistic behaviours remain largely unknown, the overall purpose of this work was to determine whether TrkB signaling cascades are disrupted in idiopathic autism, contribute to autistic behaviour and are potential therapeutic targets for this disease.

2.1 Hypothesis # 1: TrkB pathways are disrupted in brains of subjects with idiopathic autism

Objective # 1: Although recent efforts have been highly successful in identifying hundreds of genes along with interacting epigenetic and environmental factors that contribute to autism susceptibility, a more thorough understanding of the proteins and pathways that link genes to behaviour is desperately needed. Indeed, protein products of genes that have been implicated in syndromic ASD have not been examined in brains of subjects with idiopathic autism. Thus, the first objective of this work was the analysis of TrkB-mediated pathway component protein expression in postmortem fusiform gyrus of patients with idiopathic autism. This area of the cortex was chosen as it is implicated in poor social skills and face recognition deficits characteristics of autistic patients (Allison et al., 1994; Boucher et al. 1992; Pierce et al., 2001; Schultz et al., 2000).

2.2 Hypothesis # 2: Mice prenatally exposed to valproic acid (VPA) exhibit autistic-like behaviour and have molecular deficits similar to those seen in brains of individuals with idiopathic autism

Objective # 2: After documenting molecular changes in brain tissue of patients with idiopathic autism, it is crucial to move to an animal model and translational work in order to further investigate the contribution of these molecular deficits to autistic traits. Hence, the second objective of this work was to determine whether autistic-like behaviours were associated with disruptions of TrkB downstream pathway components in an animal model of autism. Because of its construct and face validity as a model for the pathways and behaviours under study (Roullet et al., 2013), the VPA-induced mouse model was chosen. Moreover, since prenatal exposure to VPA leads to transient hyperacetylation of H3 and H4 histones in the embryonic mouse brain (Kataoka et al., 2013), VPA mice appear to be a model of epigenetic changes and hence they might better represent idiopathic autism cases having no known genetic basis than transgenic mice carrying mutations in single autism-linked genes.

2.3 Hypothesis # 3: Treatment with TrkB-regulated pathway activators, LM22A-4 and (1-3)IGF-1, improves autistic-like behaviour and restores TrkB-mediated pathway disruptions in VPA-exposed mice

Objective # 3: As shown with animal models of Rett syndrome, a syndromic ASD which arises from a mutation in an epigenetic regulatory gene (Castro et al., 2014; Kron et al., 2014; Schmid et al., 2012; Tropea et al., 2009), there is potential for pharmacological intervention in TrkB-regulated pathways. Treatment with the partial TrkB agonist LM22A-4 increased TrkB and Akt phosphorylation and improved respiratory deficits in *Mecp2* deficient mice (Kron et al., 2014; Schmid et al., 2012). Furthermore, administration of human recombinant full-length IGF-1 and (1-3)IGF- increased Akt phosphorylation, excitatory postsynaptic marker PSD-95 levels and dendritic spine density in the visual cortex and improved breathing and social impairments in *Mecp2* deficient mice (Castro et al., 2014; Tropea et al., 2009). In light of these findings, the third objective of this work was to test whether either the partial TrkB agonist LM22A-4 or the N-terminal IGF-1 tripeptide fragment (1-3)IGF-1 could ameliorate autistic-like behaviours and increase TrkB-Akt signaling in the offspring of VPA-injected mothers.

CHAPTER 3: MATERIALS & METHODS

3.1 OBJECTIVE 1

3.1.1 Human Brain Tissue Samples

All experimental protocols were approved by the Research Ethics Board of McMaster University. Frozen samples consisting of eleven postmortem fusiform gyrus samples from subjects with idiopathic autism (3 females, 8 males) and thirteen control brain samples (3 females, 10 males) were provided to us by the Autism Speaks' Autism Tissue Program (Princeton, NJ, USA) via the Harvard Brain Tissue Resource Centre (Belmont, MA, USA) and the National Institute of Child Health and Human Development (NICHD) Brain and Tissue Bank (University of Maryland, Baltimore, MD, USA) and were stored at -80°C before use. Clinical information about each tissue sample was obtained through the Autism Tissue Program online portal [http://www.autismbrainnet.com/about-us/portal/] (Garcia et al., 2012; Nicolini et al., 2015) (**Table 1 & 2**). Samples were matched as much as possible for age, gender and postmortem interval (PMI). There were no significant differences between groups for these variables (Garcia et al., 2012). Cause of death, however, differed between samples. It was not possible to match the cause of death due to scarcity of available tissue. All of the tissues were from fusiform gyrus because this area expresses BDNF and is implicated in autism (Allison et al., 1994; Boucher & Lewis, 1992; Gauthier et al., 1997; Schultz et al., 2000). Hypoactivation of the "Fusiform Face Area" is thought

to be responsible for abnormalities in face recognition skills and difficulty with face perception in autistic patients (Boucher and Lewis, 1992). The diagnosis of autism was confirmed using the Autism Diagnostic Interview-Revised (Lord et al., 1994) postmortem through interviews with the parents and/or caregivers. Samples from subjects with known genetic causes of autism spectrum and related disorders (Rett, Asperger etc.) were excluded.

Table 1. Characteristics of Autism Tissue Samples

Sample ID#	CASE #	Age (years)	Gender	PMI (hours)	Primary Cause of Death	ADI-R	Drug Treatment History
A1	AN01093	56	Male	19.48	Anoxic Encephalopathy	48	N/A
A2	UMB1174	7	Female	14	Seizure, Hypotension	44	Depakote, Dilantin, Tegretol
A3	AN00764	20	Male	23.7	Auto Trauma	50	Minocin
A4	AN08792	30	Male	20.3	GastroIntestinal Bleeding	41	Phenobarbital, Mysoline, Dilantin, Depakote, Cisapride, Clorazepate, Prolosec, Propulsid, Reglan, Tranxene
A5	AN06420	39	Male	13.95	Cardiac Tamponade	41	Synthroid, Depakote, Risperidol, Paxil, Blood pressure medication
A6	AN00493	27	Male	8.3	Drowning	-	Synthroid
A7	AN08873	5	Male	25.5	Asphyxia Due To Drowning	47	Prozac
A8	UMB797	9	Male	13	Drowning	50	Desipramine
A9	UMB1182	9	Female	24	Smoke Inhalation	-	N/A
A10	AN16641	9	Male	27	Seizure Disorder	46	Clonidine, Depakote, Dilantin, Lamictal, Ritalin, Tegretol
A11	AN16115	11	Female	12.88	Seizure & Drowning in Tub	44	Adderall, Dexadrine, Dilantin, Klonopin, Lamictal, Tegretol, Topomax
	Mean	**20.1**		**18.3**			
	SEM (+/-)	**4.9**		**1.8**			

N/A = Information Not Available; **PMI** = Postmortem Interval; **ADI-R** = Autism Diagnostic Interview-Revised.

Table 2. Characteristics of Control Tissue Samples

Sample ID#	CASE #	Age (years)	Gender	PMI (hours)	Primary Cause of Death	Secondary Cause of Death
C1	AN12552	56	Male	23.61	Traumatic asphyxia and crush injury	Cardiac Arrest
C2	AN17344	46	Male	25.9	Unknown	N/A
C3	AN14771	30	Male	23	Cardiac Arrhythmia	N/A
C4	UMB818	27	Male	10	Multiple injuries	Accident
C5	AN17425	16	Male	26.16	Unknown	N/A
C6	AN15240	36	Female	18.08	Unknown	N/A
C7	AN19760	28	Male	23.25	Unknown	N/A
C8	AN12240	51	Male	4.75	MI	N/A
C9	AN10606	56	Male	23	Myocardial infarction	N/A
C10	UMB1706	8	Female	20	Rejection of Cardiac Allograft Transplantation	N/A
C11	UMB1860	8	Male	5	Cardiac Arrhythmia	N/A
C12	UMB1407	9	Female	20	Asthma	N/A
C13	UMB1649	20	Male	22	Multiple injuries	N/A
	Mean	**28.5**		**18.8**		
	SEM (+/-)	**4.8**		**1.8**		

N/A = Information Not Available; **PMI** = Postmortem Interval.

3.1.2 Protein Extraction from Brain Tissue

Eleven autism and thirteen control samples from fusiform gyrus were used for Western blot analysis. Approximately one gram of tissue was homogenized on ice without thawing using a sonic dismembrator in homogenization buffer (HB) (0.05 M Tris pH 7.5, 0.5% Tween-20, 10mM EDTA) with protease (complete, Mini, EDTA-free) and phosphatase (PhosSTOP) inhibitor cocktail tablets (Roche, Indianapolis, IN, USA). Homogenates were incubated for 15 minutes on ice and then centrifuged at 12000 xg for 20 minutes at 4°C to precipitate insoluble debris. The supernatants containing solubilized protein were collected, aliquoted and stored at -80°C prior to use. Protein concentrations were determined using the DC™ protein assay (Bio-Rad Laboratories, Mississauga, ON, Canada) as described by the manufacturer.

3.1.3 Western Blotting & Densitometry

Samples containing from 10 to 35 μg total protein each (depending on the target) were separated on 8%-12% sodium dodecyl sulfate (SDS)-polyacrylamide gels under reducing conditions. Proteins were transferred onto polyvinylidene difluoride membrane (PVDF) (Immobilon-FL, Millipore, Billerica, MA, USA) in transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol) for 1.5 hour at 250 mA, at 4°C. Membranes were then blocked with a 1:1 solution of phosphate-buffered saline (PBS) pH 7.4 and Odyssey® Blocking Buffer (BB) (Cedarlane, Burlington, ON, Canada) for 1 hour at room temperature. After blocking, the blots were probed overnight at 4°C in 4ml

BB/PBS (1:1) and 10 μl of 0.2% Tween-20 with the following primary antibodies: TrkB (Cell Signaling Technology, Danvers, MA, USA; diluted 1:700), GFAP (Cell Signaling Technology, diluted 1:2000), βIII-Tubulin, PI3K p85, Akt, mTOR , phospho-mTOR (Ser2448), 4E-BP1, eIF4E, eIF4B (Cell Signaling Technology, diluted 1:1000), p70S6K (Santa Cruz Biotechnology, Dallas, TX, USA; diluted 1:500), Eps8 (generous gift from Dr. Michela Matteoli, Humanitas Clinical & Research Centre, Milan, Italy; dilution 1:1000), $p75^{NTR}$ and PSD-95 (Millipore, Billerica, MA, USA; diluted 1:1000). The mTOR antibody detects levels of total mTOR protein which is present in two complexes, mTORC1 and mTORC2, thus references to mTOR throughout the text encompass both mTORC1 and mTORC2. Similarly, the phospho-mTOR antibody detects levels of phosphorylated mTOR at serine 2448 (Ser2448) in mTORC1 and mTORC2 complexes; therefore, references to phospho-mTOR throughout the text refer to Ser2448 phosphorylated mTOR within both complexes. Membranes were simultaneously probed with mouse monoclonal anti-β-actin antibody (Sigma, St. Louis, MO, USA; diluted 1:5000) as a loading control. After washing with PBS-0.1% Tween-20 (PBS-T), the blots were incubated with the secondary antibodies IRDye® 680RD Goat Anti-Rabbit and IRDye® 800CW Goat Anti-Mouse (LI-COR Biosciences, Lincoln, NE, USA; diluted 1:8000) for 1 hour at room temperature in a 5ml solution of PBS-T and BB (1:1). Lastly, after another wash with PBS-T, all membranes were scanned using an Odyssey® Infrared Imaging System (LI-COR, Biosciences). Band intensities were quantified by

densitometry with local background subtraction using LI-COR® Odyssey Software, version 2.0.

3.1.4 RNA Isolation & cDNA Synthesis

Seven autism and eleven control samples from fusiform gyrus were used for RT-PCR. RNA was isolated from 0.08 to 0.13 g of tissue per sample. Yield and purity of total cellular RNA were determined by absorbance at 260 and 280 nm, and RNA integrity was verified by agarose gel electrophoresis.

cDNA was generated in a reaction mixture containing 1 μg of DNase-treated sample, 10mmol/L each of dATP, dCTP, dGTP and dTTP (Invitrogen, Burlington, ON, Canada), and 300 ng of random primers (Invitrogen). Samples containing double-distilled water in place of reverse transcriptase were included as negative controls (no-RT).

3.1.5 Quantitative Real-Time Polymerase Chain Reaction

Quantitative real-time polymerase chain reaction (qPCR) was performed using the MX3000P real-time PCR system (Stratagene, La Jolla, CA, USA). The reaction mixture was composed of cDNA from 100 ng of RNA or reference standard for absolute quantification, 300 nM of forward and reverse primers, 10 μL of SYBR Green qPCR Supermix UDG™ (Invitrogen, Burlington, ON, Canada), and 30 nM of ROX reference dye™ (Stratagene) in a total volume of 20 μL. Primers were designed using Primer3 Software, version 0.4.0 (http://frodo.wi.mit.edu) and synthesized by MOBIX (McMaster

University). Forward primer, 5'-GGC CCA GAT GCT GTC ATT AT-3', and reverse primer, 5'-TTC TGC TCA GGA CAG AGG TT-3', were used to detect full-length TrkB (TrkB-FL), while truncated TrkB (TrkB-T1) was detected using forward primer, 5'-TGC CTT TTG GTA ATG CTG TTT-3', and reverse primer, 5'-GGC TTC ATA TAG TAC AGC CTC CA-3'. Lastly, detection of the truncated isoform TrkB-Shc was performed using forward primer, 5'-GGC CCA GAT GCT GTC ATT AT-3', and reverse primer, 5'-AGG CAT GGA TTT AGC CTC CT-3'. Standards for each target were generated by RT-PCR of target-containing (brain) tissue using the above primers and spectrophotometric quantification of gel-purified product. "No-RT" and "No template" controls containing double-distilled water in place of cDNA were included, and all samples, standards and controls were run in triplicate. The following thermal profiles were used: 2 minutes at 50°C, 2 minutes at 95°C followed by 40 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 45 seconds, for TrkB-FL and TrkB-Shc; 2 minutes at 50°C, 2 minutes at 95°C followed by 40 cycles of 95°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds and 84°C for 45 seconds, for TrkB-T1. Dissociation curves were run to verify that no secondary products were formed. Copy numbers using absolute quantification and PCR efficiencies were calculated with MXPro Mx3000P Software (Stratagene). Only experiments in which the real-time PCR efficiency was between 90%-100% and standard curves yielded a R^2>0.990 were used for analysis.

3.1.6 Statistical Analysis

Each Western blot contained a standard curve consisting of different amounts of protein per lane (from 1 to 80 μg) from a single normal human cortex sample to allow normalization between blots and to ensure that the sample loading amount was in the linear range of detection for all targets tested. Amounts of protein from unknown samples (both autism and control) were chosen so as to fall within the linear range of the standard curve.

For both qPCR and Western blot experiments, each sample was expressed as a ratio to its corresponding β-actin value.

Differences in the target mRNA or protein levels between autism and control samples were calculated by 2-tailed Student's *t*-test with statistical significance set at $p<0.05$.

3.2 OBJECTIVES 2 & 3

3.2.1 Animals & Treatments

All experimental procedures were performed in compliance with standards of the Canadian Council on Animal Care after approval from the McMaster University Animal Research Ethics Board. CD-1 female mice were mated until a plug was detected. CD-1 mice are an outbred stock. Outbred stocks are colonies of genetically variable animals that are bred to maintain maximum heterozigosity as opposed to inbred strains where all mice are genetically identical (Chia et al, 2005). It follows that the genetically heterogeneous CD-1 stock is a good model of the heterogeneity present in the

human autism population. Day 1 of gestation was determined as the day following the sperm plug detection (E0). On day 12.5 after conception (E12.5), pregnant females received a single intraperitoneal (i.p.) injection of 500 mg/kg sodium valproate (VPA; Sigma, Oakville, ON, Canada) dissolved in 0.9% NaCl solution, while controls were injected with only saline. Prenatal exposure to 500 mg/kg VPA on E12.5 was previously shown to transiently increase acetylated H3 and H4 histones in CD1 mouse embryonic brains by Kataoka and colleagues (2013). Therefore, this concentration was used here. Pups were weaned on postnatal day (PD) 21 and were administered once daily i.p. either saline, the small-molecule BDNF loop-domain mimetic LM22A-4 (50 mg/kg [Schmid et al., 2012]; generous gift from Dr. Frank Longo, Stanford University School of Medicine, Stanford, CA, USA) or the active N-terminal tripeptide fragment of IGF-1 (1-3)IGF-1 (20 mg/kg [Tropea et al., 2009]; Bachem Biosciences, Torrance, CA, USA) until PD 35 (**Table 3**). Athough Massa et al. (2010) initially reported that LM22A-4 had low blood brain barrier (BBB) penetration, later reports demonstrated that LM22A-4 crosses the BBB. Specifically, Schmid and colleagues (2012) determined, using liquid chromatography with tandem mass spectrometry, that an hour after an i.p. injection of 50 mg/kg LM22A-4, brainstem and forebrain concentrations of LM22A-4 were within the range at which this compound exhibits its biological activity in TrkB function assays (Massa et al., 2010). Interestingly, while the 50 mg/kg LM22A-4 dose increased TrkB and Akt phosphorylation and improved respiratory function in Mecp2 deficient mice, it

had no effect on either phosphorylated TrkB or Akt levels or respiratory frequency in wild-type mice (Schmid et al., 2012).

Offspring of VPA- and saline-injected mothers were tested behaviourally between PD 29-34. A summary of the drug treatment and behavioural testing timeline is reported in Figure 10. Mice that were not subjected to behavioural testing and did not receive any of the treatments are referred to as naïve. To avoid VPA exposure and treatment effects being confounded by natural variation between litters, only up to two males and two females from each VPA-exposed and saline-exposed litter were used per treatment group (naïve, saline, LM22A-4, (1-3)IGF-1). Animals were killed by decapitation on PD 35 and brains were rapidly harvested and dissected. Brain tissue samples were snap-frozen in liquid nitrogen and stored at -80 °C prior to use. As the fusiform gyrus, which is part of the temporal and occipital lobes in humans, is not present in rodents, parietal/temporal neocortices were used for analysis.

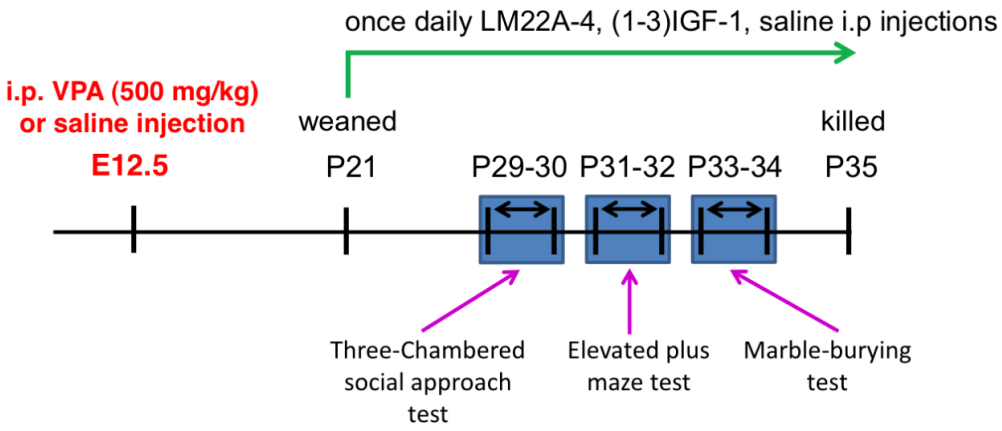

Figure 10. Timeline of drug treatments and behavioural tests. i.p., intraperitoneal; P, postnatal day.

Table 3. Treatment Groups

Treatment Groups	Experimental Conditions
Naïve	Saline- and VPA-exposed mice that were not subjected to behavioural tests and did not undergo any of the treatments
Saline	Saline- and VPA-exposed mice that were tested behaviourally and received an i.p. injection of saline once daily from PD 21-35
LM22A-4	Saline- and VPA-exposed mice that were tested behaviourally and received an i.p. injection of LM22A-4 once daily from PD 21-35
(1-3)IGF-1	Saline- and VPA-exposed mice that were tested behaviourally and received an i.p. injection of (1-3)IGF-1 once daily from PD 21-35

VPA = valproic acid; **i.p.** = intraperitoneal; **PD** = postnatal day.

3.2.2 Social Approach Behaviour

Social approach behaviour was assessed using the three-chambered social approach task (adopted form: Crawley et al., 2004; Moy et al., 2007; Yang et al., 2011) on PDs 29 and 30. The social approach apparatus consisted of a rectangular, clear Plexiglas three-chambered box. Each chamber measured 20 cm (length) X 20 cm (width) x 40 cm (height) and had small openings with removable doorways which allowed access into each side compartment. Test mice were first individually placed in the middle chamber with the two doorways closed and allowed a 10-min habituation time. Next, mice were habituated to all three chambers for 10 minutes. Following the second 10-min habituation phase, a same-sex novel mouse ('stimulus') was placed in one of the side compartments, while the other was left empty. Novel mice ("stimulus") were located inside an inverted wire pencil cup to ensure that social approaches were initiated only by the test mice and to prevent potential confounding factors such as aggressive and sexual interactions (Moy et al., 2007; Yang et al., 2011). The wire pencil cup allowed visual, tactile, olfactory and auditory interactions. An identical clean empty inverted wire pencil cup was placed in the empty compartment. Test mice were presented with the choice of spending time either in the chamber with the same-sex novel conspecific or in the empty chamber, and sociability was quantified. Sociability is defined as the tendency to spend more time in the chamber containing the novel mouse than in the empty chamber (Yang et al., 2011). Each subject mouse was given 10 minutes to explore freely all three chambers. To avoid side preference bias, the side containing the novel object or the novel

mouse was alternated between subjects. Time spent in each chamber was scored. Number of transitions across chambers was also recorded as a measure of exploratory locomotion.

3.2.3 Marble-Burying

Repetitive digging behaviour was scored using the marble-burying test on PDs 33 and 34 (Deacon, 2006). Clean standard mouse cages were filled with approximately 4 cm-deep fresh wood chip bedding, and 15 glass marbles were arranged in a regular pattern on the surface. Animals were then placed individually in these cages and allowed to freely explore for 30 minutes. At the end of the 30-minute session, the number of marbles that were not buried in bedding by 2/3 their depth was counted. This value was next subtracted from 15 to obtain the number of buried marbles.

3.2.4 Elevated Plus Maze

Anxiety-like behaviour was measured using an elevated plus maze (KinderScientific, Poway, CA, USA; generously provided by Dr. Jane Foster, McMaster University, St. Joseph's Healthcare, Hamilton, ON, Canada) on PDs 31 and 32 (Neufeld et al., 2011; Sidor et al., 2010). The apparatus, made from black Plexiglas and raised 76.2 cm above floor level, was configured in the shape of a "plus" and comprised two open arms perpendicular to two closed arms and a center platform. The open arms had 0.5 cm-raised ledges, while the closed arms were enclosed by 15.2-cm sides. Each mouse was

videotaped using a camera placed above the apparatus for 5 minutes. Number of entries into open and closed arms, and time spent in open arms, closed arms and centre were scored (Sidor et al., 2010).

3.2.5 Protein Extraction from Mouse Brain Tissue

Mouse temporal/parietal neocortices were used for Western blot analysis. Protein extraction from mouse brain tissue samples was carried out as described in section 3.1.2.

3.2.6 Western Blotting & Densitometry

Western blotting and densitometry for mouse brain samples were performed as described in section 3.1.3. 50 μg total protein was separated on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels under reducing conditions. The following primary antibodies were used to detect mouse protein targets: Akt, phospho-Akt (Ser473), S6 ribosomal protein (54D2), phospho-S6 ribosomal protein (Ser240/244) (Cell Signaling Technology, Danvers, MA, USA; diluted 1:1000). Membranes were simultaneously probed with mouse monoclonal anti-β-actin antibody (Sigma, Oakville, ON, Canada; diluted 1:5000) as a loading control. All blots were scanned using an Odyssey® Infrared Imaging System (LI-COR, Biosciences) and band intensities were quantified by densitometry as described in section 3.1.3.

As described in section 3.1.6, each Western blot contained a standard curve consisting of different amounts of protein per lane (from 1 to 80 μg)

from a single normal mouse cortex sample to allow normalization between blots and to ensure that the sample loading amount was in the linear range of detection for all targets tested.

3.2.7 Statistical Analysis

Main effects of variables such as VPA exposure, drug treatment and sex were evaluated with two-way ANOVA (unless otherwise specified all further references to 'ANOVA' assume two-way ANOVA). Whenever a significant difference was revealed by two-way ANOVA, post-hoc analyses were done using the Student-Newman-Keuls (SNK) test for multiple comparisons. Prior to analyzing the data, significant outliers were identified using the Grubb's test and removed when found. Linear regressions (Pearson's Product Moment correlations) were conducted to assess whether significant relationships existed between behavioural and molecular data. Statistical significance was set at $p<0.05$. All analyses were done using Statistica software (v6, Tulsa, OK, USA).

CHAPTER 4: RESULTS

4.1 OBJECTIVE 1

4.1.1 Imbalance in TrkB Protein Isoforms in Idiopathic Autism

As TrkB involvement in autism pathogenesis is supported by the finding of an association between *trkB* (*NTRK2*) gene variants and autism (Correia et al., 2010), protein levels of TrkB isoforms were measured by Western blotting in postmortem fusiform gyrus of subjects with idiopathic autism, that is, patients without a known genetic cause and without a related disorder on the spectum. Fusiform gyrus is an area of the brain implicated in face discrimination and perception difficulties of autistic subjects (Boucher & Lewis, 1992; Schultz et al., 2000). TrkB bands migrating at about 140 kDa and 90 kDa were detected in all samples. The 140 kDa band corresponds to the molecular weight of the full-length form of TrkB receptors (Wong et al., 2013). The 90 kDa band corresponds to TrkB-T1 and TrkB-Shc isoforms (Wong et al., 2013) and is large and diffuse, as the two truncated TrkB isoforms, having similar size, migrate together. A significant reduction in full-length TrkB (TrkB-FL)/truncated TrkB (TrkB-T1 + TrkB-Shc) isoform ratio and increase in the ratio of truncated TrkB isoforms/TrkB-FL (*$p<0.05$, **Figure 11A**; *$p<0.05$, **Figure 11B**; 2-tailed *t* tests) were observed in the fusiform gyrus of individuals with idiopathic autism versus controls. This may be due to significantly decreased TrkB-FL (*$p<0.05$, 2-tailed *t* test; **Figure 11**

C,D) as well as a trend towards increased truncated TrkB (p=0.09, 2-tailed *t* test; **Figure 11 E,F**).

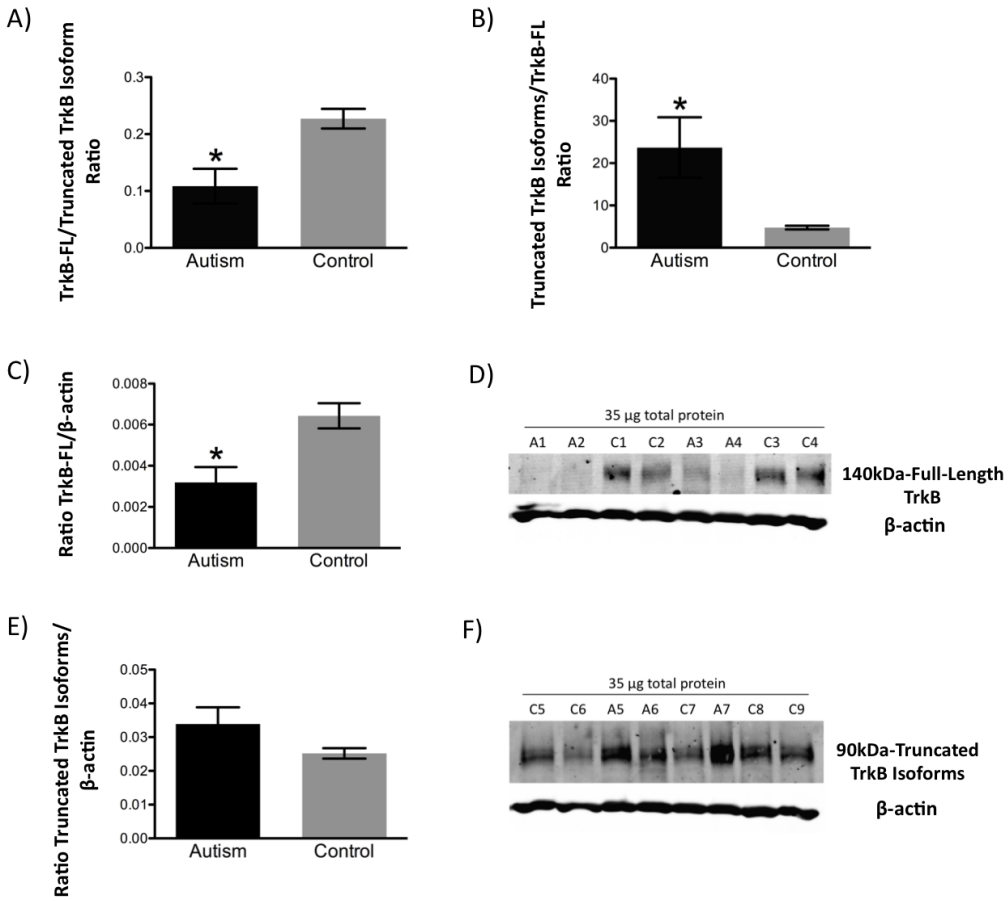

Figure 11. Quantification of (**A**) full-length (FL)/truncated and (**B**) truncated/full-length (FL) TrkB isoform protein expression ratios in fusiform gyrus of autism and control samples. *p<0.05 for FL/truncated and truncated/FL TrkB isoform ratios, 2-tailed *t* tests. Bars indicate mean ± SE. Autism, n=11; control, n=13. (**C**) Quantification of full-length TrkB (TrkB-FL) and (**E**) truncated TrkB isoform protein expression in fusiform gyrus of autism and control samples by Western blotting. Each sample was normalized

to its β-actin. *p<0.05 for TrkB-FL, p=0.09 for truncated TrkB isoforms, 2-tailed *t* tests. Bars indicate mean ± SE. Autism, n=11; control, n=13. (**D**) and (**G**) Representative Western blots of fusiform gyrus showing autism (A) and control (C) cases. Sample numbers correspond to samples listed in Tables 1 and 2. 35 μg of total protein from each autism and control sample was loaded.

TrkB-FL and truncated TrkB are differentially expressed in different cell types: TrkB-FL is mainly neuronal whereas truncated TrkB isoforms are expressed in both neurons and glia. Therefore, protein levels of βIII-Tubulin (neuronal) and GFAP (glial) markers were measured. There were no significant differences in βIII-Tubulin or GFAP protein expression in the fusiform gyrus (p=0.5, **Figure 12A,B**; p=0.4, **Figure 12C,D**; 2-tailed *t* tests) between control and idiopathic autism subjects, which precludes differential cell loss as a mechanism for TrkB isoform alterations.

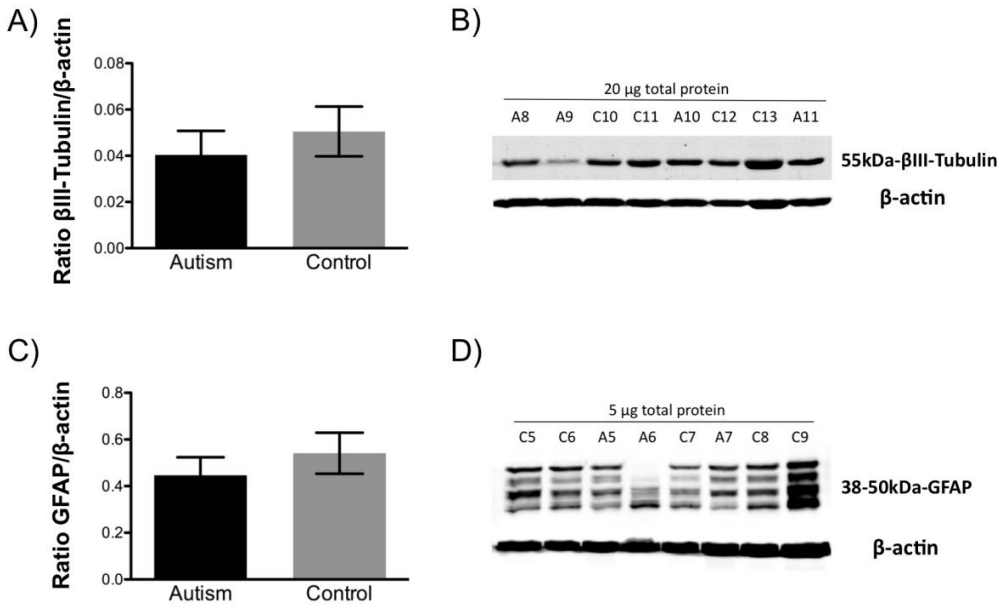

Figure 12. Quantification of (**A**) Beta-III Tubulin and (**C**) GFAP protein expression in fusiform gyrus of autism and control samples by Western blotting. Each sample was normalized to its β-actin. As reported by other studies (Newcombe et al., 1986; Fatemi & Folsom, 2011), four bands ranging from 38 kDa to 50 kDa were detected for GFAP in all samples and quantified together. p=0.5 for Beta-III Tubulin, p=0.4 for GFAP, 2-tailed *t* tests. Bars indicate mean ± SE. Autism, n=11; control, n=13. (**B**) and (**D**) Representative Western blots of fusiform gyrus showing autism (A) and control (C) cases. Sample numbers correspond to samples listed in Tables 1 and 2. 20 µg for Beta-III Tubulin and 5 µg for GFAP of total protein from each autism and control sample were loaded.

No significant differences in TrkB isoform mRNA levels were found in the fusiform gyrus between control and idiopathic autism samples (TrkB-FL: p=0.1; TrkB-T1: p=0.3; TrkB-Shc: p=0.7; 2-tailed *t* tests), suggesting that post-transcriptional mechanisms might be responsible for the TrkB protein isoform imbalance seen in autistic brains (see Discussion).

Finally, it is worth mentioning that, contrary to Luberg and colleagues' findings, we did not observe significant changes with age in the expression levels of TrkB isoform mRNA (**Figure 13A-C** in Appendix) or protein (**Figure 14A-B** in Appendix) in our human fusiform gyrus samples from patients aged 5-56 years old. This suggests that age-related expression patterns of TrkB isoforms might be specific to brain areas. It is also possible that no correlation between age and expression of TrkB isoforms was observed here due to our smaller sample size (n=24) compared to the Luberg's group (n=52).

4.1.2 Reduced PI3K p85, Akt, mTOR, phosho-mTOR, p70S6K, eIF4B & Eps8 Protein Expression in Idiopathic Autism

Studies of single-gene disorders with a high prevalence of autism such as Rett and fragile X syndromes, tuberous sclerosis, neurofibromatosis, and macrocephaly point to the mTOR pathway as a good candidate for involvement in autism pathogenesis. Thus, protein levels of mTOR upstream regulators (PI3K and Akt), mTOR, phospho-mTOR, and mTOR downstream effectors (p70S6K, eIF4B, 4E-BP1 and eIF4E) were examined in the fusiform gyrus of subjects with idiopathic autism versus controls. Bands migrating at approximately 85 kDa, 60 kDa, 290 kDa, 70 kDa, 80 kDa, 20 kDa and 25 kDa

and corresponding to the molecular weights of the p85 subunit of PI3K, Akt, mTOR, phospho-mTOR, p70S6K, eIF4B, 4E-BP1 and eIF4E, respectively (Bergeron et al., 2014; Ochs et al., 2002; Sheikh et al., 2010; Xu et al., 2013), were detected in all postmortem human brain tissue samples. Western blotting revealed a statistically significant decrease in PI3K p85 and Akt (*$p<0.05$, **Figure 15A,B**; *$p<0.05$, **Figure 15C,D**; 2-tailed *t* tests) in the fusiform gyrus of subjects with idiopathic autism relative to controls.

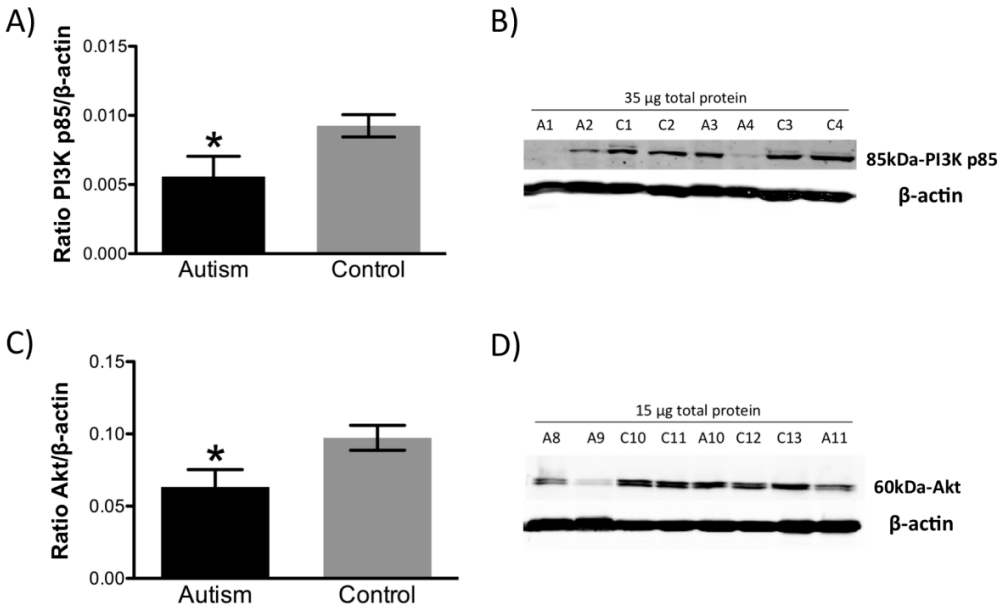

Figure 15. Quantification of (**A**) PI3K p85 and (**C**) Akt protein expression in fusiform gyrus of autism and control samples by Western blotting. Each sample was normalized to its β-actin. *p<0.05 for PI3K and Akt, 2-tailed *t* tests. Bars indicate mean ± SE. Autism, n=11; control, n=13. (**B**) and (**D**) Representative Western blots of fusiform gyrus showing autism (A) and control (C) cases. Sample numbers correspond to samples listed in Tables 1 and 2. 35 µg for PI3K p85 and 15 µg for Akt of total protein from each autism and control sample were loaded.

Total and phosphorylated mTOR (phospho-mTOR) were also reduced in idiopathic autism *vs.* control brain (*p<0.05, **Figure 16A**; *p<0.05, **Figure 16B**; 2-tailed *t* tests). Furthermore, the ratio of phospho-mTOR to total mTOR was significantly decreased in fusiform gyrus from patients wih idiopathic autism compared to controls (*p<0.05, **Figure 16C**; 2-tailed *t* test).

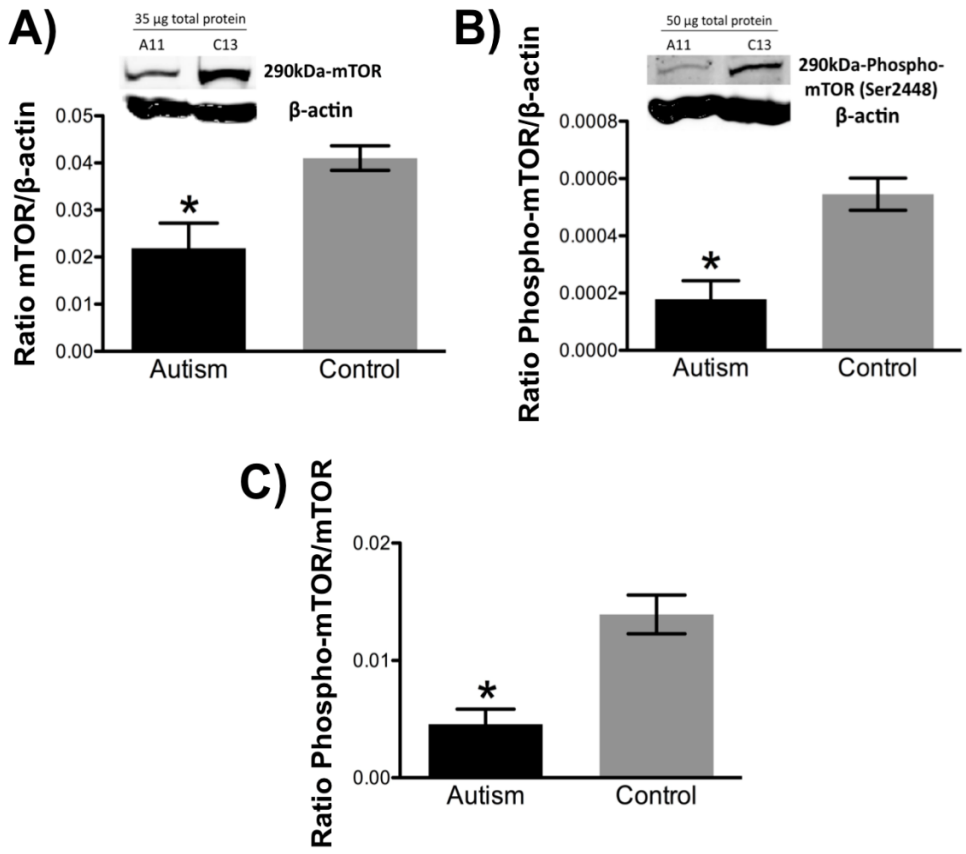

Figure 16. (**A**) and (**B**) Quantification by Western blotting and representative Western blots of total mTOR and phospho-mTOR protein expression in fusiform gyrus of autism and control samples. Each sample was normalized to its β-actin. *p<0.05 for mTOR and phospho-mTOR, 2-tailed *t* tests. Bars indicate mean ± SE. Autism, n=11; control, n=13. 35 µg for mTOR and 50 µg for phospho-mTOR of total protein from each autism (A) and control (C) sample were loaded. Sample numbers correspond to samples listed in Tables 1 and 2. (**C**) Ratio of phospho-mTOR to total mTOR in fusiform gyrus samples from autism and control subjects. *p<0.05, 2-tailed *t* test. Bars indicate mean ± SE. One outlier was identified (Grubb's test) and removed from the autism group. Autism, n=10; control, n=13.

mTOR influences protein translation at dendritic spines via two different downstream effector pathways, one involving p70 kDa-ribosomal protein S6 kinase (p70S6K), ribosomal protein S6 (rp S6), and eukaryotic initiation factor 4B (eIF4B), and the other involving regulators of translation initiation, eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) and eukaryotic initiation factor 4E (eIF4E). Both rp S6 and eIF4B can be measured as a read-out of p70S6K (**Figure 2**). Here, eIF4B total protein levels were assessed because, similar to eIF4E, eIF4B is involved in translation initiation (Dennis et al., 2012; Rogers et al., 2001). Western blotting revealed a statistically significant decrease in p70S6K and eIF4B protein expression (*$p<0.05$, **Figure 17A,B**; *$p<0.05$, **Figure 17C,D**; 2-tailed *t* tests) in the fusiform gyrus of autistic subjects relative to controls. However, there were no significant differences in protein levels of 4E-BP1 and eIF4E ($p=0.6$, **Figure 17E,F**; $p=0.4$, **Figure 17G,H**; 2-tailed *t* tests) between idiopathic autism and control fusiform gyrus samples, supporting the specificity of the p70S6K/eIF4B pathway deficit.

No clear correlation between the ADI-R score of autistic patients and the reduction in mTOR, phospho-mTOR, p70S6K or eIF4B protein expression was found at present. Patients with less drastic decreases in mTOR pathway components had ADI-R scores comparable to patients with the greatest reductions in these targets. This could be due to the limited number of autistic samples (n=9; ADI-R score was unavailable for two autistic patients). Thus, future experiments will have to establish whether the severity of the ADI-R

score is correlated with the magnitude of the reduction in mTOR pathway components.

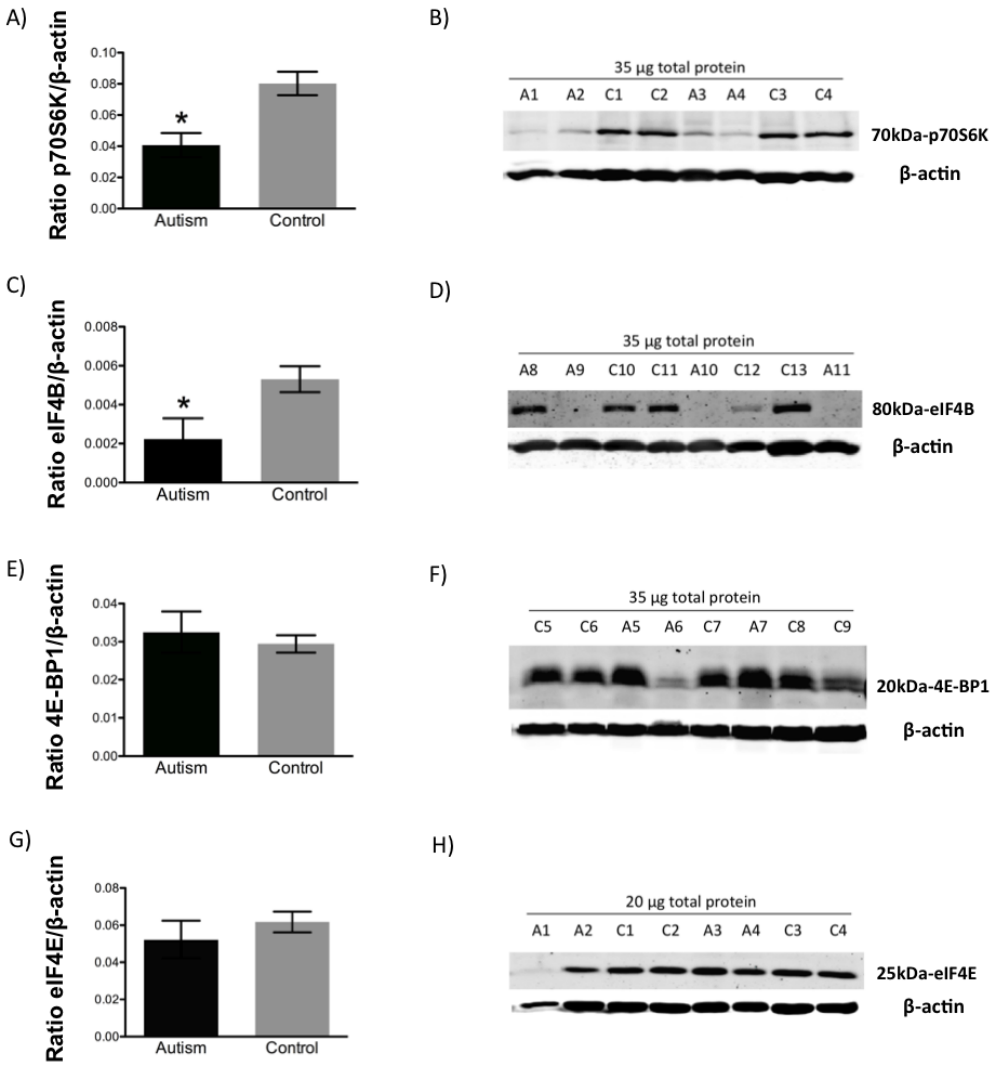

Figure 17. Quantification of (**A**) p70S6K, (**C**) eIF4B, (**E**) 4E-BP1 and (**G**) eIF4E protein expression in fusiform gyrus of autism and control samples by Western blotting. Each sample was normalized to its β-actin. *$p<0.05$ for p70S6K and eIF4B, $p=0.6$ for 4E-BP1 and $p=0.4$ for eIF4E, 2-tailed *t* tests. Bars indicate mean ± SE. Autism, n=11; control, n=13. (**B**), (**D**), (**F**) and (**H**) Representative Western blots of fusiform gyrus showing autism (A) and

control (C) cases. Sample numbers correspond to samples listed in Tables 1 and 2. 35 µg for p70S6K, eIF4B and 4E-BP1 and 20 µg for eIF4E of total protein from each autism and control sample were loaded.

A separate pathway downstream of PI3K activates Eps8, an actin-capping molecule that controls spine stability and filopodial motility through Rac (Innocenti et al., 2003; Offenhäuser et al., 2004). To test whether PI3K down-regulation negatively impacts Eps8, protein levels of Eps8 were measured by Western blotting in fusiform gyrus of autism *vs.* control subjects. Bands migrating at approximately 95 kDa corresponding to the molecular weight of Eps8 (Menna et al., 2013) were detected in all postmortem human brain tissue samples. A significant reduction in Eps8 protein expression was found in idiopathic autism patients compared to controls (*p<0.05, 2-tailed *t* test; **Figure 18A,B**).

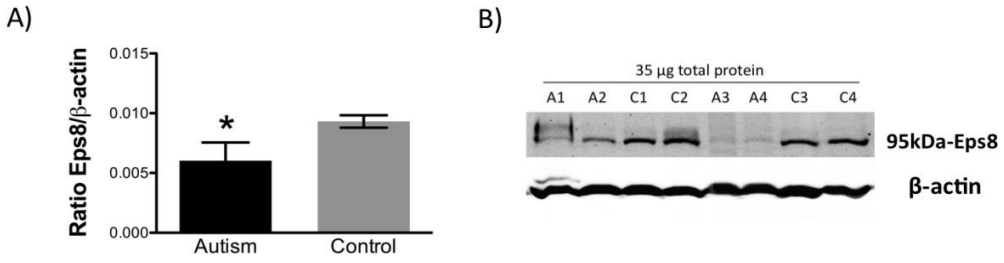

Figure 18. (**A**) Quantification of Eps8 protein expression in fusiform gyrus of autism and control samples by Western blotting. Each sample was normalized to its β-actin. *p<0.05, 2-tailed *t* test. Bars indicate mean ± SE. Autism, n=11; control, n=13. (**B**) Representative Western blot of fusiform gyrus showing

autism (A) and control (C) cases. Sample numbers correspond to samples listed in Tables 1 and 2. 35 μg of total protein from each autism and control sample was loaded.

4.1.3 Trend Towards Increased p75^{NTR} in Idiopathic Autism

proBDNF binds to p75^{NTR} and activates RhoA, which opposes Rac and destabilizes dendritic spines (Lin & Koleske, 2010). Since proBDNF is increased in fusiform gyrus of subjects with idiopathic autism compared to controls (Garcia et al., 2012), p75^{NTR} protein expression was examined in the same cohort. Immunoreactive bands migrating at approximately 75 kDa corresponding to the molecular weight of p75^{NTR} (Kanning et al., 2003) were detected in all postmortem human brain tissue samples. Western blotting revealed a trend towards elevated p75^{NTR} receptor protein (p=0.07, 2-tailed *t* test; **Figure 19A,B**) in the fusiform gyrus of autistic subjects relative to controls.

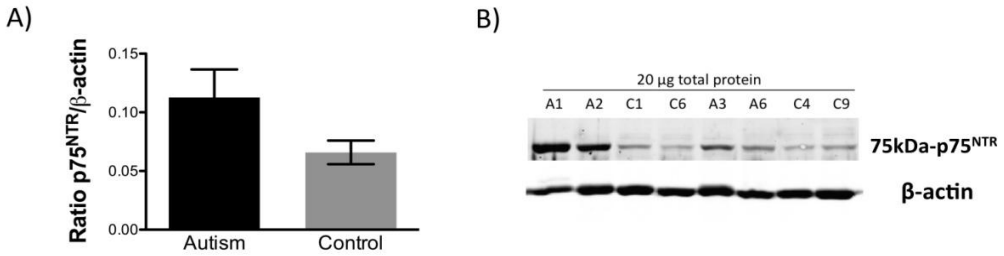

Figure 19. (**A**) Quantification of p75^{NTR} protein expression in fusiform gyrus of autism and control samples by Western blotting. Each sample was normalized to its β-actin. p=0.07, 2-tailed *t* test. Bars indicate mean ± SE.

Autism, n=11; control, n=13. (**B**) Representative Western blot of fusiform gyrus showing autism (A) and control (C) cases. Sample numbers correspond to samples listed in Tables 1 and 2. 20 μg of total protein from each autism and control sample was loaded.

4.1.4 Decreased PSD-95 Protein in Idiopathic Autism

The TrkB/PI3K/Akt pathway is known to influence PSD-95 trafficking to dendritic spines (excitatory synapses) (Yoshii & Constantine-Paton, 2007), while the mTOR pathway influences spine protein synthesis, and its de-regulation is associated with spine deficits in monogenic disorders with high rates of autism (Troca-Marín et al., 2012). Therefore, protein levels of PSD-95, a marker of excitatory synapses, were examined by Western blotting in the fusiform gyrus of autism versus control subjects. Immunoreactive bands migrating at approximately 95kDa corresponding to the molecular weight of PSD-95 (Glantz et al., 2007) were detected in all postmortem human brain tissue samples. A significant decrease in PSD-95 protein expression ($*p<0.05$, 2-tailed *t* test; **Figure 20A,B**) was found in individuals with idiopathic autism versus controls.

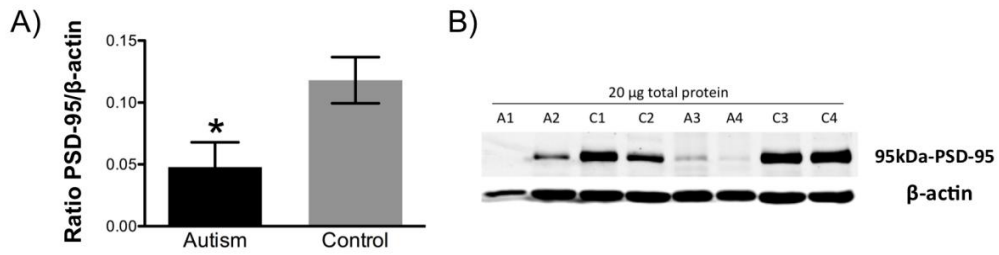

Figure 20. (**A**) Quantification of PSD-95 protein expression in fusiform gyrus of autism and control samples by Western blotting. Each sample was normalized to its β-actin. *$p<0.05$, 2-tailed *t* test. Bars indicate mean ± SE. Autism, n=11; control, n=13. (**B**) Representative Western blot of fusiform gyrus showing autism (A) and control (C) cases. Sample numbers correspond to samples listed in Tables 1 and 2. 20 µg of total protein from each autism and control sample was loaded.

4.2 OBJECTIVES 2 & 3

4.2.1 VPA Female Mice Lack Sociability

Deficits in social interaction are the identifying feature of autistic patients. Here, social approach behaviour in mice was measured on postnatal days 29 and 30 using a modified 3-chambered arena (**Figure 21A**). Sociability is defined as the test mouse spending more time in a chamber containing a novel mouse (located under an inverted wire cup) compared to time spent in a chamber containing an empty wire cup (Moy et al., 2004; Nadler et al., 2004; Yang et al., 2011).

Rather unexpectedly, saline-treated control males displayed a complete lack of social behaviour. In fact, they spent more time in the empty chamber (13% greater, n.s.; see **Table 5A** in Appendix) than in the chamber with the novel mouse, suggesting avoidance of social approach. In retrospect, this was likely an artifact of the experimental design (explanation provided in Discussion). Regardless, it is not possible at present to determine whether prenatal exposure to VPA impairs social approach behaviour in male mice. Consequently, male data is omitted here and reported in the Appendix (**Table 5A**).

Saline-treated female control mice exhibited the expected strong social preference, as they spent significantly more (80%) time in the chamber containing the novel mouse (*$p<0.05$, ANOVA post-hoc SNK; **Figure 21B**). Conversely, saline-treated VPA-exposed females showed no preference towards either chamber ($p=0.8$, ANOVA post-hoc SNK; **Figure 21B**),

suggesting that they were not interested in social interactions any more than at the level of indiscriminate exploratory behaviour (empty chamber). Remarkably, VPA females that were treated with the partial TrkB agonist LM22A-4 displayed sociability, since they spent 33% more time in the chamber containing the novel mouse than in the chamber containing the empty wire cup (*p<0.05, ANOVA post-hoc SNK; **Figure 21B**). Contrary to LM22A-4, the tripeptide fragment (1-3)IGF-1 did not restore sociability in VPA females. Despite spending 26% more time in the chamber with the novel mouse than in the empty wire cup compartment, (1-3)IGF-1-treated VPA females fell short of statistical significance (p=0.1, ANOVA post-hoc SNK; **Figure 21B**). Social behaviour of control mice was not affected by either LM22A-4 or (1-3)IGF-1.

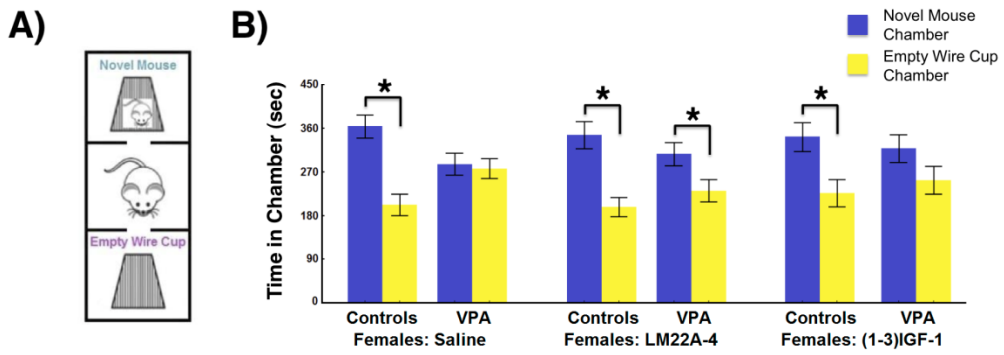

Figure 21. Three-Chambered Social Approach Task. (**A**) Schematic diagram of the social approach apparatus. (**B**) Time spent in the chamber containing a novel mouse located under an inverted wire cup and in the chamber containing an identical empty wire cup was recorded during a 10-minute trial on postnatal days 29 and 30. Data for **female** mice (saline-treated

controls, n=14; saline-treated VPA mice, n=13; LM22A-4-treated controls, n=14; LM22A-4-treated VPA mice, n=14; (1-3)IGF-1-treated controls, n=13; (1-3)IGF-1-treated VPA mice, n=12). *p<0.05, ANOVA post-hoc SNK. Bars indicate mean ± SE.

4.2.2 VPA Mice Exhibit Increased Repetitive Digging Behaviour

Repetitive and restricted behaviours, another core symptom domain of autism, were assessed in mice using the marble-burying test on postnatal days 33 and 34. This test measures spontaneous digging activity, which is a compulsive behaviour unrelated to anxiety (Gyertyan, 1995; Njung'e & Handley, 1991; Thomas et al., 2009; Witkin, 2008). No significant differences between sexes were observed (p=0.4, ANOVA), hence female and male data were pooled. Saline-treated VPA-exposed mice buried significantly more (~ 34%) marbles over 30 minutes compared to saline-treated controls, (*p<0.05, ANOVA post-hoc SNK; **Figure 22B**), suggesting that VPA exposure increased digging behaviour. Notably, LM22A-4-treated VPA mice buried significantly fewer marbles (20% decrease) than saline-treated VPA mice (*p<0.05, ANOVA post-hoc SNK; **Figure 22B**), consistent with a decrease in repetitive digging. Lastly, (1-3)IGF-1-treated VPA mice buried only 16% fewer marbles than saline-treated VPA mice, suggesting that (1-3)IGF-1's therapeutic effect on repetitive digging was weaker than LM22A-4's (p=0.13, ANOVA with post-hoc SNK; **Figure 22B**). Repetitive digging behaviour of control mice was not affected by either LM22A-4 or (1-3)IGF-1.

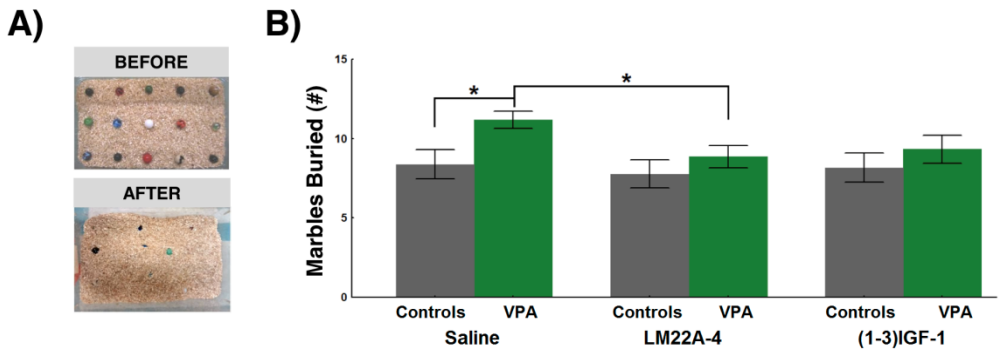

Figure 22. Marble-Burying Test. (**A**) Pictures showing glass marbles before and after a 30-minute testing session. (**B**) The number of marbles buried over a 30-minute trial on postnatal days 33 and 34 was calculated. Data pooled for both sexes (saline-treated controls, n=32; saline-treated VPA mice, n=30; LM22A-4-treated controls, n=30; LM22A-4-treated VPA mice, n=30; (1-3)IGF-1-treated controls, n=30; (1-3)IGF-1-treated VPA mice, n=27). *p<0.05, ANOVA post-hoc SNK. Bars indicate mean ± SE. One outlier was identified (Grubb's test) and removed from the saline-treated VPA group.

4.2.3 VPA Mice Do Not Display Anxiety-Related Behaviour

Anxiety-related behaviours, one of the most common co-occurring symptoms in autism, were assessed in mice using the elevated plus maze test on postnatal days 31 and 32. Mice have a natural aversion for unprotected and elevated areas such as the open arms of the plus maze, but they also have a spontaneous tendency to explore novel environments (Lister, 1987; Rodgers & Dalvi, 1997; Sidor et al., 2010). Time spent in the open arms of the plus maze as well as frequency of entries into the open arms reflect levels of

open/elevated space-induced anxiety e.g. less time and fewer entries into open arms indicate enhanced anxiety-like behaviour (Sidor et al., 2010). No differences between sexes were found (time in zones: $p=0.92$; open arm entries: $p=0.72$; closed arm entries: $p=0.68$; ANOVA), hence data were pooled for females and males. Time spent in open arms did not differ between saline-treated VPA and control mice ($p=0.2$, ANOVA with post-hoc SNK; see **Table 6** in Appendix), suggesting that prenatal exposure to VPA did not affect anxiety-related behaviour. At the same time, saline-treated VPA mice made fewer entries into the open arms of the plus maze compared to saline-treated controls (*$p<0.05$, ANOVA with post-hoc SNK; see **Table 6** in Appendix). The latter may indicate increased open space-induced anxiety. However, this could be an artifact of decreased exploratory locomotion as suggested by the finding that saline-treated VPA mice also made fewer closed arm entries than saline-treated controls (*$p<0.05$, ANOVA with post-hoc SNK; see **Table 6** in Appendix). Lastly, there were no differences in either open or closed arm entries between saline-, LM22A-4- and (1-3)IGF-1-treated VPA mice (open arm entries: $p=0.67$, LM22A-4; $p=0.68$, (1-3)IGF-1; closed arm entries: $p=0.94$, LM22A-4; $p=0.60$ (1-3)IGF-1; ANOVA with post-hoc SNK), suggesting no measurable impacts of treatments on anxiety-related behaviour and decreased exploratory locomotion here.

4.2.4 VPA Mice Have Decreased Phosphorylated Akt and Ribosomal Protein S6 Levels

To establish whether molecular targets found to be decreased in human idiopathic autism were similarly altered in mice prenatally exposed to VPA, total protein and phosphorylation levels of the serine/threonine kinase Akt were measured by Western blotting in postnatal day 35 temporal/parietal neocortices from VPA and control mice (**Figure 23A-B**). Several receptors converge on this kinase including metabotropic glutamate receptors (Hou & Klann, 2004), NMDA receptors (Sutton & Chandler, 2002) and full-length TrkB receptors (Yoshii & Constantine-Paton, 2010) whose protein levels we demonstrated to be reduced in patients with idiopathic autism. No significant differences between sexes were observed ($p=0.37$, two-way ANOVA), hence female and male data were pooled. As sensorimotor enrichment has been demonstrated to impact levels of several proteins involved in synaptic function including neurotrophins (Ickes et al., 2000), we included a naïve group which comprised mice that were prenatally exposed to either saline (controls) or VPA but that were not subjected to behavioural tests. Thus, these mice represented the baseline group for assessing whether VPA exposure had an effect on the molecular targets under investigation in this study. Contrary to our findings in rats (Nicolini et al., 2015), prenatal VPA exposure had no effect on total Akt levels in mice. Indeed, a main effect of VPA across all groups was entirely absent ($p=0.83$, two-way ANOVA). Specifically, while naïve VPA-exposed rats had significantly decreased total Akt compared to controls (Nicolini et al., 2015), no differences in total Akt levels between

naïve VPA-exposed and control mice were found (p=0.84, ANOVA post-hoc SNK; see **Table 7** in Appendix). Also, no changes in total Akt between saline-, LM22A-4- or (1-3)IGF-1-treated VPA mice and their respective controls were determined (p=0.66, saline; p=0.97, LM22A-4; p=0.68, (1-3)IGF-1; ANOVA post-hoc SNK; see **Table 7** in Appendix). Finally, there were no differences in total Akt levels between naïve VPA mice and saline-, LM22A-4- or (1-3)IGF-1-treated VPA mice (p=0.82 saline; p=0.98, LM22A-4; p=0.41, (1-3)IGF-1; ANOVA post-hoc SNK; see **Table 7** in Appendix).

Since no effect of *in utero* VPA exposure on total Akt was observed in our mice, we investigated whether maternal challenge with VPA resulted in decreased Akt phosphorylation as seen in rats (Nicolini et al., 2015). Similar to naïve VPA-exposed rats (Nicolini et al., 2015), naïve VPA-exposed mice showed a significant 16% reduction in phosphorylated Akt levels compared to naïve controls (*p<0.05, ANOVA post-hoc SNK; **Figure 23A**). Notably, a 6% (n.s.) elevation in phosphorylated Akt levels was observed in VPA mice that received saline (sham) injections and underwent behavioural testing, suggesting that the associated enrichment may have a positive influence on Akt phosphorylation. A further 7% (n.s.) increase in phosphorylated Akt was determined in LM22A-4-treated VPA mice compared to saline-treated VPA mice (p=0.23, ANOVA post-hoc SNK). Additionally, VPA mice that were subjected to behavioural testing and treated with LM22A-4 had significantly higher (13% increase; *p<0.05, ANOVA post-hoc SNK) phosphorylated Akt levels compared to naïve VPA mice. This finding suggests that LM22A-4 contributed to higher Akt phosphorylation, resulting in greater levels of

activated Akt than those obtained via behavioural enrichment alone (saline-treated VPA mice).

A 7% (n.s.) increase in phoshorylated Akt was observed in VPA-exposed mice treated with (1-3)IGF-1, suggesting that (1-3)IGF-1 does not elevate Akt phosphorylation beyond the effects of behavioural enrichment.

Phosphorylated Akt levels of control mice were not affected by either LM22A-4 or (1-3)IGF-1.

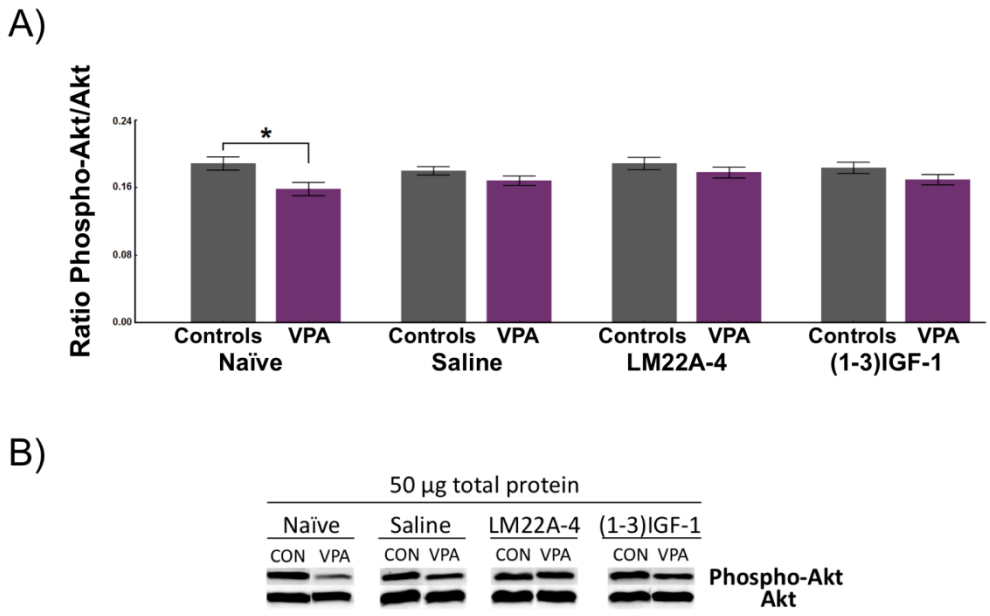

Figure 23. (**A**) Quantification of phosphorylated Akt levels by Western blotting in postnatal day 35 temporal/parietal neocortices from VPA and control mice. Groups were either not subjected to behavioural testing or treatments (naïve), or underwent behavioural tests while treated with either saline, LM22A-4 or (1-3)IGF-1 for 15 days. Each sample was normalized to its total Akt protein. *$p<0.05$, ANOVA post-hoc SNK. Bars indicate mean ±

SE. Pooled data for males and females: Naïve controls, n=31; naïve VPA mice, n=28; saline-treated controls, n=32; saline-treated VPA mice, n=31; LM22A-4-treated controls, n=30; LM22A-4-treated VPA mice, n=30; (1-3)IGF-1-treated controls, n=30; (1-3)IGF-1-treated VPA mice, n=26. (**B**) Representative Western blot of mouse control and VPA temporal/parietal neocortex samples. 50 μg of total protein from each sample were loaded.

To establish whether reduced activated Akt is associated with decreased activation of the mTOR pathway in VPA mice, phosphorylation levels of the mTOR effector ribosomal protein S6 (rp S6) were measured (**Figure 24A-B**). As no differences between sexes were found (p=0.7, ANOVA), data were pooled for females and males. As for the Akt measurements, a group of mice (naïve) that were prenatally exposed to either saline (controls) or VPA but that did not undergo behavioural testing was included. This was done in order to avoid potential masking of *in utero* VPA exposure effects on rp S6 levels by sensorimotor enrichment. Similar to our findings in rats (Nicolini et al., 2015), prenatal exposure to VPA had no effect on total rp S6 levels in mice. Indeed, a main effect of VPA across all groups was entirely absent (p=0.49, two-way ANOVA). Specifically, as in naïve rats (Nicolini et al., 2015), no differences in total rp S6 levels between naïve VPA-exposed and control mice were found (p=0.72, ANOVA post-hoc SNK; see **Table 7** in Appendix). Also, no changes in total rp S6 between saline-, LM22A-4- or (1-3)IGF-1-treated VPA mice and their respective controls were determined (p=0.89, saline; p=0.25, LM22A-4;

p=0.45, (1-3)IGF-1; ANOVA post-hoc SNK; see **Table 7** in Appendix). Lastly, there were no differences in total rp S6 levels between naïve VPA mice and saline-, LM22A-4- or (1-3)IGF-1-treated VPA mice (p=0.43 saline; p=0.25, LM22A-4; p=0.30, (1-3)IGF-1; ANOVA post-hoc SNK; see **Table 7** in Appendix).

Since no effect of *in utero* VPA exposure on total rp S6 was observed in our mice, we investigated whether maternal challenge with VPA resulted in decreased rp S6 phosphorylation as seen in rats (Nicolini et al., 2015). Similar to naïve VPA-exposed rats (Nicolini et al., 2015), naïve VPA mice exhibited a statistically significant decrease (11%) in phosphorylated rp S6 levels in temporal/parietal neocortices of compared to naïve controls (*$p<0.05$, ANOVA post-hoc SNK; **Figure 24A**). However, despite expectations stemming from the Akt findings (see above), no effect of either behavioural enrichment alone (saline-treated VPA mice), LM22A-4 or (1-3)IGF-1 on the levels of activated rp S6 was observed in VPA-exposed mice (p=0.97, saline; p=0.70 LM22A-4; p=0.68, (1-3)IGF-1; ANOVA post-hoc SNK).

Phosphorylated rp S6 levels of control mice were not affected by either LM22A-4 or (1-3)IGF-1.

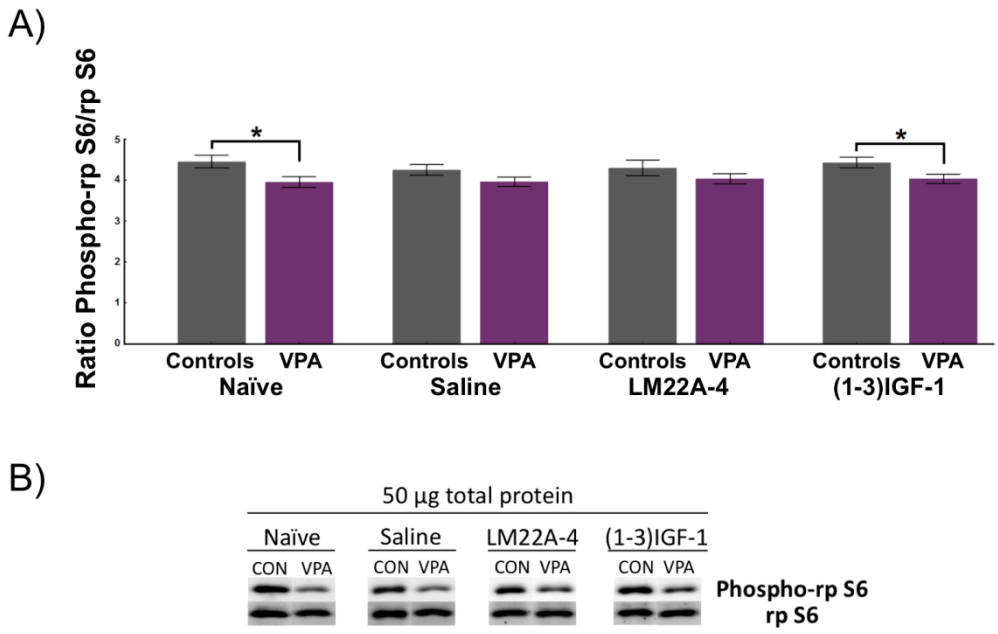

Figure 24. (**A**) Quantification of phosphorylated ribosomal protein S6 (rp S6) levels by Western blotting in postnatal day 35 temporal/parietal neocortices from VPA and control mice. Groups were either not subjected to behavioural testing or treatments (naïve), or underwent behavioural tests while treated with either saline, LM22A-4 or (1-3)IGF-1 for 15 days. Each sample was normalized to its total S6 ribosomal protein. *$p<0.05$, ANOVA post-hoc SNK. Bars indicate mean ± SE. Pooled data for males and females: Naïve controls, n=22; naïve VPA mice, n=18; saline-treated controls, n=19; saline-treated VPA mice, n=20; LM22A-4-treated controls, n=20; LM22A-4-treated VPA mice, n=20; (1-3)IGF-1-treated controls, n=20; (1-3)IGF-1-treated VPA mice, n=19. (**B**) Representative Western blot of mouse control and VPA temporal/parietal neocortex samples. 50 µg of total protein from each sample were loaded.

CHAPTER 5: DISCUSSION

5.1 OBJECTIVE 1

While it is believed that defects in the establishment and maintenance of functional neuronal networks due to synaptic/spine dysfunction underlie the clinical symptomatology of autism, the molecular mechanisms causing these defects remain unknown. Genetic studies have implicated molecules involved in synapse development and plasticity in autism pathogenesis (Bourgeron, 2009; Lionel et al., 2013; Pinto et al, 2010; Toro et al., 2010). In particular, an association between brain-derived neurotrophic factor receptor *NTRK2* (TrkB) gene genetic variants and autism has been reported (Correia et al., 2010). However, the protein products of this gene had never been examined in postmortem brains of subjects with idiopathic autism, that is, cases of autism without a known genetic cause and thought to be of epigenetic/environmental origin. This work is the first to demonstrate strongly decreased full-length TrkB protein expression in patients with idiopathic autism. Additionally, a trend towards increased truncated TrkB isoform protein levels and significantly altered TrkB isoform ratios were found. These findings support the hypothesis that, similar to other neuropsychiatric disorders such as schizophrenia (Pillai et al., 2008; Weickert et al., 2005; Wong et al., 2013), a TrkB isoform imbalance contributes to idiopathic autism. Since only the full-length isoform of TrkB contains the intracellular catalytic tyrosine kinase domain needed to activate TrkB-mediated signaling cascades (Klein et al., 1991; Stoilov et al., 2002), reduced TrkB-FL in idiopathic autism is likely to

result in decreased TrkB effector pathways such as Akt/mTOR and Eps8/Rac regulating spine protein synthesis (Takei et al., 2004; Jaworski et al., 2005) and spine actin cytoskeletal reorganization (Innocenti et al., 2002, 2003; Offenhäuser et al., 2004), respectively. In addition, increased truncated TrkB isoforms, which are able to bind and sequester BDNF but, lacking tyrosine kinase activity, cannot activate TrkB downstream signaling cascades, might reduce even further TrkB-mediated pathways by trapping BDNF (Biffo et al., 1995; Snapyan et al., 2009) or by forming inactive heterodimers with TrkB-FL receptors (Eide et al, 1996; Haapasalo et al, 2001; Stoilov et al. 2002).

As TrkB-FL is expressed almost exclusively in pyramidal neurons and interneurons (Klein et al., 1991; Romanczyk et al., 2002), whereas the truncated isoforms are found in both neurons and glia (Frisén et al., 1993; Ohira et al., 2005), it was possible that TrkB isoform imbalances were due to alterations in these cell types between autism and control subjects. However, no differences in either βIII-Tubulin (neuronal marker) or GFAP (glial marker) levels were found between groups, demonstrating that TrkB isoform imbalances are not an artifact of a shift in the proportion of neurons versus glia. Also, as no changes were observed in TrkB isoform mRNA levels, it is likely that translational mechanisms might contribute to the abnormal ratio of TrkB protein isoforms seen in brains of patients with idiopathic autism. For example, small non-coding RNAs (microRNAs) targeting TrkB-FL mRNA could be responsible for its translational repression. MicroRNAs that have been associated with decreased TrkB-FL protein include miR-200c (Howe et al., 2011) and miR-212 (Jimenez-Gonzalez et al., 2016). Future studies will

have to investigate whether the levels of these microRNAs are increased in autism fusiform gyrus.

Studies of single-gene disorders with a high prevalence of autism such as Rett syndrome (mutations in MECP2), fragile X syndrome (mutations in FMR1), tuberous sclerosis (mutations in TSC1/TSC2), neurofibromatosis (mutations in NF1), and macrocephaly (mutations in PTEN) implicate the mTOR pathway in autism etiology (Bourgeron, 2009; Ehninger & Silva, 2011; Kelleher & Bear, 2008; Ricciardi et al., 2011; Troca-Marín et al., 2012). The current work suggests that mTOR might also be disrupted in idiopathic autism. Specifically, we demonstrated here that, in contrast to fragile X syndrome, tuberous sclerosis, neurofibromatosis type 1, and macrocephaly, which exhibit increased mTOR pathway activation (Kelleher & Bear, 2008; Troca-Marín et al., 2012), mTOR and its upstream regulators PI3K and Akt were decreased in idiopathic autism. These results are consistent with previous reports of reduced mTOR pathway in Rett syndrome (Ricciardi et al., 2011) and decreased Akt total protein and phosphorylation in frontal cerebral cortex from autistic patients (Sheikh et al., 2010). Moreover, these findings support the idea that either hyper- or hypo-activation of mTOR, leading to excessive or deficient protein synthesis at synapses, can have equally disruptive consequences and result in autistic behaviour (Zoghbi & Bear, 2012).

mTOR regulates protein synthesis at synapses via two distinct downstream pathways which are responsible for promoting translation of different pools of mRNAs (Hay & Sonenberg, 2004; Santini & Klann, 2011; Santos et al., 2010). The p70S6K and eukaryotic initiation factor 4B (eIF4B)

cascade regulates translation of mRNAs encoding translational machinery components such as elongation and initiation factors and ribosomal proteins (Jefferies et al., 1997; Meyuhas, 2000), while the eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) and eukaryotic initiation factor 4E (eIF4E) pathway control translation of 5' capped mRNAs coding for structural and functional synaptic proteins (Beretta et al., 1996; Messaoudi et al., 2007; Miller et al., 2002; Schratt et al., 2004). Single nucleotide insertions in the promoter of mTOR effector eIF4E have been found in individuals with autism (Neves-Pereira, 2009), and genome wide association studies (GWAS) have shown a linkage of the region containing the EIF4E locus on chromosome 4q to autism (Schellenberg et al., 2006; Yonan et al., 2003). In the current work, autistic subjects had significantly decreased p70S6K and eIF4B and no changes in 4E-BP1 or eIF4E. These data point to specific deficits in mTOR-dependent translation via the p70S6K/eIF4B pathway in idiopathic autism and suggest that the 4E-BP1/eIF4E pathway may be preferentially affected in genetic cases of autism. In addition, it is noteworthy that we observed no significant differences in ERK protein expression between autism and control samples (unpublished data; see **Figure 25A-C** in Appendix). This evidence is consistent with a pathway deficit via PI3K/Akt/mTOR. Decreased TrkB is likely to contribute to perturbed PI3K-Akt-mTOR. However, other systems including AMP-activated protein kinase (AMPK) (Mihaylova & Shaw, 2011) and NMDA glutamate receptors (NMDARs) (Gong et al., 2006) converge on mTOR and could contribute to its disruption. The metabolic sensor AMPK negatively regulates mTOR in response to cellular energy levels by

phosphorylating and activating TSC2 (Mihaylova & Shaw, 2011). AMPK also phosphorylates and inhibits mTOR binding partner raptor (Gwinn et al., 2008). It follows that increased AMPK could result in decreased mTOR and contribute to autism. However, while many autism patients have comorbid metabolic disorders (Frye & Rossignol, 2012), it remains to be determined whether AMPK is altered in autism. Conversely, alterations in NMDARs resulting in both reduced and excessive NMDAR function have been associated with autism (Lee et al., 2015). The mechanisms by which NMDA receptor dysfunction may impact neuronal development and synaptic plasticity and ultimately result in autistic behaviour are still unknown. At excitatory synapses, NMDARs are part of multiprotein complexes, which comprise scaffolding proteins, signaling molecules and cytoskeletal elements, and are coupled to multiple synaptic signaling pathways (Husi et al, 2000). NMDAR activation promotes TrkB signaling, which, in turn, recruits more PSD-95 to synapses via PI3K-Akt (Yoshii & Constantine-Paton, 2007). NMDARs also activate mTOR signaling through PI3K-Akt, and thereby control activity-dependent dendritic protein synthesis (Gong et al., 2006). Therefore, NMDAR dysfunction in autism patients could contribute to disrupted TrkB and mTOR signaling, and thus negatively impact TrkB-mediated PSD-95 trafficking to synapses and mTOR-dependent protein synthesis.

Along with dysfunctional mTOR and mTOR-dependent protein synthesis, changes in spine morphology, density and dynamics have been demonstrated in syndromic forms of autism such as tuberous sclerosis, fragile X and Rett syndromes (Belichenko et al. 2009; Nimchinsky et al., 2001;

Tavazoie et al., 2005; Troca-Marín et al., 2012). This suggests that decreased full-length TrkB and mTOR might be associated with disrupted dendritic spines in idiopathic autism. However, it is also possible that reduced PSD-95 might contribute to decreased mTOR signaling. Consistent with this hypothesis, PSD-95, when recruited to TrkB, has been reported to enhance PI3K-Akt-mTOR signaling (Cao et al., 2013). Specifically, overexpression of PSD-95 in SH-SY5Y cells, where TrkB was exogenously expressed, was shown to increase phosphorylation of Akt and mTOR downstream effector p70S6K, while not impacting either ERK or TrkB phosphorylation levels (Cao et al., 2013).

Reduced TrkB and PI3K may also negatively affect dendritic spine dynamics by interfering with PI3K-mediated activation of a trimeric complex that includes Eps8 (Innocenti et al., 2002, 2003; Offenhäuser et al., 2004) and controls actin cytoskeletal stability through Rac. Consistent with this model, a reduction in Eps8 protein expression was determined in patients with idiopathic autism (Menna et al. 2013). The notion that disrupted Eps8, by impairing Rac-dependent actin remodeling at spines, might be involved in dysregulation of spine dynamics and density and contribute to autistic behaviour is corroborated by the finding that Eps8 KO mice have spine abnormalities and lack sociability (Menna et al. 2013). The effects of decreased TrkB/PI3K/Eps8 on spine dynamics might be further exacerbated by increased $p75^{NTR}$, which opposes Eps8/Rac via Rho, destabilizing spines and reducing their density (Koshimizu et al., 2009; Lin & Koleske, 2010; Zagrebelsky et al., 2005). Significantly increased proBDNF protein expression

was previously identified in subjects with idiopathic autism (Garcia et al., 2012). This work shows a trend towards increased $p75^{NTR}$ protein in the same patients, suggesting increased signaling via proBDNF/$p75^{NTR}$ and an imbalance in Eps8/Rac and Rho pathways in idiopathic autism, which, together with decreased TrkB/mTOR, might contribute to dendritic spine impairments.

Alterations in dendritic spines, which are the principal site of excitatory synapses (Alvarez & Sabatini, 2007; Bourne & Harris, 2008), are likely to perturb establishment and remodeling of cortical networks that subserve cognition and behaviour thus resulting in autism's cognitive and behavioural deficits. Abnormal spine density and morphology have been reported in cortical neurons from autistic subjects (Hutsler & Zhang, 2010; Williams et al., 1980). Interestingly, the current work demonstrates a significant reduction of the postsynaptic excitatory marker PSD-95 in autistic patients, suggesting fewer dendritic spines (excitatory synapses). PSD-95 is a scaffolding protein essential for organization and function of excitatory synapses (Kim & Sheng, 2004; Kornau et al., 1995; Ehrlich & Malinow, 2004). It forms a complex with TrkB, thereby promoting signaling through PI3K/Akt, which, in turn, induces recruitment of PSD-95 to synapses (Yoshii & Constantine-Paton, 2007). It follows that decreased TrkB/PI3K/Akt might interfere with PSD-95 transport and hence negatively affect excitatory synapses. Together, the current findings suggest that decreases in TrkB-FL may disrupt dendritic spines (excitatory synapses) in idiopathic autism via several molecular mechanisms including mTOR, PI3K/Eps8 and PI3K/Akt/PSD-95. However, since the

synaptoneurosomal fraction was not extracted, the possibility stands that the decrease in PSD-95 protein observed in autistic patients does not reflect a change in total amounts of this marker but rather how tightly associated PSD-95 is within the postsynaptic density.

Alterations in TrkB isoforms and disruptions of TrkB-regulated pathways might also negatively impact GABAergic synapses. Consistent with this hypothesis, we found significantly reduced protein levels of the postsynaptic scaffolding protein gephyrin, a key organizer of inhibitory synapses (Choii & Ko, 2015; Tretter et al., 2012; Tyagarajan & Fritschy, 2014) (**Figure 26A-B** in Appendix), in autism postmortem fusiform gyrus. Disrupted neurotrophin signaling has been associated with deficiencies in cortical inhibitory interneurons in neuropsychiatric disorders such as schizophrenia (Fung et al., 2009; Hashimoto et al., 2005; Wong et al., 2013; Woo et al., 2006). Decreased TrkB-FL on interneurons could result in decreased activation of downstream signaling cascades, while increased TrkB-T1 in glia could limit the amount of BDNF available for interneurons (Wong et al., 2013). Moreover, since TrkB-T1 receptors play a role in regulating calcium release from astrocytes (Lidow, 2003), elevated TrkB-T1 levels could also adversely affect interneurons by compromising calcium signaling (Wong et al., 2013). Lastly, it has been recently demonstrated that mTOR not only plays a role in regulating development and plasticity of excitatory synapses, but also in controlling GABAergic transmission (Weston et al., 2012). Notably, Centoze et al. (2008) reported enhanced GABA-mediated synaptic

transmission in *Fmr1* KO mice. Future studies will need to investigate whether decreased TrkB and mTOR impair GABAergic synapses in idiopathic autism.

As several subjects with autism exhibited seizures (n=4), while others did not (n=6), results from these two groups of patients were compared. No differences in fusiform gyrus levels of any of the protein studied (TrkB isoforms, PI3K p85, Akt, total and phosphorylated mTOR, p70S6K, eIF4B, Eps8, PSD-95 and gephyrin) were found between autism cases with seizure disorder and those without ($p>0.05$, 2-tailed *t* tests; **Figure 27A-K** in Appendix). This is consistent with previous findings for BDNF (Garcia et al., 2012). BDNF mRNA is increased by seizures (Binder et al., 2001), and yet in that study, none of the autistic subjects with seizures, which are the same ones used in this study, exhibited higher BDNF mRNA levels than those without. Overall, nine out of ten autism subjects, regardless of whether they had concurrent seizure disorder, exhibited decreases in TrkB-FL and mTOR pathway components (**Table 4**).

The direction of change is uniform for each subject, suggesting coordinated or feedback regulation of these pathways. It is possible that decreased TrkB-FL total protein, which could originate from micro-RNA-mediated inhibition of TrkB-FL mRNA translation (see above), might result in reduced activation of TrkB and its downstream pathways, including mTOR. Since mTOR controls protein translation (Burnett et al., 1998; Hay & Sonenberg, 2004), reduced mTOR is likely to result in decreased mTOR-dependent protein synthesis, which, in turn, may be responsible for the uniform decrease in total protein levels of the molecular targets analyzed in

the present study. Moreover, these findings demonstrate that disruptions in TrkB, mTOR and their cascade components are widespread in the autism population and not limited to genetic forms of autism. Epigenetic modifications are a likely underlying mechanism of these disruptions as substantiated by the evidence that autistic-like behaviours arise from *in utero* exposure to a 500 mg/kg valproic acid dose which induces transient hyperacetylation of histones H3 and H4 in embryonic mouse brains (Kataoka et al., 2013).

Table 4. Distribution of analyzed protein targets in autism subjects relative to controls

	Autism patients with no seizure disorder							Autism patients with seizure disorder			
	AN01093 (A1)	AN00764 (A3)	AN00493 (A6)	UMB1182 (A9)	AN06420 (A5)	UMB797 (A8)	AN08873 (A7)	UMB1174 (A2)	AN08792 (A4)	AN16641 (A10)	AN16615 (A11)
TrkB-FL	-2.0 SD	-1.5 SD	-2.0 SD	-1.5 SD	0.5 SD	-0.5 SD	0.5 SD	-2.0 SD	-2.0 SD	-1.5 SD	-1.5 SD
PI3K p85	-2.0 SD	-1.5 SD	-2.0 SD	-2.0 SD	0.5 SD	-0.5 SD	0.5 SD	-2.0 SD	-2.0 SD	1.5 SD	-1.5 SD
Akt	-2.0 SD	-0.5 SD	-1.5 SD	-2.0 SD	1.5 SD	-0.5 SD	0.5 SD	-1.5 SD	-1.5 SD	-0.5 SD	-1.5 SD
mTOR	-2.0 SD	-2.0 SD	-2.0 SD	-2.0 SD	1.5 SD	-0.5 SD	0.5 SD	-2.0 SD	-2.0 SD	-1.5 SD	-2.0 SD
Phospho-mTOR	-1.5 SD	-2.0 SD	-2.0 SD	-2.0 SD	0.5 SD	-0.5 SD	-0.5 SD	-2.0 SD	-2.0 SD	-2.0 SD	-2.0 SD
p70 S6 Kinase	-2.0 SD	-1.5 SD	-1.5 SD	-2.0 SD	0.5 SD	-0.5 SD	-0.5 SD	-1.5 SD	-2.0 SD	-1.5 SD	-1.5 SD
eIF4B	-2.0 SD	-2.0 SD	-2.0 SD	-1.5 SD	1.5 SD	2.0 SD	-0.5 SD	-1.5 SD	-2.0 SD	-2.0 SD	-2.0 SD
Eps8	-0.5 SD	-2.0 SD	-0.5 SD	-2.0 SD	2.0 SD	1.5	-0.5 SD	-2.0 SD	-2.0 SD	-2.0 SD	-0.5 SD
PSD-95	-1.5 SD	-1.5 SD	-1.5 SD	-1.5 SD	0.5 SD	0.5 SD	0.5 SD	-1.5 SD	-1.5 SD	-1.5 SD	-1.5 SD

SD = Standard deviation. The mean of each target for the control group was used as a reference to calculate the corresponding standard deviation values for each target. **Dark red** = **2** standard deviations **less** than the mean of controls. **Medium red** = **1.5** standard deviation **less** than the mean of controls. **Light red** = **0.5** standard deviation **less** than the mean of controls. **Light blue** = **0.5** standard deviation **greater** than the mean of controls. **Medium blue** = **1.5** standard deviation **greater** than the mean of controls. **Dark blue** = **2** standard deviations **greater** than the mean of controls.

5.1.1 Significance of Objective 1

This work is the first to demonstrate major decreases in protein expression of TrkB-FL and its downstream effectors PI3K, Akt, mTOR, p70S6K, eIF4B (Nicolini et al., 2015) and Eps8 (Menna et al., 2013) in subjects without known genetic causes of autism and without related disorders on the spectrum. These data show that deficits in TrkB and its downstream cascades are widespread in idiopathic autism and associated with decreased excitatory postsynaptic marker PSD-95, suggesting that dendritic spines might be reduced in idiopathic autism. Furthermore, the current work demonstrates that, in constrast to neurodevelopmental disorders with high rates of autism such as tuberous sclerosis, neurofibromatosis type I, fragile X syndrome and macrocephaly, where the mutated genes cause increased mTOR pathway, mTOR is decreased in idiopathic autism. This supports that both hyper- and hypo-activated mTOR are equally disruptive and might contribute to autistic behaviour. Taken together, the present findings point to dysregulated TrkB, mTOR and their signaling pathways as primary molecular substrates in the pathogenesis of idiopathic autism and as potential therapeutic targets for this disease. As shown with animal models of Rett syndrome (Castro et al., 2014; Kron et al., 2014; Schmid et al., 2012; Tropea et al., 2009), there is potential for pharmacological intervention in these cascades.

5.2 OBJECTIVES 2 & 3

In the present study, the contribution of defective TrkB signaling to autistic behaviour and whether pharmacological intervention in this cascade could ameliorate behaviour was investigated. To this end, it was essential to move to an animal model. The valproic acid (VPA)-induced rodent was chosen because of its validity as a model for both the pathways and the behaviours studied here. Also, since autistic-like phenotypes observed in this model are the result of an environmental insult (*in utero* exposure to a chemical, valproic acid), VPA-exposed mice are a model for possible environmental/epigenetic origins of idiopathic autism. Indeed, as a model of epigenetic changes, these mice appear to better represent cases of idiopathic autism, which have no known genetic basis, than transgenic models carrying mutations in single autism-linked genes.

VPA acts as a histone deacetylase (HDAC) inhibitor (Göttlicher et al., 2001; Phiel et al., 2001). *In utero* exposure to VPA results in an increased risk of ASD in human offspring (Moore et al., 2000; Rasalam et al., 2005; Bromley et al., 2008). Furthermore, rodents prenatally exposed to VPA display autistic-like behaviours, including decreased social interaction and increased repetitive behaviour, as well as molecular alterations similar to those observed in autism (Kataoka et al., 2013; Kolozsi et al., 2009; Mehta et al., 2011; Nicolini et al., 2015; Roullet et al., 2010; Schneider & Przewlocki, 2005). In line with these findings, the current work demonstrates behavioural and molecular alterations in mice exposed to a single injection of 500 mg/kg VPA at embryonic day 12.5. This dose has been shown to transiently increase

the levels of acetylated histones H3 and H4 in embryonic mouse brains, suggesting that changes in HDAC activity during a critical period of brain development contribute to causing autistic-like behavioural impairments (Kataoka et al., 2013).

In contrast to control animals, VPA-exposed mice displayed a complete lack of interest in interacting with a novel conspecific as revealed in a social approach task (female data only). This is consistent with earlier studies of both VPA-induced rat and mouse models (Kang & Kim, 2015; Kim et al., 2011; Kim et al., 2014; Moldrich et al., 2013) and clearly shows a social impairment induced by maternal exposure to VPA. It was not possible to ascertain whether maternal exposure to VPA affected social behaviour in males, as controls systematically failed to display sociability in the three-chambered apparatus used here. In retrospect, this appears to have been an artifact of the experimental design. Stimulus mice were matched to be the same sex as subject mice. Indeed, this was needed to avoid sexual (mating) behaviours from overriding or confounding asexual sociability responses. However, male rodents tend to have dominating territorial nature (Davis, 1958; Poole & Morgan, 1973; Warne, 1947). Unlike female stimulus mice that were calm and docile and allowed all social approaches to be initiated by the test mice, male stimulus mice were visibly more active and aggressive. This likely evoked a suite of behaviours in test males that did not reflect and essentially obscured any social preferences. In the future, more inactive and docile strains such as 129/SvJ (Yang et al., 2011) or transgenic growth hormone mice (Rollo et al., 2014) should be used when testing sociability in males. Furthermore, it is

possible that stimulus mice displayed agitation and aggressive behaviours, since they had not been habituated to the wire cups. In the future, mice used as novel mice should be habituated to the inverted wire cup prior to experiments (e.g. placed in an inverted wire cup for three 10-minute sessions on three consecutive days before testing) in order to prevent or minimize potential agitation and aggressive behaviour originating from confinement in the cups. Lastly, a strain such as C57Bl/6J that has been previously demonstrated to display sociability in the three-chambered social approach task (Moy et al., 2004, 2007; McFarlane et al., 2008; Yang et al., 2007) could be used in future experiments.

Maternal exposure to VPA also resulted in increased stereotyped, perseverative behaviours, another key feature of autism. VPA-exposed mice showed higher repetitive digging as indicated by the greater number of marbles buried compared to controls. This is consistent with previous studies of VPA mice on both C57Bl/6J and ICR(CD-1) backgrounds (Kim et al., 2014; Mehta et al., 2011), and provides further support to the evidence that *in utero* exposure to VPA induces repetitive, stereotyped behaviour in mice.

The most common co-occurring symptom in autism is anxiety. Contrary to previous studies reporting enhanced anxiety-related behaviour in VPA-exposed rats (Markram et al., 2008; Schneider et al., 2006, 2008), the present work shows that maternal challenge with VPA did not increase anxiety-like behaviour in mice (assessed in an elevated plus maze). This discrepancy might be attributable to species-specific differences between rats and mice. In addition, the current findings are also in contrast with Kataoka and colleagues'

findings (2013), who claim elevated anxiety-related behaviour in mouse offspring prenatally exposed to VPA. This could be attributed to the different ages at which the mice were tested, 4 weeks (current study) *vs.* 8 weeks (Kataoka et al., 2013). It is also noteworthy that Kataoka et al. (2013) reported fewer entries made into the closed arms of the plus maze by VPA mice, pointing to a general decrease in motor activity. It follows that the increase in anxiety-related behaviour described by these authors may be entirely attributed to low overall activity levels.

Several VPA mechanisms of action may be implicated in VPA-induced autistic-like behavioural phenotypes in the offspring. These include inhibition of GSK3β and subsequent activation of Wnt signaling, which induces axonal remodeling and clustering of synaptic molecules (Hall et al., 2002), regulation of differentiation and proliferation of neural progenitor cells via β-catenin-Ras-ERK-p21 pathway (Jung et al., 2008) and elevation of GABA levels by promoting GABA synthesis and inhibiting its degradation (Löscher et al., 2002; Phiel et al., 2001). Notably, recent work demonstrated that VPA exposure at mid-gestation in rodents causes anatomical, behavioural and molecular alterations similar to human autism by inhibiting histone deacetylases and by inducing histone modifications (Kataoka et al., 2013; Moldrich et al., 2013). Yet, it remains unclear which molecular pathways are affected by VPA's HDAC inhibitory activity. We recently determined that, similar to the molecular changes seen in human idiopathic autism, brains of rats prenatally exposed to VPA have decreased Akt and mTOR pathway components (Nicolini et al. 2015). Consistently, the current work shows that

TrkB signaling via Akt and mTOR signaling via rp S6 are decreased in temporal/parietal neocortices of naïve VPA-exposed mouse offspring. This evidence suggests that decreases in TrkB and mTOR pathways might be implicated in lack of sociability and increased repetitive behaviours of VPA mice. Support for this hypothesis emerges in the relationship between sociability and levels of phosphorylated Akt. Higher levels of phospho-Akt corresponded to greater time spent in the chamber with the novel mouse (social cue) (**Figure 28A** in Appendix). Likewise, increased asocial behaviour was seen with the lowest levels of phospho-Akt (**Figure 28B** in Appendix). This suggests that deficits in phospho-Akt preclude sociability. However, no similar correlation patterns emerged between measures of sociability and levels of phosphorylated S6 ribosomal protein (**Figure 29A-B** in Appendix), suggesting that activation levels of rp S6 might not influence social behaviour.

The involvement of activated Akt in decreased repetitive behaviour remains inconclusive. No significant correlation between number of marbles buried (digging activity) and Akt phosphorylation levels (**Figure 30** in Appendix) was found, suggesting that other molecular mechanisms (see below) are likely more impactful. Similarly, the complete lack of correlation between perseverative behaviour and phosphorylated rp S6 (**Figure 31** in Appendix) suggests that activated rp S6 is not likely to contribute to increased repetitive behaviour and corroborates the hypothesis that additional molecular pathways (independent of activated rp S6 but parallel to activated Akt) might contribute to this phenotype. Potential pathway candidates may include the mTOR-activated 4E-BP1/eIF4E pathway and the Eps8 pathway. Despite no

changes in 4E-BP1 or eIF4E protein levels having been found in our cohort of patients with idiopathic autism (see Objective 1), it is possible that the 4E-BP1/eIF4E pathway is disrupted by *in utero* exposure to VPA and that deregulated 4E-BP1/eIF4E might specifically underlie VPA-induced autistic-like behaviours in rodents. Indeed, support for this hypothesis comes from our previous findings demonstrating a significant reduction in 4E-BP1 total and phosphorylated protein levels in VPA-exposed rats (Nicolini et al., 2015). The Eps8 pathway might also be perturbed in VPA mice and hence contribute to behavioural deficits by negatively impacting spine density and maturation. This hypothesis is supported by the present work showing significantly decreased Eps8 protein levels in autism fusiform gyrus where TrkB is reduced (see Objective 1). Furthermore, the involvement of this pathway in autistic behaviour is corroborated by the evidence that Eps8 KO mice exhibit spine abnormalities and lack sociability (Menna et al., 2013). Lastly, another molecular candidate that might contribute to the behavioural changes of VPA mice is glycogen synthase kinase-3β (GSK3β). GSK3β is a constitutively active serine-threonine kinase which is inhibited following phosphorylation of the serine 9 residue in its amino-terminal domain (Cross et al., 1994). Akt phosphorylates this site and leads to inhibition of GSK3β kinase activity (Cross et al., 1994, 1995). GSK3β activation has been shown to impair social behaviour in mice expressing a mutant form of the brain serotonin synthesis enzyme, tryptophan hydroxylase 2 (Beaulieu et al., 2008). Also, the selective serotonin reuptake inhibitor fluoxetine, which enhances GSK3β phosphorylation by increasing serotonin levels (Beaulieu et al., 2008), has

been demonstrated to suppress marble-burying (digging activity) in mice (Li et al., 2006). This evidence suggests that GSK3β activation resulting from reduced Akt-mediated phosphorylation may play a role in increasing repetitive digging behaviour in VPA mice. Future experiments will need to examine whether these additional pathways are disrupted in the VPA-induced mouse model.

Currently, there is no cure available for autism. Promisingly, autistic-like behaviour and TrkB signaling deficits have been partially ameliorated in animal models of Rett syndrome, a syndromic form of autism which arises from a mutation in an epigenetic regulatory gene, by administration of full-length recombinant human IGF-1 (Castro et al., 2014), its N-terminal tripeptide (1-3)IGF-1 (Tropea et al., 2009) and the partial TrkB agonist LM22A-4 (Schmid et al., 2012). The present work investigated the effects of LM22A-4 and (1-3)IGF-1 on sociability, repetitive behaviour and TrkB signaling in the VPA-induced mouse model. The results of these experiments are the first to show that treatment with the TrkB partial agonist LM22A-4 restored sociability and reduced repetitive behaviour in VPA-exposed mice. Furthermore, LM22A-4, together with enrichment from behavioural testing, normalized the deficits in phosphorylation of the TrkB downstream effector Akt, suggesting that TrkB activation might contribute to restoring sociability and repetitive behaviours. It is noteworthy that a 6% non-significant increase in phosphorylated Akt levels was observed in sham-injected VPA mice that were subjected to behavioural testing, suggesting that the associated sensorimotor stimulation might have had an impact on Akt phosphorylation.

Sensorimotor enrichment has been demonstrated to increase levels of several proteins involved in synaptic function including neurotrophins (Ickes et al., 2000), PSD-95 (Nithianantharajah et al., 2004), and NMDA (Tang et al., 2001) and AMPA (Naka et al., 2005) receptor subunits. Consistent with these findings, the present work suggests that even a limited time of sensorimotor enrichment may have some positive influence on activated Akt levels. This evidence raises the possibility that the restoration of Akt phosphorylation seen in VPA mice receiving LM22A-4 might have been the result of a combined effect of behavioural enrichment and drug treatment. To establish whether LM22A-4 treatment alone is sufficient for normalizing Akt phosphorylation, further experiments, in which LM22A-4 is administered to mice not subjected to any behavioural tests, should be carried out.

The current work also shows that, despite expectations originating from the restoration of Akt phosphorylation, neither enrichment alone nor combined with LM22A-4 reversed the rp S6 phophorylation deficits of VPA-exposed mice. rp S6 is phosphorylated at five serine residues (Ser235, Ser236, Ser240, Ser244, Ser247), located at the carboxylic end of the protein (Bandi et al., 1993; Krieg et al., 1988). Here, only phosphorylation of rp S6 at Ser240/244, which is mainly mTOR-dependent (Pende et al., 2004; Roux et al., 2007), was measured. This leaves room for the possibility that LM22A-4, like Haloperidol (Valjent et al., 2011), might increase phosphorylation of rp S6 at the Ser235/236 site. Future experiments are needed to ascertain this hypothesis. Moreover, as mentioned above, it is also possible that, similar to rats (Nicolini et al., 2015), the 4E-BP1/eIF4E pathway and thus translation of mRNAs

coding for structural and functional synaptic proteins might be disrupted in mice by prenatal exposure to VPA. Indeed, contrary to idiopathic autism which exhibits only p70S6K/rp S6 pathway deficits (Nicolini et al., 2015), deregulated 4E-BP1/eIF4E might specifically underlie VPA-induced autistic-like behaviours in rodents. Therefore, total protein and phosphorylation levels of 4E-BP1 and eIF4E in VPA *vs.* control mouse temporal/parietal neocortices should be measured in future experiments. If changes in 4E-BP1/eIF4E are found, whether treatment with LM22A-4 improves them should be tested next.

Lastly, the present findings demostrate that (1-3)IGF-1 treatment neither successfully rescued behavioural impairments nor had an effect beyond enrichment on molecular deficits induced by VPA. This evidence suggests that compounds leading to TrkB activation such as LM22A-4 might be better therapeutic candidates than activators of other receptors in ameliorating behavioural and molecular deficits arising from prenatal VPA exposure. In summary, these data point to the small-molecule Trk agonist LM22A-4 as a promising therapeutic candidate and to TrkB as a potential therapeutic target for autism.

5.2.1 Significance of Objectives 2 & 3

This work shows that exposure to the HDAC inhibitor VPA at a critical period of embryonic brain development (E12.5) negatively impacted sociability and elevated repetitive behaviour in mice. It also provides evidence that maternal challenge with VPA resulted in decreased Akt phosphorylation levels. This suggests that, similar to idiopathic autism, TrkB and mTOR

signaling were decreased in VPA-exposed mouse offspring and might have contributed to their behavioural deficits. Furthermore, the present work is the first to show that intraperitoneal injections of the partial TrkB agonist LM22A-4 restored social behavioural deficits and reduced repetitive, stereotyped behaviour in VPA-exposed mice. It also demonstrates that while LM22A-4 administration combined with behavioural enrichment normalized phosphorylated Akt levels, it did not reverse the deficits in rp S6 phosphorylation induced by prenatal VPA exposure. Additionally, this study reveals for the first time that, contrary to LM22A-4, the active tripeptide fragment of IGF-1 (1-3)IGF-1 did not ameliorate behavioural dysfunctions in VPA mice. These results are consistent with the hypothesis that deficits in TrkB signaling contribute to social impairments and increased repetitive, perseverative behaviour in VPA-exposed mice. They also support the possibility that behavioural deficits can be improved by the use of TrkB activators in adulthood and point to a potential therapeutic role for LM22A-4 in treating autism. Lastly, VPA's ability to induce behavioural and biochemical deficits similar to idiopathic autism implicates epigenetic mechanisms in the pathogenesis of this disease.

CHAPTER 6: CONCLUSIONS & FUTURE DIRECTIONS

6.1 CONCLUSIONS

Recent years have seen major advances in both the genetics and the behavioural aspects of autism. Hundreds of genes associated with autism susceptibility as well as interacting epigenetic and environmental factors have been identified. Yet, relatively little is known about the molecular pathways that link genes to autistic behaviour. Full knowledge of the molecular signature underlying autistic behaviours is key to gaining a better understanding of autism pathogenesis and to identifying molecules that can be targeted for therapy. Here, protein expression analysis of signaling pathway components, which act as crucial regulators of synapse development and plasticity, was carried out in postmortem brain tissue from human patients with idiopathic autism. Decreased TrkB and downstream effectors PI3K, Akt, mTOR, Eps8, and PSD-95 were found in autistic brains, supporting the hypothesis that changes in these markers contribute to behavioural deficits associated with autism. This differs from neurodevelopmental disorders with high rates of autism such as tuberous sclerosis, neurofibromatosis type I, macrocephaly and fragile X syndrome which are characterized by enhanced PI3K/Akt/mTOR signaling. Hence, this work demonstrates that molecular dysfunction might differ between genetic and epigenetic forms of autism and supports the growing realization that either too much or too little signaling can result in aberrant autistic behaviour. The contribution of these molecular changes to autistic behaviour was further investigated in the VPA-induced

mouse model of autism. Deficits in sociability and increased repetitive, stereotyped behaviour were determined together with reduced TrkB and mTOR signaling in this model. Moreover, treatment in adult animals with the partial TrkB agonist LM22A-4 was demonstrated to restore sociability, reduce repetitive, stereotyped behaviour and, in combination with behavioural enrichment, to improve TrkB signaling deficits. These results suggest that decreased TrkB signaling might be implicated in autistic behaviour, and suggest that pharmacological interventions occurring in adulthood and leading to TrkB activation, such as administration of LM22A-4, might help in treating autism symptomatology. In light of these findings, the NMDA receptor coagonist D-serine could be a compound with therapeutic potential for treating behavioural and molecular deficits in autism. D-serine treatment has been reported to normalize TrkB, Akt, mTOR, p70S6K and GSK3β phosphorylation in a mouse model of schizophrenia (Balu et al., 2013). Future studies will be needed to test whether D-serine can rescue TrkB signaling, restore sociability and decrease repetitive, perseverative behaviour in VPA-exposed mice.

The current work provides a mechanistic insight into the pathogenesis of autistic behaviour, including social deficits and repetitive, stereotyped behaviour, and implicates reduced TrkB-mTOR in autism pathophysiology. However, other pathways such as Eps8/Rac, 4E-BP1/eIF4E and GSK3β are likely to be at play. Future experiments are needed to better elucidate the molecular mechanisms underlying autism symptomatology.

One limitation of the present findings is the generalizability of the VPA murine model to the ASD population. Although maternal challenge with VPA recapitulates many behavioural and molecular deficits of idiopathic autism, the majority of autistic patients were not prenatally exposed to this drug. Hence, the validity of the VPA rodent model might be limited to autism cases caused by exposure to drugs with HDAC inhibition activity. Yet, valuable inferences can be drawn nonetheless. Another difference between the VPA-induced rodent model and human autism, which was confirmed by the present findings, should be acknowledged here. Contrary to the general population where autism is four times more common in males than in females (Fombonne, 2002), maternal challenge with VPA appears to have a negative impact on both females and males in rodents. Consistently, a 1:1 male to female ratio has been observed in children exposed to VPA during pregnancy, suggesting that valproate may carry an increased risk for females (Rasalam et al., 2005). Lastly, we demonstrated differences in down-regulated mTOR pathways between VPA-exposed rats and individuals with idiopathic autism (Nicolini et al., 2015). Specifically, while autism patients had only p70S6K/rp S6 pathway deficits, prenatal exposure to VPA in rats adversely affected phosphorylation of both mTOR downstream effectors, rp S6 and 4E-BP1 (Nicolini et al., 2015).

Despite these limitations, this work highlights the importance of combining human and animal studies to gain a better understanding of the molecular mechanisms associated with the aberrant behavioural phenotypes characteristics of autism. Animal models are indeed essential to perform

experimental manipulations (e.g. testing of mechanistic hypotheses and compounds with potential therapeutic effects) that cannot be carried out in human subjects. In conclusion, full documentation of signaling cascades in human postmortem brain tissue followed by further investigation of these pathways in animal models that mirror these molecular changes (such as the VPA murine model) will lead to the identification of new therapeutic targets and novel drugs for this increasingly prevalent, lifelong, handicapping disorder.

6.2 FUTURE DIRECTIONS

This work provides mechanistic insights into the pathophysiology of behavioural impairments such as social deficits relevant to autism. Decreased TrkB signaling emerged as an important underlying mechanism and a promising therapeutic target for this disease. The TrkB partial agonist LM22A-4 showed potential therapeutic implications for treating associated social deficits and repetitive behaviours. However, additional pathways that might contribute to autistic behaviour will need to be investigated. Future studies will also have to determine whether LM22A-4 would be well tolerated in the clinical population. Promisingly, since this compound binds exclusively to TrkB and does not activate $p75^{NTR}$, LM22A-4 as a potential autism treatment might have a reduced risk of adverse effects originating from excessive activation of TrkB and $p75^{NTR}$ (Massa et al., 2010). Future studies will need to examine whether disrupted TrkB-mediated signaling pathways, which regulate actin cytoskeletal remodeling and spine protein synthesis, have

adverse consequences on dendritic spine (excitatory synapse) number, morphology, and dynamics. Changes in spine density, shape and plasticity accompany synapse formation, stability and elimination and hence are intimately linked to establishment and maintenance of functional high-order neuronal networks which subserve cognition and behaviour. It follows that establishing whether disrupted TrkB signaling negatively impacts spines will provide us with critical insights into the synaptic alterations underlying perturbed connectivity, which constitutes a hallmark of autistic brains and is thought to be ultimately responsible for symptoms of the disease. Additionally, since TrkB receptors are involved in the formation of both glutamatergic and GABAergic synapses (Spenger et al., 1995; Seil, 1999; Seil & Drake-Baumann, 2000), whether the latter are affected by decreased TrkB signaling should be investigated in future experiments. Lastly, while the VPA-induced mouse model appears to be a valuable tool to study the molecular mechanisms underlying autistic behaviour and test novel potential therapeutic compounds, it might not fully mirror human idiopathic autism. Future studies will be needed to find a model which explains the major decreases in total protein expression of TrkB and its downstream pathway components seen in the autistic brain.

To conclude, a summary of the questions that remain to be answered and the proposed experiments to address them is provided below:

- In the current study, we demonstrated a decrease in TrkB-FL protein with no change in TrkB-FL mRNA in fusiform gyrus samples from patients with idiopathic autism. We hypothesize

that microRNAs targeting TrkB-FL mRNA could be responsible for its translational repression. Therefore, we propose to measure the levels of miR-200c and miR-212, two microRNAs that have been associated with decreased TrkB-FL protein (Howe et al., 2011; Jimenez-Gonzalez et al., 2016), in fusiform gyrus of patients with idiopathic autism compared to controls.

- In the present work, we demonstrated decreased PI3K/Akt/mTOR in fusiform gyrus samples from patients with idiopathic autism. TrkB-FL acts via PI3K/Akt/mTOR (Santos et al., 2010), thus decreased TrkB-FL is likely to contribute to perturbed PI3K/Akt/mTOR in human idiopathic autism. However, other systems including NMDA glutamate receptors (Gong et al., 2006) and AMP-activated protein kinase (AMPK) (Mihaylova & Shaw, 2011) converge on mTOR and could contribute to its disruption (see Discussion). Thus, we propose to measure protein levels of NMDAR subunits, GluN1 and GluN2, and total and phosphorylated AMPK in fusiform gyrus of patients with idiopathic autism compared to controls.
- Here, we demonstrated reduced protein levels of the inhibitory and excitatory postsynaptic markers gephyrin and PSD-95 in fusiform gyrus samples from patients with idiopathic autism, suggesting fewer inhibitory and excitatory synapses. We next propose to quantify the number of inhibitory and excitatory synapses in fusiform gyrus from patients with idiopathic autism

compared to controls by measuring the total number of gephyrin- and PSD-95-immunoreactive punctae.

- This work showed that the reduction in Akt phosphorylation observed in temporal/parietal neocortices from VPA-exposed mice was normalized by LM22A-4 treatment together with enrichment from behavioural testing. To establish whether LM22A-4 treatment alone is sufficient for restoring Akt phosphorylation, we propose to measure phosphorylated Akt levels in brains of VPA-exposed mice that are not subjected to behavioural testing (naïve) and are given intraperitoneal injections of LM22A-4 for 15 days.
- In the present study, we determined that phosphorylation levels of Akt and rp S6 are reduced in temporal/parietal neocortices from VPA-exposed mice. This suggests that decreases in these targets might be implicated in lack of sociability and increased repetitive behaviours displayed by VPA mice. However, no correlation between measures of sociability and phosphorylated rp S6 protein was found. We also observed a lack of correlation between number of marbles buried and either Akt or rp S6 phophorylation levels. These findings suggest that additional molecular targets might contribute to social deficits and increased repetitive behaviours induced by prenatal VPA exposure in mice. We hypothesize that the mTOR-activated 4E-BP1/eIF4E pathway, the Eps8 pathway and GSK3β might be

potential candidates (see Discussion). Hence, we propose to measure total and phosphorylated protein levels of 4E-BP1, eIF4E, Eps8 and GSK3β in temporal/parietal neorcortices of VPA-exposed mice compared to controls. If reduced phosphorylated 4E-BP1, eIF4E and GSK3β levels are determined in VPA-exposed mice, whether treatement with LM22A-4 improves 4E-BP1, eIF4E and GSK3β phosphorylation deficits in these mice should be assessed.

- Decreases in Akt/mTOR have been associated with changes in dendritic spine number and shape in mouse models of RTT syndrome (Troca-Marín et al., 2012). Therefore, to establish whether decreased phosphorylated Akt and mTOR downstream effector rp S6 negatively impact dendritic spines in VPA mice, we propose to examine spine density and morphology (thin, stubby, mushroom) in VPA-exposed *vs.* control temporal/parietal neorcortices.

Finally, two potential deficiencies of the present work should be addressed in future experiments.

- A homogenization buffer that does not solubilize postsyanaptic densities (synaptoneurosomal fraction) was used here (see Materials & Methods). The possibility thus stands that solubilization of proteins that reside within postsynaptic densities such as PSD-95 might not have been complete and that some portion of these proteins might have been discarded in the non-

solubilized fraction. Hence, we propose to measure protein levels of PSD-95, gephyrin, Akt, mTOR and mTOR pathway components in fusiform gyrus synaptoneurosomal fractions from patients with idiopathic autism compared to controls.

- Control males displayed a complete lack of sociability in the three-chambered apparatus used in this study, making it impossible at present to determine whether prenatal exposure to VPA impairs social behaviour in male mice. We think this might have been an artifact of the experimental design, as novel mice were matched to be the same sex as test mice. Novel males proved to be more active and aggressive than novel females (see Discussion), likely due to the dominating territorial nature of male mice (Davis, 1958; Poole & Morgan, 1973; Warne 1947). Additionally, the aggressive and more active behaviours of males could have been exacerbated by the fact that mice were not habituated to confinement in wire cups. We therefore propose to evaluate sociability in male mice prenatally exposed to valproic acid compared to controls following the changes to the protocol suggested in the Discussion (e.g. male novel mice will be habituated to confinement in wire cups prior to experiments). If a lack of sociability is found in VPA-exposed male mice, whether treatement with LM22A-4 restores sociability in these mice should be assessed.

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APPENDIX

Table 5A. Three-Chambered Social Approach Task

Social Approach Task Measures	Treatment	Males			
		Controls		VPA	
		Novel Mouse	Empty Wire Cup	Novel Mouse	Empty Wire Cup
Time in Chamber (sec)	Saline	256.78 ± 24.78	288.78 ± 24.84	258.39 ± 27.40	271.44 ± 26.40
	LM22A-4	258.65 ± 34.10	296.81 ± 31.33	269.19 ± 36.83	281.81 ± 32.61
	(1-3)IGF-1	271.59 ± 33.47	283.94 ± 32.48	279.53 ± 33.08	270.74 ± 31.85
Entries (#)	Saline	9.33 ± 0.67	9.39 ± 0.60	10.89 ± 0.92	10.56 ± 0.82
	LM22A-4	8.06 ± 0.83	9.56 ± 0.48	8.88 ± 0.95	10.13 ± 1.00
	(1-3)IGF-1	9.06 ± 0.80	9.47 ± 0.93	10.53 ± 1.11	10.73 ± 1.22
Total Entries (#)	Saline	18.72 ± 1.18		21.44 ± 1.66	
	LM22A-4	17.63 ± 1.19		19.00 ± 1.58	
	(1-3)IGF-1	18.53 ± 1.56		21.27 ± 2.19	

Table 5B. Three-Chambered Social Approach Task

Additional Social Approach Task Measures	Treatment	Females			
		Controls		VPA	
		Novel Mouse	Empty Wire Cup	Novel Mouse	Empty Wire Cup
Entries (#)	Saline	10.57 ± 0.96	9.71 ± 1.17	10.38 ± 0.80	10.00 ± 1.06
	LM22A-4	9.29 ± 0.73	9.29 ± 0.71	10.64 ± 0.87	10.57 ± 1.08
	(1-3)IGF-1	9.23 ± 0.73	8.92 ± 0.78	9.08 ± 0.95	9.58 ± 0.82
Total Entries (#)	Saline	20.29 ± 2.05		20.38 ± 1.80	
	LM22A-4	18.57 ± 1.31		21.21 ± 1.92	
	(1-3)IGF-1	18.15 ± 1.46		18.67 ± 1.61	

Table 5C. Two-way ANOVA p-values

Effect	Treatment	Time in Chamber	Entries
Sex*Chamber Preference	Saline	**0.009 ***	0.698
	LM22A-4	**0.001 ***	0.244
	(1-3)IGF-1	**0.042 ***	0.877

All mice were tested on postnatal days 29 and 30. The social approach apparatus featured 3 chambers: (a) chamber containing a novel mouse under an inverted wire cup, (b) chamber containing an identical empty wire cup and (c) central chamber connecting the others. Time spent in the novel mouse and

the empty wire cup chambers was measured. Number of entries into the novel mouse and the empty wire cup chambers and total number of transitions were also scored. These measures reflect exploratory locomotion and represent an important built-in control parameter for inactivity, a confounding factor in sociability evaluation (Yang et al., 2011). A sex by chamber preference interaction effect was found for time spent in chamber (sociability) (*$p<0.05$, two-way ANOVA). Hence, females and males were analyzed separately. Effects of treatments (saline, LM22A-4, (1-3)IGF-1) across groups (VPA and controls) were assessed and presented here. Data for male mice: Saline-treated controls, n=18; saline-treated VPA mice, n=18; LM22A-4-treated controls, n=16; LM22A-4-treated VPA mice, n=16; (1-3)IGF-1-treated controls, n=17; (1-3)IGF-1-treated VPA mice, n=15. No statistically significant differences were found (two-way ANOVA). Mean ± SE. Data for female mice: Saline-treated controls, n=14; saline-treated VPA mice, n=13; LM22A-4-treated controls, n=14; LM22A-4-treated VPA mice, n=14; (1-3)IGF-1-treated controls, n=13; (1-3)IGF-1-treated VPA mice, n=12. Number of entries and total entries: No statistically significant differences were found (two-way ANOVA). Mean ± SE. Time spent in chambers is reported in Results: section 4.2.1.

Table 6. Elevated Plus Maze Test

Elevated Plus Maze Test Measures		Treatment	Controls	VPA
Time (sec)	Open Arms	Saline	26.75 ± 4.16	16.00 ± 2.72
		LM22A-4	19.89 ± 4.39	13.22 ± 2.39
		(1-3)IGF-1	15.96 ± 3.34	22.76 ± 4.42
	Closed Arms	Saline	174.28 ± 8.29	176.95 ± 8.03
		LM22A-4	180.72 ± 10.06	192.97 ± 8.03
		(1-3)IGF-1	185.96 ± 10.35	182.30 ± 9.35
	Centre	Saline	67.53 ± 3.92	79.64 ± 5.89
		LM22A-4	72.26 ± 6.68	65.34 ± 5.98
		(1-3)IGF-1	67.53 ± 6.00	68.35 ± 4.43
Entries (#)	Open Arms	Saline	**4.03 ± 0.55 ***	**2.20 ± 0.29 ***
		LM22A-4	3.03 ± 0.55	1.93 ± 0.34
		(1-3)IGF-1	3.07 ± 0.68	2.50 ± 0.47
	Closed Arms	Saline	**17.25 ± 0.85 +,#**	**14.00 ± 0.90 +**
		LM22A-4	15.27 ± 0.96	13.90 ± 0.80
		(1-3)IGF-1	**14.63 ± 1.04 #**	13.33 ± 0.70

All animals were tested on postnatal days 31 and 32. Time (sec) spent in open arms, closed arms and centre as well as entries (#) into open and closed arms were measured. Effects of treatments (saline, LM22A-4, (1-3)IGF-1) and groups (VPA and controls) were evaluated for each measure and reported here. No significant differences between sexes were observed (two-way ANOVA), hence female and male data were pooled. Saline-treated controls, n=32; saline-treated VPA mice, n=31; LM22A-4- treated controls, n=30; LM22A-4- treated VPA mice, n=30; (1-3)IGF-1-treated controls, n=30; (1-3)IGF-1-treated VPA mice, n=27. Values designated by the same symbols are significantly different from each other ($p<0.05$, two-way ANOVA with post-hoc SNK). Mean ± SE. Outliers were identified using the Grubb's test. One single outlier was removed from each of the following groups: saline-treated control and VPA, LM22A-4-treated VPA, (1-3)IGF-1-treated control and VPA groups (entries into open arms); saline-treated VPA, LM22A-4-treated VPA, and (1-3)IGF-1-treated control groups (time in open arms).

Table 7. Western Blots

Western Blot Targets	Treatment	Controls	VPA
β-actin	Naïve	40.901 ± 1.859	42.636 ± 2.085
	Saline	42.350 ± 1.974	39.819 ± 1.837
	LM22A-4	41.029 ± 1.653	39.608 ± 1.385
	(1-3)IGF-1	40.387 ± 1.564	41.591 ± 2.264
Total Akt/β-actin	Naïve	0.850 ± 0.035	0.839 ± 0.037
	Saline	0.803 ± 0.040	0.827 ± 0.039
	LM22A-4	0.829 ± 0.032	0.840 ± 0.032
	(1-3)IGF-1	0.840 ± 0.039	0.795 ± 0.041
Total S6-Ribosomal Protein/β-actin	Naïve	0.089 ± 0.003	0.085 ± 0.003
	Saline	0.089 ± 0.002	0.088 ± 0.003
	LM22A-4	0.088 ± 0.002	0.093 ± 0.003
	(1-3)IGF-1	0.093 ± 0.002	0.089 ± 0.003

All parietal/temporal neocortex samples were collected on postnatal day 35. Total protein levels of β-actin, Akt and S6-ribosomal protein were measured by Western blotting. Integrated Intensity values were quantified using the LI-COR® Odyssey Software, version 2.0 (LI-COR Biosciences, Lincoln, NE, USA) and are reported here. For Akt and S6-ribosomal protein, each sample was normalized to its β-actin. No significant differences between sexes were observed (two-way ANOVA), hence female and male data were pooled. Effects of treatments (saline, LM22A-4, (1-3)IGF-1) and groups (VPA and controls) were evaluated for each measure and reported here. Naïve mice were neither subjected to behavioural tests nor received any treatments or sham injections. β–actin and total Akt/β-actin: Naïve controls, n=31; naïve VPA mice, n=28; saline-treated controls, n=32; saline-treated VPA mice, n=31; LM22A-4-treated controls, n=30; LM22A-4-treated VPA mice, n=30; (1-3)IGF-1-treated controls, n=30; (1-3)IGF-1-treated VPA mice, n=26. Total S6-ribosomal protein: Naïve controls, n=22; naïve VPA mice, n=18; saline-treated controls, n=19; saline-treated VPA mice, n=20; LM22A-4-treated

controls, n=20; LM22A-4-treated VPA mice, n=20; (1-3)IGF-1-treated controls, n=20; (1-3)IGF-1-treated VPA mice, n=19. No statistically significant differences were found (two-way ANOVA). Mean ± SE.

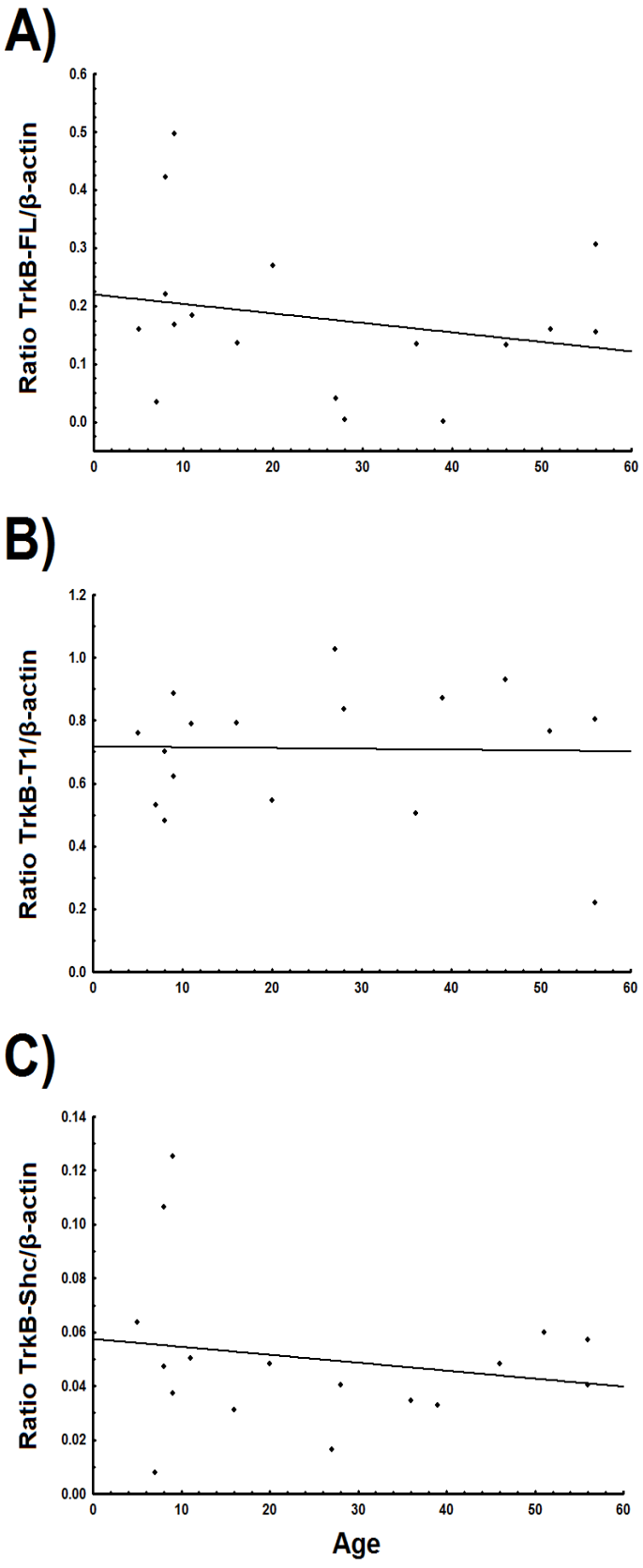

A)
Ratio TrkB-FL/β-actin
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
50
60
B)
Ratio TrkB-T1/β-actin
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
C)
Ratio TrkB-Shc/β-actin
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0
10
20
30
40
50
60
Age

Figure 13. Correlations between (**A**) full-length TrkB (TrkB-FL), (**B**) truncated TrkB-T1 and (**C**) truncated TrkB-Shc mRNA levels and age. TrkB-FL: r=-0.22, p=0.39, r^2=0.0494; TrkB-T1: r=-0.02, p=0.93, r^2=0.0006; TrkB-Shc: r=-0.19, p=0.47, r^2=0.0359; Pearson's correlations.

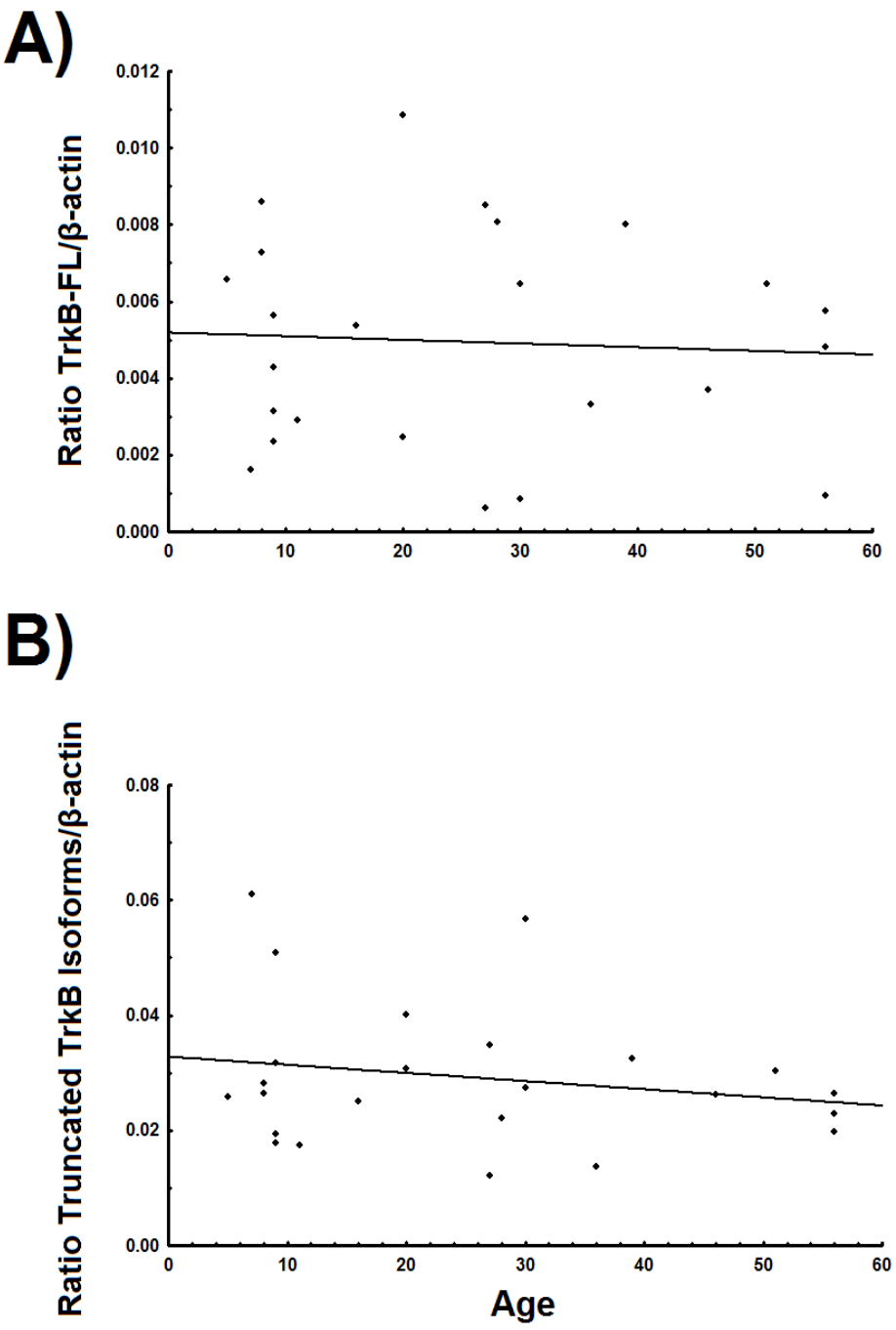


Figure 14. Correlations between (**A**) full-length TrkB (TrkB-FL) and (**B**) truncated TrkB isoform protein levels and age. TrkB-FL: r=-0.06, p=0.78, r^2=0.0036; truncated TrkB isoforms: r=-0.20, p=0.35, r^2=0.0396; Pearson's correlations.

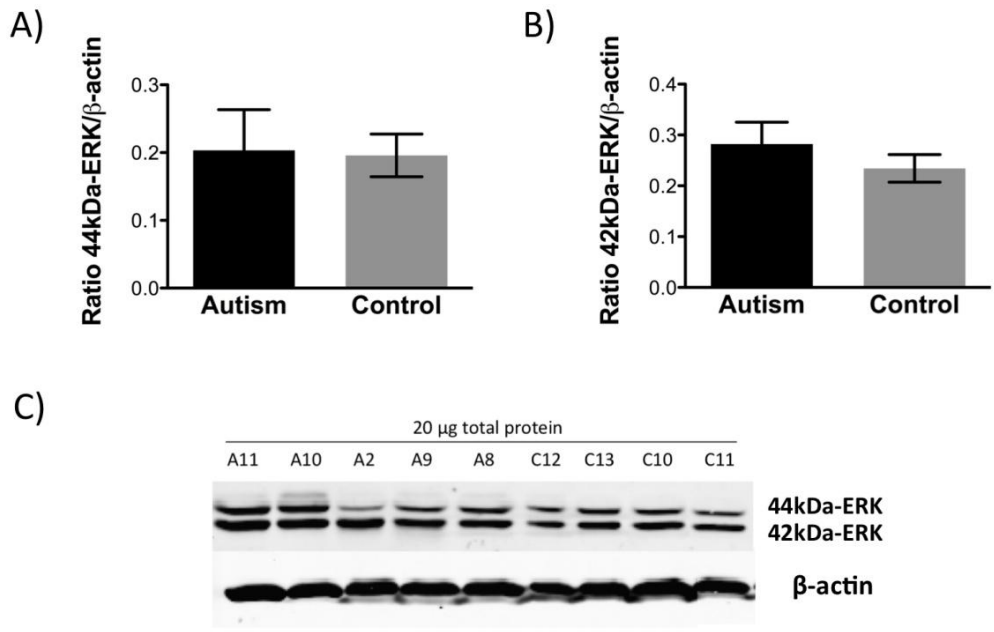


Figure 25. Quantification of (**A**) 44kDa- and (**B**) 42kDa-ERK protein expression in fusiform gyrus of autism and control samples by Western blotting. Each sample was normalized to its β-actin. p=0.9 for 44kDa-ERK and p=0.3 for 42kDa-ERK, 2-tailed *t* tests. Bars indicate mean ± SE. Autism, n=11; control, n=13. (**C**) Representative Western blot of fusiform gyrus showing autism (A) and control (C) cases. Sample numbers correspond to samples listed in Tables 1 and 2. 20 µg of total protein from each autism and control sample was loaded. This work was carried out by Farhia Haque.

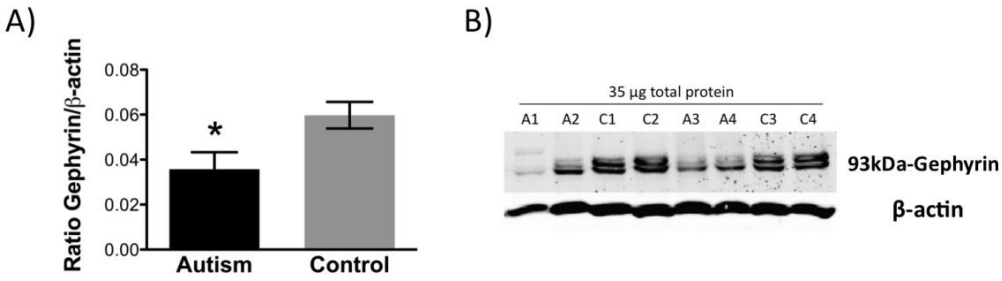
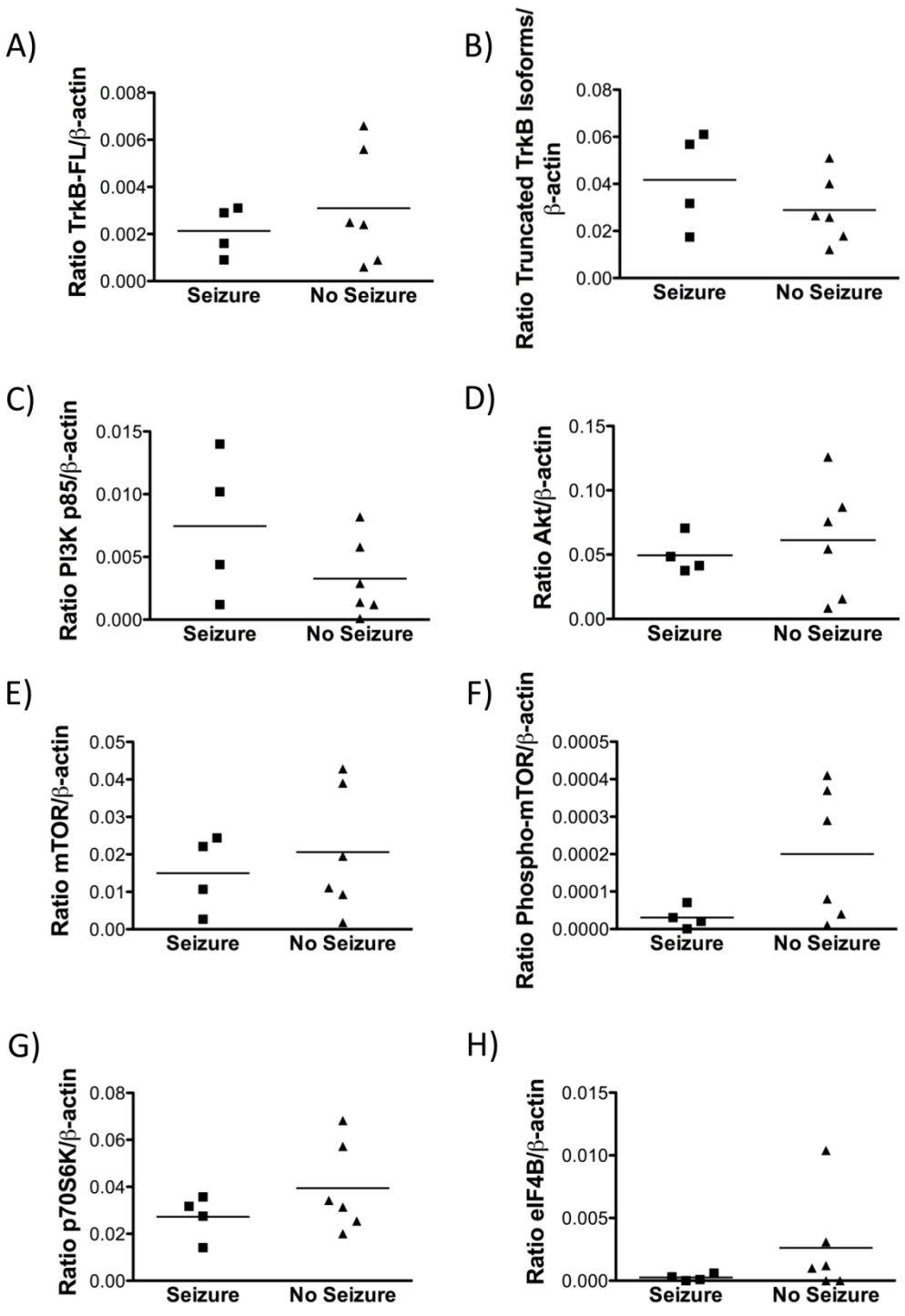


Figure 26. (**A**) Quantification of Gephyrin protein expression in fusiform gyrus of autism and control samples by Western blotting. Each sample was normalized to its β-actin. p=0.02, 2-tailed *t* test. Bars indicate mean ± SE. Autism, n=11; control, n=13. (**B**) Representative Western blot of fusiform gyrus showing autism (A) and control (C) cases. Sample numbers correspond to samples listed in Tables 1 and 2. 35 µg of total protein from each autism and control sample was loaded.


A)
Ratio TrkB-FL/β-actin
0.008
0.006
0.004
0.002
0.000
Seizure
No Seizure
B)
Ratio Truncated TrkB Isoforms/ β-actin
0.08
0.06
0.04
0.02
0.00
Seizure
No Seizure
C)
Ratio PI3K p85/β-actin
0.015
0.010
0.005
0.000
Seizure
No Seizure
D)
Ratio Akt/β-actin
0.15
0.10
0.05
0.00
Seizure
No Seizure
E)
Ratio mTOR/β-actin
0.05
0.04
0.03
0.02
0.01
0.00
Seizure
No Seizure
F)
Ratio Phospho-mTOR/β-actin
0.0005
0.0004
0.0003
0.0002
0.0001
0.0000
Seizure
No Seizure
G)
Ratio p70S6K/β-actin
0.08
0.06
0.04
0.02
0.00
Seizure
No Seizure
H)
Ratio eIF4B/β-actin
0.015
0.010
0.005
0.000
Seizure
No Seizure

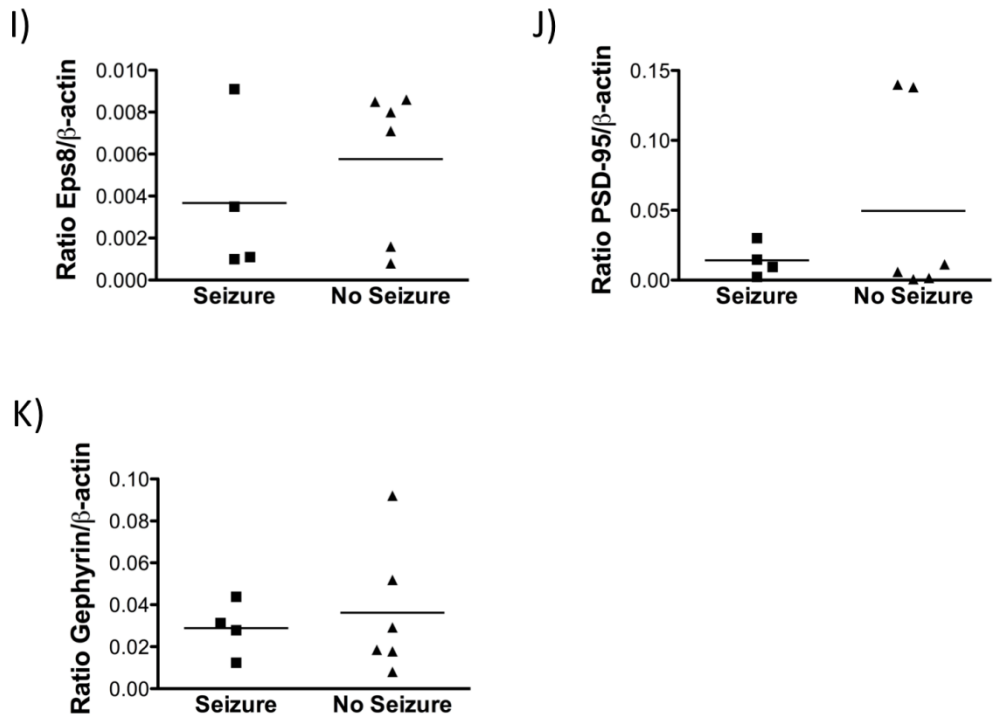


Figure 27. Quantification of (**A**) full-length TrkB (TrkB-FL), (**B**) truncated TrkB isoform, (**C**) PI3K p85, (**D**) Akt, (**E**) total mTOR, (**F**) phospho-mTOR, (**G**) p70S6K, (**H**) eIF4B, (I) Eps8, (J) PSD-95 and (K) gephyrin protein expression in fusiform gyrus of autism cases wih seizure disorder and those without by Western blotting. Each sample was normalized to its β-actin. p=0.48 for TrkB-FL, p=0.28 for truncated TrkB isoforms, p=0.17 for PI3K p85, p=0.63 for Akt, p=0.57 total mTOR, p=0.1 phospho-mTOR, p=0.27 for p70S6K, p=0.28 for eIF4B, p=0.40 for Eps8, p=0.35 for PSD-95 and p=0.67 for gephyrin, 2-tailed *t* test. Bars indicate mean ± SE. Autism patients with seizure disorder, n=4; autism patients with no seizure disorder, n=6.

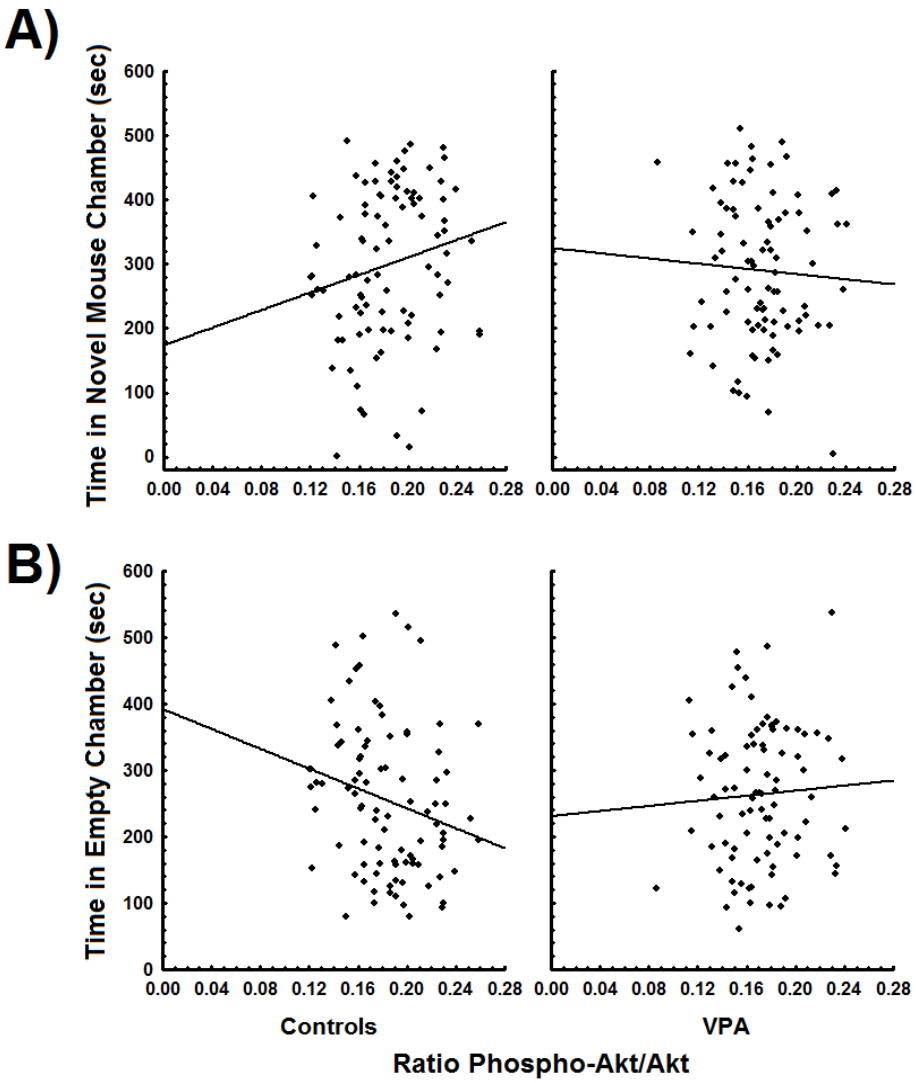


Figure 28. Correlations between social approach task measures and phosphorylated Akt levels: (**A**) Time spent in the chamber with the novel mouse, controls: r=0.19, p=0.07, r^2=0.0359; VPA: r=-0.05, p=0.62, r^2=0.0030; Pearson's correlation. (**B**) Time spent in the empty chamber, controls: r=-0.22, p=0.03, r^2=0.0494; VPA: r=0.06, p=0.61, r^2=0.0031; Pearson's correlation.

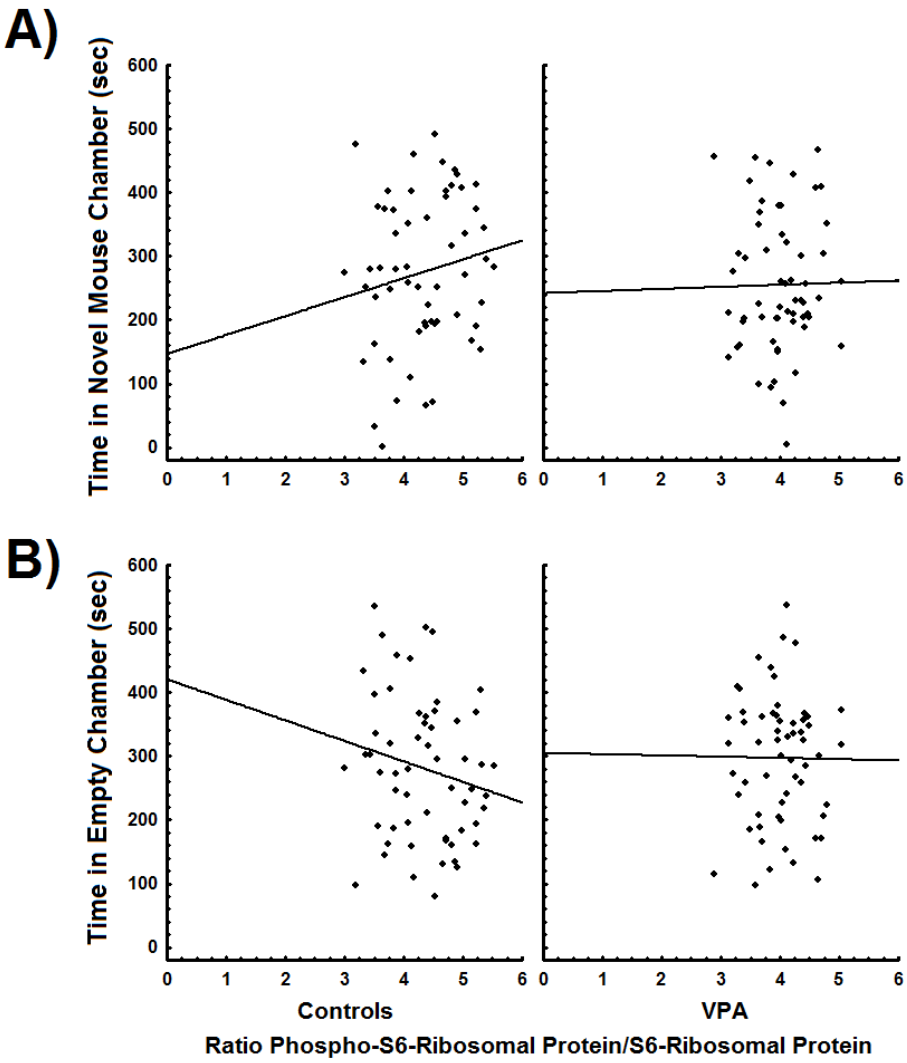


Figure 29. Correlations between social approach task measures and phosphorylated S6 ribosomal protein levels: (**A**) Time spent in the chamber with the novel mouse, controls: r=0.16, p=0.22, r^2=0.0263; VPA: r=0.02, p=0.91, r^2=0.0002; Pearson's correlation. (**B**) Time spent in the empty chamber, controls: r=-0.19, p=0.16, r^2=0.0345; VPA: r=-0.01, p=0.94, r^2=0.0001; Pearson's correlation.

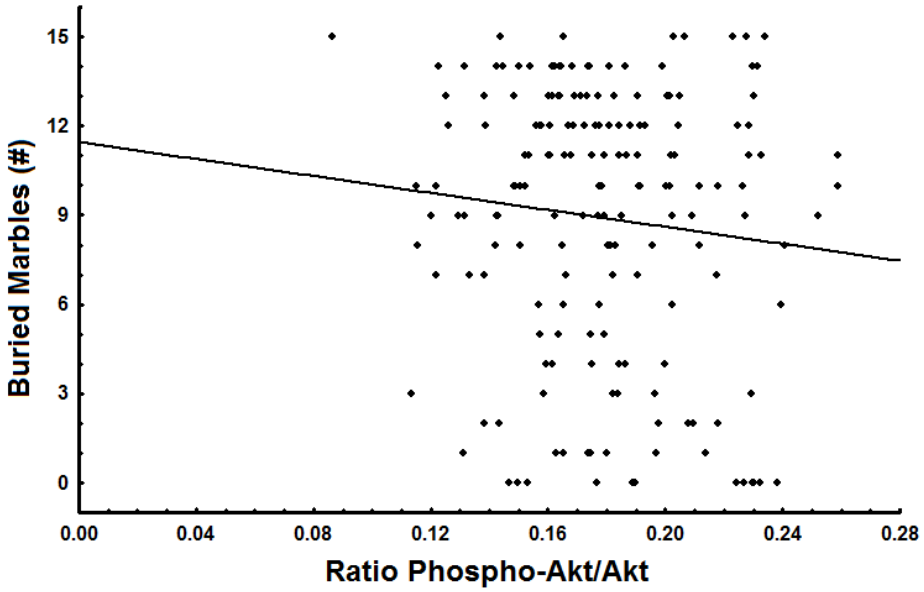


Figure 30. Correlations between number of buried marbles and phosphorylated Akt levels: r=-0.10, p=0.18, r^2=0.0104; Pearson's correlation.

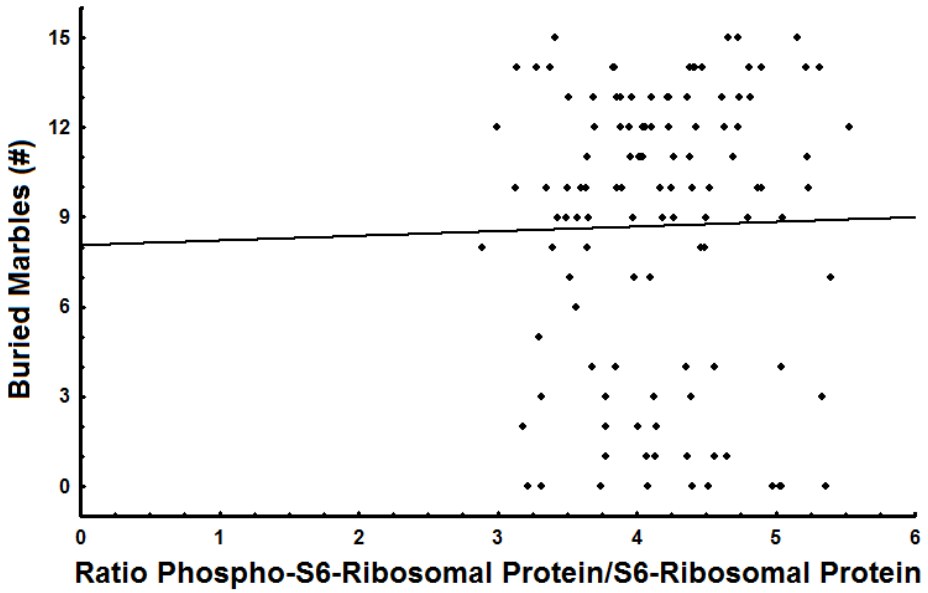


Figure 31. Correlations between number of buried marbles and phosphorylated S6 ribosomal protein levels: r=0.02, p=0.83, r^2=0.0004; Pearson's correlation.