INVESTIGATION OF STRIATAL SOLUBLE N-ETHYLMALEIMIDE SENSITIVE FACTOR ATTACHMENT RECEPTOR (SNARE) PROTEINS IN SCHIZOPHRENIA

by

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B.Sc., Queen's University, 2004

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY
in
THE FACULTY OF GRADUATE STUDIES
(Neuroscience)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

October 2010

Abstract

Synaptic dysfunction likely contributes to abnormal brain function in schizophrenia. Patient symptoms indicate that the striatum is involved in this disease. The three neuronal soluble-NSF-attachment receptor (SNARE) proteins (SNAP-25, syntaxin-1 and VAMP) interact at the presynaptic neuronal membrane to facilitate neurotransmission, and are thus key players in synaptic function. SNARE abnormalities have already been reported in cortical and hippocampal brain regions in schizophrenia. Their involvement in striatal dysfunction has not been investigated. Normal synaptic function requires the SNAREs to physically interact with each other, but little is known about how altered SNARE protein levels in schizophrenia relate to SNARE protein interactions. Multiple isoforms of each SNARE exist in the brain, may affect SNARE protein interactions and synaptic transmission differently, and may diverge functionally. SNARE isoform expression in schizophrenia is unknown. Thus, abnormalities in SNARE protein expression or function may underlie or contribute to brain dysfunction and disease.

In this thesis, SNARE protein levels were measured in human post mortem brain samples of schizophrenia subjects for the first time in the striatum. The functional consequences of SNARE alterations were investigated by developing a novel ELISA assay to measure SNARE protein interactions. The possible confounding effects of medications were addressed in several ways, including the use of striatal tissue from animals exposed to antipsychotic medications.

Alterations in SNAP-25 and syntaxin-1 protein levels were further dissected by measuring protein isoforms. Syntaxin-1 isoforms were assayed by quantitative immunoblotting. A mass-spectrometry based assay was developed and used to measure SNAP-25 protein isoform levels.

The results of these investigations suggest that SNARE protein alterations in schizophrenia are restricted to distinct functional regions of the striatum, perturb SNARE protein

interactions, involve specific protein isoforms, and may occur independent of patient treatment with antipsychotic medications. Furthermore, these studies contribute a new, high-throughput method for measuring SNARE protein interactions, and for the first time, a means of detecting and quantifying SNAP-25 isoforms in brain tissue.

Preface

Working with my supervisor, Dr. William Honer, I was responsible for the design, organization, execution, analysis and interpretation of the experiments described herein. Several experiments were conducted in collaboration with other investigators, mainly Dr. Clare Beasley, Dr. Alasdair Barr and Dr. Gregg Morin. In these cases study design was developed with their guidance, and I was responsible for the laboratory work and data analysis. Each research chapter represents a multi-author manuscript. I took primary responsibility for conducting the experiments, but received assistance in several areas. In Chapter 2, H. Li was involved in assay development and collected the capture ELISA data for human samples. Animal handling, injection and tissue preparation was performed collaboratively with A. Ypsilanti, C. Beasley and A. Barr. The UBC Animal Care Committee approved protocols involving animals (certificate #A06-272). In Chapter 4, A. Moradian operated the mass spectrometer, analyzed part of the MRM data, and wrote the description of MS and HPLC running conditions. I was involved in all aspects of the assay design and development, but was not responsible for maintenance or operation of the mass spectrometer. I also took primary responsibility for the statistical analysis, assembly of figures and tables and drafting of manuscripts. Manuscript drafts were re-written collaboratively with W. Honer. I sought advice for statistical analyses (H. Wong, A. Thornton), but conducted all analyses myself. All co-authors had an opportunity to make edits, and these were incorporated in the final drafts. W. Honer made substantial edits and additions in response to co-author and reviewer suggestions prior to and after submission. Human samples were obtained via collaboration with Dr. Andrew Dwork. I was not involved in human sample collection or dissection. Additional co-authors were involved in human sample collection and study conception. Studies involving human samples were approved by the UBC Biohazards Committee (protocol #H02-0079 and B07-0043).

A version of Chapter 2 has been published:
Barakauskas VE, Beasley CL, Barr AM, Ypsilanti AR, L, HY, Thornton AE, Wong H, Rosokilja G, Mann JJ, Mancevski B, Jakovski Z, Davceva N, Ilievski B, Dwork AJ, Falkai P, Honer WG. (2010). A novel mechanism and treatment target for presynaptic abnormalities in specific striatal regions in schizophrenia. *Neuropsychopharmacology*. 35(5): 1226-38. doi:10.1038/npp.2009.228

Chapters 3 and 4 will be submitted for publication shortly.

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Abbreviations

2D-DIGE	two-dimensional difference gel electrophoresis
5-HT	serotonin (5-hydroxytryptamine)
5HTT	serotonin transporter (also known as SERT)
3' UTR	3-prime untranslated region
aa.	amino acid
ADHD	attention-deficit hyperactivity disorder
AJD	Andrew J. Dwork
AMPA	alpha-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
AnCg	anterior cingulate
AnCgCtx	anterior cingulate cortex
APD	antipsychotic drugs
BA	Brodmann area
Bdr	*blind-drunk* mutant mouse
BM	B. Mancevski
BSA	bovine serum albumin
CamK	calcium/calmodulin-dependent protein kinase
Ch	chromosome
Cm	*coloboma* mutant mouse
CNS	central nervous system
CNV	copy number variation
COMT	catechol *O*-methyl transferase
CTL	control (non-psychiatric comparison subject)
ctx	cortex
CV	coefficient of variation
D2R	dopamine D2 receptor
Da	Dalton
DA	dopamine
DAO	D-amino acid oxidase gene (human; also known as DAAO)
DAOA	D-amino acid oxidase activator gene (human; also known as G72)
DAT	sodium-dependent dopamine transporter
DB	dot blot
DCd	dorsal caudate
dlPFC	dorsolateral prefrontal cortex
DSM	diagnostic and statistical manual of mental disorders
DTNBP1	dystrobrevin binding protein 1 gene (human; also known as dysbindin)
EBI	European Bioinformatics Institute
EEG	electroencephalography
ELISA	enzyme-linked immunoadsorbant assay
EM	electron microscopy
EP10	α-synaptophysin antibody
EPP	end-plate potential
EPSC	excitatory post-synaptic current
EPSP	excitatory post-synaptic potential
ERP	event-related potential
fMRI	functional magnetic resonance imaging
FPLC	fast protein liquid chromatography

GABA	γ-aminobutyric acid
GAD	glutamic acid decarboxylase
GAT	GABA reuptake transporter Official gene name: SLC6A1; solute carrier family 6 (neurotransmitter transporter, GABA), member 1
GR	G. Rosokilja
GRM3	mGluR3 gene
GSK3β	glycogen synthase kinase 3 beta
Hipp	hippocampus
HPLC	high performance liquid chromatography
IB	B. Ilievski (section 2.5 only)
IB	immunoblot
ICD	international classification of diseases
IFM	Institute of Forensic Medicine (Skopje, Macedonia)
IgG	immunoglobulin G
IHC	immunohistochemistry
IPSP	inhibitory post-synaptic potential
IRB	institutional research ethics board
JJM	J.J. Mann
kDa	kilodalton
KO	knock-out
L-DOPA	L-3,4-dihydroxyphenylalanine
LC	liquid chromatography
LDCV	large dense core vesicle
LSD	lysergic acid diethylamide
LTP	long term potentiation
LVA	low-voltage activated
MAO	monoamine oxidase
MD	mediodorsal thalamic nucleus
mEPP	miniature end-plate potential
mEPSP	miniature excitatory post-synaptic potential
MFCtx	medial frontal cortex
mGluR	metabotropic glutamate receptor
MRI	magnetic resonance imaging
MRM	multiple reaction monitoring
MS	mass spectrometry
MSN	medium spiny neuron
Munc	mammalian uncoordinated
MWCO	molecular weight cut-off
n.s.	not statistically significant
NAA	N-acetyl-aspartate
NAc	nucleus accumbens
NCBI	National Centre for Biotechnology Information
ND	N. Davceva
NE	norepinepherine (noradrenaline)
NGF	nerve growth factor
NMDA	N-methyl-D-aspartate
NMDAR	NMDA receptor
NPC	non-psychiatric comparison subject; called ‘control/CTL’ in Chapter 2

NRG1	neuregulin-1 gene (human)
NS0	non-secreting mouse myeloma cell line (antibody supernatant control)
NSF	N-ethylmaleimide sensitive factor
OFC	orbitofrontal cortex
PBS	phosphate-buffered saline
PC12	pheochromocytoma cell line
PCP	phencyclidine
PET	positron emission tomography
PFC	prefrontal cortex
Physin	synaptophysin
PI	protease inhibitors
PKA	protein kinase A
PKC	protein kinase C
PMI	post mortem interval
PNS	peripheral nervous system
PPD	paired-pulse depression
PPF	paired-pulse facilitation
PPI	paired-pulse inhibition
PVDF	polyvinylidene fluoride
qDot	quantum dot fluor
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
SCZ	schizophrenia
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SERT	serotonin reuptake transporter Offical gene name: SLC6A4; solute carrier family 6 (neurotransmitter transporter, serotonin), member 4
SNAP-25	25-kD synaptosomal associated protein
SNAP25	SNAP-25 gene (human)
SNARE	soluble NSF-attachment receptor
SN	substantia nigra
SNc	substantia nigra pars compacta
SNr	substantia nigra pars reticulata
SNP	single nucleotide polymorphism
SNr	substantia nigra pars reticulata
Sp1	specificity protein 1 transcription factor
SP6	α-syntaxin-1 antibody
SP7	α-syntaxin-1 antibody
SP10	α-VAMP antibody
SP11	α-VAMP antibody
SP12	α-SNAP-25 antibody
SP15	α-synaptophysin antibody
Stx	syntaxin
STX1A	syntaxin-1A gene (human)
STX1B	syntaxin-1B gene (human)
STXBP1	syntaxin binding protein 1 (a.k.a. Munc-18)
TBS	tris-buffered saline

TBST	tris-buffered saline containing 0.05% v/v Tween-20
ThalA	anterior thalamus
Tx100	tritonX-100
u.d.	undetermined
UPS	ubiquitin proteosome system
v/v	volume by volume
VA	ventroanterior thalamic nucleus
VGAT	vesicular GABA transporter official gene name: SLC32A1; solute carrier family 32 (GABA vesicular transporter), member 1
VGlutT1	vesicular glutamate transporter official gene name: SLC17A7; solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 7
VMAT	vesicular monoamine transporter Official gene name: SLC18A2; solute carrier family 18 (vesicular monoamine), member 2
VMC	ventromedial caudate
VTA	ventral tegmental area
w/v	weight by volume
WCST	Wisconsin card sorting task
WGH	William G. Honer
ZJ	Z. Jakovski

With respect to gene name usage in this thesis:
Many of the current approved gene name abbreviations do not allude to the protein product the gene encodes. As this thesis focuses on protein-level assessment of the SNAREs, is not conducted in the context of a genetics study, and to improve flow and comprehension, usage of the common/prevalent names of genes (and their encoded proteins) has been retained. In cases where the current approved gene name differs from that which is used in the document, the current approved gene name is documented in the abbreviations list. In the body of the document human gene names are capitalized and italicized, mouse gene names are italicized only, and references to proteins are not italicized.

Acknowledgements

A very large number of individuals had an impact on my time as a graduate student, which I would like to acknowledge. I thank my supervisor Dr. William Honer for his patience, composure and generosity and for knowing when I needed a coach, a mentor or a guide. I am grateful for the opportunity to work with the exceptional people at the Centre for Complex Disorders. I appreciated the opportunity to think about both basic and clinical science, attend a variety of meetings and conferences and be supported in my efforts to develop teaching and learning skills outside of the laboratory.

Sincere thanks to my supervisory committee members: Dr. Elizabeth Simpson, Dr. Weihong Song and Dr. Gregg Morin for their guidance and support over the past six years. I thank Dr. Steven Vincent and the Graduate Program in Neuroscience for accepting me into the Strategic Training Program in Neurobiology and contributing to my first year of funding. I thank Drs. Tim Murphy and Liisa Galea and their lab members for the opportunity to learn from them as part of the training program. Thanks also to Liz Wong for helping me through the many, many degree and finance-related details that have needed sorting over the years.

I gratefully acknowledge my funding sources, which provided stipend and travel funds over the past six years. These include the National Science and Engineering Research Council of Canada, the Michael Smith Foundation for Health Research, and the Michael Smith Foundation for Health Research/Canadian Institute for Health Research Strategic Training Program in Neurobiology. I am also grateful for an award received from the Michael Stahl Memorial Fund, and am honoured that my research may contribute to a better understanding of mental health and addiction.

I am fortunate to be surrounded by knowledgeable and generous individuals from whom I learned many laboratory methods, organizational strategies and data analysis techniques. I would like to thank past and present members of our lab: Clare Beasley, Hong Ying Li, Ming Yang, Jenny Yan, Athena Ypsilanti, Fidel Vila, Alasdair Barr, Jehannine Austin, Donna Lang and others who have crossed my path for their patience, willingness to help, share good news, lament bad news, save samples on a Friday night when the -80 broke down, track down equipment that grew legs, proof-read my applications, listen to my presentations and for taking me seriously. Thanks also to Carri-Lyn Mead, Grace Cheng, Annie Moradian, Gary Wilson, Taka Ichiu, Geoff Smith, Allen Thornton, Paul Pavlidis, Rob Holt, Todd Woodward and Liang Wang for patiently sharing their expertise, experience and advice on a variety of topics.

Thank you to my friends and fellow graduate students. To Carri-Lyn Mead, Aldona Businskas and Jen Barker-Helli for showing me it can be done, for empathizing and for encouraging me to forge ahead. Thanks to Paul Metzak, Jen Whitman, Josh Rizak, Babak Khorram and Fidel Vila for their encouragement, kind words and interesting conversations during the evenings and weekends that we hadn't planned to be, but were, in the lab.

To my family and Geoff, for their unending and unconditional support, encouragement and care. For believing that I could undertake and complete this thesis work, and reminding me of this when I had forgotten. I could not have done it without your love.

Dedication

To Geoff: I know this document means as much to you as it does to me. May this be the end of something good and the beginning of something new that we share.

1 Introduction

1.1 Thesis overview

The objectives of this thesis stem from the current understanding of synaptic alterations in schizophrenia and the overarching hypothesis that synaptic pathology is associated with the disease. Experiments were designed to characterize protein-level abnormalities of the three presynaptic SNARE proteins for the first time in the striatum, a region involved in persistent patient symptoms and that is targeted by disease treatments. The functional implications of SNARE alterations were addressed by considering SNARE protein interactions and protein isoform expression in schizophrenia. In order to do so, methods were developed and characterized, helping to extend our understanding of synaptic function in schizophrenia, but also contributing new tools and approaches to SNARE protein quantitation. Finally, a collateral benefit of this work is the contribution to our [limited] knowledge of SNARE proteins and their properties in healthy human striatum.

1.2 Schizophrenia

While diseases of the mind and brain have likely been the curiosity and fear of society for centuries, if not millennia, it was not until the late nineteenth century that classification of schizophrenia was formally undertaken [1]. Initially termed “dementia praecox”, Emil Kraeplin described a brain disease with an early onset, poor prognosis and deteriorating course [2, 3]. The disease description was broadened by Eugene Bleuler, who re-coined it “schizophrenia” to describe the “splitting of psychic functions” he perceived as defining the illness [4]. Current diagnostic guidelines reflect a shift back towards a narrower definition of schizophrenia, with psychotic symptoms and deterioration of function being at the core of disease classification [3, 5].

1.2.1 Disease description

Schizophrenia is a severe mental illness characterized by impaired reality testing (psychosis). Patient symptoms are often classified as positive or negative. Symptoms that appear to be exaggerations of normal functions are termed "positive" and include hallucinations (perception in the absence of a stimulus), delusions (strongly held false beliefs) and disorganized speech. Negative symptoms suggest a loss of normal function and include alogia (poverty of speech), affective blunting/flattening, avolition (reduced motivation) and may also include anhedonia (inability to experience pleasure), inappropriate affect, labile mood/emotion and poor insight [1, 5]. Cognitive symptoms such as impaired executive functions, attention, memory and social cognition are also prominent, and may even precede illness onset [6, 7]. Symptoms typically surface during late adolescence or early adulthood, although they may be present in attenuated forms prior to the first episode of illness [6, 8], and severely impair a person's day-to-day function, social interactions and quality of life.

Current diagnostic criteria are outlined in the International Classification of Disease (ICD-10) and Diagnostic and Statistical Manual of Mental Disorders version IV (DSM-IV). Besides requiring the presence of at least two characteristic symptoms, a DSM-IV diagnosis of schizophrenia also requires significant social or occupational dysfunction to be present and symptoms to persist for at least 6 months. The precise subset of symptoms identified leads to classification into the paranoid, disorganized, catatonic, undifferentiated or residual sub-type [5] and the specific constellation of symptoms and symptom severity varies between individuals.

Lifetime morbid risk for schizophrenia has recently been estimated at 7.2 per 1000 persons, disease prevalence may vary between centres and incidence is higher in males than females [9]. Patients have an increased risk of mortality, including suicide [9, 10]. Studies of twins and adoptees indicate that schizophrenia is highly heritable, and risk of disease is increased

in first-degree relatives [11]. Twin-concordance rates suggest that environmental factors also play a role in disease manifestation [11, 12]. The severity, chronicity and early presentation of schizophrenia result in a high socioeconomic burden, requiring substantial medical and social resources to treat and support patients [13, 14].

1.2.2 Mechanistic theories

Attempts to explain the underpinnings of schizophrenia have incorporated clinical, genetic, pathological, epidemiological and brain imaging data. Many different theories have been put forth. Three major perspectives and their variants are discussed here.

1.2.3 The neurodevelopmental hypothesis

Two "versions" of the neurodevelopmental hypothesis exist. The first posits that one or more insults occurring early in life (pre- and perinatal) underlie the disease symptoms that surface during early adulthood [15], while the second suggests that such insults occur closer to adolescence, during a period of substantial brain maturation and reorganization [16]. It has also been proposed that developmental abnormalities occurring during these two critical developmental time points may act in concert to produce disease [17].

Evidence to support a developmental model is varied. Abnormalities in cortical cell migration, congenital anomalies, increased prevalence of obstetric complications, perinatal exposure to viruses, and neurological soft signs in children support an early-life event [18-21]. As children, patients may also exhibit subtle movement abnormalities [21], developmental delays [19], and behavioural problems [22]. Additional support comes from animal models of perinatal brain insult. In rodents, ventral hippocampal lesions or exposure to influenza *in utero* results in behavioural and brain abnormalities later in life [20, 23].

Increased ventricular volume and decreased cortical grey matter have been repeatedly reported in studies of the brain in schizophrenia [24, 25] and may support the neurodevelopment model, as the reduction is evident at disease onset [26-28]. It has been suggested that lower grey matter volume may be the result of over-maturation of the brain. Synaptic remodeling and pruning occurs during late adolescence, and grey matter volume decreases with age in the general population [29, 30]. Increased ventricular size and reduced grey matter volumes observed by MRI in schizophrenia may represent alterations in the trajectory of brain maturation [16, 17, 19], implicating synaptic pruning and reorganization in the pathophysiology of the disorder.

Finally, a number of genes that have been associated with schizophrenia in recent years are expressed during various developmental time points and encode proteins that function in neuronal or glial development, migration and survival [19, 20, 31, 32].

1.2.4 Neurotransmitter-based hypotheses

Up until the late nineteenth century, electrical conduction was thought to underlie signal propagation in the brain [33, 34]. The discovery that adrenaline (by T.R. Elliot) and acetylcholine (by H. Dale and H. Dudley) produce effects similar to peripheral nerve stimulation, and the demonstration (by O. Loewi) that perfusate from a stimulated frog heart could affect a denervated heart, provided evidence of chemical signaling in the nervous system [35]. The possibility of chemical transmission in the brain was strengthened by the isolation and identification of these compounds in animal organs [35] and identification of membrane receptors to which chemical transmitters bind [36, 37]. The discovery of chlorpromazine, and re-discovery of reserpine's anti-psychotic effects prompted neuropsychopharmacological research into the endogenous receptors of antipsychotic medications [36, 38]. Since then,

accumulating evidence has implicated a number of different chemical transmitters in the pathophysiology of schizophrenia. The major prevailing theories are described here, with examples of evidence used to support each of them. It must be noted that these are all theories, so evidence opposing each theory also exists, and the interaction of all of these systems in the brain means that each neurotransmitter will likely be involved, to some extent, in the disorder.

1.2.4.1 Dopamine

Several lines of evidence lent support to the original dopamine hypothesis of schizophrenia. The fact that early antipsychotic medications induced Parkinsonian side effects and prolactinemia suggested that these drugs target the dopamine system in the brain. In addition, typical antipsychotic drugs (APDs) increase catecholamine metabolites, with rare exception their clinical efficacy correlates with their affinity for the dopamine D_2-receptor (D2R), and drugs whose action mimics dopamine (such as L-DOPA and amphetamine) produce psychotic symptoms reminiscent of schizophrenia [39]. Reports of increased D2Rs in the brain of patients also supported this initial hypothesis, although confounding effects of antipsychotics weaken this line of evidence (reviewed in [40]). These observations suggested that dopaminergic over-activity is involved in schizophrenia.

The dopamine hypothesis was later refined to address some inconsistencies and incorporate new findings. Among them, the introduction and superior efficacy of clozapine, an atypical APD with a lower affinity for the D2R, and the observation that negative symptom-like behaviour as well as poor performance on tasks of frontal brain function (such as the Wisconsin card sorting task) could be ameliorated by amphetamine [41]. In addition, animal studies demonstrated that increased prefrontal dopamine results in decreased striatal dopamine

turnover/release [41, 42]. Thus, the dopamine hypothesis was re-stated to include striatal hyperfunction but cortical hypofunction of dopamine in schizophrenia.

Advances in understanding schizophrenia genetics and environmental risk factors combined with new brain imaging techniques have prompted additional refinement of the dopamine hypothesis. Recent PET studies, genetic association of genes modulating monoamines (such as *COMT* and *MAO*), as well as evidence that stressors which increase risk of schizophrenia also dysregulate striatal dopamine release, imply that multiple routes may lead to dopamine dysfunction, and that "presynaptic dopamine dysregulation is the 'final common pathway' [to psychosis]" [43].

Whatever the underlying cause, dopamine dysregulation is a part of the disease profile of schizophrenia, and its modulation has been shown to improve at least part of patient symptoms.

1.2.4.2 GABA/Glutamate

The limited efficacy of APDs in treating negative or cognitive symptoms called into question completeness of the older dopamine hypotheses of schizophrenia. Pharmacological, post mortem, genetic and animal data suggest that other types of neurotransmitters may also be involved. Glutamate and γ-aminobutyric acid (GABA) are two major small molecular transmitters found throughout the brain. Via their respective receptors, they exert excitatory (propagation of action potentials/nerve signals) or inhibitory (reduction in signal propagation) effects in the brain, respectively.

Glutamatergic involvement in schizophrenia is suggested by the psychomimetic properties of NMDA-receptor (NMDAR) antagonists such as ketamine and phencyclidine (PCP) [44]. Low doses of these drugs may also produce negative and cognitive symptoms, suggesting that NMDAR-hypofunction might provide a disease model that better accounts for all patient

symptoms [45, 46]. Several schizophrenia candidate genes encode products involved in NMDAR function, including *DAO* and *DAAO* (which are needed for the breakdown of D-serine, an NMDAR co-agonist) [47], *GRM3* (which encodes a metabotropic glutamate receptor which reduces glutamatergic transmission presynaptically) [48], as well as *DTNBP1* and *NRG1* (which affect glutamatergic transmission in cell cultures) [49, 50]. While genetic association of these individual genes has not been replicated by all studies (compiled in [51]), they present functional and positional candidates that converge on and provide support for glutamatergic disruption in the disease. Reduced N-acetylaspartate levels reported *in vivo* in schizophrenia are thought to reflect decreased glutamatergic neuron activity [45]. Some presynaptic markers of glutamatergic terminals are also altered in some brain regions in post mortem patient samples [52-54].

Abnormalities of GABAergic neurons in the cortex and hippocampus have been repeatedly reported in schizophrenia and include decreased expression of GAD (GABA synthesis enzyme) and GAT1 (GABA transporter involved in clearance from the synapse) with compensatory upregulation of GABA(A) receptors also described [46, 55]. Some of these abnormalities may be restricted to subsets of interneurons [46, 56, 57]. Disruption of GABA transmission may be associated with patient symptoms, as GABA neurons are involved in generating some of the rhythmical electrical patterns in the brain (for example, gamma and theta-band oscillations), which are associated with cognitive performance [46]. Expression of GABA-related genes in schizophrenia is disrupted in many different cortical regions, suggesting widespread GABA-signaling disruption in the brain [58].

The involvement of GABA and glutamate in schizophrenia likely does not occur in isolation, as the close apposition of each terminal type in most brain regions means that dysfunction of one neurotransmitter will affect the other. Alterations in the balance of excitation and inhibition reconcile NMDAR hypofunction with GABA neuron abnormalities [59, 60]. In addition, while GABA gene expression might be uniformly affected across the brain, the pattern

of glutamate-related protein and mRNA changes in various brain regions and subregions appears to differ. For example, glutamatergic afferents are increased in the anterior cingulate but not in the dlPFC, while GABAergic interneurons are decreased in both regions [55]. Such discrepancies have suggested that "extrapolation of findings across widely dispersed brain areas should be avoided" [55], a notion that has influenced the research design of this thesis.

While leading models of GABA-glutamate interactions have concentrated on cortical and limbic microcircuitry [46, 55], the human striatum is also a site where glutamate and GABA transmission converge to integrate and filter cortical activity, and therefore is particularly important when interpreting patient symptoms in the context of neurotransmitter system alterations [61]. Dopaminergic signaling onto GABAergic striatal cells can affect incoming glutamatergic cortical information, altering both striato-nigral and striato-pallidal signaling and changing the information relayed back to the cortex [36, 61].

1.2.4.3 Other neurotransmitter systems

The involvement of other neurotransmitters in schizophrenia has been implied, as newer antipsychotic medications (APDs) and some psychomimetic drugs also target other systems.

Several lines of evidence suggest that serotonin dysfunction might occur in some patients. Hallucinogenic properties of lysergic acid diethylamide (LSD) and psilocybin produce symptoms similar to acute schizophrenia episodes by activating serotonin receptors, in particular 5-HT2A receptors. LSD produces impairments in behavioural plasticity and sensorimotor gating (such as pre-pulse inhibition) resembling impairments seen in patients. Some APDs such as clozapine and risperidone antagonize the 5-HT2A receptor [62]. There is also post mortem evidence for 5-HT2A receptor abnormalities in schizophrenia. A summary of ligand-binding assays and mRNA expression studies in brain tissue concluded that this receptor is lower in

cortical areas, in particular the dlPFC, in schizophrenia (reviewed in [63]). Initial studies of receptor binding in first-episode patients assessed using PET produced conflicting results [64, 65], but a recent study with a larger sample size supports the post mortem findings of lower 5-HT2A receptors in frontal cortex [66], indicating that abnormalities in serotonin transmission may occur early in the disorder.

Some APDs have anticholinergic properties, suggesting that abnormal cholinergic transmission may play a role in schizophrenia [67]. Studies of the muscarinic and nicotinic acetylcholine receptors have produced varied results (reviewed in [68]). Studies in post mortem brain have reported lower levels of α-7 nicotinic receptors in prefrontal cortex, but no differences at the mRNA level, in schizophrenia [69-71]. High rates of nicotine use combined with genetic association between the α-7 nicotinic receptor subunit gene and P50 ERP abnormalities in schizophrenia subjects and first-degree relatives suggests that cholinergic modulation plays some role in patient symptoms [67]. Post mortem studies of α2β4-type nicotinic receptors are less conclusive [68], with reports of both increases and decreases in receptor mRNA and/or ligand binding in schizophrenia samples (for example, [71, 72]). Muscarinic receptors have also been studied post mortem, and a recent review of these studies concluded that receptors assayed using [^{3}H]pirenzepine (which binds to M1 and M4-type receptors) are lower in frontal cortex and hippocampus in schizophrenia, but not in the thalamus or parietal cortex [68]. This is supported by *in vivo* imaging of total muscarinic receptors in the brain, which detected lower levels of receptors in cortex and subcortical structures in patients [73]. Lower levels of cortical muscarinic receptors post mortem have been suggested to delineate a subgroup of patients [74]. Thus, cholinergic abnormalities appear to be involved in the disease and perhaps more so in subsets of patients.

Finally, some of the evidence implicating dopamine in psychosis is derived from psychomimetic effects of amphetamine, a substance that causes the release of catecholamine

stores, including noradrenaline. Combined with adrenergic antagonism of newer APDs, this may indicate noradrenergic involvement in patient symptoms [75, 76].

1.2.4.4 Neurotransmitters and patient treatment

Currently, the primary treatment strategy for schizophrenia is pharmacological. All compounds target neurotransmitter receptors. Conventional antipsychotics can be divided into two classes, first-generation or "typical" antipsychotics and second-generation "atypical" agents.

Typical antipsychotics primarily target D2-receptors, are effective at ameliorating positive symptoms in a majority of, but not all patients, and generally do not improve other symptoms [38]. Atypical antipsychotics are equally efficacious but produce less extra pyramidal (motor) side effects than typical APDs [77]. D2-receptor blockade in the striatum results in decreased activation of medium spiny neurons (MSNs), and inhibition of the thalamus [78]. D2-receptors are present post-synaptically (on MSNs and cholinergic interneurons). They also exist as presynaptic autoreceptors or heteroreceptors (for example, on corticostriatal inputs) and negatively regulate neurotransmitter release [79-82]. Different receptor isoforms may be involved at these different synaptic locations (long and short forms, respectively) [83].

Development of newer antipsychotic agents has been motivated by the need for better management of negative and cognitive symptoms, and has targeted other neurotransmitter systems. Modulation of glutamatergic transmission has been attempted using NMDAR co-agonists (D-cycloserine or D-serine) [84], and also using metabotropic glutamate receptor (type 2/3) agonists which reduce presynaptic glutamate release [85]. Cholinergic transmission has also been targeted (reviewed in [68]). For example, a selective muscarinic receptor agonist recently studied appears to improve symptoms and some aspects of cognition [86], and one trial of a nicotinic receptor agonist suggested improvement in negative symptoms at the high treatment

dose, although this agent also acts at 5-HT3 receptors [87]. Use of a drug that increases cholinergic transmission by several mechanisms (galantamine), in conjunction with antipsychotic treatment, may improve aspects of cognition, though the reported effects differed between studies (reviewed in [68]). The use of agents modifying GABA transmission is less clear, although it has been suggested that GABA(A) agonists selective for the alpha-2 receptor subunit might improve cognitive symptoms [60].

1.2.5 Connectivity

Aberrant brain connectivity has been postulated to account for many patient symptoms [55, 88-91]. In patients, functionally connected brain regions may not operate in concert. This is illustrated by functional brain imaging studies, where patients and control subjects recruit different brain areas when performing the same cognitive tasks [92-95]. Disconnectivity in the brain can occur in at least two different ways – at the synaptic level, with alterations in synaptic function, or via compromised anatomical connections between brain regions [91].

1.2.5.1 Synaptic connectivity

Synaptic connectivity refers to the transmission and modulation of signals from one neuron to the next, across the synapse. Pre- and post-synaptic neuronal molecules are involved, including those necessary for neurotransmitter release (for example, the SNAREs, calcium channels, transmitter synthesis enzymes), neurotransmitter detection (receptors), synapse stabilization (for example, active zone proteins, post-synaptic density components and trans-synaptic molecules) and modulation of release (presynaptic receptors, signaling proteins) [96, 97]. In addition to neurons, astrocytes are found at the synapse and can respond to and modulate synaptic signaling

[98]. Several of these synaptic elements have been implicated in schizophrenia, suggesting that brain dysfunction may occur at the level of the synapse.

Gene expression studies in post mortem brain of schizophrenia patients have identified gene groups involved in synaptic function. The first large post mortem gene expression study comparing schizophrenia and control subjects identified reductions in expression of presynaptic genes as well as RGS4, a GTPase which modulates some post-synaptic receptors [99]. The precise combination of presynaptic genes that were down regulated varied between individual patients, suggesting that neurotransmitter release is disrupted, but a single molecular abnormality is not causative [99]. N-ethylmaleimide sensitive factor (NSF) (essential for vesicle fusion and recycling) and synapsin II (involved in vesicle tethering), however, were downregulated in a majority of samples [99-101]. A recent study by Maycox *et al*. also identified an overrepresentation of neurotransmission-related gene abnormalities in schizophrenia [102]. A number of other studies have identified expression changes in individual genes or gene families associated with synaptic function, including neuropeptides, GABA-synthesis, GABA and glutamate receptors and vesicular transport molecules [103-106].

Post mortem histological and protein studies also implicate synapses in the pathophysiology of the disease. Reduced cortical neuropil, [107, 108], changes in spine density and morphology (reviewed in [109, 110]), altered cell numbers (as discussed earlier), and changes in mRNA and protein levels of synaptic proteins (reviewed in [111-113]) all point to synaptic dysfunction.

1.2.5.2 White matter

Regional connectivity in the brain depends on the white matter tracts that link different regions together and consist of projecting axons ensheathed by myelin-producing oligodendrocytes.

Altered functional connectivity between brain regions in schizophrenia has prompted study of the physical connections between them. Evidence of white matter and oligodendrocyte abnormalities include alterations in myelin-related gene and protein expression in both grey and white matter, reduced oligodendrocyte numbers and metabolic abnormalities [106, 114-117], although changes may not be the same in all brain regions [118, 119]. *In vivo* imaging techniques that assess the integrity/organization of white matter lend support to post mortem findings [120]. White matter deficits have been identified in tracts that connect the cortex with the anterior striatum in schizophrenia [118, 121] and white matter integrity in the brain may be related to cognitive function [119].

1.2.6 Brain areas affected

Given the heterogeneity and number of patient symptoms, it is unlikely that a focal brain lesion can account for the entirety of the disorder [122]. Structural and functional brain imaging, together with anatomical correlates of patient symptoms suggest that several brain regions are involved.

Alterations in brain activity, volume or shape have been reported in patients and have implicated temporal, limbic and prefrontal areas, as well as subcortical brain regions, in the disease [123-127]. In addition, activity in these regions (either at rest or while performing cognitive tasks) has been associated with different types of patient symptoms [92, 93, 124, 128]. Symptoms of schizophrenia involve brain circuitry that includes the striatum; this is supported by functional brain imaging and by the fact that neuropsychological deficits in schizophrenia patients can resemble deficits seen in humans with focal lesions affecting fronto-striatal circuitry [88, 93, 129-132]. Thus, several lines of evidence suggest that multiple cortical and subcortical brain regions are involved in patient symptoms.

1.2.6.1 Rationale for studying the striatum

By virtue of its connectivity, the striatum is implicated in positive, negative and cognitive symptoms of schizophrenia. The striatum may also be a point of convergence for connectivity-based, neurochemical and neurodevelopmental theories of schizophrenia [133].

Many patient symptoms can be attributed to dysfunction of various parts of the striatum [134, 135]. Disruption of cognitive functions subserved by fronto-striatal circuitry occurs if the striatum is lesioned in animals [61]. In humans, diseases that affect the striatum illustrate the behavioural and cognitive consequences of striatal lesions. For example, personality changes such as emotional lability observed with orbitofrontal lesions, are also observed in Huntington's disease when medial caudate is involved. Apathy/reduced motivation is seen in Parkinson's and Huntington's patients [88, 130].

In schizophrenia, abnormal ventral striatal activation, determined by fMRI, is associated with negative symptoms [95, 124], higher anhedonia symptoms when presented with emotional stimuli [136], and when loss was avoided during a monetary reward paradigm [137]. Abnormal activity in the caudate may underlie psychomotor symptoms [92] and cognitive performance [138], while changes in striatal shape have been associated with negative symptoms [126]. A site of dopaminergic projections, PET imaging has found increased dopamine transmission in the striatum, which may be related to positive symptoms in patients [139-142].

Post mortem studies of the striatum in schizophrenia have been fewer, but several demonstrate disease-associated abnormalities, including lower cell numbers [143], lower cholinergic interneuron density [144], and alterations of neurotransmitter-specific markers [110]. Ultrastructure studies indicate increased dopamine inputs and glutamatergic terminals to subsections of the striatum in schizophrenia, which may be related to treatment response or patient symptoms [145-147].

The striatum contains a high concentration of dopamine receptors [148], targets of current pharmacological treatment of schizophrenia. Structural brain imaging studies suggest that plasticity in the striatum may be important for patient treatment. Striatal volumes are lower in medication-naïve patients, but treatment with antipsychotic medications results in increased basal ganglia volume (including the caudate) [133, 149]. Volume increases in some striatal regions (putamen and globus pallidus) may occur with typical, rather than atypical antipsychotics [150].

Structural, functional, neuropathological, neurochemical, anatomical, clinical and cognitive lines of evidence support investigation of the striatum in schizophrenia, in efforts to better understand patient deficits as well as treatment-mediated improvements.

1.2.6.2 Striatal anatomy

The striatum forms part of the subcortical structures of the basal ganglia. In primates it is composed of the caudate, putamen and nucleus accumbens (Figure 1.1). The GABAergic medium spiny projection neurons (MSNs), constitute ~ 75% of the cells in the human striatum [110]. Several different types of local interneurons have also been identified and include cholinergic cells as well as several types of aspiny GABAergic interneurons differentiated by the presence of different calcium binding proteins or co-transmitting neuropeptides [82, 151]. In contrast, the majority of synapses (~80%, estimate in rats) in the striatum are glutamatergic corticostriatal and thalamostriatal afferents, as cortical information converges and is integrated in the striatum [82, 152]. Dopaminergic afferents arrive from the substantia nigra pars compacta (SNc) and the ventral tegmental area (VTA) [153]. Additional afferent sources include the hippocampus, amygdala, raphe nucleus, subthalamic nucleus, pedunculopontine nucleus and locus coeruleus [151]. Striatal efferents terminate in the globus pallidus external segment,

internal segment and substantia nigra pars reticulata, ultimately affecting cortical functioning via the direct or indirect basal ganglia pathways [82]. A final level of anatomical organization in the striatum is delineated by the pattern of μ-opioid and acetylcholinesterase staining, the former delineating patches (also called striosomes), the later staining the striatal matrix, resulting in a histological "mosaic" that is visible in both rodent and primate preparations [154, 155].

The striatum has been functionally subdivided into sensorimotor, associative and limbic regions (Figure 1.1). These subdivisions reflect the ventromedial to dorsolateral topographic organization of corticostriatal inputs proposed to underlie distinct parallel processing pathways in human brain [88, 129, 134, 135]. Most relevant to the symptoms of schizophrenia are the limbic and associative striatum, consisting of the nucleus accumbens, ventral caudate and to some extent the dorsal regions of the anterior caudate head in primates, which form parts of cortical-striatal-thalamic loops important for attention and motivation, emotion and social cognition (i.e. emotional perception and theory of mind), and executive functions, respectively [88, 89, 130, 131].

The synaptic, cellular and anatomical organization of the striatum suggests that striatal dysfunction in patients could be the result of abnormal synaptic connectivity and impaired neurotransmission of one or more types of transmitters implicated in the disease.

1.3 SNARE proteins

The soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) are a family of proteins classified by the presence of a conserved 60-70 amino acid SNARE motif [156]. They are involved in vesicle fusion events in various parts of the cell [157]. There are four major classes of SNAREs (Qa, Qb, Qc and R) delineated by the central amino acid residue of the SNARE motif (glutamine or arginine) [158].

To function in membrane fusion events, different SNAREs are present in opposing membranes. Protein-protein interactions between the SNAREs produce a highly structured parallel four-helix bundle containing one of each SNARE motif (Qa, Qb, Qc and R) [159, 160]. This SNARE complex brings membranes close together, facilitating fusion [156].

There are 36 mammalian SNAREs, and while promiscuous interactions between different SNAREs can occur *in vitro* [161], SNARE pairings appear to be specific for different fusion events *in vivo*, including neurosecretion [156, 162, 163]. The neuronal SNAREs include synaptosome-associated protein of 25 kDa (SNAP-25), syntaxin-1 and vesicle-associated membrane protein (VAMP; also known as synaptobrevin), Figure 1.2, and are highly conserved between species [164-167]. There are two isoforms of each neuronal SNARE protein in humans (and in rodents), Figure 1.3. Syntaxin-1 is found at the presynaptic membrane, anchored via a C-terminal transmembrane domain. During SNARE complex formation, it contributes one Q-SNARE domain. Syntaxin-1 also possesses an N-terminal three-helix domain (H_{abc}) that is involved in regulating its availability for SNARE interactions, Figure 1.3A. SNAP-25 contains two Q-SNARE domains and is associated with the plasma membrane via four palmitoylated cysteine residues, Figure 1.3B. Both syntaxin-1 and SNAP-25 are distributed in neuropil as well as in axons [168, 169]. At the presynaptic membrane, they cluster in cholesterol-rich membrane microdomains, which define vesicle fusion sites and may also contain calcium channels and reuptake transporters [170-172]. Finally, neuronal VAMP is associated with the vesicle via a C-terminal transmembrane domain connected to an N-terminal SNARE motif, Figure 1.3C [166, 173, 174].

1.3.1 SNARE functions in the brain

Chemical transmission at synapses occurs in response to an action potential. Membrane depolarization results in the opening of and calcium influx through membrane channels, resulting in neurotransmitter release. At classical synapses (containing neurotransmitters such as GABA and glutamate) neurotransmission occurs at specialized sites on the axon – presynaptic active zones. Prior to fusion, vesicles need to be filled with neurotransmitter, localized to active zones, docked and primed at the plasma membrane. After vesicle fusion, the vesicle must be recycled [175]. A large number of presynaptic molecules are involved at various stages of synaptic transmission [96].

Genetic studies in humans provide evidence of the importance of SNAREs to human health and brain function. Genetic variation in the form of single-nucleotide polymorphisms (SNPs) has been reported within and around each SNARE gene in humans; the SNP database curated by NCBI contains over 700 SNPs in *SNAP25* alone, the vast majority occurring in non-coding regions of the gene. *SNAP25* SNPs have been associated not only with schizophrenia, but also with ADHD, human intelligence/cognition and most recently neuroticism [176-179]. *STX1A* SNPs have been associated with schizophrenia, as well as migraine, high-functioning autism and type II diabetes [180-184]. SNPs in *VAMP2* have been associated with bipolar disorder in a German sample, but not in a replication sample [185].

SNARE knock-out (KO) animals illustrate the importance of SNAREs to synaptic function. Many of the KO models show embryonic lethality, and all exhibit abnormalities of stimulus-evoked neurotransmitter release or synaptic plasticity (Table 1.1-3). In the absence of regulated neurotransmission, animals die from lack of respiratory muscle contraction [186], seizure activity [187] or other reasons [188]. The importance of SNAREs in synaptic plasticity is evidenced by disruption in the properties of long-term potentiation or depression seen in tissue

slices or cultured cells, as well as altered behaviour in KO animals including memory consolidation and pre-pulse inhibition (Tables 1.1-3).

Differences in the phenotypes of the various SNARE KO animals (Tables 1.1-3) suggest that, while the SNAREs work in concert during fusion events, they may also have additional functions during the synaptic vesicle cycle. A role for VAMP in vesicle priming and regulating fusion rate has been suggested, as has a role for syntaxin-1 and SNAP-25 in vesicle docking (Tables 1.1-2) [189-191].

Additional functional heterogeneity is suggested by recent speculation that not all SNAREs are present at all synapses. The Matteoli group demonstrated a lack of SNAP-25 expression at GABAergic synapses in rat hippocampus and cortex and in hippocampal cultures [192, 193]. They suggested that this difference in SNARE composition might underlie differences in transmission properties exhibited by excitatory versus inhibitory synapses in culture; similar expression heterogeneity was detected in human brain [192, 194, 195]. A subset of glutamatergic terminals was also found to lack SNAP-25 immunoreactivity [193]. The authors speculate that other SNAP-25 isoforms (such as SNAP-23, -29 or -47) might function at the synapse in such cells [193]. These alternate forms are thought to be involved in fusion events other than neurotransmission [156] and don't support vesicle fusion to the same extent as SNAP-25 [196-198]. However, given that loss of neuronal SNARE expression may induce compensatory expression of other SNAREs (for example, cellubrevin is upregulated in chromaffin cells of VAMP2 KO mice) [189] and changes in calcium channel expression can induce expression of syntaxin-1A in HEK cells [199], substitution of another SNARE, in the absence of SNAP-25, seems plausible. It is important to note, however, that studies using SNAP-25 KO animals suggest that SNAP-25 does exist in GABAergic terminals [200]. Also, the proteome of glutamatergic versus GABAergic vesicles is not significantly enriched for SNAP-25 [201]. The controversy surrounding SNAP-25 expression in different terminals might

reflect differences in tissue processing (for example, cryostat versus vibratome tissue sectioning) as well as the age of neurons and animals assessed (detection of SNAP-25 appears to decrease as GABAergic neurons mature) [193, 202], or perhaps reflects different protein conformation/epitope accessibility in the two different terminal types. In addition, N-terminal acetylation of SNAP-25 has recently been identified; this modification occurs on the second amino acid residue (alanine) with concomitant removal of the first amino acid (methoinine) [203]. It forms the epitope recognized by one of the SNAP-25 antibodies used pervasively in the literature [193, 203], and raises the interesting possibility that the lack of SNAP-25 in GABAergic terminals could reflect differential processing of SNAP-25 in different cell types. Proteomic and histological analyses of rodent synapses suggests differential enrichment of neuronal syntaxins between GABAergic and glutamatergic synapses [201, 204]. Finally, while not routinely discussed, differences in the location and kinetics of classical synaptic vesicle versus slower dense-core vesicle release may indicate differences in fusion machinery [205]. Different isoforms of another presynaptic protein, synaptotagmin, are associated with these different vesicle types, but SNARE components have not been systematically investigated [175]. The possibility that unique combinations of neuronal SNARE isoforms are involved in the release of dopamine, serotonin and neuropeptides, for example, would have consequences for the interpretation of presynaptic alterations in schizophrenia.

1.3.2 SNARE protein interactions

The core of the SNARE complex is composed of four SNARE domains. These protein interactions are highly stable, requiring both heat and SDS to disrupt them *in vitro* [206]. This thermodynamic stability is thought to overcome the free energy barrier to lipid bilayer mixing [207, 208]. SNARE complexes likely assemble in higher order structures; mathematical

modeling indicates that at least 3 SNARE complexes are needed for vesicle fusion to occur [209-211].

SNARE motifs assemble in an N- to C-terminal "zippering" fashion. A transition from loose to tight zippering of SNAREs on different membranes (*trans*-SNARE complexes) forces membranes together [212]. As membrane fusion progresses, the SNARE proteins end up on the same membrane (*cis*-SNARE complexes), see Figure 1.2 [213]. Tight SNARE zippering is important for fast stimulus-evoked vesicle fusion [214], while loose zippering may reflect the primed vesicle state [215]. Syntaxin and SNAP-25 dimers are thought to assemble in the plasma membrane and act as acceptor complexes for VAMP [216]. Pre-assembly of the dimers, and loose zippering of the *trans*-SNARE complex presumably decreases the time needed for evoked fusion [215, 217, 218].

While SNARE proteins constitute the minimal fusion machinery *in vitro* [217], the requirement for very fast vesicle fusion at central synapses, combined with a need for regulation and specificity of SNARE protein interactions at the synapse, suggests that other factors play a role in SNARE regulation and vesicle fusion. For example, synaptotagmin acts as a sensor for calcium-triggered release [219]. Prior to this, assembled SNARE complexes may be stabilized by complexins, which hold the SNARE complex until the appropriate trigger and sensor appears [220]. Munc-18 stabilizes preassembled SNARE complexes too, but may also act upstream of vesicle priming, limiting the availability of syntaxin-1 to interact with other SNAREs [221]. Additional proteins, such as tomosyn, which contain SNARE-like motifs, may help stabilize acceptor complexes by contributing a SNARE domain to an otherwise less stable SNARE composition [156]. Stabilization of intermediates may be important for functional SNARE complex formation, as off-pathway SNARE interactions (such as 2:1 syntaxin/SNAP-25 complexes) can occur [216, 222, 223]. Once fusion occurs, NSF and soluble NSF-attachment proteins (SNAP) co-factors disassemble *cis*-SNARE complexes [221, 224].

For normal synaptic transmission and SNARE function, the three SNAREs must interact. Botulinum toxins cleave within the SNARE domain of monomeric SNAREs, altering their ability to interact with each other and abolishing neurotransmission [100, 225]. The use of antibodies or SNARE protein fragments also impairs vesicle fusion [226-228]. Mutations within SNARE binding domains affect the properties of neurotransmission [215, 229].

SNARE complex assembly can reflect brain activity. Excessive brain activity, as occurs during seizures, produces a sustained increase in hippocampal SNARE complexes [230, 231], while chronic morphine administration reduces SNARE complexes [232].

Thus, SNARE protein interactions are integral for proper SNARE function in the brain.

1.3.3 SNARE protein isoforms

There are two isoforms of each neuronal SNARE protein in some organisms, including humans. These include syntaxin-1A and 1B, and SNAP-25A and B and VAMP-1 and 2 (Figure 1.3). In addition, other SNARE proteins that are ubiquitously expressed, or expressed in other cells, also exist in the brain, such as SNAP-23, SNAP-29, cellubrevin and many different syntaxins [233-235]. Isoform and SNARE redundancy is thought to account for some of the milder SNARE KO phenotypes (see references in Tables 1.1-3). Syntaxin and SNAP-25 isoforms are the focus of experiments in this thesis.

1.3.3.1 Syntaxin isoforms[1]

Syntaxin-1A and 1B are encoded by separate genes (located at ch7q11.23 and ch16p11.2, respectively, in humans), and code for proteins that are 84% identical [169, 236] (Figure 1.3A), which may play different roles in the nervous system.

[1] Syntaxin-1A and syntaxin-1B are most accurately described as paralogs. However, they are commonly referred to as isoforms in the literature and this convention has been retained throughout this thesis.

Syntaxin-1A is the only isoform in rat pituitary endocrine cells, is higher in noradrenergic versus adrenergic bovine chromaffin cells, and is enriched in perivascular [autonomic/sensory] terminals of the PNS [237-239]. In contrast, motor neurons preferentially express syntaxin-1B [238]. Cellular localization in the dorsal root ganglia and chromaffin cells differs between the two isoforms, with 1A at the membrane and 1B in cell bodies [239, 240]. In the rat CNS, both syntaxin isoforms are present in the neuropil as well as fiber tracts. Syntaxin-1A expression is lower in some layers of the olfactory bulb, in the globus pallidus, Purkinje cell layer of the cerebellum and in some brainstem nuclei, when compared to other brain regions. Syntaxin-1B immunostaining is low in other olfactory layers, hippocampal fimbria and thalamus. While isoform expression overlaps, several areas show complementary staining patterns [237]. High levels of 1B staining in the medial forebrain bundle may reflect isoform-specific involvement in monoamine transmission [237]. Other interpretations of tissue distribution patterns suggest that syntaxin-1B may be associated with fast neurotransmitter release, and 1A associated with slow [neuropeptide] secretion [241]. In addition, it was suggested that the lack of syntaxin-1A immunoreactivity in the region of GABAergic Purkinje cell terminals indicates that 1A might be preferentially associated with excitatory synaptic transmission [237]. Recent immunohistochemical studies support this, as syntaxin-1A was not detected in rat cortical GABAergic terminals [204] and is enriched more than 2-fold in glutamatergic vesicles as compared to GABAergic vesicles [201]. Syntaxin-1 isoform expression patterns are only known in rodent brain, and have not been investigated in humans.

Functional divergence between syntaxin-1A and 1B is also implied by differential binding affinities for VAMP isoforms. *In vitro* binding assays using recombinant rat syntaxin and VAMP isoforms demonstrated that syntaxin-1A has a higher affinity for VAMP-2 than VAMP-1. Syntaxin-1B/VAMP affinities varied with protein concentration, with greater 1B/VAMP-1 interactions at higher syntaxin amounts, but greater 1B/VAMP-2 affinity at lower

syntaxin concentrations [241]. Syntaxin-1 and VAMP protein sequences differ only slightly between rodents and humans (human and rat protein sequences are 95-100% identical). Whether sequence differences impact the interaction patterns described using recombinant rat proteins is unknown. SNARE protein isoform affinities have not been investigated in human brain.

The ability of one syntaxin-1 isoform to compensate for the other has not been directly tested (i.e. through over-expression studies or protein-binding studies). However, it has been postulated that the viability of some homozygous STX1A KO mice [242] (Table 1.2) may be due to functional compensation by syntaxin-1B. Their ability to compensate functionally may be limited, however, by expression patterns in the brain, given that some regions preferentially express one isoform [237].

Finally, syntaxin isoforms may be involved in different processes in the brain. Following amphetamine-sensitization syntaxin-1B but not 1A mRNA was increased in the NAc [243], while syntaxin-1B mRNA expression increased in the dentate gyrus following LTP induction [244]. Although only syntaxin-1B mRNA was measured, increased 1B expression has been associated with learning; expression changes were isolated to distinct brain regions and associated with specific learning tasks [245, 246].

Thus, differential expression patterns, SNARE protein binding affinities and response to plasticity, point to a possible divergence in function of the syntaxin-1 isoforms in the brain.

1.3.3.2 SNAP-25 isoforms

SNAP-25 isoforms result from alternative splicing of the same gene (located at ch20p12-p11.2), with alternate usage of exons 5a and 5b (Figure 1.3B) [247, 248]. The evolutionary duplication of exon 5, observed in vertebrate but not invertebrate species [247], suggests the two isoforms may have distinct roles. The two isoforms differ by only 9 amino acids (out of a total of 206

amino acid residues) (Figure 1.3B). Amino acid differences result in the rearrangment of cysteine residues thought to be involved in membrane anchoring and also occur in one of the SNARE protein interaction domains (Figure 1.3B) [247, 249]. The two isoforms have been studied almost exclusively at the mRNA level [248]. In mice, isoform expression is developmentally regulated, with SNAP-25A mRNA being predominant pre- and perinatally, and SNAP-25B mRNA expression beginning first week post-natal with levels 40-fold higher by early adulthood [250]. The pattern of mRNA changes may differ between regions, as seen within the hippocampus [251]. Expression of SNAP-25 is required for normal development and survival (Table 1.1). Animals who cannot express SNAP-25B present with seizures during early adulthood and show behavioural impairments [252], suggesting that SNAP-25A cannot compensate completely for SNAP-25B. The developmental expression pattern of SNAP-25 mRNA has been interpreted to mean that SNAP-25A is associated with synapse development and SNAP-25B may be associated with mature synaptic function [250].

In-situ hybridization studies showed isoform expression overlap in some rat brain regions, while in other areas only one isoform was detectable. For example, only SNAP-25B mRNA was detected in the caudate/putamen and globus pallidus, layer IV of the cortex, most cerebellar layers, and many brainstem nuclei. Interestingly, SNAP-25A mRNA was very strong in the substantia nigra pars compacta [251]. When overexpressed in differentiated PC12 cells, the two isoforms translocate to cell processes, although SNAP-25B is concentrated in varicosities, while SNAP-25A is expressed diffusely [250].

SNARE complexes containing SNAP-25B have increased thermal stability, and support larger exocytotic bursts when overexpressed in chromaffin cells. This is likely due to differences in interactions with modulatory proteins [249]. Increased stability of SNAP-25B containing complexes may have neurological and behavioural consequences (Table 1.1) [229, 253].

The developmental expression pattern of A and B mRNA and predominance of SNAP-25A mRNA in PC12 cells suggests a more general role for SNAP-25A in neurosecretion and axon outgrowth; SNAP-25B may mediate transmission at central synapses [250]. After hippocampal damage SNAP-25A but not B mRNA is upregulated, suggesting a role for the A isoform in synaptic remodeling and synapse development [251]. Differentiation of cultured PC12 cells is associated with an increase in SNAP-25B mRNA and protein, implicating the B isoform in the function of mature neurons [254]. Subcellular fractionation of neuroblastoma cells overexpressing the two isoforms found SNAP-25A and B to be associated with different vesicle pools; SNAP-25A eluted preferentially with lower density fractions (where small synaptic vesicles might be found) and B eluted with higher density fractions (possibly associated with large dense-core vesicles) [255]. This somewhat contradicts the idea that SNAP-25A mediates neurosecretetory events (as suggested by its predominance in neuroendocrine cells [250]), but could also be an artifact of protein overexpression and fractionation techniques.

Ample evidence exists to support differences in function between the SNAP-25 isoforms. Unfortunately, most evidence is derived from mRNA studies, and it is unclear if similar patterns of expression and plasticity-associated changes are maintained at the protein level. Since SNAP-25B knock-out mice exhibit seizure activity, impaired paired-pulse facilitation at synapses and behavioural abnormalities such as anxiety and reduced locomotion (Table 1.1) [252], it appears that SNAP-25A cannot fully compensate for SNAP-25B expression postnatally. Given that animals are viable, and lethal seizures do not start until later in life, SNAP-25A appears to be sufficient for early (and possibly basic) synaptic function. When overexpressed in SNAP-25 -/- chromaffin cells, both isoforms restore vesicle secretion. However, a larger RRP is supported by the B isoform, when compared to SNAP-25A [196]. Thus, while performing similar actions at synapses, each isoform confers slightly different properties on synaptic release.

1.4 SNAREs in schizophrenia

Given their location and function, SNARE proteins are excellent candidate molecules for studying synaptic pathology in schizophrenia. SNAREs provide a logical link between several etiological theories [256]. The importance of developmentally regulated SNARE protein and isoform expression points to a potential role of SNAREs in neurodevelopmental models of schizophrenia. Evidence for differential association of SNAREs with different neurotransmitters and brain regions in rodents (as outlined in section 1.3.3) may provide a link to neurochemical models of disease (as discussed in section 1.2.4). SNAREs are also integral to theories of synaptic disconnectivity (section 1.2.5.1). SNARE alterations in schizophrenia have been studied at the DNA, RNA and protein level, in living patients and using post mortem tissue, with abnormalities detected in many studies. A brief overview is given below.

1.4.1 SNARE proteins in schizophrenia

SNARE proteins have been quantified in patient samples using a number of different techniques (Table 1.4). In general, studies that detect disease-associated differences show lower protein levels in schizophrenia, particularly in the hippocampus and frontal cortex. However, studies of anterior cingulate cortex and CSF found higher SNAP-25 protein levels in schizophrenia (Table 1.4). Disease-associated differences in all three SNAREs in a single region have generally not been detected (Table 1.4), supporting the notion that presynaptic changes in schizophrenia are not simply due to a general decrease in synaptic density. It also appears that SNARE abnormalities can differ between even adjacent brain regions (for example, BA9 and BA10, [257]). Discrepancies between studies (for example, [258] and [259]) may be due to differences in tissue dissection in larger Brodmann areas (i.e. BA9). Some studies have been conducted in elderly patients, where age-related changes may have had an influence (i.e. [260]). The use of

different methods in various studies (such as immunoblotting, immunohistochemistry and ELISA) may also contribute to discrepancies. In addition, many brain, plasma and CSF proteomic studies have been conducted using patient samples (reviewed in [261] and [262]), however, most do not report SNARE protein data. Failure to report this information can be due to several reasons. For example, protein spots do not differ between groups and therefore are not selected for identification by mass spectrometry, or proteins are not detectable (i.e. present below the level of detection, outside of the isoelectric focusing range, poorly resolved or limited protein coverage).

Animal models have been created and used to test specific causative or mechanistic hypotheses of schizophrenia, using experimental manipulations that elicit behavioural or neurochemical changes similar to those seen in patients. Limited information regarding SNARE protein changes in animal models of the disease exists, and suggests that SNARE protein alterations seen in patients are not adequately modeled in animals. For example, neonatal ventral hippocampal lesions produce behavioural changes in adult animals including impaired pre-pulse inhibition, increased locomotion to stressors and other changes reminiscent of striatal hyperdomapinergia and cortical hypodominergia [263]. However, immunoblot analysis of SNARE and other synaptic proteins in frontal cortex of female rats with neonatal hippocampal lesions (tissue from P70 rats) showed increased levels of VAMP protein only [259]. This finding is in contrast to VAMP changes observed in schizophrenia subjects, where lower levels or no change has been found in frontal cortex (Table 1.4) [258, 259, 264]. In a second model, acute systemic administration of an NMDAR antagonist (MK801) increased SNAP-25 in the somatosensory cortex of rats [265]. One study has assessed SNAP-25 levels in parietal (sensory) cortex in schizophrenia, and found no difference between patients and controls [260], in conflict with the MK801 findings. Increased SNAP-25 expression in the MK801-treated animals is likely a response to neuronal damage [266], as this treatment damaged neurons [265].

Neurodegeneration is generally not considered a hallmark of schizophrenia, suggesting this model does not well represent synaptic changes in disease. *DTNBP1* is a strong schizophrenia candidate gene and dysbindin protein levels are reduced in post mortem brain of schizophrenia patients [267]. The *sandy* mouse carries a deletion in *dtnbp1* and does not express any dysbindin protein, and thus is a genetics-based animal model of schizophrenia [268]. SNARE protein levels were reportedly normal in these mice, although neurotransmission, assessed in hippocampal and adrenal slices was abnormal [269]. Vesicle release was slower, the RRP was smaller and vesicles were larger in *sandy* mice [269]. The *pallid* mouse, which carries a mutation in pallidin, a protein which interacts with dysbindin in the BLOC-1 complex, also exhibits reduced dysbindin levels, but SNAP-25 and syntaxin-1 protein levels appear normal [270]. Studies in cell cultures, however, had previously suggested that SNAP-25 expression could be affected by dysbindin expression [49]. Thus, modeling these genetic and mechanistic aspects of the disorder does not appear to replicate SNARE protein alterations seen in post mortem patient samples. This is not surprising, given that schizophrenia is postulated to be a polygenic disease, with multiple genetic as well as environmental factors contributing to disease susceptibility. In order for an animal model to reproduce behavioural and molecular changes seen in patients, it may be necessary to combine several animal models together. Recently, a gene-environment interaction has been demonstrated in the *blind-drunk* mouse (Table 1.1). Prenatal stress combined with a mutation in the SNAP-25 gene enhanced behavioural abnormalities such as impaired pre-pulse inhibition and abnormal social interaction, supporting the use of the *Bdr* mouse as a schizophrenia model [253]. While SNAP-25 protein levels were normal, cortical slice electrophysiology showed spontaneous neurotransmission to be less frequent, while evoked neurotransmission showed abnormal plasticity (Table 1.1 and [229]). Failure of animal models of schizophrenia to reproduce SNARE protein abnormalities seen in patients suggests that post mortem studies may be more informative.

1.4.2 SNAREs at the mRNA level in schizophrenia

The first large gene-expression study conducted in post mortem brain revealed perturbations in presynaptic molecules in schizophrenia [99]. Only a few recent studies have focused on SNARE gene expression, but lend support to this finding. In a study of elderly subjects, higher SNAP-25, syntaxin-1A and VAMP-1 mRNA levels were detected by RT-PCR in temporal cortex of 'younger '(< 79.5 years) schizophrenia subjects, while levels were lower than normal when subjects were older still [271]. VAMP-2 mRNA was higher in schizophrenia subjects, irrespective of age, although this was attributed to non-neuronal expression of VAMP-2 [271]. Northern blotting of prefrontal tissue in an independent study, however, did not find differences in SNAP-25 mRNA levels in schizophrenia, despite a decrease at the protein level [272].

Recent gene expression studies have not identified individual neuronal SNARE mRNA expression changes, but have implicated the vesicle cycle, presynaptic proteins and neurotransmission-related gene families [105, 273, 274]. Results of one study identified downregulation of VAMP-2 expression in the anterior prefrontal cortex among the perturbations [102]. Gene expression studies have been conducted in a number of brain regions (discussed in [273]), but predominantly in cortex and hippocampus. At least one gene expression study has included striatal regions, and while the number of differentially expressed genes in schizophrenia in the caudate outnumbered that of the dlPFC, the identify of these genes was not catalogued [275]. SNARE related gene expression differences in the striatum would be of great interest. It is unclear whether the paucity of SNARE gene expression information in the literature represents minimal perturbation of mRNA expression of these genes, or whether the negative findings are the result of the analysis methods used. For example, SNARE protein level changes in schizophrenia are often less than 50% relative to control subjects (Table 1.4). Some mRNA quantitation methods (such as microarrays) set thresholds above this (for example, only 2-fold

signal differences are considered). Subtle changes in mRNA levels may thus never be detected or reported. In addition, many studies have employed custom microarrays, which may not contain probes for the SNARE genes at all [276].

1.4.3 SNAREs at the DNA level in schizophrenia

Many linkage and association studies have been conducted in efforts to understand the genetic contributions to disease pathology [51]. SNARE genes have been among the candidates studied.

A recent Irish family-based study found an association between disease and several single-nucleotide polymorphisms (SNPs) within the SNAP-25 gene, some of which appear to be associated with prominent psychotic symptoms [176]. Association was tested using several different definitions of schizophrenia, and several SNPs showed association irrespective of definition stringency. Several of the associated SNPs were predicted to perturb transcription factor binding sites, suggesting a mechanism by which the associated variation might affect SNAP-25 expression in schizophrenia. SNP association, however, was not replicated in a second [case-control] sample set [176]. Using an initial screening set of 377 Japanese patients and an equal number of control subjects, modest association between schizophrenia and two *SNAP25* SNPs were also identified by Kawashima *et al* [277]. While 7 of the 29 *SNAP25* SNPs genotyped in this study were the same as in the Irish study (4 of which had shown some association with schizophrenia in the Irish sample), none of these were significant in the Japanese population. The nominal association was not replicated in a second set of samples [277]. An earlier family-based study of Canadian and Portuguese trios did not detect an association between *SNAP25* and schizophrenia [278], nor did a case-control study of a tandem repeat element in the *SNAP25* promoter in Japanese subjects [279]. SNAP-25 has also been implicated in the etiology of ADHD, a disorder that occurs at higher frequency amidst youth at-

risk for schizophrenia [280]. Recent evidence suggests that alleles associated with a lower risk of ADHD may be associated with a higher risk of schizophrenia [177]. Finally, two studies have shown an association between *SNAP25* SNPs and weight gain during antipsychotic treatment. Both studies genotyped the same SNPs, but different SNPs were associated with weight gain in the different sample sets [281, 282]; SNPs were also associated with treatment response in only one of these studies [281]. Both the ADHD low-risk alleles and treatment-associated SNPs were found in the 3'UTR of *SNAP25*.

Recent studies indicate that genetic variation in *SNAP25* may influence behaviour and cognition in general. In a genome-wide association study of personality dimensions in a large Sardinian cohort, a SNP within intron 1 of SNAP25 showed a strong association with neuroticism [178]. Follow-up studies in American and Dutch cohorts found a similar trend, but did not reach statistical significance [178]. Several association studies link *SNAP25* to cognitive function. Different SNPs within intron 1 of *SNAP25* have been associated with IQ in a family-based study of two Dutch cohorts [179]. Analysis of the same sample sets using additional SNPs within the associated region indicated significant association of four SNPs and IQ. Interestingly, different haplotypes were associated with IQ in adult versus child cohorts, reflecting the complex nature of this trait [283]. In schizophrenia cohorts, it appears that *SNAP25* variation also affects cognition, despite disease and treatment. Golimbet *et al*. recently assessed the association of one of the 3'UTR SNPs with memory and executive functions in patients with psychosis (primarily schizophrenia and schizoaffective patients) as well as subjects with a family history of psychosis. The same genotype was associated with poorest task performance in all subject groups, although subtle differences between performance and genotype existed when each task was considered separately [284]. This was interpreted to mean that the *SNAP25* gene influences cognition independently of disease [284]. A study of German patients initially off medications then followed during 12 weeks of treatment with atypical APDs, found a different 3'UTR SNP to be

associated with better global cognitive indices and neurocognitive test performance over the three time points measured [285]. Thus, it appears that association of *SNAP25* with cognition may also occur independently of patient treatment. Interestingly, the SNPs associated with cognition in these two studies are also the SNPs that were associated with disease treatment effects discussed above. Treatment respose and weight gain were not reported in the association studies of cognition in patients.

Other SNARE genes have also been implicated in schizophrenia, albeit through different avenues. Meta-analyses of genome association studies have identified a region of ch16p as a possible schizophrenia linkage region; this genomic region contains the gene for syntaxin-1B (16p11.2) [286, 287]. Copy number variation (CNV) is another mechanism by which genome-level variation might be associated with disease and *STX1B* is located proximal to a CNV region recently associated with schizophrenia [288, 289].

The syntaxin-1A gene is located amidst the genes deleted in Williams-Beuren syndrome [290], a disorder associated with cognitive impairment [291, 292]. Variation in an intronic region was associated with schizophrenia in North American samples, in both family-based and case-control sample sets [180]. However, a recent association study of SNARE-gene SNPs did not detect statistically significant associations between *STX1A* markers and schizophrenia in a Japanese cohort. This same study also looked at SNPs in the *VAMP2* gene, and did not find any significant association with disease [277].

1.4.4 SNARE protein interactions in schizophrenia

SNARE complexes have been less studied in schizophrenia, despite their functional importance. Two studies measured SNARE protein-protein interactions in human brain tissue and came to different conclusions. In the first, SNARE complexes were higher in anterior frontal cortex

homogenates from schizophrenia subjects who had died by suicide as opposed to those who died of other causes [264]. However, a recent study of syntaxin-1 phosphorylation state in post mortem samples found that reduced phospho-syntaxin-1 in schizophrenia might contribute to reduced SNARE protein interactions [293]. Finally, the *blind-drunk* mouse (*Bdr*) contains a mutation in the SNAP-25B specific exon resulting in an amino acid substitution that increases the stability of the SNARE complex (Table 1.1). The phenotype associated with this biochemical change in the SNARE complex includes anxiety, apathy and impaired sensorimotor gating [229]. When subject to prenatal stress these behavioural abnormalities are amplified [253], suggesting that SNARE protein interactions may be modulated by developmental stressors that increase risk of schizophrenia in humans, producing behavioural deficits similar to those seen in patients.

Presynaptic molecules known to modulate SNARE protein interactions have been studied in schizophrenia in several brain regions. These include the complexins (1 and 2), munc-18, septin-5 (CDCrel-1), synaptotagmin and synaptophysin. Two of three studies of hippocampal complexins found the balance between complexin-2/1 protein or mRNA expression altered in at least some subregions in schizophrenia [294-296]. This may be associated with cognitive deficits [296]. Other studies found complexin-1 protein to be decreased in prefrontal cortex (BA9 and/or BA10) [297], complexin-1 and -2 proteins unchanged in anterior cingulate (~ BA24) [298] and complexin-2 reduced in cerebellar granule (glutamatergic) cells in schizophrenia [299]. Because complexin-1 and 2 are associated with inhibitory and excitatory neurotransmission, respectively, interpretation of data has included the idea that select terminal types are affected in patients [53, 298]. Complexins are thought to act as clamps, stabilizing the SNARE complex prior to vesicle fusion [220]. Reductions in complexin levels might therefore reflect reduced SNARE complex stability in some brain regions, potentially only in subsets of terminals. Munc-18/STXBP1 interacts with syntaxin in two different ways, preventing syntaxin

interactions, but later stabilizing the SNARE complex [300]. Increased munc-18 levels in dlPFC in schizophrenia [301] could result in less efficient formation or increased stability of SNARE complexes. Septin-5 is located at ch22q11. Deletion of this chromosomal region results in velocardiofacial syndrome and increased risk of schizophrenia [302]. Septin-5-syntaxin-1 protein interactions have a negative effect on vesicle exocytosis [303] and septin-5 KO animals show reduced anxiety behaviours and increased pre-pulse inhibition, suggesting that septin-5 modulation of SNARE protein interactions may be related to patient symptoms [304]. Two-dimensional gel electrophoresis of the dlPFC detected increased protein expression of two septin-5 isoforms (identified by mass spectrometry) in schizophrenia samples relative to controls [305]. At least three synaptotagmin isoforms function as calcium sensors during neurotransmitter release [175, 219, 221]. Increased synaptotagmin-1 mRNA was found in temporal cortex of elderly schizophrenia subjects [271]. Synaptotagmin-11 has been identified as a potential schizophrenia candidate gene, although whether this has significance for synaptic function is unclear [219, 306]. Finally, synaptophysin is present in the vesicle membrane near VAMP and, at least in adult synaptic vesicles, the two proteins interact [307]. This interaction may facilitate SNARE protein interactions [308]. Synaptophysin levels have been measured in many post mortem studies at the protein and mRNA level, in many brain regions, and have produced varying results (reviewed in [111, 256]).

Despite the paucity of SNARE protein interaction data in schizophrenia, indirect evidence from alterations in SNARE-modulating proteins supports the idea that SNARE complexes function abnormally at the synapse in the disease.

1.4.5 SNARE isoforms in schizophrenia

Despite evidence of SNARE isoform functional divergence and developmental expression, the involvement of SNARE protein isoforms in schizophrenia is largely unknown. Protein studies have not distinguished between protein isoforms, using antibodies that detect both forms of each protein. One study did resolve syntaxin as two bands by immunoblotting, although statistically significant differences between schizophrenia and control subjects were detected only when both protein bands were added together, and the identity of the two bands was not confirmed [258]. Separate mRNA probes for syntaxin-1A and 1B and VAMP-1 and VAMP-2 exist in gene expression microarrays, but comparisons between probes have not been reported. SNAP-25 probes may not differentiate between the two isoforms. Genetic association of SNAP-25 has thus far involved SNPs in the 3'UTR and therefore does not distinguish between the isoforms. However, given that the genetic variation occurs in a regulatory region of the gene, these SNPs could potentially affect transcription, splicing and translation. Evaluation of putative splice sites or splicing factor binding sites in the regions of schizophrenia-associated SNPs may shed light on the possible involvement of aberrant SNAP-25 isoform splicing. Association or linkage studies of syntaxin-1B or VAMP-1 have not been reported in schizophrenia cohorts.

1.4.6 Effects of antipsychotic medications on SNAREs

Antipsychotic drugs (APDs) used to treat schizophrenia block receptors in the brain, altering brain activity. Some evidence that APDs modulate SNARE protein expression or protein-protein interactions exists.

Effects of antipsychotics on SNARE expression have been investigated using animals. Increases in hippocampal SNAP-25 protein with typical antipsychotic administration (haloperidol 1 mg/kg, chlorpromazine 10 mg/kg, trifluoperazine 6 mg/kg, 21 days) [309], no

changes in SNARE proteins in prefrontal cortex (haloperidol 1 mg/kg, chlorpromazine 10 mg/kg, trifluoperazine 7 mg/kg, 21 days) [297], increases in all SNARE mRNAs with a typical and atypical antipsychotic in prefrontal cortex but not striatum (haloperidol 0.2 or 0.5 mg/kg, clozapine 8 or 20 mg/kg, 26 days) [310] and decreases in syntaxin-1A and VAMP-2 mRNA in NAc (25 mg/0.5 ml/kg haloperidol decanoate, 28 days) [311] have been reported. APD-associated changes in SNARE proteins have not been assessed in human striatum, a major site of drug action. However, ultrastructural studies suggest that APDs normalize the increased density of corticostriatal synapses in schizophrenia subjects [312].

The effects of APDs on SNARE protein complexes are also unknown. Psychoactive drugs and brain activity can modulate SNARE complexes, including morphine and seizures [230, 232], therefore it is likely that APDs can modify SNARE protein interactions as well. This could occur indirectly via modulation of brain/neuron activity through post-synaptic receptor binding, via presynaptic receptor antagonism, or via second messenger cascades that result in post-translational modification of SNAREs. Alternatively, APDs or their metabolites could bind SNAREs directly. A number of SNARE-modulating compounds have recently been identified, which affect the ability of SNAREs to interact with each other [313-315]. Modulation of SNARE complex stability and/or specific SNARE protein isoforms may present novel presynaptic treatment targets.

1.5 Post mortem protein studies in schizophrenia

The use of post mortem tissue in schizophrenia research is invaluable given that disease etiology remains elusive, among other reasons. However, its use, as with any experimental system, brings unique challenges to study design and analysis.

1.5.1 Advantages of studying proteins in human brain

Post mortem human brain tissue allows researchers to study a "snap-shot" of the human brain experiencing the disease state of schizophrenia. It incorporates the disease in its entirety, irrespective of the limits of our understanding of its underpinnings. It allows for the direct qualitative and quantitative assessment of parameters involved in brain function (molecular, cellular and anatomical), in contrast to *in vivo* techniques such as brain imaging that evaluate the brain indirectly [316]. In post mortem tissue, the precise molecules that are involved in disease in the brain might be identified and their alterations quantified. This can be performed in distinct anatomical structures, leading to new hypotheses of how molecular abnormalities affect brain function.

Schizophrenia is inherently a human disease – it involves the disruption of human capacities such as perception of self and others and relies on language and self-report for its diagnosis. Thus, while aspects of the disease can be modeled in other systems, disease complexity and biological variation is not incorporated into such models. Existing animal models are based on etiological hypotheses, candidate genes or schizophrenia risk factors and model validity is partly assessed by determining how well the model reproduces behavioural or cognitive deficits observed in humans with the disease (for example, pre-pulse inhibition or ventricular enlargement), although many of these deficits are not unique to schizophrenia [317]. In the absence of a clear understanding of disease etiology, animal models of schizophrenia remain valuable tools for testing etiological hypotheses, rather than accurate disease models. Their validity remains in question due to an inability to diagnose an animal using DSM-IV or ICD criteria. Finally, schizophrenia is a disease of the brain, and therefore must be studied in the brain [316]. Human and other animal brains share structural similarities, however, some brain circuitry is species-specific. Brain circuitry underlying negative and cognitive symptoms in

patients is different in non-human primates [61]. Use of human tissue results in the study of the complete disease entity and avoids difficulties associated with translating results between species.

The study of proteins as opposed to DNA or mRNA may also be advantageous. Practically, mRNA can be very unstable during laboratory manipulations of tissue samples [271], and can be more sensitive to tissue conditions than some proteins [318]. Discordance between mRNA and protein expression levels is known to occur and mRNA expression may not be directly proportional to the amount of final protein product [319-321]. Proteins represent the end product of gene expression, and therefore reflect the molecular and functional consequences of alterations of precursor molecules, whether the perturbation occurs at the DNA, RNA or protein level.

1.5.2 Limitations of using post mortem human brain

Post mortem brain tissue represents the disease state. However, the use of human tissue comes with many limitations as well. Because post mortem sample sets usually represent a naturalistic/observational (rather than experimental) study design, variation in many sample characteristics and reliability of sample information must be taken into consideration. Prospective participant recruitment provides an opportunity for antemortem diagnostic interviews [322], but has traditionally involved older, chronically hospitalized patients, limiting the degree to which results obtained from such samples can be extrapolated to different [in particular, community] patient populations [316]. Without the ability to assess the patient directly, post mortem diagnosis is greatly improved by the use of multiple information sources. Systematic evaluation of patient chart information can be achieved using tools such as the diagnostic evaluation after death (DEAD) [323]. Collateral information is often obtained

through a structured interview with family members or other personal informants, using tools such as the psychological autopsy (PA) [324]. Post mortem diagnosis of schizophrenia shows good consensus between multiple raters (Kappa coefficients indicate moderate to excellent interrater agreement), and suggests that post mortem chart review maybe as reliable as live patient interviews [323-325]. However, reliance solely on chart review may result in biased diagnoses and for samples collected prior to the DSM-IV, may reflect differences in diagnostic practice in different centres [3, 326-328]. For samples studied in this thesis, a modified version of the DEAD as well as a PA was conducted in addition to chart review. A consensus diagnosis was reached by several raters or else additional information sought to facilitate the diagnosis.

Brain collections often rely on samples identified by medical examiners' offices [329]. This may not identify patients who die of natural causes in the care of family or friends, or deaths that are not investigated by the medical examiners office, and may therefore over-represent suicide victims, severely ill/refractory patients and individuals with less family/social supports [325]. In a similar manner, 'control' subjects identified through the medical examiners office may not represent the general population. In addition, due to the length of time needed for the diagnosis of schizophrenia, tissue sets are less likely to include first-episode patients or those who are early in their illness, and therefore represent primarily chronic illness states. Collection of samples in multicultural centres can also present unique challenges for specific types of studies. For example, genetic studies may require more detailed ethnicity information or genetic analysis to account for population stratification [330, 331]. Whether such sample characteristics are important in post mortem protein studies remains unknown. The samples studied in this thesis were all obtained in Macedonia, a country where autopsy is mandatory for all deaths. This increases the likelihood that the patient and comparison subjects are representative of their respective populations. Furthermore, the Macedonian population is primarily Caucasian, with most individuals of Macedonian or Albanian descent. Within sample groups, natural biological

variation will also contribute to heterogeneity of findings. Differences in illness course, illness symptoms, health, lifestyle and co-morbid conditions exist between each person and could impact findings. In the current sample set, individual patients had different sub-types of schizophrenia. Finally, post mortem tissue is obtained at a finite time point of the subject's life and disease course, potentially increasing biological variation.

Other sample characteristics such as gender, age, post mortem interval (PMI), agonal state, brain pH, sample storage time and medications are potential confounding factors in post mortem studies [316, 332, 333]. Their effects in schizophrenia research have been greatly studied in the context of gene expression studies, which have suggested that all of these factors can have an influence on gene expression, including some of the genes implicated in this disease [310, 331, 334, 335]. Impact of these factors on protein studies is less documented.

The post mortem interval (PMI) is the time between death and tissue collection and storage. After death, brain encasement may help preserve DNA, mRNA and protein but this is likely to depend on the specific molecule in question, and the length of PMI [259, 332, 336]. For example, some PMI-associated changes might occur rapidly, ceasing at later time points and changes may differ between brain regions [316, 336]. Sampling within a narrow PMI interval might limit PMI effects on results.

Brain pH appears to be linked to agonal state, with slower modes of death and hypoxia producing lower pH measures [332, 337, 338]. Agonal state/pH associated gene-expression changes appear to involve specific transcripts and not generalized mRNA degradation [337]. Correlations between pH-associated and age-associated gene-expression changes have been noted, illustrating the increased likelihood of prolonged death in older subjects and the complexity in attributing sample variation to specific sample characteristics/confounders [334, 335]. Less information is known about pH/agonal state effects on protein levels, although

available studies indicate that protein products of some affected mRNA are stable at different pH [318].

The potential influence of sample characteristics on protein study results can be minimized, or at least identified, by several approaches. The use of sample pairs matched for relevant characteristics (such as age, PMI, pH, co-morbid conditions) has been recommended [333], although it likely takes longer to assemble a matched sample set and it assumes that the factor affects protein measures in both groups equally. As an alternative, ensuring that sample groups do not differ on these variables (equal means and variances) makes it easier to statistically covary for sample characteristics [316]. Indiscriminant use of covariates, however, may over-fit the data, modeling random error rather than the effects of interest [339]. In addition, it requires the researcher to choose which variables should be covariates, and assumes they are aware of all the underlying factors that might influence results [339], which is unlikely. Selection of covariates is aided by first identifying relationships between sample characteristics and the protein measures using correlations or other measures of association [53, 340, 341], and was the approach taken in this thesis, in light of an un-matched sample set. *A priori* inclusion of covariates can also be informed by previous human or animal studies. For example Siew *et al.* specifically investigated the effects of post mortem interval on syntaxin levels, and determined that syntaxin immunoreactivity was stable up to 24 hours post mortem in deceased rats kept at 20°C or 4°C, and in human frontal cortex [336]. Results from human temporal cortex of the same study, however, did not find a similar reduction with prolonged PMI, illustrating that effects of potential covariates may not be the same in all brain regions, or with all molecules studied, which can complicate covariate use [339]. Halim *et al.* also investigated PMI effects on SNAREs using rodents. Syntaxin levels did not differ significantly over the 70 hour PMI tested, while SNAP-25 levels were significantly lower at 48 hrs, and VAMP at 24 hrs [259]. In comparison, the sample set studied in this thesis contained only one individual with a PMI

greater than 24 hours. However, caution must be exercised when drawing conclusions from simulated post mortem studies, as cooling rate, circumstances of death and body storage during the PMI may not be accurately reproduced in a lab setting. Other post-mortem studies of SNARE proteins in schizophrenia have investigated the association of protein measures with PMI and/or covaried for PMI in statistical anaysis in human samples. PMI was not significantly associated with SNAP-25, syntaxin and/or VAMP protein levels in many [260, 264, 342-344] but not all studies [345]. However, possible differences in the PMI range as well as sample collection and storage procedures mean that PMI [and other] effects should be investigated in each sample set, as has been undertaken here.

In the experiments described in this thesis, correlation analysis was used to identify potential confounding factors associated with sample characteristics. Thus for characteristics such as PMI, Spearman correlations were used to first determine if a statistically significant association with protein measures exists. If so, the primary analysis was then re-run co-varying for PMI (i.e. analysis of covariance, ANCOVA, was used with diagnosis as the between subjects factor, striatal area as the within subjects factor, and PMI as a covariate) to determine whether significant effects of diagnosis were affected by the covariate.

Finally, the presence of APDs in patient but not control samples prevents the use of APDs as a covariate. However, APD effects can be addressed by including samples from other patient groups that may have had exposure to similar medications [316] or comparing patients who were on or off medications at time of death [146]. Another approach involves modeling the effects of APD treatment in animals (reviewed in [346]). If medications produce changes in animals similar [in direction and magnitude] to the changes observed in schizophrenia patients, disease-associated changes may be confounded by patient treatment. If medications do not affect the experimental measures or do so in the opposite direction to the changes seen in patients, it is thought that the molecular alterations in patients may reflect the disease state.

Caveats associated with the study of drug-treated animals include the limitations of modeling drug effects in non-diseased systems as well as differences in drug metabolism and therefore dosage needed when administering APDs to rodents [347]. If selecting the former approaches, due to the possibility of non-compliance [316], it may be better to determine medication use at time of death via post mortem toxicological analysis as opposed to chart review [348]. The half-life of haloperidol measured in post mortem human brain is approximately 7 days; because concentrations in the brain exceed those in serum, drugs persist longer in brain, facilitating detection [349]. A final alternative is to investigate potential APD effects in the patient group alone, and this is often done by converting lifetime dosage of antipsychotics into equivalent units, creating a continuous variable for statistical analyses [350]. In addition to antipsychotic medications, other medications or comorbid substance use may also complicate post mortem studies. Substance abuse in patients occurs at a higher frequency than the general population and includes alcohol, nicotine and illicit substances including cannabis [351, 352]. These compounds can affect brain structure and function either acutely or in the long term. For example, nicotine use can improve cognition but modifies synapses [353, 354]; cannabis use has been suggested to increase the risk of psychosis [355] and affects cannabinoid receptor density in brain [356]; and alcohol abuse may reduce grey matter volume [357]. The ability to account for such effects may depend on the rate of substance use in comparison subjects. Based on some theories of how comorbid substance use arises, substance use in comparison subjects may indicate risk of psychiatric diagnosis [351], complicating the use of sample matching for such characteristics. APD effects in this thesis were evaluated in two ways – by performing similar experiments in tissue from animals treated with antipsychotic medications, and by statistical re-analysis of only patients who did not have antipsychotic medications on board at time of death.

Many of the strengths of using post mortem tissue also contribute to the limitations of its use. However, several strategies for identifying, overcoming or addressing biological variation

between individuals and variation between sample characteristics can be applied to post mortem studies to aid in interpreting results that are very pertinent to this uniquely human disease.

1.5.3 Post mortem samples used in this thesis

The following is a brief summary of the characteristics of the sample set studied throughout this thesis, in the context of the strengths and limitations of post mortem studies as discussed above (Section 1.5.1 and 1.5.2). Sample characteristics are detailed in Table 2.1. The samples were collected in Macedonia, and all subjects were Caucasian. All samples were processed in the same manner (detailed in Section 2.5.1). Groups did not differ in age or pH. Schizophrenia samples had longer sample storage times and PMI as compared with non-psychiatric comparison subjects. However, only one individual had a PMI greater than 24 hours, an unusual strength of this sample set. Indeed, some of most widely used sample sets in schizophrenia research include samples with PMIs as great as 80 hours [305, 329]. As described above, possible association of sample characteristics with experimental measures was determined using the correlation approach to identifying covariates. Prolonged death was not common among subjects, reducing agonal-state and hypoxia-related brain changes. Groups were not balanced for gender, precluding analysis of sex-effects. Information regarding specific patient symptoms or clinical measures was not available. Modest group size precluded analysis of results in the context of schizophrenia sub-types. Co-morbid alcohol use was minimal in subjects, and potential drug use was determined through toxicological analysis. Antipsychotic medications were detected in only two subjects at time of autopsy, reducing the likelihood that patient treatment accounts for our results. APD effects were investigated both statistically, and by modeling APD effects in animals. Finally, all samples were dissected by one investigator (WGH), which reduced sampling heterogeneity between subjects.

1.5.4 Methods for studying proteins in the brain

Protein quantification is often performed using antibody-based methods. Antibodies bind to specific regions (epitopes) of the protein of interest. Detection of the protein-antibody complex is then achieved directly if the detection antibody is labeled, or indirectly by using a labeled secondary antibody which binds the first one [358]. Signal-producing labels are often enzymes such as horseradish peroxidase which, in the presence of the appropriate substrate, can produce colorimetric or chemiluminescent signal that can be measured. Fluorescently labeled secondary antibodies are also used, and detection has been improved in new generations of stable fluors [359].

Immunoblotting and enzyme-linked immunoadsorbant assays (ELISA) are widely-used antibody-based assays. Sensitivity and specificity of both methods depend greatly on the specificity and sensitivity of the antibodies employed. Monoclonal antibodies afford increased specificity as they detect only one epitope of the protein, minimizing cross-reactivity, as opposed to polyclonals which detect many different epitopes [358]. Sensitivity is affected by the strength of antibody-epitope binding and partly depends on whether the assay conditions produce epitopes similar to those used for antibody generation. If the antibody detects an epitope created by the protein's tertiary structure, it may not perform well in an assay where protein is denatured (such as SDS-PAGE) or modified (such as tissue fixation). ELISAs may have a greater dynamic range in detecting synaptic protein differences as compared to immunoblotting [360]. Use of microtitre plates and robotic liquid handlers has increased the throughput of ELISAs, which facilitated the analysis of samples in this thesis. Antibody specificity is still an issue with ELISAs, but can be verified by immunoblotting. The linear range of detection is also important, regardless of the method used. It must be confirmed that the amount of antigen present in the sample does not saturate the antibody, and that the signal intensity is directly proportional to the

amount of antigen present. Detection limits and dynamic range are also affected by detection methods; enzyme-substrate detection methods is are influenced by enzyme kinetics and substrate stability [361].

Protein interactions in biological samples are commonly assayed by immunoprecipitation. This antibody-based method "fishes" out the protein of interest, and any other molecules that interact with the target [362]. This is not a very high-throughput method, as this is a heterogeneous assay that requires a number of wash steps and care must be taken to avoid loss of proteins. SNARE protein interactions can also be studied via SDS-PAGE and immunoblotting, without immunoprecipitation, owing to their stability in SDS. Under specific conditions SNARE complexes migrate as higher molecular weight bands, usually between 100-200 kDa [206, 230, 293]. The aforementioned limitations of antibody-based detection apply here as well. In addition, the exact composition of bands is difficult to discern as apparent molecular weights of non-denatured protein complexes are not accurate, and epitope masking is likely to occur.

Protein isoform measurement depends on the ability to detect isoforms as separate entities. If isoforms differ in size, they may be resolved by SDS-PAGE. However, if isoforms do not differ greatly, it can be difficult to resolve them, and even to make isoform-specific antibodies. This can be overcome by using techniques that do not rely on antibodies for detection. Mass spectrometry (MS) identifies proteins based on the fragmentation patterns of ionized proteolytic peptides. In schizophrenia research, MS has been widely used to identify proteins that are differentially expressed between groups, detected by two-dimensional gel electrophoresis [261]. Protein identification is achieved by matching the peptide fragmentation mass/charge ratio (m/z) spectra to a database of predicted spectra for known proteins. Reducing sample complexity aids in identifying and detecting proteins by MS. This is commonly achieved by pre-fractionation of the sample using methods such as immunoprecipitation, gel

electrophoresis, and/or chromatography [363]. Beyond identification, MS offers the ability to quantify peptides as well. Multiple-reaction monitoring (MRM)-MS has been widely used to quantify small molecules for decades, and has been successfully applied to proteins [364]. In protein MRM-MS, the protein of interest is proteolytically digested and a representative peptide is monitored by the MS. The selected peptide is fragmented, and one or more fragments are monitored. The amount of signal corresponding to the selected fragment is proportional to the amount of total protein in the sample. Specificity is achieved at multiple levels in the assay - peptide elution time (if using chromatography prior to MS), monitoring only a peptide of a specific m/z and monitoring a fragment of a specific m/z. While two peptides may have the same m/z, if they have different amino acid sequences, they will produce unique fragments. The MRM-MS strategy has aptly been described as the mass spectrometrist's ELISA, in reference to the level of specificity achievable by this approach [365]. Absolute quantitation is facilitated by including a known amount of internal standard that is identical to the peptide to be quantified, differing only in mass (achieved by using amino acids containing stable isotopes) so that it behaves exactly like the peptide of interest, but can be resolved due to its different m/z [366]. While MRM-MS avoids some of the limitations associated with antibody use, it carries a few of its own. MRM design may be limited by the ability to synthesize isotopically labeled internal standards, selection of an appropriate peptide to monitor (for example, avoiding easily-oxidized methionine-containing peptides), the efficiency of protein digestion and other preparation steps (for example, reduction, alkylation and peptide recovery) [367, 368].

1.6 Thesis chapter summaries and specific hypotheses

Synaptic abnormalities hypothesized to be involved in schizophrenia were investigated in several ways in the work described here. SNARE proteins were the subject of study, given their

importance for synaptic function, and prior evidence that SNAREs are altered in some brain regions in patients with schizophrenia. The primary aims of this thesis were to extend the investigation of SNARE proteins in schizophrenia to the striatum, and to identify some of the possible functional implications of SNARE protein alterations by measuring SNARE protein interactions as well as isoform levels in the same samples. To do so, methods were developed or optimized when necessary.

Chapter 2 describes the quantitation of SNARE proteins and protein-protein interactions in human striatum in schizophrenia. Three regions of the anterior striatum were subdissected and analyzed separately, based on their involvement in patient symptoms and the functional subdivisions of the striatum. These included the nucleus accumbens, ventromedial caudate (VMC) and dorsal caudate. It was hypothesized that protein levels of one or more SNARE proteins are abnormal in striatal regions involved in positive, negative and cognitive symptoms of schizophrenia. This study design also provided an opportunity to determine the distribution of SNARE proteins within the anterior striatum (limbic and associative regions) in humans, which has not been previously published. The use of a sensitive and linear indirect ELISA assay using monoclonal antibodies against SNAP-25, syntaxin-1 and VAMP allowed quantitative measurement of each protein. SNAP-25 and syntaxin-1 were lower in the VMC in schizophrenia. To determine if SNARE protein interactions were affected, a high-throughput protein-protein interaction assay was developed to measure syntaxin-SNAP-25 protein interactions (Appendix 1). It was hypothesized that SNARE protein-protein interactions would differ between diagnostic groups in the striatal areas that show protein level alterations. Syntaxin-1-SNAP-25 protein interactions were higher in the VMC in patient samples. It was also hypothesized that any group differences observed would reflect the disease state and not patient treatment. The possible confounding effect of APD treatment was assessed by statistical reanalysis of subsets of subjects, and by performing similar experiments in striatal samples of

rats treated subchronically with some of the most prevalent medications used to treat patients. Both approaches indicated that antipsychotic exposure did not account for the decrease in SNAP-25 and syntaxin-1 or the increase in SNARE protein interactions we observed in the human samples.

Chapter 3 begins to delve into the possible mechanisms underlying increased SNARE protein interactions by better characterizing the changes in syntaxin-1. If syntaxin-1 expression changes were restricted to a single isoform, additional information regarding the functional consequences and underlying causes of SNARE protein abnormalities might be gained. Syntaxin-1 isoforms (1A and 1B) were measured by quantitative immunoblotting. Syntaxin-1 isoforms were resolved as separate bands using Tris-Urea-SDS-PAGE followed by immunoblotting. To increase dynamic range and reduce variability associated with chemiluminescent detection, the stable qDot fluors were used. Creating a ratio of the two isoforms facilitated within-lane normalization. Explorations of the relationship of this ratio with sample characteristics revealed a significant negative correlation with age. The ratio of syntaxin-1A:1B was significantly lower in the striatum overall in schizophrenia. Drawing on the early studies characterizing sytnaxin-1 isoform expression in rodent brain, as well as newer studies of vesicle proteomes, these results may indicate that glutamatergic terminals in the striatum are affected more so in schizophrenia than striatal inhibitory terminals.

Chapter 4 continues the characterization of SNARE isoforms in schizophrenia by measuring SNAP-25A and B levels in the VMC. If SNAP-25 protein changes in schizophrenia are restricted to a single protein isoform, the effects on synaptic and brain function might be inferred from what is known about isoform function in model systems. Because of the high degree of similarity between the two isoforms and the fact that isoform-specific antibodies are not available, a novel MRM-based method was developed, characterized and used to quantitate SNAP-25A and B proteins in tissue and cell lysates. An isotopically labeled internal standard

was used for absolute quantification of SNAP-25 in the VMC. We developed a novel approach to quantifying A and B isoforms by constructing an external calibration curve of recombinant SNAP-25A and B. The two methods were applied to human samples in the VMC to quantify and compare the amount of SNAP-25A and B between schizophrenia and comparison subjects. We report, for the first time, that SNAP-25 protein isoform ratios in human striatum are approximately 1:1. Furthermore, SNAP-25A was significantly lower in the schizophrenia subjects. SNAP-25B was also lower, but not significantly so. Combining these findings with those from Chapter 2, it appears that lower levels of SNAP-25 in the VMC in schizophrenia are primarily due to SNAP-25A reduction. Possible functional consequences of this include increased SNARE complex stability and might explain the increased SNARE protein interactions observed in Chapter 2. In addition, the isoform assay provides a valuable tool to continue more detailed studies of SNAP-25 proteins in psychiatric illness, and in neuroscience in general, as our current understanding of endogenous SNAP-25 isoforms does not extend past mRNA expression.

1.7 Chapter 1 tables

Table 1.1 - SNAP-25 knock-out models.
Brain, synapse and behavioural alterations reported in various SNAP-25 gene knock-out animal models. Note, two versions of the SNAP-25B knock-out exist (with and without removal of the neo-gene cassette used for developing the knock-out); characteristics unique to the neo-removed version are underlined. Also, the characteristics tabulated for SNAP-25 and SNAP-25B KOs were generally derived from homozygous KO animals, while heterozygote *coloboma* and *blind-drunk* mice were studied. *Abbreviations*: PPF – paired-pulse facilitation; PPI – paired pulse inhibition; LTP – long term potentiation; EPSP – excitatory post-synaptic potential; EPSC – excitatory post-synaptic current; LVA – low-voltage activated; mEPPs – miniature end-plate potential; NAc – nucleus accumbens; NE – norepinephrine (noradrenaline); DA – dopamine; 5-HT – serotonin; VGluT-vesicular glutamate transporter; VGAT – vesicular GABA transporter.
*http://www.mousebook.org

	Coloboma *Cm*	**SNAP-25 KO** *Snap25^{tm1Mcw}*	**SNAP-25B KO** *Snap25$^{tm2Mcw[369]}$* *Snap25^{tm1Bark}; Snap25$^{tm1.1Bark\ [252]}$*	***Blind-drunk* (*Bdr*)** *Snap25^{Bdr}*
Extent of gene knockout	Chromosomal deletion[370]	Disruption of exons 5a and 5b[186]	Disrupted splicing event [369] SNAP-25B exon replaced with chicken exon 5a[252]	Point mutation in exon 5b; results in I67T[229]
Viability			Lethal seizures postnatally[252, 369]	Not identified (likely lethal)*
Homozygote	Embryonic lethal	Embryonic lethal	(Seizure onset later)[252]	
Heterozygote	Viable[370]	Viable[186]	Presumed viable[369]	Viable[229]
Physical abnormalities	Small eyes, head bobbing, hyperactive[370] Smaller size[371]	Small, tucked No spontaneous movement Skin vasculature abnormal[186]	Smaller, disheveled, Infertile No spontaneous activity[369] Slower growth[252] Normal growth [252]	Ataxic Smaller size[229]
Gross brain anatomy	Unremarkable[370]	Unremarkable[186] Normal embryonic thalamocortical development [372] Some embryos showed abnormal cortical plate pattern with concomitant reduction in calbindin-positive neurons[372]	Unremarkable[369] Progressive hippocampal pathology [252]	Unremarkable[229]
EM terminal morphology		Larger NMJ endplate diameter[186] Normal E18 cortical synapses[186]		
Tissue		Fewer docked vesicles in adrenals[191]		

Table 1.1 continued

	Coloboma	SNAP-25 KO	SNAP-25B KO	Blind-drunk
EM terminal morphology Cultured cells		Normal vesicle size and number in hippocampal cultures[373] Impaired growth, survival and arborization in cultured neurons[374] Normal docking and vesicle numbers in chromaffin cells[196] Fewer docked vesicles in chromaffin cells[191]		
Behavioural	Normal sleep/wake cycle Increased activity, reversed by amphetamine or inhibition of NE transmission [370, 375, 376] Developmental delays[377] Hyperactivity [377] Increased stress response [378]	Heterozygotes showed normal sensorimotor gating, anxiety, motivation and social interaction as well as normal behaviour in the force-swim test and fear conditioning paradigms [253]	Heterozygote behaviour grossly normal[369] Spontaneous seizures[252, 369] Increased anxiety Impaired spatial learning, Lower locomotor activity[252]	Impaired sensorimotor gating; increased anxiety; apathy[253] Environmental stress induces social deficits and augments sensorimotor gating impairment[253]
Presynaptic proteins	50% reduction in SNAP-25 mRNA and protein[370] SNAP-25 transgene increases expression in striatum and cerebellum[375]	SNAP-25 not detected Other proteins normal[186]	SNAP-25A mRNA higher but protein is lower syntaxin-1 protein normal[252, 369] SNAP-25 protein is higher than WT, normal mRNA levels[252] SNARE complexes less stable[252]	SNAP-25 protein levels normal[253]
Neurotransmitter-specific info	Reduced cortical glutamate[378] Stimulus-evoked DA and 5-HT lower in dorsal striatum but higher in cortex [378] Lower neuropeptide release in hypothalamus[378] Abnormal catecholamine regulation in the striatum and NAc[371, 379] Serotonin normal[379] Amphetamine increases PPI[380]	Tyrosine hydroxylase normal in brainstem[186] Glutamate and GABA-mediated responses affected[200] Lower phospho-VGAT in hippocampal cultures (VGluT and unmodified VGAT normal)[200]	Altered hippocampal neuropeptide expression[252]	

Table 1.1 continued

	Coloboma	**SNAP-25 KO**	**SNAP-25B KO**	**Blind-drunk**
Neurotransmission ***Spontaneous*** *In vivo*	Absence-type seizures/abnormal cortical EEG activity starting at ~P14[381]			
Tissue slices		mEPPs (diaphragm) larger amplitude, variable frequency[186] mIPSCs (cortex) reduced amplitude and frequency[200]		Cortical mEPSCs less frequent, normal amplitude[229]
Cultured neurons		mEPSCs and mIPSCs less frequent[186, 373, 374] mEPSCs smaller amplitude[373, 374]		
Chromaffin cells				
Evoked *In vivo*	Reduced stimulus-evoked hippocampal theta rhythm; PPF normal; increased PPI; reduced LTP[382]			
Tissue slices	LVA calcium currents increased in thalamus[381]	No evoked cortical EPSCs [200]	Increased hippocampal PPF at low stimulus strengths and low inter-pulse intervals[369] Hippocampal PPF reduced at low frequency stimulation[252]	Stimulation frequency-dependent cortical EPSP plasticity[229]
Cultured neurons		No evoked release[186, 373, 374] Reduced RRP size [373] Ca2+-sensitivity of release reduced [373]		
Cultured chromaffin cells		No Ca^{2+}-triggered fast exocytosis; minimal slow secretion[196] Faster fusion pore opening [196]		

Table 1.2 - Syntaxin knock-out models.
Brain, synapse and behavioural alterations reported in various syntaxin gene knock-out animal models. *Abbreviations*: PPF – paired-pulse facilitation; PPI – paired pulse inhibition; LTP – long term potentiation; EPSC – excitatory post-synaptic current; mEPSC- miniature excitatory post-synaptic current; Stx- syntaxin; PPD – paired pulse depression; 5-HT - serotonin. * Heterozygotes were investigated in some studies, results that hold for both heterozygotes and homozygotes are indicated by '*', otherwise statements pertain only to complete KO animals.

	Stx1A KO[242] *Stx1a*^{tm1Tfuj}	**Stx1A KO**[383] *Stx1a*^{tm1Gmz}	**Stx1AKO + mutant Stx1B**[187] *Stx1a*$^{tm2.1Sud}$ + *Stx1b*$^{tm1.1Sud}$
Extent of gene knockout	Exons 9-10 disrupted[242]	Exons 3-6 disrupted premature STOP in exon 2	Exons 2-9 Stx1B in constitutively "open" conformation
Viability			Stx1A-/- were viable
Homozygote	Viable[242]	Embryonic lethal	Stx1B mutant: lethal at P28
Heterozygote	Viable	Viable	Double mutant: lethal at P28
Physical abnormalities	Indistinguishable from WT[242]	Developmentally delayed Surviving homozygotes were smaller	Stx1A-/- : no obvious defects Double mutant: ataxic, lethal seizures
Gross brain anatomy	Unremarkable[242]		
EM Terminal Morphology	Normal structure, vesicle number and distribution[242]		Fewer docked vesicles in chromaffin cells
Tissue			
Cultured cells			More docked vesicles in cortical cultures
Behavioural	Normal locomotion and anxiety levels Impaired consolidation of contextual and cued fear memory Impaired extinction of cued fear memory[242] Greater acoustic startle response and attenuated latent inhibition*[384] Abnormal social interaction and novel object exploration*[384] Enhanced allodynia following sciatic nerve damage[385]	Abnormal horizontal ladder task performance* Normal performance on many other tasks*	

Table 1.2 continued

	Stx1A KO	Stx1A KO[383]	Stx1AKO + mutant Stx1B[187]
Other presynaptic proteins	No detectable Stx1A in homozygotes 50% less Stx1A in heterozygotes Other presynaptic proteins normal*[242]		No detectable Stx1A Reduction in Stx1B Decreased Munc18 levels Reduced Munc18-syntaxin-1 complexes Normal SNARE complexes
Neurotransmitter-specific info	Increased 5-HT in hypothalamus and hippocampus but not other regions [384]		
Neurotransmission			
Spontaneous *In vivo*			
Tissue slices	Increased frequency of mEPSCs in spinal cord after nerve injury[385]		
Cultured neurons	mEPSCs amplitude was smaller in immature neurons[242]		Stx1A-/-: mEPSCs normal Double mutant: increased mEPSCs frequency
Chromaffin Cells			
Evoked *In vivo*			
Tissue slices	Impaired hippocampal LTP[242] Larger EPSC amplitude and more frequent PPD[385] Glutamate-induced 5-HT release lower in hippocampus [384]		
Cultured Neurons	Normal paired pulse inhibition[242]		Double mutant: normal evoked EPSCs Enhanced use-dependent synaptic depression Smaller RRP but vesicles fuse more readily

Table 1.3 - VAMP knock-out models.
Synaptic alterations reported in various VAMP gene knock-out animal models. *Abbreviations*: PPF – paired-pulse facilitation; PPI – paired pulse inhibition; LTP – long term potentiation; EPSC – excitatory post-synaptic current; mEPSC- miniature excitatory post-synaptic current; PPD – paired pulse depression. Heterozygotes were minimally investigated; results that hold for both heterozygotes and homozygotes are indicated by '*', otherwise statements pertain only to KO animals.

	Synaptobrevin 2 (VAMP-2) KO *Vamp2*^{tm1Sud}	***Lethal-wasting* (*Lew*)** [386] *Vamp1*^{lew}
Extent of gene knockout	Disruption of 5' end plus two point mutations in coding region[188]	G190T nonsense mutation in VAMP1 gene
Viability Homozygote	Die at birth	Die ~P15
Heterozygote	Normal[188]	Not reported
Physical abnormalities	Rounded body shape Brown fat shoulder hump[188]	Immobile No purposeful limb movement
Gross brain structure	Unremarkable[188]	Unremarkable
EM Terminal Morphology		
Tissue	Increased total vesicle numbers in chromaffin cells[187]	
Cultured cells	Elongated and larger vesicles in neurons[387] Reduced number of primed vesicles in chromaffin cells [189]	
Behavioural		Cannot right themselves when placed on back
Other presynaptic proteins	VAMP-2 undetectable Other presynaptic proteins are normal*[188] Cellubrevin upregulated in chromaffin cells[189]	VAMP-1 mRNA reduced 4-fold Other SNARE mRNA normal
Neurotransmitter-specific info		

Table 1.3 continued

		Synaptobrevin 2 (VAMP2) KO	**Lethal-wasting (Lew) [386]**
Neurotransmission	*In vivo*		
Spontaneous	Tissue slices		
	Cultured neurons	Reduced frequency, normal amplitude and slope[188]	
	Chromaffin Cells		
Evoked	*In vivo*		
	Tissue slices		
	Cultured neurons	100-fold reduction in action-potential evoked release[188] Impaired fast Ca^{2+} triggered release[188] Frequency-dependent facilitation of synaptic responses[387] Delayed endocytosis after fusion[387] Activity-dependent and spontaneous release vesicle pools not segregated[190]	
	Cultured chromaffin cells	~50% reduction in Ca^{2+} evoked release[189, 388] Reduced RRP size due to increased depriming[189] Longer time for fusion pore expansion[189]	

Table 1.4 - SNARE protein findings in schizophrenia.
Results of studies that measured SNARE proteins or protein-protein interactions in post mortem brain samples from schizophrenia patients. Tissue source is given when described in the literature. Several studies included additional subject groups; differences and samples sizes are given only for schizophrenia and comparison subjects. *Abbreviations*: "-" – not measured; n.s. – not significant; u.d. - undetected; CTL – non-psychiatric comparison subjects; SCZ – schizophrenia; BA – Brodmann area; IB – immunoblot; IHC – immunohistochemistry; DB – dot blot; 2D-GE+MS – two-dimensional gel electrophoresis coupled with mass spectrometry-based protein identification.

Footnotes:

*Many brain, plasma and CSF proteomic studies have been conducted (reviewed in [261]), however, they are not included in this table because they do not report SNARE protein data. Failure to report this information may be due to several reasons including: protein spots are not different between groups and therefore are not selected for identification by mass spectrometry or proteins are not detectable.

** Phosphorylated syntaxin-1 was significantly lower by 22% in schizophrenia subjects.

*** Immunoblots were used only for assaying SNARE complexes.

Table 1.4

Ref	Brain Region	Change relative to controls			SNARE complexes	N CTL/ SCZ	Method	Tissue Source
		SNAP-25	Syntaxin	VAMP				
118	ALIC	n.s.	-	-	-	13/15	ELISA	Macedonian/NYSPI Brain Collection
389	Cingulate cortex	n.s. (35% ↑increase)	55% ↑	-	-	24 / 18	ELISA	Multiple sources
344	Anterior cingulate cortex*	1.72-fold ↑	<1.5 fold or u.d.	<1.5 fold or u.d.	-	10/10	2D-GE +MS	NSW Tissue Resource Centre
111	Anterior cingulate (BA24)	34% ↑	47% ↑	-	-	16/19	ELISA	Mt. Sinai Brain Bank
260	Temporal (BA20)	n.s.	n.s.	-	-			
	Frontal (BA7)	n.s.	n.s.	-	-			
	Parietal (BA8)	n.s.	n.s.	-	-			
342	Visual association cortex (BA19)	-	-	n.s.	-	15/15	ELISA	Stanley Foundation
257	Occipital (BA17)	n.s.	-	-	-	10/9	IB	McLean Hospital Brain Collection
	Temporal (BA20)	33% ↓	-	-	-	7/4		
	Prefrontal (BA10)	55% ↓	-	-	-	5/5		
	Prefrontal (BA9)	30% ↑	-	-	-	6/10		
390	Parietal (BA 40)	n.s.	n.s.	n.s.	-	20/20	IB	Rebecca L Cooper Research Laboratory CNS Repository
	Prefrontal (BA10)	n.s.	n.s.	n.s.	-			
	Prefrontal (BA 46)	n.s.	n.s.	n.s.	-			
272	BA10	50% ↓	-	-	-	12/14	IB	
293	BA10 PFC	-	n.s. **	-	34% ↓ in subset	15/15	IB	Dallas Brain Collection
258	BA9	n.s.	10% ↓	n.s.	-	20/20	IB	Rebecca L Cooper Research Laboratory CNS Repository
259	Prefrontal cortex	n.s.	n.s.	22% ↓	-	23/18	IB	NIMH Clinical Brain Disorders Branch
264	Anterior frontal cortex	28% ↓	n.s.	n.s.	↑ in suicide	11/13	ELISA IB***	NIMH Clinical Brain Disorders Branch & NYPSI collection
391	Hippocampus	49% ↓	-	-	-	8/7	IB	NIMH Neuroscience/St. Elizabeth Hospital
392	Hippocampus	n.s.	-	-	-	13/13	ELISA	NIMH clinical autopsy series
	(subregions)	↓in: subiculum, CA1, CA3, CA4, DG	-	-	-	12/12	IHC	
343	Hippocampus (subregions)	51% ↓ granule cell layer	-	-	-	15/15	IHC	Stanley Foundation
345	Cerebellum (synaptosomes)	25% ↓	n.s.	-	-	8/8	ELISA	Cambridge Brain Bank
393	CSF	2.1-fold ↑	-	-	-	6/8	DB	
394	CSF	2.4-fold ↑	-	-	-	25/25	DB	

1.8 Chapter 1 figures

Figure 1.1 - Divisions of the anterior striatum investigated in this thesis.
A: Location of the basal ganglia, including striatum, superimposed on the human cortex. The striatum is an internal structure and is found below the cortex. **B:** Coronal view of the anterior striatum, at the level of interest in this thesis, indicated by the dashed line in panel A. **C:** Regions of the anterior caudate investigated in this thesis are outlined, and include the nucleus accumbens (NAc), ventromedial caudate (VMC) and dorsal caudate (DCd). The ventral striatum receives cortical inputs from the anterior cingulate, while dorsolateral prefrontal cortex and orbitofrontal cortex project to the associative striatum. Note, the most dorsolateral regions of the striatum receive inputs from the premotor/motor cortex, although this may occur in sections posterior to the view diagrammed here [61, 134, 151, 395]. **D:** Coronal section of human brain stained for synaptophysin to illustrate the approximate anatomical location and divisions of the anterior striatum.

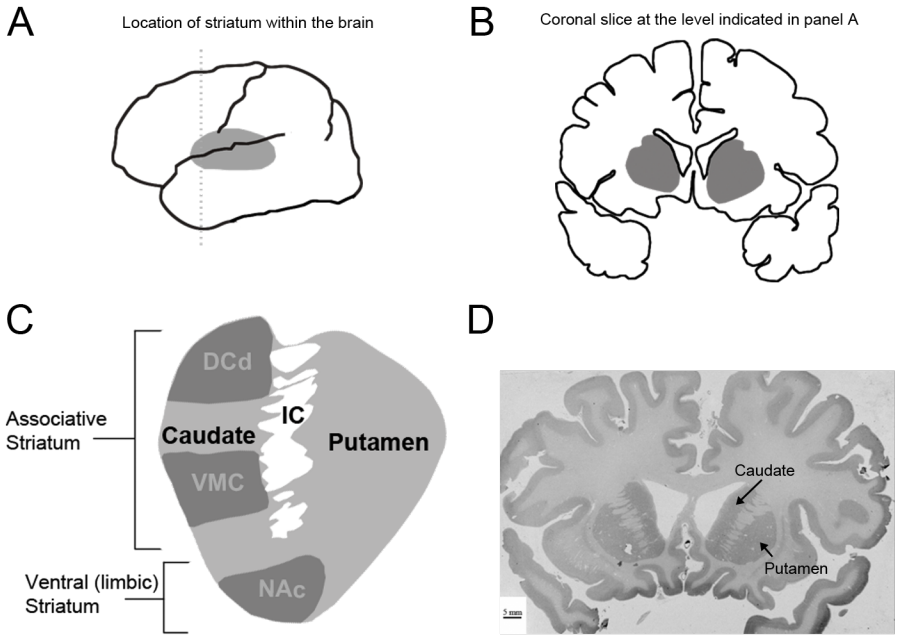


Figure 1.2 - SNARE proteins physically interact to bring membranes together.
VAMP and syntaxin-1 are anchored to their respective membranes via a transmembrane domain. Each contributes one SNARE motif to the final SNARE complex. Syntaxin-1 also contains an additional three-helix domain involved in other protein interactions. SNAP-25 is associated with the presynaptic membrane via palmitoylated cysteine residues and contributes two SNARE motifs to the final complex. Syntaxin and SNAP-25 likely form an acceptor complex prior to VAMP binding (not shown). The interaction of the three proteins brings the synaptic vesicle membrane close to the plasma membrane, resulting in bilayer mixing and neurotransmitter release. During this process, the SNAREs transition from a *trans*-conformation to a *cis*-SNARE complex, with all three proteins on the same membrane (third panel).

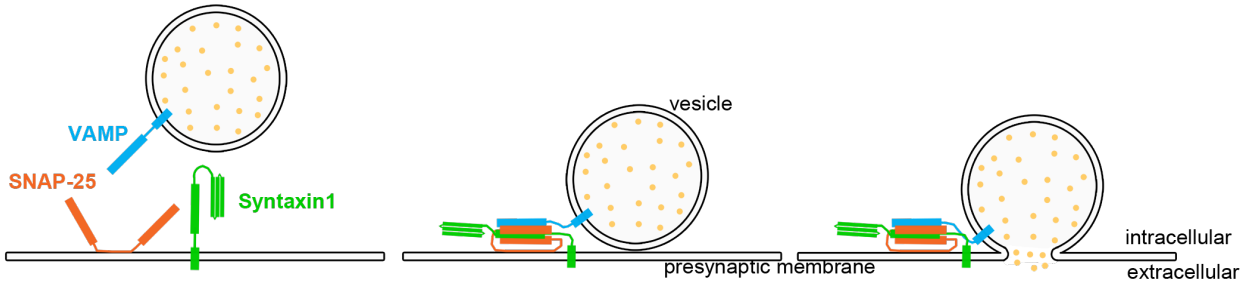


Figure 1.3 - There are multiple isoforms of each neuronal SNARE.
Major protein domains are indicated [158, 169, 396, 397]. Transmembrane domains are involved in membrane anchoring of VAMP and syntaxin-1 proteins. The SNARE motif is involved in protein-protein interactions necessary for SNARE complex formation. Amino acid differences are marked by "*". **A: Alignment of human syntaxin-1A and 1B.** Sequences correspond to NCBI sequence # NP_004594.1 (STX1A) NP_443106.1 (STX1B). The H_{abc} domain is composed of three alpha-helices that interact with a number of modulator proteins. The H_{abc} domain can also fold back onto the syntaxin SNARE domain, forming a "closed" syntaxin conformation [300]. **B: Alignment of human SNAP-25A and B.** Sequences correspond to NCBI sequence #: NP_003072 (A) NP_570824 (B). SNARE interaction domains are indicated. Cysteine residues putatively involved in membrane anchoring are underlined [247, 397]. Protein sequence encompassed by alternative exon 5 usage is indicated. **C: Alignment of human VAMP-1 and VAMP-2**. Sequences correspond to NCBI sequence # NP_055046.1 (VAMP 1) and NP_055047.2 (VAMP 2). Major protein domains are indicated [397, 398]. Additional splice variants have been identified [399, 400].

A

```
Syntaxin-1A  1   MKDRTQELRT AKDSDDDDDV AVTVDRDRFM DEFFEQVEEI RGFIDKIAEN 50
Syntaxin-1B      MKDRTQELRS AKDSDDEEEV –VHVDRDHFM DEFFEQVEEI RGCIEKLSED 49
                       *       ***    *    *                 * *  ***

Syntaxin-1A      VEEVKRKHSA ILASPNPDEK TKEELEELMS DIKKTANKVR SKLKSIEQSI 100
Syntaxin-1B      VEQVKKQHSA ILAAPNPDEK TKQELEDLTA DIKKTANKVR SKLKAIEQSI 99
                   *  **        *          *   * **                 *

Syntaxin-1A      EQEEGLNRSS ADLRIRKTQH STLSRKFVEV MSEYNATQSD YRERCKGRIQ 150
Syntaxin-1B      EQEEGLNRSS ADLRIRKTQH STLSRKFVEV MTEYNATQSK YRDRCKDRIQ 149
                                                 *        *   *   *

Syntaxin-1A      RQLEITGRTT TSEELEDMLE SGNPAIFASG IIMDSSISKQ ALSEIETRHS 200
Syntaxin-1B      RQLEITGRTT TNEELEDMLE SGKLAIFTDD IKMDSQMTKQ ALNEIETRHN 199
                           *           **   ***      ***     *      *

Syntaxin-1A      EIIKLENSIR ELHDMFMDMA MLVESQGEMI DRIEYNVEHA VDYVERAVSD 250
Syntaxin-1B      EIIKLETSIR ELHDMFVDMA MLVESQGEMI DRIEYNVEHS VDYVERAVSD 249
                       *          *                      *

Syntaxin-1A      TKKAVKYQSK ARRKKIMIII CCVILGIVIA STVGGIFA- 288
Syntaxin-1B      TKKAVKYQSK ARRKKIMIII CCVVLGVVLA SSIGGTLGL 288
                                     *  * *   **  ***
```

Major protein domains
Transmembrane
H_{abc}
SNARE motif

B

```
SNAP-25A   1   MAEDADMRNE LEEMQRRADQ LADESLESTR RMLQLVEESK DAGIRTLVML 50
SNAP-25B       MAEDADMRNE LEEMQRRADQ LADESLESTR RMLQLVEESK DAGIRTLVML

               ----------------------alternative exon----------------------------
SNAP-25A       DEQGEQLDRV EEGMNHINQD MKEAEKNLKD LGKCCGLFIC PCNKLKSSDA 100
SNAP-25B       DEQGEQLERI EEGMDQINKD MKEAEKNLTD LGKFCGLCVC PCNKLKSSDA
                      * *     **  *          *     *   **

SNAP-25A       YKKAWGNNQD GVVASQPARV VDEREQMAIS GGFIRRVTND ARENEMDENL 150
SNAP-25B       YKKAWGNNQD GVVASQPARV VDEREQMAIS GGFIRRVTND ARENEMDENL

SNAP-25A       EQVSGIIGNL RHMALDMGNE IDTQNRQIDR IMEKADSNKT RIDEANQRAT 200
SNAP-25B       EQVSGIIGNL RHMALDMGNE IDTQNRQIDR IMEKADSNKT RIDEANQRAT

SNAP-25A       KMLGSG  206
SNAP-25B       KMLGSG
```

Major protein domains
SNARE motif

C

```
VAMP-1  1   MSAPAQPPAE GTEGTAPGGG PPGPPPNMTS NRRLQQTQAQ VEEVVDIIRV 50
VAMP-2      MSATAATAPP --AAPAGEGG PPAPPPNLTS NRRLQQTQAQ VDEVVDIMRV 48
               * *****   ******     *    *                    *

VAMP-1      NVDKVLERDQ KLSELDDRAD ALQAGASQFE SSAAKLKRKY WWKNCKMMIM 100
VAMP-2      NVDKVLERDQ KLSELDDRAD ALQAGASQFE TSAAKLKRKY WWKNLKMMII 98
                                              *             *    *

VAMP-1      LGAICAIIVV VIVIYFFT 118
VAMP-2      LGVICAIILI IIIVYFST 116
              *     ** * **  *
```

Major protein domains
Transmembrane
SNARE motif

2 A novel mechanism and treatment target for presynaptic abnormalities in specific striatal regions in schizophrenia[2]

2.1 Introduction

Abnormalities of presynaptic terminals contribute to the mechanism of illness in schizophrenia [46]. Postmortem studies indicate deficits in terminal numbers, and in terminal-enriched molecules such as transporters and proteins involved with vesicular neurotransmission [40, 111]. Current models of functional disturbances in schizophrenia emphasize disinhibitory processes, acting through GABA, glutamate and dopamine neurotransmission to affect the firing patterns of local networks of cortical neurons [46, 401]. Striatal processes are also critically important. Functional imaging provides evidence of frontostriatal dysfunction in schizophrenia, with altered patterns of activation and impaired cognition [94, 402]. Presynaptic terminals in striatum have lower than expected levels of glutamate transporters [52, 403]. Functional studies of dopaminergic presynaptic terminals carried out with *in vivo* imaging techniques indicate increased synthesis and release of dopamine in schizophrenia [404].

Families of molecules enriched in presynaptic terminals could play important roles in these disturbances. One such family is the SNARE proteins, SNAP-25, syntaxin[3] and VAMP [163]. These proteins form a trimeric complex that is vital to the mechanism of vesicular neurotransmission. Most, but not all studies of individual SNARE proteins in frontal cortex and hippocampus in schizophrenia indicate lower than expected levels of SNARE proteins [257-260, 264, 272, 343, 345, 389, 392]. One study linked lower postmortem levels of SNAP-25 in

[2] **A version of this chapter has been published:**
Barakauskas VE, Beasley CL, Barr AM, Ypsilanti AR, L, HY, Thornton AE, Wong H, Rosokilja G, Mann JJ, Mancevski B, Jakovski Z, Davceva N, Ilievski B, Dwork AJ, Falkai P, Honer WG. (2010). A novel mechanism and treatment target for presynaptic abnormalities in specific striatal regions in schizophrenia. *Neuropsychopharmacology*. 35(5): 1226-38. doi:10.1038/npp.2009.228

[3] All mention of *syntaxin* in this chapter refers to *syntaxin-1* (both isoforms)

hippocampus to poorer antemortem cognitive function in schizophrenia [309, 392]. Functional aspects of SNARE proteins may also be affected in schizophrenia. A study indicated higher SNARE complex formation in anterior frontal cortex samples from patients who died by suicide [264]. Investigation of samples of CSF from living patients indicated higher than expected levels of SNAP-25, and some correlations with symptom severity [393, 394]. Genetic studies in humans provide indirect evidence for a role for SNARE proteins in brain function, and in psychiatric illness. Variation in the SNAP-25 gene may increase the risk for schizophrenia, contributes to cognitive function in healthy subjects and patients with schizophrenia, is associated with metabolic measures in hippocampus, antipsychotic treatment response, and with weight gain related to antipsychotic medications [177, 179, 280-282, 285, 405]. Variation in *STX1A* (coding for syntaxin-1A) may be associated with a diagnosis of schizophrenia [180].

Animal models indicate that abnormalities of amount, or function of SNARE proteins have consequences for neurotransmission and behavior. The *coloboma* mouse (*Cm*/+) has a chromosomal deletion that spans the mouse *Snap25* gene, and results in 50% less SNAP-25 protein in brain [370]. These mice show greater levels of noradrenaline but lower glutamate, dopamine and serotonin neurotransmission in a regionally specific manner, delayed acquisition of motor milestones, and hyperactivity as adults [371, 377, 378]. Hyperactivity in these animals responds to amphetamine treatment [375]. Microdialysis studies indicate *coloboma* mice have high basal levels of synaptic dopamine in the striatum, and increased dopamine release after amphetamine challenge [406]; both these features are similar to schizophrenia [404]. Another mutant mouse helps to understand the implications of functional changes in SNARE proteins, rather than differences in amount. The *blind-drunk* mouse (*Bdr*/+) has motor abnormalities and impaired prepulse inhibition [229]. Characterization of the molecular basis of this mutation indicated a T-to-C transversion resulting in an isoleucine-to-threonine amino acid change [229].

This change in amino acid sequence in SNAP-25 results in greater affinity for the partner SNARE protein syntaxin, greater stability of the SNARE complex, and decreased exocytosis in glutamatergic terminals [229]. Interestingly, in the *blind-drunk* mouse, the prepulse inhibition abnormalities in adulthood are enhanced further if the mice are exposed to prenatal stress [253]. Treatment of *blind-drunk* mice with clozapine restored prepulse inhibition to near normal levels; haloperidol was also moderately effective [253]. Interestingly, earlier studies of rat brain slice preparations found that administration of dopamine increased SNARE complex formation, an effect that could be blocked by haloperidol [407].

The present study was designed to investigate SNARE protein levels and SNARE protein-protein interactions in schizophrenia, and to study the effects of antipsychotic drugs on these properties of SNARE proteins in rat brain. SNARE protein levels were measured in three striatal regions involved in cognitive and limbic circuitry (dorsal caudate, ventromedial caudate and nucleus accumbens) [134, 135]. To determine whether altered SNARE protein-protein interactions were present, we developed a modified sandwich ELISA to measure interactions between syntaxin and SNAP-25. Finally, to determine if antipsychotic treatment affects SNAREs and SNARE complex formation, we performed similar measures in the striatum of rodents treated with haloperidol or clozapine.

2.2 Materials and methods

2.2.1 Human subjects

Samples were obtained from the Macedonian/New York State Psychiatric Institute Brain Collection. Tissue collection and screening details can be found in Section 2.5 (Supplementary materials and methods I). The frozen tissue series consisted of 15 subjects with schizophrenia or related illness (13 schizophrenia, 2 schizoaffective disorder) and 13 non-psychiatric comparison

subjects (Table 2.1). All subjects died of acute causes, and only one subject had a post mortem interval greater than 24 hours. Over their lifetimes, all patients were exposed to high-potency typical antipsychotics in addition to lower-potency drugs; only three patients were prescribed an atypical antipsychotic (clozapine). Post mortem toxicological analyses detected antipsychotics in only two patients. Caffeine and benzodiazepines were the most frequently detected compounds, and were found in both groups.

Samples were dissected from frozen coronal tissue slabs of the right hemisphere, using a standard human brain atlas [408] by a single investigator (WGH). Striatal regions were dissected from the same tissue block at the level of the nucleus accumbens (NAc) (approximating slice 12, Mai, 1997). The most dorsal caudate region was dissected (DCd), as well as a medial portion adjacent to the body of the lateral ventricle (VMC) and the grey matter ventral to the internal capsule (NAc).

Formalin-fixed tissue from 4 control subjects and 4 schizophrenia subjects was also obtained (Table 2.3). Fixed tissue from the VMC was dissected using similar landmarks as for the frozen tissue by a single investigator (AJD). Samples were coded to mask investigators to diagnosis and sample characteristics.

2.2.2 Animals and antipsychotic drug administration

Adult male Sprague-Dawley rats (Charles River, Montreal, Canada) were pair housed, with access to food and water *ad libitum*. Animals were treated with vehicle or one of two antipsychotic medications: haloperidol, a high-potency typical antipsychotic agent or clozapine, an atypical antipsychotic, both commonly used in the schizophrenia cases (Table 2.1). Rats were divided into three groups balanced for starting weight (270-320 g range), and administered haloperidol (1 mg/kg), clozapine (20 mg/kg) or pH-adjusted saline (1 ml/kg) daily for 28 days

intraperitoneally [309, 310] (see appendix Appendix 2 for additional details). Haloperidol or clozapine was dissolved in 0.9% acetic acid/saline, adjusted with NaOH to pH 5.0-5.5 and final volume adjusted with water to give a drug concentration of 1 mg/ml. Due to complications at the injection site, the clozapine group was injected subcutaneously with larger volumes of dilute clozapine (0.4-0.5 mg/ml, 20 mg/kg) for the final 16 days. Saline and haloperidol-treated animals gained weight at a similar rate during the 4 weeks, while clozapine-treated animals did not gain weight, in agreement with others' findings [409].

Animals were sacrificed by decapitation 24 hours after the final injection. Brains were quickly removed, cooled in artificial cerebrospinal fluid (148 mM NaCl, 3 mM KCl, 1.4 mM $CaCl_2$, 0.8 mM $MgCl_2$, 10 mM D-Glucose, 0.8 mM Na_2HPO_4, 0.2 mM NaH_2PO_4) and dissected. The brain was cut coronally, at the level of the optic chiasm and again, 3 mm anterior to this. This produced a 3 mm thick section at approximately Bregma 0.48 μm [410]. The tissue inferior to the ventral striatum was removed, and the nucleus accumbens dissected as the most ventral portion of striatum, at the level of the anterior commissure. The remaining caudate/putamen was divided in half, to give medial and lateral portions. A similar dissection protocol was described previously [411]. Regions were dissected bilaterally, frozen rapidly on dry ice and stored at -70°C.

All procedures were approved by the UBC Animal Care Committee and were conducted in accordance with the Canadian Council on Animal Care guidelines.

2.2.3 Antibodies

We previously prepared monoclonal antibody-producing hybridoma cell lines by fusing B-lymphocytes from immunized mice to the non-secreting mouse myeloma cell line NS0. The monoclonal antibodies produced by the individual hybridoma cell lines detect SNAP-25 (SP12,

IgG_1 subclass), syntaxin (SP6, IgG_1; SP7, IgG_{2A}), VAMP (SP10, IgM; SP11, IgG_1) and synaptophysin (EP10, IgG_1) [264, 389]. For each antibody, previously reported characterization studies used: immunoblotting to demonstrate single bands at the expected molecular weights for the target antigens, immunohistochemistry to demonstrate staining patterns consistent with a presynaptic pattern of distribution, and studies of reactivity with fusion proteins prepared in bacterial or mammalian cell systems [264, 389]. Additional studies of antibody specificity appear in Figure 2.4. For immunohistochemistry, a polyclonal calretinin antibody (Swant, Bellinzona, Switzerland) was also used. Secondary antibodies included: peroxidase-conjugated anti-mouse IgG and IgM (Jackson Immunoresearch Laboratories Inc., West Grove PA), peroxidase-conjugated anti-mouse IgG_{2A} (Southern Biotech Ltd., Birmingham AL) and goat anti-mouse or anti-rabbit conjugated to Alexa-488 or Alexa-555 fluors (Molecular Probes, Eugene OR).

Antibody specificity was confirmed by immunoblotting (Figure 2.4). For a negative control, we prepared conditioned tissue culture medium by growing the parent mouse myeloma line (NS0) and collecting the medium, which contains the same percentage of fetal bovine serum as was used to grow the antibody-secreting hybridomas, derived by fusing B-lymphocytes with NS0 cells.

2.2.4 Direct ELISA studies

Frozen samples were rapidly thawed and homogenized in tris-buffered saline (TBS), aliquoted and stored at –70°C. Protein concentrations were determined using a Lowry-based method (DC assay; Biorad, Mississauga ON). Samples were diluted to a standard concentration for the ELISA, then all were serially diluted in duplicate on the ELISA plate over a 1 to 128-fold range.

For the present series of studies, hybridoma cell culture supernatants prepared in our laboratory were used as the source of antibodies. These tissue culture supernatants do not contain

any other mouse immunoglobulin, which could result in increased non-specific background signal in the ELISA. This is in contrast to many commercial monoclonal antibodies prepared from mouse ascites fluid, which can contain non-specific mouse immunoglobulin. The tissue culture supernatants contain fetal bovine serum, and small amounts of bovine immunoglobulin are present. Secondary antibodies specific for mouse immunoglobulin, with minimal cross reactivity with bovine or human immunoglobulin were used. We also prepared tissue culture supernatants by growing the non-secreting parent myeloma line NS0 in the same culture conditions and fetal bovine serum concentration used to grow the antibody-secreting hybridomas. Substitution of the NS0 conditioned-tissue culture supernatant for antibody-containing supernatant was used as a negative control in the ELISA.

Assays were performed using previously published protocols [264, 342]. SNARE proteins were detected by incubating with SP12 (α-SNAP-25), SP6 (α-syntaxin) or SP10 (α-VAMP) diluted (1:10, 1:10 and 1:5, respectively) in 5% milk/TBS (Appendix 1). Based on human sample data, modal values for the linear range of antigen detection (after log transformation) were 64-fold (SNAP-25), 32-fold (syntaxin) and 8-fold (VAMP). Immunoreactivity was compared between samples as the amount of protein required to give a fixed optical density within the linear range. Each experiment was performed twice and a mean value calculated for each sample.

Samples were assayed twice, on separate days, with run-to-run correlations (Pearson's r) ≥ 0.8 for each measure. Each plate also contained a reference sample, not part of the study sample set. The multiple replicates of reference samples also allowed calculation of variability in the assays within a sample run. The intra-run coefficient of variation (standard deviation for reference samples divided by mean of reference samples) was ≤ 10% for all runs.

2.2.5 Capture ELISA

The amount of syntaxin interacting with SNAP-25 was measured using a modified sandwich ELISA. Assay development and validation is detailed in Section 2.6 (supplementary materials and methods II). Capture and primary detection mouse monoclonal antibodies were prepared from tissue culture supernatants, as described above, that were free of non-specific mouse immunoglobulin.

To measure syntaxin-SNAP-25 complexes, 384-well plates (Nalge Nunc International, Rochester NY) were first coated with purified SP12 antibody (an IgG_1 subclass α-SNAP-25, capture antibody, 0.075 μg per well). Residual binding sites on the coated plates were then blocked with 5% milk/TBS. Brain homogenates were diluted over a 12-fold range in TBS, and incubated in the antibody-coated plates overnight at 4°C. The capture SP12 antibody immobilized on the ELISA plate pulled out SNAP-25 from the homogenates, as well as any protein interacting with SNAP-25. After washing, syntaxin bound to SNAP-25 was assayed by incubating with antibody SP7 (IgG_{2A} subclass α-syntaxin, primary detection antibody, 1:10), then with a peroxidase-conjugated secondary antibody specific for mouse IgG_{2A}, and ABTS substrate (KPL Inc., Gaithersburg MA) (Appendix 1). Negative controls performed on each plate included wells with: a) all steps with omission of the homogenate, b) all steps with omission of the primary detection antibody SP7, c) all steps with omission of both the homogenate and the primary detection antibody SP7. The optical density signal in the negative control wells was < 0.15, which was the same as the background signal in an empty well.

Analysis was similar to that of the direct ELISA. Immunoreactivity was compared between samples as the amount of protein required to give a chosen optical density within the linear range (12-fold range, modal value). Intra-run coefficient of variation, determined using a reference sample, was ≤ 10% and the run-to-run correlation was > 0.8. Each experiment was

completed twice and a mean calculated for each sample. Because the amount of captured syntaxin was related to the amount of total SNAP-25 in the sample (Section 2.6, supplementary materials and methods II, Figure 2.7), the raw capture values were normalized to total SNAP-25 as measured in the direct ELISA.

2.2.6 Immunohistochemistry

Colocalization of SNARE proteins in human striatum was confirmed by confocal microscopy on tissue slices of VMC (demographics in Table 2.3). Sections were cut 30 μm in thickness with a vibratome. Tissue was stained using previously published protocols [296] with modifications. To reduce autofluorescence, sections were incubated for 15 min in Sudan Black (0.3% in 70% ethanol) (Sigma-Aldrich Inc., St. Louis MO) prior to processing. Antigen retrieval was performed for SP7-stained sections only, by heating sections in sodium acetate (200 mM $NaCH_3COO$, pH 4) for 10 min at 95°C. Primary antibody dilutions were as follows: SP12 1:10, SP7 1:10, SP11 1:10, calretinin 1:500. Sections were incubated with fluorescent secondary antibodies (1:500 dilution) for 1 hour at 37°C, washed and mounted in 70% glycerol/TBS.

The degree of autofluorescence was ascertained by including sections processed using culture-conditioned media instead of primary antibody. These were imaged using identical acquisition parameters used for stained sections of that sample. Fluorophores were reversed in some samples.

A laser scanning confocal microscope (LSM 5 Pascal, Zeiss) with a 63x/1.2W C-Apochromat water immersion objective was used. Excitation wavelengths of 488 nm and 543 nm were used with 505-530BP and 585LP emission filters. Acquisition parameters were optimized for each image so that signal in each channel was not saturated and images were obtained at a 512 x 512 pixel resolution. Image processing (auto contrast, brightness and cropping) was

performed using Photoshop 7.0 (Adobe, San Jose CA). All confocal microscopy was carried out blinded to diagnosis.

2.2.7 Statistical analysis

Data analysis was performed using JMP 5.0.1 for Macintosh (SAS Institute Inc., Cary NC) and SPSS 11.0 for Macintosh (SPSS Inc, Chicago IL). Data were graphed using GraphPad Prism 4 (GraphPad Software Inc., San Diego CA).

For protein measures, consistency with the normal distribution was assessed by Shapiro-Wilk's tests and data was $\log_e$-transformed where necessary. Equality of variances was confirmed by Levene's and/or Brown-Forsythe tests. For human studies, two-tailed t-tests were used to determine if post mortem interval (PMI), sample storage time, age and pH differed between groups (Table 2.1). The ELISA data values (total protein at a fixed optical density) are inversely related to the amount of target antigen present in a sample. For graphing purposes only, we employed a simple algebraic transformation to plot the data in the intuitively simpler fashion where greater values represent greater amounts of the target antigen. This results in no distortion of the distribution of values.

Separate two-way ANOVAs were used to determine the effects of diagnosis and striatal region for each protein measure. Striatal region was entered as the within-subjects factor, and diagnosis as the between-subjects factor. For the animal data, striatal region was entered as the within-subjects factor, and treatment group as the between-subjects factor. Multivariate F-values are reported. For human data, statistically significant diagnosis-by-region interactions were followed up by F-tests to determine in which region there was a statistically significant difference between the diagnostic groups. Raw *P*-values are reported; statistically significant effects are those that withstand Bonferonni correction for three comparisons (three striatal

regions). For animal data, statistically significant effects were followed up by contrasts to determine which drug treatment group showed statistically significant differences from controls. Significance criteria was Bonferonni-adjusted for two comparisons (each of two drugs versus control).

To determine if sample characteristics or demographics may account for statistical differences we observed, associations between the protein measure in a given area and PMI, pH, sample storage time and age were investigated using Pearson or Spearman correlations, as appropriate. A similar approach to identifying potential confounding factors has been used by others [340, 341].

Additional follow-up analyses were conducted using paired t-tests to determine if the levels of SNARE proteins differed between striatal regions.

2.3 Results

2.3.1 Human studies

As seen in Table 2.1, post mortem interval (PMI) was longer in the control group ($t = 3.73$, df = 25, $P = 0.001$) and sample storage time was longer in the schizophrenia group ($t = 4.21$, df = 26, $P < 0.001$). Age and pH did not differ between the groups ($P > 0.05$). Following a $\log_e$-transformation, the SNAP-25, syntaxin and VAMP protein measures were normally distributed, except for the dorsal caudate in the control group where two values for syntaxin immunoreactivity were found to be lower than 1.5 times the interquartile range and therefore possible outliers. However, there were no differences in variances between groups. Transformed values were used for all subsequent analyses. The amount of captured syntaxin, normalized to total SNAP-25, was normally distributed, and therefore data was not transformed.

2.3.2 SNAP-25 protein levels

Investigation of SNAP-25 protein levels using ANOVA revealed a significant effect of region ($F = 7.44$, df = 2,25, $P = 0.003$) and a region-by-diagnosis interaction ($F = 4.90$, df = 2,25, $P = 0.016$). Follow-up analyses indicated a lower amount of SNAP-25 protein in the VMC in schizophrenia as compared with control subjects (32% lower; $F = 6.86$, df = 1,26, $P = 0.015$) (Figure 2.1A). SNAP-25 protein was also 25% lower in the NAc in schizophrenia, although this difference was not significant after Bonferonni correction ($F = 5.24$, df = 1,26, $P = 0.030$). There were no statistically significant correlations between possible confounding factors (pH, PMI, sample storage time and age) and SNAP-25 in the VMC (all $P > 0.09$).

2.3.3 Syntaxin protein levels

A significant effect of striatal region ($F = 10.50$, df = 2,25, $P < 0.001$) and a region-by-diagnosis interaction ($F = 6.59$, df = 2,25, $P = 0.005$) were detected for syntaxin protein levels. Syntaxin was lower in the VMC in schizophrenia as compared with controls (26% lower, $F = 9.18$, df = 1,26, $P = 0.006$) (Figure 2.1B). Correlation analysis of syntaxin protein levels in the VMC did not indicate any statistically significant associations between pH, PMI, sample storage time or age with protein (all $P > 0.1$).

2.3.4 VAMP protein levels

A significant effect of striatal region on VAMP protein levels was observed ($F = 10.01$, df = 2,25, $P < 0.001$). The region-by-diagnosis interaction was also significant ($F = 5.88$, df = 2,25, $P = 0.008$). While VAMP levels were 25% lower in schizophrenia in the VMC, this did not reach

statistical significance ($F = 3.84$, df = 1,26, $P = 0.061$) (Figure 2.1C). There were no statistically significant associations with possible confounding variables.

2.3.5 SNARE protein interactions

The amount of syntaxin interacting with SNAP-25 (normalized to the total level of SNAP-25 in each sample) was compared between groups and striatal regions. Significant effects of region ($F = 5.89$, df = 2,25, $P = 0.008$) and a region-by-diagnosis interaction ($F = 6.80$, df = 2,25, $P = 0.004$) were observed. Follow-up *F*-tests indicated that syntaxin-SNAP-25 interactions were higher in schizophrenia in the VMC ($F = 8.39$, df = 1,26, $P = 0.008$) relative to controls (Figure 2.1D). No statistically significant correlations with sample characteristics were identified. We also examined the effect of covarying for the amount of synaptophysin present in the VMC, as an index of the total number of synapses. Synaptophysin levels measured by ELISA were not different in the VMC between schizophrenia and control samples ($t = 1.12$, df = 26, $P = 0.27$), Appendix 3. The covariance analysis still demonstrated syntaxin-SNAP-25 interactions were greater in schizophrenia than in control samples (diagnosis $F = 6.62$, df = 1,25, $P = 0.016$; synaptophysin $F = 7.09$, df = 1,25, $P = 0.013$).

2.3.6 Comparison of striatal regions

To determine how SNARE protein expression varies between striatal regions, within-subject analyses were performed using paired t-tests. Because we had identified region-by-diagnosis interactions in the omnibus ANOVA analyses, paired t-tests were performed separately for control and schizophrenia samples. No protein measures showed a significant difference between striatal areas in the control group (all *P*-values > 0.05), suggesting that SNARE protein levels are homogenous throughout the striatum in control samples. Interestingly, similar pair-wise

comparisons within the schizophrenia group indicated significant differences between all three striatal regions for SNAP-25, syntaxin and VAMP (all *P*-values < 0.008) (Table 2.2).

A similar analysis for the capture ELISA data indicated a nominally significant difference in syntaxin-SNAP-25 interactions between the DCd and NAc in the control group ($t = 2.28$, $P = 0.042$), but not between the NAc and VMC, nor between VMC and DCd. In contrast, in schizophrenia, syntaxin-SNAP-25 interactions were different in the NAc versus VMC, as well as the VMC versus DCd (all *P*-values < 0.008) but not between the DCd and NAc (Table 2.2).

2.3.7 Immunohistochemical studies of SNARE proteins in the VMC

Because our measurements of SNARE interactions relied on the use of brain homogenates (where protein localization is disrupted), we confirmed the colocalization of these proteins in intact human striatum. Stained tissue sections showed no qualitative differences in staining between four control and four schizophrenia subjects. To aid orientation within slices, we double-stained for calretinin, a calcium-binding protein present in the cytoplasm of a subset of striatal interneurons. This provided a reference for cell body size and shape that remained unstained by presynaptic markers (Figure 2.2A-B). SNAP-25 and syntaxin staining appeared diffuse, consistent with their presence not only at synapses but also along axons (Figure 2.2A-C). Syntaxin staining appeared more uniform than SNAP-25 staining. VAMP staining appeared more punctate, consistent with predominantly synaptic and vesicular locations (Figure 2.2D). Incomplete colocalization of SNARE proteins was observed.

2.3.8 Antipsychotic drug studies

We examined the possible effects of psychotropic medications present in toxicological analyses at time of death on the key findings in the VMC. All results remained statistically significant

after removing the two samples with clozapine ($P < 0.016$), or after removing the seven samples with benzodiazepines from the analyses ($P \leq 0.017$). There were no statistically significant differences in any of the primary findings between smokers and non-smokers.

We analyzed the possible effects of antipsychotic drugs further, by carrying out studies in rats. To improve the normality of the distribution and variances between groups, SNAP-25 protein measures were $\log_e$-transformed. For all other measures, untransformed values were used in analyses.

For SNAP-25 protein levels, ANOVA revealed a significant effect of treatment ($F = 5.71$, df = 2,27, $P = 0.009$) and striatal region ($F = 6.34$, df = 2,26, $P = 0.006$), but no interaction effect. Follow-up contrasts indicated that SNAP-25 was increased by a mean amount of 24% in haloperidol-treated rats as compared with saline controls ($F = 11.01$, df = 1,27, $P = 0.003$) but not different between clozapine-treated animals and controls (Figure 2.3A).

Drug treatment had a significant effect on syntaxin protein levels ($F = 3.79$, df = 2,27, $P = 0.035$), but region did not. Syntaxin levels were higher by a mean amount of 18% in the haloperidol group than in controls ($F = 7.58$, df = 1,27, $P = 0.010$) (Figure 2.3B).

Drug treatment had a significant effect on VAMP levels ($F = 7.82$, df = 2,27, $P = 0.002$), as did striatal region ($F = 7.14$, df = 2,26, $P = 0.003$). VAMP protein was significantly higher by a mean amount of 16% in the haloperidol group ($F = 13.38$, df = 1,27, $P = 0.001$), and by a mean amount of 13% in the clozapine group ($F = 9.80$, df = 1,27, $P = 0.004$) (Figure 2.3C) compared with controls.

The amount of syntaxin interacting with SNAP-25 did not differ between drug treatment groups, nor between striatal regions (Figure 2.3D).

Because the group-by-treatment interaction was not significant, we investigated the effect of region with the treatment groups combined, for protein measures that showed a significant

effect of region. SNAP-25 levels were significantly higher in the lateral as compared with the medial striatum, or with the NAc (paired t-test, all *P*-values ≤ 0.009). VAMP levels were higher in the medial striatum as compared with NAc or lateral striatum (paired t-test, all *P*-values < 0.005). Syntaxin levels were not significantly different between regions.

2.4 Discussion

We observed lower SNARE protein levels in the ventromedial caudate in schizophrenia, but no significant differences in the nucleus accumbens, or the dorsal caudate. These results demonstrate that adjacent but functionally distinct brain regions can be differentially affected in schizophrenia. Further, although SNARE protein levels were lower, protein-protein interactions forming SNARE complexes in schizophrenia were greater. Haloperidol administration to rats was associated with higher levels of SNARE proteins, but did not appear to affect protein-protein interactions. Clozapine administration had more selective effects, with only VAMP levels being higher than expected.

2.4.1 SNARE protein levels

Topographically organized corticostriatal and thalamostriatal inputs account for a majority of synapses in the striatum [134]. Relevant to our findings, and in the context of patient symptoms, the VMC receives projections from the medial orbitofrontal cortex, mediodorsal and medial ventral anterior thalamic nuclei [134], areas important for social cognition. In contrast, the NAc receives inputs from medial cortex as well as limbic regions, in addition to the thalamus (MD and VA) [134]. Differential SNARE protein changes in these striatal regions could reflect their diverse synaptic inputs. In brain homogenates the synaptic origin of the measured proteins is unknown. It is possible that not all SNAREs are downregulated in the same synapse in the VMC.

Qualitative assessment of double-labeled VMC sections (Figure 2.2) suggested incomplete colocalization of the SNAREs. SNARE isoforms do show some differential distribution in the CNS, supporting this hypothesis [173, 237, 251]. Alternatively, the changes we observe may be restricted to a specific synapse or terminal type.

Ultrastructural and immunohistochemical studies in the striatum indicate that specific types of synapses may be altered in schizophrenia. Higher numbers of corticostriatal synapses were reported in anterior caudate in schizophrenia [312]. Alterations may differ between schizophrenia subtypes, with a higher density of excitatory inputs in striatal patches in schizophrenia, but increases in the matrix only in subjects with undifferentiated schizophrenia [145]. The relationship of ultrastructural findings to levels of proteins directly involved in presynaptic function is unclear. Compensatory down-regulation of specific SNARE proteins in corticostriatal afferents due to increased synaptic density is one possibility.

Alterations in cell numbers may also contribute to lower SNARE levels. Lower density of cholinergic interneurons was reported in ventral striatum (corresponding to the NAc in our study) in schizophrenia [144], lower NOS-positive interneurons in the putamen [412], and lower total neuron number in the caudate (with no alterations in density) [143]. We remain cautious in interpreting our findings in the context of such literature, as anatomical locations of the studies might differ, and it is unclear if SNARE protein distribution is the same in all synapses [193].

The balance of SNARE proteins between striatal regions may also be important. In schizophrenia, there is evidence for differences in the relationships between striatal compartments [146], in the balance between excitatory and inhibitory transmission [401], and in relative activity of different brain regions [413]. Structural imaging suggests the associative striatum (consistent with VMC and DCd in our study) is most affected in schizophrenia, and also shows the greatest abnormalities of presynaptic functional activity of the dopamine system [126,

142]. The subregional distribution of abnormalities of dopamine release in schizophrenia is unknown. Basal and amphetamine stimulated dopamine release is higher than normal in striatum of *coloboma* mice, where SNAP-25 is low [414]. The low levels of SNAP-25 observed in VMC in our samples could implicate SNAP-25 in the abnormalities of release in patients with schizophrenia.

2.4.2 Syntaxin-SNAP-25 protein-protein interactions

One approach to assessing the consequences of lower SNARE protein levels is to examine a functional unit – the SNARE complex. In the VMC in schizophrenia we observed higher SNAP-25-syntaxin interactions, even though levels of SNAP-25 and syntaxin were lower. SNARE complexes were reported to be increased 4-fold in rat striatal slices following exogenous dopamine application [407]. The consequences of greater SNARE complex formation/stability for neurotransmission may include reduced vesicular release, as shown for glutamatergic terminals in the *blind-drunk* mouse [229]. A greater amount of dopamine release in schizophrenia would be consistent with lower levels of SNAP-25, as observed in *coloboma* mice [414]. The observed increased SNARE complex formation in VMC in schizophrenia could represent a compensatory mechanism, attempting to dampen overactive dopamine release directly, or indirectly via reduced glutamate release (which normally facilitates striatal dopamine release) [415].

At a molecular level, greater SNARE complex formation could occur if SNAREs have increased affinity for each other. Post-translational modifications, different isoforms and changes in amino acid sequence affect SNARE complex stability and synaptic function [196, 229, 397]. Accessory factors also modulate SNARE complex formation, stability and disassembly. Some interacting proteins are already known to be altered in schizophrenia, such as septin-5 and

complexins [297, 305]. Future work is needed to determine the possible roles for these factors in the increase in SNARE complexes we observed.

2.4.3 Antipsychotic drug effects

The effects of antipsychotic treatment in rats on striatal SNARE protein levels and protein-protein interactions are different from the findings in schizophrenia tissues. In rats, haloperidol increased levels of all three proteins throughout the striatum, while clozapine increased VAMP protein levels. These results are consistent with previous reports in other rat brain areas [297, 309], and with ultrastructural studies of the effects of haloperidol in striatum [416-418]. Our findings do not seem to replicate decreases in synaptic density observed with longer exposure to haloperidol in rodents [419], although compensatory up-regulation of SNAREs due to antipsychotic drug-related decreases in synapse number cannot be ruled out. In the latter study, synaptic terminal density returned to normal upon 4 weeks of drug withdrawal [419]. This scenario may well reflect our human data as antipsychotic drugs were detectable in only two patients at time of autopsy. Given a 6.8 day half-life of haloperidol in human brain [349], it appears that many patients were not taking medications for a number of weeks prior to death. The effects of clozapine appeared to be limited to increasing VAMP levels; SNAP-25 was unchanged. This finding is consistent with the recently reported effects of clozapine on VAMP and SNAP-25 mRNA levels in human neural cell aggregates exposed to these drugs for three weeks [420]. Overall, the greater effect of haloperidol versus clozapine on SNARE proteins could relate to differences in mechanism of drug action. Previous ultrastructural studies described increased overall glutamate labeling in striatal asymmetric synapses associated with both drugs, and additional labeling in a subset of synapses associated with haloperidol only, suggesting different synaptic effects of the two drugs [421].

Greater SNARE complex formation in the VMC was identified in schizophrenia samples in the present study. In rats, haloperidol administration was associated with lower SNARE complex formation in the NAc, however these effects were not statistically significant. One previous study investigated the effects of dopamine, and dopamine plus haloperidol on SNARE complex formation in a slice preparation [407]. Dopamine increased SNARE complex formation, an effect that was blocked by haloperidol. The effects of dopamine antagonists on SNARE complex formation may be different in the presence of elevated dopamine levels.

SNARE proteins were suggested to be targets for development of therapeutics in psychiatric illness [422], and modifying SNARE complex formation could be a fruitful strategy. The effects of several classes of psychoactive drugs on SNARE complexes have been studied. Morphine was reported to reduce SNARE complex formation [232]. Although the antidepressants fluoxetine and reboxetine reduced the levels of all three SNARE proteins, SNARE complex formation was unchanged by these drugs [423]. Several other studies used screening strategies in peptide libraries, and from plant extracts to identify compounds that alter SNARE complex formation [228, 313, 314, 424, 425]. Synthetic peptides that modify SNARE complex formation can protect against neurotoxicity in experimental systems [228], and could also be investigated for activity in animal models of psychiatric illness.

2.4.4 Possible limitations

Human brain tissue is invaluable to schizophrenia research, but carries with it inherent limitations. Post mortem interval is one possible confounding factor, although samples we obtained had short PMIs compared to other sample sets (Table 2.1). Since protein levels show little change or decrease with PMI [336], the larger PMI of control subjects would lessen protein differences between diagnostic groups. While pH has been implicated in the quality of gene

expression data [318, 426], there is less evidence for an effect of pH on protein levels [318]. Neither of these sample characteristics had an impact on the group differences we observed. It is possible that differences in life experience (hospitalization, social interaction, active psychosis) could contribute to SNARE alterations, as SNARE expression and complexes can be modified by neuronal activity [231, 246]. The SNARE protein-protein interactions we observed here were likely formed *in vitro*, and the relationship with SNARE mechanisms in living patients is uncertain. Overall, our results could indicate synaptic consequences, not mechanisms, of the disorder, providing important information and a possible treatment target. Commonly, post mortem sample sets are also limited by differences in ethnicity between subjects. Heterogeneity of this kind is minimized in our sample set, procured exclusively in Macedonia. Finally, we recognize that this sample set is relatively small. Replication in a different sample set will strengthen our conclusions.

In summary, alterations in SNARE proteins and SNARE protein-protein interactions provide a possible novel molecular mechanism contributing to abnormal brain function in schizophrenia. These alterations appear to be related to the disease state, and suggest novel targets for the development of therapeutics.

2.5 Supplementary materials and methods I

2.5.1 Tissue collection and screening

Brain tissue was collected from three chronic care psychiatric hospitals in the Republic of Macedonia, and from subjects with sudden deaths in the region of Skopje, Macedonia. Autopsies were performed by the Institute for Forensic Medicine (IFM) at the School of Medicine in Skopje. For deaths at the two psychiatric hospitals outside of Skopje, pathologists from IFM traveled to perform autopsies locally. Toxicological analysis was performed on blood, urine and

hair. The right cerebral hemisphere and cerebellum were sliced on glass plates, rapidly frozen and stored at –80°C. Frozen tissues were transported to the New York State Psychiatric Institute on dry ice and stored at -80°C.

Blood alcohol levels were measured at IFM, on every case. Screening for drugs of abuse or toxic levels of medications in serum was performed using a fluorescence polarization immunoassay. In New York, a cerebellar sample of frozen brain tissue from all cases was tested for psychotropic medications using capillary gas chromatography and mass spectrometry; tissue pH was also determined. Gross and microscopic pathology was investigated on fixed tissue of the opposite hemisphere by a neuropathologist (AJD) in New York.

Clinical information was procured by Macedonian research psychiatrists (BM, ZJ, ND), using the modified diagnostic evaluation after death (mDEAD) for all patients and a psychological autopsy (PA) interview with personal informants [322-324]. The final diagnosis using DSM-IV criteria was determined by a panel of clinicians in New York (BM, JJM, GR, AJD and others), as well as the Macedonian interviewer, from all available information and was never based solely on existing clinical diagnoses. Informed consent was obtained from family members and the study was approved by the Columbia/New York State Psychiatric Institute research ethics board.

2.6 Supplementary materials and methods II

2.6.1 Capture ELISA assay development and validation

Because minimal characterization of SNAREs has been undertaken using proteins obtained from human brain [264], we confirmed that SNARE proteins in human brain can and do interact as a complex (Figure 2.5). SDS-resistant complexes were detected within the molecular weight range

reported by others (60-200 kDa) [171, 231, 264, 427-429], although some studies report the major band to be at ~ 75 kDa [171, 206, 430].

2.6.2 Assay development using a microtitre plate

To better understand how alterations in individual SNARE levels affect synaptic function we developed a high-throughput method of measuring binary SNARE interactions in a microtitre plate (Appendix 1) as an alternative to bead-based immunoprecipitation methods. Benefits of such assays include higher throughput, better reproducibility and decreased assay time [431]. The microplate application enabled: assay of 44 samples in duplicate, per 384-well plate, assurance of signal linearity using a dilution curve for each sample, repetition of each experiment twice, and relation of each SNARE protein ELISA measurement directly to the capture assay measurements of protein-protein interactions.

Optimal assay conditions were determined in several ways. First, the amount of capture antibody (SP12) coated on a 384-well plate was optimized for high binding to the ELISA plate but with reduced chance of steric hindrance, allowing Fab portions of the antibody to be oriented away from the plate surface (Figure 2.6A). The effect of coating antibody concentration was assessed by measuring bound syntaxin (Figure 2.6B). At 5 μg/ml coating antibody concentration, syntaxin bound to SNAP-25 showed a log-linear relationship with total protein concentration.

The use of serially diluted samples ensured log-linear responses with varying amounts of captured SNAP-25. Sample dilutions gave a linear response over a 3 to 12-fold range (Figure 2.7A); smaller linear ranges were usually due to low signal at low concentration steps rather than saturation at higher concentrations.

The maintenance of increasing signal with higher homogenate concentrations confirmed that the amount of capture antibody (SP12) on the ELISA plate was not limiting. The amount of

captured SNARE complexes correlated with the total SNAP-25 measured in the direct ELISA (Figure 2.7B), suggesting that normalization to total SNAP-25 levels was necessary to account for differences in SNAP-25 levels in individual samples.

An experiment was performed to confirm that the detection of SNARE complexes was dependent on the presence of the α-SNAP-25 capture antibody, and not non-specific adherence of protein aggregates to the ELISA plate. The capture assay format was scaled up into 96 well plates. Instead of using a secondary antibody detection system, we extracted the total protein present in the wells using sample buffer, and used an immunoblotting assay to identify captured and interacting proteins. As seen in Figure 2.8, in the absence of any capture antibody, incubation of the blocked well with homogenate did not result in any adherence of SNARE proteins. In the presence of capture antibody but no homogenate (to control for possible signal from extraction of capture antibody and detection on the blot), no heavy or light chain was seen on the blot at the detection settings used. With the capture antibody present, incubation of homogenate in the wells revealed specific pull-down of SNAP-25 and syntaxin. The ubiquitous synaptic vesicle protein synaptophysin was not detected, potentially indicating specificity of the interactions detected. Under these detection conditions, VAMP could not be seen, although this is most likely due to low levels of protein (as illustrated by the fact that even after immunoprecipitation, VAMP signal is low relative to the other SNAREs, Figure 2.5).

Pull-down of SNARE complexes was further confirmed using the scaled-up capture assay, by probing unboiled well extracts with specific antibodies. Complexed proteins were captured from CTL and SCZ samples, wells were extracted and equal amounts of boiled and non-boiled samples were separated by electrophoresis, transferred to PVDF and probed for SNAP-25, syntaxin and VAMP. Stripping and re-probing the blot confirmed the existence of SDS-stable higher molecular weight SNARE complexes (Figure 2.9).

2.7 Chapter 2 tables

Table 2.1 - Frozen tissue sample characteristics.
All subjects received an autopsy. Clinical diagnoses were made using DSM-IV criteria using all available information by a panel of clinicians. All subjects were Caucasian. *Post mortem interval (PMI) was different between groups ($P = 0.001$). **Sample storage time was different between groups ($P < 0.001$).

Abbreviations: CTL – control, SCZ – schizophrenia, SA – schizoaffective, F – female, M – male, MI – myocardial infarction, MVA – motor vehicle accident, CO- carbon monoxide, CVA – cerebral vascular accident, TB – tuberculosis, BZ – benzodiazepine, AS – asthma medication, Caf – caffeine, alc – alcohol, N/A – not available/unknown, storage time – time between brain extraction and homogenization for ELISA assay; PMI – postmortem interval between death and autopsy
Drug abbreviations: L – levopromazine, P – promazine, C – chlorpromazine, H – haloperidol, F – fluphenazine, S – sulpiride, T – thioridazine, TF – trifluoperazine, CZ – clozapine, PM- pimozide, BZ – benzodiazepine (available without prescription in Macedonia).

Subject No.	Diagnosis	Sex	Age (yrs)	PMI (hours)	Storage time (wks)	Brain pH	Cause of death	Toxicology findings	Alcohol abuse	Smoker	Antipsychotic exposure
1	CTL	M	53	16	239	5.90	Homicide	Caf	No	Yes	
2	CTL	M	65	18	68	6.76	MI	Caf	No	No	
3	CTL	M	37	7	238	6.20	Heart failure (chronic myocarditis)	Negative	No	Yes	
4	CTL	M	81	22	255	6.28	MVA, pulmonary Contusion	Caf	No	N/A	
5	CTL	M	56	14	106	6.40	MI	Caf	No	No	
6	CTL	F	41	15	105	5.84	Homicide, gun shot	Caf	No	Yes	
7	CTL	M	36	20	239	6.30	MVA, cerebral contusion	BZ	No	Yes	
8	CTL	M	73	21	248	6.09	MI	Negative	No	Yes	
9	CTL	F	56	19	68	6.07	Traffic accident (pedestrian)	BZ, Caf	No	No	
10	CTL	M	26	15	256	5.44	Homicide	Caf	No	Yes	
11	CTL	F	79	33	238	6.00	CO intoxication (accidental)	Negative	No	No	
12	CTL	M	30	6	249	6.30	MVA	Negative	No	Yes	
13	CTL	M	35	10	238	6.50	MVA	Caf	No	Yes	
Mean, SD		3F: 10M	51.4, 18.8	16.6, 7.1*	196, 77**	6.16, 0.33				8 Yes, 4 No, 1 N/A	
14	SCZ	M	35	6	218	6.40	Ileus	Caf	Yes	Yes	L, P, C, H, F
15	SCZ	F	45	7	417	6.19	Pneumonia	Negative	No	Yes	C, P, H
16	SA	F	60	9	236	6.40	MI	BZ, CZ	No	No	L, S, T, F
17	SCZ	M	45	N/A	413	6.50	Accidental drowning	BZ, caf	No	Yes	N/A
18	SCZ	M	58	6	326	6.00	Pneumonia	Negative	No	Yes	C, L, T, F, H, CZ
19	SCZ	M	45	8	320	6.40	Undetermined	BZ, caf	No	Yes	N/A
20	SCZ	F	65	6	312	6.50	Cardio-respiratory insufficiency	Caf	No	No	C, L, P, TF, F
21	SCZ	M	33	15	390	6.50	Peritonitis	Negative	No	N/A	L, P, F, H
22	SCZ	F	77	6	396	5.90	MI	BZ, AS	No	Yes	L, P, T, H, F, CZ
23	SCZ	F	65	7	302	5.90	Cardio-respiratory insufficiency, cardiomyopathy	Negative	No	No	C, L, P, F
24	SA	M	53	8	235	6.30	MI	BZ, CZ	No	Yes	C, L, P, F, H, CZ
25	SCZ	M	46	14	392	6.40	Pancreatitis	Negative	No	Yes	L, C, P, F, H
26	SCZ	F	63	7	325	6.60	MI, TB, lobectomy	Negative	No	N/A	C, L, P, F
27	SCZ	M	61	9	236	6.50	TB bronchiectasis	Negative	No	No	L, C, T, H, F
28	SCZ	M	53	15	220	6.30	Asphyxia, pharyngeal bolus	Negative	Yes	Yes	C, P, L, F, PM
Mean, SD		6F: 9M	53.6, 12.1	8.8, 3.2	316, 74	6.32, 0.22				9 Yes, 4 No, 2 N/A	

Table 2.2 - Within-subject comparisons of SNARE measures.
Comparisons are made in each striatal area CTL and SCZ groups separately. *T*- and *P*-values are reported. *P*-values < 0.008 are statistically significant (*), correcting for 6 comparisons within a protein, or the protein-protein interaction.

		SNAP-25		Syntaxin		VAMP		SNAP-25 – syntaxin interaction	
		CTL	SCZ	CTL	SCZ	CTL	SCZ	CTL	SCZ
NAc - VMC	*t* =	-2.08	-3.11	-.485	-4.56	-1.03	-4.53	-.194	-3.77
	P =	0.06	0.0077*	0.64	0.0004*	0.32	0.0005*	0.85	0.0021*
DCd - VMC	*t* =	-.599	-5.37	-.174	-6.62	-.513	-6.12	1.78	-3.76
	P =	0.56	<0.0001*	0.87	<0.0001*	0.62	<0.0001*	0.10	0.0021*
DCd - NAc	*t* =	.931	-5.08	-.519	-4.63	.497	-3.34	2.28	-.538
	P =	0.37	0.0002*	0.61	0.0004*	0.63	0.0048*	0.04	0.60

Table 2.3 - Sample characteristics of fixed tissue.

Supplementary Table S1: At time of autopsy, tissue was sliced, placed in custom cassettes, fixed for 5 days in 10% formalin in phosphate-buffered saline (PBS), transferred to PBS with 0.02% sodium azide at 4°C until used. All samples were from Caucasian subjects. *Abbreviations*: CTL – control, SCZ – schizophrenia, SF – schizophreniform, F – female, M – male, CVA – cerebral vascular accident, TB – tuberculosis, AS – asthma medication, Caf – caffeine, alc – alcohol, N/A – not available/unknown, storage time – time between brain extraction and fixed tissue slicing; PMI – postmortem interval between death and autopsy. *Drug abbreviations*: L – levopromazine, P – promazine, C – chlorpromazine, H – haloperidol, F – fluphenazine, S – sulpiride, PM- pimozide.

Subject No.	Diagnosis	Sex	Age (yrs)	PMI (hours)	Storage time (wks)	Brain pH	Cause of death	Toxicology findings	Alcohol abuse	Smoker	Antipsychotic exposure
29	CTL	M	57	19	120	6.70	Lung contusions	Negative	No	N/A	
30	CTL	M	21	21	132	6.70	Hemorrhagic shock, homicide (stabbing)	Caf	No	N/A	
31	CTL	F	72	18	131	6.20	Heart failure	Caf	No	N/A	
32	CTL	F	50	22	82	6.10	Ileus (peritonitis)	Caf, Alc	Probable	N/A	
Mean, SD		2F: 2M	42.5, 24.4	20.0, 1.8	116, 24	6.38, 0.38					
33	SCZ	M	56	25	76	6.00	Pulmonary TB, respiratory failure	Caf, AS	No	N/A	C, L, S, F, PM
34	SF	M	24	21	127	6.70	Suicide (hanging)	Caf, Alc	No	N/A	C,P
35	SCZ	F	70	12	113	6.10	CVA, bronchopneumonia	N/A	No	N/A	C, L, P, F, H
36	SCZ	F	20	9	126	6.70	Suicide (jump from height)	Nicotine	No	N/A	H
Mean, SD		2F: 2M	50.0, 21.4	16.8, 7.5	111, 24	6.43, 0.32					

2.8 Chapter 2 figures

Figure 2.1 - SNARE protein levels and protein-protein interactions in human striatum. Significant post-hoc *F*-tests are indicated (*), $P < 0.05$ Bonferonni-adjusted. Differences that do not survive Bonferonni correction are indicated (*a*). All three SNARE protein levels were lower in the VMC of subjects with schizophrenia (A-C). Syntaxin-SNAP-25 protein-protein interactions were increased in the VMC (D). Horizontal lines indicate group means. Raw (untransformed) but re-scaled values are plotted for the three SNARE measures. *Abbreviations*: NAc – nucleus accumbens, VMC – ventromedial caudate, DCd – dorsal caudate, CTL – controls, SCZ – schizophrenia.

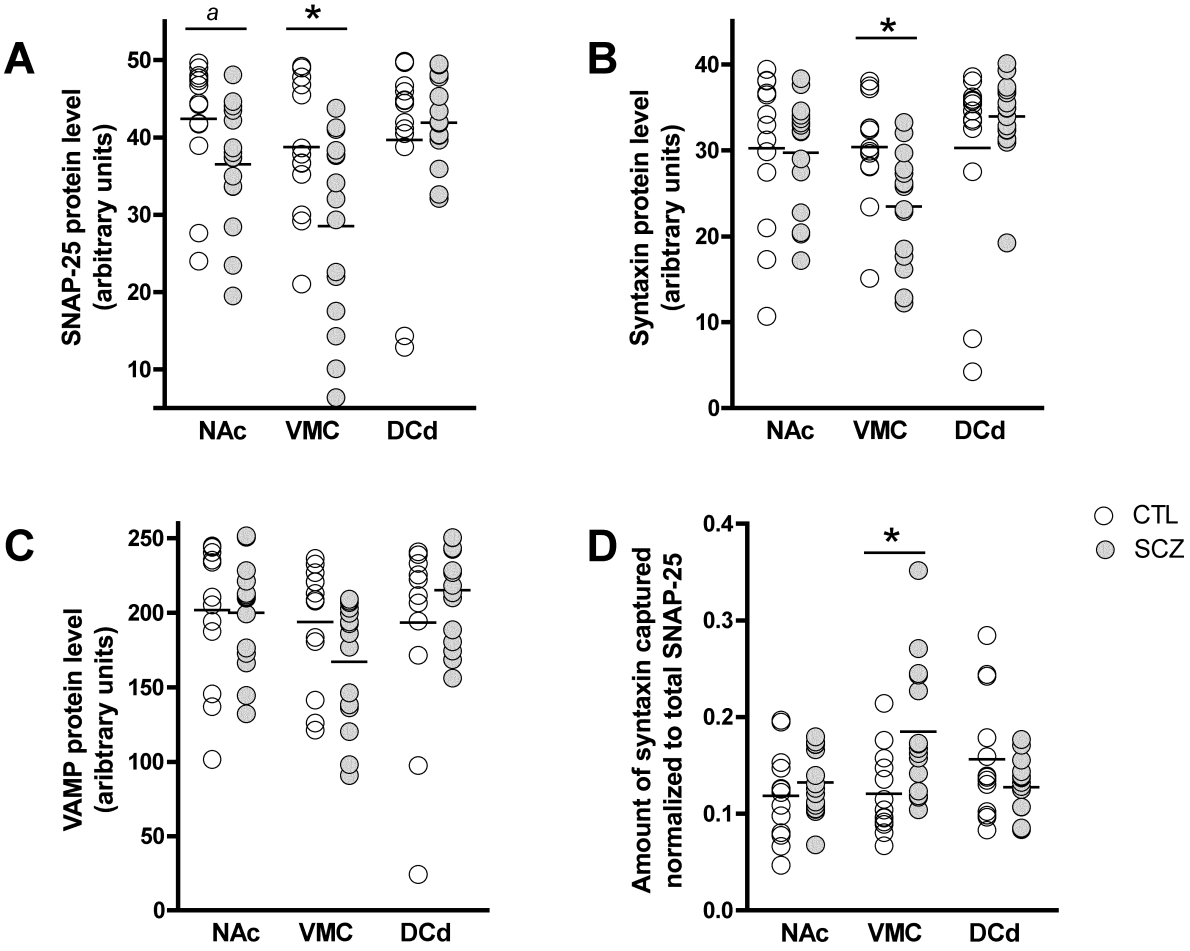


Figure 2.2 - Distribution of SNARE proteins in human VMC.
Single-channel images appear in the first two columns, with merged images in the third column. Arrows indicate the region of interest highlighted in the inset. Differences in staining intensity may be the result of slight differences in antibody concentration, antibody penetration, and image capture/processing conditions. Calretinin staining appears not to colocalize with presynaptic markers (A and B) and was used as a cell body stain for orientation within the tissue slice. Syntaxin, VAMP and SNAP-25 show some colocalization with each other (C and D, inset). *Abbreviations*: S25 – SNAP-25, CR – calretinin, STX – syntaxin.

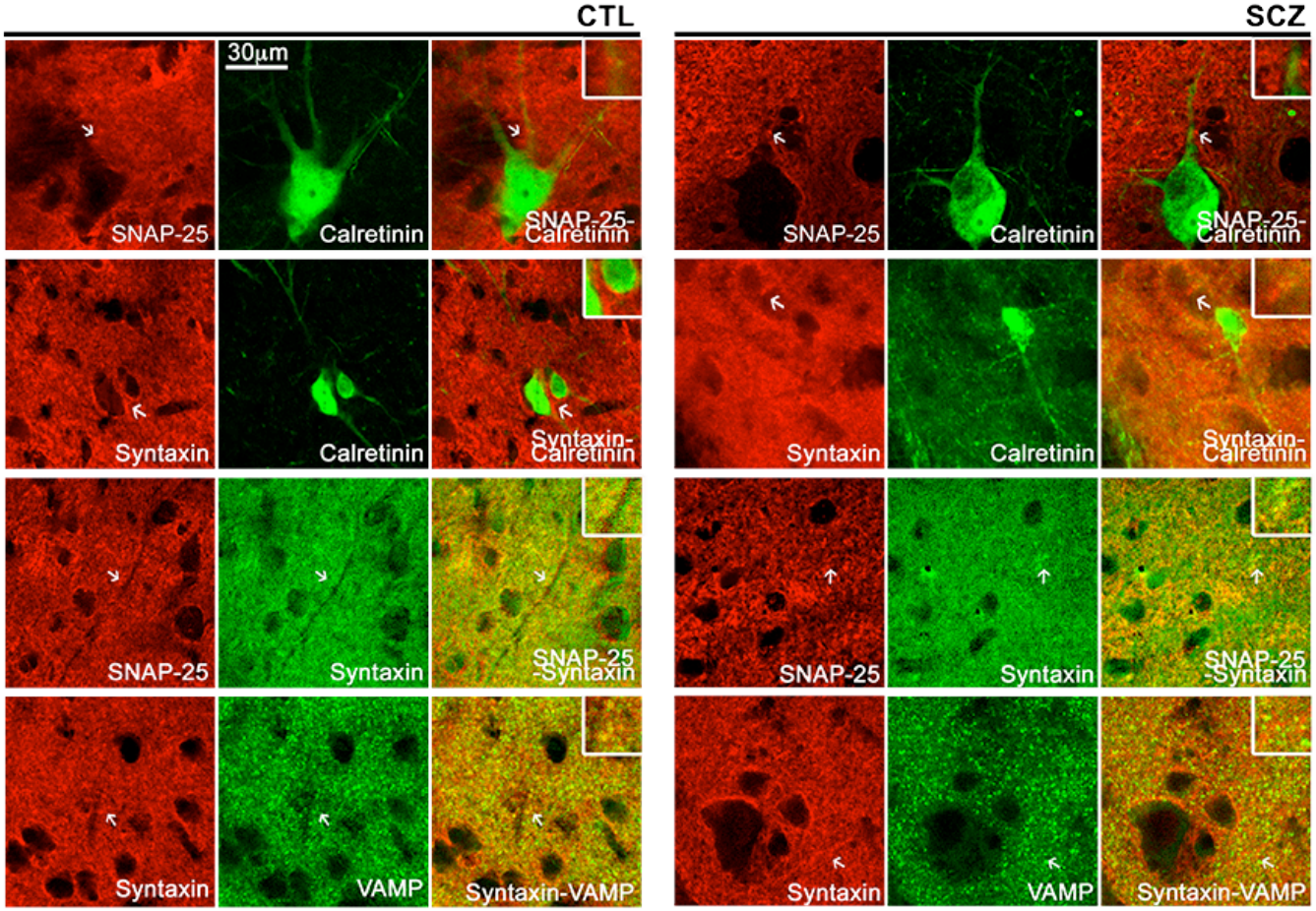


Figure 2.3 - SNARE protein levels and interactions in APD treated animals.
Significant effects of drug treatment ($P < 0.05$) were investigated using contrasts. Statistically significant differences between treatment groups and saline controls are indicated (all $P < 0.02$, Bonferonni-adjusted). Syntaxin-SNAP-25 protein interactions were not different between groups or striatal regions ($P > 0.05$) (D). Horizontal lines indicate group means (all regions combined). Raw (untransformed) but re-scaled values are plotted for the three SNARE measures.

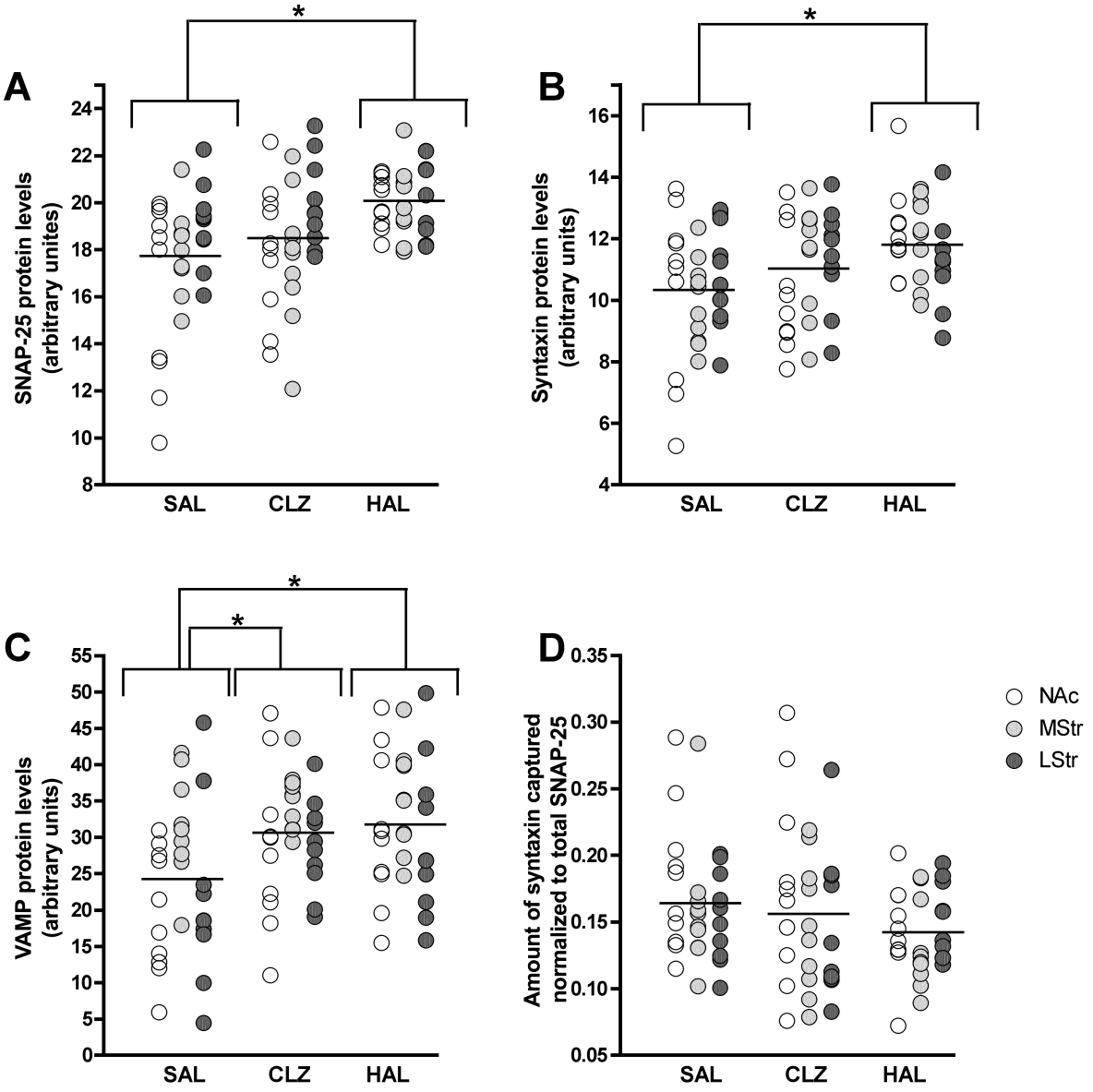


Figure 2.4 - Antibody specificity.

Supplementary Figure S1: Equal amounts of human cortical brain homogenate and rat brain homogenate were separated by SDS-PAGE and transferred to PVDF. Eight separate membranes were created and stained with individual antibodies. Peroxidase-conjugated secondary antibody was diluted 1:5000 and blots visualized by chemiluminescence.

For SNAP-25 (antibody SP12, 1:10), syntaxin (antibodies SP6 and SP7, 1:10), and synaptophysin (EP10, 1:10) immunostaining, 5 μg total protein was loaded. In the ELISA studies these antibodies were used at the same dilution, but the maximum amount of total protein loaded was 1.8 μg per well, with a linear range down to 0.03 μg per well. For VAMP (antibodies SP10 and SP11) immunoblotting, 30 μg total protein was loaded. In the ELISA studies with SP10, the maximum amount of total protein loaded was 3.6 μg per well, with a linear range down to 0.45 μg per well. The ELISA is more sensitive than the immunoblotting assay. For calretinin 10 μg of total protein was loaded. Calretinin antibody was used at 1:1000 dilution. All antibodies detected protein bands at expected molecular weights and gave one band, except calretinin that also produced fainter bands at higher molecular weights, more so in rat tissue than human tissue. Antibody specificity is illustrated by the single bands detected, as well as the greater prevalence of synaptic proteins in grey matter as opposed to white matter. Previous studies indicate SP12 detects a region of SNAP-25 shared between SNAP-25A and SNAP-25B, SP6/SP7 detect regions shared between syntaxin-1A and 1B, and SP10 detects VAMP 1 and VAMP 2 [264, 389]. The presence of syntaxin and SNAP-25 in white matter is due to the presence of these proteins in axons as well as terminals [118, 237, 432, 433].

Figure 2.4

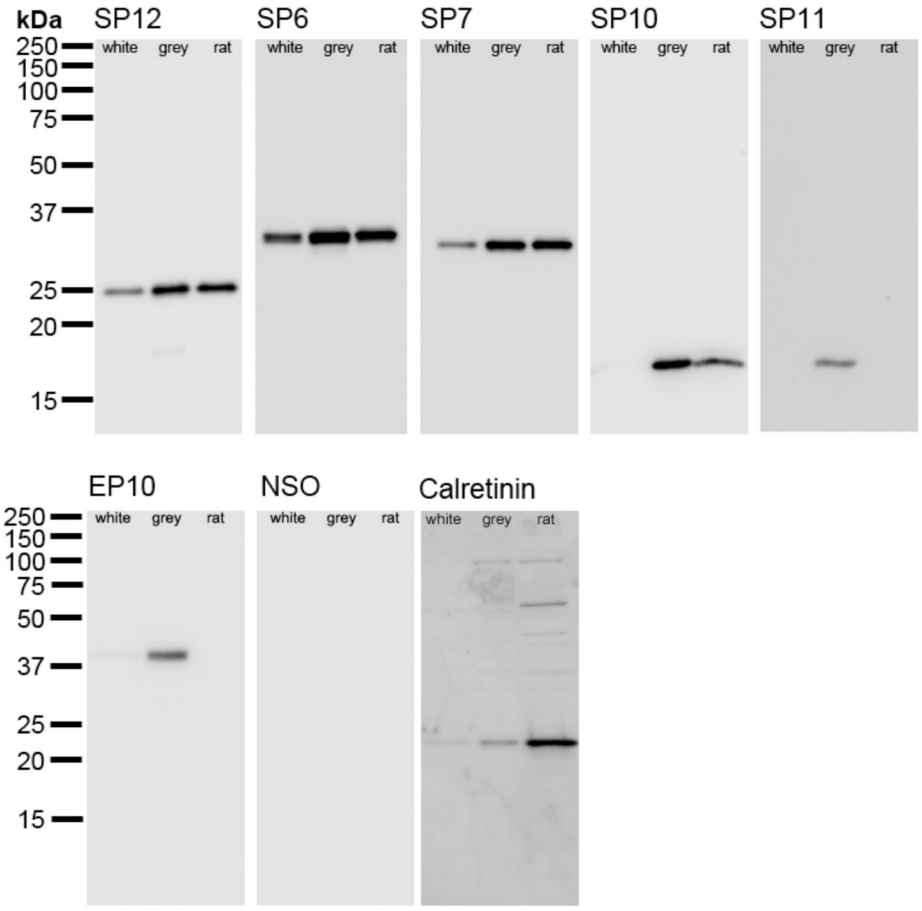

kDa
SP12
SP6
SP7
SP10
SP11
white
grey
rat
250
150
100
75
50
37
25
20
15
EP10
NSO
Calretinin

Figure 2.5 - SNARE complexes in human brain.
Supplementary Figure S2: (A) SDS-stable SNARE complexes in human brain homogenates. Human brain samples were diluted in sample buffer and either reduced and boiled (+) or left at room temperature (-) for five minutes prior to separation by SDS-PAGE. After transfer to PVDF, the membrane was cut, and the two halves were stained for either syntaxin or SNAP-25. The two pieces of membrane were imaged simultaneously. The presence of each SNARE in the ternary complex was ascertained by immunoblotting to detect monomeric SNARE proteins (lower bands) or complexed SNAREs (higher-molecular weight bands between 60 and 200 kDa) [264, 427, 434]. VAMP was detectable in boiled samples, but not in unboiled samples (not shown). This is likely due to epitope stability or low protein levels, and may require higher protein loading [264]. **(B)** Ternary SNARE complexes are detectable in human brain samples by immunoprecipitation. The blot has been stained for SNAP-25, syntaxin and VAMP, simultaneously. The identity of immunoprecipitated material was confirmed by loading immunoprecipitates on multiple gel lanes and probing each with antibodies against a single SNARE protein (not shown). Faint bands at approximately 60 and 18 kDa are detected with anti-SNAP-25 antibody, suggesting possible dimerization, post-translational modification and/or breakdown products of SNAP-25. Specificity of pull-down was confirmed by the failure to detect synaptophysin in immunoprecipitates (not shown here). Heavy and light chain from the SP12 (SNAP-25 antibody) are identified by comparing to antibody-coupled beads (IgG-SP12). In the absence of antibody (cntrl IP) no SNAREs are immunoprecipitated.

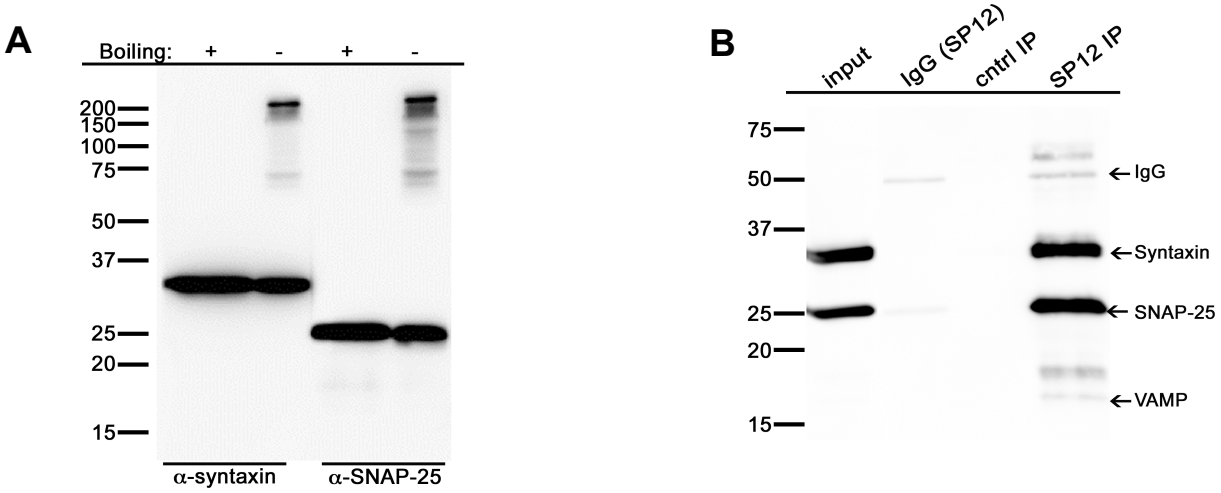


Figure 2.6 - Capture assay optimization.
Supplementary Figure S3: (**A**) Antibody binding-capacity of the ELISA plate. 15 µl of serially diluted coating antibody in TBS was incubated for 3 hours at 37°C. After blocking, bound antibody was detected with peroxidase-conjugated secondary antibody. Substantial binding occurred at antibody concentrations of 5 and 10 µg/ml. (**B**) Effect of varying amounts of coating antibody on amount of syntaxin bound to captured SNAP-25. Each well of a microtitre plate was incubated with 15 µl of α-SNAP-25 antibody at varying concentrations. Capture ELISA was performed as described, detecting bound syntaxin using a biotinylated-α-syntaxin antibody followed by streptavidin-conjugated HRP. Low concentrations of coating antibody result in insufficient pull-down of SNARE complexes, illustrated by the lack of a dilution effect when varying homogenate concentration (x-axis) at 2 and 0 µg/ml conditions.

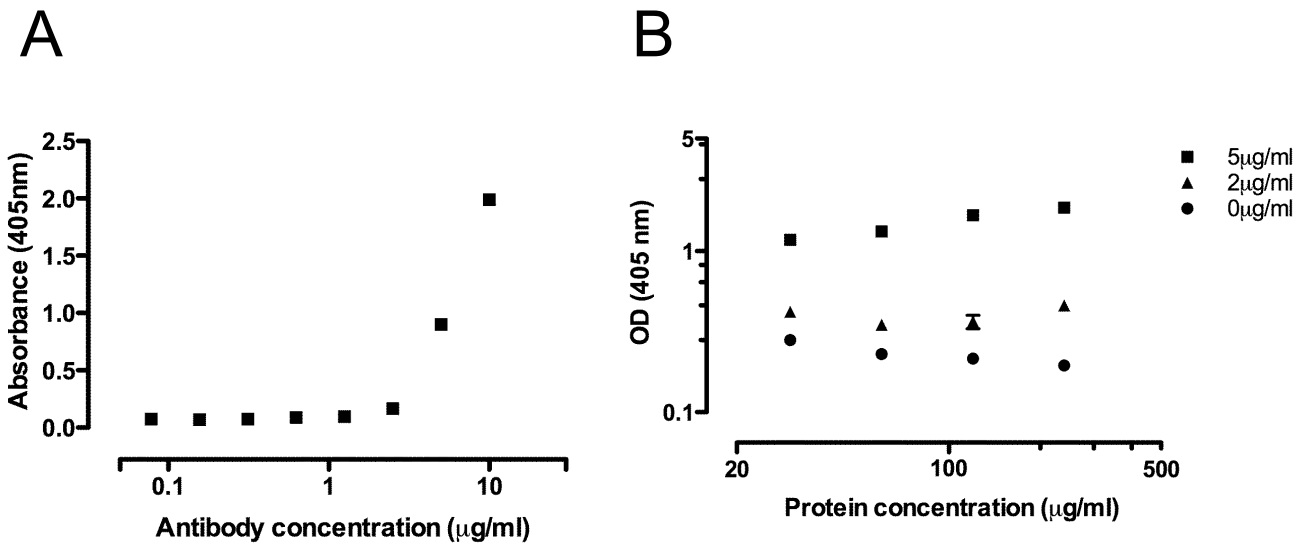


Figure 2.7 - Capture ELISA measurements.
Supplementary Figure S4: **(A)** Raw optical density of syntaxin interacting with bound SNAP-25 at various homogenate concentrations. Each well was coated with 15 μl of SP12 and incubated with varying dilutions of homogenate. Error bars represent the standard mean error of a sample run in duplicate on three plates. **(B)** The relationship between the amount of SNAP-25 measured by direct ELISA and the amount of syntaxin detected with a capture ELISA. Data represents values of 28 VMC samples. In general, less syntaxin was bound to captured SNAP-25 in samples containing less SNAP-25.

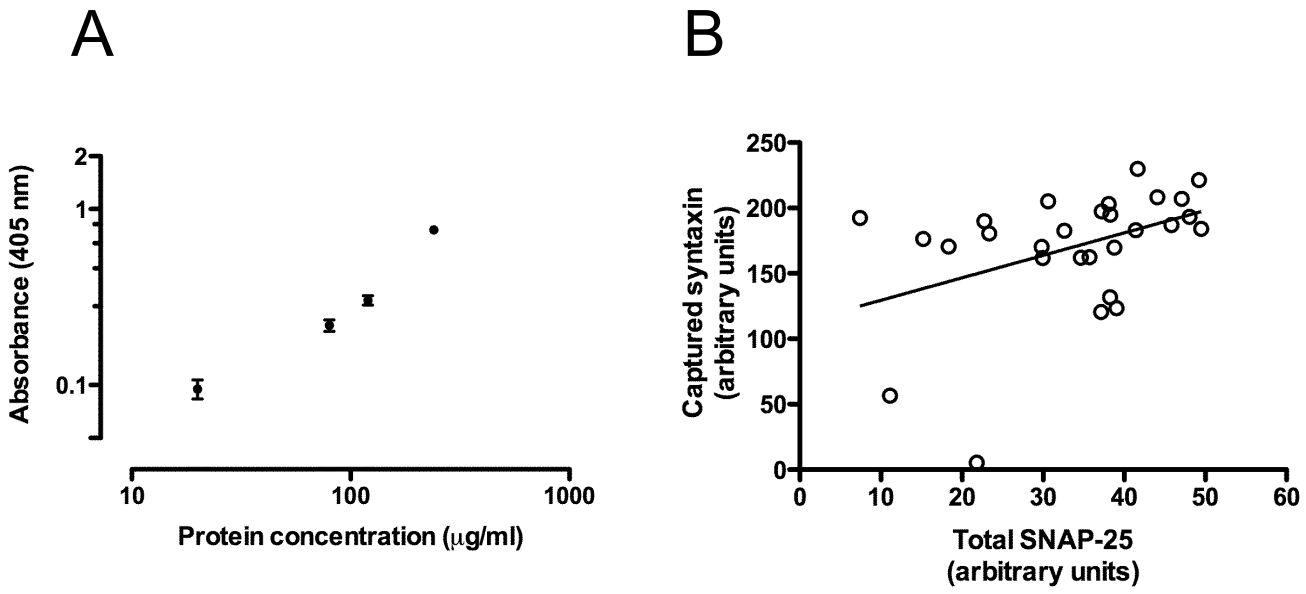


Figure 2.8 - Specificity of protein interactions detected by capture ELISA.
Supplementary Figure S5: Samples were prepared on 96-well plates as follows: A – no capture antibody in the sample well; B – no homogenate added to a coated sample well; C- regular assay conditions with both capture antibody and homogenate added. Protein complexes captured were extracted with SDS-sample buffer, equal volumes of conditions A, B and C were loaded multiple times and separated by SDS-PAGE. After transfer, the membrane was cut and single pieces probed for SNAP-25, syntaxin or synaptophysin. Specificity of pull-down is confirmed by the lack of synaptophysin detected in lane C.

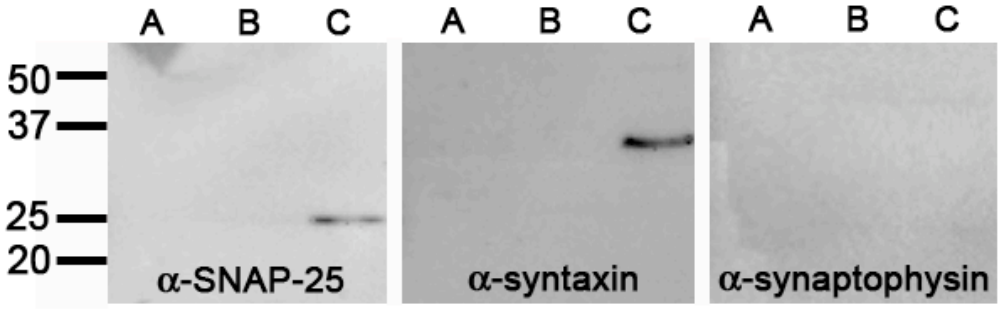
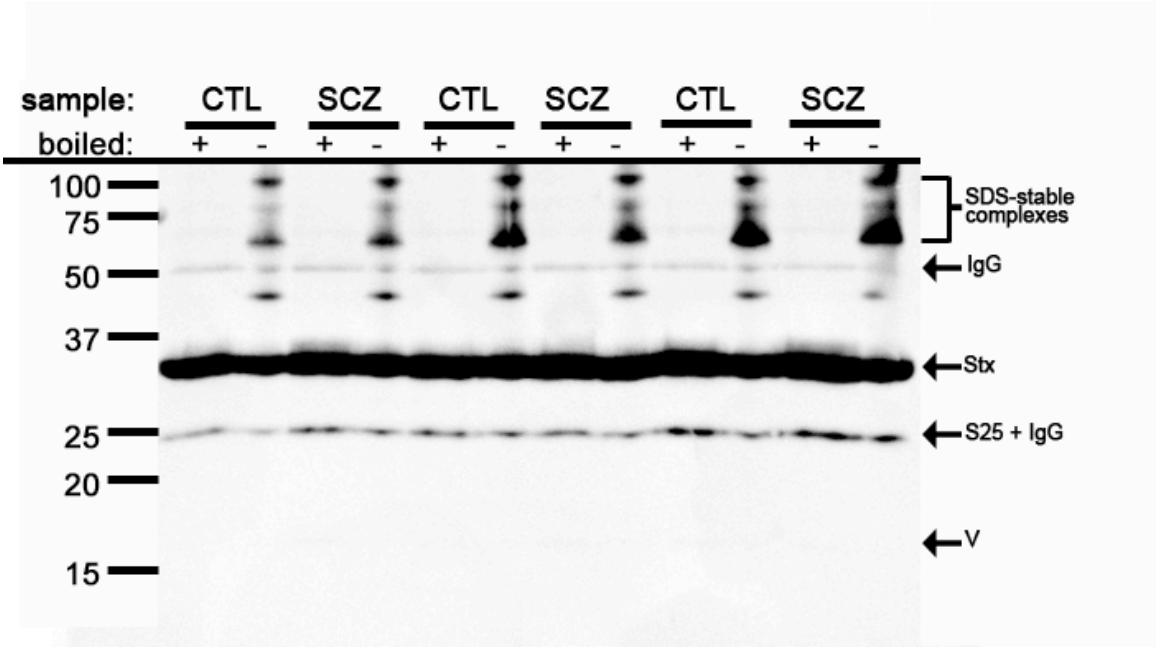


Figure 2.9 - Complexed SNARE proteins are pulled-down in the capture ELISA assay. Supplementary Figure S6: Captured samples from CTL and SCZ subjects were either boiled (+) or left at room temperature (-) prior to separation by SDS-PAGE. Blots were probed for all three SNARE proteins, and stripping and re-probing the blot confirmed higher molecular weight bands contained syntaxin and SNAP-25. VAMP was detectable in monomeric form in boiled samples when the sensitivity of the detector or exposure time was increased. *Abbreviations:* S25 – SNAP-25, Stx – syntaxin, V – VAMP, CTL – control, SCZ – schizophrenia



3 The relative expression of striatal syntaxin-1 isoforms is altered in schizophrenia[4]

3.1 Introduction

Synaptic dysfunction is postulated to underlie the behavioural abnormalities and cognitive deficits observed in schizophrenia [91, 296]. The balance between glutamatergic and GABAergic neurotransmission, and abnormalities in dopamine release may contribute to brain dysfunction [45, 59, 139], and may alter fronto-striatal circuit function [94, 139, 402]. Molecules involved in synaptic transmission, plasticity and connectivity may therefore contribute to the pathophysiology, progression and treatment of the disease. Converging evidence implicates the presynaptic protein syntaxin-1 in this regard.

Syntaxin-1 is one of the three soluble-NSF-attachment receptor (SNARE) proteins that interact to facilitate neurotransmission [96, 163]. There are two isoforms of syntaxin-1 – 1A and 1B, coded for by different genes [169, 235]. Both are important for synaptic and brain function and may be involved in synaptic plasticity [159, 242, 245]. Syntaxin isoforms are differentially expressed in rat brain [237], within some cells and between cell types [239, 435]. Syntaxin isoforms are differentially enriched in glutamatergic and GABAergic terminals; syntaxin-1A is present primarily in glutamatergic synapses in rat cortex, while 1B is present in both types of synapses [201, 204]. Isoform expression in dopaminergic terminals remains unknown. However, sensitizing the dopamine system in rats with a paradigm of amphetamine administration then withdrawal, resulted in up-regulation of syntaxin-1B mRNA in the nucleus accumbens shell, with no effect on syntaxin-1A [243]. Additional evidence of functional divergence between isoforms is plentiful. Syntaxin-1A and 1B show differential affinity for isoforms of another

[4] **A version of this chapter will be submitted for publication:**
Barakauskas VE, Beasley CL, Barr AM, Rosokilja G, Mann JJ, Mancevski B, Jakovski Z, Davceva N, Ilievski B, Dwork AJ, Falkai P, Honer WG. (2010). The relative expression of striatal syntaxin-1 isoforms is altered in in schizophrenia.

SNARE protein, VAMP [241]. Syntaxin-1A interacts with other presynaptic molecules such as calcium channels as well as dopamine and GABA reuptake transporters [199, 406, 436-440] and is important in fear memory consolidation and extinction, as well as hippocampal LTP [242]. In contrast, learning a working memory task is associated with an increase in syntaxin-1B, but not 1A mRNA expression in the hippocampus, and learning a spatial reference memory task increases syntaxin-1B in the rodent prefrontal cortex and nucleus accumbens shell [245, 246].

In schizophrenia, lower than expected levels of syntaxin protein were reported in ventromedial caudate [441], with evidence for increased interaction between syntaxin-1 and SNAP-25, another SNARE protein. In contrast to this finding in striatum, other reports indicated higher levels of syntaxin in cingulate cortex [260, 389] and unchanged levels in frontal cortex of patients [260, 264, 293]. Genetic studies provide additional evidence for a role of syntaxin in human illness. Williams-Beuren syndrome is associated with a chromosomal deletion encompassing the syntaxin-1A gene [290], and involves significant cognitive and behavioural abnormalities that include impaired fronto-striatal processing [291]. Syntaxin-1A expression in lymphoblast cells derived from patients with Williams-Beuren syndrome correlates with IQ [292]. Variation in the syntaxin-1A gene has also been associated with schizophrenia [180].

The distribution of syntaxin-1A and 1B in human brain is unknown. Previous investigations of syntaxin-1 protein in schizophrenia did not distinguish between the two isoforms. One study appeared to resolve the two isoforms, but statistical analaysis of the two immunoreactive protein bands did not detect differences between groups (the sum of the two bands, however, was statistically lower in schizophrenia subjects) [258]. Using the same series of samples in which we observed lower levels of total syntaxin-1 protein in the ventromedial caudate, we examined syntaxin-1A and 1B isoforms. The amount of syntaxin-1A, enriched in glutamatergic terminals, was compared with syntaxin-1B, found in glutamatergic as well as

GABAergic terminals. A lower ratio of syntaxin-1A to 1B in schizophrenia would be consistent with lower than expected levels of striatal glutamate transporters reported in schizophrenia [52, 403]. The possible association between relative isoform expression and SNARE protein interactions was investigated to determine whether an imbalance in syntaxin isoforms could contribute to the altered interactions observed in schizophrenia [441]. The possibility of differential distribution of syntaxin-1A and 1B within synapses of one striatal region was addressed using confocal microscopy.

3.2 Methods

3.2.1 Tissue samples

Post mortem tissue samples were obtained from the Macedonian/New York State Psychiatric Institute Brain Collection. The sample set included 15 subjects with schizophrenia or related illness (13 schizophrenia, 2 schizoaffective disorder; SCZ) and 13 non-psychiatric comparison subjects (NPC) (Table 3.1). Tissue collection and screening is detailed elsewhere [441] (Section 2.5). Briefly, clinical information was collected by Macedonian research psychiatrists using the modified diagnostic evaluation after death (mDEAD) and a psychological autopsy interview with personal informants [322-324]. A consensus diagnosis was determined using DSM-IV criteria by clinicians in New York and the Macedonian interviewer, obtained from all available information. Informed consent was obtained from family members and tissue collection based on a protocol approved by the Columbia/New York State Psychiatric Institute research ethics board.

Toxicological analyses for drugs of abuse and psychotropic agents were performed on each case. All SCZ subjects had exposure to multiple first-generation (typical) antipsychotics (including: levopromazine, promazine, chlorpromazine, haloperidol, fluphenazine, sulpiride, thioridazine, trifluoperazine, and/or pimozide) and 4 SCZ subjects also had exposure to the

second-generation antipsychotic clozapine (as determined by mDEAD or toxicology). Toxicological analysis revealed caffeine and benzodiazepines (BZ) in both NPC and SCZ subjects; BZ are available without prescription in Macedonia and their use in NPC subjects may not be due to psychiatric illness. Only two SCZ subjects had detectable levels of antipsychotic medications (clozapine) at time of death. More than half were smokers (8/13 NPC and 9/15 SCZ).

Striatal samples were dissected from frozen coronal tissue slabs of the right hemisphere, using a standard human brain atlas [408]. Subregions included dorsal caudate (DCd), nucleus accumbens (NAc) and ventromedial caudate (VMC), Figure 3.1. Prior to analyses, frozen tissue was rapidly thawed, homogenized in tris-buffered saline (TBS: 10mM Tris-HCl, 140 mM NaCl, pH 7.4; 1.5 ml per mg of tissue), aliquoted and frozen at -70°C until further use. Total protein concentration of one aliquot was measured with a modified Lowry assay (Biorad, Mississauga ON) using BSA as a reference standard.

3.2.2 Immunoblotting

Syntaxin-1 isoforms were resolved using 10% SDS-PAGE gels containing 6 M urea [163, 237]. Samples were diluted in sample buffer (62.5 mM Tris-HCl, pH 6.8; 25% v/v glycerol, 2% SDS v/v, 0.01% v/v bromophenol blue, 5% v/v β-mercaptoethanol) to a final protein concentration of 0.3 μg/μl and heated at > 95°C for 5 minutes prior to gel loading. Five microlitres (1.5 μg total protein) per sample was loaded onto each gel. Gels were run so that both NPC and SCZ samples were present on one gel; 5-9 samples were run on each gel. To control for blot-to-blot variation and to account for potential differences in antibody-binding affinity, one sample within the set was used as a reference sample. The reference sample was serially diluted over a 39-fold range,

diluted in sample buffer, heated and the dilution series was loaded on each gel (6.25 μg to 0.16 μg total protein).

After electrophoresis, gels were transferred to nitrocellulose (1 hr, 250 mA). Membranes were blocked in 5% w/v non-fat milk powder dissolved in 0.05% v/v Tween-20/TBS (TBST) for one hour at room temperature (RT). Syntaxin-1A and 1B were detected using the same antibody (SP6) [389] which detects both isoforms (Figure 3.2). SP6 from hybridoma tissue culture supernatants was diluted 1:10 in milk/TBST, and membranes were incubated for 1 hour at RT. After washing, membranes were incubated with biotinylated goat-anti-mouse secondary antibody (1:5000 dilution in milk/TBST) (Jackson Immunoresearch Laboratories Inc., West Grove PA) and washed again. Bands were visualized by incubating with streptavidin-conjugated qDot705 (Molecular Probes, Eugene OR) diluted 1:1000 in TBST. After a final TBST wash, blots were dried. Membranes were imaged using a Fuji LAS-3000 imager (Fuji Lifesciences, Tokyo Japan) in fluorescence mode, with 488 nm excitation, a 710AF40 bandpass filter and flat-frame correction. Blots were imaged at multiple exposure times to ensure that an image without saturated pixels was obtained for quantification (Figure 3.3A). To control for loading using an approach that provided additional biologically relevant data, blots were re-probed for synaptophysin using the EP10 antibody [389, 442] following the same procedure as for syntaxin. It was confirmed using an independent assay that synaptophysin levels did not differ between groups or between striatal regions (Appendix 3).

Samples were assayed twice, on different days. Samples were coded to mask investigators to diagnosis and sample characteristics.

3.2.3 Blot analysis

Fluorescence intensity of the detected bands was analyzed using the "profile mode" in ImageGaugeV4.22 software for Macintosh (Fuji Lifesciences, Tokyo, Japan). Because the synaptophysin band was at times not fully resolved from syntaxin-1B, for synaptophysin, half of the intensity peak (the lower half of the band) was quantified. Overlap of synaptophysin and syntaxin-1B did not affect syntaxin-1B quantification because EP10 was used only after syntaxin-1 staining was quantified. A linear standard curve of intensity versus total protein loaded was constructed using the serially diluted reference sample, on each blot, for each protein separately (Figure 3.3B). The equivalent amount of reference protein needed to give the same band intensity as the sample (in μg) was interpolated for each lane. Higher values indicate higher antigen amount present. The ratio of syntaxin-1A to 1B, as well as each isoform to synaptophysin was constructed using these protein values, for each sample, on each experiment day. The mean value of the two experimental days was taken; paired t-tests indicated that the replicate data did not differ significantly from each other (data not shown). Four samples in the VMC had immunoblot data for only one day (due to assay failures related to complications including air bubbles or gel anomalies).

3.2.4 Statistical analysis

The ratio of syntaxin-1A to 1B, and the individual isoform values were analyzed separately. Normality and equal variance between groups were evaluated using Shapiro-Wilk's and Brown-Forsythe tests, respectively. A two-way ANOVA, with diagnosis as the between-subjects factor and striatal region as the within-subjects factor was performed; multivariate F-values are reported. Statistical significance was set at $p < 0.05$ for main effects. Associations of each variable with sample set characteristics (age, PMI, pH, sample storage time) were investigated

using Spearman correlations with both groups combined. Analyses were performed using JMP 8.0 (SAS Institute Inc., Cary NC).

3.2.5 Immunohistochemistry

Formaldehyde-fixed 30 μm sections from a non-psychiatric comparison subject and a schizophrenia subject were used to investigate the distribution of syntaxin isoforms in human VMC, employing isoform-specific antibodies to differentiate between syntaxin-1A and 1B. Tissue was stained using previously published protocols with some modifications [441]. Primary antibody dilutions were as follows: EP10 (anti-synaptophysin) 1:10, syntaxin-1A and syntaxin-1B 1:250 (Synaptic Systems, Gottingen Germany). Sections were incubated with subtype and species-specific fluorescent secondary antibodies (Alexa-488, 555 or 647) at 1:500 dilution for 1 hour at 37°C, washed and mounted in FluorSave (Calbiochem, EMD Chemicals, Gibbstown NJ).

The degree of autofluorescence was ascertained by including a section processed using culture-conditioned media instead of primary antibody. These were imaged using identical acquisition parameters used for the stained section.

A laser scanning confocal microscope (LSM 5 Pascal, Zeiss) with a 63x/1.2W C-Apochromat water immersion objective was used. Excitation wavelengths of 488 nm, 543 nm and 633 nm were used with 505-530BP, 560LP and 650LP emission filters. Scanning was performed sequentially, with line averaging of 16 times, pinhole size ≤ 1 Airy unit and resolution of 512 x 512 pixels. 63x images were initially taken to orient within the tissue slice, and then 5 subfields were imaged using an electronic zoom factor of 5 (0.056 μm/pixel resolution). Image acquisition parameters (gain and offset) were set so as to minimize pixel saturation. Colocalization of syntaxin isoforms and synaptophysin was explored using colocalization

estimates. Automatic threshold selection using a non-parametric method for bi-level image segmentation was first applied to each image [443, 444]. Colocalization coefficients were then calculated for thresholded images using the JaCOPv2.0 plugin for ImageJ (NIH, Bethesda, MD) (*http://rsb.info.nih.gov/ij/plugins/track/jacop.html*) [445]. The overlap coefficient, OC, as described by Manders [446], was used as an alternative to Pearson's correlation coefficient, as it is describes the degree of overlap of signals from two channels, represents true colocalization, and is less affected by differences in intensity between two antigens [447]. OC values range from 0 (no colocalization) to 1.0 (complete colocalization). Manders' colocalization coefficients, M1 and M2 were also calculated. These coefficients describe the percentage of signal of a given antigen that is colocalized with the second antigen, and therefore allowed us to determine the percentage of each syntaxin-1 isoform colocalizing with a synaptic marker (synaptophysin) [447]. A total of 25 fields of view from one tissue slice were assessed for colocalization in two subjects. For figure assembly only, images were processed and assembled using Photoshop CS4 (Adobe, San Jose CA).

3.3 Results

3.3.1 Sample characteristics

A summary of the demographic characteristics of the sample set appears in Table 1. We previously investigated SNARE protein levels and interactions in this exact sample set; detailed sample-by-sample descriptions can be found elsewhere, [441] or Table 2.1. Briefly, age and pH were similar between groups ($P > 0.05$). NPC samples had a longer mean PMI: 16.6 versus 8.8 hrs ($t = 3.73$, df = 25, $P = 0.001$), while SCZ samples were stored longer: 316 versus 196 weeks ($t = 4.21$, df = 25, $P < 0.001$).

3.3.2 Syntaxin-1 isoform ratio in schizophrenia

Syntaxin-1A:syntaxin-1B (Stx1A:1B) data were normally distributed with equal variances between groups. Possible associations between age, postmortem interval, duration of storage and pH and the isoform ratio were investigated using Spearman correlations. Positive correlations were observed between age and Stx1A:1B in all three striatal regions (Table 3.2). No other associations were observed, and age was therefore added as a covariate to examine differences between the diagnostic groups, and between striatal regions. Two-way analysis of variance was carried out with diagnosis as a between-subjects factor, region as a within-subjects repeated measure, and age as a covariate. Statistically significant effects were observed for diagnosis (F = 6.76, df = 1,25, P = 0.016) and age (F = 27.35, df = 1,25, $P < 0.0001$) (Figure 3.4A and Table 3.3). No differences between striatal regions, or interaction between diagnosis and region were observed. Overall, in schizophrenia the mean value of Stx1A:1B was decreased slightly, an effect that appeared consistent across the age range studied (Figure 3.4B).

Primary analyses were repeated to assess the effect of smoking status on syntaxin isoform measures in the striatum. Combining the NPC and SCZ groups (n = 25; 3 subjects were missing smoking data), smoking status was entered as the between-subjects factor and striatal area as the within-subjects factor. There was no difference in striatal Stx1A:1B ratio in subjects who were smokers as compared to those who were not ($P > 0.05$). We also examined the possible effects of drugs detected in the toxicological analysis. Excluding samples where benzodiazepines were detected (n = 2 NPC, n = 5 SCZ) the effect of diagnosis on Stx1A:1B remained significant (F = 7.41, df = 1,18, P = 0.014). Similarly, excluding the two SCZ samples where antipsychotics (both clozapine) were detected did not alter the effect of diagnosis on Stx1A:1B (F = 6.81, df = 1, 23, P = 0.016).

We also analyzed the levels of the individual isoforms of syntaxin. Stx1A and Stx1B (expressed as μg of serially diluted reference sample) were normally distributed with equal variances. There were no statistically significant differences between levels of syntaxin-1 isoforms related to diagnosis (Table 3.3). Normalizing the syntaxin isoform values by the amount of synaptophysin immunoreactivity detected in the same lane (as a loading control) made no difference. The total mean levels of syntaxin-1 assessed by immunoblotting were lower in the schizophrenia samples, however differences were not statistically significant. There was a statistically significant correlation between total syntaxin-1 as measured by immunoblotting and the previously published syntaxin ELISA data (Chapter 2) for the same samples ($r_s = 0.48$, $P < 0.0001$).

3.3.3 Syntaxin isoform ratio and syntaxin-SNAP-25 protein-protein interactions

The interaction between syntaxin-1 and a second SNARE protein, SNAP-25 was previously measured in the striatum in the same sample set [441] and demonstrated greater complex formation in schizophrenia. We carried out an exploratory analysis of the relationship between the Stx1A:1B ratio and syntaxin-SNAP-25 interactions using Spearman correlations in each striatal area (Table 3.4). Syntaxin-SNAP-25 protein complexes were negatively correlated with the Stx1A:1B ratio in the VMC; the greater complex formation (as reported in schizophrenia) was associated with lower Stx1A:1B ratio. Correlations were not statistically significant in the diagnostic groups analysed separately, or in the NAc or DCd.

3.3.4 Colocalization of syntaxin isoforms in VMC

Immunostaining in human VMC using isoform-specific antibodies against syntaxin-1A and syntaxin-1B demonstrated that syntaxin-1 staining is more diffuse than that of synaptophysin (Figure 3.5), consistent with its presence in axons as well as terminals. Stain contrast between white-matter tracts and grey matter was less obvious for syntaxin-1B than for syntaxin-1A. As expected, synaptophysin staining was punctate, consistent with its presence in synaptic vesicles, and was absent from white-matter tracts and cell bodies. Colocalization was explored using conventional colocalization measures, the overlap coefficient (OC) and Manders' colocalization coefficients (M1, M2) [446, 447]. Overlap coefficients were greater than 0.9 for all pair-wise comparisons (Stx1A-Stx1B, Stx1A-physin, Stx1B-physin) indicating a high degree of antigen colocalization. Manders' colocalization coefficients indicated that approximately 55% of synaptophysin signal overlapped with syntaxin-positive pixels (mean M1, similar % for Stx1A and Stx1B). Twenty-two percent of syntaxin isoform signal overlapped with synaptophysin (mean M2), suggesting that a large proportion of syntaxin-1 is not present in synaptic sites delineated by synaptophysin. Syntaxin-1 isoform colocalization involved only ~50% of signal from each channel (mean M1 = 52%, mean M2 = 51%), suggesting that isoforms may be differentially distributed even within a brain region.

3.4 Discussion

Our results indicate that the ratio of syntaxin-1A to 1B in striatum is significantly lower in schizophrenia, and decreases with age in both the schizophrenia and NPC samples. The balance between syntaxin isoforms is not significantly different between striatal regions, but appears related to SNARE protein-protein interactions in the VMC.

3.4.1 Syntaxin isoform ratio in schizophrenia

The Stx1A:1B ratio was slightly lower in schizophrenia subjects in the striatum as a whole, as compared with the NPC group (Figure 3.4), and may reflect changes in the balance of excitatory/inhibitory neurotransmission in the striatum. Recent work suggests that syntaxin isoforms are differentially expressed in glutamatergic and GABAergic synapses [201, 204]. Colocalization and immunoprecipitation studies in rat cortex found syntaxin-1A to be present in VGluT1-positive puncta, but virtually absent from VGAT- positive puncta, while syntaxin-1B was present in both terminal types [204]. Similarly, proteomic analysis of synaptic vesicles found that syntaxin-1A expression is significantly higher in VGluT1-positive vesicles as compared with VGAT-positive vesicles, while syntaxin-1B is not differentially enriched between the two [201]. The possibility that different syntaxin-1 isoforms are associated with different terminal types in the human striatum is supported by our immunohistochemical investigation. Only 55% of synaptophysin-positive synaptic terminals in the VMC contained a syntaxin isoform, and syntaxin isoforms colocalized only 50% of the time, suggesting partial separation into distinct types of striatal terminals.

Recent hypotheses of striatal synaptic dysfunction in schizophrenia include integrated abnormalities of glutamate and dopamine. Lower levels of glutamate transporters [52, 403] and lower density of cortical afferents projecting to the striatum are described in schizophrenia [312, 448]. Our results may reflect this reduced cortical input. Since syntaxin-1A is enriched in glutamatergic synapses [204], lower numbers of glutamatergic inputs to the striatum would produce a lower Stx1A:1B ratio. An additional source of synaptic number/density changes in the striatum may include dopaminergic inputs, which are increased in schizophrenia subjects who respond to antipsychotic treatment [147]. The distribution of syntaxin-1 isoforms in dopamine terminals in humans remains unknown. Interestingly, following an amphetamine sensitization

protocol, syntaxin-1B mRNA is increased in the rat nucleus accumbens shell, with unchanged syntaxin-1A mRNA [243]. This would correspond to a decrease in the relative amount of syntaxin-1A:1B mRNA. Thus, the lower Stx1A:1B ratio observed in the present striatal samples could reflect a lower level of syntaxin-1A with no difference in 1B (which would be consistent with an increase in glutamatergic terminal numbers), but it is also consistent with a higher syntaxin-1B expression with no difference in 1A (perhaps due to changes at non-glutamatergic terminals). Parcelation of the Stx1A:1B ratio into its components (Table 3.3) alludes to the possibility that that each scenario could occur in different striatal regions.

3.4.2 Syntaxin isoforms and age

Age and the Stx1A:1B ratio were correlated in all three regions of the striatum, although correlations were stronger in more ventral regions (Table 3.2). Age-related changes in glutamate-induced release of dopamine or GABA have been documented, and occur in a region-specific manner, with decreases in the former but increases in the later occurring in ventral but not dorsal striatum in rodents [449]. While this phenomena may partially be explained by differences in the types of glutamate receptors involved [449], pre-synaptic mechanisms could also play a role. Interestingly, the correlation of Stx1A:1B with age was more pronounced in NPC as compared with SCZ samples (Table 3.2). If the Stx1A:1B ratio changes along with the glutamate-GABA-dopamine balance in the aging striatum, this suggests that the balance of neurotransmitters in schizophrenia may be more similar to that of older NPC individuals in the VMC. Investigation of possible interactions between age and disease on syntaxin-1 isoform expression in a larger sample set may be warranted.

3.4.3 Syntaxin isoforms and SNARE protein interactions

One of the ways that syntaxin-1 isoforms may affect brain function is via the SNARE complex. If the two isoforms differentially incorporate into a SNARE complex, interact differently with SNAREs or other modulating proteins, or confer different properties on the SNARE complex, synaptic transmission and brain function could be affected. For this reason we related the balance between Stx1A:1B in striatum with previously measured syntaxin-SNAP-25 protein interaction levels (Chapter 2) [441]. We observed a moderate negative correlation between the Stx1A:1B ratio and the amount of syntaxin-1 interacting with SNAP-25 in the VMC (Table 3.4). While little is known about the affinity of different syntaxin isoforms for SNAP-25, syntaxin-1A and 1B have different affinities for VAMP isoforms [241], suggesting one potential mechanism behind this association.

Recent evidence indicates that syntaxin-1 phosphorylation may be lower in prefrontal cortex of schizophrenia patients [293]. Less phosphorylation was associated with lower SNARE protein interactions (in disagreement with an earlier study reporting increased interactions between SNARE proteins in a subset of patients [264]). Whether a similar relationship between syntaxin phosphorylation and SNARE protein interactions also holds in the striatum, and whether syntaxin-1 isoforms are differentially phosphorylated, is unknown.

Although our interaction analysis focused on total syntaxin-SNAP-25 interactions, syntaxin-1A is known to interact with many other presynaptic molecules including ion channels and neurotransmitter transporters, such as the dopamine transporter. In schizophrenia, presynaptic dopaminergic terminals are metabolically overactive, and demonstrate greater dopamine release [139, 404]. In a *Caenorhabditis elegans* model system, interference with the interaction between the homologous molecules for syntaxin-1A and the dopamine transporter resulted in excessive synaptic dopamine release [438], suggesting a possible link between the

lower Stx1A:1B ratio we observed here, and striatal dopamine over-activity observed in living patients.

3.4.4 Limitations

The possible effects of drug exposure on the measures of syntaxin-1 isoforms must be considered. We previously reported that haloperidol administered to rats increased levels of total syntaxin, in contrast to the overall lower level of syntaxin detected by ELISA in the present series of samples [441]. While few of the samples used in the present study had detectable levels of antipsychotic drugs, it is possible that drug exposure could alter isoform expression. Many of the subjects smoked cigarettes, and nicotine may affect synapses in the striatum [354, 450, 451]. However, we did not detect differences in isoform ratios of smokers and non-smokers.

The immunoblotting assay used here was adequate to detect a relative difference in isoform expression. Absolute values of total syntaxin in the immunoblot assay correlated with the previously reported values using an ELISA, however the immunoblot assay was unable to detect statistically significant differences in absolute amounts of syntaxin-1A or 1B. The immunoblotting assay was linear over a six-fold range, similar to human presynaptic protein immunoblot assays reported by others [259, 452]. Our previously reported finding of lower total syntaxin-1 in the same samples assayed by ELISA likely reflects the superior sensitivity and linear range of the ELISA assay [452], which was 32-fold for syntaxin [441].

3.4.5 Conclusions

In conclusion, these results suggest that the balance of syntaxin-1 isoform expression in the striatum is disrupted in schizophrenia in the VMC, and may contribute to synaptic dysfunction via the SNARE complex. The balance of syntaxin-1 isoform expression appears to be age-

dependent in human striatum. Future work to determine syntaxin-1 isoform distribution within striatal terminal types, and analyse age-related changes, would improve our understanding of how syntaxin-1 affects human brain function and disease.

3.6 Chapter 3 tables

Table 3.1 - Tissue sample characteristics.
Diagnoses were established using DSM-IV criteria using all available information. Two subjects had a diagnosis of schizoaffective disorder. Post mortem interval (PMI) and sample storage time was different between groups ($P \leq 0.001$). PMI is unavailable for one individual. Detailed sample information has been previously described [441] or Table 2.1. *Abbreviations*: M - male; F - female; MVA - motor vehicle accident; N/A - not available.

Mean ± SD	**Non-psychiatric comparison subjects (NPC)**		**Schizophrenia (SCZ)**	
Gender	10M: 3F		9M: 6F	
Age (years)	51.4 ± 18.8		53.6 ± 12.1	
Postmortem interval (hours)	16.6 ± 7.1		8.8 ± 3.2	
Sample storage time (weeks)	196 ± 77		316 ± 74	
Brain pH	6.16 ± 0.33		6.32 ± 0.22	
Smoker	8 Yes:4 No:1 N/A		9 Yes: 4 No: 2 N/A	
Cause of death	MVA	5	Pulmonary	3
	Cardiac	4	Cardiac	6
	Homicide	3	Gastrointestinal	3
	Accidental	1	Accidental	2
			Undetermined	1

Table 3.2 - Association of age with syntaxin isoform ratio.
The ratio of syntaxin-1A to syntaxin-1B was negatively correlated with age in the nucleus accumbens (NAc), ventromedial caudate (VMC) and the dorsal caudate (DCd). Spearman correlations are reported for the whole sample set (NPC and SCZ combined) and also explored in each group separately.

Striatal	**All, n=28**		**NPC n=13**		**SCZ, n=15**	
Area	**R_s**	**P-value**	**R_s**	**P-value**	**R_s**	**P-value**
NAc	-0.54	0.003	-0.72	0.005	-0.40	0.14
VMC	-0.70	<0.0001	-0.82	0.0006	-0.62	0.01
DCd	-0.39	0.04	-0.61	0.03	-0.28	0.32

Table 3.3 - Relative abundance of syntaxin-1 isoforms in the striatum.
The band intensities for each isoform were expressed relative to the reference sample (in μg of reference sample total protein). Mean and standard deviation (SD) for each group are given below. Absolute values of syntaxin-1 isoforms are unknown and therefore abundance cannot be compared between syntaxin-1A and syntaxin-1B. *The Stx1A:1B ratio was constructed from the μg values and is therefore unitless. § The Stx1A:1B ratio was significantly different between schizophrenia and comparison subjects ($P < 0.05$, ANCOVA). No effect of diagnosis was observed for individual isoforms.

	NAc		VMC		DCd	
syntaxin	NPC	SCZ	NPC	SCZ	NPC	SCZ
1A:1B*§	1.17 (0.14)	1.15 (0.13)	1.24 (0.14)	1.13 (0.15)	1.28 (0.16)	1.15 (0.11)
1A	1.74 (0.30)	1.72 (0.25)	1.88 (0.32)	1.76 (0.36)	2.06 (0.43)	1.95 (0.37)
1B	1.50 (0.16)	1.51 (0.19)	1.55 (0.27)	1.53 (0.30)	1.62 (0.28)	1.72 (0.30)

Table 3.4 - Association of syntaxin isoform ratio with SNARE protein interactions.
We previously reported greater syntaxin-SNAP-25 interaction in the VMC in schizophrenia (Chapter 2). The Stx1A:1B ratio measured here was associated with the syntaxin-SNAP-25 interaction; a smaller ratio (as observed in schizophrenia) was associated with greater syntaxin-SNAP-25 interaction.

Striatal	All, n=28		NPC n=13		SCZ, n=15	
Area	R_s	P-value	R_s	P-value	R_s	P-value
NAc	-0.34	0.08	0.01	0.99	-0.73	0.002
VMC	-0.57	0.002	-0.45	0.12	-0.54	0.04
DCd	-0.02	0.93	-0.04	0.690	-0.29	0.30

3.7 Chapter 3 figures

Figure 3.1 - Dissection diagram of frozen human striatal regions.
Nucleus accumbens (NAc), dorsal and ventral caudate (DCd, VMC) were dissected from the same block of frozen striatal tissue. Dissected areas are illustrated in black. *Abbreviations*: IC-internal capsule.

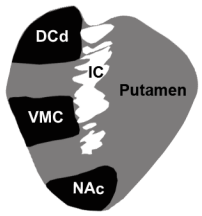


Figure 3.2 - SP6 detects both syntaxin-1 isoforms.
Human brain homogenate (5 and 10 μg total protein) was separated by Tris-Urea-SDS-PAGE, transferred to nitrocellulose, probed with either SP6 (α-syntaxin-1 antibody) or antibodies against syntaxin-1A or syntaxin-1B (Synaptic Systems, Gottingen, Germany) and visualized using enhanced chemiluminescence.

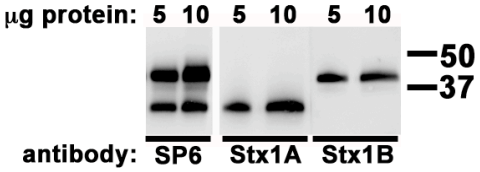
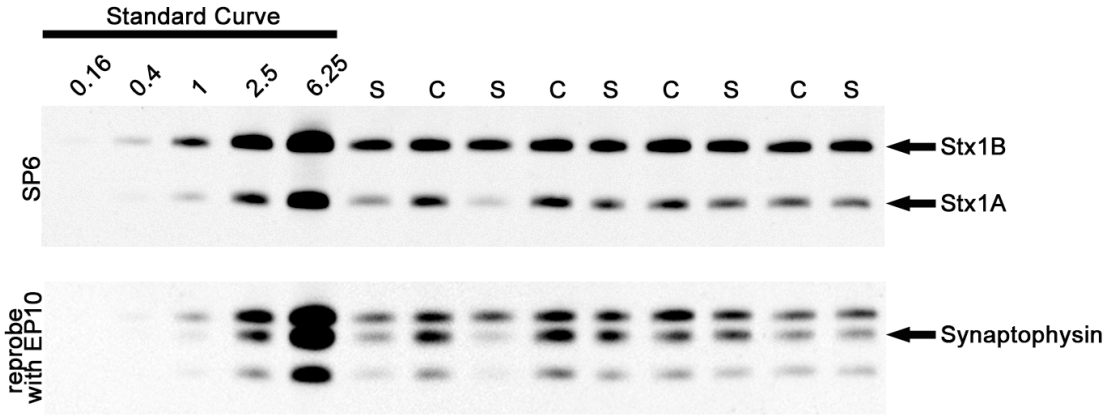


Figure 3.3 - Quantification of syntaxin-1A and 1B protein levels.
A: Representative immunoblot illustrating signal intensity obtained from a blot probed with α-syntaxin antibody SP6 (top) and later re-probed with anti-synaptophysin antibody EP10 (bottom). **B**: Standard curves were constructed from a serially diluted reference sample. Each curve has a different slope, demonstrating that the use of raw density values to calculate a ratio is not a valid means of reducing variance associated with sample loading or blot processing. The equation of each line was used to interpolate the amount of total protein (μg of the reference sample) required to obtain the measured immunoreactivity, for syntaxin-1A, syntaxin-1B or synaptophysin. The ratio of Stx1A:1B was calculated using the interpolated 'μg of reference sample' values. Curves were constructed for each blot separately.

A



B

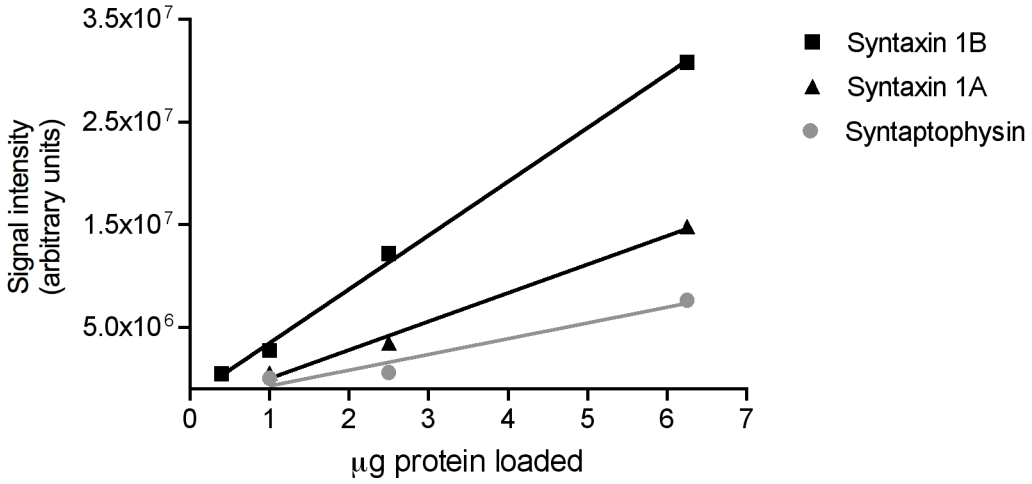


Figure 3.4 - Altered syntaxin-1 isoform ratio in schizophrenia.
A: Ratio of the amount of syntaxin-1A to 1B in striatal regions studied. Mean values were lower in the SCZ samples. Horizontal lines denote the mean. **B:** Ratio of syntaxin-1A:1B in the context of age. Since there was no systematic effect of striatal region, mean values were calculated for each NPC or SCZ case across regions. The linear relationship between age and syntaxin isoform ratio is illustrated for the NPC group (slope = -0.0052, intercept = 1.496, R^2 = 0.682) and the SCZ group (slope = -0.0049, intercept = 1.402, R^2 = 0.338) separately. *Abbreviations*: NPC- non-psychiatric comparison subject, SCZ- schizophrenia; NAc – nucleus accumbens; VMC – ventromedial caudate; DCd – dorsal caudate. '*' indicates $P < 0.05$, two-way ANCOVA.

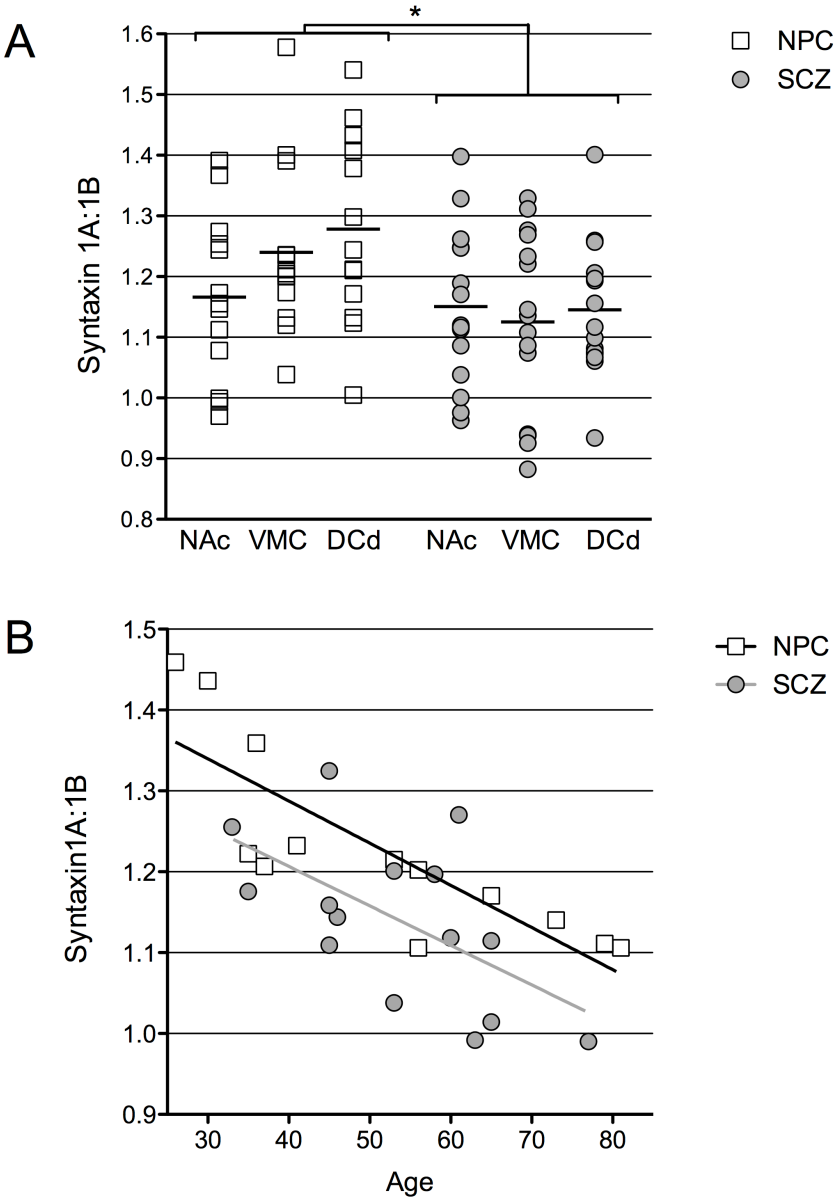
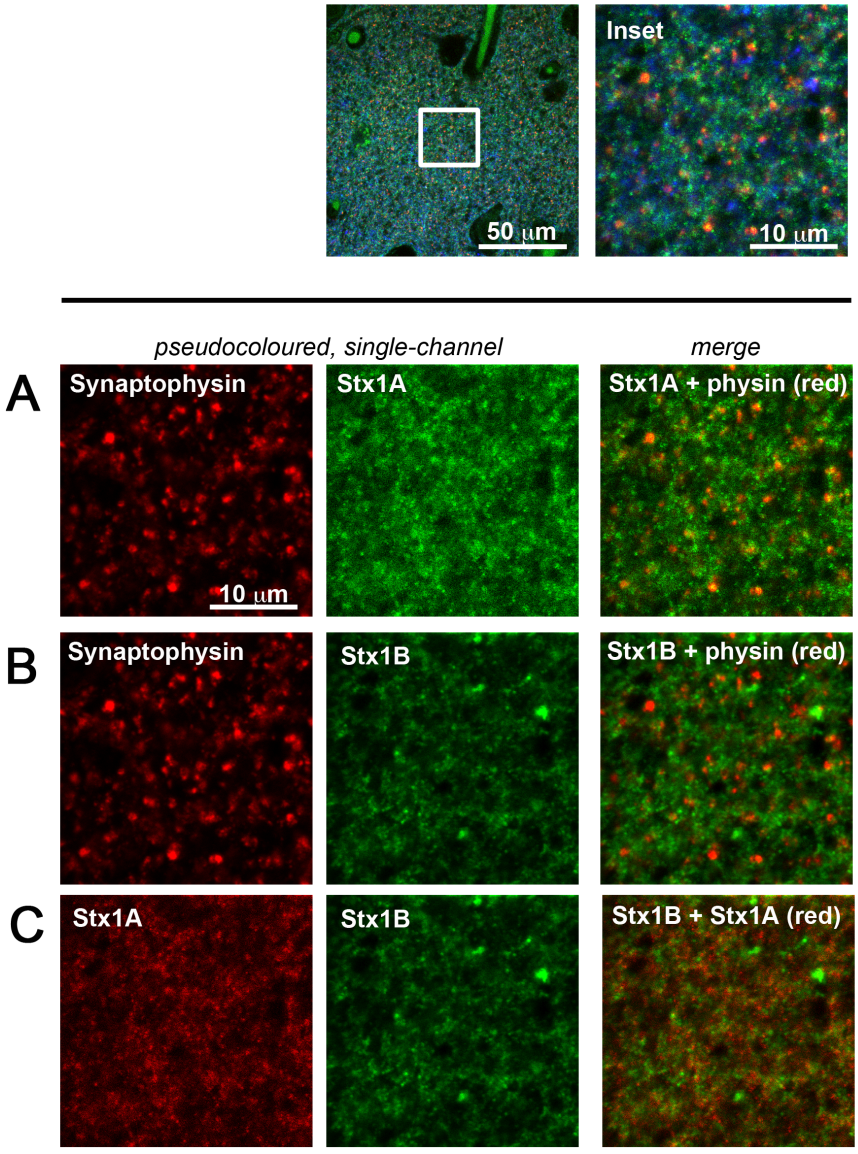


Figure 3.5 - Incomplete colocalization of syntaxin-1 isoforms in human VMC.
Top: Tissue was triple-labeled for synaptophysin (red), syntaxin-1A (green) and syntaxin-1B (blue) using syntaxin isoform-specific antibodies. For colocalization assessment, a survey image at 63x magnification (top left) was initially performed and sub-fields were then imaged at higher magnification (one sub-field is indicated, inset, shown at top right). Single-channel images of the inset are shown below, A-C. *A-C:* Colocalization/signal co-occurrence was assessed pair-wise for each combination of antigens. Overlay of each pair-wise combination is illustrated below, pseudo-coloured for easier viewing.



4 Quantitative mass spectrometry reveals changes in SNAP-25 isoforms in schizophrenia[5]

4.1 Introduction/results/discussion[6]

Individuals at risk of schizophrenia, patients in the acute phase of illness, and cognitively impaired patients with schizophrenia all exhibit functional or molecular abnormalities of presynaptic terminals [141, 142, 296]. These terminals are enriched with specific proteins such as SNAP-25, one of three neuronal soluble NSF-attachment receptors (SNAREs) essential for evoked neurotransmitter release [186, 249]. Through highly stable interactions with partner SNARE proteins (syntaxin and VAMP), SNAP-25 participates in docking and fusion of synaptic vesicles, vital to normal brain function. In schizophrenia, SNAP-25 protein expression is lower in the ventromedial caudate [441], a region where abnormalities of dopamine release have been identified by imaging studies [139]. In contrast to the lower protein levels, the interaction of SNAP-25 with syntaxin is increased in schizophrenia, providing further support for dysfunctional presynaptic terminals.

The SNAP-25 gene transcript undergoes mutually exclusive alternative splicing of exons 5a and 5b, producing protein isoforms of equal length that differ at only 9 residues [250, 369], Figure 4.1A. A switch between predominant SNAP-25A mRNA expression early in life, to predominant SNAP-25B mRNA expression postnatally, is necessary for survival in mice. SNARE protein complexes containing SNAP-25B are more stable than those containing SNAP-25A, altering the properties of neurotransmitter release [196, 252, 374]. Interestingly, the *blind drunk* (*Bdr*) mouse contains an isoleucine-to-threonine substitution within the alternative exon of

[5] **A version of this chapter will be submitted for publication:**
Barakauskas VE, Moradian A, Barr AM, Beasley CL, Rosokilja G, Mann JJ, Ilievski B, Stankov A, Dwork AJ, Falkai P, Morin GB, Honer WG. (2010). Quantitative mass spectrometry reveals changes in SNAP-25 isoforms in schizophrenia.

[6] This research chapter has been formatted as a brief communication, therefore conventional sub-sections are not used and detailed methodology is found at the end of the chapter.

SNAP-25B that further increases SNARE complex stability [229]. The *Bdr* mouse shares phenotypic similarities with schizophrenia [253].

We investigated SNAP-25 protein isoform levels in postmortem brain in schizophrenia using multiple-reaction monitoring (MRM), a quantitative mass spectrometry (MS) assay, (4.2 Supplementary Methods) that monitors peptides specific to each SNAP-25 isoform, as well as a peptide common to both (Figure 4.1B-D). MRM involves the fragmentation of selected peptides (parent ions) and detection of specific peptide fragments (transitions), producing ion chromatograms with separate peaks for different parent ions, and overlapping peaks corresponding to multiple transitions of the same parent ion (Figure 4.1E). Peak areas are proportional to peptide amounts.

For initial studies of SNAP-25 isoforms, an external ratio calibration curve relating MRM peak area ratios to the percentage of SNAP-25A in the sample was constructed using mixtures of recombinant SNAP-25A and B proteins (Figure 4.2A). This allowed interpolation of the %SNAP-25A in analyzed samples. Detection of isoform-specific peptides was log-linear over a 50 to 150-fold range (Figure 4.6). SNAP-25A/B ratios were highly reproducible in biological samples (CV ≤ 6%, 4.3 Supplementary Results). To test the feasibility of detecting biologically meaningful changes in SNAP-25A/B protein expression, we investigated the effects of depolarization and differentiation of PC12 pheochromocytoma cells on SNAP-25 isoform expression. The MRM assay was able to detect the expected increase in SNAP-25B [254] (Figure 4.2B). We also detected differences in the SNAP-25A/B ratio between human brain regions (Figure 4.2C).

To quantify the absolute amounts of SNAP-25 peptides in human striatum SNAP-25 was immunoprecipitated from homogenized samples and prepared for MRM analysis (Figure 4.3). An isotopically-labeled peptide (heavy peptide) identical to the common SNAP-25 peptide was

added to the trypsin digested samples as an internal quantification standard [366]; the heavy peptide co-chromatographs with the common peptide but is resolved by MS due to its 10 Da mass difference (Figure 4.7). The absolute amount of total SNAP-25 in each sample was determined using a three point external standard curve, run with each sample batch (Figure 4.2D). The absolute amounts of SNAP-25A and B were then calculated using the percentage of SNAP-25A interpolated from the ratio calibration curve (Figure 4.2A).

Samples of ventromedial caudate were studied, from the same cases of schizophrenia (SCZ, $n = 15$) and non-psychiatric controls (NPC, $n = 13$) that were previously reported to have low levels of total SNAP-25, and greater SNAP-25-syntaxin interactions (Chapter 2 and Table 4.1). Total SNAP-25 was 27% lower in SCZ subjects ($P = 0.002$; two-tailed *t*-test) as compared with NPCs (Figure 4.2E), in agreement with previous findings in this exact sample set using ELISA (Chapter 2). The absolute amount of SNAP-25A was 31% lower in SCZ as compared to the NPC group ($P = 0.002$; two-tailed *t*-test); SNAP-25B was 20% lower in SCZ but was not significantly different ($P = 0.10$; Welch's ANOVA for samples with unequal variances). Lower levels of SNAP-25A were neither related to smoking status, nor to the presence of benzodiazepines or antipsychotic drugs detected by post-mortem toxicology (see 4.3 Supplementary Results for additional analyses). To further explore the possible effects of antipsychotic drugs, rats were treated sub-chronically with haloperidol, clozapine, or saline control (Section 4.2.1.7). SNAP-25A and B proteins in the medial striatum did not differ between treatment groups (Figure 4.2F, for all measures $P > 0.05$, ANOVA). Previously, an ELISA-based analysis of the same human ventromedial caudate samples used in this study demonstrated increased protein interactions between total SNAP-25 and syntaxin in the schizophrenia samples (Chapter 2). We used the present data to examine the relationship between the previously measured SNAP-25/syntaxin protein interactions and the SNAP-25

isoform amounts. An overall negative correlation was observed with SNAP-25A ($P = 0.03$, Pearson correlation coefficient r=-0.41), but not with SNAP-25B ($P = 0.27$, Pearson correlation coefficient r = -0.22).

The central finding indicates that lower levels of SNAP-25 in ventromedial caudate in schizophrenia are primarily due to less SNAP-25A and that the remaining SNAP-25 is associated with greater syntaxin protein interactions. The absence of a link to recent treatment effects in the human samples, or to administration of antipsychotic drugs in rats suggests the findings are illness- and not treatment-related. Both SNAP-25A and B protein isoforms appear to be expressed at similar levels; approximately 60% SNAP-25A in NPC and SCZ samples. Previous estimates of isoform expression using mRNA may not reflect protein isoform expression in human striatum [453].

These isoform-specific findings increase the level of specificity at which we can characterize alterations in striatal presynaptic proteins in schizophrenia. Functionally, lower levels of SNAP-25A and increased overall SNAP-25/syntaxin interaction may increase the stability of the SNARE complex in schizophrenia compared with NPC terminals [249, 252]. The present results suggest that experiments measuring altered SNAP-25 isoform expression in schizophrenia cohorts and studying SNAP-25 isoform expression concurrently with SNARE complex stability in cell lines and mouse models [229, 414] will be relevant to understanding the mechanisms underlying synaptic dysfunction in schizophrenia.

4.2 Supplementary methods

4.2.1 Reagent and sample preparation

4.2.1.1 Cloning of SNAP-25A and B

Human cDNAs encoding the open reading frames for SNAP-25A and B (OpenBiosystems, Thermo Fisher; clone ID: LIFESEQ2152214 and LIFESEQ1399540, respectively) were each amplified using two different primer sets (set1: sense 5'-ATCGGGCGCGCCGCCGAAGACGCAGACATGCGC-3', antisense 5'-ATCGTTAATTAATTAACCACTTCCCAGCATCTTTGTTGCACGTTG-3'; set2: sense 5'-ATCGGGCGCGCCATGGCCGAAGACGCAGACATGCGC-3', antisense 5'-ATCGTTAATTAAACCACTTCCCAGCATCTTTGTTGCACGTTG-3') and ligated into the V954 donor vector of the Creator Splice system [454]. V954 constructs were sequence verified. Cre-lox recombination was used to transfer cDNA to vectors for mammalian expression of SNAP-25 with either N- or C-terminal 3xFLAG tag (V180 and V181 vectors). For bacterial expression, cDNA was ligated directly into a pGEX4T-2 (V1544) vector to generate an N-terminal GST fusion protein.

4.2.1.2 Expression and purification of SNAP-25 isoforms in HEK293 cells

HEK293 cells (human embryonic kidney) were maintained in DMEM with glutamax (Invitrogen, Burlington ON), containing 10% v/v fetal bovine serum (Gibco, Invitrogen, Carlsbad, CA, USA). FLAG-SNAP-25A, B or empty vector (control) was transfected using Lipofectamine 2000 (Invitrogen) according to manufacturers instructions. Approximately 48 hours post-transfection, cells were harvested in phosphate-buffered saline (PBS: Gibco), pelleted and stored at -80°C until further use. FLAG-tagged proteins were isolated by

immunoprecipitation using anti-FLAG M2 agarose (Sigma-Aldrich Ltd., St. Louis, MO USA) using previously published protocols [455].

4.2.1.3 Bacterial expression and purification of recombinant SNAP-25A and B

BL21 *E. coli* (Invitrogen) were transformed with GST-SNAP-25A or B plasmids according to manufacturers instructions. Overnight cultures were diluted 1/25 in 500 ml Luria Broth and protein expression induced with isopropyl β-D-1-thiogalactopyranoside (1 mM final concentration) once the culture reached an OD of 0.4-0.6. Cultures were grown for ~ 4 hours post-induction, cells were pelleted by centrifugation and stored at -80°C until further use. Pellets were resuspended in 15 ml PBS containing 1% TritonX-100, 500mM NaCl and protease inhibitors (PIs) (Roche, Mississauga, ON Canada). Bacteria were lysed by five freeze-thaw cycles followed by sonication (3 x 30sec, 2min rests, 40% amplitude) and 30 min of solubilization, rotating end-over-end at 4°C. Extracts were centrifuged to remove insoluble material (15 min, 12,000xg, 4°C) and supernatants clarified by passing through a 0.45 μm filter. Lysate was diluted ~1:3 in PBS containing PIs to reduce viscosity. GST-fusion proteins were purified on a GSTrap glutathione sepharose column (1ml bed volume; GE Lifesciences, Baie d'Urfe, QC Canada) with automated sample loading, washing and eluting using an AKTApurifier FPLC system (GE Lifesciences). FPLC conditions were: 0.3ml/min flow rate, 10 column volumes wash with PBS containing PIs following sample loading, step elution of bound protein using 5 column volumes of freshly made 20mM reduced glutathione (Sigma-Aldrich) in 50mM Tris-HCl, pH 8 and collection of 1 ml fractions. Eluted protein was monitored by UV at 280nm. Fractions containing GST-SNAP-25 (as determined by SDS-PAGE/Coomassie staining) were pooled and dialyzed overnight against 10mM ammonium bicarbonate (200x volume) with

three changes of dialysis buffer, using a 2,000 MWCO dialysis cassette (Slide-a-lyzer, Pierce, Rockford, IL USA). Dialyzed protein samples were aliquoted, lyophilized and stored at -80°C. Prior to use, one or more aliquots were resuspended in a small volume of HPLC-grade water and protein concentration calculated using the Bradford method (Bio-Rad, Mississauga, ON Canada) with bovine serum albumin (BSA) as the reference standard. Quantification of full-length protein was further refined by separating equal volumes of GST-SNAP-25A and GST-SNAP-25B by SDS-PAGE followed by staining with colloidal Coomassie (20% v/v ethanol, 1.6% v/v phosphoric acid, 8% w/v ammonium sulfate, 0.08% w/v Coomassie brilliant blue G-250). The gel was de-stained in water, and imaged at high resolution (300 dpi) using an Odyssey infrared imaging system (Li-Core Biosciences, Lincoln, NB USA) with 700nm excitation. Band intensity was quantified using the 'profile' mode in ImageGauge v4.22 software (Fuji Lifesciences, Tokyo Japan), and full-length SNAP-25A and B intensities as well as lower molecular weight bands were quantified to determine the percentage of full-length protein.

4.2.1.4 Synthetic peptides

Crude synthetic peptide standards were purchased (Peptide 2.0, Chantilly, VA USA). The common peptide (residues 104-119 of SNAP-25) was dissolved in HPLC-grade water. Cysteine-containing peptides (residues 84-94 of SNAP-25A and B) were each dissolved in 6 mM dithiothreitol (DTT), 12% v/v acetonitrile, 0.2% v/v tri-fluoro acetic acid (TFA) with mild heating and sonication. Cysteine-containing peptides were reduced and alkylated using DTT in 10-fold excess followed by iodoacetamide in 20-fold excess. Solubilized peptides were diluted 1:200 with MS buffer (3% v/v acetonitrile, 1% v/v TFA), desalted on C18 STAGE-tips [456], lyophilized and stored. The heavy peptide internal standard was a high-purity (> 90%) version of the common peptide, residues 104-119 (H104-119), containing an isotopically labeled C-

terminal arginine residue ([$^{13}C_6{}^{15}N_4$]Arg) (University of Victoria Proteomics Facility, Victoria, BC Canada). Peptide concentration was determined by amino acid analysis by the vendor. The heavy peptide co-elutes with the common peptide when added to SNAP-25 that was immunoprecipitated from human brain (Figure 4.7).

4.2.1.5 Depolarization and differentiation of PC12 cells

PC12 (pheochromocytoma cells) were maintained in 5%CO_2 in DMEM (containing 10% v/v horse serum, 5% v/v fetal bovine serum, 1% v/v minimal non-essential amino acids and 1% v/v penicillin-streptomycin) (Gibco). To induce SNAP-25B expression, cells were treated with 35 mM KCl or 35 mM KCl plus 50 ng/ml nerve-growth factor (NGF; Sigma-Aldrich), using 35 mM NaCl as the control condition [254]. Equal numbers of cells were plated on 10 cm plates (Cellbind; Corning Inc. Life Sciences, Lowell, MA USA). Cells were allowed to adhere to the plates overnight and treated with NaCl, KCl, or KCl+NGF the next day. After 3.5 days, media was aspirated, cells washed twice with cold PBS and collected using a cell scraper. After centrifugation, PBS was removed and the cell pellets stored at -80ºC. For immunoprecipitation, pellets were resuspended in 0.1% v/v TritonX-100 in tris-buffered saline (TBS: 10 mM Tris-HCl, 140 mM NaCl, pH 7.4) with PIs, 0.5 ml per plate of cells, passed through a 20-gauge needle three times, solubilized for 1.5 hrs, and centrifuged (20 min, 15 000 x g, 4ºC). Protein concentration was determined using the Bradford method as above. Approximately 3-4 plates of lysate were diluted to produce equal protein concentrations, and SNAP-25 was immunoprecipitated as outlined below. SNAP-25 was not completely depleted from lysates under these experiment conditions.

4.2.1.6 Human brain samples

Post-mortem tissue samples from 15 subjects with schizophrenia (SCZ) and 13 non-psychiatric comparison subjects (NPC) were obtained from the Macedonian/New York State Psychiatric Institute Brain Collection. Tissue collection, screening and post-mortem diagnosis is detailed elsewhere (Table 2.1), and summarized in Table 4.1. Informed consent was obtained from family members and tissue collection was approved by the Columbia/New York State Psychiatric Institute IRB.

Toxicological analyses for drugs of abuse and psychotropic agents were performed on each case. All SCZ subjects had exposure to multiple antipsychotic agents, according to chart review. Caffeine and benzodiazepines (available without prescription in Macedonia) were detected during post-mortem toxicological analyses in both NPC and SCZ subjects. Only two SCZ subjects had detectable levels of antipsychotic medications (clozapine). More than half of subjects were smokers at time of death (8/13 NPC and 9/15 SCZ).

Frozen coronal tissue slabs that included pre-commissural striatum were sampled, and the ventromedial caudate (VMC) dissected using a standard human brain atlas [408, 441]. Tissue samples were homogenized in TBS, aliquoted and frozen at -70°C until further use. Total protein concentration of one aliquot was measured with a modified Lowry assay (Bio-Rad) using BSA as a reference standard.

Frozen tissue of two different subjects (one outside of the sample set) sampled from several different brain regions was used during the method validation stages.

4.2.1.7 Antipsychotic treated animals

Adult male Sprague-Dawley rats (Charles River, Montreal, QC Canada) were pair housed, with access to food and water *ad libitum*. Animals were habituated to their environment for one

week. At the start of the experiment, rats weighed 270 to 320 g and were divided into three groups (n = 10 per group used in this study) balanced for starting weight. All procedures were approved by the University of British Columbia Animal Care Committee and were conducted in accordance with the Canadian Council on Animal Care guidelines.

Rats were administered high doses of haloperidol (1 mg/kg) or clozapine (20 mg/kg), or pH-adjusted saline daily (1 ml/kg) for 28 days intraperitoneally. Drug administration and preparation are detailed elsewhere (Chapter 2 or [441]). Clozapine was injected subcutaneously with larger volumes of dilute clozapine (0.4-0.5 mg/ml, still 20 mg/kg administration) for the final 16 days, due to irritation at the intraperitoneal injection site. One day after the final injection, animals were sacrificed by decapitation. Whole brain was rapidly removed from the skull, cooled briefly in artificial cerebrospinal fluid (148 mM NaCl, 3 mM KCl, 1.4 mM $CaCl_2$, 0.8 mM $MgCl_2$, 10 mM D-Glucose, 0.8 mM Na_2HPO_4, 0.2 mM NaH_2PO_4) and dissected. Coronal cuts at the level of the optic chiasm and 3 mm anterior gave a section at approximately Bregma 0.48 μm [410]. The tissue inferior to the anterior commissure was removed and the remaining caudate/putamen was divided in half, to give a medial and lateral portion. This dissection protocol was described previously (Chapter 2 and [411, 441]). Regions were dissected bilaterally, frozen rapidly on dry ice and stored at -70°C.

4.2.1.8 Immunoprecipitation of endogenous SNAP-25

For measurement of SNAP-25 isoforms in brain tissue and cell lysates, SNAP-25 was immunoprecipitated from solubilized homogenates, using a monoclonal antibody that detects both isoforms (SP12) [389, 441]. Samples were coded to mask investigators to diagnosis and sample characteristics. Purified SP12 was coupled to magnetic beads (Goat-anti-mouse IgG Dynabeads; Invitrogen) according to manufacturers instructions. For each sample, 4.5 $x10^7$

SP12-coated beads (corresponding to 4.5 μg of SP12 antibody) were used for immunoprecipitation. For human brain samples, 150 μg of crude homogenate protein was solubilized in 750 μl 0.1% (v/v) TritonX-100/TBS for 90 minutes rotating end-over-end at 4ºC. Insoluble material was removed by centrifugation (20 min, 15 000 x g, 4ºC). The supernatant was pre-cleared with 0.5 $x10^7$ empty beads (beads that were not coupled with SP12) for 30min at 4ºC. Pre-cleared samples were then incubated with SP12-coupled beads (4.5 μg of antibody per sample) for 10 hours, rotating end-over-end at 4ºC. Immunoprecipitates were washed once with 0.1% v/v TritonX-100/TBS/PIs, twice with 0.1%TritonX-100/TBS and twice with TBS. The remaining beads were resuspended in 15 μl Laemelli sample buffer without reducing agent (62.5 mM Tris-HCl, pH 6.8; 25% v/v glycerol, 2% v/v SDS, 0.01% v/v bromophenol blue) and stored at -20ºC in preparation for SDS-PAGE and sample processing for MS. The sample preparation workflow is diagrammed in Figure 4.3. The amount of antibody used for immunoprecipitation was optimized to extract total SNAP-25 from human brain, as determined by immunoblot comparison of equal volumes of pre and post-IP supernatant with increasing amounts of antibody used for IP (data not shown). Monitoring of one sample illustrates the removal of SNAP-25 during immunoprecipitation of VMC samples, Figure 4.4.

4.2.1.9 Preparation of samples for MS/MS or MRM-MS analysis

Recombinant or immunopreciptated SNAP-25 was prepared for MS/MS or MRM analysis in the following manner. Protein samples in Laemelli sample buffer (without reducing agent) were heated for 10 min at ~95ºC. In the presence of heat, and absence of reducing agent, the SDS-stable SNARE complex is dissociated [206], but the antibody used for immunoprecipitation remains relatively intact (Figure 4.4), reducing the amount of IgG light-chain present in the 25 kDa region of the gel.

Samples were separated on NuPage 4-12% Bis-Tris SDS-PAGE gels (Invitrogen). Gels were stained with colloidal Coomassie overnight, destained in water and imaged for gel-cutting purposes. The bands corresponding to SNAP-25 (~25kD) were excised (see Appendix 5) and cut into ~1mm^3 pieces. Gel pieces were placed in a well of a 96-well plate, one well per sample. Samples were prepared for MS by automated in-gel dehydration, alkylation, trypsin digestion (Sigma-Aldrich), and extraction (Progest; Genomic Solutions, Ann Arbor, MI, USA). Extracted peptides were transferred to a BSA-coated plate, lyophilized and stored at -20ºC until further use. Prior to MS analysis, samples were resuspended in 8.5 µl of 1x MS buffer (1% v/v formic acid, 2% v/v acetonitrile) or in 8.5 µl of 1x MS buffer containing the isotopically labeled H104-119 peptide as an internal standard (80 fmol H1014-119 peptide per 4 µl). For MRM analysis in the isoform assay, 4 µl was analyzed for human samples, 8 µl was analyzed for rat samples.

4.2.2 Isoform assay development and characterization

MS method development and optimization, liquid chromatography tandem MS (LC-MS/MS), and final MRM collection were performed on a 4000 QTrap (Applied Biosystems/Sciex, Foster City, CA USA) with a nano-electrospray ionization source. The instrument was coupled to a high-performance liquid chromatography system (Agilent 1000 Nano-HPLC; Agilent, SantaClara, CA USA). Samples were desalted online using a reverse-phase trap column (Agilent, Zorbax, 300SB-C18, 5µm, 5x0.3mm) for 5 min in solvent A (5% acetonitrile, 0.1% formic acid) then directed onto a reverse phase analytical column (75 µm x 15 cm, packed in-house with 3 µm-diameter Reprosil-Pur C18; Dr. Maisch, Ammerbuch-Entringen, Germany) and coupled to an uncoated fused silica emitter tip (20 µm inner diameter, 10 µm tip; New Objective, Woburn, MA USA). Chromatographic separation was achieved using a 300 nl/min flow rate and a linear gradient of 0 to 23% solvent B (90% acetonitrile, 0.1% formic acid) over 23 min

followed by a 9 min gradient from 23 to 39% solvent B and a 4 min gradient from 39% to 80% solvent B. A 30 min gradient with blank solvent injection was run in between samples.

For MRM experiments, 4000 QTrap acquisition parameters were as follows: 1800-2200 V nano-spray voltage, curtain gas setting of 10 (~1 L/min) and nebulizer gas setting of 2 (~0.5 L/min), interface heater at 200° C, 3.4 x 10^{-5} torr base pressure, and Q1 and Q3 set to unit resolution (0.6-0.8 Da full width at peak half-height). For spray stability and improved tip lifespan a postcolumn, prespray makeup solvent (90% MeOH) at a flow rate of 50-150nl/min was introduced using a Harvard PicoPlus syringe pump.

4.2.3 Tandem mass-spectrometry, database searching and peptide selection

FLAG-SNAP-25, GST-SNAP-25 and endogenous SNAP-25 from human brain were analyzed by LC-MS/MS to determine which isoform-specific peptides were detectable by MS and to obtain their fragmentation patterns (Figures 4.1B-D). Data were collected using a 400-1600 m/z Enhanced MS scan followed by an Enhanced Resolution scan to select the top five +2 and +3 ions for collision-induced dissociation and a final Enhanced Product Ion MS scan. Raw mass spectral data was analyzed using Mascot (ver.2.2.2; Matrix Science, Boston, MA, USA) and searching against European Bioinformatics Institute human International Protein Index database, which contains both isoforms of SNAP-25. Searches were performed with carbamidomethylation of cysteine as a fixed modification, allowing for one missed cleavage and a peptide mass tolerance of 0.5 Da. Variable modification search parameters were: oxidation of methionine, deamidation (asparagine, glutamine) and phosphorylation (serine, threonine, tyrosine). SNAP-25 sequence coverage ranged from 55-83%. MS/MS spectra and database searches were used to choose MRM peptides. Peptides were selected based on their presence in

the MS/MS spectra, absence of methionine residues (which are easily oxidized resulting a large mass difference which would affect detection and quantitation by MRM), lower likelihood of missed-cleavages and other modifications (as identified by Mascot). While the chosen peptides contained proline residues, fragmentation at this site did not overly predominate the MS/MS spectra (Figure 4.1 B-D) and may have also been controlled through subsequent optimization steps (Section 4.2.4).

4.2.4 MRM optimization

Synthetic A, B and common peptides (Figure 4.1) were resuspended in 30% acetonitrile/1% formic acid and directly infused into the 4000 QTrap using a Harvard PicoPlus syringe pump and Hamilton syringe at a flow-rate of 300 nL/min. Q1 scans were centered on a 5 or 10 Da wide mass window and optimal declustering potential (DP) was determined over a 0-200 V range. Optimal collision energy (CE) between 0-90V for each transition was determined with Q1 and Q3 set at unit resolution [457]. Collision cell exit potential was held at 15 V for all transitions. The top 2 or 3 transitions for doubly-charged parent ions with low cross-reactivity with the other isoform or control IP conditions were selected for final monitoring. The final MRM acquisition conditions are given in Figure 4.5. Despite optimization, the intensity of isoform specific transitions remained unequal when a mixture of equal amounts of SNAP-25A and B was analyzed (Figure 4.1E, inset).

4.2.5 MRM data analysis

MRM data were processed using MultiQuant v.1.0 (Applied Biosystems, Foster City CA). Default values for noise percentage and minimum peak height were used (40% and 50 CPS, respectively). A peak-splitting factor of 5, base-line subtraction window of 3 min and 3-point

smoothing were used. Manual inspection ensured that correct peaks were integrated for all samples. Integrated values were transferred to Excel, peak areas for multiple transitions summed [367, 457], and a ratio constructed of summed peak areas for SNAP-25A versus summed peak areas for SNAP-25B transitions (referred to as A/B ratio).

4.2.6 Linearity and limit of detection

The linear range and limit of detection of isoform-specific peptides was estimated using recombinant SNAP-25A and B. Protein was serially diluted, processed in-gel and analyzed using the isoform assay. For the common peptide, the purified synthetic peptide was serially diluted in 1x MS buffer and analyzed (Figure 4.6).

4.2.7 Reproducibility of isoform ratios

Reproducibility of A/B ratios over time was assessed by injecting the sample multiple times over a span of ~12 hours. One human brain sample IP was scaled up 5-times, and the equivalent of half an IP was analyzed during each MRM run.

4.2.8 Ratio calibration curve

To relate A/B ratios to the percentage of each isoform in the sample, an external calibration curve was constructed using recombinant protein. GST-SNAP-25A and B were mixed in known ratios, and different total protein amounts loaded on to gels (1, 2 and 4 μg, determined by Bradford assay). Samples were processed and analyzed as described above, injecting half of each sample. The relationship between A/B summed peak areas and %SNAP-25A was plotted and the log-linear portion of the graph was curve fit ($\log(y) = 0.0008x + 0.0635$; $r^2 = 0.9752$, 1/y

weighting), Figure 4.2A. The %SNAP-25A in endogenous samples was then interpolated using this curve. Curve fitting and interpolation were performed using Prism 5.0 for Macintosh (GraphPad Software Inc., La Jolla, CA USA).

4.2.9 Recovery of protein from gels and absolute quantification of total SNAP-25

To estimate the amount of SNAP-25 recovered from gel pieces, extracted, lyophilized peptides were resuspended in MS sample buffer containing a constant amount (80 fmol per 4 μl injection) of the heavy peptide (isotopically labeled common peptide, H104-119). The ratio of endogenous (unlabelled) common peptide originating from the sample, to the amount of signal from the added heavy peptide was calculated. An external three-point serially diluted standard curve (20, 80 and 320 fmol) of the heavy peptide was constructed, and this was expressed as a ratio relative to the 80fmol point on the curve (i.e. peak area of the 20, 80 or 320 fmol sample/peak area of the 80 fmol sample), Figure 4.2D. For rat samples, twice as much heavy peptide was present, since twice as much sample (IP and standard curve samples) was injected. Log-log regression (with 1/x weighting) [367, 457], of the calibration curve was performed and the femtomole recovery of SNAP-25 (based on the ratio of endogenous common peptide to H104-119 peptide) was interpolated using Prism software. A new heavy peptide standard curve was run with each batch of samples. The transition peak area ratio for the common peptide/heavy peptide for each ventromedial caudate sample was compared to the external curve for each batch to calculate the absolute amount of total SNAP-25 in each sample. We confirmed that ion suppression of the heavy peptide was negligible (Figure 4.8).

4.2.10 Absolute quantification of A and B isoforms

In order to accurately quantify total SNAP-25 in human brain samples, immunoprecipitation methods were optimized to completely deplete SNAP-25 from the samples, as determined by immunoblot analysis (Figure 4.4 and data not shown). The heavy peptide internal standard was added to each sample prior to MRM analysis. The relative amount of each SNAP-25 isoform was calculated using the A/B ratio for each sample to estimate the %SNAP-25A using the ratio calibration curve (Figure 4.2A), then the absolute amount of SNAP-25A was determined as (%SNAP-25A/100) x fmol of total SNAP-25 (calculated using the heavy peptide external standard curve, Figure 4.2D). Fmol of SNAP-25B was calculated as fmol total SNAP-25 minus fmol SNAP-25A for each sample.

4.2.11 Statistical analysis

Assay variability was assessed by calculating the coefficient of variation (CV: standard deviation/mean) of replicate measurements. SNAP-25 isoform expression in human and rat striatum was assessed using ANOVA. Distribution and equality of variance were assessed using Shapiro-Wilk's and Brown-Forsythe tests, respectively. Data was $\log_e$-transformed where necessary to improve distributions and variances.

For human samples, disease-associated differences in the absolute quantities of SNAP-25A and SNAP-25B were assessed using two-tailed t-tests (or Welch's ANOVA if variances remained unequal even after $\log_e$-transformation). Possible association of sample demographics with SNAP-25 isoform expression was explored using Spearman correlations and ANCOVA.

For rat samples, treatment-associated differences in total SNAP-25, SNAP-25A and B were investigated using ANOVA. Analyses were run with and without one extreme value that was not remedied by $\log_e$-transformation, to confirm it did not influence statistical conclusions.

Untransformed data was used for figures, unless indicated on axis labels.

4. 3 Supplementary results

4.3.1 Assay reproducibility

We assessed the reproducibility of the isoform ratio over time, by repeatedly analyzing the same sample. The CV of the A/B ratios calculated from 8 replicate runs of a single brain sample was 4.5%. The %SNAP-25A was interpolated using the ratio calibration curve (Figure 4.2A); the CV of %SNAP-25A was 2.4%.

The CV of technical replicates was assessed by independently assaying the same human brain sample three times, in parallel, starting from IP through to MRM analysis. The CV of these replicates was 6.0% (A/B ratio) and 3.0% (%SNAP-25A, interpolated).

4.3.2 SNAP-25 isoforms in human VMC

4.3.2.1 Associations with sample characteristics

Possible associations between age, post-mortem interval, storage time and pH and each isoform were investigated using Spearman correlations. Storage duration was negatively correlated with total SNAP-25 ($P = 0.02$, Spearman correlation $r_s = -0.43$) and SNAP-25B ($P = 0.02$, Spearman correlation $r_s = -0.426$) only. Therefore analyses of SNAP-25 and SNAP-25B differences were re-run, covarying for storage time. The significant difference between diagnostic groups in total SNAP-25 and lack of statistically significant difference in SNAP-25B were unchanged when storage sample time was added to the analysis as a covariate using ANCOVA.

Because the ventromedial caudate samples were run over the span of several days, and potentially affected by variation in machine sensitivity and/or stability of the internal standard,

the possible association of such factors (represented by the peak area of the heavy peptide internal standard) with SNAP-25 isoform measures was assessed using correlation. No significant correlation was detected ($P > 0.05$, Spearman correlation). Covarying for internal standard signal (to remove variation associated with signal stability over time) in the primary analyses did not alter statistical conclusions.

While the IP conditions were optimized to pull out total SNAP-25 from human brain samples, this was not optimized for each brain sample individually. While unlikely, it is possible that SNAP-25 extraction, IP efficiency or recovery from the beads varied from sample to sample. Since investigators were blinded to diagnosis throughout, experimental error is random. If disease-associated differences in IP efficiency occur, they are likely to lessen the differences detected between groups due to a ceiling effect if higher SNAP-25 amounts in NPC samples are not completely depleted from the sample.

4.3.2.2 Exploratory analyses of SNAP-25 isoform associations

Medications and other drugs that affect brain function could have an effect on SNAP-25 isoform expression. We explored the possible effects of antipsychotic exposure, benzodiazepines and smoking to see if these factors could account for the group differences we observed.

All patients had exposure to first generation or "typical" antipsychotic drugs during their lifetimes, while a subset were also exposed to the "atypical" antipsychotic drug clozapine that is used for refractory patients. There was no difference in the level of total SNAP-25, SNAP-25A or B between SCZ subjects who had exposure to clozapine (n = 4) as compared to those who did not (n = 9; uncertain exposure status n = 2) ($P > 0.05$, t-tests). The effect of diagnosis remained statistically significant for total SNAP-25 and for SNAP-25A when primary analyses were re-run

excluding the two subjects with detectable clozapine in toxicological assays. No other samples had any detectable antipsychotic drugs present.

There was no difference in any of the SNAP-25 measures between subjects taking benzodiazepines at time of death as compared to those who were not (7 versus 21 respectively, as determined by post mortem toxicology, $P > 0.05$, t-tests). If analyses are re-run excluding the 7 subjects who tested positive for benzodiazepines, statistical differences between SCZ and NPC remain for total SNAP-25 and SNAP-25A ($P < 0.002$, t-test), and the lower SNAP-25B levels in SCZ become marginally statistically significant ($t = -2.45$, df = 19, $P = 0.024$, two-tailed).

There was also no difference in any of the SNAP-25 measures between smokers and non-smokers (16 versus 9 subjects, $P > 0.05$, t-tests).

Synaptophysin is a ubiquitous synaptic vesicle protein with similar levels in the schizophrenia and NPC samples in the VMC (Appendix 3). Controlling for the level of synaptophysin present in each sample (measured and reported in Chapter 2), results for the SNAP-25 total and isoform analyses remained statistically significant (total SNAP-25: diagnosis $F = 9.86$, df = 1,25, $P = 0.004$, synaptophysin $F = 1.21$, df = 1,25, $P = 0.28$, ANCOVA; SNAP-25A: diagnosis $F = 10.17$, df = 1,25, $P = 0.004$, synaptophysin $F = 1.32$, df = 1,25, $P = 0.26$, ANCOVA; SNAP-25B: diagnosis $F = 2.72$, df = 1,25, $P = 0.11$, synaptophysin $F = 0.36$, df = 1,15, $P = 0.56$, ANCOVA).

4.4 Chapter 4 tables

Table 4.1 - Tissue sample characteristics.
Supplementary Table 1: Diagnoses were established using DSM-IV criteria using all available information. Two subjects had a diagnosis of schizoaffective disorder. Post mortem interval (PMI) and sample storage time were different between groups ($P \leq 0.001$, t-test). PMI was unavailable for one individual. Detailed sample information was previously described [441].
Abbreviations: M – male; F – female; MVA – motor vehicle accident; NPC – non-psychiatric comparison subjects; SCZ – schizophrenia

Mean (SD)	**NPC**		**SCZ**	
Age (years)	51.4 (18.8)		53.6 (12.1)	
Postmortem interval (hours)	16.6 (7.1)		8.8 (3.2)	
Sample storage time (weeks)	196 (77)		316 (74)	
Brain pH	6.16 (0.33)		6.32 (0.22)	
Gender (M:F)	10 : 3		9 : 6	
Smoker (yes:no:N/A)	8 : 4 : 1		9 : 4 : 2	
Cause of death	MVA	5	Pulmonary	3
	Cardiac	4	Cardiac	6
	Homicide	3	Gastrointestinal	3
	Accidental	1	Accidental	2
			Undetermined	1

4.5 Chapter 4 figures

Figure 4.1 - SNAP-25 peptides used for MRM-MS based isoform assay.
A: SNAP-25 protein sequence schematic. Approximate locations of isoform-specific amino acid residues are indicated by vertical lines in the region encoded by the alternative exons (residues 54-96). Peptides chosen for MRM-monitoring in the isoform assay are indicated in color (red = A-specific; blue = B-specific; green = peptide common to both isoforms) and correspond to the MS/MS spectra in panels B-D. **B-D: Peptides and MS/MS spectra used for the isoform assay.** MS/MS spectra obtained from ectopically expressed SNAP-25 for the peptides monitored in the MRM isoform assay are shown. Peptide fragments (transitions) observed by MS/MS are labeled on the spectra and peptide fragmentation diagram. Transitions monitored in the isoform assay are circled. CPS = counts per second, m/z = mass/charge. **B:** SNAP-25A peptide; **C**: SNAP-25B peptide; **D**: Common SNAP-25 peptide. **E: Extracted ion chromatograms of MRM signals for one human brain sample.** Traces correspond to SNAP-25A (red), SNAP-25B (blue) and total SNAP-25 (green) ions detected in a VMC sample. For MRM quantification, peak areas for each co-eluting transition were summed for each peptide (A, B, and Common). **E (inset): Extracted ion chromatogram for a balanced mixture (50/50) of recombinant SNAP-25A and B**. The traces correspond to the most intense transition peak for each peptide. The A and B peptides show different inherent ionization efficiency, as illustrated by the difference in heights and areas of the A and B-specific peaks, thus necessitating the use of an external calibration curve to determine the corresponding percentage of A and B in the sample.

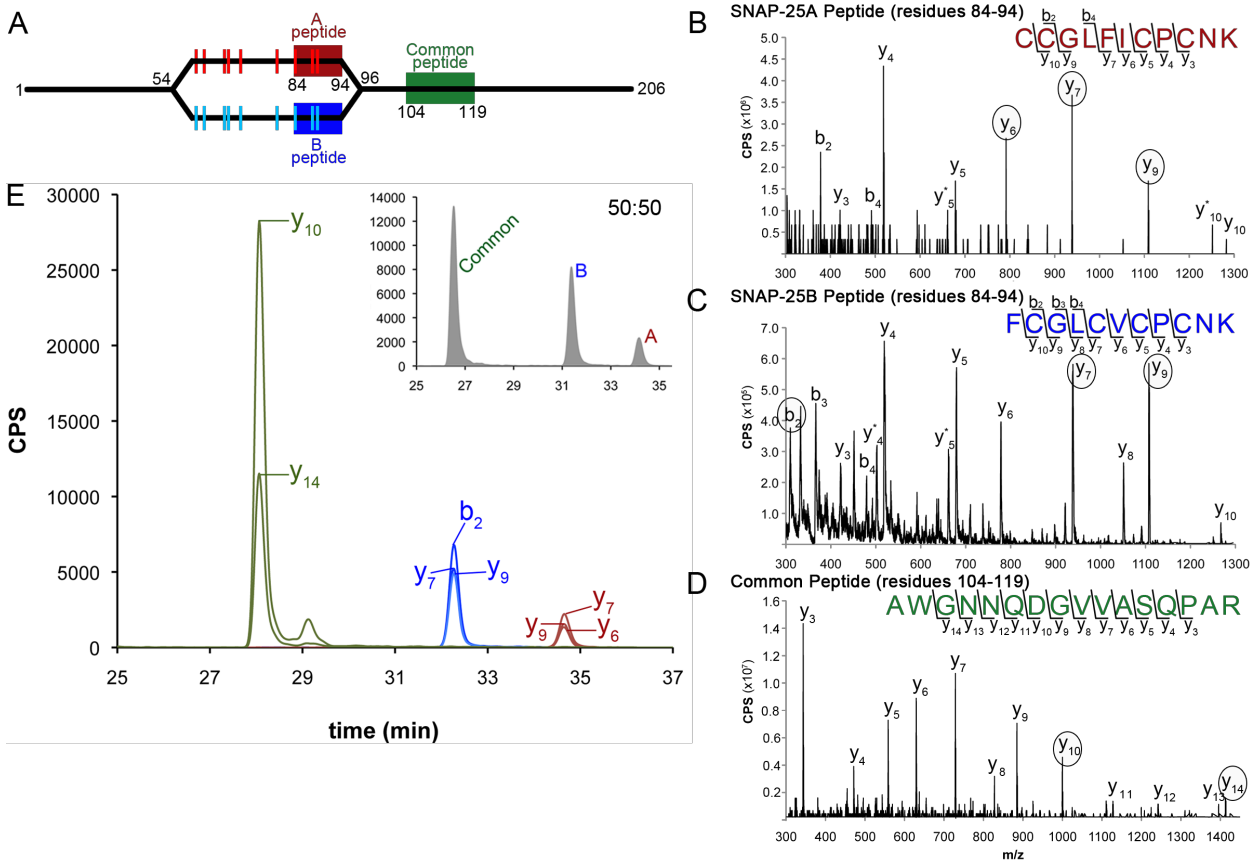


Figure 4.2 - The MRM-isoform assay is able to detect changes in isoform expression. **A:** SNAP-25 isoform ratio calibration curve. Recombinant SNAP-25A and B were mixed in known amounts (%SNAP-25A, x-axis) and analyzed by MRM. The summed peak areas of the A and B transitions were expressed as a ratio (A/B, y-axis). The general relationship between A/B and %SNAP-25A is exponential, but is approximated by a semi-log linear fit in the 5-90% SNAP-25A range (filled circles only, line of best fit has been weighted by 1/y). Error bars represent standard deviation of two or three replicates at different total protein amounts (0.5, 1, 2 μg, determined by Bradford assay). The A/B values for the human ventromedial caudate samples analyzed fell within the linear range (shaded grey). **B:** Depolarization (KCl) or depolarization plus differentiation (KCl+NGF, nerve growth factor) of PC12 cells results in an increase in SNAP-25B expression, measured as the decrease in A/B ratio for the isoform-specific transitions (left axis, line graph) and a change in %SNAP-25B expression (right axis, grey bars) relative to control treatment (NaCl). Error bars on grey columns represent SEM of three biological replicates. **C:** Relative expression of SNAP-25 protein isoforms in human brain may differ between regions. SNAP-25 isoforms were assayed in two individuals (circles - subject #1, squares - subject #2) in different brain regions (Hipp – anterior hippocampus, MFCtx – medial frontal cortex, AnCgCtx – anterior cingulate cortex, anterior thalamus – ThalA, NAc – nucleus accumbens). Plotted are the A/B ratios for the isoform-specific transitions corresponding to SNAP-25A and B peptides. **D:** Standard curves used for absolute quantification of SNAP-25 isoforms in the ventromedial caudate. Three separate amounts of the isotopically labeled heavy peptide (20, 80, 320 fmol) were run with each batch of ventromedial caudate samples (4-12 samples per batch) to create an external standard curve, within the known linear range of peptide detection (Figure 4.6). All ventromedial caudate samples quantified fell within this range. The transition peak area ratios for each heavy peptide sample were normalized to the peak area for the 80 fmol sample and curve fit (log-log fit, 1/x weighting). The common peptide/heavy peptide transition peak area ratio for each ventromedial caudate sample was compared to the curve to interpolate the total amount of SNAP-25 in each sample. **E:** Absolute quantities of SNAP-25 isoforms in samples of human ventromedial caudate. The fmol amount of each isoform was calculated using the A/B ratio for each sample to estimate the %SNAP-25A using the external ratio calibration curve (Panel A), then the absolute amount of SNAP-25A was determined as (%SNAP-25A/100) x fmol of total SNAP-25 (calculated using the heavy peptide external standard curve, Panel D). Fmol of SNAP-25B was the difference between fmol total SNAP-25 and fmol SNAP-25A for each sample. *- Total SNAP-25 and SNAP-25A were 27% and 31% lower in SCZ, respectively ($P = 0.002$; two-tailed t-test). Horizontal lines represent group median, crosses represent means, boxes represent the 25th and 75th percentiles and whiskers represent 10th and 90th percentiles. *Abbreviations*: NPC – non-psychiatric comparison subject; SCZ – schizophrenia. **F:** Absolute quantities of SNAP-25 isoforms in rats with subchronic exposure to antipsychotic medications. SNAP-25 isoforms were measured in medial striatum of rats treated with medications or saline control for 28 days ($n = 10$ per group). Drug treatment did not have a significant effect on SNAP-25A or B levels.

Figure 4.2

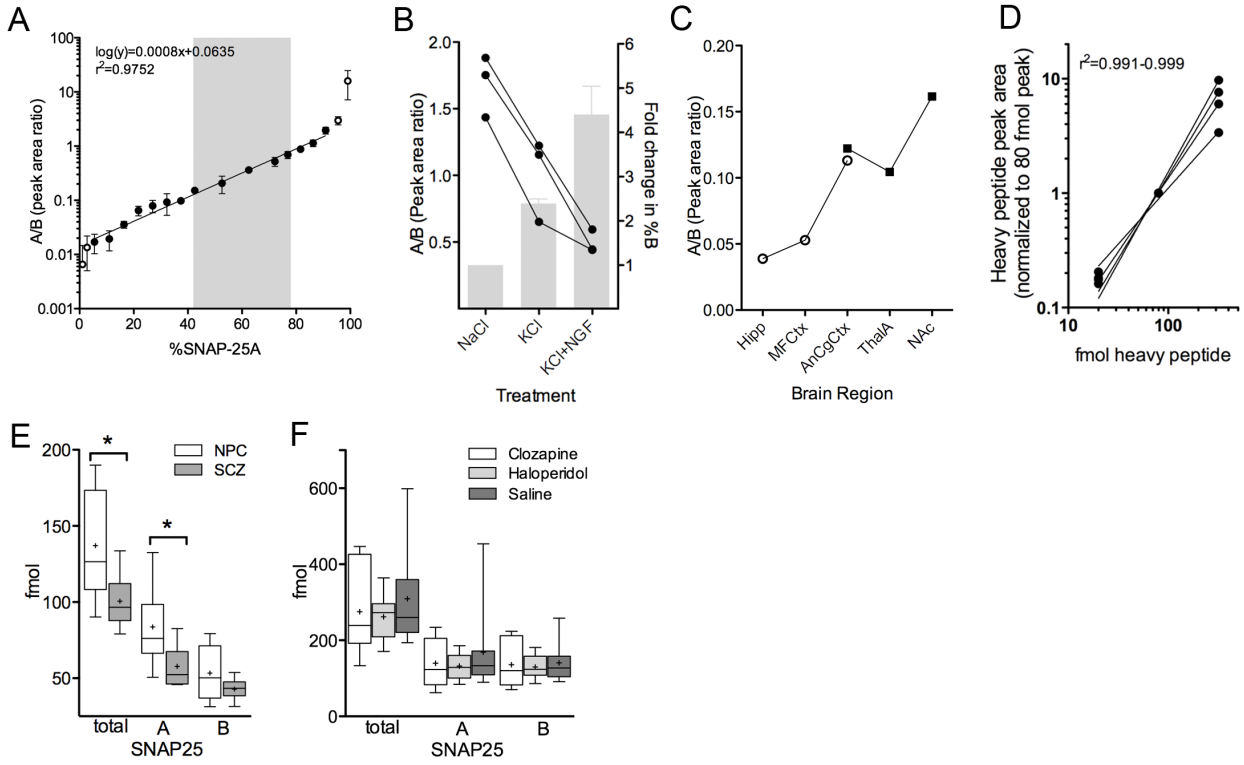

A
log(y)=0.0008x+0.0635
r2=0.9752
A/B (peak area ratio)
%SNAP-25A
B
A/B (Peak area ratio)
Fold change in %B
NaCl
KCl
KCl+NGF
Treatment
C
A/B (Peak area ratio)
Hipp
MFCtx
AnCgCtx
ThalA
NAc
Brain Region
D
r2=0.991-0.999
Heavy peptide peak area (normalized to 80 fmol peak)
fmol heavy peptide
E
NPC
SCZ
fmol
total
A
B
SNAP25
F
Clozapine
Haloperidol
Saline
fmol
total
A
B
SNAP25

Figure 4.3 - SNAP-25 isoform assay sample preparation and analysis workflow.

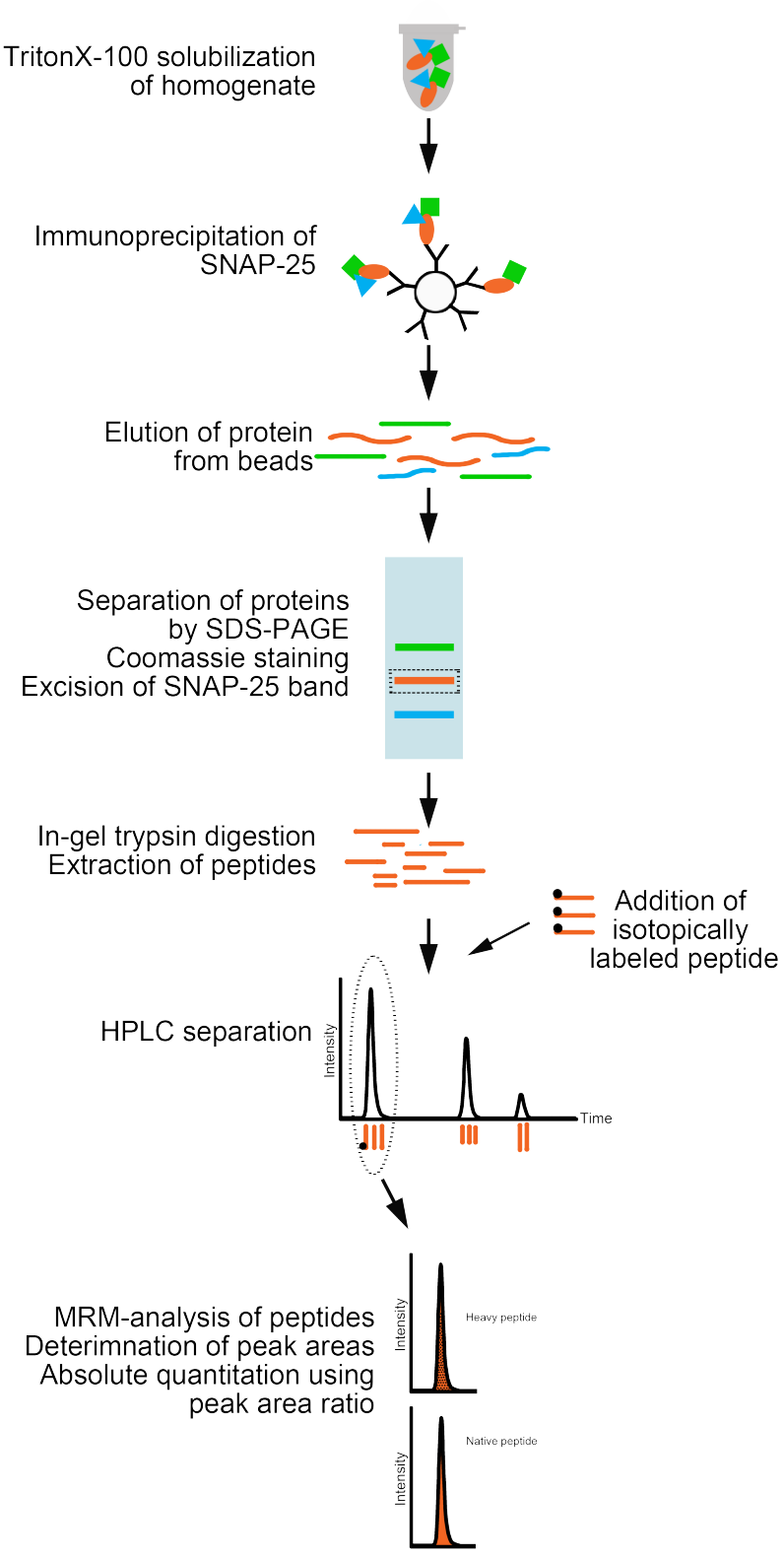


Figure 4.4 - Efficiency of SNAP-25 immunoprecipitation (IP).
For NPC and SCZ samples, equal total protein amounts of crude brain homogenate were solubilized and spun down. IP efficiency of the SCZ sample was monitored by immunoblot. 1/20th of the input was kept to compare with 1/20th of the post-IP supernatant (SN) to confirm that SNAP-25 was specifically removed from the sample. A control IP (beads that are not coupled with α-SNAP-25 antibody, "empty beads") did not immunoprecipitate SNAP-25 from a different brain sample. Efficiency of bead washes was also monitored; wash volume was equal to the volume of IP input, so input, SN and wash lanes can be compared directly. Empty beads correspond to magnetic beads not coupled to α-SNAP-25 antibody, the same bead conditions used in the control IP. Antibody-coupled beads correspond to SP12 (α-SNAP-25 antibody) coupled beads, used for IP. High molecular weight bands correspond to antibody eluting off the beads (IgG). Minimal heavy-chain and no antibody light-chain was detectable in eluted samples (comparing the SCZ or NPC IP lanes with antibody-coupled beads illustrates bands originating from antibody). *Abbreviations:* SCZ – schizophrenia; NPC- non-psychiatric comparison subject; SN – supernatant; IP- immunoprecipitation.

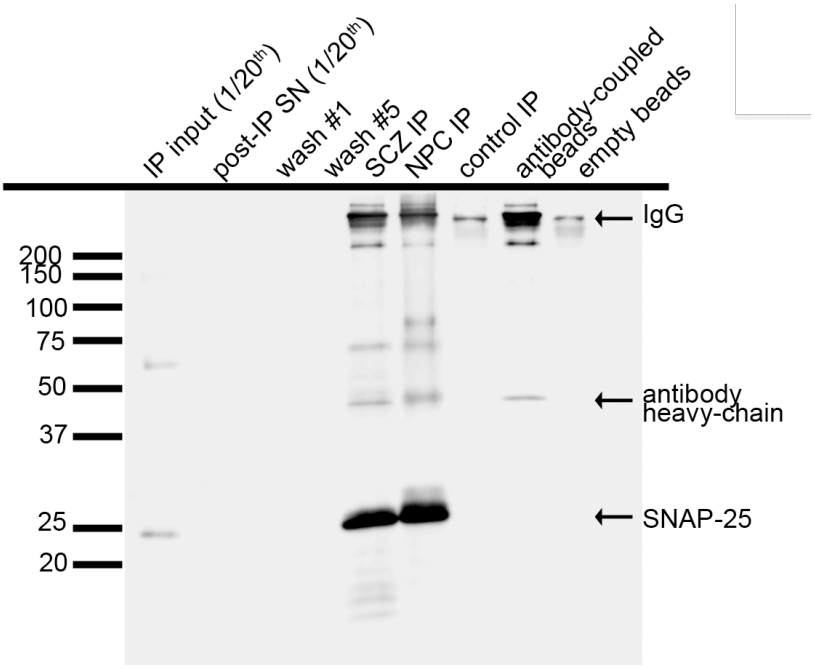
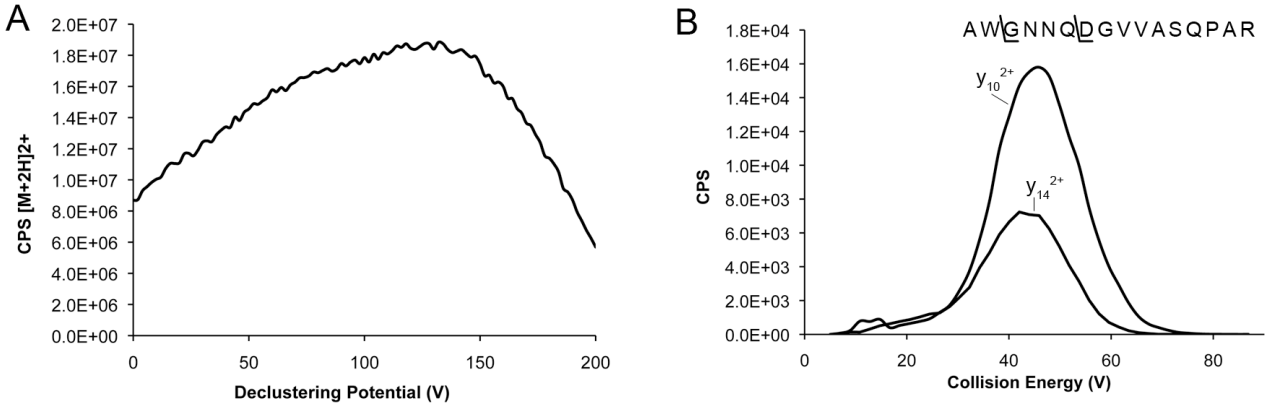


Figure 4.5 - Optimization of declustering potential (DP) and collision energy (CE). **A:** For each peptide monitored, optimal DP was determined for each parent ion by ramping voltage from 0-200V in 2V increments. Optimal DP was selected as the voltage that gave highest signal intensity (counts per second, CPS). **B:** Using the optimal DP, CE was ramped from 0-90V for each potential transition to be monitored, and optimal CE was chosen as the voltage that gave highest signal intensity (counts per second, CPS). Example curves are given for the SNAP-25 common peptide. Similar procedures were performed for SNAP-25A and B peptides. **C:** The table shows the final MRM parameters used.



C. Final MRM Parameters							
	SNAP-25 Peptide	**Residue**	**Charge state**	**Parent Ion m/z**	**Fragment m/z**	**DP (V)**	**CE (V)**
A	CCGLFICPCNK	84-94	2+	714.8	1108.7 (y_9) 938.8 (y_7) 791.6 (y_6)	100	33 33 33
B	FCGLCVCPCNK	84-94	2+	707.8	308.3 (b_2) 1107.8 (y_9) 937.7 (y_7)	100	37 33 33
Common	AWGNNQDGVVASQPAR	104-119	2+	835.4	999.8 (y_{10}) 1413 (y_{14})	120	45 44

Figure 4.6 - Linearity and detection limit of SNAP-25 peptides.
A: Detection range of the SNAP-25 common peptide. The heavy peptide (H104-119) was serially diluted in 1x MS buffer. Summed transition peak areas were normalized against one point in the curve (~80 fmol dilution step) and plotted against the absolute quantity of the peptide. Points omitted from the curve fit are unfilled. Relative peak areas were log-linear over 2049-fold range (2.4 – 5000 fmol, filled circles). The dilution range encompassed by the external standard curves used to quantify human brain samples (Figure 4.2D) is shaded in grey. **B & C: Detection range of SNAP-25A and B** was assessed using recombinant SNAP-25 and in-gel trypsin digestion. Isoforms were serially diluted and run on an SDS-PAGE gel, stained with Coomassie (bands are illustrated) and digested. Sums of the raw peak areas for isoform-specific transitions are plotted (left axis, black points) against the amount of recombinant SNAP-25 loaded onto the SDS-PAGE gel (x-axis). Extracted peptides were run in the presence of the heavy peptide (H104-119) internal standard to allow quantification of the amount of SNAP-25 (common peptide) extracted from the gel (right axis, grey shading). Isoform specific-peptide signals were log-linear over a 50 to 150-fold range, and were detectable by MRM even at protein amounts below the detection limit of Coomassie staining. Note that the amount of the A or B specific peptide measured is directly proportional to the total SNAP-25A or B measured using the common peptide, suggesting that peptide recovery is not biased. Note: X and Y parameters in equations refer to log values and log values are used in plots.

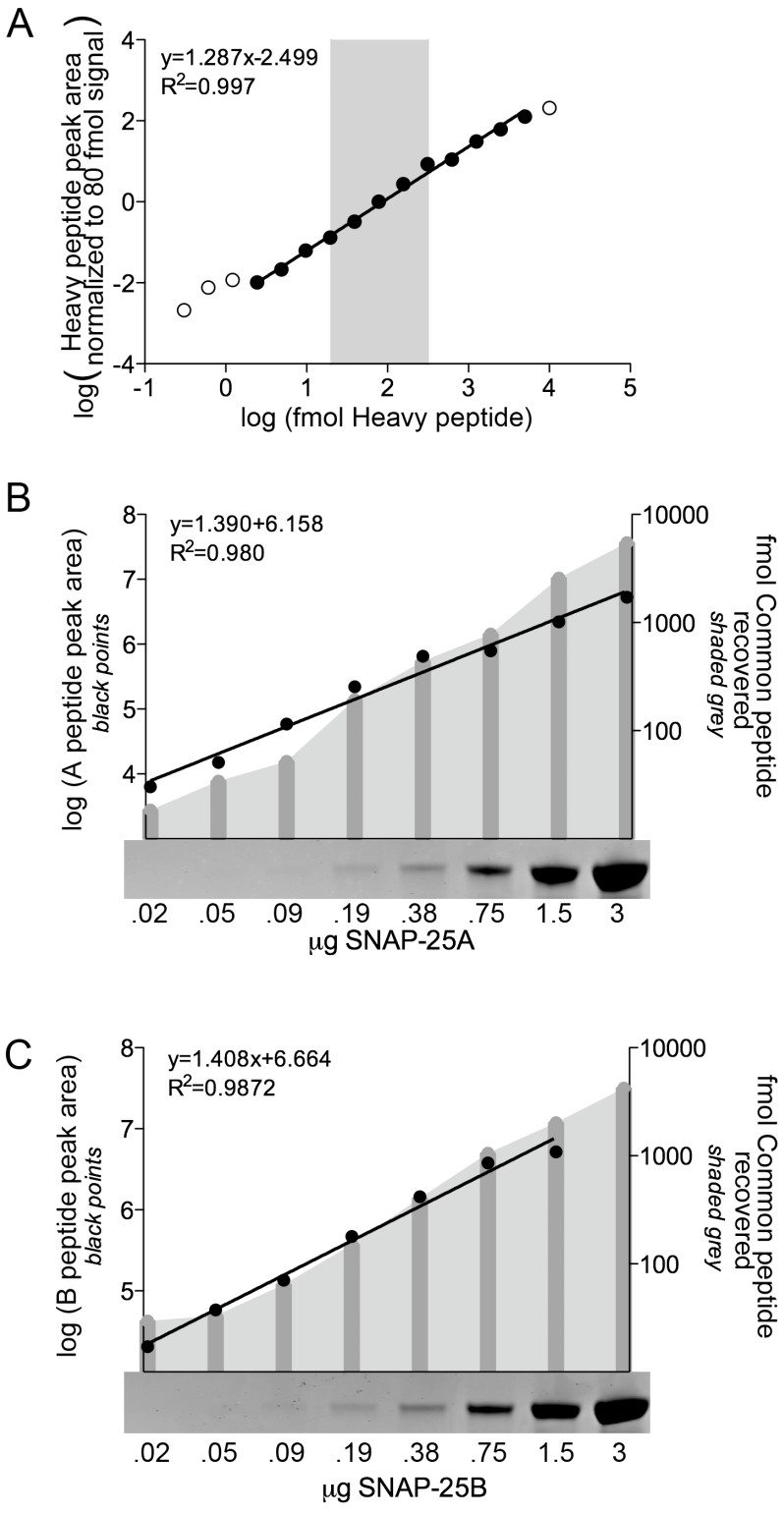

A
y=1.287x-2.499
R2=0.997
log(Heavy peptide peak area normalized to 80 fmol signal)
log (fmol Heavy peptide)
B
y=1.390+6.158
R2=0.980
log (A peptide peak area) black points
fmol Common peptide recovered shaded grey
.02 .05 .09 .19 .38 .75 1.5 3
µg SNAP-25A
C
y=1.408x+6.664
R2=0.9872
log (B peptide peak area) black points
fmol Common peptide recovered shaded grey
.02 .05 .09 .19 .38 .75 1.5 3
µg SNAP-25B

Figure 4.7 - Co-elution of heavy internal standard and endogenous common SNAP-25 peptide in a human brain sample. Prior to MRM analysis the heavy peptide (H104-119) was added to the tryptic peptides of immunoprecipitated SNAP-25. The MRM traces for the common and heavy peptide transitions from a brain sample are shown (heavy = yellow traces, common = green traces). The heavy and common peptides co-chromatograph but are distinguishable by MS due to the 10 Da greater mass of the heavy peptide relative to the common peptide. 'C' denotes common peptide, 'H' denotes heavy peptide.

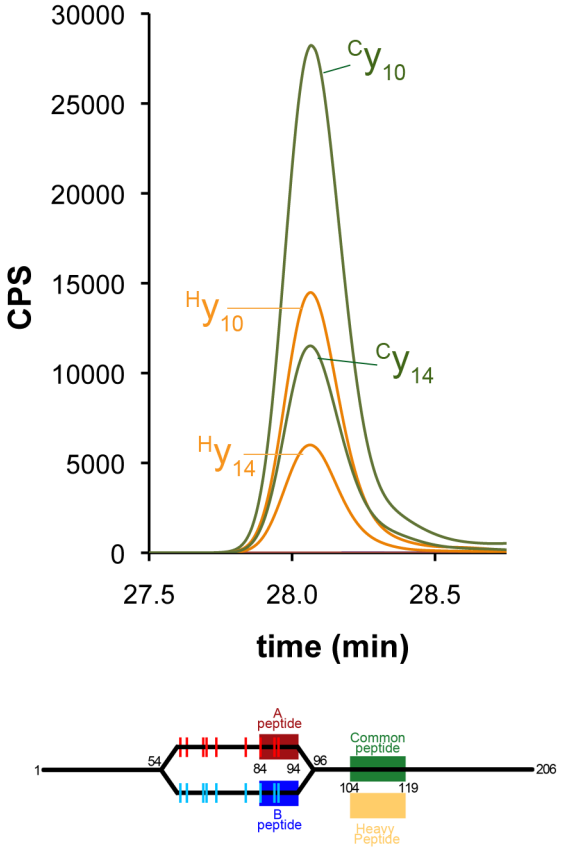
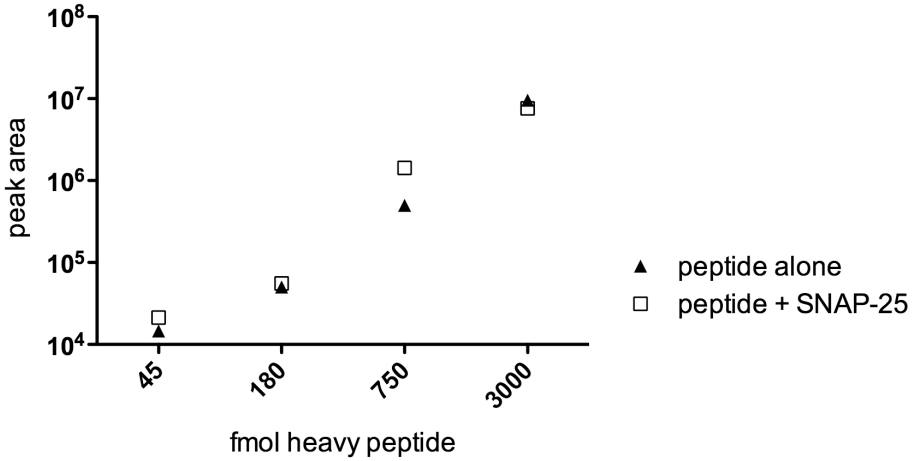


Figure 4.8 - Ion suppression of the isotopically labeled peptide is minimal in the presence of SNAP-25 protein. The peptide was run alone, or added into a digested sample of SNAP-25 (~ 1µg starting material) to determine if the heavy peptide signal is suppressed in the presence of native SNAP-25.



5 Discussion

5.1 Major findings

SNARE protein measurements in human striatum revealed significantly lower levels of SNAP-25 and syntaxin-1 protein in the ventromedial caudate in schizophrenia patients. In contrast, antipsychotic administration in rats produced changes in the opposite direction. Significant differences in the ratio of syntaxin-1A to syntaxin-1B were detected by quantitative immunoblotting, and results indicated that syntaxin-1 isoform expression in schizophrenia might be an exaggeration of normal age-related changes. A novel quantitative mass spectrometry approach demonstrated that significantly lower levels of SNAP-25A protein contributed to the overall lower SNAP-25 level in schizophrenia. In addition, a unique ELISA assay indicated that syntaxin-SNAP-25 protein interactions were significantly higher in the VMC in patients (Table 5.1).

5.2 Striatal SNARE abnormalities in the context of schizophrenia

Characterization of protein expression, protein-protein interactions and isoform-specific differences in SNAREs in schizophrenia was undertaken to better understand the involvement of these proteins in synaptic dysfunction in patients. The potential functional consequences of SNARE abnormalities identified in Chapters 2-4 are discussed further in section 5.2, drawing on what is known in the literature about SNARE function and relating this to brain function and disease in patients. In addition, possible underlying mechanisms that could produce the SNARE alterations we observed are put forth. In doing so, it becomes apparent that there are gaps in our understanding of SNARE function, limiting the interpretation of the present findings and pointing to areas in need of future research.

5.2.1 Lower SNARE protein levels in the VMC

The significantly lower levels of SNAP-25 and syntaxin in the VMC observed here (Chapter 2) are consistent with other lines of research indicating abnormal striatal function in schizophrenia [61, 139, 402, 458, 459]. The level of resolution of post mortem dissection versus brain imaging is quite different however, some studies have distinguished between ventral and associative striatum which helps put our findings into context of imaging and cognitive studies of schizophrenia [139, 141]. Increased dopamine release in associative striatum [139] and overall striatal activity during tasks that require maintenance of working memory [402] as well as decreased striatal activity during motor inhibition tasks [459] have been reported in patients. Such findings indicate that baseline and evoked dopamine transmission in the striatum is abnormal in schizophrenia, and that patients recruit different neural circuits during fronto-striatal tasks. Reduction in syntaxin-1 and SNAP-25 in the VMC, as we observed, could be involved in one or both of these phenomena.

5.2.1.1 Functional consequences of lower SNAREs

The functional consequences of lower SNARE protein levels are illustrated by SNARE KO animals as well as genetic and biochemical manipulations in cells. Given that complete genetic KO is lethal in many models (SNAP-25, VAMP2 and some syntaxin-1A KO animals) [186, 188, 383], large reductions in SNARE protein levels were not expected in schizophrenia.

Knock-out mice created by Fujiwara *et al*. illustrate the consequences of syntaxin-1A deletion. These mice exhibit impaired memory consolidation and extinction, abnormal social interaction, impaired latent inhibition and a heightened startle response, while spontaneous and evoked GABAergic and glutamatergic neurotransmission appear normal (Table 1.2) [242]. Spontaneous neurotransmission, however, is altered in immature neuronal cultures from these

animals. This could mean that the functional consequences of syntaxin-1A reduction during times of synapse maturation may manifest as behavioural deficits later on in life. This possibility provides a link between our finding of reduced syntaxin-1 expression in the VMC and the neurodevelopmental hypothesis of schizophrenia (bearing in mind that post mortem protein measurement in adults may not reflect protein levels during development). In addition, at least some of the behavioural impairments (including attenuated latent inhibition, also seen in schizophrenia patients) are also observed in heterozygote KOs (where syntaxin-1A expression may be more comparable to that seen in patients) and abnormal behaviour can be ameliorated by a selective serotonin reuptake inhibitor, but not dopamine or noradrenaline reuptake inhibitors [384], suggesting that syntaxin-1A may be important for the release of some, but not all, neurotransmitters. Another syntaxin-1A KO mouse, created by McRory *et al*., however, possessed a completely different phenotype. Only 4 of 224 animals were born homozygous knock-outs, but these animals showed limited behavioural abnormalities (direct effects on neurotransmission were not assessed in these animals) (Table 1.2) [383]. The different effect of syntaxin-1A KO in the two models was suggested to be due to differences in the extent of gene disruption [460, 461]. This may be relevant when considering sources of SNARE protein variation in schizophrenia, as complete deletion of SNARE genes does not occur, much variation is contained within non-coding regions of the genes, and epigenetic mechanisms have been postulated to be involved in disease expression [180, 281, 462, 463]. In addition, differences in mouse strain background may also contribute to phenotype differences [253, 460, 464]. The mouse strain used by McRory *et al*. is not documented, thus the contribution of strain effects remains speculative. However, it seems plausible that changes in SNARE protein expression in our human samples might have different effects on (or be related to differences in) brain function

and behaviour due to differences in the genetic make up of each individual, just as the strain background of KO mice can affect the phenotypes expressed.

Consequences of reduced SNAP-25 expression are illustrated by several different animal models (Table 1.1) and provide a perspective for interpreting our VMC findings (Figure 2.1). Complete ablation of SNAP-25 eliminates evoked neurotransmitter release while spontaneous, calcium-independent release is still present (Table 1.1). Thus, SNAP-25 expression level affects neurotransmission. Lower levels of SNAP-25 detected in Chapter 2 could make patient synapses less responsive to action potentials or other sources of presynaptic calcium, reducing synaptic transmission. This could contribute to the functional dysconnectivity observed in patients during cognitive task performance, and could account for the need to use different combinations of neural circuits to complete the same task [402]. The *coloboma* mouse exhibits a 50% reduction in SNAP-25 protein levels (Table 1.1). Less extreme than SNAP-25 KO animals, SNAP-25 expression in *Cm*/+ mice is more in line with SNARE protein levels observed in schizophrenia samples (Table 1.4), and may therefore be more relevant in interpreting consequence of SNAP-25 reductions observed in the VMC in our sample set. Synaptic plasticity is altered in *Cm*/+ animals, and this includes inhibitory neuron function (as illustrated by reduced hippocampal theta rhythms). Animals are hyperactive and respond abnormally to amphetamine [375, 382]. Expression of a *Snap25* transgene[7] (encoding mouse SNAP-25B) in the brain ameliorated the hyperactivity phenotype in a gene-dose-dependent manner and also improved the abnormal amphetamine response of *Cm*/+ mice, indicating that SNAP-25 likely contributes to abnormal dopamine release in the striatum in schizophrenia [378, 380, 382]. In addition, the striatum may be particularly sensitive to changes in SNAP-25 protein levels as transgene expression in the

[7] While the transgene was not designed for tissue-specific expression, the founder line selected for study by Hess *et al*. did not show transgene [mRNA] expression in liver, kidney and spleen [339]. Transgene expression was not inducible and therefore presumed to be expressed during development as well as in adulthood. Transgene expression did not ameliorate head-bobbing and eye dysmorphology in these mice.

coloboma mouse was greatest in the striatum and cerebellum, and less noticeable in other brain regions [375]. Reduction of SNAP-25 in the striatum, as we observed in Chapter 2, may have greater functional implications than in other regions.

SNARE KO animals provide a glimpse of how SNARE reductions might affect brain and behaviour, albeit to an extreme. The severity of SNARE KO is difficult to relate or compare to the smaller magnitude SNARE perturbations seen in schizophrenia patients. However, this is somewhat reconciled by evidence of allele-dose effects. Heterozygous SNARE KO phenotypes are less severe than their homozygous counterparts (Table 1.1-1.3), and allele-dose effects also occur with SNARE gene SNPs and human brain function. For example, some phenotypes of high-functioning autistic subjects were dependent on allele-dose of the associated *STX1A* SNPs, with more impairment in subjects homozygous for the disease-associated allele, heterozygotes showing less severe impairment [183]. In addition, weight-gain in schizophrenia subjects was most pronounced in patients who were homozygous for the overtransmitted SNP, while weight-gain in heterozygote individuals was intermediate [281]. Schizophrenia is a complex disease. Homozygosity of a genetic mutation in SNARE (or other) genes therefore may not be sufficient or necessary for disease expression, although could contribute to disease risk. SNARE KO animals therefore model only one possible genetic contribtion to the disease. Divergence of SNARE gene sequence between species may also contribute to the difference in phenotypic severity.

Reduced SNARE protein levels in animals affect evoked, more so than spontaneous, neurotransmission. However, changes in amplitude and frequency of spontaneous neurotransmission (or "minis") are also reported (see Tables 1.1-1.3). Minis have been postulated to be a means of shaping evoked synaptic responses [465]. It seems plausible that the

partial reductions in SNAREs in schizophrenia observed in our sample set might cause shaping to occur to a different extent, contributing to abnormal synaptic function.

The means by which reduced SNARE protein levels in knock-out animals affect synaptic function are unclear. SNARE protein interactions may be affected, as might other steps of the vesicle cycle. There is insufficient evidence in the literature to conclude the direction in which (i.e. an increase or decrease in) neurotransmission might be changed in the patients with reduced SNARE protein levels observed here. Directionality may also vary between terminal/neurotransmitter types. For example, in the *Cm*/+ mouse, glutamate release from cortical synaptosomes is *decreased*, as is evoked monoamine release in dorsal striatum (Table 1.1). However, evoked monoamine release is *higher* in the cortex of the same animals (Table 1.1) [378]. Studies of *Snap25* -/- animals and cultured cells indicated that evoked transmitter release is most sensitive to SNAP-25 reductions, with spontaneous neurotransmitter release still present, but at reduced frequency [186]. *Snap25* +/- (heterozygote) animals have not been studied extensively, and synaptic function is assumed to be normal[8], however, differences in behaviour or neuron function likely exist, or have been compensated for during development. SNARE complexes were not studied in these animals.

Intuitively we postulated that decreased SNARE protein levels in the VMC in schizophrenia subjects (Chapter 2) would limit SNARE complex formation, and inhibit neurotransmission. The results of our SNARE protein interaction measures (Figure 2.1D), however, do not support this, as syntaxin-SNAP-25 protein interactions were increased in

[8] Western blots reported by Washbourne et al. 2002 indicate that SNAP-25 protein is reduced in Snap25 +/- animals. Interestingly, electrophysiological and cell-culture experiments used +/+ and +/- animals as controls in various experiments. In particular +/+ and -/- mice were compared using cortical slices while +/- and -/- mice were compared in hippocampal cultures. Whether this is due to impaired synaptic transmission at heterozygote synapses when neurons are present in more complex networks (such as brain slices or intact brain) as opposed to autapses of cultured neurons, was not reported [161].

schizophrenia in the face of lower SNAP-25 and syntaxin-1 levels in the VMC. But, as discussed later on, increased SNARE complexes do not definitively mean increased neurotransmission, as they can represent dead-end assembly pathways that will not be used for transmitter release [222], or impaired SNARE dissociation and vesicle recycling [466], leading to reduced synaptic transmission. Potential functional consequences of our findings of lower syntaxin-1 and SNAP-25 protein in the VMC include alterations in neurotransmission, synaptic plasticity, behaviour and cognition.

5.2.1.2 Possible mechanisms underlying SNARE protein differences

Mechanisms that may underlie the lower syntaxin-1 and SNAP-25 levels we observed in the VMC include regulation of expression at the gene or protein level. Evidence for gene-level mechanisms altering SNARE expression in schizophrenia include the genetic association of SNARE SNPs with disease in some patient populations [176, 180] and perturbed SNARE expression and function in SNARE KO animals. However, gene deletions in animals may have different phenotypic effects as compared to single nucleotide polymorphisms or mutations seen in humans due to pleiotropy or compensatory mechanisms. Extensive disruption of a SNARE gene is only known to occur in William's syndrome in humans (where chromosomal deletion encompasses *STX1A*) and is certainly a severe syndrome with many developmental and cognitive consequences [290]. Differences between patient and SNARE KO animal phenotypes also reflect the polygenic and environmental components of disease. Indeed, more complex phenotypes reminiscent of disease are produced when SNARE mutations and other disease risk factors co-occur in animals, as seen in the *blind-drunk* mouse (Table 1.1) [253], suggesting that environmental factors might also contribute to the present findings. Finally, association of SNARE gene variation with schizophrenia has not been replicated in all samples groups studied,

as detailed in Section 1.4.3 [176, 277-279, 282]. This suggests that genetic variation in SNARE genes may not account for protein-level changes and is probably not primarily responsible for disease symptoms. Alterations in synaptic proteins may also occur as the result of other disease-associated changes, including gene expression (mRNA) changes or protein turnover as well as more global sources such as altered brain function [467].

Regulation at the mRNA level, either by reduced transcription, translation or mRNA stability could account for our findings in Chapter 2, and seems likely given that disease-associated SNARE SNPs occur in non-coding regions of the gene [176, 180]. Areas of the prefrontal cortex, specifically the orbitofrontal regions (approximately BA10/47) are thought to project to the VMC [129, 134]. However, SNAP-25 mRNA levels were not different in schizophrenia when assayed in BA10 [272], suggesting that altered SNAP-25 expression in corticostriatal projection neurons may not account for the present findings. Attempts to interpret protein changes in the VMC based on mRNA changes in areas that project to the striatum assume that mRNA resides in the cell body, and not distal regions of the neuron. This assumption, however, may not always be valid, as we now know that of mRNA can be localized and translated in synaptic terminals, and not just cell bodies [468, 469]. Expression levels of SNARE mRNA in the striatum in schizophrenia are unknown. The possibility that SNAP-25 gene expression might be decreased locally in the striatum is indirectly supported by recent reports of lower expression of the Sp1 transcription factor in striatal tissue of subjects with schizophrenia [470]. Sp1 regulates SNAP-25 expression [471], and its expression levels are correlated with the expression of genes it regulates [470]. Lower SNAP-25 transcript levels also occur after spinal nerve injury, and in early development [266]. Whether axonal damage is responsible for striatal SNAP-25 regulation remains unknown, but the possibility is supported by

reports of white matter alterations in patients, both post mortem and *in vivo* [119]. It is unknown if syntaxin-1 mRNA is altered in cells which terminate in the striatum.

Support for protein-level regulation of SNARE expression also exists. One of the methods of degrading proteins in eukaryotic cells involves post-translational modification of the protein via enzymatic addition of ubiquitin. Ubiquitin-tagged proteins are targeted to the proteosome for degradation. This is a multi-step process involving several different enzymes [472]. Problems with this process underlie conditions such as juvenile-onset familial Parkinson's disease and Angelman syndrome [473]. Dysregulation of the ubiquitin-proteasomal system (UPS) in schizophreina has been suggested by gene expression studies of thalamic and blood samples from patients [474, 475], and might account for reduced SNARE protein levels if dysregulation extends to the striatum. Both SNAP-25 and syntaxin-1 can be degraded via the proteasomal pathway [476, 477]. Interestingly, proteasomal protein degradation is thought to be involved in synapse elimination [478] and plasticity [479], suggesting a point of convergence between UPS dysregulation, SNAREs and the dysconnectivity hypotheses of schizophrenia.

Besides protein-specific changes, lower levels of SNAP-25 and syntaxin-1 we observed in the VMC could be due to reductions in synaptic terminal numbers, although this possibility seems less likely for number of reasons. Only a few studies have shown changes in cell numbers in the striatum [143, 144]. Whether this produces a change in synapse numbers is not known. Increased density of glutamatergic and dopaminergic terminals has been described in some patients [147, 312], arguing against synaptic loss as the underlying cause of lower SNARE protein levels. Furthermore, VAMP and synaptophysin protein levels were not different between groups in our studies (Figure 2.1 and Appendix 3). Our findings might also reflect the differential expression of syntaxin-1 and SNAP-25 in different terminal types selectively affected in schizophrenia [194, 204]. We observed incomplete colocalization of SNAREs in the

VMC, supporting this possibility (Figure 2.2). Others have shown that neuronal transporters involved in reuptake of glutamate from synapses are downregulated in the striatum in schizophrenia [52, 403]. Gene expression of vesicular glutamate transporters is also downregulated in dlPFC of schizophrenia subjects [53]. Some of these cells likely project to the VMC; whether similar expression changes occur in the OFC is unknown. It is somewhat difficult to reconcile the decreased expression levels of glutamatergic markers or SNAREs with the increase in glutamatergic synapse density reported in ultrastructural analyses [312]. Compensatory down-regulation of proteins in the face of increased synaptic density, or up-regulation of synapse numbers to accommodate lower protein expression levels cannot be ruled out. Future work to determine if individual SNAREs are proxies for specific terminals seems warranted, and may help resolve such inconsistencies.

5.2.2 Lower SNAP-25A expression

It appears that lower levels of SNAP-25 protein in the VMC are primarily due to a decrease in SNAP-25A protein expression, which suggests additional regulatory mechanisms might be involved. The preferential decrease in one isoform allows the functional consequences of SNAP-25 reductions to be interpreted with more specificity, as discussed in the following section.

The functional consequences of lower SNAP-25A protein levels observed in the VMC can be estimated from cell culture and animal studies. SNAP-25B KO mice (Table 1.1) show altered synaptic facilitation in hippocampal slices, indicating that changes in the amount of only one isoform can perturb the balance of activity in larger circuitry, as would occur in schizophrenia. Overexpression of SNAP-25B in *Snap25* -/- chromaffin cells supports a larger vesicle pool size and increased SNARE complex stability compared to SNAP-25A [196, 249],

suggesting that a greater reliance on SNAP-25B results in increased neurotransmission. Overexpression in and subcellular fractionation of cultured neuroblastoma cells indicates that the B isoform is associated with LDCV (like those involved in neuropeptide release), while SNAP-25A is associated more with small synaptic vesicles (similar to glutamate and GABA-containing vesicles) [250]. Thus, our findings might indicate dysfunction of a subset of terminals.

The means by which SNAP-25A levels are lower in schizophrenia include the mechanisms described in section 5.2.1, as well as control at the level of splicing. This possibility is supported by evidence of aberrant mRNA splicing of other schizophrenia-related genes in post mortem brain [480, 481]. The possibility of aberrant SNAP-25 splicing in disease is also supported by the fact that disease and cognition-associated SNPs occur in regulatory, not coding, regions of the gene [281, 282, 285]. Of particular relevance to the neurodevelopmental model of schizophrenia, the alternative splicing of SNAP-25 is developmentally regulated; mRNA of the B isoform is upregulated two weeks postnatally in rodents [250] and is estimated to account for up to 90% of SNAP-25 mRNA in adult rat cortical neurons [453]. Despite the preponderance of B mRNA in adulthood, however, regulation of isoform expression may also differ at the protein level. In some versions of the SNAP-25B KO mouse, total SNAP-25 protein levels are 50% lower despite the fact that SNAP-25A mRNA levels are 4-fold higher than in wild-type mice [252]. Our measurements in Chapter 4 indicate that in rodent and human striatum isoform expression at the protein level is approximately equal, in contrast to the reported mRNA proportions (90%B mRNA in adult rodent brain) [453]. There may be an optimal balance between A and B protein levels or a maximum amount of a given isoform that can be expressed, accounting for the mRNA/protein expression discord.

The interpretations of SNAP-25A protein reduction detected in our samples and discussed above are limited by incomplete understanding of the isoforms at the protein level, and

point to an area for future research. Existing information and the present findings will help guide future hypotheses surrounding the involvement of SNAP-25 in illness.

5.2.3 Greater levels of syntaxin-SNAP-25 protein interactions

Higher SNARE protein interactions detected in the VMC in schizophrenia could have several functional implications, some of which would be consistent with increased or decreased striatal activity seen in patients, and are discussed here. The possible molecular and systems-level phenomena that could contribute to these findings are also outlined.

Increased SNARE complex stability is associated with larger evoked synaptic responses [196, 229, 249] suggesting that action potential-induced signal propagation or synaptic strength might be higher in the VMC in schizophrenia. Increased stability has been demonstrated in SNAP-25B containing SNARE complexes [196], and may underlie the increases observed here, as suggested by the negative association of SNAP-25A protein levels with SNARE protein interactions (Chapter 4).

From an alternate perspective, more SNAREs in complexed form could reflect reduced SNARE complex dissociation and neurotransmission [466] if SNARE proteins are not available for re-use and are in *cis*-conformation on recycling vesicles. This would support *in vivo* studies that find reduced striatal activation in patients [459].

Increased complex stability is likely due to differences in interactions between SNAP-25A and B containing SNARE complexes and SNARE accessory factors [249]. Several SNARE-modulating proteins have been studied in schizophrenia. Molecules with altered mRNA or protein expression in schizophrenia include the complexins, Munc-18, septin-5, NSF and synaptophysin. Synaptophysin measurement in the striatum (Appendix 3) indicated no differences between groups or striatal regions, suggesting that modulation of VAMP availability

by synaptophysin levels may not underlie the increase in SNARE protein interactions detected in the VMC [307]. However, complex stabilization by complexins, or decreased dissociation by NSF could account for the increased syntaxin-SNAP-25 interactions detected here. Interestingly, complexin-2 mRNA levels may be lower in the dlPFC in schizophrenia [53]. Given that complexin-2 is associated with glutamatergic terminals this could result in altered complexin-2 protein in corticostriatal terminals. Higher levels of NSF in anterior cingulate of schizophrenia subjects detected by using 2D-gels and mass spectrometry also suggest that SNARE protein interactions could be abnormal due to changes in accessory factors [344], although such a change would be consistent with decreased SNARE complexes since NSF is involved in SNARE complex dissociation [224]. Whether these molecules are altered in terminals in the VMC and contribute to our findings remains to be determined.

The capture ELISA measures syntaxin bound to SNAP-25 and cannot discriminate between protein complexes of different composition. Thus, the trimeric SNARE complex, as well as binary syntaxin-SNAP-25 interactions are likely being measured. Different functional consequences would be inferred depending on whether the SNARE trimer or dimers are increased in the VMC. During the vesicle cycle, syntaxin and SNAP-25 form a 1:1 acceptor complex to which VAMP later binds [215, 216]. Increased acceptor complexes might result in increased efficiency of SNARE complex assembly, vesicle priming and fusion. SNAP-25-syntaxin-1A protein interactions have been shown to decrease the uptake of GABA from the synaptic cleft via GABA transporter binding [406], indicating an additional functional consequence of increased syntaxin-SNAP-25 complexes. However, off-pathway SNARE protein interactions have also been documented. These represent non-functional combinations of SNAREs, including syntaxin-SNAP-25 2:1 complexes [222]. If the increased complexes we

observed are actually off-pathway products, they might reflect reduced neurotransmission or inefficient formation of the functional SNARE trimer.

Systems-level phenomena could also contribute to increased protein interactions in schizophrenia. Increased brain activity in the hippocampus is associated with increases in SNARE complexes [230, 231] and dopamine has similar effects in striatal slice preparations [407]. While the findings in Chapter 2 suggest that haloperidol and clozapine do not affect SNARE complex levels, other psychoactive substances may. Morphine-induced SNAP-25 phosphorylation decreases SNARE complex levels in rat hippocampus, but not frontal cortex [232]. Phosphorylation of individual SNARE proteins appears to be one way of modulating SNARE protein interactions [397], and decreased syntaxin-1 phosphorylation in the dlPFC may be related to reduced SNARE complex levels in schizophrenia subjects [293]. Volatile anesthetics can bind SNARE complexes and treatment of cultured cells with isoflurane decreased exocytosis in a syntaxin-1A-dependent manner [482, 483]. Peroxynitrite, a reactive oxidant formed from nitric oxide, can modify SNAP-25. When synaptosomes are exposed to peroxynitrite, SNARE complex levels and glutamate release increases [484]. Thus, redox-sensitive states may change SNARE protein interactions, an interesting possibility in light of reported alterations in antioxidants and nitric oxide species in patients [485-488].

Differences in the affinity of SNAREs for each other (due to isoform differences, post-translation modifications, or accessory factors) likely affect SNARE complex levels. Indeed, the findings in Chapter 3 and Chapter 4 support this possibility, as the syntaxin isoform ratio and SNAP-25A levels were negatively correlated with syntaxin-1-SNAP-25 protein interaction levels. While statistically significant, some of these correlations were of modest effect size, suggesting that SNARE isoforms are likely not the only factor contributing to SNARE protein interactions in human brain samples, supporting the possible involvement of additional factors.

Whether syntaxin-1-SNAP-25 isoforms have different affinities for each other remains unknown. *In vitro* binding assays would be useful in investigating this possibility.

5.2.4 Altered syntaxin isoform ratio

The possibility of differences in SNARE isoforms influencing SNARE complex levels was also investigated for syntaxin, and revealed a significantly lower Stx1A:1B ratio in the striatum in schizophrenia, and a moderate negative association between the syntaxin ratio and SNARE complex levels in ventral striatal areas (Chapter 3). Functional implications and possible reasons for this difference can be inferred from proteomic and immunohistochemical studies in the literature, and are discussed in this section.

Recent studies found syntaxin-1A preferentially associated with vGlutT1-positive vesicles [201, 204]. The functional consequences of changes in the syntaxin-1A:1B ratio in schizophrenia may therefore be changes in the balance of glutamate and GABA transmission in the striatum. These conclusions were based on immunohistochemical analysis of cortex, and proteomic analysis of synaptic vesicles [presumably] originating from total brain preparations [201, 204]. Whether the distribution is similar in striatal terminals needs to be determined in future studies. The excitatory/inhibitory split between syntaxin-1 isoforms may be region or cell-type specific, given that LTP induction of syntaxin-1B in mossy fiber terminals of the hippocampus is associated with increased glutamate release from synaptosomes of those terminals [489]. Additionally, in the PNS syntaxin-1A expression is concentrated in areas containing substance P, while 1B is more uniformly distributed in the dorsal root ganglia [238]. Pancreatic islet cells which use LDCV for insulin release express only syntaxin-1A [490], as does the pituitary [237]. In adrenal cells, syntaxin-1A is found at the plasma membrane, while 1B is associated with cytosolic granules [239]. This distribution pattern has been interpreted to

mean that syntaxin-1A may be preferentially associated with neuroendocrine and neuropeptide release. Exploratory microscopy results in Chapter 3 indicate partial differential distribution of syntaxin-1 isoforms in the VMC. Because *in vivo* imaging studies found increased striatal dopamine release in schizophrenia patients, it might be hypothesized that the reduced Stx1A:1B ratio in the VMC observed in our sample contributes to increased dopamine transmission in patients. Measuring striatal dopamine neurotransmission in syntaxin-1A KO animals could test this hypothesis. Use of heterozygous STX1A KOs would more appropriately approximate our post mortem findings, and would produce a reduced Stx1A:1B ratio, since syntaxin-1B levels are not altered in STX1A KO mice [242].

The syntaxin-1A:1B ratio appeared to increase in a ventral-dorsal pattern in the striatal regions assayed; this gradient was less pronounced in the schizophrenia group (Figure 3.4). Interestingly, this type of gradient is observed with several other markers or properties in the striatum, including dopamine availability. Evoked dopamine release in primate caudate is highest in dorsal regions and lowest in ventral regions [395]. Involvement of syntaxin-1A, as might be implied by the Stx1A:1B pattern could be tested in a STX1A KO model. It could also be tested by interfering with syntaxin-1A function (by using isoform-specific antibodies or neurotoxins) in the same primate slice preparations used to measure dopamine release (as used by Crag *et al*. [395]). Involvement of syntaxin-1A in dopamine release would be demonstrated if this perturbation then altered dopamine release.

While modest, the association between Stx1A:1B and SNARE complex levels might reflect a difference in affinity between syntaxin and SNAP-25 isoforms, although this has not been reported in the literature. Syntaxin-1A and 1B have different affinities for VAMP isoforms [241]. In rat brain, VAMP-2 protein is widely expressed while VAMP-1 expression is more restricted [491]. Changes in the syntaxin-1A:1B balance could result in a change in the relative

strength of SNARE interactions in the CNS, since syntaxin-1A preferentially binds to VAMP-2, while syntaxin-1B -VAMP interactions differ depending on syntaxin-1B concentration [241].

Synaptic activity itself could cause the differences in Stx1A:1B observed here in schizophrenia. Different sources of brain plasticity (LTP, learning, amphetamine sensitization) modulate syntaxin-1 isoform expression [243, 246, 489, 492]. Changes in Stx1A:1B observed in our sample set may reflect differences in brain activity, whether a cause or result of illness. Induction of syntaxin-1B mRNA expression following LTP and learning has already been demonstrated in some brain regions in the rat [246, 489]. If this extends to the protein level, this would lead to decreased Stx1A:1B, similar to that seen in our samples. Future work to identify protein-level changes in syntaxin-1 isoforms in response to brain activity is needed. Modulation of brain activity in order to return the Stx1A:1B ratio to healthy levels could be an attractive treatment target. Development of pharmacological agents that target terminals expressing syntaxin-1A or 1B presents one means of doing so. Alternatively, modulating brain activity at a systems level (such as behaviours, cognitive tasks or brain stimulation) is a potentially less invasive alternative.

A change in the balance of syntaxin-1 isoforms may have functional consequences outside of the SNARE complex. Syntaxin-1A binds to and modulates the function, expression and localization of a number of transporters involved in neurotransmitter clearance [437, 493, 494]. For example, a direct interaction between syntaxin-1A and the dopamine transporter modulates amphetamine-induced dopamine efflux from cultured midbrain neurons [437] and has been associated with enhanced dopamine release in *C. elegans* [438]. Binding of the C-terminal domain of syntaxin-1A to the GABA transporter reduces the rate of GABA transport in cultured cells [406, 493]. Syntaxin-1A also modulates serotonin and norepinephrine transporters [494-496]. The extent to which such reports apply to these proteins in human brain is unknown, and

may limit their usefulness in interpreting our findings. In particular, many of these interactions have been demonstrated *in vitro* (for example, yeast-two hybrid screens [436]), employing ectopic protein expression in cultured [and not necessarily neuronal] cells (for example, [437]) or in non-mammalian systems (for example, [438, 494]). In addition, syntaxin is known to be a "sticky" protein, increasing the likelihood of non-specific or non-physiological protein-protein interactions to be identified [497]. Thus some caution must be exercised in interpolating these findings to the human brain. Nonetheless, these studies point to the need for similar work to be conducted in intact animals and using post mortem human tissue. Other molecular associations include syntaxin-1A-phospholipid interactions which affect vesicle fusion dynamics. Interference with these interactions reduces the frequency of vesicle fusion, size of the fusion pore, and slows fusion pore opening [498]. Finally, evidence of interactions with calcium channels exists [439]. Syntaxin-1A expression can be induced by activation of P/Q-type calcium channels, in a calcium-dependent manner [199], another mechanism possibly underlying the changes in syntaxin-1 isoform expression we observed in the VMC. Thus, in addition to changes in the SNARE complex, alterations in Stx1A:1B ratio might affect brain function and the timing of synaptic transmission in several other ways. One caveat to this interpretation, however, is that similar experiments have not been conducted with the 1B isoform, and it its therefore unknown if syntaxin-1B mediates similar processes. Even if this is the case, the confocal results in Chapter 3 indicate that the two isoforms are not always present in the same synapses/cells, and therefore individual isoforms likely affect transmission in different terminals in several ways. It is likely that some of the extra-SNARE-complex functions of syntaxin-1 isoforms are abnormal in the striatum of schizophrenia subjects. This possibility is particularly important in the context of neurotransmitter –based hypotheses of schizophrenia and evidence of alterations in GABA and dopamine transporters in schizophrenia [103, 499]. Identification of concomitant changes in

syntaxin-1 isoforms and such transporters would support a mechanistic link between syntaxin-1 isoform changes and abnormal GABA and dopamine transmission in schizophrenia.

5.3 Significance and contributions to the field

The present results contribute to the understanding of synaptic changes in schizophrenia. The findings provide additional and new examples of synaptic abnormalities in the disease, including the abundance of presynaptic proteins, but also suggest possible functional consequences of these changes. The experiments also contribute to our knowledge of SNARE proteins in human striatum and add to the tools available for the study of SNARE protein interactions and SNAP-25 isoforms isolated from brain.

This work extends SNARE protein measurements in schizophrenia to the basal ganglia for the first time, illustrating the extent to which presynaptic terminals are affected in the disease. As in the cortex, the present results indicate that striatal synapses are not affected *en mass*, and the molecular alterations show specificity – VAMP and synaptophysin were not affected in the VMC. The findings also illustrate that disease-associated changes are subregion specific, just as was implied by previous studies in adjacent cortical regions (Table 1.4). The abnormalities were concentrated in the VMC, providing a possible molecular mechanism underlying the abnormalities in associative striatum observed in living patients. Given that the different striatal regions mediate different symptoms seen in patients [61, 88], the current results suggest that different molecular mechanisms underlie dysfunction of different striatal regions. SNARE protein abnormalities do not contribute to dorsal caudate dysfunction, but play more of a role in the ventral regions of the striatum (Figure 2.1). Given the functional connectivity of distinct cortical and striatal regions, patient symptoms mediated by other striatal regions may be caused by synaptic alterations in other, connected, brain regions. Such a possibility is supported by the

multiple reports of SNARE abnormalities in dlPFC, a cortical region with substantial projections to the anterior part of the dorsal caudate [129, 134]. Determining the location of synaptic alterations in different functional circuits involved in patient symptoms will be important for understanding how patient symptoms are generated and for applying more targeted treatment. Future work to establish patterns of synaptic alterations across connected brain regions (such as components of fronto-striatal or fronto-hippocampal circuitry, which function abnormally in patients) would help locate the level at which presynaptic malfunction occurs.

It must also be noted that SNARE protein alterations reported in cortical brain regions (Table 1.4) are not necessarily unique to schizophrenia, with abnormalities reported in bipolar and depressed subjects by some studies [258, 342, 390]. This may indicate shared mechanisms or downstream pathways involved in brain dysfunction. This may also reflect the syndromal nature of these disorders, as symptoms, including cognitive deficits, can overlap between patient groups [5, 500]. However, the precise nature of SNARE abnormalities in these disorders appears to differ between. SNAP-25 protein levels were reported to be higher in bipolar subjects as compared to controls in dorsolateral prefrontal cortex [258] and parietal but not prefrontal cortex [390], with no changes in syntaxin-1 or VAMP, and none detected in schizophrenia patients (Table 1.4). Lower levels of VAMP were reported in bipolar and depressed subjects in visual association cortex [342], suggesting heterogeneity in SNARE protein alterations in affective disorders, just as occurs in schizophrenia (Table 1.4). In the striatum, moderate reductions in SNAP-25 and VAMP proteins (but not syntaxin-1) have been reported in Huntington's patients post mortem [501]. Whether striatal SNARE protein expression levels are altered in bipolar or major depressive disorder is unknown. If the SNARE abnormalities observed in the present studies reflect symptoms/deficits that are also observed in bipolar and depressed patients, similar

presynaptic alterations would be conceivable. Future studies to determine the specificity of the present striatal findings seem warranted.

The current findings shed light on the functional consequences of SNARE protein alterations reported in schizophrenia (Table 1.4), supporting the initial hypothesis that SNARE protein-protein interactions would be altered in regions where SNARE protein levels are abnormal (the VMC in this sample set). Implementation of the capture ELISA provides an alternative to lower-throughput approaches of measuring SNARE protein interactions (such as immunoprecipitation or immunoblotting of SDS-resistant complexes). The directionality of the difference (higher syntaxin-SNAP-25 complexes in schizophrenia, despite lower protein levels) indicates that lower levels of SNAREs in disease do not limit protein interactions, and helps reconcile the reduction in syntaxin-1 and SNAP-25 protein levels in the VMC with reports of increased dopamine release in the same region in patients [139]. Future work to determine if SNARE abnormalities are directly or indirectly involved in dopamine dysregulation in the striatum in schizophrenia would help link the current post mortem findings to striatal dysfunction seen *in vivo*.

Syntaxin-1 and SNAP-25 isoform studies contribute new and needed information about SNARE isoforms in human brain and disease. For the first time, the different properties and putative roles of SNARE isoforms gleaned from *in vitro*, cell culture and animal studies can be used to understand synapses in human striatum. Indeed, SNAP-25 isoforms were quantified at the protein level for the first time, providing a tool that should be of use to a wide variety of researchers, since endogenous SNAP-25 protein isoform expression is largely unknown in any system that expresses both forms. The quantitation of SNAP-25 isoforms in Chapter 4 indicates that protein-level expression of the two isoforms is much different from mRNA expression in adult brain. Previous estimates suggested up that ~ 90% of SNAP-25 mRNA expressed in adult

rodent cortex is of the B isoform [250, 453]; here we demonstrate that SNAP-25B accounts for ~ 50% of SNAP-25 protein in rodent striatum (Figure 4.2F). Our results indicate that SNAP-25A is expressed in adult brain, and therefore is not only associated with brain/synapse development and regeneration. The discrepancy between mRNA and protein expression could indicate a more static role for SNAP-25A at the synapse, with a more dynamic role for SNAP-25B, perhaps similar to the plasticity-associated changes seen with select syntaxin-1 isoforms (Section 1.3.3.1 and 5.2.4). Irrespective of disease, the persistent expression of SNAP-25A in adulthood suggests that SNAP-25A-associated properties of neurotransmission (such as less stable SNARE complexes and smaller RRP size) are still required during adulthood. Perhaps lability of the SNARE complex is needed for plasticity. Future work to better understand the *in vivo* implications of synaptic use of one or the other SNAP-25 isoform will be extremely valuable, and might be undertaken in animals or tissue preferentially expressing one isoform, or under wild-type conditions to determine which isoforms are involved in different synaptic activities (for example, LTP). Use of the assay developed here will be integral to such research, as protein-level expression of isoforms can now be assessed. In addition, our unique approach of using an external calibration curve constructed from recombinant protein in the MRM assay will make this proteomic technique accessible to more researchers.

The use of a relatively new, well-characterized sample set that was collected, catalogued and dissected in a uniform manner adds confidence to the experimental conclusions. In addition, results documented in Chapters 2 and 4 likely reflect the disease state, given that differences associated with antipsychotic treatment in animals, if any, occurred in the opposite direction to that observed in human samples. This suggests that the mechanisms of APD action may work through or at least mitigate SNARE protein abnormalities in patients. This also means that lower protein levels observed in schizophrenia patients may be underestimates of SNARE

perturbations in disease if antipsychotic treatment has partially remedied disease-associated changes.

5.3.1 Implications for disease etiology

The results of these experiments support the idea that synapses are dysfunctional in schizophrenia and at least in the VMC, involve a presynaptic mechanism. Abnormal SNARE protein levels (and the isoform-specific contribution to the decreases) as well as protein-protein interaction increases in the VMC integrate a number of etiological theories, given that SNARE protein and isoform expression is developmentally regulated, preferentially associated with different neurotransmitters and important for synaptic and brain function.

It has been suggested by some that abnormal brain morphology (in particular lower grey matter volumes and smaller hippocampus) as well as lower neuropil volume and reduced dendritic spines in schizophrenia, may reflect an exaggeration of normal developmental processes in the cortex [16, 19]. Alternatively, the possibility of late maturation, at least in some brain areas, has also been suggested [502, 503]. The current findings of reduced syntaxin-1 and SNAP-25 as well as lower SNAP-25A expression are consistent with neurodevelopmental hypotheses of schizophrenia, and support the possibility of altered developmental trajectories. The expression of SNARE proteins, including SNAP-25 isoforms (although this is known only at the mRNA level), is developmentally regulated, so lower levels of proteins observed in the VMC may reflect developmental perturbations. Syntaxin-1 levels, measured in rat synaptosomes, reach peak expression during the first week post-natal, decrease substantially by the second week and are relatively stable after that [504]. In rats, total SNAP-25 protein expression increases during development and decreases somewhat with advancing age [504, 505]. SNAP-25A mRNA is expressed during embryonic and postnatal development in rat brain,

and may decrease after P21 [250, 251]. The B isoform, however, is only detected postnatally starting around the second week, and peaking during early adulthood [250, 506]. The implications of this trajectory for protein expression remain unknown, and must be interpreted with caution, as age-related changes in mRNA may not parallel changes in protein [507]. Future work should measure SNAP-25 protein isoforms during development. This will complement developmental mRNA studies in the literature, and will determine if the SNAP-25B protein is a hallmark of adult/mature synapses as has been assumed from mRNA studies, or rather expressed dynamically, as proposed here. While the developmental profile of syntaxin-1 isoforms is less known, the correlation of isoform ratio with age observed in the striatum in Chapter 3 supports the idea that syntaxin-1 isoform expression in schizophrenia reflects "accelerated aging", as the Stx1A:1B ratio at a given age is lower in the patient samples as compared with controls (Figure 3.4). Whether this is a proxy for age-related changes known to occur in the dopaminergic system remains to be determined [508, 509]. If this is the case, SNARE perturbations would be a link between developmental and neurotransmitter theories of schizophrenia. Age-related modulation of SNARE protein availability also occurs. In rodents, VAMP-synaptophysin interactions are only detected at approximately P25 (early adolescence), and are thought to regulate the availability of VAMP for interactions with the other two SNAREs [307]. The developmental trajectory of VAMP is similar to synaptophysin, with increasing expression up until adolescence, and decreasing expression with age in adulthood [504, 510], although the pattern may differ in different brain regions or subregions [507]. Thus, at least in rodents, SNARE expression levels change during adolescence, when synaptic elimination and other final brain maturation steps are occurring. Because the expression profiles of SNAP-25 and syntaxin show both an increase and subsequent decrease in expression, the lower levels of SNAP-25 and syntaxin observed the VMC in schizophrenia could reflect over or under-maturation of the normal trajectory.

Since syntaxin-1A, -1B and SNAP-25 may be differentially expressed in excitatory and inhibitory terminals, the current findings support neurotransmitter-based etiological theories as well. Assuming that SNAP-25 and syntaxin-1A preferentially mark glutamatergic terminals [192, 194, 201, 204, 511], our results may indicate presynaptic glutamatergic dysfunction in the VMC. Whether differential expression accounts for dopaminergic dysfunction in this brain region is more difficult to determine as SNARE protein expression in dopamine terminals has not been investigated. However, interaction of glutamate, GABA and dopamine is known to occur in the striatum [449], so evidence of glutamatergic dysfunction also supports the dopamine hypothesis [43]. The SNARE protein and isoform abnormalities uncovered in our work suggest convergence of developmental and neurotransmitter-based hypotheses of schizophrenia. It seems reasonable to expect that different SNAREs, isoforms and alterations in their expression and function in different brain regions will be associated with specific patient symptoms. Future work to delineate which terminals express which SNARE isoforms and which of these terminals experience increased SNARE complexes in patients in the VMC will help determine how our current findings relate to patient symptoms and whether SNARE alterations contribute directly or indirectly to abnormal dopamine/glutamate/GABA transmission.

Differential changes in SNAP-25 isoforms detected in the VMC of patients raise the possibility that abnormal splicing events are involved in the disease as well. Isoform variants of several other schizophrenia candidate molecules such as neuregulin and the D2R exist, and may be of functional relevance to the disorder [512, 513]. Abnormalities in splicing in schizophrenia could present a plausible disease mechanism linking several etiological theories together, given that splicing events can be regulated developmentally, could be influenced by genetic variation in non-coding regions, and may occur in molecules already implicated in disease.

Finally, SNARE protein abnormalities may not represent a completely independent disease mechanism, and our findings may be influenced by other molecular alterations that occur in patient brains. Several schizophrenia candidate proteins are known to interact with or modulate SNARE proteins and vice versa. Dysbindin, a strong schizophrenia candidate gene, has been shown to modulate SNAP-25 protein expression and trafficking in cell cultures. Increases and decreases of dysbindin expression result in parallel changes in SNAP-25 expression [49], and the two proteins have been shown to interact *in vitro* [270]. Relevant to the NMDAR-hypofunction hypothesis of schizophrenia, SNAP-25 is involved in PKC-dependent NMDAR membrane insertion [514]. Relevant to the dopamine hypothesis, syntaxin-1A has been linked to dopamine transporter function [437, 438]. There is also evidence of lower GSK3β expression in schizophrenia, as well as changes in phosphorylation state related to dopamine signaling [515]. Interestingly, this serine/threonine kinase may also affect SNARE complexes; activation of GSK3β decreases SNARE protein interactions, possibly by preventing VAMP from dissociating from synaptophysin [516]. Finally, evidence of disturbed phospholipid composition in the brain and periphery of patients has given rise to the membrane hypothesis of schizophrenia [517]. Membrane composition may affect SNARE protein localization, levels or function, given that these proteins are localized to distinct parts of the membrane, defined by the local lipid environment [172, 518]. SNARE abnormalities identified in this thesis may not represent a completely independent disease mechanism, but may be linked to other molecules implicated in schizophrenia.

Schizophrenia risk factors such as prenatal stress may affect SNARE function as well (a "protein x environment" interaction). Prenatal stress enhanced sensorimotor gating impairments and induced social deficits in *blind-drunk* mice [253]. Rats raised in isolation had lower levels of septin-5 in the striatum as compared with rats raised in groups [519]. Therefore

environmental risk factors for schizophrenia may increase SNARE protein interactions. Thus, it might be anticipated that reducing environmental risk factors would mitigate abnormal SNARE protein function and preventing abnormal SNARE protein function might reduce the effects of risk factors.

Finally, in addition to psychiatric and cognitive symptoms, schizophrenia is associated with an increased risk of comorbid conditions including metabolic syndrome and diabetes, which may be associated with antipsychotic treatment [520, 521]. SNARE proteins provide a point of convergence between this comorbidity and disease. SNARE proteins are present in pancreatic β-cells and are involved in insulin secretion [226, 490]. Interestingly, both SNAP-25A and B, but only syntaxin-1A is expressed in pancreatic cells [435, 522]. Whether the isoform specific changes observed in striatum extend to or affect insulin secretion would be valuable in understanding the link between schizophrenia and increased diabetes risk. It may also be fruitful to study the effects of patient treatment on SNAREs in the periphery, as insulin secretion may be altered by patient treatment [523-525], and SNARE genes may be associated with weight-gain side effects [281] and with risk of diabetes [184].

Thus, the SNARE protein abnormalities we have detected in the VMC in this sample set may contribute to the disease, but may also reflect presynaptic perturbations secondary to other disease-associated molecules and processes. The examples above suggest that SNARE protein abnormalities fit well into existing disease etiology hypotheses. Future studies should focus on defining the terminals and brain regions in which SNARE isoforms and SNARE protein interactions are altered, as this will help determine whether the different molecular alterations are associated with distinct mechanism theories, or lend support to all of them.

5.3.2 Implications for disease treatment

Current pharmacological treatments for schizophrenia target and block neurotransmitter receptors, molecules downstream of transmitter release [526]. Some of the treatment side effects are due to blockade of receptors that do not need to be blocked, indicating a lack of specificity of these compounds. Modulating presynaptic release may be a more appropriate treatment target [43, 527], and might be achieved by targeting the SNARE proteins.

SNAREs are currently being manipulated in the periphery using botulinum toxins (Botox) which cleave SNAREs, preventing SNARE complex formation and signaling at neuromuscular junctions [528]. Toxin use in the CNS is likely to be risky, however, the specificity of toxin types for specific SNAREs [206], and the possibility that SNAREs are differentially distributed in different synapse types, may provide a means by which to manipulate presynaptic release in only subsets of terminals. Of particular interest is the possibility that different toxins/compounds might affect SNARE isoforms differently. Indeed, botulinum toxin type-A appears to cleave SNAP-25B more readily than SNAP-25A [529], and may be useful in identifying if isoform-specific SNARE modulation is a viable treatment target. Based on our results, neurotransmission in the VMC in schizophrenia may rely more heavily on SNAP-25B than A (given that SNAP-25A was significantly lower in patient samples; Chapter 4). Since SNAP-25B is associated with increased neurotransmission [196, 249] specifically reducing SNAP-25B-mediated neurotransmission might be an appropriate treatment strategy in the VMC. Alternatively, increasing SNAP-25A expression would also be an option, and may pose less risk of seizures (which are seen in SNAP-25B deficient mice, Table 1.1). Evidence of naturally occurring compounds that modulate SNARE protein interactions suggest that other, potentially milder, agents might also be used [313, 314]. Screening for compounds that selectively modulate specific SNARE protein isoform function might increase treatment specificity.

SNARE involvement in current patient treatment is supported by genetic association of SNAP-25 SNPs with treatment response and weight gain in some patients [281]. In addition, SNARE protein expression may be affected by current treatments, with changes that act against some of the disease-associated SNARE expression alterations (Chapter 2) and [309, 310]. A preliminary study of an mGluR2/3 agonist suggested that presynaptic molecules maybe a viable treatment target [85], although this has yet to be replicated in larger clinical studies. Modulation of presynaptic receptors may well affect SNARE function because signaling pathways downstream of receptor activation can influence kinases (for example, PKA, PKC and CamK), signaling proteins (G-proteins affiliated with metabotropic receptors) and the production of lipids (for example, arachidonic acid), all known to affect SNARE protein interactions [397, 518, 530]. Whether mGluR (or other presynaptic receptor) activation affects SNARE protein function is unknown, although mGluR1 activity has been associated with SNAP-25 dependent NMDAR trafficking [531]. Studies in PC12 cells indicate that Gβγ proteins (involved in G-protein coupled receptor signaling) inhibit vesicle exocytosis by binding to the SNARE complex [530], indicating that the SNAREs may be downstream targets of metabotropic receptor signaling. It also seems plausible that presynaptc ligand-gated channels (such as some of the nicotinic receptors [532]) could also affect SNARE protein expression or function, as calcium-dependent changes in syntaxin-1A expression have been linked to calcium channel activity [199]. This is likely an important avenue for future research, given that some of the receptors targeted by current and novel patient treatments are presynaptic receptors [48, 79, 83, 86] and therefore could modify SNAREs.

Findings in Chapter 4 suggest that SNAP-25 isoforms may not be affected differentially by haloperidol or clozapine. The possibility that gene splicing events could be a treatment target may be a future reality, and is currently being explored in the treatment of muscular dystrophy

[533]. Separate genes encode syntaxin-1 isoforms, and therefore their expression is likely independently controlled. Understanding the regulation of gene expression and ways in which to stabilize individual protein levels might be an alternate way to treat SNARE protein abnormalities.

Finally, results in Chapter 2 suggest that when there are effects of APDs on striatal SNARE proteins, the effect is the same in all regions. However, our studies in human samples suggest that changes in striatal SNAREs are region specific. Selectively targeting the VMC for treatment may ameliorate specific symptoms that involve the VMC, and may carry less risk of side effects.

The studies of SNARE isoforms and SNARE protein interactions reported in this thesis provide more detailed information regarding SNARE abnormalities in disease, and identify new ways of targeting SNARE functions for patient treatment.

5.4 Limitations of the current studies

The samples and methods used throughout this thesis provide new insights and approaches to studying the synapse in schizophrenia. However, these come with a number of caveats, some of which were discussed in Chapter 1, and some elaborated further here. Effects of potential limitations were minimized in the methods used, as pointed out below. Limitations on the interpretation of results are discussed. Finally, opportunities for future improvements are identified.

Measurement in post mortem tissue provides only a "snap-shot" of SNARE proteins and synapses. Protein interactions, modifications and expression are likely dynamic (as evidenced by the activity and plasticity-related changes observed in animals) and are not accounted for by static measurements. Some of these protein properties may exhibit differential stability post

mortem or between individuals (for example, phosphorylation states) and may affect protein measurements. Also, it is not possible to distinguish between disease causation and consequence; hence many of the disease-associated changes observed in the VMC have been interpreted from both perspectives.

Effects of gender were not investigated in the present study owing to the small number of females in the control group ($n = 3$) and the possibility that the effect of gender is confounded by other sample characteristics (for example, age of females is higher than males in the SCZ group ($t = -2.80$, $df = 10.25$, $P = 0.018$), but not in the NPC group ($t = -0.750$, $df = 3.32$, $P = 0.502$). Restricting current studies to male subjects only would have reduced the power of statistical comparisons [534]; the study was not designed to investigate gender effects. However, men have a significantly increased risk of developing schizophrenia (odds ratio = 1.4) [9, 125, 535], and may experience an earlier age of onset [536]. Whether SNARE protein expression and SNARE interactions are sexually dimorphic is less clear, but there is evidence that sex hormones can influence SNAP-25 mRNA expression in adult rat cortex and pituitary, supporting such a possibility [537, 538]. Given our current findings of lower SNAP-25A levels in schizophrenia, and the possible link to developmental, synaptic and neurotransmitter-based etiological theories, future work both in humans and in animals may provide needed information regarding sex-related differences in SNARE protein and isoform expression in the striatum.

Heterogeneity within subgroups of patients sharing the syndrome of schizophrenia (for example, illness course, symptom constellation and severity) could limit the ability to statistically detect illness-related synaptic changes. The sample size and study design did not allow patients to be subgrouped or clinical measures to be investigated. Heterogeneity in patient symptoms may mean that different brain circuitry is affected differently in individual patients, which could lead to varying degrees of synaptic pathology in different people, perhaps occurring

to a greater extent in brain regions directly involved in specific symptoms. Thus, patients with whose prominent symptoms or deficits differ may show synaptic pathology in different brain regions and to a different extent. Future studies designed to look within patient groups would help determine if SNARE protein and isoform expression as well as protein-protein interactions reflect specific aspects of illness.

Reliance on brain homogenates is also a limitation of these studies given the heterogeneity of cell types in the striatum, and because protein localization is disrupted and protein origin is difficult to discern. Whether disease-associated differences occur in the same synapses, all synapses, or are restricted to different terminals, remains unknown. Until SNARE composition in a variety of terminals and brain regions is better characterized, it is uncertain whether the measurements made here are proxies for specific types of striatal synapses. Furthermore, subcellular localization may also be important to SNARE function, as SNAREs are localized to membrane domains that contain calcium-channels and regulatory proteins, may be endocytosed, and are trafficked down axons as well [172, 505, 539, 540]. Also, as in all experiments involving extraction and separation steps, it is assumed that proteins from both control and schizophrenia subjects behave the same way. However, it is possible that the conformation, modifications or local environment of the SNARE proteins could differ in disease, affecting the recovery, solubility and therefore quantitation of these proteins. While this does not invalidate the disease-associated differences observed here, it may mean that protein levels are not different, but rather, the state of the protein, affecting its detection, differs in disease.

The use of highly specific antibodies increases sensitivity and specificity of protein detection and quantitation. However, lower levels of antibody binding occur not only if protein expression is reduced, but also if the epitope detected by the antibody is altered/inaccessible, as might be the case with post-translational modifications or proteins in complexes. Such issues

have come to the forefront of SNARE research recently, and the controversy surrounding the lack of SNAP-25 in GABAergic terminals could well be related to the antibodies used and epitopes detected [202, 203]. The precise nature of the epitopes of the monoclonal antibodies used throughout this thesis are unknown, although all antibodies had been previously characterized in intact tissue and using fusion proteins to confirm their specificity and partially map the immunogenic region of the protein [264, 389, 442]. It is likely that SP12 recognizes an N-terminal epitope, as it is unable to detect N-terminally tagged GST-SNAP-25 by immunoblot (data not shown). All antibodies can detect both monomeric and complexed SNAREs (Figure 2.5). However, all SNAREs can be phosphorylated and some can be palmitoylated [397, 541] potentially affecting SNARE detection with the methods used here.

Several limitations are associated with quantitative immunoblotting techniques, some of which were addressed and minimized in Chapter 3. Others seem inherent to the method and likely affected the results obtained. To attempt to control for variation within and between blots, it is common practice to normalize the band intensity of the protein of interest to the band intensity of a "housekeeping" protein. This approach, however, is only valid if both antigens, the protein of interest and the loading control, behave the same way – mainly, the slope of the curve of a serially diluted sample is the same for both bands. This means that the ratio of the bands does not change when total protein load changes and the ratio of the band intensities is equal to the ratio of the protein amounts. This however, is hardly ever investigated, and as illustrated in Chapter 3, may not hold true even when the *same* antibody is used to detect both bands. The ability to convert band intensity values to absolute protein amounts prior to creating a ratio helps to overcome this issue, but requires the use of a purified protein standard [542]. Using a purified protein, likely generated in a non-mammalian system, and outside of the biological matrix being assayed, carries with it the limitations discussed above regarding epitopes and protein extraction.

To avoid these limitations, a native sample can be used to create a reference curve, and band intensities of the samples of interest can be expressed relative to the reference sample. This approach was employed here, and by others (for example, [391]), but does not permit the absolute amount of protein to be determined. Results are quantitative, but expressed relative to the reference sample. An additional benefit of this approach is the ability to compare results between samples and brain regions that have been quantified using the same reference curve. The second condition for valid use of a loading control is the selection of an appropriate control protein. Traditional loading controls include proteins such as tubulin and GAPDH. However, at least two difficulties arise when using such controls. First the abundance of "housekeeping" proteins is often much different than that of the protein of interest. The amount of protein loaded onto a gel to ensure the target protein is within the linear range of detection is likely to be very different than the amount needed for the loading control, and therefore one of the proteins may be assayed outside its linear range. Second, when studying an illness, it is difficult to know whether the loading control is affected in disease or not. Much evidence has accumulated for cytoskeletal and metabolic disturbances in schizophrenia, questioning the use of traditional "housekeeping" proteins as loading controls [543, 544]. The use of ratios may also impede analysis and interpretation of results, and can reduce the sensitivity of some statistical analyses [545, 546]. In addition to statistical sensitivity, immunoblotting appears to be a less sensitive technique as compared to ELISA for measurement of syntaxin (for example, syntaxin-1 as measured by ELISA was detectable at total protein amounts at least as low as 0.05 μg, while immunoblot detection was difficult past 0.4 μg). In addition, there are multiple stages during which protein may be lost/not fully recovered (i.e. during electrophoresis, transfer and blotting stages), which likely contributes to increased variation and decreased sensitivity.

In Chapters 2 and 3, synaptophysin levels were used as a means of normalization for synapse numbers in some of the complementary data analyses. Synaptophysin measures have traditionally been used as an index of synaptic terminal numbers [547]. Recent colocalization studies suggest that it may not be a marker of all synapses [194], and confocal results in Chapter 3 also support this possibility. Thus, normalizing capture ELISA results or syntaxin-1 isoform measures to synaptophysin may account for only some striatal terminals. It is also possible that synaptophysin-positive terminals may not necessarily contain the molecules that are being normalized, making it difficult to interpret the meaning of such ratios.

Several limitations of the MRM isoform assay also exist. First, despite best efforts, the assay is still a relatively low-throughput one, requiring multiple time consuming steps. This primarily stems from the use of immunoprecipitation and gel separation to reduce sample complexity. Initial assay development stages included attempts to prepare and digest samples in-solution, which would eliminate the need for a gel-separation step. However, the SNARE complex is resistant to trypsin digestion [548] necessitating the use of SDS and heat to dissociate it prior to proteolytic digestion of its components [206]. Trypsin digestion requires the removal of strong denaturants such as SDS [549]; in-solution digestion would then require detergent removal either by precipitation or size exclusion. Both methods are associated with sample loss, and initial tests suggested recoveries of approximately 40% following SDS removal using a spin column/centrifugal filtration (see Appendix 4). In addition, MRM signal intensities of isoform-specific peptides obtained for samples prepared in-solution after detergent removal were not as high as those obtained by in-gel digestion (see Appendix 5). For these reasons in-solution digestion was abandoned and samples were prepared in-gel prior to MRM analysis. Limitations of in-gel peptide preparation include poor or biased peptide recovery from gels and peptide loss due to absorption to plastics when drying down extracted peptides [368, 550]. These limitations

were accounted for through the use of the recombinant protein curve of isoform mixtures, as biased peptide recovery should be reflected in the curve. Speicher *et al*. suggest that sample concentration using a speedvac should be avoided as peptides can absorb to plastic [368], however, to facilitate sample storage after processing, samples needed to be dried down. To minimize absorption of sample peptides to plastic, sample wells were pretreated with BSA. Thus, several steps were taken to mitigate the limitations associated with the quantification of proteins prepared in-gel. Additional methodological limitations include the length of time needed for sample analysis (total run time per sample is 1.5 hours), and could be improved by modifying the HPLC elution gradient to encompass only the portion during which the target peptides are eluting. Addition of the isotopically labeled peptide after sample processing means that variability due to sample handling and processing (i.e. immunoprecipitation, digestion efficiency, extent of reduction/ alkylation, peptide recovery from gel) is not accounted for during quantitation. This is assumed to occur randomly, as the experimenter was blind to sample characteristics. In future this could be accounted for by introducing a source of labeled peptide (either synthetic peptide, or through the use of isotopically labeled recombinant SNAP-25) at an earlier stage of sample preparation.

The current studies were undertaken in a single sample set composed of 28 subjects. While a small sample size, it reflects the availability of tissue for post mortem research and was in keeping with studies in the field which used tissue from homogenous groups of subjects to study SNARE protein expression levels at the time this thesis work was undertaken (see Table 1.4). It is unclear whether these findings will extrapolate to other sample sets collected in different parts of the world, with different age and PMI ranges. Replication in additional sample sets will help determine the degree to which SNARE protein alterations reflect disease, as opposed to the temporary state of the striatum. Finally, the current experiments indicate an

association between SNARE protein abnormalities and disease, and do not establish whether these differences are the cause or result of disease.

5.5 Future directions

The current findings indicate that synaptic pathology in the VMC in schizophrenia includes SNARE protein alterations likely affecting SNARE function in neurotransmission. Future work to test the hypothesis that SNARE complex function is abnormal in patients in the VMC should include experiments that uncover the mechanism behind increased SNARE protein interactions observed here. Areas of potentially interesting and valuable research have been identified in previous sections, however gaps in our understanding of SNARE protein regulation and expression patterns limit the interpretation of our current findings. Means of ameliorating these knowledge gaps are detailed here.

Only one of the possible mechanisms underlying the increased SNARE protein interactions was investigated in this thesis (syntaxin-1 and SNAP-25 isoform involvement). However, SNARE protein interactions can be modulated by accessory factors or SNARE protein level changes and increased SNARE complexes could occur in different terminals. Furthermore, the findings of Chapter 4 raise the possibility that SNARE isoform expression in schizophrenia is controlled at the level of splicing. Future studies to determine which of these scenarios occurs will provide evidence of the mechanism behind striatal SNARE dysfunction in patients. Three major aims of future investigations are put forth here.

5.5.1 Characteristics of SNARE complexes in schizophrenia

The current studies detected an association between SNARE protein interactions and protein isoforms in Chapters 3 and 4, but did not directly address the relationship between altered

SNARE isoform expression and SNARE protein interactions. Future research is needed to determine if differential isoform incorporation and/or association of modulator proteins in SNARE complexes is directly responsible for the increased protein interactions we observed. One might hypothesize that in schizophrenia, SNARE isoforms differentially incorporate into the SNARE complex, resulting in different SNARE complex stability and accounting for differences in SNARE protein interactions detected in Chapter 2. Direct evidence of isoform effects on SNARE complexes in human samples is needed. This can be investigated with the capture ELISA assay, using antibodies specific for syntaxin-1A or 1B to determine if the increased syntaxin-SNAP-25 interactions in SCZ are associated with one isoform. To test the inverse, in the absence of SNAP-25 isoform-specific antibodies, the MRM isoform assay would be applied to measure SNAP-25A and B co-immunoprecipitated with either syntaxin-1A or -1B. While syntaxin-1 and SNAP-25 were the focus of isoform-specific studies in this thesis, differential involvement of VAMP isoforms in schizophrenia may also contribute to synaptic abnormalities. Functional differences between VAMP-1 and VAMP-2 are suggested in the literature. *In situ* hybridization studies in rat brain demonstrated highest VAMP-1 mRNA expression in regions of the nervous system associated with somatomotor functions. VAMP-2 was more widely expressed in the CNS [173]. Differential expression of VAMP-1 and VAMP-2 also occurs at the protein level, with inverse expression patterns in some brain regions (such as the hippocampal dentate gyrus) and congruent expression in others (such as the cerebellum) [491]. In addition, the lethality of VAMP-2 KO in mice suggests that VAMP-1 cannot compensate for VAMP-2 function [188]. Preferential affinity between VAMP and syntaxin-1 isoforms has been demonstrated *in vitro* [241]. Thus, similar to SNAP-25 and syntaxin-1 isoforms, VAMP-1 and 2 appear to diverge in function, and could therefore affect SNARE complex stability and function. To determine if differential incorporation of VAMP isoforms into SNARE complexes

contributes to increased SNARE protein interactions in schizophrenia, similar analyses should be applied to measure VAMP-1 and VAMP-2.

To understand how our findings impact brain function it is necessary to determine if the increased SNARE protein interactions reflect increased or decreased neurotransmission. Studies of neurotransmitter release from synaptosomes prepared from post mortem brain, combined with SNARE protein interaction measurements could be undertaken, and would link the increase in SNARE complexes directly to altered neurotransmitter release. The tissue series used here would be well suited, as short PMI is required for successful synaptosome preparation and retention of activity [551]. Additional evidence of the direction in which neurotransmission is affected by increased SNARE complex stability could also be obtained by electrophysiological and behavioural measurements in animals where SNARE complex stability is altered, such as the *Bdr*/+ and SNAP-25B KO mice. The capture assay developed in Chapter 2 could be used to confirm that the predicted increase in SNARE protein interactions does indeed occur in these animals. Microdialysis experiments would determine if specific types of transmission (glutamate, dopamine) were also affected in these mice and concurrent changes in SNARE protein interactions would implicate the SNAREs in the underlying mechanism.

Dead-end or intermediate complexes are thought to consist of non-ternary SNARE complexes (for example, 1:2 SNAP-25-syntaxin complexes), while SNARE complexes used in fusion events should occur at a 1:1:1 stoichiometry [222]. If the increased SNARE protein interactions observed in schizophrenia represent functional SNARE complexes, they should be composed of syntaxin-SNAP-25-VAMP in a 1:1:1 ratio. Stoichiometry of SNARE complexes from human brain samples could be measured by immunoprecipitating SNARE complexes and quantifying each protein/isoform by MRM. The molar equivalent of each SNARE could then be

determined using internal standards and external standard curves of synthetic peptides representing each protein, the same approach used to measure total SNAP-25 in Chapter 4.

Differential stability of SNARE complexes in the presence of SNAP-25A or B may be due to different interactions of the SNAREs with accessory proteins [249]. SNARE modulating proteins are altered in some brain regions in schizophrenia. Thus, increased complexes in the VMC in SCZ may be a point of convergence between differential SNARE isoform expression and altered SNARE accessory protein expression seen in post mortem brain. Identifying which SNARE accessory factors are associated with SNARE complexes in patients will help interpret findings of altered modulator factors (such as the complexins, septin-5 and munc-18) reported in some studies [293, 297, 305]. Multiple proteins are co-immunoprecipitated with SNAP-25 (Appendix 5). These interacting proteins could be identified by standard MS/MS combined with database searching. Data analysis and filtering approaches would then be used to determine if the proteins identified in a sample group are different from those identified from control samples [455]. It is hypothesized that lower levels of proteins that prevent formation or facilitate dissociation of the SNARE complex will be associated with SNARE proteins in SCZ samples. Thus, lower levels of proteins such as NSF and septin-5 in SCZ are predicted to occur in the striatum; changes in mRNA or protein-level expression have already been reported in cortical regions in schizophrenia in some studies [99, 305]. Proteins identified by MS/MS would then be validated using a quantitative method, such as MRM analysis and/or IP-immunoblot. It is anticipated that different accessory proteins will preferentially associate with SNAP-25A and B containing-complexes, accounting for different SNARE complex stability and protein interaction levels. Correlation analysis of SNAP-25 protein isoform levels and the amount of each interacting protein would support this hypothesis. To determine how the combination of SNARE isoforms and modulatory proteins seen in patient samples affect synaptic transmission (increased

or decreased) different combinations of SNARE isoforms and modulatory proteins could b expressed in cultured cells and vesicle fusion assessed through electrophysiological recordings.

Based on the findings of Chapter 2, it is hypothesized that lower SNARE proteins do not limit complex formation. To test this hypothesis, SNARE protein interactions (using the capture ELISA) could be assessed in heterozygous SNARE KO animals that express lower amounts of individual SNAREs. Because compensatory changes in SNARE expression or function may occur in KO animals during development, the effects of transient knock-down of individual SNAREs (in culture or by developing conditional KO animals) would help differentiate between the effects of low SNARE levels early in development, versus later on in life. This distinction is important in the context of early and late neurodevelopmental hypotheses of schizophrenia; differences in SNARE complex levels between traditional and conditional KO animals could indicate the developmental time-point at which SNARE protein expression perturbation in schizophrenia occurs to yield increased SNARE complexes observed in adult post mortem VMC.

Finally, the significance of increased SNARE protein interactions in the VMC should also be assessed at the systems level. If the findings of this thesis affect brain function, this should be reflected in patient symptoms and behavioural abnormalities in animals. Replication studies in post mortem tissue could use sample sets that document patient symptoms and cognitive measures that assess VMC function. If SNARE protein abnormalities in the VMC impact patient function, these measures should be related to ante mortem functioning in tasks subserved by this region of the striatum. Associations between SNARE complex abnormalities and behaviour could be corroborated in the animal models. One would expect patients and animal models with increased SNARE complexes (such as the *Bdr*/+ mouse) to perform poorly on reversal learning or delayed reward/gambling tasks, which rely on frontostriatal circuits that involve the VMC [89, 131, 552].

5.5.2 Determination of SNARE isoform distribution in synaptic terminals

An important hypothesis for future research and in interpreting the current findings is the idea that different combinations of SNARE isoforms mediate neurotransmission of different neurotransmitters/ at different terminal types. Differential distribution of isoforms at the protein and mRNA level reported in the literature alludes to this possibility. Immunohistochemical studies of tissue co-stained for markers of terminal types (such as vesicular glutamate and dopamine transporters) are likely to be difficult due to both synaptic and non-synaptic localization of syntaxin and SNAP-25, and would not be possible for SNAP-25, since isoform-specific antibodies are not readily available. A recent proteomic approach that isolated synaptic vesicles using specific markers such as VGlutT and VGAT and then quantified different presynaptic proteins, would be more appropriate [201]. This approach could be applied to purify synaptosomes based on neurotransmitter transporters such as DAT and 5HTT, or vesicles marked with VMAT. Differential centrifugation techniques could be used to separate small and dense-core vesicles, allowing neuropeptide-containing vesicles to be assessed as well. The use of our MRM-isoform assay would allow SNAP-25 isoforms to be monitored in these different terminal populations. Other SNARE proteins could be measured concurrently as well. Identification of the specific SNARE isoforms that function at different terminal types will help localize the findings of the current studies to specific neurotransmitters. Different cell groups of the same neurotransmitter type often project to different brain regions. For example, dopaminergic inputs from the substantia nigra are topographically organized, as are cortico-striatal projections. In addition, there is evidence for heterogeneity of presynaptic machinery within the same terminal types originating from different cell bodies. For example, Rab3B mRNA expression differs between dopaminergic cell groups of the VTA and SN in rats [553].

For this reason, the above-mentioned studies should be conducted in sub-dissected tissue. Separate analysis of DCd, VMC and NAc tissue in humans would distinguish between dopamine terminals originating from ventral and dorsal tiers of the SNc and the VTA [134]. It would also enrich for glutamatergic terminals originating from different cortical areas (DLPFC, orbitofrontal and anterior cingulate cortex, respectively) [129, 134]. Thus, it would be possible to determine if neurotransmitter-specific combinations of SNARE isoforms are constant throughout the brain, or if they vary between cell groups/brain regions. Given the heterogeneity of glutamatergic alterations in various cortical regions in schizophrenia [55], and differential expression of some presynaptic markers in dopaminergic terminals of different sources [553], it might be predicted that neuronal SNARE combinations will vary between dorsal and ventral striatal regions.

By determining which terminals/neurotransmitters are associated with which SNARE proteins and isoforms, the functional consequences of our present results might indicate specific neurotransmitter and brain circuitry aberrations in patients.

5.5.3 Investigation of splicing in schizophrenia

The results in Chapter 4 (SNAP-25 isoform studies) raise the question of whether gene splicing is abnormal in schizophrenia. Investigation of splicing regulation in schizophrenia could provide evidence that unifies (or alternatively, classifies) the diversity of genetic associations and protein-level changes observed in the disease. Isoform-specific expression differences of schizophrenia candidate genes have been detected in post mortem brain [480, 481, 512, 554], splice variants of candidate genes are known or predicted to exist (for example, [555]) and many schizophrenia-associated SNPs occur in non-coding regions of genes, supporting the possibility that abnormal splicing events are involved in the disease. Abormal splicing may underlie our findings in Chapter 4. Besides SNAP-25, alternative splicing of both VAMP-1 and VAMP-2

genes may occur [556-558], although it is unclear if the splice variants are expressed at the protein level. Predicted protein sequences indicate that alternative splicing produces variation in the C-terminal transmembrane region (Figure 1.3C), important for VAMP sorting and cellular localization [557, 558]. Investigation of VAMP gene splice variants in schizophrenia seems warranted. Also, an alternative splice variant of syntaxin-1A may occur in glial cells [559]. Combined with the results of Chapter 4, future research into splicing regulation in schizophrenia may be fruitful and it is plausible that aberrant splicing events in schizophrenia will share common splicing factors or regulatory domains, such that similar disturbances underlie aberrant splicing of different genes in schizophrenia. To address this hypothesis, it is necessary to determine if SNARE and other schizophrenia candidate genes contain common regulatory sequences. This could be performed *in silico* by identifying regulatory regions and splice junctions of genes to determine if specific sequences are over-represented or preferentially disrupted (for example, disease-associated mutations in intronic regions) in genes/proteins implicated in schizophrenia, or could be identified experimentally using array or sequencing-based methods [560-562]. To determine if SNAP-25 splicing is regulated by the same splicing factors used for other candidate genes, potential splicing factors could be screened to determine which factors control SNAP-25A and B expression *in vitro* or in cell culture [563, 564]. A similar assay could then be used to determine if the identified splicing factors also control the splicing of other disease-associated genes. Evidence that splicing regulation is common among SNARE and other candidate genes and is perturbed in disease could be gathered through experimental means. Co-expression analysis of splice variants [565] in post mortem tissue, schizophrenia animal models, or other systems that mimic aspects of disease (for example, dopamine application to striatal slice preparations to model a hyperdopaminergic state [407]), or which are known to perturb isoform expression (for example, hippocampal LTP [489], or

disruption of the expression/function of splicing factors) would provide additional evidence of splicing events contributing to presynaptic and other candidate molecule abnormalities in schizophrenia. Further investigtions to determine if the identified splicing regulatory regions/factors of candidate genes are affected by SNPs that have been associated with schizophrenia (as has been shown for other schizophrenia candidate genes [462, 500]) would provide a link between the findings in Chapter 4 and genetic association of SNAREs with disease.

Thus, the findings in this thesis raise several questions and point to a need for better knowledge of basic properties of SNARE protein expression and function in the brain, as well as direct evidence of how these properties relate to protein abnormalities and symptoms seen in patients.

5.6 Conclusions

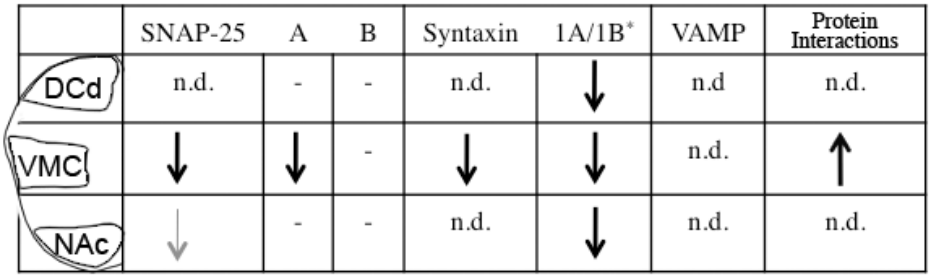
The results of these studies indicate that dysfunction of the associative striatum in schizophrenia involves SNARE protein abnormalities, likely due to changes in the ability of SNARE proteins to interact with each other. This supports the hypothesis that synaptic pathology occurs in schizophrenia, although, it appears that when SNARE proteins are used as a means to assess synapses, abnormalities may be restricted to certain striatal regions. A change in the balance of syntaxin-1 isoform expression, and/or decreased SNAP-25A expression is associated with increased SNARE complex levels. As the expression pattern of SNAREs and their isoforms in distinct terminal types in the striatum becomes better understood, our present findings will help elucidate which neurotransmitters are affected presynaptically in the striatum. Given that at least one SNARE isoform is developmentally regulated, increased knowledge of expression patterns in humans will elucidate whether SNARE protein abnormalities contribute to the development of

illness. Finally, that the current findings reflect the disease state and not patient treatment with antipsychotic medications is supported by statistical and experimental investigation of antipsychotic medication effects on striatal SNAREs. The present findings point to specific molecules that could be targeted to improve patient symptoms.

5.7 Chapter 5 tables

Table 5.1 - Summary of findings

Summary of results of statistical analyses reported in Chapters 2-4. Black arrows denote statistically significant findings of individual tests following significant main effects. Differences that did not meet statistical significance criteria (for example, Bonferroni correction) are illustrated in grey. 'n.d.' denotes no statistical differences ($P > 0.05$). Measurements that are not available are indicated by '-'. * For syntaxin-1 ratios, the statistical differences between schizophrenia and control subjects occurred in the striatum overall.



	SNAP-25	A	B	Syntaxin	1A/1B*	VAMP	Protein Interactions
DCd	n.d.	-	-	n.d.	↓	n.d	n.d.
VMC	↓	↓	-	↓	↓	n.d.	↑
NAc	↓	-	-	n.d.	↓	n.d.	n.d.

References

1. Andreasen NC. (1995). Symptoms, signs, and diagnosis of schizophrenia. *Lancet*, 346(8973): 477-81.

2. Ion RM and Beer MD. (2002). The British reaction to dementia praecox 1893-1913. Part 1. *Hist Psychiatry*, 13(51 Pt 3): 285-304.

3. Andreasen NC. (1989). The American concept of schizophrenia. *Schizophr Bull*, 15(4): 519-31.

4. Black DW and Boffeli TJ. (1989). Simple schizophrenia: past, present, and future. *Am J Psychiatry*, 146(10): 1267-73.

5. American Psychiatric A. (1994). *Diagnostic and statistical manual of mental disorders : DSM-IV*. Washington, DC: American Psychiatric Association.

6. van Os J and Kapur S. (2009). Schizophrenia. *Lancet*, 374(9690): 635-45.

7. Fioravanti M, Carlone O, Vitale B *et al*. (2005). A meta-analysis of cognitive deficits in adults with a diagnosis of schizophrenia. *Neuropsychol Rev*, 15(2): 73-95.

8. Yung AR and McGorry PD. (1996). The initial prodrome in psychosis: descriptive and qualitative aspects. *Aust N Z J Psychiatry*, 30(5): 587-99.

9. McGrath J, Saha S, Chant D *et al*. (2008). Schizophrenia: a concise overview of incidence, prevalence, and mortality. *Epidemiol Rev*, 30: 67-76.

10. Caldwell CB and Gottesman, II. (1990). Schizophrenics kill themselves too: a review of risk factors for suicide. *Schizophr Bull*, 16(4): 571-89.

11. Cardno AG, Marshall EJ, Coid B *et al*. (1999). Heritability estimates for psychotic disorders: the Maudsley twin psychosis series. *Arch Gen Psychiatry*, 56(2): 162-8.

12. Kety SS. (1999). Mental illness and the sciences of brain and behavior. *Nat Med*, 5(10): 1113-6.

13. McEvoy JP. (2007). The costs of schizophrenia. *J Clin Psychiatry*, 68 Suppl 14: 4-7.

14. Goeree R, Farahati F, Burke N *et al*. (2005). The economic burden of schizophrenia in Canada in 2004. *Curr Med Res Opin*, 21(12): 2017-28.

15. Weinberger DR. (1987). Implications of normal brain development for the pathogenesis of schizophrenia. *Arch Gen Psychiatry*, 44(7): 660-9.

16. Feinberg I. (1982). Schizophrenia: caused by a fault in programmed synaptic elimination during adolescence? *J Psychiatr Res*, 17(4): 319-34.

17. Keshavan MS and Hogarty GE. (1999). Brain maturational processes and delayed onset in schizophrenia. *Dev Psychopathol*, 11(3): 525-43.

18. Falkai P, Schneider-Axmann T and Honer WG. (2000). Entorhinal cortex pre-alpha cell clusters in schizophrenia: quantitative evidence of a developmental abnormality. *Biol Psychiatry*, 47(11): 937-43.

19. Rapoport JL, Addington AM, Frangou S *et al*. (2005). The neurodevelopmental model of schizophrenia: update 2005. *Mol Psychiatry*, 10(5): 434-49.

20. Fatemi SH and Folsom TD. (2009). The neurodevelopmental hypothesis of schizophrenia, revisited. *Schizophr Bull*, 35(3): 528-48.

21. Schiffman J, Walker E, Ekstrom M *et al*. (2004). Childhood videotaped social and neuromotor precursors of schizophrenia: a prospective investigation. *American Journal of Psychiatry*, 161(11): 2021-7.

22. Cannon M, Caspi A, Moffitt TE *et al*. (2002). Evidence for early-childhood, pan-developmental impairment specific to schizophreniform disorder: results from a longitudinal birth cohort. *Arch Gen Psychiatry*, 59(5): 449-56.

23. Lipska BK, Swerdlow NR, Geyer MA *et al*. (1995). Neonatal excitotoxic hippocampal damage in rats causes post-pubertal changes in prepulse inhibition of startle and its disruption by apomorphine. *Psychopharmacology (Berl)*, 122(1): 35-43.

24. Van Horn JD and McManus IC. (1992). Ventricular enlargement in schizophrenia. A meta-analysis of studies of the ventricle:brain ratio (VBR). *Br J Psychiatry*, 160: 687-97.

25. Wright IC, Rabe-Hesketh S, Woodruff PW *et al*. (2000). Meta-analysis of regional brain volumes in schizophrenia. *Am J Psychiatry*, 157(1): 16-25.

26. Boos HB, Aleman A, Cahn W *et al*. (2007). Brain volumes in relatives of patients with schizophrenia: a meta-analysis. *Arch Gen Psychiatry*, 64(3): 297-304.

27. Steen RG, Mull C, McClure R *et al*. (2006). Brain volume in first-episode schizophrenia: systematic review and meta-analysis of magnetic resonance imaging studies. *Br J Psychiatry*, 188: 510-8.

28. Hopkins R and Lewis S. (2000). Structural imaging findings and macroscopic pathology. In: *The neuropathology of schizophrenia : progress and interpretation* (Harrison PJ and Roberts GW, eds.), pp 5-56. Oxford; New York: Oxford University Press.

29. Smith CD, Chebrolu H, Wekstein DR *et al*. (2007). Age and gender effects on human brain anatomy: a voxel-based morphometric study in healthy elderly. *Neurobiol Aging*, 28(7): 1075-87.

30. Bourgeois JP, Goldman-Rakic PS and Rakic P. (1994). Synaptogenesis in the prefrontal cortex of rhesus monkeys. *Cereb Cortex*, 4(1): 78-96.

31. Torkamani A, Dean B, Schork NJ *et al*. (2010). Coexpression network analysis of neural tissue reveals perturbations in developmental processes in schizophrenia. *Genome Res*, 20(4): 403-12.

32. Lang UE, Puls I, Muller DJ *et al*. (2007). Molecular mechanisms of schizophrenia. *Cell Physiol Biochem*, 20(6): 687-702.

33. Bennett MR. (1999). The early history of the synapse: from Plato to Sherrington. *Brain Res Bull*, 50(2): 95-118.

34. Todman D. (2008). John Eccles (1903-97) and the experiment that proved chemical synaptic transmission in the central nervous system. *J Clin Neurosci*, 15(9): 972-7.

35. Tansey EM. (2006). Henry Dale and the discovery of acetylcholine. *C R Biol*, 329(5-6): 419-25.

36. Carlsson A. (2001). A paradigm shift in brain research. *Science*, 294(5544): 1021-4.

37. Kandel ER, Schwartz JH and Jessell TM. (ed.^eds.). (1991). *Principles of neural science*. New York: New York : Elsevier.

38. McKenna PJ. (1994). *Schizophrenia and related syndromes*. Oxford; New York: Oxford University Press.

39. Seeman P. (1987). Dopamine receptors and the dopamine hypothesis of schizophrenia. *Synapse*, 1(2): 133-52.

40. Harrison PJ. (1999). The neuropathology of schizophrenia. A critical review of the data and their interpretation. *Brain*, 122(Pt 4): 593-624.

41. Davis KL, Kahn RS, Ko G *et al*. (1991). Dopamine in schizophrenia: a review and reconceptualization. *Am J Psychiatry*, 148(11): 1474-86.

42. Kolachana BS, Saunders RC and Weinberger DR. (1995). Augmentation of prefrontal cortical monoaminergic activity inhibits dopamine release in the caudate nucleus: an in vivo neurochemical assessment in the rhesus monkey. *Neuroscience*, 69(3): 859-68.

43. Howes OD and Kapur S. (2009). The dopamine hypothesis of schizophrenia: version III--the final common pathway. *Schizophr Bull*, 35(3): 549-62.

44. Olney JW and Farber NB. (1995). Glutamate receptor dysfunction and schizophrenia. *Arch Gen Psychiatry*, 52(12): 998-1007.

45. Coyle JT. (2006). Glutamate and schizophrenia: beyond the dopamine hypothesis. *Cell Mol Neurobiol*, 26(4-6): 365-84.

46. Lewis DA and Sweet RA. (2009). Schizophrenia from a neural circuitry perspective: advancing toward rational pharmacological therapies. *J Clin Invest*, 119(4): 706-16.

47. Chumakov I, Blumenfeld M, Guerassimenko O *et al*. (2002). Genetic and physiological data implicating the new human gene G72 and the gene for D-amino acid oxidase in schizophrenia. *Proc Natl Acad Sci U S A*, 99(21): 13675-80.

48. Harrison PJ, Lyon L, Sartorius LJ *et al*. (2008). The group II metabotropic glutamate receptor 3 (mGluR3, mGlu3, GRM3): expression, function and involvement in schizophrenia. *J Psychopharmacol*, 22(3): 308-22.

49. Numakawa T, Yagasaki Y, Ishimoto T *et al*. (2004). Evidence of novel neuronal functions of dysbindin, a susceptibility gene for schizophrenia. *Hum Mol Genet*, 13(21): 2699-708.

50. Hahn CG, Wang HY, Cho DS *et al*. (2006). Altered neuregulin 1-erbB4 signaling contributes to NMDA receptor hypofunction in schizophrenia. *Nat Med*, 12(7): 824-8.

51. Allen NC, Bagade S, McQueen MB *et al*. (2008). Systematic meta-analyses and field synopsis of genetic association studies in schizophrenia: the SzGene database. *Nat Genet*, 40(7): 827-34. SzGene website accessed October 5, 2010.

52. Nudmamud-Thanoi S, Piyabhan P, Harte MK *et al*. (2007). Deficits of neuronal glutamatergic markers in the caudate nucleus in schizophrenia. *Journal of neural transmission.Supplementum*, (72)(72): 281-5.

53. Eastwood SL and Harrison PJ. (2005). Decreased expression of vesicular glutamate transporter 1 and complexin II mRNAs in schizophrenia: further evidence for a synaptic pathology affecting glutamate neurons. *Schizophr Res*, 73(2-3): 159-72.

54. Bauer D, Gupta D, Harotunian V *et al*. (2008). Abnormal expression of glutamate transporter and transporter interacting molecules in prefrontal cortex in elderly patients with schizophrenia. *Schizophr Res*, 104(1-3): 108-20.

55. Benes FM. (2000). Emerging principles of altered neural circuitry in schizophrenia. *Brain Res Rev*, 31(2-3): 251-69.

56. Beasley CL and Reynolds GP. (1997). Parvalbumin-immunoreactive neurons are reduced in the prefrontal cortex of schizophrenics. *Schizophr Res*, 24(3): 349-55.

57. Beasley CL, Zhang ZJ, Patten I *et al*. (2002). Selective deficits in prefrontal cortical GABAergic neurons in schizophrenia defined by the presence of calcium-binding proteins. *Biol Psychiatry*, 52(7): 708-15.

58. Hashimoto T, Bazmi HH, Mirnics K *et al*. (2008). Conserved regional patterns of GABA-related transcript expression in the neocortex of subjects with schizophrenia. *Am J Psychiatry*, 165(4): 479-89.

59. Kehrer C, Maziashvili N, Dugladze T *et al*. (2008). Altered Excitatory-Inhibitory Balance in the NMDA-Hypofunction Model of Schizophrenia. *Front Mol Neurosci*, 1: 6.

60. Lewis DA, Hashimoto T and Volk DW. (2005). Cortical inhibitory neurons and schizophrenia. *Nat Rev Neurosci*, 6(4): 312-24.

61. Simpson EH, Kellendonk C and Kandel E. (2010). A possible role for the striatum in the pathogenesis of the cognitive symptoms of schizophrenia. *Neuron*, 65(5): 585-96.

62. Geyer MA and Vollenweider FX. (2008). Serotonin research: contributions to understanding psychoses. *Trends Pharmacol Sci*, 29(9): 445-53.

63. Dean B. (2003). The cortical serotonin2A receptor and the pathology of schizophrenia: a likely accomplice. *J Neurochem*, 85(1): 1-13.

64. Erritzoe D, Rasmussen H, Kristiansen KT *et al*. (2008). Cortical and subcortical 5-HT2A receptor binding in neuroleptic-naive first-episode schizophrenic patients. *Neuropsychopharmacology*, 33(10): 2435-41.

65. Hurlemann R, Matusch A, Kuhn KU *et al*. (2008). 5-HT2A receptor density is decreased in the at-risk mental state. *Psychopharmacology (Berl)*, 195(4): 579-90.

66. Rasmussen H, Erritzoe D, Andersen R *et al*. (2010). Decreased frontal serotonin2A receptor binding in antipsychotic-naive patients with first-episode schizophrenia. *Arch Gen Psychiatry*, 67(1): 9-16.

67. Hyde TM and Crook JM. (2001). Cholinergic systems and schizophrenia: primary pathology or epiphenomena? *J Chem Neuroanat*, 22(1-2): 53-63.

68. Scarr E and Dean B. (2009). Role of the cholinergic system in the pathology and treatment of schizophrenia. *Expert Rev Neurother*, 9(1): 73-86.

69. Guan ZZ, Zhang X, Blennow K *et al*. (1999). Decreased protein level of nicotinic receptor alpha7 subunit in the frontal cortex from schizophrenic brain. *Neuroreport*, 10(8): 1779-82.

70. De Luca V, Likhodi O, Van Tol HH *et al*. (2006). Regulation of alpha7-nicotinic receptor subunit and alpha7-like gene expression in the prefrontal cortex of patients with bipolar disorder and schizophrenia. *Acta Psychiatr Scand*, 114(3): 211-5.

71. Martin-Ruiz CM, Haroutunian VH, Long P *et al*. (2003). Dementia rating and nicotinic receptor expression in the prefrontal cortex in schizophrenia. *Biol Psychiatry*, 54(11): 1222-33.

72. Breese CR, Lee MJ, Adams CE *et al*. (2000). Abnormal regulation of high affinity nicotinic receptors in subjects with schizophrenia. *Neuropsychopharmacology*, 23(4): 351-64.

73. Raedler TJ, Knable MB, Jones DW *et al*. (2003). In vivo determination of muscarinic acetylcholine receptor availability in schizophrenia. *Am J Psychiatry*, 160(1): 118-27.

74. Scarr E, Cowie TF, Kanellakis S *et al*. (2009). Decreased cortical muscarinic receptors define a subgroup of subjects with schizophrenia. *Mol Psychiatry*, 14(11): 1017-23.

75. Tajima K, Fernandez H, Lopez-Ibor JL *et al*. (2009). Schizophrenia treatment. Critical review on the drugs and mechanisms of action of antipsychotics. *Actas Esp Psiquiatr*, 37(6): 330-42.

76. Yamamoto K and Hornykiewicz O. (2004). Proposal for a noradrenaline hypothesis of schizophrenia. *Prog Neuropsychopharmacol Biol Psychiatry*, 28(5): 913-22.

77. Kapur S and Remington G. (2001). Dopamine D(2) receptors and their role in atypical antipsychotic action: still necessary and may even be sufficient. *Biol Psychiatry*, 50(11): 873-83.

78. Carlsson A, Waters N and Carlsson ML. (1999). Neurotransmitter interactions in schizophrenia--therapeutic implications. *Biol Psychiatry*, 46(10): 1388-95.

79. Feuerstein TJ. (2008). Presynaptic receptors for dopamine, histamine, and serotonin. *Handbook of experimental pharmacology*, (18064418): 289-338.

80. Mizuno T, Schmauss C and Rayport S. (2007). Distinct roles of presynaptic dopamine receptors in the differential modulation of the intrinsic synapses of medium-spiny neurons in the nucleus accumbens. *BMC Neurosci*, 8: 8.

81. Calabresi P, De Murtas M and Bernardi G. (1997). The neostriatum beyond the motor function: experimental and clinical evidence. *Neuroscience*, 78(9135088): 39-60.

82. Wilson CJ. (2004). Basal Ganglia. In: *The synaptic organization of the brain* (Shepherd GM, ed., pp 361-414. New York: Oxford University Press.

83. Usiello A, Baik JH, Rouge-Pont F *et al*. (2000). Distinct functions of the two isoforms of dopamine D2 receptors. *Nature*, 408(6809): 199-203.

84. Javitt DC. (2004). Glutamate as a therapeutic target in psychiatric disorders. *Mol Psychiatry*, 9(11): 984-97, 79.

85. Patil ST, Zhang L, Martenyi F *et al*. (2007). Activation of mGlu2/3 receptors as a new approach to treat schizophrenia: a randomized Phase 2 clinical trial. *Nat Med*, 13(9): 1102-7.

86. Shekhar A, Potter WZ, Lightfoot J *et al*. (2008). Selective muscarinic receptor agonist xanomeline as a novel treatment approach for schizophrenia. *Am J Psychiatry*, 165(8): 1033-9.

87. Freedman R, Olincy A, Buchanan RW *et al*. (2008). Initial phase 2 trial of a nicotinic agonist in schizophrenia. *Am J Psychiatry*, 165(8): 1040-7.

88. Tekin S and Cummings JL. (2002). Frontal-subcortical neuronal circuits and clinical neuropsychiatry: an update. *J Psychosom Res*, 53(2): 647-54.

89. Chudasama Y and Robbins TW. (2006). Functions of frontostriatal systems in cognition: comparative neuropsychopharmacological studies in rats, monkeys and humans. *Biol Psychol*, 73(1): 19-38.

90. Pantelis C and Brewer W. (1995). Neuropsychological and olfactory dysfunction in schizophrenia: relationship of frontal syndromes to syndromes of schizophrenia. *Schizophr Res*, 17(1): 35-45.

91. Stephan KE, Baldeweg T and Friston KJ. (2006). Synaptic plasticity and dysconnection in schizophrenia. *Biol Psychiatry*, 59(10): 929-39.

92. Liddle PF, Friston KJ, Frith CD *et al*. (1992). Patterns of cerebral blood flow in schizophrenia. *Br J Psychiatry*, 160: 179-86.

93. Frith C. (1995). Functional imaging and cognitive abnormalities. *Lancet*, 346(8975): 615-20.

94. Tost H, Ende G, Ruf M *et al*. (2005). Functional imaging research in schizophrenia. *Int Rev Neurobiol*, 67: 95-118.

95. Juckel G, Schlagenhauf F, Koslowski M *et al*. (2006). Dysfunction of ventral striatal reward prediction in schizophrenia. *NeuroImage*, 29(2): 409-16.

96. Sudhof TC. (2004). The synaptic vesicle cycle. *Annu Rev Neurosci*, 27: 509-47.

97. Dresbach T, Qualmann B, Kessels MM *et al*. (2001). The presynaptic cytomatrix of brain synapses. *Cell Mol Life Sci*, 58(1): 94-116.

98. Araque A, Parpura V, Sanzgiri RP *et al*. (1999). Tripartite synapses: glia, the unacknowledged partner. *Trends Neurosci*, 22(5): 208-15.

99. Mirnics K, Middleton FA, Marquez A *et al*. (2000). Molecular characterization of schizophrenia viewed by microarray analysis of gene expression in prefrontal cortex. *Neuron*, 28(1): 53-67.

100. Hayashi T, Yamasaki S, Nauenburg S *et al*. (1995). Disassembly of the reconstituted synaptic vesicle membrane fusion complex in vitro. *EMBO J*, 14(10): 2317-25.

101. Cesca F, Baldelli P, Valtorta F *et al*. (2010). The synapsins: Key actors of synapse function and plasticity. *Prog Neurobiol*. doi: 10.1016/j.pneurobio.2010.04.006.

102. Maycox PR, Kelly F, Taylor A *et al*. (2009). Analysis of gene expression in two large schizophrenia cohorts identifies multiple changes associated with nerve terminal function. *Mol Psychiatry*, 14(12): 1083-94.

103. Hashimoto T, Arion D, Unger T *et al*. (2008). Alterations in GABA-related transcriptome in the dorsolateral prefrontal cortex of subjects with schizophrenia. *Mol Psychiatry*, 13(2): 147-61.

104. Vawter MP, Crook JM, Hyde TM *et al*. (2002). Microarray analysis of gene expression in the prefrontal cortex in schizophrenia: a preliminary study. *Schizophr Res*, 58(1): 11-20.

105. Mudge J, Miller NA, Khrebtukova I *et al*. (2008). Genomic convergence analysis of schizophrenia: mRNA sequencing reveals altered synaptic vesicular transport in post-mortem cerebellum. *PLoS One*, 3(11): e3625.

106. Prabakaran S, Swatton JE, Ryan MM *et al*. (2004). Mitochondrial dysfunction in schizophrenia: evidence for compromised brain metabolism and oxidative stress. *Mol Psychiatry*, 9(7): 684-97, 43.

107. Selemon LD and Goldman-Rakic PS. (1999). The reduced neuropil hypothesis: a circuit based model of schizophrenia. *Biol Psychiatry*, 45(1): 17-25.

108. Selemon LD. (2004). Increased cortical neuronal density in schizophrenia. *Am J Psychiatry*, 161(9): 1564.

109. Honer WG, Young C and Falkai P. (2000). Synaptic Pathology. In: *The neuropathology of schizophrenia : progress and interpretation* (Harrison PJ and Roberts GW, eds.), pp 105-36. Oxford; New York: Oxford University Press.

110. Perez-Costas E, Melendez-Ferro M and Roberts RC. (2010). Basal ganglia pathology in schizophrenia: dopamine connections and anomalies. *Journal of neurochemistry*, 113(2): 287-302.

111. Honer WG and Young CE. (2004). Presynaptic proteins and schizophrenia. *Int Rev Neurobiol*, 59: 175-99.

112. Eastwood SL. (2004). The synaptic pathology of schizophrenia: is aberrant neurodevelopment and plasticity to blame? *Int Rev Neurobiol*, 59: 47-72.

113. Reynolds GP and Harte MK. (2007). The neuronal pathology of schizophrenia: molecules and mechanisms. *Biochem Soc Trans*, 35(Pt 2): 433-6.

114. Barley K, Dracheva S and Byne W. (2009). Subcortical oligodendrocyte- and astrocyte-associated gene expression in subjects with schizophrenia, major depression and bipolar disorder. *Schizophr Res*, 112(1-3): 54-64.

115. Martins-de-Souza D, Gattaz WF, Schmitt A *et al*. (2009). Proteomic analysis of dorsolateral prefrontal cortex indicates the involvement of cytoskeleton, oligodendrocyte,

energy metabolism and new potential markers in schizophrenia. *J Psychiatr Res*, 43(11): 978-86.

116. Haroutunian V and Davis KL. (2007). Introduction to the special section: Myelin and oligodendrocyte abnormalities in schizophrenia. *Int J Neuropsychopharmacol*, 10(4): 499-502.

117. Kubicki M, McCarley RW and Shenton ME. (2005). Evidence for white matter abnormalities in schizophrenia. *Curr Opin Psychiatry*, 18(2): 121-34.

118. Beasley CL, Dwork AJ, Rosoklija G *et al*. (2009). Metabolic abnormalities in fronto-striatal-thalamic white matter tracts in schizophrenia. *Schizophr Res*, 109(1-3): 159-66.

119. Dwork AJ, Mancevski B and Rosoklija G. (2007). White matter and cognitive function in schizophrenia. *Int J Neuropsychopharmacol*, 10(4): 513-36.

120. Kyriakopoulos M, Bargiotas T, Barker GJ *et al*. (2008). Diffusion tensor imaging in schizophrenia. *Eur Psychiatry*, 23(4): 255-73.

121. Lang DJ, Khorram B, Goghari VM *et al*. (2006). Reduced anterior internal capsule and thalamic volumes in first-episode psychosis. *Schizophr Res*, 87(1-3): 89-99.

122. Robbins TW. (1990). The case of frontostriatal dysfunction in schizophrenia. *Schizophr Bull*, 16(3): 391-402.

123. Fornito A, Yucel M and Pantelis C. (2009). Reconciling neuroimaging and neuropathological findings in schizophrenia and bipolar disorder. *Curr Opin Psychiatry*, 22(19365187): 312-9.

124. Goghari VM, Sponheim SR and MacDonald AW. (2010). The functional neuroanatomy of symptom dimensions in schizophrenia: a qualitative and quantitative review of a persistent question. *Neurosci Biobehav Rev*, 34(3): 468-86.

125. MacDonald AW and Schulz SC. (2009). What we know: findings that every theory of schizophrenia should explain. *Schizophr Bull*, 35(3): 493-508.

126. Ballmaier M, Schlagenhauf F, Toga AW *et al*. (2008). Regional patterns and clinical correlates of basal ganglia morphology in non-medicated schizophrenia. *Schizophr Res*, 106(2-3): 140-7.

127. Csernansky JG, Schindler MK, Splinter NR *et al*. (2004). Abnormalities of thalamic volume and shape in schizophrenia. *Am J Psychiatry*, 161(5): 896-902.

128. Lahti AC, Weiler MA, Holcomb HH *et al*. (2006). Correlations between rCBF and symptoms in two independent cohorts of drug-free patients with schizophrenia. *Neuropsychopharmacology*, 31(1): 221-30.

129. Alexander GE, DeLong MR and Strick PL. (1986). Parallel organization of functionally segregated circuits linking basal ganglia and cortex. *Annu Rev Neurosci*, 9: 357-81.

130. Levy R and Dubois B. (2006). Apathy and the functional anatomy of the prefrontal cortex-basal ganglia circuits. *Cereb Cortex*, 16(7): 916-28.

131. Rolls ET. (2004). The functions of the orbitofrontal cortex. *Brain Cogn*, 55(1): 11-29.

132. Pantelis C, Barnes TR, Nelson HE *et al*. (1997). Frontal-striatal cognitive deficits in patients with chronic schizophrenia. *Brain*, 120(Pt 10): 1823-43.

133. Brandt GN and Bonelli RM. (2008). Structural neuroimaging of the basal ganglia in schizophrenic patients: a review. *Wien Med Wochenschr*, 158(3-4): 84-90.

134. Haber SN. (2003). The primate basal ganglia: parallel and integrative networks. *J Chem Neuroanat*, 26(4): 317-30.

135. Voorn P, Vanderschuren LJ, Groenewegen HJ *et al*. (2004). Putting a spin on the dorsal-ventral divide of the striatum. *Trends Neurosci*, 27(8): 468-74.

136. Dowd EC and Barch DM. (2010). Anhedonia and emotional experience in schizophrenia: neural and behavioral indicators. *Biol Psychiatry*, 67(10): 902-11.

137. Schlagenhauf F, Sterzer P, Schmack K *et al*. (2009). Reward feedback alterations in unmedicated schizophrenia patients: relevance for delusions. *Biol Psychiatry*, 65(12): 1032-9.

138. Rubin P, Holm S, Madsen PL *et al*. (1994). Regional cerebral blood flow distribution in newly diagnosed schizophrenia and schizophreniform disorder. *Psychiatry Res*, 53(1): 57-75.

139. Kegeles LS, Abi-Dargham A, Frankle WG *et al*. (2010). Increased synaptic dopamine function in associative regions of the striatum in schizophrenia. *Arch Gen Psychiatry*, 67(3): 231-9.

140. Abi-Dargham A, van de Giessen E, Slifstein M *et al*. (2009). Baseline and amphetamine-stimulated dopamine activity are related in drug-naive schizophrenic subjects. *Biol Psychiatry*, 65(12): 1091-3.

141. Laruelle M, Abi-Dargham A, Gil R *et al*. (1999). Increased dopamine transmission in schizophrenia: relationship to illness phases. *Biol Psychiatry*, 46(1): 56-72.

142. Howes OD, Montgomery AJ, Asselin MC *et al*. (2009). Elevated striatal dopamine function linked to prodromal signs of schizophrenia. *Arch Gen Psychiatry*, 66(1): 13-20.

143. Kreczmanski P, Heinsen H, Mantua V *et al*. (2007). Volume, neuron density and total neuron number in five subcortical regions in schizophrenia. *Brain*, 130(Pt 3): 678-92.

144. Holt DJ, Bachus SE, Hyde TM *et al*. (2005). Reduced density of cholinergic interneurons in the ventral striatum in schizophrenia: an in situ hybridization study. *Biol Psychiatry*, 58(5): 408-16.

145. Roberts RC, Roche JK and Conley RR. (2008). Differential synaptic changes in the striatum of subjects with undifferentiated versus paranoid schizophrenia. *Synapse*, 62(8): 616-27.

146. Roberts RC, Roche JK and Conley RR. (2005). Synaptic differences in the patch matrix compartments of subjects with schizophrenia: a postmortem ultrastructural study of the striatum. *Neurobiol Dis*, 20(2): 324-35.

147. Roberts RC, Roche JK, Conley RR *et al*. (2009). Dopaminergic synapses in the caudate of subjects with schizophrenia: relationship to treatment response. *Synapse*, 63(6): 520-30.

148. Levey AI, Hersch SM, Rye DB *et al*. (1993). Localization of D1 and D2 dopamine receptors in brain with subtype-specific antibodies. *Proc Natl Acad Sci U S A*, 90(19): 8861-5.

149. Lang DJ, Kopala LC, Vandorpe RA *et al*. (2001). An MRI study of basal ganglia volumes in first-episode schizophrenia patients treated with risperidone. *Am J Psychiatry*, 158(4): 625-31.

150. Lang DJ, Kopala LC, Vandorpe RA *et al*. (2004). Reduced basal ganglia volumes after switching to olanzapine in chronically treated patients with schizophrenia. *Am J Psychiatry*, 161(10): 1829-36.

151. Nakano K, Kayahara T, Tsutsumi T *et al*. (2000). Neural circuits and functional organization of the striatum. *J Neurol*, 247 Suppl 5: V1-15.

152. Ingham CA, Hood SH, Taggart P *et al*. (1998). Plasticity of synapses in the rat neostriatum after unilateral lesion of the nigrostriatal dopaminergic pathway. *J Neurosci*, 18(12): 4732-43.

153. Haber SN, Fudge JL and McFarland NR. (2000). Striatonigrostriatal pathways in primates form an ascending spiral from the shell to the dorsolateral striatum. *J Neurosci*, 20(6): 2369-82.

154. Gerfen CR. (1984). The neostriatal mosaic: compartmentalization of corticostriatal input and striatonigral output systems. *Nature*, 311(5985): 461-4.

155. Graybiel AM and Ragsdale CW, Jr. (1978). Histochemically distinct compartments in the striatum of human, monkeys, and cat demonstrated by acetylthiocholinesterase staining. *Proc Natl Acad Sci U S A*, 75(11): 5723-6.

156. Jahn R and Scheller RH. (2006). SNAREs--engines for membrane fusion. *Nat Rev Mol Cell Biol*, 7(9): 631-43.

157. Novick P, Ferro S and Schekman R. (1981). Order of events in the yeast secretory pathway. *Cell*, 25(2): 461-9.

158. Fasshauer D, Sutton RB, Brunger AT *et al*. (1998). Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q- and R-SNAREs. *Proc Natl Acad Sci U S A*, 95(26): 15781-6.

159. Sutton RB, Fasshauer D, Jahn R *et al*. (1998). Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. *Nature*, 395(6700): 347-53.

160. Canaves JM and Montal M. (1998). Assembly of a ternary complex by the predicted minimal coiled-coil-forming domains of syntaxin, SNAP-25, and synaptobrevin. A circular dichroism study. *J Biol Chem*, 273(51): 34214-21.

161. Yang B, Gonzalez L, Prekeris R *et al*. (1999). SNARE interactions are not selective. Implications for membrane fusion specificity. *J Biol Chem*, 274(10026182): 5649-53.

162. Sollner T, Bennett MK, Whiteheart SW *et al*. (1993). A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. *Cell*, 75(3): 409-18.

163. Sollner T, Whiteheart SW, Brunner M *et al*. (1993). SNAP receptors implicated in vesicle targeting and fusion. *Nature*, 362(6418): 318-24.

164. Catsicas S, Larhammar D, Blomqvist A *et al*. (1991). Expression of a conserved cell-type-specific protein in nerve terminals coincides with synaptogenesis. *Proc Natl Acad Sci U S A*, 88(1992470): 785-9.

165. Risinger C, Blomqvist AG, Lundell I *et al*. (1993). Evolutionary conservation of synaptosome-associated protein 25 kDa (SNAP-25) shown by Drosophila and Torpedo cDNA clones. *J Biol Chem*, 268(32): 24408-14.

166. Sudhof TC, Baumert M, Perin MS *et al*. (1989). A synaptic vesicle membrane protein is conserved from mammals to Drosophila. *Neuron*, 2(5): 1475-81.

167. Cerezo JR, Jimenez F and Moya F. (1995). Characterization and gene cloning of Drosophila syntaxin 1 (Dsynt1): the fruit fly homologue of rat syntaxin 1. *Brain Res Mol Brain Res*, 29(2): 245-52.

168. Oyler GA, Polli JW, Higgins GA *et al*. (1992). Distribution and expression of SNAP-25 immunoreactivity in rat brain, rat PC-12 cells and human SMS-KCNR neuroblastoma cells. *Brain Res Dev Brain Res*, 65(2): 133-46.

169. Bennett MK, Calakos N and Scheller RH. (1992). Syntaxin: a synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones. *Science*, 257(5067): 255-9.

170. Salaun C, Gould GW and Chamberlain LH. (2005). Lipid raft association of SNARE proteins regulates exocytosis in PC12 cells. *J Biol Chem*, 280(20): 19449-53.

171. Gil C, Soler-Jover A, Blasi J *et al*. (2005). Synaptic proteins and SNARE complexes are localized in lipid rafts from rat brain synaptosomes. *Biochem Biophys Res Commun*, 329(1): 117-24.

172. Lang T. (2007). SNARE proteins and 'membrane rafts'. *J Physiol*, 585(17478530): 693-8.

173. Trimble WS, Gray TS, Elferink LA *et al*. (1990). Distinct patterns of expression of two VAMP genes within the rat brain. *J Neurosci*, 10(4): 1380-7.

174. Elferink LA, Trimble WS and Scheller RH. (1989). Two vesicle-associated membrane protein genes are differentially expressed in the rat central nervous system. *J Biol Chem*, 264(19): 11061-4.

175. Sudhof TC. (2008). Neurotransmitter release. In: *Pharmacology of neurotransmitter release* (Sudhof TC and Starke K, eds.), pp 1-22. Berlin: Springer.

176. Fanous A, Zhao Z, Van Den Oord E *et al*. (2010). Association study of SNAP25 and schizophrenia in Irish family and case-control samples. *American Journal of Medical Genetics Part B: Neuropsychiatric Genetics*, 153B(2): 663-74.

177. Carroll LS, Kendall K, O'Donovan MC *et al*. (2009). Evidence that putative ADHD low risk alleles at SNAP25 may increase the risk of schizophrenia. *Am J Med Genet B Neuropsychiatr Genet*, 150B(7): 893-9.

178. Terracciano A, Sanna S, Uda M *et al*. (2010). Genome-wide association scan for five major dimensions of personality. *Mol Psychiatry*, 15(6): 647-56.

179. Gosso MF, de Geus EJ, van Belzen MJ *et al*. (2006). The SNAP-25 gene is associated with cognitive ability: evidence from a family-based study in two independent Dutch cohorts. *Mol Psychiatry*, 11(9): 878-86.

180. Wong AH, Trakalo J, Likhodi O *et al*. (2004). Association between schizophrenia and the syntaxin 1A gene. *Biol Psychiatry*, 56(1): 24-9.

181. Corominas R, Ribases M, Cuenca-Leon E *et al*. (2009). Contribution of syntaxin 1A to the genetic susceptibility to migraine: a case-control association study in the Spanish population. *Neurosci Lett*, 455(2): 105-9.

182. Lemos C, Pereira-Monteiro J, Mendonca D *et al*. (2010). Evidence of syntaxin 1A involvement in migraine susceptibility: a Portuguese study. *Arch Neurol*, 67(4): 422-7.

183. Nakamura K, Anitha A, Yamada K *et al*. (2008). Genetic and expression analyses reveal elevated expression of syntaxin 1A (STX1A) in high functioning autism. *Int J Neuropsychopharmacol*, 11(8): 1073-84.

184. Tsunoda K, Sanke T, Nakagawa T *et al*. (2001). Single nucleotide polymorphism (D68D, T to C) in the syntaxin 1A gene correlates to age at onset and insulin requirement in Type II diabetic patients. *Diabetologia*, 44(11): 2092-7.

185. Abou Jamra R, Gobina CM, Becker T *et al*. (2008). Association study between genetic variants at the VAMP2 and VAMP3 loci and bipolar affective disorder. *Psychiatr Genet*, 18(4): 199-203.

186. Washbourne P, Thompson PM, Carta M *et al*. (2002). Genetic ablation of the t-SNARE SNAP-25 distinguishes mechanisms of neuroexocytosis. *Nat Neurosci*, 5(1): 19-26.

187. Gerber SH, Rah JC, Min SW *et al*. (2008). Conformational switch of syntaxin-1 controls synaptic vesicle fusion. *Science*, 321(5895): 1507-10.

188. Schoch S, Deak F, Konigstorfer A *et al*. (2001). SNARE function analyzed in synaptobrevin/VAMP knockout mice. *Science*, 294(5544): 1117-22.

189. Borisovska M, Zhao Y, Tsytsyura Y *et al*. (2005). v-SNAREs control exocytosis of vesicles from priming to fusion. *EMBO J*, 24(12): 2114-26.

190. Sara Y, Virmani T, Deak F *et al*. (2005). An isolated pool of vesicles recycles at rest and drives spontaneous neurotransmission. *Neuron*, 45(4): 563-73.

191. de Wit H, Walter AM, Milosevic I *et al*. (2009). Synaptotagmin-1 docks secretory vesicles to syntaxin-1/SNAP-25 acceptor complexes. *Cell*, 138(5): 935-46.

192. Verderio C, Pozzi D, Pravettoni E *et al*. (2004). SNAP-25 modulation of calcium dynamics underlies differences in GABAergic and glutamatergic responsiveness to depolarization. *Neuron*, 41(4): 599-610.

193. Garbelli R, Inverardi F, Medici V *et al*. (2008). Heterogeneous expression of SNAP-25 in rat and human brain. *J Comp Neurol*, 506(3): 373-86.

194. Bragina L, Candiracci C, Barbaresi P *et al*. (2007). Heterogeneity of glutamatergic and GABAergic release machinery in cerebral cortex. *Neuroscience*, 146(4): 1829-40.

195. Frassoni C, Inverardi F, Coco S *et al*. (2005). Analysis of SNAP-25 immunoreactivity in hippocampal inhibitory neurons during development in culture and in situ. *Neuroscience*, 131(4): 813-23.

196. Sorensen JB, Nagy G, Varoqueaux F *et al*. (2003). Differential control of the releasable vesicle pools by SNAP-25 splice variants and SNAP-23. *Cell*, 114(1): 75-86.

197. Holt M, Varoqueaux F, Wiederhold K *et al*. (2006). Identification of SNAP-47, a novel Qbc-SNARE with ubiquitous expression. *J Biol Chem*, 281(16621800): 17076-83.

198. Su Q, Mochida S, Tian JH *et al*. (2001). SNAP-29: a general SNARE protein that inhibits SNARE disassembly and is implicated in synaptic transmission. *Proc Natl Acad Sci U S A*, 98(24): 14038-43.

199. Sutton KG, McRory JE, Guthrie H *et al*. (1999). P/Q-type calcium channels mediate the activity-dependent feedback of syntaxin-1A. *Nature*, 401(6755): 800-4.

200. Tafoya LC, Mameli M, Miyashita T *et al*. (2006). Expression and function of SNAP-25 as a universal SNARE component in GABAergic neurons. *J Neurosci*, 26(30): 7826-38.

201. Gronborg M, Pavlos NJ, Brunk I *et al*. (2010). Quantitative comparison of glutamatergic and GABAergic synaptic vesicles unveils selectivity for few proteins including MAL2, a novel synaptic vesicle protein. *J Neurosci*, 30(1): 2-12.

202. Matteoli M, Pozzi D, Grumelli C *et al*. (2009). The synaptic split of SNAP-25: different roles in glutamatergic and GABAergic neurons? *Neuroscience*, 158(1): 223-30.

203. Connell E, Darios F, Peak-Chew S *et al*. (2009). N-Terminal Acetylation of the Neuronal Protein SNAP-25 Is Revealed by the SMI81 Monoclonal Antibody. *Biochemistry*, 48(40): 9582-9.

204. Bragina L, Giovedi S, Barbaresi P *et al*. (2010). Heterogeneity of glutamatergic and GABAergic release machinery in cerebral cortex: analysis of synaptogyrin, vesicle-associated membrane protein, and syntaxin. *Neuroscience*, 165(3): 934-43.

205. Langley K and Grant NJ. (1997). Are exocytosis mechanisms neurotransmitter specific? *Neurochem Int*, 31(6): 739-57.

206. Hayashi T, McMahon H, Yamasaki S *et al*. (1994). Synaptic vesicle membrane fusion complex: action of clostridial neurotoxins on assembly. *EMBO J*, 13(21): 5051-61.

207. Abdulreda MH, Bhalla A, Rico F *et al*. (2009). Pulling force generated by interacting SNAREs facilitates membrane hemifusion. *Integr Biol (Camb)*, 1(4): 301-10.

208. McNew JA, Weber T, Parlati F *et al*. (2000). Close is not enough: SNARE-dependent membrane fusion requires an active mechanism that transduces force to membrane anchors. *J Cell Biol*, 150(1): 105-17.

209. Montecucco C, Schiavo G and Pantano S. (2005). SNARE complexes and neuroexocytosis: how many, how close? *Trends Biochem Sci*, 30(7): 367-72.

210. Hua Y and Scheller RH. (2001). Three SNARE complexes cooperate to mediate membrane fusion. *Proc Natl Acad Sci U S A*, 98(14): 8065-70.

211. Lu X, Zhang Y and Shin YK. (2008). Supramolecular SNARE assembly precedes hemifusion in SNARE-mediated membrane fusion. *Nat Struct Mol Biol*, 15(7): 700-6.

212. Pobbati AV, Stein A and Fasshauer D. (2006). N- to C-terminal SNARE complex assembly promotes rapid membrane fusion. *Science*, 313(5787): 673-6.

213. Jahn R, Lang T and Sudhof TC. (2003). Membrane fusion. *Cell*, 112(4): 519-33.

214. Bretou M, Anne C and Darchen F. (2008). A fast mode of membrane fusion dependent on tight SNARE zippering. *J Neurosci*, 28(34): 8470-6.

215. Walter AM, Wiederhold K, Bruns D *et al*. (2010). Synaptobrevin N-terminally bound to syntaxin-SNAP-25 defines the primed vesicle state in regulated exocytosis. *J Cell Biol*, 188(3): 401-13.

216. Fasshauer D and Margittai M. (2004). A transient N-terminal interaction of SNAP-25 and syntaxin nucleates SNARE assembly. *J Biol Chem*, 279(9): 7613-21.

217. Weber T, Zemelman BV, McNew JA *et al*. (1998). SNAREpins: minimal machinery for membrane fusion. *Cell*, 92(6): 759-72.

218. Rickman C, Meunier FA, Binz T *et al*. (2004). High affinity interaction of syntaxin and SNAP-25 on the plasma membrane is abolished by botulinum toxin E. *J Biol Chem*, 279(1): 644-51.

219. Sudhof TC. (2002). Synaptotagmins: why so many? *J Biol Chem*, 277(10): 7629-32.

220. Giraudo CG, Eng WS, Melia TJ *et al*. (2006). A clamping mechanism involved in SNARE-dependent exocytosis. *Science*, 313(5787): 676-80.

221. Lang T and Jahn R. (2008). Core proteins of the secretory machinery. In: *Pharmacology of neurotransmitter release* (Sudhof TC and Starke K, eds.), pp 107-28. Berlin: Springer.

222. Fasshauer D. (2003). Structural insights into the SNARE mechanism. *Biochim Biophys Acta*, 1641(2-3): 87-97.

223. Weninger K, Bowen ME, Choi UB *et al*. (2008). Accessory proteins stabilize the acceptor complex for synaptobrevin, the 1:1 syntaxin/SNAP-25 complex. *Structure*, 16(2): 308-20.

224. Littleton JT, Barnard RJ, Titus SA *et al*. (2001). SNARE-complex disassembly by NSF follows synaptic-vesicle fusion. *Proc Natl Acad Sci U S A*, 98(21): 12233-8.

225. Breidenbach MA and Brunger AT. (2005). New insights into clostridial neurotoxin-SNARE interactions. *Trends Mol Med*, 11(8): 377-81.

226. Vikman J, Ma X, Hockerman GH *et al*. (2006). Antibody inhibition of synaptosomal protein of 25 kDa (SNAP-25) and syntaxin 1 reduces rapid exocytosis in insulin-secreting cells. *J Mol Endocrinol*, 36(3): 503-15.

227. Tomes CN, Michaut M, De Blas G *et al*. (2002). SNARE complex assembly is required for human sperm acrosome reaction. *Dev Biol*, 243(2): 326-38.

228. Blanes-Mira C, Merino JM, Valera E *et al*. (2004). Small peptides patterned after the N-terminus domain of SNAP25 inhibit SNARE complex assembly and regulated exocytosis. *J Neurochem*, 88(1): 124-35.

229. Jeans AF, Oliver PL, Johnson R *et al*. (2007). A dominant mutation in Snap25 causes impaired vesicle trafficking, sensorimotor gating, and ataxia in the blind-drunk mouse. *Proc Natl Acad Sci U S A*, 104(7): 2431-6.

230. Matveeva EA, Whiteheart SW and Slevin JT. (2003). Accumulation of 7S SNARE complexes in hippocampal synaptosomes from chronically kindled rats. *J Neurochem*, 84(3): 621-4.

231. Matveeva EA, Vanaman TC, Whiteheart SW *et al*. (2007). Asymmetric accumulation of hippocampal 7S SNARE complexes occurs regardless of kindling paradigm. *Epilepsy Res*, 73(3): 266-74.

232. Xu NJ, Yu YX, Zhu JM *et al*. (2004). Inhibition of SNAP-25 phosphorylation at Ser187 is involved in chronic morphine-induced down-regulation of SNARE complex formation. *J Biol Chem*, 279(39): 40601-8.

233. Wilhelm A, Volknandt W, Langer D *et al*. (2004). Localization of SNARE proteins and secretory organelle proteins in astrocytes in vitro and in situ. *Neurosci Res*, 48(3): 249-57.

234. Chen D, Minger SL, Honer WG *et al*. (1999). Organization of the secretory machinery in the rodent brain: distribution of the t-SNAREs, SNAP-25 and SNAP-23. *Brain Res*, 831(1-2): 11-24.

235. Teng FY, Wang Y and Tang BL. (2001). The syntaxins. *Genome Biol*, 2(11): reviews3012.1-.7.

236. Smirnova T, Miniou P, Viegas-Pequignot E *et al*. (1996). Assignment of the human syntaxin 1B gene (STX) to chromosome 16p11.2 by fluorescence in situ hybridization. *Genomics*, 36(3): 551-3.

237. Ruiz-Montasell B, Aguado F, Majo G *et al*. (1996). Differential distribution of syntaxin isoforms 1A and 1B in the rat central nervous system. *Eur J Neurosci*, 8(12): 2544-52.

238. Aguado F, Majo G, Ruiz-Montasell B *et al*. (1999). Syntaxin 1A and 1B display distinct distribution patterns in the rat peripheral nervous system. *Neuroscience*, 88(2): 437-46.

239. Baltazar G, Carvalho AP and Duarte EP. (2003). Differential expression of syntaxin 1A and 1B by noradrenergic and adrenergic chromaffin cells. *Neurochem Res*, 28(10): 1453-7.

240. Aguado F, Majo G, Ruiz-Montasell B *et al*. (1996). Expression of synaptosomal-associated protein SNAP-25 in endocrine anterior pituitary cells. *Eur J Cell Biol*, 69(4): 351-9.

241. Perez-Branguli F, Ruiz-Montasell B and Blasi J. (1999). Differential interaction patterns in binding assays between recombinant syntaxin 1 and synaptobrevin isoforms. *FEBS Lett*, 458(1): 60-4.

242. Fujiwara T, Mishima T, Kofuji T *et al*. (2006). Analysis of knock-out mice to determine the role of HPC-1/syntaxin 1A in expressing synaptic plasticity. *J Neurosci*, 26(21): 5767-76.

243. Bhardwaj SK, Cassidy CM and Srivastava LK. (2006). Changes in syntaxin-1B mRNA in the nucleus accumbens of amphetamine-sensitized rats. *Int J Neuropsychopharmacol*, 9(6): 751-9.

244. Hicks A, Davis S, Rodger J *et al*. (1997). Synapsin I and syntaxin 1B: key elements in the control of neurotransmitter release are regulated by neuronal activation and long-term potentiation in vivo. *Neuroscience*, 79(2): 329-40.

245. Davis S, Rodger J, Hicks A *et al*. (1996). Brain structure and task-specific increase in expression of the gene encoding syntaxin 1B during learning in the rat: a potential molecular marker for learning-induced synaptic plasticity in neural networks. *Eur J Neurosci*, 8(10): 2068-74.

246. Davis S, Rodger J, Stephan A *et al*. (1998). Increase in syntaxin 1B mRNA in hippocampal and cortical circuits during spatial learning reflects a mechanism of trans-synaptic plasticity involved in establishing a memory trace. *Learn Mem*, 5(4-5): 375-90.

247. Bark IC and Wilson MC. (1994). Human cDNA clones encoding two different isoforms of the nerve terminal protein SNAP-25. *Gene*, 139(2): 291-2.

248. Bark IC. (1993). Structure of the chicken gene for SNAP-25 reveals duplicated exon encoding distinct isoforms of the protein. *J Mol Biol*, 233(1): 67-76.

249. Nagy G, Milosevic I, Fasshauer D *et al*. (2005). Alternative splicing of SNAP-25 regulates secretion through nonconservative substitutions in the SNARE domain. *Mol Biol Cell*, 16(12): 5675-85.

250. Bark IC, Hahn KM, Ryabinin AE *et al*. (1995). Differential expression of SNAP-25 protein isoforms during divergent vesicle fusion events of neural development. *Proc Natl Acad Sci U S A*, 92(5): 1510-4.

251. Boschert U, O'Shaughnessy C, Dickinson R *et al*. (1996). Developmental and plasticity-related differential expression of two SNAP-25 isoforms in the rat brain. *J Comp Neurol*, 367(2): 177-93.

252. Johansson JU, Ericsson J, Janson J *et al*. (2008). An ancient duplication of exon 5 in the Snap25 gene is required for complex neuronal development/function. *PLoS Genet*, 4(11): e1000278.

253. Oliver PL and Davies KE. (2009). Interaction between environmental and genetic factors modulates schizophrenic endophenotypes in the Snap-25 mouse mutant blind-drunk. *Hum Mol Genet*, 18(23): 4576-89.

254. Hepp R, Dupont JL, Aunis D *et al*. (2001). NGF enhances depolarization effects on SNAP-25 expression: induction of SNAP-25b isoform. *Neuroreport*, 12(4): 673-7.

255. Andersson J, Fried G, Lilja L *et al*. (2000). Differential sorting of SNAP-25a and SNAP-25b proteins in neuroblastoma cells. *Eur J Cell Biol*, 79(11): 781-9.

256. Johnson RD, Oliver PL and Davies KE. (2008). SNARE proteins and schizophrenia: linking synaptic and neurodevelopmental hypotheses. *Acta Biochim Pol*, 55(4): 619-28.

257. Thompson PM, Sower AC and Perrone-Bizzozero NI. (1998). Altered levels of the synaptosomal associated protein SNAP-25 in schizophrenia. *Biol Psychiatry*, 43(4): 239-43.

258. Scarr E, Gray L, Keriakous D *et al*. (2006). Increased levels of SNAP-25 and synaptophysin in the dorsolateral prefrontal cortex in bipolar I disorder. *Bipolar Disord*, 8(2): 133-43.

259. Halim ND, Weickert CS, McClintock BW *et al*. (2003). Presynaptic proteins in the prefrontal cortex of patients with schizophrenia and rats with abnormal prefrontal development. *Mol Psychiatry*, 8(9): 797-810.

260. Gabriel SM, Haroutunian V, Powchik P *et al*. (1997). Increased concentrations of presynaptic proteins in the cingulate cortex of subjects with schizophrenia. *Arch Gen Psychiatry*, 54(6): 559-66.

261. Taurines R, Dudley E, Grassl J *et al*. (2010). Proteomic research in psychiatry. *J Psychopharmacol*. doi: 10.1177/0269881109106931.

262. English JA, Pennington K, Dunn MJ *et al*. (2010). The Neuroproteomics of Schizophrenia. *Biol Psychiatry*.

263. Marcotte ER, Pearson DM and Srivastava LK. (2001). Animal models of schizophrenia: a critical review. *J Psychiatry Neurosci*, 26(5): 395-410.

264. Honer WG, Falkai P, Bayer TA *et al*. (2002). Abnormalities of SNARE mechanism proteins in anterior frontal cortex in severe mental illness. *Cereb Cortex*, 12(4): 349-56.

265. Lema Tome CM, Nottingham CU, Smith CM *et al*. (2006). Neonatal exposure to MK801 induces structural reorganization of the central nervous system. *Neuroreport*, 17(8): 779-83.

266. Jacobsson G, Piehl F, Bark IC *et al*. (1996). Differential subcellular localization of SNAP-25a and SNAP-25b RNA transcripts in spinal motoneurons and plasticity in expression after nerve injury. *Brain Res Mol Brain Res*, 37(1-2): 49-62.

267. Tang J, LeGros RP, Louneva N *et al*. (2009). Dysbindin-1 in dorsolateral prefrontal cortex of schizophrenia cases is reduced in an isoform-specific manner unrelated to dysbindin-1 mRNA expression. *Hum Mol Genet*, 18(20): 3851-63.

268. Li W, Zhang Q, Oiso N *et al*. (2003). Hermansky-Pudlak syndrome type 7 (HPS-7) results from mutant dysbindin, a member of the biogenesis of lysosome-related organelles complex 1 (BLOC-1). *Nat Genet*, 35(1): 84-9.

269. Chen XW, Feng YQ, Hao CJ *et al*. (2008). DTNBP1, a schizophrenia susceptibility gene, affects kinetics of transmitter release. *J Cell Biol*, 181(5): 791-801.

270. Ghiani CA, Starcevic M, Rodriguez-Fernandez IA *et al*. (2010). The dysbindin-containing complex (BLOC-1) in brain: developmental regulation, interaction with SNARE proteins and role in neurite outgrowth. *Mol Psychiatry*, 15(2): 115, 204-15.

271. Sokolov BP, Tcherepanov AA, Haroutunian V *et al*. (2000). Levels of mRNAs encoding synaptic vesicle and synaptic plasma membrane proteins in the temporal cortex of elderly schizophrenic patients. *Biol Psychiatry*, 48(3): 184-96.

272. Karson CN, Mrak RE, Schluterman KO *et al*. (1999). Alterations in synaptic proteins and their encoding mRNAs in prefrontal cortex in schizophrenia: a possible neurochemical basis for 'hypofrontality'. *Mol Psychiatry*, 4(1): 39-45.

273. Bowden NA, Scott RJ and Tooney PA. (2008). Altered gene expression in the superior temporal gyrus in schizophrenia. *BMC Genomics*, 9: 199.

274. Weidenhofer J, Bowden NA, Scott RJ *et al*. (2006). Altered gene expression in the amygdala in schizophrenia: up-regulation of genes located in the cytomatrix active zone. *Mol Cell Neurosci*, 31(2): 243-50.

275. Katsel P, Davis KL, Gorman JM *et al*. (2005). Variations in differential gene expression patterns across multiple brain regions in schizophrenia. *Schizophr Res*, 77(2-3): 241-52.

276. Iwamoto K and Kato T. (2006). Gene expression profiling in schizophrenia and related mental disorders. *Neuroscientist*, 12(4): 349-61.

277. Kawashima K, Kishi T, Ikeda M *et al*. (2008). No association between tagging SNPs of SNARE complex genes (STX1A, VAMP2 and SNAP25) and schizophrenia in a Japanese population. *Am J Med Genet B Neuropsychiatr Genet*, 147B(7): 1327-31.

278. Wong AH, Macciardi F, Klempan T *et al*. (2003). Identification of candidate genes for psychosis in rat models, and possible association between schizophrenia and the 14-3-3eta gene. *Mol Psychiatry*, 8(2): 156-66.

279. Tachikawa H, Harada S, Kawanishi Y *et al*. (2001). Polymorphism of the 5'-upstream region of the human SNAP-25 gene: an association analysis with schizophrenia. *Neuropsychobiology*, 43(3): 131-3.

280. Corradini I, Verderio C, Sala M *et al*. (2009). SNAP-25 in neuropsychiatric disorders. *Ann N Y Acad Sci*, 1152: 93-9.

281. Muller DJ, Klempan TA, De Luca V *et al*. (2005). The SNAP-25 gene may be associated with clinical response and weight gain in antipsychotic treatment of schizophrenia. *Neurosci Lett*, 379(2): 81-9.

282. Musil R, Spellmann I, Riedel M *et al*. (2008). SNAP-25 gene polymorphisms and weight gain in schizophrenic patients. *J Psychiatr Res*, 42(12): 963-70.

283. Gosso MF, de Geus EJ, Polderman TJ *et al*. (2008). Common variants underlying cognitive ability: further evidence for association between the SNAP-25 gene and cognition using a family-based study in two independent Dutch cohorts. *Genes Brain Behav*, 7(3): 355-64.

284. Golimbet VE, Alfimova MV, Gritsenko IK *et al*. (2010). Association between a synaptosomal protein (SNAP-25) gene polymorphism and verbal memory and attention in patients with endogenous psychoses and mentally healthy subjects. *Neurosci Behav Physiol*, 40(4): 461-5.

285. Spellmann I, Muller N, Musil R *et al*. (2008). Associations of SNAP-25 polymorphisms with cognitive dysfunctions in Caucasian patients with schizophrenia during a brief trail of treatment with atypical antipsychotics. *Eur Arch Psychiatry Clin Neurosci*, 258(18347838): 335-44.

286. Lewis CM, Levinson DF, Wise LH *et al*. (2003). Genome scan meta-analysis of schizophrenia and bipolar disorder, part II: Schizophrenia. *Am J Hum Genet*, 73(1): 34-48.

287. Ng MY, Levinson DF, Faraone SV *et al*. (2009). Meta-analysis of 32 genome-wide linkage studies of schizophrenia. *Mol Psychiatry*, 14(8): 774-85.

288. McCarthy SE, Makarov V, Kirov G *et al*. (2009). Microduplications of 16p11.2 are associated with schizophrenia. *Nat Genet*, 41(11): 1223-7.

289. Tam GW, van de Lagemaat LN, Redon R *et al*. (2010). Confirmed rare copy number variants implicate novel genes in schizophrenia. *Biochem Soc Trans*, 38(2): 445-51.

290. Pober BR. (2010). Williams-Beuren syndrome. *N Engl J Med*, 362(3): 239-52.

291. Mobbs D, Eckert MA, Mills D *et al*. (2007). Frontostriatal dysfunction during response inhibition in Williams syndrome. *Biol Psychiatry*, 62(3): 256-61.

292. Gao M, Bellugi U, Dai L *et al*. (2010). Intelligence in Williams Syndrome is Related to STX1A Which Encodes a Component of the Presynaptic SNARE Complex. *PLoS One*, 5(4): e10292.

293. Castillo MA, Ghose S, Tamminga CA *et al*. (2010). Deficits in syntaxin 1 phosphorylation in schizophrenia prefrontal cortex. *Biol Psychiatry*, 67(3): 208-16.

294. Harrison PJ and Eastwood SL. (1998). Preferential involvement of excitatory neurons in medial temporal lobe in schizophrenia. *Lancet*, 352(9141): 1669-73.

295. Eastwood SL and Harrison PJ. (2000). Hippocampal synaptic pathology in schizophrenia, bipolar disorder and major depression: a study of complexin mRNAs. *Mol Psychiatry*, 5(4): 425-32.

296. Sawada K, Barr AM, Nakamura M *et al*. (2005). Hippocampal complexin proteins and cognitive dysfunction in schizophrenia. *Arch Gen Psychiatry*, 62(3): 263-72.

297. Sawada K, Young CE, Barr AM *et al*. (2002). Altered immunoreactivity of complexin protein in prefrontal cortex in severe mental illness. *Mol Psychiatry*, 7(5): 484-92.

298. Eastwood SL and Harrison PJ. (2001). Synaptic pathology in the anterior cingulate cortex in schizophrenia and mood disorders. A review and a Western blot study of synaptophysin, GAP-43 and the complexins. *Brain Res Bull*, 55(5): 569-78.

299. Eastwood SL, Cotter D and Harrison PJ. (2001). Cerebellar synaptic protein expression in schizophrenia. *Neuroscience*, 105(1): 219-29.

300. Sudhof TC and Rothman JE. (2009). Membrane fusion: grappling with SNARE and SM proteins. *Science*, 323(5913): 474-7.

301. Behan AT, Byrne C, Dunn MJ *et al*. (2009). Proteomic analysis of membrane microdomain-associated proteins in the dorsolateral prefrontal cortex in schizophrenia and bipolar disorder reveals alterations in LAMP, STXBP1 and BASP1 protein expression. *Mol Psychiatry*, 14(6): 601-13.

302. Williams NM, O'Donovan MC and Owen MJ. (2006). Chromosome 22 deletion syndrome and schizophrenia. *Int Rev Neurobiol*, 73: 1-27.

303. Beites CL, Xie H, Bowser R *et al*. (1999). The septin CDCrel-1 binds syntaxin and inhibits exocytosis. *Nat Neurosci*, 2(5): 434-9.

304. Suzuki G, Harper KM, Hiramoto T *et al*. (2009). Sept5 deficiency exerts pleiotropic influence on affective behaviors and cognitive functions in mice. *Hum Mol Genet*, 18(9): 1652-60.

305. Pennington K, Beasley CL, Dicker P *et al*. (2008). Prominent synaptic and metabolic abnormalities revealed by proteomic analysis of the dorsolateral prefrontal cortex in schizophrenia and bipolar disorder. *Mol Psychiatry*, 13(12): 1102-17.

306. Inoue S, Imamura A, Okazaki Y *et al*. (2007). Synaptotagmin XI as a candidate gene for susceptibility to schizophrenia. *Am J Med Genet B Neuropsychiatr Genet*, 144B(3): 332-40.

307. Becher A, Drenckhahn A, Pahner I *et al*. (1999). The synaptophysin-synaptobrevin complex: a hallmark of synaptic vesicle maturation. *J Neurosci*, 19(6): 1922-31.

308. Yelamanchili SV, Reisinger C, Becher A *et al*. (2005). The C-terminal transmembrane region of synaptobrevin binds synaptophysin from adult synaptic vesicles. *Eur J Cell Biol*, 84(4): 467-75.

309. Barr AM, Young CE, Phillips AG *et al*. (2006). Selective effects of typical antipsychotic drugs on SNAP-25 and synaptophysin in the hippocampal trisynaptic pathway. *Int J Neuropsychopharmacol*, 9(4): 457-63.

310. MacDonald ML, Eaton ME, Dudman JT *et al*. (2005). Antipsychotic drugs elevate mRNA levels of presynaptic proteins in the frontal cortex of the rat. *Biol Psychiatry*, 57(9): 1041-51.

311. Nakahara T, Nakamura K, Tsutsumi T *et al*. (1998). Effect of chronic haloperidol treatment on synaptic protein mRNAs in the rat brain. *Brain research.Molecular brain research*, 61(1-2): 238-42.

312. Roberts RC, Roche JK and Conley RR. (2005). Synaptic differences in the postmortem striatum of subjects with schizophrenia: a stereological ultrastructural analysis. *Synapse*, 56(4): 185-97.

313. Jung CH, Yang YS, Kim JS *et al*. (2009). Inhibition of SNARE-driven neuroexocytosis by plant extracts. *Biotechnol Lett*, 31(3): 361-9.

314. Riley LG, Roufogalis BD, Li GQ *et al*. (2006). A radioassay for synaptic core complex assembly: screening of herbal extracts for effectors. *Anal Biochem*, 357(1): 50-7.

315. Rickman C and Davletov B. (2005). Arachidonic acid allows SNARE complex formation in the presence of Munc18. *Chem Biol*, 12(5): 545-53.

316. Lewis DA. (2002). The human brain revisited: opportunities and challenges in postmortem studies of psychiatric disorders. *Neuropsychopharmacology*, 26(2): 143-54.

317. Chen J, Lipska BK and Weinberger DR. (2006). Genetic mouse models of schizophrenia: from hypothesis-based to susceptibility gene-based models. *Biol Psychiatry*, 59(12): 1180-8.

318. Harrison PJ, Heath PR, Eastwood SL *et al*. (1995). The relative importance of premortem acidosis and postmortem interval for human brain gene expression studies: selective mRNA vulnerability and comparison with their encoded proteins. *Neurosci Lett*, 200(3): 151-4.

319. Tian Q, Stepaniants SB, Mao M *et al*. (2004). Integrated genomic and proteomic analyses of gene expression in Mammalian cells. *Mol Cell Proteomics*, 3(10): 960-9.

320. Chen G, Gharib TG, Huang CC *et al*. (2002). Discordant protein and mRNA expression in lung adenocarcinomas. *Mol Cell Proteomics*, 1(4): 304-13.

321. Gygi SP, Rochon Y, Franza BR *et al*. (1999). Correlation between protein and mRNA abundance in yeast. *Mol Cell Biol*, 19(3): 1720-30.

322. Deep-Soboslay A, Akil M, Martin CE *et al*. (2005). Reliability of psychiatric diagnosis in postmortem research. *Biol Psychiatry*, 57(1): 96-101.

323. Keilp JG, Waniek C, Goldman RG *et al*. (1995). Reliability of post-mortem chart diagnoses of schizophrenia and dementia. *Schizophr Res*, 17(2): 221-8.

324. Kelly TM and Mann JJ. (1996). Validity of DSM-III-R diagnosis by psychological autopsy: a comparison with clinician ante-mortem diagnosis. *Acta Psychiatr Scand*, 94(5): 337-43.

325. Deep-Soboslay A, Iglesias B, Hyde TM *et al*. (2008). Evaluation of tissue collection for postmortem studies of bipolar disorder. *Bipolar Disord*, 10(7): 822-8.

326. Roberts SB, Hill CA, Dean B *et al*. (1998). Confirmation of the diagnosis of schizophrenia after death using DSM-IV: a Victorian experience. *Aust N Z J Psychiatry*, 32(1): 73-6.

327. Hill C, Keks N, Roberts S *et al*. (1996). Problem of diagnosis in postmortem brain studies of schizophrenia. *Am J Psychiatry*, 153(4): 533-7.

328. Wilkinson G. (1986). Political dissent and "sluggish" schizophrenia in the Soviet Union. *Br Med J (Clin Res Ed)*, 293(6548): 641-2.

329. Torrey EF, Webster M, Knable M *et al*. (2000). The stanley foundation brain collection and neuropathology consortium. *Schizophr Res*, 44(2): 151-5.

330. Campbell CD, Ogburn EL, Lunetta KL *et al*. (2005). Demonstrating stratification in a European American population. *Nat Genet*, 37(8): 868-72.

331. Li JZ, Meng F, Tsavaler L *et al*. (2007). Sample matching by inferred agonal stress in gene expression analyses of the brain. *BMC Genomics*, 8: 336.

332. Hynd MR, Lewohl JM, Scott HL *et al*. (2003). Biochemical and molecular studies using human autopsy brain tissue. *J Neurochem*, 85(3): 543-62.

333. Benes FM. (1988). Post-mortem structural analyses of schizophrenic brain: study designs and the interpretation of data. *Psychiatr Dev*, 6(3): 213-26.

334. Mistry M and Pavlidis P. (2010). A cross-laboratory comparison of expression profiling data from normal human postmortem brain. *Neuroscience*, 167(2): 384-95.

335. Lipska BK, Deep-Soboslay A, Weickert CS *et al*. (2006). Critical factors in gene expression in postmortem human brain: Focus on studies in schizophrenia. *Biol Psychiatry*, 60(6): 650-8.

336. Siew LK, Love S, Dawbarn D *et al*. (2004). Measurement of pre- and post-synaptic proteins in cerebral cortex: effects of post-mortem delay. *J Neurosci Methods*, 139(2): 153-9.

337. Li JZ, Vawter MP, Walsh DM *et al*. (2004). Systematic changes in gene expression in postmortem human brains associated with tissue pH and terminal medical conditions. *Hum Mol Genet*, 13(6): 609-16.

338. Mexal S, Berger R, Adams CE *et al*. (2006). Brain pH has a significant impact on human postmortem hippocampal gene expression profiles. *Brain Res*, 1106(1): 1-11.

339. Tabachnick BG and Fidell LS. (2007). *Using Multivariate Statistics, 5th Ed*. New York: Pearson Education Inc.

340. Burnet PW, Eastwood SL, Bristow GC *et al*. (2008). D-amino acid oxidase activity and expression are increased in schizophrenia. *Mol Psychiatry*, 13(7): 658-60.

341. Narayan S, Head SR, Gilmartin TJ *et al*. (2009). Evidence for disruption of sphingolipid metabolism in schizophrenia. *J Neurosci Res*, 87(1): 278-88.

342. Beasley CL, Honer WG, Bergmann K *et al*. (2005). Reductions in cholesterol and synaptic markers in association cortex in mood disorders. *Bipolar Disord*, 7(5): 449-55.

343. Fatemi SH, Earle JA, Stary JM *et al*. (2001). Altered levels of the synaptosomal associated protein SNAP-25 in hippocampus of subjects with mood disorders and schizophrenia. *Neuroreport*, 12(15): 3257-62.

344. Clark D, Dedova I, Cordwell S *et al*. (2006). A proteome analysis of the anterior cingulate cortex gray matter in schizophrenia. *Mol Psychiatry*, 11(5): 459-70, 23.

345. Mukaetova-Ladinska EB, Hurt J, Honer WG *et al*. (2002). Loss of synaptic but not cytoskeletal proteins in the cerebellum of chronic schizophrenics. *Neurosci Lett*, 317(3): 161-5.

346. Harrison PJ. (1999). The neuropathological effects of antipsychotic drugs. *Schizophr Res*, 40(2): 87-99.

347. Kapur S, VanderSpek SC, Brownlee BA *et al*. (2003). Antipsychotic dosing in preclinical models is often unrepresentative of the clinical condition: a suggested solution based on in vivo occupancy. *J Pharmacol Exp Ther*, 305(2): 625-31.

348. Lehrmann E, Afanador ZR, Deep-Soboslay A *et al*. (2008). Postmortem diagnosis and toxicological validation of illicit substance use. *Addict Biol*, 13(1): 105-17.

349. Kornhuber J, Schultz A, Wiltfang J *et al*. (1999). Persistence of haloperidol in human brain tissue. *Am J Psychiatry*, 156(6): 885-90.

350. Andreasen NC, Pressler M, Nopoulos P *et al*. (2010). Antipsychotic dose equivalents and dose-years: a standardized method for comparing exposure to different drugs. *Biol Psychiatry*, 67(3): 255-62.

351. Lybrand J and Caroff S. (2009). Management of schizophrenia with substance use disorders. *Psychiatr Clin North Am*, 32(4): 821-33.

352. Koskinen J, Lohonen J, Koponen H *et al*. (2009). Rate of Cannabis Use Disorders in Clinical Samples of Patients With Schizophrenia: A Meta-analysis. *Schizophr Bull*. doi: 10.1093/schbul/sbp031

353. Mobascher A and Winterer G. (2008). The molecular and cellular neurobiology of nicotine abuse in schizophrenia. *Pharmacopsychiatry*, 41 Suppl 1: S51-9.

354. Even N, Cardona A, Soudant M *et al*. (2008). Regional differential effects of chronic nicotine on brain alpha 4-containing and alpha 6-containing receptors. *Neuroreport*, 19(15): 1545-50.

355. Barkus E and Murray RM. (2010). Substance use in adolescence and psychosis: clarifying the relationship. *Annu Rev Clin Psychol*, 6: 365-89.

356. Dean B, Sundram S, Bradbury R *et al*. (2001). Studies on [3H]CP-55940 binding in the human central nervous system: regional specific changes in density of cannabinoid-1 receptors associated with schizophrenia and cannabis use. *Neuroscience*, 103(1): 9-15.

357. Mathalon DH, Pfefferbaum A, Lim KO *et al*. (2003). Compounded brain volume deficits in schizophrenia-alcoholism comorbidity. *Arch Gen Psychiatry*, 60(3): 245-52.

358. Stryer L. (1995). *Biochemistry*. New York: New York : W.H. Freeman.

359. Makrides SC, Gasbarro C and Bello JM. (2005). Bioconjugation of quantum dot luminescent probes for Western blot analysis. *Biotechniques*, 39(4): 501-6.

360. Gottschall PE, Ajmo JM, Eakin AK *et al*. (2010). Panel of synaptic protein ELISAs for evaluating neurological phenotype. *Exp Brain Res*, 201(4): 885-93.

361. Kemeny DM and Challacombe SJ. (ed.^eds.). (1998). *ELISA and Other Solid Phase Immunoassays*. Chichester: John Wiley & Sons.

362. Kaboord B and Perr M. (2008). Isolation of proteins and protein complexes by immunoprecipitation. *Methods Mol Biol*, 424: 349-64.

363. Gygi SP and Aebersold R. (2000). Mass spectrometry and proteomics. *Curr Opin Chem Biol*, 4(5): 489-94.

364. Yocum AK and Chinnaiyan AM. (2009). Current affairs in quantitative targeted proteomics: multiple reaction monitoring-mass spectrometry. *Brief Funct Genomic Proteomic*, 8(2): 145-57.

365. Proteomics AJ. Webinar. (2009). *Targeted Proteomics: Revolutionizing protein analysis in cellular pathways and biomarker research*. November 17

366. Kirkpatrick DS, Gerber SA and Gygi SP. (2005). The absolute quantification strategy: a general procedure for the quantification of proteins and post-translational modifications. *Methods*, 35(3): 265-73.

367. Han B and Higgs R. (2008). Proteomics: from hypothesis to quantitative assay on a single platform. Guidelines for developing MRM assays using ion trap mass spectrometers. *Brief Funct Genomic Proteomic*, 7(5): 340-54.

368. Speicher K, Kolbas O, Harper S *et al*. (2000). Systematic analysis of peptide recoveries from in-gel digestions for protein identifications in proteome studies. *J Biomol Tech*, 11(2): 74-86.

369. Bark C, Bellinger FP, Kaushal A *et al*. (2004). Developmentally regulated switch in alternatively spliced SNAP-25 isoforms alters facilitation of synaptic transmission. *J Neurosci*, 24(40): 8796-805.

370. Hess EJ, Jinnah HA, Kozak CA *et al*. (1992). Spontaneous locomotor hyperactivity in a mouse mutant with a deletion including the Snap gene on chromosome 2. *J Neurosci*, 12(7): 2865-74.

371. Jones MD, Williams ME and Hess EJ. (2001). Abnormal presynaptic catecholamine regulation in a hyperactive SNAP-25-deficient mouse mutant. *Pharmacol Biochem Behav*, 68(4): 669-76.

372. Molnar Z, Lopez-Bendito G, Small J *et al*. (2002). Normal development of embryonic thalamocortical connectivity in the absence of evoked synaptic activity. *J Neurosci*, 22(23): 10313-23.

373. Bronk P, Deak F, Wilson MC *et al*. (2007). Differential effects of SNAP-25 deletion on Ca2+ -dependent and Ca2+ -independent neurotransmission. *J Neurophysiol*, 98(2): 794-806.

374. Delgado-Martinez I, Nehring RB and Sorensen JB. (2007). Differential abilities of SNAP-25 homologs to support neuronal function. *J Neurosci*, 27(35): 9380-91.

375. Hess EJ, Collins KA and Wilson MC. (1996). Mouse model of hyperkinesis implicates SNAP-25 in behavioral regulation. *J Neurosci*, 16(9): 3104-11.

376. Jones MD and Hess EJ. (2003). Norepinephrine regulates locomotor hyperactivity in the mouse mutant coloboma. *Pharmacol Biochem Behav*, 75(1): 209-16.

377. Heyser CJ, Wilson MC and Gold LH. (1995). Coloboma hyperactive mutant exhibits delayed neurobehavioral developmental milestones. *Brain Res Dev Brain Res*, 89(2): 264-9.

378. Raber J, Mehta PP, Kreifeldt M *et al*. (1997). Coloboma hyperactive mutant mice exhibit regional and transmitter-specific deficits in neurotransmission. *J Neurochem*, 68(1): 176-86.

379. Jones MD, Williams ME and Hess EJ. (2001). Expression of catecholaminergic mRNAs in the hyperactive mouse mutant coloboma. *Brain research.Molecular brain research*, 96(1-2): 114-21.

380. Steffensen SC, Henriksen SJ and Wilson MC. (1999). Transgenic rescue of SNAP-25 restores dopamine-modulated synaptic transmission in the coloboma mutant. *Brain Res*, 847(2): 186-95.

381. Zhang Y, Vilaythong AP, Yoshor D *et al*. (2004). Elevated thalamic low-voltage-activated currents precede the onset of absence epilepsy in the SNAP25-deficient mouse mutant coloboma. *J Neurosci*, 24(22): 5239-48.

382. Steffensen SC, Wilson MC and Henriksen SJ. (1996). Coloboma contiguous gene deletion encompassing Snap alters hippocampal plasticity. *Synapse*, 22(3): 281-9.

383. McRory JE, Rehak R, Simms B *et al*. (2008). Syntaxin 1A is required for normal in utero development. *Biochem Biophys Res Commun*, 375(3): 372-7.

384. Fujiwara T, Snada M, Kofuji T *et al*. (2010). HPC-1/syntaxin 1A gene knockout mice show abnormal behavior possibly related to a disruption in 5-HTergic systems. *Eur J Neurosci*.

385. Takasusuki T, Fujiwara T, Yamaguchi S *et al*. (2007). Enhancement of synaptic transmission and nociceptive behaviour in HPC-1/syntaxin 1A knockout mice following peripheral nerve injury. *Eur J Neurosci*, 26(8): 2179-87.

386. Nystuen AM, Schwendinger JK, Sachs AJ *et al*. (2007). A null mutation in VAMP1/synaptobrevin is associated with neurological defects and prewean mortality in the lethal-wasting mouse mutant. *Neurogenetics*, 8(1): 1-10.

387. Deak F, Schoch S, Liu X *et al*. (2004). Synaptobrevin is essential for fast synaptic-vesicle endocytosis. *Nat Cell Biol*, 6(11): 1102-8.

388. Deak F, Shin OH, Kavalali ET *et al*. (2006). Structural determinants of synaptobrevin 2 function in synaptic vesicle fusion. *J Neurosci*, 26(25): 6668-76.

389. Honer WG, Falkai P, Young C *et al*. (1997). Cingulate cortex synaptic terminal proteins and neural cell adhesion molecule in schizophrenia. *Neuroscience*, 78(1): 99-110.

390. Gray LJ, Dean B, Kronsbein HC *et al*. (2010). Region and diagnosis-specific changes in synaptic proteins in schizophrenia and bipolar I disorder. *Psychiatry Res*, 178(2): 374-80.

391. Thompson PM, Egbufoama S and Vawter MP. (2003). SNAP-25 reduction in the hippocampus of patients with schizophrenia. *Prog Neuropsychopharmacol Biol Psychiatry*, 27(3): 411-7.

392. Young CE, Arima K, Xie J *et al*. (1998). SNAP-25 deficit and hippocampal connectivity in schizophrenia. *Cereb Cortex*, 8(3): 261-8.

393. Thompson PM, Rosenberger C and Qualls C. (1999). CSF SNAP-25 in schizophrenia and bipolar illness. A pilot study. *Neuropsychopharmacology*, 21(6): 717-22.

394. Thompson PM, Kelley M, Yao J *et al*. (2003). Elevated cerebrospinal fluid SNAP-25 in schizophrenia. *Biol Psychiatry*, 53(12): 1132-7.

395. Cragg SJ, Hille CJ and Greenfield SA. (2002). Functional domains in dorsal striatum of the nonhuman primate are defined by the dynamic behavior of dopamine. *J Neurosci*, 22(13): 5705-12.

396. Fernandez I, Ubach J, Dulubova I *et al*. (1998). Three-dimensional structure of an evolutionarily conserved N-terminal domain of syntaxin 1A. *Cell*, 94(6): 841-9.

397. Snyder DA, Kelly ML and Woodbury DJ. (2006). SNARE complex regulation by phosphorylation. *Cell Biochem Biophys*, 45(1): 111-23.

398. Archer BT, 3rd, Ozcelik T, Jahn R *et al*. (1990). Structures and chromosomal localizations of two human genes encoding synaptobrevins 1 and 2. *J Biol Chem*, 265(28): 17267-73.

399. Isenmann S, Khew-Goodall Y, Gamble J *et al*. (1998). A splice-isoform of vesicle-associated membrane protein-1 (VAMP-1) contains a mitochondrial targeting signal. *Mol Biol Cell*, 9(7): 1649-60.

400. Friedland DR, Eernisse R and Popper P. (2008). Identification of a novel Vamp1 splice variant in the cochlear nucleus. *Hear Res*, 243(1-2): 105-12.

401. Lisman JE, Coyle JT, Green RW *et al*. (2008). Circuit-based framework for understanding neurotransmitter and risk gene interactions in schizophrenia. *Trends Neurosci*, 31(5): 234-42.

402. Manoach DS, Gollub RL, Benson ES *et al*. (2000). Schizophrenic subjects show aberrant fMRI activation of dorsolateral prefrontal cortex and basal ganglia during working memory performance. *Biol Psychiatry*, 48(2): 99-109.

403. McCullumsmith RE and Meador-Woodruff JH. (2002). Striatal excitatory amino acid transporter transcript expression in schizophrenia, bipolar disorder, and major depressive disorder. *Neuropsychopharmacology*, 26(3): 368-75.

404. Lyon G.J A. (2009). Presynaptic regulation of dopamine transmission in schizophrenia. *Schizophr Bull*: 1-10.

405. Scherk H, Backens M, Zill P *et al*. (2008). SNAP-25 genotype influences NAA/Cho in left hippocampus. *J Neural Transm*, 115(11): 1513-8.

406. Fan HP, Fan FJ, Bao L *et al*. (2006). SNAP-25/syntaxin 1A complex functionally modulates neurotransmitter gamma-aminobutyric acid reuptake. *J Biol Chem*, 281(38): 28174-84.

407. Fisher H and Braun JE. (2000). Modulation of the SNARE core complex by dopamine. *Can J Physiol Pharmacol*, 78(10): 856-9.

408. Mai JK, Assheuer J and Paxinos G. (1997). *Atlas of the Human Brain*. San Diego, CA: Academic Press.

409. Albaugh VL, Henry CR, Bello NT *et al*. (2006). Hormonal and metabolic effects of olanzapine and clozapine related to body weight in rodents. *Obesity (Silver Spring)*, 14(1): 36-51.

410. Paxinos G and Watson C. (1986). *The rat brain in stereotaxic coordinates*. Sydney; Orlando: Academic Press.

411. Meyer DK and Protopapas Z. (1985). Studies on cholecystokinin-containing neuronal pathways in rat cerebral cortex and striatum. *Ann N Y Acad Sci*, 448: 133-43.

412. Lauer M, Johannes S, Fritzen S *et al*. (2005). Morphological abnormalities in nitric-oxide-synthase-positive striatal interneurons of schizophrenic patients. *Neuropsychobiology*, 52(3): 111-7.

413. Whalley HC, Simonotto E, Marshall I *et al*. (2005). Functional disconnectivity in subjects at high genetic risk of schizophrenia. *Brain*, 128(Pt 9): 2097-108.

414. Fan X and Hess EJ. (2007). D2-like dopamine receptors mediate the response to amphetamine in a mouse model of ADHD. *Neurobiol Dis*, 26(1): 201-11.

415. West AR, Floresco SB, Charara A *et al*. (2003). Electrophysiological interactions between striatal glutamatergic and dopaminergic systems. *Ann N Y Acad Sci*, 1003: 53-74.

416. Benes FM, Paskevich PA, Davidson J *et al*. (1985). The effects of haloperidol on synaptic patterns in the rat striatum. *Brain Res*, 329(1-2): 265-73.

417. Kerns JM, Sierens DK, Kao LC *et al*. (1992). Synaptic plasticity in the rat striatum following chronic haloperidol treatment. *Clin Neuropharmacol*, 15(6): 488-500.

418. Uranova NA, Orlovskaya DD, Apel K *et al*. (1991). Morphometric study of synaptic patterns in the rat caudate nucleus and hippocampus under haloperidol treatment. *Synapse*, 7(4): 253-9.

419. Roberts RC, Gaither LA, Gao XM *et al*. (1995). Ultrastructural correlates of haloperidol-induced oral dyskinesias in rat striatum. *Synapse*, 20(3): 234-43.

420. Chana G, Lucero G, Salaria S *et al*. (2009). Upregulation of NRG-1 and VAMP-1 in human brain aggregates exposed to clozapine. *Schizophr Res*, 113(2-3): 273-6.

421. Meshul CK, Bunker GL, Mason JN *et al*. (1996). Effects of subchronic clozapine and haloperidol on striatal glutamatergic synapses. *J Neurochem*, 67(5): 1965-73.

422. Lesch KP and Schmitt A. (2002). Antidepressants and gene expression profiling: how to SNARE novel drug targets. *Pharmacogenomics J*, 2(6): 346-8.

423. Bonanno G, Giambelli R, Raiteri L *et al*. (2005). Chronic antidepressants reduce depolarization-evoked glutamate release and protein interactions favoring formation of SNARE complex in hippocampus. *J Neurosci*, 25(13): 3270-9.

424. Blanes-Mira C, Pastor MT, Valera E *et al*. (2003). Identification of SNARE complex modulators that inhibit exocytosis from an alpha-helix-constrained combinatorial library. *Biochem J*, 375(Pt 1): 159-66.

425. Jung CH, Yang YS, Kim JS *et al*. (2008). A search for synthetic peptides that inhibit soluble N-ethylmaleimide sensitive-factor attachment receptor-mediated membrane fusion. *FEBS J*, 275(12): 3051-63.

426. Bahn S, Augood SJ, Ryan M *et al*. (2001). Gene expression profiling in the post-mortem human brain--no cause for dismay. *J Chem Neuroanat*, 22(1-2): 79-94.

427. Kubista H, Edelbauer H and Boehm S. (2004). Evidence for structural and functional diversity among SDS-resistant SNARE complexes in neuroendocrine cells. *J Cell Sci*, 117(Pt 6): 955-66.

428. Xu T and Bajjalieh SM. (2001). SV2 modulates the size of the readily releasable pool of secretory vesicles. *Nat Cell Biol*, 3(8): 691-8.

429. Pellegrini LL, O'Connor V, Lottspeich F *et al*. (1995). Clostridial neurotoxins compromise the stability of a low energy SNARE complex mediating NSF activation of synaptic vesicle fusion. *EMBO J*, 14(19): 4705-13.

430. Tolar LA and Pallanck L. (1998). NSF function in neurotransmitter release involves rearrangement of the SNARE complex downstream of synaptic vesicle docking. *J Neurosci*, 18(24): 10250-6.

431. Craig TJ, Ciufo LF and Morgan A. (2004). A protein-protein binding assay using coated microtitre plates: increased throughput, reproducibility and speed compared to bead-based assays. *J Biochem Biophys Methods*, 60(1): 49-60.

432. Oyler GA, Higgins GA, Hart RA *et al*. (1989). The identification of a novel synaptosomal-associated protein, SNAP-25, differentially expressed by neuronal subpopulations. *J Cell Biol*, 109(6 Pt 1): 3039-52.

433. Tao-Cheng JH, Du J and McBain CJ. (2000). Snap-25 is polarized to axons and abundant along the axolemma: an immunogold study of intact neurons. *J Neurocytol*, 29(1): 67-77.

434. Lawrence GW and Dolly JO. (2002). Multiple forms of SNARE complexes in exocytosis from chromaffin cells: effects of Ca(2+), MgATP and botulinum toxin type A. *J Cell Sci*, 115(Pt 3): 667-73.

435. Nagamatsu S, Fujiwara T, Nakamichi Y *et al*. (1996). Expression and functional role of syntaxin 1/HPC-1 in pancreatic beta cells. Syntaxin 1A, but not 1B, plays a negative role in regulatory insulin release pathway. *J Biol Chem*, 271(2): 1160-5.

436. Lee KH, Kim MY, Kim DH *et al*. (2004). Syntaxin 1A and receptor for activated C kinase interact with the N-terminal region of human dopamine transporter. *Neurochem Res*, 29(7): 1405-9.

437. Binda F, Dipace C, Bowton E *et al*. (2008). Syntaxin 1A interaction with the dopamine transporter promotes amphetamine-induced dopamine efflux. *Mol Pharmacol*, 74(4): 1101-8.

438. Carvelli L, Blakely RD and DeFelice LJ. (2008). Dopamine transporter/syntaxin 1A interactions regulate transporter channel activity and dopaminergic synaptic transmission. *Proc Natl Acad Sci U S A*, 105(37): 14192-7.

439. Sheng ZH, Westenbroek RE and Catterall WA. (1998). Physical link and functional coupling of presynaptic calcium channels and the synaptic vesicle docking/fusion machinery. *J Bioenerg Biomembr*, 30(4): 335-45.

440. Bennett MK, Calakos N, Kreiner T *et al*. (1992). Synaptic vesicle membrane proteins interact to form a multimeric complex. *J Cell Biol*, 116(3): 761-75.

441. Barakauskas VE, Beasley CL, Barr AM *et al*. (2010). A novel mechanism and treatment target for presynaptic abnormalities in specific striatal regions in schizophrenia. *Neuropsychopharmacology*, 35(5): 1226-38.

442. Honer WG, Kaufmann CA and Davies P. (1992). Characterization of a synaptic antigen of interest in neuropsychiatric illness. *Biol Psychiatry*, 31(2): 147-58.

443. Liao Ping-Sung C. (2001). A fast algorithm for multilevel thresholding. *J Inform Sci Eng*, 17(5): 713-27.

444. Otsu N. (1979). A threshold selection method from gray-level histogram. *IEEE Transactions on System Man Cybernetics*, SMC-9(1): 62-6.

445. Bolte S and Cordelieres FP. (2006). A guided tour into subcellular colocalization analysis in light microscopy. *J Microsc*, 224(Pt 3): 213-32.

446. Manders EMM, Verbeek FJ and Aten JA. (1993). Measurement of co-localzation of objects in dual-colour confocal images. *J Microsc*, 169(Pt 3): 375-82.

447. Zinchuk V, Zinchuk O and Okada T. (2007). Quantitative colocalization analysis of multicolor confocal immunofluorescence microscopy images: pushing pixels to explore biological phenomena. *Acta Histochem Cytochem*, 40(4): 101-11.

448. Kung L, Conley R, Chute DJ *et al*. (1998). Synaptic changes in the striatum of schizophrenic cases: a controlled postmortem ultrastructural study. *Synapse*, 28(2): 125-39.

449. Mora F, Segovia G and Del Arco A. (2008). Glutamate-dopamine-GABA interactions in the aging basal ganglia. *Brain Res Rev*, 58(2): 340-53.

450. Calabresi P and Di Filippo M. (2008). ACh/dopamine crosstalk in motor control and reward: a crucial role for alpha 6-containing nicotinic receptors? *Neuron*, 60(1): 4-7.

451. Isola R, Zhang H, Tejwani G *et al*. (2009). Acute nicotine changes dynorphin and prodynorphin mRNA in the striatum. *Psychopharmacology (Berl)*, 201: 507-16.

452. O'Callaghan JP, Imai H, Miller DB *et al*. (1999). Quantitative immunoblots of proteins resolved from brain homogenates: underestimation of specific protein concentration and of treatment effects. *Anal Biochem*, 274(1): 18-26.

453. Tafoya LC, Shuttleworth CW, Yanagawa Y *et al*. (2008). The role of the t-SNARE SNAP-25 in action potential-dependent calcium signaling and expression in GABAergic and glutamatergic neurons. *BMC Neurosci*, 9: 105.

454. Colwill K, Wells CD, Elder K *et al*. (2006). Modification of the Creator recombination system for proteomics applications--improved expression by addition of splice sites. *BMC Biotechnol*, 6: 13.

455. Mead CL, Kuzyk MA, Moradian A *et al*. (2010). Cytosolic Protein Interactions of the Schizophrenia Susceptibility Gene Dysbindin. *J Neurochem*, 113(6): 1491-503.

456. Rappsilber J, Ishihama Y and Mann M. (2003). Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. *Anal Chem*, 75(3): 663-70.

457. Kuzyk MA, Smith D, Yang J *et al*. (2009). Multiple reaction monitoring-based, multiplexed, absolute quantitation of 45 proteins in human plasma. *Mol Cell Proteomics*, 8(8): 1860-77.

458. Siegel BV, Jr., Buchsbaum MS, Bunney WE, Jr. *et al*. (1993). Cortical-striatal-thalamic circuits and brain glucose metabolic activity in 70 unmedicated male schizophrenic patients. *Am J Psychiatry*, 150(9): 1325-36.

459. Vink M, Ramsey NF, Raemaekers M *et al*. (2006). Striatal dysfunction in schizophrenia and unaffected relatives. *Biol Psychiatry*, 60(1): 32-9.

460. Hitzemann R, Demarest K, Koyner J *et al*. (2000). Effect of genetic cross on the detection of quantitative trait loci and a novel approach to mapping QTLs. *Pharmacol Biochem Behav*, 67(4): 767-72.

461. Willott JF, Tanner L, O'Steen J *et al*. (2003). Acoustic startle and prepulse inhibition in 40 inbred strains of mice. *Behav Neurosci*, 117(4): 716-27.

462. Owen MJ, Craddock N and O'Donovan MC. (2005). Schizophrenia: genes at last? *Trends Genet*, 21(9): 518-25.

463. Roth TL, Lubin FD, Sodhi M *et al*. (2009). Epigenetic mechanisms in schizophrenia. *Biochim Biophys Acta*, 1790(9): 869-77.

464. Doetschman T. (2009). Influence of genetic background on genetically engineered mouse phenotypes. *Methods Mol Biol*, 530: 423-33.

465. Otsu Y and Murphy TH. (2003). Miniature transmitter release: accident of nature or careful design? *Sci STKE*, 2003(211): pe54.

466. Schweizer FE, Dresbach T, DeBello WM *et al*. (1998). Regulation of neurotransmitter release kinetics by NSF. *Science*, 279(5354): 1203-6.

467. Brose N. (2008). Altered complexin expression in psychiatric and neurological disorders: cause or consequence? *Mol Cells*, 25(18319609): 7-19.

468. Steward O and Schuman EM. (2003). Compartmentalized synthesis and degradation of proteins in neurons. *Neuron*, 40(2): 347-59.

469. St Johnston D. (2005). Moving messages: the intracellular localization of mRNAs. *Nat Rev Mol Cell Biol*, 6(5): 363-75.

470. Ben-Shachar D and Karry R. (2007). Sp1 expression is disrupted in schizophrenia; a possible mechanism for the abnormal expression of mitochondrial complex I genes, NDUFV1 and NDUFV2. *PLoS One*, 2(9): e817.

471. Cai F, Chen B, Zhou W *et al*. (2008). SP1 regulates a human SNAP-25 gene expression. *J Neurochem*, 105(2): 512-23.

472. Segref A and Hoppe T. (2009). Think locally: control of ubiquitin-dependent protein degradation in neurons. *EMBO Rep*, 10(1): 44-50.

473. Jiang YH and Beaudet AL. (2004). Human disorders of ubiquitination and proteasomal degradation. *Curr Opin Pediatr*, 16(4): 419-26.

474. Chu TT, Liu Y and Kemether E. (2009). Thalamic transcriptome screening in three psychiatric states. *J Hum Genet*, 54(11): 665-75.

475. Bousman CA, Chana G, Glatt SJ *et al*. (2010). Preliminary evidence of ubiquitin proteasome system dysregulation in schizophrenia and bipolar disorder: convergent pathway analysis findings from two independent samples. *Am J Med Genet B Neuropsychiatr Genet*, 153B(2): 494-502.

476. Chin LS, Vavalle JP and Li L. (2002). Staring, a novel E3 ubiquitin-protein ligase that targets syntaxin 1 for degradation. *J Biol Chem*, 277(38): 35071-9.

477. Ma Z, Portwood N, Foss A *et al*. (2005). Evidence that insulin secretion influences SNAP-25 through proteasomal activation. *Biochem Biophys Res Commun*, 329(3): 1118-26.

478. Ding M, Chao D, Wang G *et al*. (2007). Spatial regulation of an E3 ubiquitin ligase directs selective synapse elimination. *Science*, 317(5840): 947-51.

479. Hegde AN. (2010). The ubiquitin-proteasome pathway and synaptic plasticity. *Learn Mem*, 17(7): 314-27.

480. Nakata K, Lipska BK, Hyde TM *et al*. (2009). DISC1 splice variants are upregulated in schizophrenia and associated with risk polymorphisms. *Proc Natl Acad Sci U S A*, 106(37): 15873-8.

481. Zhao C, Xu Z, Wang F *et al*. (2009). Alternative-splicing in the exon-10 region of GABA(A) receptor beta(2) subunit gene: relationships between novel isoforms and psychotic disorders. *PLoS One*, 4(9): e6977.

482. Nagele P, Mendel JB, Placzek WJ *et al*. (2005). Volatile anesthetics bind rat synaptic snare proteins. *Anesthesiology*, 103(4): 768-78.

483. Herring BE, Xie Z, Marks J *et al*. (2009). Isoflurane inhibits the neurotransmitter release machinery. *J Neurophysiol*, 102(2): 1265-73.

484. Di Stasi AM, Mallozzi C, Macchia G *et al*. (2002). Peroxynitrite affects exocytosis and SNARE complex formation and induces tyrosine nitration of synaptic proteins. *J Neurochem*, 82(2): 420-9.

485. Virit O, Altindag A, Yumru M *et al*. (2009). A defect in the antioxidant defense system in schizophrenia. *Neuropsychobiology*, 60(2): 87-93.

486. Dietrich-Muszalska A, Olas B, Glowacki R *et al*. (2009). Oxidative/nitrative modifications of plasma proteins and thiols from patients with schizophrenia. *Neuropsychobiology*, 59(1): 1-7.

487. Do KQ, Cabungcal JH, Frank A *et al*. (2009). Redox dysregulation, neurodevelopment, and schizophrenia. *Curr Opin Neurobiol*, 19(2): 220-30.

488. Djordjevic VV, Stojanovic I, Stankovic-Ferlez D *et al*. (2010). Plasma nitrite/nitrate concentrations in patients with schizophrenia. *Clin Chem Lab Med*, 48(1): 89-94.

489. Helme-Guizon A, Davis S, Israel M *et al*. (1998). Increase in syntaxin 1B and glutamate release in mossy fibre terminals following induction of LTP in the dentate gyrus: a candidate molecular mechanism underlying transsynaptic plasticity. *Eur J Neurosci*, 10(7): 2231-7.

490. Jacobsson G, Bean AJ, Scheller RH *et al*. (1994). Identification of synaptic proteins and their isoform mRNAs in compartments of pancreatic endocrine cells. *Proc Natl Acad Sci U S A*, 91(26): 12487-91.

491. Raptis A, Torrejon-Escribano B, Gomez de Aranda I *et al*. (2005). Distribution of synaptobrevin/VAMP 1 and 2 in rat brain. *J Chem Neuroanat*, 30(4): 201-11.

492. Salin H, Maurin Y, Davis S *et al*. (2002). Spatio-temporal heterogeneity and cell-specificity of long-term potentiation-induced mRNA expression in the dentate gyrus in vivo. *Neuroscience*, 110(2): 227-36.

493. Deken SL, Beckman ML, Boos L *et al*. (2000). Transport rates of GABA transporters: regulation by the N-terminal domain and syntaxin 1A. *Nat Neurosci*, 3(10): 998-1003.

494. Quick MW. (2003). Regulating the conducting states of a mammalian serotonin transporter. *Neuron*, 40(3): 537-49.

495. Dipace C, Sung U, Binda F *et al*. (2007). Amphetamine induces a calcium/calmodulin-dependent protein kinase II-dependent reduction in norepinephrine transporter surface expression linked to changes in syntaxin 1A/transporter complexes. *Mol Pharmacol*, 71(1): 230-9.

496. Binda F, Lute BJ, Dipace C *et al*. (2006). The N-terminus of the norepinephrine transporter regulates the magnitude and selectivity of the transporter-associated leak current. *Neuropharmacology*, 50(3): 354-61.

497. Fletcher S, Bowden SE and Marrion NV. (2003). False interaction of syntaxin 1A with a Ca(2+)-activated K(+) channel revealed by co-immunoprecipitation and pull-down assays: implications for identification of protein-protein interactions. *Neuropharmacology*, 44(6): 817-27.

498. Lam AD, Tryoen-Toth P, Tsai B *et al*. (2008). SNARE-catalyzed fusion events are regulated by Syntaxin1A-lipid interactions. *Mol Biol Cell*, 19(2): 485-97.

499. Lee F, Pei L and Liu F. (2009). Disruption of the dopamine transporter-dopamine D2 receptor interaction in schizophrenia. *Synapse*, 63(8): 710-2.

500. Bora E, Yucel M and Pantelis C. (2010). Cognitive impairment in schizophrenia and affective psychoses: implications for DSM-V criteria and beyond. *Schizophr Bull*, 36(1): 36-42.

501. Morton AJ, Faull RL and Edwardson JM. (2001). Abnormalities in the synaptic vesicle fusion machinery in Huntington's disease. *Brain Res Bull*, 56(2): 111-7.

502. Keshavan MS, Anderson S and Pettegrew JW. (1994). Is schizophrenia due to excessive synaptic pruning in the prefrontal cortex? The Feinberg hypothesis revisited. *J Psychiatr Res*, 28(3): 239-65.

503. Saugstad LF. (2009). Kraepelin's dichotomy is true: contrasting brain dysfunction at the extremes of human growth and maturation. Excitability, the fundamental property of nervous tissue, is affected. *World J Biol Psychiatry*, 10(4 Pt 3): 998-1015.

504. Shimohama S, Fujimoto S, Sumida Y *et al*. (1998). Differential expression of rat brain synaptic proteins in development and aging. *Biochem Biophys Res Commun*, 251(1): 394-8.

505. Oyler GA, Polli JW, Wilson MC *et al*. (1991). Developmental expression of the 25-kDa synaptosomal-associated protein (SNAP-25) in rat brain. *Proc Natl Acad Sci U S A*, 88(12): 5247-51.

506. Spear LP. (2000). The adolescent brain and age-related behavioral manifestations. *Neurosci Biobehav Rev*, 24(4): 417-63.

507. Eastwood SL, Weickert CS, Webster MJ *et al*. (2006). Synaptophysin protein and mRNA expression in the human hippocampal formation from birth to old age. *Hippocampus*, 16(8): 645-54.

508. Reeves S, Bench C and Howard R. (2002). Ageing and the nigrostriatal dopaminergic system. *Int J Geriatr Psychiatry*, 17(4): 359-70.

509. Stark AK and Pakkenberg B. (2004). Histological changes of the dopaminergic nigrostriatal system in aging. *Cell Tissue Res*, 318(1): 81-92.

510. Biranowska J, Dziewiatkowski J, Ludkiewicz B *et al*. (2002). Developmental changes of synaptic proteins expression within the hippocampal formation of the rat. *Anat Embryol (Berl)*, 206(1-2): 85-96.

511. Sesack SR and Snyder CL. (1995). Cellular and subcellular localization of syntaxin-like immunoreactivity in the rat striatum and cortex. *Neuroscience*, 67(4): 993-1007.

512. Law AJ, Lipska BK, Weickert CS *et al*. (2006). Neuregulin 1 transcripts are differentially expressed in schizophrenia and regulated by 5' SNPs associated with the disease. *Proc Natl Acad Sci U S A*, 103(17): 6747-52.

513. Bertolino A, Fazio L, Caforio G *et al*. (2009). Functional variants of the dopamine receptor D2 gene modulate prefronto-striatal phenotypes in schizophrenia. *Brain*, 132(Pt 2): 417-25.

514. Lau CG, Takayasu Y, Rodenas-Ruano A *et al*. (2010). SNAP-25 is a target of protein kinase C phosphorylation critical to NMDA receptor trafficking. *J Neurosci*, 30(1): 242-54.

515. Jope RS and Roh MS. (2006). Glycogen synthase kinase-3 (GSK3) in psychiatric diseases and therapeutic interventions. *Curr Drug Targets*, 7(11): 1421-34.

516. Zhu LQ, Liu D, Hu J *et al*. (2010). GSK-3 beta inhibits presynaptic vesicle exocytosis by phosphorylating P/Q-type calcium channel and interrupting SNARE complex formation. *J Neurosci*, 30(10): 3624-33.

517. Horrobin DF, Glen AI and Vaddadi K. (1994). The membrane hypothesis of schizophrenia. *Schizophr Res*, 13(3): 195-207.

518. Davletov B, Connell E and Darios F. (2007). Regulation of SNARE fusion machinery by fatty acids. *Cell Mol Life Sci*, 64(13): 1597-608.

519. Barr AM, Young CE, Sawada K *et al*. (2004). Abnormalities of presynaptic protein CDCrel-1 in striatum of rats reared in social isolation: relevance to neural connectivity in schizophrenia. *Eur J Neurosci*, 20(1): 303-7.

520. Oud MJ and Meyboom-de Jong B. (2009). Somatic diseases in patients with schizophrenia in general practice: their prevalence and health care. *BMC Fam Pract*, 10: 32.

521. Stahl SM, Mignon L and Meyer JM. (2009). Which comes first: atypical antipsychotic treatment or cardiometabolic risk? *Acta Psychiatr Scand*, 119(3): 171-9.

522. Gonelle-Gispert C, Halban PA, Niemann H *et al*. (1999). SNAP-25a and -25b isoforms are both expressed in insulin-secreting cells and can function in insulin secretion. *Biochem J*, 339(Pt 1): 159-65.

523. Best L, Yates AP and Reynolds GP. (2005). Actions of antipsychotic drugs on pancreatic beta-cell function: contrasting effects of clozapine and haloperidol. *J Psychopharmacol*, 19(6): 597-601.

524. Scheen AJ and De Hert MA. (2007). Abnormal glucose metabolism in patients treated with antipsychotics. *Diabetes Metab*, 33(3): 169-75.

525. Melkersson K. (2004). Clozapine and olanzapine, but not conventional antipsychotics, increase insulin release in vitro. *Eur Neuropsychopharmacol*, 14(2): 115-9.

526. Virani A, Bezchlibnyk-Butler KZ and Jeffries JJ. (2009). *Clinical handbook of psychotropic drugs, 18th edition*. Toronto; Lewiston, NY: H. Huber Publishers.

527. Ito C. (2009). Histamine H3-receptor inverse agonists as novel antipsychotics. *Cent Nerv Syst Agents Med Chem*, 9(2): 132-6.

528. Scott AB. (2004). Development of botulinum toxin therapy. *Dermatol Clin*, 22(2): 131-3.

529. Puffer EB, Lomneth RB, Sarkar HK *et al*. (2001). Differential roles of developmentally distinct SNAP-25 isoforms in the neurotransmitter release process. *Biochemistry*, 40(31): 9374-8.

530. Blackmer T, Larsen EC, Bartleson C *et al*. (2005). G protein betagamma directly regulates SNARE protein fusion machinery for secretory granule exocytosis. *Nat Neurosci*, 8(4): 421-5.

531. Lan JY, Skeberdis VA, Jover T *et al*. (2001). Activation of metabotropic glutamate receptor 1 accelerates NMDA receptor trafficking. *J Neurosci*, 21(16): 6058-68.

532. Wevers A. (2010). Localisation of pre- and postsynaptic cholinergic markers in the human brain. *Behav Brain Res*.

533. Wood MJ, Gait MJ and Yin H. (2010). RNA-targeted splice-correction therapy for neuromuscular disease. *Brain*, 133(Pt 4): 957-72.

534. Cohen J. (1992). A Power Primer. *Psychol Bull*, 112(1): 155-9.

535. Aleman A, Kahn RS and Selten JP. (2003). Sex differences in the risk of schizophrenia: evidence from meta-analysis. *Arch Gen Psychiatry*, 60(6): 565-71.

536. McGrath JJ. (2005). Myths and plain truths about schizophrenia epidemiology--the NAPE lecture 2004. *Acta Psychiatr Scand*, 111(1): 4-11.

537. Lustig RH, Hua P, Wilson MC *et al*. (1993). Ontogeny, sex dimorphism, and neonatal sex hormone determination of synapse-associated messenger RNAs in rat brain. *Brain Res Mol Brain Res*, 20(1-2): 101-10.

538. Jacobsson G, Razani H, Ogren SO *et al*. (1998). Estrogen down-regulates mRNA encoding the exocytotic protein SNAP-25 in the rat pituitary gland. *J Neuroendocrinol*, 10(9576603): 157-63.

539. Mitchell SJ and Ryan TA. (2004). Syntaxin-1A is excluded from recycling synaptic vesicles at nerve terminals. *J Neurosci*, 24(20): 4884-8.

540. Walch-Solimena C, Blasi J, Edelmann L *et al*. (1995). The t-SNAREs syntaxin 1 and SNAP-25 are present on organelles that participate in synaptic vesicle recycling. *J Cell Biol*, 128(4): 637-45.

541. Prescott GR, Gorleku OA, Greaves J *et al*. (2009). Palmitoylation of the synaptic vesicle fusion machinery. *J Neurochem*, 110(4): 1135-49.

542. Pitre A, Pan Y, Pruett S *et al*. (2007). On the use of ratio standard curves to accurately quantitate relative changes in protein levels by Western blot. *Anal Biochem*, 361(2): 305-7.

543. Beasley CL, Pennington K, Behan A *et al*. (2006). Proteomic analysis of the anterior cingulate cortex in the major psychiatric disorders: Evidence for disease-associated changes. *Proteomics*, 6(11): 3414-25.

544. Martins-De-Souza D, Gattaz W, Schmitt A *et al*. (2009). Proteomic analysis of dorsolateral prefrontal cortex indicates the involvement of cytoskeleton, oligodendrocyte, energy metabolism and new potential markers in schizophrenia. *J Psychiatr Res*, 43(11): 978-86.

545. Anderson D and Lydic R. (2002). On the effect of using ratios in the analysis of variance. *Biobehavioural Reviews*, 1(4): 225-9.

546. Atchley WR and Anderson D. (1978). Ratios and the Statistical Analysis of Biological Data. *Syst Zool*, 27: 71-8.

547. Walaas SI, Jahn R and Greengard P. (1988). Quantitation of nerve terminal populations: synaptic vesicle-associated proteins as markers for synaptic density in the rat neostriatum. *Synapse*, 2(5): 516-20.

548. Poirier MA, Hao JC, Malkus PN *et al*. (1998). Protease resistance of syntaxin.SNAP-25.VAMP complexes. Implications for assembly and structure. *J Biol Chem*, 273(18): 11370-7.

549. Zhang N and Li L. (2004). Effects of common surfactants on protein digestion and matrix-assisted laser desorption/ionization mass spectrometric analysis of the digested peptides using two-layer sample preparation. *Rapid Commun Mass Spectrom*, 18(8): 889-96.

550. Havlis J and Shevchenko A. (2004). Absolute quantification of proteins in solutions and in polyacrylamide gels by mass spectrometry. *Anal Chem*, 76(11): 3029-36.

551. Hardy JA, Dodd PR, Oakley AE *et al*. (1982). Use of post-mortem human synaptosomes for studies of metabolism and transmitter amino acid release. *Neurosci Lett*, 33(3): 317-22.

552. Hampshire A and Owen AM. (2006). Fractionating attentional control using event-related fMRI. *Cereb Cortex*, 16(12): 1679-89.

553. Chung CY, Koprich JB, Hallett PJ *et al*. (2009). Functional enhancement and protection of dopaminergic terminals by RAB3B overexpression. *Proc Natl Acad Sci U S A*, 106(52): 22474-9.

554. Law AJ, Kleinman JE, Weinberger DR *et al*. (2007). Disease-associated intronic variants in the ErbB4 gene are related to altered ErbB4 splice-variant expression in the brain in schizophrenia. *Hum Mol Genet*, 16(2): 129-41.

555. Colelli V, Fiorenza MT, Conversi D *et al*. (2010). Strain-specific proportion of the two isoforms of the dopamine D2 receptor in the mouse striatum: associated neural and behavioral phenotypes. *Genes Brain Behav*.

556. Mandic R, Trimble WS and Lowe AW. (1997). Tissue-specific alternative RNA splicing of rat vesicle-associated membrane protein-1 (VAMP-1). *Gene*, 199(1-2): 173-9.

557. Mandic R and Lowe AW. (1999). Characterization of an alternatively spliced isoform of rat vesicle associated membrane protein-2 (VAMP-2). *FEBS Lett*, 451(2): 209-13.

558. Berglund L, Hoffmann HJ, Dahl R *et al*. (1999). VAMP-1 has a highly variable C-terminus generated by alternative splicing. *Biochem Biophys Res Commun*, 264(3): 777-80.

559. Nakayama T, Mikoshiba K, Yamamori T *et al*. (2003). Expression of syntaxin 1C, an alternative splice variant of HPC-1/syntaxin 1A, is enhanced by phorbol-ester stimulation in astroglioma: participation of the PKC signaling pathway. *FEBS Lett*, 536(1-3): 209-14.

560. Mockler TC, Chan S, Sundaresan A *et al*. (2005). Applications of DNA tiling arrays for whole-genome analysis. *Genomics*, 85(1): 1-15.

561. Mortazavi A, Williams BA, McCue K *et al*. (2008). Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat Methods*, 5(7): 621-8.

562. Morin R, Bainbridge M, Fejes A *et al*. (2008). Profiling the HeLa S3 transcriptome using randomly primed cDNA and massively parallel short-read sequencing. *Biotechniques*, 45(1): 81-94.

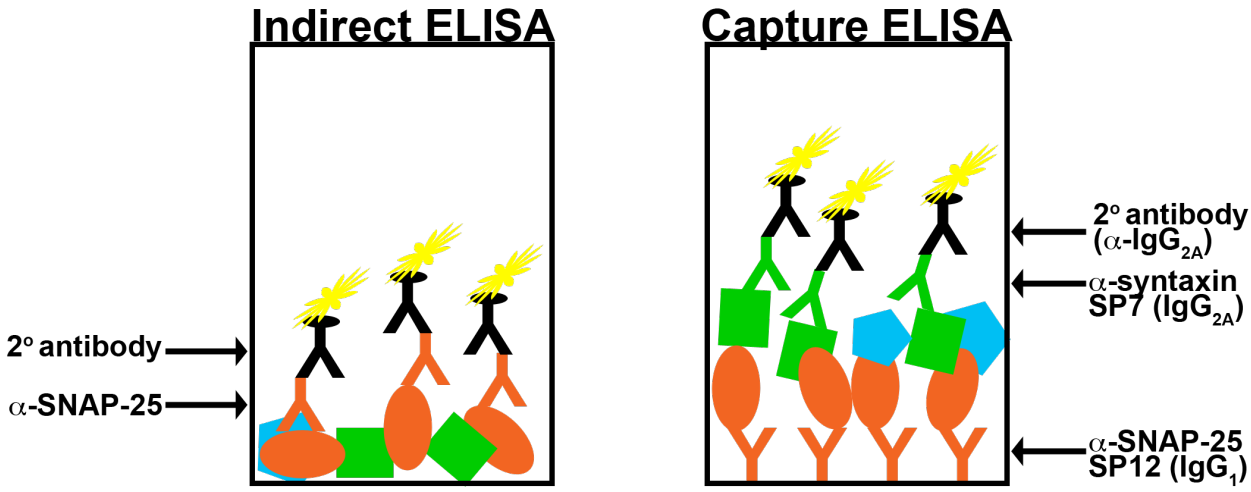
563. Hicks MJ, Lam BJ and Hertel KJ. (2005). Analyzing mechanisms of alternative pre-mRNA splicing using in vitro splicing assays. *Methods*, 37(4): 306-13.

564. Ule J. (2009). High-throughput sequencing methods to study neuronal RNA-protein interactions. *Biochem Soc Trans*, 37(Pt 6): 1278-80.

565. Castle JC, Zhang C, Shah JK *et al*. (2008). Expression of 24,426 human alternative splicing events and predicted cis regulation in 48 tissues and cell lines. *Nat Genet*, 40(12): 1416-25.

Appendix 1 – Illustration of ELISA methods used

SNARE protein levels were assessed using an indirect ELISA (which we have called a 'direct ELISA' in Chapter 2, to increase clarity in relation to the capture assay), whereby brain protein was adhered to a microtitre plate, specific proteins detected using a primary antibody (SP12, α-SNAP-25, is illustrated here in orange) followed by enzyme-linked secondary antibody (black). The capture ELISA developed in Chapter 2 is a modified sandwich ELISA. SNAP-25 is immobilized in the SP12-coated well. After washing, syntaxin (and other interacting proteins) remain. Syntaxin is detected with SP7 antibody of a different subclass (α-syntaxin, green). A subclass-specific secondary antibody prevents detection of SP12. In both assays, addition of substrate results in a colour change (indicated by yellow bursts) that is measured by a spectrophotometer.

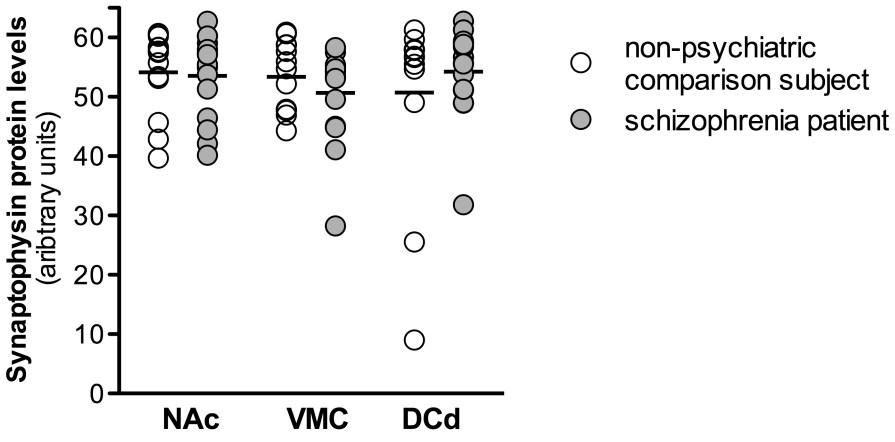


Appendix 2 – Additional drug dosage details

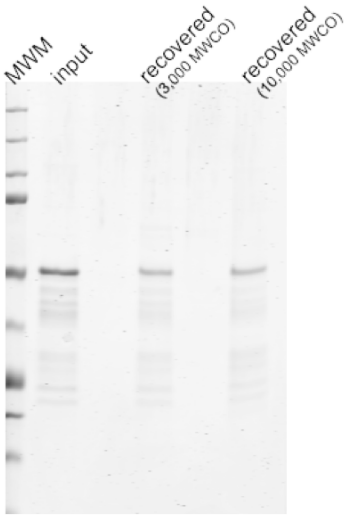
In studies of APD effects in animals, drug doses were similar to those used in previous studies of presynaptic proteins [309, 310]. The route of administration was selected to reduce effects of first-pass metabolism. Daily administration and high drug doses were necessary due to the high rate of drug metabolism in rodents as compared with humans [347]. Four weeks of administration was selected as this is often the time needed for clinical response to be observed in patients, has been used previously [309, 310] and to maximize chances of observing changes that reflect repeated, as opposed to single-dose, drug exposure.

Appendix 3 – Synaptophysin protein levels in the striatum

Synaptophysin protein levels were measured in the same samples using the EP10 antibody (Figure 2.4), and the ELISA protocol detailed in Chapter 2. Synaptophysin protein levels were not different between groups in the striatum overall ($F = 0.002$, df = 1,26, $P = 0.835$, two-way ANOVA) nor in each region separately (all $P > 0.25$, two-tailed t-test). Data was $\log_e$-transformed prior to statistical analysis, but not graphing. Statistical significance remained the same when analyses were repeated removing two extreme values in the DCd. For graphing purposes only, data was re-scaled to facilitate interpretation. *Abbreviations*: NAc – nucleus accumbens, VMC – ventromedial caudate, DCd – dorsal caudate.

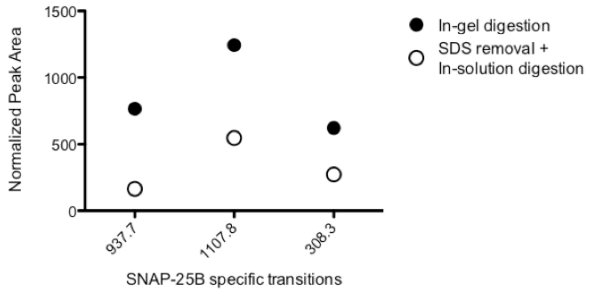


Appendix 4 – Additional figures from MRM assay development



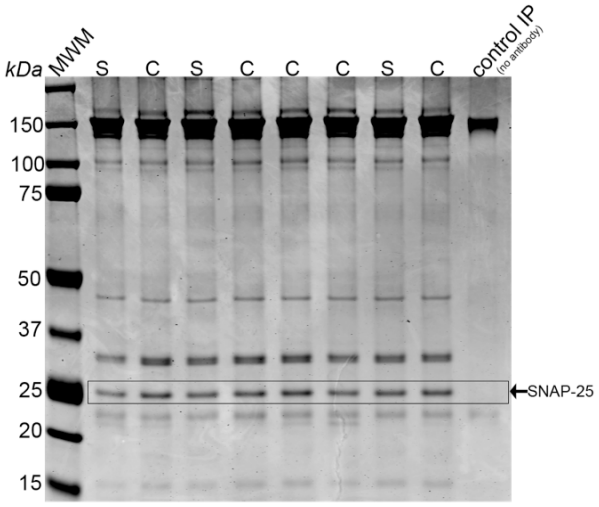
Removal of SDS and sample recovery using a spin column. Equal amounts of recombinant protein was resuspended in Laemelli sample buffer (input). Detergent was removed using Microcon spin columns with two molecular weight cut-offs (MWCO), according to manufacturer's instructions. Retentate was collected. Samples were separated by SDS-PAGE and stained with Coomassie. Recovered protein was ~ 40% of initial input. Detergent removal using spin columns appears to result in a > 50% loss of protein.

Comparison of signal intensity between in-gel versus in-solution sample preparation. SNAP-25 was immunoprecipitated from a human brain sample and either processed in-gel or, after SDS removal using a spin-column, digested with trypsin in-solution. In agreement with the image above, higher signal intensity was obtained for the sample digested in-gel. Note, optimal MRM parameters and sample IP conditions were not used during this test. In the final assay, higher signal intensity was generally obtained.



Appendix 5 – Example of gel-separated immunoprecipitates

Immunoprecipitation of SNAP-25 from human VMC. After IP, samples were eluted in Laemelli sample buffer, separated by SDS-PAGE and stained with Coomassie. The band corresponding to SNAP-25 was excised for each sample separately then digested in-gel. Multiple proteins were pulled down during the IP, likely the other SNARE proteins and SNARE interactors. Specificity of pull-down is illustrate here by including a sample which was IP'd using "empty" beads (beads which were not coupled with α-SNAP-25 antibody). The bands at ~150kD represent intact antibody. As illustrated by the control IP lane, some material (likely mouse IgG) elutes from the beads themselves. Note, image contrast was enhanced in a non-linear fashion to facilitate band cutting. *Abbreviations*: MWM – molecular weight marker; S – schizophrenia; C – non-psychiatric comparison subject.



Appendix 6 – Ethics certificates

UBC ethics approval certificates for protocols involving animals and human samples.

THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A06-0272

Investigator or Course Director: Alasdair M. Barr

Department: Psychiatry

Animals:

Rats Sprague-Dawley 100

Start Date: **June 15, 2006** **Approval Date:** **July 7, 2007**

Funding Sources:

Funding Agency: Canadian Institutes of Health Research (CIHR)
Funding Title: Complexins as a neural substrate for cognitive function and dysfunction

Funding Agency: Canadian Institutes of Health Research (CIHR)
Funding Title: Characterisation of oligodendrocyte abnormalities in schizophrenia

Funding Agency: Vancouver Coastal Health Research Insitute (VCHRI)
Funding Title: A role for neuroligins in schizophrenia

Funding Agency: Michael Smith Foundation for Health Research
Funding Title: Characterisation of oligodendrocyte abnormalities in schizophrenia

Unfunded title: N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

The University of British Columbia

Biohazard Approval Certificate

PROTOCOL NUMBER: **H02-0079**

INVESTIGATOR OR COURSE DIRECTOR: **Honer, William**

DEPARTMENT: **Psychiatry**

PROJECT OR COURSE TITLE: **Developmental and Synaptic Abnormalities in Schizophrenia**

APPROVAL DATE: **03-06-20**

APPROVED CONTAINMENT LEVEL: **1**

FUNDING AGENCY: **Canadian Institutes of Health Research**

The Principal Investigator/Course Director is responsible for ensuring that all research or course work involving biological hazards is conducted in accordance with the Health Canada, Laboratory Biosafety Guidelines, (2nd Edition 1996). Copies of the Guidelines (1996) are available through the Biosafety Office, Department of Health, Safety and Environment, Room 50 - 2075 Wesbrook Mall, UBC, Vancouver, BC, V6T 1Z1, 822-7596, Fax: 822-6650.

Approval of the UBC Biohazards Committee by one of:
Chair, Biosafety Committee
Manager, Biosafety Ethics
Director, Office of Research Services

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required.

A copy of this certificate must be displayed in your facility.

Office of Research Services
102, 6190 Agronomy Road, Vancouver, V6T 1Z3
Phone: 604-827-5111 FAX: 604-822-5093

The University of British Columbia

Biohazard Approval Certificate

PROTOCOL NUMBER: **B07-0043**

INVESTIGATOR OR COURSE DIRECTOR: William G. Honer

DEPARTMENT: **Psychiatry**

PROJECT OR COURSE TITLE: **Neural Connectivity in Schizophrenia**

APPROVAL DATE: **March 26, 2009** START DATE: **January 31, 2007**

APPROVED CONTAINMENT LEVEL: **2**

FUNDING TITLE: **Developmental and Synaptic Abnormalities in Schizophrenia**
FUNDING AGENCY: **Canadian Institutes of Health Research (CIHR)**

FUNDING TITLE: **Epidemiologic Study of Neural Reserve and Neurobiology of Aging Epidemiologic Study of Brain Vitamin E, Diet, and Age-Related Neurologic Diseases**
FUNDING AGENCY: **National Institutes of Health - National Institute on Aging**

FUNDING TITLE: **Neural Connectivity in Schizophrenia**
FUNDING AGENCY: **Canadian Institutes of Health Research (CIHR)**

UNFUNDED TITLE: **Neural Connectivity in Schizophrenia**

The Principal Investigator/Course Director is responsible for ensuring that all research or course work involving biological hazards is conducted in accordance with the University of British Columbia Policies and Procedures, Biosafety Practices and Public Health Agency of Canada guidelines.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there are no changes. Annual review is required.

A copy of this certificate must be displayed in your facility.

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