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Quantitative mass spectrometry reveals changes in SNAP-25 isoforms in schizophrenia

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Abstract

SNAP-25 and syntaxin are presynaptic terminal SNARE proteins altered in amount and function in schizophrenia. In the ventral caudate, we observed 32% lower SNAP-25 and 26% lower syntaxin, but greater interaction between the two proteins using an in vitro assay. SNAP-25 has two isoforms, SNAP-25A and B, differing by only 9 amino acids, but with different effects on neurotransmission. A quantitative mass spectrometry assay was developed to measure total SNAP-25, and proportions of SNAP-25A and B. The assay had a good linear range (50- to 150-fold) and coefficient of variation (4.5%). We studied ventral caudate samples from patients with schizophrenia (n=15) previously reported to have lower total SNAP-25 than controls (n=13). We confirmed 27% lower total SNAP-25 in schizophrenia, and observed 31% lower SNAP-25A ($P=0.002$) with 20% lower SNAP-25B amounts ($P=0.10$). Lower SNAP-25A amount correlated with greater SNAP-25-syntaxin protein-protein interactions ($r=-0.41$, $P=0.03$); the level of SNAP-25B did not. Administration of haloperidol or clozapine to rats did not mimic the changes found in schizophrenia. The findings suggest that lower levels of SNAP-25 in schizophrenia may represent a greater effect of the illness on the SNAP-25A isoform. This in turn could contribute to the greater interaction between SNAP25 and syntaxin, and possibly disturb neurotransmission in the illness.

Keywords

Neural plasticity; SNARE proteins; Postmortem; Neurotransmission; Synapse

1. Introduction

Neurotransmitter release from presynaptic terminals is dependent on the functional interaction of three neuronal soluble NSF-attachment receptors (SNAREs), SNAP-25, syntaxin and VAMP (vesicle-associated membrane protein) (Nagy et al., 2005; Washbourne et al., 2002). Abnormalities of the amount of SNARE proteins, or modifications to these proteins that alter protein-protein interactions, can result in greater or less neurotransmitter release. The consequences of quantitative and functional abnormalities of SNAP-25 may have important implications for schizophrenia and other mental illnesses (Ramos-Miguel et al., 2015).

SNAP-25 appears to be enriched in glutamatergic terminals, while relatively lower amounts of the protein are detectable in GABAergic terminals (Matteoli et al., 2009). In the striatum in schizophrenia, lowered levels of glutamate transporters are reported, suggesting abnormalities of presynaptic glutamatergic terminals (McCullumsmith and Meador-Woodruff, 2002; Nudmamud-Thanoi et al., 2007). In contrast, dopamine transporter levels are not altered, suggesting the numbers of dopaminergic terminals are unchanged (Fusar-

Poli and Meyer-Lindenberg, 2013a). However, greater dopamine synthesis and release occurs in individuals at risk of psychosis, in first episode schizophrenia, and in patients with a good response to antipsychotic drug treatment (Abi-Dargham et al., 2009; Demjaha et al., 2012; Fusar-Poli et al., 2010; Fusar-Poli and Meyer-Lindenberg, 2013a; Howes et al., 2009; Kegeles et al., 2010). Impaired cortical function could alter the balance of activity between glutamatergic and dopaminergic terminals, resulting in greater dopamine synthesis and release (Kegeles et al., 2000; Meyer-Lindenberg et al., 2002).

Presynaptic function in three striatal regions (nucleus accumbens, ventromedial and dorsal caudate) involved with cognitive and limbic circuitry, including the reward system, could play a key role in the mechanism of illness in schizophrenia (Lee et al., 2015; Richter et al., 2015). The amounts of two SNARE proteins, SNAP-25 and syntaxin, were lower in the ventromedial caudate (Barakauskas et al., 2010), a component of the associative striatum reported to have greater dopamine synthesis and release in the illness. Abnormalities in the nucleus accumbens were similar, but smaller in magnitude. Absence of change in the dorsal caudate suggested some specificity to the observations. In addition to lower amounts of these proteins, greater SNAP-25-syntaxin protein interactions were observed, further suggesting function abnormalities of presynaptic terminals in associative striatum in schizophrenia. Greater SNARE protein-protein interactions are also reported in other brain regions in schizophrenia, and appear sensitive to antipsychotic drug treatment (Gil-Pisa et al., 2012; Honer et al., 2002; Ramos-Miguel et al., 2015).

Studies of the expression and function of SNAP-25 provide a context for further investigations in schizophrenia. Lower levels of SNAP-25 result in multiple neurochemical and behavioural abnormalities in animal models. Hyperactive motor behaviour, delayed acquisition of motor milestones, as well as impaired learning and memory were reported during development in mice with heterozygous deficiency in SNAP-25 (Raber et al., 1997). Generalized hyperexcitability resulted in frequent EEG spikes, and greater sensitivity to kainate-induced seizures (Corradini et al., 2014). Terminals lacking all SNAP-25 displayed spontaneous transmitter release, but evoked release was absent (Washbourne et al., 2002). Animals with heterozygous SNAP-25 deficiency showed greater dopamine release in striatum after amphetamine challenge (Fan and Hess, 2007). Modification of SNAP-25 can also alter the functional properties of neurons and animal behaviour. Impaired phosphorylation of SNAP-25 reduces the SNAP-25-syntaxin protein-protein interaction (Nakata et al., 2012). These animals are anxious, may have seizures, as well as impaired dopamine and serotonin release in the amygdala (Kataoka et al., 2011).

The SNAP-25 gene transcript undergoes mutually exclusive alternative splicing of exons 5a and 5b, producing protein isoforms of equal length, that differ by only 9 amino acids (C. Bark et al., 2004; I. C. Bark et al., 1995). In mice, SNAP-25A is expressed more highly in rostral brain regions (Yamamori et al., 2011). A switch between predominant SNAP-25A mRNA expression early in life to predominant SNAP-25B mRNA expression postnatally is necessary for survival in this species (C. Bark et al., 2004). Transgenic mice expressing more SNAP-25A than normal had increased synaptic facilitation, but not vesicle release probability (C. Bark et al., 2004). However, total SNAP-25 expression was lower in these animals, complicating the interpretation of the findings. A different line of SNAP-25B

deficient mice (with normal total SNAP-25) showed multiple behavioural and electrophysiological abnormalities, including anxiety, seizures and altered synaptic plasticity (Johansson et al., 2008). SNARE protein complexes containing SNAP-25B are more stable than those containing SNAP-25A (Johansson et al., 2008). Other studies show SNAP-25B more effectively maintained vesicles in a primed state prior to fusion, than did SNAP-25A (Nagy et al., 2004; Sørensen et al., 2003).

Another model, the *blind-drunk* (*Bdr*) mouse, contains an isoleucine-to-threonine substitution within the SNAP-25B exon. As noted above, the interaction of SNAP-25B with syntaxin is stronger than that observed between SNAP-25A and syntaxin. The change in the *Bdr* mouse SNAP-25B increases SNARE complex stability even further, and alters terminal function by reducing spontaneous and evoked glutamate exocytosis (Jeans et al., 2007). The *Bdr* mouse shares phenotypic similarities with schizophrenia, including impaired prepulse inhibition, increased sensitivity to prenatal stress, and show grossly abnormal circadian rhythm (Jeans et al., 2007; Oliver and Davies, 2009; Oliver et al., 2012).

To further evaluate the possible role for SNAP-25 in schizophrenia, we developed an assay to quantify levels of the SNAP-25A and B proteins using mass spectrometry. We hypothesized that previously reported SNAP-25 alterations in ventromedial striatum might be restricted to one isoform, possibly contributing to the increased interaction between SNAP-25 and syntaxin observed in the same samples (Barakauskas et al., 2010).

2. Methods

2.1 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 postmortem diagnosis are detailed elsewhere (Barakauskas et al., 2010), and are summarized in Table 1. Fixed tissues were examined by a neuropathologist; no clinically significant pathology was detected. 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) was dissected from the right hemisphere using a standard human brain atlas (Barakauskas et al., 2010; Mai et al., 1997). Tissue samples were homogenized in tris-buffered saline (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.

2.2 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. The doses and duration of treatment were chosen to result in occupancy of dopamine D2 receptors in rats that would be as comparable as possible to those observed in patients with schizophrenia at steady state (Crocker and Hemsley, 2001; Kapur et al., 2003; Turrone et al., 2003), and were similar to those used in other studies of the effects of antipsychotic drugs on presynaptic proteins (Barr et al., 2006). Drug administration and preparation are detailed elsewhere (Barakauskas et al., 2010). 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, and cooled briefly in artificial cerebrospinal fluid (148 mM NaCl, 3 mM KCl, 1.4 mM CaCl₂, 0.8 mM MgCl₂, 10 mM D-Glucose, 0.8 mM Na₂HPO₄, 0.2 mM NaH₂PO₄) and dissected. Coronal cuts at the level of the optic chiasm and 3 mm anterior gave a section at approximately Bregma 0.48 μ m (Paxinos and Watson, 1986). 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. A similar dissection protocol was described previously (Barakauskas et al., 2010; Meyer and Protopapas, 1985). Regions were dissected bilaterally, frozen rapidly on dry ice and stored at -70°C.

2.3 SNAP-25A and B isoform assay

The overall workflow is illustrated in Figure 1. More detailed description of the assay development and validation appears in the Supplemental Methods. For measurement of SNAP-25 isoforms, SNAP-25 was immunoprecipitated from solubilized homogenates, using a monoclonal antibody that detects both isoforms (SP12) (Barakauskas et al., 2010; Honer et al., 1997). Samples were coded to mask investigators to diagnosis and sample characteristics. 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 2.

Following sample preparation (see Supplemental Methods SNAP-25 protein isoforms were detected and quantified using a multiple-reaction monitoring (MRM) mass spectrometry (MS) method. This approach detects peptides specific to each SNAP-25 isoform, as well as a peptide common to both (Figure 3A-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 3E). Peak areas are proportional to peptide amounts.

An external ratio calibration curve relating MRM peak area ratios to the percentage of SNAP-25A in samples was constructed using mixtures of recombinant SNAP-25A and B proteins (Figure 3F). This allowed interpolation of the %SNAP-25A in analyzed samples. To quantify the absolute amounts of SNAP-25 peptides, SNAP-25 was immunoprecipitated from homogenized samples and prepared for MRM analysis. An isotopically-labeled peptide (heavy peptide, see Supplemental Methods) identical to the common SNAP-25 peptide was added to the trypsin digested samples as an internal quantification standard (Kirkpatrick et al., 2005); the heavy peptide co-elutes with the common peptide by chromatography but is resolved by MS due to its 10 Da mass difference. 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 3G). The absolute amounts of SNAP-25A and B were then calculated using the percentage of SNAP-25A interpolated from the ratio calibration curve. The CV of technical (intra-run) 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% (%A, interpolated).

2.4 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-Wilks and Brown-Forsythe tests, respectively. Data was log-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-transformation). The raw *P*-values for these two primary tests of significance are reported. Possible association of sample demographics with SNAP-25 isoform expression was explored using Spearman correlations, and ANCOVA was used where significant correlations were observed.

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 which was not remedied by log-transformation, to confirm it did not influence statistical conclusions.

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

3. Results

3.1 Assay performance

Detection of isoform-specific peptides was log-linear over a 50 to 150-fold range (Figure 4). We assessed the reproducibility of the isoform ratio over time, by repeatedly analyzing the

same sample. The CV of the A/B ratio calculated from 8 replicate runs of a single brain sample was 4.5%. The %SNAP-25A was interpolated using the ratio calibration curve; the CV of %A values was 2.4%.

3.2 Schizophrenia samples

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 (Barakauskas et al., 2010). Total SNAP-25 was 27% lower in SCZ subjects ($P = 0.002$; two-tailed t -test) as compared with NPCs (Figure 5C), in agreement with previous findings in this exact sample set using ELISA (Barakauskas et al., 2010). 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).

Synaptophysin is a ubiquitous synaptic vesicle protein present in similar amounts in the schizophrenia and NPC VMC samples (Barakauskas et al., 2010). Controlling for the level of synaptophysin present in each sample, results for the SNAP-25 total and isoform analyses remained statistically significant (total SNAP-25: diagnosis $P = 0.004$, synaptophysin $P = 0.28$, ANCOVA; SNAP-25A: diagnosis $P = 0.004$, synaptophysin $P = 0.26$, ANCOVA; SNAP-25B: diagnosis $P = 0.11$, synaptophysin $P = 0.56$, ANCOVA).

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 (Barakauskas et al., 2010). 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 $r = -0.22$).

3.3 Associations of SNAP-25 isoforms in human ventromedial caudate with sample characteristics

Possible associations between age, post-mortem interval, storage time, pH and gender with each isoform were investigated using Spearman correlations or a t -test. 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 $r_s = -0.43$), but not SNAP-25A ($P = 0.12$, Spearman correlation $r_s = -0.29$). The significant difference between diagnostic groups in total SNAP-25, and in SNAP-25A, 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. None of the other variables tested showed a relationship with isoform levels.

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.

3.4 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, a subset were 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 ($P = 0.024$, t-test). 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).

3.5 Effects of antipsychotic drugs in rats

To further explore the possible effects of antipsychotic drugs, rats were treated subchronically with haloperidol or clozapine, or saline control. SNAP-25A and B proteins in the medial striatum did not differ between treatment groups (Figure 5D, for all measures $P > 0.05$, ANOVA).

4. Discussion

The central finding indicates that lower SNAP-25 in ventromedial caudate in schizophrenia is primarily due to less SNAP-25A, and that the remaining SNAP-25 is associated with greater protein-protein interaction with syntaxin. 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. SNAP-25 A and B isoform expression in human adult striatum is consistent with that reported in mice (SNAP-25A ~60%) (Yamamori et al., 2011).

The observation of a differential SNAP-25A isoform expression adds to characterization of presynaptic abnormalities in striatum in schizophrenia. Several electron microscopy studies

indicate abnormalities of synapses in the striatum in schizophrenia (Roberts et al., 1996; 2005; 2009). Although a recent meta-analysis of studies of the dopamine transporter concluded there were no consistent abnormalities in schizophrenia (Fusar-Poli and Meyer-Lindenberg, 2013a), the synthesis and release properties of dopamine terminals were reported to be abnormal more consistently (Fusar-Poli and Meyer-Lindenberg, 2013b). Subtle neuropathological changes in the substantia nigra in schizophrenia are also consistent with dopaminergic overactivity (Williams et al., 2014). Glutamatergic presynaptic terminals are also reported to be abnormal (McCullumsmith and Meador-Woodruff, 2002; Nudmamud-Thanoi et al., 2007), and could contribute to increased dopamine release. Dysfunction of presynaptic proteins may be a molecular mechanism of increased neurotransmitter release. The specific effects of lower levels of SNAP-25A on neurotransmission are uncertain. However, mice deficient in total SNAP-25 showed greater dopamine release in striatum at baseline and in response to amphetamine challenge than control mice (Fan and Hess, 2007).

Preservation of SNAP-25B expression, with lower levels of SNAP-25A may increase the stability of the SNARE complex in schizophrenia compared with normal presynaptic terminals (Delgado-Martínez et al., 2007; Nagy et al., 2005). More stable SNARE complexes found in the *Bdr* mouse model produced lower spontaneous and evoked glutamate release (Jeans et al., 2007). In healthy humans, impairing NMDA receptor function with ketamine resulted in greater amphetamine stimulation of dopamine release, and can precipitate psychotic-like symptoms, suggesting a mechanism by which increased SNARE complex stability could increase dopaminergic transmission in schizophrenia (Kegeles et al., 2000).

Although there are no known human mutations of the SNAP-25B exon as found in the *Bdr* mouse (isoleucine-to-threonine substitution, Ile67Thr), two mutations (Ile67Asn and Val48Phe) are reported in association with intellectual disability and other neurological disorders (Rohena et al., 2013; Shen et al., 2014). The effects of these mutations on SNARE complex stability are unknown.

Methodological limitations were minimized wherever possible in the present study. However, while the IP conditions were effective in pulling 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 was 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 non-psychiatric control samples are not completely depleted from the sample. Finally, the similar mean difference in total SNAP-25 from our previous ELISA study and the present measurements with mass spectroscopy provide some converging evidence of assay reliability. Other limitations of the study include a relatively small sample size, and the generally unavoidable complication of medication treatment at unknown doses over the lifetime of the patients.

The present results suggest investigation of SNAP-25 protein isoform expression in other schizophrenia cohorts, and the regulation of SNAP-25 isoform expression and SNARE complex stability in cell lines and in animal models will be important to furthering our understanding of the mechanisms of illness in schizophrenia (Fan and Hess, 2007; Jeans et al., 2007).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
SNAP-25 isoform assay sample preparation and analysis workflow. See text for description.

Figure 2.

Efficiency of SNAP-25 immunoprecipitation (IP) from human brain samples. For NPC and SCZ samples, equal total protein amounts of crude brain homogenate were solubilized and clarified by centrifugation. IP efficiency of the SCZ sample was monitored by immunoblot. An aliquot of the input was kept to compare with the post-IP supernatant (SN) to confirm that SNAP-25 was specifically removed from the sample. Efficiency of bead washes was also monitored, the input, SN and wash #1 and #5 lanes can be compared directly. A control IP (beads that are not coupled with anti-SNAP-25 antibody, “empty beads”) did not immunoprecipitate SNAP-25 from a different brain sample. Empty beads correspond to magnetic beads not coupled to anti-SNAP-25 antibody, the same bead conditions used in the control IP. Antibody-coupled beads correspond to SP12 (anti-SNAP-25 antibody) coupled beads, used for IP. High molecular weight bands correspond to antibody eluting off the beads. 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 3.

SNAP-25 peptides for MRM 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 recombinant 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-to-charge ratio. **(B)**: SNAP-25A peptide; **(C)**: SNAP-25-B 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. **(F):** 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). **(G):** 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. 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.

Figure 4.

Linearity and detection limit of SNAP-25 peptides. **(A):** Detection range of the SNAP-25 common peptide. The heavy peptide internal standard (H104-119) was serially diluted in $1\times$ 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 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 in-gel. 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 MRM 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.

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Figure 5.

The MRM isoform assay detects changes in SNAP-25 isoform expression. **(A):** 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 (Fig. 3F), then the absolute amount of SNAP-25A was determined as $(\% \text{SNAP-25A}/100) \times \text{fmol of total SNAP-25}$ (calculated using the heavy peptide external standard curve, Fig. 3G). 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). *Abbreviations:* NPC – non-psychiatric comparison subject; SCZ – schizophrenia. **(B):** 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. Horizontal lines represent group median, crosses represent means, boxes represent the 25th and 75th percentiles and whiskers represent 10th and 90th percentiles.

Table 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 were shorter in the patient group ($P = 0.001$, t-test). PMI was unavailable for one individual. Detailed sample information was previously described.¹⁴

<i>Mean (SD)</i>	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

Abbreviations: M, male; F, female; MVA, motor vehicle accident; NPC – non-psychiatric comparison subjects; SCZ – schizophrenia