# Increased SNARE Protein-Protein Interactions in Orbitofrontal and Anterior Cingulate Cortices in Schizophrenia

# Supplement 1

## Supplementary Methods

**Human postmortem brains.** The present research work presents neurochemical findings in two independent postmortem brain collections (Cohort1 and Cohort 2), obtained from different sources (see Table S1). Samples included in Cohort 1 were obtained from the Macedonian/New York State Psychiatric Institute Brain Collection (1). This postmortem series comprises dissected tissues from persons with and without schizophrenia suffering accidental or unexpected, sudden deaths, collected between 1997 and 2004. Suicide victims were excluded for the present study. In schizophrenia group, only Caucasian individuals with antemortem diagnoses of schizophrenia ($n$ = 13) or schizoaffective disorder ($n$ = 2) according to DSM-IV criteria (2) were included. Subjects with no history of mental illness or any chronic disease, and closely matched for age, sex, and postmortem interval (PMI) were selected for comparison ($n$ = 13). In order to confirm clinical diagnoses, medical records, police reports and family interviews were reviewed by three independent psychiatrists.

To replicate the major findings in Cohort 1, and thus meet the primary goals of the present study, a secondary postmortem series (Cohort 2) was obtained from the Stanley Foundation Neuropathology Consortium (Chevy Chase, MD, USA). This collection included 15 schizophrenia and 15 control donors. Collective and individual features are listed in Tables S1 and S3, respectively. Further details were described elsewhere (3; 4).

Gray matter samples from the right anterior orbitofrontal [Brodmann area (BA) 10/47; Cohorts 1 and 2] and cingulate (BA 24; only available for Cohort 1) cortices were dissected

quickly on dry ice, avoiding thawing, following a standard atlas (5). Brain specimens (109-204 mg) were homogenized in glass grinders with an automated Teflon pestle in pre-chilled Tris-buffered saline (TBS), pH 7.4, supplemented with a 1% protease inhibitor cocktail (Sigma-Aldrich, Saint Louis, MO, USA), in a weight/volume ratio of 1:1. Protein concentration was quantified with DC assay (Bio-Rad, Hercules, CA, USA), and additional volumes of homogenization buffer were added to reach equal protein concentration (10 μg/μl) in all cortical samples. Aliquots of brain homogenates were stored at –80ºC until used for quantitative assays involving co-immunoprecipitation (co-IP), standard sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE), or blue-native (BN) PAGE.

**Effect of PMI on cortical SNARE complexes.** The potential effect of PMI on SNARE complex was modeled in a series of control human postmortem brains selected from the National Specimen Brain Bank (Los Angeles, CA, USA) to span the range of postmortem intervals relevant to the primary study. These included nine males (mean age 66±5, range 40-86; mean PMI 15±2 h; range 7-25 h). Cause of death was sudden in all cases (i.e. motor-vehicle accidents, gunshot murders), with no previous report of mental illness or any serious disorder, and presenting negative blood toxicology for psychotropic drugs and ethanol. Tissue blocks from the right temporal cortices were initially dissected and immediately stored at -80ºC. Small pieces from inferior temporal gyrus (approximating BA 20) were carefully separated while still frozen to obtain only gray matter tissue, and homogenized in TBS, pH 7.4, supplemented with 1% of a protease inhibitor cocktail (Sigma-Aldrich) as indicated above.

**Animals and antipsychotic treatments.** Adult male Sprague-Dawley rats ($n$ = 10 per group) were supplied by Charles-River (Montreal, QC, Canada). All procedures were approved by UBC's Animal Care Committee, and the NIH Guidelines for the Care and Use of Laboratory Animals were followed at all times. Haloperidol hydrochloride (1 mg/kg) and clozapine (20 mg/kg) (both from Tocris, Bristol, UK) were dissolved in saline (.9% NaCl), and administered intraperitoneally (i.p.) for 28 consecutive days as previously described (1). Rats were killed by decapitation 24 h after last injection; brains were quickly removed and rinsed in ice-cold artificial cerebrospinal fluid. Frontal cortices were immediately dissected, frozen onto dry ice bricks, and stored at –80ºC. Tissue homogenization was performed as described above.

**Antibodies.** A detailed list of primary antibodies used in the study is provided in Table S4. Production and characterization of mouse monoclonal antibodies against Stx1 (SP6, SP7), SNAP25 (SP12, SP14), VAMP (SP10, SP11), Cplx1 (SP33), Cplx2 (LP27) and Stg (MAB48) was described elsewhere (1; 6–8). Other commercial antibodies targeting Munc18-1, Stx1A, Stx1B, SNAP25 (SMI81), β-actin, and nonspecific phosphoserine residues (pSer) were also used. Peroxidase-conjugated secondary antibodies against mouse IgG+M, rabbit IgG and goat IgG were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Other secondary antibodies selectively recognizing mouse IgG1, IgG2a, IgG2b, or IgM were from Southern Biotech (Birmingham, AL, USA).

**IP and co-IP assays.** Target proteins were immunoprecipitated using sheep anti-mouse IgG-coated magnetic Dynabeads (Novex, Carlsbad, CA, USA). In each reaction, 150 μg of beads were incubated (2 h) in .1% Triton X-100 (TX100)-TBS alone (negative control), or containing 1

μg of anti-Stx1 (SP7) or anti-SNAP25 (SP12), antibodies. Nonspecific binding sites were blocked in the same buffer supplemented with 3% bovine serum albumin (BSA). In parallel, human or rat brain homogenates were solubilized in .5% TX100-TBS (1 h, 4ºC), and cell debris cleared by centrifugation (16,000 × *g*, 30 min, 4ºC). To control for variability in the assay across control and schizophrenia subjects, equal volumes from all control subjects were pooled into one standard sample. Three aliquots of this standard were separated and processed along with samples. Unless otherwise stated, 750 μg of solubilized brain proteins were combined with the antibody-conjugated beads and incubated overnight at 4ºC. After washing, IP products were eluted in 50 μl of SDS-loading buffer (50 mM Tris, pH 6.8, 1% SDS, 2.5% β-mercaptoethanol, 20% glycerol, .1% bromophenol blue). An alternative elution strategy was followed when the IP products were designated for further native electrophoresis. This elution was achieved with 25 μl of a mild acidic solution, containing 50 mM 6-aminohexanoic acid, pH 2.3, followed by addition of 75 μl of restoring buffer (50 mM Bis-tris, pH 7.0, 50 mM NaCl, 1 mM EDTA, 1% TX100), in order to preserve native protein structures and interactions.

**Standard SDS-PAGE and Western blotting.** Brain homogenates were solubilized and centrifuged as above, then combined with equal volumes of 2× SDS-loading buffer. For quantitative analyses, 5- or 10-μg protein aliquots (or 5 μl of the immunoprecipitated products) of every sample (including the three pooled standards) were boiled for 5 min (unless otherwise stated) and loaded into 12% polyacrylamide gels. These amounts were previously determined to be within the linear range for densitometric quantifications (data not shown). After electrophoresis, proteins were transferred to PVDF membranes, and subsequently blocked (1 h), and incubated with primary (overnight, 4ºC; Table S4) and secondary (1 h; 1:5,000) antibodies,

in TBS containing 5% milk and .1% Tween-2. Chemiluminescence was induced with commercial ECL reagents (Perkin Elmer, Waltham, MA, USA), and images were digitized using a LAS-3000 Image Reader (Fujifilm, Tokyo, Japan). Membranes were finally stripped and reprobed with antibodies targeting β-actin (or SNAP25 in IP assays), allowing data normalization. ImageGauge (version 4.22, Fujifilm) was used for densitometric quantification. To calculate the immunoreactivity of a given lane, the raw signal was first estimated using the rectangle tool in Image Gauge software. To subtract background, the rectangle selection was duplicated and located in a "clean" area nearby. This process was repeated for all samples in a gel. The specific signal of a sample was the result of the intensity in former rectangle minus the total intensity in the background rectangle. This approach takes into account both the size and the intensity of the band analyzed, and accurately removes background noise. Every sample was assessed in three different gels, and the immunoreactivity was calculated as a percentage of in-gel standards. This procedure was reported to reduce variability between subjects (9; 10).

**BN-PAGE and two-dimension (2-D) BN+SDS-PAGE.** Unlike SDS-gels, BN-PAGE allows the separation of protein complexes while preserving their native conformations. In the present study, we optimized previously published protocols (11; 12) to characterize and quantify presynaptic complexes in postmortem human and rat brains. To this end, aliquots of crude cortical homogenates were combined with equal volumes of an ice-cold 2× solubilization buffer that reached 50 mM Bis-tris, pH 7.0, 50 mM NaCl, 1 mM EDTA, 2 mM 6-aminohexanoic acid, .5% TX100, and 1% protease inhibitor cocktail final concentrations. Proteins were solubilized for 1 h at 4ºC with gentle rotation, and the insoluble fraction was separated by centrifugation (16,000 × *g*, 30 min, 4ºC). Supernatants were diluted in 1× solubilization buffer to a protein

concentration of 2 µg/µl, and stored at -80ºC until use. To account for putative TX100-insoluble SNARE complexes, pellets (P1 fraction) were washed, centrifuged and resuspended in equal volumes of the same solution. Before electrophoretic separation, BN-loading buffer was added to yield .5% TX100, .125% Coomassie Brilliant Blue G-250 (CBB), 5% glycerol, and 1 µg/µl protein final concentrations (a detergent-dye ratio of 4:1 was found optimal for SNARE complex visualization). In characterization assays, aliquots from the same brain sample were either boiled for 5 min, incubated with .1% SDS, or both denaturing conditions together. As in regular SDS-PAGE, for quantitative studies, a standard pooled control sample was also loaded in triplicate in BN-gels. Preliminary studies determined loading 5 µg of protein was optimal to quantify SNARE and other presynaptic protein complexes (data not shown). BN-PAGE was conducted in 4-16% gradient NativePAGE precast gels (Novex). Electrophoresis was run under constant voltage (150 V), at 4ºC, with pre-chilled anode (50 mM Bis-tris, pH 7.0) and cathode (15 mM Bis-tris, 50 mM tricine, pH 7.0, .02% CBB) buffers. After 30 min, cathode buffer was replaced for a 10 times CBB-diluted (.002%) solution, containing the same salt composition. Upon completion of electrophoresis, gels underwent through protein transfer to PVDF membranes, or 2-D SDS-PAGE. In the first procedure, BN-gels were initially incubated for 10 min in an ice-cold mild denaturing solution (12 mM Tris, 96 mM glycine, pH 8.3, .1% SDS) to increase the transfer efficiency. Protein transfer was completed in SDS-free, alkaline buffer (25 mM Tris, 25 mM glycine, pH 9.2), with constant voltage (110 V) at 4ºC for 2 h. To visualize molecular markers (NativeMARK, Novex, range 1240-20 kDa), membranes were partially de-stained in 25% methanol, 10% glacial acetic acid for 15 min. Fully removal of CBB dye was achieved by rinsing the membranes in 100% methanol, followed by immediate rehydration in TBS for 15 min, and finally immunoblotted with the standard procedure described for SDS-PAGE. In

quantitative studies, complex density was normalized with the total amount of the corresponding protein quantified in the same brain sample using the same detection antibody. For 2-D SDS-PAGE, the desired BN-gel strips were sliced out and incubated in 30 ml of SDS-loading buffer for 30 min. When indicated, this mixture was heated in a microwave for 20 seconds (or until boiling). Gel strips were then carefully placed onto discontinuous 3-12% stacking-separating SDS-gels. Electrophoresis was set at 80 V until proteins fully exited the stacking gel, and then shifted to 120 V. Proteins were then transferred to PVDF membranes and sequentially blotted (as indicated for standard SDS-PAGE) with antibodies against VAMP (SP10), Stx1 (SP7) and SNAP25 (SP12). To avoid cross-reactions, specific secondary antibodies against mouse IgM, IgG2a and IgG1 respectively were used, and harsh stripping solutions were applied to the membranes between re-probing procedures. Similarly, to identify other complexes, membranes were sequentially blotted with anti-Cplx1 (SP33), anti-Stx1 (SP7), and anti-Munc18-1, using anti-mouse IgG1, IgG2a and anti-goat IgG respectively, as secondary antibodies. To enhance signal intensities of both 1-D and 2-D native immunoblots, the ECL method was used, and images were captured with LAS-3000 Reader at various exposure times. Quantification of the resulting immunoreactive bands was done as above.

**Enzymatic dephosphorylation of brain proteins.** The effect of full protein dephosphorylation on SNARE protein-protein interactions was assessed essentially as previously described (13). Pooled rat cortical homogenates were incubated in triplicate in the presence (1 U/μl) or absence of active, calf alkaline phosphatase (Millipore, Billerica, MA, USA), at 37ºC for 1 h. Reactions were stopped with 50 mM sodium pyrophosphate, and chilled on ice. Brain samples were then prepared for BN-PAGE, SDS-PAGE or IP, as indicated in the corresponding section.

**Chemical disruption of SNARE complexes.** Some phenolic compounds have been shown to dampen neurotransmitter release through selective inhibition of SNARE complex assembly (14). We used the flavonol myricetin (3,3',4',5',5,7-hexahydroxy-2-phenylchromen-4-one; Sigma-Aldrich), a potent SNARE disruptor, to characterize functional SNARE complexes by BN-PAGE. To this end, pooled rat cortical samples were initially solubilized in 50 mM Bis-tris, pH 7.0, 50 mM NaCl, 1 mM EDTA, 2 mM 6-aminohexanoic acid, .5% TX100, and 1% protease inhibitor cocktail final concentrations 5% for 1 h at 4ºC, and cell debris cleared by centrifugation (16,000 × *g*, 30 min, 4ºC). Supernatants were then incubated overnight at 4ºC in the presence or absence of .1, 1, 10, or 100 μM myricetin (Sigma-Aldrich). Samples were then split and the aliquots were combined with equal volumes of 2× BN- or SDS-loading buffers, and separated by BN- or SDS-PAGE, respectively.

## Supplementary Results

**Identification of Cplx1/2, Munc18-1 and Stg presynaptic protein complexes by BN-PAGE.** The putative existence of stable protein complexes containing Cplx1/2, Munc18-1, or Stg in human and rat brains was also assessed by BN-PAGE. In 1-D gels, Cplx1 immunoblots revealed two major bands at ~200 and ~550 kDa, respectively (Figure S4A). These bands were sensitive to harsh thermochemical (boiling+SDS) denaturation, indicating the presence of protein complexes dissociated upon loss of native conformations. The 14-kDa monomeric Cplx1 was not always recognized in native blots, possibly due to migration out the gels (for comparison, see e.g., Figure S2A). Notably, the ~200-kDa Cplx1 band overlapped with the 200-kDa SNARE complex detected (more diffusely) with the three SNAREs (Figures S1 and S2A), possibly representing a tetrameric structure of SNARE proteins and Cplx1. In fact, this complex co-immunoprecipitated with anti-Stx1 and anti-SNAP25 antibodies (Figure S2A) and, similar to SNARE protein-protein interactions, was partially resistant to the 2-D SDS-PAGE denaturing conditions, yielding a ~75-kDa spot that overlapped with Stx1 (Figure S4C).

Antibodies against Cplx2 (not shown) and Munc18-1 (Figure S4A) did not react against any particular band in 1-D BN-PAGE. Interestingly, in 2-D gels, Munc18-1 antibody detected two spots/lines, both at its corresponding size (68 kDa) in SDS-PAGE (Figure S4C). The smaller one expanded from 66-146 kDa markers in BN-PAGE, and may be derived from the monomeric form in BN strip. The larger spot/line originated between the 146-242 kDa BN-markers, and was found to underlie both 150-kDa SNARE and 200-kDa SNARE-Cplx1. Thus, Munc18-1 could be a component of these complexes.

Anti-Stg antibody reacted against two major complexes of ~130 and ~350 kDa in BN-PAGE, both eliminated by denaturing conditions (Figure S4A), and revealed as ~65-kDa migrating spots in 2-D gels (Figure S4B).

**Enzymatic dephosphorylation alters native SNARE associations.** An important characteristic of SNARE physiological regulation is the ability to switch binding affinities upon changes in the phosphorylation status of SNARE monomers and/or their binding partners (15). Alkaline phosphatase was used to remove all phosphate groups from rat brain proteins and the impact on SNARE interactions was compared in BN- *versus* SDS-PAGE, or after Stx1 IP (Figure 3A-C). An antibody detecting non-specific phosphoserine sites (pSer) was used in the IP products to confirm that complete removal of phosphate groups was achieved (Figure 3C). As shown by BN-PAGE, sample dephosphorylation induced an increment of SNARE apparent molecular mass (Figure 3A). This effect is unlikely attributable to the removal of phosphate residues, since these positively charged groups are hindrances that slow protein migration towards the cathode. Consistent with the hindrance effect, in denatured samples, dephosphorylated SNARE monomers showed the typical molecular shift down described for most phosphoproteins (Figure 3A). Instead, the apparently enhanced weight of the SNARE heterotrimer following dephosphorylation may be due to the attachment of an additional partner to the complex, putatively Cplx1. A Cplx1 immunoreactive band overlaps with the shifted SNARE complex (~200 kDa), and its density was dramatically increased upon phosphatase reaction, with concomitant reductions in the monomeric and 550-kDa species (Figure 3A, right panel). None of these effects could be observed in standard SDS-gels (Figure 3B). Analysis of the Stx1 IP and co-IP products from control and phosphatase-exposed samples showed that removal of

phosphate groups did not interfere with SNARE (Stx1-SANP25-VAMP) interactions (Figure 3C). In contrast, Stx1 interactions with Munc18-1, Cplx2 (but not Cplx1), and Stg were enhanced in dephosphorylated brain homogenates. Subtle differences between BN-PAGE and IP assay conditions may modulate Stx1 affinity for Cplx1 or Cplx2 respectively, which compete *in vitro* for a binding site in the SNARE complex.

**Effects of PMI, antipsychotic drugs, and other variables.** In Cohort 1, control samples had longer PMI than schizophrenia cases (Table S1), although in both groups these values were relatively short (mean PMI = 12.6 h); for comparison, mean PMI values of most commonly used postmortem brain collections in schizophrenia research range between 12.1-34.3 h (16). The mean difference in PMI between the groups in Cohort 1 complicates the interpretation of the observed relationships between protein complexes and PMI. The following results indicate that findings related to diagnostic group do not appear to be attributable to the difference in PMI:

1) In the combined diagnostic groups (schizophrenia and controls) of Cohort 1, the amounts of SNARE protein-protein interactions in the orbitofrontal cortex (OFC), quantified either by co-IP (SNAP25-Stx1 interaction) or BN-PAGE, were negatively correlated with PMI (Table S5). However, in the same combined group, in the anterior cingulate cortex there were no statistically significant correlations between the same neurochemical measures and PMI, and a difference related to diagnostic group was still present (see main text). None of the observed relationships between neurochemical measures and PMI in the total group were statistically significant when the diagnostic groups (schizophrenia and controls) were examined separately.

2) In the independent Cohort 2, there was no difference in mean PMI between schizophrenia and controls, and the range of PMI was larger than in Cohort 1. There were no statistically significant correlations between neurochemical measures and PMI, and the same pattern of differences between neurochemical measures related to diagnostic group in Cohort 1 was observed (see main text).
3) The 150-kDa SNARE complex was additionally quantified in an independent series of control samples selected to have a similar range of PMI (7-25 h) to the schizophrenia samples (6-33 h). Within this range, no association was found between postmortem degradation and the immunodensity of native 150-kDa SNARE (Figure S6A), or other presynaptic complexes (data not shown). This is consistent with a previous study (9) also reporting no association between postmortem time, and SNARE proteins or SDS-resistant complexes in a cohort with a broader range of PMI (5-102 h).
4) Where statistically significant correlations are found between potential confounds such as PMI and neurochemical outcome studies, ANCOVA is often used to "control" for the possible effects of the potential confound. However, when the mean value of the potential confound differs between groups, the suitability of this strategy is questionable (17), and the approaches described above in (1)-(3) may be more informative. With this caveat in mind, in order to allow comparison to other literature, we provide the results from ANCOVA models for the OFC samples from Cohort 1, including diagnosis (control, schizophrenia) as a main effect, and PMI as a covariate (Table S5). All overall models were highly statistically significant. The contributions of the PMI covariate were not statistically significant in any model. The effects of diagnosis remained statistically

significant ($p$ = .0089–.0012), in six of seven models, including the primary hypothesis testing the 150-kDA SNARE (average).

Finally, we pooled the 150-kDa SNARE complex data across brain regions and cohorts in order to increase the number of available observations and power for multiple correlation analyses with the potential confounding variables, including PMI, age at death and brain pH (Figure S5). These combined analyses showed no relationship between the amount of SNARE interactions and PMI, when analyzing diagnostic groups separately, or together (Figure S5). As previously observed in non-combined analyses, no associations were found between the 150-kDa SNARE complex cortical levels, and brain pH or age at death.

The potential effect of other categorical variables (sex, presence of antipsychotics and/or benzodiazepines, smoking habit) was assessed by ANCOVA. Adding these variables (individually or together) to the analyses did not modify any of the reported findings (not shown). Increased SNARE protein-protein interactions might be the result of prior-to-death, long-term medication, perhaps even in the absence of detectable antipsychotic drugs in 13/15 schizophrenia cases from Cohort 1. Possible effects of typical (haloperidol) or atypical (clozapine) antipsychotics on cortical SNARE complex were assessed in rats. Compared with saline treatment, exposure to haloperidol or clozapine for 28 days did not significantly alter cortical 150-kDa SNARE complex associations or the total immunodensity of SNARE monomers (Figure S6B).

**Table S1.** Characteristics of control and schizophrenia groups of subjects in Cohorts 1 and 2 included in the study.[a]

| | **Cohort 1** | | **Cohort 2** | |
|---|---|---|---|---|
| Brain collection source | Macedonian/New York State Psychiatric Institute | | Stanley Foundation Neuropathology Consortium | |
| Available brain areas | BA 10/47, BA 24[b] | | BA 10/47 | |
| Group (size) | Control ($n$ = 13) | Schizophrenia ($n$ = 15) | Control ($n$ = 15) | Schizophrenia ($n$ = 15) |
| Sex | 3F / 10M | 6F / 9M | 9F / 6M | 9F / 6M |
| Age at death (years ± SD) | 51.4 ± 18.8 | 53.6 ± 12.1 | 48.1 ± 1.7 | 44.5 ± 13.1 |
| PMI (hours ± SD) | 16.6 ± 7.1 | 8.8 ± 3.4* | 23.7 ± 9.9 | 33.7 ± 14.6 |
| Brain pH (± SD) | 6.16 ± .33 | 6.32 ± .22 | 6.27 ± .24 | 6.16 ± .26 |
| Storage (months ± SD) | 137 ± 23 | 164 ± 19* | N.K. | N.K. |
| Psychotropics[c] ($n$) | | | | |
| Antipsychotics | none | 2 | none | 12 |
| Antidepressants | none | none | none | 5 |
| Mood stabilizers | none | none | none | 3 |
| Benzodiazepines | 2 | 2 | none | none |
| Anticholinergics | none | none | none | 3 |
| Alcohol abusers | none | none | none | 3 |
| Drug abusers | none | none | none | 3 |
| Cause of death ($n$) | | | | |
| Suicide | none | none | none | 4 |
| Natural | 4 | 12 | 13 | 8 |
| Accidental | 6 | 3 | 2 | 3 |
| Homicide | 3 | none | none | none |

BA, Brodmann area; F, female; M, male; N.K., not known; PMI, postmortem interval; SD, standard deviation.
[a]Individual characteristics are provided in Tables S2 (Cohort 1) and S3 (Cohort 2).
[b]BA 24 was not available for one of the control subjects in Cohort 1.
[c]The presence of psychotropic drugs in brain specimens of Cohort 1 was confirmed by standard toxicological procedures, whereas positive cases in Cohort 2 were based on medical records.
*Significantly different from control group (Student $t$-test).

**Table S2.** Individual demographic characteristics and brain toxicological data of subjects with schizophrenia and matched controls of Cohort 1 obtained from the Macedonian/New York State Psychiatric Institute.

| Group and case number | Sex | Age (years) | PMI (hours) | Brain pH | Storage (months) | Cause of death | Brain toxicology |
|---|---|---|---|---|---|---|---|
| *Control (n =13)* | | | | | | | |
| 1 | M | 26 | 15 | 5.44 | 155 | Homicide | caf |
| 2 | M | 81 | 22 | 6.28 | 155 | MVA | caf |
| 3 | M | 30 | 6 | 6.30 | 153 | MVA | negative |
| 4 | M | 73 | 21 | 6.10 | 150 | Myocardial infarction | negative |
| 5 | M | 53 | 16 | 5.90 | 151 | Homicide | caf |
| 6[a] | M | 36 | 20 | 6.30 | 151 | MVA | dzm, mdz |
| 7 | F | 79 | 33 | 6.00 | 151 | CO intoxication | negative |
| 8 | M | 35 | 10 | 6.50 | 151 | MVA | caf |
| 9 | M | 37 | 7 | 6.20 | 151 | Heart failure | negative |
| 10 | M | 56 | 14 | 6.41 | 109 | Myocardial infarction | caf |
| 11 | F | 41 | 15 | 5.84 | 108 | Homicide | caf |
| 12 | M | 65 | 18 | 6.77 | 100 | Myocardial infarction | caf |
| 13 | F | 56 | 19 | 6.08 | 100 | MVA | dzm, caf |
| *Schizophrenia (n =15)* | | | | | | | |
| 14 | M | 61 | 9 | 6.50 | 150 | Bronchiectasis | negative |
| 15 | F | 60 | 9 | 6.40 | 150 | Myocardial infarction | dzm, clo |
| 16 | M | 53 | 8 | 6.30 | 150 | Myocardial infarction | dzm, clo |
| 17 | M | 53 | 15 | 6.30 | 135 | Asphyxia, pharyngeal bolus | negative |
| 18 | M | 35 | 6 | 6.40 | 134 | Ileus | caf |
| 19 | M | 45 | n.d. | 6.50 | 191 | Accidental drowning | ndz, caf |
| 20 | F | 45 | 7 | 6.20 | 192 | Pneumonia | negative |
| 21 | F | 77 | 6 | 5.90 | 187 | Myocardial infarction | dzm, eto |
| 22 | M | 46 | 14 | 6.40 | 186 | Pancreatitis necrotica | negative |
| 23 | M | 33 | 15 | 6.50 | 174 | Peritonitis | negative |
| 24 | M | 58 | 6 | 6.00 | 159 | Pneumonia | negative |
| 25 | F | 63 | 7 | 6.60 | 159 | Myocardial infarction | negative |
| 26 | M | 45 | 8 | 6.40 | 158 | n.d. (non confirmed CVA) | dzm, ndz, caf |
| 27 | F | 65 | 6 | 6.50 | 168 | CRI | caf |
| 28 | F | 65 | 7 | 5.90 | 166 | Myocardiopathy, CRI | negative |

caf, caffeine; clo, colzapine; CO, carbon monoxide; CRI, cardio-respiratory insufficiency; CVA, cerebral vascular accident; dzm, diazepam; eto, etophylline; F, female; M, male; mdz, midazolam, MVA, motor vehicle accident; n.d., not determined; ndz, nordiazepam.
[a]BA 24 was not available for this subject.

**Table S3.** Individual demographic characteristics of subjects with schizophrenia and matched controls included of Cohort 2 obtained from the Stanley Foundation Neuropathology Consortium.

| Case # | Sex | Race | Age (y) | PMI (h) | Brain pH | Cause of death[a] | Prescribed psychotropics | Lifetime APD[b] | Alcohol use[c] | Drug use[d] |
|---|---|---|---|---|---|---|---|---|---|---|
| *Control group (n = 15)* | | | | | | | | | | |
| 1 | F | W | 52 | 28 | 6.5 | Cardiac arrest | none | 0 | 2 | 0 |
| 2 | M | W | 44 | 25 | 6.3 | Cardiac arrest | none | 0 | 1 | 0 |
| 3 | F | W | 59 | 26 | 6.4 | Cardiac arrest | none | 0 | 3 | 0 |
| 4 | F | W | 52 | 8 | 6.5 | Cardiac arrest | none | 0 | 1 | 0 |
| 5 | F | W | 52 | 22 | 6.2 | Cardiac arrest | none | 0 | 3 | 0 |
| 6 | F | W | 53 | 28 | 6.2 | Cardiac arrest | none | 0 | 2 | 1 |
| 7 | F | W | 44 | 10 | 6.4 | Cardiac arrest | none | 0 | 1 | 0 |
| 8 | M | W | 35 | 23 | 6.6 | Cardiac arrest | none | 0 | 1 | 0 |
| 9 | F | NW | 41 | 11 | 6.0 | PE | none | 0 | 1 | 0 |
| 10 | F | W | 42 | 27 | 6.6 | Cardiac arrest | none | 0 | 0 | 0 |
| 11 | M | W | 35 | 40 | 5.8 | PE | none | 0 | 0 | 0 |
| 12 | M | W | 68 | 13 | 6.3 | PE | none | 0 | 0 | 0 |
| 13 | F | W | 58 | 27 | 6.0 | Cardiac arrest | none | 0 | 1 | 0 |
| 14 | M | W | 29 | 42 | 6.2 | MVA | none | 0 | 0 | 0 |
| 15 | M | W | 57 | 26 | 6.0 | MVA | none | 0 | 0 | 0 |
| *Schizophrenia group (n = 15)* | | | | | | | | | | |
| 16 | M | W | 30 | 60 | 6.2 | Jumped (s) | thx, dpm | 6000 | 2 | 3 |
| 17 | F | W | 52 | 61 | 6.0 | Cardiac arrest | none | 9000 | 0 | 0 |
| 18 | F | W | 30 | 32 | 5.8 | Pneumonia | thz, ris | 50000 | 1 | 0 |
| 19 | M | NW | 62 | 26 | 6.1 | MVA | none | 50000 | 1 | 0 |
| 20 | M | W | 60 | 40 | 6.2 | Cardiac arrest | none | 0 | 0 | 0 |
| 21 | F | W | 60 | 31 | 6.2 | Drowning | thz, clo, ami | 80000 | 0 | 0 |
| 22 | F | NW | 32 | 19 | 6.1 | Overdose | clo | 15000 | 5 | 4 |
| 23 | F | W | 31 | 14 | 5.8 | Jumped (s) | clo | 4000 | 1 | 0 |
| 24 | M | W | 58 | 26 | 5.9 | Cardiac arrest | hal | 35000 | 4 | 0 |
| 25 | F | W | 25 | 32 | 6.6 | Hanging (s) | ris, par | 4000 | 4 | 0 |
| 26 | F | W | 44 | 50 | 6.5 | Cardiac arrest | hal, czp, flu, bnz | 100000 | 3 | 0 |
| 27 | F | W | 44 | 29 | 5.9 | COPD | chl, clo, lit | 130000 | 2 | 3 |
| 28 | M | NW | 56 | 12 | 6.4 | Overdose (s) | hal, lit | 150000 | 0 | 1 |
| 29 | F | W | 35 | 35 | 6.5 | Cardiac arrest | chl, clo, map, bnz | 50000 | 3 | 2 |
| 30 | M | W | 49 | 38 | 6.2 | Medical | clo | 150000 | 0 | 0 |

ami, amitriptyline; APD, antipsychotic drugs; bnz, benztropine; chl, chlorpromazine; clo, colzapine; COPD, chronic obstructive pulmonary disease; czp, carbamazepine; dzm, diazepam; eto, etophylline; F, female; flu, fluoxetine; h, hours; hal, haloperidol; lit, lithium; M, male; MVA, motor vehicle accident; n.d., not determined; NW, nonwhite; par, paroxetine; PE, pulmonary embolism; ris, risperidone; s, suicide; thx, thiothixene; thz, thioridazine; W, white; y, years.

[a]Suicide cases are indicated (s).

[b]Estimation of lifetime APD consumption was based on medical records.

[c]Lifetime alcohol use scaled as follows: 0, little or none alcohol use (<1 drink/day); 1, social drinker (1-2 drinks/day); 2, moderate drinker in the past (>2 drinks/day); 3, moderate drinker by the time of death; 4, heavy drinker in the past (met criteria for alcohol abuse/dependence); 5, heavy drinker by the time of death.

[d]Lifetime drug use scaled as follows: 0, little or none drug use; 1, moderate use in the past; 2, moderate use by the time of death; 3, heavy abuse in the past (met criteria for substance abuse/dependence); 4, heavy abuse by the time of death.

**Table S4.** Antibodies used for characterization and quantification presynaptic proteins and complexes in present study.

*Commercially available antibodies*

| Target protein | Immunogen | Host | Clone | Subtype | Dilution | Cat. No. | Vendor |
|---|---|---|---|---|---|---|---|
| Syntaxin-1A | Rat recombinant Stx1A (N-t residues) | Mouse | 78.3 | IgG2a | 1:5,000 | 110 111 | Synaptic Systems |
| Syntaxin-1B | Stx1B synthetic peptide (residues 171-187) | Rabbit | Polyclonal | - | 1:5,000 | 110 402 | Synaptic Systems |
| SNAP25 | Human SNAP25 (acetylated N-t residues) | Mouse | SMI 81 | IgG1 | 1:2,000 | SMI-81R | Covance |
| Munc18-1 | Munc18-1a peptide (residues 591-603) | Goat | Polyclonal | - | 1:2,000 | PAB6504 | Abnova |
| Phosphoserine | Phosphoserine containing proteins | Rabbit | Polyclonal | - | 1:750 | 61-8100 | Invitrogen |
| β-actin | β-actin synthetic peptide (residues 2-16) | Mouse | AC-15 | IgG1 | 1:10,000 | ab6276 | Millipore |

*Locally produced mouse monoclonal antibodies*

| Target protein | Immunogen | Host | Clone | Subtype | Dilution | Reference |
|---|---|---|---|---|---|---|
| Syntaxin-1 | Crude human brain immunoprecipitate | Mouse | SP7 | IgG2a | 1:100 | (6) |
| Syntaxin-1 | Crude human brain immunoprecipitate | Mouse | SP6 | IgG1 | 1:100 | (6) |
| SNAP25 | Crude human brain immunoprecipitate | Mouse | SP12 | IgG1 | 1:100 | (6) |
| SNAP25 | Crude human brain immunoprecipitate | Mouse | SP14 | IgG1 | 1:100 | (6) |
| VAMP | Crude human brain immunoprecipitate | Mouse | SP10 | IgM | 1:10 | (6) |
| VAMP | Crude human brain immunoprecipitate | Mouse | SP11 | IgG1 | 1:10 | (6) |
| Complexin 1 | Purified synthetic Cplx1 | Mouse | SP33 | IgG1 | 1:10 | (7) |
| Complexin 2 | Purified synthetic Cplx2 | Mouse | LP27 | IgG1 | 1:10 | (7) |
| Synaptotagmin[a] | Rat brain synaptic membranes | Mouse | MAB48 | IgG2b | 1:50 | (8) |

[a]Hybridomas were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242.

**Table S5.** Altered proteins or complexes in the OFC (BA 10/47) of schizophrenia subjects in Cohort 1 potentially influenced by postmortem interval (PMI), and analyses of the covariance (ANCOVA).

| Neurochemical finding | | Pearson's correlation with PMI | | ANCOVA | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Whole model effects | | | | Diagnose | | PMI | |
| **Technique** | **Protein or complex** | ***r*-value** | ***p*-value** | $r^2$ | ***df*** | ***F*-ratio** | ***p*-value** | ***F*-ratio** | ***p*-value** | ***F*-ratio** | ***p*-value** |
| SNAP25-IP | Stx1 | –.4959 | .0085 | .4752 | 2, 24 | 10.864 | .0004 | 10.481 | .0035 | .5680 | .4584 |
| | Cplx1 | –.4335 | .0239 | .4235 | 2, 24 | 8.8152 | .0013 | 11.891 | .0021 | .0197 | .8895 |
| BN-PAGE | 150-kDa SNARE (Stx1) | –.4770 | .0119 | .5512 | 2, 24 | 12.605 | .0002 | 13.399 | .0012 | .3220 | .5757 |
| | 150-kDa SNARE (SNAP25) | –.5057 | .0071 | .3329 | 2, 24 | 5.9877 | .0076 | 3.6905 | .0667 | 1.344 | .2577 |
| | 150-kDa SNARE (VAMP) | –.4182 | .0300 | .3959 | 2, 24 | 7.8649 | .0024 | 9.4105 | .0053 | .0330 | .8573 |
| | 150-kDa SNARE (average) | –.4923 | .0091 | .4894 | 2, 24 | 11.503 | .0003 | 11.327 | .0026 | .5305 | .4734 |
| | 200-kDa Cplx1 | –.4575 | .0164 | .4196 | 2, 24 | 8.6740 | .0015 | 8.0997 | .0089 | .5431 | .4683 |

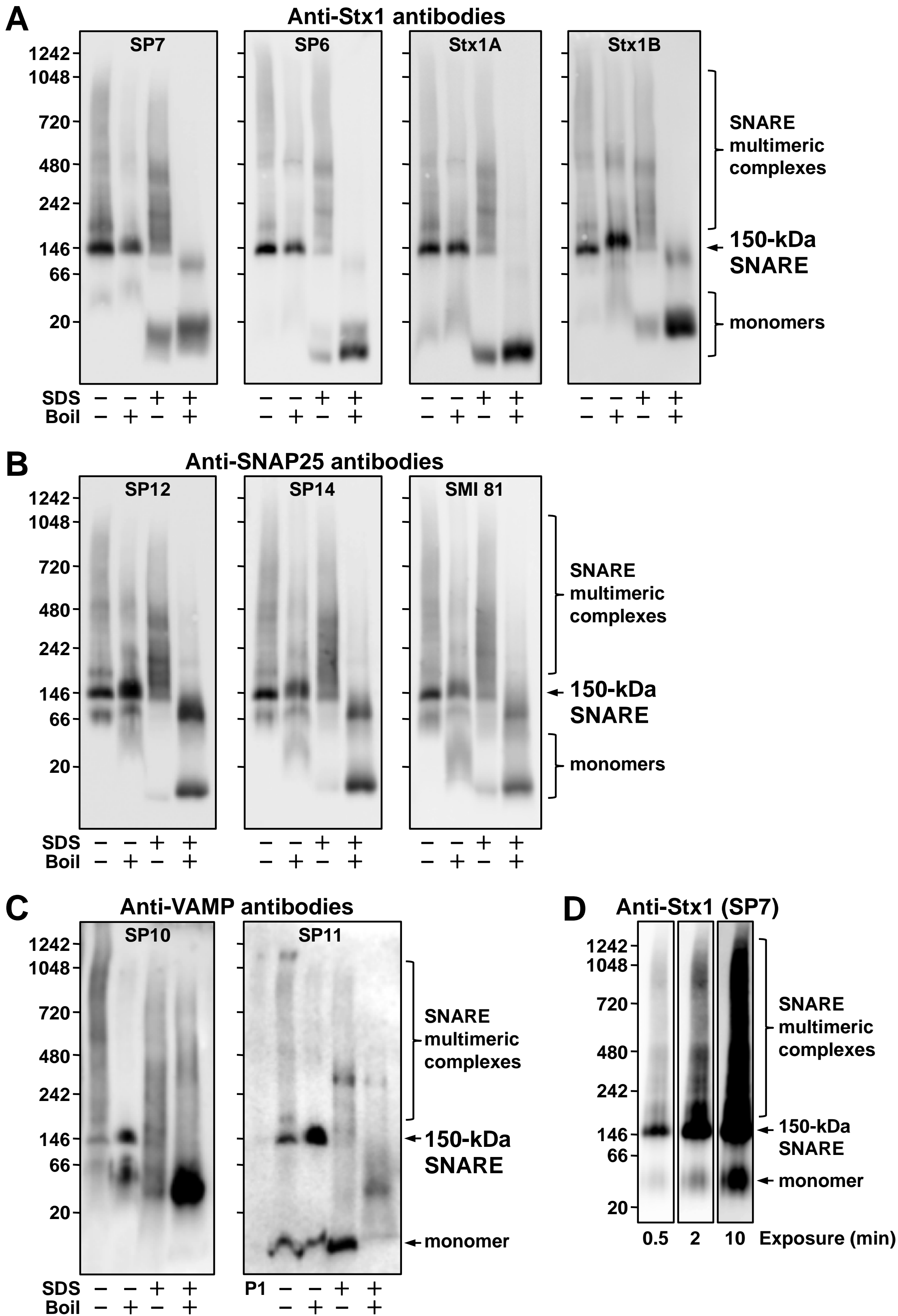
A
Anti-Stx1 antibodies
SP7
SP6
Stx1A
Stx1B
1242
1048
720
480
242
146
66
20
SNARE multimeric complexes
150-kDa SNARE
monomers
SDS − − + +
Boil − + − +
B
Anti-SNAP25 antibodies
SP12
SP14
SMI 81
C
Anti-VAMP antibodies
SP10
SP11
monomer
P1
D
Anti-Stx1 (SP7)
0.5 2 10 Exposure (min)

**Figure S1.** Characterization of Stx1, SNAP25 and VAMP complexes by blue-native (BN)-PAGE. **(A-C)** BN gradient gels (4-16% acrylamide) were loaded with 5-µg protein aliquots from TX100-solubilized human cortical samples under native conditions or after harsh denaturation (.1% SDS and/or 5 min boiling). Protein complexes were transferred to PVDF membranes and immunoblotted with anti-Stx1 (SP7, SP6) or with Stx-1A/1B isoform-specific antibodies **(A)**, anti-SNAP25 (SP12, SP14, SMI 81) **(B)**, or anti-VAMP (SP10, SP11) **(C)**. Very similar staining patterns were observed across the antibodies targeting one particular SNARE monomer. The putative ~150-kDa SNARE complex was detected regardless of the SNARE protein probed with all antibodies used. Note that SP7, SP12 and SP10 immunoblots are replicates from Figure 2A. For comparison, the TX100-insoluble fraction (P1) is also shown in the SP11 immunoblot. The anti-VAMP (SP10) staining detected in the P1 fraction in Figure 2A did not result in a discrete pattern of bands, a characteristic of non-specific binding profiles (especially for IgM antibodies), and an anti-VAMP IgG1 (SP11) showed no staining on the same P1 sample. Therefore, the anti-VAMP signal on the TX100-unsoluble fraction was considered spurious. **(D)** Comparison at increasing exposure times (.5-10 min) of the same immunoblot (SP7) containing a human brain sample analyzed by BN-PAGE as above. Monomeric forms were only detected after high exposure times. **(A-D)** Molecular masses (in kDa) of native and SDS-PAGE prestained standards are shown on the left.

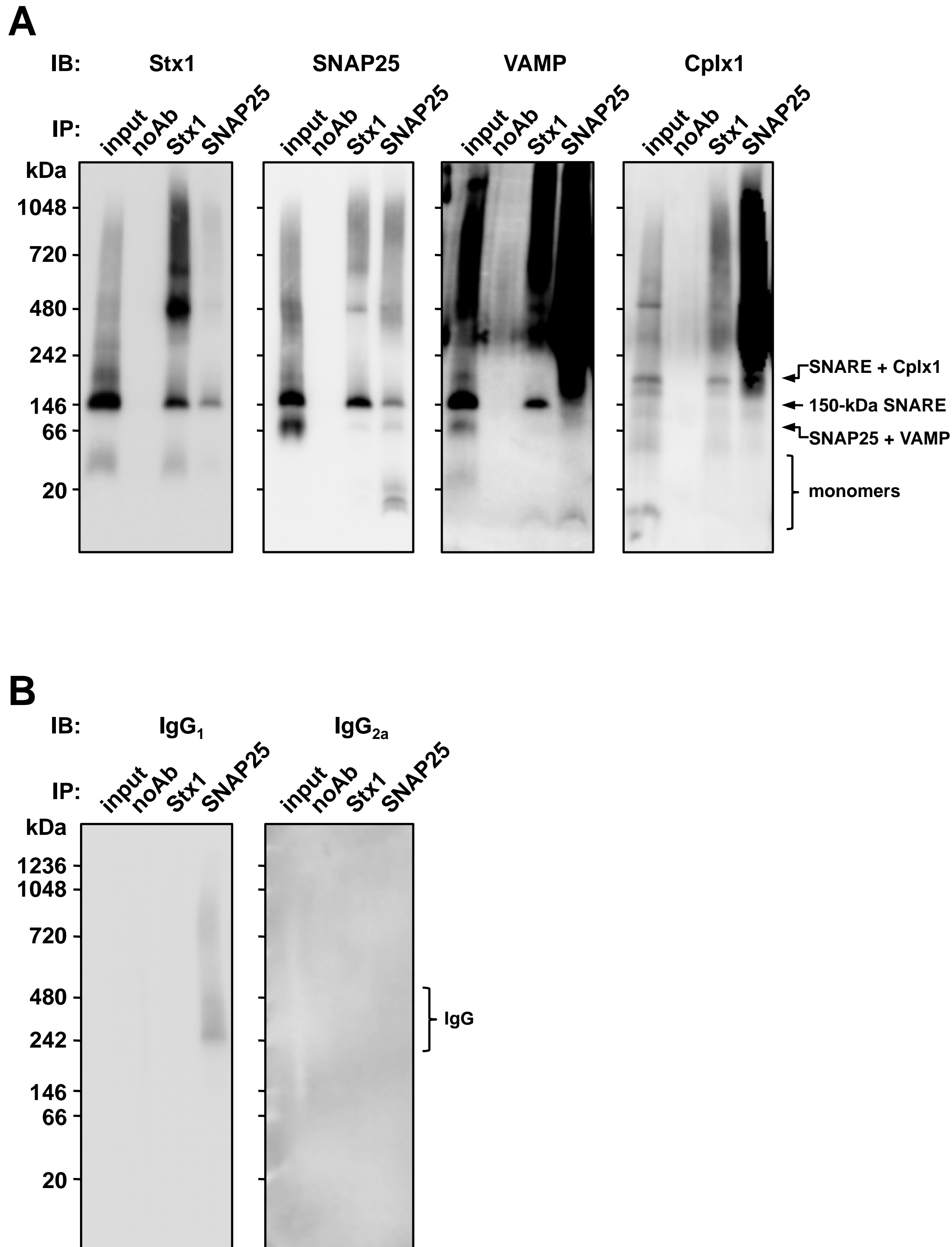


**Figure S2.** Characterization of SNARE and SNARE-Cplx1 oligomers by BN-PAGE after immunoprecipitation (IP) with antibodies against Stx1 (SP7) and SNAP25 (SP12) from human orbitofrontal cortex. **(A)** Solubilized human brain proteins were incubated with magnetic beads pre-coated in absence (noAb; negative control) or presence of one of the antibodies above, and extracted in native elution buffer (pH 2.3). Five µl of the resulting pellets, alongside 5 µg of IP

input sample, were resolved by BN-PAGE followed by immunoblotting (IB). The ~150-kDa SNARE heterotrimer was precipitated with both anti-Stx1 and anti-SNAP25 antibodies, and detected with all SNARE antibodies. The ~200-kDa Cplx1 major band was also precipitated with both anti-Stx1 and anti-SNAP25 antibodies. For all tested antibodies, null immunoreactivity was detected in negative control samples. **(B)** The same samples were processed as above and immunoblots were exposed to peroxidase-conjugated anti-mouse $IgG_1$ or $IgG_{2a}$, omitting the primary antibody incubation. Minimal ($IgG_1$) or null ($IgG_{2a}$) signals were obtained even after long exposure times (10 min). The spurious staining at large molecular weights in VAMP and Cplx1 immunoblots **(A)** may correspond to IgG-bound SNARE/Cplx1, which resisted mild elution, or reassembly during the BN-PAGE procedure. **(A and B)** Molecular mass (in kDa) of native and SDS-PAGE prestained standards are shown on the left.

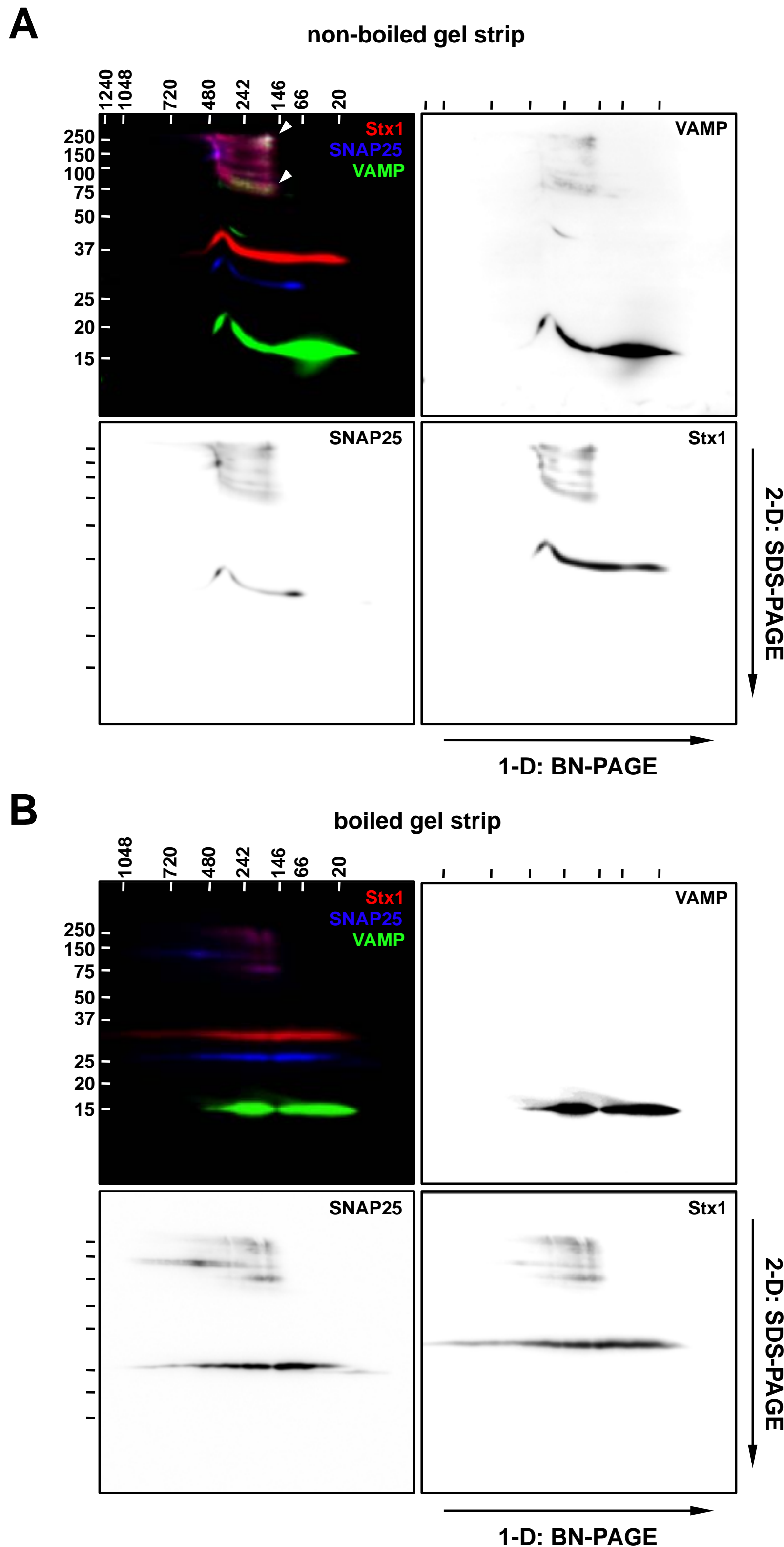


**Figure S3.** Characterization of rat cortical SNARE complexes by two-dimension (2-D) blue-native (BN)/SDS-PAGE. BN gradient gels (4-16% acrylamide) were loaded with 5-µg protein aliquots of solubilized rat cortical samples. After BN-PAGE, two gel strips were separated and incubated for 15 min in SDS-loading buffer at room temperature **(A)** or after brief boiling **(B)**. Both gel strips were further separated by SDS-PAGE and proteins were transferred to PVDF membranes and sequentially probed with antibodies against VAMP (SP10), Stx1 (SP7) and SNAP25 (SP12). Captured images were arbitrarily assigned to one RGB channel and merged to visualize overlapping spots (indicated with white arrowheads). **(A and B)** Molecular masses (in kDa) of native and SDS-PAGE prestained standards are shown above and at left, respectively.

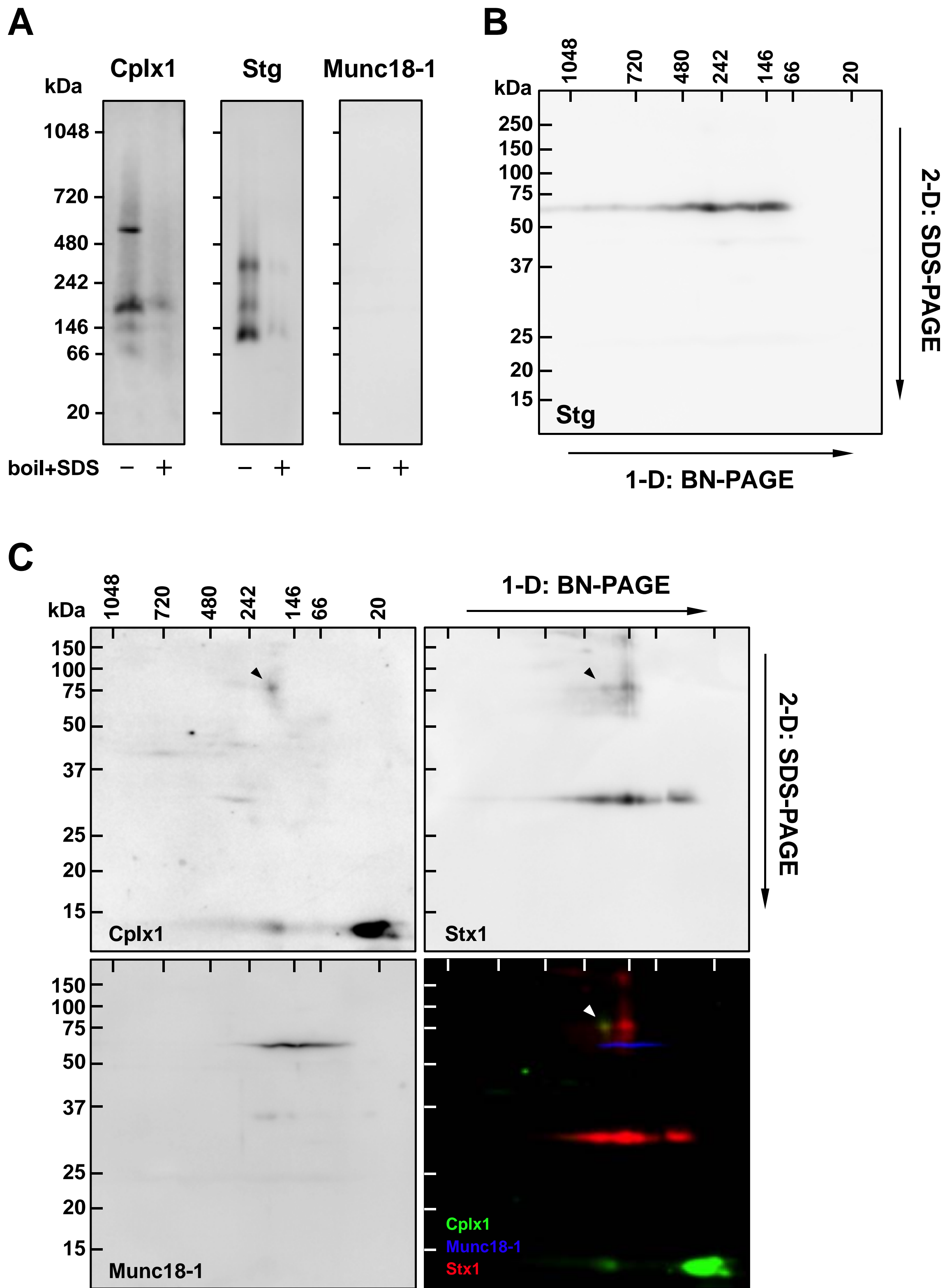


**Figure S4.** Characterization of Cplx1, Stg, and Munc18-1 complexes by one-dimension (1-D) **(A)** and two-dimension (2-D) **(B and C)** blue-native (BN)/SDS-PAGE. **(A)** BN gradient gels (4-16% acrylamide) were loaded with 10-µg protein aliquots of solubilized human cortical samples,

under native conditions or after harsh denaturation (.1% SDS and 5 min boiling). Protein complexes were transferred to PVDF membranes and immunoblotted with anti-Cplx1 (SP33), anti-Stg (MAB48), or anti-Munc18-1 antibodies. **(B and C)** Two BN-gel strips containing non-denatured samples from **(A)** were sliced out prior to transference, incubated 15 min in SDS-loading buffer, and mounted onto a 12% SDS-PAGE for 2-D electrophresis. Proteins were then transferred to PVDF membranes and sequentially probed with anti-Stg (MAB48) alone **(B)**, or sequentially with anti-Cplx1 (SP33), anti-Stx1 (SP7), and Munc18-1 antibodies **(C)**. Captured images were arbitrarily assigned to one RGB channel and merged to visualize overlapping spots (arrowheads), indicating the presence of a Cplx1-SNARE complex, which corresponds to the ~200-kDa band observed in Cplx1 immunoblots after 1-D BN-PAGE. **(A-C)** Molecular masses (in kDa) of native and SDS-PAGE prestained standards are shown on the left and above.

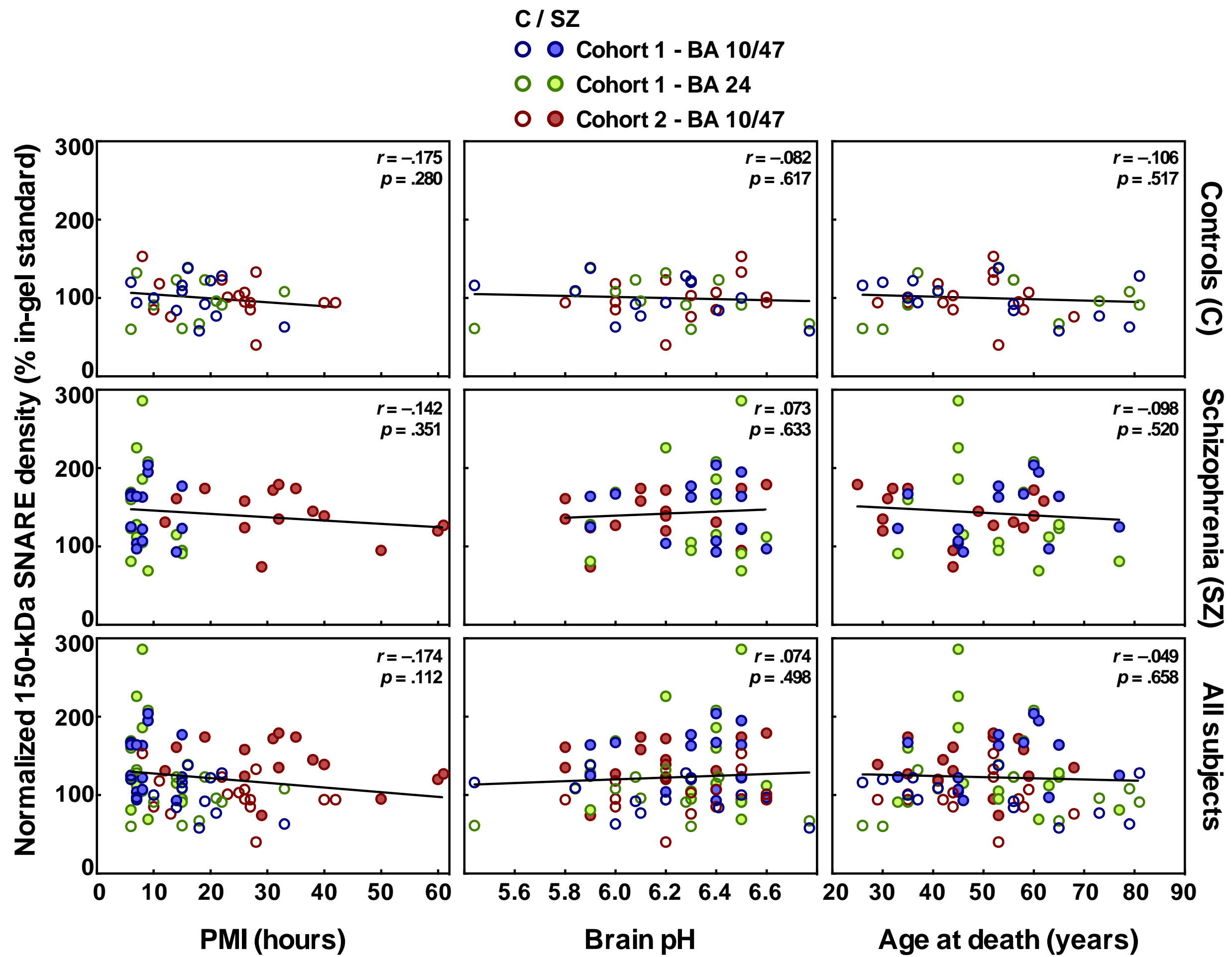


**Figure S5.** Scatterplots depicting the absence of correlation between postmortem interval (PMI, left panels), brain pH (center panels) or age at death (right panels), and the immunodensity of 150-kDa SNARE complex (average of three anti-SNARE antibodies) in the orbitofrontal and/or the anterior cingulate cortices of control (C, upper panels), schizophrenia (SZ, middle panels), or all subjects together (bottom panels) from Cohorts 1 and 2 combined. For each analysis Pearson's coefficient and *p*-value are provided.

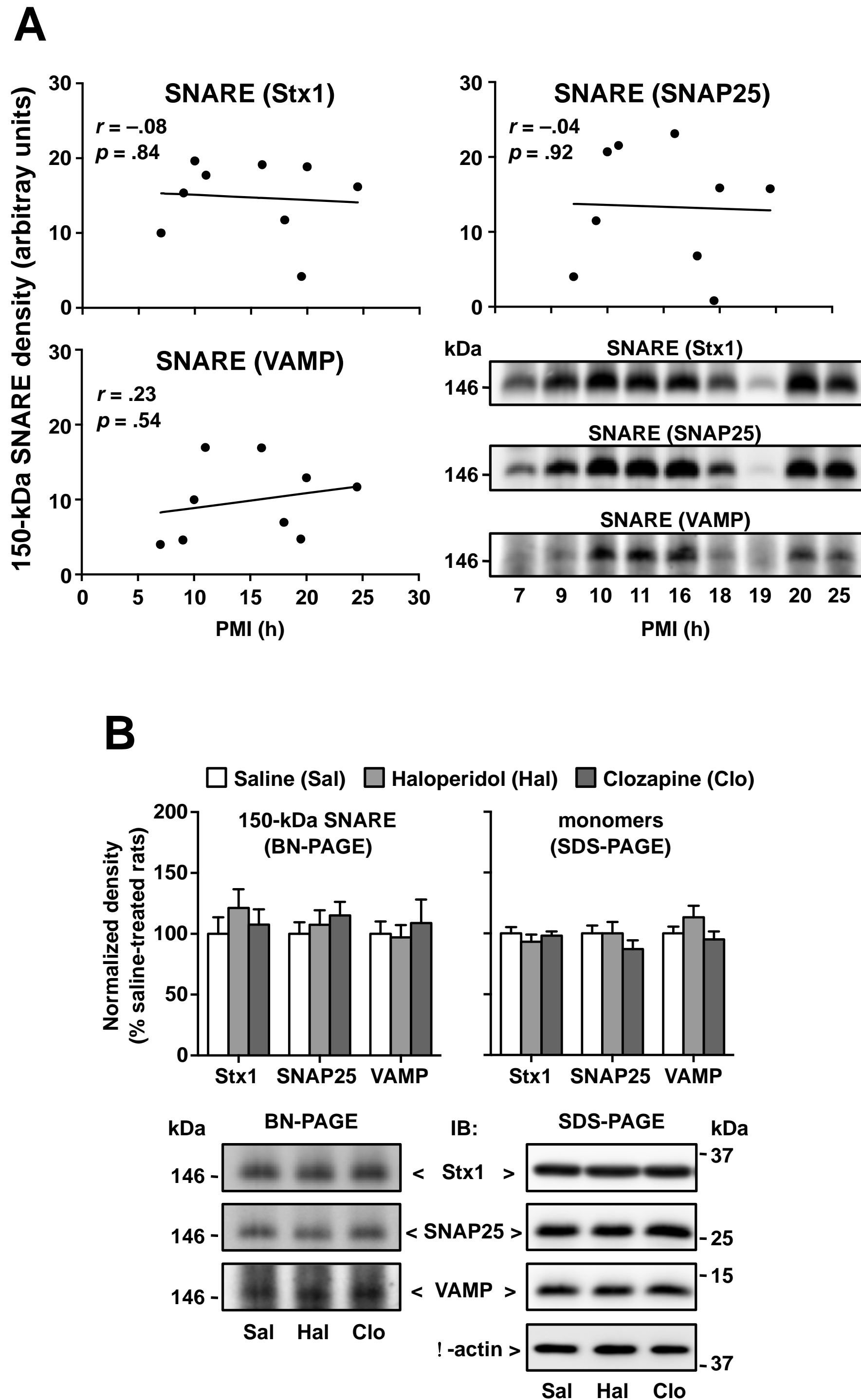


**Figure S6. (A)** Effect of postmortem degradation on the preservation of 150-kDa SNARE complexes in human brain tissue. Cortical samples were collected from 9 previously healthy men suffering sudden and unexpected deaths, and SNARE complex density was quantified by blue native (BN)-PAGE followed by immunoblotting with anti-Stx1 (SP7), anti-SNAP25 (SP12) or

anti-VAMP (SP10) antibodies. Raw individual densities (in arbitrary units) are represented versus sample postmortem interval value (PMI; range: 7-25 h). Linear decay models do not show a significant effect of postmortem interval on native 150-kDa SNARE density. **(B)** Effect of chronic antipsychotic treatments on rat cortical 150-kDa SNARE complexes. Animals were treated with saline-vehicle (Sal; $n$ = 10), haloperidol (Hal, 1 mg/kg; $n$ = 10) or clozapine (Clo, 20 mg/kg; $n$ = 10) for 28 days. Cortical densities of native 150-kDa SNARE complex (left) and monomers (right) were quantified by BN- or SDS-PAGE, respectively, followed by immunoblotting (IB) with anti-Stx1 (SP7), anti-SNAP25 (SP12) or anti-VAMP (SP10) antibodies. β-actin content was used as a loading control. Columns represent mean ± SEM values in percent to saline-treated animals. For each antibody, representative immunoblots of 150-kDa SNARE complex (left) and monomers (right) are shown below the plots.

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