Markedly Lower Glutamic Acid Decarboxylase 67 Protein Levels in a Subset of Boutons in Schizophrenia

Supplemental Information

Table S1. Detailed demographic and postmortem characteristics of human subjects. There were no statistical differences between schizophrenia and comparison subjects in age (45.05 ± 7.27 yrs and 45.75 ± 8.69 yrs, respectively), PMI (9.53 ± 3.39 hrs and 9.92 ± 4.04 hrs, respectively), and storage time (11.69 ± 5.29 yrs and 13.78 ± 5.28 yrs, respectively). Previously studied designates histology or protein expression studies that included the pair (1-6). Numbers designate the associated reference(s).

Healthy Comparison Subjects							Subjects with Schizophrenia										
Pair	Case	Sex/ Race	Age (yrs)	PMI[a]	Storage Time[b]	Cause of Death	Case	DSM IV diagnosis	Sex/ Race	Age (yrs)	PMI[a]	Storage Time[b]	Cause of Death	Antipsy-chotic ATOD	Antide-pressant ATOD	Benzodiaze-pine/ Anticon-vulsant ATOD	Nicotine Use ATOD	Previously studied
1	727	M/B	19	7.00	12.90	Trauma	829	Schizoaffective disorder; ADC; OAR	M/W	25	5.00	11.34	Suicide by salicylate overdose	N	N	Y	Y	1,2,3,6
2	852	M/W	54	8.00	10.90	Cardiac tamponade	781	Schizoaffective disorder; ADR	M/B	52	8.00	12.16	Peritonitis	Y	Y	N	Y	1,2,3
3	1307	M/B	32	4.80	5.20	ASCVD	10024	Paranoid schizophrenia	M/B	37	5.98	5.96	ASCVD	N	N	N	N	1
4	567	F/W	46	15.00	14.80	Mitral valve prolapse	537	Schizoaffective disorder	F/W	37	14.50	15.25	Suicide by hanging	N	N	N	U	1,2,6
5	1047	M/W	43	13.80	8.10	ASCVD	1209	Schizoaffective disorder	M/W	35	9.1	6.50	Suicide by diphenhydramine overdose	Y	N	N	N	1
6	739	M/W	40	15.8	13	ASCVD	933	Disorganized schizophrenia	M/W	44	8.30	9.66	Myocarditis	Y	Y	Y	N	1,3
7	451	M/W	48	12.00	16.30	ASCVD	317	Chronic undifferentiated schizophrenia	M/W	48	8.30	18.9	Bronchopneumonia	Y	Y	N	N	5
8	178	M/W	48	7.80	20.50	ASCVD	377	Chronic undifferentiated schizophrenia	M/W	52	10.00	18.1	Gastrointestinal bleeding	Y	N	N	N	4,5
9	452	F/W	40	14.30	16.30	ASCVD	341	Chronic undifferentiated schizophrenia	F/W	47	14.50	18.6	Suicide, chlor-promazine overdose	Y	N	N	Y	6
10	449	F/W	47	4.30	16.30	Accidental CO poisoning	517	Chronic disorganized schizophrenia	F/W	48	3.70	15.10	Intracerebral hemorrhage	Y	N	N	Y	4,5
11	681	M/W	51	11.60	14.10	Hypertrophic cardiomyopathy	234	Chronic paranoid schizphrenia	M/W	51	12.80	21.00	Cardio-myopathy	N	N	N	N	4,5,6
12	395	M/W	42	12.30	18.80	Pericardial tamponade	322	Chronic undifferentiated schizophrenia	M/W	40	8.50	20.20	Suicide, combined drug overdose	Y	Y	N	N	NPS
13	575	F/B	55	11.30	15.40	ASCVD	597	Schizoaffective disorder	F/W	46	10.10	15.1	Pneumonia	Y	Y	N	Y	6
14	278	M/W	50	4.50	20.40	ASCVD	640	Chronic paranoid schizphrenia	M/W	49	5.20	14.5	Pulmonary Embolism	Y	Y	N	U	NPS
15	1284	M/W	55	6.40	5.60	ASCVD	1105	Schizoaffective disorder	M/W	53	7.90	7.47	ASCVD	Y	N	N	Y	NPS
16	1122	M/W	55	15.40	7.30	Cardiac Tamponade	930	Disorganized schizophrenia; ADR; OAR	M/W	47	15.30	9.70	ASCVD	Y	N	Y	Y	NPS
17	250	F/W	47	5.30	19.70	ASCVD	398	Schizoaffective disorder	F/W	41	10.30	17.7	Pulmonary embolus	Y	N	Y	U	4,5,6
18	412	M/W	42	14.20	17.50	Aortic stenosis	422	Chronic paranoid schizphrenia	M/W	54	11.00	17.20	ASCVD	Y	N	Y	U	4,5
19	344	M/W	50	6.80	18.60	ASCVD	1296	Undifferentiated schizophrenia	M/W	48	7.80	5.42	Pneumonia	Y	Y	N	Y	NPS
20	1391	F/W	51	7.8	4	ASCVD	1189	Schizoaffective disorder; AAR	F/W	47	14.4	7	Suicide by combined drug overdose	Y	Y	Y	Y	1

[a]PMI, postmortem interval (hours).
[b]Years stored in 30% glycerin/30% ethylene glycol solution at -30°C.
ASCVD, arteriosclerotic cardiovascular disease; ATOD, at time of death; U, unknown; M, male; F, female; W, white; B, black; NPS, not previously studied.

Table S2. *F*-statistics and *p*-values for the data presented in Figures 2, 3, 4, and S1 on vGAT+ and vGAT+/GAD67+ bouton densities and protein levels, and ChC bouton protein levels within different PFC cortical layers. Bolded values indicate significant differences.

	vGAT+ Boutons		**vGAT+/GAD67+ Boutons**			**ChC Boutons**	
Layer	**Boutons/μm^3**	**vGAT Levels**	**Boutons/μm^3**	**vGAT Levels**	**GAD67 Levels**	**GAD67 Levels**	**vGAT Levels**
1	$F_{1,19}$ = 0.26 p = 0.618; $F_{1,37}$ = 0.32 p = 0.574	$F_{1,19}$ = 1.25 p = 0.278; $F_{1,37}$ = 1.32 p = 0.258	$F_{1,19}$ = 3.15 p = 0.092; $F_{1,38}$ = 3.17 p = 0.083	$F_{1,19}$ = 0.01 p = 0.979; $F_{1,38}$ = 0.01 p = 0.978	**$F_{1,19}$ = 4.66** **p = 0.044**; **$F_{1,38}$ = 4.22** **p = 0.047**	No cartridges	No cartridges
2/3s	$F_{1,19}$ = 2.51 p = 0.129; $F_{1,38}$ = 1.84 p = 0.183	$F_{1,19}$ = 1.70 p = 0.208; $F_{1,38}$ = 2.10 p = 0.156	**$F_{1,19}$ = 9.60** **p = 0.006;** **$F_{1,38}$ = 9.18** **p = 0.004**	$F_{1,19}$ = 0.31 p = 0.587; $F_{1,38}$ = 0.29 p = 0.595	**$F_{1,19}$ = 7.24** **p = 0.014**; **$F_{1,38}$ = 7.17** **p = 0.011**	$F_{1,19}$ = 1.58 p = 0.224; $F_{1,38}$ = 1.81 p = 0.186	$F_{1,19}$ = 1.48 p = 0.238; $F_{1,38}$ = 1.07 p = 0.307
3d/4	$F_{1,19}$ = 0.88 p = 0.359; $F_{1,38}$ = 0.61 p = 0.439	$F_{1,19}$ = 2.52 p = 0.129; $F_{1,38}$ = 2.84 p = 0.100	**$F_{1,19}$ = 10.08** **p = 0.005**; **$F_{1,38}$ = 10.65** **p = 0.002**	$F_{1,18}$ = 0.34 p = 0.569; $F_{1,38}$ = 0.08 p = 0.779	**$F_{1,19}$ = 10.26** **p = 0.005**; **$F_{1,38}$ = 9.99** **p = 0.003**	$F_{1,19}$ = 0.66 p = 0.427; $F_{1,38}$ = 0.81 p = 0.374	$F_{1,19}$ = 0.49 p = 0.494; $F_{1,38}$ = 0.51 p = 0.478
5	$F_{1,19}$ = 0.04 p = 0.848; $F_{1,38}$ = 0.03 p = 0.876	**$F_{1,19}$ = 5.09** **p = 0.04**; **$F_{1,38}$ = 5.46** **p = 0.025**	**$F_{1,19}$ = 13.17** **p = 0.002**; **$F_{1,38}$ = 17.06** **p<0.0005**	$F_{1,18}$ = 0.01 p = 0.962; $F_{1,38}$ = 0.05 p = 0.822	**$F_{1,18}$ = 11.14** **p = 0.004**; **$F_{1,37}$ = 8.53** **p = 0.006**	$F_{1,19}$ = 2.67 p = 0.118; $F_{1,38}$ = 1.87 p = 0.179	$F_{1,19}$ = 0.21 p = 0.653; $F_{1,38}$ = 0.23 p = 0.638
6	$F_{1,19}$ = 0.25 p = 0.621; $F_{1,37}$ = 0.18 p = 0.673	**$F_{1,19}$ = 4.83** **p = 0.041**; $F_{1,37}$ = 3.64 p = 0.064	**$F_{1,19}$ = 9.05** **p = 0.007**; **$F_{1,38}$ = 10.98** **p = 0.002**	$F_{1,19}$ = 0.01 p = 0.965; $F_{1,37}$ = 0.03 p = 0.865	**$F_{1,19}$ = 7.61** **p = 0.013**; **$F_{1,37}$ = 4.77** **p = 0.035**	$F_{1,19}$ = 2.88 p = 0.106; $F_{1,38}$ = 2.68 p = 0.110	$F_{1,19}$ = 0.04 p = 0.850; $F_{1,38}$ = 0.02 p = 0.881

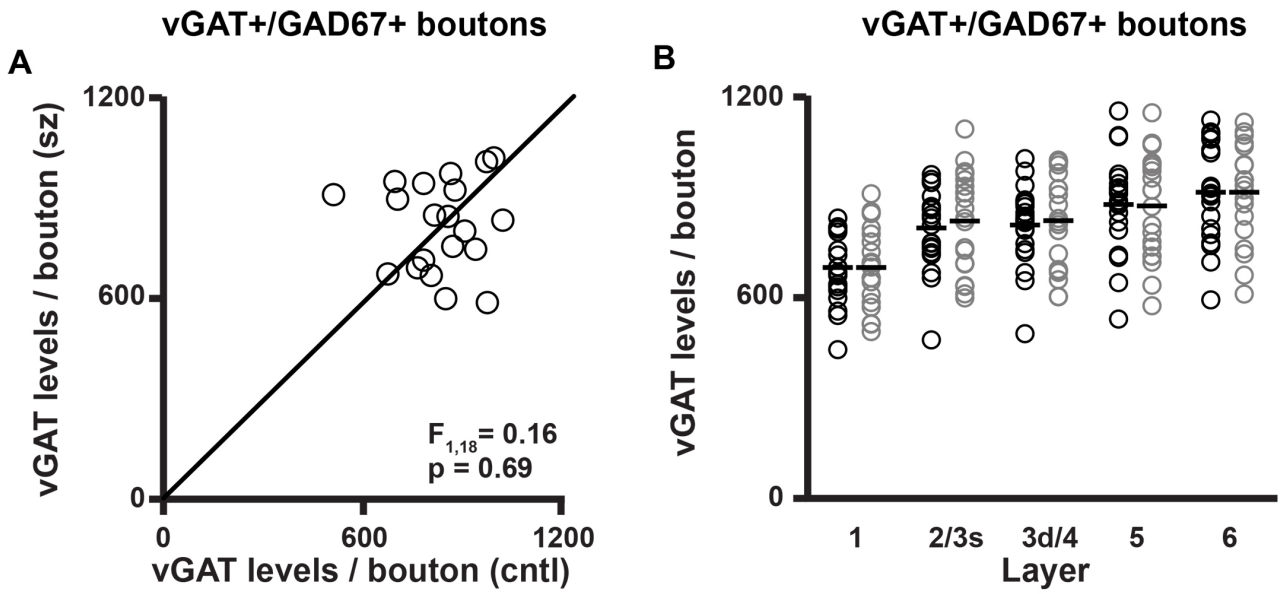

Figure S1. vGAT+/GAD67+ bouton vGAT protein levels. (**A**) PFC total gray matter vGAT protein levels per bouton. The data points in each scatterplot represent a matched pair of schizophrenia (sz) and comparison (cntl) subject. Points below the unity line reflect pairs in which the measures are lower for the schizophrenia relative to the comparison subject. (**B**) Laminar analysis of vGAT protein levels per bouton in the PFC of schizophrenia (gray) and comparison (black) subjects. *F*-statistics and *p*-values are provided in Table S2.

Supplemental Methods & Materials

Immunohistochemistry

Sections were incubated in 0.01M sodium citrate in distilled H_2O at 80ºC for 75 minutes (7), cooled to room temperature (RT), and rinsed with 0.1M phosphate buffer (PB). Next, sections were incubated in 1% sodium borohydride in PB for 30 minutes at RT and then rinsed in 0.1M phosphate buffered saline (PBS). Sections were permeabilized with 0.3% Triton X-100 in PBS for 30 minutes at RT, incubated in 20% donkey serum in PBS for 2 hours at RT, and then incubated for ~72 hours at 4ºC in PBS containing 2% donkey serum and primary antibodies that recognize vGAT (mouse host; 1:500, Synaptic Systems, Goettingen, Germany; product # 131011, Lots 131011/41 and 131011/42) and GAD67 (goat host; 1:100, R&D Systems, Minneapolis, MN, USA; product # AF2086, Lot KRD0110031). The specificity of each antibody was verified by Western blot in our laboratory (data not shown and (8)) or other laboratories (vGAT (9); GAD67 (10, 11)). Sections were then rinsed for 2 hours in PBS and incubated for 24 hours in PBS containing 2% donkey serum and secondary antibodies (donkey host) conjugated to Alexa 488 (vGAT) or 647 (GAD67; Invitrogen, Grand Island, NY, USA; 1:500 for all) at 4ºC. After washing, sections were mounted (ProLong Gold antifade reagent, Invitrogen) on slides, which were coded to conceal diagnosis and subject number, and stored at 4ºC until imaged. Secondary antibody specificity was verified by omitting the primary antibody in control experiments. Multiple pilot studies were performed to determine if any primary/secondary combinations influenced the outcome; results from these studies indicated that the ability to detect each antigen was not dependent on the secondary antibody spectra.

Microscopy

TetraSpeck microspheres (fluorescent blue/green/orange/dark red; Invitrogen) were used to confirm the absence of alignment issues between wavelengths.

Image Processing

For data segmentation, a Gaussian channel was made for each deconvolved channel by calculating a difference of Gaussians using sigma values of 0.7 and 2. The Gaussian channel was used for data segmentation only. Data segmentation was performed as described (12), with a few exceptions. The Ridler-Calvard iterative thresholding algorithm (13) was used to obtain an initial value for iterative segmentation for each channel within each image stack. Multiple iterations with subsequent threshold settings increasing by 25 gray levels were performed in MATLAB (R2012). After each iteration, the object masks were size-gated within a range of 0.05-0.7 μm^3. For analyses, the image stacks were virtually cropped in the x-, y-, and z-dimensions using the center x-, y-, and z-coordinates of the immunoreactive puncta object masks. In the x- and y-dimensions, the center of each object mask had to be contained in the central 490 x 490 pixels of the image. To select the z-dimension used for analyses, the z-position of each object mask was normalized by the following equation:

$$Z_{coordinate} \text{ (\# of z-planes for image stack / 40).}$$

Next, each object mask was placed in one of 40 z-bins based on its normalized z-position. The mean object mask density and mean fluorescence intensity for vGAT and GAD67 were determined within each z-bin, and used for an analysis of variance with post-hoc comparison via Tukey's honestly significant difference test. The maximum number of adjacent z-bins that were not significantly different for both intensity and object mask number across all channels were used for analyses (n = 17 bins, which corresponded to 8.5 µm of the cut tissue thickness). By taking this approach we controlled for possible edge effects (i.e., all puncta assessed were fully represented in the virtual space), differences in antibody penetration and differences in fluorochromes. The final object masks were then used to collect information on the deconvolved channels.

Supplemental References

1. Glausier JR, Kimoto S, Fish KN, Lewis DA (2015): Lower glutamic acid decarboxylase 65-kDa isoform messenger RNA and protein levels in the prefrontal cortex in schizoaffective disorder but not schizophrenia. *Biol Psychiatry*. 77:167-176.

2. Glausier JR, Fish KN, Lewis DA (2014): Altered parvalbumin basket cell inputs in the dorsolateral prefrontal cortex of schizophrenia subjects. *Mol Psychiatry*. 19:30-36.

3. Curley AA, Arion D, Volk DW, Asafu-Adjei JK, Sampson AR, Fish KN, et al. (2011): Cortical deficits of glutamic acid decarboxylase 67 expression in schizophrenia: clinical, protein, and cell type-specific features. *Am J Psychiatry*. 168:921-929.

4. Volk DW, Pierri JN, Fritschy JM, Auh S, Sampson AR, Lewis DA (2002): Reciprocal alterations in pre- and postsynaptic inhibitory markers at chandelier cell inputs to pyramidal neurons in schizophrenia. *Cerebral Cortex*. 12:1063-1070.

5. Pierri JN, Chaudry AS, Woo TU, Lewis DA (1999): Alterations in chandelier neuron axon terminals in the prefrontal cortex of schizophrenic subjects. *Am J Psychiatry*. 156:1709-1719.

6. Eggan SM, Stoyak SR, Verrico CD, Lewis DA (2010): Cannabinoid CB1 Receptor Immunoreactivity in the Prefrontal Cortex: Comparison of Schizophrenia and Major Depressive Disorder. *Neuropsychopharmacology*.

7. Jiao Y, Sun Z, Lee T, Fusco FR, Kimble TD, Meade CA, et al. (1999): A simple and sensitive antigen retrieval method for free-floating and slide-mounted tissue sections. *JNeurosciMeth*. 93:149-162.

8. Fish KN, Sweet RA, Lewis DA (2011): Differential distribution of proteins regulating GABA synthesis and reuptake in axon boutons of subpopulations of cortical interneurons. *Cereb Cortex*. 21:2450-2460.

9. Guo C, Stella SL, Jr., Hirano AA, Brecha NC (2009): Plasmalemmal and vesicular gamma-aminobutyric acid transporter expression in the developing mouse retina. *J Comp Neurol*. 512:6-26.

10. Gottlieb DI, Chang YC, Schwob JE (1986): Monoclonal antibodies to glutamic acid decarboxylase. *Proc Natl Acad Sci U S A*. 83:8808-8812.

11. Chang YC, Gottlieb DI (1988): Characterization of the proteins purified with monoclonal antibodies to glutamic acid decarboxylase. *J Neurosci*. 8:2123-2130.

12. Fish KN, Sweet RA, Deo AJ, Lewis DA (2008): An automated segmentation methodology for quantifying immunoreactive puncta number and fluorescence intensity in tissue sections. *Brain Res*. 1240:62-72.

13. Ridler TW, Calvard S (1978): Picture thresholding using an iterative selection method. *IEEE Transactions on Systems, Man, and Cybernetics*. SMC-8:630-632.