Data Supplement for Curley et al., Cortical Deficits of Glutamic Acid Decarboxylase 67 Expression in Schizophrenia: Clinical, Protein, and Cell Type-Specific Features, American Journal of Psychiatry (doi: 10.1176/appi.ajp.2011.11010052)

Supplemental Method and Results Sections

Supplemental Method Section

GAD67 mRNA levels

Tissue Collection and Preparation. The right hemisphere of each brain was blocked coronally, frozen immediately, and stored at –80°C (1). Cryostat sections from the anterior-posterior level corresponding to the middle portion of the superior frontal sulcus were cut serially and confirmed to contain DLPFC area 9 from adjacent Nissl-stained sections using cytoarchitectonic criteria (1). Cortical gray matter was dissected in a manner that excluded white matter contamination and provided excellent RNA preservation, and collected into tubes containing Trizol reagent (Invitrogen, Carlsbad, CA). Total RNA was isolated from Trizol homogenates, cleaned by RNeasy columns (Qiagen, Valencia, CA), and used for qPCR, as described below. RIN and pH were determined as described previously (2).

Quantitative PCR. cDNA was synthesized from total RNA for each subject using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). All primer pairs (supplemental Table S2) demonstrated: 1) high amplification efficiency (>96%) across a wide range of cDNA dilutions; 2) specific single products in dissociation curve analysis; and 3) melting temperatures similar to those predicted by oligonucleotide software. Quantitative PCR was performed with Power SYBR Green dye and an ABI StepOne Plus Real Time PCR System (Applied Biosystems). The difference in cycle

threshold (dCT) for GAD67 mRNA expression was calculated by subtracting the geometric mean of the cycle threshold for the three reference genes from the cycle threshold of GAD67 mRNA. Because this dCT represents the log2-transformed expression ratio of the target transcript to the geometric mean of the 3 reference genes, the relative expression level of GAD67 mRNA was determined as 2^{-dCT} (3,4).

Total GAD67 protein levels

Tissue Collection and Preparation. Gray matter from the right hemisphere of each brain was obtained as described above and collected into tubes for total protein analysis (average of 22 mg of tissue per tube). Tissue was homogenized by sonication in a volume of SDS buffer (125mM Tris pH 7, 2% SDS, 10% glycerol) equal to 10 times the tissue weight. Protein was extracted from homogenates by incubation at 70°C for 10 min with occasional vortexing, followed by centrifugation at 16,000 g for 10 min. This method allows for optimal protein extraction with minimal degradation (5). The supernatant was collected and an aliquot was used for total protein concentration determination in triplicate using a BCA assay kit (Thermo Fisher Scientific, Rockford, IL) according to the manufacturer's protocol. The remaining supernatant from protein extraction was frozen in aliquots. A single aliquot was then used to prepare samples in 1X Protein Loading Buffer (LI-COR Biosciences, Lincoln, NE) containing 10% β-mercaptoethanol that were stored in single-use aliquots at –20ºC. To limit its effect on protein levels, samples were exposed to only 2 freeze-thaw cycles.

Western Blotting. Unless otherwise noted, all reagents were obtained from Thermo Fisher Scientific, all incubation steps were performed at room temperature, and all washes were

performed for 5 minutes each in Tris-buffered saline with Tween-20 (TTBS; 10mM Tris pH 7.5, 150mM NaCl, 0.05% Tween 20).

After denaturing at 70ºC for 5 min, samples were resolved by SDS PAGE in 10% Precise Protein Gels in Tris-HEPES SDS running buffer (100 mM Tris, 100 mM HEPES, 3 mM SDS) at 60–62 volts for 2.5 hrs. Proteins were transferred to nitrocellulose membrane (LI-COR Biosciences) at 85 volts for 45 min in Tris-Glycine blotting buffer (25mM Tris, 192mM glycine, 20% methanol). Membranes were blocked for 1 hr in 50% Odyssey Blocking Buffer (LI-COR Biosciences) and 50% Tris-buffered saline, and washed once. Membranes were then incubated in a primary antibody mixture containing β-tubulin (mouse, 1:20,000, Millipore, Billerica, MA) and GAD67 (goat, 1:800, R&D Systems, Minneapolis, MN) in SuperBlock Blocking Buffer with 0.05% Tween 20 overnight at 4°C, and subsequently washed 5 times before incubation for 1 hr in a mixture of secondary antibodies containing IRDye donkey anti mouse 680 and IRDye donkey anti goat 800 (both 1:20,000; LI-COR Biosciences) in 50% Odyssey Blocking Buffer and 50% TTBS containing 0.01% SDS. Following 5 washes, signal was detected using the LI-COR Odyssey Infrared Imaging System.

GAD67 protein levels at PV puncta

Tissue Collection and Preparation. The left hemisphere of each brain was processed in 4% paraformaldehyde (6) and tissue blocks containing the superior frontal gyrus were sectioned coronally at 40 µm on a cryostat and stored in antifreeze solution at −30°C until processing for immunohistochemistry.

Immunohistochemistry. Tissue sections were processed for fluorescent

immunohistochemistry as described previously (7). Sections were incubated for 48 hrs in the following primary antibodies: GAD67 (mouse, 1:500, Millipore), GAD65 (rabbit, 1:1000, Millipore), and PV (goat, 1:1000, Swant, Bellinzona, Switzerland). Tissue sections were then incubated for 24 hrs with secondary antibodies (donkey) which were conjugated to Alexa 488 (1:500), 568 (1:500), or biotin (1:200; all from Invitrogen). For the biotin secondary, a tertiary incubation (24 hrs) with streptavidin 647 was performed. All sections were processed together in a single immunohistochemical run, and slides were coded so that subsequent steps were performed in a blinded fashion.

Microscopy. Image stacks (512x512 pixels; 0.23 μm z-step) were collected on an Olympus IX71 inverted microscope (Center Valley, PA) equipped with an Olympus DSU spinning disk confocal using a 60X 1.42 N.A. oil immersion objective and a Hamamatsu C9100 CCD camera (Bridgewater, NJ). SlideBook 4.2 software (Intelligent Imaging Innovations, Inc., Denver, CO) was used to control the microscope and for image processing. A total of 6–12 sites per section were imaged, with the number determined by the size of the region of interest. Running means were used to confirm that the number of sites per section was sufficient to adequately sample the region.

Z axis determination. Given differences in antibody penetration across the z axis, only a portion of the z axis was selected for analysis. The upper and lower ~1 μm of z planes were excluded due to irregularity in the surface of the tissue and compression at the slide/coverslip. Fluorescence intensity, and thus puncta number, is greatest at the coverslip and therefore the z plane located closest to the coverslip (after exclusion of ~1 μm as described above) was selected as the starting point of the quantified region.

Regression analyses were performed as planes were progressively added from the starting point to determine the largest number of z planes for which the slopes of the mean GAD65 intensity, coefficient of variation (CV) of mean GAD65 intensity, and number of GAD65 puncta regression lines were not statistically significantly different from 0. The selected region comprised 8% of the total thickness of the tissue.

Exclusion of lipofuscin autofluorescence. To exclude lipofuscin autofluorescence from our analyses, and thus eliminate the potential confounds it presents (see supplemental Figure S2), lipofuscin was imaged in the 403 nm channel (which does not overlap with channels used to image GAD65, GAD67, or PV) and then masked for subsequent quantification and exclusion from the GAD67 data. Any GAD67+/GAD65+/PV+ puncta that overlapped with 403 nm masks were excluded. In addition, a 403 nm maximum intensity cut off of 200 was applied to the data set to eliminate any lipofuscin that was not masked by our automated approach.

Antibody Specificity

All antibodies are commercially available and have had specificity demonstrated by multiple approaches per the manufacturers' data sheets. In addition, the specificity of each antibody was verified by Western blot in our laboratory (data not shown), with the exception of the PV antibody which has been verified by others (8).

Supplemental Results Section

Effect of PMI on Total GAD67 Protein Levels

To assess the effects of PMI on GAD67 protein levels, we used tissue samples from 6 male, white comparison subjects, 53–55 years of age. Subjects were divided into 2

triads, each containing a subject with a short, medium, and long PMI (Triad 1 PMIs: 8.0, 15.5, and 23.2 hrs; Triad 2 PMIs: 6.4, 15.4, and 28.0 hrs). Members of a triad were processed together throughout all steps. Tissue collection, tissue preparation, and Western blotting was performed as described in the Methods and Supplemental Appendix 1. Mean tubulin-corrected GAD67 ODs for the short, medium, and long PMIs were 0.49, 0.43, and 0.40, respectively. GAD67 protein levels were reasonably well-preserved across PMI, with 88 and 82% of the level present at the short PMI group (7.2 hrs) still present at 16 and 26 hrs, respectively.

In order to verify and extend the human findings, we performed a similar analysis in DLPFC tissue from an adult male macaque monkey in which in vivo levels (i.e., PMI of 0 hr) could be obtained. Monkey DLPFC tissue was collected as described previously (9). Briefly, the animal was sedated with ketamine hydrochloride, intubated, anesthetized with 1% halothane in 28% oxygen, and placed in a stereotaxic apparatus. The skull was opened and biopsy blocks were removed from the prefrontal cortex. A single block containing the DLPFC was frozen immediately in isopentane (0 hr PMI), and 3 others were stored in room temperature artificial cerebrospinal fluid for 6, 12, or 24 hrs and then frozen (6, 12, and 24 hr PMI). Tissue sections were collected from the blocks on a cryostat, protein was extracted, and samples prepared for Western blotting as described above.

Mean tubulin-corrected GAD67 ODs for the 0, 6, 12, and 24 hr PMIs were 0.39, 0.30, 0.27, and 0.30, respectively. GAD67 protein levels in monkey DLPFC were relatively well-preserved across all PMIs with 78, 69, and 76% of the baseline GAD67 level retained at the 6, 12, and 24 hr time points, respectively. However, the greater loss of GAD67 protein across PMI in the monkey (in which comparison was made to a true 0 hr PMI)

relative to the human indicates that the true effect of PMI on GAD67 protein in human tissue may be slightly larger than what was observed. Consequently, we selected pairs of schizophrenia and comparison subjects with PMIs <20 hrs (n=19) for GAD67 protein quantification.

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