Supplement 1

Supplemental Table 1: Overview on previous studies of white matter neurons in schizophrenia and affective disorder subjects. Red shading demarcates studies demonstrating significant increases in the clinical cohorts.

Study	Cohort	Brain region	Marker & counting	White matter (WM) space examined	Disease-specific alterations
Akbarian *et al.* (1)	Control 5 Schizophr. 5	Frontal lobe, superior frontal gyrus (BA 9)	NADPH-d 2-dimens.	WM space 0 - 4.8 mm beneath cortex divided into six 0.8 mm wide bins.	**3 out of 5 cases showed significant increase** in one or more deeper WM compartments.
Akbarian *et al.* (2)	Controls 7 Schizophr. 7	I) Hippocampus and entorhinal ctx. II) Temporal neocortex (BA 21)	NADPH-d 2-dimens.	I) **Lateral temporal neocortex**: Three 1.0 mm compartments. II) **Pre-/Parasubiculum**: Two 1.0 mm compartments. III) **Entorhinal cortex**: Two 1.0 mm compartments.	I) **45-90% increase** in NADPH-d neuron density in lateral temporal WM, 1-3 mm beneath cortex. II) No alterations in WM space of hippocampus and entorhinal cortex.
Akbarian *et al.* (3)	Controls 20 Schizophr. 20	Frontal lobe, middle frontal gyrus (BA 46)	a) MAP2-ir, b) NF-H, ir c) NADPH 2-dimens.	WM space beneath cortex divided into six 1.0 mm wide bins.	**Altered distribution in schizophrenia** cohort due to deficits in superficial bins and **increased density in deep WM**. (Effect primarily due to 7 out of 20 schizophrenia cases.)
Anderson *et al.* (4)	Controls 5 Schizophr. 5	Frontal lobe, middle frontal gyrus (BA9/46)	MAP2-ir, 2-dimens.	WM space 0-4.8 mm beneath cortex divided into six 0.8 mm wide bins.	**44% increase in** MAP2+ neuron density in schizophrenia; significant changes confined to WM < 3.2mm beneath gray matter.
Kirkpatrick *et al.* (5)	Controls 9 Schizophr. 9 *Deficit S. 3* *Non-deficit S. 6*	Parietal lobe (BA 39)	MAP2-ir, 3-dimens.	WM space < 2.25 mm beneath gray matter at depth of sulcus.	**115% increase** in MAP2+ neuron density in deficit syndrome schizophrenia.
Beasley *et al.* (6)	Controls 15 Schizophr. 15 Bipolar Dis. 15 Major Dep. 15	Frontal lobe (BA9/46) Middle frontal sulucs	MAP2-ir, 2-dimens.	WM space beneath cortex divided into five 0.5 mm wide bins.	No significant differences between control and psychiatric groups.
Rioux *et al.* (7)	Controls 15 Schizophr. 41	Anterior parahippo-campal gyrus	MAP2-ir, 2-dimens.	Measured distance of all neurons to closest gray/white boundary; cells were then grouped into 0.03 mm bins.	Altered neuronal distribution, with more MAP2+ neurons **located in deeper WM** in schizophrenia.
Eastwood & Harrison (8)	Controls 14 Schizophr. 12	Temporal lobe, Sup.temporal gyrus, (BA 22)	NeuN-ir, 2-dimens.	WM space < 1.5 mm beneath gray matter.	**16% increase** in NeuN+ neuronal density in superficial WM in schizophrenia. (Effect mainly due to 4 subjects with negative syndrome schizophrenia.)
Kirkpatrick *et al.* (9)	Controls 5 Schizophr. 7 *Deficit S. 3* *Non-deficit S. 4*	Frontal lobe (BA 46)	MAP-2 ir., 3-dimens.	WM space < 2.5 mm beneath gray matter.	**60% increase** in MAP-2 neurons in schizophrenia subjects with deficit syndrome**.**
Molnar *et al.* (10)	Controls 18 Bipolar Dis. 8 Major Dep. 10	Frontal lobe (BA 9)	NADPH-d Enzyme 2-dimens.	WM space beneath cortex divided into six 1.0 mm wide bins.	No significant differences between cases and controls.

Ikeda *et al.* (11)	Controls 6 Schizophr. 13 *Disorgan. 7* *Paranoid 6*	Frontal lobe (BA 9)	NPY-ir. 2-dimens.	WM space divided into 0-0.8 and 0.8 – 1.6 mm beneath gray matter.	**23% increase** in proportion of NPY+ neurons in the deeper WM of disorganized schizophrenia subjects.
Eastwood & Harrison (12)	Controls 12 Schizophr. 11	I) Frontal lobe (BA 9/46) II) Parahippoc.gyrus	NeuN-ir, 2-dimens.	Superficial WM space < 2 mm beneath gray matter.	**20% increase** in NeuN+ neuronal density in superficial WM of frontal lobe of schizophrenia; Effect mainly in 4 schizophrenia subjects.
Bertram *et al.* (13)	Controls 22 Schizophr. 22 Bipolar Dis. 5 Major Dep. 7	I) DLPFC II) Orbitofrontal gyrus III) Cingulate cortex	NRG-1α-ir, 2-dimens.	Not specified.	I) **~50% reduction** in NRG-1α-ir neuronal density in superficial & deep WM in schizophrenia. II) **~60% reduction** in gray matter in schizophrenia and **~50%** in major depression.

Methods

Immunohistochemistry: Given the large number of specimens included in this study (N = 96), it was not possible to process the entire cohort simultaneously; however, to reduce inter-assay variability, multiple sets of randomly mixed cases and controls were processed in parallel, using the same antibodies and reagents. Incubation times were kept constant across the entire study. Adjacent sections were stained for (i) Nissl, or immunoreactivity for, (ii) Neuron-specific nuclear protein (NeuN), (iii) Neuregulin 1 alpha (NRG), (iv) Ionized calcium-binding adaptor molecule 1 (Iba1). Sections were incubated in mouse anti-NeuN antibody (Upstate; Billerica, MA) diluted 1:500, rabbit anti-NRG antibody (Lab Vision; Fremont, CA) diluted 1:200, or mouse anti-Iba1 antibody 1:500 (a generous gift from Dr. A. Chang and Dr. B.D. Trapp) overnight and processed using a standard protocol for immunoperoxidase-based labeling (Vector Laboratories; Burlingame, CA). For two subjects, double-immunofluorescence was performed for NeuN and NRG in conjunction with AlexaFluor 488 and 594-conjugated secondary antibodies (Invitrogen; Carlsbad, CA). For a subset of 6 cases and 6 controls, NeuN immunohistochemistry was conducted for the superior frontal gyrus (BA9), using 5 μm thick sections from paraffin-embedded blocks.

Microscopy: Two-dimensional (2D) cell counts for NeuN or NRG immunostained sections were conducted under 1.25x and 20x objectives, using an Olympus BX51 upright microscope in conjunction with a motorized stage and BIOQUANT Life Science software version 8.00.20. Under the 1.25x objective, the gray/white matter border along BA33 was demarcated, and the white matter space (subcortical white matter and cingulum bundle but excluding the corpus callosum) was divided into compartments each 500 microns deep and 2000 microns wide (**Fig. 1C**). After compartments were drawn, the NeuN+ neurons were

counted using the 20x objective. A cell was defined as NeuN+ if immunoreactivity was detectable in the nucleus with weaker stained neuron-like cytoplasm or processes visible (**Fig. 1D**). A cell was defined as NRG+ if cut through the level of nucleus with robust staining confined to cytoplasm and processes (**Fig. 2D**). For each specimen, NeuN+ and NRG+ cells were counted in each of the 5 white matter compartments in two tissue sections per marker by a counter blind to diagnosis. For sections collected from Brodmann's Area 9, sampling was limited to the upper 3 white matter compartments, because of difficulties to identify deeper white matter space. To assess potential differences in cell size between diagnostic groups, average somal area of NeuN+ neurons in cingulate white matter was estimated by tracing 5 cells per subject using Bioquant software and 20x magnification (n = 5 per group)**.** All NeuN counts were performed by Y.G. NRG-counts and microglial ratings (see below) were done by C.C. and S.A., and no significant differences between raters were observed. For the NeuN counts, intra-rater reliability (counting the same subject, but not necessarily the same section, twice) approached 90%.

In addition, NRG+ cells were counted in the gray matter of BA33; NeuN+ cells were counted in BA33 gray matter for a subset of subjects (n = 5 for each of the 3 cohorts). For the NeuN counts, the clinical cases were chosen randomly, and then matched to controls with similar age and postmortem interval, and same sex. As in the white matter, a two-dimensional counting method was used. For each section, a rectangular box 2000 microns wide was placed across the full vertical thickness of BA33 and the numbers and density of NeuN+ or NRG+ neurons were calculated per mm^2.

Finally, all cases (bipolar = 15; schizophrenia = 22) and a subset of controls (n = 21) were immunostained with the anti-microglial antibody, Iba1, and blindly graded as 0 (staining

indistinguishable from background), 1 (occasional fiber staining), 2 (numerous fibers and occasional cell bodies), 3 (numerous fibers and cell bodies), and 4 (intensely stained fibers and cell bodies) (**Fig. S2**).

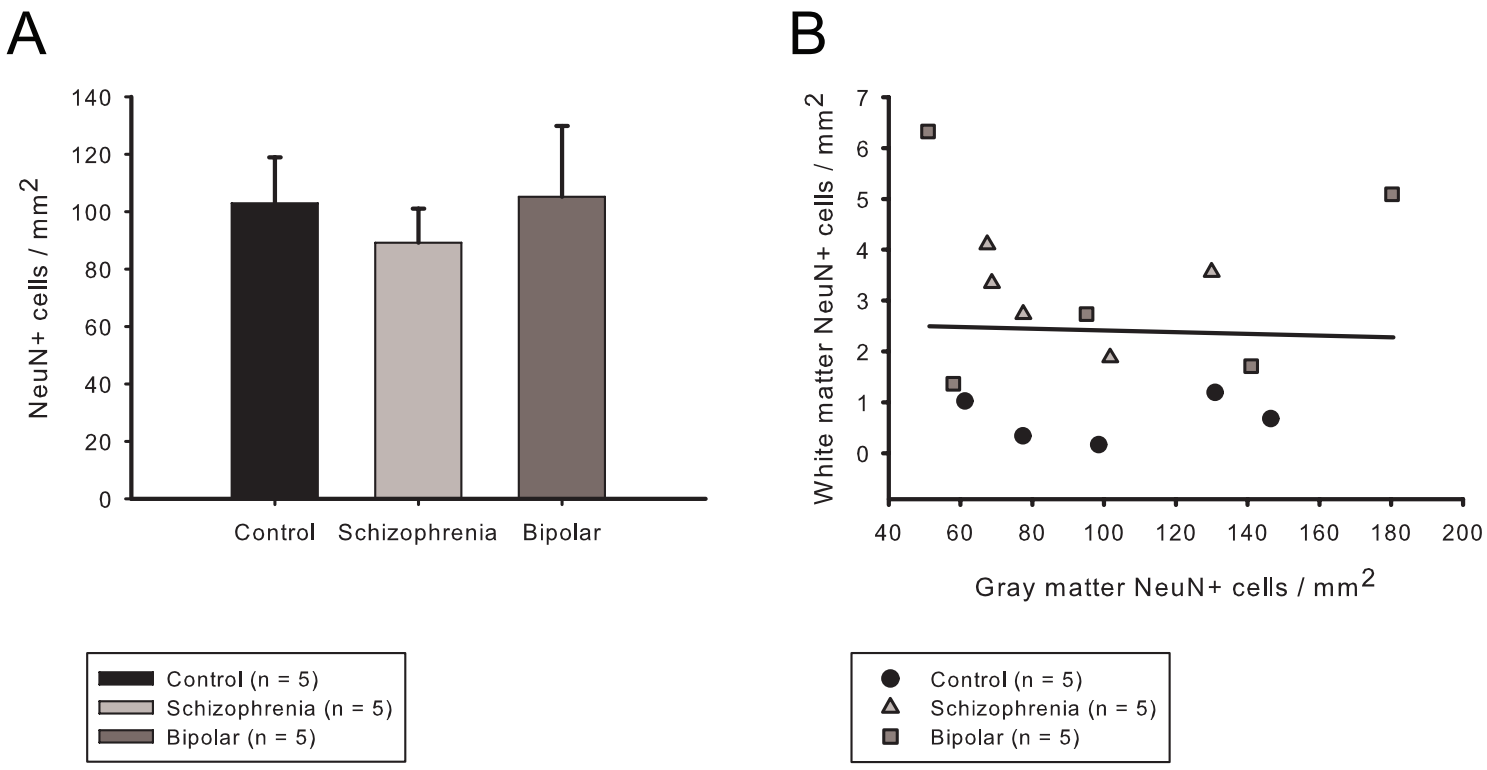
Isolation and quantification of nuclei in cingulate white matter: For a subset of subjects (**Table 1**), a column of white matter adjacent to the gray/white border of the ventral portion of BA24 from frozen cingulate was isolated with a metal borer with a diameter of 3.15 mm and the length measured with calipers. The borer was placed perpendicular to the gray/white matter border after visual inspection ensured that the border had not shifted on the opposite of the block. Subsequently, tissue was dounced in lysis buffer (14), layered onto a sucrose cushion, and ultracentrifuged at 28,000 rpm at 4°C for 2.5 hrs. The nuclei pellet was dissolved in 500 μl PBS, incubated with 200 μl antibody solution (anti-NeuN, 1:500; anti-mouse AlexaFluor 488) for 45 min at 4°C, and fixed with 70 μl 10% formalin. A 5 μl aliquot was removed, diluted with 495 μl PBS, and ten 5 μl aliquots from this solution applied to 10 separate slides and coverslipped with Vectashield Mounting Medium with the nucleophilic dye, DAPI.

The total number of NeuN+ and DAPI+ nuclei was counted for each the 10 slides and then averaged. The following formula was used to estimate the total number of NeuN+ and DAPI+ nuclei per tissue column:

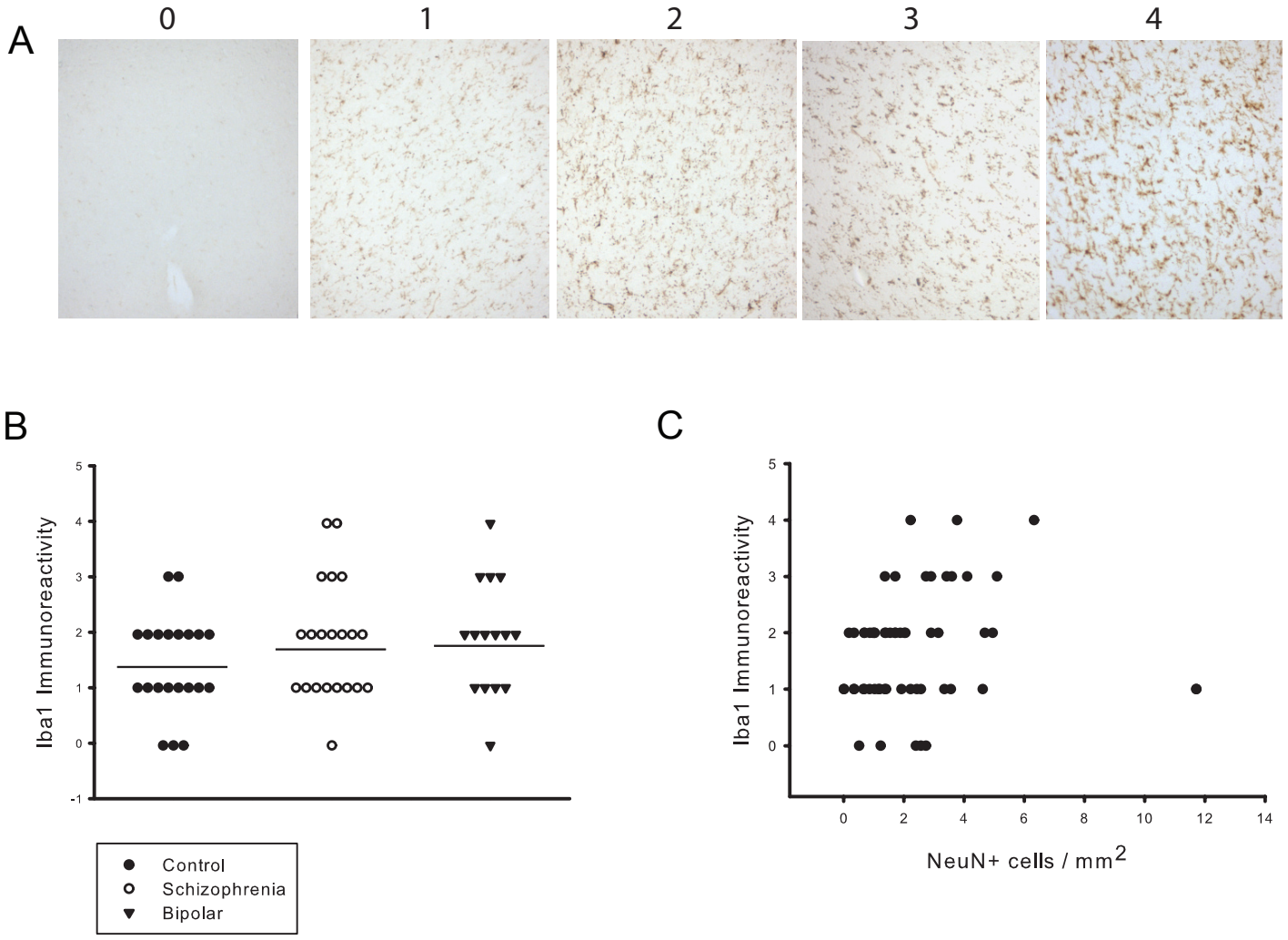
$$[(\#\ \text{NeuN+ or DAPI+ nuclei counted} / 5\ \mu l)\ (500\ \mu l) / 5\ \mu l] \times 770\ \mu l$$

To determine the number of nuclei per mm tissue cubed (mm^3), the estimated total number of nuclei was divided by the volume of tissue for that particular sample.

Statistics: For each subject, NeuN+ and NRG+ cell densities were calculated for each white matter compartment as cells per square millimeter and the densities from the two slides per subject averaged. A Kruskal-Wallis one-way ANOVA was used to compare NeuN+ or NRG+ cell densities between bipolar, schizophrenia, and control groups. The Dunn's test was utilized to conduct post-hoc comparisons between diagnostic categories. Additionally, presence or absence of NeuN+ cells in deeper white matter (compartments III-V) were determined for each subject and each patient group was compared to the control group with Pearson chi-square. Effects of potential confounds on overall white matter NeuN density—including postmortem interval (PMI), subject age, gender, hemisphere, and medication status—were examined by Pearson correlation, Mann-Whitney U-test, and also with ANCOVA for a mixed model by REML (restricted estimation by maximal likelihood).



Supplemental Figure 1: **NeuN+ neuronal density is unaltered in gray matter in schizophrenia and bipolar disorder.** (**A**) NeuN+ neuronal density (presented as cells/mm^2, y-axis) in BA33 gray matter of controls (N = 5, black), schizophrenia (N = 5, light gray), and bipolar disorder (N = 5, dark gray) subjects. There was no significant difference in NeuN+ neuronal density between diagnostic groups. (**B**) Correlation between NeuN+ neuronal densities in gray matter (x-axis) versus white matter (y-axis) for control, schizophrenia, and bipolar subjects. Notice that there was no appreciable correlation between gray and white matter NeuN+ neuronal densities (r = 0.0013).



Supplemental Figure 2: **Iba1 immunoreactivity is unaltered in cingulate white matter in schizophrenia and bipolar disorder**. (**A**) Digitized images from cingulate white matter processed for Iba1 immunoreactivity with DAB/peroxidase labeling, depicting the grading scale used for assessment of microglia. 0 = indistinguishable from background; 1 = occasional fiber staining; 2 = numerous fibers and occasional cell bodies; 3 = numerous fibers and cell bodies; 4 = intensely stained fibers and cell bodies. (**B**) Frequency distribution of Iba1 immunoreactivity levels for control (N = 21), schizophrenia (N = 22), and bipolar disorder (N = 15) subjects. Note that the mean Iba1 immunoreactivity levels were not significantly different between the 3 diagnostic groups. (**C**) Correlation between Iba1 immunoreactivity levels and NeuN+ neuronal densities in white matter. There was no appreciable correlation between these two cell populations.

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