Decreased Numbers of Somatostatin-Expressing Neurons in the Amygdala of Subjects with Bipolar Disorder or Schizophrenia: Relationship to Circadian Rhythms

Supplemental Information

Supplemental Methods & Materials

Statistical Analysis of Potential Confounding Factors

Age, gender, postmortem time interval, inflammation (classified as positive or negative for inflammatory condition at time of death), hemisphere, cause of death, brain weight, exposure to alcohol, nicotine, electroconvulsive therapy, and lifetime, as well as final six months', exposure to antipsychotic drugs, exposure to selective serotonin reuptake inhibitors classified as positive or negative for exposure, exposure to valproic acid and lithium treatment were tested systematically for their effects on the main outcome measures, and included in the model if they significantly improved the model goodness of fit. Postmortem interval (PMI) was used as an indicator of tissue quality, as this factor has been reported to correlate with protein levels, possibly in a protein and brain region specific manner (1-3). In addition, cause of death was used as a further indicator of tissue integrity, as prolonged agonal states have been reported to correlate with tissue integrity (1). Potential effects of PMI and cause of death, categorized as acute vs chronic, were tested on our outcome measures in stepwise linear regression analysis.

Data on nicotine and alcohol exposure was only available for subjects with SZ or BD; on the basis of the subjects' record, exposure was considered as high (scored as 4), moderate (scored as 3-2), low (scored as 1) and absent (scored as 0), as well as present or absent during the last 10 years of life. We analyzed medical records for exposure to various classes of psychotropic and neurotropic drugs. Estimated daily mg doses of antipsychotic drugs were converted to the approximate equivalent of chlorpromazine as a standard comparator (4), and corrected on the basis of a qualitative assessment of treatment-adherence based on taking

prescribed psychotropic medicines more or less than approximately half of the time, as indicated by the extensive antemortem clinical records. These values are reported as lifetime, as well as last six months' of life, grams per patient (Tables 1 and S3). Exposure to lithium salt and valproic acid was estimated in the same manner (Tables 1 and S3). Exposure to other classes of psychotropic drugs was reported as present or absent (Tables 1 and S3). In addition to testing the potential effects of exposure to antipsychotics and lithium salt within our stepwise linear regression process, the effects of these variables, together with other psychotropic and neurotropic drugs, adherence to pharmacological treatment (good or poor), age of onset of the disease and duration of the illness, were tested directly in separate ANOVA analyses.

Antibody Specificity

Primary antibody monoclonal rat anti-SST (1:500, Millipore, MAB354, lot# NG1934075) raised against synthetic SST peptide corresponding to amino acids 1-14, and rabbit anti-NPY (1:1000, Chemicon, AB1915, lot # 0604027825), raised against synthetic porcine neuropeptide tyrosine were used for these studies. Previous studies have demonstrated the specificity of these antibodies using immuno-absorption testing with synthetic SST and NPY peptides (5). We confirmed the specificity on human amygdala protein samples first by incubating each antibody with 100 mirograms their respective peptide (human neuropeptide-Y, cat # 05-23-2005; somatostatin, cat # 05-23-0850; EMD Millipore Corp., Billerica, MA) for 48 hours, followed by immunohistochemistry procedures described in the main text. Peptide pre-incubation resulted in absence of specific labeling in amygdala sections from control subjects (Supplemental Figure S2A-D).

We further confirmed the specificity of each antibody using Western blotting, which demonstrated a single band for each antibody, corresponding to the predicted molecular weights of approximately 13 kDa for SST (Supplemental Figure S2E) and approximately 11 kDa for NPY (Supplemental Figure S2F). For Western blot analysis, fresh amygdala blocks were

immediately sliced into 2 mm coronal slabs and frozen with liquid nitrogen vapor. Slabs were sectioned on a freezing microtome into 30 µm sections, and the amygdala was dissected out of four of these sections and incubated in NP40 lysis buffer (Invitrogen, cat # FNN0021) with 1 mM PMSF and protease inhibitor cocktail (Sigma cat # P-2714) for 20 minutes at 4°C. Samples were then sonicated, centrifuged, and the supernatant containing the protein extract was collected. Protein extracts were quantified using Bradford protein assay (6). Protein samples (30 µg per sample) were heated at 70°C for 10 minutes in NuPAGE LDS sample buffer (Invitrogen) and NuPAGE reducing agent (Invitrogen), and loaded into a NuPAGE Bis-Tris 4-12% gel (Invitrogen). The XCell SureLock Mine-Cell system (Invitrogen) with 4–12% Bis–Tris gradient polyacrylamide gels (Invitrogen) was used and 10 µg of protein was added per lane. Gels were incubated in NuPAGE SDS running buffer (Invitrogen) with 500 µl NuPAGE antioxidant (Invitrogen) during electrophoresis. Proteins were then transferred onto Immobilon-FL PVDF membranes (Millipore, cat # IPFL00010) using an XCell II Blot Module (Invitrogen). Membranes were then washed three times and incubated with Li-Cor Blocking Buffer (Li-Cor Biosciences) for 1 h at room temperature with rocking to block nonspecific antibody binding. Membranes were incubated with either anti-SST (1:500) or anti-NPY (1:1000) overnight at 4°C in blocking buffer with 0.1% Tween. Membranes were then washed 4 times with 0.01M PBS with 0.1% Tween, followed by incubation for 2 hours at room temperature with anti-rabbit (IRDyes 680RD donkey anti-Rbt cat # 926-68073 or IRDye 680RD goat-anti-Rat cat # 926-68076) secondary antibodies (1:20,000), washed with 0.1M phosphate buffer (PB), and visualized using a LiCor Odyssey CLx system interfaced with Image Studio version 4.0.

Effects of Pharmacological Treatments on SST Expression

In rodents, chronic haloperidol treatment has been shown to increase SST and NPY protein levels in multiple cortical brain regions (7), and in the striatum (8). Clozapine treatment has been shown to have similar effects, rescuing SST levels in a mouse model of SZ (9). Furthermore,

chronic lithium treatment was also shown to increase SST and NPY mRNA in several brain regions in rodent (10, 11). In our study, SST-IR N_t and N_d were not affected by exposure to antipsychotics, SSRIs, or lithium as tested in stepwise linear regression models, and did not correlate with levels of exposure to these treatments. Although we cannot rule out possible effects of antipsychotics on SST or NPY-IR neurons, there is no indication that these treatments were responsible for, or possibly corrected for, decreases in these markers in our subjects.

Potential effects of alcohol and nicotine exposure on SST-IR neurons were tested using estimates of alcohol and nicotine use per subject, rated 0 for no exposure, and 1-4 for severity of exposure, with 1 representing low and 4 representing high. These measures for alcohol were tested first by comparing subjects with exposure to subjects without (Supplemental Fig. S3A,B), followed by statistical correlations of ratings of alcohol exposure to numbers of SST-IR neurons in subjects with BD or SZ (Supplemental Fig. S3C,D). Potential effects of nicotine exposure were tested in the same manner (Supplemental Fig. S3E-H). No statistical relationship was observed for alcohol or nicotine exposure with SST-IR neurons (Supplemental Fig. S3) or with NPY-IR neurons (data not shown).

Tissue Processing for Immunohistochemistry

Tissue blocks were dissected from fresh brains and post-fixed in 0.1M PB containing 4% paraformaldehyde and 0.1M Na azide at 4°C for 3 weeks, cryoprotected at 4°C for 3 weeks (30% glycerol, 30% ethylene glycol and 0.1% Na azide in 0.1M PB), embedded in agar, and pre-sliced in 2 mm coronal slabs using an Antithetic Tissue Slicer (Stereological Research Lab., Aarhus, Denmark). Each slab was exhaustively sectioned using a freezing microtome (American Optical 860, Buffalo, NY). Sections were stored in cryoprotectant at −20°C. Using systematic random sampling criteria, sections through the amygdala were serially distributed in 26 compartments (40 µm thick sections; six-ten sections/compartment; 1.04 mm section separation within each compartment). All sections within one compartment/subject were

selected for immunocytochemistry (i.e., SST or NPY), thus respecting the 'equal opportunity' rule (12, 13).

Table S1. Percentages of SST and NPY-IR Neurons in Comparison to Total Numbers of Amygdala Neurons in These Subjects Reported Previously (14).

	SST Total Number (% of All Neurons)	**NPY Total Number (% of All Neurons)**	**% NPY-IR / SST-IR**
Lateral Nucleus	28277 (1.4%)	14994 (0.7%)	53%
Basal Nucleus	20614 (1.7%)	10661 (0.9%)	52%
Accessory Basal	11423 (0.62%)	3755 (0.6%)	33%
Cortical Nucleus	6096 (0.44%)	1816 (0.4%)	30%
Total (LN-BN-AB-CO)	1.5%	0.7%	47%

Table S2. Total Numbers of SST and NPY-IR Neurons[a] and Total Volumes[a]

Nucleus	Control		Schizophrenia				Bipolar Disorder			
	TN Neurons Mean	TN Neurons SD	TN Neurons Mean	TN Neurons SD	*t* Ratio	*p* Value	TN Neurons Mean	TN Neurons SD	*t* Ratio	*p* Value
Total Numbers of SST-IR Neurons										
LN	28277.6	10138.9	18018.0	9354.3	**-2.26**	**0.03** [b]	20890.1	7418.6	**-3.04**	**0.005***
BN	20614.5	9378.6	16553.3	9010.7	-1.36	0.19	17612.4	7903.6	-1.23	0.23
AB	11422.7	7936.6	10068.5	5660.3	0.31	0.77	11660.1	5492.5	0.75	0.48
CO	6096.1	3555.7	6088.3	3144.34	0.05	0.96	7503.6	4064.4	-0.71	0.49
Total Numbers of NPY-IR Neurons										
LN	14994.6	6986.7	11528.8	4969.0	-1.32	0.20	13601.5	6693.9	-0.58	0.57
BN	10661.9	4716.1	9405.5	4042.3	-0.85	0.41	8690.9	4581.9	-1.25	0.22
AB	3755.1	1782.5	3512.2	2392.7	-1.02	0.32	3170.3	2127.2	-1.27	0.22
CO	1816.0	770.2	1397.5	777.2	-1.66	0.11	1296.5	899.1	**-2.11**	**0.04** [c]

Nucleus	Total Volume Mean	Total Volume SD	Total Volume Mean	Total Volume SD	*t* Ratio	*p* Value	Total Volume Mean	Total Volume SD	*t* Ratio	*p* Value
Total Volumes (in mm^3)										
LN	225.5	64.9	193.9	36.5	-0.44	0.67 [d]	176.6	57.8	**-3.01**	**0.005** [e]
BN	153.76	60.36	135.9	37.1	-0.15	0.88 [d]	125.2	53.9	-1.66	0.10
AB	73.9	21.5	66.6	24.5	-0.04	0.97 [d]	58.3	22.5	-1.94	0.06
CO	57.8	23.4	40.9	12.2	-1.70	0.10 [d]	40.0	17.6	**-2.88**	**0.007**

AB, accessory basal nucleus; BN, basal nucleus; CO, cortical nucleus; CPZ, antipsychotic dosage lifetime in grams; LN, lateral nucleus; PMI, postmortem interval; TN, total number.

Mean and SD were obtained from original values. Statistical analysis was performed on logarithmic transformation of all original values because the data were not normally distributed. *t*-ratio and *p*-values were calculated based on values after logarithmic transformation.

[a]Significance values were derived from stepwise regression models. Significance values refer to comparisons with the control group.

[b] Adjusted for effect of hemisphere.

[c] Adjusted for effect of PMI.

[d] Adjusted for effect of hemisphere.

[e] Adjusted for effects of lifetime exposure to CPZ in grams.

*Adjusted for effect of time of death.

Table S3. Sample Demographic and Descriptive Characteristics of the Cohort Used for Immunohistochemical Investigation

Case	Cause of Death/ Inflammation	Brain Weight (g)	PMI (hrs)	Hemisphere	Time of Death
	Schizophrenia				
3/31/M	unknown	1450	15.0	L	11:30
7/49/M	Suicide (A, N)	1440	19.1	R	01:00
9/32/M	Cardiac arrest and pancreatitis (A, I)	1400	7.8	L	13:10
11/82/F	Cancer (C, N)	1110	23.9	L	00:28
21/55/M	Cardiac arrest (A, N)	1380	21.4	L	10:00
22/60/F	Cancer (C, N)	1220	19.6	R	05:25
24/73/F	Cancer (C, N)	1170	24.1	L	NA
28/61/F	Pneumonia (A, I)	1200	14.08	L	12:40
32/72/F	Renal failure (C, N)	1065	21.75	L	12:50
36/73/F	Cancer (C, N)	1130	28.8	R	15:53
39*/62/M	Sepsis (C, I)	1340	25.3	L	05:30
42*/58/M	COPD (C, N)	1160	32.38	R	15:30
Total/mean ± SD 59.0 ± 15.7/ 6F, 6M		1255.4 ± 137.9	21.1 ± 6.7	8L/4R	
	Bipolar Disorder				
5/76/F	Cardiac arrest (C, N)	1170	22.8	R	15:22
6/74/M	Pneumonia (A, I)	1270	24.8	L	08:15
8/86/F	unknown	1040	11.3	L	06:26
10/73/M	Pneumonia (A, I)	1190	7.2	R	09:00
19/25/M	Pulmonary edema (A, N)	1480	12.56	R	04:30
20/66/M	Pneumonia (C, I)	1480	17.4	R	20:45
25/73/F	Sepsis (C, I)	1060	20.8	L	19:10
27/75/F	unknown	1310	36.0	R	00:31
29/73/F	Cancer (C, N)	1020	17.0	L	06:45
34/83/M	unknown	860	17.5	L	18:43
37*/62/F	Congestive heart failure (C, N)	1210	13.4	R	22:19
35/67/F	Pneumonia (A, I)	1150	25.3	L	08:55
38*/40/F	Sepsis (C, I)	1340	21.92	L	23:30
40*/51/F	Ischemic heart disease (A, N)	1260	35.1	L	10:25
41*/70/M	Renal failure (C, N)	1215	17.25	L	11:45
Total/mean ± SD 66.3 ± 16.2/ 9F, 6M		1203.7 ± 167.7	20.0 ± 8.1	9L/6R	

Case	Cause of Death/ Inflammation	Brain Weight (g)	PMI (hrs)	Hemisphere	Time of Death
		Controls			
1/36/M	Myocardial infarction (A, N)	1470	24.5	L	12:37
2/68/F	Coronary artery disease (A, N)	1330	14.75	R	09:30
4/70/M	Myocardial infarction (A, N)	1360	23.2	R	12:17
12/52/M	Myocardial infarction (A, N)	unk	32.1	L	03:07
13/71/M	Cardiac arrest (A, N)	1580	24.0	R	10:10
14/37/M	Electrocution (A, N)	1460	18.75	R	21:00
15/65/M	Cardiac arrest (A, N)	1240	17.3	R	06:45
16/53/F	Cancer (C, N)	1330	24	R	08:32
17/62/M	Myocardial infarction (A, N)	1300	29.2	R	21:18
18/70/M	Aortic aneurysm (A, N)	1400	17.3	R	20:46
23/58/F	COPD (A, N)	1345	17.8	R	00:35
26/72/M	Myocardial infarction (A, N)	1560	28.2	R	07:35
30/85/M	Cancer (C, N)	1225	20.3	L	05:30
31/78/F	Cancer (C, N)	1100	23.9	L	05:00
33/74/F	Cancer (C, N)	1145	12.2	L	14:00
Total/mean ± SD 63.4 ± 14.0/ 5F, 10M		1346.1 ± 141.8	21.8 ± 5.6	5L/10R	

A: acute, no prolonged agonal period; C: chronic, with agonal period; I: infection/inflammatory condition present at time of death; N: no significant infection/inflammation present at time of death.

* denotes subjects for whom SST-IR data from the hippocampus was available.

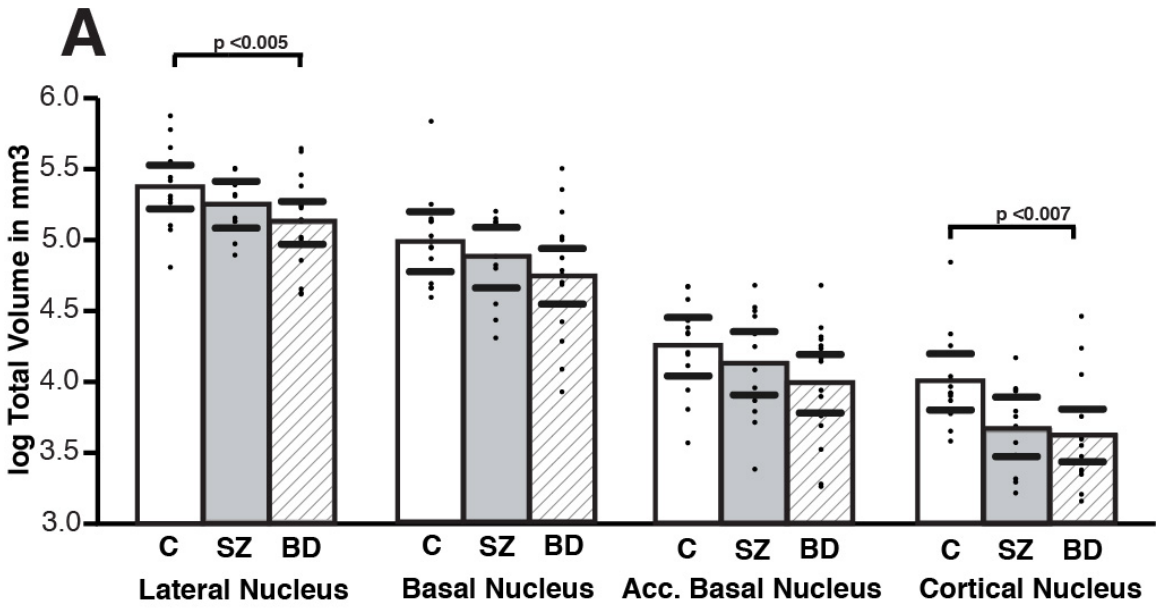

Figure S1. Decreased Amygdala Volume in BD. Scatterplots depicting total volumes in mm^3 of the amygdala nuclei in control, SZ, and BD subjects. Significant decreased volume was detected in the lateral and cortical nuclei of BD subjects. Significance values are derived from stepwise linear regression models. Scatterplots show the mean (histogram) and 95% confidence intervals (black lines). *Adjusted for significant effect of lifetime exposure to CPZ in grams.

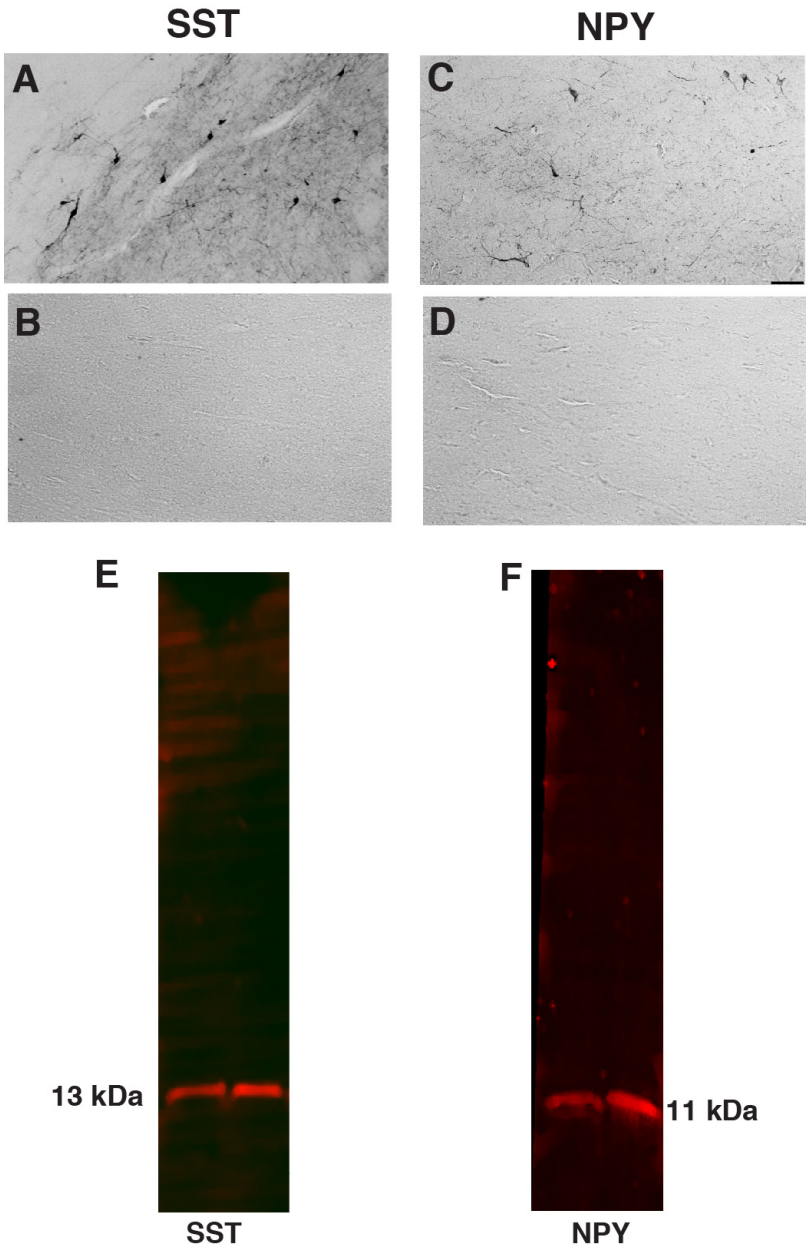

Figure S2. Antibody Specificity. The specificity of the SST and NPY antibodies was tested by incubating the primary antibodies with SST or NPY peptides 100 µg of SST or NPY respectively. Pre-incubation with the specific peptide resulted in absence of positive labeling for SST compared to positive control sections (**A**&**B**) and for NPY (**C**&**D**). In addition, antibody specificity was tested using western blots, which showed a single band corresponding to approximately 13 kDa for SST (**E**) and 11 kDa for NPY (**F**).

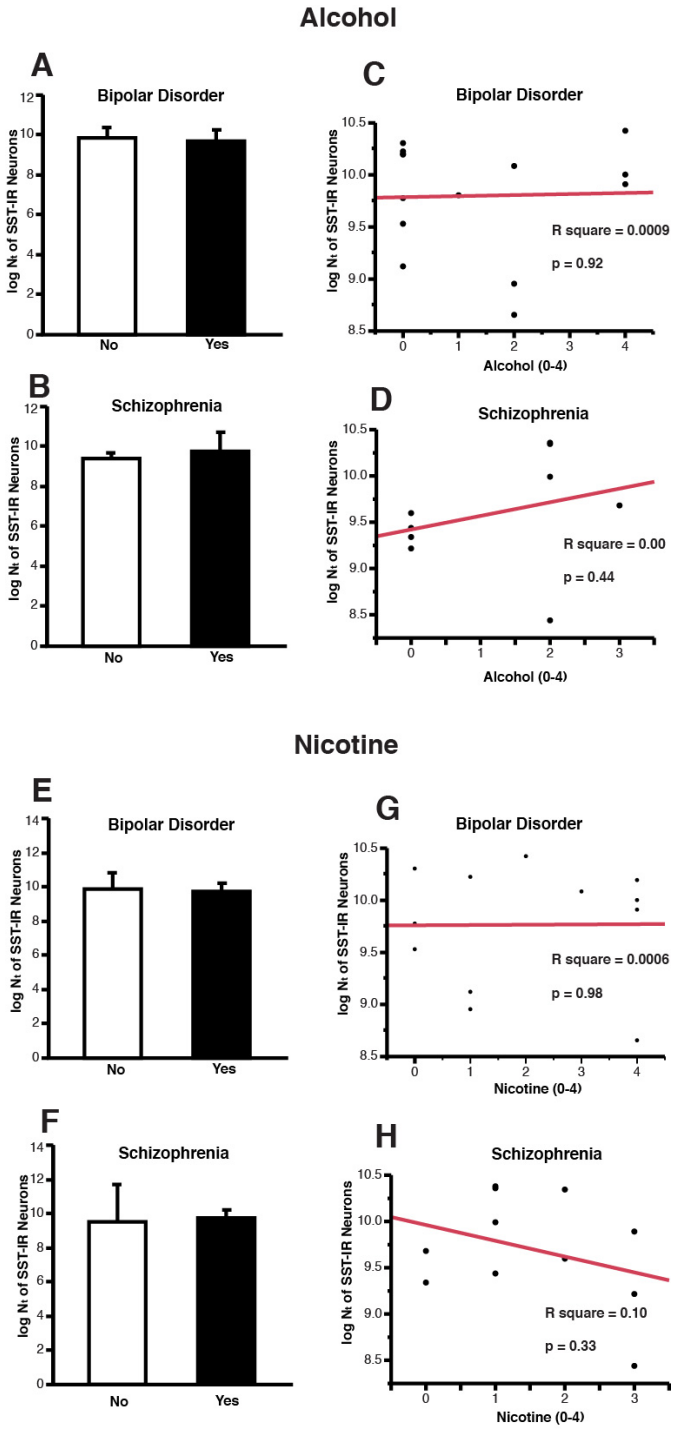

Figure S3. Relationship with Alcohol and Nicotine. For subjects with BD or SZ, exposure to alcohol was rated 0 for no exposure, and 1 (low) to 4 (high) for severity of exposure on the basis of medical records. Measures of SST-IR neurons from subjects with and without exposure were first compared using ANOVA (**A**&**B**), followed by correlations of alcohol ratings with numbers of SST-IR neurons (**C**&**D**). No statistical effect of alcohol exposure on numbers of SST-IR neurons was observed. The same process was used to examine nicotine use (**E**-**H**), which showed no statistical relationship of nicotine use with numbers of SST-IR neurons in SZ or BD. Error bars represent 95% confidence intervals.

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