Molecular evidence for BDNF- and GABA-related dysfunctions in the amygdala of female subjects with Major Depression

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1 SUPPLEMENTARY METHODS

1.1 Total RNA processed for microarray analysis

1µg of total high quality total RNA was reverse transcribed into single stranded cDNA, that was then converted into double stranded cDNA and purified using the Illumina TotalPrep RNA Amplification Kit. An in vitro transcription reaction was carried out overnight in the presence of biotinylated UTP and CTP to obtain biotin-labeled cRNA from the double stranded cDNA. The cRNA was purified using the same Amplification Kit, and quality control was assessed. 1.5 µg of cRNA was mixed with the hybridization controls and further hybridized onto Human Whole-Genome Expression Beadchips (Human HT12 v4.0), assessing around 31,000 annotated genes with more than 47,000 probes derived from the National Center for Biotechnology Information Reference Sequence (NCBI) RefSeq Release 38 (November 7, 2009). Hybridation was performed in an oven during 16 hours at 58°C. After a series of washes, array were stained with streptavidin-Cy3, washed again and dried before scanning using the Illumina BeadArray reader. To reduce the influence of technical variability, paired samples were processed together, but different pairs were randomly distributed at each experimental step. Probeset signals (i.e., transcript levels) were extracted with the Beadarray software following the default quality control parameters and batch-normalized.

1.2 Statistical analysis

Microarray analysis. Effects of MDD on gene expression in the amygdala : Analysis using Random intercept model (RIM) and Multiple factors correction integration :

To account for paired design (MDD samples paired with corresponding controls with no disease) and existence of MDD related clinical variables, we apply a random intercept model (RIM). For a
given gene g, we fit the model

\[ Y_{gik} = \mu_g + \beta_{g0} X_{0ik} + \sum_{i=1}^{L} \beta_{g1} X_{l_{ik}} + \alpha_k + \epsilon_{gik} \]

where \( Y_{gik} \) is the gene expression value of gene g (1≤g≤G) and sample i (i=1 for controls and i=2 for MDD) in pair k (1≤k≤K), \( X_{0ik} \) takes one if the sample is MDD and zero if the sample is in control group. \( X_{l_{ik}} \) represents values for clinical variable l (e.g. 0-1 binary for whether taking alcohol or not, or numerical for pH values of brain environment) and \( \alpha_k \) is the random intercept, which are the deviations of average of all expression values at k\(^{th}\) pair from the average in the whole population and is assumed to follow normal distribution with mean zero and variance \( \ell^2_g \). Finally, \( \epsilon_{gik} \) are independent random noises with normal distribution with mean zero and variance \( s^2_g \).

Under this model, \( \beta_{g0} \) is the disease effect of gene g of interest and to be estimated. To obtain a MDD-associated biomarker list, likelihood ratio test (LRT) is used to assess the p-values of testing \( H_0: \beta_{g0} = 0 \) and p-values are then corrected by Benjamini-Hochberg procedure for multiple comparison,\(^1\) with a false discovery rate (FDR) set at 5%.

1.3 Functional analysis using the Ingenuity knowledge database

Functional annotation analyses were carried out by submitting list of genes to the Ingenuity Pathway Analysis tool (Ingenuity Systems, Mountain View, CA, USA; http://ingenuity.com/), a system with a web-based interface providing computational algorithms to functionally analyze large datasets and identify or generate gene/protein networks that are formed by the genes of interest. To test the robustness of our IPA analysis, 47 different lists of genes generated with increasing absolute fold-change (0 to 50%) and more stringent statistical values cut-offs (0.05 to 0.0001) obtained by either RIM alone or in combination with paired t-test and Wilcoxon analysis were generated and ran through IPA.
1.4 Real-time quantitative Polymerase Chain Reaction (qPCR)

Small PCR products (80–150 base-pairs) were amplified in quadrupletts using universal PCR conditions (65°C to 59°C touch-down, followed by 35 cycles: 15s at 95°C, 10s at 59°C and 10s at 72°C) as previously described on an Eppendorf Mastercycler.\(^2\) cDNA (150 pg) was amplified in 20 \(\mu\)l reactions (0.3X Sybr-green, 3 \(\mu\)M MgCl\(_2\), 200 \(\mu\)M dNTPs, 200 \(\mu\)M primers, 0.5 unit Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA)). Primer dimers were assessed by amplifying primers without cDNA. Primers were retained if they produced no primer dimers or nonspecific signal only after 35 cycles. Results were calculated as the geometric mean of relative intensities compared to three internal controls (actin, glyceraldehyde-3-phosphate dehydrogenase, and cyclophilin G), previously shown not to be altered in MDD.\(^3\)

1.5 In situ hybridization

Templates for the synthesis of the antisense and sense riboprobes for human SST mRNA were generated by PCR.\(^4\) The specific primers amplified a 337 base pair (bp) fragment of human SST. These fragments corresponded to bases 112–448 of the human SST gene (GenBank NM_001048). Nucleotide sequencing confirmed 100% homology of the amplified fragment to the previously reported sequence. The fragment was then subcloned into a plasmid (pSTBlue-1, Novagen, Madison, WI). The antisense and sense riboprobes were transcribed in the presence of 35S-CTP (Amersham Biosciences, Piscataway, NJ) using T7 and SP6 RNA polymerase, respectively. DNase I was used to digest the DNA template. The riboprobes were purified using RNeasy mini spin columns (Qiagen, Valencia, CA). One section from each pair was processed during a single run with the sections from each pair processed side by side, and with the location of the slides in the hybridization container counterbalanced between diagnostic groups during each run. A total of three representative sections of the anterior part of the amygdala were used per subjects. Prior to the hybridization reaction, tissue sections were fixed with 4%
paraformaldehyde in phosphate-buffered saline solution, acetylated with 0.25% acetic anhydrate in 0.1 M triethanolamine/0.9% NaCl for 10 min, and dehydrated with a graded alcohol series. The sections were then hybridized with [\(^{35}\)S]-labeled riboprobes (10\(^{6}\) cpm/\(\mu\)L) in hybridization buffer at 56 °C for 16h. The hybridization buffer contained 50% formamide, 0.75 M NaCl, 20 mM 1,4-piperazine diethane sulfonic acid, pH 6.8, 10 mM ethylenediaminetetraacetic acid (EDTA), 10% dextran sulfate, 5X Denhardt’s solution (0.2 mg/mL Ficoll, 0.2 mg/mL polyvinylpyrrolidone, 0.2 mg/mL bovine serum albumin), 50 mM dithiothreitol, 0.2% sodium dodecyl sulfate, and 100 \(\mu\)g/mL yeast transfer RNA. Following the hybridization reaction, sections were washed in a solution of 0.3 M NaCl, 20 mM Tris–HCl, pH 8.0, 1 mM EDTA, pH 8.0, and 50% formamide at 63 °C, treated with RNase A (20 \(\mu\)g/mL) at 37°C, washed in 0.1X SSC (1.5 mM NaCl, 150 mM sodium citrate) at 67°C, dehydrated through a graded ethanol series, and air dried. After 3 days of exposition, autoradiographics were developed, captured, digitized, and analyzed with a Microcomputer Imaging Device (MCID; Imaging Research Inc., London, Ontario, Canada). The lateral, basal and laterobasal nuclei were localized on the original slide using the contrast difference between respective nuclei on brightfield images and confirmed in parallel with Nissl and Acetylcholine-esterase staining on adjacent serial sections. Films and slides were then overlaid and signal density quantifications was performed on the total area of each nucleus.

1.6 qPCR and in situ hybridization statistical analysis

Diagnosis-related expression differences in genes signal was determined by analysis of covariance (ANCOVA) using SPSS (SPSS, Inc., Chicago, IL). The qPCR data were averaged across the four replicates and transformed into relative expression levels (2\(^{-\Delta\Delta Ct}\)) so that higher values represent greater relative expression. To determine relevant covariates, scale covariates (such as age, pH, PMI, RIN) were tested by Pearson correlation. Multiple comparisons with
covariates were controlled by adjusting for simultaneous inference of significance levels using the Bonferroni-Holm method.$^5,6$

1.7 Protein isolation, Prepro-SST and BDNF Immunoblotting

Acetone precipitation of proteins was carried out following RNA extraction from the TRIzol samples of brain tissues. The lower red phenol-chloroform phase was used for protein isolation, using ethanol to precipitate DNA, and acetone to extract protein from the supernatant. Following extensive washes, the dried pellet was dissolved in 1x SDS buffer. The supernatant was collected after 5 min centrifugation at 14,000 rpm. An aliquot was used for protein quantification using PIERCE BCA assay (PIERCE, Rockford, IL). Confirmation of expected sizes for pre-pro SST (14kD), pro-BDNF (25kD), m-BDNF (15kD) and beta actin (37kD) were performed with a HEPES-based buffer system. For comparative quantification, 5µg of protein samples were resolved by SDS PAGE in 10% Tris/glycine gels and transferred to PVDF membrane. Western blot analysis was performed using the Odyssey system (LI-COR Biosciences, Lincoln, NE, USA) as described.$^7$ In brief, membranes were blocked in LI-COR blocking buffer and incubated with mouse anti-actin at 0.5µg/mL (Sigma #A 2228) and rabbit polyclonal primary antibody for prepro-somatostatin (prepro-SST) at 0.5µg/mL (ab53165, Abcam, Cambridge, MA, USA) or mouse monoclonal primary antibody for BDNF at 0.25µg/mL (MAB248, R&D Systems, Minneapolis, MN, USA). Fluorescent IR Dye 680 anti-Rabbit and fluorescent IR Dye 800 anti-mouse (LI-COR Biosciences) secondary antibodies were used in signal detection. Dual signals were detected using the LI-COR Odyssey Infrared imaging system, and prepro-SST/Actin signal ratios were calculated.
1.8 Immunoblot statistical analysis

To control for gel-to-gel variability, two parallel statistical approaches were applied. First, paired t-tests were run in age- and sex-matched pairs, and group results were combined across the three runs using the Stouffer’s approach, which considers p-values, sample sizes, and effect directions. This analysis was chosen for statistical descriptions on Figures 1 & 2. Second, an ANCOVA model with age, pair, PMI and batch processing as covariate factors was applied and group results were similarly combined across the three runs using the Stouffer’s approach. Significance was set at $\alpha=0.05$.

1.9 Unpredictable chronic mild stress (UCMS) and antidepressant treatment reversal

UCMS was used to model the role of socio-environmental stressors in precipitating a depressive-like syndrome that shares characteristics with human depression, such as increased fearfulness/anxiety-like behavior, decreased consumption of palatable food and physiological changes. Here, UCMS consisted of a 6-week regimen of pseudo-random unpredictable mild stressors: forced bath, wet bedding, predator odor, light cycle changes, social stress, tilted cage, mild restraint and bedding changes. Stressed animals were group-housed, as for control animals (4-5 animals/cage). Fluoxetine treatment (16 mg/kg, p.o.) was administered to mice during 4 weeks after the first 2 weeks of UCMS. Changes in behavior were monitored using elevated plus maze (EPM), open field (OF) and novelty suppressed feeding (NSF) tests. Results are described in Guilloux et al., 2011. In the OF, no significant effects of UCMS or fluoxetine were observed on either time in the center or ratio of ambulatory distance in the center. In the EPM, UCMS-exposed females spent significantly less time ($p < 0.001$) and entered proportionately less often ($p < 0.01$) into the open arms compared to controls, while
chronic fluoxetine treatment blocked the development of those effects for both parameters (p < 0.05). In the NSF, fluoxetine-treated animals displayed lower latency to start eating the food pellet compared to saline treated UCMS-exposed mice (p<0.05). No difference between control unstressed mice and fluoxetine-treated stressed mice was observed for all experiments. We used advantage of emotionality score calculation to evaluate convergent and consistent changes in behavior in this cohort. Using this tool, we conclude that final z-score integration revealed a significant effect of UCMS, suggesting a stable underlying effect of stress on behavior. As expected, chronic SSRI treatment reversed the elevated stress-induced z-score measures of emotionality (p < 0.01). After behavioral assessment, brains were harvested and amygdalae micropunctured and stored as mentioned in the manuscript.

Total RNA was extracted from frozen samples stored in TRIzol (Invitrogen, Carlsbad, Calif.), processed for microarray analysis according to the microarray manufacturer's protocol (Illumina, San Diego, Calif.) and hybridized onto Mouse Whole-Genome Expression Beadchips (MouseWG-6 v2.0), assessing over 19,100 annotated genes with more than 47,000 probes derived from the National Center for Biotechnology Information Reference Sequence (NCBI RefSeq) database (Build 36, Release 22). Probeset signals (i.e., transcript levels) were extracted with the Beadarray software following the default quality control parameters and batch-normalized. For statistical analysis between UCMS and UCMS+fluoxetine, we used ANOVA model with UCMS exposure and UCMS+Flx as separate groups.
2 SUPPLEMENTARY TABLES

Supplementary Table ST1: Full q-list Core Genes Significantly Affected in Female Major Depression

(Separate download)

This list contains the 116 genes differentially expressed in major depressive disorder (MDD) versus control. Changes were assessed by the random intercept model ("RIM") with the associate p-values (adjusted for confounding clinical, demographic and technical variables) and q-values obtained after multiple testing correction. "WM/GM Fold Change" indicates relative neuronal (low fold change values, top rows) to neuronal (high fold change values, high rows) enrichment of gene transcript, as previously described.16

Supplementary Table ST2: Genes observed differentially expressed in MDD in at least one statistical test and linked to BDNF in Ingenuity Pathway Database

(Separate download)

Supplementary Table ST3: Overall genes tested and/or confirmed by qPCR

(Separate download)

As mentioned in the results sections, microarray results for 14 genes differently expressed in MDD (q-list : AMPH, CDK5RAP2, CORT, GFAP, KCNG1, MBP, MOBP, NPY, NSF, RGS4, SLC32A1, SST, SSTR1, TAC1) were confirmed by qPCR. 27 other transcripts selected because of their role in GABA and/or BDNF signaling were tested (such as BDNF, GAD1, GAD2, GABA-receptors subunits (GABRA2 and GABRA5), calretinin (CALB2) and HTR3A).
Supplementary Table ST4: Pearson Coregulation Values for transcript levels between any-two BDNF-related Genes Across Overall Samples

Pearson correlation values of expression of between BDNF, SST, NPY, RGS4 and TAC1 across the whole cohort, confirmed that these genes are co-expressed. This suggests that the expression of these genes is tightly linked together and that these genes may participate together in common biological functions.\textsuperscript{17}

|          | BDNF-IxD | SST   | NPY   | RGS4  | TAC1  |
|----------|----------|-------|-------|-------|-------|
| BDNF-IxD | 1        |       |       |       |       |
| SST      | 0.39     | 1     |       |       |       |
| NPY      | 0.33     | 0.86  | 1     |       |       |
| RGS4     | 0.53     | 0.50  | 0.41  | 1     |       |
| TAC1     | 0.29     | 0.60  | 0.54  | 0.49  | 1     |

Supplementary Table 4: Pearson Coregulation Values Between BDNF-related Genes Across Overall Samples
3 SUPPLEMENTARY FIGURES

Supplementary Figure S1: Functional analysis using the Ingenuity knowledge database

(A) The top biological functions over-represented using an exploratory gene list (307 genes differentially expressed following these criteria fold change>20% and p<0.05 in either RIM, Wilcoxon and Paired Student T-test analyses) were associated with function of the nervous system, neurological disorder, psychological disorders and behavior. (B) Notably, the second network, entitled "Neurological Disease, Genetic Disorder, Psychological Disorders" was associated with the "GABA receptor signaling" top canonical pathway. (C) To assess the robustness of these functional results, we performed analyses on different lists of genes generated with increasing threshold for absolute fold-change (from 0 to 50%) and different statistical values cut-offs (from 0.05 to 0.0001), obtained by either RIM alone or in combination with paired t-test and Wilcoxon analysis. Thus, 47 different lists including 13 to 4131 genes were generated and ran through IPA analysis. The low mean rankings of our top identified gene groups confirmed the robustness of the findings, as "Genetic Disorders", "Neurological Disease" and "Psychological Disorders" appears in the 3 most recurrent findings over all iterations.
Figure S1

### Top Biological Functions

| Diseases & Disorders                       | Name                                | p-value              | # Molecules |
|-------------------------------------------|-------------------------------------|----------------------|------------|
| Neurological Disease                      |                                     | 4.02E-11 - 3.25E-02 | 118        |
| Genetic Disorder                          |                                     | 6.75E-11 - 3.25E-02 | 154        |
| Psychological Disorders                   |                                     | 6.75E-11 - 3.25E-02 | 61         |
| Molecular & Cellular Functions            | Cellular Assembly and Organization  | 2.32E-06 - 3.25E-02 | 53         |
|                                            | Cell-To-Cell Signaling and Interaction | 3.07E-06 - 3.25E-02 | 30         |
|                                            | Cellular Function and Maintenance   | 4.36E-05 - 3.25E-02 | 23         |
| Physiological System Development & Function | Nervous System Development and Function | 3.07E-06 - 3.25E-02 | 61         |
|                                            | Behavior                            | 9.73E-05 - 2.91E-02 | 23         |

### Top Canonical Pathways

| Name                                | p-value   | Ratio         |
|-------------------------------------|-----------|---------------|
| GABA Receptor Signaling             | 9.80E-05  | 6/55 (0.109)  |
| Huntington's Disease Signaling      | 6.94E-04  | 11/240 (0.046)|
| Mitochondrial Dysfunction           | 2.70E-03  | 7/169 (0.041) |

### Top Biological Function

| Diseases and Disorders               | # of appearance over 47 iterations | Mean Rank over 47 iterations |
|--------------------------------------|------------------------------------|-----------------------------|
| Genetic Disorder                     | 47                                  | 2.0                         |
| Neurological Disease                 | 46                                  | 1.7                         |
| Psychological Disorders              | 39                                  | 3.3                         |
| Skeletal and Muscular Disorders      | 35                                  | 3.5                         |
**Supplementary Figure S2: BDNF exons and Trk-B expression in MDD**

mRNA levels BDNF non-coding exons I, IV and IXa as well as the different forms of the Trk-B receptors were assessed in 10 pairs on concentrated solutions of cDNA to increase detection of exons, and no differences were observed between MDD (red bars) and CTRL (blue bars) subjects. All p-values >0.05.
Figure S2

The bar chart illustrates the qPCR (DCt) values for various BDNF Exons and Trk-B constructs:

- BDNF Exon I
- BDNF Exon IV
- BDNF Exon IXa
- Full Length
- Truncated 1
- Truncated 2
Supplementary Figure S3: Effect of age on the BDNF-related genes

We assessed the effect of age on 52 BDNF related-gene set as a whole group. Within subject, we first calculated an individual corrected log-ratio of increase or decrease of each gene expression compared to its expression in the whole cohort. We then calculated the average of the absolute value of these ratios across genes within subject, defined here as a "BDNF signal". A Pearson correlation revealed a global effect on this BDNF signal across the whole cohort (R=0.44, p<0.004)

Furthermore, using an ANCOVA analysis, with "BDNF signal" as a dependent variable, MDD as a fixed factor and age as a covariate gave the following results: age was considered as a strong covariate that predicts the effect of MDD over the BDNF signal results (p=7.2E-4). Nonetheless, when effect of age was removed, effect of MDD was still highly significant (p=8.8E-4).
Figure S3

A scatter plot showing the relationship between BDNF signal and age, with two groups indicated: Ctrl (blue) and MDD (red). The correlation coefficient is r=0.44**.
Supplementary Figure S4: Hormonal Status

(A-F) Expression values of genes implicated in hormone regulation or biomarkers of menopause in function of the age of the subjects. Each bar represents a subject (CTRL or MDD). Gene selection was realized based on the enzymes involved in the steroidogenesis pathway (CYP19A1, SRD5A2, HSD17B1, UGT1A4, SULT1A1), estradiol receptors (ESR1, ESR2), key-elements of the ovarian cycle (PGR, PRL, TSHR) and genes already reported in the literature as peripheral biomarkers of menopause (AMH\textsuperscript{18} or of HRT (PAX8\textsuperscript{19}). Only AMH, CYP19A1, ESR1, ESR2, PGR and SULT1A1 were the mostly expressed throughout the cohort in the amygdalae. None of the genes' expression measured correlates with age (G) Pearson correlation values of expression of between BDNF-related genes and genes related to hormonal status across the whole cohort (inside brackets [ ] is the number of subjects for which genes expression was detected), None of the genes' expression measured correlates with any of the expression levels of BDNF-related genes (G).
Figure S4

| GENES          | BDNF-IX [42] | SST [42] | NPY [42] | RGS4 [42] |
|---------------|--------------|----------|----------|-----------|
| CYP19A1 [29]  | 0.09         | 0.26     | 0.36     | 0.13      |
| ESR1 [29]     | 0.16         | 0.26     | 0.15     | 0.29      |
| ESR2 [14]     | -0.26        | 0.36     | 0.41     | -0.10     |
| PGR [25]      | -0.21        | -0.22    | -0.17    | 0.02      |
| SULT1A1 [42]  | -0.12        | 0.15     | 0.22     | -0.31     |
Supplementary Figure S5: Suicide: cofactor of BDNF related genes regulation in MDD

Distribution histograms of the 52 BDNF-related genes depending on their alr values in the overall MDD population (A), in MDD-subjects who died by accidental or natural causes (n=14) (B), and MDD-subjects died by suicide (n=7) (C). The narrowing of the histogram around the no-change axis indicates higher frequency of BDNF-related genes with low alr values in suicide MDD subjects (n=7) compared to the non-suicide MDD subjects (B).
Figure S5

A. Overall MDD

B. MDD - Non Suicide

C. MDD - Suicide

- Size effect < 20%
- Upregulated genes > 20%
- Downregulated genes > 20%

Alr (MDD/Control)
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