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MODELING DEPRESSION: SOCIAL DOMINANCE–SUBMISSION GENE EXPRESSION PATTERNS IN RAT NEOCORTEX

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Abstract—Gene expression profiles in the cortex of adult Long-Evans rats as a function of a stressful social loss and victory in inter-male fighting encounters were examined. This social dominance and subordination model has been postulated to simulate early changes in the onset of depression in the losers. Microarrays were fabricated containing 45mer oligonucleotides spotted in quadruplicate and representing 1178 brain-associated genes. Dynamic range, discrimination power, accuracy and reproducibility were determined with standard mRNA "spiking" studies. Gene expression profiles in dominant and subordinate animals were compared using a "universal" reference design [Churchill GA (2002) Fundamentals of experimental design for cDNA microarrays. Nat Genet 32 (Suppl):490–495]. Data were analyzed by significance analysis of microarrays using rank scores [Tusher VG, Tibshirani R, Chu G (2001) Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci USA 98:5116–5121; van de Wiel MA (2004) Significance analysis of microarrays using rank scores. Kwantitatieve Methoden 71:25–37]. Ontological analyses were then performed using the GOMiner algorithm [Zeeberg BR, Feng W, Wang G, Wang MD, Fojo AT, Sunshine M, Narasimhan S, Kane DW, Reinhold WC, Lababidi S, Bussey KJ, Riss J, Barrett JC, Weinstein JN (2003) GoMiner: a resource for biological interpretation of genomic and proteomic data. Genome Biol 4(4): R28]. And finally, genes of special interest were further studied using quantitative reverse transcriptase polymerase chain reaction. Twenty-two transcripts were statistically significantly differentially expressed in the neocortex between dominant and subordinate animals. Ontological analyses revealed that significant gene changes were clustered primarily into functional neurochemical pathways associated with protein biosynthesis and cytoskeletal dynamics. The most robust of these were the increased expression of interleukin-18, heat shock protein 27, β3-tubulin, ribosome-associated membrane protein 4 in subordinate animals. Interleukin-18 has been found to be over-expressed in human depression and panic disorder as well as other physiological stress paradigms [Takeuchi M, Okura T, Mori T, Akita K, Ohta T, Ikeda M, Ikegami H, Kurimoto M (1999) Intracellular production of interleukin-18 in human epithelial-like cell lines is enhanced by hyperosmotic stress in vitro. Cell Tissue Res 297(3):467–473] and heat shock proteins have been shown to be involved in the pathogenesis of many neurodegenerative and psychiatric disorders [Iwamoto K, Kakiuchi C, Bundo M, Ikeda K, Kato T (2004) Molecular characterization of bipolar disorder by comparing gene expression profiles of postmortem brains of major mental disorders. Mol Psychiatry 9(4):406–416; Pongrac JL, Middleton FA, Peng L, Lewis DA, Levitt P, Mirnics K (2004) Heat shock protein 12A shows reduced expression in the prefrontal cortex of subjects with schizophrenia. Biol Psychiatry 56(12):943–950]. Thus, the gene expression changes that we have observed here are consistent with and extend the observations found in the clinical literature and link them to the animal model used here thereby reinforcing its use to better understand the genesis of depression and identify novel therapeutic targets for its treatment. © 2005 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: aggression, depression, genes, microarray, qRT-PCR, vocalization.

It is a commonplace observation that there are "winners" and "losers" in all forms of social competition. In fact, the idea that social defeat, which typically leads to subordination, may be a major contributory factor in the emergence of depression has recently crystallized within evolutionarily oriented segments of the psychiatric community (Gardner, 2001; Gardner and Wilson, 2004). A related idea is that early social loss is a major contributor to future depression (Heim and Nemeroff, 2001; Nelson and Panksepp, 1998).

The purpose of the present work was to identify gene expression changes using an animal model that shows prototypical forms of socio-emotional arousal accompanying social loss that may be related to the etiology of depression and lead to the development of novel therapeutics. It is widely recognized that the chronic stress that occurs in the presence of persistent loss in aggressive social encounters (Agid et al., 2000; Nemeroff, 1998), especially in the absence of social support (Ruis et al., 1999; de Jong et al., 2005), is a major factor that sets in motion incompletely understood neurobiological events that leads to depressive disorders (Lopez et al., 1999; Nemeroff, 1998; Nestler et al., 2002; Price et al., 1994; Tornatzky and Miczek, 1993). In this work we analyzed the genetic consequences of social loss using a well-standardized dominance–submission model in adult male rats, a model of depression originally proposed by Price (1967) and advanced by Sloman and Gilbert (2000). In typical resident–intruder models, the intruder uniformly becomes the loser (Blanchard et al., 1993).

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Abbreviations: FCTX, frontal cortex; FDR, false discovery rate; FGF, fibroblast growth factor; GO, gene ontology; IL-18, interleukin-18; LOWESS, locally weighted scatterplot smoothing; PCTX, posterior cortex; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; RAMP4, ribosome-associated membrane protein 4; SAM, significance analysis of microarrays; SAM-RS, significance analysis of microarrays using rank scores; USVs, ultrasonic vocalizations.

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While little is known about the molecular biological changes set in motion by social defeat, among the neurochemical possibilities suggested by animal models are an initial arousal of biogenic amines (Higley et al., 1993; Tidey and Miczek, 1996), endogenous opioids (Miczek et al., 1991), related neuropeptides such as corticotrophin releasing factor (CRF) (Nemeroff, 1998), cytokines (Dantzer et al., 1999), and increases in cortical cholecystokinin (CCK) neurotransmission (Becker et al., 2001). Moreover, recent studies have demonstrated that changes in social status following the formation of stable dominance hierarchies can change neural architectures associated with survival of new neurons (Kozorovitskiy and Gould, 2004). In contrast, antidepressant agents (esp. serotonin specific reuptake inhibitors) decrease irritability and increase confidence in humans (e.g. Knutson et al., 1999; Raleigh et al., 1991), and can modulate neuronal survival (Mattson et al., 2004; Post et al., 2004).

Studies employing *in situ* hybridization have highlighted major differences in *c-fos* gene expression patterns in winners and losers in inter-male aggression encounters (Kollack-Walker et al., 1997). Because such immediate-early gene expression induces poorly understood "downstream" cascades of gene-induction, it is expected that there will be many gene expression changes associated with this form of social competition. It is also expected that some of these genetic changes may be critical in the progression toward depression.

The use of microarray techniques in neurological systems is rapidly evolving, and their value in the study of psychiatric disorders is well appreciated (Watson and Akil, 1999). For example, recent studies of sleep cycles (Cirelli et al., 2004) have yielded provocative findings indicating waking-specific and sleep-specific profiles of gene expression. Preliminary results are also emerging for other complex brain processes and disorders such as aging and cognition (Blalock et al., 2003), development (Mody et al., 2001), learning and memory (Cavallaro et al., 2002; Thompson et al., 2003), Alzheimer's disease (Blalock et al., 2004), schizophrenia (Mirnics et al., 2001), and depression (Sibille et al., 2004). However, this technology has not yet been applied systematically in the social dominance/psychological depression models described above.

Here we report on a carefully selected cohort of six pairs of male rats from inter-male aggression encounters that exhibited the strongest asymmetries in dominance and submission. mRNAs from neocortical tissues were examined by microarray analysis. Utilizing significance analysis of microarrays using rank scores (SAM-RS) (Tusher et al., 2001; van de Wiel, 2004), 18 genes were found to be predominantly expressed in submissive neocortex compared with dominant neocortex and one gene predominantly expressed in dominant neocortex compared with submissive neocortex. Ontological analyses on these differentially expressed genes were then performed using the GOMiner algorithm (Zeeberg et al., 2003). Genes regulating protein synthesis and cytoskeleton formation were significantly enriched. A subset of these genes was further examined and quantified by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR): interleukin-18 (IL-18), heat shock protein 27, β3-tubulin, and ribosome-associated membrane protein 4 (RAMP4).

EXPERIMENTAL PROCEDURES

Behavioral analysis

Subjects. This research was approved by the Bowling Green State University IACUC, which included measures to reduce suffering and total number of animals used in this study, and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The 18 animals for this study were derived from a larger group of 29 Long-Evans adult male rats that were evaluated for behavioral dominance in an isolation-induced resident–intruder aggression paradigm, after which they were killed, and brain tissues harvested. From this larger group, the six male pairs from different litters that exhibited the clearest dominance–submission patterns and six littermate control males that were treated the same way, except for aggression testing, were selected for further genetic analysis. This winnowing procedure was deployed to assure the highest-quality tissues from our samples. Animals were 3–4 months old weighing 435–573 g at the time of kill. All animals had been bred and born at the BGSU animal facility, pups were weaned at 21 days of age, and isolate housed in 20×40×20 cm plastic cages until testing. Animals were maintained on a 12-h light/dark cycle, with lights on at 7 a.m. Animals were given *ad libitum* access to food and water throughout the study and were tested during the light cycle.

Behavioral testing. Prior to testing, animals were weighed and assigned to either the experimental resident or intruder groups, or the control group. Animals were approximately matched for weight, with residents being approximately 10% heavier than the intruders. All subjects were paired with former littermates, but the animals had no social interactions since weaning. All animals were habituated to the testing environment (a separate room) for two consecutive days for 30 min each day. Habituation consisted of removing the animal's cage lid and placing a translucent plastic lid on top of the cage. During testing, ultrasonic vocalizations (USVs) were also recorded using a Petterssen D980 ultrasonic detector (Uppsala, Sweden; frequency division 1/10), and the animals were videotaped using a commercially available camera and VCR. Videotape records were hand scored by a single observer for three behaviors: 1) freezing, 2) bites, and 3) dorsal contacts. Inter-observer correlations for these behavioral measures have previously been reported, with the inter-observer Pearson correlations for dorsal contacts $r=+.87$, for freezing $r=+.99$, and for bites it was $r=+.98$ (Panksepp et al., 2004). USVs were scored via sonogram (Avisoft Bioacoustics, Berlin, Germany) with 50-kHz USVs scored from heterodyne (tuned to 55-kHz with a range of ±5-kHz) and 20-kHz calls scored from frequency division recordings. All behaviors monitored were scored blind with respect to biochemical results.

Testing occurred on the days immediately after habituation, and consisted of two consecutive days of placing the intruder in the resident's cage and video recording their social interaction for 30 min for each day. This initial period was followed by 4 days of no testing and concluded with two additional days of testing. In order to appropriately account for changes due to handling and exploration of a novel environment, control animals were placed alone into the home cage of another adult male rat during testing, and transported to the same test room at the same time as the experimental rats. Animals were killed with carbon dioxide six hours after the last encounter. Their brains were rapidly removed, olfactory bulbs were discarded, and the rest of the brain was dissected and immersed into an RNAse inhibitor (RNAlater, Ambion, Austin, TX, USA). Prior to analysis, brain samples were further dissected into nine sub-areas: 1) frontal cortex

(FCTX, tissue anterior to the frontal pole of the basal ganglia); 2) posterior cortex (PCTX, including all the posterior neocortical areas dorsal to the rhinal sulcus behind the FCTX sample); 3) basal ganglia (including caudate nuclei, globus pallidus and nucleus accumbens); 4) temporal-amygdaloid area; 5) hippocampus; 6) diencephalon (including most of the thalamus and hypothalamus); 7) the cerebellum, 8) the mesencephalon, and 9) the brainstem. This study focused on cortical changes and the remaining tissue samples were frozen at −80 °C and banked for potential future analysis.

Gene expression analysis

Microarray fabrication, validation, and quality control. The genes comprising our rat CNS microarrays were compiled from currently available NCBI/EMBL/TIGR rat sequence databases and commercially available CNS arrays (Clontech, Mountain View, CA, USA/Affymetrix, Santa Clara, CA, USA), and provided representation from greater than 90% of the major gene ontological categories.

Individual 45-mer oligonucleotides complementary to sequences of 1178 cloned rat CNS mRNAs were designed and prioritized based on combining very stringent selection criteria (minimal secondary structure, minimal homology to other genes in the available rat genomic databases, no low complexity or repeat regions, defined T_m [ArrayDesigner v2.03]) with a statistical ranking algorithm (Lockhart et al., 1996). Control oligonucleotides representing the most traditionally-accepted and commonly-utilized housekeeping genes (Lee et al., 2002) were similarly designed and prioritized.

These optimal oligonucleotides were individually synthesized on a PolyPlex™ 96-well oligonucleotide synthesizer (GeneMachines®, San Carlos, CA, USA), utilizing standard phosphoramidite chemistry. Addition of a 5′-amino linker (C6-TFA, Glen Research, Sterling, VA, USA) onto each oligonucleotide facilitates covalent attachment of only full length, properly synthesized oligos to aldehyde-treated glass microarray slides. The oligonucleotide probes were spectrophotometrically quantitated and immobilized on the microarrays.

Array manufacture utilized an OmniGrid™ robotic microarrayer (GeneMachines®) and a four pin configuration. Each oligonucleotide, suspended in 3× SSC/1.5 M betaine buffer at a concentration of 500 ng/μl was covalently linked in quadruplicate to aldehyde-coated glass microscope slides (GeneMachines®) at a spacing of 250 μm. The arrays were then baked *ad vacuo* at 80 °C for 2 h and stored desiccated until use.

The dynamic range, discrimination power, accuracy, reproducibility, and specificity of the oligonucleotide microarrays used in these studies were evaluated by exogenous mRNA spiking experiments (Baum et al., 2003). Each of the five exogenous bacterial RNAs were spiked into varying background rat reference RNA (Stratagene, La Jolla, CA, USA) concentrations (0.5 μg, 2 μg and 10 μg) at molar ratios ranging from 10^{-3} to 10^{-7}. Each combination of transcript dilution and RNA background was individually amplified, labeled, and hybridized to the arrays, as described below. The dynamic range, defined as the range of transcript abundance over which hybridization intensity was linearly correlated in six independent experiments and was found to be between two and three orders of magnitude. The data presented in this report fell within this dynamic range. We used discrimination power, or the ability to discriminate authentic signal from background at the low end of the dynamic range, to set appropriate cutoffs prior to statistical analysis of the data (described below). Any data obtained that were below this cutoff were eliminated. The reproducibility of both the raw, preprocessed data (unnormalized, not corrected for background, no elimination of outliers or low intensity features) and appropriately normalized data was determined by comparison of the coefficients of variation across all levels of expression for each exogenous transcript. Reproducibility levels in our microarrays, as estimated by coefficients of variation, are typically CV=0.09, which is consistent with all published microarray reports (Mirnics, 2001). The accuracy of the microarray results was determined by direct comparison to individual mRNA abundance determined by qRT-PCR analysis of the spiked mRNA samples. Conformity between the two datasets (i.e. qRT-PCR and the spiked microarray samples) was measured, with a Pearson correlation coefficient of +0.92, which is in good agreement with reported values (Baum et al., 2003). Hybridization specificity was evaluated using a range of one to six sequence mismatches synthesized within the gene-specific 45mer oligonucleotide immobilized on the array. The mismatched oligonucleotides were also pretested for cross-hybridization, as above. The results of this study demonstrated that the oligonucleotides that we synthesized were gene specific since adverse effects on hybridization efficiency were not found with less than three mismatches.

Target preparation; RNA extraction and labeling, and microarray hybridization. Total RNA was extracted from defined brain regions with guanidine isothiocyanate and CsCl-ultracentrifugation, purified (Qiagen, Valencia, CA, USA) and used as the substrate for RNA amplification and labeling using a procedure based on the Eberwine protocol (Van Gelder et al., 1990). Specifically, reverse transcription of 5 μg RNA primed with an oligo(dT) primer bearing a T7 promoter was followed by *in vitro* transcription in the presence of amino-allyl dUTP (3:2 with unmodified dUTP). We utilized universal rat reference RNA (Stratagene) in our analyses and treated identical aliquots concurrently with the tissue samples. 10 μg each of the Cy5-labeled (experimental) and purified Cy3-labeled (reference) amplified RNA (aRNA) targets (each labeled to 15–18% incorporation) were combined in a hybridization solution containing 8 μg poly(dA)40–60, 10 μg rat C_0t-1 DNA, 4 μg yeast tRNA, 1× Denhardt's solution, 0.2% SDS and 2.4× SSC in a final volume of 50 μl, subsequently denatured and hybridized in a humidified hybridization chamber at 46 °C for 16 h. Following sequential high-stringency washes, individual Cy3 and Cy5 fluorescence hybridization to each spot on the microarray was quantitated by a high resolution confocal laser scanner.

Data acquisition and statistical analysis. Arrays were scanned using two lasers (633 nm and 543 nm) at 5 μm resolution on the ScanArray 4000XL (Packard Biochip Technologies, Billerica, MA, USA) utilizing QuantArray software [v3.0] at the maximal laser power that produced no saturated spots. Data from these scans were collected as two 16-bit .tiff images and analyzed using the adaptive threshold method to differentiate the spot from the background. Spot intensity was determined using median pixel intensity. Prior to normalization, quality confidence measurements (spot diameter, spot area, array footprint, spot circularity, signal:noise ratio, spot uniformity, background uniformity, and replicate uniformity) were calculated for each scanned array to assess overall quality and to ensure that acceptable tolerance limits are not exceeded. Spots were flagged that did not pass stringent selection criteria. Along with each of the 16-bit .tiff files relating to both samples, raw data files containing spot intensity values were uploaded to an in-house server running GeneTraffic (v2.8, Iobion Informatics, La Jolla, CA, USA). The data from each channel were normalized using the locally weighted scatterplot smoothing (LOWESS) curve-fitting equation on a print-tip specific basis. Statistical analyses were performed using the permutation-based SAM-RS (van de Wiel, 2004) within the traditional significance analysis of microarrays (SAM) software package (v1.13, Stanford University, Tusher et al., 2001). This software utilizes an algorithm based on the Student's *t*-test to derive statistically significantly differentially expressed genes between two groups of samples using a permutation-based determination of the median false discovery rate (FDR). The SAM algorithm reports the FDR as the percentage of genes in the identified gene list (rather than in the entire cohort of genes present on the microarray) that are falsely reported as showing statistically significant differential expression. The threshold of differential expression can be adjusted to identify different sizes of

sets of putatively significant genes, and FDRs are modified accordingly. Although SAM has been successfully used in a wide variety of experimental paradigms, the one major disadvantage of traditional SAM analyses is that the estimation of the number of significant genes may actually be biased (Efron et al., 2000; Pan et al., 2003). SAM-RS has been recently developed, using rank scores to provide an unbiased estimate of the expected number of falsely called genes (van de Wiel, 2004). Thus, using normal rank scores, or inverse standard normal transformations of the ranks, within the SAM algorithm clearly allows for better control of the FDR than SAM does. In our analyses, appropriately normalized data were analyzed utilizing the two class, unpaired analysis on a minimum of 500 permutations and was performed comparing expression data derived from dominant animals vs. subordinate animals. The cutoff for significance in these experiments was set at a FDR of $<5\%$ at a specified 1.1-fold change.

Ontological analysis. The genes identified in this study were examined for their biological association to gene ontology (GO) categories (Harris et al., 2004). Here, we use the ontological mapping software GOMiner (Zeeberg et al., 2003, available at (http://discover.nci.nih.gov/gominer). GOMiner calculates the enrichment or depletion of individual ontological categories with genes that have changed expression and identifies cellular pathways potentially relevant to social dominance and submission. Pathways within three independent functional hierarchies, namely, biological process, molecular function and cellular component, were queried. Statistical analysis (via Fisher's exact test) highlights and ascribes a level of significance to individual GO annotations and potential biological characteristics of a given coregulated gene set.

Quantitative real-time PCR analysis. The expression levels of selected genes were analyzed by real-time PCR. Reverse transcription of 1 μg of DNAsed, total RNA that was used in the microarray analyses was primed with oligo(dT) and random hexamers, utilized SuperScriptIII, and was performed according to manufacturer's specifications (Invitrogen, Carlsbad, CA, USA). A 1:10 dilution of cDNA was used as a template for real-time PCR and was performed with Brilliant SYBR Green qRT-PCR Master Mix (Stratagene) on an Mx3000P Real-Time PCR System. ROX reference dye was included in all reactions. All primer sets were designed across intron:exon boundaries to derive ~100 bp amplicons, and individual primer concentrations were optimized for each gene. Final amplification conditions were also individually optimized based upon the T_m of the primer set and initially assessed by gel electrophoresis. In addition, dissociation curves were performed on all reactions to assure product purity. Original input RNA amounts were calculated by comparison to standard curves using purified PCR product as a template for the mRNAs of interest and were normalized to amount of H3.3. The minimum expression level of most genes detectable by qRT-PCR was approximately 1×10^{-8} pg, well below the limit of detectability on the microarrays. Experiments were performed in triplicate for each data point.

The sequences of the qRT-PCR primers used in the study were as follows:

Histone H3.3 (X73683), forward 5′-GACTTGAGGTTTCAAAGTGC-3′ and reverse 5′-GGCACACAGATTGGTATCTT-3′

IL-18 (AJ222813), forward 5′-CCCTCTCCTGTAAAAACAAA-3′ and reverse 5′-GCACACGTTTTTGAAAGAAT-3′

β3 Tubulin (AF459021), forward 5′-CTCTAGCCGAGTGAAGTCAG-3′ and reverse 5′-GTCACTGATGACCTCCCA-3′

RAMP4/SERP1 (AB018546), forward 5′-CCTCTTCATTTTTGTCGTTT-3′ and reverse 5′-GGAGAATGGAAACATCTCAA-3′

HSP27-1 (M86389), forward 5′-GCTCACAGTTAAGACCAAGG-3′ and reverse 5′-TGAAGCACCGAGAGATGTA-3′

RESULTS

Behavioral analyses

Differences in dorsal contacts, bites, and freezing behavior were statistically contrasted using within subject *t*-tests. All of the subordinate animals exhibited zero bites. Thus, given the skewness of the biting data, a square root transformation was performed prior to statistical analysis. 50-kHz and 20-kHz USVs were analyzed separately with repeated measures ANOVA. All statistical tests utilized two-tailed comparisons. Dominant animals exhibited more dorsal contacts ($t(5)=4.27$, $P<0.01$) and bites ($t(5)=2.99$, $P<0.05$) than subordinate animals (Fig. 1), and subordinate animals exhibited more

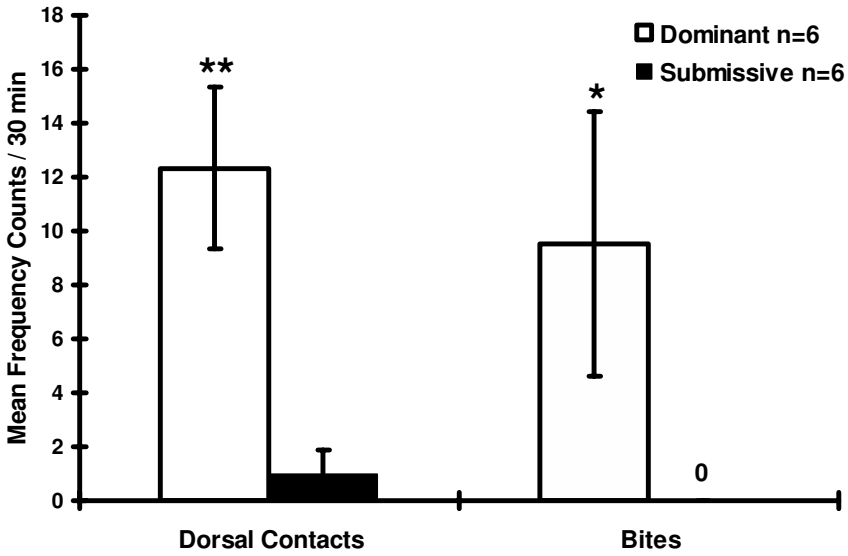


Fig. 1. Mean (±S.E.M.) frequency of dorsal contacts and bites exhibited by the dominant and submissive animals during the final 30 min resident–intruder encounter. [** $P<0.01$, * $P<0.05$ (between subjects two-tailed *t*-test).]

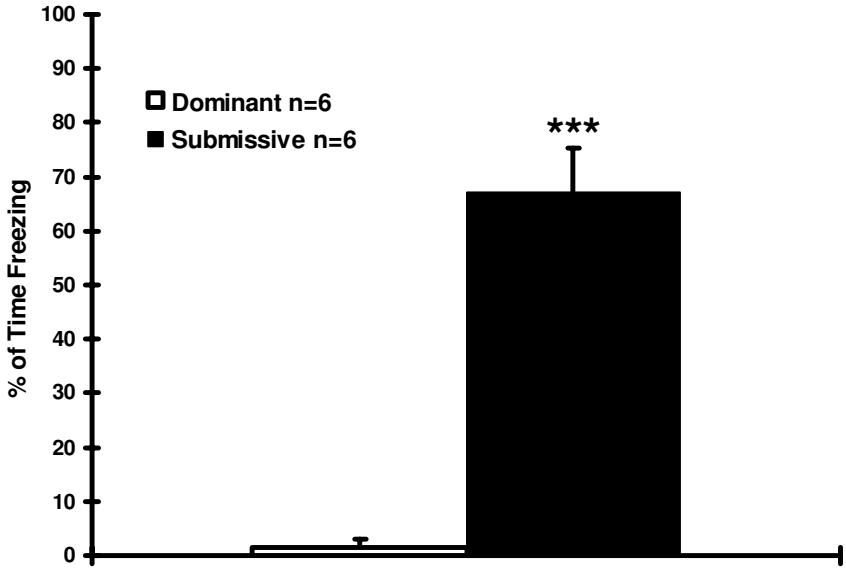


Fig. 2. Mean (±S.E.M.) percentage of time spent freezing during the final 30 min resident intruder encounter for submissive and dominant animals. [*** $P<0.0005$ (between subjects two-tailed *t*-test).]

freezing behavior than dominant animals on the final test day ($t(5)=8.29$, $P<0.0005$; Fig. 2). At the beginning of the test session, animals exhibited only 50-kHZ USVs, but by 10 min into the session 20-kHz USVs prevailed, probably emitted mostly by the subordinate animal. Thus, across the test session, levels of 50-kHz vocalizations decreased markedly across trial blocks ($F(5,25)=2.94$, $P<0.05$), while levels of 20-kHz USVs increased dramatically across trial blocks on the final test day ($F(5,25)=6.92$, $P<0.0005$; Fig. 3). Weight gain across testing was significantly greater for dominant (mean±S.E.M.; 9.5±3.2 g) rats than submissive (3.0±3.8 g) rats ($t(5)=2.80$, $P<0.05$). Inspection of dominance–submission videotapes indicated that in each case when potential thoracic movements in both animals could be observed while 20-kHz USVs were being emitted, it was the submissive animals' 20-kHz USVs that were coincident with thoracic movements (a methodology that cannot be used for the 50-kHz USVs). Indeed, past data using such procedures indicate that practically all 20-kHz USV are generated by the intruders (Panksepp et al., 2004; Takahashi et al., 1983).

Gene expression analyses

Identification of dominance-associated genes. The microarrays described in the Experimental Procedures

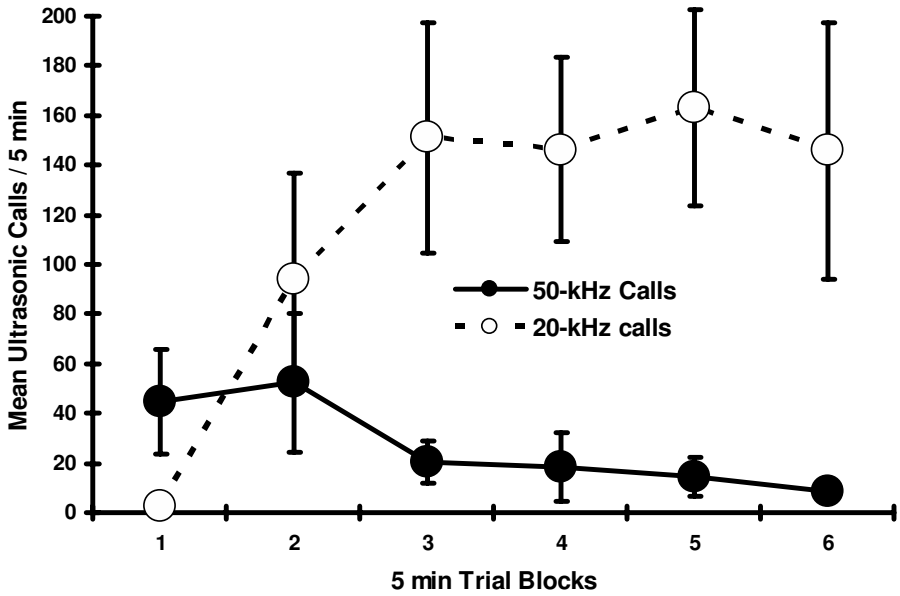


Fig. 3. Mean (±S.E.M.) USVs for animals in the resident–intruder paradigm, summarized in 5 min blocks during the final 30 min encounter.

were used to identify genes expressed in cortical tissue associated with dominance and submission by comparing the expression profile of dominant animals and subordinate animals. Analysis was performed with six individual specimens per group to decrease bias that may be introduced by donor-specific gene expression patterns. A reference experimental design was used. Dominant (n=6), subordinate (n=6), and control (n=6) RNA samples were studied in triplicate with three microarray slides for each sample. As each oligonucleotide is spotted in quadruplicate on the array, there are a total of 72 expression measurements for each gene in each group. Study samples were labeled with Cy5 and universal rat reference samples (Stratagene) were labeled with Cy3.

Initial statistical evaluation at a moderately stringent FDR of 10% for the dominant–subordinate comparison identified 13 and 28 genes preferentially expressed in dominant and subordinate PCTX, respectively. However, in order to focus more on individual differentially expressed genes, so as to select genes of potential interest for further evaluation, we reanalyzed the data with a more stringent FDR. With FDRs <5%, one and 18 genes were identified in dominant and subordinate cortex, respectively (Table 1). Thus, and very importantly, at this stringent FDR, only one of these changes was expected to be a false positive. Table 1 shows the identities, functional annotations, and relative expression ratios of these genes. Although the abundance ratios of these genes did not exceed 1.5-fold, these results clearly indicate that statistically significant changes could be detected. Many functional categories of genes were represented in theses analyses, including signal transduction, synaptic transmission and cytoskeletal reorganization. Several of the identified genes were representative of pathways potentially involved in the depressogenic cascade, including genes in the dopaminergic, GABAergic, and growth factor pathways. However, many genes and pathways not yet described in the context of depression or anxiety were also identified.

The directionalities of all of the significant neocortical gene expression changes, derived from expression ratio differences in dominant or submissive animals relative to controls, were compared (Fig. 4). The majority (>90%) of the differences were due either to changes of opposite directionality or to significant changes exhibited in only the dominant or submissive animals. However, a minor fraction of the significant expression differences between dominant and submissive animals was due to similar changes in directionality, differing only in magnitude. The vast majority of the gene expression differences observed were found in the submissive animals.

In order to provide additional statistical stringency to the identification of potential targets, we then analyzed the datasets generated by the SAM-RS analysis of the microarray data for dominance-associated co-regulation of multiple, functionally related genes. The 19 genes identified at a FDR of <5% were examined for their biological association to GO categories. Using GOMiner software, three independent category structures (biological process, molecular function, and cellular component) based on the 1178 rat genes represented on the microarray (of which, 783 currently carry GO annotations) were initially constructed and used as Query gene files. The genes identified in this study were then loaded as a “Query Changed Gene File” into the program to examine the distribution of these genes within the GO category structures. All of these queried genes carried current GO annotations. Among the most significant of the functionally clustered genes, several involved in biological processes associated with (1) protein metabolism (biosynthesis, folding, and poly-

Table 1. Dominance-associated transcripts in the posterior cortex (<5% FDR)

RGD[a] symbol	Fold change[b]	Gene ID	Function/ontology
Ppia	1.45	Peptidylprolyl isomerase A	Protein folding and transport
Tubal	−1.43	α-Tubulin	Microtubule-based process
Hspb1	−1.29	Heat shock 27kDa protein 1	Heat shock response/chaperone activity
Igf2r	−1.23	Insulin-like growth factor 2 receptor	Insulin-like growth factor receptor activity
Ill8	−1.19	IL-18	Immune response
RAMP4	−1.17	RAMP4/SERP1	Protein maturation/glycosylation
Tubb3	−1.16	β3-Tubulin	Microtubule-based process
Syngr1	−1.16	Synaptogyrin 1	Synaptic vesicle transport
Igf1	−1.15	Insulin-like growth factor 1 precursor	Insulin-like growth factor receptor signaling pathway
Nnat	−1.14	Neuronatin	Development
Gnb1	−1.13	Guanine nucleotide binding protein, β1	G-protein-coupled receptor protein signaling pathway
Rck5	−1.12	Potassium channel subunit (RCK5)	Potassium transport
Actb	−1.11	β-Actin	Cytoskeletal structure
Gabrd	−1.08	GABA-A receptor, delta	Neurotransmitter receptor activity
Cyp2b15	−1.08	Cylochrome P-450c	Electron transport pathway
Ctxn	−1.08	Neuron-specific cortexin	CNS development
Thra	−1.06	Thyroid hormone receptor-α	Ligand-dependent nuclear receptor activity
Pdap1	−1.06	PDGF-associated protein 1 (HASPP28)	PDGF receptor signaling pathway
Drd3	−1.06	Dopaminergic receptor D3	G-protein-coupled receptor protein signaling pathway

[a] Rat genome database.

[b] The fold change was calculated between mean values of dominant (n=6) and subordinate (n=6) rats. Positive values are indicative of an increase, and a negative a decrease, in gene expression in dominant relative to subordinate cortex.

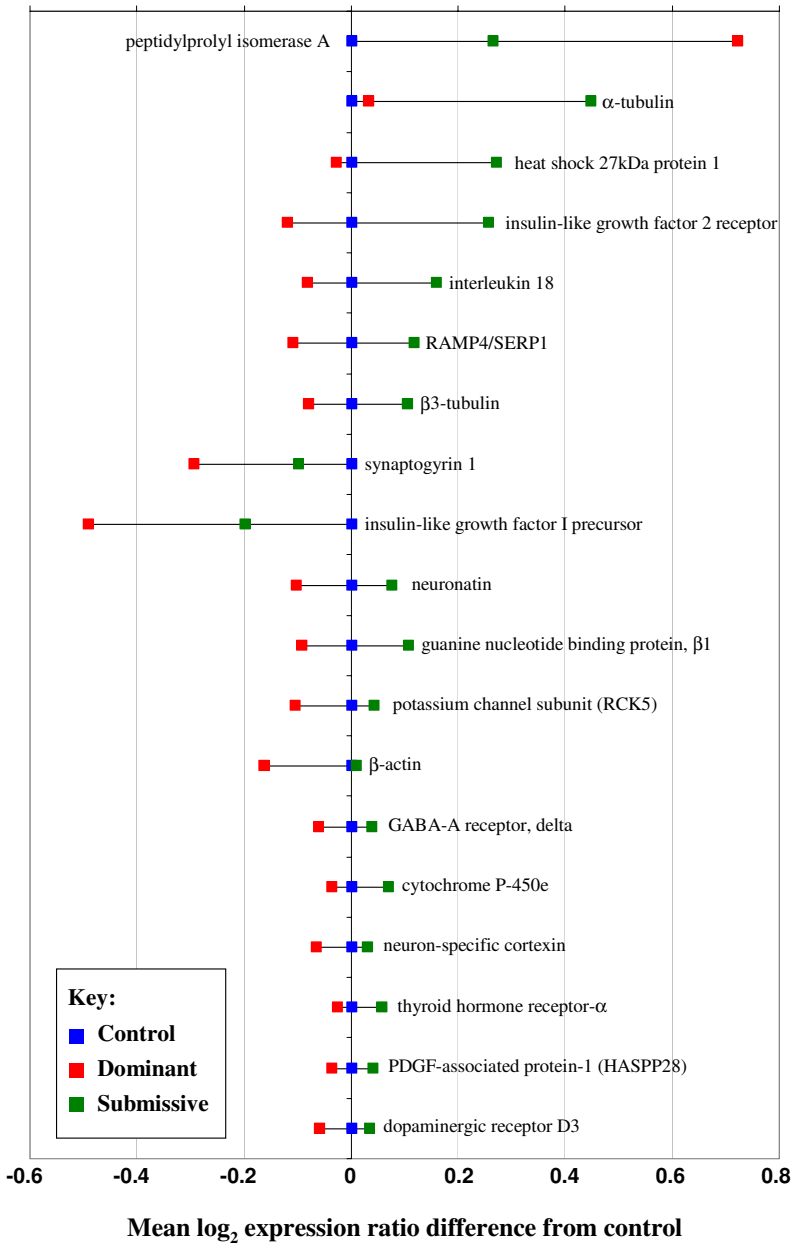


Fig. 4. Directionality of significant gene expression changes in the PCTX of dominant and submissive animals with respect to controls. The $\log_2$ expression ratio difference was calculated between mean values of dominant ($n=6$), submissive ($n=6$), and control ($n=6$) rats. Positive values are indicative of an increase, and a negative a decrease, in gene expression relative to control neocortex.

merization) and (2) small GTPase mediated signal transduction were enriched greater than two-fold ($P<0.05$, see Fig. 5). These genes were also significantly clustered, as expected, in GO categories associated with the molecular functions of GTPases and cytoskeletal structure elements.

The expression pattern of four of the genes involved in protein biosynthesis and cytoskeletal structure, identified by stringent SAM analysis of the microarray data *and* that clustered into a functionally related GO category, was further studied by quantitative real-time RT-PCR. Heat shock 27 kD protein 1, IL-18, RAMP4, and β3-tubulin were found to be robustly elevated in submissive animals compared with dominant ones and in close agreement with the microarray results (Fig. 6).

DISCUSSION

Depression is a complex phenotype, clearly heterogeneous in both its biology and in its etiology (Mayberg, 1997, 2004). The role of positive social relationships in both physical and mental health is becoming widely recognized, as is the power of social stress, in its various forms, for the genesis of depression (Gardner and Wilson, 2004; Heim and Nemeroff, 2001; Ornish, 1997). Mean-

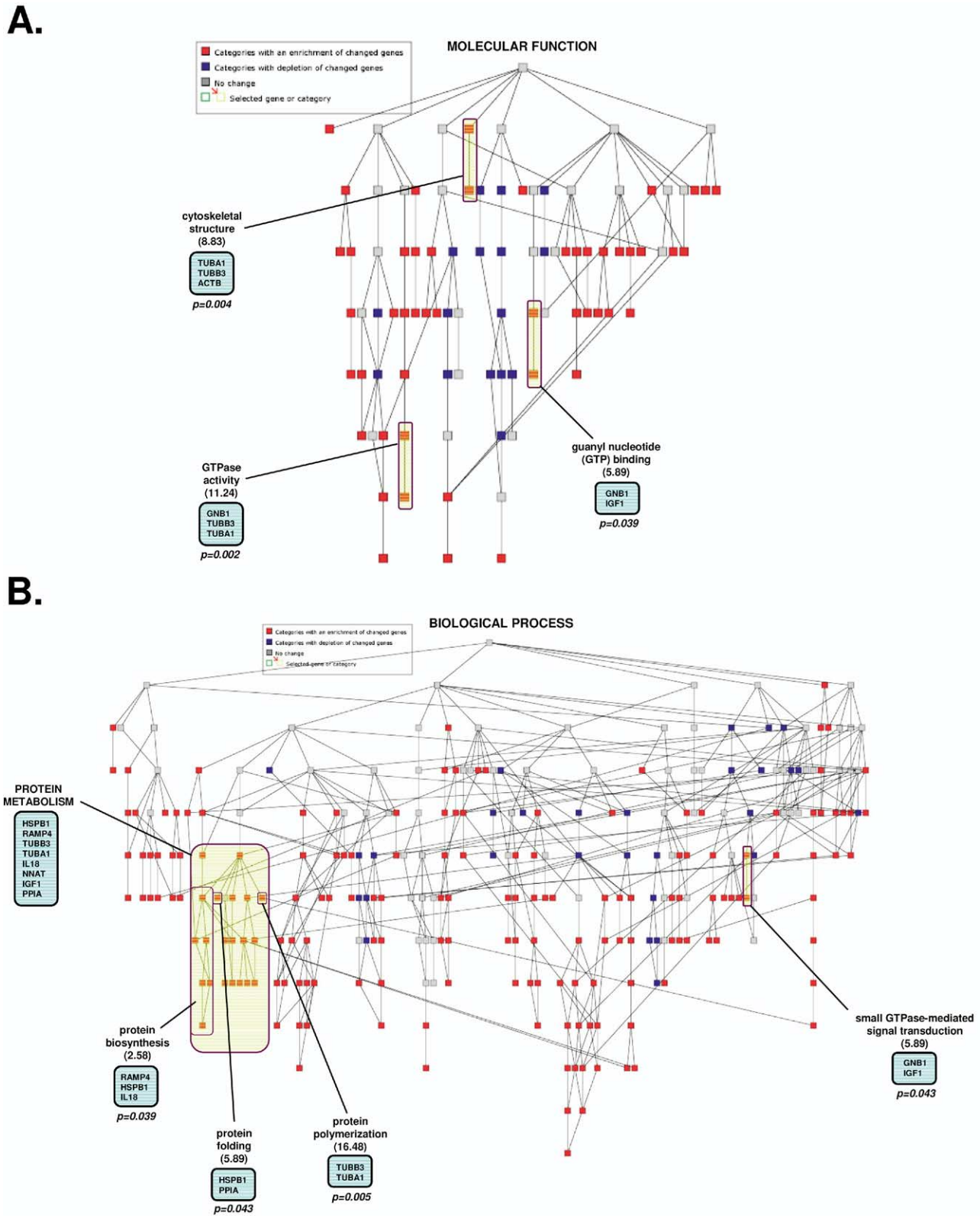
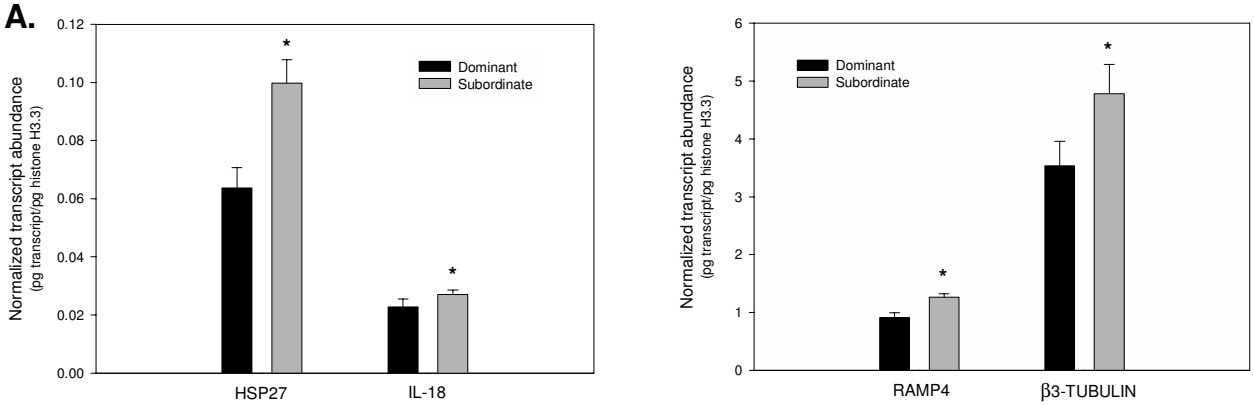


Fig. 5. Distribution of asymmetrically regulated genes at various levels of GO categories. The genes identified in this study were imported into the GoMiner program to generate directed acylic graphs (DAG) for the biological processes and molecular functions represented by the changes and were based on current annotations in the GO database. Each node of the DAG represents one GO category at various levels. The 19 genes differentially expressed in dominant vs. submissive cortex (panels A, B) were used as queries and loaded into GoMiner to classify these genes into each GO category. The distribution of these genes is represented by a different color at each node. The color of the node indicates enrichment (red), depletion (blue) or no change (gray) of the genes compared with the distribution over all identified genes at that level. Several critical GO categories are labeled. The fold enrichment for each category is shown in parentheses.



B.

Accession #	*Gene ID*	*Fold change (μarray)*	*Fold change (qRT-PCR)*[a]	*p-value*[b]
M86389	HSP27	-1.29	-1.57	0.020
AJ222813	interleukin 18	-1.19	-1.19	0.048
AB018546	RAMP4/SERP1	-1.17	-1.39	0.001
AF459021	β3-tubulin	-1.16	-1.35	0.017

[a] The fold change was calculated between mean values of dominant (n=6) and submissive (n=6) rats. Positive values are indicative of an increase, and a negative a decrease, in gene expression in dominant relative to submissive cortex.
[b] Statistical comparison was made by student t-test (two-tailed).

Fig. 6. qRT-PCR validation of selected targets in PCTX. (A) For each mRNA, transcript abundance, normalized to histone H3.3, was calculated by qRT-PCR, as described in the Experimental Procedures. Data presented for each group ($n=6$) represent mean±S.D. Significant differences between dominant and subordinate animals were observed for all four genes (* $P<0.05$). (B) Direct comparison of the microarray and qRT-PCR analyses for the selected genes.

while, our knowledge about what happens in the brain as a function of such social variables at the molecular level remains preliminary (Kollack-Walker et al., 1997). The present work was initiated with the expectation that a study of social loss in a rodent aggression model will shed some light on genetic variables that may be important in the emergence of depression (Gardner, 2001; Price, 1967; Rygual et al., 2005).

MRI evidence for brain changes associated with major depression have been reported in many interconnected regions, including the hippocampus, amygdala, caudate nucleus, putamen, as well as in limbic and anterior cingulate cortex (reviewed in Sheline, 2000 and Harrison, 2002). Among the hallmarks of affective diseases such as schizophrenia and depression are alterations in the emotional circuitry within the brain, and are thus likely to involve disturbed executive functions within the cerebral cortex. In support of this, the pivotal role of cortical abnormalities in the onset and development of depression have been described (reviewed in Drevets, 2001 and Davidson et al., 2002). Several studies demonstrating altered cortical expression of specific candidate genes in affective disorders (Lopez-Figueroa et al., 2004; Laifenfeld et al., 2005) have provided strong evidence for initially focusing on cortical changes. Additionally, recent microarray analyses of cortical tissues both using postmortem clinical specimens (Evans et al., 2004; Iwamoto et al., 2004) and in animal model systems (Nakatani et al., 2004; Kromer et al., 2005) are providing a more comprehensive identification of depression-related gene expression changes. Thus, we have also chosen to initially focus on alterations in cortical regions that have also been highlighted as most active in previous studies involving *c-fos* activation in this social loss model (Gordon et al., 2002; Kollack-Walker et al., 1997).

Statistical analyses of microarray data that stringently control family-wise type I error rate (FWER), (e.g. Bonferroni) will derive gene lists that demonstrate high confidence in all selected genes. Such analyses have been shown to be excessively stringent for microarray analysis of complex neurological diseases, where overly aggressive loss of power occurs due to large number of tests. Thus, many differentially expressed genes may not appear significant. Conversely, procedures based on FDR, (e.g. SAM-RS) are more flexible and allow the individual researcher to decide how many candidate genes to select, based on less stringent and more practical considerations. It provides less conservative control than the traditional Bonferroni-type corrections and is able to handle the interdependence (e.g. co-regulation of functionally-related genes) among gene expression profiles. Recent evaluation of gene selection techniques demonstrated that SAM and patterns of gene expression (PaGE) were more accurate than simple *t*-test analysis (Singhal et al., 2003). Such FDR-based methodology has been demonstrated to provide reliable results in a wide variety of systems (see Xu et al., 2002).

The utility of statistical algorithms, such as GOMiner, Gene Set Enrichment Analysis (GSEA), or Expression Analysis Systematic Explorer (EASE) that provide statistical rigor to analyses of co-regulation of multiple genes (gene sets) that are functionally related or related by involvement in a given biological pathway, is yielding pro-

vocative data and is giving rise to testable hypotheses in complex disease states such as diabetes (Mootha et al., 2003) and Alzheimer's disease (Blalock et al., 2004). These ontological analyses focus on the behavior of gene groups and pathways rather than single genes and are capable of demonstrating significant correlations between the expression of specific gene sets and complex phenotypic distinctions even if individual genes do not. With such analyses, however, the potential impact of applying arbitrary selection thresholds on the significant gene list generated by SAM must be considered. Balancing the level of stringency (FDR) and the accompanying level of acceptable false positives with the size of the final dataset is central to the interpretation of subsequent post hoc ontological analyses that are dependent upon this dataset. At low stringency (i.e. high FDR), the increased number of false positives may identify significance where there is none. On the other hand, higher stringencies generate smaller, higher confidence gene sets, and, although this strategy may miss some biologically relevant ontological correlations, it will clearly identify major pathways. With these critical concepts in mind, coupling the GOMiner-based ontological analyses with stringent statistical microarray data analyses provided a very powerful platform to identify fundamental cellular pathways significantly altered in the resident–intruder paradigm.

Ontological analysis of the microarray data derived in these studies revealed significant enrichment in gene categories primarily related to protein synthesis and cytoskeleton assembly (Fig. 5A, B). Within the protein metabolism GO term, several genes related to protein biosynthesis, polymerization and folding were represented. Most notably, the differential expression of four of the genes among the members of this category was verified by qRT-PCR (IL-18, HSPB1, RAMP4, and TUBB3). These changes may be reflective of alterations in cellular number or metabolism, or alternatively, a change in cellular architecture or morphology. The latter is particularly intriguing as changes in morphology clearly underlie changes in neurochemistry in cortical tissue of depressed patients. For example, cortical neuroimaging has demonstrated structural and functional abnormalities in adults (Drevets et al., 1998) and adolescents (Steingard et al., 2002) with depression. Hypotheses considering the association of depression with structural changes, especially in the hippocampus, have been proposed (reviewed in Sapolsky, 2001 and McEwen, 2000). Neuronal cytoskeletal alterations were observed in an experimental animal model of depression (Reniés et al., 2004) as well as in rats exposed to restraint stress (Bianchi et al., 2003) and in mouse lines selected for differences in aggressive behavior (Feldker et al., 2003). In addition, alterations in genes related to cytoskeletal organization and biogenesis were significantly over-represented in the temporal cortex of patients with major depressive disorder compared with matched controls (Aston et al., 2004). At a more detailed structural level, exposure to chronic behavioral stress (either restraint stress or maternal separation) significantly alters dendritic spine morphology and reorganizes cortical synaptic networks (Radley et al., 2004 and Poeggel et al., 2003). Although it is unclear as to whether these changes represent predisposing factors to the illness or are a consequence of the disease process itself, based on the data from our study, we can hypothesize that aberrant regulation of cortical structure is likely an early event in the onset of depression.

The full elucidation of the molecular mechanisms that underlie depression must take into consideration the fact that mRNA levels are not always directly correlated with protein levels. Moreover, protein function itself can be readily affected by a host of post-translational modifications. For example, altered RNA editing of the serotonin 2C receptor (HTR2C) has been reported in postmortem brains of depressed patients that committed suicide (Gurevich et al., 2002) and recent results by Iwamoto et al (2005), using an animal model of depression, also showed significant RNA editing of the HTR2C receptor which could be modified by anti-depressants. On the other hand, Evans et al. (2004), using microarray technology, have observed alterations in the expression of the fibroblast growth factor (FGF) family of mRNAs in the frontal cortices of humans with major depressive disorder. These transcript expression levels appeared to be directly modulated by serotonin reuptake inhibitors suggesting that the regulation of FGF transcript expression levels itself may be part of the mechanism of action of SSRIs. We have begun to address this complex problem of evaluating protein changes associated with depression directly and linking these data with our gene expression data. We routinely isolate brain tissue from each animal, in each experiment, and have begun to subject them to both mRNA analysis and protein/peptide analysis (Panksepp et al., 2004; Burgdorf et al., 2005).

Nevertheless, as the primary focus of most major genomic efforts shifts toward the identification of targetable molecular *pathways*, the potential impact of the identification of a single gene change (and thus the potential of creating therapeutics by modulating the expression of a single gene) must not be neglected. For example, the altered expression of single genes in the human monoamine oxidase pathway (either monoamine oxidase A or specific alleles of the serotonin transporter) dramatically influences complex antisocial violent behaviors manifested only in adults maltreated as children (Caspi et al., 2002, 2003). In addition, increasing the expression of the vasopressin 1a receptor in a specific forebrain region, either with pharmacological agents or by direct molecular viral vector gene transfer, profoundly altered social behaviors associated with stable pair bond formation in male voles (Lim et al., 2004). As such, evidence implicating the involvement of individual genes identified in this study in the genesis of depression becomes equally important. As an example, expression of IL-18, significantly higher in the submissive than in the dominant animals, has been found to be over-expressed in other physiological stress paradigms (Takeuchi et al., 1999) and might help submissive animals to better cope with the social stress. IL-18 can directly modify brain information processing (Curran and O'Connor, 2001), and is elevated in patients with major depression and panic disorders (Kokai et al., 2002; Merendino et

al., 2002). Similarly, heat shock proteins have also been shown to be involved in the pathogenesis of many neurodegenerative and psychiatric disorders (Iwamoto et al., 2004; Pongrac et al., 2004) and their expression is modulated by antidepressants (Khawaja et al., 2004).

CONCLUSION

In summary, the present studies were undertaken as part of a larger program aimed at utilizing molecular biological technologies with appropriate animal models to give some insight into the molecular mechanisms underlying the emergence of psychiatric disorders. Of course, a host of additional issues still needs to be addressed, including the regional brain issues, the effects of a variety of other behaviors on the genes that were differentially expressed in these animals, the role of social-support factors, and kin-relations in modulating the effects in resident–intruder models, and a host of other variables. Our preliminary results indicate that many of these issues may be worth pursuing.

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