BEHAVIORAL AND MOLECULAR CORRELATES OF MAJOR DEPRESSION IN MICE LACKING THE SEROTONIN TRANSPORTER

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Major depressive disorder (MDD) is responsive to serotonin transporter reuptake inhibitors (SSRIs), and a functional polymorphism in the serotonin transporter (SERT) putative promoter region impacts vulnerability to develop MDD. Paradoxically, mice lacking SERT (knock-out; SERT-KO) display an increased anxiety-like phenotype, which has been demonstrated to have developmental origins; however, the extent to which systems adaptations that have occurred in SERT-KO mice recapitulates a broader anxious depressive-like phenotype at the behavioral and molecular levels is not known. We investigated SERT-KO in a panel of behavioral tests, and analyzed WT and KO gene expression in amygdala (AMY) and cingulate cortex (CC) by microarray. We then compared the SERT-KO gene expression results to human MDD and Control microarray in AMY and anterior cingulate cortex (ACC), areas shown to be functionally, structurally and molecularly affected in MDD. Gene expression changes were confirmed by real-time quantitative polymerase chain reaction (qPCR). RESULTS: SERT-KO behaviors across several tests denote a robust anxious depressive-like syndrome, which is reminiscent of the human MDD syndrome. Some gene expression changes were conserved between mouse and human in AMY and ACC/CC as measured by microarray (29 genes; 19 genes), and a subset were selected for qPCR validation. Differential gene expression confirmed by qPCR in both mouse and human (2 genes in AMY) included an upregulation in AMY of adenylate cyclase VII (ADCY7), a gene previously implicated in MDD. Increased expression of two genes that display significant coregulation in mouse and human suggests the recruitment of a conserved functional unit related to cyclic adenosine monophosphate (cAMP) signaling, a signal transduction pathway

implicated in MDD. CONCLUSIONS: The SERT-KO mouse recapitulates behavioral and selected molecular features of a rodent syndrome homologous to human MDD. Therefore, it provides a useful model for investigating molecular mechanisms in AMY which are relevant to the pathology of MDD. These results support altered cAMP signaling pathway as a cross-species conserved feature of the pathophysiology of MDD.

PREFACE

Yingjie Wang assisted in working with DNA and RNA samples for microarray and qPCR, and Nicole Edgar and Bhavani Ramesh assisted in behavioral assays. Etienne Sibille, Xing bin Wang and Christopher Gaiteri provided support for computational analysis of microarray data. Human tissue was provided by the University of Pittsburgh Brain Donation Program (D.A. Lewis, PI). This work was supported by the National Institute of Mental Health (MH085111 & MH077159; E. Sibille, PI)

TABLE OF CONTENTS

List of tables

List of figures

1.0 INTRODUCTION

The serotonin transporter (SERT), the protein responsible for the reuptake of serotonin from the synaptic cleft back into presynaptic serotonergic neurons, is the therapeutic target protein of serotonin transporter reuptake inhibitors (SSRIs) which are used to treat major depressive disorder (MDD). Furthermore, the vulnerability to develop MDD in response to life events is modulated by a putatively lower-expressing short allele (s-allele) in the human SERT-linked-promoter-region (5HTTLPR) polymorphism (Caspi, et al. 2003), a tandem-repeat polymorphism in the promoter region. This is known as a gene-by-environment interaction (G x E). Many attempts were made to replicate this finding, with mixed results, and a meta-analysis later claimed that there was no 5HTTLPR G x E effect (Risch, et al. 2009). However, the s-allele is robustly associated with increased amygdala reactivity (Hariri, et al. 2002; Heinz, et al. 2005) and anxiety-related personality traits (Lesch, et al. 1996). The s-allele was shown to decrease SERT expression *in vitro* (Lesch, et al. 1996), but does not translate to a change in SERT protein quantity in adult human postmortem brain tissue (Mann, et al. 2000) or in adult human SERT binding *in vivo* in the brain (Christian, et al. 2009). Therefore, it has been suggested the behavioral, structural, and functional changes due to the 5HTTLPR polymorphism are caused by altered SERT during development, which results in altered neural network connectivity in the adult (Sibille and Lewis, 2006). S-allele carriers appear to have decreased volume and functional coupling in ACC and AMY, which may mediate the behavioral effects of the polymorphism

(Pezawas, et al. 2005). A functional decoupling of ACC and AMY is also seen in MDD by fMRI (Matthews, et al. 2008), supporting the possibility of a genetic predisposition to develop MDD in response to life stress (pro-depressive state). We investigated both of these brain areas at the gene expression level in human and mouse in order to further assess changes in these brain regions due to genetics, disease, and gene manipulation.

Unexpectedly, an increased emotion-related phenotype is observed in the SERT knock-out mouse (SERT-KO), which has no SERT activity. This effect is thought to be mediated by the developmental role of serotonin as a trophic factor, supported by results showing that early-life blockade of SERT activity during a critical developmental window results in a similar phenotype in mice (Ansorge, et al. 2004). Thus, the behavioral phenotype due to SERT-KO appears to originate from altered SERT activity during development rather than during adulthood, similar to human functional changes due to the 5HTTLPR polymorphism. Behavioral tests on the emotional phenotype of SERT-KO yielded mixed results, as some researchers showed a behavioral difference in tests of anxiety-like behaviors such as the open field and elevated plus maze (Holmes, et al. 2003), while others have reported a behavioral difference in tests of depressive-like behaviors such as novelty-suppressed feeding, forced swim test, and the "learned helplessness" shock escape paradigm (Lira, et al. 2003). Curiously, Lira, et al. reported null results in the open field and elevated plus maze tests, but this result may be confounded by the effect of genetic background. The results from Lesch, et al. (1996), Lira, et al. (2003) and Ansorge, et al. (2004) were measured using cohorts of SERT-KO mice on the 129Sv inbred strain, while Holmes, et al. (2003) used the C57BL/6J inbred strain. 129Sv mice appear to be more anxious than C57BL/6J mice in OF and EPM and may mask increases in anxiety due to the "floor effect" in both tests (when mice mostly or completely avoid the aversive areas). As a

result, several hypotheses emerged: (1) the SERT-KO phenotype may be similar to MDD (Lira, et al. 2003), or (2) to the anxiety-related personality traits associated with the s-allele in SERT (Lesch, et al. 1996; Holmes, et al. 2003). The behavioral data are complicated by differences in protocols, genetic background, and "genetic drift" (genetic divergence of inbred strains which have not been backcrossed for several generations) across research groups, so *the SERT emotional phenotype would be better evaluated by a comprehensive panel of behavioral tests performed under uniform conditions on directly related cohorts of mice.*

Because of the heterogeneous nature of MDD and the poorly understood disease mechanism, animal models are required to study the induction and reversal of MDD-related syndromes. Animal models of MDD should meet these criteria: (1) Face validity (relevant factors, such as genetics or environment, are included in the manipulation, and induce a long-term syndrome); (2) Pharmacological validation (predictive validity) by chronic antidepressant reversal; (3) Construct validity, which requires that a model syndrome exhibits behavioral and biological correlates of MDD symptoms (Tripp and Sibille, 2010). For example, the olfactory bulbectomy model of MDD lacks face validity because the removal of the olfactory bulb has nothing to do with developing human MDD. Furthermore, the FST has itself been called a "model" of MDD, even though it is used to screen antidepressant drugs by acute administration in normal animals with no long-term syndrome. Unpredictable Chronic Mild Stress (UCMS) is a model which does meet these three criteria, because it induces a long-term syndrome by introducing stressful life events. The syndrome is reversible by chronic antidepressant treatment, and displays similarities with the human MDD syndrome in several behavioral dimensions (Belzung, et al. 2008; Surget, et al. 2008), altered physiology (body weight and neuroendocrine changes) and altered gene expression measures (Sibille, et al. 2009). As a model of MDD,

SERT-KO has modest face validity due to the artificial absence of SERT, which is not altered in MDD or in s-allele carriers (Mann, et al. 2000). Furthermore, it cannot be evaluated for predictive validity using SSRIs because of the absence of the target SERT protein. SERT-KO is not responsive to SSRIs in tests predictive of AD activity, but responds to tricyclic ADs (Holmes, et al. 2002). However, it has not been fully evaluated in terms of construct validity, which is targeted by the approach described in this work.

Emotionality is defined as an observable measure related to emotion (which cannot be directly measured). Anxiety-like emotionality in rodents consists of behaviors which are attenuated by anxiolytic drugs, while depressive-like emotionality consists of behaviors which are attenuated by chronic antidepressant exposure. Commonly used behavioral tests which are predictive of antidepressant efficacy (forced swim test and tail suspension test) are defined as antidepressant-like behavior and have no orthologous human behavior. Because MDD is a complex disease which is defined as a cluster of symptoms (emotional, cognitive, and physiological), animal models of MDD are best characterized by a panel of behavioral tests which evaluate multiple symptom dimensions relevant to MDD, such as increased anxiety and depressed mood. For example, anxiety-like emotionality can be measured by the elevated plus maze and open field tests, but represents only one symptom out of the full MDD syndrome. Our previous results have shown that SERT-KO mice display an emotionality phenotype which crosses over multiple symptom dimensions (anxiety-like and depressive-like), which we define as a syndrome of increased emotionality. SERT-KO mice also reach a higher level of anxious emotionality in response to the pharmacologically validated UCMS mouse model of depression, especially in females (Joeyen-Waldorf, et al. 2009). Thus, SERT-KO demonstrates similarity to human MDD in terms of sexual dimorphism, as well as environmental life stress, which could

mimic the G x E reported by Caspi, et al (2003). Hence, *we addressed here current ambiguities as to the "depressive" quality of the SERT-KO behavioral phenotype and further assess the validity of the SERT-KO model for investigating putative molecular mechanisms that are relevant to MDD*. We hypothesize that *a panel of pharmacologically-validated tests measuring anxiety-like and depressive-like behavior, anhedonia-like, and antidepressant-like response will clarify the exact nature of the SERT-KO emotional phenotype (1)*.

To begin investigating putative underlying mechanisms, we tested three hypotheses regarding similarities between SERT-KO and MDD (Holmes, et al. 2003) vs. SERT-KO and the 5HTTLPR human s-allele (Lesch, et al. 1996) at the molecular level via *a comparison of gene transcript changes between SERT-KO and WT to those measured in human MDD patients relative to control, s-allele carriers relative to non-carriers, and in the UCMS mouse model of MDD (2).* UCMS is a pharmacologically validated behavioral model of a syndrome induced by chronic stress, which appears to be similar to MDD behaviorally and at the molecular level in the amygdala (Sibille, et al. 2009). SERT-KO shows an increased vulnerability to develop this syndrome in response to UCMS (Joeyen-Waldorf, et al. 2009). Thus, we compare the microarray data of SERT-KO to existing human and mouse microarray datasets to evaluate gene expression similarities across-species to the disease and putative pro-depressive states, as well as within-species to the disease state.

2.0 METHODS

2.1 ANIMALS

SERT KO and wild-type (WT) C57BL/J6 littermates (Bengel, et al. 1998) were obtained from Taconic (Hudson, New York) and bred at our mouse colony via heterozygous breeding to avoid genetic drift and variation in parental care. Experimental cohorts were comprised of male WT and KO littermates. *Cohort 1* included 36 WT and 37 SERT-KO male mice at 3-6 months of age, which were tested in open field, novelty-suppressed feeding, and sucrose preference in order to evaluate SERT-KO baseline behavior relative to WT. *Cohort 2* included 19 WT and 19 SERT-KO male mice at 3-6 months of age which were tested in elevated plus maze, forced swim test, and tail suspension test (Joeyen-Waldorf, et al. 2009). *Cohort 3* included 5 WT and 5 KO male mice at 3-5 months of age, from which AMY and CC tissue samples were collected for microarray analysis of gene expression. *Cohort 4* included 10 WT and 10 KO male mice at 3-5 months of age, from which AMY and CC tissue samples were collected for qPCR validation of differential gene expression and gene coregulation measured by microarray. All mice were maintained on a 12-hour light cycle with access to food *ad libitum*, and all procedures received Institutional Animal Care and Use Committee (IACUC) approval.

2.2 BEHAVIORAL TESTS INVESTIGATING ANXIETY-LIKE, DEPRESSIVE-LIKE, AND ANTIDEPRESSANT-LIKE EMOTIONALITY

SERT-KO and WT mice were compared in a panel of behavioral tests evaluating multiple symptom dimensions. The Open Field (OF) and Elevated Plus Maze (EPM) are classic tests of anxiety-like emotionality which are based on the behavioral inhibition of an "aversive" environment on the exploratory drive of the mouse. The Novelty Suppressed Feeding (NSF) test evaluates both anxiety-like and depressive-like emotionality by measuring the behavioral inhibition of a novel environment on the feeding behavior of the mouse. The Sucrose Preference (SP) test evaluates anhedonia-like (depressive-like) behavior by measuring the drive to consume a palatable sucrose solution. Finally, the Forced Swim Test (FST) and Tail Suspension Test (TST) quantify immobility in an inescapable aversive environment, which is robustly decreased by acute antidepressant treatment and represents antidepressant-like emotionality. Together, these behavioral tests provide a profile of complex disease-related behavior.

2.2.1 Open Field (OF)

Time in center and activity in center (normalized to total activity) was measured relative to a grid on the floor of the 30" x 30" open field. Behavior in the OF was measured as previously described (Sibille et al, 2000). The OF chamber was divided in 16 even-size squares. The total number of gridline crosses was used as an index of locomotor activity. Amount of time and gridline crosses within the squares of the aversive center were recorded for 10 minutes, and the ratio of crosses into the center compared to the total number of crosses was calculated to evaluate anxious emotionality. The total number of crosses is a control measure of locomotor activity.

2.2.2 Elevated Plus Maze (EPM)

Time in open arms and number of entries into open arms (normalized to total activity) was measured in EPM as previously described (Sibille et al, 2000), using a cross maze with two open and two closed 30x5cm arms. The total number of entries was used as a second index of locomotor activity. Entries and time spent in the open and closed arms was recorded for 10 minutes to evaluate anxious emotionality. The total number of entries is a control measure of locomotor activity.

2.2.3 Novelty Suppressed Feeding (NSF)

Mice were food deprived for 16 hours and then placed in a novel, empty and brightly-lit chamber with one food pellet placed in the center. Latency to begin feeding was measured. In NSF, the latency to feed in a threatening novel environment correlates with fearfulness and decreases after acute treatment with anxiolytic drugs (Bodnoff et al., 1988) or chronic antidepressant exposure (Santarelli et al., 2003), suggesting that mechanisms underlying changes in the latency to start feeding involve anxiety-like and antidepressant-like processes, which we also refer to here as anxious or depressive emotionality. The test was applied as previously described (Santarelli et al, 2003) with an increased session duration. During testing, a food pellet was placed in the brightly-lit center of the 30x60cm chamber. The drive to overcome the aversive center of the apparatus was increased by 16-hour food deprivation and the latency to start eating was recorded in a 30 minute session. A control measure of food consumption was monitored in the home cage after the test over the next 24 hours.

2.2.4 Sucrose Preference Test (SP)

After 24 hours of training to drink from customized tubes filled with sweetened condensed milk, mice were provided with identical tubes of 2% sucrose and water, and the consumption was measured by weight following a 16-hour period. The ratio of sucrose solution to total volume consumed indicates a preference for sucrose solution when greater than 0.50, and mice with a ratio of sucrose solution consumption lower than control are thought to display anhedonia-like emotionality.

2.2.5 Forced Swim Test (FST)

Duration of immobility of the mouse was measured in a 3-liter beaker of water at room temperature in a 6-minute trial (2-minute intervals were measured). Immobility was defined as floating with all four paws relatively inactive. Swimming and climbing behaviors were observed and considered to be periods of mobility. Because no changes over time were measured, the results are reported as a total duration of immobility out of a 6 minute trial.

2.2.6 Tail Suspension Test (TST)

Duration of immobility was measured while each mouse was suspended by the tail in a 6-minute trial (2-minute intervals were measured). Immobility was defined as hanging without struggling or attempting to climb. Because no changes over time were measured, the results are reported as a total duration of immobility out of a 6 minute trial.

2.2.7 Statistical Analysis

Behavioral test data were analyzed by one-way analysis of variance (ANOVA) to define the effect of genotype (SERT-KO vs. WT).

2.3 GENE EXPRESSION IN CINGULATE CORTEX AND AMYGDALA MEASURED BY MICROARRAY

AMY and ACG samples were collected from WT and KO mice by micropunch (n=5 per are and per genotype group; total, n=20 arrays). A 1 mm diameter punch was used to dissect AMY, corresponding to rostral AMY (atlas fig. 52; Paxinos, et al. 1997). This included the basolateral and lateral nuclei of AMY, areas which process sensory information and project to the central nucleus of AMY. A 2 mm diameter punch was used for CC at the region with closest orthology to human ACC, which includes frontal cortex and cingulate cortex (atlas fig. 35; Paxinos, et al. 1997) Total RNA transcripts were extracted from tissue homogenized in Trizol reagent (Invitrogen, Carlsbad, California). Reversed-transcribed cDNA and labeled copy RNA (cRNA) were hybridized to MOE430-Plus 2.0 microarray (Affymetrix, Santa Clara, California; N=5 arrays per area) as previously described (Surget, et al. 2008). Light-intensity laser scans of each chip provided quantitative measurements of transcript levels. Data were extracted and normalized with the Robust Multi Array (RMA) algorithm (Irizarry, et al. 2003), prior to comparisons.

The changes in gene expression transcript levels between SERT-KO and WT in AMY and ACG ($p<.05$) were compared with the gene expression correlates of human MDD, human

5HTTLPR s/l polymorphism, and mouse UCMS model in existing mouse and human gene expression datasets (in orthologous brain regions) from Sibille, et al. (2009). To reduce the heterogeneity of the cohort, we analyzed 16 MDD and 19 Control white male subjects. All MDD subjects had familial depression, 8 had recurrent episodes, and 9 died by suicide, while Control subjects never experienced MDD or other psychiatric disorders and did not die by suicide. For the SERT-KO/MDD comparison, MDD subjects were matched by age with pair Control subjects. For the SERT-KO/s-allele comparison, the same subjects were regrouped by 5HTTLPR genotype and matched groupwise for age and disease condition. Human data were analyzed in paired matched groups for MDD (n=16 pairs), and in aged-matched SERT genotype-determined subgroups for s/l effect (n=16 MDD and 19 controls). Statistical models were described in Sibille, et al. (2009) and included clinical and demographic parameters of covariate factors. Preliminary results indicate that heterozygous and homozygous s-allele carriers were similar in gene expression, so comparisons were made between s/x vs. l/l subjects. For the SERT-KO/UCMS comparison, SERT-KO array results were compared with microarray data from BALB/c mice who received UCMS for 7 weeks (Surget, et al. 2009). Because we compared differential expression across multiple arrays, we maintained criteria at moderate stringency to determine differential expression within array groups, and did not require that individual genes survive controls for multiple testing via a comparison to randomized permutated data. Instead, we relied on cross-species significance of effects and on qPCR validation of individual genes in both mouse and human to confirm microarray results. As an internal control, we measured SERT mRNA levels by qPCR.

2.4 QPCR VALIDATION OF DIFFERENTIAL GENE EXPRESSION AND COREGULATION MEASURED BY MICROARRAY

Concordant gene expression changes measured by microarray were verified by qPCR in mouse (independent cohort) and human samples. mRNA transcripts from independent samples were extracted from Trizol and reverse-transcribed to produce cDNA samples. Primers were designed targeting specific genes (Table 1), and amplification of cDNA was quantified in quadruplicates by SYBR green fluorescence signal (Invitrogen) using the Opticon Monitor DNA Engine (Bio-Rad, Berkeley, California). Primers for three control genes (actin, GAPDH, and cyclophilin in both mouse and human) were used as a reference for comparison across subjects. As previously described (Sibille, et al. 2009), qPCR provides a sigmoidal curve for each gene which quantifies the amount of cDNA in a lognormal distributrion. For each gene, the geometric mean of the three control genes was subtracted to provide $\Delta C(t)$ values (the number of amplification cycles relative to baseline required for this gene's signal to reach threshold), which can be converted to signal intensity (SI) in arbitrary units on a linear scale ($SI=100*2^{-\Delta C(t)}$). Because this is a nonlinear transformation which affects the detection of linear Pearson correlations, the correlations of gene expression were calculated using SI and not $\Delta C(t)$. Data are reported as fold-changes in SI across groups.

Coexpression values for all genes in a data matrix of p genes by n samples can be estimated by computing the "expression profile" of fluctuation of a given gene across samples, with the fluctuations of all other genes, resulting in a p x p matrix of correlations between all possible combinations of genes. Out of this matrix, we only examine the coexpression between ADCY7, KCTD9, and KCNIP4 (resulting in a 3x3 correlation matrix). To find the likelihood of generating a given coexpression value between two genes, we generate a null distribution by

individually row-permutating the *p* gene by *n* samples data matrix and generating a series of such pseudo correlation matrices. This process removes any synchronous fluctuations between genes, beyond those generated by random effects, providing a null distribution from which to estimate the significance of the actual correlation values. This resampling technique is superior to exact p-value tests for correlation because it makes no assumptions concerning the distribution structure of the original data and is robust against outlying data points, thus yielding reliable and conservative p-value estimation, given the limited number of array samples per experimental group (n=5). Furthermore, we applied Bonferroni correction to all p-values to compensate for the selection of 3 genes.

3.0 RESULTS

3.1 SERT-KO EMOTIONALITY IS CONSISTENT WITH A SYNDROME HOMOLOGOUS TO MDD SYMPTOMS

SERT KO and WT control mice were studied in a comprehensive panel of behavioral tests for anxiety-like (OF, EPM) and depressive-like emotionality (NSF, SP), and for antidepressant-like behavior (FST, TST) in order to characterize the SERT-KO behavioral phenotype as it relates to major depression (MDD).

3.1.1 Confirmed increased anxiety-like emotionality of SERT-KO mouse in EPM and OF and anxiety-like/depressive-like emotionality in NSF

The previously reported anxiety-like emotionality of SERT-KO was confirmed in OF, EPM, and NSF. The SERT-KO group spent less time in the "aversive" center of the OF (Fig. 1c) and also displayed lower activity in the center region as measured by a ratio of center gridline crosses out of the total number of crosses (Fig. 1d), suggesting higher anxiety-like emotionality than wildtype. This is also supported by the SERT-KO group's lower average time in the "aversive" open arms of the EPM (Fig. 1e). While it has been suggested that the OF and EPM behavior could be a result of the known hypolocomotion phenotype of SERT-KO (Kalueff, et al. 2006),

anxiety-like emotionality is confirmed in the locomotion-independent NSF test (Fig. 1d) and suggests increased depressive-like emotionality.

3.1.2 SERT-KO mouse shows depressive-like (anhedonia-like) emotionality in SP

The SERT-KO group displayed increased latency to feed in the NSF (Fig. 1a), suggesting elevated anxiety-like and/or depressive-like emotionality relative to wildtype because latency to feed in NSF responds to both acute anxiolytic drug and chronic antidepressant drug treatments. In contrast to the results of Kalueff, et al. 2006, the SERT-KO group showed a robust decreased preference for sucrose in SP in both percentage sucrose solution consumption (-27%; Fig. 1b) and absolute sucrose solution consumption (-72%; Fig. 1c), suggesting that they do display a depressive-like or anhedonia-like state relative to WT, independent of their known hypolocomotion phenotype (confirmed, data not shown).

3.1.3 No change in antidepressant-like behavior in FST and TST

Mice spent the same amount of time immobile across groups (WT and SERT-KO) in both FST and TST. We measured no difference across genotypes in the FST (Fig. 1g) and TST (Fig. 1h). No changes were measured over the three 2-minute intervals and no differences across genotype were measured at any point (not shown), so they are reported as duration of immobility out of a total of six minutes.

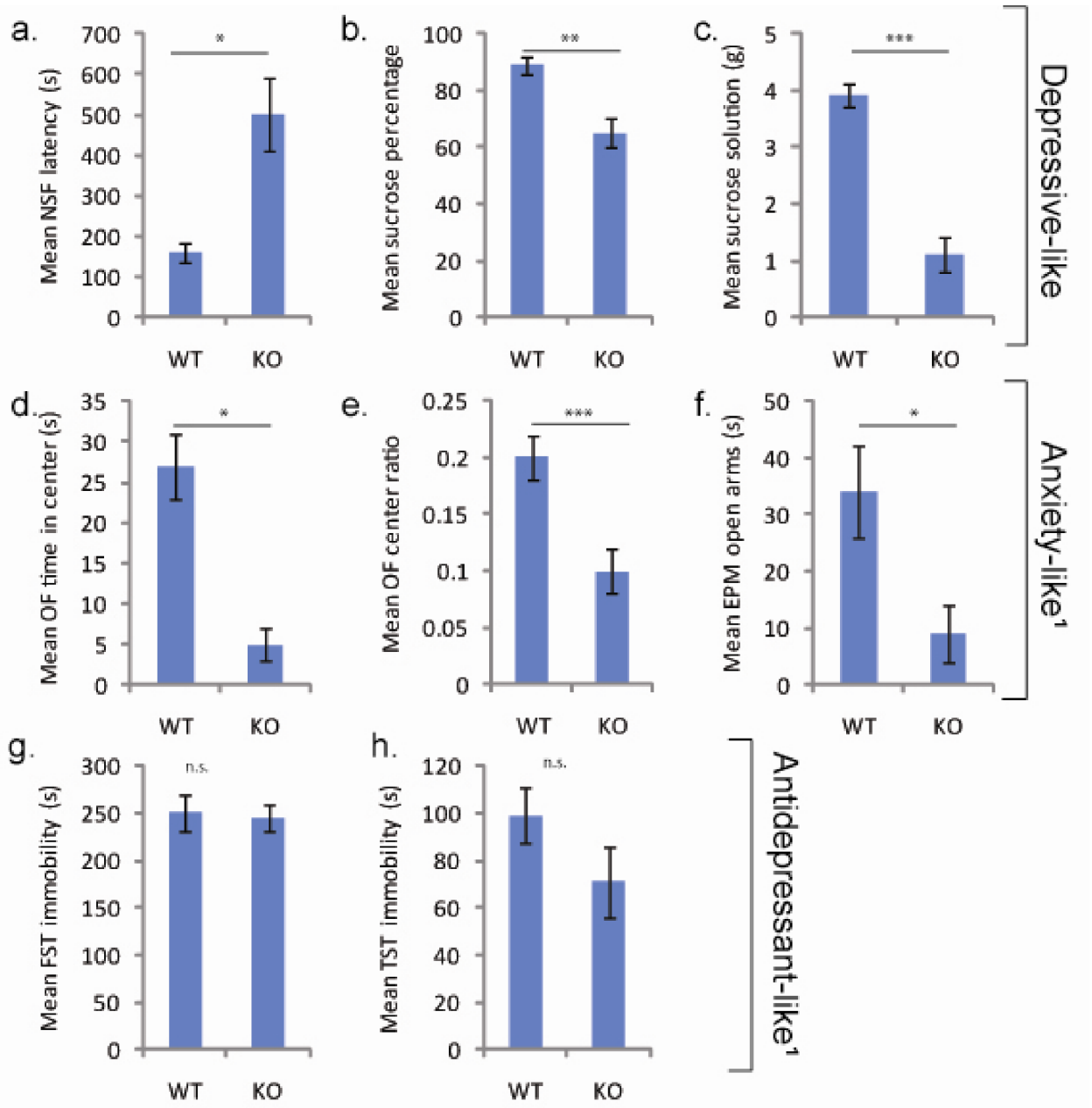

Fig 1. SERT-KO behavioral correlates of MDD (1) Increased depressive-like behavior in SERT-KO: a. NSF (latency); WT=19; KO=19; p=.02; **b.** SP (percentage); WT=36; KO=37; p=.003; **c.** SP (sucrose solution); WT=36; KO=37; p=4x10^-5 **(2) Confirmed increased anxiety-like behavior in SERT-KO: d.** OF (time in center); WT=19; KO=18; p=.002; **e.** OF (center ratio); WT=19; KO=17; p=.0007; **f.** EPM (time in open arms); WT=19; KO=19; p=.032 **(3) No change in tests predictive of antidepressant activity: g.** FST (immobility); WT=19; KO=19; n.s. **h.** TST (immobility); WT=18; KO=18; n.s.; * p<.05; ** p<.005; *** p<.0005; error bars represent

standard error on the mean. *Not shown:* SERT-KO hypolocomotion was confirmed in OF and EPM. OF and NSF results were replicated in independent cohorts. No differences in weight loss or home-cage feeding in NSF. No difference in H2O consumption in SP. [1]adapted from Joeyen-Waldorf, et al. 2009

3.2 GENE EXPRESSION CHANGES ARE PHYLOGENETICALLY CONSERVED ACROSS SPECIES IN AMYGDALA

To test various hypotheses regarding the similarity between the SERT-KO emotionality phenotype and MDD or s-allele-related phenotypes at the molecular level, the changes in gene expression transcript levels between SERT-KO and WT in AMY and ACC ($p<.05$, at least 20% change) were quantified by Affymetrix microarray (N=5 per group and area) and compared with the gene expression correlates of human MDD, human 5HTTLPR s/l polymorphism, and mouse UCMS model in existing mouse and human gene expression datasets (in orthologous brain regions). Gene expression changes between WT and KO were compared with gene expression changes from existing datasets representing human MDD, human SERT polymorphism, and mouse UCMS model. Concordant gene expression changes measured by microarray were independently verified by qPCR in mouse and human samples. Here we tested three hypotheses (SERT-KO is similar to MDD, s-allele and/or UCMS?) and generated cellular and molecular hypotheses of altered function in SERT-KO (Fig. 1).

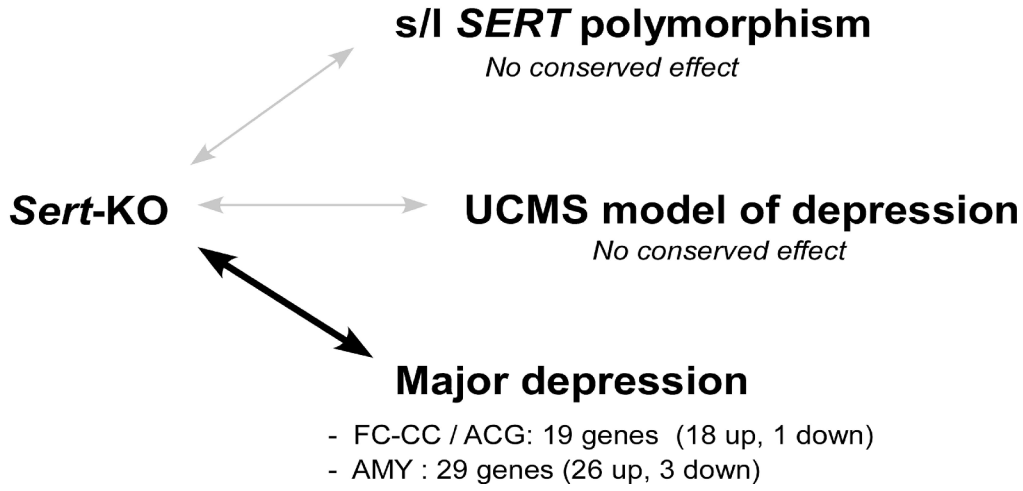

Fig. 2. Phylogenetically conserved differential gene expression as assessed by microarray. We tested three hypothesis of similar or conserved changes in gene expression between experimental groups. Do molecular adaptations that occurred in SERT KO mice mimic gene expression changes associated with human MDD (1), with the presence of the s-allele of the 5-HTTLPR polymorphism (2), and/or with the depressive-like phenotype induced in mice by UCMS (3)?

3.2.1 Phylogenetically conserved gene expression changes in SERT-KO emotionality syndrome and MDD

In a comparison of differential gene expression as measured by microarray in AMY and CC, no changes were conserved between SERT-KO and UCMS. Furthermore, no changes were conserved between SERT-KO and human s-allele carriers (unpublished results), but 29 genes in AMY and 19 genes in CC/ACC were differentially expressed in both SERT-KO and human MDD in AMY as measured by microarray (N=5 per mouse genotype; N=13 MDD subjects, N=16 control; Table 1). About 45-50% of the genes in each area (such as adenylate cyclase 7) are related to receptor function and signal transduction.

Amygdala

Gene	Gene Symbol	Mouse (alr)	Human (alr)
adenylate cyclase	ADCY7	0.60	0.40
voltage-dependent calcium channel	CACNA1D	0.33	0.41
potassium channel-interacting protein 4	KCNIP4	0.43	0.41
lin-7 homolog	LIN7C	0.48	0.31
nuclear receptor 1D2	NR1D2	0.45	0.47
potassium channel tetramerisation domain	KCTD9	0.17	0.55
mitogen-activating protein kinase kinase 2	MAP2K2	-0.42	-0.32
amyloid beta (A4) precursor-like protein 2	APLP2	0.45	0.47
ankyrin repeat domain 6	ANKRD6	0.42	0.46
calcium activated nucleotidase 1	CANT1	0.22	0.45
choline phosphotransferase 1	CHPT1	0.56	0.28
coiled-coil domain containing 50	CCDC50	0.33	0.32
coxsackie virus andadenovirus receptor	CXADR	0.27	0.27
fibroblast growth factor	FGF14	0.56	0.52
golgi SNAP receptor complex member 1	GOSR1	0.37	0.47
nucleoporin 50	NUP50	0.36	0.31
nucleoporin-like 1	NUPL1	0.35	0.31
PBX/knotted 1 homeobox	PKNOX1	0.38	0.44
pleckstrin homology domain	PLEKHF2	0.59	0.38
Ras-like without CAAX 2	RIT2	0.48	0.31
ribonucleotide reductase M1	RRM1	0.68	0.27
ring finger protein 170	RNF170	0.32	0.28
solute carrier family 25	SLC25A27	0.30	0.28
sulfiredoxin 1 homolog	SRXN1	0.30	0.31
TBC1 domain family 4	TBC1D4	0.25	0.63
WD repeat domain 1	WDR1	0.36	0.63
TAF15 RNA polymerase II	TAF15	-0.30	-1.42
zinc finger protein 444	ZFP444	-0.36	-0.44

Table 1. Differentially expressed genes in AMY and CC/ACC as detected by microarray and confirmed by qPCR. Gene expression changes are reported as average log ratio (alr), which is the base-2 logarithm of the ratio of the average value in one group versus the average value in another group ($p<0.05$ for all values). Positive values (in red) indicate an upregulation, while negative values (in green) indicate a downregulation. The first 7 genes listed in each area were evaluated by qPCR.

Cingulate Cortex

Gene	Gene Symbol	Mouse	Human
aminoadipate-semialdehyde dehydrogenase-phosphopantetheinyl transporter	AASDHPPT	0.34	0.88
CCAAT/enhancer binding protein	CEBPG	0.21	0.39
E1A binding protein p300	EP300	0.27	0.29
EPH receptor A5	EPHA5	0.21	0.50
protein phosphatase 1A	PPM1A	0.28	0.54
Rho GTPase activating protein 5	ARHGAP5	0.24	0.52
sorting nexin 13	SNX13	0.34	0.35
arestin domain containing 3	ARRDC3	0.36	0.46
coproporphyrinogen oxidase	CPOX	0.27	0.57
fucose-1-phosphate guanylyltransferase	FPGT	0.33	0.46
metal response element binding transcription factor 2	MTF2	0.26	0.69
protein-L-isoaspartate O-methyltransferase domain	PCMTD1	0.35	0.45
sperm associated antigen 9	SPAG9	0.28	0.38
splicing facor proline/glutamine rick	SFPQ	0.27	0.32
transmembrane and tetratricopeptide repeat containing 2	TMTC2	0.37	0.57
transmembrane protein 64	TMEM64	0.31	0.28
Yip1 domain 5	YIPF5	0.26	0.44
PDZ domain 4	PDZD4	-0.32	-0.30

Table 1. Differentially expressed genes in AMY and CC/ACC as detected by microarray and confirmed in qPCR (cont.)

3.2.2 ADCY7 and KCTD9 are upregulated in SERT-KO syndrome and MDD in AMY

Consistent with our moderate stringency in initial statistical and fold change criteria, only some gene changes confirmed by qPCR. Selected concordant gene expression changes detected by microarray in CC/ACC were validated by qPCR in mouse but not human (CEBPG, ARHGAP5, EP300). However, an upregulation in ADCY7 expression in AMY was confirmed in both mouse and human (+68%, p=0.03; +32%, p=0.04; Table 2). An increase KCNIP4 was

validated in mouse but qPCR revealed no difference in gene expression in human. KCTD9 was significantly increased in mouse and reached a trend-level increase in human. As an internal control, we measured SERT mRNA levels by qPCR. In SERT-KO, there was actually an unexpected large increase in the amount of SERT transcript (SLC4A6) mRNA as measured by both microarray and qPCR (+245%, $p<0.0005$; +2,460%; $p<0.0005$). This may represent an attempted upregulation of SERT transcript levels as a feedback mechanism for the absence of SERT protein. Notably, the coding section of the SERT transcript is defective (Bengel, et al. 1996). Genes were selected for qPCR evaluation based on their potential function as it relates to receptor function and signal transduction, so we next evaluated the functional relationships of validated genes of interest.

		Mouse				**Human**			
Gene symbol	Gene name	**array (fold change)**	**p-value**	**qPCR (fold change)**	**p-value**	**array (fold change)**	**p-value**	**qPCR (fold change)**	**p-value**
ADCY7	adenylate cyclase VII	1.52	p<.0005	1.68	p=0.03	1.25	p=0.02	1.32	p=0.04
KCNIP4	potassium channel interacting protein	1.26	p=0.05	1.53	p=0.04	1.01	p=0.02	-1.14	p=0.30
KCTD9	potassium channel tetramierization domain	1.47	p=0.03	1.23	p=0.04	1.03	p=0.03	1.09	p=0.10
SLC6A4 (SERT)	serotonin transporter	24.5	p<0.0005	246	p<0.0005	N/A	N/A	N/A	N/A

Table 2. Differential expression of genes in AMY

3.2.3 Upregulated AMY genes identify a coregulated module related to cAMP signal transduction

Despite the lack of qPCR verification of altered transcript levels for two genes in the human dataset, we further investigated the putative relationship between the identified genes, in view of their robust changes in the mouse and of conserved changes in ADCY7. Correlation of gene

expression across samples (coregulation) has been shown to signify related function (Lee, et al. 2004). In mouse, ADCY7, KCNIP4 and KCTD9 expressions were significantly correlated. In human, ADCY7 and KCTD9 were significantly correlated (Table 2; Fig. 2).

The coregulation of these genes suggests a functional module related to cellular signaling which may be affected in MDD and in the SERT-KO model. Thus, we provide evidence for gene expression changes which may act in a common functional pathway and explain some behavioral differences observed in SERT-KO.

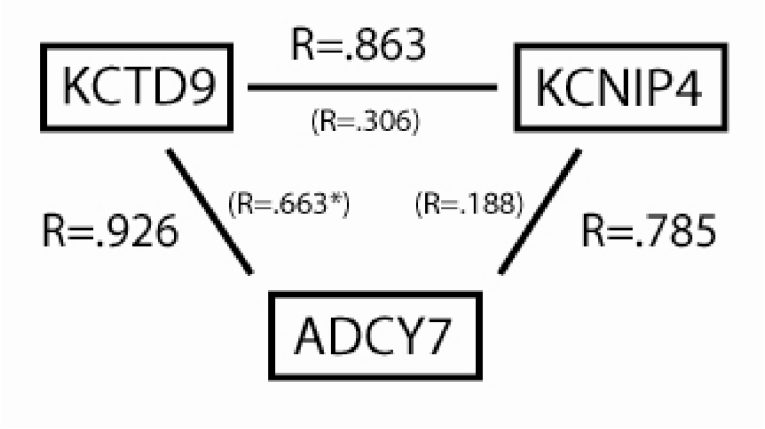

Fig. 3 A tightly coregulated functional module upregulated in SERT-KO. Outer R-values are from mouse qPCR and inner R-values (in parentheses) are from human qPCR. *p<0.0005

In order to evaluate the cell-type origin of mRNAs, we calculated a ratio of white matter (WM) microarray from neighboring tissue with gray matter (GM) of interest (WM/GM ratio). Because WM contains mainly glial cell bodies while GM contains both glial and neuronal cell bodies, this ratio evaluates mRNA enrichment in either glia or neurons. This approach showed that KCTD9 and KCNIP4 were highly enriched in expression in neurons over glia (184%, 1220% enriched), while ADCY7 was moderately enriched (156%) in neurons (Sibille, et al. 2008).

Mouse

	qPCR		Array	
Genes	correlation	p-value	correlation	p-value
ADCY7xKCTD9	0.926	p<0.0005	0.770	p=0.013
ADCY7xKCNIP4	0.785	p=0.02	0.861	p=0.043
KCTD9xKCNIP4	0.863	p<0.0005	0.738	p=0.023

Human

	qPCR		Array	
Genes	correlation	p-value	correlation	p-value
ADCY7xKCTD9	0.663	p<0.0005	0.252	n.s.
ADCY7xKCNIP4	0.188	n.s.	0.334	n.s.
KCTD9xKCNIP4	0.302	n.s.	0.223	n.s.

Table 3. Coregulation of gene expression in AMY

4.0 DISCUSSION

These results confirmed an increase in anxiety-like emotionality (OF, EPM) in SERT-KO and revealed a robust depressive-like emotionality (NSF, SP), together suggesting the presence of an overall anxious depressive-like syndrome. No change in tests denoting behaviors that are predictive of antidepressant response (FST, TST) were observed. Furthermore, the differential gene expression profile of SERT-KO as measured by microarray in AMY and CC showed no similarity to those of UCMS-treated mice or human s-allele carriers, but did reveal potential concordant gene expression changes seen in MDD subjects. Subsequent analysis focused on genes validated by qPCR in AMY in human (ADCY7, KCTD9) and mouse (ADCY7, KCTD9, KCNIP4). The three genes upregulated in mouse AMY are tightly coregulated in both microarray and qPCR data, while ADCY7 and KCTD9 are more modestly coregulated in human qPCR data but not human microarray. Changes to this module of functionally related genes including an isoform of adenylate cyclase suggest a change in the cyclic AMP (cAMP) signaling pathway.

4.1 SERT-KO'S EMOTIONALITY SYNDROME CONTAINS SYMPTOM DIMENSIONS SIMILAR TO MDD

The increased anxiety-like behavior SERT-KO shows in the EPM and OF, as well as increased depressive-like emotionality in SP and NSF, are consistent with MDD symptoms of anxiety and depressed mood. The anxiety-like EPM and OF results are consistent with previous data reported by Holmes et al. in C57/B6 (2003), while contradicting the results of Lira in 129Sv, et al. (2003). This discrepancy may be due to differences in strain baseline emotionality in C57/B6 and 129Sv mice, as 129Sv mice are highly anxious and may not be useful for measuring increases in anxiety-like emotionality. The SERT-KO rat also shows increased anxiety in EPM and OF, demonstrating conservation of function across rodent species (Olivier, et al. 2008). The increased depressive-like emotionality measured in NSF is consistent with the data reported by Lira, et al. (2003) and the increased depressive-like emotionality in the SERT-KO rat in NSF (Olivier, et al. 2008), but the increased depressive-like emotionality measured in SP conflicts with previous data (Kalueff, et al. 2006; Perona, et al. 2008). The SP test as a measure of anhedonia-like emotionality was first implemented in rats (Willner, et al. 1987) and may yield less consistent results in mice. The discrepancy with Kalueff report may be due to variation in experimental paradigms including different concentrations of sucrose solution and different training methods. We used the most commonly reported 2% sucrose solution and a high-reward training method using sweetened condensed milk. Although it has been shown in the SERT-KO rat (Olivier, et al. 2008), this is the first positive evidence of an anhedonia-like behavioral difference of SERT-KO in SP in mice, suggesting an additional *depressive-like feature to the SERT-KO increased emotionality syndrome*. Finally, the absence of a change in immobility in the FST and TST in SERT-KO may appear to contradict previous results in 129Sv and rat (Lira,

et al. 2003; Olivier, et al. 2008). However, differential response in FST and TST due to SERT-KO have been shown to be strain-specific and does not appear in C57BL/6J mice (Holmes, et al. 2002; Perona, et al. 2008), suggesting that differences in FST and TST in SERT-KO may be more variable, less robust and difficult to replicate. Moreover, FST and TST results have limited utility in the context of changes in emotionality due to gene manipulation rather than acute antidepressant administration. For a review of SERT-KO rodent behavior, see Kalueff, et al. (2010) and Lesch and Murray (2008). Thus, the SERT-KO syndrome exhibits increases in both anxiety-like and depressive-like emotionality, suggesting that it may effectively model the complex multidimensional features seen in MDD. This evidence supports the construct validity of SERT-KO as a model of MDD.

4.2 PHYLOGENETICALLY CONSERVED GENE EXPRESSION CHANGES IN AMY OF SERT-KO AND MDD SUBJECTS SUGGEST AN UPREGULATED FUNCTIONAL MODULE RELATED TO CAMP SIGNAL TRANSDUCTION

Two genes (ADCY7 and KCTD9) were upregulated in AMY in both the SERT-KO and human MDD subjects, and a related gene (KCNIP4) was upregulated only in mice. ADCY7 encodes adenylate cyclase 7, a membrane-bound protein that catalyzes the synthesis of cyclic AMP (cAMP), creating a long-term amplified signal cascade within the cell. KCTD9 encodes a potassium channel tetramerization domain, and KCNIP4 encodes a potassium channel-interacting protein. Interestingly, these genes may belong to a shared functional module, as revealed by significant coregulation patterns in mice, and to a lesser extent in humans. Due to different probesets and probe-binding efficiencies as well as real species-specific biological

relationships, coexpression relationships between species have relatively small overlap (Wang, et al. 2009) although they share very similar global network characteristics such as connectivity distribution (frequency of nodes with each possible connectivity value in the network) and modular structure (most networks are inhomogeneous and have many links within modules and few links between different modules) (Tsaparas, et al. 2006). Given these broad statistical characteristics, and the similar correlation levels indicated by array and qPCR, it appears that ADCY7, KCTD9, and KCNIP4 are part of a biological module in the mouse. This situation is produced when all the genes are under common control by other biological factors, either within cells (transcriptional and/or translational regulation or across cells (e.g. through common signaling drive), or when they are part of a small network with a common functional goal (Lee, et al. 2004). Since there are significant interspecies differences in coexpression networks, and array and qPCR indicate different levels of coexpression, there may or may not be a persistent link between ADCY7 and KCTD9 in humans.

The cAMP pathway has been investigated in MDD, including downstream targets Protein Kinase A and cAMP response element binding protein (CREB) (Duman, 2002). Multiple types of ADs are known to increase both CREB expression and CREB phosphorylation in the brain (Donati, et al. 2003). CREB and phosphorylated CREB (pCREB) were also diminished by MDD models such as UCMS and chronic swim stress, and the molecular and behavioral changes caused by these models were both reversed by AD administration (Li, et al. 2009; Qi, et al. 2008). Specific upregulation of CREB in the dentate gyrus of the hippocampus produced antidepressant-like behavior in FST and in the "learned helplessness" fear conditioning paradigm (Andrew, et al. 2001). However, specific upregulation of CREB in the basolateral amygdala revealed underlying complexities, as it produced an antidepressant-like response in the "learned

helplessness" shock escape paradigm, but produced the opposite result in FST and increased anxiety-like behavior in OF and EPM and enhanced cued fear conditioning (Wallace, et al. 2004). While it is known that a cAMP response element (CRE) binding site is located in the promoter region for BDNF, and increased BDNF is reported to coincide with long-term AD treatment, it has been shown in mice lacking CREB that behavioral and endocrine antidepressant-like responses can be induced even though upregulation of BDNF is blocked (Conti, et al. 2002), suggesting that antidepressant efficacy is not solely dependent on an upregulation of BDNF by CREB. Thus, the effect of CREB upregulation may be brain-region-specific, and AD efficacy may depend on changes in cAMP signaling which are independent of increased CREB and BDNF.

In spite of the support from animal models, there are only three reports supporting a primary pathology in CREB or cAMP signaling in MDD, all of which were investigated in cerebral cortex. Reduced pCREB was found in the orbitofrontal cortex of postmortem MDD patients (Yamada, 2003). Reduced CREB was reported in the temporal cortex of MDD subjects who died by suicide (Dowlatshahi, et al. 1999). Curiously, *increased* CREB and pCREB were measured in the prefrontal cortex of MDD subjects not treated with ADs, but not in MDD subjects treated with ADs (Odagaki, et al. 2001), suggesting that alterations in cAMP signaling may be brain-region-specific. No reports indicate changes in cAMP-related mRNAs or proteins in AMY in MDD subjects. Thus, the present report may *provide the first evidence (although indirect) of altered cAMP signaling as a primary pathology in AMY in MDD subjects.*

A tandem repeat polymorphism with a putative role of regulation of ADCY7 expression has been implicated as a genetic risk factor for familial MDD in females, and FST and TST results in two mutant mouse models (heterozygous ADCY7 +/-, and upregulated human

ADCY7) associated higher expression of ADCY7 with increased antidepressant-like behavior, and conversely, lower expression with decreased antidepressant-like behavior in females (Hines, et al. 2006), suggesting that ADCY7 may be associated with the increased risk of females to develop MDD and that blocking or downregulating ADCY7 may have an antidepressant effect. These authors identified ADCY7 through quantitative trait locus (QTL) mapping in mice evaluated in FST and TST, and the chromosome region was identified by QTL in human MDD vs. control (Maes, et al. 1994). Thus, *the independent identification of ADCY7 by an unbiased microarray survey of gene expression provides further evidence for a role of ADCY7 in MDD by providing the first evidence of an upregulation of ADCY7 in MDD.*

How could an upregulation in ADCY7 result in the decrease in cAMP signaling anticipated in MDD? In AMY, increased fMRI activity in MDD suggests an increased recruitment of neurons (Matthews, et al. 2008), which would indicate an increase in Ca++ influx, leading to increased activity in some ADCY isoforms and decreased activity in others. Notably, an important contrast has to be made between adenylate cyclases (ADCYs) which are activated by calcium (isoforms 1 and 8) and those which are, like ADCY7, inhibited by calcium (ADCY5). Ca-activated ADCY1 and ADCY8 double knock-outs showed increased depressive-like behavior and in SP, while Ca-inhibited ADCY5 knock-outs showed normal SP but decreased depressive-like behavior in FST and decreased anxiety-like behavior in the elevated plus maze and light/dark box tests (Krishnan, et al. 2008). Thus, the authors suggest that pharmacotherapies blocking ADCY5 may promote resilience against MDD and anxiety disorders. ADCY5 knock-out mice also have increased longevity and other improved health factors (Yan, et al. 2007). Interestingly, ADCY5 and ADCY7 are both targets of the mood stabilizer Lithium (Mann, et al. 2008).

The potassium channel-related genes (KCTD9 and KCNIP4) are coregulated with each other and with ADCY7, suggesting that they *form a functional unit which is related to the pathology of MDD.* This could be accomplished by increased activity of a common transcription factor, or functional feedback regulating the expression of these genes. Because ADCY7 is calcium-inhibited, the KCNIP4 and KCTD may function to localize potassium influx, regulating ADCY7 via calcium influx. *It is possible that an upregulation of these genes results in a net decrease in cAMP signaling in AMY by altering the balance between calcium-activated and calcium-inhibited ADCYs.*

4.3 LIMITATIONS

The animals and human subjects in this study are all male, which limits our ability to generalize results to female subjects, who represent the majority of MDD patients. Results from this study should be replicated in cohorts of female MDD subjects and female mice, especially in light of the sexually dimorphic nature of MDD, in order to determine whether the outcome can be generalized across sexes.

The mRNA transcripts used to measure gene expression were collected from multiple cell types, diluting the signal from individual cell types and limiting our ability to infer cellular changes. This can be addressed in follow-up studies using laser-capture to extract mRNAs from individual identified cells. However, the transcripts for KCTD9 and KCNIP4 are robustly enriched and ADCY7 is moderately enriched in neurons, as measured by a ratio of gray matter to white matter gene expression (Sibille, et al. 2008).

These results were not investigated at the protein level, and further data is required to know whether changes in mRNA quantity translate to changes in protein quantity and/or function. From these results, we would anticipate that ADCY7 is robustly upregulated in AMY of MDD subjects and SERT-KO at the protein level.

The SERT-KO mouse is devoid of serotonin transporter protein, which represents an extreme gene manipulation not seen in humans, and the heterozygous (HZ) mouse was not included, although it has been put forth as a more realistic model. Our results suggest that the HZ mouse shows an intermediate phenotype not qualitatively different from SERT-KO, and is sometimes indistinguishable from WT (Joeyen-Waldorf, et al. 2009). Comparing the SERT-KO and WT maximizes the possibility of finding behavioral and molecular differences and greatly simplifies analysis.

4.4 CONCLUSIONS

The SERT-KO genetic mutant model shows an emotionality phenotype which is consistent with MDD symptoms, in terms of increased anxiety-like, depressive-like and anhedonia-like behaviors, overall suggesting an increased emotionality behavioral syndrome that recapitulates apects of the human syndrome. Some of the molecular changes at the gene expression level in the amygdala of SERT-KO are also seen in the human postmortem brains of MDD patients, suggesting that a phylogenetically conserved mechanism may be responsible for aspects of both MDD symptoms and the SERT-KO emotionality phenotype. The affected genes include an upregulation of ADCY7 transcripts, a gene which has been previously implicated in MDD (Hines, et al. 2006), and upregulation of at least one gene which is related to potassium channel

function. The coregulation of these genes is increased in MDD and SERT-KO, suggesting the strengthening of a signal transduction-related functional unit in the pathological condition. Because ADCY7 is a calcium-inhibited ADCY, its upregulation may be consistent with a decrease in cyclic AMP activity which has been observed in MDD (Yamada, et al. 2003; Dowlatshahi, et al. 1999).

4.5 FUTURE DIRECTIONS

Several experiments in both human and mouse remain, to investigate whether ADCY7 human polymorphism impacts fMRI amygdala reactivity, whether the SERT-KO emotionality phenotype can be rescued via ADCY7 regulation, and whether the ADCY7 knock-out mouse shows a positive change in tests of anxiety-like and depressive-like emotionality. The SERT-KO provides a useful model for investigating changes in ADCY7 at the protein level and cellular morphological changes in the amygdala which may be relevant to MDD.

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