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Research Report

The roles of sex and serotonin transporter levels in age- and stress-related emotionality in mice

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ABSTRACT

Mood disorders are influenced by genetic make-up and differentially affect men and women. The s/l promoter polymorphism in the serotonin transporter (SERT) gene moderates both trait emotion and the vulnerability to develop depressive states in humans. Similarly, male mice lacking SERT (Knockout/KO) display an elevated emotionality phenotype. We now report that the SERT-KO phenotype is maintained throughout late-adulthood, and that female KO mice develop a larger emotionality phenotype with increasing age. Thus, to test the hypothesis that these findings reflected a putative sexual dimorphism in SERT-mediated modulation of emotionality, we submitted adult male and female wild-type, heterozygous (HZ) and KO mice to unpredictable chronic mild stress (UCMS) and assessed behavioral changes. In males, the elevated SERT-KO emotion-related behavior converged with other groups after UCMS. Conversely, female SERT-KO displayed a normal non-stressed baseline, but highest UCMS-induced emotionality. SERT-HZ displayed variable and intermediate phenotypes in both experiments. Thus, consistent results across different biological modalities (age, stress) revealed a high contribution of SERT genotype for baseline "trait" emotionality in males, and low contribution for females. In contrast, age-correlated and stress-induced behavioral changes resulted in a high SERT genotype-mediated behavioral variance in females, but low in males. This suggests that high emotionality states associated with low SERT were differentially achieved in males (high baseline/trait) compared to females (increased vulnerability to develop high emotionality). This sex-by-SERT double dissociation provides a framework to investigate molecular substrates of emotionality regulation in concert with serotonin function and may contribute to the sexually dimorphic features of mood disorders.

1. Introduction

A short promoter variant (s-allele) in the SERT gene is associated with anxiety-related personality traits (Lesch et al., 1996), increased probability to develop depressive episodes when faced with stressful life events (Caspi et al., 2003) and robustly correlates with increased amygdala reactivity (Hariri et al., 2002; Heinz et al., 2005). The s-allele correlates with low

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SERT levels in cell lines (Lesch et al., 1996), although associations with reduced RNA or binding levels in the adult brain have not been established (Mann et al., 2000; Shioe et al., 2003; Parsey et al., 2006; Lim et al., 2006), likely reflecting the presence of additional factors contributing to SERT regulation (Serretti et al., 2006) or, of a developmental role for SERT in modulating emotions (Ansorge et al., 2004; Sibille and Lewis, 2006). Subsequent association studies between SERT polymorphisms and mood regulation have been numerous and conflicting at times (Caspi and Moffitt, 2006; Serretti et al., 2006), potentially due to the lack of an established conceptual framework for the contribution of SERT genetic variability to baseline personality traits, to states of altered mood regulation, and to the well-documented observation of higher rates of mood disorders in females.

In addition to its role in the etiology and treatment of depression and anxiety-related disorders, altered serotonin signaling is considered a contributing factor to age-related processes and a risk factor for late-life mood disorders (Mattson et al., 2004; Meltzer et al., 1998). We have previously shown that mice lacking the regulatory presynaptic HTR1B receptor displayed an early profile of age-related gene expression changes and a significant reduction in longevity (Sibille et al., 2007), thus hinting at a potential causative role for serotonin in age-related processes, and suggesting a pathogenic pathway linking serotonin, aging, and potentially mood disorders. In this report, defining "emotionality" as the expression of anxiety-like and/or depressive-like behaviors we focused on aspects of emotionality regulation between young and older adult mice with altered serotonin homeostasis through genetically manipulated SERT levels. SERT mutant mice display a gene dosage-dependent downregulation in SERT levels, as demonstrated by absent binding in homozygous SERT-KO mice, compared to 50% in HZ and 100% levels in wild-type (WT) controls (Bengel et al., 1998).

Thus, to test the hypothesis that low SERT levels moderates the regulation of emotionality and to model potential sex-by-SERT gene interactions, changes in anxiety-like and depressive-like behaviors were investigated in male and female WT, HZ and SERT-KO mice during normal aging and after being submitted to unpredictable chronic mild stress (UCMS). UCMS is an informative model to study emotionality regulation in mice, as it mimics the role of socio-environmental stress in precipitating a syndrome that is reminiscent of symptoms of human depression, including increased emotion-related behaviors in several behavioral paradigms (Willner, 2005; Ducottet et al., 2004).

Thus, a central objective of this study was to assess the trajectory of changes in emotionality between non-stressed baseline in young mice, defined here as "trait" (as it is less likely to be affected by cumulative events across the lifespan), and higher emotionality states induced through physiological aging or socio-environmental stress, defined here as "induced-state". Accordingly, investigating dynamic changes in emotionality and taking into consideration three factors important for human emotion regulation (baseline trait/induced-state, sex and genetic vulnerability), we confirm that female sex and low SERT levels are both associated with elevated emotionality, and report a double dissociation between the impact of SERT levels and sex on behavioral variance, where high emotionality "states" were differentially achieved in males with low SERT (due to high baseline/trait) compared to females with low SERT (due to increased susceptibility to develop age- or stress-induced high emotionality).

2. Results

2.1. *Sustained SERT-KO hypolocomotion phenotype between young and older adult male and female mice*

As changes in the serotonin system can induce early signs of aging (Sibille et al., 2007), we initially assessed putative changes in general health and activity in WT, SERT-HZ and SERT-KO male and female mice at 2–4, 6–8 and 12–14 months of age (referred to a 3-, 6- and 12-month time-points) (Fig. 1). Weight increased with age, but did not differ as a function of genotype in males or females (Fig. 2a). Body temperature was not different between groups or ages (not shown). Locomotor activity decreased significantly with age in the open field test (OF), as measured by the total crosses in OF ($F_{2,251}=8.6$, $p<0.0005$) and a trend towards a decrease in total number of arm entries in the elevated plus maze test (EPM) ($F_{2,250}=2.6$, $p=0.07$) (Figs. 2b–c). The previously described hypolocomotor phenotype of KO mice (Lira et al., 2003; Holmes et al., 2003) was present at all three time-points relative to WT in males and females (KO<WT and HZ at all ages, $p<0.001$; Figs. 2b–c). The total activity of SERT-HZ mice varied between WT and KO levels in males and was indistinguishable from WT levels in females. In the rotarod test, maximal motor coordination was observed in all groups at all ages using a slow 20 rpm rotating speed (not shown). At higher speed (40 rpm), WT and HZ mice appeared to improve their performance at 12-month of age, while KO male and female mice displayed stable or lower performance levels, resulting in significantly shorter latencies to fall from the rotating rod, compared to WT and HZ mice (Fig. 2d). No early mortality was reported in any experimental groups. Together these results confirmed the presence of a stable and robust hypolocomotion phenotype in

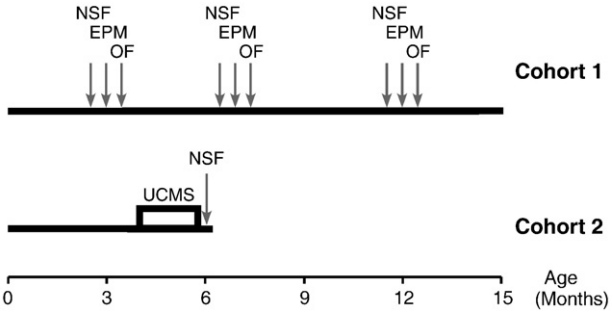
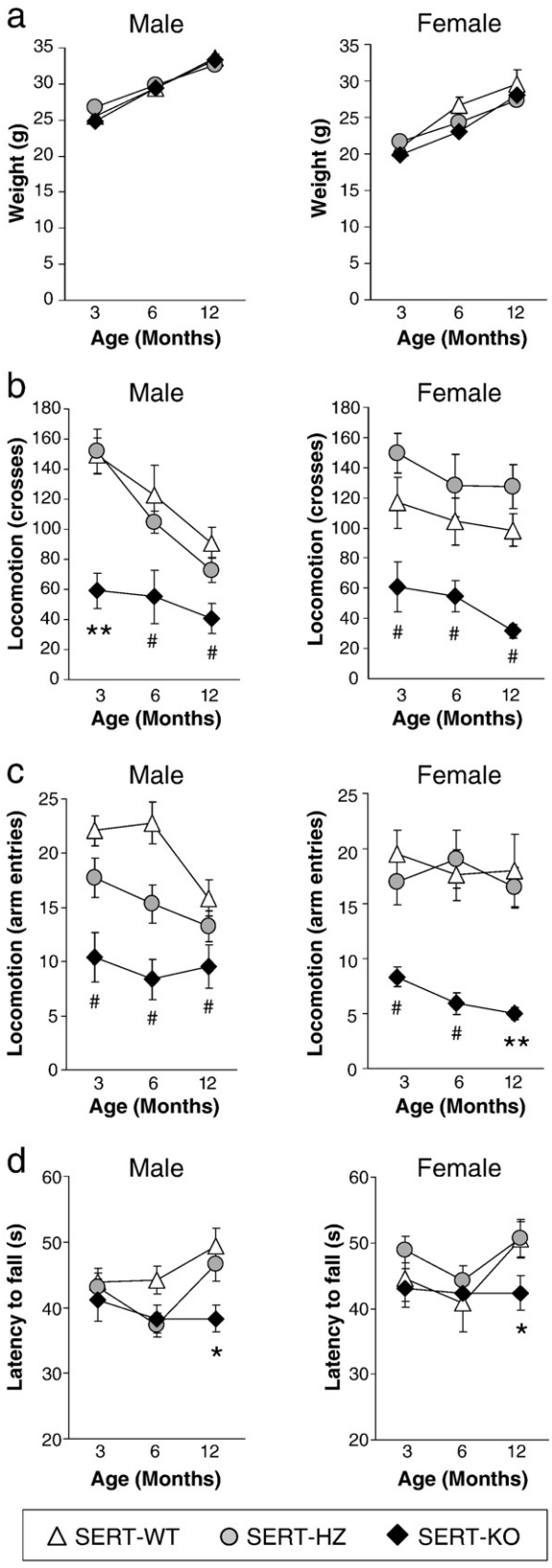


Fig. 1 – Experimental design. Cohort 1 ($n=94$ mice) was used longitudinally and exclusively for the age-related behavioral assessments. Accordingly, cohort 1 was tested in the open field (OF), elevated plus maze (EPM) and novelty-suppressed feeding (NSF) tests at 2 months of age and twice thereafter at 4–5 months intervals. Cohort 2 ($n=54$ mice) was submitted to a 4-week UCMS paradigm and tested in the NSF during the 5th week.

SERT-KO mice, and suggested the appearance of a reduced motor coordination phenotype with increasing age in SERT-KO mice.



2.2. *Conserved male SERT-KO elevated emotionality phenotype with increasing age and appearance of a similar robust female SERT-KO phenotype*

Changes in emotionality with increasing age were assessed in the OF, EPM and novelty-suppressed feeding (NSF) tests (Fig. 3). The OF and EPM tests rely on behavioral inhibition, where low activity in the threatening compartments denotes elevated anxiety-related behaviors. Significant reductions in activity in center of the OF and in time in open arms of the EPM were observed with increasing age for all groups combined (Age effect: OF, $F_{2,248}=27.0$ $p<0.0005$; EPM, $F_{2,251}=4.4$ $p=0.013$). As previously reported (Holmes et al., 2003; Lira et al., 2003), 3-month-old male SERT-KO mice displayed significant elevated anxiety-related behaviors compared to WT mice in OF and EPM (Figs. 3a–b). SERT-KO differences were reduced to non-significant trends by 12 months of age, although both tests were limited by low activities, or floor effects, in the threatening compartments of the tests (see below). Male HZ behavior was similar to WT behavior in OF, but comparable to KO in the EPM, thus suggesting an intermediate phenotype. Female mice as a group displayed a lower activity in the threatening compartments of both tests (OF, $p<0.005$; EPM, $p<0.001$), and a further reduction with increasing age (Figs. 3a–b). 12-month-old female SERT-KO mice were the least active in the center of OF compared to all other groups (Fig. 3b), denoting highest anxiety-related behavior from all male and female groups. However, the dynamic ranges of the OF and EPM tests are typically limited for high-anxiety groups due to very low activity in the threatening compartments, thus making quantification of anxiety-related behavior less reliable. For instance, the average time spent in EPM open arms for all female groups was less than 1.5% of the total assay time (Fig. 3b). Time in OF

Fig. 2 – Normal weight gain, sustained hypolocomotion phenotype and appearance of a motor coordination phenotype in male and female SERT-KO mice with increasing age. (a) Weight increased with age ($F_{2,252}=124$, $p<0.0005$). Sex effect: Male>Female ($F_{1,252}=157$, $p<0.0005$). (b) OF total activity decreased with age ($F_{2,251}=8.6$, $p<0.0005$) and was lower in SERT-KO mice (Genotype effect: Male KO<WT, $p<0.0005$; KO<HZ, $p<0.0005$; Female: KO<WT, $p=0.0005$, KO<HZ, $p<0.0005$ and WT<HZ, $p=0.015$). (c) EPM total activity displayed a trend towards a decrease with age ($F_{2,250}=2.6$, $p=0.07$) and was lower in SERT-KO mice (Male: KO<WT, $p<0.0005$; KO<HZ, $p<0.0005$; HZ<WT, $p=0.001$. Female: KO<WT, $p<0.0005$; KO<HZ, $p<0.0005$). Significant sex*genotype interactions in OF ($p=0.004$) and EPM ($p=0.018$) suggested that total activity in female WT and SERT^{HZ} decreased at a slower rate compared to males. (d) Motor coordination in the rotarod test (40 rpm) improved at the 12-month time-point in WT and HZ male and female mice ($p=0.006$), but remained flat in SERT-KO mice, resulting in a significant lower motor coordination in 12-month-old male and female SERT-KO mice (Genotype effect: $F_{2,251}=4.8$, $p=0.009$; KO<WT, $p=0.06$; KO<HZ, $p=0.05$).

center and relative activity in open arms of the EPM were even further affected by floor effects (not shown). Despite these limitations, results from the EPM and OF suggested a trend for a different contribution of SERT levels to behavioral variance between males and females, where genotype differences converged in males, but were revealed in females, with increasing age.

In contrast to OF and EPM, behavioral measurements in the NSF test (latency to feed) increase with elevated anxiety-related and depressive-like behavior and are thus not limited by floor effects. The latency to feed in NSF significantly increased with age (Main age effect, $F_{2,251}=36.2$, $p<1\times e^{-10}$; Fig. 3c). Consistent with the elevated baseline emotionality phenotype of male SERT-KO, NSF latency in 3-month-old male mice was elevated in SERT-KO males (Fig. 3c) and remained higher compared to WT and HZ mice at all ages tested. Female baseline behaviors were more variable, with HZ mice displaying shorter latency, while young WT and KO female groups did not differ. Older female SERT-KO mice displayed remarkably higher latency compared to all other experimental groups, representing a ~4-fold increase between 3 and 12 months of age (Fig. 3c). Control measures indicated that 12-month-old female SERT-KO mice, the most affected group, consumed less food after NSF (−15%, $p<0.05$), which we interpreted as residual emotionality, since weight (Fig. 2a) and home-food cage feeding ($p>0.05$) was not different at that age.

In summary, converging results across three behavioral tests confirmed the high emotionality phenotype in young male SERT-KO, highlighted age-correlated increases in emotionality in males and females, and revealed a robust and highest emotionality phenotype induced by age in female SERT-KO mice. Results also suggested a potential sexual dimorphism in the regulation of emotionality according to sex and SERT genotype. Namely, OF behavioral differences in correlation with SERT genotype were larger for males under baseline conditions in young males compared to age-correlated states, as genotype differences were maintained (NSF) or converged (OF, EPM) in older adult subjects. Conversely, female groups did not differ under baseline conditions in terms of emotionality, but display a significant genotype effect with increasing age, as older female KO mice displayed highest emotionality compared to all other male and female experimental groups.

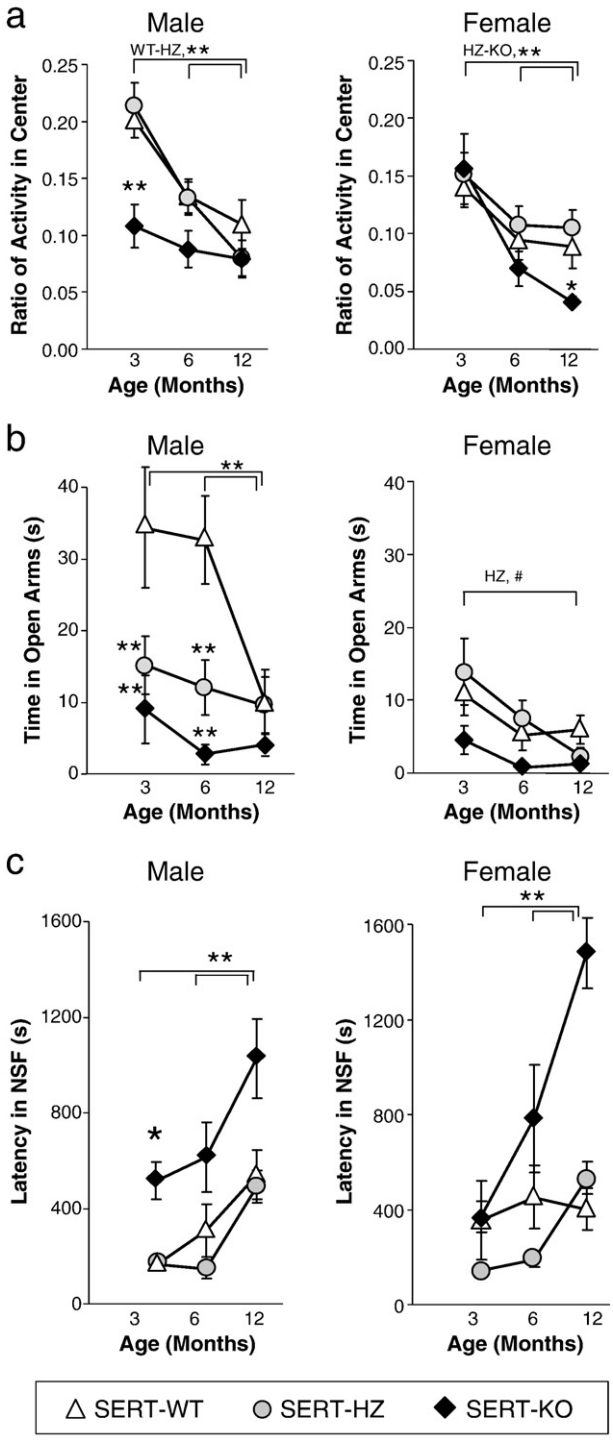


Fig. 3 – Emotion-related behavioral profile with increasing age in OF, EPM and NSF tests. (a) Reduced activity in the center of the OF indicated elevated anxiety-related behavior in young male KO mice and progressive increases with age in WT and HZ mice (, $p<0.001$). Young female groups were indistinguishable, but 12-month-old female KO mice displayed highest anxiety-related behaviors compared to all other groups, including male KO mice (KO *versus* WT or HZ, *, $p<0.01$; 12-month-old KO female vs KO male, $^{\#}p=0.05$). (b) Reduced time in the open arms of the EPM indicated elevated anxiety-related behaviors in young male HZ and KO mice (HZ or KO *versus* WT, **, $p<0.001$) and progressive increases with age in WT mice (**, $p<0.001$). Female mice displayed very low activity in the open arms of the EPM at all ages, but displayed a similar trend of increasing anxiety-related behavior with age ($^{\#}$, $p<0.05$). (c) Increased latency to feed in NSF indicated elevated emotionality in young male KO mice compared to WT and HZ male mice (*, $p=0.001$). Latencies to feed increased with age within ($^{\#}p=0.04$; $^{\#\#}$ $p<0.001$) and across sex and genotype groups (*, $p=0.001$; **, $p<0.005$). Older female mice took significantly longer to start feeding in NSF compared to all other experimental groups (**, $p<0.005$), indicating highest levels of emotionality.**

It is important to note that aspects of the interpretation of these results were limited for several reasons, including SERT-KO hypolocomotion, floor effects of emotionality measurements in OF and EPM, and post-NSF feeding. Moreover, minimal memory saving is expected at 3–4 month testing intervals, but we cannot exclude that repeated exposure may have contributed to the overall increase in emotionality with age, although it would not explain the differences in genotype effect between males and females. Nevertheless, to address these limitations and test the hypothesis that the present findings represented a putative sexual dimorphism in SERT-mediated regulation of emotionality, we submitted a separate cohort of age-matched adult mice to a UCMS paradigm and assessed concomitant increases in emotionality as a function of sex and SERT genotype (Fig. 1).

2.3. *UCMS exposure increased emotionality, reduced behavioral differences of male SERT-KO mice, and induced a robust and highest emotionality phenotype in female SERT-KO mice*

WT, HZ and KO male and female littermate mice were submitted to a 4-week UCMS paradigm (Fig. 1). Since the UCMS has time constraints for acute testing and in view of the observed floor effects of the OF and EPM, we focused on the NSF test, due to the broader dynamic range of this test. Moreover, the latency to feed in NSF was not correlated with locomotor activity in our previous experiments [Pearson correlations between OF or EPM locomotion and NSF latency were less than 0.2 ($p>0.5$)] and was thus less likely to be influenced by the hypolocomotion phenotype of SERT-KO mice.

Consistent with results in Fig. 3c, baseline latency to feed was elevated in male SERT-KO mice (Fig. 4). Female WT, HZ and KO control groups (i.e. non-stressed) were indistinguishable. UCMS significantly *(i)* increased the latency to feed ($F_{1,39}=57.7$, $p<1\times e^{-5}$), *(ii)* reduced behavioral differences between WT, HZ and KO groups in males, *(iii)* induced greater latencies to feed in females compared to males (UCMS-by-sex, $F_{1,39}=10.1$, $p<0.005$), and *(iv)* induced the longest latencies ($p<0.001$ compared to all other groups) and highest relative increase (~8 fold UCMS/control increase) in female SERT-KO mice (Fig. 4). Control measures of weight (males, $p=0.23$; females, $p=0.38$) and post-NSF food consumption (males, $p=0.77$; females, $p=0.89$) were not different. Together, these results validated the previous age-related findings and further suggested that changes in emotionality induced by UCMS were differentially regulated in males and females with low SERT levels.

2.4. *Sexually dimorphic vulnerability to altered emotionality in mice with low SERT*

To further characterize this putative sexually dimorphic effect, we calculated relative increases in NSF behavior (i.e. latency to feed) in the different sex and genotype groups, either during aging or after UCMS, as a measure of "vulnerability" to develop high emotionality. NSF latencies of 12-month-old or UCMS-exposed groups exposure were

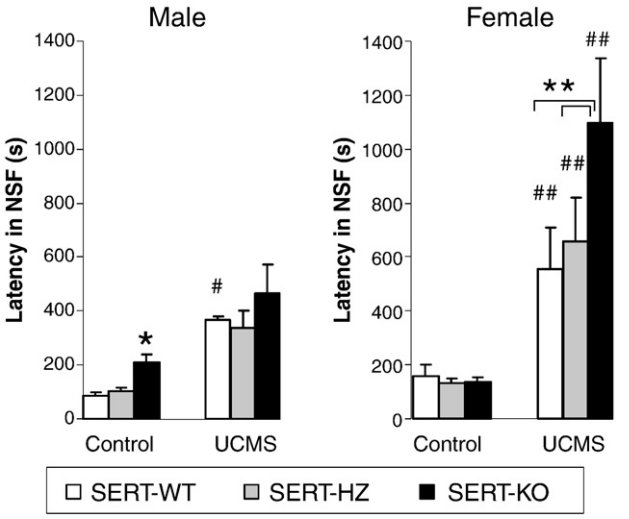


Fig. 4 – UCMS-induced latencies to feed in NSF by sex and SERT genotype groups. Baseline male genotype differences (*, KO>HZ and WT, $p=0.001$). UCMS increased latencies in both groups ($F_{1,39}=57.7$, $p<1\times e^{-5}$; Males, $p<0.0001$; Females, $p<0.0001$). UCMS effects (i.e. compared to respective non-stressed control group) within genotype groups ($^{\#}p=0.04$; $^{\#\#}p<0.001$). UCMS-exposed female mice took significantly longer to start feeding in NSF compared to all other experimental groups (, $p<0.005$), indicating highest levels of emotionality.**

expressed in percentage of their respective control groups (i.e. induced behavior level relative to basal activity) (Fig. 5). NSF behavior increased by 335% in WT between 3 and 12 months of age, but only by ~200% in SERT-KO. HZ displayed an intermediate ~290% increase. The lower relative increase of KO mice reflected the higher baseline emotionality in that group. In females, highest relative changes in NSF behavior were observed in KO (~400%) and lowest in WT (~113%). HZ displayed intermediate values. Further analyses revealed that the relative behavioral changes across genotype groups followed significant linear fits in males ($p=0.0009$) and displayed a trend in females ($p=0.08$) (dashed lines in Fig. 5a), together yielding a significant sex-by-SERT genotype interaction ($F_{2,79}=8.5$, $p<0.0005$) (Fig. 5a). Specifically, normalized UCMS-induced latency increased during aging in females with low (HZ) or absent (KO) SERT levels, but did not change (or decreased) in older males with lower SERT levels. Consistent with the age-related results, normalized UCMS-induced latency to feed progressively increased in females from WT (~220%) to HZ (~500%) and KO (~800%), but remained stable (or decreased) in males (WT, ~437%; HZ, 334% and KO, ~223%) in concert with SERT levels (Fig. 5b). Similarly, analyses across genotype groups revealed significant linear fits in males ($p=0.006$) and females ($p=0.05$) (dashed lines in Fig. 5b) and a significant sex-by-SERT genotype interaction ($F_{2,47}=5.3$, $p=0.026$) (Fig. 5b). The lower relative changes in NSF behavior in HZ and KO males did not translate in decreases in absolute emotion-related behavioral level, but rather in a reduced rate of increased behavior between baseline "trait" and high emotionality "states". Similar analyses could not be performed

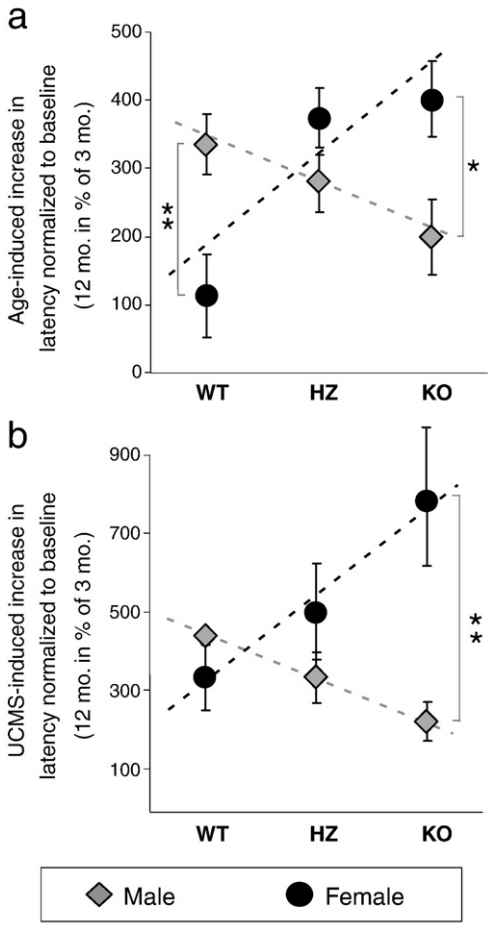


Fig. 5 – Sexual dimorphism in proportional increases in emotionality according to SERT genotype. (a) Age cohort: when controlling for baseline group differences in young mice, relative increases in the latency to feed (i.e. 12-month/3-month × 100) progressed in opposite directions, as revealed by a significant sex-by-SERT genotype interaction ($F_{2,79}=8.5$, $p<0.0005$). Female KO mice differed significantly from male KO mice (*, $p<0.05$). (b) Similarly, when controlling for baseline group differences, analyses of relative increases in latencies (i.e. UCMS/baseline × 100) confirmed the significant sex-by-SERT genotype interaction ($F_{2,20}=5.4$, $p=0.01$) and the male–female difference in KO mice in terms of "vulnerability" to develop high emotionality (, $p<0.01$). Dashed lines represent trend lines (See text for significance of linear fit).**

with OF and EPM results due to floor effects in groups displaying highest anxiety-related behaviors.

3. Discussion

Combining three parameters that are essential to emotionality regulation (trait/induced-state, sex and SERT genetic vulnerability) in two studies with very distinct experimental designs (normal aging and UCMS), we confirm that low SERT levels and female sex are both associated with elevated emotionality in mice. Moreover, we report a sex-by-SERT interaction, whereas states of elevated emotionality associated with low SERT and induced by aging or UCMS were attained through different trajectories between male and female mice. Overall, aging or UCMS increased emotionality regardless of SERT levels; however, baseline and relative increases in emotionality were moderated in a sex-dependent manner in mice with low or absent SERT levels (Fig. 5). Specifically, male SERT-KO displayed an high baseline emotionality, but normal rates of increase, whereas female SERT-KO mice displayed normal baseline but highest vulnerability to develop high emotionality states in correlation with aging or induced by UCMS. This sex difference suggests a shift in SERT genetic influence on emotion-related behavior between baseline non-stressed "trait" and induced "states" of elevated emotionality. These convergent findings also suggested that the observed changes in the "vulnerability" to develop high emotionality occurred in a SERT gene dosage manner in correlation with age or after UCMS (i.e. linear effects in Fig. 5). Hence, results across different experimental modalities suggest that sex and SERT interact to moderate the expression and degree of emotionality in mice. Fig. 6 proposes a schematic model for this interaction. Summarizing our findings in a non-quantitative manner, this model proposes that female rodents with low SERT develop high emotionality states through normal baseline but increased "vulnerability" to environmental or biological factors (i.e. rate of increase in Fig. 5), whereas male rodents with low SERT reached elevated high emotionality states due to an elevated baseline but normal vulnerability.

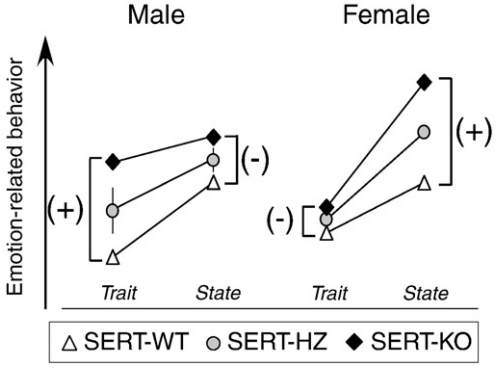


Fig. 6 – Proposed model of sex-by-SERT interaction in emotionality regulation. This schematic model summarizes in a non-quantitative manner the results from this study. Sex elicited different effects on behavioral variance associated with SERT genetic variation (Brackets): In males, the genetic influence of SERT on emotionality variance was high in baseline non-stressed "trait" behavior and low in high emotionality "states" induced by age or UCMS; Conversely, in females, the genetic contribution of SERT to behavioral variance was low for "trait" behavior, but higher for high emotionality "states". HZ male behavior varied along the WT-KO spectrum (See Figs. 2 and 3). A critical feature of this model is that high emotionality states were reached through different trajectories in males (due to high baseline) compared to females (due to high vulnerability; See Discussion).

A strength of these findings is that convergent results were obtained in two studies that used either a naturalistic paradigm (normal aging) or a more constrained experimental design (UCMS). In both cases, robust increases in emotionality were observed, but according to different timeframes (weeks *versus* months) and potentially through different mechanisms (physiological aging *versus* socio-environmental stress). This suggests the presence of a modality-independent mechanism of emotionality regulation, which was moderated here in an interactive way by sex and serotonin function. Potential mechanisms for this interaction may include sex-based neuroendocrine modulation, differential recruitment of the stress hypothalamic–pituitary–adrenal axis (Barr et al., 2004; Kalueff et al., 2007), or differences in baseline compensatory mechanisms in monoamine regulation between male and female SERT-KO mice (reviewed in Li, 2006). Although memory saving is expected to be minimal for repeated testing at 3–4 month intervals, we cannot exclude that it may have represented an additional minor contributing mechanism underlying the observed sex-by-SERT interaction in these studies.

With regard to aging it should be noted that while it robustly increased emotionality in our study, it has only been associated with an increased risk for developing mood disorders in human subjects. Interactions between age, mood disorders and serotonin have been documented and hypothesized to mediate this increased risk (Lerer et al., 1996; Meltzer et al., 1998; Gareri et al., 2002). Here our preliminary findings on overall health and general locomotor activity confirmed the presence of a sustained hypolocomotion phenotype in SERT-KO mice and of a general decrease in activity with age in all groups (Figs. 2b–c), and revealed only minor additional KO differences by 12 months of age (Rotarod behavior in Fig. 2d), which were unlikely to explain the observed differences in the emotionality phenotypes of male and female KO mice. On the other hand, our results provide supporting evidence for a role for serotonin in the regulation of emotionality during aging in mice, and further suggest that it is partially modulated by SERT function (Fig. 3).

Together, aging and UCMS induced changes in emotionality that mimicked the elevated prevalence of mood disorders in human female populations. Indeed, the presence of the highest age-related/UCMS-induced emotionality states in female KO mice is consistent with the more robust clinical findings in SERT genetic association studies in human female subjects, especially for symptoms of mood disorders in response to environmental risk (Eley et al., 2004) or to social and physical burden (Grabe et al., 2005). Sjoberg et al. (2006) have also reported an increased susceptibility to depression in response to environmental stress factors in female s-allele carriers only, and further suggested that male s-allele carriers might be protected. In clinical settings, reaching a threshold for a diagnostic of mood disorders depends on at least three factors: baseline levels of mood-related symptoms, vulnerability to develop symptoms (i.e. rate of increase or severity), and location of the diagnosis threshold along a gradient of increasing intensity of mood-related symptoms. Here, projecting from this rodent study to potential clinical settings, our results may shed light on apparent sex differences. Indeed, the combined output of a high baseline and normal or low rate of increase in male KO mice still resulted in higher emotionality in males, although the impact of SERT genetic variance was low compared to baseline non-stressed "trait" conditions. On the other hand, female mice with low SERT were identified as the group with highest vulnerability to develop robust and high emotionality states (Fig. 5).

It is important to note that the current studies do not directly model the role of the commonly investigated s/l SERT polymorphism in human subjects. Indeed, based on cell culture studies, low SERT expression associated with the s-allele has been hypothesized to modulate the anxiety-related personality traits and increased probability to develop stress-induced depressive episodes; however, correlations between the s-allele and SERT levels have been difficult to establish in the adult human brain (Mann et al., 2000; Shioe et al., 2003; Lim et al., 2006; Parsey et al., 2006). Rather, we have investigated the contributions of SERT levels, sex and environmental factors in the regulation of emotionality using a comprehensive experimental design and behavioral paradigm that allowed us to distinguish interactions between these three factors that are of critical importance in human mood disorders. Our studies also did not investigate the role of early life stress and SERT in the modulation of adult emotionality. All mice were reared under non-stressful conditions, thus the baseline behavioral performance in young adult mice (~2–4 months of age) was equated with "trait" behavior. However, baseline trait and age-related/UCMS-induced states were assessed in mice that displayed reduced SERT levels (HZ and KO) throughout development and adult life (Bengel et al., 1998) and it is likely that developmental adaptations may have contributed to the occurrence of the adult phenotype. Indeed, the fact that low-expressing SERT groups (including HZ males and females) displayed elevated emotionality during aging or after UCMS was in clear contrast with the use of selective serotonin reuptake inhibitors (SSRI) in adult mice, since reduced SERT availability through chronic SSRI exposure has anxiolytic/antidepressant effects and completely blocks the development of the UCMS syndrome (Ducottet et al., 2004; Surget et al., 2008). Therefore, our results clearly demonstrated that low SERT in the adult brain is not sufficient to induce a therapeutic effect. Rather our results were reminiscent of developmental SERT blockade, where SSRI treatment during a critical postnatal period recreated the elevated baseline emotionality that is observed later in adult males with constitutive SERT deletion (Ansorge et al., 2004). Therefore, we provide additional evidence that altered developmental trajectories and compensatory changes in neural networks that are remote from SERT genetic variance may mediate the increased vulnerability to develop elevated emotionality regulation during physiological aging or after a chronic unpredictable stress.

Several additional limitations of our studies are worth noting. *First*, although we report clear sexual dimorphic effects, we have not controlled our studies for estrous cycles. In view of the group sizes and of the fact that testing occurred over a period of several days, all phases of the estrous cycles were likely to be represented. The expected effect would have been to increase the behavioral variance and to underestimate the actual effect size, thus the full effect of low SERT in female mice may be greater than reported here. *Second*, our age-related

study included re-testing at 4–5 months interval. Although minimal memory savings is expected at these intervals, we cannot exclude it. Residual memory would typically increase fearfulness; thus a memory effect would have accelerated the "floor effect" of the OF and EPM tests in high anxiety-related groups (Fig. 3). On the other hand, food is a significant motivator in the NSF test and re-exposed mice typically display a shorter latency to feed. Since our results describe very large increases in latencies, a re-exposure effect may have actually slightly underestimated the full effect and was therefore unlikely to affect the conclusions. *Third*, our choice of behavioral tests was directed by time constraints in the UCMS paradigm. Acute testing on large groups of mice need to be performed in a relatively short period of time (~1 week) to ensure that control mice do not get stressed. Thus, due to the potential for floor effects in OF and EPM, we opted for the maximal sensitivity of the NSF, rather than adding other tests that are also stressful. In the independent aging cohort, such time constraint did not apply and we included the OF and EPM tests. Results from these tests in the age cohort also determined our choice of the NSF as the main test, since both OF and EPM displayed much reduced sensitivity in high emotionality groups. Thus, it remains to be seen if the double dissociation reported here will extrapolate to other dimensions of the depressive-related syndrome induced by UCMS, including for instance anhedonia-related behavior and neuroendocrine dysregulation. *Fourth*, plotting data as ratios normalized to baseline (Fig. 5) may have over-emphasized smaller underlying differences. While not standard, this approach clearly uncovered a relationship between SERT levels and the intrinsic capacity of the different groups of mice to develop higher emotionality (e.g. linear trends in Fig. 5). In fact, the different effects in male and female KO mice strongly suggest that SERT mutant mice may represent a useful tool in investigating the molecular bases of sex-based differences in mood disorders (Tripp and Sibille, 2009). Moreover, despite the presence of substantial adaptive changes in monoamine regulation (Li, 2006), the linear trajectories of proportional changes in altered behavior between WT, HZ and KO groups (Fig. 5) validate the use of KO mice in investigating relevant mechanisms of emotionality regulation. *Fifth*, the sex-by-SERT interaction effect will have to be replicated in independent groups, and using additional tests with broad dynamic ranges, such as the sucrose preference test for instance.

In conclusion, the sex-by-SERT double dissociation presented here is consistent with higher rates of mood disorders and depression and more robust clinical findings in SERT association studies in female subjects. This is the first demonstration that the genetic impact of SERT on emotionality is sexually dimorphic in a high/low emotionality or trait/state dependent manner. In female mice, this effect manifested in increased behavioral variance according to SERT genotype in the UCMS-induced high emotionality "state" *versus* baseline non-stressed "trait" behavior, while males displayed an opposite trend. Together, our findings reveal a dynamic role of genetic influence on emotionality regulation and provide a framework to investigate the complexity of the molecular substrates of mood disorders in concert with serotonin function. Of course, the clinical relevance of this hypothesis remains be tested in humans.

4. Experimental procedures

4.1. Animals

C57BL/6 $SERT^{KO}$ and WT mice (Bengel et al., 1998) were obtained from Taconic (Hudson, NY). Littermates from $SERT^{HZ}$ breeding were used for all experiments. The mice were maintained under standard conditions (12/12 h light/dark cycle, 22±1 °C, food and water *ad libitum*), in accordance with the University of Pittsburgh Institutional Animal Care and Use Committee.

4.2. Experimental design

(Fig. 1) Cohort 1 consisted of 94 mice (Female: WT n=10, HZ n=22, KO n=12; Male: WT n=19, HZ n=19, KO n=12) and was used longitudinally and exclusively for the age-related behavioral assessments. Accordingly, cohort 1 was tested in NSF, EPM and OF at 2 months of age and twice thereafter at 4–5 months intervals. Since the tests were applied at least one week apart and due to the large number of mice, each testing period covered 4–5 weeks. Accordingly, due to variable timing in litters to generate large experimental groups and due to extended period of testing, the ages at the different behavioral assessment covered the following ranges: 2–4, 6–8 and 12–14 months of age. Cohort 2 consisted of 54 mice of 3–5 months of age and was used exclusively for the UCMS experiment [Control non-stressed female, n=12 (WT n=3, HZ n=5, KO, n=4); Control non-stressed male, n=13 (WT, n=5, HZ, n=4, KO n=4); UCMS female, n=12 (WT n=3, HZ n=5, KO n=4); UCMS male, n=15 (WT n=5, HZ n=5, KO n=5)]. The mice were submitted to a 4-week UCMS paradigm and tested in the NSF during the 5th week. Although the cohorts differed in size of experimental groups, a power analysis indicated that cohort 1 had a >95% power to detect differences larger than 40% between any two groups, and that cohort 2 had a ~80% power to detect differences larger than 60% between any two groups.

4.3. Rotarod

Each mouse was submitted to 10 trials, in which it was placed on a slowly accelerating rotating rod (Rotamex-5, Columbus Instruments, Columbus, OH). In the 0–20 RPM condition, the rod accelerated at 0.3 rpm/s ending at 20 rpm, and in the 0–40 RPM condition, the rod accelerated at 0.7 rpm/s ending at 40 rpm (all mice were tested in both conditions). In each trial, the latency to fall from the accelerating rod was recorded as a measure of coordination. Maximum speed was achieved at 60 s, at which time the clock was stopped and a 60 s performance was attributed to a mouse if it was still on the rod. Comparisons were made using measures from the tenth trial.

4.4. Open field test (OF)

Behavior in the OF was measured as previously described (Sibille et al., 2000). A 76×76 cm OF chamber was divided in 16 even-size squares. The total number of crosses was used

as an index of locomotor activity. Crosses into the squares of the aversive center were recorded for 10 min, and the ratio of crosses into the center compared to the total number of crosses was calculated to evaluate anxiety-related behaviors.

4.5. *Elevated plus maze (EPM)*

Behavior in the EPM was measured as previously described (Sibille et al., 2000), using a cross maze with 2 open and two closed 30×5 cm 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 were recorded for 10 min to evaluate anxiety-related behaviors.

4.6. *Novelty-suppressed feeding test (NSF)*

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), thus suggesting that mechanisms underlying changes in the latency to start feeding involve anxiety-related and antidepressant-related processes, which we refer to here as 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 30×60 cm chamber. The drive to overcome the aversive center of the apparatus was increased by 16 h food deprivation and the latency to start eating was recorded in a 30 min session. A control measure of food consumption was monitored in the home cage after the test.

4.7. *Unpredictable chronic mild stress (UCMS)*

The UCMS consisted of a 4-week long regimen of unpredictable mild stressors according to a pre-determined "random" schedule (Ducottet et al., 2004; Surget et al., 2008). Two to three different stressors were applied per day, including forced bath (~2 cm of water in cage for 15 min), wet bedding, aversive smell (1 h exposure to fox urine), light cycle reversal or disruption, social stress (rotate mice into previously occupied cages), 45° tilted cage, restraint (50 ml falcon tube with air hole for 15 min), repeated bedding change, or no bedding. Experimental groups also had the additional stress of frequent handling by the experimenter. Control animals were group-housed to eliminate the stress of single-housing.

4.8. *Statistics*

Three way analyses of variance (ANOVA) with genotype, sex and treatment (age or UCMS) as co-factors were performed. For significant interactions, ANOVA with genotype and treatment co-factors was applied within sex groups and followed by post-hoc least-square difference (LSD) analyses. For three comparisons (WT/KO, WT/HZ and KO/HZ), the Holm-Bonferroni correction for multiple testing establishes significance at $p<0.025$ for $\alpha=0.05$. Linear fits in Fig. 5 were tested with the following linear model equation: $y_i = \alpha + \beta \cdot x_i + \varepsilon_i$, where WT: $x_i = 0$, HZ: $x_i = 1$ and KO: $x_i = 2$.

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REFERENCES

Ansorge, M.S., Zhou, M., Lira, A., Hen, R., Gingrich, J.A., 2004. Early-life blockade of the 5-HT transporter alters emotional behavior in adult mice. Science 306, 879–881.

Barr, C.S., Newman, T.K., Schwandt, M., Shannon, C., Dvoskin, R.L., Lindell, S.G., Taubman, J., Thompson, B., Champoux, M., Lesch, K.P., Goldman, D., Suomi, S.J., Higley, J.D., 2004. Sexual dichotomy of an interaction between early adversity and the serotonin transporter gene promoter variant in rhesus macaques. Proc. Natl. Acad. Sci. U. S. A. 101, 12358–12363.

Bengel, D., Murphy, D.L., Andrews, A.M., Wichems, C.H., Feltner, D., Heils, A., Mossner, R., Westphal, H., Lesch, K.P., 1998. Altered brain serotonin homeostasis and locomotor insensitivity to 3, 4-methylenedioxymethamphetamine ("Ecstasy") in serotonin transporter-deficient mice. Mol. Pharmacol. 53, 649–655.

Bodnoff, S.R., Suranyi-Cadotte, B., Aitken, D.H., Quirion, R., Meaney, M.J., 1988. The effects of chronic antidepressant treatment in an animal model of anxiety. Psychopharmacology (Berl.) 95, 298–302.

Caspi, A., Moffitt, T.E., 2006. Gene-environment interactions in psychiatry: joining forces with neuroscience. Nat. Rev. Neurosci. 7, 583–590.

Caspi, A., Sugden, K., Moffitt, T.E., Taylor, A., Craig, I.W., Harrington, H., McClay, J., Mill, J., Martin, J., Braithwaite, A., Poulton, R., 2003. Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. Science 301, 386–389.

Ducottet, C., Aubert, A., Belzung, C., 2004. Susceptibility to subchronic unpredictable stress is related to individual reactivity to threat stimuli in mice. Behav. Brain Res. 155, 291–299.

Eley, T.C., Sugden, K., Corsico, A., Gregory, A.M., Sham, P., McGuffin, P., Plomin, R., Craig, I.W., 2004. Gene-environment interaction analysis of serotonin system markers with adolescent depression. Mol. Psychiatry 9, 908–915.

Gareri, P., De Fazio, P., De Sarro, G., 2002. Neuropharmacology of depression in aging and age-related diseases. Ageing Res. Rev. 1, 113–134.

Grabe, H.J., Lange, M., Wolff, B., Volzke, H., Lucht, M., Freyberger, H. J., John, U., Cascorbi, I., 2005. Mental and physical distress is modulated by a polymorphism in the 5-HT transporter gene interacting with social stressors and chronic disease burden. Mol. Psychiatry 10, 220–224.

Hariri, A.R., Mattay, V.S., Tessitore, A., Kolachana, B., Fera, F., Goldman, D., Egan, M.F., Weinberger, D.R., 2002. Serotonin transporter genetic variation and the response of the human amygdala. Science 297, 400–403.

Heinz, A., Braus, D.F., Smolka, M.N., Wrase, J., Puls, I., Hermann, D., Klein, S., Grusser, S.M., Flor, H., Schumann, G., Mann, K., Buchel, C., 2005. Amygdala-prefrontal coupling depends on a

genetic variation of the serotonin transporter. Nat. Neurosci. 8, 20–21.
Holmes, A., Murphy, D.L., Crawley, J.N., 2003. Abnormal behavioral phenotypes of serotonin transporter knockout mice: parallels with human anxiety and depression. Biol. Psychiatry 54, 953–959.
Kalueff, A.V., Ren-Patterson, R.F., Murphy, D.L., 2007. The developing use of heterozygous mutant mouse models in brain monoamine transporter research. Trends Pharmacol. Sci. 28, 122–127.
Lerer, B., Gillon, D., Lichtenberg, P., Gorfine, M., Gelfin, Y., Shapira, B., 1996. Interrelationship of age, depression, and central serotonergic function: evidence from fenfluramine challenge studies. Int. Psychogeriatr. 8, 83–102.
Lesch, K.P., Bengel, D., Heils, A., Sabol, S.Z., Greenberg, B.D., Petri, S., Benjamin, J., Muller, C.R., Hamer, D.H., Murphy, D.L., 1996. Association of anxiety-related traits with a polymorphism in the serotonin transporter gene regulatory region. Science 274, 1527–1531.
Li, Q., 2006. Cellular and molecular alterations in mice with deficient and reduced serotonin transporters. Mol. Neurobiol. 34, 51–66.
Lim, J.E., Papp, A., Pinsonneault, J., Sadee, W., Saffen, D., 2006. Allelic expression of serotonin transporter (SERT) mRNA in human pons: lack of correlation with the polymorphism SERTLPR. Mol. Psychiatry 11, 649–662.
Lira, A., Zhou, M., Castanon, N., Ansorge, M., Gordon, J., Francis, J., Bradley-Moore, M., Lira, J., Underwood, M., Arango, V., Kung, H. F., Hofer, M.A., Hen, R., Gingrich, J.A., 2003. Altered depression-related behaviors and functional changes in the dorsal raphe nucleus of serotonin transporter deficient mice. Biol. Psychiatry 15, 960–971.
Mann, J.J., Huang, Y.Y., Underwood, M.D., Kassir, S.A., Oppenheim, S., Kelly, T.M., Dwork, A.J., Arango, V., 2000. A serotonin transporter gene promoter polymorphism (5-HTTLPR) and prefrontal cortical binding in major depression and suicide. Arch. Gen. Psychiatry 57, 729–738.
Mattson, M.P., Maudsley, S., Martin, B., 2004. A neural signaling triumvirate that influences ageing and age-related disease: insulin/IGF-1, BDNF and serotonin. Ageing Res. Rev. 3, 445–464.
Meltzer, C.C., Smith, G., DeKosky, S.T., Pollock, B.G., Mathis, C.A., Moore, R.Y., Kupfer, D.J., Reynolds III, C.F., 1998. Serotonin in aging, late-life depression, and Alzheimer's disease: the emerging role of functional imaging. Neuropsychopharmacology 18, 407–430.
Parsey, R.V., Hastings, R.S., Oquendo, M.A., Hu, X., Goldman, D., Huang, Y.Y., Simpson, N., Arcement, J., Huang, Y., Ogden, R.T., Van Heertum, R.L., Arango, V., Mann, J.J., 2006. Effect of a triallelic functional polymorphism of the serotonin-transporter-linked promoter region on expression of serotonin transporter in the human brain. Am. J. Psychiatry 163, 48–51.
Santarelli, L., Saxe, M., Gross, C., Surget, A., Battaglia, F., Dulawa, S., Weisstaub, N., Lee, J., Duman, R., Arancio, O., Belzung, C., Hen, R., 2003. Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. Science 301, 805–809.
Serretti, A., Calati, R., Mandelli, L., De Ronchi, D., 2006. Serotonin transporter gene variants and behavior: a comprehensive review. Curr. Drug Targets 7, 1659–1669.
Shioe, K., Ichimiya, T., Suhara, T., Takano, A., Sudo, Y., Yasuno, F., Hirano, M., Shinohara, M., Kagami, M., Okubo, Y., Nankai, M., Kanba, S., 2003. No association between genotype of the promoter region of serotonin transporter gene and serotonin transporter binding in human brain measured by PET. Synapse 48, 184–188.
Sibille, E., Lewis, D.A., 2006. SERT-ainly involved in depression, but when? Am. J. Psychiatry 163, 8–11.
Sibille, E., Pavlides, C., Benke, D., Toth, M., 2000. Genetic inactivation of the Serotonin(1A) receptor in mice results in downregulation of major GABA(A) receptor alpha subunits, reduction of GABA(A) receptor binding, and benzodiazepine-resistant anxiety. J. Neurosci. 20, 2758–2765.
Sibille, E., Su, J., Leman, S., Le Guisquet, A.M., Ibarguen-Vargas, Y., Joeyen-Waldorf, J., Glorioso, C., Tseng, G.C., Pezzone, M., Hen, R., Belzung, C., 2007. Lack of serotonin(1B) receptor expression leads to age-related motor dysfunction, early onset of brain molecular aging and reduced longevity. Mol. Psychiatry 12, 1042–1056.
Sjoberg, R.L., Nilsson, K.W., Nordquist, N., Ohrvik, J., Leppert, J., Lindstrom, L., Oreland, L., 2006. Development of depression: sex and the interaction between environment and a promoter polymorphism of the serotonin transporter gene. Int. J. Neuropsychopharmacol. 9, 443–449.
Surget, A., Wang, Y., Leman, S., Ibarguen-Vargas, Y., edgar, N.M., Griebel, G., Belzung, C., Sibille, E., 2009. Corticolimbic transcriptome changes are state-dependent and region-specific in a rodent model of depression and of antidepressant reversal. Neuropsychopharmacology 34, 1363–1380.
Tripp, A., Sibille, E., 2009. SERT models of emotional dysregulation. In: Kalueff, A.V., Laporte, J.L (Eds.), Experimental Models in Serotonin Transporter Research. InCambridge University Press, Cambridge, UK, pp. 105–135.
Willner, P., 2005. Chronic mild stress (CMS) revisited: consistency and behavioural-neurobiological concordance in the effects of CMS. Neuropsychobiology 52, 90–110.