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RESEARCH ARTICLE

# Increased vulnerability to psychosocial stress in heterozygous serotonin transporter knockout mice

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## SUMMARY

Epidemiological evidence links exposure to stressful life events with increased risk for mental illness. However, there is significant individual variability in vulnerability to environmental risk factors, and genetic variation is thought to play a major role in determining who will become ill. Several studies have shown, for example, that individuals carrying the *S* (short) allele of the serotonin transporter (5-HTT) gene-linked polymorphic region (5-HTTLPR) have an increased risk for major depression following exposure to stress in adulthood. Identifying the molecular mechanisms underlying this gene-by-environment risk factor could help our understanding of the individual differences in resilience to stress. Here, we present a mouse model of the 5-HTT-by-stress risk factor. Wild-type and heterozygous 5-HTT knockout male mice were subjected to three weeks of chronic psychosocial stress. The 5-HTT genotype did not affect the physiological consequences of stress as measured by changes in body temperature, body weight gain and plasma corticosterone. However, when compared with wild-type littermates, heterozygous 5-HTT knockout mice experiencing high levels of stressful life events showed significantly depressed locomotor activity and increased social avoidance toward an unfamiliar male in a novel environment. Heterozygous 5-HTT knockout mice exposed to high stress also showed significantly lower levels of serotonin turnover than wild-type littermates, selectively in the frontal cortex, which is a structure that is known to control fear and avoidance responses, and that is implicated in susceptibility to depression. These data may serve as a useful animal model for better understanding the increased vulnerability to stress reported in individuals carrying the 5-HTTLPR *S* allele, and suggest that social avoidance represents a behavioral endophenotype of the interaction between 5-HTT and stress.



## INTRODUCTION

Major depression is a severe, life-threatening and widespread psychiatric disorder (Mathers and Loncar, 2007) (see also World Health Organization report: www.who.int/chp/chronic_disease_report/en/). The link between exposure to stressful life events and increased risk for major depression has been confirmed in several studies (Risch et al., 2009; McEwen, 2007; de Kloet et al., 2005; Caspi et al., 2003; Kendler et al., 1999). Maladaptive mechanisms by which stress exposure could increase the risk for depression have been proposed, including deficient neuromodulatory homeostasis (Krishnan and Nestler, 2008); alterations of neural structure and function (McEwen, 2007; McEwen, 1999); a hyperactive hypothalamus-pituitary-adrenocortical (HPA) axis (Lupien et al., 2009; Sapolsky et al., 2000); and hyperactivity of the immune system (Dantzer et al., 2008). It has been shown that certain individuals are better able to cope with stressful life events by engaging protective psychological strategies (Feder et al., 2009; Kaufman et al., 2004). Other individuals may show resilience owing to an altered functional capacity in the adaptive molecular pathways that are engaged by stress (Feder et al., 2009). Identifying genetic risk factors that contribute to either an increased susceptibility to stress or an inability to engage successful coping strategies has the potential to uncover the molecular mechanisms involved in stress vulnerability, and could suggest novel therapeutic approaches to depression (Caspi and Moffitt, 2006; Lesch, 2004).

Recently, several studies have reported that individuals carrying the *S* (short) allele of the serotonin transporter (5-HTT) gene-linked polymorphic region (5-HTTLPR) are more likely to develop major depression following exposure to life stress (Caspi et al., 2003; Kendler et al., 2005; Pezawas et al., 2005) (but see Risch et al., 2009). The 5-HTTLPR *S* and *L* (long) alleles are associated with low and high transcriptional activity of the 5-HTT (*SLC6A4*) gene, respectively (Canli and Lesch, 2007; Lesch et al., 1996). Imaging studies have associated the *S* allele with increased neural activity in several forebrain areas during resting (Canli et al., 2006) and during the execution of emotionally arousing tasks (Dannlowski et al., 2007; Pezawas et al., 2005; Canli et al., 2005; Hariri et al., 2002; Fallgatter et al., 1999). In addition, a synergistic effect of the 5-HTTLPR *S* allele and life stress events has been reported on neural activity in multiple brain regions (Canli et al., 2006). At the same time, brain regions were also identified in 5-HTTLPR *L* allele subjects that might explain the resilience of these individuals to stress (Canli and Lesch, 2007; Canli et al., 2006). An analogous 5-HTT promoter repeat polymorphism has also been shown to interact with rearing environment to affect similar physiological and behavioral alterations in rhesus macaques (Champoux et al., 2002; Lesch et al., 1997), suggesting that 5-HTT function may play a conserved role in regulating adaptations to stress across species.

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Although mice do not carry a 5-HTT regulatory region that is orthologous to 5-HTTLPR, several studies have used 5-HTT knockout mice as a model of human allelic variation in 5-HTT function (for a review, see Murphy and Lesch, 2008). These mice show an altered ability to cope with stress, and increased anxiety and depression-like behaviors (Wellman et al., 2007; Holmes et al., 2002; Holmes et al., 2003; Jansen et al., 2010). 5-HTT knockout mutations have also been shown to moderate the adaptive response to early adverse environmental factors (Carroll et al., 2007; Heiming et al., 2009) and poor maternal care (Carola et al., 2008). The latter study used heterozygous 5-HTT knockout mice (having a 50% gene dose-dependent reduction of 5-HTT expression) and found that, although these mice did not show behavioral deficits when raised by mothers providing high maternal care, they developed increased anxiety and depression-related behavior in adulthood when raised by mothers providing poor maternal care. It was proposed that such a gene-by-environment interaction could serve as a model for the increased vulnerability to early life stress in individuals with the 5-HTTLPR *S* allele (Caspi et al., 2003).

However, most human studies have focused on the increased vulnerability of individuals carrying the 5-HTT *S* allele (Canli and Lesch, 2007) to stressful events during adulthood, and no attempts has been made in animals to model the increased vulnerability to adult chronic psychosocial stressors conferred by a partial genetic deficiency in 5-HTT. Thus, we examined the physiological and behavioral responses of heterozygous 5-HTT knockout (5-HTT+/–) mice in an established animal model of chronic psychosocial stress-induced depression-related disorders (Bartolomucci et al., 2005).

## RESULTS

### Normal psychosocial stress-induced alterations in 5-HTT+/– mice

We tested the hypothesis that male mice with a gene dose-dependent reduction of 5-HTT expression (5-HTT+/– mice) might show altered physiological and behavioral responses to chronic psychosocial stress. Psychosocial stress involved continuously housing a male mouse in a cage with a removable wire mesh barrier that allowed sensory contact with an aggressive male CD-1 mouse. Each day, the barrier was briefly removed and the mice were allowed to physically interact for up to ten minutes (Fig. 1A) (Bartolomucci et al., 2009a; Bartolomucci et al., 2004). In all cases, wild-type and 5-HTT+/– littermates were vigorously attacked by the CD-1 mouse and showed clear signs of social subordination (Fig. 1B). Importantly, the aggression received by the experimental animals during the first four days of the procedure, when the hierarchy was being established (Bartolomucci et al., 2001; Bartolomucci et al., 2004), did not correlate with aggression performed by the experimental mice during this same time period (supplementary material Fig. S1). This allowed us to conclude that the psychosocial stress experienced by the experimental mice did not depend significantly on their individual coping style.

Consistent with previous findings, stressed mice showed a significant increase in body weight gain (Fig. 1C) and a transient increase in resting body temperature (Fig. 1D) during the experimental procedure. The resting plasma concentration of corticosterone, which was determined 24 hours after the last social defeat, was also elevated in stressed mice when compared with controls (Fig. 1E). No significant effect of genotype on these measures was observed in either control or stressed mice. Moreover, acute social defeat-induced elevations in body temperature were similar in wild-type and 5-HTT+/– mice (Fig. 1F), as were the number of attacks received (Fig. 1B). These findings argue that 5-HTT gene variation does not alter the physiological consequences of chronic psychosocial stress.

### Depression of locomotor activity and increased social avoidance in 5-HTT+/– mice exposed to psychosocial stress

It has been argued that depression of locomotor activity is an animal equivalent of the locomotor disturbances and depressed mood that are observed in depressed human individuals (Bartolomucci et al., 2009a; Fuchs et al., 1996; Meerlo et al., 1996). We observed a significant reduction of home cage locomotor activity in stressed 5-HTT+/– mice, but not stressed wild-type mice, during the dark (active) phase (Fig. 2A). The circadian amplitude of locomotor activity, however, was similarly reduced in both stressed wild-type and 5-HTT+/– mice (Fig. 2B).

Social withdrawal and diminished interest in social contact are hallmarks of major depression (Stein et al., 2001). To examine social anxiety in stressed wild-type and 5-HTT+/– mice, we measured exploratory investigation of a novel open field before and after the introduction of an unfamiliar adult CD-1 male into a wire mesh container, located on one side of the arena (Berton et al., 2006) (Fig. 3A). Stressed 5-HTT+/– mice showed clear social avoidance, as evident by a reduction in the time spent in the interaction zone relative to the time spent in the corners during the presence of the intruder (preference/avoidance ratio) (Fig. 3B), and an increase in the time in the distal corners of the arena (avoidance zone) (Fig. 3C) when the intruder mouse was present. All stressed mice showed an overall decrease in time spent in the interaction zone when compared with controls (Fig. 3D), suggesting increased stress-induced novelty avoidance. 5-HTT+/– mice also showed significantly greater hyperthermia during the social avoidance test when compared with both control and stressed wild-type littermates (Fig. 3E). No effect of psychosocial stress or genotype was seen on the total distance traveled in the open arena (supplementary material Fig. S1), arguing that these effects reflected altered emotional responses to the intruder rather than changes in general exploratory drive. These findings demonstrate that, despite the normal physiological consequences of psychosocial stress, 5-HTT+/– mice show exaggerated depression of locomotor activity and social avoidance of an unfamiliar male in a novel environment.

### Severity of psychosocial stress predicts social avoidance in 5-HTT+/– mice

In humans, the risk for major depression may be proportional to the severity of stress exposure (Kendler et al., 2005; Caspi et al., 2003). Accordingly, we sought to determine whether the intensity of aggression received during daily interactions (Fig. 4A) might have affected the physiological and behavioral responses to psychosocial stress in our mice. Consistent with a measured effect of stress, the number of attacks received correlated negatively with home cage activity during the dark (active) phase (Fig. 4B), correlated positively with resting body temperature in the third week of stress (Fig. 4C), and correlated marginally with body weight gain (Fig. 4D). Importantly, social avoidance, which was measured at the end of the stress paradigm, was negatively correlated with the number of

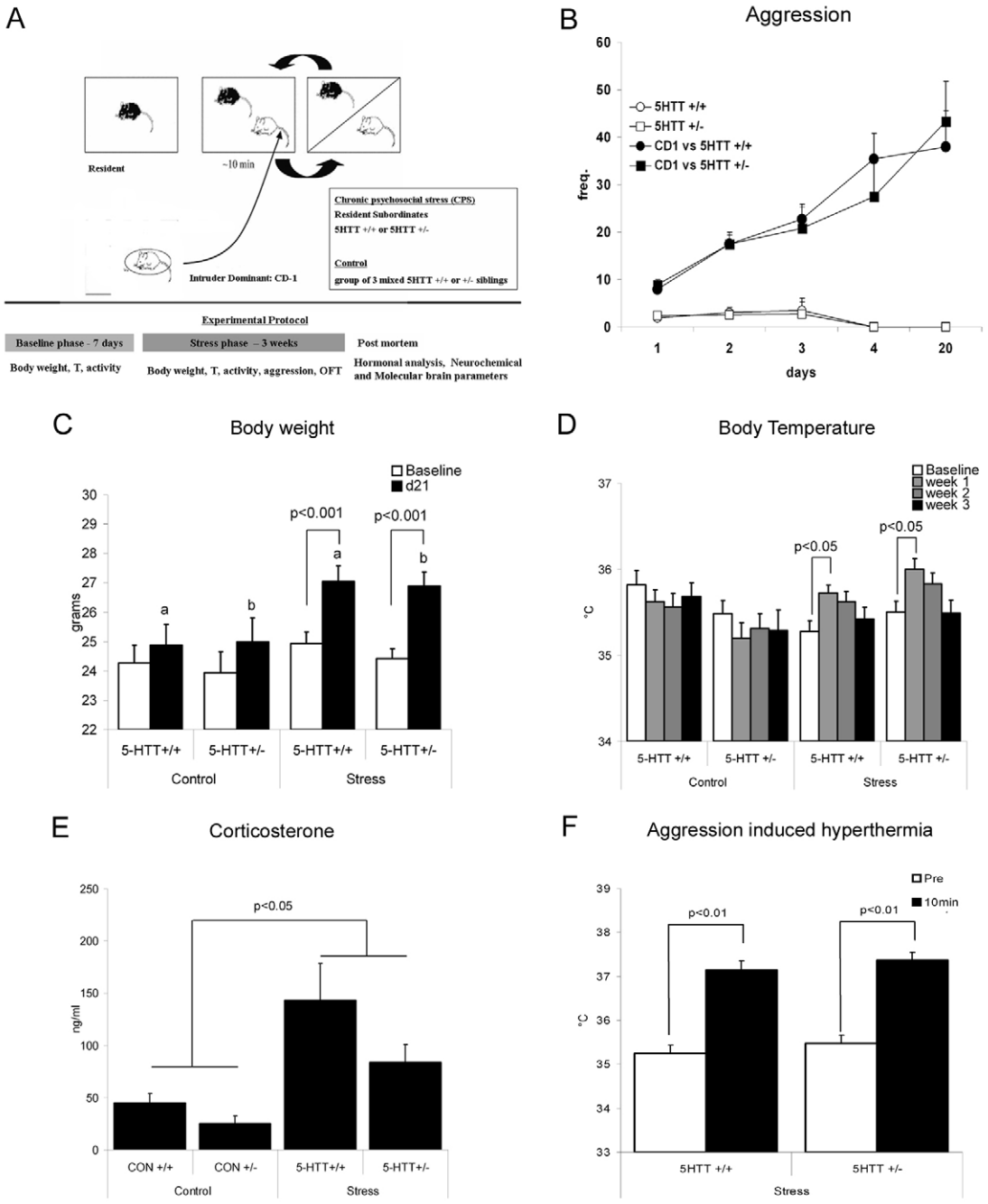


**Fig. 1. Physiological changes induced by chronic psychosocial stress.** (A) A schematic overview of the experimental procedure of chronic psychosocial stress, as well as a timetable and list of parameters assessed. T, temperature; OFT, open-field test. (B) All 5-HTT+/– and +/+ mice were reliably attacked by CD-1 intruders and, by day 4, did not show any aggressive behavior. No significant difference was observed in the mean number of attacks received or performed by 5-HTT+/+ mice and +/– mice. (C) Experimental mice showed an increased weight gain when compared with baseline or controls ($F_{(1,51)}$=18.2, $P$<0.0001; a or b indicates $P$<0.01 versus control mice with the same genotype; $n$=16-20). (D) Basal body temperature measured in the early light phase increased during the first week in stressed mice when compared with baseline ($F_{(3,147)}$=11.5, $P$<0.0001; $n$=12-20). (E) Basal corticosterone levels measured in the early light phase were increased by chronic psychosocial stress exposure ($F_{(1,30)}$=7.8, $P$<0.01; $n$=8-17). (F) Aggression-induced hyperthermia was measured during the last 5 days of the stress procedure by measuring body temperature, daily, before opening the wire mesh partition and 10 minutes after the completion of the agonistic interaction. Both 5-HTT+/+ and +/– mice showed a significant aggression-induced increase in body temperature ($F_{(1,39)}$=53.9, $P$<0.0001; $n$=14-18).

attacks received by 5-HTT+/– mice, but not wild-type mice (Fig. 4E,F). This finding suggested that the effect of the 5-HTT genotype might be greatest in animals receiving the highest aggression. To investigate this possibility, we divided the experimental mice into two groups (median split) according to the mean number of attacks received during the stress procedure (HA=high aggression, LA=low aggression) (Fig. 5A). HA mice, but not LA mice, of both genotypes showed decreased home cage activity during the stress

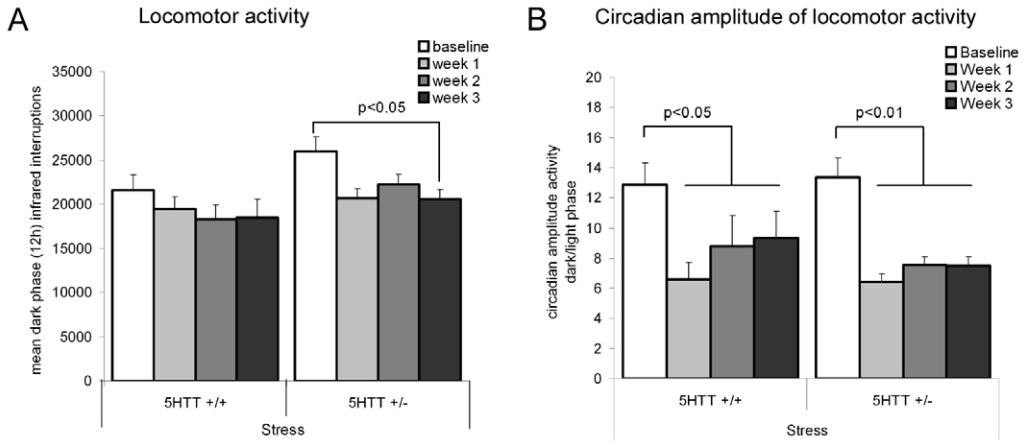


**Fig. 2. Depression of locomotor activity induced by chronic psychosocial stress.** (A) 5-HTT+/–, but not +/+, mice exposed to chronic stress showed a depression of locomotor activity during the dark phase of the third week of stress when compared with baseline ($F_{(3,102)}$=13.9, $P$<0.0001; $n$=16-20). (B) Circadian amplitude of the locomotor activity rhythm (dark phase/light phase) was similarly depressed in all mice under chronic psychosocial stress when compared with baseline ($F_{(3,102)}$=5.0, $P$<0.01; $n$=16-20).

procedure when compared with baseline (Fig. 5B). These data argue that the selective decrease of activity that was observed in the overall group of 5-HTT+/– mice, which was not observed in wild-type mice (Fig. 2A), was primarily dependent on the amount of aggression received and only to a limited extent on the gene deficiency. Consistent with this interpretation, a significant interaction between 5-HTT genotype and stress severity (HA or LA) was observed in terms of the effect on social avoidance. Whereas the severity of stress did not significantly moderate social avoidance in wild-type mice, 5-HTT+/– mice experiencing high aggression showed significantly greater social avoidance than non-stressed controls (Fig. 5C,D). These findings confirm that the 5-HTT genotype moderates the impact of psychosocial stress in a dose-dependent manner. Finally, the level of aggression received did not modulate the physiological consequences of chronic psychosocial stress exposure (data not shown).

### Decreased serotonin turnover in the frontal cortex of stressed 5-HTT+/– mice

To examine whether changes in monoamine homeostasis might explain the increased social avoidance seen in stressed 5-HTT+/– mice, we measured serotonin and norepinephrine metabolites from tissue punches of the striatum, hippocampus and frontal cortex of wild-type and 5-HTT+/– mice that were exposed to high levels of aggression (HA group). Consistent with previous data (Carola et al., 2008; Murphy and Lesch, 2008), 5-HTT+/– mice showed significantly reduced serotonin turnover (ratio of 5-hydroxyindole acetic acid to serotonin: 5-HIAA/5-HT) compared with wild-type mice in all three regions (Fig. 6). At the same time, psychosocial stress was associated with significantly reduced serotonin turnover, specifically in the frontal cortex, and stressed 5-HTT+/– mice showed significantly lower serotonin turnover than all other groups in this brain region (Fig. 6A). Notably, changes in frontal cortex serotonin turnover were determined by a combination of increased 5-HT and decreased 5-HIAA in this group (Fig. 6B,C). This finding suggests that selective changes in serotonin neurotransmission in the frontal cortex could represent a neurochemical endophenotype that increases vulnerability upon subsequent exposure to adverse stressful events.

Next, we examined whether psychosocial stress might impact serotonin homeostasis by altering the expression of 5-HTT. As expected, 5-HTT+/– mice showed decreased 5-HTT protein levels in all brain areas tested (Fig. 7). However, no effect of stress was seen on 5-HTT protein levels in any brain area, arguing that the changes in serotonin turnover seen in the frontal cortex of stressed mice (Fig. 6) were not mediated by changes in 5-HTT expression or 5-HT innervation. In addition, we observed a significant positive correlation between serotonin turnover in the frontal cortex and 5-HTT binding in the central (R=0.61, $P$<0.001) and basolateral (R=0.58, $P$<0.001) amygdala, but not in the infra- or pre-limbic (INF-PRE) frontal cortex or hippocampus (supplementary material Fig. S2). This correlation suggested that there might be a link between changes in serotonin homeostasis in the frontal cortex and the levels of serotonin transporter binding or innervation in the amygdala.

Finally, we examined the levels of norepinephrine and its metabolite in several brain regions. Like serotonin, norepinephrine is implicated in the etiology of depression and is a major target of antidepressant drugs (Gainetdinov and Caron, 2003). Psychosocial stress, but not the 5-HTT genotype, was associated with a significant decrease in norepinephrine turnover selectively in the frontal cortex (supplementary material Fig. S3). Moreover, serotonin and norepinephrine turnover were significantly correlated in all brain structures assayed (supplementary material Fig. S3), suggesting a strong coupling of these monoamine systems.

## DISCUSSION

The main finding of the present study is that exposure of adult male mice to an ethological model of chronic stress induced physiological and behavioral changes that were modulated by genetic variation in the 5-HTT (*Slc6a4*) gene. Repeated exposure to high levels of aggression coupled with constant sensory exposure to the aggressor over a period of three weeks led to physiological alterations (weight gain, hyperthermia and increased corticosterone) that were independent of 5-HTT deficiency. Although 5-HTT+/– mice showed similar physiological consequences of stress compared with wild-type littermates, they exhibited an increase in social avoidance of an unfamiliar male mouse when encountered in a novel environment. Thus, reduced 5-HTT function appeared to increase emotional responses to a social threat, while not affecting the response to chronic psychosocial stress itself.

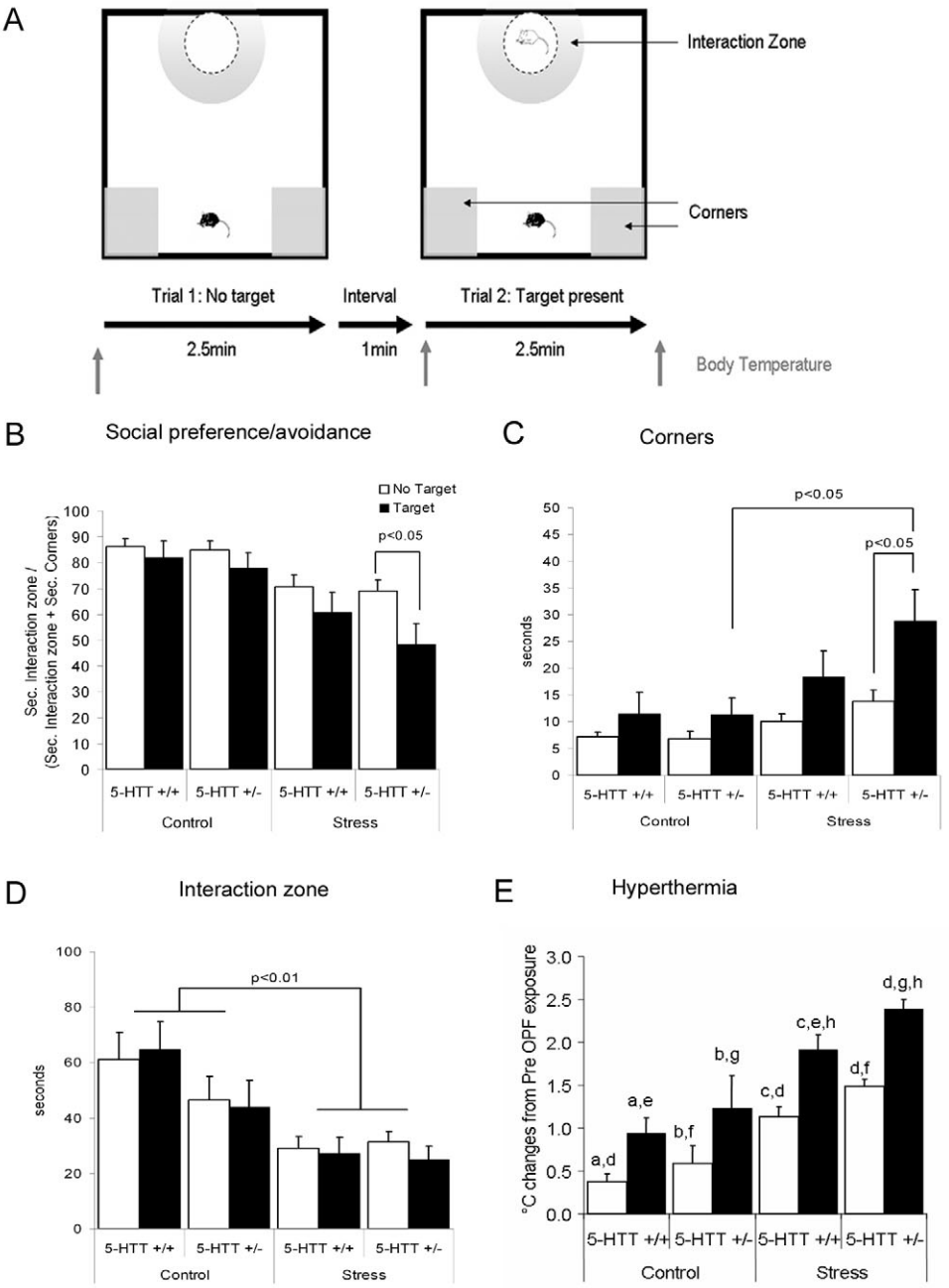


**Fig. 3. Social avoidance in stressed 5-HTT+/– mice.** (A) A schematic view of the experimental procedure and variables measured. (B) In the presence of the intruder, stressed 5-HTT+/– mice showed a significantly lower social preference/avoidance ratio ($F_{(1,50)}$=19.1, $P$<0.001) and (C) spent more time in the corners, which was considered the avoidance zone ($F_{(1,51)}$=7.9, $P$<0.01). (D) All mice exposed to chronic stress spent less time in the interaction zone than controls ($F_{(2,52)}$=2.1, $P$=0.12). (E) Immediately following behavioral testing, 5-HTT+/– mice showed the largest degree of hyperthermia, both when the intruder male was absent and present, when compared with controls and wild-type mice ($F_{(2,53)}$=31.1, $P$<0.00001; $n$=16-19; letters a-h indicate $P$<0.05 or $P$<0.01). OPF, open field.



In our experimental model, stressed mice showed a strong avoidance of the interaction zone even in the first session of the open-field test, which may be considered as a direct measure of increased anxiety and novelty avoidance (Morellini et al., 2007). Importantly, when an unfamiliar male CD-1 mouse was introduced into the arena, defeated 5-HTT+/– mice responded with an increase in time spent in the corners far away from the interaction zone (Figs 3 and 5). Overall, quantification of the relative time spent in the interaction zone (preference/avoidance ratio) demonstrated a clear preference for the area that was far from the stimulus mouse. Previous studies established that social avoidance in this behavioral test has face and predictive validity for social anxiety and depression (Berton et al., 2006; Krishnan et al., 2007). However, the parameters we used to quantify social avoidance (preference/avoidance ratio; time in the avoidance zone) do differ somewhat from the measures reported by other studies using similar social interaction tests, where avoidance/approach was measured as: (1) lower absolute time in the interaction zone when the intruder was present (Krishnan et al., 2007; Covington et al., 2009); (2) a lack of the increased time in the interaction zone, shown by controls, when the intruder male was present (Berton et al., 2006; Razzoli et al., 2009); (3) reduced time spent in the interaction zone expressed as a percentage of the time spent there when no intruder was present (in this case, controls showed the same duration of interaction when the intruder was present or absent) (Tsankova et al., 2006); and (4) increased time in the corners when the intruder male was present

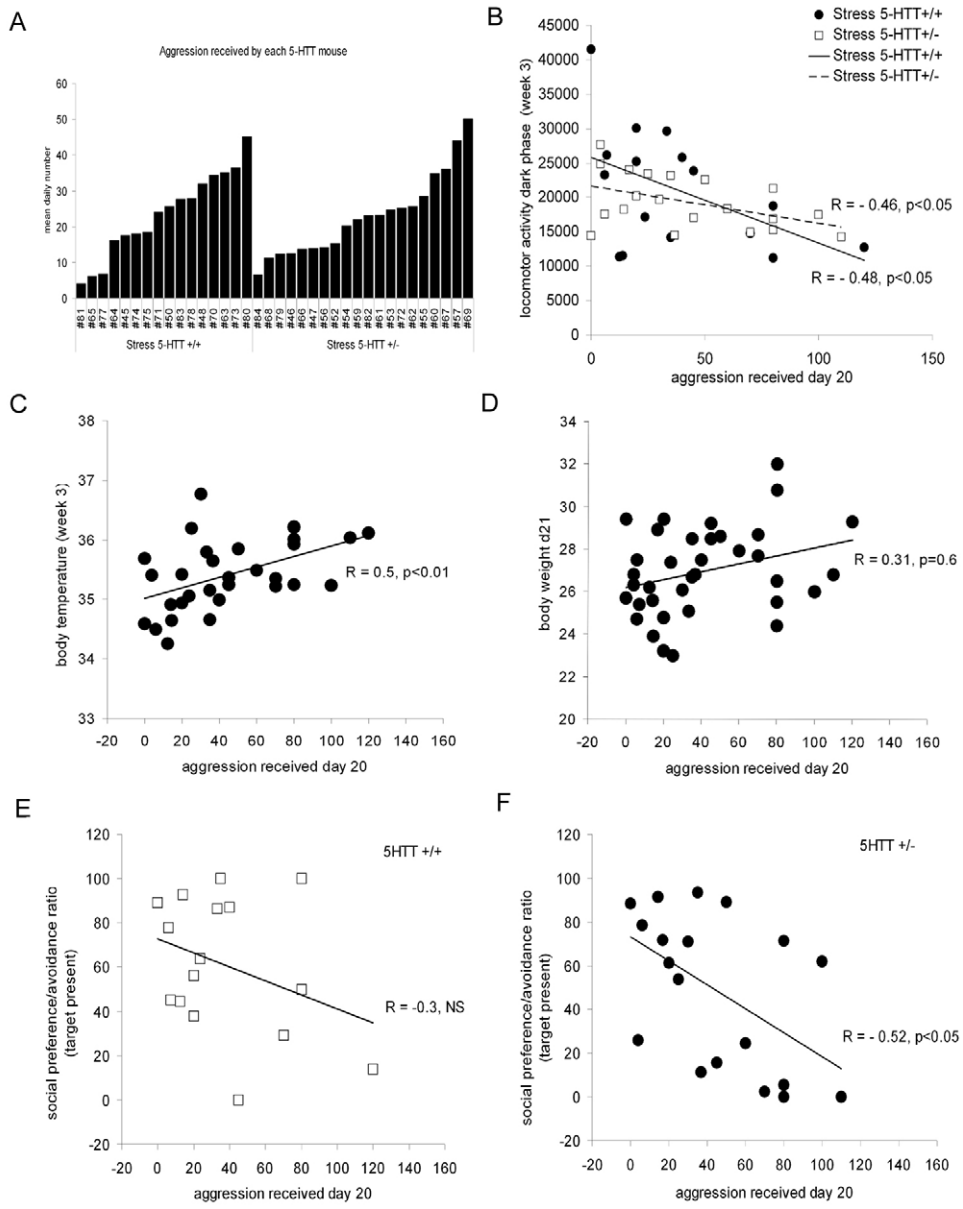


**Fig. 4. The level of aggression received predicts behavioral and physiological consequences of psychosocial stress.** (A) Mean number of daily attacks received by each individual mouse exposed to chronic psychosocial stress. (B) Average home cage activity during the last week of psychosocial stress showed a significant negative correlation with the number of attacks received by experimental animals on day 20. Day 20 was considered representative of the entire stress phase because social hierarchy is not fully established during days 1-4 (see Fig. 1B). (C) A significant positive correlation emerged between the amount of aggression received and body temperature, measured during the third week of chronic stress. (D) The mean number of daily attacks received by individual mice exposed to chronic psychosocial stress was associated mildly with final body weight. (E,F) 5-HTT+/– mice (F), but not +/+ mice (E), showed a significant negative correlation between the number of attacks received on day 20 of the stress procedure and the social preference/avoidance ratio.

(Krishnan et al., 2007). The apparent lack of a significant increase in preference/avoidance ratio in the control mice following introduction of the stimulus mouse is not surprising given the relatively lack of sociability of male mice toward unfamiliar males (Brain and Parmigiani, 1990). Therefore, we propose that increased time spent in the avoidance zone together with an overall decrease of preference/avoidance ratio (see also Krishnan et al., 2007) could be considered a valid measure of social withdrawal in this test.

Two features argue that our findings reflect a measured gene-by-environment risk factor in which genetic variation in serotonin neurotransmission moderates the long-term consequences of stress (Caspi and Moffitt, 2006; Lesch, 2004). First, the severity of social withdrawal from an unfamiliar male was directly proportional to the number of attacks received during the stress procedure in 5-HTT+/– mice only (Fig. 4). In addition, a median split analysis conducted on the average number of attacks received during the stress procedure allowed us to demonstrate that exposure to high aggression was required to elicit social avoidance in 5-HTT+/– mice (Fig. 5). This measured effect of stress suggests that social avoidance was a direct consequence of persistent neural adaptation to repeated aggression in susceptible individuals. Second, although 5-HTT+/– and wild-type mice showed differences in social interaction in a novel environment following chronic stress, they showed similar physiological and behavioral adaptations to the stress itself (Figs 1 and 3). These findings argue that the 5-HTT genotype did not moderate the immediate impact of chronic stress, but rather that it altered plastic changes in neural circuits that control the generalization of responses to potential threats.

Our neurochemical findings demonstrate that serotonin neurotransmission in the frontal cortex was influenced in a synergistic way by stress and 5-HTT genotype. Although stress had little effect on serotonin turnover in the frontal cortex of wild-type

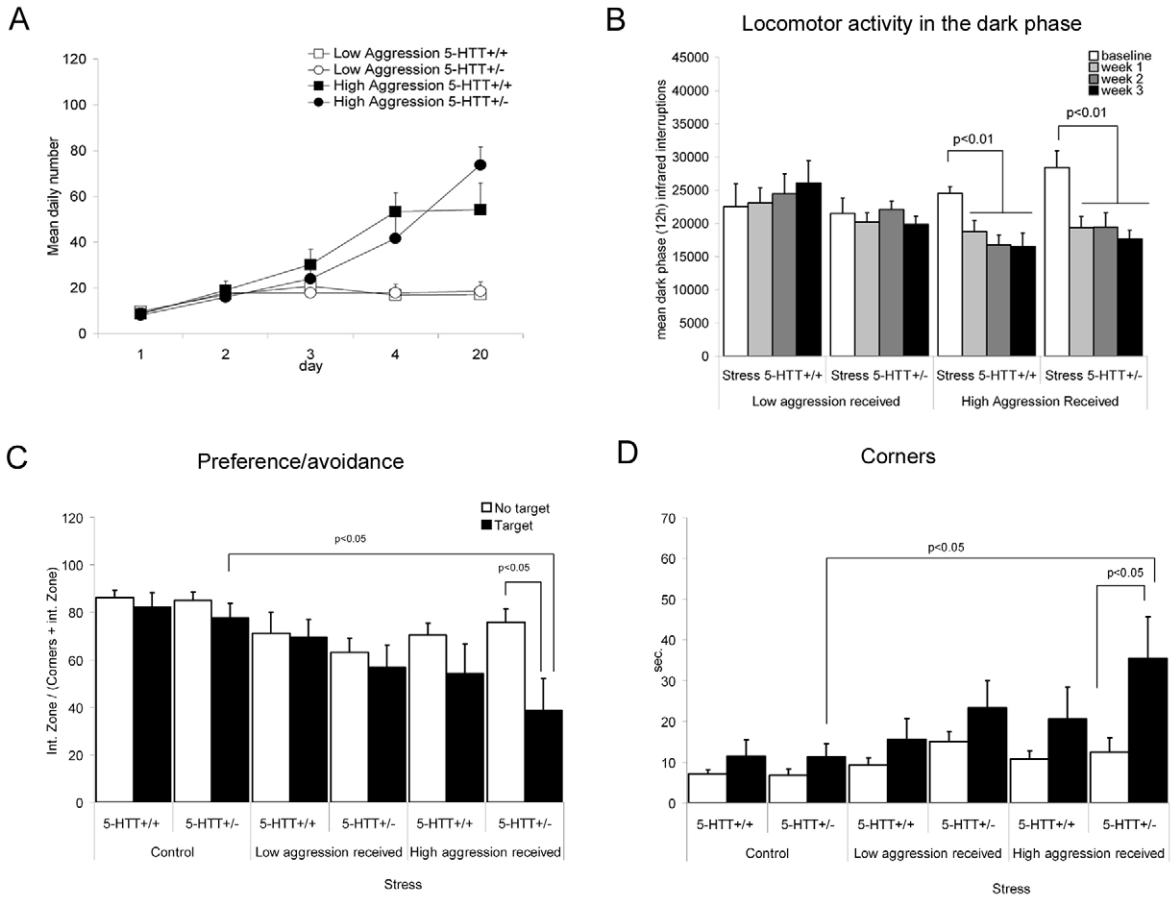


**Fig. 5. Increased social avoidance in 5-HTT+/– mice receiving a high level of daily aggression.** (A) A median split within each group was used to produce classes of mice receiving high and low aggression. (B) Mice of both genotypes receiving high, but not low, aggression showed significantly decreased home cage activity during the entire stress phase ($F_{(9,96)}$=3.4, $P$<0.01; $n$=7-10). (C,D) 5-HTT+/– mice receiving high aggression showed increased social avoidance, as evidenced by a decreased preference/avoidance ratio (C) ($F_{(5,49)}$=4.6, $P$<0.01; $n$=7-12) and an increase in the time spent in the corners (D) ($F_{(5,49)}$=2.7, $P$<0.05; $n$=7-12) when the intruder CD-1 mouse was present.

mice, it was associated with a significant reduction in stressed 5-HTT+/– mice. In 5-HTT+/– mice, lower serotonin turnover is caused by reduced synaptic re-uptake, increased extracellular serotonin levels (Daws et al., 2006; Mathews et al., 2004), and a subsequent increase in inhibitory feedback via serotonin autoreceptors (Gobbi et al., 2001). However, the mechanism behind the reduction of serotonin turnover in stressed 5-HTT+/– mice is less clear. Notably, the synergistic effects of psychosocial stress and 5-HTT genotype on serotonin turnover were driven by similar impacts on both 5-HT and its metabolite 5-HIAA (Fig. 6). However, stress did not alter 5-HTT protein levels (Fig. 7), suggesting that its effect on turnover was not caused by a simple change in 5-HTT expression.

Because increased extracellular 5-HT in the frontal cortex of 5-HTT+/– mice (Daws et al., 2006; Mathews et al., 2004) is associated with increased inhibition of pyramidal neurons (Puig et al., 2005; Hajos et al., 2003), low 5-HT turnover in these mice might result in a suppression of frontal cortex neural processing, and impairment of executive and motivational functions that are known to be exerted by this brain region (Arnsten, 2009; Wellman et al., 2007). Our data are also consistent with a model of risk for depression in which reduction of 5-HTT function leads to a failure of cortical systems to exert sufficient inhibitory control over the amygdala during stressful experiences, thereby catalyzing the development of chronic pathological states such as anxious avoidance and depression (Wellman et al., 2007; Hariri and Holmes, 2006; Pezawas et al., 2005; Puig et al., 2005; Hariri et al., 2002). Low 5-HTT expression and serotonin turnover has been observed in several brain structures, including the frontal cortex and the amygdala, in other animal models of depression (Hellweg et al., 2007; McKittrick et al., 2000). Moreover, functional magnetic resonance imaging studies found greater fear-induced amygdala responses in human carriers of the 5-HTTLPR *S* allele (Hariri et al., 2002), an effect that was enhanced by life stress exposure (Canli et al., 2006). Subsequent studies found that amygdala hyperactivity was related to functional uncoupling between amygdala and frontal cortical structures (Pezawas et al., 2005; Heinz et al., 2005), an endophenotype that is correlated significantly with trait anxiety. Thus, we speculate that defective functional connectivity between frontal cortex and downstream limbic targets plays a role in the enhanced social threat responses seen in 5-HTT+/– mice exposed to stress (Holmes, 2008).

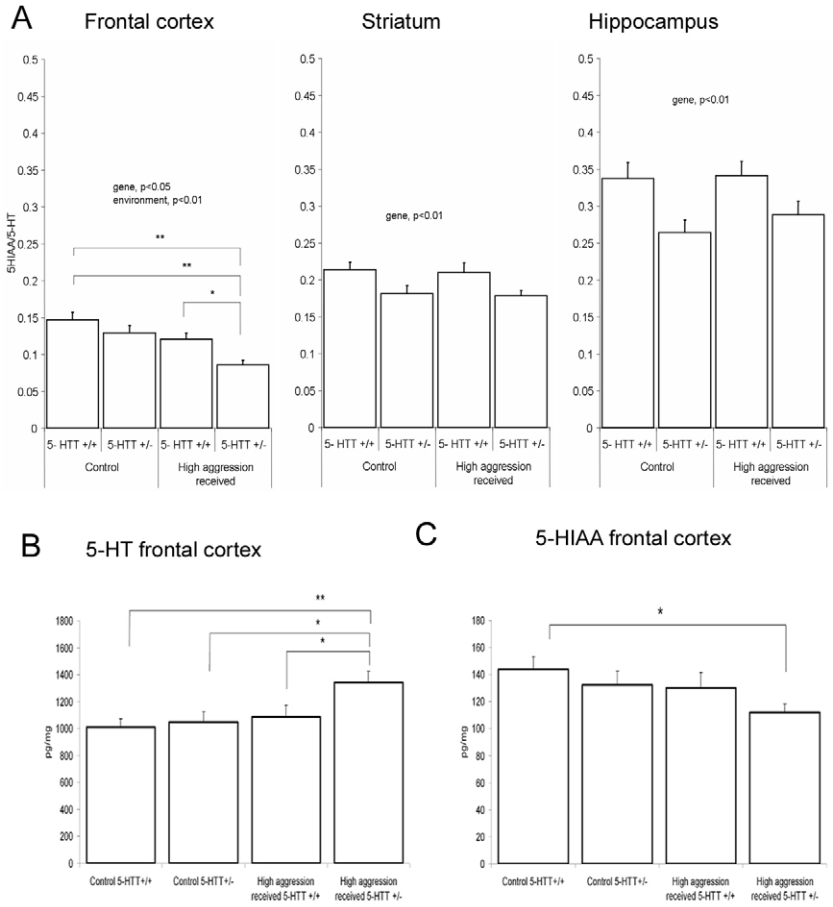


**Fig. 6. Decreased serotonin turnover in the frontal cortex of stressed 5-HTT+/– mice.** (A-C) Heterozygous 5-HTT knockout mice showed decreased serotonin turnover (5-HIAA/5-HT) in the frontal cortex ($F_{(1,31)}$=8.1, $P$<0.01), striatum ($F_{(1,31)}$=9.3, $P$<0.01) and hippocampus ($F_{(1,36)}$=9.1, $P$<0.01). However, chronic psychosocial stress caused a significant decrease in serotonin turnover in only the frontal cortex ($F_{(1,31)}$=14.1, $P$<0.001), such that stressed 5-HTT+/– mice showed a selective decrease in serotonin turnover in the frontal cortex when compared with all other groups ($n$=7-19). (B) 5-HTT+/– mice showed a selective increase in serotonin (5-HT; $F_{(1,36)}$=5.6, $P$<0.05) and (C) a decrease in the metabolite 5-hydroxyindole acetic acid (5HIAA; $F_{(4,36)}$=3.4, $P$=0.07) in the frontal cortex ($n$=8-13). *$P$<0.05; **$P$<0.001.

Several features suggest that our findings may serve as a relevant mouse model for the 5-HTTLPR-by-stress risk factor for major depression reported in some human studies (Caspi et al., 2003) (but see Risch et al., 2009). First, our use of heterozygous knockout mice that express 50% of the normal level of transporter mRNA and protein (Murphy and Lesch, 2008; Carola et al., 2008; Bengel et al., 1997) is approximately equal to the 30-50% reduction in 5-HTT mRNA that has been reported in subjects carrying the 5-HTTLPR *S* allele (Ridge et al., 2008; Lesch et al., 1996). Crucially, unlike homozygous knockout mice that have dramatic alterations in serotonin neurotransmission and behavior, heterozygous 5-HTT knockout mice have a modest but significant increase in extracellular serotonin levels under baseline conditions (Mathews et al., 2004), but show normal anxiety and depression-related behavior in the absence of stress (Carola et al., 2008; Holmes et al., 2002) or in the presence of mild stressors (Adamec et al., 2006). Similarly, 5-HTTLPR has not been associated consistently with an increased risk for depression in the absence of stressful life events (Kendler et al., 2005; Caspi et al., 2003). Second, our psychosocial stress procedure, in which a mouse was housed in continuous sensory contact with a dominant male interspersed by direct social defeats, is likely to tap into similar social stress mechanisms to those implicated in human depression (Bartolomucci, 2007; Sapolsky, 2005). Indeed, sustained suppression of locomotor activity, body weight gain, increased corticosterone level and hyperthermia are found both in animals exposed to chronic psychosocial stress and individuals with major depression (Bartolomucci et al., 2009a; Fuchs et al., 1996; Meerlo et al., 1996; Bartolomucci, 2007). Moreover, the social avoidance exhibited by stressed 5-HTT+/– mice may be related to the decreased motivation for social interaction, or increased fear of social threat, seen as a syndromal dimension of depression (Berton et al., 2006; Stein et al., 2001). Despite determining a subset of depression-related phenotypes, altogether, our findings point to an association between individual variation in serotonin homeostatic set point and behavioral endophenotypes linked to social coping strategies and risk for depression (Watson et al., 2009; Domschke et al., 2009; Brune et al., 2006; Gould and Gottesman, 2006; Jacob et al., 2004; Hasler et al., 2004). By studying animal models of the 5-HTTLPR risk factor, we may be able to identify the neural and behavioral circuits that are involved, and produce translatable hypotheses to guide human epidemiological studies.

Finally, the findings of this study are complementary to our earlier experiments showing that 5-HTT+/– mice are more susceptible to the increased anxiety and depression-related behavior seen in mice following exposure to poor maternal care during development (Carola et al., 2006; Carola et al., 2008). However, the different common neural substrates of gene and environment that were identified in the two studies suggests that different molecular circuits are involved in the genetic moderation of early and adult stress by 5-HTT. These differences suggest that genetically driven variation in serotonin function has pleiotropic effects on brain

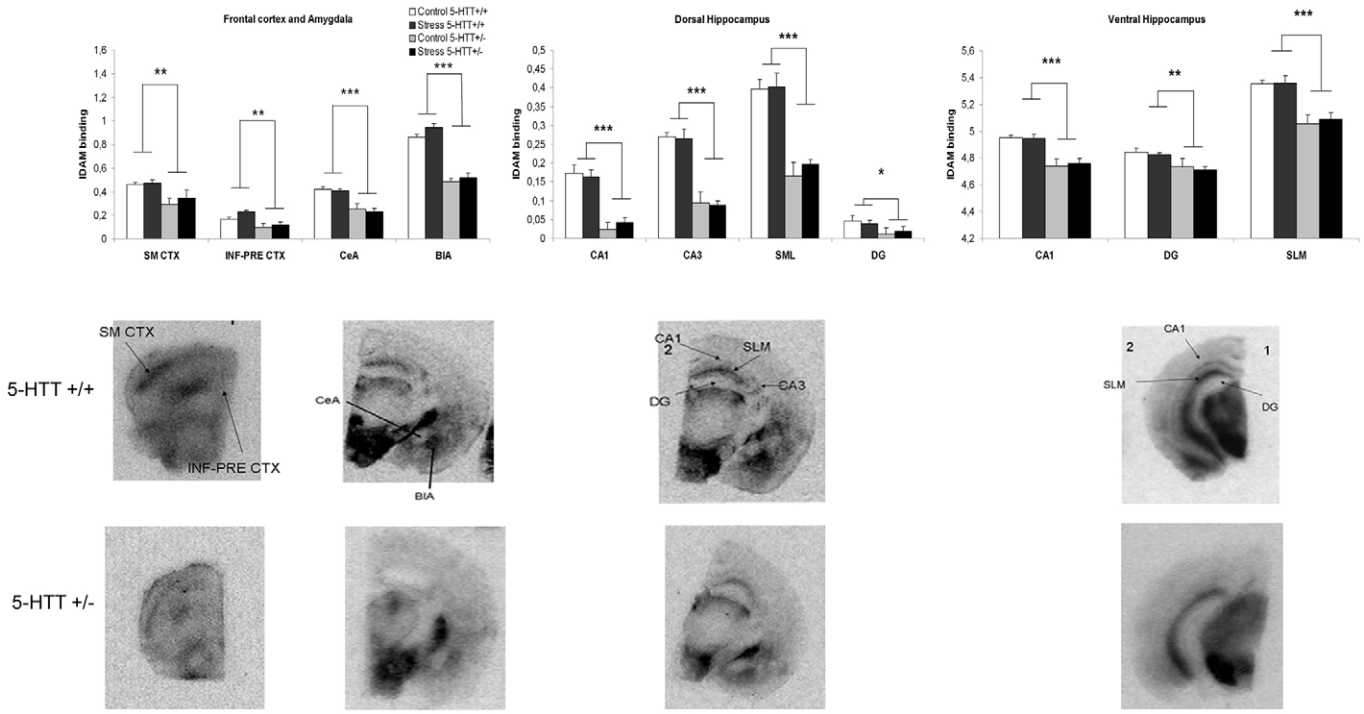


**Fig. 7. Effect of genotype and stress on 5-HTT binding.** The level of 5-HTT protein, which was determined by ligand autoradiography ($^{125}$IDAM binding) in tissue sections, was lower in 5-HTT+/– mice compared with wild-type mice. No interaction between genotype and stress exposure emerged. SM CTX, somatomotor cortex; INF-PRE CTX, inferior-prelimbic cortex; CeA, central amygdala; BIA, basolateral amygdala; CA, cornu ammonis; DG, dentate gyrus; SLM, stratum lacunosum moleculare. *n*=5-8. **P*<0.05; ***P*<0.01; ****P*<0.001.

function that can moderate multiple environmentally responsive neural circuits, and is consistent with the broad serotonergic modulation of neural circuits seen in functional brain imaging studies of individuals with the 5-HTTLPR *S* allele (Canli et al., 2006; Fallgatter et al., 1999).

In conclusion, our findings add to the growing body of data arguing that genetic variation in 5-HTT alters adaptive responses to environmental challenges that influence social behavior and contribute to the risk for psychiatric disorders. Our data serve as a mouse model of the human 5-HTTLPR-by-stress risk factor for major depression and other disorders of emotional regulation with high face and construct validity, and point to alterations in frontal cortical serotonin homeostasis in its etiology.

## METHODS

### Animals

Wild-type and heterozygous male 5-HTT knockout littermates were produced by mating wild-type females (C57BL/6J, JAX mice; Charles River Italia, Calco, Italy) and heterozygous 5-HTT mice [B6.129(Cg)-*Slc6a4*$^{tm1Kpl}$/J] (Bengel et al., 1998). Male Swiss CD-1 mice were from an outbred stock that was originally obtained from Charles River Italia and maintained at the University of Parma. Mice were born and reared at the University of Parma in a 12-hour light-dark cycle (lights on at 07:00 h) and maintained at 22±2°C. After weaning on postnatal day 21 (day 28 for CD-1 mice), mice were housed in same-sex groups of siblings (3-6 per cage) in plexiglass cages (38 × 20 × 18 cm) with wood shaving bedding that was changed weekly. All animal experimentation was conducted in accordance with the European Communities Council Directive of November 24, 1986 (86/EEC), and was approved by the ethical committees of the University of Parma and the Italian Institute of Health.

### Chronic psychosocial stress

The procedure was a modified version of our standard procedure (Bartolomucci et al., 2009a; Bartolomucci et al., 2004). Wild-type or 5-HTT+/– littermate males were used as residents and CD-1 male mice of the same age were used as intruders. CD-1 males were selected as intruders because of their clear competitive advantage over C57BL/6J mice (Parmigiani et al., 1999; Bartolomucci et al., 2009b), and our experience is that, most if not all, C57BL/6J mice become subordinates when repeatedly exposed to CD-1 males. Three-month-old male mice were housed individually in plexiglass cages (38 × 20 × 18 cm) for a 7-day baseline period. The 3-week stress procedure started on day 7 with each resident mouse receiving a CD-1 intruder mouse in its cage for 10 minutes. After the interaction, the two animals were separated by means of a wire mesh partition, which allowed continuous sensory contact but no physical interaction. Between 10:00 h and 12:00 h, the partition was removed daily for a maximum of 10 minutes. To prevent injuries, the mice were separated by the partition if fighting escalated (when the dominant mouse persistently bit the opponent). Body weight was monitored weekly. Food and water were always available ad libitum. The number of attack bouts performed by each animal was quantified by direct observation (Bartolomucci et al., 2004). Age-matched 5-HTT+/–

or +/+ mice housed in groups of three littermates were included as the non-stressed control group. Stressed and control males were littermates to reduce the possible confounds of inter-litter variability. Our choice of controls was based on previous experiments showing that, despite being grouped, siblings maintain normal social behavior and social hierarchy, and show no metabolic, immune, endocrine or behavioral evidence of stress activation or anxiety (for details, see Bartolomucci et al., 2001; Bartolomucci et al., 2002; Bartolomucci et al., 2003; Bartolomucci et al., 2004).

### Home cage phenotyping

Body temperature was recorded using temperature-sensing subcutaneous transponders (Bio Medic Data Systems, Seaford, DE). Sensors were implanted at least 15 days before the beginning of the experiment. The assessment of individual locomotor activity in the home cage was carried out by means of an automated system that uses small passive infrared sensors positioned on the top of each cage (TechnoSmart, Rome, Italy), as described previously (Bartolomucci et al., 2009a).

### Social approach test

The test was performed between 16:00 h and 19:00 h on day 20 of the stress procedure. The experimental mouse was introduced into a square open field (54 × 54 cm) for two consecutive sessions of 2.5 minutes each (Fig. 3) (adapted from Berton et al., 2006). During the first session (T1, 'No target') the open field contained an empty wire mesh cage (10-cm diameter) located at one end of the field. During the second session (T2, 'Target'), an unfamiliar CD-1 male mouse of the same age was introduced into the cage. Between sessions, the experimental mouse was placed back into its home cage for approximately one minute. Locomotion was quantified with a video tracking system (Ethovision, Noldus, Wageningen, Netherlands). Total dwell time, frequency of entry and latency to enter were all determined for the interaction area (an 8-cm-wide ring region around the target cage) and the corners of the open field (8-cm-square regions in the corners at the opposite end of the open field to the target cage). A social preference ratio was computed as: time in interaction zone/(time in interaction zone + time in corners).

### Plasma corticosterone

On day 21 of the stress procedure, mice were not exposed to social defeat and were sacrificed between 10:00 h and 12:00 h. Trunk blood was collected in heparin-coated tubes, centrifuged at 4000 rpm for 10 minutes at 4°C, and plasma was then frozen at –20°C for later corticosterone analysis by radioimmunoassay (ICN, Orangeburg, NY; sensitivity 0.017 ng/ml; the intra-assay coefficient was 6.1%).

### Neurochemical analysis and IDAM binding

After sacrifice (decapitation following brief $CO_2$ exposure), brains were immediately collected on ice and snap frozen. Neurochemical analyses were carried out as reported previously (Puglisi-Allegra et al., 2000). Briefly, punches were obtained from brain slices (coronal sections) that were no thicker than 300 µm. Stainless steel tubes with an inside diameter of 1.0 or 1.5 mm were used. The coordinates were measured according to Sidman et al. (Sidman et al., 1971) as follows: frontal cortex, two slices from section 80 to section 130 (1.5-mm tube); caudate putamen, four slices from section 151 to section 230 (1.5-mm tube); hippocampus, three slices from section 301 to section 350 (1.0-mm tube; including CA1, CA2 and CA3 fields). The punches were stored in liquid nitrogen until the day of analysis when they were weighed and homogenized in 0.05 M $HClO_4$. The homogenates were centrifuged at 14,000 rpm for 20 minutes at 4°C. Aliquots of supernatant were transferred to a reverse-phase high-performance liquid chromatography (HPLC) system for analysis. The tissue levels of 5-hydroxytryptamine (5-HT), norepinephrine (NE) and their metabolites [5-hydroxyindoleacetic acid (5-HIAA) and 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG)] were assessed simultaneously by using an HPLC system consisting of an Alliance (Waters, Milford, MA) system and a coulometric detector (ESA Model 5200A Coulochem II, ESA, Chelmsford, MA) with a 5011 high-sensitivity analytical cell and a 5021 conditioning cell. The

## TRANSLATIONAL IMPACT

### Clinical issue

Major depression (MD) is a severe, life-threatening and widespread psychiatric disorder. The link between exposure to stressful life events and increased risk for MD has been confirmed in several studies. Transition from stress exposure to MD may be gradual and the degree of individual vulnerability may be explained by genetic predisposition. *SLC6A4* is a gene associated with depression that encodes for the serotonin transporter (5-HTT). Within *SLC64A*, the 5-HTT gene-linked polymorphic region (5-HTTLPR) is of particular medical interest. The 5-HTTLPR is expressed in humans and non-human primates with two alleles, *S* and *L*, which are associated with low and high transcriptional activity, respectively. Individuals carrying the *S* allele are more likely to develop MD following exposure to life stress and to respond poorly to antidepressant treatments.

### Results

Here, the authors define a mouse model to understand the influence of 5-HTT on the stress response and susceptibility to depression. Their 5-HTT heterozygous mouse shows a 50% reduction of 5-HTT expression, which recapitulates the 50% reduction of 5-HTT that occurs in humans with the 5-HTTLPR *S* allele, relative to *L* allele carriers. Exposure of adult male mice to an ethological model of chronic stress over a period of three weeks led to physiological alterations, including weight gain, hyperthermia and increased corticosterone, which are compatible with changes observed in MD patients. These characteristics were independent of 5-HTT deficiency. However, 5-HTT heterozygous mice experience increased suppression of locomotor activity and an increase in social avoidance. This is similar to the psychomotor disturbances and social withdrawal characteristic of people with MD. Importantly, these behavioral changes reflect the amount of stress experienced: higher stress causes greater depression-like behavioral disorders. Stressed 5-HTT heterozygous mice also show reduced serotonin turnover in the frontal cortex, which was not associated with an additional modulation in 5-HTT protein expression.

### Implications and future directions

This mouse model at least in part recapitulates both the genetic and behavioral changes associated with MD in humans through mutation of 5-HTT. It should prove valuable for subsequent studies to identify the mechanisms by which reduced 5-HTT levels may predispose individuals to develop MD. Further work should reveal the molecular effectors that unbalance serotonin transmission in the frontal cortex and establish a functional link with the depression disorders. These findings may open a new venue of research for therapeutic interventions in depressed 5-HTTLPR *S* allele carriers who are resistant to treatment.

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potentials were set at –350 mV and +250 mV, respectively. The columns were a Nova-Pack Phenyl column (3.9 × 50 mm) and a Sentry Guard Nova-Pack pre-column (3.9 × 20 mm; Waters). The flow rate was 1 ml/min. The mobile phase consisted of 5% methanol in 0.1 M Na-phosphate buffer, pH 2.5, 0.1 mM EDTA and 1.5 mM 1-octane sulphonic acid Na salt (Sigma-Aldrich, Milan, Italy). 5-HTT protein expression levels were determined by quantitative autoradiographic binding to frozen brain tissue sections of the left hemisphere using $^{125}$I-IDAM, a kind gift of Karl Ploessl and Hank Kung, as described previously (Gross et al., 2002).

### Statistical analysis

Data were analyzed with analysis of variance (ANOVA) followed by Tukey's HSD post hoc test, which can also be used in case of a non-significant F value (Wilcox, 1987). Correlations were performed with the parametric Pearson test.

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#### COMPETING INTERESTS

The authors declare no competing financial interests.

#### AUTHOR CONTRIBUTIONS

The project was initiated as a collaboration between A.B., V.C. and C.G. A.B. designed the physiological and behavioral experiments with the help of V.C., C.G., P.P. and S.P.; A.B. carried out all physiological and behavioral procedures, and analyzed the resulting data; V.C. processed tissue samples for autoradiography and corticosterone analysis; S.P.-A. processed samples for HPLC analysis; T.P. performed HPLC analysis; S.C., S.P.-A. and T.P. analyzed HPLC data; K.-P.L. provided reagents; A.B. and C.G. wrote the manuscript with input from other authors.

#### SUPPLEMENTARY MATERIAL

Supplementary material for this article is available at
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