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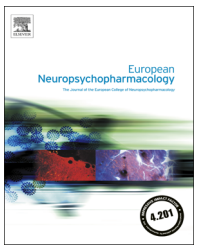

Lifelong disturbance of serotonin transporter functioning results in fear learning deficits: Reversal by blockade of CRF_1 receptors

Elisabeth Y. Bijlsma[a,*], Hendrikus Hendriksen[a], Johanna M.P. Baas[b], Mark J. Millan[c], Lucianne Groenink[a]

[a]*Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences and Brain Center Rudolf Magnus, Utrecht University, The Netherlands*
[b]*Department of Experimental Psychology and Psychopharmacology, Utrecht University, The Netherlands*
[c]*Institut de Recherches Servier, Croissy-sur-Seine, France*

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Abstract
The inability to associate aversive events with relevant cues (i.e. fear learning) may lead to maladaptive anxiety. To further study the role of the serotonin transporter (SERT) in fear learning, classical fear conditioning was studied in SERT knockout rats ($SERT^{-/-}$) using fear potentiation of the startle reflex. Next, fear acquisition and concomitant development of contextual conditioned fear were monitored during training. To differentiate between developmental and direct effects of reduced SERT functioning, effects of acute and chronic SSRI treatment were studied in adult rats. Considering the known interactions between serotonin and corticotropin-releasing factor (CRF), we studied the effect of the $CRFR_1$ antagonist CP154,526 on behavioral changes observed and determined CRF_1 receptor levels in $SERT^{-/-}$ rats. $SERT^{-/-}$ showed blunted fear potentiation and enhanced contextual fear, which resulted from a deficit in fear acquisition. Paroxetine treatment did not affect acquisition or expression of fear-potentiated startle, suggesting that disturbed fear learning in $SERT^{-/-}$ results from developmental changes and not from reduced SERT functioning. Although CRF_1 receptor levels did not differ significantly between genotypes, CP154,526

*Corresponding author. Tel.: +31 6 10031691.
E-mail address: e.y.bijlsma@uu.nl (E.Y. Bijlsma).

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treatment normalized both cue- and contextual fear in SERT$^{-/-}$ during acquisition, but not expression of fear-potentiated startle. The disrupted fear acquisition and concomitant increase in contextual conditioned fear-potentiated startle fear in SERT$^{-/-}$ resembles the associative learning deficit seen in patients with panic disorder and suggests that normal SERT functioning is crucial for the development of an adequate fear neuro-circuitry. Moreover, the normalization of fear acquisition by CP154,526 suggests a role for central CRF signaling in the generalization of fear.

1. Introduction

Classical fear conditioning is the process by which a previously neutral stimulus comes to evoke fear following its repeated pairing with an aversive unconditioned stimulus. The inability to learn these fear contingencies results in unpredictability of the aversive event and consequently in maladaptive fear, reflected in enhanced contextual fear (Baas et al., 2008; Grillon, 2002). Literature suggests that this type of associative learning deficit plays a crucial role in the development of several anxiety disorders, including panic disorder (Lissek et al., 2009). The serotonin system is involved in fear regulation (Burghardt et al., 2004; Grillon et al., 2007a). In addition, serotonin has been implicated in both the pathology and the treatment of panic disorder. First, selective serotonin re-uptake inhibitors (SSRIs), acting on the serotonin transporter, are medication of choice for panic disorders (Andrews and Hunt, 1998; Romano et al., 2004). Further, panic disorder has been associated with a polymorphism in the serotonin transporter gene (SLC6A4) (Strug et al., 2010) increased serotonin turnover (Esler et al., 2007) and both decreased and increased serotonin transporter availability (Maron et al., 2004; Maron et al., 2011).

Another important mediator in fear learning is the neuropeptide corticotropin-releasing factor (CRF). For example, local repeated administration of CRF into the basolateral amygdala potentiates the acquisition of cue-conditioned fear (Bijlsma et al., 2011) and CRF_1 receptor antagonists effectively block the acquisition and expression of contextual conditioned fear (Hubbard et al., 2007; Walker et al., 2009).

Several studies suggest direct interactions between serotonin and CRF in the regulation of anxiety-like responses (Lukkes et al., 2009; Meloni et al., 2008). In addition, central administration of CRF decreases activity of serotonin neurons in the raphe and serotonin release in forebrain regions in a dose-dependent manner (Kirby et al., 2000; Price and Lucki, 2001). Interestingly, a recent study within our department showed that interactions between serotonin transporter and CRF_1 receptor polymorphisms are associated with deficient associative fear learning in healthy subjects (Heitland et al., 2013). Together, these studies suggest that especially the interplay between these two brain systems may be important for adequate fear learning.

This study aimed at further studying the role of the serotonin transporter in classical fear conditioning deficits using a SERT knockout (SERT$^{-/-}$) model in rats. This SERT$^{-/-}$ rat was created by N-ethyl-N-nitrosurea (ENU)-driven target-selected mutagenesis resulting in a premature stop codon (Smits et al., 2006). This premature stop codon results in a complete ablation of SERT in the SERT$^{-/-}$ rat (Homberg et al., 2007a). This SERT$^{-/-}$ rat shows selective disturbances in 5-HT homeostasis, including nine-fold higher extracellular 5-HT levels in the hippocampus and decreased intracellular availability of 5-HT (Homberg et al., 2007a). Behaviorally, the SERT$^{-/-}$ rat shows increased anxiety-like behavior in exploration-driven paradigms (Olivier et al., 2008), decreased memory performance in an object recognition paradigm (Olivier et al., 2009), but improved inhibitory control (Homberg et al., 2007b). Here we studied classical fear conditioning in SERT$^{-/-}$ rats by measuring potentiation of the acoustic startle response (i.e. fear-potentiated startle), a robust measure of defensive states in both humans and rodents (Bijlsma et al., 2011; Grillon, 2008). Recently, it was reported that panic disorder patients show an associative fear learning deficit in this fear-potentiated startle paradigm, resulting fear-like responding to safety cues (Lissek et al., 2009). To differentiate between developmental and direct effects of reduced SERT functioning, the effect of pharmacological SERT inhibition in adulthood on the acquisition and expression of fear-potentiated startle were studied following acute and chronic paroxetine treatment in Wistar rats. In addition, because of above mentioned interactions between serotonin and CRF and the putative inhibitory effects of $CRFR_1$ antagonists on contextual conditioned fear, we studied changes in CRF_1 receptor levels in SERT$^{-/-}$ rats and tested the hypothesis that the $CRFR_1$ antagonist CP154,526 was able to normalize the fear learning deficits found.

2. Experimental procedures

2.1. Subjects

SERT knockout rats (Slc6a4 [1Hubr]) on a Wistar rat genetic background were generated by ENU-driven mutagenesis (Smits et al., 2006). All males were derived from crossings between heterozygous (SERT$^{+/-}$) rats and genotyped as described previously [36]. In Experiments 1, 2, 4 and 5, SERT$^{+/+}$, SERT$^{+/-}$ and SERT$^{-/-}$ rats were compared. Rats were housed in groups of four, with mixed genotype. In Experiment 3, male Wistar rats were used (Harlan, Zeist, the Netherlands), which were housed in groups of four and were allowed to acclimate to the facilities for two weeks before the start of the experiment. All animals were housed in a temperature (21 °C ±2), humidity (55% ±5), and light controlled environment (lights on from 6 a.m. to 6 p.m.). Food and water were freely available in the home cages. The experiments were carried out during the light phase of the day-night cycle between 8 a.m. and 4 p.m. This study was approved by the ethical committee of the Academic Biomedical Center (DEC-ABC), Utrecht University, The Netherlands.

2.2. Startle apparatus

Eight startle devices were used simultaneously (SR-lab, San Diego instruments, San Diego CA, USA). The startle devices consisted of a Plexiglas cylinder (9 cm diameter and 20 cm length) placed on a Plexiglas base in a ventilated sound-attenuated cubicle. During the FPS experiments this startle device was equipped with a stainless steel grid floor. Cage movements were measured with a piezoelectric film attached to the Plexiglas base of the startle device. Startle stimuli (50 ms white-noise bursts) were presented through a piezoelectric tweeter situated 15 cm from the top of the cylinder. Background noise was set at 70 dB. Startle amplitudes were sampled each ms during a period of 65 ms beginning at the onset of the startle stimulus. The startle response was measured as the maximum peak-to-though waveform during the 65 ms period. Each startle device was equipped with a stimulus light (180 lx) in the ceiling situated 15 cm from the top of the cylinder for FPS conditioning. There was no background illumination in any of the experiments.

2.3. Expression of fear-potentiated startle

Animals were trained for 2 consecutive days. During both training sessions, animals were presented with 10 cue-shock pairings (0.6 mA during the last 500 ms of the 3700 ms light cue) at an average interval of 4 min (range: 3–5 min). 24 h later, rats were tested for fear expression using the FPS test. Test procedure was as follows: After an acclimation period of 5 min, 10 habituation stimuli were presented (105 dB), followed by 40 startle stimuli (20×100 and 105 dB, ISI 30 s). Half of the 40 startle stimuli were presented during the last 50 ms of a 3250 ms light cue (cued); the other half were delivered in darkness (non-cued). Expression of fear-potentiated startle was analyzed both on basis of absolute startle values as well as percentage potentiation (percentage fear-potentiated startle: [(cued – non-cued)/non-cued * 100%].

2.4. Fear acquisition

Acquisition of fear-potentiated startle was assessed during an adjusted training session. To optimally study fear learning, and its modulation by pharmacological treatment, over the course of training startle responses to cued and non-cued trials was assessed during training. To this end, animals were exposed to two startle stimuli (cued and non-cued, 105 dB) before the first cue-shock pairing and in between the other cue-shock pairings. To visualize the training effect in Experiment 2, responses from each subject to cued and non-cued trials during fear acquisition training were collapsed into three phases: The initial phase (cued and non-cued response before the first cue-shock pairing); '1st phase' (cued and non-cued response during the first set of 5 cue-shock pairings); and'2nd phase' (cued and non-cued response during the second set of 5 cue-shock pairings).

2.5. Drugs

Paroxetine hydrochloride hemi-hydrate (10 and 20 mg/kg, Pharmacy Mediq, Bergen op Zoom) was dissolved in water and administered by daily injections (*p.o.*) in a volume of 2 ml/kg.

CP154,526 (butyl-ethyl-(2,5-dimethyl-7-(2,4,6-trimethylphenyl)-7H-pyrrolo(2.3-d)pyrimidin-4-l)amine)-HCl; gift from Servier, Croissy/Seine France) was suspended in 2% Tween80 in distilled water and administered intraperitoneally in volume of 2 ml/kg 30 min before FPS training (Experiment 4B, 10 and 30 mg/kg) or test (experiment 5, 10 mg/kg).

2.6. In situ hybridization

CRF 1 receptor mRNA levels in basolateral amygdala (bilateral, Bregma −2, 30, Paxinos) and dorsal raphe nucleus (Bregma −7, 30, −7, 64, Paxinos) were determined in drug-naive $SERT^{+/+}$ and $SERT^{-/-}$ rats. Rats were decapitated and brains were removed and stored at −80 °C until further use. Brains were sliced into coronal sections, sections were directly placed onto SuperFrost plus slides (Gerhard Menzel GmbH, Germany) and immediately frozen at −80 °C until further use. For in situ hybridization, the RNAscope RED 2.0 FFPE assay (Advanced Cell Diagnostics Inc., Hayward, CA) was performed as described in the user manual (Rev.20121022). Specific probes targeted at CRFR1 (NM_030999.3, catalog 318931), a positive control (housekeeping gene Peptidyl-prolyl cis-trans isomerase B (PPIB NM_022536), and the bacterial gene dihydrodipicolinate reductase (dapB; EF191515) as a negative control were obtained from Advanced Cell Diagnostics Inc., Hayward, CA (Wang et al., 2012).

Analyses of expression levels: The slides were analyzed with a bright-field microscope (Olympus, BX50). The pictures were made with a Leica camera (type DFC320) and the counting was performed using Image-Pro Plus version 630.0.512 (1993–2008, Media Cybernetics, Inc.) for Windows XP/Vista. Expression levels represented by red dots were manually counted twice for each area by an observer who was blind for genotype. For the amygdala, four or five squares of 8934 μm^2 (95.91 $\mu m \times 93.15$ μm), spread over the area, were counted. For the dorsal RN, three rectangles of 31,244 μm^2 (258.75 $\mu m \times 120.75$ μm) were vertically placed under the aquaduct and counted using the same strategy as for the BLA.

2.7. Overview of experiments

All experiments were carried out in separate experimental groups. 4 days before the start of an experiment, animals were habituated to the startle set-up during a habituation session (30 startle stimuli (10×100, 105 and 115 dB, ISI 30 s)). Three intensities were used during this habituation session in order to capture individual differences in startle reactivity. In Experiments 2, 3A, and 4 mean startle amplitude during this habituation session was used to evenly distribute animals over experimental conditions.

Experiment 1. Expression of fear-potentiated startle was assessed using different shock intensities during training (0.3 mA vs 0.6 mA). Based on the results from Experiment 1, all subsequent studies were performed using a shock intensity of 0.6 mA.

Experiment 2. The level of fear acquisition was assessed during the first FPS training using the fear acquisition training protocol. Mean startle amplitude during the habituation session was used to evenly distribute animals over experimental conditions. To control for general deficits in sensory information processing and to assess the specificity of the fear-related deficit, prepulse inhibition and light-enhanced startle, a measure of sustained anxiety, were assessed (see Supplement 1).

Experiment 3. To differentiate between developmental and direct effects of SERT blockade, the effects of acute and chronic (21 days) paroxetine treatment (0, 10 and 20 mg/kg) on fear acquisition (A) and expression of FPS (B) were studied in Wistar rats. To assess the acute effects on fear acquisition, paroxetine was administered 1 h before fear acquisition and effects were analyzed during FPS expression 24 h later. To assess the chronic effects on fear acquisition, animals were treated for 21 days, starting one week after the habituation session. On day 21, animals were trained 1 h after drug administration and effects were analyzed during FPS expression 24 h later. In Experiment 3A the mean startle amplitude during the habituation session was used to evenly distribute animals over treatment conditions. The acute and chronic effects on fear

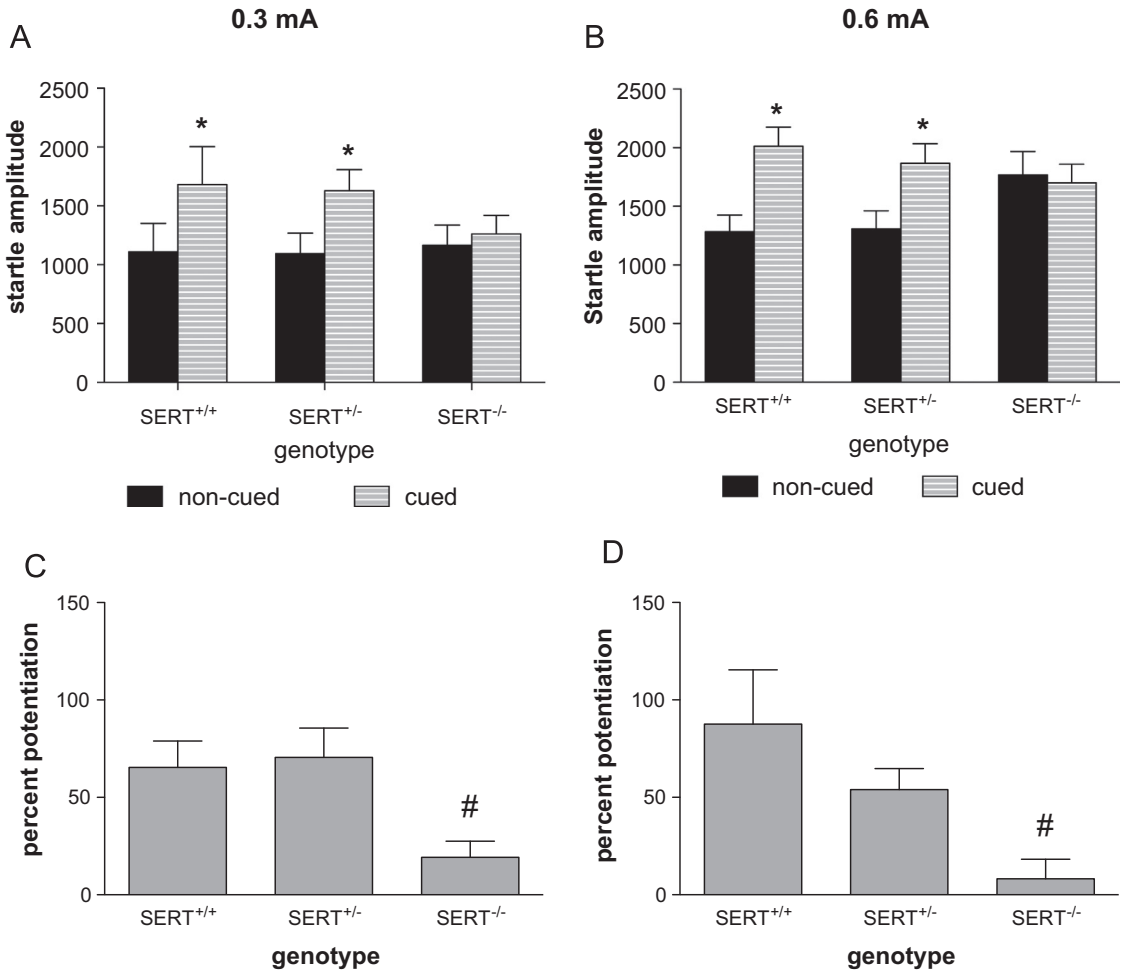

Figure 1 The expression of fear-potentiated startle in $SERT^{+/+}$, $SERT^{+/-}$ and $SERT^{-/-}$ rats following fear-potentiated startle training with 0.3 (A, C) or 0.6 mA (B, D) foot shock. (A and C) shows mean startle amplitude (±SEM) to non-cued and cued trials during fear-potentiated startle (n=12-17 per genotype for 0.3 mA condition and n=19-20 per genotype for 0.6 mA condition). (B) and (D) shows percent fear-potentiated startle (mean±SEM). $^{*}P<0.05$ for cued versus non-cued trials. $P<0.05$ compared to $SERT^{+/+}$.

expression were studied in pre-trained rats. Animals were trained for two consecutive days and tested for baseline FPS 24 h later. Animals were assigned to treatment groups on basis of their baseline FPS response. A week later, animals were exposed to another training session and 24 h later drug treatment started. Animals were treated for 21 days and on day 1 (acute) and day 21 (chronic) animals were tested for FPS 1 h after drug administration. Animals did not receive additional training during the treatment period to prevent possible interference of drug treatment with further acquisition.

Experiment 4. To adequately assess potential differences in the development of contextual conditioned fear between genotypes, an additional control group was included (Experiment 4A). Standardly trained vehicle groups (cue-shock group) were compared to vehicle control groups that were not shocked during fear acquisition (cue-no-shock group). In addition, we studied whether the blunted FPS in $SERT-/-$ rats could be normalized by CRF_1 receptor blockade during fear acquisition (Experiment 4B). To this end, the CRF_1 receptor antagonist CP154,526 was administered once, 30 min before fear acquisition and effects were analyzed during FPS expression 24 h later. The mean startle amplitude during habituation was used to evenly distribute animals over experimental groups.

Experiment 5. The effect of CP154,526 (10 mg/kg versus vehicle) on expression of FPS in pre-trained rats was studied in a within-subject design. A cue-no-shock control group was included to differentiate between drug effects on contextual fear and baseline startle reactivity. Assignment of animals to the cue-shock and cue-no-shock condition was based on mean startle amplitude during the habituation session. Pre-trained animals were tested twice, with a one-week interval. In both test weeks, animals were trained once and 24 h later, administered with CP154,526 or vehicle and tested for FPS expression. Treatment was counterbalanced over the two test sessions.

Experiment 6. To evaluate whether the disrupted fear acquisition in $SERT^{-/-}$ directly results from changes in $CRFR_1$ expression, we studied $CRFR_1$ mRNA expression in the basolateral amygdala and dorsal raphe nucleus of $SERT^{-/-}$ and $SERT^{+/+}$ rats using in situ hybridization.

2.8. Data analyses

All data were analyzed using repeated measures ANOVAs with stimulus intensity and trial type (cued versus non-cued) as within-subject factors. Specific additional contrasts per experiment: Experiments 1, 2, 4 and 5: genotype as between-subjects factor; Experiment 1: shock intensity as between-subjects factor;

Experiment 2: phase as within-subject factor; Experiment 4A and 4: condition (cue-no-shock group and cue-shock group) as between-subjects factor; Experiment 3 and 4B: drug (three levels) as a between-subjects factor. In Experiment 5, drug (2 levels) was added as a within-subject factor. For analyses of CRF_1 receptor mRNA expression in Experiment 6, expression levels were calculated by correcting the counted amount of dots for the total area. Mean expression levels for the BLA were analyzed using repeated measured ANOVA with hemisphere as within-subject factor and genotype as between-subject factor. Two animals were excluded from analysis due to damage to the sections. Mean expression levels for the dorsal raphe nucleus were analyzed using independent students *t*-test. *P*-values of 0.05 or smaller were considered significant. For complete statistical reports, see Tables 1 to 4 in Supplement 2.

3. Results

Experiment 1: $SERT^{-/-}$ rats show no fear-potentiated startle.

With both shock intensities, genotypes differed in their response to cued and non-cued trials (trial × genotype interaction: [$F_{2,42}=4.9$, $p<0.05$ and $F_{2,52}=8.9$, $p<0.001$ for 0.3 and 0.6 mA respectively]): $SERT^{+/+}$ and $SERT^{+/-}$ showed significant potentiation of the startle response whereas $SERT^{-/-}$ did not ($SERT^{+/+}$: $F_{1,11}=12.4$, $p<0.01$, $SERT^{+/-}$: $F_{1,15}=18.6$, $p=0.001$, $SERT^{-/-}$: $F_{1,16}=1.2$, NS; Figure 1A and B). The response to non-cued trials was increased in $SERT^{-/-}$ following 0.6 mA, but not 0.3 mA FPS training. However, this effect did not reach significance (effect genotype on non-cued trials [$F_{2,42}<1$] and [$F_{2,52}=2.8$, $p<0.1$] for 0.3 and 0.6 mA respectively). The blunted FPS response in $SERT^{-/-}$ under both shock intensities was also reflected in a significantly lower percentage FPS (Effect of genotype [$F_{2,42}=6.5$, $p<0.01$] and [$F_{2,52}=4.4$, $p<0.05$] for 0.3 and 0.6 mA respectively, Figure 1C and D).

Experiment 2: Blunted fear-potentiated startle due to deficient fear acquisition.

$SERT^{+/+}$ and $SERT^{+/-}$ showed significant acquisition of the cue-shock association, whereas $SERT^{-/-}$ did not (trial × genotype interaction: $F_{2,50}=3.3$, $p<0.05$; effect of trial: $SERT^{+/+}$ $F_{1,17}=10.1$, $p<0.01$; $SERT^{+/-}$ $F_{1,18}=5.0$, $p<0.05$; and $SERT^{-/-}$ $F_{1,15}<1$, Figure 2A and C). In addition, whereas $SERT^{+/+}$ differentiated between cued and non-cued trials during both the first and second phase of acquisition (1st phase [$T_{1,17}=-2.6$, $p<0.05$], 2nd phase [$T_{1,17}=-3.0$, $p<0.01$]), $SERT^{+/-}$ only differentiated between cued and non-cued trials during the first phase of acquisition (1st phase [$T_{1.18}=-2.9$, $p<0.001$], 2nd phase $T_{1,18}=-1.7$, NS]). The response to non-cued trials increased over time independently of genotype [phase × genotype interaction and overall effect phase on non-cued trials $F_{4,106}<1$ and $F_{2,106}=36.6$, $p<0.001$].

Experiment 3: Neither acute nor chronic pharmacological SERT inhibition affects the acquisition and expression of fear-potentiated startle.

Acquisition: Following fear acquisition significant FPS was induced (Effect of trial [$F_{1,30}=39.3$, $p<0.001$] and [$F_{1.32}=32.0$, $p<0.001$] in acute and chronic group respectively) and neither acute nor chronic paroxetine treatment significantly affected the level of fear acquisition, as measured 24 h later ([drug × trial interaction $F_{1,30}<1$ and $F_{2,32}<1$ in acute and chronic group respectively]; Figure 3A and C). Rather, acute paroxetine treatment during acquisition increased overall startle responding 24 h later [main effect dose $F_{1,30}=3.6$, $p<0.05$], an effect that was primarily mediated by the 10 mg/kg dose [$p=0.053$].

Expression: Neither acute nor chronic paroxetine treatment affected the expression of FPS in pre-trained rats (trial × dose interaction $F_{2,30}<1$ for both time points; Figure 3B and D). Significant FPS was established at both time points measured (Effect of trial [$F_{1,30}=19.0$, $p<0.001$] and [$F_{1,30}=21.6$, $p<0.001$] for acute and chronic treatment respectively).

Experiment 4A: $SERT^{-/-}$ rats show exacerbated contextual conditioned fear in the fear-potentiated startle paradigm.

The level of contextual conditioned fear was measured during non-cued trials. Separate analyses of non-cued trials in cue-no-shock and cue-shock groups showed that the level of contextual conditioned fear differed between genotypes [condition × genotype interaction $F_{2,66}=5.1$, $p<0.01$]. More specifically, $SERT^{-/-}$ and $SERT^{+/-}$ in the cue-shock group showed clear contextual conditioned fear, which was reflected in a significant increase in response to non-cued trials compared to the cue-no-shock group ([$F_{1,21}=12.9$, $p<0.01$] and [$F_{1,23}=7.7$, $p<0.05$] in $SERT^{-/-}$ and $SERT^{+/-}$ respectively, Figure 4B and C). $SERT^{+/+}$ rats, on the other

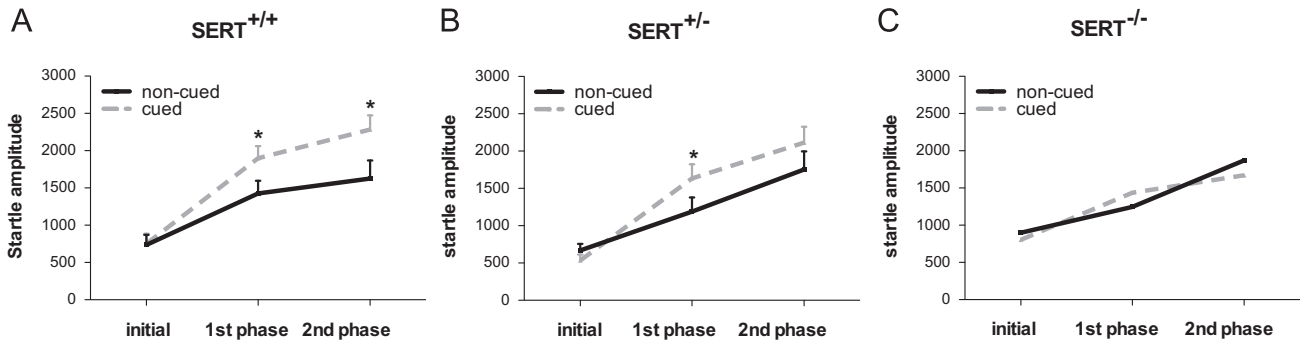

Figure 2 Acquisition of cue-conditioned fear during fear-potentiated startle training in $SERT^{+/+}$ (A, $n=19$), $SERT^{+/-}$ (B, $n=20$) and $SERT^{-/-}$ (C, $n=17$) rats. 'Initial' represents startle amplitude (±SEM) in response to non-cued and cued trials before the first cue-shock pairing. '1st phase' represents mean startle amplitude (±SEM) in response to non-cued and cued trials presented in between the first set of 5 cue-shock pairings. '2nd phase' represents mean startle amplitude (±SEM) in response to non-cued and cued trials presented in between the second set of 5 cue-shock pairings. $^{*}p<0.05$ for cued versus non-cued trials.

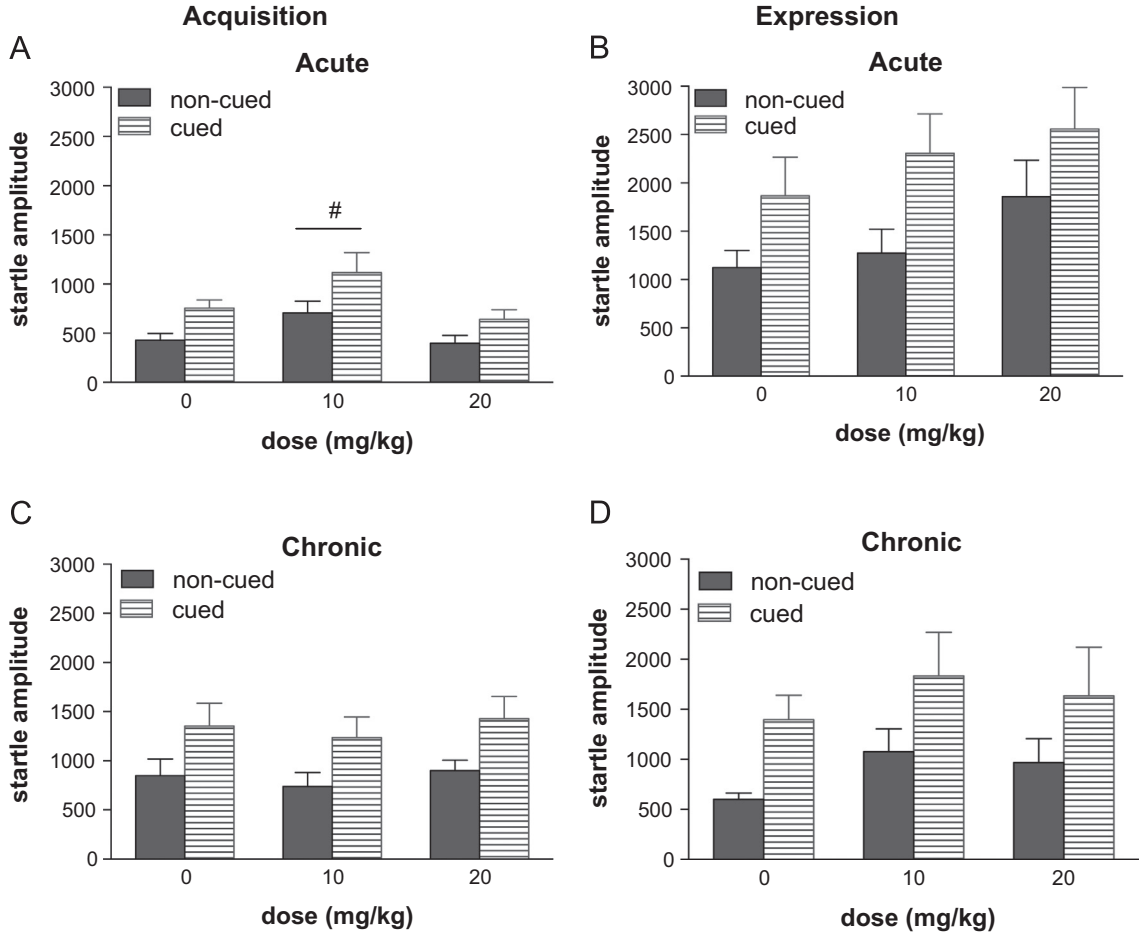

Figure 3 The effect of acute and chronic treatment with paroxetine on the acquisition (A, C) and expression (B, D) of fear-potentiated startle (n=11-12 per experimental group). Data is presented in absolute startle values (mean ± SEM) during non-cued and cued trials. $^{*}p<0.05$ for cued versus non-cued trials. $p<0.05$ compared to vehicle. In all 4 experiments, significant difference between cued and non-cued trials was induced. These significant effects are not depicted in the graphs.

hand, did not show contextual conditioned fear ([$F_{1,21}$=1.5, NS], Figure 4A). Direct comparison of the three genotypes in the cue-shock group showed that SERT$^{-/-}$ rats showed exacerbated contextual conditioned fear during non-cued trials compared to SERT$^{+/+}$, whereas SERT$^{+/-}$ did not (effect of genotype [$F_{2,33}$=10.5, p=0.001]; post-hoc analyses SERT$^{-/-}$ vs SERT$^{+/+}$ [p=0.001], SERT$^{+/-}$ vs SERT$^{+/+}$ [p=0.953]).

Furthermore, overall analyses of the FPS response in all genotypes confirmed the previously found disturbance in FPS in SERT$^{-/-}$ rats [condition × genotype × trial interaction: $F_{2,66}$=3.7, $p<0.05$]. Both SERT$^{+/+}$ and SERT$^{+/-}$ in the cue-shock group showed a significant increase in response to cued trials relative to cue-no-shock group (trial × condition interaction [$F_{2,12}$=8.0, p=0.01] and [$F_{1,23}$=9.7, $p<0.01$] in SERT$^{+/+}$ and SERT$^{+/-}$ respectively). These genotypes also clearly differentiated between cued and non-cued trials, reflecting normal FPS. On the other hand, SERT$^{-/-}$ rats in the cue-shock group showed an overall increase in startle reactivity relative to the cue-no-shock control group (trial × condition interaction [$F_{1,21}<1$] and overall effect of condition [$F_{1,21}$=13.5, p=0.001]). In addition, these SERT$^{-/-}$ in the cue-shock group did not differentiate between cued and non-cued trials.

Experiment 4B: CP154,526 during fear acquisition normalizes fear-potentiated startle and prevents exacerbation of contextual conditioned fear in SERT$^{-/-}$ rats.

As genotypes differed markedly in basal fear acquisition, drug effects could differ strongly between genotypes. Therefore, all effects in this experiment were analyzed separately for each genotype. In SERT$^{+/+}$ and SERT$^{+/-}$, administration of CP154,526 during acquisition had no effect on fear expression 24 h later, nor on the startle response per se (SERT$^{+/+}$: [drug × trial interaction $F_{2,32}<1$, overall effect of drug $F_{2,32}<1$]; SERT$^{+/-}$: [drug × trial interaction $F_{2,37}<1$, overall effect of dose $F_{2,37}$=1.1, NS]; Figure 4D and E, respectively). In SERT$^{-/-}$, on the other hand, CP154,526 treatment during acquisition had a differential effect on cued and non-cued trials ([drug × trial interaction $F_{2,33}$=3.8, $p<0.05$]; Figure 4F). Administration of 10 mg/kg CP154,526 during acquisition resulted in a significant potentiation of the startle response in response to cued trials (effect trial: [T_{11}, $p<0.01$]), whereas the response to cued and non-cued trials was similar following treatment with vehicle or 30 mg/kg CP154,526 (effect trial: vehicle [T_{10}=0.3, NS]; 30 mg/kg [T_{12}=−0.2, NS]). This significant fear potentiation following 10 mg/kg CP154,526 in SERT$^{-/-}$ was reflected in the absolute difference scores, as these scores were significantly increased compared to

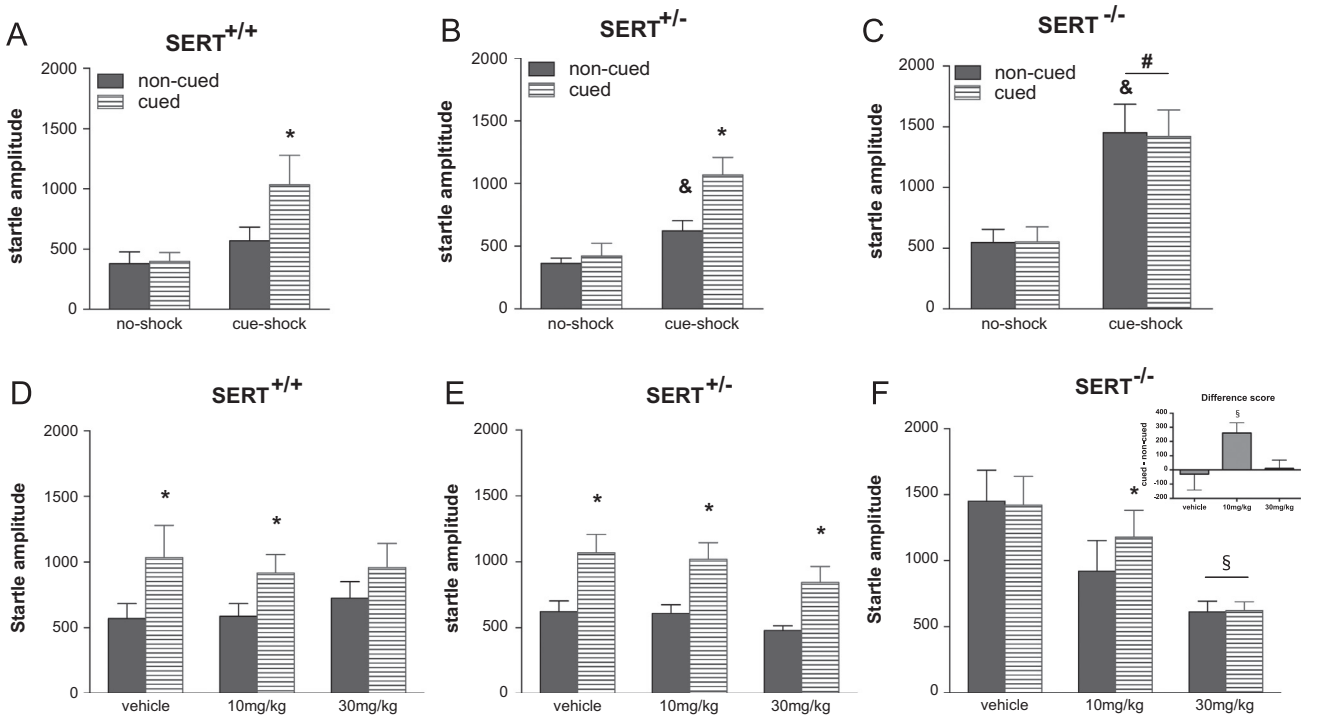

Figure 4 Effect of CP154,526 during acquisition on expression of fear-potentiated startle in $SERT^{+/+}$, $SERT^{+/-}$ and $SERT^{-/-}$ rats (n=11–15 per experimental group). Data is presented in absolute startle values (mean±SEM) during non-cued and cued trials. (A–C) represent the effect of fear acquisition on expression of fear-potentiated startle in the cue-shock condition compared to the no-shock control condition in $SERT^{+/+}$, $SERT^{+/-}$ and $SERT^{-/-}$ rats, respectively. (D–F) represent the effect of acute CP154,526 administration before acquisition on the expression of fear-potentiated startle 24 h later in $SERT^{+/+}$, $SERT^{+/-}$ and $SERT^{-/-}$ rats, respectively (inset in (F) difference score between cued and non-cued trials). $^{*}p<0.05$ for cued versus non-cued trials; $p<0.05$ compared to no-shock control group; $^{\S}p<0.05$ compared to vehicle control; $^{\&}p<0.05$ compared to non-cued trials of cue-no-shock control group.

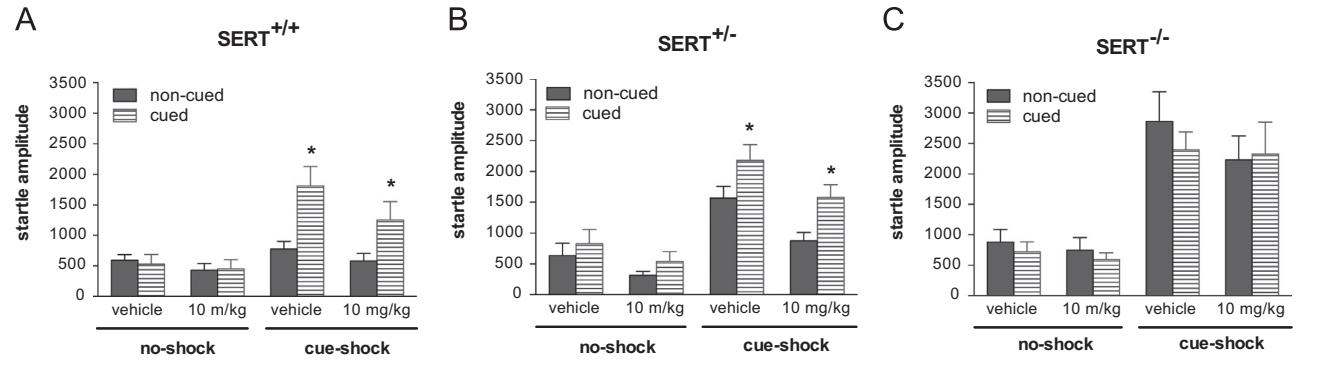

Figure 5 Effect of CP154,526 on expression of fear-potentiated startle in pre-trained $SERT^{+/+}$ (A), $SERT^{+/-}$ (B) and $SERT^{-/-}$ (C) rats (n=7–9 per genotype for cue-no-shock control groups and n=9–10 per genotype for cue-shock groups). CP154,526 was administered 30 min before the fear-potentiated startle test. Data is presented in absolute startle values (mean±SEM) during non-cued and cued trials. $^{*}p<0.05$ for cued versus non-cued trials. Effect of CP154,526 on overall startle reactivity is not depicted in (A, C).

$SERT^{-/-}$ vehicle controls (Effect of drug [$F_{2,33}$=3.8, $p<0.05$]; post-hoc analyses: 10 mg/kg vs vehicle [p=0.033], 30 mg/kg vs vehicle [p=0.913], inset Figure 4F), whereas they were unaltered in $SERT^{+/+}$ and $SERT^{+/-}$ rats [effect of drug $F_{2,32}<1$ and $F_{2,37}<1$ respectively]. In addition, in $SERT^{-/-}$, a main effect of CP154,526 on overall startle responding revealed that CP154,526 administration during acquisition also prevented the development of contextual conditioned fear, as measured 24 h later (main effect drug [$F_{2,33}$=4.6, $p<0.05$]). This effect was primarily due to the 30 mg/kg dose.

Experiment 5: CP154,526 does not affect expression of fear-potentiated startle.

CP154,526 had no specific effect on the level of fear potentiation when administered before the fear-potentiated startle test in pre-trained rats, rather it significantly decreased the overall startle response (main effect drug [$F_{1,47}$=21.5, $p<0.001$], dose × condition × trial interaction [$F_{1,47}<1$]); Figure 5). This effect tended to be stronger in the cue-shock group, compared to the cue-no-shock group (drug × condition interaction [$F_{1,47}$=3.86, p=0.055]). The genotype differences

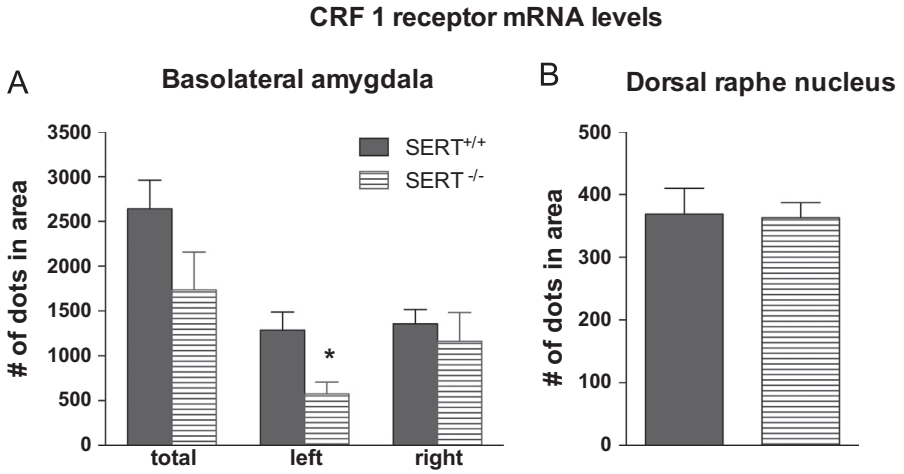

Figure 6 CRF_1 receptor mRNA expression level in the basolateral amygdala (A, $n=9$ for both genotypes) and dorsal raphe nucleus (B, $n=10$ for both genotypes) of $SERT^{+/+}$ and $SERT^{-/-}$ rats. $^{*}p<0.05$ compared to $SERT^{+/+}$.

in expression of FPS, as detected earlier, were confirmed (trial × genotype interaction in cue-shock group [$F_{2,25}=10.0$, $p=0.001$]; effect trial in $SERT^{+/+}$ [$F_{1,9}=18.2$, $p<0.01$], $SERT^{+/-}$ [$F_{1,8}=19.0$, $p<0.01$] and $SERT^{-/-}$ {$F_{1,8}=1.4$, NS]). In the cue-no-shock control group, on the other hand, no fear potentiation was present and genotypes did not differ in startle reactivity (effect of trial [$F_{1,22}<1$], effect of genotype [$F_{2,22}<1$], genotype × trial interaction [$F_{2,22}=2.7$, $p<0.1$]).

Experiment 6: CRF_1 receptor mRNA expression in BLA and DR was not significantly altered in $SERT^{-/-}$ rats.

CRF_1 receptor levels in the basolateral amygdala, represented by number of dots, were not significantly different between $SERT^{+/+}$ and $SERT^{-/-}$ rats (main effect genotype [$F_{1,16}=1.3$, NS], Figure 6A). Because of a trend towards a difference between hemispheres (main effect hemisphere [$F_{1,16}=4.1$, $p<0.1$], genotype × hemisphere interaction [$F_{1,16}=3.4$, $p<0.1$]), genotype effects were also analyzed in both hemispheres separately. This analysis showed a significant decrease in CRF_1 receptor levels in the left BLA of $SERT^{-/-}$ rats ($t_{16}=-2.468$, $p<0.05$), whereas CRF_1 receptor levels in the right BLA did not differ between genotypes ($t_{16}=-0,116$, NS). No significant differences CRF_1 receptor levels in the dorsal raphe nucleus were found between $SERT^{+/+}$ and $SERT^{-/-}$ rats ([$t_{18}=0.125$, NS], Figure 6B).

4. Discussion

This study evaluated the role of SERT in classical fear conditioning using a SERT knockout rat model. We found a clear disruption of cue-conditioned fear in $SERT^{-/-}$ rats, as measured with the fear-potentiated startle. This disruption was due to a deficit in fear acquisition and was accompanied by development of enhanced contextual conditioned fear. Neither the acquisition nor the expression of fear-potentiated startle was affected by pharmacological SERT inhibition. Moreover, both the deficit in acquisition of cue-conditioned fear and the development of exacerbated contextual conditioned fear in $SERT^{-/-}$ rats could be reversed by treatment with the CRF_1 receptor antagonist CP154,526 during acquisition.

The failure to learn the cue-shock contingency resulted in increased contextual fear in $SERT^{-/-}$ rats. This was specifically related to the fear learning deficit, as $SERT^{-/-}$ rats show normal unconditioned anxiety in the light-enhanced startle paradigm (Supplement 1). Furthermore, it is unlikely that the fear learning deficit is a result of increased contextual fear, because results from Experiment 4 show that the prevention of contextual fear by 30 mg/kg CP-154,526 did not result in reinstatement of the cued fear expression. In humans, unawareness of the cue-shock contingency also increases contextual fear (Baas et al., 2008), a phenomenon that is specifically associated with maladaptive fear processing in panic disorders patients (Grillon et al., 2007b; Lissek et al., 2009). Interestingly, these patients shown normal startle responding in unconditioned measures of fear and anxiety (Grillon et al., 1994; Melzig et al., 2007). Thus, the current findings in $SERT^{-/-}$ rats fit well with aforementioned human data and resemble both fear- and anxiety-related potentiated startle data in panic disorder patients. Interestingly, several studies indicate altered SERT functioning in panic disorder (Esler et al., 2007; Strug et al., 2010), further strengthening the link between SERT availability and fear learning deficits in anxiety disorders.

To the best of our knowledge, this is the first report on the effect of chronic SSRI treatment on the acquisition and expression of fear-potentiated startle in rodents. The lack of effect on expression of cued fear is in line with the single human fear-potentiated startle study available (Grillon et al., 2009). The finding that neither acute nor chronic paroxetine treatment affected the acquisition and expression of cued conditioned fear suggests that the fear learning deficit found in $SERT^{-/-}$ is due to developmental changes and does not result from compromised SERT functioning during fear learning. Serotonin is known to modulate neurodevelopment (Gaspar et al., 2003; Lauder, 1990) and the neural systems regulating anxiety-like behavior are especially sensitive to changes in serotonergic functioning during early development (Gross et al., 2002; Vinkers et al., 2010). It has already been reported that $SERT^{-/-}$ mice show increased spine density and excitatory drive of the BLA (Wellman et al., 2007). Such changes in neuronal structure and excitability may also be responsible for the learning deficit observed in $SERT^{-/-}$ rats. However, the exact

mechanism underlying this deficit in $SERT^{-/-}$ rats is subject of further investigation.

Interestingly, acute administration of paroxetine during fear acquisition did increase overall startle responding 24 h later, which suggests that acute SERT inhibition potentiates the acquisition of contextual conditioned fear. To the best of our knowledge, this is the first time the acute anxiogenic effect of SSRIs in humans, as measured with fear-potentiated startle, is shown in rodents (Burghardt et al., 2004; Grillon et al., 2007a). Together with findings from our concurrent study in humans that SERT S/S carriers show exaggerated contextual fear learning (Heitland et al., 2013), these results suggest that increased extracellular serotonin levels may also be responsible for the increased contextual conditioned fear seen in $SERT^{-/-}$ rats.

Findings from the present study indicate that CRF_1 receptors are especially important in fear acquisition. CP154,526 given before acquisition training, at least partly, reinstated the fear-potentiated startle response and blocked the development of contextual conditioned fear in $SERT^{-/-}$ rats. CP154,526 administered to pre-trained rats however, neither normalized the expression of fear-potentiated startle nor blocked contextual conditioned fear in $SERT^{-/-}$ rats. This absence of effect of CP154,526 on fear expression in pre-trained rats is consistent with accumulating evidence that CRF_1 receptor antagonists do not affect the expression of fear-potentiated startle (de Jongh et al., 2003; Walker et al., 2009). In addition, together with the findings from our concurrent study in humans that interactions between SERT and the CRF_1 receptor play a pivotal role in the regulation of conditioned fear acquisition in healthy subjects (Heitland et al., 2013), this study not only supports the idea that especially the interplay between the CRF and serotonin systems regulates fear learning, but also emphasizes the translational value of the fear-potentiated startle paradigm.

A putative locus for both the basal deficits in cued fear learning as well as its normalization by CP154,526 may be the serotonergic projections originating from the dorsal raphe nucleus that innervate several brain areas implicated in fear learning, including the central amygdala and hippocampus. CRF modulates serotonin release in the raphe (De Souza, 1995; Kirby et al., 2000; Van Pett et al., 2000) and $SERT^{-/-}$ rats show continuous high serotonergic tone in serotonergic projections (Homberg et al., 2007a). The current study did not show any changes in CRF_1 receptor expression in the dorsal raphe. This suggests that altered CRF1 receptor signaling within this area does not underlie the fear learning deficit found in $SERT^{-/-}$ rats. Because this study looked into the anterior part of the DR, it can not be excluded that changes in CRF_1 receptor expression occurred in the more posterior regions of the DR that have been implicated in fear regulation as well. However, several studies have suggested that CRF-serotonin interactions in these posterior regions are selectively mediated by CRF2, and not CRF1, receptors (for review, see Fox and Lowry (2013)).

Other possible loci for the deficits in cued fear learning and normalizing effects of CP-154,526 may be the basolateral amygdala (BLA) and hippocampus. Both brain areas have been implicated in fear conditioning (Malin and McGaugh, 2006; Phillips and LeDoux, 1992) and receive strong serotonergic input from the dorsal raphe (Hensler, 2006). In addition, $SERT^{-/-}$ rats show increased activation of the BLA (Nonkes et al., 2010) and in mice, lifelong disturbance of SERT functioning is associated with increased contextual conditioned fear, increased spine density within, and excitatory drive of the BLA (Kalueff et al., 2010; Wellman et al., 2007). Moreover, CRF_1 receptor blockade in both the BLA and hippocampus prevents the development of contextual conditioned fear (Hubbard et al., 2007; Roozendaal et al., 2002; Roozendaal et al., 2008), whereas CRF_1 receptor activation within the hippocampus enhances fear learning (Blank et al., 2003; Radulovic et al., 1999). Chronic activation of the CRF system within the BLA has also been associated with increased excitatory drive within this region (Rainnie et al., 2004). Decreased CRF_1 receptor expression in the left BLA, as observed within the current study, may be indicative of adaptive changes in response to chronic CRF_1 receptor activation and may suggest that lifelong disruption of SERT functioning mediates part of its effects via local changes in CRF signaling. In addition, blockade of CRF_1 receptors by CP154,526 may have resulted in the reinstatement of adequate fear learning in $SERT^{-/-}$ rats by inhibiting the excitatory drive of BLA neurons in $SERT^{-/-}$ rats. In line with this hypothesis, it has already been shown that repeated activation of CRF receptors in the BLA potentiated the acquisition of fear-potentiated startle (Bijlsma et al., 2011) and CRF_1 receptor blockade in the BLA prevents the development of contextual conditioned fear (Roozendaal et al., 2002; Roozendaal et al., 2008). The relevance of the hemispheric differences in CRF1 receptor expression found is currently unknown. Although human research has implicated hemispheric differences in activation patterns in the regulation of emotion, few animal studies have investigated lateralized amygdala involvement and findings are inconsistent (Adamec and Morgan, 1994; Baker and Kim, 2004).

Heterozygous SERT knockout showed an intermediate level of FPS in Experiment 1, but normal FPS in Experiments 4 and 5. Apparently, high redundancy exists in the involvement of SERT in normal fear acquisition, as availability of only 60% of normal SERT levels (Homberg et al., 2007a) is still sufficient to show relatively normal fear learning.

The deficit in fear acquisition in $SERT^{-/-}$ rats is very consistent, as it was replicated in all separate experimental groups throughout the study. In addition, it is unlikely that the absence of fear-potentiated startle in $SERT^{-/-}$ rats was due to a ceiling effect because fear-potentiated startle was also absent under experimental conditions were $SERT^{-/-}$ did not show exacerbated contextual fear and overall startle levels were still at baseline levels (Figures 1B and 2). The finding that prepulse inhibition (Supplement 1), a measure of sensorimotor gating, is intact shows that the deficits found are not due to general deficits in information processing, rather they seem specific for the responses to threatening stimuli.

In conclusion, we showed that deletion of the SERT results in a marked fear learning deficit, which shows similarity to associative fear learning deficits in panic disorder patients. These findings further implicate the serotonin transporter in the fear learning deficits seen in panic disorder patients. The finding that CRF_1 receptor blockade during fear acquisition could normalize fear learning and prevented the exacerbation of contextual fear, suggests a specific role for central CRF signaling and CRF-serotonin interactions in fear learning. As such, current findings suggest that the CRF_1 receptor may be an interesting pharmacological target to prevent fear generalization and subsequent symptom exacerbation in panic disorder patients.

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Contributors

The study was designed by EYB and LG. CP-154,526 was provided by MJM. The experiments were executed by EYB and HH. Statistical analyses were done by EYB and LG. The first draft of the manuscript was written by EYB. All authors contributed to and have approved the final manuscript.

Conflict of interest

EYB, HH, JMPB and LG have no conflicts of interest to disclose. JMPB received research grants/support from Utrecht University focus area Neuroscience and Cognition Utrecht and NWO Aspasia. LG received research grants/support from Utrecht University focus area Neuroscience and Cognition Utrecht, Grünenthal GmBh, Institut de Recherches Servier and Psychogenics Inc., New York. MJM is a full time employee of the Institut de Recherches Servier.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.euroneuro.2015.07.004.

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