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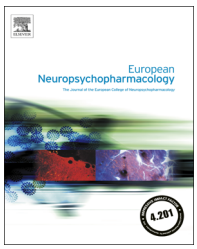

Lifelong disturbance of serotonin transporter functioning results in fear learning deficits: Reversal by blockade of CRF_1 receptors

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.