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Short communication

Acute inescapable stress alleviates fear extinction recall deficits caused by serotonin transporter abolishment

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ABSTRACT

Life stress increases risk for developing post-traumatic stress disorder (PTSD), and more prominently so in short-allele carriers of the serotonin transporter linked polymorphic region (5-HTTLPR). Serotonin transporter knockout (5-HTT$^{-/-}$) rats show compromised extinction (recall) of conditioned fear, which might mediate the increased risk for PTSD and reduce the therapeutic efficacy of exposure therapy. Here, we assessed whether acute inescapable stress (IS) differentially affects fear extinction and extinction recall in 5-HTT$^{-/-}$ rats and wildtype controls. Surprisingly, IS experience *improved* fear extinction recall in 5-HTT$^{-/-}$ rats to the level of wildtype animals, while wildtypes were unaffected by this IS. Thus, whereas 5-HTT$^{-/-}$ rats evidently were more responsive to the stressor, the behavioral consequences presented themselves as adaptive.

Severe life adversity has been linked to increased risk for developing post-traumatic stress disorder (PTSD) [1]. A large body of evidence suggests that the serotonergic system plays a role in mediating these detrimental effects of stress. Genetic variation in serotonin transporter (5-HTT) expression is known to alter stress sensitivity in humans, non-human primates and rodents, with genetic variants conferring a reduction in function (such as the 5-HTTLPR s-allele) exacerbating the effects of stressful life experiences on the incidence of PTSD [2]. Critically, traumatic life events modulate the strength and neural basis of fear acquisition and extinction in a 5-HTT dependent manner, which may underlie the increased vulnerability to psychopathology [3,4]. As fear acquisition and extinction processes are key in both the development and treatment of PTSD [5], understanding 5-HTT by stress interactions is essential for the development of therapeutic interventions attuned to these individuals.

5-HTT knockout (5-HTT$^{-/-}$) rodents are characterized by a behavioral profile of generalized anxiety (e.g. [6], and impaired fear extinction memory recall (e.g. [7])), modeling symptoms of stress-related psychopathology. While 5-HTT abolishment results in a wide array of anatomical and physiological changes and adaptations in the brain, perhaps the most prominent of these is a constitutive sevenfold increase in extracellular serotonin levels [8]. This is relevant, given that acute inescapable stress (IS), an experimental stressful life experience, impairs fear extinction by increasing dorsal raphe nucleus (DRN) serotonergic signaling and subsequent serotonin release in the basolateral amygdala (BLA) [9]. Expression of conditioned fear is associated with phasic elevation of BLA serotonin [10], and terminating serotonergic inputs into the amygdala reduces its expression, but only in repeated inescapable stress (IS) experienced mice [11], implicating a critical role for serotonin in mediating the behavioral fear phenotype induced by IS. Combining these findings with the constitutively increased extracellular serotonin levels in 5-HTT$^{-/-}$ rats raises the expectation that IS-induced fear extinction impairment is exacerbated in those with inherited 5-HTT down-regulation, explaining the 5-HTTLPR related clinical findings for PTSD.

To investigate how the effects of IS on fear extinction are modulated by 5-HTT genotype, we here assessed fear extinction and extinction recall in both naïve and IS-experienced 5-HTT$^{-/-}$ rats and their wildtype (5-HTT$^{+/+}$) counterparts [8]. We first subjected a substantial group of adult males of both genotypes to IS consisting of one session of 100 unpredictable tail shocks of randomized duration under restraint (n_5-HTT$_{-/-}$ = 20, n_5-HTT$_{+/+}$ = 19), or a control manipulation (n_5-HTT$_{-/-}$ = 20, n_5-HTT$_{+/+}$ = 16), followed by cued fear conditioning 24 h later. This stressor (albeit given after conditioning) was previously shown to increase freezing during extinction [12]. Animals were then re-exposed to the fear conditioned stimulus to measure fear extinction

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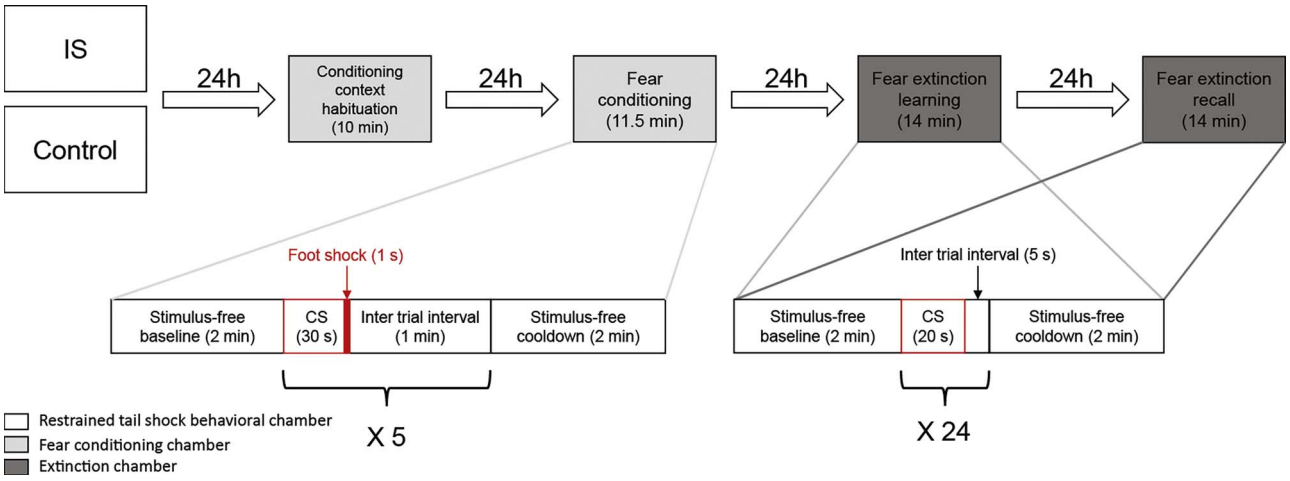

Fig. 1. Experimental outline.
All animals underwent habituation to the fear conditioning apparatus, fear conditioning, fear extinction learning and fear extinction recall testing respectively 24, 48, 72 and 96 h after IS, which consisted of 100 unpredictable tail shocks under restraint, or a control manipulation consisting of two hours of mild restraint in the behavioral apparatus used for tail shock administration.

learning and subsequent recall, by means of behavioral freezing (see Fig. Figure 1 for the experimental timeline).

Serotonin transporter knockout rats (Slc6a41Hubr) were generated on a Wistar background by N-ethyl-N-nitrosurea (ENU)-induced mutagenesis. Experimental animals were derived from crossing heterozygous 5-HT transporter knockout (5-HTT$^{+/-}$) rats that were outcrossed for at least twelve generations with wild-type Wistar rats obtained from Harlan Laboratories (Horst, The Netherlands). Ear punches were taken at the age of 21 days after weaning for genotyping, which was done by Kbiosciences (Hoddesdon, United Kingdom). We tested male adult 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats which ranged from 16 to 24 weeks of age. The animals were housed in pairs, in open cages. All animals had *ad libitum* access to food and water. A 12-h light-dark cycle was maintained, with lights on at 08.00 A.M. All behavioral experiments were performed between 08.00 A.M. and 18:00 P.M. All experiments were approved by the Committee for Animal Experiments of the Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands, and all efforts were made to minimize animal suffering and to reduce the number of animals used.

IS tail shocks were given in a triadic chamber (large) measuring 18.3 × 11.4 × 18.5 cm with grid floors (Med Associates, St. Albans, VT, USA). The grid floors were covered with vinyl to minimize injury to the animal. Shocks were delivered by a shock generator (model ENV-412, Med Associates). A 30.5 cm × 24.1 cm × 21 cm operant conditioning chamber (Model VFC-008, Med Associates) was used for fear conditioning and sham conditioning. The box was housed within a sound-attenuating cubicle and contained a white LED stimulus light, a white and near infrared house light as well as a speaker capable of producing an 85 dB 2.8 kHz tone. The metal grid floor of the apparatus was connected to a scrambled shock generator (model ENV-412, Med Associates) configured to deliver shocks at 0.6 mA intensity. Fear extinction and extinction recall were tested in a novel context. The novel context consisted of a 25 cm × 25 cm × 30 cm Plexiglas cage, the bottom of which was covered in a +/−0.5 cm thick layer of black bedding. In this context, 85 dB (measured at the center of the floor) 2.8 kHz auditory stimuli were delivered through a set of external speakers.

Animals in the IS group were restrained by the tail in the triadic chamber using disposable finger electrodes, under which electrolytic gel was applied. 100 shocks of increasing intensity (30 shocks at 0.8 mA, 30 shocks at 1.0 mA, 40 shocks at 1.2 mA) and of randomized duration (1 – 30 s, 5 s average) were given on a variable interval schedule ranging from 50–70 seconds (60 s average). The IS procedure took 2 h. Control animals were restrained by the tail (while they were still able to move all limbs) for 2 h in the apparatus using disposable finger electrodes, but were not given shocks. 24 h after IS or the control procedure, animals were habituated to the fear conditioning environment for 10 min. The house light was on during habituation and conditioning. For the fear conditioning itself, after a 2 min habituation period, a 30 s 85 dB 2.8 kHz auditory stimulus co-terminated with a 1 s 0.6 mA foot shock, followed by a 1 min inter-trial interval. A total of 5 of these tone – shock pairings were given. 24 h and 48 h after conditioning, fear extinction and extinction recall were tested, respectively. After a 2 min habituation period, 24 20-s presentations of the auditory stimulus were given, with an inter-trial interval of 5 s. Conditioning and extinction sessions were recorded and freezing was manually assessed by a trained observer who was blind to genotype and treatment. For the IS or control procedures, the conditioning and the habituation to the fear conditioning chamber, the apparatus was cleaned before and after each animal using a tissue slightly dampened with 70% EtOH. Water was used for cleaning during the extinction and extinction recall. Due to equipment malfunction, the conditioning session could be recorded only for half the animals of each group.

All statistical analyses were performed using IBM SPSS Statistics version 20.0 (SPSS Inc., Chicago, Illinois, USA). Data are presented as mean ± standard error of the mean (SEM). Effects of genotype and treatment on freezing during conditioning and extinction were analyzed using a 2-way repeated measures ANOVA (F); development of freezing behavior was assessed across extinction sessions and trial blocks within the extinction recall session. Significant genotype x treatment interactions were further explored using *post hoc* Student's *t*-tests. Probability p-values below 0.05 were considered significant.

When assessing freezing during the stimulus free 2-minute period preceding the tone-shock pairings in the conditioning session through 2-way ANOVA, we found an effect of genotype ($F_{(1,36)} = 4.591$, $p = 0.039$), with 5-HTT$^{-/-}$ spending more time on freezing. No effect of IS ($F_{(1,36)} = 0.155$, $p = 0.696$), nor a genotype x IS interaction effect ($F_{(1,36)} = 0.123$, $p = 0.728$) was found. Analyzing total time spent freezing during cue presentation in the fear conditioning session using repeated measures 2-way ANOVA analysis yielded no effect of genotype ($F_{(1,36)} = 0.021$, $p = 0.884$), IS ($F_{(1,36)} = 0.707$, $p = 0.406$), or genotype x IS interaction ($F_{(1,36)} = 0.1358$, $p = 0.716$) (Fig. Figure 2A).

Analysis of time spent freezing during the stimulus free baseline-period preceding the extinction sessions revealed no significant effect of genotype, IS or genotype x IS interaction in the extinction learning session ($F_{(1,74)} = 2.153$, $p = 0.147$; $F_{(1,74)} = 3.592$, $p = 0.062$; and

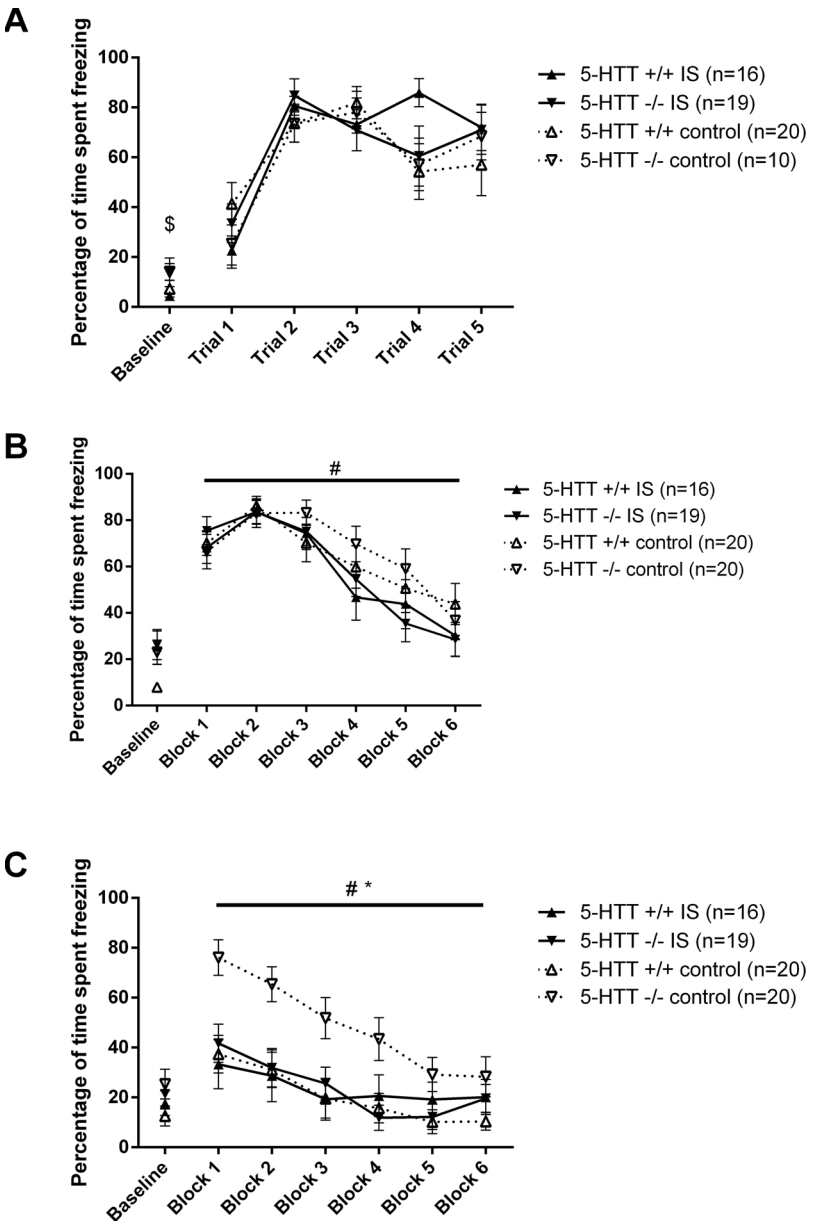

Fig. 2. Fear conditioning, fear extinction, and extinction recall.
(A) Behavioral freezing during stimulus-free baseline and stimulus presentations in the fear conditioning session. During the stimulus-free baseline period, 5-HTT$^{-/-}$ animals froze slightly more than wildtype animals. Freezing increased across trial blocks but was not significantly affected by genotype, stress, or genotype x stress interaction. (B) Behavioral freezing during baseline and in response to the conditioned stimulus 24 h after fear conditioning per block of 4 stimulus presentations. Freezing decreased across the trial blocks but was not affected by genotype, stress, or genotype x stress interactions. (C) Behavioral freezing during baseline and in response to the conditioned stimulus 24 h after the fear extinction learning session per block of 4 stimulus presentations. Impaired fear extinction recall in 5-HTT$^{-/-}$ rats was normalized by IS experience. Data are expressed as mean percentage of the duration of stimulus presentation spent freezing ± SEM. #, significant effect of trial block # ($p < 0.001$). *, significant genotype × stress interaction ($p < 0.05$). $, significant effect of genotype ($p < 0.05$).

$F_{(1,74)} = 1.816$, $p = 0.182$ respectively), nor in the extinction recall session ($F_{(1,74)} = 2.393$, $p = 0.126$; $F_{(1,74)} = 0.013$, $p = 0.910$; and $F_{(1,74)} = 0.716$, $p = 0.400$ respectively). We found that time spent freezing during cue presentations decreased across extinction sessions ($F_{(1,71)} = 112.086$, $p < 0.001$). A trend-level significant session x genotype x IS interaction was found ($F_{(1,71)} = 3.623$, $p = 0.061$). Analyzed across both sessions, there was a trend level significant effect of genotype ($F_{(1,71)} = 3.165$, $p = 0.079$) and no significant effect of IS ($F_{(1,71)} = 01.123$, $p = 0.293$). No effects of genotype ($F_{(1,71)} = 0.108$, $p = 0.744$), IS ($F_{(1,71)} = 1.222$, $p = 0.273$) or genotype x IS interactions ($F_{(1,71)} = 0.26$, $p = 0.873$) were observed in total time spent freezing during extinction training (24 h post-conditioning) (Fig. Figure 2B). However, we observed a significant genotype x IS interaction in total freezing during the presentation of the conditioned stimulus in the extinction recall test (48 h post-conditioning) ($F_{(1,74)} = 3.967$, $p = 0.050$) (Fig. Figure 2C). Exploring this effect using *post hoc* t-tests revealed that we replicated earlier reports [7] by showing that stress naïve 5-HTT$^{-/-}$ animals displayed impaired retention of conditioned fear extinction compared to 5-HTT$^{+/+}$ animals ($t_{(1,38)} = 2.969$, $p = 0.005$). However, no difference was found in freezing during CS presentation in the extinction recall test between IS-exposed and control 5-HTT$^{+/+}$ rats ($t_{(1,36)} = 0.318$, $p = 0.752$), but, surprisingly, IS *improved* extinction retention in 5-HTT$^{-/-}$ animals ($t_{(1,38)} = 3.437$, $p = 0.001$).

Here, we show that – contrary to our hypothesis – a single session of severe IS does not affect freezing behavior during fear conditioning, extinction, or extinction recall in 5-HTT$^{+/+}$ animals and normalizes the typically impaired recall of fear extinction memory in 5-HTT$^{-/-}$ rats, in the absence of effects on freezing behavior during conditioning and extinction learning. Although freezing during the stimulus free baseline period preceding the fear conditioning was higher in 5-HTT$^{-/-}$ animals, baseline freezing during all behavioral sessions was not affected by IS. This indicates IS did not induce nonspecific fear or generalized anxiety. Successful recall of extinction memory of conditioned fear is a critical adaptive response in the face of changing environmental conditions. Accordingly, normalized freezing during the extinction recall test indicates that 5-HTT$^{-/-}$ animals successfully updated the contingency of the fear conditioned stimulus from signifying the onset of danger to a neutral cue. The fact that the behavioral effects of IS were limited to the extinction recall session suggests that IS improved

consolidation or retrieval of extinction memory, but not extinction learning itself, in a manner dependent on 5-HTT expression.

The finding that a single session of IS exposure did not affect the acquisition of conditioned fear implies that fear learning is not affected by IS, neither in 5-HTT$^{+/+}$ nor in 5-HTT$^{-/-}$ rats. However, contrasting our finding, Baratta et al. found enhanced fear acquisition 7 days after IS in wildtype rats [12]. A potential explanation is that we used a conditioning paradigm consisting of five tone-shock pairings, while Baratta et al. used a single tone-shock pairing fear conditioning protocol. Potentially, the stronger conditioning in the present study obscured effects of the stressor on fear acquisition through a ceiling effect in freezing during the extinction learning session. We chose the five tone-shock protocol as it is known to robustly demonstrate induce extinction recall deficits in 5-HTT$^{-/-}$ rats (e.g. [13]). Animal species may also crucially influence the effects of stress on behavioral readouts relating to fear memory. While effects of prior stress experience on fear acquisition and extinction have been reported in Sprague Dawley and Long Evans rats and C57BL/6 NCrl mice [12,14,15], no effects of similar stressors on fear behavior have been documented in Wistar rats. There is evidence that rat strain crucially modulates coping with and sensitivity to stress [16–18], complicating direct comparison of the present findings with reports of results obtained from Sprague Dawley animals.

Our observation that animals with compromised 5-HTT availability displayed an increase in extinction recall corresponds to the previous finding that 5-HTT deficient rats showed a reduction in IS-induced escape deficits compared to control animals when they had undergone early life stress (maternal separation) [19]. While these findings seem counterintuitive, they seem to suggest adaptive behavioral sequelae of stress. The mechanisms underlying these findings remain to be investigated. IS-induced elevation in serotonin release in the DRN is thought to be a key mediator of its behavioral effects [20]. The increased release of serotonin in DRN target regions, modulating fear memory processes, is of a transient nature [9], and the effects of IS persist well past the duration of this initial elevation of 5-HT. The IS-induced transient rise in serotonin levels is thought to cause desensitization of the 5-HT1a receptor in the DRN itself, which has been demonstrated to amplify subsequent serotonergic responses to new challenges [21]. As this DRN 5-HT1a receptor is desensitized in 5-HTT$^{-/-}$ rodents [22], changes beyond the 5-HT1a receptor or even the DRN may be involved in the beneficial effects of IS in these animals [23].

Before designating the IS-induced adaptations in the regulation of fear behavior in 5-HTT$^{-/-}$ rats as strictly beneficial, further study is necessary. It is presently not known whether the improvements in extinction recall seen in 5-HTT$^{-/-}$ are of an enduring or transient nature, what mechanisms underlie them, and whether they are part of a larger array of (mal)adaptive behavioral effects. In addition, whether exposure to this stressor affected any other learning or extinction processes in 5-HTT$^{-/-}$ rats was not assessed. Abolishment or diminution of 5-HTT expression has been shown to enhance cognitive flexibility [24], and in a wide range of settings; whether and how these benefits of reduced 5-HTT expression are affected by IS in the 5-HTT$^{-/-}$ animals remains to be investigated. Though it may be premature to suggest to implement measures similar to the ones employed here (i.e., stress exposure) to improve treatment success in psychiatric practice, “shock to the system” approaches to treating depression and anxiety have been suggested previously and may indeed be of merit in combating these disorders, particularly in 5-HTTLPR s-allele carriers, who typically poorly respond to cognitive behavioral therapy [25]. While our understanding of the phenomenon and its relation to psychiatric disorders has a long way to go still, our findings lend credence to the notion that Paracelsus’ adage “the dose makes the poison” may apply to stress (or its molecular mediators), and that we may be able to wield its adaptive properties for therapeutic benefit before long.

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