ACS Chemical Neuroscience

Letter

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Improved Stress Control in Serotonin Transporter Knockout Rats: Involvement of the Prefrontal Cortex and Dorsal Raphe Nucleus

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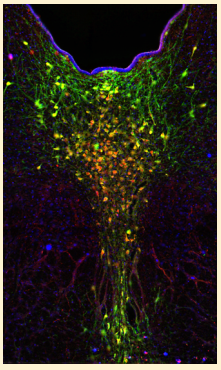
Supporting Information

ABSTRACT: Variations in serotonin transporter (5-HTT) expression have been associated with altered sensitivity to stress. Since controllability is known to alter the impact of a stressor through differential activation of the medial prefrontal cortex (mPFC) and dorsal raphe nucleus (DRN), and that these regions are functionally affected by genetic 5-HTT down-regulation, we hypothesized that 5-HTT expression modulates the effect of controllability on stressor impact and coping. Here, we investigated the effects of a signaled stress controllability task or a yoked uncontrollable stressor on behavioral responding and mPFC and DRN activation. 5-HTT$^{-/-}$ rats proved better capable of acquiring the active avoidance task than 5-HTT$^{+/+}$ animals. Controllability determined DRN activation in 5-HTT$^{+/+}$, but not 5-HTT$^{-/-}$, rats, whereas controllability-related activation of the mPFC was independent of genotype. These findings suggest that serotonergic activation in the DRN is involved in stress coping in a 5-HTT expression dependent manner, whereas mPFC activation seems to be implicated in control over stress independently of 5-HTT expression. We speculate that alterations in serotonergic feedback in the DRN might be a potential mechanism driving this differential stress coping.



KEYWORDS: *Serotonin, serotonin transporter, stress, controllability, avoidance, dorsal raphe nucleus*

The etiology of stress-related disorders is complex and poorly understood, but stress is one factor which certainly plays a role in its pathogenesis. However, the impact of a stressor depends on the vulnerability of the individual, as conferred by genetic factors, as well as properties relating to the stressor itself. Elucidating the neural mechanisms mediating such gene-environment interactions will increase our understanding of the disorders, and may lead to opportunities for the development of new therapeutic strategies.

An important category of genetic factors determining individual vulnerability is those influencing the expression of the serotonin transporter (5-HTT). Human carriers of the short (s) allelic variant, displaying reduced 5-HTT transcription and expression,[1] have been shown to be more anxious[2] and extra vulnerable to stress-related mental disorders, such as major depression.[3] In order to study this genetic variant and the vulnerability it confers with regard to affective disorders, serotonin transporter knockout (5-HTT$^{-/-}$) mice and rats have been developed.[4] These animals are characterized by altered susceptibility to various stressors. Data from mouse models have, for instance, revealed increased anxiety-like behavior in response to a chronic resident intruder paradigm in mice with reduced expression of 5-HTT.[5] Furthermore, repeated social defeat stress was shown to impair fear extinction learning in 5-HTT deficient mice,[6] and brief exposure to predator odor was shown to induce long-lasting anxiogenesis in the light-dark box and elevated plus maze assays in 5-HTT$^{-/-}$ mice selectively.[7] Moreover, exaggerated epinephrine responses have been noted in response to stress,[8] while hypothalamic−pituitary−adrenal axis responsivity seems to be unaffected by altered 5-HTT expression.[5a]

However, all these studies addressed the interaction between 5-HTT signaling and stressors that were in fact uncontrollable. A yet unexplored facet of 5-HTT-dependent stress sensitivity is how it is modulated by this exact controllability of the stressor. Control over a stressor (as reviewed by Maier et al.[9]) diminishes its impact, such that the typically stress-induced behavioral phenotype that is characterized by neophobia, increased expression of fear behavior, and increased anxiety, does not occur in response to controllable stress. These features have also been reported in naïve 5-HTT rodents.[10] Uncontrollable stress activates serotonergic neurons in the dorsal raphe nucleus (DRN), while mPFC activation during controllable stress is known to inhibit DRN activation.[11] It has been demonstrated that serotonin depletion in the mPFC increases active stress coping,[12] suggesting that prefrontal serotonin levels play an important role in steering the behavioral response to controllable stress. Because intracellular prefrontal serotonin levels are reduced in 5-HTT$^{-/-}$ rats[13] (while extracellular serotonin levels are increased in 5-HTT$^{-/-}$ mice; see ref 14), it is plausible that these animals cope more actively with stressors, when they are controllable.

Special Issue: Serotonin Research

Published: July 1, 2015

DOI: 10.1021/acschemneuro.5b00126
ACS Chem. Neurosci. 2015, 6, 1143−1150

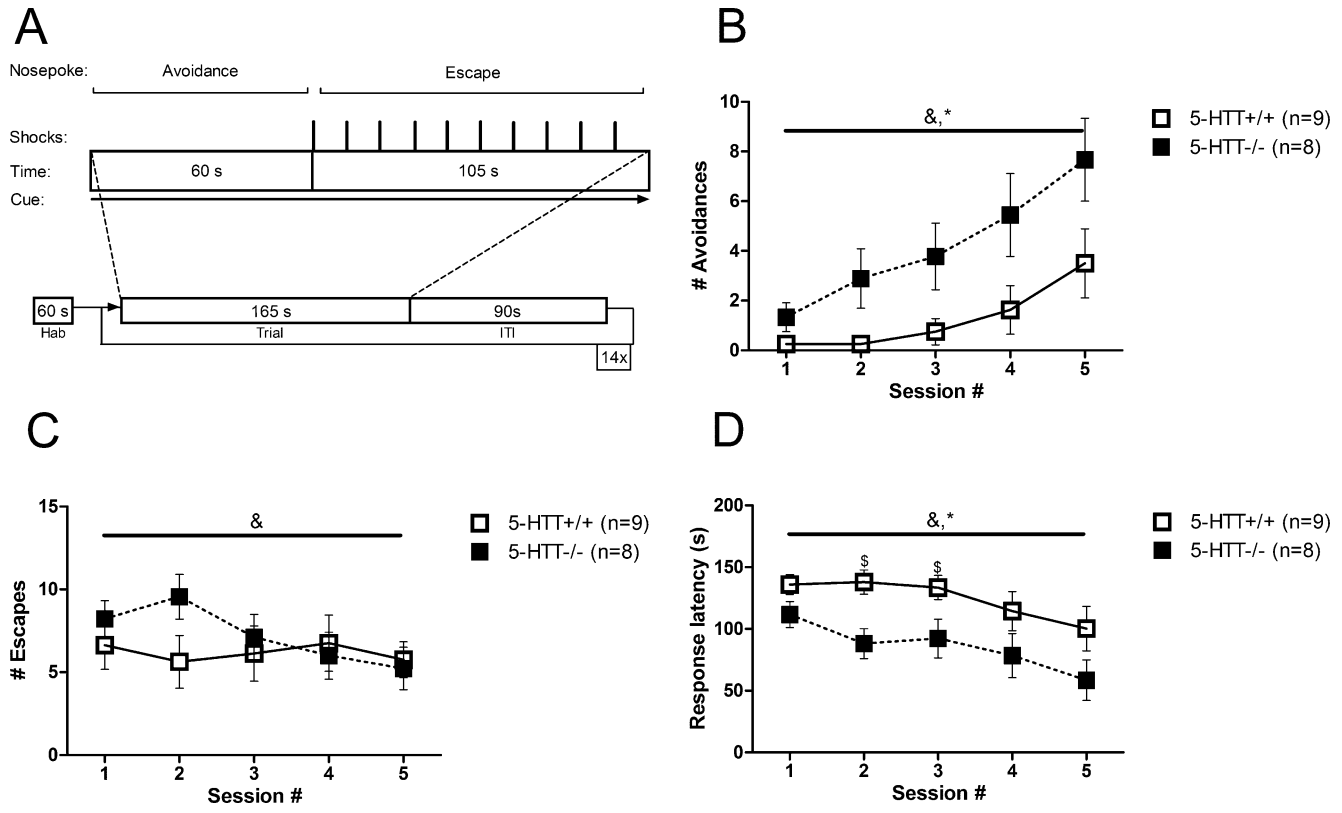


Figure 1. Active avoidance behavioral assay. (A) Outline of a signaled stress controllability session. During 14 trials, animals were presented with a compound stimulus, consisting of illumination of the nose-poke hole and a constant tone. During the first 60 s of this signal (i.e., the avoidance period), animals were able to nose-poke to avoid shocks. The cue would be discontinued immediately, and the trial would move on to the intertrial interval phase; this was considered an *avoidance* response. If animals failed to respond during the avoidance phase, a 1 s 0.6 mA scrambled foot shock was administered, followed by another foot shock every 10 s until 10 foot shocks were administered or the animal responded. If an animal nose-poked during this period, the compound stimulus and foot shocks were discontinued immediately and the trial moved on to the intertrial interval; this was considered an *escape response*. Failure to respond during this phase was considered a *non*-response. (B), Development of avoidance responses during the two-chamber sessions. (C) Development of escape responses across daily sessions did not differ between genotypes in the two-chamber test. (D) Mean nose-poke response latency across daily sessions was significantly lower in 5-HTT$^{-/-}$ rats during the two-chamber sessions. Sessions were 24 h apart. Data are expressed as mean ± SEM. * indicates a significant effect of genotype ($p > 0.05$), & or && indicates a significant effect of session ($p < 0.05$ or $p < 0.01$, respectively), + or ++ indicates a significant genotype by session interaction ($p < 0.05$ or $p < 0.01$, respectively), and $ indicates a significant genotype effect in a single session ($p > 0.05$, Student's t test).

To evaluate how 5-HTT genotype affects coping with a controllable stressor, and if stress controllability affects serotonergic signaling in the DRN and activity of the mPFC in response to stress in a 5-HTT expression dependent manner, we exposed 5-HTT$^{-/-}$ rats and their wild-type counterparts to a self-designed triadic controllability experiment. Previous studies had already shown that 5-HTT$^{-/-}$ rats show persistent "maladaptive" freezing in response to signaled uncontrollable stressors in a fear conditioning paradigm.[15] Here, using a similar stressor (i.e., signaled foot shock), we tested whether these animals show "adaptive" active responding when comparable signaled stressors are controllable. For an equal measure of controllable and uncontrollable stressor exposure we subjected rats to either a signaled controllable stress (CSt) paradigm, or a yoked uncontrollable stress (USt) paradigm in which the timing and intervals of the given stressors were matched to those of active avoidance participants, but no actual control was given. Afterward, activation of serotonergic neurons in the DRN was assessed by evaluating coexpression of immediate early gene c-Fos and 5-HT through immunohistochemistry. To explore genotype differences in mPFC activity during controllable and uncontrollable stressor exposure, we also determined neuronal activation in the infralimbic (IL) and prelimbic (PrL) subareas of the mPFC using c-Fos immunohistochemistry.

In our triadic controllability experiment, rats were first trained in one chamber of a shuttle box. Upon presentation of a conditioned stimulus/signal, the animals were enabled to avoid or escape a foot shock by active nose poking (Figure 1A). Once the response criterion of a genotype group average of 70% avoidance responding was met for both genotypes, the rats switched to a two-chamber setting and the paradigm was repeated, with the additional requirement of shuttling over to the opposite shuttle box compartment before an avoidance or escape nose-poke response could be made. Repeated measures ANOVA analysis revealed a significant genotype effect in the number of avoidance responses ($F_{(1,15)}$= 5.486, $p < 0.05$) in the two-chamber paradigm, with 5-HTT$^{-/-}$ animals displaying more avoidance responses than wild-types (Figure 1B). No effect of genotype was seen in the number of escape responses ($F_{(1,15)}$ = 0.459, $p > 0.05$, Figure 1C). 5-HTT$^{-/-}$ animals responded also significantly faster to the cue than 5-HTT$^{+/+}$ animals ($F_{(1,15)}$ = 5.333, $p < 0.05$) (Figure 1D). These data show that 5-HTT$^{-/-}$ rats, in line with what we hypothesized, display enhanced avoidance acquisition and lower response latency in a signaled controllable stress test. Similar results were

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ACS Chem. Neurosci. 2015, 6, 1143−1150

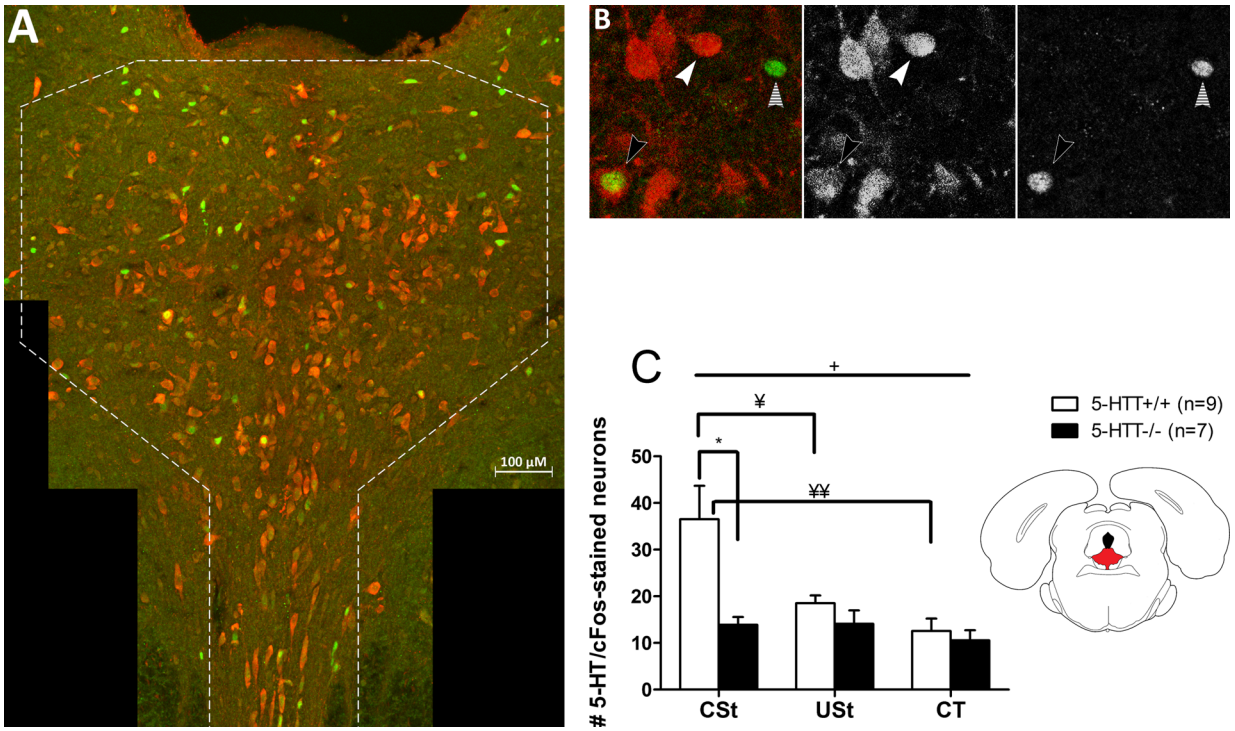

Figure 2. Activation of serotonergic neurons was assessed through measuring 5-HT + c-Fos colocalization. (A) Colocalization was assessed in the region depicted here, in coronal sections corresponding to −8.00 mm from bregma. The fluorescence channel corresponding to c-Fos is displayed as green, and 5-HT is colored red. Scale bar: 100 μm. (B) Close-up view of a 5-HTergic neuron colocalizing with a c-Fos immunoreactive nucleus (black arrow), non colocalizing 5-HTergic neuron (white arrow) and non colocalizing c-Fos positive nucleus (striped arrow) as visualized in combined Cy3 (red) and Alexa488 (green) signal (left panel), Cy3 signal only (middle panel) and Alexa488 signal only (right panel). (C) Colocalization of 5-HT and c-Fos immunoreactivity was increased in 5-HTT$^{+/+}$ rats, but only after controllable stress. Data are expressed as mean number of colocalizations ± SEM. CSt, controllable stress. USt, uncontrollable stress. CT, control treatment. * indicates a significant effect of genotype ($p < 0.05$), ¥ or ¥¥ indicates a significant effect of stress ($p < 0.05$ or $p < 0.01$, respectively). + indicates a significant genotype by stress interaction ($p < 0.05$).

obtained in the one-chamber training; these data are presented in Supporting Information Figure 1.

Intuitively, this observation of improved acquisition of avoidance behavior under controllable stress conditions might seem at odds with the pattern of heightened basal emotional behavior found in both 5-HTT$^{-/-}$ rats and mice, and their increased sensitivity to uncontrollable stress.[10] A previous study reported on impaired shock escape in unstressed 5-HTT$^{-/-}$ mice in an unsignaled single session shock escape assay.[16] Potentially, differences in the experimental (signaled vs unsignaled) and behavioral (multiple vs single session) setups may determine the differences in stress coping responses. We here show that 5-HTT$^{-/-}$ rats display enhanced coping behavior in a controllable stress setting. Possibly, this improved active stress coping is facilitated by the improved cognition seen in these animals, as evidenced by the findings of enhanced reversal learning and extradimensional set-shifting;[17] elevated awareness of environmental cues could contribute to increased performance in the present behavioral task.

Ninety minutes after conclusion of the last behavioral session the rats were transcardially perfused. We also included a control treatment (CT) group which was exposed to the same handlings and signals as the controllable stress and uncontrollable stress groups, but not the foot shocks. Brains were used for c-Fos (recent neuronal activity marker) and 5-HT fluorescence immunostaining in the DRN (Figure 2A,B), and c-Fos immunostaining in the mPFC (Figure 3A). In the DRN, we found a significant genotype × stressor interaction ($F_{(2,42)} = 5.3$, $p < 0.01$) for 5-HT+c-Fos coexpressing neurons (Figure 2C). 5-HTT$^{+/+}$ rats exposed to controllable stressors showed more double-labeled neurons compared to controllable stress exposed 5-HTT$^{-/-}$ subjects ($t_{(1,7.8)} = -3.1$, $p < 0.05$). Bonferroni-corrected post hoc analysis revealed significant differences between controllable and uncontrollable stressor exposed 5-HTT$^{+/+}$ rats ($p < 0.05$) and between controllable stress exposed 5-HTT$^{+/+}$ and control 5-HTT$^{+/+}$ rats ($p < 0.01$). Thus, controllable stress, but not uncontrollable stress, increased activation of serotonergic neurons in 5-HTT$^{+/+}$ rats, which was not observed in 5-HTT$^{-/-}$ rats. Serotonergic activation of subdivisions of the DRN is specified in Supporting Information Figure 2.

The inhibitory 5-HT$_{1A}$ autoreceptors in the DRN potentially play a role in this observation. It has been reported that their purported function, namely autoinhibition of 5-HTergic signaling, is still intact and in fact hyperresponsive in 5-HTT$^{-/-}$ mice,[18] although 5-HT$_{1a}$ mRNA was found to be decreased.[19] This finding suggests that changes in within-DRN signaling may contribute to the altered activity of serotonergic neurons in 5-HTT$^{-/-}$ animals in response to controllable stressors, although further specification of signaling in the DRN after stressor exposure would be necessary to elaborate on this. It should be noted that the DRN is a heterogeneous area in terms of cellular makeup,[20] and additional information on the inhibitory or excitatory nature of the activated cells could also contribute to a better understanding of how the local network within the DRN is affected by 5-HTT abolishment.

DOI: 10.1021/acschemneuro.5b00126
ACS Chem. Neurosci. 2015, 6, 1143−1150

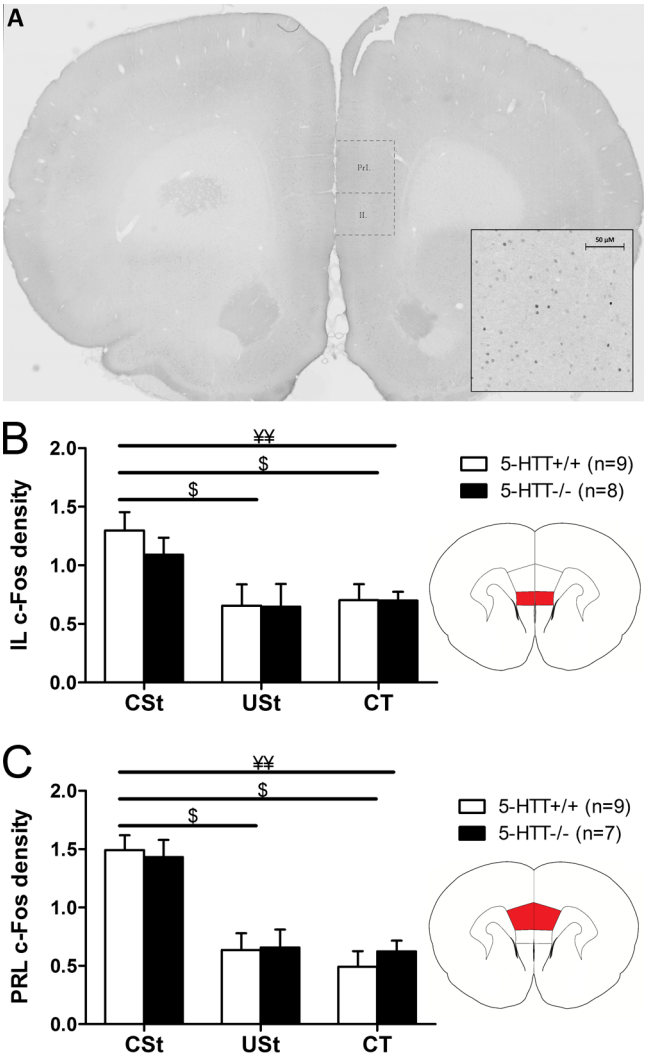

Figure 3. Neuronal activation in the mPFC was measured by quantifying c-Fos immunoreactivity. (A) Density of c-Fos immunoreactive nuclei was determined in the IL and PrL regions of the mPFC in coronal sections between −4.20 mm and −2.20 mm distance from bregma. The density of c-Fos immunoreactivity in the IL region (B) as well as the PrL region (C) was increased after exposure to controllable, but not uncontrollable stressors, in both genotypes. Data are expressed as number of c-Fos positive nuclei detected per 10.000 pixels + SEM. CSt, controllable stress. USt, uncontrollable stress. CT, unstressed control. ¥¥ indicates a significant effect of stress ($p < 0.01$). $ indicates that a significant difference was found between two stress conditions in Bonferroni post hoc analysis ($p > 0.01$).

Another noteworthy aspect of the present findings is the lack of increased serotonergic activity in the DRN following uncontrollable stressor exposure in either genotype, as has been reported previously by others.[11,21] However, the chronic component in this experiment (i.e., animals were exposed to uncontrollable stressors for 10 consecutive days) may be of critical importance here, and makes comparisons with findings from experiments using acute stressors difficult. Repetition of the stressor may have caused a habituation-like effect on serotonergic circuitry in the DRN, thereby diminishing the effect of the stressor on the serotonergic response. Such habituation of activation has been observed in multiple studies wherein neuronal activation in the DRN or serotonin release after acute and chronic stress was compared.[22]

To investigate whether differential serotonergic signaling in 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ rats related to distinct activation of the mPFC in response to controllable and uncontrollable stress, we next analyzed c-Fos expression levels in this region, divided into the IL and PrL cortices (Figure 3A). A two-way ANOVA revealed a significant main effect of stressor ($F_{(2,43)} = 27.23$, $p < 0.01$), but no significant effect of genotype ($F_{(1,43)} = 0.08$, NS) or genotype × stressor interaction effect ($F_{(2,43)} = 0.25$, NS) on the density of c-Fos immunopositive nuclei in the PrL subregion of the mPFC (Figure 3b). Similarly, in the IL, a significant main effect of stressor ($F_{(2,41)} = 7.99$, $p < 0.01$), but no effect of genotype ($F_{(1,41)} = 0.35$, NS), or genotype × stressor interaction ($F_{(2,41)} = 0.30$, NS) was found (Figure 3c). Bonferroni post hoc analysis showed that neuronal activation in the IL and PrL was significantly higher in the CSt group than in the USt and CT groups ($p < 0.01$ in both comparisons), while it did not differ between USt and CT conditions (non-significant in both comparisons). These data show that the mPFC is activated after exposure to controllable stressors, but not uncontrollable stressors, in 5-HTT$^{+/+}$ and 5-HTT$^{-/-}$ rats alike. The finding of increased c-Fos expression in the mPFC only in our controllable stress group is consistent with other reports of recruitment of prefrontal regions during controllable stressor experience.[11,21,23] The lack of a genotype effect on recent mPFC activation could be explained by the similar performance of the genotypes during the final behavioral session.

To test the relationship between the behavioral and brain activation data under controllable stress conditions, we next tested for any significant correlations between behavioral outcomes obtained during the last session of the double-chamber signaled controllable stress test, as well as mean response latency across all session and immunohistochemical data from the DRN (5-HT + c-Fos coexpressing cells) and the mPFC (c-Fos positive nuclei) (Table 1). While correlations between these behavioral parameters and IL/PrL activation could not be detected in either genotype, avoidance behavior during the last test session correlated positively with activation of serotonergic neurons in the DRN in 5-HTT$^{+/+}$, but not in 5-HTT$^{-/-}$ rats. This indicates that the lower performance of 5-HTT$^{+/+}$ rats in this task was accompanied by increased activity of serotonergic neurons in 5-HTT$^{+/+}$ rats, whereas performance seemed unrelated to activity of serotonergic neurons in 5-HTT$^{-/-}$ rats. Mean escape latency during the last behavioral session correlated with serotonergic DRN activation as well in these animals. In sum, whereas there seemed to be a clear link between DRN activity and behavior under conditions of controllable stress and behavioral output in terms of active avoidance task performance in the 5-HTT$^{+/+}$ rats, no such associations were found in the 5-HTT$^{-/-}$ rats.

It seems remarkable that, although 5-HTT$^{-/-}$ animals have repeatedly been shown to suffer from impaired extinction of conditioned fear responses,[15,24] they excel at acquiring the escape and avoidance responses to the "conditioned stimulus" that predicts the incoming stressor in our experiment. Apparently, 5-HTT$^{-/-}$ rats were able to overcome their impairment in the presence of operant control over the foot shock stress, implying that the possibility to control stress takes precedence over a conditioned stimulus predicting uncontrollable stress. In our experimental set,up the conditioned stimulus contingency is gradually updated from a danger signal toward a stimulus that signals controllability and, secondary, safety. This process may resemble extinction of the fear-predicting value of

DOI: 10.1021/acschemneuro.5b00126
ACS Chem. Neurosci. 2015, 6, 1143−1150

Table 1. Relation between Activation of Serotonergic Neurons in the DRN and Behavioral Markers of Stress Controllability Task Performance Was Investigated through Comparing Pearson Correlation Outcomes between Genotypes[a]

		genotype		
		5-HTT$^{-/-}$ 5-HT + c-Fos double labeled cells	5-HTT$^{+/+}$ 5-HT + c-Fos double labeled cells	significance of comparison
escape latency during the final session	Pearson correlation	0.085	0.808	0.100
	sig. (two-tailed)	0.841	**0.015**	
mean escape latency	Pearson correlation	0.095	0.732	0.186
	sig. (two-tailed)	0.824	**0.039**	
avoidances during the last session	Pearson correlation	−0.251	−0.648	0.418
	Sig. (two-tailed)	0.549	0.083	
nonresponses during the last session	Pearson correlation	−0.087	0.858	**0.030**
	sig. (two-tailed)	0.837	**0.006**	
c-Fos density in IL	Pearson correlation	0.389	0.542	0.757
	Sig. (two-tailed)	0.341	0.165	
c-Fos density in PrL	Pearson correlation	0.456	0.131	0.569
	sig. (two-tailed)	0.257	0.758	

[a]Comparisons of correlations between genotypes were made using two-tailed Fisher r-to-z analysis. Behavioral markers correlated with serotonergic activation in the DRN in 5-HTT$^{+/+}$, but not 5-HTT$^{-/-}$ rats.

the conditioned stimulus in cued fear-extinction paradigms. Given that fear extinction is mediated by the mPFC (see Quirk et al. for review),[25] genotype differences in IL/PrL neuronal activity in response to controllable stress exposure in our task could be expected. However, the improved performance of the 5-HTT$^{-/-}$ animals was not reflected in increased neuronal activation in these mPFC subareas, nor did performance in the behavioral test correlate with IL/PrL neuronal activation. Improved signaled active avoidance acquisition was previously shown to correspond with altered behavior-dependent ΔFosB protein expression in the mPFC of behaviorally inhibited Wistar Kyoto rats,[26] although the use of a chronic neuronal activation marker in this study[27] prevents direct comparison.

Some limitations to this study should be mentioned. First of all, freezing and locomotion were not measured during the behavioral proceedings; therefore, we cannot exclude differences in mobility contributed to the genotype effects found in the acquisition of avoidance behavior. However, alterations in 5-HTT expression are reported not to influence general locomotion in rats,[28] although modest effects have been reported in mice.[29] Second, because successful avoidances prevented shock administration and 5-HTT$^{-/-}$ animals acquired the task more effectively, 5-HTT$^{+/+}$ received more shocks during most sessions (Supporting Information Figure 3). Therefore, it is possible that the findings of 5-HTergic activation in the DRN were affected by differences in shock quantity between genotypes. Furthermore, the readout of neuronal activation in the mPFC carries some ambiguity, as a lack of colabeling for cell-type leaves open the possibility that different stress conditions or genotypes favor recruitment of different neuronal populations.

■ CONCLUSION AND FUTURE DIRECTIONS

Although genetic 5-HTT downregulation is known to be associated with poor stress resilience and persistent negative emotional behavior, 5-HTT$^{-/-}$ rats were shown to outperform their wild-type counterparts during the acquisition of a signaled controllable stress task. We did not include 5-HTT$^{+/-}$ rats in this experiment, which is regarded as a closer model for 5-HTTLPR s-allele carriers in terms of gene dose-dependency. Since the s-allele has been associated with increased trait anxiety,[2] and has been linked to the emergence of affective disorders in these individuals,[3] most research has focused on poor stress resilience. However, in line with our findings, it has also been demonstrated that healthy s-allele carriers display improved active avoidance.[30] Evolutionary biology predicts that the high prevalence of the 5-HTTLPR s-allele reflects overall positive or adaptive effects of this s-allele.[31] Improved coping with (signaled) controllable stress may reflect such an adaptive effect.

Although present and other findings strongly suggest that stressors drive the DRN differently in animals with compromised 5-HTT expression, more work is needed to elucidate what mechanisms are at the basis of this. Further characterization of the neuronal activation in the mPFC and DRN after CSt and USt, including identifying the type of neurons being activated through costaining for inhibitory and excitatory markers, as well as the circuits they connect to through tracer imaging, will help clarify how the development and function of the mPFC and DRN depend on 5-HTT expression. Moreover, functional manipulations of the serotonergic circuits in these regions, using optogenetic or pharmacological interventions, could demonstrate their functional involvement in mediating the behavioral effects observed in the present paradigm. Furthermore, assessing 5-HT$_{1a}$ receptor expression, function and ligand binding qualities in naïve and stressed 5-HTT$^{-/-}$ animals will inform us on the role of both the autoreceptors in the DRN and the heteroreceptors in the mPFC in driving the stress response and its persistent consequences. Moreover, assessing emotional and cognitive behavioral parameters within a certain time interval after CSt and USt will reveal the trans-situational and persistent impact of stressor controllability on emotion regulation and cognitive functioning. Finally, it remains unclear to what degree the effects of 5-HTT abolishment on active avoidance behavior and associated controllability-dependent DRN activation effects are

DOI: 10.1021/acschemneuro.5b00126
ACS Chem. Neurosci. 2015, 6, 1143−1150

mediated through acute alterations in 5-HTergic neurotransmission in adult life, or through altered 5-HT-mediated neurodevelopment.[32] Additional experiments using conditional 5-HTT knockdown models are necessary to dissociate these effects.

METHODS

Animals. 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. Serotonin transporter knockout rats (Slc6a41Hubr) were generated on a Wistar background by *N*-ethyl-*N*-nitrosurea (ENU)-induced mutagenesis[4b] and have been described previously.[28] Experimental animals were derived from crossing heterozygous 5-HT transporter knockout (5-HTT$^{+/-}$) rats that were outcrossed for at least 12 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). Since stress sensitivity in females is dependent on their estrous cycle phase,[33] we here restricted ourselves to the gender with the most robust and stable stress response. All animals had ad libitum access to food and water. A 12 h light−dark cycle was maintained, with lights on at 0800 AM. All behavioral experiments were performed between 0800 AM and 1800 PM.

Apparatus. A 40.6 cm (width) × 15.9 cm (depth) × 21.3 cm (height) rectangular shuttle box (model ENV-010MD, Med Associates, St. Albans, VT) was used, which was split into two identical chambers with an automated door and housed within a sound-attenuating cubicle. Each compartment was equipped with a circular nose-poke hole of 2.5 cm circumference containing an infrared detection mechanism and a white LED light, as well as a speaker capable of producing an 85 dB 2.8 kHz tone. Eight infrared beams were installed in order to detect the position of the animal. The grid floor of the apparatus was connected to a scrambled shock generator (model ENV-412, Med Associates).

Yoked Triadic Controllability Design. The signaled active avoidance paradigm is briefly detailed in Figure 1A. For an in-depth description, we refer to the methods in the Supporting Information. In order to differentiate controllable and uncontrollable stressors, responses from the controllable stress test were recorded and used to create "yoked" uncontrollable stress groups; these rats were exposed to foot shocks and signals of the same duration and intervals as rats from the controllable stress group. Since these animals were not able to influence the stressor with their behavior, no behavioral parameters were recorded for this group. The animals that were subjected to this yoked paradigm are referred to as the uncontrollable stress (USt) group. The USt treatment was performed after the active avoidance behavioral proceedings of the CSt group. We included a control treatment group to dissociate the effect of controllability from the effects of the stressor. The animals belonging to this control group were exposed to the same visual and auditory signals of the controllable paradigm, but not the foot shocks. Uncontrollable stress and control rats were individually matched to rats of their own genotype from the controllable stress group in terms of the number of shocks administered (uncontrollable stress only) and time spent in the shuttle box. This paradigm differs in several key aspects from classic wheel-turning paradigms that have been employed to determine the influence of controllability of stressors, such as predictability,[34] methods of shock administration, freedom of movement, and method of control over the stressor. The triadic yoked element is transferred fully intact from that paradigm, however; every animal from the controllable stress group was matched up with an animal from the uncontrollable stress group and one from the control treatment group of its own genotype, ensuring that controllability of the stressor was the only aspect in which the treatment of animals from the controllable and uncontrollable groups differed.

Immunohistochemistry. Ninety minutes after conclusion of the last behavioral session, rats were anesthetized and perfused transcardially with 0.1 M phosphate buffered saline (PBS), and subsequently by 4% paraformaldehyde in 0.1 M PBS. Brains were collected, postfixed in the same fixative for 30 min and subsequently stored in 0.1 M PBS at 4 °C until sectioning. Before sectioning brains were put in a 30% sucrose in 0.1 M PBS solution. When brains were saturated (and had sunk) (∼2 days) 40 μm thick coronal sections were frozen and cut on a sliding microtome (Microm HM 440 E, Thermo Fisher Scientific Inc., Waltham, MA). Sections were stored at 4 °C in 0.1 M PBS with 0.01% NaN_3 (antimicrobial) until use. DRN sections were then stained for 5-HT and c-Fos, and mPFC sections were stained for c-Fos. A detailed description of the staining and quantification protocols can be found in the Supporting Information methods.

Statistical Analysis. All statistical analyses were performed using SPSS Statistics version 17.0 (SPSS Inc., Chicago, IL). Data are presented as mean ± standard error of the mean (SEM). Behavioral and immunohistochemical data was analyzed using a repeated-measures analysis of variance (ANOVA) and a two-way ANOVA, respectively, with genotype and stress (uncontrollable stress, controllable stress, control (no stress)) as between-subject factors. When appropriate, subsequent Bonferroni post hoc tests were performed to further specify genotype or stress condition effects, or Student's *t* tests to explore interacting effects. Probability *p* values of less than 0.05 were considered significant. Pearson's correlations were used to assess relations between behavioral and immunohistochemistry outcomes, and compared across genotypes using Fisher r-to-z transformation.

ASSOCIATED CONTENT

Supporting Information

Development of avoidance responses, escape responses, and nose-poke reponse latency from the one-chamber signaled active avoidance task; 5-HT + c-Fos colabeling in subregions of the DRD, DRV, and DRVL; additional methods. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschemneuro.5b00126.

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Behavioral experiments: P.S., D.L., R.J.R., J.J.; immunohistochemical work: P.S., D.L., R.J.R.; data analysis: P.S., D.L., R.J.R., M.J.A.G.H.; experimental design: P.S., J.J., T.K., J.R.H.; manuscript writing: P.S., M.J.A.G.H., J.R.H.

Funding

This work was supported by The Netherlands Organization for Scientific Research (NWO), Grant No. 864.10.003 awarded to J.R.H.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Anthonieke Middelman for the breeding and genotyping of the animals, Jos Dederen for his assistance for the immunostaining, and Jesse Stoop for his contributions in the quantification of c-Fos immunoreactivity. Funding organizations had no further role in the design of the study, nor in the collection, analysis, and interpretation of data.

DOI: 10.1021/acschemneuro.5b00126
ACS Chem. Neurosci. 2015, 6, 1143−1150

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