Smart coping:

serotonin transporter gene variation and environment interact in determining the behavioral adaptation to stress

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General introduction

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Stress

Stress, in the broadest sense, is defined as an environmental stimulus that threatens homeostasis in an organism. Stress-inducing stimuli, or stressors, can take on many forms but be they physical, social, psychological, the aforementioned description applies to all of them. The stress response is defined as the efforts the organism undertakes in order to restore homeostasis, including neuroendocrine, neuroplasticity and behavioral adjustments. Respectively, these help prepare the individual for action and increase vigilance, learn and store relevant information about the stressor to optimize future encounters with it, and protect the organism, either actively by initiating a fight/flight response in order to remove or escape the stressor, or passively to minimize harm resulting from the stressor and conserve energy (Chrousos and Gold 1992). These mechanisms of the stress response provide vital benefits to the organism, allowing it to survive and thrive in threatening environments. However, prolonged or repeated exposure to stress, or extreme stress, have been shown to turn the stress response maladaptive and contribute to the pathogenesis of psychiatric disorders, such as major depressive disorder (MDD) and post-traumatic stress disorder (PTSD). A substantial degree of inter-individual variation exists in the susceptibility to these detrimental effects of stress. This may be caused by differences in endocrine as well as behavioral responses to stress due to disparities in genetic and environmental circumstances. In this thesis, we will examine how a genetic factor that regulates the reuptake of serotonin in the brain interacts with properties of a stressor to affect stress coping behavior and the consequences of stress exposure.

The neuroendocrine stress response

Detection of a stressor initiates a cascade of stress hormone release, orchestrated primarily by the hypothalamus, the pituitary and the adrenal glands (i.e., the hypothalamic-pituitary-adrenal (HPA) axis). The neuroendocrine stress response is initiated when sensory systems in the central nervous system perceive a stimulus or situation as threatening. This triggers the release of corticotropin-releasing hormone (CRH) from the neurons originating in the paraventricular nucleus of the hypothalamus. CRH subsequently activates the anterior pituitary, causing it to secrete adrenocorticotropic hormone (ACTH), which then stimulates cortisol (CORT) release from the adrenal gland (De Kloet et al. 1998). CORT provides the energy required to cope with the stressor by increasing blood sugar levels through gluconeogenesis and accelerating metabolism of nutrients and lipid reserves (Ray et al. 1964, Nieuwenhuizen and Rutters 2008). In addition, CORT dampens its own release; it is instrumental in the negative feedback loop that controls activity of the HPA axis and inhibits further release of CRH and ACTH. While CORT is an essential component of the acute adaptive stress response, it is also strongly implicated in the detrimental effects of prolonged and excessive stress

exposure; abnormalities in basal CORT and responsivity of the HPA axis are implicated in both MDD and PTSD (Plotsky et al. 1998, Meewisse et al. 2007). Parallel to the actions of the HPA axis, sensory detection of a stressor initiates sympathetic activation and induces the synthesis and peripheral release of noradrenalin from the adrenal glands, mediating the effects of stress on heart rate and blood pressure (Iversen 2000), as well as central release from the locus coeruleus, eliciting a state of arousal (Aston-Jones and Cohen 2005, Sara and Bouret 2012).

The neuroendocrine stress response affects mnemonic function; acute stress exposure is known to enhance the encoding and consolidation of new memory traces while impairing memory retrieval (Roozendaal and McGaugh 2011). This effect is thought to be mediated by the actions of CORT and noradrenalin on plasticity in the basolateral amygdala (BLA). Experimental evidence from rodent studies report that arousal through noradrenergic activation of the BLA is a necessary prerequisite for CORT-mediated enhancement of memory function (Okuda et al. 2004). Stress-enhanced formation and storage of memory may contribute to the pathogenesis of psychiatric disorders resulting from a traumatic experience (further discussed in section *Stress-related disorders* of this chapter).

The behavioral stress response: coping strategies

Stress coping is defined as the actions an individual undertakes to mitigate the effects of the stressor it encounters. The manner in which a stressor is coped with strongly affects its influence on the individual, and is also thought to determine the stressor's potency for contributing to the pathogenesis of psychiatric disorders (Koolhaas et al. 1999). Broadly, coping strategies can be divided into active (i.e., problem-focused) coping, and passive (i.e., emotion-focused) coping. Active coping strategies aim to remove the stressor or escape from it (fight/flight), while passive coping strategies (e.g., freeze) aim minimize harm from the stressor and conserve energy, potentially until an opportunity arises to fight or escape it (Lazarus 1993a). Adopting a coping style that suits the situation best can minimize the harmful effects of a stressor, whereas a maladaptive coping style can worsen the situation. Adopting a passive coping style in the face of a controllable stressor (i.e., one that can be avoided or escaped from) exacerbates its effects through unnecessarily prolonging exposure to it, while choosing an active coping style to confront an uncontrollable stressor (which cannot be avoided or escaped from) expends energy unnecessarily, thereby reducing the individual's capacity to respond to situational changes. Therefore, it is not the general tendency in coping strategy, but rather the flexibility in coping style that seems to determine how stress coping defines resilience (Austenfeld and Stanton 2004). Previous stressor coping experience may determine subsequent coping responses - e.g., an individual that has learned to cope passively in a certain situation

may have trouble adopting active coping strategies in the future, even if the context requires it - thereby decreasing coping flexibility and stress resilience (Homberg 2012, Nederhof and Schmidt 2012).

Stress-related disorders

Major depressive disorder

Major depressive disorder (MDD) is a debilitating mood disorder which is characterized by low mood, anhedonia, disturbed eating and/or sleeping patterns, reduced energy levels and in some cases even suicidal ideation (American Psychiatric and American Psychiatric Association 2013). MDD is one of the most prevalent and deleterious mental disorders across the world. In the majority of countries 8-12% of the population encounters a depressive episode during their lives (Andrade et al. 2003), and the global burden of disease caused by depressive disorders is the highest among all mental illnesses (Whiteford et al. 2013), with women having a higher risk of both onset and recurrence of the disorder (Kessler et al. 1993). Although the precise mechanisms behind the pathogenesis of depression remain elusive, much is already known about the factors that contribute to its development. The principal risk factors for depression can be divided into genetic and environmental categories, while some genetic and environmental factors only pose a risk in interaction with each other (Lesch 2004). These gene-environment interactions make up a promising new avenue of research that could well lead to new insights into the variation in susceptibility to depression that exists within the population. Furthermore, this type of research can provide opportunities for the development of novel therapeutic interventions. The wide range of risk factors contributing to depression and their interacting effects point to a heterogeneity in pathogenic mechanisms, which could warrant a more personalized approach in its treatment. This is urgent, considering that existing therapies are effective in a mere 50% of patients suffering from depression (Rush et al. 2006). Despite the ambiguity surrounding risk factors for developing MDD, it has long been clear that severe stress, both in early and later life, is an important contributor to MDD pathogenesis (Lazarus 1993b, Heim et al. 1997).

MDD is diagnosed according to the criteria stated in the fifth edition of the Diagnostic and Statistics Manual of Mental Disorders (DSM-5); these include:

- Subjectively reported or observed depressed mood
- Disinterest in activities and stimuli generally considered pleasurable (anhedonia)
- Significant gain or loss of body weight
- Insomnia or hypersomnia
- Psychomotor agitation or retardation

- Fatigue, loss of energy
- Diminished self-worth, excessive and/or delusional self-blaming
- Cognitive impairment, indecisiveness
- Recurrent and pervasive thoughts of death, suicidal ideation

Five or more of these criteria must be present throughout a 2-week period, and the presence of either depressed mood or anhedonia is a necessary prerequisite for the diagnosis. The principal lines of treatment for MDD consist of pharmacological treatment, cognitive therapy and electroconvulsive therapy (American Psychiatric 2013). The most commonly used pharmacological treatment consists of chronic administration of selective serotonin reuptake transmitters (SSRIs) (Reid and Barbui 2010), which elevate the level of serotonin (5-HT) in the brain by preventing its reuptake (Sarkissian et al. 1990).

With regards to the pathophysiology occurring in MDD, our understanding is currently limited. The monoamine hypothesis of depression states that its biological basis consists of a monoaminergic imbalance in the brain, specifically a deficiency of norepinephrine and the neurotransmitter 5-HT (discussed extensively below) (Hirschfeld 2000). This hypothesis was formulated on the back of experimental findings in users of the antihypertensive reserpine, which has the side effect of causing depression-like symptoms (Muller et al. 1955). These were found to be reversible by monoamine precursor supplementation, implicating reserpine's effect on 5-HT levels as a mediator of these side effects (Carlsson et al. 1957). This finding led to the development of the presently used first line pharmaceutical treatment for depression; SSRIs. However, the limited efficacy of monoamine-focused pharmacological treatment of MDD has prompted the proposition of alternative and supplemental hypothesis for the pathogenesis of the disorder (Trivedi et al. 2006). It has been suggested that alterations in neural plasticity lie at the basis of the pathogenesis of depression, and that enhancing plasticity may mediate the efficacy of existing and novel therapeutic strategies (Pittenger and Duman 2008). Brain-derived neurotrophic factor (BDNF), an important signalling molecule in synaptic plasticity, appears to be decreased in patients suffering from MDD (Autry and Monteggia 2012), as well as animal models exerting a depressive-like phenotype (Duman and Monteggia 2006). In line with this, animal models of genetically downregulated BDNF exert depressive-like symptoms (Duman et al. 2007), and the beneficial effect of chronic SSRI administration on rats coincides with its influence on BDNF signalling (Duman 1998). Further supporting the plasticity hypothesis are enhancing effects of BDNF (Lee et al. 2002) and successful antidepressant treatment on synaptic plasticity and neurogenesis (Eisch and Petrik 2012), i.e., the formation of new functional neurons in the dentate gyrus of the hippocampus. Reduction of hippocampal neurogenesis is proposed to lie at the basis

of the hippocampal atrophy that is observed in long-time sufferers of MDD (Campbell et al. 2004); an increase in neurogenesis is seen as a result of antidepressant treatment using electroconvulsive shock therapy (Rotheneichner et al. 2014), serotonin norepinephrine reuptake inhibitors (SNRIs) (Mostany et al. 2008) and SSRIs (where preventing the neurogenic effects disrupted their behavioral antidepressant effects) (Santarelli et al. 2003). Pharmacological treatment of depression using SSRIs needs to be sustained for several weeks before any benefits can be observed (2000); this proposed route of action of SSRIs (i.e., through enhanced neurogenesis) could explain this.

An entirely different approach to the treatment of MDD is targeting nutrition, specifically lipid intake. Omega-3 polyunsaturated fatty acids (n-3 PUFA's) are dietary components that cannot be synthesized from other nutrients. Docosahexanoic acid (DHA) is crucial to healthy neuronal membrane physiology, and deficiencies in its intake affect signal transduction, monoamine neural transmission and the formation of the lipid rafts that compartmentalize cell membranes, separating integrated receptors, transporters and signalling molecules (Chalon 2006, Innis 2007). In addition, DHA stimulates synaptic plasticity and neurogenesis (Dagai et al. 2009). Low intake of dietary n-3 PUFA's is associated with increased prevalence of depression (Golding et al. 2009), and dietary supplementation has been proposed as both a monotherapy for depression as well as a complementary therapy. However, therapeutic efficacy of n-3 PUFA supplementation for the treatment of MDD has been limited (Appleton et al. 2015); potentially, the mechanisms that mediate its beneficial effects are only relevant in a subset of MDD patients.

Post-traumatic stress disorder

PTSD is a stress-related disorder that can arise after an individual experiences a severe traumatic event. It is considered an occupational disease for those working in professions that regularly deal with violence and trauma, such as military personnel on active duty, law enforcement and emergency responders. Reports of lifetime prevalence of PTSD are estimated to be around 3.3% for men and 8.5% for women (McLean et al. 2011). PTSD is diagnosed on the basis of the criteria as stated in the DSM-5 which include:

- Exposure to a traumatic event personally or being witness to one, or learning of one occurring to a close friend or family member
- Experiencing intrusive memories, dreams, flashbacks related to the traumatic occurrence, and/or severe psychological / physiological reactions to internal or external cues related to the event
- Persistent avoidance of cues related to the event since its occurrence
- Negative and/or aberrant cognition and emotionality since occurrence of the event, including trouble retrieving memories relating to the event, exaggerated

negative beliefs about oneself, others or the world, distorted cognitions pertaining to cause and consequence traumatic event (e.g. self-blaming), general negative emotional state, reduced interest in significant activities, detachment from others and/or inability to experience happiness or satisfaction
- Significant changes in emotional reactivity and arousal since the event, including proneness to anger, self-destructive behavior, hypervigilance, increased startle response, trouble with mental focus and disturbed pattern of sleep

The symptoms must persist for at least one month, cause significant distress and/or deterioration of social/professional functioning to the individual, and must not be attributable to intoxication, substance abuse or another medical condition (American Psychiatric 2013). First line treatment of the disorder typically consists of cognitive behavioral therapy (CBT), eye movement desensitization and reprocessing (EMDR) and exposure therapy (Ursano et al. 2004), all of which appear to be equally effective (Bisson et al. 2013). Of these, CBT is a therapy aimed at identifying and correcting negative cognitive biases pertaining to the patients themselves, their trauma and their environment. EMDR, in contrast, is a trauma-focused therapy in which the senses are stimulated in multiple sensory dimensions (e.g., using directing visual and auditory attention to a discrete stimulus) while the patient focuses on their aversive memories. Although EMDR has been determined to be as effective as other first line therapies in treating PTSD, its underlying mechanisms remain relatively poorly understood (McGuire et al. 2014). Finally, exposure therapy focuses on extinguishing the physiological and emotional response to stimuli related to the trauma by repeatedly presenting them to the patient in a safe, therapeutic environment. This therapy is based on the theory that PTSD is caused by exacerbation and generalization of aversive associations between the trauma and stimuli present during the individual's encounter with it; i.e., maladaptive persistence of a Pavlovian fear-conditioned association (further discussed in section *Fear, anxiety disorders and 5-HTTLPR*, Box 2) (Shin and Liberzon 2010). The primary first line pharmacological treatment of PTSD consists of SSRI administration (Cuijpers et al. 2013); while novel insights into the mechanistic underpinnings of PTSD have suggested a multitude of potential new avenues for pharmacological treatment of PTSD, most of these are still under investigation and not yet being put into clinical practice (Murrough et al. 2015, Mithoefer et al. 2016). For instance, a novel therapy consisting of trauma recollection followed by propranolol administration aims to destabilize and prevent reconsolidation of trauma memory, and has proven somewhat successful (Kindt and van Emmerik 2016). Furthermore, administration of CORT shortly after a traumatic experience has been shown to reduce the risk of developing PTSD (Schelling et al. 2006), whereas chronic low-dose CORT treatment seems to reduce intrusive symptoms in patients (Aerni et al. 2004).

Findings from neuroimaging studies suggest that PTSD is characterized by a number of anatomical and physiological aberrations. These findings help to give credence to the fear conditioning hypothesis of PTSD, as many of these are located in brain regions and circuits that are involved in the regulation of Pavlovian fear associations; mainly the amygdala, the hippocampus and the prefrontal cortex (PFC) are implicated (Rauch et al. 2006). The amygdala, involved in emotional processing and vigilance (Davis 1992), has been reported to be hyperactive in patients suffering from PTSD, both at rest (Chung et al. 2006), when exposed to aversive stimuli (Linnman et al. 2011), and during the acquisition of conditioned fear (Bremner et al. 2005). The hippocampus, which is generally understood to mediate the encoding and retrieval of episodic memory, serves to link the trauma to contextual cues in PTSD. Reduced activation of the hippocampus has been observed in PTSD patients during failed fear extinction recall (Milad et al. 2009). Moreover, reactivity of the ventromedial PFC (vmPFC), exerting inhibitory control over the amygdala (Quirk et al. 2000), to fearful faces and trauma-related stimuli is decreased in PTSD subjects (Shin et al. 1999, Phan et al. 2006, Felmingham et al. 2010). Structural analysis show that both amygdala and hippocampus volume are decreased in PTSD patients, compared to healthy controls (Ahmed-Leitao et al. 2016). In addition, grey matter reductions were found in the mPFC of PTSD patients (Li et al. 2014).

While PTSD is widely understood to be caused by severe trauma experience, not all individuals who experience a severe traumatic incident develop the disorder. Environmental and genetic factors greatly influence the risk for PTSD. Early life adversity, such as childhood abuse or neglect, has been designated to be an important risk factor for PTSD (Bremner et al. 1993). Similarly, severe stressful experiences during adult life, prior to the traumatic experience from which PTSD originates, enhance risk for PTSD as well as the severity of its symptoms (Breslau et al. 2008). Numerous genetic risk factors have also been identified for PTSD (see Smoller et al. for review (Smoller 2016)); in the interest of brevity, only the genetic variations in the 5-HTT linked polymorphic region (5-HTTLPR) will be discussed here.

Serotonin

Serotonin in MDD

Serotonin is a monoaminergic neurotransmitter that is synthesized from the amino acid L-tryptophan by the enzyme tryptophan hydroxylase (TPH) (Wang et al. 2002). Outside the central nervous system, serotonin serves regulatory functions in the gastrointestinal and cardiovascular system (Kaumann and Levy 2006, Tecott 2007). In the brain, it is an important modulator of neuronal signaling, and is involved in a plethora

of central nervous system functions such as cognition, sleep and emotionality. The role of 5-HT as a regulator of emotionality came to prominence when it was discovered that the efficacy of early antidepressants were mainly attributable to their effect on the brain metabolism of serotonin (Lapin and Oxenkrug 1969). Through the newly emergent practice of disease mechanism-driven drug design, the new antidepressant zimelidine was developed, which selectively reduced the reuptake of serotonin with the aim of enhancing circulating serotonin levels in the brain (Siwers et al. 1977). This entirely new class of antidepressant drugs, the SSRIs, has become a staple in the treatment of MDD, with fluoxetine as its most prominent example (Fuller et al. 1974). SSRI's route of action is to reduce function of the serotonin transporter (5-HTT) by binding to it, thereby reducing the reuptake of 5-HT into synaptic boutons after neurotransmission (Marcusson and Ross 1990).

5-HTTLPR, stress and MDD

The establishment of serotonergic mechanisms in the pathogenesis and treatment of MDD has engendered interest for the influence of genetic variations in the serotonergic systems on the predisposition for mood disorders. It was found that a variable repeat sequence in the promotor of the gene that encodes the human 5-HTT, the 5-HTT linked polymorphic region (5-HTTLPR), determines its expression and function; the short (s) allele of 5-HTTLPR results in reduced transcription and function compared to the long (l) allele (Greenberg et al. 1999). As 5-HTT is the only transporter capable of transporting 5-HT from the extracellular space back to the intracellular compartment, 5-HTT expression and function are expected to affect serotonergic signaling and thus emotionality (Smith et al. 2004). In line with this, carriers of low-functioning alleles of 5-HTTLPR were found to display increased anxiety- and neuroticism-related traits (Lesch et al. 1996), but more pressingly, were also overrepresented in samples of patients suffering from MDD (Collier et al. 1996). The effect of 5-HTTLPR allelic variation on the risk for developing mood disorders appeared to be heavily modulated by traumatic experiences, especially early life stress; the risk of low-functioning variants of 5-HTTLPR for developing MDD is potentiated by early life adversity (Taylor et al. 2006). This association has made 5-HTTLPR the prime example of a genetic factor that produces different outcomes depending on environmental factors, i.e., a gene x environment interaction. In particular, 5-HTTLPR is often used to illustrate vulnerability factors in the diathesis stress model. This model states that the likelihood to develop mood disorders as a result of stress is dependent on the presence of vulnerability and resilience factors; certain "diathesis" traits that may be genetic or other environmental factors that predispose for or protect against psychiatric illness. While converging evidence has indeed confirmed that low-functioning allelic variations of 5-HTTLPR interact with life adversity to increase vulnerability to mood disorders (Karg et al. 2011), it was also proposed that the s-allele in fact enhances receptivity to positive

environmental factors as well (Belsky and Pluess 2009). In this 'differential susceptibility' model, low expressing variants of 5-HTTLPR reflect 'plasticity' rather than 'susceptibility', and actually increase an individual's receptivity to *positive* environmental elements. Therefore, while adversity may produce poorer outcomes in these individuals compared to l-allele carriers, s-allele carriers may reap additional benefits from environmental enrichment and social support.

The s-allele as an environment-dependent vulnerability factor is exemplified by its effects on the neuroendocrine stress response. A meta-analysis has shown that the cortisol response to acute stressors is exaggerated in s-allele carriers, which was most pronounced in individuals with a history of severe life adversity (Miller et al. 2013). This link was proposed to be mediated by increased functional connectivity between the amygdala and hypothalamus (Alexander et al. 2012). However, as discussed previously, detrimental effects of a stressor on an individual are not only dependent on HPA axis homeostasis and reactivity, but also on the appropriateness and efficacy of the behavioral stress coping response to that stressor. Accumulating evidence suggests that differential preferences and tendencies in coping strategy may be a mechanism by which 5-HTTLPR modulates stress susceptibility. For example, trait worry, a form of anxiety predisposing one to - among other psychiatric illnesses - PTSD, is more prevalent in s-allele carriers (Bredemeier et al. 2014). In addition, S-allele carriers newly diagnosed with breast cancer exerted greater anxious preoccupation compared to their l-allele peers (Schillani et al. 2012). These genetic differences in stress (coping) responses seem to hinder responsivity to first-line behavioral cognitive therapies for both MDD and PTSD (Bryant et al. 2010). Drinking-to-cope, or alcohol consumption as a coping mechanism was reported less frequently as a motivation for consuming alcohol by carriers of low functioning allelic variants of 5-HTTLPR (Armeli et al. 2008). This is somewhat surprising, as these allelic variants are also associated with increased alcohol abuse (Thompson and Kenna 2016). In addition, 5-HTTLPR genotype affected the degree to which children internalized and externalized problems (Cline et al. 2015).

In addition to its status as a risk factor for developing depression, the s-allelic variant of the 5-HTTLPR has also been shown to exacerbate the effects of severe combat-related trauma on the development of PTSD symptoms (Telch et al. 2015). However, other studies have found the s-allele to confer increased risk for PTSD only in the presence of pre-existing trauma experience (Xie et al. 2012, Gressier et al. 2013). In addition, preliminary data shows that first line therapeutic interventions to treat PTSD are ineffective in 5-HTTLPR s-allele carriers (Bryant et al. 2010), making the development of therapies specifically suiting the needs of this group of patients urgent.

Stress coping in animal models of 5-HTTLPR

Evidently, coping strategies in humans are complex behaviors; the fact that their underlying mechanisms are as yet poorly described, makes it difficult to speculate how 5-HTTLPR intercedes with them. By comparison, investigating stress coping behavior in animals is relatively straightforward; stressors can be more easily standardized in an experimental environment, the coping behavior of the subjects can be observed directly, and invasive methods of determining neural correlates are available. Work in animal models for genetically induced reduction of 5-HTT expression (either by complete knock-out (KO) of the gene or its partial knock-down (KD)) has replicated its earlier-mentioned interacting effect with (early) life stress in mediating vulnerability to stress-related mental disorders in humans (to some degree). A body of research has shown that 5-HTT heterozygous (5-HTT$^{+/-}$) mice are more susceptible to the anxiogenic effects of early life stress than their wild type counterparts (Carola and Gross 2012). Reduction of 5-HTT expression enhanced the effect of unpredictable chronic mild stress exposure during adulthood, increasing feeding delay in a novelty-suppressed feeding assay and decreasing open arm time in the elevated plus maze test (Joeyen-Waldorf et al. 2009). Similar results were observed after social stress in a resident intruder paradigm during adulthood, suggesting 5-HTT reduction potentiates vulnerability to stressors in the social as well as the physical dimension (Jansen et al. 2010). Moreover, social stress experience resulted in a greater increase in social avoidance in 5-HTT$^{+/-}$ mice compared to wild types. However, in a remarkable example of early life and later life stress interaction with 5-HTT genotype, early life adversity bred resilience to later life inescapable shock-induced behavioral helplessness in 5-HTT$^{+/-}$, but not in wild type or 5-HTT$^{-/-}$ rats. Interestingly, under both naïve and early life stress conditions, 5-HTT$^{-/-}$ animals were more resilient to inescapable-shock induced escape deficits than wild type rats (van der Doelen et al. 2013). As yet, many aspects of the mechanisms by which variation in 5-HTT expression regulate stress sensitivity remains to be elucidated. Contrasting the exclusivity of its behavioral effects to 5-HTT$^{+/-}$ animals, maternal separation affected basal CORT levels in all genotypes *except* 5-HTT$^{+/-}$; it lowered basal CORT in 5-HTT$^{-/-}$ rats and increased it in wild type animals (van der Doelen et al. 2014b). This implies that the altered stress coping and sequelae seen in these animals may be orchestrated by factors outside of the HPA axis.

Stressor controllability

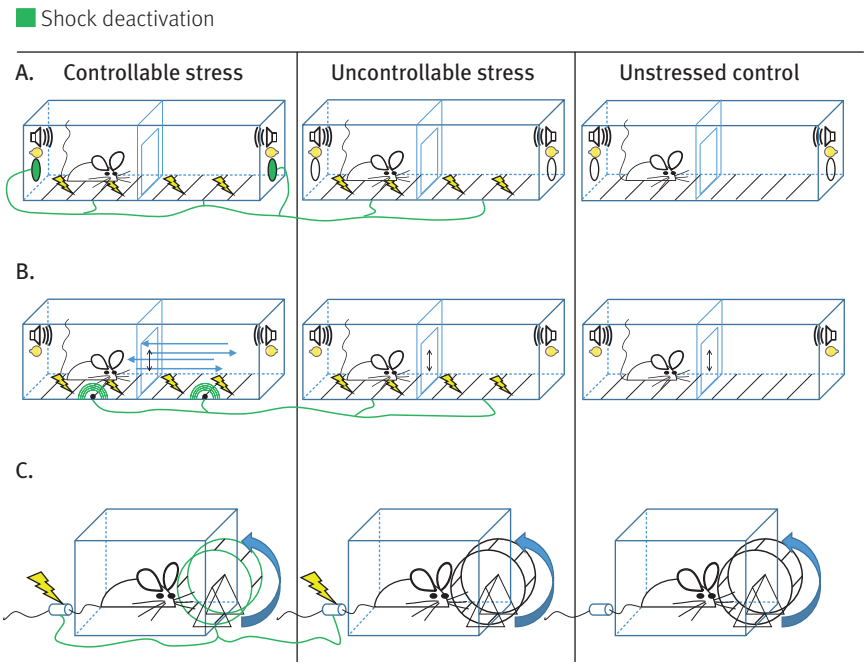
Stressor controllability is a factor that profoundly affects the harmful persistent effects of stress exposure on an individual. An uncontrollable stressor (USt) is defined as a stressor that the individual cannot influence through its own behavior; the nature, severity, duration and frequency of the stressor are wholly determined by external

factors (i.e., beyond control). USt experience affects subsequent stressor coping behavior in a way that is generally considered maladaptive; an encounter with a severe USt is known to promote a persistent, more passive overall coping style, which can prevent an individual from coping with a stressor actively when the situation permits; a phenomenon known as learned helplessness (Overmier and Seligman 1967). The most prominent preclinical assay for investigating this phenomenon is the classical learned helplessness shock-escape test. Typically, such an assay begins with a prolonged inescapable foot shock experience, followed by behavioral challenge in the same environment in which the previously inescapable foot shocks can be escaped by shuttling across the cage (Box 1). While nearly all animals without prior stressor experience are able to acquire an (active) escape response, a prior USt experience instills escape learning deficits in a subset of animals (Seligman and Maier 1967).

While an escape learning deficit is the best known sequela of inescapable stress experience, it is part of a series of behavioral adaptations, which will from here on out be referred to as the "helpless" phenotype. These adaptations are persistent and trans–situational as they are be observed during behavioral assays that have little or no resemblance to the setting in which the stressor experience was obtained, unlike the sequelae of conditioned fear (Bouton and King 1983, Overmier 1996). The helpless behavioral phenotype includes increased acquisition of conditioned fear (Grahn et al. 2000), impaired fear extinction learning (Baratta et al. 2007) and decreased social interaction (Short and Maier 1993); features that are induced by uncontrollable, but not controllable stressor experience. Investigating the effect that stressor controllability has on its sequelae requires a behavioral assay in which control over the stressor is the only factor distinguishing the experimental group from the control group. For this purpose, the yoked triadic wheel turning assay was developed (Box 1), but different behavioral assays have also been used to study controllability; signaled two-way active avoidance vs. cued fear conditioning to pair a conditioned stimulus (CS) to an escapable or inescapable stressor (Box 1), respectively (Lee et al. 2008), and controllable and uncontrollable variants of the forced swim test (Brown et al. 2001). In another proposed method of providing control, restrained animals in the controllable stress group undergo shocks while having access to a piece of bark to chew on, providing them with an additional means of stress coping behavior, whereas animals in the uncontrollable stress group do not. This is an interesting take on controllability; while the animals in the controllable stress group have no control over actual exposure to the stressor (i.e. the restraint and the foot shocks), in the ability to chew they receive a means of exerting coping behavior, and indeed an effect of controllability is demonstrated (Helmreich et al. 2012). This raises the question of how to define controllability, and to which degree findings in one paradigm can be generalized across others.

Box 1 Triadic stress controllability assays

Various assays are used to determine the effect of stressor controllability in rodents. In all these assays, the controllable stress (CSt) and uncontrollable stress (USt) animal are exposed to stressors that are similar in dimension, predictability, intensity, and duration. However, the CSt animal can influence the stressor through its behavior, while the USt animal, although given the same behavioral options in the experimental environment, cannot influence the stressor. Animals in the unstressed control group are placed in the same experimental environment as their CSt and USt counterparts for the duration of the session, but are not exposed to the stressor. Occasionally, animals that remain in their home cage during stress testing are used as an additional control group, to account for any stress the "unstressed" control animals might endure (such as novelty exposure, or restraint in the case of wheel turning experiments).



A. Signaled operant active avoidance. In this paradigm, the CSt animal learns to escape and eventually avoid footshocks by shuttling over to the other side of the skinner box and nosepoking in response to the shock-predicting signal, which consists of a tone and/or light. Correct responding aborts or prevents foot shocks.

The USt animal receives all shocks and signals that the CSt animal receives, but cannot influence the shocks by nose poking. The unstressed animal receives only the signals.

B. Signaled shuttle avoidance. Here, CSt animals learn to escape and eventually avoid footshocks by shuttling across to the other side of the skinner box twice in response to the shock-predicting signal which consists of a tone and/or light. A guillotine door separates the compartments between trials. Correct responding aborts or prevents foot shocks. Again, the USt animal receives all shocks and signals that the CSt animal receives, but cannot influence the shocks by shuttling. The unstressed animal receives only the signals.

C. Wheel turning stress escape. CSt animals receive unpredictable shocks which can be aborted by turning the wheel. The shocks are administered via a restraining electrode on the tail. Successfully aborting the shocks increases the amount of wheel turning required to abort the next trial. In this type of assay, the shocks are not signaled, nor can they be prevented by wheel turning. The USt animal receives all the shocks that the CSt animal receives and has access to a similar wheel, but turning it has no effect on shock exposure. The unstressed animal is restrained using the tail electrode for the duration of the session and does not receive shocks.

Mechanisms mediating uncontrollable and controllable stress

The dorsal raphe nucleus (DRN), where 5-HT is synthesized and from where all serotonergic innervation originates, is a key brain region in orchestrating the effects of USt. In the absence of a severe stressor, DRN activity is suppressed through glutamatergic innervation originating from the vmPFC (Celada et al. 2001). However, USt causes activation of 5-HT immunoreactive neurons in the DRN (Berner et al. 1999) and elicits a large, immediate efflux of 5-HT from the DRN (Maswood et al. 1998), extending to the mPFC (Bland et al. 2003), BLA (Amat et al. 1998a), the ventral hippocampus and dorsal periaqueductal gray (Amat et al. 1998b). 5-HTergic projections originating from the DRN inhibit mPFC activity (Hajos et al. 2003) and promote reactivity of the amygdala (Baratta et al. 2016).

The outflow of 5-HT is thought to contribute to the desensitization of the serotonin receptor 1A (5-HT1A) following USt (Rozeske et al. 2011). The presynaptic 5-HT1A receptor plays a central role in the negative feedback system that regulates 5-HT levels in the brain; presynaptic 5-HT1A binding to 5-HT reduces 5-HT release (Bonvento et al. 1992) and inhibits 5-HTergic neuron firing (Sprouse and Aghajanian 1987). Considering

the instrumental role of 5-HT in the mechanisms orchestrating the effects of controllability in stress, it is highly likely that they are modulated by genetic factors that influence 5-HT homeostasis, such as 5-HTTLPR.

Fear, stress-related disorders and 5-HTTLPR

Mechanisms of conditioned fear and extinction

The mechanisms that mediate the acquisition and extinction of classical Pavlovian fear conditioning are thought to be instrumental to the pathogenesis and treatment of PTSD, respectively (Rosen and Schulkin 1998) (Box 2). In the acquisition of a classical Pavlovian fear response, an unconditioned stimulus (US), i.e., severe negative event or trauma, is associated with a neutral stimulus (e.g., sight, sound, or environment), which becomes the conditioned stimulus (CS). Like Pavlov's bell would cause a dog to salivate as it would to the presence of food, reencountering the CS will elicit a fear response as if the unconditioned stimulus itself were present. In PTSD, an association is formed between a trauma and environmental cues present at the time of the experience. This associative learning is mediated by the BLA, and is dependent on modulation of long-term potentiation through a Hebbian learning process (Johansen et al. 2011). The BLA receives inputs from brain regions that process and integrate sensory information, such as the hippocampus and the neocortex (McDonald 1998). In addition, it receives input directly from the auditory thalamus and auditory association cortex (Romanski et al. 1993). The former pathway provides more detailed sensory information that has been weighted for relevance and salience, while the latter pathway provides a more reflexive means for sensory information to access the fear system. Arrival of sensory information corresponding with a known threat subsequently activates projections from the BLA to the central nucleus of the amygdala (CeA). Activation of the CeA triggers the behavioral fear response, consisting of fear potentiated startle, freezing and changes in autonomic nervous system parameters, such as heart rate, blood pressure and body temperature via its projections to the hypothalamus, bed nucleus of the stria terminalis and brainstem (LeDoux et al. 1988, Fanselow 1994, Critchley et al. 2002).

Apart from the amygdala, cortical regions play an important role in the regulation and expression of fear. The prefrontal cortex can functionally be divided in the vmPFC and a dorsolateral area (dlPFC). Its analogs in rodents are the infralimbic (IL) and prelimbic (PrL) cortices, respectively. These regions serve opposite functions in the regulation of fear. Upon encountering a CS, glutamatergic projections from the PrL drive activation of the CeA, thereby promoting a fear response. If the CS has been (partially or completely) extinguished through non-reinforced encounters, the IL will be activated

when the CS is presented, stimulating the intercalated cell (ITC) region of the amygdala through excitatory innervation (Sotres-Bayon et al. 2006). The intercalated cells, in turn, inhibit activation of the CeA, decreasing the fear response. Successful extinction is dependent on plasticity occurring in the IL during non-reinforced CS presentation, and can be prevented by inhibiting protein synthesis after non-reinforced CS presentation (Santini et al. 2004). It should be noted that extinction of a conditioned fear response does not equal erasure of its memory trace; instead, the extinction and fear memory exist independently from one another and can be manipulated individually (Quirk 2002). Moreover, the IL controls fear by exerting inhibitory control over the PrL through a local GABAergic circuit (Saffari et al. 2016).

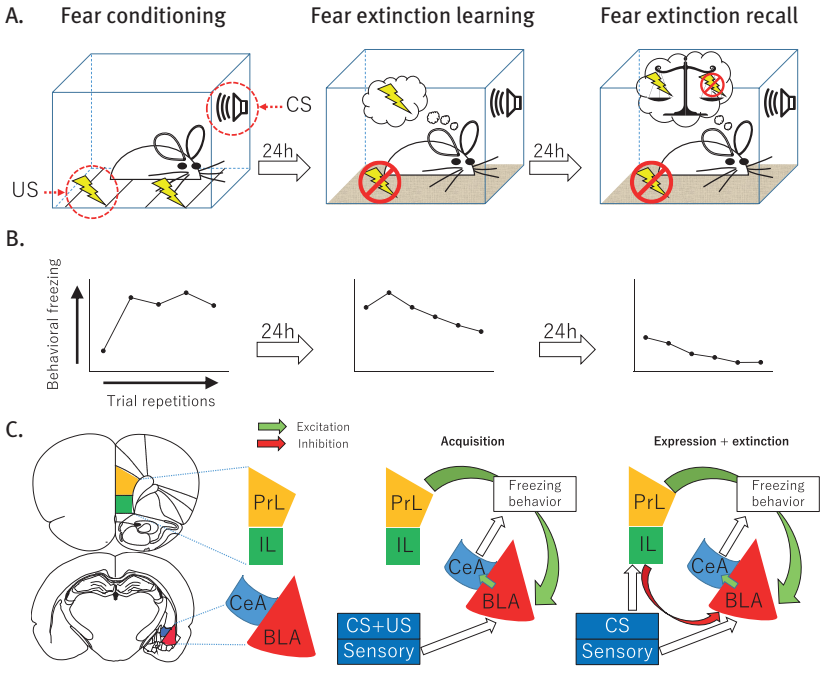
Perhaps relevant to the predisposing effect of life adversity for PTSD (Cabrera et al. 2007), chronic stress is known to have profound effects on the anatomy and function of the hippocampus, PFC and amygdala. Reduction of hippocampal volume is a hallmark pathology of stress-related disorders, has been reported in those suffering from MDD and PTSD (Bremner et al. 1995, Sheline et al. 2002), and may be related to altered neuronal turnover and neuroplasticity in that brain region (Lucassen et al. 2006, Malykhin and Coupland 2015). Similar volume loss accompanied by neuroplastic changes is observed in the PFC (Cerqueira et al. 2005), while chronic stress causes hypertrophy and increased dendritic arborization in the amygdala (Vyas et al. 2002).

The role of serotonin in fear mechanisms

While exposure to severe trauma is known to be the main causating factor in the development of PTSD, not all individuals that are confronted with it develop the disorder. Genetic factors are known to contribute to inter-individual variations in resilience to the effects of trauma. The 5-HTTLPR s-allele is known to potentiate the effect of severe trauma on the development of PTSD (Telch et al. 2015). In addition, preliminary evidence has shown that the s-allele diminishes the efficacy of cognitive behavior therapy, a first line treatment for PTSD (Bryant et al. 2010) (although conflicting reports exists, see Andersson et al. (Andersson et al. 2013)). The amygdala has been shown to be hyper-reactive in s-allele carriers (Munafo et al. 2008), while top-down control of cortical areas over it is reduced, as evidenced by the finding of reduced functional coupling between frontocortical regions and the amygdala (Pezawas et al. 2005). This has been proposed as a mechanism mediating the increased vulnerability in s-allele carriers to stress-related disorders, as these alterations suggest that impaired extinction of conditioned fear underlie the vulnerability to trauma seen in s-allele carriers. Acquisition of fear in cued fear conditioning has been reported to be stronger in carriers of the s-allele (Garpenstrand et al. 2001, Lonsdorf et al. 2009, Wendt et al. 2015), and to be potentiated by stressful life events (Hermann et al. 2012). Moreover, the s-allele was shown to potentiate the

Box 2 Acquisition and extinction of Pavlovian fear conditioning

Pavlovian fear acquisition and extinction processes are thought to be important in the pathogenesis and treatment of PTSD. These processes are studied in rodents by means of fear conditioning paradigms, as depicted below. In the paradigm discussed here and used throughout this thesis, a discrete, unfamiliar neutral signal (in this case, an auditory stimulus), the conditioned stimulus (CS), is paired to an aversive stimulus (in this case, a mild foot shock). This creates an aversive association with the CS, causing it to elicit a behavioral fear response upon subsequent re-exposure. This behavioral response to the CS subsides upon repeated non-reinforced presentations of the CS; this is called extinction of the conditioned fear response. Note that the context in which the US is encountered also elicits a fear response upon re-encountering it. To separate contextual associations from the CS contingency, evaluation of the response to the CS after conditioning in done in a novel, unfamiliar context. Variations in 5-HTT expression are known to cause alteration in the regulation of fear. In 5-HTT-/- rats, this manifests as impaired recall of fear extinction.



A. Schematic outline of a cued fear conditioning experiment. CS and US are paired by (usually multiple) presentations of the CS (tone) ending with a US (mild foot shock) in a familiarized environment. Subsequently, non-reinforced presentations of the CS are given in a novel context.

B. The behavioral freezing response across conditioning and extinction. To determine in response to the CS, defined as the absence of all motion except for that needed for respiration. Freezing increases quickly during conditioning and then decreases gradually through nonreinforced presentations of the CS.

C. Simplified model of the prefrontal and amygdala circuitry involved in fear conditioning and extinction. Acquisition and extinction of conditioned fear responses are dependent on interactions between the prelimbic (PrL) and infralimbic cortices and the basolateral and central nuclei of the amygdala. Acquisition is dependent on plasticity in the amygdala, while plasticity in the IL cortex mediates extinction. During extinction, the original fear memory is not degraded; instead, behavior during nonreinforced CS exposure is determined by competing responses from the IL and the Prl/BLA.

reconsolidation of conditioned fear (Agren et al. 2012). However, no effects on the efficacy of extinction were found (Garpenstrand et al. 2001), although several studies have shown that the neural correlates of extinction, as determined through functional magnetic resonance imaging, are affected by the s-allele in interaction with stressful life events (Hermann et al. 2012, Klucken et al. 2013a, Klucken et al. 2015). In addition, increased severity of the fear response could be an important factor exacerbating the effects of trauma in 5-HTTLPR s-allele carriers (Garpenstrand et al. 2001, Klumpers et al. 2012).

Work in animal models has solidified the relation between variations in the expression of 5-HTT and abnormalities in the regulation of fear. 5-HTT$^{-/-}$ rats and mice have been repeatedly demonstrated to display impaired recall of conditioned fear extinction (Wellman et al. 2007, Nonkes et al. 2012a, Shan et al. 2014). Several studies report that extinction learning is however not impaired as a result of 5-HTT abolishment (Wellman et al. 2007, Nonkes et al. 2012a). Moreover, when an appetitive conditioned distractor stimulus was presented interspersed with shock-paired CS presentations throughout the extinction session, recall of extinction was markedly improved in 5-HTT$^{-/-}$ rats compared to those that had received neutral distractors, while distractor type had no effect in wild type animals (Nonkes et al. 2012a). This lends credence to the notion that

reduced 5-HTT expression in fact enhances sensitivity to all external stimuli, both negatively and positively valenced (Homberg and Lesch 2011). This is further substantiated by the finding of increased reactivity of the BLA to appetitive conditioning in s-allele carriers (Klucken et al. 2013b).

The mechanisms at a molecular/cellular level underlying altered regulation of fear resulting from variations in 5-HTT expression are still poorly understood. Serotonin is involved in the mechanisms of conditioning, expression and extinction of fear in a multitude of ways, offering many possible routes of action by which altered serotonergic homeostasis resulting from variations in 5-HTT expression can interfere with fear regulation. Activation of serotonergic neurons in the DRN is seen in response to aversive physical stimuli, but also after expression of conditioned fear (Spannuth et al. 2011). 5-HT release is also gradually enhanced in the DRN and BLA in response to fear memory retrieval, peaking around 30 minutes after CS exposure (Zanoveli et al. 2009). Consequentially, acute administration of the SSRI citalopram, which elevates the serotonin level in the amygdala (Bosker et al. 2001), enhances the fear-potentiated startle response (Browning et al. 2007), while chronic administration does not (Grillon et al. 2009), and has even been reported to reduce the behavioral fear response to conditioned stimulus presentation in rats (Burghardt et al. 2004). This effect of chronic administration was found to be mediated through N-methyl-D-aspartic acid (NMDA) receptor unit 2B (NR2B) downregulation (Burghardt et al. 2013); fear learning and extinction both depend on activation of NMDA receptors in BLA, and specifically blocking subunit 2B has been demonstrated to impair these types of learning (Rodrigues et al. 2001). Furthermore, freezing in response to a fear CS is diminished when serotonergic inputs into the BLA are selectively lesioned by local intracerebral injection of 5,7 –dihydroxytryptamine (Izumi et al. 2012).

Serotonin in neural development

Influences of variations in 5-HTT expression on neural development may offer supplemental or even alternative explanations for their effect on fear regulation. Regional expression of 5-HTT varies greatly throughout development, but is much more prominent during than after gestation, when expression is mostly restricted to the DRN and to axon terminals of projection neurons originating from it (Zhou et al. 2000). Selective, transient blockage of 5-HT reuptake during development produces an anxious and emotional phenotype that has many similarities to that seen in animals characterized by genetically induced (i.e., chronic) reduction of 5-HTT expression (Ansorge et al. 2004, Glover and Clinton 2016). The effects of prenatal reduction of 5-HTT function on brain anatomy are most prominent in the somatosensory cortex;

5-HTT$^{-/-}$ rodents show reduced anatomical definition of somatosensory "barrel fields", neuronal columns that serve an important function in the processing of tactile information, and as a result show impaired performance in gap crossing assays (Persico et al. 2001). Similar, but less pronounced effects were reported in rodents after neonatal SSRI exposure (Lee 2009). In addition, it is probable that altered development contributes to the structural abnormalities seen in corticolimbic connectivity as well. 5-HTTLPR genotype affects the structural integrity of the uncinated fasciculus, an important white matter tract connecting the PFC to the amygdala, thought to be instrumental in suppression conditioned fear responses (Pacheco et al. 2009). This might cause impaired functional connectivity between PFC and amygdala in s-allele carriers as well (Heinz et al. 2005). Examining the neuronal morphology in relevant brain regions of 5-HTT$^{-/-}$ mice uncovered aberrant neuronal morphology in the IL, but not the BLA, indicating that 5-HT availability affects morphology on the neuronal level as well (Wellman et al., 2007). These findings imply that besides the effects of acutely altered serotonin signaling in response to stress, developmental effects of altered serotonergic signaling contribute to this phenotype in 5-HTT knockout models as well, although it is not clear to what extent.

Aim and outline of this thesis

Genetically-induced variation of 5-HTT has been demonstrated to modulate the risk for developing psychiatric disorders such as MDD and PTSD. This association seems to be mediated by altered susceptibility to stress. What is perceived as 5-HTTLPR-determined stress resilience in population-wide studies may in reality be reflective of 5-HTT expression dependent differences in stress coping tendencies and adaptability, and variation in the acquisition, extinction and expression of conditioned fear responses, which may develop during severely stressful experiences and lead to psychiatric disorders, such as PTSD. Although there is initial evidence suggesting that 5-HTTLPR affects stress coping, it is not known precisely to what degree the relation between 5-HTT expression and stress susceptibility is dependent on the nature of the stressor and appropriateness of the coping response. Moreover, the effect of 5-HTT expression on the ability to flexibly adapt the coping style to situational demands has not been explored. In addition, experimental data from animal models strongly implicate serotonergic mechanisms in the modulating effects of a stressor's controllability on its impact; this suggests that factors affecting 5-HT homeostasis, such as 5-HTTLPR, could alter the effects of control over the stressor. Furthermore, the question remains to what degree developmental abnormalities contribute to impaired stress resilience in carriers of low-expressing variants of 5-HTTLPR and corresponding animal models. Finally, it remains to be investigated whether nutritional interventions targeting the

intake of essential fatty acids have an impact on the emotional and depressive-like behavioral profile of rodents lacking 5-HTT.

In **chapter 2**, we investigate how full and partial abolishment of 5-HTT affects the extinction and extinction recall of conditioned fear across development by subjecting 5-HTT$^{-/-}$, 5-HTT$^{+/-}$ and wild type rats to a fear conditioning and extinction paradigm. Subsequently, we assess glutamate decarboxylase (GAD) 65 and 67 positive cell populations in the infralimbic cortex and the amygdala, to determine how the development of fear extinction relates to variations in inhibitory cell populations in these brain regions. In **chapter 3** we examine whether severe uncontrollable stress affects fear expression in a 5-HTT expression dependent manner by exposing 5-HTT$^{-/-}$ and wild type rats to a restrained tail shock paradigm and then assessing fear extinction and extinction recall. In **chapter 4**, we explore whether the neural underpinnings of the effects of controllable and uncontrollable stress are 5-HTT expression dependent by subjecting 5-HTT$^{-/-}$ and wild type animals to a triadic yoked active avoidance paradigm, wherein animals undergo either signaled active avoidance learning, receive the same quantity of signaled shocks in an inescapable setting, or receive only signals. Subsequently, we measure activation in the prelimbic and infralimbic cortices by determining c-Fos immunoreactivity, and serotonergic activation in the DRN by assessing co-expression of 5-HT and c-Fos immunoreactivity in that region. In **chapter 5** we investigate 5-HTT dependent stress coping flexibility. In order to learn whether and how the prior acquisition of a passive coping response to a stressor affects subsequent learning of an active coping response to it, we subject 5-HTT$^{-/-}$ and wild type animals to a fear or sham conditioning paradigm, and then a signaled active avoidance paradigm using the fear conditioned stimulus to predict oncoming shocks. Following these procedures, we assess the behavioral coping response to this stimulus in a novel, neutral environment. Finally, **in chapter 6**, we investigate whether the emotional and depressive-like behavioral pattern of 5-HTT$^{-/-}$ rats is affected by dietary supplementation with n-3 PUFA's by performing various emotionality-related behavioral assays on 5-HTT$^{-/-}$ and wild type animals after prolonged intake of either a diet rich in DHA and eicosapentanoic acid (EPA) or a control diet.

2

Impaired fear extinction recall in serotonin transporter knockout rats is transiently alleviated during adolescence

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Abstract

Adolescence is a developmental phase characterized by emotional turmoil and increased risk-seeking behavior. Evidence from neurodevelopmental studies suggests that during adolescence the amygdala, an important brain region for driving anxiety and fear-driven behavior, develops more quickly than the medial prefrontal cortex (mPFC), the brain area controlling the amygdala's activity. Altered function of the mPFC – amygdala circuit is thought to be important factor in the pathogenesis of many anxiety disorders. Several aspects of the adolescent behavioral phenotype are shared with carriers of the 5-HTTLPR short allele, which confers reduced expression of the serotonin transporter (5-HTT) in the brain, which clears serotonin from the extracellular space. Prefrontal control over the amygdala is reduced in 5-HTTLPR s-allele carriers, which could be due to increased availability of serotonin affecting the development of limbic areas and the cortical regions that exert control over them. This raises the question whether and how the regulation of fear processes develops differently across adolescence as a result of reduced 5-HTT expression. To address this question we tested serotonin transporter knockout (5-HTT$^{-/-}$) rats, heterozygous (5-HTT$^{+/-}$) and wildtype rats of preadolescent, adolescent and adult age for cued fear extinction and extinction recall, which critically depend on a well-functioning mPFC-amygdala circuit, and are known to be impaired in adult 5-HTT$^{-/-}$ rats. We quantified inhibitory neuron populations in the IL (the rat analog of the human mPFC) and basolateral amygdala (BLA), regions known to be functionally active in gating fear and extinction. We find that the impaired recall of conditioned fear that characterizes preadolescent and adult 5-HTT$^{-/-}$ rats is transiently normalized during adolescence. Moreover, during all phases of development IL inhibitory neuron populations are reduced in 5-HTT$^{-/-}$ rats, while the number of inhibitory neurons in the BLA was not altered. As IL inhibitory cells regulate the activity of a cortical area that directs the fear response via inhibition of the prelimbic cortex (PrL), this reduction may contribute persistence of conditioned fear seen in 5-HTT$^{-/-}$ rats, although its stability across development suggests it is not related to the transient improvement of fear extinction seen during adolescence. The improvement of fear extinction recall during adolescence suggests corticolimbic development is paced or executed differently in 5-HTT$^{-/-}$ rats.

Introduction

Adolescence is a period of physical and brain maturation that is characterized by an increase in pervasive fears and risky behaviors, and coincides with the emergence of anxiety and other affective disorders (Pine et al. 1998, Dahl 2004, Steinberg 2005, Somerville and Casey 2010, Somerville et al. 2010a, Somerville et al. 2010b, Britton et al. 2011). Recent data implicates organizational changes of the cognitive control circuitry regulating emotional behavior in this vulnerability during adolescence. More specifically, there is evidence for relative immaturity of the prefrontal cortex (PFC) and its top-down control over subcortical areas mediating emotion and motivation such as the amygdala, which' development precedes that of the PFC. According to the developmental mismatch hypothesis, the delayed maturation of the PFC in comparison to the amygdala results in a temporary imbalance between emotion and its regulatory processes (Heller et al. 2016). However, there are substantial individual differences in this transient "imbalance" in function of cortical and subcortical regions during adolescence, and the underlying mechanisms and factors influencing the maturation process are not yet clear. As the PFC-amygdala circuit is dysfunctional in anxiety disorders (Shin and Liberzon 2010) that frequently emerge during adolescence and often persist into adulthood (Kim-Cohen et al. 2003), the understanding of the maturation of the PFC-amygdala circuit in healthy subjects is expected to inform the pathophysiology of stress-related neuropsychiatric disorders.

Cortical–subcortical connectivity has been shown to be essential for the learning and recall of extinction of conditioned fear, which is known to depend on successful suppression of amygdala activation by the infralimbic (IL) cortex (Sotres-Bayon et al. 2006). As such, fear extinction is a behavioral marker that reflects the cortical-subcortical developmental imbalance in both humans and rodents; importantly, fear extinction appears to be diminished in adolescents, compared to pre-adolescents and adults, of both species (McCallum et al. 2010, Pattwell et al. 2012). Inhibitory signaling plays an important role in the regulation of fear and anxiety. While the IL is most known for inhibiting the fear response in the central amygdala (CeA) after successful fear extinction via its excitatory projections to the intercalated cells of the amygdala, it has recently become apparent that the IL through a local GABAergic circuit also inhibits the prelimbic cortex (PrL), responsible for activating the CeA (Saffari et al. 2016), which may be another route through which the IL exerts control over fear. The excitability of the basolateral amygdala (BLA), the amygdalar subnucleus responsible for maintaining the learned fear-association (LeDoux et al. 1990), is also regulated by inhibitory signaling of local GABAergic interneurons, a mechanism by which fear and anxiety are attenuated (Ehrlich et al. 2009). Patients suffering from post-traumatic stress disorder, a disorder of aberrant fear extinction, are characterized by abnormalities in GABAergic

signaling within the prefrontal cortex (Michels et al. 2014), implicating these local inhibitory circuits in its pathology. However, as of yet, their exact contribution to the impaired fear extinction in adolescents remains to be investigated.

Interestingly, the adolescent behavioral and supposed neural phenotype shows striking similarities to that seen in carriers of the low activity variant short (s) allele of the serotonin transporter linked polymorphic region (5-HTTLPR). Adult s-allele carriers, which presumably display increased extracellular serotonin levels, show increased acquisition (Garpenstrand et al. 2001) and reduced extinction (Klucken et al. 2013a) of conditioned fear, together with amygdala hyper-reactivity (Hariri et al. 2002), and attenuated anatomical and functional coupling between the mPFC and amygdala (Pezawas et al. 2005, Pacheco et al. 2009). Thus, the behavioral and brain phenotypes seen in adult carriers of the s-allele of the 5-HTTLPR may also imply a cortical-subcortical functional imbalance. Serotonin acts as a neurotrophic factor during development, and variations in serotonin availability occurring due to limited availability of 5-HTT are thought to affect the development of circuits involved in the regulation of emotional behavior (Gaspar et al. 2003, Homberg et al. 2010c, Witteveen et al. 2013). This poses the hypothesis that 5-HTTLPR may affect the development of the cortical-subcortical circuit, such that the transitions from preadolescence to adolescence and from adolescence to adulthood are altered in 5-HTTLPR s-allele carriers.

Serotonin transporter knockout (5-HTT$^{-/-}$) rats are used as a model organism for the 5-HTTLPR s-allele in humans, and show many phenotypical similarities, both adaptive and maladaptive, to s-allele carriers (see Homberg et al. (Homberg and Lesch 2011)). Similar to humans and rodents during adolescence and adult 5-HTTLPR s-allele carriers, 5-HTT$^{-/-}$ rats display impaired recall of extinction of conditioned fear (Nonkes et al. 2012a). Since the level of 5-HTT influences neuronal development in a multitude of ways (Homberg et al. 2010c), it is possible that development of prefrontal and amygdala inhibitory circuits are altered, potentially influencing the fear extinction recall deficit seen in 5-HTT$^{-/-}$ rodents across developmental stages.

Here, we employ a fear extinction paradigm to evaluate how differential 5-HTT expression affects the development of fear extinction learning and recall across adolescence using the serotonin transporter knockout (5-HTT$^{-/-}$) and heterozygous (5-HTT$^{+/-}$) rat, and compare them to wildtype animals (5-HTT$^{+/+}$). We then quantify the population of inhibitory cells in the IL and BLA by measuring the number of cells expressing the inhibitory markers glutamic acid decarboxylase 65 and 67 (GAD65/67) to assess development of the IL's capacity to inhibit fear through the PrL and the BLA's capacity to regulate the amygdala's excitability.

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 (Smits et al. 2006b). Experimental animals were derived from crossing heterozygous 5-HT transporter knockout (5-HTT$^{+/-}$) rats that were outcrossed for at least twelve generations with wildtype 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. Male adult 5-HTT$^{-/-}$, 5-HTT$^{+/-}$ and wildtype rats entered the experiment at p24 (preadolescent), p35 (adolescent) or p70 (adult). The adult animals were housed in pairs, while the adolescent and preadolescent animals were housed three per cage, in open cages. All animals had *ad libitum* access to food and water. A 12-hr light-dark cycle was maintained, with lights on at 08.00 AM. All behavioral experiments were performed between 08.00 AM and 18:00 PM.

Apparatus

A 30.5 cm x 24.1 cm x 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 x 25 cm x 30 cm Plexiglas cage, the bottom of which was covered with 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.

Procedure

On the day on which the animals entered the experiment (p24 for the preadolescent group, p35 for the adolescent group and p70 for the adult group) the animals were habituated to the conditioning context for 10 minutes. 24 hours after habituation, animals were given a cued fear conditioning session. Fear conditioning began with a two minute habituation period, followed by 5 instances of a 30 second 85 dB 2.8 kHz auditory stimulus co-terminating with a 1 second 0.6 mA foot shock, followed by a 1 minute inter-trial interval. 24, 48 and 72 hours after conditioning, fear extinction and

two sessions of extinction recall were given, respectively. In each of these sessions, rats were exposed to a 2 minute habituation period, after which 24 20-second presentations of the auditory stimulus were given, with an inter-trial interval of 5 seconds. Sessions were recorded and freezing was automatically assessed by a software program. For 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 in between the extinction and extinction recall sessions.

Assessment of behavior

For assessing the time spent freezing during the extinction learning and both the extinction recall sessions, we used the Ethovision 9.0 behavioral software package (Noldus Information Technology B.V., Wageningen, the Netherlands). Freezing was determined using the Activity Monitor feature of the software package. The threshold for pixel change between frames was set between 0.05% and 0.09% (depending on the specific camera in use, but not different between groups). Automatic assessment was compared to manually scored samples, as made by a trained observer blind to the genotype of the animal, and proved to be a reliable assessment of freezing behavior (correlation between manual and automatic outcomes: $r = 0.7397$).

GAD65/67 immunostaining

The immunostaining procedure was adopted from Olivier et al. (2008) and Nonkes et al. (2010) (Olivier et al. 2008, Nonkes et al. 2010). 90 minutes following either the extinction learning session or the second extinction recall session, anesthetized rats were perfused transcardially with 0.1 mol/l PBS, pH 7.3, followed by 400 ml 4% para-formaldehyde dissolved in 0.1 mol/l phosphate buffer (PB), pH 7.2. Subsequently, the brains were removed from the skull and postfixed overnight in 4% paraformaldehyde at 4°C. Before sectioning, the brains were cryoprotected with 30% sucrose in 0.1 mol/l PB. Forty micrometer thick brain sections were cut on a freezing microtome, and collected in six parallel series in 0.1 mol/l PBS containing 0.1% sodium azide. One series from each rat was used for every staining. The free-floating sections were washed three times in PBS and preincubated with 0.3% perhydrol (30% H_2O_2, Merck, Darmstadt, Germany) for 30 min. After washing three times in PBS the sections were presoaked for 30 min in an incubation medium consisting of PBS with 0.1% bovine serum albumin and 0.5% Triton X-100. The sections were then incubated with goat anti-GAD65/67, 1:2000 (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) overnight on a shaker, at room temperature, and consecutively incubated for 90 min at room temperature with biotinylated donkey-anti-goat (Jackson Immuno Research Laboratories, West Grove, Pennsylvania, USA) diluted 1 : 1500 in incubation medium for 90 minutes and for 90 min at room temperature with ABC-elite, diluted 1:800 in PB

(Vector Laboratories, Burlingame, California, USA). Between incubations, sections were rinsed three times with PBS. The GAD65/67–antibody peroxidase complex was made visible using 3,3-diaminobenzidine tetrahydrochloride staining. Sections were incubated for 10 min in a chromogen solution consisting of 0.02% 3,3-diaminobenzidine tetrahydrochloride and 0.03% nickel–ammonium sulfate in 0.05 mol/l Tris-buffer (pH 7.6), and subsequently for 10 min in chromogen solution containing 0.006% hydrogen peroxide. This resulted in a blue–black staining. Then, the sections were rinsed three times in PBS and mounted on gelatin chrome alum-coated glass slides, dried overnight in a stove at 37 °C, dehydrated in an increased series of ethanol, cleared in xylene, embedded with Entellan (Merck) and coverslipped.

Quantification

Numbers of GAD65/67-immunopositive cells were quantified using the software program Fiji ImageJ, a public domain image-processing program (http://rsb.info.nih.gov/ij/) (Schindelin et al. 2012). Cells were counted in the IL in equally framed sections across groups at 2.20 from Bregma at ×40 magnification using an Axio Imager.A2 microscope (Zeiss, Oberkochen, Germany). BLA GAD65/67 immunoreactivity was measured in sections at -1.88 or -1.80 from Bregma at ×40 magnification. The results for each subject are expressed as the total amount of cells counted in each section.

Statistics

All statistical analyses were performed using SPSS Statistics version 24.0 (SPSS Inc., IBM, Armonk, NY, USA). Data are presented as mean ± standard error of the mean (SEM). Behavioral and immunohistochemical data were analyzed using two-way analysis of variance (ANOVA) and repeated-measures ANOVA, respectively, with genotype and age (preadolescent, adolescent, adult) as between-subject factors. When appropriate, subsequent Bonferroni *post hoc* tests were performed to further specify genotype effects. Probability p values of less than 0.05 were considered significant.

Results

Freezing behavior

Basal anxiety. To measure baseline anxiety, we assessed freezing during the two minute stimulus free period preceding the extinction learning session. Freezing in response to the novel context was significantly affected by age ($F_{(2, 309)} = 42.182$, $p < 0.001$), but not genotype ($F_{(2, 309)} = 0{,}737$ $p = 0.473$), and no significant genotype x age interaction was found ($F_{(4, 309)} = 0.581$, $p = 0.677$). Bonferroni *post-hoc* analysis revealed that adolescent animals froze more upon novel context exposure than adult animals

(p = 0.02), while preadolescent animals froze more than adolescent and adult animals (p < 0.01).

Fear extinction across sessions. Freezing in response to the CS+ decreased across the three extinction sessions ($F_{(2,141)} = 168.046$, p < 0.001), indicating that exposure to the non-reinforced CS was successful in diminishing the conditioned fear response. Session # did not significantly interact with genotype ($F_{(4, 141)} = 0.761$, p = 0.544) or age ($F_{(4, 141)} = 2.251$, p = 0.068), nor did we find a genotype x age x session interaction ($F_{(8, 141)} = 1.367$, p = 0.215), indicating that the learning effect of extinction training and recall across sessions was not age- or genotype-dependent. However, over all sessions together we did find main effects of genotype ($F_{(2,141)} = 8.509$, p < 0.001)), age ($F_{(2,141)} = 6.123$, p = 0.003) and a trend-level significant genotype x age interaction ($F_{(4,141)} = 2.032$, p = 0.093). Examining freezing across sessions per age group revealed significant overall genotype effects in the preadolescent ($F_{(2,44)} = 5.566$, p = 0.007) and adult groups ($F_{(2,45)} = 6.324$, p = 0.004) (driving the main effect of genotype), but not the adolescent group ($F_{(2,52)} = 0.405$, p = 0.668). Bonferroni *post hoc* testing revealed that these genotype effects were driven by differential freezing of 5-HTT$^{-/-}$ rats compared to both 5-HTT$^{+/-}$ and wildtypes in the preadolescent group (p = 0.007 and p = 0.034 respectively) and differential freezing in 5-HTT$^{-/-}$ animals compared to 5-HTT$^{+/-}$ (p = 0.003), but not wildtype animals (p = 0.226) in the adult group (Figure 1). Freezing was significantly affected by age in 5-HTT$^{-/-}$ rats ($F_{(2, 35)} = 5.735$, p = 0.007), but not in 5-HTT$^{+/-}$ ($F_{(2, 71)} = 2.123$, p = 0.127) or wildtype animals ($F_{(2,37)} = 0.214$, p = 0.809). Within the 5-HTT$^{-/-}$ group, adolescent animals froze less compared to preadolescent animals (p = 0.007), and a trend-level significant decrease was found compared to adult animals (p = 0.053). Freezing between preadolescent and adult animals was not different (p = 0.657).

Fear extinction learning. In the extinction learning session, total freezing during all cue presentations was significantly affected by genotype ($F_{(2, 309)} = 3.090$, p = 0.047) and age ($F_{(2, 141)} = 4.367$, p = 0.013), but no genotype x age interaction was observed ($F_{(4, 309)} = 0.352$, p = 0.842). Exploration of the genotype effect through Bonferroni *post-hoc* revealed that 5-HTT$^{-/-}$ animals froze more during the extinction learning session than 5-HTT$^{+/-}$ animals (p = 0.004), but not wildtype animals (p = 0.107). Freezing in 5-HTT$^{+/-}$ and wildtype animals was not significantly different (p = 1.000). Remarkably, in this population consisting of all three 5-HTT genotypes, adolescent animals froze less than preadolescent (p = 0.003) and adult animals (p = 0.010), while these latter age groups did not differ in freezing from one another (p = 1.000), an effect that is likely to be mainly driven by the findings of improved extinction and recall in 5-HTT$^{-/-}$ rats. We analyzed within-session extinction learning by assessing the development of freezing behavior during the extinction learning session by dividing its

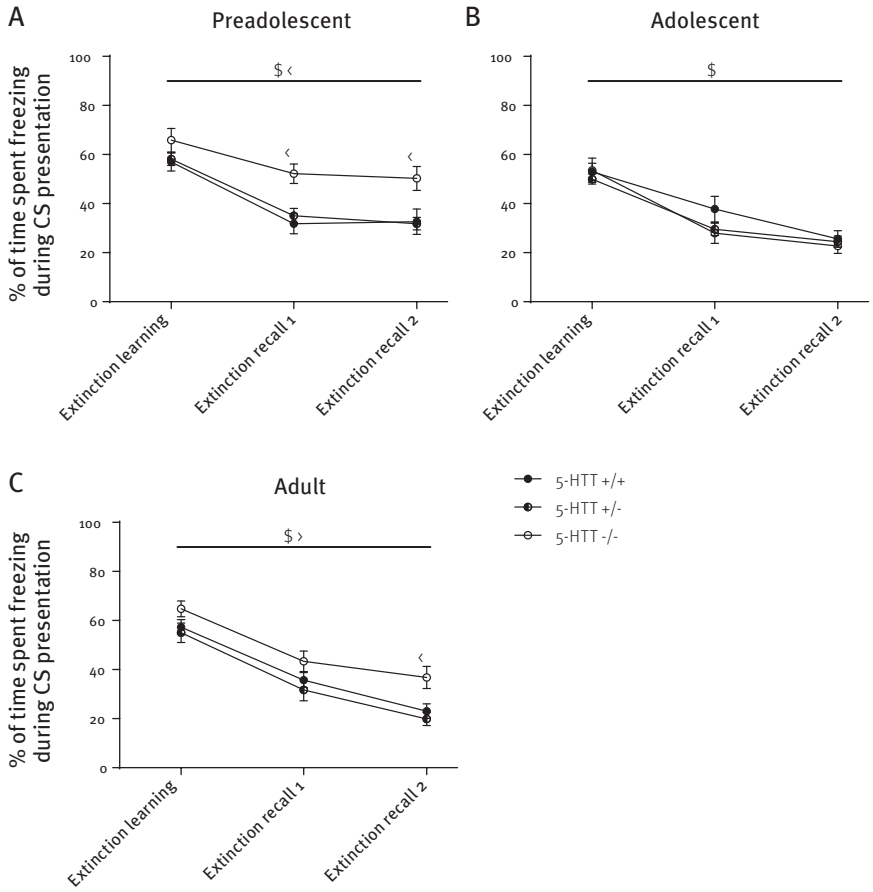

Figure 1 Fear conditioning behavioral data across extinction learning and the two extinction recall sessions. (**A**, **B**, **C**) Fear extinction recall is impaired in preadolescent 5-HTT$^{-/-}$ rats, then normalized in this genotype during adolescence, to be impaired again in adulthood. Data are expressed as mean % of time spent freezing during stimulus presentations ± standard error of the mean. $ indicates a significant effect of session number ($p < 0.05$), ‹ indicates a significant effect of genotype (5-HTT$^{-/-}$ vs. 5-HTT$^{+/-}$ and wildtype, $p < 0.05$); › indicates a significant effect of genotype (5-HTT$^{-/-}$ vs. 5-HTT$^{+/-}$, $p < 0.05$). Group sizes: 5-HTT$^{+/+}$ preadolescent n = 13 - 25 , adolescent n = 10 - 25, adult n = 17 - 35; 5-HTT$^{+/-}$ preadolescent n = 26 - 51, adolescent n = 35 - 79, adult n = 16 - 31; 5-HTT$^{-/-}$ preadolescent n = 10 - 25 , adolescent n = 10 - 17, adult n = 18 - 30.

24 trials into 6 blocks of 4 cue presentations and analyzing freezing through 2-way repeated measures ANOVA using trial block #, age and genotype as factors. An overall significant effect of trial block # was found ($F_{(2, 309)}$ = 295.241, p ‹ 0.001), indicating successful extinction learning. Trial block # interacted significantly with genotype ($F_{(4, 309)}$ = 6.903 p ‹ 0.001) and age ($F_{(4, 309)}$ = 3.704, p = 0.001), but no genotype x age x trial block was detected ($F_{(8, 309)}$ = 0.620, p = 0.825) (Figure 2).

First fear extinction recall. Total freezing during the first extinction recall session was used as a behavioral indicator of the recall of the extinction memory acquired during the first fear extinction learning session. We observed a genotype x age interaction for this parameter ($F_{(4, 141)}$ = 2.731, p = 0.032), which appeared to be driven by a significant effect of genotype in the preadolescent ($F_{(2, 48)}$ = 6.145, p = 0.004), but not the adolescent ($F_{(2, 52)}$ = 1.401, p = 0.255) and adult animals ($F_{(2, 48)}$ = 2.328, p = 0.108). The genotype effect in the preadolescent group was driven by 5-HTT$^{-/-}$ rats, which froze significantly more than 5-HTT$^{+/-}$ (p = 0.009) and wildtype (p = 0.006) animals, while freezing was not different between 5-HTT$^{+/-}$ and wildtype animals (p = 1.000).

Second fear extinction recall. We found a genotype x age interaction in freezing behavior during the second extinction recall session ($F_{(4, 141)}$ =2.968, p = 0.022). Here, we found a significant effect of genotype in the preadolescent ($F_{(2, 46)}$ = 5.754, p = 0.006) and the adult group ($F_{(2, 48)}$ = 6.537, p = 0.003), but not in the adolescent animals ($F_{(2, 52)}$ = 0.137, p = 0.873). 5-HTT$^{-/-}$ rats froze more than 5-HTT$^{+/-}$ and wildtype animals in both the preadolescent (p = 0.009 and p = 0.006 respectively) and the adult (p = 0.005 and p = 0.024 respectively) age groups, while freezing between 5-HTT$^{+/-}$ and wildtype animals was not different in either age group (p = 1.000 in both age groups).

GAD65/67 immunoreactivity

Infralimbic cortex. The number of GAD65/67 immunopositive cells in the IL was significantly affected by genotype ($F_{(2, 36)}$ = 9.747, p ‹ 0.001), but not age ($F_{(2, 36)}$ = 2.226, p = 0.123), and no genotype x age interaction could be detected ($F_{(4, 36)}$ = 0.663, p = 0.622). Further analysis of the genotype effect revealed that the number of cells expressing GAD65/67 was significantly reduced in 5-HTT$^{-/-}$ animals compared to 5-HTT$^{+/-}$ (p = 0.001) and wildtype animals (p = 0.002), while the latter two genotypes did not differ from one another (p = 1.000).

Basolateral amygdala. No effects of genotype ($F_{(2, 36)}$ = 1.574, p = 0.221) or age ($F_{(2, 36)}$ = 1.291, p = 0.287) were found in the number of GAD65/67 immunopositive cells in the BLA, but an interaction between these factors was found to be trend-level significant ($F_{(4, 36)}$ = 2.239, p = 0.084). Exploring this effect using a one way ANOVA revealed a

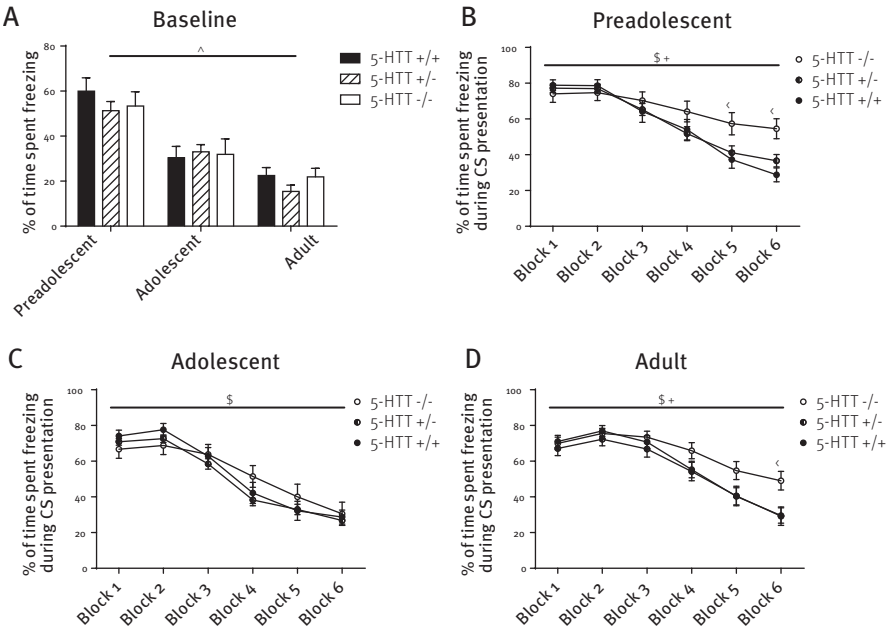

Figure 2 Fear extinction learning. (**A**) Freezing during the 2-minute stimulus free baseline period preceding extinction learning decreased across age in all genotypes. (**B**,**C**,**D**) Fear extinction learning is impaired in preadolescent 5-HTT$^{-/-}$ rats, then normalized in this genotype during adolescence, to be impaired again in adulthood. Data are expressed as mean % of time spent freezing during the baseline period (**A**) or mean % of time spent freezing during stimulus presentations (**B**,**C**,**D**) ± standard error of the mean. ^ indicates a significant effect of age ($p < 0.05$), $ indicates a significant effect of trial block number ($p < 0.05$), + indicates a significant trial block number x genotype interaction ($p < 0.05$), ‹ indicates a significant effect of genotype (5-HTT$^{-/-}$ vs. 5-HTT$^{+/-}$ and wildtype, $p < 0.05$). Group sizes: 5-HTT$^{+/+}$ preadolescent n = 25, adolescent n = 25, adult n = 35; 5-HTT$^{+/-}$ preadolescent n = 51, adolescent n = 79, adult n = 17; 5-HTT$^{-/-}$ preadolescent n = 31, adolescent n = 25, adult n = 30.

genotype effect in the adolescent ($F_{(2, 12)} = 3.952$, $p = 0.048$) but not the preadolescent ($F_{(2, 12)} = 0.888$, $p = 0.437$) or adult groups ($F_{(2, 12)} = 0.528$, $p = 0.603$). Within the adolescent group, a trend-level significant increase in GAD65/67 immunoreactivity was seen in 5-HTT$^{+/-}$ over wildtype animals ($p = 0.052$), while 5-HTT$^{-/-}$ GAD65/67 immunoreactivity in the BLA was not different from that in 5-HTT$^{+/-}$ ($p = 0.267$) or wildtype animals ($p = 1.000$).

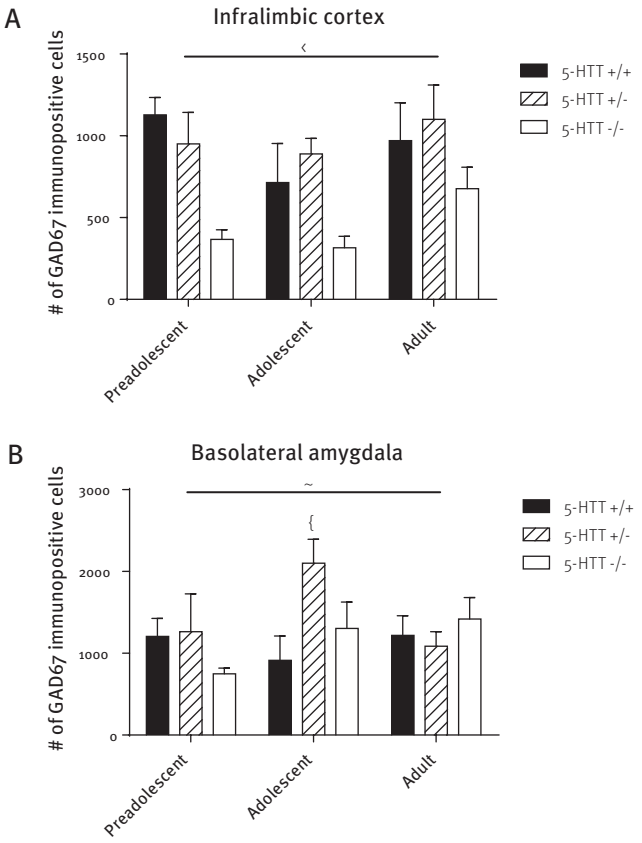

Figure 3 GAD65/67 immunoreactivity in the IL and BLA. A) IL GAD 65/67 immunoreactivity is significantly reduced in preadolescent, adolescent and adult 5-HTT$^{-/-}$ animals. B) BLA GAD 65/67 immunoreactivity is not different in 5-HTT$^{-/-}$ rats across all developmental phases. ‹ indicates a significant effect of genotype (5-HTT$^{-/-}$ vs. 5-HTT$^{+/-}$ and wildtype, $p < 0.05$), ~ indicates a trendline significant genotype x age interactions ($p = 0.084$), { indicates a trendline significant effect of 5-HTT$^{+/-}$ vs wildtype animals ($p = 0.052$). Group size: $n = 5$ (all genotype / age combinations).

Discussion

Here, we confirm that fear extinction recall is impaired in 5-HTT$^{-/-}$ rats, an established and oft replicated phenomenon (Schipper et al. 2011a, Schipper et al. 2011b, Nonkes et al. 2012a, Shan et al. 2014), in addition to finding impaired extinction learning in this genotype. Strikingly, an effect of age on fear extinction recall was seen only in

5-HTT$^{-/-}$ rats, which enjoyed a transient normalization (i.e., improvement) of fear extinction recall during adolescence. Augmented fear extinction learning seems to be responsible for the improved fear extinction observed in 5-HTT$^{-/-}$ rats during adolescence. The number of GAD65/67 positive cells, indicative of inhibitory circuit function, was decreased in the IL of 5-HTT$^{-/-}$ rats, regardless of age, while no clear effect of age or genotype were seen on the number of GAD65/67 positive cells in the BLA.

A number of developmental abnormalities arising from 5-HTT abolishment have been described in literature. The development of several motor and sensory functions, namely reflexes, motor coordination and olfactory discrimination, is delayed in 5-HTT$^{-/-}$ rats, but normalized upon reaching adulthood. Remarkably, other deficiencies seen in adult 5-HTT$^{-/-}$ animals, i.e., impaired object recognition, object directed behavior and sensorimotor gating, do not arise until after adolescence (Kroeze et al. 2016). The present results suggest that the abnormal emotional profile seen in 5-HTT$^{-/-}$ rats is subject to a nonlinear developmental trajectory as well, implying that 5-HTT abolishment influences neural maturation depending on the developmental phase and locus. The finding of transiently alleviated recall of fear extinction during adolescence in 5-HTT$^{-/-}$ rats suggests that the pacing of development of cortical and subcortical regions may be altered in this genotype. Congruent with our findings, a study in 5-HTT$^{-/-}$ mice has demonstrated that increased anxiety, another hallmark trait of the 5-HTT$^{-/-}$ rodent phenotype, is not present during adolescence (Sakakibara et al. 2014). Also the typically observed increased spine density in the BLA in adult 5-HTT$^{-/-}$ and 5-HTT$^{+/-}$ animals was not observed in adolescence in the same study (Sakakibara et al. 2014), which is in line with the behavioral phenotype. Furthermore, IL (but not PrL) spine density was increased in adolescent and adult 5-HTT$^{-/-}$ rats compared to wildtypes (Sakakibara et al. 2014), suggesting a relative reduction of the BLA – IL imbalance during adolescence in 5-HTT$^{-/-}$ animals. However, at the same time aberrant dendritic branching is observed in the PrL of adult 5-HTT$^{-/-}$ mice (Wellman et al. 2007), complicating the picture. These altered spine densities may be directly related to abnormalities in serotonin levels during development as serotonin plays a critical role in the pruning of synapses; a “trimming” of excess synaptic connections that occurs throughout development (Gaspar et al. 2003). Pruning is prevalent in cortical areas during adolescence (see Brenhouse et al. for review (Brenhouse and Andersen 2011)) and the IL inputs to the BLA are specifically pruned during late adolescence (Cressman et al. 2010). Alterations in neuronal morphology are thought to contribute to the network abnormalities seen in a variety of psychiatric and developmental disorders (Penzes et al. 2011). In addition, altered connectivity between regions may affect neural network function. In addition, a prenatal diminishing of 5-HTT expression through a pharmacological intervention influences expression of a number of genes that regulate nervous pathway myelination in the brain (Kroeze et al. 2015). 5-HTTLPR

s-allele carriers show altered functional connectivity between cortical-subcortical connectivity (Fang et al. 2013) and abnormalities in white matter structure. It remains to be determined to which degree abnormalities in the development of tracts that physically connect IL and BLA resulting from 5-HTT abolishment contribute to the findings reported here.

The inhibitory neuron population in the IL is reduced in 5-HTT$^{-/-}$ rats across all age groups. These cells are known to be functionally active in a local cortical circuit with the PrL, and contribute to the regulation of the expression of conditioned fear via the attenuation of the excitability of PrL. Reduced inhibition of the PrL may drive generalized anxiety seen in 5-HTT$^{-/-}$ rodents (Olivier et al. 2008), but may also be a causative factor of the reduced efficacy of fear extinction observed in this genotype. However, as the reduction in inhibitory neurons appears to remain stable across development from preadolescence to adulthood, it seems unlikely that altered development of local prefrontal inhibitory circuits contributes to the remarkable development of fear- and extinction behavior seen in these animals.

This study does not replicate findings from other studies that suggest fear extinction recall deficits in adolescent animals and humans with normal 5-HTT expression (McCallum et al. 2010, Pattwell et al. 2012), as our results indicate that in wildtype animals fear extinction recall is not significantly affected by age ($F_{(2,35)} = 0.214$, $p = 0.809$). Differences in details of the experimental procedures may crucially determine whether an effect of age presents itself. For instance, the experiments may differ in the degree to which contextual cues from the conditioning session are present during the extinction, which determines the additional involvement of the hippocampus on fear expression and extinction (Maren et al. 2013). We do corroborate the findings of another earlier study, in which extinction learning was found to be similar between adolescent and adult C57BL/6J mice (Hefner and Holmes 2007). This variability in the reported findings necessitates additional investigation towards the exact circumstances under which adolescent fear extinction (recall) is impaired.

Some limitations of the study require attention. First, animals that had undergone one and three days of fear extinction were pooled to determine the number of GAD65/67 positive neurons in the IL and BLA to obtain sufficient statistical power for a comparison. Since GAD65/67 expression is influenced by recent fear conditioning, it is possible that levels of expression were affected by this variation in time between conditioning and sacrifice of the animal. However, all GAD65/67 positive cells were included in the assessment regardless of expression level; given the high signal to background ratio of the DAB-Ni, variations in expression due to the varying recency of fear conditioning is unlikely to have affected the findings. In addition, in the absence of data describing

the total number of neurons present in the IL, the possibility that the reduction in GAD65/67 immunoreactivity in 5-HTT$^{-/-}$ rats reflects a lower overall neuron count cannot be fully excluded. Furthermore, housing conditions varied between the age groups; although no animals were kept in isolation, preadolescent and adolescent animals were housed with more cage mates than adults for practical and ethical reasons. Although this aspect is often overlooked in animal research concerning stress and psychiatric illness, social elements in housing conditions have been shown to influence emotional behavior (Hunter 2014), and social factors are known to be especially influential and instrumental to psychiatric wellbeing during adolescence (Crone and Dahl 2012).

In conclusion, the present findings show that the influence of genetic reduction of 5-HTT expression on the development of fear extinction recall manifests in a non-linear pattern, temporarily normalizing during adolescence, to become deficient again at adulthood. This discovery raises as many questions as it answers; delayed or aberrant maturation of cortical or subcortical regions or interconnecting tracts is a likely cause, but exploiting this finding for therapeutic benefit will require further specification of their nature and functional implications. The involvement of brain-derived neurotrophic factor (BDNF) in particular is an interesting avenue to explore in this regard, since it contributes to the development of the IL and its expression is dependent on serotonergic influences (Homberg et al. 2014). In addition, anatomical and functional development of excitatory neurons in the IL projecting to the amygdala are of interest for future study. As it stands, the data suggest that reduced inhibitory signaling within the IL is a potential cause for the impaired control over the amygdala seen in individuals with reduced expression of 5-HTT, but the finding of consistently reduced inhibitory neuronal populations in the IL across developmental phases implies the transient alleviation of the extinction recall deficit during adolescence has a different neural correlate.

Acknowledgements

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3

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

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Abstract

Life stress increases risk for developing stress-related mental disorders like post-traumatic stress disorder (PTSD), and more prominently so in carriers of the 5-HTTLPR short-allele, an allelic variant that confers reduced expression of the serotonin transporter. Findings from animal models have shown that severe inescapable stressor (IS) experience has a detrimental effect on the extinction of fear, a memory updating mechanism critical for adequate fear/trauma recovery and necessary for the successful treatment of PTSD. This effect is mediated by an elevation of serotonin levels resulting from IS. Accordingly, serotonin transporter knockout (5-HTT$^{-/-}$) rats and mice show compromised extinction (recall) of conditioned fear. This raises the expectation that IS will exacerbate the fear extinction (recall) deficit. To test this, we assessed whether 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 point where it was indistinguishable from that of wildtype animals, while wildtypes were unaffected by IS. Thus, 5-HTT$^{-/-}$ rats evidently were more sensitive to the effects of the stressor than wildtypes, although the behavioral consequences presented themselves as adaptive. These results focus attention on the under-investigated adaptive, potentially even beneficial sequelae of stress on fear memory in reputedly stress-sensitive individuals, which may eventually yield new avenues of therapy.

Introduction

Stress has long been understood to play a role in the development of psychiatric disorders, even if the underlying mechanisms still mystify us. For example, severe life adversity has been linked to increased risk for developing post-traumatic stress disorder (PTSD). A large body of evidence suggests that the serotonergic system plays a role in mediating these detrimental effects of stress. For instance, 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 {Gressier, 2013 #2272}. Critically, traumatic life events modulate the strength and neural basis of fear acquisition and extinction in a 5-HTT dependent manner, potentially underlying this increased vulnerability to psychopathology (Hermann et al. 2012, Klucken et al. 2013a). As fear acquisition and extinction processes are key in respectively the development and treatment of PTSD (Shin and Liberzon 2010), understanding 5-HTT – stress interactions is essential for the development of therapeutic interventions attuned to these individuals. This is especially urgent given that 5-HTTLPR s-allele carriers show poor response to cognitive behavioral therapy, a first line treatment for PTSD (Bryant et al. 2010).

Rodent models of 5-HTT deficiency, modeling both the neurodevelopmental and neurophysiological effects of reduced 5-HTT function, are characterized by a behavioral profile of generalized anxiety (e.g. (Mohammad et al. 2016), and impaired fear extinction memory recall (e.g. (Wellman et al. 2007)), 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 (Homberg et al. 2007a). This is relevant, given that acute inescapable stress (IS) potentially impairs fear extinction (Baratta et al. 2007) potentially by increasing dorsal raphe nucleus (DRN) serotonergic signaling and subsequently serotonin release in the basolateral amygdala (BLA) (Amat et al. 1998a). Expression of conditioned fear is associated with phasic elevation of BLA serotonin (Zanoveli et al. 2009), and terminating serotonergic inputs into the amygdala reduces the expression of conditioned fear, but only in IS experienced mice (Baratta et al. 2016). Since 5-HTT is of vital importance in the clearance of serotonin from the extracellular space, the detrimental effects of IS on fear extinction are likely affected by 5-HTT expression (Jasinska et al. 2012), and IS-induced fear extinction impairment is expected to be 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 a pre-existing state of high extracellular 5-HT, we assessed fear extinction and extinction recall in both naïve and IS-experienced 5-HTT$^{-/-}$ rats and their wildtype (5-HTT$^{+/+}$) counterparts (Homberg et al. 2007a). We subjected adult males of both genotypes to IS consisting of one session of 100 unpredictable tail shocks of randomized duration under restraint ($n_{5\text{-HTT-/-}}$ = 20, $n_{5\text{-HTT+/+}}$ = 19), or a control manipulation ($n_{5\text{-HTT-/-}}$ = 20, $n_{5\text{-HTT+/+}}$ = 16), followed by a cued fear conditioning paradigm 48 hours later. 24 and 48 hours after fear conditioning, animals were re-exposed to the fear conditioned stimulus to measure fear extinction learning and recall by means of behavioral freezing.

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. 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-hr light-dark cycle was maintained, with lights on at 08.00 AM. All behavioral experiments were performed between 08.00 AM and 18:00 PM.

Apparatus

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 x 24.1 cm x 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 x 25 cm x 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.

Procedure

The timeline of the procedure is displayed in Figure 1. 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 seconds, 5 seconds average) were given on a variable interval schedule ranging from 50 to 70 seconds (60 seconds average). The IS procedure took 2 hours. Control animals were restrained by the tail (while they were still able to move all limbs) for 2 hours in the apparatus using disposable finger electrodes, but were not given shocks. 24 hours after IS or restraint, animals were habituated to the fear conditioning environment for 10 minutes. The house light was on during habituation and conditioning. For the fear conditioning itself, after a two minute habituation period, a 30 second 85 dB 2.8 kHz auditory stimulus co-terminated with a 1 second 0.6 mA foot shock, followed by a 1 minute inter-trial interval. A total of 5 of these tone – shock pairings were given. 24 hours and 48 hours after conditioning, fear extinction and extinction recall were tested, respectively. After a 2 minute habituation period, 24 20-second presentations of the auditory stimulus were given, with an inter-trial interval of 5 seconds. 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.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). All statistical analyses were performed using IBM SPSS Statistics version 20.0 (SPSS Inc., Chicago, Illinois, USA). Effects of genotype and treatment were analyzed using a 2-way ANOVA (F). Significant genotype x treatment interactions were further explored using *post hoc* Student's t-tests. Probability p-values below 0.05 were considered significant.

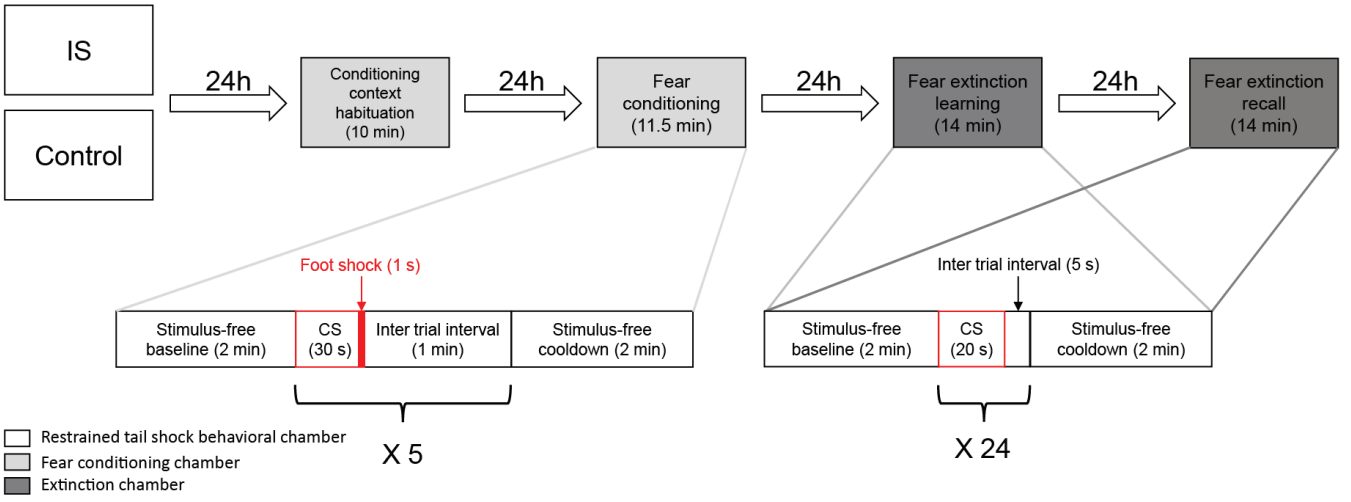


Figure 1 Experimental outline. All animals underwent habituation to the fear conditioning apparatus, fear conditioning, fear extinction learning and fear extinction recall testing respectively 24, 48, 72 and 96 hours after IS, which consisted of 100 unpredictable tail shocks under restraint, or a control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

Results

Analyzing freezing behavior during cue presentation in the fear conditioning session using 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) (Figure 2A). Moreover, 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 time spent freezing during extinction training (24 hours post-conditioning) (Figure 2B). However, we observed a significant genotype x IS interaction in freezing during the presentation of the conditioned stimulus in the extinction recall test (48 hours post-conditioning) ($F_{(1,74)}$ = 3.967, p = 0.050). Exploring this effect using *post hoc* t-tests revealed that in line with earlier reports (Wellman et al. 2007), 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). 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) (Figure 2C).

A

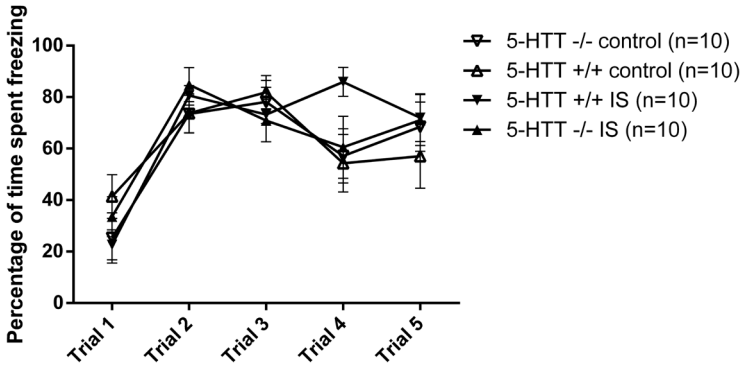
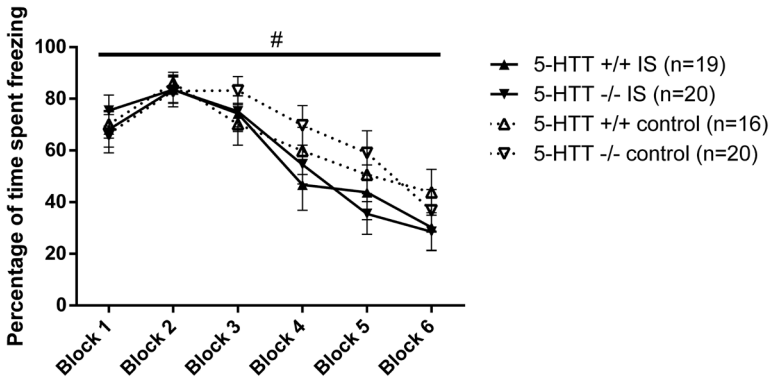


Figure 2 Fear conditioning and extinction. A. Behavioral freezing during stimulus presentations in the fear conditioning session. Freezing increased across trial blocks but was not significantly affected by genotype, stress or genotype x stress interaction.

B



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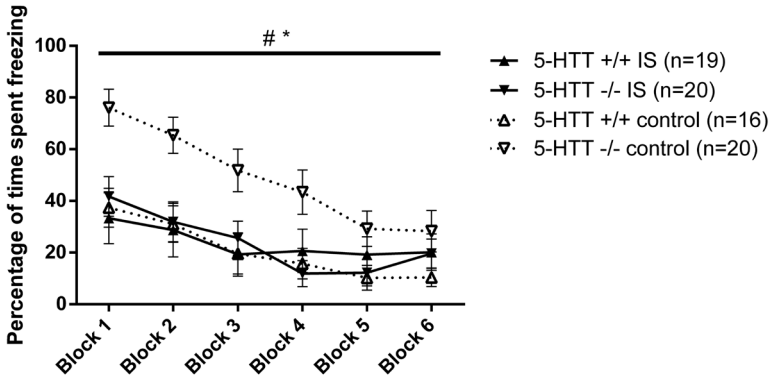

Figure 2 Continued. B. Behavioral freezing in response to the conditioned stimulus 24 hours after fear conditioning per block of 4 stimulus presentations. Freezing decreased across the trial blocks but was not affected by genotype, stress or genotype x stress interactions. **C.** Behavioral freezing in response to the conditioned stimulus 24 hours 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 x stress interaction ($p < 0.05$)

Discussion

Here, we show that severe IS normalizes the typically impaired recall of fear extinction memory in 5-HTT$^{-/-}$ rats, whereas extinction recall in 5-HTT$^{+/+}$ animals was unaffected by a history of acute stress exposure. 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 serotonin transporter expression.

These results are in striking contrast with majority of studies reporting on detrimental effects of IS experience on the extinction of conditioned fear. A single session of exposure to uncontrollable foot shocks has been shown to potentiate the acquisition of fear in several rodent models for PTSD (e.g. (Rau et al. 2005, Herrmann et al. 2012)). Several others reported IS to increase fear acquisition and impair fear extinction learning, using an acute stress paradigm similar to the one used here (Maier 1990, Grahn et al. 2000, Baratta et al. 2007). Here, we demonstrate that this inescapable stressor may also produce adaptive behavioral effects and improve fear extinction recall depending on serotonin transporter genotype. It is possible that the enhancing effects of IS on the acquisition of fear in wild type animals were obscured by a ceiling effect in our experiment, as the conditioning paradigm used here induced a maximal level of freezing upon exposure of the first CS presentations during the extinction learning session. Furthermore, differences in baseline behavior and stress-sensitivity between Sprague Dawley rats predominantly used to describe effects of the IS tail shock paradigm utilized by Baratta et al. and the Wistar rats used here may have played a role as well. Upon repeated stress exposure rats were found to display normal fear acquisition and extinction learning, but impaired extinction recall (Knox et al. 2012). Yet, other studies reported that chronic IS (repeated restraint), administered 48 hours before fear conditioning, enhanced the acquisition of conditioned fear, impaired extinction learning (Hoffman et al. 2014), and hindered extinction recall (Miracle et al. 2006) in (wildtype) rats. As acute and chronic stressors exert effects through very different neural mechanisms, and repeated stress exposure causes a wide arrange of (mal)adaptations (McEwen 2004), it is expected that the chronicity of the stressor and the duration between stressor exposure and the assessment of fear conditioning and extinction behavior crucially affect the precise nature of the interaction between stress experience and the regulation and expression of conditioned fear.

Previous experiments have demonstrated 5-HTT$^{-/-}$ rats to be resilient to the detrimental effect of IS on subsequent escape learning (van der Doelen et al. 2013). In that study, animals with compromised 5-HTT availability displayed stress resilience, and were found to be even more resistant to IS-induced escape deficits than control animals when they had undergone early life stress (maternal separation). Similar instances of adaptive behavioral sequelae of stress have been reported previously, albeit much more emphasis is put on its maladaptive behavioral consequences. Cortisol for example, a highly important neuroendocrine mediator of the stress response, is known for its detrimental effects on hippocampal function upon chronic exposure and its acute impairing effects on memory retrieval (Wingenfeld and Wolf 2014). However, at the same time it acutely enhances the encoding and the consolidation of emotional memory (Roozendaal et al. 2009), prioritizing the storage of emotional memories benefiting the survival of the individual. Furthermore, a moderate amount of life stress contributes to resilience and improves psychiatric wellbeing, yet a high amount of life stress experience clearly predicts increased risk for developing psychiatric disorders (Seery et al. 2010).

A key feature of 5-HTT abolishment is a profound constitutive enhancing effect on the level of extracellular 5-HT in the brain (Homberg et al. 2007c). Pharmacological interventions that aim to alter extracellular 5-HT levels, such as selective serotonin reuptake inhibitors (SSRIs), have previously been described to alter the regulation of conditioned fear. Interestingly, acute and constitutive increases in extracellular 5-HT have an opposite effect on the acquisition of fear, with acute administration of the SSRI citalopram enhancing it and chronic administration reducing it (Burghardt et al. 2004). This reduction appears to be mediated through downregulation of N-methyl-D-aspartate (NMDA) receptor subunit NR2b in the amygdala (Burghardt et al. 2013), which is instrumental in the physiology of fear acquisition (Rodrigues et al. 2001). However, reduced 5-HTT function throughout development (as present in 5-HTT$^{-/-}$ animals) seems to exert differential effects; NR2b levels in the amygdala were found to be similar in 5-HTT$^{-/-}$ and wild-type animals (Karel et al., in press), implying that mechanisms by which chronic SSRI exposure modulates fear do not mediate fear extinction deficits in 5-HTT$^{-/-}$ rats. In addition, acute treatment with the SSRI fluoxetine potentiated freezing in a signaled shock escape assay (Greenwood et al. 2008). In 5-HTT$^{-/-}$ rats, however, high central 5-HT levels are seen in conjunction with extinction-resistant conditioned fear and *improved* signaled shock escape / avoidance (Shan et al. 2014, Schipper et al. 2015). Potentially, alterations in neural development that result from genetic ablation of 5-HTT contribute to this apparent inconsistency. In particular, genetic abolishment of 5-HTT resulted in altered development of the serotonergic projections from DRN to PFC (Witteveen et al. 2013), which are functionally relevant in mediating the behavioral effects of stress (Waselus et al. 2011). Furthermore,

5-HTT abolishment has been shown to induce desensitization of 5-HT1a autoreceptors in the DRN at baseline (Homberg et al. 2008). 5-HT1a desensitization has also been found to be a requirement for IS-induced behavioral despair to occur (Rozeske et al. 2011), but remarkably has also been theorized to mediate the therapeutic effects of SSRIs (Millan 2003).

The mechanisms underlying our findings remain to be investigated. As the elevation in serotonin in response to IS is thought to be a key mediator of its behavioral effects (Amat et al. 2006), future studies must address how serotonin transporter expression affects this exact serotonergic response. However, although the increased release of serotonin in the target regions of the DRN, modulating fear memory processes, is of a transient nature (Amat et al. 1998a), the effects of IS persist past the duration of their 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 (Rozeske et al. 2011). Putatively, serotonergic receptors in DRN-projection sites are affected as well; the 5-HT2c receptor in the amygdala is of particular interest due to its modulatory role in fear memory consolidation (Baratta et al. 2016). Yet, although data from the amygdala are not available, both PFC and hippocampal 5-HT2c receptor populations in 5-HTT$^{-/-}$ and wild type rats were similar in quantity and post-translational editing (Lyddon et al. 2010). Additional studies are needed to determine the role of altered serotonin receptor function in both the impaired retention of extinction memory in 5-HTT$^{-/-}$ animals, but importantly, also in its alleviation through IS experience. It is also possible that factors that lie outside the direct influence of the serotonergic system contribute to the differential effects of IS on fear regulation. 5-HTT$^{-/-}$ rats have been reported to feature a number of functional adaptations in the hypothalamic-pituitary-adrenal axis, an important regulatory system of the stress response (van der Doelen et al. 2013, van der Doelen et al. 2014a, van der Doelen et al. 2014b, van der Doelen et al. 2015, van der Doelen et al. 2016). Among these, the finding of increased mineralocorticoid (MR) receptor expression in the mPFC and decreased glucocorticoid receptor (GR) expression in the dlPFC may be especially relevant in this context (van der Doelen et al. 2014a). GRs in the mPFC contribute to the consolidation of memory (Barsegyan et al. 2010), and expression levels have been shown to increase in male rats in response to stress (Karandrea et al. 2002, Wang et al. 2012), potentially contributing to stress-enhanced memory consolidation. mPFC MRs seem to serve functions in the retrieval of fear memory (Souza et al. 2014) and are downregulated following stress (Wang et al. 2012). It is tempting to speculate that IS provokes adaptations in corticosteroid receptor expression in the 5-HTT$^{-/-}$ brain, to make it resemble that of wild type animals. However, it is currently not known what sort of adaptations in the localization and expression of these receptors may have resulted

from IS in the 5-HTT$^{-/-}$ animals, and additional studies are required to explore their role in post IS fear regulation.

As a limitation of this study, it should be noted that while the control group did not receive shocks during the control manipulation, they were restrained by the tail like the IS group. While a single session of restraint is not expected to affect fear acquisition or extinction in a cued fear conditioning paradigm (Cordero et al. 2003), it is possible that 5-HTT$^{-/-}$ rats were more sensitive to this stressor. However, as behavior during fear extinction learning and recall in the control animals was similar to that seen in previous assessments of fear extinction in 5-HTT$^{-/-}$ rats (e.g. (Nonkes et al. 2012a)), it is unlikely that the single restraint session greatly impacted behavior in the control group.

Before designating the adaptations in the regulations of fear behavior that result from IS 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. Abolishment or diminution of 5-HTT expression has been shown to enhance cognitive flexibility in a wide range of species, 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, which typically poorly respond to cognitive behavioral therapy. For instance, it has been proposed that sky-diving may provide therapeutic benefits (van Roekel et al. 2016). 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.

Acknowledgements

We thank Anthonieke Middelman for the breeding and genotyping of the animals. This work was supported by the Netherlands Organization for Scientific Research (NWO), grant # 864.10.003 awarded to Judith R. Homberg. Funding organizations had no further role in the design of the study, nor in the collection, analysis and interpretation of data.

4

Improved stress control in serotonin transporter knockout rats: involvement of the prefrontal cortex and dorsal raphe nucleus

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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.

Introduction

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 – have been shown to be more anxious (Lesch et al. 1996) and extra vulnerable to stress-related mental disorders, such as major depression (Lesch and Gutknecht 2005). In order to study this genetic variant and the vulnerability it confers with regards to affective disorders, serotonin transporter knockout (5-HTT$^{-/-}$) mice and rats have been developed (Bengel et al. 1998, Smits et al. 2006b). 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 (Bartolomucci et al. 2010, Jansen et al. 2010). Furthermore, repeated social defeat stress was shown to impair fear extinction learning in 5-HTT deficient mice (Narayanan et al. 2011), 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 (Adamec et al. 2006). Moreover, exaggerated epinephrine responses have been noted in response to stress (Tjurmina et al. 2002), while hypothalamic–pituitary–adrenal axis responsivity seems to be unaffected by altered 5-HTT expression (Jansen et al. 2010).

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. (Maier et al. 2006)) 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 (Kalueff et al. 2010). Uncontrollable stress activates serotonergic neurons in the dorsal raphe nucleus (DRN), while mPFC activation during controllable stress is known to inhibit DRN activation (Amat et al. 2005). It has been demonstrated that serotonin depletion in the mPFC increases active

stress coping (Andolina et al. 2013), 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 (Homberg et al. 2007b) (while extracellular serotonin levels are increased in 5-HTT$^{-/-}$ mice; see (Mathews et al. 2004, Shen et al. 2004)), it is plausible that these animals cope more actively with stressors, when they are controllable.

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 (Schipper et al. 2011a). 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. Afterwards, activation of serotonergic neurons in the DRN was assessed by evaluating co-expression 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.

Results and Discussion

In our triadic controllability experiment rats were first trained in one chamber of a shuttlebox. 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 shuttlebox compartment before an avoidance or escape nosepoke 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 obtained in the one-chamber training; these data are presented in Supplementary 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 (Kalueff et al. 2010). A previous study reported on impaired shock escape in unstressed 5-HTT$^{-/-}$ mice in an unsignaled single session shock escape assay (Lira et al. 2003). Potentially, differences in the experimental

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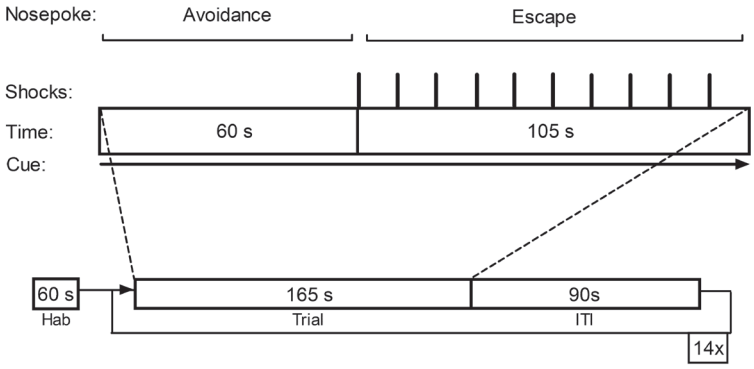

Figure 1 Active avoidance behavioral assay. (**A**) Outline of a signaled stress controllability session. During 14 trials, animals were presented with a compound stimulus, consisting of illumination of the nose-poke hole and a constant tone. During the first 60 seconds of this signal (i.e., the avoidance 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 seconds 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.

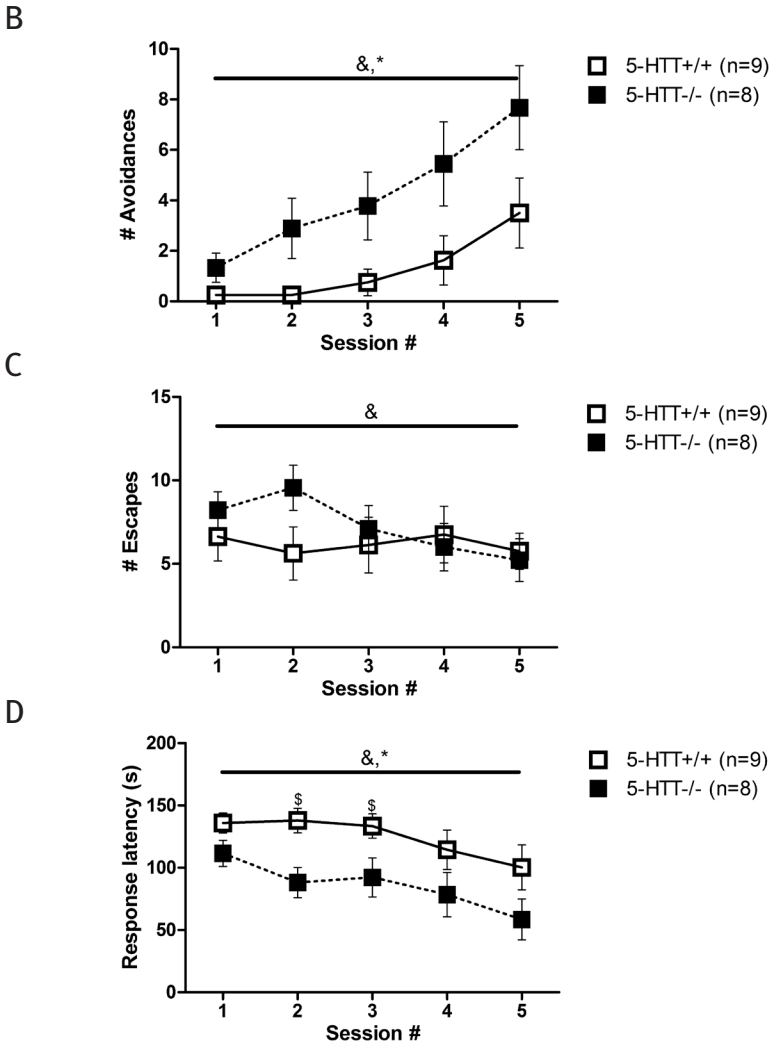

Figure 1 Continued. (B) Development of avoidance responses during the two-chamber sessions. (C) Development of escape responses across daily sessions did not differ between genotypes in the two-chamber test. (D) Mean nose-poke response latency across daily sessions was significantly lower in 5-HTT$^{-/-}$ rats during the two-chamber sessions. Sessions were 24 hours 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). $ indicates a significant genotype effect in a single session ($p > 0.05$, Student's t-test).

(signaled vs. unsignaled) and behavioral (multiple vs. single session) set-ups 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 extra-dimensional set-shifting (Nonkes et al. 2011, Nonkes et al. 2012b); 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 x stressor interaction ($F_{(2,42)} = 5.3$, $p < 0.01$) for 5-HT+c-Fos co-expressing 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 *posthoc* 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 Supplementary 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 (Araragi et al. 2013), although 5-HT$_{1a}$ mRNA was found to be decreased (Li et al. 2000). 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 make-up (Calizo et al. 2011), 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.

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 (Amat et al. 2005, Liu et al. 2009). However, the chronic component in this experiment (i.e., animals were exposed to uncontrollable

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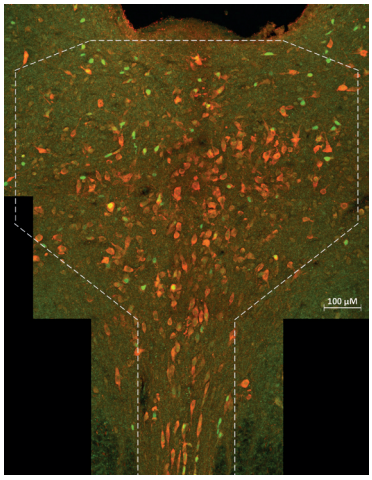

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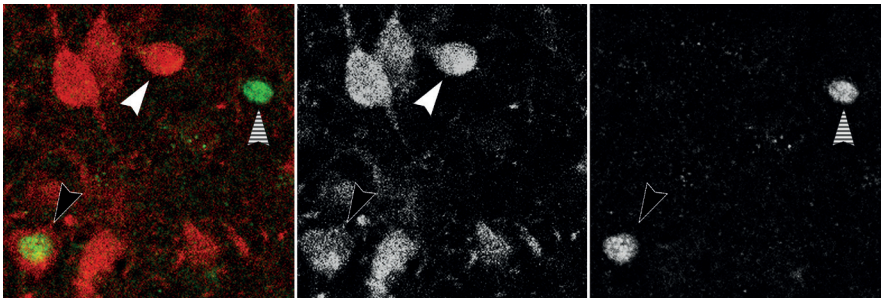

Figure 2 Activation of serotonergic neurons was assessed through measuring 5-HT + c-Fos co-localization. (**A**) Co-localization was assessed in the region depicted here, in coronal sections corresponding to -8.00 mm from Bregma. The fluorescence channel corresponding to c-Fos is displayed as green, 5-HT is colored red. (**B**) Close-up view of a 5-HTergic neuron co-localizing with a c-Fos immunoreactive nucleus (black arrow), non co-localizing 5-HTergic neuron (white arrow) and non co-localizing 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).

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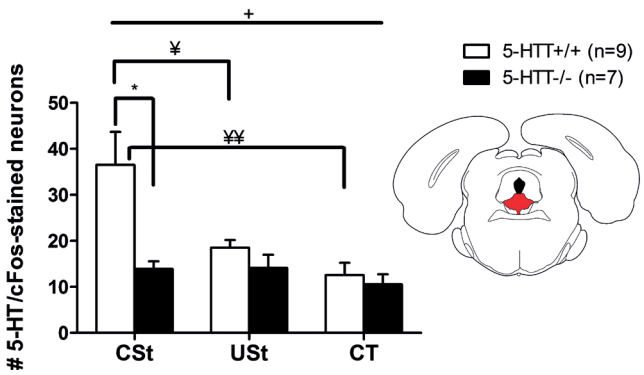

Figure 2 Continued. (C) Co-localization of 5-HT and c-Fos immunoreactivity was increased in 5-HTT$^{+/+}$ rats, but only after controllable stress. Data are expressed as mean number of co-localizations ± SEM. CSt, controllable stress. USt, uncontrollable stress. CT, control treatment. * indicates a significant 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 ($p < 0.05$).

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 (Stamp and Herbert 2001, Price et al. 2002, Hajos-Korcsok et al. 2003).

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 x 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 x stressor interaction ($F_{(2,41)} = 0.30$, NS) was found (Figure 3c). Bonferroni *posthoc* 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

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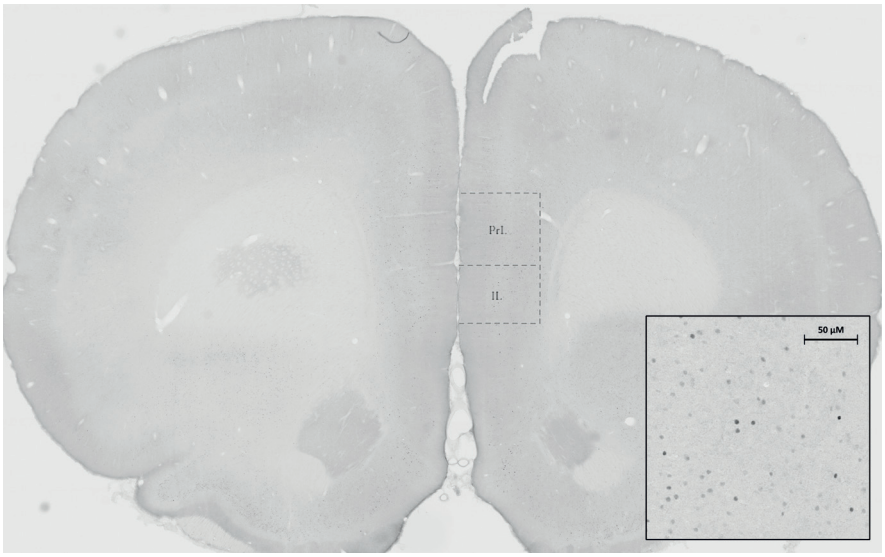

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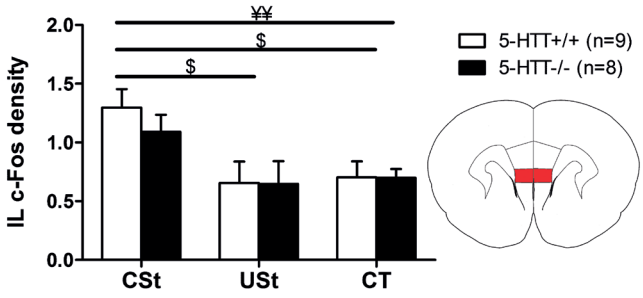

Figure 3 Neuronal activation in the mPFC was measured by quantifying c-Fos immunoreactivity. (A) Density of c-Fos immunoreactive nuclei was determined in the IL and PrL regions of the mPFC in coronal sections between -4.20mm and -2.20mm distance from Bregma. The density of c-Fos immunoreactivity in the 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 *posthoc* analysis ($p > 0.01$).

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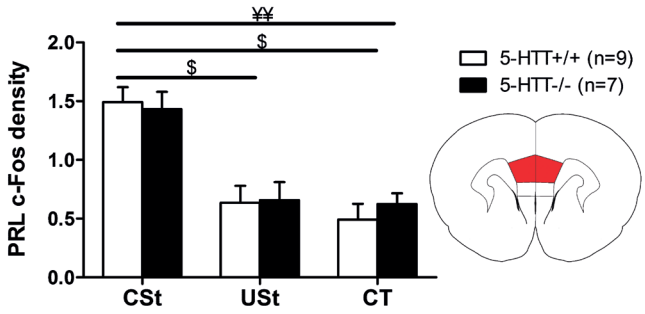

Figure 3 Continued.

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 (Amat et al. 2005, Liu et al. 2009, Moscarello and LeDoux 2013). 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 co-expressing 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.

Table 1 The 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.

Genotype		5-HTT$^{-/-}$	5-HTT$^{+/+}$	
		5-HT + c-Fos double labeled cells	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. (2-tailed)	0.841	**0.015**	
Mean escape latency	Pearson Correlation	0.095	0.732	0.186
	Sig. (2-tailed)	0.824	**0.039**	
Avoidances during the last session	Pearson Correlation	-0.251	-0.648	0.418
	Sig. (2-tailed)	0.549	0.083	
Non-responses during the last session	Pearson Correlation	-0.087	0.858	**0.030**
	Sig. (2-tailed)	0.837	**0.006**	
c-Fos density in IL	Pearson Correlation	0.389	0.542	0.757
	Sig. (2-tailed)	0.341	0.165	
c-Fos density in PrL	Pearson Correlation	0.456	0.131	0.569
	Sig. (2-tailed)	0.257	0.758	

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.

It seems remarkable that, although 5-HTT$^{-/-}$ animals have repeatedly been shown to suffer from impaired extinction of conditioned fear responses (Wellman et al. 2007, Schipper et al. 2011a), 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 towards a stimulus that signals controllability, and secondary,

safety. This process may resemble extinction of the fear-predicting value of the conditioned stimulus in cued fear-extinction paradigms. Given that fear extinction is mediated by the mPFC (see Quirk et al. for review (Quirk et al. 2000)), 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 (Perrotti et al. 2013), although the use of a chronic neuronal activation marker in this study (Nestler 2001) 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 (Homberg et al. 2007a), although modest effects have been reported in mice (Kalueff et al. 2007). Secondly, because successful avoidances prevented shock administration and 5-HTT$^{-/-}$ animals acquired the task more effectively, 5-HTT$^{+/+}$ received more shocks during most sessions (Supplementary 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 co-labeling 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 down-regulation 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 (Lesch et al. 1996), and has been linked to the emergence of affective disorders in these individuals (Lesch and Gutknecht 2005), 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 (Finger et al. 2007). Evolutionary biology predicts that the high prevalence of the 5-HTTLPR s-allele reflects overall positive or

adaptive effects of this s-allele (Homberg and Lesch 2011). 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 co-staining 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 transsituational 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 mediated through acute alterations in 5-HTergic neurotransmission in adult life, or through altered 5-HT-mediated neurodevelopment (Gaspar et al. 2003, Kinast et al. 2013). Additional experiments using conditional 5-HTT knockdown models are necessary to dissociate these effects.

Materials & 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 (Smits et al. 2006b) and have been described previously (Homberg et al. 2007a). 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). Since stress sensitivity in females is dependent on their estrous cycle phase (Devall et al. 2015), 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-hr light-dark cycle was maintained, with lights on at 08.00 AM. All behavioral experiments were performed between 08.00 AM and 18:00 PM.

Apparatus

A 40.6 cm (width) x 15.9 cm (depth) x 21.3 cm (height) rectangular shuttlebox (model ENV-010MD, Med Associates, St. Albans, VT, USA), was used, which was split into two identical chambers by 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 Supplementary methods. 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(Weiss 1968), 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, post-fixed in the same fixative for 30 minutes 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, USA). 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 Supplementary Methods.

Statistical analysis

All statistical analyses were performed using SPSS Statistics version 17.0 (SPSS Inc., Chicago, IL, USA). 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 *posthoc* 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.

Acknowledgements

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5

Serotonin transporter knockout reduces passive coping following active retraining of a fear conditioned stimulus in rats

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Abstract

Stressors can be actively or passively coped with, and adequate adaption of the coping response to environmental conditions can reduce their potential deleterious effects. One major factor influencing stress coping behavior is serotonin transporter (5-HTT) availability. Abolishment of 5-HTT is known to impair fear extinction but facilitate signaled active avoidance (AA). Flexibility in adapting coping behavior to the nature of the stressor shapes resilience to stress-related disorders. Therefore, we investigated the relation between 5-HTT expression and ability to adapt a learned coping response to changing environmental conditions. To this end, we first established and consolidated a cue-conditioned passive fear response in 5-HTT$^{-/-}$ and wildtype rats. Next, we used the conditioned stimulus (CS) to signal oncoming shocks during signaled AA training in 5-HTT$^{-/-}$ and wildtype rats to study their capability to acquire an active coping response to the CS following fear conditioning. Finally, we investigated the behavioral response to the CS outside of the AA training context. To this end, we exposed the animals to the CS in a novel environment and measured freezing and exploration. We found that fear conditioned and sham conditioned 5-HTT$^{-/-}$ animals acquired the signaled AA response faster than wildtypes, while prior conditioning briefly delayed AA learning in both genotypes. Subsequent exposure to the CS outside the AA training context elicited reduced freezing in 5-HTT$^{-/-}$ compared to wildtype rats. This indicates that their improved AA performance resulted in a weaker residual passive fear response to the CS in a novel context. Fear conditioning prior to AA training did not affect freezing upon re-encountering the CS, although it did reduce exploratory behavior in 5-HTT$^{-/-}$ rats. We conclude that independent of 5-HTT signaling, prior fear conditioning does not greatly impair the acquisition of subsequent active coping behavior when the situation allows for it. Abolishment of 5-HTT results in a more active coping style when the CS is encountered in a novel context after AA learning. This is partially undone when the 5-HTT$^{-/-}$ animals are fear conditioned to the CS prior to AA learning.

Introduction

Stress is recognized as one of the foremost contributors to the development of psychiatric disorders such as posttraumatic stress disorder (PTSD) and major depression (MD). However, large inter-individual variation exists in vulnerability to stress; not all individuals who are faced with severe trauma succumb to anxiety or mood disorders. The capability of an individual to appropriately adapt one's coping response to a stressor has a great influence on its potentially deleterious sequelae. Therefore, it has been proposed that varying levels of stress susceptibility may in part be mediated by differences in stress coping strategies (Veenema et al. 2003). Stress coping is defined as the actions an individual undertakes to reduce the impact of a stressor. Coping can be done either actively in an effort to remove the stressor, or passively by conserving energy while enduring a stressor. It has been suggested that both styles can be adaptive or maladaptive and thus can confer resilience or vulnerability. Whether a stress coping style is adaptive depends on its appropriateness to the exact environmental setting; stress coping *flexibility* has been proposed as an important factor in resilience (Homberg 2012, Nederhof and Schmidt 2012).

Certain genetic factors modulating serotonergic neurotransmission are known to affect stress coping behavior, and thereby influence vulnerability to stress-induced psychopathology. The short (s) allelic variant of the serotonin transporter linked polymorphic region (5-HTTLPR) is thought to compromise the availability of the serotonin transporter (5-HTT) in the brain (although conflicting evidence exists as well (Willeit and Praschak-Rieder 2010)). The s-allele is well known for increasing susceptibility to MD in conjunction with the presence of early life adversity (Taylor et al. 2006, Karg et al. 2011), and to PTSD following severe trauma (Gressier et al. 2013). Since associations between 5-HTTLPR and these stress-related disorders have been found exclusively in the presence of previous stressful life experience it is likely that modulation of coping behavior is key to understanding these gene x environment interactions (Markus 2013). This is further supported by the finding that 5-HTT binding is influenced by prior adverse experience in depressed subjects (Miller et al. 2009). Several studies point out differences in stress coping behavior between 5-HTTLPR s- and long (l)-allele carriers. Trait worry, a coping style that constitutes a cognitive focus on undesirable future outcomes that predisposes one to - among other psychiatric illnesses - PTSD (Holeva and Tarrier 2001, Berenbaum 2010), is more prevalent in s-allele carriers (Bredemeier et al. 2014). In line with this, greater anxious preoccupation was observed in s-allele carriers newly diagnosed with breast cancer compared to their l-allele peers (Schillani et al. 2012). Furthermore, coping style has been shown to be a mediator in the interaction between 5-HTTLPR genotype and stress regarding the susceptibility to MD (Wang et al. 2016). For both MD and PTSD these genetic differences

in stress (coping) responses also seem to hinder responsivity to first-line behavioral cognitive therapies (Bryant et al. 2010). Although the mechanisms directing these alterations in stress coping behavior and resilience are not fully understood, it is known that genetic variations in 5-HTT expression result in functional adaptations of the hypothalamic-pituitary-adrenal axis. The 5-HTTLPR s-allele is associated with increased basal levels of the stress hormone cortisol (Wankerl et al. 2010), and increased cortisol response to stress (Way and Taylor 2010). Furthermore, serotonin plays an important role in the neural circuits involved in managing fear learning, expression and extinction (Bocchio et al. 2016).

Work in animals with genetically altered levels of 5-HTT has solidified the association between serotonin and stress coping strategy, though many of the underlying mechanisms remain unclear. 5-HTT$^{-/-}$ animals display impaired extinction memory, and thus prolonged expression of a passive stressor coping response (i.e., freezing) in a cued fear conditioning paradigm (Wellman et al. 2007, Schipper et al. 2011a, Shan et al. 2014). At the same time, 5-HTT abolishment was shown to improve the acquisition of an active stressor coping task, signaled active avoidance (AA) (Schipper et al. 2015). The discrepancy between impaired fear extinction and improved AA performance in 5-HTT$^{-/-}$ animals is peculiar, as overcoming the freezing response induced by the shock-predicting signal is a prerequisite to proactively respond to it. During initial unsuccessful AA trials, signal–shock pairings induce conditioned freezing. The animal then has to overcome this conditioning in order to subsequently avoid or escape the shock (Lazaro-Munoz et al. 2010, Moscarello and LeDoux 2013). While successful fear extinction is dependent on updating the contingency of the conditioned stimulus (CS) by *passive* exposure to it, signaled AA learning allows the individual to reevaluate the CS contingency by *actively* interacting with it. Therefore, AA could be considered the "controllable" counterpart of fear extinction, in the sense that it could achieve a behavioral outcome similar to fear extinction training (i.e., reduced freezing to a CS presentation) by altering the coping response elicited by the CS.

Here, we further explore whether the updating of a passive coping stimulus contingency to an active one is modulated by 5-HTT expression. To this end, we assessed signaled AA performance in previously fear conditioned and sham conditioned 5-HTT$^{-/-}$ and wildtype rats, using the CS to signal incoming shocks during AA. We then measured freezing in response to the CS in a novel environment to evaluate the effects of 5-HTT genotype on the carry-over of the conditioned fear response to different environmental conditions (Figure 1). Using fear conditioning, we induce a pre-existent behavioral freezing response to the CS. This is expected to reduce the ability to acquire an active coping response (i.e., impair AA learning), due to the animals having to overcome their acquired freezing response to the CS in order to respond actively to it. Therefore,

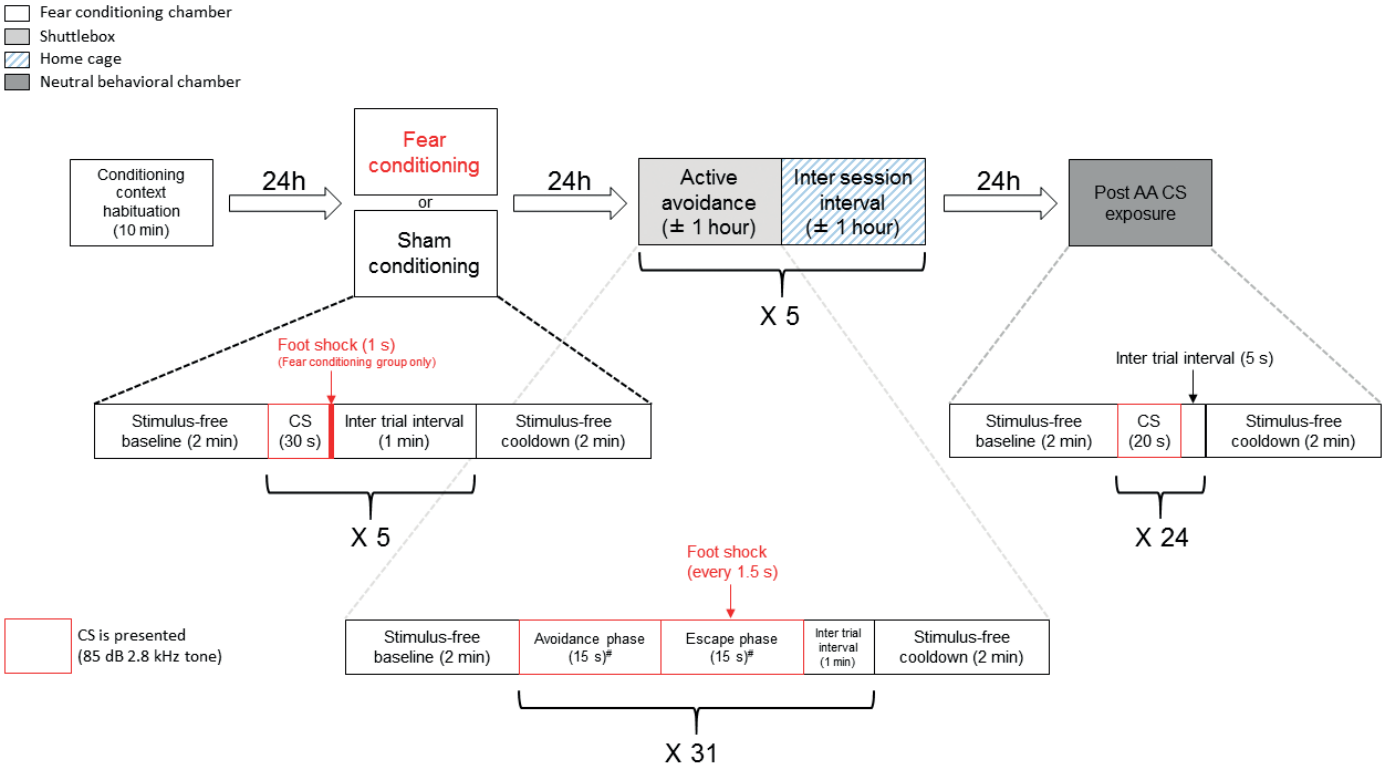


Figure 1 Outline of the experimental procedures. #: When an animal shuttled across during the avoidance or escape phase, the remainder of the trial was suspended and the inter trial interval was initiated.

we hypothesized that AA performance would decrease in both genotypes as a result of prior fear conditioning. We expected that 5-HTT$^{-/-}$ rats would be relatively resistant to these effects of prior fear conditioning, as they have previously been demonstrated to be resilient to stressor induced escape learning deficiencies (van der Doelen et al. 2013). Consequently, we expected a greater freezing response to the CS after AA learning in a novel context in wildtype animals, since improved AA learning of 5-HTT$^{-/-}$ rats would strengthen the active coping contingency of the CS and reduce their fear response.

Methods and materials

Animals

Serotonin transporter knockout rats (Slc6a41Hubr) were generated on a Wistar background by N-ethyl-N-nitrosurea (ENU)-induced mutagenesis (Smits et al. 2006b) as described previously (Homberg et al. 2007a). 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). Since stress sensitivity in females is dependent on their estrous cycle phase (ter Horst et al. 2012, Devall et al. 2015), we here restricted ourselves to the gender with the most stable stress response, i.e., males. Twenty homozygous knockout (5-HTT$^{-/-}$) and twenty wildtype animals were used for this experiment; half of each group received fear conditioning before signaled AA training, while the remaining animals received sham conditioning. All animals had *ad libitum* access to food and water and were housed in pairs in standard Makrolon type 3 open cages. A 12-hr light-dark cycle was maintained, with lights on at 08.00 AM. For consistency with previous experiments performed in this rat line (e.g. (Schipper et al. 2011a, Shan et al. 2014, Schipper et al. 2015)), all behavioral experiments were performed between 08.00 AM and 18:00 PM. At the time of entering the experiments, the animals were between 12 and 20 weeks old. All experiments were approved by the Committee for Animal Experiments of the Radboud University, Nijmegen, The Netherlands, (application # RU-DEC 2013-149) and all efforts were made to minimize animal suffering and to reduce the number of animals used.

Fear conditioning

A 30.5 cm x 24.1 cm x 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. Animals were habituated to the fear conditioning chamber for the duration of 10 minutes, 24 hours prior to conditioning. For the conditioning and habituation, the apparatus was cleaned before and after each animal using a tissue slightly dampened with 70% EtOH. The house light was on during habituation and conditioning. For the fear conditioning itself, after a two minute habituation period, a 30 second 85 dB 2.8 kHz auditory stimulus (the CS) co-terminated with a 1 second 0.6 mA foot shock, followed by a 1 minute inter-trial interval. A total of 5 of these tone – shock pairings were given. For the sham conditioning groups, the foot shock was omitted.

Signaled active avoidance

A rectangular shuttlebox (model ENV-010MD, Med Associates, St. Albans, VT, USA), consisting of two 20.3 cm x 15.9 cm x 21.3 cm compartments, was used for AA learning. It was divided into two identical chambers by an automated door and housed within a sound-attenuating cubicle. Each compartment was outfitted with 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 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. All animals underwent five AA sessions, each consisting of 31 trials, on a single day, the day after fear conditioning or sham conditioning. Each trial consisted of an avoidance phase, an escape phase, and a one minute inter-trial interval phase, in that exact order. During the avoidance phase, an 85 dB, 2.8 kHz tone (i.e., the CS) sounded continuously, and the door opened, enabling the animal to escape through the door and terminate the trial by breaking the infrared detection beams at the other end of the shuttlebox. If the animal failed to do so within 15 seconds, the escape phase would begin, during which a 0.5 second 0.6 mA foot shock was administered every second. The shocks and the CS would continue until the animal escaped to the opposite compartment or until 15 seconds (and thus 15 shocks) had passed. A session lasted 40 – 60 minutes, depending on AA performance and the inter-session interval was one hour, making the total AA procedure last 7 – 9 hours. Each session began and ended with a 2 minute stimulus free period. Avoidance, escape, and response latency were assessed automatically through laser beam detection during every session, while freezing during stimulus presentation, defined as the absence of all movement except that necessary for respiration, was assessed by a trained observer blind to the experimental condition of the animal from digital video recordings of the last session. In between sessions, the apparatus was cleaned using a tissue slightly dampened with 70% EtOH.

Post AA training CS exposure

The day after AA, all animals were exposed to the CS used in the prior sessions delivered in a novel, distinctive context. The novel context was located in a different experimental room from the conditioning and AA environments, and consisted of an unfamiliar 25 cm x 25 cm x 30 cm Plexiglas cage, the bottom of which was covered in a +/- 0.5 cm thick layer of black bedding. In this behavioral chamber, 85 dB (measured at the center of the floor) 2.8 kHz auditory stimuli (i.e., the CS) could be delivered through a set of external speakers. After a 2 minute habituation period, 24 20 second presentations of the CS were given, with an inter-trial interval of 5 seconds. The chamber was cleaned using water between animals to create olfactory cues distinctive from those during the prior experiments. The session was recorded and freezing during stimulus presentation was manually assessed by a trained observer who was blind to genotype and treatment. In addition, exploration of the chamber was assessed using the computer program Ethovision v9.0 (Noldus Information Technology, Wageningen, the Netherlands), which determined the distance moved by tracking the animals' movement in digital video recordings of the behavioral session.

Statistics

Data are presented as mean ± standard error of the mean (SEM). All statistical analyses were performed using IBM SPSS Statistics version 20.0 (SPSS Inc., Chicago, Illinois, USA). Effects of genotype, conditioning, and session/trial number were analyzed using repeated measures ANOVA. Probability *p*-values below 0.05 were considered significant.

Results

Active avoidance (AA)

When assessing the total number of avoidances throughout the entire five-session procedure, we found a significant effect of genotype ($F_{(1,35)} = 5.555$, $p = 0.024$), with the 5-HTT$^{-/-}$ rats making significantly more avoidance responses than wildtypes (Figure 2A). No effects of conditioning ($F_{(1,35)} = 0.527$, $p = 0.473$), nor genotype x conditioning interactions ($F_{(1,35)} = 0.579$, $p = 0.452$) could be detected. A significant effect of session number was found for both 5-HTT$^{-/-}$ ($F_{(1,19)} = 5.636$, $p < 0.001$) and wildtype ($F_{(1,18)} = 9.489$, $p = 0.001$) animals, in the absence of a genotype x session number interaction (($F_{(1,35)} = 0.947$, $p = 0.416$)), indicating that the number of avoidances made increased significantly across AA sessions for both genotypes, reflecting AA learning. Similar results were found in the mean response time across all active avoidance sessions, with 5-HTT$^{-/-}$ animals responding significantly faster than wildtype animals ($F_{(1,35)} = 4.644$, $p = 0.038$) in the absence of effects of conditioning ($F_{(1,35)} = 0.354$, $p = 0.555$) or genotype x conditioning interaction effects ($F_{(1,35)} = 0.472$, $p = 0.472$, Figure 2B). AA

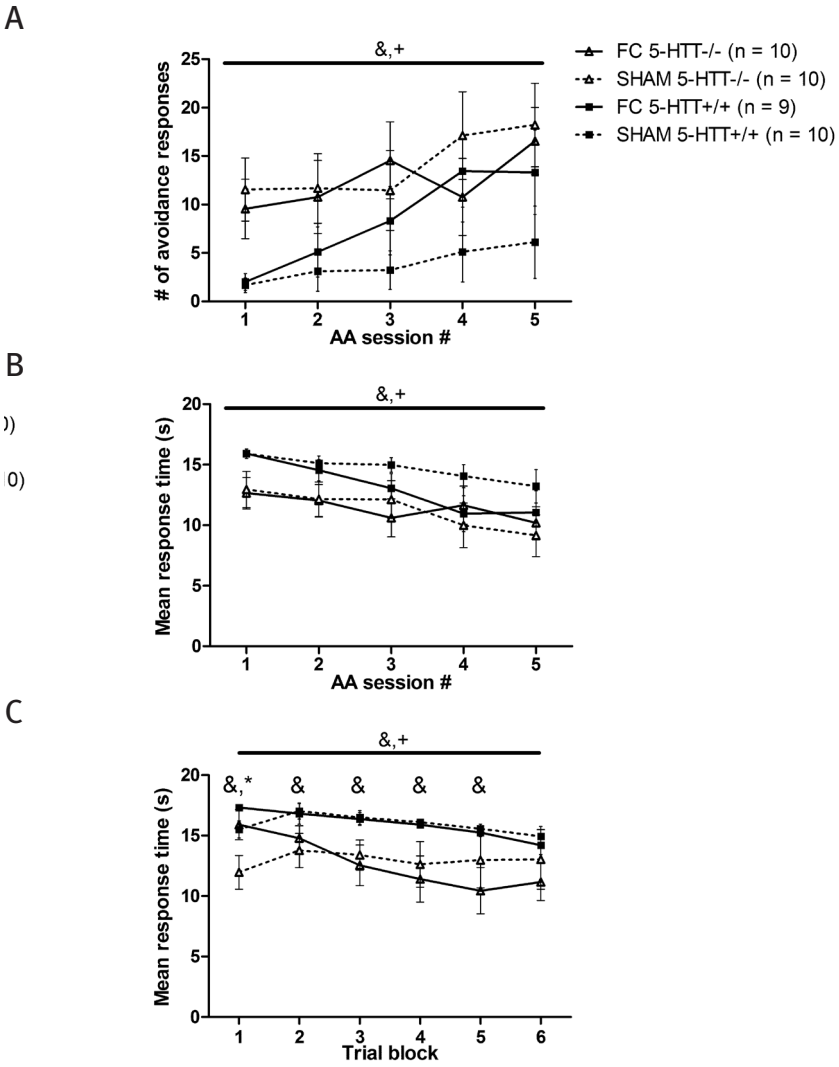
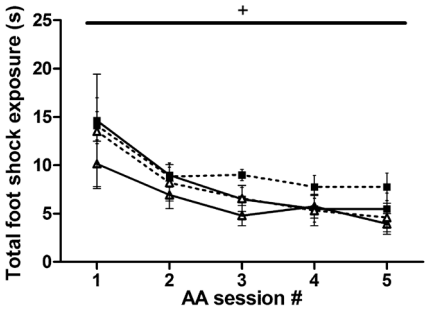


Figure 2 Active avoidance behavior. (**A**) Avoidance responses increased across AA sessions, and 5-HTT$^{-/-}$ rats made significantly more avoidance responses during the AA sessions. (**B**) Response time decreased across sessions, and was significantly lower in 5-HTT$^{-/-}$ rats. (**C**) Response time during the first AA session increased as a result of prior fear conditioning, but the effect did not persist past the first block of 5 trials. 5-HTT$^{-/-}$ rats responded significantly faster in all but one session.

A



B

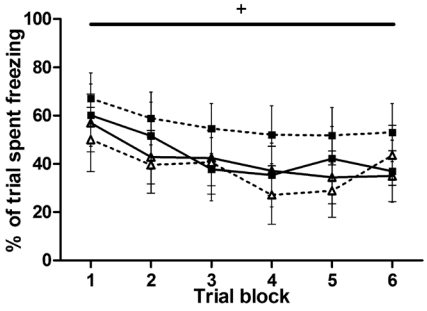


Figure 2 Continued. (**D**) Foot shock exposure during AA decreased across session and was not significantly affected by genotype or treatment. (**E**) No effects of genotype or fear conditioning were found in freezing during the last AA session. FC, fear conditioning; SHAM, sham conditioning. Data are expressed as mean ± S.E.M, freezing during AA session five was expressed as a percentage of the time during which the stimulus was presented. & indicates a significant effect of genotype on the overall number of avoidance responses and mean response time across all AA sessions, and the mean response time per trial block and across all trial blocks of the first AA session ($p < 0.05$). * indicates a significant effect of treatment on the first trial block of the first AA session ($p < 0.05$). + indicates a significant effect of session or trial number across all blocks / trials on every outcome ($p < 0.05$).

response time decreased significantly across sessions in both 5-HTT$^{-/-}$ ($F_{(1,19)} = 5.609$, $p = 0.003$) and wildtype rats ($F_{(1,18} = 10.482$, $p < 0.001$) without a significant genotype x session interaction ($F_{(1,35)} = 0.593$, $p = 0.593$), again reflecting AA learning. However, conditioning significantly increased AA response time during the first block of five trials of the first AA session ($F_{(1,35)} = 7.614$, $p = 0.009$), but not in the remaining blocks

of that session (Figure 2C). The data for the incidence of shock exposure during AA sessions correspond with those for the number of successful avoidances made subtracted from the number of trials per session (n=31), as a foot shock was initiated each time an animal failed to make an avoidance. The time exposed to foot shocks during AA per session decreased across sessions ($F_{(1,35)}$ = 17.684, p < 0.001), but was not affected by genotype ($F_{(1,35)}$ = 1.346, p = 0.254), treatment ($F_{(1,35)}$ = 0.651, p = 0.425) or displayed a genotype x treatment interaction ($F_{(1,35)}$ < 0.001, p = 0.993) (Figure 2D).

As a measure of passive coping behavior, the percentage of time spent freezing during active avoidance trials was assessed during the final session. No significant effects of genotype ($F_{(1,35)}$ = 1.376, p = 0.249), nor conditioning ($F_{(1,35)}$ = 0.250, p = 0.620), nor interactions between them ($F_{(1,35)}$ = 0.767, p = 0.387) were found regarding freezing behavior during active avoidance (Figure 2E). Freezing did however significantly decrease across the trials of the last session ($F_{(1,35)}$ = 4.064, p < 0.001), in both 5-HTT$^{-/-}$ ($F_{(1,19)}$ = 2.943, p = 0.002) and wildtype rats ($F_{(1,18)}$ = 2.318, p = 0.016), indicating successful acquisition of an active coping response to the stressor in the absence of a trial x genotype interaction ($F_{(1,35)}$ = 0.794, p = 0.585). Correlational analyses revealed that (in both genotypes) the amount of freezing correlated positively with response time in both genotypes (5-HTT$^{-/-}$; r = 0.744, p < 0.001, wildtype; r = 0.745, p < 0.001), whereas it correlated negatively with the number of avoidance responses made during the session (5-HTT$^{-/-}$; r = -0.691, p = 0.001, wildtype; r = -0.669, p = 0.002), indicating competing (i.e., passive vs. active) response strategies.

Post AA training CS exposure

In the novel environment, freezing behavior was first of all measured during the two minute pre-stimulus (i.e., habituation) period, serving as a measurement of baseline (novelty-induced) anxiety. We found that baseline freezing levels to a novel context were significantly higher in wildtype rats compared to 5-HTT$^{-/-}$ rats ($F_{(1,35)}$ = 7.219, p = 0.011, Figure 3A), whereas no effects of conditioning ($F_{(1,35)}$ = 2.118, p = 0.154), nor genotype x conditioning interaction ($F_{(1,35)}$ = 0.033, p = 0.856) were found. Subsequent CS-induced freezing across all trials was also found to be significantly higher in wildtype animals ($F_{(1,35)}$ = 5.050, p = 0.031), while no conditioning effects ($F_{(1,35)}$ = 1.527, p = 0.225) nor genotype x conditioning interactions ($F_{(1,35)}$ = 0.407, p = 0.528) were observed (Figure 3B). Freezing decreased significantly across trials ($F_{(1,35)}$ = 13.560, p < 0.001), in both 5-HTT$^{-/-}$ ($F_{(1,19)}$ = 7.724, p < 0.001) and wildtype rats ($F_{(1,18)}$ = 6.937, p < 0.001) in the absence of a trial x genotype interaction ($F_{(1,35)}$ = 0.794, p = 0.585), suggesting similar extinction of the behavioral freezing response to the CS in both genotypes. CS-induced freezing during the post AA CS exposure session correlated positively with the total response time across all active avoidance sessions, in 5-HTT$^{-/-}$ (r = 0.508, p = 0.022), but not in wildtype rats (r = 0.178, p = 0.466).

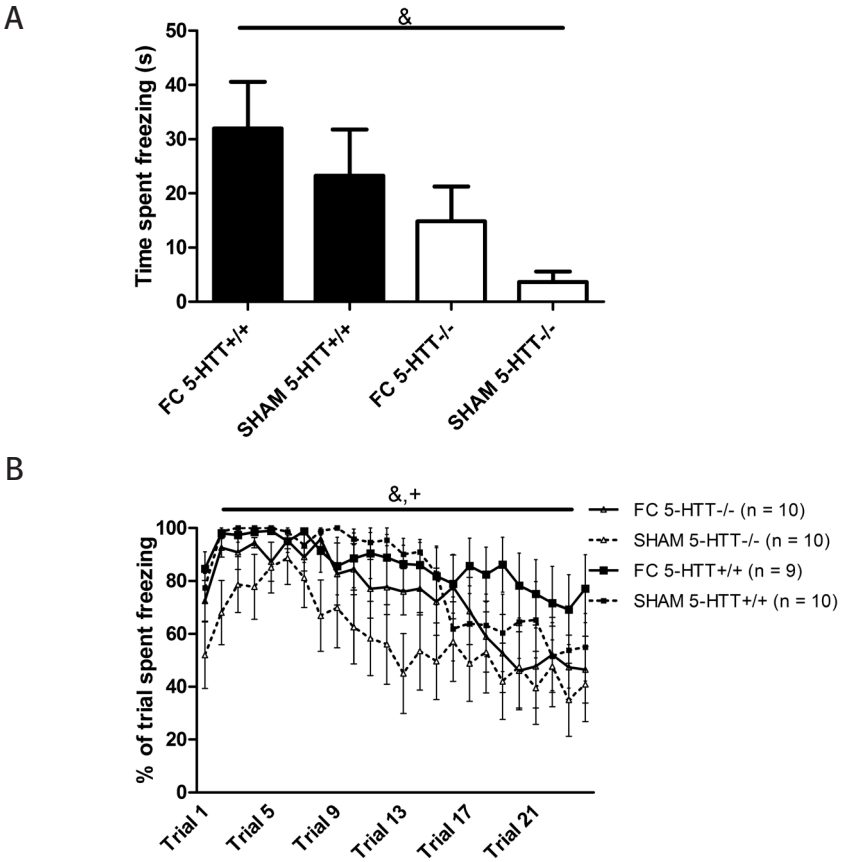


Figure 3 Freezing behavior during presentation of the CS in a novel context 24 hours after AA training. (**A**) In the 2-minute period before stimulus presentation, wildtype animals displayed significantly more freezing compared to 5-HTT$^{-/-}$ animals. (**B**) During the 24 stimulus presentations of the extinction session, wildtype rats froze significantly more than 5-HTT$^{-/-}$ rats. Data are expressed as mean percentage of time (of the 2 minute baseline period or 20 second cue presentation) spent freezing ± S.E.M. & indicates a significant effect of genotype on time spent freezing during the stimulus free baseline period and across all trials of the post AA CS exposure ($p < 0.05$). + indicates a significant effect of trial number on time spent freezing during CS exposure after AA training ($p < 0.05$).

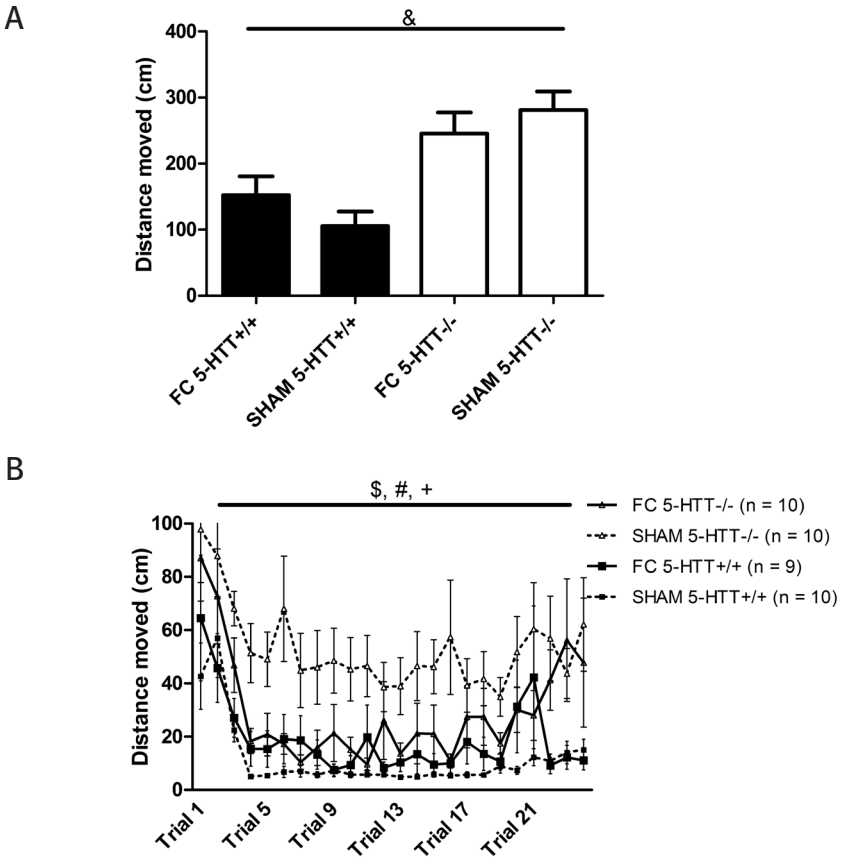


Figure 4 Locomotion during exposure to (the CS in) a novel context. (**A**) Locomotion during the 2-minute stimulus free period before CS presentation was significantly higher in 5-HTT$^{-/-}$ rats. (**B**) Fear conditioning prior to AA training significantly reduced locomotion during CS presentation in 5-HTT$^{-/-}$ rats, but not in 5-HTT$^{+/+}$ rats. Data are expressed as total distance moved in centimeters during a given time period (2 minute baseline period or 20 second CS presentation) ± S.E.M. $ indicates a significant gene x environment interaction in total distance moved across all trials of the post AA CS exposure ($p > 0.05$). & indicates a significant effect of genotype on distance moved during the stimulus free baseline period and across all trials of the post AA CS exposure ($p < 0.05$). + indicates a significant effect of trial number and # indicates a significant effect of conditioning in 5-HTT$^{-/-}$ rats ($p > 0.05$ on distance moved during the post AA CS exposure ($p < 0.05$).

Furthermore, 5-HTT$^{-/-}$ animals exhibited significantly more locomotion in the novel context during the stimulus free baseline period of the post AA CS exposure ($F_{(1,35)}$ = 20.712, p ‹ 0.001) (Figure 4A). During the subsequent CS exposures, a gene-environment interaction was found in locomotion ($F_{(1,35)}$ = 7.696, p = 0.009), driven by a reduction in locomotion during CS presentation due to prior conditioning in 5-HTT$^{-/-}$ ($F_{(1,18)}$ = 6.377, p = 0.021), but not wildtype rats ($F_{(1,17)}$ = 1.592, p = 0.224), signifying that prior conditioning reduced exploration of the novel environment only in 5-HTT$^{-/-}$ animals (but to similar levels as observed in wildtypes) (Figure 4B). In addition, an overall effect of trial number was found, indicating that exploration increased across trials ($F_{(1,35)}$ = 8.385, p › 0.001). Locomotion during the post AA CS exposure correlated with negatively with freezing in wildtype (r = -0.625, p = 0.004), but not in 5-HTT$^{-/-}$ rats (r = -0.307, p = 0.188).

Discussion

Here, we set out to test whether prior fear conditioning and the acquisition of a passive stress coping strategy interferes with subsequent acquisition of an active stress coping response in an AA task in a 5-HTT dependent manner. In addition, we assessed to what degree this passive-active stress coping interaction influenced the fear response outside of the original training context. Results confirm our previous finding that abolishment of 5-HTT in rats improves signaled AA learning (Schipper et al. 2015). However, fear conditioning prior to CS-signaled AA training only briefly impeded AA learning performance in 5-HTT$^{-/-}$ and wildtype animals. Moreover, prior fear conditioning did not influence freezing behavior during subsequent re-exposure to the CS in a novel context. However, it did reduce exploration in 5-HTT$^{-/-}$ rats to the level of wildtype rats in this setting. These findings counter our hypotheses that the acquired passive coping response (i.e. freezing behavior) would strongly interfere with the subsequent acquisition of an active coping response, and that changes in stress coping resulting from it would be more pronounced in wildtypes. Furthermore, 5-HTT$^{-/-}$ rats were shown to exhibit less freezing at baseline and to the CS in a novel context, indicating that the advantage of these animals during AA acquisition translates to reduced passive fear expression beyond the AA context.

Contrary to our expectations, prior fear conditioning (i.e., pairing the CS to brief inescapable foot shocks inducing a passive stress coping response) to the CS prior to CS-signaled AA training, had only minor effects on AA acquisition, in both 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats. Effects of prior conditioning were seen only in the first trials of the first session. This indicates that the new contingency of the stimulus acquired during those first trials quickly superseded the passive coping contingency that was attributed to it

during conditioning. Previous work has shown that exposure to a severe unpredictable, inescapable stressor induces an inflexible and generalized passive coping response known as learned helplessness (LH) (Overmier and Seligman 1967, Seligman and Maier 1967). However, the quantity and intensity of the foot shocks given in the LH paradigms exceed those given during the conditioning session in the present experiment by a large margin (e.g. (Baratta et al. 2007, Schulz et al. 2010)). This difference with LH in stressor intensity, as well as its predictability (Machida et al. 2013), may in fact crucially determine the generalization of its sequelae to other settings. Indeed, LH paradigms have been shown to not only affect subsequent acquisition of escape behavior, but also anxiety, fear extinction, and social parameters (for review see (Maier et al. 2006)) in paradigms which have little to no similarity to the context or proceedings of the LH assay. This indicates that the persistent behavioral consequences of LH are not solely mediated by the neural correlates of fear conditioning, but constitute a much broader range of physiological adaptations. Here, we aimed at potential disturbance of AA acquisition by targeting a passive coping response to a specific conditioned stimulus to measure the influence of 5-HTT expression on the ability to adapt a learned stress coping style to suit changing environmental conditions.

We show that 5-HTT genotype modulated signaled AA learning, with 5-HTT$^{-/-}$ acquiring the task faster than 5-HTT$^{+/+}$ rats, regardless of prior fear conditioning. In a previous study, we also observed improved AA in 5-HTT$^{-/-}$ rats, but the presently used AA paradigm differs in many key aspects (Schipper et al. 2015); the temporal distribution of the training sessions (all sessions same day vs. separate days), and the method of behavioral responding (crossover vs. nose poke). Replication of this finding suggests generally improved active stress coping in 5-HTT$^{-/-}$ rats. We have also found a similar advantage for 5-HTT$^{-/-}$ rats using a signaled lever press shock avoidance paradigm (unpublished findings), but not in instrumental food conditioning experiments (Nonkes et al. 2012a). This suggests that improved learning is specific to aversive learning paradigms. Successful signaled AA learning is dependent on the suppression of the (passive) fear response induced by presentation of the AA stimulus (foot shock) following unsuccessful avoidance trials (Moscarello and LeDoux 2013).

Finally, presentation of the CS in a novel context after fear conditioning and signaled AA training yielded lower freezing in 5-HTT$^{-/-}$ compared to wildtype animals. AA performance during the final AA training session was not different between genotypes ($F_{(1,35)} = 2.678$, $p = 0.110$), nor was the number of shocks received by both genotypes during the final AA session ($F_{1,35)} = 1.450$, $p = 0.236$). Yet, the resulting stimulus contingency produced a reduced passive fear response in 5-HTT$^{-/-}$ animals, which generalized to a novel environment. This is supported by the correlational data

showing that CS-induced freezing in the novel context was associated with AA performance in 5-HTT$^{-/-}$ (better performance predicted less freezing), but not wildtype animals.

The novel environment in which the post AA CS exposure took place induced an increased freezing (and reduced locomotive) response in wildtype animals, which may suggest either generalization of fear across contexts or induction of generalized anxiety. The increase in freezing in wildtype animals presumably resulted from AA, as it was not affected by prior fear conditioning in this study, and was not found previously in fear conditioned animals prior to fear extinction in a novel context (Nonkes et al. 2012a). As fear generalization is a key feature of anxiety disorders like PTSD (Lopresto et al., 2016), this may suggest controllable stressors are less likely to induce anxiety disorders in individuals with genetically reduced expression of 5-HTT.

While freezing behavior induced by post AA CS presentation was not affected by fear conditioning prior to AA training, it significantly reduced the typically increased exploratory behavior (as assessed by general locomotion) observed in non-conditioned 5-HTT$^{-/-}$ animals to levels comparable to wildtype animals. Thus, fear conditioning experience did not severely impair CS signaled AA learning in 5-HTT$^{-/-}$ rats, but it reduced the exploration of a new environment upon encountering the fear conditioned stimulus in these animals, reflecting some residual fear. Exploration of the environment may indicate that the animals are primed to interact with the CS and the environment, as they had done during AA training, even though the novel context provides no direct means of doing so. Apparently, prior association of the CS with passive stress endurance (obtained during fear conditioning) influenced coping behavior extending beyond AA training in 5-HTT$^{-/-}$ but not wildtype rats. Possibly, similar effects on exploratory behavior may be present in wildtype animals, but the high levels of freezing (and thus low exploration) observed in wildtype animals upon CS exposure during post AA CS presentation may obscure the measurement of such effects. Our data indeed suggest that high freezing interferes with locomotion in wildtypes, given the strong negative correlation between these behaviors present in this genotype.

A few limitations of the study require mention. First, since freezing during the first active avoidance session was not assessed, we cannot claim that the increased AA response time caused by conditioning in the first AA session was accompanied by increased freezing. However, given our previous experiences with the exact same conditioning procedure (Schipper et al. 2011a, Nonkes et al. 2012a, Shan et al. 2014), we expect the re-exposure to the stimulus to robustly induce a passive coping (i.e., freezing) response in these animals. Second, it is possible that the increased baseline

anxiety in wildtype animals observed post AA in a novel context affected freezing during the CS presentations, where wildtypes also exerted higher freezing. Remarkably, we previously reported lower novelty-induced freezing in wildtype rats after solely fear conditioning (Shan et al. 2014). Naïve 5-HTT$^{-/-}$ rodents normally display more anxiety-related behaviors and reduced exploration in anxiety-assays in a novel environment, such as the elevated plus maze and open field test (Kalueff et al. 2010, Mohammad et al. 2016). Therefore, the additional AA training (and thus altered stressor experience in terms of severity and controllability) in the present experiments has likely affected the behavioral reaction to novel environments. Third, sex-specific effects of 5-HTT on stressor coping transfer were not assessed here as we considered these beyond the scope of this study. Stress coping behavior has been demonstrated to be sex-dependent (e.g. (Gruene et al. 2015)), as have the risks of developing stress-related psychiatric disorders (see e.g. (Bromet et al. 2011, Shansky 2015). Therefore, future studies will have to determine whether the findings reported here can be generalized across sexes. Finally, it remains to be investigated whether the behavioral coping responses resulting from passive-active CS retraining are CS-specific, or if they are subject to fear generalization, one of the hallmark symptoms of PTSD. Therefore, coping responses during stimulus-free periods and during presentation of stimuli of various degrees of similitude should be assessed in future studies.

To conclude, in line with our suggestion that 5-HTT$^{-/-}$ rats have a greater tendency to cope actively than passively with signaled stressors, 5-HTT$^{-/-}$ animals are better able to suppress their passive coping response to the CS after gaining control over it. However, conditioned fear resists effective extinction in 5-HTT$^{-/-}$ animals when environmental conditions do not provide opportunities for active stress coping (Wellman et al. 2007, Schipper et al. 2011a, Shan et al. 2014) . Our findings of reduced freezing in response to CS exposure following AA-training advocate that behavioral therapy focused on adopting an active coping strategy may supplant or supplement extinction therapy to combat fear extinction impairments induced by variation in 5-HTT expression. We therefore propose further investigation to elucidate the therapeutic potential of approaching psychiatric treatment of trauma with an emphasis on engendering active coping, thereby reducing fear. Furthermore, we recommend an increased focus on coping style flexibility when assessing the influence of genotype – stress interactions on vulnerability and resilience.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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6

A mixed PUFA diet normalizes hippocampal neurogenesis and reduces anxiety in serotonin transporter knockout rats

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Abstract

The aim of this study was to investigate the effects of a mixed dietary intervention on behavioral symptoms in serotonin transporter knockout (5-HTT$^{-/-}$) rats modeling the human 5-HTT length polymorphic region short-allele. Twenty female 5-HTT$^{-/-}$ and 19 wild-type (5-HTT$^{+/+}$) rats were fed for 3 months on a mixed polyunsaturated fatty acid (PUFA) diet comprising n-3 PUFAs, B vitamins and phospholipids, or an isocaloric control diet, and a subgroup was subsequently tested in an array of anxiety-related behavioral tests. All brains were harvested and immunostained for doublecortin, a neurogenesis marker. In addition, hippocampal volume was measured. 5-HTT$^{-/-}$ rats on the control diet displayed increased anxiety-related behavioral responses, and impaired fear extinction. These effects were completely offset by the mixed PUFA diet, whereas this diet had no behavioral effect in 5-HTT$^{+/+}$ rats. In parallel, dentate gyrus doublecortin immunoreactivity was increased in 5-HTT$^{-/-}$ rats fed on the control diet, which was reversed by the mixed PUFA diet. Hippocampal volume was unaffected by the mixed PUFA diet in 5-HTT$^{-/-}$ subjects, whereas it increased in 5-HTT$^{+/+}$ rats. We conclude that a mixed n-3 PUFA diet ameliorates anxiety-related symptoms in a genotype-dependent manner, potentially by normalizing neurogenesis. We suggest that such a mixed diet may serve as an attractive adjuvant to treat anxiety in 5-HTT length polymorphic region short-allele carriers.

Introduction

Anxiety and depression are leading causes of disease burden in the United States (Kessler et al. 2005). As 50% of the patients do not respond to first drug treatment, suffering is prolonged and medical costs increased (Bystritsky 2006). Therefore, the need for alternative treatments for those showing treatment resistance is high.

Affective disorders are associated with disturbances of the serotonergic system (Mann 1999, Gordon and Hen 2004, Lesch and Gutknecht 2005). Because the 5-HT transporter (5-HTT) is solely responsible for reuptake of 5-HT from the synaptic cleft (Kriegebaum et al. 2010), changes in 5-HTT expression and/or function have profound consequences for the availability of 5-HT in the extracellular space and emotional control. For example, the low activity (short; s) variant of the 5-HTT length polymorphic region (5-HTTLPR) in humans, is well-known for its association with anxiety-related personality traits (Lesch et al. 1996), and increased risk for depression due to early-life stress (Caspi et al. 2003, Caspi et al. 2010). These behavioral manifestations correlate with amygdala hyper-reactivity, amygdala and hippocampus hyper-perfusion, and alterations in volume (Canli et al. 2006, Canli and Lesch 2007). It is possible that a 'gain of function' in these limbic nodes results in decreased stress-resilience (Young et al. 2008). 5-HTTLPR genotype also influences the decrease in hippocampal volume typically seen in prolonged affective disorders, although some ambiguity remains with regard to the precise nature of this effect (Bremner et al. 1995, Taylor et al. 2005, Canli et al. 2006, Frodl et al. 2008).

Meta-analyses have revealed that 5-HTTLPR short (s)-allele carriers respond relatively poor to selective serotonin reuptake inhibitors (SSRIs) (Lesch and Gutknecht 2005, Stein et al. 2006, Serretti et al. 2007), a staple in the treatment of affective disorders (Stein et al. 1998). SSRIs may exert their effects through alterations in adult hippocampal neurogenesis (Santarelli et al. 2003), potentially via altered 5-HT_{1A} signaling (Wang et al. 2010). The association between the s-allele and neurogenesis remains to be assessed, but the 'gain of function' concept suggests that SSRI mechanisms of action work out differently in s-allele carriers.

Recently, nutrients like n-3 poly-unsaturated fatty acids (n-3 PUFA's) and B-vitamins have gained interest for their influence on mood-related disorders; an inverse relation was demonstrated between n-3 PUFA intake and prevalence of anxiety and depression (Hibbeln et al. 2006, Sanchez-Villegas et al. 2007, Lin et al. 2010), and increased intake improved the outcome of regular antidepressant therapy (Nemets et al. 2002). Increasing the n-3/n-6 PUFA ratio in the neuronal membrane beneficially affects membrane fluidity (Suzuki et al. 1998), which, in turn, facilitates neuronal signaling by

increasing ion channel availability (Zimmer et al. 2000), or increasing 5-HT_{1A} receptor density (Levant et al. 2008) and binding affinity (Farkas et al. 2002). *Vice versa*, n-3 PUFA deficiency may negatively affect serotonin neurotransmission (Chalon 2006). Additionally, n-3 PUFA's have been associated with changes in hippocampal neurogenesis in rodents (Kawakita et al. 2006, Cao et al. 2009, He et al. 2009, Dyall et al. 2010), and the intake of B-vitamins is inversely related with the risk for depression (Hibbeln et al. 2006, Skarupski et al. 2010). Finally, vitamin B_9 (folate) increases the synthesis of s-adenosyl methionine (SAMe). SAMe is a methyl donor in central membrane phospholipid synthesis (Hirata and Axelrod 1980) and exerts antidepressant properties (Bressa 1994), also in SSRI-resistant depressed patients (Papakostas et al. 2010).

Since nutrients may help to compensate deficits reported for s-allele carriers, we hypothesized that a mixed diet of n-3 PUFAs, phospholipids and B-vitamins has beneficial effects in 5-HTTLPR s-allele carriers. To test this hypothesis, we used female serotonin transporter knockout (5-$HTT^{-/-}$) rats and wild-type (5-$HTT^{+/+}$) controls. Similar to human s-allele carriers, these animals show increased anxiety and depression-like symptoms (Kalueff et al. 2010). The higher prevalence of affective disorders among women compared to men (Hausken et al. 2010, Hollingworth et al. 2010) was our rationale for choosing female rats. After being fed either a mixed PUFA diet or a control diet, the animals were subjected to a series of anxiety-related tests (elevated plus maze, social interaction test, fear extinction). Because neurogenesis is implicated in depression, SSRI, and n-3 PUFA effects (Kawakita et al. 2006, Cao et al. 2009, He et al. 2009, Dyall et al. 2010), we also addressed the neurobiological correlates of genotype and diet effects using immunohistochemical staining for the neurogenesis marker doublecortin (DCX) (Brown et al. 2003). In addition, we measured hippocampal volume, as n-3 PUFA consumption may increase hippocampal volume (Venna et al. 2009).

Materials and Methods

Animals

Serotonin transporter knockout rats (*Slc6a4^{1Hubr}*) were generated by N-ethyl-N-nitrosourea-induced mutagenesis (Smits et al. 2006a). Female experimental animals were derived from crossing heterozygous 5-HTT knockout (5-$HTT^{+/-}$) rats that were outcrossed with commercial (Harlan, Ter Horst, The Netherlands) wild-type Wistar rats for at least eight generations. All animals were housed two per cage in a temperature (21 ± 1°C) and humidity-controlled room (45-60% relative humidity), and had *ad libitum* access to water and food until testing. A 12/12 h light–dark cycle was maintained, with

lights on at 08.00 a.m. All experiments were approved by the Committee for Animal Experiments of the Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands (application # 2009-095), and performed in compliance with European Communities Council Directive 86/609/EEC. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Diets

From the age of 65 days the animals were fed for 3 months with either a control diet or a mixed PUFA diet (Research Diet Services, Wijk bij Duurstede, The Netherlands) before behavioral studies started. The diets were continued during behavioral testing and in the week before sacrifice. Both diets were AIN-93M based (Reeves et al. 1993), isocaloric and identical with respect to their protein, carbohydrate, fiber, and mineral content. Differences between the diets are summarised in Table 1. The diets were stored at -20°C, and used within the expiration date.

Table 1 Diet contents.

	Control	Mixed PUFA
5% Fat		
Soy oil	1.90	
Coconut oil	0.90	0.10
Corn oil	2.20	1.87
Fish oil		3.03
Extra's		
Soy lecithin		0.755
Pyridoxine		0.00328
Folic acid		0.00067

Overview of the differences between the two isocaloric diets that were used in the current study. In contrast to the control diet, the mixed PUFA diet provided the combination of n-3 PUFA's (fish oil), phospholipids (soy lecithin) and increased levels of B-vitamins (pyridoxine and folic acid). Values are expressed as g / 100g diet.

Experimental groups

We used four groups of animals: 1] 5-HTT$^{+/+}$ rats on control diet, 2] 5-HTT$^{-/-}$ rats on control diet, 3] 5-HTT$^{+/+}$ rats on mixed PUFA diet, and 4] 5-HTT$^{-/-}$ rats on mixed PUFA diet. All groups consisted of 10 animals, except 5-HTT$^{+/+}$ rats on mixed PUFA diet

(9 animals). In order to determine whether the behavioral experiments influenced hippocampal neurogenesis, 5 rats of each group were used in the behavioral experiments and immunohistochemistry and 5 were used exclusively for immunohistochemistry. Because no differences in DCX immunostaining and hippocampal volume were detected (Table 2), the two groups were pooled for these parameters.

Table 2 Neurogenic effects of behavioral tests.

	p-value	t	df
Neurogenesis			
5-HTT$^{+/+}$ control diet	0.99	0.02	6
5-HTT$^{+/+}$ mixed n-3 PUFA diet	0.99	0.02	7
5-HTT$^{-/-}$ control diet	0.69	0.42	7
5-HTT$^{-/-}$ mixed n-3 PUFA diet	0.78	0.29	8
Hippocampal volume			
5-HTT$^{+/+}$ control diet	0.19	1.5	6
5-HTT$^{+/+}$ mixed n-3 PUFA diet	0.09	2.0	6
5-HTT$^{-/-}$ control diet	0.14	1.7	6
5-HTT$^{-/-}$ mixed n-3 PUFA diet	0.41	0.87	8

Overview of statistical comparisons of neurogenesis and hippocampal volume data between groups of participants and non-participants of behavioral tests.

Behavior

In general, the behavior observer was trained and unaware of the genotype and diet group of the animals. Behavioral tests were performed in the order of mention, from least stressful to most stressful, with at least 48 hours between tests.

Elevated plus maze

The maze was elevated to a height of 50 cm with two open (50×10) and two enclosed arms (50×10×40) arranged such that the arms of the same type were opposite to each other. The illumination intensity measured in the open arms was 80 lux. Rats were placed in the center of the maze for a free exploration period of 5 minutes, as described earlier (Olivier et al. 2008). The movements and position of the animals were recorded and registered automatically using Ethovision® 3.1 software (Noldus Information Technology B.V., Wageningen, The Netherlands). Results were expressed as the mean

of time spent in the open arms, and total distance moved. Results were expressed as the mean of time spent in the open arms, closed arm entries, and total distance moved.

Social interaction test

Social interaction was measured in a test cage (50 x 50 x 75 cm ((l x w x h)) which had transparent walls and was filled with sawdust (2 cm). The experimental room was illuminated by a 25-W fluorescent red light, mounted 60 cm above the test cage. Twenty-four hours before the test, the female rats were habituated to the test cage during 10 minutes. Social interaction pairs were designed such that both rats were genotype and diet matched, and that rats from the same litter or home cage were not paired. Thus, there were types of rat pairing: 5-HTT$^{-/-}$ and 5-HTT$^{-/-}$ fed on control diet, 5-HTT$^{-/-}$ and 5-HTT$^{-/-}$ fed on mixed PUFA diet, 5-HTT$^{+/+}$ and 5-HTT$^{+/+}$ fed on control diet, and 5-HTT$^{+/+}$ and 5-HTT$^{+/+}$ fed on mixed PUFA diet. On the test day, test pairs were isolated for 2 hours prior to the test to increase in the amount of social behavior, and subsequently tested for 15 minutes. Behavior of the animals was recorded on video tape. Using Observer 4.0 (Noldus Information Technology, Wageningen, The Netherlands), frequencies and durations of the following behaviors were scored: *contact*: sniffing or licking any body part of the test partner; *self-grooming*: forepaw licking, face washing, scratching, body grooming and genital grooming; *no contact*: none of these behaviors. Behavior was assessed per individual animal. Animals were used only once.

Forced swim test

Cylindrical glass tanks (50 cm long×18 cm in diameter), filled to a depth of 30 cm with water at 25(±1)°C, were used. After a 15-min water experience on day 1, the subjects were tested 24 h later in the water cylinders for 5 min. The movements of the rats were videotaped for offline measurement of the duration of immobility (s). The behavioral variable 'immobility' was defined as follows: making no movements for at least 2 s or making only those movements that were necessary to keep the nose above the water. Active climbing, diving and swimming along the wall were scored as mobility (s). The results of the forced swim test are not shown in the results section because several rats had to be excluded from the test for lodging themselves between the cylinder walls, a strategy that results in misleading values for immobility and swimming data. After exclusion of these rats the groups had become too small for reliable statistical analysis.

Pavlovian fear conditioning and extinction (recall)

Conditioning was conducted in a home-made chamber with transparent walls and a metal rod floor. A camera was mounted on the top of the chamber. After habituation to the chamber the animals received a conditioning session consisting of a 120 second acclimation period (baseline measurement), three pairings (60–120 s variable inter-

stimulus interval) of the conditioned stimulus (CS) (30 s, 80 dB, 3 kHz tone) and the unconditioned stimulus (US) (1 second, 0.6 mA scrambled footshock), in which the US was presented during the last 2 seconds of the CS (home-made freezing program). After a 120 seconds no-stimulus consolidation period the rats were returned to their home cage. 24 hours later, initial CS-recall and subsequent CS-extinction (test 1) was measured in a novel context and room. After a 120 second acclimation period (baseline measurement), the rats received five 30 second CS presentations (60-120 seconds variable interstimulus interval). The same procedure was repeated 24 (test 2) and 48 (test 3) hours later. Freezing (no visible movement except respiration) was scored using Observer 4.0 (Noldus Information Technology). Freezing was summed up in each session, and extinction recall during test 2 and 3 was expressed as % of freezing during test 1.

Immunohistochemistry

Immunostaining

The procedure was adopted from (Olivier et al. 2008, Nonkes et al. 2010). One week following the last behavioral test (when potential immediate effects of stress were expected to be 'washed out'), anesthetised rats were perfused transcardially with 0.1 mol/l PBS, pH 7.3, followed by 400 ml 4% paraformaldehyde dissolved in 0.1 mol/l of phosphate buffer, pH 7.2. Subsequently, the brains were removed from the skull and postfixed overnight in 4% paraformaldehyde at 4°C. Before sectioning, the brains were cryoprotected with 30% sucrose in 0.1 mol/l of phosphate buffer. Forty micrometer thick brain sections were cut on a freezing microtome, and collected in six parallel series in 0.1 mol/l PBS containing 0.1% sodium azide. One series from each rat was used for every staining. The free-floating sections were washed three times in PBS and preincubated with 0.3% perhydrol (30% H_2O_2, Merck, Darmstadt, Germany) for 30 min. After washing three times in PBS the sections were presoaked for 30 min in an incubation medium consisting of PBS with 0.1% bovine serum albumin and 0.5% Triton X-100. The sections were then incubated with goat anti-DCX, C-18 terminal, 1 : 3000 (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) overnight at room temperature, on a shaker, and consecutively incubated for 90 min at room temperature with donkey anti-rabbit, diluted 1 : 1500 in incubation medium, (Jackson Immuno Research Laboratories, West Grove, Pennsylvania, USA) and for 90 min at room temperature with ABC-elite, diluted 1 : 800 in PBS (Vector Laboratories, Burlingame, California, USA). Between incubations, sections were rinsed three times with PBS. The DCX–antibody peroxidase complex was made visible using 3,3'-diaminobenzidine tetrahydrochloride staining. Sections were incubated for 10 min in a chromogen solution consisting of 0.02% 3,3'-diaminobenzidine tetrahydrochloride and 0.03% nickel–ammonium sulfate in 0.05 mol/l Tris-buffer (pH 7.6), and subsequently for 10 min in chromogen solution containing 0.006% hydrogen peroxide. This resulted in blue–

black staining. Then the sections were rinsed three times in PBS and mounted on gelatin chrome alum-coated glass slides, dried overnight in a stove at 37°C, dehydrated in an increased series of ethanol, cleared in xylene, embedded with Entellan (Merck), and coverslipped. On account of technical difficulties during sectioning of the brains, several brains could not be used for immunohistochemistry or assessment of hippocampal volume.

Quantification

Numbers of DCX-immunopositive cells were quantified using the software program Stereo Investigator (MicroBrightfield Inc, Williston, Vermont, USA). Cells were counted in the hippocampus in sections at Bregma -3.30 mm, -3.80 mm and -4.16 mm, at 20x magnification (Paxinos 2004). The results for each subject were expressed as the total amount of cells counted in these three sections. Hippocampal volume was deduced using photos of the hippocampus at Bregma -3.80mm taken at 2.5x magnification (Paxinos 2004). Using ImageJ, a public domain image processing program (http://rsb.info.nih.gov/ij/) we subsequently drew a contour around the hippocampus of which the surface area was calculated.

Statistical analysis

Data are presented as mean±standard error of the mean (S.E.M.). All statistical analyses were performed using SPSS version 16.0 (SPSS Inc., Chicago, Illinois, USA). Data were analyzed using a two-way analysis of variance (ANOVA) or a repeated-measures ANOVA (fear extinction recall) with genotype and diet as between-subject factors. When appropriate, subsequent Student's t-tests were performed. Probability P values of less than 0.05 were considered significant.

Results

Mixed PUFA diet exhibits anxiolytic properties in the elevated plus maze in 5-HTT$^{-/-}$ rats

Two-way ANOVA revealed a significant genotype effect ($F_{(3,16)} = 9.7$, $p < 0.01$) and a significant genotype x diet interaction ($F_{(3,16)} = 9.8$, $p < 0.01$) for time spent in the open arms, but no main diet effect ($F_{(3,16)} = 0.93$, not significant (NS)) was obtained (Figure 1a). 5-HTT$^{-/-}$ rats on a control diet spent significantly less time in the open arms than their 5-HTT$^{+/+}$ counterparts ($t_{(1,8)} = 4.2$, $p < 0.005$). The mixed PUFA diet increased open arm time in the 5-HTT$^{-/-}$ group ($t_{(1,8)} = 3.2$, $p < 0.05$), whereas 5-HTT$^{+/+}$ rats were unaffected ($p > 0.05$). We observed no significant effect of genotype ($F_{(3,16)} = 0.13$, NS), diet ($F_{(3,16)} = 0.0086$, NS) or an interaction between the two ($F_{(3,16)} = 0.16$, NS) on the total distance moved during the elevated plus maze test (Figure 1b). We saw a

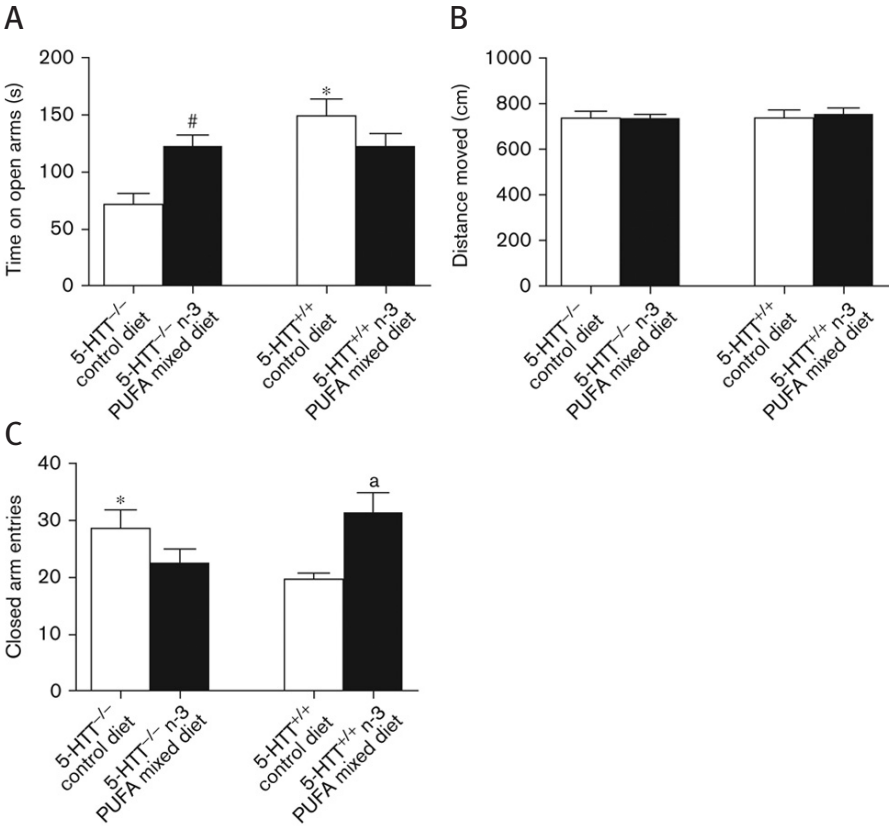

Figure 1 Effects of mixed PUFA diet in 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats on elevated plus maze performance. Data represent mean ± S.E.M. of time spent (seconds) in the open arms (**A**), total distance moved on the elevated plus maze (**B**), and the number of closed arm entries (**C**). * $p < 0.05$ 5-HTT$^{-/-}$ versus 5-HTT$^{+/+}$ rats on control diet; # $p < 0.05$ 5-HTT$^{-/-}$ rats on control diet versus mixed PUFA diet.

significant genotype–diet interaction in the amount of closed arm entries during the elevated plus maze test ($F_{(3,16)} = 9.56$, $p < 0.01$), but no separate effects of genotype ($F_{(3,16)} = 0.75$, NS) or diet ($F_{(3,16)} < 0.001$, NS). 5-HTT$^{-/-}$ rats on the control diet entered the closed arm more often than 5-HTT$^{+/+}$ rats on the same diet ($t_{(1,8)} = 2.7$, $p < 0.05$). Frequency of closed arm entry was also significantly higher in 5-HTT$^{+/+}$ rats on the mixed PUFA diet compared with their control diet peers ($t_{(1,8)} = 2.85$, $p < 0.05$) (Figure 1c).

Mixed PUFA diet enhances social behavior in 5-HTT$^{-/-}$ rats

There was a significant genotype x diet interaction ($F_{(3,12)} = 14.3$, $p < 0.005$) for total time spent in contact with the test partner (Figure 2A). In addition, there was a diet effect ($F_{(3,12)} = 5.3$, $p < 0.05$), but no main genotype effect was obtained ($F_{(3,12)} = 0.01$, NS). The 5-HTT$^{+/+}$ rats on control diet spent significantly more time on contact than 5-HTT$^{-/-}$ rats on control diet ($t_{(1,6)} = 4.8$, $p < 0.005$). Further, the mixed PUFA diet significantly increased contact time in 5-HTT$^{-/-}$ rats ($t_{(1,6)} = 3.8$, $p < 0.01$). There was no significant difference in contact time between the control and mixed PUFA diet groups

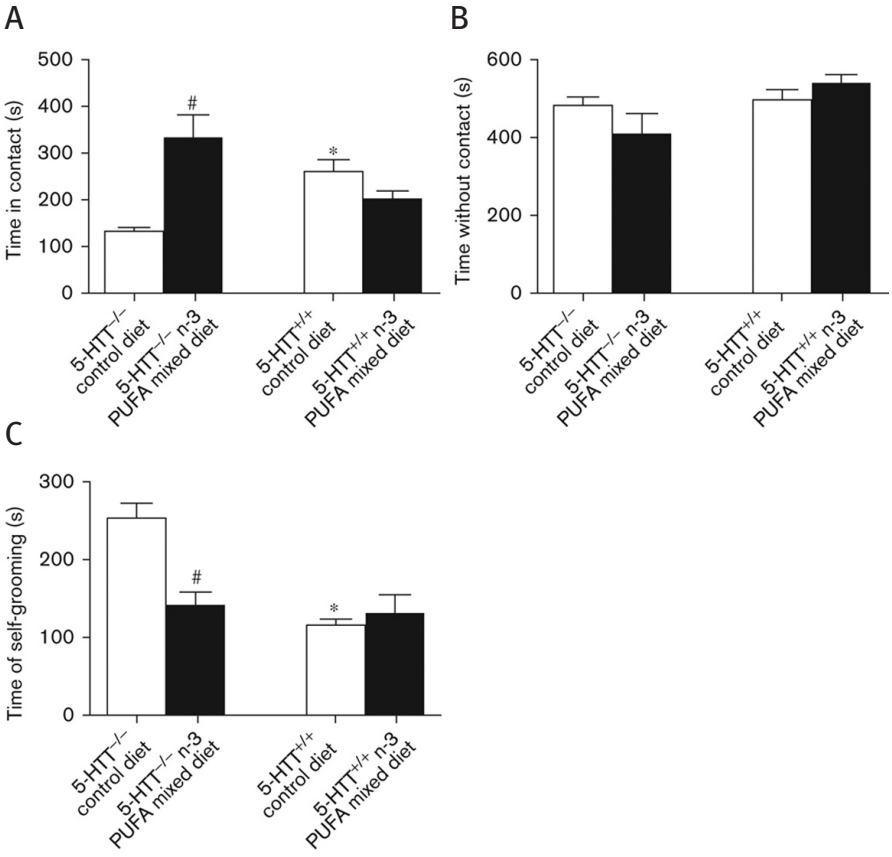

Figure 2 Effects of mixed PUFA diet in 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats on behavior in the social interaction test. Data represent mean ± S.E.M. of time spent (seconds) on social contact (**A**), no social contact (**B**), and self-grooming (**C**). * $p < 0.05$ 5-HTT$^{-/-}$ versus 5-HTT$^{+/+}$ rats on control diet; # $p < 0.05$ 5-HTT$^{-/-}$ rats on control diet versus mixed PUFA diet.

for the 5-HTT$^{+/+}$ rats ($p > 0.05$). None of these effects were found in the no-contact parameter (genotype ($F_{(3,12)} = 4.6$, NS); diet ($F_{(3,12)} = 0.24$, NS); genotype x diet interaction: ($F_{(3,12)} = 2.7$, NS; Figure 2B)). When comparing the time spent on self-grooming we found a significant genotype x diet interaction ($F_{(3,12)} = 11.0$, $p < 0.01$), a diet effect ($F_{(3,12)} = 7.0$, $p < 0.05$), and a genotype effect ($F_{(3,12)} = 14.9$, $p < 0.005$) (Figure 2C). More time was spent on self-grooming by 5-HTT$^{-/-}$ compared to 5-HTT$^{+/+}$ rats when fed on control diet ($t_{(1,6)} = 6.6$, $p < 0.005$). Comparing mixed PUFA diet fed 5-HTT$^{-/-}$ subjects to control diet counterparts revealed that the latter group spent significantly more time on self- grooming ($t_{(1,6)} = 4.2$, $p < 0.005$). This diet effect was not seen in 5-HTT$^{+/+}$ animals ($p > 0.05$).

Mixed PUFA diet facilitates fear extinction recall in 5-HTT$^{-/-}$ rats

Due to technical issues with recording equipment, three animals were excluded from this analysis (one 5-HTT$^{-/-}$ rat on mixed PUFA diet, two 5-HTT$^{-/-}$ rats on control diet). Baseline freezing in response to the conditioning environment, prior to the conditioning session, was higher in 5-HTT$^{+/+}$ rats compared to 5-HTT$^{-/-}$ rats ($F_{(3,15)} = 7.6$, $p < 0.05$). No further effects or interactions ($p > 0.05$) were found in baseline freezing upon exposure to either conditioning or extinction environment. For time spent on freezing, expressed as % of freezing during test 1, we obtained a significant genotype x diet interaction ($F_{(3,12)} = 9.4$, $p < 0.05$), and a genotype effect ($F_{(3,12)} = 5.1$, $p < 0.05$), but no main diet effect ($F_{(3,12)} = 0.25$, NS) (Figure 3). The data imply that the mixed PUFA diet facilitated fear extinction recall in the otherwise extinction recall impaired 5-HTT$^{-/-}$ rats, given that the significant diet effect in 5-HTT$^{+/+}$ rats ($t_{(1,6)} = 3.5$, $p < 0.05$) was absent during test 3 ($t_{(1,6)} = 2.3$, NS), while the significant genotype effect in animals on control diet during test 2 ($t_{(1,6)} = 2.7$, $p < 0.05$) was maintained through test 3: $t_{(1,6)} = 2.9$, $p < 0.05$). Furthermore, the significant diet effect in 5-HTT$^{-/-}$ rats was only observed during test 3 (test 2: $t_{(1,6)} = 0.93$, NS; test 3: $t_{(1,6)} = 2.4$, $p < 0.05$).

Mixed PUFA diet reduces hippocampal neurogenesis in 5-HTT$^{-/-}$ rats

In this analysis, three brains were excluded due to technical difficulties (one 5-HTT$^{-/-}$ rat on control diet (technical error during staining), one 5-HTT$^{+/+}$ rat on control diet (spoiled) and one 5-HTT$^{+/+}$ rat on mixed PUFA diet (hippocampal sections not intact)), and one rat was designated a statistical outlier (5-HTT$^{-/-}$ rat on mixed PUFA diet, > 3 standard deviation from the mean). Examples of our immunohistochemical staining are displayed in Figure 4. We obtained a significant interaction between genotype and diet ($F_{(3,31)} = 4.5$, $p < 0.05$) (Figure 5), as well as a significant effect of diet ($F_{(3,31)}=6.4$, $p<0.05$). A significant genotype effect was not found ($F_{(3,31)} = 3.9$, NS). 5-HTT$^{-/-}$ animals exhibited significantly more DCX immunostaining than 5-HTT$^{+/+}$ rats when both were fed on the control diet ($t_{(1,15)} = 2.4$, $p < 0.05$). The comparison of diet effects in 5-HTT$^{-/-}$ animals yielded a significant reduction in DCX-immunopositive hippocampal neurons

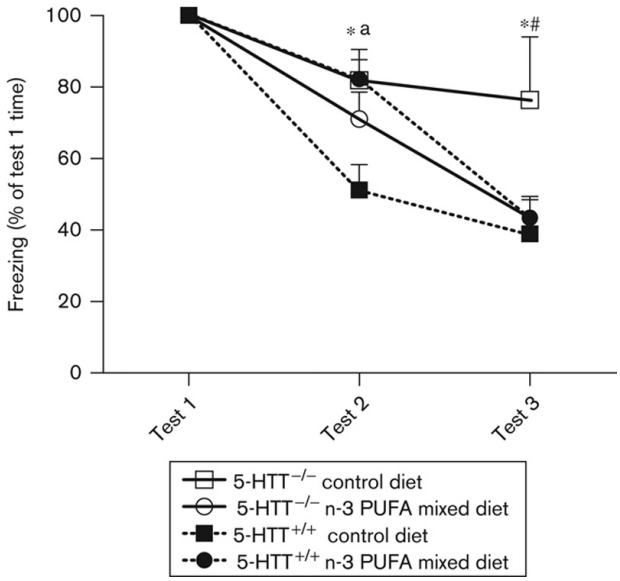

Figure 3 Effects of mixed PUFA diet in 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats on the extinction recall of conditioned fear. Data represent mean ± S.E.M. of time spent on conditioned freezing behavior on test day 2 (48 hours following conditioning) and day 3 (72 hours following conditioning), expressed as % of freezing time on day 1 (24 hours following conditioning). * $p < 0.05$ 5-HTT$^{-/-}$ versus 5-HTT$^{+/+}$ rats on control diet; # $p < 0.05$ 5-HTT$^{-/-}$ rats on control diet versus mixed PUFA diet; [a] $p < 0.05$ 5-HTT$^{+/+}$ rats on control diet versus mixed PUFA diet.

in the animals fed on n-3 PUFA ($t_{(1,17)} = 3.4$, $p < 0.005$). In 5-HTT$^{+/+}$ animals the diet had no significant effect ($t_{(1,15)} = 0.32$, NS).

Mixed PUFA diet increases hippocampal volume of 5-HTT$^{+/+}$ rats to a level indistinguishable from 5-HTT$^{-/-}$ rats

Because of technical reasons we were unable to determine hippocampal volume in four of the brains (hippocampal sections not intact: one 5-HTT$^{-/-}$ rat on control diet, and two 5-HTT$^{+/+}$ rats on mixed PUFA diet; spoiled: one 5-HTT$^{+/+}$ rat on control diet), one brain was a statistical outlier (5-HTT$^{-/-}$ rat on control diet, > 3 standard deviation from the mean). Hippocampal volume showed a genotype x diet interaction ($F_{(3,30)} = 9.4$, $p < 0.005$) (Figure 6), but no significant effects were found for diet ($F_{(3,30)} = 0.71$, NS) or genotype ($F_{(3,30)} = 2.8$, NS). Hippocampal volume was larger in 5-HTT$^{-/-}$ rats

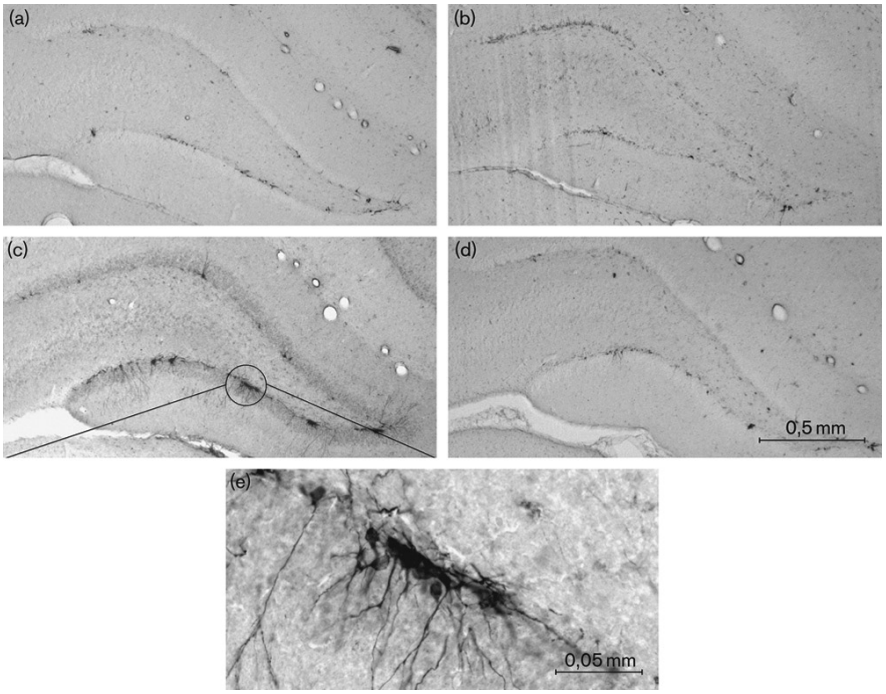

Figure 4 Doublecortin immunohistochemical staining. Displayed at 5x magnification: stained 40 µm hippocampal sections of a 5-HTT$^{+/+}$ animal on control diet (**A**), a 5-HTT$^{+/+}$ animal on mixed PUFA diet (**B**), a 5-HTT$^{-/-}$ animal on control diet (**C**), and a 5-HTT$^{-/-}$ animal on mixed PUFA diet (**D**). Displayed at 40x magnification: 5-HTT$^{-/-}$ animal on control diet (**E**).

compared to 5-HTT$^{+/+}$ rats ($t_{(1,14)} = 4.8$, $p < 0.0005$), and a significant enlargement of the hippocampus in 5-HTT$^{+/+}$ rats as a result of administering the mixed PUFA diet ($t_{(1,14)} = 3.0$, $p < 0.01$) was found. Animals from both genotypes on the mixed PUFA diet did not differ in hippocampal volume ($t_{(1,16)} = 0.84$, NS).

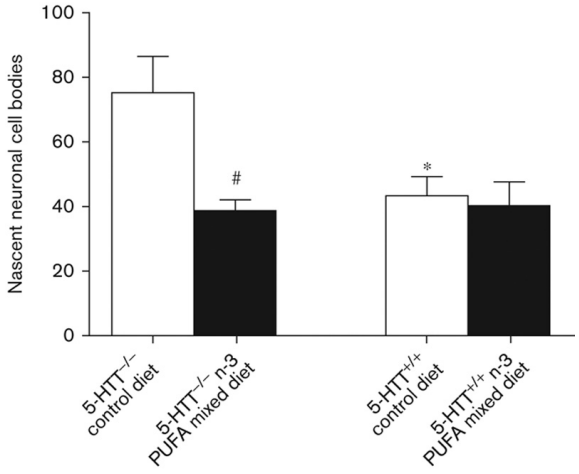

Figure 5 Effects of mixed PUFA diet in 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats on hippocampal neurogenesis. Data expressed number of as nascent neuronal cell bodies identified by DCX staining. Data represent mean ± S.E.M. number of DCX immunoreactive neurons. * $p < 0.05$ 5-HTT$^{-/-}$ versus 5-HTT$^{+/+}$ rats on control diet; # $p < 0.05$ 5-HTT$^{-/-}$ rats on control diet versus mixed PUFA diet.

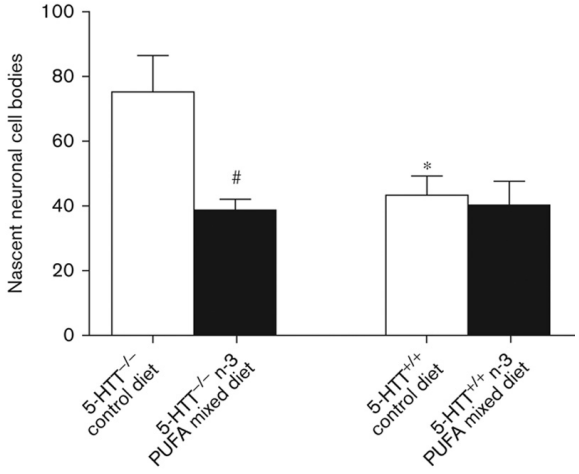

Figure 6 Effects of mixed PUFA diet in 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rats on hippocampal volume. Data represent mean ± S.E.M. surface area (mm^2). * $p < 0.05$ 5-HTT$^{-/-}$ versus 5-HTT$^{+/+}$ rats on control diet; [a] $p < 0.05$ 5-HTT$^{+/+}$ rats on control diet versus mixed PUFA diet.

Discussion

We have investigated the effects of a mixed PUFA diet in 5-HTT$^{-/-}$ rats displaying anxiety- and depression-like symptoms (Olivier et al. 2008). We show for the first time that a mixed dietary intervention completely abolished anxiety-like symptoms in these animals. The diet-induced changes in behavior were accompanied by a normalization of hippocampal neurogenesis in 5-HTT$^{-/-}$ rats, and an increase in hippocampal volume in wild-type rats.

Behavior

As previously demonstrated, 5-HTT$^{-/-}$ animals spent significantly less time on the open arms of the elevated plus maze, and spent less time on social interaction. These behavioral manifestations correspond to increased anxiety (Pellow and File 1986, Rodgers and Dalvi 1997, Cryan et al. 2002, File et al. 2004, Kalueff and Tuohimaa 2005, Milad et al. 2006, Moy et al. 2009). We also observed that fear extinction was impaired in 5-HTT$^{-/-}$ rats on control diet, potentially reflecting posttraumatic stress disorder-like emotionality (Wellman et al. 2007). Because the animals had five CS exposures during each of the conditioning/extinction/recall tests, the within session extinction was limited. Yet, we detected extinction between tests, suggesting that extinction consolidation and/or subsequent extinction recall were affected by genotype and mixed PUFA diet. Extinction recall was also found to be impaired in 5-HTT$^{-/-}$ mice (Wellman et al. 2007). Baseline freezing prior to the conditioning session was increased in 5-HTT$^{+/+}$ rats. We believe the confounding effect of the difference in baseline fear is minimal, since the direction of the baseline fear effect is opposite to the genotype / diet effects seen during extinction (recall). Possibly it even causes an underestimation of our findings.

Collectively, while 5-HTT$^{-/-}$ rats do not respond to SSRIs (Homberg et al. 2007a), the mixed PUFA diet alleviated these symptoms to such a degree that 5-HTT$^{-/-}$ and 5-HTT$^{+/+}$ rat behavior was indistinguishable. The underlying mechanisms are unclear, but could relate to parallel changes in hippocampal neurogenesis (see below), given that the hippocampus plays a critical role in fear extinction (Peters et al. 2010, Sierra-Mercado et al. 2010). These data suggest that mixed PUFA diets can serve as an adjunctive to treat anxiety-like symptoms in SSRI hyporesponsive subjects, in particular those characterised by inherited reduced 5-HTT function. Notably, our behavioral findings are not confounded by locomotor effects, given that distance moved on the elevated plus maze was not different between groups. In addition, the genotype and diet induced changes in open arm time were not paralleled by changes in closed arm entries, which is considered as another measurement of locomotor activity. Furthermore, although we conducted multiple tests in the same animals, prior testing

had no discernible effects on the outcomes since phenotypes of 5-HTT$^{-/-}$ rats on the control diet were very similar to those previously demonstrated in single-test studies (Kalueff et al. 2010).

Neurogenesis

In line with the behavioral data, the mixed PUFA diet significantly decreased hippocampal neurogenesis in 5-HTT$^{-/-}$ rats, bringing the amount of DCX immunoreactive neurons down to the level found in 5-HTT$^{+/+}$ rats. While results are very similar, DCX holds some key advantages over the 'gold standard' bromodeoxyuridine (BrdU), since it does not require a secondary staining to identify neurons. DCX has been extensively validated as a neurogenic marker (Brown et al. 2003, Rao and Shetty 2004). Yet, in 5-HTT$^{-/-}$ mice Schmitt et al. reported an increased proliferatory capacity of *in vitro* neuronal precursors using BrdU and the neuronal marker NeuN (Schmitt et al. 2007). This was, however, observed in old and not young adult mice, and was also not observed *in vivo*. Thus, although a great similarity exists between the outcomes of the two methods, there may be differences in the timeframe of expression of these markers (Brown et al. 2003). Further, it has been shown that certain behavioral tests, especially those that increase stress responses in 5 HTT$^{-/-}$ rats, can influence neurogenesis (Pham et al. 2005). However, our behavioral tests did not result in any discernible neurogenic effect. We speculate that the long-lasting effects of 3 months of mixed diet feeding overruled any potential effects of stress on neurogenesis.

It has previously been reported that n-3 PUFA exposure affects neurogenesis (Kawakita et al. 2006, Beltz et al. 2007, Cao et al. 2009, He et al. 2009). We show for the first time that the effects of a mixed PUFA diet are 5-HTT genotype-dependent. Unexpectedly, neurogenesis was decreased in 5-HTT$^{-/-}$ rats fed on the mixed n-3 PUFA diet. As touched upon earlier, it is notable that neurogenesis measures differ across n-3 PUFA studies, and DCX assessments have not been obtained before. Further, several controversies exist in the field of hippocampal neurogenesis. For instance, both decreases and increases in neurogenesis have been observed in response to stress exposure (Rodgers and Dalvi 1997, Milad et al. 2006, Parihar et al. 2009). Likewise, SSRI exposure has been associated with increases in hippocampal neurogenesis (Santarelli et al. 2003, Wang et al. 2008), but in some studies SSRIs were without effect (Cowen et al. 2008, Navailles et al. 2008). A recent study showed even that chronic fluoxetine administration to adult mice induced a dematuration of mature granule cells in the dentate gyrus (Kobayashi et al. 2010). Interestingly, 5-HTT$^{-/-}$ rats show reduced amounts of brain-derived neurotrophic factor (BDNF) in the hippocampus and prefrontal cortex (Molteni et al. 2010a). This implies that survival of hippocampal neurons may be reduced, since BDNF and neuronal survival are believed to be closely associated (Choi et al. 2009). It is possible the reduction in BDNF thereby reduces the

effectiveness of the neurogenesis. The increased neural proliferation in 5-HTT$^{-/-}$ rats in the present study may reflect a compensatory mechanism. Since n-3 PUFA supplementation increases the level of hippocampal BDNF (Jiang et al. 2009, Venna et al. 2009, Cysneiros et al. 2010), neuronal survival may have improved, reducing the need for additional neurogenesis. Clearly, investigation of the ability of new neurons to survive and integrate into existing neural networks is needed to test this hypothesis.

The mixed PUFA diet increased hippocampal volume in 5-HTT$^{+/+}$ animals, an effect that has previously been reported in mice and humans as a result of n-3 PUFA mono-supplementation (Conklin et al. 2007, Venna et al. 2009). We also observed increased hippocampal volume in 5-HTT$^{-/-}$ rats, which was not affected by the mixed PUFA diet. This indicates that the reduction in neurogenesis seen in 5-HTT$^{-/-}$ rats as a result of the dietary intervention was not accompanied by a decrease in hippocampal volume, suggesting that the reduction in new cell formation was compensated by increased neuropil production, neuron size and/or improved survival of existing neurons. It is worth noting that hippocampal volume was not corrected for whole brain volume or body weight, which may have affected the outcome.

Limitations

A limitation of this study is that we do not have data on depression-related behavior in the 5-HTT$^{-/-}$ rat as a function of the mixed n-3 PUFA diet. Because several rats were excluded for lodging themselves with the front and hind paws between opposite walls of the glass cylinder (18 cm diameter) during the forced swim test, we did not achieve the statistical power necessary to make conclusive statements about depression-related behavior. Because of the importance to test the effect of the n-3 PUFA diet in the context of depression, we aim to elaborate the present findings to depression-like symptoms in future studies. We did not measure food intake during this study. Although our previous work has shown that there is a difference in food intake between female 5 HTT$^{-/-}$ and 5-HTT $^{+/+}$ animals when expressed as calories/kg (Homberg et al. 2010b), absolute food intake, measured in grams per rat per day did not differ between genotypes (data not shown). Therefore, we deem it very unlikely that measured effects in behavior and immunohistochemistry can be attributed to differences in diet intake. Furthermore, the number of animals used for the behavioral studies was low. Nonetheless, we obtained significant effects, and in view of animal ethics and the strive to limit the amount of animals we decided not to add more animals. Finally, we used a mixed diet that promised to exert antidepressant effects in rodents (Broersen 2008). Here we show that this diet indeed reduces stress responses, but it remains to be determined whether this effect is attributed to a particular component of the diet, or the mixed nature of the diet. Administration of the separate components may be helpful to address this issue, which is also among our future aims.

Conclusion

In conclusion, we show that a mixed n-3 PUFA diet has a profound anxiolytic effect in 5-HTT$^{-/-}$ rats, and normalizes their increased neurogenesis. Although further research is required to understand the underlying mechanisms, our results strongly suggest this dietary intervention can serve as a putative therapeutic adjunctive for treating anxiety disorders. In particular, considering the resemblances between 5-HTT$^{-/-}$ rodents and the s-allelic variant of the 5-HTTLPR (Hariri and Holmes 2006, Kalueff et al. 2010), and meta-analyses showing that s-allele carriers respond poorly to SSRIs (Lesch and Gutknecht 2005, Serretti et al. 2007), our findings may have heuristic value for treating 5-HTTLPR s-allele carriers suffering from mood or anxiety disorders that respond poorly to SSRIs.

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Disclosure

All authors report no biomedical financial interests or potential conflicts of interest.

7

General discussion

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Summary of findings

The aim of this thesis was to investigate how serotonin transporter (5-HTT) gene variation and environment interact in determining the behavioral adaptation to stress. We targeted several negative environments that differed in intensity and designated optimal stress coping style, but also a positive environment (i.e., enriched diet). Moreover, we tested how this interaction between 5-HTT expression and environment evolves with ageing, targeting a critical period in brain development, i.e., adolescence. Cortical-subcortical developmental imbalance that occurs during adolescence is thought to increase the incidence of psychiatric disorders during this developmental period. A similar imbalance is seen in serotonin transporter linked polymorphic region (5-HTTLPR) short (s)-allele carriers, and may contribute to their increased risk of developing psychiatric disorders. In **chapter 2**, we set out to investigate how 5-HTT expression affects cortical-subcortical imbalance across adolescence, using fear extinction as a behavioral marker for cortical control. We assessed fear extinction and fear extinction recall behavior during pre-adolescence, adolescence and adulthood in serotonin transporter knockout (5-HTT$^{-/-}$) and wild type animals, and tested whether potential behavioral differences observed in these developmental periods are accompanied by differential development of inhibitory cell populations in the infralimbic (IL) cortex and basolateral amygdala (BLA) by measuring regional glutamic acid decarboxylase (GAD) 65/67 immunoreactivity. In accordance with previous findings, fear extinction and extinction recall were impaired in adult 5-HTT$^{-/-}$ animals compared to wild type rats. We found that the same genotype effect can be observed in pre-adolescent rats, but remarkably, the effect is not present during adolescence; fear extinction and recall appear to be transiently normalized during this developmental period in 5-HTT$^{-/-}$ animals. While this was not accompanied by differences in GAD65/67 immunoreactivity over development in IL and BLA, inhibitory cell populations were found to be decreased in the IL of 5-HTT$^{-/-}$ rats of all ages.

Since serotonin (5-HT) is known to play an important role in mediating the behavioral effects of uncontrollable stress (USt), we investigated in **chapter 3** whether variation in 5-HTT expression modulates USt-induced adaptations in emotional behavior by assessing the effects of a severe USt experience on subsequent extinction of conditioned fear 5-HTT$^{-/-}$ and wild type rats. Animals were exposed to 100 inescapable tail shocks under restraint, or a control manipulation consisting of a period of restraint similar to the duration of the shock procedure, followed by a fear conditioning, fear extinction and extinction recall session 48, 72 and 96 hours after the inescapable shock or control manipulation. While fear extinction and recall were not affected by the USt in wild type animals, USt normalized fear extinction recall in 5-HTT$^{-/-}$ rats.

Differential expression of 5-HTT is likely to affect how the dorsal raphe nucleus (DRN), a brain region that mediates (primarily maladaptive) behavioral consequences of USt, responds to USt and controllable stress (CSt). Therefore, in **chapter 4**, we explored how abolishment of the 5-HTT affects controllability-dependent stressor-induced activation of the DRN and prefrontal cortex (PFC) by exposing 5-HTT$^{-/-}$ and wild type animals to either a CSt signaled active avoidance (AA) paradigm, yoked signaled USt, or presentation of the signals used in the active avoidance context without the stressor. Subsequently we measured immediate early gene c-Fos immunoreactivity in the IL and prelimbic (PrL) region of the PFC and co-expression of 5-HT and c-Fos in the DRN to assess how CSt and USt affected neuronal activation in these regions. We found that active avoidance learning was improved as a result of 5-HTT abolishment. In both the IL and PrL cortices, c-Fos immunoreactivity was increased in both 5-HTT$^{-/-}$ and wild type rats that had undergone CSt, in the absence of genotype effects. However, no main effects of genotype were observed in terms of prefrontal or DRN (serotonergic) activation. Moreover, serotonergic activation in the DRN was controllability-dependent in wild type, but not 5-HTT$^{-/-}$ animals. Contrary to findings by others, DRN activation was increased in wild type rats that had been exposed to the *controllable* variant of the stressor paradigm, compared to those that experienced *uncontrollable* or no stress, and compared to all 5-HTT$^{-/-}$ groups. In 5-HTT$^{-/-}$ rats, DRN activation was not at all affected by stress exposure of either type.

As 5-HTT$^{-/-}$ animals have demonstrated adaptive coping behavior in CSt assays, but maladaptive coping in settings where no control over the stressor is offered, we investigated whether 5-HTT affects the flexibility of stress coping response acquisition across different stressor types in **chapter 5**. To this end, we assessed behavior in 5-HTT$^{-/-}$ and wild type rats during subsequent administration of fear- or sham-conditioning and a signaled shock active avoidance learning paradigm, in which the conditioned stimulus (CS) from the fear- or sham-conditioning was used to signal incoming shocks. Subsequently, we measured the behavioral response to that CS in a novel, neutral context. We found that prior fear conditioning had a small effect on signaled shock escape learning during AA in both genotypes, albeit only during the first few trials of the first escape learning session. In accordance with our findings described in chapter 4, we found that 5-HTT$^{-/-}$ rats acquired the escape response faster than wild types, regardless of prior fear conditioning. During the subsequent re-exposure to the CS in a novel context, we found that irrespective of sham- or fear-conditioning, CS-induced freezing was reduced in 5-HTT$^{-/-}$ animals compared to wild type animals. While fear conditioning did not affect freezing here, we found that the distance moved during the post AA CS exposure in 5-HTT$^{-/-}$ rats that had experienced fear conditioning, was reduced, while it did not affect distance moved in wild types.

Finally, in **chapter 6,** we examined the effect of a diet rich in n-3 polyunsaturated fatty acids (PUFA) on emotional behavior in 5-HTT$^{-/-}$ and wild type animals by comparing behavior of these animals fed either a diet rich in n-3 PUFA, B-vitamins and phospholipids, or an isocaloric control diet. We found that the n3-PUFA diet normalized anxiety, social behavior and fear extinction in 5-HTT$^{-/-}$ animals, while it had no effect on these behaviors in wild types. The behavioral normalization resulting from the n-3 PUFA diet in 5-HTT$^{-/-}$ rats was accompanied by a normalization of hippocampal neurogenesis, which was found to be higher in 5-HTT$^{-/-}$ rats that were fed the control diet, compared to their wild type counterparts.

Fear regulation across development and influence of genetically induced reduction of 5-HTT

Fear extinction efficacy has been reported to be variable across development. A substantial body of evidence suggests that fear extinction is transiently impaired during adolescence (McCallum et al. 2010, Pattwell et al. 2012, Baker et al. 2014, Baker et al. 2016). A general hypothesis describing the cause of this transiently impaired fear extinction is that the pacing of neural development of subcortical regions that facilitate fear conditioning and the expression of fear, and cortical regions that inhibit fear expression, are not synchronous (Casey et al. 2008); amygdala development completes before the onset of adolescence, whereas the PFC undergoes a protracted development that continues well into adulthood (Brenhouse and Andersen 2011). Specifically, during adolescence, the PFC undergoes substantial "pruning", or synaptic loss, leading to significant volumetric thinning of and gray matter reduction in the cortex (Giedd et al. 2015). This temporary imbalance is suggested to contribute to the increased incidence of psychiatric disorders seen during this developmental period (Casey et al. 2008). Rodent studies have demonstrated that the reduced efficacy of extinction during adolescence is accompanied by a reduced increase of phosphorylated mitogen-activated protein kinase (pMAPK) (Baker and Richardson 2015), as well as the absence of increased immediate early gene c-Fos expression in the IL following extinction (Pattwell et al. 2012). The finding of reduced extinction-induced pMAPK in the IL suggests adolescent extinction deficits result from reduced activity-dependent synaptic plasticity in the IL, for which pMAPK can be considered a marker (Huang et al. 2000). In the amygdala, plasticity that is induced by expression of (non-reinforced) conditioned fear is altered during adolescence as well. While pMAPK expression is upregulated in the adult BLA and IL after extinction and diminished in the central amygdala (CeA), during adolescence it is increased in CeA and reduced in BLA and IL, compared to animals that have not undergone fear extinction (Baker and Richardson 2015).

Here, we investigated whether fear extinction develops differently in 5-HTT$^{-/-}$ rats, and whether this is accompanied by abnormal development of inhibitory networks in the

PFC and amygdala. Remarkably, we found that the impairment of fear extinction seen in pre-adolescent (p24) and adult (p70) 5-HTT$^{-/-}$ rats was transiently alleviated during adolescence (p35). We propose that this is attributable to an altered developmental trajectory of frontocortical and subcortical regions due to differential availability of 5-HT during development in these animals. Previous research has indicated that the PrL frontocortical region controls expression of fear via activation of the CeA, but may be inhibited through a local inhibitory network via inhibitory GAD65-positive cells in the IL (Saffari et al. 2016), whereas inhibitory cells in the BLA, maintaining the learned fear association, control its excitability and may thereby attenuate fear behavior (Ehrlich et al. 2009). Therefore, we assessed whether 5-HTT expression dependent development of fear extinction behavior was related to altered development of inhibitory cell populations in the IL and BLA. We found that the inhibitory cell population in the IL was significantly smaller in 5-HTT$^{-/-}$ rats across all age groups. This suggests that activity of the PrL may be constitutively increased in 5-HTT$^{-/-}$ animals, which could not only contribute to the prolonged expression of conditioned fear seen in this genotype, but also to the other anxiety-related behavioral markers that appear to be enhanced in these animals (Olivier et al. 2008). However, it is unlikely that these alterations in local inhibitory networks contribute to the transient alleviation of the fear extinction impairment during adolescence, considering that this deficit in inhibitory capacity is persistent across all age groups. We observed no differences over development or due to genotype in the inhibitory cell populations in the BLA.

Recent studies appointed the reduced ability to recruit NMDA receptors located in the IL during extinction as a root cause for impaired fear extinction during adolescence; while extinction in preadolescent and adult mice causes an increase in the AMPA/NMDA receptor ratio in the IL, no such increase occurs in adolescent animals (Pattwell et al. 2012). Glutamatergic signaling in frontocortical areas has been suggested to contribute to mature higher cognitive function, and undergoes substantial development during adolescence (Flores-Barrera et al. 2014). In addition, new evidence suggests that extinction-induced plasticity operates by different mechanisms during adolescence, compared to extinction before and after adolescence. Specific disruption of the synthesis of ephrin type B receptor 2 in the IL, a receptor involved in synaptic remodeling, effectively inhibits extinction in adolescent, but not preadolescent or adult rats (Cruz et al. 2015). How these factors are modulated by available 5-HT, remains however largely unknown. A very recent study quantifying the expression of different NMDA receptor subunits revealed reduced transcription of NR1/NR2C in the prefrontal cortex of 5-HTT$^{-/-}$ rats (Karel et al. 2016). These subunits form the target receptors for pharmacological extinction stimulant d-cycloserin (Ogden et al. 2014), which has been shown to alleviate extinction deficits in adolescent rats (McCallum et al. 2010), but not adult 5-HTT$^{-/-}$ rats (Nonkes et al., unpublished observations).

Altogether, it seems that NMDA-mediated IL plasticity during extinction and a defunct local prefrontal inhibitory circuit may contribute to impaired fear regulation in 5-HTT$^{-/-}$ rats, but it remains unclear how these abnormalities develop across adolescence.

Notably, we did not replicate the finding of several others that fear extinction recall is transiently impaired during adolescence. Several possible reasons exist for this apparent discrepancy with existing literature. First, the extinction learning protocol used in chapter 2 may have obscured the effects of age on extinction recall because of its relatively long duration. Previous work has shown that utilizing an extended fear extinction protocol improved extinction in p35 rats, normalizing it to the levels seen in preadolescent and adult animals (McCallum et al. 2010), although the effects of extended extinction on preadolescent and adult rats were not reported. Second, the method of assessing fear extinction recall was different from previous paradigms, which used a single continuous tone to determine CS-induced freezing after extinction, where we utilized a protocol in which multiple CS presentations were interspersed with short stimulus-free periods, which was previously established to detect differences in fear extinction efficacy between 5-HTT$^{-/-}$ and wild type rats. Third, differences in the used rat strain may have contributed to the incongruence of findings. Here, Wistar rats were used, while other studies used Sprague Dawley rats. Rat strain has previously been shown to be an influential factor in determining the outcome of fear behavior-related experimental outcomes. For instance, lesioning of the IL, a region crucial in the extinction of conditioned fear in Sprague Dawley rats (Quirk et al. 2000), had no effect on fear extinction in Long Evans rats (Chang and Maren 2010). Beside altered effects of experimental manipulations, baseline fear acquisition and extinction may differ between strains, potentially introducing distinct floor and ceiling effects that may obscure or exaggerate the influence of other experimental factors (Sartory and Eysenck 1976). In addition, parameters of seemingly minor importance that are often adjusted during the optimization of behavioral paradigms, such as lighting conditions and habituation durations, can affect outcomes differentially depending on the animal strain that is used (Rex et al. 2004), making it difficult to predict how a change in rat strain will affect results.

These results could be relevant for interpreting findings from studies investigating the effect of the 5-HTTLPR s-allele during adolescence. However, contrary to our findings, the 5-HTTLPR also appears to be positively correlated with the incidence and severity of affective disorders during adolescence (Xia and Yao 2015). In addition, adolescent s-allele carriers were found four times more likely to develop PTSD following a traumatic experience (Tian et al. 2015). A similar discrepancy with the observations in human s-allele carriers can be found in a developmental study of anxiety in 5-HTT$^{-/-}$ mice; increased anxiety was not seen in 5-HTT$^{-/-}$ mice until after adolescence (Sakakibara et

al. 2014), whereas the s-allele was positively associated with anxiety in human adolescents (Hemmings et al. 2016, Otten et al. 2016). A possible explanation for these incongruences is that the developmental effects of 5-HTT are possibly not synchronous between humans and rodents. Furthermore, the effect of age x 5-HTTLPR interaction on fear acquisition and extinction has not directly been studied in human subjects, further complicating the comparison between findings. In addition, a potential publication bias (i.e., not publishing of null-findings), could have contributed to exaggeration of the effect of the s-allele during adolescence (Munafo et al. 2008, Mohammad et al. 2016).

Severe inescapable stress produces unexpected amelioration of impaired recall of fear extinction

Severe USt is known to produce a behavioral phenotype that is characterized by maladaptivity as manifested by escape learning deficits and enhanced generalized anxiety (e.g. Maier et al. 1993). USt also affects the acquisition and extinction of conditioned fear, potentiating the former (Maier 1990, Baratta et al. 2007), and impairing the latter (Hartley et al. 2014, Hoffman et al. 2014). The effects of acute USt are supposedly mediated by the activation of DNR 5-HT positive neurons and 5-HT release from/in the DRN (Maswood et al. 1998, Grahn et al. 1999, Takase et al. 2004), while 5-HT levels in the amygdala and dorsolateral periaqueductal grey (dlPAG) increase as well (Amat et al. 1998a, Amat et al. 1998b). Similarly increased activation of 5-HTergic neurons in the DRN and elevated levels of 5-HT in the amygdala and dlPAG are induced by the exposure to a previously fear-conditioned stimulus (Zanoveli et al. 2009, Spannuth et al. 2011). The persistence of USt-induced effects is however attributed to a desensitization of 5-HT1a autoreceptors situated in the DRN that persists as long as the behavioral adaptations resulting from USt do (Rozeske et al. 2011). Taking into account that 5-HT1a receptors are already desensitized in naïve 5-HTT$^{-/-}$ rats (Homberg et al. 2008), and that these animals are characterized by tonic high levels of extracellular 5-HT at baseline (Homberg et al. 2007c), it is to be expected that USt affects 5-HTT$^{-/-}$ and wild type rats differentially, and arguments for either exaggerated or diminished effects of USt in these animals could be put forward.

Targeting the interaction between USt and 5-HTT genotype, we found that deficits in fear extinction recall in 5-HTT$^{-/-}$ animals were normalized in the USt group, improving recall to the level seen in naïve wild type animals, while USt did not affect behavior in wild types. This absence of an effect on fear extinction recall in wild types could be explained using findings from others, which suggest the most prominent effects of USt on conditioned fear lie in the acquisition thereof instead of its extinction (Rau et al. 2005, Baratta et al. 2007, Herrmann et al. 2012). Effects in extinction learning and recall have been reported as well, but mainly following chronic stress paradigms

(Miracle et al. 2006, Hoffman et al. 2014). However, we also did not observe any effects of USt on the acquisition of fear. It is possible that such effects (in wild types, but also in 5-HTT$^{-/-}$ rats) have been obscured by our conditioning paradigm, which was sufficiently severe to induce the maximum level of freezing upon first presentation of the CS in all experimental groups.

Due to the phenomenon that the expected USt-like serotonergic adaptations (such as 5-HT1a desensitization) are already present in naïve 5-HTT$^{-/-}$ animals, it is possible that the serotonergic mechanisms described to mediate behavioral despair, operate differently or are irrelevant to the USt-induced modulation of fear behavior in these animals. For example, it is unknown whether the increase in extracellular 5-HT during and following USt exposure, or further adaptations to 5-HT1a receptor expression and function, occur in 5-HTT$^{-/-}$ animals. Studies into the effects of chronic selective serotonin reuptake inhibitor (SSRI) treatment, elevating circulating 5-HT levels, can provide valuable insight in the mechanistic effects of constitutively increased 5-HT levels (Popa et al. 2010), although importantly, they do not model the neurodevelopmental effects of genetic abolishment of 5-HTT. Moreover, chronic SSRI treatment seems to exert behavioral effects opposite to those of genetic 5-HTT abolishment and *reduce* fear acquisition (Burghardt et al. 2004). Elevation of circulating 5-HT levels (by chronic SSRI administration) suppresses the acquisition of fear by means of downregulation of NMDA receptor subunit NR2B in the amygdala (Burghardt et al. 2013), a parameter which was found to be unaffected by 5-HTT abolishment (Karel et al. 2016). Notably, chronic SSRI treatment does not reverse stress-induced deficits in fear extinction, whereas it does alleviate the anxiogenic effects of this stressor (Lin et al. 2016). As already mentioned, neurodevelopmental effects may account for this apparent discrepancy. In particular, altered development of the serotonergic projections from DRN to PFC (Witteveen et al. 2013), which are functionally relevant in mediating the behavioral effects of stress (Waselus et al. 2011), could play a role.

As we only looked at fear acquisition and extinction learning and recall, we cannot determine whether USt exposure actually induced a broader set of (adaptive) behavioral effects. Previous work has shown that USt exposure was ineffective in inducing escape learning deficits in 5-HTT$^{-/-}$ animals (van der Doelen et al. 2013), corroborating our finding that USt affects wild type and 5-HTT$^{-/-}$ rats differently. It remains to be investigated to what degree the serotonergic mechanisms to which USt-induced behavioral adaptations are ascribed are applicable in 5-HTT$^{-/-}$ animals, and how these may be affected by pre-existent serotonergic and non-serotonergic adaptations, such as desensitization of 5-HT1a receptors at baseline (Homberg et al. 2008).

The controllability-dependent effects of 5-HTT abolishment on stress-induced DRN activation

As discussed previously in chapters 1 and 3, 5-HT signaling emerging from the DRN mediates the effects of uncontrollable stress on subsequent behavior. In chapter 4, we examined activation of 5-HTergic neuron population in the DRN after a controllable stressor, a yoked uncontrollable stressor, or a control manipulation. A signaled active avoidance paradigm was used as the controllable stressor, while the uncontrollable stress cohort received the same amount of shocks and signals in the same order without any element of control. The control group was only exposed to the signals.

Acquisition of signaled active avoidance is said to be dependent on overcoming the freezing response due to the CS-US contingency that is formed during the initial, failed AA trails (Moscarello and LeDoux 2013). As passive exposure to unreinforced CS presentations (as happens during typical fear extinction paradigms) has been shown to be less effective in reducing CS-induced freezing in 5-HTT$^{-/-}$ rats, these animals were expected to display stronger CS-induced freezing and thereby impaired AA learning. However, surprisingly, 5-HTT$^{-/-}$ rats acquired the active avoidance task faster than their wild type counter parts.

5-HTergic activation in the DRN was increased in wild type animals that had undergone controllable stress compared to those that had undergone uncontrollable stress or no stress. This is incongruent with other reports, which state that DRN activation of 5-HTergic neurons, as assessed through measuring immunoreactive co-labeling for immediate early gene c-Fos and 5-HT, is increased after uncontrollable stress, while animals that have undergone controllable stress maintain the low level of 5-HT activity seen in unstressed animals (Grahn et al. 1999, Amat et al. 2005). This discrepancy can most likely be attributed to differences in experimental proceedings (refer to **Elements of controllability** further in this chapter). Contrary to our findings in wild type animals, stressors of either type had no effect on 5-HTergic activation in the DRN of 5-HTT$^{-/-}$ rats, indicating stress-dependent 5-HT signaling is altered in 5-HTT$^{-/-}$ animals. Control over a stressor has been reported to suppress 5-HT signaling from the DRN by means of activation of the PFC (Amat et al. 2005). Indeed, we found IL and PrL activation to be increased in animals that had experienced the controllable stressor. However, this IL and PrL activation was not influenced by genotype, offering no explanation for the improved AA performance or altered controllability dependent serotonergic DRN signaling in 5-HTT$^{-/-}$ rats. Moreover, we found that time to avoidance/escape was positively correlated with 5-HT/c-Fos double labeling in wild type, but not 5-HTT$^{-/-}$ rats, implying that poor performance and thereby a greater quantity of experienced stress, was related to increased 5-HTergic DRN activation in wild type animals, but not in 5-HTT$^{-/-}$ rats. Apparently, the 5-HTergic stress circuitry operates differently in 5-HTT$^{-/-}$

rats. Enhanced 5-HT signaling mediated by 5-HT1a receptor desensitization in the DRN after uncontrollable stress has found to be a requirement for the behavioral adaptations resulting from uncontrollable stress experience (Rozeske et al. 2011). However, 5-HT1a receptors are desensitized at baseline in 5-HTT$^{-/-}$ rats (Homberg et al. 2008), and extracellular levels of 5-HT are tonically elevated (Homberg et al. 2007c). Given this, it should be expected that controllability-dependent behavioral consequences resulting from these stressor experiences may be most prominent in wild type animals that underwent the controllable stressor manipulation, not the uncontrollable stress, and may be absent entirely in 5-HTT$^{-/-}$ rats.

The influence of 5-HTT abolishment on passive-to-active stress cue retraining

Acquiring signaled active avoidance training is dependent on suppressing the PrL-CeA mediated fear response that is forged during initial unsuccessful trials, wherein the stressor is perceived as inescapable and thus serves as unconditioned stimulus (US) that is paired to the AA signal that precedes it (serving as conditioned stimulus (CS)) (Lazaro-Munoz et al. 2010, Moscarello and LeDoux 2013). Thereby, animals that have acquired a fearful response to the AA signal prior to AA learning should exert delayed acquisition of the AA response compared to animals which are familiarized with the CS in a neutral setting (provided that the degree of familiarization was not sufficient to induce latent inhibition (Schauz and Koch 1998)). While fear extinction and AA acquisition are closely related in their mechanistic underpinnings (both relying on the IL to inhibit the PrL- and CeA-mediated fear response), 5-HTT$^{-/-}$ rats exert a seemingly contradictory behavioral phenotype featuring impaired fear extinction in conjunction with improved signaled AA acquisition. Apparently, 5-HTT$^{-/-}$ rats can display greater adaptability in their stress coping behavior when they are given means to actively respond to a stimulus signaling danger, while inflexibility (i.e., the delayed updating of the CS contingency in response to non-reinforced presentations) is seen when these possibilities are limited (such as observed during (passive) fear extinction) (Nonkes et al. 2012a).

We hypothesized that 5-HTT$^{-/-}$ rats would be able to overcome the passive coping response to a previously learned CS-US association by offering control over it. To test this hypothesis, fear-conditioned and sham-conditioned 5-HTT$^{-/-}$ and wild type rats were trained in a signaled AA test using the fear CS to signal oncoming shocks. Unexpectedly, the effect of fear conditioning on AA acquisition tempo was only modest, with a significant effect being discernible only within the very first AA trials. In accordance with our earlier finding (chapter 4), 5-HTT$^{-/-}$ animals acquired the AA response faster than wild type rats, regardless of prior conditioning. When we assessed the behavioral response to the CS in a neutral, novel context after fear/sham

conditioning and AA learning, wild type animals exerted a higher degree of freezing than 5-HTT$^{-/-}$ rats, both before and during presentations of the CS. The stronger passive coping response to both novelty and the CS in wild types indicates that AA training may have been more stressful for these animals. Increased freezing in response to the novel context seen in wild types suggests increased anxiety that may potentially result from the generalization of contextual cues acquired during AA learning. This finding is however not in line with other reports on novelty-induced freezing in 5-HTT$^{-/-}$ rats as recorded prior to fear extinction; reporting on higher (Shan et al. 2014), similar (Nonkes et al. 2012a), and lower (Schipper et al. 2011a) levels of freezing compared to wild types, under similar experimental conditions. Most likely, experiences prior to the novelty exposure, such as AA learning in the case of the present study, contribute to variations in novelty-induced anxiety in a 5-HTT genotype dependent manner.

Although fear conditioning prior to AA learning had no discernible effect on freezing during post AA exposure to the CS, locomotor activity - expressed as the distance moved during CS presentation - was reduced by fear conditioning in 5-HTT$^{-/-}$ rats, while the (overall low) level of mobility in wild types was not affected by fear conditioning. As baseline locomotor activity is not affected by 5-HTT genotype (Homberg et al. 2010a), these differences can likely be attributed to the stressor experience. The increased mobility as observed in the 5-HTT$^{-/-}$ animals may be interpreted as them adopting a more active coping style, which is reduced by prior fear conditioning leading to favoring more passive behavioral coping in these animals. However, the animals' motivation for the increased mobility here cannot be discerned from these data alone. Enhanced locomotion in the presence of novelty or stressors is frequently observed in rodent strains known for, or individual rodents selected for, their active coping style (Tsuda et al. 1988, Veenema et al. 2003, Boersma et al. 2009, Boersma et al. 2011), even though it is not regarded as a defining quality of an active coping style as such (de Boer et al. 2016). In summary, we conclude fear conditioning causes only minor interference with CS-signaled active avoidance learning, and that the improved acquisition of AA in 5-HTT$^{-/-}$ rats leads to a decreased residual fear response to subsequent encounters with the CS in these animals.

A diet rich in omega-3 polyunsaturated fatty acids normalizes the behavioral profile of 5-HTT$^{-/-}$ rats

n-3 PUFAs have been deemed essential nutritional elements, implicated in the healthy development and function of the brain (Rathod et al. 2016). Accumulating evidence suggests that dietary supplementation with n-3 PUFAs docosahexanoic acid (DHA) and eicosapentanoic acid (EPA) may improve therapeutic outcomes of patients suffering from MDD (Sinn et al. 2010). n-3 PUFA supplementation enhances hippocampal neurogenesis, a form of cellular plasticity that is thought to be decreased

in MDD patients (Kawakita et al. 2006). However, findings from clinical trials in which their efficacy as therapy or therapeutic adjuvant in the treatment of psychiatric disorders is tested, have been uneven, with many studies reporting absence of (therapeutically relevant) beneficial effects (Bloch and Hannestad 2012, Appleton et al. 2015). This may reflect existing heterogeneity within patients suffering from this disorder, as depression can be caused by a wide array of triggers and vulnerability factors, of both genetic and environmental nature. Possibly, a specific subset of these patients can benefit from the therapeutic actions of additional dietary intake of n3-PUFAs, while others do not.

As 5-HTT$^{-/-}$ rats exert a number of behavioral features that resemble those seen in depression, such as reduced social interaction, anhedonia, and despair-like behavior in the forced swim test (discussed later in the section **Disentangling stress coping in 5-HTT$^{-/-}$ rats**) (Kalueff et al. 2010), we hypothesized that n-3 PUFA supplementation could ameliorate these symptoms. Given that improvement of hippocampal neurogenesis is the main proposed mechanism for antidepressant action of n-3 PUFA supplementation (as well as many other antidepressant treatments) (Santarelli et al. 2003), we assessed whether any behavioral changes were accompanied by alterations in this type of plasticity. We found that while a diet rich in n-3 PUFAs had no effect on behavioral parameters in wild type rats, it normalized levels of anxiety, social interaction and fear extinction in 5-HTT$^{-/-}$ rats. Surprisingly, baseline neurogenesis was increased in 5-HTT$^{-/-}$ rats, and was normalized to the lower level seen in wild types fed on either type of diet. We hypothesized that previously observed reductions in hippocampal BDNF reduce neuronal survival in the hippocampus of 5-HTT$^{-/-}$ rats (Molteni et al. 2010b, Gray et al. 2013), which thereby requires additional compensatory neurogenesis. n-3 PUFA supplementation may have promoted neuronal survival, thereby eliminating the need for this compensation. However, as hippocampal apoptosis was not assessed, we cannot state this with certainty.

Hippocampal neurogenesis affects fear extinction in multiple ways. Ablation of hippocampal neurogenesis impairs extinction of contextual fear (Pan et al. 2012). Conversely, boosting neurogenesis enhances pattern separation, and may thereby aid in reducing the generalization of conditioned fear response (Sahay et al. 2011), which is thought to contribute to the pervasiveness of trauma-related conditioned fear present in post-traumatic stress disorder (Lopresto et al. 2016). However, the present findings suggest an opposite relationship in 5-HTT$^{-/-}$ animals, with excessive neurogenesis prolonging the conditioned fear response. Notably, abolishment of 5-HTT and chronic SSRI administration both elevate extracellular 5-HT levels as well as hippocampal neurogenesis. However, as mentioned before, in spite of this mechanistic similarity, their behavioral outcomes in terms of fear processing and anxiety are

opposite. In order to definitively determine whether neurogenesis is instrumental in causing the emotional behavioral patterns seen in 5-HTT$^{-/-}$ rats, and whether behavioral changes due to n-3 PUFA supplementation are mediated by decreased neurogenesis or changes in neuronal survival, future studies implementing an apoptotic assay and selective reduction of neurogenesis using focal irradiation may prove informative (Tada et al. 2000).

Disentangling stress coping in 5-HTT$^{-/-}$ rats

Genetically reduced expression and function of 5-HTT has previously been reported to increase an individual's sensitivity to stress (Caspi et al. 2010, Karg et al. 2011), and different tendencies in behavioral coping strategies have been suggested as a mediating factor therein (Wellman et al. 2007, Schillani et al. 2012, Cline et al. 2015). While data obtained from 5-HTT$^{-/-}$ rats corroborate the link between 5-HTT expression and altered stress coping and sensitivity, only a limited subset of stressful behavioral assays support the notion that 5-HTT abolishment increases stress sensitivity or diminishes the ability to adapt the coping response to suit the given stressor. That is, 5-HTT$^{-/-}$ rat behavior in signaled AA (chapter 4) and learned helplessness assays (van der Doelen et al. 2013) fits the interpretation of adaptive behavior, while their behavior during fear extinction (Nonkes et al. 2012a) and the Porsolt forced swim test (FST) (Olivier et al. 2008) can be interpreted as maladaptive and matches human findings. In this sense, the behavioral stress coping profile in these animals appears paradoxical at times, and whether 5-HTT$^{-/-}$ animals can adapt to the challenge a stressor imposes on them, seems to be strongly dependent on the type of stressor and the exact context. Here, we will attempt to define what stressor properties determine the effect of 5-HTT abolishment on stressor coping and subsequent behavioral adaptation.

5-HTT$^{-/-}$ rats show a persistent passive coping response to a conditioned fear response, even upon repeated non-reinforced exposure to the CS. Yet, during signaled AA learning, initial failure to escape the signaled stressor, which has been reported to induce freezing and inhibit AA acquisition (Moscarello and LeDoux 2013), does not induce a (lasting) passive coping response in these animals, as AA responding was not delayed, but in fact accelerated, in 5-HTT$^{-/-}$ rats (chapter 4, chapter 5). Similarly, establishment of a passive coping response to a CS by fear conditioning does not impair CS signaled AA learning in these animals (chapter 5). A parallel of sorts to these findings can be observed when comparing the behavior of 5-HTT$^{-/-}$ rats in assays of learned helplessness assays behavioral despair. The induction of escape deficits through inescapable shock exposure and the FST share several conceptual and mechanistic similarities; both initial stressor experiences elicit passive coping behavior in the subsequent assay (Porsolt et al. 1977, Maier 1990), which is reduced by antidepressant (but not anxiolytic) treatment, leading to the interpretation that

USt-induced passive coping can be described as 'depressive-like' (Porsolt et al. 1977, Sherman et al. 1982). However, a crucial difference between the two assays is that the conditions under which post-stress behavior is tested either allow for the animal to escape the stressor (i.e., in the shock escape test), or not (in the FST). 5-HTT$^{-/-}$ rats show increased passive coping in the FST (Olivier et al. 2008), while they are resistant to USt-induced deficits in shock escape learning (van der Doelen et al. 2013). This could imply that these animals have an improved capacity to determine whether their behavioral efforts can actually influence the stressor, i.e., whether the stressor is escapable or not. It can be argued that 5-HTT$^{-/-}$ animals appropriately adapt their coping response to the situation, conserving energy by showing passivity when behavior does not influence the outcome (in the FST), while making an effort to escape the stressor when it is escapable (in a shock escape assay). By extension, the more active coping response seen in the FST after antidepressant treatment may actually be interpreted as a shift towards a maladaptive coping response, as the additional efforts expended in attempting to escape the water basin will not improve the outcome for the animal. A recent evaluation of the FST corroborates this, and suggests that FST outcomes should instead be interpreted as an assessment of whether an animal can recall the (un)controllability of the repeated stressor (de Kloet and Molendijk 2016). A parallel can be drawn between these findings and the seemingly discrepant outcomes from fear extinction and signaled AA assays in 5-HTT$^{-/-}$ rats. While prolonged freezing in response to a fear CS is interpreted as a maladaptive coping response, whether the animal freezes or not has no real influence on the animal's wellbeing. Potentially, the 5-HTT$^{-/-}$ rat correctly determines that the environment in which post-conditioning freezing is assessed offers no opportunities to interact with the stressor, thereby causing it to adopt a conservational coping response (i.e., freezing). On the other hand, during a signaled AA assay, it correctly identifies the stressor as controllable, and appropriately adopts an active coping response.

During typical rodent fear extinction assays, conditioned stimuli are presented in a novel, neutral environment, where possibilities to exert active coping behavior other than exploration are few. In a study examining the influence of a form of behavioral therapy on fear extinction in 5-HTT$^{-/-}$ rats, it was found that when fear-CS presentations were combined with the presentation of distracting appetitively conditioned stimuli (which were previously paired with the arrival of a food pellet in an available dispenser), 5-HTT$^{-/-}$ rats did not freeze more than wild type animals during extinction training or extinction recall (Nonkes et al. 2012a). This effect was present regardless of whether the appetitive stimulus contingency was reinforced during the extinction. Here, animals had to balance a conservative freezing response with their motivation to collect the food pellet, and similar to a signaled AA paradigm, a freezing response would interfere with another competing survival oriented response (i.e., pellet

collection), yet this response does not directly influence the stressor (i.e. the aversive CS presentation). While exploration of novel contexts elicits similar activity from 5-HTT$^{-/-}$ and wild type rats (Homberg et al. 2010a), it is possible that fear interferes specifically with novelty exploration. The reduction in freezing in 5-HTT$^{-/-}$ rats due to the aforementioned 'behavioral therapy' was maintained when CS-induced freezing was assessed 5 days after the last extinction session, without the presence of the appetitive stimulus. This implies that this 'behavioral therapy' had a lasting impact on the CS-associated fear response in 5-HTT$^{-/-}$ rats. The availability of an operant response mechanisms to deactivate the stressor may have had a similar distracting function in signaled AA. However, it is presently unclear how (consolidation of) extinction memory is persistently improved in this manner. It is possible that failed extinction recall, as occurs in 5-HTT$^{-/-}$ rats in the control condition, maintains or increases the fear response to the CS due to the aversiveness of the CS presentations only in the absence of distractor stimuli. However, additional data are needed to verify this.

In conclusion, experimental observations here and elsewhere support the notion that 5-HTT$^{-/-}$ animals exert a coping profile that is not necessarily slanted towards maladaptive, passive coping, but instead may be better adjusted to the situational demands, and therefore in fact more adaptive than that seen in wild types. The lack of congruence between behavioral outcomes in different paradigms that are supposedly directed by similar mechanisms might suggest availability of control and the means to exercise it in these paradigms may be better detected by 5-HTT$^{-/-}$ animals. This may misdirect our interpretation of their behavior in certain situations (e.g., in fear extinction), leading us to conclude that 5-HTT abolishment results in favoring passive stress coping strategies. Coping behavior in these animals may be heavily dependent on the opportunities that the environment offers, but additional studies are needed to determine which environmental conditions direct active and passive coping.

Extrapolating these findings to the human situation is not straightforward, as both the coping strategies and encountered stressors differ vastly between rodents and humans. As briefly discussed in chapter 1, the human 5-HTTLPR s-allele has also been seen to modulate stress coping, although defining coping here as adaptive, maladaptive, (pro)active, or passive is rather problematic. Coping in humans primarily consists of self-reported attitudes towards adversity and mental coping mechanism and is measured retrospectively through questionnaires and interviews. In contrast, stress coping in animal models is assessed through the behavior that is displayed in response to experimental stressors; (lack of) behavioral responses and latencies are then interpreted and categorized, and attitudes towards adversity are deduced. While coping is understood to be important in mitigating the effects of adversity, finding parallels between coping studies in humans and animals is complex. Nevertheless, as

discussed in chapter 1, 5-HTTLPR seems to influence the reported coping strategies in humans in several ways. The degree to which problems are externalized was found to be greater in children carrying two s-alleles, which also utilized distraction coping styles more often (Cline et al. 2015). Emotional eating, a coping mechanism that is sometimes seen in MDD, is more frequent in s-allele carriers compared to l-allele carriers (van Strien et al. 2010). Moreover, rumination, or engaging in a negative spiral of self-deprecating thought, is a coping strategy that occurs more frequently in s-allele carriers (Canli et al. 2006, Clasen et al. 2011) (but see (Beevers et al. 2009)). Rumination is primarily seen as a maladaptive coping strategy: it elevates the level of cortisol for a prolonged period of time (Zoccola and Dickerson 2012), suggesting it greatly increases allostatic load, and it is said to amplify negativity (Nolen-Hoeksema 2000). Another coping behavior that can almost certainly be designated as maladaptive is drinking-to-cope, or (excessive) alcohol intake to help cope with adversity. Interestingly, s-allele carriers report a reduced motivation for drinking alcohol as stress coping strategy (Armeli et al. 2008). Altogether, it is clear that genetically determined variation in 5-HTT expression and function affects stress coping strategy in humans as well as rodents. However, it is presently not known to what degree these differences in coping preference mediate alterations in susceptibility to stress-related disorders.

Elements of controllability

The concept of stressor controllability has been mainly explored by means of various stress manipulations in rodents. These offer several advantages over studies in human subjects; greater control over experimental conditions (including rearing circumstances and genetic and epigenetic factors) and the availability of invasive experimental techniques that can provide a higher level of detail than what can be obtained in humans. However, translation of rodent data to the human situation can be difficult, and depends on how closely one can model the environmental and genetic factors that contribute to the pathogenesis of human stress-related disorders, as well as the relevant behavioral readouts (Homberg 2013).

Stressor controllability, and particularly its interaction with the serotonergic system, has been studied primarily using the triadic wheel turning paradigm in rats (described in chapter 1, box 1). This design elegantly equalizes the quantity of the given stressors between the CSt and USt animals by 'yoking' their behavioral experience; applying all shocks the CSt animal failed to escape from to the USt animal as well. Controllability is defined here as being able to influence stressor exposure; the CSt animal can directly terminate shocks by manipulating the wheel, while the USt animal receives shocks regardless of its efforts. In a different type of controllability assay, animals are restrained by the tail while receiving tail shocks and controllability is determined by the availability of a wooden dowel to chew on, which USt animals do not have

(Helmreich et al. 2012). Here, controllability is defined as the presence of a behavioral outlet for coping, even though the stressor itself cannot be influenced or escaped from. Yet, the realization of this type of controllability induces a similar distinction in phenotypical outcomes between CSt and USt experienced animals to that observed in triadic wheel turning experiments (Maier and Watkins 2010), with CSt animals displaying lower levels of anxiety in open field and social exploration paradigms than their USt counterparts (Helmreich et al. 2012). This raises the question of how controllability should be defined; either as control over the stressor or control over coping behavior.

In determining whether a stressor is controllable, a distinction could first of all be made on the basis of whether the subject perceives the stressor as susceptible to behavioral manipulation. To meet this criterion of controllability, behavior that leads to circumvention or aborting of the stressor should be goal-oriented, and motivated by this outcome. Goal-oriented behavior has been found to be a prerequisite for instrumental controllability (Amat et al. 2014), and is mediated through a prelimbic–dorsomedial striatal circuit (Balleine and O'Doherty 2010) both in rodents and humans (Balleine and O'Doherty 2010). In contrast, habitual behavior prevents goal-directed behavior. Habitual behavior is mediated by a circuit of the dorsolateral striatum and sensorimotor cortex (Shiflett and Balleine 2011), and appears to prevail over goal-directed behavior under stressful circumstances; an effect mediated by β-adrenergic activity (Schwabe et al. 2011). Activation of the dorsomedial striatum during CSt is necessary for its protective effects, and blocking its activity gives CSt properties and consequences similar to those of USt (Amat et al. 2014). The shift between habitual and goal-oriented behavior is mediated by the orbitofrontal cortex (Gremel and Costa 2013). In 5-HTT$^{-/-}$ rats, improved cognitive flexibility in a reversal learning test was found to be accompanied by enhanced activation of this cortical region (Nonkes et al. 2010). Potentially, this is directed by the same mechanisms that facilitate the detection of instrumental controllability in these animals.

Otherwise, the ability of the subject to engage in coping behavior that does *not* affect stressor exposure could contribute to the experience of controllability. This phenomenon has also been referred to as 'stress blunting' displacement behavior (Berridge et al. 1999), and is exemplified by the aforementioned wood dowel stress paradigm. Multiple studies have confirmed that the availability of indigestible chewing materials alters the neuroendocrine stress response; corticosterone release in response to novelty exposure was found to be decreased in the presence of non-digestible chewing materials, but not by highly palatable food (Hennessy and Foy 1987). In addition, the corticotropin releasing factor (CRF) response to restraint was diminished in animals that had a wooden dowel available (Hori et al. 2004), although

this was not the case for experiencing tail shocks (Helmreich et al. 2012). Furthermore, the restraint stress-induced inhibition of hippocampal neurogenesis was prevented by allowing mice to chew indigestible material during the restraint period (Kubo et al. 2009). In contrast, while only limited evidence is available, it is suggested that the neuroendocrine stress response in instrumental controllability assays (such as triadic wheel turning) is not different between CSt and USt experienced animals; CRF levels in the paraventricular nucleus after CSt and USt wheel turning experience are not different (Helmreich et al. 1999) nor are plasma levels of ACTH and CORT (Maier et al. 1986). However, when foot shocks were given in an escapable or inescapable fashion, plasma CORT levels were increased in rats that had experienced inescapable shock relative to those that experienced escapable ones (Swenson and Vogel 1983). In terms of stress-induced 5-HT release, differences between the CSt- and USt-elicited serotonergic response are known to occur in triadic wheel turning assays (Amat et al. 1998b), but availability of chewing material does not affect a novelty-induced serotonergic response in rats (Berridge et al. 1999). In summary, the mode of controllability has clear effects on HPA-axis mediators and stress-induced 5-HT release, but also appears to heavily depend on the dimension and severity of the stressor used. Many of the 'stress blunting' studies use restraint as a stressor, which may be less aversive than the tail and foot shocks often utilized in instrumental stressor controllability assays.

As 5-HTT$^{-/-}$ rats feature a wide array of adaptations in both functioning of the HPA-axis and serotonergic system (Homberg et al. 2007c, Homberg et al. 2008, van der Doelen et al. 2014b, van der Doelen et al. 2015), it seems likely that resilience in these animals is strongly determined by the aforementioned stressor parameters. The dimension of the stressor (be it physical or psychological), its severity, and degree and type of controllability are likely to influence how variations in 5-HTT expression contribute to vulnerability or resilience to it. The findings described in this thesis clearly indicate that describing 5-HTT expression as a factor that simply enhances or limits stress vulnerability is an oversimplification. Specifically, 5-HTT$^{-/-}$ animals may find controllability in stressors that wild type animals find unsurmountable, while (perhaps accurately) assessing the situation is out of their hands in other settings. The general consensus on how outcomes from behavioral assays are interpreted may lead to inaccurate conclusions in this regard; in assays in which 5-HTT$^{-/-}$ animals cannot control exposure to the stressor, they exert behavior that we interpret as maladaptive, e.g., freezing during fear extinction and floating during a FST. It is possible that this alternative perspective on controllability may benefit 5-HTT$^{-/-}$ animals in some situations, and harm them in others.

These findings may give nuance to stress vulnerability in 5-HTTLPR s-allele carriers as they suggest that controllability may modulate the effects of stress differently in these

individuals. However, at this time, neuropsychiatric research describing controllability-dependent effects of stress exposure on the risk of developing mood disorders in human populations is scarce. Controllability can be defined very clearly in instrumental stress escape and avoidance paradigms, in rodents (as discussed above) as well as in humans, in laboratory settings (Hartley et al. 2014, Wood et al. 2015); a stressor and some operant means of turning it off that either works or not are all that is needed. However, defining controllability in the real-life stressors that contribute to the development of stress-related disorders, like chronic stressors such as work stress, but especially in highly traumatic stressors of an acute nature such as sexual abuse and violence, can be problematic. Many traumatic stressors contain a social element, such as the interaction with an aggressor. While active and passive coping behaviors are frequently studied using paradigms of social interactions, for instance by assessing attack latency in a resident-intruder paradigm (e.g. (de Boer et al. 2016)), it is not known how these relate to controllability. Ostensibly, adopting an active coping style could be viewed as an attempt to gain control over a stressor, and upon successfully doing so the stressor may exert CSt properties. However, this is highly speculative and currently unsubstantiated by experimental findings.

Considering the high likelihood of the involvement of social components in traumatic stress, defining the boundaries of controllability in a social context is highly relevant, but a very delicate issue. As controllable stress is understood to be less harmful than uncontrollable stress (Maier and Watkins 2010), categorizing traumatic events that lead to the development of stress-related disorders as "controllable" may even be harmful to victims. After all, implying that a victim has "control" over a traumatic incident is tantamount to putting responsibility for such an incident and its sequelae in the hands of the victim. Considering that self-blaming and decreased sense of self-worth are important features of both PTSD and MDD (American Psychiatric 2013), it is even possible that a greater degree of perceived controllability in such a traumatic event is detrimental to a victim, instead of empowering.

In animal studies, the concept of controllability is generally studied with the aim of assessing how the risk of a stressor contributing to psychiatric illness is modulated by its controllability, and by which mechanisms. In human literature, however, controllability is conceptualized as a cognitive psychological coping mechanism. It has been suggested that engendering a sense of controllability over factors that generate stress in life can empower and activate those suffering from MDD (Duckworth et al. 2005, Seligman et al. 2006). The findings in this thesis suggest that "giving control" over stressors provide the greatest benefit to animals with reduced expression of 5-HTT. In parallel with this, 5-HTTLPR s-allele carriers may benefit more from cognitive therapy that emphasizes the (perception of) control over situations and stressors.

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Curriculum Vitae

Pieter Schipper (born December 17th, 1984 in Utrecht) obtained his pre-university degree (VWO) at the Johan van Olderbarnevelt Gymnasium in Amersfoort in 2002. He went on to earn his Bachelor and Master of Science degrees in the field of Biomedical Sciences at Radboud University Nijmegen. Over the course of his studies here, he completed several research internships. Pieter was introduced to the field of cognitive neuroscience by Amanda Kiliaan, under whose supervision he contributed to a study at the Radboudumc department of Anatomy. Here, he compared amyloid beta pathology in two mouse models for Alzheimer's disease, and studied the efficacy of a novel pharmacological agent in reducing it. Subsequently, a detour in the field of integrative physiology and active gaming was made. During a research internship at the department of Physiology, he investigated the activity levels associated with playing the Nintendo Wii under the supervision of prof. Maria Hopman, resulting in an internship award from the faculty as well as a presentation at the American College of Sports Medicine meeting in Seattle, USA. Craving deeper knowledge of the inner workings of the brain, Pieter returned to neuroscience for his master internship. Supervised by Judith Homberg and his former associate Amanda Kiliaan, Pieter studied the effects of a diet rich in omega-3 fatty acids on a wide array of behavioral properties of 5-HTT-/- rats at the Radboudumc dept. of Cognitive Neuroscience. His efforts were rewarded with lead authorship on the resulting publication. This paper was the start of a PhD focused on 5-HTT x stress interaction, under the supervision of Judith Homberg, and later prof. Tamas Kozicz and Marloes Henckens. The research was conducted between Februari 2011 and December 2017, and resulted in a number of publications, all of which can be found within this thesis. The findings described here have been presented at (international) scientific meetings including Donders Discussions, the Dutch Neuroscience annual meeting and the annual meetings of the Society for Neuroscience in San Diego and Washington.

Nederlandstalige samenvatting

Depressieve en angststoornissen zijn ingrijpende aandoeningen die vaak voorkomen en een hoge ziektelast veroorzaken. Hoe deze aandoeningen ontstaan is niet precies bekend, maar duidelijk is wel dat stress hierin een grote rol speelt. We begrijpen ook nog niet goed waarom sommige mensen ten gevolge van stress wel een psychiatrische aandoening krijgen, en anderen niet. Genetische factoren kunnen daarin een rol spelen. Het is bekend dat een genetische factor die beïnvloedt in welke mate de serotonine transporter (5-HTT) aangemaakt wordt, de zogenaamde "5-HTT linked polymorphic region" (5-HTTLPR), mede bepaalt hoe gevoelig een individu is voor stress. Bij dragers van korte variant hiervan, het short (s) allel, wordt minder 5-HTT aangemaakt in het brein. Klinische studies hebben aangetoond dat depressieve en angststoornissen vaker voorkomen bij dragers van dit s-allel, en dat dit effect versterkt wordt door traumatische gebeurtenissen. Een theorie over de relatie tussen 5-HTTLPR en stressgevoeligheid is dat het verband hiertussen zijn oorzaak vindt in hoe s-allel dragers met stress omgaan: dit wordt "stress coping" gedrag genoemd. Succesvol met een stressor omgaan is in sterke mate afhankelijk van of het individu zijn stijl van coping kan aanpassen aan de specifieke stressor. Sommige stressoren worden het beste benaderd met een actieve coping strategie, waarbij het individu inspanningen verricht om de stressor bij de bron aan te pakken (te confronteren), of eraan te ontkomen. Andere stressoren vereisen een passieve coping strategie, waarbij het individu de stressor "ondergaat" en daarbij probeert leed en letsel tot een minimum te beperken, en energie te conserveren.

In het onderzoek dat wordt beschreven in dit proefschrift wordt getracht de relatie tussen de expressie van 5-HTT en stress coping gedrag te beschrijven. Hiervoor wordt gebruik gemaakt van ratten die via genetische modificatie verminderd (5-HTT$^{+/-}$) of geen 5-HTT (5-HTT$^{-/-}$) aanmaken. Het uitvoeren van dit onderzoek in ratten in plaats van in menselijke proefpersonen heeft twee belangrijke voordelen. De experimentele stressoren die toegepast kunnen worden in proefdieronderzoek zijn heviger, en daarmee traumatischer, dan wat toelaatbaar is in gezonde humane proefpersonen, en beter te standaardiseren, kwalificeren en kwantificeren dan (retrospectief) mogelijk in menselijke patiënten die lijden aan stress-gerelateerde psychiatrische aandoeningen. Bovendien kunnen door middel van invasieve experimentele technieken belangrijke inzichten vergaard worden over welk effect deze stressoren hebben op het brein. Deze 5-HTT$^{-/-}$ ratten hebben veel gedragsmatige overeenkomsten met menselijke s-allel dragers: ze zijn angstiger en vertonen slechte uitdoving van een geconditioneerde Pavloviaanse angstrespons. Bij Pavloviaanse angstconditionering wordt een neutrale stimulus, zoals bijvoorbeeld een toon, gekoppeld aan een aversieve stimulus, zoals bijvoorbeeld een milde elektrische schok, door deze na elkaar te presenteren.

De daarop volgende associatie tussen beide stimuli kan worden bepaald door de angstrespons ("freezing") op de neutrale stimulus (de toon) te meten, en worden uitgedoofd door deze herhaaldelijk te presenteren in de afwezigheid van de aversieve stimulus (de schok). Het afleren van zo'n negatieve associatie is belangrijk in de succesvolle behandeling van het post-traumatisch stress syndroom, een aandoening die vaker voorkomt en moeilijker te behandelen is bij s-allel dragers. Tegelijkertijd laten 5-HTT$^{-/-}$ ratten een hogere mate van cognitieve flexibiliteit zien dan wildtype ratten, de evenknie van de 5-HTT$^{-/-}$ rat met normale 5-HTT expressie; soortgelijke cognitieve voordelen worden gezien bij menselijke dragers van het s-allel. Door deze dieren bloot te stellen aan verschillende (opeenvolgende) stressoren, en te meten welke invloed deze stressoren hebben op gedrag en het brein, is getracht in kaart te brengen hoe 5-HTT expressie stress coping en stress coping flexibiliteit beïnvloedt.

In hoofdstuk 2 wordt bezien hoe de uitdoving van geconditioneerde angst wordt beïnvloed door interacties tussen de ontwikkelingsfase waarin een individu zich bevindt en 5-HTT expressie. Eerder onderzoek in mensen heeft aangetoond dat angstuitdoving moeizamer verloopt tijdens adolescentie, wat zou kunnen bijdragen aan de toegenomen incidentie van psychiatrische stoornissen tijdens die ontwikkelingsfase. 5-HTT zou hier een sleutelrol in kunnen spelen, aangezien bekend is dat diens expressie de neurale ontwikkeling beïnvloedt: 5-HTT reguleert de hoeveelheid beschikbare serotonine in het brein, en serotonine is een belangrijke groeifactor tijdens de neurale ontwikkeling. De prefrontale cortex (PFC) en amygdala spelen een sleutelrol in de conditionering en uitdoving van angst. Mogelijk ontwikkelen deze hersendelen zich anders bij verlaagde expressie van 5-HTT. Andere studies hebben een andere verhouding van inhibitoire (onderdrukkende) en excitatoire (activerende) cellen aangetoond in corticale hersengebieden in 5-HTT$^{-/-}$ dieren. Een dergelijke afwijking in de PFC en amygdala zou ten grondslag kunnen liggen aan de verslechterde uitdoving van angst in deze dieren. In deze studie zijn 5-HTT$^{-/-}$, 5-HTT+/- en wildtype dieren van pre-adolescente, adolescente en volwassen leeftijd blootgesteld aan een angstconditionering protocol. Er werd bekeken wat het effect van 5-HTT genotype en leeftijd was op de snelheid waarmee de angstreactie kon worden uitgedoofd. Vervolgens werd de populatie inhibitoire neuronen in de basolaterale amygdala en het infralimbische gebied van de PFC bepaald door via immunohistochemie het eiwit glutaminezuur decarboxylase 65/67 (GAD65/67) aan te kleuren en te kwantificeren. De retentie van angstuitdoving, gemeten op de tweede dag van het uitdovingsprotocol, was zoals eerder al aangetoond was slechter in volwassen 5-HTT$^{-/-}$ ratten dan in 5-HTT+/- ratten en in wildtypes. Opmerkelijk genoeg vonden we dit ook in pre-adolescente 5-HTT$^{-/-}$ dieren, maar niet tijdens adolescentie: het lijkt erop dat uitdoving juist tijdelijk genormaliseerd is tijdens deze ontwikkelingsfase. GAD65/67 expressie was verlaagd in 5-HTT$^{-/-}$ ratten van alle leeftijden.

Niet-beïnvloedbare stress heeft een sterker negatief effect op het individu dan stress die beïnvloedbaar is. Experimenten in de jaren '60 hebben al aangetoond dat dieren die een ernstige traumatische ervaring hebben gehad met een niet-beïnvloedbare stressor, daarna niet of verminderd kunnen ontkomen aan een eenvoudig vermijdbare stressor. Dit fenomeen heet "learned helplessness" (aangeleerde hulpeloosheid) en wordt gemedieerd door sterke serotonine afgifte in de dorsale raphe nucleus (DRN), het hersendeel waar de serotonerge innervatie van andere hersendelen zijn oorsprong vindt. Naast een verminderd vermogen tot het aanleren van stressontwijking heeft ernstige niet-beïnvloedbare stress ook gevolgen voor geconditioneerde angst: conditionering van angst wordt erdoor versterkt en de uitdoving ervan verzwakt. In de studie beschreven in hoofdstuk 3 werd bepaald hoe verminderde expressie van 5-HTT de effecten van niet-beïnvloedbare stress op angstconditionering en uitdoving beïnvloedt, door 5-HTT$^{-/-}$ en wildtype dieren bloot te stellen aan een zware, niet beïnvloedbare stressor en vervolgens een geconditioneerde angstrespons te creëren en uit te doven. De stressor bleek geen effect op de wildtype dieren te hebben, maar was in staat de retentie van de uitdoving van geconditioneerde angst in de 5-HTT$^{-/-}$ ratten te normaliseren naar het niveau van de wildtypes. Deze opmerkelijke bevinding suggereert dat zware stress ook een therapeutisch effect teweeg kan brengen in sommige gevallen, maar nader onderzoek is nodig om het exacte werkingsmechanisme van dit effect vast te stellen.

De effecten van beïnvloedbare en niet-beïnvloedbare stress zijn tegengesteld aan elkaar: een stressor waarover het individu geen controle kan uitoefenen heeft maladaptieve effecten, maar ervaring met een beïnvloedbare stressor kan juist beschermen tegen de effecten van toekomstige stressoren en helpen bij de omgang ermee. Controleerbare stress activeert de PFC, en zorgt dat deze weer geactiveerd wordt bij een volgende stresservaring. Deze activatie van de PFC onderdrukt serotonine afgifte in de DRN, wat de maladaptieve effecten van stress zou voorkomen. In hoofdstuk 4 is beschreven hoe 5-HTT expressie de activatie van de PFC en DRN na beïnvloedbare en niet-beïnvloedbare stress moduleert. 5-HTT$^{-/-}$ en wildtype ratten werden tien dagen achtereen geleerd schokken te ontwijken door de neus in een sensor te plaatsen wanneer er een signaal van toon en licht waarneembaar was. Een niet-beïnvloedbare variant van deze stressor werd gerealiseerd door een andere groep 5-HTT$^{-/-}$ en wildtype ratten aan dezelfde signalen en schokken bloot te stellen als de eerder genoemde groep, maar zonder de mogelijkheid deze te ontwijken. Een niet-gestreste controle groep kreeg enkel de signalen gepresenteerd. Vervolgens werden via immunohistochemie serotonine en c-Fos, een eiwit dat kort na activatie van een neuron tot expressie komt, zichtbaar gemaakt en gekwantificeerd in de DRN, en enkel c-Fos in de PFC. De 5-HTT$^{-/-}$ ratten bleken het ontwijken van de stressor sneller aan te leren dan wildtypes. Activatie van serotonerge neuronen in de wildtypes bleek alleen op te

treden als de stressor beïnvloedbaar was geweest, terwijl serotonerge activatie in de DRN in 5-HTT$^{-/-}$ dieren onder alle drie de omstandigheden (beïnvloedbare, niet-beïnvloedbare en geen stress) op hetzelfde, lage niveau bleef. In beide genotypes was activiteit in de PFC op gelijke manier verhoogd na beïnvloedbare stress, maar niet na niet-beïnvloedbare stress. Er kan geconcludeerd worden dat de DRN in wildtype ratten gevoeliger is voor de beïnvloedbaarheid van de stressor dan die van 5-HTT$^{-/-}$ dieren. Welke rol de PFC speelt in de verbeterde actieve stress coping van 5-HTT$^{-/-}$ ratten wordt uit deze resultaten niet duidelijk.

Stress coping flexibiliteit, het vermogen om de stress coping stijl aan te passen aan de situatie, lijkt een belangrijke voorspeller voor iemands mate van stress-gevoeligheid. In hoofdstuk 5 wordt besproken hoe 5-HTT genotype de gedragsmatige respons op verschillende opeenvolgende stressoren beïnvloedt. Hiertoe werd bij 5-HTT$^{-/-}$ en wildtype ratten eerst een Pavloviaanse associatie tussen een toon en een onvermijdbare elektrische schok gecreëerd door deze achtereenvolgens te presenteren; de controlegroepen kregen hier enkel de tonen gepresenteerd. Een dag later werd diezelfde toon gebruikt om schokken aan te kondigen in een "active avoidance" gedragsparadigma in een nieuwe, onbekende omgeving, waarbij de ratten de schokken kunnen vermijden door tweemaal de kooi over te steken. Vervolgens werd in een derde, voor de ratten onbekende omgeving de gedragsmatige reactie op de toon gemeten, in afwezigheid van de schokken. In de active avoidance taak ontsnapten de ratten uit de controlegroep niet sneller dan de geconditioneerde ratten. De passieve coping respons die aangeleerd werd gedurende de conditionering op de eerste dag had het aanleren van de ontwijkrespons op de tweede dag blijkbaar niet kunnen verhinderen, in zowel de 5-HTT$^{-/-}$ als de wildtype ratten. De 5-HTT$^{-/-}$ ratten leerden wederom sneller te ontsnappen dan de wildtypes, conform de bevindingen in hoofdstuk 4. Het blootstellen aan de geluidssignalen in een nieuwe context in de afwezigheid van schokken resulteerde in een grotere passieve coping respons in de wildtype ratten dan in de 5-HTT$^{-/-}$ ratten, blijkens hun langer aanhoudende angstreactie. Ook legden wildtype ratten een kleinere afstand af gedurende deze test, wat aan zou kunnen duiden dat de aanwezigheid van de toon het exploreren van de kooi ontmoedigde. Bij 5-HTT$^{-/-}$ ratten bleek de afgelegde afstand (en daarmee de mate van exploratie) afhankelijk van of de ratten waren geconditioneerd op de eerste dag van het experiment: deze ratten legden een significant kleinere afstand af dan de 5-HTT$^{-/-}$ ratten uit de controlegroep. Dit kan duiden op een grotere gevoeligheid voor de angst-conditionering in deze dieren met lagere 5-HTT expressie, maar zou ook verklaard kunnen worden doordat de mobiliteit van wildtype ratten in de controlegroep al op een laag niveau zat, waardoor een verdere verlaging ten gevolge van de conditionering niet kon worden gedetecteerd. Duidelijk is dat de verbeterde actieve coping van 5-HTT$^{-/-}$ ratten leidde tot geringere passieve coping wanneer het stressor-voorspellende

signaal in een andere omgeving gepresenteerd werd, wat erop lijkt te wijzen dat de active avoidance ervaring minder traumatisch was voor de 5-HTT$^{-/-}$ dieren.

De gedragsmatige kenmerken van 5-HTT$^{-/-}$ dieren vertonen sterke overeenkomsten met gedrag dat waargenomen wordt in depressieve menselijke patiënten. Het voorschrijven van voedingssupplementen met omega-3 meervoudig onverzadigde vetzuren wordt gezien als een veelbelovende (co)therapie voor de behandeling van depressie. In hoofdstuk 6 werd bepaald of het gedragspatroon van 5-HTT$^{-/-}$ ratten, wat gekenmerkt wordt door verhoogde angst, verlaagde sociale capaciteit, en persistentie van geconditioneerde angst, beïnvloed kon worden door een dieet rijk aan omega-3 vetten. Hiertoe werden volwassen 5-HTT$^{-/-}$ en wildtype ratten drie maanden lang op een dieet rijk aan visolie gezet, of een isocalorisch controledieet, waarbij de visolie vervangen was door soja-, kokos- en maisolie. Vervolgens werden angst, sociaal gedrag en uitdoving van de geconditioneerde angstrespons bepaald in alle ratten. Ten opzichte van wildtype ratten uit de controlegroep vertoonden 5-HTT$^{-/-}$ ratten die het controledieet hadden gekregen meer angst, minder sociaal gedrag en slechtere uitdoving van geconditioneerde angst, in overeenstemming met eerder gerapporteerde bevindingen over deze dieren. In 5-HTT$^{-/-}$ ratten die het omega-3 dieet hadden gekregen bleken al deze gedragsparameters genormaliseerd naar het niveau van wildtypes, terwijl het omega-3 dieet geen effect bleek te hebben op het gedrag van de wildtype ratten. Immunohistochemische aankleuring en kwantificatie van cellen positief voor het eiwit dubbelcortine liet zien dat het niveau van neurogenese, de aanmaak van nieuwe neuronen in de hippocampus, was verhoogd in 5-HTT$^{-/-}$ dieren die het controle dieet hadden gekregen, maar dat dit werd genormaliseerd door het omega-3 dieet. Dit is opmerkelijk, omdat depressieve patiënten vaak juist een verlaging van neurogenese laten zien, en succesvolle antidepressieve behandelingen vaak samengaan met een verhoging ervan. Verondersteld wordt dat de verhoogde neurogenese in 5-HTT$^{-/-}$ ratten onderdeel is van een pathologisch compensatiemechanisme.

Concluderend kan gesteld worden dat de gedragsmatige stress coping respons in 5-HTT$^{-/-}$ ratten sterk afhankelijk is van bepaalde eigenschappen van de stressor. Stressoren die weinig mogelijkheden tot interactie (of actieve coping) bieden, zoals Pavloviaanse angstconditionering, induceren maladaptief gedrag in 5-HTT$^{-/-}$ dieren. Dit wordt gekenmerkt door een aanhoudende passieve angst/coping respons, lang nadat het gevaar is geweken. 5-HTT$^{-/-}$ dieren excelleren echter in het omgaan met stressoren waarmee ze wel kunnen interacteren, d.w.z. waarbij ze een actieve coping respons kunnen aanleren, getuige hun prestaties in de active avoidance paradigma's beschreven in hoofdstukken 4 en 5. De bevindingen beschreven in hoofdstuk 5 suggereren bovendien dat een actieve stress coping ervaring daardoor minder traumatisch is voor 5-HTT$^{-/-}$ dan voor wildtype dieren. Er zijn echter ook tegenstrijdige

bevindingen: de niet-beïnvloedbare stressor beschreven in hoofdstuk 3 produceerde een adaptieve respons in 5-HTT$^{-/-}$ ratten. De angstuitdoving trad in deze dieren sneller op dan in hun soortgenoten die niet aan de stressor waren blootgesteld. Vooralsnog is niet bekend welke hersenmechanismen ten grondslag liggen aan het afwijkende stress coping gedrag in 5-HTT$^{-/-}$ ratten. De geringere gevoeligheid van de DRN voor de controleerbaarheid van stress (beschreven in hoofdstuk 4) suggereert dat de serotonerge reactie die volgt op zware niet-beïnvloedbare stress wellicht een kleinere rol speelt in deze dieren, en dat de stressor in hoofdstuk 3 wellicht via andere mechanismen een uitwerking op latere angstuitdoving heeft. Nadere studies zijn echter nodig om dit te bevestigen. Met name het meten van de directe serotonerge respons op stress in deze dieren kan hierin informatief zijn. De bevindingen hebben mogelijk relevantie voor onderzoek naar stressgevoeligheid in menselijke s-allel dragers en de rol die de beïnvloedbaarheid van de stressoren hierin speelt. Er moet echter wel benadrukt worden dat er aanzienlijke verschillen bestaan in stress coping strategieën tussen mensen en dieren, en dat het trekken van parallellen hiertussen niet eenduidig is. Klinische onderzoeken die de relatie tussen stress en psychiatrische aandoeningen trachten te beschrijven zouden baat hebben bij een nauwkeuriger definitie van de eigenschappen van de stressor en de coping respons erop, terwijl preklinisch dieronderzoek naar stress behoefte heeft aan stress paradigma's die traumatische stressoren in mensen nauwkeuriger benaderen.

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Het uitvoeren van een promotieonderzoek kan gezien worden als een potente stressor, en zonder adequate coping strategieën zou men er wel eens aan ten onder kunnen gaan. Coping doe je niet alleen: zonder de steun van vrienden, familie en collega's was het nog slecht met me afgelopen. Hier wil ik de ruimte nemen om mijn dank te betuigen aan iedereen die die me bijgestaan heeft in deze monsterklus: degenen die me voorzien hebben van inhoudelijke input en discussie, praktische ondersteuning, onderricht in technieken en uiteraard de vele, vele schouders waar ik op heb mogen uithuilen als het me allemaal teveel werd: dit hoofdstuk is voor jullie.

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Lieve Ellen, er is niets wat ik hier op kan schrijven wat recht zou doen aan hoeveel je voor me betekent. Je bent de liefste, ik hou van je!

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