The Role of the Hippocampal GABAergic System in the Development of Posttraumatic Stress Disorder

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Contents

I Abbreviations

ACSF	Artificial cerebrospinal fluid
Ampl	Amplitude
As	Adulthood stress
CB1	Cannabinoid receptor 1
CCK	Cholecystokinin
Cl^-	Chloride anion
CNQX	6-cyano-7-nitroquinoxaline-2, 3-dione
CNS	Central nervous system
CT	Cycle threshold
Ctrl	Control
DMSO	Dimethyl sulfoxide
DSI	Depolarization-induced suppression of inhibition
D-APV	D-2-amino-5-phosphonovalericacid
EC	Entorhinal cortex
eIPSC	Evoked inhibitory postsynaptic current
GAPDH	Glycerinaldehyd-3-phosphat-dehydrogenase
HPA	Hypothalamic–pituitary–adrenal
ITI	Inter trial interval
Js	Juvenile stress
LTD	Long-term depression
mIPSC	Miniature inhibitory postsynaptic current
nACh	Nicotinic acetylcholine
NMDA	N-Methyl-D-aspartate
P	Postnatal day
PPF	Paired-pulse facilitation
PTSD	Posttraumatic stress disorder
PV	Parvalbumin
R_{in}	Input resistance
sIPSC	Spontaneous inhibitory postsynaptic current
TTX	Tetrodotoxin
5-HT	5-Hydroxytryptamin
8-OH-DPAT	(±)-8-Hydroxy-2-dipropylaminotetralin hydrobromide

1 Introduction

1.1 Definition of PTSD

PTSD is an anxiety disorder that follows a traumatic or life-threatening event, for instance natural disasters, war, terrorism, abuse or serious accidents. Causing dismay and strong anxiety in patients, these not only suffer psychologically from psychic hyperesthesia, emotional numbness, helplessness and disturbed self-conception but also physically from hyper-arousal or vegetative tumultuousness with insomnia, irritation, emotional outbursts and increased vigilance (Mancino *et al*, 2006). Commonly, patients suffer from the recurrent re-experiencing of the traumatic event in the form of incriminating thoughts, intrusive memories or illusory episodes (flashbacks). These are evoked spontaneously or by any reminder that patients associate with the traumatic events and cause serious physical stress and restriction of quality of life (Kessler *et al*, 1995). Therefore, patients often actively try to avoid potential reminders and develop emotional neglect and estrangement from formerly important aspects in their personal lives.

Diagnostic guidelines of PTSD are provided by the *DSM* (Saß *et al*, 1994) and the *ICD-10* (*International Statistical Classification of Diseases and Related Health Problems, ICD-10*, World Health Organization, 1992). Accordingly, clinical conditions that last up to three months are classified as acute, longer periods as chronic and appear immediately after the traumatic event or with a delay of numerous years. However, the diagnosis of PTSD is hindered by several circumstances, for instance variable accentuation of symptoms in diagnostic guidelines, variability in the definition of traumatic events or the fact that some symptoms of PTSD are shared by other mental disorders like depression and panic disorder.

1.2 Epidemiology of PTSD

1.2.1 Prevalence

The prevalence of PTSD strongly varies depending on environmental factors, severity of the trauma as well as genetic background and age of the person affected. Generally, women have a higher prevalence for PTSD (10%) than men (5%-6%) (Breslau *et al*, 1991; Kessler *et al*, 1995; Yehuda, 2002). Overall, the prevalence of a traumatic events is reported to be at 18%-25% and rates of PTSD following a traumatic event vary between 1.3%-2.0% (Perkonigg *et al*, 2000). Interestingly, interpersonal trauma, for instance child abuse, rape or personal violence is more likely to cause PTSD than traumatic events by force majeure like natural disasters. Further, the frequency of the occurrence of traumatic events contributes to the prevalence of the disorder. Therefore prevalence is higher in areas where subjects are naturally exposed to environmental threats, e.g. in regions plagued by natural disasters or

violent conflict. Also, professions posing special occupational risks like police, firefighter and emergency services increase the prevalence of experiencing a traumatic event by about 80% (Teegen, 1999). Factors that contribute to prevalence are controllability and predictability of traumatic events as well as the feeling of helplessness in such a condition. Importantly, young individuals are specifically vulnerable to the later development of the disorder (Ford and Kidd, 1998; Heim and Nemeroff, 2001) and this was partly related to the specific vulnerability of stress-related brain regions like the hippocampus at this age (Ben-Ari, 2013; Spear, 2000). In view of this, the exposure to a traumatic event is thought to predispose a subject to posttraumatic sequelae after later traumatic or severely stressful events (Avital and Richter-Levin, 2005; Horovitz *et al*, 2012, 2014; Tsoory *et al*, 2007).

1.2.2 Comorbidity

In over 80%, PTSD often shows a high comorbidity with psychiatric and somatic disorders. Frequent indications are a decrease in overall sense of security, elevated anxiety, major depression, panic disorder, substance abuse, dissociative amnesia or loss of memory (Kessler *et al*, 1995; Mancino *et al*, 2006; Yehuda, 2002).

1.3 Neurobiology of PTSD

1.3.1 Learning and memory

A strong association was found between trauma and memory deficits (Elzinga and Bremner, 2002; Pitman, 1989), for instance dissociative amnesia, fragmentation of memory and deficits in declarative memory. Memory refers to the coding, storage and retrieval of knowledge and learning is the process by which behavior is changed through the acquisition of knowledge. Memory is generally classified along time course, i.e. short-term and long-term, and nature of information stored (Fig. 1), i.e. implicit or explicit (Zola-Morgan and Squire, 1991).

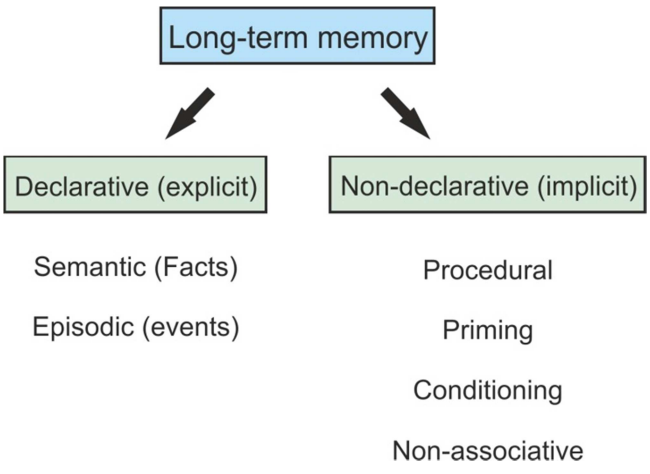

Fig. 1. A classification of memory along its nature.

Patients suffering from PTSD often exhibit deficits in the storage and retrieval of declarative (explicit) memory that relates to episodic memory in general but also to aspects of the trauma. Psychologically, this is thought to serve as a coping mechanism to protect mental health. Intrusive memories or flashbacks are assumed to reflect the pathological facilitation of aversive conditioning, and they are triggered spontaneously or by reminder cues that reflect aspects of the trauma. Therefore, patients suffering from PTSD often show increased avoidance behavior (Elzinga and Bremner, 2002).

In animal models, fear conditioning is suitable to study the neurobiology of anxiety disorders such as PTSD (Siegmund and Wotjak, 2006; Stein and Matsunaga, 2006). In this paradigm, an aversive stimulus, for example foot shock, is being associated with a neutral stimulus, for example a tone. Subsequent presentation of the neutral stimulus or reminder cue will then evoke an elevated stress response and this has been employed to induce PTSD-like behavioral changes in rodents (Albrecht *et al*, 2013; Horovitz *et al*, 2014; Olson *et al*, 2011). Vice versa, extinction of fear-related memory is used in the treatment of PTSD (Quirk *et al*, 2010).

The groundwork for understanding the cellular basis of learning and memory were laid by studies of simple forms of learning like sensitization, habituation and conditioning in the mollusk *Aplysia* (Castellucci *et al*, 1970). Long-term potentiation (LTP) and long-term depression (LTD) are the commonly accepted cellular correlates of memory. The storage of information is thought to be linked to an increased efficiency in synaptic transmission which changes the signal propagation in neural networks and that this is regulated via changes in Ca^{2+} transmission (Menzel, 2001). The NMDA receptor, blocked by Mg^{2+}, is in an inactivated state at membrane potentials close to -65 mV. After depolarization, Mg^{2+} is released and Ca^{2+} enters the cell. By activation of second messenger cascades this results in structural changes involving protein phosphorylation and synthesis as well as gene transcription and eventually results in an increase in activity-dependent synaptic efficiency. Functionally, NMDA receptors act as coincidence detectors for simultaneously occurring events at the pre- and postsynapse (Menzel, 2001). This mechanism has been proposed by Donald Hebb in 1949, hence the name Hebbian-synapse. Besides LTP and LTD, further processes that modify synaptic strength are homeostatic, activity-dependent and spike-timing-dependent plasticity as well as depolarization-induced suppression of inhibition (DSI). Taking advantage of the knowledge of these processes helps to better understand mood disorders that are related to hippocampal plasticity.

From lesions studies of the limbic system, for instance after the removal of hippocampal structures in the treatment of epilepsy, it is known that this brain region is involved in declarative (explicit) memory function (Scoville and Milner, 1957). The hippocampal

formation, the organizer of declarative memory, is the region of initial storage and computation of information. It connects to associative regions of the neocortex where information is finally stored (Fink *et al*, 1996; Markowitsch, 2000; Zola-Morgan and Squire, 1991). In turn, the hippocampus also controls retrieval of information stored in the neocortex (Elzinga and Bremner, 2002). Emotional learning during the stress response relies on the interaction of the hippocampus, the hypothalamus and the amygdala (LeDoux, 1992; Tsoory *et al*, 2008), the latter being supposed to tag memories with emotional qualities (Akirav and Richter-Levin, 1999; Avital *et al*, 2006; Hadad-Ophir *et al*, 2014; Li and Richter-Levin, 2013).

1.3.2 The hippocampal formation

The hippocampal formation can be divided into a six-layered parahippocampal region and a three-layered hippocampal region. The parahippocampal region consists of the entorhinal cortex (EC) and the pre- and parasubiculum. The hippocampus consists of the hippocampus proper or Cornu ammonis (CA1-CA3), the subiculum and the dentate gyrus (Amaral and Witter, 1995; Amaral *et al*, 2007). Some authors consider these regions together with the perirhinal cortex, the mammillary bodies, the fornix and the medial dorsal nucleus of the thalamus as the extended hippocampal formation (Aggleton and Brown, 1999).

The EC is the major source of hippocampal sensory information. Via the perforant pathway, afferent information from the EC reaches the dentate gyrus, is initially computed by high-threshold granule cells and conveyed further to the CA3 (Amaral *et al*, 2007). For the intrinsic and synaptic properties of granule cells, the dentate gyrus has been attributed a gating function for incoming information (Heinemann *et al*, 1992), however, an upstream computational role for all sensory information in the brain has also been proposed for the EC (van Strien *et al*, 2009). EC efferents terminate on dendrites of granule cells but also on dendrites of basket cells in the outer two thirds of the molecular layer (Amaral *et al*, 2007). In turn, the major hippocampal efferents from the CA1 and subiculum project back to the EC. Apart from perforant pathway input, other afferents of the hippocampus are the fornix which conveys information from the medial septum and thalamus and the commissural pathway which connects both hippocampi. Extra-hippocampal monoaminergic input is supplied by serotonergic fibers of the raphe nuclei (Leranth and Hajszan, 2007), noradrenergic fibers of the locus coeruleus (Lindvall and Björklund, 1974) and dopaminergic fibers of the tegmental area and substantia nigra (Gasbarri *et al*, 1994). Their transmitters act as neuromodulators on a diverse range of hippocampus-dependent functions such as memory and mood. While the ventral part of the hippocampus mediates the processing of information related to the motivational and homeostatic state of the animal, the dorsal part is thought to be involved in spatial learning (Moser and Moser, 1998; Segal *et al*, 2010).

Typical for the hippocampus is convergent computation and unidirectional information processing. Intra-hippocampal information flow is determined by the tri-neuronal circuit formed by the excitatory principal cells of the dentate gyrus and ammon's horn. First, mossy fibers of dentate gyrus granule cells innervate the dendrites of pyramidal cells of the CA3. Axon collaterals of CA3 pyramidal cells called Schaffer collaterals in turn project onto the dendrites of CA1 pyramidal cells and close the intra-hippocampal loop. This network is supplemented by local circuits between all types of principal cells and interneurons (Altman *et al*, 1973). Through this reciprocal wiring and lower discharge thresholds for interneurons, afferent excitation of principal cells (1) leads to excitation of interneurons (2), which in turn decreases the release probability of principal cells including those initially excited (3), hence (tri-synaptic) feed-back inhibition. In a di-synaptic feed-forward circuit, the first step is the excitation of interneurons (4), which then inhibit principal cells (5) (Fig. 2). Feed-back inhibition provides stability, feed-forward inhibition functions as a filter for afferent input and improves signal-to-noise ratio (Buzsáki, 1984). This complex wiring provides the background for the computation of sensory information in the hippocampus.

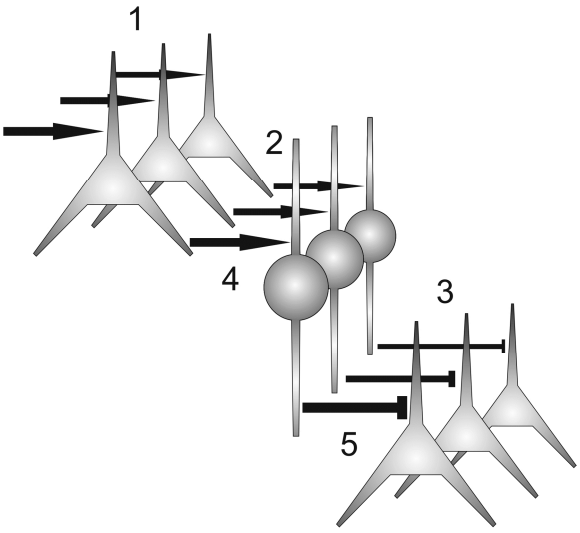

Fig. 2. Feed-back inhibition (1-3) and feed-forward inhibition (4-5) between interneurons and principal cells. For details, see text.

1.3.3 The dentate gyrus

The dentate gyrus (Fig. 3) is a U-shaped, three-layered structure located between the septal and temporal poles of the hippocampal formation. Ovoid-shaped granule cells (10-18 µM), the principal cells of the dentate gyrus, are densely packed in the middle layer, the granule cell layer. Their apical dendrites arborize in the outer, molecular layer which is mostly devoid of other neuronal structures except fibers from the perforant path (Amaral *et al*, 2007). In the inner layer, the hilus (polymorphic layer, CA4), the major cell type are mossy cells (Scharfman, 1995). The unmyelinated axons of granule cells, the mossy fibers, traverse the

hilus where they innervate local interneurons to terminate in the CA3. A considerable number of mossy fiber branches also innervate inhibitory basket cells in the granule cell layer (Acsády *et al*, 1998).

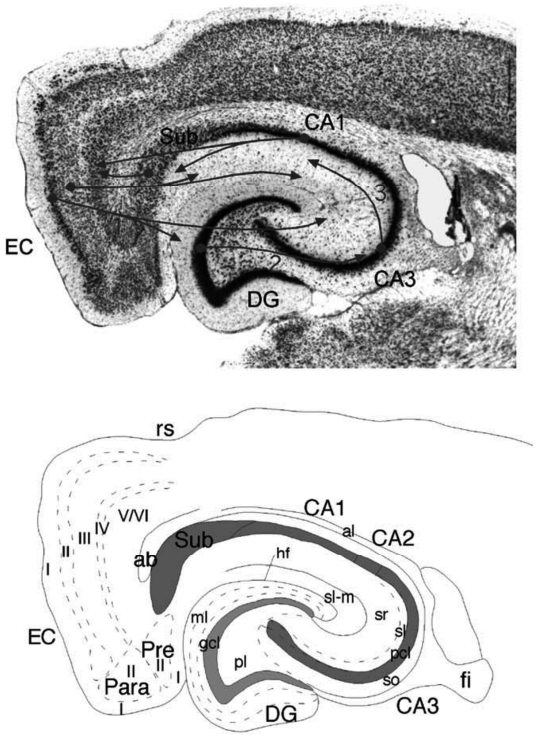

Fig. 3. Nissl-stained horizontal section (upper panel) and schematic drawing (lower panel) of the rat hippocampal formation. EC projections terminate in the molecular layer of the DG and in the CA3. Efferents of granule cells (mossy fibers) terminate within the hilus and the CA3. Efferents of the CA3 (Schaffer collaterals) innervate the CA1. The DG is divided into a molecular layer (ml), a granule cell layer (gcl) and a polymorphic layer (pl). The hippocampus is divided into CA3, CA2 and CA1. Modified from Amaral *et al*, 2007.

1.3.4 Basket cells

The hippocampus is equipped with a vast diversity of interneurons, synonymously called GABAergic non-principal cells (Freund and Buzsáki, 1996). They are classified along morphological, neurochemical and physiological features. One important class is the basket cell, named so after the basket-like morphology of its axon collaterals (Lorente De Nó, 1934). Basket cells are found throughout the hippocampus. In the dentate gyrus, they are located between the granule cell layer and the hilus. Targeting the perisomatic region of the postsynaptic cell, the ideal site for controlling action potential discharges (Miles *et al*, 1996), they mediate feedback inhibition onto granule cells, their main termination site (Amaral *et al*, 2007; Freund and Katona, 2007). They thus exert powerful control over granule cell transmission to the CA3. Their apical dendrites reach the molecular layer and their basal dendrites and axons make contacts in the hilus, allowing for integration of information from the perforant pathway as well as from mossy fiber collaterals in the hilus. Neurochemically, two major types of basket cells are distinguishable, the parvalbumin (PV)-positive (+) and cholecystokinin (CCK)+ cells. PV+ cells discharge fast and non-accommodating action potentials and have a low input resistance and fast membrane time constant (Doischer *et al*,

2008; Földy *et al*, 2007). CCK+ cells discharge slow and accommodating action potentials and have a higher input resistance and slower membrane time constant (Glickfeld and Scanziani, 2006; Lee and Soltesz, 2011). In contrast to PV+ cells, CCK+ express 5-HT3, nicotinergic and cannabinoid receptors that are thought to be mediators of anxiety (Bambico *et al*, 2012; File *et al*, 1998; Freund and Katona, 2007; Freund, 2003). Furthermore, the projection sites of PV+ cells express GABA-A receptors containing the α1 subunit, whereas those of CCK+ cells express α2 containing GABA-A receptors, thought to mediate the effects of benzodiazepines in the symptomatic treatment of mental disorders (Freund, 2003; Nyiri *et al*, 2001). Both cell types innervate principal cells and are also connected to each other (Bartos and Elgueta, 2012; Karson *et al*, 2009). These properties suggest complementary roles of PV+ and CCK+ cells in the generation and control of network oscillations (Klausberger *et al*, 2005). PV+ cells represent a non-plastic network that synchronizes principal cell networks. CCK-positive cells appear to fine-tune the network by the integration of information related to the emotional, motivational and general physiological state (Freund and Buzsáki, 1996; Freund, 2003).

1.3.5 GABAergic signaling

Besides basket cells, other major inhibitory GABAergic interneurons of the hippocampus are bistratified cells, oriens-lacunosum moleculare cells and axo-axonic cells (chandelier cells) which can be classified along their different postsynaptic projection sites, i.e. distally on dendrites or proximally, close to the soma. Via spontaneous or action potential-dependent GABA release from interneurons, an inhibitory response in postsynaptic cells is elicited and, due to electrotonic filtering, distally evoked inhibitory currents in the dendrites of postsynaptic cells have slower kinetics than those generated close to the soma (Kobayashi and Buckmaster, 2003; Maccaferri et al., 2000). This current is blocked by specific GABA-A antagonists, e.g. bicuculline, a phytotoxin of *Bicuculla cucularia* (*Dicentra cucullaria*). By binding to ionotropic GABA-A receptors, GABA opens Cl^- channels and thereby hyperpolarizes the postsynaptic cell which drives away the membrane potential from action potential initiation. At membrane potentials more negative than the Cl^- equilibrium potential, GABA has an depolarizing effect as it is the case in immature neurons (Cherubini *et al*, 1990). After strong activation of interneurons, excess GABA reaches extrasynaptically located metabotropic GABA-B receptors which mediate an inhibiting K^+ current and activation of second messenger cascades.

GABA-A receptors are important in hippocampus-dependent tasks like fear conditioning and spatial learning and pose a target in the symptomatic treatment of mental illnesses (Bergado-Acosta *et al*, 2008, 2014; Collinson *et al*, 2002; Crestani *et al*, 2002; Freund, 2003; Low *et al*,

2000; Rudolph *et al*, 1999). For instance, in anxiety-related structures like the amygdala and hippocampus, synapses of CCK+ cells are enriched with the α2-subunit-containing $GABA_A$ receptor, known to mediate the anxiolytic effect of benzodiazepines (Freund, 2003; Low *et al*, 2000; Möhler, 2006; Rudolph *et al*, 1999) and it has been shown that stress interferes with the expression of the α subunits of the GABA-A receptor (Jacobson-Pick *et al*, 2008). Anesthetics, barbiturates and alcohol act by stabilizing the GABA-A receptor channel in the open state (Möhler, 2006). GABAergic signaling is subject to neuromodulation as inhibitory cells specifically express a diverse range of respective receptors.

1.3.6 Neuromodulation of GABAergic signaling by 5-HT

Serotonin (5-Hydroxytryptamin; 5-HT) is a ubiquitous messenger and involved in a vast diversity of physiological functions, for instance nociception, motor functions, sleep, blood clotting, food intake, thermoregulation, immune responses and neurogenesis. Unsurprisingly, this messenger plays an important role in a high number of disorders. It is well established that emotional and cognitive psychiatric disorders are related to alterations in the serotonergic system (Manji *et al*, 2001; Pralonga *et al*, 2002).

In humans, 5-HT receptors are classified into seven classes (5-HT1-7), giving 14 different subtypes of 5-HT receptor in total (Barnes and Sharp, 1999). All 5-HT receptor subtypes are metabotropic G-protein-coupled receptors that act via intracellular second messenger cascades except the 5-HT3 receptor which is an ionotropic ligand-gated ion channel. In the CNS, 5-HT acts as a neuromodulator on synaptic plasticity and neurogenesis and exerts an inhibitory tone onto principal cells either directly or through excitation of interneurons. Besides GABA-A receptors, 5-HT also acts on extrasynaptic GABA-B receptors (Ghadimi *et al*, 1994; Segal, 1990). In the dentate gyrus, serotonergic afferents from the raphe nuclei, specifically targeting CCK+ basket cells, are activated by stress (Amaral *et al*, 2007; Amat *et al*, 2005; Leranth and Hajszan, 2007; Morales and Bloom, 1997).

1.3.6.1 The 5-HT1A receptor

In a simple view, according to the monoamine hypothesis, mood disorders rely on a deficiency in 5-HT signaling (Hirschfeld, 2000), however, the picture is more complex. Facilitation of 5-HTergic signaling via 5-HT1A receptors, elevated 5-HT levels or by selective serotonin re-uptake inhibitors (SSRIs) was found to have an anxiogenic effect (Blumberg *et al*, 2009; File *et al*, 1996; Kalynchuk *et al*, 2006; Oh *et al*, 2009; Sass and Wörtwein, 2012). An inverse relationship was observed in animals previously exposed to early life trauma, where later treatment with SSRIs may reverse the stress response (Joca *et al*, 2006; Uys *et al*, 2006), however, other 5-HT receptor subtypes might also be involved. Antagonizing 5-

HT1A receptors reverses spatial learning impairment in rats (Carli *et al*, 1997). This was attributed to the 5-HT1A-mediated decrease in pyramidal cell inhibition that compensates for a loss in excitatory cholinergic signaling associated with learning deficits (Barnes and Sharp, 1999). A neurotrophic function in neurogenesis thought to be involved in learning processes has also been described for 5-HT1A receptors (Jacobs *et al*, 1998; Klempin *et al*, 2010). These findings indicate that 5-HT acts beneficially or counter wise dependent on the history of stress and the interaction with other neuromodulatory systems as well as on the parameters examined, e.g. spatial navigation or anxiety-related behavior.

The amino acid sequences of human and rodent 5-HT1A receptors are homologous by 89% (Albert and Bunzow, 1990). In both species, 5-HT1A receptors are enriched in limbic regions, especially the hippocampus and entorhinal cortex, but also on 5-HTergic neurons themselves in the raphe nuclei (Barnes and Sharp, 1999; Burnet *et al*, 1995; Kung *et al*, 1995; Miquel *et al*, 1991). 5-HT receptor activation inhibits the downstream G-protein-coupled signaling cascade by cleavage of the G-protein complex. In principal cells, this leads to the activation of the inwardly rectifying K1 channel (GIRK) at the somatodendritic site, leading to an increase in conductance with, according to Ohm's law, a concurrent decrease in evoked currents (Baskys *et al*, 1989; Ghadimi *et al*, 1994; Piguet and Galvan, 1994; Schmitz *et al*, 1995). At the presynaptic site, activation of G-protein-coupled receptors inhibits transmitter release in a Ca^{2+}-dependent way (Andrade and Nicoll, 1987; Lüscher *et al*, 1997). Activation of postsynaptic 5-HT1A receptors also causes an increase in hormone levels, for instance that of corticosteroids (Fuller, 1996).

1.3.6.2 The 5-HT3A receptor

In humans and rodents, 5-HT3 receptor antagonism has facilitating effects on learning in hippocampus-dependent tasks as well as anxiolytic effects (Jones *et al*, 1988; Stäubli and Xu, 1995). Consistent with this, 5-HT3 receptor agonism inhibits LTP, a cellular correlate of memory (Bliss and Collingridge, 1993). However, in concert with other receptor subtypes, 5-HT exerts more complex effects on anxiety-related behavior. 5-HT3 receptors co-localize with other receptors indicated in mood, e.g. cannabinoid and nicotinergic receptors, on CCK+ cells (Morales and Bäckman, 2002; Morales and Bloom, 1997; Morales *et al*, 2008). In the human and rodent hippocampus, 5-HT3 receptors are only enriched on presynaptic nerve terminals of CCK+ but not PV+ interneurons where this ligand-gated ionotropic receptor mediates a rapidly activating, desensitizing inward current (Katsurabayashia *et al*, 2003; Morales and Bloom, 1997; Waeber *et al*, 1988, 1989; Yakel and Jackson, 1988). This can be measured as a burst of mIPSCs in granule cells (Dorostkar and Boehm, 2007; Ghadimi *et al*, 1994; Kawa, 1994; Piguet and Galvan, 1994; Ropert and Guy, 1991; Turner *et al*, 2004). Due

to the excitatory effect of 5-HT3 receptors and the inhibitory effect of 5-HT1A receptors on CCK+ interneurons, 5-HT exerts opposite effects on this cell type. This explains why 5-HT exerts a facilitating effect on action potential-independent mIPSCs through ionotropic 5-HT3 receptors and an inhibitory effect on action potential-dependent sIPSCs through metabotropic 5-HT1A receptors.

1.3.7 Neuromodulation of GABAergic signaling by cannabinoids

Cannabinoids have disruptive as well as facilitating effects on hippocampus-dependent tasks and are vital for the extinction of conditioned fear associations (Akirav, 2011). Both, the suppression and strong facilitation of cannabinoid-signaling have an increasing effect on stress and anxiety levels; the moderate facilitation of cannabinoid-signaling decreases stress and anxiety levels (Akirav, 2011; Crippa *et al*, 2009; Scherma *et al*, 2009). Cannabinoids, through DSI, act inhibitory and highly specific in concert with CCK via N-type voltage-gated Ca^{2+} channels on action potential-dependent GABA release in CCK+ basket cells (Akirav, 2011; Hefft and Jonas, 2005; Lee and Soltesz, 2011; Wilson *et al*, 2001).

1.4 The stress response

Stress is defined as any stimulus that presents a challenge to homeostasis which leads to an adaption to environmental challenges on many different physiological levels of an organism (Joëls and Baram, 2009). These adaptions act immediately but also serve to protect from future stressful challenges, and this necessitates learning and memory functions. Thus, the limbic system being involved in these functions is an integral part of the stress response. Stress activates the median and dorsal raphe nuclei which send a major neuromodulatory, serotonergic input to the dentate gyrus (Amat *et al*, 2005). The effects of the stress hormone corticosterone are mediated by membrane-bound mineralocorticoid and glucocorticoid receptors that are densely expressed in the hippocampus (Joëls and Baram, 2009). Corticosteroids have immediate and delayed effects. Immediate effects are mediated by low levels of corticosteroid binding to low-affinity mineralocorticoid receptors. This leads to a decrease in GABAergic transmission and 5-HT1A-mediated effects through changes in K^+ conductance (Joëls and Van Riel, 2004; Van Riel *et al*, 2003). Delayed effects of corticosterone are mediated by high levels of the hormone that bind to high-affinity glucocorticoid receptors during stress. After activation, they translocate to the nucleus and act as transcription factors (Beato and Sánchez-Pacheco, 1996; Chen *et al*, 2006; Karten *et al*, 1999; McEwen, 2007). The activation of mineralocorticoid and glucocorticoid receptors has opposite effects and includes changes in voltage-gated ion channels, ion gradients, G-

protein-coupled signaling cascades, transmitter systems, cell morphology and metabolism as well as 5-HT1A receptor binding in the dentate gyrus (Baratta *et al*, 2009; Fernandes *et al*, 1997; Halasy *et al*, 1992; Joëls and De Kloet, 1994; Joëls, 2001; Karten *et al*, 2001). Interestingly, the effects of stress are region dependent and the dorsal and the ventral part of the hippocampus are affected by stress or stress hormones in an opposite manner. Stress causes facilitation of LTP in the ventral part and suppresses LTP in the dorsal part (Segal *et al*, 2010).

2 Aim of this study

Evidence indicates a substantial role of the hippocampus in the development of PTSD. The present study applies a multi-disciplinal approach using behavioral, electrophysiological and gene expression analysis to better understand the role of the ventral dentate gyrus in the development of PTSD. Childhood trauma is a major risk factor for trauma-induced and stress-related psychopathology in adulthood. The current study takes advantage of the contribution of early-life trauma to the development of posttraumatic sequelae upon stress in adulthood and examines the effects of juvenile and/or adulthood stress on the 5-HT-mediated modulation of synaptic inhibition of ventral dentate gyrus granule cells focusing on the following questions:

1. Which behavioral alterations occur after juvenile and/or adulthood stress?
2. Which stress-related functional changes occur in the serotonergic modulation of GABAergic inhibition in the dentate gyrus?
3. Which cells mediate this effect? Are there cell-specific effects?

3 Materials and Methods

3.1 Study design

The experiments were approved by the Regional Berlin Animal Ethics Committee. Male rats were obtained from Harlan, Germany and Janvier, France, weighing 35-51 g on delivery. Rats were kept on a 12 hour light-dark cycle, room temperature 22±2 °C, in groups of 2-6 per cage, a group size reported not to influence exploratory behavior in the open field (Botelho *et al*, 2007). At postnatal day (p) 21, after weaning, rats were randomly assigned to one of four experimental conditions as previously described (Tsoory and Richter-Levin, 2006):

1) Control (ctrl): rats were not exposed to any adverse condition.

2) Juvenile stress (js): rats were exposed to the juvenile stress protocol at p27-p29.

3) Adulthood stress (as): rats were exposed to the adulthood stress protocol at p60-p69.

4) Juvenile and adulthood stress (js+as): rats were exposed to the juvenile and adulthood stress protocol.

Recent studies have shown that variable stress paradigms evoke stronger stress responses compared with repeated stressors (Garcia-Vallejo *et al*, 1998; Tsoory and Richter-Levin, 2006) and a higher susceptibility to stress in juvenile rats compared with adults (Foilb *et al*, 2011; Garcia-Vallejo *et al*, 1998; Tsoory and Richter-Levin, 2006). The stress protocols used here do not interfere with locomotor skills (Horovitz *et al*, 2012, 2014), and the development of the locomotor system is already completed at the age at which juvenile stressors commence (Altman and Sudarshan, 1975; Gramsbergen, 2001). On three consecutive days (p22-p24), all rats were handled for 5 min. One day (or 14 days when indicated) after the adulthood stress protocol was performed (or at the corresponding age in controls and rats stressed as juveniles) rats were tested in the open field and immediately afterwards killed for experimentation (Fig. 4).

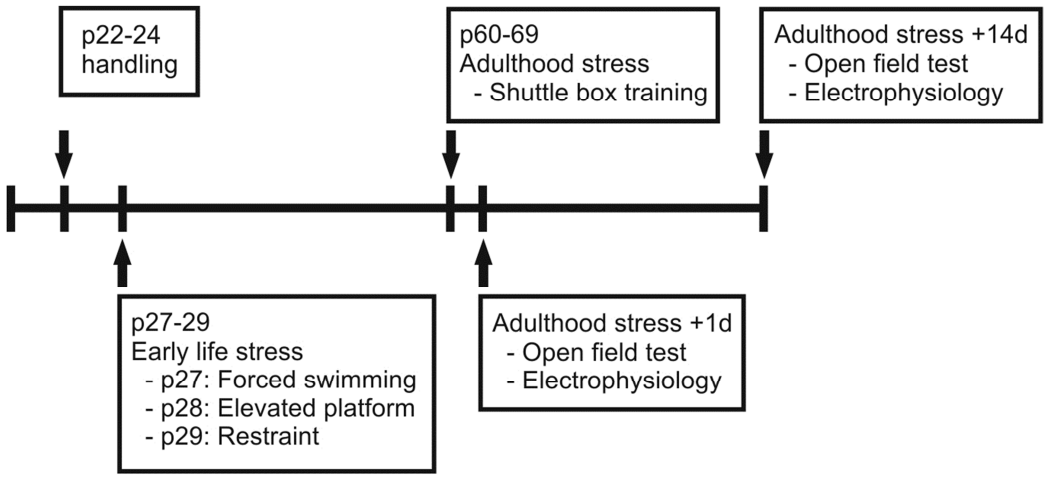

Fig. 4. Study design.

Juvenile stress paradigm

At p27-p29 rats were exposed to a stress protocol which comprised of three different stressors.

p27: Forced swimming: rats were forced to swim for 10 min in a circular water tank (diameter 0.5 m; height 0.5 m; water level 0.4 m).

p28: Elevated platform: rats were placed on a platform (10x10 cm), 70 cm above ground level, three trials for 30 min, intertrial interval (ITI) 60 min.

p29: Restraint: rats were singly placed in a small metal box that prevented the movement of the rats for 2 h.

Adult stress paradigm

At p60-p69 rats were subjected to a single two-way shuttle box experiment as an adult stressor.

Two-way shuttle box training: One experiment comprised of 100 trials, ITI 60±12 s. In a trial, rats were presented a 10 s tone and a light signal immediately followed by an electric foot shock (0.8 mA, 10 s) if not prevented by shuttling to the other compartment of the shuttle box.

Open field test

The open field arena, surrounded by black walls, consisted of a square platform divided into 49 squares of 10x10 cm. Rats were placed in a corner of the open field and their behavior was recorded for 5 min and later analyzed and double-checked by a blind experimenter. Total line crossings between the squares represent total motor activity levels of the rat (Avital *et al*, 2006). Line crossings and time spent in the center of the open field represent anxiety levels (Tsoory *et al*, 2007).

3.2 Slice preparation

Rats were decapitated under nitrous oxide/isoflurane anesthesia and brains were quickly removed. Brain slices (350 µm) containing the hippocampal formation and the entorhinal cortex were prepared in ice-cold saccharose-based artificial cerebrospinal fluid (ACSF; in mmol/l: NaCl 87, NaH_2PO_4 1.25, KCl 2.5, $NaHCO_3$ 26, $MgCl_2$ 7, $CaCl_2$ 0.5, saccharose 75, and glucose 25, saturated with 95 % O_2 and 5 % CO_2 at a pH of 7.4) using a VT1200S vibroslicer (Leica Microsystems GmbH, Germany) and stored under submerged conditions at 34 °C for 20 min before being placed in recording ACSF (in mmol/l: NaCl 129, $NaHCO_3$ 26,

KCl 3, NaH_2PO_4 1.25, $MgSO_4$ 1.8, $CaCl_2$ 1.6 and glucose 10, saturated with 95 % O_2 and 5 % CO_2 at a pH of 7.4) at room temperature under submerged conditions.

3.3 Electrophysiology

Whole-cell patch-clamp recordings were performed in the granule cell layer of the ventral dentate gyrus at room temperature. Recordings were performed using a Multiclamp 700B amplifier in conjunction with a Digidata 1440A interface and PClamp software (Molecular Devices, Sunnyvale, CA, USA) for miniature IPSCs (mIPSCs), or an EPC7 amplifier in conjunction with an InstruTECH LIH 8+8 interface and TIDA software (both HEKA, Lambrecht/Pfalz, Germany) for evoked IPSCs (eIPSCs) and input resistance (R_{in}). Only cells with resting membrane potentials more negative than −65 mV and a series resistance of less than 20 MΩ with less than 30% change under these recording conditions were accepted. Both series and input resistance were measured throughout voltage-clamp recordings using a hyperpolarizing 5 mV step of 300 ms second duration. No series resistance compensation was used. All recordings were performed in the presence of the AMPA receptor-antagonist 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX) and the NMDA receptor-antagonist D-2-amino-5-phosphonovalericacid (D-APV). Patch-clamp electrodes (3–4 MΩ) were pulled on a horizontal DMZ Universal Puller (Zeitz Instruments GmbH, Martinsried, Germany) and filled with (in mM) K-gluconate 135, KCl 20, HEPES 10, phosphocreatine 7, Mg-ATP 2, Na-GTP 0.3 and EGTA 0.2 and adjusted with KOH to a pH of 7.35 for recording monosynaptic eIPSCs and input resistance. Under these conditions, for an optimal signal-to-noise ratio, eIPSCs were recorded at the holding potential of -40 mV. eIPSCs were evoked by stimulation of the perforant pathway in the middle molecular layer at a frequency of 0.1 Hz with amplitudes set to approximately 40-50% of the maximum response. Paired-pulse facilitation (PPF) was investigated by analyzing the ratio of the second to the first synaptic response (eIPSC2/eIPSC1, interpulse interval 50 ms). For the recording of tetrodoxin (TTX)-insensitive mIPSCs at -70 mV, patch-clamp electrodes were filled with (in mM): CsCl 135, NaCl 3, $CaCl_2$ 0.1, EGTA 1, HEPES 10, Na_2ATP_2 and adjusted with CsOH to a pH of 7.35. mIPSCs were detected and analyzed using custom-designed templates (PClamp software; for details see Clements and Bekkers, 1997). This allowed for the identification of the vast majority of events with a minimal number of false positives upon visual inspection and detection of amplitudes ≥5.8 pA. Basket cells innervate the perisomatic region of the postsynaptic neuron and, due to electrotonic filtering, have faster rise times than those generated at distal sites, leading to intermediate kinetics of IPSCs (1.8±0.4 ms for uIPSCs; Kobayashi and Buckmaster, 2003; Maccaferri et al., 2000). Therefore, only events with rise

times (10-90%) ≤2.5 ms were accepted to ensure that mIPSCs generated close to the recording site were included. Signals were low-pass filtered at 2 kHz, sampled and processed at 10 kHz. In all experiments, after recording a stable 10 min baseline, 5-HT was applied for 10 min, a period which in pilot experiments was found to suffice for the effect of 5-HT to reach a plateau.

3.4 Reverse transcription and real-time PCR (in cooperation with Prof. Stork, Magdeburg)

Tissue sample lysis and isolation of total RNA was conducted using the RNeasy Micro Plus kit (Qiagen, Hilden, Germany) according to manufacturer's instructions, including steps for removal of genomic DNA. First-strand synthesis of cDNA was conducted using with the Sensiscript Reverse Transcription kit (Qiagen), specifically designed for low amounts of RNA, in the presence of 2.5 mM dNTPs, 50 µM Oligo (dT)18 and 50 µM random decamer first strand primers (Life Technologies, Darmstadt, Germany) as well as RNase inhibitor (SuperaseIN; 20 U/µl; Life Technologies) for 60 min at 37 °C. Real-time PCR was performed with a 1:5 dilution of cDNA using the ABI Prism Step One real time PCR apparatus (Life Technologies) and TaqMan® reagents with predesigned assays for the target genes (assay IDs: 5-HT1A: Rn00561409_s1; 5-HT3A: Rn00667026_m1; CCK: Rn00563214_m1; CB1: Rn00577436_m1; PV: Rn00574541_m1; Chrna7: Rn00563223_m1) and for the housekeeping gene Glycerinaldehyd-3-phosphat-dehydrogenase (GAPDH; endogenous control, Life Technologies) that was labeled with another fluorescent dye, allowing for quantitative evaluation. Multiplex PCR samples were run in triplicates with 50 cycles of 15 s at 95 °C and 1 min at 60 °C, preceded by a 2 min 50 °C decontamination step with Uracil-N-glycosidase and initial denaturation at 95 °C for 10 min. Mean cycle threshold (CT) was determined for each triplicate assay and relative quantification of each target gene was conducted with the ddCT method (Livak and Schmittgen, 2001), normalizing each sample to the overall content of cDNA using GAPDH as an internal control (dCT; dCT (target gene)=(CT (target gene))-(CT (GAPDH)). Normalization of all ddCT values was done relative to the control group with ddCT=dCT (sample)-mean dCT (control group). Transformation to RQ values for a specific target gene and area was done according to RQ %=(2^{-ddCT})*100 with RQ % (control)=100 %.

3.5 Data analysis and statistics

Data are expressed as mean±SEM. Statistical analysis was performed on raw data using GraphPad software (La Jolla, CA, USA). Statistical distribution of data was tested using

Kolmogorov-Smirnov test. Behavior: Shuttle box training was analyzed using two-tailed Mann-Whitney test. Normally distributed data from open field experiments were analyzed by one-way ANOVA followed by Bonferroni *post-hoc* test. When not normally distributed, Kruskal-Wallis test followed by Dunn's multiple comparison test was used. Electrophysiology: For the analysis of effects, raw mean data values from the last minute of each experimental condition were used. For the visualization of effects, data was normalized over the last 5 minutes of baseline. Normally distributed data was analyzed by one-way ANOVA followed by Bonferroni *post-hoc* test or by Student's *t*-test when indicated. Otherwise, Kruskal-Wallis test followed by Dunn's multiple comparison test was used. The effect on the PPF of eIPSCs was analyzed using one-way ANOVA for repeated measures followed by Bonferroni *post-hoc* test. The distribution of mIPSCs was fit with a nonlinear cumulative Gaussian equation. Comparison between groups with respect to the number of cells with a burst-like discharge of mIPSCs was performed using chi-square test followed by Bonferroni's corrected chi-square test as *post-hoc* test. Real-time PCR: Comparison of effects between groups was performed by one-way ANOVA followed by *post-hoc* LSD test. Interaction between stress protocols as factors was analyzed using two-way ANOVA with significance level set at $p<0.05$. In all *post-hoc* tests, all groups were compared with each other. Significance levels were set at $p<0.05$ (*) and $p<0.01$ (**).

3.6 Drugs

The following substances were bath-applied: 10 µM (+)-bicuculline (Sigma Aldrich, Munich, Germany), 20 µM CGP hydrochloride (Sigma Aldrich), 30 µM CNQX disodium salt, 50 µM D-APV (Ascent Ligands, Bristol, UK), 10 µM ritanserin (Biozol Diagnostica, Eching, Germany), 1 µM (100 µM for mIPSCs) serotonin creatine sulphate, 60 nM tropisetron, 0.5-1 µM TTX, 1 µM WAY 100635 maleate salt, 50 µM 1-phenylbiguanide and 1 µM 8-OH-DPAT (Sigma Aldrich). Substances were dissolved and stored as stock solutions at 1000 times the end concentration in distilled water. Ritanserin was dissolved in DMSO to 100 mM giving an end concentration of 0.01% DMSO in the ACSF, a concentration which was reported not to affect cellular properties or synaptic transmission in the dentate gyrus (Gilling *et al*, 2013).

4 Results

4.1 The effects of stress on anxiety-related behavior

4.1.1 Avoidance learning

It has been shown in rats that the exposure to stress in early life has long-lasting effects into adulthood with respect to the emotional and cognitive aspects of behavior, for instance the ability to cope with stress (Avital and Richter-Levin, 2005; Avital *et al*, 2006; Tsoory and Richter-Levin, 2006). Therefore, we analyzed the avoidance learning performance in the two-way shuttle box training of control rats (n=17) and rats stressed as juveniles (n=20). No differences were found in the number of avoidance shuttles, escape shuttles and escape failures (Fig. 5).

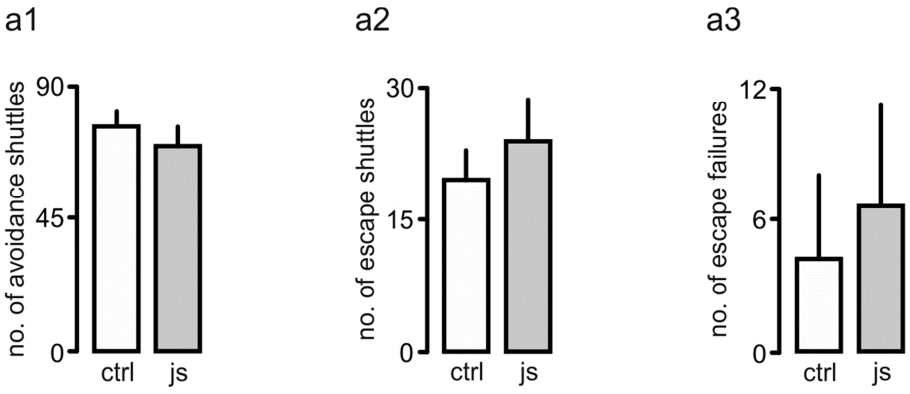

Fig. 5. Stress effects on avoidance learning. No significant differences between controls and rats stressed as juveniles were found in the two-way shuttle box training with regard to the number of avoidance shuttles (a1), escape shuttles (a2) and the number of escape failures (a3).

Rats from both groups acquired the task after approximately 20 trials, after which no improvement in learning occurred anymore. However, after having reached similar plateau levels of performance, further shuttle box training resulted in a significant decrease in avoidance shuttles in control rats (Fig. 6) as observed before (G. Richter-Levin, personal communication).

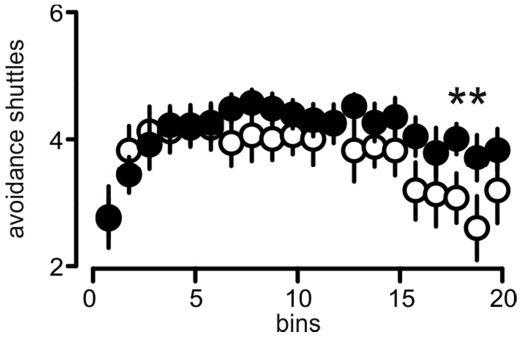

Fig. 6. Time course of avoidance learning. After 90 trials (bins 18, 19), control rats performed less avoidance shuttles compared with rats stressed as juveniles.

4.1.2 Exploratory behavior

To determine the effects of stress on exploratory and anxiety-related behavior, the rats' performance was assessed in an open field test. Rats exposed to stress as juveniles performed similar as controls (Fig. 7, a1-a3). The adult stress protocol did not significantly reduce the activity of rats in the open field (Fig. 7, a2, a3). Rats subjected to both stressors, i.e. as juveniles and in adulthood, showed a significant reduction in exploratory behavior (ctrl: n=35, js: n=32, as: n=17, js+as: n=20, F(3,100)=4.256, p=0.0071, one-way ANOVA; Fig. 7, a1) and increased anxiety levels (F(3,100)=5.109, p=0.0025, one-way ANOVA, Fig. 7, a2; (H(3)=16.79, p=0.0008, Kruskal-Wallis test Fig. 7, a3).

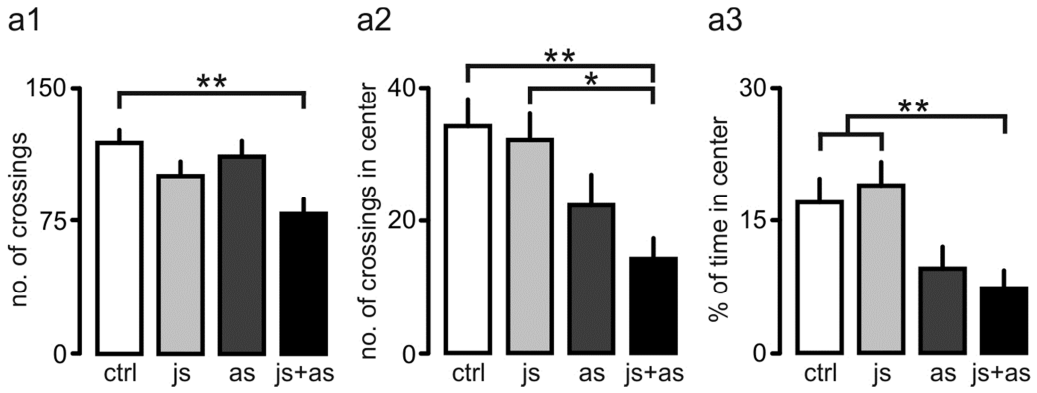

Fig. 7. Stress induces anxiety-related behavior. (b1) Compared with ctrls, only js+as-rats showed a significant reduction in total motor activity. (b2) After the js+as-stress paradigm, the activity of rats in the center of the open field was decreased compared with ctrls and js-rats. (b3) js+as-rats spent significantly less time in the center of the field compared with ctrls or js-rats.

To analyze the long-term effects of stress on exploratory and anxiety-related behavior, we performed a supplementary set of experiments in which rats were examined two weeks after the adulthood stress protocol was applied. In these experiments, the rats' activity in the open field test was similarly changed as in experiments performed one day after the adulthood stress protocol. After two weeks, the adult stress protocol did not significantly reduce the activity of rats (Fig. 8, a1, a2). Rats stressed at both ages still showed significant reductions in total motor activity (as+2: n=11, js+as+2: n=9, F(2,52)=4.963, p=0.0107, one-way ANOVA; Figure 8., a1), the activity in the center of the open field (F(2,52)=5.491, p=0.0069, one-way ANOVA; Figure 8., a2), and time spent in the center of the open field (H(2)=13.71, p=0.0011, Kruskal-Wallis test; Figure 8., a3). These data indicate that the combined exposure to stress in early life and adulthood decreases exploratory behavior and increases anxiety-related behavior. These effects last for a minimum of two weeks after the adulthood stress protocol was applied.

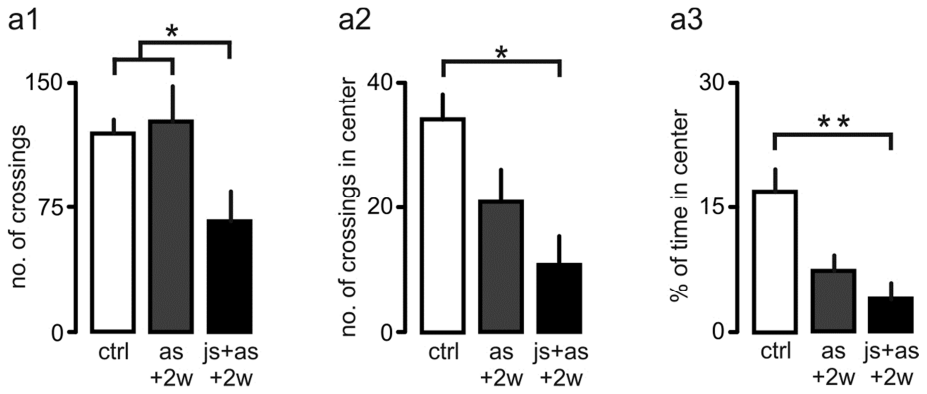

Fig. 8. Long-term effects of stress on exploratory and anxiety-related behavior. After two weeks, js+as-rats still showed significant reductions in total motor activity, activity and time spent in the center of the open field.

4.2 The effect of stress on granule cells' membrane and synaptic properties

4.2.1 Stress attenuates input resistance

In the CA3 region of the rodent tree shrew, stress was found to be able to decrease the input resistance of principal cells (Kole *et al*, 2004). In the present study, the input resistance of dentate gyrus granule cells was found to be reduced in all experimental stress groups compared with controls (ctrl: n=11, js: n=10, as: n=11, js+as: n=11, $F(3,39)=4.184$, $p=0.0116$, one-way ANOVA; Fig. 9).

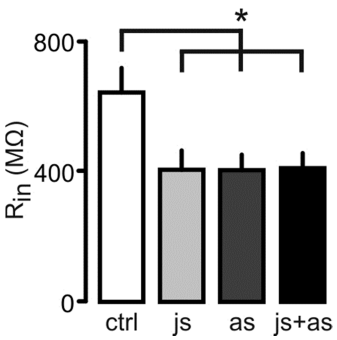

Fig. 9. Stress decreases membrane conductance. In all stressed rats, the input resistance of granule cells was reduced compared with ctrls.

The stress hormone corticosterone and the activation of mineralocorticoid receptors have been shown to reduce the 5-HT-mediated decrease in input resistance in CA1 principal cells and granule cells of the dentate gyrus (Karten *et al*, 1999, 2001). Upon application of 5-HT to slices from control rats, a transient decrease of almost 50% was found in input resistance (Fig. 10, a1, a2). In rats that were subjected to any of the stress protocols, the 5-HT-evoked

decrease in input resistance was significantly reduced to approximately 30% ($F(3,39)=4.942$, $p=0.0053$, one-way ANOVA; Fig. 10, a1, a2).

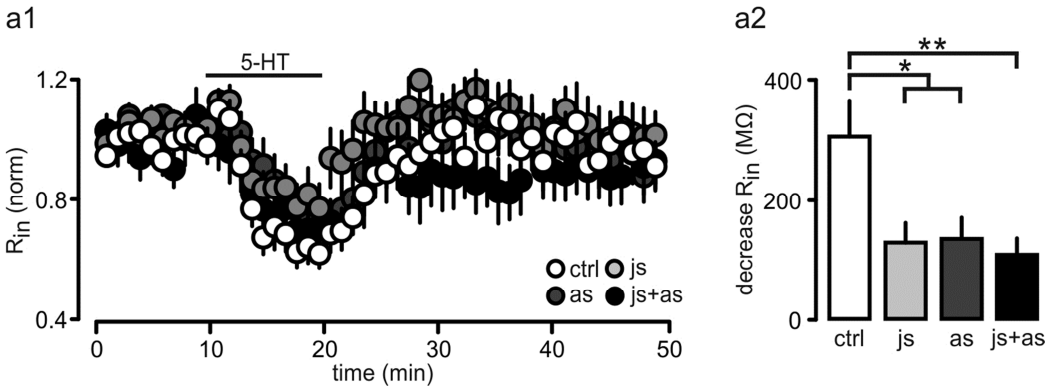

Fig. 10. 5-HT application decreases the input resistance of granule cells differently in ctrls and stressed rats. (a1) Application of 5-HT decreased the input resistance in all experimental groups. (a2) In all stressed rats, the decrease in input resistance was significantly attenuated compared with ctrls.

4.2.2 Stress attenuates 5-HT1A-mediated effects on input resistance

Activation of 5-HT1A receptors has been shown to result in activation of postsynaptic Ca^{2+}-independent inward rectifier K^+ channels (Baskys *et al*, 1989; Ghadimi *et al*, 1994; Piguet and Galvan, 1994), leading to a decrease in input resistance. Using control rats, we found that the 5-HT-mediated decrease in input resistance of granule cells could be blocked by the application of the 5-HT1A receptor antagonist WAY-100635 and mimicked by the application of the 5-HT1A receptor agonist 8-OH-DPAT (Fig. 11), confirming the involvement of this receptor subtype in this effect. In addition, the application of the 5-HT_2 receptor antagonist Ritanserin failed to block the effect of 5-HT (Fig. 11).

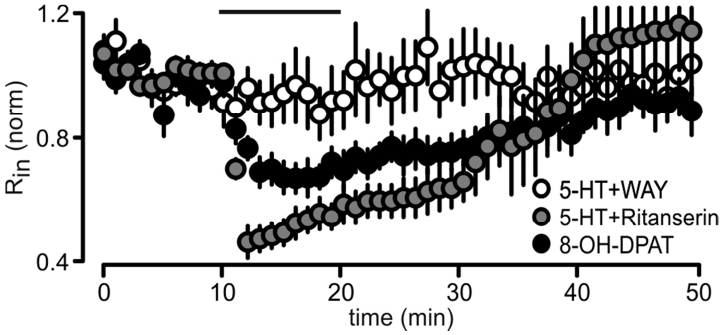

Fig. 11. The decrease in input resistance is 5-HT1A-mediated. The 5-HT1A agonist 8-OH-DPAT reproduced the effect of 5-HT. 5-HT with the 5-HT1A antagonist WAY-100635 inhibited the effect in ctrls. The 5-HT2 antagonist Ritanserin did not block the effect.

As 5-HT increases the release probability of GABA onto dentate granule cells (own data, see Fig. 18), the decrease in input resistance might derive from an increase in postsynaptic

conductance. To test this, the effect of 5-HT on input resistance in the presence of the GABA-A and GABA-B receptor antagonists bicuculline and CGP hydrochloride was studied. Under these conditions, 5-HT still caused a robust decrease in input resistance in controls (n=5, p=0.0337, two-tailed *t*-test; Figure 12.), suggesting the involvement of a 5-HT1A receptor-mediated K^+ conductance.

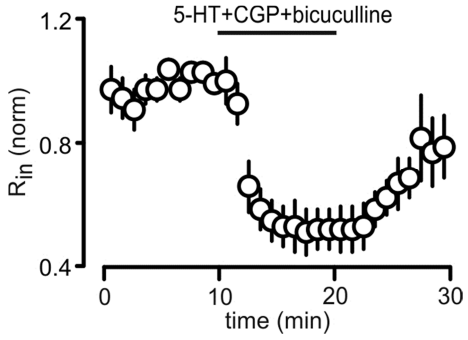

Fig. 12. The 5-HT-mediated decrease in input resistance of granule cells is not blocked by GABA-antagonism. 5-HT in the presence of the GABA-A and GABA-B receptor antagonists bicuculline and CGP hydrochloride still decreased the input resistance of granule cells in ctrls.

4.2.3 Single stress attenuates 5-HT1A-mediated effects on eIPSCs

Previous studies have shown that stress affects 5-HT1A-mediated effects on granule cells and synaptic currents (eEPSCs) in the dentate gyrus and the CA1 (Karst and Joëls, 2003; Van Riel *et al*, 2003). We utilized this in order to determine stress-specific effects on GABAergic currents in each experimental group. Application of 5-HT caused a robust biphasic decrease in the amplitude of eIPSCs (Fig. 13, a1). In the presence of 5-HT, eIPSCs decreased by 43% in slices from controls (n=11), an effect which was significantly smaller in tissue from rats stressed in early life (n=10, 25%) or adulthood (n=11, 26%; $F(3,39)=4.792$, $p=0.0062$, one-way ANOVA) but not in tissue from rats stressed both at the young and adult age (n=11, 37%; Fig. 13, a1, a2 left panel). In addition to the decrease in eIPSC amplitudes, which might be associated with a 5-HT-induced conductance increase (Ginsborg, 1973), application of 5-HT also resulted in a long-term depression (LTD) of eIPSCs, present 30 min after wash out (Fig. 13, a1, a2 right panel). The LTD was significantly smaller in tissue from rats stressed in early life (22%) or in adulthood (22%, $F(3,39)=5.946$, $p=0.0019$, one-way ANOVA; Fig. 13, a2 right panel) than in tissue from controls (37%) and rats stressed at both ages (39%).

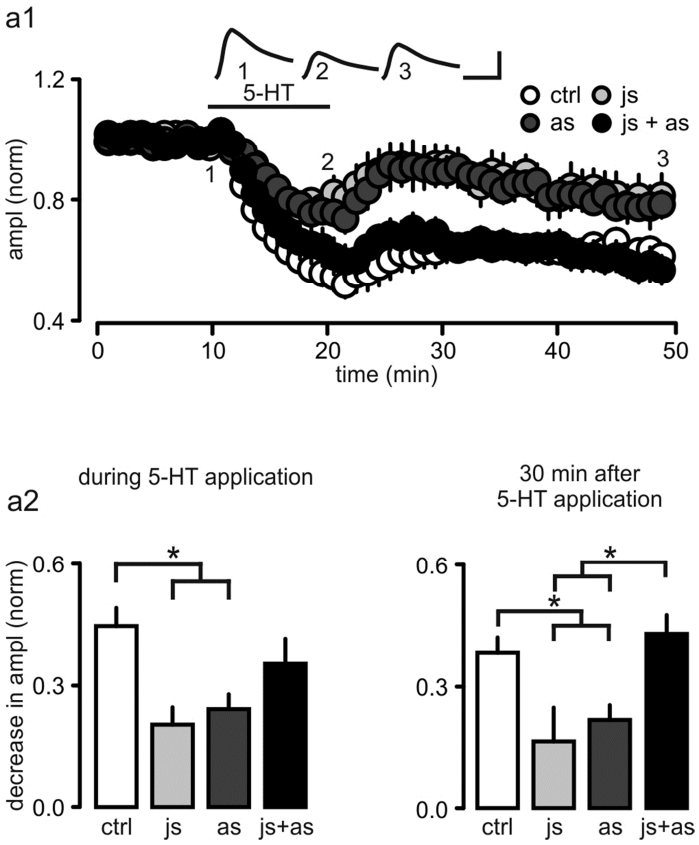

Fig. 13. 5-HT attenuates inhibitory transmission. (a1, a2, left panel) 5-HT (1 µM) attenuated eIPSC measured from granule cells from all groups. This effect was decreased in js- or as-rats. (a2, right panel) 30 min after the washout of 5-HT, eIPSC amplitudes were still reduced. This LTD was attenuated in js- and as-rats compared with ctrls and js+as-rats. Scale bars: 50 pA, 25 ms.

In controls, the 5-HT-induced decrease in the amplitude and LTD of eIPSCs could be attenuated by the application of the 5-HT1A receptor antagonist WAY-100635 (n=6) and reproduced by the application of the respective agonist 8-OH-DPAT (n=6), demonstrating that this effect is predominantly mediated by the 5-HT1A receptor subtype (Fig. 14), in accordance with previous findings (Schmitz *et al*, 1995).

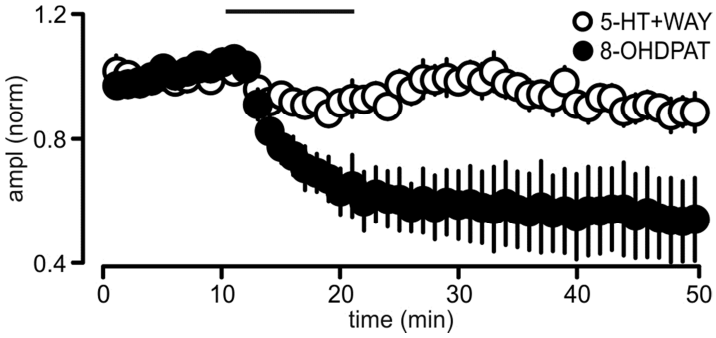

Fig. 14. The attenuation of eIPSCs is mediated by 1A receptors. The 5-HT1A agonist 8-OH-DPAT reproduced the effect of 5-HT in ctrls. 5-HT and the 5-HT1A antagonist WAY inhibited the attenuation in ctrls.

To determine whether the 5-HT-evoked short- and long-term reductions in eIPSCs are mediated by pre- or postsynaptic mechanisms, we analyzed the effect of 5-HT on the PPF of eIPSCs. Application of 5-HT resulted in a significant but transient increase in the PPF of 16±3.5% of baseline in controls ($F(2,30)=6.208$, $p=0.008$, one-way ANOVA for repeated

measures; Fig. 15, a1, a2). 30 minutes after the application of 5-HT, PPF values were not significantly different from baseline levels (103±2.8%; Fig. 15, a1, a2). These data indicate that in addition to a postsynaptic 5-HT-induced increase in total membrane conductance (Ginsborg, 1973) which causes a reduction in eIPSCs, 5-HT seems to modulate the presynaptic release probability as well (Zucker and Regehr, 2002).

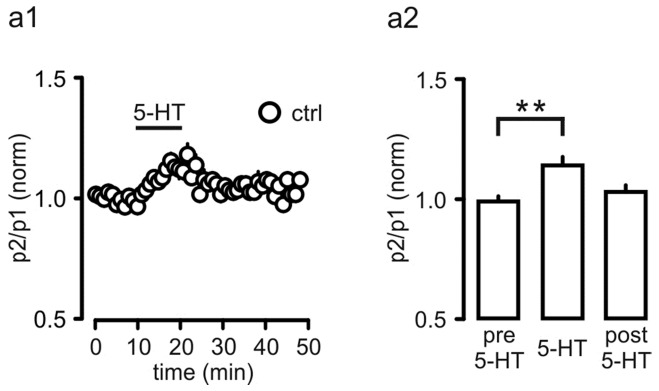

Fig. 15. The effect of 5-HT on the PPF of eIPSCs. In ctrls, application of 5-HT caused a significant increase in the PPF. After washout, responses returned to baseline values.

4.2.4 Single stress attenuates mIPSCs

To further characterize the effect of stress on the release probability of GABA, mIPSCs in dentate granule cells were studied. Previously, it has been shown that the stress hormone corticosterone, through activation of mineralocorticoid receptors, attenuates the frequency of mIPSCs (Maggio and Segal, 2009, 2012). In the present study, the frequency of mIPSC was reduced to about 60% in rats stressed once compared with controls (ctrl: n=23, 1.01±0.092 Hz, js: n=14, 0.64±0.057 Hz, as: n=17, 0.59±0.028 Hz, H(3)=15.81, p=0.0012, Kruskal-Wallis test; Fig. 16, a1, a2, c). In rats stressed at both ages, the frequency of mIPSCs was still reduced to 78% (n=14, 0.79±0.063 Hz), however, this was not statistically significant. The amplitude of mIPSCs in rats stressed once as juveniles or adults was reduced to 75% and 81%, respectively (from 64±2.7 pA to 48±1.7 pA and 52±2.6 pA, H(3)=16.56, p=0.0009, Kruskal-Wallis test; Fig. 16, b1, b2, c). In rats stressed at both ages, the amplitude of mIPSCs (62±4.7 pA) was not different from control values but from values of rats stressed as juveniles (Fig. 16, b1, b2, c).

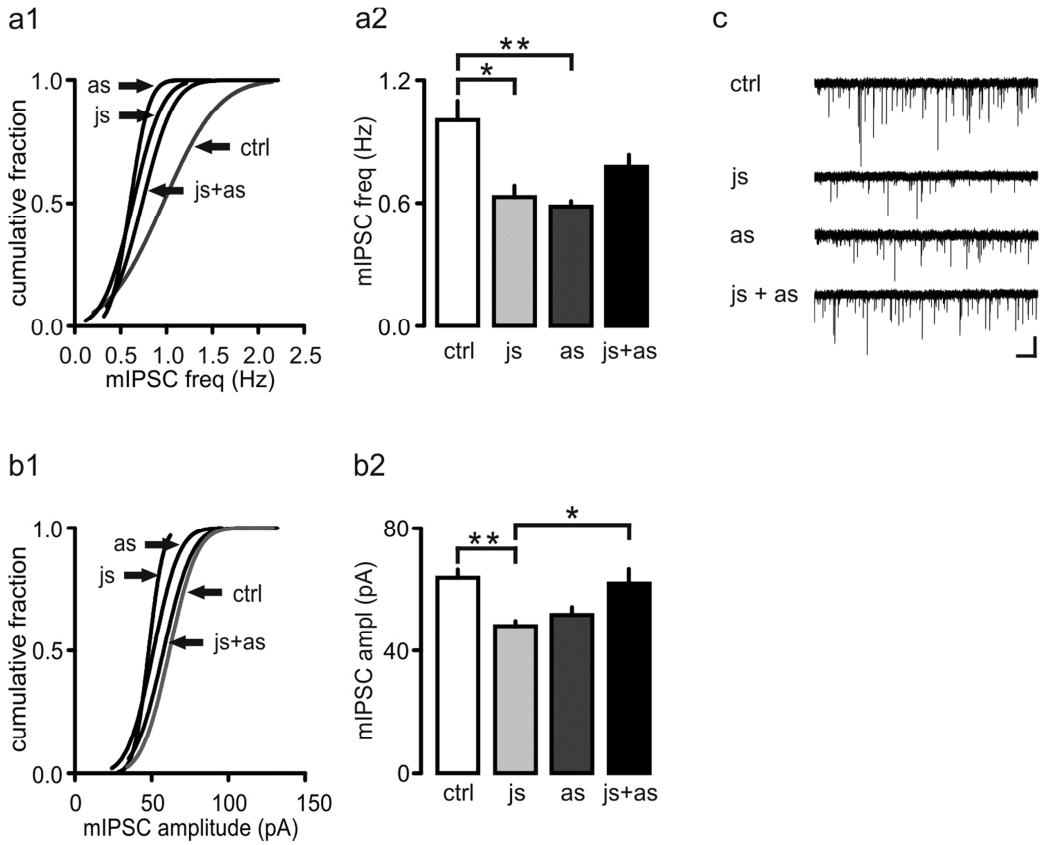

Fig. 16. Stress depresses spontaneous TTX-insensitive GABAergic transmission. (a1, a2, c) Compared with ctrls, js- and as-rats showed a reduction in the frequency of mIPSCs. (b1, b2) Compared with ctrls and js+as-rats, js-rats showed a reduction in the amplitude of mIPSCs. (c) Example traces of mIPSCs measured from ctrls, js-, as- and js+as-rats. Scale bars: 50 pA, 5 s.

To analyze the long-term effects of stress on mIPSCs, we performed a supplementary set of experiments in which rats were examined two weeks after the adulthood stress protocol was performed. In these rats, the frequency of mIPSCs was similar as in experiments performed one day after the adulthood stress paradigm (as+2: n=9, 0.53±0.038 Hz; js+as+2: n=6, 0.68±0.079 Fig. 17, a1). The amplitude of mIPSCs was not significantly different from controls (as+2: 60±4.2 pA; js+as+2: 58±6.1 pA; Fig. 17, a2).

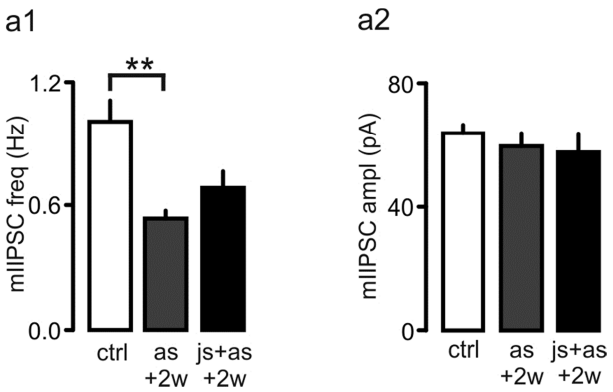

Fig. 17. Long-term effects of stress on mIPSCs. (a1) After 2 weeks (as+2w), as-rats still showed a reduction in the frequency of mIPSCs. (a2) After 2 weeks (as+2w, js+as+2w), mIPSC amplitudes were not different compared to ctrls.

The decrease in the frequency and amplitude of mIPSCs suggests that exposure to single-stress affects both the pre- and postsynaptic site. The effect on mIPSC frequency but not amplitude in rats sacrificed two weeks later indicates lasting stress-induced effects only at the presynaptic site.

4.2.5 Single stress attenuates 5-HT3-mediated effects on mIPSCs

To further analyze the effects of stress on the release probability of GABA, we studied the effect of 5-HT on mIPSCs in all groups. Earlier studies have shown a burst-like, 5-HT-induced Ca2+-dependent facilitation of GABA release (Kawa, 1994; Piguet and Galvan, 1994; Turner *et al*, 2004). In this study, in 89% (16 out of 18) of cells measured from controls, application of 5-HT resulted in a single burst-like discharge of mIPSCs with an increase in frequency (from 0.83±0.163 Hz to 3.04±0.522 Hz; Fig. 18, a) and amplitude (from 57±4.1 pA to 108±11.2 pA; Fig. 18, b). The induction of a burst-like discharge was subject to desensitization and could be induced only once per experiment. After single stress, fewer cells responded with a burst-like discharge (js: 19%, 3 out of 16, as: 43%, 21 out of 49, $\chi^2(3)=19.57$, p=0.0002; Fig. 18, c). After combined stress, the number of cells with a burst-like discharge was comparable to that in controls (js+as: 63%, 15 out of 24; Fig. 18, c).

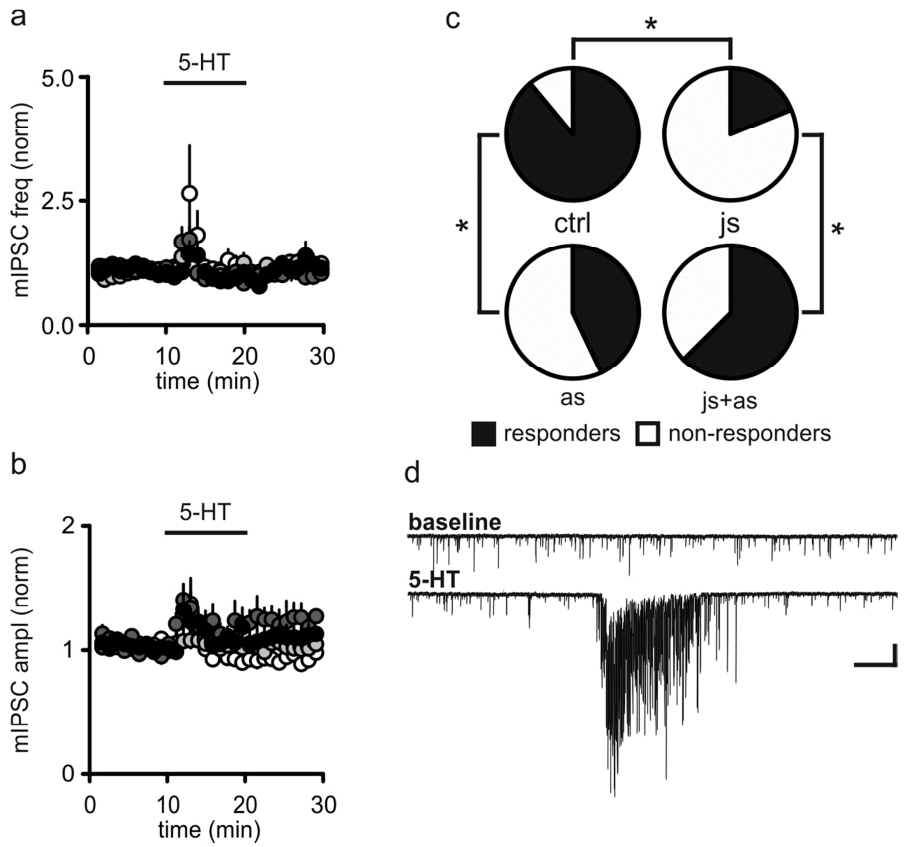

Fig. 18. 5-HT modulates GABA release probability. (a, b) During a 5HT-induced mIPSC burst-like discharge, the frequency and amplitude of mIPSCs increased robustly. (c) Circular chart of cells responding with a mIPSC burst-like discharge and non-responding cells. In js- and as-rats, fewer cells responded with burst-like discharges compared with ctrls. The number of cells responding with a burst-like discharge in js-rats was also reduced compared with js+as-rats. (d) Example traces of mIPSCs measured from a ctrl before and during application of 5-HT, showing a mIPSC burst-like discharge. Scale bars: 100 pA, 5 s.

In controls, bath-application of the 5-HT3 receptor agonist 1-phenylbiguanide closely mimicked the burst-like response of mIPSCs to 5-HT (4 out of 5 cells tested, Fig. 19, a1, a2). Application of the specific 5-HT3 receptor antagonist tropisetron blocked the burst-like response to 5-HT (0 out of 4 cells tested; Fig. 19, b1, b2).

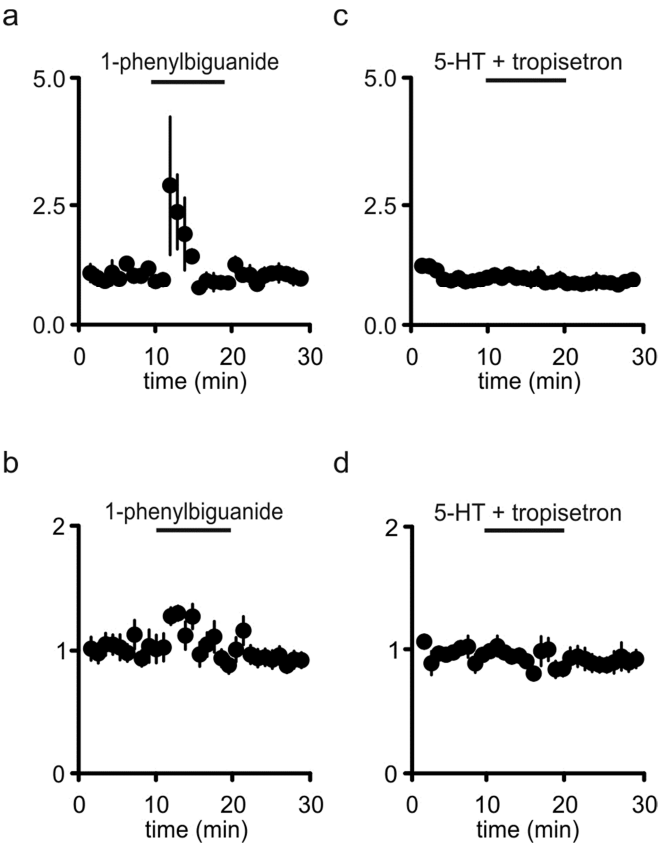

Fig. 19. mIPSC burst-like discharges are mediated by 5-HT3 receptors (a-d) In ctrls, the induction of a burst-like discharge by the 5-HT3 receptor agonist 1-Phenylbiguanide and its absence during co-application of 5-HT and the 5-HT3 receptor antagonist Tropisetron showed that this effect was 5-HT3 receptor-mediated.

Table 1 summerizes the main effects of 5-HT on intrinsic and synaptic properties. Rats that were exposed to combined stress lacked all synaptic but not intrinsic effects found after single stress.

Tab. 1. Summary of alterations found following single and combined stress. mIPSC frequency and number of cells with a mIPSC burst during 5-HT: as and js+as: 24 h or 14 d after stress. Black circle: change compared with ctrls; empty circle: no change compared with ctrls.

	js	as	js+as
R_{in}	●	●	●
R_{in} during 5-HT	●	●	●
eIPSCs after 5-HT	●	●	○
mIPSC frequency	●	●	○
no. of cells with mIPSC bursts during 5-HT	●	●	○

4.3 Effect of stress on interneuron expression profile in the dentate gyrus (in cooperation with Prof. Stork, Magdeburg)

It has been shown that stress affects the gene expression of GABAergic factors, for instance neuropeptide Y, somatostatin, the GABA-synthesizing enzymes GAD65 and GAD67, as well as CCK (Hadad-Ophir *et al*, 2014). Here, we assessed the effects of single and combined stress on 5-HT1A and 5-HT3A receptor and on basket cell expression profiles by means of real-time PCR experiments in laser microdissected samples of the granule cell layer of the ventral hippocampus. No change in the expression of the 5-HT1A receptor was detected between groups (n=8, F(3,28)=1.174, p=0.337, one-way ANOVA; Fig. 20, a). We found a significant interaction of both stressors on 5-HT3A expression (n=8, F(1,28)=6.695, p=0.0152, two-way ANOVA). In fact, only rats stressed once displayed a reduction while levels in rats stressed at both ages resembled that of controls (Fig. 20, b). A similar, even more pronounced pattern of expression changes was observed for two other markers of CCK+ basket cells, the CB1 receptor and CCK. The expression of CB1 was significantly reduced after the exposure to single stress but not after the exposure to combined stress (n=8, F(3,28)=3.361, p=0.033, one-way ANOVA; Fig. 20, c). In rats stressed at both ages, again, we found a significant interaction of both stress protocols on the expression of CB1 (F(1,28)=7.611, p=0.010, two-way ANOVA). A stress-induced effect on the expression of CCK (n=8 per group, F(3,28)=4.055, p=0.016, one-way ANOVA; Fig. 20, d) but not of parvalbumin (PV) (F(3,28)=1.677, p=0.195; Fig. 20, e), was also observed. The interaction between the two stressors on CCK expression was significant (F(1,28)=5.972, p=0.021, two-way ANOVA). The majority of CCK/CB1 receptor+ basket cells (80-90%, Morales et al, 2008)

also express the α7 nACh receptor, however, the expression of this receptor was not affected after stress (n=8 per group, F(3,28)=1.063, p=0.381 one-way ANOVA; Fig. 20, f).

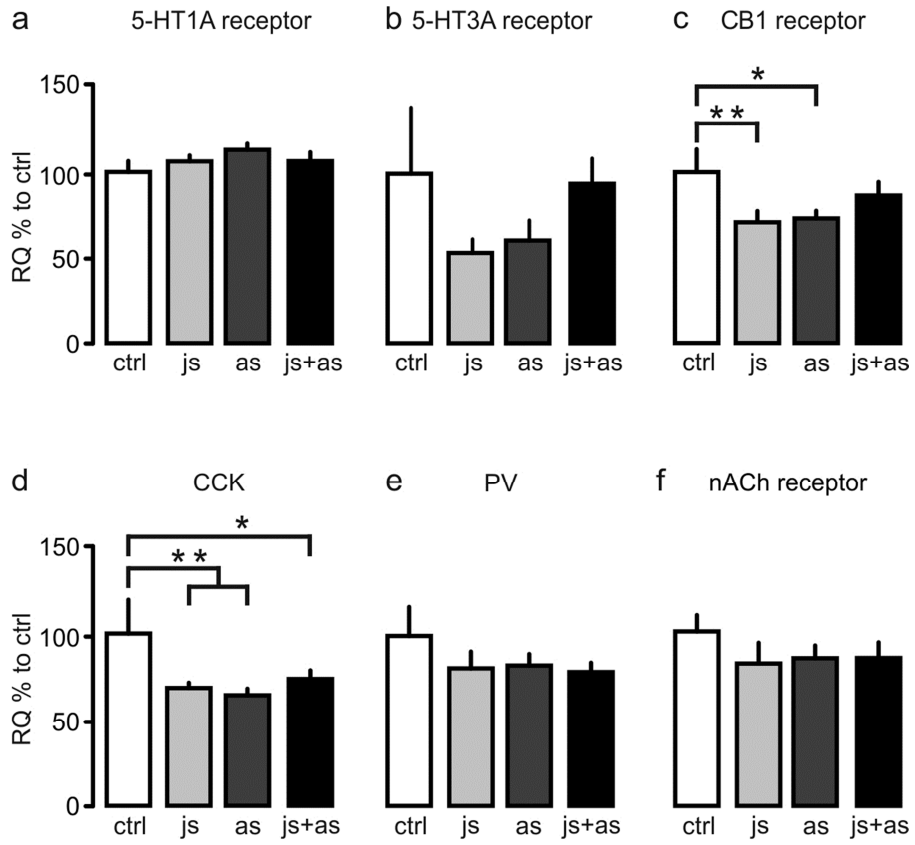

Fig. 20. Stress differentially affects mRNA expression levels of CCK+ basket cell markers. (a) The expression of the 5-HT1A receptor was not affected by any of the stress protocols. (b) The interaction of both stressors on 5-HT3A receptor expression was significant. (c) The expression of the CB1 receptor was reduced after js or as, but not after combined js+as. The interaction between both stressors on CB1 receptor expression was significant. (d) Compared with ctrls, the expression of CCK was reduced after js, as and the combination of both. The interaction between both stressors on CCK expression was significant. (e, f) None of the stress protocols altered the expression of PV and nACh receptors.

5 Discussion

During short or mild episodes of stress, decreased inhibition in the hippocampus favors a condition that facilitates learning and makes memory more accessible (Joëls and Baram, 2009). This stress response serves the animal to adapt and respond to environmental challenges but an exaggerated, maladaptive stress response may contribute to excessive fear, avoidance behavior and anxiety in mood disorders, for instance PTSD. Although in certain conditions stress-induced corticosteroid signaling in the hippocampus may be advantageous, excessive stress is likely to harm hippocampal function and imbalance hypothalamic–pituitary–adrenal (HPA) axis activity with inadequate behavioral responses. Evidence suggests that a high anxiety trait may develop into PTSD after a stressful event in adulthood (Cohen *et al*, 2007; Ford and Kidd, 1998). In humans and rodents, the hippocampus is particularly vulnerable to aversive stimuli, and in the dentate gyrus, the high density of stress hormone receptors, its connection with other stress-relevant brain regions and the HPA axis, as well as constant remodeling is thought to account for this (Bremner and Vermetten, 2001; Elzinga and Bremner, 2002; McEwen and Sapolsky, 1995; McEwen, 1999; Sapolsky, 1999; Spear, 2000; Tsoory *et al*, 2008). Previous studies have emphasized the unique role of the ventral hippocampus and GABAergic transmission under stressful conditions and their implication in mediating anxiety-like behavior together with other stress-related brain regions (Bannerman *et al*, 2004; Low *et al*, 2000; Rudolph *et al*, 1999; Segal *et al*, 2010; Zhang *et al*, 2014).

Animal models are useful tools in understanding biological mechanisms and can indicate new approaches in the symptomatic treatment of these disorders. This study suggests a crucial role for the 5-HTergic modulation CCK+ basket cells in the stress response and offers promising targets for future studies.

5.1 Anxiety-related and exploratory behavior

The present study shows that single juvenile stress did not affect exploratory behavior in the open field and avoidance learning in the two-way shuttle box experiment. In contrast, after the combined exposure to juvenile and adult stress, rats showed decreased exploratory behavior and avoided the aversive, i.e. exposed central area of the open field. Earlier studies found that the single and acute activation of the stress response can even improve hippocampal-dependent performances (Kampen *et al*, 2002) and confirm severe behavioral deficits after combined stress (Avital and Richter-Levin, 2005; Avital *et al*, 2006; Blumberg *et al*, 2009; Tsoory and Richter-Levin, 2006; Tsoory *et al*, 2007, 2008). To our current knowledge, this study is the first to prove a long-term effect of combined stress on behavior,

and confirms that the effects of juvenile stress are age-dependent, do not interfere with the locomotor system and that repeated stress in adulthood does not replicate the negative effects of juvenile stress (Altman and Sudarshan, 1975; Avital and Richter-Levin, 2005; Chen *et al*, 2006; Gramsbergen, 2001; Horovitz *et al*, 2012, 2014; Ohta *et al*, 1999; Tsoory *et al*, 2008). Taken together, these results propose the development of a resilient effect after the single exposure to juvenile stress and a long-term increase in anxiety-like behavior after further stress in adulthood. This underlines the impact of juvenile stress on behaviors related to emotional responses in adulthood (Chen *et al*, 2006; Tsoory and Richter-Levin, 2006) and supports the notion that juvenile stress predisposes to posttraumatic sequelae after stress in adulthood (Tsoory *et al*, 2007), in accordance with the 'two-hit hypothesis' of PTSD (Cohen *et al*, 2006). The specificity of these stress effects highlights the pivotal role of the history of stress.

5.2 The effects of stress on dentate gyrus granule cells

It has been shown for some of the stress protocols used in this study (immobilization, elevated platform, two-way shuttle box training) that they interfere with stress hormone levels, GABA-A receptor subunit expression and thus synaptic transmission in the dentate gyrus (Avital *et al*, 2006; Chen *et al*, 2006; Jacobson-Pick *et al*, 2008; Ohta *et al*, 1999; Umegaki *et al*, 2003; Vouimba *et al*, 2004; Yaniv *et al*, 2003). The serotonergic modulation of GABAergic transmission in the dentate gyrus plays an important role in both, coping behavior (Blumberg *et al*, 2009; File *et al*, 1996; Holmes *et al*, 2003; Joca *et al*, 2006; Oh *et al*, 2009) and/or in the mediation of the stress response measured in the open field (Holmes *et al*, 2003; Kalynchuk *et al*, 2006; Oh *et al*, 2009; Sass and Wörtwein, 2012; Stefański *et al*, 1992, 1993).

In control rats, the transient 5-HT-mediated decrease in input resistance resulted from the activation of somatodendritic 5-HT1A receptor-coupled GIRK channels that mediate an inward K^+ current (Baskys *et al*, 1989; Ghadimi *et al*, 1994; Piguet and Galvan, 1994), in agreement with previous findings (Gilling et al, 2013; Schmitz et al, 1995). In support of this, antagonizing GABA-A and GABA-B receptors did not prevent the decrease in input resistance, confirming that it was not caused by a 5-HT-induced increase in GABAergic tone. As predicted by Ohm's law, the transient increase in conductance was associated with a transient decrease in eIPSCs. 5-HT also produced a long-term depression of eIPSCs; therefore, the amplitudes of eIPSCs displayed a biphasic time course of depression. Due to the time-shift of the two effects, the 5-HT1A-mediated LTD of eIPSCs did not depend on the

increase in conductance. In addition, application of 5-HT reliably elicited 5-HT3 receptor-mediated burst-like discharges of mIPSCs (Kawa, 1994; Piguet and Galvan, 1994).
Single exposure to either juvenile or adulthood stress induced the following changes in granule cell inhibition and 5-HTergic modulation:

1. a reduction in the 5-HT1A-induced decrease in input resistance. The smaller increase in total membrane conductance was consistent with an attenuated reduction of eIPSCs amplitudes.
2. a reduction in the 5-HT1A-mediated LTD of eIPSCs.
3. a long-term reduction in mIPSC frequency.
4. a long-term reduction in the number of cells with a 5-HT3-mediated burst-like discharge of mIPSCs.

Interestingly, after combined juvenile and adulthood stress, rats still displayed a decrease in input resistance and its 5-HT-mediated decrease but lacked the effects seen at the synaptic level in rats stressed once, i.e. eIPSC amplitudes were not different from those in control animals and the number of cells that displayed 5-HT3 receptor-mediated burst-like discharges of mIPSCs was not reduced. In summary, the single exposure to juvenile stress induced long-lasting alterations in the 5-HT receptor-mediated modulation of granule cell inhibition without behavioral change compared to controls. Severe behavioral changes occurred after the exposure to a second stressful event in adulthood despite the lack of the changes in physiological parameters seen after single stress. Table 1 (p. 28) summarizes the neuronal alterations found after the single and combined exposure to stress.
Transcriptional regulation of the 5-HT1A receptor did not occur after any stressor but RNA levels do not necessarily correlate with protein levels (Gygi *et al*, 1999) and might therefore nevertheless be affected. Lower levels of functional 5-HT1A receptors or other proteins involved in related signaling cascades might also be caused by stress-induced translational insufficiency but these issues were not addressed. In previous studies (Holmes *et al*, 1995; Karten *et al*, 1999; Van Riel *et al*, 2003), stress and corticosterone also failed to alter transcriptional regulation of the 5-HT1A receptor in the dentate gyrus and CA1 but, yet, decreased 5-HT1A receptor function. It was concluded that a corticosterone-induced decrease in mineralocorticoid and glucocorticoid receptor mRNA affects 5-HT1A receptor function through alterations in genes transcribing for proteins mediating G-protein-coupled signaling or posttranslational modifications of such proteins (Karten *et al*, 1999; Van Riel *et al*, 2003). Facilitation of 5-HT1A-mediated effects via processes that involve protein synthesis are mediated by high levels of the hormone through glucocorticoid receptors, and

moderate levels of corticosterone, through mineralocorticoid receptors, decrease 5-HT1A-mediated effects as well as GABAergic transmission by alterations in K^+ conductance (Beato and Sánchez-Pacheco, 1996; Hesen and Joëls, 1996; Holmes *et al*, 1995; Joëls and Van Riel, 2004; Joëls, 2001; Karten *et al*, 2001; Van Riel *et al*, 2003). Activation of presynaptic G-protein-coupled mineralocorticoid receptors decreases the frequency of mIPSCs in the ventral hippocampus by inhibiting Ca2+-dependent GABA release (Maggio and Segal, 2009). Activation of postsynaptic mineralocorticoid receptors decreases the amplitude of mIPSCs (Olijslagers *et al*, 2008), and the additional activation of glucocorticoid receptors reverses these effects (Joëls, 2001). Finally, corticosterone and stress were further found to alter transmission by affecting transmitter systems, cell morphology, metabolites and 5-HT1A receptor binding in the dentate gyrus (Fernandes *et al*, 1997; Joëls and De Kloet, 1994; Raghupathi and McGonigle, 1997).

5.3 The effects of stress on interneuron expression profile

In the hippocampus, 5-HT3 receptors are selectively expressed by CCK+ interneurons on presynaptic nerve terminals (Morales and Bäckman, 2002; Morales and Bloom, 1997; Ropert and Guy, 1991), and activation of 5-HT3 receptors induced a burst-like discharge of mIPSCs in granule cells (Kawa, 1994; Piguet and Galvan, 1994). After single but not combined stress, the number of cells with a burst-like discharge was reduced. The interaction of the juvenile and adulthood stress protocols on 5-HT3 receptor expression and the number of granule cells showing 5-HT3 receptor-mediated burst-like discharges of mIPSCs suggest that a reduction in 5-HT3 receptor function leads to a reduction in burst-like GABA release from these cells. In this context, it is worth to note that 5-HT3 receptor antagonists exert anxiolytic effects (Jones *et al*, 1988) and enhance memory retention of hippocampus-dependent tasks (Stäubli and Xu, 1995). After all stress protocols, the mRNA levels of CCK but not of PV and alpha-7 nACh receptors were reduced, and only after single stress but not combined stress, the mRNA levels of CB1 were reduced in parallel with a change in presynaptic release probability. Therefore, steady-state regulation of mRNA is more likely to have occurred than apoptosis or reduced proliferation, although, stress was reported to affect neurogenesis and cell turnover (Heine *et al*, 2004; Karten *et al*, 2005; Lucassen *et al*, 2006; Schoenfeld and Gould, 2012). Nearly all hippocampal CCK+ basket cells express CB1 receptors (97%), and only a small portion of CB1 receptor+ cells is CCK-negative (14%; Katona 1999). Furthermore, the majority of CB1 receptor+ cells also express 5-HT3A receptors (80%; Morales and Bäckman, 2002). Accordingly, classified along morphological, chemical and functional aspects, CCK+ basket cells expressing 5-HT3A, alpha-7 nACh and CB1 receptors

are considered as a separate class of interneurons (Katona, 1999; Morales and Bäckman, 2002; Morales and Bloom, 1997). CB1, via DSI, interacts with CCK to inhibit action potential-dependent GABA release from CCK+ basket cells (Lee and Soltesz, 2011). In the present study, mIPSCs were decreased in animals stressed once, indicating that action potential-independent GABA release is not disinhibited by a decrease in CB1 receptor function. Taken together, this suggests that the selective reduction in 5-HT3A receptor mRNA levels after single stress is part of an adaptive response that involves the regulation of steady-state mRNA expression levels of modulators of CCK+ basket cell transmission. Indeed, CCK+ basket cells are uniquely equipped with receptors that integrate subcortical and cortical information which makes these cells ideal mediators of emotion (Freund, 2003) and a key regulator of neural activity and network oscillations (Klausberger *et al*, 2005). Importantly, the adaptions after single stress were attenuated after the second exposure to stress in adulthood in parallel with the recovery of spontaneous GABA release, in accordance with the 'two-hit hypothesis' of PTSD (Cohen *et al*, 2006). Whether the observed changes in mRNA levels are causally involved in the adaptive physiological response of CCK+ basket cells or merely a reflection of their activity state remains to be determined.

6 Summary

The present study shows that the changes in the 5-HT-dependent modulation of GABAergic inhibition of ventral dentate granule cells depend on the history of stress. More specifically, the decrease in 5-HT1A and 5-HT3 receptor-mediated modulation of inhibition after single stress recovered after a second stressful experience in adulthood along with severe behavioral alterations. The results show that the recovery of the 5-HTergic modulation of inhibition and the increase of anxiety-related behavior after combined stress are inversely related and suggest a key role of CCK+ basket cells for which an important role in the mediation of emotion has been proposed earlier (Freund, 2003). The findings indicate a compensatory mechanism through alterations in gene expression and GABA release modulation after single-stress that is attenuated upon combined juvenile and adult stress, in accordance with the 'two-hit hypothesis' of PTSD (Cohen *et al*, 2006). Future studies will have to determine if the changes in 5-HT-dependent modulation of inhibition after juvenile stress support the behavioral resilience in these animals.

Zusammenfassung

Die vorliegende Arbeit zeigt, dass Änderungen in der serotonergen Modulation der GABAergen Hemmung von Körnerzellen des ventralen Gyrus dentatus von der Anzahl und dem Zeitpunkt von Stressoren in der Vorgeschichte abhängig sind: Es zeigte sich, dass die nach singulärem Stress verringerte 5-HT1A und 5-HT3 Rezeptor-vermittelte Modulation der Inhibition sich nach wiederkehrendem Stress im Erwachsenenalter regenerierte. Dieser Befund ging mit deutlichen Verhaltensänderungen einher. Die Ergebnisse zeigen somit ein inverses Verhältnis zwischen der Wiederherstellung der serotonergen Modulation der Inhibition und dem gesteigerten Angstverhalten nach kombiniertem Stress. CCK+ Korbzellen, welchen eine wesentliche Rolle bei der Vermittlung von emotionalen Zuständen zugesprochen worden ist (Freund, 2003), scheinen hier eine Schlüsselfunktion einzunehmen. Die Befunde lassen auf einen kompensatorischen Mechanismus mittels veränderter Genexpression und Modulation der GABA Freisetzung nach singulärem Stress schließen, welcher nach der Kombination von juvenilem und adultem Stress unterdrückt ist. Diese Ergebnisse sind in Übereinstimmung mit der 'two-hit hypothesis' der PTSD (Cohen *et al*, 2006). Die Frage, ob diese Veränderungen der serotonergen Modulation der Inhibition nach juvenilem Stress die verhaltensrelevante Belastbarkeit in diesen Tieren fördern, bleibt in zukünftigen Studien zu klären.

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