Traces of a Trauma

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Pharmacological Interventions of PTSD

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Diese Dissertation wurde von Dr. Carsten T. Wotjak betreut.

Ort, Datum **Kathrin Henes**

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Introduction

preparation – fun – friends – alcohol – music – crowd – electronic dance – festival – tunnel – heat – narrows – euphoria – bottleneck – stumble – orientation – fall down – breathlessness – screams – way out – fear – injured people – escape – dead people – panic – crowd rush – 500 injured people (physical) – 12 people dead – one year later – ? injured people (psychologically)

1.1 Psychiatric disorders - anxiety disorders

To this day, triggers for psychiatric disorders are often unknown and the underlying physiological changes are poorly understood. Probably because of this fact, the bigger part of the general public (Western Europe) does not consider psychiatric disorders as serious diseases, and indeed, it is hard to comprehend why people are scared of facing spiders. However, anxiety disorders are widely spread with a prevalence of Europe is about 50% (Olesen, 2003; Sobocki et al., 2005), and almost everybody, directly or indirectly, is confronted with these affective diseases.

As recently as one year ago (2010), a crowd rush during the Love Parade in Duisburg, Germany led to injuries and death of people. Sadly enough, the tragedy was not over after a few days, it is still lasting. To this day, many of the people involved have to deal with nightmares, problems in concentrating, lack of motivation, emotional blunting and social withdrawal, or even the incapability of handling their everyday life.

They came down with a post-traumatic stress disorder (PTSD).

1.2 Post-traumatic stress disorder (PTSD)

PTSD was first defined by A. Kardiner in 1941 and called *physioneurosis* in which patients developed an enduring vigilance for and sensitivity to environmental threat. "*These patients cannot stand being slapped on the back abruptly; they cannot tolerate a misstep or stumble. From a physiological point of view, a state of readiness for fright reactions"* (p 95).

PTSD, as characterized in the Diagnostic and Statistical Manual for Psychiatric Disorders[1] (DSM IV), implies a traumatic (life-threatening) event (criterion A), which after an incubation time leads to three main clusters of symptoms. First, maintained trauma related memories (criterion B) including distressing recollections and dreams about the event, flashbacks, psychological distress with and physiological reactions to reminders of the event – patients simply can not stop thinking about the event. Second, emotional numbing and avoidance of trauma-related stimuli (criterion C) including the effort to avoid thoughts, feelings, or conversations as well as activities, places, or people associated with the event and diminished interest in significant activities. And third, hyperarousal (criterion D) including difficulties in falling or staying asleep as well as in concentrating, hypervigilance, and exaggerated startle response. For diagnose of PTSD, these three clusters of symptoms have to persist over one month (criterion E) and disturb functioning of everyday life performance. The life-time prevalence of experiencing a traumatic event is as high as 56%, but only 15% of confronted people develop PTSD over time and one third of these still show symptoms ten years after the traumatic event (Kessler et al., 1995). These data and the mentioned symptoms enforce the necessity for a specific therapy of PTSD which has not been identified yet.

Current therapies mostly target single symptoms, come along with side effects, and frequently lead to a relapse after the end of the treatment. Therefore, they are not satisfying options. To enhance therapeutic efficiency and the

[1] DSM IV: the current classification of anxiety disorders includes generalized anxiety (GAD), phobias, and post-traumatic stress disorders (PTSD), as well as panic and obsessive compulsive disorders (Washington, DC, American Psychiatric Association, 2000)

compliance of PTSD patients, a better understanding of the disease is indispensable. According to McKinney (McKinney, 1984), animal models are '*experimental preparations developed in one species for the purpose of studying phenomena occurring in another species. In the case of animal models in human psychopathology, one seeks to develop syndromes in animals which resemble those of humans in certain ways in order to study selected aspects of human psychopathology*.' A promising animal model for psychiatric disorders has to include three validation criteria: First, the face validity which includes the parallelism in behavioral responses of the animal to the symptoms obtained in humans. Second, the construct validity which requires similar neurological changes in the animal model as compared to the human situation. And third, the predictive validity which supposes a therapeutic effect of clinically effective drugs also in the animal model.

1.3 Animal model of PTSD

For most traumata used for the induction of PTSD-like symptoms in animal models, including physical restraint, underwater-holding, predator confrontation, social confrontations, and learned-helplessness (Table 1), the intensity of the trauma can only be increased by prolongation or repetition (onset of habituation) (Adamec and Shallow, 1993; Richter-Levin, 1998; Cohen et al., 2000). In contrast, using a single electric foot shock as the traumatic event, trauma intensity can be modified by increasing the electrical current without prolongation or repetition of the traumatic event.

Furthermore, abnormal psychophysiological reactions in PTSD occur on two different levels. First, in response to specific reminders of a trauma and second, in response to unspecific stimuli, such as loud noises, indicating a loss of stimuli discrimination (van der Kolk, 2001).

Table 1: Overview over traumata used to induce PTSD-like symptoms in rodents

Stressor	References	Time line
physical restraint	Belda et al., 2008	d0: 2 h immobilization; d1+3+7 EPM (rats)
inescapable electric shock	Siegmund and Wotjak, 2006 Pynoos et al., 1996 Servatius et al., 1995	d0: 1.5 mA/ 2 s foot shock; d28: contextual fear (mice) d0: 2 mA/ 2 s foot shock ; d1/ 21/ 42 locomotion + EPM (mice) d0: 40* 2 mA/ 200 ms tail shock; d4+7+10 ASR (rats)
social confrontations	Golden et al., 2011 Trainor et al., 2011	d0-9: 5-10 min soc. defeat stress; d10: social interaction (mice) d0: <7 min social defeat stress; 24 h/ 4 weeks social interaction (mice)
underwater-holding	Wang et al., 2000 Cohen et al., 2007 Richter-Levin, 1998	d0: 30 s underwater; 20 min water maze (rats) d0: 30 s underwater (rats) d0: 30 s underwater; 1 h: EPM + water maze (rats)
exposure to a predator	Adamec and Shallow, 1993 Adamec et al., 1998 Cohen et al., 2003	d0: 5 min cat exposure; d1, 2, 7, 14, or 21: plus maze + hole board (rats) d0: 5 min cat exposure; d7: hole board + EPM (rats) d0: 5 min cat exposure; d7 EPM (rats)
learned-helplessness	Hammak et al., 2011 Mallei et al., 2011	d0: inescapable tail shocks (rats; review) d0: 200 tail shocks in 40 min (0.8 mA; 5-15 s;); d1: LH behavior

Therefore, associative fear memories and non-associative fear sensitization seem to play an important role in the development and maintenance of PTSD (Foa et al., 1992; Charney et al., 1993; Sorg and Kalivas, 1993), but only a few animal models reflect that criterion (Siegmund and Wotjak, 2006, 2007).

Associative and non-associative memories

The basis of associative learning is the classical conditioning paradigm, first described by the experiments performed by Ivan Pavlov around 1900 (Gantt, 1927). Pavlov signalled his dog the occurrence of food (unconditioned stimulus, US) by ringing a bell (neutral stimulus, NS). After learning this link, the dog started to salivate in appearance of the acoustic cue of the bell only. So, the neutral stimulus became a conditioned stimulus (CS).

In relation to PTSD, traumatic memories and flashbacks that occur in response to a trauma cue (directly related to the trauma) are based on such associative memories (Costanzi et al., 2011). Before the traumatic event, these stimuli (the bell) are neutral – without any (negative) association – but in combination with the trauma, they become conditioned stimuli and therefore are able to evoke the reaction to the trauma by themselves.

On the other hand, habituation and sensitization are two examples for non-associative memories. Habituation is a progressive and implicit diminution of a behavioral response upon repeated stimulus presentation. After the first contact with a stimulus, the animal reflects subconsciously if the response was adequate (reward) or not (harmful or marginal). If the response was not adequate, it is reduced in a stepwise manner. Sensitization, in contrast, is a progressive amplification of a behavioral response following repeated administrations of a stimulus (Bell et al., 1995). If the response to the first stimulus seems to be adequate (meaningful), the animal will enhance the consequent response. However, sensitization also reflects an increased response to a stimulus due to a changed general situation. For example in humans the startle response increases in the darkness, whereas rats, which are crepuscular, show higher startle responses in light (Steiner et al., 2011).

1.3.1 Face validity

Siegmund and Wotjak (2007) established an animal model of PTSD, where mice receive an inescapable electric foot shock as the traumatic event and are tested for conditioned and sensitized fear, light/dark test, social interaction test, forced swim test, and modified holeboard test. Due to these results, mice develop exaggerated fear responses, generalization of fear, and increased depression-like behavior after a period of at least 28 days of fear incubation. Furthermore, mice show avoidance behavior in the conditioned odor avoidance test (Pamplona et al., 2011), as well as decreased generalized and contextual fear after extinction training (Golub et al., 2009).

Most of these behavioral changes occurring in PTSD patients as well as in shock-traumatized mice can be detected by simple observation (analysis of the behavior), but rely on a physiological background, which is essential to be investigated.

1.3.2 Construct validity

Investigation of anxiety disorders assumes that animals not only show “normal” anxiety but psychopathological (elevated) anxiety and that animal models have construct validity (the biological background of psychological disorders), a prerequisite for studying the neurobiology and therapeutic mechanisms of pathological anxiety (Sartori et al., 2011).

Changes in the neuronal activity of the brain can be assigned to either phasic or tonic activity changes. Phasic activity changes include modifications in immediate early genes (IEG) expression, like for example c-Fos expression (Plendl and Wotjak, 2010; Lim et al., 2011) or accumulation of 2-Deoxy-D-glucose (2-DG[2]) (McCasland, 1997). These phasic changes can be employed best for the monitoring of activity changes in animal models of panic attacks or specific phobias, which are short-lasting. In contrast, tonic activity is hypothesized to reflect psychopathology in animal models of depression or

[2] 2-DG is a glucose derivative which has the 2-hydroxyl group replaced by hydrogen. This modificaiton prevents further glycolysis. 2-DG is trapped into the cells and therefore a good marker for tissue glucose use, if radioactivity labelled with 14C.

PTSD. Such changes are visualized e.g. by changes in cytochrome c oxidase activity (CO[3]), the magnetic resonance imaging (MRI) (Tavanti et al., 2011), the manganese enhanced MRI (MEMRI[4]), in kinases (Dahlhoff et al., 2010), in GluR1[5] (Lin et al., 2011, Thoeringer et al., submitted), decreased serotonin (5-HT) levels in the central nervous system (Valzelli, 1982), or even the hippocampal volume (Golub et al., 2011).

For mapping regional functional activity in the brain, cytochrome c oxidase (CO) can be used as an endogenous marker of local tissue metabolic capacity (Wong-Riley, 1989; Gonzalez-Lima and Garrosa, 1991).

Cytochrome c oxidase (CO)

CO activity is directly linked to mitochondria which are organelles found in eukaryotic cells. Mitochondria are just called the *cellular power plants* because of the generation of adenosine triphosphate (ATP), the main energy source in cells (Chance, 1961). Furthermore, mitochondria are also responsible for cell differentiation, cell growth, cycle, and death, and cell signaling. The functionality of the ATP synthase (producing ATP) is based on an electrochemical gradient, consisting of protons, which is generated by the electron transport chain (Wikstroem and Saari, 1977). This chain, consisting of 4 respiratory chain complexes (complex I-IV), transports protons (H^+) in a series of redox (reduction

[3] CO ≠ COX: cyclooxygenase (COX) is an enzyme which is responsible for formation of prostanoids. Pharmacological inhibition of COX is used in inflammation and pain therapy (COX inhibitors).

CO ≠ CO_2: carbon dioxide (CO_2) is a physiological transmitter involved in the autoregulation of blood supply and the regulation of blood pH (, if converting into HCO_3^- by carbonic anhydrase in the red blood cells).

CO ≠ CC: cytochrome c (CC) is a small heme protein which is an essential component of the electronic transport chain, where it carries one electron. CC is oxidized by CO.

[4] MEMRI: Manganese ions are paramagnetic and therefore shorten the T1-times of the surrounding tissue. Based on the fact, that neuronal activity increases the Ca^{2+} influx into the cell and that Ca^{2+} is quite similar to Mn^{2+} (manganese), neuronal activity also increases Mn^{2+} influx into the cell and therefore images areas with high neuronal activity. As a result, Mn^{2+} enhances MRI.

[5] GluR1 is a subunit of Ca^{2+}-permeable AMPARs (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors).

and oxidation) reactions across the membrane to the intermembrane space (Figure 1) (Alberts et al., 1995).

The enzyme cytochrome c oxidase (CO) or complex IV, the last mitochondrial respiratory chain complex, is a large transmembrane protein complex including two atoms of each copper and iron, consuming over 90% of the oxygen (Mahad et al., 2009). It receives an electron from each of 4 cytochrome c (CC) molecules which are reduced from CC(Fe^{2+}) to CC(Fe^{3+}) and transfers the electrons to one oxygen molecule (O_2), thereby oxidizing molecular oxygen to two molecules of water (H_2O) (Figure 2).

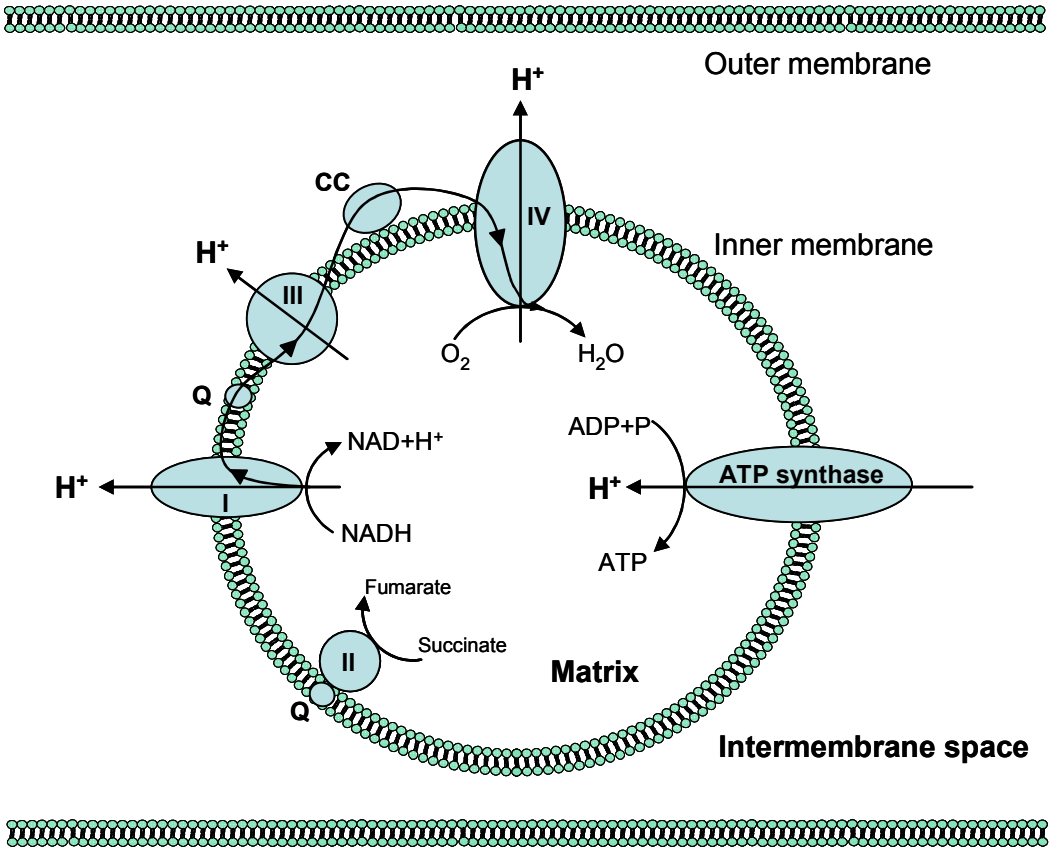

Figure 1: Schematic overview over the electron transport chain in the mitochondrion including the 4 respiratory chain complexes and the ATP synthase. I-IV respiratory chain complex I-IV, CC cytochrome c, H^+ proton, NAD^+ oxidized nicotinamide adenine dinucleotide, NAHD reduced nicotinamide adenine dinucleotide, Q quinone pool

$$4\ CC_{ox}\ (Fe^{2+}) + O_2 + 8\ H^+_{in} \rightarrow 4\ CC_{red}\ (Fe^{3+}) + 2\ H_2O + 4\ H^+_{out}$$

Figure 2: Chemical equation of CO. CC_{ox} (Fe^{2+}): oxidized cytochrome c including an ion of ferrum with the oxidation state of 2; CC_{red} (Fe^{3+}): reduced cytochrome c including an ion of ferrum with the oxidation state of 3

Furthermore, CO binds four protons (H^+) from the inner aqueous phase to create water, and in addition translocates four protons (H^+) across the membrane, thus establishing a transmembrane difference of proton electrochemical potential that the ATP (adenosine-tri-phosphat) synthase[6] subsequently uses to synthesize ATP[7] (Wikstroem, 1981; Alberts et al., 1995).

In 1979, Wong-Riley demonstrated *the changes in the visual system of monocularly sutured cats with cytochrome oxidase histochemistry* (Wong-Riley, 1979). Wong-Riley and Gonzalez-Limas groups evaluated the cytochrome c staining for other applications, e.g. in a genetic model of helpless behavior or the activity in the auditory system (Hevner and Wong-Riley, 1989; Wong-Riley, 1989; Hevner and Wong-Riley, 1990; Gonzalez-Lima and Garrosa, 1991; Hevner et al., 1993; Gonzalez-Lima and Cada, 1994; Poremba and Jones, 1998; Shumake et al., 2002).

In summary, CO is a marker for the sustained neuronal activity and might help to detect brain regions which are altered as a consequence of the traumatic event. Furthermore, CO could provide a physiological correlation to the obvious behavioral changes and therefore as a marker for treatment success.

1.3.3 Predictive validity

The treatment of PTSD has several specific goals: to reduce the severity of symptoms, to prevent and/or treat comorbid disorders, to decrease functional impairment, to modify pathogenic fear schemas, to prevent relapse, to build resilience, and to improve the quality of life (Ursano et al., 2007).

Keeping in mind the timeline of PTSD, i.e. the incubation time after the trauma and the maintenance period in which PTSD symptoms are fully developed, the therapy of PTSD normally starts months or even years after the trauma (therapeutic therapy). However, there are several preclinical studies which demonstrate that pharmacological intervention in the early aftermath of a

[6] ATP synthase transfers energies from H^+ (following the electrochemical gradient via the inner membrane – energy are released) to phosphorylate ADP (adenosine-di-phosphat) to ATP (energy are needed).

[7] ATP can provide energy within cells for metabolism. ATP → ADP + P^+.

trauma (before PTSD symptoms developed; preventive therapy) have a higher efficiency as at later time points. For example, Cohen and colleagues showed that rats treated with high-dose corticosterone immediately after the trauma reduced behavioral disruption 30 days later (Cohen et al., 2008), and Thoeringer and colleagues showed in a mouse model of PTSD that the treatment with a CRHR1[8] antagonist in the first week after the trauma attenuated the consolidation of remote fear memories (Thoeringer et al., submitted). Furthermore, the efficiency of exposure therapy crucially depends on the timing of the intervention; earlier sessions appeared to be more effective than sessions performed at a later time point (Campfield and Hills, 2001). In addition, propranolol, a beta-blocker, administered immediately after the traumatic event, reduced or perhaps even prevented the development of PTSD in humans (Vaiva, 2003; Henry and Fishman, 2007; McGhee et al., 2009).

However, in humans, the therapy of PTSD is, not only because of the high relapse rate after the end of treatment, still unsatisfying.

Nevertheless, a huge line-up of different active agents exists (Box 1) and are combined in a meta-analysis of social anxiety disorders (de Menezes et al., 2011). First-line pharmacological treatment of PTSD are selective serotonin reuptake inhibitors (SSRI) including sertaline, paroxetine[9], fluoxetine[10], fluovoxamine, citalopram, and escitalopram (Asnis et al., 2004; Schoenfeld et al., 2004; Ravindran and Stein, 2009). Furthermore, selective norepinephrine (NE) reuptake inhibitors (SNRIs, including duloxetine, venlafaxine, desvenlafaxine, and milnacipran), tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOIs), other antidepressants (burpropion SR - selective NE and dopamine reuptake inhibitor), among anti-adrenergic agents, anticonvulsants, benzodiazepines, and others are second- or third-line treatments of PTSD (Ravindran and Stein, 2009).

[8] CRHR1: corticotropin-releasing hormone (CRH) receptor 1

[9] sertaline and paroxetine: indication for PTSD in Germany (FDA approval)

[10] fluoxetine: indication for PTSD in U.S.A., in Germany only for depression (not PTSD)

As fluoxetine (a SSRI) is the first-line treatment in PTSD patients, to ensure predictive validity, fluoxetine was used in the present study.

Box 1: Overview of pharmacological agents clinically used for PTSD

SSRIs
- fluoxetine
- sertaline
- paroxetine
- fluovoxamine
- citalopram
- escitalopam

SNRIs
- ducloxetine
- venlafaxine
- desvenlafaxine
- milnacipran

MAOIs
- phenelzine
- brofaromine

Tricyclic antidepressants
- desipramine
- amitriptyline
- imipramine

Other antidepressants

Anti-adrenergic agents

Anticonvulsants

Benzodiazepines

Fluoxetine (SSRIs)

Based on the good compliance of fluoxetine treatment, it is the mostly used anti-depressive drug worldwide. Fluoxetine is also indicated of generalized fear and panic disorders. Furthermore, several studies showed an improvement of PTSD symptoms under fluoxetine treatment (van der Kolk et al., 1994; Connor et al., 1999; Martenyi et al., 2002).

Nevertheless, patients are prone for relapse of symptoms upon discontinuation of treatment. This relapse urges for a refinement of pharmacologic interventions and the identification of markers for treatment success.

1.4 Aims

The aim of this work was the identification of new approaches for the PTSD therapy and/or the sophistication of already existing therapies via mimicking the symptomatology observed in humans using a mouse model of PTSD. Additionally, a potential marker of treatment success was to be revealed.

Our working hypotheses were as follows:

(1) A traumatic event (a single electric foot shock) changes the tonic activity (cytochrome c oxidase) in the brain one month after the trauma.

(2) Chronic treatment with fluoxetine starting either right after the trauma (preventive treatment) or 28 days later (therapeutic treatment) prevents or reverses the PTSD-like symptoms.

(3) Chronic treatment with fluoxetine starting either right after the trauma (preventive treatment) or 28 days later (therapeutic treatment) reverses the changes in CO activity.

(4) The discontinuation of treatment with fluoxetine after preventive or therapeutic treatment, leads to a relapse of PTSD-like symptoms.

1.5 Experimental overview

The experimental schedules of all experiments are summarized in Figure 3.

Experiment 1: Searching for construct validity in long-lasting changes in cellular activity in the brain, CO activity was analysed in shocked compared to non-shocked mice. Mice were shocked or non-shocked (control) at day 0 (d0). They remained in their home cages until day 28 (d28) post shock, when they were tested for hyperarousal (d28), generalized and contextual fear (d29-30), and avoidance (d32-33). After an additional week, brains were collected and CO staining was performed.

Experiment 2: To confirm the CO data of experiment 1 and to test for the influence of incubation time after the trauma, mice were shocked or non-shocked (control) at d0 and tested for hyperarousal at d2 or d28. After an additional week, brains were collected and CO staining was performed.

Experiment 3: To investigate whether the increase in CO (Exp. 1 and 2) activity involves changes in AMPAR or $GABA_AR$ neurotransmission, in vitro patch-clamp recordings of AMPAR-mEPSPs and $GABA_AR$-mIPSCs in the dentate gyrus (DG) and the cornus ammonis 1 (CA1) were performed.

Experiment 4: To confirm the changes in AMPAR neurotransmission in the DG (Exp. 3), the physiological relevance of hippocampal GluR1 (subunit of AMPAR) for remote contextual fear memories was investigated. For equal levels of sensitization between different groups, mice were stratified at d1 or d27. At d3 or d29, mice were injected with philanthotoxin 433 (PhTX, 100µM intrahippocampal), a specific blocker of Ca^{2+}-permeable GluR1 containing AMPARs and tested for contextual fear 30 minutes later.

Experiment 5: To assess whether pharmacological treatment influences the PTSD-like symptoms and/or the CO activity (Exp. 1 and 2), mice were shocked or non-shocked (control) at d0 and treated either from d1 until d42 (preventive) or from d28 until d65 (therapeutic) with fluoxetine (20 mg/kg/d) or vehicle via drinking water and tested under treatment for hyperarousal and generalized and contextual fear. After an additional week, brains were collected and CO staining was performed.

Experiment 6: To control, whether long-lasting changes in CO activity (Exp. 5) predict relapse of PTSD-like symptoms, behavioral tests were performed after 4 weeks of wash-out period. Mice were shocked or non-shocked (control) at d0, remained in their home cages, and received fluoxetine (20 mg/kg/d) or vehicle via drinking water from d1-28 (preventive) or d28-56 (therapeutic). At d28, the preventively treated mice were tested for the presence of PTSD-like symptoms and for successful fluoxetine treatment (1' tone). The therapeutically treated mice were tested at d28 for the presence of PTSD-like symptoms and for stratification, and at d56 for successful fluoxetine treatment (1' tone). Four weeks after the end of treatment, mice were tested for hyperarousal and generalized and contextual fear.

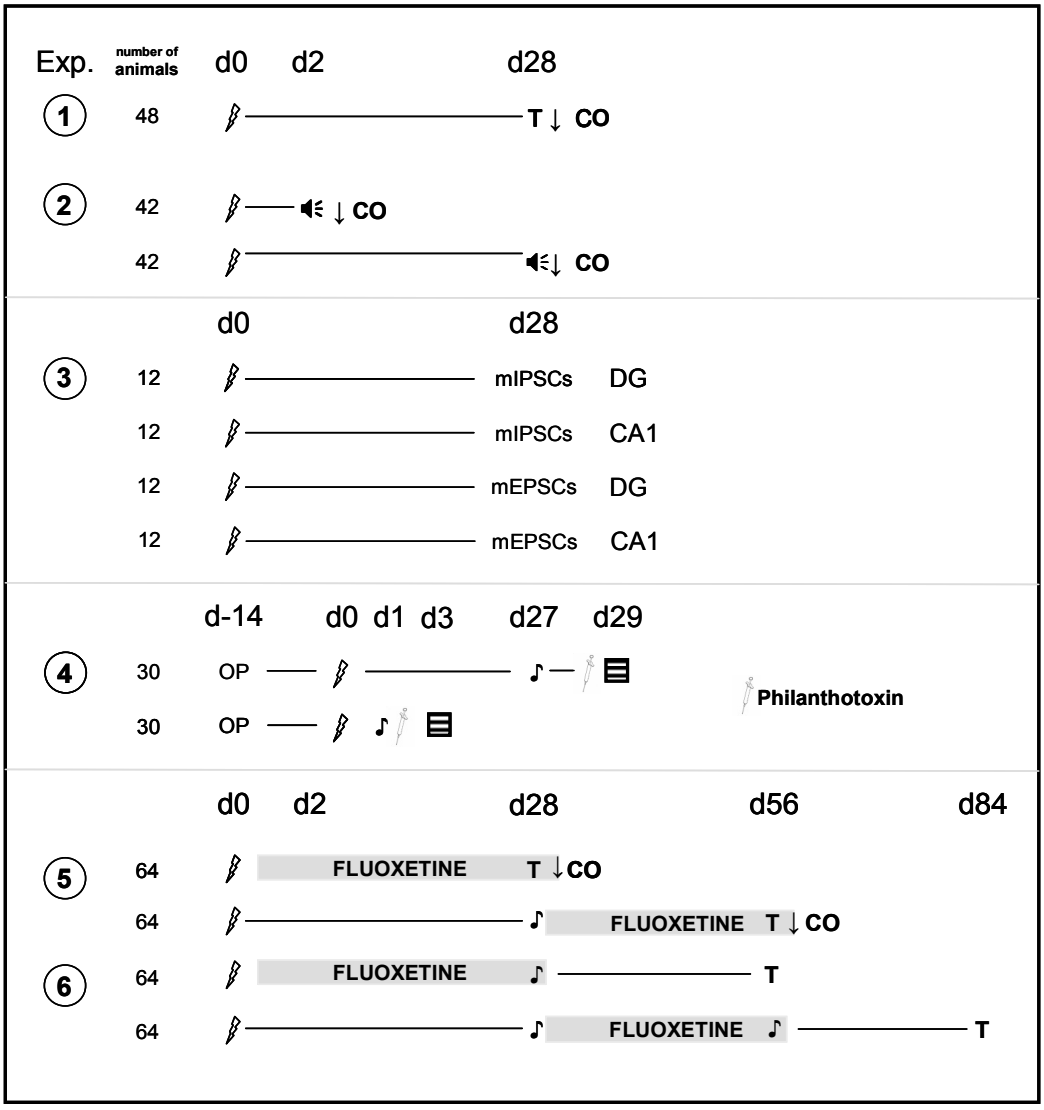

Figure 3: Experimental overview. CO cytochrome c oxidase; T: tested for PTSD-like symptoms (hyperarousal (ASR), generalized and contextual fear (cylinder/tone, hexagon, and chamber), and/or avoidance); OP surgery; ↓ collection of brains; Injection; ♪ Stratification. For further details see Figure 4**.**

Material and Methods

1.6 Animals

All experimental procedures were approved by the Committee on Animal Health and Care of the State of Upper Bavaria (Regierung von Oberbayern) and performed in strict compliance with the European Union recommendations for the case and use of laboratory animals (86/609/CEE).

A total number of 496 mice (plus additional 120 mice for the standards of the cytochrome c oxidase staining) were used. All experiments were performed with male C57BL/6NCrl mice[11] which were purchased from Charles River Germany at the age of 6 to 7 weeks.

Mice were singled housed and kept under standard housing conditions[12] in the animal facility of the Max Planck Institute of Psychiatry for at least 2 weeks before the start of the experiments or surgery and remained under these conditions until the end of the experiments. Mice were tested in the dark phase between 10:00 am and 7:00 pm. Sample sizes are indicated in the respective description of the experiments (Figure 3).

1.7 Surgery

Mice were initially anesthetized with isoflurane (Forene®, Abbott, Germany) and placed in a stereotaxic apparatus (TSE systems, Germany) where they received a sustentative inhalation anesthesia with isoflurane. Before opening the skull, mice received analgesic treatment (0.5 mg/kg meloxicam s.c., Metacam®, Boehringer Ingelheim, Germany), afterwards stereotactically guided holes were drilled and guide cannulae (23G) were implanted bilaterally above the dorsal

11 The “PTSD vulnerable” mouse strain (Siegmund and Wotjak., 2007; Siegmund et al., 2009; Dahlhoff et al., 2010).

12 Standard housing conditions: Makrolon type II cage with wood shavings and nesting material, 12:12 hours light dark schedule, lights off at 9:00 am, 22 ± 2°C room temperature, and 55 ± 5% humidity, free access to food and water.

hippocampus. Coordinates based on the stereotaxic mouse brain atlas (Franklin and Paxinos, 2001) were -1.8 mm posterior, ±1.3 mm lateral to bregma, and 1.0 mm below the surface of the skull. Cannulae were fixed to the skull with dental cement (Paladur®, Heraeus, Germany). Analgesic treatment was continued for 3 days via drinking water (0.5 mg/kg/d meloxicam). Before starting the experiment, mice were allowed to recover from surgery for 2 weeks. Correct positions of coordinates were controlled post mortem by histological examination of cryo sections, stained with cresyl violet. Mice were excluded from the analysis if coordinates were incorrect.

1.8 Intracerebral injections

Mice were slightly anaesthetised with isoflurane, and philanthotoxin 433 (PhTX, P207, Sigma-Aldrich) was infused into the hippocampus at a volume of 0.5 µl per injection site over the course of 1 min. Injections were performed by means of an injection cannula which was connected to a microliter syringe via a calibrated tubing containing an air bubble for monitoring volume progress. The injection cannula protruded from the guide cannula by 1 mm thus reaching the stratum lacunosum moleculare of the dorsal hippocampus. After completion of the injection, the cannula was left in place for another minute before removal. Mice were excluded from the experiment, if fluid or blood was flowing out of the guide cannula.

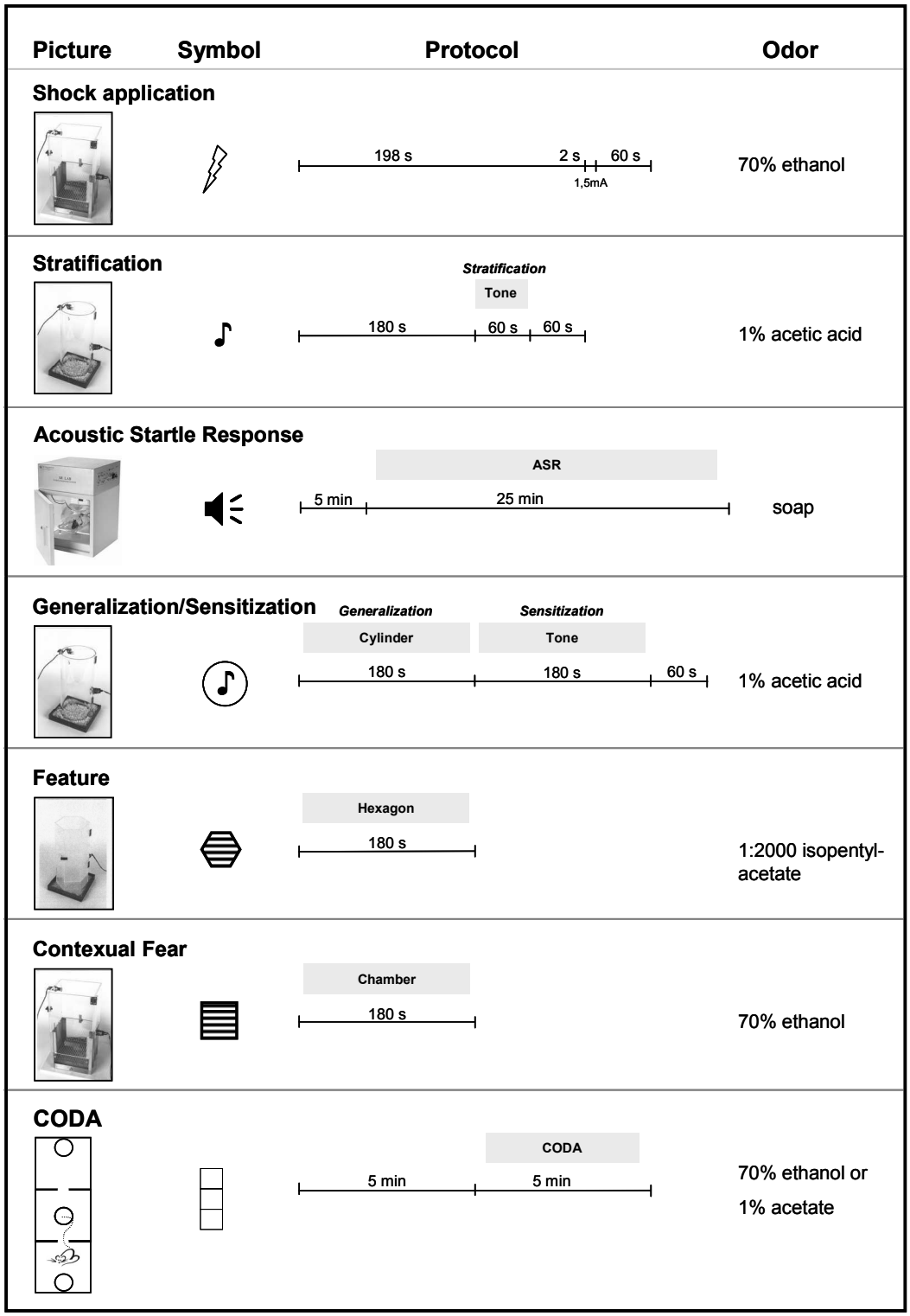

Figure 4: Overview over all behavioral setups used in this thesis. Gray bars show the analyzed part of the protocol, indicated via the symbols in the following figures.

1.9 Behavioral tests

All setups and procedures have been described previously (Siegmund and Wotjak, 2007; Golub et al., 2009; Pamplona et al., 2011) and are shown in Figure 4.

1.9.1 Shock application

Mice were placed into a chamber (MED Associates U.S.A.) (cubic shape, metal grid floor, two metal and two Plexiglas walls, odor of 70% ethanol), and after 198 s they received an inescapable electric foot shock (1.5 mA current intensity/ 2 s duration) via the metal grid at the floor. The electric foot shock constituted the traumatic event for the mice. After receiving the shock, mice remained in the shock context for another 60 s before being placed back to the home cage. Non-shocked mice underwent same procedure, but without the foot shock.

1.9.2 Stratification

To ensure equal levels of sensitisation between different groups (before the treatment or to control for the development of PTSD-like symptoms or the treatment effect), sensitized fear was measured essentially as the response to neutral tone in the cylinder (cylindrical shape, bedding on the floor, transparent and smooth Plexiglas wall, odor: 1% acetic acid). To avoid associations with the sensitized chamber, the odor was different in all environments (cylinder, hexagon, chamber, startle set up, CODA). After 180 s, a neutral tone (80 dB, 9 kHz) was presented for 60 s. After the tone, mice remained in the cylinder for another 60 s before placed back in their home cages.

1.9.3 Acoustic startle response - ASR

To measure hyperarousal symptomatology, mice were tested for acoustic startle responses (ASR). Therefore, mice were placed into a non-restrictive Plexiglas cylinder (inner diameter 4 cm, length 8 cm) mounted onto a plastic platform and placed in a sound attenuated chamber (SR-LAB, San Diego Instruments SDI, San Diego, CA, USA). The movement was detected by a piezoelectric element mounted under each platform and the voltage output of the piezo was amplified and digitized (sampling rate 1 kHz) by a computer

interface (I/O-board provided by SDI). The startle amplitude was defined as the peak voltage output within the first 50 ms after stimulus onset and quantified by means of SR-LAB software. Startle stimuli and background noise were delivered through a high-frequency speaker. Four different startle stimuli consisting of white noise bursts, 20 ms duration, and 75, 90, 105, or 115 dB intensity (INT) were presented in a constant background noise of 50 dB. Within control trials only background noise was present. After an acclimation period of 5 min, 10 control trials and 80 startle stimuli (20 stimuli each intensity) were presented in a pseudorandom order. After each session, Plexiglas cylinders were cleaned with soap water and dried.

1.9.4 Generalized and contextual fear

To measure generalized fear, mice were placed in the cylinder (as described for the stratification). After 180 s, a neutral tone (80 dB, 9 kHz) was presented for 180 s. After the tone, mice remained in the cylinder for another 60 s. Furthermore, animals were tested for 3 min in the hexagon (hexagonal shape, metal grid floor, non-transparent and rough Plexiglas walls, and odor of 1:2000 isopentyl acetate ("banana flavor")), including the metal grid as a dominant feature of the trauma context (chamber). Contextual fear was measured by exposing the mice to the sensitized chamber for 3 min. All contexts were cleaned after each trial (with the detergents used as odor in the according protocol) and bedding was changed. For further details see Figure 4.

Recent fear memories were measured 2 days after the shock application, remote fear memories after 1 month. Freezing behavior (defined as the absence of movement except for breathing and the head remaining in a horizontal position) was analyzed as a criterion for fear (Kamprath and Wotjak, 2004).

The sensitization and the tests for generalized and contextual fear were recorded by CCD cameras (Conrad Electronics, Hirschau, Germany) and analyzed offline (EVENTLOG, Robert Henderson, 1986). The experimentator was blind to treated groups and initially trained by repeated analysis of the video tapes until reaching a determination coefficient $r^2>0.9$ (Figure 5).

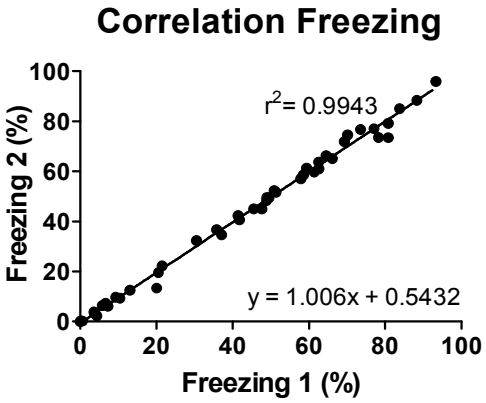

Figure 5: Evaluation of freezing analysis. Double freezing analysis of a single experiment. n = 45

1.9.5 Conditioned odor avoidance - CODA

To test for avoidance behavior, mice were tested in the conditioned odor avoidance task (CODA). CODA was conducted in a rectangular box which was divided into three compartments that were interconnected by small openings with guillotine doors. A filter paper-lined Petri dish (10 cm diameter), containing own home cage bedding (nest compartment, center), 70% ethanol, or 1% acetate (left or right compartment, counterbalanced) was placed in each compartment. For CODA testing, mice were placed in the nest compartment for 5 min (habituation phase) followed by 5 min of free exploration (test phase, open doors). During testing, the latency until the first exit of the mouse from the nest compartment and the time spent in each of the compartments were recorded. The animals' behavior was observed and rated online.

1.10 Fluoxetine treatment

Fluoxetine-ratiopharm solution (Ratiopharm GmbH, Germany) was dissolved in drinking water resulting in a daily dose of 20 mg/kg and was provided in light-proof drinking bottles (home cage). In the first days of treatment, body weight was measured every third day. Mice showed a slight, but not significant decrease in body weight during the first days of treatment, but remained stable from day 9 on. To preclude withdrawal effects after the end of treatment (experiment 5 and 6), mice received only 10 mg/kg/d fluoxetine (50% of the treatment dose) for 4 days and afterwards the drug was completely withdrawn.

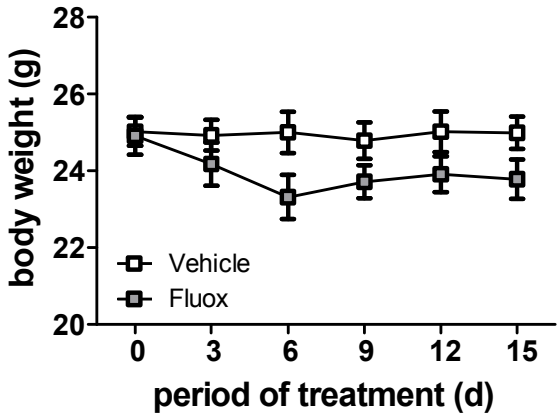

Figure 6: Changes in body weight under fluoxetine treatment.

1.11 Molecular experiments

For cytochrome c oxidase (CO) staining, mice have been killed by an overdose of isoflurane. Brains were quickly removed, frozen in chilled methylbutane on dry ice, and stored at -80°C until sectioning them on a cryostat (20 µm). Sections were mounted on microscope slides (superfrost® plus, Thermo scientific, Germany) and stored at -80°C until staining.

1.11.1 CO staining

During the whole staining protocol, slides were moved gently (using a shaker). First, slides were fixed for 5 min in cold acetone (4°C), and washed afterwards 3 times (5 min each) in 0.1 M Na^+P-buffer[13] (4°C).

Slides were transferred into 37°C staining medium[14] for 20 min. Staining was stopped by transferring the slides into 4°C 0.1 M Na^+P-buffer, changing them 2 times (5 min each) and post-fixing them for 30 min in 4% paraformaldehyde (PFA) at room temperature (RT). Sections were washed twice with 0.1 M Na^+P-

[13] 0.5M Na^+P-buffer: 14.33g NaH_2PO_4 H_2O, 85.29g Na_2HPO_4 $2H_2O$ per 1 l H_2O bidest., pH 7.6

[14] staining medium: 45 g sucrose, 390 mg DAB (2,6-Diacetylpyridine, D8801, Sigma- Aldrich), 50 mg cytochrome c (C2506, Sigma-Aldrich), 150 mg ammonium nickel sulfate (A1827, Sigma-Aldrich) per litre 0.1 M Na^+P-buffer. Staining medium was made freshly before each staining. It was heated up to ≤40°C for 30 min and filtered afterwards. Preliminary tests verified that this was within the linear range of reaction product development.

buffer for 5 min, dehydrated with ethanol (70%, 80%, 99%) and isopropyl alcohol (2 min each), and cover-slipped with histo-kit. Microscope images (AxioCam MR05®, Leica, Germany) were analyzed with Image J. Representive brain slices (left side) and the region of interest (ROI) (enhanced at the right side) are shown in Figure 7.

1.11.2 Tissue homogenization

Tissue homogenization was performed as previously described (Riddle and Forbes, 2005). Homogenates of mouse brain and liver were used to generate a standard curve of known CO activity. Tissue was cooled on ice during all procedures. After being minced into fine pieces with a scalpel, tissue was homogenized. To produce the lowest CO activity standard, the brain homogenate was transferred into a plastic tube and heated up to 60°C in a water bath for 1 h. For intermediate CO activity standards, combinations of brain (inactivated; 0% activity) and liver homogenates (100% activity) were prepared by weighting proportions of homogenates (w/w), followed by thorough mixing. A small measured amount (100 mg) from each of the final 6 standards was separated and divided into 2 ml aliquots and briefly centrifuged at 250g to remove air bubbles. The paste standards were frozen in 2-methylbutane cooled with dry ice and stored at -80°C. Sections of paste standards were cut on a cryostat and mounted as described below for tissue preparation.

1.11.3 Spectrophotometric determination of CO activity

CO activity in tissue paste homogenates was performed as described by Hess and Pope (1953; Hevner et al., 1993) with slight modifications. A 1% cytochrome solution (3 ml) was prepared in 0.05 M potassium phosphate buffer[15], pH 7.0 (K^+P-buffer). Cytochrome was reduced by addition of 40 mg sodium ascorbate and dialyzed in Spectropor 1 dialysis tubing (MWCO 6000-8000) against three changes of K^+P-buffer (3 l each) for at least 24 h at 4°C. The recovered cytochrome solution was diluted to 0.07% in K^+P-buffer and

[15] 0.5 M potassium phosphate buffer (K^+P-buffer): 68 g KH_2PO_4, 1 l H_2O bidest., pH 7.0 with 1 M KOH

checked for sufficient reduction (550/565 nm optical density (OD) ratio ≥ 6). Twenty percent (w/v) solutions of fresh tissue pastes in cold isolation buffer[16] were homogenized on ice using micro ground glass dounces.

To measure CO activity, an aliquot of the 20% tissue homogenate stock was further diluted to 0.25% in cold isolation buffer with the addition of 0.5% sodium deoxycholate (Sigma-Aldrich). Homogenates were slightly vortexed, incubated for 8 min at room temperature (RT), vortexed again, and then placed on ice. Reduced cytochrome solution (1.5 ml), oxidized by the addition of saturated potassium ferricyanide (Sigma-Aldrich), was used to zero the spectrophotometer at 550 nm. Five microliters of 0.25% tissue paste standard homogenate were added to a 1 cm path length cuvette containing 1.5 ml of 22°C reduced cytochrome solution. The cuvette was inverted twice to disperse the solubilized tissue and OD readings at 550 nm were recorded at 15 s intervals; starting at 30 s and finishing after 2.5 min. Measurements were made in triplicate for each paste standard. All reactions were linear over the initial 2.5 min assayed. The change in ODs for the five recorded 1 min intervals (0.5 - 1.5, 0.75 - 1.75 min, etc.) for each sample were averaged. CO activity was calculated by dividing the mean change in OD 550 per minute by the difference in the molar extinction coefficients for reduced (28 mM^{-1} cm^{-1}) minus oxidized (8.4 mM^{-1} cm^{-1}) cytochrome (19.6 mM^{-1} cm^{-1} or 19.6 mol^{-1} ml cm^{-1}). This result was divided by 8.3×10^{-6} g tissue sampled per ml to give CO activity, defined as micromoles of reduced cytochrome substrate oxidized per minute at 22°C (pH 7) per gram of tissue (mol/min/g). In CO figures, CO activity is denoted in mol/min/g, except otherwise mentioned (relative CO activity in Experiment 1).

Chemicals for Na^+P-buffer, K^+P-buffer, and isolation-buffer were purchased from Merck (Germany).

[16] isolation buffer: 10 mM Tris-HCl, pH 7.4, containing 0.32 M sucrose and 1 mM dipotassium EDTA

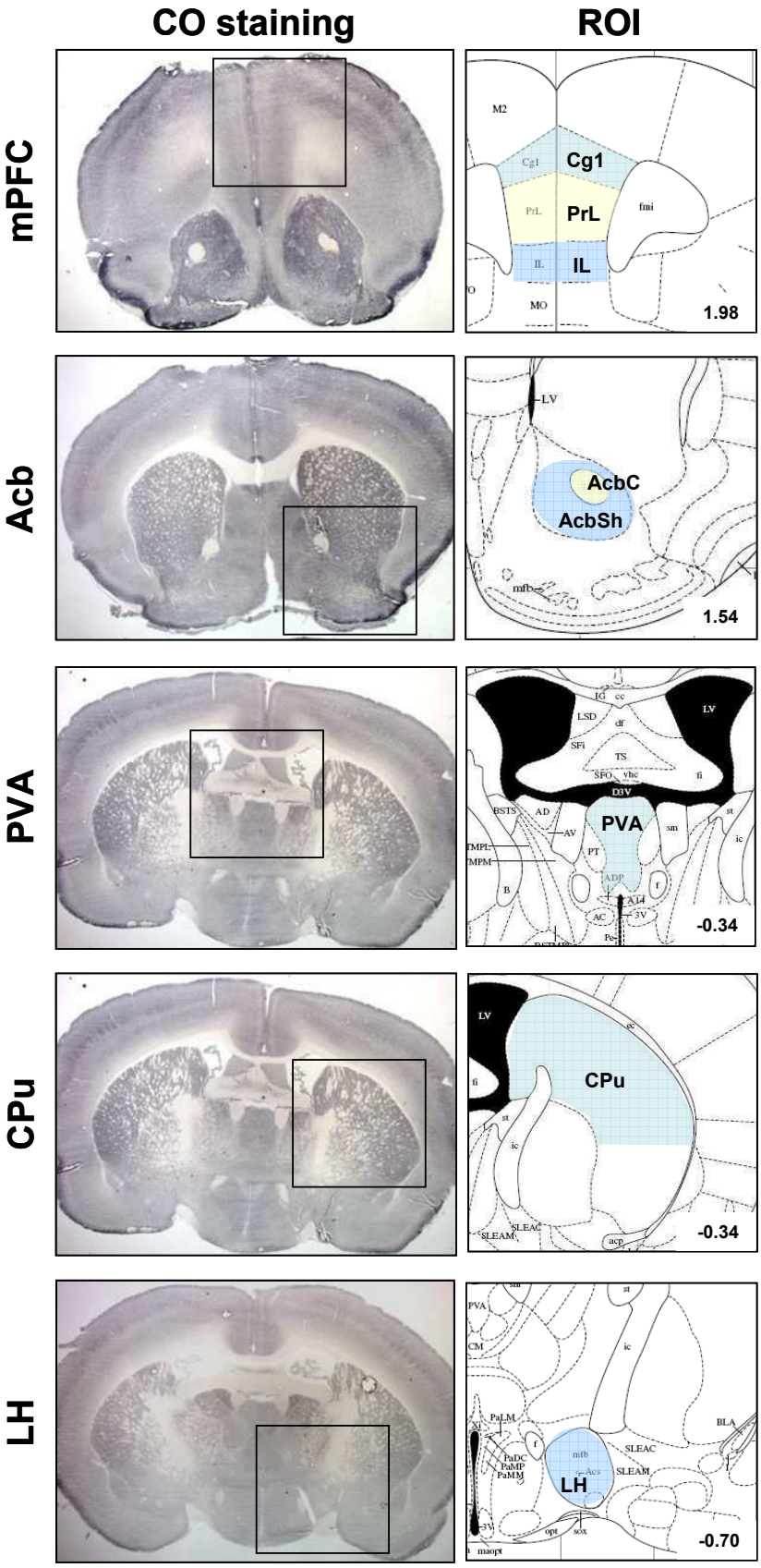

Figure 7: Representative CO stained coronal slices of the region of interest (ROI, left side). The blotted box is enlarged on the right (Franklin and Paxinos, 2001), and analyzed regions are labeled (distance to Bregma is noted down right in mm).

Acc - nucleus accumbens,
AccC - accumbens nucleus core,
AccSh - accumbens nucleus shell,
Cg1 - cingulate cortex (area 1),
CPu - caudate putamen (striatum),
IL - infralimbic cortex,
LH - lateral hypothalamic area,
mPFC - medial prefrontal cortex,
PrL - prelimbic cortex,
PVA - paraventricular thalamic nucleus (anterior)
(to be continued on the next page)

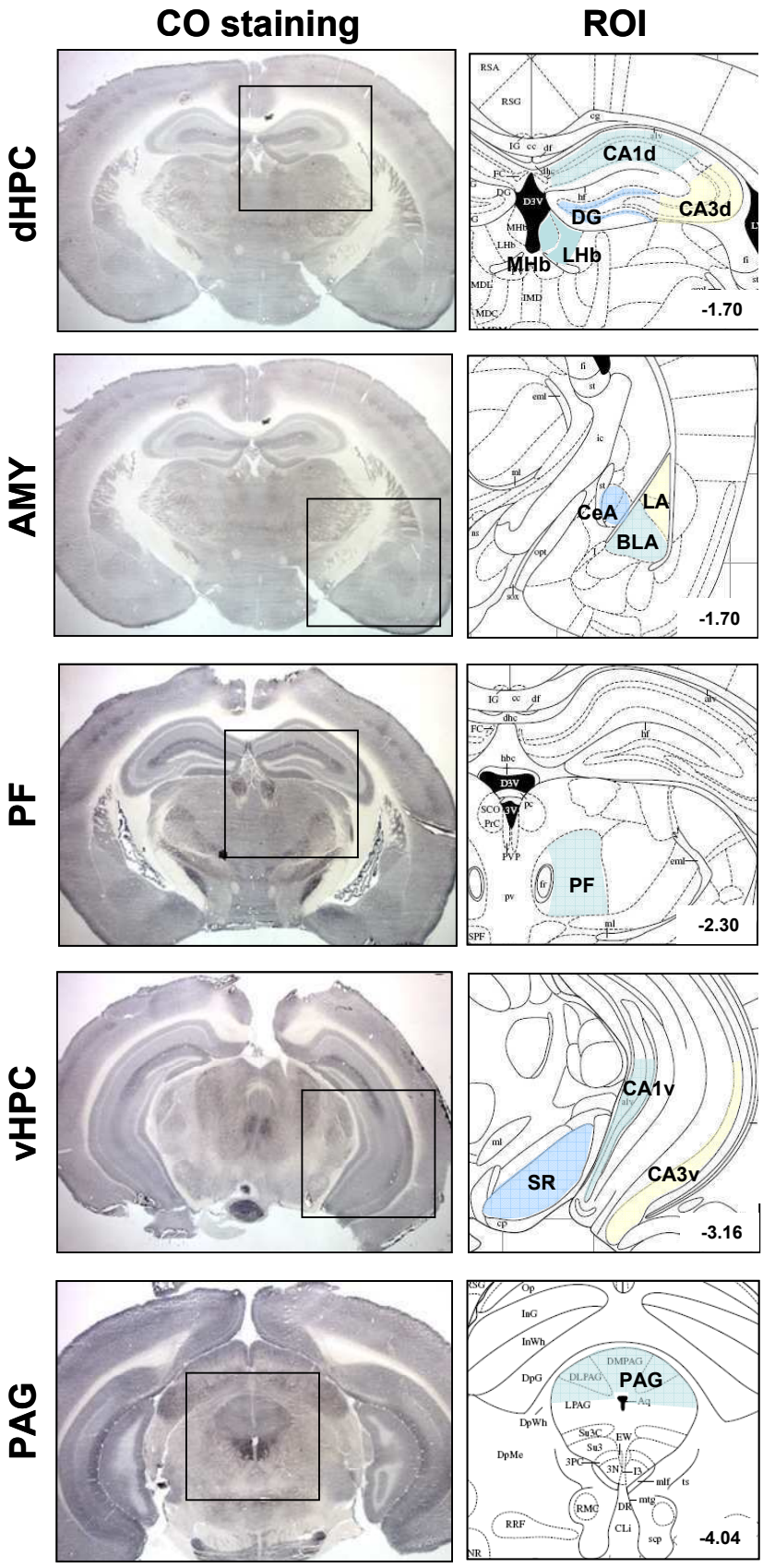

Figure 8: Typical CO stained coronal slices of the region of interest (left side; in continuation)

AMY - amgdala,
BLA - basolateral amygdaloid nucleus,
CA1 - cornus ammonis 1,
CA1v - ventral cornus ammonis 1,
CA3 - cornus ammonis 3,
CA3v - ventral cornus ammonis 3,
CeA - central amygdaloid nucleus,
DG - dentate gyrus,
dHPC - dorsal hippocampus,
LA - lateral amygdaloid nucleus,
LHb - lateral habenular nucleus,
MHb - medial habenular nucleus,
PAG - periaqueductal gray,
PF - parafascicular thalamic nucleus,
SN - substancia nigra,
vHPC - ventral hippocampus

1.12 Electrophysiology

1.12.1 Brain slice preparation

Acute 350 µm thick coronal hippocampal slices were prepared using a vibratome (HM650V, Thermo Scientific) and maintained in artificial cerebrospinal fluid (aCSF[17]) saturated with 5% CO_2 / 95% O_2. Slices were allowed to recover in a storage chamber initially at 36°C for 30 min and for another 30 min at RT before being transferred to the recording chamber.

1.12.2 AMPAR-mEPSC and $GABA_A$R-mIPSC recordings

Whole-cell patch-clamp recordings were carried out from granule cells of the dentate gyrus (DG) and the cell layer of the CA1 (cornu ammonis 1) in acute brain slices by means of a SEC-10LX amplifier (npi Electronics, Tamm, Germany). An infrared video microscope equipped with the gradient contrast system was used to visualize somata of DG and CA1 neurons. The pipette was filled with intracellular solution. Patch-clamp electrodes (open-tip resistance of 4-6 MΩ) were pulled from borosilicate glass capillaries (Harvard Apparatus, Kent, UK) on a DMZ-Universal puller. Neurons were voltage-clamped at -60 mV, and AMPAR-mEPSCs recordings (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor miniature excitatory post-synaptic current) were measured while slices were continuously superfused with artificial cerebrospinal fluid (aCSF) containing 1 µM tetrodotoxin (TTX), 50 µM D (-)-2-amino-5-phosphonopentanoic acid (AP5), and 10 µM (-)-bicuculline methiodide (BIM). For $GABA_A$R-mIPSCs recordings (γ-aminobutyric acid receptor A - miniature inhibitory post-synaptic current), neurons were voltage-clamped at -40 mV and measured while slices were continuously superfused with oxygenated aCSF containing 1 µM TTX, 50 µM AP5, and 5 µM 2,3-dihydroxy-6-

[17] aCSF (in mM): NaCl 125, KCl 2.5, NaH_2PO_4 1.25, $CaCl_2$ 2, $MgSO_4$ 1, $NaHCO_3$ 25, glucose 25 at pH 7.4

nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX). All recordings were performed at RT.

Ten minutes after breaking into the cell, AMPAR-mEPSCs or $GABA_A$R-mIPSCs were recorded in individual neurons for 5 minutes. Amplitudes and frequencies were analyzed offline using the Mini Analysis software (Synaptosoft, GA) with a detection threshold set at 4 pA.

Chemicals for aCSF preparation were purchased from Merck (Germany), TTX, NBQX, and AP5 from Ascent Scientific (UK), and BIM from Tocris Biosciences (UK).

1.13 Data analysis

Behavioral data were averaged to the testing period and presented as a percentage of the analysis interval. Regarding AMPAR-mEPSC or $GABA_A$-mIPSC recordings, mean amplitude and frequency of 5 min-recordings were analyzed and presented as absolute values. Statistical analysis of data was performed using Statistica 7 (StatSoft, USA). Graphs were created with GraphPad Prism 5 (GraphPad Inc., USA). Data were analyzed by unpaired student's t-tests or 2-way ANOVAs when appropriate with shock and treatment as independent variables as indicated in the text, or repeated measures ANOVAs when appropriate with shock and treatment in the ASR. Newman-Keuls post-hoc test was used for point-by-point comparisons in case of significant main effects. Note for the ASR-experiments: If * or $^{\#}$ are upon the data points (in the figures), the interaction was significant ($p \leq 0.05$) and Newman-Keuls post-hoc test was used for point-by-point comparisons; if * or $^{\#}$ are at the right side of the data points, the interaction was not significant and the significant effects (shown in the figures) were between the groups. All data are presented as mean ± s.e.m. Statistical significance was accepted if $p \leq 0.05$.

For clarity and brevity, only significant and relevant results of the statistical analyses are reported in the main text and the figures.

Results

1.14 Behavioral tests and CO activity one month after shock (Exp. 1)

A single electric foot shock (1.5 mA/ 2 s) increased hyperarousal (Figure 8), generalized and contextual fear (Figure 9), and avoidance behavior (Figure 10) compared to non-shocked (control) mice between day 28 (d28) and day 35 (d35).

After the behavioral tests, mice remained in their home cages for another week to prevent acute influence of testing on the CO activity.

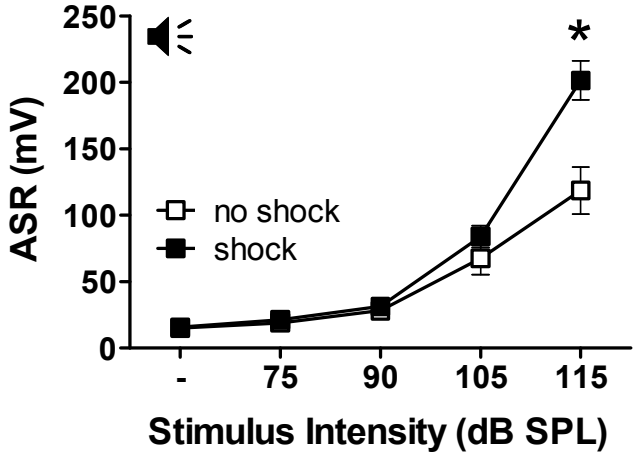

Figure 8: A single electric foot shock increased hyperarousal at d28 post shock (Exp. 1). Mice were shocked (1.5 mA/ 2 s) or non-shocked in the chamber at d0 and remained in their home cages for 28 days. At d28, mice were tested for hyperarousal. Acoustic startle responses (ASR) as a function of stimulus intensity are presented as mean ± s.e.m. Statistical analysis were performed by 2-way repeated measures ANOVA ($F_{1, 42, shock}$=6.70, $p<0.05$; $F_{4, 168, INT}$=93.87, $p<0.001$;$F_{4, 168, shock*INT}$=7.85, $p<0.001$), followed by Newman-Keuls post-hoc test. n = 12 (non-shocked) or 32 (shocked) mice; *$p<0.001$ vs. non-shocked mice

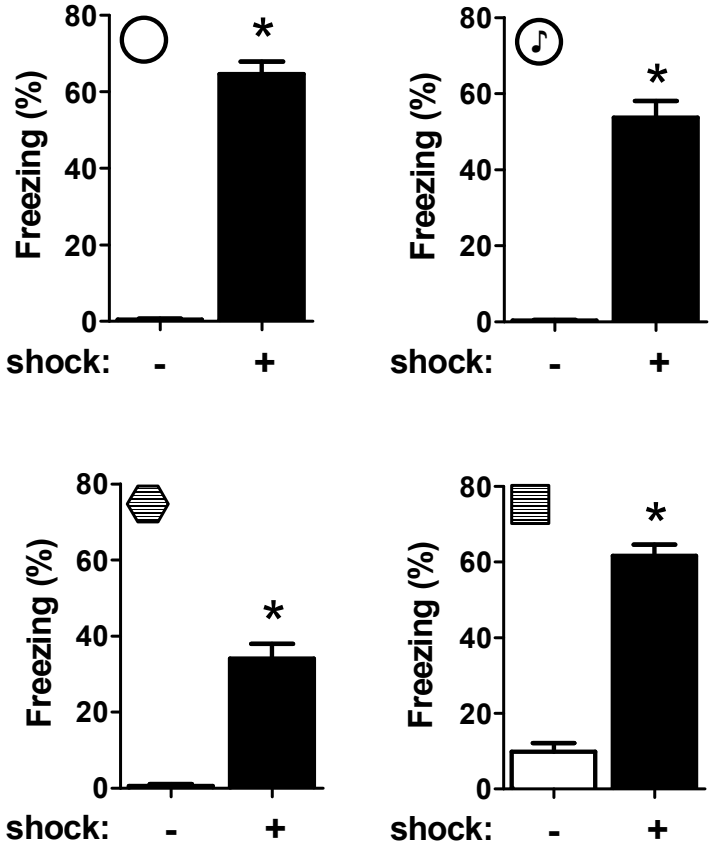

Figure 9: A single electric foot shock increased generalized and contextual freezing responses at d29-30 post shock (Exp. 1). Mice were shocked (1.5 mA/ 2 s) or non-shocked in the chamber at d0 and remained in their home cages for 28 days. At day 28, mice were tested for hyperarousal symptomatology (Figure 8) and afterwards (d29-30) exposed to a neutral context (cylinder/ 3' tone), the hexagon, and the sensitized chamber. The percentage of time mice spent in freezing behavior was analyzed. Statistical analysis were performed by unpaired t-test (cylinder: $p<0.001$, $t=13.86$, $df=44$; tone: $p<0.001$, $t=8.090$, $df=44$; hexagon: $p<0.001$, $t=5.727$, $df=45$, chamber $p<0.001$, $t=12.02$, $df=43$). n = 14-16 (non-shocked) or 29-32 (shocked) mice; *$p<0.001$ vs. non-shocked mice

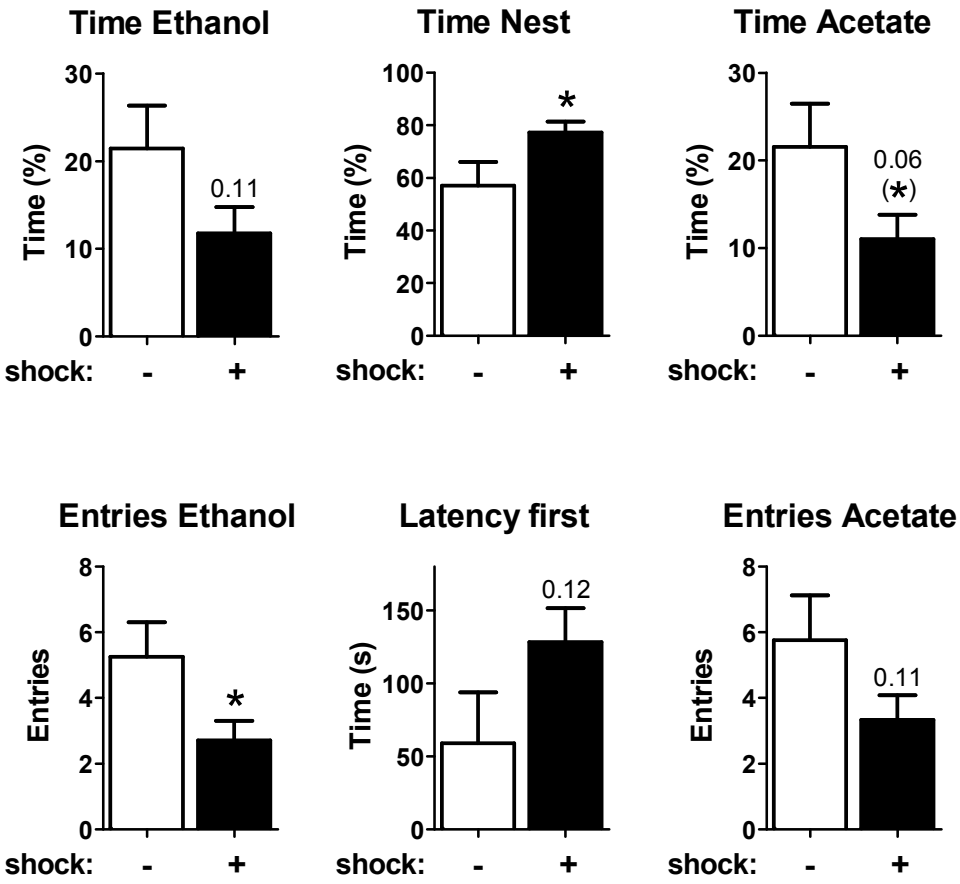

Figure 10: A single electric foot shock increased avoidance behavior at d32-33 (Exp. 1). Mice were shocked (1.5 mA/ 2 s) or non-shocked in the chamber at d0 and remained in their home cages for 28 days. At d28 and follows, mice were tested for hyperarousal symptomatology (Figure 9), generalized and contextual fear responses (Figure 10), and afterwards for avoidance behavior. Time spent in the ethanol, acetate, and nest compartments, as well as the total number of entries in the ethanol and acetate compartment and the latency of entering the first compartment were analyzed online. Data are represented as mean ± s.e.m. Statistical analysis were performed by unpaired t-test (time ethanol: $p=0.11$, $t=1.677$, $df=27$; time nest: $p<0.05$, $t=2.352$, $df=27$; time acetate: $p=0.06$, $t=1.943$, $df=27$; entries ethanol: $p<0.05$, $t=2.200$, $df=27$; latency first: $p=0.12$, $t=1.594$, $df=27$; entries acetate: $p=0.11$, $t=1.644$, $df=27$). n = 8 (non-shocked) or 21 (shocked) mice. *$p<0.05$ vs. non-shocked mice

At day 42, cytochrome c oxidase (CO) activity was significantly increased in shocked compared to non-shocked mice in the following brain regions: the prelimbic cortex (PrL), the nucleus accumbens core (AccC) and shell (AccSh), the basolateral amygdaloid nucleus (BLA), the medial habenular nucleus (MHb), the dorsal hippocampus (CA1, CA3, DG), the ventral hippocampus (vCA1 and vCA3), the lateral hypothalamic area (LH), the parafascicular thalamic nucleus (PF), and the periaqueductal gray (PAG), but not in the cingulated cortex (Cg ant.), the infralimbic cortex (IL), the caudate putamen (CPu, striatum), the lateral (LA) and central amygdaloid nucleus (CeA), the lateral habenula nucleus (LHb), the paraventricular thalamic nucleus (PVA), and the substantia nigra (SN) (Table 2).

PTSD-like symptoms and CO activity one month after shock

Four weeks after the shock (traumatic event), shocked mice showed increased hyperarousal, generalized and contextual fear response, and avoidance behavior as well as increased CO activity in most of the analyzed brain regions (PrL, CA1+3, DG, BLA, PAG, among others) compared to non-shocked (control) mice.

Table 2: A single electric foot shock increased CO activity in most of the analyzed brain areas at d42 post shock (Exp. 1). Mice were shocked or non-shocked at d0 and remained in their home cages until d28. Between d28 and d33, mice were tested for hyperarousal, generalized and contextual fear responses, and avoidance behavior (Figure 8 -Figure 10). Afterwards, they remained in their home cages for an additional week. Brains have been collected at d42 and CO staining was performed.CO activity measured in relative values (not absolute; pilot project without standards). ns non-shocked; s shocked mice

	CO activity (ns)	CO activity (s)	t, df	p-value
Prefrontal Cortex, medial				
Cg ant.	1.154 ± 0.01 n=8	1.171 ± 0.01 n=21	t=0.921 df=27	0.37
PrL	1.136 ± 0.01 n=8	1.166 ± 0.00 n=21	t=2.975 df=27	<0.001
IL	1.155 ± 0.01 n=8	1.169 ± 0.01 n=21	t=1.295 df=27	0.21
Accumbens				
Acc core	1.161 ± 0.01 n=8	1.212 ± 0.01 n=21	t=3.880 df=27	<0.001
Acc shell	1.164 ± 0.01 n=8	1.214 ± 0.01 n=21	t=3.083 df=27	<0.001
Amygdala				
LA	1.109 ± 0.01 n=8	1.109 ± 0.00 n=21	t=0.03385 df=27	0.97
BLA	1.151 ± 0.01 n=8	1.170 ± 0.01 n=21	t=2.623 df=27	<0.01
CeA	1.179 ± 0.01 n=8	1.176 ± 0.01 n=21	t=0.3956 df=27	0.70
Hippocampus, dorsal				
CA1	1.118 ± 0.01 n=8	1.143 ± 0.00 n=21	t=3.375 df=27	<0.001
CA3	1.101 ± 0.01 n=8	1.125 ± 0.00 n=21	t=3.336 df=27	<0.001
DG	1.288 ± 0.01 n=8	1.340 ± 0.01 n=21	t=3.390 df=27	<0.001
Hippocampus, ventral				
CA1	1.144 ± 0.00 n=8	1.176 ± 0.00 n=21	t=3.855 df=27	<0.001
CA3	1.142 ± 0.00 n=8	1.171 ± 0.00 n=21	t=4.078 df=27	<0.001
Miscellaneous				
PAG	1.163 ± 0.01 n=8	1.185 ± 0.00 n=21	t=2.408 df=27	<0.05
PVA	1.084 ± 0.01 n=8	1.098 ± 0.01 n=21	t=1.479 df=27	0.15
LH	1.086 ± 0.01 n=8	1.110 ± 0.00 n=21	t=2.798 df=27	<0.01
CPu	1.169 ± 0.01 n=8	1.186 ± 0.00 n=21	t=1.737 df=27	0.09
MHb	1.131 ± 0.01 n=8	1.149 ± 0.00 n=21	t=2.462 df=27	<0.05
LHb	1.239 ± 0.02 n=8	1.245 ± 0.01 n=21	t=0.3682 df=27	0.72
PF	1.141 ± 0.01 n=8	1.164 ± 0.01 n=21	t=2.209 df=27	<0.05
SN	1.187 ± 0.01 n=8	1.178 ± 0.01 n=21	t=0.6290 df=27	0.53

1.15 ASR and CO activity two days vs. one month after shock (Exp. 2)

To verify these data (exp. 1), the behavioral data as well as the CO activity, experiment 1 was repeated with modifications and an analysis of changes in the early aftermath of the traumatic event was added.

A single electric foot shock (1.5 mA/2 s) increased hyperarousal at d28, but not at d2 (Figure 11) compared to non-shocked mice.

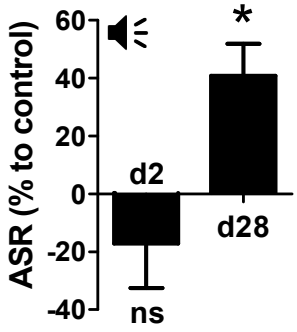

Figure 11: A single electric foot shock increased hyperarousal at d28 but not at d2 (Exp. 2). Mice were shocked (1.5 mA/ 2 s) or non-shocked in the chamber at d0 and remained in their home cages for 2 or 28 days before being tested for hyperarousal symptomatology. The difference in the ASR (to 115dB) of shocked compared to non-shocked mice (ASR = 100%) is represented as mean ± s.e.m. Statistical analysis were performed by unpaired t-test (d2: $p=0.60$, $t=0.5260$, $df=38$; d28: $p<0.001$, $t=5.422$, $df=38$). n = 16 (non-shocked) or 24 (shocked) mice. *$p<0.05$ vs. non-shocked mice (data not shown)

After the behavioral tests, mice remained in their home cages for another week to prevent acute influence of testing on the CO activity. CO activity was not affected at day 9 after shock (Table 3). In contrast, 35 days after the traumatic event, again the CO activity was increased in the following brain sections: the prelimbic cortex (PrL), the dorsal hippocampus (CA1+3, DG), the basolateral amygdaloid nucleus (BLA), and the periaque ductal gray (PAG), among others, (Table 4). These findings underscore the results of the first experiment.

ASR and CO activity two days vs. one month after shock

At d28 but not d2 post shock mice showed increased hyperarousal compared to non-shocked mice. Furthermore, in shocked mice, no brain region with increased CO activity was found at day 9 (compared to non-shocked mice), whereas at day 35 the same brain regions showed an increased CO activity in shocked compared to non-shocked mice as at day 42 (Exp. 1).

Table 3: A single electric foot shock did not change CO activity in the analyzed brain areas at d9 post shock (Exp.2). Mice were shocked or non-shocked at d0, tested for hyperarousal at d2 (Figure 11), and remained in their home cages for an additional week. Brains have been collected at d9 and CO staining was performed. ns non-shocked, s shocked mice; CO activity in mol/min/g (absolute values)

	CO activity (ns)	CO activity (s)	t, df	p-value
Prefrontal Cortex, medial				
Cg ant.	102.9 ± 0.94 n=13	103.9 ± 0.93 n=19	t=0.7353 df=30	0.47
PrL	103.0 ± 0.93 n=13	104.2 ± 0.93 n=19	t=0.8824 df=30	0.38
IL	103.2 ± 0.94 n=13	104.5 ± 0.95 n=19	t=0.9323 df=30	0.36
Accumbens				
Acc core	105.4 ± 0.88 n=13	106.3 ± 0.84 n=19	t=0.6973 df=30	0.49
Acc shell	106.6 ± 0.96 n=13	107.8 ± 0.92 n=19	t=0.8908 df=30	0.38
Amygdala				
LA	101.2 ± 0.77 n=14	102.5 ± 0.65 n=19	t=1.278 df=31	0.21
BLA	104.2 ± 0.79 n=14	105.5 ± 0.64 n=19	t=1.284 df=31	0.21
CeA	104.4 ± 0.73 n=14	105.5 ± 0.55 n=19	t=1.219 df=31	0.23
Hippocampus, dorsal				
CA1	102.0 ± 0.89 n=14	103.3 ± 0.70 n=17	t=1.146 df=29	0.26
CA3	101.4 ± 0.91 n=14	102.4 ± 0.71 n=19	t=0.8713 df=31	0.39
DG	108.5 ± 0.87 n=14	109.8 ± 0.64 n=18	t=1.214 df=30	0.23
Hippocampus, ventral				
CA1	104.3 ± 0.62 n=12	103.6 ± 0.46 n=17	t=0.9868 df=27	0.33
CA3	103.3 ± 0.40 n=14	103.1 ± 0.38 n=18	t=0.4023 df=30	0.69
Miscellaneous				
PAG	104.5 ± 0.95 n=12	106.0 ± 0.71 n=16	t=1.264 df=26	0.22
PVA	-	-	-	-
LH	101.9 ± 0.50 n=14	101.4 ± 0.39 n=19	t=0.7512 df=31	0.46
CPu	104.2 ± 0.94 n=13	105.8 ± 0.80 n=19	t=1.291 df=30	0.21
MHb	101.8 ± 1.01 n=14	103.4 ± 0.75 n=19	t=1.331 df=31	0.19
LHb	106.9 ± 1.24 n=14	109.0 ± 0.78 n=19	t=1.454 df=31	0.16
PF	103.7 ± 0.49 n=14	103.6 ± 0.46 n=19	t=0.1915 df=31	0.85
SN	102.3 ± 0.45 n=14	102.1 ± 0.44 n=18	t=0.3290 df=30	0.75

Table 4: A single electric foot shock changed CO activity in most of the analyzed brain areas at d35 post shock (Exp.2). Mice were shocked or non-shocked at d0, remained in their home cages, tested for hyperarousal at d28, and remained in their home cages for an additional week. Brains have been collected at d35 and CO staining was performed. ns non-shocked, s shocked mice; CO activity in mol/min/g (absolute values)

	CO activity (ns)	CO activity (s)	t, df	p-value
Prefrontal Cortex, medial				
Cg ant.	103.1 ± 0.81 n=16	101.8 ± 0.79 n=19	t=1.147 df=33	0.26
PrL	101.2 ± 0.80 n=16	103.6 ± 0.69 n=19	t=2.328 df=33	<0.05
IL	102.1 ± 0.81 n=16	102.3 ± 0.69 n=19	t=0.1599 df=33	0.87
Accumbens				
Acc core	103.5 ± 0.62 n=16	105.7 ± 0.62 n=19	t=2.530 df=33	<0.05
Acc shell	105.1 ± 0.72 n=16	107.3 ± 0.73 n=19	t=2.148 df=33	<0.05
Amygdala				
LA	100.7 ± 0.64 n=16	100.2 ± 0.67 n=19	t=0.5985 df=33	0.55
BLA	102.7 ± 0.59 n=16	104.5 ± 0.56 n=19	t=2.092 df=33	<0.05
CeA	104.8 ± 0.67 n=16	104.5 ± 0.64 n=19	t=0.3914 df=33	0.70
Hippocampus, dorsal				
CA1	100.6 ± 0.79 n=16	102.7 ± 0.71 n=19	t=1.986 df=33	0.05
CA3	99.36 ± 0.78 n=16	101.9 ± 0.78 n=19	t=2.258 df=33	<0.05
DG	107.5 ± 0.73 n=16	109.6 ± 0.69 n=19	t=2.044 df=33	<0.05
Hippocampus, ventral				
CA1	104.2 ± 0.82 n=15	106.8 ± 0.76 n=19	t=2.343 df=32	<0.05
CA3	103.1 ± 0.85 n=15	105.8 ± 0.72 n=19	t=2.414 df=32	<0.05
Miscellaneous				
PAG	103.3 ± 0.86 n=14	105.5 ± 0.67 n=19	t=2.058 df=31	<0.05
PVA	-	-	-	-
LH	100.7 ± 0.73 n=16	103.1 ± 0.84 n=19	t=2.061 df=33	<0.05
CPu	103.0 ± 0.82 n=16	105.2 ± 0.62 n=19	t=2.102 df=33	<0.05
MHb	100.1 ± 0.77 n=16	102.3 ± 0.68 n=19	t=2.143 df=33	<0.05
LHb	107.7 ± 0.85 n=16	108.8 ± 0.93 n=19	t=0.8167 df=33	0.42
PF	102.9 ± 0.82 n=16	105.5 ± 0.85 n=19	t=2.173 df=33	<0.05
SN	102.7 ± 0.71 n=16	103.4 ± 0.77 n=19	t=0.6443 df=33	0.52

1.16 AMPAR-mEPSCs and $GABA_A$R-mIPSCs (Exp. 3)

In vitro patch-clamp recordings revealed no differences in amplitudes and frequencies of $GABA_A$R-mIPSCs, measured 28 days after foot shock, in CA1 and DG neurons of shocked compared to non-shocked mice (Figure 12). However, amplitudes but not frequencies of AMPAR-mEPSCs were significantly increased in DG but not in CA1 neurons of shocked mice 28 days after shock compared to non-shocked mice (Figure 13 and Figure 12).

Note: AMPAR-mEPSC recordings were performed by CK Thoeringer.

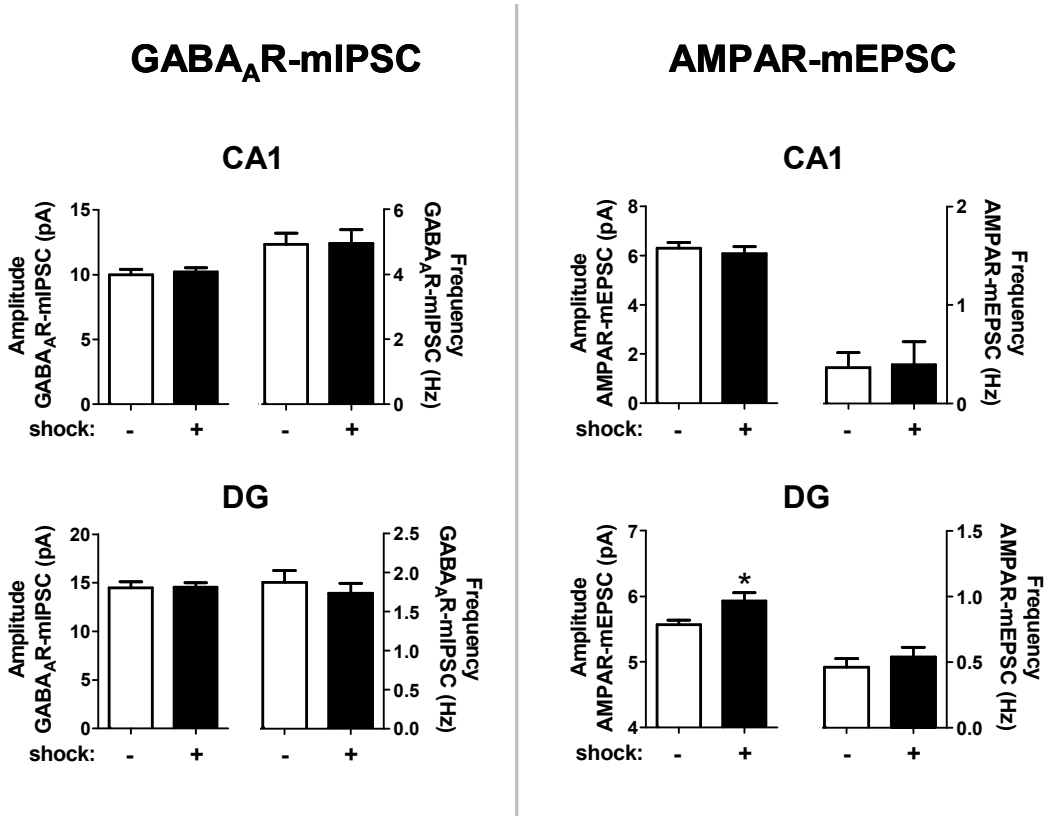

Figure 12: Amplitudes of AMPAR-mEPSCs were significantly increased in DG neurons of shocked mice 28 days after foot shock compared to non-shocked (right bottom), whereas amplitudes and frequencies of $GABA_A$R-mIPSCs in CA1 and DG neurons as well as AMPAR-mEPSCs in CA1 neurons and frequencies in DG neurons were similar (Exp. 3). Mice were shocked or non-shocked at d0, remained in their home cages until d28, acute brain slices were prepared and spontaneous activity were measured. Neurons were voltage-clamped at -60 mV for AMPAR-mEPSCs and at -40 mV for $GABA_A$R-mIPSCs. $GABA_A$R-mIPSCs: CA1: N_{mice} (non-shock/shocked) = 6/6; n_{slices} (non-shocked/shocked) = 14/17; DG: N_{mice} (non-shock/shocked) = 6/6; n_{slices} (non-shocked/shocked) = 18/17; AMPAR-mEPSCs: CA1: N_{mice} (non-shocked/shocked) = 6/7; n_{slices} (non-shocked/shocked) = 19/35; DG: N_{mice} (non-shocked/shocked) = 6/7; n_{slices} (non-shocked/shocked) = 21/30. Statistical analysis were performed by unpaired t-test (AMPAR-mEPSCs (DG): p=0.03, t=2.212, df=49). *p<0.05 vs. non-shocked

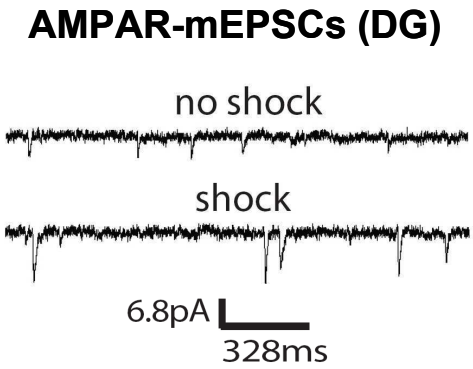

Figure 13: Amplitudes of AMPAR-mEPSC were significantly increased in DG neurons of shocked mice 28 days after foot shock compared to non-shocked (Exp. 3). Representative traces of *in vitro* patch-clamp AMPAR-mEPSCs.

1.17 Influence of PhTX on PTSD-like symptoms at d3 vs. d29 (Exp. 4)

The increase in AMPAR-mEPSCs amplitudes may result from an increased surface expression of AMPARs, especially GluR1-containing AMPARs (O'Brien et al., 1998). Therefore, the physiological relevance of hippocampal GluR1 receptors for remote contextual fear memories by intrahippocampal administration of philanthotoxin 433 (PhTX) was confirmed. Intrahippocampal application of PhTX reduced contextual fear at day 29 (remote fear memories), but not at day 3 (recent fear memories) after foot shock compared to vehicle application (Figure 14).

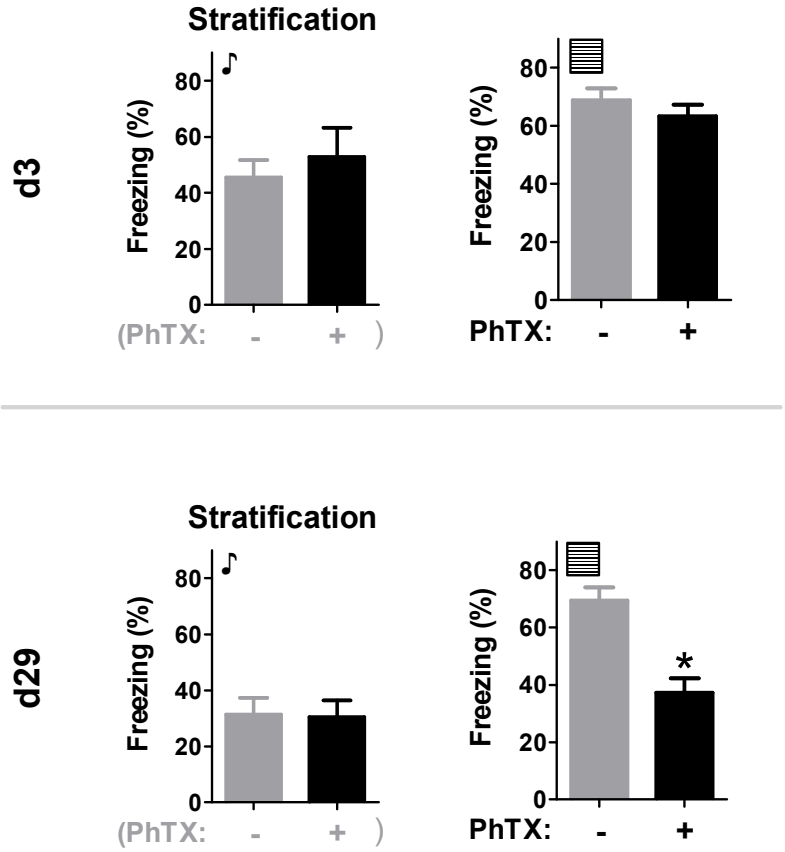

Figure 14: Philanthotoxin (intrahippocampal, 30 min before testing) decreased contextual fear at d29, but not at d3 post shock (Exp. 4). Mice were shocked (1.5 mA/ 2 s) in the chamber at d0 and remained in their home cages until testing. Mice were assigned to two groups with identical levels of sensitization on basis of their freezing responses to a 1' tone at d1 or d27 (left; prospective PhTX classification). At day 3 or d29, PhTX was injected and mice were tested for contextual fear 30 min later. The percentage of time mice spent in freezing behavior was analyzed. Statistical analysis were performed by unpaired t-test (d29: $p<0.001$, $t=4.70$, $df=23$). n = 12-13 mice; *$p<0.001$ vs. non-shocked mice

Changes in the glutamatergic system as a result of the traumatic event.
Both, electrophysiological and behavioral studies, showed a long-term influence of the shock on the glutamatergic system (increased amplitudes in AMPAR-mEPSC in the DG and reduced freezing responses at d29 after intrahippocampal injection of philanthotoxin 433).

1.18 Influence of preventive vs. therapeutic treatment with fluoxetine on PTSD-like symptoms and CO activity (Exp. 5)

Non-shocked and shocked mice were assigned to two groups with identical freezing levels at sensitization on basis to their freezing responses (Figure 15).

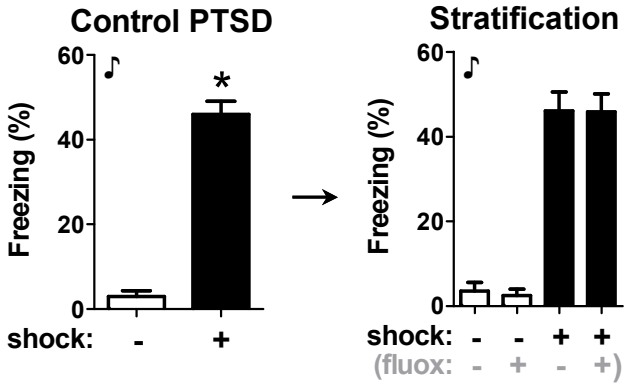

Figure 15: For stratification, mice were exposed to a 1' tone protocol at d28 (Exp. 5). Mice were shocked (1.5 mA/ 2 s) or non-shocked in the chamber at d0 and remained in their home cages for 28 days. At d28, mice were exposed to a neutral context (cylinder/ 1' tone) to control for PTSD-like symptoms (left) and group the mice in a pseudo-randomized order for fluoxetine treatment (right, prospective fluoxetine classification). Percentage of time mice spent in freezing behavior was analyzed and data are represented as mean ± s.e.m. Statistical analysis were performed by unpaired t-test ($p<0.001$, $t=10.54$, $df=60$). n = 32 (non-shocked) or 39 (shocked) mice; *$p<0.001$ vs. non-shocked mice

Preventive (d1-28) or therapeutic (d28-56) treatment with fluoxetine (20 mg/kg/d) inhibited the increase of hyperarousal (Figure 16) and generalized and contextual fear responses (Figure 17). For statistical analysis see Table 5.

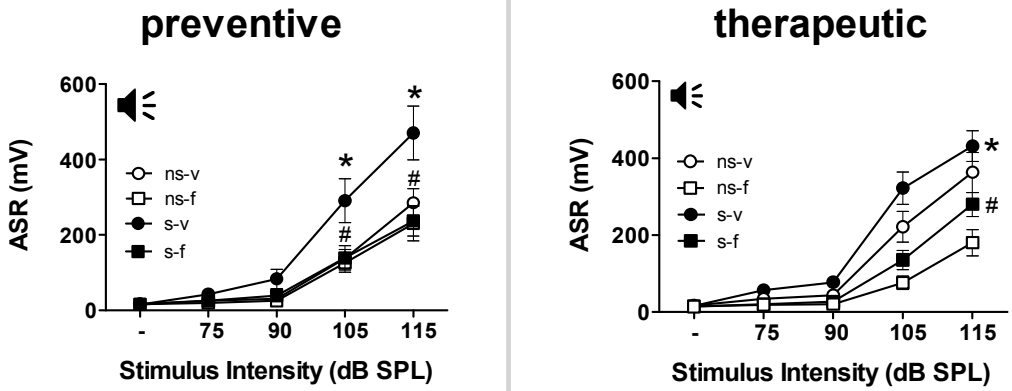

Figure 16: Four weeks of chronic fluoxetine treatment (preventive or therapeutic) reduced hyperarousal in shocked mice down to the level of non-shocked mice (Exp. 5). Mice were shocked (1.5 mA/ 2 s) or non-shocked in the chamber at d0 and remained in their home cages where they received fluoxetine (20 mg/kg/day) or vehicle via drinking water starting at d1 (preventive, left) or d28 (therapeutic, right). Statistical analysis were performed by 2-way repeated measures ANOVA (preventive: $F_{4, 240, shock*INT}=3.27$, $p<0.01$; $F_{4, 240, treat*INT}=6.34$, $p<0.001$; $F_{4, 240, shock*treat*INT}=2.86$, $p<0.05$; therapeutic: $F_{4, 236, shock*INT}=3.85$, $p<0.01$; $F_{4, 236, treat*INT}=10.77$, $p<0.001$; $F_{4, 236, shock*treat*INT}=0.8748$, $p=0.48$), followed by Newman-Keuls post-hoc test (preventive). $n_{preventive}$ = 16 per group, $n_{therapeutic}$ = 11-12 (non-shocked) or 19 (shocked) mice; *$p<0.05$ vs. non-shocked/ non-treated mice (ns-v); $^{\#}p<0.05$ vs. shocked/ non-treated mice (s-v); ns-v non-shocked, vehicle treated mice; ns-f non-shocked, fluoxetine treated mice; s-v socked, vehicle treated mice; s-f shocked fluoxetine treated mice

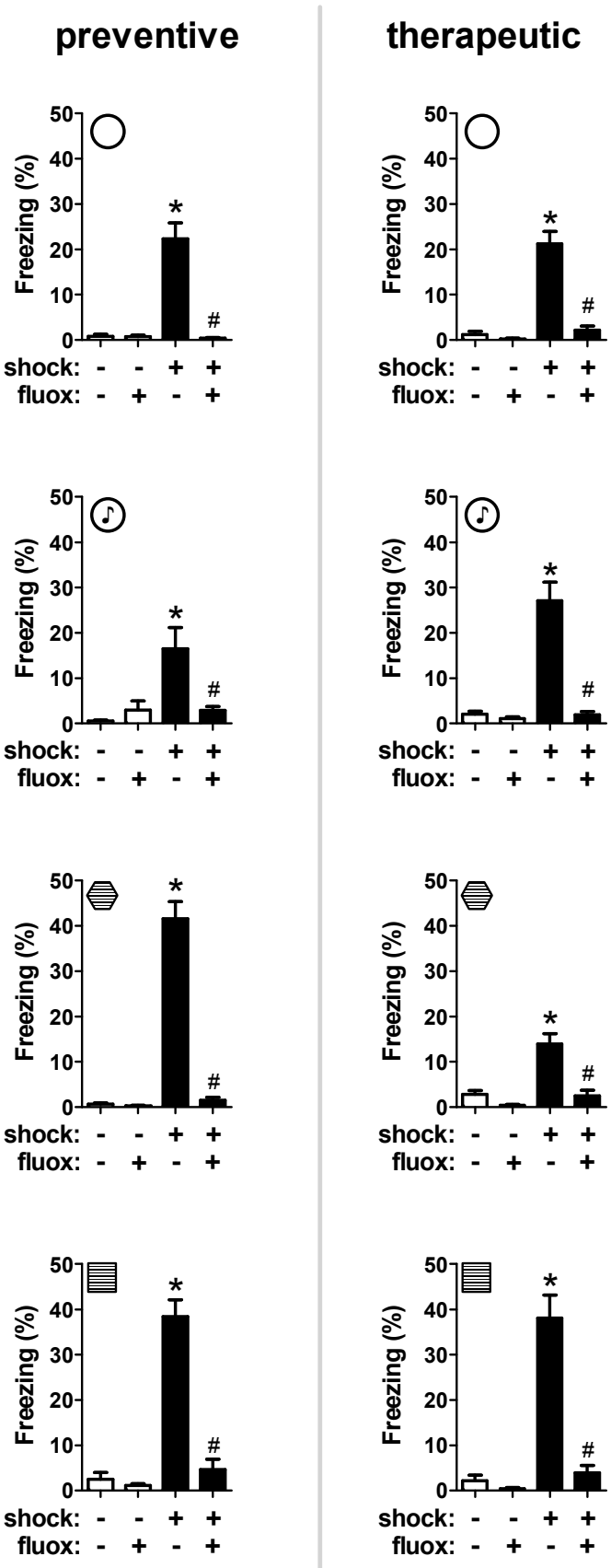

Figure 17: Four weeks of chronic fluoxetine treatment (preventive or therapeutic) reduced generalized and contextual fear in shocked mice down to the level of non-shocked mice (Exp. 5). Mice were shocked (1.5 mA/ 2 s) or non-shocked in the chamber at d0 and remained in their home cages where they received fluoxetine (20 mg/kg/day) or vehicle via drinking water starting at d1 (preventive, left) or d28 (therapeutic, right). After 4 weeks of treatment, mice were tested (under fluoxetine) for hyperarousal (Figure 16) and afterwards for their freezing responses to a neutral context (cylinder/ 3' tone), the hexagon, and the chamber. Statistical analysis were performed by 2-way ANOVA (Table 5), followed by Newman-Keuls post-hoc test. $n_{pre.}$ = 16 mice per group; $n_{ther.}$ = 11-12 (non-shocked) or 18-19 (shocked) mice; *p<0.05 vs. non-shocked/ non-treated mice; $^{\#}$p<0.05 vs. shocked/ non-treated mice

Table 5: Statistical analysis of generalized and contextual fear responses under preventive or therapeutic treatment (Figure 9, Exp. 5). Statistical analyses were performed by 2-way ANOVA.

	shock		**treatment**		**shock*treatment**	
	F	**p-level**	**F**	**p-level**	**F**	**p-level**
preventive						
Cylinder	$36.28_{(1,60)}$	<0.001	$39.03_{(1,60)}$	<0.001	$38.50_{(1,60)}$	<0.001
Tone	$9.64_{(1,60)}$	<0.01	$4.76_{(1,60)}$	<0.05	$9.91_{(1,60)}$	<0.01
Hexagon	$122.67_{(1,60)}$	<0.001	$113.02_{(1,60)}$	<0.001	$108.19_{(1,60)}$	<0.001
Chamber	$74.73_{(1,60)}$	<0.001	$59.12_{(1,60)}$	<0.001	$50.61_{(1,60)}$	<0.001
therapeutic						
Cylinder	$33.11_{(1,57)}$	<0.001	$27.34_{(1,57)}$	<0.001	$22.31_{(1,57)}$	<0.001
Tone	$18.14_{(1,56)}$	<0.001	$11.65_{(1,56)}$	<0.001	$9.85_{(1,56)}$	<0.01
Hexagon	$14.88_{(1,58)}$	<0.001	$16.62_{(1,58)}$	<0.001	$6.92_{(1,58)}$	<0.01
Chamber	$19.62_{(1,58)}$	<0.001	$26.76_{(1,58)}$	<0.001	$12.45_{(1,58)}$	<0.001

After 1 month, shocked mice showed increased CO activity in several brain regions including the prelimbic cortex (PrL), the accumbens (AccC, AccSh), the basolateral amygdaloid nucleus (BLA), the dorsal (CA1, CA3, DG) and ventral (CA1v, CA3v) hippocampus, the periaqueductal gray (PAG), the lateral hypothalamic area (LH), the striatum (CPu), and the parafascicular thalamic nucleus (PF) (Exp. 1 and 2). Interestingly, in preventive treated, shocked mice, these CO activity increases could not be detected in all structures. The following brain regions, which showed an increase in CO activity without treatment, were affected by preventive treatment with fluoxetine, i.e. the CO activity was significantly lower as in shocked, vehicle treated mice: the prelimbic cortex (PrL), the accumbens nucleus shell (AccSh), the basolateral amygdaloid nucleus (BLA), the CA1 and the DG in the dorsal hippocampus, the periaqueductal gray (PAG), the lateral hypothalamic area (LH), and the parafascicular thalamic nucleus (PF). However, there were still regions which were not affected by preventive treatment with fluoxetine, including the Acc c, the CA3 (dorsal), the ventral hippocampus (CA1, CA3), and the CPu (Figure 18 and Table 6). In contrast, therapeutic treatment with fluoxetine had no influence on the CO activity and the brain regions, which showed increased levels in CO activity due to the shock, showed no differences between fluoxetine treated and vehicle treated mice (Figure 19 and Table 7).

Influence of preventive or therapeutic treatment with fluoxetine on PTSD-like symptoms and CO activity

Preventive and therapeutic treatment with fluoxetine in shocked mice abolished PTSD-like symptoms. Under preventive treatment, shocked, fluoxetine treated mice showed increased CO activity only in a few regions (Acc core, CA3 dorsal, CA1+3 ventral), whereas under therapeutic treatment the CO activity in the analyzed brain regions of shocked, fluoxetine treated mice was significantly increased in comparison to non-shocked, vehicle treated mice and similarly to shocked, vehicle treated mice. Consequently preventive, but not therapeutic treatment prevented the development of chronic changes in CO activity.

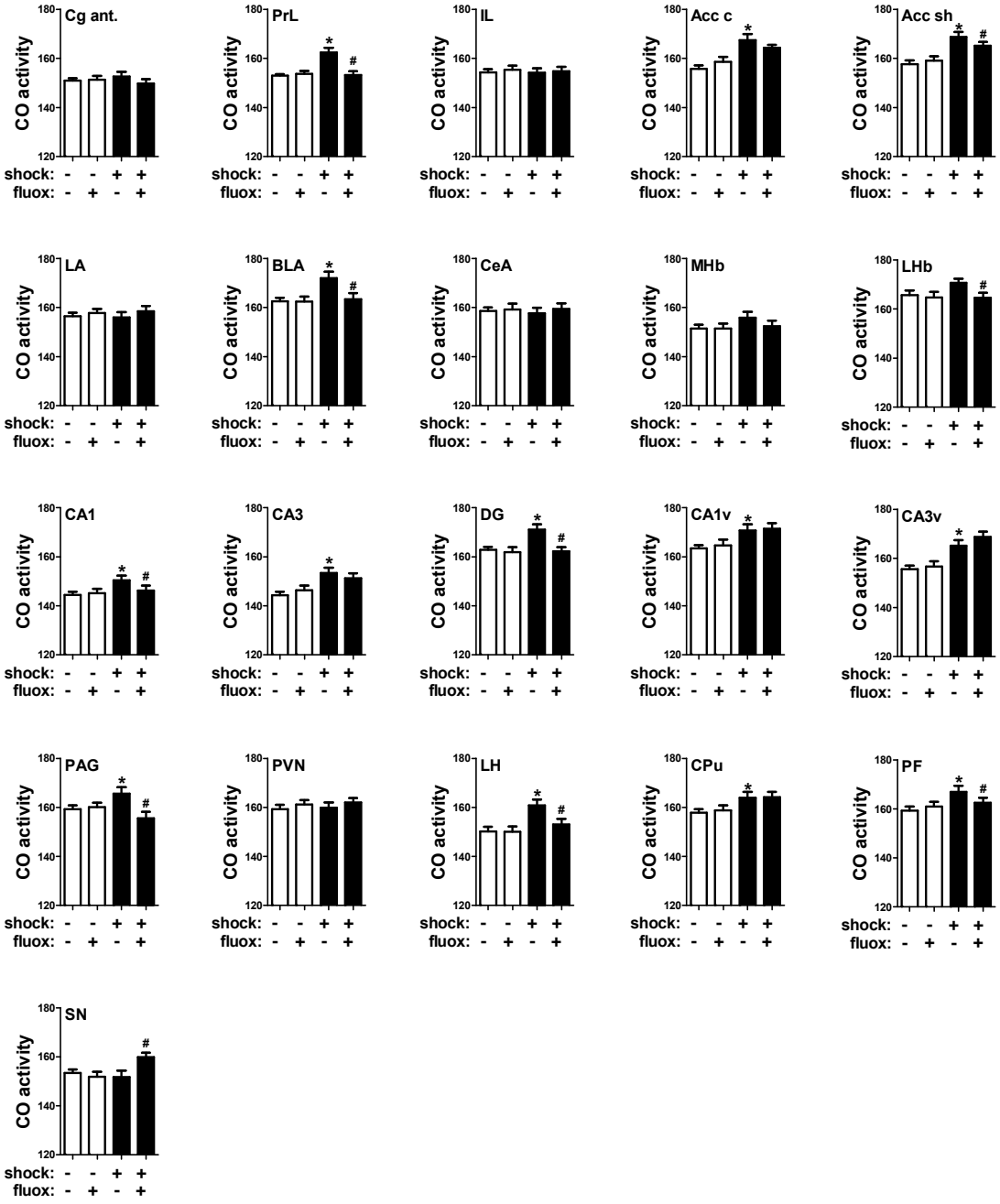

Figure 18: Preventive treatment with fluoxetine prevented the increase in CO activity in response to the shock (Exp. 5). Mice were shocked or non-shocked at d0, remained in their home cages where they were treated with fluoxetine (20 mg/kg/d) from d1 until d35. Mice were tested for hyperarousal and generalized and contextual fear responses (d28-30; Figure 16 and 18), and remained in their home cages for an additional week. Brains have been collected and CO staining was performed. Statistical analysis were performed by 2-way ANOVA (Table 6), followed by Newman-Keuls post-hoc test. ns non-shocked mice; s shocked mice; *p<0.05 vs. non-shocked/ vehicle-treated mice; $^{\#}$p<0.05 vs. shocked/ vehicle-treated mice; CO activity in mol/min/g (absolute values)

Table 6: Preventive treatment with fluoxetine prevented the increase in CO activity in response to the shock (Exp. 5). Mice were shocked or non-shocked at d0, remained in their home cages where they were treated with fluoxetine (20 mg/kg/d) from d1 until d35. Mice were tested for hyperarousal and generalized and contextual fear responses (d28-30; Figure 16 and 18), and remained in their home cages for an additional week. Brains have been collected and CO staining was performed. ns non-shocked mice; s shocked mice; S p-value of factor shock; T p-value of factor treatment; S*T p-value of the interaction; *p<0.05 vs. non-shocked/ vehicle-treated mice; #p<0.05 vs. shocked/ vehicle-treated mice; CO activity in mol/min/g (absolute values); (to be continued on the next page)

Note: Data are graphically depicted in Figure 18.

	CO activity ns/vehicle	CO activity ns/fluoxetine	CO activity s/vehicle	Co activity s/fluoxetine	S	T	S*T
Prefrontal Cortex, medial							
Cg ant.	151.0 ± 1.01 n=15	151.3 ± 1.53 n=15	152.7 ± 1.84 n=14	149.7 ± 1.80 n=15	0.99	0.68	0.40
PrL	153.0 ± 0.67 n=15	153.7 ± 1.20 n=15	162.5 ± 1.88 n=14 *	153.3 ± 1.41 n=15 #	<0.001	<0.001	<0.001
IL	154.3 ± 1.30 n=15	155.3 ± 1.69 n=15	154.2 ± 1.80 n=14	154.8 ± 1.80 n=15	0.76	0.37	0.92
Accumbens							
Acc core	155.7 ± 1.44 n=15	158.6 ± 1.97 n=15	167.5 ± 2.44 n=14 *	164.3 ± 1.17 n=15	<0.001	0.94	<0.05
Acc shell	157.6 ± 1.54 n=15	159.1 ± 1.74 n=15	168.8 ± 2.04 n=14 *	165.1 ± 1.64 n=15 #	<0.001	0.35	<0.05
Amygdala							
LA	156.5 ± 1.334 n=15	157.8 ± 1.631 n=15	156.0 ± 2.02 n=14	158.5 ± 2.21 n=15	0.75	0.33	0.94
BLA	162.6 ± 1.41 n=15	162.5 ± 1.96 n=15	172.0 ± 2.60 n=14 *	163.4 ± 2.43 n=15 #	<0.01	<0.01	<0.01
CeA	158.5 ± 1.51 n=15	159.1 ± 2.46 n=15	157.7 ± 2.24 n=14	159.4 ± 2.35 n=15	0.98	0.61	0.92
Hippocampus, dorsal							
CA1	144.3 ± 1.33 n=15	145.1 ± 1.84 n=15	150.3 ± 1.98 n=14 *	146.2 ± 2.11 n=15 #	<0.05	0.07	0.08
CA3	144.3 ± 1.38 n=15	146.3 ± 2.03 n=15	153.3 ± 2.27 n=14 *	151.3 ± 1.98 n=15	<0.001	0.48	0.15
DG	162.7 ± 1.35 n=15	161.9 ± 2.04 n=15	171.0 ± 2.10 n=14 *	162.2 ± 1.68 n=15 #	<0.01	<0.001	<0.01

Table 6: Preventive treatment with fluoxetine prevented the increase in CO activity in response to the shock (Exp. 5) (in continuation).

Hippocampus, ventral							
CA1	163.3 ± 1.45 n=15	164.7 ± 2.23 n=15	170.7 ± 2.48 n=14 *	171.4 ± 2.20 n=15	<0.001	0.96	0.60
CA3	155.6 ± 1.51 n=15	156.7 ± 2.12 n=15	165.1 ± 2.35 n=14 *	168.7 ± 2.22 n=15	<0.001	0.61	0.80
Miscellaneous							
PAG	159.1 ± 1.59 n=15	160.1 ± 1.81 n=15	165.5 ± 2.65 n=14 *	155.5 ± 2.61 n=15 #	0.39	<0.01	<0.01
PVA	159.1 ± 1.92 n=15	161.1 ± 1.86 n=15	159.8 ± 2.13 n=14	162.0 ± 1.84 n=15	0.50	0.38	0.82
LH	150.2 ± 1.83 n=15	150.0 ± 2.22 n=15	160.7 ± 2.42 n=14 *	153.0 ± 2.36 n=15 #	<0.001	<0.05	<0.05
CPu	157.8 ± 1.39 n=15	158.8 ± 1.97 n=15	164.0 ± 2.24 n=14 *	164.1 ± 2.07 n=15	<0.01	0.91	0.71
MHb	151.4 ± 1.62 n=15	151.4 ± 2.12 n=15	155.9 ± 2.22 n=14	152.4 ± 2.22 n=15	0.16	0.32	0.27
LHb	165.6 ± 1.83 n=15	164.7 ± 2.21 n=15	170.6 ± 1.83 n=14	164.6 ± 1.99 n=15 #	0.19	<0.05	0.14
PF	159.2 ± 1.71 n=15	160.9 ± 1.83 n=15	166.9 ± 2.45 n=14 *	162.4 ± 2.16 n=15 #	<0.01	0.17	<0.05
SN	153.3 ± 1.46 n=15	151.8 ± 1.99 n=15	151.7 ± 2.55 n=14	159.7 ± 1.92 n=15 #	0.06	0.18	<0.05

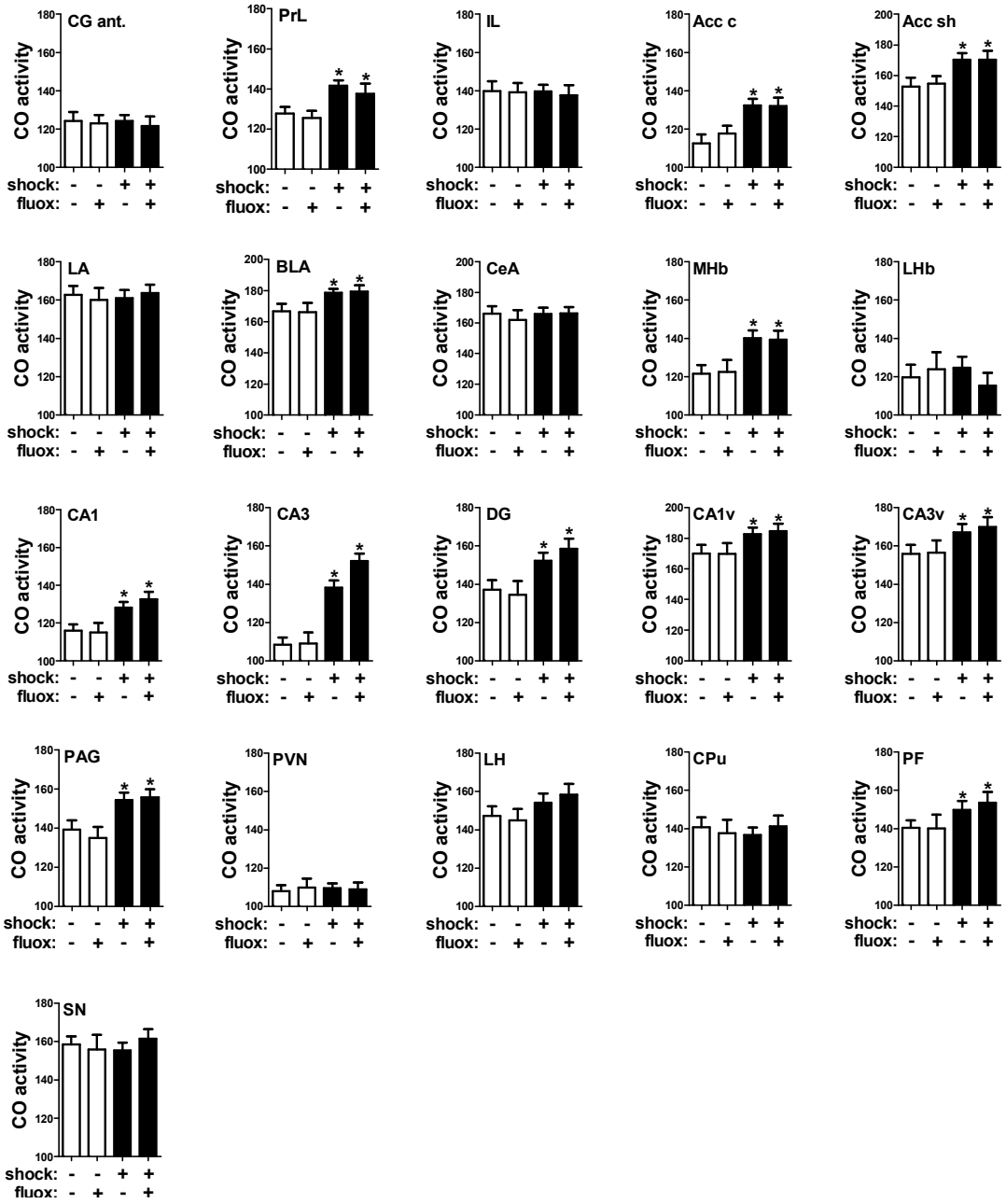

Figure 19: Therapeutic treatment with fluoxetine did not abolish the increased CO activity in response to the shock (Exp. 5) Mice were shocked or non-shocked at d0 and remained in their home cages where they were treated with fluoxetine (20 mg/kg/d) or vehicle from d28 until d72. Mice were tested for hyperarousal and generalized and contextual fear responses, and remained in their home cages for an additional week. Brains have been collected at d72 and CO staining was performed. ns non-shocked mice; s shocked mice; *p<0.05 vs. non-shocked/ vehicle treated mice (same data as Table 7); CO activity in mol/min/g (absolute values)

Table 7: Therapeutic treatment with fluoxetine did not change the increased CO activity in response to the shock (Exp. 5). Mice were shocked or non-shocked at d0 and remained in their home cages where they were treated with fluoxetine (20 mg/kg/d) or vehicle from d28 until d72. Mice were tested for hyperarousal and generalized and contextual fear responses, and remained in their home cages for an additional week. Brains have been collected at d72 and CO staining was performed. ns non-shocked mice; s shocked mice; S p-value of factor shock; T p-value of factor treatment; S*T p-value of the interaction; *p<0.05 vs. non-shocked/ vehicle treated mice; CO activity in mol/min/g (absolute values); (to be continued on the next page)

Note: Data are graphically depicted in Figure 19.

	CO activity ns/vehicle	CO activity ns/fluoxetine	CO activity s/vehicle	CO activity s/fluoxetine	S	T	S*T
Prefrontal Cortex, medial							
Cg ant.	124.3 ± 4.64 n=13	122.9 ± 4.48 n=11	124.3 ± 3.40 n=20	121.5 ± 5.12 n=18	0.87	0.64	0.88
PrL	127.8 ± 3.28 n=13	125.5 ± 3.59 n=11	141.6 ± 2.73 n=20 *	137.7 ± 4.99 n=18 *	<0.01	0.44	0.83
IL	139.8 ± 5.23 n=13	139.2 ± 4.90 n=11	139.7 ± 3.47 n=20	137.6 ± 5.23 n=18	0.87	0.78	0.88
Accumbens							
Acc core	112.6 ± 4.67 n=13	117.8 ± 3.91 n=11	125.3 ± 3.39 n=20 *	132.0 ± 4.37 n=18 *	<0.01	0.16	0.86
Acc shell	152.8 ± 5.91 n=13	154.5 ± 4.88 n=11	163.2 ± 4.38 n=20	170.4 ± 5.81 n=18	<0.05	0.42	0.63
Amygdala							
LA	162.6 ± 4.69 n=13	160.0 ± 6.23 n=11	160.9 ± 4.16 n=20	163.6 ± 4.38 n=18	0.84	0.99	0.59
BLA	166.7 ± 4.73 n=13	166.0 ± 5.95 n=11	178.5 ± 2.71 n=20 *	179.4 ± 3.96 n=18 *	<0.01	0.98	0.85
CeA	165.9 ± 4.92 n=13	161.9 ± 6.55 n=11	165.7 ± 4.16 n=20	166.2 ± 4.25 n=18	0.68	0.71	0.65
Hippocampus, dorsal							
CA1	116.0 ± 3.23 n=13	115.0 ± 5.07 n=11	128.2 ± 2.97 n=20 *	132.6 ± 4.05 n=18 *	<0.001	0.66	0.49
CA3	108.4 ± 3.80 n=13	109.1 ± 5.63 n=11	138.1 ± 3.83 n=20 *	152.1 ± 3.99 n=17 *	<0.001	0.15	0.20
DG	137.1 ± 4.85 n=13	134.5 ± 7.11 n=11	152.3 ± 4.16 n=20 *	158.5 ± 5.33 n=18 *	<0.001	0.75	0.41

Table 7: Therapeutic treatment with fluoxetine did not change the increased CO activity in response to the shock (Exp. 5, in continuation).

Hippocampus, ventral							
CA1	178.0 ± 5.47 n=13	177.6 ± 7.06 n=11	179.6 ± 4.29 n=20	184.5 ± 5.07 n=17	<0.01	0.89	0.83
CA3	163.8 ± 4.71 n=13	164.4 ± 6.55 n=11	164.0 ± 4.46 n=20	169.8 ± 5.14 n=17	<0.05	0.74	0.83
Miscellaneous							
PAG	139.2 ± 4.77 n=13	135.1 ± 5.57 n=11	154.4 ± 3.81 n=20 *	155.8 ± 4.14 n=18 *	<0.001	0.76	0.54
PVA	107.9 ± 3.25 n=13	109.9 ± 4.62 n=11	109.4 ± 2.56 n=20	108.9 ± 3.46 n=18	0.93	0.84	0.72
LH	155.3 ± 5.05 n=13	152.9 ± 5.91 n=11	151.3 ± 4.70 n=20	158.4 ± 5.54 n=17	0.07	0.87	0.55
CPu	140.7 ± 5.21 n=13	137.6 ± 7.00 n=11	136.8 ± 3.65 n=20	141.2 ± 5.65 n=17	0.97	0.90	0.48
MHb	133.4 ± 4.52 n=13	130.5 ± 6.10 n=11	133.0 ± 4.02 n=20 *	139.3 ± 4.60 n=17 *	<0.01	0.45	0.59
LHb	119.5 ± 6.64 n=13	123.8 ± 8.98 n=11	124.4 ± 5.92 n=20	115.2 ± 6.77 n=17	0.80	0.73	0.35
PF	147.3 ± 3.97 n=13	143.1 ± 7.06 n=11	149.8 ± 4.68 n=20	153.4 ± 5.70 n=17	<0.05	0.75	0.73
SN	158.4 ± 4.28 n=14	155.9 ± 7.61 n=10	155.3 ± 4.28 n=20	161.5 ± 5.05 n=17	0.84	0.54	0.57

1.19 Relapse of PTSD-like symptoms after 4 weeks of wash-out subsequent to therapeutic, but not preventive treatment (Exp. 6)

At the end of the preventive treatment, mice were tested for freezing behavior in the cylinder (1' tone) to look for the PTSD-like symptoms and the efficiency of fluoxetine treatment (Figure 20, top). The therapeutically treated mice were tested at d28, to look for the PTSD-like symptoms and assigned them into 2 groups with identical freezing levels, and at d56 to scrutinize for the efficiency of fluoxetine treatment (Figure 20, bottom).

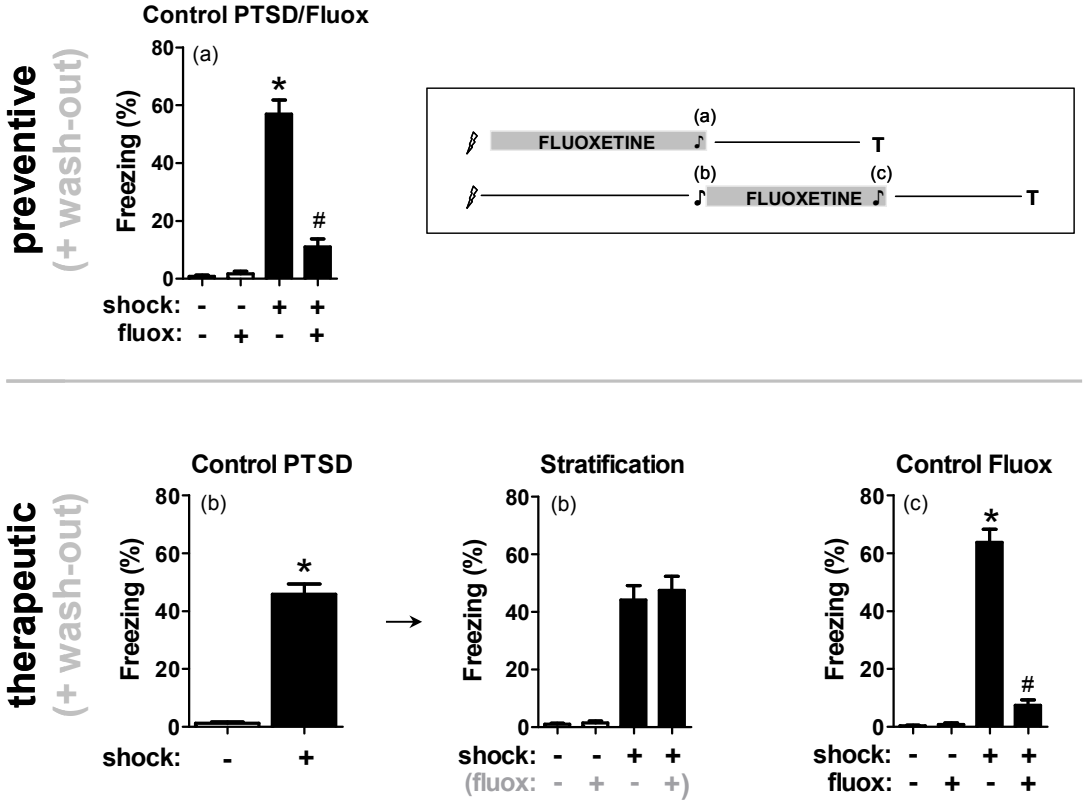

Figure 20: To look for the presence of PTSD-like symptoms and the efficiency of fluoxetine treatment, mice were exposed to a 1' tone protocol (Exp. 6). Mice were shocked (1.5 mA/ 2 s) or non-shocked in the chamber at d0 and remained in their home cages where they received fluoxetine (20 mg/kg/day) or vehicle via drinking water starting at d1 (preventive, above) or d28 (therapeutic, bottom) for 28 days. At d28 mice were exposed to a neutral context (cylinder/ 1' tone) to look for PTSD-like symptoms and the efficiency of fluoxetine treatment (preventive treatment) or the existing of PTSD-like symptoms (therapeutic treatment) and stratification for the prospective fluoxetine treatment. At d56 therapeutically treated mice were exposed to the cylinder again to measure the efficiency of fluoxetine treatment. Statistical analysis were performed with 2-way ANOVA (preventive: $F_{1, 58, shock}$=98.48, p<0.001; $F_{1, 58, treat.}$ =62.06, p<0.001; $F_{1, 58, shock*treat.}$=54.04, p<0.001; therapeutic: $F_{1, 59, shock}$=26.67, p<0.001; $F_{1, 59, treat.}$=5.58, p<0.001; $F_{1, 59, shock*treat.}$=19.03, p<0.001), followed by Newman-Keuls post-hoc test or unpaired t-test (therapeutic: p<0.001, t=12.52, df=60). $n_{preventive}$ = 15-16 mice per group, $n_{therapeutic}$ = 32 mice per group (2 groups) or $n_{therapeutic}$ = 15-16 mice per group (4 groups); *p<0.05 vs. non-shocked/ vehicle-treated mice; #p<0.05 vs. shocked/ vehicle-treated mice

Four weeks after the end of fluoxetine treatment, the PTSD-like symptoms in preventively treated mice stayed on baseline level (level of non-shocked/ vehicle treated mice) both in hyperarousal (Figure 21) and in the generalized and contextual fear (Figure 22), whereas in therapeutically treated mice symptoms relapsed to the level of shocked vehicle treated mice.

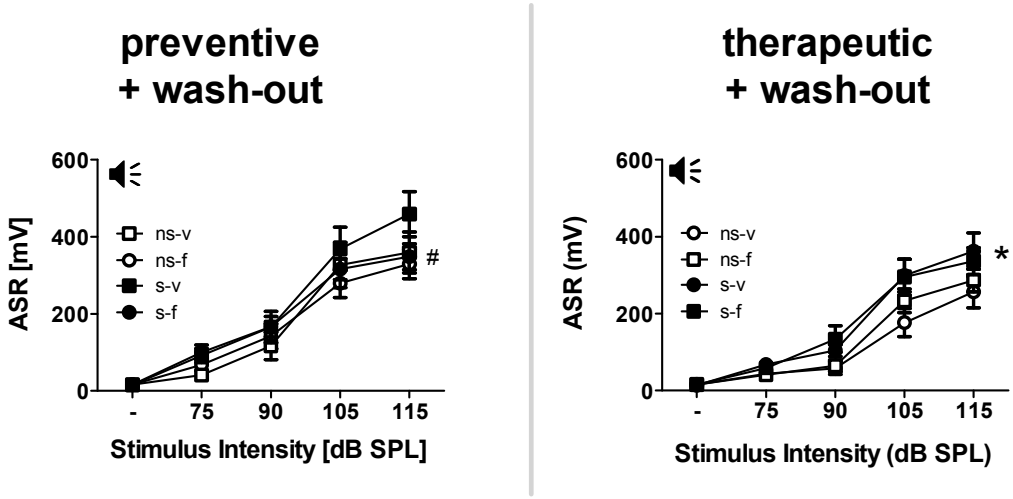

Figure 21: Four weeks after the end of the preventive or therapeutic treatment with fluoxetine, mice were tested for hyperarousal (Exp. 6). Mice were shocked (1.5 mA/ 2 s) or non-shocked in the chamber at d0 and remained in their home cages, where they received fluoxetine (20 mg/kg/day) or vehicle via drinking water, starting at d1 until d28 (preventive, left) or at d28 until d56 (therapeutic, right). After additional 4 weeks (drug-free), mice were tested for hyperarousal symptomatology (ASR). Statistical analysis were performed by 2-way repeated measures ANOVA (preventive: $F_{4,\ 236,\ shock*INT}$=0.46, p=0.76; $F_{4,\ 236,\ treat*INT.}$=3.33, p<0.01; $F_{4,\ 236,\ shock*treat*INT}$=0.19, p=0.94; therapeutic: $F_{4,\ 232,\ shock*INT}$= 3.28, p<0.01; $F_{4,\ 232,\ treat*INT}$=0.33, p=0.85; $F_{4,\ 232,\ shock*treat*INT.}$=1.45, p=0.22), followed by Newman-Keuls post-hoc test. $n_{pre.\ +\ wash\text{-}out}$ = 15-16, $n_{ther.\ +\ wash\text{-}out}$ = 14-15; ns-v non-shocked, vehicle treated mice; ns-f non-shocked, fluoxetine treated mice; s-v socked, vehicle treated mice; s-f shocked fluoxetine treated mice; *p<0.05 s-v vs. ns-v mice; $^{\#}$p<0.05 s-f vs. s-v mice

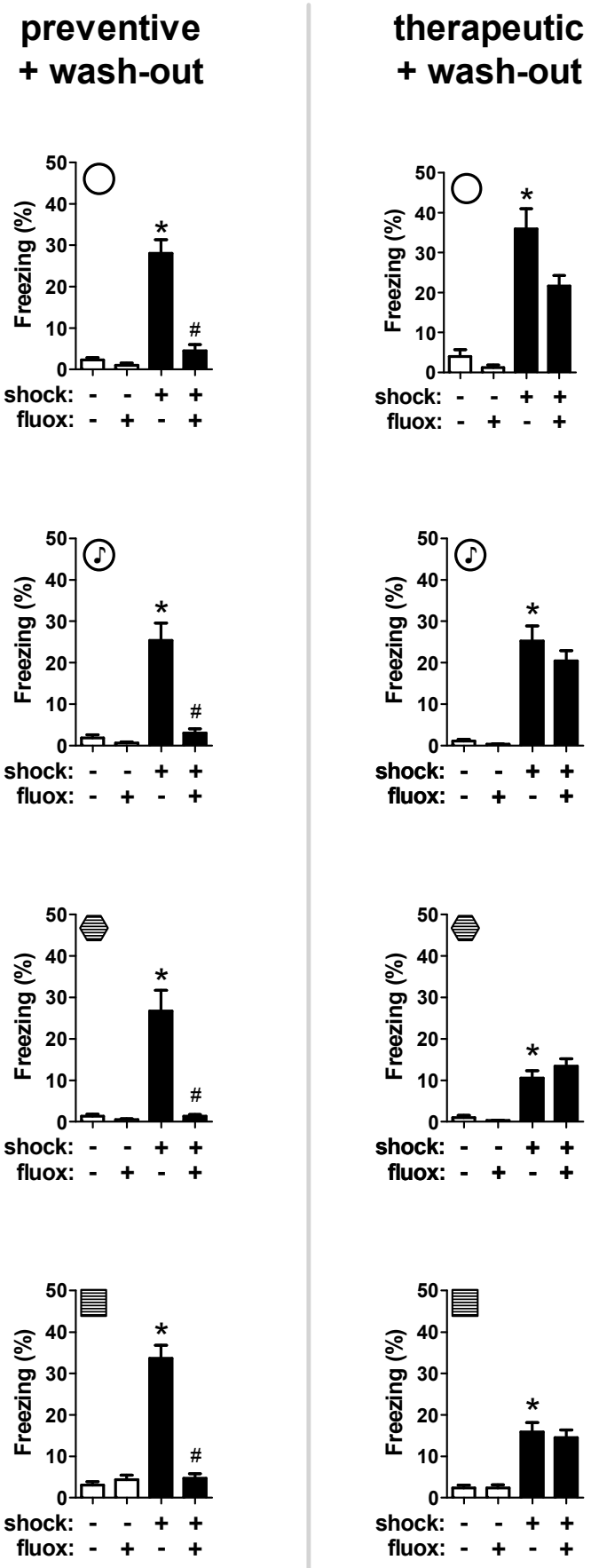

Figure 22: Four weeks after the end of preventive or therapeutic treatment with fluoxetine, mice were tested for generalized and contextual fear (Exp. 6). Mice were shocked (1.5 mA/ 2 s) or non-shocked in the chamber at d0 and remained in their home cages, where they received fluoxetine (20 mg/kg/day) or vehicle via drinking water, starting at d1 until d28 (preventive, left) or starting at d28 until d56 (therapeutic, right). After additional 4 weeks (drug-free) mice were tested for hyperarousal (Figure 22) and afterwards to a neutral context (cylinder/ 3' tone), the hexagon, and the sensitized chamber. The percentage of time mice spent in freezing behavior were analyzed. Statistical analysis were performed with 2-way ANOVA (Table 8), followed by Newman-Keuls post-hoc test. $n_{prev. + wash.}$ = 15-16, $n_{ther. + wash.}$ = 16; *p<0.05 vs. non-shocked/ vehicle treated mice. #p<0.05 vs. shocked/ vehicle treated mice.

Table 8: Statistical analysis of generalized and contextual fear responses 4 weeks after the end of preventive or therapeutic treatment (Figure 22, Exp. 6). Statistical analysis was performed with 2-way ANOVA.

	shock		treatment		shock*treatment	
	F	**p-level**	**F**	**p-level**	**F**	**p-level**
preventive						
Cylinder	$38.03_{(1,59)}$	<0.001	$28.46_{(1,59)}$	<0.001	$23.81_{(1,59)}$	<0.001
Tone	$29.01_{(1,59)}$	<0.01	$24.76_{(1,59)}$	<0.001	$20.22_{(1,59)}$	<0.001
Hexagon	$23.75_{(1,59)}$	<0.001	$23.64_{(1,59)}$	<0.001	$24.03_{(1,59)}$	<0.001
Chamber	$55.56_{(1,59)}$	<0.001	$53.09_{(1,59)}$	<0.001	$53.26_{(1,59)}$	<0.001
therapeutic						
Cylinder	$51.28_{(1,60)}$	<0.001	$2.12_{(1,60)}$	0.15	$2.62_{(1,60)}$	0.11
Tone	$79.79_{(1,60)}$	<0.001	$0.01_{(1,60)}$	0.92	$0.40_{(1,60)}$	0.53
Hexagon	$21.04_{(1,60)}$	<0.001	$0.48_{(1,60)}$	0.49	$0.50_{(1,60)}$	0.48
Chamber	$21.97_{(1,60)}$	<0.001	$0.00_{(1,60)}$	1.00	$0.87_{(1,60)}$	0.35

Changes of PTSD-like symptoms and CO activity four weeks after the end of preventive or therapeutic treatment with fluoxetine

Four weeks after the end of fluoxetine treatment, the PTSD-like symptoms in the preventively treated group were still at baseline level (i.e. were prevented), whereas there was evidence of a relapse in hyperarousal symptomatology and generalized and contextual fear responses in the therapeutically treated group.

Discussion

The motivation behind this study was to advance the development of novel PTSD therapy strategies by providing a better understanding of the neurobiological mechanisms in an animal model of the disease with high face, construct, and predictive validity.

In our mouse model of PTSD, a brief, inescapable electric foot shock, after an incubation time of 1 month, led to PTSD-like symptoms including hyperarousal symptomatology (Figure 8), generalized and contextual fear (Figure 9), and avoidance behavior (Figure 10) as well as increased CO activity in the prelimbic cortex, the hippocampus, the basolateral amygdaloid nucleus, and the periaqueductal gray, among others (Table 2 and 4). However, in the early aftermath of a trauma, neither the PTSD-like symptoms (Figure 11; Siegmund and Wotjak, 2007) nor changes in the CO activity were observed (Table 3).

Furthermore, the preventive as well as the therapeutic treatment with fluoxetine (an SSRI belonging to the first-line treatment of PTSD) inhibited the PTSD-like symptoms, hyperarousal symptomatology (Figure 16) and generalized and contextual fear (Figure 17), after 4 weeks of treatment, whereas changes in the CO activity were affected only after the preventive (Figure 18), but not after the therapeutic treatment (Figure 19).

In addition, discontinuation of treatment led to relapse of PTSD-like symptoms including hyperarousal symptomatology (Figure 21) as well as generalized and contextual fear (Figure 22) in the therapeutically, but not in the preventively treated group.

1.20 Traces of a trauma

1.20.1 Face validity

In humans, PTSD is defined by the presence of trauma related memories, hyperarousal, emotional numbing, and avoidance of trauma-related stimuli for at least 1 month (DSM IV). To mimic these obvious behavioral symptoms in our mouse model of PTSD, the present study used a brief, inescapable electric foot shock (the traumatic event for the mice) which had no influence on the hyperarousal symptomatology (Figure 11) nor the generalized and contextual fear (Siegmund and Wotjak, 2007) 2 days after the trauma. In contrast, after an incubation time of 4 weeks, the traumatic event led to PTSD-like symptoms as hyperarousal symptomatology (increased acoustic startle responses (ASR); Figure 8), generalized and contextual fear (increased freezing responses to the cylinder, the tone, the hexagon, and the chamber; Figure 9), and avoidance behavior (increased avoidance in the conditioned odor avoidance test (CODA); Figure 10) in shocked mice as compared to non-shocked. These behavioral data are in-line with previous studies in our mouse model (Golub et al., 2009; Pamplona et al., 2011) and other animal models of PTSD, for example Wang and colleagues using an underwater trauma (Wang et al. 2000), Adamec and colleagues using exposure to a predator as stressor (Adamec et al., 1998) or Cohen and colleagues using predator (scent) stress (Cohen et al., 2003, 2008).

As mentioned above, in the early aftermath of a trauma, the PTSD-like symptoms could not be detected and developed over a time course of 4 weeks. Nevertheless, if the mice only remained in their home-cage after the traumatic event, the PTSD-like symptoms peak around day 28 and are diminished with time and were not detectable after 3 to 5 months. The reason for this phenomenon might be the brief, electric foot shock (1.5 mA/ 2 s) which is probably not effective enough to elicit PTSD-like symptoms in mice for a longer time period, but more aversive foot shock protocols are precluded by the local ethics rules and the animal protection law. It is unclear whether other animal models of PTSD show PTSD-like symptoms for a longer time period. In most studies, the animals were tested within days or weeks after the traumatic event

(Adamec and Shallow, 1993; Cohen et al., 2003; Belda et al., 2008) and not after months.

This phenomenon limited the time interval after the traumatic event in which studies could be performed. Even so, the observation of significant effects between shocked vs. non-shocked and treated vs. non-treated mice almost 3 months after the traumatic event was possible (ASR - Figure 21; generalized. and contextual fear - Figure 22; right side).

Nevertheless, the decrease of PTSD symptoms might be the explanation for the marginal differences in the hyperarousal symptomatology 3 months after the traumatic event (Exp. 6: therapeutic treatment + wash-out; Figure 21).

PTSD is a clinical symptomatological diagnosis and some cardinal symptoms are reflected in our animal model; however the neurobiological basis of the disorder is still not completely understood, thus preventing the development of specific therapeutic strategies. Therefore it is necessary to investigate the neurobiological mechanism.

1.20.2 Construct validity

Although there is a huge amount of literature concerning molecular changes in PTSD patients as well as in animal models of PTSD, little is known about the time-course of these molecular changes – e.g. which are principally involved in the initiation of the pathophysiology and which are merely consequences of others. Focusing on the tonic changes of neuronal activity which are considered surrogate markers of long-lasting disorders such PTSD, a lot of molecular und structural changes after a traumatic event, both in humans and animals, have been identified. For example, several studies showed the reduction of the hippocampal volume in PTSD patients (Stein et al., 1994; Bremner et al., 1995) which have also been observed in mice (Golub et al., 2011), the enhancement of AMPA receptor signaling (Thoeringer et al., submitted), and increased kinase (pAKT and GSK-3β) activity (Dahlhoff et al., 2010) have been described. The group of Gonzalez-Lima (Gonzalez-Lima and Garrosa, 1991; Gonzalez-Lima and Cada, 1994; Poremba and Jones, 1998) and others (Hevner and Wong-Riley, 1989, 1990; Zhang et al., 2006) used CO as a neuronal activity marker

for example in the classical conditioning paradigms. Therefore CO activity has been identified as a valuable tool for detecting changes of neuronal activity.

Alterations of CO activity as a consequence of the traumatic event

For behavioral correlates to the expected CO activity changes between shocked and non-shocked mice, mice were tested for PTSD-like symptoms such as hyperarousal symptomatology, generalized and contextual fear, and avoidance behavior. To avoid acute influence of testing, CO staining was performed 1 week after the last behavioral test. Brain regions linked to emotions in general, to fear, or learning were analyzed. The brain regions with their relevant function in detail:

- The medial prefrontal cortex (mPFC) including the subregions Cg1, PrL, and IL because of its relevance for executive functions, including decision-making, attention control, working memory, stress response, behavioral inhibition, and moderating the correct social behavior (Yamasaki et al., 2002; Heidbreder and Groenewegen, 2003; Morgane et al., 2005; Rossetti and Carboni, 2005; Shad et al., 2011; Staiti et al., 2011).
- The nucleus accumbens (Acc) including both sub-nuclei (AccC and AccSh) because of its important role in fear and aggression. Additionally, this structure is critical for the acquisition and expression behavior; a number of reward related behaviors, and processes specific information about reward availability (Knutson and Cooper, 2005; Wise, 2006; Day and Carelli, 2007; Bradfield and McNally, 2010).
- The amygdala (AMY) including the lateral (LA), basolateral (BLA), and central amygdaloid nuclei (CeA) as a major structure involved in emotional learning and attention (Adolphs et al., 1995; LeDoux, 2001).
- The dorsal hippocampus including CA1, CA3, and the dentate gyrus and the ventral hippocampus (CA1, CA3) for their relevance in learning and memory consolidation (McClelland et al., 1995; Norman and O'Reilly, 2003; Yassa and Stark, 2011).

- The periaqueductal gray (PAG) which is involved in the regulation of defensive behavior (Carrive, 1993; Yamashita et al., 2011).
- The paraventricular thalamic nucleus ant. (PVA) which is activated by stress and governs control over the HPA axis (Antoni, 1986; Whitnall, 1993).
- The lateral hypothalamic area (LH) involved in fear regulation; (Wise, 1974; Elmquist et al., 1999; Swanson, 2000).
- The dorsal striatum (CPu) activated by intense and aversive stimuli and involved in learning and memory (Packard and Knowlton, 2002).
- The habenula including the medial (MHb) and lateral (LHb) part involved in pain processing, fear, learning, and stress (Sutherland and Nakajima, 1981; Benabid and Jeaugey, 1989; Murphy et al., 1996; Hikosaka, 2010).
- The parafascicular thalamic nucleus (PF) (Steriade and Deschenes, 1984; Vogt et al., 2008). And:
- the substantia nigra (SN) which is involved, among others, in learning (Da Cunha et al., 2009).

Significant increase in the CO activity in shocked compared to non-shocked mice was found in the following brain regions: the prelimbic cortex (PrL), the accumbens (core and shell), the basolateral amygdaloid nucleus, the dorsal hippocampus (CA1, CA3 and DG), the ventral hippocampus (CA1 and CA3), the periaqueductal gray (PAG), the lateral hypothalamic area (LH), the striatum (CPu), the medial part of the habenula (MHb), and the parafascicular thalamic nucleus (PF). In contrast, in the cingulate (Cg ant.) and infralimbic cortex (IL), the lateral and central amygdaloid nucleus (LA and CeA), the paraventricular thalamic nucleus (PVN), the lateral part of the habenula (LHb), and the substancia nigra (SN) showed no differences in the CO activity (Table 2, Table 3, Table 6, and Table 7).

It is interesting that in all of the analyzed brain regions either increases or no changes in CO activity were observed; no structure displayed a decrease. This observation might indicate a limitation of the method. However, there are

several points which underscore the quality of this method: First, the fact that the CO activity at day 9 after the foot shock, so in the early aftermath of the trauma, was not affected at all (Table 3). These findings give important insight into the neuronal network mechanism during the post-traumatic incubation period; further scrutiny of these mechanisms might unravel novel targets for preventive therapy. Second, the remarkable reproducibility of the CO staining (Table 9), which showed a high consistency between the measurements. Third, the fact that regions e.g. which are localized in the same slice and consequently on the same slide and in the same staining chamber, showed differences in activity changes. For example, the basolateral amygdaloid nucleus (BLA) always showed a significant increase in CO activity in shocked compared to non-shocked mice after fear incubation, whereas the lateral and central amygdaloid nuclei (LA and CeA) showed no changes in CO activity at any time. The same was observed in the medial prefrontal cortex, where the prelimbic cortex (PrL) showed a significant increase in shocked compared to non-shocked mice, whereas the cingulate cortex (Cg ant.) and the infralimbic cortex (IL) did not. In summary, the CO staining proved for analyzing tonic changes in neuronal activity in the brain in our mouse model of PTSD. Furthermore, Shumake and colleagues found in congenitally helpless rats decreased levels of CO activity compared to non-helpless rats (Shumake et al., 2002). This increase was normalized after fluoxetine treatment. In conclusion, PTSD is equitable with an increase in the CO activity.

To compare the activation patterns from this study with other studies, Lui and colleagues (2009) measured the resting state activities via functional MRI (fMRI) in (human) survivors within 25 days after the Wenchuan 8.0 earthquake in China (2008). They found increased resting state activity in the prefrontal cortex, the nucleus accumbens, the hippocampus, as well as the amygdala. (The authors did not further subdivide these brain regions.) These findings are in consent with the results from the present study, where the hippocampus, the basolateral amygdaloid nucleus (BLA), the periaqueductal gray (PAG), the prelimbic cortex (PrL), and the nucleus accumbens (AccC and AccSh) showed significantly increased CO activity.

Table 9: Overview of CO activity changes in the regions of interest (ROI) of all experiments. Left: Shocked (vehicle treated) mice were compared to non-shocked (vehicle treated) mice (control). Right: Shocked, fluoxetine treated mice were compared to non-shocked, fluoxetine treated mice (control). Red: significant increase in CO activity; green: no difference in CO activity if compared to non-shocked, vehicle treated mice and significant decrease if compared to shocked, vehicle treated mice; grey: no change in CO activity.

	shocked / non-shocked					**fluox / veh**	
Post shock	d9	d35	d37	d42	d65	d37	d65
Nr. of experiment	2	2	5	1	5	5	5
Prefrontal Cortex, medial							
Cg ant.							
PrL							
IL							
Accumbens							
Acc core							
Acc shell							
Amygdala							
LA							
BLA							
CeA							
Hippocampus, dorsal							
CA1							
CA3							
DG							
Hippocampus, ventral							
CA1							
CA3							
Miscellaneous							
PAG							
PVN							
LH					p=0.07		
CPu							
MHb							
LHb							
PF							
SN							

Considering these findings, the first working hypothesis *"A traumatic event (a single electric foot shock) changes the tonic activity (cytochrome c oxidase) in the brain one month after the trauma."* can be accepted.

However, CO activity is a marker for neuronal activity, but to this point the molecular events leading to it remain unclear. Therefore, the glutamatergic and gabaergic system in the dorsal hippocampus, CA1 region and dentate gyrus, were investigated.

Recordings of $GABA_A$-mIPSCs in the CA1 and the dentate gyrus in the dorsal hippocampus of shocked mice showed no changes in consequence of the traumatic event, neither in the amplitudes nor in frequencies, compared to non-shocked animals at d28. However, both, the increased amplitudes in AMPAR-mEPSCs in the dentate gyrus at day 28 (electrophysiological study) and the decreased freezing responses 30 min after intrahippocampal injection of philanthotoxin 433 (PhTX), a specific blocker of Ca^{2+}-permeable GluR1 containing AMPARs, at day 29, showed an influence of the traumatic event on the glutamatergic system. This increase in AMPAR-mEPSC amplitudes may result from an increased surface expression of AMPARs, especially GluR1-containing AMPARs (O'Brien et al., 1998, Thoeringer et al., submitted). In addition, the reduced freezing responses 30 min after intrahippocampal injection of philanthotoxin 433 at day 29 post shock is in accordance with reduced contextual fear after pharmacological blockade of hippocampal AMPA and kainate receptors by NBQX and reduced contextual fear after inhibition of GluR1 synthesis (Thoeringer et al., submitted). Learning-induced trafficking and translation of this specific glutamate receptor subtype in the hippocampus have consistently been shown to be required for the consolidation of context (Matsuo et al., 2008; Mitsushima et al., 2011) or auditory fear memory (Thoeringer et al., 2010). Noteworthy, treatment with philanthotoxin 433 attenuated contextual fear 29 days, but not 3 days, after conditioning, thus substantiating that CA^{2+}-permebale GluR1 containing AMPARs play also a prominent role in the retention and/or expression of remote fear memories.

1.21 Pharmacological interventions of PTSD

Every group of psychopharmacological agents has been claimed to be effective for the treatment of at least some aspects of the PTSD symptomatology. However, first-line therapies of PTSD in humans are selective serotonin reuptake inhibitors (SSRIs) including paroxetine and fluoxetine which reduce most of the PTSD symptoms (Connor et al., 1999; Van der Kolk, 2001; Tucker et al., 2001; Martenyi et al., 2002). However, the relapse rate after the cessation of treatment is high (Davidson, 1998). Therefore, this study investigated the effects of chronic treatment with fluoxetine on PTSD-like symptoms as well as CO activity as well as the relapse of behavioral symptoms after cessation. Concerning the different stage of PTSD, the incubation period and the maintenance, the treatment was started at different time points. The preventive treatment was started at the early aftermath of a trauma (day 1) and the therapeutic treatment after the incubation period, when PTSD-like symptoms already had occurred (day 28). Furthermore, the probability of a relapse after successful treatment with fluoxetine, testing 4 weeks after discontinuation the preventive as well as the therapeutic treatment was investigated.

1.21.1 Predictive validity

Preventive or therapeutic therapy with fluoxetine

Preventive and therapeutic treatment with fluoxetine in shocked mice abolished PTSD-like symptoms in the hyperarousal symptomatology (Figure 16) and the generalized and contextual fear (Figure 17), which is in-line with the reduced avoidance behavior under chronic fluoxetine treatment shown by Pamplona and colleagues (Pamplona et al., 2011), reduced freezing response to conditioned stimuli by Siegmund and Wotjak (2007), and analog to humans studies (Connor et al., 1999; Martenyi, 2002). Furthermore, this study showed that under preventive treatment shocked, fluoxetine treated mice showed increased CO activity only in a few brain regions compared to control (vehicle treated) mice. The affected regions were as follows: the nucleus accumbens core (Acc C), the CA3 of the dorsal hippocampus, the ventral hippocampus (CA1 and CA3), and the striatum (CPu) (Figure 18), whereas under therapeutic treatment in every

analyzed brain regions the CO activity in shocked, fluoxetine treated mice were at the same level as controls (shocked, vehicle treated mice; Figure 19). These results were summarized in Table 9. Consequently, preventive but not therapeutic treatment prevented the development of chronic changes in CO activity. These findings are in consent with findings from Reinés and colleagues, who treated rats chronically with fluoxetine (10mg/kg i.p.) starting at d4 after exposure to the learned helplessness (LH) paradigm and found after 21 days of treatment decreased levels of escape latencies compared to vehicle treated rats (Reinés et al., 2007). In addition, in congenitally helpless rats chronic fluoxetine treatment increased the immobility in the forced-swim test to baseline level and the CO changes were in the predicted direction of metabolic normalization (Shumake et al., 2010). The direction of metabolic normalization was also observed in our mouse model where the CO activity after preventive treatment was at baseline level (level of non-shocked mice) in most of the analyzed regions.

Therefore, the second working hypothesis *"Chronic treatment with fluoxetine starting either right after the trauma (preventive treatment) or 28 days later (therapeutic treatment) reverses the PTSD-like symptoms."* can be accepted. Whereas the third hypothesis "*Chronic treatment with fluoxetine starting either right after the trauma (preventive treatment) or 28 days later (therapeutic treatment) reverses the changes in CO activity."* was only partly confirmed. The preventive treatment with fluoxetine, but not the therapeutic, reversed the changes in CO activity.

Wash-out after preventive and therapeutic therapy with fluoxetine

Four weeks after discontinuation the fluoxetine treatment, the PTSD-like symptoms in the preventively treated group were still at baseline level (level of non-shocked mice; Figure 21 and 23), whereas in the therapeutically treated group, there was evidence of a relapse in hyperarousal symptomatology (Figure 21) as well as in generalized and contextual fear responses (Figure 22). These findings are in-line with the findings of Reinés and colleagues, who investigate the changes in synaptic markers including synaptophysin (SYN) and postsynaptic density 95 (PSD-95), in an animal of depression (Reinés et al.,

2007). Even starting the treatment at day 4, the behavioral symptoms and the changed levels of synaptic markers were present before starting the treatment and could inhibit via treatment with fluoxetine. However, after discontinuation the treatment, the behavioral symptoms as well as the changed levels of synaptic markers relapsed. Therefore, the improvement of symptoms as well as the recovered levels of synaptic and cytosceletal proteins under treatment are merely an inhibition but not a curative effect.

Therefore the fourth and last working hypothesis *"The discontinuation of treatment with fluoxetine after preventive or therapeutic treatment, leads to a relapse of PTSD-like symptoms."* is only partly true. After the therapeutic treatment with fluoxetine, which inhibited the PTSD-like symptoms, but did not affect the CO activity, a wash-out period of 4 weeks led to relapse of symptoms. However, after the preventive treatment, which prevented the increase of CO activity in most of the analyzed brain regions and therefore the development of PTSD-like symptoms, no evidence of relapse could be observed.

Conclusion

Therapeutic treatment of PTSD, as commonly used in humans, is able to inhibit the PTSD-like symptoms in our animal model of PTSD, but is not able to cure the disease and therefore relapse of symptoms occurred after the end of therapy. However, preventive treatment of PTSD is able to avoid tonic activity changes in the neuronal activity and therefore avoid the development of PTSD-like symptoms and as a result relapse of symptoms does not occur.

For the first time it could be shown, that pharmacological intervention of PTSD is possible not only as a symptomatically and therefore short-dated therapy, but rather for a curative and long-term therapy of this disease. Taken together:

The sooner the better!

(At least if you are a mouse.)

And:

The reversal of CO activity changes may serve as a marker for the development of successful treatment!

Summary

Posttraumatic stress disorder (PTSD) is characterized by exaggerated trauma-related memories (contextual fear), increased avoidance of trauma-related cues, and hyperarousal. Pharmacotherapy of PTSD is still unsatisfactory, with SSRIs being the first choice drugs. However, as known for depressed patients, PTSD patients are prone for relapse of symptoms upon discontinuation of treatment. This urges for a refinement of therapeutic interventions and the identification of markers of treatment success. These issues were addressed in our mouse model of PTSD. In this model, mice are exposed to a brief, inescapable electric foot shock. Within 1 month after the trauma, they developed PTSD-like symptoms such as generalized contextual fear, generalized avoidance, and increased hyperarousal symptomatology. This time frame allows for pharmacological interventions during maturation of PTSD-like symptoms (i.e. preventive treatment) or at time points when the symptoms have fully developed (i.e. therapeutic treatment). The work presented in this thesis revealed the following key findings:

(1) Fear incubation (i.e. simply the passage of time after trauma) was accompanied by highly selective changes in neuronal activity, as assessed by cytochrome c oxidase (CO) activity >1 month after trauma.

(2) Chronic treatment with fluoxetine via drinking water starting either right after the trauma (preventive treatment) or 28 days later (therapeutic treatment) completely reversed the PTSD-like symptoms assessed during ongoing treatment 1 (preventive treatment) or 2 months (therapeutic treatment) after trauma.

(3) Despite the similarities to PTSD-like symptoms, preventive treatment with fluoxetine abolished most of the trauma-related changes in CO activity, whereas those changes were maintained after therapeutic intervention.

(4) If fluoxetine was washed out after 1 month of treatment, PTSD-like symptoms remained absent following preventive treatment, but re-occurred after therapeutic treatment.

In conclusion, these data suggest preventive treatment with fluoxetine starting in the early aftermath of a trauma as a successful intervention strategy for preventing the development of PTSD-like symptoms. In contrast, therapeutic treatment abolishes the expression of symptoms, without curative effects. Chronic changes in CO activity reflect traces of a trauma. They might serve as an indicator of PTSD relapse.

Perspectives

In science, the fact that *'You are at the end of a project.'* only means that you're at the beginning of new ones.

(1) Research of neuronal changes in patients with PTSD, a disorder which is long-lasting and ubiquitous, should investigate tonic (long-term) neuronal changes such cytochrome c oxidase (CO) or resting state activity like Lui and colleagues did in survivors of an earthquake (Lui et al., 2009).

(2) Research of the structural changes, e.g. increased spine intensity in the basolateral amygdaloid nucleus (BLA) which may facilitate symptoms of PTSD by enhancing connectivity and modulation for fear memory (Mitra et al., 2005) should be investigated in animals as well as in humans.

(3) The CO seems to be a promising marker for successful treatment of PTSD, but can only been analyzed *ex vivo*. Nevertheless, the online monitoring of treatment success would be very helpful not only for PTSD, but a lot of other psychiatric disorders. Preliminary data already showed in an animal model for anxiety that CO activity was increased in the same regions as found with the MEMRI (manganese-enhanced MRI). Therefore, it would be interesting to measure shocked vs. non-shocked mice longitudinally up to 28 days after the traumatic event using MEMRI. If the activity is different between shocked and non-shocked mice, the investigation of activity changes after the preventive treatment with fluoxetine compared to vehicle in shocked mice must be obligatory.

Abbreviations

AccC	accumbens nucleus core
AccSh	accumbens nucleus shell
aCSF	artificial cerebrospinal fluid
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AMY	amygdala
AP5	D (-)-2-amino-5-phosphonopentanoic acid
ASR	acoustic startle response
BIM	(-)- bicuculline methiodide
BLA	basolateral amygdaloid nucleus
CA1	cornus ammonis 1
CA3	cornus ammonis 3
CC	cytochrome c
CeA	central amygdaloid nucleus
Cg1	cingulate cortex
CO	cytochrome c oxidase
CODA	conditioned odor avoidance
CPu	caudate putamen
DAB	2,6-Diacetylpyridine
DG	dentate gyrus
dHPC	dorsal hippocampus
$GABA_AR$	γ-aminobutyric acid receptor A
GluR1	subunit of AMPAR
IL	infralimbic cortex

INT	intensity
K^+P-buffer	potassium phosphate buffer
LA	lateral amygdaloid nucleus
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione
LH	lateral hypothalamic area
LHb	lateral habenular nucleus
M	molar concentration
MHb	medial habenular nucleus
mEPSC	miniature excitatory post-synaptic current
mIPSC	miniature inhibitory post-synaptic current
mPFC	medial prefrontal cortex
Na^+P-buffer	sodium phosphate buffer
OD	optical density
PAG	periaqueductal gray
PF	parafascicular thalamic nucleus
PFA	paraformaldehyde
PhTX	philanthotoxin 433
PrL	prelimbic cortex
PTSD	post traumatic stress disorder
PVA	paraventricular thalamic nucleus
ROI	region of interest
RT	room temperatur
SN	substancia nigra
TTX	tetrodotoxin
vHPC	ventral hippocampus

Chemicals

AP5	Ascent Scientific	
BIM	Tocris Biosciences	
$CaCl_2$	Merck	
CC (C2506)	Sigma-Aldrich	cytochrome c
$C_6H_7NaO_6$	Merck	sodium ascorbate
DAB (A1827)	Sigma-Aldrich	
isopentyl acetate (“banana”)	Sigma-Aldrich	
NaCl	Merck	
$NaHCO_3$	Merck	
NaH_2PO_4 H_2O	Merck	
Na_2HPO_4 $2H_2O$	Merck	
NBQX	Ascent Scientific	
$(NH_4)_2Ni(SO_4)_2 \cdot 6H_2O$ (A1827)	Sigma-Aldrich	ammonium nickel sulfate
KCl	Merck	
K_2- EDTA	Merck	
$K_3Fe(CN)_6$	Sigma-Aldrich	potassium ferricyanide
KH_2PO_4	Merck	
$MgSO_4$	Merck	
PhTX	Sigma-Aldrich	
Tris-HCl	Sigma-Aldrich	

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