Neuronal activity patterns associated with a traumatic experience in PTSD-vulnerable and -resilient mice

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Posttraumatic stress disorder (PTSD) is a clinical condition that can develop when an individual is exposed to a traumatic event, leading to significant social, occupational and interpersonal impairment. Although many individuals experience at least one traumatic event in their lifetime, only a subset of around 10 to 20% of these develop PTSD. This suggests the existence of inter-individual differences in vulnerability to developing psychopathology after trauma exposure. Discovering the exact neurobiological nature of these differences may be key to understanding the factors that constitute PTSD resilience, ultimately enabling the development of new treatment options. In this study we use an established mouse model for PTSD induction that employs a battery of behavioral tests to differentiate between subgroups of mice that show varying degrees of PTSD symptomatology after trauma exposure. Since re-experiencing of traumatic memories forms a core feature of PTSD, it is interesting to study the neuronal activity patterns that are induced by a specific traumatic experience and that are likely to be involved in the formation of a trauma memory trace. By coupling a fluorescent molecular label to the promoter of the immediate-early gene *Arc*, neurons that are active in these mice during trauma exposure can be labeled. In our study, we try to investigate **1)** if neuronal activation patterns during trauma exposure differ between the PTSD-like and resilient mice, **2)** if the neurons that are activated during trauma exposure are re-activated during subsequent re-exposure to the trauma environment and **3)** whether both subgroups of mice differed from each other in neuronal type and distribution in the hippocampus. Neuronal activation during trauma exposure did not differ between PTSD-like and resilient mice, but during re-exposure to the trauma environment PTSD-like mice showed significantly less neuronal activation in both the dorsal and ventral hippocampus. A small percentage of neurons that were activated during trauma exposure were re-activated during re-exposure to the trauma environment, although this only significantly differed between both subgroups of mice in the ventral inferior dentate gyrus. This suggests that PTSD-like and resilient mice did not differ in fear memory encoding, but did so in memory recall. To investigate whether PTSD-like and resilient mice differed from each other in the type of interneurons that were present, brain slices of these mice were stained for the GABAergic cell markers parvalbumin and somatostatin. PTSD-like mice showed significantly more parvalbumin positive cells in the ventral CA1, while the number of somatostatin positive cells in the dorsal dentate gyrus and dorsal CA1 was significantly reduced when compared to resilient mice, suggesting an altered inhibitory network between these subgroups of mice.

Introduction

Posttraumatic stress disorder (PTSD) is a clinical condition that can develop after a person is exposed to a traumatic event.[1,2] PTSD patients show involuntary and uncontrollable re-experiencing of the trauma, overgeneralization of these traumatic memories, emotional numbness, avoidance of stimuli associated with the trauma, negative cognitions and mood and hyperarousal.[1-4] People with PTSD are also more likely to develop anxiety and mood disorders and are more prone to substance abuse.[5,6] These clinical symptoms can lead to significant social, occupational and interpersonal impairment.[3,7] Although many people experience at least one traumatic event in their lifetime (estimates range from 50 to 90%)[1,2,7], the prevalence of PTSD is only around 5 to 20%.[1,5,8] This suggests differences in inter-individual vulnerability towards developing the disorder after trauma exposure.[4,7,8] PTSD is more common in women than in men and is associated with personality traits such as neuroticism and introversion.[9,10] Furthermore, intentionally inflicted trauma and experiencing of other traumatic events prior to the PTSD-causing event are important risk factors for PTSD. [2,5,6,10] Although these demographic and environmental risk factors play a role, biological differences are also likely to underlie PTSD vulnerability.

Twin studies have shown that genetic risk factors attribute to 30 to 40% of the heritability of PTSD risk.[1,6-8] Most of this genetic variation can be traced back to a small group of biological systems that play a role in PTSD: the serotonergic system, the dopaminergic system and the hypothalamic-pituitary-

adrenal (HPA) axis.[7,8,10] Serotonin is involved in the regulation of emotional responding and mood and has been implicated in PTSD pathology. Studies have indicated that people with the specific short allele polymorphism in the promoter region of the serotonin transporter gene have reduced expression of this gene and thus fewer functioning serotonin transporters. This leads to higher synaptic serotonin levels and is associated with an increased risk for developing PTSD. [1,8,10] Dopamine is involved in attention, vigilance, arousal and sleep, which are all negatively impacted by PTSD and it is therefore likely that dopamine dysregulation is involved in the development of PTSD.[7,8,10] A 9-repeat allele in the dopamine transporter gene as well as other polymorphisms in the dopamine receptor D2 and D3 genes have been implicated in PTSD pathology, although not all studies show these associations.[6,8,10] Polymorphisms in the gene that codes for catechol-O-methyltransferase, an enzyme that is involved in the breakdown of neurotransmitters such as dopamine, are also associated with increased PTSD risk.[8,10]

Dysregulation of the HPA axis, which consists of several neural and endocrine structures that together coordinate the stress response, is another key feature of PTSD pathology.[8,10,11] In response to a stressful event the amygdala stimulates neurons within the paraventricular nucleus of the hypothalamus to release corticotrophin-releasing hormone (CRH). This CRH binds to CRH_1 receptors in the anterior pituitary gland, which subsequently releases adrenocorticotrophic hormone (ACTH). ACTH is released into the blood and stimulates systemic release of cortisol from the adrenal cortex. Cortisol helps the body in coping with the stressful event by increasing glucose availability, but eventually also limits the stress response itself via feedback inhibition by binding to glucocorticoid receptors (GRs) in the hypothalamus and anterior pituitary gland. Furthermore, it is involved in coping with traumatic memories by enhancing memory consolidation and by diminishing retrieval and working memory.[7,11] The amygdala also stimulates norepinephrine release from the locus coeruleus, which plays a role in consolidation of fear memories and further stimulates the amygdala via a positive feedback loop.[7,11] Polymorphisms in the pituitary adenylate cyclase-activating polypeptide, which is also involved in the HPA-axis stress response by regulating CRH production, have been found to be associated with PTSD.[1,8-10]

GR hypersensitivity has repeatedly been associated with PTSD and several genes have been implicated in this, including CRHR1, NR3C1, FKBP5 and STAT5B.[11] CRHR1 encodes the CRH_1 receptor and is expressed in the hypothalamus, the pituitary and the amygdala. Several studies indicate that polymorphisms in the CRHR1 gene are associated with PTSD onset, although not all studies show this.[2,10] NR3C1 encodes the GR itself and single nucleotide polymorphisms (SNPs) in this gene have been significantly associated with PTSD, which suggests that NR3C1 plays an important role in PTSD development.[8,9] Also, hypomethylation of the promoter region of the NR3C1 gene was shown to increase NR3C1 expression and hippocampal GR count in rats, which caused neuroendocrine alterations that resembled those found in PTSD.[7,12] Gene expression of the glucocorticoid receptor inhibitors FKBP5 and STAT5B was found to be reduced in PTSD patients compared to controls.[2,7,8,10,11] GRs are present in the HPA-axis, where they suppress the stress response and cortisol release, but can also be found in other brain structures, such as the hippocampus, the amygdala and the PFC.[13] Hypersensitivity of hippocampal GRs increases the risk for hippocampal atrophy during stress, inhibits neurogenesis and alters dendrite morphology, which might explain the negative cognitive effects seen in PTSD.[6,11,14,15]

Besides these genetic risk factors, there are also other biological differences that are likely to be involved with PTSD vulnerability. To find these, it is helpful to look more closely at the clinical symptoms that PTSD patients develop. Re-experiencing of traumatic memories and avoidance of trauma-associated stimuli in PTSD patients can be attributed to emotional enhancement of the memory of the traumatic event, failure to extinguish this memory and overgeneralization of the trauma memory.[15,16] Since the hippocampus plays an important role in emotional memory, it is not surprising that it is hypothesized that this structure is likely to be involved in PTSD.[14,17]

The hippocampus is located in the medial temporal lobe and is involved in encoding, consolidation and retrieval of explicit memories and in the regulation of emotion, fear, anxiety and stress.[14,15,18] It consists of the dentate gyrus (DG) and the Cornu Ammonis (CA), which can be subdivided into CA1 and CA3.[19,20] Both the DG and the CA are composed of multiple layers and host both excitatory neurons and a great variety of interneurons.[19,21] The hippocampus receives projections from all regions of the association and cingulate cortex via the entorhinal cortex, which projects to the dentate gyrus (DG). In the DG, pattern separation takes place, a cognitive function in which discrimination of similar and overlapping patterns into more dissimilar patterns is performed based on minimal differences between stimuli.[14,22] After this, the information is sent to the CA3 region via mossy fibers. The information is stored in the CA3 region in auto-association circuits in which CA3 pyramidal neurons synapse onto other CA3 neurons, thus forming a recurrent network.[14,22] This circuit is thought to be involved in pattern completion, the process in which memories can be recalled after experiencing only a part of the original stimulus that was present during initial learning[14]. The information that is stored in the CA3 region can be retrieved by the CA1 region via Schaffer collateral projections and can then be sent to other brain regions. The CA1 region also receives input from the entorhinal cortex and is thought to be involved in memory generalization and in comparing memories from the CA3 input to new information from the entorhinal cortex for match-mismatch and novelty detection.[14,23] Evidence suggests a functional gradient in the hippocampus, in which the dorsal and ventral hippocampus have different functions. The dorsal part is important in cognitive functions such as spatial processing, while the ventral part is more involved in the regulation of stress and emotion.[14,18] This is also reflected in hippocampal connectivity patterns: the dorsal hippocampus receives more projections from areas involved in spatial processing, such as the retrosplenial cortex, while the ventral hippocampus has far more connections with the amygdala and other regions that are involved in fear learning and the stress response.[18] Hippocampal volumes are decreased in PTSD patients when compared to both trauma-exposed and trauma-unexposed controls. Also, PTSD symptom severity was found to be negatively correlated with hippocampal volume in both trauma-exposed and –unexposed twins, with smaller volumes corresponding to increases in symptom severity.[17]

Hippocampal neurons can be either excitatory (glutamatergic) or inhibitory (GABAergic). Although the majority of neurons are excitatory, inhibitory interneurons are of crucial importance since they control the excitability of both glutamatergic and GABAergic neurons.[24] While glutamatergic neurons are rather homogeneous, GABAergic neurons are highly diverse in both physiology and chemical markers and can be subdivided into several subgroups.[20,24,25] These chemical markers can be used to differentiate the GABAergic interneurons into, for example, parvalbumin expressing cells, somatostatin expressing cells and neuropeptide Y expressing cells. Parvalbumin (PV) expressing neurons are more numerous in the CA1 and CA3 regions than in the DG (40% in the CA1/3 compared to 20% in the DG), show no dorsoventral difference in expression and are located at the base of the granule cell layer and in the hilus of the DG.[20,24] Somatostatin (SOM) expressing neurons can be found

in both the CA1, CA3 and DG regions. In the DG virtually all SOM expressing neurons are found in the hilus, while almost no cells are located in the granule cell layer.[24] SOM expressing neurons show more expression in the ventral hippocampus compared to the dorsal hippocampus.[20,25] Neuropeptide Y (NPY) is an anxiolytic neuropeptide and low levels of NPY expression have been found in patients with PTSD.[26] Normally, NPY expressing cells are present in high levels in the hippocampus, where they account for 31% of GABAergic neurons, and show no dorsoventral expression gradient.[25] NPY expressing cells are especially prevalent in the CA1, where they regulate synaptic transmission and plasticity in feedforward pathways and reduce short-term facilitation.[26]

Exposure to stress, among other external stimuli, results in the activation of immediate-early genes (IEGs), which can be considered markers of neuronal activation.[27,28] Prominent IEGs are the proto-oncogene *c-Fos*, a transcription factor that regulates expression of other genes, and activity-regulated cytoskeleton-associated protein (*Arc*). *Arc* is suggested to play a role here by forming an endocytic complex that is involved in AMPA receptor trafficking. This trafficking causes a change in the number of AMPA receptors that are present on the membrane surface and alters neuronal excitability.[29,30] Both IEGs are found to be induced in the hippocampus after contextual fear conditioning and their expression is modulated by the basolateral amygdala.[29] While stress-induced c-*Fos* is expressed all over the brain, *Arc* is only expressed in certain brain areas, including those that are important in learning and fear memory like the hippocampus and amygdala. Coupling a transgene that expresses *Cre* to the promoter of the IEGs *c-Fos* or *Arc* induces *Cre* expression in neurons that are active during exposure to stress. Expression of *Cre* enables recombination of another transgene, allowing expression of a fluorescent molecular label such as *tdTomato*. Since non-active cells will not express *Cre*, they do not undergo recombination and will not express the fluorescent molecular label.[31,32]

Since re-experiencing of traumatic memories forms a core feature of PTSD, it is interesting to study the neuronal activity patterns that are induced by a specific traumatic experience and that are likely to be involved in the formation of a trauma memory trace. By coupling a fluorescent molecular label to the promoter of the immediate-early gene *Arc*, neurons that are active in these mice during trauma exposure are labeled.[31,32] Here we try to investigate **1)** if neuronal activation patterns during trauma exposure differ between PTSD-like and resilient mice, **2)** if the neurons that are activated during trauma exposure are re-activated during subsequent re-exposure to the trauma environment and **3)** whether both subgroups of mice differ from each other in neuronal type and distribution in the hippocampus. To accomplish this we use an established mouse model for PTSD induction that employs a battery of behavioral tests to differentiate between subgroups of mice that show varying degrees of PTSD symptomatology after trauma exposure.[33,34] Following PTSD induction and behavioral tests, the mice are sacrificed and fluorescent immunolabeling is used to determine the type and number of neurons that are active in the hippocampus during both trauma induction and re-exposure.

Material and Methods

Animal care

Mice were housed in groups of 3 to 5 animals per cage in a pathogen-free temperature-controlled mouse facility at the Central Animal Facility of the Radboud University Nijmegen on a reverse 12 hour light/dark cycle. Food and water were provided *ad libitum*. All behavioral experiments were performed during the dark period. Animals were checked every day and were euthanized when pre-defined humane endpoints were reached.

Injection of tamoxifen

To be able to pinpoint differential expression of genes to specific cell populations, the Targeted Recombination in Active Populations (TRAP) technique was employed.[31] In this experiment male transgenic Arc-CreERT2 mice from the C57BL/6J strain were used to assess patterns of neuronal plasticity. In these animals, one allele of the *Arc* promoter is coupled to *Cre*, which leads to the expression of *Cre* when *Arc* is activated. In the absence of tamoxifen, *Cre* is retained in the cytoplasm of the cell and degraded after a short while. After *i.p.* injection of one dose of 100 mg/kg tamoxifen, *Cre* is able to travel to the nucleus of a cell, where it induces recombination of a transgene that enables expression of the fluorescent protein tdTomato. This recombination is only possible 24-36 hours post-injection, during which tamoxifen is present. In this way, only cells that are active during a specific time window will be labeled with tdTomato.[31]

PTSD protocol

To induce PTSD-like behavior in mice we followed the protocol established by Lebow et al. (2012).[33] In this model the mice receive 14 foot shocks of 1 mA on the first day in variable intervals during 85 minutes in context A. This represents the 'trauma'. One day later, the same mice receive 5 foot shocks of 0.7 mA in a fixed interval during 5 minutes in context B, representing the 'trigger'. The shocks were given in a fear-conditioning apparatus. Context A consisted of a black cage with a metal grid floor, 1 lux illumination, acetic acid scent and no noise. The animals were transferred from their home cages to the experiment room in darkness in cardboard boxes. Between animals, the cages and grids were cleaned with 70% ethanol solution, after which a 1% acetic acid solution was sprayed to reinstate the context-specific scent. Context B consisted of a white cage with a metal grid floor, 70 lux illumination, ethanol scent and 70 dB white noise. In this context mice were transferred from their home cages to the experiment room in darkness in empty cages. Between animals, the cages and grids were cleaned with 70% ethanol solution. Seven days after the 'trauma' and 'trigger' the mice were subjected to a battery of behavioral tests. These tests were performed over a period of 11 days. Five days after the last behavioral test, mice were re-exposed to the trauma environment and subsequently sacrificed. Control mice were also placed in both the 'trauma' and 'trigger' context, but received no foot shocks.

Light-dark transfer

The light-dark transfer test is based on the innate aversion of mice to brightly illuminated areas and the conflicting natural tendency of the animals to explore a novel environment .[35] For the light-dark transfer test a box was used that was divided by a partition into a dark area (one third of the box) and a brightly illuminated area (two thirds of the box, 950-1200 lux). The two areas were connected by a sliding door that was located at floor level in the center of the partition. The mice were placed in the dark area of the box and the sliding door was removed to initiate a 5 minute test session. The animal's movements were recorded and the time spent in the light area, the number of visits to the light area and the total distance traveled in the light area were measured.

Marble burying

In the marble burying test mice were placed in the corner of a box containing 5 cm deep bedding with 20 marbles that were arranged 4 by 5 in a room with 10 lux illumination. The mice were then recorded

for 25 minutes and their behavior was scored by counting the number of unburied marbles each 5 minutes.[36]

Prepulse inhibition

Prepulse inhibition (PPI) is the ability of a non-startling stimulus to inhibit the reaction to a normally startling stimulus that is presented shortly after the non-startling stimulus.[37] The animals were transferred to the experiment room in their home cages and were placed in a small tubular enclosure, which was then placed in a sound-attenuated and ventilated chamber (SR-LAB) with 5 lux illumination. A high precision sensor that was integrated into the measuring platform detected movement and two high-frequency loudspeakers inside the chamber produced the audio stimuli. Each session began with 5 minute habituation, after which six startle stimuli of 120 dB were presented. After this, twelve additional startle stimuli were presented at randomly varying inter-stimulus intervals, randomly preceded by prepulses of either 75 dB, 80 dB or 85 dB. The session was concluded with six startle stimuli of 120 dB. Between animals, the enclosures were cleaned with 70% ethanol solution. Latency to peak startle amplitude and percentage PPI were measured and calculated for each mouse.

Locomotion

Locomotion was assessed using the Ethovision 3.1 phenotyper system. Mice were housed individually for 72 hours in darkness and during this time general locomotion measurements were collected at 10 minutes interval. Food and water were provided *ad libitum* during this time.

Re-exposure and sacrifice

During re-exposure the mice were subjected to the same context as that in which the 'trigger' took place. The animals were transferred from their home cages to the experiment room in darkness in empty cages and placed for 10 minutes in a white cage with a metal grid and 70 lux illumination, but did not get shocks during the re-exposure. The mice were sacrificed by perfusion fixation 90 minutes after the start of the re-exposure, after which their brains were extracted and stored in paraformaldehyde at 4°C. After 24 h, the brains were transferred to PBS + 0.01% NaN_3.

Inclusion criteria for PTSD-like versus resilient mice

The mice were subcategorized as either PTSD-like or resilient based on their behavioral results as described by Lebow et al. (2012).[33] They were assessed on risk assessment (dark-light transfer), compulsivity (marble burying), arousal (acoustic startle), attentional gating (prepulse inhibition) and insomnia (home cage locomotion). For risk assessment, arousal and attentional gating, scores were sorted from lowest to highest because lower scores reflect more extreme behavior. The 20% mice that had the lowest scores were given 3 points for risk assessment, 3 points for arousal and 3 points for attentional gating. Scores for compulsivity and insomnia were sorted from highest to lowest and the top 20% on each test were given 1 point. Mice that had a total of 0 points were termed 'resilient', while mice that had 4 or more points were termed 'PTSD-like'.

Brain slicing

Prior to brain slicing the brains were sliced in half. The right hemisphere of each brain was stored for 1 to 7 days in PBS with 30% sucrose for cryoprotection. After this, the brains were sliced into 30 µm slices using a microtome. The brain slices were stored at 4°C in 12-wells plates in PBS with 0.01% NaN_3.

Immunohistochemistry

Fluorescent immunolabeling was performed to determine which cells were active during the trauma and trigger. First, slices that included the dorsal or ventral hippocampus were selected and incubated in pre-incubation buffer (0.1 M PBS with 1% bovine serum albumin and 0.3% Triton X-100). Afterwards, the slices were incubated in primary antibody (rabbit anti-NPY (1:1000, AB_572253, Immunostar, WI, USA), rat anti-SOM (1:200, AB_2255365, Merck, MA, USA), rabbit anti-PV (1:1000, AB_298032, Abcam, United Kingdom) and guinea pig anti-*c-Fos* (1:750, AB_2619946, Synaptic Systems, Germany) diluted in incubation buffer for 16 hours at room temperature. The slices were then washed 3 times in PBS for 10 minutes and incubated in secondary antibody diluted in incubation buffer (1:200) for 3 hours in the dark at room temperature. Finally, the slices were again washed 3 times in PBS for 10 minutes in the dark and mounted on gelatin coated slides. After air drying, the slices were embedded in FluorSave (Merck, MA, USA) and stored at -20°C in the dark.

Imaging

For each mouse, an average of 4 dorsal and 3 ventral hippocampal slices per immunolabeling were photographed under fluorescent light with 10x magnification using an Axio Imager A2 that was present at the department of Anatomy of the Radboudumc in Nijmegen, the Netherlands. For each staining the same exposure time was used for all slices. Following this, pictures of the slices were stitched using Fiji ImageJ to get a picture of the full dorsal or ventral hippocampus. Unfortunately, it turned out that the NPY immunolabeling had not worked. Also, two mice were excluded from analysis after imaging because the transgenic construct was not expressed in these mice.

Analysis

Regions of interest (the inferior and superior DG, hilus, CA3 and CA1) were drawn in the dorsal and ventral hippocampal pictures and cells in these regions were counted manually. These cell counts were corrected for the relative size of their region of interest and the cell counts of all slices were averaged for each mouse. After this, Student's T-tests (independent samples, two-tailed) were used to determine whether the cell counts in each region of interest differed between animals that showed a PTSD-like or resilient phenotype.

Results

PTSD phenotyping

Five behavioral tasks were used to assess whether mice exhibited PTSD-like symptoms following the trauma + trigger. Subpopulations of PTSD-like and resilient mice were based on extreme behavior or lack thereof. PTSD-like mice show significantly less time engaging in risk assessment behavior ($p = 0.003$) and a significantly shorter latency to peak startle amplitude ($p = 0.049$), but did not differ from resilient mice in the number of marbles they buried, their activity during light phase or their response to startle with a pre-pulse stimulus (figure 1).

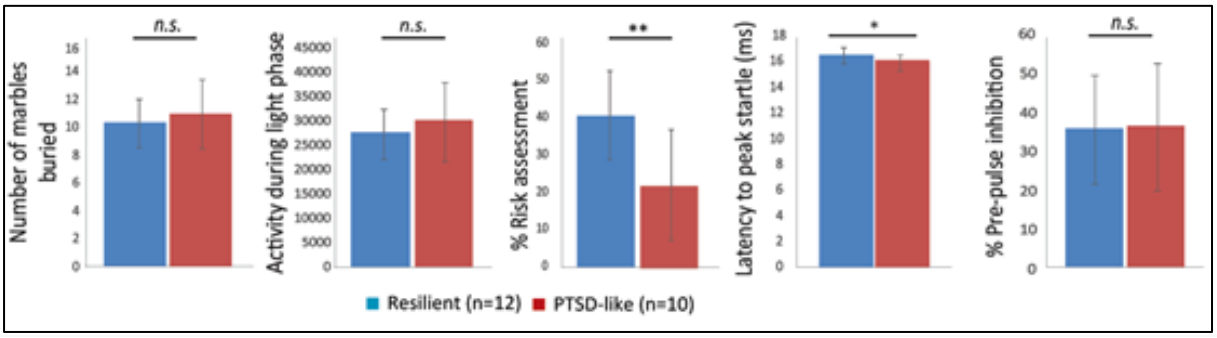

Figure 1. PTSD phenotyping. PTSD-like mice showed significantly less time engaged in risk assessment behavior ($p = 0.003$) and a significantly shorter latency to peak startle amplitude response ($p = 0.049$). There were no differences in the number of marbles buried, activity during light phase or response to startle with a pre-pulse stimulus between PTSD-like and resilient mice (*$p < 0.05$, **$p < 0.01$).

Immunohistochemistry

To measure neuronal activation during trauma expression the number of tdTomato positive cells were analyzed. There were no differences found in the number of tdTomato positive cells between PTSD-like and resilient mice in any region of interest in the dorsal and ventral hippocampus (figure 2).

To assess whether PTSD-like and resilient mice differed from each other in the amount and distribution of GABAergic neurons, hippocampal slices were stained for parvalbumin and somatostatin. Since both parvalbumin and somatostatin are structural markers, these immunolabelings could only tell us whether GABAergic cells were present in the hippocampus, but not if they were active during either trauma exposure or re-exposure to the trauma environment. PTSD-like mice showed a significantly higher number of parvalbumin positive cells in the ventral CA1 ($p = 0.02$) (figure 3). PTSD-like mice had significantly less somatostatin positive cells in the dorsal hippocampus ($p = 0.04$), an effect that was driven by significantly less somatostatin positive cells in both the dentate gyrus ($p = 0.03$) and the CA1 ($p = 0.04$) (figure 4).

Neuronal activation during re-exposure to the trauma environment was assessed by staining for the immediate early gene *c-Fos*. The number of *c-Fos* positive cells in PTSD-like mice was significantly decreased in both the dorsal ($p = 0.05$) and the ventral hippocampus ($p = 0.01$), with the latter effect being driven by a significant decrease of *c-Fos* positive cells in the ventral CA1 ($p = 0.005$) (figure 5).

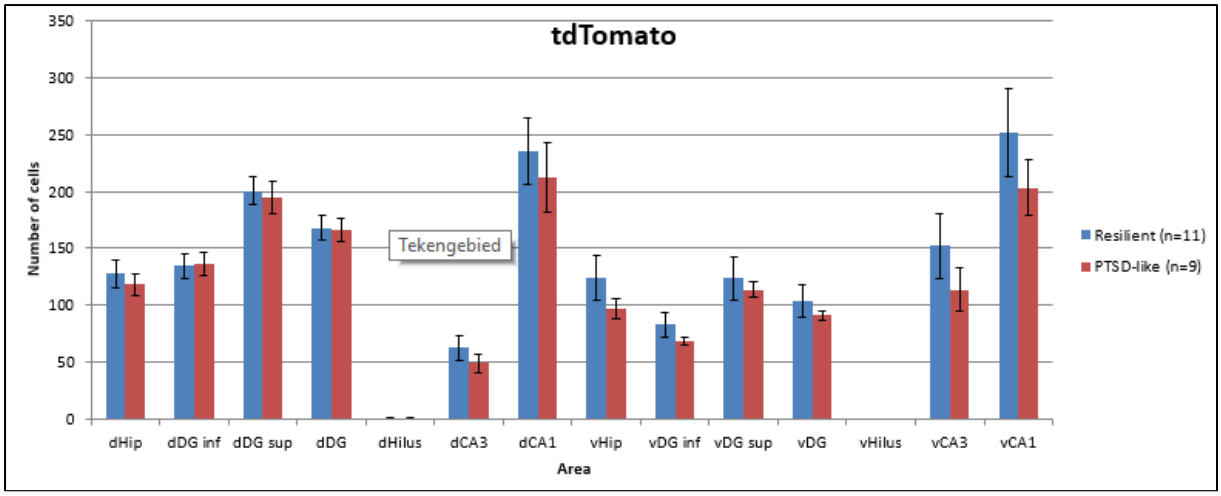

Figure 2. Neuronal activation during trauma exposure. There were no significant differences between PTSD-like and resilient mice in the number of tdTomato positive cells in any region of interest in the dorsal and ventral hippocampus.

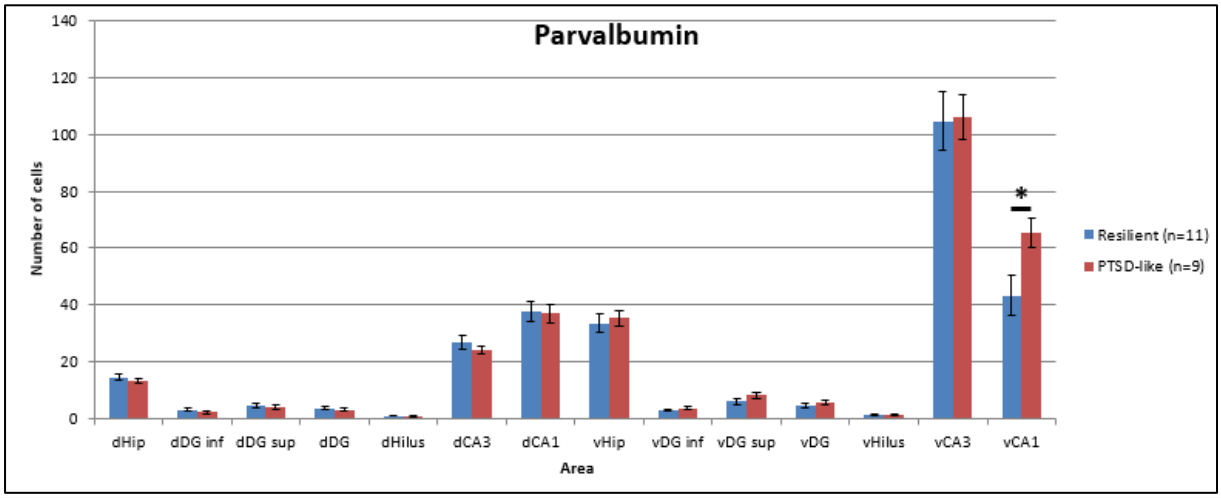

Figure 3. Number of parvalbumin positive cells after re-exposure to the trauma environment. PTSD-like mice showed significantly more parvalbumin positive cells in the ventral CA1 ($p = 0.02$) (*$p < 0.05$).

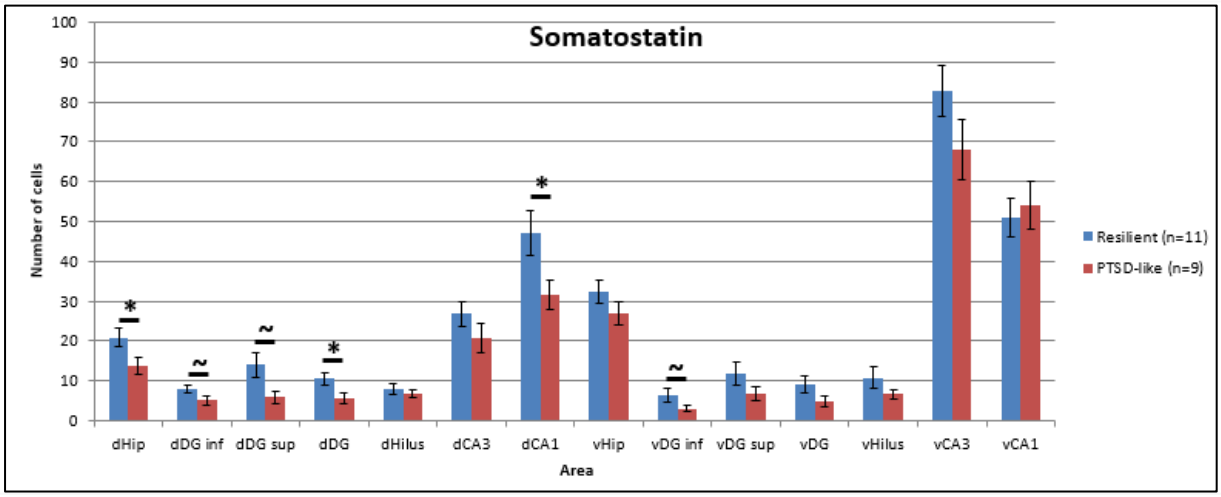

Figure 4. Number of somatostatin positive cells after re-exposure to the trauma environment. PTSD-like mice showed significantly less somatostatin positive cells in the dorsal hippocampus ($p = 0.04$), which was driven by significantly less somatostatin positive cells in the dorsal DG ($p = 0.03$) and the dorsal CA1 ($p = 0.04$). A trend effect was seen in both the inferior and superior dorsal DG and the inferior ventral DG (*$p < 0.05$, ~$p < 0.10$).

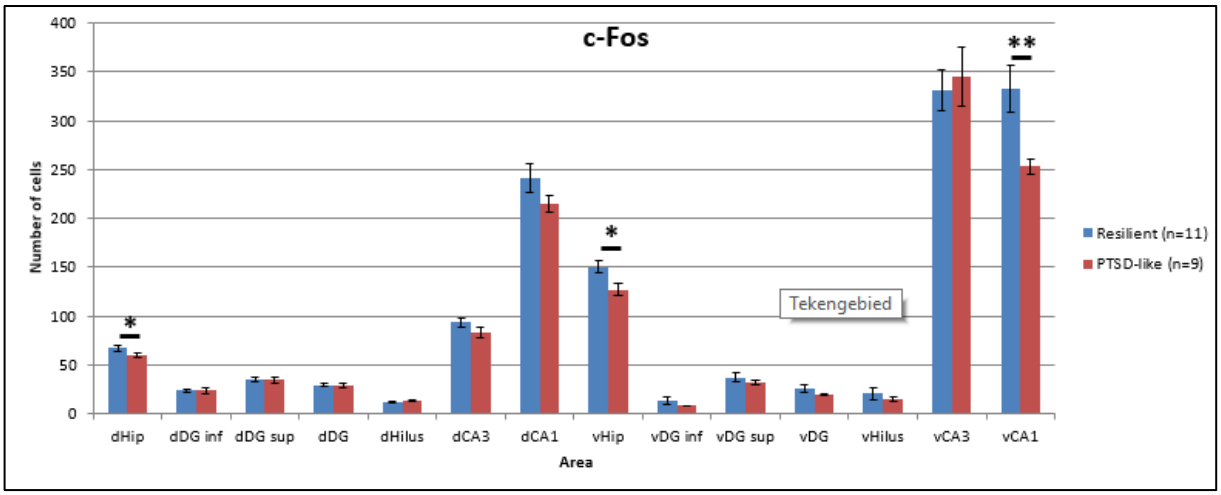

Figure 5. Neuronal activation after re-exposure to the trauma environment. PTSD-like mice showed significantly less *c-Fos* positive cells in both the dorsal ($p = 0.05$) and ventral hippocampus ($p = 0.01$), with the latter effect being driven by significantly less *c-Fos* positive cells in the ventral CA1 ($p = 0.005$) (*$p < 0.05$, **$p < 0.01$).

Less than ten percent of the cells that were activated during trauma exposure were re-activated during re-exposure to the trauma environment. Only in the ventral inferior DG there was a significant increase in re-activated cells in PTSD-like mice when compared to resilient mice ($p = 0.03$), while in all other regions of interest there were no significant differences between the two subgroups of mice.

Discussion

In this study we looked at neuronal activity patterns that are induced by a specific traumatic experience and are likely to be involved in the formation of a trauma memory trace. Our results show that neuronal activation during trauma exposure did not differ between PTSD-like and resilient mice. However, during re-exposure to the trauma environment PTSD-like mice showed significantly less neuronal activation in both the dorsal and ventral hippocampus. This suggests that PTSD-like and resilient mice do not differ from each other in fear memory encoding but do so in memory recollection. Only a small percentage of neurons that were activated during trauma exposure were re-activated during re-exposure to the trauma environment, with PTSD-like mice having significantly more re-activated neurons in the ventral inferior DG when compared to resilient mice. Since only a small percentage of neurons were re-activated, it could be that the trauma memory trace that is induced by trauma exposure is a different one than that induced by re-exposure to the trauma environment. In this sense, the mice are not remembering the initial traumatic memory during re-exposure, but are rather encoding a new memory trace. This is however not supported by behavioral data, which show that mice that were exposed to trauma exhibited more freezing behavior during trauma re-exposure than mice that were not exposed to trauma (data not shown).

A staining for the GABAergic cell markers parvalbumin and somatostatin was used to assess whether PTSD-like and resilient mice differed from each other in the type of neurons that were present. Parvalbumin expressing neurons were mainly located in the pyramidal cell layer of the CA1 and CA3, which is in line with existing literature.[20,24] Somatostatin expressing neurons were present in all hippocampal regions. In line with previous literature, these neurons were mainly located in the molecular layer of the DG and the stratum oriens of the CA1 and CA3, while almost no neurons were found in the granule cell layer.[24] However, in contrast to previous literature, we found no difference in

somatostatin expression between the dorsal and ventral hippocampus.[20,25] PTSD-like mice showed significantly more parvalbumin positive cells in the ventral CA1, while the number of somatostatin positive cells in the dorsal DG and dorsal CA1 was significantly reduced when compared to resilient mice. This suggests that the inhibitory network is altered between PTSD-like and resilient mice. Since the ventral CA1 is involved in generalization of emotional memories[14], it might be speculated that this increase in parvalbumin positive cells leads to overgeneralization of traumatic memories, which is a key symptom of PTSD.[23] Because the dorsal DG and CA1 are important in pattern separation and memory generalization, a decrease in somatostatin positive cells in these regions could suggest an alteration in the way these processes take place place in PTSD-like mice.[14]

Since all mice used in this study are of the same C57BL/6J strain and were exposed to the same trauma, the differences that were found between PTSD-like and resilient mice are likely the result of some existing biological natural variation. Because all mice had the same genetic background, this biological natural variation is likely the result of epigenetic variation between PTSD-like and resilient mice. This epigenetic variation is of interest because it could be important to understanding why some people are more susceptible to developing PTSD than others. However, it is unclear whether differences between PTSD-like and resilient mice were already present before trauma or if they are induced by the trauma exposure itself. To address this, neuronal activation patterns could be measured before trauma exposure, instead of during trauma exposure, as we did here. Also, neuronal activation patterns in control mice, which do not receive any trauma exposure, have to be analyzed to determine the effect of the trauma exposure itself.

PTSD is a multifaceted clinical condition that can present itself by many different symptoms. Because of this, animal models of PTSD cannot replicate the disease in its entirety but try to mimic certain symptoms of it. The model used here for PTSD phenotyping was adapted from Lebow et al. and consisted of five different behavioral tests, which tested risk assessment, compulsivity, arousal, attentional gating and insomnia.[33] Although previous studies showed differences between PTSD-like and resilient mice on all tests, here we only found significant differences between the two subgroups of mice on risk assessment and arousal. Because PTSD can present itself by many different symptoms, mice can show PTSD-like symptoms in one test but fail to show them in another test. This makes it hard to determine whether mice that we classified as PTSD-like are really suffering from PTSD, suggesting that the distinction we make between PTSD-like and non-PTSD-like is an artificial one.

Since PTSD is a complex disease with many different symptoms, it is difficult to say what criteria have to be met before someone is classified as having PTSD. Rather, PTSD constitutes of a spectrum of symptoms and people suffering from PTSD can do so in varying degrees.[1] To account for this, future research should also include mice that showed intermediate PTSD-like symptoms and were not classified as either PTSD-like or resilient in this study. As mentioned, neuronal activation patterns can be measured before instead of during trauma exposure to determine whether differences are already present before trauma exposure or induced by the trauma itself. Finally, since PTSD patients showed lower hippocampal volumes when compared to controls[17], it might be interesting to measure hippocampal volumes of PTSD-like and resilient mice.

Our findings show that there are differences in neuronal activation patterns between PTSD-like and resilient mice during memory recall, but not during memory encoding. Also, PTSD-like and resilient mice differ in the type of interneurons that are present in the hippocampus. These differences are

likely the result of biological natural variation, which could be caused by epigenetic variation. Future research should investigate the exact nature of this variation and in what way it leads to an increased susceptibility to PTSD.

References

1 Smoller, J. W. The Genetics of Stress-Related Disorders: PTSD, Depression, and Anxiety Disorders. *Neuropsychopharmacology* **41**, 297-319, doi:10.1038/npp.2015.266 (2016).

2 Carvalho, C. M., Coimbra, B. M., Ota, V. K., Mello, M. F. & Belangero, S. I. Single-nucleotide polymorphisms in genes related to the hypothalamic-pituitary-adrenal axis as risk factors for posttraumatic stress disorder. *Am J Med Genet B Neuropsychiatr Genet* **174**, 671-682, doi:10.1002/ajmg.b.32564 (2017).

3 Yehuda, R., Koenen, K. C., Galea, S. & Flory, J. D. The role of genes in defining a molecular biology of PTSD. *Disease Markers* **30**, 67-76 (2011).

4 Whitaker, A. M., Gilpin, N. W. & Edwards, S. Animal models of post-traumatic stress disorder and recent neurobiological insights. *Behav Pharmacol* **25**, 398-409, doi:10.1097/FBP.0000000000000069 (2014).

5 Kessler, R. C. Posttraumatic Stress Disorder: The Burden to the Individual and to Society. *J Clin Psychiatry* **61**, 4-12 (2000).

6 Voisey, J., Young, R. M., Lawford, B. R. & Morris, C. P. Progress towards understanding the genetics of posttraumatic stress disorder. *J Anxiety Disord* **28**, 873-883, doi:10.1016/j.janxdis.2014.09.014 (2014).

7 Skelton, K., Ressler, K. J., Norrholm, S. D., Jovanovic, T. & Bradley-Davino, B. PTSD and gene variants: new pathways and new thinking. *Neuropharmacology* **62**, 628-637, doi:10.1016/j.neuropharm.2011.02.013 (2012).

8 Banerjee, S. B., Morrison, F. G. & Ressler, K. J. Genetic approaches for the study of PTSD: Advances and challenges. *Neurosci Lett* **649**, 139-146, doi:10.1016/j.neulet.2017.02.058 (2017).

9 Sheerin, C. M., Mackenzie, J. L., Bountress, K. E., Nugent, N. R. & Amstadter, A. B. The genetics and epigenetics of PTSD: overview, recent advances, and future directions. *Current Opinion in Psychology* **14**, 5-11 (2017).

10 Almli, L. M., Fani, N., Smith, A. K. & Ressler, K. J. Genetic approaches to understanding post-traumatic stress disorder. *Int J Neuropsychopharmacol* **17**, 355-370, doi:10.1017/S1461145713001090 (2014).

11 Castro-Vale, I., van Rossum, E. F., Machado, J. C., Mota-Cardoso, R. & Carvalho, D. Genetics of glucocorticoid regulation and posttraumatic stress disorder--What do we know? *Neurosci Biobehav Rev* **63**, 143-157, doi:10.1016/j.neubiorev.2016.02.005 (2016).

12 Zovkic, I. B. & Sweatt, J. D. Epigenetic mechanisms in learned fear: implications for PTSD. *Neuropsychopharmacology* **38**, 77-93, doi:10.1038/npp.2012.79 (2013).

13 Mizoguchi, K., Ishige, A., Aburada, M. & Tabira, T. Chronic stress attenuates glucocorticoid negative feedback: involvement of the prefrontal cortex and hippocampus. *Neuroscience* **119**, 887-897, doi:10.1016/s0306-4522(03)00105-2 (2003).

14 Bartsch, T. & Wulff, P. The hippocampus in aging and disease: From plasticity to vulnerability. *Neuroscience* **309**, 1-16, doi:10.1016/j.neuroscience.2015.07.084 (2015).

15 Richter-Levin, G. The amygdala, the hippocampus, and emotional modulation of memory. *Neuroscientist* **10**, 31-39, doi:10.1177/1073858403259955 (2004).

16 Baldi, E. & Bucherelli, C. Brain sites involved in fear memory reconsolidation and extinction of rodents. *Neurosci Biobehav Rev* **53**, 160-190, doi:10.1016/j.neubiorev.2015.04.003 (2015).

17 Shin, L. M., Rauch, S. L. & Pitman, R. K. Amygdala, medial prefrontal cortex, and hippocampal function in PTSD. *Ann N Y Acad Sci* **1071**, 67-79, doi:10.1196/annals.1364.007 (2006).

18 Strange, B. A., Witter, M. P., Lein, E. S. & Moser, E. I. Functional organization of the hippocampal longitudinal axis. *Nat Rev Neurosci* **15**, 655-669, doi:10.1038/nrn3785 (2014).

19 Amaral, D. G., Scharfman, H. E. & Lavenex, P. The dentate gyrus: fundamental neuroanatomical organization (dentate gyrus for dummies). *Prog Brain Res* **163**, 3-22 (2007).
20 Nomura, T. *et al.* Distribution of nonprincipal neurons in the rat hippocampus, with special reference to their dorsoventral difference. *Brain Research* **751**, 64-80 (1997).
21 Scharfman, H. E. & Myers, C. E. Hilar mossy cells of the dentate gyrus: a historical perspective. *Frontiers in Neural Circuits* **6**, 1-17 (2013).
22 Madronal, N. *et al.* Rapid erasure of hippocampal memory following inhibition of dentate gyrus granule cells. *Nat Commun* **7**, 10923, doi:10.1038/ncomms10923 (2016).
23 Zhou, H. *et al.* The interhemispheric CA1 circuit governs rapid generalisation but not fear memory. *Nat Commun* **8**, 2190, doi:10.1038/s41467-017-02315-4 (2017).
24 Molgaard, S. *et al.* Immunofluorescent visualization of mouse interneuron subtypes. *F1000Res* **3**, 242, doi:10.12688/f1000research.5349.2 (2014).
25 Jinno, S. & Kosaka, T. Patterns of expression of neuropeptides in GABAergic nonprincipal neurons in the mouse hippocampus: Quantitative analysis with optical disector. *J Comp Neurol* **461**, 333-349, doi:10.1002/cne.10700 (2003).
26 Li, Q., Bartley, A. F. & Dobrunz, L. E. Endogenously Released Neuropeptide Y Suppresses Hippocampal Short-Term Facilitation and Is Impaired by Stress-Induced Anxiety. *J Neurosci* **37**, 23-37, doi:10.1523/JNEUROSCI.2599-16.2016 (2017).
27 Ons, S., Rotllant, D., Marin-Blasco, I. J. & Armario, A. Immediate-early gene response to repeated immobilization: Fos protein and arc mRNA levels appear to be less sensitive than c-fos mRNA to adaptation. *Eur J Neurosci* **31**, 2043-2052, doi:10.1111/j.1460-9568.2010.07242.x (2010).
28 Ons, S., Marti, O. & Armario, A. Stress-induced activation of the immediate early gene Arc (activity-regulated cytoskeleton-associated protein) is restricted to telencephalic areas in the rat brain: relationship to c-fos mRNA. *J Neurochem* **89**, 1111-1118, doi:10.1111/j.1471-4159.2004.02396.x (2004).
29 Huff, N. C. *et al.* Amygdala regulation of immediate-early gene expression in the hippocampus induced by contextual fear conditioning. *J Neurosci* **26**, 1616-1623, doi:10.1523/JNEUROSCI.4964-05.2006 (2006).
30 Minatohara, K., Akiyoshi, M. & Okuno, H. Role of Immediate-Early Genes in Synaptic Plasticity and Neuronal Ensembles Underlying the Memory Trace. *Front Mol Neurosci* **8**, 78, doi:10.3389/fnmol.2015.00078 (2015).
31 Guenthner, C. J., Miyamichi, K., Yang, H. H., Heller, H. C. & Luo, L. Permanent Genetic Access to Transiently Active Neurons via TRAP: Targeted Recombination in Active Populations. *Neuron* **78**, 773-784 (2013).
32 Gouty-Colomer, L. A. *et al.* Arc expression identifies the lateral amygdala fear memory trace. *Mol Psychiatry* **21**, 364-375, doi:10.1038/mp.2015.18 (2016).
33 Lebow, M. *et al.* Susceptibility to PTSD-Like Behaviour is Mediated by Corticotropin-Releasing Factor Receptor Type 2 Levels in the Bed Nucleus of the Stria Terminalis. *The Journal of Neuroscience* **32**, 6906-6916 (2012).
34 Henckens, M. J. A. G. *et al.* CRF receptor type 2 neurons in the posterior bed nucleus of the stria terminalis critically contribute to stress recovery. *Mol Psychiatry* **22**, 1691-1700, doi:10.1038/mp.2016.133 (2017).
35 Bourin, M. & Hascoët, M. The mouse light/dark box test. *European Journal of Pharmacology* **463**, 55-65, doi:10.1016/s0014-2999(03)01274-3 (2003).
36 Sztainberg, Y., Kuperman, Y., Justice, N. & Chen, A. An anxiolytic role for CRF receptor type 1 in the globus pallidus. *J Neurosci* **31**, 17416-17424, doi:10.1523/JNEUROSCI.3087-11.2011 (2011).
37 Powell, S. B., Zhou, X. & Geyer, M. A. Prepulse inhibition and genetic mouse models of schizophrenia. *Behav Brain Res* **204**, 282-294, doi:10.1016/j.bbr.2009.04.021 (2009).