The neurobiological signature of susceptibility to traumatic stress

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General Introduction

General Introduction

While stress exposure in itself is not harmful, and can in fact be adaptive in most situations[1], exposure to severe forms of stress can lead to a variety of long-lasting maladaptive symptoms[2], including anxiety, negative mood, irritability and a plethora of physical responses due to continued activation of the body's stress systems in susceptible individuals. When these symptoms persist for an extended period of time and interfere with normal day-to-day life, they can pose a large burden to both individuals and society as a whole[3]. Often, the stress symptomatology can be traced back to a (series of) traumatic event(s) in the past, which may lead to a diagnosis of post-traumatic stress disorder (PTSD)[4]. Given the ubiquity of traumatic events, it is unsurprising that the incidence of PTSD is rising, with an estimated 7.7 million European citizens currently being affected by the disorder, leading to over €9 billion in annual costs[5].

PTSD, which is classified under the clinical category of Stressor-Related Disorders[6], is typically caused by exposure to major traumatic events, like natural disasters, war and conflict, accidents and serious health problems. Symptoms can arise after exposure to a single traumatic event, but also after long-term exposure to severe daily stressors[7]. As a relevant example, the recent COVID-19 pandemic has been shown to have caused a spike in PTSD symptomatology in the general population[8-10]. This demonstrates that such a global emergency can cause significant psychological and physical distress, leading to long-lasting stress-related symptoms in a large group of affected people. As described in the DSM-V[6], the main symptom clusters of PTSD include intrusion symptoms, avoidance of trauma-related stimuli, negative alterations in cognition and mood, and increased arousal and hyperreactivity. While the development of PTSD is, by definition, linked to a certain traumatic event or an accumulation of stressful events, biological and psychosocial risk factors are increasingly considered as predictors of symptom onset[4]. Considering that over 80% of people are faced with one or multiple traumatic experiences during their lifetime[11-13], PTSD prevalence (5-10% across the general population[14]) is relatively low. This raises the question why some people are more vulnerable than others when it comes to developing PTSD symptomatology after traumatic stress exposure. First of all,

it is not clear at what point in time resilience or vulnerability to PTSD can be distinguished. Behavioral resilience may in part be innate, but may also arise due to a deviant response to the trauma. In addition, it might be the case that all animals cope with trauma exposure in a similar way, yet recover differently. This underlines the importance of longitudinal designs when studying animal models of PTSD, in order to temporally pinpoint when potential alterations arise and how they develop over time[15]. While susceptibility is likely a dynamic state comprised of pre-existing risk factors and trauma-induced sequalae[16], it is especially interesting to identify targets for early intervention and treatment and to assess whether prevention in vulnerable individuals might be possible. Elucidating the biological basis of this interindividual variability in PTSD susceptibility will be critical for understanding PTSD psychopathology, and may hold unique insights for identifying vulnerable individuals and optimizing prevention[17]. As current medication is only effective for fewer than half of the patients[18], it becomes even more important to understand this apparent heterogeneity across trauma-exposed individuals in order to optimize treatment options.

Animal models for PTSD

Using human patients to research human diseases is of course an effective way to learn. However, the acquisition of PTSD in humans is incidental, and thus rarely observed in real-time. In addition, the nature of the trauma, its remoteness, and treatment history are highly variable, and PTSD induction in healthy volunteers is obviously not ethically viable. These factors complicate using human subjects to identify the factors that are related to brain mechanisms involved in the development of PTSD after trauma exposure[19]. Recent years have seen a growing number of rodent models being used for studying the neurobiology of maladaptive stress coping and PTSD specifically[19,20]. This is pivotal, as these models allow us to simulate the induction of PTSD-like symptomatology, test causal factors in longitudinal designs, and invasively study the neuronal effects of stress in a controlled manner[15]. Crucially for the study of PTSD, the neurocircuitry involved in fear and anxiety is highly conserved throughout evolution, which makes these models particularly relevant[21].

However, these models also come with downsides. PTSD remains a complex phenotype that is difficult to model in rodents. Despite the major scientific efforts over the past years, the gained knowledge from these animal models is still largely lacking translatability, and has therefore not yet led to better treatment options. Understandably, this has made researchers propose important adaptations to these models in order to overcome current shortcomings and improve overall translational value[17]. Crucially, preclinical studies on PTSD have often disregarded the fact that only a small proportion of stress-exposed individuals later develops a long-lasting maladaptive phenotype. Typically, a group of stressed animals is contrasted directly to a group of non-stressed animals, neglecting potential interindividual differences among individuals in the stressed group and ignoring potential adaptive effects of stress exposure[22]. This asks for a paradigm shift in the use of the animal models, in which stressed individuals are further classified into 'resilient' and 'susceptible' subgroups, based on their behavioral phenotype[23]. Data from stress vulnerability studies have often shown that only individuals vulnerable to the stressful experience show the characteristic stress-related symptomatology, while resilient individuals are behaviorally much more comparable to controls[24,25]. This supports the idea that it would be an oversimplification to consider all stressed individuals as one homogenous group when studying underlying biological variation across stressed and non-stressed groups, and potentially vital information is lost by grouping maladaptive and adaptive stress responses. Specifically studying PTSD-resilient individuals may teach us which processes underly successful stress recovery; processes that might be mimicked as a method for intervention with disease.

Throughout this line of research, we employed a mouse model in which we behaviorally tested animals for PTSD-like symptoms following trauma exposure (foot shock), to dissociate susceptible from resilient mice and distinguish maladaptive from adaptive trauma coping[26]. Mice were classified as susceptible or resilient to PTSD-like symptomatology based on a compound score comprising multiple behavioral PTSD-like symptoms (i.e., risk assessment, anxiety, hypervigilance, pre-pulse inhibition and activity during the inactive phase; symptoms mostly based on the 'arousal and reactivity' cluster of the DSM-V[6]), rather than single behavioral features. This classification largely

resembles the situation in patients[27], which are also diagnosed with PTSD once they meet several criteria. Furthermore, substantial behavioral heterogeneity is seen among susceptible trauma-exposed animals[26,28], similarly to what is observed in the clinic[27]. As such, this model allowed us to study the effects of traumatic stress in a controlled setting and to do invasive brain measurements related to susceptibility to PTSD-like symptoms.

Circulating theories for the emergence of PTSD

Various theories have been proposed to explain the development and perpetuation of PTSD. Classically, PTSD has been understood in light of classical conditioning and learning theory, yielding a model where the trauma is regarded as an unconditioned stimulus, which involuntarily evokes a reaction of distress[29]. Overconsolidation of the traumatic memory occurs, leading to conditioned fear responses during re-exposure to stimuli that remind the individual of the trauma. Over time, learning theory has evolved with the incorporation of more cognitive aspects. For example, the dual representation theory[30] proposes the existence of two distinct types of memory, that occur in parallel: verbally accessible memory (VAM), which can easily be recalled, and situationally accessible memory (SAM), which cannot be deliberately accessed. The theory states that trauma exposure leads to impairment of the VAM, because conscious attention is narrowly drawn to threat-related information. This leads to pathobiological chronic emotional processing, where the trauma-related memory is heavily focused on the fear response. On the other hand, the cognitive model[31] of PTSD focuses more on the idea that exposure to traumatic events skews one's appraisal of external and internal threats, viewing them with excessive negativity. This would lead to misinterpretation of situations and a constant sense of current threat. While all slightly different, what all these theories have in common is that they point to aberrant memory processing as the key underlying factor for development of PTSD pathophysiology. Still, it is unclear whether aberrant memory processing is caused by deviating memory acquisition or consolidation, and to what degree it is influenced by pre-existing neurobiological vulnerability factors[32,33].

It is often argued that that the scope of functional abnormalities shown in PTSD (and other psychiatric disorders for that matter) cannot be captured by referring to abnormalities in singular neuronal processes or brain regions. Instead, a broader integrative approach is necessary to capture the complexity of such disorders[34]. As such, another theory has gained substantial traction over the past few years, especially among neuroimaging researchers. Evidence suggests that the brain can be organized into functionally distinct brain networks with high intrinsic functional connectivity[35]. It is thought that an imbalance or other disruptions in these networks may underlie the complex pathophysiology of PTSD. In this thesis, both theories of memory and triple network dysfunction are explored in more detail.

Memory deviations in PTSD

Even though the symptoms of PTSD are relatively well-defined, there is a large heterogeneity in the clinical profile of PTSD patients[27], which has led researchers to define multiple subtypes (e.g., externalizing/internalizing PTSD, and dissociative/non-dissociative PTSD) of the disorder[36]. Interestingly however, over 90% of all patients face intrusive memories, including flashbacks and nightmares[37], making these hallmark symptoms of PTSD. In fact, these alterations in memory are very specific to PTSD and specifically distinguish PTSD from other stress-related disorders. Not unsurprisingly, the therapeutic strategies that are regularly used to treat patients with PTSD are also generally based on facing the traumatic memories, in order to better cope with them, suppressing involuntary memories[38], and making trauma memories less emotionally salient (in the case of Eye Movement Desensitization and Reprocessing therapy)[39]. However, despite their centrality to the disorder, the basic cognitive and neural mechanisms underlying these involuntary memories in PTSD are not well understood[40]. As such, increasing interest has gone out to understand the specific memory trace that is created upon trauma exposure, hypothesizing that it is the lasting memory of a traumatic event, rather than exposure to the event itself, that determines PTSD symptoms[32]. Building on this, it is important to understand how such maladaptive trauma memory differs from an adaptive trauma memory.

Normal adaptive trauma memory allows individuals to learn from dangerous situations and prevent them in the future[41]. Maladaptive memories in PTSD, on the other hand, are characterized by excessive fear expression, and a continuous state of readiness, likely related to uncontrollable, vivid recollections of trauma-related images in ordinary, safe situations[42]. The latter reflects an impairment of the important discrimination between safe and dangerous cues[29,43]. This increased generalization has been found to correlate with the severity of re-experiencing symptoms in war veterans with PTSD[44]. In addition, while memories of a traumatic event are often perceived as vivid and full of perceptual detail, they tend to be disorganized and full of gaps[45,46]. Memory for central aspects of the event is often retained quite explicitly for a long period of time, but the details are remembered with less accuracy[47], lacking temporal[48] and factual context[49]. The combination of these observations leads to the seemingly paradoxical conclusion that PTSD is characterized by emotional hypermnesia[47] (i.e., the amplification of emotional and sensory content of the traumatic memory) and contextual hypomnesia[41] (i.e., the relative reduction of peri-trauma contextual memory). This has made clinicians postulate the dual memory representation theory of PTSD[30]. This theory distinguishes normal episodic trauma memory from flashbacks. Normal episodic trauma is supported by flexible, contextualized representations that are proposedly adaptive, as they ensure restricted recall of the traumatic memory only if the context requires. In contrast, flashbacks are supported by representations that are inflexible and lack context[49], making that this maladaptive trauma memory escapes voluntary control as it is automatically reactivated, in whatever context, by the sole presence of salient cues somewhat related to the traumatic event[49]. Importantly, therapeutic success is associated with the integration of sensory memory traces into structured, contextual narratives[38], arguably altering the maladaptive trauma memory trace into an adaptive one[50-53]. Yet, this re-contextualization process is still poorly understood mechanistically, making that enhanced insight into the underpinnings of maladaptive memories in PTSD absolutely critical.

A role for the hippocampus and amygdala in PTSD

Given their involvement in memory processing and emotional regulation[54], the hippocampus and amygdala, among other brain regions, have long been implicated in the pathophysiology of PTSD[55]. Morphologically, atrophy of the hippocampus[56,57], as well as decreased amygdalar volume[58,59], have been consistently reported in PTSD patients. Preclinical and clinical studies have furthermore shown dysfunction of these limbic brain regions in the memory alterations regularly seen in PTSD patients[55,60]. The hippocampus, a brain area involved in declarative and episodic memory, is sensitive to the effects of stress. Chronic psychosocial stress in animals has been associated with damage to neurons in the dentate gyrus (DG) and cornu Ammonis 1 and 3 (CA1 and CA3) regions of the hippocampus[61], as well as inhibition of neurogenesis[62,63]. Animal studies have furthermore shown deficits in hippocampal-based memory function (a reduction in long-term potentiation and memory performance), alterations in hippocampal morphology[64], and altered functional connectivity between the hippocampal subregions[65] following traumatic stress exposure. Although it is still debated which mechanisms lead to these alterations, one popular theory is that the hippocampus is particularly affected by elevated levels of glucocorticoids released during stress[66,67], which have also been associated with deficits in new learning[68,69]. The idea that PTSD patients are particularly vulnerable to this stress-induced rise in glucocorticoid level may be partially explained by the observation that animal models of PTSD have increased expression of the glucocorticoid receptor in the hippocampus post-stress[70-72], while PTSD patients show enhanced glucocorticoid receptor sensitivity[73]. Furthermore, PTSD has earlier been associated with lower basal glucocorticoid levels[74,75], potentially contributing to glucocorticoid system sensitivity to stress-induced fluctuations.

Studies have specifically implicated impaired pattern separation and completion in the hippocampus as an underlying mechanism associated with risk for PTSD psychopathology through increased fear generalization[76]. These processes enable the hippocampus to differentiate between new, incoming stimuli, and old, known memories, for example in order to distinguish between potentially threatening and safe situations, based on prior experiences[77]. In PTSD, it is hypothesized that there is a bias

towards pattern completion in the face of a partial threat cue, and difficulty in absorbing contextual nuances necessary for proper pattern separation[78]. This suggests potential abnormalities in the information stream from the DG to the CA3 subregion, which underlies the process of pattern separation and completion, as well as in the CA1 subregion, which is involved in context-specific memory retrieval and fear extinction[79]. These findings support the idea that hippocampal abnormalities may underlie the contextual hypomnesia that is so often observed in PTSD[80].

At the same time, fMRI studies in PTSD patients have observed hyperactivity of the amygdala in response to trauma-specific stimuli[60,81-83], correlating with symptom severity[84]. Activity of specific amygdalar subregions (i.e., the basolateral (BLA) and central (CeA) nucleus) is required for responding to fearful stimuli, as well as to predictors of conditioned fear[85,86]. However, hyperactivity of these regions may lead to exaggerated fear responses, attentional bias for negative stimuli and dysfunction in regulating responses to negative emotions overall[87,88]. Many studies have implicated poor top-down control of the amygdala from the anterior cingulate and ventromedial prefrontal cortex as part of the fear neurocircuitry underlying this amygdalar hyperactivity and thereby PTSD symptomatology[89]. As such, these amygdalar abnormalities seem to, at least partially, underly the characteristic emotional hypermnesia that is observed in PTSD[47]. Still, several cases of PTSD have been reported where the amygdala fails to activate in response to emotional stimuli, leading to emotional disengagement and numbing[90]. This trait amygdalar hypoactivity is associated with a more dissociative subtype of PTSD. Interestingly, individuals may present both hyper- and hyporeactivity of the amygdala, depending on which emotional stimuli are presented to them[91,92].

Taken together, the current literature on hippocampal functional impairments and amygdalar hyperactivity support a model of cognitive imbalance between the two memory systems, in favor of amygdala-based emotional hypermnesia[93]. Therapeutic strategies may restore this imbalance by enabling hippocampus-dependent ‘re-contextualization’ of the traumatic experience through contextualized re-exposure to trauma-related cues within the appropriate context in the absence of threat, thereby aiming to re-establish normal fear memory expression. To test the theory of

hippocampal/amygdalar imbalance, it is important to observe brain activity specifically during trauma memory formation, and to focus on how the specific memory trace is stored and recalled at a later stage. Hence, this is a key aim underlying the experiments described in this thesis.

The memory engram of trauma

During trauma processing, the complex configuration of trauma-related information triggers the activity of neural ensembles that communicate through neuronal synapses, which are subsequently strengthened and stabilized through synaptic plasticity on the neuronal and circuit level[94]. These neural ensembles - in which the memory is physically stored - are referred to as the memory engram[95-99]. Although many attempts have been made to localize engrams in the brain, they have remained largely elusive, partly because of the dynamic nature of memory representations. Furthermore, studies in PTSD patients lack the possibility of accessing the actual physical memory trace of a certain traumatic experience.

However, excitingly, recent advances in the field of neurobiology have enabled the identification, long-term labeling, and manipulation of trauma memory engram neurons in transgenic mice[95,97]. This allows researchers to identify exactly which neurons are active at the time of trauma exposure (i.e., during learning), as well as during recall of the originally formed memory, making them very likely involved in the storage of that specific memory trace. Furthermore, it enables us to study animals in a longitudinal fashion and assess neuronal activity at multiple time points in the same animals without the need to sacrifice them, e.g., directly after stress. Most of these techniques are based on labeling of cells that express certain immediate early genes (IEGs). IEGs are genes which are expressed transiently and rapidly in response to cellular activation, thereby activating a down-stream cascade of subsequent 'late response' gene expression[100]. In neurons, this may eventually lead to synaptic changes which are necessary for memory formation. As such, measuring IEG expression in the brain may serve as a proxy for neuronal activity[101].

In this thesis, neurons that were active at specific time points before, during or after the trauma were labelled and later identified in a Targeted Recombination in Active Populations (TRAP) transgenic

mouse line[102]. This genetic construct (Figure 1) expresses the tamoxifen-dependent recombinase CreER^{T2} in an activity-dependent manner from the locus of the immediate early gene *Arc*. Active (i.e., *Arc*-expressing) neurons undergo recombination in the presence of the compound tamoxifen, allowing these cells to be permanently labeled by the fluorescent protein tdTomato[102]. This construct offers much more flexibility in assessing specific brain activity at several time points during a longitudinal animal experiment, as there is no need to prematurely sacrifice the animals (i.e., prior to behavioral characterization). This has previously always been necessary to measure IEG expression specifically related to neuronal activity during a short period prior to sacrifice. While previously impossible, the TRAP mice allowed us to label neuronal activity at specific time points surrounding the trauma, while also allowing for extensive behavioral testing and classification before eventually harvesting the brain material. This crucially allowed us to not only identify neuronal activity differences between susceptible and resilient animals, but also to pinpoint when these differences occur.

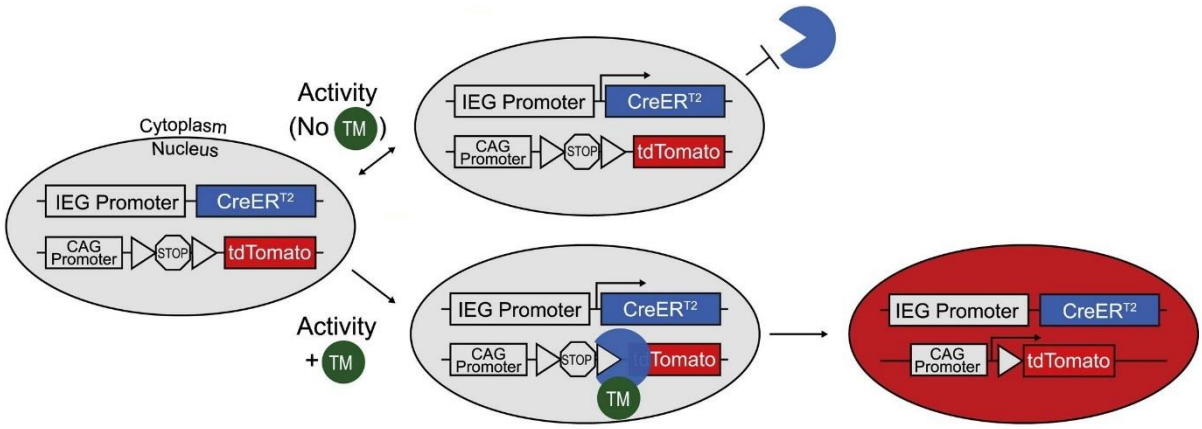

Figure 1. Targeted Recombination in Active Populations (TRAP)[102]. TRAP makes use of two transgenes: one that expresses CreERT2 upon activation of the IEG (i.e., *Arc*) promoter; and one that expresses the effector gene tdTomato in a Cre-dependent manner. CreERT2 is retained in the cytoplasm, but can readily relocate to the nucleus in the presence of tamoxifen (TM). In such case, CreERT2 recombination can occur, which induces expression of the fluorescent tdTomato protein specifically in activated cells.

Moving towards a brain-wide understanding of PTSD

Together, the hippocampus and amygdala are crucial for controlling the effects of emotion and arousal on memory consolidation, especially of memories that include spatial and contextual information[103]. Using the earlier mentioned labeling and tagging technologies, researchers have been able to identify specific memory engrams localized to the hippocampus[104-106] and amygdala[107], which are preferentially reactivated during the recall of that event[95]. Hence, it is no surprise that there is a large and continuously growing body of literature studying potential abnormalities in these areas with regards to PTSD and abnormal fear memory in general. However, it is also important to broaden our view and do exploratory research into other areas of the brain that may potentially be involved in PTSD, especially as we are trying to elucidate small interindividual differences that make some individuals slightly more vulnerable to developing PTSD after a trauma than others. Supporting this is the growing idea - mainly within the neuroimaging field - that psychiatric disorders, including PTSD, may also be understood as a disorder of circuits, rather than of single brain regions[34,108-112]. Still, preclinical work has largely remained its focus on the earlier discussed well-known regions of interest.

A good starting point to unbiasedly study potential neuronal abnormalities underlying PTSD is to assess brain-wide activity, for example by mapping expression of the IEG *cFos* in preclinical animal models for PTSD. Recently, Azevedo and colleagues[113] did exactly that in a mouse model of single prolonged stress, and they found stress-induced activity in a wide variety of brain regions, including the amygdala, pallidum, bed nucleus of the stria terminalis, paraventricular hypothalamus, thalamus, periaqueductal gray, habenula, and cuneiform nucleus. In a related study, researchers studied activity induced by remote fear memory attenuation in 16 brain regions, identifying the amygdala, nucleus reuniens and ventral hippocampal CA1 and CA3 as targets that were specifically activated by recall of a fearful memory[114].

The triple network model

PTSD symptoms have previously been successfully mapped to activity and functional connectivity of a variety of cortical and subcortical regions, yielding a model that could predict PTSD symptom score

with significantly higher accuracy than chance[115]. Altogether, these and other studies give considerable evidence that the heterogenous phenotypes in PTSD may potentially be explained by large-scale network dysfunction[34]. The vast majority of evidence has fallen under one of three networks: the default mode (DMN), executive control (ECN) and salience (SN) networks. The theory that disruptions in these three networks may underlie PTSD has been dubbed the triple network model[116] (Figure 2). Generally, PTSD may be characterized by a weakly interconnected and hypoactive DMN[117-119], putatively destabilized by an overactive and hyperconnected SN[120-122]. The latter appears to have a low threshold for saliency, and to be incapable of efficient DMN-ECN modulation. Abnormalities within the ECN may underlie some of the cognitive, executive, and emotional regulatory dysfunctions in PTSD[118,123]. An enhanced ECN to DMN connectivity[124], which has been linked to treatment response, may be an acquired resilience or bypass mechanism by which trauma-exposed individuals adapt to/overcome a specific circuit or nodal aberrancy.

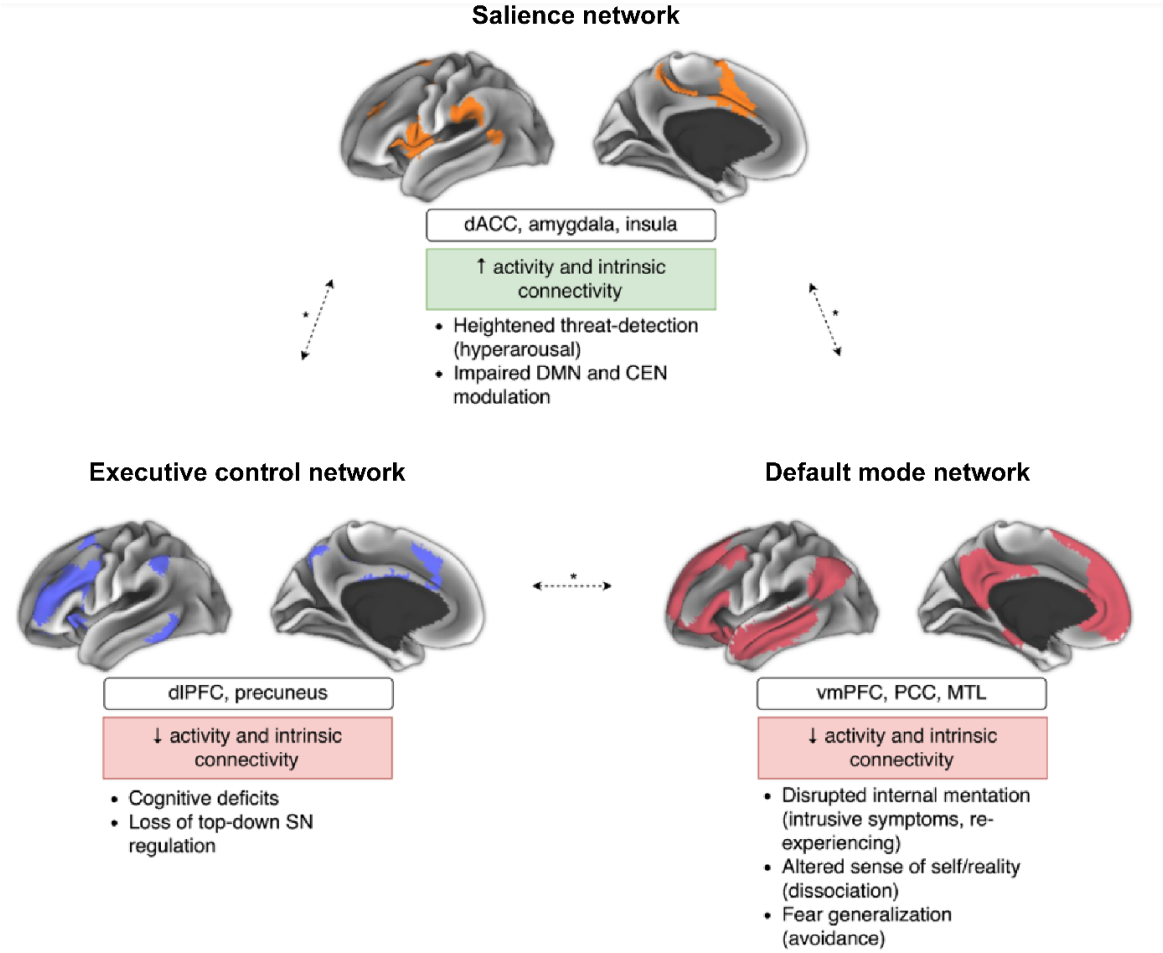

Figure 2. Triple network model of PTSD[34]. Shown here are the cortical representations of the salience network (SN; orange), executive control network (ECN; blue) and default mode network (DMN; red). The triple network theory of PTSD proposes that the SN is hyperconnected and hyperactive, and is incapable of sufficiently modulating DMN and ECN activity. Contrarily, the ECN and DMN are supposedly weakly interconnected and hypoactive, resulting in impaired cognition and top-down SN regulation (in case of the ECN) and intrusive, dissociative and avoidance symptoms (in case of the DMN). * Altered between-network connectivity.

Brain-wide exploratory studies in rodents

Exploratory studies into brain-wide activity, especially in the context of the SN, DMN and ECN networks, are necessary to advance our understanding of the neurobiological underpinnings of PTSD at the neural network level. However, these approaches are scarce in current literature, in part by technical limitations. Recent advances in the field of whole brain staining, embedding, and clearing, combined with the imaging of intact brain tissue, have now provided us with the tools to unbiasedly study protein expression throughout the rodent brain[125-127]. In this thesis, we employed a novel technique, called iDISCO+, to clear and fluorescently label entire mouse brain hemispheres and capture 3D image stacks using a light-sheet microscope[126]. This allowed us to get activity measurements throughout the entire hemisphere and specifically study the activity of regions within the SN, DMN and ECN networks.

Epigenetic regulation

A meta-analysis published in 2019, based on over 30,000 PTSD cases and 170,000 controls, concluded that the phenotypic variation observed in PTSD could be explained for 5-20% by genetic differences, varying by sex[128]. This heritability figure is very similar to that for major depression[129], and is consistent with earlier findings from twin studies[130,131]. However, that means that over 80% of PTSD variability is unaccounted for by genetics. As we know that gene expression is extremely sensitive to stress and trauma[132], epigenetic alterations have received growing attention, forming an important mechanism by which environmental experiences can induce long-lasting changes in the brain. Epigenetics confer transcriptional memory of exposure to environmental stress conditions[133,134],

regulate memory formation[135] and shape long-term behavioral adaptations[136-138]. As such, they may contribute to the development and persistence of PTSD symptoms, especially as they reflect both genetic and environmental influences[139].Several studies have shown that early life stress can induce epigenetic changes in various genes, such as those coding for the glucocorticoid receptor (GR)[140], brain-derived neurotrophic factor (BDNF)[141] and vasopressin[142], leading to different maladaptive behavioral alterations in adulthood, including increased stress reactivity and vulnerability to stress-related neuropsychiatric diseases. This suggests that epigenetic alterations may precede traumatic stress susceptibility and confer individual susceptibility to negative outcomes after a trauma.

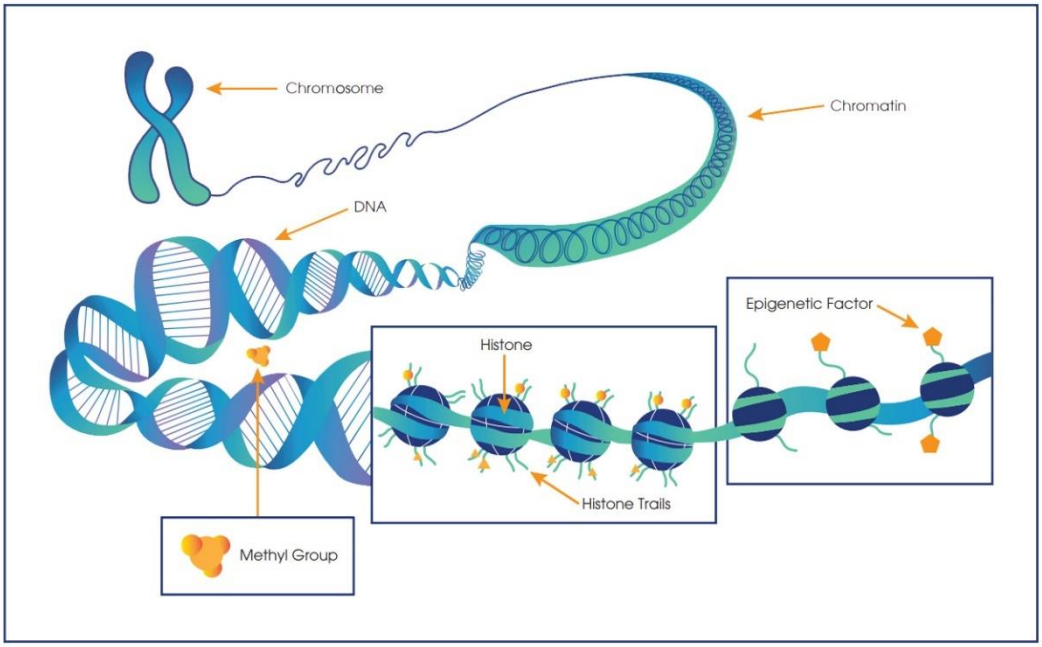

Figure 3. Epigenetic changes[143]. Epigenetic modifications include methyl tags that attach to DNA bases, and alterations to the histone proteins that DNA wraps around for compaction. DNA methylation is what occurs when methyl groups tag DNA and activate or repress genes. Histones are proteins around which DNA can wind for compaction and gene regulation. Histone modification occurs when the binding of epigenetic factors to histone tails alters the extent to which DNA is wrapped around histones, influencing the availability of genes in the DNA to be activated. Furthermore, other epigenetic factors, like micro-RNAs, may bind to DNA or histones to influence DNA accessibility and expression.

At the same time, evidence is growing that traumatic stress exposure itself may also induce long-lasting epigenetic changes that may underlie PTSD psychopathology. A longitudinal study in US military service members has for example reported increased DNA methylation of immune-related genes pre- vs. post-deployment in individuals later diagnosed with PTSD[144]. Some excellent review articles have described the current body of evidence for epigenetic alterations in PTSD[145], as well as how these alterations may influence fear learning and memory[146]. These processes include not only DNA methylation, but also histone modifications (e.g., acetylation, methylation, and phosphorylation), and microRNAs. Histone acetylation is most robustly associated with memory formation[147]. In the hippocampus, chronic stress has been found to downregulate histone deacetylase (HDAC) 2[148] and increase the expression of genes that are required for synaptic plasticity and long-term memory formation[149,150]. Interestingly, lower hippocampal HDAC2 expression after chronic stress was found to be related to stress resilience[151]. Stress exposure also seems to change DNA methylation state[152], with both increased expression of several hippocampal DNA methyl transferases[153,154] and lower hippocampal DNA methylation[155] being observed following stress. 5-hydroxymethylcytosine (5hmC), another oxidative product within the DNA methylation pathway, is not only a key intermediate in cytosine demethylation, but also a stable epigenetic modification[156], modulating gene transcription independently from 5-methylcytosine (5mC)[157]. The abundance of 5hmC specifically in the mammalian brain, its high interindividual variability, and its upregulation by stress[158], make it an interesting candidate for involvement in stress-related memory engrams. Specifically relevant to the concept of resilience, a study from 2010 showed a correlation between hippocampal methylation of the Disks Large-Associated Protein (Dlgap2) gene and behavioral stress responses after trauma exposure in rats[159]. However, despite the growing body of literature, this important contrast between PTSD-vulnerable and PTSD-resilient individuals in a trauma-exposed group is largely lacking within preclinical studies of epigenetics in PTSD. Hence, it will be interesting to further study potential epigenetic alterations in neurons involved in trauma memory processing that differentiate susceptible from resilient animals.

Outline of the thesis

This thesis aims to elucidate differences in neuronal (engram) activity and epigenetic regulation in mice vulnerable and resilient to PTSD.

In **Chapter 2**, I provide further background into current literature describing the epigenetic changes that may occur in response to adult life stress, like a traumatic experience. The focus lies on specific epigenetic alterations within the body's stress system: the hypothalamic-pituitary adrenal (HPA) axis. In addition, we discuss the implications of this knowledge for the identification and treatment of stress-related psychiatric disorders.

In **Chapter 3**, I tested the hypothesis that susceptible mice display alterations in the relative size and composition of trauma-related hippocampal engrams in susceptible compared to resilient mice. I studied activity of the different hippocampal subregions during trauma, but also during recall of the trigger context three weeks after initial exposure. Furthermore, levels of the epigenetic markers HDAC2, 5mC and 5hmC were determined in hippocampal tissue as a whole, but also specifically in the engram cells. Lastly, parvalbumin-positive hippocampal interneuron presence and recall-specific activity were assessed, as these are potentially important regulators of the trauma memory engram.

In **Chapter 4**, I investigated neuronal activity within the different amygdalar subregions before, during, and after exposure to the traumatic event. I aimed to address whether susceptible and resilient animals could be contrasted based on altered amygdalar activity either before trauma exposure, during trauma, or post-trauma. In addition, I assessed amygdalar activity in response to three different trauma-related contexts to investigate its reactivity to different trauma-reminders. Lastly, I assessed the amygdalar presence of somatostatin-positive interneurons, as well as their relative activity during trauma context re-exposure, as the somatostatin system plays a crucial role in the acquisition and expression of contextual fear memory.

The aim of **Chapter 5** was to move beyond the candidate-based approaches, and test the hypothesis that brain-wide trauma-related neuronal activity is different between susceptible and resilient animals.

As in the previous chapter, I assessed neuronal activity before, during, and after trauma exposure to temporally define potential activity differences and identify risk factors and potential targets for early intervention and treatment. By employing iDISCO+, I cleared and fluorescently labeled entire mouse brain hemispheres, which allowed me to obtain activity measurements throughout the entire hemisphere and study network activity and correlations of multiple areas of the brain.

All study findings are summarized and discussed in **Chapter 6**.

Chapter 2

Epigenetic programming of the neuroendocrine stress response by adult life stress

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ABSTRACT

The hypothalamic-pituitary adrenal (HPA) axis is critically involved in the neuroendocrine regulation of stress adaptation, and the restoration of homeostasis following stress exposure. Dysregulation of this axis is associated with stress-related pathologies like major depressive disorder, post-traumatic stress disorder, panic disorder, and chronic anxiety. It has long been understood that stress during early life can have a significant lasting influence on the development of the neuroendocrine system and its neural regulators, partially by modifying epigenetic regulation of gene expression, with implications for health and well-being in later life. Evidence is accumulating that epigenetic plasticity also extends to adulthood, proposing it as a mechanism by which psychological trauma later in life can long-lastingly affect HPA-axis function, brain plasticity, neuronal function, and behavioral adaptation to neuropsychological stress. Further corroborating this claim is the phenomenon that these epigenetic changes correlate with the behavioral consequences of trauma exposure. Thereby, epigenetic modifications provide a putative molecular mechanism by which the behavioral phenotype and transcriptional / translational potential of genes involved in HPA-axis regulation can change drastically in response to environmental challenges, and appear an important target for treatment of stress-related disorders. However, improved insight is required to increase their therapeutic (drug) potential. Here, we provide an overview of the growing body of literature describing the epigenetic modulation of the (primarily neuroendocrine) stress response as a consequence of adult life stress and interpret the implications for - and the challenges involved in applying this knowledge to - the identification and treatment of stress-related psychiatric disorders.

GLOSSARY

Restraint stress: A stress paradigm in which the animal is restrained in a confined space for a certain period of time, during which it is unable to move.

Social defeat: A stress paradigm that entails the (repeated) exposure of an animal to losing a confrontation with a dominant con-specific. It is most commonly established by the resident-intruder paradigm, in which the animal (the intruder) is repeatedly placed in the cage of a dominant animal (the resident) in a manner that allows for non-lethal contact.

Chronic variable mild stress (CVMS): A paradigm in which the animal is exposed to various mild stressors for a prolonged period of time (usually twice daily for 14 consecutive days). Stressors include relatively mild sessions of social isolation, cold swim, cold isolation, wet bedding, food and water deprivation, overnight illumination, alteration of light-dark cycle, and restraint stress. All stressors are applied in a fixed order and only repeated twice, to avoid habituation to the stressor.

Chronic variable stress (CVS): A paradigm in which the animal is exposed to various moderate stressors for a prolonged period of time (usually twice daily for 14 consecutive days). Stressors include social isolation, social crowding, warm swim, cold swim, cold isolation, and cage rotation. All stressors are applied in a semi-randomized manner and only repeated twice, to avoid habituation to the stressor.

Chronic unpredictable stress (CUS): A paradigm in which the animal is exposed to various stressors for an extended period of time (usually once a day for 28 consecutive days). Stressors include cold swim, thermal environment, wet bedding, food and water deprivation, cage tilting, noise, overnight illumination, and alteration of light-dark cycle. All stressors are applied in a semi-randomized manner and only repeated twice, to avoid habituation to the stressor.

1. INTRODUCTION

Adequate responding to stress and restoration of homeostasis requires a widespread activation of different response systems in the body. Crucial to the stress response is the neuroendocrine system, which tightly regulates adaptive processes following stress exposure[160]. The primary endocrine effectors of the neuroendocrine response are located in the paraventricular nucleus (PVN) of the hypothalamus, the anterior pituitary, and the adrenal gland. This collection of structures, called the hypothalamic-pituitary-adrenal (HPA) axis, is critically involved in the regulation of a variety of body processes, including the immune system, energy storage and expenditure, digestion, mood, and emotional responsivity to stress[161]. The neuroendocrine stress response should be adequate for coping with the specific stressor and should be of limited duration to prevent hyperactivity after stress cessation. Dysregulation of the HPA-axis is associated with stress-related pathologies like major depressive disorder (MDD), post-traumatic stress disorder (PTSD), panic disorder, and chronic anxiety[162]. While depression pathology is linked to basal hyperactivation of the HPA-axis[163,164] and impaired negative feedback of the HPA-axis[165], PTSD is thought to be characterized by increased sensitivity of glucocorticoid receptors (GRs), moderating enhanced negative feedback and overall decreased cortisol levels[166]. This endocrine dysregulation might be mediated by lasting neurobiological alterations caused by extreme or repeated stress exposure; especially in the case of PTSD, where trauma exposure is directly linked to disease development.

Recent advances in stress research have implicated epigenetic modifications in the central nervous system as mechanisms by which environmental stimuli (such as stress) can induce long-lasting alterations in neurobiological systems[167], including the neuroendocrine system[168]. The term ‘epigenetics’ refers to reversible chemical modifications to the chromatin structure that alter gene transcription without altering the DNA sequence. These include DNA methylation, DNA hydroxymethylation, and histone modifications (i.e., methylation, acetylation, and phosphorylation). Other important epigenetic modulators that influence protein expression are microRNAs (miRNAs), which act as translational repressors (see Table 1). While miRNAs do not alter chromatin structure and therefore technically do not follow the classical definition of epigenetics, they are, more often than not, considered important players in the epigenetic control of posttranscriptional gene expression. Altogether, these epigenetic modifications

constitute important mechanisms by which transient environmental stimuli can induce persistent changes in gene expression and ultimately behaviour[169]. However, the exact consequences of epigenetic modifications for gene transcription are not that straight-forward, but seem to be context-dependent and determined by both the location and the nature of the modification. For example, decreasing the accessibility of a gene regulatory element by DNA methylation could either decrease or increase nearby gene transcription, depending on whether a repressor or activator binds at that site[170].

It has long been understood that stress during early life can have a significant lasting influence on the development of neural and neuroendocrine systems, with implications for health and well-being in later life[171-174]. Alterations in epigenetic regulation have been suggested to contribute to this increased risk on neuropsychiatric disease by aberrant gene expression and cell differentiation during early developmental stages[175,176]. Early in development, each cell in the body starts placing epigenetic marks during differentiation under the influence of perinatal environmental cues, with the goal of establishing an adaptive long-term phenotype that meets the probable demands later in life[177]. This process, i.e., transdifferentiation[178] or epigenetic reprogramming[179], may last for weeks, months, and even years, depending on the cell or tissue type. Altered environmental cues (e.g., stress) may therefore greatly affect brain development, as well as regional gene-expression throughout life, in an attempt to meet environmental demands. In later life, these epigenetic changes can proof either adaptive or maladaptive, protecting from or increasing risk on mental disease depending on the later life environment[180,181]. While it is thus clear that there is a window of sensitivity for environmentally induced epigenetic changes during perinatal development, influencing risk on psychopathology, evidence is accumulating that epigenetic plasticity also extends into adulthood[182-185]. It has been shown that psychological trauma during adulthood can induce epigenetic changes that affect brain plasticity, neuronal function, and behavioral adaptation to neuropsychological stress[186,187]. Hence, these epigenetic changes may provide a molecular mechanism for the phenotypical development observed e.g., after trauma exposure in PTSD, explaining how phenotype and transcriptional potential can change drastically and long-lastingly in response to environmental challenges, even when experienced in adulthood. As such, more recent advancements in the fields of epigenetics have focused on the presence of stress-mediated epigenetic modifications in adulthood. The

ability of stressful events to affect epigenetic regulation in the brain has been illustrated in fear conditioning and extinction paradigms in rodents, where contextual fear learning induced altered methylation patterns in memory- and plasticity-related genes[182,183]. Altered hippocampal DNA methylation levels have also been observed in rodent models for PTSD[159,187]. These modifications of DNA transcription were shown to be persistent[188] and even transmissible across generations[189,190], underlining their importance as mediators of the imprinting of stressor experience on brain and behavior. Enhancing our understanding of the epigenetic mechanisms that occur following stress exposure has far-reaching clinical potential. Stress exposure in adulthood not only contributes to the development of stress-related mental disorders, it can also precipitate or perpetuate other psychiatric disorders (e.g. addiction, dementia, and schizophrenia) and can negatively affect the course of non-psychiatric conditions like cancer and cardiovascular disease[170]. As such, being able to improve the ability to treat neuropsychiatric disorders, would not only decrease world-wide stress-related disability, but would also significantly reduce the ever-increasing health-care costs.

Here, we provide a review of recent studies in humans and rodents on epigenetic modulation of the (primarily neuroendocrine) stress response as a consequence of adult life stress. We first summarize evidence for the global changes in epigenetic markers as a consequence of stress exposure in adulthood in rodents and humans. Although (chronic) stress exposure has been clearly linked to increased risk on MDD[191], studies in depressed patients were left out of consideration here, as prior stress exposure is no prerequisite for MDD diagnosis, and resulting pathology can therefore not be causally linked to the experience of (adult) life stress (as is the case for PTSD). We then offer an overview of scientific evidence for stress-induced epigenetic alterations in HPA-axis function and in stress-related neurotransmitter systems. Finally, we discuss the implications of these data for and the challenges of applying this knowledge to the identification and treatment of stress-related psychiatric disorders.

2. STRESS-RELATED GENERAL EPIGENETIC CHANGES

2.1 HUMAN STUDIES

Blood samples of PTSD patients constitute the primary evidence for long-lasting epigenetic modifications due to (adult) stress exposure in humans. Studies have indicated that PTSD patients display increased levels of trimethylation in histone 3 lysine 4 (H3K4), H3K9, and H3K36 in peripheral blood mononuclear cells[192], suggesting altered activity of histone methyl transferases (HMTs) and demethylases (HDMTs), which most likely affects the expression of a plethora of genes. Moreover, a global increase in DNA methylation at thousands of DNA CpG sites was found to be associated with PTSD[193]. These changes were independent of age, ethnicity and, most importantly, early life stress, suggesting that stress during adulthood can alter global DNA methylation patterns, likely through differential regulation of DNA methyl transferases (DNMTs).

Whereas the aforementioned studies relied on retrospective data and thus were unable to demonstrate a causal relationship between stress exposure and the observed epigenetic profiles, a recent longitudinal study by Sipahi, et al. [194] actually did indicate such a causal link. Here, pre- and post-trauma DNA methylation profiles were compared in PTSD patients and age-, gender-, and trauma exposure-matched controls. Trauma-exposure was found to be associated with increased DNA methylation at multiple CpG loci in *DNMT1*, *DNMT3A*, and *DNMT3B* genes. However, remarkably, these epigenetic responses to trauma did not differ between healthy subjects and patients, except for the increased *DNMT1* methylation, which was only observed in patients, suggesting that the majority of these epigenetic changes occurred in response to stress regardless of eventual behavioral symptoms. Moreover, pre-trauma DNA methylation was higher in the patients compared to controls at a single *DNMT3B* CpG site, reflecting a pre-existing risk factor for the development of PTSD in response to trauma. This finding highlights the importance of longitudinal studies for the identification of (epigenetic) risk markers for PTSD, and to distinguish these from pathology-related epigenetic changes that should be targeted in evidence-based interventions (Box 1).

Besides these well-known alterations in gene methylation patterns, recent studies of the epigenetic regulation of the stress response have increasingly implicated miRNAs as important mediators of environmentally-induced alterations in gene expression. miRNA expression levels in rodents and human cells have been found to be altered in response to various environmental factors, such as light, sound, nutrients, drugs, and stress[195]. Preliminary results have demonstrated upregulation of several serum miRNAs directly after an acute social stress task in healthy participants[196] and have associated transiently altered expression of serum miRNAs with chronic academic stress[197]. Abnormalities in miRNA expression have also been implicated in PTSD, with several miRNAs being significantly downregulated in PTSD cases vs. age-matched healthy controls[198]. Lower expression of DICER1, an enzyme that contributes to the generation of mature miRNAs, has been proposed as a molecular mechanism for this decrease in global miRNA levels[199]. Expression of DICER1 and other DICER-like proteins themselves might also be epigenetically regulated, as is suggested by multiple studies investigating RNA-directed DNA methylation in plants[200,201].

While examination of DNA extracted from peripheral blood from patients has provided us with important indications of epigenetic changes induced by stressful life events, stress-related disorders are disorders of the brain. Interindividual variation in whole blood is not a strong predictor of interindividual variation in the brain[202] and epigenetic patterns vary substantially across functionally distinct brain regions[203], making that blood- or saliva-based epigenetic studies provide only limited information on the actual pathological neural processes. Therefore, additional research in brain tissue is important for assessing the epigenetic plasticity of neural cells as a consequence of adult life stress. Human post-mortem studies are most suitable in this respect, but data available is limited as these studies face multiple practical issues in the collection of tissue from a sufficient amount to appropriate subjects, together with a detailed subject history of SLEs, stress-related pathology, and use of medication. Longitudinal studies[204], as well as increased storage and application of human post-mortem data in biobank tissue repositories, like the U.S.A. National Institutes of Health Neurobiobank[205], are necessary to increase insight in the brain region-specific epigenetic profiles associated with stress-related psychopathology.

2.2 ANIMAL STUDIES

A useful remedy to study the epigenetic effects of stress exposure associated with the pathology of stress-related mental disorders in brain tissue is the use of animal models. Animal models provide us with a means to study stress in organisms that i) have a homogeneous genetic and environmental background, ii) can be exposed to standardized stress paradigms in a controlled fashion, iii) can easily be longitudinally studied and iv) allow for more invasive (direct) measurements of brain tissue rather than peripheral blood. Therefore, animal studies allow for the investigation of the causal relationship between stress exposure and changes in the epigenome and thereby to dissect whether epigenetic patterns reflect psychological states (as a consequence of stress) that contribute to psychopathology (Box 2). When studying the stress response in rodents, multiple brain regions are of importance. First of all, the regions involved in the HPA-axis are relevant. These include the paraventricular nucleus (PVN) of the hypothalamus, which contains neuroendocrine neurons that synthesize and secrete corticotropin-releasing hormone (CRH) and vasopressin, and the pituitary, which secretes adrenocorticotropic hormone (ACTH)[161]. Limbic structures of the forebrain, like the amygdala, hippocampus, and prefrontal cortex (PFC) contribute to the regulation of the HPA-axis, by mediating glucocorticoid-induced activation and inhibition of the HPA-axis, respectively[206]. As the neuronal populations in these regions also form the respective anatomical substrates for emotional responding, memory formation, and emotion regulation, they may serve as a link between the stress system and the emotional and cognitive abnormalities observed in neuropsychiatric disorders[207]. Besides these 'classical' regulators, an emerging neurobiological substrate of the stress response is the nucleus accumbens (NAc), where CRH facilitates cue-elicited motivation and social bonding through dopaminergic transmission[208]. Chronic stress has been reported to induce drastic neurochemical alterations in the NAc, leading to a depressive phenotype[209].

2.2.1 GENERAL EPIGENETIC EFFECTS OF ACUTE STRESS EXPOSURE

Research in rodents has indicated that epigenetic modulation and corresponding changes in gene expression as a consequence of stress exposure critically depend on the frequency of the stressor[210]. For example, differential histone methylation patterns in rat hippocampus were observed resulting from either 1 day (acute), 7 days (subchronic), or 21 days (chronic) of restraint stress[186]. H3K9 and H3K27

trimethylation, associated with transcriptional silencing[211,212], were increased by both acute and subchronic stress, but decreased by chronic stress. Conversely, H3K4 trimethylation, a known activator of gene transcription[212], was unaffected by acute and subchronic stress, but significantly increased after chronic exposure. It could be hypothesized that general transcriptional silencing in response to acute stress exposure may avoid the brain from overreacting to the stimulus, whereas activating specific genes in response to chronic stress may allow the brain to properly adapt to the new stressful environment. However, no behavioral data were collected in this study, leaving the functional (i.e., behavioral) relevance of these alterations open for future investigation. Interestingly, repetition of the acute stressor seems to increase its potential to evoke epigenetic alterations. Four consecutive 15-minute sessions of social defeat stress on one day, but not one single 15-minute session, increased hippocampal H3 acetylation in a rat model of social defeat, accompanied by increased depressive-like behaviour[213]. However, H3 acetylation in the defeated animals returned to baseline levels 72 hours after the stress episode, even though the depressive behavior remained present for at least 6 weeks. While this might argue against the histone modification as a potential underlying mechanism for the behavioral profile, transient changes in histone acetylation have previously been proposed to induce long-term changes in gene activity[214,215] and behaviour[216] by inducing transcription of genes that influence the transcription of other downstream targets that are more long-lasting. This emphasizes that their modulation, albeit transiently, can have long-lasting consequences. In line with this modulatory role for stressor frequency, Renthal, et al. [217] showed that a single 10 minute session of social defeat stress was insufficient to alter levels of the histone deacetylases (HDACs) 1, 2, 3, 4, 5, and 9 in the NAc of adult mice, but that a 10-day repetition of the paradigm downregulated HDAC5 in the NAc by almost 25%. This regulation of HDAC5 expression likely contributed to the behavioral consequences of the stressor, as in this same study it was found that HDAC5 knockout mice developed more severe social avoidance and anhedonia in response to the stress paradigm than wild-type littermate controls. Interestingly, knockout and wild-type mice did not differ in their behavioral responses to an acute defeat episode, indicating that HDAC5 is involved in the epigenetic regulation of behavioral adaptations to chronic, but not acute, stress. These findings suggest that the regulatory systems involved in the brain's innate response to stress differ between acute and chronic

exposure. This is especially interesting for understanding vulnerability to PTSD, as both acute (e.g., violent personal assault and severe car accidents) and chronic stress (e.g., war and child neglect) exposure can precipitate PTSD-associated psychopathology[218].

The effectiveness of acute stress to induce epigenetic changes seems to not only depend on stressor frequency, but also on stressor dimension and severity, as 15 minutes of forced swimming and 30 minutes of predator exposure, but not 3 minutes of ether vapor exposure or 4 hours of cold exposure, were found to increase H3 phosphorylation in the rat dentate gyrus (DG)[219]. One hour of acute restraint stress also appeared to be sufficient to significantly decrease global DNA methylation levels in rat hippocampus, medial prefrontal cortex (mPFC), and periaqueductal grey[155]. Possibly, stressors with a strong psychological component (such as restraint and predator exposure) might be more effective at inducing epigenetic changes than primarily physical stressors (such as cold and vapor exposure)[219].

Epigenetic involvement in the persistent behavioral consequences induced by acute stressors is also apparent in the formation of long-lasting, recurring traumatic memories, characteristic for PTSD[220]. Animal models have identified a critical contribution of epigenetic modifications in the hippocampus and amygdala to the encoding and expression of fear memory[132,221]. DNMT inhibition in the rat hippocampal CA1 region[222] and lateral amygdala[223] following fear conditioning was shown to disrupt the consolidation of contextual and cued fear, respectively. This indicates an important role of DNA methylation in trauma memory formation. Moreover, histone acetylation, especially in hippocampal H3[135] and H4[224], as well as amygdalar histone trimethylation of H3K4[225], have been found to also promote fear encoding. Extensive reviews describing the involvement of epigenetic mechanisms in fear memory formation have been performed by Roth, et al. [226], Zovkic, et al. [169], Kwapis and Wood [227], Rudenko and Tsai [228], and Blouin, et al. [229].

2.2.2 GENERAL EPIGENETIC EFFECTS OF CHRONIC STRESS EXPOSURE

The epigenetic effects of chronic stress have been more elaborately studied. At the histone level, many changes in methylation and acetylation status have been found following repeated stress exposure. Wilkinson, et al. [230] observed increased accumbal H3K9 and H3K27 dimethylation in rats exposed to either

10 days of social isolation or social defeat stress compared to controls, which was associated with depressive-like avoidance behavior. As animals that were behaviorally resilient to the social avoidant phenotype displayed histone methylation levels resembling those of control animals, these epigenetic effects seem to be directly related to the behavioral consequences of this chronic stressor. Both the increase in histone dimethylation and the avoidant phenotype remained stable 28 days post stress-termination, indicating that the changes are rather long-lasting. Moreover, the increases in methylation level were significant even after averaging across the entire genome, lending credence to the idea that widespread stress-induced epigenetic changes in the NAc occur throughout the entire genome. In contrast to the *increased* histone methylation in the NAc, 10-day socially defeated animals were shown to display *decreased* global DNA methylation levels in the mPFC[231], which were accompanied by an anxious phenotype. This reduction in global methylation levels was associated with an increased expression of mPFC DNMT3A. Further confirming the region-specific nature of the epigenetic changes in the brain, DNMT3A was upregulated in the central nucleus of the amygdala (CeA), while DNMT3B levels, which were not altered in the mPFC, were downregulated in this region. Other studies have reported that DNMT3A is upregulated in the NAc[232] and downregulated in the hippocampus[154] of defeated vs. control mice. Additionally, DNMT3B was found to be reduced in the paraventricular nucleus (PVN) of the hypothalamus[233] of vulnerable vs. resilient mice after chronic social defeat. Chronically stressed animals also show differential histone acetylation patterns when compared to controls. Ferland and Schrader [234] reported on decreased rat hippocampal H3K9 and H4K12 acetylation as a consequence of 14-day chronic variable stress (CVS). Application of HDAC inhibitors to hippocampal slices induced a stronger increase in histone acetylation in the CVS animals compared to the controls, implying higher HDAC activity as a consequence of chronic stress. Similar decreases in hippocampal H3K9 and H4K12 acetylation were observed in rats following 28 days of chronic unpredictable stress (CUS)[235], which was accompanied with a significant increase in HDAC5 in hippocampal tissue. Interestingly, HDAC5 was found to be downregulated in the amygdala[236], as well as the NAc[217] in chronically stressed rats, again pointing towards region-specific epigenetic modulations. Lastly, HDAC2 was found to be downregulated by 10-day social defeat stress in the NAc of defeated vs. control mice, coinciding with increased accumbal H3K14

acetylation in the NAc[237] and in the PVN of social avoidant vulnerable compared to resilient mice[233] (see Table 2 for an overview of all reported region-specific stress-induced epigenetic changes).

Rodent models have also demonstrated altered region-specific miRNA levels in response to both acute[238-241] and chronic stressors[242-244]. Moreover, altered miRNA expression levels have been observed as a consequence of a model for PTSD-induction in rats[245] and have been proposed as mediators of resilience to chronic stress[246,247]. Yet, determining the role of miRNAs in regulatory processes remains a major challenge, as miRNAs often have a wide range of direct molecular targets and might indirectly influence the expression of even more genes by altering the levels of transcription factors[248]. Hence, identifying important target genes for miRNAs often relies on *in silico* target prediction.

3. STRESS-INDUCED EPIGENETIC MODIFICATION OF THE HPA-AXIS

While it is clear that there is a myriad of epigenetic modifications occurring after stress exposure, those occurring in genes involved in the regulation of the HPA-axis are of particular importance. As mentioned before, stress-related psychopathology is associated with HPA-axis dysfunction[162], which has clear clinical relevance; elevated basal cortisol has for example been shown predictive of the risk for depressive episodes[249], whereas successful antidepressant treatment is associated with the resolution of the impaired HPA-axis negative feedback[250] by restoring corticosteroid receptor expression in the brain[251], which also predicts the patient's long-term clinical outcome[250]. In PTSD, low cortisol levels following trauma have been shown predictive of subsequent PTSD symptomatology[252-255], whereas elevating these levels reduced PTSD incidence[256-259]. Corticosteroid administration prior to trauma was shown to reduce PTSD symptoms[260,261], whereas preliminary work indicated that chronic corticosteroid treatment of PTSD patients reduces symptomatology[262]. In this section, we will discuss how stress-induced epigenetic alterations in adult life can mediate changes in HPA-axis function through affecting CRH and glucocorticoid signaling, mainly in the hypothalamic PVN, hippocampus, and PFC.

3.1 CORTICOTROPIN-RELEASING HORMONE SIGNALLING

CRH expression in the PVN, amygdala and bed nucleus of the stria terminalis is related to a wide range of stress-adaptive responses, including the autonomic, immune, and behavioral domain[263]. Stress exposure generally increases PVN *CRH* mRNA and peptide levels, peaking at 30 minutes post-stress and slowly declining thereafter[264]. Increased stress-induced *CRH* transcriptional responses have been linked to both early and adult life trauma exposure[265-269], and epigenetic mechanisms may underlie these changes. Sterrenburg, et al. [236] reported on demethylation of the *Crh* promoter region and subsequent CRH upregulation in the PVN of stressed rats compared to controls as a consequence of 14-day chronic variable mild stress. Similar alterations have been observed in the mouse PVN following chronic social defeat stress in vulnerable vs. resilient animals[233], demonstrating a direct link between the epigenetic alterations and the observed social avoidant phenotype. DNMT3B and HDAC2 in the PVN were decreased and the demethylation-promoting factor GADD45 was substantially upregulated 1 hour after the last social defeat

session in defeated vs. control animals, suggesting their involvement in *Crh* demethylation. The increased CRH levels, demethylation of *Crh*, and the decrease in HDAC2 remained present for at least 2 weeks after the end of social defeat. CRH is thought to exert its overall anxiogenic effects by binding to CRH receptor 1 (CRHR1)[270]. A recent study showed that 21 days of CUS decreased hypothalamic H3K9 trimethylation in the rat, which was correlated with elevated levels of local CRHR1 expression and avoidance behaviour[271]. Moreover, Sotnikov, et al. [272] showed that amygdalar CRHR1 expression was regulated by *Crhr1* methylation and correlated with trait anxiety, substantiating the link between epigenetic regulation of the CRH-CRHR1 system and the anxious phenotype induced by stress. A growing body of evidence demonstrates that also miRNAs can regulate the expression of HPA-axis-related target genes. Haramati, et al. [241] reported on decreased levels of amygdalar miR-34c following acute social defeat, which was found to target *Crhr1* via a complementary site on the 3' untranslated region of the receptor transcript. Overexpression of miR-34c appeared to reduce cell responsiveness to CRH by inhibiting CRHR1 expression and induce an anxiolytic phenotype. Among the predicted targets of the miR-34c family were also other stress-related proteins, including brain-derived neurotrophic factor (BDNF) and 5-HT and glutamate receptors. These data suggest that miR-34c plays a role in regulating multiple amygdalar genes that collectively modulate the behavioral response to stress.

An important modulator of CRH expression is the BDNF. BDNF is able to induce expression of CRH in the PVN by binding to hypothalamic tropomyosin receptor kinase B (TrkB) receptors. TrkB activation induces expression of cAMP response element-binding protein, which binds to the *Crh* promoter region and acts as a transcriptional activator[273]. BDNF in the rat PVN has been found to be upregulated by chronic restraint stress, concurrent with elevated *Crh* mRNA levels[274]. Upregulation of PVN BDNF by stress-induced epigenetic modifications could therefore contribute to the increased CRH expression and the HPA-axis dysfunction that is observed in rodents following chronic stress in adulthood[275] and in human stress-related pathology[162]. In contrast, both acute and chronic stress have been found to reduce BDNF expression in the mouse and rat hippocampus, which was associated with increased local H3K27 methylation[215], decreased H3 acetylation[276], and enhanced *Bdnf* promoter methylation[187,277]. Furthermore, hippocampal expression levels of TrkB were reduced following forced swim stress, which increased

methylation of *Trkb*[277]. Decreased hippocampal BDNF has been hypothesized to underlie hippocampal dysfunction in response to traumatic stress[278,279], as BDNF is an important neurotrophic factor that enhances long-term potentiation and other forms of synaptic plasticity in the hippocampus[280]. Indeed, overexpression of hippocampal BDNF has been found to mediate behavioral resilience to chronic mild stress in rats[281]. Despite evidence for altered *Bdnf* methylation levels in rodent PVN and hippocampus, plasma BDNF levels and *BDNF* methylation status were not found to be altered after acute psychosocial stress in healthy human subjects[282].

3.2 CORTICOSTERONE SIGNALLING

3.2.1 GLUCOCORTICOID RECEPTOR

Many of the behavioral effects of stress-induced corticosteroid release are thought to be mediated by activation of GRs[283,284]. Moreover, corticosteroid binding to GRs contributes to the negative feedback inhibition of the HPA-axis, which is important in the termination of the stress response. This negative feedback loop is disrupted in PTSD, thought to be mediated by increased GR expression levels in the PFC and hippocampus[285]. This implies that altered regulation of GR transcription by epigenetic modifications serves as a potential underlying mechanism. Demethylation of *NR3C1*, the gene coding for GR, was observed in blood and saliva from PTSD patients vs. trauma-matched healthy controls[286-288]. *NR3C1* methylation levels even inversely correlated with PTSD symptom severity, emphasizing its relevance to psychopathology. Although these patient studies do not provide evidence for a causal role of trauma-exposure to these differences, rodent work has reported on increased DNA hydroxymethylation (5-hmC) of the *Nr3c1* promoter in mouse hippocampus after acute stress exposure[158]. Since 5-hmC is associated with active gene transcription[289], these data suggest that the observed increased 5-hmC is likely associated with elevated local GR expression. This would be in line with previous findings that acute stress in adulthood increases hippocampal *Nr3c1* mRNA levels in mice[290]. The study by Li, et al. [158] did not detect a stress-related change in total methylation levels (i.e., 5-mC + 5-hmC), suggesting that the increase in 5-hmC was paralleled by a decrease in 5-mC, which collectively induced the stress-related NR3C1

upregulation. Stress exposure may additionally induce alterations in the epigenetic regulation of FKBP5, a known regulator of GR sensitivity[291], as corticosterone administration during adulthood was shown to increase anxiety-like behavior and elevate mouse hippocampal FKBP5 expression (and thus potentiate GR-sensitivity) by decreasing DNA methylation at the *Fkbp5* locus[292]. These findings collectively suggest that disrupted negative glucocorticoid feedback, as observed in PTSD, is characterized by elevated hippocampal and PFC GR levels, mediated by epigenetic mechanisms on the DNA and RNA level.

In contrast, Uchida, et al. [293] reported on the downregulation of GR expression by miRNAs in the rat PVN following repeated restraint stress, a paradigm commonly used to induce a depressive-like phenotype[294,295]. Protein, but not mRNA levels of PVN GR, were found to be significantly lower in repeatedly stressed vs. control rats, suggesting the involvement of regulatory mechanisms at the post-transcriptional level. Indeed, miR-18a, targeting two sites of the 3' untranslated region of *Nr3c1* and downregulating gene expression, was found to be upregulated in the PVN. The finding that GR expression is elevated by acute stressors, but decreased by repeated stressors, might reflect earlier observations that GR expression (and thereby negative feedback regulation) is oppositely affected in MDD and PTSD[296].

3.2.2 MINERALOCORTICOID RECEPTOR

Whereas the role of GR in stress response reactivity and regulation has been extensively studied, the mineralocorticoid receptor (MR), has received less attention. While the GR is associated with regulation of HPA negative feedback and termination of the stress response, the MR, which in humans is encoded by the *NR3C2* gene, is thought to be involved in the appraisal process and onset of the stress response upon binding of glucocorticoids[297]. Co-localization of both receptors is found in the hippocampus of almost all species[298]. The receptors collectively orchestrate the stress response as an altered GR/MR balance has been implicated in persistent dysregulation of the HPA-axis[299]. Recently, the possibility of dynamic regulation of MR expression in response to stress was demonstrated in a preclinical study showing an increase in rat hippocampal MR density after a forced swimming task[300], which served to restrain the HPA-axis. Hippocampal *Nr3c2* mRNA levels were however found to be decreased by almost 20% due to CUS[301], whereas local MR (but not GR) protein levels were reduced following the chronic administration of

corticosterone[302], which was accompanied by depressive-like symptomatology. These results indicate that MR expression is highly responsive to stress exposure, which likely has important consequences for neuroendocrine control of the stress response. *NR3C2* is also subject to epigenetic regulation, but, in contrast to the case of *NR3C1*, only few studies have investigated this. Perroud, et al. [303] reported on lower methylation of several CpGs located within the *NR3C2* promoter in trauma-exposed women. While plasma MR levels were significantly elevated in these same individuals, no significant correlation was found with the altered *NR3C2* methylation status. Recent findings in rodents[304] have also implicated miRNAs (miR-135a and miR-124) as potential regulators (i.e., suppressors) of NR3C2 protein expression. An independent study by Mannironi, et al. [239] showed that these miRNAs were downregulated in the mouse amygdala following acute restraint stress, which increased amygdalar MR expression.

4. STRESS-RELATED EPIGENETIC MODIFICATION OF STRESS-RELATED NEUROTRANSMITTERS

Besides modulating the neuroendocrine response to stress, epigenetic modifications may alter neurotransmitter release and signaling in brain circuits that orchestrate the stress response and are known to be altered in PTSD[305]. Alterations in dopamine (DA)[306], norepinephrine (NE)[307], and serotonin (5-HT)[308] transmission are thought to contribute to the symptoms commonly observed in PTSD patients, including hypervigilance, impulsivity, exaggerated startle, and depressed mood, and may be subject to epigenetic regulation. For example, levels of the enzymes tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH), responsible for creating precursor metabolites for the production of DA, epinephrine, NE, and 5-HT, were found to be significantly decreased in the hippocampus of chronically stressed rats[235]. This decreased TH and TPH expression was blunted by the administration of an HDAC5 inhibitor, implicating epigenetic mechanisms in mediating these effects. Direct evidence for altered epigenetic regulation of stress-related neurotransmitters as a consequence of adult life stress exposure primarily exists for the serotonergic system. The serotonin transporter (5-HTT), which in humans is encoded by *SLC6A4*, is an integral membrane protein in the central and peripheral nervous system that transports 5-HT back from the synaptic cleft into the pre-synaptic neuron, thereby waning serotonergic transmission. Reduced expression of this transporter incites high basal 5-HT levels, which has been associated with enhanced vulnerability to chronic stress[309] and increased risk for life time depression[310]. Resilience to clinical depression under chronic high stress conditions was however found to be associated with reduced methylation of the *SLC6A4* promoter[311], which is expected to increase 5-HTT expression[312]. Increased reuptake of 5-HT by 5-HTT and subsequent basal 5-HT decrease might therefore be a mechanism of stress adaptation, contributing to chronic stress resilience. Chronic stress was also found to induce a long-lasting upregulation of *5-Ht1a* RNA and 5-HT1A protein levels in the mouse mPFC and dorsal raphe nucleus[313], corroborating the evidence of altered epigenetic regulation of serotonergic transmission as a consequence of adult life stress exposure. This stress-induced increase in *5-Ht1a* mRNA was paralleled by the increased methylation of a uniquely conserved CpG site in *5-Ht1a* that serves as a binding site for the transcriptional repressor Sp4, explaining the observed upregulation in expression. Yet, it is unknown how these changes

in 5-HT1A expression affect serotonergic transmission, as they may upregulate 5-HT1A in different cell (interneurons vs. pyramidal cells) and receptor types (post-synaptic receptors vs. autoreceptors) which regulate serotonergic network activity in an opposite manner.

Findings from human studies have indicated that epigenetic modifications, besides having a direct modulatory effect, can also interact with the genotype to shape the stress response. DNA methylation profiles within *SCL6A4* were found to moderate the association of the 5-HTT linked polymorphic region (5-HTTLPR) and stress coping[314,315]. High serum *SCL6A4* methylation was associated with an increased risk of unresolved responses to loss or other trauma in carriers of the usually protective 5-HTTLPR long allelic variant, while low levels of methylated *SCL6A4* predicted unresolved loss or trauma in short allele carriers.

5. CONCLUSION AND FUTURE DIRECTIONS

In this review, we have provided a comprehensive overview of several lines of evidence suggesting that epigenetic modifications form an important link between stress exposure in adult life and the resulting persistent changes in gene expression and behavior associated with stress-related psychopathology. This epigenetic regulation can be found at the level of many mediators of the stress response, including neuroendocrine components of the HPA-axis and stress-related neurotransmitter system. Epigenetic mechanisms have been shown to underlie the stress-induced alterations in the HPA-axis that are observed in PTSD patients and rodent models of acute and chronic stress. This includes increased CRH expression in the PVN, decreased hippocampal CRH and MR levels, and elevated hippocampal and prefrontal GR expression. This knowledge can be of critical importance to treat stress-related symptomatology.

While the reviewed rodent studies provide valuable insights into the relatively short-term epigenetic response to adult life stress, a thorough assessment of persistent changes over prolonged periods of time is required to better model the lasting and intrusive nature of stress and trauma-exposure on neuroendocrine function and the associated neuropsychiatric symptomatology. All studies into acute and chronic stress investigated epigenetic marks relatively shortly (1-28 days) after the last stressor. It would, however, be interesting to test for the involvement of epigenetic mechanisms in the long-lasting behavioral effects of transient stress exposure. This type of longitudinal research is already being performed to study the epigenetic consequences of early life stress during adulthood, for example by Bockmuhl, et al. [316] and Pusalkar, et al. [317], who followed up rats and mice for 6 and 15 months after perinatal stress, respectively. Following up adult rodents for several months after stress induction could yield valuable information about the epigenetic processes and marks that play a role in the induction of long-term depressive and anxious phenotypes.

While evidence is accumulating for a crucial role of epigenetic modifications in the pathology of stress-related disorders, the next step should be to apply this knowledge to prevent and treat these disorders by targeted interventions. Once we have an overview of the maladaptive epigenetic changes that occur after stress exposure that are linked to neuropathology; is it possible to revert these changes and to remodel

the stress-vulnerable brain to a stress-resilient brain? Preliminary findings have focused on five possible intervention / treatment strategies:

i) Antidepressants. The tricyclic antidepressant imipramine and the selective serotonin reuptake inhibitor fluoxetine have been shown to revert stress-induced histone demethylation[186] and methylation[230], demethylation of *Crh*[233], methylation of *Bdnf*[215] and *5-Ht1a*[313] and decreased levels of HDAC5[217], which all reduced depressive and anxiety-like behavior induced by the respective stress protocols.

ii) HDAC inhibitors. The HDAC inhibitors sodium valproate and MS-275 have been shown to reduce depressive and anxiety-like behavior by reverting stress-induced increases in HDAC2 and HDAC5 and subsequent histone acetylation marks on H3K9, H3K14 and H4K12[235,237].

iii) DNMT inhibitors. The DNMT inhibitor RG108 has been shown to reduce depressive and anxiety-like behavior by reverting stress-induced increases in DNMT3A[231].

iv) miRNAs. The amygdalar miRNA-34 has been identified as a repressor of stress-induced anxiety[241]. As such, miRNA-34 and other stress-related miRNAs pose potential novel targets for treatment of stress-related disorders.

v) Exercise. Physical exercise has been shown to improve cognitive responses to psychosocial stress and rescue rats from social defeat-induced anxiety-like behavior and memory impairment[318]. This beneficial effect might potentially be mediated by epigenetic mechanisms, including exercise-induced H3 acetylation and modulation of methylation in the hippocampus[319].

However, it is currently mechanistically unclear whether the behaviorally beneficial effects of these treatments are mediated directly through an effect on the epigenome, or through another external mediator affecting both behavior and epigenetic markers independently. Because these treatment strategies all have a broad scope and potentially affect a wide range of processes in the body, higher precision DNA editing might be necessary to specifically target epigenetic marks in the brain and enable personalized medicine. Still, these preliminary results show that it possible to attend to the behavioral consequences of stress exposure by pharmacological and therapeutic interventions targeting epigenetic profiles.

DECLARATION OF INTEREST

The authors have no conflict of interest to report.

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FIGURES AND BOXES

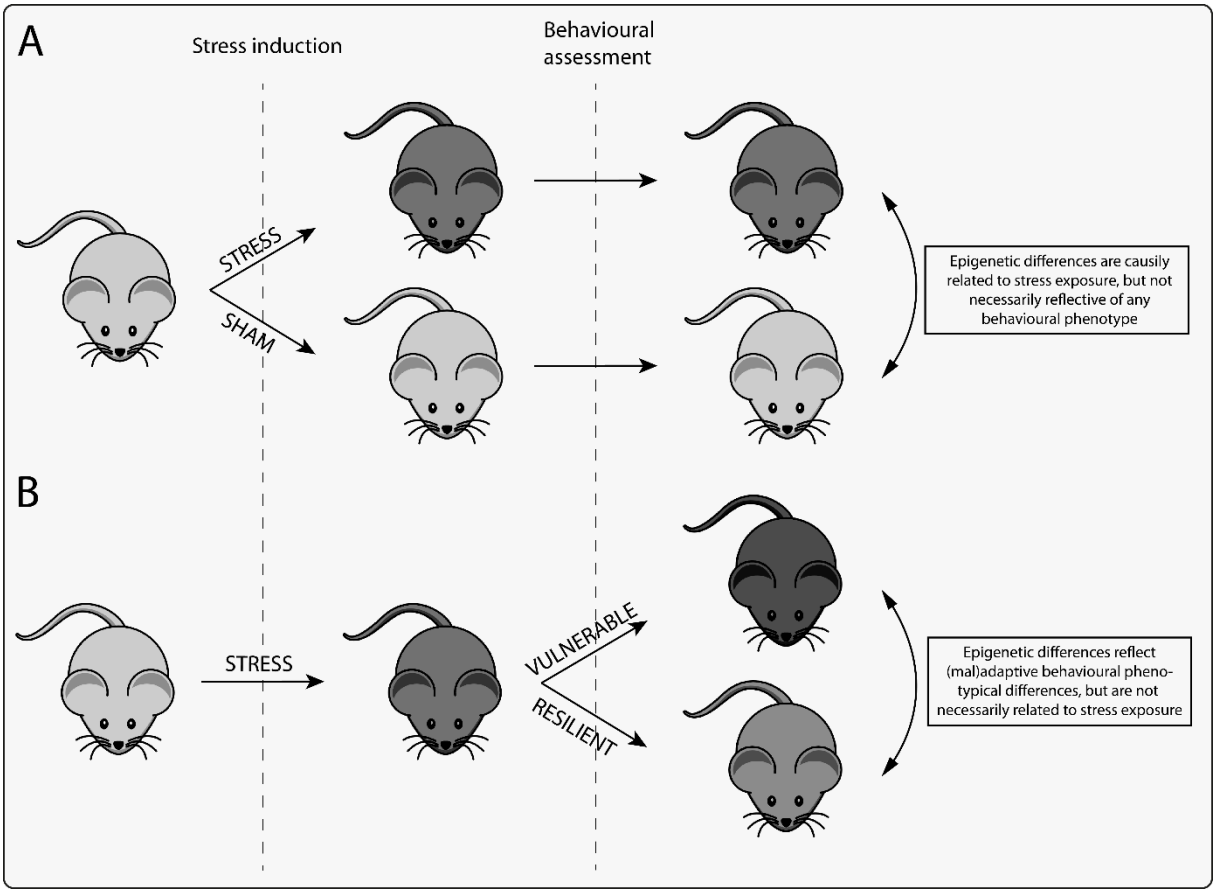

Fig 1. Animal models of stress. (A) Stressed vs. control contrast: half of the animals from a genetically homogeneous group undergoes a certain stress procedure, while the other half receives a sham procedure. **(B)** Vulnerable vs. resilient contrast: all animals from a group undergo the same stress procedure and are tested on stress-related symptomatology afterwards. The behaviorally most resilient animals are compared to the behaviorally most vulnerable animals.

Box 1. Epigenetic contributions to individual stress vulnerability

Stressful life events (SLEs), caused by environmental, psychological, or social situations, are important risk factors for the development of neuropsychiatric disorders, including MDD, PTSD, and anxiety disorders[320]. While an estimated 90% of individuals in the general population are faced with one or multiple SLEs at some point in their lives, only a small percentage of these individuals ultimately develop psychiatric symptoms. This implicates interindividual differences in the underlying mechanisms constituting (natural) vulnerability or resilience to stress-induced pathology[321]. Influential studies on monozygotic twins have demonstrated that stress vulnerability can be explained partially (30-70%) by genetic variation, mainly mediated by single nucleotide polymorphisms[322,323]. In addition, epigenetic patterns, either inherited or resulting from the cumulative environmentally-induced alterations that occurred throughout life, can shape vulnerability (i.e., the induction of pathological processes following stressor exposure) and resilience (i.e., the absence of psychiatric symptoms despite stressor exposure) to the development of psychopathology following future stressors[170]. As such, neuropsychiatric disorders which develop during adulthood are most likely caused by a combination of pre-existing genetic and epigenetic vulnerability factors and alterations that are caused as a consequence of adult life stress exposure itself[324], as suggested by the diathesis-stress model for psychiatric illnesses[325] and the three-hit concept of vulnerability to stress-related mental disorders[326]. In line with this idea of differential (pre-existing) epigenetic patterns reflecting vulnerability, DNA methylation of *SKA2* and *BDNF* prior to trauma exposure was found to predict suicidal behavior and PTSD symptomatology[327-329], while methylation of *SLC6A4*[330] and *GRIN1*[331], which encodes subunit zeta-1 of the N-methyl-D-aspartate (NMDA) glutamate receptor, predicted depression. Furthermore, other human studies have linked the basal state of the DNA methylome to substance abuse[332], aggression[333], and depressive behaviour[334].

Box 2. Epigenetic contributions to a stress-related phenotype

When investigating the epigenetic 'backbone' of stress-related disorders to improve treatment, it is important to consider the causal relationship between the epigenetic signature and the observed behavioral phenotype. Yet, it is difficult to establish i) which epigenetic marks are directly linked to a certain stressful event (or instead reflect inborn differences (see Box 1)) and ii) which epigenetic marks directly contribute to pathology, by mere *post-hoc* comparisons in human studies. However, rodent studies can be specifically designed to yield information about the exact factors contributing to a stress-related phenotype. Two important contrasts are studied (Fig. 1):

i) Stressed vs. control. Half of the animals from a genetically homogeneous group undergo a certain stress procedure, while the other animals receive a sham procedure. Afterwards, differences in epigenetic regulation between the two groups are assessed. Notably, the observed differences reflect epigenetic changes that can be directly linked to stress exposure, and are likely reflective of the mean behavioral differences between the stressed and control animals, but not necessarily directly related to any stress-induced phenotype.

ii) Resilient vs. vulnerable. All animals from a group undergo the same stress procedure and are tested on stress-related symptomatology afterwards. The behaviorally (most) resilient animals are compared to the behaviorally (most) vulnerable animals to distinguish potential adaptive from maladaptive epigenetic changes as a consequence of stress exposure. More so than in the stressed vs. control contrast, this contrast links epigenetic signature directly to the behavioral phenotype (i.e., psychopathology). However, the observed epigenetic signature is not necessarily linked to any alterations induced by the stress procedure in itself, as the animals' epigenetic profiles might have already been distinct before the procedure (and reflect innate susceptibility (Box 1)). Still, studying which epigenetic marks underlie the behaviorally adaptive responses of the resilient animals that distinguish them from the behaviorally maladaptive ones, may provide useful starting points for treating stress-related disorders.

Box 3. Mechanisms for stress-induced epigenetic alterations

While it has been known for quite some years that stress exposure can induce epigenetic modifications in a variety of genes and brain regions, it is still largely unclear by which molecular pathways these effects are exactly established. Recent studies have however started to elucidate these mechanisms by implicating a novel, non-genomic mechanism by which glucocorticoids act to (amongst others) facilitate consolidation of memories associated with a specific adverse event through epigenetic pathways. Gutierrez-Mecinas, et al. [335] observed that binding of glucocorticoids to GRs in rat hippocampal DG granule neurons activated the extracellular signal related kinase (ERK) / mitogen-activated protein kinase (MAPK) signaling pathway. Downstream kinases of this pathway induced serine 10 phosphorylation and lysine 14 acetylation at histone H3 (H3S10p-K14ac) via recruitment of histone acetyl-transferases[336]. This epigenetic mark has been associated with the activation of silent genes, possibly through chromatin remodeling, making them accessible for transcription[337,338]. This glucocorticoid-induced H3S10p-K14ac could long-lastingly activate genes that were silent before stress exposure, thereby offering a possible mechanism by which stress could induce stable epigenetic and (eventually) behavioral alterations. Indeed, the interaction of the H3S10p-K14ac mark with the promoter region of the immediate-early genes (IEGs) *c-Fos* and *Egr-1* was found to facilitate the induction of these genes[335]. Injection of a GR-occupying dose of corticosterone in rat hippocampus was however ineffective to form H3S10p-K14ac and induce IEG expression, suggesting the required involvement of another molecular pathway in mediating these effects[336]. The NMDA receptor was later identified as a co-activator of the MAPK pathway, whose synchronized activation is necessary for formation of H3S10p-K14ac and IEG induction[339]. For an extensive review describing this glucocorticoid control over epigenetic modifications, see Reul, et al. [340].

Chapter 3

The hippocampal memory engram coding traumatic stress susceptibility

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ABSTRACT

While the majority of the population is ever exposed to a traumatic event during their lifetime, only a small fraction of them develops post-traumatic stress disorder (PTSD). Previous work has implicated disrupted trauma memory processing as a core factor underlying of PTSD symptomatology. Here, we used transgenic Targeted Recombination in Active Populations (TRAP) mice to investigate potential alterations in trauma-related hippocampal memory engrams being associated with the development of PTSD-like symptomatology. Mice were exposed to a stress-enhanced fear learning paradigm, in which prior exposure to a stressor (severe, unpredictable foot shock) affects the learning of a subsequent fearful event (contextual fear conditioning using mild, predictable foot shocks), during which neuronal activity was labeled. One week later, mice were behaviorally phenotyped and classified into subgroups of resilient and susceptible to developing PTSD-like symptomatology. Three weeks post-learning, the mice were re-exposed to the conditioning context to induce memory recall, and recall-induced neuronal activity in the hippocampus was analyzed. While no differences in the size of the hippocampal neural ensemble activated during fear learning were observed between the groups, susceptible animals were characterized by a smaller ensemble activated upon remote fear memory recall in the ventral CA1, as well as higher regional hippocampal PV^+ neuronal density and a relatively lower activity of PV^+ interneurons upon remote memory recall. Investigation of potential epigenetic regulators of the engram, revealed rather generic, instead of engram-specific differences between groups, with susceptible mice displaying lower hippocampal histone deacetylase 2 expression, as well as higher methylation and hydroxymethylation levels. Altogether, these finding implicate variation in epigenetic regulation within the hippocampus, as well as reduced regional hippocampal activity during remote fear memory recall in interindividual differences in susceptibility to traumatic stress.

INTRODUCTION

Post-traumatic stress disorder (PTSD) is a debilitating disorder one can develop after exposure to a traumatic event. One of the hallmark features of PTSD is the re-experiencing of the trauma by flashbacks, spontaneous recollections, and recurrent nightmares of the trauma, which affect over 90% of patients[6,341]. Behavioral treatment strategies in which the trauma memory is targeted are among the most effective clinical treatments for PTSD[38,342], implicating disrupted trauma memory processing in PTSD. Interestingly, whereas the majority of the population is ever exposed to a traumatic event during their lifetime, only a small fraction of them develops PTSD[321]. We hypothesize that resilience may be characterized by adaptive trauma memory processing, which turns maladaptive in susceptible individuals. During trauma processing, the complex configuration of trauma-related information triggers the activity of neural ensembles that communicate through neuronal synapses, which are subsequently strengthened and stabilized through synaptic plasticity at the neuronal and circuit level[94]. These neural ensembles in which the memory is physically stored are referred to as the memory engram[98,99]. The development of new genetic tools provides current, unprecedented opportunities to capture and study these engrams[95]. Here, we make use of Targeted Recombination in Active Populations (TRAP) to investigate whether PTSD-like symptomatology is associated with an aberrant hippocampal trauma memory engram.

Decades of work have implicated the hippocampus as an important site for memory engrams, through its role in contextual memory processing[343,344], and its modulation by the amygdala in case of emotionally salient events[54,345,346]. Neuroimaging studies have observed smaller hippocampal volume[347] and impaired function[344] in PTSD patients, while animal models for PTSD have shown increased hippocampal apoptosis[348], reduced levels of brain-derived neurotrophic factor[349] and increased glucocorticoid receptor expression[72], implicating aberrant hippocampal function in PTSD pathophysiology. Furthermore, reduced hippocampal activity during exposure to trauma-related stimuli has been positively correlated with PTSD severity[350] and trauma-related memory distortions in PTSD-affected combat veterans[351]. Yet, it remains unclear how these rather generic hippocampal abnormalities

relate to potential deviations in the memory engram for the traumatic event itself. Here, we investigated whether deviations in the hippocampal trauma memory engram code vulnerability to the long-term consequences of trauma exposure in terms of PTSD-like symptomatology in mice, dissociating ventral from dorsal hippocampus[352-354], as well as hippocampal subregion (i.e., dentate gyrus (DG), Cornu Ammonis areas 1 (CA1) and 3 (CA3))[355].

As potential modulators of the engram, we investigated parvalbumin positive (PV^+) interneurons, which innervate large numbers of hippocampal pyramidal neurons and are spatially well-positioned to coordinate neuronal ensemble activity[356]. Moreover, PV^+ neurons are vulnerable to the effects of prolonged stress[357,358], and their activity in the CA1 has been shown required for the stabilization of hippocampal connectivity networks upon learning of a novel experience[359]. Additionally, we investigated epigenetic regulation, which confers transcriptional memory of exposure to environmental stress conditions[133,134], regulates memory formation[135] and shapes long-term behavioral adaptations[136-138]. Histone acetylation is most robustly associated with memory formation[360] and the expression of particularly hippocampal histone deacetylase (HDAC) 2 is negatively related to memory performance and hippocampal plasticity[149,150]. Prior reports have shown that chronic stress downregulates hippocampal HDAC2 levels, causing depressive-like symptomatology in mice[148]. Yet, others reported on a stress protective effect HDAC2 reductions[151]. Similarly, stress exposure changes DNA methylation state[152], with both stress-induced increases[153,154] and reductions[155] in hippocampal DNA methylation being observed.. Also 5-hydroxymethylcytosine (5hmC) levels, a stable epigenetic modification[156] modulating gene transcription independently from 5mC[157], have been shown to be modulated by prior stress exposure[158].

We here used a mouse model to test our hypothesis that alterations in trauma-related hippocampal engrams are associated with the development of PTSD-like symptomatology, and investigated aforementioned key engram regulators potentially at the core of these alterations. The PTSD mouse model used is based on the phenomenon of stress-enhanced fear learning (SEFL[28,361]), with prior stress

exposure altering fear learning and memory. Mice were therefore first exposed to a stressor (severe, uncontrollable, unpredictable foot shocks), followed by contextual fear conditioning (mild foot shock) the next day. Critically, in this PTSD model, mice were behaviorally tested for PTSD-like symptoms to dissociate susceptible from resilient mice[26,362,363] and delineate distinct fear memory formation and recall in these subgroups, respectively. Engram neurons activated during the encoding of SEFL were identified by using the TRAP transgenic mouse model[102], whereas engram neurons supporting remote fear memory recall were identified by conditioning context re-exposure three weeks later by immunohistochemistry. PV^+ interneuron presence and activity, as well as HDAC2, 5mC and 5hmC expression levels in both engram and non-engram neurons were assessed by immunohistochemistry as well.

MATERIALS & METHODS

Animals.

Two founder mouse lines, ArcCreER^{T2} (B6.129(Cg)-*Arc*$^{tm1.1(cre/ERT2)Luo}$/J) and conditional tdTomato (B6.Cg-*Gt(ROSA)26Sor*$^{tm9(CAG-tdTomato)Hze}$/J, 007909), were purchased from The Jackson Laboratory and bred as described before[102] to generate heterozygote ArcCreER^{T2}xROSA offspring, referred to as ArcTRAP. This genetic construct allows *Arc*-expressing (i.e., active) neurons to be labeled by the fluorescent protein tdTomato in a 36 hour time window after injection with the compound tamoxifen. Because the PTSD model[26,362] has only been validated in males, experiments were restricted to male mice. Mice were group housed (3-4 mice per cage) in individually ventilated cages on a reverse 12 h light/dark cycle (09:00 - 21:00 h) at the Central Animal Facility of the Radboud University Nijmegen, The Netherlands, according to institutional guidelines. Food and water were provided *ad libitum*. Unless otherwise stated, behavioral testing was performed during the animal's active phase (i.e., the dark) between 13.00 - 18.00 h. The experimental protocols were in line with international guidelines, the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council 2003), the principles of laboratory animal care, as well as the Dutch law concerning animal welfare and approved by the Central Committee for Animal Experiments, Den Haag, The Netherlands.

General procedure.

44 ArcTRAP mice were injected with tamoxifen to induce fluorescent labeling of all *Arc*-expressing neurons and subsequently exposed to a PTSD mouse model as described before[26,362] (Figure 1A). The model is based on stress-enhanced fear learning (SEFL), which builds on the clinical observation that prior stress exposure precipitates PTSD[364,365]. In SEFL, prior stress exposure is observed to affect the learning of future aversive events, creating traumatic-like memories characterized by exaggerated fear responses and resistance to extinction[28,361,366,367]. Importantly, the SEFL paradigm induces persistent behavioral symptoms in a subset of mice[26,362,363], resembling observations in PTSD patients, while also recapitulating the hypothalamic-pituitary-adrenal (HPA)-axis abnormalities as observed in subgroups of PTSD patients (i.e., reduced glucocorticoid release upon challenge[368]) in susceptible mice. These

consequences are not observed if mice are only exposed to the initial stressor[26], emphasizing aberrant fear learning to be at the core of the development of symptomatology.

To induce a PTSD-like phenotype in susceptible mice, all mice were exposed to an initial stressor, followed by fear learning (contextual fear conditioning) the next day. After a week, mice were subjected to a subset of behavioral tests over the course of two weeks to assess PTSD-like symptomatology. One week after the final behavioral test, mice were re-exposed to the conditioning context for 10 minutes to induce fear memory recall and sacrificed by perfusion-fixation 90 minutes later.

Tamoxifen.

Tamoxifen was dissolved in a 10% ethanol / corn oil solution at a concentration of 10 mg/mL by overnight sonication and stored at -20°C until further use. Solutions were heated to body temperature and intraperitoneally injected at a dosage of 150 mg/kg to induce activity-dependent neuronal labeling. Mice were injected with tamoxifen on the morning of day 1 - seven hours before the stressor - to induce SEFL-dependent active neuronal labeling. Fear learning was conducted 21 hours post-stressor. This allowed both the stressor and fear learning to fall within the 36-hour labeling window, capturing neuronal activation during both events. To reduce labeling of neuronal activity that was non-SEFL-related, mice were kept in their home cage without external disturbances during the rest of the labeling period.

PTSD protocol.

Seven hours after the tamoxifen injection, mice were individually placed in *Context A* boxes, in which they received 14 1 second 1.0 mA shocks (i.e., the stressor) over 85 minutes in variable intervals. Mice were first moved to the dark experimental room in groups of two to three animals in dark carton boxes before being placed in the fear-conditioning boxes, which were connected to a shock generator (Campden Instruments). *Context A* consisted of a black, triangular shaped Plexiglas box with a steel grid and metal tray. The boxes were sprayed with 1% acetic acid, not illuminated, and 70 dB background noise was presented.

On the second day, 28 hours after the tamoxifen injection, mice were individually placed in *Context B* boxes, in which they received 5 1 second shocks of 0.7 mA over a period of five minutes (i.e., the fear learning), presented over fixed intervals. For this trigger session, mice were moved to the 70 lux illuminated experimental room in see-through cages in groups of two to three animals. The *Context B* boxes contained curved white walls and a steel grid with a white tray underneath. The boxes were furthermore cleaned with 70% ethanol and during the session the house lights in the boxes were turned on. No background noise was presented.

Mice were allowed to recover for a week, after which their behavioral response to trauma was assessed by testing for PTSD-like behavior: impaired risk assessment (dark-light transfer test), increased anxiety (marble burying), hypervigilance (acoustic startle), impaired sensorimotor gaiting (pre-pulse inhibition), and disturbed circadian rhythm (locomotor activity during the light phase)[26].

Behavioral testing.

Dark-light transfer test. On day 8 of the protocol, mice were tested in the dark-light transfer test[26]. The test was executed in a box that was divided into a dark compartment (DC, 29 x 14 cm) and brightly illuminated (ca. 1100 lux) compartment (LC, 29 x 29 cm), connected by a retractable door. The mice were individually placed in the DC, and the door was opened to initiate a 5-minute test session. Movement of the mice was recorded and scored automatically with EthoVision XT (Noldus). An additional area of 6 x 3 cm surrounding the opening of the LC was programmed into the software tracking measurements. Time spent in the LC as well as time spent in this 'risk assessment' zone was measured. Percentage risk assessment was calculated as the amount of time spent in the risk assessment zone as a percentage of total time spent in the LC.

Marble burying. On day 10, mice were individually placed in a 10 lux illuminated black open box (30 x 28 cm), containing a 5 cm deep layer of corn cobs, on top of which 20 marbles were centrally arranged in a 4 x 5 grid formation. Each mouse was placed in the corner of the box to initiate the task. Mice were videotaped for 25 minutes. Videos were scored by assessing the number of buried marbles after 25 minutes.

Startle response and pre-pulse inhibition. On day 12, mice were moved to the experimental room in their home cage and individually placed in small, see-through Plexiglas constrainers mounted on a vibration-sensitive platform inside a ventilated cabinet that contained two high-frequency loudspeakers (SR-LAB, San Diego Instruments). Movements of the mice were measured with a sensor inside of the platform. The pre-pulse inhibition test (PPI) started with an acclimatization period of 5 minutes, in which a background noise of 70 dB was presented, which was maintained throughout the entire 30 minute session. Thirty-two startle cues of 120 dB, 40 ms in duration and with a random varying ITI (12-30 s), were presented with another 36 startle cues preceded by a 20 ms pre-pulse of either 75 dB, 80 dB or 85 dB. Sessions were scored by assessing the latency to peak startle amplitude of the 12 middle startle trials, and the pre-pulse inhibition; i.e., the percentage of startle inhibition response to the different pre-pulse stimuli [1 - (mean pre-pulse startle response / mean startle response without pre-pulse) x 100].

Homecage locomotion. Immediately after the pre-pulse inhibition test, mice were individually housed in phenotyper cages (45 x 45 cm, Noldus) for 72 hours while their locomotion was being recorded by an infrared-based automated system (EthoVision XT, Noldus). The first 24 hours were considered habituation time and data were discarded. Total locomotion time during the subsequent two light phases (21:00 - 09:00 h) was assessed.

Behavioral categorization.

In order to categorize mice as either susceptible or resilient, one compound measure was generated based on the five behavioral outcome scores. Mouse behavior on each of the tests was sorted, and the 20% of mice that had the lowest values were attributed 3 points for percentage risk assessment, 3 points for latency to peak startle amplitude, and 2 points for percentage PPI. Similarly, the 20% of mice showing the highest values were attributed 1 point for light locomotor activity and marble burying[362]. Points for each test were determined by factor analysis in which tests were clustered in three separate groups: (1) latency to peak startle amplitude and percentage risk assessment, (2) percentage PPI, and (3) marble burying and total light activity[26]. Ties in the marble burying test were resolved by also assessing the number of marbles buried after 15 minutes, and assigning points to the mice that buried most marbles

then. The points per animal were tallied to generate and overall PTSD-like symptom score. Mice that had a total of four or more points (necessitating extreme behavior in multiple tests) were termed susceptible. Only mice that had zero points (indicating no abnormal behavior within any of the tests) were termed resilient.

Re-exposure and sacrifice.

On the final day of the experiment, day 23, mice were re-exposed to the *Context B* box (i.e., the fear conditioning context) for 10 minutes to induce fear memory recall, following the exact same procedures as during the fear conditioning session. However, no shocks were administered during this context re-exposure session. Mice were sacrificed 90 min post re-exposure under anesthesia (5% isoflurane inhalation followed by intraperitoneal injection of 200 μL pentobarbital) by perfusion with phosphate buffered saline (PBS) followed by 4% *paraformaldehyde* solution (PFA). The brains were surgically removed and post-fixed for 24 hours in 4% PFA, after which they were transferred to 0.1 M PBS with 0.01% sodium azide and stored at 4°C.

Freezing behavior.

Mice were videotaped during fear conditioning (day 2) and the re-exposure to the conditioning context (day 23) to assess fear memory encoding and remote recall. Freezing behavior was manually scored by an observer blinded to the experimental condition using The Observer XT12 software (Noldus). Consistent with previous literature, mice were considered to freeze when they were immobile for more than two consecutive seconds[369,370].

Immunofluorescence.

Right hemispheres of susceptible (n = 10) and resilient (n = 12) animals were sliced at 30 μm thickness using a freezing sliding microtome (Microm HM440E, GMI Inc., Ramsey, MN, USA) and stored in PBS with 0.01% sodium azide. Floating sections were used for immunohistochemistry of the hippocampus. For each animal, 4-6 sections were collected between anterior-posterior coordinates -1.46

mm and -1.94 mm relative to Bregma for the dorsal hippocampus, and between -2.92 mm and -3.52 mm relative to Bregma for the ventral hippocampus. tdTomato, as a proxy for the immediate early gene Arc, was used to measure neuronal activity during SEFL, while cFos immunofluorescence was assessed to measure recall-related activity. We used c*Fos*, rather than Arc, because Arc labeling is primarily dendritic in some hippocampal subregions[371], and both cFos and Arc expression have earlier been found to strongly overlap in neurons[372,373] - and specifically in the hippocampus[374,375] - in response to a challenge.

Immunolabeling of cFos and parvalbumin (PV) or histone deacetylase (HDAC) 2. Sections were washed three times in 1x PBS and blocked in PBS-BT (1x PBS with 0.3% Triton X-100 and 1% bovine serum albumin) for 30 minutes at room temperature (RT). Incubation of the primary antibodies was performed overnight (guinea pig anti-cFos, 1:750, 226004, Synaptic Systems; rabbit anti-PV, 1:1000, ab11427, ITK; or rabbit anti-HDAC2, 3 µg/µL, AB_2533908, Thermo Fisher) in PBS-BT for 18 hours at RT. Then, sections were washed three times in 1x PBS, and incubated with the secondary antibodies (Alexa 647-conjugated donkey anti-guinea pig, 1:200, AP193SA6, Merck Chemicals; Alexa 488-conjugated donkey anti-rabbit, 1:200, A-21206, Thermo Fisher) in PBS-BT for 3 hours at RT. Lastly, slices were washed three times in 1x PBS, mounted on gelatin-coated slides using FluorSave™ reagent (345789, Merck Chemicals) and cover slipped. The slices were stored at -20°C until image acquisition and cell counting.

Immunolabeling of cFos, 5-methylcytosine (5mC) and 5-methylhydroxycytosine (5hmC). Sections were washed three times in 1x PBS and permeabilized in 1x PBS with 0.1% Triton X-100 for 5 minutes at RT. Then, slices were incubated in 1 M HCl for 2 hours, washed three times in 1x PBS and blocked in PBS-NT (1x PBS with 0.3% Triton X-100 and 8% normal goat serum) for 50 minutes, all at RT. Because this process bleaches endogenous fluorescence - here the tdTomato fluorescent signal - these slices had to be immunolabeled for red fluorescent protein (RFP) in addition to the other markers. After again washing the slices three times in 1x PBS, incubation of the primary antibodies was performed overnight (guinea pig anti-cFos, 1:750, 226004, Synaptic Systems; rat anti-RFP, 1:1000, 5f8, Chromotek; mouse anti-5mC, 1:500, GWB-BD5190, GenWay Biotech; rabbit anti-5hmC, 1:1000, AB_10013602, Active

Motif) in PBS-NT for 18 hours at 4°C. Then, sections were washed three times in 1x PBS, and incubated with the secondary antibodies (Alexa 647-conjugated donkey anti-guinea pig, 1:200, AP193SA6, Merck Chemicals; Alexa 555-conjugated donkey anti-rat, 1:200, ab150154, Abcam; Alexa 488-conjugated goat anti-mouse, 1:200, A11001, Thermo Fisher; Alexa 405-conjugated anti-rabbit, ab175651, Abcam) in PBS-NT for 2 hours at RT. Lastly, slices were washed three times in 1x PBS, mounted on gelatin-coated slides using FluorSave™ reagent (345789, Merck Chemicals) and cover slipped. The slices were stored at -20°C until image acquisition and cell counting.

Image acquisition and cell counting.

Images of the tdTomato/cFos/PV and tdTomato/cFos/HDAC2 signals were captured through a light microscope (Axio Imager 2, Zeiss) using a 10x (for tdTomato/cFos/PV) or 40x (for tdTomato/cFos/HDAC2) objective lens and a LED module (Colibri 2, Zeiss). Images of the tdTomato/cFos/5mC/5hmC staining were captured through a confocal microscope (LSM900, Zeiss) using a 40x objective lens. For the tdTomato/cFos/PV signal, as well as the tdTomato/cFos/HDAC2 signal, whole hippocampi were photographed. For the tdTomato/cFos/5mC/5hmC staining, the entire DG was photographed, while for the CA1 and CA3 regions three representative photos each were taken, with locations being consistent across slices and animals (Figure S1). Separate photos were stitched and $cFos^+$, $tdTomato^+$ and PV^+ cells were manually counted per region in Fiji software[376] by an experimenter blinded to the experimental group. Hippocampal surface areas in each slice were assessed and corrected for to obtain standardized measures of cell density. Normalized cell counts were averaged per hippocampal subregion per animal and subjected to statistical testing. Note that the CA2 and CA1 regions were segmented together. This combined region will henceforth be referred to as 'CA1'.

Fluorescent signal intensity analysis

Expression levels of HDAC2, 5mC and 5hmC per cell were assessed by measuring signal intensity, and four cell clusters were identified by masks per hippocampal subregion per slice[376]: 1) all $tdTomato^+cFos^-$ cells, 2) all $cFos^+tdTomato^-$ cells, 3) all $tdTomato^+cFos^+$ cells, and 4) all $tdTomato^-cFos^-$ $DAPI^+$ cells.

Furthermore, a mask was generated for the background signal, which was obtained by inverting the $DAPI^+$ mask. Within mask 1-4, the mean signal intensity of HDAC2, 5mC and 5hmC was assessed. Here, masks 1-3 define the fear memory engram cells, while mask 4 defines the non-engram cells. In the background mask, the mean of the signal intensity of HDAC2, 5mC and 5hmC was assessed to exclude potential inter-slice differences in background intensity. These background values were very consistent across slices, hippocampal axis, and subregion and did not show any group differences. Specifically, background HDAC2 signal did not show any effect of group (main effect; $F(1,19.324) = .007$, $p = .936$, all group interactions; p's > .508), hippocampal axis ($F(1,46.241) = 1.113$, $p = .297$), or subregion ($F(2,42.178) = .698$, $p = .503$). Similarly, the 5mC and 5hmC signals did not show any group differences (5mC; $F(1,72) = .311$, $p = .610$, 5hmC; $F(1,72) = .428$, $p = .898$). Hence, fluorescent signals were not background-corrected.

Statistical Analyses.

Data were analyzed using IBM SPSS Statistics 23. Normality was checked using the Shapiro-Wilk test. For normally distributed data, data points deviating more than two standard deviations from the mean were considered outliers and removed from further analysis, and statistical testing was performed by independent t-tests or one-way ANOVAs. Freezing behavior over time was analyzed by repeated measures ANOVA (with time as within-subjects factor, and group as between-subjects factor), whereas immunohistochemistry data was analyzed using linear mixed modelling implementing the restricted maximum likelihood estimation. In the latter, the factors axis (dorsal, ventral) and region (DG, CA3, CA1) were included as within-subjects variables, and group as between-subjects variable. For the epigenetic data, the factor engram type (non-engram ($tdTomato^-cFos^-$), encoding ($tdTomato^+$), recall ($cFos^+$), reactivated ($tdTomato^+cFos^+$) engram) was additionally included as within-subjects variable. For non-parametric data, the Mann-Whitney U test or Kruskal-Wallis test was used. Differences were considered statistically significant if $p < 0.05$. Figures show mean ± standard of the mean (SEM).

RESULTS

Behavioral differences between susceptible and resilient animals

To assess potential differences in hippocampal trauma-related engram activity associated with differential susceptibility to PTSD-like symptoms, a cohort of 44 ArcTRAP mice was exposed to the PTSD induction protocol. Following a week of recovery, mice were assessed on PTSD-like symptomatology to yield a group of susceptible (n = 10) and resilient animals (n = 12), which significantly differed on their overall PTSD-like symptom score (U = 120, p < .001) (Figure 1B). Symptomatology was rather heterogeneous across susceptible animals (Figure 1C), sharing some symptoms (percentage risk assessment (t(19) = 4.280, p < .001) and reaction time to peak startle (t(18) = 2.110, p = .025)), yet differing on others (marble burying (t(20) = .739, p = .234), percentage pre-pulse inhibition (t(17) = 1.210, p = .121) and locomotor activity in the light phase (t(14.633) = .864, p = .201). Thus, resembling observations in PTSD patients, individual symptom profiles across susceptible mice differed.

Behavior during stress exposure was checked by assessing beam break data. Susceptible and resilient mice did not differ in their overall locomotor activity during the stressor (F(1,14) = .041, p = .843, nor in its reduction over time (main effect of time: F(9.490, 132.857) = 25.682, p < .001, group x time interaction: F(9.490, 132.857) = 1.022, p = .427), indicating no gross differences in stress coping behaviors. During the subsequent fear learning session, no overall group differences were observed in freezing rates (F(1,18) = .629, p = .438), yet the increase in freezing behavior over time (F(4,72) = 13.534, p < .001) significantly differed across groups (F(4,72) = 3.172, p = .019) (Figure 1D). Freezing levels tended to start lower in resilient mice, but also seemed to plateau sooner. *Post hoc* tests revealed only significant differences in the third minute of the fear learning session, when resilient mice displayed higher freezing levels than susceptible mice (t(18) = 2.870, p_{corr} = .05). Freezing behavior upon re-exposure to the fearful context - to induce fear memory recall - was not different between resilient and susceptible animals (Figure 1E). Neither overall freezing levels (F(1,19) = 1.308, p = .267), nor the

observed reduction in freezing over time (F(3.542,67.297) = 3.323, p = .019) differed between groups (group x time interaction: F(3.542,67.297) = .703, p = .576).

Susceptible animals show a smaller activated neuronal ensemble within the CA1 upon fear memory recall, but not during encoding

In the ArcTRAP mice, the neuronal ensemble active during SEFL, i.e., those neurons expressing the immediate early gene *Arc*, was permanently labelled by the reporter gene *tdTomato* (Figure 2ABC). No significant differences in the total number of activated hippocampal neurons during SEFL were observed between susceptible and resilient mice (F(1, 33.255) = .715, p = .404), nor was there any interaction effect between group and axis (F(1,40.938) = .880, p = .354), group and hippocampal subregion (F(2,30.033) = .295, p = .747) or group x axis x subregion interaction (F(2,30.033) = .255, p = .776), suggesting that hippocampal activity between groups was not different during initial memory formation. Neuronal activity associated with fear memory recall was measured by immunolabelling cFos$^+$ neurons (Figure 2ABD); cells that were active during remote fear memory recall induced by re-exposure to the conditioning context. For the number of hippocampal neurons active upon recall, a trend-level significant main effect of group was found (F(1, 4.034) = 4.100, p = .051), as well as a group x hippocampal subregion interaction (F(2,20.586) = 5.055, p = .016), whereas all other group interaction effects failed to reach significance (all p's > .577). These effects were caused by lower neuronal activity during memory recall in the CA1 of susceptible vs. resilient animals (F(1,14.693) = 5.298, p = .036), most notably within the vCA1 (vCA1; p = .013, dCA1; p = .100).

Susceptible and resilient animals show no difference in hippocampal fear memory reactivation

To investigate which encoding-related (i.e., tdTomato$^+$) cells eventually remained incorporated in the hippocampal memory engram for the fearful experience, overlap between the tdTomato$^+$ and cFos$^+$ neurons was assessed. These overlapping signals represent neurons that were active both during trauma encoding and recall, and therefore reflect the stable memory trace. Neuronal reactivation is expressed

as the Reactivation Rate (RR), which is calculated by dividing the number of $cFos^+tdTomato^+$ overlapping neurons by the number of $tdTomato^+$ neurons[377,378].

An average of 4.2% of hippocampal $tdTomato^+$ neurons were reactivated during the trigger context re-exposure, with RRs in the different subregions ranging between 1% (dDG) to 12% (vCA1). Reactivation rates were not statistically different between the groups, and did not show any significant interactions between group, subregion, and/or axis (all *p*'s = 1.00) (Figure 2E).

Susceptible animals show an increased number of vCA1 PV^+ neurons that is recruited relatively less during fear memory recall

Given the influence of PV^+ interneuronal activity on the excitability and firing behavior of surrounding neurons, we investigated densities of PV^+ neurons, as well as its relative activity during fear memory recall. The latter was calculated by assessing the number of PV^+cFos^+ overlapping neurons divided by the number of PV^+ neurons. This number, henceforth called the PV 'Activation Rate' (AR), is a way to express which percentage of the total interneuronal PV^+ population was active during remote fear memory recall. In line with prior work indicating that Arc-expression in restricted to glutamatergic neurons[379], the population of tdTomato-labeled ('TRAPped') neurons was exclusively glutamatergic, which prevented us from also investigating relative activity of PV^+ neurons during SEFL.

The overall number of hippocampal PV^+ neurons was not found to be significantly affected by group ($F(1,35.978) = 1.533$, $p = .224$), but revealed a trend-level significant group x hippocampal axis interaction ($F(1,32.529) = 3.160$, $p = .085$), caused by a tendency towards higher PV^+ density in the ventral hippocampus of susceptible vs. resilient animals ($p = .059$) (Figure 2F), an effect that seemed driven by higher densities in the vCA1 (vCA1; $p = .026$, vDG; $p = .227$, vCA3; $p = .993$). Additionally, a significant main effect of group ($F(1,35.454) = 8.613$, $p = .006$), together with a group x axis ($F(1,35.681) = 9.045$, $p = .005$), group x subregion ($F(2,33.512) = 4.728$, $p = .016$), and group x axis x subregion ($F(2,33.512) = 5.172$, $p = .011$) interaction effects were found for the PV AR (Figure 2G). Follow up tests revealed no significant group effects in the dorsal hippocampus (*p*'s > .543), but a significant main effect of group ($F(1,18.823) = 14.541$, $p = .001$) as well as a group x subregion

interaction (F(2,15.804) = 7.056, p = .006) in the ventral hippocampus. This interaction effect was driven by significantly reduced PV activation of susceptible mice in the vCA1 (p = .001), but not other hippocampal subregions (both p's > .340). Thus, these findings suggest that susceptibility to PTSD-like symptoms post-trauma is associated with an increase in PV^+ neurons in the vCA1, of which a relatively smaller part is active during remote fear memory recall.

Susceptible mice display altered HDAC2 expression patterns in the ventral hippocampus

The intensity of HDAC2 fluorescence in engram and non-engram cells was measured to quantify HDAC2 expression within these neurons (Figure 3AB)[380,381]. HDAC2 expression was dependent on hippocampal axis (F(1,207.453) = 159.497, p < .001), subregion (F(2,145.718) = 41.926, p < .001) and engram type (F(3,142.060) = 28.686, p < .001), but did not reveal a significant main effect of group (F(1,18.223) = 2.496, p = .131) (Figure 3C). Pair wise comparisons revealed that engram type effects were caused by significantly higher HDAC2 expression in memory encoding ($tdTomato^+$; p < .001), recall ($cFos^+$; p < .001) and reactivated ($tdTomato^+cFos^+$; p < .001) neurons, compared to non-engram cells, whereas the engram types amongst each other did not show overall differences in HDAC2 expression (all p's > .320) (Figure 3CDE), suggesting histone acetylation is overall reduced in memory engram-related cells compared to non-engram cells.

Critically, we observed a significant group x hippocampal axis x subregion interaction in HDAC2 levels (F(2,145.718) = 3.467, p = .034). Follow up tests revealed no significant effects of group in the dorsal hippocampus (all p's > .227), but a significant group x hippocampal subregion interaction (F(2,80.421) = 3.368, p = .039) in the ventral hippocampus. This interaction seemed to be caused by a tendency towards reduced HDAC2 levels in the vCA1 (p = .057) in susceptible compared to resilient mice, in the absence of differences in the vDG (p = .169) and vCA3 (p = .382). Noteworthy, HDAC2 levels in the vCA1 appeared modulated by an engram x group interaction (F(3,22.337) = 3.254, p = .041), which appeared caused by lower HDAC2 expression in engram neurons specifically (non-engram; p = .202, encoding; p = .059, recall; p = .080, reactivated; p = .033).

Susceptible animals show rather generic increases in hippocampal 5mC and 5hmC levels

The intensity of 5mC and 5hmC fluorescence in engram and non-engram cells was measured to determine the DNA methylation status of these neurons[381,382] (Figure 4AB). 5mC levels appeared modulated by hippocampal subregion ($F(2,96.738) = 3.116$, $p = .049$), engram type ($F(3,46.479) = 27.426$, $p < .001$) and group ($F(1,14.271) = 5.324$, $p = .037$), without a main effect of hippocampal axis ($p = .567$) (Figure 4C). Moreover, a significant group x engram type interaction was observed ($F(3,46.479) = 3.389$, $p = .026$), whereas the group x subregion ($F(2,96.738) = 2.430$, $p = .093$) and group x axis ($F(1,103.217) = 3.573$, $p = .062$) interactions failed to reach significance. All higher order interactions with group were non-significant (all p's > .589). Pair wise comparisons revealed significant differences in 5mC levels between all types of engram cells, with memory encoding and reactivation cells displaying higher 5mC levels than non-engram cells ($p < .001$ and $p = .004$, respectively), whereas memory recall cells displayed significantly lower 5mC levels compared to non-engram cells ($p = .005$). Follow up tests on the group x engram type interaction revealed significant upregulation of 5mC levels of susceptible mice in memory encoding ($p = .019$), recall ($p = .015$) and non-engram cells ($p = .029$), without significant differences in reactivated cells ($p = .107$).

5hmC levels depended on engram type ($F(3,97.708) = 24.770$, $p < .001$) and group ($F(1,16.006) = 6.837$, $p = .019$), without a main effect of hippocampal axis ($p = .457$) or subregion ($p = .431$) (Figure 4D). Moreover, a significant group x axis interaction was observed ($F(1,183.505) = 28.105$, $p < .001$), whereas the group x subregion interaction ($F(2,139.631) = 2.822$, $p = .063$) just failed to reach significance. All other interactions with group were non-significant (all p's > .369). Pair wise comparisons revealed significantly lower 5hmC levels in all types of engram cells compared to non-engram cells (all p's < .001), whereas the different type of engram cells (encoding, recall and reactivation) did not differ from each other (all p's > .425). Follow up tests for the group x hippocampal axis interaction revealed that susceptible displayed significantly higher 5hmC levels in in the ventral hippocampus specifically ($F(1,14.694) = 8.419$, $p = .011$), whereas this effect failed to reach significance in the dorsal hippocampus ($F(1,14.190) = 3.295$, $p = .091$).

While 5mC and 5hmC levels have been linked to decreased and increased gene expression respectively[383,384], the 5hmC/5mC ratio might actually be most informative with regard to a cell's gene expression profile, with high ratios coding increased gene expression[385]. Therefore, 5hmC/5mC ratios were calculated as well (Figure S2). 5hmC/5mC ratio data revealed a significant effect of engram type ($F(3,71.552) = 65.954$, $p < .001$), without any effects of hippocampal axis ($p = .194$), subregion ($p = .540$) or group ($p = .210$). Moreover, a significant group x engram type interaction was found ($F(3,71.552) = 6.833$, $p < .001$). Pair wise comparisons of 5hmC/5mC ratios revealed significantly lower ratio in engram vs. non-engram cells (encoding; $p < .001$, recall; $p = .010$, reactivation; $p < .001$), with encoding and reactivation cells displaying lowest ratio's (both p's $< .001$ compared to recall cells). This suggests that engram neurons are transcriptionally less active than neurons that are not incorporated into the engram, which is in line with previous studies marking increased DNA methylation in engram cells as a key mechanism in stabilizing memory engrams during memory consolidation[386]. Follow up analyses on the group x engram type interaction however failed to indicate clear differences between susceptible and resilient mice, as group comparisons per engram type revealed tendencies towards reduced 5hmC/5mC ratios in susceptible mice, but these failed to reach significance (non-engram; $p = .195$, encoding; $p = .523$, recall; $p = .249$, reactivation; $p = .963$). Paired comparisons of group effects across engram types revealed group x engram type interactions for all engram type comparisons.

DISCUSSION

Here, we tested the hypothesis that susceptibility to traumatic stress is characterized by interindividual differences in hippocampal activation upon fear memory encoding and recall and its epigenetic regulation. We examined potential alterations in the hippocampal memory engram for a stress-enhanced fear memory in mice that were susceptible and resilient to developing PTSD-like symptoms as a consequence of it. While no differences in the size of the engram activated during trauma encoding were observed between the groups, susceptible mice displayed a smaller engram activated in the vCA1 upon fear memory recall, as well as higher PV^+ neuronal density and a relatively lower activity of PV^+ neurons in the vCA1 upon memory recall. Epigenetic data revealed rather generic instead of engram-specific differences across groups, with susceptible animals displaying generally lower hippocampal HDAC2 expression, as well as higher 5mC and 5hmC signal.

Mice were classified as susceptible or resilient based on a compound score comprising multiple behavioral PTSD-like symptoms (i.e., impaired risk assessment, increased anxiety, hypervigilance, impaired pre-pulse inhibition and higher activity during the inactive phase, potentially linking to sleep disturbances), rather than single behavioral features. This classification more closely resembles the situation in PTSD patients[27], which are also diagnosed based on a compound score of symptomatology. Behaviorally, no differences were observed in how susceptible and resilient mice behaved during the encoding and recall of the fear memory, reflected by similar freezing levels over time. Earlier work has indicated that susceptible mice show extinction-resistant fear memory and generalization in a stress-enhanced cued fear learning paradigm[367], leading us to expect altered freezing behavior upon fear memory recall. This fits observations of emotional hypermnesia in PTSD patients, as well as context-nonspecific recall of the trauma memory by sensory cues. Yet, PTSD-like memory impairments not only comprise emotional hypermnesia, but also contextual amnesia[47,387]. Importantly, we here implemented stress-enhanced contextual fear learning[28], rather than cued fear learning. Therefore, one could speculate that impaired contextual fear memory retrieval, combined with excessive fear upon recall, cancel each

other out in susceptible mice, yet future studies need to confirm this by dissociating both aspects of fear memory.

Despite the absence of differences in freezing behavior, we did find a significant reduction in vCA1 engram size and a relative decrease in PV^+ cell activation during remote memory recall in susceptible animals. Previous work has implicated the vCA1 in contextual fear memory[58,275,388-390] and the subsequent contextual modulation of fear recall and expression[391,392]. Ventral CA1 neurons have been shown to convey contextual information through monosynaptic projections to the basolateral amygdala[388,389,393,394]. As such, the reduction in vCA1 engram size might reflect impaired functionality in the recall of contextual information, which may lie at the core of the context-nonspecific recall of trauma memories as observed in PTSD[395-397]. Moreover, it is in line with contextual amnesia as reported for PTSD[53]. As we did not find any differences in engram size during fear memory encoding (and reactivation), data suggests that initial memory encoding is not different between groups, but it is rather the (systems) consolidation process during which differences arise. The memory engram is not static, but rather dynamic over time, reorganizing both within and across brain regions[398-400], ultimately resulting in different storage sites of the memory following its consolidation[97,398-402]. This is especially relevant as we employed a remote recall paradigm, whereas most previous studies focused on more recent memory recall.

Parvalbuminergic network plasticity has been shown critical in the regulation of learning[403], with PV^+ interneurons contributing to memory consolidation by stabilizing functional connectivity patterns among CA1 neurons[359] and mediating coherent hippocampal-neocortical communication[404]. We observed a higher number of PV^+ neurons in the vCA1 of susceptible animals as well as a relatively smaller portion of these being activated during memory recall. Although not consistently reported[405,406], prior research has indicated a loss of PV^+ neurons following chronic stress[358,405], which might contradict our findings in the most stress-susceptible mice. Yet, notably, previous studies have ignored interindividual differences, posing the possibility that this observed reduction in PV^+ density may actually reflect an adaptive phenomenon. Alternatively, the differences between susceptible and resilient

mice may have been present already before PTSD induction, and as such do not reflect a differential effect of trauma itself. The lower recruitment of these neurons in susceptible mice may reflect a compensatory effect, resulting in similar absolute activity levels of the total PV^+ population in both susceptible and resilient animals. Regardless, these alterations in PV^+ interneuron presence and recruitment might relate to disrupted consolidation of the traumatic memory in PTSD[220], proposing it as a target for dedicated future studies.

Hippocampal HDAC2 expression was higher in engram compared to non-engram cells, and reduced in susceptible compared to resilient animals. Histone acetylation is most robustly associated with promoting memory formation. It is increased following neuronal activity, and promotes a chromatin structure permissive to gene transcription, necessary for synaptic plasticity[407]. HDACs, in particular HDAC2, induce the removal of acetyl groups, and their pharmacological or genetic inhibition was found to facilitate learning and memory[149] and improve extinction learning[408]. Our finding of increased HDAC2 levels in engram vs. non-engram cells seems to be at odds with these reports. Yet, one could speculate that plasticity should be suppressed once a memory is formed, with memory-related gene silencing serving to stabilize the memory engram[409]. This interpretation is supported by our findings in terms of DNA methylation patterns, with engram cells having overall higher levels of 5mC and lower levels of 5hmC, decreasing 5hmC/5mC ratio, suggesting an overall decrease in transcriptional activity within the engram. Prior reports implicating DNA methylation in stabilizing engrams during consolidation and aiding successful memory recall support this notion[386]. Reduced HDAC2 levels as observed in susceptible mice may also indicate a less stable trauma memory engram. Moreover, it is in line with prior reports on HDAC2 downregulation following acute stress being related to increased stress susceptibility[410] and a stronger fear memory[411]. In terms of methylation, we found susceptible animals to be characterized by overall higher hippocampal levels of 5mC and lower levels of 5hmC, both in engram and non-engram cells. As these markers are inversely related to gene expression, we conclude that both groups, despite the slight differences in hippocampal methylation profile, do likely not differ in terms of overall gene expression. This is supported by the observation that the 5hmC/5mC ratio -

most informative with regard to a cell's gene expression profile[385] - was not statistically different between susceptible and resilient individuals. This limits the group differences to the observed reduction of HDAC2 levels in susceptible mice. Considering HDAC2 expression is negatively related to the expression of cFos[149], it might seem contradictory that susceptible animals show both lower HDAC2 levels and a reduced number of cFos expressing cells. However, the temporal dynamics of HDAC2 and cFos are likely different[412,413] (i.e., recall-induced HDAC2 alterations will occur at a later timescale than cFos expression) which means it is hard to correlate both markers within 90 minutes after the context re-exposure. Furthermore, HDAC2 is of course only one of the many regulators of the epigenetic profile, and other regulatory pathways may influence cFos expression as well. One of these might be HDAC5, which has been previously found to be upregulated post-trauma in the bed nucleus stria terminalis of susceptible animals in this same mouse model[26]. Future studies should assess such alternative regulators of the engram.

Some limitations should be noted. Firstly, assessment of the memory engram related to memory encoding was restricted to glutamatergic neurons in the ArcTRAP mice[379]. Thus, the role of GABAergic neurons in the engram and their role in traumatic stress susceptibility still needs to be elucidated. We preferred ArcTRAP mice over the available FosTRAP mice based on superior labeling sensitivity in the hippocampal CA3 and CA1, which are typically devoid of labeled cells in the FosTRAP mouse lines[102,414]. Yet, the ArcTRAP line has substantial background labeling (i.e., fluorescent tagging of neurons in the absence of tamoxifen) in the hippocampal DG[102], which may explain why we did not recapitulate prior findings of peri-trauma DG activation being predictive of fear memory generalization and stress susceptibility in general[414,415]. Furthermore, the tamoxifen-induced labeling window in our TRAP mice comprised ~ 36 hours, capturing both the trauma and trigger experiences. We opted for this approach as it is currently unknown whether the interindividual differences in SEFL and its long-term consequences originate from differential responding to the first stressor or from later fear learning. We hypothesized the latter, as the behavioral consequences of this PTSD-model are not observed to a similar degree if mice are only exposed to the initial stressor[26], emphasizing aberrant fear learning to be at the

core of the development of symptomatology. However, others have shown that PTSD-like memories can also be induced by stress exposure post-learning[93,387], leaving this issue unresolved. Moreover, while we assume that the tdTomato-tagged and cFos-labelled neurons represent the trauma memory, it will require experimental manipulation of these populations to show that their activity is necessary and/or sufficient for memory expression.

Finally, while immunofluorescence of epigenetic markers is more often used to draw preliminary conclusions about changes in transcriptional processes[416,417], it is not possible to draw a one-to-one relationship between the observed differences in HDAC2, 5mC and 5hmC levels and actual alterations in histone acetylation, DNA methylation and gene expression. Different studies have shown transcriptional alterations in response to stress[418], and in PTSD specifically[419,420], but it would require future studies to causally link such changes to the alterations in histone acetylation, DNA methylation and hydroxymethylation that have been observed.

Concluding, we have shown PTSD-like symptomatology to be related to alterations in remote fear recall-specific engram size and PV^+ interneuronal activity - as well as overall PV^+ density - in the ventral CA1. These findings propose an important role for aberrant memory recall, resulting from an altered (systems) consolidation process, in mediating traumatic stress susceptibility. Epigenetically, we found marked differences in HDAC2 expression and DNA methylation and hydroxymethylation between susceptible vs. resilient mice, suggestive of overall higher hippocampal transcriptional activity. These changes were however not restricted to neurons involved in the memory engram, indicating epigenetic changes throughout the entire hippocampus as an important target for further research into the pathophysiology of PTSD. These overall alterations could potentially contribute to deviations in memory consolidation by destabilizing hippocampal memory representations, although future research is needed to determine such causal relationship.

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Conflict of Interest

The authors declare no competing financial interests.

FIGURES

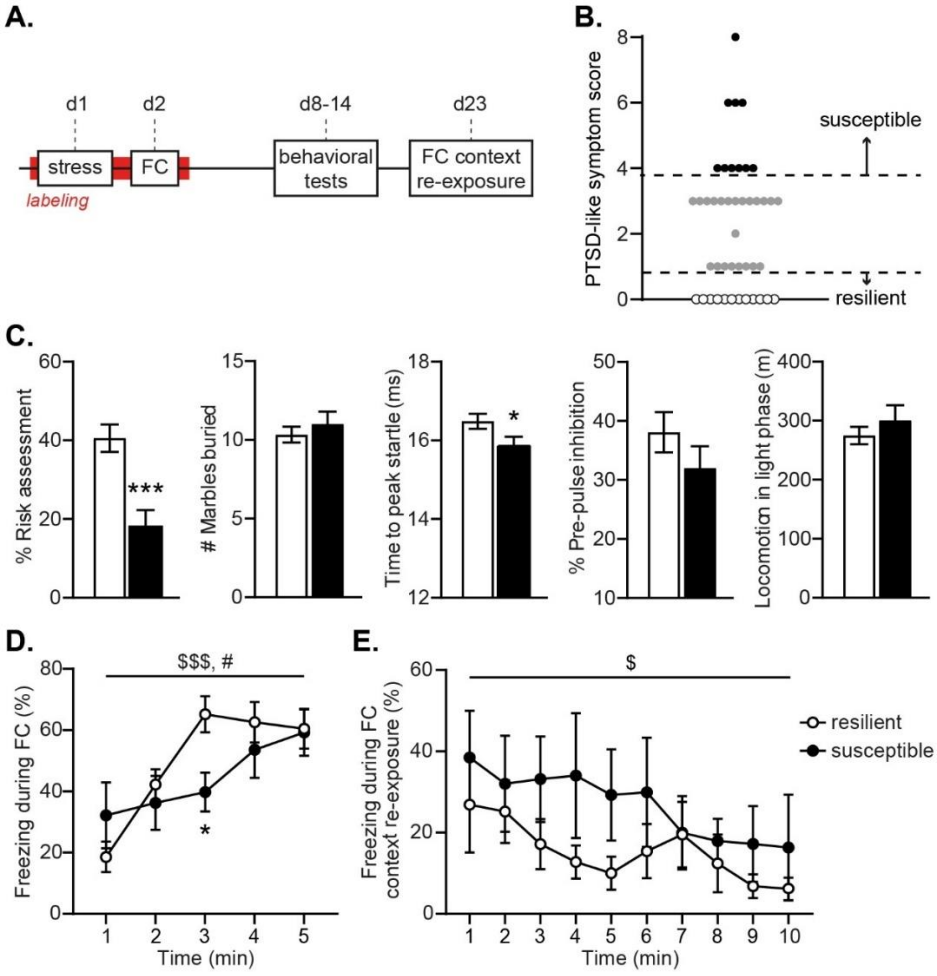

Figure 1. Experimental schedule and behavioral assessments. Mice were exposed to a stressor and then subjected to contextual fear conditioning (FC). PTSD-like symptomatology was assessed in a set of behavioral tests, mice were re-exposed to the conditioning context and then sacrificed (**A**). Susceptible mice were defined by PTSD-like symptom scores >=4 (necessitating extreme behavior in multiple tests), whereas resilient mice did not show any aberrant behavior (score = 0) (**B**). Susceptible mice displayed significantly reduced risk assessment behavior and shorter latencies to peak startle compared to resilient animals at the group level. No group differences were observed in marble burying, pre-pulse inhibition or locomotor activity during the light phase (**C**). Increases in freezing behavior during contextual FC differed across groups (shock administration at 1, 2, 3, 4 and 5 min) (**D**), whereas no differences in freezing levels were observed upon later FC context re-exposure (no shocks) (**E**). Data are presented as

mean +/- SEM. ###: $p < .001$, #: $p < .05$, main effect of time; $: $p < .05$, group x time interaction; ***: $p < .001$, *: $p < .05$ effect of group

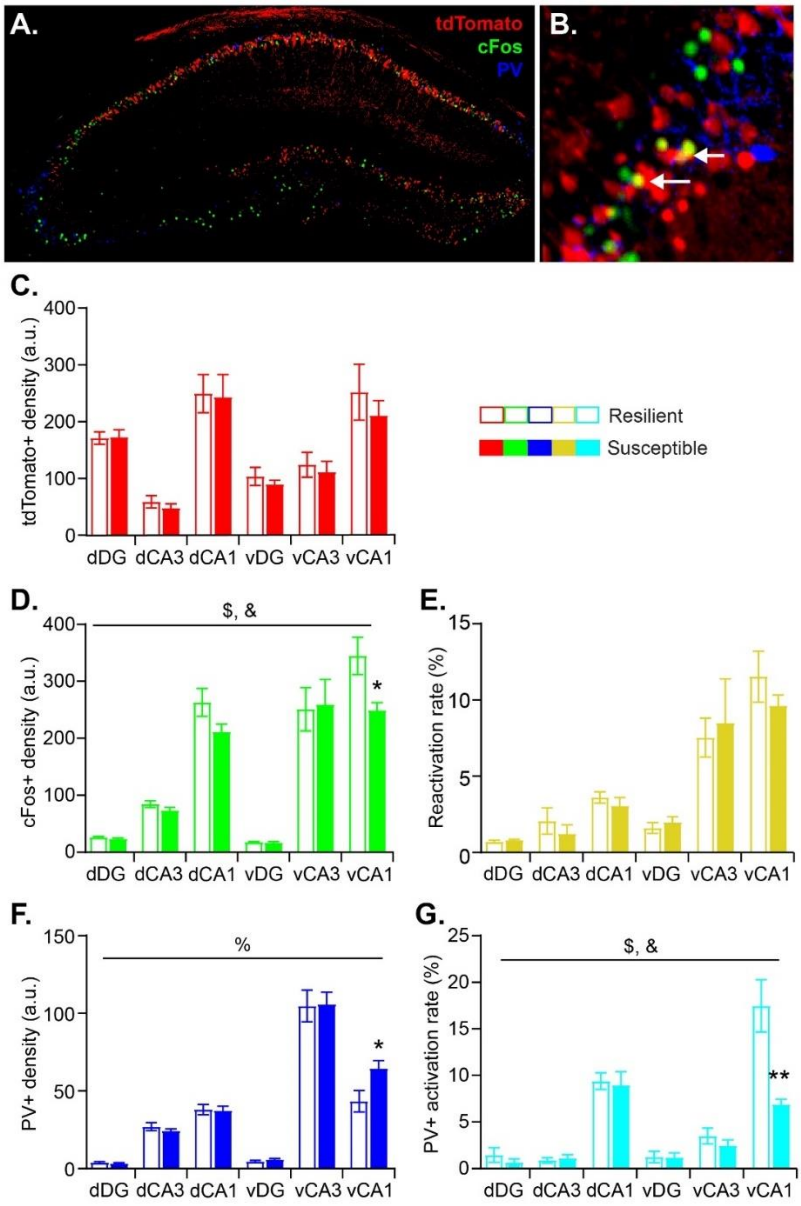

Figure 2. Hippocampal activity during fear memory encoding (marked by tdTomato expression), remote fear memory recall (marked by cFos expression), as well as parvalbumin (PV) interneuron density were assessed by immunohistochemistry. Arrows indicate tdTomato^{+}cFos^{+} double-positive cells. (**AB**). No group differences were observed in the size of the engram recruited during stress-enhanced fear learning (**C**). However, susceptible animals displayed a smaller population of ventral hippocampal neurons active during remote fear memory recall (**D**), without any group differences in neuronal reactivation rate (**E**). Lastly, susceptible animals showed a significantly increased PV^{+} density in (mainly the ventral) CA1 (**F**), yet a decrease in ventral hippocampal activity of PV^{+} neurons

specifically during remote memory recall (**G**). Data represent mean +/- SEM. **: $p < .01$, *: $p < .05$, $: $p < .05$, main effect of group, &: $p < .005$, group x subregion interaction, %: $p < .05$, group x axis interaction

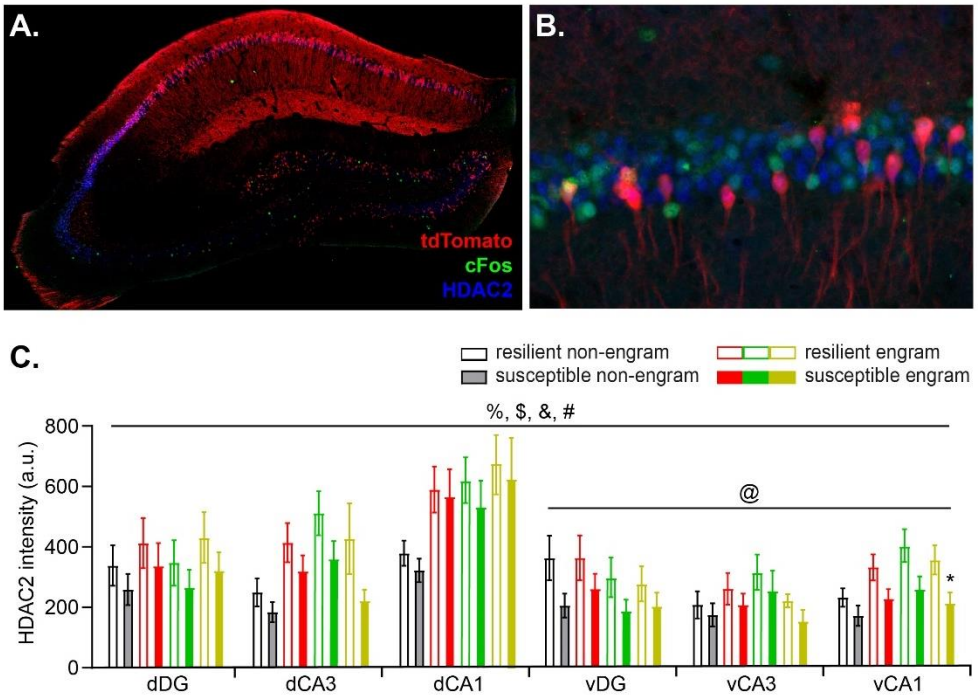

Figure 3. Hippocampal HDAC2 fluorescence in cells active during fear memory encoding (marked by tdTomato expression) and remote fear memory recall (marked by cFos expression) were assessed by immunohistochemistry (**AB**). Neurons involved in memory encoding ($tdTomato^+$) and recall ($cFos^+$), as well as reactivated ($tdTomato^+cFos^+$) neurons, were characterized by overall higher HDAC2 fluorescence than non-engram cells. HDAC2 levels in the ventral hippocampus were modulated by a subregion x group interaction, which seemed to be caused by a tendency towards lower HDAC2 levels in the vCA1 in susceptible animals (**CDE**). Data represent mean +/- SEM. %: $p < .001$, main effect of axis, $: $p < .001$, main effect of subregion, &: $p < .001$, main effect of engram type, #: $p < .05$, group x axis x subregion interaction, @: $p < .05$ group x subregion interaction, *: $p < .05$, effect of group

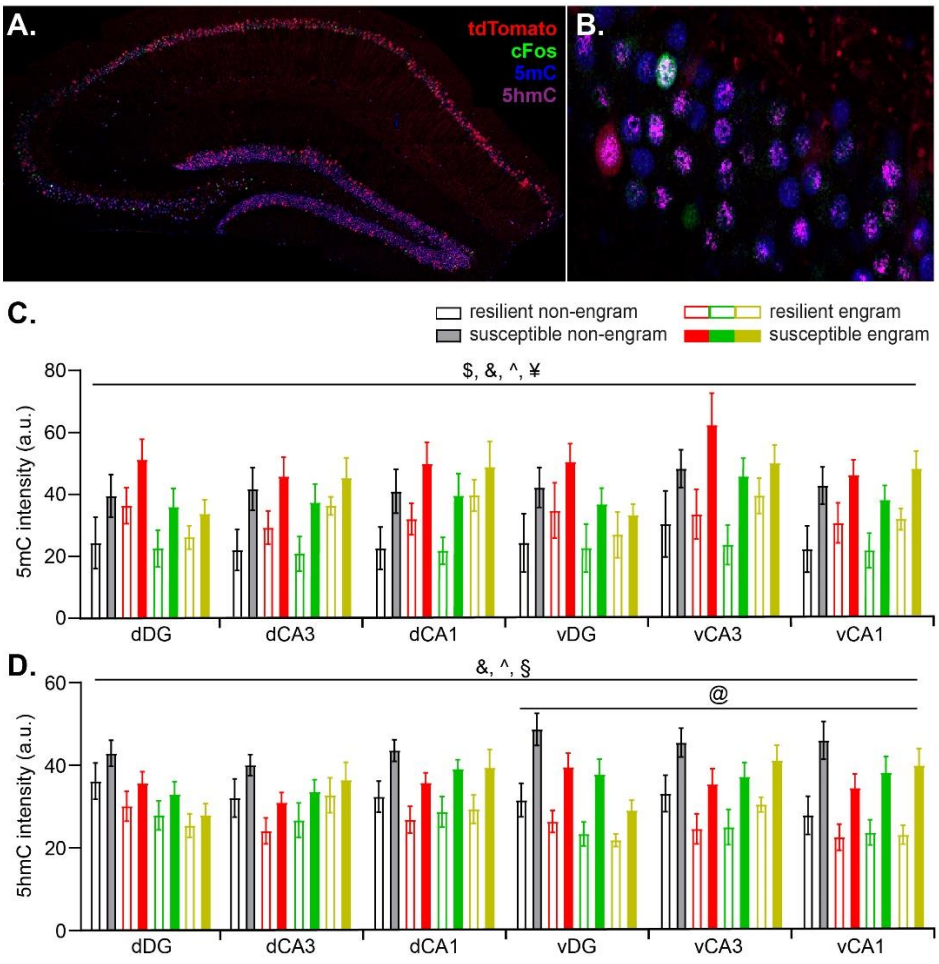

Figure 4. Hippocampal 5mC and 5hmC fluorescence in cells active during fear memory encoding (marked by tdTomato expression), remote fear memory recall (marked by cFos expression) and both were assessed by immunohistochemistry and compared to non-engram cells (tdTomato and cFos negative cells) (**AB**). 5mC levels were higher in encoding and recall cells compared to non-engram cells. In contrast, reactivated cells displayed lower 5mC levels than non-engram cells. Importantly, susceptible mice displayed higher 5mC levels in memory encoding, recall, and non-engram cells, compared to resilient mice, without significant differences in reactivated cells (**C**). 5hmC levels were lower in all types of engram cells compared to non-engram cells, and susceptible mice displayed higher 5hmC levels in in the ventral hippocampus (**D**). Data represent mean +/- SEM. $: $p < .05$, main effect of subregion, &: $p < .001$, main effect of engram type, ^: $p < .05$, main effect of group, ¥: $p < .05$, group x engram type, §: $p < .001$, group x axis interaction, @: $p < .05$, main effect of group

SUPPLEMENTARY FIGURES

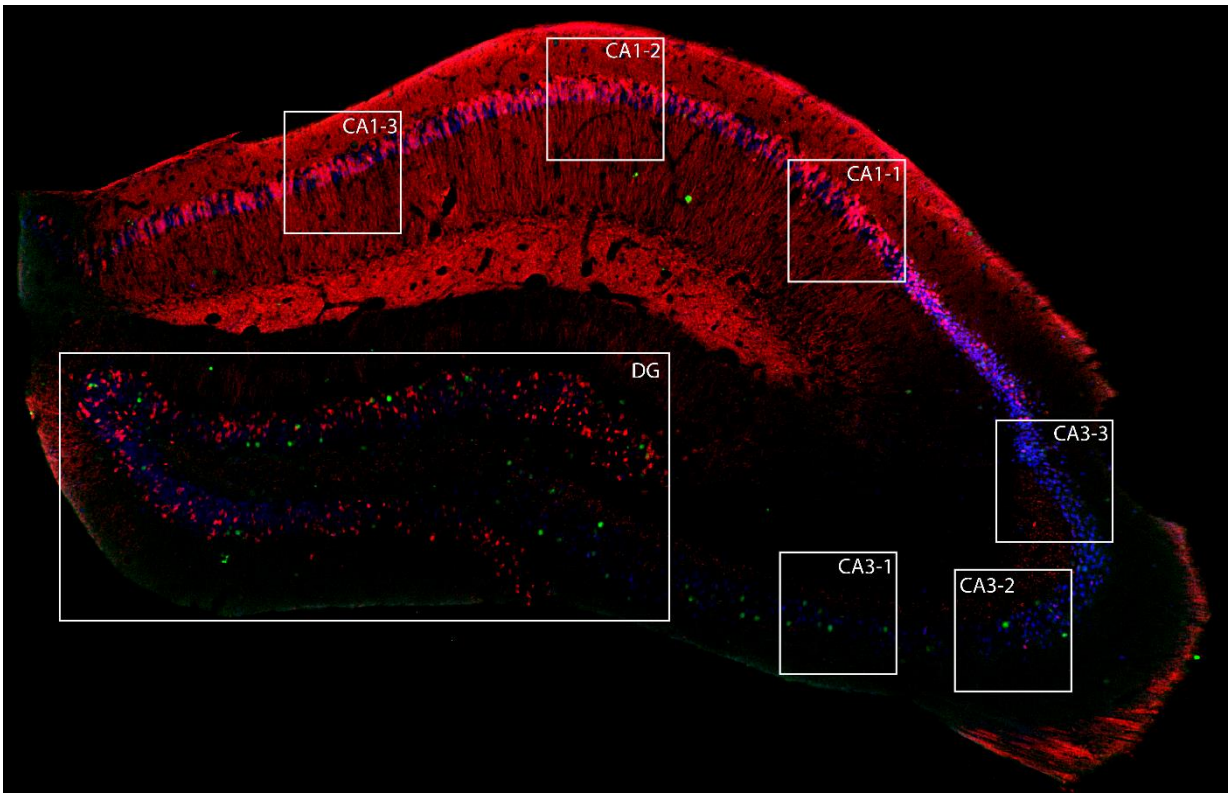

Figure S1. Hippocampal HDAC2 fluorescence was assessed in 40x microscopic frames. For the DG, multiple frames were stitched to obtain a photo of the entire structure. For the CA3 and CA1, three representative photos each were taken in locations consistent across slices and animals.

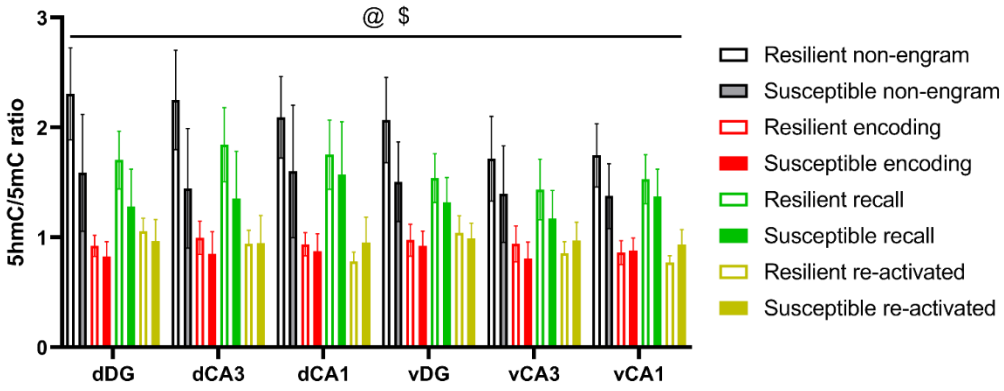

Figure S2. Hippocampal 5hmC/5mC fluorescence ratio in cells active during fear memory encoding (marked by tdTomato expression) and remote fear memory recall (marked by cFos expression) as assessed by immunohistochemistry. All types of engram neurons displayed lower ratio than non-engram cells, with encoding-activated ($tdTomato^+$) and reactivated ($tdTomato^+cFos^+$) neurons displaying lowest 5hmC/5mC ratios, indicative of a relative decrease in gene transcription. Data represent mean +/- SEM. @: $p < .001$, main effect of engram type, $: $p < .001$, group x engram type interaction

Chapter 4

Longitudinal assessment of amygdala activity in mice susceptible to trauma

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ABSTRACT

Resilience to consequences of trauma exposure contains relevant information about the processes that contribute to the maintenance of mental health in the face of adversity; information that is essential to improve treatment success of stress-related mental diseases. Prior literature has implicated aberrant amygdala (re)activity as potential factor contributing to traumatic stress susceptibility. However, it remains to be resolved which amygdalar subregions and neuronal subclasses are involved, and when - i.e., pre-, peri- or post-trauma exposure - and under what conditions changes in amygdala (re)activity manifest themselves. Here, we implemented a preclinical rodent model for PTSD that entailed exposure to a traumatic event (severe, unpredictable foot shock) followed by a trigger (mild, predictable foot shock). Using behavioral phenotyping, trauma susceptible vs. resilient mice were identified and pre-, peri- or post-trauma amygdala activity was compared. Neuronal activity was tagged in living mice by the use of the ArcTRAP transgenic mouse line, labeling all activated (i.e., *Arc*-expressing) neurons by a systemic injection of tamoxifen. Furthermore, we assessed amygdala responses during fear memory recall, induced by either (re-)exposure to the trauma, trigger, or a novel, yet similar context, and analyzed behavioral fear responses under these conditions, as well as basal anxiety in the mice. Results revealed no major differences dissociating susceptible vs. resilient mice prior to trauma exposure, but exaggerated activity in specifically the basolateral amygdala (BLA) peri-trauma that predicted susceptibility to later PTSD-like symptoms. Post-trauma, susceptible mice did not display altered resting amygdala activity, but BLA hyperreactivity in response to trigger context re-exposure, and BLA hyporesponsivity in response to the trauma context. Exposure to the novel, similar context evoked a differential temporal pattern of freezing behavior in susceptible mice and an increased activity of amygdalar somatostatin-expressing neurons specifically. As such, these results for the first time show that deviant BLA activity during fear learning predicts susceptibility to its long-term consequences and that aberrant subsequent BLA responses to stressful contexts depend on the exact context.

1. INTRODUCTION

Posttraumatic stress disorder (PTSD) is a debilitating disorder one can develop after exposure to a traumatic event. Importantly, whereas the majority of the population is ever exposed to a traumatic event during their lifetime, only a small fraction of them develops PTSD[321]. This observed natural resiliency likely contains important information on PTSD etiology and can be key to generate new leads for improved PTSD diagnostics and treatment[421]. However, what dissociates vulnerable vs. resilient individuals, under what circumstances these differences surface, and when they develop, is largely unknown.

One important factor associated with PTSD is the function of the amygdala. The amygdala is a key coordinator involved in responding to threat and subsequent trauma-related behavioral alterations [98]. In line with their symptoms of hyperarousal and anxiety, PTSD patients display exaggerated amygdala responses to threatening stimuli[81,84]; a response that correlates with symptom severity [84]. Moreover, this amygdala hyperreactivity predicts poor treatment response[422], whereas symptom attenuation is associated with a suppression of its reactivity[423]. However, due to the retrospective nature of the majority of studies in PTSD patients, it is currently unclear whether this amygdala hyperreactivity is a pre-existing risk factor for PTSD, or a consequence of acquired pathology[424]. Moreover, it is currently unknown how the distinct amygdalar subnuclei and neuronal subpopulations contribute to this response, as patient studies lack the required spatial resolution and specificity. Sensory information primarily flows through the lateral amygdala (LA) into the basolateral amygdala (BLA), where long-term potentiation key to associative fear learning takes place, after which the signal is conveyed to the central amygdala (CeA) that regulates the output of fear behavior[425]. The LA and BLA primarily exist of glutamatergic pyramidal neurons (80-85%)[426], but within the CeA fear output is regulated mainly by an intrinsic network of GABAergic inhibitory neurons[427], which are classified based on the expression of specific neurochemical markers[428,429]. Of these, particularly somatostatin-expressing CeA neurons have been associated with the generation of a fear response[430,431].

Preclinical animal models for PTSD allow one to study amygdala function in a subregion- and cell-type specific manner over time. To improve validity of current models for PTSD, and hence enhance

translational value of insights obtained, acknowledging the interindividual variability in susceptibility to develop PTSD has been argued to be key[17]. Therefore, we here longitudinally investigated the differences in amygdalar subregional activity between vulnerable mice and those resilient to the development of PTSD-like symptoms following trauma exposure (i.e., electrical foot shocks). To investigate amygdala neuronal activity in living animals, we used the ArcTRAP mouse line crossed with a reporter line in which the injection of tamoxifen induces the indelible fluorescent labeling of activated (i.e., *Arc*-expressing) neurons[102]. Specifically, we wanted to assess in which amygdalar subregions and cell types trauma susceptible and resilient animals show differential activity and, if so, at what point in time these arise and under which conditions these differences become apparent. To answer these questions, we compared neuronal activity in the major subregions of the amygdala between groups i) pre-trauma, ii) peri-trauma, and iii) post-trauma. Three weeks after the trauma, animals were re-exposed to three different trauma-related contexts to assess amygdala activity during fear recall.

2. MATERIALS & METHODS

2.1. Animals.

This study consisted of three separate experiments: cohort 1 (n = 48) to assess amygdalar neuronal activity under resting (i.e., home cage) conditions before trauma, cohort 2 (n = 44) to assess amygdalar neuronal activity during trauma, and cohort 3 (n = 48) to assess amygdalar neuronal activity under resting conditions after trauma (Figure 1A). ArcTRAP mice (see Supplementary Material for details) were used to label activated (i.e., *Arc*-expressing) neurons upon tamoxifen injection[102]. Since the implemented PTSD model has only been validated in males, experiments were restricted to male mice. Mice were group housed (3-4 mice per cage) in individually ventilated cages on a reverse 12 h light/dark cycle (09:00 - 21:00 h) at the Central Animal Facility of the Radboud University Nijmegen, The Netherlands, according to institutional guidelines. Food and water were provided *ad libitum*. Unless otherwise stated, behavioral testing was performed during the animal's active phase (i.e., the dark) between 13.00 - 18.00 h. The experimental protocols were in line with international guidelines, the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council 2003), the principles of laboratory animal care, as well as the Dutch law concerning animal welfare and approved by the Central Committee for Animal Experiments, Den Haag, The Netherlands.

2.2. General procedure.

All mice were exposed to a PTSD mouse model as described before[362,432] (Figure 1A). To induce a PTSD-like phenotype, mice were exposed to a traumatic event (severe, unpredictable foot shocks) followed by a less severe trigger event (mild, predictable foot shocks) the next day. This model is based on stress-enhanced fear learning, which builds on the clinical observation that prior stress exposure precipitates PTSD[364,365]. In this model, prior stress exposure (the 'trauma') is observed to affect the learning of future aversive events (the 'trigger'), creating traumatic-like memories characterized by exaggerated fear responses and resistance to extinction[28,361,366,367]. Importantly, the stress-enhanced fear learning paradigm induces persistent behavioral symptoms in a subset of mice[362,432,433] resembling observations in PTSD patients, while also recapitulating the hypothalamic-pituitary-adrenal (HPA)-axis

abnormalities as observed in subgroups of PTSD patients (i.e., reduced glucocorticoid release upon challenge[166]) in susceptible mice. These consequences are not observed if mice are only exposed to the initial stressor[432], emphasizing aberrant fear learning to be at the core of the development of symptomatology. After a week of recovery, mice were subjected to a subset of behavioral tests over the course of two weeks to assess PTSD-like symptomatology. One week after the final behavioral test, mice were re-exposed to a trauma-related context for 10 minutes to trigger fear memory recall and sacrificed by perfusion-fixation 90 minutes later.

2.3. Tamoxifen.

All mice were intraperitoneally injected with tamoxifen to induce fluorescent labeling of all *Arc*-expressing neurons. Tamoxifen was dissolved in a 10% ethanol / corn oil solution at a concentration of 10 mg/mL by overnight sonication and stored at -20°C until further use. Solutions were heated to body temperature and i.p. injected at a dosage of 150 mg/kg to induce activity-dependent neuronal labeling. Mice in cohorts 1 and 3 were injected with tamoxifen under homecage conditions either pre-trauma or post-trauma exposure (Figure 1). Mice in cohort 2 were injected on the morning of day 1 -seven hours before the trauma session- to induce trauma-dependent active neuronal labeling.

2.4. PTSD protocol and behavioral testing.

Mice were individually placed in trauma context boxes, in which they received 14 1 second 1.0 mA shocks (the 'trauma') over 85 minutes at variable intervals. Mice were moved to the dark experimental room in groups of two to three animals in dark carton boxes before being placed in the fear-conditioning boxes, which were connected to a shock generator (Campden Instruments). The trauma context consisted of a black, triangular shaped Plexiglas box with a steel grid and metal tray. The boxes were sprayed with 1% acetic acid, not illuminated and 70 dB background noise was presented. On the second day, mice were individually placed in trigger context boxes, in which they received 5 1 second shocks of 0.7 mA over a period of five minutes (the 'trigger'), presented over fixed intervals. This context was created by placing curved white walls in the box and a steel grid with a white tray underneath. The boxes

were furthermore cleaned with 70% ethanol and during the session the house lights in the boxes were turned on. No background noise was presented. For this trigger session, mice were moved to the 70 lux illuminated experimental room in see-through cages in groups of two to three animals.

Mice were allowed to recover for a week, after which their behavioral phenotype was assessed by testing for PTSD-like behavior: impaired risk assessment (in the dark-light transfer test), increased anxiety (by marble burying), hypervigilance (by acoustic startle), impaired sensorimotor gaiting (by pre-pulse inhibition (PPI)), and disturbed circadian rhythm (by locomotor activity during the light phase). In addition to the behavioral testing to assess PTSD-like symptomatology, mice in cohort 1 were tested in the open field and elevated plus maze for assessing pre-trauma anxiety. Details on all behavioral tests can be found in the Supplementary Material.

2.5. Behavioral categorization.

In order to categorize mice as either trauma susceptible or resilient, one compound measure was generated based on the five behavioral outcome scores. Mouse behavior on each of the tests was sorted, and the 20% of mice that had the lowest values were attributed 3 points for percentage risk assessment, 3 points for latency to peak startle amplitude, and 2 points for percentage PPI. Similarly, the 20% of mice showing the highest values were attributed 1 point for light locomotor activity and marble burying[362]. The points per animal were tallied to generate an overall PTSD-like symptom score. Mice that had a total of four or more points (necessitating extreme behavior in multiple tests) were termed susceptible. Only mice that had zero points (indicating no extreme behavior within any of the tests) were termed resilient.

2.6. Re-exposure and sacrifice.

On the final day of the experiment, day 23, mice were re-exposed to a trauma-related context for 10 minutes to induce fear memory recall. No shocks were administered during this context re-exposure session. Mice in cohort 3 were re-exposed to the trauma context, following the exact same procedures as during the trauma session. Mice in cohort 2 were re-exposed to the trigger context, following the exact same procedures as during the trigger session. Mice in cohort 1 were exposed to a novel context,

which was similar, yet distinct, from the two other contexts. The novel context consisted of a Plexiglas box with triangular white walls and a steel grid with a white tray underneath. The boxes were sprayed with 1% lactic acid, there was an 80 dB 10 kHz continuous tone, and during the session the house lights in the boxes were turned on.

Mice were videotaped during the (re-)exposure to assess fear memory recall. Freezing behavior for cohort 1 was automatically scored using EthoVision software (Noldus), whereas freezing for cohorts 2 and 3 was manually scored by an observer blinded to the experimental condition using The Observer XT12 software (Noldus), since the quality of several videos in these cohorts did not comply to the standards needed for automatic scoring. Mice were considered to freeze when they were immobile for more than two consecutive seconds. Manual and automatic scorings were compared for a subset of the videos, and were highly correlated ($r(15) = .89$, $p = .012$).

Mice were sacrificed 90 min post re-exposure under anesthesia (5% isoflurane inhalation followed by intraperitoneal injection of 200 µL pentobarbital) by perfusion with phosphate buffered saline (PBS) followed by 4% *paraformaldehyde* solution (PFA). The brains were surgically removed and post-fixed for 24 hours in 4% PFA, after which they were transferred to 0.1 M PBS with 0.01% sodium azide and stored at 4°C.

2.7. Immunofluorescence and microscopy.

Right hemispheres of susceptible and resilient animals of each cohort were sliced at 30 µm thickness using a freezing sliding microtome (Microm HM440E, GMI Inc.) and stored in PBS with 0.01% sodium azide. Left hemispheres were used for whole-brain assessments beyond the scope of this manuscript. Based on earlier reports on hemispheric asymmetry in amygdala deviations in PTSD (e.g., Kaouane et al., 2012[387]; Mutluer et al., 2018[434]), we chose not to counterbalance hemisphere selection across animals, but select the hemisphere with the most consistently reported aberrancies in PTSD. Floating sections were used for immunohistochemistry of the amygdala, which was stained for cFos and somatostatin (SOM) expression (see Supplementary Material for further details). Images of the cFos and SOM stainings and tdTomato signal were captured through a light microscope (Axio Imager 2, Zeiss)

using a 10x objective lens and a LED module (Colibri 2, Zeiss), and cells counted manually by an experimenter blinded to the experimental group. Amygdalar subregion surface areas in each slice were assessed and corrected for to obtain standardized measures of cell density in each cohort. Normalized cell counts were averaged per subregion per animal and subjected to statistical testing. Amygdala data acquisition was successfully completed for 8 resilient and 10 susceptible mice of cohort 1, 10 resilient and 9 susceptible mice of cohort 2, and 11 resilient and 7 susceptible mice of cohort 3.

2.8. Statistical analyses.

Data were analyzed using IBM SPSS Statistics 23. Data points deviating more than three inter-quartile ranges from the median were considered outliers and removed from further analysis. Additionally, tdTomato$^+$ cell counts of 2 mice (1x susceptible, 1x resilient) in cohort 1 were excluded, because of exceptionally low neuronal labelling, potentially due to suboptimal tamoxifen injection. Normality was checked using the Shapiro-Wilk test. For normally distributed data, independent t-tests were carried out, while for non-parametric data, the Mann-Whitney U test was used. Analyses of normally distributed data which also included intra-subject variables (e.g., time or amygdalar subregion) were performed using linear mixed modelling. Significant interactions were followed up by Fisher's LSD *post hoc* tests. An overview of all statistical tests and their results can be found in Supplementary Table S1. To assess correlations, bivariate Pearson or Spearman correlation coefficients were computed, depending on compliance to normal distribution. Differences were considered statistically significant if $p < .05$. Data are depicted as bars reflecting medians together with single data points, as well as line graphs depicting means ± standard error of the mean (SEM) created in GraphPad Prism (version 9.3.1).

3. RESULTS

3.1. Behavioral differences between susceptible and resilient mice

To assess potential differences in amygdalar activity associated with differential susceptibility to PTSD-like symptoms following trauma, three cohorts of 44-48 mice were exposed to the PTSD induction protocol and, following a week of recovery, assessed on PTSD-like symptomatology to yield groups of susceptible (n = 12, n = 10, n = 8, for cohorts 1, 2, and 3, respectively) and resilient (n = 12, n = 12, n = 11, for cohorts 1, 2, and 3, respectively) animals. Resilient and susceptible groups within each cohort significantly differed on their overall PTSD-like symptom score (cohort 1: $U = 144$, $p < .001$, cohort 2: $U = 120$, $p < .001$), cohort 3: $U = 88$, $p < .001$, Figure 1B). Results from the separate behavioral assessments are plotted in Supplementary Figure S1 as well as described in the Supplementary results.

3.2. Pre-trauma anxiety measures did not predict traumatic stress susceptibility

To test whether pre-trauma trait anxiety predicted later PTSD development and correlated with resting amygdala activity, all animals of cohort 1 were tested for anxiety-like behavior prior to trauma exposure. Animals later categorized as trauma susceptible did not display different behavior in the open field test prior to trauma exposure compared to resilient animals (Figure 2A). The total distance traveled ($t(21) < 1$, $p = .827$), the time spent in the center ($U = 78$, $p = .729$), and the number of visits to the center ($U = 74.5$, $p = .887$) were not different between groups. Also, behavior on the elevated plus maze did not differ significantly between groups, with susceptible mice traveling similar distance on the maze ($t(17.439) < 1$, $p = .430$), spending similar amount of time on the open arms ($U = 45$, $p = .128$; Figure 2B), and visiting the open arms as frequently ($t(22) = .980$, $p = .338$) as resilient mice.

3.3. Increased peri-trauma BLA activity codes susceptibility to trauma-induced PTSD-like symptomatology

Neuronal activity either before, during or after the trauma, as reflected by expression of the immediate early gene *Arc*, was permanently labelled in three independent cohorts by expression of the reporter gene *tdTomato* (Figure 3A). As *Arc*-expression is largely restricted to glutamatergic neurons[379] the

number of neurons co-expressing tdTomato and the GABAergic subclass marker somatostatin, was negligible, and therefore not included into analyses.

Resting neuronal activity pre-trauma was not different between groups (main effect of group: $F(1,13.576) = .764$, $p = .397$; group x subregion interaction: $F(2,25.997) = 2.484$, $p = .103$; Figure 3B). However, amygdala activity during trauma and trigger exposure did significantly differ between susceptible and resilient mice in a subregion-specific manner (group x subregion interaction: $F(2,32.921) = 4.140$, $p = .025$; Figure 3C). *Post hoc* tests revealed that neuronal activity was increased in susceptible vs. resilient animals specifically in the BLA ($p = .020$), in which activity also correlated with PTSD-like symptom score ($\rho(18) = .495$, $p = .037$). No differences were observed for the other amygdala subregions (both p's > .537). Levels of amygdala activity under resting conditions post-trauma were however again not different between groups (main effect of group: $F(1,15.996) = .004$, $p = .950$; group x subregion interaction: $F(2,31.248) = .079$, $p = .924$; Figure 3D).

3.4. Fear recall in susceptible and resilient mice

To study the conditions under which memory recall for the foot shock experiences is provoked, as well as the behavioral responses and amygdala activity associated with it, mice were either re-exposed to a novel context they had not seen before, but which resembled the trauma/trigger contexts in a number of features, the trigger context, or the trauma context.

3.4.1. Exposure to a novel context, similar to the trauma/trigger context.

When exposed to a novel context resembling the trauma/trigger context in a number of features, both susceptible and resilient mice showed substantial freezing behavior, yet differential freezing patterns over time (group x time interaction: $F(1,171) = 2.452$, $p = .012$), in the absence of a main effect of group ($F(1,19) = 1.082$, $p = .311$; Figure 4A). Remarkably, whereas resilient mice did not show significant alterations in freezing behavior over time ($F(2.969,29.693) = 1.94$, $p = .144$), susceptible mice reduced their freezing behavior as time passed ($F(9,81) = 4.970$, $p < .001$). These data suggest that both groups of animals initially fear the novel (yet alike to trauma) context to a similar extent, but that susceptible animals reduce their fear response faster than resilient ones.

This subtle difference in behavioral responding was however not related to differential overall neural activity in the amygdala evoked by the novel context between groups (main effect: $F(1,15) = .967$, $p = .341$, group x subregion interaction: $F(2,30) = .440$, $p = .648$; Figure 4B). Yet, the recruitment of amygdalar somatostatin neurons during the exposure to this novel, similar context (assessed by cFos-somatostatin co-expression; Supplementary Figure S2A) was significantly increased in susceptible mice (main effect of group: $F(1,42) = 8.244$, $p = .006$; group x subregion interaction: $F(2,42) = .080$, $p = .923$; Supplementary Figure S2B).

3.4.2. Trigger context re-exposure.

When re-exposed to the trigger context, total freezing behavior was not different in resilient vs. susceptible animals (Figure 5A). Neither overall freezing levels ($F(1,163.690) = .150$, $p = .699$) nor the observed reduction in freezing over time ($F(9,167.649) = 4.194$, $p < .001$) differed between groups (group x time interaction: $F(9,167.649) = .602$, $p = .794$). Neuronal activity induced by trigger context re-exposure however revealed a trend-level significant group x amygdalar subregion interaction ($F(2,32.473) = 2.752$, $p = .079$, in the absence of a main effect of group ($F(1,16,926) = 1.741$, $p = .205$)), which was further explored using *post hoc* tests. These exploratory analyses unfolded a significantly higher re-exposure-induced neuronal activity in specifically the BLA of susceptible vs. resilient animals ($p = .003$), which strongly correlated with overall PTSD-like symptom score ($\rho(17) = .627$, $p = .007$). No group differences were observed in the other amygdalar subregions (LA: $p = .413$; CeA: $p = .198$; Figure 5B). Interestingly, BLA activity during trigger re-exposure strongly correlated with BLA activity observed during the initial trauma/trigger processing in these mice ($\rho(16) = .700$, $p = .003$). The relative activation of somatostatin-expressing neurons in response to trigger context re-exposure did not differ between groups (main effect of group: $F(1,17) = .057$, $p = .814$; group x subregion interaction: $F(2,34) = 1.535$, $p = .230$; Supplementary Figure S2C).

3.4.3. Trauma context re-exposure.

Re-exposure to the trauma context initiated equally strong freezing behavior across groups. There was no difference in overall times spent freezing between susceptible and resilient animals ($F(1,16) = .052$, $p = .824$), nor in reduction of freezing behavior over time (main effect time: $F(9,144) = 3.210$, $p = .001$;

group x time interaction: $F(9,144) = .623$, $p = .776$, Figure 6A). Trauma context re-exposure did also not evoke overall distinct amygdala activation (as assessed by cFos immunohistochemistry; Figure 6B) between groups (main effect of group: $F(1,15.898) = 3.112$, $p = .097$), but revealed a trend-level significant group x subregion interaction ($F(2,27.066) = 3.288$, $p = .053$; Figure 4C). Surprisingly, exploratory *post hoc* tests revealed significantly reduced recall-induced activity in the BLA ($p = .027$), whereas no significant differences were observed in LA ($p = 0.057$) and CeA ($p = .812$) neuronal activity. The recruitment of somatostatin-expressing neurons did not significantly differ between susceptible and resilient mice (main effect of group: $F(1,14.739) = 3.635$, $p = .076$; group x subregion interaction: $F(2,28.968) = 1.955$, $p = .160$; Supplementary Figure S2D).

4. DISCUSSION

Here, in three independent experiments, we set out to longitudinally assess amygdala activity in mice susceptible and resilient to the long-term behavioral consequences of trauma exposure. Results revealed no major differences in either trait anxiety or amygdala activity dissociating susceptible vs. resilient mice prior to trauma exposure, but exaggerated activity in specifically the basolateral amygdala (BLA) peri-trauma that predicted susceptibility to later PTSD-like symptoms. Post-trauma, susceptible mice did not display altered resting amygdala activity, but PTSD-like symptomatology was associated with BLA hyperreactivity in response to trigger context re-exposure, and BLA hyporesponsivity in response to the trauma context, in the absence of altered behavioral manifestation of fear (i.e., freezing). Exposure to a novel, similar context evoked a differential temporal pattern of freezing behavior in susceptible mice and an increased activity of amygdalar somatostatin-expressing neurons specifically.

To refine pathophysiological models of PTSD, it is crucial to distinguish between adaptive vs. maladaptive responses to trauma, as well as between pre-existing factors conferring mere risk for PTSD vs. neurobiological markers of psychopathology. Therefore, we used a mouse model to longitudinally investigate amygdala neuronal activity coding differential susceptibility to trauma exposure. To enhance translational value, we dissociated mice resilient to the behavioral consequences of trauma exposure from those susceptible. This dissociation was based on a compound score comprising multiple behavioral PTSD-like symptoms, rather than single behavioral features. As such, this classification resembles the situation in patients[27], which can be diagnosed with PTSD based on 20 criteria across four distinct symptom categories, resulting in a highly heterogeneous patient population (DSM-V[6]). Similarly, mice coined susceptible in this mouse model can display a complete different set of behavioral symptoms. Whereas this may arguably increase variance within the susceptible group (hence, the large spread as observed in some of our outcomes), this approach aims at common mechanisms for the diverse set of PTSD-like traits that may be more predictive for clinical practice. Using this mouse model, we assessed pre-existing risk factors indicative of traumatic stress susceptibility, focusing on trait anxiety

and resting amygdala activity pre-trauma. Trait anxiety in humans has been found predictive of PTSD risk and symptom severity[435,436] and associated with heightened amygdala responsivity to emotional stimuli[437], which might mediate increased risk for PTSD. Interestingly, previous animal work has indicated that pre-existing susceptibility only becomes evident in increased anxiety following exposure to a mild stressor, not at baseline[438]. Accordingly, prior work has found no association between stress susceptibility and BLA activity under resting conditions[439] or in response to a novel environment[440], matching our findings.

In contrast, mice susceptible for the long-term behavioral consequences of trauma displayed exaggerated neuronal activity in the BLA during trauma and trigger processing. Prior animal studies have already implicated hyperactivity of the amygdala during fear memory encoding and consolidation into processes of fear generalization[441] and deviant fear memory quality[387]. In humans, neural processing of actual trauma exposure is typically inaccessible, restricting studies into peri-trauma predictors of PTSD to (mostly retrospective) questionnaire data. These studies have however implicated differences in peri-trauma psychological (emotional) processing as strongly predictive of PTSD[442], with elevated arousal soon after trauma being predictive of later symptom severity[443]. As amygdala reactivity has been implicated in states of hypervigilance and arousal[444], these observations match our findings. Moreover, amygdala reactivity to fearful faces relatively soon after trauma exposure, was found positively associated with PTSD and predicted symptoms 1 year later[445]. Whereas the latter findings were not directly related to fear memory encoding, studies in healthy volunteers have indicated that besides post-encoding anxiety[446], elevated amygdala activity at the time of aversive memory encoding[447] predicts the occurrence of intrusive memories. Our data are in line with this by implicating exaggerated amygdala activity during trauma processing into increased susceptibility to the development of the overall behavioral consequences of trauma exposure. As such, we also provide first evidence for models suggesting emotional hypermnesia in PTSD, proposedly centered around the amygdala and associated with perceptionally driven, situationally accessible (rather than verbally accessible) trauma memory[448].

The PTSD model implemented here is based on stress-enhanced fear learning, which builds on the clinical observation that prior stress exposure precipitates PTSD[442]. In these rodent models, prior stress exposure is observed to affect the learning of future aversive events, creating traumatic-like memories characterized by exaggerated fear responses and resistance to extinction[28,367]. Similar to other mouse models for PTSD, multiple 'hits' are required to induce aberrant fear memory[367,387]. Whereas the tamoxifen-induced labeling did not allow for the association between amygdala activity observed during the initial stressor and later trigger, we could dissociate later amygdala responses to these two events upon memory retrieval. Interestingly, we observed *increased* cFos expression in the BLA in response to re-exposure to the trigger context to be associated with PTSD-like symptomatology. This corresponds with a previous report modelling PTSD by initial stress exposure and later fear conditioning, showing increased BLA activity upon remote fear memory retrieval in stress susceptible mice[367]. Moreover, it seems in line with increased excitatory activity in the BLA upon a contextual reminder of underwater trauma in rats that developed an anxious phenotype as a consequence of it, in contrast to those unaffected[449]. It also fits observations of increased amygdala responses to (trauma-related and -unrelated) emotional stimuli in PTSD patients[81,84,450] Whereas others have suggested that this hyperreactivity is related to trauma exposure per se rather than PTSD symptomatology[451], our results of exaggerated BLA reactivity being related to PTSD-like symptoms in animals with identical stress exposure links this feature to symptomatology. In contrast, we observed no alterations in amygdala response to the novel context, and *hypoactivity* of the BLA in response to re-exposure to the initial stress context. This suggests that the BLA response in affected individuals greatly depends on the exact stimulus/context, rather than that it is characterized by generally exaggerated fear responses or increased attentional bias for negative stimuli[87,450]. Also human work has reported on suppressed amygdala responses in PTSD[90] and has explained this by emotional numbing or dissociation[452]. Potentially related to this, others have reported on a bidirectional tuning of amygdala responsivity in PTSD, displaying hyper-reactivity to low-arousing stimuli and hypo-reactivity during highly arousing states[91]. In terms of resting amygdala activity post-trauma, we did not observe differences between susceptible and resilient groups, which seems to correspond to other reports on no overall differences in BLA excitability in a

rat model for PTSD[453]. Altogether, these findings suggest that PTSD is not simply characterized by increased sensitivity of the amygdala towards emotional stimuli, but indicate complex deviations that depend on the exact context and stimulus to which it is assessed.

Noteworthy, the observed deviations in amygdala function were specific to the BLA, implicating aberrant fear memory acquisition and recall in traumatic stress susceptibility[425] instead of generally exaggerated fear- and arousal-related amygdala output. Yet, these deviations did not clearly translate to differential behavioral profiles of fear (encoding or recall) in response to the fear contexts. This is in contrast with prior reports on exaggerated, extinction-resistant, and generalized fear memory in PTSD-susceptible mice[367] in studies implementing stress-enhanced cued fear learning. Considering that PTSD has been linked to impairments in hippocampal context processing[397] and memory[387], it is tempting to speculate that impaired contextual fear memory retrieval, combined with excessive fear upon recall in susceptible mice, might cancel each other out on the behavioral level. In turn, the BLA hyperreactivity during fear encoding and recall may reflect a features-based representation of context as introduced by theoretical models of Rudy and Fanselow[352,353], in which the BLA integrates sensory information to generate a representation of the context. Here, we set out to assess potential deviations in the quality of context processing by measuring fear responses to a novel context. Interestingly, freezing behavior over time in the novel context differed between groups, but reduced over time in susceptible mice, whereas resilient mice showed no such decline in fear. This altered temporal pattern of freezing behavior was not associated with differences in overall amygdala activity, but susceptible mice displayed higher activity of amygdalar somatostatin-expressing neurons. Whereas somatostatin neurons in the BLA have been shown to inhibit local pyramidal neurons and modulate fear learning and responding[454], their exact contribution to fear recall requires further investigation.

Some limitations to the work also need to be mentioned. TdTomato expression in ArcTRAP mice is restricted to glutamatergic cells[379], making that the contribution of GABAergic interneurons to resting

activity pre- and post-trauma, as well as peri-trauma responses, and their role in traumatic stress susceptibility was not assessed. Whereas amygdala responses related to fear recall do involve both glutamatergic and GABAergic subpopulations (cFos is expressed in both), we only dissociated somatostatin-expressing cells. This choice was based on their well-established role within the CeA in mediating fear learning[430] and expression[431], as well as prior reports implicating alterations in CeA somatostatin neuronal activity in stress susceptibility[455]. Future work should also investigate the involvement of the other amygdala neuronal populations in traumatic stress susceptibility. Moreover, it is increasingly acknowledged that even within the currently defined amygdalar subregions (i.e., LA, BLA and CeA), even smaller functional subregions exist which seem to serve different functions by projecting to distinct brain sites[456]. Here we aimed at targeting subregions in the most consistent manner, yet subtle variation in slice selection may have led to slight differences in regional assessments, potentially contributing to the observed data spread. Future work should target these specialized subregions in dedicated studies. Also, we only included the study of the right amygdala. Laterality effects in emotional processing in the amygdala[457] and its association with PTSD-like symptoms[387] have been reported before, so future work should assess whether similar associations can be found in the left amygdala. Furthermore, we only tested male mice here. Future studies should assess whether susceptible females share similar deviations in BLA activity, by using PTSD models validated in females and implementing relevant PTSD-like symptomatologies, as these differ across sexes[458]. Also, we did not include non-shock exposed control groups. Applying the TRAP method in control mice would have been of particular interest for the post-trauma cohort, to investigate whether activity in the amygdala is increased under resting conditions due to the PTSD-induction protocol. Yet, since susceptible and resilient mice do not differ in amygdala activity post-trauma, control mice are not essential for the interpretation of our data. Lastly, our results may be influenced by the PTSD model used. Our model, focusing on impaired risk assessment, high anxiety, hypervigilance, attention disturbances and insomnia, is strongly based on the 'trauma-related arousal and reactivity' symptom cluster of PTSD in the DSM-V[6]. While assessing multiple symptoms, it likely does not capture the full, complex human PTSD-symptomatology, and other models may be better suited to study the other symptom clusters.

Concluding, this study revealed differences in BLA responses during trauma processing, as well as its subsequent recall in trauma susceptible vs. resilient mice. Our findings indicate that excessive trauma-related BLA activity predicts the development of later PTSD-like symptoms, proposing it as an early biomarker for intervention (secondary prevention). Following trauma exposure, BLA activity is modulated bi-directionally as a function of traumatic stress susceptibility, displaying hypo- and hyperactivity depending on the exact context. Together, these findings provide first evidence for differential trauma memory encoding, followed by deviations in fear memory recall in trauma susceptible individuals, and further highlight the central role for the BLA in these processes.

AUTHOR CONTRIBUTIONS

Bart Dirven: Conceptualization, Data acquisition, Data analyses, Writing - original draft, Writing - review & editing. **Andriana Botan:** Data acquisition, Data analyses. **Dewi van der Geugten:** Data acquisition, Data analyses. **Blom Kraakman:** Data acquisition, Data analyses. **Lennart van Melis:** Data acquisition, Data analyses. **Sanne Merjenburgh:** Data acquisition, Data analyses. **Rebecca van Rijn:** Data acquisition, Data analyses. **Liz Waajen:** Data acquisition, Data analyses. **Judith Homberg:** Conceptualization, Writing - review & editing. **Tamas Kozicz:** Conceptualization, Funding acquisition, Writing - review & editing. **Marloes Henckens:** Conceptualization, Funding acquisition, Writing - original draft, Writing - review & editing.

ACKNOWLEDGEMENTS

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DECLARATION OF INTEREST

None.

FIGURES

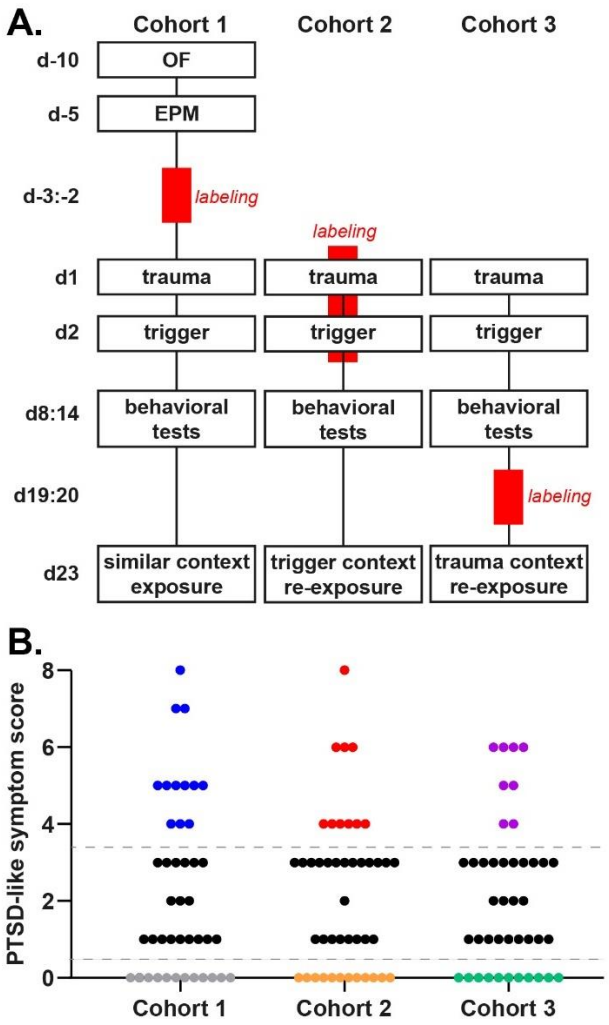


Figure 1. Timeline of the behavioral protocols as implemented for cohorts 1-3 (**A**). In cohort 1, pre-trauma resting amygdala activity and response to a novel context (similar to the trauma and trigger context) were assessed. In cohort 2, integrated amygdala responses to the trauma and trigger session were measured to capture neuronal activity during PTSD-induction. Furthermore, amygdala responses during trigger memory recall were assessed in this cohort. Cohort 3 was used to measure resting amygdala activity post-trauma, as well as activity induced by trauma memory recall. Behavioral test results were combined to generate overall PTSD-like symptom scores, used to define susceptible (PTSD-like symptom score >= 4) and resilient (PTSD-like symptom score = 0) mice (**B**). D, day; EPM, elevated plus maze; OF, open field; Red blocks indicated time windows during which neuronal activity was labeled by tamoxifen injections, which took place on either day -3, day 1 or day 19.

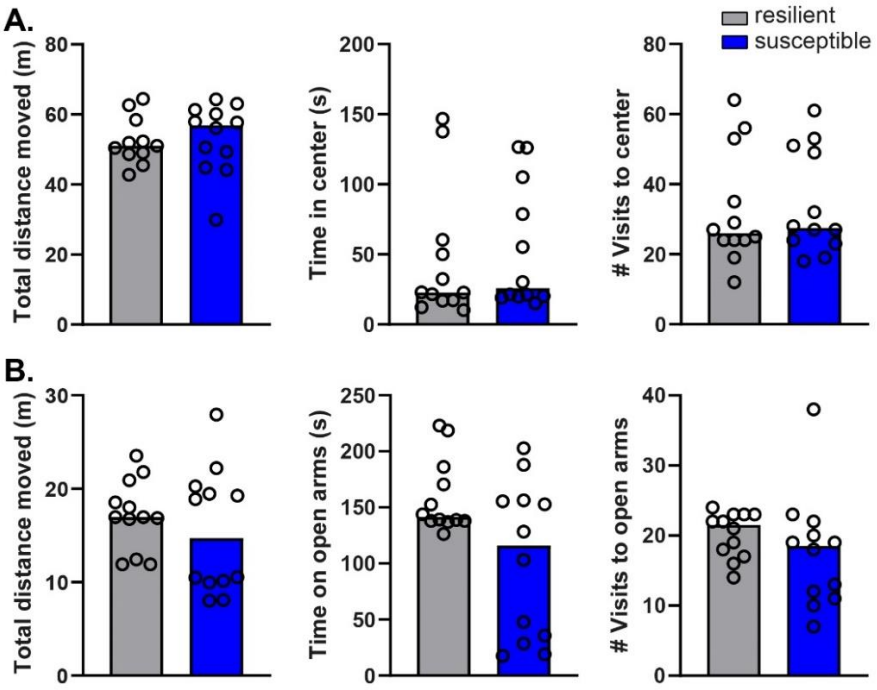


Figure 2. Pre-trauma anxiety-like behavior did not predict traumatic stress susceptibility. Susceptible and resilient mice did not behave any differently in the open field test (**A**) or elevated plus maze (**B**) prior to trauma exposure. $n_{resilient} = 12$, $n_{susceptible} = 12$

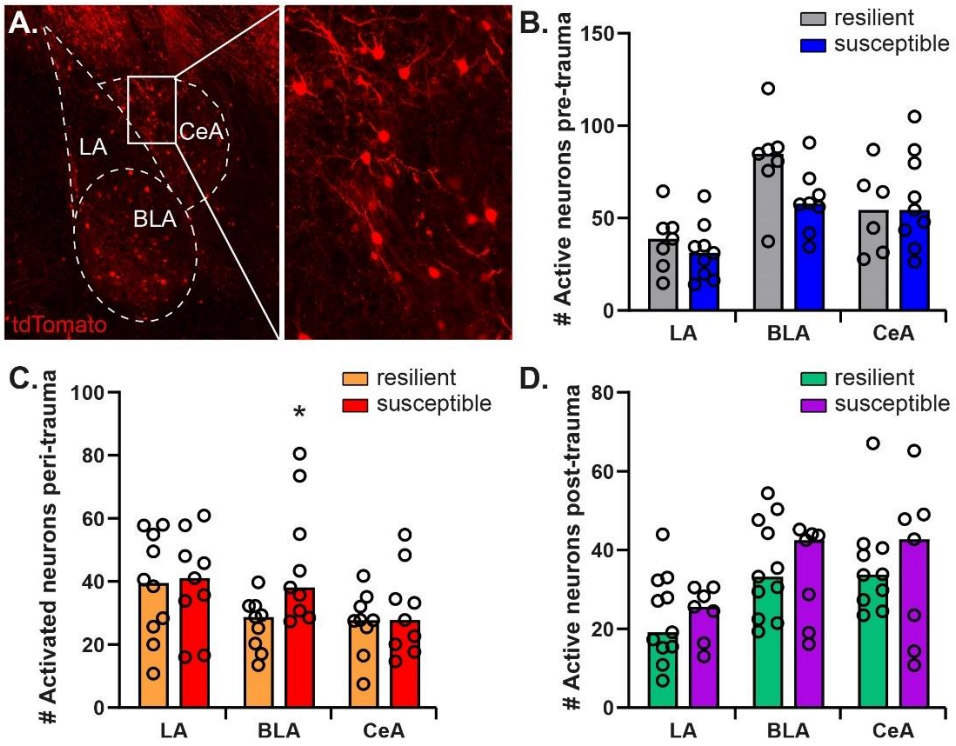


Figure 3. Amygdalar neuronal activity mapped by tdTomato-expression triggered by *Arc* transcription in ArcTRAPxtdTomato mice (**A**). Neuronal activity pre- ($n_{resilient}$ = 7, $n_{susceptible}$ = 9; **B**) and post- ($n_{resilient}$ = 11, $n_{susceptible}$ = 7; **D**) trauma was not different between resilient and susceptible mice. Susceptible mice however tended to display increased neuronal activity in the basolateral amygdala (BLA) during trauma+trigger exposure ($n_{resilient}$ = 10, $n_{susceptible}$ = 9; **C**). CeA, central amygdala; LA, lateral amygdala. *: $p < .05$, effect of group.

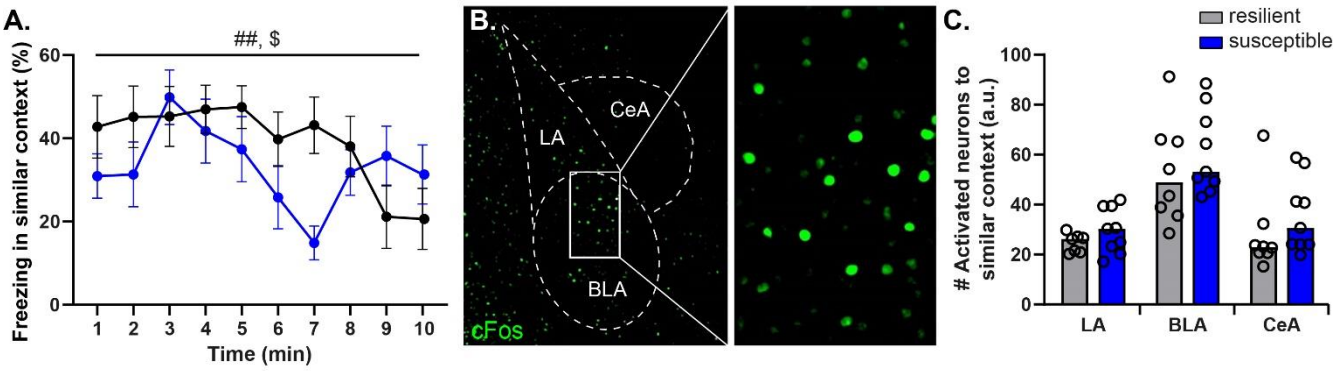


Figure 4. Behavioral fear responses and amygdalar neuronal activity induced by re-exposure to a novel context, similar to the trauma and trigger context in susceptible vs. resilient mice. Groups displayed no difference in overall freezing levels averaged over the entire session, but differential dynamics of the freezing response over time, with only susceptible mice displaying a time-dependent reduction ($n_{resilient}$ = 11, $n_{susceptible}$ = 10; **A**). Amygdalar neuronal activity as assessed by cFos expression (**B**), revealed no differences in amygdalar neuronal activity in response to the similar context (($n_{resilient}$ = 8, $n_{susceptible}$ = 9; **C**). BLA, basolateral amygdala; CeA, central amygdala; LA, lateral amygdala. ##: $p < .01$, effect of time; $: $p < .05$, group x time interaction.

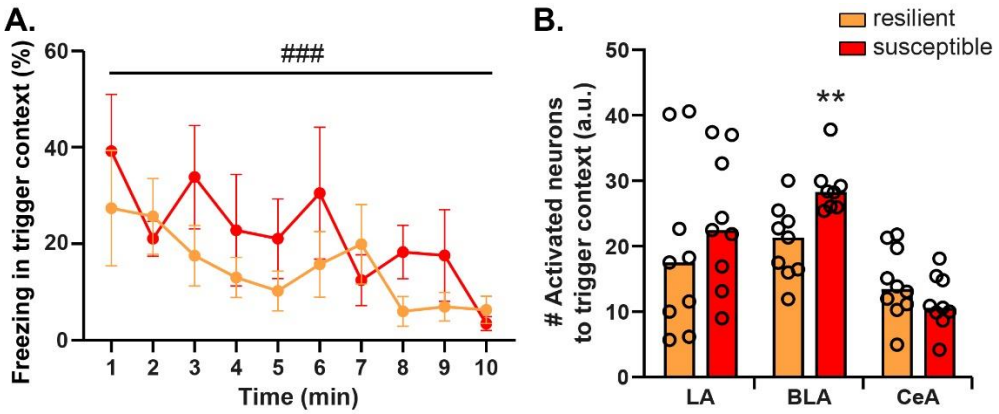


Figure 5. Behavioral fear responses and amygdalar neuronal activity induced by re-exposure to the trigger context revealed similar freezing responses in susceptible vs. resilient mice, which overall decreased over time ($n_{resilient}$ = 12, $n_{susceptible}$ = 9; **A**), but significantly increased neuronal activity within the basolateral amygdala (BLA) ($n_{resilient}$ = 10, $n_{susceptible}$ = 9; **B**) of susceptible mice compared to those resilient to trauma. CeA, central amygdala; LA, lateral amygdala. ###: $p < 0.001$, effect of time; **: $p < .01$, effect of group.

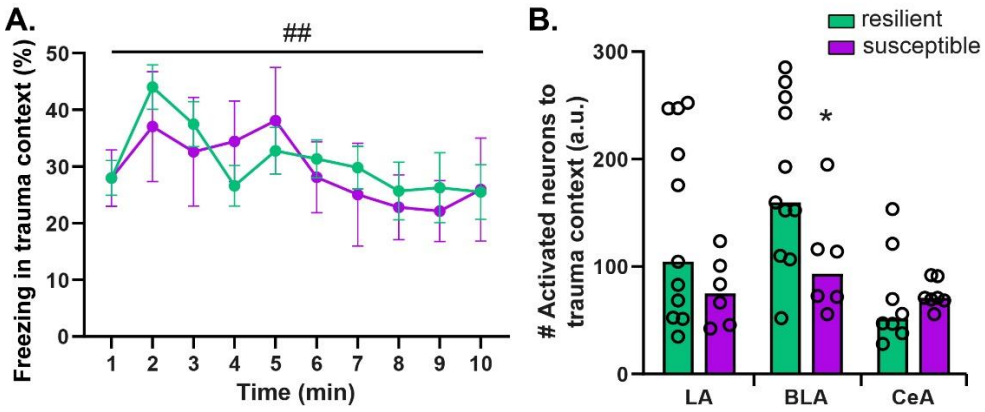


Figure 6. Behavioral fear responses induced by re-exposure to the trauma context, revealed similar freezing behavior in susceptible vs. resilient mice, which overall decreased over time ($n_{resilient}$ = 11, $n_{susceptible}$ = 7; **A**). Neuronal activity in the basolateral amygdala (BLA) in response to the trauma context tended to be reduced in susceptible mice, without differences in the lateral amygdala (LA), and the central amygdala (CeA) ($n_{resilient}$ = 11, $n_{susceptible}$ = 7; **B**). ##: $p < .01$, effect of time; *: $p < .05$, effect of group.

SUPPLEMENTARY MATERIALS

Supplementary methods

Labeling of neuronal activity in living mice.

Two founder mouse lines, ArcCreER^{T2} (B6.129(Cg)-*Arc*$^{tm1.1(cre/ERT2)Luo}$/J) and conditional tdTomato (B6.Cg-*Gt(ROSA)26Sor*$^{tm9(CAG-tdTomato)Hze}$/J, 007909), were purchased from The Jackson Laboratory and bred to generate heterozygote ArcCreER^{T2}xROSA offspring, referred to as ArcTRAP. In these mice, the tamoxifen-dependent recombinase CreER^{T2} is expressed in an activity-dependent manner from the locus of the immediate early gene *Arc*. Active cells that express CreER^{T2} can undergo recombination, and express the fluorescent marker tdTomato, only when tamoxifen is present. This allows for the fluorescent labeling of activated neurons in a 36 hour time window after injection with the compound tamoxifen[102]. ArcTRAP mice were preferred over FosTRAP mice because of their increased labeling sensitivity[102] as well as high selectivity of labeling within the amygdala. Tamoxifen was chosen over its active derivate 4-hydroxytamoxifen (inducing instant labeling over a shorter temporal window[102]) to prevent potentially confounding effects of injection stress in labeling, and because there was no need for high temporal specificity in labeling.

Behavioral testing for PTSD-like behaviors.

Dark-light transfer test. On day 8 of the protocol, mice were tested in the dark-light transfer test. The test was executed in a box that was divided into a dark compartment (DC, 29 x 14 cm) and brightly illuminated (ca. 1100 lux) compartment (LC, 29 x 29 cm), connected by a retractable door. The mice were individually placed in the DC, and the door was opened to initiate a 5 minute test session. Movement of the mice was recorded and scored automatically with EthoVision XT (Noldus). An additional area of 6 x 3 cm surrounding the opening of the LC was programmed into the software tracking measurements. Time spent in the LC as well as time spent in this 'risk assessment' zone was measured. Percentage risk assessment was calculated as the amount of time spent in the risk assessment zone as a percentage of total time spent in the LC.

Marble burying. On day 10, mice were individually placed in a 10 lux illuminated black open box (30 x 28 cm), containing a 5 cm deep layer of corn cobs, on top of which 20 marbles were centrally arranged in a 4 x 5 grid formation. Each mouse was placed in the corner of the box to initiate the task. Mice were videotaped for 25 minutes. Videos were scored by assessing the number of buried marbles after 25 minutes.

Startle response and pre-pulse inhibition. On day 12, mice were moved to the experimental room in their home cage and individually placed in small, see-through Plexiglas constrainers mounted on a vibration-sensitive platform inside a ventilated cabinet that contained two high-frequency loudspeakers (SR-LAB, San Diego Instruments). Movements of the mice were measured with a sensor inside of the platform. The pre-pulse inhibition test (PPI) started with an acclimatization period of 5 minutes, in which a background noise of 70 dB was presented, which was maintained throughout the entire 30 minute session. Following acclimatization, 6 startle cues of 120 dB were presented, 40 ms in duration and with a random varying ITI (12-30 s). Then, a block of 12 120 dB startle cues were presented without pre-pulse, 12 preceded by a 20 ms pre-pulse of either 75 dB, 12 with 80 dB pre-pulse, and 12 with 85 dB pre-pulse in random order. The session ended with another 6 startle cues of 120 dB without pre-pulse. Sessions were scored by assessing the latency to peak startle amplitude of the 12 middle 120 dB startle trials, and the averaged pre-pulse inhibition observed for the 36 pre-pulse trials; i.e., the percentage of startle inhibition response to the different pre-pulse stimuli [1 - (mean pre-pulse startle response / mean startle response without pre-pulse) x 100].

Homecage locomotion. Immediately after the pre-pulse inhibition test, mice were individually housed in phenotyper cages (45 x 45 cm, Noldus) for 72 hours while their locomotion was being recorded by an infrared-based automated system (EthoVision XT, Noldus). The first 24 hours were considered as habituation period and data were discarded. Total locomotion time during the subsequent two light phases (21:00 - 09:00 h) was assessed.

Pre-trauma anxiety tests.

The open field apparatus consisted of a 120 lux illuminated white Plexiglas box (50 x 50 x 40 cm). Each mouse was placed in the corner of the apparatus to initiate a 10 min test session. Time spent in the center (the inner 25 x 25 cm), visits paid to the center, and total distance traveled, were captured using a camera mounted above the apparatus and analyzed by EthoVision software (Noldus).

As a second test for pre-trauma anxiety, the slightly more aversive elevated plus maze was used. The 5 lux illuminated elevated plus maze comprised a central part (5 x 5 cm), two opposing open arms (30.5 x 5 cm), and two opposing Plexiglas closed arms (30.5 x 5 x 15 cm), elevated at a height of 53.5 cm. Mice were placed in one of the closed arms facing the center to initiate a 5 min test session. The number of visits to the open arms, time spent in the open arms, as well as total distance traveled were captured using a camera mounted above the apparatus and analyzed by EthoVision software (Noldus).

Immunohistochemistry.

For each animal, 4-6 sections including the amygdala were selected between anterior-posterior coordinates -1.25 mm and -1.75 mm relative to Bregma. Sections were washed three times in 1x PBS and blocked in PBS-BT (1x PBS with 0.3% Triton X-100 and 1% bovine serum albumin) for 30 minutes at room temperature (RT). Incubation of the primary antibodies was performed overnight (guinea pig anti-cFos, 1:750, 226004, Synaptic Systems; rat anti-somatostatin (SOM), 1:200, MAB354, Merck Chemicals) in PBS-BT for 18 hours at RT. Then, sections were washed three times in 1x PBS, and incubated with the secondary antibodies (Alexa 647-conjugated donkey anti-guinea pig, 1:200, AP193SA6, Merck Chemicals; Alexa 488-conjugated donkey anti-rat, 1:200, A-21206, Thermo Fisher) in PBS-BT for 3 hours at RT. Lastly, slices were washed three times in 1x PBS, mounted on gelatin-coated slides using FluorSave™ reagent (345789, Merck Chemicals) and cover slipped. The slices were stored at -20°C until image acquisition and cell counting.

Image acquisition and cell counting.

Images of the cFos/SOM stainings and tdTomato signal were captured through a light microscope (Axio Imager 2, Zeiss) using a 10x objective lens and a LED module (Colibri 2, Zeiss). Separate photos were

stitched and cFos^{+}, tdTomato^{+} and SOM^{+} cells were manually counted per region in Fiji software [376] by an experimenter blinded to the experimental group.

Supplementary results

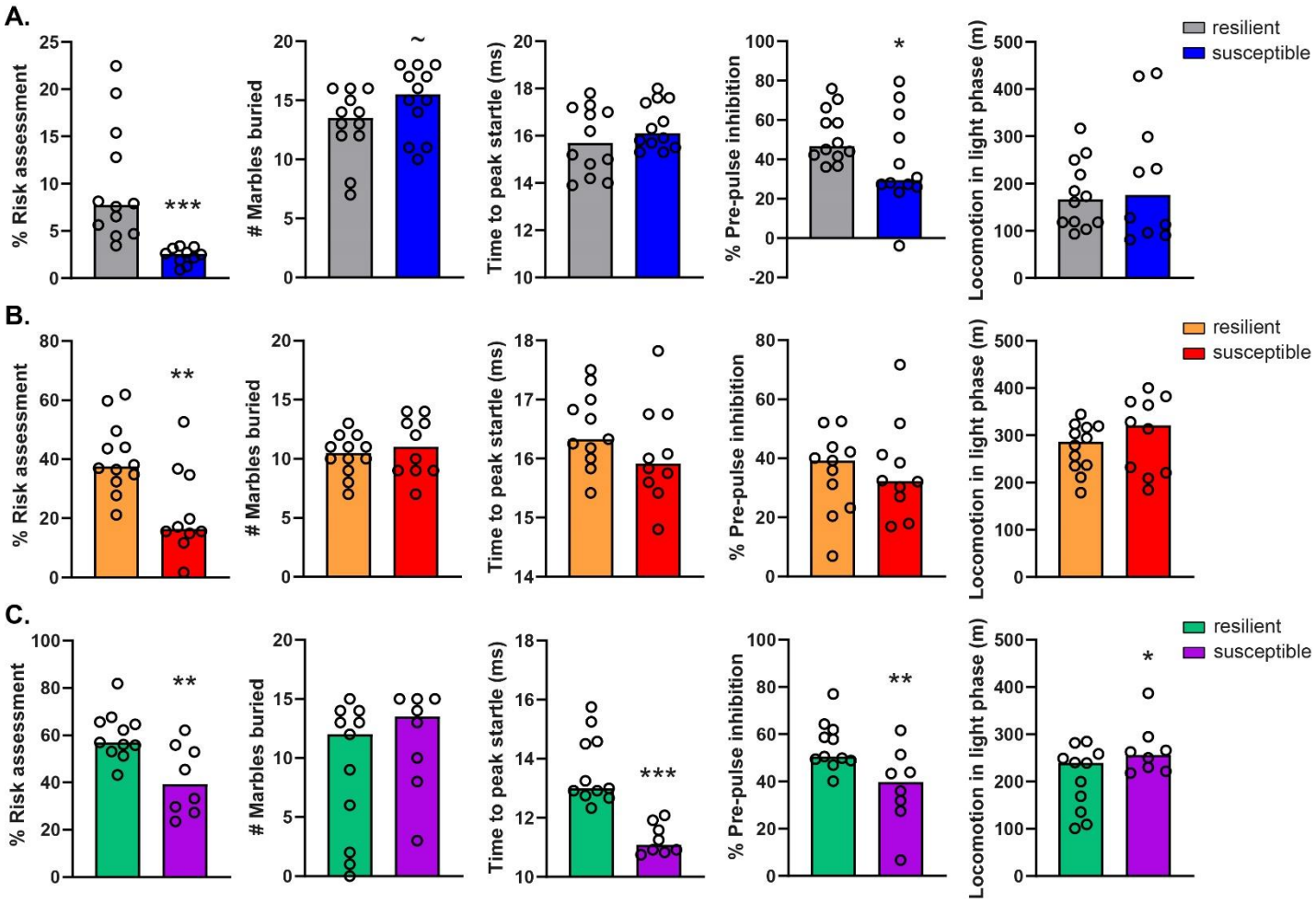
PTSD-like symptomatology

Cohort 1: Susceptible mice were characterized by significantly higher PTSD-like symptom scores than resilient mice ($U = 144$, $p < .001$). In terms of isolated behaviors for each individual test for PTSD-like symptomatology (Figure S1A), we observed that susceptible mice displayed significantly lower risk assessment ($U = 0$, $p < .001$), and reduced pre-pulse inhibition ($U = 41$, $p = .039$), as well as trend-level significant higher marble burying behavior ($t(22) = 1.667$, $p = .055$). Reaction times to peak startle ($t(19.608) = 1.245$, $p = .114$) and locomotor activity in the light phase ($U = 61$, $p = 1.00$) were overall not significantly affected in this cohort.

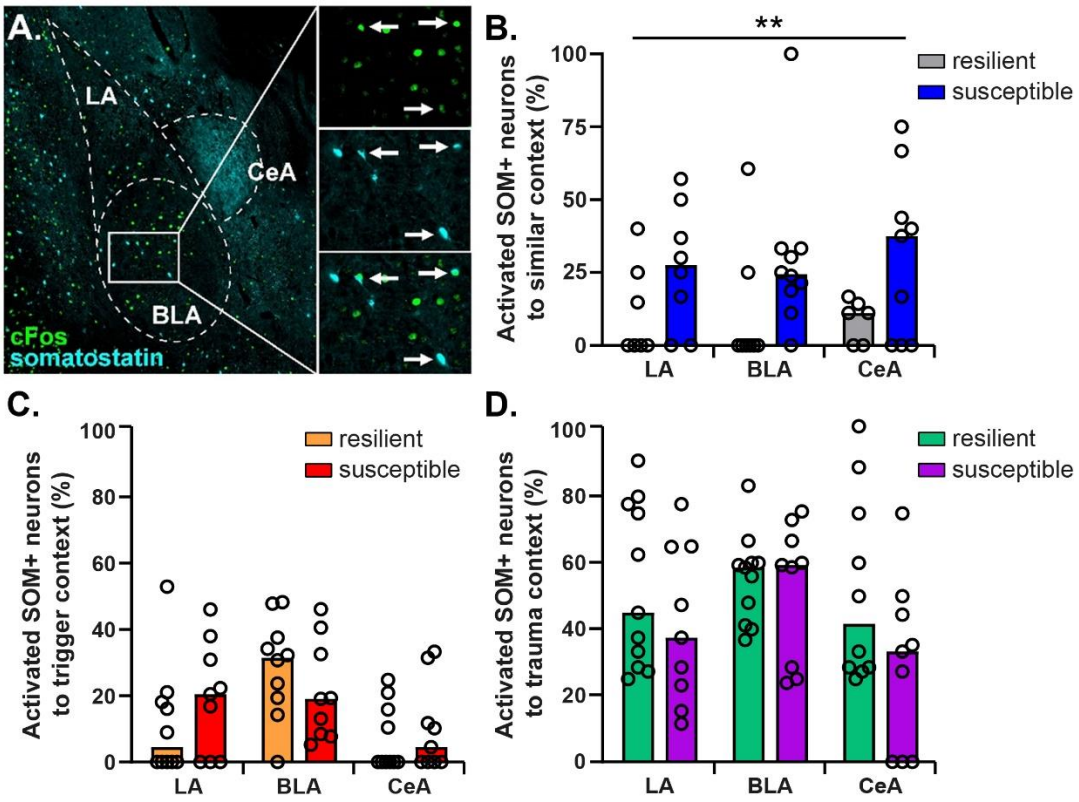
Cohort 2: PTSD-like symptom scores were significantly higher in susceptible vs. resilient mice ($U = 120$, $p < .001$). Susceptible mice displayed strongly reduced risk assessment behavior ($t(20) = 3.221$, $p = .002$). Overall differences in the other isolated behaviors did not reach significance (all p's > .05) (see Figure S1B and Table S1 for all statistical results).

Cohort 3: Susceptible mice displayed significantly higher PTSD-like symptom scores than resilient mice ($U = 88$, $p < .001$). In terms of specific PTSD-like symptoms (Figure S1C), susceptible mice showed lower risk assessment behavior ($t(17) = 3.261$, $p = .003$), a shorter reaction time to peak startle ($t(14.604) = 5.901$, $p < .001$), reduced pre-pulse inhibition ($t(17) = 2.811$, $p = .006$), as well as higher locomotor activity in the light phase ($t(17) = 2.067$, $p = .027$) compared to resilient ones. Only marble burying behavior was not different between groups ($U = 28.5$, $p = .103$).

Supplementary figures



Supplementary Figure S1. Behavioral assessment of separate behavioral traits reflecting PTSD-like symptomatology over the three behavioral cohorts (See Supplementary Results and Figure 1; Cohort 1 ($n_{resilient}$ = 12, $n_{susceptible}$ = 12; **A**), Cohort 2 ($n_{resilient}$ = 12, $n_{susceptible}$ = 10; **B**), Cohort 3 ($n_{resilient}$ = 11, $n_{susceptible}$ = 8; **C**)). ~: $p < .10$, *: $p < .05$, **: $p < .01$, ***: $p < .001$



Supplementary Figure S2. Relative activity of somatostatin neurons in the amygdala. Activated somatostatin neurons upon fear memory recall were identified by co-expression of somatostatin and cFos (**A**). Susceptible mice showed increased relative activity of somatostatin (SOM+) neurons upon exposure to a novel, yet similar context ($n_{resilient}$ = 8, $n_{susceptible}$ = 10; **B**) compared to resilient ones. Groups did not differ in activity of somatostatin neurons upon re-exposure to the trauma ($n_{resilient}$ = 10, $n_{susceptible}$ = 9; **C**) and trigger ($n_{resilient}$ = 11, $n_{susceptible}$ = 9; **D**) contexts. BLA, basolateral amygdala; CeA, central amygdala; LA, lateral amygdala. **: $p < .01$, main effect of group.

Supplementary tables

Readout	Statistical test	Outcome	P value
PTSD-like behaviors			
Cohort 1			
PTSD-like symptom score	Mann-Whitney U	$U = 144$	$p < .001^{\#}$
% Risk assessment	Mann-Whitney U	$U = 0$	$p < .001^{\#}$
# Marbles buried	Independent samples t-test	$t(22) = 1.667$	$p = .055^{\#}$
Reaction time to peak startle	Independent samples t-test	$t(19.608) = 1.245$	$p = .114^{\#}$
% Pre-pulse inhibition	Mann-Whitney U	$U = 41$	$p = .039^{\#}$
Light phase locomotion	Mann-Whitney U	$U = 61$	$p = 1.00^{\#}$
Cohort 2			
PTSD-like symptom score	Mann-Whitney U	$U = 120$	$p < .001^{\#}$
% Risk assessment	Independent samples t-test	$t(20) = 3.221$	$p = .002^{\#}$
# Marbles buried	Independent samples t-test	$t(20) < 1$	$p = .234^{\#}$
Reaction time to peak startle	Independent samples t-test	$t(19) = 1.246$	$p = .114^{\#}$
% Pre-pulse inhibition	Independent samples t-test	$t(19) < 1$	$p = .456^{\#}$
Light phase locomotion	Independent samples t-test	$t(14.633) < 1$	$p = .201^{\#}$
Cohort 3			
PTSD-like symptom score	Mann-Whitney U	$U = 88$	$p < .001^{\#}$
% Risk assessment	Independent samples t-test	$t(17) = 3.261$	$p = .003^{\#}$
# Marbles buried	Mann-Whitney U	$U = 28.5$	$p = .103^{\#}$
Reaction time to peak startle	Independent samples t-test	$t(14.604) = 5.901$	$p < .001^{\#}$
% Pre-pulse inhibition	Independent samples t-test	$t(17) = 2.811$	$p = .006^{\#}$
Light phase locomotion	Independent samples t-test	$t(17) = 2.067$	$p = .027^{\#}$
Pre-trauma anxiety			
Open field test			
Total distance moved	Independent samples t-test	$t(21) < 1$	$p = .827$
Time spent in center	Mann-Whitney U	$U = 78$	$p = .729$
# Visits to center	Mann-Whitney U	$U = 74.5$	$p = .887$
Elevated plus maze			
Total distance moved	Independent samples t-test	$t(17.439) < 1$	$p = .430$
Time spent on open arms	Mann-Whitney U	$U = 45$	$p = .128$
# Visits to open arms	Independent samples t-test	$t(22) = .980$	$p = .338$
Freezing behavior			
Freezing per minute in similar, novel context (*cohort 1*)	Linear mixed model	Main effect time: $F(9,171) = 3.091$ Main effect group: $F(1,19) = 1.082$ Time x group: $F(1,171) = 2.452$	$p = .002$ $p = .311$ $p = .012$
Freezing per minute in trigger context (*cohort 2*)	Linear mixed model	Main effect time: $F(9,167.649) = 4.194$ Main effect group:	$p < .001$

		$F(1,163.690) < 1$ Time x group: $F(9,167.649) < 1$	$p = .699$ $p = .794$
Freezing per minute in trauma context (*cohort 3*)	Linear mixed model	Main effect time: $F(9,144) = 3.210$ Main effect group: $F(1,16) < 1$ Time x group: $F(9,144) < 1$	 $p = .001$ $p = .824$ $p = .776$
Tdtomato labeling (TRAP)			
Pre-trauma resting (*cohort 1*)	Linear mixed model	Main effect group: $F(1,13.576) < 1$ Group x subregion: $F(2,25.997) = 2.484$	 $p = .397$ $p = .103$
Peri-trauma (*cohort 2*)	Linear mixed model *Post hoc* Fisher's LSD test	Main effect group: $F(1,17.278) = 2.131$ Group x subregion: $F(2,32.921) = 4.140$ - LA - BLA - CeA	 $p = .162$ $p = .025$ $p = .881$ $p = .020$ $p = .537$
Post-trauma resting (*cohort 3*)	Linear mixed model	Main effect group: $F(1,15.996) < 1$ Group x subregion: $F(2,31.248) < 1$	 $p = .950$ $p = .924$
cFos induced by re-exposure			
Similar context (*cohort 1*)	Linear mixed model	Main effect group: $F(1,15) = .967$ Group x subregion: $F(2,30) = .440$	 $p = .341$ $p = .648$
Trigger context (*cohort 2*)	Linear mixed model *Post hoc* Fisher's LSD test	Main effect group: $F(1,16,926) = 1.741$ Group x subregion: $F(2,32.473) = 2.752$ - LA - BLA - CeA	 $p = .205$ $p = .079$ $p = .413$ $p = .003$ $p = .198$
Trauma context (*cohort 3*)	Linear mixed model *Post hoc* Fisher's LSD test	Main effect group: $F(1,15.898) = 3.112$ Group x subregion: $F(2,27.066) = 3.288$ - LA - BLA - CeA	 $p = .097$ $p = .053$ $p = .057$ $p = .027$ $p = .812$
SOM+ neuron activation rates			

Similar context (*cohort 1*)	Linear mixed model	Main effect group: $F(1,42) = 8.244$ Group x subregion: $F(2,42) < 1$	 $p = .006$ $p = .923$
Trigger context (*cohort 2*)	Linear mixed model	Main effect group: $F(1,17) = .057$ Group x subregion: $F(2,34) = 1.535$	 $p = .814$ $p = .230$
Trauma context (*cohort 3*)	Linear mixed model	Main effect group: $F(1,14.739) = 3.635$ Group x subregion: $F(2,28.968) = 1.955$	 $p = .076$ $p = .160$

Supplementary Table S1. Statistical tests and outcomes. Normality was checked using the Shapiro-Wilk test. In case of single dependent variables, independent t-tests were carried out for normally distributed data, while for non-parametric data, the Mann-Whitney U test was used. Levene's test for equality of variances was carried out to determine equal variances across groups. In case of repeated within-subject measurements (e.g., freezing levels over time, or neuronal activity over amygdalar subregions) linear mixed models were used to estimate between and within subject effects. Groups x subregion interactions were followed up by *post hoc* Fisher's LSD tests.

#: Based on our clear hypotheses on group differences in PTSD-like symptoms, these data were tested using one-tailed instead of two-tailed tests.

Chapter 5

Susceptibility to stress: Temporally specific changes in brain-wide neuronal activity and functional network connectivity at cellular resolution

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ABSTRACT

Understanding the biological basis of susceptibility to traumatic stress is key to improve insight into stress-related psychopathology like post-traumatic stress disorder (PTSD). As a result of decades of clinical research, PTSD is recognized as a disorder that involves aberrant activity and intra- and inter-network connectivity of large-scale functional brain networks; i.e., the salience (SN), default mode (DMN) and executive control (ECN) network. Yet, it is unclear when - i.e., pre-, peri- or post-trauma exposure - and how these changes in brain activity and connectivity are exactly manifested. Preclinical research offers the unique possibility to study PTSD's mechanistic underpinnings and carefully study susceptibility profiles at different time points surrounding trauma exposure, but the vast majority of preclinical studies has been restricted to single time point assessments of the traditional brain regions of interest (the prefrontal cortex, hippocampus and amygdala). Here, we implemented a preclinical rodent model for PTSD that entails exposure to a traumatic event (severe, unpredictable foot shock) followed by a trigger (mild, predictable foot shock). Using behavioral phenotyping for PTSD-like symptoms, traumatic stress susceptible vs. resilient mice were identified and pre-, peri- and post-trauma brain-wide activity was compared - in three independent cohorts - by tagging neuronal activity in living mice using the ArcTRAP transgenic mouse line. Immunolabeling-enabled three-dimensional imaging of solvent-cleared organs (iDISCO+) was used to unbiasedly identify brains regions that displayed differential activity in mice identified as either susceptible or resilient to the long-term behavioral consequences of trauma exposure. Results implicated increased resting activity of the (lateral) orbitofrontal cortex both post- and pre-trauma in susceptible mice, indicating it as a potential driver of susceptibility. Susceptible mice showed increased activity of the retrosplenial cortex peri- and post-trauma, reflecting an acquired alteration. Furthermore, susceptibility was associated with increased peri-trauma activity of sensory- and memory-related regions, including the somatosensory, visual, and auditory cortex, as well as the subiculum. Functional neural network connectivity within groups was approximated by assessing intra- and inter-network cross-subject correlations in regional activity, focusing on the SN, DMN and lateral cortical networks (LCN, the rodent homologue of the ECN). Susceptible mice showed increased correlations in neuronal activity between the DMN and LCN pre- and peri-trauma, increased DMN-SN

correlations peri- and post-trauma, and increased SN intra-network correlations post-trauma, the latter recapitulating observations in PTSD patients. We used state-of-the-art fluorescent labeling and 3D imaging techniques, combined with sophisticated individual behavioral profiling in male mice and report on aberrant large-scale functional network activity and connectivity pre- and peri-trauma. We also show that these alterations could predict later PTSD-like symptom development and that specific connectivity patterns are associated with susceptibility to stress.

1. INTRODUCTION

Every individual has to cope with stressful situations, trauma, and adversity during their lifetime. In cases of severe acute or chronic stress, this may lead to the development of fear, anxiety, or mood disorders[459,460]. Still, most people are able to successfully adapt in the face of stress and are resilient to its long-term deleterious effects[461,462]. While effects of acute and chronic stress on an individual's physiology and behavior have been studied in detail, much less is known about the biological basis of interindividual differences in stress responses[463]; information that may be key for gaining improved insight into stress-related disorders and generating new leads for their improved detection, prevention and treatment.

Human neuroimaging work over the past decades has indicated that the brain is organized as a set of large-scale functional networks, which are reciprocally connected and carry out specialized functions[464]. Psychopathology is characterized by deficits in access, engagement and disengagement of these networks[116]. This is encapsulated in theoretical models, notably the triple network model of psychopathology[323]. This model posits that aberrant function of the salience network (SN), executive control network (ECN), and default mode network (DMN) and their dynamic cross-network interactions encode a wide range of psychopathological mechanisms, and thus could explain differences in brain function between susceptible and resilient individuals. Specifically, in patients suffering from post-traumatic stress disorder (PTSD), weak intrinsic intra-network connectivity within the DMN[111,117] and ECN[123,465], and a hyperactive and strongly intra-connected SN[120,121] have been observed, as well as increased inter-network connectivity between the SN and DMN networks[121]. These network connectivity changes are thought to contribute to a relative dominance of threat-oriented and emotional self-reflective processing in PTSD.

However, it is currently unknown *when* these deviations in network activity and connectivity become apparent, as differences could either exist as a risk factor before trauma exposure, emerge as aberrant response to trauma exposure, or originate from inadequate trauma recovery. Discerning these distinct scenarios is essential, as they each carry unique implications for prevention, early intervention, and

treatment of PTSD. Moreover, the neurobiological underpinnings of the network imbalance, as well as the detailed dissection of the network deviations, still need to be elucidated to be able to intervene.

Animal models offer the unique opportunity to fill these knowledge gaps as they allow for more invasive measurements and manipulations to identify biological determinants of resilience vs. susceptibility in controlled, prospective studies. These models increasingly acknowledge the relevance of incorporating interindividual differences in stress resilience/susceptibility to enhance the translational value of the results derived from the animals and have begun to identify neural circuits and molecular pathways that mediate these distinct phenotypes[462,466,467]. Traditionally, these models have, however, strongly focused on the prefrontal cortex, hippocampus and amygdala as core implicated brain structures, because of their established role in emotional processing and memory[323,468], lagging behind on advancing clinical insights[34]. Excitingly, new development of transgenic constructs in mice now also allow for the identification of active neurons across the whole brain in living animals[95,97], offering the unprecedented opportunity to elucidate the neuronal populations active at specific time points before and after, but critically also during traumatic stress in mice resilient or susceptible to developing PTSD-like symptoms. In combination with brain clearing and light-sheet microscopy technologies, this allows for the brain-wide assessment of neuronal activity and approximated connectivity throughout the various stages of PTSD development. Importantly, the DMN and SN have also been identified in the rodent brain[469,470], as well as a lateral cortical network (LCN), which' activity is anti-correlated with the DMN[469,471,472], and thereby resembles the human ECN. This allows for more invasive research on these networks, which should add translational value to current insights based on neuroimaging studies in PTSD patients.

In this study, we combined fluorescent labeling of active neurons and immunolabeling-enabled three-dimensional imaging of solvent-cleared organs (iDISCO+)[126] with a preclinical mouse model for PTSD to assess brain-wide neuronal activity at cellular resolution peri-, as well as during rest pre- and post-trauma exposure. Using this powerful approach, we aimed to unbiasedly identify brain regions that

display differential activity and investigate network connectivity in mice identified as either susceptible or resilient to the long-term behavioral consequences of trauma exposure. We did so by a) making voxel-wise comparisons of activity heatmaps, b) comparing labelled active cell counts in 92 anatomically defined brain regions of the mouse brain, and c) exploring cross-animal activity correlations between brain regions that are part of the DMN, SN and LCN in resilient and susceptible groups as a proxy for network functional connectivity[473]. The findings contribute to a better understanding of the brain-wide spatial and temporal profile by which aberrant neuronal activity and network connectivity encode stress resilience or susceptibility and pave the way towards future mechanistic investigations of the neurobiological mechanisms underlying these deviations.

2. MATERIALS & METHODS

2.1 Animals.

This study builds on a previous study that assessed amygdalar neuronal activity in animals susceptible to PTSD-like symptomatology[474]. While the current study has a broader aim and employs different techniques, the biological samples that have been analyzed were obtained from the same animals as were used in that study targeting the detailed assessment of neuronal activity in amygdalar subregions. The animal cohorts were part of three separate experiments: cohort 1 (n = 48) to assess brain-wide neuronal activity under resting (home cage) conditions pre-trauma, cohort 2 (n = 44) to assess neuronal activity during (peri) trauma exposure, and cohort 3 (n = 48) to assess neuronal activity under resting (home cage) conditions post-trauma. Heterozygote ArcCreER^{T2}xROSA offspring, referred to as ArcTRAP mice, were generated from crossing two founder mouse lines: ArcCreER^{T2} (B6.129(Cg)-*Arc*$^{tm1.1(cre/ERT2)Luo}$/J) and conditional tdTomato (B6.Cg-*Gt(ROSA)26Sor*$^{tm9(CAG-tdTomato)Hze}$/J, 007909). These were purchased from The Jackson Laboratory and bred as described before[102]. The ArcTRAP genetic construct allows *Arc*-expressing (i.e., active) neurons to be labeled by the fluorescent protein tdTomato in a 48-hour time window after injection with the compound tamoxifen. Only male mice were used for this study, as this PTSD model[26,362] has only been validated in males. Mice were group housed (3-4 mice per cage) in individually ventilated cages on a reverse 12 h light/dark cycle (09:00 - 21:00 h) at the Central Animal Facility of the Radboud University Nijmegen, The Netherlands, according to institutional guidelines. Food and water were provided *ad libitum*. Unless otherwise stated, behavioral testing was performed during the animal's active phase (i.e., the dark) between 13.00 - 18.00 h. The experimental protocols were in line with international guidelines, the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council 2003), the principles of laboratory animal care, as well as the Dutch law concerning animal welfare and approved by the Central Committee for Animal Experiments, Den Haag, The Netherlands.

2.2. General procedure.

All mice were exposed to a PTSD paradigm (Figure 1A) as described before[26,362,474]. To induce a PTSD-like phenotype, mice were exposed to a traumatic event (severe, unpredictable foot shocks) followed by a less severe trigger event (mild, predictable foot shocks) the next day. This trigger event is necessary for causing the long-term behavioral phenotype that is observed in this mouse model[26]. The premise that this relatively mild event can trigger PTSD-like symptomatology after earlier exposure to a trauma is clinically relevant, as it is comparable to how exposure to stressors and previous traumas predispose individuals to showing abnormal stress responses to later experiences[475]. The trauma can therefore be seen as 'opening the window' for PTSD-like symptom development, with the trigger as the second hit that eventually leads to the development of PTSD-like symptoms[476]. this model has been used in the light of studying the phenomenon of stress-enhanced fear learning, in which initial stress exposure enhances memory for subsequent mild, fear-learning experience[28]. After the PTSD induction and a week of recovery, mice were subjected to a set of behavioral tests over the course of two weeks to assess PTSD-like symptomatology. One week after the final behavioral test, mice were re-exposed to a trauma-related context for 10 minutes and sacrificed by perfusion-fixation 90 minutes later (data not included here).

2.3. Tamoxifen.

All mice were injected with tamoxifen to induce fluorescent labeling of all *Arc*-expressing neurons at different time points during the protocol. Mice in cohort 1 were injected with tamoxifen on day -3 - four days before the trauma session - to label pre-trauma active neurons under home cage conditions. Mice in cohort 2 were injected on the morning of day 1 -seven hours before the trauma session- to induce peri-trauma active neuronal labeling. Mice in cohort 3 were injected on day 19 - eighteen days after the trauma and four days before sacrifice - to label post-trauma active neurons under home cage conditions. Tamoxifen was dissolved in a 10% ethanol / corn oil solution at a concentration of 10 mg/mL by overnight sonication and stored at -20°C until further use. Solutions were heated to body temperature and intraperitoneally injected at a dosage of 150 mg/kg to induce activity-dependent neuronal labeling.

2.4. PTSD protocol.

Mice were individually placed in *Context A* boxes, in which they received 14 1 second 1.0 mA shocks (the 'trauma') over 85 minutes in variable intervals. For this, mice were first moved to the dark experimental room in groups of two to three animals in dark carton boxes before being placed in the fear-conditioning boxes, which were connected to a shock generator (Campden Instruments). *Context A* consisted of a black, triangular shaped Plexiglas box with a steel grid and metal tray. The boxes were sprayed with 1% acetic acid, not illuminated and 70 dB background noise was presented.

On the second day, mice were individually placed in *Context B* boxes, in which they received 5 1 second shocks of 0.7 mA over a period of five minutes (the 'trigger'), presented over fixed intervals. For this trigger session, mice were moved to the 70 lux illuminated experimental room in see-through cages in groups of two to three animals. The *Context B* boxes contained curved white walls and a steel grid with a white tray underneath. The boxes were furthermore cleaned with 70% ethanol and during the session the house lights in the boxes were turned on. No background noise was presented.

Mice were allowed to recover for a week, after which their behavioral response to trauma was assessed by testing for PTSD-like behavior: impaired risk assessment (in the dark-light transfer test), increased anxiety (by marble burying), hypervigilance (by acoustic startle), impaired sensorimotor gaiting (by pre-pulse inhibition), and disturbed circadian rhythm (by locomotor activity during the light phase)[26].

2.5. Behavioral categorization.

In order to categorize mice as either susceptible or resilient, one compound measure was generated based on the five behavioral outcome scores. Mouse behavior on each of the tests was sorted, and the 20% of mice that had the lowest values were attributed 3 points for percentage risk assessment, 3 points for latency to peak startle amplitude, and 2 points for percentage PPI. Similarly, the 20% of mice showing the highest values were attributed 1 point for light locomotor activity and marble burying[362]. Points for each test were determined by factor analysis in which tests were clustered in three separate groups: (1) latency to peak startle amplitude and percentage risk assessment, (2) percentage PPI, and (3) marble burying and total light activity[26]. Ties in the marble burying test were resolved by also assessing the

number of marbles buried after 15 minutes. The points per animal were tallied to generate an overall PTSD-like symptom score. Mice that had a total of four or more points (necessitating extreme behavior in multiple tests) were coined susceptible. Only mice that had zero points (indicating no abnormal behavior within any of the tests) were coined resilient. Notably, this approach allows for differential symptom profiles across susceptible mice.

2.6. Re-exposure and sacrifice.

On the final day of the experiment, day 23, mice were re-exposed to a trauma-related context for 10 minutes to induce fear memory recall (data not included in this manuscript). No shocks were administered during this context re-exposure session. Mice were sacrificed 90 min post re-exposure under anesthesia (5% isoflurane inhalation followed by intraperitoneal injection of 200 µL pentobarbital) by perfusion with phosphate buffered saline (PBS) followed by 4% paraformaldehyde solution (PFA). The brains were surgically removed and post-fixed for 24 hours in 4% PFA, after which they were transferred to 0.1 M PBS with 0.01% sodium azide and stored at 4°C.

2.7. Whole-brain immunostaining and clearing.

Left hemispheres of susceptible and resilient animals of each cohort were processed following the iDISCO+ protocol for adult brains[126] (Figure 1B). While most steps were followed in accordance with the existing protocol, two notable exceptions were made. First of all, no heparin was added to the PTwH buffer, as initial testing did not show any qualitative differences in clearing or staining upon addition or omission of this chemical. Furthermore, the samples were not incubated in 66% DCM / 33% methanol after the first dehydration series, as this was deemed unnecessary for proper delipidation of the brain, shortening the protocol by one day. In summary, the hemispheres were dehydrated using a methanol gradient, bleached in 5% H_2O_2 in methanol at 4°C overnight and subsequently rehydrated. As endogenous fluorescence was bleached during these steps, the hemispheres had to be relabeled for tdTomato. Additionally, cFos-positive cells were labelled to assess neuronal activity during trauma-related context re-exposure (data not reported here). To do so, the hemispheres were permeabilized for

5 days at RT, blocked for 4 days at 37°C and then incubated with primary antibodies (rabbit anti-RFP, 1:750, 600-401-379, Rockland; guinea pig anti-cFos, 1:2.500, 226004, Synaptic Systems) for 6 days at 37°C. Subsequently, brains were washed 5 × 1 h + 1 × overnight at RT, and incubated for 7 days with secondary antibodies (goat anti-rabbit Alexa-555, 1:200, A27039, Thermo Fisher; donkey anti-guinea pig, Alexa-647, 1:400, 706-605-148, Jackson ImmunoResearch) at 37 °C. Following 5 × 1 h + 1 × overnight washing at RT, samples were dehydrated in a methanol gradient and then incubated 2 x 1 h in 100% methanol, followed by 3 h in 66% DCM / 33% methanol and 2 × 15 min 100% DCM. Finally, the hemispheres were cleared in 100% dibenzyl ether (DBE, Sigma) in airtight glass vials. Brains were typically transparent within 2 h, and completely cleared overnight.

2.8. Whole-brain imaging.

The cleared hemispheres were imaged on a LaVision Ultramicroscope II light-sheet microscope, equipped with a NTK Photonics white-light laser and filter sets for 488 nm and 568 nm, imaged through a long-working distance objective (LaVision) at 1.1 × magnification (effective 2.2x, NA 0.1), and recorded with an Andor Neo 5.5 cooled sCMOS camera. Imaging was performed at 647 nm for capturing the cFos signal and at 555 nm to record the tdTomato signal. The emission light consisted of a triple light-sheet from the dorsal side of the brain at 0.54 NA, scanning at 2.95/2.95/3 µm x/y/z resolution (3 µm z-steps) with the "horizontal focus" method and 17-18 horizontal focus steps. The sample was imaged submerged in DBE in sagittal configuration, and the entire cerebrum fit inside a single field of view (x/y), with a typical brain producing ~ 1600 z-planes of 3 µm each.

2.9. Image preprocessing.

The resulting image stacks were downsampled with a customized version of ClearMap 1[21]. Subsequently, each downsampled image stack was manually aligned with a template brain from the Allen Brain Atlas using the Bigwarp tool in FIJI, a landmark-based tool for deformable image alignment, by matching ca. 200 landmarks between each sample brain and the template brain. The experimenter was blinded to the experimental group. The tdTomato signal yielded sufficient spatial information for

aligning the brains to the atlas, obviating the need for also capturing autofluorescence signal during imaging. See Figure S1 for a high-resolution image of the tdTomato signal in one of the sagittal sections from a representative image stack. A warped version of the atlas was exported for each hemisphere, and overlain with the downsampled image stack of that particular brain for visual inspection of the quality of the alignment. Cell segmentation was performed in Arivis Vision4D software (Arivis GmbH, https://www.arivis.com) using the "Machine Learning Segmenter" plugin. For the purpose of this paper, only the tdTomato$^+$ cells were considered. Cell coordinates and landmark coordinates were re-imported to ClearMap for mapping of the cells to the atlas and calculating cell counts per brain region. To guarantee that potential differences in cell counts would not be caused by inter-animal variation in signal quality, a brain mask was constructed that contained only areas of the brain covered by all image volumes and analysis was only performed therein (Figure S2).

2.10. Data analyses and statistics.

Preprocessing of the image stacks in ClearMap yielded two main outputs per animal: a heatmap of tdTomato$^+$ cell density across the brain; and a dataset with raw tdTomato$^+$ cell counts, allocated to 1241 brain regions as defined by the Allen Brain Atlas. Total cell counts across animals and across cohorts differed (effect of cohort: $F(2,44) = 12.377$, $p < .001$), due to slight variations in staining, clearing and imaging quality (cohorts were analyzed in separate batches). Tukey's HSD test for multiple comparisons found that total cell counts in the pre- and peri-trauma cohorts differed from those in the post-trauma cohort (pre vs. post: $p < .001$, 90% CI = [0.60, 1.77], peri vs. post: $p < .001$, 90% CI = [0.68, 1.83]), but not from each other (pre vs. peri: $p = .96$, 95% CI = [-0.60, 0.46]). However, average total cell counts across groups within each cohort were similar (effect of group: $F(1,44) = .016$, $p = .899$, cohort x group interaction: $F(2,44) = .509$, $p = .605$, pre-trauma: $\bar{x}_{resilient}$ = 1,902,730, $SD_{resilient}$ = 343,825, $\bar{x}_{susceptible}$ = 2,152,472, $SD_{susceptible}$ = 297,040, peri-trauma: $\bar{x}_{resilient}$ = 2,186,506, $SD_{resilient}$ = 499,202, $\bar{x}_{susceptible}$ = 1,933,022, $SD_{susceptible}$ = 744,623, post-trauma: $\bar{x}_{resilient}$ = 775,338, $SD_{resilient}$ = 273,459, $\bar{x}_{susceptible}$ = 863,989, $SD_{susceptible}$ = 341,145), with susceptible animals having on average 113.1%, 88.4% and 111.4% of the cell count of the resilient animals in the three cohorts respectively (Figure S3).

Unfortunately, not all brain samples were cleared and/or stained successfully, meaning that some animals had to be excluded from further analysis. This exclusion encompassed 8 animals in the pre-trauma cohort (2 resilient, 6 susceptible) and 8 animals in the post-trauma cohort (5 resilient, 3 susceptible).

The heatmaps from all remaining animals per group were resampled to 100 μm isotropic resolution, all signals were corrected for the total signal strength in the sample (i.e., total cell count, to account for differences in cell detection caused by variance in clearing quality), after which spatial smoothing was applied with a 200 μm full-width half-maximum kernel to enhance signal-to-noise ratio and to account for small mis-registrations. Heatmaps were loaded into R ('oro.nifti' package) and the effect size between susceptible and resilient animals was estimated using Hedge's g ('effectsize' package) for every voxel. This is motivated by a) the mass univariate nature of the tests which are prone to false positives with p-value based inferences, and b) Hedge's g being a better indicator over other standardized effect size indicators for group with $n < 20$.

Cell counts for each segregated region were corrected for the total cell count within the sample, and clustered into 92 larger anatomical regions (Table S4), to promote spatial accuracy and functional relevance. Then, Hedge's g values were calculated per brain region to contrast susceptible and resilient animals. Effects were considered of relevance if the 90% confidence interval did not contain 0 itself.

Lastly, the DMN (7 regions), SN (13 regions), or LCN (11 regions) were defined (Table S5) based on previous viral tracer studies targeting these networks[477-479], injecting virus in their core region (i.e., the anterior cingulate area, anterior insular area, and primary motor area, respectively) and assessing labels in projection regions. Brain regions identified as (i.e., labeled by) part of multiple brain networks (i.e., the claustrum, orbital area, prelimbic area, agranular insular area, frontal pole of the cerebral cortex, mediodorsal nucleus of the thalamus, substantia nigra compact part, central medial nucleus of the thalamus, caudoputamen, ventral medial nucleus of the thalamus, paracentral nucleus, secondary motor area, and gustatory areas) were assigned to the network to which they most strongly contributed (i.e., correlated with). Bivariate Pearson correlation coefficients were computed between the cell counts for

these regions in susceptible and resilient animals separately and plotted as correlation heatmaps (Figure S9). Hedge's g values were estimated using the Pearson's r values within or between networks as the statistical unit. Correlations between entire networks were determined by averaging all Pearson's r values between the regions comprising those networks.

2.11. Data and code availability.

The pre-processed data, consisting of cell counts per ROIs and 3-dimensional cell heat maps, as well as the code to reproduce the analyses, is available freely here: https://gitlab.socsci.ru.nl/preclinical-neuroimaging/stat_bart.

3. RESULTS

3.1. Interindividual differences in traumatic stress susceptibility

To assess potential differences in brain-wide neuronal activity associated with susceptibility to developing PTSD-like symptoms following trauma exposure, three cohorts of 44-48 mice were exposed to the PTSD induction protocol. Susceptible and resilient mice differed in their display of PTSD-like symptoms , as evidenced by significant differences in their PTSD-like symptom scores[474] (Figure S6,S7).

3.2. Neuronal activity differences distinguish susceptible from resilient individuals

Neuronal activity pre-, peri- and post-trauma was assessed by calculating brain-wide tdTomato$^+$ cell counts in the three different mouse cohorts.

Neuronal activity counts of 92 anatomically defined regions revealed that pre-trauma ($n_{resilient}$ = 10, $n_{susceptible}$ = 6, Figure 2), susceptible animals showed higher activity in specifically the lateral orbital cortex (g = 1.26, 90% CI = [0.35, 2.13]), ventral striatum (g = 0.88 [0.02, 1.72]) and medial pallidum (g = 1.11 [0.22, 1.96]), as well as lower activity in the ventral anterior cingulate cortex (g = -0.86 [-1.70, 0.00]), ventral retrosplenial area (g = -0.96 [-1.80, -0.09]), dorsal hippocampal CA2 (g = -0.87 [-1.70, 0.00]) and ventral group of the dorsal thalamus (g = -1.17 [-2.04, -0.27]), compared to resilient animals. Peri-trauma ($n_{resilient}$ = 9, $n_{susceptible}$ = 8, Figure 3), susceptible animals showed more activity in several regions in the somatosensory cortex (lower limb area: g = 1.20 [0.32, 2.04], trunk area: g = 0.93 [0.08, 1.74]), auditory cortex (dorsal auditory area: g = 1.12 [0.25, 1.95], ventral auditory area: g = 0.87 [0.03, 1.68]), visual cortex (anterolateral visual area: g = 1.45 [0.53, 2.33], lateral visual area: g = 1.06 [0.20, 1.88], primary visual area: g = 1.28 [0.39, 2.13], posterolateral visual area: g = 1.17 [0.30, 2.00], posteromedial visual area: g = 0.86 [0.03, 1.67]), retrosplenial cortex (lateral agranular retrosplenial area: g = 1.55 [0.61, 2.44], and dorsal retrosplenial area: g = 1.71 [0.74, 2.62]), subiculum (postsubiculum: g = 0.91 [0.07, 1.72], presubiculum: g = 1.41 [0.50, 2.28]) and temporal association areas (g = 0.85 [0.01, 1.65]). Contrarily, the olfactory region (anterior olfactory nucleus (g = -1.13 [-1.97, -0.26]), taenia tecta (g = -0.89 [-1.70, -0.05]), dorsal peduncular area (g = -1.02 [-1.84, -0.16]),

ventral agranular insular area (g = -0.89 [-1.70, -0.05]) and dorsal striatum (g = -0.85 [-1.66, -0.02]) showed relatively lower activity in susceptible compared to resilient animals peri-trauma.

Post-trauma ($n_{resilient}$ = 7, $n_{susceptible}$ = 5, Figure 4), susceptible animals showed higher activity in the orbital cortex (lateral orbital area: g = 1.02 [0.04, 1.97], ventral orbital area: g = 1.09 [0.09, 2.03]), taenia tecta (g = 1.12 [0.12, 2.07]), dorsal retrosplenial area (g = 1.37 [0.32, 2.36]) and the parasubiculum (g = 1.31 [0.27, 2.29]) than their resilient counterparts. Results on all cohorts are summarized in Table S8.

3.3. Neuronal activity correlation differences reflect altered neural network connectivity

Next, we investigated potential differences between susceptible and resilient mice in terms of functional connectivity between and within the DMN, SN, and LCN by comparing cross-subject correlations in regional activity. This revealed increased correlations between the DMN and LCN regions in susceptible vs. resilient animals both pre- (g = 0.50 [0.18, 0.82]) and peri-trauma exposure (g = 0.65 [0.32, 0.97]). Intriguingly, this difference was caused by overall negative correlations in the resilient animals (pre: r = -0.55, p = .10, peri: r = -0.62, p = .08), while the susceptible animals on average showed a positive correlation between these networks (pre: r = 0.60, p = .21, peri: r = 0.76, p = .03, Figure S10). Peri-trauma, susceptible animals furthermore showed stronger correlations between the DMN and SN brain regions than their resilient counterparts (g = 0.28 [-0.01, 0.57]). This finding was also replicated in the post-trauma cohort (g = 0.43 [0.14, 0.73]). Post-trauma, stronger correlations were also observed between regions within the SN (g = 0.34 [0.02, 0.65]) in susceptible vs. resilient animals.

4. DISCUSSION

Here, we implemented a preclinical rodent model for PTSD where mice were behaviorally phenotyped on PTSD-like symptomatology after exposure to a traumatic event (severe, unpredictable foot shock) followed by a trigger (mild, predictable foot shock), and classified as either trauma-susceptible or -resilient. Brain-wide neuronal activity differences between these groups were compared under resting (i.e., home cage) conditions pre-trauma, peri-trauma, and under resting conditions post-trauma, by tagging neuronal activity in living mice using the ArcTRAP transgenic mouse line. Results implicated increased activity of the lateral orbitofrontal cortex, ventral striatum and medial pallidum, and reduced activity of the ventral anterior cingulate area, ventral retrosplenial area, dorsal CA2 and ventral group of the dorsal thalamus pre-trauma under home cage conditions in forecasting later PTSD-like symptomatology. During trauma exposure, increased activity of sensory and memory-related regions, including the retrosplenial cortex and subiculum, was observed in susceptible vs. resilient mice, as well as reduced activity of olfactory areas, the ventral agranular insula and dorsal striatum. The relative increase in activity of the retrosplenial cortex in susceptible mice remained present under home cage conditions after trauma, accompanied by increased orbitofrontal activation, similar to the pre-trauma condition. Furthermore, susceptible mice showed increased correlations between DMN and LCN activity pre- and peri-trauma, increased DMN-SN activity correlations peri- and post-trauma, and increased correlations within the SN post-trauma.

To elucidate the neurobiological underpinnings of PTSD pathophysiology, it is crucial to distinguish between adaptive vs. maladaptive trauma responses, as well as between pre-existing factors conferring mere risk for PTSD vs. neurobiological underpinnings of psychopathology that could be targeted in treatment. In this study, we employed a PTSD mouse model to investigate brain-wide neuronal activity coding differential susceptibility to trauma exposure in three different timepoints surrounding trauma exposure. To specifically study traumatic stress susceptibility, and to enhance translational value, we classified mice as either resilient or susceptible to the behavioral consequences of trauma exposure. This stratification was based on a compound behavioral outcome score comprising of multiple behavioral

PTSD-like symptoms, rather than a single behavioral feature. As such, this classification resembles the situation in patients[27], which can be diagnosed with PTSD based on 20 criteria across four distinct symptom categories, resulting in a highly heterogeneous patient population[6]. Here, we report on altered activity of several brain regions in susceptible vs. resilient animals either pre-, peri-, or post-trauma. Notably, several findings were replicated across cohorts, suggesting shared neural correlates even across behaviorally heterogeneous groups. In this discussion, we will not discuss every finding individually, but rather focus on the more robust findings and those that parallel human literature on PTSD.

Firstly, we observed increased activation of the ventrolateral orbital cortex in susceptible mice both prior to trauma exposure and post-trauma. The orbitofrontal cortex is involved in learning, processing sensory input related to reward, regulating emotions, and reversing of stimulus-reinforcement associations[480,481]. Interestingly, structural and functional alterations in the orbitofrontal cortex have been reported before in PTSD[482], most notably in relation to altered mood symptomatology[483]. Patient studies have reported on reductions in orbitofrontal cortex activity upon re-exposure to trauma-related and emotionally valent stimuli[52,450,484], and emotional memory tasks[481], whereas increased recruitment of the orbitofrontal cortex during unpredictable stress was found associated with increased risk for affective disorders[485]. Our findings propose resting ventrolateral orbital activation as a predisposing factor for PTSD. Early-life stress, which is a major risk factor for PTSD development later in life[486], has been shown to negatively impact orbitofrontal volumes[487]. Furthermore, increased resting activity of the region may reflect deficits in reward processing, which have long been posed as risk factors for substance abuse and mood disorders like PTSD[480]. This is particularly interesting, as we observed a similar effect on pre-trauma activity of the ventral striatum, an area that is also strongly linked to reward processing and whose dysfunction has been implicated in PTSD[119].

We also observed increased activity of the retrosplenial cortex post-trauma in susceptible mice. The retrosplenial cortex is known for its role in contextual memory, acting as a hub that integrates and coordinates the activity of distinct brain regions to mediate acquisition and time-independent retrieval of contextual memories[488]. Furthermore, it has been linked to self-reflection and is especially implicated

in the retrieval of episodic memory[489]. In line with this, stimulation of neural ensembles activated in the retrosplenial cortex with contextual learning has been found to be sufficient to induce contextual fear memory retrieval[378]. There is evidence showing increased activation of the retrosplenial cortex and precuneus in response to trauma-related stimuli in PTSD patients vs. trauma-exposed controls[490,491]. Interestingly, increased activity of the dorsal retrosplenial area in susceptible vs. resilient animals was also observed during trauma exposure itself. This is in line with prior work showing that increased retrosplenial activation in response to traumatic film imagery predicted later development of intrusion symptoms[492]. These findings collectively suggest that alterations in activity of the retrosplenial cortex may be acquired through the trauma and persist afterwards.

In addition to these post-trauma observations, we were able to perform unprecedented assessments of peri-trauma neuronal activity, a timepoint which is typically inaccessible in humans. Substantial alterations were found in the activity of the somatosensory, visual, and auditory areas, with susceptible animals having a notably higher activation of these regions. One hallmark feature of PTSD is that patients exhibit intrinsic sensory hyperactivity, and are prone to sensory overload following trauma[493]. These hallmarks may in part underlie the exaggerated response to traumatic stress[494], and could even relate to intrusion symptoms[495]. Yet, sensory hyperactivation could also be a direct consequence of an exaggerated stress response, which, through noradrenaline modulation, can potentiate early perception of visual cues[496,497], and possibly other sensory modalities. The current findings support the idea that aberrant sensory processing during trauma exposure, potentially due to an abnormally strong stress response, may relate to later development of PTSD-like symptoms.

During and after trauma, we also observed increased activity in different subregions of the subiculum in susceptible vs. resilient animals. The subiculum is part of the hippocampal formation and is the main hub for hippocampal afferents from the neocortex, specifically conferring spatiovisual information. As such, it plays an important role in different memory processes[498,499], such as rapid memory updating and retrieval-driven instinctive fear responses[500]. Roles for the subiculum, particularly the ventral subiculum, in the response to fear, stress and anxiety are however largely elusive. The subiculum exerts a dynamic

and inhibitory influence on the HPA axis, and thereby orchestrates the endocrine stress response[501]. A disruption of interneuronal regulation of the ventral subiculum is proposed to lead to an overdrive of the dopamine system, rendering the system in a constant hypervigilant state[501]. It is possible that a similar process drives hypervigilant behavior seen in drug abuse and stress-related disorders like PTSD[502], which fits the current observation of increased subiculum activation specifically in susceptible animals. While we observed increased peri- and post-trauma activation in all subiculum subregions in susceptible vs. resilient animals, the peri-trauma differences were greatest in the pre- and postsubiculum, while the parasubiculum was specifically more active after trauma. Although these subregions may have slightly different functions[503-505], they are often considered together - especially the pre- and postsubiculum[503] - , making it difficult to substantiate the implications for the current findings.

When correlating regional activation across animals, we observed increased inter-network correlations between the DMN and SN, as well as increased intra-network correlations within the SN post-trauma in susceptible vs. resilient animals. Prior resting-state functional neuroimaging studies already reported on a hyperactive and strongly intra-connected SN[120,121] in PTSD patients, as well as increased inter-network connectivity between the SN and DMN networks[121], matching our work. It is hypothesized that these disruptions in network balance may lead to exaggerated attention to external stimuli, thereby contributing to the hyperarousal and hypervigilance symptomatology of PTSD[121]. Interestingly, the increase in DMN-SN correlations was also observed peri-trauma, suggesting that it might be an acquired alteration that surfaces during trauma, and persists after the trauma.

We also observed increased correlations between regions of the DMN and LCN in susceptible vs. resilient animals, both pre- and peri-trauma exposure. The DMN and ECN are typically anti-correlated[506,507], as are the DMN and LCN in animals[471,479]. Interestingly, such negative correlations were only observed in the resilient animals, while the susceptible animals on average showed a positive correlation between networks. It has been proposed that disrupted DMN-ECN coupling is associated with episodic memory deficits and could form the basis for intrusive trauma memory recollection[111]. While we did not find evidence of altered DMN-LCN coupling post-trauma, our study does suggest that

pre-trauma abnormalities in this circuitry may play a role during trauma exposure in modulating subsequent memory processing.

Our findings indicate that several neurobiological factors, including increased orbitofrontal activity and DMN-LCN correlation, may predict susceptibility to the development of PTSD-like symptoms. Yet, these are still early findings in a relatively new field of stress research, and they will need to be followed up by further mechanistic animal studies, as well as prospective longitudinal studies in humans, which - although challenging - are feasible with current technologies[451]. It is important to note that the pre- and post-trauma neuronal activity measurements were performed under home cage conditions. This was done to compare our findings to resting-state studies of PTSD patients that have been reported in literature, as well as to specifically study basal brain function. For future research, it will be interesting to instead expose the animals to a stress-related challenge, as previous animal work has indicated that pre-existing susceptibility becomes primarily evident in increased anxiety following exposure to a mild stressor, not at baseline[16,438,508]. This would make potential results - at least those acquired post-trauma - more comparable to most studies that are currently being performed in human PTSD patients, where patients and trauma-exposed controls are often exposed to trauma-related imagery or sounds[509,510].

Some limitations to the current work need to be noted. First, the current approach is not optimally fit for dissecting very small regions, like the basolateral amygdala or nucleus reuniens. This is mainly due to a lack of proper tools to align each downsampled image stack to a template brain. This registration was now performed manually, based on ca. 200 anatomical landmarks throughout the brain. However, it is possible that small regions - especially those not close to any clear landmarks - get slightly misaligned during image warping. Hence, the current study design is not (yet) a replacement for dedicated studies into regions of interest, but is rather a valuable addition to them. Furthermore, with regards to the activation correlation data, it should be noted that we inferred network connectivity from calculating cross-subject activity correlations[511-513], rather than construing connectivity from correlating changes in signal strength across time within individual animals. This is unfortunately a given shortcoming when

being limited to one measurement in time, unlike with fMRI. And finally, while the human ECN and rodent LCN networks seem similar in terms of their correlations to other networks, it is not entirely clear to what extent they can be functionally compared.

Regardless, the current study shows that state-of-the-art fluorescent labeling and 3D clearing and staining techniques can be used to do fundamental research on brain activity at very specific timepoints and in response to various challenges, including traumatic stress. This approach has yielded an unprecedented assessment of pre- and especially peri-trauma neuronal activity, typically inaccessible in humans. Not only did this brain-wide approach lead to the identification of new brain region targets, but it also allowed us to replicate observations from human PTSD patients in an animal model. Replicating the activity and network observations that have been reported in human studies, like the triple network theory, will not only increase the translational value of rodent models of PTSD, but facilitate future mechanistic studies aiming at their neurobiological underpinnings.

FIGURE AND TABLES

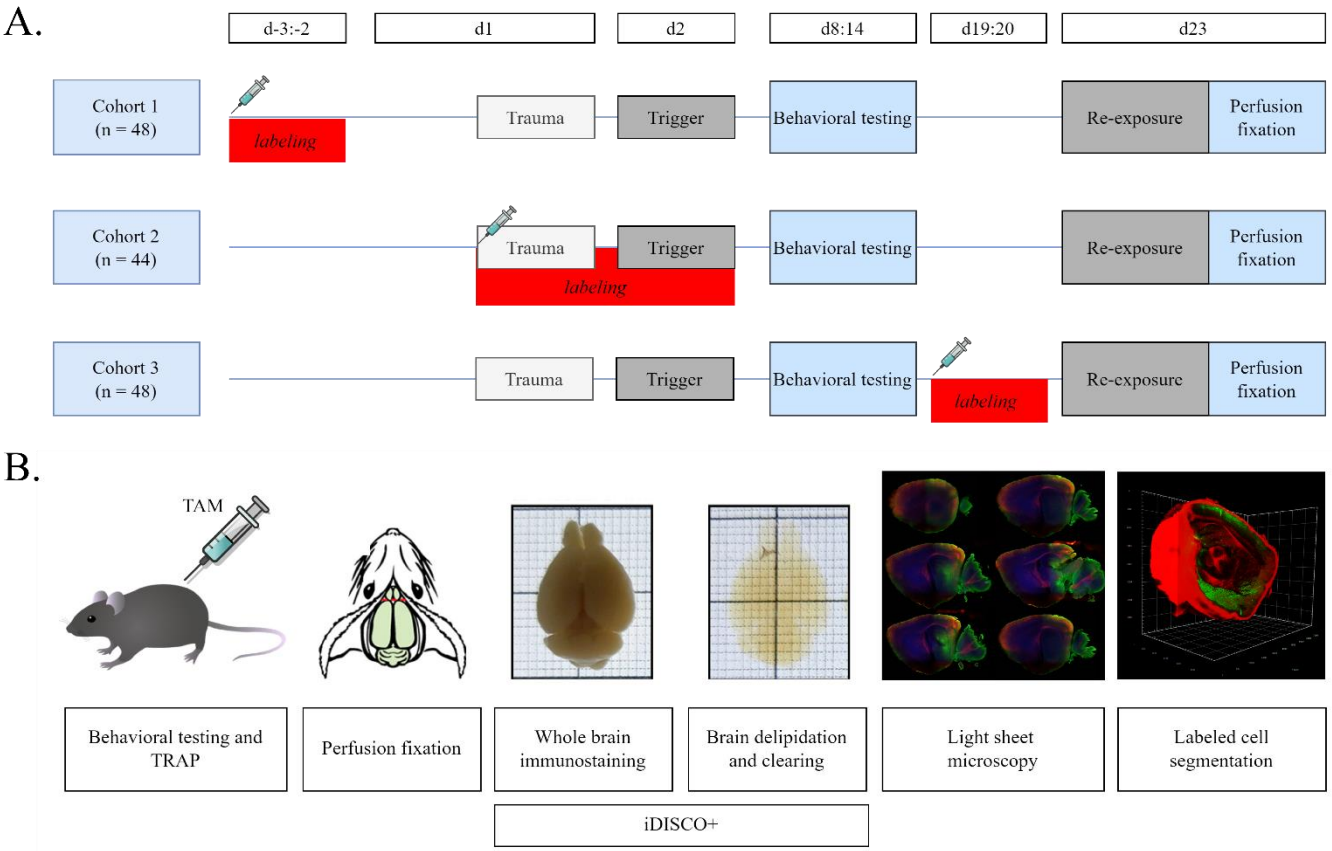

Figure 1. Timeline of the behavioral protocols as implemented for the three cohorts. TAM, tamoxifen injection (**A**). Overview of the labeling, staining and clearing procedure (**B**). TAM, tamoxifen injection

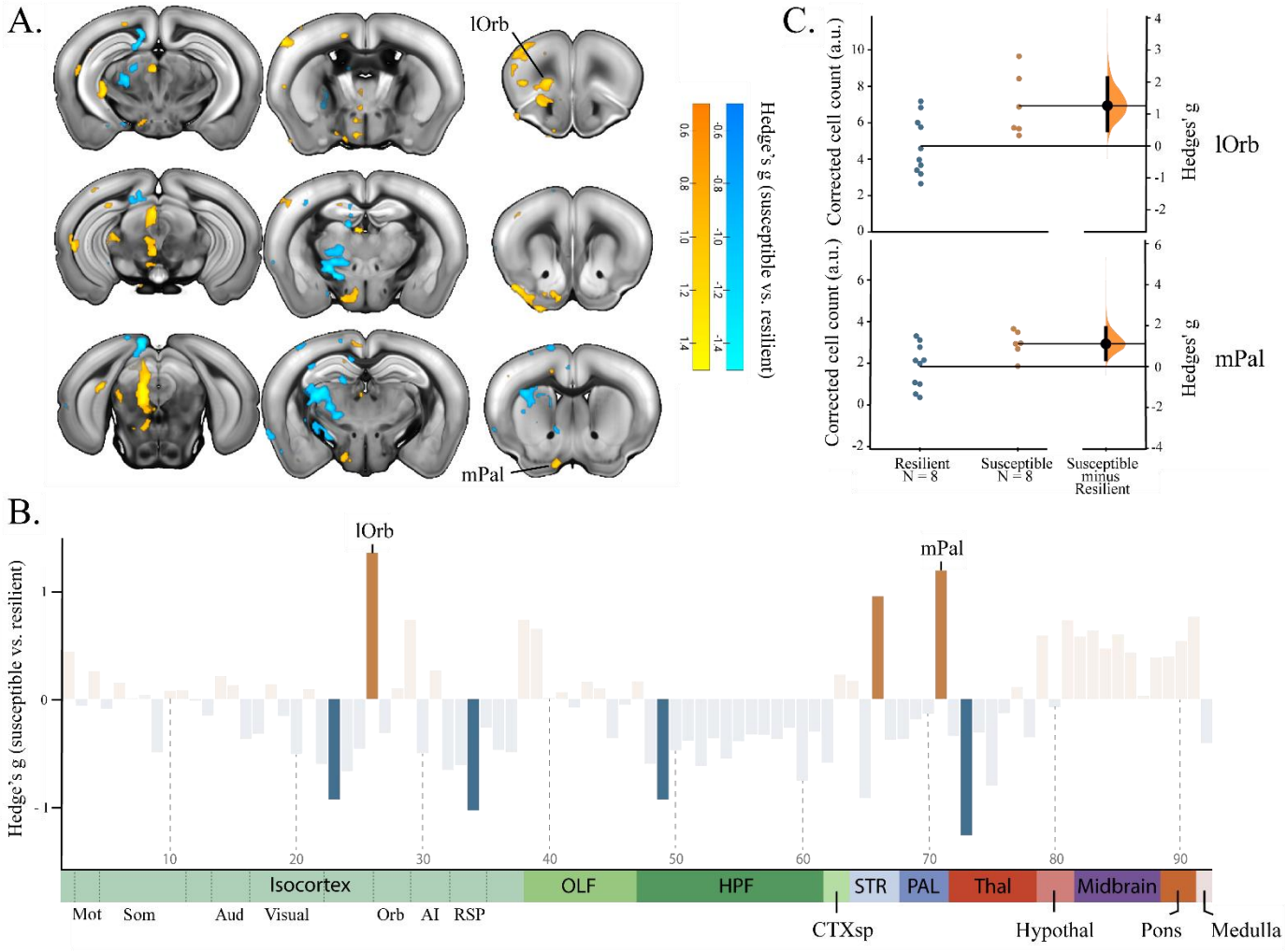

Figure 2. Pre-trauma neuronal activity differences in susceptible vs. resilient animals. Voxel-wise comparisons (**A**) show normalized Hedge's g values as overlays with color-coding over the Allen Institute for Brain Science template. Images are thresholded such that only voxels are shown with an effect size where the 90% confidence interval does not contain 0. In a different approach, Hedge's g values of tdTomato$^+$ labeled (i.e., active) cell counts were calculated for 92 brain region clusters (**B**). Opaque orange and blue bars indicate all regions with an effect size where the 90% confidence interval does not cross 0. Pre-trauma, susceptible animals showed an increase in tdTomato$^+$ neuronal density in the lateral orbital area and medial pallidum (**C**). The left part of each graph shows the corrected cell count in the resilient and susceptible animals. The right part of each graph shows the Hedge's g value (i.e., effect size) with a 90% confidence interval.

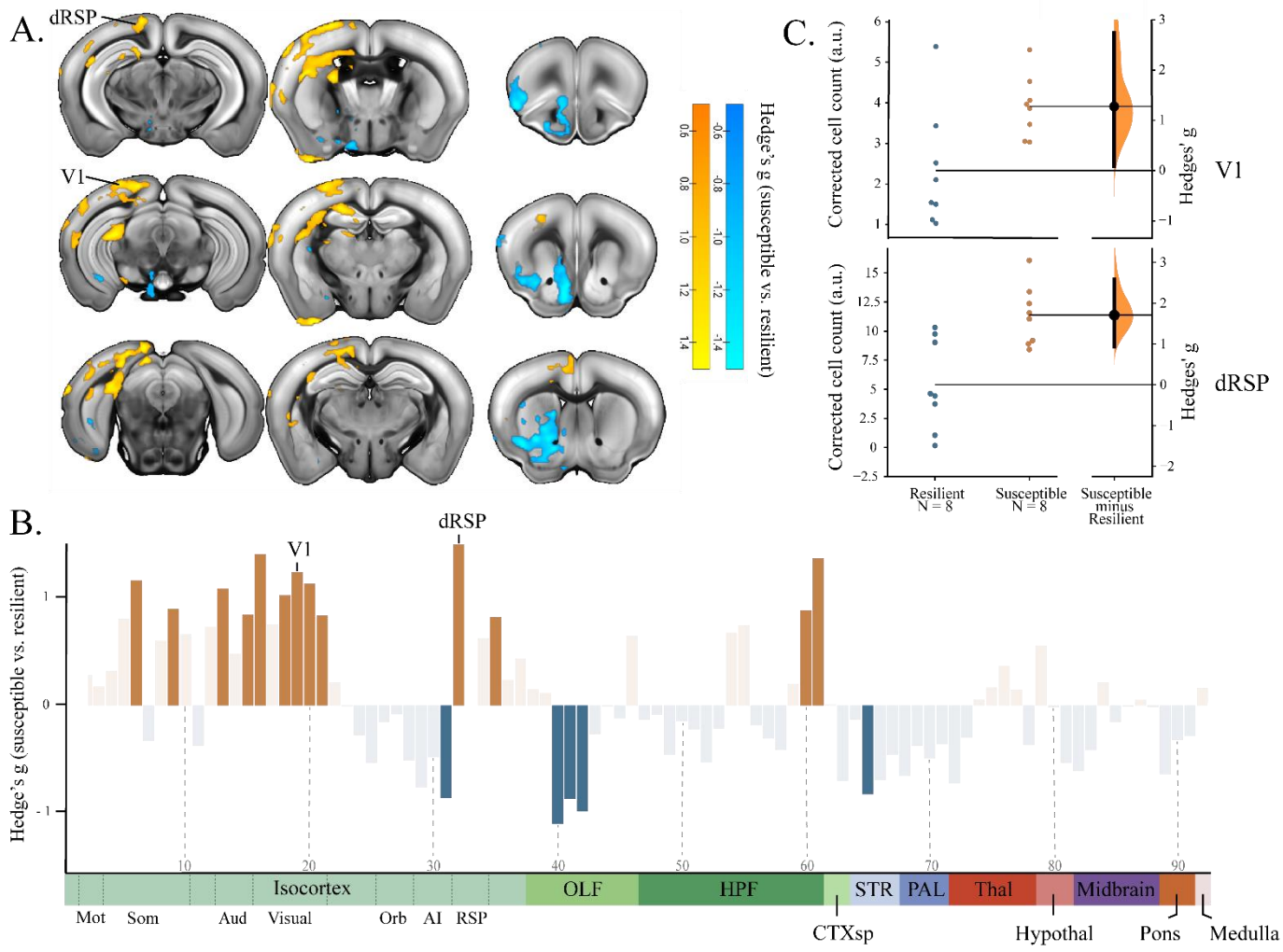

Figure 3. Peri-trauma neuronal activity differences in susceptible vs. resilient animals. Voxel-wise comparisons (**A**) show normalized Hedge's g values as overlays with color-coding over the Allen Institute for Brain Science template. Images are thresholded such that only voxels are shown with an effect size where the 90% confidence interval does not contain 0. In a different approach, Hedge's g values of tdTomato$^+$ labeled (i.e., active) cell counts were calculated for 92 brain region clusters (**B**). Opaque orange and blue bars indicate all regions with an effect size where the 90% confidence interval does not cross 0. Peri-trauma, susceptible animals showed an increase in tdTomato$^+$ neuronal density in the primary visual area and dorsal retrosplenial cortex (**C**). The left part of each graph shows the corrected cell count in the resilient and susceptible animals. The right part of each graph shows the Hedge's g value (i.e., effect size) with a 90% confidence interval.

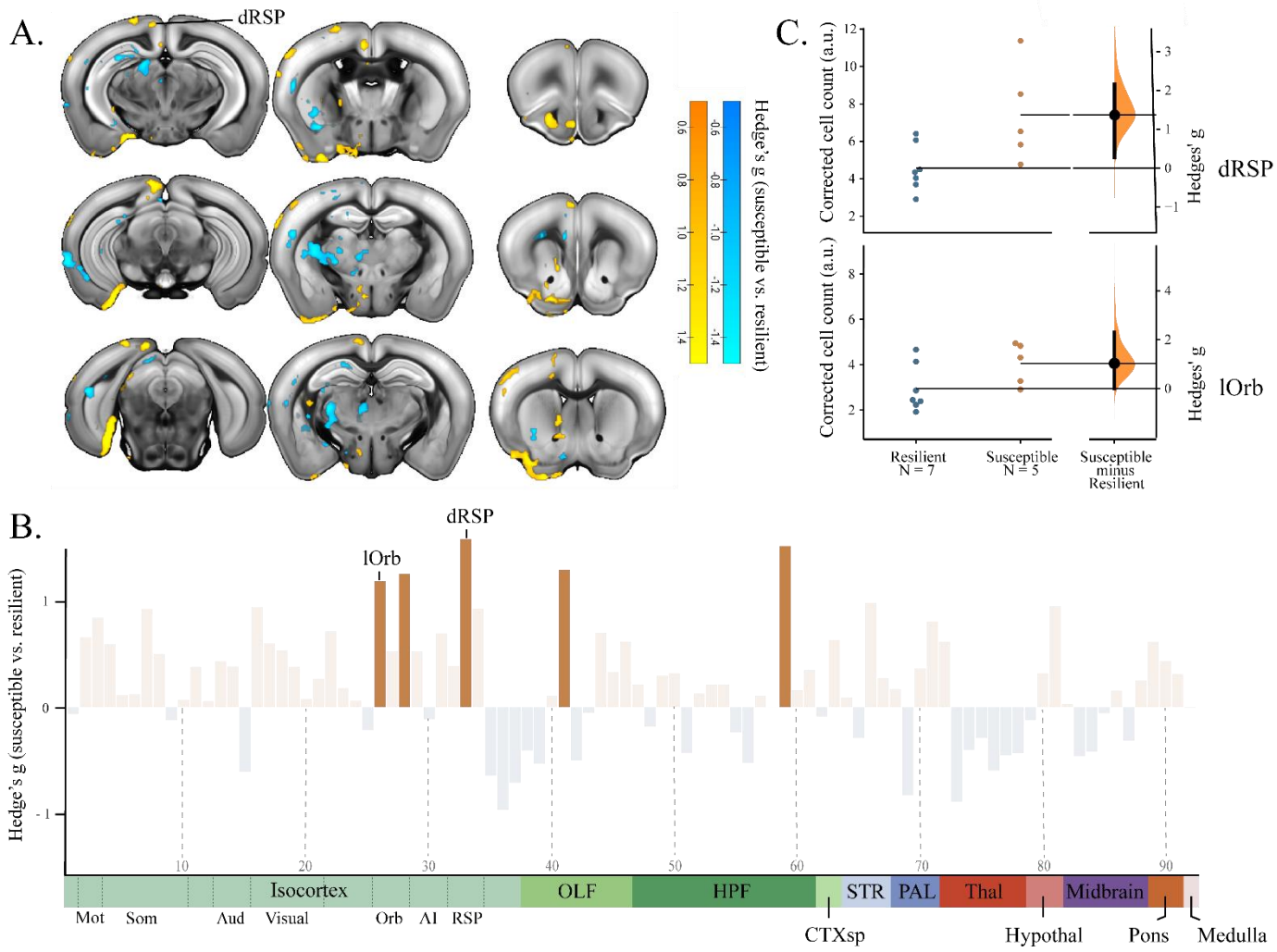

Figure 4. Post-trauma neuronal activity differences in susceptible vs. resilient animals. Voxel-wise comparisons (**A**) show normalized Hedge's g values as overlays with color-coding over the Allen Institute for Brain Science template. Images are thresholded such that only voxels are shown with an effect size where the 90% confidence interval does not contain 0. In a different approach, Hedge's g values of tdTomato$^+$ labeled (i.e., active) cell counts were calculated for 92 brain region clusters (**B**). Opaque orange and blue bars indicate all regions with an effect size where the 90% confidence interval does not cross 0. Post-trauma, susceptible animals showed an increase in tdTomato$^+$ neuronal density in the dorsal retrosplenial cortex and lateral orbital area (**C**). The left part of each graph shows the corrected cell count in the resilient and susceptible animals. The right part of each graph shows the Hedge's g value (i.e., effect size) with a 90% confidence interval.

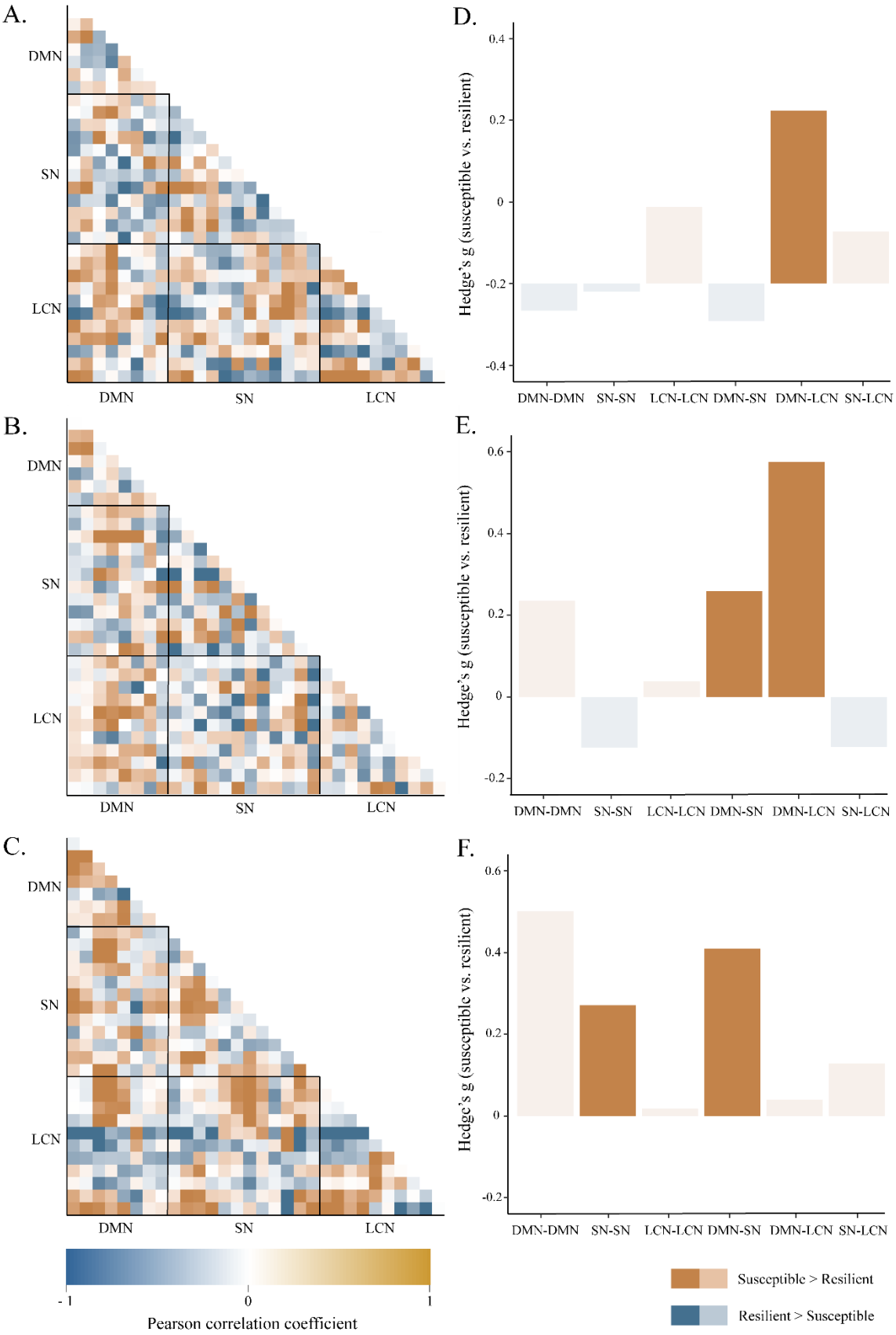
A.
DMN
SN
LCN
DMN
SN
LCN
B.
DMN
SN
LCN
DMN
SN
LCN
C.
DMN
SN
LCN
DMN
SN
LCN
- 1
0
1
Pearson correlation coefficient
D.
Hedge's g (susceptible vs. resilient)
0.4
0.2
0
-0.2
-0.4
DMN-DMN
SN-SN
LCN-LCN
DMN-SN
DMN-LCN
SN-LCN
E.
Hedge's g (susceptible vs. resilient)
0.6
0.4
0.2
0
-0.2
DMN-DMN
SN-SN
LCN-LCN
DMN-SN
DMN-LCN
SN-LCN
F.
Hedge's g (susceptible vs. resilient)
0.6
0.4
0.2
0
-0.2
DMN-DMN
SN-SN
LCN-LCN
DMN-SN
DMN-LCN
SN-LCN
Susceptible > Resilient
Resilient > Susceptible

Figure 6. Correlations of neuronal activity in susceptible vs. resilient animals, based on Pearson correlation coefficients between regions in the default mode network (7 regions), salience network (13 regions), and lateral cortical network (11 regions). Correlations showing susceptible vs. resilient differences are shown pre- (**A**), peri- (**B**), and post-trauma (**C**). Furthermore, Hedge's g values of intra- and inter-network correlation differences between susceptible and resilient animals are shown for each time point (**DEF**). Opaque orange and blue bars indicate all correlations where the 90% confidence interval does not cross 0.

SUPPLEMENTARY FIGURES AND TABLES

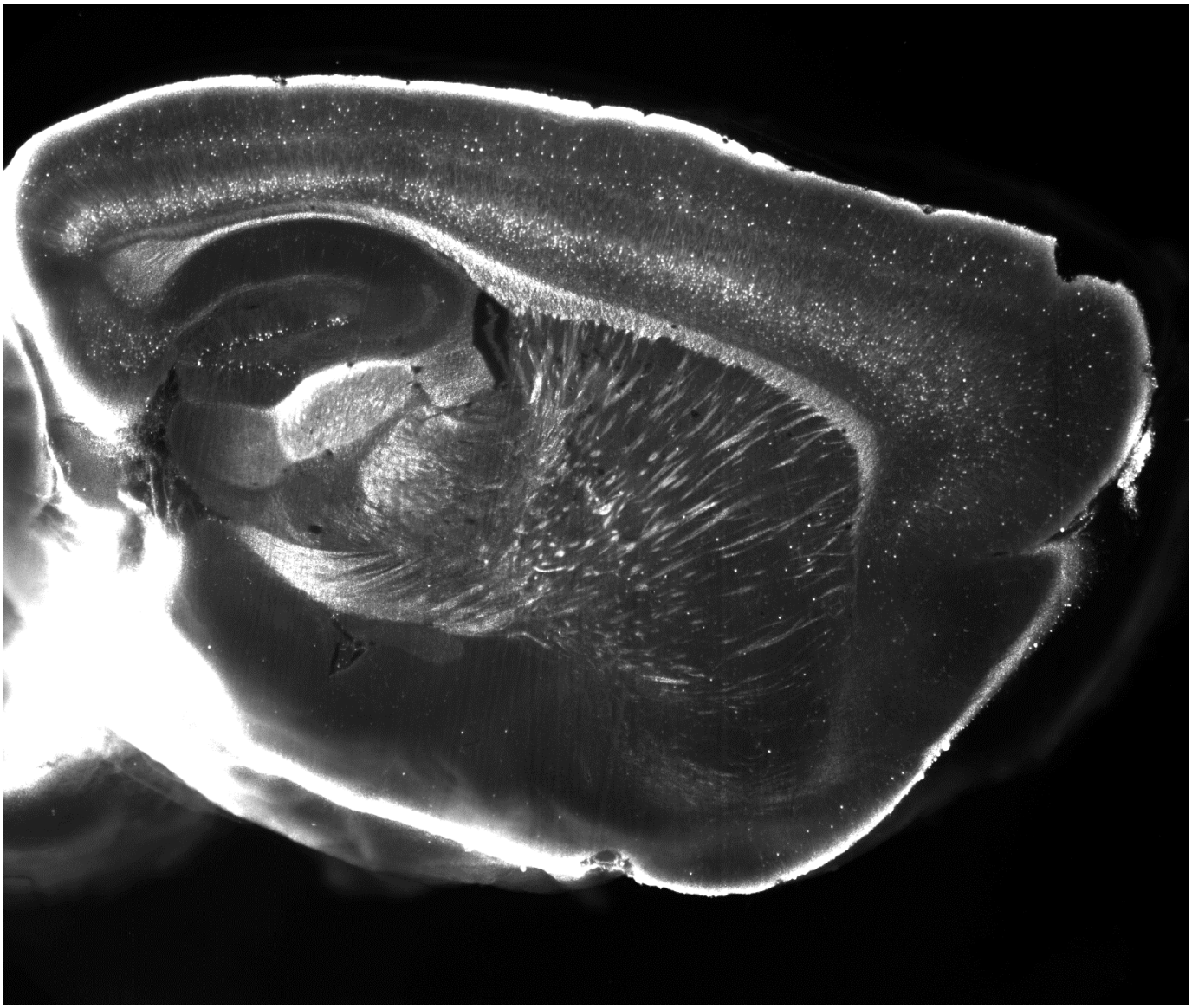

Figure S1. High-resolution image of tdTomato signal in a sagittal section from a representative 3D image stack.

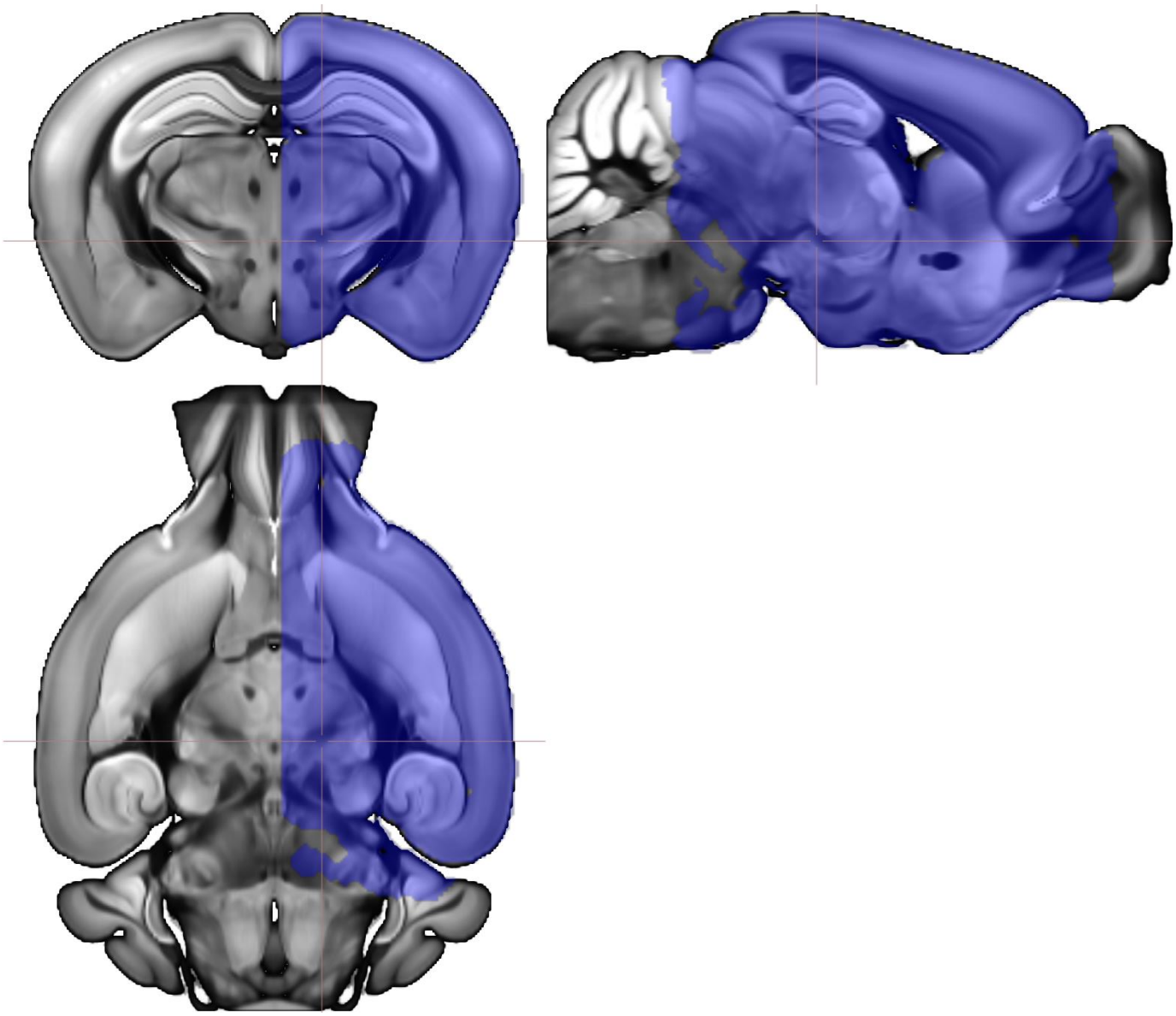

Figure S2. Mask of included brain regions.

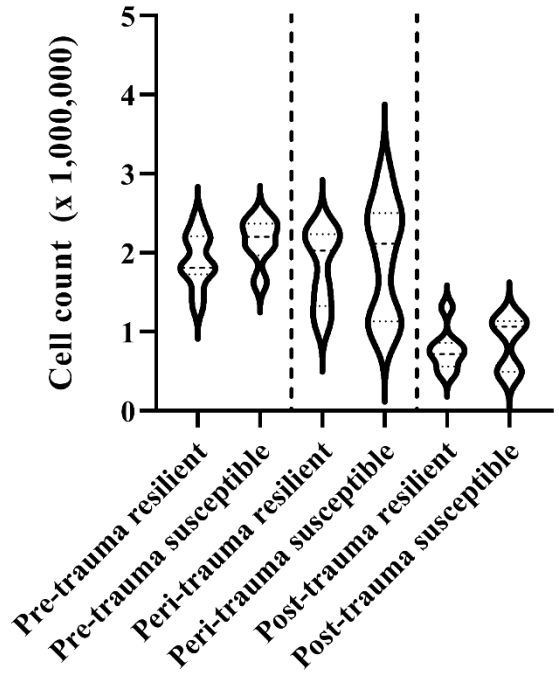

Figure S3. Total cell counts in the three cohorts.

Parent region	Subregion	Region #	Region
Isocortex		1	Frontal pole, cerebral cortex
	Motor cortex	2	Primary motor area
		3	Secondary motor area
	Somatosensory cortex	4	Primary somatosensory area, nose
		5	Primary somatosensory area, barrel field
		6	Primary somatosensory area, lower limb
		7	Primary somatosensory area, mouth
		8	Primary somatosensory area, upper limb
		9	Primary somatosensory area, trunk
		10	Supplemental somatosensory area
		11	Gustatory areas
		12	Visceral area
	Auditory cortex	13	Dorsal auditory area
		14	Primary auditory area
		15	Ventral auditory area
	Visual cortex	16	Anterolateral visual area
		17	Anteromedial visual area
		18	Lateral visual area
		19	Primary visual area
		20	Posterolateral visual area
		21	posteromedial visual area
		22	Anterior cingulate area, dorsal part
		23	Anterior cingulate area, ventral part
		24	Prelimbic area
		25	Infralimbic area
	Orbital cortex	26	Orbital area, lateral part
		27	Orbital area, medial part
		28	Orbital area, ventral part
	Agranular insula	29	Agranular insular area, dorsal part
		30	Agranular insular area, posterior part
		31	Agranular insular area, ventral part
	Retrosplenial cortex	32	Retrosplenial area, lateral agranular part
		33	Retrosplenial area, dorsal part
		34	Retrosplenial area, ventral part
		35	Temporal association areas
		36	Perirhinal area
		37	Ectorhinal area
Olfactory bulb		38	Main olfactory bulb
		39	Accessory olfactory bulb
		40	Anterior olfactory nucleus
		41	Taenia tecta
		42	Dorsal peduncular area
		43	Piriform area
		44	Nucleus of the lateral olfactory tract
		45	Cortical amygdalar area
		46	Piriform-amygdalar area
		47	Postpiriform transition area
Hippocampal formation		48	Dorsal CA1
		49	Dorsal CA2
		50	Dorsal CA3

	51	Ventral CA1
	52	Ventral CA2
	53	Ventral CA3
	54	Dorsal DG
	55	Ventral DG
	56	Lateral entorhinal cortex
	57	Medial entorhinal cortex
	58	Ventral entorhinal cortex
	59	Parasubiculum
	60	Postsubiculum
	61	Presubiculum
	62	Subiculum
Cortical subplate	63	Cortical subplate
	64	Amygdala
Striatum	65	Dorsal striatum
	66	Ventral striatum
	67	Lateral septum complex
	68	Striatum-like amygdalar nuclei
Pallidum	69	Dorsal pallidum
	70	Ventral pallidum
	71	Medial pallidum
	72	Caudal pallidum
Thalamus	73	Ventral group of the dorsal thalamus
	74	Geniculate group of the dorsal thalamus
	75	Lateral thalamus
	76	Anterior thalamus
	77	Medial thalamus
	78	Interlaminar thalamus
	79	Geniculate thalamus
Hypothalamus	80	Periventricular zone
	81	Periventricular region
	82	Medial hypothalamus
	83	Lateral hypothalamus
Midbrain	84	Midbrain, sensory
	85	Midbrain, motor
	86	Periaqueductal gray
	87	Pretectal area
	88	Midbrain, behavioral state
Pons	89	Pons, sensory
	90	Pons, motor
	91	Pons, behavioral state
Medulla	92	Medulla

Table S4. List of clustered regions.

Network	Region #	Region	Abbreviation
Default mode network	1	Central lateral nucleus of the thalamus	CL
	2	Anterior group of the dorsal thalamus	ATL
	3	Anterior cingulate area	ACA
	4	Infralimbic area	ILA
	5	Retrosplenial area	RSP
	6	Anteromedial visual area	VISam
	7	Pons, behavioral state related	P-sat
Salience network	8	Anterior amygdalar area	AAA
	9	Hypothalamic lateral zone	LZ
	10	Medial group of the dorsal thalamus	MED
	11	Basolateral amygdalar nucleus, anterior part	BLAa
	12	Pallidum, ventral region	PALv
	13	Agranular insular area	AI
	14	Visceral area	VISC
	15	Orbital area	ORB
	16	Gustatory areas	GU
	17	Striatum ventral region	STRv
	18	Frontal pole, cerebral cortex	FRP
	19	Prelimbic area	PL
	20	Claustrum	CLA
Lateral cortical network	21	Intralaminar nuclei of the dorsal thalamus	ILM
	22	Posterior complex of the thalamus	PO
	23	Midbrain, motor related	Mbmot
	24	Ventral group of the dorsal thalamus	VENT
	25	Caudoputamen	CP
	26	Pallidum, dorsal region	PALd
	27	Pontine gray	PG
	28	Primary somatosensory area, mouth	SSp-m
	29	Supplemental somatosensory area	SSs
	30	Primary motor area	MOp
	31	Secondary motor area	MOs

Table S5. List of network regions.

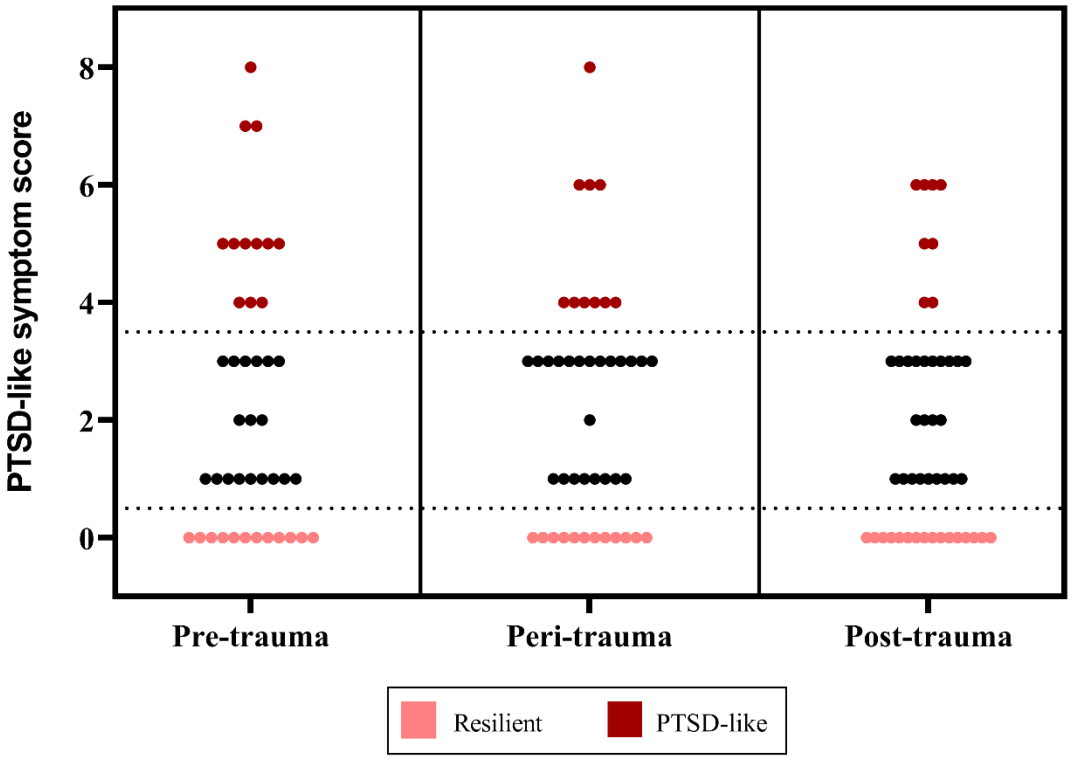

Figure S6. PTSD-like symptom scores across the three cohorts. Mice that had a total of four or more points (necessitating extreme behavior in multiple tests) were termed susceptible. Only mice that had zero points (indicating no abnormal behavior within any of the tests) were termed resilient.

		Mean resilient	SD resilient	Mean susceptible	SD resilient	Significance
Pre-trauma	Time to peak startle (ms)	16.42	0.99	15.79	1.43	
	% Pre-pulse inhibition	51.99	13.58	38.51	23.61	
	# Marbles buried	13.00	2.95	15.00	2.92	
	Locomotion in light phase (km)	17.68	7.22	21.25	13.56	
	% Risk assessment	9.88	6.25	2.39	0.85	***
Peri-trauma	Time to peak startle (ms)	16.49	0.64	16.08	0.84	
	% Pre-pulse inhibition	35.25	13.88	35.99	16.30	
	# Marbles buried	10.33	1.72	11.00	2.49	
	Locomotion in light phase (km)	27.49	5.12	30.05	8.13	
	% Risk assessment	40.55	12.07	22.05	14.91	**
Post-trauma	Time to peak startle (ms)	13.63	1.17	11.28	0.52	***
	% Pre-pulse inhibition	55.06	10.22	37.78	16.61	
	# Marbles buried	9.00	5.74	11.63	4.34	
	Locomotion in light phase (km)	20.62	6.76	26.64	5.50	
	% Risk assessment	170.30	101.65	80.10	48.45	**

Table S7. Behavioral assessment of separate behavioral traits reflecting PTSD-like symptomatology over the three behavioral cohorts (See Figure 1). For normally distributed data, independent t-tests were carried out, while for non-parametric data, the Mann-Whitney U test was used. *: $p < .05$, **: $p < .01$, ***: $p < .001$, effect of group

Parent region	Subregion	Pre-trauma	Peri-trauma	Post-trauma
Isocortex	Primary somatosensory area, lower limb		1.20 [0.32, 2.04]	
	Primary somatosensory area, trunk		0.93 [0.08, 1.74]	
	Dorsal auditory area		1.12 [0.25, 1.95]	
	Ventral auditory area		0.87 [0.03, 1.68]	
	Anterolateral visual area		1.45 [0.53, 2.33]	
	Lateral visual area		1.06 [0.20, 1.88]	
	Primary visual area		1.28 [0.39, 2.13]	
	Posterolateral visual area		1.17 [0.30, 2.01]	
	posteromedial visual area		0.86 [0.03, 1.67]	
	Anterior cingulate area, ventral part	-0.86 [-1.70, 0.00]		
	Orbital area, lateral part	1.26 [0.35, 2.13]		1.03 [0.04, 1.97]
	Orbital area, ventral part			1.09 [0.09, 2.03]
	Agranular insular area, ventral part		-0.89 [-1.70, -0.05]	
	Retrosplenial area, lateral agranular part		1.55 [0.61, 2.44]	
	Retrosplenial area, dorsal part		1.71 [0.74, 2.62]	1.37 [0.32, 2.36]
	Retrosplenial area, ventral part	-0.96 [-1.80, -0.09]		
	Temporal association areas		0.85 [0.01, 1.65]	
Olfactory bulb	Anterior olfactory nucleus		-1.13 [-1.97, -0.26]	
	Taenia tecta		-0.89 [-1.70, -0.05]	1.12 [0.12, 2.07]
	Dorsal peduncular area		-1.02 [-1.84, -0.16]	
Hippocampal formation	Field CA2	-0.87 [-1.70, -0.01]		
	Parasubiculum			1.31 [0.27, 2.29]
	Postsubiculum		0.91 [0.07, 1.72]	
	Presubiculum		1.41 [0.50, 2.28]	
Striatum	Striatum dorsal region		-0.85 [-1.66, -0.02]	
	Striatum ventral region	0.88 [0.02, 1.72]		
Pallidum	Pallidum, medial region	1.11 [0.22, 1.96]		
Thalamus	Ventral group of the dorsal thalamus	-1.17 [-2.04, -0.27]		

Table S8. Neuronal activity differences pre-, peri- and post-trauma between susceptible and resilient animals. Hedge's g values and 90% confidence intervals are shown for all regions where the 90% confidence interval of the effect size did not contain 0.

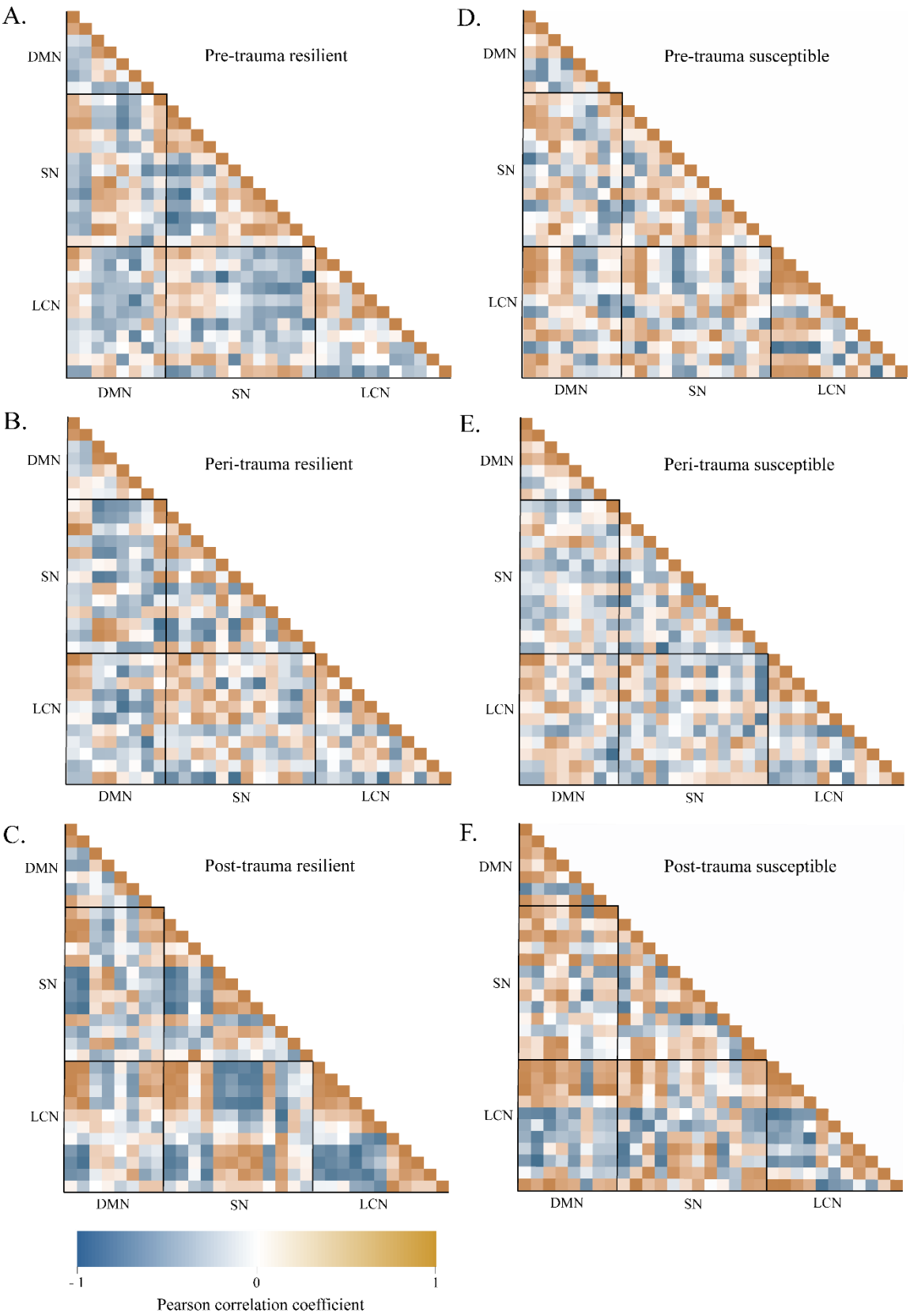

Figure S9. Correlations of neuronal activity in susceptible vs. resilient animals, based on Pearson correlation coefficients between regions in the default mode network (7 regions), salience network (13

regions), and lateral cortical network (11 regions). Correlations for resilient and susceptible animals are shown pre-trauma (**AB**), peri-trauma (**CD**) and post-trauma (**EF**).

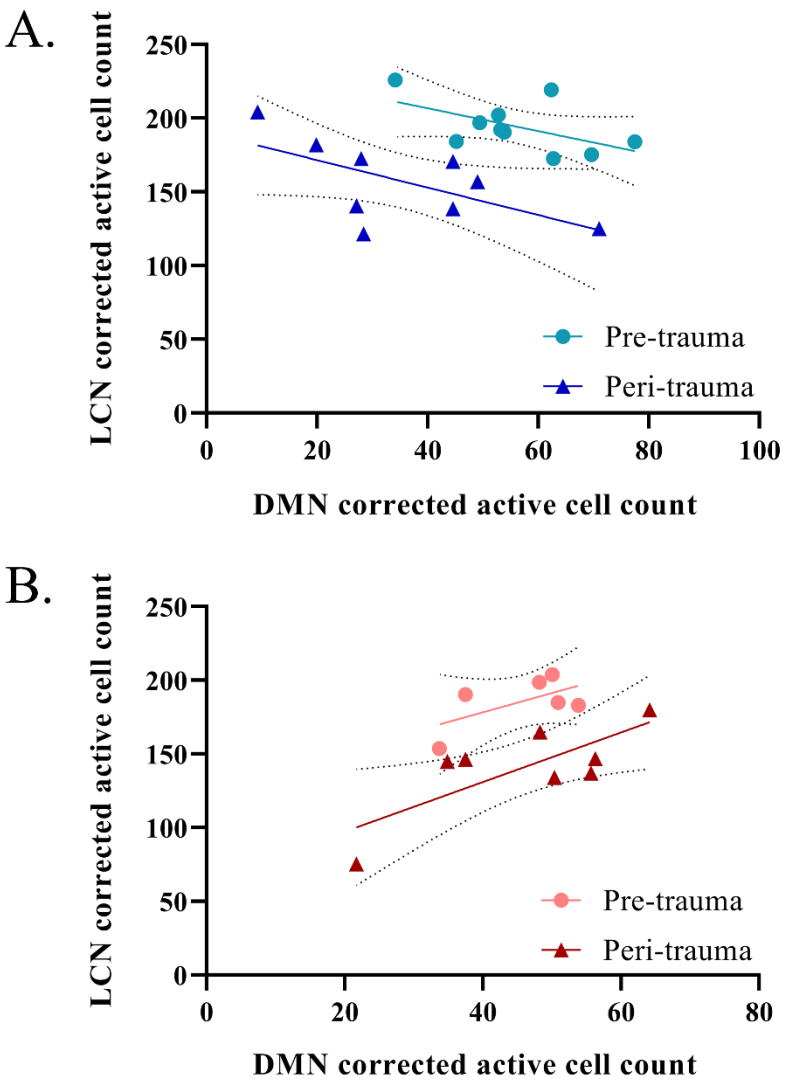

Figure S10. Average total-corrected cell counts for all LCN regions, plotted against the average-total corrected cell counts for all DMN regions in resilient (**A**) and susceptible (**B**) animals. Linear best-fit lines with 90% confidence bands are shown on top of the data points.

Chapter 6

Summary of the chapters

General Introduction

1. Summary of the chapters

Stress-related disorders, like post-traumatic stress disorder (PTSD), constitute an increasing burden on society[2,5], but current treatment strategies are only effective in fewer than half of the patients[17,18]. Interestingly, only ca. 5-10% of the general population develops PTSD[14], even though the majority experiences at least one traumatic experience during life time[11-13]. Apparently, some individuals are more vulnerable than others. Elucidating the biological basis of this interindividual variability in PTSD susceptibility and resilience will be critical for understanding PTSD psychopathology, as it dissociates responses contributing to disease from those maintaining health. As such, it may hold unique insights for identifying vulnerable individuals and optimizing prevention, early intervention and treatment strategies in PTSD[17]. Therefore, in this thesis, we studied how interindividual behavioral heterogeneity in the long-term consequences of stress exposure is reflected in multiple neurobiological factors, including neuronal activity, connectivity and epigenetic regulation across multiple brain regions.

While studies of PTSD patients have come a long way in providing evidence for alterations in the activity and functional connectivity of certain brain regions, human studies lack the possibilities for invasive research and tests for causality. This is, however, crucial to elucidate the complex neurobiological effects of stress, and to mechanistically explore the effects that trauma exposure exerts on brain structure and function[19,514], in order to be able to intervene with these or target these in treatment. Furthermore, there is still a relative lack of longitudinal studies into the effects of (traumatic) stress exposure, making it hard to pinpoint the origin of potential deviations in patients and determine whether they represent risk factors or acquired anomalies[32,33]. To his end, we exposed male ArcTRAP mice to a PTSD induction paradigm, in which they were exposed to a traumatic event, followed by a less severe trigger event the next day[26]. By making use of the ArcTRAP genetic construct[102], we were able to label active neurons under resting (i.e., home cage) conditions before, during, and under resting conditions after trauma exposure. As such, we aimed to show at what point in time neuronal alterations, that give rise to susceptibility to PTSD-like behavioral symptoms, arise.

Pre-trauma, we did not observe any differences in amygdalar (**Chapter 4**) or hippocampal (unpublished findings) neuronal activity, showing that alterations in resting activity of these regions do not contribute to traumatic stress susceptibility. Peri-trauma, we found that exaggerated activity in specifically the basolateral amygdala (BLA) predicted susceptibility to later PTSD-like symptoms (**Chapter 4**), while no group differences were observed in hippocampal activity (**Chapter 3**). The differences in BLA activity were not present after trauma, nor did we observe group differences in activity of any of the other amygdalar subregions (**Chapter 4**).

In addition, we aimed to study neuronal activity in response to contextual recall of the traumatic memory and assess context specificity of memory recall as assessed by freezing behavior. We re-exposed mice to one of three different contexts, three weeks after the initial trauma; either the trauma context, trigger context, or a novel context (resembling the trigger context only in some aspects). We demonstrated that mice displaying PTSD-like symptomatology activated fewer neurons in the hippocampal CA1 region (**Chapter 3**), yet showed BLA hyperreactivity (**Chapter 4**) when re-exposed to the trigger context. This was accompanied by higher numbers of hippocampal parvalbumin (PV) positive neurons and a relatively lower activity of PV^+ interneurons during re-exposure (**Chapter 3**). Exposure to the trauma context, on the other hand, evoked BLA hyporesponsivity. Neither re-exposure to the trigger or trauma context evoked differential freezing responses between groups. Yet, exposure to the novel context evoked a differential temporal pattern of freezing behavior in susceptible mice and an increased activity of BLA somatostatin-expressing neurons specifically, in the absence of overall differences in BLA activity (**Chapter 4**). These findings suggest that traumatic stress susceptibility is specifically characterized by aberrant BLA fear memory encoding, as well as altered recall-specific activity in the hippocampal CA1 and BLA. The exact BLA responses to the stressful contexts, however, depend on the exact context in which they are assessed.

Epigenetic modulation has received growing attention in explaining differential stress susceptibility, as well as for being a mechanism by which stress may lead to long-term biological and behavioral alterations[139]. In **Chapter 3**, we performed immunohistochemical stainings for epigenetic markers, to

study the epigenetic profile of hippocampal neurons that were active peri-trauma (i.e., during memory encoding) and/or during trigger context re-exposure (i.e., during remote memory recall). Specifically, we investigated the epigenetic markers histone deacetylase 2 (HDAC2), 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC), whose relation to stress research is discussed in **Chapter 2**. Susceptible animals displayed significantly lower hippocampal HDAC2 expression, as well as higher 5mC and 5hmC signal, suggestive of overall higher hippocampal transcriptional activity. However, the epigenetic differences were independent of whether the hippocampal neurons were active during initial memory encoding, remote recall or neither.

It is often argued that the scope of functional abnormalities shown in PTSD (and other psychiatric disorders for that matter) cannot be captured by abnormalities in singular neuronal processes or brain regions, like the typical regions of interest, the hippocampus and amygdala. Instead, a broader integrative approach is necessary to capture the complexity of such disorders[34]. There is growing evidence - mainly within the neuroimaging field - that psychiatric disorders, including PTSD, may be better understood as disorders of circuits, rather than of single brain regions[34,108-112]. In **Chapter 5**, as in Chapter 4, we assessed neuronal activity before, during, and after trauma exposure to temporally define potential activity differences and identify risk factors, as well as potential targets for early intervention and eventual treatment. Here, we employed the iDISCO+ technique[126] to label and clear entire brain hemispheres, thereby moving our analyses beyond singular regions of interest. We report on altered activity in a multitude of brain regions either pre-, peri- or post-trauma, related to susceptibility to PTSD-like symptomatology. Most notably, we observed increased resting activity of the orbitofrontal cortex (OFC) both pre- and post-trauma, identifying this as a potential risk factor. Furthermore, both during and after trauma exposure, the retrosplenial cortex (RSP) was substantially more active in susceptible animals, which may reflect an acquired alteration in neural processing. By organizing our regions into neural networks, we were also able to analyze correlations within and between the three most well-studied brain networks: the salience (SN), default mode (DMN) and lateral cortical (LCN, the rodent homologue of the executive control network) networks, as a proxy for

their functional connectivity. Susceptible mice showed increased correlations in neuronal activity between the DMN and LCN pre- and peri-trauma, as well as increased resting DMN-SN correlations peri- and post-trauma. Furthermore, the SN was substantially more intra-correlated post-trauma in susceptible vs. resilient animals, recapitulating observations in PTSD patients. These results pose increased OFC activity and DMN-LCN functional connectivity as pre-trauma risk factors, while highlighting increased RSP activity and DMN-SN functional connectivity as acquired maladaptive pathologies that arise during trauma.

Considering these results, we observed that stress resilience and susceptibility are influenced by alterations in neuronal activity, epigenetic regulation and inter- and intra-network correlations, that may present themselves either before trauma (i.e., risk factors), during trauma or after trauma (i.e., acquired factors). The current chapter aims at further integrating and discussing these results, as well as to their limitations, followed by some suggestions for future research.

2. A rodent model of PTSD - Individual profiling for enhanced translational value

Using human patients to study PTSD comes with clear limitations. The acquisition of PTSD in humans is incidental, and thus never observed in real-time. Furthermore, controlled exposure to trauma is ethically unviable, and invasive measurements can only be performed post-mortem[19]. The latter are crucial to obtain a better mechanistic understanding of the neurobiological alterations that occur in response to trauma, and that affect brain structure and function at the microscale level. Therefore, *in vivo* animal models are still of great importance for studying the brain mechanisms involved in the development of PTSD-related symptomatology[15,19,20]; insights that are essential to optimize both pharmacotherapy and psychotherapy approaches, which generally lack empirical support[38,515,516]. Rodent models for stress-related disorders offer unprecedented opportunities to elucidate the mechanisms and underlying sources for interindividual variance in stress susceptibility in human psychopathology[20,517].

It has long been a challenge to design a PTSD animal model that is both effective and translationally valid. Not only is there a large overlap with other disorders, like mood disorders, anxiety disorders and drug abuse[518,519], but the definition and diagnosis of PTSD (like all other psychopathologies) in humans is based on behavioral symptoms and self-reports, without any (neuro)biological parameters[17]. Here, we employed a mouse model based on the principle of stress-enhanced fear learning (SEFL)[28], implementing electrical foot shocks, given on two separate days and in two different contexts. The mice are exposed to a set of unpredictable shocks on day 1, which leads to a long-lasting non-associative sensitization. This in turn results in increased fear learning to a mild conditioning regiment, i.e., exposure to a set of predictable and relatively mild shocks in a different context on day 2. Inappropriately strong fear responses to relatively mild stressors form a key component of PTSD symptomatology[520], and a subset of mice subjected to the SEFL model develop several PTSD-like symptoms, including hypervigilance, heightened startle response, reduced risk assessment, and insomnia[19]. These symptoms are mainly in line with the 'arousal and reactivity' cluster (i.e., criterion E) of the DSM-V[6]. Moreover, the model recapitulates the neuroendocrine abnormalities observed in PTSD, like attenuated corticosterone levels in response to stress (i.e., hypocortisolemia)[368]. This is a great strength of this model over other PTSD models, using e.g., single-prolonged stress, foot shock stress or predator scent stress[17,19].

Yet, we here did not directly observe evidence of behavioral display for an aberrant trauma memory (indexed by deviant freezing behavior upon context re-exposure), as could be expected from PTSD's intrusion symptoms and avoidance (**Chapters 3 and 4**), which would have been in line with the 'physical reactivity after exposure to traumatic reminders' symptom defined in criterion B of the DSM-V criteria for PTSD[6]. This seeming lack of behavioral outcome is discussed in more detail later (Section 3).

A major current trend in stress research is to consider the spectrum of responses by different individuals to the same stressful stimulus or environment. The motivation for such an approach is to dissociate the adaptive vs. maladaptive responses to stress exposure[521]. In human studies, PTSD

patients are often contrasted to stress-exposed healthy controls, in order to assess pathology rather than effects of life history. Most animal models of PTSD, however, homogenize all trauma-exposed animals as having the same maladaptive phenotype[22,522]. This is thought to be one of the main reasons for low construct and predictive validity of these models, which hinders their translational value[523]. It is imperative to consider that individual animals, even in inbred strains, may be more or less susceptible to experimental manipulations. Rather than viewing this as a limitation of preclinical research, it should be seen as an opportunity to better understand interindividual differences in stress susceptibility. Over the last decade, multiple PTSD models have been developed that classify susceptible and resilient individuals on the basis of specific behavioral readouts, like hyperarousal[524], anxiety and risk-taking behavior (e.g., open field and elevated plus maze)[522], social interaction[525], or freezing behavior[526]. Used as the only behavioral readout, however, these behaviors do not capture the full complexity of PTSD, but rather capture excessive fear instead of PTSD. This underlines the need for a model that incorporates multiple behavioral readouts to generate a translationally valid model of a complex disorder like PTSD[522,527].

The SEFL model, that we employed in this thesis, can be used to study the effects of stress itself, but also to assess differences in the consequences of stress exposure between individuals vulnerable to the behavioral symptoms and those that are resilient[26]. To do so, factor analyses were implemented to yield a behavioral compound score for each animal, based on the five behavioral readouts of PTSD-like symptoms mentioned earlier. This allows animals with a high PTSD-like symptom score to be contrasted to animals with a low PTSD-like symptom score, which resembles the classification of PTSD patients, who are also stratified based on a compound score of symptomatology[27]. In this thesis, we performed this PTSD paradigm on three cohorts of ca. 50 mice (**Chapters 3, 4 and 5**), to label neuronal activity at different timepoints (i.e., pre-trauma, peri-trauma or post-trauma). It should be noted that the behavioral profile of the mice across these experiments was slightly different. Even though the compound score was based on five separate behavioral outcomes, contrasting the resilient and susceptible groups post-classification did not always yield significant differences in all of

outcomes. In addition, statistical differences in a certain behavioral test in one cohort were no guarantee for similar differences in that same test in the other two cohorts. For example, the difference in startle response across phenotypes is much stronger in the post-trauma cohort than in pre- and peri-trauma cohorts. Still, we believe that exactly this observed variability in behavioral symptom profiles underlines the strength of this PTSD model, as the behavioral profile of PTSD of patients is similarly heterogeneous[27]. As such, we would indeed expect variation not only within, but also across cohorts, with not all behavioral outcomes being equally affected.

3. The hippocampal and amygdalar memory engram of trauma

The dual memory representation theory of PTSD

The hallmark feature of PTSD is the re-experiencing symptom cluster, i.e., intrusive memories, nightmares, flashbacks and emotional distress upon trauma reminders[6], which affects >90% of patients. This implicates alterations in fear memory in PTSD, which may distinguish PTSD from other stress-related disorders[37]. It is important to note that maladaptive, intrusive memories observed in PTSD qualitatively differ from normal, adaptive trauma memory, which allows individuals to learn from dangerous situations and prevent them in the future[41]. The high prevalence of intrusions, together with the fact that current therapies most effective in treating PTSD (i.e., exposure therapy and Eye Movement Desensitization and Reprocessing) are aimed at modifying this memory[93,342], has made researchers postulate that an aberrant trauma memory lies at the core of the disease. Traditionally, the hippocampus and amygdala have received particular attention with regards to the pathophysiology of PTSD[55] for their known role in memory processing and emotional regulation of memory[54]. The hippocampus is sensitive to stress, and its dysfunction has been proposed to partially underlie the contextual hypomnesia often observed in PTSD[80]. That is, PTSD patients display fear memory recall that is little context-dependent, fragmented and contains memory gaps, and is triggered by trauma-related sensory cues[528,529]. The observation of this contextual hypomnesia, in conjunction with emotional hypermnesia - the intensification of the emotional and sensory content of the traumatic memory, which is modulated by the amygdala[530] - has made clinicians postulate the dual memory

representation theory of PTSD[30]. This theory distinguishes normal episodic trauma memory and flashbacks, with the former being supported by flexible, contextualized representations that are proposedly adaptive, as they ensure restricted recall of the traumatic memory only if the context requires. In contrast, flashbacks are supported by representations that are inflexible and lack context[49], making that these maladaptive trauma memories escape voluntary control as they are automatically reactivated, in whatever context, by the sole presence of salient cues somewhat related to the traumatic event[49]. However, convincing experimental evidence in patients for this theory is currently lacking.

Evidence for hippocampal and amygdalar alterations

Our observations suggest that hippocampal activity during trauma exposure is not per se different between resilient and vulnerable individuals (**Chapter 3**). However, during remote memory recall, the CA1 area (mainly the ventral part) was significantly less active in susceptible vs. resilient animals. CA1 alterations in PTSD patients, like lower CA1 subfield volume[531,532], have been reported before, and the area itself has been known to be necessary for retrieval of contextual fear memory[533]. Pharmacological disruption of the CA1 has also been demonstrated to ameliorate PTSD-like behaviors in mice[534,535], while activation of the same region was found to restore adaptive contextual fear memory[93]. These findings support the idea that, at least at the hippocampal level, it is specifically the memory consolidation process which seems affected in vulnerable individuals, which may play a role in the contextual hypomnesia posited by the dual memory representation theory. In our experiments, we also observed that susceptible animals had more PV^+ neurons in the ventral hippocampus, again mainly in the CA1, than their resilient counterparts. As we performed these measurements under resting conditions post-trauma, it is not clear if these differences were already present before trauma exposure. In contrast to this overall increase in PV^+ density, a relatively lower proportion of trigger recall-activated neurons consisted of PV^+ neurons. This might in fact be a compensatory mechanism for the increased overall PV^+ density, as activated PV^+ neuron counts were similar across groups. However, the higher PV^+ density remains interesting, as it is seemingly in conflict with earlier findings[358,405], which report decreased numbers of hippocampal PV^+ neurons in tree shrews exposed to

five weeks of psychosocial conflict stress[358], and no changes at all in rats exposed to 8 weeks of chronic mild stress[405,536]. In both cases, differences may be explained by the use of different animal models and stress paradigms, especially as chronic stress might influence PV^+ differently than the SEFL paradigm that we employed. This is in line with the observation that acute stress is not sufficient to induce changes in rat hippocampal PV expression[357,537]. Additionally, we only show a relative difference in PV^+ density between susceptible and vulnerable individuals, but were not able to relate these to trauma exposure per se, as the experiments lacked non-trauma exposed control groups. This shortcoming is discussed later in more detail (Section 6).

In **Chapter 4**, we reported on amygdalar (BLA) hyperactivity specifically during trauma exposure, but not under resting conditions pre- or post-trauma. This is in line with prior animal studies implicating exaggerated activity of the amygdala during fear memory encoding and consolidation in the development of fear generalization[441], aberrant fear memory quality[387] and intrusive memories[447]. In addition, we observed increased amygdala activation in susceptible vs. resilient animals when re-exposing them to the trigger context. In humans, similar amygdalar hyperactivity has been observed when exposing PTSD patients to trauma-specific stimuli[60,81-83]. Our findings were specific to the BLA, which is involved in associative fear learning[425]. This suggests that susceptible animals suffer from aberrant fear memory acquisition and recall, rather than from generally exaggerated fear- and arousal-related amygdala output (which would have likely involved the central amygdala (CeA) as well). This overall increase in involvement of the amygdala in both fear learning and recall, specifically in susceptible animals, may connect to the emotional hypermnesia that is observed in PTSD patients. Together, the findings from the hippocampus and amygdala seem to fit within the context of the dual memory representation theory, proposing adaptive episodic trauma memories in resilient vs. maladaptive memories in susceptible mice (Figure 1).

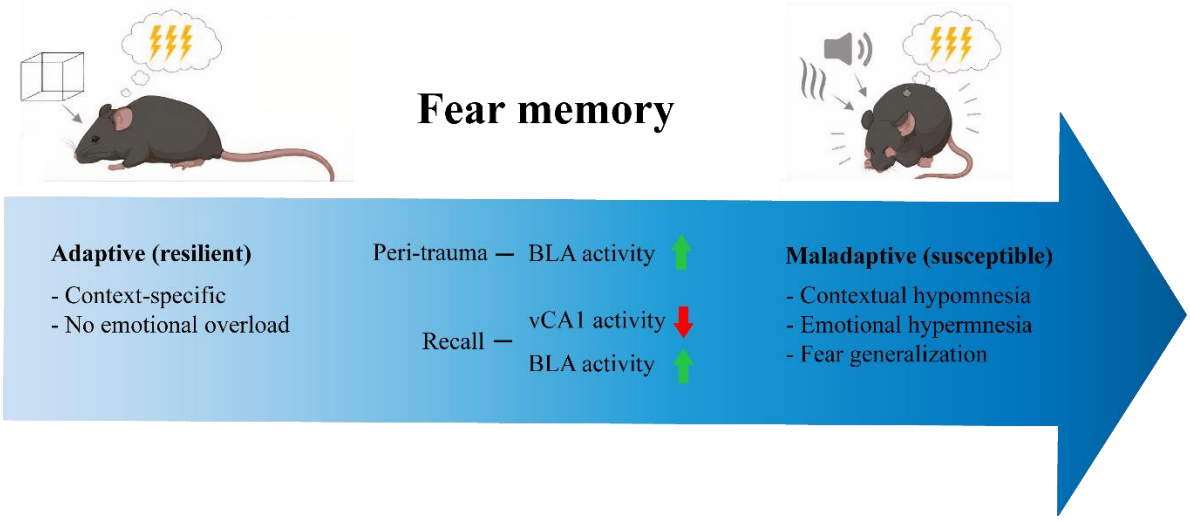

Figure 1. Visual representation of the distinction between adaptive and maladaptive memories. Maladaptive memories are characterized by contextual hypomnesia, which is reflected by a reduction in vCA1 activity upon traumatic context re-exposure, as well as emotional hypermnesia, which is underlain by increased BLA activity both during trauma and during fear memory recall. Together, these phenomena induce fear generalization.

Tracer studies have shown that the BLA has prominent reciprocal projections to and from the ventral CA1[389,538]. This hippocampal-amygdalar pathway has been implicated in the retrieval of contextual fear memory, and it has been shown that activity in ventral CA1 (vCA1) projections to the basal amygdala contributes to the encoding of conditioned fear[394]. Previous research has highlighted an essential role for BLA input to the hippocampus for adequate contextual fear learning, including the BLA as both the main integrator of sensory representations and direct modulator of hippocampal function by sending the integrated information back[539]. Moreover, the BLA is known to modulate the consolidation of the context representation in the hippocampus[103,540,541], with high BLA activity being proposed to inhibit the hippocampus, causing a shift in the locus of memory consolidation away from the hippocampus to the amygdala[41,542]. The fact that we found recall-specific alterations in both vCA1 and BLA activity in susceptible vs. resilient animals supports the idea that the trauma memory is differentially processed/stored in susceptible vs. resilient mice. Interestingly, additional analyses on our data revealed that vCA1 and BLA activity upon trigger context re-exposure were significantly negatively correlated ($r = -.533$, $p = .009$), as were correlations between BLA activity during trauma

encoding and vCA1 activity upon trigger context re-exposure (r = -.602, p = .002), all independent of group. This lends support to the idea that both regions should be considered together when examining PTSD susceptibility, and that functional connectivity might be affected in susceptible individuals. Future studies should directly address vCA1-BLA projections, and how these are activated during trauma memory recall in susceptible vs. resilient individuals. For example, TRAP may be combined with rabies virus-based genetically targeted transsynaptic tracing methods[543,544], to identify neurons that connect to TRAPped cells. This would be especially useful, as it would enable us to visualize direct anatomical connections between engram cells in the vCA1 and BLA, and further investigate the theory that maladaptive fear memory may be caused by alterations in BLA modulation of hippocampal fear memory consolidation.

Shortcomings

Despite finding evidence for reduced CA1 and increased BLA activity during trigger context re-exposure, these deviations did not clearly translate to differential behavioral profiles (i.e., altered freezing behavior). Freezing behavior is mainly regulated through output of the CeA[545], which sends direct projections to the periaqueductal grey[546]. Although we found no differences in CeA activity during fear recall, the CA1 and BLA activity alterations were indicative of altered fear memory processing, which could result in altered freezing behavior. Importantly, the fact that no differences in freezing were observed when re-exposing the mice to the trigger context does not necessarily mean that their memory is not affected. Both resilient and susceptible animals may have similar memory strength for the trigger context, but differential quality of the memory. This theory is supported by literature showing that stress does not only influence memory strength, but also impacts the quality and accuracy of memory[47,547,548]. The latter might be differentially affected in susceptible and resilient individuals. We set out to test this hypothesis by re-exposing another cohort of mice to a novel, unfamiliar context, which resembled the trigger context only in some aspects (**Chapter 4**). However, this did not induce substantial differential freezing behavior between susceptible and resilient mice. Yet, the dynamics of the freezing response over time were different, accompanied by increased

activity of somatostatin (SOM) positive cells. The fact that overall freezing strength was not affected, may be explained by the presence of some relatively salient contextual cues (e.g., a tight space, the presence of a shock grid, etc.) in both contexts[549,550]. Alternatively, it could be that the mouse model does not optimally model PTSD-like fear memory aggravations, because, as mentioned earlier, the behavioral classification is mainly based on hyperarousal and -reactivity symptoms[26]. As such, we encourage additional research to better describe the behavioral memory-related alterations present in this mouse model.

One final observation with regards to the memory engram is that only a relatively low percentage of tdTomato-tagged hippocampal (4.2% on average) and amygdalar (6.1% on average) neurons was reactivated upon trigger context re-exposure (**Chapters 3 and 4**). These low percentages are in line with previous rodent capture-and-tag studies in the same ArcCreER^{T2} animal model[371], but also in models employing TeTTag[551]. Engram contraction - a reduction in engram size upon encoding and subsequent consolidation - is a possible explanation for the low overlap[552,553]. Another explanation could be that, because only glutamatergic neurons were tagged with tamoxifen[102], GABAergic engram cells were missed, reducing the overlap with the cFos^{+} cells, which did encompass both glutamatergic and GABAergic cells. Memory engrams have traditionally been defined as neuronal ensembles that are activated during learning and that, when reactivated, lead to recall of the stored memory trace[96,97,99]. However, the term is also more loosely used to mean all neurons that form the physical substrate of a memory in the brain[554-556]. By this definition, neurons involved in memory encoding do not necessarily have to be reactivated upon fear recall before being considered part of the engram. Indeed, the engram is not static, and the representation of a memory may shift from regions supporting recent memories (e.g., the hippocampus and amygdala) to the neocortex over time during systems consolidation[557-559]. We also chose to term all neurons that were active during trauma encoding, during memory recall upon context re-exposure, or during both, as engram cells (**Chapter 3**). However, it should be noted that experimental stimulation of the tdTomato- or cFos-labeled cells, e.g., using optogenetics, would be necessary to determine if their activation is sufficient to induce memory recall.

4. Epigenetic regulation underlying stress susceptibility

As we found evidence for altered activity of the hippocampus in susceptible vs. resilient animals upon trigger memory recall, our next goal was to identify factors that could explain this interindividual variability. Considering the animals all originated from an inbred mouse line, they can be coined genetically identical. However, as mentioned before, that is not to say that gene expression is unaltered, as we, and others, have found clear differences in local gene expression between resilient and susceptible mice[560-562]. Noteworthy, the animals may still differ in their epigenetic makeup. Especially in the study of PTSD susceptibility, which is estimated to be only 5-20% heritable[128], epigenetic modulation has received growing attention in explaining differential stress susceptibility, as well as for being a mechanism by which stress may lead to long-term biological and behavioral alterations[139]. In **Chapter 3**, we measured HDAC2, 5mC and 5hmC immunofluorescence within engram and non-engram cells in the hippocampus. Engram cells, compared to non-engram cells, generally contained higher levels of HDAC2 and 5mC, and lower levels of 5hmC, suggesting reduced histone acetylation and increased DNA methylation related to memory. These factors are both indicative of a relative reduction in gene transcription[407]. Memory encoding has been linked to increased gene transcription and chromatin modifications, resulting in a substantially altered epigenomic and transcriptomic profile of engram neurons[563]. This in turn drives synaptic plasticity, which is necessary for long-term memory formation[564,565]. Yet, previous studies have indicated increased hippocampal DNA methylation as a key mechanism in stabilizing memory engrams during memory consolidation, supporting successful memory retrieval[386], matching our findings. Retrieval of fear memory has also been associated with increases in histone acetylation in the hippocampal CA1[566,567] and lateral amygdala[568], thereby transiently inducing transcriptional activity. This is necessary to allow for the reconsolidation of the memory, but it also opens up a window for potential intervention and restructuring of the engram[569,570]. Importantly, we here assessed these epigenetic markers in neurons that were either initially recruited during memory encoding, but only after memory consolidation had taken place, or in neurons that supported memory recall, but at a timepoint too early

for these reconsolidation mechanisms to be detected. Based on previous work, as well as our current findings, we hypothesize that trauma exposure may lead to an immediate and initial increase in gene transcription in neurons that become part of the engram. During consolidation, long-term and persistent synaptic and transcriptional changes occur[571], which guarantee successful long-term memory formation. During the consolidation process, epigenetic mechanisms are induced, which stabilize the neuronal memory representations[386], supporting successful memory retrieval. It is only during memory recall that the memory may become destabilized and open for reconsolidation. This hypothesis is depicted in Figure 2A.

Interestingly, we observed that susceptible animals showed lower HDAC2, but higher 5mC and 5hmC fluorescence in the hippocampus. These findings were not limited to the engram cells, but were independent of whether the cells were active during either memory encoding, memory recall, or neither. As such, we speculate that these differences do not reflect engram-related alterations per se. The implications in terms of transcription levels are also unclear, especially as 5mC and 5hmC are theoretically inversely related to gene expression[383,384]. Furthermore, HDAC2 is only one of the many regulators of histone markers, let alone the entire cellular epigenetic profile, and other regulatory pathways may play a role. Purely looking at the observed differences in 5mC and HDAC2 levels in susceptible vs. resilient animals, one might hypothesize that consolidated hippocampal engram cells are transcriptionally more active in susceptible than in resilient animals after trauma memory consolidation (Figure 2B). As such, the memory that these engram cells represent may be relatively unstable, allowing for memory generalization[572] and involuntary memory expression[95].

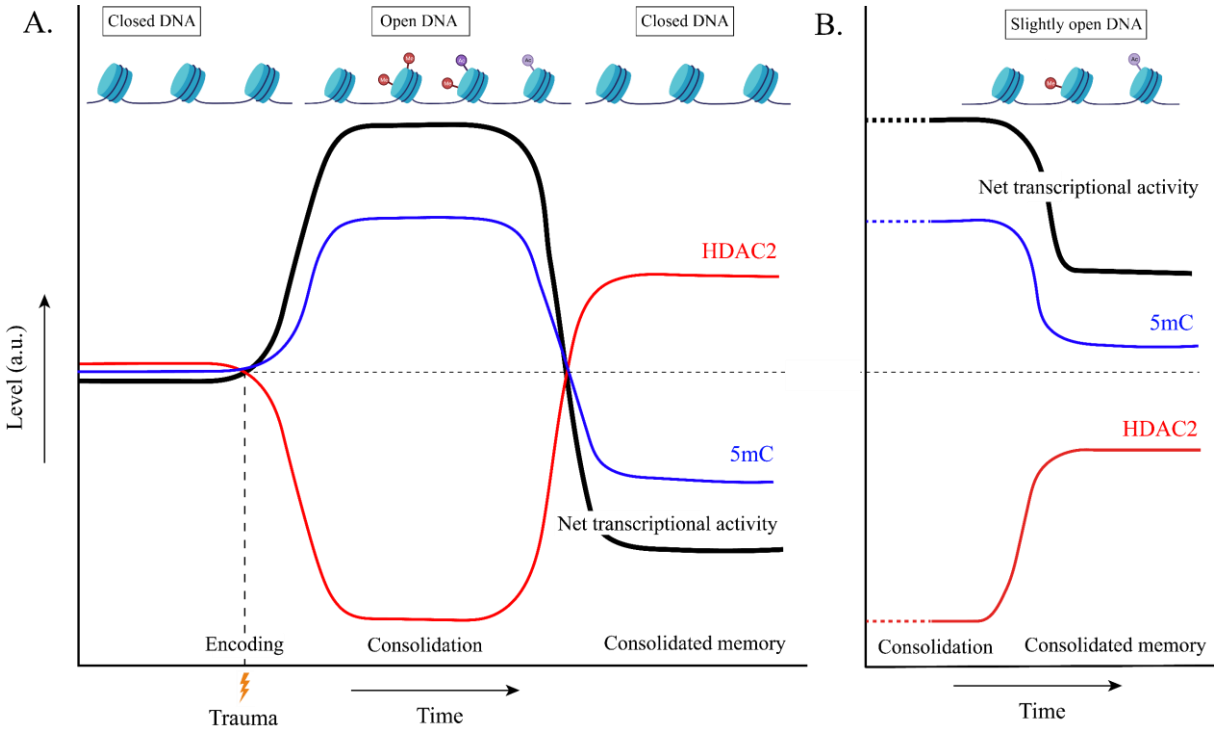

Figure 2. Hypothetical timeline of how transcriptional activity might develop within hippocampal memory engram cells shortly after trauma exposure, upon consolidating the trauma memory, and once the memory is fully consolidated. Shortly after trauma exposure, HDAC2 is downregulated and 5mC is upregulated, leading to increased methylation and acetylation of DNA in engram cells. This in turn leads to increased transcriptional activity, necessary for synaptic plasticity and successful long-term memory formation. In resilient animals (**A**), in time, 5mC and HDAC2 levels decrease and increase, respectively, leading to demethylation and deacetylation of the engram neuronal DNA. This in turn reduces transcriptional activity, leading to a stable consolidated memory representation. We hypothesize that this reversion of 5mC and HDAC2 levels upon memory consolidation does not happen to a similar degree in susceptible animals (**B**), leaving the DNA in those engram cells methylated and acetylated to a higher degree and rendering the cells transcriptionally more active.

In **Chapter 2**, we described a wide body of evidence showing epigenetic changes following stress exposure. However, as mentioned there, evidence describing specific changes in stress-susceptible vs. resilient animals is still lacking. A previous study has correlated reduced resting hippocampal HDAC2 expression with resilience to chronic unpredictable stress, whereas experimentally overexpressing HDAC2 would increase depressive-like behavior[151]. However, because of the differences in stress paradigm, it is hard to connect these findings to our current results. Furthermore, as we did not include

naïve unstressed groups in our experiments, we cannot exclude the possibility that the differences that we observed between resilient and susceptible animals originated already before trauma exposure and memory formation in the first place. And lastly, we should realize that, although we currently only investigated global changes in levels of HDAC2, 5mC and 5hmC, there are many more regulators of the epigenome. It would therefore be too simplistic to draw a one-to-one relationship between the observed levels of these markers and gene expression in general, nor can we be sure which exact genes' expression would be altered. This would require more sophisticated approaches, like bisulfite sequencing or methylated DNA immunoprecipitation.

Despite all this, it is interesting to speculate about when differing epigenetic profiles develop. As mentioned earlier, the mice used in these studies were genetically similar, yet they showed clearly different behavioral profiles in response to traumatic stress, as well as different protein expression in their brains. While we assume that epigenetics may play an important role in explaining the observed behavioral variation, this does not yet explain when these epigenetic differences emerge in the first place. Monozygotic twin studies in humans have shown that, even though twins are epigenetically indistinguishable during the early years of life, remarkable differences in content and genomic distribution of 5-mC and histone acetylation arise later in life[573,574]. Furthermore, epigenetic markers are more distinct in twins who are older, have different lifestyles, and/or have spent less of their lives together[573]. This suggests that the environment in which one is brought up is a key determining factor in shaping the epigenome. In laboratory animals, environmental factors are kept as uniform as possible across different individuals and litters. However, as is apparent in our study, substantial differences in behavioral profiles are manifested. Differences in maternal care have been shown to shape epigenetic profiles, and are a source of interindividual variation[216,575]. However, differences may also already arise *in utero*, and even already during maturation of the parental germline[576]. As such, it is in the end not surprising to find behavioral differences between individuals, even in controlled laboratory settings. In the end, the fact that the animals from an inbred line are genetically similar makes them ideal to study the specific effects of epigenetic variation on traumatic stress susceptibility; hence why

we chose to study these animals over animals from an outbred line, which would more likely result into studying genetic contributions to risk.

5. Brain-wide activity alterations underlying stress susceptibility

For the past few decades, countless human neuroimaging studies have been performed to study the neural underpinnings of psychiatric disorders. In the case of PTSD, the majority of studies have focused on measuring resting-state and task-based brain activity in PTSD patients vs. either trauma-exposed or non-exposed controls, aiming to identify aberrant activity in specific regions or brain-wide networks in the PTSD-affected brain[577]. Still, there is a continued need for longitudinal studies, to not only assess PTSD-related alterations *post hoc*, but also identify potential risk factors and alterations occurring at the moment of trauma exposure to be able to target prevention and early intervention in the development of PTSD. Additionally, most follow-up animal studies have been restricted to the study of candidate brain regions, and would benefit from adopting brain-wide designs similar to those in human neuroimaging. The latter is necessary to further our understanding of potential network level aberrations in the pathophysiology of PTSD. To overcome both challenges, we employed iDISCO+ in the three cohorts discussed earlier, to study susceptibility-related differences in brain-wide neuronal activation before, during, and after trauma (**Chapter 5**). We identified a number of brain regions that were differentially activated between susceptible and resilient animals, with the most robust differences being found at the time surrounding trauma exposure. Interestingly, we not only corroborated prior literature on areas that have been implicated in the pathology of PTSD, like the retrosplenial cortex (RSP)[490,491,578], orbitofrontal cortex (OFC)[52,450,480-482,484] and sensory regions[493,494,496,497], but we also highlighted regions that have not been extensively studied before in relation to stress susceptibility, like the subiculum and pallidum. In addition, we also identified regions that have been previously linked to disorders that are often comorbid with PTSD[579]; e.g., the striatum, whose activity has previously been linked to major depressive disorder[580,581] and addiction[582].

It is now increasingly recognized that the analysis of brain networks, rather than singular regions, can help to explain the complex neurobiological mechanisms underlying psychiatric disorders[116,583]. Our

work shows increased SN intra-connectivity, as well as increased inter-connectivity between the SN and DMN in susceptible vs. resilient animals post-trauma. This is in line with resting-state neuroimaging studies contrasting PTSD patients to trauma-exposed controls[120,121,584]. Abnormal functional communication between the SN and DMN has also been attributed to the pathophysiology of other disorders, like schizophrenia[470], bipolar disorder[585], mania[585] and obsessive-compulsive disorder[584]. Interestingly, the altered SN-DMN connectivity was already observed during trauma, suggesting that the trauma may induce a long-lasting shift in network balance that leads to post-trauma hypervigilance and -arousal symptomatology[111,121]. A stronger connection between the DMN, which is involved in self-referential mental activity[586,587], and the SN, which integrates emotional and sensory stimuli and mediates a "switch" between the DMN and ECN[588], may induce a more self-referenced reading of the environment and self-conscious processing of potential arousal states[589]. Apparently, this represents a maladaptive strategy to react to trauma and makes an individual susceptible to negative behavioral outcomes.

We also observed a near-significant positive correlation between DMN and LCN activity specifically in susceptible animals, both pre- and peri-trauma exposure. This is remarkable, as the DMN and ECN are typically anti-correlated in activity in humans[506,507], as are the DMN and LCN in rodents[471,479]. The DMN, involving regions that activate in the absence of external task demands[590,591], is known to be associated with internally-directed cognitive processes[592,593], whereas the ECN is mainly engaged in the cognitive processes that involve externally-directed attention[594,595]. The anticorrelation between both networks reflects a switching balance between internally and externally directed cognition[596,597]. Disruption of this anticorrelation, as observed in the susceptible animals, has previously been associated with episodic memory deficits[111]. and as such could form the basis for intrusive trauma memory recollection. A summary of the observed alterations in network connectivity between susceptible and resilient animals is shown in Figure 3. It should be noted that, while we discuss our findings in terms of connectivity differences, we were only able to infer network connectivity from calculating cross-subject activity correlations[511-513]. This is obviously not an optimal assessment of

functional connectivity, which could be better estimated from correlating changes in signal strength across time within individual animals. Still, other approaches would have come with their own downsides too - e.g., confounds of anesthesia (in the case of fMRI), or being limited to the study of a small number of regions (in the case of electrophysiology) -, hence why we opted for this approach.

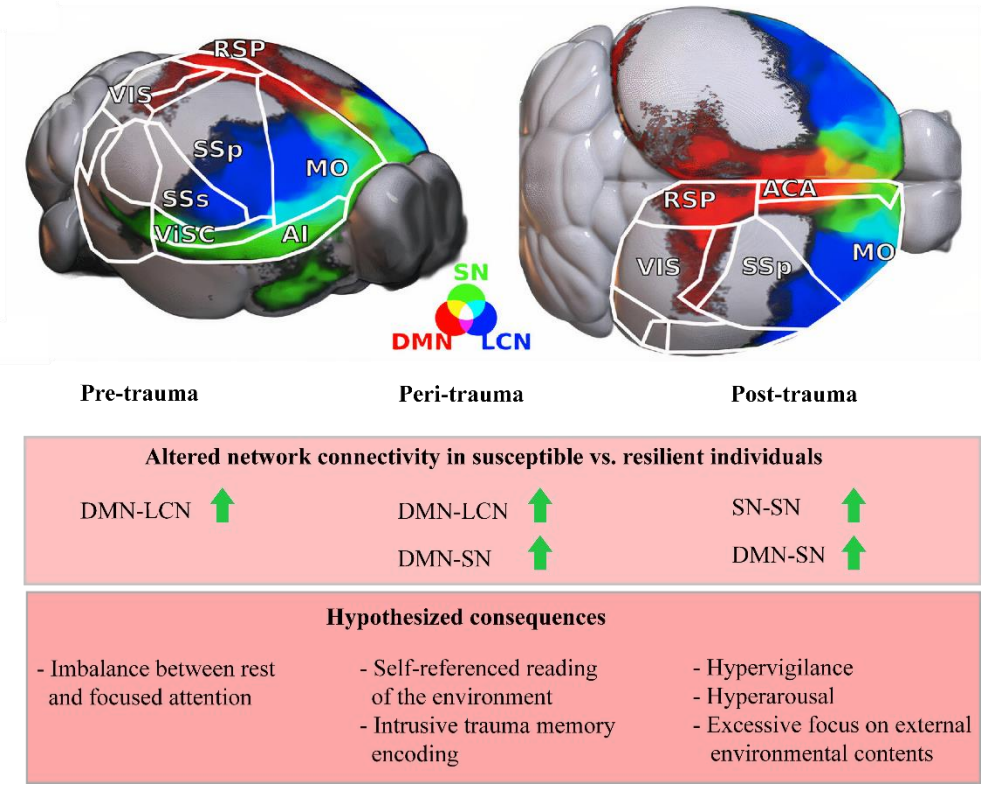

Figure 3. Alterations in network connectivity, as observed in susceptible vs. resilient animals pre-, peri- and post-trauma. Potential functional consequences of these changes in network balance are shown underneath. Figure adapted from Mandino et al. (2021)[479]. DMN: default mode network, SN: Salience network, LCN: lateral cortical network

Noteworthy, we were not able to replicate findings of BLA hyperactivity during trauma exposure (**Chapter 4**) in the iDISCO+ experiments. One potential explanation is that differences may exist between the activity and function of corresponding regions in the left and right hemisphere, with the right hemispheres being used for immunostainings, and left hemispheres for the iDISCO+ experiments. Especially the amygdala has been known to be affected by lateralization[457,598], and specifically the structure and function of the right amygdala has been associated with avoidance,

hyperarousal and re-experiencing symptoms in PTSD related to childhood abuse[434] and aberrant fear memory in mice[387]. Another explanation is that the current approach for mapping signal to the brain atlas was not yet optimized for dissecting very small regions, like the BLA, as was already noted in **Chapter 5**. In general, we should remark that the iDISCO+ technique, as well as the successive data analysis, can still be optimized to better deal with small regions, as well as for correcting for variations in signal strength throughout the brain - an unavoidable consequence of differences in antibody penetration between cortical and core brain regions. All in all though, combining the iDISCO+ and TRAP methods has proven to be a significant development in enabling brain-wide neuronal activity assessments in rodents.

6. Limitations and future directions

The results of these chapters have to be interpreted with some limitations. Below, I will highlight a few of these limitations and give some ideas for future studies that could help address these matters.

Resilience as an active process

The PTSD animal model that we used (**Chapters 3, 4 and 5**) has many benefits. It allows for behavioral classification of susceptible and resilient individuals, rather than considering all stress-exposed animals as a single homogeneous group. Although we were able to pinpoint several neurobiological differences between both groups, it remains unclear whether these were the result of maladaptive processes in the susceptible animals, or rather of adaptive processes as a consequence of stress exposure in the resilient animals. Resilience is often defined as the absence of behavioral symptoms in a subset of stress-exposed animals, which suggests that it is a passive process whereby the lack of a maladaptive response is actually adaptive. There is increasing evidence that stress resilience arises from active adaptations and coping strategies, both at the biomolecular and behavioral level[599]. To make this distinction, one should contrast susceptible and resilient animals to a control group, which is not exposed to stress, to determine a baseline control condition[600]. Future studies in this mouse model should consider adding these control groups to clarify how altered behavior and

neurobiology differ from non-stress baseline and distinguish adaptive from maladaptive adaptations to stress exposure.

Neuronal activity labeling using TRAP

In the current study, we employed targeted recombination in active populations (TRAP) to label active neurons at specific time points (i.e., pre-, peri- and post-trauma)[102]. Activity-dependent expression of immediate early genes (IEGs), like *cFos* and *Arc*, has been exploited in numerous methods for studying neural circuits[551,601]. TRAP adds to those methods by introducing an inducible CreERT2-*loxP* construct, which can be transiently activated by administering tamoxifen to the animal. However, as already noted in **Chapters 3, 4 and 5**, the technique still has some shortcomings, that should be considered. The transgene that we employed labeled neurons upon the expression of the IEG *Arc*, which is implicated in various forms of synaptic plasticity[602], and is necessary for memory consolidation[603,604]. However, *Arc* is expressed mainly in glutamatergic cells[102], which hindered the identification and analysis of GABAergic cells in our experiments. Furthermore, *Arc* is widely expressed within the hippocampal dentate gyrus (DG)[605], which caused substantial background labeling in this subregion. This might have affected labeling specificity, which may in turn be the reason that we did not replicate earlier findings of increased dorsal DG activity upon trauma exposure in mice susceptible to PTSD-like symptomatology[414]. We decided to use the ArcTRAP over the FosTRAP construct, as FosTRAP mice, despite showing higher labeling specificity, had very low overall labeling sensitivity. Recently, a new construct, FosTRAP2, has been developed, which has remedied the above drawbacks[606]. It promises high specificity (96%), as well as efficiency (65%), and does not show the relatively high background labeling that is observed in ArcTRAP mice. Hence, we would recommend future studies to consider this model, to overcome the limitations that we faced.

TRAP constructs can be induced by injecting the animal with tamoxifen, or its downstream metabolite 4-hydroxytamoxifen (4-OHT)[102]. While both show similar results in terms of labeling, the temporal window in which these compounds work is different. Tamoxifen leads to neuronal labeling from 6 hours prior to injection until 36 hours after injection, with maximal TRAPping being observed 24

hours after injection. For 4-OHT, the time window only stretches from 6 hours before injection until 6 hours after injection, as this compound is metabolized much faster by the body[607]. This would put forward 4-OHT as the preferable option (reducing the temporal window during which potentially unwanted neuronal background activity may be captured), we opted to use tamoxifen in our experiments for two reasons. First, as none of the animals were subjected to habituation to handling prior to the experiments, we did not want neuronal activity induced by injection stress to confound our readouts (which would be captured in case of 4-OHT injection). Secondly, as it is currently unclear how SEFL is exactly manifested, we wanted to capture both the trauma and trigger events within the labeling window. As these were 21 hours apart, we needed the extended labeling window provided by tamoxifen. Still, for future research, it is interesting to isolate these two events, and specifically study neuronal activation during either the trauma or trigger event, which would require the use of 4-OHT. As injections would need to occur within ca. 2 hours of the trauma, we would advise to conduct pilot experiments to rule out any potential negative influences on behavior and neuronal activity during the trauma session.

Sex differences in PTSD susceptibility

One caveat of the current study is that we only used male subjects[608,609]. This limitation holds for the majority of preclinical research into stress-related disorders[610]. This is particularly unfortunate as females are generally more prone to develop stress and anxiety disorders[611], including PTSD[612,613]. Females, compared to males, show different HPA axis activity, glucocorticoid feedback and emotional reactivity, and tend to react differently both physiologically and behaviorally to biological or psychological stress. This may explain sex bias in disease prevalence[611,614,615]. Additionally, the female estrous cycle has been observed to affect acquisition and expression of fear conditioning[616,617]. This confound complicates their inclusion in behavioral paradigms established in males, such as the model used by us. To ensure that new insights and potential treatment are applicable to both sexes, it is important to extend the current research to also include female mice. In the current study, we only used male mice, mainly because both the behavioral protocol[26], as well as the ArcTRAP construct[102],

were only validated in males. To adapt the model for inclusion of females, the rationale underlying behavioral categorization would need to be changed, as females behaviorally react differently to stress[618,619], are sensitive to different types of stressors[620], and present different symptoms[458,621]. With regards to TRAP, it was mainly uncertain whether tamoxifen injection would differentially affect male and female mice, especially as tamoxifen is an estrogen receptor antagonist[622]. Because tamoxifen is widely used to induce CreERT2-*loxP* transgenic mouse constructs, and because of the increasing efforts to include both sexes in biomedical studies, multiple studies have since been undertaken to unravel potential sex-specific effects[623]. Accordingly, no long-lasting or sex-divergent effects of tamoxifen were found in the brain epigenome and transcriptome[623], nor in adult neurogenesis, learning and anxiety[624]. Moving forward, ArcTRAP study designs should therefore not be hindered anymore in including both sexes. However, behaviorally, dedicated paradigms and readouts should still be designed for the proper study of females, as most current models have been validated in males only. With regards to our PTSD model, we know through personal communication that the model implemented here is not successful in inducing long-term behavioral symptoms in female mice. Hence, the model would have to be adapted to include readouts that are validated for both sexes.

Manipulations and pharmacological interventions

The observational data described in this thesis allowed us to infer correlations between post-trauma behavior and neurobiological alterations. Crucially, however, it will require experimental manipulations to prove a causal link between the two. This is especially relevant if we want to translate our findings to novel pharmacological interventions for the treatment of PTSD. Here, we would like to shortly propose two different avenues of further research. Firstly, neurons may be chemogenetically inhibited or activated, e.g., by the use of designer receptors exclusively activated by designer drugs (DREADD)[625]. This has allowed researchers to manipulate the memory engram, for example by enforcing or preventing memory recall[371,378], changing the information encoded in an endogenous memory[601], or stimulating extinction neurons to suppress fear recall[94,371]. These techniques could also be used to study the behavioral consequences of inhibiting BLA activity in trauma memory

engram cells during fear memory encoding. It would be interesting to see if this would decrease heterogeneity across individual PTSD-like symptom profiles post-trauma.

In this thesis, we show that susceptible animals are characterized by reduced hippocampal levels of HDAC2, and increased levels of 5mC and 5hmC post-trauma (**Chapter 3**), suggesting that persistent changes in epigenetic profiles may underlie PTSD symptomatology. Emerging evidence shows that drugs that target epigenetic processes could improve extinction of traumatic memories and prevent potential relapse. For example, administration of HDAC inhibitors has been shown to enhance extinction of conditioned fear[147,626,627]. This fits with our findings that engram cells in general have higher HDAC2 levels than non-engram cells, potentially making them transcriptionally less active and more stable. Making these cells transcriptionally more active - by administering HDAC inhibitors - would indeed possibly open a window for extinction of the memory stored in those cells. Combining pharmacological intervention with controlled re-exposure to trauma-related stimuli might also be an interesting therapeutic avenue[628]. Hypothetically, inducing memory reconsolidation might open a window of opportunity where the memory representation is flexible and amenable to intervention. DNA methylation could be therapeutically targeted in a similar manner, for example by administering DNA methyltransferase inhibitors, like zebularine[141]. We encourage actively studying these and other interventions, as this will be key to translating insights from animal research into improved treatment strategies for stress-related disorders like PTSD.

7. Concluding remarks

Overall, in this thesis, we have demonstrated that traumatic stress susceptibility is characterized by interindividual differences in neuronal activity, connectivity, and epigenetic regulation across multiple brain regions. By implementing longitudinal study designs, combined with state-of-the art *in vivo* neuronal labeling techniques, we have shown some of these differences to be already present prior to trauma exposure (i.e., representing risk factors), while others arise during, or as a consequence of the trauma. Crucially, however, we have demonstrated that the complex phenotype of PTSD cannot be explained by only considering deviations in a handful of high-profile regions, like the amygdala and

hippocampus. Instead, we have provided a proof of concept to show that brain-wide clearing and labeling techniques may be used to identify large scale brain networks, whose aberrant activity pre-, peri-, or post-trauma may play a role in shaping stress susceptibility. Additional research, however, will be necessary to show their causal involvement in the development of PTSD symptomatology. Still, studies like these are of utmost importance to enhance understanding of the biological basis of interindividual variability in PTSD susceptibility and resilience and will be critical for identifying vulnerable individuals and optimizing prevention, early intervention and treatment strategies.

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Appendices

Dutch summary | Nederlandse samenvatting

Stressgerelateerde stoornissen, zoals post-traumatische stressstoornis (PTSS), hebben een steeds grotere invloed op onze samenleving[2,5], met name omdat bestaande behandelingsstrategieën slechts in minder dan de helft van de patiënten effectief zijn[17,18]. Bijna iedereen maakt één of meerdere trauma's mee gedurende zijn/haar leven[11-13]. Het is daarom opvallend dat slechts 5-10% van de bevolking PTSS ontwikkelt[14]. Dit laat zien dat sommige individuen kwetsbaarder zijn voor het ontwikkelen van stressgerelateerde stoornissen dan anderen. Het ontrafelen van de biologische basis van deze interindividuele variatie in gevoeligheid voor PTSS is cruciaal voor een beter begrip van de pathofysiologie van PTSS, en kan belangrijke inzichten geven voor het beter onderscheiden van kwetsbare en weerbare individuen en het optimaliseren van preventie[17]. Daarom hebben we in dit proefschrift onderzocht hoe interindividuele heterogeniteit in stressgevoeligheid verklaard kan worden door afwijkingen in verschillende neurobiologische factoren, waaronder neuronale activiteit, connectiviteit en epigenetische regulatie in verschillende hersengebieden.

Hoewel studies naar PTSS steeds meer bewijs hebben geleverd voor afwijkingen in de activiteit en connectiviteit van bepaalde hersengebieden, is het in humane studies slechts beperkt mogelijk om invasief onderzoek te doen en causaliteit aan te tonen. Dit is echter cruciaal voor het verder ophelderen van de neuronale effecten van stress, en voor een beter mechanistisch begrip van het effect van trauma op hersenstructuur en -functie[19,514]. Daarnaast is er een relatief gebrek aan longitudinale studies die de langetermijneffecten van blootstelling aan (traumatische) stress onderzoeken, waardoor het lastig is om de precieze oorsprong aan te wijzen van deze afwijkingen die stressgevoeligheid karakteriseren. Zo blijft het grotendeels onduidelijk of de afwijkingen risicofactoren representeren - welke reeds vóór het trauma aanwezig zijn - of het directe gevolg zijn van blootstelling aan het trauma[32,33]. Om dit op te helderen, hebben we mannelijke ArcTRAP muizen blootgesteld aan een PTSS-inductie paradigma, waarin ze op twee opeenvolgende dagen werden blootgesteld aan een reeks elektrische schokken, in een zogenaamde trauma- en triggersessie[26]. Het ArcTRAP genetisch construct[102] stelde ons in staat om actieve neuronen te labelen die vóór, tijdens en ná blootstelling aan dit gecombineerde trauma actief

waren. Op deze manier hoopten we aan te tonen op welk moment neuronale afwijkingen, die ten grondslag liggen aan gevoeligheid voor PTSS, ontstaan. Voorafgaand aan het trauma vonden we geen verschillen in neuronale activiteit van de amygdala (**Hoofdstuk 4**) of hippocampus (niet-gepubliceerde data), wat aantoont dat afwijkingen in basale activiteit van deze gebieden geen een risicofactor vormen voor stressgevoeligheid. Tijdens het trauma vonden we dat een sterk verhoogde activiteit in specifiek de basolaterale amygdala (BLA) gecorreleerd was met gevoeligheid voor het ontwikkelen van PTSS-achtige symptomen (**Hoofdstuk 4**). Wederom zagen we geen verandering in hippocampale activiteit tussen groepen (**Hoofdstuk 3**). De veranderingen in BLA activiteit waren niet aanwezig basaal ná het trauma, noch vonden we activiteitsverschillen in de andere amygdalaire subgebieden (**Hoofdstuk 4**).

Aanvullend op deze bevindingen wilden we neuronale activiteit bestuderen die optrad wanneer muizen enkele tijd na het trauma opnieuw blootgesteld werden aan een context die gelinkt was met de herinnering aan het oorspronkelijke trauma. Hierbij waren we specifiek geïnteresseerd of de specifieke context waaraan de muizen blootgesteld werden resulteerde in verschillen in bevriezingsgedrag. Hiertoe hebben we de muizen drie weken na het trauma blootgesteld aan verschillende contexten; de trauma context, de trigger context, en een nieuwe context, welke in slechts enkele aspecten leek op de trigger context. We toonden aan dat stressgevoelige muizen tijdens hernieuwde blootstelling aan de trigger context minder neuronen in de hippocampale CA1 activeerden (**Hoofdstuk 3**), maar juist een hyperactiviteit van de BLA lieten zien (**Hoofdstuk 4**). Dit werd vergezeld door een hogere aanwezigheid van hippocampale parvalbumine (PV) positieve neuronen en een relatief verlaagde activiteit van deze interneuronen specifiek bij de hernieuwde blootstelling (**Hoofdstuk 3**). Blootstelling aan de trauma context daarentegen zorgde juist voor een verlaging in BLA activiteit in deze dieren. Tijdens blootstelling aan een nieuwe context, welke in enkele aspecten leek op de trigger context, zagen we subtiele verschillen in het patroon waarin de dieren bevroren, evenals een verhoogde activiteit van somatostatine positieve neuronen in de BLA (**Hoofdstuk 4**). Deze resultaten tonen voor de eerste keer aan dat afwijkende activiteit in de BLA tijdens blootstelling aan een trauma

individuen vatbaar kan maken voor het ontwikkelen van PTSS-achtige symptomen, en dat afwijkende activiteit van de BLA tijdens hernieuwde blootstelling aan een trauma-gerelateerde context afhankelijk is van de precieze context waaraan de muizen blootgesteld worden (**Hoofdstuk 4**).

Epigenetische modulatie heeft de afgelopen decennia steeds meer aandacht gekregen als een mechanisme dat verschillen in stressgevoeligheid kan verklaren, omdat het een link kan vormen tussen blootstelling aan stress en langdurige biologische en gedragsmatige veranderingen [139]. In **Hoofdstuk 3** hebben we immunohistochemische kleuringen uitgevoerd voor verschillende epigenetische markers, om zo het epigenetische profiel te bestuderen van hippocampale neuronen die actief waren tijdens het trauma of gedurende hernieuwde blootstelling aan de trigger context. Specifiek hebben we de epigenetische markers histone deacetylase 2 (HDAC2), 5-methylcytosine (5mC) en 5-hydroxymethylcytosine (5hmC) onderzocht, wier relatie tot stressonderzoek reeds in **Hoofdstuk 2** is beschreven. Stressgevoelige dieren vertoonden een significant lagere hippocampale expressie van HDAC2, evenals een sterker 5mC en 5hmC signaal. Dit suggereert dat de hippocampus gemiddeld genomen transcriptioneel actiever is in deze dieren, vergeleken met weerbare dieren. Deze epigenetische verschillen waren echter onafhankelijk van of de hippocampale neuronen actief waren tijdens het initiële trauma, tijdens de hernieuwde blootstelling aan de trigger context, of tijdens geen van beide.

Er wordt vaak beargumenteerd dat de verscheidenheid aan gedragsmatige afwijkingen in PTSS (of van andere psychiatrische aandoeningen) niet slechts verklaard kan worden door deviaties in losse neuronale processen of hersengebieden, zoals de hippocampus en amygdala. Er is juist een bredere integratieve aanpak nodig om de complexiteit van zulke aandoeningen volledig te verklaren[34]. Dit beeld wordt ondersteund door het groeiende idee - met name binnen het neuroimaging veld - dat psychiatrische aandoeningen, waaronder PTSS, ook gezien kunnen worden als aandoeningen van neuronale netwerken, in plaats van enkelvoudige hersengebieden[34,108-112]. In **Hoofdstuk 5**, evenals in het vorige hoofdstuk, hebben we hersenactiviteit vóór, tijdens en ná blootstelling aan het trauma onderzocht, om zo een temporeel profiel van hersenactiviteit te vormen en potentiële risicofactoren en

trauma-gerelateerde afwijkingen aan te wijzen. We gebruikten de iDISCO+ techniek[126] om hele hemisferen immunofluorescent te labelen en doorzichtig te maken, om zo inzicht te krijgen in activiteit door het gehele brein. Onze resultaten laten groepsverschillen in activiteit zien in een veelvoud aan hersengebieden, zowel vóór, tijdens, als ná trauma. We observeerden een toegenomen basale activiteit van de orbitofrontale cortex (OFC), zowel vóór als ná het trauma in stressgevoelige vs. -weerbare dieren, wat suggereert dat dit een risicofactor is voor het ontwikkelen van PTSS-achtige symptomen. Zowel tijdens als ná het trauma zagen we ook dat stressgevoelige dieren meer activiteit vertoonden van de retrospleniële cortex (RSP). Dit lijkt dus een afwijking te zijn die ontstaat tijdens het trauma. Door onze hersengebieden te organiseren in functionele netwerken, was het verder mogelijk om correlaties binnen en tussen de drie meest bestudeerde neuronale netwerken te analyseren: het salience- (SN), default- (DMN) en lateraal corticaal netwerk (LCN, het knaagdierhomoloog van het humaan centraal-executief netwerk). Stressgevoelige muizen lieten verhoogde correlaties in neuronale activiteit zien tussen het DMN en LCN netwerk, zowel vóór als tijdens het trauma, evenals verhoogde DMN-SN correlaties tijdens en ná het trauma. Daarnaast was het SN netwerk sterker geïntracorreleerd in stressgevoelige vs. -weerbare muizen ná het trauma, waarmee we vergelijkbare observaties in PTSS-patiënten wisten te bevestigen. Deze resultaten laten zien dat toegenomen activiteit van de OFC, evenals toegenomen DMN-LCN connectiviteit risicofactoren vormen voor het ontwikkelen van PTSS-achtige symptomen. Een verhoogde RSP activiteit en een sterkere DMN-SN connectiviteit kunnen daarentegen als maladaptieve aanpassingen worden gezien, die ontstaan gedurende het trauma.

Concluderend hebben we gezien dat stressgevoeligheid en -weerbaarheid beïnvloed worden door een veelvoud aan verschillen in neuronale activiteit, epigenetische regulatie en intra- en inter-netwerk correlaties in en tussen verschillende hersengebieden. Sommige van deze verschillen blijken al vóór het trauma aanwezig te zijn (i.e., risicofactoren), terwijl anderen ontstaan tijdens het trauma, of zich pas ná het trauma uiten. Een belangrijke conclusie is echter dat we het complexe fenotype van PTSS niet kunnen verklaren door afwijkingen in een handjevol veel bestudeerde hersengebieden, zoals de amygdala en hippocampus. Door het combineren van nieuwe technieken voor het doorzichtig maken

en fluorescent labelen van hersenweefsel, hebben we aangetoond dat er veel meer gebieden zijn die afwijkende activiteit vertonen voor, tijdens of na het trauma, en die dus ook een rol in stressgevoeligheid kunnen spelen. Aanvullend onderzoek zal nodig zijn om daadwerkelijke causale verbanden te leggen tussen de geobserveerde afwijkingen en het PTSS-achtige fenotype. Deze studie heeft echter een belangrijke basis gelegd voor een beter begrip van de biologische basis van interindividuele variatie in gevoeligheid voor PTSS.

List of abbreviations

4-OHT	4-hydroxytamoxifen
5-HT	Serotonin
5-HTT	Serotonin transporter
5-HTTLPR	5-HTT linked polymorphic region
5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
ACTH	Adrenocorticotropic hormone
BDNF	Brain-derived neurotrophic factor
BLA	Basolateral amygdala
CA	Cornu Ammonis
CeA	Central amygdala
CMS	Chronic mild stress
CRH	Corticotropin-releasing hormone
CUS	Chronic unpredictable stress
CVMS	Chronic variable mild stress
CVS	Chronic variable stress
DA	Dopamine
DG	Dentate gyrus
Dlgap2	Disks Large-Associated Protein
DMN	Default mode network
DNMT	DNA methyl transferase
ECN	Central executive network
GR	Glucocorticoid receptor
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HDMT	Histone demethylase
HMT	Histone methyl transferase
HPA	Hypothalamic-pituitary adrenal
iDISCO+	Immunolabeling-enabled three-dimensional imaging of solvent-cleared organs
IEG	Immediate early gene
LA	Lateral amygdala
LCN	Lateral cortical network
MDD	Major depressive disorder
miRNA	MicroRNA
MR	Mineralocorticoid receptor
NAc	Nucleus accumbens
NE	Norepinephrine
OFC	Orbitofrontal cortex
PaS	Parasubiculum
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
PFC	Prefrontal cortex

PoS	Postsubiculum
PPI	Pre-pulse inhibition
PrS	Presubiculum
PTSD	Post-traumatic stress disorder
PV	Parvalbumin
PVN	Paraventricular nucleus
RFP	Red fluorescent protein
RSP	Retrosplenial cortex
RT	Room temperature
SAM	Situationally accessible memory
SD	Standard deviation
SEFL	Stress-enhanced fear learning
SEM	Standard of the mean
SN	Salience network
SOM	Somatostatin
TH	Tyrosine hydroxylase
TM	Tamoxifen
TPH	Tryptophan hydroxylase
TRAP	Targeted recombination in active populations
VAM	Verbally accessible memory

Acknowledgements | Dankwoord

Curriculum vitae

Bart Christiaan Joseph Dirven werd op 7 september 1992 geboren in Oosterhout, Noord-Brabant. Na het *cum laude* behalen van het gymnasiumdiploma op het Sint Oelbert-gymnasium te Oosterhout in 2010, startte hij met de bachelor Moleculaire Levenswetenschappen aan de Radboud Universiteit in Nijmegen. Tijdens zijn bachelor rondde hij tevens het Interdisciplinaire Honoursprogramma af aan dezelfde universiteit. Na het *cum laude* afronden van zijn bachelor in 2013, vervolgde hij zijn studie met de Research Master Cognitive Neuroscience, eveneens aan de Radboud Universiteit, waar hij in 2015 zijn diploma behaalde.

Tijdens zijn bachelor- en masteropleiding liep Bart twee stages. Tijdens zijn bachelorstage, aan de afdeling Behavioural Neurogenetics van het Radboudumc, onderzocht hij het effect van maternaal gebruik van specifieke antidepressiva (SSRIs, serotonin reuptake inhibitors) tijdens de zwangerschap op de groei en reflexontwikkeling van jonge ratten. Hij werd hierbij begeleid door dr. Yvet Kroeze en prof. dr. Judith Homberg. Zijn masterstage liep Bart op de afdeling Neurochemie van het Translationeel Metabool Laboratorium in het Radboudumc, onder begeleiding van Elisanne Biemans en dr. Marcel Verbeek. Gedurende deze stage onderzocht hij de aggregatiekarakteristieken en toxiciteit van het bèta-amyloïd eiwit in cerebrovasculaire gladde spiercellen. Specifiek onderzocht hij hoe beide factoren beïnvloed werden door de lengte van bèta-amyloïd, alsmede potentiële interacties van dit eiwit met apolipoproteïne E. Dit onderzoek gaf relevante inzichten in de rol van bèta-amyloïd in de Ziekte van Alzheimer. Tijdens deze stage heeft Bart ruime ervaring opgedaan met o.a. celkweek, assays voor cellulaire levensvatbaarheid, SDS-PAGE, Western blotting, aggregatiemetingen, ELISA en PCR.

In september 2016 begon Bart als promovendus op de afdeling Anatomie (tegenwoordig: Beeldvorming) in het Radboudumc te Nijmegen, onder begeleiding van prof. dr. Tamás Kozicz, prof. dr. Judith Homberg en dr. Marloes Henckens. Gedurende zijn promotieonderzoek heeft hij zes studenten begeleid bij hun bachelor- en masterstages. Enkele van Bart's onderzoeksresultaten zijn gepubliceerd en gepresenteerd bij verschillende internationale conferenties, middels het geven van

poster- en mondelinge presentaties. Hieronder vallen onder andere de Munich Winter Conference on Stress, Dutch Neuroscience Meeting, Donders Discussions en de Society for Neuroscience meeting.

List of publications

Used in this thesis:

1. **Dirven BCJ**, Homberg JR, Kozicz T, Henckens MJAG. Epigenetic programming of the neuroendocrine stress response by adult life stress. *J Mol Endocrinol*. 2017 Jul;59(1):R11-R31. doi: 10.1530/JME-17-0019. Epub 2017 Apr 11. PMID: 28400482.
2. **Dirven BCJ**, van Melis L, Daneva T, Dillen L, Homberg JR, Kozicz T, Henckens MJAG. The hippocampal memory engram coding traumatic stress susceptibility. *In preparation*.
3. **Dirven BCJ**, Botan A, van der Geugten D, Kraakman B, van Melis L, Merjenburgh S, van Rijn R, Waajen L, Homberg JR, Kozicz T, Henckens MJAG. Longitudinal assessment of amygdala activity in mice susceptible to trauma. *Psychoneuroendocrinology*. 2022 Nov;145(105912). doi: 10.1016/j.psyneuen.2022.105912. Epub 2022 Aug 31.
4. **Dirven BCJ**, Negwer M, Mahadevan HM, Maas R, van Melis L, Merjenburgh S, van Rijn R, Botan A, Grandjean J, Homberg JR, Kozicz T, Henckens MJAG. Susceptibility to stress: Temporally specific changes in brain-wide neuronal activity and functional network connectivity at cellular resolution. *In preparation*.

Other:

1. Kroeze Y, **Dirven BCJ**, Janssen S, Kröhnke M, Barte RM, Middelman A, van Bokhoven H, Zhou H, Homberg JR. Perinatal reduction of functional serotonin transporters results in developmental delay. Neuropharmacology. 2016 Oct;109:96-111. doi: 10.1016/j.neuropharm.2016.05.012. Epub 2016 May 18. PMID: 27208789.
2. **Dirven BCJ**, van der Geugten D, Filipe ACTM, van Bodegom M, Madder L, van Agen L, Homberg JR, Kozicz T, Henckens MJAG. Aberrant ventral dentate gyrus structure and function in trauma susceptible mice. *Transl Psych* (under revision). Available at bioRxiv: https://doi.org/10.1101/2020.10.01.321893.

PhD portfolio

Name PhD student: B.C.J. Dirven	**PhD period:** 01-09-2016 until 31-08-2020
Department: Medical Imaging, Anatomy	**Supervisors:** Prof. dr. L.T. Kozicz Prof. dr. J.R. Homberg
Graduate school: Donders Center for Medical Neuroscience (DCMN)	**Co-supervisor:** dr. M.J.A.G. Henckens

Training activities	**Year(s)**	**ECTS**
a) Courses & Workshops		
- Course on Laboratory Animal Science	2016	3
- Donders Graduate School Introduction Day	2016	0.25
- Summer School Molecules, Mice and Math: A Statistical Toolbox for the lab	2017	2
- Course Programming Skills: Python	2018	3
- Course Scientific integrity	2018	0.25
- Course Education in a nutshell	2018	1
- Donders Graduate School Day	2020	0.25
b) Seminars & Lectures		
- Scientific meetings department Anatomy	2016-2020	4
- Scientific meetings department Cognitive Neuroscience	2016-2020	4
c) (Inter)national Symposia & Congresses		
- Munich Winter Conference on Stress	2017	0.86
- Dutch Neuroscience Meeting	2017, 2018	1.14

- Donders Discussions	2018	0.5
- Stress-NL Meeting	2017, 2019	0.5
- Society for Neuroscience Meeting	2019	2.14

d) Supervision of internships

- MSc Lennart van Melis	3
- MSc Riv Maas	3
- BSc Lieke Dillen	2
- BASc Andriana Botan	2
- MSc Hariharan Murali Mahadevan	1
- BSc Teya Daneva	3

TOTAL	**36.89**

Research Data Management

Data acquired during my PhD at the Radboudumc and Donders Institute for Cognitive Neuroscience are archived according to the Findable, Accessible, Interoperable, and Re-usable (FAIR) principles. The laboratory journals, protocols, and data can be obtained upon request by contacting the corresponding author from the specific chapters as well as by contacting the Department of Medical Imaging, Anatomy at the Radboudumc in Nijmegen, the Netherlands.

Animal experiments described in this thesis were performed according to the appropriate Dutch federal regulations for animal protection and welfare, and were approved by the Central Authority for Scientific Procedures on Animals (CCD), as well as by the Veterinary Authority of the Radboudumc. All efforts were made to minimize animal suffering and to reduce the number of animals used, according to the National Institutes of Health guide for the care and use of laboratory animals.

Donders Graduate School for Cognitive Neuroscience

For a successful research Institute, it is vital to train the next generation of young scientists. To achieve this goal, the Donders Institute for Brain, Cognition and Behaviour established the Donders Graduate School for Cognitive Neuroscience (DGCN), which was officially recognized as a national graduate school in 2009. The Graduate School covers training at both Master's and PhD level and provides an excellent educational context fully aligned with the research programme of the Donders Institute.

The school successfully attracts highly talented national and international students in biology, physics, psycholinguistics, psychology, behavioral science, medicine and related disciplines. Selective admission and assessment centers guarantee the enrolment of the best and most motivated students.

The DGCN tracks the career of PhD graduates carefully. More than 50% of PhD alumni show a continuation in academia with postdoc positions at top institutes worldwide, e.g., Stanford University, University of Oxford, University of Cambridge, UCL London, MPI Leipzig, Hanyang University in South Korea, NTNU Norway, University of Illinois, North Western University, Northeastern University in Boston, ETH Zürich, University of Vienna etc. Positions outside academia spread among the following sectors: specialists in a medical environment, mainly in genetics, geriatrics, psychiatry and neurology. Specialists in a psychological environment, e.g., as a specialist in neuropsychology, psychological diagnostics or therapy. Positions in higher education as coordinators or lecturers. A smaller percentage enters business as research consultants, analysts or head of research and development. Fewer graduates stay in a research environment as lab coordinators, technical support or policy advisors. Upcoming possibilities are positions in the IT sector and management positions in pharmaceutical industry. In general, the PhD graduates almost invariably continue with high-quality positions that play an important role in our knowledge economy. For more information on the DGCN, as well as past and upcoming defenses, please visit: http://www.ru.nl/donders/graduate-school/phd.