The Role of Hippocampal Memory Engram in Mediating Stress Susceptibility in an Animal Model of Depression

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Abstract

Apart from mood changes, depression has been associated with cognitive changes such as biased memory for negative stimuli. Imaging studies suggest the bias in processing of negative information in the brain is related to the enhanced functioning of the hippocampus. We hypothesize that the facilitated formation of hippocampal engram cells, cellular substrates for memory, is related to the cognitive bias for negative stimuli in depression.

We employed a chronic social defeat model to examine the relationship between hippocampal engram cells and depression-related behaviours. We used a transgenic TetTag mouse model that allows the tagging of activated neurons by a reporter gene LacZ at an earlier time point for studying their reactivation at a later time point by examining the co-expression of LacZ with an activity related immediate early gene cFos. TetTag mice were stressed by social defeat, consisting of daily attacks by and co-housing with an aggressive mouse. After 8 days of social defeat, mice were separated into susceptible (exhibiting social avoidance) and resilient groups according to their social behaviour. Engram cells were reactivated by an extra episode of social defeat to induce cFos expression. Neurons with both LacZ and cFos labeling represent engram cells. Due to the differential roles of the dorsal and ventral hippocampus in spatial and emotional memory formation, we looked at changes in engram cells in these two regions separately.

We found no statistical significant changes in the density of LacZ- and cFos- labeled hippocampal CA1 neurons in susceptible mice compared to resilient and non-stressed control mice in either the dorsal or ventral hippocampus. However, susceptible mice displayed higher LacZ cell density than other mouse groups when we compared the dorsal and ventral data together. Intriguingly, we found significantly more engram cells in susceptible mice than other

mouse groups in both the dorsal and ventral hippocampus. No difference in LacZ labeled and engram cells between mouse groups was found in the dentate gyrus. When we stopped the labeling of hippocampal neurons by LacZ before social defeat, we did not see the increase in engram cell density in the dorsal hippocampus of susceptible mice. However, we still observed higher engram cell density in these mice than resilient and control mice in the ventral hippocampus when LacZ labeling was terminated before social defeat.

Our findings suggest susceptible mice may have an enhanced hippocampal memory for social stress, which may underlie the development of depressive behaviours in these animals.

Résumé

Autre que les changements d'humeur, la dépression a été associée à des biais cognitifs tels que la mémoire biaisée des stimulus négatifs. Des études d'imagerie du cerveau suggèrent que le biais dans le traitement des informations négatives est lié au fonctionnement augmenté de l'hippocampe. Nous émettons l'hypothèse que la formation facilitée des cellules engrammes de l'hippocampe, soit le substrat cellulaire des souvenirs, est lié au biais cognitif pour les stimulus négatifs liés à la dépression.

Nous employons un modèle de défaite sociale chronique pour examiner la relation entre les cellules engrammes de l'hippocampe et les comportements liés à la dépression. Nous utilisons un modèle de souris transgéniques TetTag qui permet le marquage de neurones activés par le gène rapporteur LacZ à un moment antérieur afin de comparer avec des neurones actifs plus tard. Les souris TetTag sont stressées selon le paradigme de la défaite sociale, qui consiste d'attaques quotidiennes suivies par la cohabitation avec une souris agressive. Après 8 jours de défaite sociale, les souris ont été séparées en 2 groupes : les souris susceptibles (qui évitent le contact social) et les souris résilientes. Les cellules engrammes sont réactivées par une épisode supplémentaire de défaite sociale, afin d'induire l'expression du gène précoce-immédiat cFos. Les neurones marqués à la fois par LacZ et cFos représentent les cellules engrammes.

Nous avons observé plus de neurones marqués LacZ dans les neurones hippocampiques du CA1 chez les souris susceptibles par rapport aux souris résilientes et les souris contrôles, non-stressées. Cette différence entre les groupes est par contre disparue lorsque nous avons analysé séparément les données de l'hippocampe dorsal et ventral. Curieusement, nous avons trouvé significativement plus de cellules engrammes chez les souris susceptibles par rapport aux autres groupes de souris dans à la fois l'hippocampe dorsal et ventral. Aucune différence n'a été

observée entre les groupes de souris dans le gyrus denté, ni dans le nombre de cellules marquées LacZ ou le nombre de cellules engrammes. Nos résultats suggèrent que les souris susceptibles pourraient avoir une mémoire hippocampique améliorée pour le stress social.

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Contributions of Authors

I wrote this thesis with editing from Dr. Tak Pan Wong. The manuscript in Chapter 2 is written by Dr. Tak Pan Wong and I.

All experiments and analyses were conducted by myself unless stated otherwise. Perfusion of TetTag animals was done by Alice Wong. Viral injections were performed by myself, Alice Wong and Dr. Tak Pan Wong. Neuronal counting was completed in joint with Amanda Larosa, Vanessa Wong, Alice Wong and Dr. Tak Pan Wong.

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Chapter 1: Introduction

1.1 Framing the question

Depression is a common and severe mental disorder. Nearly 30% of the population has one depressive episode at some points in their lives (Clark and Beck 2010) and 75% will go on to have recurrences of depressive episodes (Boland et al. 2002). Common symptoms of depression include mood changes, inability to feel pleasure (anhedonia) and having suicidal tendencies. There is an imperative need to understand the biological mechanisms underlying depression for the development of effective treatments and therapies.

There are cognitive deficits in depressed individuals including the inability to concentrate, slow responsive time and recurrent negative thoughts (Kircanski et al. 2012). Interestingly, according to the cognitive theory of depression, maladaptive cognitive functions in turn lead to depression onset and maintenance. Depressed individuals have a negative style of thinking that leads to a negative perception of their life events, a stronger memory of the negative events and easier recall of the negative memory (Disner et al. 2011). Negative information processing underlies the formation of the above cognitive changes in depression. Previous studies found that negative information processing in the brain is altered in depressed patients: attention, and memory to negative information is enhanced and inhibition of negative thoughts is impaired in depressed patients (Berman et al. 2011; Clark and Beck 2010; Joormann et al. 2015). Neuroimaging studies found that the hippocampus is one of the brain areas involved in negative information processing (Mayberg et al. 1999). The hippocampus, well known for its roles in learning and memory, may be a critical player in the formation and maintenance of depression.

The hippocampus processes negative and stressful information and is also involved in encoding episodic memory (VarghaKhadem et al. 1997). The hippocampus may mediate both the

encoding and retrieval of the negative memory in depressed individuals. Mechanistically speaking, there are engram cells in the hippocampus responsible for memory storage and recall. Hippocampal engrams cells are activated by learning and are re-activated during memory retrieval (Tonegawa et al. 2015). Does hippocampal engram cell activation differ in individuals with depression, or depression-related symptoms, compared with those that with out?

There are animal models available to examine the neuronal basis of depression. Inbred mice exhibit depression-related behaviours after stress that are analogous to depressive symptoms in humans, including the decreased ability to feel pleasure and social avoidance (Krishnan and Nestler 2008). Transgenic mouse models, such as the TetTag mice we employed in this study, are available for examining hippocampal engram activation.

In this study, we used TetTag mice to examine hippocampal neuronal engram activation and reactivation between animals with depressive-related behaviours compared to those that with out.

1.2 Depression

Major depressive disorder, often referred to simply as depression, is a devastating mood disorder. Major depressive disorder is characterized by symptoms including: depressed mood, anhedonia (reduced ability to feel pleasure from rewards), irritability, abnormalities in appetite and sleep and more (Krishnan and Nestler 2008; Berton et al. 2006; Golden et al. 2011). Affecting approximately 350 million people worldwide (World Health Organization 2016), the World Health Organization Global Burden of Disease Study has long ranked depression as the single most burdensome disease worldwide in terms of total disability-adjusted years (Murray and Lopez 1996). This is because depression is frequently comorbid with a number of other

chronic illnesses including other psychiatric disorders, type II diabetes, acute coronary syndrome, and even cancer (Kang et al. 2015). A report by the Conference Board of Canada in 2016 found depression cost $32.3 billion in gross domestic product annually (The Conference Board of Canada, 2016).

On an individual level, Pratt and Brody (2008) found that eighty percent of depressed individuals are impaired in daily functioning. It has also been shown that depressed individuals lose 5.6 hours of productive work each week to depression (Stewart et al. 2005). About 50% of the loss of productivity is due to absenteeism and short-term disability (Kessler et al. 2006). Depressive individuals are also more likely to be out of work or not looking for work (Greenberg et al. 2015)

Moreover, there is an increase in the depressive population, therefore a growing burden on society (Patten et al. 2017). From 2005 to 2010, depression prevalence rose from 13.8 million to 15.4 million adults in the United States, subsequently increasing the economic burden of depressive individuals from $173.2 billion to $210.5, a 21.5% increase (Greenberg et al. 2015).

Thus, it is imperative to identify factors contributing to the cause of depression, to develop effective treatment, if not preventative measures.

1.3 Depression and Cognition

Depression is characterized by not only mood changes of the affected individual, but also changes in cognitive functions. There are specific aspects of cognition that are impaired in depressed individuals, including difficulties engaging in tasks that require significant mental effort (Ellis and Ashbrook 1989), deficits in executive function, and lack of inhibition (Joormann and Gotfib 2008). Weingartner (1986) proposed that depression is selectively associated with a

dysfunction in cognitive processing that demands much mental effort and many depression studies found executive dysfunction to be a key feature of major depressive disorder. Executive functions are cognitive processes that integrate and regulate other cognitive processes, according to Bryan and Luszcz (2000). Both phonetic and categorical fluency are affected in depression, although the impairments are mild (Stordal et al. 2004). Performance on the Stroop test which examines selective allocation of attention to eligible responses (Lamers et al. 2010) is decreased in depressive cohort compared to controls (Stordal et al. 2004). Depressed patients perform worse than control individuals in memory measures of working memory and on delayed free recall, but not of verbal learning and recognition (Egeland et al. 2003). The same study assessed learning style and found depression is associated with enhanced verbal memory acquisition while having difficulties with memory retrieval and forgetting (Egeland et al. 2003).

1.4 Cognitive Theory of Depression

In addition to cognitive deficits, there are studies that indicate the cognitive impairments actually underlie the development and maintenance of depression. Therefore depression is also classified as a cognitive disorder, first proposed by Aaron Beck.

Aaron Beck is an American psychiatrist well known for his cognitive theory of depression. Beck argued that "early adverse events, or stressors, contribute to the establishment of a depressive schema" (Beck 1967). A schema is a cognitive framework or concept that helps organize and interpret information. Individuals with negative schemas will interpret situations in a negative light and limit their attention to the negative aspects of events (Boury et al. 2001). The focus on negative events then perpetuates the negative schema, which then sustains depression

(Disner et al. 2011). Essentially, alterations in negative information processing are responsible for both the development and maintenance of depression.

Related to the cognitive theory of depression is the differential activation hypothesis (DAH). In addition to the negative schema that Beck proposed to be persistent in depressed patients in both the depressed and non-depressed state, the DAH proposes that vulnerability to severe and persistent depression is related to differential thinking patterns while individuals are currently in the depressed state (Teasdale 1988). The DAH proposes that negative cognitive processes determine both the time course and severity of depressive episodes (Teasdale 1988). Teasdale postulated while mild depressive episodes are very common, a sad mood can interact with a negative thinking style reciprocally and push a mild dysphoria into a severe depressive episode.

Clearly, negative information processing plays a key role in the depressive disorders according to both Beck's cognitive theory of depression and Teasdale's DAH. An overview of the biases in negative information processing in depressed patients is such: (1) there is a bias in attention to negative stimuli in depressed individuals; (2) the negative stimuli is then remembered better in memory; (3) depressed individuals have a harder time casting away or moving on from the negative information after it becomes irrelevant.

Researchers have found depressed individuals pay preferential attention to negative information and have trouble disengaging from negative stimuli once they are aware (Kircanski, Joormann, and Gotlib 2012). Gotlib and Cane (1987) showed that depressed individuals exhibit longer response latencies to negative words in an emotional Stroop test, a test for information processing of emotions. However, other researchers were not able to consistently replicate this finding (Mathews and MacLeod 2005). Using a spatial-cueing task to measure higher stages of

emotional processing, Koster et al. (2010) found patients with depressive symptoms showed negative emotional bias only under conditions that allowed for elaborate emotional processing.

Once attention is given to negative information, studies have also found that depressed individuals have a biased memory for negative events. In the same spatial-cueing task conducted by Koster et al. (2010), they found a coherence between attention bias and memory bias in patients with depressive symptoms. Depressed individuals recall sad faces or words more than non-depressed individuals in both recognition and familiarity tasks (Ridout et al. 2003; Koster et al. 2010; Kensinger and Corkin 2003). In a negative mood, depressed individuals recall more negative self-related adjectives (Hedlund and Rude 1995), less vivid happy memories (Werner-Seidler and Moulds 2011), but had more vivid and distressing intrusive negative memories than control subjects (Newby and Moulds 2011; Matt et al. 1992).

Depressive individuals have decreased ability to inhibit the processing of negative irrelevant information. Even after the negative information is no longer useful, depressive individuals have difficulties shifting their thoughts away from it. Depressed individuals are impaired in tasks assessing flexibility to switch between tasks, such as the Wisconsin Card Sorting Task (WCST) or the Go-No-Go task (Jones et al. 1988). For individuals to actively disengage from emotional information that is no longer relevant, individuals need to exercise inhibition. Depressed or previously diagnosed depressed individuals have reduced inhibition of irrelevant emotional material, as shown by the negative affective priming (NAP) task (Goeleven et al. 2006; Joormann 2004). Joormann and Gotlib (2008) used a modified Sternberg task to examine the ability of depressed individuals to actively reject previously relevant material from working memory. Depressed individuals have more difficulties rejecting negative emotional words from working memory compared to non-depressed control individuals. In addition,

negative autobiographical events often intrude into the minds of depressed individuals at high frequency (Brewin et al. 1999). Kircanski et al. (2012) suggested that cognitive difficulties in inhibiting and actively rejecting negative material may be part of the reason depressed individuals are prone to experience recurrent, persistent and uncontrollable negative thoughts. Other studies also suggest that depressed patients have trouble forgetting information, which may lead to increased rumination (Hertel and Gerstle 2003).

Rumination is a common symptom in depression or in people at risk for depression (Nolen-Hoeksema, et al. 2008). Rumination is defined as obsessive and perseverative thinking about one's emotions and problems rather than specific event details (Nolen-Hoeksema, et al. 2008). Rumination is thought to maintain and exacerbate depression by enhancing negative thinking, impairing problem solving, interfering with instrumental behavior, and eroding social support (Nolen-Hoeksema, et al. 2008). Disner et al. (2011) proposed that rumination is associated with altered emotion and memory processing, increased self-referential processing and decreased executive inhibition of these processes. Clinically depressed patients think more negatively about the past, present and future. Depressed patients recall more negative memories and are biased in thinking the negative events occurred more frequently than reality (Lyubomirskyet al. 1998; McFarland and Buehler 1998). Questionnaire measures of cognitive attitude found increased negative thinking, in both measures of negative automatic thoughts and measures at assumptions, in patients currently undergoing a depressive episode (Weissman and Beck 1978).

Overgeneralization is another feature commonly found in depressed individuals. Suicidal patients fails to retrieve specific memory for both positive and negative cue words (Williams and Broadbent 1986). Meta-analysis of studies examining performances of depressed individuals in

cued memory retrieval found strong evidence for a close association between memory overgeneralization and depression or depressive symptoms (Williams et al. 2007). In addition, overgeneralized memory recall was strongly associated with failure to recover from depression. Overgeneralization usually does not improve over time, suggesting that it is a trait marker associated with depression (Brittlebank et al. 1993).

Changes in negative information processing, including inability to inhibit negative thoughts, underlie rumination and other cognitive deficits exhibited by depressed individuals. A neuroimaging study using positron emission tomography (PET) suggests that the biased processing of negative and stressful events is related to enhanced functioning of the limbic system in the brain, including the hippocampus (Mayberg et al. 1999). Hamilton and Gotlib (2008) also showed that the increase in negative memory sensitivity in depressed individuals is related to an increase in functional connectivity between the hippocampus and the amygdala. Therefore the hippocampus is one of the brain areas of interest to study the link between negative information processing and the development of depressive symptoms.

1.5 The Hippocampus

The hippocampus is one of the key brain regions involved in cognitive functions such as learning and memory. The case of patient H.M. first raised the attention of the memory functions of the temporal lobe. Patient H.M. had bilateral surgical removal of the medial temporal lobe, including both hippocampi, and onwards had severe anterograde and retrograde amnesia (Scoville and Milner 1957). Patient H.M was unable to form long-term memories of either facts or events without significant changes in intelligence. Another patient with medial temporal lesion specifically of the CA1 subregion had difficulties with forming both semantic and episodic

memory (Zolamorgan, Squire, and Amaral 1986). Patients with damage to the medial temporal lobe due to Alzheimer's disease often have deficits in spatial and episodic memory (Kolb and Wishaw, 1996). Furthermore, Vargha-Khadem et al. (1997) found patients with early on-set hippocampal damage have severe anterograde amnesia.

The hippocampus is also unique in its neuroanatomical organization. In contrast to the neocortex, principle neurons in the hippocampus culminate in one layer and form the canonical trisynaptic circuit (Anderson et al. 2007). Information from sensory cortical regions is relayed into the entorhinal cortex (EC) and passes on to the granule cells of the dentate gyrus (DG). Granule cell axons called mossy fibers synapse on to the CA3 subregion that sends projections via the Schaffer collateral axons onto the CA1 pyramidal cell. Finally, the CA1 sends information unidirectionally to the subiculum as an output centre.

In terms of subregion functionalities, the DG plays an important role as a pattern separator. The DG granule neurons are under constant strong interneuron inhibition with a resting membrane potential lower than either the CA1 or CA3 (Scharfman 1992; Sik et al. 1997; Soltesz and Mody 1994) and fires sparsely to encode information (Guzowski et al. 1999). Granule cells have been shown to contain place cells that encode spatial information (Muller, Kubie, and Ranck 1987). Studies also found differential activation of the DG to subtle changes in context, indicating the DG as a pattern separator (Deng et al. 2013; Denny et al. 2014; Leutgeb et al. 2007).

The CA3 region is involved in pattern completion; Treves and Rolls (1992) used a computational model to suggest that the CA3 is a powerful auto-associator. The CA3 recurrent pathway is activated by a strong afferent input from the entorhinal cortex and sends the information back on itself to finally relay a specific enough signal for information retrieval. The

CA3 functional impairment via NMDA receptor knock-out or lesions showed a deficit in contextual and spatial learning (Lee and Kesner 2004; Nakazawa et al. 2003).

The CA1 is the main output region of the hippocampus, critical for the main hippocampal roles of learning and memory. Focal lesions of the CA1 in human patients significantly altered their autobiographical and detailed episodic memory retrieval (Bartsch et al. 2011). The CA1 is also shown to be involved in maintaining the sequence of memories with long intervals (Farovik et al. 2010). Place cells in the CA1 not only encode for a physical location but also an intended destination (Ainge et al. 2009).

1.6 Engram Cells

The mechanisms of hippocampal memory encoding have been extensively researched. One neuronal mechanism for storing and retrieving memory is through the formation and reactivation of engram cells.

German biologist Richard Semon first coined the term engram in 1908, meaning a physical change of neurons in the brain responsible for a certain memory. Engram cells are now considered as neurons that are activated by experience. Reactivation of engram cells is believed to mediate memory retrieval (Tonegawa et al. 2015). The formation of engram cells during learning could be related to associative firing of neuronal ensembles and strengthening of synaptic connections between them. During memory retrieval, the same neurons are reactivated due to the strengthened connection between engram neurons (Josselyn et al. 2015).

One common method of engram cells identification is to use immediate early genes to examine neurons that were activated by a specific memory and the retrieval of said memory. Immediate early genes are proteins induced by Ca^{2+} influx due to high levels of synaptic

stimulation and are indications of neuronal activation (Sheng and Greenberg 1990). Normally quiescent, immediate early genes are transcribed minutes after neuronal stimulation and protein expression lasts for hours (Schilling et al. 1991). Observational studies used immediate early genes to guide the tagging of activated neurons during learning to compare with neuronal activation during memory retrieval. Using this approach, previous studies found engram cells in the amygdala (Reijmers et al. 2007), hippocampus (Denny et al. 2014; Tayler et al. 2013), somatosensory cortex (Yokoyama and Matsuo 2016) and the prefrontal cortex (Kitamura et al. 2017). The studies showed that engram cells are reactivated significantly above chance levels to encode and retrieve the memory for a particular event.

The necessity and sufficiency of engram cells for memory retrieval was demonstrated by loss of function and gain of function studies respectively. Han et al. (2009) allocated certain lateral amygdala neurons to preferentially become engram cells encoding for contextual fear information by over-expressing the transcription activator cAMP response element-binding protein (CREB). Following learning, the specific ablation of these artificial engram cells interfered with the fear memory recall. Furthermore, fear memory recall impairment was found in mice when previously active hippocampal CA3, DG (Denny et al. 2014) or CA1 neurons (Tayler et al. 2013) were inhibited. Place preference to cocaine was also abolished when neurons active during cocaine-association learning were inactivated (Cruz et al. 2014). Meanwhile, Liu et al. (2012) showed that optogenetically activating the hippocampal engram for fear memory leads to fear-response behaviour in mice, even in contexts they did not learn to associate with fear. Similarly, activating neurons recruited in fear memory encoding via chemogenetics also induced fear behaviour in a novel context (Kim et al. 2014).

The above evidence all demonstrate engram cells are essential to memory retrieval and therefore engram cells are termed cellular substrates of memory.

1.7 Animal Models of Depression

Although depression is fundamentally a human disorder, studying the underlying biological mechanism for depression in humans is a difficult task. Therefore, animal models of stress and depression have been developed to mimic symptoms of depression while allowing for examination of neuronal substrates and molecular mechanisms. One important animal model for depression is the rodent social defeat model.

Most animal studies of depression focused on comparisons between stressed and non-stressed individuals, while few of them examined the neuronal mechanisms underlying individual differences in susceptibility to depressive-symptoms following stress. In reality, the majority of the general population do not develop psychiatric disorders such as depression, post-traumatic stress disorder (PTSD), and anxiety disorder following stressful or traumatic events (Franklin et al. 2012). These individuals who exhibit resilience are able to recover from stressful experiences and behave similar to naïve individuals never exposed to the same traumas. On the other hand, there are susceptible individuals who are more prone to attaining psychiatric disorders after stress. Therefore it is important to study individual differences in stress susceptibility in order to prevent and treat disorders such as the PTSD, anxiety disorders and depression. The rodent social defeat model allows studying of not only the acute effect of chronic stress, but also individual differences in response to stress. Under the chronic social defeat stress, only a portion of inbred C57 mice become susceptible to this stressor and develop depression-

related symptoms such as social avoidance, anhedonia and blunted circadian amplitude, while the remaining stressed mice are resilient to this stressor (Krishnan et al. 2007).

The social defeat model has ethological relevance in examining social subordination (Malatynska and Knapp 2005) and it generates long-lasting depression-like behaviour (Krishnan et al. 2007) that can be reversed by chronic, not acute, anti-depressant treatment (Tsankova et al. 2006).

1.8 Differences in Depressive-Symptom Susceptibility

As previously stated, negative and stressful events play a key role in the development of depression according to the cognitive theory of depression. Interestingly, the hippocampus is also involved in mediating the body's stress response. When faced with a stressful event, the hippocampus is one of the first brain regions activated to coordinate the body's immediate response. The hippocampus has reciprocal connections with the stress regulator, the hypothalamus-pituitary-adrenal axis (HPA-axis) (Pariante and Lightman 2008). In fact, the communication between the hippocampus and the HPA-axis is impaired in depression, causing increased activation of the HPA-axis that has an effect on the acquisition of new memories and the emotional appraisal of events (Lupien et al. 2002).

Recent studies suggest that the hippocampus also regulates stress susceptibility. Examining hippocampal volume in a chronic social defeat stress model revealed a deficit in hippocampal growth only in susceptible mice (Tse et al. 2014). Increased neurogenesis has been found in mice (Lagace et al. 2010) and monkeys (Lyons et al. 2010) that are susceptible to stress. In addition, ventral hippocampal transmission to nucleus accumbens is increased in susceptible animals (Bagot et al. 2015).

The hippocampus, therefore, makes an ideal brain region of interest to determine whether individuals exhibiting depression-related symptoms have an enhanced memory of stressful events.

1.9 Research Question and Objectives

Given the importance of the hippocampus in mediating stress susceptibility, and the known contribution of the biased cognitive processing and memory formation in the vulnerability to depression, we proposed that hippocampal engram cells could be cellular substrates that underlie stress susceptibility and resilience.

Stemming from the research questions are two objectives to be addressed by the current study:

Objective (1): to examine whether the hippocampus regulates stress susceptibility from a cognitive perspective, specifically whether memory engram formation and reactivation is related to depressive behaviour.

Objective (2): to examine whether hippocampal engram cells are causally linked to the development of depressive behaviours.

We hypothesize that: (1) susceptible animals may have a hippocampus more sensitive to stress, as indicated by an increase in neuronal activation at stress onset and that susceptible animals will have more hippocampal engram cells related to stress to reflect the enhanced negative information processing in depressed individuals; (2) activating engram cells related to stressful experience will lead to depressive behaviour in animals

Chapter 2: Manuscript

The methodology and findings for the current masters project are summarized and presented in a manuscript by Zhang et al. in preparation. The manuscript includes an introduction that states the rationale for the study, which is elaborated in the Chapter 1: Introduction in the current thesis. Detailed methodology, results and related figures are presented in the Chapter 2: Zhang et al. Manuscript. There are data not shown in the manuscript that will be presented and discussed in Chapter 3: Supplemental Data. The discussion in the manuscript is also expanded upon in Chapter 4: Discussion. A bibliography of all references cited in the thesis and the manuscript is present at the end of this thesis.

Susceptibility to chronic social defeat stress is related to increased hippocampal CA1 engram cell formation

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Introduction

Alterations in the structure and function of the hippocampus have been highly implicated in depression. Meta analyses have revealed reduced hippocampal volume in depressed patients (Videbech and Ravnkilde, 2004; McKinnon et al. 2009). The therapeutic effects of classical antidepressants (e.g. fluoxetine) could be abolished by inhibiting hippocampal neurogenesis (Santarelli et al. 2003). The fast acting antidepressant action of ketamine was associated with altered phosphorylation status and functional properties of glutamate receptors in the hippocampus (Maeng et al. 2008; El Iskandrani et al. 2015). These findings strongly suggested that the manifestation of depression symptoms is related to both structural and functional changes of the hippocampus. Given the important roles of the hippocampus in learning and memory (Squire, 1992), the hippocampus could be a major culprit for cognitive symptoms of depression, especially those related to memory.

Apart from anxiety, sad mood, anhedonia, hopelessness and low self-esteem, cognitive symptoms are also common in depression. Cognitive symptoms in depressed patients include slow cognitive speed and reaction time, impairments in executive function and deficit in episodic memory (McDermott and Ebmeier, 2009; Marazziti et al. 2010). Depression is also known for the negative bias in cognitive processing and memory formation (for review, see (Disner et al. 2011; Joormann and Quinn, 2014)). Depressed patients exhibited biased attention to negative stimuli and mood-congruent interpretation of emotion-related events. Regarding memory, depression is associated with enhanced encoding and recall of mood-congruent negative memory (Koster et al. 2010), less forgetting of negative memory (Hertel and Gerstle, 2003), and impaired recall of positive memory (Gaddy and Ingram, 2014). Ruminative thoughts of negative emotions are also common in depression (Nolen-Hoeksema, 2000). Negative biased in cognitive

processing and rumination predicts the vulnerability to depression (Alloy et al. 1999; Rude et al. 2003; Abela and Hankin, 2011), suggesting a causal relationship between biased cognitive processing and depression. Imaging findings support the notion that increased hippocampal responses to negative stimuli could be related to the memory bias in depression. Depressed patients exhibited increased responses of the neural network including the hippocampus, amygdala, striatum and cingulate cortex to sad faces (Fu et al. 2004). The connectivity between the amygdala and the hippocampus during encoding of negative information in depressed patients was also stronger than control subjects (Hamilton and Gotlib, 2008). Hippocampal responses to negative stimuli could be attenuated by antidepressant treatment (Mayberg et al. 2000; Fu et al. 2004). Indeed, a lower than control hippocampal response to negative stimuli was found in remitted depressed patients (Thomas et al. 2011), suggesting a contribution of suppressing hippocampal function to maintain remission. Rumination has also been associated with increased functioning of several cortical regions and the hippocampus (Denson et al. 2009; Mandell et al. 2014). These human findings, which strongly supported the importance of enhanced hippocampal function in mediating depression-related cognitive bias, warranted further studies in animal models using techniques with higher resolution and precision to reveal underlying mechanisms.

In this study, we aim to examine the role of the hippocampus in cognitive processing using the chronic social defeat stress model. C57 mice that experienced daily and repeated social defeat due to attacks by aggressive CD1 mice display various depression-related behaviours, including anhedonia and the avoidance of aggressive mice in a social interaction test (Krishnan et al. 2007). The chronic social defeat stress model also has construct validity, since depression-related behaviours in susceptible mice can be ameliorated by chronic, but not acute selective

serotonin reuptake inhibitors (SSRI) treatment (Tsankova et al. 2006), similar to the slow-onset therapeutic effect of SSRIs in depressed patients. Finally, similar to individual differences in stress susceptibility in humans (McEwen and Stellar, 1993), not all stressed mice will express depression-related behaviours after chronic social defeat. We have previously shown that mice that were 'susceptible' to chronic social defeat stress (i.e. developed social avoidance post-defeat) have different stress-related hippocampal volume trajectories than 'resilient' mice that did not express depression-related behaviours after stress (Tse et al. 2014). Notably, social avoidance developed after chronic social defeat stress is context dependent, since defeated mice exhibited lower levels of avoidance if an anaesthetized aggressive mouse or a non-CD1 strain mouse was used in the social interaction test (Krishnan et al. 2007; Venzala et al. 2012). We hypothesized that compared to resilient mice, susceptible mice that exhibit social avoidance after stress would show enhanced contextual memory related to social defeat. To test this, we used TetTag mice with cFos promoter-dependent expression of reporter gene LacZ to examine the formation and activity of hippocampal ensembles during social defeat. Enduring changes in the activity of neuronal ensembles in the hippocampus have been suggested to underlie memory formation (Josselyn et al. 2015; Tonegawa et al. 2015). Using TetTag mice, we labeled activated hippocampal neurons during social defeat and compared the reactivation of defeat-related hippocampal ensembles in TetTag mice that were either susceptible or resilient to chronic social defeat stress.

Materials and Methods

TetTag mice

Male TetTag mice were obtained from the Jackson Laboratory (stock no. 008344). TetTag mouse is a bi-transgenic strain with a cFos driven tetracycline-controlled transactivator (tTA) protein construct and a tetracycline-responsive regulatory element (tetO) driven beta-galactosidase (LacZ) construct. cFos promoter can be activated by neuronal activity. This strain has been used for labeling activated neurons by the expression of LacZ via a doxycycline off (Dox-off) mechanism as previously described (Reijmers et al. 2007). Double hemizygote TetTag mice were bred with wild type C57 mice. Only male double hemizygote offspring (approximately 1/8 of all offspring) were used in this study. Breeding pairs and offspring were fed with Dox-containing food (40 mg/kg, Envigo) ad libitum in a 12 hour light/dark cycle (light on from 8AM to 8PM). LacZ labeling can be induced by feeding TetTag mice with Dox-free food (Dox off), which allows the cFos-driven expression of tetracycline transactivator (tTA) to activate the tetO-LacZ construct. The activation of tetO during Dox off also triggered the expression of a tetracycline-insensitive tTA (with a H100Y point mutation), which sustained the expression of LacZ even after the reintroduction of Dox to maintain long-term labeling of activated neurons. Average age of mice was 3 months. All experiments were approved by the Facility Animal Care Committee at Douglas Institute and followed the guidelines from Canadian Council on Animal Care.

Chronic Social Defeat Stress

The chronic social defeat stress protocol was modified from a previously used protocol (Tse et al. 2014). Briefly, the protocol consists of 4 days of habituation (Day -4 to -1), 8 days of social defeat (Day 1 to 8), a social interaction test (Day 9) and an extra episode of social defeat before sacrificing the mice for immunohistochemical staining (Day 10). Mice were housed in a

rat cage divided by a perforated transparent partition during habituation and social defeat. They were off Dox during habituation and the first two days of social defeat to allow the labeling of hippocampal ensembles by LacZ. Mice were habituated while the systemic Dox levels remain high to reduce the labeling of hippocampal neurons from being housed in a novel environment (Radulovic et al. 1998). During habituation, two TetTag mice were housed in neighbour compartments in each rat cage.

TetTag mice were defeated by male retired breeders of the CD1 strain during social defeat. Resident CD1 mice were housed in a partitioned compartment of a rat cage, identical to that used in habituation, before social defeat. Each CD1 mouse was screened for their aggressiveness by attacking intruders with a latency of less than 60 seconds. The chronic social defeat stress paradigm consisted of 8 episodes of defeat. In each defeat episode, a CD1 mouse was allowed to attack a TetTag mouse for up to 12 attacks in a maximum period of 5 minutes. Following each social defeat episode, each TetTag mouse was housed next to the CD1 mouse in the neighbour compartment there was separated by a perforated partition for 24 hours. Without physical contact, TetTag mice were stressed during cohousing by the presence of visual and odour stimuli from the CD1 mice. Each TetTag mouse was paired with a new CD1 mouse in each of the 8 episodes of social defeat to prevent reduced attack numbers mediated by animal familiarity. LacZ labeling was enabled in the first 2 episodes of social defeat only. Using cFos and LacZ staining, we found that 2 episodes of social defeat were sufficient to induce cFos (Figure 1A, page 48) and LacZ (Figure 1B, page 48) expression in the hippocampus of defeated mice. LacZ labeling was stopped after 2 episodes of social defeat by providing a highly concentrated Dox food (1 g/kg) for 1 day, followed by regular Dox food (40 mg/kg) to prevent further LacZ labeling. There was no neuronal LacZ expression in the hippocampus of TetTag

mice that were always on Dox (Figure 1B, page 48), suggesting the shutdown of LacZ expression by Dox. After 8 episodes of social defeat, stress susceptibility of stressed mice was examined by a social interaction test.

Control non-stressed mice were pair-housed in neighbouring compartments in a rat cage for 8 more days after habituation before the social interaction test. Dox was off during habituation and in the first two days after habituation in these control animals to maintain a similar length of LacZ expression as stressed mice. Control mice were handled and weighed daily during pair-housing after habituation.

Social Interaction Test

The social interaction test consisted of two 150-second-long exploration sessions in a Plexiglas open field (44 cm x 44 cm). An empty perforated enclosure (10 cm x 5 cm x 30 cm) was placed in the center of the north side of the open field during the first open field session. After the end of the first open field session, a CD1 mouse was put into the enclosure. Both open field sessions were performed under ambient red light, with static white noise at 60 dB. Time mice spent in the interaction zone (10 cm around the enclosure) during the first (empty) and second (with a CD1 mouse) open field sessions were estimated from recorded videos of these sessions using the software TopScan LITE (Clever system Inc.). The social interaction ratio was calculated by dividing the time mice spent in the interaction zone in the second open field session with the time they spent in the interaction zone in the first open field session. We also measured the time TetTag mice spent in the two corners zones (10 cm x 10 cm) on the opposite side of the enclosure, farthest away from the CD1 mouse. Corner ratios were calculated by dividing time TetTag mice spent in those corners in the second open field session by the time that was spent in

the first session. Susceptible mice were defined as animals having a social interaction ratio of less than 1, indicating they spent less time in the interaction zone when a CD1 mouse was present. Resilient mice were defined to have a social interaction ratio of greater than 1 and spent at least 50 seconds in the social interaction zone during the second open field session.

Ensembles reactivation

After the social interaction test, both stressed and control mice were housed singly in mouse cages. To reactivate ensembles that were related to social defeat in stressed mice, we gave stressed mice an extra episode of social defeat, followed by co-housing in the neighbouring compartment with a CD1 mouse in a partitioned rat cage for 90 minutes. Ensembles related to contextual information of the rat cage were reactivated in control mice to express cFos by co-housing them with another control mice in neighbouring compartments of a partitioned rat cage for 90 minutes. After ensembles reactivation, mice were anesthetised and perfused by heparin-containing phosphate-buffered saline (PBS) and 4% paraformaldehyde solution (PFA). Brains were extracted from the skulls, post-fixed in PFA overnight and cryoprotected in 30% sucrose-containing PBS.

Immunohistochemistry

Fixed brains were snap-frozen in dry ice-chilled isopentane before cut into 35 μm thick sections using a cryostat (Leica). Brain sections were washed with PBS (five 5-minute washes; similar washing procedure was used between all antibody incubations), followed by a 30-minute incubation in 0.3% $NaBH_4$ (Sigma) to quench endogenous fluorescence. After PBS washes, sections were incubated for one hour in a blocking solution (3% normal goat serum and 0.1%

Triton in PBS (PBS-T), this blocking solution was also used for diluting antibodies). For triple immunofluorescent staining, sections were incubated overnight at 4˚C with the first primary antibody (mouse monoclonal LacZ antibody, 1:2000 (MP Biomedicals, 08633651)). The next day sections were washed by PBS-T and incubated with the first secondary antibody (donkey anti-mouse Alexa 674 antibody, 1:2000 (abcam, Ab150107)) for three hours at room temperature. In the same fashion, incubations were done for the second primary antibody (rabbit polyclonal cFos antibody, 1:40,000 (Sigma, F137)) and the corresponding secondary antibody (goat anti-rabbit Alexa 488 antibody, 1:4000 (Life Technologies, A11034)). Finally, sections were incubated with 600 nM 4',6-Diamidino-2-Phenylindole, Dilactate (DAPI) (Life Technologies, D3571) for 10 minutes. Triple labeled sections were mounted on a slide, covered with VectaShield anti-fade mounting medium (Vector Laboratories) and sealed with nail polish. The stained sections were scanned using a slide scanner (Olympus VS120) with the VS-ASW acquisition software to a magnification of 20X with eleven 15 μm thick z-sections. Sections were stitched together by the VS-ASW software.

For 3,3'diaminobenzidine (DAB, Sigma) staining of cFos, after primary antibody incubation and washes, slices were incubated for 1 hour with a biotinylated goat anti-rabbit antibody (1:500, Vector Laboratories, BA-1000), followed by an hour long incubation with the ABC reagent (1:250, Vector Laboratories, PK-7200). Sections were finally incubated with DAB (0.6%) and H2O2 for 2 minutes to visualize staining.

Data Analysis

Analysis of the digital slides from the slide scanner were done manually with the help of Fiji (ImageJ). As there are regional differences in inputs, projections and functions between

dorsal and ventral and hippocampus (Fanselow and Dong, 2010), cell counting was performed separately in each region. For CA1 counting, a 400 μm (width) by 200 μm (height) counting window was used for counting LacZ-, cFos- and DAPI-labeled cells in the dorsal and ventral hippocampus. Density of single (LacZ or cFos)- and double (LacZ and cFos)-labeled cells were determined by dividing their numbers with the number of DAPI-labeled cells in the counting window. Only neurons in the *stratum pyramidale* were counted. Since we found no LacZ-labeled cells in the pyramidal layer of the CA3 region in both control and stressed mice, the CA3 was excluded from further analysis. Finally, due to the low number of double-labeled cells in the DG, the entire DG granule cell layer in the dorsal and ventral hippocampus in each section was counted. To compare the density of DAPI-labeled cells between animal groups, we controlled for the differences in the size of DG between sections by normalizing the density of DAPI-labeled cells by the length of the granule cell layer. Three to five sections from each hippocampal region of each mouse were used for counting. Data from these sections were averaged and the mean densities of single- and double-labeled cells for each mouse were used for statistical analysis.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 7. Normality of data was examined by the Shapiro-Wilk's test. One-way ANOVA was performed to examine the effect of animal groups (control, resilient and susceptible) on the density of LacZ-, cFos- and double-labeled engram cells. Tukey's test was used for post hoc analyses. Student's t-tests were used to compare data between two groups. All data were presented as mean ± SEM.

Results

Using TetTag mice, we labeled hippocampal ensembles that were activated during the first 2 episodes of social defeat by LacZ and studied their reactivation one day after the social interaction test determined their stress susceptibility. To find out whether LacZ expression lasted long enough for examining ensembles reactivation after chronic social defeat stress, we examined the density of LacZ-labeled cells (LacZ/DAPI) in the CA1 region of the dorsal and ventral hippocampus at 1 day, 4 days and 8 days after 2 episodes of social defeat (Figure 1C, page 48). We found a significant effect of time after labeling in the ventral hippocampus only (dorsal hippocampus: $F(2,14) = 1.66$, $p = 0.225$; ventral hippocampus: $F(2,16) = 4.64$, $p = 0.032$). Post-hoc pairwise comparisons revealed a decrease in the density of LacZ-labeled cells from 1 to 4 days after social defeat (Tukey's test: 1 day vs. 4 days: 0.032; 4 days vs. 8 days: 0.903). No change in the density of LacZ-labeled cells was found after this early reduction. We concluded that despite a decrease in LacZ signal in the ventral hippocampus in the first few days after social defeat, long-lasting LacZ expression can be induced by 2 episodes of social defeat in TetTag mice for examining ensemble reactivation.

Susceptibility to social defeat was revealed by the expression of social avoidance

Figure 2A (page 50) summarizes the chronic social defeat stress protocol, which consisted of 4 days of habituation (off Dox, Day -4 to -1), two episode of social defeat off Dox (Day 1 to 2), and six episodes of social defeat on Dox (Day 3 to 8). One day after chronic social defeat stress, we examined the susceptibility of mice using the social interaction (SI) test (Day 9). Using this protocol, we identified 17 susceptible mice that displayed social avoidance (i.e. a social interaction ratio (SI ratio) < 1) and 12 resilient mice that showed normal social behaviour after

stress (Figure 2B, C page 50). In addition, 9 control non-stressed mice were habituated and fed with Dox and normal food like the stressed mice. These mice were pair-housed with another TetTag or nontransgenic littermates for 8 days after habituation and handled daily. One-way ANOVA revealed a significant difference in SI ratio between animal groups ($F(2,35) = 15.7$; $p = 1.37E-05$) with susceptible mice having lower SI than control (post-hoc Tukey's test: $p = 5.24E-04$) and resilient mice ($p = 4.28E-05$).

During the SI test, we also examined the time mice spent in the corners of the open field, which represented the regions that are farthest from the CD1 mouse containing enclosure (Figure 2B, C page 50). Susceptible mice spent significantly more time in corner zones than control and resilient mice in the second open field session when a social object was present in the enclosure. Comparing the corner ratios revealed a significant group difference ($F(2,35) = 4.56$; $p = 0.0173$). The corner ratio of susceptible mice was significantly higher than control ($p = 0.0319$). Although susceptible and resilient mice displayed distinct behaviours during the SI test, we did not observe differences in the number of attacks (11.8 ± 0.2 for susceptible mice vs. 11.5 ± 0.3 for resilient mice) and the duration of each social defeat episode (i.e. time used for attacks: 153.4 ± 13.0 seconds for susceptible mice vs. 143.6 ± 13.9 seconds for resilient mice) between these two groups. Finally, all three mouse groups showed similar weight gain from Day 1 to Day 8 after habituation (1.44 ± 0.30 g for control mice; 1.85 ± 0.34 g for resilient mice; 1.38 ± 0.39 g for resilient mice).

Susceptible mice displayed more engram cells in the hippocampal CA1 region than resilient and control mice

To find out if stress susceptibility is related to the reactivation of hippocampal ensembles that were labeled during social defeat, we examined the reactivation of LacZ ensembles formed during the first 2 episodes of social defeat by an extra episode of social defeat in stressed mice. cFos induced by the additional episode of social defeat reaches its peak 90 minutes after induction (Schilling, Luk et al. 1991), at which time we perfused the animals. Control mice were only weighed and exposed to the context during LacZ expression (i.e. pair-housed in a partitioned rat cage) for 90 minutes to examine the reactivation of neutral context-related LacZ ensembles. The overlapping of LacZ and cFos ensembles was represented by cells expressing both LacZ and cFos (Figure 3, page 52). The formation of these double-labeled cells cannot be explained by probabilistic reasons. When we compared the density of double-labeled cells with the estimated chance levels of their occurrence (LacZ/DAPI x cFos/DAPI), the density of double-labeled cell in all mouse groups was significantly higher than chance in both the dorsal (control: $t(8) = 4.23$, $p = 0.003$; resilient: $t(11) = 7.63$, $p = 1.03E-05$, susceptible: $t(15) = 7.45$, $p = 2.06E-05$) and the ventral hippocampus (control: $t(8) = 5.10$, $p = 9.35E-04$; resilient: $t(11) = 3.87$, $p = 0.003$, susceptible: $t(15) = 6.62$, $p = 8.13E-06$). We therefore named these double-labeled cells as engram cells, which represented the reactivation of LacZ ensembles in these mice.

Densities of LacZ, cFos and engram cells in the dorsal and ventral hippocampus were separately compared in order to reveal region specific differences. Although only resilient and susceptible mice were stressed by social defeat, we did not observe significant differences of the density of LacZ (Figure 4A, page 53) and cFos cells (Figure 4B, page 53) between the three mouse groups (non-stressed control, resilient and susceptible animals) in both the dorsal and ventral hippocampal CA1 regions. However, we found that the density of engram cells in

susceptible mice was significantly higher than control and resilient mice in both the dorsal (Figure 4C, page 53, $F(2,35) = 18.4$; $p = 3.54E-06$; post-hoc Tukey's test: control vs. susceptible, $p = 8.88E-05$, resilient vs. susceptible, $p = 2.21E-05$) and the ventral hippocampus ($F(2,35) = 16.2$; $p = 1.07E-05$; post-hoc Tukey's test: control vs. susceptible, $p = 0.00180$, resilient vs. susceptible, $p = 1.52E-05$). Since we have previously shown that chronic social defeat stress has different impacts on hippocampal volume in susceptible and resilient mice (Tse et al. 2014), we asked if changes in the density of CA1 neurons were responsible for the increase in engram cell density in susceptible mice (Figure 4D, page 53). However, we did not observe differences in the density of DAPI-labeled CA1 cells between these mouse groups.

Although we did not observe significant changes in the density of LacZ cells between the three animal groups when we analyzed data from the dorsal and ventral hippocampus separately, two-way ANOVA analysis of the effect of dorsal and ventral regions and the animal groups on the density of LacZ cells revealed a significant effect of animal groups (Effect of animal groups: $F(2,70) = 4.23$, $p = 0.0185$), and a significantly higher LacZ cell density in susceptible mice than in resilient mice (post-hoc Tukey's test: control vs. susceptible, $p = 0.121$, resilient vs. susceptible, $p = 0.025$). This slight but significant increase in LacZ cell density may underlie the increased engram cell formation in susceptible mice. To test this, we normalized the engram cell density by dividing engram cell density with the LacZ cell density and compared the data between the 3 animal groups. However, susceptible mice still have more normalized engram cells than both control and resilient mice in the dorsal hippocampus ($F(2,35) = 9.06$; $p = 6.72E-04$; post-hoc Tukey's test: control vs. susceptible, $p = 0.00256$, resilient vs. susceptible, $p = 0.00423$). Susceptible mice also have more normalized engram cells than resilient mice in the ventral hippocampus ($F(2,35) = 6.78$; $p = 6.72E-04$; post-hoc Tukey's test: control vs. susceptible, p =

0.0738, resilient vs. susceptible, p = 0.00321). These findings strongly suggest that susceptible mice have more social defeat-related CA1 engram cells in both the dorsal and ventral hippocampus than resilient and control mice.

The higher engram cell density in susceptible mice than resilient and control mice suggest that engram cell density is related to the expression of depression-related behaviour of these mice. Indeed, we found that CA1 engram cell density in both the dorsal (Figure 5A, page 55, R^2 = 0.192, p = 0.00598) and ventral hippocampus (R^2 = 0.166, p = 0.0110) of all tested mice correlated negatively with the SI ratio. When we examined the relationship between CA1 engram cell density and the corner ratio, we also found a significant correlation between dorsal CA1 engram cell density and corner ratios (Figure 5B, page 55, R^2 = 0.176, p = 0.00865). However, the correlation between ventral CA1 engram cell density and corner ratios did not reach a significant level (R^2 = 0.0627, p = 0.129). These findings suggested that high CA1 engram cell density in susceptible mice is related to the expression of social avoidance.

Social defeat stress reduced engram cell density in the hippocampal dentate gyrus region

Fear memory formation and recall has been associated with engrams in the DG (Liu et al. 2012; Deng et al. 2013; Denny et al. 2014). We next examined if the susceptibility to social defeat stress is also related to the activity of DG engram cells. Similar to findings we observed from the CA1 region, we did not find changes in the density of LacZ (Figure 6A, page 56) and cFos cells (Figure 6B, page 56) in the DG between the three mouse groups. Interestingly, we saw a trend that engram cell density in control mice may be higher than that of both resilient and susceptible mice in the ventral DG (Figure 6C, page 56, F(2,34) = 3.21; p = 0.0529). The fact that both the resilient and susceptible groups displayed similar changes in engram cell density

suggested a stress effect. Confirming the prediction, engram cell density in control mice remained higher than the data pooled from susceptible and resilient groups (control vs. stressed mice: $t(35) = 3.33$, $p = 0.00204$). Similarly, in the dorsal hippocampus, we observed a trend-level stress effect to decrease engram cell density in stressed mice (control vs. stressed mice: $t(36) = 1.86$, $p = 0.0717$). Finally, we compared the density of DAPI-labeled neurons in the DG of the three mouse group and revealed no between group differences (Figure 6D, page 56), even after we pooled data of susceptible and resilient mice together. Taken together, our data suggest that unlike the CA1 region, DG engram cells may not contribute to stress susceptibility.

Engram cell formation in susceptible mice caused by neutral stimuli

Even after habituation, we saw overlapping LacZ and cFos ensembles in control non-stressed mice. The density of engram cells was higher than chance (dorsal hippocampus: $t(8) = 4.23$, $p = 0.003$; ventral hippocampus: $t(8) = 5.10$, $p = 9.35E-04$). These findings suggested that during Dox off, the exposure of neutral contextual information triggered the formation of LacZ ensembles in the CA1 region. These ensembles were reactivated by the re-exposure of the same context. Thus, in stressed mice, engram cells we observed in Figure 4 (page 53) were likely due to the reactivation of ensembles that are related to neutral (context) and negative (social defeat) stimuli. To find out whether mice with different stress susceptibility exhibited differences in the reactivation of neutral stimuli-related LacZ ensembles, we stopped LacZ labeling before social defeat and studied ensembles reactivation in control, susceptible and resilient mice (Figure 7A, page 58). Both control and stressed mice were habituated off Dox in the partitioned rat cage for 6 days to maintain a similar duration of LacZ expression as in previous experiments (see Figure 4, page 53, 4 days habitation plus 2 days of social defeat). LacZ expression was stopped one day

before social defeat by Dox (Figure 7A, page 58). Stressed mice were then received 8 episodes of social defeat, while control mice were pair-housed with another control mice in rat cages for 8 days. After the social interaction test, mice were housed singly in mouse cages. One day later, we triggered ensembles reactivation in stressed and control mice by an extra episode of social defeat and pair-housing, respectively. We identified 13 susceptible mice and 5 resilient mice in this experiment. Compared with control mice (n = 10), again we did not observe differences in the density of LacZ (Figure 7B, page 58) and cFos cells (Figure 7C, page 58) between the 3 animal groups. Interestingly, we also did not find significant differences in the density of engram cells in the dorsal hippocampus between the animal groups. However, we found that susceptible mice expressed more engram cells in the ventral CA1 region than control mice (Figure 7D, page 58 $F(2,22) = 5.05$; $p = 0.0156$; post-hoc Tukey's test: control vs. susceptible, $p = 0.0259$). When we compared the density of DAPI-labeled neurons in the CA1 of the three mouse groups, we found no between group differences (Figure 7E, page 58), suggesting no changes in neuronal density. These findings suggest that in the ventral hippocampus, susceptible mice also exhibited higher reactivation of neutral stimuli-related ensembles than resilient and control mice.

Since we found no change in the reactivation of neutral stimuli-related LacZ ensembles in the dorsal hippocampus between the three mouse groups, our findings suggested that there was limited overlap of neutral stimuli- and negative stimuli-related engram cells in susceptible mice. Indeed, compared to the density of engram cells that are related to neutral stimuli (Figure 7, page 58), we found more engram cells that are related to negative stimuli (Figure 4, page 53) in both the dorsal ($t(26) = 7.41$, $p = 7.17E-08$) and ventral hippocampus of susceptible mice ($t(26) = 7.22$, $p = 1.16E-07$). These findings suggested that the increased density of engram cells after social defeat in susceptible mice were primarily induced to negative stimuli.

Discussion

Hippocampal engrams have been regarded as cellular substrates for learning and memory. Here, we provide evidence that hippocampal engrams are also related to the susceptibility to a chronic stressor. Using TetTag mice to examine the reactivation of hippocampal ensembles that were formed during social defeat, a chronic stressor that can induce depression-related behaviours such as social avoidance, we found that mice that were susceptible to chronic social defeat stress have higher reactivation of CA1 engram cells in both the dorsal and ventral hippocampus than resilient mice. Indeed, reactivation of CA1 engram cells in resilient mice was similar to that in non-stressed control mice, whose engram cells represented memory for neutral contextual information. The density of engram cells was negatively correlated with social interaction performance of mice, supporting a functional contribution of high CA1 engram cell activity to the social avoidance of susceptible mice. Chronic social defeat stress decreased engram cell reactivation in the dorsal and ventral DG of both susceptible and resilient mice. Finally, compared with CA1 engram cells that were triggered by neutral contextual information before social defeat, engram cells that were induced by negative stimuli during social defeat in both the dorsal and ventral hippocampus of susceptible mice exhibited stronger reactivation. Taken together, our findings suggest that stress-related CA1 engram cell reactivation, or stress engram, is important for determining the susceptibility to chronic social defeat stress.

To our knowledge, this is the first report to reveal a contribution of hippocampal CA1 engrams to stress susceptibility. CA1 neurons are known for their crucial roles in memory formation. Selective lesion of the CA1 region impaired new memory formation in rodents (Volpe et al. 1992) and humans (Zola-Morgan et al. 1986). Normal functioning of the CA1 region is crucial for memory retrieval; focal CA1 lesion from transient global amnesia strongly impaired

the retrieval of autobiographical memory (Bartsch et al. 2011). Indeed, optogenetic inhibition of CA1 neurons in mice strongly impaired contextual fear acquisition and retrieval (Goshen et al. 2011). Using the cFos-tTA technology like the current study, the formation of engram cells has been detected in the CA1 region after fear conditioning (Tanaka et al. 2014; Cai et al. 2016; Roy et al. 2017). Activating and inhibiting these CA1 engram cells was sufficient to induce and suppress, respectively, fear memory retrieval (Tanaka et al. 2014; Cai et al. 2016). By studying the reactivation of CA1 LacZ ensembles, which corresponded to neurons activated during the first two episode of social defeat and reactivated during the extra episode of social defeat 8 days later, we showed that susceptible mice have higher ensembles reactivation than resilient and control mice. These findings suggest that the retrieval of social-defeat-related stress engram may underlie the susceptibility to this stressor.

CA1 engram formation in susceptible mice shows that these animals have higher sensitivity towards stimuli with a negative valence. By shutting down LacZ labeling at an earlier time to exclude labeling of the social defeat event, we found that the reactivation of negative social defeat-related CA1 engram cells (Figure 4, page 53) in both the dorsal and ventral hippocampus were higher than neutral context-related engram cells (Figure 7, page 58) in susceptible mice. The increased reactivation of negative stimuli-related CA1 engram cells cannot be explained by more LacZ cells in susceptible mice than resilient and control mice, since engram cell density normalized by LacZ cell density in susceptible mice remained higher than other mouse groups. Since we observed a slight time-dependent decrease of LacZ signal after stopping its expression by Dox (Figure 1, page 48), one would argue the lower engram cell density caused by the reactivation of neutral stimuli-related ensembles was due to a longer time period between the end of LacZ expression and engram cell reactivation (10 days in Figure 7,

page 58, compared to the 8 days between the end of the 2nd episode of social defeat and ensembles reactivation in Figure 2, page 52). However, comparing the LacZ cell density resulted from neutral (Figure 7, page 58) and negative stimuli (Figure 4, page 53) in either the dorsal or ventral hippocampus of susceptible mice revealed no differences, therefore it is unlikely that the reduction in LacZ expression contributed to our findings. Mechanisms for increased engram formation, while remaining unclear, could include changes in inhibitory neurons (Morrison et al. 2016; Stefanelli et al. 2016) and neural networks (Yamamoto and Tonegawa, 2017) that regulate engram size and reactivation.

Unlike the CA1 region, we did not find differences in DG engrams between susceptible and resilient mice. Instead, we observed lower engram cell density in these stressed mice when compared to non-stressed control mice. The DG plays important roles in pattern separation and is likely sensitive to changes in context (Leutgeb et al. 2007). Using TetTag mice, it has been shown that while engrams were formed in both the CA1 and DG during contextual learning, subsequent exposure to the same context favoured the reactivation of CA1, but not DG engrams (Deng et al. 2013). Since mice were kept in a similar context for multiple days during chronic social defeat stress, The CA1 instead of the DG ensembles may be preferentially reactivated under this behavioural paradigm. Indeed, compared to 4-9% reactivation of DG cells in a relatively short behavioural task such as fear conditioning (Liu et al. 2012; Denny et al. 2014; Stefanelli et al. 2016), only ~0.5% of DG cells were reactivated by social defeat. While chronic stress is known to modulate the structural and functional properties of both the DG and the CA1 regions (de Kloet et al. 2005; McEwen et al. 2016), it is unclear why chronic social defeat stress reduced engram cell density only in the DG. Although prolonged restraint stress for 6 weeks has been shown to reduce the density of granule cells in the DG (Pham et al. 2003), we did not

observe changes in granule cell density after chronic social defeat stress (Figure 6D, page 56). Since adult neurogenesis in the DG was transiently reduced after chronic social defeat stress (Lagace et al. 2010), our findings may suggest a link between weakened neurogenesis and reduced ensembles reactivation in the DG.

Similar to a recent report using the same mouse model (Deng et al. 2013), we were not able to detect CA3 engram cells in TetTag mice due to low LacZ expression in this hippocampal region. In the experiment for detecting the duration of LacZ expression after social defeat, we found a large number of LacZ-labeled cells in the CA3 region 1 day after social defeat. However, LacZ signals seemed to disappear quickly in the CA3 region so that few or none LacZ cells were found in the pyramidal layer of the CA3 region at 4 days after social defeat. Indeed, we saw high levels of cFos expression in the CA3 region during reactivation of CA1 and DG engram cells, suggesting the activation of CA3 cells during memory recall. It is unclear why long-term LacZ expression can be found in the CA1 and DG regions but not in the CA3 region. Since LacZ expression is sustained by the tetracycline-insensitive tTA after the reintroduction of Dox food, the lack of CA3 LacZ signal may be due to poor expression of this mutated tTA in the CA3 region. The role of CA3 engrams in stress susceptibility cannot be ruled out, since CA3 engrams have been shown to be more sensitive to fear-related contextual information than a neutral novel context (Denny et al. 2014). Using the Cre-dependent ArcCreERT2 mouse line may reveal the contribution of CA3 neurons to stress susceptibility.

We observed similar increases in engram cell density in both the dorsal and ventral hippocampal regions of susceptible mice. Although the dorsal and ventral have been suggested to contribute distinct functions in spatial and emotional memory formation (Fanselow and Dong, 2010), chronic social defeat stress is a complex stressor such that spatial (e.g. partitioned rat

cage) and emotional information (olfactory, visual and other sensory stimuli from the CD1 aggressor) is likely to be encoded by defeated mice to avoid future attacks. There were however some subtle dorsal-ventral differences between the three mouse groups. For instance, compared to control mice, both susceptible and resilient mice had lower density of engram cells in the ventral, but not the dorsal DG (Figure 6C, page 56). The density of CA1 engram cells that are related to the re-exposure of neutral context in susceptible mice was higher than resilient and control mice in the ventral hippocampus only (Figure 7, page 58). Even though the dorsal and ventral hippocampus in susceptible mice underwent similar changes in the current study, these hippocampal subregions project to different brain regions that have distinct functions. For instance, ventral hippocampal projects to the nucleus accumbens and plays a crucial role in determining stress susceptibility and resilience (Bagot et al. 2015). Stress engram formation in the dorsal and ventral hippocampus may contribute differently to the stress responses and behaviour of susceptible mice.

The difference in stress engram formation between susceptible and resilient mice has important implications for depression. Changes in these memory functions could be related to the bottom up changes from a hypersensitive medial temporal lobe, including hyperfunctioning of the amygdala and the hippocampus. We found a stress engram in the mice that are susceptible to social defeat stress suggested that stress engrams could be a hippocampal mechanism that contribute to the negative bias of memory formation in depression. Stress engrams correlated with the expression of social avoidance, suggesting their role in mediating cognitive symptoms of depression. Using optogenetic approaches to reactivate memory engrams that were associated with a positive experience could reduce depression-related behaviour in stressed mice (Ramirez

et al. 2015). Our findings suggest that inhibiting stress engrams in the hippocampus could be a novel therapeutic approach for treating cognitive symptoms in depression.

It is unclear why the stress engram seems to be absent in resilient mice. One possible explanation is that compared to susceptible mice, resilient mice have impaired encoding of negative information for the formation of stress engrams. Alternatively, resilient mice may have actively suppressed the reactivation of the stress engram after repeated social defeat. Retrieval of mood congruent memory, which is commonly found in depression, has been suggested to reduce the ability of depressed patients in problem-solving and sparing attention to positive information and memory (Conway and Pleydell-Pearce, 2000). Persistent recall of negative memory underlies rumination, which has been associated with the vulnerability to depression and the severity of depression symptoms (Alloy et al. 1999; Rude et al. 2003; Abela and Hankin, 2011) and perhaps most importantly, with hippocampal activation (Denson et al. 2009; Mandell et al. 2014). Suppressing the reactivation of the stress engram, probably through a top down inhibitory control from the frontal lobe (Disner et al. 2011; Kircanski et al. 2012), may help resilient mice to cope with stressful situations. Future experiments could focus on comparing stress engram formation between susceptible and resilient mice, and determining whether forgetting mechanisms that affect memory retrieval were employed by resilient mice.

List of Figures

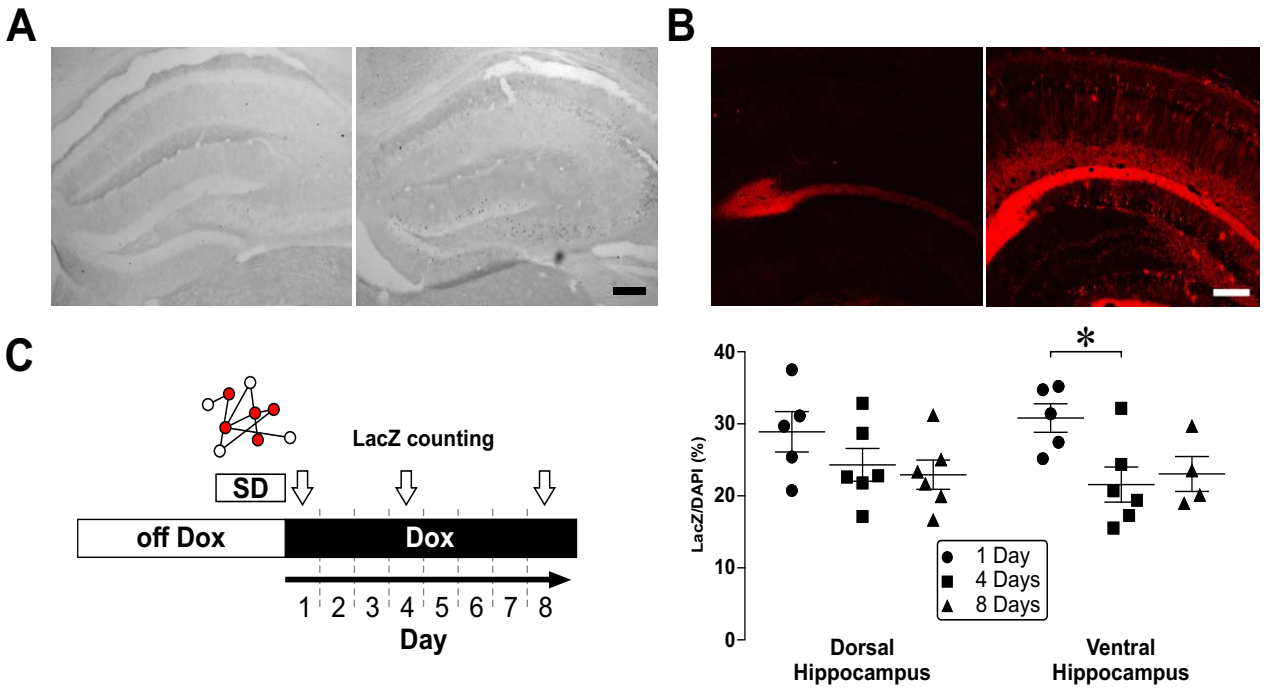

Figure 1: Social defeat triggers the formation of hippocampal ensembles. A) cFos stained dorsal hippocampal sections from a control mouse from its home cage (Left) and 1 day after a single episode of social defeat (SD, Right). Scale bar = 200 µm. B) LacZ staining of ventral hippocampal neurons from TetTag mice that were off doxycycline-containing food during labeling (Dox off, Right). A stained section from a mouse that was on doxycycline-containing food during labeling was shown on the Left (Dox on). Note that apart from non-specific staining near the hippocampal fissure, LacZ labeled neurons and processes cannot be found in tissue from the Dox on mouse on the Left. Scale bar = 200 µm. C) A schematic diagram of the experimental design. TetTag mice were off Dox for 4 days. After two episodes of social defeat (SD), labeling

was blocked by putting defeated mice on Dox. TetTag mice were sacrificed 1 day, 4 days and 10 days later (white arrows). Scatter plots on the right summarize the density of LacZ positive neurons in the CA1 region of the dorsal and ventral hippocampus of TetTag mice at different time points after labeling. * $p < 0.05$, post hoc Tukey's test vs. data from day 3 group in each hippocampal region after ANOVA.

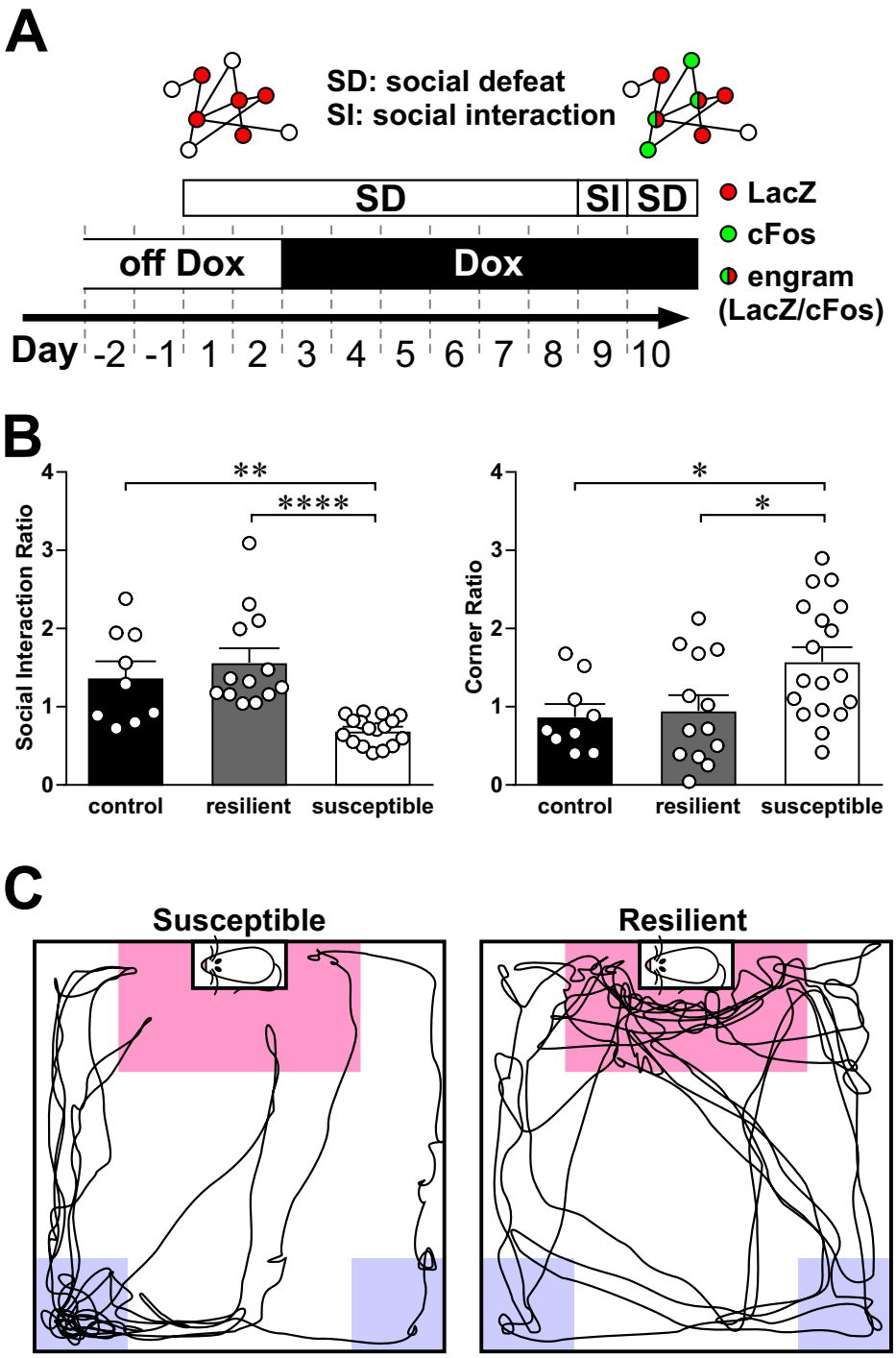

Figure 2: Susceptible but not resilient mice expressed social avoidance after chronic social defeat stress. A) A schematic diagram of the experimental design. TetTag mice were off Dox for

4 days. After two episodes of social defeat (SD) on day 1 and 2, labeling was blocked by putting defeated mice on Dox-containing food. Mice were then stressed by 6 more episodes of SD. The interaction between TetTag mice and a CD1 mouse, the strain of aggressive mice used for SD, was examined in a social (SI) interaction test. One day after the SI test, mice underwent one more episode of SD to trigger neuronal activation. Mice were sacrificed 90 minutes after the last episode of social defeat. Cartoons above the experimental plan depict the labeling of activated neurons during the first two days of chronic SD (red, LacZ), during the last episode of SD (green, cFos), and engram cells that expressed both signals (red/green). B) Histograms summarize the social interaction ratio (Left) and the corner ratio (Right) of susceptible, resilient and unstressed control mice. * $p < 0.05$, **** $p < 0.0001$, post hoc Tukey's test after ANOVA. C) Example tracks of a susceptible (Left) and a resilient mouse during the second open field session of the SI test. The pink and purple zones are the virtual interaction and corner zones, respectively. Note the cluster of tracks in the interaction and corner zones for the resilient and susceptible mice, respectively.

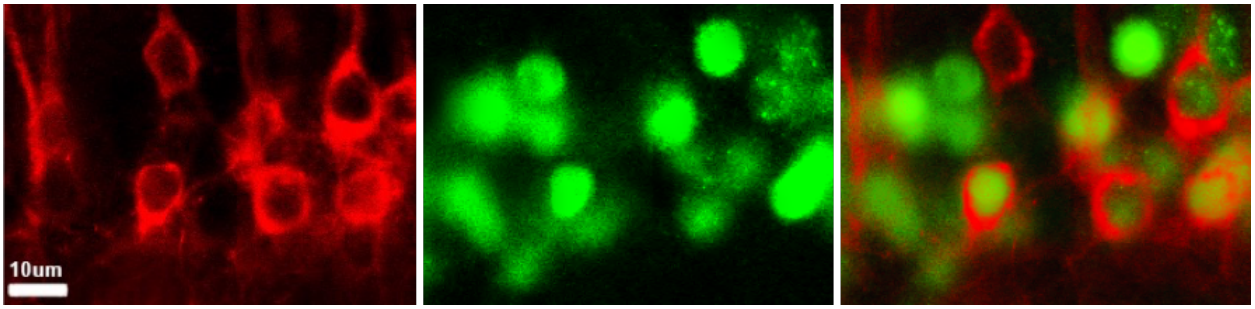

Figure 3: LacZ and cFos staining in the CA1 region of a TetTag mouse. Florescent micrographs of dorsal hippocampal CA1 neurons that were stained for A) LacZ, B) cFos. Panel C) shows the overlapping of LacZ and cFos in engram cells (arrowheads). Scale bar = 10µm.

Figure 4

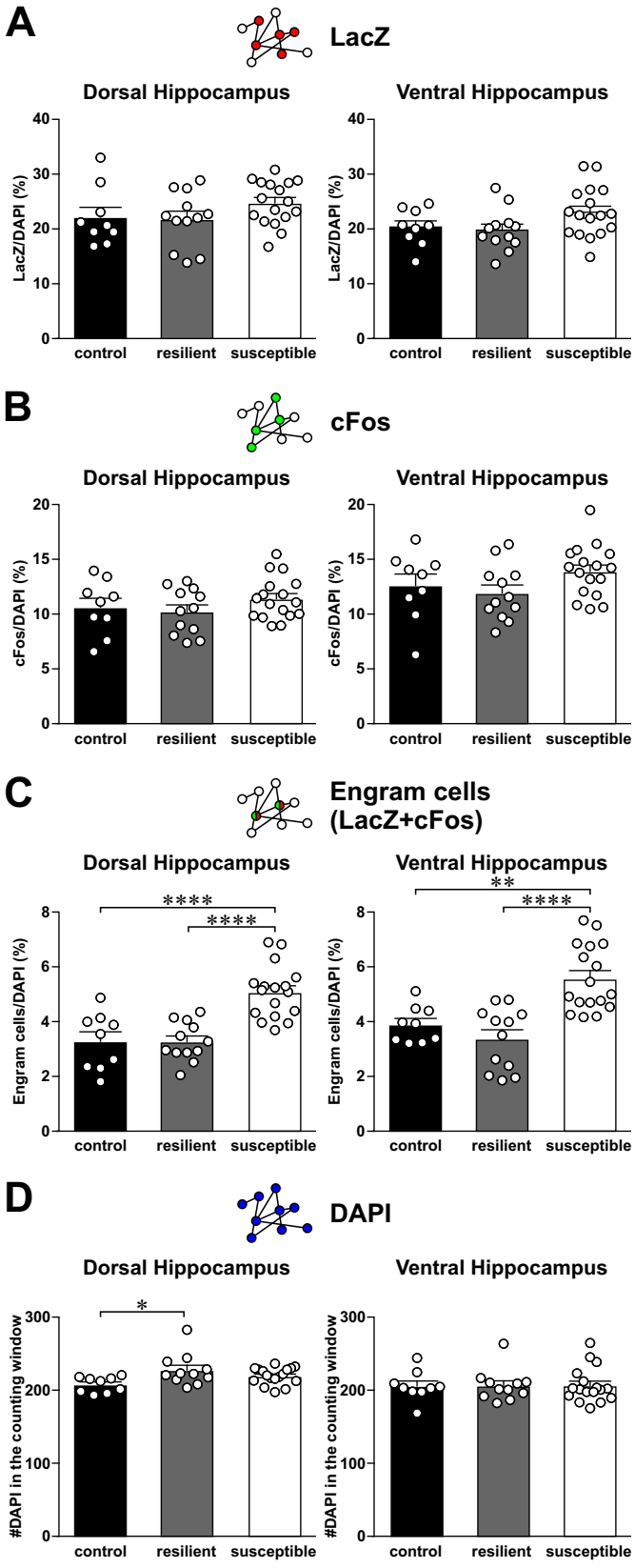
A
LacZ
Dorsal Hippocampus
Ventral Hippocampus
LacZ/DAPI (%)
control
resilient
susceptible
B
cFos
cFos/DAPI (%)
C
Engram cells
(LacZ+cFos)
Engram cells/DAPI (%)
****
**
D
DAPI
#DAPI in the counting window
*

Figure 4: Expression of LacZ, cFos and engram cells in the CA1 region of the dorsal and ventral hippocampus of control, resilient and susceptible mice. A) Histograms show the density of LacZ labeled neurons in the CA1 region of dorsal (Left) and ventral hippocampus (Right) of control, resilient and susceptible mice. B) Histograms show the density of cFos labeled neurons in the CA1 region of dorsal (Left) and ventral hippocampus (Right) of control, resilient and susceptible mice. C) Histograms show the density of engram cells (double labeled for both LacZ and cFos) in the CA1 region of dorsal (Left) and ventral hippocampus (Right) of control, resilient and susceptible mice. ** $p < 0.01$, **** $p < 0.0001$, Tukey's test after ANOVA. D) Histograms show the density of DAPI (double labeled for both LacZ and cFos) stained cells in the CA1 region of dorsal (Left) and ventral hippocampus (Right) of control, resilient and susceptible mice. * $p < 0.05$, Tukey's test after ANOVA.

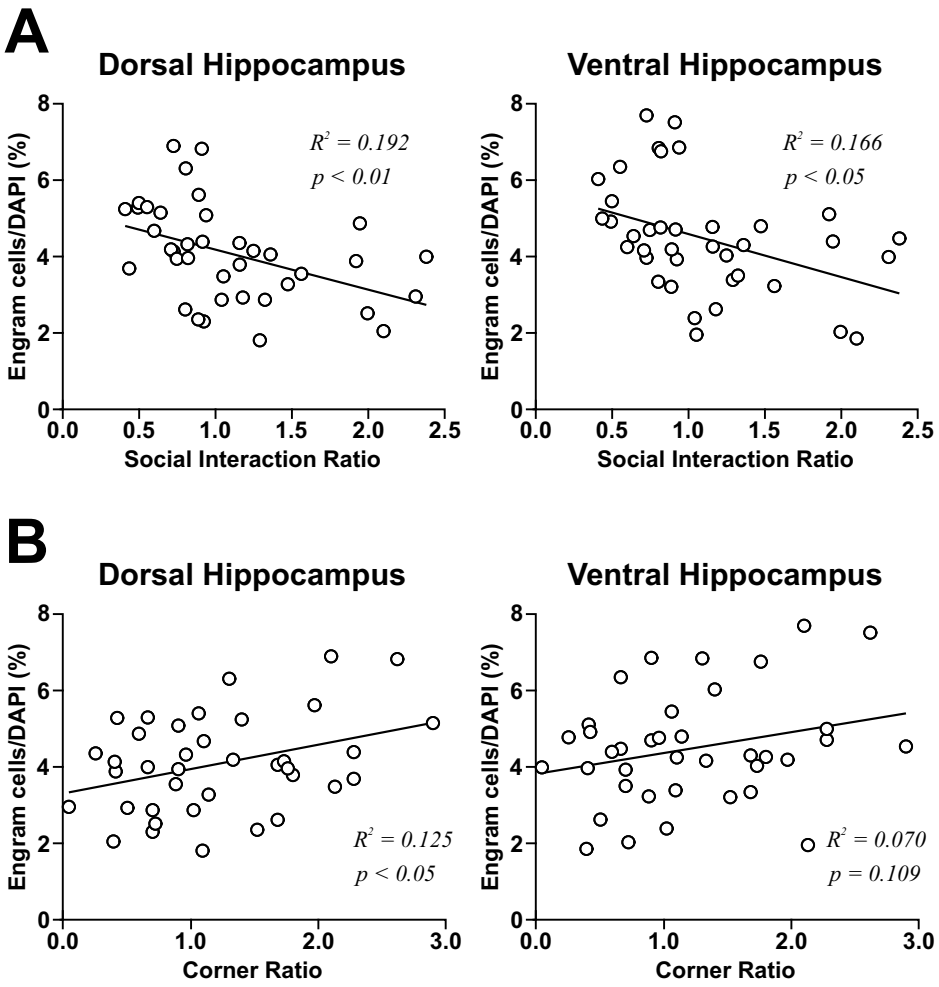

Figure 5: Density of CA1 engram cells correlates with depression-related behaviors. A) Scatter plots of engram cell density in the CA1 region of dorsal (Left) and ventral hippocampus (Right) vs. social interaction ratio of control and defeated mice. B) Scatter plots of engram cell density in the CA1 region of dorsal (Left) and ventral hippocampus (Right) vs. corner ratio of control and defeated mice.

Figure 6

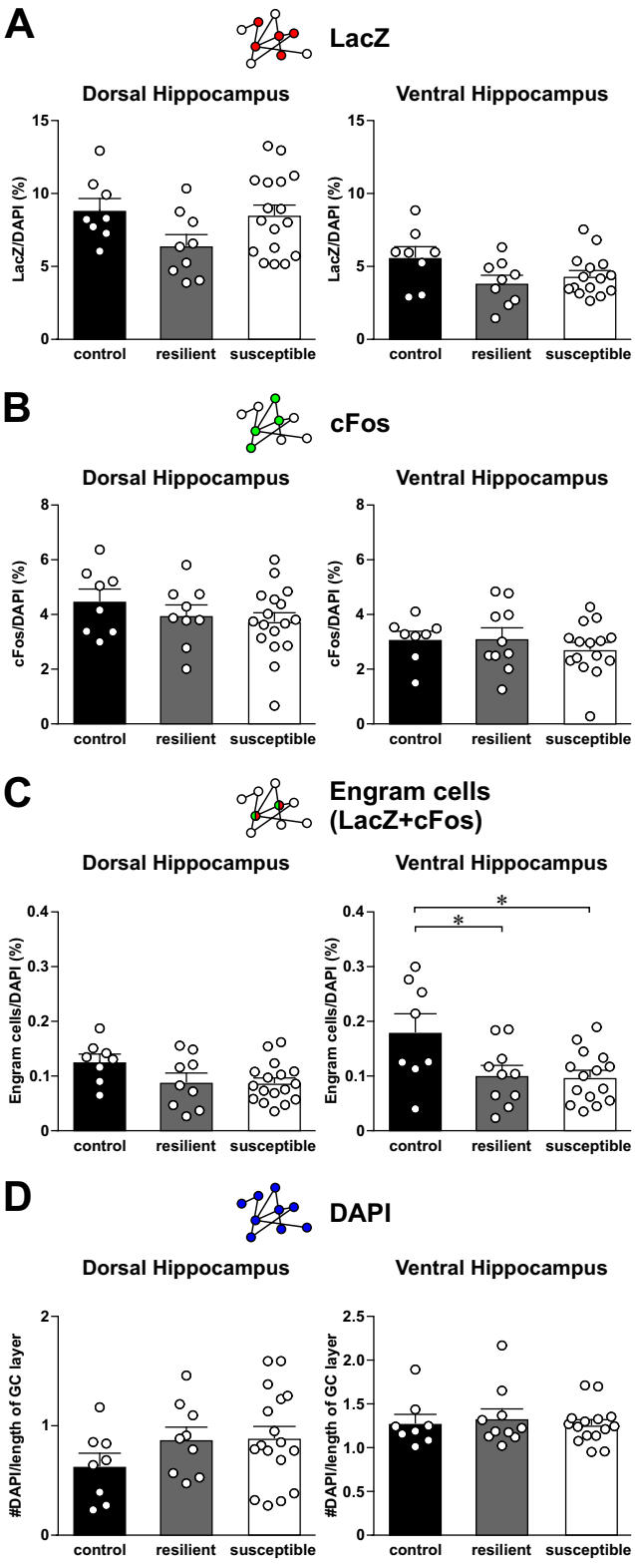
A
LacZ
Dorsal Hippocampus
Ventral Hippocampus
LacZ/DAPI (%)
15
10
5
0
control
resilient
susceptible
B
cFos
Dorsal Hippocampus
Ventral Hippocampus
cFos/DAPI (%)
8
6
4
2
0
control
resilient
susceptible
C
Engram cells
(LacZ+cFos)
Dorsal Hippocampus
Ventral Hippocampus
Engram cells/DAPI (%)
0.4
0.3
0.2
0.1
0
*
*
control
resilient
susceptible
D
DAPI
Dorsal Hippocampus
Ventral Hippocampus
#DAPI/length of GC layer
2
1
0
2.5
2.0
1.5
1.0
0.5
0
control
resilient
susceptible

Figure 6: Expression of LacZ, cFos and engram cells in the DG of the dorsal and ventral hippocampus of control, resilient and susceptible mice. A) Histograms show the density of LacZ labeled neurons in the DG region of dorsal (Left) and ventral hippocampus (Right) of control, resilient and susceptible mice. B) Histograms show the density of cFos labeled neurons in the DG region of dorsal (Left) and ventral hippocampus (Right) of control, resilient and susceptible mice. C) Histograms show the density of engram cells (double labeled for both LacZ and cFos) in the DG region of dorsal (Left) and ventral hippocampus (Right) of control, resilient and susceptible mice. * $p < 0.05$, Tukey's test after ANOVA. D) Histograms show the density of DAPI (double labeled for both LacZ and cFos) stained cells in the DG region of dorsal (Left) and ventral hippocampus (Right) of control, resilient and susceptible mice.

Figure 7

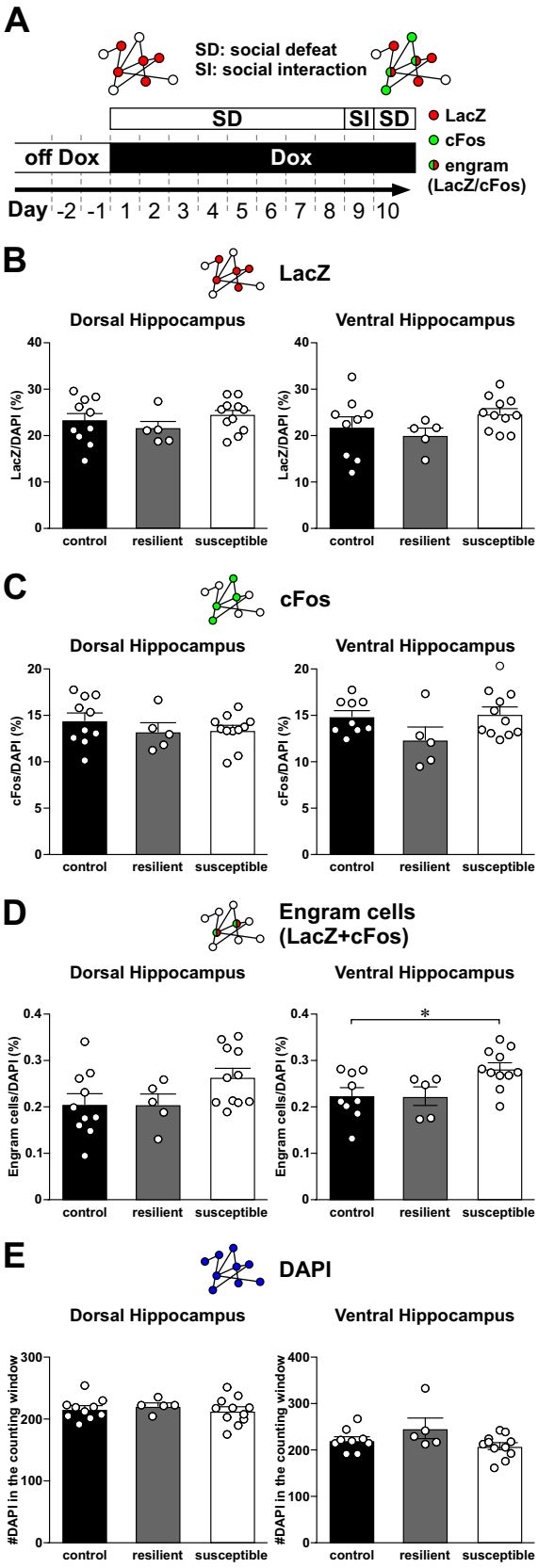
A
SD: social defeat
SI: social interaction
SD
SI
SD
LacZ
cFos
engram
(LacZ/cFos)
off Dox
Dox
Day -2 -1 1 2 3 4 5 6 7 8 9 10
B
LacZ
Dorsal Hippocampus
Ventral Hippocampus
LacZ/DAPI (%)
control
resilient
susceptible
C
cFos
cFos/DAPI (%)
D
Engram cells
(LacZ+cFos)
Engram cells/DAPI (%)
*
E
DAPI
#DAPI in the counting window

Figure 7: Expression of LacZ, cFos and engram cells in the CA1 region of the dorsal and ventral hippocampus of control, resilient and susceptible mice, when labeling was stopped before social defeat. A) A schematic diagram of the experimental design. Tet-Tag mice were off Dox for 4 days. Labeling of neurons was stopped a day before the beginning of social defeat by feeding TetTag mice with doxycycline-containing food. Mice were then stressed by 8 episodes of social defeat (SD). The interaction between TetTag mice and a CD1 mouse, the strain of aggressive mice used for SD, was examined in a social (SI) interaction test. One day after the SI test, mice underwent one more episode of SD to trigger neuronal activation. Mice were sacrificed 90 minutes after the last episode of social defeat. Cartoons above the experimental plan depict the labeling of activated neurons during the first two days of chronic SD (red, LacZ), during the last episode of SD (green, cFos), and engram cells that expressed both signals (red/green). B) Histograms show the density of LacZ labeled neurons in the CA1 region of dorsal (Left) and ventral hippocampus (Right) of control, resilient and susceptible mice. C) Histograms show the density of cFos labeled neurons in the CA1 region of dorsal (Left) and ventral hippocampus (Right) of control, resilient and susceptible mice. D) Histograms show the density of engram cells (double labeled for both LacZ and cFos) in the CA1 region of dorsal (Left) and ventral hippocampus (Right) of control, resilient and susceptible mice. * $p < 0.05$, Tukey's test after ANOVA. E) Histograms show the density of DAPI (double labeled for both LacZ and cFos) stained cells in the CA1 region of dorsal (Left) and ventral hippocampus (Right) of control, resilient and susceptible mice.

Chapter 3: Supplemental Data

Not included in the manuscript are data further exploring the comparison of neuronal activation between the contextual labeling experiment and the original social defeat experiment. We also have preliminary data exploring a causal relationship between engram activation and depressive behaviours and these data are presented in the current thesis.

3.1 Comparison between Contextual Labeling and Original Social Defeat

In addition to the manuscript, we compared changes in LacZ, cFos and engram cell expression caused by context encoding alone (excluding social defeat) and context with social defeat (including social defeat) in the CA1 region of both the dorsal and ventral hippocampus of the three mouse groups. We performed two-way ANOVA with each labeling using neuronal labeling time (including or excluding the social defeat event) and animal groups as the two factors.

We found no difference between the density of LacZ positive neurons in the CA1 of any animals between the two neuronal labeling conditions (Figure 8, page 63, effect of labeling dorsal hippocampus $F(1,58) = 0.0027$, $p = 0.962$; ventral hippocampus $F(1, 57) = 0.969$, $p = 0.329$). This shows that either context itself or the context and social defeat events together activate similar densities of neurons in the CA1.

When we compared cFos labeling in the dorsal hippocampus caused by the last episode of social defeat, we observed no effect of animal groups, but a significant effect of labeling time after two-way ANOVA interaction between labeling time and mouse groups: $F(2,58) = 26.69$, $p = 3E\text{-}6$). Post hoc analyses revealed a significant reduction of cFos labeling in control mice when labeling was stopped after 2 days of social defeat compared to control mice when labeling

was stopped before social defeat (Tukey's test: $p = 0.00301$). We did not observe differences in cFos labeling in the ventral hippocampus after two-way ANOVA ($F(2, 57) = 3.687$, $p= 0.0598$). As the events leading to the cFos activation are identical in both the contextual and social defeat experiments, it is uncertain what caused the differences in cFos activation in the two experiments. One possible explanation is that the animals used in the contextual labeling experiment are slightly younger (around 2 month-old at the start of social defeat, approximately about two weeks younger) than animals used in the original social defeat experiment. The slight age difference may cause more neuronal firing due to the same stimuli; it has been show that hippocampal neuron's intrinsic excitability decreases with age (Oh, Oliveira, and Disterhoft 2010) but the time-scale for such age-related experiments are usually much greater than a few weeks.

Finally, two-way ANOVA comparison of engram cell density in both the dorsal and ventral hippocampus revealed significant effects of labeling time (dorsal hippocampus: $F(1,58) = 55.5$, $p = 5.12E-10$; ventral hippocampus: $F(1,57) = 58.5$, $p = 2.64E-10$), significant effects of mouse groups (dorsal hippocampus: $F(2,58) = 15.6$, $p = 3.82E-6$; ventral hippocampus: $F(1,57) = 14.5$, $p = 7.89E-6$), and significant interactions between these two factors ($f(2,58) = 3.99$, $p = 0.0238$; ventral hippocampus: $F(2,57) = 4.34$, $p = 0.0176$).

Compared to control mice when LacZ labeling was stopped before social defeat, we found more engram cells in both the dorsal and ventral hippocampus of control mice when labeling was stopped after 2 days of social defeat (dorsal hippocampus: 59.3% increase, $p = 0.0208$; ventral hippocampus: 73.3% increase, $p = 0.00338$). The increase in engram density is likely due to the handling that was present when labeling was stopped after 2 days of handling, compared to the contextual labeling where animals were not handled. Although handling is not

stressful like the social defeat event, handling itself may be encoded in the engram cells in the control animals.

Compared with susceptible mice when the social defeat events were excluded from the LacZ labeling, there was a significant increase in engram cell activation in susceptible mice when labeling was stopped after 2 days of defeat (dorsal hippocampus: 92.0% increase, $p = 3.10E-09$; ventral hippocampus: 97.2% increase, $p = 1.24E-09$). Interestingly, the engram cell activation was higher when labeling of the social defeat events was included, even when the cFos activation was lower in these animals. Specific reactivation of neurons, indicated by cFos, are much higher in LacZ labeled neurons when labeling is stopped after social defeat than LacZ labeled neurons when labeling is stopped before social defeat. Therefore the engram cell increase we observed in susceptible mice was likely due to engram cells established in the 2 days of social defeat rather than those that encode neutral contextual information before defeat.

Our findings strongly suggest that an increase in social defeat related ‘stress engrams’ are related to the expression of stress susceptibility.

Figure 8

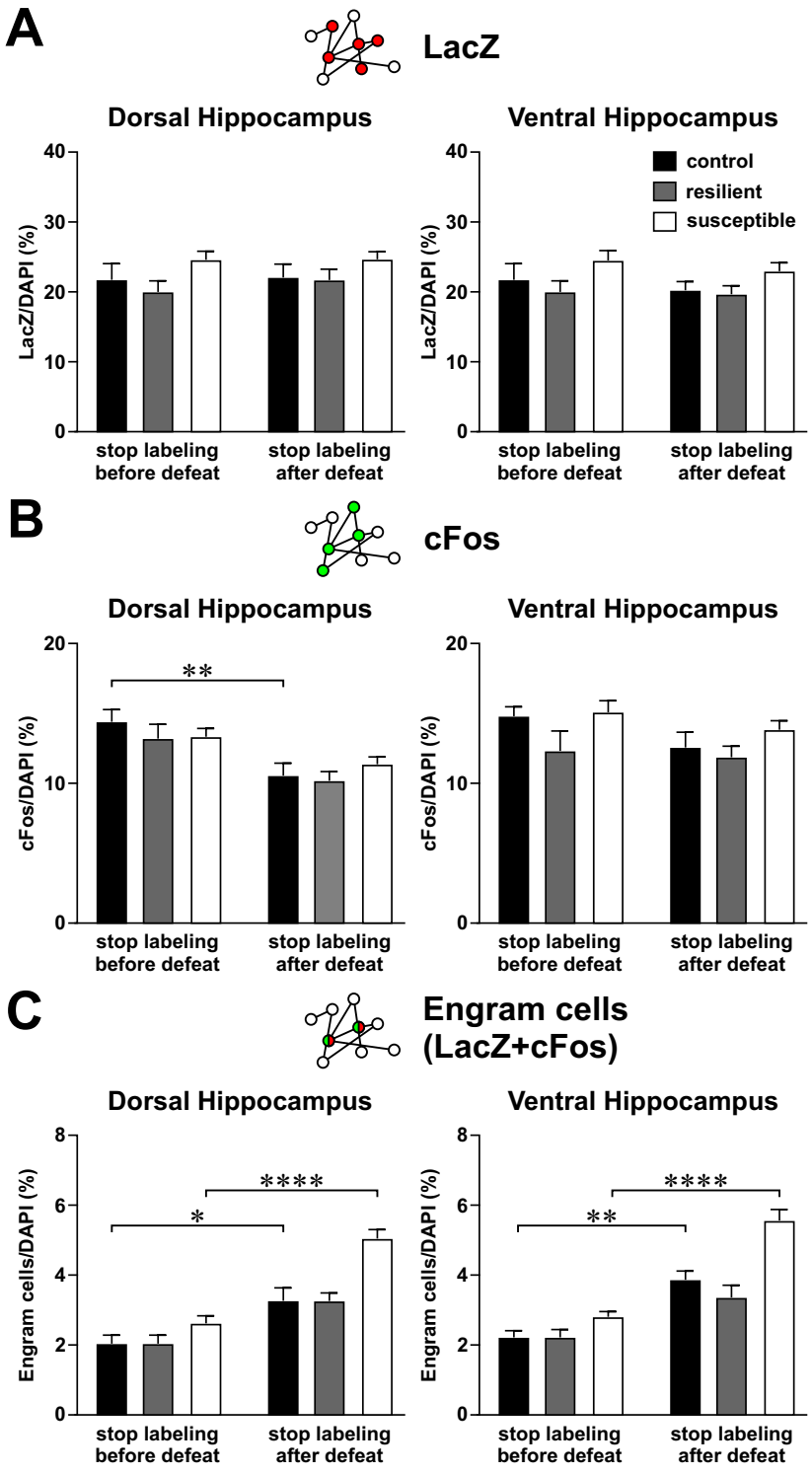
A
LacZ
Dorsal Hippocampus
Ventral Hippocampus
control
resilient
susceptible
LacZ/DAPI (%)
40
30
20
10
0
stop labeling before defeat
stop labeling after defeat
B
cFos
Dorsal Hippocampus
Ventral Hippocampus
cFos/DAPI (%)
20
10
0
**
stop labeling before defeat
stop labeling after defeat
C
Engram cells (LacZ+cFos)
Dorsal Hippocampus
Ventral Hippocampus
Engram cells/DAPI (%)
8
6
4
2
0
*
****
**
****
stop labeling before defeat
stop labeling after defeat

Figure 8. Comparison of LacZ, cFos and engram cell density between animals excluding and including labeling of social defeat with LacZ. Two-way ANOVA analysis was conducted using animal groups (non-stressed control, resilient and susceptible) and labeling time (excluding or including LacZ labeling of social defeat) as the two factors. (A) Histograms show the density of LacZ labeled neurons in the CA1 region of dorsal (Left) and ventral hippocampus (Right) of control, resilient and susceptible animals by excluding or including labeling of social defeat (B) Histograms show the density of cFos labeled neurons in the CA1 region of dorsal (Left) and ventral hippocampus (Right) of control, resilient and susceptible mice. ** $p < 0.01$, Tukey's test after ANOVA. (C) Histograms show the density of engram cells (double labeled for both LacZ and cFos) in the CA1 region of dorsal (Left) and ventral hippocampus (Right) of control, resilient and susceptible mice. * $p < 0.05$, Tukey's test after ANOVA, ** $p < 0.01$, Tukey's test after ANOVA, **** $p < 0.0001$, Tukey's test after ANOVA.

3.2 DREADD Activation of Stress Engram Cells

Since a significant correlation has been established between CA1 engram activation and degree of depression-related behaviours manifested in the animals, as a next step we decided to manipulate the engram activation to find a causal relationship between hippocampal stress engrams and depressive behaviour. We used a chemogenetic approach with an excitatory designer receptor exclusively activated by designer drugs (DREADD) to selectively activate neurons that were activated during social defeat. We hypothesized that activating stress engrams in the hippocampus will lead to the animals displaying depressive behaviours.

To do so, we used a related strain of the TetTag mouse with only the transgene cFos-tTA. We bilaterally injected an AAV virus that expresses TRE-HM3Dq-mCherry into the dorsal CA1.

In these animals, instead of the LacZ expression in the TetTag animals, cFos activation drives the expression of an excitatory DREADD. Examining mCherry expression as an indicator for DREADD expression (Figure 9, page 65), we found DREADD expression limited to the CA1, primarily in the dorsal hippocampus, but extended slightly into the ventral hippocampus.

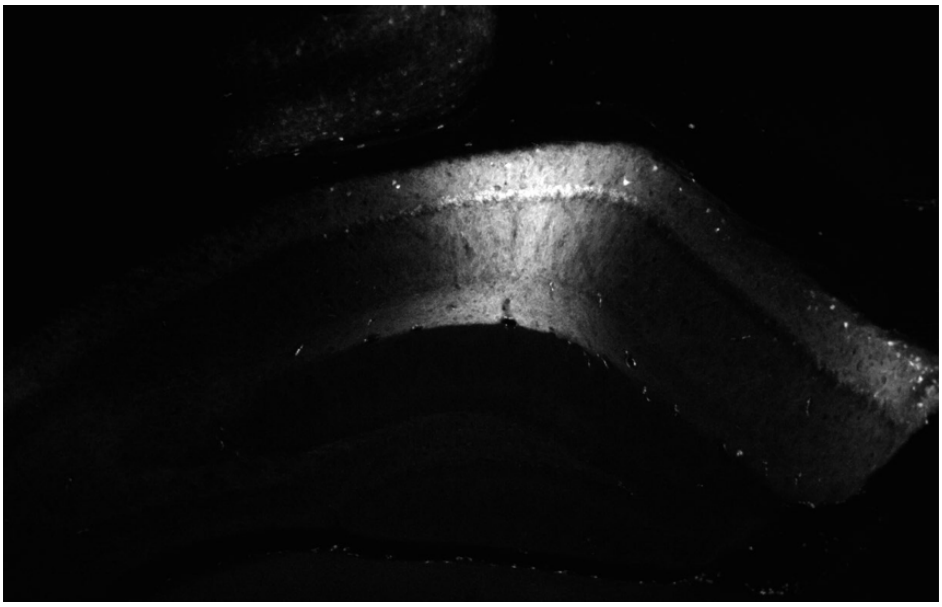

Figure 9. Excitatory DREADD expression in the dorsal CA1 of cFos-tTA animals. DREADD expression is visualized by examining the conjugated fluorescent protein mCherry expression.

We bilaterally injected 0.5µl of AAV-HM3Dq virus into the dorsal CA1 of the animals while they were on doxycycline-containing food a week prior to social defeat. We took the animals off doxycycline for two days prior to the start of social defeat and then conducted social defeat experiments on these animals for two days to allow the expression of the excitatory DREADD in activated neurons according to the schematic shown in Figure 10 (page 66). After two days, the animals were put back on doxycycline and no more social defeat episodes were conducted. Two days of social defeat provides a sub-threshold level of stress that does not

normally induce depression-like behaviour in animals. The social interaction test was conducted 9 days after the start of social defeat .We injected (intraperitoneal) the animals with either vehicle (saline, n=6) or the DREADD agonist Clozapine-N-oxide (CNO 1mg/kg in saline solution n=8) one hour before the social interaction test to activate neurons labeled during social defeat. We then analyzed the social behaviour of animals during the social interaction test.

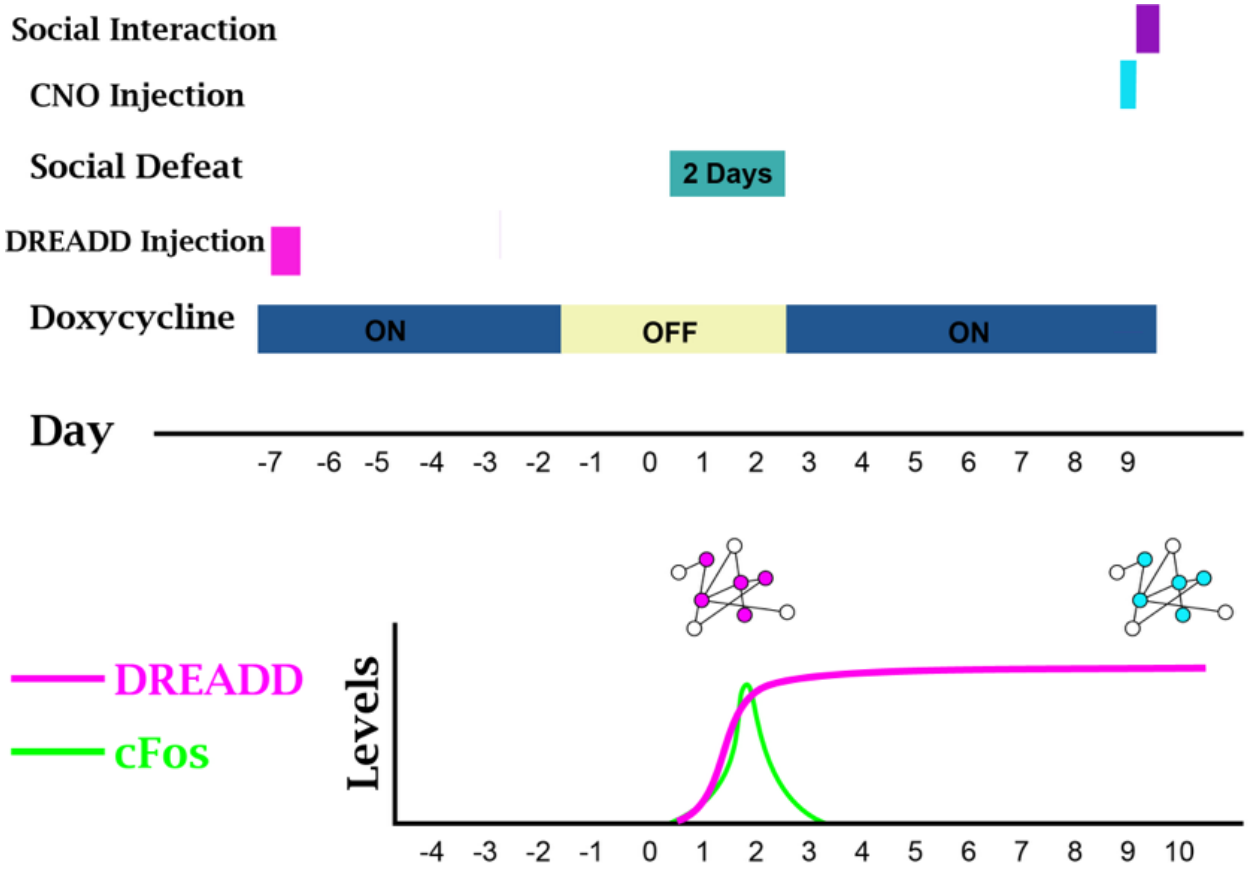

Figure 10. A schematic diagram of the experimental design for DREADD-mediated activation of stress engrams in the CA1 of animals receiving sub-threshold level stress. Animals were kept on a doxycycline-containing diet and injected with DREADD a week prior to social defeat. They were taken off doxycycline and defeated for only two days. On the third day the animals were no longer socially defeated and were given doxycycline-containing food to prevent further

DREADD expression. On the 9th day, the animals undergo a social interaction test, with CNO or vehicle injected an hour prior to activate the neurons that were labeled during social defeat. The schematic experimental plan depicts the labeling of DREADD neurons during the two days of chronic SD (pink), during the two episodes of SD (green, cFos). The subsequent activation of DREADD neurons is labeled in blue.

We found that there were no significant differences in social interaction ratio between the vehicle and CNO group (t-test p=0.155). However, there was a decrease in total amount of time the animal spends interacting with the CD1 aggressor in the second trial, comparing animals that received vehicle injection with animals that received CNO. The animals that received CNO to activate the stress engram spent significantly less time in the social interaction zone when the social object is present (t-test p=0.0322). The activation of neurons that were previously activated during social defeat may induce depressive behaviour and suggests a causal relationship between stress engram activation and social avoidance behaviour.

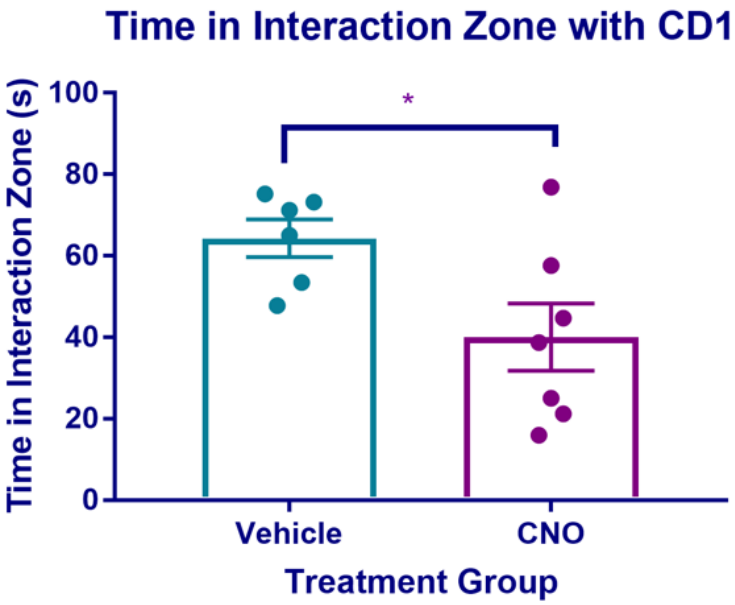

Figure 11. Time (in seconds) DREADD injected animals spent in the interaction zone with the CD1 present in the enclosure. Compared to animals that receive vehicle, animals that received CNO to activate stress engrams showed a decrease in time spent in the interaction zone when the CD1 is present (t-test * $p < 0.05$).

Chapter 4. Discussion

In the current study, we stressed animals using chronic social defeat and examined whether hippocampal neurons are differentially activated in animals susceptible or resilient to social defeat stress. We found that in the CA1 and the DG of the hippocampus, there are similar densities of neurons that were activated in the beginning or the end of social defeat, in non-stressed control, susceptible and resilient animals. We then examined the reactivation of neurons that were labeled during encoding when the animals are challenged with the same stressors; we refer to the reactivated neurons as stress engram cells. We found that engram cells in the CA1 are reactivated more in the susceptible animals than either the resilient or non-stressed control animals. There is a negative correlation between CA1 engram cell activation and the social interaction ratio, indicating animals that exhibit more social avoidance behaviour have more engram cell reactivation. In the ventral DG, there are decreases in engram cell activation in stressed animals, both susceptible and resilient, compared to non-stressed controls, without any decrease in total number of neurons. Artificially activating stress engrams in the CA1 also induced depressive social avoidance behaviour in animals receiving a sub-threshold amount of stress.

4.1 Role of CA1 in Stress Susceptibility

We saw differences in engram cell activation in the CA1 between animal groups, perhaps due to the role that the CA1 plays in encoding events. It has been shown that patients with bilateral CA1 lesions have enduring declarative memory deficits (Zolamorgan et al. 1986). The CA1 has also been associated with encoding novel information, with a neuronal density that correlates with neuronal response to novel words (Grunwald et al. 1999). In animal studies, rats

with CA1 bilateral neuronal degeneration showed significant impairments in working memory (Ordy et al. 1988). Animals lacking NMDA receptors in the CA1 are also impaired in both spatial (Morris and Frey 1997) and non-spatial learning and memory (Rampon et al. 2000). Loss of CA1 function is also found to mediate deficits in declarative memory in normal aging by a failure to link memories close in time together (Sellami et al. 2017). Differences in CA1 engram activation among susceptible and resilient animals suggest that susceptible and resilient animals have differences in declarative episodic memory; in particular, susceptible animals either encode or recall the social defeat event more than resilient animals.

4.2 Stress Engrams and Stress Susceptibility

We refer to the engram reactivation we found as a stress engram because the engrams are related to the stressful social defeat event, rather than only the context in which animals were stressed in. In a control experiment, we shifted the neuronal labeling to before social defeat to examine neuronal activation and engram cells formed due to the housing context, instead of the social defeat event. Engram cell activation was much lower in animals with neuronal labeling for the context but not the social defeat episodes, supporting the notion that the increased engram activation in susceptible animals is primarily mediated by the social defeat stress . It should be noted that even when we induced neuronal labeling corresponding to only the housing context, susceptible animals showed a small but significant increase in engram activation in the ventral CA1. This finding suggests that susceptible animals may have a more sensitive or easily excitable hippocampus, in which they encode or retrieve more of both contextual and stressful attack information. Supporting an increased neuronal excitability in susceptible animals, it has been found that animals with CREB overexpression in the amygdala had enhanced social defeat

memory formation and facilitated acquisition of submissive behaviour following social defeat (Jasnow et al. 2005). Yiu et al. (2014) showed that neurons with CREB overexpression had increased excitability and are allocated to be engram cells. Overexpressing CREB facilitated long-term memory following a procedure that normally does not produce fear behaviour. Therefore, if susceptible animals have a more excitable hippocampus, potentially mediated by an increase in CREB expression, they may form a greater density of hippocampal engram cells to encode stress-related experiences such as social defeat. When animals are challenged with adverse events again, the large density of allocated engram cells reactivate thus lead to the increase in engram cell density we found in susceptible animals. Examination of CREB or other signaling molecules related to enhanced neuronal activity in the hippocampus immediately after social defeat could reveal if susceptible animals have increased hippocampal neuronal excitability compared to resilient animals.

However, it should first be ascertained whether the engram increase we found at the end of social defeat is due to an increase in encoding or retrieval of the stressful event. The increased neuronal excitability hypothesis favours the notion that susceptible animals have increased encoding of stress engram, but it is also possible that susceptible animals recall fearful information better, even when the event is encoded similarly as resilient animals. Patients with depression show easier accessibility to recall negative information, for example recalling more negative self-referent adjectives (Bradley and Mathews 1983). This is not an issue of encoding, because in the same experiment when the patient is asked to recall non-self-referent adjectives, they performed similarly as control individuals, suggesting an activation of negative self schema is behind the enhanced negative self recall. It is also possible that susceptible animals exhibit overgeneralization, a trait often found in depressed patients, such that during the social

interaction test the presence an unfamiliar CD1 mouse is sufficient as a cue to retrieve memory of the previous social defeat events.

Interestingly, resilient animals had similar levels of CA1 engram cell activation as non-stressed control animals even though they had undergone social defeat. It is not clear whether resilient animals do not form engrams at all or the engrams are formed but not re-activated over time as a part of active forgetting. Active forgetting does not mean that the resilient animals do not form memory, but the animals do not access the memories with normal cues. Yoshii et al. (2017) found that artificial activation of an extinguished fear memory induced fear behaviour in mice, even when natural contextual cues no longer activate the contextual engram. Resilient animals may also use decreased retrieval of negative memories as a coping mechanism as opposed to susceptible animals that cannot inhibit negative information. Functional MRI neuroimaging studies of human showed that the anterior cingulate cortex (ACC) is a brain area activated in the inhibition of negative information (Shafritz et al. 2006). Coincidentally, ACC activity differs between control and depression individuals: ACC is more highly activated in control individuals when inhibiting positive words while ACC is higher activated in depressed individuals to inhibit negative words (Eugene et al. 2010). Hence, the resilient animal's stress engram reactivation over the course of social defeat can be examined to determine whether resilient animals use active inhibition of memory retrieval as a coping mechanism.

The relationship between engram cells and fear memory has been extensively studied. Previous studies found engrams coding for contextual fear memory in the CA1 (Deng et al. 2013), CA3 (Denny et al. 2014), DG (Liu et al. 2012) and the amygdala (Rashid et al. 2016). The activation of fear memory, often through optogenetic techniques, in these areas resulted in fear-related behaviour in the animals. In the above studies, researchers compared engram cell

activation between the non-conditioned control animals and fear-conditioned animals. In the current study, we explored engram activation in stressed animals with different behavioural outcomes, in addition to comparison with non-stressed control animals. We stressed animals equally and found two behaviourally distinct animal groups: susceptible animals responded negatively to the stressful event and resilient animals behaved similar to non-stressed control animals. Interestingly, we found that susceptible animals had preferential activation of the stress engram, which may underlie the basis for their depressive behaviour. There is a continuous distribution of stress engram size and the size of the stress engram is negatively correlated with the animal's amount of interaction with a social object. Tanaka et al. (2014) found that the amount of time the animals spend freezing following contextual fear conditioning is negatively correlated with dorsal CA1 engram reactivation, in agreement with our results suggesting the magnitude of engram activation may mediate the severity of the behavioural response.

When we explored a causal relationship between CA1 engram activation and behaviour by activating CA1 stress engram with excitatory DREADD, we saw a decrease in the time animals spend in the interaction zone. Artificial activation of the stress engram may reinstate the memory of the stressful experience in the hippocampus of these animals and subsequently lead to a depressive social avoidance behaviour. Our data suggests that CA1 stress engram activation possibly plays a causal role in mediating the depressive behaviour.

Our study showed an involvement of both the dorsal and ventral CA1 in mediating a depressive behaviour following chronic stress, with a correlation between engram activation and behaviour in both subregions. The involvement of both the dorsal and ventral hippocampal engram cells may be due to the complex nature of social defeat involving multiple environmental cues such as context, the presence of the attacker, the physical interaction between the mouse and

its attacker, etc. Therefore both the dorsal and ventral CA1 were likely engaged to encode the stress engram, even though there are functional and connective differences along the hippocampal dorsal-ventral axis. The dorsal hippocampus is essential for declarative and spatial memory. Lesion of the dorsal, not the ventral hippocampus, impaired performance in the Morris water maze (Moser et al. 1995) and radial arm maze (Pothuizen et al. 2004). The ventral hippocampus has been shown to mediate emotional learning, via projections to brain regions including the amygdala (Anagnostaras et al. 2002). Ventral hippocampal lesions impaired fear memory acquisition and or expression. During tests of contextual freezing, ventral hippocampal lesions produced the same deficits as whole hippocampal lesions (Richmond et al. 1999). Therefore the increase in both dorsal and ventral CA1 engrams in the susceptible animals is likely due to the multiple aspects of the social defeat events, ranging from contextual and spatial cues to social emotional learning.

4.3 Role of Dentate Gyrus in Stress Susceptibility

We found that the DG engram activation does not differ between susceptible and resilient animals. The DG has been shown to have sparse neuronal firing that is sensitive to changes in context (Leutgeb et al. 2007), playing the role of pattern separation. Optogenetic activation of the DG contextual engram has been able to generate fear response for a context the animals were not conditioned in (Ramirez et al. 2015). In our experiment stressed animals showed a decrease in engram activation in the DG compared to non-stressed controls without a decrease in total neuronal number. The lack of engram cell difference between resilient and control animals suggests that DG engram cells do not play a role in mediating the individual differences in stress susceptibility.

4.4 Hippocampus and Depressive Behaviour

Our data further supports the relationship between hippocampal function and individual differences in the manifestation of depressive behaviour. Ventral hippocampal activation and transmission to the nucleus accumbens is increased in susceptible animals (Bagot et al. 2015), in line with our data finding that susceptible animals had increased ventral hippocampal engram cell activation. It remains to be determined if the projection between hippocampal engram cells and the nucleus accumbens is increased in susceptible animals.

Although examining hippocampal volume revealed a deficit in hippocampal growth in susceptible mice (Tse et al. 2014), functional studies suggest greater hippocampal usage in depressed patients or individuals at risk of developing depression. Joormann and Gotlib (2008) found depressed individuals have more difficulties rejecting negative emotional words from working memory compared to non-depressed control individuals. Similarly, susceptible animals may find it difficult to cast away its stress engram following social defeat and lead to persistent social avoidance behaviour. Patients with major depressive disorders who responded to antidepressant fluoxetine treatment had a decreased activation of the limbic system, including the hippocampus, as shown by PET-glucose studies (Mayberg et al. 2000). In the social defeat model, it has been shown that susceptible animals increase social interaction in response to anti-depressant fluoxetine treatment (Krishnan et al. 2005), so it would be worth exploring whether fluoxetine decreases engram cell activation in mice. The hippocampus of young people with familial risk to depression also showed stronger hippocampal activation to episodic memory under magnetic resonance spectroscopy for glutamate and glutamine (Mannie et al. 2014). This finding suggests that the hippocampus may be hyperactivated in high-risk individuals prior to clinical symptom onset. In an animal setting, a hyperactivated hippocampus may encode and

retrieve the traumatic event better by facilitating engram cells formation, which underlies the expression of depression-related behaviors after social defeat

4.5 Mechanism for Controlling Engram Size

The neuronal mechanism underlying the differential engram activation between susceptible and resilient animals remains unclear. There has been data showing that interneuron inhibition may control the size of engrams in the hippocampus and amygdala. Disinhibiting the pyramidal neurons by inhibiting interneurons increased the size of engrams in these areas (Fuchs et al. 2017; Stefanelli et al. 2016; Morrison et al. 2016). Therefore, it would be interesting to examine whether there are differences in interneuron inhibition onto CA1 pyramidal neurons between susceptible and resilient animals.

4.6 Limitations

There are limitations of using the TetTag model to examine engram cell activation. First of all, there is a lack of CA3 cells with LacZ labeling in all animals, in the experiment in which we tested the stability of the LacZ signal. We saw some LacZ labeling in the CA3 one day after we stopped neuronal labeling (day 3 of social defeat), but not 4 days after (day 6 of defeat) or 8 days after (day 10 of defeat). For reasons unclear, the LacZ signal in CA3 decays very quickly and is no longer present when we examine after the entire 8-days of social defeat. This limitation may be caused by the mutated tTA protein that is insensitive to doxycycline and continues to drive LacZ expression when the animals are put back on Dox. It is possible that the mutated tTA is less stable in the CA3 and the mutated tTA becomes degraded very fast. Another limitation is also revealed by the experiment testing the stability of LacZ. We found that the level of LacZ

signal decreased over time in the ventral hippocampus, with higher levels of LacZ one day after neuronal labeling was stopped compared to levels of LacZ on day 6 or 10. Therefore, the neuronal labeling by LacZ is long-lasting but not permanent.

4.7 Future Directions

Further examination of engram cell activation during the course of chronic social defeat should be conducted to examine if any changes occur in engram formation over time. Examination of engram activation during the social interaction test should also be completed to confirm stress engram activation when the animals are faced with a situation that is similar but not identical to the encoded stressful event. Expansion on the causal studies should also be conducted, including exploring the effect of inactivating the stress engrams on animals' behaviour. Depressive behaviours can also be studied beyond the social interaction test, though not all depressive symptoms may be related to hippocampal engram cells. Observationally, we noted animals that failed to make their bedding tend to turn out as susceptible. Other behavioural measures, for example fear generalization, could provide a fuller picture of the animal's behavioural and psychological change. After all, depression is a multi-faceted disorder; multiple behavioural changes need to take place for a diagnosis of major depressive disorder.

Chapter 5: Conclusion

In conclusion, the current study found that there is an increase in stress engram activation in the hippocampus of mice exhibiting depressive behaviour after chronic social defeat stress. The degree of stress engram activation is negatively correlated with the magnitude of social interaction behaviour. Our study suggests hippocampal engram activation as a cellular mechanism for the negative information processing and cognitive symptoms found in depressed individuals.

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