THE ROLE OF FEAR GENES IN STRESS AND SOCIAL BEHAVIOR

By

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ABSTRACT OF THE DISSERTATION

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The role of genes, regulating fear, in behaviors critical for survival remains unclear and requires further studies at the molecular, cellular and neural circuitry levels. Towards this end, this thesis is focused on how the anatomic neural circuits expressing the gastrin-releasing peptide (GRP) regulate two types of behaviors, those that are related to individual's threat and those that are related to the survival of the species. Individual's threat is studied using fear memory extinction in Aim 1. Behaviors related to the species' survival are studied using maternal behaviors in Aims 2 and 3. The main conclusion from this work is that the molecular mechanisms and neural circuits regulating fear memory are also involved in behaviors critical for the survival of the species.

Acknowledgement

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Specific aims

Fear processing is at the core of emotional and affective behaviors in humans and animals. Growing evidence shows that genes and their signaling pathways are critical for brain function. Therefore, dissecting the molecular and cellular mechanisms of fear processing would greatly improve our understanding of the basic events regulating emotional behavior and will help clinical applications. More specifically, our laboratory and other investigators have found several genes critical for threat, fear and anxiety responses. Using this earlier knowledge, my project has focused on the role of the *Grp* gene, GRPergic neural circuitry and microtubules (MTs) in post-traumatic stress disorder (PTSD)-like behavior in males and maternal care in females. Previous research showed that the GRP is highly enriched in the basolateral amygdala (BLA)-associated neural circuitry of learned fear and is critical for regulating amygdala synaptic plasticity and memory of fear. Another amygdala-enriched gene, encoding stathmin protein, an inhibitor of MT formation, was shown to control innate and learned fear as well as maternal care.

Main Hypothesis: The GRPergic neural circuitry regulates processing of stress-regulated emotional behaviors, including fear extinction and postpartum behaviors.

Specific Aim 1: Examine the role of the GRP and its neural circuitry in stress-enhanced fear

Rationale: Research shows that adding mild stress to fear extinction protocols separates animals into two groups, those that have difficulty to extinguish fear memory (susceptible group) and those that are able to do so (resilient group). Previous work also suggested that the GRP is involved in fear memory and fear extinction. Therefore, the GRP knockout (KO) mice might be a good model for examining the mechanisms of stress-enhanced fear extinction.

Hypothesis: Enhanced neuronal activity in the basolateral amygdala of the GRP KO mice leads to difficulties in extinguishing fear memory.

Approach: To understand the molecular mechanisms and neural circuits involved in extinction of fear, and how deficiency in extinction may lead to enhanced fear and anxiety, we focused on the *Grp* gene. We employed the $Grp^{-/-}$ mice generated in our lab to investigate the role of the GRPergic neural circuits in extinction of fear memory using the stress-enhanced fear learning (SEFL) paradigm, a recently developed behavioral model of PTSD.

Results: The $Grp^{-/-}$ male mice have enhanced neuronal activity in the BLA following fear conditioning. They also show stronger memory of fear as well as a deficiency in extinction in SEFL. Interestingly, transcription of dopamine-related genes was decreased in the BLA of $Grp^{-/-}$ mice following SEFL extinction recall, suggesting that the GRP may be an upstream molecule in the dopamine-signaling pathway, and thus the GRP and dopamine might work together to regulate fear extinction.

Specific Aim 2: Examine the role of microtubules in the GRPergic neural circuitry in regulating maternal care.

Rationale: For Aims 2 and 3, we have been studying stathmin protein, a major inhibitor of microtubule (MT) formation, which our lab identified as regulating innate and learned fear in mice. We also showed that memory processing leads to and requires changes in MT stability.

Hypothesis: Activity-induced changes in MTs are important for maternal care and affective behaviors postpartum.

Approach: MTs mediate transport of molecules and organelles between the synapse and cell body. Stathmin protein is a major negative regulator of MTs. Stathmin function is controlled by its phosphorylation. Wildtype unphosphorylated stathmin binds tubulin and let it go once phosphorylated. We employed stathmin to control MT function. We used unphosphorylatable and thus constitutively-active stathmin4A (Stat4A) mutant protein, which irreversibly binds tubulin, causing destabilization of MTs, to make MTs more stable overall and less dynamic in response to neuronal activity. We expressed Stat4A in the GRPergic neural circuitry and studied maternal care and postpartum anxiety- and depressive-like behaviors in Stat4A transgenic females. We also employed virus-delivery of the Stat4A to test several brain regions in these behaviors. The results of these experiments suggest that various parts of the GRPergic neural circuits differentially regulate maternal care and affective behaviors.

Results: Wildtype female mice exhibit changes in stathmin and MTs during pregnancy and after the delivery. Also, *Stat4A* transgenic female mice have deficits in maternal care, anxiety- and depressive-like behaviors postpartum.

Specific Aim 3: Examine the role of the dorsal subiculum in maternal care and affective behaviors postpartum.

Rationale: The *Stat4A* transgene is strongly expressed in several brain areas of *Stat4A* transgenic mice, including the anterior cingulate cortex (ACC) and dorsal subiculum (DS), which are located next to each other. See Rationale for Aim 2 above for more details.

Hypothesis: Stathmin-mediated microtubule deficiency in the dorsal subiculum regulates maternal care, anxiety and depressive like behaviors.

Approach: We used adeno-associated virus (AAV) to deliver Stat4A in the brain areas of WT female mice and tested them in maternal care and affective behaviors postpartum.

Results: The DS, but not ACC, has a role in maternal care and anxiety postpartum.

Background

Deciphering the molecular and cellular mechanisms underlying the neural circuitry of fear may give us a deeper insight of how the brain processes PTSD-like and maternal behaviors. This is because both PTSD and maternal care are behaviors critical for survival of the organism and involve assessment of threat in the environment. Fear and maternal behaviors and their corresponding neural circuits are highly conserved in mammals. In our lab, we focus on the mouse work, hoping that we may learn the fundamental mechanisms of behavior and at the same time may shed light on the mental states in humans related to fear and maternal care.

Most behaviors with time (through an experience of the interaction with the outside world) become a combination of innate and learned responses. Therefore, it is not surprising to find an overlap between the mechanisms being discovered now as underlying maternal care and those controlling memory processes (Kandel et al., 2014; Poo et al., 2016).

Threat helps animals and humans to survive in the world as long as they can adapt their initial innate behavior response through learned fear (Kandel *et al.*, 2014; Tinbergen, 1970). When threat is excessive, prolonged fear takes place and the organism develops an abnormal response to threatening events (Herry et al., 2010; Maren and Holmes, 2016).

There is a distinction between innate and learned fear. Even though, both of them are part of the survival responses, innate fear is an emotional feeling that is hard-wired in brain circuits and does not require previous experience. In rodents, innate fear can be a predator smell or fear of open spaces. In contrast, learned fear results from experiencing an aversive event at any point in the past, and Pavlovian or classical fear conditioning is a powerful tool used in laboratory conditions (LeDoux, 2014; Shumyatsky et al., 2005). Conditioned fear responses can also subdue through fear extinction. During fear extinction, the shock US is absent and the animal learns anew that the CS no longer predicts the US, and the conditioned fear response is suppressed. Extinction does not erase the original fear memory, but creates a new memory representation.

Amygdala, as a main brain region that integrates information from other areas, is responsible for learning of fear. Importantly, the lateral nucleus of the amygdala (LA) is one of the brain areas where long-term potentiation, a cellular model of memory formation, has been shown to be linked to memory. Fear extinction depends on the communication between several brain regions: the amygdala, hippocampus, medial prefrontal cortex (mPFC) and some other brain regions (Luchkina and Bolshakov, 2019; Maren and Holmes, 2016). Deficiency in fear extinction contributes to PTSD in humans (Lebois et al., 2019; Sangha et al., 2020). Even though PTSD is a human disorder, it can be studied in rodents that have impaired fear extinction because the brain regions, neural circuits, genes

and behaviors are largely overlapped between humans and other mammals (Fenster et al., 2018; Singewald and Holmes, 2019). However, the genetic characterization of the neural circuits involved in both fear extinction and PTSD remains largely unresolved (Duman and Girgenti, 2019; Lonsdorf and Kalisch, 2011; Singewald and Holmes, 2019). Defining the logic by which genes operate on specific cell types and in turn direct the neural output is therefore a central issue in understanding etiology of fear extinction and may also be crucial to individualized approaches to diagnosis and treatment of PTSD (Yehuda et al., 2015).

Maternal care is an example of affiliative social behaviors, but its role is more than just survival of individual organisms – it is essential for the survival of the species as a whole. Not surprisingly, deficits in care for the progeny may result in their neglect and death. Throughout the animal kingdom, maternal care plays a fundamental role in keeping the offspring safe and healthy (Numan and Insel, 2003). The maternal brain is highly plastic and the ability of the mother to respond to often changing and potentially dangerous to the progeny environment is critical to the progeny survival. Laboratory mice have become increasingly popular due to our ability to apply the tools of mouse genetics. In particular, classical and conditional gene knockout approaches have provided important information to studies in maternal care. Classical gene knockout methodology is critical as it “cleanly” removes the gene from all cells in the body. This may be a problem with conditional knockout approaches where it is not always easy to

assess how many cells are affected by the knockout. The drawback is the possibility of indirect effects of the deleted gene as the classical gene knockout may affect the developmental stages and have compensatory mechanisms (expression of other genes may change as a result of the missing gene), thus complicating the interpretation of gene of interest function in an adult mother (Kuroda et al., 2011; Nelson and Young, 1998). Conditional gene technology provides spatial and temporal resolution, allowing to disturb gene expression at the adult stage only, in a particular brain region and even cell type as well as restore at various time points gene expression or test other genes on the background of the original gene knockout.

In this work, we focus mainly on the molecular mechanisms of maternal behavior and the activity of which is changed during maternal care. However, it is important to put this into perspective of brain regions involved. There is extensive work describing the topic of brain areas involved in maternal behavior (Kohl et al., 2017; Numan and Insel, 2003; Pawluski et al., 2016). Different experience-dependent changes have been described in various brain areas during maternal care (Alsina-Llanes and Olazábal, 2020; Champagne et al., 2001; Champagne et al., 2004; Champagne et al., 2003; de Moura et al., 2015; Francis et al., 2000; Francis et al., 2002; Gammie et al., 2016; Ray et al., 2015; Stamatakis et al., 2015; Tsuneoka et al., 2013). Therefore, it is important to continue studying and comparing the molecular changes in separate brain areas.

Seminal studies in rodents have shown that maternal care can be positively or negatively regulated by specific brain areas (Dulac et al., 2014; Numan and Insel, 2003). Some key brain areas important for stimulation of maternal behavior include the medial preoptic area (mPOA), the ventral bed nucleus of stria terminalis (vBNST) and lateral septum. The mPOA direct projections to the nucleus accumbens (NAc), or indirect via retrorubral field (RRF) and via ventral tegmental area (VTA) would also mediate parental responses. The mPOA-VTA-NAc network, which is part of the motivational circuit, is regulated by projections from the paraventricular nucleus of the hypothalamus (PVN), lateral habenula (lHb) and the dorsal raphe nucleus (Kohl *et al.*, 2017).

Activity of the medial amygdala inhibits young virgin female rats to approach pups, which has been interpreted as a way how in nature young female rats stay away from eating or hurting newborn pups (Lévy and Keller, 2009). Olfactory cues coming from the pups are processed by the accessory and main olfactory bulbs which increase the activity of the medial amygdala in virgin females. In turn mPOA neurons are inhibited together with the onset of care-giving behaviors. As will be described later, the identity of medial amygdala cells is critical to understand their role in maternal care.

The role of the hippocampus in maternal behavior has received some attention, but perhaps not enough due to its importance for synaptic plasticity and sensitivity to hormones. Maternal care induces neurogenesis in the dentate gyrus

as well as changes in dendritic spine morphology in the hippocampus and several other brain areas (Pawluski *et al.*, 2016). Cell proliferation is decreased during the early postpartum and exogenous treatment with corticosterone produces a higher decrease in proliferation (Pawluski *et al.*, 2016). Hippocampal log-term potentiation (LTP) is increased in mothers, an effect that is abolished by gestational stress (Pawluski *et al.*, 2016). Moreover, $GABA_A$ receptors (GABAR role is discussed in more detail in one of the chapters), whose expression in the hippocampus is modulated during pregnancy, are involved in pup retrieval and affective behaviors in the postpartum but not in virgin females (Maguire and Mody, 2008).

Maternal behavior relies on the hormonal state of the mother and sensory cues coming from the progeny. The mother's brain is continuously processing external sensorial information and must adjust its activity to meet the demands of the offspring (Olazabal et al., 2013a; b). Activity-dependent genes and proteins may play a key role in initiating and maintaining neuronal activity elicited by the mother-progeny interaction and later processing it into long-term memory.

Recent evidence indicates that the maternal brain is highly dynamic and susceptible to both internal and external influence. This plasticity is displayed at various levels, intra- and intercellular signaling and pathways. It may be instructive to compare activity-regulated processes in maternal care and memory. By definition, memory processing is dynamic and plastic. In a

somewhat similar manner, the motherhood enhances plasticity of the female brain inducing neurogenesis, changes in dendritic spine morphology, LTP and hippocampus-dependent memory (Leuner and Sabihi, 2016; Pawluski and Galea, 2006) and these processes are affected during perinatal stress and maternal disturbance in animals and peripartum depression in humans (Qiu et al., 2020). The hippocampus, amygdala and prefrontal cortex are some of the overlapping brain regions involved in memory, postpartum states and depression in humans (Leuner and Shors, 2006; 2013).

LTP is a widely accepted model of the cellular mechanisms of activity-dependent synaptic plasticity leading to memory formation (Bliss and Lomo, 1973; Malenka and Nicoll, 1997; Martin et al., 2000; Poo *et al.*, 2016; Siegelbaum and Kandel, 1991; Stevens, 1998). Activity-dependent genes are critical for both LTP and memory processing and somewhat similar links can be expected between genes, synaptic plasticity and maternal care. Maternal experience-dependent cortical plasticity was found to be related to the ability to retrieve pups (Lau et al., 2020) and related to a gene knockout of the *Mecp2*, which loss-of-function mutations cause the neurodevelopmental disorder Rett syndrome (Amir et al., 1999).

Changes in dendritic spines are believed to be a critical part of memory processing (Helm et al.). These changes are also consistently found during normal motherhood and fatherhood (Glasper et al., 2016) as well as in animal models of maternal disturbance (Workman et al., 2013). Galanin-expressing

neurons in the mPOA govern preprogrammed innate maternal care as well as may serve as a switch from paternal aggression towards pups to paternal care (Wu et al., 2014). Therefore, galanin-expressing cells may be involved in both innate and learned maternal responses.

Learned responses to pups' auditory cues are critical for mothers' efficient maternal care. There is evidence for the role of maternal physiological state (virgin females vs. mothers) in creating memory for ultrasonic vocalizations from pups (USVs) (Lin et al., 2013); this memory may transform and shape innate maternal care responses into learned behavior with time and experience, making them more adaptive to the current situation surrounding the mother and her progeny. With daily pup exposure, virgin females learn maternal care and perform it well in comparison to mothers (Fleming and Rosenblatt, 1974).

The sensory cues elicited by pups change the neural activity in the female brain. In the postpartum, the representation of the pup calls in the primary somatosensory cortex increases and is later refined, likely due to activity-dependent plasticity elicited during nursing. Pup calls produce a stronger activation of the auditory cortex in mothers compared to virgin females, and the balance between excitation and inhibition is changed (Valtcheva and Froemke, 2019). The temporal association cortex receives inputs from the auditory cortex and exhibits activity-dependent changes that improve the discrimination of pup calls in mothers compared with females (Tasaka et al., 2020). Auditory-driven

plasticity was found in the temporal association cortex (TeA) in mothers in response to USVs from pups. Tasaka et al. suggest that TeA's processes USVs to support the memory of pup cries by the parents, somewhat similar to how TeA processes information for auditory memory in fear conditioning (Quirk et al., 1997; Romanski and LeDoux, 1992).

In addition to mother's own experience in nursing, learned adaptations that improve maternal care include learning by "social transmission" from other experienced mothers (Carcea et al., 2021; Schiavo et al., 2020). Learning maternal care from other females makes biological sense as both wild and laboratory mice as well as rats prefer to rear their young in communal nests and nurse both their own and other mother's pups (Branchi, 2009; Heiderstadt and Blizard, 2011; Weidt et al., 2014).

Significance

Neuroscience research in the last 30 years has made it clear that many molecular and synaptic events important for learning and memory are also critically involved in several other brain processes, such as drug addiction, food consumption and many others, such social and affiliative behaviors. Similarly, the genes implicated in parental behavior overlap significantly with those already established as regulating memory.

The most recent focus in neuroscience has been on systems and circuitry neuroscience. However, genes and proteins define and regulate short- and long-term events in neural circuits and their role therefore should not be ignored (Sudhof, 2017). In addition, changes in genes and proteins predispose to or cause mental disorders including maternal dysfunction.

Learning and memory depend on neuronal plasticity originating at the synapse following an exogenous stimulus and requiring gene transcription in the nucleus to persist. RNA and protein products following these transcription events are transported back to synapses strengthening synaptic connections. While we are beginning to understand activity-regulated processes and how synapse-to-nucleus back and forth communication supports long-term plasticity as well as learning and memory, the role of these processes in maternal behavior remains unclear.

The microtubule-mediated synapse-to-nucleus transport is just one of many activity-regulated intracellular events. Others include changes in the blood flow, spine dynamics, synaptic connections, intracellular movement of organelles via synaptic and nuclear trafficking, and changes in protein structure and function, to name a few. This is clearly not a full list as we are learning all the time that some of the molecular and cellular events that were considered stable are in fact dynamic in the adult brain (for example, neurogenesis and microtubule stability). The genetic characterization of the neural circuits in PTSD and maternal behavior remains largely unresolved. The genes and neural circuits underlying this behavior, and especially which processes malfunction and turn normal maternal care into postpartum depression, are understudied. Knowing these processes would benefit the approaches to treat postpartum depression. Still, our understanding of the molecular mechanisms and neural circuits they serve on to mediate maternal care and its links to anxiety and depression remains surprisingly incomplete. In particular, details of how genes control maternal behavior and how their products at synapses and in the nucleus are involved in taking care of the young during normal maternal behavior and when the behavior turns into pathological are too poorly understood to be harnessed for therapeutic applications. We propose to address this gap in knowledge from a new angle featuring a new genetically modified mouse line that displays behaviors reminiscent of postpartum depression. We will also use a behavioral paradigm where pup-naïve and virgin females are assessed in comparison to mothers in

maternal care, anxiety and depressive-like behaviors. We have developed a multidisciplinary approach that uniquely positions us to address three aims that together will markedly advance understanding of the biology of maternal care and its links to anxiety and depression.

Neural Circuitry of Fear and PTSD

i. Fear, fear conditioning, and anxiety

The ability to remember threatening events is critical for an organism's survival since fear memory triggers a behavioral response that will help to escape from a dangerous situation. The experience of fear varies among species. For instance, in humans fearful and aggressive faces of other humans can trigger a defense response, while the smell of urine from a predator is enough to release a threat response in a mouse. Despite the differences in dangerous stimuli, the neuroanatomic circuits and adaptive responses to the environment are highly conserved among mammalian species (LeDoux, 2012a).

There are several types of fear, some of them involve a survival circuit activity and the observable behavior, whether it is freezing, jumping, or running, is an indicator of an animal experiencing fear (LeDoux, 2014). In mammals, fear can be innate or learned. The amygdala is the major brain structure controlling both types of fear (Duvarci and Pare, 2014; Likhtik et al., 2014; Shumyatsky *et al.*, 2005).

The neural circuits for learned fear include several brain areas such as the amygdala, medial prefrontal cortex (mPFC) and hippocampus (HPC). The amygdala participates in all phases of fear memory processing. Several

behavioral approaches exist to study memory of fear in the laboratory conditions with Pavlovian fear conditioning being one of the most extensively used (LeDoux, 2000). Conditioned fear acquisition requires projections to the amygdala that originate from the auditory inputs in the thalamus, auditory cortex and the contextual inputs from the ventral HPC. The neurons that comprise the amygdala are excitatory glutamatergic spiny projection neurons and inhibitory GABAergic interneurons, and the balance between excitation and inhibition in the amygdala is critical for fear learning (Reviewed in (Tovote et al., 2015)).

Synaptic plasticity in the amygdala is necessary for acquisition and expression of learned fear (Rogan et al., 1997). The rodent amygdala has 13 different nuclei and several of them can undergo physiological changes in response to fear conditioning. For instance, neurons in the lateral nucleus of the amygdala (LA) have an increase in the frequency and amplitude of spontaneous postsynaptic currents (sPSC) (Butler et al., 2018). Even subgroups of cells within the lateral amygdala can have different electrophysiological properties (Repa et al., 2001). Fear conditioning induces robust plastic changes also in other nuclei of the amygdala. For instance, excitatory synapses onto inhibitory neurons in the central amygdala (CeA) are highly prominent, and when synaptic potentiation of excitatory cells onto somatostatin-positive neurons is diminished, fear memory formation is impaired (Li et al., 2013). These and other data suggest that the amygdala is very plastic during fear conditioning.

Even though amygdala's main function is to acquire and store information about an aversive experience (Reviewed in (Fanselow and LeDoux, 1999)), it also modulates consolidation of other information in different brain regions and might be a critical link in a subcortical circuit that mediates formation and more permanent storage of conditioned fear (Cahill et al., 1999). As novel techniques have emerged, it has been possible to identify more neural connections between brain areas that are involved in fear memory. For example, optogenetic manipulation of the basal amygdala (BA) showed a functional connectivity between BA neurons and pre-limbic (PL) cortex neurons, where BA projects to PL during fear conditioning (Senn et al., 2014). Also, PL integrates information from BLA and ventral hippocampus (vHPC). Inactivation of BLA neurons using pharmacological approach causes a decrease in activity of PL pyramidal neurons in response to fear conditioning by eliminating fear responses, while inactivation of vHPC decreases activity of PL inhibitory interneurons in response to fear conditioning at the same time increasing the conditioned tone response (Sotres-Bayon et al., 2012).

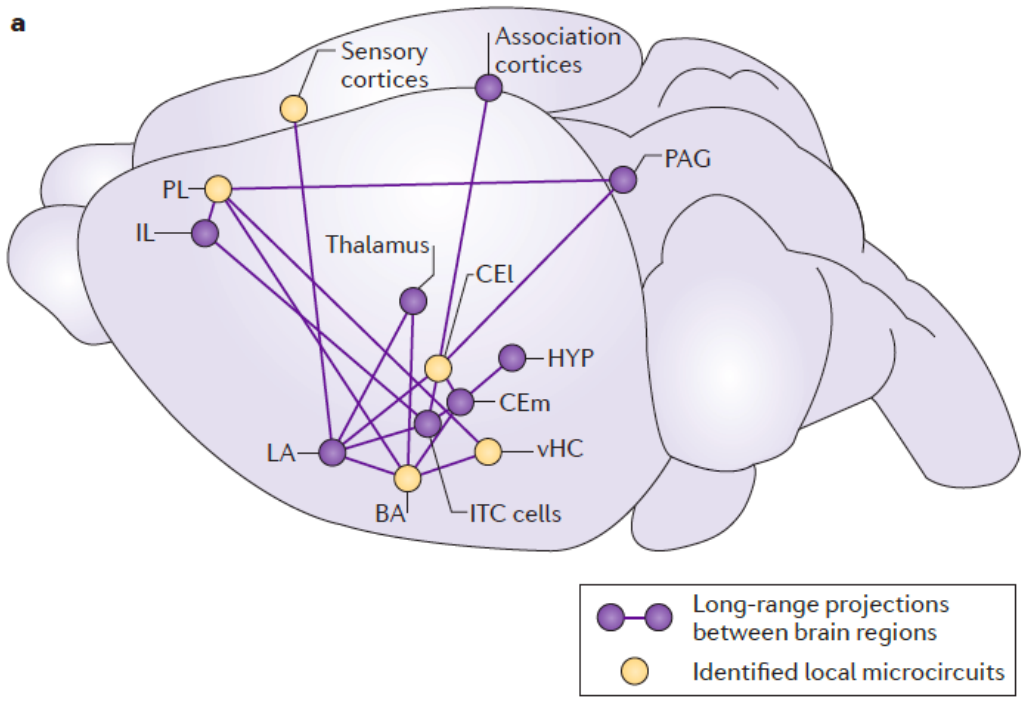

Figure 1. Neural circuitry of learned fear (Tovote et al. 2015)

Fear conditioning is used in laboratory settings to assess memory of fear in many species, including humans and rodents (Fanselow, 1998). Fear conditioning is one of the variations of Pavlovian or associative conditioning. Since 1960's, Pavlovian conditioning has been used to study the cellular and molecular mechanisms of learning in different species (Kandel and Spencer, 1968), the classic paradigm consists in presenting a neutral conditional stimulus (CS) which can be a tone followed by an unconditional stimulus (US), like an electric shock, which is aversive. The subject learns that the CS predicts something harmful and later shows an avoidant response to the CS even in the absence of the US. The avoidant response is usually measured as the level of freezing. The freezing

response is innate in animals, what is learned is the association of the CS with the US, in other words the subjects learn that the CS is a warning of something dangerous (LeDoux, 2012b). The field of fear conditioning has evolved to the extent that Pavlovian fear conditioning paradigm has been modified to asses a wider range of fear experiences, for instance, fear extinction, resilience to stressful conditions, anxiety, and hormone-behavior relationships (Indovina et al., 2011; Li and McNally, 2014; Revest et al., 2005).

ii. Post-traumatic Stress Disorder

It is important to distinguish between fear and anxiety because they generate different sets of behaviors in mammals, including humans. Therefore, while fear behaviors respond to a specific single event, anxiety does not have a specific triggering event. In this sense, fear is caused by a stimulus that predicts danger and the behavioral response will be a defense activated by the circuits responding to that behavior. Acute versus sustained threat could also explain the difference between fear and anxiety, since they trigger different brain areas. When an acute threat is present, BLA-CeA-brainstem circuit is activated. When a subject experiences sustained threat, in addition to CeA, the BLA projects to the BNST to sustain the fear response giving the BNST a complementary role in the circuitry (Reviewed by (Perusini and Fanselow, 2015)).

Another way to differentiate fear and anxiety is through the concept of fear overgeneralization. Aversive memories can and very often become generalized. When a subject learns that a stimulus is threatening, the subject can in other context or situation, making a generalization of it. Fear generalization can be helpful because it prepares the organism to make an assessment whether the event is dangerous or not, which helps to survive in a threatening situation. However, when fear becomes overgeneralized, and the response based on a high level of fear is the only way of responding to multiple events, the fear response becomes dysfunctional. Therefore, fear overgeneralization is a distinctive feature of anxiety disorders and stress-related behavior in humans (Asok et al., 2018).

In humans, Post-traumatic Stress Disorder (PTSD) is part of the anxiety disorders. By definition, PTSD symptoms develop after an exposure to a traumatic event, and the symptoms include re-experience of the traumatic event, avoidance, and hyperarousal. PTSD is considered to be a very disabling disease since patients experience a high degree of functional impairment (Reviewed in (Skelton et al., 2012)). Animal studies on fear conditioning provide important knowledge about the cellular and molecular mechanisms that regulate PTSD (Hersman et al., 2019; Ressler et al., 2011; Sillivan et al., 2017). Genetic studies in mice suggest vulnerability to the development of PTSD, for instance, there are genes that increase sensitivity of glucorticoid receptors (GR) which are known to be involved in the PTSD response: *FKBP5* gene impacts the Hypothalamic-

Pituitary-Adrenal (HPA) axis function by regulating GR activity in PTSD both in mice (Hubler and Scammell, 2004) and humans with PTSD (Koenen et al., 2005).

Stress-enhanced fear learning (SEFL) is a behavioral paradigm to study PTSD symptoms and produce extinction resistance in mice. In this paradigm, rodents are first exposed to a series of foot-shocks in a distinct context and tested on auditory fear conditioning (tone-shock pairing) one to 7 days later in a novel context. The goal of this procedure is to potentiate the conditional fear response that is acquired during the second learning experience. Extinguishing fear to the first context does not eliminate SEFL, rather there is a non-associative sensitization of fear that is resistant to extinction (Reviewed in (Maren and Holmes, 2016)). In this project, we use a modification of the SEFL paradigm to precipitate traumatic-like memories where we included a brief stressor, rather than a chronic stressor in order to split the mice into resilient and susceptible subgroups. This variation of SEFL where a short acute stress is followed by single pairing fear conditioning and fear extinction is a useful tool to understand PTSD (Sillivan *et al.*, 2017). This SEFL paradigm is used in my dissertation work.

iii. The GRP and GRPergic neural circuitry of learned fear

Many aspects of memory in mammals involve the molecular and cellular mechanisms that are triggered by gene expression (Tonegawa et al., 2003).

Genetic manipulations in mice allowed for dissection of neural circuits in mammals and understanding of several understudied psychological states. Learned fear is an excellent model to study the basics of memory processes as it is simple, robust, quickly learned, conserved between species and amenable for laboratory investigation. To understand how the brain processes fear information is also crucial from a biological and psychological perspective since it is so basic for survival and is the critical component in many mental states in humans, such as phobias, anxiety disorders, PTSD, schizophrenia, autism and many others (Acheson et al., 2015; Mahan and Ressler, 2012; Steinberg et al., 2015). Genetic manipulations in mice have allowed for identification of neural circuits underlying fear conditioning processes and, by extension, related types of fear memory (Mayford et al., 2012). This work is focused on studying the molecular and cellular mechanisms of learning and memory, and social behavior.

The *Grp* gene, which encodes the gastrin releasing peptide, was found to be highly expressed in the lateral nucleus of the amygdala and the regions that convey the auditory conditioned stimulus (CS) information of fear (Shumyatsky et al., 2002). In addition, GABAergic interneurons in the lateral nucleus of the amygdala have receptors for GRP (GRPR).

Deletion of GRPR gene in mice shows alterations in cued fear conditioning but no alterations in innate fear. Figure 2 shows RNA in situ hybridization of mouse coronal sections of the Grp gene in the brain areas that respond to fear learning.

The Grp gene is highly expressed in the lateral and basal nucleus of the amygdala. However, there is also expression of Grp gene in the medial, ventral, and dorsal subdivisions of the medial geniculate body, the posterior intralaminar nucleus of the auditory thalamus, the TE3 subregion of the auditory cortex, and the perirhinal cortex (Shumyatsky *et al.*, 2002). This pattern of Grp expression is intriguing because the amygdala has afferent projections with the auditory thalamus, auditory cortex, and HPC, and these brain areas control processing of the conditioned stimulus (Reviewed in (Tovote *et al.*, 2015)). Thus, the GRP can be a marker of the brain areas sending the conditioned stimulus information to the amygdala.

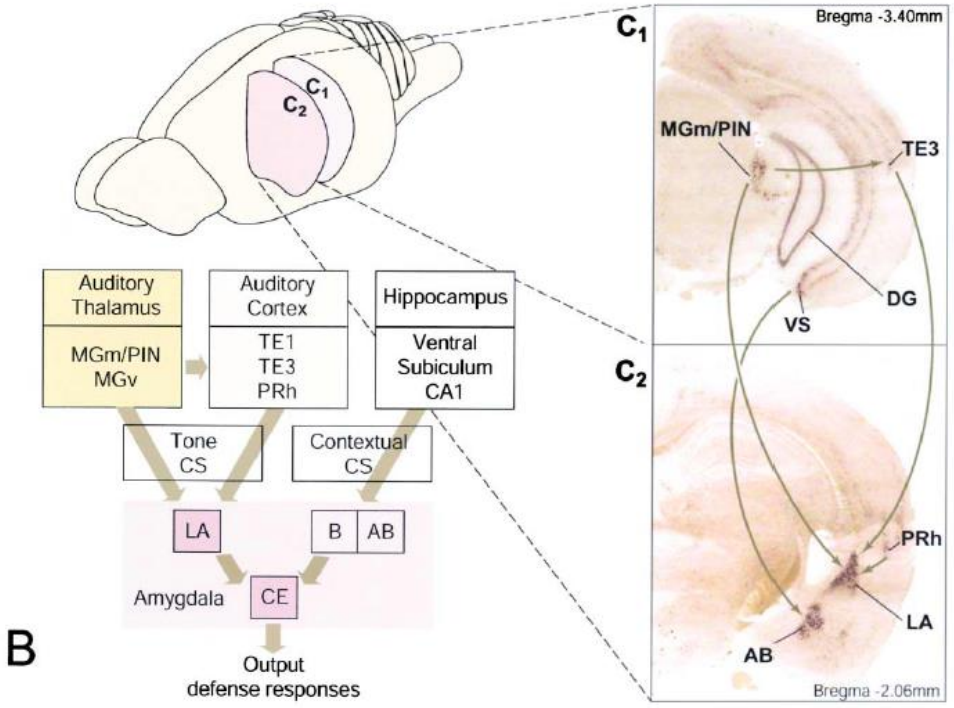

Figure 2. Expression of the GRP in brain areas involved in fear learning (Shumyatsky et al, 2002)

The GRP acts as a negative regulator of fear memories in the amygdala (Figure 3). GRP is released from principal neurons in the lateral amygdala in WT animals during fear response and excites GABAergic inhibitory neurons through GRPR. Thus, interneurons get activated and in turn inhibit principal neurons reducing their excitability through an inhibitory feedback loop. When this inhibitory molecular network is disrupted, there is an increase in LTP in the lateral nucleus of the amygdala and stronger and longer lasting fear memory. Thus, it is very likely that the GRPR has a role in modulating the balance between excitation and inhibition in the neural circuitry related to learned fear.

Through the development of a knockout mouse that lacks the Grp gene ($Grp^{-/-}$) and instead the gene is replaced with the enhanced green fluorescent protein (GFP), it is possible to study different stages of fear, such as fear conditioning, fear extinction, and stress related to fear. The different brain areas where the Grp gene is expressed in the mouse brain, and they include the amygdala (lateral and basolateral nuclei), vHPC, mPFC, retrosplenial cortex, dorsal subiculum (the focus of Specific Aim 3), auditory cortex, and the entorhinal/perirhinal cortex.

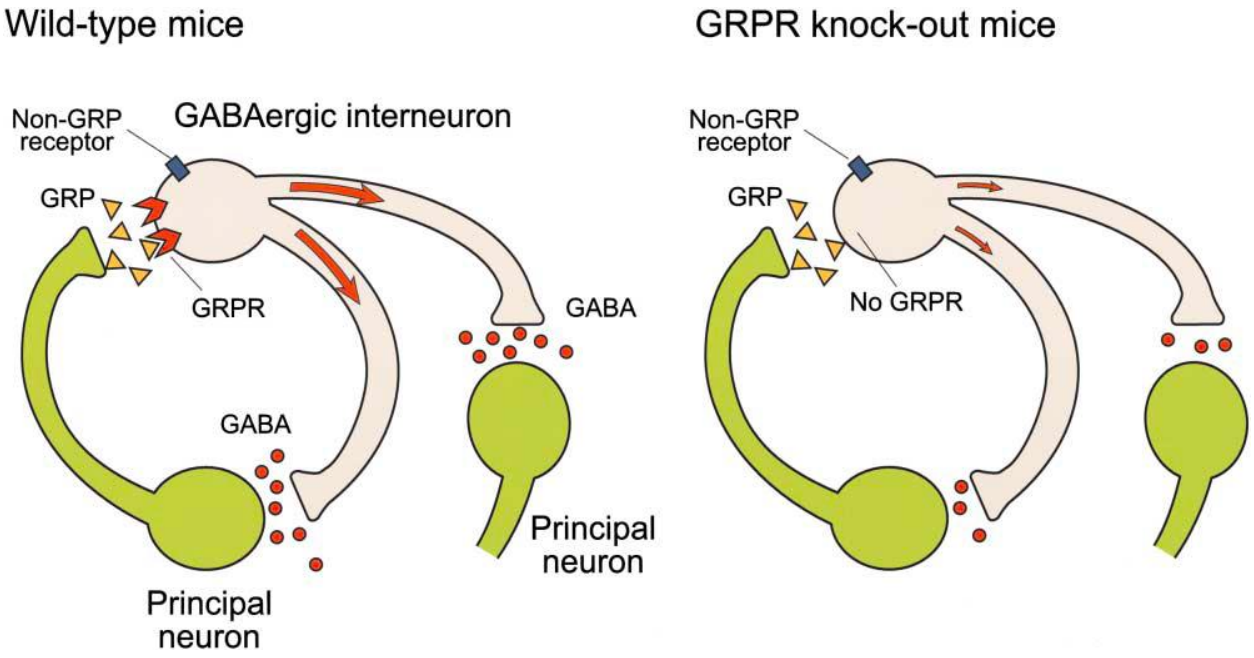

Figure 3. A model for GRP-dependent negative feedback to principal neurons in the amygdala in wild-type and GRPR knockout mice (Shumyatsky et al, 2002)

iv. Stathmin and its control of fear and anxiety

The gene stathmin/oncoprotein 18, which encodes for the stathmin protein, was identified as an amygdala-enriched gene (Shumyatsky *et al.*, 2002). It was found in vitro that stathmin is a cytosolic phosphoprotein that binds to tubulin dimers preventing MT formation (Curmi et al., 1997) and after phosphorylation it releases tubulin allowing MTs to be formed (Belmont and Mitchison, 1996). Therefore, stathmin regulates MT dynamics via control of their polymerization (growth) and depolymerization (shrinkage).

MTs are one of the major cytoskeletal structures that are critical for every cell function. Specifically, neural MTs are important for neurodevelopment, axonal pathfinding, and synapse trafficking. An important characteristic of MTs is their ability to undergo cycles of rapid growth and disassembly, this polarity is known as dynamic instability and it allows MTs to search the cell's three-dimensional space finding the specific target sites on the cell periphery (Conde and Caceres, 2009). Tubulin is the main globular protein which is assembled into MTs (Mohri, 1968). In every cell type, α and β tubulin make heterodimers that interact in a head-to-tail manner giving rise to linear polymers known as protofilaments. MTs are made of 13 protofilaments in most cell types, including neurons (Kapitein and Hoogenraad, 2015).

Stathmin is highly expressed in the lateral nucleus (LA) of the amygdala (Shumyatsky *et al.*, 2005). Stathmin is also expressed in the brain areas that process the tone CS, which include the posterior intralaminar nucleus (PIN) and medial geniculate nucleus (MGm), which send afferent projections to the BLA. These findings show that the expression pattern of the stathmin gene in the mammal brain resembles the neural circuitry of innate and learned fear (LeDoux, 2000) and it can be a powerful genetic tool to study fear-related events in mammals. Indeed, stathmin KO mice have an increase in MT stability in the amygdala. The fact that MTs stay longer in the assembled form made them hyperstable, and even though there were no morphological changes in the

neurons of the stathmin KO mice, there was a deficiency in innate and learned fear (Shumyatsky *et al.*, 2005).

Neural Circuitry of Fear Extinction

i. Concept of fear extinction

The concept of extinction refers to the decrease in strength or frequency of a specific learned behavior that was associated with a specific context, commonly known as learned associative behavior. A particular characteristic of extinction is that the decrease of the learned associative behavior is only observed when the context is changed (Bouton and Bolles, 1979; Todd et al., 2014).

The conceptualization of extinction has evolved through years. Initially, in Pavlovian conditioning, there was the idea that the loss of the conditioned response (CR) in extinction is the result of forgetting or unlearning the CS-US association (reviewed by (Dunsmoor et al., 2015)). However, spontaneous recovery phenomenon dismisses the idea that extinction is the same as erasure, and implies that initial learning can remain even after extinction (Rescorla, 2004).

The context plays a critical role in extinction. Some experiments suggest that the loss of the CR during extinction is attributed to a change in the context of the CS-US, opposite to the idea that extinction is the result of losing the CS-US

association (Bouton and Bolles, 1979). For instance, behavioral responses can return or relapse when the CS is removed from the extinction context and tested in a novel one. It is even suggested that extinction can leave the strength of the original CS-US association more or less “fully preserved” (Rescorla, 2003; 2004).

The behavioral response is actively inhibited in the context of extinction. Such inhibition can be observed in different ways. For instance, in the associative learning model of Rescorla-Wagner, extinction with the CS in a neutral context could make the associative strength of the context become negative, which means that the context will no longer predict the US. A more supported idea of inhibition in Pavlovian extinction is the one called “negative occasion setting” where the context of extinction is used as hierarchical information cue that acts as the signal: CS will not be paired with the US (Bouton, 2004).

Thus, extinction is not the same as erasure, and this was demonstrated by the following phenomena: spontaneous recovery was shown in several experiments where if time is past after extinction, the response can return by itself; in renewal, the response that was extinguished can return if the CS is tested in a different context; in reinstatement, the behavior can reappear if the animal is presented with the US after extinction; and rapid reacquisition of the fear response can occur if the CS-US pairings are presented after extinction (reviewed in (Todd *et al.*, 2014).

The phenomena above confirm that extinction is a learning process and, as any memory related mechanism, it involves several brain circuits. The neurobiological underpinnings of extinction are discussed here, as well as future directions that can extend our knowledge of extinction as a learning process.

ii. Neural circuitry of extinction

Extinction memory is a complex process that requires more than one brain structure and its representation in the brain involves more than one neural circuit.

Major brain areas involved in fear extinction include the amygdala, the medial prefrontal cortex (mPFC) and the hippocampus (reviewed in (Todd *et al.*, 2014)). Studies focusing on the molecular mechanisms of the amygdala provide insights about the role of this brain area in fear extinction. Similar to fear conditioning, extinction depends on NMDA receptor activity in the lateral amygdala, local injection of antagonist NR2B before extinction training impairs the initial acquisition and following retrieval of fear extinction (Sotres-Bayon et al., 2007).

Different subgroups of neurons in the amygdala may be specialized in the mechanisms of extinction. For instance, by lesioning intercalated amygdala neurons using a peptide-toxin conjugate that targets cell expressing μ-opioid receptors, causes a strong inverse correlation between freezing levels during

extinction and the number of surviving ITC cells, producing a deficit in extinction (Likhtik et al., 2008).

The role of medial prefrontal cortex (mPFC) in acquisition and extinction of fear is critical. For instance, when mPFC is disrupted, the emotional associations that are triggered by the amygdala are not inhibited (Morgan et al., 1993). In addition, a decrease in freezing during extinction recall is highly correlated with an increase in infralimbic (IL) mPFC neuronal response, therefore the IL mPFC is essential for the suppression of fear during extinction (Milad and Quirk, 2002).

Cortical control of the basolateral nucleus of the amygdala (BLA) and interactions between the BLA and the IL mPFC are thought to be critical as an inhibitory mechanism that controls fear response after extinction (Milad and Quirk, 2002). One study examined the projections of prelimbic and infralimbic brain areas to the BLA during extinction of fear (Bloodgood et al., 2018). Using two different dye conjugates of the retrograde tracer CTB and physiological recordings, the authors found that IL-BLA projecting cells had higher activation than PL-BLA projecting cells, which confirmed the critical role of IL neuronal projections to BLA for fear extinction.

Prefrontal projections to brain areas, other than amygdala, are known to participate in the regulatory mechanisms of fear extinction (Giustino et al., 2019; Ramanathan et al., 2018). A study found that prefrontal projections to the

thalamic nucleus reuniens regulates fear extinction in the way that nucleus reuniens suppresses extinguished fear through the mPFC pathway (Ramanathan *et al.*, 2018).

There is also evidence of morphological changes in specific brain areas after fear extinction (Ago et al., 2017). Genetic deletion of *VIPR2* which encodes a receptor involved in the circuitry of fear: VPAC2, was shown to impair fear extinction. The PL and IL mPFC of the VIPAC2 deficient mice exhibit morphological abnormalities in neuronal cell bodies, total branch number, length of apical and basal dendrites, and abnormal increase of dendritic material proximal to the soma in apical dendrites even though the BLA dendritic morphology or cell body size were not altered in VIPAC2-KO mice.

In addition to mPFC and amygdala, other brain areas, are involved in extinction. Since context plays a critical role in the mechanism of extinction, studies where the hippocampus is inactivated provide a deeper understanding of the neurobiology of fear extinction (Corcoran et al., 2005; Hobin et al., 2006).
For instance, fornix lesions, which is part of the hippocampal formation, were tested in fear conditioning, extinction, renewal, reinstatement, and spontaneous recovery (Wilson et al., 1995). The results show that fornix lesions impair the reinstatement process (when animals are exposed to the US alone after extinction, which causes recovery of the CS response) suggesting an

involvement of the hippocampal formation in one of the mechanisms of extinction.

Synaptic properties of hippocampal neurons can provide insights of the mechanisms involved in fear learning and extinction. Rats that receive intraperitoneal injections of carbenoxolone (Cbx), a general gap junction blocker of connexin 36(Cx36), display deficits in context fear learning and an accelerated decrease of freezing on the first day of extinction (Bissiere et al., 2011). The study showed that blocking gap junctions of hippocampal neurons before contextual fear training affects c-fos expression within the amygdala-hippocampal network and facilitates fear extinction.

One of the suggested mechanisms of extinction is that a new memory is formed together with the original fear memory. To test this hypothesis, Lacagnina et al. studied neuronal representations of fear memory and fear extinction using activity-dependent neural tagging in the dentate gyrus of the hippocampus (Lacagnina et al., 2019). The ArcCreERT2 transgenic mouse line was used to tag and manipulate neurons during contextual fear acquisition or fear extinction, it was found that fear extinction prevents reactivation of a subgroup of neurons that were activated during fear memory acquisition. Next, Lacagnina et al. examined whether retrieval of fear memory and retrieval of extinction reactivate distinct ensembles by tagging neurons before the behavioral paradigms. The experiment showed that the extinction response is associated with reactivation of

neurons that were previously active during extinction training, while the spontaneous recovery response is associated with reactivation of the group of neurons that was active during fear learning.

Given the complex interaction between neural circuits in extinction learning, it is very likely that the specific involvement of a certain brain region or neuronal ensemble in extinction is closely related to the phase of memory extinction the organism is engaged in at the moment of study. Therefore, it is necessary that future research focuses on the neural mechanisms that participate on distinct phases of extinction learning.

iii. Extinction and the dopaminergic signaling

Under the framework of learning theory, extinction represents a way of building new memories on top of previous ones. The dopamine system has emerged as being critically involved in fear extinction. Dopamine neurotransmitters have a wide range of brain functions, and it regulates reward learning, motivation behavior, motor control, and overall cognitive functioning (Berke, 2018).

Specifically, in fear extinction, the dopaminergic system is thought to initiate the extinction of memories through a prediction error coding (Kalisch et al., 2019). The concept of prediction error is linked to the dopamine reward function. The reward system in the brain has three main specific functions: it acts as positive

reinforcer that helps the individual to learn, it provides economic utility which helps the individual to move towards the desired object, and it has an emotional component that provides pleasure every time the individual reaches the expected goal. The reward is a process that requires a series of sequential mechanisms. First, an object will trigger an attentional response from the individual through the sensory system. Second, the individual makes a comparison between the new and previous objects to determine its novelty. And third, the individual provides a value to the object that will be essential in the future to distinguish the chosen object among others (Schultz, 2016). Thus, reward is a complex cognitive process where the individual engages in an initial detection of an object, followed by the discrimination of the novel object vs. known objects, to finally assign value to the new object that will trigger motivation.

Dopamine prediction error can initiate fear extinction learning. According to the Rescorla-Wagner model, when successful extinction, the animal learns a new association that the presence of the CS no longer predicts the US, meaning that the new outcome does not match the prediction (Dunsmoor *et al.*, 2015; Rescorla, 2003). Increasing evidence suggests that fear extinction may be mediated by the reward learning system driven by dopaminergic activity, since not experiencing an aversive event can be considered rewarding (Abraham et al., 2016; Bouchet et al., 2018; Kalisch *et al.*, 2019; Luo et al., 2018). To support this, several studies showed that successful extinction of fear triggers DA neuronal firing when the expected aversive outcome does not happen (Badrinarayan et al.,

2012; Bouchet *et al.*, 2018; Holtzman-Assif et al., 2010; Salinas-Hernandez et al., 2018).

The VTA is one of the main brain areas with the largest population of dopaminergic neurons in reward behaviors (Morales and Margolis, 2017). During fear extinction, the VTA sends major dopaminergic projections to BLA, NAc, and ILmPFC. Evidence indicates that dopaminergic activity regulates acquisition of fear extinction in BLA, specifically dopamine receptors are important modulators of the initial phase of fear extinction learning (Hikind and Maroun, 2008; Shi et al., 2017) whereas the phase of fear extinction consolidation seems to be highly regulated by the dopaminergic signaling in the mPFC specifically the IL subregion of the mPFC, since this area receives an large amount of dopaminergic inputs (Luo *et al.*, 2018; Mueller et al., 2010; Pfeiffer and Fendt, 2006).

Important to note, the dopaminergic system includes several groups of neurons that can participate in specific brain functions. In addition, due to the multiple dopamine receptors and the large signaling cascades trigger by these receptors, it can be challenging to assign a specific function to each receptor type. However, findings on specific dopamine receptors provide promising information about their role in fear extinction. Agonists and antagonists of dopamine receptors are an excellent tool to assess the role of these receptors in different types of learning, including extinction.

Infusion of D1 (D1) receptor antagonist SCH23390 in the IL mPFC at two different time points, before acquision and after fear extinction, impaired acquision of extinction fear memory (Hikind and Maroun, 2008). Interestingly, the hippocampus is not known to be specialized in fear extinction, however, when novelty is applied to the extinction context, several molecular mechanisms are triggered in the hippocampus (de Carvalho Myskiw et al., 2014; Nachtigall et al., 2019). Evidence suggests that D1 receptor in the hippocampus regulates avoidance behavior in the fear extinction paradigm, furthermore, novelty increases hippocampal dopamine signaling and facilitates extinction (Menezes et al., 2015).

The IL mPFC is known to be heavily innervated by dopaminergic inputs coming from the midbrain (Starkweather et al., 2018). Blocking Dopamine 2 (D2) receptors in the IL mPFC with the antagonist raclopride, results in an impairment in the consolidation of extinction memory and in a reduction of extinction-related tone responses in IL area (Mueller *et al.*, 2010). D2 receptors also regulate fear extinction in BLA. Before extinction training, injections in BLA with quinpirole (a D2 receptor agonist) enhances extinction learning in rats (Shi *et al.*, 2017).

In humans, there is interesting evidence suggesting that the dopaminergic pathway is highly involved in the extinction of traumatic memories. Same as in rodents, reward behavioris are modulated by different brain areas in the human

brain, and several regions get heavily innervated by dopaminergic inputs. For instance, dopaminergic signaling is activated in the ventral medial prefrontal cortex (vmPFC), the NAc and the VTA in humans during fear extinction, providing a different input pattern according to the extinction phase: vmPFC (the human homologue of the IL in mice (Nieuwenhuis and Takashima, 2011)) participates on decreasing the expectancy to the US during extinction learning, while omission of an expected aversive memory is regulated by the NAc, and connections between NA and VTA happen when the US is omitted (Esser et al., 2021). Interestingly, dopaminergic activity in the vmPFC exherts an important role in the prediction of extinction memory at the retrieval phase providing evidence that dopamine-depent vmPFC activity pattern in humans is a key brain area for extinguishing long-term traumatic memories (Gerlicher et al., 2018).

A posible model of dopaminergic signaling in GRP KO mice is proposed (Figure 4). In WT condition, dopamine is released from the presynaptic neuron triggering dopamine signaling in the postsynaptic neuron. However, the Grp gene knockout may affect the dopamine signaling during SEFL. This might explain the susceptibility in SEFL in the GRP-/- mice.

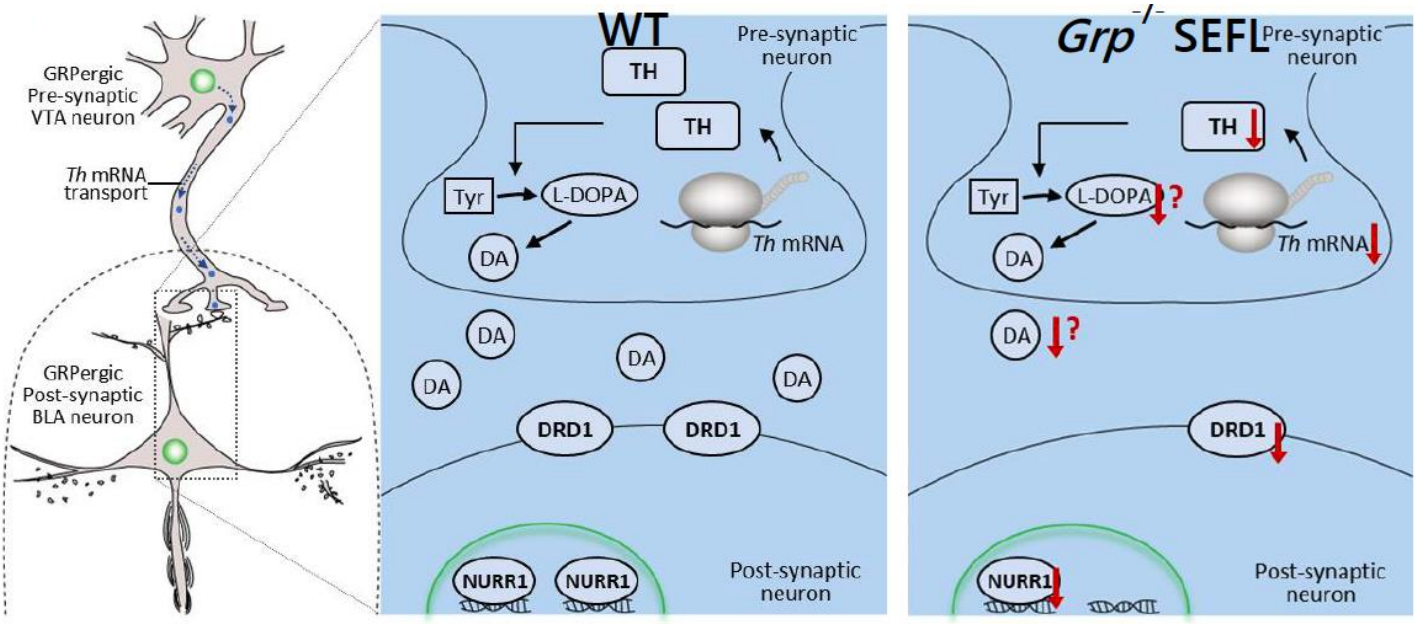

Figure 3. Proposed model for the dopamine signaling in the GRPergic circuit in extinction learning after SEFL.

Neural Circuitry of Fear in Postpartum Depressive-like Behavior

i. Maternal care

Maternal (or parental) care is critical for the survival of the progeny and includes behaviors by the mother (or mother and father) that keeps the offspring safe and healthy (Gammie, 2013). In mice and rats, which are often used to study maternal care in laboratory conditions, maternal care is measured by the ability to nest building, pup retrieving, pup licking, nursing and defending of the young (Francis *et al.*, 2002; Kuroda *et al.*, 2011). Virgin female rats eventually become maternal after several days of continuous exposure to foster newborn pups

(Cosnier, 1963; Rosenblatt, 1967). This process (called pup-induction or sensitization of maternal behavior) leads to a pattern of behavior very similar to that of the lactating mother (Numan et al., 2006). During that continuous exposure to foster newborn, the animal often must overcome an initial aversion to pups before they approach, contact and start displaying all components of maternal behavior (Fleming et al., 1989). Opposite to virgin rats, laboratory virgin female mice are “spontaneously parental”; even though they are not as efficient as mothers, they improve significantly within just a few minutes of pup exposure (Kuroda *et al.*, 2011; Lonstein and De Vries, 2000).

Importantly, behavioral differences between strains of mice may provide useful information about the mechanisms involved in maternal care. Two strains of mice are characterized as poor or good mothers: females of BALB/c strain have high anxiety (Francis et al., 2003) and spend little time in the nest, less time grooming pups and have longer latency to retrieve pups, in comparison to mothers of C57BL/6 strain (Priebe et al., 2005). It is important to note that both virgin females and mothers of laboratory mice are able provide maternal care (Kuroda *et al.*, 2011).

ii. Brain areas involved in maternal behavior

Some key brain areas important for maternal behavior include medial preoptic area of the hypothalamus (MPOA), nucleus accumbens (NAc), medial amygdala,

BLA, bed nucleus of the stria terminalis, olfactory bulb, periaqueductal gray, mPFC, paraventricular nucleus of hypothalamus, HPC, and ventral tegmental area (VTA) (Brunton and Russell, 2008; Francis *et al.*, 2002).

Studies in rodents provide knowledge about the brain areas involved in stimulation and inhibition of maternal care (Dulac *et al.*, 2014). The main olfactory epithelium (MOE) is activated by the olfactory infant stimuli, and MPOA direct projections to the nucleus accumbens (NAc), or indirect, via retrorubral field (RRF) and via ventral tegmental area (VTA) mediate parental responses, which is part of the motivational circuit regulated by projections from the paraventricular nucleus of the hypothalamus (PVN), lateral habenula (lHb) and the dorsal raphe nucleus (reviewed in (Kohl *et al.*, 2017)).

The MPOA is one of the most critical areas governing paternal care, however, the mechanism is still unclear by which this brain region responds to maternal responses. To understand better the role of the MPOA in females and males with different social experience, subpopulations of neurons were examined using the immediate-early gene c-fos in virgin females, virgin males and paternal males after prolonged exposure to pups (Wu *et al.*, 2014). The authors found that after parental care, fathers and virgin females showed a robust brain activation pattern in MPOA *c-fos*+ cells that was not shown in virgin males. Next, Wu et al. characterized active cells involved in parental behavior using double fluorescent in situ hybridization with *c-fos* and different molecular markers with distinct

MPOA expression, and they found that a candidate marker for MPOA *c-fos* cells in virgin females, mothers and fathers is the neuropeptide galanin (Gal). By silencing MPOA Gal+ neurons, the authors found that ablation impaired all major aspects of parental behavior including pup retrieval, nest building, retrieval to nest, and pup interaction in mothers, fathers and virgin females.

Even though maternal behavior can be induced in males, the question remains whether there is a neural circuitry underlying sex differences in parental care. To test this idea a recent study examined the role of tyrosine hydroxylase (TH)-expressing neurons in the anteroventral periventricular nucleus (Scott et al., 2015). The authors found that the number of TH+ AVPV cells was higher in virgin females compared to virgin males, and the number of TH+ AVPV cells of postpartum females compared to virgin females was also higher. When the authors ablated the TH-expressing neurons in the anteroventral periventricular nucleus, the virgin females and postpartum females showed an impairment in parental behavior, however, ablation of TH-expressing neurons in virgin males and fathers did not show any change in behavior.

Surprisingly, pup retrieval was found to require the left auditory cortex while testing the role of oxytocin in mothers responding to infant distress calls (Marlin et al., 2015). This study showed that oxytocin receptor expression is lateralized to the left auditory cortex of specifically mothers and virgin females. Marlin et al. showed that functional lateralization in the mammalian brain is important for

responding to pups' distress calls, which in turn would allow mothers to attend their progeny faster and more efficiently. There is evidence that deficiency in the mPFC can impair maternal behavior, which was shown by lesioning (Afonso et al., 2007) and silencing neurons (Febo et al., 2010) in this brain area.

Mother-pup interactions are highly regulated by the levels of neuropeptides and neurotransmitters in specific brain circuits (Dulac *et al.*, 2014; Pawluski et al., 2017). For example, oxytocin (see more about oxytocin below) in the VTA regulates dopamine levels in the NAc of mothers during pup care, once again confirming that these brain areas are important for mother-to-pup communications (Shahrokh et al., 2010).

During postpartum depression, the ability to take care of the progeny decreases, and anxiety, agitation and suicidal thoughts increase, followed by cognitive impairment (Abramowitz et al., 2010). Studies in women have shown that there is a decrease in connectivity between the amygdala, anterior cingulate cortex, dorsal lateral PFC and HPC (Pawluski *et al.*, 2017).

There are also brain changes during postpartum such as plasticity (Fleming and Korsmit, 1996; Pawluski *et al.*, 2016). Some of these areas are also involved in memory, cognition, anxiety and depression, for instance, some abnormalities were found in HPC during pregnancy and postpartum (Afonso *et al.*, 2007).

Importantly for my work, the neural circuitry of fear is also important for maternal behavior. Earlier research from our lab showed that female mice carrying a deletion for the amygdala-enriched stathmin display deficiency in pup retrieval and enhanced anxiety (Martel et al., 2008). The maternal deficit is due to the lack in threat assessment, motivation and anxiety, and this work for the first time showed BLA involvement in parental care.

iii. Neurotransmitters in maternal behavior

Biochemical changes at the level of neurotransmitters happen at multiple levels during pregnancy and postpartum period. There are several neurotransmitters critical for maternal care: dopamine, norepinephrine, serotonin, GABA.

In rats, the extracellular dopamine (DA) signal in the NAc is increased with licking and grooming behavior, and the magnitude and duration of the increase in the DA signal is highly correlated with the time of licking and grooming of rats towards their pups. A deficiency in low licking and grooming rats can be rescued using injections of selective DA uptake inhibitor GBR 12909 (Champagne *et al.*, 2004). Another study investigated the effect of using an antagonist for D1 and D2 receptors in the NAc and showed that disruption of D1 receptor in postpartum rats impaired their ability to retrieve pups (Numan et al., 2005). This effect was not observed when the receptor was blocked in the MPOA or ventral pallidum, showing that D1 receptors in NAc only are required for maternal retrieval.

Norepinephrine (NE) is another neurotransmitter that was shown to be involved in maternal behavior. A genetic study showed that disruption of the dopamine beta-hydroxylase (Dbh) gene that does not allow synthesis of the NE, which is a ligand of the adrenergic receptor, leads to lower pup survival rate and poor ability of females *Dbh-/-* and males *Dbh-/-* to retrieve pups to the nest (Thomas and Palmiter, 1997). Another study looked at the downregulation of noradrenergic activity in the vBNST in maternal responses and found that infusion of yohimbine (an α2 autoreceptor antagonist that increases NE release) in the vBNST of postpartum mothers impaired almost completely retrieval of pups but no other maternal behaviors such as initial contact, licking, or nursing, suggesting an important role of downregulation of NE activity in vBNST in maternal care (Smith et al., 2012).

Mice deficient in the tryptophan hydroxylase, an enzyme that synthesizes serotonin, have abnormalities in maternal responses. The removal of the olfactory bulb in males or females leads to a significantly decrease in the tryptophan hydroxylase, resulting in an increase in infanticide (Neckers et al., 1975). Also, a mouse lacking the tryptophan hydroxylase 2 exhibits impaired maternal care, which leads to poor survival of progeny (Alenina et al., 2009).

GABA receptors are also involved in postpartum behavior. For example, mothers with a knockout of the δ subunit of the GABA receptor, which leads to a

deficiency in synaptic function in the dentate gyrus of the HPC, neglected pups and had a decrease in pup survival (Maguire and Mody, 2008). Also, work from our laboratory showed that mothers of the stathmin transgenic mice (Stat4A) also have deficiency in pup retrieval, anxiety- and depressive-like behaviors (Uchida, unpublished). Intriguingly, these Stat4A transgenic mice have a deficiency in synapse transport of AMPA and NMDA receptors (Martel et al., 2016; Uchida et al., 2014). Further demonstrating the importance of stathmin MT dynamics and synaptic trafficking in maternal care, our earlier research showed that stathmin knockout mice display deficiency in pup retrieval and enhanced anxiety (Martel *et al.*, 2008).

The studies described above provide strong evidence that there are neurobiological and genetic factors contributing to optimal species-specific maternal care. Further studies need to focus on the specific neuroanatomic and molecular mechanisms defining states of good or poor motherhood.

iv. The role of stathmin in maternal behavior

MTs are one of the major cytoskeletal structures in every type of cells including neurons. MT dynamics are a constant shift between MT stability and instability, the critical importance of this process is well-established in cell division, neurodevelopment, cell injury, axonal pathfinding, and synaptic trafficking (Di Paolo et al., 1997).

In mature neurons, however, MTs have been considered to be stable and non-dynamic structures that are present in dendritic shafts but not in dendritic spines. Interestingly, work in hippocampal primary neuronal cultures showed recently that MTs can be dynamic during neuronal activity and can move from the dendritic shaft to dendritic spines (Gu et al., 2008; Hu et al., 2008; Jaworski et al., 2009; Kapitein et al., 2011; Merriam et al., 2011). Despite this new information, the role of MT dynamics in memory formation remains unclear. Recently however our lab has shown that learning processes, such as fear conditioning, induce biphasic shifts in MT stability/instability (dynamics) in the dentate gyrus of the HPC (Martel *et al.*, 2016; Uchida *et al.*, 2014). These shifts in MT dynamics are controlled by stathmin protein, an inhibitor of MT formation, which is involved in innate and learned fear responses in rodents and humans.

Because stathmin was identified as an amygdala-enriched gene (Shumyatsky *et al.*, 2005; Shumyatsky *et al.*, 2002), our lab has asked whether stathmin and the amygdala can control maternal care. Stathmin knockout females are deficient in their ability to take care of their progeny due to their inability to properly assess danger (Martel *et al.*, 2008). This is consistent with stathmin being expressed in the BLA and having a role in innate and learned fear (Shumyatsky *et al.*, 2005). This was the first genetic demonstration of the role of the BLA role in maternal care and it was an important demonstration of how neural circuits of innate and learned fear that are enriched in stathmin are involved in maternal care.

Stathmin participates in the intracellular transport since it controls MTs. There is genetic demonstration that stathmin has a role in adult hippocampal neurogenesis, spinogenesis, and NMDA receptor dependent memory, and Stat4A mice overexpressing the mutant form of stathmin that cannot be phosphorylated, exhibit impaired hippocampal neurogenesis and reduced spine density in the dentate gyrus (Martel *et al.*, 2016).

Stathmin is involved in regulation of memory and shows a decrease in its expression in aging. In the HPC of the mouse brain, stathmin regulates MT stability by changing its affinity to α-tubulin in two distinct phases in contextual fear conditioning: during the first phase (0.5-1 h following learning) MTs become unstable and during the second phase (8 h following learning) MTs become hyperstable. Furthermore, aged mice show decrease stathmin levels and lack of learning-induced increase of GluA2 levels in synaptosomal and MT fractions. This suggests that learning-dependent regulation of GluA2 dendritic transport by stathmin-mediated changes can be one of the signaling pathways of age-related processes (Uchida *et al.*, 2014).

In mature neurons, MTs have an important role in synaptic structure, synaptic plasticity, and memory. Stathmin phosphorylation plays a critical role in learning-induced changes in MTs. Fear learning can induce biphasic shifts in MT stability in the dentate gyrus of the HPC: MTs become unstable 0.5-1 h and hyperstable 8

h following learning. Changes in MT stability after fear conditioning were observed in synaptosomal fractions of the dentate gyrus of the HPC. α-amino-3-ydroxy-5methyl-4isoxazolepropionic acid (AMPA) receptors are glutamate receptors that mediate fast synaptic transmission (Malinow and Malenka, 2002). AMPA have four receptor subunits, GluA2 subunit is one of them (Matsubara et al., 1996). Our lab showed that stathmin via the regulation of MTs dynamics controls GluA2 subunit trafficking to the synapse during fear conditioning. More specifically, learning increases GluA2 in the synaptosomal and MT fractions during the late phase of learning (8 h following learning acquisition), and during this late phase, phosphorylation of stathmin is increased, thus the amount of tubulin dimers increases as well. A greater amount of tubulin triggers the release of GluA2 subunit from the soma to synaptic sites, promoting binding of the KIF5-GluA2 subunit complex to MTs (Uchida *et al.*, 2014). The evidence showing that stathmin can regulate the molecular events that happen at a later phase of learning is critical for our understanding of long-term memory since both synaptic function and memory require the coordinated transport of molecules between the cell body and synapses.

Also, N-methyl-D-aspartate receptors (NMDARs) is critical for learning and memory (Bauer et al., 2002; Ben Mamou et al., 2006; Zhao et al., 2005). NMDARs must be transported by MTs into dendrites and synaptic sites, for instance, MT-depolymerizing molecules can inhibit NMDA receptor-mediated ionic and synaptic currents in cortical pyramidal neurons (Yuen et al., 2005).

Stathmin has an important role in adult hippocampal neurogenesis, spinogenesis, and NMDA receptor-dependent and neurogenesis-associated memory. Stathmin controls its downstream target, NMDARs as measured by levels of phosphorylated CREB 30 min after fear conditioning. Stathmin also regulates NMDAR-dependent-memory, but not NMDAR-independent memory (Martel *et al.*, 2016).

Stathmin can undergo phosphorylation at its four serine (Ser) amino acid sites: Ser16, Ser25, Ser38 and Ser63 (Larsson et al., 1997). To investigate the role of stathmin phospohorylation in memory, we employed the unphosphorylatable constitutively active stathmin4A (Stat4A) mutant, generously provided by Dr. Andre Sobel. The four amino acid sites of phosphorylation are changed from Ser to alanine(Ala), and thus, stathmin4A mutant protein cannot be phosphorylated and thus it constitutively binds tubulin dimers: which means stathmin4A mutant protein binds but does not release tubulin (Kuntziger et al., 2001). We generated tTA/tetO-regulated bi-transgenic Stat4A mice expressing the Stathmin4A mutant fused to the enhanced green fluorescent protein (Stat4A:EGFP); both proteins are translated in one open reading frame (ORF). In the Stat4A mutant mice, the constitutively active Stahmin4A mutation permanently binds to α-tubulin, which causes depletion of cellular levels of free tubulin and destabilizes MTs. Also, the mutation can be regulated by the doxycycline system (Figure 5), in the absence of doxycycline the tTA transcriptional activator binds tetO promoter and Stat4A:EGFP is expressed (Uchida *et al.*, 2014). Thus, a mutation where

stathmin cannot be phosphorylated is an excellent genetic tool to test MT role in learning, memory, and social behaviors.

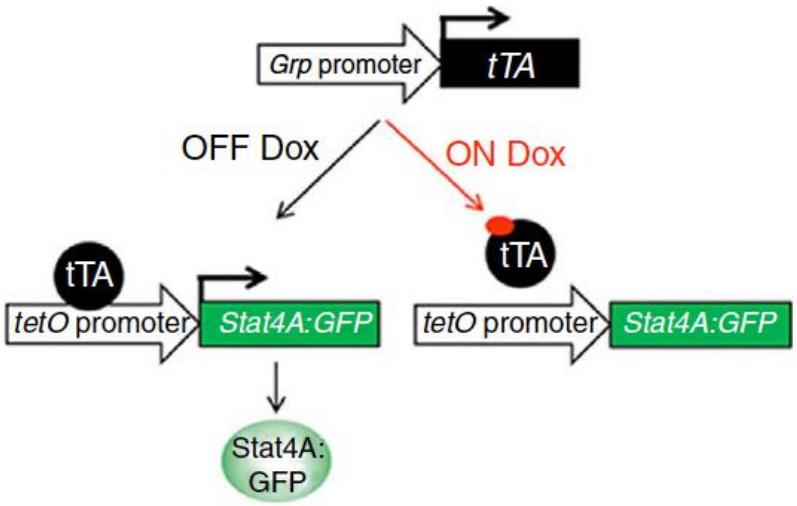

Figure 5. Diagram illustrating transgenic design and control of transgene expression by doxycycline (dox) in Stat4A transgenic mice (Uchida et al, 2014).

v. Microtubule and neuropsychiatric diseases

There is a growing evidence demonstrating the role of microtubule-associated proteins involved in cognitive impairments (Martel et al., 2012; Shumyatsky *et al.*, 2005). Because of the role of MTs in neuronal plasticity, MTs can be directly linked to neuropsychiatric disorders. Evidence from clinical studies and animal models support the concept that deficiencies in MT cytoskeleton underlie behavioral abnormalities and cognitive dysfunction observed in neuropsychiatric diseases (Reviewed in (Marchisella et al., 2016).

MT stabilizing factors, also known as microtubule-associated proteins (MAPs), play a critical role in MT assembly and dynamics. For instance, in neurons the

protein MAP2 helps to differentiate and maintain dendrites, and other MAPs are known to participate in MT loop formation during synaptogenesis (Reviewed in (Conde and Caceres, 2009)). MAPs have been associated in a wide range of developmental, neurodegenerative, and psychiatric disorders. MAPs can be responsible for decreased dendritic spine density and abnormalities in dendritic arborization, leading to synapse loss and disturbed feedback loops that are seen in intellectual disability, depression, and schizophrenia (Reviewed in ((Marchisella *et al.*, 2016)). Also, MAP2 immunoreactivity has been proposed as a hallmark of schizophrenia (DeGiosio et al., 2019) and MAP6 mRNA, which is highly involved synaptic connectivity, was found to be enriched in the prefrontal cortex of patients with schizophrenia (Shimizu et al., 2006).

Abnormalities in MTs can increase the risk of psychiatric disorders. In Schizophrenia, findings show disorganized MTs in human olfactory neuroepithelial cells, while in cells of bipolar disorder patients MTs are shortened, suggesting that abnormalities in MTs are potential markers that can differentiate schizophrenia from bipolar disorder (Solis-Chagoyan et al., 2013).

Increased amount of literature show that reduced MT stability is observed in several neurodegenerative diseases, since neurodegeneration happens in great part due to the progressive loss of structure and/or function of neurons (Reviewed in (Dubey et al., 2015)). Tau is a MT-related protein that contributes to neuronal morphology and axon outgrowth. The primary function of tau is to

stabilize MTs, however, under pathological conditions there is abnormal disengagement of tau that causes axonal transport defects. High concentrations of unbound tau cause that tau undergoes misfolding and aggregation which leads to formation of neurofibrillary tangles (Ballatore et al., 2007). Rodent models of Alzheimer's disease show age-related memory loss due to the presence of neurofibrillary tangles that are made of phosphorylated or modified tau (Lu et al., 1999). Tau is also involved in other neurodegenerative diseases. In Frontotemporal dementia with parkinsonism-17, over 50 mutations are found in exonic and intronic domains that affect the sequence of tau and tau isoforms, weakening the ability of tau to interact with MTs and increasing tau aggregation (Hong et al., 1998). Also, the 3-repeat (3R tau) isoform is linked to the progression of pick disease (Rockenstein et al., 2015). The progressive supranuclear palsy and the corticobasal degeneration are less common neurodegenerative diseases, however, they are considered to be in the spectrum of the 4-repeat taupathy disease where there is accumulation of tau-positive inclusions in neuronal and glial cells (Yamada et al., 1992). Therefore, tau is considered a biomarker for several neurodegenerative diseases.

Also, stathmin, a focus of my thesis work, is involved in anxiety, PTSD symptoms, and in panic disorders and phobias in humans (Brauer et al., 2013; Brocke et al., 2010; Cao et al., 2013). To understand the association between stathmin and PTSD symptoms in humans, a study assessed a sample of Chinese victims of a recent earthquake using correlation of the C-allele with

cognitive-affective symptoms of PTSD. Those subjects who were rs182445 C-allele carriers had a higher level of recalling past traumatic experience (Cao *et al.*, 2013). Stathmin is also involved in fear and anxiety responses in humans. Intriguingly, the STMN1 variation of stathmin impacts fear and anxiety response in a sex dependent manner in laboratory conditions using the psychosocial stress protocol. Female T- and G- allele carriers had a tendency to have stronger startle response than males. In contrast, male carriers of the T+ allele of the STMN1 showed significantly larger cortisol increase in response to stress that female T+ carriers (Brocke *et al.*, 2010).

Molecular Mechanisms Underlying Maternal Behavior

i. Cell-type specificity of activity-regulated events

Studies using immediate early genes (IEG) activated following mother's interaction with pups have helped mapping brain regions involved in maternal care (Lonstein et al., 2000; Numan, 2007; Tsuneoka *et al.*, 2013), but the identity of the cell types has only recently started to be explored.

In the mPOA, more than 75% of the cells that become active during maternal care are GABAergic (Tsuneoka *et al.*, 2013). These GABAergic cells express estrogen receptor alpha, galanin, neurotensin, and tachykinin2 (Lonstein *et al.*, 2000; Tsuneoka *et al.*, 2013). Studies using chemo- and optogenetic approaches

recently showed that the activation of mPOA cells expressing the estrogen receptor alpha induces pup approach and retrieval (Fang et al., 2018; Wei et al., 2018). The activation of galanin-expressing cells that project from the mPOA to the periaqueductal grey matter or VTA promotes grooming and motivation to seek pups (Kohl et al., 2018). GABAergic mPOA cells, different from those expressing the estrogen receptor alpha, might be involved in nest building behavior but their identity is unclear (Li et al., 2019). On the other hand, inhibitory neurons in the arcuate nucleus of the hypothalamus that express the agouti-related neuropeptide (AGRP) form synapses with mPOA cells and their activation decreases nest building (Li *et al.*, 2019). In the medial amygdala, the activation of GABAergic cells, but not of glutamatergic ones, promotes pup grooming and to a lesser extent pup retrieval (Chen et al., 2019).

Fiber photometry studies show that pup sniffing or grooming increase intracellular calcium in the galanin-expressing mPOA cells while approach and pup retrieval increases calcium signal in the mPOA cells expressing the estrogen alpha receptor (Fang *et al.*, 2018; Kohl *et al.*, 2018; Wei *et al.*, 2018). Intracellular calcium in the GABAergic cells from the medial amygdala is also increased during pup grooming (Chen *et al.*, 2019). Together with work showing that ERK phosphorylation is increased following the interaction with pups (Kuroda et al., 2007), these studies suggest that maternal care initiates calcium-dependent signaling cascades, which often lead to activity-dependent intracellular changes as is well described in memory processes (Cohen et al., 2018).

ii. Activity regulated genes in maternal behavior

Transcriptomic profiling has demonstrated that similar to the learning processes, hundreds of genes are activated during the maternal states or behaviors, such as pregnancy, giving birth or the sensory stimuli of nest building, pup retrieval and pup care (Gammie *et al.*, 2016).

More than 700 core genes were found to be changed between naïve and postpartum states across four brain regions, mPOA, mPFC, lateral septum and nucleus accumbens (Gammie *et al.*, 2016). We will focus on those that are known to be regulated by neuronal activity during maternal care. We will also consider some other activity-regulated genes that might be of interest to maternal behavior.

Activity-dependent synaptic and nuclear events have been described quite extensively for learning and memory and several excellent reviews exist (Alberini, 2009; Klann and Dever, 2004; Mayford *et al.*, 2012; Nonaka et al., 2014; Yap and Greenberg, 2018). It is important to note that it is challenging to completely separate the basal processes at synapses and in the nucleus from activity-dependent events, as most of the genes and proteins involved are active to a certain degree not only as a result of activity but also in the basal state.

iii. Synaptic molecules involved in maternal care

Complex behaviors, such as maternal care, are guided by the interaction of molecules in the pre- and post-synaptic sites of the neuron in several brain areas. Biochemical changes at the level of neurotransmitters happen at multiple levels during pregnancy and postpartum period.

Neurotrophins are well established stimulators of neuronal IEG response and they play a major role in memory and depression (Yang et al., 2020). Synthesis and release of the brain derived neurotrophic factor (BDNF) is regulated by neuronal activity (Cunha et al., 2010; Lu, 2003), therefore it is likely that activity-dependent mechanisms elicited during mother-progeny interactions involve this neurotrophin.

Studies focused on BDNF role in the mother's brain are limited but they point to a prominent role of this neurotrophin during maternal care as well. Maynard et al., 2018, showed that *Bdnf* deletion decreases maternal behaviors in virgin females and mothers; knock out females even showed more harmful behaviors towards the pups such as biting them (Maynard et al., 2018). Chronic unpredictable stress (CUS) applied after giving birth produced depression-associated behaviors, which was accompanied by a decrease in the *Bdnf* mRNA and protein levels in the mPFC of the mothers (Liu et al., 2020). The *Bdnf* gene knockout in the mPFC also led to changes in FoxO1 expression, which was previously

involved in major depressive disorders (Liu *et al.*, 2020). As CUS and other stress procedures are known to alter maternal behavior (Leuner et al., 2014; Maguire and Mody, 2016), it is probable that the reduction in *Bdnf* and FoxO1 might also be involved in these behavioral disturbances. Moreover, rat females exposed to opium during pregnancy showed reduced maternal behaviors in the postpartum period that were accompanied by decreased BDNF expression in the hippocampus (Rezaei et al., 2021). Supporting the hypothesis that activity-dependent events produced by bouts of maternal care involve BDNF, Zhang et al., 2020 recently showed that mothers that spent more time grooming and licking pups had higher levels of BDNF in the hippocampus and the nucleus accumbens (Zhang et al., 2020). This work also showed that oxytocin-induced regulation of B might be mediating the observed behavioral differences.

Neurotrophic factors can bind to specific receptors and influence plastic events in the brain. Maynard et al. has recently shown that the disruption of the BDNF-TrKB signaling in oxytocin neurons leads to reduced maternal care in female mice (Maynard *et al.*, 2018). The authors suggest that BDNF could be a modulator of sex-specific social behaviors and be a new activity-dependent molecule critical for oxytocin neuron function (Maynard *et al.*, 2018).

Materials and Methods

Animals. Female C57BL/6J mice are purchased from Jackson Laboratory. Generation of Stat4A mice is done by crossing the GRP-tTA knock-in mouse line and the tetO-Stat4A:GFP transgenic mouse line as previously described (Martel *et al.*, 2016; Uchida *et al.*, 2014). Experimental mice (wild-type [WT], Stat4A double mutant mice, GRP-tTA single mutant mice, and tetO-Stat4A:GFP single mutant mice) are generated by crossing the GRP-tTA with tetO-Stat4A:GFP mice.

Intracranial surgery is performed as previously reported (Martel *et al.*, 2008). Mice are anaesthetized with avertin (250 mg/kg) and Hamilton syringe is implanted into the dorsal subiculum, retrosplenial cortex and pre limbic cortex. Injections are bilateral. Coordinates: dorsal subiculum: AP -3.3, ML ±0.65, DV −0.8; retrosplenial cortex: AP +3.3, ML ±0.65, DV −0.7; pre limbic cortex: AP +1.7, ML ±0.3, DV −2.2. Mice are injected with AAV-DJ-CaMKII-HA-Stat4A-WPRE or AAV-DJ-CaMKII-GFP, titer 1.0 x 10^{13} GC/ml. Three weeks after surgery, females are mated with C57 males and following pup delivery are then subjected to behavioral experiments.

Adeno-Associated Virus (AAV) contains two inverted terminal repeats (ITRs), CaMKIIalpha promoter (it is used to drive expression of the transgene in the principal neurons), HA-Stat4A cDNA (HA is a tag for anti-HA antibody to

recognize the transgenic protein), woodchuck hepatitis virus (WHP) posttranscriptional regulatory element (WPRE; increases transgene expression in viral vectors), human growth hormone polyA signal (improves the stability of mRNA transcripts); 5.9 Kb (gift from Dr. Shusaku Uchida).

Retrograde tracing was performed using rAAV2-retro-CaMK2-tdTomato (rAAV2), a mutant form of AAV2 that shows highly efficient retrograde transport in neurons (Tervo et al., 2016).

Auditory fear conditioning and extinction: 7 days after restraint stress, mice were exposed to training context A three times in one day for a total of 12 minutes to habituate them to the context. 24 hours later, mice underwent the following FC protocol: two minutes of exploration followed by two, 30 second CS-US pairings that coterminated with a 0.5mA footshock (US) separated by a 60 or 120 second inter-tone interval (ITI; both produce the same results). The CS was an 85dB, 10khz tone. Mice were removed from the training context 1 minute after the second shock and immediately returned to their home cages. Context A consisted of grid floors, a dim corner light in the room, no overhead lights, and 70% ethanol used for cleaning. Extinction training (4 days post shock) and remote memory retrieval tests (30 days post shock) were performed in novel context B, consisting of smooth plastic flooring, a plastic insert on the walls of the chamber, bright overhead lights, chamber lights on, orange scent, a 65dB white noise and isopropanol for cleaning. Following a 2-minute exploration in Context

B, animals were given 5 (recall) or 30 (extinction) CS presentations in the absence of the US (tone only), each separated by a 60 second ITI.

Stressed enhanced fear learning (SEFL): Restraint stress, fear conditioning, extinction and memory recall were performed as described (Sillivan et al. 2017). Stress exposure consisted of two hours of immobilization/restraint, followed by auditory fear conditioning 7 days later and two days of fear extinction four days later. Fear extinction recall is done 30 days after auditory fear conditioning. Fear conditioning included two CS-US pairings using a 0.5 mA footshock. This moderate protocol was used to avoid a ceiling effect in controls and the potential for induction of a depressive-like phenotype in stressed animals.

Elevated plus maze. This test is performed as previously reported (Uchida *et al.*, 2014). The elevated plus maze (1 m above the floor) consists of a center platform (10 cm X 10 cm), two open arms (40 cm X 10 cm), and two closed arms (40 cm X 10 cm) within walls (height 30 cm). Mice are individually placed in the center of the maze, and then time spent on each arm was measured using track vision system (Limelight software, Coulbourn Instruments). Results are expressed as percentage of time spent in closed arms over the total time spent in the maze.

Sucrose preference test. This test is performed as previously reported (Tervo *et al.*, 2016). A mouse is habituated to drink water from two bottles for 3 days. The

mice are subjected to sucrose preference test 24 hours after habituation. Two pre-weighed bottlers, one containing tap water and the other containing 1% sucrose solution, are presented to each animal for 4 hours. The bottles are then weighed, and the weight difference represents the amount consumed by the animal.

Pup retrieval. This test is performed as previously reported (Martel *et al.*, 2008). Females are housed individually 1 week before the experiment with the nest material placed in the cage. Each behavior is videotaped and then analyzed by an observer blind to the genotype. Three pups (foster pups for virgin females and the female's own pups for postpartum females) are placed in three corners away from the nest in the home cage. Sniffing and retrieval of each pup by the female is recorded for 20 minutes. Retrieval is defined as a female picking up a pup in her mouth and transporting it to the nest.

Immunohistochemistry. This test is performed as previously reported (Uchida *et al.*, 2014). Mice are anesthetized with Avertin and transcardially perfused with 4% paraformaldehyde (PFA). The brains are kept in PFA for 4 hours at 4ºC and transferred to 30% sucrose overnight. They are frozen in OCT compound at -80ºC. The brains are sectioned (40 μm) using a cryostat, and double immunofluorescence was performed on free-floating sections. Primary antibody in use is Anti-HA tag antibody (Rabbit) (1:1000; Abcam). Secondary antibody in use is goat anti-rabbit Alexa Fluor 594 (1:500, ThermoFisher). Images are

acquired with a Zeiss Observer Z1 microscope with multichannel excitation and detection options.

Hormonal assessment. The level of corticosterone, estradiol and progesterone are tested using urine samples during estrous cycle, pregnancy and postpartum periods using Enzyme-Linked ImmunoSorbent Assay (ELISA). The oxytocin-positive cells in hypothalamic PVN are stained using fluorescent IHC and analyzed using ImageJ and Neurolucida. Alternatively, we examine receptors for estrogen and oxytocin using immunohistochemistry.

Western Blotting. The DG of the HPC is isolated from Stat4A mothers to test NMDARs, AMPARs, and GABARs, which are receptors critical for synaptic function and which synaptic localization is regulated by MTs (Martel *et al.*, 2016; Uchida *et al.*, 2014). Isolation of synaptosomal fractions and Western Blot is performed as previously reported (Uchida *et al.*, 2014). In most cases the brain tissue from one animal is treated as one sample (if there is enough tissue). Equal amount of protein (10-20 μg) is separated on 7% or 12% Bis-Tris gels (Invitrogen) and transblotted onto prolyvinylidene difluoride membranes according to the manufacture's instructions (GE Healthcare Bio-Sciences). After blocking the membrane with 5% skim milk or 5% BSA, the membranes were incubated with antibodies directed against β-actin, pS16-stathmin, pS25-stathmin, pS38-stathmin, total stathmin, GluN1, GluN2A, GluA1, GluA2, GABAR δ and γ subunits. After incubation with HRP-conjugated secondary antibodies

(HRP-linked anti-mouse IgG or HRP-linked anti-rabbit IgG antibody, Cell Signaling). We will develop the blots using the ECL-Plus Detection Kit (GE Healthcare Bio-Sciences or Thermo Scientific). All buffers will include sodium fluoride (50mM) for the phosphor-specific antibodies. Western blots are visualized using KwikQuant Imager.

Statistical analysis: Analyses of the data were performed using two-way ANOVA. Significant effects were determined using Fisher's post hoc test or Bonferroni's correction. Unpaired Student's t tests were used for two-group comparisons. In all cases, p values were two-tailed, and the comparisons were considered to be statistically significant when $p < 0.05$. All data are presented as the mean ± SEM.

Results

1. The neural circuitry of PTSD-like behavior

Experiment 1.1: Do the *Grp*$^{-/-}$ mice exhibit increased susceptibility to Stress-Enhanced Fear Learning (SEFL)?

Rationale: Since the GRP gene is an amygdala-enriched gene and the GRPergic neural circuits are expressed in the conditioned stimulus (CS) pathways, we hypothesized that it plays a role in processing of sensory information related to PTSD-like behavior, which was assessed by SEFL, a behavioral model in rodents to assess some of the PTSD-like symptoms in humans.

To examine *Grp*$^{-/-}$ mice in conditions where stress is combined with fear learning, we turned to SEFL (Sillivan *et al.*, 2017). Stress exposure consisted of two hours of immobilization/restraint (Figure 5A), which is considered an acute stress (Yasmin et al., 2016). Following fear conditioning, the stressed group was separated into two subgroups, resilient and susceptible, based on their freezing performance during one minute of post-shock freezing during fear conditioning training. Post-shock freezing was used as an index of stress susceptibility (Figure 5B), as it was shown previously (Sillivan *et al.*, 2017). Animals that froze above the mean % freezing for the stressed group were classified as stress-susceptible (SS), while those that fell below the mean were classified as stress-resilient (SR) (Figure 5B). Interestingly, in *Grp*$^{-/-}$ mice, the ratio of the amount of animals in the

susceptible group to that in the resilient group was increased compared to WT mice. GRP KO mice showed increased susceptibility in SEFL. The course of extinction and recall test in SEFL shown in Figure 5C. Shown are five bins (6 tones each) of the conditioned stimulus (CS) presentations during extinction. These results suggest that the enhancement of freezing level in KO mice resulted from the difference in stress susceptibility.

Grp$^{-/-}$ mice exhibit increased susceptibility to PTSD-like behaviors: they had enhanced fear memory and slower extinction of fear memory in SEFL compared to unstressed-fear conditioned *Grp*$^{-/-}$ mice and their both stressed- and unstressed-fear conditioned wildtype counterparts.

Experiment 1.2: Do GRP-positive neurons send their projections to the BLA by direct (auditory thalamus) or indirect auditory pathway (auditory cortex)?
Rationale: The distribution of the Grp-GFP signal in the mouse brain in the *Grp*$^{-/-}$ mice. It is not clear whether GRP-positive neurons follow an indirect auditory pathway to the BLA. We used AAV2-retro (Tervo *et al.*, 2016) to perform retrograde tracing in order to determine if GRP-positive neurons have projections to the BLA via direct pathway (auditory thalamus) or indirect pathway (auditory cortex).

To map the GRPergic positive neurons onto the amygdala-associated neural circuitry, we injected retrograde neuronal tracer rAAV2-retro-CaMK2-tdTomato

(rAAV2) (Tervo *et al.*, 2016) in several brain regions in the *Grp*$^{-/-}$ mice. When rAAV2 was injected in the lateral nucleus of the amygdala (LA), the areas labeled with tdTomato were the TE3 area of the auditory cortex and the MGm/PIN area of the auditory thalamus, two major regions sending projections to the LA (LeDoux, 2000). Only the TE3 area showed co-localization of the tdTomato and GFP (Figure 6B); the MGm/PIN area had no GRP-positive cells co-labeled with the tdTomato (Figure 6A). When rAAV2 was injected in the basal nucleus of the amygdala (BA), the co-localization of tdTomato and GFP was observed in the vHPC. Finally, we injected rAAV2 in the mPFC to look at the BLA projections to mPFC (Figure 6C). There was no co-localization between GRP-positive cells and those labeled with tdTomato in the BA area (Figure 6D) or any other areas of the amygdala projecting to the mPFC.

These results confirm our previous work suggesting that the GRPergic neural circuits are expressed in the conditioned stimulus (CS) pathways and may play a role in processing of sensory information related to fear memory. However, only one pathway of the two is utilized by the GRPergic cells, the indirect pathway, going from the MGm/PIN in the auditory thalamus to the auditory cortex area TE3 and then to the LA.

2. The neural circuitry of fear in postpartum depressive-like behavior

Rationale: Previous unpublished results in our lab showed that female mice that have an unphosphorylatable form of the protein stathmin (Stat4A mutation), an amygdala enriched gene (Shumyatsky *et al.*, 2005; Shumyatsky *et al.*, 2002), have depressive- and anxiety-like behaviors the first week after delivery. More specifically, postpartum but not virgin Stat4A mice showed deficiency in sucrose preference test, forced swim test, elevated plus maze, pup retrieval and pup survival (Shusaku Uchida, unpublished). Because dendritic spines are implicated in depression, and spine formation is dependent on MTs, we tested spine density in postpartum and virgin *Stat4A* female mice. We found that spine density was significantly reduced in the HPC of virgin (naïve) Stat4A female mice as compared to their wildtype (WT) littermates (Itzamarie Chevere-Torres, unpublished). In postpartum, spine density has not changed in Stat4A females, while it went down in WT postpartum females. Additionally, postpartum WT females have an increase in stathmin phosphorylation as compared to naïve virgin WT females (Shusaku Uchida, unpublished). These observations suggest that disruption of stathmin and MT function may lead to depression-like symptoms postpartum. The results show a link between stathmin-dependent MT dynamics, learning, and depression-like behaviors. Importantly, mice with the deletion of the δ subunit of the GABA receptor (GABAR) behave very similar to the Stat4A mice: virgin females are normal in maternal care, anxiety and depression, but mothers are deficient (Maguire and Mody, 2008). To our knowledge, this is the only genetically modified mouse line similar to the Stat4A mice postpartum.

Experiment 2.1: Do Stat4A mothers have postpartum depressive-like symptoms long term?

We assessed whether the depressive- and anxiety-like behaviors are present long term. *Stat4A* females were tested in sucrose preference test, forced swim test, elevated plus maze, and open field. *Stat4A* females had increased depressive-like behavior in sucrose preference test and forced swim test 40 days after delivery (Figure 7) (WT vs Stat4A; sucrose preference test p=0.019, percentage of immobility in forced swim test p=0.016, latency to stop swimming p=0.0127). However, the *Stat4A* mice were normal in anxiety tests, the elevated plus maze and open field.

These observations suggest that disruption of stathmin and MT function in the GRPergic pathway may lead to depressive- but not anxiety-like symptoms in mothers in a long-term fashion. The results show a link between activity-dependent stathmin-mediated MT dynamics and postpartum depressive-like behaviors.

Experiment 2.2: What is the neural circuitry responsible for the behavioral deficits in Stat4A mice postpartum?

Rationale: Because the *stathmin4A* transgene is expressed in several brain areas, it is critical to understand which of these areas are responsible for the development of the postpartum depressive-like behaviors. Our main focus is on

the dentate gyrus, amygdala, cingulate cortex and prefrontal cortex, which are the main areas of Stathmin4A expression in *Stat4A* transgenic mice.

Method: Using local brain injections with the *Stat4A*-expressing virus, we are in the process of examining these brain areas. To this end, we are injecting adeno-associated virus (AAV; the DJ serotype, which infects effectively excitatory neurons) expressing the *Stat4A* (*AAVDJ-Stat4A*) in different brain areas of wild type mice and examine their behavior after the delivery.

Maternal care: Three weeks after the viral injections (this time is necessary to allow full expression of an AAV in the brain) the female mice are mated with wildtype males. Their maternal care and anxiety behavior is examined in the first week after pup delivery. Control group will consist of wildtype mice injected with AAVDJ-GFP virus and be processed exactly like the AAVDJ-Stat4A-injected group. The brain areas we have tested are the retrosplenial gyrus (Figure 8) and pre-limbic area (PrL) of the mPFC (Figure 9). Separate injections in these two brain areas led to no changes in the behavioral tests.

In addition, we are using neurotracers to examine projections between stathmin4A-expressing brain areas and those regulating maternal care (similar to Experiment 1.2).

Experiment 2.3: Is the dorsal subiculum involved in postpartum depressive-like symptoms?

Rationale: The dorsal subiculum is one of the major regions expressing the *Stathmin4A* transgene in the *Stat4A* transgenic mice showing postpartum behavioral deficits. The dorsal subiculum is involved in stress-induced responses and memory retrieval (Roy et al., 2017). However, the role of the dorsal subiculum in maternal behavior is unknown. we hypothesized that the dorsal subiculum controls anxiety-like behaviors in a stressful and challenging condition, postpartum. We decided to test whether or not the dorsal subiculum is involved in maternal behavior or postpartum depressive- and anxiety-like behavior.

Method: we performed injections of the adeno-associated virus (AAV) expressing the Stathmin4A transgene (AAVDJ-Stat4A) in the dorsal subiculum of wild type female mice and tested maternal effects as described in Experiment 2.2. Control group is wildtype mice injected with AAVDJ-GFP virus and was processed exactly like the AAVDJ-Stat4A-injected group.

After injection in the dorsal subiculum of an adeno associated virus that expresses either the mutation stathmin4A (AAVDJ-Stat4A) or green fluorescent protein (AAVDJ-EGFP), we tested post-partum females on pup retrieval, survival of pups, elevated plus maze, and sucrose preference test.

Result: Smaller litter size in mothers injected in dorsal subiculum with AAV-Stat4A

Mothers injected with AAV-Stat4A in the dorsal subiculum had smaller litter size (AAV-EGFP vs AAV-Stat4A; number of pups that survived the first 21 days after birth $p=0.039$) (Figure 10).

Result: Decreased anxiety in mothers injected in dorsal subiculum with AAV-Stat4A

Mothers injected with AAV-Stat4A in the dorsal subiculum had decreased anxiety in the elevated plus maze (AAV-EGFP vs AAV-Stat4A; increased ratio open to close arms p=0.042) (Figure 10).

Experiment 2.4: Are hormonal changes involved in the behavioral deficits in *Stat4A* mice postpartum?

Rationale: It is well known that significant hormonal changes occur and are critical during peripartum period in both humans and rodents. Oxytocin is a hormone critically involved in social and affiliative behaviors including maternal care. Other hormones, such as corticosterone, progesterone and estradiol are also important in maternal care and stress. Because Stat4A mothers, but not virgin Stat4A females, have deficits in maternal care, anxiety and depressive-like behaviors, we hypothesize that Stat4A mothers have deficiency in hormonal changes compared to their wildtype counterparts. Therefore, these hormonal changes might be critical for our mouse model of PPD. We test potential hormonal changes in Stat4A mutant mothers using ELISA and immunohistochemistry approaches.

Discussion

Clinical implications for PTSD and PPD

Disturbed microtubule cytoskeleton by disrupting synapse-nucleus connectivity is supported by evidence from clinical studies and animal models (Marchisella *et al.*, 2016). Changes in tubulin expression are found in the hippocampus and prefrontal cortex of psychiatric patients. Genetic linkage studies associate tubulin-binding proteins such as the dihydropyrimidinase family with an increased risk of developing schizophrenia and bipolar disorder. For many years, altered immunoreactivity of microtubule associated protein-2 (MAP2) has been a hallmark found in the brains of individuals with schizophrenia.

We generated the *Grp*$^{-/-}$ mice and showed that they are deficient in fear extinction in the SEFL behavioral paradigm, designed to model aspects of PTSD in humans. Retrograde tracing showed the GRPergic connections between the BLA, mPFC and hippocampus, the areas critically involved in fear extinction. Transcription of several genes related to the dopamine signaling was downregulated in the BLA of the *Grp*$^{-/-}$ mice following the recall of fear memory in SEFL. These data point to the GRP and its neural circuitry as a link to dopamine in regulating fear extinction. Moreover, the *Grp*$^{-/-}$ mice hold promise as a genetic model of PTSD-like symptoms.

The deficiency in extinction in the *Grp*$^{-/-}$ mice is persistent as evident in their enhanced freezing during the recall test 14 days following extinction. Confirming our finding that the GRP removal prolongs fear extinction in the *Grp*$^{-/-}$ mice, the GRP decreases fear memory reconsolidation when applied following recall in rats (Murkar et al., 2018). Also, GRP protein and mRNA are increased following pharmacological treatment leading to facilitation of fear extinction (Hashimoto et al., 2018). These findings support earlier observations that a removal of the GRP signaling *in vivo* leads to enhanced and prolonged fear memory and deficient fear extinction in *Grpr*$^{-/Y}$ mice (Chaperon et al., 2012; Martel *et al.*, 2016; Shumyatsky *et al.*, 2002). Therefore, our current work suggest that the GRP may be involved in integrating processing of stress and memory of fear, having important implications for PTSD treatments in humans (Inoue et al., 2018; Roesler et al., 2014).

Single nucleotide polymorphisms (SNPs) and increased mRNA have been identified for MAP6/STOP in the prefrontal cortex of patients with schizophrenia (Shimizu *et al.*, 2006). Because MAP6/STOP knockout females demonstrate deficits both in maternal care and those that were described as related to the “schizophrenia-like” phenotype, there is a possibility that this gene is involved in human maternal care.

Our qPCR data (not shown here) shows downregulation of genes expressed in the dopamine-signaling pathway following SEFL protocol in the GRP-/- mice. To

confirm these data, we will perform experiments to test mRNA levels in the GRP-/- mice after SEFL. The experiment is described in more detailed in the Future work section (Future experiment 3.1).

It is important to note that the analysis of the top 700 maternal genes against genes from autism, bipolar disorder and schizophrenia databases found overlapped genes for each of the disorders (Gammie *et al.*, 2016). Importantly, there is an increase incidence of bipolar disorder in mothers. Given that these disorders include social deficits, these genes' findings warrant a follow up in both animal and human studies. Genes found in schizophrenia and bipolar disorder had altered expression in the highly social maternal phenotype in the mPFC (Eisinger et al., 2014).

PPD is a type of major depression that starts in the perinatal period and can persist after the baby is born for several months. The depressive symptoms that occur in the peripartum period have detrimental effects on the mother itself and the development of the child.

Studying the molecular and cellular mechanisms in animal models has important implications for the design of psychological and pharmacological treatments. While several molecules have been found to be changed in the peripartum, only a few have been shown to be critical to maternal care. The GABA-Rs are among those few that are important as discussed in the previous chapters. Changes in

GABA-R are associated with neuroendocrine disruptions during PPD, and neuroactive steroids such as allopregnanolone can affect GABAergic signaling by modulating $GABA_A$ receptors (Walton and Maguire, 2019). Lower levels of serum allopregnanolone can predict symptoms of PPD in women (Osborne et al., 2017) and the relationship between GABA and allopregnanolone is important in the pathophysiology of PPD (Deligiannidis et al., 2019).

The discovery of GABA-R deficiency and its connection to progesterone has allowed the development of the drug that is approved to use by FDA (Mody, 2019). Pre-clinical research supports the role of neuroactive steroid (NAS) $GABA_A$ receptor ($GABA_AR$)–positive allosteric modulator (PAM) in PPD-like symptoms in rodents (Melon et al., 2018).

The first FDA-approved drug to treat PPD was Brexanolone, an analog of the neurosteroid allopregnanolone. Women with PPD on Brexanolone displayed a reduction of the depressive symptoms compared to the placebo group (Kanes et al., 2017; Meltzer-Brody and Kanes, 2020). Another FDA-approved is Zuranolone. It is similar to Brexanolone, also a $GABA_AR$ positive allosteric modulator and has a similar mechanism of action as allopregnanolone. The study showed a rapid reduction of depressive symptoms (by day 3) in women under zuranolone treatment, which lasted for at least 45 days. Importantly, care for the child was improved in women who took Zuranolone (Deligiannidis et al., 2021).

These pharmacological interventions show a quick reduction of depressive symptoms. In addition, the knowledge gained from the molecular and cellular studies can be employed in a more sophisticated design of psychotherapy methods. More research is needed to find specific molecules and synaptic mechanisms that allow us to have better treatments.

Brain areas regulating anxiety postpartum

Our results suggest that the dorsal subiculum is regulating anxiety postpartum. The subiculum is part of the hippocampal formation, that together with CA1, CA2, CA3 subareas of the HPC and the dentate gyrus is known to control memory processes in mammals. The subiculum receives and sends projections to several cortical and subcortical structures. The subiculum has afferent projections from CA1 area, entorhinal cortex, retrosplenial cortex, basal amygdala, and nucleus reuniens; whereas some of the efferent targets of the subiculum include the entorhinal cortex, pre-/para-subiculum, retrospleanial cortex, perirhinal/postrhinal cortex, CA1 area, nucleus accumbens, hypothalamic nucleus, thalamic nucleus, mammillary body, and lateral septum (reviewed by (Matsumoto et al., 2019).

There has been an effort to identify those genes that are important for subiculum function. It was found that the subiculum can be divided into proximal and distal regions according to their genetical expression. In this study, population RNA-Seq showed that there are specific genes encoding groups of neurons that

project either to nucleus accumbens (NAc) or retrosplenial cortex (RSC) (Cembrowski et al., 2018).

The subiculum has been proposed to be a mediator of hippocampal-HPA interactions, and several studies (reviewed by (Herman and Mueller, 2006) indicated that lesions in ventral subiculum and ventral CA1 of the rat induced several changes in the HPA axis function. However, the role of the dorsal subiculum in emotional responses is unknown. There is only one report by Roy et al. that examined the dSub – mammillary body (MB) circuit in retrieval-induced stress hormone responses (Roy *et al.*, 2017). The authors measured the levels of the stress hormone corticosterone (CORT) in the blood right after contextual fear conditioning (CFC) and after recall, Roy et al. found increased CORT levels in the blood in both phases of memory. Next, Roy et al. assessed the dSub – mammillary body circuit role in retrieval-induced stress hormone responses by inhibiting the dSub to MB projections. Optogenetic inhibition of the dSub to MB projections following CFC recall prevented the CORT increase, however, this effect was not shown following CFC training (Roy *et al.*, 2017). Interestingly, Roy et al. also found that optogenetic activation of the dSub to MB projections following CFC recall increased CORT levels, which confirmed that the dSub – MB circuit is necessary to induce CORT (Roy *et al.*, 2017).

Our future work is focused on exploring the function of the dorsal subiculum. To this end, it is important to determine the link between dorsal subiculum and

hormonal changes, as well as the link to other brain areas such as the mamillary bodies. The proposed experiments are explained in detail the Future work section (Future experiment 3.2 and 3.3).

Future work

Future Experiment 3.1: Is the susceptibility to SEFL accompanied by gene expression changes in *Grp*$^{-/-}$ mice?

Rationale: Our preliminary data using qPCR show that several genes in the dopamine-signaling pathway are down-regulated following the recall test in SEFL. Since dopamine is critical for fear extinction, we might have found that the GRP regulates dopamine function during this process. To examine other potential gene changes in an unbiased way, we now perform the computer analysis of the RNA-seq data that we obtained a few weeks ago from *Grp*$^{-/-}$ mice following SEFL memory recall. This is performed in collaboration with the Premal Shah lab (Genetics department, Rutgers).

Future Experiment 3.2: Does the expression of Stat4A in the dorsal subiculum lead to hormonal changes?

Rationale: Oxytocin is a hormone critically involved in social and affiliative behaviors including maternal care. Other hormones, such as corticosterone, progesterone and estradiol are also important in maternal care and stress. Changes in corticosterone levels were previously reported when dorsal subiculum was manipulated in stress induced response, therefore, the hormonal changes can be an indicator of dorsal subiculum function in anxiety postpartum

(Roy *et al.*, 2017). Using ELISA, we test potential hormonal changes in mothers injected with the AAV virus which expresses the Stathmin4A transgene (AAVDJ-Stat4A) in the dorsal subiculum in comparison with SHAM mothers (injected with AAVDJ-GFP). We use immunohistochemistry to test oxytocin and its receptor in the brain.

Future Experiment 3.3: Is the dorsal subiculum – mammillary body circuit involved in postpartum anxiety?

Rationale: Because the dorsal subiculum – mammillary body neural circuit was found to be involved in stressed induced responses (Roy *et al.*, 2017), we hypothesize that anxiety responses can be regulated by the subiculum under the stressful conditions of the motherhood. We test the role of the dorsal subiculum-mamillary body neural circuit of mothers after pup delivery in an anxiety-related behavioral task and compare it with naïve females in the same behavioral task. we will do optogenetic inhibition of the dorsal subiculum – mammillary body circuit to test whether this circuit is involved in postpartum anxiety behavior.

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