EMOTIONAL REGULATION AND THE LIMBIC SYSTEM ASSOCIATED MEMBRANE PROTEIN

By

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Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

In partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Neuroscience

August 2008

Nashville, Tennessee

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To my parents, Susan and Barry,

who have always been there for me in every possible way.

ACKNOWLEDGEMENTS

First, I would like to thank my advisor, Pat Levitt, for his mentorship. Pat provided me with the freedom to explore my ideas while offering an unbelievable wealth of knowledge and advice. He taught by demonstrating: knowledge of a great body of scientific literature, careful work, thorough troubleshooting, and ardent exploration of the possible meanings and larger place of experimental results. His high scientific standards combined with his consummate open-mindedness provided me the confidence to trust my interpretations even when they broke with obvious tradition. I learned by his example and encouragement the great importance and gift of discussing my work with other scientists, both within my own lab and through the entire scientific community, to foster collaborations and new ideas. But, perhaps more importantly than all of that, his monumental enthusiasm and energy for science is contagious and will continue to be a source of inspiration for me as I move forward in my career.

I am also very grateful to the rest of my committee, Craig Kennedy, Elisabeth Dykens, and Ed Weeber. Because of them my meetings were fun and stimulating discussions of my work. Their support and excitement about my project always came when most needed.

I am deeply indebted to everyone in my lab. Every person in our group has shared advice, help, technical and/or emotional support, questions, ideas and encouragement along my way. It was an amazing place to do a PhD— everyone is enthusiastic and curious.

Luckily for me (and possibly not so luckily for her), I moved to a desk directly adjacent to Barbara Thompson halfway through my graduate career. She possesses the unique combination of a PhD in behavioral neuroscience, a lot of overlap with my scientific interests and the patience to be bothered nearly everyday by a confused, excited, and/or frustrated graduate student. Barb assisted me with experimental design, acted as a sounding board for troubleshooting in nearly every experiment and made herself generally available to gab about whatever science goodie I was pondering at the moment. She also was always there to offer perspective and talk me down when things spun wildly out of control. I'd like to thank Barb as a colleague and a friend. Whoever gets my desk next is very lucky.

I'd like to thank Kathie Eagleson for her open door. She had helped me with data analysis and microscope foibles. Without her I may never even have started counting c-Fos profiles, let alone created a set of finished data. She was always available and enthusiastic to discuss and ponder the latest results. She helped me to navigate the politics of a large lab and has become a trusted friend.

Finally, although he's no longer a part of our lab, I'd like to thank Gregg Stanwood. Gregg too always made himself available, even during the very busy transition of starting his own lab. He's offered great advice and much experimental assistance.

I am grateful to all of the collaborators I have worked with throughout the last six years. I owe special thanks to Kathleen Gordon, who very quickly ran all of my plasma samples and returned to me fabulous data that made for fascinating results.

I would be remiss if I didn't remember my first science mentor, Gig Levine. It is because of my time in his lab that I decided to pursue a PhD in neuroscience. He was a

great teacher, a great scientist, and a great person and I feel very lucky to have known him and worked with him.

Of course, there are a huge number of friends who have supported and encouraged me along the way. Thank you to all of my fellow graduate students for commiserating and understanding. Thank you to all of my non-science friends for all the pep talks and cheering on I got while writing. A special thanks to Mary Kibbe and Michelle Jacobs, who have both helped me maintain my scientific and personal sanity on more than one occasion.

My parents are two of my best friends. They have always been there, to support me in whatever crazy thing I've decided to do. They've taught me integrity, that nothing is beyond my reach and that quitting should never be an option. Without these things I wouldn't have made it to or through my PhD.

And, finally, to my husband, Ken. I owe Ken thanks for just about everything. Because he listened when I was frustrated or dejected, because he distracted and cheered me up when nothing else could, because he tolerated my graduate school induced (and worse, dissertation induced) moodiness, because of all the good advice he's given, because he brought me sushi while I wrote, because he proofread for me, because his enthusiasm and creativity for science reminded me what fun it can be when I had forgotten, and because he always makes me feel that I'll have his support no matter what happens or what direction I go.

This work was made possible by financial support from: NIMH Grant MH45507 to Pat Levitt, the Vanderbilt Kennedy Center core grant NICHD HD15052, and the Vanderbilt Neuroscience Program's pre-doctoral fellow training grant T32 MH065215.

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CHAPTER I

INTRODUCTION

Neuropsychiatric disorders such as generalized anxiety disorder, panic disorder, post-traumatic stress disorder, and depression all involve disruptions in emotional regulation. These disruptions encompass not only disturbances in mood and anxiety, but also disturbances in the physiological response to stress (Chrousos & Gold, 1992; Mello, Mello, Carpenter, & Price, 2003; Rosen & Schulkin, 1998). These behavioral and physiological alterations are correlated with dysfunctions in the limbic circuitry of the brain, but the underlying neural causes are complex and there is no evidence for gross defects in the brain. Instead, reported central nervous system (CNS) alterations are multiple and subtle (Millan, 2003). The origins of complex neuropsychiatric disorders such as anxiety disorders and depression are also not well understood. However, it has become apparent through both human and animal investigations that their etiologies are developmental, dependent upon complex interactions of genes and environment (Gross & Hen, 2004; Leonardo & Hen, 2008).

To improve the treatment and outcome of disorders of emotional regulation it is necessary to better understand both the changes in brain signaling that underlie the behavioral and physiological components of the disorders and the origin of those changes. One way to tackle these questions is by using animal models to investigate the neural basis of anxiety behaviors and the stress response, using either environmental or genetic perturbations to disrupt emotional regulation. In this chapter I review the current

state of knowledge about emotional regulation in animal models from a behavioral, neurobiological and developmental perspective. I introduce a specific example of a developmentally relevant protein from a class of molecules whose roles in emotional regulation are, thus far, infrequently considered.

The Stress Response

In order to define the response to stress, it is first necessary to define stress itself. Here, stress is defined as any challenge to homeostasis (maintaining the internal environment in a constant state). When homeostasis is challenged an animal mounts both a hormonal and autonomic response. Challenges to homeostasis are not always marked by threat (the prototypical "flight-or fight" response). A stress response is mounted to any number of "stressors", including environmental challenges that might not be considered threatening, such as exposure of an animal to another of the opposite sex (Marchlewska-Koj & Zacharczuk-Kakietek, 1990), birth (Liggins, 1994), suckling (Uribe, Redondo, Charli, & Joseph-Bravo, 1993), and feeding (Rovirosa, Levine, Gordon, & Caba, 2005). Stress responses are also mounted to potentially dangerous or harmful stimuli such as novelty (Grootendorst, de Kloet, Dalm, & Oitzl, 2001; Rodgers et al., 1999), social defeat (Buwalda et al., 1999), cold stress (Bligh-Tynan, Bhagwat, & Castonguay, 1993), and immune challenge (Mekaouche et al., 1996).

There are two main legs to the physiological response to stress, autonomic and endocrine. The autonomic response is immediate and is primarily mediated by the release, from the sympathetic nervous system (SNS), of epinephrine into the bloodstream. This causes an increase in heart rate, blood pressure and a number of other cardiac

functions as well affecting brain function (for review of the SNS role in stress see: Wortsman, 2002; Wurtman, 2002). The endocrine response is slower, longer lasting and has been greatly studied for its role in behavior, disease and emotional regulation. The neuroendocrine response will be the focus of this chapter.

A cascade of three hormones from the hypothalamic-pituitary-adrenal (HPA) axis is responsible for producing the neuroendocrine stress response (Figure 1). The response begins with the release of corticotropin releasing hormone (CRH) from the para-ventricular nucleus of the hypothalamus (PVN) into the portal bloodstream to the anterior pituitary. CRH stimulates the release of adreno-corticotropin hormone (ACTH) from the pituitary into the peripheral bloodstream. ACTH acts on the adrenal medulla to excite the release of Corticosterone (CORT). CORT release is basally regulated on a circadian cycle, with the highest levels of circulating CORT during the active period (night for rodents) and the lowest levels at the beginning of the rest period (day for rodents). In response to stress CORT release is greatly up regulated. The surge of CORT into the peripheral bloodstream begins to occur within minutes, generally peaks within half an hour and has numerous effects including altering metabolism to release glucose stores and working in concert with the SNS response to alter heart rate and blood flow (Miller & O'Callaghan, 2002). CORT feedback to the brain turns off the stress response by acting at a number of structures including the hippocampus, the PVN and the pituitary (Herman, Ostrander, Mueller, & Figueiredo, 2005). The magnitude and duration of CORT stress response varies in proportion with intensity of the stressor (Campeau & Watson, 1997)

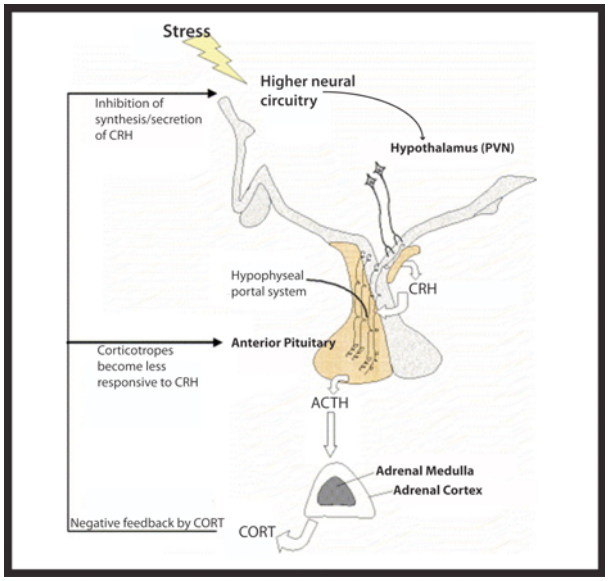

Figure 1. The hypothalamic-pituitary-adrenal axis. (Adapted from Tilbrook & Clarke, 2006).

and pathological states are frequently associated with increased HPA reactivity to stress or disturbances in normal feedback (Chrousos & Gold, 1992).

The effects of CORT in the brain are mediated through two known receptors, the mineralcorticoid receptor (MR) and the glucocorticoid receptor (GR) (Reul & de Kloet, 1985, 1986). GRs are more widely distributed than MRs and, because of their much lower affinity for CORT, are considered to be the mediators of negative feedback in the brain (de Kloet, Karst, & Joels, 2008; Reul & de Kloet, 1985, 1986). MRs, on the other hand are involved in regulating basal levels of CORT. Traditionally both GRs and MRs are considered to be nuclear receptors—binding of CORT causes translocation to the nucleus, where the receptors can alter transcription (de Kloet et al., 2008; Joels, 2006). This mode of signaling allows them to have prototypical slow effects: modulating basal CORT levels for MR and shutting CORT off after stress, which can take hours, for GR. However, there is accumulating evidence that there are fast effects of CORT in the brain. Membrane bound MR receptors have been identified as having non-genomic effects in the hippocampus (Karst et al., 2005) and there is evidence of fast-feedback in the amygdala, hypothalamus and pituitary as well (Dallman, 2005). The ability of CORT to mediate fast, non-genomic effects in the brain has implications not only for neuronal signaling (Joels, Karst, DeRijk, & de Kloet, 2008; Karst et al., 2005), but also for behavior: there are examples of acute increases of CORT increasing behaviors such as aggression and locomotion within minutes (Haller, Halasz, Makara, & Kruk, 1998; Joels et al., 2008).

The development of the HPA axis and stress response is well-defined (Levine, 2005; Meaney et al., 1993; Meaney et al., 1996; Rosenfeld, Suchecki, & Levine, 1992),

and there are critical periods in development during which alterations in the peri-natal environment can alter the adult HPA axis response to stress. Newborn rodents already have the ability to mount a CORT response to stress, but experience a stress-hyporesponsive period (SHRP) early postnatally (post-natal days 4-14 in a rat) during which they cannot normally mount a CORT response to stress (Schapiro, Geller, & Eiduson, 1962; Schmidt, Enthoven et al., 2003). The SHRP is mediated by licking and grooming of the pups by the damand, separating the pups from the dam for 24 hours can disinhibit the dampening of CORT responsiveness (Levine, Stanton, & Gutierrez, 1988; Stanton, Gutierrez, & Levine, 1988) and alter responsiveness throughout the life of the offspring, indicating the importance of maternal care in mediating a sensitive period for adult functioning of the HPA axis. Meaney and colleagues have demonstrated that even slight variations in the quality of maternal care during a critical period (post-natal days 1-7) that overlaps with the SHRP can have deleterious effects on the response to stress by the offspring when they are adults (Liu et al., 1997), with the offspring of dams that give lower quality care (low licker-groomers) having more reactive HPA axes as adults. In addition, as discussed in the following sections, the changes caused by this environmental alteration are accompanied by changes both in the behavioral response to stress and in the circuits that mediate the stress response.

Stress-Related Behaviors

Generally, when measuring the behavioral response to stress in a rodent, the goal is to mimic or better understand the way that some manipulation (environmental or genetic) may be relevant to human behavioral states after stress. It is from this goal that

measuring “anxiety” in rodents has become universal. In an attempt to describe anxiety in a way applicable to both humans and rodents, Leonardo and Hen recently defined anxiety as “a state of cognitive and behavioral preparedness that an organism mobilizes in response to a future or distant potential threat” (Leonardo & Hen, 2008). There are many tests used to measure “anxiety” in rodents. These tests fall into two main categories: those that use conditioned response (e.g. fear-potentiated startle, shock probe burying) and those that instead rely on spontaneous behavior to determine the state of the animal (Rodgers, 1997). Tests of anxiety should ideally meet three measures of validity (predictive, face, and construct). The most commonly used tests of anxiety were developed for, and therefore have, predictive validity of the ability of anxiolytics (most notably Diazepam) to reduce anxiety in humans. Many of these tests also have face validity; that is, they appear to be measuring anxiety-like behaviors to an untrained eye. However, it is not apparent that they are necessarily models with construct validity (the basis of the elicited behavior is the same as that trying to be modeled) (Rodgers & Dalvi, 1997). Generally, those behavioral tests that rely on spontaneous behavior (and exploration of a novel environment— certainly a “stressor”) are considered more ecologically valid (that is they are measuring responses that are more natural and are more likely to have construct validity) and I will utilize an example of one of these to discuss the measurement of anxiety behavior in more depth.

The elevated plus maze (EPM), like almost all other commonly used anxiety-related behavioral tasks, relies on an approach-avoidance conflict (Crawley, 2008) and was developed to test the effects of anxiolytic drugs in rats (Pellow & File, 1986). The

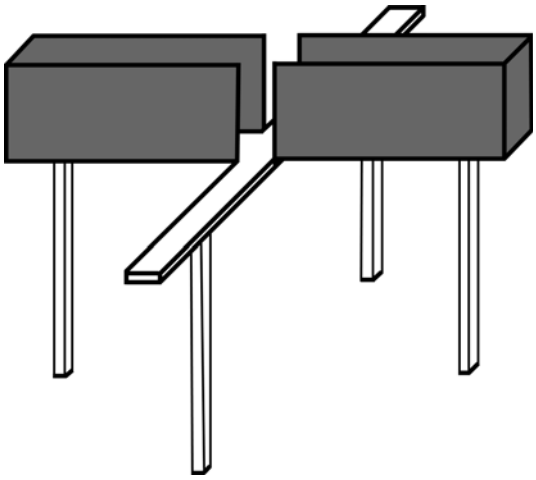

Figure 2. The elevated plus maze.

maze is shaped like a cross with two enclosed arms and two open arms that form narrow platforms (Figure 2). The enclosed arms are considered more safe and the open arms more threatening, thus a rodent's desire to explore a novel area is counteracted by the danger and fear evoked by an unenclosed space. Originally, the measures of interest were time spent in the open arms and closed arms and the number of entries in the open arms and closed arms. Rodents generally show a strong aversion to the "anxiety provoking" open arms and spend a greater amount of time and make a greater number of entries into the closed arms (Hogg, 1996; Pellow & File, 1986; Rodgers, 1997). Anxiolytic Benzodiazapines increase the preference of rodents for the open arms as measured by both duration and number of entries (Pellow & File, 1986).

In an effort to increase the ecological validity of the test, other behaviors that take place during maze exploration have been defined (Rodgers, 1997). These behaviors include stretch-attend postures and head-dipping, two measures of risk-assessment. Several factor analyses of EPM behaviors have suggested that these two behaviors load with traditional measures of anxiety, but are inversely correlated with open time and entries (Cruz, Frei, & Graeff, 1994; Rodgers & Johnson, 1995), supporting the idea that risk assessment is increased in response to threat (Rodgers, 1997). Using these measures of ethologically relevant defense behaviors should increase the construct validity of exploratory tests, as clinical anxiety states in humans have been proposed to be inappropriately activated defense responses (Rodgers, 1997).

It is important to note that the behaviors elicited by the EPM and similar approach-avoidance tests (open field, light/dark, zero maze, open field emergence) are driven by the balance between behavioral/motor activation (needed in order to explore)

and behavioral/motor inhibition (needed to inhibit entry in potentially threatening parts of the maze). Generally, animals with greater "anxiety" (elicited by a drug, or genetic or environmental manipulation) will show more behavioral inhibition, and less exploration, whereas those with less anxiety will show greater behavioral activation (more exploration). To account for the complication of using motor behavior to measure anxiety state, an overall change in motor behaviors, i.e. hyper-activity, is normally measured by using behaviors that are not typically thought to fluxuate with anxiety state, such as the number of closed entries on the EPM (Rodgers, 1997). Even given the similarities in these tests and controls for motor activation, anxiety may be detected on one test and not another in the same animal. However, when interpreting the results of any exploration based "anxiety" test it is important to remember that validity is based on pharmacological manipulations and that the meaning of the exploratory/motor driven behaviors on the maze should be interpreted in the context of other behaviors (risk-assessment, freezing, etc) in order to best interpret the basis of the behavior with the most validity.

Not surprisingly, exposure to novel environments in the form of behavioral tests of anxiety, such as EPM, induces a neuroendocrine response (Matzel et al., 2006; Pellow, Chopin, File, & Briley, 1985; Qin & Smith, 2008). As is the case of many human anxiety disorders (Chrousos & Gold, 1992; Rosen & Schulkin, 1998), animals that demonstrate increased "anxiety" also often have increased HPA reactivity. This is seen with manipulations of both environmental (Liu et al., 1997; Stankevicius, Rodrigues-Costa, Camilo Florio, & Palermo-Neto, 2008) and genetic components (Koster et al., 1999; Raber et al., 2000). The converse is also true; rodents that demonstrate decreases in "anxiety" often have a correspondingly blunted HPA axis response to stress (Bale et al.,

2002; Timpl et al., 1998). The behavioral and physiological phenotype is not just a correlational relationship. Acute injections of physiological levels of CORT cause an increase in anxiety-like behavior on the EPM (Mitra & Sapolsky, 2008), indicating that increases in CORT can mediate increased anxiety-like behavior.

Human anxiety disorders are now considered to be developmental in their origins (Leonardo & Hen, 2008) and, just as HPA axis reactivity is sensitive to developmental perturbations, so are anxiety behaviors. Maternal separation during the SHRP can induce long-term upregulation of HPA axis reactivity (Plotsky & Meaney, 1993), and it also effects long-term increases in anxiety-like behavior (Romeo et al., 2003). For example, the offspring of high licking/grooming rats not only display decreased HPA axis reactivity, but also demonstrate reduced anxiety behaviors on a number of tests. The offspring of high licking-grooming rats demonstrate both reduced HPA axis reactivity as adults, and decreased anxiety-like responding in several behavioral paradigms (Caldji, Francis, Sharma, Plotsky, & Meaney, 2000; Caldji et al., 1998). The developmental effects appear to be mediated by altering methylation state of the gene encoding GR, resulting in altered levels of GRs in the hippocampus (Weaver et al., 2004). Greater expression (less methylation) is more effective in mediating the negative feedback response to shut down the HPA axis response. These examples demonstrate that there is a clear relationship between emotional regulation and the neuroendocrine stress response in adults, and that they are developmentally linked, such that perturbations during critical periods of their development cause long-term alterations in function. To understand why the behavioral and endocrine stress responses are intertwined and to begin to investigate

how they are altered in pathological states, it is necessary to explore the neurobiology that coordinates and activates both behavioral and the endocrine responses to stress.

The Circuitry of Emotional Regulation

Is there a neuronal circuit for emotional regulation? Broca described a set of structures he named "the limbic lobe" in 1878 (Broca, 1878) and in 1937, Papez employed many of those structures and published "A Proposed Mechanism of Emotion" in which he proposed that an inter-connected set of structures were responsible for emotion and emotional expression (Papez, 1937). Included in the "Papez loop" were the circuits that interconnect the cingulate cortex, the hippocampus, anterior thalamic nuclei, and the hypothalamus (along with "connecting structures" such as the mammilary bodies and cingulum). Ten years later, MacLean furthered the description of the brain circuitry involved in emotional mechanisms, describing what would become known as the limbic system, including Papez's loop and adding other structures such as the frontal lobes and the amygdala (MacLean, 1949). Today a pubmed search with the words "limbic system" returns over 10,000 results, including nearly 1,500 reviews. However, there is no single agreed upon definition of what comprises the limbic system, and the structures included, ranging from cortical areas to brain stem nuclei have varied over time. A number of researchers have suggested that there is no limbic system (Dalgleish, 2004; Heimer & Van Hoesen, 2006; Pessoa, 2008). One argument for this view is that each area included is involved in several functions and therefore not "primarily" responsible for emotional regulation (Pessoa, 2008). A counter-argument could be made that emotional regulation is so important to survival and, therefore, so intertwined with the performance of other

functions, such as memory and cognition, that it's no surprise at all that it's circuitry is involved in mediating such actions. There is abounding physiological, anatomical and molecular evidence that an interconnected set of brain structures are integral for defining emotional regulation and these structures are capable of mediating both the behavioral and endocrine response to stress (Charney & Deutch, 1996; Herman et al., 2005; Levitt, 1984; Petrovich, Canteras, & Swanson, 2001). For the purposes of this discussion, I will continue to use limbic to suggest an interconnected set of structures that include both the historical limbic lobe, and also interconnected circuitry (to be introduced) that has a well-defined role in behavioral, endocrine, and autonomic emotional regulation.

Parvocelluar neurons of the PVN are responsible for releasing CRH into the portal blood stream (Whitnall, 1993). These neurons receive direct inputs from many hypothalamic, sub-cortical and brainstem nuclei (e.g. bed nucleus stria terminalis (BNST), dorsal medial and lateral hypothalamus, pre-optic area, raphe nucleus, sub-fornical organ) which are involved in regulating autonomic function, allowing the HPA to make a rapid response to homeostatic threats of an immune or physiological nature (Herman et al., 2003). Many of the areas of the brain that are responsible for mediating the HPA axis response to psychological threat (the limbic structures and circuitry) do not project directly to the PVN, but instead influence parvocellular neurons by indirect connections (Herman et al., 2003) (figure 3). In the following sections, I will concentrate on three main limbic structures, the amygdala, the hippocampus and the pre-frontal cortex and their circuitry to the parvocellular neurons.

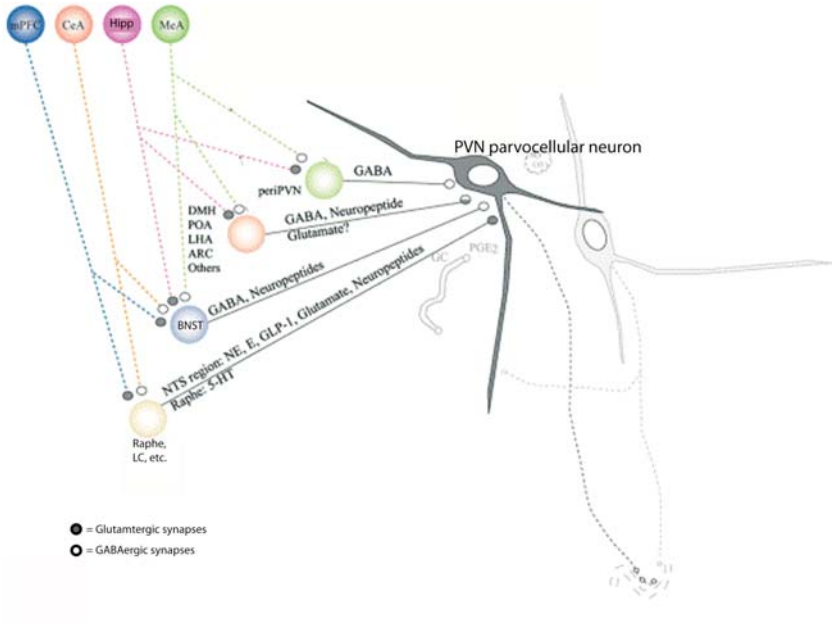

Figure 3. Inputs to the PVN of the hypothalamus.
(Adapted from Herman et al., 2003).

The amygdala plays a central role in mediating autonomic, endocrine and behavioral responses to stress through reciprocal connections with thalamus, sensory cortices, brainstem structures, orbitofrontal cortex, hippocampus, BNST and hypothalamus (Charney & Deutch, 1996; Herman et al., 2003). The central nucleus (CeA) of the amygdala excites the HPA axis through connections with the lateral group of the BNST, which has glutamatergic (excitatory) projections to the PVN (Dong, Petrovich, & Swanson, 2001). The CeA also projects directly to the lateral hypothalamic area where it influences the autonomic response to stress. Finally, CRH-containing neurons project from CeA to the locus coeruleus (LC), a major source of norepinephrine (NE) in the brain, which itself is a major mediator of stress responsiveness (Morilak et al., 2005). The medial nucleus of the amygdala (MeA) projects to the transverse and interfascicular nuclei of the BNST; however, both of these connections appear to be largely GABAergic and therefore, may be excitatory to the PVN via disinhibition (Dong et al., 2001; Herman et al., 2003). There are amygdalar projections to the peri-PVN, which in turn has a significant GABAergic input to the PVN (Sawchenko & Swanson, 1983). Finally, the lateral nucleus (LA) of the amygdala has projections through the hippocampus which indirectly influences hypothalamic nuclei involved in coordinating defensive behavior, such as the anterior nucleus (Petrovich et al., 2001).

The hippocampal formation (including dentate gyrus and subiculum) is also interconnected with sensory cortices, cingulate cortex, the BNST, and the hypothalamus (Herman et al., 2003). It is an important part of the circuit of negative feedback to shut down the HPA axis response (Feldman & Weidenfeld, 1999). These inhibitory actions are most likely mediated through the subiculum's projections to several areas including

the BNST, peri-PVN, and other hypothalamic areas that relay to the PVN itself (Herman et al., 2003; Sawchenko & Swanson, 1983). There are also sparse projections of neurons from CA1 to the PVN itself (Cenquizca & Swanson, 2006).

The medial prefrontal (mPFC) cortex also is implicated in negative feedback of the HPA axis (Diorio, Viau, & Meaney, 1993; Herman et al., 2003). However, it is involved in the HPA axis response to stress as well, with evidence for both excitatory and inhibitory functions. This is due to the roles of multiple areas of mPFC in modulating the stress response. Lesioning of dorsal mPFC (pre-limbic) suggests that it inhibits PVN neurons, while ventral mPFC lesions (including infralimbic cortex (IL)) excite those neurons during stress (J. J. Radley, Arias, & Sawchenko, 2006). The mPFC also does not directly project to the PVN. Pre-limbic mPFC contains projections to pre-optic area and the peri-PVN, both inhibitors of the stress response. Alternatively, IL mPFC has connections with anterior BNST, lateral hypothalamus, MeA and CeA of the amygdala, all implicated in excitation of the PVN (Herman et al., 2005). mPFC also has reciprocal connections with LC and it has been recently demonstrated that at least part of the excitatory effect of NE on HPA axis reactivity is mediated through mPFC (J. J. Radley, Williams, & Sawchenko, 2008).

The circuitry involved in the behavioral response to stress completely overlaps with that discussed as involved in HPA axis reactivity. Partially, this is due CORT's ability to directly affect behavior (see The Stress Response section above). However, the limbic circuitry as a whole is involved in mediating all aspects of emotional regulation, including behavioral, autonomic and neuroendocrine (Charney & Deutch, 1996). As would be expected of any circuitry regulating complex functions, the brain structures

involved are numerous (Figure 4); in order to functionally modulate anxiety or fear states the ciruitry must include sensory areas for the detection of threats, integrative areas, and output areas to elicit the physiological and behavioral response. This discussion will focus on a few key structures that are involved in integrating input and coordinating all aspects of the stress response. Charney and Deutch provide a comprehensive review of these structures and some of their major connections (Charney & Deutch, 1996). As mentioned above, the amygdala is central to emotional regulation due to its mostly reciprocal connections with cortical areas, the hippocampus, LC, the periaqueductal gray (PAG) and the hypothalamus (Figure 4). The CeA sends projections to several brainstem nuclei and has an excitatory effect on NE producing neurons of LC, as well as on nuclei regulating cardiovascular and respiratory control. BLA sends projections to striatum, situating it to modulate reward and motoric output. Stimulation of the amygdala in humans provokes feelings of fear and lesions in animals reduce fear related behaviors along with aggression. The mPFC also receives and sends projections to the amygdala, and to striatum. As with neuroendocrine stress responsiveness, the mPFC can both increase and decrease behavioral reactivity to stress. Ventromedial PFC (in a pre-limbic area) seems to function to decrease anxiety-like behavior in some situations, while IL increases anxiety-like responses in others (Wall, Blanchard, Yang, & Blanchard, 2004). Besides its role in the HPA axis, the hippocampus is important in consolidating memory associated with emotional events (for review see- (Phelps, 2004)) and therefore, is likely part of a circuit important in attaching salience to stress and possibly altering subsequent behavioral responses. The LC is connected with the circuitry of emotional regulation in several ways (see above) and is considered a direct activator of the HPA axis. Drugs that

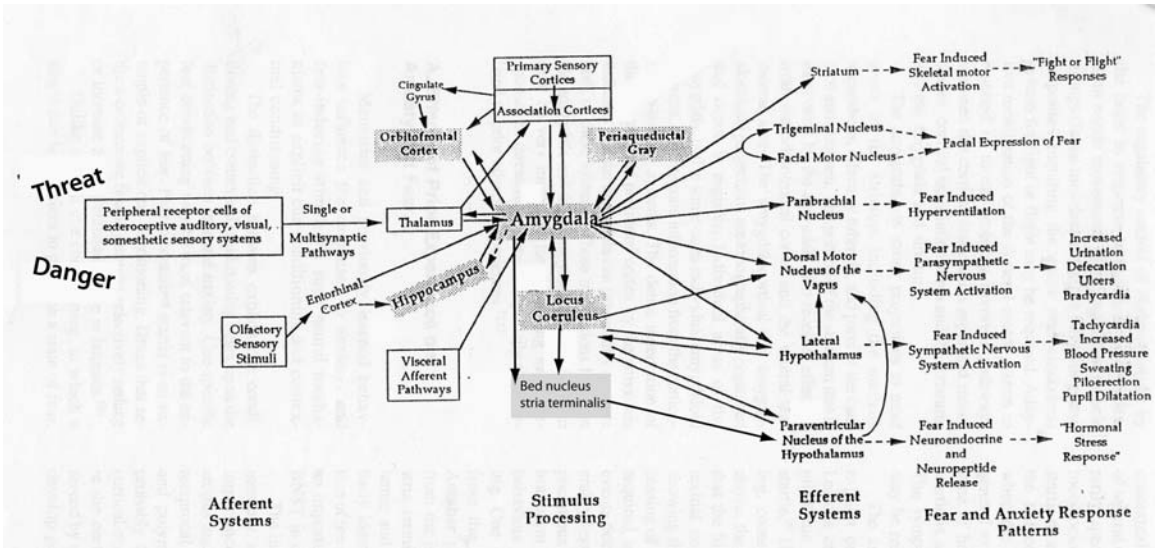

Figure 4. The circuitry of emotional regulation.
(Adapted from Charney & Deutch, 1996).

increase the output of NE from the LC cause increased fear behavior in rodents and increased feelings of anxiety in humans.

These structures (amygdala, mPFC, hippocampus, LC, and hypothalamus) form the circuits that play a major role in all aspects of emotional regulation across mammalian species. Disrupting these circuits by modification of neurotransmitter function is common. There are many neurotransmitters that modulate the effects of stress, but there are a small number of molecules mentioned above that are important in mediating stress responsiveness and that have also been demonstrated to play a role in the development of the stress response. CRH is not only expressed by neurons in the PVN, but is also used as a neurotransmitter throughout the rest of the brain (one major site of release is the amygdala CeA). Alterations of the CRH system by genetically deleting the CRH receptor 1 (CRHR1) have demonstrated its importance in regulating adult responsiveness to stress and anxiety (Muller, Keck, Zimmermann, Holsboer, & Wurst, 2000; Muller et al., 2003; Timpl et al., 1998), and also in modulating the activity of the HPA axis during the developmental sensitive period (SHRP) that is responsible for setting up adult responsiveness to stressors (Schmidt, Oitzl et al., 2003). NE has long been known to have an activating effect on the HPA axis and to be involved in anxiety-like behaviors (Morilak et al., 2005). Again, genetic deletion of the adrenergic alpha-2 receptor is associated with increased anxiety in adults (Lahdesmaki et al., 2002) and reduction specifically during the early post-natal critical period resulted in decreased anxiety along with increased expression of the receptor in adult animals (Shishkina, Kalinina, & Dygalo, 2004). Serotonin (5-HT) released from the raphe and other brainstem nuclei is another neurotransmitter that has been demonstrated to be both a modulator in emotional

regulation and to have involvement in human neuropsychological disorders (Kusserow et al., 2004; Leonardo & Hen, 2008). In addition, as with the other modulators of emotional regulation that have been discussed, disrupting 5-HT signaling through receptor knockout specifically during the critical period for developing stress responsiveness resulted in more anxious animals—developmental regulation of the neuromodulator was enough to change the animal's stress response throughout it's life (Gross et al., 2002).

The major excitatory and inhibitory neurotransmitters in the brain, glutamate and GABA, respectively, are very important in the circuits of stress responsiveness. Many of the excitatory projections to the PVN are glutamatergic (Figure 3) but glutamate is often missing from lists of genes important in anxiety or stress (Gratacos et al., 2007; Muller & Keck, 2002; Wood & Toth, 2001) because disruptions of glutamatergic signaling causes major changes in all parts of the brain and makes interpretation of phenotypes difficult. However, there are several examples of specific disruptions in glutamatergic signaling effecting emotional regulation (Alt et al., 2007; Du et al., 2008; Kiryk et al., 2008) and drugs that alter glutamtergic signaling through metabatropic receptors are being pursued as possible treatments for anxiety and depression (Palucha & Pilc, 2007).

The role of GABA in both anxiety-like behavior and the neuroendocrine response to stress is better characterized. Disturbances of the $GABA_A$ receptor system have been implicated extensively in clinical studies of depression and anxiety (for review see-(Brambilla, Perez, Barale, Schettini, & Soares, 2003; Nutt & Malizia, 2001)) and the most commonly prescribed anxiolytics for anxiety disorders are benzodiazepines ((McLaughlin, Geissler, & Wan, 2003)), agonists to the benzodiazepine (BZ) receptors (i.e. $GABA_A$ receptors with subunits composing BZ sites). Decreased sensitivity to BZs

and decreased levels of BZ binding have been reported in the brains of patients with panic disorder and generalized anxiety disorder (Abadie et al., 1999; Kaschka, Feistel, & Ebert, 1995; Malizia et al., 1998; P. Roy-Byrne, Wingerson, Radant, Greenblatt, & Cowley, 1996; P. P. Roy-Byrne, Cowley, Greenblatt, Shader, & Hommer, 1990; Tiihonen et al., 1997) suggesting that alterations in BZ receptors may be a causative mechanism underlying anxiety disorders in humans. There also is evidence from animal studies that $GABA_A$ receptor levels and composition are related to the stress response and its development.

For example, in the maternal care paradigm of Meaney and colleagues, offspring of high licking-grooming mothers display reduced anxiety and HPA axis reactivity as adults. In addition offspring exhibit increased BZ binding in limbic areas that modulate the stress response, including amygdala nuclei (CeA, BLA, and LA), LC, and nucleus tractus solitarius (NTS) (Caldji et al., 2000; Caldji et al., 1998). The modified BZ binding in these animals is accompanied by permanent changes in the subunit composition of the $GABA_A$ receptors in the same structures. Levels of the α1, γ1 and γ2 $GABA_A$ receptor subunits are significantly increased in the CeA, BLA, LA and LC (α1 and γ2 only) of adult offspring of high L-G rats (Caldji, Diorio, & Meaney, 2003). The γ subunits are required for formation of the BZ binding site and α subunits determine the affinity of different benzodiazepines for the receptor and the sensitivity of the receptor to GABA (for review of $GABA_A$ receptors see- (Macdonald & Olsen, 1994)). Inclusion of the α1 subunit creates the BZ receptor subtype 1, which has a heightened affinity for certain BZ site ligands ((Macdonald & Olsen, 1994)). These changes in adult subunit composition

and BZ binding in the offspring of high licker-groomer mothers are due to alterations in the development of the system during an early postnatal critical period.

The demonstration that alterations of limbic circuit development can result in altered emotional regulation manifests the idea that the development of the circuitry itself, the anatomical connections between relevant structures, should play an important role in the development of the stress response. Alterations in molecules that regulate circuit formation, such as cell adhesion molecules (CAMs), are interesting candidates for examining the role of circuit formation on emotional regulation. Guidance molecules are responsible for facilitating appropriate connectivity in forebrain circuitry during development through a combination of attractive and repulsive cues (for review see - (Lopez-Bendito & Molnar, 2003)). Cell adhesion molecules (CAM's) of the immunoglobulin super-family (IgSF) are guidance molecules with multiple functions including neurite outgrowth, axon guidance, and synapse formation (Rougon & Hobert, 2003). There is evidence that genetic disruption of a widely expressed CAM, the neural cell-adhesion molecule (NCAM) results in disrupted HPA-axis responsiveness (Stork, Welzl, Cremer, & Schachner, 1997). It is reasonable, therefore, to hypothesize that disruption of guidance molecules that are enriched in limbic circuitry may result in altered neurodevelopment of those circuits, with outcomes that disrupt emotional regulation.

The Limbic System Associated Membrane Protein

The limbic system associated membrane protein (protein: LAMP, gene: *Lsamp*) is of compelling interest to the study of the biological mechanisms of emotional regulation

because it is a developmentally relevant molecule that is expressed primarily in limbic circuitry. LAMP is another CAM of the IgSF family. It is a 64- to 68-kDa protein with three immunoglobulin domains, a glycosyl-phosphatidylinositol (GPI) anchor to the cell membrane (Pimenta, Fischer, & Levitt, 1996; Pimenta et al., 1995) and three possible isoforms (Pimenta & Levitt, 2004). The gene is highly conserved among species. There is 99% homology in LAMP protein between human and rodent (Pimenta, Fischer et al., 1996) and similar distribution patterns in birds (Yamamoto & Reiner, 2005; Yamamoto, Sun, Wang, & Reiner, 2005), rat (Levitt, 1984; Reinoso, Pimenta, & Levitt, 1996; Zacco et al., 1990), monkey (Cote, Levitt, & Parent, 1995, 1996) and human (Prensa, Gimenez-Amaya, & Parent, 1999; Prensa, Richard, & Parent, 2003). In the adult, LAMP resides on the somata and dendrites of neurons (Levitt, 1984) and *Lsamp* expression is moderate to heavy in classic limbic areas and associated midbrain and hindbrain structures (Table 1) (Reinoso et al., 1996). More specifically, in the amygdala, *Lsamp* expression is moderate in the CeA and MeA, and high in BLA. PFC expression is high, as it is in other limbic cortical areas, such as cingulate cortex. *Lsamp* is expressed densely throughout all subfields of the hippocampus. *Lsamp* is expressed densely throughout parts of the hypothalamus including the PVN, although expression in the parvocelluar part is more moderate. The BNST and LC have moderate expression levels. There is sparse expression in the periaqueductal gray.

Lsamp expression begins early in embryonic development in post-mitotic neurons, around E13 in the rat, and has a distribution pattern similar to that of the adult (Pimenta, Reinoso, & Levitt, 1996). During early development, when limbic pathways

Table 1. Distribution of *Lsamp* expression in the adult brain.
(Adapted from Reinoso et al., 1996).

Site	Density
Olfactory system	
Main olfactory bulb	
Glomerulus	0
External plexiform layer	0
Mitral cell layer	+++
Internal plexiform layer	0
Internal granular layer	0
Accessory olfactory bulb	++
Olfactory tubercle	+++
Cerebral Cortex	
Sensorimotor	+
Prefrontal	+++
Perirhinal	++
Infralimbic/prelimbic	++
Anterior cingulate	
Superficial	+++
Deep	++
Posterior cingulate	
Superficial	++
Deep	+
Insula	+++
Piriform	+++
Auditory	+
Visual	0
Taenia tecta	+++
Induseum griseum	+++
Presubiculum	+++
Parasubiculum	+++
Subiculum	++
Entorhinal	
Medial	++
Lateral	+
Hippocampus	
CA1	+++
CA2	++++
CA3	++++
CA4	++++
Dentate	
Anterodorsal	++
Caudoventral	++++
Striatum	
Caudate-Putamen	+
Nucleus Accumbens (ventral striatum)	++
Globus Pallidus	++
Septal and basal forebrain regions	
Medial septal nucleus	++
Lateral septal nucleus	
Dorsal part	++
Intermediate part	+
Ventral part	++
Nucleus of the diagonal band	++
Subfornical organ	++
Bed nucleus of the stria terminalis	++
Encapsulated	+++
Anteroventral periventricular nucleus	++
Medial preoptic nucleus	+++
Medial preoptic area	+++
Lateral preoptic area	++
Substantia innominata	++
Amygdala	
Cortical nucleus	+++
Medial nucleus	++
Posterodorsal part	++++
Lateral nucleus	++
Basolateral nucleus	+++
Central nucleus	++
Epithalamus	
Medial habenular nucleus	+++
Lateral habenular nucleus	
Medial part	++
Lateral part	+
Thalamus	
Anterodorsal nucleus	++++
Anteroventral nucleus	+
Anteromedial nucleus	+++
Paratenial nucleus	++++
Mediodorsal nucleus	+++
Laterodorsal nucleus	
Rostral	+++
Posterior	++
Paraventricular nuclei	+++
Rhomboid nucleus	++++
Reuniens nucleus	++
Ventrolateral nucleus	+
Ventromedial nucleus	+
Ventral posterolateral nucleus	++
Ventral posteromedial nucleus	++
Centromedial nucleus	+++

Site	Density
Parafascicular nucleus	++
Lateroposterior nucleus	+
Posterior nuclei	++
Dorsal lateral geniculate nucleus	++
Ventral lateral geniculate nucleus	0
Medial geniculate	++
Thalamic reticular nucleus	+++
Subthalamus	
Subthalamic nucleus	++
Zona incerta	0
Hypothalamus	
Suprachiasmatic nucleus	++
Anterior hypothalamus	++
Lateral hypothalamus	++
Supraoptic nucleus	++++
Paraventricular nucleus	
Magnocellular	++++
Parvocellular	++
Periventricular nuclei	++
Arcuate nucleus	+
Median eminence	0
Ventromedial nucleus	+++
Dorsomedial nucleus	++
Dorsal hypothalamic area	++
Posterior hypothalamic nucleus	+
Premammillary nucleus	++
Medial mammillary nucleus	
Medial part	
Median	++
Lateral	0
Lateral part	++
Supramammillary nucleus	+
Midbrain	
Anterior pretectal nucleus	0
Red nucleus	+
Substantia nigra pars compacta	++
Substantia nigra pars reticulata	+
Ventral tegmental area	++
Interpeduncular nucleus	++
Superior colliculus	++
Inferior colliculus	++
Edinger-Westphal nucleus	++
Oculomotor nucleus	++
Trochlear nucleus	++
Periaqueductal gray	++
Dorsal raphe	++
Pons	
Pontine nuclei	++
Pontine tegmental reticular nucleus	++
Parabrachial nuclei	++
Superior olive	++
Nucleus of the trapezoid body	+++
Nucleus of the lateral lemniscus	++
Principal sensory trigeminal nucleus	++
Motor trigeminal nucleus	++
Dorsal tegmental nucleus	+++
Ventral tegmental nucleus	++
Locus coeruleus	+++
Nucleus raphe magnus	++
Medulla Oblongata	
Spinal trigeminal nucleus	++
Abducens nucleus	++
Facial nucleus	+++
Vestibular nucleus	
Superior	+
Medial	++
Lateral	+
Hypoglossal nucleus	++
Cochlear nucleus	
Dorsal	+
Ventral	+++
Nucleus ambiguus	++
Nucleus of the solitary tract	++
Dorsal motor nucleus of the vagus	+++
Lateral reticular nucleus	+++
Cerebellum	
Dentate nucleus	++
Interpositus nucleus	+
Fastigial nucleus	0
Molecular layer	+
Granule cell layer	0
Purkinje cell layer (basket cells)	++

0 = negligible
+ = sparse
++ = moderate
+++ = heavy
++++ = dense

are forming, LAMP is present not only on neuron soma and dendrites, but also is present on axons (Horton & Levitt, 1988). The presence of LAMP on axonal growth cones provides the ability for LAMP to guide the formation of limbic pathways and there is much evidence to support this. In vitro analysis has shown that antibody perturbation of LAMP disrupts axonal targeting from septum to hippocampus, without affecting general axon outgrowth (Keller, Rimvall, Barbe, & Levitt, 1989). Addition of LAMP increases axonal branching in explants of cortical and thalamic limbic neurons, but has no effect on branching in non-cortical areas (Mann, Zhukareva, Pimenta, Levitt, & Bolz, 1998; V. V. Zhukareva, N. Chernevskaya, A. Pimenta, M. Nowycky, & P. Levitt, 1997). LAMP also promotes neurite outgrowth in limbic neurons while inhibiting outgrowth of non-limbic neurons (Eagleson et al., 2003; Pimenta et al., 1995; V. Zhukareva, N. Chernevskaya, A. Pimenta, M. Nowycky, & P. Levitt, 1997). In vivo, antibody perturbation of LAMP causes aberrant projection of mossy fibers in the developing hippocampus (Pimenta et al., 1995). These results demonstrate that LAMP has an important role in the assembly of limbic circuitry and that disruptions in LAMP will cause alterations in that circuitry.

There are several examples of additional evidence linking LAMP to the modulation of emotional regulation. In a study of the exploratory behavior of rats on the elevated plus maze, rats that demonstrated reduced anxiety, as measured by entries and time in the open arms, also exhibited reduced *Lsamp* expression in the periaqueductal grey, amygdala and hippocampus (Nelovkov, Areda, Innos, Koks, & Vasar, 2006; Nelovkov, Philips, Koks, & Vasar, 2003). *Lsamp* expression is also down-regulated during the critical period in which maternal care is responsible for modulating development of the behavioral and physiological responses to stress. Champagne et. al.

recently demonstrated that *Lsamp* expression is decreased in the PVN of the offspring of high licking-grooming dams on postnatal days 1 and 7 (unpublished data); the period when maternal care regulates the development of limbic circuits responsible for mediating the stress response. These investigators hypothesized that differential expression of *Lsamp* during this critical period of development may cause altered patterning and wiring of limbic circuitry, which subsequently creates the structural basis for early "programming" of individual differences in HPA axis activity (Danielle Champagne, personal communication). There have also been several recent studies of polymorphisms in *Lsamp* associated with panic disorder, and male suicide, both disorders of emotional regulation (Koido et al., 2006; Maron et al., 2006; Must et al., 2008). Taken together, these studies provide strong evidence that LAMP is involved in the development of the circuits that underlie emotional regulation.

Our laboratory has developed mice in which the *Lsamp* gene is deleted, creating a unique model to examine the functional consequences of disrupting limbic circuit assembly. We propose to examine *Lsamp*$^{-/-}$ mice for alterations in emotional regulation and in the limbic circuitry that is responsible for creating and modulating the behavioral and physiological responses to stress. Utilizing behavioral tests that measure exploratory behavior and activity in response to a novel environment will reveal what behavioral disruptions occur in response to stress when *Lsamp* is not expressed. Examining the timing and magnitude of the HPA axis stress response in *Lsamp*$^{-/-}$ mice will demonstrate if LAMP is necessary for a normal neuroendocrine response to stress. In addition, studies to determine if *Lsamp*$^{-/-}$ mice have altered stress-induced activation patterns in the circuits that are involved in stress-related behavior and that normally express LAMP, will

be the first step in determining the effect of disrupting LAMP on limbic circuit development and structure. We hypothesize that *Lsamp*$^{-/-}$ mice will have developmental disruptions in limbic circuit formation that have long-term effects on limbic circuit structure and functional response to stress.

CHAPTER II

GENETIC DELETION OF *LSAMP* CAUSES EXAGGERATED BEHAVIORAL ACTIVATION IN NOVEL ENVIRONMENTS

Introduction

The limbic system is comprised of interconnected brain structures responsible for emotional regulation, cognitive function and autonomic responses. Although first described more than 120 years ago (Broca, 1878), hypotheses regarding the functional organization and specific contributions of the basic circuitry to complex behaviors are under continuous refinement. There is even debate regarding the limbic system as a unifying concept (Dalgleish, 2004; Heimer & Van Hoesen, 2006). Yet, specific frontal and temporal cortical areas, forebrain regions (septum, amygdala, hypothalamus) and brainstem nuclei (locus coeruleus, raphe, vagal nuclei) are implicated in the behavioral and physiological disruptions that cause neuropsychiatric diseases such as anxiety, depression and psychosis. However, the underlying, complex changes at the circuit level remain ill-defined (Millan, 2003). A neurodevelopmental etiology has been hypothesized for many psychiatric disorders (Ansorge, Hen, & Gingrich, 2007; Gross & Hen, 2004; Lewis & Levitt, 2002; Weinberger, 1995), suggesting that the functional impact may occur through the disruption of the assembly of limbic circuitry. One approach to examine the development, maintenance and disruption of limbic-related behaviors is to manipulate the expression of molecules that mediate the development and function of the underlying neural circuitry.

The limbic system-associated membrane protein (LAMP), a cell adhesion molecule (CAM) of the IgLON family expressed in cortical and sub-cortical limbic-associated regions of the developing and adult brain (Cote et al., 1995, 1996; Horton & Levitt, 1988; Levitt, 1984; Pimenta, Reinoso et al., 1996; Reinoso et al., 1996; Zacco et al., 1990) is one such molecule. The protein exhibits 99% homology between rodent and human (Pimenta, Fischer et al., 1996) and there is a close correlation between *Lsamp* mRNA and protein distribution patterns in rat (Levitt, 1984; Pimenta, Reinoso et al., 1996; Reinoso et al., 1996; Zacco et al., 1990), monkey (Cote et al., 1995, 1996), and human (Prensa et al., 1999; Prensa et al., 2003). Experimental manipulations of LAMP in vitro result in altered axon targeting and neurite growth (Eagleson et al., 2003; Keller et al., 1989; Mann et al., 1998; Pimenta et al., 1995; V. V. Zhukareva et al., 1997). In the analysis of different rat substrains, Nelovkov and colleagues correlated lower expression of *Lsamp* mRNA in the amygdala and hippocampus with decreased anxiety and increased exploration (Nelovkov et al., 2006; Nelovkov et al., 2003). Moreover, there is genetic association of a polymorphism in the *Lsamp* gene with panic disorder in humans (Koido et al., 2006; Maron et al., 2006). Both studies suggested that alterations in LAMP may have functional consequences on complex behaviors.

We have developed mice in which the *Lsamp* gene is deleted constitutively. Here, we have evaluated gross neuroanatomical organization and characterized the behavioral phenotype of *Lsamp*$^{-/-}$ mice by providing an assessment of their response to novel, stressful environments as measured by activity and exploratory behavior. The results of this study support the importance of LAMP in limbic function and provide the basis for further anatomical, physiological and biochemical phenotyping of *Lsamp*$^{-/-}$ mice.

Materials and Methods

Lsamp targeting

To disrupt the *Lsamp* gene, we generated a targeting vector that replaced 69 nt of exon 2 including the 3' splice site and 31 nt of intron 2 with a neo cassette inserted in the opposite transcription/translation frame relative to the *Lsamp* gene (Figure 1a). To generate the targeting vector, a mouse 129/ReJ genomic library constructed in the λFIX II vector (provided by Dr John Pintar, UMDNJ-RWJMS, Piscataway, NJ) was screened using probes derived from the rat *Lsamp* cDNA (Pimenta et al., 1995). Among the *Lsamp* genomic clones isolated and characterized, the m*Lsamp*-λ-11a clone was selected for containing a 13.2 kb insert, including the partial nucleotide sequence of the first intron, exon 2 and ~4.0 kb of intron 2. The linearized vector was electroporated into R1 ES cells ((129X1/SvJ x 129S1/Sv)F1-*Kitl*$^{+}$). Targeted ES cells were injected into C57BL/6J blastocysts to generate chimeric mice. One heterozygous *Lsamp* founder was obtained and back-crossed into the C57BL/6J strain for more than 10 generations for all experiments reported here. Initial genotyping was done by Southern Blot analysis. All subsequent mice were genotyped by polymerase chain reaction (PCR) amplification using primers to identify the presence of the wild type allele (5`- GTC CTG ATT GGT CTT GTT GAG TCC -3` and 5`- TCT TAT CCC ACT TCC CCC TTA CC -3`) and the targeted allele (5`-CTC CTG CCG AGA AAG TAT CCA TC-3` and 5`-CTC TGG AAT ACA GCC TCC GAA TC-3`). PCR reactions were performed using the AmpliTaq gold system (Applied Biosystems, Foster City, CA).

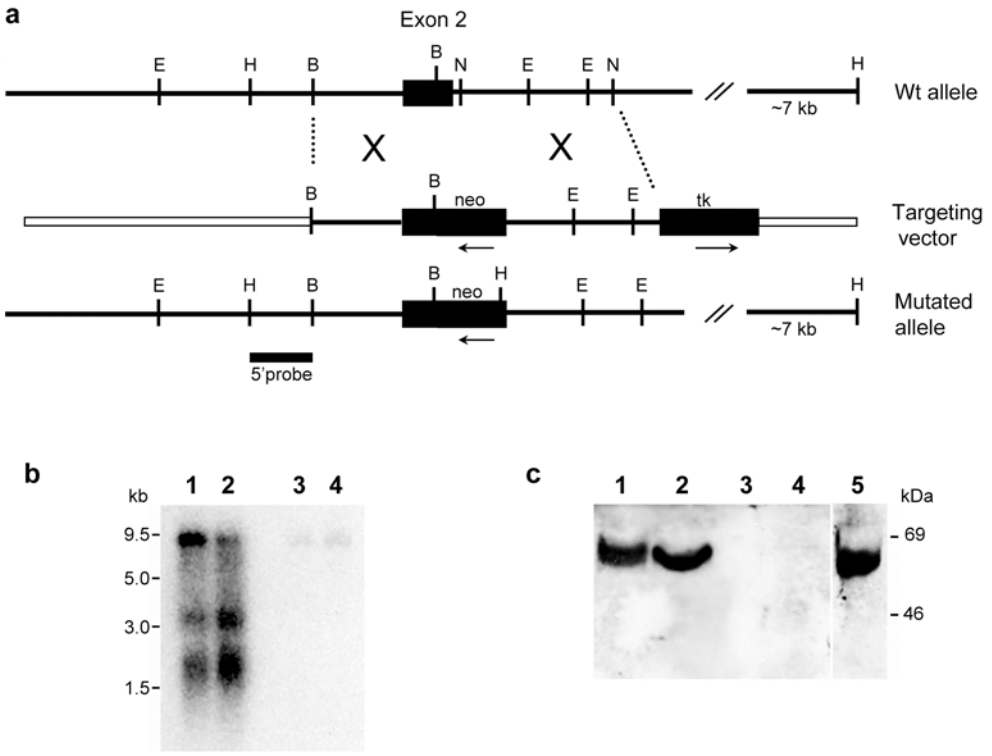

Figure 1. Targeted disruption of the *Lsamp* gene. (a) Restriction map of the *Lsamp*$^{+/+}$ genomic nucleotide sequence surrounding *Lsamp* exon 2 indicates the region of homology selected for construction of the targeting vector. The schematic representation of the mutated allele represents the homologous recombination event that disrupted the *Lsamp* locus. The location of the 5' probe used for the screening of the targeted event is indicated. (b) Northern blot of *Lsamp*$^{+/+}$ (lanes 1,2) and *Lsamp*$^{-/-}$ (lanes 3,4) mRNAs from hippocampus (lanes 1,3) and cerebellum (lanes 2,4). Three bands representing different sized *Lsamp* transcripts are evident in the *Lsamp*$^{+/+}$ samples. Note the absence of message in the samples harvested from null mice. (c) Membrane extracts from cerebellum (lanes 1,3) and hippocampus (lanes 2,4) were analyzed by Western blotting. Samples from *Lsamp*$^{+/+}$ mice (lanes 1,2) exhibit a single band of approximately 64-68kD, whereas samples harvested from *Lsamp*$^{-/-}$ mice (lanes 3,4) do not have this band. As a control, lane 5 depicts LAMP recombinant protein that is the same molecular mass as the native protein. Abbreviations for restriction enzymes: B, *Bam*H I; E, *Eco*R I; H, *Hind* III; N, *Nco* I. Figure credit: Dr. Aurea Pimenta.

GeneBank accession number for the rat *Lsamp* nucleotide sequence is U31554. *LSAMP/Lsamp* are respectively the symbols for the human and rodent gene encoding LAMP, its mRNAs and cDNAs, approved by the human and mouse gene nomenclature committees. LAMP is the designation for the protein (Zacco et al., 1990)."

Unless otherwise indicated, all standard molecular biology techniques were performed as described by Ausubel et. al. (1998) and Sambrook et. al. (1989). All animal experiments were approved by the Vanderbilt University Institutional Animal Care and Use Committee and were conducted following the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Publication No. 80–23, revised 1996).

Northern Blot Analysis

Total cellular RNA was isolated from adult mouse hippocampus and cerebellum using the TRIzol reagent (Invitrogen, Carlshad, CA) following manufacturer's protocol. The poly(A)$^+$ RNA fraction was purified using the Oligotex mRNA isolation system (Qiagen). Poly(A)$^+$ RNA (1 mg) was separated on a 1.5% agarose-formaldehyde gel, transferred to a nylon membrane (Nytran SuperCharge, Schleicher and Schuel, Keene, NH), UV cross-linked, and hybridized overnight under stringent conditions with ^{32}P-labeled cRNA probes. Antisense probes were transcribed in vitro using T7 RNA polymerase from rat *Lsamp* cDNA template (Pimenta et al., 1995) linearized with *Msc*I (nt 464-1238). GeneBank accession number for the rat *Lsamp* nucleotide sequence is U31554.

Western Blot Analysis

Crude membrane preparations of hippocampus and cerebellum from adult mice were solubilized with 4% CHAPS (Zacco et al., 1990; Zhukareva & Levitt, 1995), separated on 10% PAGE using standard methods (Laemmli, 1970) and blotted onto nitrocellulose membranes. LAMP immunoreactivity was detected using a chicken anti-LAMP polyclonal antibody, produced by Ames Laboratory (Tigand, OR) against recombinant protein that was purified to homogeneity in our laboratory. Specificity of the purified IgY fraction was characterized in our laboratory using Western Blot analysis. This polyclonal antibody specifically recognizes recombinant LAMP as well as a single, 64-68kDa band corresponding to native LAMP from crude membrane brain extracts.

Histological Analysis

Standard cresyl violet and Kluver-Barrera stains (Kluver & Barrera, 1953) were used for analysis of general gray matter cytoarchitecture and myelination. A monoclonal antibody (4A11) that recognizes neurofilament-H (NF-H) (Pimenta, Strick, & Levitt, 2001) was used to map the general organization of forebrain fiber tracts. The antibody was used at a 1:100 dilution, followed by a standard HRP/DAB reaction (Pimenta et al., 2001). Acetylcholinesterase histochemistry (Robertson, Mostamand, Kageyama, Gallardo, & Yu, 1991) was used for the assessment of the organization of the septo-hippocampal cholinergic pathway. The fixation, sectioning of tissue and all standard histological procedures and stains, unless otherwise indicated, were performed as described by Hockfield et al. (1993). Complete serial sections from 3-5 animals of each genotype were examined at postnatal day (P) 6, P16 and adults.

Behavior

As noted above, all adult mice used for the behavior studies were backcrossed for more than 10 generations the C57BL/6J background. Mice were housed on ventilated racks in Plexiglas shoebox cages filled with CareFresh shredded paper bedding (Absorbtion Corp., Bellingham, WA). Mice were housed in groups of 2-5 per cage and given access to food (Lab Diet Rodent Chow 5001, PMI Nutrition International, Brentwood, MO) and water *ad libitum*. The colony was temperature (22.22±1°C) and light controlled (12 hour light/dark cycle, lights on at 6 a.m. CST). All animals for these studies were obtained by heterozygous breedings. *Lsamp*$^{-/-}$ mice and their wildtype (*Lsamp*$^{+/+}$) littermates were used for all behavioral tests. Behavioral testing was performed in the Vanderbilt Kennedy Center and Center for Molecular Neuroscience Murine Neurobehavioral Core. Mice were tested between 3-6 months of age and were behaviorally naïve at the time of testing except for those in the 30 minute activity chamber experiment (see section 2.5.3.2). In all adult testing, males and females were tested in separate groups. All testing took place between 12-6 p.m. Housing and testing procedures were approved by the Vanderbilt University Institutional Animal Care and Use Committee. For all behavioral testing, sample sizes for each genotype and sex ranged from 7-16 animals/group. Specific group sizes are noted for each test in the figure legends. Strategies for analytical and statistical procedures were developed with consultants in the Vanderbilt Kennedy Center Statistics and Methodology Core. In all cases where coding was done by hand, coders (blind to genotype) achieved inter-rater reliability of greater than 95%. In all tests where there was a main effect of sex, the sexes

were then split, a new omnibus was performed and subsequent analysis was performed separately.

Postnatal development

The first litters born from *Lsamp* heterozygous breeding pairs were monitored daily in their homecage during the first postnatal week for nesting, feeding and postnatal lethality. Sensorimotor responses and body weight were used to assess the postnatal development of the *Lsamp* mice. Mice were tested and scored at P3-P5, P7, P14 and P21, as described by Fox (1965): righting reflex, postural flexion and extension, limb grasping, negative geotaxis, bar holding, cliff drop-aversion, tail suspension and visual placing.

Acoustic startle and prepulse inhibition

Apparatus. The acoustic startle reflex and prepulse inhibition (PPI) of the acoustic startle reflex were evaluated using four identical, ventilated, sound-attenuated acoustic chambers (51×55×31 cm; Med Associates, St. Albans, VT, USA), each equipped with two speakers, a mouse holder and a transducer system through which startle responses were recorded. Chambers were connected to an amplifier and to a computer equipped with the Startle Reflex software (MED Associates).

Testing Procedures. Group housed mice were handled for the three days preceding testing and were acclimated for one hour in an adjacent room on testing day. PPI was performed following the behavioral core protocol as previously described (Howard et al., 2002). Each mouse was placed in a holder that was then mounted on the response platform. Test sessions were preceded by a 5 minute acclimation period in the startle chambers during which a 65-dB background noise was continuously present,

followed by 54 trials in 9 blocks of 6 trials each. Each six-trial block contained one startle trial (40-ms, 120-dB burst of white noise), one null trial (no stimulus) and four prepulse trials (20-ms bursts of 70-, 76-, 82-, and 88 dB white noise; followed 100 ms later by the startle stimulus). The trials in which no stimulus was presented were used to measure baseline movement in the cylinders. The six different trial types were pseudo-randomly assigned. The inter-trial interval ranged from 10-20 s with an average of 15s. Mice were exposed to the EPM for 5 minutes ten days prior to this test.

Analysis and Statistics. PPI was calculated as the percent reduction in maximal startle on prepulse versus startle only trials. PPI data were analyzed using repeated measures ANOVA.

Activity Chamber

Apparatus. The activity chambers (MED Associates, Georgia, VT) were square arenas (27cm x 27cm x 20cm) with clear Plexiglas walls and white floors. An infrared beam break system positioned 1 cm above the floor on both the x and y axis was used to monitor mouse horizontal movements.

Testing Procedures. Group-housed mice were handled once daily for the three days preceding testing. Mice were transported into the testing room one at a time from an adjacent room, placed in the middle of the novel activity chamber and allowed to explore the chamber for 30 minutes. The chambers were illuminated at 550-650 lux and a white noise generator was placed in the room. Activity chambers were cleaned with water and 70% ethanol between each animal. Male and female mice were run on the y-maze for 8 minutes one week prior to this experiment. Exploration and alternation in the y-maze is dependent on novelty; therefore, y-maze was run prior to measuring activity. However, a

second cohort of behaviorally naïve, unhandled male mice was used to monitor activity over a one-hour time period.

Analysis and Statistics. The beam break data collected using the Med Associates software was used to measure the total distance (cm) traveled per ten-minute block. For the 30-minute trial a repeated measures ANOVA was used with genotype and sex as between subject factors and distance traveled as within subjects factor. For the 1-hour test (males only) data were analyzed using repeated measures ANOVA with genotype as the between subjects factor. If the omnibus test detected a significant effect for genotype, or genotype*behavior, a post-hoc t-test was performed to determine at which time points the differences occurred.

Elevated Plus Maze

Apparatus. The elevated plus maze (EPM) was a plus shaped apparatus consisting of two open arms (platforms with no sides) and two closed arms (platforms with tall walls) connected by a small center square. Both the open and closed arms of the maze were 30 cm long x 5 cm wide with white Plexiglas floors. The closed arms had 15 cm high walls made of black Plexiglas and the open arms were equipped with a 0.25cm high Plexiglas edge on the sides and ends to decrease the chance of mice falling off the maze. The center box was 5 x 5 cm. The maze was built on 40 cm high legs and placed on the floor for testing. Four white screens were placed around the maze in order to reduce spatial cues from the room.

Testing Procedures. In all EPM experiments, animals were brought into the testing room one at a time from a neighboring room, placed in the center of the maze and allowed to freely explore for 5 minutes. Mice were naïve and not handled prior to

exposure to the EPM. A white noise generator was present in the room for all experiments and a camera was placed directly above the maze to record the behavior of each animal. The maze was illuminated at approximately 250 lux. The maze was cleaned with water and 70% ethanol between each animal. Additional cohorts of animals were run under dimmer lighting conditions (~ 60lux) and after being handled, but there was no statistical effect of these environmental manipulations on any of the standard EPM measures.

Analysis and Statistics. The number of entries into the open and closed arms, and duration of time spent in open arms, closed arms and center of the maze were measured for each animal. Entries and exits from maze arms were defined as all four paws crossing into or out of the arm. Additionally, as an indication of risk assessment, we measured both unprotected and protected head-dipping. Unprotected head-dips were defined as the head, neck and shoulders of the mouse crossing off the edge of an open arm while all four paws were in an open arm. Protected head-dips were defined as the head, neck and shoulders of the mouse crossing off the edge of an open arm while at least one paw was in either the center or closed arms of the maze. Entries and durations measurements were automated using the MazeScan suite of TopScan video analysis software (CleverSys Inc., Reston VA). TopScan measurements were validated by comparison to hand scoring by a trained, observer blind to genotype. The hand coding was performed from video using ProcoderDV (Vanderbilt University, Nashville TN) and correlations between TopScan and hand coding were greater than 0.90 for all measurements. A trained observer blind to genotype manually coded head-dips. Each of the three specific behavior categories (entries, duration and head-dips) was analyzed separately. An omnibus repeated measure

ANOVA was performed for each with genotype and sex as between subject factors and area of the maze (i.e. open/closed arm, protected/unprotected area) as the within subject factors. To control for analyzing data from multiple EPM measures, a Bonferroni-corrected alpha (0.0167) was used. Significant genotype or genotype*behavior effects were followed by post-hoc t-tests.

Y-maze

Apparatus. The y-maze was a y-shaped apparatus in which the three arms were of equal length. The three enclosed arms were made of clear circular Plexiglas and the bottom of the Plexiglas tube was removed so that the maze sat flat on a grey rubber surface. Each arm was 30.5cm long x 4.8 cm wide x 4.3 cm tall. The end of one arm was removable for placement of mice in the maze. The arms of the maze joined in the center with each arm at a 120° angle from the next. Spatial cues were available to the mice during testing (e.g. walls, door to room, shelving in room).

Testing Procedures. Male and female mice were naive prior to the y-maze test. All mice were handled for three days prior to testing. Mice were transported into the room one at a time from a neighboring room and placed in the end of one arm of the y-maze. They were allowed to freely explore the chamber for 8 minutes. A camera was placed directly above the maze to record the animal's behavior. The room was illuminated at approximately 200 lux. The maze was cleaned with water and 70% ethanol between each animal.

Analysis and Statistics. A trained observer blind to genotype scored the number and sequence of arm entries in the y-maze. An arm entry was defined as all four paws crossing into an arm. The number of spontaneous alternations, same arm returns and

Table 1: *Lsamp* $^{-/-}$ mice are fertile and show normal growth.

Measures	***Lsamp*** $^{-/-}$	***Lsamp*** $^{+/+}$
Body weight, P6	3.5 +/- 0.6 (n=13)	3.6 +/- 0.4 (n=10)
Body weight, P7	4.0 +/- 0.5 (n=36)	4.1 +/- 0.7 (n=53)
Body weight, P14	7.4 +/- 1.0 (n=19)	7.2 +/- 0.6 (n=20)
Body weight, P21	10.0 +/- 1.4 (n=6)	10.4 +/- 1.2 (n=12)
Body weight, Adult males	29.3 +/- 2.0 (n=19)	28.4 +/- 2.0 (n=17)
Body weight, Adult females	22.1 +/- 1.7 (n=50)	22.5 +/- 1.9 (n=52)
Brain weight, Adult male	0.48 +/- 0.01 (n=20)	0.48 +/- 0.02 (n=20)
Fertility	Male and Female mice are fertile	
Nesting and feeding (P0-P7)	All mice in nest; milk plaque present	
Lethality	No neonatal lethality associated with genotype	

Body and brain weight values in grams

alternate arm returns were then determined. Spontaneous alternations were defined as entries into each of the three arms in a sequential manner. Same arm returns (SAR) were defined as re-entering the same arm that was just visited after all four paws left the arm and before any other arm was entered. Alternate arm returns (AAR) were defined as entry into two of three arms in three sequential entries where the same arm was entered at the beginning and end of the triplet (e.g. arm A,B,A). Percent spontaneous alternation for each animal was calculated as the ratio between the number of actual spontaneous alternations (#SA) and the total number of possible spontaneous alternations (total entries – 2) multiplied by 100: (#SA/(total entries-2))*100. Alternate arm returns and same arm returns were calculated as a ratio of returns to total number of entries multiplied by 100 (e.g. (AAR/total entries)*100). A MANOVA was calculated using four measures (total entries, %SA, %AAR and %SAR) including sex and genotype as factors.

Results

Initial characterization of *Lsamp*$^{-/-}$ mice

The targeting event resulted in disruption of the *Lsamp* locus (Figure 1a). As a consequence of this genetic manipulation, the mutant mice lack both *Lsamp* transcripts and LAMP protein as determined by Northern and Western Blot analysis, respectively (Figure 1b and c). Analysis of over 250 litters, congenic into C57BL/6J strain, revealed an expected Mendelian ratio of +/+, +/- and -/- genotypes, reflecting normal viability *in utero* and postnatally. *Lsamp* $^{-/-}$ mice were normal in appearance, size, growth and development. There were no differences in monitored weight gain and brain weight was

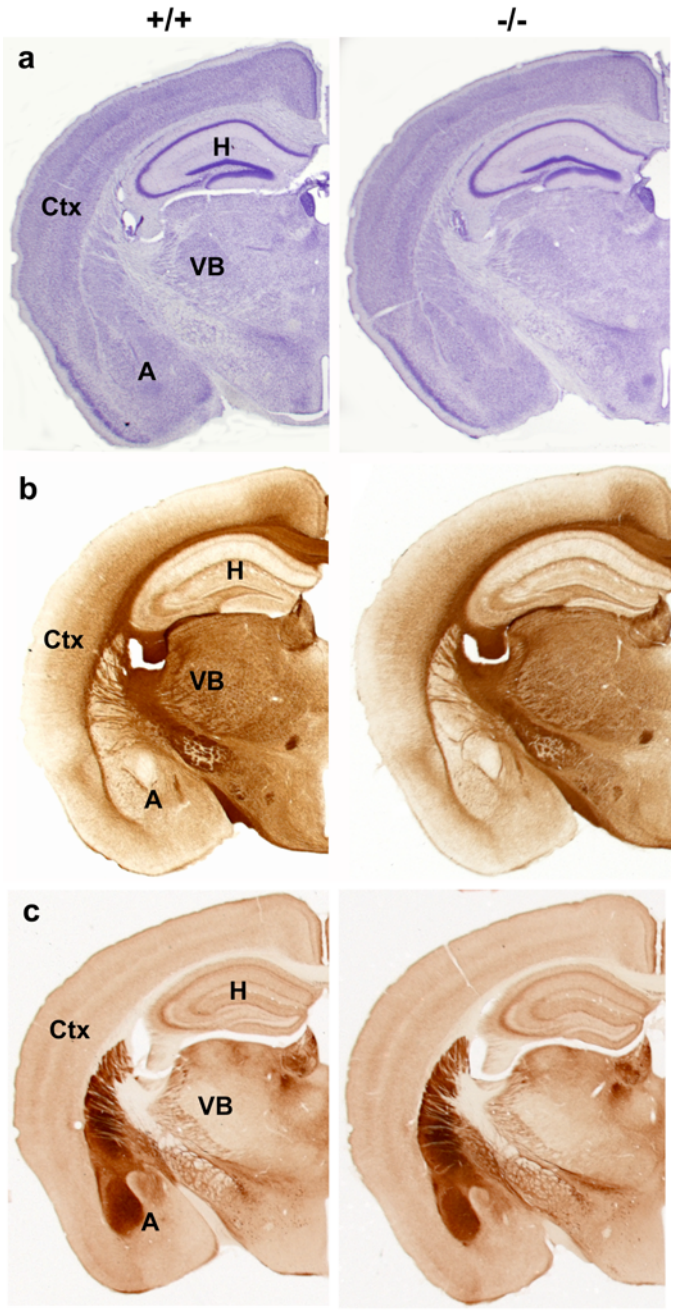

Figure 2. Histological analysis of adult *Lsamp*$^{-/-}$ mice. Coronal section images taken at the level of rostral hippocampus of *Lsamp*$^{+/+}$ and *Lsamp*$^{-/-}$ littermates. Note the normal cytoarchitecture of the cerebral cortex (ctx), hippocampus (H), amygdala (A), thalamic ventral basal complex (VB) and hypothalamus viewed by cresyl violet staining (a). Major fiber tracts, viewed by immunostaining with a neurofilament-H antibody (b), also appear normal in *Lsamp*$^{-/-}$ mice compared to their *Lsamp*$^{+/+}$ counterparts. Limbic structures and fiber tracts show normal distribution of AChE histochemistry in both genotypes (c). N=5/genotype for each histological staining. Scale bar =1mm. Figure credit: Dr. Aurea Pimenta.

identical for *Lsamp* $^{-/-}$ and *Lsamp* $^{+/+}$ male littermates (Table 1). The gross anatomy of the brain was normal. Thus, white matter staining, general gray matter cytoarchitecture and the appearance of forebrain cortical and subcortical structures did not differ across genotypes. For this initial screening, several immuno- and histochemistry stains were used. Normal cytoarchitecture and fiber tracts are illustrated in Figure 2. Cortical lamination patterns and amygdala and thalamic nuclei all appear intact and well-delineated. In addition, commissural pathways throughout the forebrain were intact. AChE histochemistry indicated a normal patterning of the septo-hippocampal cholinergic pathway, differing from experimental studies in vitro (Keller et al., 1989; Zhukareva & Levitt, 1995). Normal AChE reactivity (Figure 2c) is depicted in the hippocampus, amygdala, caudal striatum, limbic thalamic nuclei and lateral hypothalamus. Strong reactivity identifies cholinergic fibers in the internal capsula and the mammillothalamic tract in both genotypes. Thus, there currently is no indication from basic neuroanatomical examination of major alterations in brain organization and gross connectivity in the *Lsamp*$^{-/-}$ mouse.

Behavioral Analysis

Lsamp$^{-/-}$ mice were indistinguishable from littermates from the day of birth. The overall motor and sensory development of *Lsamp*$^{-/-}$ mice was evaluated from P3-P21 using a selected battery of tests (Fox, 1965). In all sensorimotor tests used, *Lsamp*$^{-/-}$ mice acquired and performed mature responses at the same rate and ability as did their wildtype littermates (Table 2), demonstrating normal reflex maturation and normal gross sensory and motor abilities.

Table 2: Sensory and motor development of *Lsamp*$^{-/-}$ mice.

Measures of development	Mature Responses in *Lsamp* $^{-/-}$ mice				
	P5	P7	P14	P21	Adult
Righting	85	100			
Postural flexion/extension	normal	normal			
Forelimb grasping		100	100		
Hindlimb grasping			100		
Inverted screen holding			100	100	
Negative geotropism		100	100		
Bar holding			100	100	
Cliff drop aversion			100	100	
Tail suspension			100	100	
Visual Placing				100	
Eyelid opening			open		
Startle					normal
Pre-pulse inhibition					normal

Acquisition of mature responses in *Lsamp* $^{-/-}$ are expressed as percentage of animals expressing a mature response (Score = 9).

Acoustic startle response and prepulse inhibition of acoustic startle

The acoustic startle response and sensory motor gating were evaluated in adult mice. There was no difference in the amplitude of the startle responses between *Lsamp* $^{-/-}$ and *Lsamp* $^{+/+}$ mice, and no deficits in PPI (Figure 3). In addition, no differences in baseline levels of movement were found between genotypes in the "no stimulus" trials (Figure 3a). There was a significant decrease in startle with increasing prepulse stimuli in both genotypes ($F_{(3,78)} = 33.38$, $p < 0.0001$), demonstrating the effectiveness of the PPI protocol (Figure 3b). These data are consistent with intact circuitry involved in the acoustic startle reflex and in those circuits that modulate PPI in the *Lsamp* $^{-/-}$ mice.

Activity Chamber

Lsamp$^{-/-}$ mice displayed hyperactivity during exposure to a novel open arena. Activity data during the 30-minute test for females and males are displayed in Figure 4. The initial repeated measures ANOVA demonstrated main effects of sex ($F_{(1,54)} = 5.47$, $p = 0.023$), genotype ($F_{(1,54)} = 18.58$, $p < 0.0001$), and distance traveled over time ($F_{(2,54)} = 129.21$, $p < 0.0001$). There also was a significant interaction of time X genotype ($F_{(2,108)} = 11.172$, $p < 0.0001$). Because there was an effect of sex, male and female data were split for the subsequent analyses. There was a main effect of genotype on distance traveled over time for both females ($F_{(1,28)} = 4.84$, $p = 0.0002$) and males ($F_{(1,26)} = 14.73$, $p = 0.0007$), and an interaction of genotype X distance traveled ($F_{(2,56)} = 10.33$, $p = 0.0001$ and $F_{(2,52)} = 4.10$, $p = 0.022$, respectively). Both female and male *Lsamp*$^{-/-}$ mice traveled a significantly greater distance than did their *Lsamp* $^{+/+}$ littermates during the first ten minutes of the test ($t(28) = 3.71$, $p = 0.0009$ and $t(26) = 3.86$, $p = 0.0007$). During the

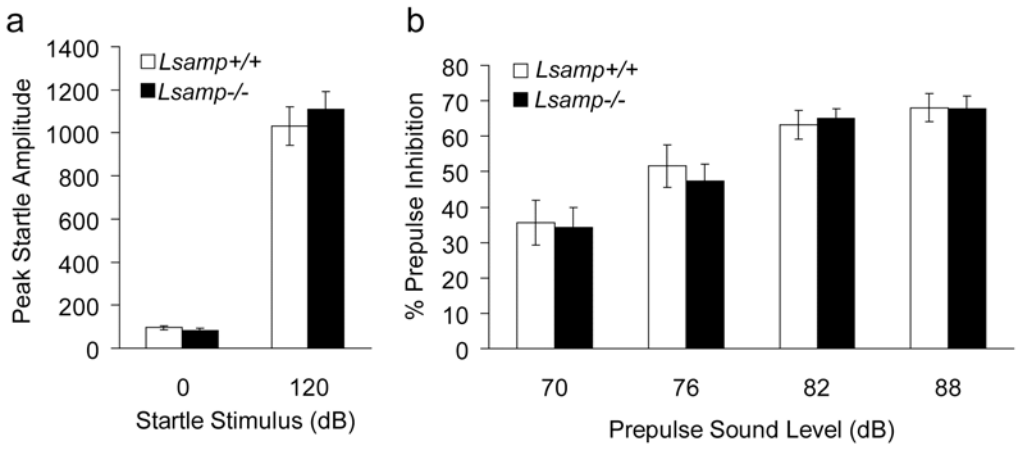

Figure 3. Normal acoustic startle response and sensorimotor gating in *Lsamp*$^{-/-}$ mice. Startle amplitude (a) and prepulse inhibiton of acoustic startle responses over varying prepulse intensities (b) are shown for *Lsamp*$^{-/-}$ mice (closed bars) and wildtype littermates (open bars) represented as mean±SE (n=14/group). Figure credit: Dr. Aurea Pimenta.

second and third ten-minute blocks of the test, female *Lsamp* $^{-/-}$ mice habituated to normal activity levels and did not significantly differ from *Lsamp* $^{+/+}$ during either time block (Figure 4a). However, male *Lsamp* $^{-/-}$ mice continued to display hyperactivity throughout the remainder of the 30-minute test (Figure 4b).

The highest levels of hyperactivity occurred during the first 10 minutes in both female and male *Lsamp* $^{-/-}$ mice. Female *Lsamp* $^{-/-}$ habituated to their environment during the 30-minute testing period. We thus hypothesized that the male *Lsamp* $^{-/-}$ mice may also habituate to normal activity levels if provided with an extended testing period. Therefore, we repeated the experiment with a new group of males but extended the length of time in the chamber to one hour (Figure 4c). Again, there were main effects of genotype ($F_{(1,13)}$ = 7.04, p = 0.0199), distance traveled over time ($F_{(5,13)}$ = 42.39, p < 0.0001) and an interaction between the two ($F_{(5,65)}$ = 4.37, p = 0.0017). In the new group of mice, the period of greatest hyperactivity also occurred during the first 10 minutes of exposure to the novel chamber ($t(13)$ = 3.56, p = 0.0035) and the male *Lsamp* $^{-/-}$ mice still displayed hyperactivity 30 minutes into the test (Figure 4c). During the ten minute bins of the final 30 minutes of the test, however, *Lsamp* $^{-/-}$ mice did not travel a greater distance than did their *Lsamp* $^{+/+}$ littermates, indicating that like the females, the male *Lsamp* $^{-/-}$ mice eventually habituated to the novel environment.

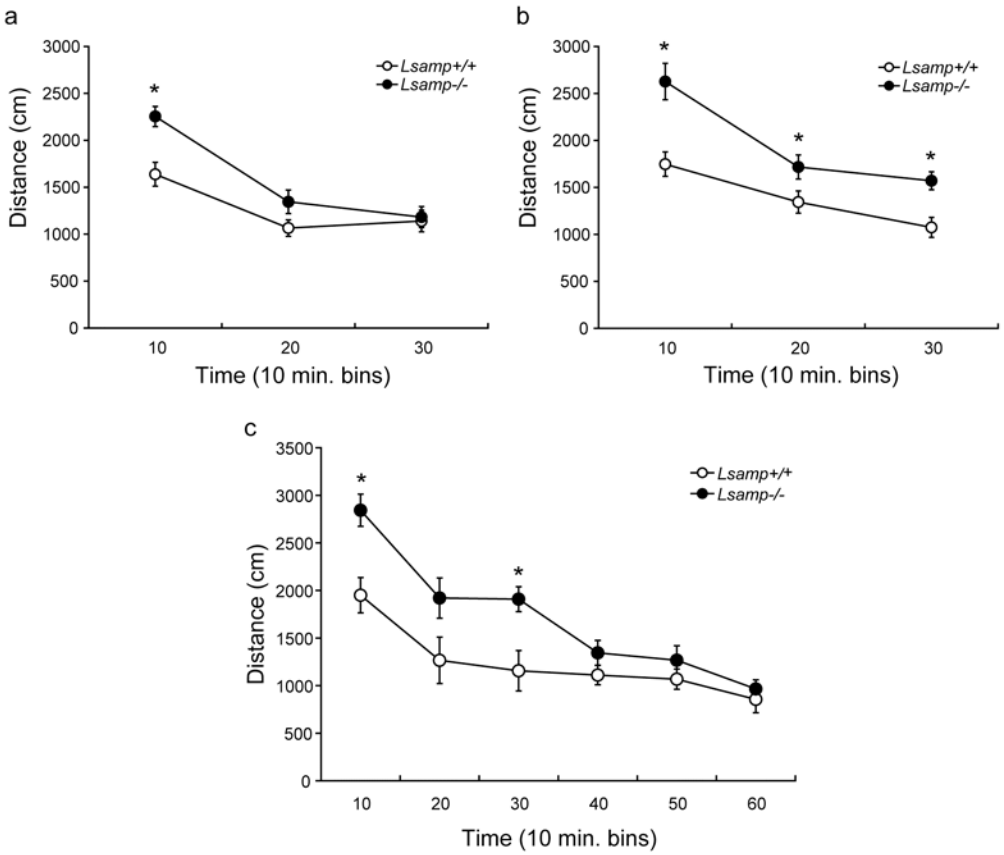

Figure 4. Open field activity of *Lsamp*$^{-/-}$ mice. The distance traveled, measured in centimeters (cm), by *Lsamp*$^{-/-}$ mice and their *Lsamp*$^{+/+}$ littermates is displayed in 10 minute bins (a&b). Both female (a) and male (b) *Lsamp*$^{-/-}$ mice exhibit hyperactivity when exposed to the novel arena. Female *Lsamp*$^{-/-}$ mice habituate to normal levels of activity within 20 minutes of being placed in the arena, but male *Lsamp*$^{-/-}$ mice remain hyperactive for the duration of the 30 minute test. (n=13-15/group). Examination of activity in the open field for a 60 minute period (c) revealed that the null mice return to normal levels of activity by the second half of the test period. The increase in distance traveled by male *Lsamp*$^{-/-}$ mice during the second 10 min bin (20) was not significant at p = 0.06 (n=7 WT, n=8 *Lsamp*$^{-/-}$).*$p<0.05$

Elevated Plus Maze

There was no main effect of sex on either of the traditional measures used for EPM (entries and durations); therefore the sexes were combined for the remainder of the analyses. The results of the EPM are presented in Figures 5 (entries, duration) and 6 (head-dips). There was a main effect of genotype ($F_{(1,28)} = 39.44$, $p < 0.0001$) on the number of entries and an interaction between category of entry and genotype ($F_{(1,28)} = 22.37$, $p < 0.0001$). *Lsamp*$^{-/-}$ mice demonstrated an increased number of entries into both the open ($t(30) = 6.59$, $p < 0.0001$) and closed ($t(30) = 2.27$, $p < 0.031$) arms of the maze (Figure 5a). The *Lsamp* $^{+/+}$ mice made more than double the number of closed entries as open entries (means±SE of 11±0.8 vs. 5±0.6), whereas *Lsamp* $^{-/-}$ mice made a similar number of entries into the closed and open arms (14±0.72 and 16±1.5). There was no main effect of genotype on durations; however, there was an interaction between genotype and category of duration ($F_{(2,56)} = 67.20$, $p < 0.0001$). This interaction occurred because *Lsamp* $^{-/-}$ mice spent more time in the open arms ($t(30) = 3.88$, $p = 0.0005$), with a corresponding decrease in center time ($t(30) = 2.78$, $p = 0.0093$), but no significant difference in time spent in the closed arms of the maze (Figure 5b).

Risk-assessment behavior on the EPM also was monitored by examining head-dips. There were main effects of sex ($F_{(1,28)} = 11.31$, $p = 0.0022$), genotype ($F_{(1,28)} = 25.28$, $p < 0.0001$) and area of the maze ($F_{(1,28)} = 75.38$, $p < 0.0001$) on the number of head-dips. The omnibus test also detected interactions between genotype and area of the maze ($F_{(1,28)} = 35.82$, $p < 0.0001$), and sex and area of the maze ($F_{(1,28)} = 6.73$, $p < 0.0149$). Since there was a main effect for sex, we split males and females for subsequent analyses on the contribution of genotype to altered head-dipping behavior. Omnibus

testing of each sex separately still detected an effect of genotype and an interaction between genotype and area of the maze in which head-dips occurred. Both female and male *Lsamp* $^{-/-}$ mice demonstrated a large increase in the number of unprotected head-dips ($t(15) = 3.02$, $p = 0.0086$ and $t(13) = 5.70$, $p < 0.0001$), while maintaining a normal number of protected head-dips as compared to *Lsamp* $^{+/+}$ littermates (Figure 6). Because the EPM was the only test in which animals were not handled before being exposed to the apparatus, we repeated the test with both handled male and female mice under slightly different environmental conditions (new room and reduced light levels). We combined data and analyzed across groups (taking into account sex, handling, and testing environment) and genotype. There was no effect of testing procedures on either entries or duration and the effect of genotype remains highly significant for both measures ($F_{(1,28)} = 6.73$, $p < 0.0001$ and $F_{(1,28)} = 6.73$, $p < 0.0001$). Thus, regardless of environmental manipulations, *Lsamp*$^{-/-}$ mice displayed hyperactivity, increases in open arm time, increases in open arm entries and increases in unprotected head-dips.

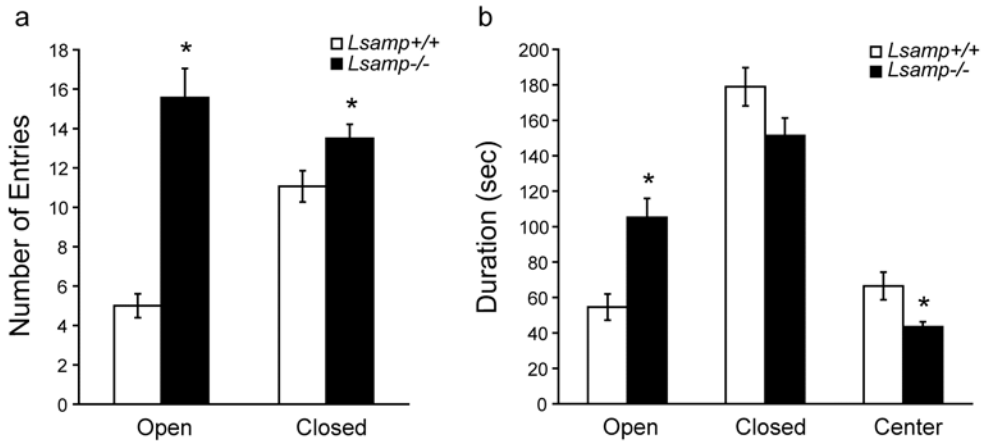

Figure 5. *Lsamp*$^{-/-}$ mice behavior on the EPM. *Lsamp*$^{-/-}$ mice make a significantly greater number of entries into both the open and closed arms of the EPM (a), indicating hyperactivity. *Lsamp*$^{-/-}$ mice also reside for a longer duration (sec) in the open arms of the maze, with a corresponding decrease in the amount of time spent in the center (b). Because there was no effect of sex on traditional EPM measures, male and female mice were combined in these analyses. (n=16/group)*$p<0.01$

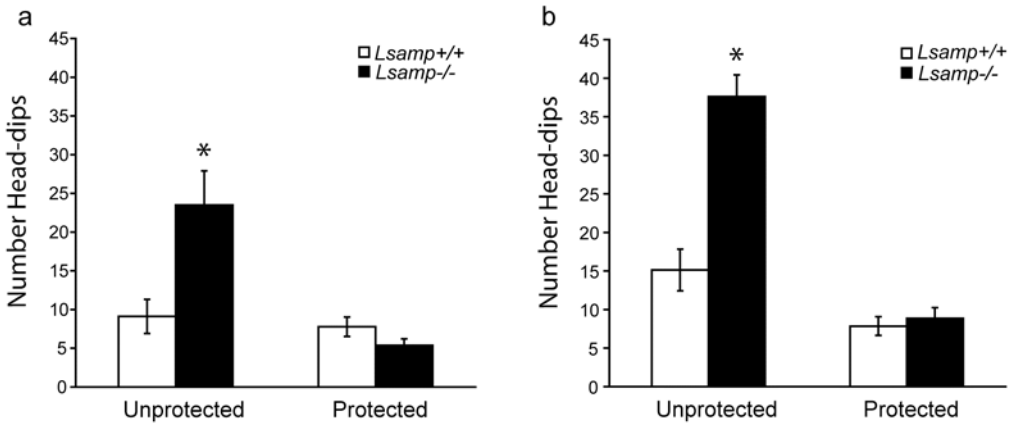

Figure 6. Exploratory behavior of *Lsamp*$^{-/-}$ mice on the EPM. Measures of head-dips were quantified as a measure of risk assessment. Both female (a) and male (b) *Lsamp*$^{-/-}$ mice make more than double the number of unprotected head-dips, compared to their *Lsamp*$^{+/+}$ littermates. There is no difference in the number of protected head-dips in null mice of either sex. (n=7-9/group)*$p<0.01$

Y-maze

Y-maze data are presented in Figure 7. There was no main effect of sex on the y-maze measurements, so data were combined across sex for the analyses. There was a main effect of genotype on y-maze measurements ($F_{(4,49)} = 17.70$, $p < 0.0001$). As expected, *Lsamp* $^{-/-}$ mice were hyperactive during the 8-minute exposure to the novel y-maze, as measured by a significant increase ($F_{(1,52)} = 53.43$, $p < 0.0001$) in the total number of arm entries (Figure 7a). *Lsamp* $^{-/-}$ mice also demonstrated a small, but significant decrease ($F_{(1,52)} = 7.27$, $p = 0.011$) in the percent spontaneous alternation (Figure 7b). The deficit in spontaneous alternation was accounted for by an increase ($F_{(1,52)} = 17.44$, $p = 0.0001$) in alternate arm returns (Figure 7c). There is no change in the proportion of same arm returns (data not shown).

Discussion

We found no evidence of gross changes in brain organization or connectivity in *Lsamp* $^{-/-}$ mice. This differs from previously reported findings using in vitro experimental paradigms in which LAMP mediated axon targeting and growth (Keller et al., 1989; Zhukareva & Levitt, 1995). The current analyses are more consistent with a role for LAMP mediating finely specialized aspects of circuit formation and maturation in regions of the limbic system. LAMP, a cell-surface molecule, is not a receptor for any known neurotransmitter or neuromodulator, consistent with the hypothesis that altered expression of the protein is likely to lead to differences in connectivity rather than direct

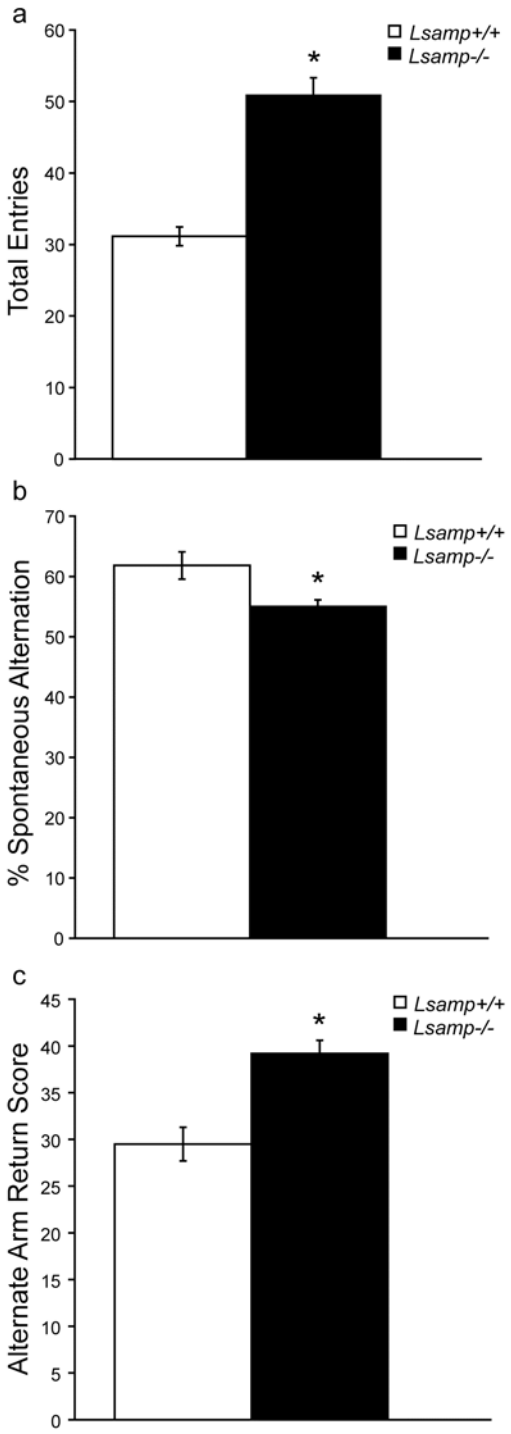

Figure 7. Spontaneous alternation by *Lsamp*$^{-/-}$ mice in the Y-Maze. The *Lsamp*$^{-/-}$ mice exhibit hyperactivity in the y-maze (a). There was a modest, but statistically significant decrease in spontaneous alternation in null mice (b), and an increase in alternate arm returns (c). (n=27-29/group)*$p<0.05$

changes in traditional neurotransmitter signaling. There are wide-ranging developmental effects of deletion of axon guidance and cell adhesion molecules, resulting in very subtle to gross changes (Barallobre et al., 2000; Sahay, Molliver, Ginty, & Kolodkin, 2003; Wiencken-Barger, Mavity-Hudson, Bartsch, Schachner, & Casagrande, 2004). Cell adhesion molecules such as L1, NCAM, neurexins/neuroligins, and ephBs/ephrins are involved in the regulation of synapse formation and stability (Dalva, McClelland, & Kayser, 2007). Deletion of these genes in model systems tends to result in more subtle defects that are consistent with most neuropathology found in psychiatric disorders, in which only modest changes at the cellular level (e.g. spine density, neuropil size, synaptic density) have been discerned (Lewis & Levitt, 2002). Thus, it is possible that for brain regions in which LAMP is expressed, neuronal signaling is only subtly disturbed, but may lead to measurable changes in functional output of the circuits that are disrupted. Accordingly, the initial analysis of the *Lsamp*$^{-/-}$ mice demonstrates alterations of certain behaviors that relate to emotional reactivity in novel situations, without disruption of the development or maintenance of basic sensory and motor behaviors. This is reflected both by the normal developmental timing of sensorimotor responses and normal adult auditory startle and sensorimotor gating as measured by pre-pulse inhibition.

Select Changes in Behavior in *Lsamp*$^{-/-}$ Mice

The behavior of mice in a novel environment reflects a balance between the desire to explore (motor and behavioral activation) and fear (motor and behavioral inhibition) (Crawley, 1985). In three of the behavioral tasks that we used, both male and female *Lsamp*$^{-/-}$ mice demonstrated heightened behavioral activation as measured by their

activity in a novel apparatus (distance in the activity chamber, entries on the EPM and entries in the y-maze). *Lsamp*$^{-/-}$ mice displayed hyperactivity when first placed in an open field activity chamber, but habituated to the same baseline activity levels as their *Lsamp*$^{+/+}$ littermates over time. This is consistent with our hypothesis that the *Lsamp*$^{-/-}$ mice are not generally hyperactive, but rather are hyper-responsive to different novel environments. Increased locomotor activity as a response to a novel, stressful environment could be interpreted as increased behavioral activation, exploratory drive or as an inability to properly inhibit behavior in a threatening situation (Crawley et al., 1997). Although these domains are likely to be linked both behaviorally and neurobiologically, our data suggest that in the absence of *Lsamp*, mice are at least exhibiting heightened behavioral activation. During the first 10 minutes of the test, mice of both genotypes demonstrate increased activity above baseline, reflecting genotype-independent increased exploratory behavior during this time period. Male and female *Lsamp*$^{-/-}$ mice, however, have a heightened level of activity compared to their *Lsamp*$^{+/+}$ littermates even during this portion of the test, indicating an increase in behavioral activation.

Increased activity also was evident in the EPM test. *Lsamp*$^{-/-}$ mice made a greater number of total arm entries, with a small increase in closed entries, exhibiting hyperactivity in the 5-minute exposure to a novel environment. Mice generally show a strong preference for the closed arms as measured by entries (Hogg, 1996), which was evident in the analyses of the *Lsamp*$^{+/+}$ mice. However, *Lsamp*$^{-/-}$ mice showed no preference in entries for either open or closed arms. One reasonable interpretation of the greater proportion of open entries, even in the context of the overall increase in entries, is

a reduction in anxious behavior (Pellow & File, 1986). Alternatively, if the *Lsamp*$^{-/-}$ mice have an increased arousal state in response to stress, a lack of preference for either arm may reflect reduced behavioral inhibition and/or increased activation. *Lsamp*$^{-/-}$ mice also spend significantly more time in the open arms, which also is generally viewed as reflecting reduced anxiety. This conclusion follows from an ethological interpretation of the test and from the results of pharmacological manipulations, in which mice receiving anxiolytic drugs increase open arm time with a corresponding decrease in closed arm time, whereas anxiogenic drugs induce the opposite behaviors (Pellow & File, 1986). We propose an alternative hypothesis, one in which interpreting the EPM data as reflecting an altered anxiety state in the *Lsamp*$^{-/-}$ mice may not be accurate. For example, the increase in open arm time is accounted for by a significant decrease in time spent in the center of the maze. When *Lsamp*$^{+/+}$ mice do spend time in the center, they generally inhibit motor behavior, remaining still, and appear to be "sizing up" the open arms prior to deciding which area to enter. In contrast, the *Lsamp*$^{-/-}$ mice do not inhibit their movement and, therefore, enter the open arms of the maze much more frequently than the *Lsamp*$^{+/+}$ mice. There is precedence for this view, as the Ts65Dn mutant mouse also demonstrates greatly increased motor output with corresponding anxiolytic-like EPM results. Because of the hyperactivity, however, these changes have been interpreted not as anxiety, but as a lack of behavioral inhibition or reduced attention to environmental stimuli (Coussons-Read & Crnic, 1996; Martinez-Cue, Rueda, Garcia, & Florez, 2006). While recognizing that a simple explanation would involve primary disruption of the anxiety state, we suggest that *Lsamp*$^{-/-}$ mice exhibit heightened reactivity to stressful stimuli, revealed by hyper-activation and lack of appropriate behavioral inhibition.

Consistent with this view, heightened reactivity in the *Lsamp* $^{-/-}$ mice is evident in other behaviors, such as unprotected head-dips. Although there is a sex difference in degree of increase of unprotected head-dips, both male and female *Lsamp* $^{-/-}$ mice demonstrate increased risk assessment. Based on reports in the literature, the co-segregation of increased unprotected head-dips and open arm exploration in the *Lsamp* $^{-/-}$ mice is unusual. For example, factor analyses of mouse and rat behavior in the EPM revealed that increased head-dips load on the same factor as the traditional measures of anxiety, but is inversely correlated with open time and entries (Cruz et al., 1994; Rodgers & Johnson, 1995). In one study (Rodgers & Johnson, 1995), when protected and unprotected head-dips were analyzed separately, protected head-dips loaded on the anxiety factor, while unprotected head-dips did not fall into the categories discovered. *Lsamp* $^{-/-}$ mice do not show reduced head-dips in the protected portion of the maze, which would be associated with decreased anxiety. Instead, the increase in head-dips on the open arms of the EPM may be due to a combination of the increased time spent in the open arms of the maze and increased behavioral activation in open, more anxiety provoking areas of the maze. This interpretation again is consistent with the hypothesis that *Lsamp* $^{-/-}$ mice may experience behavioral hyper-activation or disinhibition in stressful environments. To gain further understanding and clarification of the underlying neurodevelopmental and molecular basis for these behavioral changes our laboratory is examining the integrity of the neural regulatory systems in which LAMP is expressed.

Spontaneous alternation in the y-maze takes advantage of the exploratory drive of rodents (Lalonde, 2002), in which animals typically investigate the newest area in an environment. The interpretation of deficits in alternation behavior can be complex

because quantitative differences in the pattern of exploration have been interpreted in other studies as a reflection of decreased attention, deficits in short-term memory, changes in arousal or anxiety (Hughes, 2004; Lalonde, 2002). Along with their characteristic hyperactivity, *Lsamp* $^{-/-}$ mice exhibit a significant, though very modest decreased level of spontaneous alternation during the period when novelty-induced hyperactivity peaks. It is unlikely that this indicates altered anxiety in the traditional sense, because such measures of anxiety are decreased in the *Lsamp* $^{-/-}$ mice. Instead, we suggest that the altered performance on the maze may be due to a disrupted state of arousal or deficits in working memory. Consistent with the interpretation of altered arousal is the finding that *Lsamp* $^{-/-}$ mice displayed heightened reactivity in other novel environments. However, in order to definitively address the underlying cause of spontaneous alternation deficits, including the possibility of deficits in working memory, further detailed testing will be required.

Taken together, our data suggest that there is a complex behavioral deficit caused by the targeted deletion of the *Lsamp* gene. Rather than a primary defect in the regulation of anxiety state, we hypothesize that the mutation results in heightened and possibly maladaptive response to novel environmental stressors. This interpretation of the animal model experiments is consistent with the recent human genetic studies in which a polymorphism in the human *Lsamp* gene is associated with panic disorder in certain environments (Koido et al., 2006; Maron et al., 2006). *Lsamp* is expressed robustly throughout the limbic circuitry responsible for mediating an animal's behavioral response to novelty (including circuitry that mediates fear, stress and exploratory behavior). Alterations in emotional regulation can be due both to direct changes in neurotransmitter

function (Hariri & Holmes, 2006; Howell & Muglia, 2006; Southwick, Vythilingam, & Charney, 2005; Wood & Toth, 2001) and to alterations of synaptic connectivity (Sandi & Bisaz, 2007; Wood & Toth, 2001). Because *Lsamp* is expressed from the time that neurons become postmitotic prenatally and throughout the life of the animal, conditional and reversible manipulation of gene expression will be necessary to determine whether the behavioral dysfunction exhibited by the *Lsamp* $^{-/-}$ mice are due to differential development of limbic circuitry, direct modulation of mature synaptic function in the adult, or even both.

CHAPTER III

GENETIC DELETION OF LAMP CAUSES HEIGHTENED RESPONSIVENESS OF THE HPA AXIS IN NOVEL ENVIROMENTS

Introduction

An animal's ability to respond to stress appropriately, both physiologically and behaviorally, is critical to its success and survival. A major component of the stress response, the hypothalamic-pituitary-adrenal (HPA) axis is responsible for the neuroendocrine response to stress, in which corticosterone (CORT) is ultimately released into the bloodstream to modulate metabolism, blood flow and brain function (Armario, 2006; Miller & O'Callaghan, 2002; Sapolsky, Romero, & Munck, 2000). Several limbic brain structures and related circuits modulate the likelihood of the para-ventricular nucleus (PVN) of the hypothalamus to release corticotropin releasing factor (CRF), the first step in the cascade (Herman et al., 2005).

It is well established that CORT can effect the expression and function of cell adhesion molecules (CAMs), such as L1 and NCAM (Grootendorst, Oitzl et al., 2001; Sandi & Loscertales, 1999; Venero et al., 2002). Furthermore, alterations in HPA-axis reactivity of the NCAM knockout mouse has provided evidence that CAMs can play an important role in stress regulation (Stork et al., 1997). The limbic system associated membrane protein (protein: LAMP, gene: *Lsamp*) is another CAM that is expressed embryonically and throughout adulthood in cortical and subcortical limbic structures and circuitry (Cote et al., 1995, 1996; Horton & Levitt, 1988; Levitt, 1984; Pimenta, Reinoso et al., 1996; Reinoso et al., 1996; Zacco et al., 1990). It is well established the alterations

during the period when limbic circuits are developing can cause long-term alterations in anxiety-like behaviors and HPA axis reactivity (Card, Levitt, Gluhovsky, & Rinaman, 2005; Meaney, Aitken, Bodnoff, Iny, & Sapolsky, 1985; Meaney et al., 1993). Both its distribution and its role in axon guidance and neurite outgrowth in the developing brain (Eagleson et al., 2003; Keller et al., 1989; Mann et al., 1998; Pimenta et al., 1995; V. V. Zhukareva et al., 1997) provide LAMP the potential to alter the circuitry that underlies the stress response.

We have previously reported that *Lsamp*$^{-/-}$ mice display exaggerated behavioral activation in novel environments, including hyperactivity and increased exploratory behaviors (Catania, Pimenta, & Levitt, 2008). We hypothesized that these behaviors are caused by a heightened, and possibly maladaptive, response to environmental stressors. Here, we investigate the stress responsiveness of *Lsamp*$^{-/-}$ mice by examining their HPA axis response and brain activity when they are exposed to novel environment.

Methods

Animals

Fully backcrossed male, adult (3-6 months) *Lsamp*$^{-/-}$ C57BL/6J mice and their wildtype littermates were used for all experiments (except one EPM experiment in which females were used. *Lsamp*$^{-/-}$ mice were generated by homologous recombination as described in chapter 2, page 30 (Catania et al., 2008). All animals were obtained by heterozygous breedings. Mice were genotyped by polymerase chain reaction (PCR)

amplification as described in Chapter 2 (p. 30). By conformity, the protein is abbreviated LAMP and the gene name, as archived in Genbank, is noted as *Lsamp*. Mice were group housed on ventilated racks in Plexiglas shoebox cages filled with CareFresh shredded paper bedding (Absorption Corp., Bellingham, WA) and given access to food (Lab Diet Rodent Chow 5001, PMI Nutrition International, Brentwood, MO) and water *ad libitum*. The colony was temperature (22±1°C) and light controlled (12 hour light/dark cycle, lights on at 6 a.m. CST). The Vanderbilt University Institutional Animal Care and Use Committee approved housing and testing procedures.

Corticosterone Assay

All testing and blood collection was done between 8 and 10 am (2-4 hours after lights on). Mice either remained undisturbed in their home cage (basal) or were placed individually in a standard homecage that had no bedding or wire lid (novel environment) until time of sacrifice. Mice were sacrificed by cervical dislocation followed by rapid decapitation either from the home cage or at 5, 15, 30, or 60 minutes after placement in the novel cage. Trunk blood was collected in 1.5 ml ethylenediametetraacetic acid-coated microcentrifuge tubes (VWR, West Chester, PA) and kept on ice until centrifuged for 15 minutes at 2000 r.p.m. at -20° C. Plasma was then transferred to clean, 0.5 ml microcentrifuge tubes and stored at -80° C. Plasma CORT levels were determined using a commercially available radioimmunoassay (RIA) kit (MP Biomedicals, Orangeburg, NY, performed at University of Delaware, Newark, DE). Assay standards were run in duplicate and samples were run in double duplicate. The CORT intra-assay variability was 6.8%. Duplicates that had a coefficient of variation greater than 15% were not used

for analysis. In all cases except one, at least one set of duplicates from a sample was usable. When both sets were usable, all four values were averaged to create a final mean value for that sample. Specific group sizes are noted in the figures.

Data were analyzed using ANOVA with genotype and condition as factors. For significant main effects or interactions, a Fisher's PLSD post-hoc was used to determine within genotype differences between time-points and a t-test with Bonferroni correction (significance = $p < 0.01$) was used to compare genotypes within a time point. All data are expressed as mean±SE.

Stress-induced Hyperthermia

Stress-induced hyperthermia is one measure to determine physiological response to stress (Koshibu, Ahrens, & Levitt, 2005; Zethof, Van der Heyden, Tolboom, & Olivier, 1994). Mice were individually housed for 10 days prior to the experiment. Eight *Lsamp*$^{+/+}$ and 7 *Lsamp*$^{-/-}$ mice were used. Mice were picked up and rectal temperature (T1) was measured for 20 seconds using a Thermalert-5 (PhysiTemp, Clifton NJ). Mice were then returned to the home cage for 10 minutes, at which point rectal temperature was taken again (T2) for 20 seconds. The change in temperature (T2-T1) was calculated (dT). Data were analyzed using a 2-way ANOVA including genotype and stress condition as factors. Data are expressed as mean ± SE.

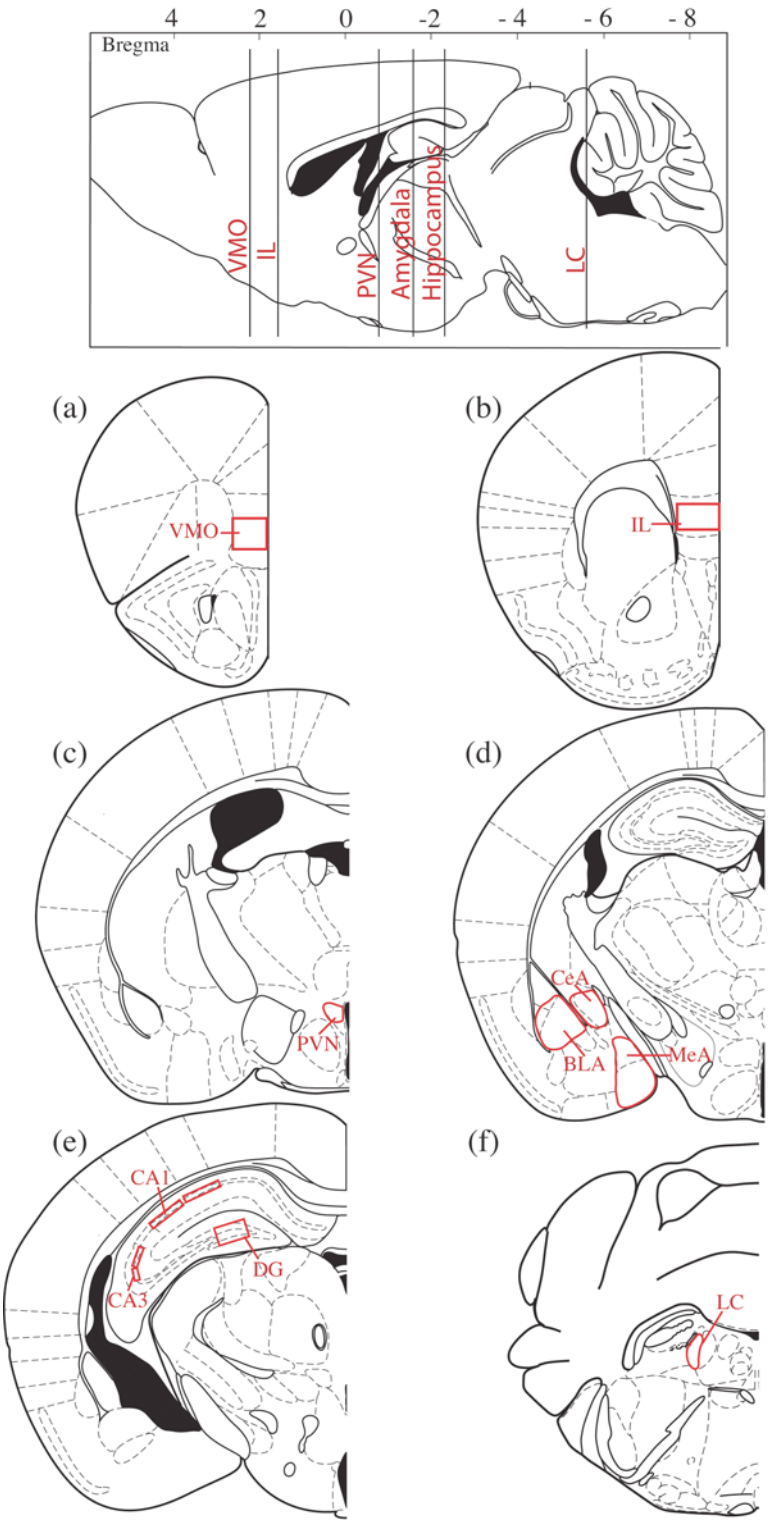

Figure 1. Structures quantified for c-Fos activation.

C-Fos Immunocytochemistry

Mice were singly housed for 13-16 days prior to novelty exposure and brain collection. Two groups were used for c-Fos measures. Control, non-stressed, mice (*lsamp*$^{+/+}$ = 12, *lsamp*$^{-/-}$ = 9) were sacrificed directly after removal from the home-cage. Novelty exposed mice (*lsamp*$^{+/+}$ = 13, *lsamp*$^{-/-}$ = 10) were placed in a square arena (27cm x 27cm x 27cm) with clear Plexiglas walls and white floors for 10 minutes and then returned to their home-cage until time of sacrifice, 2 hours after placement in the novel arena. Novelty exposure for all animals was between 8 and 10 am. Mice were deeply anesthetized with sodium pentobarbital (60mg/kg i.p.) and perfused transcardially with 4% paraformaldehyde in phosphate-buffered saline (PBS, 7.2 pH). Brains were removed and post-fixed overnight at 4° C, and then cryoprotected in sequential 24-hour incubations of 10, 20, and 30% sucrose in PBS. Coronal sections of 50μm were cut on a sliding microtome in 4-6 series and collected into freezing medium at -20°C until processing.

Briefly, free-floating sections were washed several times in PBS, incubated for 10 min in 0.5% H_2O_2 in PBS, rinsed again in PBS, incubated for 30min in 0.1 M Tris-glycine (pH 7.4), and washed several times in Blotto (4% Carnation dried milk in PBS with .2% Triton-X 100). Sections were incubated in rabbit anti-c-Fos (Oncogene, Cambridge, MA) at 1:20,000 in Blotto for 48-72 hours at 4°C. After several Blotto washes for 30 minutes, sections were incubated in biotin-SP-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA) at 1:1000 in Blotto for 1 hour at room temperature. After 30 minutes of washing in Blotto sections were processed using

the ABC method (Vector, Burlingame, CA) and rinsed in PBS before visualization by application of 0.5% 33-diaminobenzidine (DAB) and .05% H_2O_2 for 4 minutes. Sections were then washed in PBS, mounted onto gelatin-subbed slides, dehydrated with alcohols, cleared with xylene and counterstained with Cresyl-violet in order to visualize anatomy. Slides were coverslipped using DPX (Fisher, Pittsburg PA).

Slides were coded that so that the investigator was blinded to genotype. C-Fos positive nuclei were counted in the following brain areas: ventromedial (VMO) and infralimbic (IL) pre-frontal cortex, CA1, CA3, and dentate gyrus (DG) of the hippocampus, central (CeA), medial (MeA) and basal lateral (BLA) nuclei of the amygdala, locus coeruleus (LC) and the paraventricular nucleus of the hypothalamus (PVN). Because not every brain had sections at a representative Bregma level, sample size for each analysis is noted below. Specific group sizes are noted in the figures and tables. Images were acquired under brightfield illumination with a Zeiss AxoCam HRc camera and Axiovision 4.1 software. For all areas counts were c-Fos positive cell counts were obtained bilaterally on one section. In VMO cortex (Bregmas: 2.2 – 2.6), positive nuclei were counted within a 30.5 μm by 44 μm rectangle, drawn with one long end laid against the pial surface (Figure 1a). In IL cortex (Bregmas: 1.4 – 1.8), a box that was 36.6 μm in the dorsal-ventral direction was defined from the pial surface to the edge of the white matter and used for counting (Figure 1b). In CA1 (for all hippocampal areas Bregmas: -2.2 – -2.4) positive nuclei were counted within a strip (mean width = 68 μm) of the pyramidal layer (Figure 1e). In CA3 positive nuclei were counted within a strip of the pyramidal layer (Figure 1e). All positive nuclei within the boundaries of a rectangle extending from the dorsal to the ventral surface of the DG on one section were counted

(Figure 1e). Within the amygdala (for all amygdalar bregmas: -1.5 -- -1.7) and LC (Bregmas: -5.3 -- -5.68) positive nuclei were counted within the anatomical boundaries of the nucleus of interest (Figure 1d and 1f). Within PVN (Bregma: -0.6 -- -0.8) positive nuclei were counted within the boundaries of the nucleus that was contained in a rectangle defined as 1 x 1.5 times the widest width of the PVN on that section (Figure 1c). The average number of cells in PVN was obtained by counting CV positive nuclei in the same area in which c-Fos positive counts were made. The average number of c-Fos positive nuclei in each area and the average number of cells in PVN was calculated with Abercrombie's formula (Abercrombie & Johnson, 1946). To measure average profile size, the diameter of c-Fos positive nuclei or CV positive nuclei was measured using ImageJ (US National Institutes of Health, Bethesda, MD).

For all areas except PVN, data were analyzed as number of c-Fos positive nuclei per unit area (μm^2). For the PVN data were also expressed and analyzed as % increase of c-Fos positive nuclei/μm^2 over mean wildtype basal c-Fos positive nuclei/μm^2. Data were analyzed using a 2-way ANOVA including genotype and stress condition as factors. Data are expressed as mean± standard error (SE).

CRF and GAD-67 Immunocytochemistry

Rabbit anti-CRF (1:10,000, generously donated by AJ Silverman) and mouse anti-glutamic acid decarboxylase-67 (GAD-67) (1:2000, Chemicon, Temecula, CA) were used for staining with selected series of brain sections from the c-Fos experiment. Sections were processed as described for c-Fos with the following alterations: anti-CRF was blocked with 5% Blotto and processed in DAB for 2 minutes; anti-GAD-67 was

blocked in 4% Blotto with no Triton-X 100 added in any steps, secondary antibody was biotin-SP-conjugated donkey anti-mouse IgG (Jackson Immunoresearch, West Grove, PA) at 1:1000, the ABC Elite kit was used, and DAB processing was 3 minutes.

Images were acquired as described for c-Fos staining. For CRF staining macroscopic images of the whole brain and 20x images of PVN were obtained. For GAD-67 staining, macroscopic images of the whole brain and high magnification images of PVN were obtained. Images from the PVN were taken at 63x for approximation of the density of GAD-67 in the PVN. A single rectangle was placed on each image and, after thresholding, area fraction was determined using ImageJ (US National Institutes of Health, Bethesda, MD). The same threshold level was used for each section. Threshold level (pixel intensities of 0 – 148) was determined by choosing the mean of the ideal threshold for the lightest and darkest image.

Elevated Plus Maze

To test for possible pharmacological manipulations that would ameliorate the stress response differences in *Lsamp*$^{-/-}$ mice, we performed pilot studies using elevated plus maze (EPM) experiments. Animals were run on the EPM in order to determine if there were genotype dependent effects on behavior in the maze. Two drugs were used: Antalarmin (Sigma-Aldrich, St. Louis, MO) is a CRF receptor 1 (CRFR1) antagonist (Webster et al., 1996) and, Diazepam (Sigma-Aldrich, St. Louis, MO), is a commonly used anxiolytic that is a Benzodiazepine receptor (BZR) agonist (McLaughlin et al., 2003). For the Antalarmin EPM, 4 *Lsamp*$^{+/+}$ mice were used for each treatment and 6-7 *Lsamp*$^{-/-}$ mice were used for each treatment. For the Diazepam EPM, 4-5 *Lsamp*$^{+/+}$ female

mice were used for each treatment and 5 *Lsamp*$^{-/-}$ female mice were used for each treatment.

The EPM experiments were performed in the Vanderbilt Kennedy Center and Center for Molecular Neuroscience Murine Neurobehavioral Core. Testing took place between 12-6 p.m. The apparatus and basic testing procedures were the same as described in chapter 2 (p. 37). All the mice in both experiments received an interperitoneal injection of either drug or vehicle at a volume of 20ml/kg 30 minutes prior to testing. Antalarmin (Sigma-Aldrich, St. Louis, MO), dissolved in a solution of distilled water with 0.5% Tween-80, was given at a dose of 10 mg/kg body weight. Diazepam (Sigma-Aldrich, St. Louis, MO), dissolved in a solution of distilled water with 0.5% Tween-80, was given at a dose of 1.5mg/kg body weight.

EPM data was analyzed as described in chapter 2 (p. 38). For these experiments, we measured only the number of entries into the open and closed arms, and duration of time spent in the open arms, closed arms and center of the maze. Small n's were used in order to obtain initial observations of the drug effects.

Results

Corticosterone Response to Novel Environments

There were no differences in morning basal CORT levels between *Lsamp*$^{+/+}$ and *Lsamp*$^{-/-}$ mice (Figure 2). In all mice, exposure to a novel environment caused a robust increase in peripheral corticosterone levels (Figure 2). In both *Lsamp*$^{+/+}$ and *Lsamp*$^{-/-}$ mice there was a significant increase in CORT levels within 15 minutes after placement

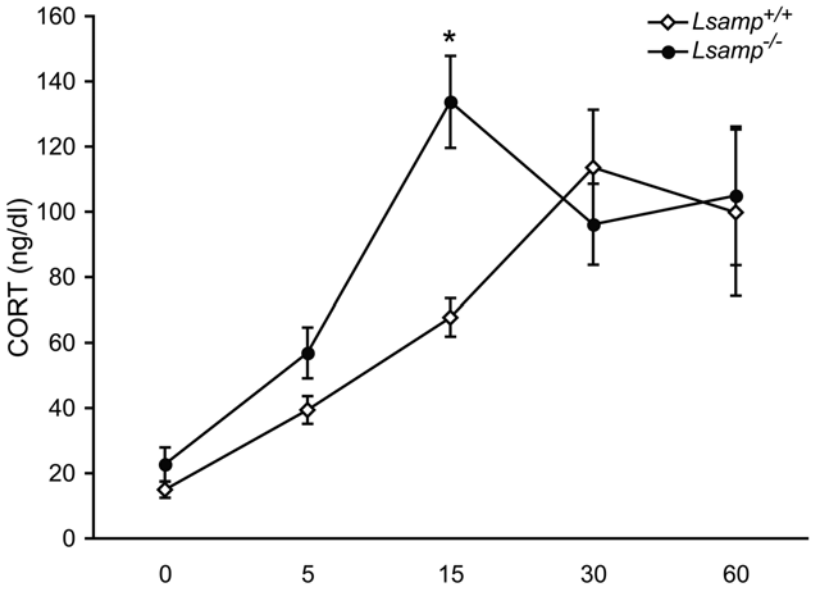

Figure 2. CORT response in *Lsamp*$^{-/-}$ mice to novelty exposure. *Lsamp*$^{-/-}$ mice have normal basal levels of CORT. After exposure to a novel environment, *Lsamp*$^{-/-}$ mice reached peak levels of CORT response within 15 minutes, while *Lsamp*$^{+/+}$ mice didn't reach peak levels until 30 minutes after exposure to the novel environment. All mice of both genotypes had significantly increased CORT levels by 15 minutes and levels remained elevated for the entire hour measured (n = 8-12/group) *p = 0.0002 compared to littermates at the same timepoint.

in the novel environment (*Lsamp*$^{+/+}$ $p < 0.05$ and *Lsamp*$^{-/-}$, $p < 0.0001$) and levels were still increased above baseline 60 minutes after novelty exposure (Figure 2).

The stress response of the *Lsamp*$^{-/-}$ mice, however, was distinct from *Lsamp*$^{+/+}$ mice. *Lsamp*$^{-/-}$ mice reached peak CORT levels twice as fast as their wildtype littermates. The highest level of CORT observed in *Lsamp*$^{-/-}$ mice (mean ± SE, 133.72±14.10) was 15 minutes after exposure to the novel environment, whereas in their wildtype littermates peak CORT levels (113.61 ± 17.70) were reached by 30 minutes after placement in the novel cage (Figure 2). *Lsamp*$^{-/-}$ mice had significantly increased levels of CORT compared to wildtype littermates at the 15 minute time-point ($t(21) = -4.463$, $p < 0.0002$) but not at any other time-point measured.

Stress-Induced Hyperthermia

There was a normal hyperthermia response to stress in both genotypes. Thus, there were no genotype-dependent effects on T1 or T2 (Figure 3a). And there was no difference in dT between *Lsamp*$^{+/+}$ and *Lsamp*$^{-/-}$ mice (Figure 3b).

C-Fos Activation

We examined activation of neuronal cell groups following a mild stressor by evaluating changes in C-Fos protein expression in different forebrain and brain stem regions that are well-known to activate in stressful situations (Kovacs, 1998; Martinez, Calvo-Torrent, & Herbert, 2002; Singewald, Salchner, & Sharp, 2003). Basal (home-cage controls) levels of c-Fos activation (Table 1) between *Lsamp*$^{+/+}$ and *Lsamp*$^{-/-}$ mice

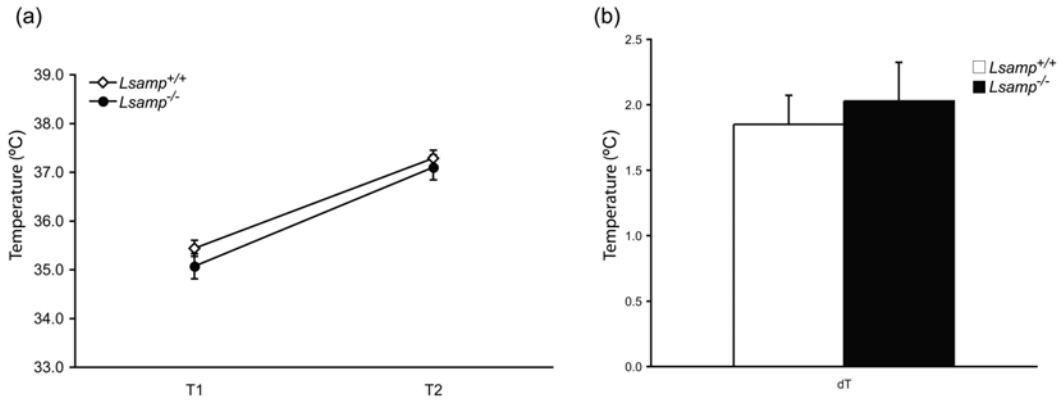

Figure 3. Stress-induced hyperthermia in *Lsamp*$^{-/-}$ mice. *Lsamp*$^{-/-}$ mice did not differ from wildtype littermates in either their basal (T1) or stressed (T2) temperature (a). The change in temperature (dT) (b) was also no different between genotypes. (n = 7-8/group).

were not different in any of the brain areas measured, as there were no main effects of genotype or genotype by condition interactions in any area except PVN, where further analysis demonstrated no difference in basal levels of activation between genotypes (Figure 4).

Based on the quantitative analysis of the number of immunoreactive nuclei in different brain regions, there was a significant increase in c-Fos protein expression after exposure to a novel environment in mice of both genotypes. A main effect of condition was found in VMO ($F_{(1,26)} = 54.30$, $p < 0.0001$) and IL ($F_{(1,29)} = 66.5$, $p < 0.0001$), CA1 ($F_{(1,30)} = 15.84$, $p < 0.001$) and CA3 ($F_{(1,14)} = 43.79$, $p < 0.0001$) of the hippocampus, BLA ($F_{(1,30)} = 56.46$, $p < 0.0001$) and MeA ($F_{(1,30)} = 155.85$, $p < 0.0001$) of the amygdala, PVN ($F_{(1,40)} = 92.05$, $p < 0.0001$) and in LC ($F_{(1,30)} = 30.89$, $p < 0.0001$). Exposure to a novel environment did not cause a rise in c-Fos protein expression in either hippocampal DG ($F_{(1,30)} = 0.534$, $p = 0.48$), or CeA of the amygdala ($F_{(1,30)} = .281$, $p = 0.60$). There were no differences between Lsamp$^{+/+}$ and *Lsamp*$^{-/-}$ mice (no effect of genotype, or genotype x condition interactions) in the numbers of c-Fos positive cells after exposure to a novel environment in any areas measured except the PVN of the hypothalamus.

Within the PVN, there was a main effect of genotype ($F_{(1,40)} = 8.287$, $p < 0.01$) and a genotype x condition interaction ($F_{(1,40)} = 5.14$, $p < 0.05$). While there was a trend, under basal conditions the number of c-Fos positive cells did not differ statistically between *Lsamp*$^{+/+}$ and *Lsamp*$^{-/-}$ mice ($p = 0.06$) (Figure 4). However, after a ten-minute exposure to a novel environment, *Lsamp*$^{-/-}$ mice exhibited significantly larger percent change from basal counts (mean diff= 1639.33%, $p < 0.05$) in c-Fos activation compared to their wildtype littermates (Figure 4).

Table 1. Basal levels of c-Fos activation did not differ between *Lsamp*$^{+/+}$ and *Lsamp*$^{-/-}$ mice in any areas measured. Data presented are the mean number of c-Fos positive nuclei/$\mu m^{2(10-4)}$ ± SE.

Area	***Lsamp*$^{+/+}$**	***Lsamp*$^{-/-}$**
VMO	42.67±4.54	46.74±12.09
IL	20.32±8.98	16.60±3.84
CA1	5.87±0.89	5.62±1.10
CA3	0.65±0.19	0.58±0.20
DG	1.29±0.29	1.50±0.15
CeA	1.55±0.40	1.93±0.35
MeA	0.40±0.07	0.49±0.10
BLA	0.35±0.09	0.39±0.10
PVN	1.09±0.14	0.77±0.00
LC	0.76±0.22	0.50±0.26

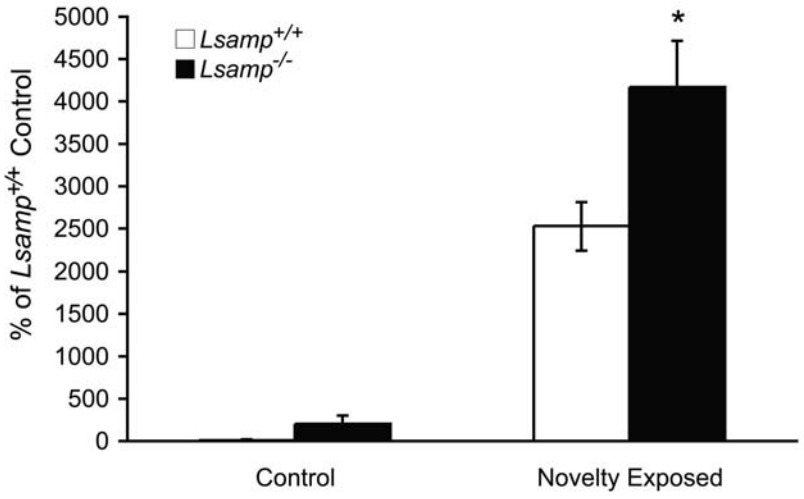

Figure 4. C-Fos activation in the PVN of *Lsamp*$^{-/-}$ mice. Basal levels of c-Fos protein expression were normal in *Lsamp*$^{-/-}$ PVN. *Lsamp*$^{-/-}$ mice had an exaggerated increase in c-Fos activation after exposure to a novel environment, nearly double the increase of *Lsamp*$^{+/+}$ mice. (n = 9-11/group) *$p < 0.05$ compared to littermates in same group.

CRH and GAD-57 Immunocytochemistry

Because of the key role that CRH plays in mediating the central stress response, we used immunocytochemistry to examine possible differences in the expression or organization of CRH-positive neurons and processes. The entire forebrain was examined, and we found no gross differences in CRH staining throughout these regions between *Lsamp*$^{+/+}$ and *Lsamp*$^{-/-}$ mice (Figure 5a) or within the PVN at higher power (Figure 5b). The PVN of both genotypes was characterized by moderate fiber staining, along with a few cell bodies. As expected from previous studies of CRF immunoreactivity, dense staining was observed in CeA and BNST where there are CRF-producing cell bodies (Asan et al., 2005) (Gray, 1990).

Because there is a large GABAergic inhibitory drive to the PVN, we used immunocytochemistry for GAD-67 to examine if GABAergic input to the PVN is altered. GAD-67 staining appeared to be decreased throughout the PVN of *Lsamp*$^{-/-}$ mice (Figure 6a). Using area fractionation we determined that there was a non-significant trend for a decreased density of GAD-67 in the PVN of *Lsamp*$^{-/-}$ mice, with a 73% decrease in GAD-67 staining (Figure 6b).

Elevated Plus Maze

While the neuroanatomical data failed to detect differences in CRH expression between genotypes, physiological alterations might account for changes in stress responsiveness in the *Lsamp*$^{-/-}$ mice. Antalarmin is a well-characterized CRH-R1 receptor antagonist that we hypothesized would reduce the response to novelty normally exhibited by the mutant mice. There was no main effect of treatment with Antalarmin or a genotype x treatment

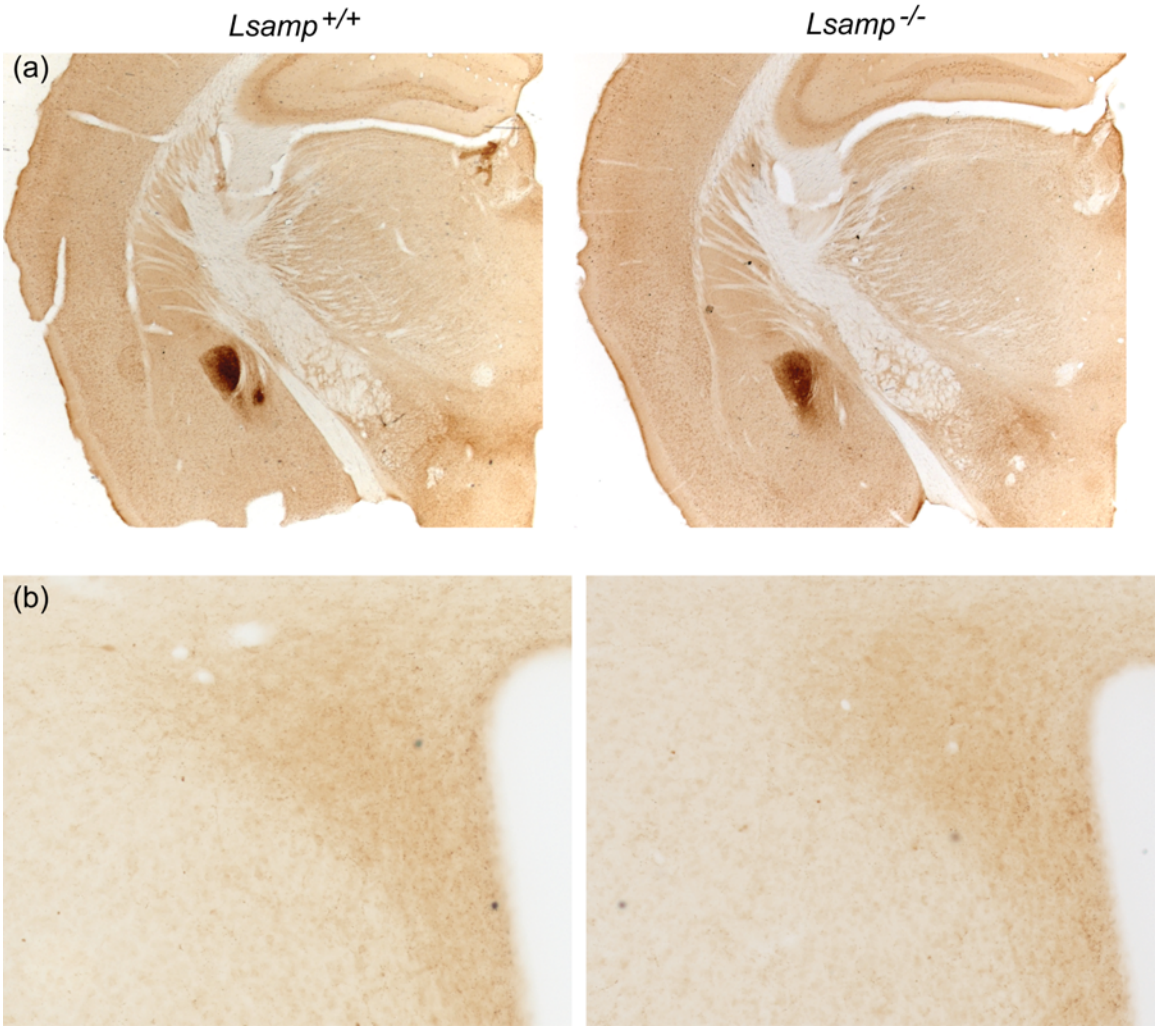

Figure 5. *Lsamp*$^{-/-}$ mice appear to have normal CRF distribution. *Lsamp*$^{-/-}$ mice did not appear to differ from their wildtype littermates in either amount or distribution of CRF in limbic areas of the brain.

interaction on either maze entries or duration spent in different areas of the maze. As expected from our previous studies, there was a main effect of genotype ($F_{(1,21)} = 14.27$, $p < 0.01$), with *Lsamp*$^{-/-}$ mice making significantly more open entries ($t(19) = 3.96$, $p < 0.001$) than *Lsamp*$^{+/+}$ mice.

The differential response to novelty between genotypes may also reflect differences in GABAergic modulation of stress-response circuitry. Diazepam is a well-known anxiolytic that reduces anxiety-like behavior on the EPM. Administration of low dose (1.5mg/kg) diazepam resulted in several novel alterations in behavior. There were main effects of treatment ($F_{(1,19)} = 7.19$, $p < 0.05$) and a treatment x genotype interaction ($F_{(1,19)} = 4.96$, $p < 0.05$) on number of entries. These effects were accounted for by significant decreases in both open ($t(8) = 4.52$, $p < 0.002$) and closed ($t(8) = 8.4$, $p < 0.0001$) entries by Diazepam-treated *Lsamp*$^{-/-}$ mice (Figure 7a) as compared to vehicle treated *Lsamp*$^{-/-}$ mice. *Lsamp*$^{+/+}$ mice entries were not affected by drug treatment (Figure 7a).

There was no main effect of genotype or treatment on time spent within each arm (durations). There was a three-way interaction between treatment, genotype and category of duration ($F_{(2,19)} = 3.64$, $p < 0.05$). Diazepam treated *Lsamp*$^{-/-}$ mice spent significantly more time on the open arms ($t(8) = -3.76$, $p = 0.0056$), with a corresponding decrease in center time ($t(8) = 5.13$, $p = 0.009$) compared to vehicle treated *Lsamp*$^{-/-}$ mice (Figure 7b). Drug treatment did not affect the durations that *Lsamp*$^{+/+}$ mice spent in any portion of the maze (Figure 7b).

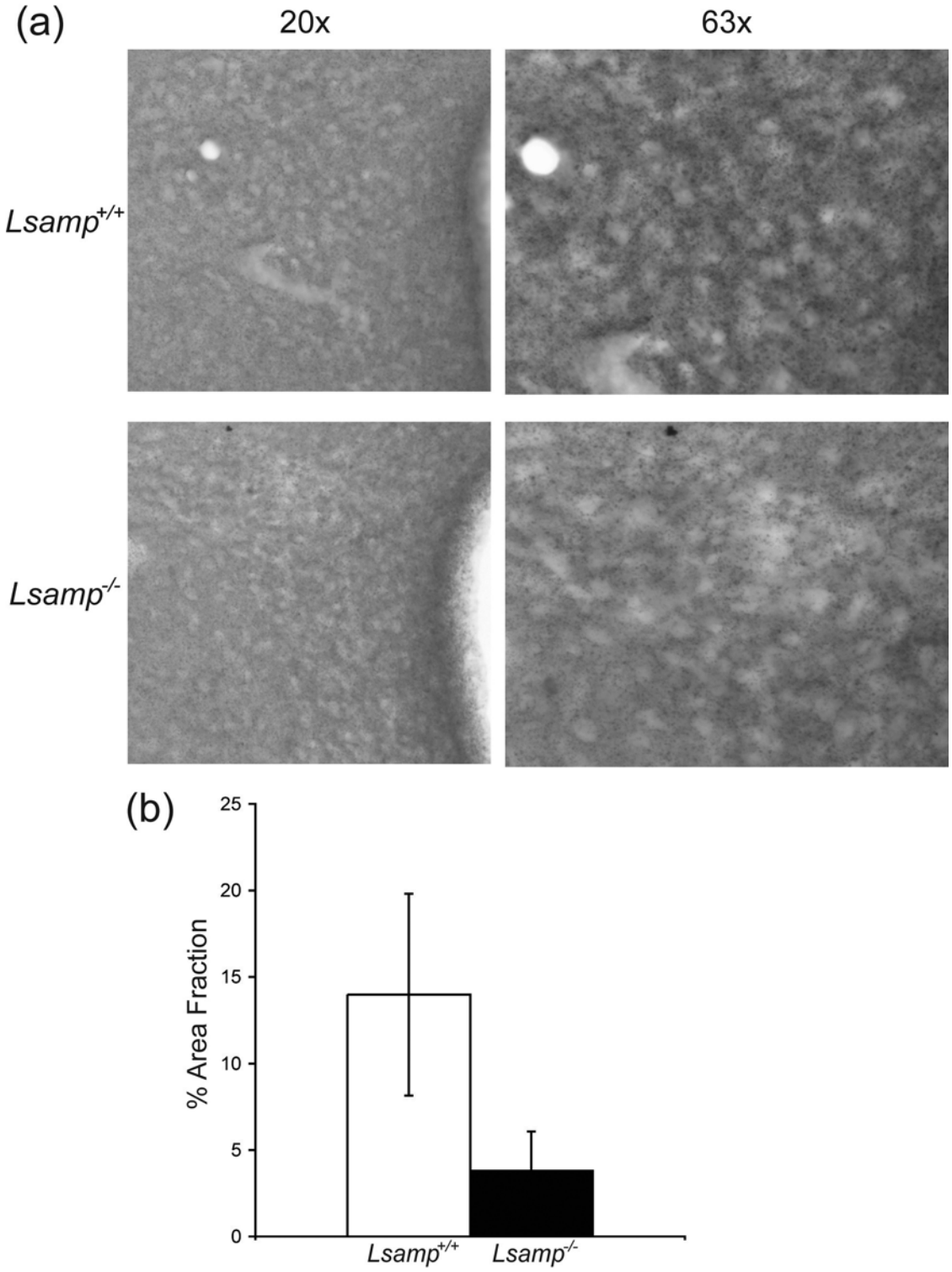

Figure 6. *Lsamp*$^{-/-}$ mice have alterations in the density of GAD-67 in the PVN. There appears to be a decrease in density of GAD-67 positive fibers in the the PVN of *Lsamp*$^{-/-}$ mice (a). There is a non-significant trend for decreased density of GAD-67 fibers in the PVN as measured by area fractionation (b).

Discussion

In a novel environment, *Lsamp*$^{-/-}$ mice are hyperactive and exhibit increased exploratory behaviors that also are atypical in their nature (Catania et al., 2008). Some of these behaviors (increased entries and duration on the open arms of the EPM) are generally interpreted as decreased anxiety, though in the *Lsamp*$^{-/-}$ mice, increased unprotected head dips and an overall increase in activity accompanied the unusual exploratory behavior. Because of this combination of atypical behaviors, we hypothesized that in *Lsamp*$^{-/-}$ mice, the phenotype was driven by heightened emotional reactivity to stressful environments, indicated by hyper-activation and/or lack of appropriate inhibition when placed in a novel situation. Multiple studies indicate that in general, animals that display decreased anxiety have a correspondingly blunted HPA axis response to stress (Kalinichev, Easterling, & Holtzman, 2002; Meaney, 2001; Timpl et al., 1998). If *Lsamp*$^{-/-}$ mice are more reactive to novel environments and not simply less anxious, we hypothesized that *Lsamp*$^{-/-}$ mice would also demonstrate a more sensitive, or reactive, neuroendocrine stress response in these environments, accompanied by changes in brain function that would be permissive of both the altered behavioral and physiological responses.

We found that the HPA axis of *Lsamp*$^{-/-}$ mice does display heightened reactivity in response to a novel environment. Although the absolute peak level of CORT response was normal, *Lsamp*$^{-/-}$ mice mounted a CORT response much more quickly than wildtype mice. This resulted in an alteration in the kinetics of the stress response. Increased HPA

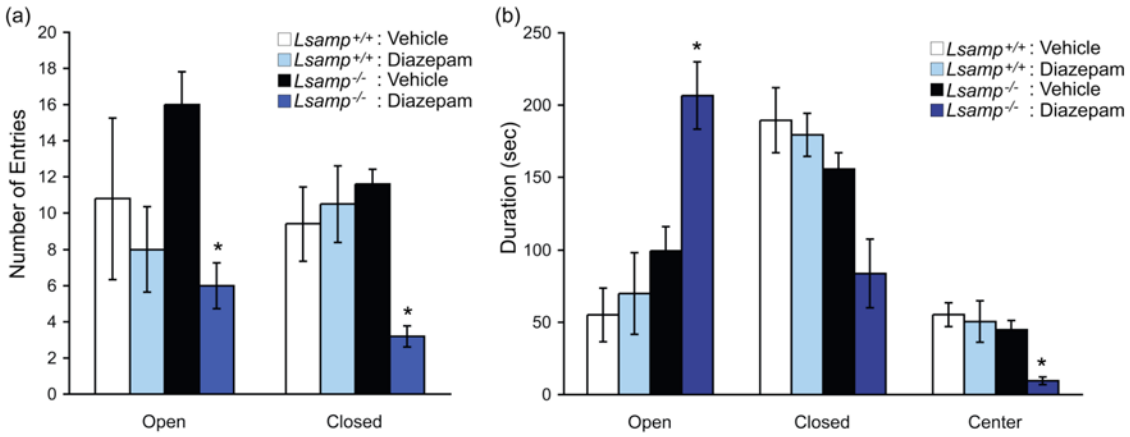

Figure 7. Effect of Diazepam on *Lsamp*$^{-/-}$ mice behavior in the EPM. A dose of 1.5mg/kg Diazepam had no effect on the anxiety or motor behavior of female *Lsamp*$^{+/+}$ mice. However, *Lsamp*$^{-/-}$ mice had greatly reduced motor activity as measured by decreased entries (a) into all parts of the maze. They also had a corresponding increase the duration of time spent in the open arms (b), probably because they spent much of the test not moving in the open arm. (n = 4-5/group). * $p < 0.005$ compared to vehicle treated mice.

responsiveness is frequently seen in conjunction with increases in anxiety behaviors (Meaney, 2001; Mitra & Sapolsky, 2008). There is thus a mismatch that we find between exploratory behavior on the EPM, an indicator of reduced stress, and more rapid HPA response, an indicator of increased stress. This lends support to the hypothesis that the absence of LAMP via constitutive deletion of the *Lsamp* gene, leads to a heightened and maladaptive response to novel environmental stressors and that the behavioral phenotype is caused more by hyper-reactivity to the stressful environment than to decreased anxiety. Several recent human genetic studies demonstrate a role for LAMP in human disorders of emotional regulation. Polymorphisms in the *Lsamp* gene have been associated both with panic disorder and male suicide (Koido et al., 2006; Maron et al., 2006; Must et al., 2008), highlighting the importance of discovering LAMPs role in defining and/or modulating the circuitry that underlies emotional regulation.

There are many potential loci along the HPA axis at which stress-induced CORT release can be altered (Miller & O'Callaghan, 2002; Rosenfeld et al., 1992) ranging from alterations in the brain circuitry responsible for exciting the PVN, to changes in pituitary or adrenal sensitivity or response. However, SIH, a gross measure of the peripheral physiological stress response (Veening et al., 2004; Zethof et al., 1994) was normal in *Lsamp*$^{-/-}$ mice, suggesting that at least some parts of the peripheral physiological stress response are intact. Given that it is an alteration in the speed of the CORT response as opposed to an alteration in absolute level, it seems possible that the increased sensitivity of HPA axis is due to a permissive state in the brain, created by either decreased inhibitory tone, or increased excitatory drive. This possibility is supported by the heightened behavioral reactivity to novelty observed in *Lsamp*$^{-/-}$ mice because there is

overlap in the brain circuitry that regulates the behavioral response to novelty and the stress response (Lopez, Akil, & Watson, 1999) and LAMP is normally heavily expressed in the limbic structures (Cote et al., 1996; Pimenta, Reinoso et al., 1996; Reinoso et al., 1996) responsible for regulating these responses. Disruption of this circuitry could potentially underlie both phenotypes.

Exaggerated c-Fos induction in the PVN of *Lsamp*$^{-/-}$ mice after exposure to a novel environment also suggests that regulation of the peripheral stress response may be altered at the level of limbic circuitry. The PVN is the first direct responder (parvocellular PVN neurons release CRF), and only CNS structure, of the HPA axis cascade (Miller & O'Callaghan, 2002). Basal levels of the number of c-Fos-positive PVN neurons were not statistically different between wild type and *Lsamp*$^{-/-}$ mice, though we do note that there was a trend towards a very modest difference in basal activation state. The very robust changes seen after the mild stressor suggest that the *Lsamp*$^{-/-}$ mice are not in a pathological state of a heightened activation under normal conditions, but there is a dramatic state change during exposure to a novel environment. It has been demonstrated that as CORT increases in response to stimulus intensity, c-Fos induction within the PVN also increases (Campeau & Watson, 1997). Given this, it seems likely that the activity within the PVN may be responsible for the increased CORT response exhibited by *Lsamp*$^{-/-}$ mice.

Release of CRF from the PVN is mediated either directly or indirectly by many of the brain structures involved in mediating the behavioral response to stress, including the other structures that we examined in the c-Fos experiment. It is important to note that while c-Fos induction is an excellent way to measure gross changes in brain activation in

response to stress, it has limitations (Kovacs, 1998; Martinez et al., 2002). For example, most of the areas we measured have neurons of several types (e.g. excitatory and inhibitory cells), and it is possible that subtle changes in activation of a certain cell type could alter the function of downstream circuitry but would be missed by assaying for c-Fos induction in the entire nucleus. Therefore, it is possible and even likely, given that the other areas we examined have either direct or indirect projections to the PVN, that there are alterations in signaling in other parts of the stress pathway, but that the differences are too specific or subtle to perceive using c-Fos induction as a marker.

CRF released from the PVN and other brain structures, such as CEA, is also involved in modulating anxiety and stress related behaviors (Muller et al., 2000). One possible mechanism for both a sensitized CORT response and increased behavioral reactivity would be an increased total amount of CRF or CRFR1, the receptor primarily responsible for mediating both the neuroendocrine and anxiety related functions of CRF (Bale, 2005). However, immunocytochemistry did not reveal any gross alterations in either levels or distribution of the CRF protein in *Lsamp*$^{-/-}$ mice. Moreover, *Lsamp*$^{-/-}$ mice displayed no alterations in sensitivity to Antalarmin, an antagonist to the CRFR1 receptor, on the EPM. This is not definitive evidence that there is no alteration in CRF signaling in *Lsamp*$^{-/-}$ mice, but it does suggest that there may be a more robust alteration in the anatomy or signaling of the stress related brain circuitry that may underlie the phenotype of *Lsamp*$^{-/-}$ mice.

Inhibitory regulation of the PVN by GABAergic cells comes from peri-PVN, other hypothalamic areas and the BNST (Herman et al., 2003; Herman, Mueller, & Figueiredo, 2004). GABAergic neurons provide tonic inhibiton to the HPA axis under

basal conditions and also regulate stress reactivity. Stress-induced c-Fos activation in the PVN is reduced by stimulation of GABA-A receptors, indicating that GABAergic inhibition can reduce HPA axis reactivity to stress (Kovacs, Miklos, & Bali, 2004). There appeared to be a decrease in GABAergic fibers in the parvocellular region of the PVN of *Lsamp*$^{-/-}$ mice, indicating that there may be decreased GABAergic tone to the PVN. A decreased inhibitory drive on the PVN could create a cellular environment that was more permissive to excitatory input and explain why *Lsamp*$^{-/-}$ mice have greater stress induced c-Fos activation and mount a much faster CORT response.

Lsamp$^{-/-}$ mice also exhibited a high level of motoric depression on the EPM when administered a low dose of Diazepam. This dose had no motor or open/closed arm exploratory effects on *Lsamp*$^{+/+}$ mice. Generally, Diazepam does not have sedative effects until much higher doses (McKernan et al., 2000; Zeller et al., 2008), suggesting that *Lsamp*$^{-/-}$ mice have extremely heightened sensitivity to the drug. This increased sensitivity could result from upregulation of BZRs in *Lsamp*$^{-/-}$ mice in order to compensate for decreased GABAergic tone throughout the brain. GABAergic signaling is also critical in the expression of anxiety related behaviors and increases levels of BZRs in GABAergic circuitry have been demonstrated to underlie decreased anxiety-like behaviors and HPA axis responsiveness in both mouse and rat models (Caldji, Diorio, Anisman, & Meaney, 2004; Caldji et al., 2003; Caldji et al., 2000). Therefore, it is possible that alterations in GABAergic tone may underlie both the behavioral deficits and the heightened HPA axis reactivity seen in *Lsamp*$^{-/-}$ mice response to novel environments. To investigate this possibility, the Diazepam experiment will be replicated with a larger cohort of animals and radioligand binding will be performed to determine both total

GABAR and BZR levels in order to examine what alterations in the GABAR system exist in *Lsamp*$^{-/-}$ mice. To further address the hypothesis that deletion of *Lsamp* may alter GABAergic tone, further experiments to elucidate both the integrity and functionality of GABAergic signaling are necessary.

The present data demonstrate that constitutive deletion of the *Lsamp* gene leads to increased reactivity to novel environments, both through heightened HPA axis sensitivity and increased hyper-activation of circuitry regulating the stress response. Combined with our previous data, this establishes a definitive role for LAMP in modulating the development and functioning of the circuitry that mediates emotional regulation. Identifying the specific circuitry alterations that underlie the behavioral and neuroendocrine phenotypes of *Lsamp*$^{-/-}$ mice will better elucidate the role of the LAMP protein in the modulation of stress and behavior.

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

We have demonstrated that the genetic deletion of *Lsamp* increases the behavioral, physiological and neurobiological responses to the stress of novelty exposure. *Lsamp*$^{-/-}$ mice are markedly more hyperactive than their littermates when exposed to novel environments, but when allowed to acclimate, they return to normal levels of motoric output. During the period when the hyperactive response is at its peak, *Lsamp*$^{-/-}$ mice display disruptions in normal exploratory behaviors. These include anxiety-related behavioral changes such as increased proportion of entries into the open arms and increased time in the open arms of the EPM. Increased exploratory behavior in the unprotected areas of the maze was accompanied by increased head-dips in the same portions of the maze and by decreased spontaneous alternation in the y-maze. In concert, these behaviors suggest that *Lsamp*$^{-/-}$ mice experience hyper-activation or lack of appropriate inhibition when exposed to the stress of a novel environment. This is mirrored in the neuroendocrine response to novelty. Although *Lsamp*$^{-/-}$ mice have normal basal levels of CORT, the HPA axis response to exposure to a novel environment is augmented, with *Lsamp*$^{-/-}$ mice reaching peak CORT levels twice as quickly as wildtype littermates. Finally, hyper-reactivity to novelty stress is reflected in the brain, with *Lsamp*$^{-/-}$ mice demonstrating greater than normal activation of the PVN, the first structure of the HPA axis, after placement in a novel environment. Taken together, these data

demonstrate that genetic deletion of *Lsamp*$^{-/-}$ causes disrupted emotional regulation that is reflected in brain function, physiological response and behavior.

The next step to understanding how LAMP is involved in emotional regulation will be to examine what changes in brain circuitry underlie the phenotypes of increased behavioral, endocrine, and neural activity in *Lsamp*$^{-/-}$ mice. Our preliminary data suggest that *Lsamp*$^{-/-}$ mice have decreased GABAergic tone in the PVN, such that the increased stress activation of the PVN and its downstream effects on the HPA axis may be due to decreased inhibitory drive to the nucleus. If deletion of LAMP somehow results in decreased GABAergic tone throughout limbic circuitry, it could explain the behavioral, neuroendocrine and neural activity changes we see in *Lsamp*$^{-/-}$ mice. The PVN is under strong inhibitory drive from several limbic brain structures that counteract excitatory drive in times of stress (Herman et al., 2003; Wall et al., 2004). Disruption of this inhibition by decreased GABA input to the PVN could explain why the HPA axis response can be elicited much more quickly in *Lsamp*$^{-/-}$ mice. Less inhibition would permit the acceleration of excitation, also arising from disparate brain areas (Herman et al., 2003), to reach a threshold more quickly. Combined with the pilot study demonstrating that *Lsamp*$^{-/-}$ mice have heightened motor sensitivity to a BZR agonist, it is possible that GABAergic tone is altered throughout the brain by both changes in GABA levels and in either numbers or types of GABA receptors. In order to explore this possibility further we will first need to replicate the sensitized motor depression seen in *Lsamp*$^{-/-}$ mice in response to Diazepam in a larger cohort of male mice. We also will need to expand on the PVN findings, both by demonstrating that there is significantly less GAD-67 in the PVN and also by expanding our consideration to the GABA receptors,

using radioligand binding to probe both the total number of GABA receptors and the number of BZRs. Determining what alterations in limbic GABAergic function are cause by genetic deletion of LAMP will inform the next steps in understanding how LAMP effects limbic circuit function.

LAMP is expressed from very early in development throughout the life of the animal and it is therefore not possible to determine if the disrupted stress response we observed in the *Lsamp*$^{-/-}$ mice, which carry a constitutive deletion of the gene, is due to differential development of the limbic circuitry that mediates these behaviors, or if the lack of LAMP in the adult animal has a direct effect on circuit function. There are many examples of developmental perturbations during the critical period of limbic circuit formation causing long-term alterations in stress induced behavior, endocrine response and/or neural microciruitry (Leonardo & Hen, 2008; Levine, 2005; Meaney, 2001; Moriceau & Sullivan, 2004). Alternatively, other CAM's are known to play a role in adult plasticity, such as LTP (reviewed by Rougon & Hobert, 2003) and it is possible that LAMP plays a similar role. Whether the effect is developmental, results from absence of LAMP in the adult brain, or is due to a combination of both, cannot be definitively determined in a constitutive knockout animal.

However, because LAMP's role modulating the development of the circuitry of emotional regulation has been established in vitro, examining *Lsamp*$^{-/-}$ mice for alterations of behavior, endocrine function, and limbic circuit formation during development may help to shed light on its role in vivo. It would be interesting to examine *Lsamp*$^{-/-}$ mice for behavioral alterations at several stages of development in order to determine if there is a consistent alteration in stress responsive behaviors. For example, it

has been demonstrated that mice with reduced levels of TGFα have normal anxiety-related behavior until puberty (Koshibu et al., 2005), so the behavioral paradigms which we have already used could be repeated in pre-pubertal *Lsamp*$^{-/-}$ mice to determine if the stress related disruptions are already present. Stress responsive behavior could also be tested early postnatally by examining ultra-sonic vocalizations induced by separating the pup from the dam. In rodents this behavioral response to stress is predictive of adult anxiety-like behavior (Brunelli & Hofer, 2007). The development of the HPA axis response could also be examined early postnatally. The SHRP occurs in the mouse from post-natal day (pnd) 1 to pnd 12 (Schmidt, Enthoven et al., 2003). During the SHRP stressors that would normally activate the HPA axis do not, but this suppression of stress response can be lifted by a 24-hour separation from the dam (Levine et al., 1988; Stanton et al., 1988). Employing this paradigm of would reveal whether *Lsamp*$^{-/-}$ mice undergo a normal SHRP and if the brain is functionally able to respond to stressors once inhibition by the dam is lifted. There also is an overlap in the period during which the SHRP occurs in the mouse and the timing of the formation of the telencephalic circuits that regulate HPA axis responsiveness. Structures like the amygdala (pnd 4 - 6.5), the BNST (pnd 8 – 10.5) and the pre-frontal cortex (pnd 8 – 10.5) come “on-line” during the SHRP (Rinaman, Levitt, & Card, 2000) and are in place to regulate HPA axis reactivity as the SHRP ends and pups become responsive to environmental challenges. Card et. al. have demonstrated that handling and separation from the dam during the SHRP, the same paradigms used to alter adult behavioral and endocrine stress responsiveness, either delay or decrease the input from these limbic circuits into the PVN (Card et al., 2005). If the formation of these circuits also is altered in *Lsamp*$^{-/-}$ mice, it would demonstrate an in

vivo role of LAMP in forming the circuits of emotional regulation and suggest that the alterations that we see in the adult mice may be at least partially due to developmental alterations of the underlying circuitry. Given the purported role of LAMP in constructing limbic circuitry, it seems likely that *Lsamp*$^{-/-}$ mice would show disruptions of stress related behavior, endocrine response, and circuit formation early postnatally, as this is when LAMP would be guiding circuit formation and when the critical developmental periods of stress response behavior and physiology are being established.

Another way to determine how LAMP mediates its role in emotional regulation would be to create a conditional knockout mouse in which *Lsamp* expression can be temporally regulated. The effectiveness of determining when a molecule is important in regulating stress responsiveness using this method has been demonstrated with a conditional deletion of the serotonin 1A receptor (Gross et al., 2002). Gross et. al. determined that eliminating expression of the receptor for only the first two weeks of life was sufficient to induce anxiety-like behaviors in the adult, and that deleting the receptor only during adulthood had no effect on anxiety-like behaviors. Using temporally controlled gene deletion would create the opportunity to discriminate between the developmental and adult effects of genetically deleting *Lsamp* and aid in the understanding the role of LAMP during development and in the modulation of adult circuits.

There is also an intriguing possibility that LAMP may be involved in human disorders of emotional regulation. There are human genetic studies that implicate *Lsamp* polymorphisms in both panic disorder and male suicide (Koido et al., 2006; Maron et al., 2006; Must et al., 2008). Human anxiety disorders have been proposed to involve

abnormal exaggeration of defensive behaviors such as hypervigilence and increased escape responses (Rodgers, 1997) which would mimic the alterations we observed in *Lsamp*$^{-/-}$ mice. Of course, genetic deletion of a gene is not the same as modulating the levels or function of a molecule and it is possible that more subtle changes in LAMP may have differential effects. Investigating the functional effects of *Lsamp* polymorphisms is an important next step for understanding their role in human disease. It will be useful to know if these changes are associated with increases or decreases in *Lsamp* expression to better understand the role of LAMP in both normal and disrupted human anxiety states.

Lsamp$^{-/-}$ mice provide a valuable tool for better understanding how limbic networks regulate stress response and for examining the role of LAMP in emotional regulation. Although the mechanism of LAMP's role in the circuitry of emotional regulation is still unclear, further studies should determine the subtle circuit changes resulting when LAMP is absent, elucidate its function in the normal development and maintenance of stress responsiveness, and may provide insight into how LAMP may be altered in neuropsychological disease.

Studies of *Lsamp*$^{-/-}$ mice also are an example of the importance of careful use of rodent behavioral tasks. The tests that are commonly used to determine the phenotypes of mice with genetic mutations are not based on the animal's ethology but were developed to test pharmaceuticals for use in humans. This makes them very useful for screening novel drugs but the measures used to predict drug effectiveness do not automatically translate to measures of a human psychiatric state in an animal. When we genetically modify an animal and use behavioral tasks to determine how the molecule may be involved in normal behavior, we are trying to understand their behavioral state, not their

reactivity to drugs for human neuropsychiatric disease. So, for example, in *Lsamp*$^{-/-}$ mice increased open arm time and increased proportion of open arm entries on the EPM would typically be interpreted as reduced anxiety because that effect is typical of anxiolytics in rodents. However, our subsequent experiments to determine the effects of deleting *Lsamp* on emotional regulation showed that *Lsamp*$^{-/-}$ mice are more reactive to stress, not less as a state of decreased "anxiety" would suggest. Behavioral tests of rodent "anxiety" are useful for examining the behavioral response to stress and can be informative of emotional reactivity. But, we must interpret the results based on the actual behaviors of the animal (which include motoric activation and inhibition), taking into account all of the behaviors that they display and what those behaviors mean in an ethological framework. This is currently rarely done and there is need both for more careful interpretation of rodent behavioral tests and the development of new methods of measuring stress responsiveness (e.g. Blanchard, Griebel, & Blanchard, 2001) if we want to correctly determine the role of specific molecules in the development or maintenance of emotional regulation.

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