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14. ABSTRACT This is our annual report for the project: P11, A Biomarker for Memory Retrieval: A Possible Role in Traumatic Stress. We carried out all of the experiments based on the proposed research design. We tested memory retrieval performance with wild type mice, since p11 knock-out mice were not bred. We found that both footshock and glucocorticoid treatment affect memory retrieval of mice. Mice had impaired performance in the water-maze spatial task after being given footshock for 30 min. In addition, systemic corticosterone was administered to non-stressed mice 30 min before retention testing induced retention impairment. We found that stress results in increases of p11 expression in the wild type mouse brain. Therefore, the experimental procedures were tested and validated in the wild type mice and the role of p11 in memory retrieval will be further determined with p11 knockout mice in our ongoing experiments.					
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INTRODUCTION

Subject:

This is our annual report for the project: P11, A Biomarker for Memory Retrieval: A Possible Role in Traumatic Stress. We have carried out all of the experiments based on the proposed research design with a minor modification. Our pre-clinical and clinical studies have shown the important role of p11 in PTSD (Zhang 2008, 2009. CSTS, News from the center, 2008; CSTS, 2008 annual report; Army Annual report 2009 and Psychiatric News Oct 2, 2009). Therefore, using wild type and p11 knock-out mice, this study will attempt to elucidate the possible molecular mechanism of p11 as a potential biomarker for memory retrieval to facilitate the development of a therapeutic intervention for PTSD. The most common characteristic of Post-Traumatic Stress Disorder (PTSD) is the reexperiencing syndrome, when the patient's memory seems to be fixed on a traumatic event whereas the processing of non-trauma-related memories is often impaired. These alterations in PTSD patients indicate that there is significant impairment of memory including memory retrieval, which is influenced by stress and glucocorticoids. Extensive evidence from animal studies supports the view that stress influences learning and memory. For example, it has been found that the retrieval process of memory is impaired by both stress and glucocorticoids (GC) in rats (de Quervain et al., 1998). They demonstrated that footshock given 30 minutes before memory testing impaired rats' retention performance in a water-maze spatial task, compared to non-stressed controls. This impaired retention performance corresponded to the levels of circulating corticosterone at the time of retention testing. In addition, they showed that the stress-induced retention impairment was blocked by metyrapone, a synthetic inhibitor of corticosterone. Furthermore, systemic corticosterone administered to non-stressed rats 30 min before retention testing induced dose-dependent retention impairment (de Quervain et al., 1998). Over the last decade, many studies have reported abnormal hypothalamic-pituitary adrenocortical (HPA) axis activity in PTSD, but these studies do not always report changes in the same direction. Both higher and lower concentrations of circulating levels of glucocorticoid in PTSD patients have been reported (Yehuda et al., 1995) (Lindauer et al., 2006). For example, Holocaust survivors with PTSD have low urinary cortisol excretion (Yehuda et al., 1995). Another study found high early morning salivary cortisol levels in police officers with PTSD (Lindauer et al., 2006). The precise role of glucocorticoid or traumatic stress in memory retrieval performances is unknown. However, and importantly, recent clinical studies have indicated that the administration of high doses of cortisone shortly after experiencing a traumatic event may prevent the development of PTSD, possibly by impairing the retrieval of the traumatic experience.

Purpose:

We investigated whether glucocorticoid hormones regulate memory retrieval through p11, a calcium-binding protein using p11 knock-out mice. The study has not been reported previously.

Scope:

The hypothesis that p11 may play a critical role in mediating the modulatory effects of glucocorticoids on memory retrieval is based on the following observations: 1) p11 is expressed in the central nervous system (CNS) and up-regulated by dexamethasone (Dex), a synthetic glucocorticoid; 2) stress increases the induction of p11 in the prefrontal cortex (PFC); 3) p11 overexpression is mediated by glucocorticoids acting via two glucocorticoid response elements in the p11 promoter region, indicating that p11 is a possible target for glucocorticoids; 4) p11 mRNA expression is increased in the postmortem PFC of PTSD patients and is also associated with depression (Zhang et al., 2008) (Su et al., 2009).

- **Innovation:** This study will examine the molecular mechanism underlying memory in stress and fill a knowledge gap in the current research on PTSD, which occurs in military personnel. This study will also provide the information facilitating drug development for the treatment of PTSD.
- **Application:** Interventions of p11 gene expression have the potential for use both in military populations (those on active duty, reservists and veterans) and in civilian populations exposed to traumatic stress (natural disasters, vehicle crashes, etc.).

BODY

Task 1: Animal protocol approvals and p11 knockout mice development

Announcement of Concept Award W81XWH-08-2-0202 to Lei Zhang 2008. The Certificate of Environmental Compliance for this project was awarded by the USUHS Environmental Compliance Officer in 2008 and the PI Assurance Document was signed April 29, 2008. Our animal protocol was approved on September 29, 2008. A copy of the IACUC approved animal protocol was submitted to the USAMRMC ACURO for review and got ACURO approval. Since this experiment is using p11 knockout mice and the mice are not available in the market, we have to develop a p11 knockout animal protocol by ourselves. Originally, we were planning to develop p11 knock-out mice at Children's National Medical Center in Washington, DC, considering their low cost and expertise. Unfortunately, they were changing personnel who were responsible for breeding our p11 knock-out mice in the hospital, so we had to search for a new knock-out facility. We have searched several possible companies, but the cost for development p11 knock-out mice is much higher than we had proposed. Fortunately, through long negotiations and discussions of the technical issue on the development of p11 knock-out mice, we found that the Jackson Laboratory can develop p11 knock out mice with our proposed cost. The Jackson Laboratory is an independent, nonprofit organization focusing on mammalian genetics research to advance human health. Their mission is to discover the genetic basis for preventing, treating and curing human disease, and to enable research for the global biomedical community. They have more than 1,400 employees located in Bar Harbor, Maine, and in Sacramento, California and conduct genetic research, providing scientific services and genetic resources to laboratories around the world. They have used wild type and knock-out mice as research tools. Jackson Laboratory is the major provider of knock-out mice in US. Together, we will develop p11 knock-out mice and have customer strain: B6J.129-S100A10tm1Jnw/H EMMA: 02152 & JAX stock #005359, B6.Cg-Tg(Camk2a-cre)T29-1Stl/J for p11 knock-out mice breeding. This will allow us to finally test the role of p11 in memory retrieval, one of the proposed key tasks of our research project.

Task 2 for Aim 1. To determine the effects of pretest administration of footshock stress or systemic corticosterone on memory retrieval performance and p11 expression in the mouse brain. In this experiment, after a footshock or treatment with corticosterone, memory performance was examined using a spatial water-maze procedure. Then, p11 expression levels in the hippocampus, amygdala and cortex of the mouse were determined by Western blot and real-time PCR. This experiment used wild-type mice only till our p11 knockout mice were not available yet. It is expected to that footshock or corticosterone will impair memory retrieval and alter the expression of p11 in discrete regions of the mouse brain. If stress-induced memory retrieval impairment does not alter p11 expression, such findings would indicate that stress-induced retrieval impairment is independent of glucocorticoid-regulated P11 expression. The results will also be confirmed in the proposed study with p11 knockout mice, when they are available in our lab. Briefly, a mouse was placed in a dark shock chamber and is acclimatized for 5 Min. The mouse was then subjected to an inescapable footshock. Each shock (the unconditioned stimulus) is 0.8 mA, and lasts 10 seconds. In the memory test, the animals were placed into the water at and facing the sidewalls of the pool, at different start positions across trials, where they quickly learn to swim to the correct location with decreasing escape latencies by more direct swim paths. The tracking system measures the gradually declining escape latency across trials, and parameters such as path-length, swim-speed, and directionality in relation to platform location. Mice received one training sessions for 10 ds. Each daily session was four training trials followed by a probe trial, which the platform was moved so that it was inaccessible. The mice were placed in the water facing the pool wall at one of four start points (north, south, east, or west). The start points were counterbalanced across trials for all animals. Upon release into the water, the mouse was allowed to swim for 60 sec, at which point the platform was raised to within 1.5 cm of the water surface. After escaping, the mouse remained on the platform for 10 sec before being removed. If the mouse fails to escape, it was guided to the platform and remained there for 10 sec. On completion of training, mice were assigned to an immediate stress or non-stressed group. Stressed mice were stressed with food shock. Non-stressed mice were used as controls. We conducted a probe trial in which the escape platform was removed from the pool and each animal allowed to swim for 60 sec. A well-trained mouse swam to the target quadrant of the pool and repeatedly across the former location of the platform until starting to search elsewhere. This spatial

bias will be used to constitute evidence for spatial memory. We found that footshock resulted a significant decrease in the time spent on target, while producing no effect on time spent on opposite, indicating that stressed mice had significant impaired performance in the water-maze spatial task compared to control in p11 wild type (Fig 1).

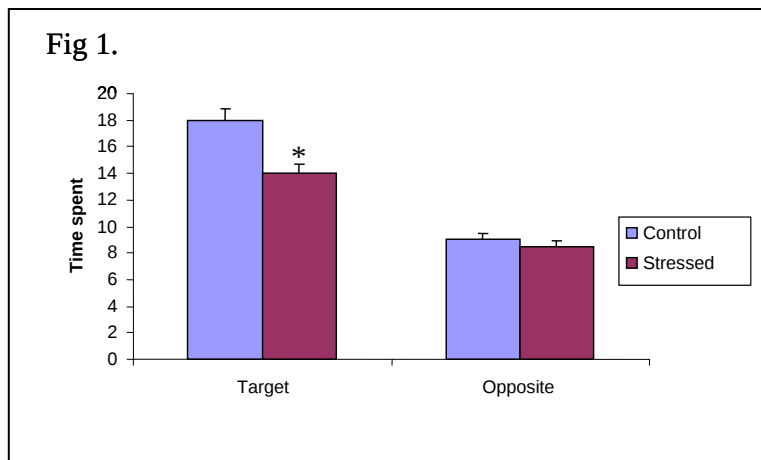


Fig 1. The effect of stress on memory retrieval. Mice had impaired performance in the water-maze spatial task after being given footshock 30 min compared to that of the control in p11 wild type mice. $p < 0.05$

In this experiment, we also carried out the water-maze spatial task performance of animals treated with saline and corticosterone; we found corticosterone treatment resulted significant decrease of time spent, indicating a glucocorticoid-induced impaired memory retrieval performance (Fig 2).

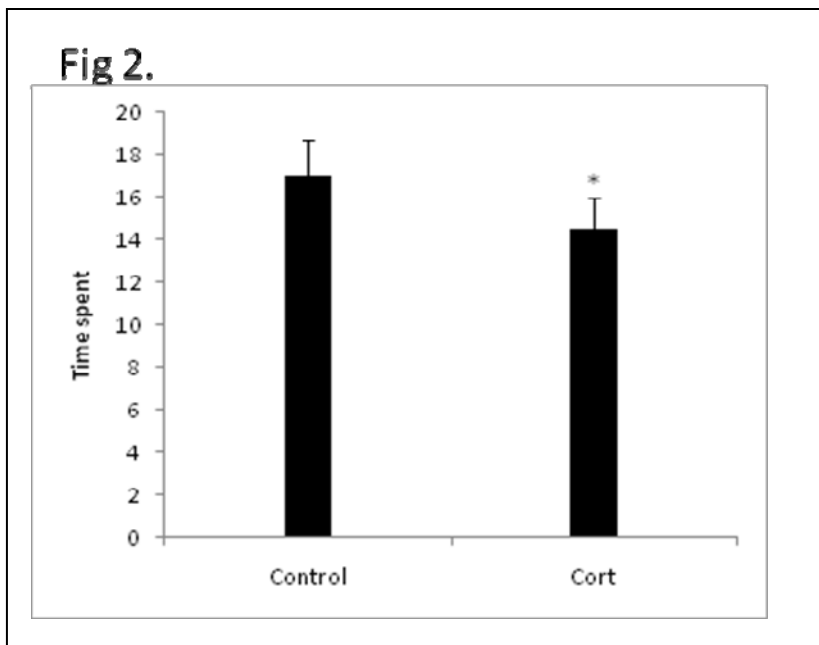


Fig 2. Effect of corticosterone on retention impairment. Cort, corticosterone. $p < 0.05$

We also examined the effect of stress on p11 expression in the hippocampus and amygdala by Western blot. The procedure for this experiment follows in the proposed methods. Briefly, control (n=10) and stressed mice (n=10) were decapitated. The brain was excised from each and sliced coronally using a vibratome. The hippocampus and amygdala were dissected from the slices and placed on dry ice immediately for Western blot and p11 mRNA expression studies. Protein concentration in the samples was determined by Bio-Rad Protein Concentration Reagent (Hercules, CA). Equal amounts of total protein (20 μ g per lane) were resolved in 10%

SDS polyacrylamide gels and blotted onto PVDF membranes for immunoblotting analysis. Protein expression was detected using a 1:500 dilution of mouse anti-p11 monoclonal antibody (BD Transduction Laboratories, Franklin Lakes, NJ) with a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG as a secondary antibody (Bio-Rad Laboratories, Hercules, CA). The density values are presented as means $\pm$ S.D. from three experiments. The density was used to quantify immunoreactivity in terms of percentage of p11 induction relative to control (non-stressed mice).

Using Western blot, we found that stress results in increases of p11 expression in the three brain regions, indicating that p11 was up-regulated by stress exposure in wild type mice (Fig3).

Fig 3. The effects of stress on p11 expression in the brain of wild type mouse

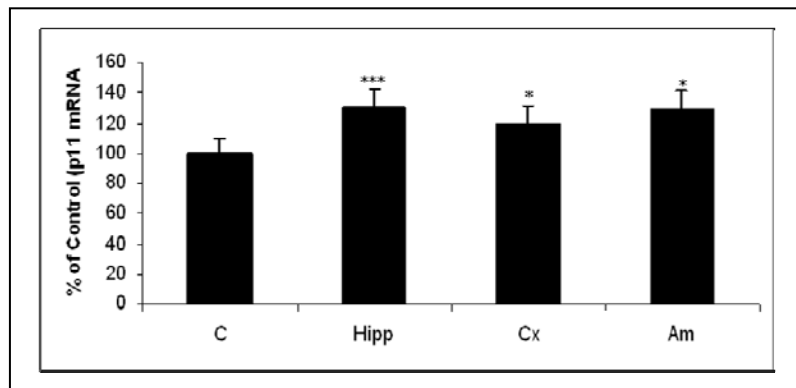


Fig 3. Stress resulted in p11 up-regulation, as determined by Western Blot in the hippocampus and amygdala of wild type mice. The data were analyzed by Student's *t*-test; * $P < 0.05$, *** $P < 0.001$. C, Control; Hipp, Hippocampus; Cx, Cortex, Am, Amygdala.

Task 3 for Aim 2. To determine the effects of footshock on water-maze spatial task performance in p11 knockout mice. This study will determine whether p11 expression is necessary to mediate footshock-induced impairment of memory retrieval in a water-maze spatial task. This task was not performed this year, since p11 knockout has not been developed in our Lab.

Task 4 for Aim 3. To determine whether corticosterone-induced impairment of memory retrieval performance is blocked in p11 knockout mice. In this experiment, p11 knockout or wild-type mice will be treated with corticosterone before retention testing and memory retrieval will be examined using a water-maze spatial task. This task was not performed this year, again, since p11 knockout has not been developed.

Summary of Experimental Results:

- All experiments were conducted in wild type mice. A P11 knockout mouse is developing.
- Footshock and corticosterone significantly decreased the time spent on target and produced no effect on time spent on opposite, indicating a significant impaired performance in the water-maze spatial task in stressed mice compared to control in wild type.
- Stress resulted in p11 protein up-regulation, as determined by Western Blot in the hippocampus, cortex and amygdala of wild type mice.

KEY RESEARCH ACCOMPLISHMENTS May 1, 2008 through Dec 30, 2009

- Animal protocol approvals at USUHS.
- Design and negotiate with companies to develop our own p11 knockout mice with several institutes.
- Examine the effect of footshock and corticosterone on memory preference in wild type mice using

proposed protocol.

- Examine the effect of footshock on p11 protein expression in the three brain regions which are associated with memory in wild type mice using Western Blot.
- P11 antibodies and Western Blot procedure were tested and validated in wild type mice.
- Behavioral procedures for Specific Aim 1, 2 and 3 were tested and validated in wild type mice.

REPORTABLE OUTCOMES

- Oral and poster presentation at the Military Health Research Forum, August 31-September 3, 2009 in Kansas City, MO.

CONCLUSION

We have got our animal protocol approvals at USUHS and first step to develop p11 knockout mice. We also conducted experiments which proposed in Specific Aim 1, 2 and 3 in p11 wild type, although p11 knockout has not been available in the early stage of the study. Our results indicate that besides the well described effects of stress and corticosterone on the acquisition and consolidation processes, stress and corticosterone also affect memory retrieval and p11 protein expression in the hippocampus, cortex and amygdala in mice. Stress and corticosterone resulted in p11 over expression in the mouse brain. Our experimental procedures for knockout mice studies were well tested, and validated in wild type mice.

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