ABSTRACT

The role of offspring in mitigating extreme stress in lactating female mice

Mothers of many species find great comfort in their offspring, which in turn has been found to alleviate mild stress in the mother. In the face of a life-threatening event, however, do offspring still act as effective stress reducers? Using mice as test subjects, I investigated how variable exposure time to pups affects a mother's anxiety levels after being subjected to a major stressor. It was found that exposure to pups alleviates fear and anxiety in lactating females after an extreme stressor. Additionally, mothers who recovered without their pups in their home cage, with only the smell of their pups, experienced the least amount of stress reduction and the highest anxiety levels compared to the other treatment groups. We believe the absence of pups in the home cage created a "double stressor", which added to the stress created by the major stressor, ultimately leading to a greater increase in anxiety.

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COVER SHEET

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The role of offspring in mitigating extreme stress in lactating female mice

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Abstract

Mothers of many species find great comfort in their offspring, which in turn has been found to alleviate mild stress in the mother. In the face of a life-threatening event, however, do offspring still act as effective stress reducers? Using mice as test subjects, I investigated how variable exposure time to pups affects a mothers' stress and anxiety levels after the lactating female is subjected to a threatening event, a forced swim test. The mother mice were subjected to a 2-minute swim test as the major stressor and their anxiety levels were measured using the light/dark box test. It was found that exposure to pups alleviates fear and anxiety in lactating females after an extreme stressor. Additionally, the mothers who recovered without their pups in the original home cage, with only the smell of their pups, experienced the least amount of stress reduction and had the most anxiety compared to the other treatment groups. We believe this absence of pups in the home cage created a "double stressor", which added to the stress created by the swim test, ultimately leading to a greater increase in anxiety for the mother mice. This study may provide insight in the treatment of post-traumatic stress, post-partum depression, and expand the understanding of mothers' responses to stress.

Introduction

A new mother stands on the side of the road, staring blankly at the remains of her totaled car. She is lucky to have made it out alive. She is shaking uncontrollably, head to

toe. She needs to get home, and soon. It is not until she returns home and cradles her baby in her arms that she finally calms down. Can contact between the mother and child really act as an effective stress reliever after a traumatic incident?

Pups have long been known to modify a mouse mother's fearfulness and anxiety when confronted with ordinary stressors (Lonstein & Gammie, 2002), but will pups modify anxiety after a life-threatening event? Having been faced with death, can your offspring enhance your ability to alleviate this stress? This experiment quantified stress relief from infants after a major stressor.

Significant decreases in anxiety were seen in postpartum rats as compared to virgin or pregnant mice, which is associated with pup contact after giving birth. (Lonstein, 2005). It was also found that lactating mice had attenuated responses to stress, as measured by heart rate before, during and after being submitted to a stressor, as compared to control or weaning animals. Lactating females also had the lowest levels of corticosterone as compared to the other groups (Silbolboro-Mezzacappa, Tu & Myers, 2003). Even in humans, it was found that there is a mean decrease in anxiety and depressive symptoms from pregnancy to the postpartum period (Heron et al., 2004).

In a number of studies, reduction in fear and anxiety in lactating females were connected to recent contact with pups. (Hard & Hansen, 1985; Lonstein & Gammie, 2002). This reduction in fear was effectively measured using a light/dark (L/D) box test (Maestripieri & D'Amato, 1991). It was also found that mothers need somatosensory input from their pups in order to experience a reduction in anxiety. Suckling is an important somatosensory cue, but it was found that this is not the only mode of stress relief in the mothers. Distal cues from the pups, such as smell and noise, were not alone

sufficient for lowering stress levels in the mother mice (Lonstein, 2005). These studies have investigated the effect of pups on mothers' anxiety levels, but nothing has been done measuring stress relief after the administration of a severe stressor.

There are many different theories regarding the neurological systems and chemicals at play in this response to stress, including corticotropin-releasing factor (CRF), the [gamma]-aminobutyric acid (GABA) and serotonin systems, neural sites like caudal periaqueductal gray (cPAG), and other systems related to hypothalamic-pituitary-adrenal (HPA) function. The CRF system is known to play a major role in the fear and anxiety response. CRF mRNA is decreased in the brains of lactating versus virgin females and previous work suggests this altered CRF plays a role in how a female responds to a stressor during lactation (Gammie, Negron, Newman & Rhodes, 2004). It has also been suggested that changes in the activity of brain (GABA) and serotonin systems might underlie reduced anxiety and enhanced aggression during lactation (Maestripieri, Badiani & Puglisi-Allegra, 1991). Additionally specific neural sites, such as the cPAG, respond to cues from pups (Lonstein & Gammie, 2002). Overall, the neurochemical basis of stress is not completely understood but likely involves a number of different systems including norepinephrine, oxytocin, vasopressin, and CRF, which are all related to changes in HPA function (Lonstein & Gammie, 2002). Contact with pups also induces changes in these systems, so it seems logical to assume that the modification of stress may be triggered in part by a mothers' contact with her pups.

Maternal anxiety was quantified using the L/D box test. This test is considered a reliable test of anxiety (Maestripieri, Badiani & Puglisi-Allegra, 1991). The L/D box is a 2-chambered apparatus made of plastic that contains a black, enclosed box on one side

with a small opening that faces the light side of the box, which is clear and open to the surroundings. The test begins by placing the mouse into the dark side of the box and then measuring how much time it spends in the dark and in the light, as well as how many times it pokes its head out. There is a natural conflict between the tendency to explore and the tendency to avoid the unfamiliar (Bourin & Hascoet, 2003). Mice usually prefer the dark side of the box because they are nocturnal animals and the dark is familiar to them, whereas, the light side of the box is a novel environment. Therefore, the more time spent on the light side, the more exploratory and less anxious they are considered to be. (Maestripieri, Badiani & Puglisi-Allegra, 1991). So the behaviors on the light side of the box are considered to be a good index of anxiolytic activity (Bourin & Hascoet, 2003).

There is a notable difference in certain behaviors when animals are subjected to moderate and extreme stressors. This is evident in tests done on maternal aggression. It was found that a moderate stressor, such as daily restraint performed hours before aggression testing, has only a modest effect on aggressive behavior of a female towards a male intruder mouse (Gammie & Stevenson, 2006). In contrast, an extreme stressor, like a swim test, significantly decreases maternal aggression behavior when applied just prior to an aggression test (Gammie, Seasholtz & Stevenson, 2008). This would suggest a neurobiological difference in the brains of these mice, depending on whether they receive a moderate or major stressor. The major stressor, a 2-minute swim test, was chosen because it is strong enough to elicit a strong anxiety response in the lactating female mouse, leading to an extinction of normal behaviors in the mouse. In the forced swim test, the mouse is placed in a large beaker of water and swims/floats for two minutes before being retrieved. The swim test can be used in lab to induce high levels of stress in

the mouse, thereby reducing or eliminating the amount of time spent in the light side of the L/D box and reducing aggressive behavior in the mice (Gammie, Seasholtz, & Stevenson, 2008).

This study investigated whether variable exposure time to pups during the lactating mother's recovery from a major stressor impacted her stress and anxiety as quantified by the light/dark box test. Based on the relationship found, examining the neural basis behind this response could lead to advances in the field of behavioral neuroscience. In a larger realm, this study could give insight to the treatment of post-traumatic stress, post-partum depression, and further the understanding of a mother's stress and anxiety responses to contact with her offspring.

It was hypothesized that the longer the mother was exposed to her pups, after being submitted to a major stressor, the lower her stress levels would be. In addition, it was hypothesized that recovery in a home cage will result in a greater reduction of stress, as compared to a clean cage, because the mother will have had indirect contact with her pups in the home cage by being exposed to the their smell.

Methods

Phase I

Phase I was completed in order to determine the amount of time it took for the lactating female to recover from the major stressor and return to their normal behavior. On day 4 after birth, the mice were given a L/D test to establish baseline performance. On day 5, the mice were submitted to a 2-minute swim test and given various amounts of recovery time (either 20, 40 or 60 minutes) in a clean cage without their pups, then given a 5-minute L/D box test to quantify their stress level. The data taken for each L/D box

test included latency to come into the light side for the first time, number of entries into the light side of box, the total time spent on the light side of the box, and the number of times the mouse comes halfway out of the box (head pokes).

Performance after the swim-test was compared to baseline performance to see which recovery time was necessary to return the mothers to normal behavior and performance. This time was to be considered the standard or full recovery time in testing of Phase II.

Phase II

The pups from Phase I were weaned 21 days after birth and were paired with males at 19 days later. They were paired for 2 weeks to become pregnant and the day they gave birth was counted as day 0. On Day 4, each animal received a L/D test to record the lactating females' baseline performance. 67 mice were used in the study, with 13 or 14 mice in each group.

On day 5, every animal was given a 2-minute forced swim test as a standardized major stressor. The lactating females from Day 4 were randomly divided into five groups, allowing for variable recovery time with their pups. The first group spent the 30-minute full recovery time alone in a clean cage, so 0 minutes of contact with their pups. The second spent the full recovery time alone in their home cage, with 0 minutes of contact with pups. The third group spent the first 20 minutes alone in their home cage, and the last 10 minutes of the recovery time with their pups. The fourth group spent 10 minutes alone in the home cage, then the last 20 minutes with the pups in the cage. The fifth group spent the entire 30 minutes with their pups in their home cage. After the full

recovery time (regardless of the exposure time), the mice were submitted to a 5-minute L/D box test.

The L/D tests were scored in real-time with stopwatches and the data was recorded on an Excel spreadsheet. After testing and scoring, difference scores were determined for each individual's performance on Day 5 (test day) versus Day 4 (baseline test). Difference scores were analyzed using the program SigmaStat. The statistical analysis consisted of one way ANOVA. The tests were used to determine the relationship between the three behaviors quantified (number of light entries, the total time spent in the light side of the L/D box, and the time to first light entry) and the amount of recovery time exposed to pups/treatment group.

Results

Phase I Data

After performing statistical analysis on the data from Phase I, it was found that there was no significant difference in performance in the L/D box test between any of the recovery groups, whether they spent 20, 40 or 60 minutes with their pups. There were no significant differences between the groups in terms of time to first light entry ($p=0.242$), total time spent in light ($p=0.291$), number of light entries ($p=0.294$), or number of head pokes ($p=0.690$). Although these results suggested that 20 minutes was sufficient for some recovery from stressor, we adopted a 30-minute recovery time to use in Phase II because this would allow for more manipulations in terms of time spent with or without pups.

Table 1: Light/Dark box results from Phase I showing the mean difference from baseline values for all treatment groups. No differences between groups were found. (Negative numbers refer to a decrease in the time measured for each quantified behavior as compared to baseline performance.)

Recovery Time	Head Pokes	Time to First Light (sec)	Total Time in Light (sec)	Number of Light Entries
20 min	1.28 ± 2.08	-1.94 ± 22.8	13.89 ± 20.52	-0.50 ± 1.09
40 min	3.33 ± 1.66	1.94 ± 20.8	0.72 ± 16.29	-8.78 ± 6.35
60 min	3.69 ± 2.67	53.25 ± 31.4	-25.56 ± 14.68	-2.06 ± 1.77

Phase II Data

Significant results were found in the number of times the mouse entered the light side of the box ($F_{4,62}$=3.452, p=0.013). There were significant differences between the following groups: 30 min. of contact vs. 10 min. (p=0.007), 30 min. vs. 0 min. home cage (p=0.007), 0 min. clean cage vs. 10 min. (p=0.018) and 0 min. clean cage vs. 0 min. home cage (p=0.019) (See figure 1). From the graph, one can see that the 30 min. treatment group showed an increase in the number of times they entered the light side of the box.

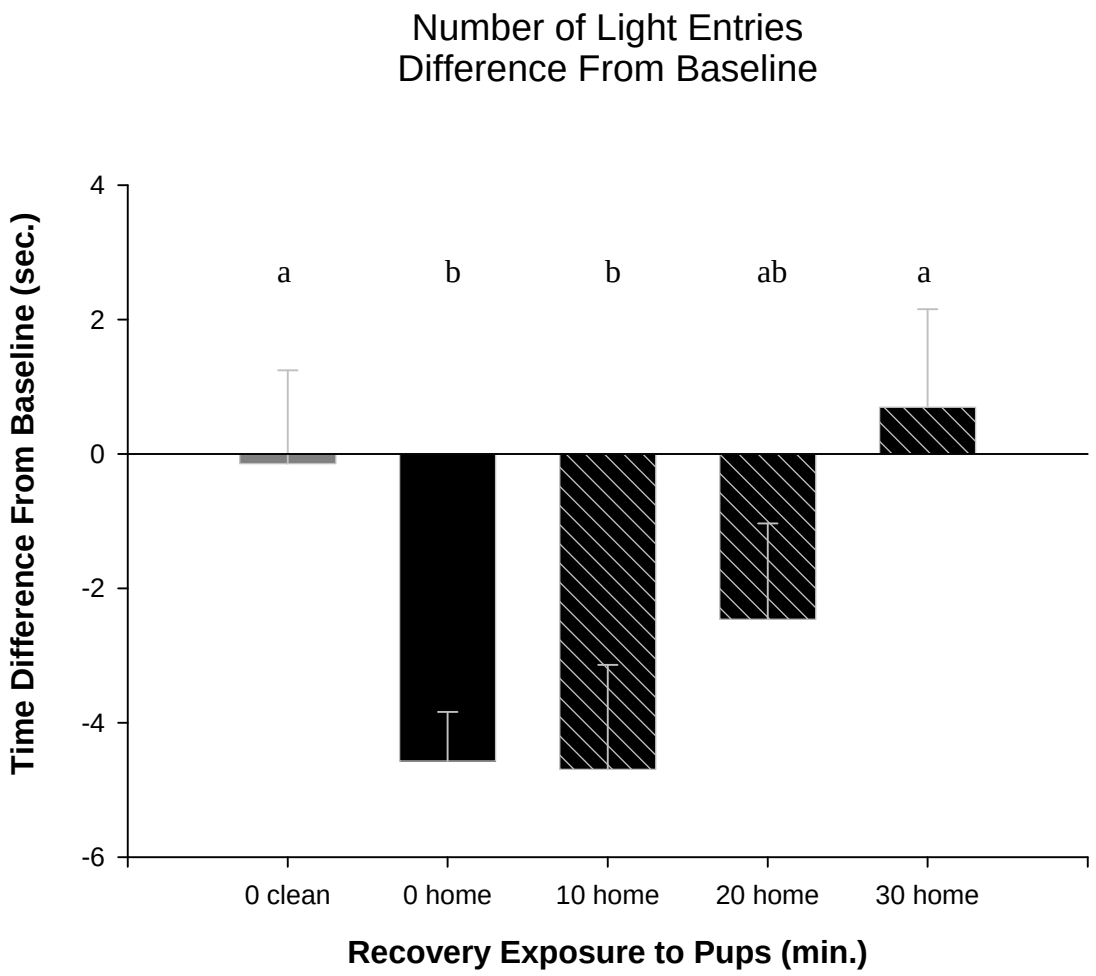

Figure 1: Graph of Number of Light Entries, Time Difference from Baseline vs. Recovery Exposure to Pups. Different letters indicate significant differences (p,0.05) between groups (one-way ANOVA).

There were also significant results in the total time spent on the light side of the box ($F_{4,62}$=4.067, P=.005). 0 min. home cage was significantly different from 0 min. clean cage (p=0.001), 30 minutes (p=0.004), and 20 minutes (p=0.035) and 0 min. clean cage from 10 minutes (p=0.049) (See figure 2). Once again, the 30 min. treatment group showed an increase in the time the spent in the light side of the box. The 0 min. with

pups in clean cage also showed an increase in time spent on the light side of the box. Notice the large difference between recovery without pups in home vs. clean cage.

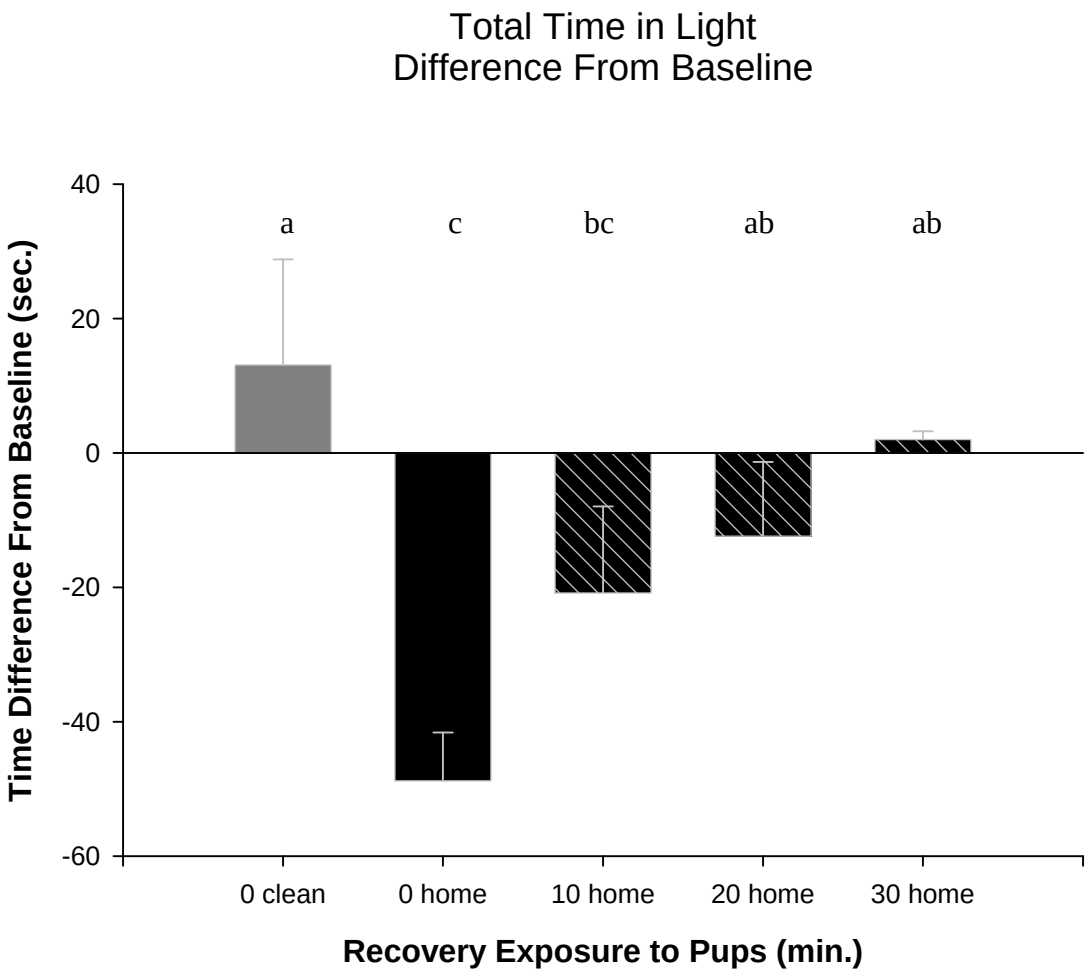

Figure 2: Graph of Total Time in Light, Time Difference from Baseline vs. Recovery Exposure to Pups. Different letters indicate significant differences (p,0.05) between groups (one-way ANOVA).

Non-significant results were found for both number head pokes compared to baseline performance (p=0.110) and latency to enter the light side compared to baseline performance (p=0.443) (See figure 3).

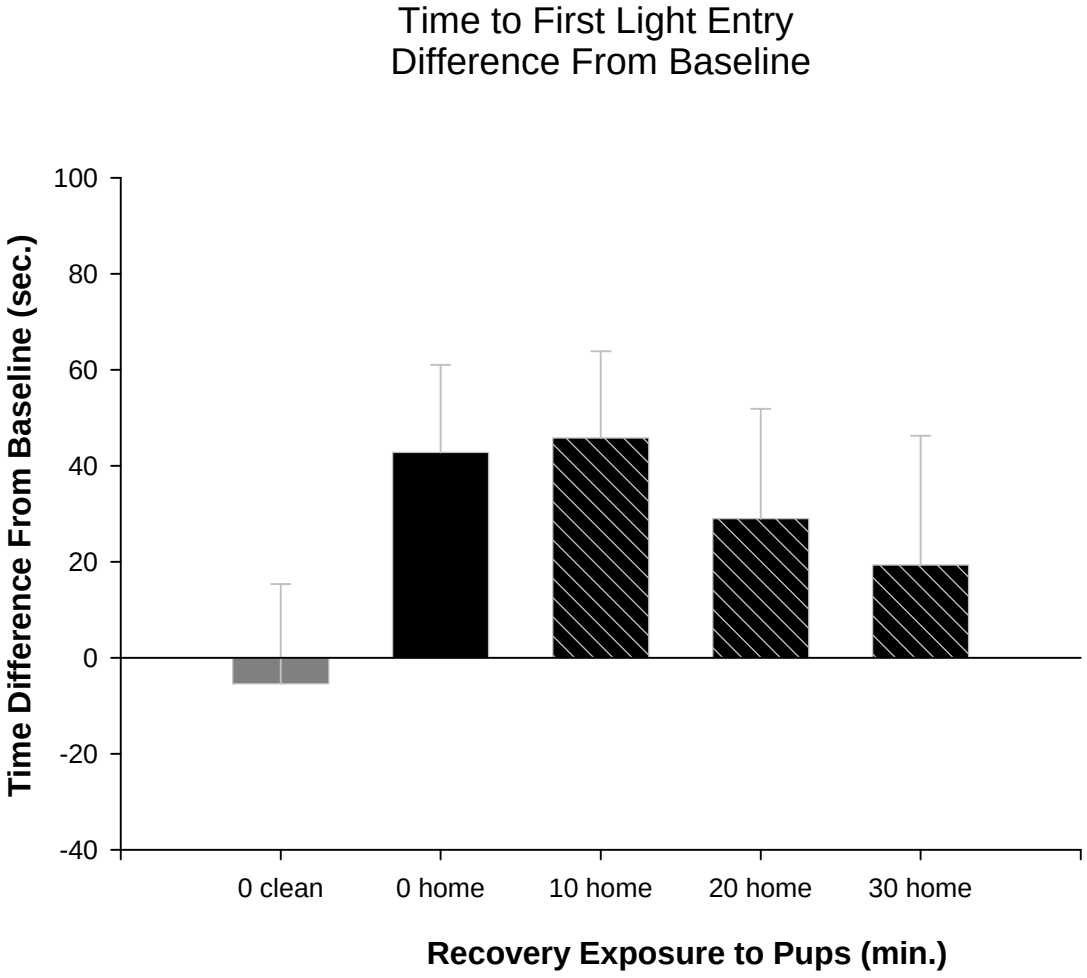

Figure 3: Graph of Time to First Light Entry, Time Difference from Baseline vs. Recovery Exposure to Pups.

Discussion

This experiment investigated how a major stressor greatly alters behavior and how exposure to pups can return that behavior to normal. It was found that pups indeed modified stress levels after a major stressor, as quantified by the L/D box. The 30 min. treatment group showed an increase in both the number of light entries and the total time

spent in the light. This shows that exposure to pups alleviates fear and anxiety in lactating females after an extreme stressor.

Another result that stood out was the group that recovered alone in the home cage compared to a clean cage. It was predicted that the mice that had no exposure to their pups and spent all 30 minutes alone in a clean cage would be the most stressed because they would not receive the stress-relieving effects that accompany contact with pups. But in reality, it was the group who recovered in their home cage without pups present who had the largest decrease in number of light entries and total time in light as compared to its baseline. This indicated that they had the least amount of recovery from the major stressor compared to all of the other groups in the experiment.

The mothers who spent time in their home cage without direct contact with their pups had higher levels of stress than those who recovered without their pups in a clean cage. From this, the hypothesis of the "double stressor" was explored. The absence of pups in the home cage might have added stress on top of the stress created in the swim test, so they had the highest anxiety levels and least stress reduction. The smell of the pups was still in the cage, but they were not physically there. A human example to better explain this stressor is if a mother returned home to her children and her children's toys were there, their snacks were sitting out and the TV on, she would become extremely stressed and worried because her kids were not where she expected them to be.

The mothers that had variable contact with pups had reduced stress, but it is possible that it was actually the elimination of the stress the procedure was unknowingly creating. For example, after the swim test, the mother was put in her home cage alone for 10 minutes. During the 10 minutes, not only did she not get any relief from the stress of

the swim test, but the stress was supplemented by the absence of her pups. By replacing her pups for the last 20 minutes of the recovery, her stress from the swim test may not have been affected, but the stress from the absence of her pups was alleviated, causing the reduction in stress levels measured by the L/D box test.

Overall, it was found that exposure to pups reduces the anxiety of lactating females after being submitted to a major stressor. We know now that pups can act as stress relievers to their mother. But considering the "double stressor" hypothesis, the absence of pups can be a stressor and in turn can modify the mothers' behavior.

From this study, there are many opportunities for future research projects to further investigate the mode of stress relief associated with pup contact and the idea of double stressors. One approach might be to examine TRP2 knockout mice, a strain of mice whose pheromonal sensing channel has been modified (Stowers, Holy, Meister, Dulac & Koentges, 2002). These mice would not be able to receive pheromonal cues from their pups in the home cage and it would be interesting to see if they are less stressed than ones that can sense pup pheromones in the cage. This would solidify the idea of the presence of pheromone and absence of pups as a trigger for stress. Another option would be to eliminate the double stress confound and do the whole experiment with all clean cages to determine the effect of physical contact with pups, with that variable alone. It would also be interesting to look at the neurobiological aspects of this behavior. By using pharmacological approaches, one could see if there are any specific chemicals at work in the connection between stress and pups. In addition, performing a c-fos antibody study after the behavior and analyzing cross-sectional slices would make it

possible to determine the brain areas showing altered activity in association with this behavior.

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