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Cross Sensitization of Depressive-Like Behavior through Two Depression Related Paradigms: Maternal Separation and Its Effect on the Forced Swim Test In the Guinea Pig

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CROSS SENSITIZATION OF DEPRESSIVE-LIKE BEHAVIOR BETWEEN
TWO DEPRESSION RELATED PARADIGMS:
MATERNAL SEPARATION AND ITS EFFECT ON THE
FORCED SWIM TEST IN THE GUINEA PIG

A thesis submitted in partial fulfillment of the
requirements for the degree of
Master of Science

By

AMANDA DANIELLE SCHREIBEIS
B.S., University of Dayton, 2013

2016
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WRIGHT STATE UNIVERSITY

GRADUATE SCHOOL

November 30, 2016

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Amanda Danielle Schreibeis ENTITLED Cross Sensitization of Depressive-Like Behavior Between Two Depression Related Paradigms: Maternal Separation and Its Effect on the Forced Swim Test in the Guinea Pig BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIRMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Schreibeis, Amanda Danielle. M.S. Department of Neuroscience, Cell Biology, and Physiology, Wright State University, 2016. Cross Sensitization of Depressive-Like Behavior through Two Depression Related Paradigms: Maternal Separation and Its Effect on the Forced Swim Test In the Guinea Pig

Early-life stress such as parental neglect, absence, or abandonment, has been hypothesized to increase the susceptibility for developing depression later in life via sensitization of stress-responsive physiological systems (e.g., pro-inflammatory cytokines, hypothalamic-pituitary-adrenal axis). Guinea pigs offer a potential model, but study has been limited to behavioral observations obtained during maternal separation tests. This thesis examined the generalization of this response by asking whether it would cross-sensitize to behavior in another depressive-related paradigm, the forced swim test. In three experiments, pups underwent three forced swim trials, in shallower or deeper water, 24 h or 9 days after 3-h separation period(s). Immobility duration and latency served as the primary dependent measures. I observed cross sensitization of depressive-like behavior (longer duration of immobility) when pups were tested in the deeper water forced swim test 24 h following maternal separation. Results further confirm use of the guinea pig separation model and suggest sensitization of an underlying depressive-like state rather than particular depressive-like behaviors.

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I. INTRODUCTION

Melancholia, translated from the Greek “black bile”, has long been a devastating mental illness in humans. The Center for Behavioral Health Statistics and Quality (2014) have estimated 7.1% of the United States population, ages 18 to 55, suffer from the disease now known as major depressive disorder (MDD). The symptoms of MDD such as general hopelessness, anhedonia, low energy or fatigue, and changes in appetite or sleeping habits may eventually lead to suicide if not treated (Hariri & Weinberger, 2009; Nierenberg, Gray, & Grandin, 2001; Voracek & Loibl, 2007). Although identified in the days of Hippocrates, there is still no defined cause for this disease and the treatments are largely symptomatic. The morbidity rate and inability to “cure” has made this disease particularly distressing not only to the afflicted but to their family, friends, and surrounding community members.

Development of major depressive disorder is attributed to both biological and environmental factors. Modern meta-analyses have estimated heritability for MDD upwards of 55%, with women being at higher risk than men to develop the disorder (Kendler, K., Gardner, C., Neal, M., & Prescott, C., 2001; Kendler, K., Gatz, M., Gardner, C., & Pedersen, N., 2006; Voracek & Loibl, 2007). Familial study data have also shown a highly significant rate of occurrence of depression between first-degree relatives providing further evidence of the genetic component of major depression (Sullivan, Neale, & Kendler, 2000). There are many hypothesized mechanisms to explain the observed genetic influence. One such mechanism

implicates the protein involved in reuptake of serotonin into a neuron, specifically the serotonin-reuptake transporter (5-HTT). The gene responsible for this protein exists in several alleles, most commonly a short "S" or long "L" allele. Those homozygous for the S allele produce less 5-HTT product than either S/L or L/L individuals (Lesch et al., 1996; Hariri et al., 2002). The finding of those homozygous for the S allele experiencing greater amounts of MDD symptoms compared to L/L homozygotes supports the 5-HTT mechanism (Caspi et al., 2003; Levinson, 2006; Wurtman, 2005).

Another mechanism involves a gene product responsible for the vesicular degradation of serotonin, Monoamine Oxidase A (MAOA). The typical function of the MAOA enzyme is breaking down of vesicular serotonin as well as norepinephrine and dopamine. A polymorphism in the MAOA gene's regulatory region can result in a more active enzyme. Heightened enzymatic activity amplifies break down of serotonin, contributing to development of depression and other affective disorders (Levinson, 2006). There also are data implicating mutations in the enzyme tryptophan hydroxylase (hTPH2), the rate-limiting enzyme for synthesis of serotonin. A polymorphism of this protein causes a significant decrease in serotonin production, possibly contributing to unipolar major depression (Harari & Weinberger, 2009; Levinson, 2006; Wurtman, 2005; Zhang et al., 2005). These findings have significantly furthered understanding of biological mechanisms in MDD and other affective disorders.

Environmental factors also play an integral role, together with genetics, in a range of psychiatric disorders. Stress, both physical and mental, is the most

recognized environmental factor for promoting MDD (Reinherz, Giaconia, Carmola Hauf, Wasserman & Silverman, 1999; Gilmer & McKinney, 2003; Levinson, 2006). Stress is a physical or imagined stimulus that disturbs or threatens to disturb homeostasis. In humans, stressors can range from physical, such as incurring an injury, to psychological, such as death of a loved one or work place difficulties. These stressors can lead to physical symptoms (i.e. aches and pains, insomnia, fatigue) along with mental symptoms associated with MDD (decreased self-esteem and hopelessness). Stressful life events are usually intermittent and when times of particularly high stress pass, mental and physical signs of depression may subside. However, the vast majority of those diagnosed with depression do not experience such relief in the aftermath of these stressors. This persistence of stress among MDD patients after stressors conclude indicates environmental factors have lasting effects, but are not the only factors contributing to the development of MDD.

Most psychiatrists today accept a gene x environment interaction as a model for the development of MDD. Various studies show those already genetically vulnerable can develop more-severe symptoms of MDD when exposed to one or more significant life stressor (Agid et al., 1999; Kendler, K., Kuhn, J., & Prescott, C., 2004; Wurtman, 2005; Mann & Currier, 2010). Caspi et al. (2003) published the most compelling evidence of this interaction. Eight hundred forty seven subjects were tested and categorized into groups based upon their serotonin reuptake protein genotype: S/S, S/L, and L/L. All subjects reported stressful events that happened to them over the past year and current psychological diagnosis, if any. A majority of S/S subjects with at least one stressful life event reported developed

symptoms or were diagnosed with MDD within a year of the stressful event.

Moreover, most S/L and L/L subjects did not develop symptoms of depression nor were they diagnosed with any depressive disorders. Such findings have furthered understanding of genetics and environment as cofactors for developing depression (Wong & Licinio, 2001; Hariri et al., 2002; Caspi et al., 2003; Eley, et al., 2004; Flint & Kendler, 2014).

Stress has a profound effect on physical and mental health of children as well as adults. In the past century, it has become clear that early life stress (ELS) promotes depression in infants and preadolescent children. ELS, specifically neglect, abuse, or loss of a social attachment figure, is strongly associated with childhood depression (Bowlby, Robertson & Rosenbluth, 1952; Robertson, 1953; Bowlby, 1960). French psychologist Rene Spitz was the first to observe the integral role early attachment-figure loss played in promoting depression during infancy. During brief periods of maternal separation, infants would display active/protest behaviors (e.s, crying) as an attempt to reestablish contact with the mother. If separations were prolonged, active behaviors typically transitioned into passive/depressive-like behaviors (i.e. lack of engagement with either social or physical environments, little to no crying, etc.; Spitz, 1946). Upon the return of the mother, the depressive-like behaviors would subside and the child would become content. However, if separation from the mother persisted, depressive-like behaviors would worsen. Spitz would go on to coin the term “anaclitic depression” for such effects in children.

Depressive- like behaviors in infants observed by Spitz and Bowlby were the immediate reaction to the loss of a care giver. Since these first experiments, there

has been an increasing number of studies examining stress experienced during childhood as a predictor for later psychopathology (Brewerton, 2007; Windom, DuMont, & Czaja, 2007; Kessler et al., 2008; Brodsky & Stanley, 2008; Martins, Tofoli, Baes & Juruena, 2011; Juruena, 2014). Such experiments have revealed ELS exposure before adolescence to be associated with a vast range of psychiatric disorders including PTSD, generalized anxiety, substance abuse, and MDD (Martins et al., 2011; Juruena, 2014; Nemeroff, 2016). However, human experiments are largely correlational in nature, with subjective reporting of past stressful events. With these constraints, a definitive, causal relationship can be difficult to demonstrate.

Animal models, including non-human primates such as chimpanzees and marmosets, have addressed this problem. Non-human primate studies have identified numerous immediate and long-term consequences of ELS inducing significant behavioral changes comparable to depression in humans (Kaufman & Rosenblum, 1967; Mineka & Suomi, 1978; Gilmer & McKinney, 2003). In the early 1970s, infant monkey experiments revealed immediate effects strikingly similar to those observed by Spitz in human infants thirty years prior. After an initial, active period displaying distress behaviors (i.e. screaming, aggression towards experimenters, & physical attempts to regain the mother) the infant monkeys started demonstrating depressive-like characteristics (hunched body posture, little to no movement, social withdrawal, no vocalizations, etc.; Kaufman & Roseblum, 1967). These depressive-like behaviors would not subside until the mother and infant were reunited. Subsequent longitudinal studies found that even short

separations during the first year of life had significant effects months or years later on social behavior and ability to cope with psychological stressors (Mitchell, Harlow, Griffin & Moeller, 1967; Hennessy, 1986).

Non-human primate studies, though informative, soon fell out of favor due to expense and animal welfare concerns regarding separating infant monkeys for such prolonged periods. Alternative animal models were developed for further research including mice, rats, and guinea pigs. Unlike the more commonly used rodent species (i.e. mice & rats) which are altricial at birth, guinea pigs are born fully furred, with eyes and ears open, and can locomote within minutes. Though guinea pigs are not weaned until around 25 days of age, newborn pups are able to nibble solid food and can drink from a water bottle a couple days after birth (Harper, 1976; Schiml & Hennessy, 1990). Guinea pigs can also form a complex social structure, in which social bonds, including filial attachment, buffer stress responses throughout infant, adolescent, and adult life (Sachser, 1986; Sachser, Dürschlag & Hirzel, 1998; Hennessy, O'Leary, Hawke & Wilson, 2002; Hennessy, 2003; Hennessy, Kaiser & Sachser, 2009). Of particular importance, is guinea pigs exhibit many behavioral similarities to primates in regards to maternal separation, including a two-stage, active/passive behavioral response. When isolated in a novel environment, pups display behaviors (i.e. high rates of vocalization and movement) to re-establish contact with the mother. After an hour or so, they quiet and assume a hunched or crouched posture, with their eyes closed, and piloerection over most of the body (Hennessy, Long, Nigh, Williams & Nolan, 1995; Hennessy, Paik, Caraway, Schiml & Deak, 2011). The passive/depressive-like response occurs within hours in guinea

pigs in contrast to days or weeks in primates. However, these effects do not occur if the pup and mother are together in the unfamiliar environment, which is analogous to findings in non-human primates (Mineka & Suomi, 1978; Hennessy, Deak, Schiml-Webb, Carlisle & O'Brien, 2010). This shows the separation from the filial attachment figure (i.e. the mother) mediates the passive/depressive-like stage. These behavioral characteristics of the guinea pig differentiate them from other laboratory rodents; making them ideal alternatives to primates for studies of the effects of disruption of attachment at an early age.

In recent years it has become clear that stressors, such as maternal separation, can induce a systemic proinflammatory reaction involving the innate immune system. Cytokines are small peptide signalers which play an integral role in cell-to-cell communication and the innate immunity response. Pro-inflammatory cytokines [e.g. interleukin-1 (IL-1), IL-6, tumor necrosis factor- α (TNF- α), & interferon- α (INF- α) among others] are secreted from monocytes/macrophages and microglia upon detection of antigens and stressors, triggering the acute phase immune response. Physiological changes (i.e., fever, increases in liver protein production, & HPA axis activation) accompany behavioral changes. Behavioral changes include reductions in feeding, drinking, socio-sexual activity, and overall interaction with the environment, as well as seeking of warmth, shivering, piloerection, sleepiness, cognitive impairments, and assumption of a hunched posture (Hart, 1988; Yirmiya, 1996; Hennessy, Deak, & Schiml-Webb, 2010b). These behaviors are typically adaptive for animals exposed to pathogens by conserving energy and promoting fever, thus hastening physical recovery. In addition, sick

animals can appear “depressed”, disengaging from their environment, being asocial, and projecting an image of sadness.

Moreover, current evidence suggests that stress-induced inflammatory mechanisms can produce depression in humans. Several sources indicate depressed patients have heightened concentrations of circulating, proinflammatory cytokines, specifically TNF- α and IL-6, in comparison to their non-depressed counterparts (Kronfol, 2002; Dowlati et al., 2010; Dantzer, O’Connor, Lawson & Kelley, 2011; Miller, Haroon, Raison & Felger, 2013). An abundance of findings suggest social stressors (conflict, threat, isolation, or rejection) experienced in adolescence and adulthood are associated with increasing levels of inflammatory activity (Herbert & Cohen, 1993; Segerstrom & Miller, 2004; Kiecolt-Glaser, Gouin & Hantsoo, 2010; Slavich & Irwin, 2014). Furthermore, recent evidence indicates early-life psychosocial stress produces lasting elevations in inflammatory activity, and that these effects may be linked to depression later in life (Danese, Pariante, Caspi, Taylor & Poulton, 2007; Carpenter et al., 2010; Miller & Chen, 2010, Yusko-Osborne, Schiml, Hennessy, & Deak, 2014).

Exposing laboratory animals, like the guinea pig, to stressors such as maternal separation has been found to elicit responses that parallel those in humans including increased proinflammatory activity, fever, and depressive-like behaviors (Maier & Watkins, 1998; Hennessy et al., 2004; Hennessy, Deak, Schiml-Webb & Barnum, 2007; Yusko, Hawk, Schiml, Deak & Hennessy, 2012; Hennessy et al., 2015). In the guinea pig, both the depressive-like behavioral response and the proinflammatory activity sensitize with repeated exposure to separations. For

example, pups separated on two consecutive days and then nine days later, showed more depressive-like passive behavior and increased fever responses during the second and third separations compared to the first (Hennessy et al., 2010b; Schneider, Schiml, Deak, & Hennessy, 2012). In addition, Yusko-Osborne et al. (2014) found that naproxen, a commonly used anti-inflammatory agent, attenuated both depressive-like responses and fever during repeated maternal separations. These results suggest sensitization of inflammatory factors may contribute to the sensitization of the depressive-like responses as well. However, until recently these results have been limited to observations using only the response to maternal separation as an indicator of depressive-like behavior. This study seeks to examine if sensitization of depressive-like behavior can be generalized to another depression-related paradigm, the forced swim test.

The forced swim test (FST) has been implemented successfully for many years as a screen for anti-depressant drug effectiveness. When first applied, the experimenters observed that if a rat is forced to swim in a small, enclosed space from which it cannot escape, it will eventually cease attempting to escape and become immobile apart from small movements necessary to keep its head above water. This originally was interpreted as the rat perceiving escape as impossible, and therefore remaining immobile as a result of despair/depressive-like behavior (Porsolt, Le Pichon, & Jalfre, 1977; Porsolt, Anton, Blavet, & Jalfre, 1978). Recently however, this explanation has come under scrutiny. Some studies have viewed immobility in the mouse/rat is now commonly viewed as an adaptation, or learned behavior, used to confront an acute stressor, not an indication of depression

(Molendijk & de Kloet, 2015). Despite the controversy among the scientific community on this issue, the FST is still a selective and efficient anti-depressant screening tool in the research and pharmaceutical communities.

While the forced swim test has been used extensively in both rats and mice, we are only the second laboratory to employ it with the guinea pig (Wicke et al., 2007). In the past, our laboratory has observed sensitization of depressive-like behavior during repeated maternal separation. To examine if sensitization of depressive-like behavior is test specific or if it can be generalized across paradigms I asked if previous maternal separation would increase immobility in the forced swim test. The majority of the methods employed were based on those of Wicke et al.'s (2007), such as water depth, water temperature, and testing container size. In addition to these, pilot data determined three forced swim trials produced more reliable levels of immobility compared to two as in Wicke et al. The overall goal of the initial experiment was to determine if cross-sensitization of depressive-like/passive behavior would occur over a long term period (9 days) consistent with our laboratory's previous results of sensitization of depressive-like behavior over repeated separations. Experiments 2 and 3 were then conducted to further test sensitization of depressive-like behavior over a shorter time period (24 h) in separated pups. These experiments only differed in whether pups could touch the bottom of the tank.

II. METHODS

Animals

Albino Hartley guinea pigs (*Cavia porcellus*) were bred and housed in our laboratory. Mother and litter were maintained in an opaque plastic cage (73 x 54 x 24 cm), with a wire front, containing sawdust bedding. Food and water were available *ad libitum*. The colony room was kept at ~21° C on a 12:12 light/dark schedule with lights on at 0700h. Pups were kept continuously with the mother with the exception of behavioral testing to be described below. In each of the three experiments, 8 male and 8 female pups were tested in either of two conditions (n=32 each experiment). All procedures were in compliance with NIH guidelines and were approved by the Wright State University Laboratory Animal Care and Use Committee.

Behavioral Testing

Separation. Both male and female pups were assigned to separated (SEP) or non-separated (NSEP) conditions with only one pup per litter assigned to a particular condition. Pups in the NSEP condition remained in their home cage with mother and siblings while pups in the SEP condition were transported in a clean carrying cage to an adjoining observation room. Each SEP pup was placed into an empty, clear, plastic test cage (47 x 24 x 20 cm) individually for 3 h. Behaviors were observed during minutes 0-30, 60-90, and 150-180 from behind one-way glass by

observers trained to a minimum of 85% inter-observer reliability. For each 1-min interval, the observer noted if the pup engaged in any of the three passive, depressive-like behavioral categories characteristic of separated pups: (1) distinctive crouched stance with feet pulled close to body-sometimes transitioning into lying on the cage floor with the trunk supporting the body (crouch/lying down); (2) complete or near complete closure of one or both eyes for greater than 1 s (eye close); (3) piloerection over more than half of the body (piloerection). The number of 1-min intervals in which a pup displayed all three passive behaviors (crouch/lying down, eye close, and piloerection) were counted as the main measure of “full passive” in each of the three experiments (Figure 1). In addition, “whistle” vocalizations were tallied using a hand-held counter. A microphone positioned within 15 cm of the test cage broadcast the sound to headphones to assist the observer. All separation test were conducted between the hours of 0800 and 1200.

Forced Swim Test. Animals in both NSEP and SEP conditions underwent forced swim tests for three consecutive trials at 24-hour intervals. A clear, glass cylinder (20 x 45 cm) was filled with $30 \pm 1^\circ\text{C}$ fresh water (Figure 2). Each trial lasted five min and was recorded via a Logitech c920 webcam positioned directly above the testing area. Using the recordings, behaviors were scored via a personalized Java Script program by a trained observer. The main measures were total immobility time and latency until immobility was observed. Immobility was defined as the guinea pig maintaining a near stationary posture with the following characteristics: (1) holding its hind legs in an outward direction to maintain balance and (2) its front paws no more than slightly moving to keep the nose above water.

Total climbing time and total swimming time were also monitored. Climbing was defined as the pup floating and actively attempting to climb out with front paws extending above the water. Swimming was identified as the pup pedaling through the water, with all four paws under the surface of the water. Immediately following each forced swim test, the pup was placed in a drying cage placed on a heating pad for 30min. The cage contained two clean, dry towels positioned under an infrared heat lamp (120 watts). All FSTs were performed between 0800h and 1200h.

Experiments

Experiment 1

Male and female pups assigned to the SEP condition were separated twice between 20 and 23 days of age, the second separation occurring 24h after the first. Pups in both SEP and NSEP conditions were exposed to three forced swim trials the first beginning 9 days following the second separation test at 24 h intervals. Water depth was set at 20 cm, allowing the vast majority (~90%) of pups of this age to barely touch the bottom of the tank with one foot.

Experiment 2

Pups in the SEP condition were separated only once between 21 and 23 days of age. Both conditions were tested over three trials, the first occurring 24 h \pm 30 m after the separation. To accommodate pups being tested at a younger age the water depth was decreased to 18 cm. This enabled all pups to easily touch the bottom of the tank with one foot firmly supporting their heads above water.

Experiment 3

The separations and forced swim trials were kept consistent with Experiment Two with the exception of water depth. Water depth was increased 23 cm, preventing any of the pups from touching the bottom of the tank with either feet.

Data Analysis

Passive behaviors during separation data analysis were examined using a 2 x 2 repeated measures ANOVA. Forced swim test data was initially examined using a repeated measures 2 x 2 x 3 ANOVA (group x sex x day). If sex was not found to be significant data was grouped for a 2 x 3 repeated measures ANOVA (group x day). Latency to immobility was further analyzed using independent samples Mann-Whitney over concerns for normality of distribution. For all experiments a probability level of $p < 0.05$ was accepted throughout. Forced swim test and separation statistical analyses were conducted using SPSS Statistics for Windows [Version 23.0. Armonk, NY; IBM Corp.].

III. RESULTS

Experiment 1

The 2 x 2 ANOVA revealed an increase in full passive behavior from the first to second separation, $F(2, 16) = 6.32$; $p < 0.05$, (First Separation: $M = 11.88 (\pm 3.41)$, Second Separation: $M = 17.81 (\pm 2.84)$). This increase in full passive behavior indicated sensitization with repeated separations in pups. Pups vocalized at a high rate during both separations (First Separation: $M = 1798.06 (\pm 375.54)$, Second Separation: $M = 1178.81 (\pm 286.72)$), showing a slight, but not statistically significant, decrease in active behavior. Overall, sensitization of full passive, but not active, behavior occurred in SP pups.

Analysis of our main measure during the forced swim, immobility duration, revealed a main effect of Day, $F(2, 56) = 9.54$; $p < 0.001$ (Fig. 3). This result reflected a general increase in total immobility time over the course of the three trials. There was no indication of an effect of previous separations or sex on a pup's time spent immobile. Analysis of our secondary measure during the forced swim, immobility latency, via Mann-Whitney U tests showed no effects of Sex or Group. Latency data were therefore combined and examined for effect of Day via a Friedman ANOVA. This test revealed no significant difference across days. Analysis of climbing duration data yielded a significant effect of Day, $F(2, 56) = 5.46$; $p < 0.001$, indicating pups showed a general decrease in time spent climbing over the three

forced swim trials. There were no effects of Sex, Group, or Day for total swimming time.

Results in Experiment 1 indicate that pups show a general increase in time spent immobile in conjunction with a decrease in time spent climbing over three forced swim tests. In contrast to these findings, latency to immobility and total swimming time remained consistent in all pups, regardless of prior separation.

Experiment 2

Analysis of immobility duration data yielded a main effect of Day, $F(2, 56) = 28.65$; $p < 0.001$, again showing a general increase in time a pup spent immobile over the three consecutive forced swim trials (Fig. 4). In addition to the main effect, analysis revealed a significant interaction between Day and Sex, $F(2, 56) = 3.76$; $p < 0.05$. Male pups increased immobility time over each trial culminating at the highest level during Trial 3. In contrast, female pups increased immobility time reaching a plateau during Trial 2 then decreasing in Trial 3 (Table 1). Immobility latency yielded an effect of Day, $p < 0.001$, indicating all pups regardless of sex or previous separation, generally became immobile earlier over the three forced swim trials. Total swimming time showed a main effect of Day, $F(2, 56) = 14.60$; $p < 0.001$, reflecting a general decrease in time spent swimming by all pups over consecutive forced swim trials. Analysis of total climbing time revealed no significant effects.

Overall in Experiment 2, pups increased time spent immobile, decreased time spent swimming, and went immobile earlier over the course of successive forced swim trials. The main passive/depression-like behaviors, immobility duration and latency, were seen in greater amounts than in Experiment 1 but there was still no

effect of previous separation. Another observation of interest was the interaction between Sex and Day in pups on immobility duration. Analysis showed males typically showing greater immobility than females across the forced swim trials.

Experiment 3

Immobility duration analysis revealed a main effect of Group, $F(1, 28) = 4.59$; $p < 0.05$ (Fig. 5). This finding indicated pups exposed to separation prior to forced swim showed greater immobility than non-separated pups. This result was unlike previous experiments which found only a main effect of Day. Immobility duration analysis also showed a main effect of Day, $F(2, 56) = 35.34$, $p < 0.001$. Analysis of immobility latency data yielded an effect of Day, $p < 0.001$, consistent with previous experiments. Swimming duration also revealed a main effect of Day, $F(2, 56) = 14.66$; $p < 0.001$, with pups showing a general decrease in time spent swimming over the three consecutive trials. There were no significant effects on total climbing time. Overall Experiment 3 results reflect that pups previously separated spent more time immobile compared to non-separated pups.

IV. GENERAL DISCUSSION

The goal of the current investigation was to examine the connection between early-life stress and later life onset of depression. Previous experiments found that if guinea pig pups were separated from their mothers in a novel environment, they showed physiological stress responses (e.g. increased fever, activation of the HPA axis) and depressive-like behavior akin to that shown by non-human primate infants. The depressive-like behaviors increased, or sensitized, if the pup was separated repeatedly. However, it has remained unclear whether maternal separation only sensitizes depressive-like behavior or if the behavior reflects an underlying depressive-like state. To address this, we asked whether sensitization occurred between responses during maternal separation and forced swim. If such sensitization occurred, it would suggest an underlying depressive-like state, possibly mediated by a proinflammatory mechanism, and not sensitization of a particular behavioral response.

In Experiment 1, pups in the separated condition underwent two, 3-h maternal separation periods, the second occurring 24 hours after the first, while non-separated pups remained in the home cage. Across separations, pups displayed increasing amounts of passive/depressive-like behaviors (i.e. piloerection, eye closure, crouching, and lying down) as expected. Nine days following the second separation period, pups in both conditions were observed over three forced swim

trials. Regardless of previous separation, all pups exhibited increases in both depressive-like behaviors (increased immobility duration and reduced latency) from the first to the third trial. However, there were no significant differences in immobility duration or latency between those pups previously separated and those which remained in their home cage. Thus while sensitization occurred within the forced swim trials, there was not cross-sensitization between the two depression-related paradigms.

In Experiments 2 and 3, the time between separation and forced swim was reduced to 24 hours as there was no evidence of long-term cross sensitization occurring during Experiment 1. Pups in the separated condition were separated only once over a 3 h period. We also considered the possibility of water depth during the forced swim affecting the depressive-like behaviors of immobility duration and latency. In previous mouse and rat studies, experimenters observed greater immobility duration and reduced latency to immobility when water depth was increased enough to prevent animals from touching the bottom of the testing tank (Cryan, Valentino, & Lucki, 2005; Castagne, Moser, Roux, & Porsolt, 2010; Bogdanova, Kanekar, D'Anci, & Renshaw, 2013). Therefore, in contrast to Experiment 1, in which some guinea pigs could touch bottom and others could not, we systematically examined two water depths, one in which pups could touch the bottom of the testing tank (Experiment 2, 18 cm), and another where none of the pups could touch (Experiment 3, 23 cm).

At the decreased water depth in Experiment 2, pups of both conditions displayed similar increases in depressive-like behavior across forced swim trials.

However, there were no significant differences between the separated and non-separated groups, in either measures of duration of immobility or latency to immobility. Therefore there was no evidence to support cross-sensitization. Moreover, pups in both conditions also showed substantial increases in active behaviors of swimming and climbing, as compared to Experiment 1.

Experiment 3, in which water depth was increased, was the only experiment in which cross-sensitization between maternal separation and forced swim was observed, as previously separated pups were immobile longer within each forced swim trial compared to non-separated pups. Over the course of these three experiments we observed several noteworthy findings. In Experiment 1, we found the passive/depressive-like response of guinea pigs showed sensitization across repeated maternal separation, as evidenced in several reports (Hennessy et al., 2010a; Schneider et al., 2012; Yusko et al., 2012). Comparing Experiment 2 to Experiment 3 raises the possibility that behavior in forced swim was affected by water depth. At a water depth at which pups could touch bottom/stabilize themselves (Experiment 2), pups showed appreciably less depressive-like behavior and more active swimming and climbing than did pups in Experiment 3, in which water was deep enough that none could touch the bottom of the testing container. Moreover, this finding is in agreement with those of previous studies in rat and mouse models (e.g. Castagne et al., 2010; Bogdanova et al., 2013), in which researchers observed similar decreases in depressive-like behavior when animals were able to touch bottom of the testing tank.

We are only the second lab to test guinea pigs using the forced swim procedure. While we did model our methods on Wicke et al (2007) methodology, our results, particularly for immobility latency, differed greatly. Whereas those authors observed an increase in latency from 21 to 75 seconds from the first to the second daily, 5-min trial, subjects in the current study exhibited a decrease from 195 to 75 seconds across the first two daily, 5 min trials in Experiment 3, the experiment that most resembled Wicke et al (2007) in other details. The explanation for this discrepancy is not clear and must await further study.

However, the core result of the current study was that pups separated from their mother in Experiment 3 displayed significantly greater depressive-like behavior (i.e. immobility duration) during the forced swim than did non-separated pups. This observation is indicative of cross-sensitization. This is the first observation of depressive-like behavior in guinea pigs becoming sensitized across distinct paradigms. Essentially this indicates the increase in depressive-like behavior is not conditioned as the animal experiences the stress repeatedly, but is evidence of an underlying depressive-like state occurring and intensifying with each stress incurred.

Conversely, findings of Experiment 1 did not indicate cross-sensitization occurring over a long-term period. This could have been due to the fact that some guinea pigs could touch the bottom of the testing tank. But until future studies can demonstrate cross sensitization over a longer interval conclusions should regarding lasting effects need to be interpreted cautiously. Nonetheless, the present study, together with prior results in which later depressive-like behavior was reversed

with an anti-inflammatory drug (e.g. Yusko-Osborne et al., 2015), do suggest that proinflammatory mechanisms may be mediators of the later depressive-like outcomes of early life stressors, in guinea pigs. These findings are consistent with observations made in human studies suggesting stressors experienced during early-life or adolescence can lead to lasting elevations in pro-inflammatory activity as well as later depression (Danese et al., 2008; Carpenter et al., 2010; Slavich & Irwin, 2014). Perhaps findings in the guinea pigs can complement human research in identifying the detection, and prevention, of development of depression later in life.

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VI. FIGURES



Figure 1: Guinea pig showing “full passive” behavior. Note eye closure, piloerection, and the crouch posture.



Figure 2: Guinea pig in forced swim apparatus.

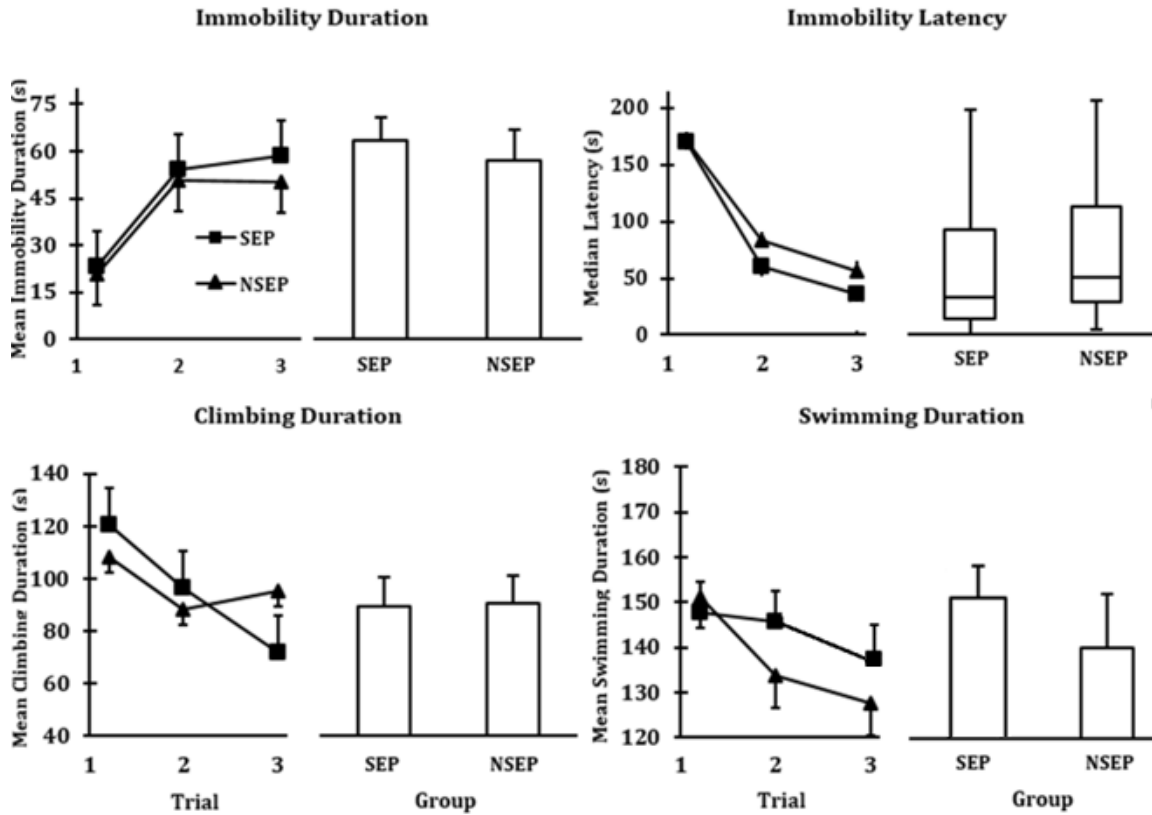


Figure 3: Mean levels of immobility, climbing, and swimming duration and median immobility latency are shown across the three trials of Experiment 1. Paired histograms show means of indicated behavior across all trials (SEP: separated; NSEP: non-separated). Error bars indicate Standard Error of the Mean. Immobility latency box and whisker plot shows median and semi-interquartile range. Immobility duration exhibited an effect of Day ($p < 0.001$) but there was no difference in any behavior between separated and non-separated pups.

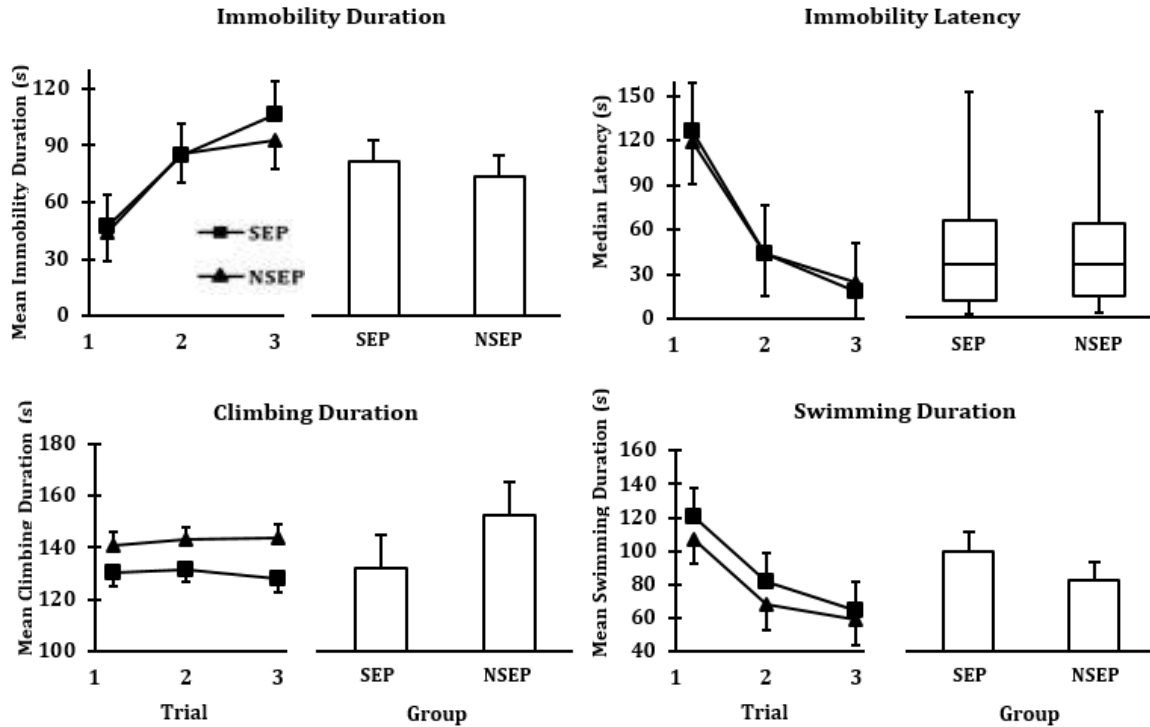


Figure 4: Mean levels of immobility, swimming, and climbing duration and median immobility latency are shown across the three forced swim trials of Experiment 2. Paired histograms show means of behavior across trials (SEP: separated; NSEP: non-separated). Error bars indicate Standard Error of the Mean. Immobility latency box and whisker plot shows median and semi-interquartile range. Immobility duration, swimming duration, and immobility latency exhibited effect of Day, $F(2, 56) = 28.65$; $p < 0.001$ but there was no difference in any behavior between separated and non-separated pups.

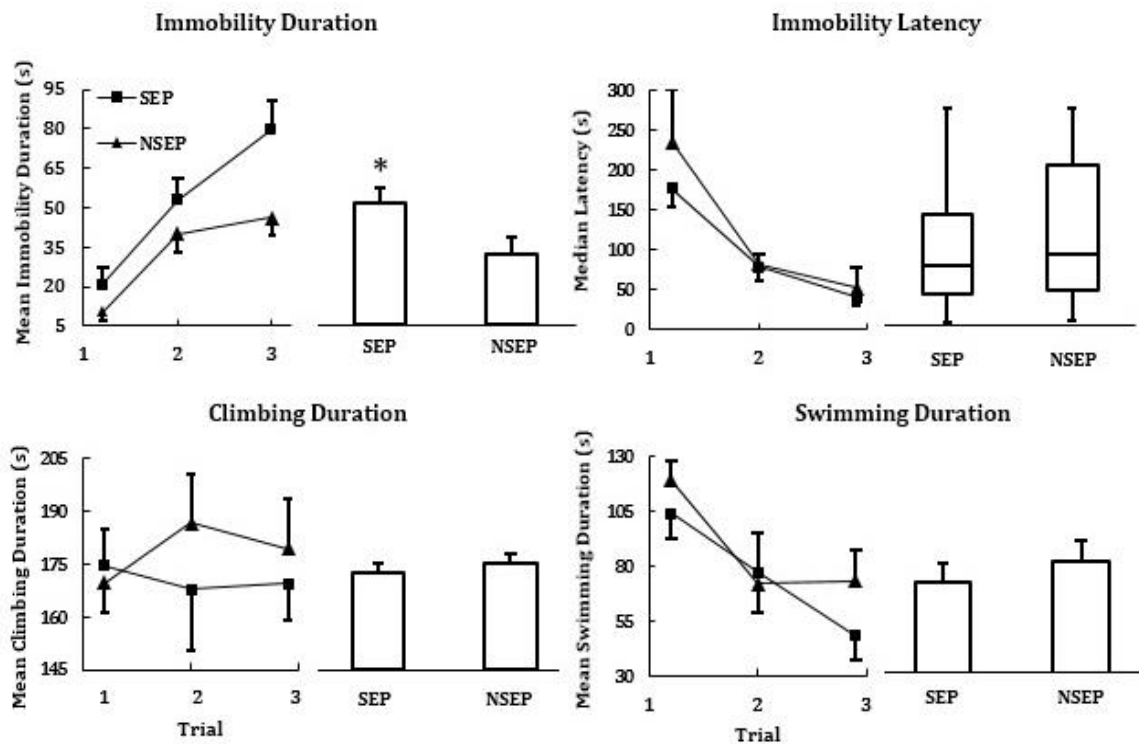


Figure 5: Mean levels of immobility, climbing, and swimming duration and median immobility latency are shown across the three forced swim trials of Experiment 3. Paired histograms show means of behavior across trials (SEP: separated; NSEP: non-separated). Error bars indicate Standard Error of the Mean. Immobility latency box and whisker plot shows median and semi-interquartile range. Swimming duration, immobility duration, and immobility latency showed a significance of Day ($p < 0.001$). Immobility duration also showed an effect of Group with exposed to separation prior to forced swim exhibiting greater immobility than non-separated pups.

*: $p < 0.05$

Table 1: Experiment 2 Sex Differences within Measure of Immobility Duration across Three Forced Swim Trials in Seconds. Analysis revealed a significant interaction between Day and Sex, $F(2, 56) = 3.76$; $p < 0.05$ in pups across both SEP and NSEP conditions.

<i>Sex</i>	<i>Day 1</i>	<i>Day 2</i>	<i>Day 3</i>
<i>Male (n=16)</i>	40.21(6.8)	73.70(11.2)	107.45(10.3)
<i>Female (n=16)</i>	50.99(7.4)	97.89(10.8)	91.71(11.2)