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Sensitization of Behavioral Response to Maternal Separation: Persistence of The Effect And Role of Proinflammatory Activity

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SENSITIZATION OF THE BEHAVIORAL RESPONSE TO MATERNAL SEPARATION:
PERSISTENCE OF THE EFFECT AND ROLE OF PROINFLAMMATORY ACTIVITY.

A thesis submitted in partial fulfillment

of the requirements for the degree of

Master of Science

By

JESSICA DANIELLE CARAWAY

B.S., Northern Kentucky University, 2008

2010

Wright State University

WRIGHT STATE UNIVERSITY

SCHOOL OF GRADUATE STUDIES

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Jessica Danielle Caraway ENTITLED Sensitization of the behavioral response to maternal separation: persistence of effect and role of proinflammatory activity BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Caraway, Jessica Danielle. M.S., Anatomy Program, Department of Neuroscience, Cell Biology, and Physiology, Wright State University, 2010. Sensitization of behavioral response to maternal separation: persistence of the effect and role of proinflammatory activity.

Maternal separation in guinea pigs produces a biphasic response consisting of an active behavior phase followed by a phase of passive behavior (crouched stance, piloerection, and eye closure). Previous studies suggest that pro-inflammatory cytokines mediate passive behavior during the passive phase. It is also known that guinea pig pups separated on two consecutive days show a significant increase (sensitization) in full passive behavior on the second day. The current study examined the persistence and role of pro-inflammatory activity in the sensitization of passive behavior in maternally separated guinea pigs. Guinea pig pups were assigned to one of four groups differing in their treatment on Day 1. The groups were either: (1) injected with saline and not separated; (2) injected with saline and separated; (3) injected with lipopolysaccharide (LPS) to induce cytokine activation and not separated; or, (4) injected with LPS and separated. All pups were separated on Day 2 and again on Day 5. By Day 5, sensitization of passive behavior was observed for all groups. LPS on Day 1 did not increase passive behavior on Day 2, but appeared to enhance the effect of separation on Day 5. These data indicate that sensitization of the passive behavior of maternally separated pups persists beyond a single day. Further the results show that LPS induced activation of pro-inflammatory activity is not sufficient to account for the initial sensitization, but does appear to enhance later sensitization effects.

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Sickness Behavior and Pro-inflammatory Cytokines

As a first line of defense against injury or invading pathogens, cells of the

immune system (macrophages, monocytes) release numerous proteins or cytokines

(Baumann & Gauldie, 1994). These cytokines produce different actions in the body

including recruitment of white blood cells, lymphocyte turnover, growth and

differentiation, and at times act as anti-viral agents (Hopkins, 2003). The cytokines can

be classified based on their action: pro-inflammatory such as Interleukin-1 (IL-1), IL-6

and Tumor necrosis factor-α (TNF-α) or anti-inflammatory such as IL-4, IL-10 and IL-13

(Cannon, 2000; Dinarello, 2000). The pro-inflammatory cytokines initiate a cascade of

events leading to some characteristic physiological and behavioral responses together

called the acute phase response (Baumann & Gauldie, 1994). The acute phase response

(APR) is an early and non-specific response of the body to injury, infection, or trauma

(Koj, 1985; Moshage, 1997). Some characteristic physiological components that

accompany the APR are fever (Bernheim, Block, & Atkins, 1979), increased

hypothalamic-pituitary-adrenal (HPA) activity and an alteration of proteins produced by

the liver (Baumann & Gauldie, 1994; Hart, 1988). Behaviors associated with the APR

include shivering, piloerection, hunched posture, eye closure and sleepiness (Hart, 1988).

The APR involves a coordinated suite of metabolic and physiological changes

that help fight off the infection (Hart, 1988). Fever, for example, is a means of

maintaining an elevated body temperature that is favorable for the production of immune

cells and unfavorable for the growth and maintenance of bacterial and viral pathogens

(Kluger, 1979). Shivering, a behavioral component of the APR, may occur to aid in the production of fever. Sickness behaviors are not a result of debilitation but a motivated action of the host (Aubert, 1999).

Accumulating research in the past decades has demonstrated the effects of pro-inflammatory cytokines on the central nervous system. To study the APR in the laboratory, lipopolysaccharide (LPS), derived from the cell wall of gram-negative bacteria, can be administered. Intraperitoneal (IP) injection of LPS in mice causes a rapid increase in pro-inflammatory cytokines, such as IL-1 and TNF-α, in the blood (Zuckerman, Shellhas, & Butler, 1989) resulting in the physiological and behavioral components of the APR. Following injection of LPS, there also is an up-regulation of IL-1 and an increase of bioactive IL-1 in the brain (Takao et al., 1990). Konsman et al. (2008) found that circulating IL-1β acts on the brain to produce the behavioral components of the APR. However, peripherally produced pro-inflammatory cytokines must find access to or signal the central nervous system. Due to their size and hydrophilicity, it would be difficult for cytokines to cross the blood-brain-barrier (BBB; Blatteis, 2000). Some general mechanisms for cytokines to gain access to or signal the CNS are described below.

Cytokines may access the CNS through areas devoid of functional BBB, also known as the circumventricular areas (Dantzer and Wollman, 2003). Circumventricular organs (CVO's) include the median eminence, posterior pituitary gland, pineal gland, subfornical organ, organum vasculosum of the lamina terminalis and area postrema.

Cytokines may also act on the CNS via a neural pathway. Cytokines can stimulate peripheral sensory nerves that innervate structures of the immune system (Dantzer, 1994). The vagus nerve is important in transmitting peripheral cytokine signals to the brain. Direct stimulation of the vagus nerve up-regulates IL-1β in the brain (Hosoi et al., 2000). Bluthe et al. (1996) found that the behavioral effects of peripheral injection of IL-1 can be abolished with a vagotomy. The vagotomy does not reduce behavioral effects of centrally injected IL-1. This shows that IL-1 uses the vagus nerve to communicate with the brain. To activate the vagus nerve, IL-1 binds to paraganglia surrounding it. The activated vagus nerve then sends signals to the nucleus of the solitary tract which projects to other areas of the brain, such as the hippocampus and hypothalamus (Maier & Watkins, 1998). IL-1 is then released by cells of the nervous system. Van Dam, Brouns, Louisse, and Berkenbosch (1992) found the source of IL-1 in the brain to be from macrophages of the choroid plexus and meninges as well as microglial and perivascular cells. IL-1 can then act on local neural tissue inducing the behaviors of the APR.

Cytokines and Stress

Certain stressors can elicit elements of the acute phase response without the presence of a virulent pathogen (Maier & Watkins, 1998). Inescapable tail shock induces fever and production of pro-inflammatory cytokines such as IL-6 and IL-1β (Deak et al.,1997; Maier & Watkins, 1998; Zhou, Kusnecov, Shurin, DePaoli & Rabin, 1993; Nguyen et al., 1998) In fact, even exposure to a novel environment can cause an increase in circulating cytokines. Mice introduced to a novel environment show an increase in levels of IL-6 (LeMay, Vander & Kluger, 1990). These and other stressors increase

levels of circulating cytokines and induce sickness-like behaviors (Dantzer, 2009). Maier

and Watkins (1995) showed that administration of an IL-1 antagonist to the brain results

in a blockade of the behavioral effects of stressors. These studies show that stress can

trigger an increase in circulating cytokines and the brain recognizes these cytokines as

molecular signals of sickness (Dantzer, 2007).

Circulating cytokines such as TNF-α, IL-1, and IL-6 can also have an effect on

the stress system by activating the hypothalamic-pituitary-adrenal (HPA) axis (Turnball

& Rivier, 1999). Besedovsky and colleagues (1986) have shown IL-1 to activate the

HPA axis by stimulating neurons of the paraventricular nucleus of the hypothalamus to

release corticotropin-releasing factor (CRF) which also stimulates the release of

adrenocorticotropin hormone (ACTH) from the pituitary gland causing an elevation of

corticosterone (Berkenbosh, 1987). IP administration of TNF-α activates the HPA axis

in mice as well as increases the levels of tryptophan in several regions of the brain and

the breakdown of norepinephrine (NE) in the hypothalamus (Ando and Dunn, 1999).

However, pro-inflammatory cytokines also enhance the enzyme kynurenine-3-

monooxygenase enzyme (KMO), which breaks down tryptophan into neurotoxic

tryptophan metabolites resulting in neurodegeneration and reduced serotonin (Wichers et

al., 2005). As demonstrated in studies reviewed above, cytokine release can have a

myriad of effects, some pathological. Recent findings show that cytokines acting on the

brain are involved in the pathophysiology of some mental health disorders, including

depression (Dantzer & Kelley, 2007).

Depression and Cytokines

Similarities between behaviors of the acute phase response and behaviors of depression led investigators to believe that the two were linked. In fact, behaviors listed by the American Psychiatric Association DSM-IV for major depression include inactivity, loss of interest in social interaction, loss of energy, and hypersomnia all of which are similar to APR behaviors. Miller et al. (2009) note that a large portion of depressed patients show activation of innate immunity, including pro-inflammatory cytokines, without any other underlying medical cause.   IFN-α was administered to patients for treatment of a malignant melanoma.   Forty-five percent of patients administered IFN-α were diagnosed with medical depression in the following weeks (Capuron et al., 2001). None of the patients had a current diagnosis of major depression. A recent meta-analysis of cytokines in major depression showed significantly higher levels of TNF-α and IL-6 in clinically depressed patients (Dowlati et al., 2010). In fact, tricyclic anti-depressants reversed the effects of LPS on behaviors such as withdrawal from the environment, reduction in activity, eye closure, etc. (Yirmiya, 1996). Cytokines, as a result of stress or sickness, can have a significant impact on our well-being.

Maternal Separation as a Stressor

Stressful occurrences early in development have been shown to alter responses to stress later in life (Ader & Friedman, 1965). A frequently studied and important example of early life stress is maternal separation. Maternal separation can have lasting effects on behavior and physiology in adulthood (Kohman, Tarr, Day, McLinden & Boehm, 2008). A recent study analyzed the stress response of humans who were separated from their parents at an early age. Participants were subjected to a social stress test involving public

speaking. The individuals who had been separated from both parents responded with higher levels of salivary cortisol and plasma ACTH (Pesonen et al., 2010). A study by Hinde and Spencer-Booth (1971) using rhesus monkeys showed that even a brief maternal separation can have effects lasting months to years. Meaney et al. (1991) have also shown that early interaction with the mother can alter the gene expression of glucocorticoid receptors in the hippocampus, resulting in altered HPA function later in life.

The effects of separation from attachment objects was first recognized by Spitz in the 1940's. Spitz (1946) documented the effects of maternal separation on children who had been hospitalized or quarantined (resulting in a long period of time without parental care). The children were socially withdrawn, inactive and had little to no interest in food. Spitz termed their behavior anaclitic depression. The children also appeared sick. Research involving maternal separation continued in later decades using non-human primates. Some species were found to display a distinct two-phase reaction to separation remarkably similar to that of the separated children (Kaufman & Rosenblum, 1967). The first phase was termed the "protest" stage. During the protest stage the animals would vocalize and move around the cage frantically as if searching for maternal contact. The later phase, "despair", would come about in a portion of the infant monkeys. Primates in the despair stage were socially withdrawn, hunched in posture, and even displayed an expression suggesting grief (Kaufman & Rosenblum, 1967). Weiner et al. (1990) found significantly higher plasma cortisol levels in infant squirrel monkeys when separated from the mother. Clarke (1993) also showed elevated levels of cortisol and ACTH following maternal separation of rhesus monkeys. These observations demonstrate that

the stress of maternal separation can activate the HPA axis as well as evoke behavioral responses, some of which are reminiscent of the APR. Recently, our laboratory has examined the effects of maternal separation in a rodent species, the guinea pig.

Maternal Separation in the Guinea Pig

The guinea pig offers an alternative animal model for studying maternal separation and depression. Unlike other laboratory rodents, the guinea pig shows a strong filial attachment to the mother (Hennessy, 2003). The guinea pig pup is born fully furred with eyes and ears open. It can locomote and ingest food and water within hours of birth (Schiml & Hennessy, 1990). Lactating females do not have a permanent nest nor do they display many active maternal behaviors (Konig, 1985). With such passive care by the mother, it is the attachment of the pup to the mother that maintains the mother-pup proximity (Hennessy, 2003). There are a number of similarities between the guinea pig and nonhuman primate in their response to separation (Hennessy, 2003). Particularly important for the current study, the pup displays a biphasic response similar to that of the primate (Hennessy & Morris, 2005).   The guinea pig first displays active behaviors: vocalizations and movement around the cage. After an hour or so, the pup begins to display passive behaviors. Characteristic passive behaviors includes a crouched stance, extensive piloerection, and eye closure (figure 1). The passive behaviors seen during maternal separation have been suggested to be mediated by pro-inflammatory cytokines acting on the central nervous system (Hennessy et al., 2007). When administered the anti-inflammatory agent, α-MSH, pups undergoing maternal separation showed a significant decrease in the level of all three passive behaviors (Schiml-Webb, Deak, Greenlee, Maken & Hennessy, 2006). Indomethacin, a prostaglandin synthesis inhibitior,

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produced similar results (Hennessy, Schiml-Webb, Miller, Maken, Bullinger & Deak, 2007).   Finally, administration of IL-10, a cytokine with strong anti-inflammatory properties, was found to significantly reduce all measures of passive behavior (Perkeybile et al., 2009). These findings suggest pro-inflammatory cytokines may be involved in the passive, depressive-like behavior seen in maternally separated guinea pigs.

Figure 1: The guinea pig displaying full passive behavior: crouched stance, piloerection, and partial to full eye closure during a 3-hr maternal separation.

Sensitization

A growing database indicates a relationship between early life stress or trauma and psychiatric disorders, such as depression, later in life (Nemeroff, 2004). Hyperactivity of the HPA axis is a prominent feature of major depression and appears to

often come about as a result of early stressors such as maternal separation, neglect, or abuse (Heim, Newport, Mletzko, Miller & Nemeroff, 2008). O'Connor et al. (2004) found that the stress of a tail shock sensitizes the HPA axis. A sensitization of the HPA axis enhances the response to the second occurrence of a stressful situation so that even a mild stressor will produce a full stress response (Fernandes et al., 2002). Post et al. (1981) proposed a kindling-sensitization hypothesis, that predicts that limbic structures exposed to early stressful experiences undergo a permanent change that plays a role in affective disorders later in life. Subsequent findings support this hypothesis (Schulkin, McEwen, & Gold, 1994). A behavioral sensitization also occurs after repeated stress due to a lowered stress threshold (Antelman, Eichler, Black, & Kocan, 1980; Antelman & Eichler, 1979; MacLennan & Maier, 1983). HPA axis disturbances have been attributed to cytokines and their pathways including the NF-kappaB and MAPK pathways which can also lead back to an alteration in glucocorticoid function as found following early stress (Anisman et al., 2008; Zunszain, et al., 2010). In sum, early life stress can senstitize the response to stress and illness later in life.

Pro-inflammatory cytokines are strongly linked to depression and an enhancement of later HPA responsiveness to stress or immune challenge (Schmidt et al., 2003). Hayley, Brebner, Lacosta, Merali & Anisman (1999) investigated the sensitizing effects of repeated cytokine exposure. Mice were exposed initially to TNF-α, a pro-inflammatory cytokine, and then again 14-28 days later. Upon re-exposure to TNF-α, the mice that received a pre-treatment of TNF-α, as opposed to saline, showed a significant increase in sickness behaviors related to depression such as reduced activity and a decrease in food intake. As described above, early maternal separation can induce

passive behaviors in children (Spitz, 1946), nonhuman primates (Kauffman & Rosenblum, 1967) and guinea pigs (Hennessy et al., 1995). Results in guinea pigs suggest this is as a result of pro-inflammatory activation. These findings raise the question of whether pro-inflammatory cytokines contribute to sensitization. A previous study in our laboratory showed that sensitization of passive behavior can occur in maternally separated guinea pigs. When guinea pigs were separated for 3 hr on two consecutive days, passive behavior significantly increased on the second day (Figure 2, Hennessy et al., 2010).
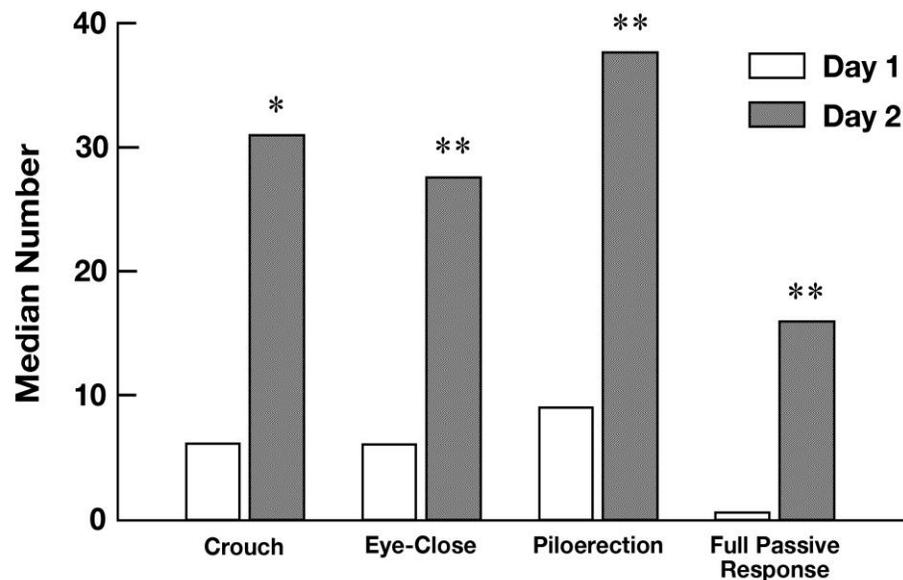
Figure 2: The median number of 60-s intervals in which pups displayed full passive behavior when separated for a duration of 3 hours on 2 consecutive days. Behavior on day 2 demonstrates sensitization. $*p < 0.05$, $**p < 0.01$. (Figure derived from Hennessy, Deak, Schiml-Webb, Carlisle, and O'Brien, 2010).

To determine if pro-inflammatory cytokines are involved in the sensitization of passive behavior in guinea pigs, Paik (2009) administered an anti-inflammatory agent,

IL-10, into the ventricles of pups prior to separation on Day 1. IL-10 blocked the increase of passive behavior seen on the second day of separation (Figure 3). These results indicate pro-inflammatory cytokines are *necessary* for sensitization of passive behavior in separated guinea pig pups, but are pro-inflammatory cytokines *sufficient* to account for the sensitization effect?
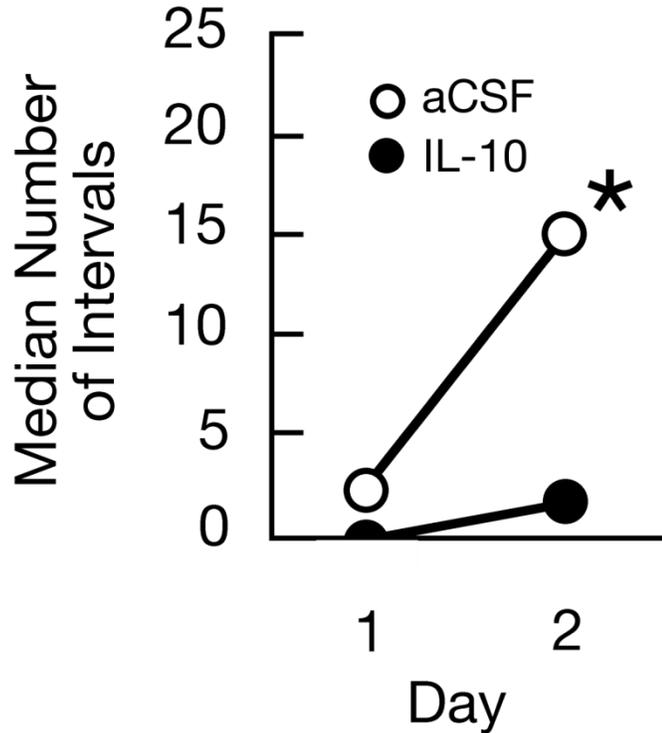

Figure 3: The median number of 1-min intervals in which separated guinea pigs displayed full passive behavior on Day 1 and Day 2 when given a central infusion of aCSF or IL-10 prior to separation on Day 1. $*p < 0.05$.

Integration and Predictions

Previous investigations of the sensitization of the passive behavior in maternally separated guinea pigs have been performed only on Days 1 and 2. To determine if sensitization of passive behavior persists, separations will be performed on Day 1, Day 2 and again on Day 5. Thus, the first goal of this experiment was to determine if the sensitization of full passive behavior persists past the second day.

The second goal was to determine the role of pro-inflammatory cytokines in the sensitization process. To determine if activation of pro-inflammatory cytokines is *sufficient* to account for sensitization on Day 2, LPS was administered to half of the pups on Day 1. If LPS is sufficient, the expected result would be an increase in passive behavior when separated the second day as compared to controls. Therefore, I compared the effect of LPS versus separation on the response to separation in later days.

II. METHODS

Subjects

Albino guinea pigs of the Hartley strain (*Cavia porcellas*) were bred in Wright State University's Laboratory Animal Resources facility. Each lactating female and her litter were housed in a polycarbonate cage (73 x 54 x 24cm) with sawdust bedding. Water and food were available continuously. The colony room was maintained on a 12 hour light-dark cycle (lights on at 7:00AM). The temperature in the colony room was maintained between 65-70° F. The day of birth was designated as Day 0. Pups remained with the mother and littermates throughout the duration of the study except for injection procedures and behavioral testing. The Wright State University Laboratory Animal Care and Use Committee approved all procedures prior to experimentation.

Experimental Design

Forty-eight guinea pigs, 24 male and 24 female, from 33 different litters were used in this study. The pups were randomly assigned to one of four experimental conditions. No more than one pup from a single litter was assigned to the same experimental condition. The experimental conditions differed based on treatment on the first day (Day 21-23 of age). Twenty-four pups received an IP saline injection and 24 pups received an IP injection of 75 µg/kg body weight LPS in saline. Half of each injection group was then separated for 3 hours on Day 1 whereas the other half was not. On Day 2 and day 5, all 48 pups underwent a 3-hr separation. Figure 4 demonstrates the experimental design as described above.

Testing Procedure

For testing, the guinea pig was transferred from the home cage to a nearby testing room via a transport cage (< 10s). Once in the testing room, the guinea pig was placed into a clear, empty, plastic cage (47 x 24 x 20 cm) located on a table under full room lighting. The cage was covered with a clear, well-ventilated lid. The cage was cleaned with detergent before each test.

Behavioral Scoring

A trained observer recorded behavior from behind one-way glass. The observer used paper and pencil to score the number of 60-s intervals in which the pup displayed a characteristic crouched stance (body hunched down with head lowered and feet tucked beneath), eye-closing (more than 1 second of sustained complete or near complete closure one or both eyes), and extensive piloerection (occurring over most of the visible body surface). The primary measure in this study was the "full passive" response. The full passive score was defined as the number of 60-s intervals in which all three passive behaviors occurred. The results for the individual passive behaviors showed a similar general pattern as the full passive response and will not be discussed further. The number of vocalizations (high-pitch whistles) was tallied with a hand counter as a measure of active behavior. The observer scored behaviors at the following intervals: 0-30, 60-90, and 150-180 min. All separations began between 7:00am and 9:00am.
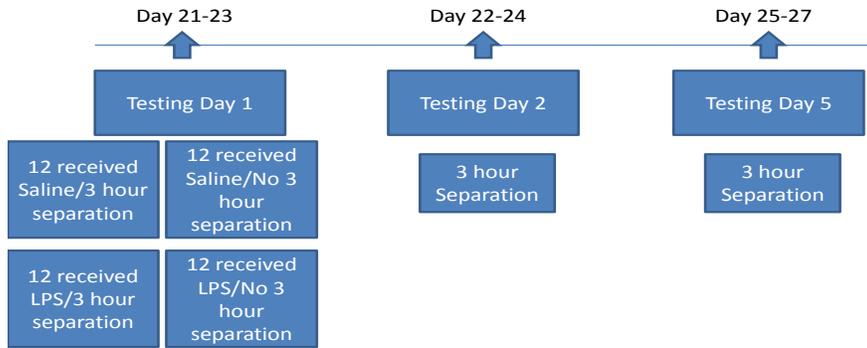
Figure 4: Experimental design showing four groups based on Day 1 treatment, pup age at time of testing, and subsequent testing time frames.

Data Analysis

For Day 1, full passive behavior and vocalizations were evaluated by 2-way (Injection x Sex) analyses of variance (ANOVAs). Full passive behavior and vocalizations on Day 2 and 5 were evaluated with 2 (Injection) x 2 (Separation) x 2 (Sex) x 2 (Day) ANOVAs with Day treated as a repeated measure. Follow-up tests were conducted with simple main effects and interaction tests (Winer, 1971).   For the ANOVA of vocalizations, data were subjected to a square root transformation prior to analysis due to heterogeneity of variance. Raw data are shown in the tables. A significance level of $p < 0.05$ (2-tailed) was accepted throughout.

III. RESULTS

Full Passive Behaviors

For Day 1, the ANOVA for full passive behavior yielded a main effect for Injection, $F(1, 20) = 18.17$, $p < 0.01$. When injected with LPS, pups displayed significantly more full passive behavior (Figure 5). However, the main effect was qualified with an interaction with sex, $F(1, 20) = 4.66$, $p < 0.05$. Although both sexes had an elevated mean for full passive behavior when injected with LPS, the values were significant only for the females, $p < 0.01$ (Table 1).

**Response to Separation on
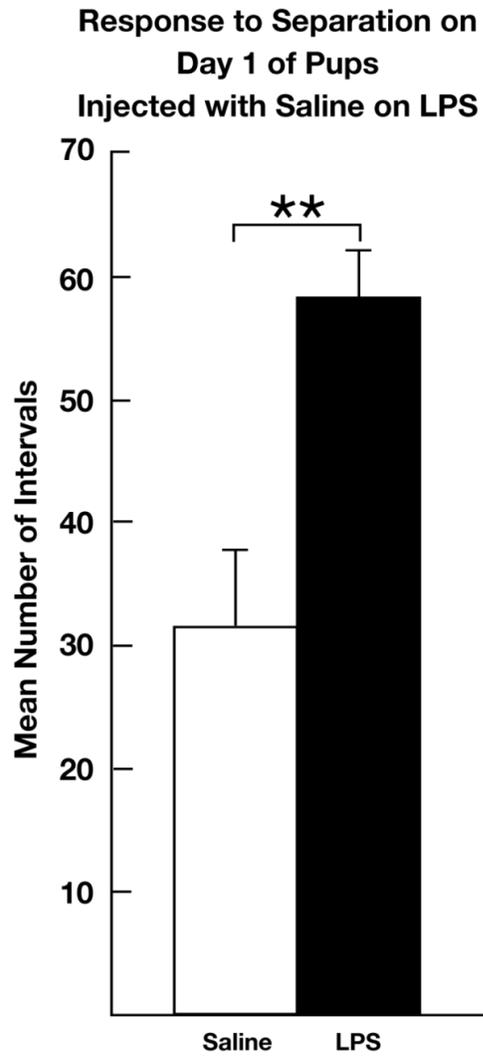Day 1 of Pups
Injected with Saline on LPS**

Figure 5: Mean number of 1-min intervals in which guinea pig pups displayed full
passive behavior during separation following injection of Saline or LPS. **$p < 0.01$.

Table 1: Mean (SE) number of 1-min intervals in which pups displayed full passive behavior during separation on Day 1 when preceded by an injection of LPS or saline.

| | Injection | |
Sex	Saline	LPS
Male	39.8 (6.9)	53.0 (6.4)
Female	23.0 (7.2)	63.2 (4.0)

The ANOVA for full passive behavior on Day 2 and 5 yielded significant effects for Day, F (1, 40) = 48.96, $p < 0.01$, Separation x Day, F (1, 40) = 8.63, $p < 0.01$, and Injection x Separation x Day, F (1, 40) = 5.40, $p < 0.05$. Separate follow-up tests on Day 2 and 5 were used to further analyze the significant 3-way interaction. On Day 2 there was a significant main effect of separation ($p < 0.01$). Pups that were separated on Day 1 exhibited significantly more full passive behavior on Day 2 than those pups not separated on Day 1 (Figure 6). There was no main or interaction effect found for Injection. Follow-up tests showed no significant effects on Day 5. In sum, pups separated on Day 1 showed a greater amount of passive behavior on Day 2, indicating sensitization. LPS injection on Day 1 did not produce an increase in passive behavior on Day 2.

Response to Separation on Day 2 of Pups Undergoing Different Treatments on Day 1
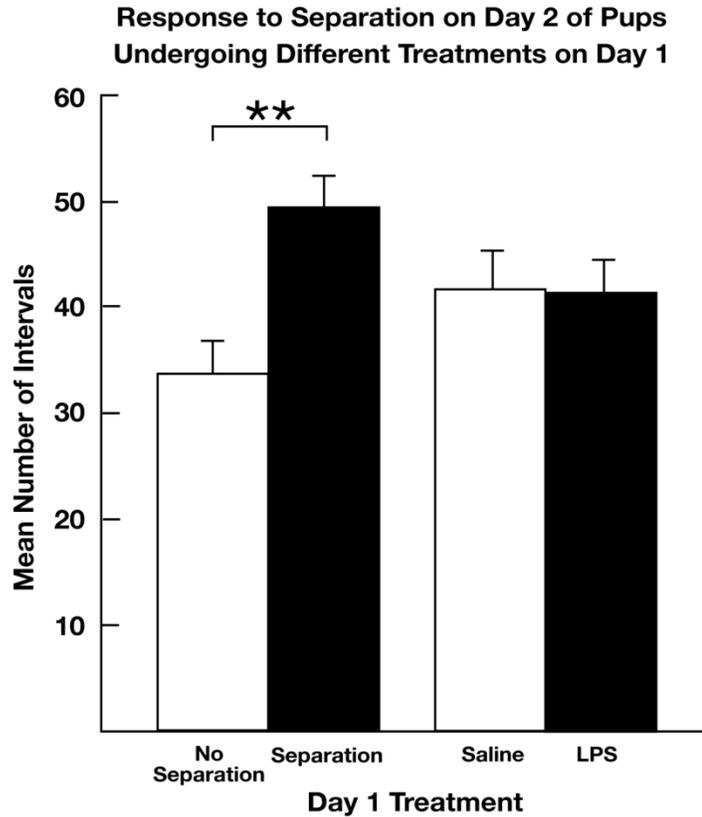
Figure 6: Mean (SE) number of 1-min intervals in which pups displayed full passive behavior during separation on Day 2 based on day 1 treatment. $**p < 0.01$.

Further follow-up tests examined possible sensitization from Day 2 to Day 5 for each of the four Separation x Injection groups. A significant increase of full passive behavior from Day 2 to 5 was seen in both groups *not* separated on Day 1 regardless of injection of saline, $F(1, 11) = 38.50$, $p < 0.01$, or LPS, $F(1, 11) = 10.91$, $p < 0.01$. The group separated and injected with LPS on Day 1 also showed a significant increase, $F(1, 11) = 7.81$, $p < 0.05$. However, the pups separated and injected with saline on Day 1 showed no change in passive behavior from Day 2 to 5. That is, pups separated on Day 1 did not show a significant increase in passive behavior from Day 2 to 5 unless they also were injected with LPS on Day 1 (Figure 7).

Change in Response from Day 2 to Day 5 of Pups Undergoing Different Treatments on Day 1

Day 1 Treatment
○ No separation/Saline
● No separation/LPS
△ Separation/Saline
▲ Separation/LPS
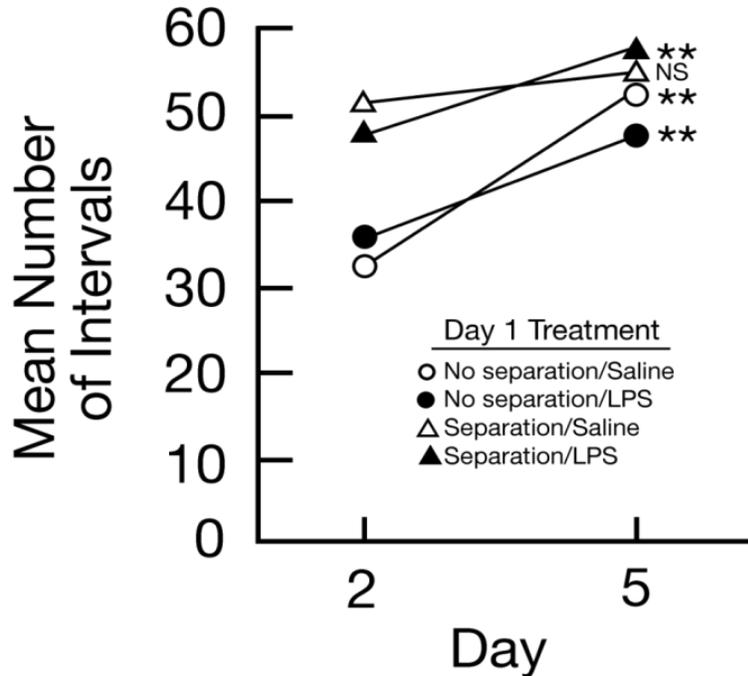
Figure 7: Mean number of 1-min intervals in which separated pups exposed to different Day 1 treatments displayed full passive behavior on Days 2 and 5. **$p < 0.01$.

Vocalizations

The ANOVA for vocalizations on Day 1 yielded no significant effects, although pups injected with LPS on Day 1 had a lower mean value than pups injected with saline (Table 2). For Day 2 and 5, the 4-way ANOVA for vocalizations yielded effects for Day, $F(1, 40) = 5.56$, $p < 0.05$, Separation x Day, $F(1, 40) = 5.00$, $p < 0.05$, and Separation x Sex x Day, $F(1, 40) = 10.17$, $p < 0.01$. Separate follow-up tests on Day 2 and 5 were used to further analyze the significant 3-way interaction. On Day 2, there was

a significant main effect of separation ($p < 0.05$). Pups that were separated on Day 1

vocalized more than those not separated on Day 1(Table 3).

Table 2: Mean (SE) vocalizations during separation for pups on Day 1 when preceded by an injection of LPS or Saline.

Injection	
Saline	LPS
709.6 (277.7)	474.3 (163.3)

Table 3: Mean (SE) vocalizations during separation on Day 2 by pups that were separated or not separated on Day 1.

Day 1 Separation	Day 1 No Separation
724.2 (131.9)*	501.8 (140.0)

* $p < 0.05$.

On Day 5, there was an interaction between Separation x Sex ($p < 0.01$).

Vocalizations for males on Day 5 were lower if they had been separated on Day 1than if

they had not been separated, whereas females vocalized more when separated on Day 1

(Table 4).   Comparisons between Days 2 and 5 for each of the four Separation x Sex

groups yielded significant effects only for the Males separated on Day 1 ($p < 0.01$),

which showed a significant decrease in vocalizations from Day 2 (729.3 ± 196.5) to Day

5 (196.4 ± 151.8).

Table 4: Mean (SE) number of vocalizations during separation on Day 5 by male and female pups that were separated or not separated on Day 1.

	Separation	
Sex	Day 1 Separation	Day 1 No Separation
Males	196.4(86.6)	678.8(197.9)
Females	646.0(200.1)	265.1(77.0)

IV. DISCUSSION

Maternal separation in guinea pigs produces a biphasic response, the later phase characterized by passive behaviors such as crouched stance, piloerection, and eye closure. The passive behaviors are thought to be induced by pro-inflammatory cytokines. Administration of anti-inflammatory agents such as α-MSH and IL-10 significantly reduce passive behavior of maternally separated guinea pigs (Schiml-Webb, Deak, Greenlee, Maken & Hennessy, 2006; Perkeybile et al., 2009). Guinea pigs separated on two consecutive days show a significant increase in passive behavior on the second day (Hennessy et al. 2010). The possibility that the sensitization of passive behavior was mediated by cytokines was tested by Paik (2009) who administered IL-10 to guinea pigs prior to separation on Day 1. IL-10 prevented the increase in full-passive behavior on Day 2. These findings indicate that pro-inflammatory cytokines are *necessary* for the sensitization seen in maternally separated guinea pigs.

The current study was designed to determine if pro-inflammatory cytokines are sufficient to produce sensitization of passive behavior. To induce pro-inflammatory cytokine activation, LPS was administered. LPS activates the innate immune system initiating a pro-inflammatory cytokine cascade. IP injections of LPS on Day 1 did not significantly affect passive behavior on Day 2. However, separation on Day 1 did cause a significant increase in passive behavior on Day 2. The sensitization seen in guinea pigs separated for two consecutive days cannot be mimicked by injection of LPS on Day 1. These results indicate that pro-inflammatory activation alone is not sufficient to account for the sensitization effect. It also is possible that different pro-inflammatory cytokine reactions are produced by separation and LPS injection. If so, it remains

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possible that a particular pattern of cytokine activation is sufficient to produce the sensitization effects.

In the present study, all groups showed more passive behavior in a subsequent separation than they did on their first. These results are consistent with earlier findings in our laboratory (Hennessy et al., 2010). Moreover, the present results demonstrate that the sensitization effect is not limited to an increase in passive behavior over just two consecutive days. However, the full passive behavior levels in the current study were considerably higher than those of Paik (2009). This effect is evident in comparison of figures 3 and 7 (though note that Figure 3 presents medians, whereas Figure 7 presents means). The reason for the discrepancy is not clear, but may be related to the surgery and central infusion procedures of the earlier but not current study.

Three of the four groups showed a significant increase in passive behavior from Day 2 to 5. Regardless of injection, both non-separation groups (groups separated for the first time on Day 2) had a significant increase in passive behavior from Day 2 to 5. Of the separation groups, only the group injected with LPS showed a significant increase in passive behavior from Day 2 to 5. Thus, while LPS activated pro-inflammatory cytokines were not initially sufficient to lead to sensitization on Day 2, they may have enhanced passive behavior during separation on Day 5.

Vocalization data were used as a measure of active behavior. Vocalizations on Day 1 did not differ between LPS and saline-injected animals although pups injected with LPS had a lower mean value. Vocalizations on Day 2 were affected by separation on Day 1. The pups separated on Day 1 vocalized more on Day 2 than those not separated

on Day 1. By Day 5, level of vocalizing varied in a complex manner depending on the sex of the pup. This pattern of effects is difficult to interpret. Nonetheless, it is interesting that an increase in passive behavior in later days was not simply accompanied by a reduction in the primary active behavior of vocalizing.

Pro-inflammatory cytokines (TNF-α, IL-6) have been found to be elevated in major depression (Dowlati et al. 2010). These peptides appear to be involved in the passive behavior seen in maternally separated guinea pigs. Various forms of attachment disruption such as maternal separation increase the risk of later depression (Heim et al., 2008). The involvement of pro-inflammatory activity in the sensitization of passive behavior as seen in the earlier study (Paik, 2009), and, to a lesser extent, in this study, suggests that guinea pigs might be effectively used to assess the involvement of pro-inflammatory factors in the development of depression in individuals exposed to early stress.

In sum, this study has shown that sensitization of passive behavior persists beyond a single day and that pro-inflammatory cytokine activation via the innate immune system is not sufficient to account for the sensitization effect. However, evidence of an enhancement of later sensitization (Day 5) in pups injected with LPS was observed. Future studies might focus on the longer-term effects of LPS and maternal separation in guinea pig pups.

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