Inhibition of Pro-inflammatory Processes Reduces Sensitization of the Behavioral Response to Maternal Separation

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INHIBITION OF PRO-INFLAMMATORY PROCESSES REDUCES SENSITIZATION OF THE BEHAVIORAL RESPONSE TO MATERNAL SEPARATION

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

By

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The current study examined the behavioral sensitization of guinea pig pups in response to consecutive days of maternal separation. In the first experiment, guinea pigs that received centrally administered artificial cerebrospinal fluid or IL-10 exhibited sensitization of passive behaviors from Day 1 to Day 2. IL-10 decreased the levels of passive behaviors on Day 1, as well as the increase on Day 2. The second experiment used unoperated pups, which also showed sensitization of the passive response from Day 1 to Day 2, though the effect appeared reduced relative to control pups of Experiment 1. Collectively, this investigation confirms previous evidence that passive behaviors are due in part to pro-inflammatory cytokines. It also provides evidence that the increase in passive measures from the first separation to the second may be caused by a sensitization of pro-inflammatory mechanisms.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Sickness Behaviors and Pro-inflammatory cytokines</td>
<td>1</td>
</tr>
<tr>
<td>Pathway of Cytokines to the CNS</td>
<td>3</td>
</tr>
<tr>
<td>Cytokines and Stress</td>
<td>5</td>
</tr>
<tr>
<td>Maternal Separation as a Stressor</td>
<td>6</td>
</tr>
<tr>
<td>Maternal Separation in Guinea Pigs</td>
<td>7</td>
</tr>
<tr>
<td>IL-10 as an anti-inflammatory agent</td>
<td>10</td>
</tr>
<tr>
<td>Depression and Cytokines</td>
<td>12</td>
</tr>
<tr>
<td>Integration and Predictions</td>
<td>14</td>
</tr>
<tr>
<td>II. GENERAL METHODS</td>
<td>16</td>
</tr>
<tr>
<td>Subjects</td>
<td>16</td>
</tr>
<tr>
<td>Behavior Test Procedures</td>
<td>16</td>
</tr>
<tr>
<td>Behavior Scoring</td>
<td>18</td>
</tr>
<tr>
<td>Data Analyses</td>
<td>19</td>
</tr>
<tr>
<td>III. EXPERIMENT 1</td>
<td>20</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Guinea Pig pup displaying passive behaviors</td>
<td>9</td>
</tr>
<tr>
<td>2. Timeline of Procedures for each subject</td>
<td>17</td>
</tr>
<tr>
<td>3. Passive behaviors of pups receiving aCSF or IL-10 (Exp. 1)</td>
<td>23</td>
</tr>
<tr>
<td>4. Passive behaviors of Unoperated pups (Exp. 2)</td>
<td>26</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Mean values and standard errors of active behaviors (Exp. 1)</td>
<td>24</td>
</tr>
<tr>
<td>2. Median values and SIR of active behaviors (Exp. 2)</td>
<td>27</td>
</tr>
</tbody>
</table>
I. Introduction

*Sickness Behavior and Pro-inflammatory cytokines*

When macrophages and monocytes encounter a foreign antigen, they secrete numerous cytokines as an initial line of defense against disease. These cytokines are large glycoproteins (8-40,000 d.) that facilitate cell-cell communication, growth, differentiation, white blood cell recruitment, and lymphocyte turnover, and often serve as anti-viral agents (Hopkins, 2003; Whiteside, 2007). Currently, scientists have identified scores of cytokines and their unique roles during progression of a disease. Cytokines can be classified as being either *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 (Dinarello, 2000). The cytokines that are considered pro-inflammatory promote a cascade of events that precede inflammation, and those that are anti-inflammatory inhibit pro-inflammatory cytokines and down regulate their genetic expression (Dinarello, 2000).

There is a large body of evidence supporting the relationship between immune challenge and cytokine release. In numerous studies, investigators have successfully induced pro-inflammatory cytokine production through administration of bacterial endotoxin (lipopolysaccharide or LPS) in rodent models. LPS is a necessary component of all viable gram-negative bacteria and has been found to elicit the innate immune response, including pro-inflammatory cytokine release. For instance, an early study by
Zuckerman, Shellhaas, and Butler (1989) reported that intraperitoneal injections of LPS in mice rapidly increased circulating levels of TNF-α and IL-1 in the blood. Recently, it was found that peripheral and central injections of LPS increased levels of IL-6 secreted by glial cells (Beurel & Jope, 2009). These studies, in addition to many others in the literature, provide indisputable evidence of pro-inflammatory cytokine production during a non-specific immune reaction.

It is well documented that during infection from invading microorganisms and tissue damage, pro-inflammatory cytokines coordinate homeostatic and physiological mechanisms to diminish the virulence of pathogens. The physical changes in the body associated with illness constitute what is known as the acute phase response (APR) characterized by fever, hyperalgesia, changes in liver proteins (C-reactive protein, fibrinogen, alpha2-macroglobulin), malaise, and reduced appetite (Baumann & Gauldie, 1994; Moshage, 1997). With these physical symptoms, however, exists an element of behavioral change, termed “sickness behavior”, that is also believed to be a defense mechanism to protect the body during a weakened state (Hart, 1988; Kent, Bluthe, Kelley & Dantzer, 1992). Some of these responses appear to be motivated behaviors. For instance, there is shivering or seeking of warmth to promote increased body temperature, anhedonia (lack of interest in pleasure), and reduced goal oriented activity such as lethargy, exploration, and socio-sexual interaction to conserve energy or to reduce the spread of pathogens (Hart, 1988). It is hypothesized that these behaviors are adaptive to nullify the detrimental effects of a pathogen. In addition, there are also cognitive deficits or reduced learning that can manifest during sickness.
Research in the past few decades has highlighted the effects pro-inflammatory cytokines on the central nervous system, which can help account for the behaviors observed during sickness. The mere fact that an immune challenge can alter the behavioral aspect of the acute phase response suggests that pro-inflammatory cytokines involved in mediating a peripheral response (e.g. inflammation) also modify central neural activity (Dantzer, 2001).

Pathway of Cytokines to the CNS

The interplay between pro-inflammatory cytokines and the central nervous system is fairly complex because of the existence of the blood-brain barrier (BBB). This barrier is maintained by tight junctional complexes of endothelial cells, consequently discouraging the passage of numerous substances such as large molecules and drugs (Gartner & Hiatt 2007). Cytokines are highly unlikely to pass through the BBB because of their size and hydrophilic nature (Blatteis, 2000). Knowing this, investigators have tried to identify how peripherally released cytokines influence central activity. There are at least two general mechanisms to help explain how peripheral cytokines reach targets in the central nervous system.

The first possibility is that pro-inflammatory cytokines travel to the brain via a humoral pathway. If cytokines were to cross the BBB, they might do so at regions of the brain where the BBB is weak or deficient (Banks, Kastin & Broadwell, 1995; Dunn, 2006; Maier & Watkins, 1998). These areas of the brain consist of the circumventricular organs (CVO’s), which include the subfornical organ, pineal gland, median eminence, organum vasculosum laminae terminalis, and the area postrema (Dunn, 2006). Once
cytokines pass this barrier, they would be able to bind to receptors on neural tissue, for example, the hippocampus, and act locally.

There is also evidence suggesting that peripheral cytokines may stimulate cells of the brain’s parenchyma to produce pro-inflammatory cytokines themselves (Dantzer, 2004). Van Dam, Brouns, Louisse, and Berkenbosch (1992) discovered possible sources of IL-1β from macrophages of the meninges and choroid plexus, microglial cells, and perivascular cells. These locally produced cytokines can then volume diffuse to neuronal targets. In addition to diffusion, there is reasonable evidence suggesting an active transport system exists for cytokines trying to enter the brain. Using radioiodinated murine IL-1α and IL-1β, one study found that the rate at which the murine interleukins crossed the BBB exceeded the rate at which radioidinated albumin crossed by approximately 50 times (Banks & Kastin, 1991). Transport of IL-1α and IL-1β was also found to be self-inhibiting, suggesting that a transport system exists, but also that it is a saturable, carrier-mediated transport.

The second possible way cytokines may signal the brain is neurally. Systemic pro-inflammatory cytokines can bind directly to afferent nerve fibers that innervate organs of the immune system. IL-1 receptors have been located on chemoreceptor-like structures called paraganglia surrounding vagus nerve terminals (Maier & Watkins, 1998). Once pro-inflammatory cytokines bind, paraganglia synapse and release neurotransmitters on the vagus nerve thereby stimulating it. Vagal nerve fibers terminate on the nucleus tractus solitarius of the medulla from which axons then project to areas of the brain related to mood and stress, such as the paraventricular nucleus of the hypothalamus, central nucleus of the amygdala, and the bed nucleus of the stria.
terminalis (Ericsson, Kovacs & Sawchenko, 1994; O’Keane, Dinana, Scott & Corcoran, 2005). Experiments providing compelling evidence for the role of the vagus nerve found that peripheral injections of IL-1β activated the marker c-fos in the vagus nerve, and that cutting the vagus below the diaphragm inhibited fever and sickness behavior induced by administration of a pro-inflammatory cytokine (Goehler et al., 1999; Watkins et al., 1995; Bluthe, 1996).

**Cytokines and Stress**

Accruing evidence demonstrates that there is an intimate connection between stress and cytokines. Certain stressors can trigger the release of pro-inflammatory cytokines, which subsequently produce behaviors characteristic of an acute phase response. Physical stressors such as tail shock appeared to activate elements of the APR including increased core body temperature (fever), and elevated serum levels of the pro-inflammatory cytokines IL-1β and IL-6 (Maier & Watkins, 1998; Zhou, Kusnecov, Shurin, DePaoli & Rabin, 1993; Nguyen et al., 1998). Immobilizing and restraining of rats coupled with insulin-induced hypoglycemia increased levels of IL-1 expression in the hypothalamus (Deak et al., 2005). Even exposure to a novel environment, a psychological stressor, induced production of IL-1β (LeMay, Vander & Kluger, 1990).

Furthermore, the presence of pro-inflammatory cytokines activates the hypothalamic-pituitary-adrenal (HPA) axis, a defining characteristic of stress.

Besedovsky, del Rey, Sorkin, and Dinarello (1986) were the first to establish the role of cytokines in the activity of the HPA axis in rats. Levels of adrenocorticotropin hormone (ACTH) and corticosterone (the principal glucocorticoid of rats) in serum increased
rapidly following small doses of IL-1 delivered intraperitoneally. ACTH is an endogenous secretion of the anterior pituitary that signals the adrenal cortex to release glucocorticoids. These glucocorticoids have a plethora of effects including suppression of inflammatory activity, and increased metabolism of carbohydrates. Clearly, cytokines in this manner can influence behavioral and endocrine responses to stressors.

**Maternal Separation as a Stressor**

Certain early experiences during critical periods of development can influence the susceptibility of the organism to stress, illness, and mood disorders (Ader & Friedman, 1965). For instance, in rodent species, brief separation from the mother, often combined with responses to novelty, affects behavior and physiology well into adulthood (Kohman, Tarr, Day, McLinden & Boehm, 2008). A recent study provides evidence that maternal separation during infancy impacts the ability of an adult mouse to cope with immune challenges. Avitsur and Sheridan (2009) reported that pups that were separated from their mothers during the pre-weaning period exhibited more sickness behaviors as adults (anorexia and reduced sucrose intake) after injections of LPS or influenza virus than pups that were not separated from their mothers. In this scenario, neonatal stress appeared to enhance the sensitivity of the pup’s immune system.

Spitz (1946) was among the first to thoroughly analyze behavioral changes due to the stress of maternal separation and novel environment. Particularly, Spitz extensively studied the effects of prolonged maternal separation in hospitalized, quarantined children. These children had little to no contact with their primary caregivers for days and even weeks at a time. As a result, a subset of children became socially withdrawn, inactive,
and generally appeared to be sick. In time, Spitz would introduce the term “anaclitic depression” as a diagnosis for these children. Continuing research concerning maternal separation used nonhuman primate models in the 1960’s to help define current principles of attachment and separation. Following separation there initially is a “protest” stage in which young vocalize and actively search for maternal contact; and, after some time, a “despair stage” in which a percentage of the infants will display depressive-like behaviors such as apathy, withdrawal, and unresponsiveness to stimuli (Kaufman & Rosenblum, 1967). In further investigations, biochemical analysis of isolated monkey infants revealed clear indication of stress during separation. HPA activity increased, and there were excessive levels of tyrosine hydroxylase, phenylethanolamine-n-methyl transferase, and choline acetylase in the adrenal gland, as well as increases in concentrations of tyrosine hydroxylase and choline acetylase in the sympathetic chain ganglion (Mineka & Suomi, 1978). Thus, the despair stage appeared associated with activation of both the HPA and sympathetic nervous system in the course of stress response.

*Maternal Separation in Guinea Pigs*

Guinea pigs will be used as a model in this investigation for studying the psychobiological response to maternal separation. Unlike other common laboratory rodents, guinea pig pups possess evidence of a strong filial attachment. They are born fully furred, with eyes and ears open, allowing them to locomote on the day of birth. In addition, pups are able to eat solid food and drink water within a few days of birth (Hennessy, 2003). In contrast to many rodents, the mother is unusually passive; therefore,
the pup has a strong attraction to the mother that serves to maintain mother-pup proximity.

Research has documented similarities in the response of guinea pigs and of infant primates during brief maternal separation (Carter & Marr, 1970; Hennessy, 2003). When placed in a novel environment alone, guinea pig pups will exhibit “active” and “passive” behaviors, suggestive of the “protest” and “despair” stages of primate separation (Hennessy & Morris, 2005). Immediately following isolation, a guinea pig pup will begin to display the active behaviors of vocalizing and locomotor activity. After some time, the pup will no longer display active behaviors, but will exhibit passive behaviors, notably prolonged eye closure, a characteristic crouched stance, and extensive piloerection (Figure 1). Previous studies with infant guinea pigs suggest these passive behaviors are mediated centrally by pro-inflammatory cytokines (i.e. are sickness behaviors). Administration of several anti-inflammatory agents attenuated passive behaviors induced by protracted maternal separation. For instance, α-MSH, an endogenous neuropeptide with numerous anti-inflammatory properties, reduced incidence of eye-close, crouch, and piloerection in maternally isolated pups (Schiml-Webb, Deak, Greenlee, Maken & Hennessy, 2006). Additionally, indomethacin, a prostaglandin synthesis inhibitor, reduced passive behaviors observed during separation (Hennessy, Schiml-Webb, Miller, Maken, Bullinger & Deak, 2007). These results indicate that depressive-like behaviors following maternal separation may be sickness behaviors mediated by pro-inflammatory cytokines in certain species.
Figure 1. A guinea pig pup displaying sickness behaviors of eye-closure, characteristic crouched posture, and extensive piloerection after protracted maternal separation.
IL-10 as an anti-inflammatory agent

In the present study, IL-10, a potent anti-inflammatory agent, was used to inhibit pro-inflammatory influences on behavior. IL-10 was originally discovered in 1989 as a cytokine synthesis inhibitory factor that blocked cytokine production from type 1 T-helper cells. Although all important producers of IL-10 are unknown, sources of IL-10 include B cells, dendritic cells, macrophages, monocytes, type 2 T-helper cells, type 1 regulatory T cells, subsets of regulatory T-cells (Tr1, Th1, Th17, CD4+, and CD8+), keratinocytes, and epithelial cells (Moore, de Waal Malefyt, Coffman & O’Garra, 2001). It appears that antigenic stimulation causes the production of IL-10, most notably in antigen presenting cells (APC) such as macrophages and dendritic cells (Howard & O’Garra, 1992).

IL-10 has an immense array of biological properties that can both suppress and costimulate numerous types of cells. If IL-10 is present, macrophages/monocytes and CD4+ cells will decrease production of various pro-inflammatory cytokines including IL-1α, IL-1β, IL-2, IL-5, IL-6, IL-12, interferon-α (IFN-α), as well as multiple stimulating factors. Antigen presenting capabilities of macrophages are impeded from down regulation of major histocompatibility complex class II proteins, and costimulatory molecules such as CD54 and CD80. IL-10 also inhibits the differentiation and maturation of dendritic cells from monocyte precursors. On the other hand, stimulatory actions of IL-10 include promoting the proliferation/cytotoxicity of CD8+ cells, and activating natural killer cells. Finally, B-cell activity is enhanced for prolonged antibody production (de Waal Malefyt, 1998).
IL-10 has also been found to modulate endocrine stress responses. Accordingly, investigators have focused on the activity of cells derived from the hypothalamus, pituitary gland, and adrenal gland. Data from such experiments attest that in murine models, IL-10 not only is produced by cells of these glands, but actually enhances hormonal secretions by them (Hughes, Cadet, Rady, Tyring, Chin & Smith, 1994; Rady, Smith, Cadet, Opp, Tyring & Hughes, 1995; Smith, Cadet, Stefano, Opp & Hughes, 1999). For instance, IL-10 caused ACTH production in pituitary and spleen cells, and also up regulated levels of corticotropin-releasing factor (CRF) from the hypothalamus. With DNA microarray technology, researchers were also able to confirm that cells of the hypothalamus, pituitary gland, and adrenal gland modified gene expression when treated with IL-10 (Tu et al., 2007). That is, it was possible to detect alterations in mRNA expression of specific genes in cells of the HPA axis in the presence of IL-10.

IL-10 can also reduce sickness behaviors that are produced in response to cytokines or stressors. For example, in experiments in which rodents received peripheral or central injections of LPS, IL-10 was found to counteract the depressive behavioral changes following administration of LPS. Bluthe et al. (1999) reported that lateral ventricle delivery of IL-10 successfully restored body weight, exploration, and mobility in rats 3 hours after LPS infusion. In addition, IL-10 knockout mice were found to be less competent in a hippocampus-dependent learning and memory task (Morris water maze) than control mice following a peripheral dose of LPS (Richwine, Sparkman, Dilger, Buchanan & Johnson, 2009). Further, Mesquita et al. (2008) also found that administration of IL-10 reversed depressive-like behaviors displayed in IL-10 knockout mice during a forced swimming test. There was a significant increase in swimming
activity instead of immobile helplessness as seen in IL-10 knockout females. Finally, a recent study in our lab reported that centrally delivered IL-10 reduced the passive behaviors of guinea pigs pups during protracted maternal separation (Perkeybile, Schiml-Webb, O’Brien, Deak & Hennessy, 2009). These data suggest that IL-10 is a potent antagonist to pro-inflammatory mediated behaviors.

*Depression and Cytokines*

Not long ago, researchers began to consider the relation of sickness behaviors to depressive illness. Indeed, there are overriding similarities between the behavioral patterns observed during sickness and the behaviors of depression. During sickness, there often is loss of ability to experience pleasure, chronic fatigue, hypomotility, cognitive deficits, and weight loss. Interestingly, clinicians include these same symptoms, according to the Diagnostic and Statistical Manual (DSM-IV-R), as part of the criteria to diagnose patients with depression. Thus, it has been suggested that pro-inflammatory cytokines may be a major contributor to depressive disorders. Substantial clinical evidence includes higher levels of cytokines in depressed patients compared to control subjects. For example, levels of IL-1β and IL-6 (when detected), increased significantly in patients diagnosed with major depression (Anisman, Ravindran, Griffiths & Merali, 1999; Thomas, Davis, Morris, Jackson, Harrison & O’Brien, 2005; Berk, Wadee, Kuschke & O’Neill-Kerr, 1997; Maes, 1995). Moreover, therapeutic administration of IL-2 and IFN-α for cancer and Hepatitis C produced “sickness behaviors”, such as sluggishness, fatigue, depressed mood, and social withdrawal (Bonaccorso, Marino, Biondi, Grimaldi, Ippoliti & Maes, 2002; Capuron et al., 2001). IL-2 and IFN-α are
cytokines used for their antiviral and antitumor properties of stimulating lymphocyte activity that can have pro-inflammatory effects. These findings raise the possibility that part of the mechanism by which maternal separation might lead to depression is by inducing a pro-inflammatory reaction.

It is now well established that early maternal separation and other forms of attachment disruption (e.g. abuse, neglect) increases the odd of developing depression in later life (Heim, Newport, Mletzko, Miller & Nemeroff, 2008). If early separation or other early stressors increase the risk of later depression, perhaps this is due in part to a sensitization of the cytokine response to stressors so that later exposure to stressors produces a greater cytokine mediated depressive response. There is some evidence supporting this possibility.

Several publications have reported the effects of cytokine re-exposure on increased HPA activity. Rats that were pretreated with a single dose of IL-1β, and later given an additional dose of IL-1β, increased production, storage, and secretion of arginine vasopressin (AVP) and CRF in secretory terminals of the external zone of the median eminence (Schmidt, Janszen, Wouterlood & Tilders, 1995). AVP is a neuropeptide that augments ACTH responsiveness to CRF in the pituitary gland. Pretreatment with IL-1β also increased ACTH and corticosterone levels after foot shock compared to rats pre-treated with saline (Schmidt, Janszen, Wouterlood & Tilders, 1995). Importantly, this study shows there is hyperactivity in the HPA response, a putative contributor to depressive illness, to repeated cytokine exposure, and suggests that there may even be a long-lasting effect of pro-inflammatory cytokines.
In another related study, Hayley, Brebner, Lacosta, Merali & Anisman (1999) investigated the sensitizing effects repeated exposure to TNF-α, a pro-inflammatory cytokine, can have on the HPA response and sickness behaviors. After mice received an initial administration of TNF-α, subsequent re-exposure 14-28 days later produced an increase in sickness behaviors compared to mice that received pre-treatment and re-exposure to saline. The sickness behaviors included decreased food intake, decreased consumption of palatable substances (chocolate milk), immobility, curled posture, and reduced exploration. In addition, there was an increase in plasma corticosterone. These findings clearly suggest that the response to repeated stimulation can, in at least some circumstances, produce a greater cytokine mediated depressive response.

Recently, our laboratory reported behavior sensitization in guinea pigs pups that were maternally separated for two consecutive days. There was an unexpected 4-fold increase in passive behaviors of pups on the second day of separation (Hennessy et al., 2008). We hypothesized that this augmented passive response might be due to sensitization of pro-inflammatory processes during maternal separation. The present study was designed to test this hypothesis.

Integration and Predictions

In the first experiment, animals received central infusion of an anti-inflammatory agent (IL-10) or control vehicle prior to an initial three-hour separation. Pups were separated in a similar way on the second day. If the pro-inflammatory response on Day 1 contributes to sensitization of the passive response, then we predicted blocking pro-inflammatory processes with IL-10 would reduce the sensitization process.
Behavior sensitization has only been seen in pups that underwent surgery. That is, in the Hennessy et al. (2008) study, pups had received surgery several days earlier to implant a telemetry probe to measure body temperature. Therefore, the next experiment examined behavior sensitization in pups that did not receive surgery. Unoperated pups were separated for three hours on consecutive days without prior infusions of any substance.
II. General Methods

Subjects

Male and female albino guinea pigs (*Cavia porcellus*) of the Hartley strain were bred in our laboratory. Each remained with the mother and littermates in polycarbonate cages (75cm x 54cm x 24cm) throughout the duration of the study, except for brief periods for placement of cannulae, routine laboratory management procedures (such as weighing), and behavioral testing. Each cage was filled with sawdust bedding, and guinea pig food and water were available *ad libitum*. The testing and colony rooms were both maintained between 65 and 70° F. The lights in the colony room operated on a 12-hour light/dark cycle with lights on at 7 am and off at 7 pm. The Wright State University Laboratory Animal Care and Use Committee approved all procedures prior to experimentation.

Behavior Test Procedures

The first behavior test occurred between Days 21-23 postnatal, while the second behavior test was conducted on the following day. Figure 2 represents a timeline of events for each subject.
Figure 2. Timeline (in days) of procedures for each infant guinea pig.
Pups were exposed to the novel environment for 3 hours between 8:30 am and 12:00 pm. An observer scored behaviors at the following intervals: 0-30 minutes, 60-90 minutes, and 150-180 minutes. To begin the test, a pup was quietly transferred to an adjacent room and placed in a clear, plastic cage (47cm x 24cm x 20cm) with a vented lid. The testing room was maintained between 65-70°F, and adjusted with space heaters as necessary.

**Behavior Scoring**

A trained observer scored active and passive behaviors behind a one-way glass. Active behaviors included vocalizations (high pitched “whistles”) and line-crossings (all four feet of the pup cross any one of four lines that equally divides the cage; Berryman, 1976). Passive behaviors included eye-close (near or complete closure of one or both eyes for at least one second), crouch (body hunched over, head tucked in between shoulders, and feet tucked beneath body), and piloerection (hair standing up over majority of the body surface). Passive behaviors, which tend to occur over protracted periods, were scored in a one-zero fashion each minute. It is important to note that the term “full passive” is used when all three passive behaviors occurred in the same minute interval. All occurrences of active behaviors (vocalizations and line crossings) were scored. A microphone set up above the test cage broadcasted vocalizations to an observer wearing headphones, who counted them with a hand counter. Line-crossings were tallied on the score sheet with paper and pencil.
Data Analyses

For passive behaviors, the number of 60-s intervals in which the pup displayed a particular behavior was analyzed, as well as the number of 60-s intervals of the “full passive” response. Because of non-normal distributions, non-parametric tests were conducted to analyze passive behaviors. These were Mann-Whitney U tests for between-group comparisons, and Wilcoxon signed-ranks tests for within-group comparisons.

Because the data for active behaviors in Experiment 1 were normally distributed, vocalization and line-crossings were examined with analyses of variance (ANOVAs). In Experiment 2, heterogeneity of variances was significant for vocalization ($p<0.01$; Levene’s Test). Therefore, non-parametrics were used for active behaviors in Experiment 2. The level of significance was set at $p<.05$ (2-tailed).

For passive measures, central tendency and variability were expressed as medians and semi-interquartile ranges when non-parametrics were used. Central tendency and variability were expressed as means and standard errors when parametric analyses were used.
III. Experiment 1

Method

Experimental Conditions. Twenty-three pups were randomly assigned to two experimental conditions. No more than one pup per litter was assigned to either condition. In the aCSF condition, 6 females and 5 males were tested, while the IL-10 condition included 6 females and 6 males. Each pup was tested twice with either 5 μl of aCSF, or 50 ng of IL-10 dissolved in 5 μl aCSF, given centrally on the first day only. This dose of IL-10 was chosen because it was the median effective dose that previously reduced passive behaviors in isolated guinea pigs (Perkeybile et al., 2009).

ICV Surgery. Between Days 16-19, pups underwent aseptic intracerebral ventricular (ICV) surgery for placement of an indwelling cannula targeting the lateral ventricle. Following pretreatment with atropine (0.05 mg/kg, intraperitoneal), pups were anesthetized with 3-5% isoflurane. A local analgesic (0.25mg/0.1ml 0.25% bupivicaine) was administered subcutaneously to the scalp before the first incision. Using a stereotaxic device, guide cannulae (26 gauge) were implanted relative to bregma at -3.0 mm anterior-posterior, -3.0 mm medial-lateral, and -4.0 mm dorsal-ventral. Adjacent to the cannula’s entry, a stainless steel metal screw was embedded in the calvaria to help anchor the cranioplastc cement. All surgical equipment was sterile prior to use. Cannulation supplies were purchased from Plastics One® (Roanoke, VA). Post-operatively, pups were
given intraperitoneal injections of buprenorphine (0.015mg/0.05ml) immediately after surgery and again 12 hours later for pain management. At least 4 days were allowed for recovery prior to the first behavior test. Pups were weighed daily after surgery, and cannulae were checked daily for integrity.

*Drugs and Infusions.* Recombinant murine IL-10 (American Research Products) was prepared in our laboratory, and both the IL-10 and vehicle solution were stored at -80° C until administration. For the central infusion, a pup was gently removed from its home cage one hour prior to the test. It was infused with 5 μl of either artificial cerebrospinal fluid (aCSF) or IL-10 in aCSF vehicle using a Hamilton Gas Tight® syringe. Administration occurred slowly over the course of 2 minutes, and the tip remained in place for an additional 30 seconds to ensure full delivery and diffusion of the substance. After infusion, the pup was returned to the home cage for one hour prior to testing. To control for handling effects, on the second day of testing pups received no infusion but were handled for 2.5 minutes, one hour earlier, before being returned to their cages to await testing.

*Results*

*Passive behaviors.* No significant effects of sex in either experimental condition were found; therefore, data were collapsed across sex. Overall, IL-10 tended to reduce each measure of passive behavior on each day (Figure 3). On Day 1, median levels of passive behaviors for pups of the IL-10 condition appeared lower than the median levels of passive behaviors for pups of the control condition. This difference was statistically
reliable, however, only for piloerection ($U=34.50, p<.05$; 1-tailed test). A decrease in passive behaviors after IL-10 administration was expected, and replicated previous results by Perkeybile et al. (2009).

In terms of sensitization, passive behaviors were more frequent on Day 2 relative to Day 1; in the aCSF condition, all passive behaviors tended to increase on Day 2. Pups showed a greater number of intervals in crouch ($p<.05$), piloerection ($p<.05$), and the full passive response ($p<.05$) on the second separation, though no significant difference between days was noted for eye-close. In contrast, pups treated with IL-10 on Day 1 only showed an increase in piloerection ($p<.05$) on Day 2.

Active behaviors. A preliminary ANOVA with sex as a variable revealed no main or interaction effect. Therefore, data were subsequently analyzed with a 2-way Drug x Day ANOVA with Day treated as a repeated measure. No significant main or interaction effects for either variable (IL-10/aCSF or Day) were found for the active behavior of vocalizations and line-crossings. Mean scores for active behaviors are listed in Table 1.
Figure 3. Median number of 60-s intervals in which pups exhibited eye close, crouch, piloerection, and full passive response following administration of either aCSF or IL-10. IL-10 reduced levels of piloerection relative to pups treated with aCSF. * differs from Day 1; p<0.05, † differs from aCSF Day 1; p<0.05, 1-tailed test. Vertical lines indicate semi-interquartile ranges.
Table 1
*Mean levels (SE) of Active Behaviors of Guinea Pigs receiving aCSF and IL-10*

<table>
<thead>
<tr>
<th>Measure</th>
<th>Day 1</th>
<th>Day 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vocalizations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>aCSF</td>
<td>2789 (±598)</td>
<td>2791 (±907)</td>
</tr>
<tr>
<td>IL-10</td>
<td>2802 (±573)</td>
<td>3040 (±868)</td>
</tr>
<tr>
<td>Line-Crossings</td>
<td></td>
<td></td>
</tr>
<tr>
<td>aCSF</td>
<td>201 (±46)</td>
<td>145 (±60)</td>
</tr>
<tr>
<td>IL-10</td>
<td>184 (±44)</td>
<td>162 (±57)</td>
</tr>
</tbody>
</table>
IV. Experiment 2

Method

Two replications of 12 pups each (6 females and 6 males) were tested in this experiment. Each pup was unoperated and tested twice on consecutive days.

Results

Passive behaviors. No effects of sex or replication were found for any measure on any day. Therefore, passive behavior scores were combined for both groups and across sex, and comparisons were made between Day 1 and Day 2 for all measures. Consecutive days of maternal separation sensitized passive behaviors of unoperated pups. Generally, the median levels of passive behaviors increased from Day 1 to Day 2 (Figure. 4). Pups showed significantly more eye-closure ($p<.002$) on Day 2 relative to Day 1, while crouch ($p<.095$) and the full passive response ($p<.06$) were marginally significant. No sensitization occurred for piloerection.

Active behaviors. In a preliminary analysis, there was no difference for sex or replication. There were no significant differences between days after combining the active behaviors of both replications. Median scores for active behaviors are listed in Table 2.
Passive Behavior of Unoperated Pups

Figure 4. Median number of intervals of eye-close, crouch, piloerection, and full passive response for unoperated pups. A significant difference was reported only for eye-close. * differs from Day 1; p<0.05. Vertical lines indicate semi-interquartile ranges.
Table 2
*Median and Semi-Interquartile Range (SIR) of Active Behaviors in Experiment 2*

<table>
<thead>
<tr>
<th>Measure</th>
<th>Day 1</th>
<th>Day 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vocalizations</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>585.5</td>
<td>888.5</td>
</tr>
<tr>
<td>SIR</td>
<td>774</td>
<td>804</td>
</tr>
<tr>
<td><strong>Line-Crossings</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>7</td>
<td>14.5</td>
</tr>
<tr>
<td>SIR</td>
<td>24</td>
<td>15</td>
</tr>
</tbody>
</table>
V. General Discussion

It has become increasingly evident that pro-inflammatory cytokines can induce passive “sickness behaviors” in some species. These sickness behaviors are adaptive reactions that often occur during stress as well as direct activation of the immune system. The passive, not active, behaviors of isolated guinea pigs may be an example. The present study replicates the results of Hennessy et al. (2008) by showing that passive behaviors are greater during the second daily separation in guinea pig pups. It was hypothesized that the increase in behaviors from Day 1 to Day 2 may be due to a sensitization of the cytokine response. As such, we predicted that administration of an anti-inflammatory agent on the first isolation would reduce the increase of passive behaviors on the second.

On the first separation, the levels of passive behaviors tended to be lower in pups that received IL-10. These data are in agreement with previous findings by our laboratory that anti-inflammatory agents can significantly reduce passive behaviors (Perkeybile et al., 2009). The reductions in passive behaviors on Day 1 were not as robust as in the preceding experiment. These differences may be due to a smaller sample size, or analysis of between-subject comparisons in the present study rather than within-subject comparisons as in the previous experiment.

Although passive behaviors tended to increase between Day 1 and Day 2 in both aCSF and IL-10 groups, the effects were stronger for the aCSF condition. Three of the
four passive measures significantly increased in pups of the aCSF group, whereas only one of the four passive measures significantly increased in pups of the IL-10 group. Subjects that were given control vehicle exhibited significant increases in levels of crouch, piloerection, and full passive behavior on the second separation. An increase in the full passive response is of particular interest because it is the most stringent measure of passive behavior. Pups of the IL-10 condition demonstrated less sensitization, significant for one measure, than did pups of the aCSF condition. This supports our hypothesis that maternal isolation on Day 1 might increase the behavioral response on Day 2 by means of a pro-inflammatory mechanism. As predicted, the effect of an anti-inflammatory agent was to produce less sensitization of passive behaviors.

Test subjects in the first experiment and in the previous study by Hennessy et al. (2008), all underwent surgical procedures that may have contributed to instances of passive behaviors. It is reasonable to believe that surgery could increase pro-inflammatory activity, and might eventually influence the passive response and the ensuing behavioral sensitization. Therefore, we tested two replications of unoperated pups to see if sensitization occurred without the effects of surgery. Pups of this second experiment showed some evidence of sensitization, though it appeared to be weaker than that shown by aCSF pups. Further, on Day 1, pups that had undergone surgery and received vehicle control tended to show higher levels of passive behaviors (other than eye-close) than did pups of the unoperated condition. This suggests that an interaction exists whereby behavior is affected by experience, as well as the immune status of the pup at the time of the experience.
It has not yet been determined which cytokines may be involved in the sensitization response. Given the eclectic bioactivity of various pro-inflammatory cytokines, there are numerous possibilities regarding the specific cytokine or groups of cytokines responsible for mediating sensitized behaviors. Previous studies have detected increases of TNF-α in the spleen of guinea pigs, but no increases were detected in central structures of the brain (Hennessy, Deak, Schiml-Webb & Barnum, 2007). IL-1 has been extensively studied as an effective promoter of cytokine-induced sickness behaviors in other rodent models. However, work to date with ELISA and RT-PCR procedures has failed to detect separation-induced changes in IL-1 or other pro-inflammatory markers in the brains of guinea pigs. Moreover, the present results do not address the specific targets of IL-10 that are involved in attenuating the passive response. IL-10 has long been known to suppress pro-inflammatory production in peripheral immune cells such as macrophages and dendritic cells, but central targets of IL-10 remain unclear. IL-10 may reduce pro-inflammatory cytokine expression in the hypothalamus and hippocampus, regions of the brain that are believed to have receptors for pro-inflammatory cytokines, and also possess the capability to mediate pro-inflammatory cytokine sickness behaviors (Maier & Watkins, 1998).

Evidence indicates that early stressors such as maternal separation can have permanent effects throughout adulthood. Various forms of attachment disruption can lead to increased risk for depression (Heim et al., 2008). There is also evidence indicating that an increased frequency of childhood illness may predispose an individual to adult depression. Key, Brown, Marsh, Spratt, and Recknor (2001) have found that adolescents with chronic illnesses reported nearly twice the occurrence of depressive symptoms
compared to control subjects. Especially male children, who suffered from ear infections, fever, and surgery, appeared to be at a higher risk for depression during the transition into adulthood (Reinherz, Giaconia, Carmola Hauf, Wasserman & Silverman, 1999). In essence, the overall well being of the child appeared to influence the onset of depressive symptoms after childhood (Raikkonen, Schubert, Pesonen, Heinonen, Viikari & Keltikangas-Jarvinen, 2004). Current literature suggests this might involve sensitization of neurochemical systems responsive to stress (e.g. CRF; Gillespie & Nemeroff, 2007).

Recent work with guinea pigs, including the present study, suggest that sensitization of cytokines may also provide a potential mechanism for the enduring effects of maternal separation. Avitsur and Sheridan (2009) recently found that neonatal maternal separation in rats could be a predisposing factor for the time and severity of LPS- or influenza virus-induced sickness behaviors. These findings also are in accord with the proposal that sensitization of cytokine systems can produce long-term effects. However, the present study provides some evidence for involvement of cytokines in behavioral sensitization over only a short 2-day period; implications for longer separations are speculative at this time. In sum, our results are consistent with the idea that sensitization may involve a pro-inflammatory effect. Future studies might examine the longer-term effects in separated guinea pigs.
VI. References


