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Electrical Brain Stimulation and Depressive-like Behavior in Guinea Pigs

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Electrical Brain Stimulation and Depressive-like Behavior in Guinea Pigs

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By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Nadia Kardegar ENTITLED Electrical Brain Stimulation and Depressive-like Behavior in Guinea Pigs BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

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Maternal separation in guinea pigs produces a biphasic response consisting of an active behavior phase (vocalizations and locomotor activity) followed by a phase of passive depressive-like behavior (crouched stance, piloerection, and eye closure). The mechanism for the transition from the active to the passive phase is unknown. One suggestion is that continual activity of neural circuitry producing active behavior eventually leads to the expression of passive behaviors. The purpose of this study was to test this possibility. Guinea pigs were assigned to experimental and control groups. The experimental group received daily stimulation of the bed nucleus stria terminalis (BNST) to produce vocalizations. Controls included non-operated animals and those which received daily electrical stimulation of a brain area not expected to produce vocalization (parietal cortex). Stimulation of the BNST elicited vocalizations, which decreased across 10 days of testing. However, BNST-stimulated animals did not show more passive depressive-like behavior than the controls.

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I. Introduction

Relation of Maternal Separation to Depression

Major depression is the leading cause of disability between the ages of 14 to 44 in the U.S. and Canada (Landers, 2007), affecting about nineteen million Americans. About 10 to 25% of women and 5 to 12 % of men will become clinically depressed sometime during their lives (Prentiss, 2011). Depressive symptoms are frequent causes of emotional and physical suffering that leads to a decrease in quality of life (Berkman et al., 1986); therefore, it is important to expand our understanding of the neural and behavioral mechanisms of depression. It is also important to study possible contributing factors throughout development that influence neuronal systems leading to depression. Clinical studies suggest that both genetic and environmental factors contribute to the risk for depression (Heim, Plotsky and Nemeroff, 2004).

A previous study estimated that 40-50% of the risk for developing depression is accounted for by genetic factors (Nestler, Barrot, DiLeone, Eisch, Gold, and Monteggia, 2002). However, genetic factors are only contributors and not the only cause for depression (Caspi and Moffit, 2006). For instance, a classic study revealed a functional polymorphism in the promoter region of the serotonin transporter (5-HT T) gene as a candidate, which moderates the influence of stressful life events on depression (Caspi et al., 2002). In their experimental study, individuals with one or two copies of the short 5-HT T allele exhibited more depressive symptoms than those individuals with two long alleles when exposed to environmental stress.

Studies have shown environmental factors including sexual, physical, and emotional maltreatment, as well as social isolation and parental loss can increase the risk for depression in adulthood (Heim, Plotsky and Nemeroff, 2004). Previous studies have suggested women who

have been abused in childhood are four times more likely to exhibit symptoms of major depression in adulthood than are those women who have not been abused (Mullen, Martin, Anderson, Romans, and Herbiosn, 1996). Another experimental study that examined about 2000 women with a history of childhood abuse found that this population attempted suicide in adulthood more often that those women without a history of childhood abuse (McCauley et al., 1997). The United States has the highest poverty rate among the wealthy nations (Belle Doucet, 2003), and depressive symptoms are very common among individuals with low incomes (Radloff, 1975). Poverty, inequality and discrimination are sources of depression among women in U.S. (Belle Doucet, 2003). Poor women more often experience stressful life events than those wealthier women. For example, in one study, about 83% of the depressed individuals were low-income mothers who experienced physical or sexual abuse in their lifetime (Bassuke, Buckner, Perloff, and Bassuk, 1998).

It is hypothesized that early life stress may induce sensitization to stress, so that vulnerability to depression in adulthood is enhanced by continued stress (Kendler, Neale, Kessler, Health, and Eaves, 1992). Early life stress also can influence developing brain regions. Various stressors and emotional trauma may shape brain development, leading to altered emotional processing and sensitization to stress (Heim, Plotsky and Nemeroff, 2004). Prolonged maternal separation is a primary example of an early stressor that appears to affect brain development to lead to depression in adulthood (Gillespie and Nemeroff, 2007).

Spitz and Wolf (1946) and Robertson and Bowlby (1952) studied the behavioral changes caused by the stress of maternal separation. They specifically studied the influence of maternal separation in hospitalized quarantined children. They discovered that the infant's initial response to maternal separation was crying (separation calls) but this stage often was followed by

withdrawal from the environment, loss of appetite, and an appearance suggesting the infant was depressed. Both studies characterized the initial response as the "protest" stage (crying) and the second response as the "despair" (depression) stage. Spitz introduced the term "anaclitic depression" to describe the second stage.

Separation calls, such as seen during protest, are the most common type of distress vocalization across mammalian species. Many studies use separation calls to study the underlying emotional dynamics of social isolation (e.g., MacLean, 1985). It is hypothesized that because separation calls reflect emotional activity of the brain, understanding this system will help to understand the nature of human psychopathologies (Panksepp, Normansell, Herman, Bishop, and Crepeau, 1988).

Mechanisms of the initial active stage of separation

One experimental technique used to evoke distress vocalizations in animals is electrical stimulation of the brain (ESB). Vocalizations have complex neural mechanisms. Motor neurons controlling separation vocalizations are located in various nuclei of the pons, medulla and spinal cord. This motor network needs input from the periaqueductal gray (PAG) of the midbrain to produce vocalizations. PAG input appears to convey emotional information. On the other hand, voluntary control of these vocalizations needs proper functioning of the forebrain (Jürgens 2002). Studies have confirmed that ESB of the PAG of the midbrain produces separation vocalizations in rat, guinea pig, bat, cat, squirrel monkey, rhesus monkey, gibbon and chimpanzee (Jürgens, 2002). Previous studies showed that lesions of the PAG lead to mutism in both animals and humans (Adametz and O'Leary, 1959).

Panksepp (1995) used electrical stimulation to map brain sites that produced vocalizations in the guinea pig. These brain areas included PAG, medial diencephalon and several basal nuclei such as the amygdala and the bed nucleus of stria terminalis (BNST) and nucleus accumbens. These brain sites include part of the limbic system, which is thought to enhance the generation of the emotions that mediate pro-social behaviors as well as separation distress vocalizations (Panksepp, 1995). Panksepp concludes these brain sites are major areas in a circuitry that he calls the PANIC system. Panksepp hypothesized this neural circuitry generates feeling of loneliness, grief and separation distress. He suggests that this circuit enhances these emotions as a result of separation and early life stress. He concludes that the PANIC system starts from the amygdala, hypothalamic area and BNST and runs down to dorsomedial thalamus and PAG. Of relevance here, the BNST has a high density of active distress vocalization sites and is a key component of the PANIC system.

Mechanism of the passive stage of separation

As Spitz and Wolf (1946) described in their study, infants will stop crying and enter a second stage of depressive like-behavior characterized by withdrawal from the environment following prolonged maternal separation. Infants of other species, including monkeys and guinea pigs show responses to separation that are reminiscent of responses in humans (Hennessy 2003; Kaufman and Rosenblum, 1967). Studies of guinea pigs may help reveal underlying mechanisms of the second stage. Guinea pigs separated from their mothers exhibit a biphasic active-passive reaction. The active and passive stages resemble the "protest" and "despair" stages seen in primates (Hennessy and Morris, 2005). The second passive stage includes a behavioral syndrome of eye closure, crouched stance and piloerection. The guinea pig is inactive and exhibits little apparent interest in its surroundings (Fig. 1).

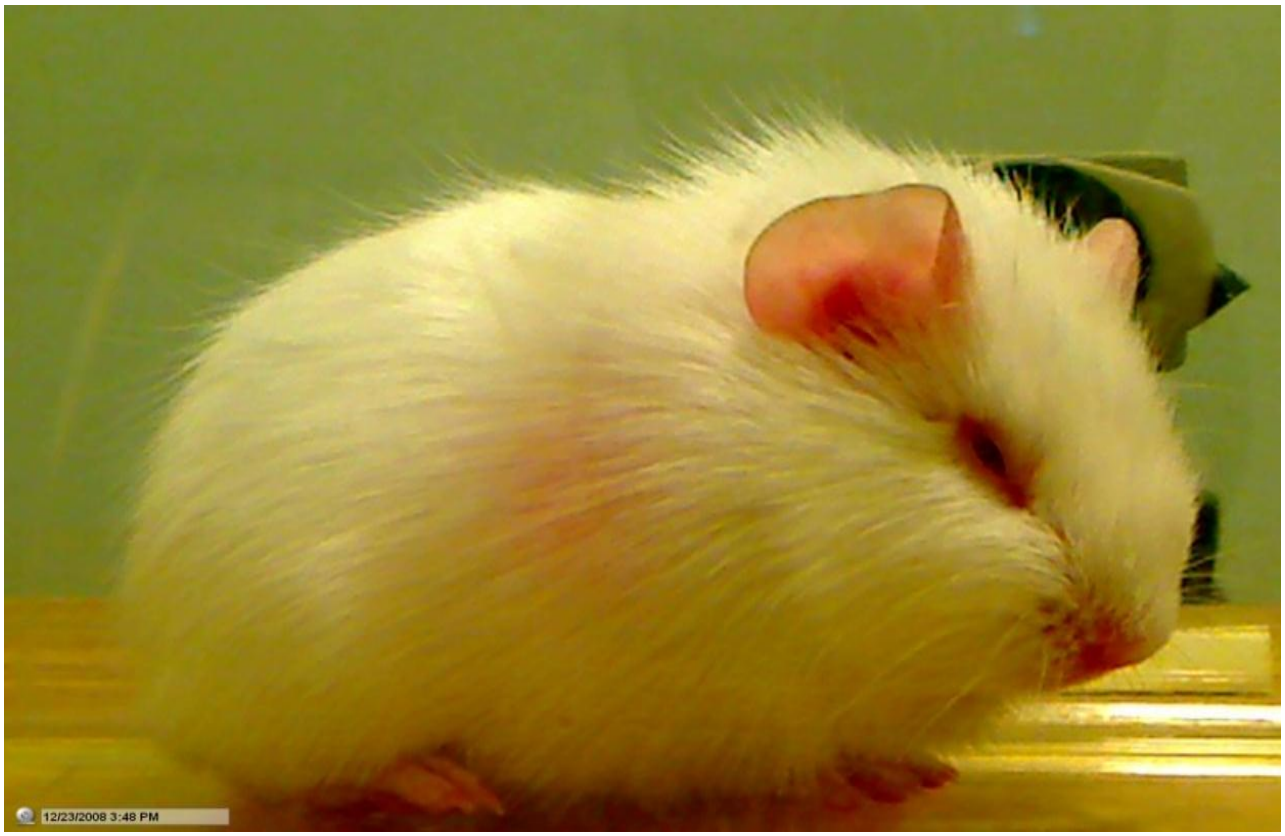

Figure 1: The guinea pig displaying passive behavior: crouched stance, piloerection, and partial eye closure.

Evidence indicates that the passive stage in the guinea pig is mediated by the immune system as part of the acute phase response (APR). The APR is the body's reaction in response to infection or injury. Cells of the immune system release cytokines as the first line of defense against invading pathogens (Baumann and Gauldie, 1994). Well-known stimulators of the APR are pro-inflammatory cytokines such as: interleukin (IL)-1, IL-6, tumor necrosis factor-α (TNF-α), interferon-γ (INF-γ), transforming growth factor β (TGF-β) and possibly IL-8 (Kushner 1993; Wigmore, Fearon, Maingay, and Lai, Ross, 1997).

Cytokines are produced and secreted by almost all immune cells of the body. Cytokines can be secreted in small amounts having localized effects or in large amounts in blood to have systemic effects. The liver has several cells that produce cytokines. When the liver has been damaged, the immune cells of the liver called Kupffer cells become activated and release cytokines allowing regeneration of the liver tissue. Along with the beneficial effects, excess production of cytokines can also be harmful by leading to scar tissue formation (Neuman, 2003). Cytokines released from the immune system can also communicate with the brain through the blood-brain barrier (Rothwell and Hopkins, 1995). When animals or humans are given cytokines intravenously, dramatic changes in behavior and brain function occur (Maes, Smith and Sciharpe, 1995; Maier and Watkins, 1998). Some physiological changes include: fever, increased slow-wave sleep, alterations in plasma ions, and increased levels of circulating white blood cells (leukocytosis); (Maier and Watkins, 1998). The behavioral changes include anorexia, adipsia (decreased drinking), loss of interest and pleasure in normal activities (anhedonia), social isolation, piloerection, hunched posture, eye closure and depressed mood (Maier and Watkins, 1998, Kent, Kelley, and Dantzer, 1992).

However, evidence shows that pathogens activating the immune system are not the only way to produce increased cytokine activity. Exposure to stressors can also increase the cytokine activity in a similar manner. Studies with guinea pigs suggest that the stressor of maternal separation may increase pro-inflammatory signaling inducing behavioral changes (Hennessy, Deak, and Schiml-Webb, 2001). Lipopolysacchride (LPS), which is a product of the cell wall of gram-negative bacteria, is well-known to induce an APR. When a guinea pig is injected with LPS it exhibits a passive response like that seen during prolonged separation. Administration of compounds with anti-inflammatory effects [α-MSH, IL-10 and indomethacin, (a prostaglandin synthesis inhibitor)] significantly reduces the passive response in guinea pigs over a prolonged separation (Hennessy, Schiml-Webb, Miller, Maken, Bullinger and Deak 2007). Separation also induces physiological signs of an inflammatory response such as fever and increased expression of the pro-inflammatory cytokine TNF-α in the spleen of the guinea pig (Hennessy, Deak, Schiml-Webb, and Barnum, 2007).

The passive stage could also represent the result of activity of specific brain circuits. Panksepp (1995) introduced the FEAR system, which generates emotions that are characterized by rapid heartbeat, sweating and increased muscle tension. He indicates activation of this system produces a freezing response (immobility), which is similar to the passive response. Panksepp found that electrical stimulation of the lateral and central amygdala and anterior and medial hypothalamus and PAG area of the midbrain projecting into the autonomic component of the brainstem will lead to expression of fearful behavior.

Panksepp's findings regarding the FEAR system and underlying brain sites are similar to those of earlier studies describing neurocircuitry of fear in the rat. Davis (1992) notes stimulation of the amygdala will induce a freezing response (immobility), therefore highlighting

the importance of the amygdala in development of fearful behavior. There are several connections between the amygdala, BNST and other brain structures involved in fear. Previous experiments suggest the central nucleus of the amygdala has direct projections to hypothalamic and brainstem areas that are involved in fearful behavior (Gray, 1993). Also there are direct projections from the central nucleus of the amygdala to the lateral hypothalamus activating the sympathetic autonomic nervous system during the fearful situation (Shiosaka et al., 1980). Indirect projections of BNST and preoptic area (which also receives input from the amygdala) to the paraventicular nucleus of the hypothalamus may also mediate fearful behavior (Sawchenko and Swanson, 1983).

How does the second stage emerge?

One of the first ideas of how the second stage emerges was in terms of Engel's (1962) "Conservation-Withdrawal" hypothesis. This hypothesis suggests that when individuals experience stressful life events, their first response is to use all their energy to combat the situation. But eventually they become exhausted and energy expenditure reaches a maximum. Individuals then feel less in control and unable to function in an accustomed manner. Therefore, they begin to hold the environment responsible for the stressful state and for their diminished capacity (feelings of helplessness and hopelessness); (Engel, 1967). Thus, they give up, become isolated from the environment, and conserve energy.

For example, during separation infants at first are active and cry to regain their mother, but eventually they become exhausted. At this point, they become inactive and withdraw from the environment. Engel (1962) suggests the first stage (crying) is a biological analog of anxiety and the second stage is an analog of depression-withdrawal. Menahem (1994) also considered

conservation-withdrawal as a biological process. His study included four infants that did not receive enough nutrients from their mothers. The infants' initial reaction was crying in effort to regain their mothers. Later infants seemed to pass into a second stage conserving their energy and biologically adapting to their mothers' inability to provide breast feeding. This study matches Engel's conservation-withdrawal theory well; in both cases infants protest (cry) in an effort to gain emotional and nutritional needs. When energy is exhausted infants stop crying, become inactive and withdraw from the environment.

Another idea of how the second stage might emerge derives from the hypothesized pro-inflammatory mechanism. The second stage of separation is thought to be due to increased pro-inflammatory activity; therefore, it may just take some time for the pro-inflammatory activity to reach a level sufficient to influence behavior (Hennessy, Deak and Schiml-Webb, 2001). One possible mechanism by which stress might initiate a pro-inflammatory response is by release of corticotropin-releasing factor (CRF). CRF is a stress hormone that can stimulate inflammation and affect behavior during separation. Subcutaneous injection of CRF in the guinea pig has been shown to suppress the active response (vocalizing and locomotor activity) and increase the passive response (Becker and Hennessy, 1993). It is hypothesized that CRF secreted during separation inhibits the active phase (Hennessy, Deak and Schiml-Webb, 2001). It appears that pro-inflammatory activity and CRF might work together to bring on the second behavioral stage of separation. A study by Gillespie and Nemeroff (2005) found elevated concentrations of CRF in the cerebrospinal fluid of several depressed individuals.

Another possible way the passive stage might emerge is based on Panksepp's idea of the two neural circuitries in the brain (the PANIC and FEAR systems). Isolation from close social figures and loss of emotional support often leads to depression. When animals are separated

from their mothers, they react by producing distress vocalizations. Thus, activating the brain areas underlying distress vocalizations (PANIC system) might lead to depression. Furthermore, there is an overlap between the brain areas underlying the PANIC and FEAR system described by Panksepp. Therefore, it is possible that prolonged activation of the PANIC system might also ultimately result in activation of the FEAR system to produce the passive depressive stage (Panksepp and Watt, 2011). In sum, continued activity of the PANIC system is thought to produce depressive-like behavior, perhaps through effects on the FEAR system.

Hypothesis

The purpose of this study was to test Panksepp's idea that overstimulation of the PANIC system produces depressive-like behavior. Our experiment used electrical stimulation of the brain (ESB) to daily stimulate a region of the PANIC system known to produce vocalization in guinea pigs. Stimulation was applied for 30 min for several daily sessions. Interspersed were probe days in which no stimulation occurred during the 30 min. Using this method we repeatedly stimulated the PANIC system, measured vocalizing, and recorded the passive response shown before and after stimulation and during probe days.

As described earlier by Panksepp, the BNST is a brain site with a high density of active distress vocalization sites and also a key component of the PANIC system. Therefore we chose the BNST as the area for applying ESB. Studies suggest the BNST receives stressor input from other regions of the brain, integrates this information, and regulates stress and reward systems (Egli and Winder, 2003). Other work suggests the BNST, basolateral nucleus of the amygdala (BLA), and central nucleus of the amygdala (CeA) are the key players for regulating fear and anxiety disorders (Radke, 2009). The CeA and BNST receive projections from the BLA and

project to brainstem regions leading to fearful behavior. These findings emphasize the importance of BNST as a site involved in producing distress vocalizations, and potentially producing depressive-like behavior.

We used female guinea pigs for our experiment because the rate of depression is higher in women than in men. This study was to specifically test how the active stage (vocalization) transitions into the passive stage. We predicted that there would be more passive behavior before and after stimulation and during the probe days in animals receiving ESB of the BNST. This study was also the first to examine effects of repeated daily stimulation of an area mediating vocalizing in awake guinea pigs.

II. Methods

Subjects

Female guinea pigs (*Cavia porcellus*) arrived in laboratory when they were 34 days old. Animals were housed in pairs in opaque plastic cages with wire fronts and sawdust bedding. Water and food were provided *ad libitum*. Lights were maintained on a 12:12 light: dark cycle; lights were on at 0700 hr. The testing and colony rooms were maintained between 65 and 75° F. Cages were changed twice per week. The Wright State University Laboratory Animal Care and Use Committee approved all procedures. Following arrival, guinea pigs were maintained in the colony room, being removed only for electrode implantation surgery, behavioral testing, and brief routine colony management procedures (such as weighing of animals). Guinea pigs were assigned to one of three separate groups: the BNST group (n=12), the cortical control group (n=8), and the non-operated control group (n=11).

Surgery

Between 38-39 days of age, each guinea pig in both the BNST and cortical control groups underwent surgery for electrode implantation. Each animal was pretreated with atropine (0.05 mg/kg, IP) and anesthetized with isoflurane (3-5%) before being placed in a stereotaxic apparatus. Surgery was performed with the skull oriented level in the apparatus. The stereotaxic device was equipped with modified incisor bar/nose cone that delivered a constant dose of isoflurane throughout the surgery. A local anesthetic was administered to the scalp (0.1 ml 0.25% bupivicaine, SC) prior to incision.

The three channel electrode array consisted of two straight stainless steel wires insulated except for the 1.5 mm at the tip. These comprised the anterior and posterior electrodes, and a third flexible ground wire wrapped around a screw secured to the skull. This stainless steel metal screw was embedded in the calvaria adjacent to the hole drilled for the electrode and also served to anchor the cranioplastic cement that secured the electrode array to the skull. The electrodes were implanted using a sterotaxic device. For the BNST group, the electrode array was directed towards the anterior and posterior BNST. The coordinates (using the posterior electrode) were +2 mm anterior-posterior, +2 mm medial- lateral and -6.5 mm dorsal-ventral relative to bregma. For the stimulation control group (stimulation of parietal cortex) the coordinates were +2 mm anterior-posterior, +5.5 mm medial-lateral and -1 mm dorsal-ventral. All surgical equipment was sterile prior to use. Throughout surgery, heart rate, and blood oxygen level were recorded.

After electrode implantation was complete, the incision was closed with absorbable suture. Animals were kept warm with a heating pad during recovery. Animals were given intraperitoneal injections of buprenorphine (0.015 mg/0.05ml) immediately after surgery and

again 12 hours later for pain management. Two to three days were allowed for recovery prior to threshold testing. Animals were weighed daily after surgery and electrodes were checked daily for stability.

Threshold determination and testing procedure

Guinea pigs were placed in a clean plastic cage in the testing room under full room lighting for a series of electrical stimulations. The embedded electrode array was connected by an insulated cable to the commutator centrally positioned above the testing chamber to allow for freedom of movement. The commutator was connected to a stimulus isolation unit, which was connected to the output of a stimulator. A 10-s train of 0.1 ms electrical pulses was delivered at increasing intensities to help identify the optimal current level for further testing. The levels of stimulation were applied in an ascending order beginning at 200 μA to a maximum of 700 μA (in increments of 100 μA) and each current was applied once. After 10 s of stimulation each animal was allowed 2 min to recover before the next stimulus was applied. During the 2 min recovery, the number of vocalizations was recorded.

For the purpose of this study, threshold was defined as the current level at which clear stimulus-bound vocalizations were observed without any abnormal behavior (e.g. distinct involuntary movement, aberrant motor responses). When such vocalizations were observed, stimulation for threshold testing was terminated. Level of the threshold and the number of vocalizations helped determine the current level for behavioral testing. If at threshold an animal emitted fewer than 150 vocalizations, then the testing intensity was set to 100 μA above threshold. Otherwise the threshold value was used for testing. The levels of stimulation for animals in the cortical control group were chosen to match those for the BNST group. Both

electrodes (anterior and posterior) were tested, and the electrode that yielded the clearest vocalization behavior was chosen.

Beginning 1 to 2 days after threshold determination, when animals were 43-47 days old, they were tested for 10 consecutive daily trials. On testing day 1, animals were quietly carried in a transport cage from the colony room to the testing room and placed in a Plexiglas chamber 9" W x 11" L x 11" D. This chamber had distinctive patterns made by black electrical tape on the outside of 3 walls, with the fourth wall remaining clear for observation. After remaining undisturbed for 5 min, the animals in the BNST and cortical control groups underwent a series of stimulations for 30 min (current was applied for 10 sec at 2 min intervals). This electrical stimulation took place during trials 1-4 and 6-9. Trials 5 and 10 were the probe days in which no stimulation occurred during the 30 min. After 30 min, each animal was returned alone to the home cage for 15 minutes of behavioral observation. All testing occurred between 9am and 3pm.

Scoring behavior

During the testing procedure, a trained observer recorded the total number of high and low whistle vocalizations (Berryman, 1976). Vocalizations were scored with a hand-held counter. The observer also scored passive behavior: the characteristic crouched posture in which the feet are tucked beneath the body, complete or near complete closure of one or both eyes, and extensive piloerection (over half of the body). The measure employed was the number of 1-min intervals in which the guinea pigs simultaneously exhibited all three passive behaviors (designated “full passive” response). Because single instances of crouch, eye-close, and piloerection typically occur over an extended period of time, these behaviors were scored with

one-zero sampling as in previous studies (e.g. Schiml-Webb, Deak, Greenlee, Maken, and Hennessy, 2006). The passive behavior was scored with pencil and previously prepared scoring sheets. Behaviors were observed and scored during the 5 min prior to stimulation, the 30 min stimulation or probe period and the 15 min following return to the home-cage. The testing chamber was cleaned with detergent prior to each use.

Histology

At the conclusion of the 10^{th} day of behavioral testing, the animals in the BNST and cortical control group were overdosed with 3-5 ml of Euthosol (subcutaneous). A 10 sec pulse of 1200-1300 μA was used to mark the stimulation site. Guinea pigs then were perfused intracardially with saline followed by 10% formalin. The brains were removed and stored in 10% formalin for 48 hours, then embedded in a 9% gelatin mold and stored in 10% formalin for at least 48 hours. This allowed the brain tissue to be fixed before sectioning on a freezing microtome. Every other 90 micron coronal section was collected through the target forebrain region and stained with cresyl violet. Prussian blue was used to confirm final electrode placement by staining the iron deposited at the electrode tip where the marking lesion was made.

Figure 2 illustrates an example of a stained slide (BNST animal) marking the final electrode placement that corresponds to the estimated coordinates for BNST.

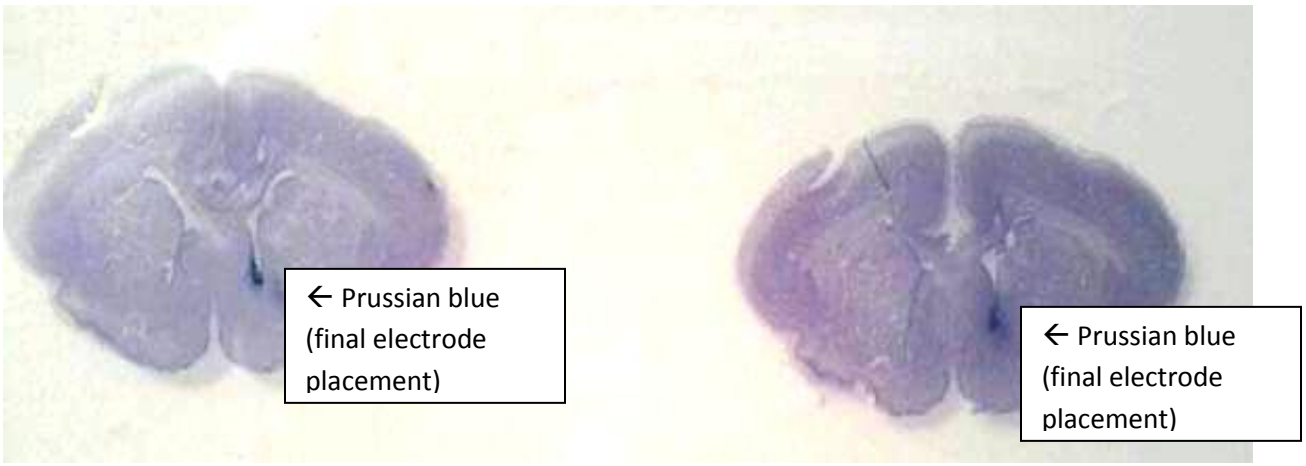

Figure 2: Cresyl violet staining of a coronal section of the brain (BNST animal).

III. Results

Data Analysis

In each test period analyzed, the total number of vocalizations and 1-min intervals in which animals exhibited the full passive response was recorded. Preliminary results showed that the cortical control group and non-operated group did not differ at any measure; therefore, data from the 2 groups were combined for further analysis. Because the data displayed a large number of zero scores and violated assumptions for parametric tests, the data were analyzed with non-parametric tests. For within group comparisons, Friedman tests and Wilcoxon matched pairs tests were used for multiple and paired comparisons respectively. Mann-Whitney U tests were used when the two separate groups were compared.

Vocalizations

During the pre-stimulation period, no differences in vocalizing were observed (Fig. 3). Mann-Whitney U tests indicated there was no significant difference in vocalizations between the two groups on any trial. Friedman's analysis of variance yielded no significant difference across the trials for either the experimental or control groups.

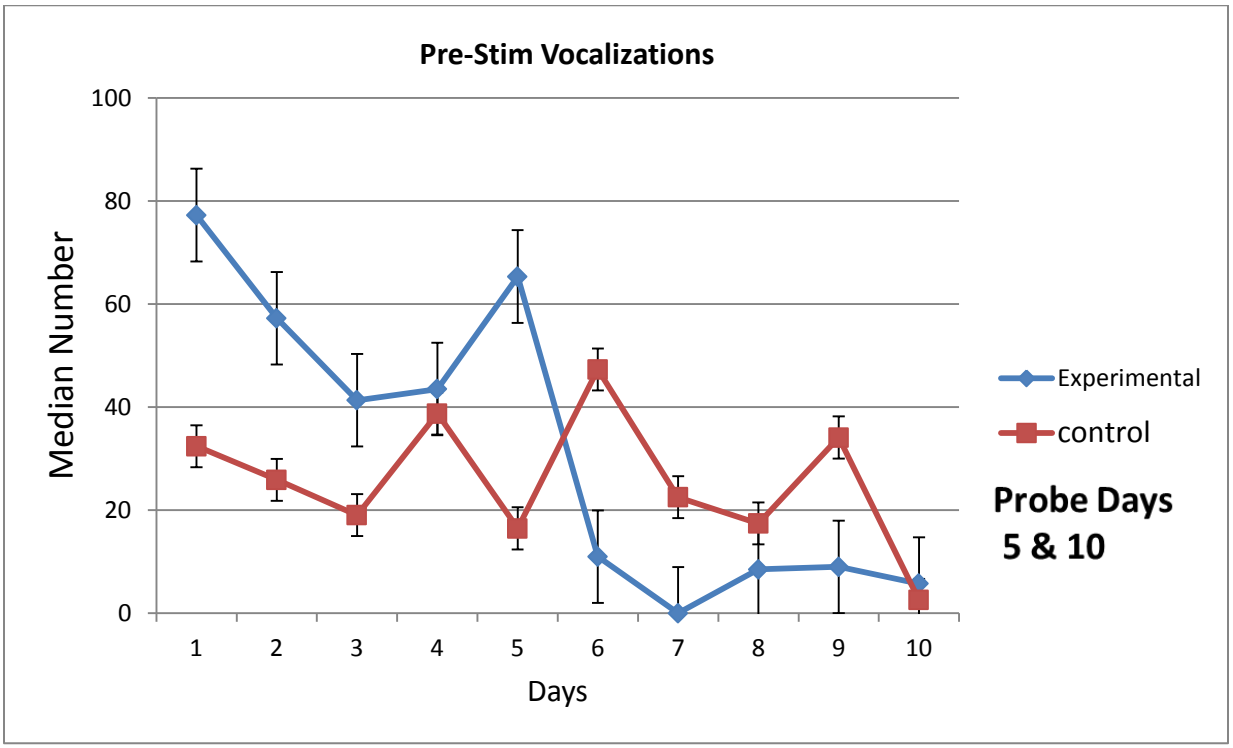

Figure 3: Median number of vocalizations during the pre-stimulation period for the experimental and control groups throughout the 10 trials.

Figure 4 illustrates the vocalizations during the stimulation period. The experimental group emitted more vocalizations than the control group on all trials excluding probe trials (p's < 0.05). Within group comparisons for the experimental group yielded a significant difference across the trials (p's < 0.05). Wilcoxon matched pairs tests were used for post-hoc comparisons. For the experimental group, there were significantly more vocalizations on days 1 and 2 than day 10 (p's < 0.05; Figure 3). There were more vocalizations on day 1 than days 5 through 9 [day 5 ($p < 0.01$), 6 ($p < 0.01$), 7 ($p < 0.05$), 8 ($p < 0.05$), 9 ($p < 0.05$)]. Within the group comparison for the control group revealed that animals vocalized more on day 1 than days 8 through 10 [day 8 ($p < 0.01$), 9 ($p < 0.05$), 10 ($p < 0.01$)].

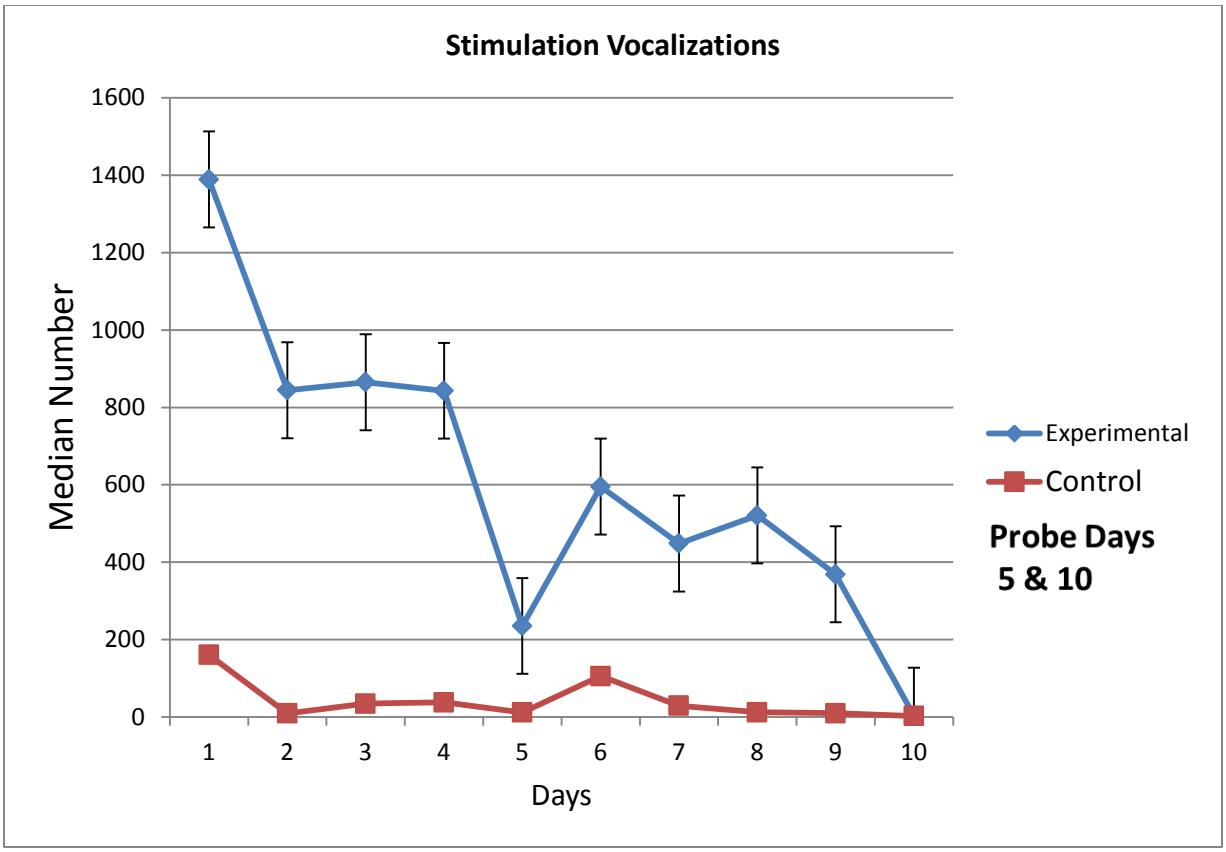

Figure 4: Median number of vocalizations during the stimulation period for the experimental and control group throughout the 10 trials.

Figure 5 illustrates the vocalizations throughout the post stimulation period. There was no significant difference between the experimental group and control group on any day. Also, there was no change in vocalizing across trials for the experimental group. For the control group vocalizing declined over days ($p < 0.05$). There was less vocalizing on day 9 compared to days 1 through 4 [day 1 ($p < 0.01$), 2 ($p < 0.01$), 3 ($p < 0.01$) and 4 ($p < 0.05$). There was less vocalizing on day 10 compared to days 1 through 4 [day 1 ($p < 0.01$), 2 ($p < 0.01$), 3 ($p < 0.01$) and 4 ($p < 0.05$)].

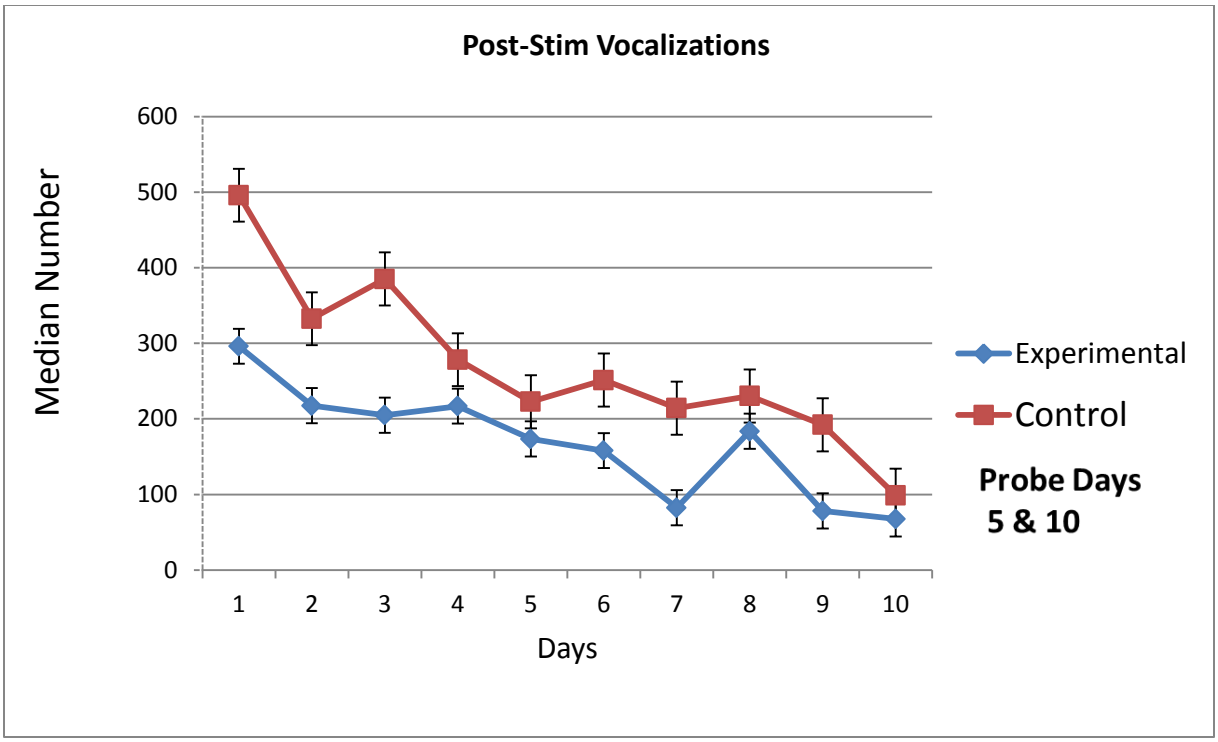

Figure 5: Median number of Vocalizations during the post-stimulation period for the experimental and control group throughout the 10 trials.

Full Passive Response:

As Figure 6 illustrates, during the pre-stimulation period few of the animals exhibited the full passive response (only one median score of 1). There was no difference between the groups on any trials. Within group comparison for both groups yielded no significant difference across the trials for either group.

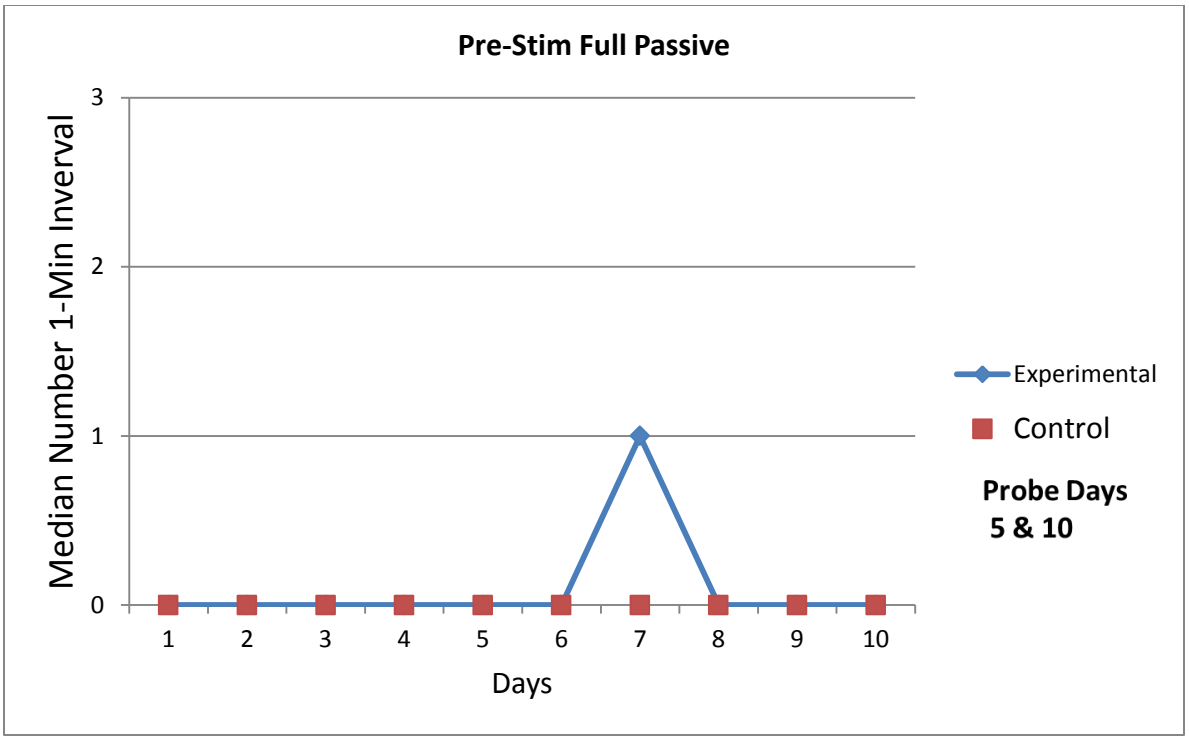

Figure 6: Median number of full passive responses during the pre-stimulation period for the experimental and control group throughout the 10 trials.

During the stimulation period, between group comparison revealed there was more full passive response in the control group than the experimental group on days 1 through 4 (p's < 0.05). As illustrated in Figure 7, the full passive response increased across the trials for both groups resulting in a significant difference for within group comparisons (p's < 0.05). For the experimental group, post hoc comparison showed there was more full passive response on day 10 than days 1 through 4 (p's < 0.01). For the control group there was more full passive response on day 9 than days 1 and 2 [day 1 ($p < 0.05$), 2 ($p < 0.01$)]. There was more full passive response on day 10 than day 1 ($p < 0.05$).

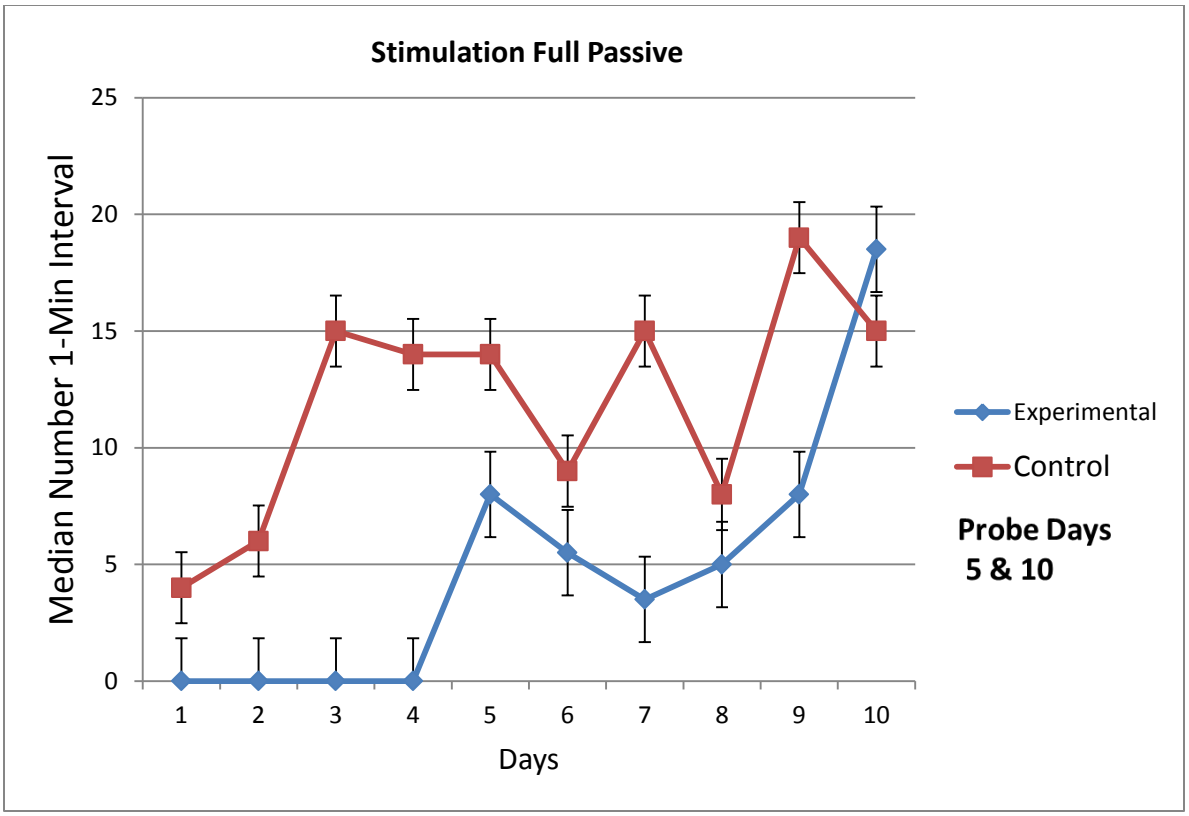

Figure 7: Median number of full passive responses during the stimulation period for the experimental and control group throughout the 10 trials.

During the post stimulation period, the full passive response was rarely observed in either group (Fig. 8). There was no difference between the 2 groups on any trial and no difference across trials for either group.

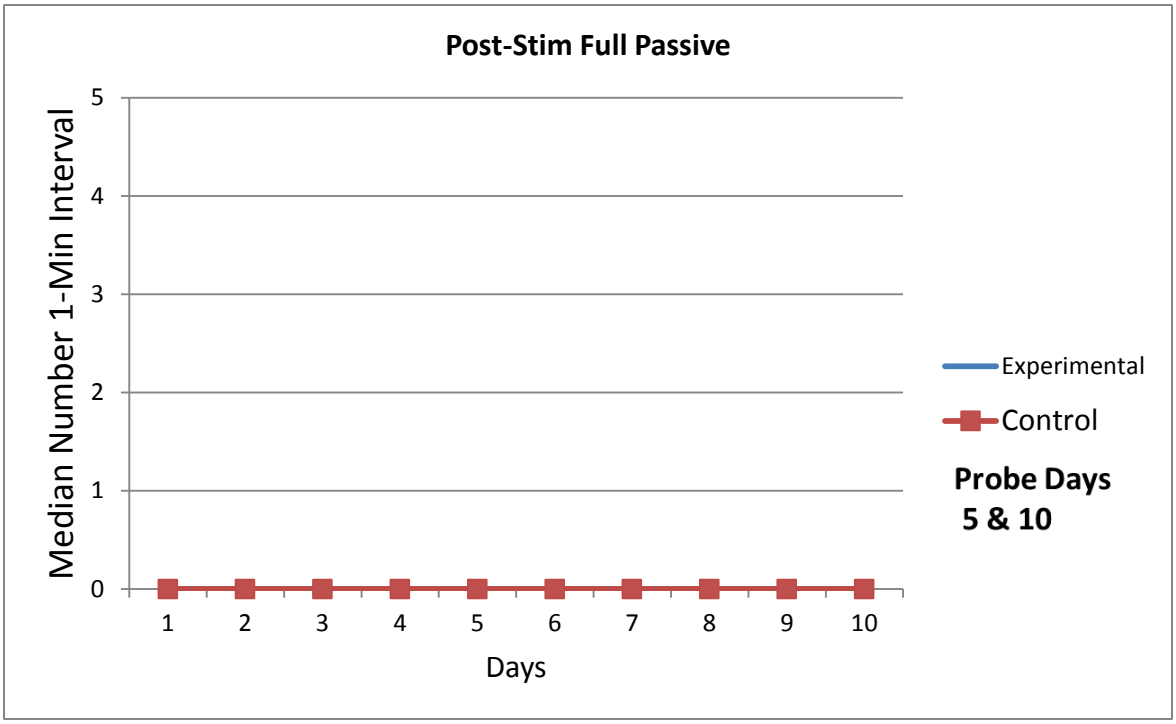

Figure 8: Median number of full passive responses during the post-stimulation period for the experimental and control group throughout the 10 trials.

IV: Discussion

Some mammals including non-human primates and guinea pigs show a biphasic active-passive response to the stress of the maternal separation. In the first stage (protest), animals actively vocalize. In the second stage (despair), passive depressive-like behavior appears. This study specifically tested how the active stage transitions to the passive stage. The mechanism for the transition is unclear, but, in guinea pigs, previous studies suggest two main hypotheses. One is that the pro-inflammatory activity that underlies the passive stage could take some time to develop following separation. After an hour or so, pro-inflammatory activity may reach a level at which it begins to influence behavior. Another suggestion specifically proposed by Panksepp is that overstimulation of the PANIC system, which is thought to generate distress vocalizations, eventually produces depressive-like behavior. This study tested Panksepp's idea of how depressive-like behavior is derived.

ESB was used to repeatedly stimulate the BNST, an area of the brain considered part of the PANIC system, in order to actively produce distress vocalizations. Our controls (non-operated and cortical), as expected, did not produce as many vocalizations. We predicted that the repeated stimulation would increase the passive behaviors seen on subsequent days in the experimental group. We tested the guinea pigs for 10 consecutive days. During each daily trial, active and full passive behaviors were observed throughout the pre-stimulation, stimulation and post-stimulation periods. Behavior also was observed on probe days, when the animals were placed into the test area but no stimulation was applied. The number of vocalizations recorded during stimulation was greater for the experimental group than for the control group. Thus, the procedure achieved the first goal of producing vocalizations by stimulating the BNST. Both groups showed a significant reduction in the number of vocalizations over days though the

decline was much greater for the experimental group. The sharp decline in the experimental group does not appear to be due to fatigue because the response takes relatively little energy and animals have a day to recover between trials. It is possible that the electrodes damaged neurons though the level of stimulation used was set at minimal level that elicited vocalizations in order to avoid damage. It seems likely instead that changes with repeated stimulation in downstream neural connections reduced the impact on the vocal apparatus.

During observation periods when no stimulation occurred (pre-stimulation, probe days, post-stimulation), there was no difference in the number of vocalizations between the experimental and control groups. However, both groups showed a substantial number of vocalizations during the post-stimulation period particularly during the early trials. This might be due to the absence of the cage-mate in the home cage or excitement of returning to the safety of the home cage.

During the pre-stimulation period there was a negligible amount of passive behavior observed in either group. The pre-stimulation observation was only for 5 min. It is possible that the pre-stimulation period was too brief to detect any differences between the two groups. The full passive behavior of the two groups also was similar during the post-stimulation period and during probe sessions. During stimulation there was an increase in full passive response in both groups across the trials. However, the experimental group showed significantly less full passive responding than the control group during the first four trials. Because we predicted that the experimental group would show *more* passive behavior with repeated BNST stimulation, our results do not support our prediction, and suggests instead that overstimulation of the PANIC system does not produce the second stage. Another alternative suggestion is that the BNST was not the best area of the PANIC system to be stimulated for this study. To test this possibility a

member of our laboratory recently stimulated a different area within the PANIC system, the PAG. She tested the guinea pigs using the same experimental procedure but only with this different brain site. Findings were similar: when animals were stimulated, vocalizations were produced, but no change in passive behavior was observed (Dazey, unpublished). In all, these findings suggest that the transition from the active stage to the passive stage is not due to the overstimulation of the PANIC system alone and that other factors are involved. Future studies might attempt to further test the involvement of pro-inflammatory factors in the transition to the second stage.

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