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The Effects of Rodent Maternal Separation on Consumption and Motivation for Sucrose Reward and Its Relationship to Anxiety

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Abstract

The Effects of Rodent Maternal Separation on Consumption and Motivation for Sucrose Reward and Its Relationship to Anxiety

By Kelly Dixon Watts

There is evidence that an adverse early-life environment is associated with susceptibility to anxiety and depression later in life. Separation of rat pups from their mother during early development may lead to an exaggerated stress response and increased anxiety in adulthood. Recent studies suggest that maternal separation also alters reward behavior. These experiments tested whether maternal separation would alter both anxiety and motivation to obtain sucrose reward in adulthood. On postnatal days 2-14, Long-Evans rat pups were subjected to either 180 min of maternal separation (HMS 180), 15 min of separation (HMS 15), animal facility raised (AFR), or unhandled (UNH). Adult male offspring were tested for anxiety-like behaviors on the open field and elevated plus maze (EPM), locomotor activity, sucrose preference, and operant responding for sucrose at increasing fixed-ratio (FR) requirements. HMS 180 had increased anxiety in both the open field and elevated plus maze compared to AFR and UNH. UNH had increased locomotion compared to HMS 15 and HMS 180. No rearing group differences were observed in preference for sucrose solution in the home cage. However, HMS 180 rats took significantly longer than UNH to eat freely available sucrose pellets in the operant chamber. Anxiety measures correlated with initial sucrose consumption in the novel chamber. There were no significant differences in acquisition of FR1 responding, in maintenance responding at any fixed-ratio requirement, or in extinction. However, anxiety measures on the EPM and the open field correlated significantly with operant responding at both low and high ratio requirements across groups. General activity did not correlate with responding at high fixed ratios. This suggests that even in rats habituated to the test setting, anxiety is associated with lower levels of responding for sucrose. Together, these observations suggest that maternal separation consistently leads to increased anxiety, which correlates with delayed initial response to sucrose reward in a novel environment. While no group differences were observed on operant responding for sucrose, anxiety was associated with lower incentive motivation across animals.

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The Effects of Rodent Maternal Separation on Consumption and Motivation for Sucrose Reward and Its Relationship to Anxiety

Introduction

There is strong evidence that an adverse early-life environment is associated with susceptibility to anxiety and depression later in life (Heim, et al, 2002). While good maternal care is associated with resilience to affective disorders (Gourion et al., 2008), early life stressors such as poverty, abuse, and neglect correspond with increased stress-reactivity and increased risk for psychopathology in adulthood. (e.g. Lupien et al., 2000;., De Bellis, 2005, Gilmer and McKinney, 2003; Gunnar et al., 2001, 2009; Heim, et al, 2002; Kendler et al., 2002; 2003).

Separation of rat pups from their mother during a critical period of early development is thought to lead to a constellation of behavioral, endocrine, and molecular changes in the brain that are proposed to model stress and depression in the adult offspring (Plotsky and Meaney, 1993; Caldji et al., 1998; Wigger and Neumann, 1999; Ladd et al., 2000; Huot et al., 2002; Aisa et al., 2007; Arborelius et al., 2004). Brief periods (15 min) of separation of rat pups from the mom has been shown by some to decrease stress reactivity in these rats as adults, presumably because of an increase in sensory stimulation from the mom upon return of her pups (Plotsky and Meaney, 1993; Bhatnagar and Meaney, 1995; Sanchez et al, 2001). However, animals experiencing a longer duration of separation (3-6 hours around postnatal days 2-14) frequently show a hyperactive stress hormone response to an acute stressor and anxiety behaviors as adults. (Plotsky and Meaney, 1993; Caldji et al., 1998; McIntosh et al., 1999; Wigger and Neumann, 1999; Ladd et al., 2000). Additionally, recent experiments suggest maternally

separated rats show altered reward behavior in adulthood, indicative of anhedonia, which is a hallmark symptom of depression (Matthews & Robbins, 2003; Ruedi-Bettschen et al., 2005; 2006). However, results from the separation procedure are not always consistent, making interpretation of findings difficult. We proposed that multiple measures of anxiety might be used to ensure that the maternal separation procedure led to a robust phenotype. Then we examined whether maternal separation differentially affected consumption of reward versus motivation, or willingness to work for reward.

Background

Impaired Stress Response after Maternal Separation

Stress increases release of corticotropin-releasing factor (CRF) from the paraventricular nucleus (PVN) of the hypothalamus which triggers the release of adrenocorticotropin hormone (ACTH) from the pituitary gland (Liu, et al, 2000a). ACTH causes the synthesis and release of glucocorticoids (GC, or corticosterone in the rat) from the adrenal gland into circulation.

Studies have shown that as adults, long-separated rats (HMS 180) exhibit an exaggerated HPA axis response to an acute stressor in adulthood, with prolonged elevations of ACTH and/ or CORT (Plotsky and Meaney, 1993; Ladd et al., 1996, 2000, 2004; Schmidt et al., 2004). Short-separated rats (HMS 15) or offspring of dams with high frequencies of licking and grooming show enhanced suppression of the stress response during adulthood (Sanchez et al., 2001; Fenoglio et al., 2006b). These short-separated rats are thought to be stress-resilient.

Feedback and Feed-forward Regulation of the Stress Response

There are two types of glucocorticoid receptors in the hippocampus and cortex, mineralocorticoid receptors (MR) and glucocorticoid receptors (GR). Mineralocorticoid receptors have a much higher affinity for circulating glucocorticoids than GRs, and most MRs are occupied basally. During stress, MRs become saturated and GRs bind glucocorticoids which triggers a negative feedback reduction of the stress response by inhibiting PVN CRF expression (Tottenham and Sheridan, 2010).

HMS 180's have increased hippocampal MR expression and binding and decreased GR expression and binding in the hypothalamus, hippocampus, and frontal cortex (Plotsky and Meaney, 1993; Ladd et al. 2000, 2004, 2005; Huot et al 2004; Francis et al., 2002). This decrease in hippocampal or cortical GR is associated with reduced negative feedback control of CRF expression in HMS 180's. Conversely, HMS 15 rats have increased GR expression in the hippocampus and reduced CRF expression in the hypothalamus (Plotsky and Meaney, 1993; Avishai-Eliner et al., 2001a). Long-separated adult offspring have impaired negative feedback control over the stress response, while short-separated rats have enhanced negative-feedback. Functional variants of the brain mineralocorticoid and glucocorticoid receptor (GR) genes have also been identified in humans (de Kloet et al, 2007), suggesting that genetics can predispose individuals to HPA axis hyperactivity.

In contrast to the negative feedback initiated in the hippocampus and frontal cortex, glucocorticoids acting in the amygdala can increase CRF production. The amygdala and bed nucleus of stria terminalis (BNST) play an important role in the initiation of the stress response to a threatening or emotional stimulus (Tottenham and

Sheridan, 2010). Aside from increases in hypothalamus CRF, HMS 180 rats also have increased CRF immunoreactivity levels in the central nucleus of the amygdala (CeA), the bed nucleus of stria terminalis (BNST), the locus coeruleus (LC), and the parabrachial nucleus, plus increased CRF receptor 1 mRNA in the locus coeruleus and the raphe nucleus (Ladd et al., 1996, Plotsky et al., 2005). This excess in CRF activity helps explain the prolonged stress response in long-separated rats. Mice over-expressing CRF exhibit reduced hippocampus, PVN, and amygdala volumes compared to wild-type mice (Goebel et al, 2010).

Stress also increases norepinephrine (NE) release from the locus coeruleus (LC) and nucleus tractus solitarius (NTS) to regions including the PVN, amygdala, and medial prefrontal cortex (Liu et al, 2000b; Morilak et al, 2005). The central nucleus of the amygdala (CeA) releases CRF to the locus coeruleus to trigger NE release, which, in turn, may activate further CRF release from the CeA in a feedforward circuit (Lui, et al., 2000b). These LC neurons receive presynaptic CRF input (Van Bockstaele et al., 1996). Early experience can alter both the number of cells and the volume of the LC nucleus, thus causing stable structural changes in this region (Lucion et al, 2003).

NE release in the PVN and other brain regions is attenuated by glucocorticoids acting in the medial prefrontal cortex (mPFC) and benzodiazepines (BDZ) acting at $GABA_A$ / BDZ receptors in the amygdala and LC (Lui et al 2000b). HMS 15 rats have higher levels of $GABA_A$ and BDZ receptors in the CeA, LC, and NTS which is associated with decreased anxiety (Caldji et al., 2000, 2003). Conversely, in HMS 180 rats, the combination of increased CRF and NE and reduced BDZ binding sites in the amygdala, frontal cortex, LC, and NTS facilitate anxiety (Caldji et al., 2000, 2003;

Morilak et al, 2005; Plotsky et al., 2005). Increased anxiety on measures such as the open field, elevated plus maze, and the startle response is a well replicated finding in HMS 180 rats (Caldji et al., 1998, 2000; Wigger and Neumann, 1999; Ladd et al., 2000; Huot et al., 2001; Kalinichev et al., 2002).

Long-separated rats also show impairments on hippocampal-dependent memory (Aisa et al., 2007; 2009; Liu et al., 2000; Fenoglio et al., 2005). Low levels of glucocorticoids in the hippocampus enhance long-term potentiation and memory, but excess glucocorticoids impair both (Sapolsky, 1996). Furthermore, long-separated rats show decreased brain-derived neurotrophic factor (BDNF) and neurogenesis in the hippocampus (Branchi et al. 2006; Lippman et al. 2007; Roceri et al. 2002, 2004; Liu et al. 2000; Mirescu, et al 2004). Decreases in hippocampal BDNF and neurogenesis are thought to be a hallmark feature of depression, and their reversal may play a role in antidepressant response (reviewed in Pittenger and Duman, 2008, but see Krishnan & Nestler, 2008, 2010). However, maternal separation also increases BDNF in the Ventral Tegmental Area (VTA), an area involved in reward and motivation (Lippman et al, 2007). One study found that injecting BDNF directly to the VTA caused depression-like behavior on the forced-swim test (Eisch et al, 2003). Therefore, differential regulation of BDNF expression in the hippocampus and the reward system may be involved in depressive behaviors.

Influence of Touch on Life-Long Stress Reactivity

One critical difference researchers have found between short maternal separation and longer intervals is a burst of licking, grooming, and arched-back nursing from the dam after a short separation (Plotsky and Meaney, 1993; Bhatnagar and Meaney, 1995;

Sanchez et al., 2001). In fact, touch may account for up to 70% of the variability in HPA axis response to a stressor in adult offspring (Liu et al., 1997; Francis et al., 1999; but see Feldon & Pryce, 2003). Even substituting the dam's touch by stroking pups with a brush is sufficient to increase hippocampal GR expression and decrease HPA hyperactivity due to long separation (Suchecki et al, 1993; Gonzalez et al, 2001).

Higher GR expression in offspring of dams with high licking and grooming levels is at least partially mediated by increases in the transcription factor nerve growth factor-inducible protein A (NGFI-A) (Weaver et al, 2004). NGFI-A interacts with other proteins to essentially "open up" the DNA to allow transcription. Conversely, pups from low licking-grooming dams show increased methylation of the GR gene promoter, which makes the glucocorticoid receptor DNA "unavailable" for transcription (Weaver et al, 2004).

Tactile stimulation from licking and grooming increases serotonin release in the hippocampus (Weaver et al, 2004). Serotonin binds to 5-HT 7 receptors and ultimately leads to increased expression of NGFI-A and CREB-binding protein (Weaver et al, 2004). NGFI-A and CREB-binding protein then act at the GR exon 1-7 promoter to increase GR transcription (Weaver et al, 2004). NGFI-A may also regulate the increase in GABA seen in short-separated offspring by upregulating the glutamic acid decarboxylase enzyme needed for GABA production (Zhang et al, 2009). So, at least one putative mechanism by which maternal touch acts through serotonin receptors is to regulate expression of glucocorticoid receptors and GABA, thereby altering feedback sensitivity to stress in short-separated rats. Serotonin levels are reduced in the hippocampus and medial prefrontal cortex of long-separated rats (Matthews et al, 2001).

BDNF expression, which is altered by maternal separation, is also regulated by methylation status of the promoter region of the BDNF gene (Roth et al. 2009). Such epigenetic modifications might be responsible for mediating other changes resulting from altered maternal care.

While these findings point to important effects of touch and serotonin on the development of stress reactivity, the role of serotonin in anxiety and depression is complex. Serotonin can be anxiogenic or anxiolyitic depending on which brain region and specific receptor subtype is activated, among other factors (Charney, 2004). Furthermore, there are complex interactions between serotonin and other neuromodulators, such as dopamine and CRF. Stress or high doses of CRF activate a specific subset of serotonin neurons in the dorsal part of the dorsal raphe (DRD) that project to anxiety-related regions (Lowry et al, 2008). Blocking serotonin release from the DRD is anxiolytic. Maternal separation increases CRF receptor density and serotonin transporter mRNA in the DRD (Gardner et al, 2009).

Reward

Both maternal separation and studies of human depression suggest alterations in the reward system (Matthews et al, 1996a,b; Krishnan & Nestler, 2008, 2010), although results are not always consistent and sometimes contradictory. The reward system is traditionally characterized by the mesocorticolimbic dopamine system projecting from the ventral tegmental area (VTA) to the nucleus accumbens (NA), amygdala, and medial prefrontal cortex (mPFC) (Yap and Miczek, 2008; Krishnan & Nestler, 2010). Dopamine is released in response to drugs of abuse or natural rewards, as well as to cues predicting reward (Schultz, 1996, 2000). As opposed to mediating pure hedonics, current studies

suggest dopamine is necessary for incentive motivation, or willingness to work for reward (Neill and Justice, 1981; Berridge, 1996; Robbins and Everitt, 1996; Aberman and Salamone, 1999). However, dopamine is also released from the VTA in response to a stressor or glucocorticoid administration (Horger & Roth, 1996; Piazza, et al 1996; Dichiara et al, 1999). Specifically, stress increases dopamine release in the mPFC and the shell region of the NA, but not to the core or dorsal striatum (Deutch, et al, 1993; Barrot, et al, 2000). This dopamine release may be adaptive in the short term, but chronic stress can lead to long-term changes in the reward system (Pittenger and Duman, 2008). Alterations in this reward system have been noted in both maternally separated rats and in human depression (Krishnan and Nestler, 2008).

Human Reward and Depression

Studies of human depression suggest altered reward processing. One functional MRI study found reduced NA and caudate activation to monetary reward in unmedicated depressed subjects (Pizzagalli et al, 2009). Also from the same lab, anhedonia but not other depression or anxiety symptoms correlated with reduced NA activation to reward and with lower baseline activity in the rostral anterior cingulate cortex (ACC) (Wacker et al, 2009). Kumar and colleagues (2008) also found reduced activation of the NA and ACC to reward cues in medicated depressed subjects. The short allele of the serotonin transporter gene (*5-HTTLPR)* may be a risk factor for altered ACC activation (Holmes et al., 2010). A missense iso/val polymorphism of the mineralocorticoid receptor (MR) gene is associated with less of a cortisol-induced increase in MR expression, a greater reactivity to stress, and more depressive symptoms (Bogdan et al, 2010). A recent study found that carriers of the val polymorphism had enhanced learning of reward associations

at baseline, but showed greater impairment of shifting response towards the more frequently rewarded stimulus under stress (Bogdan et al, 2010). Dillon and colleagues (2009) found that subjects with childhood adversity were more anhedonic, rated reward cues less positively, and had weaker activation of the globus pallidus to reward cues.

Changes in dopamine function have been found in human depression, but the results are more equivocal. A subset of depressed patients with psychomotor slowing showed increased binding of D2/ D3 receptors in the striatum (Ebert et al, 1996; D'Haenen and Bossuyt, 1994). However, other studies have not found differences in these receptors, perhaps because they did not include a significant number of subjects with psychomotor slowing (as reviewed in Martin-Soelch, 2010). Studies have reported both increased (Laason-Balk et al, 1999; Brunswick et al, 2003; Yang et al, 2008) and decreased DAT binding in the striatum (Meyer et al, 2004; Sarchiapone, et al, 2006) or no differences (Argyelan et al, 2005). Tremblay and colleagues (2005) found that depressed subjects were hypersensitive to the rewarding effects of d-amphetamine, which correlated with severity of anhedonic symptoms and with decreased activation of the ventrolateral PFC and premotor cortex. Of particular relevance to the maternal separation paradigm, Pruessner and colleagues (2004) found enhanced dopamine release to stress in individuals with a history of poor maternal care. The magnitude of DA enhancement correlated with salivary cortisol. Together these studies provide support for the hypothesis of altered reward functioning in depression, but also highlight the heterogeneity of the disorder.

Reward and Rodent Maternal Separation

Some of the initial studies of rodent maternal separation and reward, led by Matthews and colleagues, found an apparent decrease in responsivity of the dopamine system in adult offspring (Matthews et al, 1996a; 1996b). They found a decrease in the initial, but not the habituated, locomotor response to novelty (Matthews et al, 1996a), and a blunted locomotor response to d-amphetamine in females (Matthews et al, 1996b). Similarly, Li and colleagues (2003) did not find an effect of maternal separation on locomotor response to novelty or cocaine. Male and female MS rats showed less of an increase in "anticipatory" conditioned locomotion to an environment paired with food (Matthews et al, 1996b). Additionally, they found a retarded acquisition for cocaine self-administration at low doses in both sexes (Matthews et al, 1999). During the maintenance phase, MS males had only a tendency to administer less cocaine, while females responded significantly more for low-dose cocaine (Matthews et al, 1999). While these studies may point to decreased sensitivity of the dopamine system, differences in MS rats were largely confined to the initial testing phase and Pavlovian conditioned actions or were sex-specific.

Later studies have found just the opposite: an apparent increased reactivity of the dopamine system in maternal separation. Using the same female rats that exhibited a blunted locomotor response to amphetamine (Matthews, 1996a), Hall and colleagues (1999) found enhanced nucleus accumbens dopamine release in response to high potassium or amphetamine using microdialysis. Also, Matthews and colleagues (2001) found increased tissue levels of dopamine in the dorsal and ventral striatum, with decreased dopamine turnover in the mPFC. Consistent with this finding, Brake and

colleagues (2004) found a lower density of dopamine transporter in the nucleus accumbens and dorsal striatum, and greater increases in NA dopamine release to stress. Behaviorally, they found an increased locomotor response to cocaine and behavioral sensitization to amphetamine (Brake et al, 2004; Kikusui et al, 2005) which is consistent with human data (Tremblay et al, 2005). Moffett and colleagues (2006) found that only MS 180 acquired cocaine self-administration at the lowest dose, while both HMS 180 and unhandled rats had higher maintenance-phase responding than HMS 15. Some of the different findings may be accounted for by the behavioral state of the animal, with separated rats more sensitive to the effects of novelty or stress.

Changes in dopamine receptor binding have been more consistently found altered in short separation offspring (HMS 15) versus long separation and AFR. HMS 15 are reported to have increased D2 receptor binding in the VTA compared to HMS 360 and AFR (Ploj et al, 2003; Meaney et al, 2002). Hippocampal D1 receptor density was increased in HMS 15 compared to HMS 360 (Ploj et al, 2003). Reduced D3 receptor binding in the NA shell was reported in HMS 15 (Brake, et al 2004). Both HMS15 and HMS 180 had increased D1 receptor binding the nucleus accumbens compared to AFR (Moffett et al, 2007). These findings mimic the heterogeneity of dopamine receptor changes in humans.

Alcohol

Several studies have looked at the effects of alcohol intake after maternal separation. There is consensus that handled (HMS 15) rats generally consume the least amount of alcohol (reviewed in Roman and Nylander, 2005). Several studies have

reported that HMS 180's consume significantly more alcohol than HMS 15 (Hout et al., 2001; Jaworski et al., 2005; Roman et al, 2003), but not all studies reported differences between HMS 180 and AFR or UNH (Jaworski et al., 2005; Roman et al., 2003; Francis and Kuhar, 2008). In fact, Ploj and colleagues (2003) only found significantly higher intake in maternally separated rats than handled and AFR if they looked at a subset of "responders" that initiated and maintained alcohol consumption alcohol (reviewed in Roman and Nylander, 2005). Importantly, Hout and colleagues (2001) found that increased alcohol consumption in HMS 180's correlated positively with cortisol response to stress and correlated negatively with time spent in the open arm of the elevated plus maze. This suggests a relationship between anxiety, stress response, and alcohol consumption.

ICSS

Intracranial brain self-stimulation (ICSS) has traditionally been used as a measure of pure reward state, although it has recently been suggested to tap into drive rather than pure hedonics (Berridge & Kringelbach, 2008). Matthews and Robbins (2003) studied only female rats and found no differences in baseline thresholds in medial forebrain bundle ICSS. However, separated rats were less responsive to ICSS reward-enhancing properties of heroin and more sensitive to the reward-diminishing properties of the D2 antagonist raclopride. Sweet and colleagues (unpublished data) found an acquisition deficit for ICSS in maternally separated offspring but no differences in maintenance responding across a range of frequencies and intensities. Michaels and colleagues (2007) also found an acquisition deficit. Only female rats showed lower levels of responding across frequencies compared to unhandled rats. Recently, Der-Avakian and Markou

(2010) found no significant differences in baseline threshold currents over the first 14 days, though amphetamine lowered the threshold for responding to a greater extent in HMS 180 rats versus unhandled. They also tested whether the additional stressor of social defeat just before ICSS testing would unveil differences in responding. After the initial social defeat, HMS 180 rats did not show a stress-induced elevation of ICSS threshold like unhandled rats. However, after the seventh defeat, HMS 180 had significant elevations in threshold compared to non-socially defeated HMS 180. Together, these data suggest subtle alterations in responding for rewarding brain stimulation, but the nature of that change depends on sex and subsequent stressors.

Specific Aims

While studies suggest maternal separation leads to alterations in reward behavior, there is disagreement in the degree of change. Furthermore, not all separation procedures yield the expected HPA axis hyperactivity and resultant increase in anxiety behaviors. We proposed that measures of anxiety on digiscan activity, open field, and the elevated plus maze might be used to ensure that the maternal separation procedure led to a robust phenotype. The following experiments were designed to test the hypothesis that long bouts of maternal separation in early development would alter both anxiety and responding to reward in the adult offspring. We hypothesized that the four experimental groups would fall on a continuum with long-separated HMS 180 being the most anxious, followed by completely unhandled rats, then animal facility-raised rats, and finally short-handled HMS 15 as the least anxious as assessed by digiscan activity, open field activity, and elevated plus maze. Additionally, maternal separation may alter both reward preference and motivation for reward. We hypothesized that maternally separated rats

would have a decreased preference for sucrose solution over water compared to the other groups, indicative of anhedonia. Further, we hypothesized that long-separated rats would have decreased motivated responding for sucrose and that short-separated rats would have the highest level of operant responding. Finally, we correlated measures of anxiety with measures of reward and motivation to determine if anxiety levels predicted a decreased willingness to work for reward.

Material and Methods

Animal Care and Maternal Separation

Timed pregnant Long Evans rats [Crl: (LE)BR; Charles River Laboratories, Portage, Mich.] arrived on day 12 of gestation. Pregnant dams were house individually with access to nesting material. On postnatal day (PND) 2, all pups were pooled, sexed, and culled to litters of six males and two females per dam (Hout, et al, 2001). Litters were randomly assigned to one of four rearing groups:

(1) unhandled (UNH)—pups were not exposed to any handling or experimenter manipulation, including cage bedding changes, from PND2 to PND14

(2) animal facility rearing (AFR)—pups were briefly handled twice a week during routine cage changes beginning on PND 4

(3) handled (HMS15)—pups were separated from the dam for 15-minutes from PND2-14

(4) maternal separation (HMS180)—pups were separated from the dam for 180-minutes from PND2-14

During separation, the dam was transferred to an adjacent cage. Then each litter was transferred to a small container with nesting material and placed in an incubator maintained at 32±0.5°C (PND 2–5) or 30±0.5°C (PND 6–14). Together with huddling behavior, this helped the pups thermoregulate. At the end of the separation period, pups were returned to their home cage, rolled in bedding to prevent rejection, and then reunited with the dam. Handling and separation were initiated between 0800 and 1600h in a semi-randomized order to prevent the dam from habituating to separation time of day.

Approximately half of the cage bedding material was changed once per week beginning on PND5 when the dam and pups were out of the home cage. Beginning on PND18, bedding was completely changed twice a week. Pups were weaned on PND21, housed with their foster littermates until PND30, and then pair-housed with foster siblings. Behavioral testing began at 5 months of age, at which time the animals were housed individually Behavioral testing was conducted between 09:00 and 16:00 h.

All animals were maintained on a 12 h/12 h light/dark cycle (lights on 0700 hours) at 22°C, 45–55% humidity, with food and water available ad libitum. All experiments were performed with approval by the Emory University IACUC and complied with Federal requirements (NIH Guidelines for the Care and Use of Laboratory Animals).

Digiscan Locomotor Activity

Adult male offspring were assessed for long-term alterations in locomotor activity in a novel, low-stress environment. The testing chamber was a square, clear plexiglass box (39 x 39 x 30.5 cm) surrounded on all four bottom edges by infrared photobeams (7

per side) (Omnitech Electronics Columbus, Ohio). This apparatus was housed inside a larger wooden box with a white noise ventilation fan and a dim light mounted on the ceiling of the chamber.

Rats were individually placed inside the testing chamber for 30 minutes. The number of beam breaks was recorded by computer. Consecutive breaks of two adjacent beams were scored as "horizontal" movement. These types of movements include both ambulatory and non-ambulatory movements, such as grooming. Consecutive breaks of a different beam that the last one were scored as "ambulatory" or locomotor movement around the box. Data were analyzed for total horizontal and ambulatory counts to test the hypothesis that HMS 180 rats would have lower activity levels.

Open Field

The open field apparatus was a circular wooden structure 82.5cm in diameter with walls a height of 31 cm. The flooring and walls were white laminate. The floor was marked with a center circle, 17cm diameter, surrounded by a circle of 50cm in diameter. The area between the 17cm and 50cm circles was divided into 6 equal sectors, while the area between the 50cm circle and the outer 82.5cm circle was divided into 12 equal sectors. The testing room was brightly lit at about 765 lux. All sessions were recorded and fed to a monitor in the adjacent room for observation.

Each rat was placed in the center of the open field and allowed to explore freely for 15 minutes. The apparatus was cleaned with quatricide solution between tests. Behavior was taped and later scored using Stopwatch (Center for Behavioral Neuroscience, Atlanta, GA). Any entry into a sector was counted as 2 paws over the line

(because large rat size meant that at this point he was half-way in a sector). Behavior was scored for latency to return to the center ring, and number of outer, middle, and center ring entries. Total entries were considered a measure of overall locomotor activity, whereas entries into the middle and center rings were considered a measure of anxiety (fear of open spaces). We expect maternally separated rats to show the fewest entries to the middle and center, followed by UNH, AFR, and then HMS 15 in order from lowest to highest.

Elevated Plus Maze

A PathFinder Maze System (Model 89000 Lafayette Instrument Co, Lafayette, IN) was converted into a plus configuration with two opposing closed arms (70cm x 10cm x 20 cm) and two opposing open arms (70cm x 10cm x 20cm) elevated 89cm from the ground. Additionally, there was a distinct plus-shaped center (35cm X 10cmx 35cm in each direction) segregated from the arms by clear polycarbonate doors elevated to create a 15cm high opening to the arms. The maze floor was white metal and walls were clear polycarbonate lined with black posterboard along the exterior to render them opaque. The room was dimly lit (~100 lux) by a single bulb pointed away from the maze. The behavior of each rat was digitally recorded into Pinnacle Studio Version 9 and was observed by experimenter in adjacent room. Each rat was placed in the center of the plus maze facing a closed arm and allowed to explore for 15 minutes. The maze was cleaned with quatricide solution between runs.

Behavior was scored using Stopwatch (Center for Behavioral Neuroscience) for number of closed and open arm entries, duration of arm entries, duration of time in the

center, and latency to enter open arm. An arm entry was defined as all four paws in an arm. One unhandled rat fell off the plus maze and was not included in the analysis. Entry and time spent in the open arm were the critical measures of anxiety, which were expected to be reduced in maternally separated rats.

Sucrose Solution Preference Test

Rats were tested for sucrose solution preference in a two bottle, free-choice design over four 24-h periods in the home cage. A decreased preference of sucrose solution is considered to be a measure of anhedonia. We expected HMS 180 rats to have a lower preference for sucrose solution than UNH, AFR, and HMS 15.

Initially, rats were given two water bottles, one on the right and one on the left side of the cage, for 48 hours to determine side preference and basal water consumption level. After 48 hours, rats were given two new pre-weighed bottles: one containing 1% sucrose and one with water. The sucrose solution was placed on the non-preferred side of the cage, and then the position was switched every 24 hours. At the end of four days, the same experiment was repeated with 4 % sucrose solution. Bottle weights were taken at the beginning of the experiment and at 24 hour intervals to determine amount drunk in grams (converted to mL). The results of sucrose consumption were expressed as amount consumed per day and as a preference ratio. Preference ratios were calculated as the percentage of sucrose solution consumed of total fluid intake (mL per 24 h). Occasionally a bottle leaked, and when this occurred, data were discarded for that day. One HMS 15 rat and one UNH rat were deleted from analysis after a cage-flooding leak

caused an apparent strong aversion to the sucrose solution (and sharp drop in intake compared to day before the leak).

Operant Responding

Rats were tested on operant responding for sucrose to determine how hard they were willing to work under increasing response requirements. We expected maternally separated rats to make fewer responses as the ratio requirement increased. We hypothesize that HMS 15 would have the highest levels of operant responding, followed by AFR, and then UNH, with HM 180 making the fewest. The operant conditioning chamber (31x31x25cm) was constructed of clear Plexi-glass© and housed inside a plywood box. A single lever was located 3 cm above the floor, and 6 cm from the front wall of the chamber. A computer program controlled a daily 20-min session, where each rat could earn food pellets by depressing the lever. Rats were not food-deprived to avoid additional stress.

At the start of training, rats were placed in the operant chamber and 45mg sucrose pellets (NOYES Precision Pellets, Research Diets, New Brunswick, NJ) were delivered to a food hopper non-contingently every minute for 20 minutes (fixed time, FT). Rats were kept on this schedule for a minimum of two days or until 19 of 20 pellets were consumed in one session. Once criterion was met, rats advanced to a fixed-ratio 1 (FR) schedule where each lever press was rewarded with a pellet. Rats were maintained on FR1 for a minimum of 12 days or until 4 consecutive days of responding were stable. Then the schedule of reinforcement was increased to FR2 (2 lever presses = 1 pellet) for 4 days, followed by FR4 and FR 8 for four days each, then FR16, FR32, and FR64 for 6

days each. Extinction, during which no lever presses were rewarded, was tested for 6 days. Data were analyzed for (1) days to FT criterion, (2) acquisition—total responses each day over the first 12 days of FR1, and days to reach criterion of 50 and 100 bar presses (3) mean responses per rat over the last for days of each fixed ratio requirement, (4) total responses each day of extinction. One rat was dropped from analyses after FR16 because of an illness, confirmed by a veterinarian, that affected lever pressing.

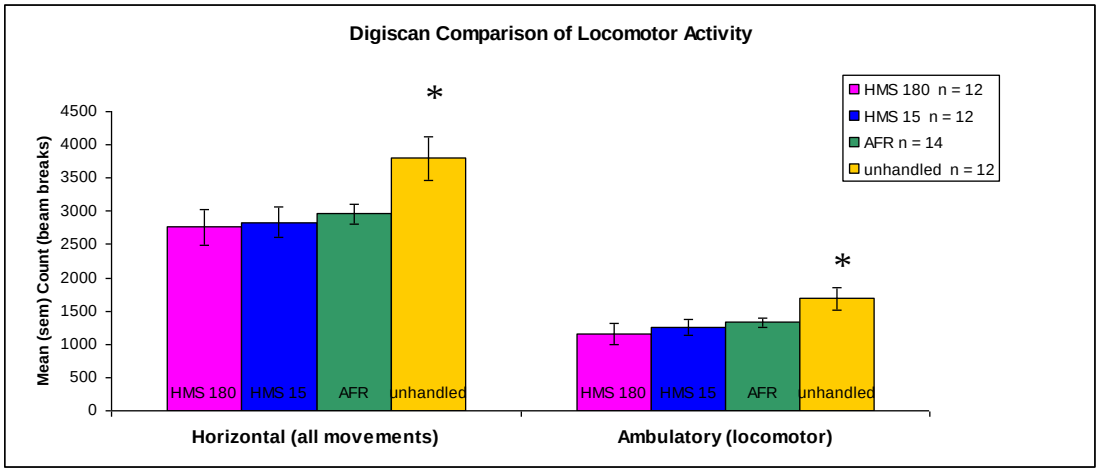
Statistical Analyses

Digiscan activity, elevated plus maze, criterion to 50 operant responses, and fixed ratio (FR) responding were analyzed with analysis of variance (ANOVA) with the between subjects factor of *Separation group* (HMS180, HMS15, AFR, UNH). Post hoc analyses were done with Tukey HSD and alpha set at $p < .05$. Sucrose solution preference, FR1 acquisition, and extinction were analyzed using repeated measures with day as the within-subjects factor and separation group as the between-subjects factor. Where appropriate, post hoc analyses were done with Tukey HSD for between-subjects factor. Because of unequal variances among groups, open field, FT criterion, criterion of 100 bar presses, FR8, FR16, and FR32 were analyzed using nonparametric Kruskal-Wallis, followed by Mann-Whitney with α set at $p \leq 0.008$ for 6 group comparisons. Correlations between elevated plus maze measures and operant responding were preformed using simple linear regression within each group individually. T-tests comparing differences in slopes between these regression lines did not yield any significant differences, so correlations were also performed on all rats together.

Results

Digiscan Activity Arena

We hypothesized that the novel environment of the digiscan would be somewhat anxiogenic. We predicted HMS 180 would show the lowest levels of activity, followed by UNH, AFR, and with HMS 15 showing the highest activity. This prediction was partially confirmedMale rat offspring of the maternal separation procedures were tested as adults for long-term alterations in basal locomotor activity (Fig 1). There was a significant difference in total "horizontal" motor activity among groups ($F_{3,46} = 3.95$, $p = 0.014$). The average number of total beam breaks was significantly increased in Unhandled (UNH) compared to both HMS 15 ($p = 0.035$) and HMS 180 ($p = 0.02$). There was also a significant group difference in "ambulatory" locomotor movements ($F_{3,46} = 3.286$, $p = 0.029$). As predicted, the average locomotor activity was significantly lower in HMS180 compared to UNH ($p = .027$). Contrary to our predictions, UNH had the highest locomotor activity and HMS 15 had lower levels of activity.



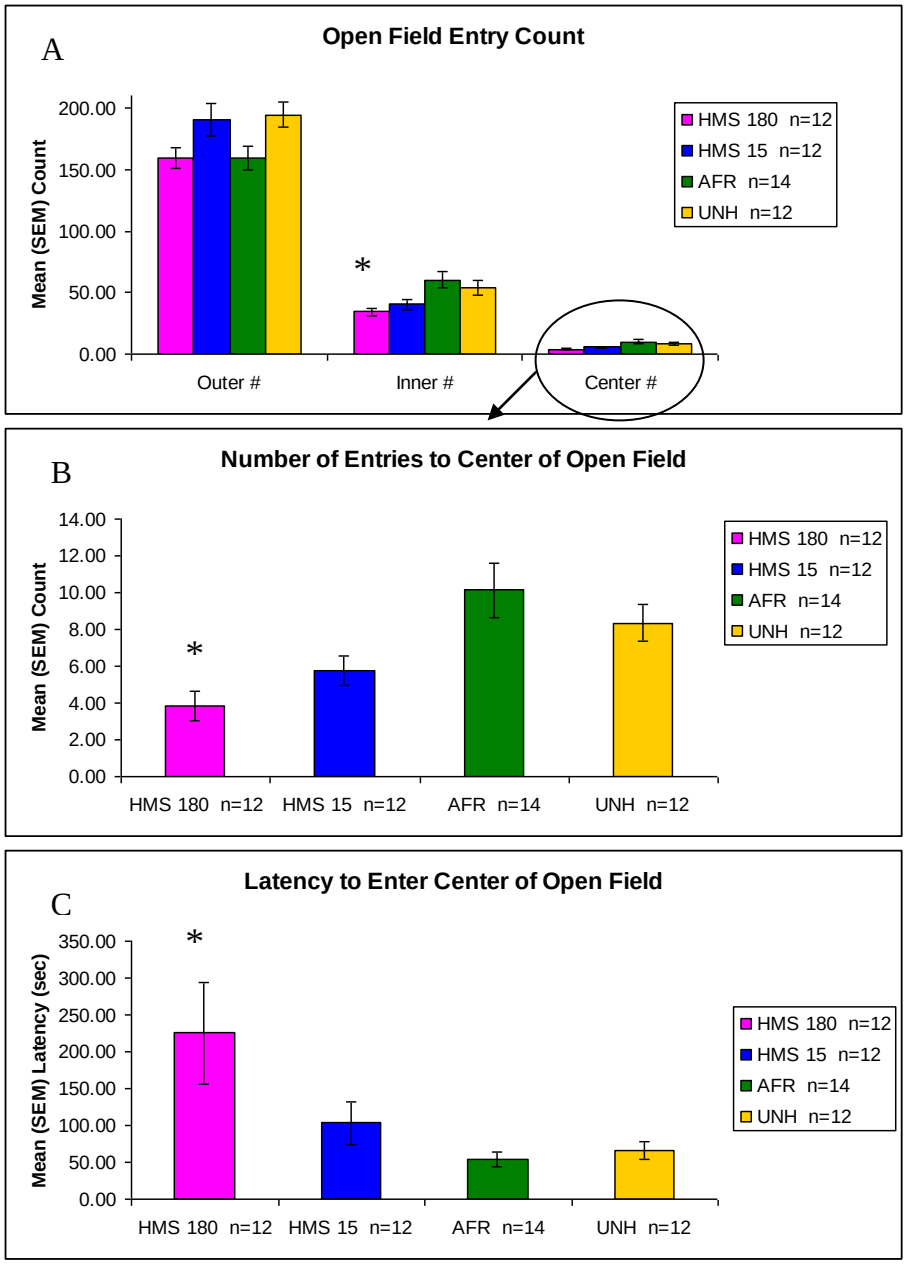
HMS 180 = maternally separated, HMS 15 = 15 min separation, AFR = animal facility raised, UNH = unhandled

Figure 1. Effects of maternal separation on baseline locomotor activity in adulthood. Mean (*SEM*). Unhandled rats had significantly higher horizontal (total) movements than HMS 15 and HMS 180 ($p < 0.05$). Unhandled rats also made significantly more ambulatory movements than HMS 180 (* $p<0.05$).

Open Field

We expect maternally separated rats to show the fewest entries to the middle and center, followed by UNH, AFR, then HMS 15 in order from lowest to highest. Levene's test indicated inequality of variance, so non-paramtetric statistics were used. Kruskal-Wallis showed a main effect of separation group on number of center rings crossed (χ^2_3= 15.54, p = .001). A Mann-Whitney test indicated that HMS 180 made significantly fewer center entries than UNH (*U* = 20.0, p = .002) or AFR (*U* = 22.5, p = .001, Fig 2B). There was also a main effect of separation group on number of inner rings crossed (Fig 2a, χ^2_3= 13.56, p = .004). Mann-Whitney showed that HMS 180 made significantly fewer inner ring entries than AFR (*U* = 18.5, p = .001, Fig 2A). There was a trend for HMS 180 to make fewer inner ring entries than UNH (*U* = 29.0, p = .012) that did not reach significance. Kruskal-Wallis revealed a significant group effect of number of outer ring entries (χ^2_3= 10.47, p = .015). Follow-up with Mann-Whitney only showed trends for HMS 180 to make fewer outer ring crossings than HMS 15(*U* = 33.0, p = .024) or UNH (*U* = 33.0, p = .024), or for AFR to make fewer outer ring crossings than HMS 15(*U* = 41.0, p = .027) or UNH(*U* = 38.5, p = .017).

As shown in Figure 2C, there was a significant main effect of separation group on latency to enter the center ring (χ^2_3= 14.33, p = .002). Mann-Whitney indicated that HMS 180 took significantly longer to return to the center than AFR (*U* = 18.0, p = .001) or UNH (*U* = 21.0, p = .003). Fewer entries to the inner and center rings and a longer latency to return to the center indicate higher levels of anxiety in the HMS 180 maternally separated rats versus AFR and UNH. HMS 15 unexpectedly had lower inner and center entries than UNH and AFR.



HMS 180 = maternally separated, HMS 15 = 15 min separation, AFR = animal facility raised, UNH = unhandled

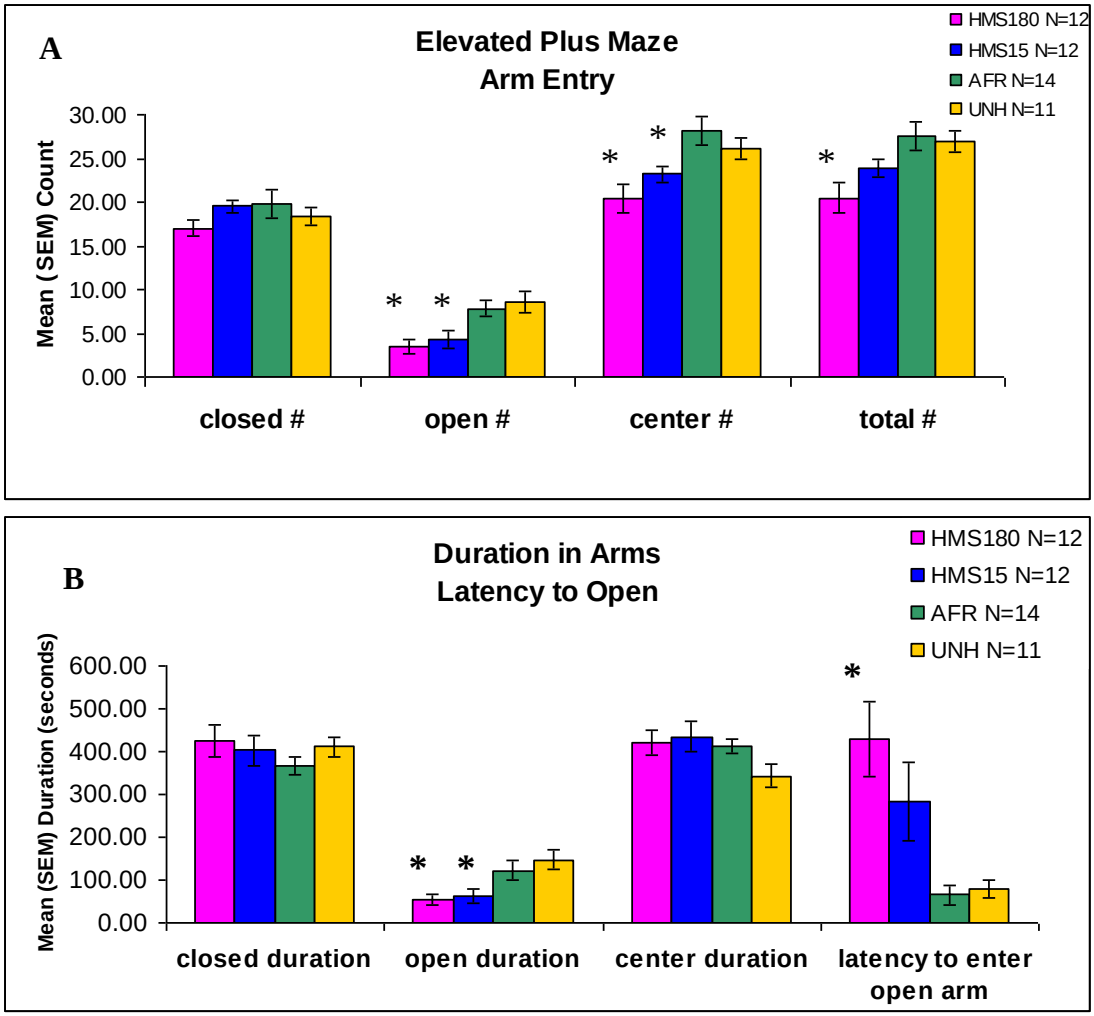
Figure 2. Effects of maternal separation on Open Field activity in adulthood. Mean (*SEM*).

- **A.** HMS 180 made significantly fewer inner ring entries than AFR.
- **B.** HMS 180 made significantly fewer center entries than UNH or AFR.
- **C.** HMS 180 took significantly longer to return to the center than AFR or UNH. (* $p < .008$)

Elevated Plus Maze

We hypothesize that HMS 180 would show the highest levels of anxiety as demonstrated by longer latencies to enter the open arm, fewer open arm entries, and shorter open arm duration. We predicted UNH would follow HMS 180 in open arm measures, then AFR and HMS 15. Analysis of variance showed a main effect of separation group on number of open arm entries ($F_{3,45} = 6.32$, $p = 0.001$). Post-hoc analyses using Tukey's HSD indicated that HMS 180 rats made fewer open arm entries than AFR ($p = .013$) or UNH ($p = .006$), while HMS 15 rats made fewer open entries than UNH ($p = .027$). There was also a main effect of separation group on center entries ($F_{3,45} = 6.99$, $p = 0.001$), with both HMS 15 ($p = .044$) and HMS 180 ($p = .001$) making fewer center entries than AFR. There were no group differences in number of closed arm entries. However, there was a significant effect of group on total arm entries ($F_{3,45} = 5.96$, $p = 0.002$). HMS 180 made fewer total entries than AFR ($p = .002$) or UNH ($p = .01$), which is presumably due to fewer open arm entries.

As shown in Figure 3B, there was a significant main effect of separation group on time spent the open arm ($F_{3,45} = 5.76$, $p = 0.002$). HMS 180 spent significantly less time in the open arm than AFR ($p = .047$) or UNH ($p = .007$). HMS 15 also spent less time in the open arm than UNH ($p = .017$). There were no significant group effects on closed arm duration ($F_{3,45} = .84$, $p = 0.478$) or center duration ($F_{3,45} = 2.07$, $p = 0.118$). There was a significant main effect of separation group on latency to enter the open arm ($F_{3,45} = .8.24$, $p < .001$). HMS 180 took significantly longer to enter the open arm than AFR ($p<.001$) or UNH ($p = .002$). HMS 180 showed the expected decrease in open arm activity, while HMS 15 unexpectedly also had significantly lower open arm activity.



HMS 180 = maternally separated, HMS 15 = 15 min separation, AFR = animal facility raised, UNH = unhandled

Figure 3. Effects of maternal separation on Elevated Plus Maze Anxiety in adulthood. Mean (*SEM*).

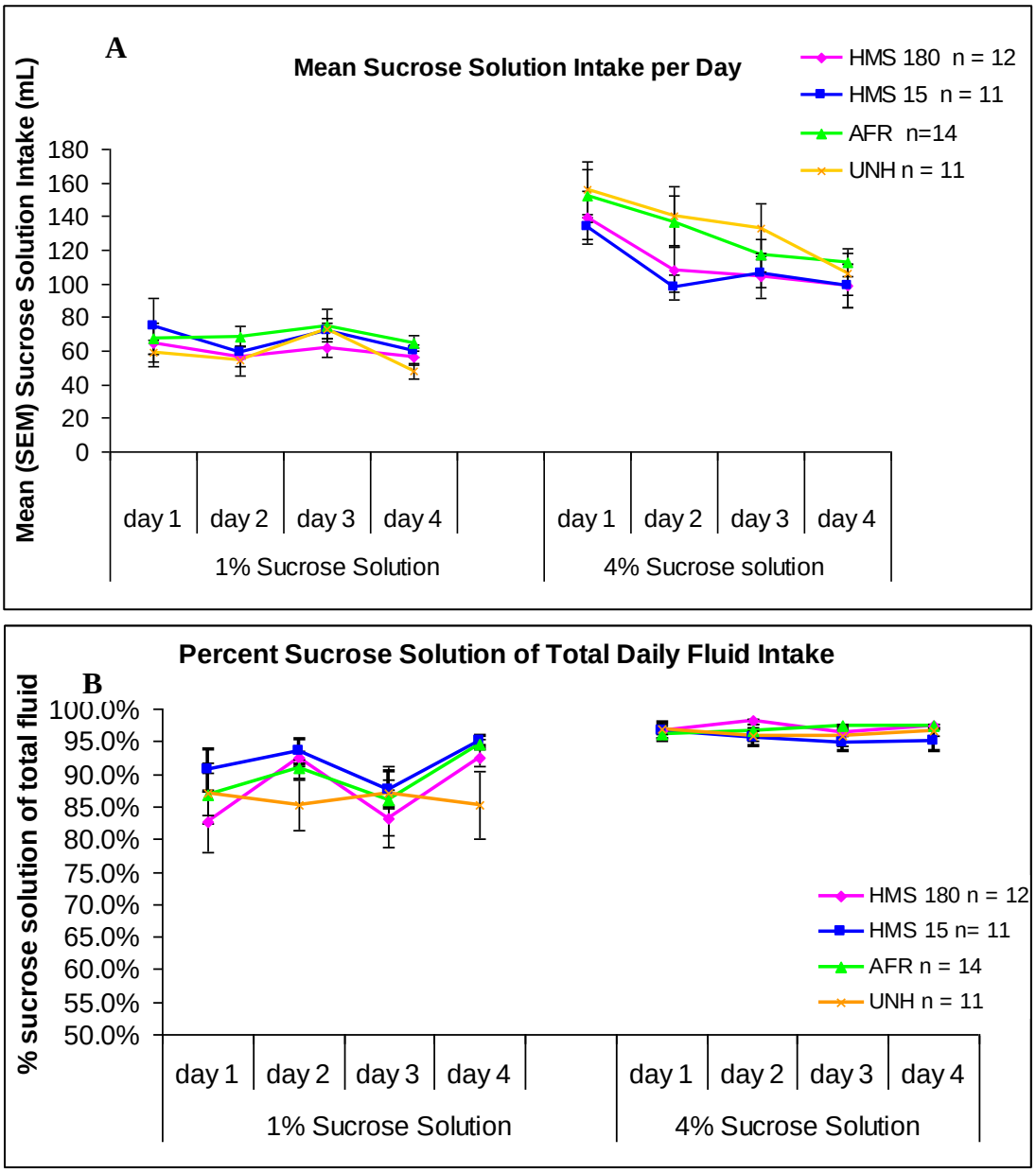
A. HMS 180 made significantly fewer open arm entries than AFR or UNH. HMS 15 made fewer open entries than UNH. Both HMS 180 and HMS 15 made fewer center entries than AFR. HMS 180 had fewer total entries than AFR or UNH.

B. HMS 180 spent significantly less time in the open arm than UNH or AFR. HMS 15 spent less time than UNH in the open arm. HMS 180 also took significantly longer to enter the open arm than UNH or AFR. * $p < .05$

Sucrose Solution Preference Test

We hypothesized that HMS 180 rats would have a lower preference for sucrose solution than UNH, AFR, and HMS 15. Contrary to expectations, repeated measures analysis for mean sucrose solution consumption over 4 days at 1% and 4% revealed no group differences (Fig 4A). At 1%, Mauchly's test indicated that the assumption of sphericity had been violated for day ($\chi 2_5 = 63.99$, $p < .001$), therefore degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity ($\varepsilon = 0.55$). There was no separation group effect ($F_{3,42} = 1.43$, $p = .248$) or interaction between group and day. At 4%, Mauchly's test indicated that the assumption of sphericity had been violated for day ($\chi 2_5 = 15.51$, $p = .008$), and again degrees of freedom were corrected using Greenhouse-Geisser ($\varepsilon = 0.80$). There was no separation group effect ($F_{3,45} = 1.26$, $p = .30$) or interaction between group and day. When averaged across days, consumption of 4% sucrose solution was significantly greater than consumption of 1% ($F_{1,44} = 184.69$, $p<.001$).

Similarly, there were no group differences in preference for sucrose solution over water, as measured by percentage of sucrose solution consumed of total fluid intake (Fig 4B). For percent total consumption of 1% sucrose, Mauchly's was significant for day ($\chi 2_5 = 19.59$, $p = .001$), and Greenhouse-Geisser correction ($\varepsilon = 0.76$) was applied. For percent total consumption of 4% sucrose solution, there were no significant differences across days or separation groups. All rats showed a strong preference for sucrose solution.



HMS 180 = maternally separated, HMS 15 = 15 min separation, AFR = animal facility raised, UNH = unhandled

Figure 4. Sucrose Solution Preference. Mean (*SEM*).

A. There were no significant differences in mean sucrose solution intake at either 1% or 4%. All rats showed a stronger preference for 4%.

B. There were no significant differences in preference for sucrose solution over water.

Operant Responding for Sucrose

We hypothesize that HMS 15 would have the highest levels of operant responding, followed by AFR, and then UNH, with HM 180 making the fewest. This hypothesis was only confirmed for HMS 180 responsiveness on fixed-time criterion of sucrose consumption. Fixed Time (FT) criterion of eating 19 of 20 sucrose pellets delivered non-contingently every minute for 20 minutes was examined using nonparametric statistics because of non-homogenous variance (Levine's median test, Fig 5). Kruskal-Wallis showed a main effect of separation group on days to criterion (χ^2_3= 11.67, p = .009). A Mann-Whitney test indicated that HMS 180 took significantly longer to meet criterion than UNH (U = 25.0, p = .004).

Repeated measures was used to examine acquisition—total responses each day over the first 12 days of fixed ratio 1 (FR1). Mauchly's test indicated that the assumption of sphericity had been violated for day ($\chi 2_{65}$= 238.91, p < .001), therefore degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity (ε = 0.46). There was a significant main effect for day ($F_{5.0,220.4}$ = 57.32, p <.001), with responding significantly higher on day 12 than the first 5 days (p<.0004). There were no significant interactions with separation group ($F_{15.0,220.4}$ = .973, p = 4.84). Nor was there a main effect of separation group ($F_{3,44}$ = 1.6, p= .202). Although HMS 180 had lower average levels of responding from days 3 to 12, this difference did not reach significance (Fig 6A). As shown in Figure 6B, number of days to reach criterion of 50 bar presses did not differ significantly among groups ($F_{3,44}$ = .987, p =.408). One HMS 180 rat never met the criterion of 100 bar presses and was assigned an arbitrary cut-off of 25 days to reach

criterion. Days to reach criterion of 100 bar presses did not differ among groups (χ^2_3= 4.83, p = 1.85).

Mean responding over the last four days of each fixed ratio was not significantly different among groups for any Fixed Ratio (FR) requirement (Fig 7). However there was a trend towards significance at FR1 ($F_{3,44}$ = 2.43, p= .078) and FR2 ($F_{3,44}$ = 2.30, p= .090), with the biggest mean difference between HMS180 and AFR (42.96 for FR1 and 80.68 for FR2). There was also a trend towards significance at FR32 (χ^2_3= 6.392, p = .094, nonparametric for unequal variance) with the biggest mean difference between HMS15 and UNH (602.88).

Repeated measures analysis for lever presses over 6 days of extinction revealed no group differences (Fig 8). Mauchly's test indicated that the assumption of sphericity had been violated for day ($\chi 2_{14}$= 204.01, p < .001), therefore degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity for day (ε = 0.32). There was a significant main effect of day ($F_{1.59, 68.29}$ = 50.22, p <.001) and a significant day x separation group interaction ($F_{4.76, 68.29}$ = 2.64, p <.033). There was a trend for separation group differences that did not reach significance ($F_{3,43}$ = 2.52, p <.070). As can be seen in Figure 8, HMS 15 started out at a noticeably lower response rate on day 1 than the other groups. All rats decreased responding across days, with the biggest decreases on days 2 and 3. Although there was a trend for acquisition deficits and lower responding in HMS 180 than AFR on FR 1 and FR 2, this did not reach significance. Against our hypothesis HMS 180 did not demonstrate clear evidence of decreased motivation on operant responding. Unexpectedly, HMS 15 did not have the highest performance levels.

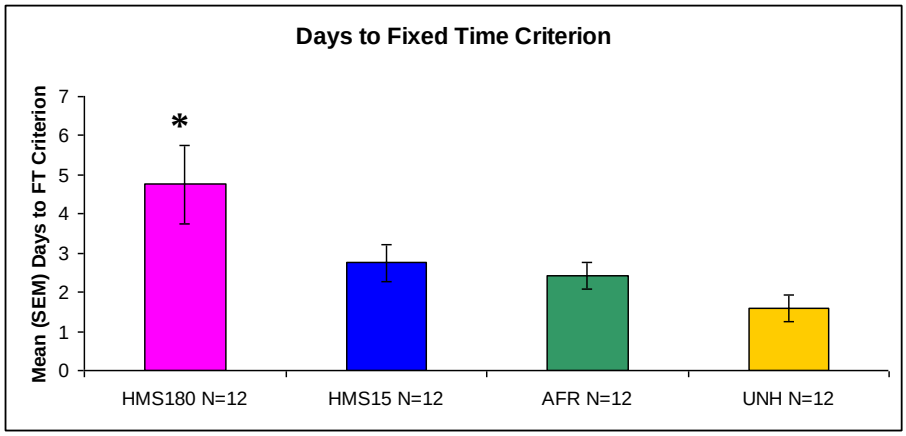
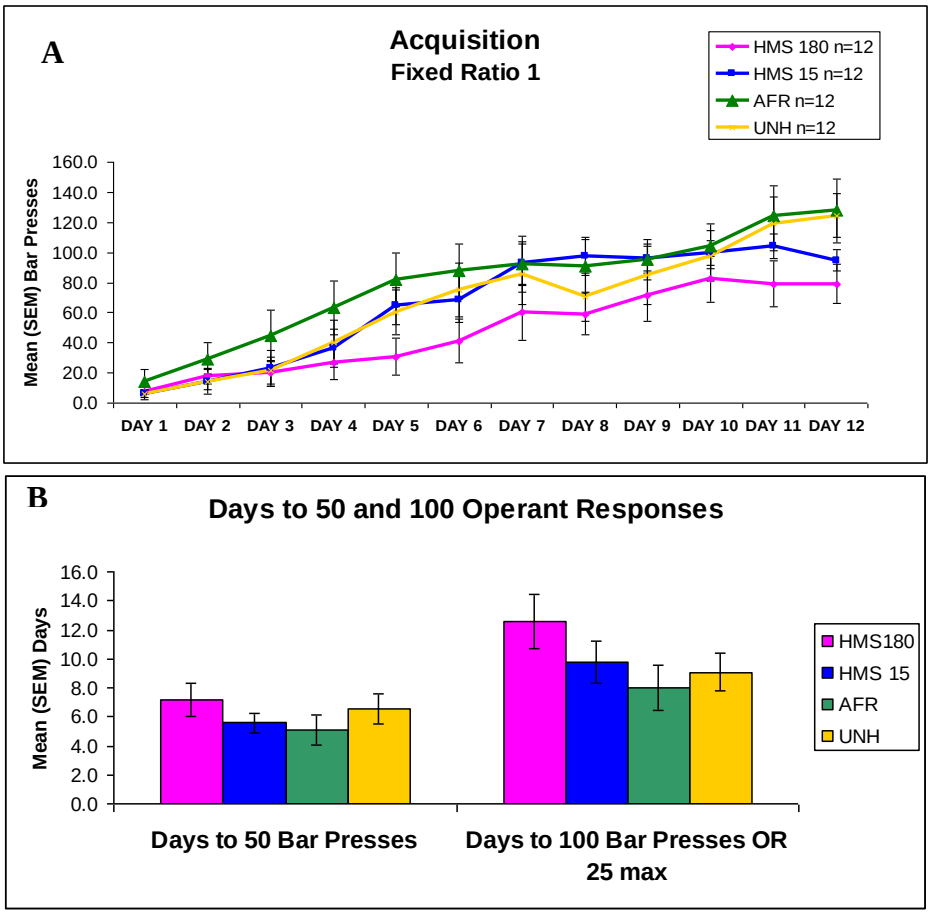


Figure 5. Sucrose Pellet Consumption in Operant Chamber Mean (*SEM*).

HMS 180 took significantly longer than UNH to meet the criterion of eating 19 of 20 sucrose pellets available non-contingently in the operant chamber. ***** $p < .008$

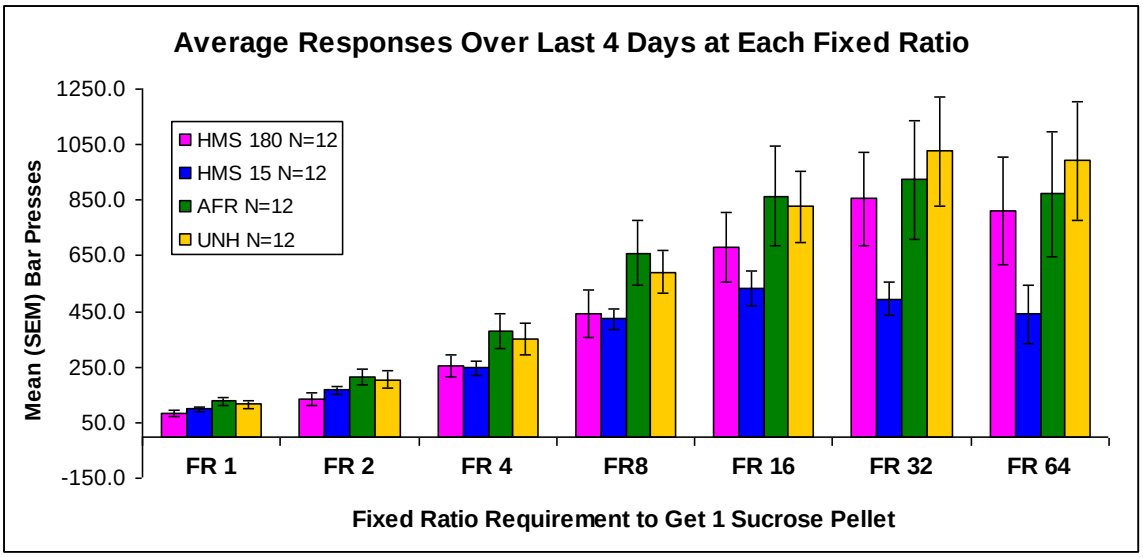


HMS 180 = maternally separated, HMS 15 = 15 min separation, AFR = animal facility raised, UNH = unhandled

Figure 6

A. Acquisition of Responding for Sucrose Pellets—Each bar press was reinforced with one sucrose pellet (FR1). Although HMS180 had lower level of responding from days 3 to 12, this difference did not reach significance.

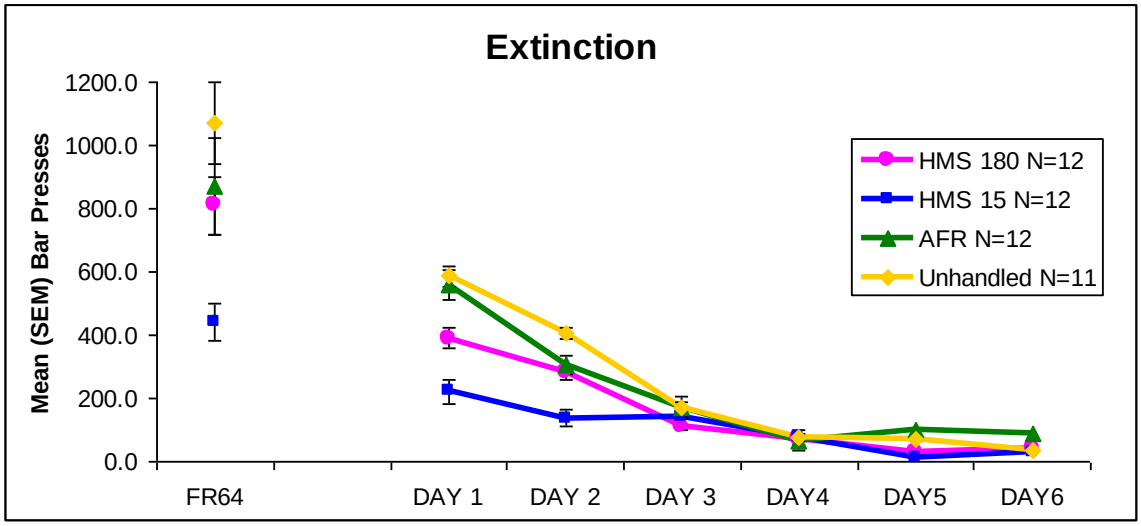
B. Criterion of reaching 50 or 100 operant responses per session did not differ significantly among groups.



HMS 180 = maternally separated, HMS 15 = 15 min separation, AFR = animal facility raised, UNH = unhandled

Figure 7 Progressively Increasing Fixed Ratio Requirements for Sucrose Pellets. Mean (*SEM*) of 4 days

There were no significant differences among groups at any fixed ratio. There was only a trend for HMS 180 to respond lower than AFR at FR1 and FR2. At FR32, there was a trend for HMS15 to respond lower than UNH. This trend did not reach significance because of high variability in the other groups.



HMS 180 = maternally separated, HMS 15 = 15 min separation, AFR = animal facility raised, UNH = unhandled

Figure 8 Extinction—Lever Presses No Longer Reinforced After FR 64.
Mean (*SEM*) responses per day over 6 days.
There was a non-significant trend for separation group differences, due to a low starting response rate in HMS15 on Day 1. All rats extinguished responding.

Correlations

We hypothesized that maternally separated rats would be more anxious and less motivated to respond for sucrose, especially at high fixed-ratio requirements. Therefore, we predicted that high anxiety on the elevated plus maze and open field would correlate with lower operant responding across fixed-ratio requirements. However, anxiety to the novel context of the operant chamber may only affect initial sucrose consumption and FR acquisition. If anxiety is only related to sucrose responding in a novel environment and not motivation, then there should be no correlation between plus maze and open field anxiety and responding at fixed ratio 64.

Individual Group Correlations

Correlations were performed per separation group on measures of anxiety on the elevated plus maze and operant responding for sucrose using simple linear regression. There were no significant differences in the slopes of any correlation between separation groups. However, correlations that reached significance in one group (see below) did not reach significance in the other groups. Therefore, it seems that different aspects of anxiety measured on the elevated plus maze differentially predict performance on operant responding, depending on maternal separation experience.

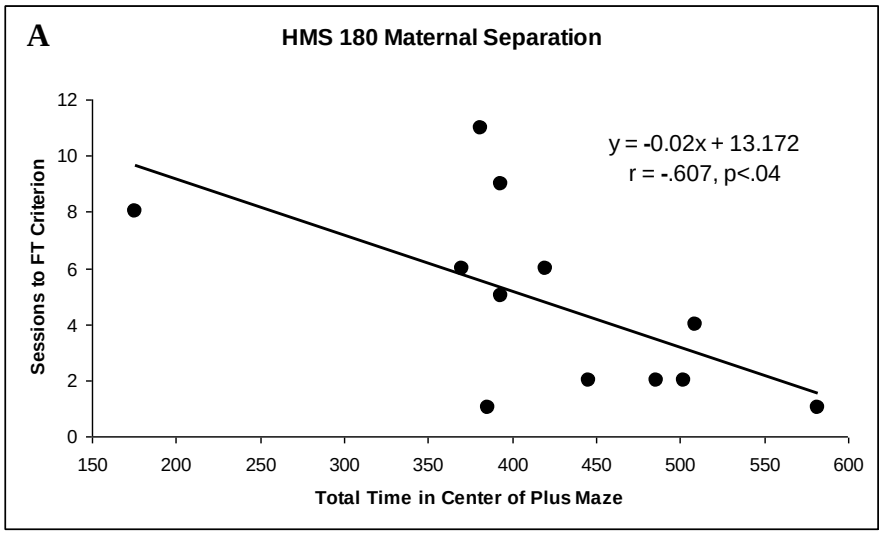
For HMS 180 and Unhandled rats, anxiety measures correlate with the number of sessions to criterion of eating sucrose pellets on a fixed time schedule (Fig 9). For HMS 180 rats, total time in the center of the plus maze correlates inversely with number of sessions to FT criterion ($F_{1,10} = 5.83$, $r = -.607$, $p = .036$). For Unhandled rats, latency to enter open arm of the plus maze correlates positively with number of sessions to FT criterion ($F_{1,9} = 8.25$, $r = .692$, $p = .018$). HMS 15 showed only a trend towards

significant correlation between number of open arm entries and sessions to FT criterion ($r= -.546$, $p = .067$).

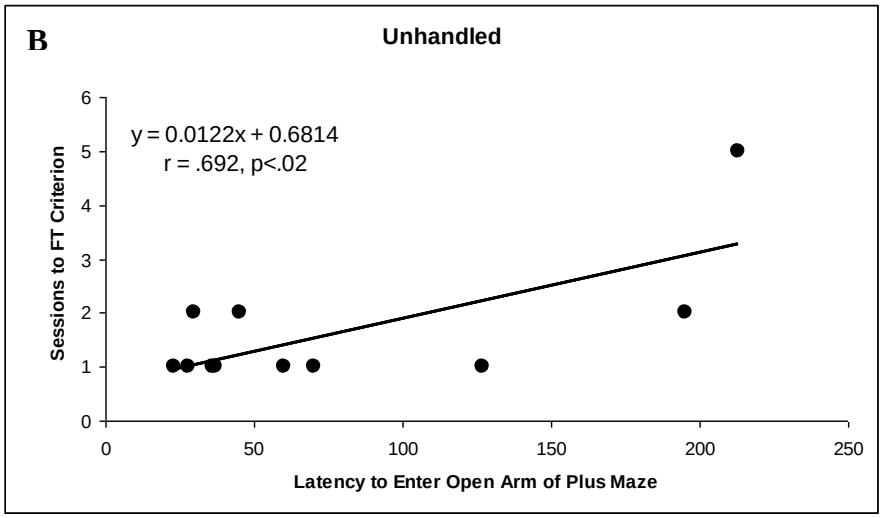
For HMS 180 and AFR rats, elevated plus maze measures correlate with days to reach criterion of 100 operant responses for sucrose (Fig 10). Both number of open arm entries ($F_{1,10} = 6.69$, $r = -.633$, $p = .027$) and total time in the open arm of the plus maze ($F_{1,10} = 8.37$, $r = -.675$, $p = .016$) correlate inversely with sessions to reach criterion of 100 operant responses for HMS 180. While in AFR rats latency to enter the open arm correlates with number of sessions to both 50 ($F_{1,10} = 6.78$, $r = .636$, $p = .026$) and 100 bar presses ($F_{1,10} = 7.42$, $r = .653$, $p = .02$).

For Unhandled rats, the total time spent in the open arm of the plus maze positively correlates with average responding for sucrose pellets at both FR1 ($F_{1,9} = 7.70$, $r = .679$, $p = .022$) and FR 64 ($F_{1,8} = 7.98$, $r = .707$, $p = .022$) (Fig 11). This suggests that in this group, the less anxious the rat, the more vigorously it responds for rewarding sucrose at both low and high work requirements.

Figure 9 Anxiety Measures Correlate with Sessions to Eat Freely Available Sucrose Pellets

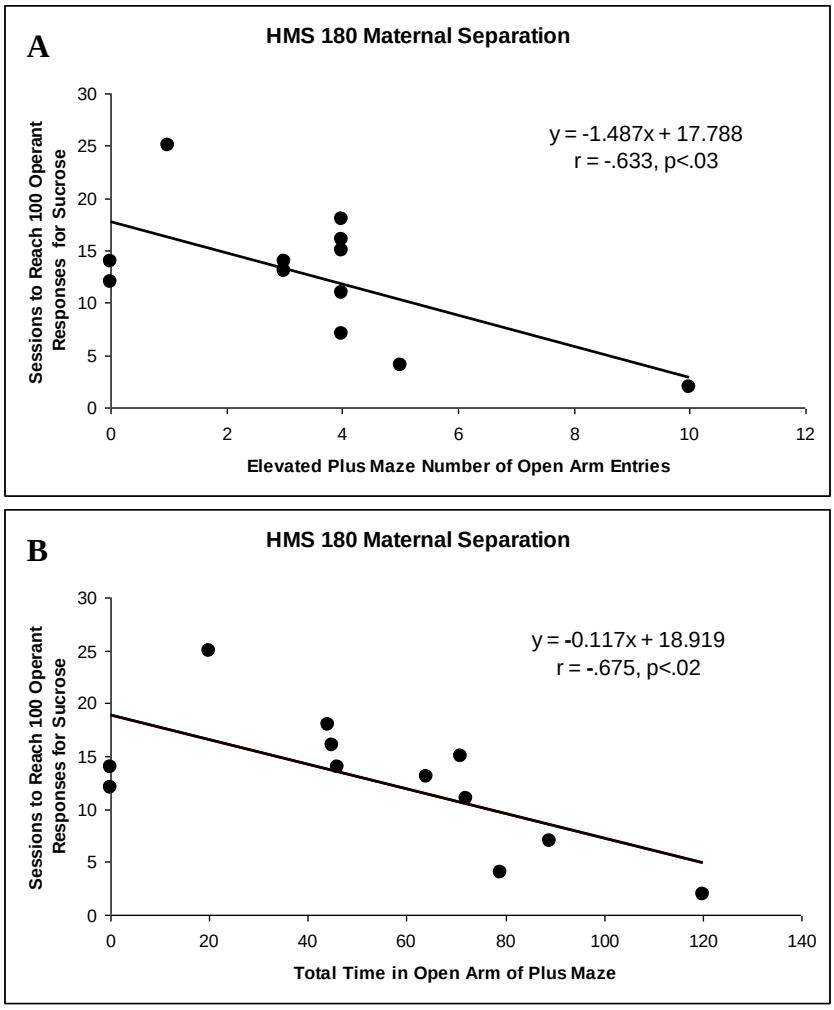


A For HMS 180 rats, total time in the center of the plus maze, which is associated with risk-assessment behavior, correlates inversely with number of sessions to FT criterion of eating 19 of 20 freely available sucrose pellets.

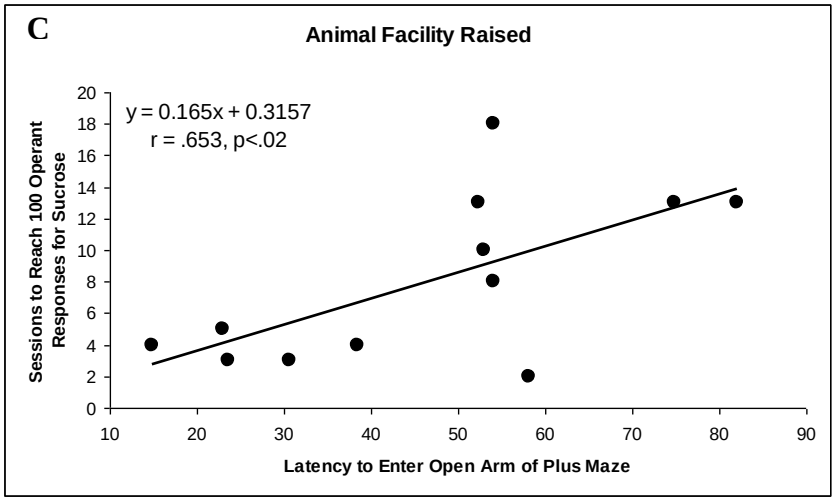


B For Unhandled rats, latency to enter open arm of the plus maze correlates positively with number of sessions to FT Criterion. The longer the rat takes to enter the open arm, the longer he tends to take to eat the pellets.

Figure 10 Anxiety Measures Predict Days to Reach 100 Bar Presses for Sucrose

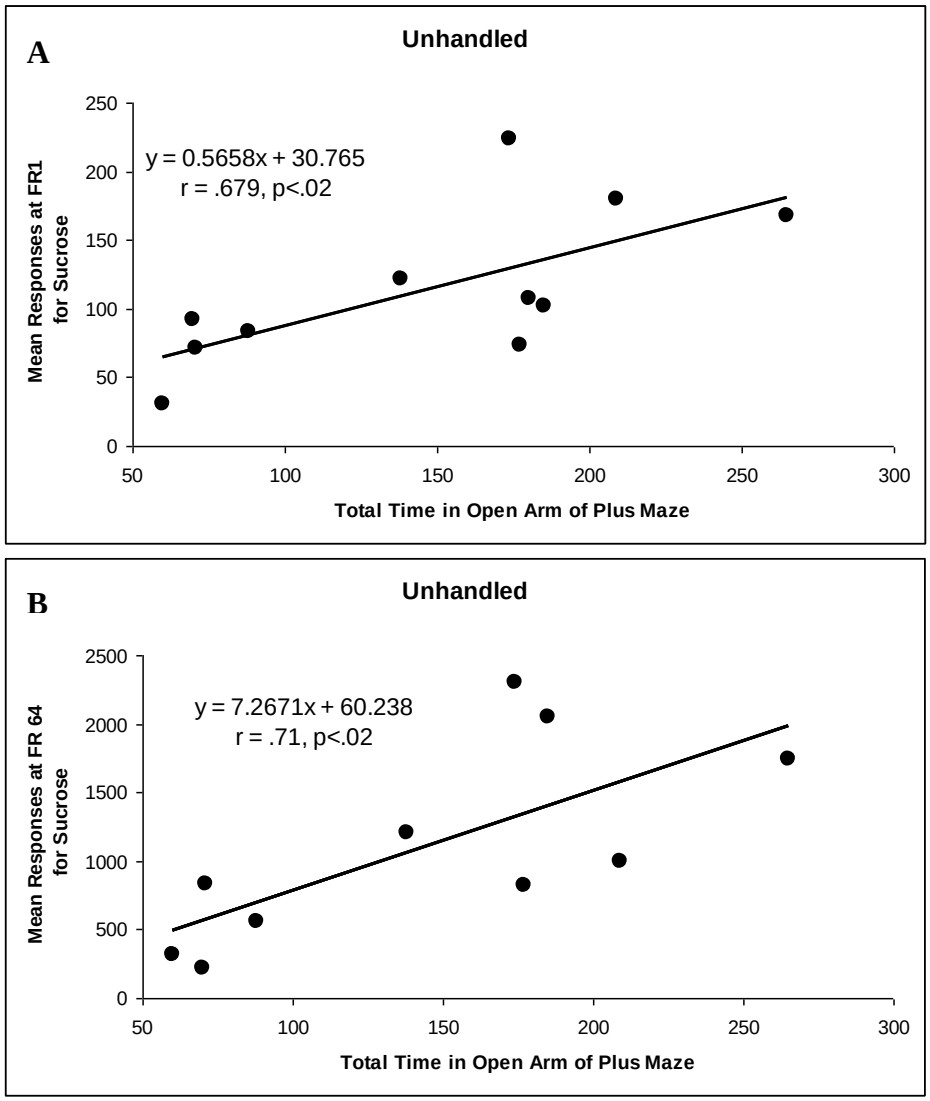


Both number of open arm entries (**A**) and total time in the open arm of the plus maze (**B**) predict how many sessions it will take to reach the criterion of 100 operant responses for sucrose pellets for HMS 180 rats, with more anxious rats taking longer to meet criterion.



C Latency to enter the open arm predicts number of sessions to 100 bar presses in AFR rats.

Figure 11 Anxiety Measures and Vigor of Responding at Low and High Ratio Requirements



For Unhandled rats, the total time spent in the open arm of the plus maze positively correlates with average responding for sucrose pellets at both fixed ratio 1 (FR 1, **A**) and FR 64 (**B**). This suggests that in this group of rats, the less anxious or more curious the rat is, the more vigorously it responds for rewarding sucrose regardless of amount of work required.

Combined Groups Correlations

Since there were no significant differences in the slopes of any regression lines between separation groups, combined group correlations between EPM measures and operant responding were examined. As shown in Table 1, operant responding correlated significantly with several parameters of anxiety and locomotion. Number of sessions to fixed-time criterion correlated inversely with elevated plus maze open arm entry and duration (fig 12), closed arm duration, and total arm entries. Open field total entries and digiscan horizontal activity also correlated inversely with sessions to FT criterion (Table 1). This suggests that consumption of food in a novel environment relates to anxiety and possibly general activity levels.

As seen in Table 1, responding at all fixed ratios correlated significantly with anxiety measures EPM open arm duration (fig 13) and open arm count (except FR 16). Open field anxiety measured by inner ring entry also correlated significantly with FR 1, FR 8, and FR 64 lever presses. So, rats that went out on the EPM open arm and inner ring of the open field were more likely to have greater operant responding at both low and high fixed ratios. Time in EPM closed arm correlated inversely with FR 32 responding, and closed arm entry correlated inversely with FR 64. Rats entering or spending more time in the protected arm tended to bar press less at high ratio requirements. Importantly, operant responding at fixed ratios higher than FR 2 did not correlate with general locomotor activity as measured by EPM total arm entries, open field total entries, or digiscan horizontal counts. This data suggests that operant responding at both low and high ratio schedules of reinforcement is more closely associated with anxiety than general locomotor activation.

Table 1

Correlations of Anxiety Measures and Operant Responding

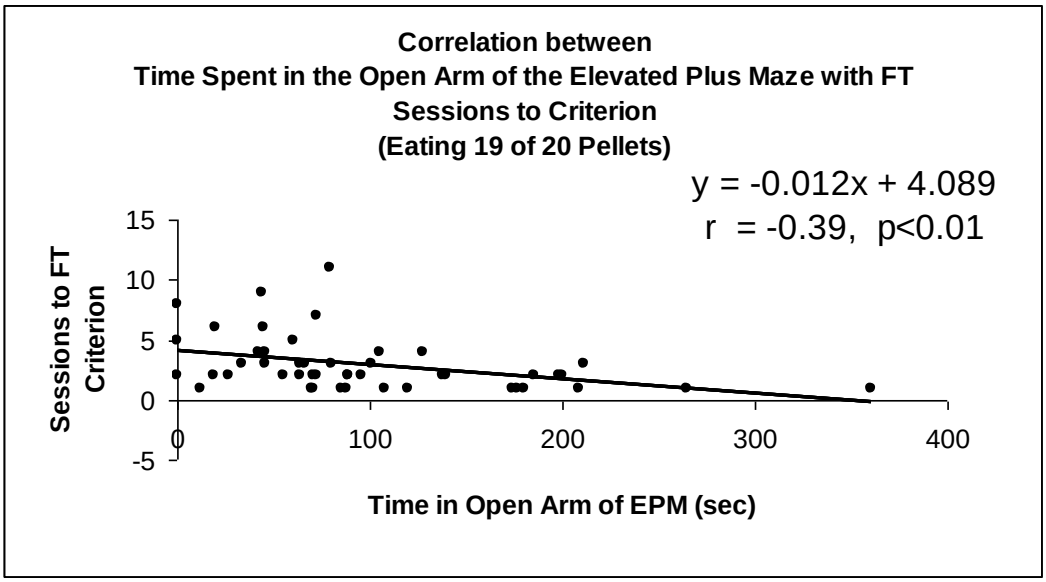
	Elevated Plus Maze					Open Field		Digiscan
	Open Time	Open #	Closed Time	Closed #	Total #	Inner #	Total #	Horizontal
FT	**-0.39****	**-0.38****	**+0.38****	-0.02	**-0.30***	-0.28	**-0.31***	**-0.30***
FR1	**0.45****	**0.39****	-0.27	-0.06	0.25	**0.37***	0.26	-0.1
FR2	**0.45****	**0.42****	-0.23	-0.01	**0.31***	0.31	0.25	-0.11
FR4	**0.36***	**0.36***	-0.25	-0.06	0.23	0.28	0.18	-0.1
FR8	**0.36****	**0.35***	-0.18	-0.03	0.24	**0.33***	0.17	-0.05
FR16	**0.31***	0.29	-0.24	-0.16	0.10	0.25	0.08	-0.02
FR32	**0.44****	**0.34***	**-0.29***	**-0.26**	0.07	0.28	0.13	-0.01
FR64	**0.41****	**0.30***	-0.23	**-0.29***	0.02	**0.30***	0.17	0.06

Pearson's Correlation r

* $p < 0.05$

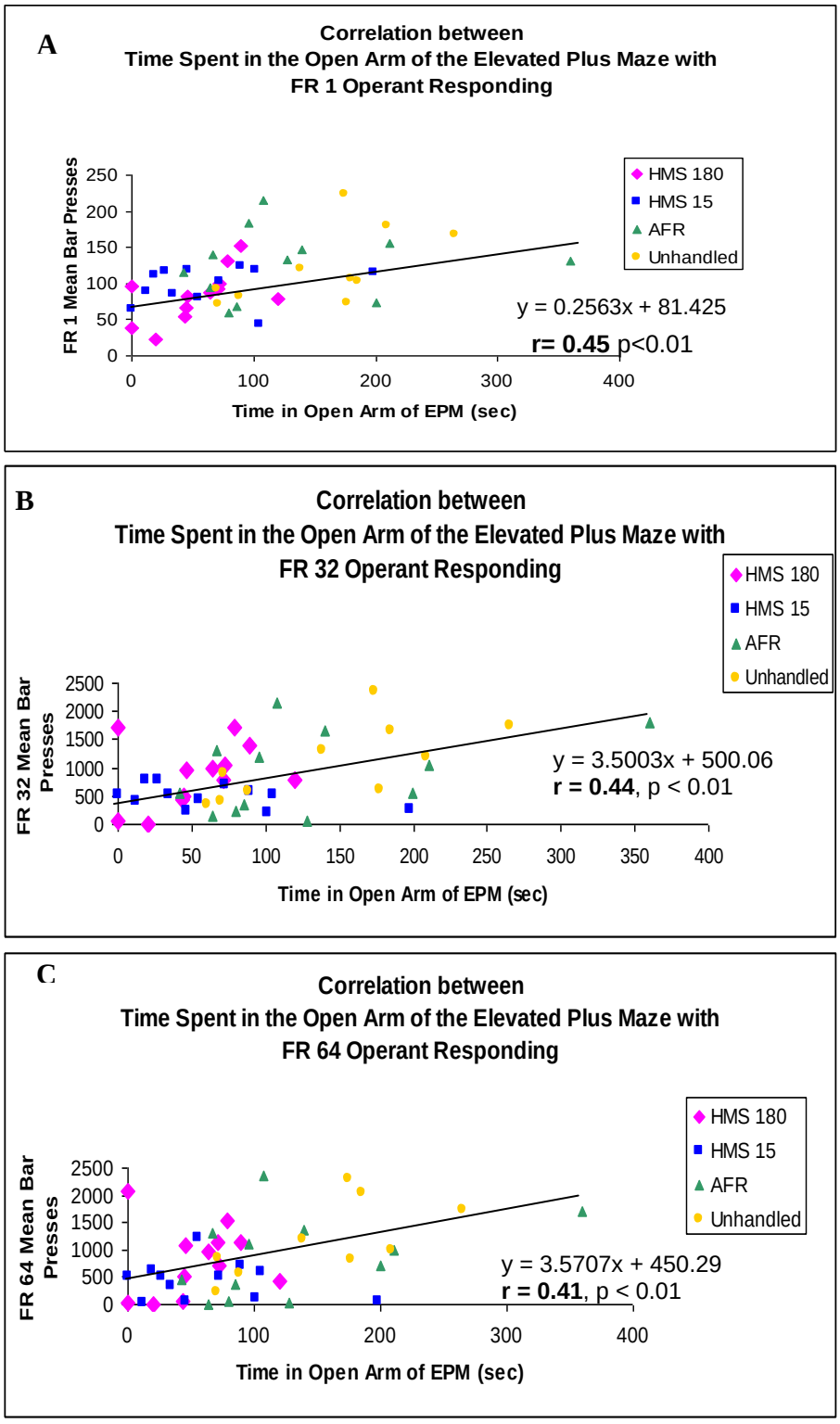
**$p < 0.01$

Figure 12 EPM Open Arm Time Correlates with Sessions to FT Criterion



For all rats, total time in the open arm of the plus maze correlates inversely with number of sessions to FT criterion of eating 19 of 20 freely available sucrose pellets, with more anxious rats taking longer to meet criterion.

Figure 13 EPM Open Arm Time Correlates with Operant Responding



For all rats, total time in the open arm of the plus maze correlates with both low and high fixed-ratio responding: **FR 1** (**A**), **FR 32** (**B**), and **FR 64** (**C**) $p<0.01$. This suggests across groups, more anxious rats make fewer operant responses for sucrose reward.

Discussion

This study examined the effects of maternal separation on consummatory and appetitive responding for a natural reward. Anxiety tests verified the effectiveness of the maternal separation procedure. As predicted, long-separated HMS 180 rats were more anxious than unhandled (UNH) or animal facility raised (AFR) rats in both the open field and the elevated plus maze. In the open field, HMS 180 had a longer latency to enter and made fewer entries into the center ring compared to AFR and UNH. On the elevated plus maze, HMS 180 made fewer open arm entries and spent less time in the open arm compared to AFR and UNH. These results are consistent with studies that have found maternally separated rats to be more anxious and suggest that our maternal separation procedure was successful (Caldji et al., 1998, 2000; Wigger and Neumann, 1999; Ladd et al., 2000, Huot et al., 2001; Kalinichev et al., 2002). One unexpected finding is that the short-separated (HMS 15) rats were also more anxious than unhandled rats on the elevated plus maze. Studies consistently find HMS 15 to be less anxious and have decreased stress reactivity as compared to HMS 180 and UNH (Plotsky and Meaney, 1993; Bhatnagar and Meaney, 1995; Caldji et al., 1998, 2000; Wigger and Neumann, 1999; Ladd et al., 2000). However, since dam behaviors were not scored, it is possible that HMS 15 did not receive the better maternal care that is associated with their resilience to stress. Also, unhandled rats had higher basal levels of locomotor activity compared to both HMS 180 and HMS 15. This may either reflect either greater general activity levels or lower anxiety levels in unhandled rats.

Sucrose Preference

Contrary to our expectations, long-separated rats did not show alterations in preference for sucrose solution over water in the home cage. These findings are, however, consistent with other studies that have found no differences in sucrose preference in HMS 180 measured across varying concentrations (Matthews et al,1996a; Shalev & Kafkafi, 2002; Michaels & Holtzman, 2006). Hout and colleagues (2001) found a decreased preference of sucrose solution in HMS 180, but this was concomitant with increased intake ethanol in a two-bottle choice. Sweet and colleagues (unpublished data) found a decrease in sucrose solution preference versus water, while Michaels and Holtzman (2007) found an increased sucrose preference. The magnitude of increase was quite small: approximately 85 % preference over water in HMS 180 versus 75% preference in UNH. Matthews and colleagues (1996a) found that maternally separated rats did not alter their lick rates as much as controls to increasing and decreasing concentrations of sucrose solutions over a brief time-period. This suggests separated rats had impaired perception of contrasting rewards. Together these data suggest maternal separation does not consistently lead to changes in preference for and consumption of sucrose in the home cage.

Operant Responding for Sucrose

We tested rats' motivation to work for sucrose reward under increasing response requirements. While we expected long-separated rats to have deficits at high ratio work requirements, we did not predict that initial approach and consumption would be affected. Maternally separated rats took significantly longer than unhandled rats to eat freely available sucrose pellets in the operant chamber. Although long-separated rats had lower

average levels of responding during the 12 day acquisition period at FR l, this difference did not reach significance. Number of days to reach criterion of 50 or 100 bar presses also did not differ. There was only a slight trend for HMS 180 rats to respond lower than AFR at fixed ratios 1 and 2. In contrast to our hypothesis, there were no group differences in responding at any higher fixed ratio requirement. Additionally, no significant differences emerged during extinction.

These data seem to suggest that maternally separated rats were hesitant in their initial approach to sucrose in a novel environment. After acquisition, no overt group differences in motivation to respond for sucrose were seen. This hypothesis is supported by significant within-group correlations between anxiety and acquisition of operant responding. For long-separated HMS 180 and UNH rats, anxiety measures on the elevated plus maze correlated with how long it took rats to approach and consume palatable sucrose pellets in a novel environment, with more anxious rats taking longer. Also, for HMS 180 and AFR, anxiety on the plus maze predicted length of time to meet acquisition criterion of 100 bar presses. Hesitance in HMS 180 initial consumption of sucrose in the operant chamber is consistent with data from Caldji and colleagues (2000), who found that maternally separated rats took longer than handled rats to approach and begin feeding in a novel environment but not in the home cage.

Combined-group correlations confirm a relationship between FT criterion and anxiety, with more anxious rats taking longer to reach criterion. However, unlike single-group correlations, combined-group correlations show a significant relationship between anxiety measures and fixed-ratio responding. There is a positive correlation between time spent in the open arm of the elevated plus maze with operant responding at all fixed

ratio requirements. EPM open arm entries and open field inner entries show the same positive relationship with operant responding at low and high ratios, although not at with all fixed-ratios. Also EPM closed arm time and entries correlate inversely with responding at high FR requirements. Notably, measures presumably more reflective of general activity—digiscan horizontal count, EPM total entries, and open field entries—only correlated with FT criterion and not with responding at high work requirements. This suggests that the positive correlations between open arm duration and activity at FR 32 and FR 64 are not merely accounted for by non-specific locomotor activity. While the correlations are not very strong, they show a consistent picture of high anxiety relating to lower operant responding across FR requirements. It is interesting to speculate that this might represent a subgroup of rats across groups with both high anxiety and decreased incentive motivation that persists in spite of habituation to novelty.

The significant correlation may be partially driven by the UNH group, who showed a much stronger correlation between anxiety and operant responding at low and high FR requirements when examined alone. No other single-group correlations showed a significant relationship between fixed-ratio responding and anxiety, aside from acquisition responding. On the other hand, combining groups may give more power to detect underlying relationships given the high variability within the groups.

Two current studies examining elevated plus maze activity in non-stress rats and subsequent operant responding fit the hypothesis that more anxious rats have lower incentive motivation. Alsiö and colleagues (2009) found a strong correlation between EPM open arm activity and subsequent acquisition of operant responding ($r_s = 0.883$, $p <$

0.001). Further, progressive ratio responding was predicted by time in the open arm (r_s = 0.691, $p < 0.02$), with less anxious rats having higher break-point responding. Davis and colleagues (2009) showed that rats that spent more time in the open arms of the EPM were more likely to become dominant after group housing, and they had increased operant responding for food on a progressive ratio schedule.

The lack of group differences in operant responding is consistent with other maternal separation studies. Moffett and colleagues (2006) found no deficits in acquisition or maintenance on a FR1 schedule of responding for food pellets after food restriction in maternally separated rats. Also, Shalev and Kafkafi (2002) did not find a maternal separation effect in acquisition or progressive ratio responding for various concentrations of sucrose solutions. Contrary to our findings, Shalev and Kafkafi (2002) did not find a significant difference in open field anxiety in HMS 180 compared to HMS 15 and unhandled rats. Studies examining early deprivation, which involves separation of rat pups from both the dam and the litter, have found evidence of reduced motivation to obtain sucrose solution on a progressive ratio schedule. However, this effect was only seen in pups isolated at cold temperatures and in stress-hyperresponsive Fischer rats (Ruedi-Bettschen et al, 2005; 2006). Pup isolation may be more of a physiological stressor than maternal separation.

In our experiment, there was a high level of variability in each group on operant responding. Some rats "gave up" or extinguished readily at higher ratio requirements, while others continued to meet the increasingly high demands for reinforcement. Specifically, we noted increased aggression, or "frustration," in some rats at higher ratios. These rats may have been the ones more motivated to respond at high ratios. Similarly,

Roman and Nylander (2005) found high levels of variance in long-separated offsprings' response to alcohol consumption. They suggested the presence of subgroups of responders and non-responders. In a different stressor paradigm, the Nestler lab found that even inbred mice subjected to social defeat could be segregated into resilient and susceptible subgroups based on depression-like social avoidance (Krishnan et all, 2007) . While it might be controversial to split data into bimodal subgroups and then compare them, the variability in human stress response and depression suggests subgroups as well (Krishnan and Nestler, 2010).

Stress Coping

Besides early life experience, the impact of a stressor also depends on numerous other environmental factors, such as coping ability, social support, age, and type of stressor (Anisman and Matheson, 2005). One caveat in our study is that the rats were aged (approximately one year) by the end of testing. Aged rats have elevated basal levels of glucocorticoids and prolonged CORT response to a stressor (Keck et al, 2000; Sapolsky, 1986). This could have added to the variability seen in all groups on operant responding. Also, rats were single-housed at the beginning of behavioral testing, which is also a stressor from lack of social interaction (eg., Weiss et al, 2004). As mentioned above, we did not record maternal behaviors in this experiment. Natural variations in licking, grooming and arched-back nursing or unexpected dam reactions in response to the separation procedure may have contributed to offspring variability. Also, since the dams arrived timed-pregnant, we could not control for possible effects of prenatal stress. While there is good evidence that dam behavior at least partially mediates the effects of

maternal separation (Huot et al, 2004), not all experiments find less active maternal behavior in long-separation dams (Macri and Würbel, 2004; 2006). In these experiments, environmental stress of separation itself explained part of the variance in HMS 180 HPA axis reactivity and anxiety. Somewhat related to variability issues is controversy over which control group to use. For example, unhandled rats may be exposed to too little stimulation or stress compared to rats in the wild, whose dams are off the nest for up to two or three hours (Lehmann and Feldon, 2000; Pryce and Feldon, 2003; Macri and Würbel, 2006). In our experiment, having three control groups increased the stringency of statistical significance because of the increased number of comparisons.

Stress is a common precipitating factor in human depression. However, not everyone exposed to stress becomes depressed. Rather, stress interacts with a person's genetic makeup to influence his or her risk for developing major depressive disorder (aan het Rot et al, 2009). While early life stress increases susceptibility to anxiety and depression (Heim et al, 2002), this vulnerability also depends on genetic background. For example, having a short allele of the serotonin transporter gene (*5-HTTLPR)* interacts with early-life stress to increase vulnerability to depression (Caspi et al., 2003), although recent studies question the strength of this finding (Munafò et al, 2009). Also, the presence of a single nucleotide polymorphism (SNP) in the corticotrophin releasing factor receptor 1 gene (*Crhr1*) interacts with childhood abuse to increase risk for adulthood depression (Bradley, et al., 2008). Other polymorphisms in genes related to CRF functioning have also recently been identified (Binder et al., 2004; 2010). Ressler and colleagues (2010) have recently identified a gene x gene x environment interaction between the *5-HTTLPR* S allele, *CRHR1* SNP, and child abuse on depressive symptoms.

Current evidence suggests that multiple genes might interact with life stressors and epigenetic modification to lead to depression (Krishnan and Nestler, 2010).

It is difficult to interpret rodent manifestations of emotion. For example, anxiety-like avoidance behaviors could also be interpreted as anhedonia. Furthermore, even when there is evidence of anhedonia or other signs of depression, these effects are often transient (Krishnan and Nestler, 2010). This might contribute to the inconsistent findings in maternal separation and reward.

Our study suggests maternal separation is better model of anxiety than depression. However, the distinction between anxiety and depression is difficult to define even in humans. While there is an abundance of evidence of HPA axis hyperactivity in pressed clinical populations that is reversed with successful antidepressant treatment (*eg,* Ising et al, 2005; Binder et al, 2009), this might represent only a severe sub-type of depression (Krishnan and Nestler, 2010). Stokes and colleagues (1984) found that depressed patients with HPA axis abnormalities may represent as little as 35% of the depressed population. Strickland and Deakin (2002) found that increased cortisol was associated with adverse life events but not depression in a general population sample.

Krishnan and Nestler (2010) suggest that depressive subtypes can be classified by a patient's reaction to proinflammatory cytokines. Cytokines are typically elevated in depression and can cause glucocorticoid receptor resistance. This GR resistance may result in either increased or decreased secretion of cortisol. Hyper-secretion is associated with decreased eating and insomnia, while hypo-secretion is associated with increased eating and fatigue (in Krishnan and Nestler, 2010). This hypothesis is interesting in the

light of recent studies that suggest increased eating may help reduce the stress response and anxiety (Teegarden & Bale, 2007; Dallman et al, 2003; Foster et al 2009).

Both acute and chronic stress have been shown to stimulate intake of palatable "comfort foods" in humans (Foster, 2009; Dallman et al, 2003). Even individuals who eat less during stress still show an increased preference for palatable and fatty foods (Teegarden & Bale, 2007). Fat intake decreases anxiety-like behaviors and facilitates stress recovery (Teegarden & Bale, 2007). Depressed people who overeat have decreased HPA activity and cerebrospinal CRF (Dallman et al, 2003).

Similarly in rats, eating palatable foods that produce abdominal obesity decreases the HPA response to restraint stress and reduces CRF mRNA in the hypothalamus and BNST (Dallman et al, 2003, Foster et al, 2009). Furthermore, palatable foods are more salient with high levels of glucocorticoids (Dallman et al, 2003). Pecina and colleagues (2006) found that CRF injected in the nucleus accumbens shell increases motivation for sucrose reward. Reduced stress-response following intake of comfort foods may reinforce the eating habit (Dallman et al, 2003). In rats exposed to chronic mild stress model of depression, motivation for food responding on progressive ratio was increased only for sucrose pellets and not for sugar-free pellets (Willner et al, 1998). In both rodents and humans consumption of "comfort foods" may ameliorate the effects of chronic stress.

It is interesting to speculate whether or not sucrose consumption and appetitive responding may have been partially driven by stress-reactivity in maternally separated rats. Future studies could examine whether or not palatable food consumption has an ameliorative effect on the HPA axis and CRF expression in maternally separated rats.

Conclusion

This experiment demonstrated that while maternally separated rats had increased anxiety, no overt differences were seen in preference or motivation for sucrose reward. In agreement with other studies, these data suggest that maternal separation does not consistently lead to anhedonia. However, the data do suggest a link between anxiety and motivated responding for sucrose. Anxiety correlated with both initial consumption of sucrose and acquisition of responding in the operant chamber in HMS 180. Further, across groups, anxiety measures correlated with operant responding at both low and high work requirements. While the relationship was not strong, it still suggests that at least a subset of rats had both higher levels of anxiety and decreased incentive motivation. The data underscores the importance of segregating out the effects of acute stress on reward responding. High variability in operant responding suggests there are a variety of factors influencing individual differences in motivated behavior.

There is evidence that stress and even depression can have a bimodal influence on palatable food consumption and drive. This needs to be taken into account when interpreting measures of anhedonia and motivation to obtain palatable food. Both animal models and human studies could benefit from a better distinction of different subtypes of anxiety and depression. This study is in agreement with an extensive literature that maternal separation increases anxiety behaviors. Further research on how genetic and epigenetic background interacts with a life-time of stressors and coping ability could lead to a better understanding of individual variability in reward responsiveness.

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