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Maternal Behavior Influences the Development of a Reflexive Action Pattern
In the Newborn Rat

Sierra Kauer

Idaho State University

A thesis

submitted in partial fulfillment

of the requirements for the degree of

Master of Science in the Department of Psychology

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Committee Approval

To the Graduate Faculty:

The members of the committee appointed to examine the thesis of Sierra Kauer find it satisfactory and recommend that it be accepted.

Michele Brumley, PhD
Major Advisor

Erin Rasmussen, PhD
Committee Member

James Lai, PhD
Graduate Faculty Representative

September 4, 2012

Michele Brumley, PhD
MS 8112
Psychology Department
Pocatello, ID 83209

RE: Your application dated 9/1/2012 regarding study number 699: Modulation of Action Patterns in Developing Rats

Dear Dr. Brumley:

Thank you for your response to requests from a prior review of your application for the new study listed above.

This is to confirm that your application is now fully approved. The protocol is approved through 9/4/2015 with an annual review at 9/4/2013.

You are granted permission to conduct your study as most recently described effective immediately. The study is subject to continuing review on or before 9/4/2013, unless closed before that date.

Please note that any changes to the study as approved must be promptly reported and approved. Some changes may be approved by expedited review; others require full board review. Contact Patricia Hunter (208-282-2179; fax 208-282-4529; email: anmlcare@isu.edu) if you have any questions or require further information.

Sincerely,

Curt Anderson, PhD
IACUC Chair

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List of Abbreviations

AG: anogenital

AGL: anogenital licking

°C: degrees Celsius

Cm: centimeters

CRF: Corticotrophin releasing factor

E20: embryonic day 20: two days before birth

E21: embryonic day 21: the day before birth

E20: embryonic day 22: the day of birth

G: grams

Ga: gauge

Hz: hertz

LER: leg extension response

Min: minute

Ml: milliliter

Mm: millimeter

P1: postnatal day 1: 24 hours after birth

P2: postnatal day 2: 2 days after birth

P3: postnatal day 3: 3 days after birth

P4: postnatal day 4: 4 days after birth

P5: postnatal day 5: 5 days after birth

P6: postnatal day 6: 6 days after birth

P7: postnatal day 7: 7 days after birth

P8: postnatal day 8: 8 days after birth

P9: postnatal day 9: 9 days after birth

P10: postnatal day 10: 10 days after birth

ROM: range of motion

S: seconds

SEM: standard error of the mean

SNB: spinal nucleus of the bulbocavernosus

ZnSO₄: zinc sulfate

Abstract

In neonatal rats, the leg extension response (LER) is a bilateral hyperextension of the hindlimbs in response to maternal anogenital licking (AGL). Past research has found that intranasal application of zinc sulfate (ZnSO_4) to the dam induces hyponosmia, thereby reducing the incidence of AGL. This study examined the effects of reduced AGL on the expression and postnatal development of the LER in rat pups. Pregnant dams received intranasal application of air (control), distilled water (control), or ZnSO_4 on the day before birth and every other day thereafter until postnatal day 9 (P9). The LER was experimentally evoked in pups, using a vibrotactile device, at P1, P5, or P10. Pups born to ZnSO_4 -treated dams showed significantly shorter bilateral LER durations and significantly smaller ankle angles than pups born to control dams. Thus, maternal behavior influenced development of the LER in rat pups, demonstrating the influence of maternal care on behavioral

Maternal Behavior Influences the Development of a Reflexive Action Pattern In the Newborn Rat

Altricial species are those that are very immature at birth (e.g., rats, cats, dogs, etc.). These newborns are fairly helpless, relatively behaviorally incompetent, and completely dependent upon a parent for survival after birth. Precocial species, while still immature, are somewhat more mature at birth than altricial species. For instance, newborn precocial young (e.g., horses and chicks) are able to walk at birth. Nonetheless, all mammals require some level of parental care. For most mammals, the primary caregiver is the mother, upon whom the young rely on for food, shelter, and protection. Not only does the individual require adequate care for survival, but it must be able to observe and learn the behavioral repertoire necessary for reproductive success as well. Our understanding of behavioral development can be better understood by exploring the earliest occurrences of experience, which are largely influenced by parental caregiving during the newborn period. For example, as discussed later in this paper, much research has demonstrated the importance of maternal caregiving on physical, behavioral, emotional, social, and sexual development in the offspring.

Does maternal behavior influence the development of motor behavior? And if so, in what way does maternal behavior affect motor functioning? Cross cultural studies examining the development of locomotion in humans have shown that different rearing practices can lead to different time courses in the onset of walking (Adolph & Robinson, 2013). For example, in Uganda mothers engage in rearing practices that are intended to facilitate the onset of walking (Bril & Sabatier, 1986; Hopkins & Westra, 1988; Raban-Jamin & Wornham, 1993; Super, 1976). Bouncing, shaking, and rubbing the legs of their

infants quickens the onset of motor development with the children walking as early as 7 months of age. In contrast, in the Shandong and Hebei regions of China mothers engage in “sandbag rearing” where their infants lie on their backs in a bag filled with sand for several hours a day until they are toilet trained (Mei, 1994; Xie & Young, 1999). Since the infants’ legs are confined to the sandbag they receive little experience moving their legs, and the child may not start walking until 15 months of age. Taken together, these studies provide examples of the importance of maternal care in influencing motor development. However, walking first emerges in human infants in the first to second year after birth. What about the expression of earlier occurring functional behaviors?

Rats are an altricial species, and like humans, continue much of their motor and behavioral development after birth. Although it takes a couple of weeks during the postnatal period for rats to develop quadrupedal walking (Altman & Sudarshan, 1975), newborns show evidence of other coordinated action patterns which can be expressed during the perinatal period. One of these action patterns is called the leg extension response (LER) and is evoked by anogenital stimulation, which typically occurs in the context of maternal-pup interactions. When the pup is stimulated by the dam licking its anogenital area, the pup rigidly extends the hindlimbs, thus providing better access to the perineum for continued maternal stimulation (which necessitates urination and defecation) (Moore & Chadwick-Dias, 1986). In this study, we reduced the amount of maternal stimulation that pups received, to test the idea that maternal-pup interactions influence development of the LER in newborn rats. Thus when we reduce the ability of the mother rat to perform a species-typical behavior (anogenital licking that is directed towards her pup), how is the behavior of the pup impacted? This is the question that was

addressed by this study. We examined this question by using an intranasal application of a zinc sulfate solution to reduce the mother rat's ability to smell, which preferentially decreases the amount of anogenital licking the dam performs on her pups. We then tested the pup's motor coordination during expression of the LER over the first 10 days of postnatal development. Therefore, this project directly examines the role of maternal care on pup motor behavior immediately after birth.

Maternal Behavior in Rats

Although they are born with closed eyes, ears, and limited motor capabilities rat pups are not completely deprived of sensory experience. On the contrary the pups are exposed to a variety of tactile, thermal, vestibular, and olfactory sensory stimulation from their mother, littermates, and the nest environment. Because the cutaneous, vestibular, olfactory, and kinesthetic senses begin developing during the prenatal period in rats, newborn pups can respond to stimulation in these domains. Previous research supports the contribution of maternal behavior as being a major source for early neural and behavioral growth and development, as well as learning, in the rat. Researchers previously have identified several different maternal behaviors in the rat that can vary in frequency of occurrence and quality depending on the mother and the pup.

Different Maternal Behaviors

There are several maternal behaviors that researchers have identified in rats that have different functions. Dams may exhibit different types of nursing when feeding their young. These categories of nursing include arched-back nursing, blanket nursing, and passive nursing. Arched-back nursing is when the dam is over her pups, her legs are spread apart and her back is in an overarching posture (Myers, Brunelli, Squire,

Shindeldecker, & Hofer, 1989). Blanket nursing is when the mother is over the pups, but she has a flatter back posture than in arched-back nursing. Passive nursing can be seen when the mother is laying on her side and the pups are suckling (Myers et al., 1989). Dams may also rest in top of her pups, not nursing them (blanketing). This helps to keep her pups warm.

Since the mother is active it is not uncommon for her to stir the pups from the nest at times. When this happens she will engage in retrieval of the pup. The mother will carry the pup by its body in her mouth back to the nest (Corodimas, Rosenblatt, Matthew-Feltons, & Morrel, 1995).

Mother rats frequently lick the bodies of their pups, which benefits the pups by grooming them and providing them with tactile stimulation. Like all other behavior, licking and grooming is variable among mother rats. Some mothers tend to lick more frequently than others; these mothers are considered high licking/grooming while mothers who lick and groom their offspring at lesser rates are low licking/grooming mothers. To determine whether a dam is a high licking/grooming mother or a low licking/grooming mother her interactions with her litter are observed during the first 10 days postpartum. Typically, observed percentage of the frequency of licking/grooming is calculated and high licking/grooming mothers are determined as being those that are one standard deviation above the mean, while low licking/grooming mothers are those that are one standard deviation below the mean (Champagne, Francis, Mar, & Meaney, 2003). This maternal licking of the pups frequently occurs within the context of nursing.

Researchers have observed that mother rats engage in different types of licking of their offspring. The mothers will lick and groom the body of their pups regularly, but if it

is directed toward the perineum of the pup then, as opposed to other parts of the body, it is categorized as anogenital licking (AGL). When the dam is engaging in AGL, she uses her forepaws to turn the pup over into a supine posture and steadily licks the pup in the anogenital area. The pups receive AGL from the mother frequently during the first two weeks after birth, but then it declines to low levels by the end of the third postnatal week (Moore & Chadwick-Dias, 1986). AGL provides tactile stimulation to the perineum and elicits a leg extension response (LER) where the pup extends its hindlegs away from the body in a “stretching” like manner. AGL also stimulates urination and defecation, which typically is preceded by expression of the LER. Because newborn rats are not able to urinate and defecate on their own, receiving AGL from the dam is crucial for their survival.

Not only is AGL necessary for the pups, but it is beneficial to the mother as well. One of the important functions of AGL is that it allows the pups to urinate and defecate (Moore, 1992). When the mother performs AGL she ingests the pup’s urine, which can replenish essential nutrients that were lost from giving birth and nursing (Moore, 1992). Importantly, it has been shown that the mother is attracted to a chemical (dodecyl propionate) contained in pup urine and feces (Moore, 1981; Brouette-Lahlou, Amouroux, Chastrette, Cosnier, Stoffelsma, & Vernet-Maury, 1991; Brouette-Lahlou, Godinot, & Vernet-Maury, 1998;). Additionally, male pups typically receive AGL more frequently than female pups (Moore & Morelli, 1979). Moore (1982) examined the reason behind the AGL preference directed towards males by treating female pups with testosterone. She found that the female pups treated with testosterone received significantly more AGL than control females. This indicates that dams may have a

preference for licking pups that have increased levels of testosterone, which is why they would be biased towards the males (Moore, 1982). In addition to chemical cues, it has been found that vocalizations produced by the pups can also elicit AGL from the dam (Brouette-Lahlou, Vernet-Maury, & Vigouroux, 1992).

Intergenerational Transfer of Maternal Behavior

The maternal behavior that a female rat demonstrates toward her own pups is more likely to be similar to the caregiving that she received when she was a newborn, thus showing an intergenerational transfer of maternal behavior. These behaviors seem to develop through experience since cross-fostering studies show that female offspring will exhibit the behaviors of the mother they are fostered to as opposed to their biological mother. Cross-fostering studies with rats have shown that mothers born to high licking/grooming mothers, but fostered to low licking/grooming mothers tended to become low licking/grooming mothers (Champagne et al., 2003). Likewise, female pups from mothers who were low licking/grooming, but fostered to a dam that was high on licking/grooming were prone to being high on licking and grooming when they had their own litters.

Effects of Non-Specific Licking & Grooming on Offspring

The role of maternal caregiving in providing sensory stimulation to offspring has important implications for development. Mother rats vary in the frequency of maternal behavior that is directed toward their offspring. As previously mentioned, these variations can be observed in the licking/grooming that is directed toward the pups. Studies examining the differences between high and low licking/grooming mothers show that the amount of sensory stimulation that the mother provides for the pup can influence its

physiological and anatomical development, as well as reproductive success and behavioral regulation.

In one study observing the differences between high licking and low licking mothers, the offspring of high licking mothers opened their eyes sooner than low licking mothers (Uriarte, Briegiron, Benetti, Rosa, & Lucion, 2007). Additionally, females that were born to high licking mothers tended to have a reduction in reproductive function. The experimenters observed the sexual receptivity of the female offspring to male copulations; this receptive position is called lordosis. Although there were no significant differences in the frequency of the females to perform the lordosis posture, females that were licked less were mounted more by males than females that had been licked more. As adults, both male and female offspring born to high licking mothers also tend to be less fearful and more prone to exploration.

Sexual development can be influenced in females if they receive higher levels of licking. These changes are not only evident in sexual behaviors, but also in reproductive capabilities. Uriarte and colleagues (2007) demonstrated that the ability to become pregnant for females could be affected by the level of licking they received as neonates. They separated females into groups based on the amount of licking that they received during the early postnatal period. After the females were sacrificed their oocytes were examined. The females who were born to high licking mothers had significantly fewer oocytes than females from low licking dams (Uriarte, et al., 2007).

Several studies have shown that rats that receive more licking and grooming and arched-back nursing tend to show less stress reactivity when tested as adults (Francis, Diorio, Liu, & Meaney, 1999; Caldji, Diorio, & Meaney, 2000; 2003). Corticotrophin

releasing factor (CRF) is associated with endocrine and behavioral responses to stress. GABA_A (gamma-aminobutyric acid) receptor activation inhibits CRF activity, and by doing this, it reduces stress reactivity. Pups that are born to mothers that are high in licking/grooming and arched-back nursing show increased GABA_A activity in the prefrontal cortex, the hippocampus, and the amygdala (Caldji et al., 2003). In contrast, pups that are born to mothers that are low in licking/grooming and arched back nursing have increased CRF gene expression in the hypothalamus and amygdala (Caldji et al., 2003). In a cross-fostering study, pups fostered from a low licking/grooming arched-back nursing mother to a mother that was high on performing the behaviors showed increased GABA_A production, whereas pups born to dams high on maternal care and fostered to mothers that were low on caregiving showed decreased GABA_A production (Caldji et al., 2003).

Maternal deprivation studies show that in the absence of typical levels of maternal care, female rats may learn to deprive their young of important experiences. When a female offspring is isolated from her mother before being weaned, and then has her own litter, she shows reduced levels of crouching over her pups, licking, as well as retrieving (Lovic, Gonzalez, & Fleming, 2001). These observations support the findings from other studies that have looked at the intergenerational transfer of maternal behaviors. Additionally, it has been shown that isolation from the mother can result in an increase in pup ultrasonic vocalizations (Wohr & Schwarting, 2008). The pups will also perform behaviors, such as raising their head and pivoting, while making the ultrasonic vocalizations. When the mother hears the vocalizations she performs “searching behaviors”, even when the calls are recorded and played back for the mother to hear.

Offspring born to low licking mothers tend to produce more ultrasonic calls when they are separated from their mothers.

Effects of AGL on Offspring

Maternal pup-directed AGL specifically (while excluding general body licking and grooming) also has been shown to significantly affect offspring behavior, physiology, and anatomy. Lenz and Sengelaub (2006) used an intranasal application of zinc sulfate to reduce the ability of the dam to smell. Reducing the ability of the mother rat to smell has been shown to preferentially decrease the amount of AGL she performs (Fleming & Rosenblatt, 1974; Mayer & Rosenblatt, 1992; Moore et al., 1992; Slotnick, Glover & Bodyak, 2000; Lenz & Sengelaub, 2006). This is likely due to her reduced ability to smell the salt and chemical signals that typically serve to attract dams to licking their pups. When AGL was reduced, there was a reduction in the number of motoneurons, smaller somata, and shorter dendritic length in the spinal nucleus of the bulbocavernosus (SNB) of the male offspring. The SNB is a motor nucleus in the lumbar spinal cord that controls penile reflexes. The reduction of the motoneurons in the SNB has a negative effect on copulation for males (Lenz & Sengelaub, 2006). Male rat pups born to dams that experimenters used polyethylene tubing to line the nasal passages, in order to reduce olfactory capabilities, showed longer latencies to ejaculate and longer latencies to resume copulation after ejaculating (Moore, 1984).

Increased AGL can alter sexual development of females as well, making them display sexual behavior that is more typical of males (Moore, 1985; 1992). For example, in one study female pups were stroked with a brush in order to receive extra perineal stimulation and to mimic maternal pup-directed AGL. As adults these females were given

testosterone injections. Compared to siblings that only received shoulder stimulation, they were more likely to perform male-typical mounting behaviors on another female (Moore, 1985; 1992).

Variations in the frequency a pup receives AGL from its mother also can predict differences in play and grooming behavior when the pup is a juvenile. Moore and Power (1992) found that either using zinc sulfate to induce maternal hyponosmia or giving the dams a dietary saline to reduce the appetite for pup urine, reduced the amount of AGL directed toward the pups. Male offspring in the treatment conditions that had hyponosmic moms received AGL less frequently and were more prone to play in a group than control males. Males that received more AGL also were more likely to engage in genital grooming than males in the control groups (Moore & Power, 1992).

Gerbils perform AGL on their young, and many of the physiological differences associated with AGL in rats also can be seen in gerbils. For example, the mother gerbil licks her male offspring more than her female offspring. Individual males that are licked more show longer latencies to urinate and produce more urine when the mother performs AGL (Clark, Bone, & Galef Jr., 1988). Although the mother tends to show a preference for licking males over females, some females actually tend to be treated more like their male siblings and receive either an equal amount of AGL or may even receive more AGL than some of their brothers. Uterine position effects may account for this phenomenon (reviewed in Ryan & Vandenberg, 2002).

In rodents, siblings are positioned in a uterine horn so that they are next to one another. Hormones can be transferred from sibling to sibling if they are next to each other in the uterine horn. If two males in the horn flank a female, she may receive more

androgens and develop masculinized features. Intrauterine position effects in many rodents result in many prominent physical and behavioral sexual differences (Ryan & Vandenberg, 2002). Female rodents that have been flanked by two male littermates in utero show masculine characteristics into adulthood. Not only do their levels of testosterone tend to be higher, but also physical traits such as the anogenital distance (distance from the anus to the genital tubercle) tends to be longer in these females which is a characteristic seen in males. Uterine position effects account for preference for mothers to individuals that are to receive AGL. Females flanked by more than one male receive more AGL than females that are not positioned by any males (Clark, et al., 1988). Similar results are found with males that are positioned in utero next to other males. Males that are positioned next to more than one female receive less AGL from the mother than males that are positioned next to one or two other males. Taken together, this research suggests that a fetus's position within the uterus influences the quantity and quality of maternal care (e.g., maternal licking) the pup will receive during the newborn period. Maternal care then influences development of several systems (i.e., brain, spinal, stress, reproductive, etc.) in the pup. Thus, maternal-pup interactions are a non-trivial aspect of growth and development.

Leg Extension Response

An Early Form of Motor Coordination

The present study manipulated maternal behavior, specifically pup-directed AGL, to examine the effects of maternal care on the development of the LER in her pups. The LER has been shown to be elicited by rhythmic, stroking stimulation of the anogenital (AG) area on the pup and not from stimulation of other parts of the body (Moore &

Chadwick-Dias, 1986). When the mother performs AGL she typically holds the pup supine between her forepaws, putting slight pressure on the pup's midsection. The pup's body is in a reverse orientation to the mother. The pup becomes immobilized while the mother licks the AG area until the legs extend outward from the body, and the pup may then urinate and/or defecate. The LER also can be evoked experimentally by rhythmically stroking the pup's AG region using a paintbrush (Smotherman & Robinson, 1987), glass rod (Moore & Chadwick-Dias, 1986), or latex-tipped electronic vibrotactile device (Roberto & Brumley, 2014).

Sex differences have been found in the expression of the LER. During maternal pup-directed AGL, male pups show shorter latencies to perform the LER than their female littermates (Moore & Chadwick-Dias, 1986). This may be due to the mother's preference for performing AGL on male pups. The preference for licking males and the increased sensory stimulation in turn contributes to their sexual development (Moore, 1992). AGL starts to decline rapidly at about postnatal week three (Moore & Chadwick-Dias, 1986), when the pup's motor coordination is more developed and weaning occurs.

Evoked Experimentally

To date there are not many studies that have experimentally studied the LER. The few studies that have looked at the LER have shown that the experimenter is able to evoke the LER using either a small, camel hair paintbrush (Smotherman & Robinson, 1987; Salas, Torrero, Regalado, & Loranca, 1998), glass rod (Moore & Chadwick-Dias, 1986), or electronic vibrotactile device (Roberto & Brumley, 2014). The paintbrush and glass rod method requires the experimenter to move their wrist/hand to perform the rhythmic stroking. The electronic vibrotactile device does not require this: the device

makes contact with the pup at the tip, which is a latex strip, and performs an automated stroking motion (120 Hz), more or less simulating maternal licking.

Moore and Chadwick-Dias (1986) first characterized the LER in detail using a glass rod to stimulate pups in the AG region for the purposes of evoking the LER. They found that the LER is elicited on average 3 seconds prior to the release of urine. They also examined whether the LER can be elicited by stimulating various regions of the body, other than the perineum. They used a paintbrush to stimulate multiple areas of the pup's body. They found that stimulation of other regions of the body (the head, midsection, neck, etc.) were not as likely to evoke the LER as stimulation of the perineum.

Smotherman and Robinson (1987) experimentally evoked the LER in spinal transected fetal rats *in vivo*. They externalized rat fetuses from the uterus into a saline bath, two days before birth (on gestational day 20, or E20). After mid-thoracic severing of the spinal cord, the experimenters used a paintbrush to stimulate the AG region of fetuses. They reported that spinal fetuses expressed the LER. Thus, at least during early development, expression of the LER does not require input from the brain. The authors further state the response appears to develop in advance (prenatally) of its apparent function (during the newborn period).

More recently, Roberto and Brumley (2014) examined the effects of an additional day of postnatal experience on motor coordination in newborn rat pups. Subjects were delivered via caesarean section one day before term (on E21), and then fostered to another dam that had recently given birth. On E22, the typical day of birth in rats, the behavior of prematurely delivered pups was compared to that of normal, vaginally

delivered pups and pups that underwent caesarean section on E22 (before the onset of labor). Pups that were delivered prematurely and received one extra day of postnatal experience showed differences in LER expression. In particular, they showed more bilateral coordination during expression of the LER. This study therefore suggests that experience around the time of birth influences the development of motor coordination.

To directly examine the idea that sensorimotor experience can influence newborn behavior, Belnap et al. (2014) studied the influence of sensorimotor training on LER expression in P1 (postnatal day 1; 24 hours after birth) rats. They did this by using a range of motion (ROM) restriction paradigm. To implement ROM restriction they placed a Plexiglas plate placed beneath pups at 50% of limb length and they repeated AG stimulation of the pups using a vibrotactile device, thus providing pups with motion-restricted LER training. This stimulation was repeated for 15 training trials over the course of an hour. After the 15th trial the Plexiglas plate was removed and the pup was stimulated for a final LER. The pups that had received the ROM restriction showed shorter durations in the LER at test (compared to controls), as well as smaller ankle and hip angles during LER expression. This study shows the importance of proprioceptive and cutaneous feedback in determining LER expression. Furthermore, it indicates that expression of this seemingly reflexive action pattern is modified by experience. This is relevant to the current study since we manipulated experience of the pup developmentally to examine postnatal development of the LER.

Neural Mechanisms

The neural mechanisms underlying the LER have yet to be elucidated. However, we know that spinal rat fetuses (Smotherman & Robinson, 1987) and newborns (Kauer et

al., 2013) can perform the LER, and therefore the neural control of this action pattern must be at least partially supported by the spinal cord. Additionally, stimulation of the AG area includes activation of the lumbosacral bladder reflex pathways, and we know that the LER typically precedes urination in pups (Moore & Chadwick-Dias, 1986). Thus, there is likely to be some overlap between the circuitry for the LER and bladder reflex pathways.

Regulation of voiding in rats involves reorganization of neurons in the spinal cord and brain during early development (de Groat, 2002). For example, rats are dependent on their mother for voiding through AGL, up through the third postnatal week. This caudal spinal cord reflex eventually comes under supraspinal control as the animal matures. Eventually, synaptic connections are reorganized and voiding is regulated by the spinobulbospinal pathway (de Groat, 2002). It has been shown that pups that are underfed tend to show longer latencies to perform the LER and reduced hindlimb activity (Salas, Torrero, Regalado & Loranca, 1997). These findings may be related to a delay in neuronal maturation and development in general.

Purpose of Current Study

The purpose of the current study was to examine the influence of maternal behavior on offspring motor behavior. We did this by studying the development of the LER in pups from normal litters compared to pups from litters with low AGL moms. There were three maternal olfactory conditions: hyponosmia and two control conditions. Hyponosmia, which is the reduced ability to smell, was induced in some of the dams (hyponosmia group) by using an intranasal application of ZnSO_4 , throughout the neonatal period. This treatment has been shown to specifically reduce the occurrence of maternal

pup-directed AGL, while not reducing other forms of maternal behavior (Alberts & Galef, 1971; Mayer & Rosenblatt, 1977, 1992; Moore & Power, 1992; Slotnick, Glover & Bodyak, 2000; Lenz & Sengelaub, 2006). AGL still is performed at low rates, apparently enough for pup survival not to be at risk. Females in the two control groups were treated with either air, or distilled water, on the same schedule as females in the hyponosmia group. In previous research using the hyponosmia paradigm, Mayer and Rosenblatt (1977) had found that using saline as a control causes nasal irritation in dams thereby impairing olfaction. It is because of this that when using intranasal applications of ZnSO₄ to induce hyponosmia in rats, researchers typically use air as a control. In this experiment we have introduced a new control of distilled water. Since it is liquid and the ZnSO₄ is also a liquid, any differences in reductions of AGL cannot be attributed to having additional liquid in the nasal cavity. Thus, we had two types of litters: those that received AGL as usual (with the control moms) and those that received reduced AGL (with the hyponosmia moms). Then the LER was experimentally evoked by vibrotactile stimulation of the AG region in pups on P1, P5, or P10, to examine the development of this early action pattern. In this study, only male pups were tested. The reason for this is that previous research has shown that male pups receive more AGL than female pups (Moore & Morelli, 1979; Moore, 1982; 1992). By testing only male pups we eliminated possible sex differences that may be due to maternal preference, and also hopefully increased the difference in levels of licking between the hyponosmia and control conditions.

The primary hypothesis of this study was that pups born to hyponosmic moms would show altered postnatal development of the LER due to receiving low levels of

AGL. Thus if pups receive less AGL, they should also express the LER less frequently. If every expression of the LER offers the pup an opportunity to improve motor coordination expressed during the action pattern (e.g., from proprioceptive feedback of the limbs, from coordinating the tactile inputs with motor output, etc.), a sort of “motor practice” that occurs naturally in the context of maternal-pup interactions, then we should expect to see “slower” development of the LER in pups that receive less AGL compared to pups from control litters. Pups were tested at one of three ages after birth—P1, P5, or P10—to observe the differences in the LER following altered AGL experience. Latency to express the LER and LER duration were examined, as well as limb positioning during LER. This is the first study to examine maternal behavior influences on offspring motor development during the perinatal period.

In addition, we had to verify that our manipulation of maternal behavior was effective. Thus, we also observed and recorded maternal behavior throughout the period of testing, to ensure that the hyposmemic moms were actually showing less AGL than control moms. Our prediction here was that we would replicate the finding that intranasal ZnSO₄ treatment specifically reduces the occurrence of maternal pup-directed AGL (Alberts & Galef, 1971; Fleming & Rosenblatt, 1977, 1974; Mayer & Rosenblatt, 1992; Moore & Power, 1992; Slotnick, Glover & Bodyak, 2000; Lenz & Sengelaub, 2006), compared to controls.

Method

Subjects

Adult subjects were 24 female, multiparous, Sprague-Dawley rats purchased from Simonsen Laboratories and housed in the Brumley Lab at Idaho State University (ISU). Test subjects were 72 newborn males: 24 tested at P1, 24 tested at P5, and 24 tested at P10. Animals were kept in a temperature-controlled room on a 12 hr light: 12 hr dark (lights on at 0900) cycle and provided ad libitum food and water. Pregnant females were housed in pairs until 2 days before they gave birth, after which they were housed individually. All animals were cared for and used in accordance with guidelines established by the National Institutes of Health, and by the Institutional Animal Care and Use Committee at ISU.

Design

There were 8 dams in the hyponosmia group, 8 dams in the air control group, and 8 dams in the distilled water control group. If litters were born with more than 8 pups they were culled after testing on P1 to 8 pups (4 males and 4 females). A total of 3 pups per litter were tested once, at one of the 3 different ages—P1, P5, or P10—for the LER. Thus all litters contained 6-8 pups. We employed a cross-sectional design for testing pups here and thus did not re-test the same subject at different ages. This is because we required pups to show the LER or they would not be included in the study. We used a cross-sectional design so that if one pup did not show the LER, that subject could be replaced by another pup of the same litter; whereas if a pup tested repeatedly did not show the LER at P5 or P10, the entire litter would have to be replaced. Thus, a total of 8 pups was tested for each of the maternal olfactory conditions at each age group ($n = 8$

subjects per group x 3 maternal olfactory conditions x 3 ages = 72 pups total). Sex effects were avoided by using all male pups, and litter effects were avoided by choosing subjects from different dams for the same groups.

Maternal Hyponosmia

Dams in the hyponosmia group were treated with zinc sulfate (ZnSO_4) on the day before they gave birth (E21) and again at the ages of P1, P3, P5, P7, and P9 of the pups to ensure hyponosmia throughout the course of the study. The female rat was placed on a heating pad and put under isoflurane anesthesia (Mayer & Rosenblatt, 1977, 1992; Slotnick et al., 2000). In experimental dams, 0.5 ml of 5% ZnSO_4 was administered to the female rat using a blunted 20-ga hypodermic needle with a pinched tip. The ZnSO_4 was diluted in saline. The solution was sprayed into each nostril at a 60° angle pointed toward the nasal mucosa (Mayer & Rosenblatt, 1992; Moore & Power, 1992; Brouette-Lahlou et al., 1998). In place of ZnSO_4 , control dams received air, or distilled water. The animals were then gently held so that the head tilted downward to drain the excess fluid if applicable (Moore & Power, 1992; Brouette-Lahlou et al., 1998). The female was then returned to her cage following recovery from anesthesia. The litter was kept warm in a thermoneutral-controlled infant incubator while the mother received the treatment until she fully recovered. Then the pups were returned to their mother's home cage. Pups were separated from the mother for 10-15 minutes.

Behavioral Testing of Pups

Subjects were healthy and had fed recently, as evidenced by a milk band across the abdomen. On the day of testing, subjects were transferred from their home cage into an incubator with a temperature of 30°- 35°C (35°C at P1; 33° at P5; 30° at P10). They

were manually voided using a small paintbrush, to standardize the time since last bladder expression. Pups were weighed and their length was measured from crown to rump. Subjects were then placed in a small plastic dish with at least 2 littermates to allow acclimation to incubator conditions for 30 minutes. To begin testing, subjects were placed in a fitted jacket that supports the neck and abdomen, but leaves the limbs free for movement. Subjects were fitted into a jacket appropriately sized for their age group. They were then attached in a prone position to a vinyl covered horizontal bar, to allow the limbs to hang pendently.

The LER was evoked by stimulating the AG area of the pup with an electronic vibrotactile device (120 Hz) with an 8 mm long, 1.5 mm wide latex sheet attached to the tip. After a 1-minute pre-stimulation period (baseline), the tip of the latex sheet was applied to the AG region of the subject until 5 seconds after they showed a bilateral LER or until a maximum of 20 seconds if they did not show the LER. If the LER wasn't shown, the stimulus was applied 3 minutes later. However, all pups showed the LER within 2 presentations of the stimulus. Test sessions were recorded onto DVDs from 2 synchronized camera views (a posterior and lateral view of the hindlimbs) and played back in reduced time for behavioral scoring.

Observation of Maternal Behavior

Maternal behavior was observed for three 30-minute sessions on the days where the pups were P2, P4, P6, and P8: once at the end of the dark cycle (8:30 am; morning, lights off), once at mid-day (2:30 pm; afternoon, lights on) and once at the end of the light cycle (8:30 pm; evening, lights on), for each of those ages. Thus, maternal behavior was observed for a total of 360 minutes per dam over the course of the study.

Observations were sampled throughout the day since previous research has shown that frequency of maternal behaviors vary at different times in the day. For example, nursing occurs more frequently during the light cycle (Champagne et al., 2003). However, licking and grooming has been shown to occur mostly just prior to the beginning of the light cycle and just before the dark cycle begins. Thus our morning and evening observation times were arranged to capture when the most licking should occur.

Scoring of maternal behavior happened live. Cages were placed on individual racks in the animal colony room before observation began; cages were not transported to the lab. To avoid disturbing the dark cycle of the rats, night vision goggles were used by the researchers while recording maternal behavior. Maternal behaviors observed and scored included the following: (1) licking and grooming the body of a pup, (2) licking directed towards the perineum of the pup (AGL), (3) arched-back nursing of pups, (4) passive nursing, (5) blanket nursing, (6) pup retrieval, (7) dam lying on pups (blanketing), and (8) dam not on pups.

Data Analysis

Behavior was scored using JWatcher, which is a real-time event-recording program that categorizes behavior and the time it is expressed ($\pm 0.01s$). The scorer was blind to pup group association. Maternal observation scorers were blind to maternal group association for the distilled water and $ZnSO_4$ treatment groups, but not for the air treatment vs distilled water or $ZnSO_4$ treatment groups. A 90% inter-rater reliability rate was required before scoring pup behavior. For maternal observations, a minimum of three training sessions where inter-rater agreement of maternal behavior was at 100% was

required before actual scoring. Data was analyzed using StatPlus statistical software. A significance level of 5% was adopted for all tests.

Pup Behavior

The LER is defined as the movement of one or both of the pup's hindlimbs from a resting position to hyperextension and positioned at a right angle to the body (Roberto & Brumley, 2014; Moore & Chadwick-Dias, 1986). From the posterior camera view, subjects were scored for the presence of a bilateral or unilateral LER during the AG stimulation period. Latency to expression of the LER (unilateral or bilateral), and latency to express the maximum bilateral LER (the full hyperextension measured by colored lines on a Plexiglas plate placed anterior to the subject) was recorded. Screen shots were taken at maximum LER to measure interlimb distance, hip angle, and ankle angle, following the methods of Belnap et al. (2014). From the posterior camera view the interlimb distance between the two hindlimbs was calculated by measuring the distance between the paws at maximum LER expression (Figure 1A). Using the lateral camera view, ankle and hip angles were calculated for the right hindlimb during maximum LER expression (Belnap et al., 2014). The ankle angle was determined from two lines drawn on the lateral camera shot. One line was drawn from the base of the tail to the point at which the subject's back made contact with the bar. The second was drawn from the tip of the subject's longest hindlimb digit and the tarsal pad. The angle formed between these lines was measured using a protractor moving counter clockwise. The hip angle was determined by two lines as well: one line was drawn along the subject's ventrum and a line tangent with the tip of the subject's longest digit that ran along the subject's thigh

and intersected with the ventral line (Figure 1B). The angle was formed between these lines (moving counter clockwise) was the hip angle.

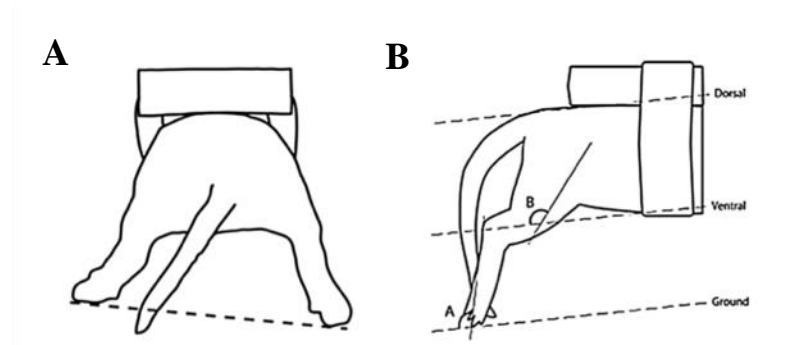


FIGURE 1: Interlimb distance and hindlimb angle measurements of maximum bilateral LER. **A:** A picture was taken using the posterior camera, a line was then drawn between the ends of the longest digits of each hind foot. Interlimb distance was measured using a ruler (mm). **B:** Ankle (angle A) and hip angles (angle B) were both measured using the lateral camera view for the right hindlimb. Three lines were drawn as reference points for the ankle and hip angles; the first was a dorsal line drawn from the base of the tail to where the subject made contact with the bar. The second line was the ventral line drawn along the subject's ventrum, through the ankle, and parallel to the dorsal line. The third line (ground line) was drawn parallel to the dorsal and ventral line and tangent with the tip of the subject's longest digit. The angles were then measured using a protractor. Figure is taken from Belnap et al (2014).

Two-way ANOVAs were used to compare group means, and Tukey's HSD was used for post hoc comparisons. Independent variables were maternal olfactory condition (a between subjects factor) and pup age (a between subjects factor). Dependent variables were latency to LER, duration of LER, interlimb distance, and ankle and hip angles.

Maternal Behavior

Each dam was observed continuously for three 30-min periods, when her pups were P2, P4, P6, and P8. Experimenters recorded the frequency or duration of specific maternal behaviors: (1) frequency of licking and grooming the body of a pup, (2) frequency of licking directed towards the perineum of the pup (AGL), (3) Duration of

arched-back nursing of pups, (4) duration of passive nursing, (5) duration of blanket nursing, (6) frequency of pup retrieval, (7) duration of dam lying on pups (blanketing), and (8) duration of dam not on pups. These categories are not necessarily mutually exclusive (Champagne et al., 2003). The duration or frequency of performing each behavior was averaged for subjects in each group, for each day (P2, P4, P6, P8). While we did examine all maternal behaviors, we were most interested in AGL.

Maternal behaviors were analyzed using repeated measures ANOVA, with Tukey's HSD for post hoc comparisons. Independent variables were maternal olfactory condition (a between subjects factor) and day/age of observation (a within subjects factor here, because the same dam and litter will was watched over the course of the study).

Results

Pup Behavior

Pup Weight and Length

A two-way ANOVA (3 maternal olfactory conditions x 3 ages) for pup weight revealed no main effect of maternal olfactory condition. A main effect of age was found ($F(2, 63) = 525.09, p < 0.001$) where pup weight increased as age increased. An interaction of maternal olfactory condition and age also was found ($F(4, 71) = 2.28, p < 0.04$), where P10 pups in the air condition weighed more than P10s in the ZnSO_4 group. Means of pup weight are displayed in Figure 2A.

For pup length, there was no significant main effect of maternal olfactory condition. A significant main effect of age on length was found ($F(2, 63) = 252.68, p < 0.001$) where, as age increased so did pup length. An interaction of maternal olfactory condition and age also was found ($F(4, 71) = 3.54, p = 0.01$). P10s in the air group had significantly longer bodies than P10s in both the distilled water and ZnSO_4 groups. Means of pup length are displayed in Figure 2B.

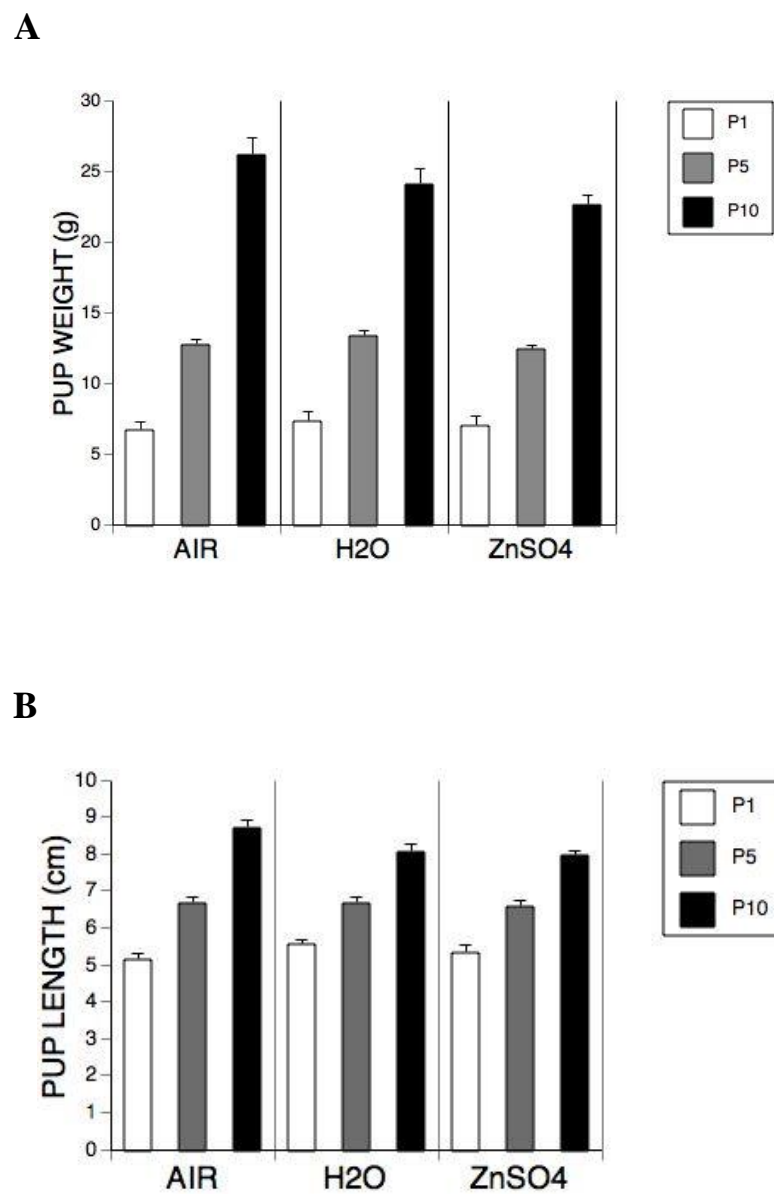


FIGURE 2: Pup weights and body lengths. **A:** Mean weight of pups measured in grams. **B:** Mean length of pups measured in centimeters. Bars depict means; vertical lines show SEM.

Unilateral LERs

While all pups showed a bilateral LER in order to be included in the study, not all pups showed a unilateral LER. There were no treatment effects for unilateral LERs, but there was an effect of age. As age increased, fewer pups showed a unilateral LER, with 21 pups showing a unilateral LER at P1, 11 at P5, and 5 pups at P10. A chi-square test revealed that this difference in unilateral LER expression was significant ($\chi^2 (2, N=72) = 21.79, p < 0.001$). Significantly fewer P5 and P10 pups showed unilateral LERs compared to P1 pups (both $ps < 0.001$). The remaining analyses on the LER focus only on the bilateral LER.

Bilateral LERs

For latency to bilateral LER expression, a two-way ANOVA (3 maternal olfactory conditions x 3 ages) revealed no significant effects. Group means for bilateral LER latency are shown in Figure 3A. For bilateral LER duration, there was a main effect of maternal olfactory condition ($F (2, 63) = 4.362, p < 0.001$). There was no significant difference between the two control conditions (air and distilled water); however, pups in the ZnSO_4 group had significantly shorter LER durations than both control groups ($F (2, 63) = 4.362, p < 0.001$). This can be seen in Figure 3B.

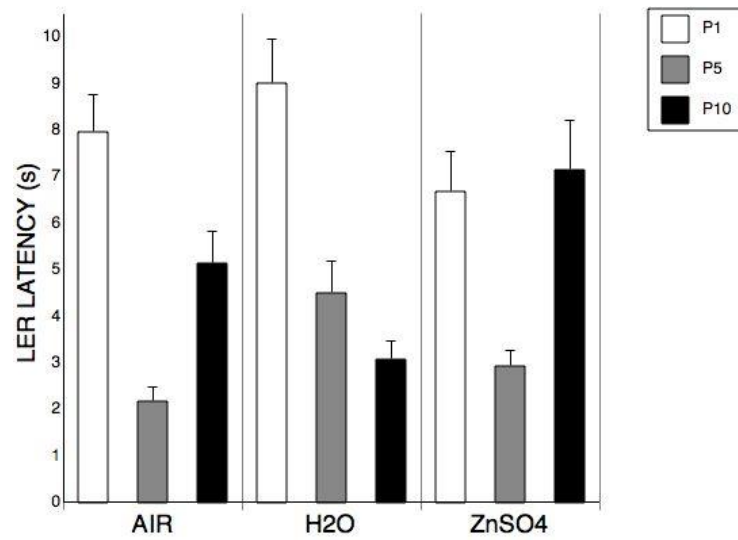
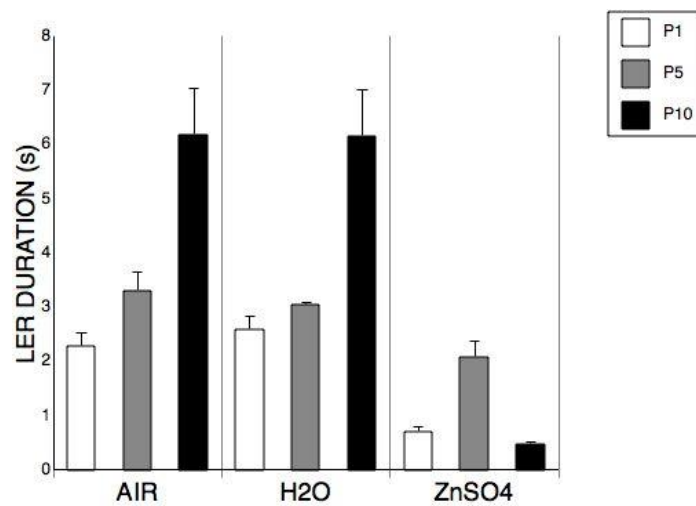
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FIGURE 3: Latency and duration of the bilateral LER following anogenital stimulation for each age group. **A:** Latency to express maximum bilateral LER. **B:** Duration of bilateral LER. Bars depict means; vertical lines show SEM.

Interlimb Distance and Limb Angles

For interlimb distance, a two-way ANOVA (3 maternal olfactory conditions x 3 ages) revealed a main effect of age ($F(2, 63) = 35.21, p < 0.001$). As shown in Figure 4A, as age increased so did interlimb distance. There were no effects of maternal olfactory condition or age on hip angle (Figure 4B). However, there was a main effect of maternal olfactory condition on ankle angle ($F(2, 63) = 4.957, p < 0.001$). Ankle angle for pups in the ZnSO₄ group was significantly smaller than the ankle angle for pups in the air group. The ankle angles for the ZnSO₄ group was smaller than for the distilled water group, but this difference did not reach statistical significance ($p = 0.08$).

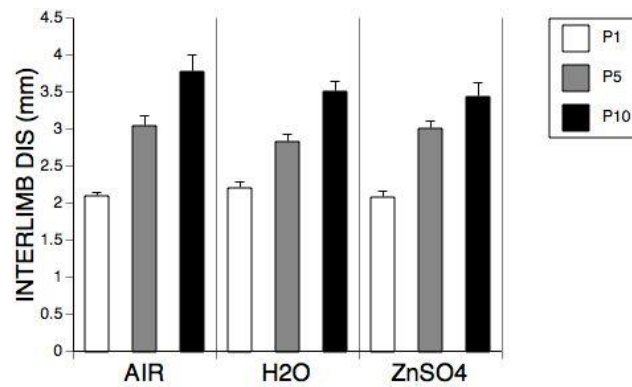
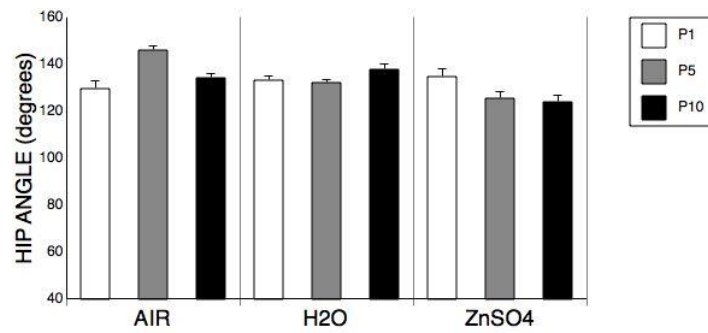
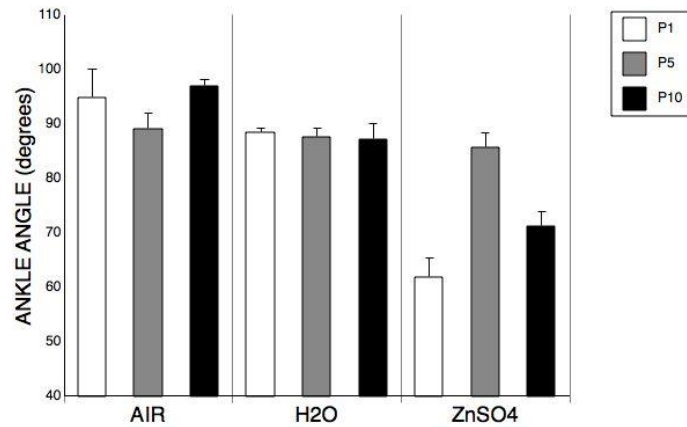
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FIGURE 4: Interlimb distance, hip angle, and ankle angle measurements for maximum bilateral LER. **A:** Interlimb distance (cm) of bilateral LER taken from posterior camera angle. **B:** Hip angle (degrees) of bilateral LER taken from lateral camera angle. **C:** Ankle angle (degrees) of bilateral LER taken from lateral camera angle. Bars depict means; vertical lines show SEM.

Maternal Behavior*Anogenital Licking*

A repeated measures ANOVA (3 maternal olfactory conditions x 4 age groups) revealed that dams in the distilled water group and air group performed more AGL on pups than dams in the ZnSO₄ group. However, this effect only approached significance ($F(2, 21) = 3.31, p = 0.056$). A main effect of age was found where dams performed AGL on P2s more than any other age group ($F(3, 63) = 2.91, p = 0.04$). Means for maternal AGL are shown in Figure 5A. Additional analyses were also done for maternal AGL across the different observation times cycle (8:30 am, morning; 2:30 pm, afternoon; 8:30 pm, evening). No significant effects of treatment were found for the morning and evening observation sessions. However, a main effect of maternal hyponosmia was found for the afternoon session where maternal AGL was performed more on pups in the air control group compared to the ZnSO₄ group ($F(2, 21) = 5.509, p = 0.05$) and AGL was performed on pups more in the distilled water control group compared to the ZnSO₄ group ($F(2, 21) = 5.509, p = 0.01$). Means for maternal AGL for the afternoon observation session are displayed in Figure 5B and 5C.

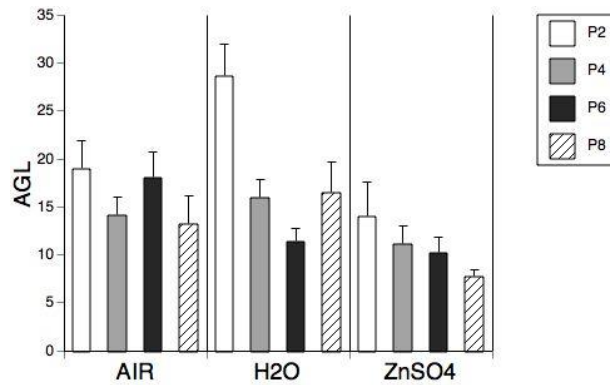
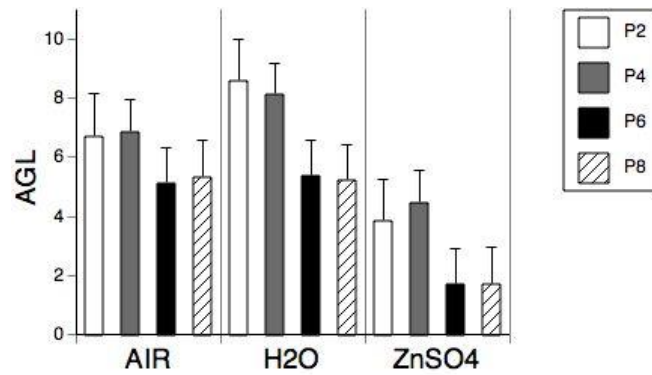
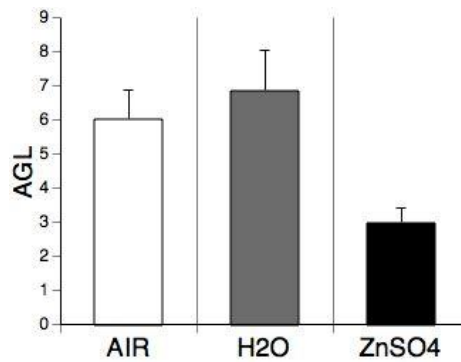
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FIGURE 5: Frequency of maternal AGL. **A:** Frequency of maternal AGL among maternal olfactory condition groups and across age groups averaged across all observation times. **B:** Frequency of maternal AGL among maternal olfactory condition groups and across age groups for the afternoon observation session. **C:** Frequency of maternal AGL among maternal olfactory condition groups averaged across age groups for the afternoon observation session. Bars depict means; vertical lines show SEM.

Nursing and Licking/Grooming

Repeated measures ANOVAs (3 maternal olfactory conditions x 4 age groups) revealed no interaction of maternal olfactory condition and age on any of the three types of nursing or licking and grooming. There also was no main effect of maternal olfactory condition or age on licking/grooming (Figure 6A). Furthermore, no main effect of maternal olfactory condition on any of the three types of nursing was found (Figure 6B). However, there was a significant main effect of age on nursing ($F(11, 264) = 20.65, p < 0.001$). Dams spent more time arched-back nursing for P2s and P4s than they did for the P6 and P8 age groups ($F(3, 95) = 10.57, p < 0.001$). Dams spent more time blanket nursing P2s than they did P4s and P8s ($F(3, 95) = 3.48, p = 0.02$). There were no differences between age groups for passive nursing. The means for time spent nursing are displayed in Figure 6C.

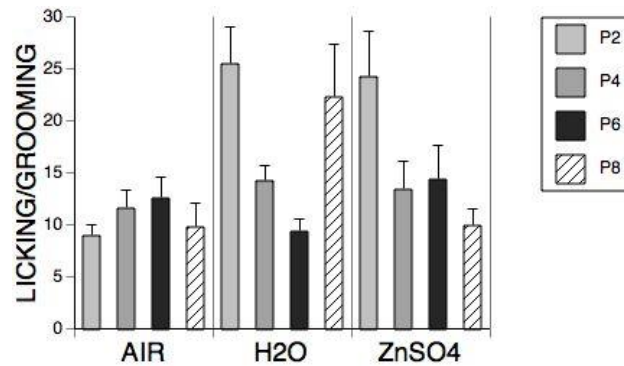
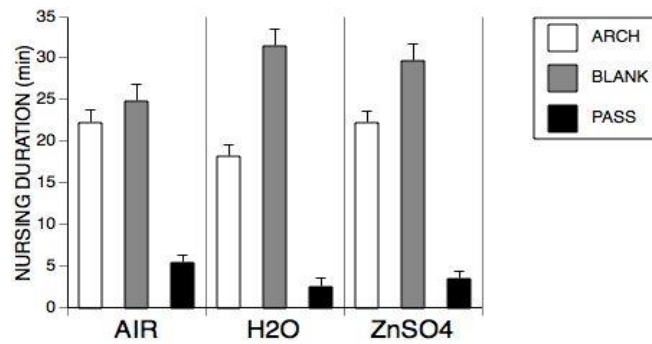
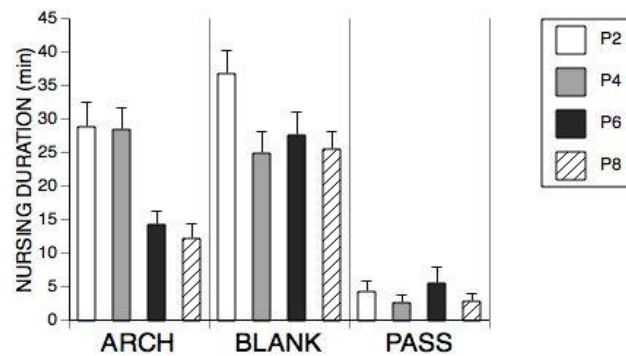
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FIGURE 6: Maternal behavior on P2, P4, P6, and P8. **A:** Frequency of licking/grooming for pups in each maternal olfactory condition and across age groups at observation. **B:** Comparison of three different nursing types (arched-back, blanket, and passive) across maternal olfactory conditions. Means are averaged across three nursing conditions. **C:** Comparison of age groups across three types of nursing. Bars depict means; vertical lines show SEM.

Blanketing, Retrieval, and Time off Pups

Repeated measures ANOVAs revealed no significant main effects or interactions of maternal olfactory condition or age on blanketing or retrieval. No main effect of maternal olfactory condition on the amount of time dams spent off pups was found. However, there was a main effect for age where dams spent more time off P6 and P8 pups than P2 and P4 pups ($F(3, 72) = 9.16, p < 0.001$). The means for these behaviors are displayed in Figure 7.

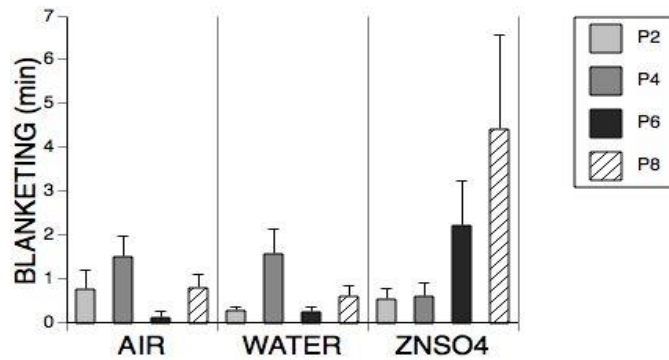
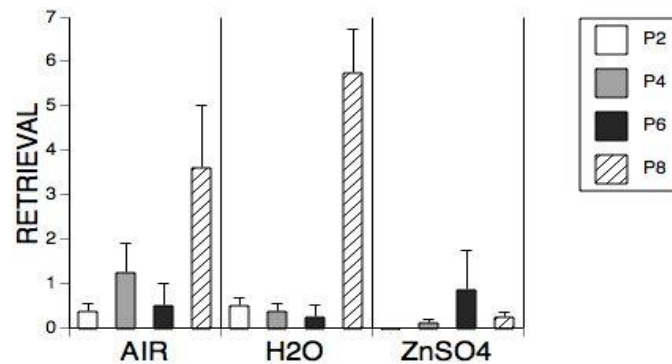
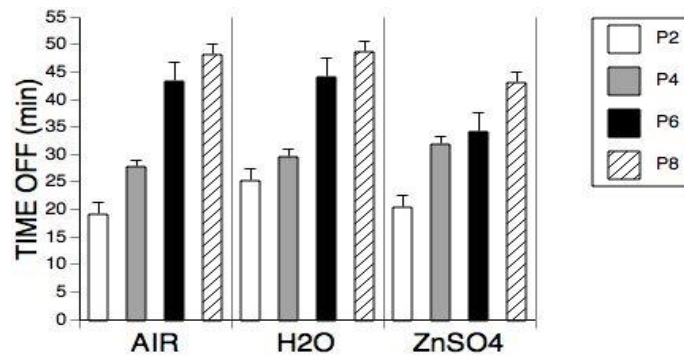
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FIGURE 7: Duration and frequency for blanketing, retrieval, and time spent off pups. **A:** Duration of blanketing in minutes for each maternal olfactory condition across age groups. **B:** Frequency of retrieval for each of the maternal olfactory conditions and across age groups. **C:** Time spent off pups represented in minutes for each maternal olfactory condition across age groups. Bars depict means; vertical lines show SEM.

Discussion

The purpose of this experiment was to examine the role of maternal behavior on the development of the LER in the newborn rat. This was done by altering a maternal behavior (AGL) by inducing maternal hyponosmia via an intranasal application of ZnSO₄. In general, the hypothesis that inducing maternal hyponosmia via an intranasal application of ZnSO₄ in dams would alter LER expression was supported. Pups born to dams in the ZnSO₄ group showed shorter LER durations and smaller ankle angles during LER expression, compared to pups in the air and distilled water control groups. Although reduction of AGL gathered from maternal observations across all observation times did not yield statistically significant results ($p = 0.056$), means of AGL for dams in the ZnSO₄ group were lower than that of the air control and distilled water control groups. Thus, there was a trend towards conventional significance levels ($p = 0.056$ vs 0.05), and furthermore, AGL results were in the predicted direction (AGL was lower for ZnSO₄-treated dams). When examining differences in maternal AGL for the different observation time points in the day (8:30 am, morning; 2:30 pm, afternoon; 8:30 pm, evening) there was a significant difference for maternal olfactory condition where the frequency in AGL for dams in the air control group and distilled water control group were both greater than that of dams in the ZnSO₄ group, but no significant differences were seen in the morning and evening observation sessions. One explanation for this occurrence is that maternal behavior differentially occurs across the day and night cycles. For example, Champagne et al. (2003) found that there is a circadian rhythm for licking/grooming where dams showed more licking/grooming just prior to the light cycle and just prior to the dark cycle. If all types of maternal licking (AGL included) occur less

frequently during the day it may be possible that the observed effects of reduced maternal AGL due to intranasal application of ZnSO₄ are more pronounced during the afternoon observations where licking occurs less naturally.

While it may be possible to obtain a statistically significant effect of maternal olfactory condition on AGL for all observation time points by increasing sample size, this would not be the most ethical or necessary course of action. For instance, increasing the sample size would require sacrificing the lives of more animals. Reducing the amount of animal deaths in research is an ethical obligation of all animal scientists. Furthermore, it is unnecessary to increase the sample size since our area of primary interest is with pup behavior, where statistically significant results were established. Maternal observations were obtained for the purpose of establishing the effectiveness of ZnSO₄ in reducing AGL and evaluating whether or not other maternal behavior was affected by the treatment. In this regard our maternal observation measurements were sufficient in establishing that the most likely determinant of altered bilateral LER expression was due to reduced maternal AGL, since no other maternal behavior yielded a main effect nor approached a main effect of maternal olfactory condition.

In the present study, none of the types of nursing, (arched-back, blanket, or passive) were affected by maternal olfactory condition. Time spent off pups also was not affected by maternal olfactory condition. We did find a main effect of age on arched-back and blanket nursing, where pups were nursed more at earlier ages than at later ages. We also found that dams spent more time on pups at earlier ages than at later ages. The effects of age on nursing and time spent off pups are consistent with the observations of Moore and Power (1992). However, Moore and Power (1992) also found that dams

treated with ZnSO₄ spent more time in the nest and more time nursing than controls. This is in contrast to our findings that treatment did not affect nursing or time spent off pups.

Reduced maternal AGL influenced both bilateral LER duration and ankle angle in pups. Pups in the ZnSO₄ group showed shorter bilateral LER durations and smaller ankle angles (i.e., relatively more flexed limb posture) than pups in the air and distilled water control groups. This supports our hypothesis that reducing maternal AGL influences expression of the LER. Differences found with LER durations and ankle angles are consistent with previous research showing that a range of motion (ROM) restriction can alter expression of the LER (Belnap et al., 2014). The ROM restriction study used changes in proprioceptive and cutaneous feedback to alter LER expression, and also found changes in LER duration and limb angles, but not latency or interlimb distance. This is the same pattern of findings for the present study.

Why do we see alterations in LER duration and angles? In the Belnap et al. (2014) study, changes in expression of the LER were seen after multiple LER expressions while receiving ROM restriction which induced a flexed limb posture. In other words, pups were receiving LER practice, with altered limb proprioceptive and cutaneous feedback during training. In the current study, pups in the ZnSO₄ group received less AGL that presumably also led to less practice of the LER, which means pups would have had less experience hyperextending their hindlimbs during anogenital stimulation. This in turn may have led to a more brief and flexed response. This may be because with each LER expression, or length of each LER expression, strength of the LER increases. There are several mechanisms that may account for this observation. One possible explanation is differences in pup arousal. Research has shown that pups that have received more

licking/grooming show less stress reactivity as adults (Francis, Diorio, Liu, & Meaney, 1999; Caldji, Diorio, & Meaney, 2000; 2003). If AGL affects stress reactivity in pups, it is possible that an increase in arousal of pups who are licked less may result in a decrease of LER responsiveness (i.e., more general arousal or activity, but less sustained LER). Differences seen in LER duration and ankle angle could also be attributable to differences in muscle strength. The more AGL a pup receives, the more it consequently should perform the LER. This in turn will work the muscles in its hip and legs, increasing muscle density and thereby increasing muscle strength and responsiveness. Pups born to dams in the ZnSO₄ group likely did not receive as much LER practice as the pups in either control condition, which could have influenced (i.e. slowed) muscle growth and affected LER expression as a result. Another possible explanation is differences in neural connections. Variations in maternal AGL have been shown to lead to neurological changes in the SNB of male rats, resulting in differences of penile reflexes (Lenz & Sengelaub, 2006). It is logical to assume that if the neural connections responsive for a penile motor reflex can be altered, then the neural mechanisms responsible for LER motor expression could also be impeded with less AGL experience for the ZnSO₄ group. If these neural mechanisms are not receiving the sensory stimulation necessary for growth during development, then the LER behavior may be compromised as well.

Although maternal olfactory condition did not influence interlimb distance, we found that as age increased so did interlimb distance. No effect of experience on interlimb distance is consistent with the Belnal et al. study (2014), in that only changes in LER expression were found for duration and limb angles. However the age effect reported here is most likely due to developmental changes in pup size, since as pup age

increased, so did body weight and limb length. Weight and lengths of pups were measured in the present study to ensure that any group differences observed in LER expression were not attributable to what may be referred to as “pup size”. All pups were within a normal and healthy range of weight and body length. Furthermore, there was no main effect of maternal olfactory condition on pup weight or length. However, there was an interaction of maternal olfactory condition and age on weight and length where P10s in the ZnSO₄ group were smaller than P10s in the air and distilled water control groups. The effect found for maternal olfactory condition in this study on pup size are consistent with effects found in previous research using the maternal ZnSO₄ olfactory treatment (Jirik-Babb, Manaker, Tucker, & Hofer, 1984; Brouette-Lahlou et al., 1998). However, these results are in contrast with Moore and Power (1992) where pups born to ZnSO₄ dams weighed more in general than pups in control conditions. These differences in weight between studies could be attributable to the differences in observations of nursing and time spent off pups. Nonetheless, in regards to pup size possibly explaining differences in LER expression between the ZnSO₄ group and control groups instead of maternal olfactory condition in the current study: this is unlikely to be the case, as LER differences in duration and ankle angle were seen for all age groups, not just for P10s where the differences in size were observed.

Olfactory Control Condition

Previous research has shown that intranasal application of ZnSO₄ is not only effective in inducing maternal hyposomnia, but also reducing AGL in rats (Alberts & Galef, 1971; Mayer & Rosenblatt, 1977, 1992; Moore & Power, 1992; Slotnick, Glover & Bodyak, 2000; Lenz & Sengelaub, 2006). In each of the experiments using an

intranasal application of ZnSO_4 to induce hyponosmia, there have been variations on the methodology, one of which is the control condition that was used in the study. In some experiments researchers have used an intranasal application of saline as a control (Alberts & Galef, 1971; Jirik-Babb et al., 1984; Mayer & Rosenblatt, 1977, 1992; Brouette-Lahlou et al., 1998; Lenz & Sengelaub, 2006), while others have used an intranasal application of air (Mayer & Rosenblatt, 1992) or did not use an intranasal application using a hypodermic needle in their sham condition (Moore & Power, 1992). In the present study we chose to introduce a distilled water control group that to our knowledge has not been used before. We chose to introduce this control group to compare possible differences with an air control group in the same fashion as Mayer and Rosenblatt (1992). We chose to forego the use of a saline control since Mayer and Rosenblatt (1977) reported that an intranasal application of saline caused minor congestion in dams. Although an air control has been found to be an effective control in previous research, as well as in our own, we propose that distilled water may be an appropriate control condition to use for this paradigm as well. We propose this for two reasons: the first is that ZnSO_4 is a liquid, as is distilled water. By using a control and treatment that are both liquid, we can discount any differences seen in maternal behavior as being attributable to the treatment alone simply being a liquid. The second reason is that by having a treatment and control as a liquid the experimenter can more effectively stay blind to experimental conditions. Using air in a hypodermic needle is distinguishable from using a liquid ZnSO_4 treatment, therefore using air as a control may inadvertently cause experimenter biases. In the current study we observed no congestion in dams that had received an intranasal application of distilled water. Furthermore, no differences in maternal behavior

were observed between dams in the air control olfactory condition and dams in the distilled water olfactory condition. Therefore, we have concluded that intranasal application of distilled water is an effective control condition for comparison with an intranasal application of ZnSO_4 .

Limitations

As with any study, the current research has several limitations, including the design we chose to use. For the current study we employed a cross-sectional design instead of a longitudinal design, because we had planned to exclude pups from the study if they did not show an LER during testing. Our primary concern with using a longitudinal design was that if a single pup in the litter did not show the LER in one of the three test sessions, we would have had to replace the entire litter. As it turns out, all pups in the study showed the LER. Thus, after completion of this study we now know that it is probable that pups will show the LER for each of the selected ages (P1, P5, and P10), and future experiments on this topic could explore differences in LER development through longitudinal design. Using a longitudinal design would show continuous developmental change for the same pups between the ages of P1, P5, and P10 as opposed to showing differences between pups of the same litter at the different age groups.

Another possible limitation to this study is that pups were briefly separated from dams when dams were anesthetized to receive the intranasal applications of ZnSO_4 , air, or distilled water. While this separation was brief (10-15 min) it is possible that this separation could have caused stress on dams and/or pups and could have affected AGL and/or the LER. Pups also were not returned to the dam after testing occurred, as they were being used in another study. This changed litter composition after the testing ages of

P1 and P5. This could have resulted in possible changes in maternal behavior towards pups.

There also are possible limitations in our measurement of AGL. In previous research ZnSO₄ has been found to significantly reduce AGL compared to controls (Moore et al., 1992). While we did see a trend towards significance in this direction, we were unable to replicate standard statistical significance in this regard. One possibility for this difference is how and when we measured AGL. Moore et al. (1992) measured duration of AGL for four hours during the dark cycle before the lights came on while we chose to measure frequency of AGL at three separate time points (8:30 am; morning, lights off), once at mid-day (2:30 pm; afternoon, lights on) and once at the end of the light cycle (8:30 pm; evening, lights on). Had we measured duration of AGL or chosen different time points it is probable that we would have seen a more pronounced difference in AGL between maternal olfactory conditions. However, we were limited in our measurement of maternal behavior during observations since we did them live as opposed to recording them for playback. We chose to do observations live because it provides better mobility for researchers while doing observations of an ever-changing nest environment. While cameras may have the benefit of playback and time stamps, they are stationary and are limited in the field of vision of what they can capture. While doing live observations we used JWatcher, a live event recorder, to score maternal behavior. This program is limited in that recording the duration is conditional, in that only one duration of a particular behavior can be recorded at once. In our study nursing, blanketing, and off pup conditions (conditional events) were recorded in duration since these behaviors are mutually exclusive. Since AGL and these conditional events were not mutually exclusive

it was necessary to record AGL (as well as other non-mutually exclusive behaviors) as frequencies. Also, the measurement of AGL frequency is beneficial in its own right, since to our knowledge this has not been done before and it may be advisable for future research to examine differences in duration vs. frequency of AGL.

Implications

The current research has significant implications regarding the influence of maternal behavior on pup motor development. While the importance of maternal care is well established for the survival of the pup, it is less well known how motor functioning develops in the context of maternal-infant interactions. Maternal AGL is a species-specific behavior that has been shown to be important for neurological, social, and sexual development in rats (Moore, 1984, 1985, 1992; Moore & Power, 1992; Lenz & Sengelaub, 2006). The current study contributes to the growing body of literature on effects of maternal AGL by adding a motor behavior, the LER. The combination of these studies lends support to the necessity of an adequate caregiver and how variations in caregiving practices can either facilitate or impede development. However, previous research examining the effects of AGL on physiology and behavior tend to look at more long-term effects while this study examined the effect of AGL on a shorter time-scale for a behavior that is expressed perinatally. Thus, it does not take weeks upon weeks for maternal behavior to influence pup development. It happens over the course of even 1-10 days.

The LER is considered a reflex, and early on (up to the first 2 weeks postnatally) is likely mediated by a purely spinal pathway. However, this research shows that even a motor pattern that is reflexive is altered by experience. The LER is a reflex that pups

exhibit early in development, but is no longer displayed as they get older. This early reflex is similar to some reflexes that are observed in human infants. The Moro, Babinski, stepping, and rooting reflexes are all examples of motor patterns that are exhibited in response to environmental stimuli in human infants that disappear later in development. These reflexes are commonly used to evaluate atypical neurological development in infants. However, the current research suggests that even seemingly automatic reflexes such as the LER are altered by even the slightest alterations in the environment, including caregiving practices. This suggests that deficits seen in primitive reflexes may not simply be due to neurological problems, but also may be due to differences in motor practice or experience. For example, Colson, Meek, and Hawdon (2008) found that maternal posture while breastfeeding can affect performance of reflexes in infants such as rooting, sucking, and swallowing. Not much unlike the LER seen in neonatal rats, these are adaptive reflexes in human infants that rely on maternal behavior for efficient expression. If this is the case, then rehabilitating motor functioning may be facilitated by an understanding of how these reflexes normally develop (Brumley, Kauer, & Swann, 2015).

Conclusions

The objective of this study was to examine if maternal behavior affects the development of offspring motor behavior. The behavior that we were interested in was a species-specific motor behavior, the LER. We wanted to see if expression of the LER was influenced by alterations of a species-specific maternal behavior, AGL. The alteration in expression of LER duration and ankle angle seen here in response to maternal olfactory condition supports the role of maternal behavior in LER development. Development of any motor behavior is a complex, integrated process that involves many

biological and environmental contributions, and the LER is no exception to this. This study shows that even a reflex is not predetermined by biological processes alone, but requires environmental stimulation to develop in the typical manner. While there may be many more biological and environmental contributions to LER development that remain to be seen, we have shown here that maternal behavior plays an influential role in its expression. Previously it has been shown that LER expression can be altered by environmental experience (Belnap et al., 2014; Roberto & Brumley, 2014). However, this is the first time that a more naturalistic manipulation, maternal behavior, has been used to alter the expression of the LER. The alterations in motor expression seen here demonstrate the importance of early experience in the development of behavior, including experience that may come from interactions with a caregiver.

References

- Adolph, K., & Robinson, S. (2013). The road to walking: What learning to walk tells us about development. In P. Zelazo (Ed.) *Oxford Handbook of Developmental Psychology*. NY: Oxford University Press.
- Alberts, J., & Galef, B. (1971). Acute anosmia in the rat: a behavioral test of a peripherally-induced olfactory deficit. *Physiology & Behavior*, 6(5), 619-621.
- Altman, J., & Sudarshan, K. (1975). Postnatal development of locomotion in the laboratory rat. *Animal Behavior*, 23, 896-920.
- Belnap, S., Allmond, J., Boomhower, S., Roberto, M., & Brumley, M. (2014). Sensorimotor training during expression of the leg expression response (LER) in 1-day-old rats. *Developmental Psychobiology*, 56(7), 1553-1563.
- Bril, B., & Sabatier, C. (1986). The cultural context of motor development: Postural manipulations in the daily life of Bambara babies (Mali). *International Journal of Behavioral Development* 9, 439-453.
- Brouette-Lahlou, I., Godinot, F., & Vernet-Maury, E. (1998). The mother rat's vomeronasal organ is involved in detection of dodecyl propionate, the pup's preputial gland pheromone. *Physiology & Behavior*, 66, 427-436.
- Brouette-Lahlou, I., Amouroux, R., Chastrett, I., Cosnier, J., Stoffelsma, J., & Vernet-Maury, E., (1991). Dodecyl propionate, the attractant from rat pups preputial gland, characterization and identification. *Journal of Chemical Ecology*, 17, 1343 – 1354.
- Brouette-Lahlou, I., Vernet-Maury, E., & Vigouroux, M. (1992). Role of pups' ultrasonic calls in a particular maternal behavior in Wistar rat: Pups' anogenital licking.

Behavioural Brain Research, 50, 147-154.

Brumley, M., Kauer, S., & Swann, H. (2015). Developmental plasticity of coordinated action patterns in the perinatal rat. *Developmental Psychobiology*.

Caldji, C., Diorio, J., & Meaney, M. (2000). Variations in maternal care in infancy regulate the development of stress reactivity. *Biological Psychiatry*, 48, 1164 – 1174.

Caldji, C., Diorio, J., & Meaney, M. (2003). Variations in maternal care alter GABA_A receptor subunit expression in brain regions associated with fear. *Neuropsychopharmacology* 28, 1950-1959.

Champagne, F. A., Francis, D. D., Mar, A., & Meaney, M. J. (2003). Variations in maternal care in the rat as a mediating influence for the effects of environment on development. *Physiology & Behavior*, 79, 359-371.

Clark, M. M., Bone, S., & Galef Jr., B. G. (1988). Uterine positions and schedules of urination: Correlates of differential maternal anogenital stimulation. *Developmental Psychobiology*, 22, 389-400.

Colson, S., Meek, J., & Hawdon, J. (2008) Optimal positions for the release of primitive neonatal reflexes stimulating breastfeeding. *Early Human Development*, 84(7), 441-449.

Corodimas, K., Rosenblatt, J., Matthews, T., & Morrell, J. (1995). Neuroanatomical tract tracing provides histological verification of neuron loss following cytotoxic lesions. *Journal of Neuroscience Methods*, 56, 71-5.

Fleming, A., & Rosenblatt, J. (1974). Olfactory regulation of maternal behavior in rats: Effects of peripherally induced anosmia and lesions of the lateral olfactory tract in

- pup-induced virgins. *Journal of Comparative and Physiological Psychology*, 86, 233-246.
- Francis D., Diorio J., Liu D., & Meaney J. (1999). Nongenomic transmission across generations in maternal behavior and stress responses in the rat. *Science*, 286, 1155–1158.
- de Groat, W. (2002). Plasticity of bladder reflex pathways during postnatal development. *Physiology & Behavior*, 77, 689-692.
- Hopkins, B., & Westra, T. (1988). Maternal handling and motor development: An intracultural study. *Genetic, Social, and General Psychology Monographs*, 114, 379-408.
- Jirik-Babb, P., Manaker, S., Tucker, A. M., & Hofer, M. A. (1984). The role of the accessory and main olfactory systems in maternal behavior of the primiparous rat. *Behavioral and neural biology*, 40(2), 170-178
- Konner, M. (1976). Metaphors for development: How important are experiences early in life? *Developmental Psychobiology*, 21, 671-678.
- Lenz, K., & Sengelaub, D. (2006). Maternal licking influences dendritic development of motoneurons in a sexually dimorphic neuromuscular system. *Brain Research*, 1092, 87–99.
- Lovic, V., Gonzalez, A., & Fleming, A. (2001). Maternally separated rats show deficits in maternal care in adulthood. *Psychobiology*, 39, 19-33.
- Mayer, A., & Rosenblatt, J. (1992). Peripheral olfactory deafferentation of the primary olfactory system in rats using ZnSO₄ nasal spray with special reference to maternal behavior. *Physiology & Behavior*, 53: 587-592.

- Mayer, A., & Rosenblatt, J. (1977). Effects of intranasal zinc sulfate on open field and maternal behavior in female rats. *Physiology & Behavior*, 18, 101-109
- Mei, J. (1994). The Northern Chinese custom of rearing babies in sandbags: Implications for motor and intellectual development. In J. H. A. van Rossum & J. I. Laszlo (Eds.), *Motor development: Aspects of normal and delayed development*. Amsterdam: VU Uitgeverij.
- Moore, C. (1992). The role of maternal stimulation in the development of sexual behavior and its neural basis. *Ann N Y Academic Science*, 662, 160–177.
- Moore, C., & Power K (1992). Variation in maternal care and individual differences in play, exploration, and grooming of juvenile Norway rat offspring. *Developmental Psychobiology*, 25, 165–182.
- Moore, C., Chadwick-Dias, A. (1986). Behavioral responses of infant rats to maternal licking: Variations with age and sex. *Developmental Psychobiology*, 19, 427-438.
- Moore, C. (1985). Development of mammalian sexual behavior. In *The Comparative Development of Adaptive Skills: Evolutionary Implications*. E. S. Gollin, Ed.: 19-55. Erlbaum. Hillsdale, N. J.
- Moore, C. (1984). Maternal contributions to the development of masculine sexual behavior in laboratory rats. *Developmental Psychobiology*, 17, 347-356.
- Moore, C. (1982). Maternal behavior of rats is affected by hormonal condition of pups. *Journal of Comparative Physiological Psychology*, 96, 123–129.
- Moore, C. (1981). An olfactory basis for maternal discrimination of sex of offspring in rats (*Rattus norvegicus*). *Animal Behavior*, 22, 679-681.

- Moore, C., & Morelli G. (1979). Mother rats interact differently with male and female offspring. *Journal of Comparative Physiological Psychology*, 93, 677-84.
- Myers, M., Brunelli, S., Squire, J., Shindeldecker, R., & Hofer, M. (1989). Maternal behavior of SHR rats and its relationships to offspring blood pressure. *Developmental Psychobiology*, 22, 29-53.
- Rabain-Jamin, J., & Wornham, W. L. (1993). Practice and representations of child care and motor development among West Africans in Paris. *Early Development and Parenting*, 2, 107-119.
- Roberto, M., & Brumley, M. (2014). Prematurely delivered rats show improved motor coordination during sensory-evoked motor responses compared to age-matched controls. *Physiology & Behavior* 130, 75-84.
- Ryan, B., & Vandenbergh, J. (2002). Intrauterine position effects. *Neuroscience and Biobehavioral Reviews*, 26, 665-678.
- Salas, M., Torrero, C., Regaldo, M., & Loranca, A. (1998). The hindlimb hyperextension associated to the urogenital response in newborn underfed rats. *Archives of Medical Research* 29, No. 2, 125-132.
- Singh, R., Strain, M., Harris, S., Kao, T., & Brumley, M. (2012). Development of the leg extension response (LER) in spinally transected male and female rat pups. *Poster presented at the Annual meeting for the International Society for Developmental Psychobiology. New Orleans, LA.*
- Slotnick, B., Glover, P., & Bodyak, N. (2000). Does intranasal application of zinc sulfate produce anosmia in the rat? *Behavioral Neuroscience*, 114, 814-829.

- Smotherman, W., & Robinson, S. (1987). Fetal expression of the leg extension response to anogenital stimulation. *Physiology and Behavior*, 43, 243-244.
- Super, C. M. (1976). Environmental effects on motor development: The case of African infant precocity. *Developmental Medicine & Child Neurology*, 18, 561-567.
- Uriarte, N., Breigeiron, M., Benetti, F., Rosa, X., & Lucion, A. (2007). Effects of maternal care on the development, emotionality, and reproductive functions in male and female rats. *Developmental Psychobiology*, 49, 451-462.
- Wohr, M., & Schwarting, R. (2008). Maternal care, isolation-induced infant ultrasonic calling, and their relations to adult anxiety-related behavior in the rat. *Behavioral Neuroscience*, 122, 310-330.
- Xie, Q., & Young, M. E. (1999). *Integrated child development in rural China*. Washington, D. C.: The World Bank