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Opioid mediated behavioral effects and learning in the neonatal rat: comparison between amniotic fluid and milk

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OPIOID MEDIATED BEHAVIORAL EFFECTS AND LEARNING IN THE NEONATAL RAT: COMPARISON BETWEEN AMNIOTIC FLUID AND MILK

by

Valerie Mendez-Gallardo

An Abstract

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of the requirements for the
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Thesis Supervisors: Senior Research Professor Scott R. Robinson
Assistant Professor Julie Gros-Louis

ABSTRACT

The purpose of this study was to explore the behavioral effects of amniotic fluid (AF) and milk in the newborn rat. Previous research has documented behavioral effects in the fetal and neonatal rat. For example, oral exposure to AF and milk reduces the response to chemosensory stimulation in rat fetuses (Korthank & Robinson, 1998) and newborns (Méndez-Gallardo & Robinson, 2010). In addition, some of the behavioral effects of AF and milk are mediated by the endogenous opioid system in the perinatal rat, including modulation of the facial wiping response (Korthank & Robinson, 1998; Méndez-Gallardo & Robinson, 2010), the stretch response induced by milk in the fetal rat (Smotherman & Robinson, 1992b), and the effect of milk as an unconditioned stimulus (US) during associative learning in the fetal rat (Robinson et al., 1993).

Taking into account the literature that suggests similarities between AF and milk, this study aimed to evaluate whether transnatal continuity in the behavioral effects of AF and milk could be found and whether mediation by the endogenous opioid system is the underlying mechanism of these effects. To fulfill this purpose, overall behavioral activation, crawling locomotion, oral responses to an artificial nipple, and associative learning were investigated in the newborn rat. Results showed that, (a) oral exposure to AF resulted in higher levels of behavioral activation than oral exposure to milk, (b) exposure to the odor of AF or milk did not produce significant behavioral activation, although the odor of milk seemed to evoke higher levels of behavioral activity than exposure to the odor of AF, (c) both AF and milk odor elicited crawling locomotion, (d) odor of AF or milk did not promote oral grasping of an artificial nipple, but promoted

mouthing responses and distinctive movements of the forepaws, (e) contingent presentations of an artificial nipple as the conditioned stimulus (CS), with AF or milk as the US, promoted mouthing responses during reexposure to the CS, but facial wiping after CS reexposure was not modified as a result of conditioning, and (f) mediation of the opioid system was evident only during hindlimb activity after oral exposure to AF or milk and during mouthing responses to the CS after associative learning. These findings suggest that oral exposure to AF or milk consistently evoke opioid responses in the neonatal rat, but exposure to the odor of AF or milk alone does not.

Through postnatal testing and the direct comparison of the behavioral effects of AF (a feature of the prenatal environment) with milk (a feature of the postnatal environment), this study contributes to a better understanding of mechanisms that promote behavioral continuity before and after birth.

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PH.D. THESIS

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To my dear family and to all my friends for supporting me and walking along with me during this arduous journey. Especially, to that girl that found courage, determination, and confidence to overcome all of the challenges that were encountered. Para ustedes, para ti y para mi, gracias

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INTRODUCTION

When studying prenatal development from a physiological and behavioral perspective, amniotic fluid (AF) represents a very interesting feature for several reasons. First, AF is a very important factor for the physiological development of the fetus that has nutritive and protective value (Ross & Brace, 2001; Underwood, Gilbert, & Sherman, 2005). In addition, research suggests that AF also plays an important role in the behavioral development of the fetus, as demonstrated by research in rat fetuses that describes the role AF may play in modulating fetal responses to chemosensory stimulation (Korthank & Robinson, 1998) or research with humans that suggests that AF prepares the fetus for the transition to birth and postnatal life (Porter, Winberg, & Varendi, 2005; Schaal, 2005). Finally and more interestingly, although AF is a feature present in the prenatal environment and during parturition, research suggests that AF also plays an important role in postnatal development and continues to exert effects on the behavior of the newborn. For instance, in newborn rats, as well as in fetal rats, an oral infusion of AF can result in a diminished facial wiping response to aversive stimulation (Korthank & Robinson, 1998). In addition, lambs, rats, and human neonates show head turning toward the odor of AF in preference over a control stimulus of water, which demonstrates the capacity to identify the odor (Hepper, 1987; Schaal, Marlier, & Soussignan, 1995; Schaal, Orgeur, & Arnould, 1995).

In a similar way as AF, milk represents an interesting chemosensory stimulus that also seems to exert behavioral effects in both fetuses and newborns. Milk is an important feature that has biological significance after birth. Not only does milk have nutritive

value, but it also exerts important behavioral effects on the newborn. In addition, research has shown that some of these effects can be observed in the fetus under experimental conditions, suggesting that the underlying mechanisms that mediate the behavioral effects of milk are present before birth. Some of these effects include reducing the facial wiping response after an intraoral infusion of milk (Korthank & Robinson, 1998; Méndez-Gallardo & Robinson, 2010), the capacity of milk to evoke a stretch response (Robinson & Smotherman, 1992c; Smotherman & Robinson, 1987), and the capacity of milk to evoke a conditioned response to an artificial nipple after serving as an unconditioned stimulus (Robinson, Arnold, Spear, & Smotherman, 1993; Smotherman & Robinson, 1994).

In addition, previous research has provided evidence that some of the behavioral effects of AF and milk are mediated by the endogenous opioid system in the perinatal rat. Some of the behavioral responses mediated by the endogenous opioid system include the effects of AF and milk on facial wiping in rat fetuses (Korthank & Robinson, 1998) and rat newborns (Méndez-Gallardo & Robinson, 2010), the stretch response induced by milk in the fetal rat (Smotherman & Robinson, 1992b), the effect of milk on antinociception in the infant rat (Blass & Fitzgerald, 1988), and the effect of milk during associative learning in the fetal rat (Arnold, Robinson, Spear, & Smotherman, 1993; Robinson et al., 1993).

Considering all the research that describes behavioral effects of AF and milk, there is growing evidence that suggests that AF and milk share chemical components that evoke similar, and perhaps identical, behavioral responses in both fetuses and newborns

of different species of mammals, including human newborns. In addition, AF and milk, as stimuli of ecological relevance for the perinate, may share similar neural mechanisms that contribute to the expression of similar behaviors as described in the literature. This is the main argument of this study. Although some research has described how AF and milk exert similar behavioral effects (Korthank & Robinson, 1998; Méndez-Gallardo & Robinson, 2010; Schaal, 2005), there is not much information comparing how these two biological fluids may produce similar behavioral responses in newborns. Building upon earlier findings, this study comprises a set of experiments to systematically explore the behavioral effects evoked by AF and milk in the newborn rat and whether activity in the endogenous opioid system constitutes an underlying mechanism of these behaviors.

The purpose of this study was to explore the behavioral relationship of AF and milk. To attain this aim, a total of 12 experiments were conducted to explore four main areas: (1) how AF and milk may evoke behavioral activation in the newborn rat (Experiments 1 and 3), (2) how AF and milk may affect behavior in tasks that reflect the attractive properties of both fluids (Experiments 5 and 7), (3) if AF and milk can act as an unconditioned stimulus to support classical conditioning in the newborn rat (Experiments 9 and 11), and (4) if the endogenous opioid system plays a role during the expression of behavioral responses to AF and milk (Experiments 2, 4, 6, 8, 10, and 12). Through postnatal testing and the direct comparison of the behavioral effects of AF (a feature of the prenatal environment) with milk (a feature of the postnatal environment), this study contributes to a better understanding of mechanisms that promote behavioral continuity before and after birth.

This study will start with Chapter 1 that includes a description of the reported research findings about the effects of AF and milk in fetuses and newborns of different species including rats, the species that were used in this study. Chapter 2 will follow with an exploration of the role of the endogenous opioid system in the expression of behavior in the perinate and will conclude with a summary of the purpose of this study, a description of the experiments conducted, and the hypotheses of this study. Chapter 3 includes a detailed description of the general methods employed throughout this study. Chapter 4 reports the first part of this study that explores behavioral activation of the newborn rat to exposure of AF and milk and the role of the endogenous opioid system during these behavioral responses. This part includes four experiments, with each one providing a description of the specific methods employed in each experiment, its results, and a short discussion of the results. Chapter 5 reports the second part of this study that explores the attractive properties of AF and milk within tasks of ecological relevance (i.e., responses to an artificial nipple and crawling locomotion) and the role of the opioid system during these responses. Chapter 5 includes four experiments, with each one providing a description of the specific methods employed in each experiment, its results, and a short discussion of the results. Chapter 6 reports the third and final part of this study that explores the role of AF and milk in mediating associative learning in the newborn rat, as well as the role of the opioid system during learning. This last part includes four experiments, with each one providing a description of the specific methods employed in each experiment, its results, and a short discussion of the results. Finally, Chapter 7 presents a general discussion of the results that were found in all of the

experiments of this study, as well as the relation of these findings to the existing literature and the future directions that these results imply could be beneficial in future research.

CHAPTER 1: BEHAVIORAL EFFECTS OF AMNIOTIC FLUID AND MILK

Perinatal effects of amniotic fluid

AF represents a feature of the prenatal environment of much relevance for the physiological and behavioral development of the fetus. On one side, AF circulates throughout the system of the fetus, providing a continuous sensory experience that supports its physical development. On the other side, research has demonstrated that regulation of AF and consequent sampling of its chemical components also plays an important part in the development of the fetus. Studies with different species of animals, especially sheep, have shown that the composition of AF changes dramatically throughout gestation. Fetuses regulate their own AF via swallowing, the main pathway of AF removal in the prenatal environment (Underwood et al., 2005). When swallowing is reduced as a consequence of gastrointestinal obstruction or because of some neurological abnormalities, it can result in lower absorption of some nutrients and growth factors by the fetus (Ross & Brace, 2001), thus providing evidence of the nutritive importance of AF. In addition, it has been suggested that fetal swallowing contributes to regulation of AF volume (Ross & Nijland, 1997). Swallowing is necessary to maintain normal levels of AF regulation.

Studies with human pregnancies provide some insight into the different components present in AF and of its regulation throughout the fetal system. In humans, during the first half of pregnancy, AF increases in volume, both in absolute terms and relative to fetal growth (Gilbert & Brace, 1993). Early in gestation, AF volume and

composition are mostly determined by water and solutes transferred from the placenta (Ross & Brace, 2001) and AF is isotonic with maternal or fetal plasma (Bonsnes, 1966; Campbell et al., 1992; Gillibrand, 1969; Lind, Parkin, & Cheyne, 1969). Later in gestation, some of the components in AF are carbohydrates, proteins and peptides, lipids, lactate, pyruvate, electrolytes, enzymes, and hormones (Underwood et al., 2005). During the second half of pregnancy, AF volume remains constant, and then is reduced sharply at the end of gestation (Gilbert & Brace, 1993). In contrast, during the second half of gestation, although water continues to be important during pregnancy, AF is mainly a product of fetal micturition and needs to be regulated through a balance between the different routes of inflow and outflow of AF (Beall, van den Wijngaard, van Gemert, & Ross, 2007). During late gestation, the two main sources of AF production are fetal urine and lung liquid secretion and the two primary routes of clearance and absorption of AF are through fetal swallowing and intramembranous absorption (Brace, 1997). The fetus regulates the flow of AF volume through the amniotic sac in relation to its own fluid needs (Ross & Brace, 2001). In this way, regulation of AF results in a constant process in which the fetus swallows AF and then urinates it to then be swallowed again. AF is then moved from the outside environment surrounding the fetus to its interior and back to the outside again in a continuous cycle.

Research about the composition of AF and about its regulation in the prenatal environment has continued to establish the importance of AF in basic developmental functions that if absent can lead to abnormalities in the fetus. As an example, AF is believed to be important in fetal nutrition because it has been found to contain a variety

of components of nutritive value (Underwood & Sherman, 2006). Among these are amino acids, proteins, vitamins, minerals, hormones, and growth factors, some of which also seem to be present in human milk (Underwood et al., 2005; Underwood & Sherman, 2006). From a diagnostic perspective, another important role of AF is that it can be used clinically to detect chromosomal abnormalities and fetal abnormalities through amniocentesis (Underwood et al., 2005). Abnormal changes in AF volume can result in oligohydramnios (i.e., deficiency of AF) or polyhydramnios (i.e., too much AF) and these deficits are related to higher occurrence of perinatal diseases and abnormal fetal development (Ross & Brace, 2001). Also, AF protects fetuses by providing a cushioning environment where the fetus can move. And as source of protection, AF contains a series of components that are part of the fetal immune system, including some enzymes and antimicrobial peptides (Underwood et al., 2005).

In summary, AF represents a very important feature of the prenatal environment that contributes to the well being of the fetus and its development. As discussed above, if AF composition or regulation its altered in some way, it can lead to developmental complications that can affect not only the prenatal environment but that can also continue to exert adverse effects after birth. Although research has seemed to concentrate on understanding the role of AF in the physiological development of the fetus (Ross & Brace, 2001), this is not the only function that has been attributed to AF. AF also has been suggested to play an important role in fetal behavior and, moreover, in some of the events happening outside the womb during postnatal life.

AF and nipple attachment

Going beyond prenatal effects of AF, an example of how AF can exert an effect on behavior during postnatal life includes studies that suggest that AF is important for the first nipple attachment. Teicher and Blass (1977) reported the effects of AF on suckling in rats. They reported that washing the nipples resulted in no nipple attachment by their offspring and that painting the nipples with AF reinstated nipple attachment. AF, then, seems to help guide neonatal rats to the first nipple attachment (Teicher & Blass, 1977). In a similar study, Varendi, Porter and Winberg (1996) suggested that AF could play a similar role in the human newborn. In their study, conducted only minutes after birth, more newborns selected a breast treated with AF than an untreated breast after they had been placed in prone position on their mother's chest. This study suggests that AF also could help human newborns during their first suckling experience, thus providing another example of how AF helps in the transition from birth to postnatal life.

AF attraction

Another example of the influence of AF on behavior is the prenatal development of positive hedonic reactions towards chemosensory stimuli experienced *in utero*. Research suggests that AF represents the vehicle of exposure to stimuli presented prenatally. In this sense, it has been reported that neonates express an attraction towards AF and seem to recognize AF hours to days after birth. Studies have described the ability of neonates of different species to detect the odor of AF, including rats, lambs, piglets, and human newborns. For example, newborn piglets spent more time in nasal contact with gauze pads soaked with birth fluids or sow's milk over a control stimulus of water

(Parfet & Gonyou, 1991). Similarly, lambs presented with cotton pads moistened with AF and water just 1-hour after birth oriented longer to AF (Schaal, Orgeur et al., 1995). In the case of human neonates, studies have shown that newborns prefer the odor of AF when presented against no odor. When tested 2 days after birth, newborn babies oriented their nose to the odor of AF for a longer period than to a control stimulus of distilled water (Schaal, Marlier et al., 1995).

In addition to showing attraction for AF over a control stimulus, newborns are capable of recognizing their own AF. This capacity has been demonstrated through comparison of their own AF with AF collected from a different, non-familiar pregnancy. Results from studies conducted with rats (Hepper, 1987), lambs (Schaal, Orgeur et al., 1995), and human newborns (Schaal, Marlier, & Soussignan, 1998) have shown that newborns preferentially oriented toward their own AF. Research has suggested that in rats, preference for familiar AF may be related to the development of kin recognition (Hepper, 1987; Robinson & Smotherman, 1991a). This capacity of discrimination between familiar and unfamiliar AF has been documented in many mammalian species (Hepper, 1991; Tang-Martinez, 2001). Research has shown that AF is so salient that human mothers and fathers can identify AF collected from their own newborn and discriminate between AF collected from a different pregnancy (Schaal & Marlier, 1998). All this research suggests that AF has specific and salient odor properties that mediate the development of preferences and attraction towards this stimulus.

AF role in flavor preferences

In addition to exploring the preference and recognition of their own AF, other research has revealed that exposing fetuses to flavor compounds during gestation induces postnatal behavioral preferences, positive behaviors, and attraction towards the novel compound. For example, in Smotherman (1982), pregnant female rats received an injection of a solution of apple juice directly into AF on day 20 of gestation. Their offspring were then tested 60 days after birth in a two-bottle preference-drinking test. Results showed that those pups that were prenatally exposed to apple juice had a greater intake of apple juice than a control stimulus of water. This study suggests that experiencing specific flavor compounds *in utero* — specifically through AF — could influence postnatal experiences with that flavor and potentially lead to a preference that could be found long after birth. In a similar way to Smotherman (1982), several reports have described that neonates of different species reliably show acceptance or attraction towards stimuli presented before birth. This capacity has been described extensively in the literature with different species of mammals, including rats (Arias & Chotro, 2005a; Chotro & Molina, 1990; Domínguez, López, & Molina, 1998; Hepper, 1988; Pedersen & Blass, 1982; Smotherman, 1982), rabbits (Bilkó, Altbäcker, & Hudson, 1994; Coureaud, Schaal, Hudson, Orgeur, & Coudert, 2002; Semke, Distel, & Hudson, 1995), dogs (Wells & Hepper, 2006), pigs (Langendijk, Bolhuis, & Laurenssen, 2007), lambs (Schaal, Orgeur et al., 1995; Simitzis, Deligeorgis, Bizelis, & Fegeros, 2008), and humans (Mennella, Jagnow, & Beauchamp, 2001; Schaal, Marlier, & Soussignan, 2000). In general, this behavior is described as a preference, although it encompasses a variety of

behaviors that reflect attraction towards the stimulus, acceptance, recognition, and positive hedonic responses after prenatal exposure.

Research has described this behavioral preference with different stimuli and by measuring preferential behavior in a variety of ways. The behaviors used to measure postnatal preference after prenatal exposure to a specific flavor compound include using a double-choice paradigm where newborns move toward a source with the preferential odor (Hepper, 1988; Semke et al., 1995), turn or move their head toward the preferred odor (Schaal et al, 1995; Schaal et al, 2000; Wells & Hepper, 2006), or eat or drink more food that contains the preferred stimulus (Arias & Chotro, 2005a; Chotro & Molina, 1990; Domínguez et al., 1998; Bilkó et al., 1994; Chotro & Molina, 1990; Langendijk et al., 2007; Simitzis et al., 2008; Smotherman, 1982). In a different way to assess preference, Pedersen and Blass (1982) tested rat newborns on the day of birth (P0; P1 = 1 day after birth) by exposing them to nipples coated with citral after exposure to this lemon-odor stimulus on day 20 of gestation. Results showed that pups preferentially attached to the nipple that was painted with citral. This last report suggests that experiencing specific odorants in AF during gestation could have an impact not only on the preference toward that stimulus, but also on tasks of important ecological relevance, such as suckling. It also demonstrates that while *in utero*, fetuses are incidentally exposed to stimuli within their amniotic environment, and such exposure has behavioral consequences, eventually leading to memories that last until after birth. Research suggests that the memory of the flavor experienced *in utero* is stronger and can be retained better when exposure happens at the end of gestation, leading to the development

of a preference for the stimulus (Mickley, Remmers-Roeber, Crouse, Walker, & Dengler, 2000).

AF and alcohol exposure

While a significant amount of research has been dedicated to study the effects of presenting different flavor compounds into the prenatal environment and specifically AF, other research has investigated how contaminating the prenatal environment with noxious agents can also have postnatal effects. In this sense, a very important line of research about the impact of chemosensory prenatal experience on postnatal behavior involves studies about prenatal alcohol exposure (see Chotro, Arias, & Laviola, 2007 for a review). However, the case of prenatal ethanol exposure represents a special case in the development of postnatal behavioral preferences, because fetuses are exposed not only to the chemosensory properties of ethanol but also to the pharmacological and toxic properties of the drug (Chotro et al., 2007). In general, these studies have shown that prenatal exposure to ethanol results in greater intake or preferential responses toward ethanol in infant rats (Arias & Chotro, 2005a, 2005b; Chotro & Molina, 1990; Domínguez et al., 1998).

As an example that demonstrates the postnatal effects of prenatal exposure to alcohol, Arias and Chotro (2005b) conducted a study in which pregnant rats received one daily intragastric administration of ethanol during days 17, 18, 19, and 20 of gestation. At P14, their offspring were observed for responses of general activity, wall climbing, passive drips, paw licking, and mouthing after intraoral infusion of alcohol to measure behavioral appetitive responses to ethanol and intake of the drug. Their study found that

those subjects that had been exposed prenatally to ethanol showed a higher intake of the drug and displayed more behavior that corresponds to appetitive responses and less aversive behaviors to ethanol. This and other studies about prenatal ethanol exposure suggest that exposure to this drug also promotes fetal learning about ethanol, as measured by an enhanced palatability of the taste (Arias & Chotro, 2005a, 2005b) and a postnatal increase in intake of the drug after prenatal exposure (Chotro & Arias, 2003; Domínguez et al., 1998). Although as discussed above, alcohol does represent a special case since experience with this compound prenatally can cause negative developmental outcomes, this research continues to suggest that experiencing specific chemosensory stimuli *in amnio* affects behavior after birth.

AF effects on antinociception

AF has been associated with having antinociceptive and soothing properties. One study reported that human newborns exposed to a cloth moistened with AF showed significantly less crying than controls or babies exposed to a cloth containing their mother's breast odor (Varendi, Christensson, Porter, & Winberg, 1998). While this study demonstrates that infants can discriminate between two naturally occurring odors, it also suggests that AF is a familiar stimulus to the neonate. The authors interpret these results as AF exerting calming effects on the newborn.

However, in an associated finding, Kristal and colleagues have reported that AF has the capacity of enhancing analgesia in adult rats (Kristal, Thompson, & Abbott, 1986). The study involved applying a morphine injection to adult rats and giving them AF for consumption (AF used was collected on day 21 of gestation). After ingestion of

AF, rats were tested in a tail-flick latency test where the rat has to move its tail away from a point source of heat. Results showed that those rats that ingested AF after the morphine injection showed an increased tail-flick latency. According to the authors, this behavioral response can be interpreted as showing greater analgesia. These results were not found if rats received exposure to AF only. In this study, AF seems to enhance the antinociceptive properties of morphine, rather than producing it by itself. Further research revealed that exposure to as little as 0.25 ml of AF is enough to enhance analgesia in the rat (Kristal, Abbott, & Thompson, 1988), which suggests that consuming even small amounts of AF during parturition may help the female tolerate pain and discomfort by enhancing endorphin-mediated analgesia (Kristal et al., 1986).

Although the capacity of AF to enhance analgesia in adult rats has not been described in fetuses or newborns, it is still relevant for understanding of the behavioral importance of AF. In this sense, it demonstrates another way in which AF continues to exert effects on behavior beyond the prenatal period. As the behavioral effects of AF have been described in fetuses, newborns, and with this example, in adults, they continue to support research that suggests that the biological importance of AF is much more than serving as a protective barrier for the fetus during gestation. In this case, research findings also show how AF is important during the birth transition from prenatal to postnatal life. Not only does AF help the pregnant dam to give birth by enhancing naturally occurring analgesia, but — as already described above — it also assists the newborn by providing an olfactory signature that helps direct it to the nipple to engage in suckling (Teicher & Blass, 1977).

AF effects on facial wiping

AF also can play a role during behavioral responses to chemosensory stimulation and affect the typical motor expression of behavior in response to a novel stimulus. An example described in the literature that shows the behavioral effects of AF is facial wiping. Facial wiping is a behavior that has been used to study the effects of chemosensory stimulation in rat fetuses (Robinson & Smotherman, 1992a) and rat newborns (Méndez-Gallardo & Robinson, 2010). This behavior involves moving the forepaws toward the head and wiping the face (Robinson & Smotherman, 1991b). Rat fetuses reliably express facial wiping in response to oral chemosensory stimulation, such as a lemon odor extract (Smotherman & Robinson, 1987).

However, although infusion of a lemon solution into the mouth of the rat fetus (Smotherman & Robinson, 1987) and rat newborn (Smotherman & Robinson, 1989) nearly always evokes facial wiping, prior exposure to AF can alter this response. A study by Korthank and Robinson (1998) demonstrated that infusing a small amount of AF first and then presenting fetuses with lemon reduces the wiping response dramatically and almost eliminates the number of wiping strokes to lemon. The same finding was reported in a study with rat newborns in which AF exposure reduced facial wiping to lemon in P1 rat pups (Méndez-Gallardo & Robinson, 2010). These findings suggest that AF exerts effects on facial wiping to chemosensory stimulation in the rat fetus and newborn.

Whereas AF affects behavior *in utero* and after birth, research has reported that milk, as a stimulus of the postnatal environment, also affects behavior of the neonate in many ways that are similar to the effects of AF.

Perinatal effects of milk

Like AF, milk is a stimulus of major biological significance not only in terms of nutritional importance, but also in terms of its consequences for behavior. In terms of its composition, research on different species, including humans and rats, has shown that the composition of the mother's milk changes throughout the lactation period (Emmett & Rogers, 1997; Keen, Lönnerdal, Clegg, & Hurley, 1981). In the case of human milk, its components provide the vulnerable newborn infant with antimicrobial protection including antibodies, fatty acids, growth factors, and hormones (Field, 2005), as well as components of nutritional value such as vitamins, minerals, and proteins (Macy, 1949). These components provide the neonate with factors that help with the development of the immune system (Hanson et al., 2003; Lönnerdal, 2003). However, although the composition of human milk changes throughout the lactation period and varies among women, research suggests that human mother's milk provides the newborn with components important for development — such as some fatty acids, proteins, and immunoglobulins — that are not present in formula milk (Emmett & Rogers, 1997), suggesting that breast milk cannot be fully substituted by formula milk.

However, milk not only provides the infant with nutrients and immune protection, but it also plays a significant role in a series of behavioral responses in the newborn in its natural environment, as well as in the fetus under experimental conditions. Some of the effects of milk include evoking behavior that displays attraction or preference (Allam, Marlier, & Schaal, 2006; Goursaud & Nowak, 1999; Marlier & Schaal, 2005), having properties of antinociception and comfort (Blass, 1997; Blass & Fitzgerald, 1988),

evoking a stretch response in rat fetuses and neonates (Hall & Rosenblatt, 1977; Smotherman & Robinson, 1987), modulating the facial wiping response in fetal and neonatal rats (Korthank & Robinson, 1998; Méndez-Gallardo & Robinson, 2010; Smotherman & Robinson, 1992a), having reinforcing properties (Arias, Spear, Molina, & Molina, 2007; Johanson & Hall, 1979; Domínguez, Bocco, Chotro, Spear, & Molina, 1993), and having the capacity to serve as an unconditioned stimulus to support classical conditioning in the perinate (Brake, 1981; Johanson & Hall, 1982; Johanson & Teicher, 1980).

Milk and suckling

The first example of how milk affects behavior is suckling. Although suckling may sound like a passive and straightforward activity, it actually constitutes a well organized example of coordinated behavior in which obtaining milk is the powerful motivation. In order for rat pups to be able to access their mother's nipples, they engage in motor behaviors such as crawling towards the mother, shifting to a supine posture, performing foreleg stepping to move to the nipple area (Eilam & Smotherman, 1998), and moving their heads from side to side (termed 'rooting' or 'scanning') and vigorously probe their noses into the fur of the mother to locate and grasp the nipple (Blass & Teicher, 1980). Once the nipple is located, they push with their hindlimbs (Blass & Teicher, 1980) and briefly show treading movements in which the forelegs rhythmically push against the mammary gland (Drewett, Statham, & Wakerley, 1974; Lau & Henning, 1985). Following orientation to the nipple, pups extend their tongue, start licking, grasp the nipple, and finally engage in rhythmic sucking and mouthing behavior that stimulates

milk letdown by the mother (Blass & Teicher, 1980). Contacting the nipple and engaging in suckling does not represent the end of the behavioral process, but on the contrary, suckling and ingesting milk leads to an array of other behavioral effects. For instance, research has shown that in newborn rats the first contact with the nipple, and consequently with milk, alters and influences subsequent contacts with the nipple and with suckling (Brake, Sullivan, Sager, & Hofer, 1982; Petrov, Nizhnikov, & Smotherman, 2000; Petrov, Varlinskaya, Bregman, & Smotherman, 1999; Smotherman, Petrov, & Varlinskaya, 1997).

Milk and the stretch response

After commencement of suckling, pups also start to express other behaviors. Once they have access to the nipple and begin sucking, exposure to milk evokes a series of behavioral responses in the pup. Among these, a well-recognized response described in newborn rats is the stretch response (Drewett et al., 1974; Hall & Rosenblatt, 1977). This is a stereotypical behavior that happens only in response to exposure to milk through suckling (Lau & Henning, 1985) or during experimental exposure to milk through an intraoral infusion (Robinson & Smotherman, 1992c). After exposure to milk, rats express stretching by lifting the body and extending the forelimbs and hindlimbs while the body becomes extended and rigid (Hall & Rosenblatt, 1977). Although this response is characteristic of the suckling process in newborn rats, it also has been observed in rat fetuses (Smotherman & Robinson, 1987). The stretch response is a distinctive behavior that can be easily observed, but there are other non-obvious examples in which milk continues to affect behavior and to demonstrate its strong influence on learning.

Milk attraction

As another example of its behavioral effects, milk — like AF — also induces behaviors that reflect attraction and preference. For example, 3- to 4-day-old human infants prefer to orient their heads towards a cotton pad moistened with the odor of human milk when presented in a paired-choice test with the odor of formula milk (Marlier & Schaal, 2005). Similarly, human newborns tested around 86 hours after birth moved their head and showed increased mouthing in response to the odor of breast milk when presented with the odor of chamomile (Allam et al., 2006). Research with lambs suggests that the first milk (i.e., colostrum) has rewarding properties that help newborns establish a preference towards their mother (Goursaud & Nowak, 1999). These findings demonstrate that infant animals, including humans, can recognize and orient toward the odor of milk.

Milk role in flavor preferences

Apart from a direct preference for milk, milk also can act as a medium for exposure to other flavor and aromatic compounds that leads to the development of other preferences in the newborn. Several examples in the literature have described that flavors ingested by the mother such as garlic, mint, and vanilla, can alter the sensory characteristics of breast milk (see Mennella, 1995 for a review) in a similar way as food compounds can be transferred to AF during gestation (Nolte, Provenza, Callan, & Panter, 1992). For example, in a study by Mennella and Beauchamp (1991a) mothers ingested capsules containing garlic, leading to a change in the odor of milk where the odor of garlic could be perceived after consumption. In addition, mothers that drank carrot juice

during the first 2 months of lactation resulted in a positive response by their infants (i.e., fewer negative facial expressions) when eating carrot-flavored cereal (Mennella et al., 2001). The same effect has been reported in rabbits whose mothers consumed juniper berries during lactation and showed a preference for that food (Bilkó et al., 1994). Similarly, rat pups tested at weaning preferred to ingest the diet their mother ingested while they were still nursing (Galef & Henderson, 1972). And in lambs, exposure to a specific flavor in milk (in this study onion or garlic flavor) resulted in preference for food containing that flavor (Nolte & Provenza, 1991). All of these studies suggest that exposure to different flavors through their mother's milk can modify the responses of infants towards the experienced flavor (Mennella & Beauchamp, 1993) and could play an important role during early learning of flavors and during the development of feeding preferences (Beauchamp & Mennella, 2009).

Milk and alcohol exposure

However, as in AF, infants also can be exposed to harmful stimuli through exposure to milk. In the same way as food flavors can alter the sensory qualities of milk, alcohol consumed during nursing also can be detected in milk and alter the behavior of the infant consuming milk contaminated by alcohol (Mennella & Beauchamp, 1991b). For example, in the case of human infants, research has shown that they consumed less breast milk after their mothers drank orange juice containing ethanol (Mennella, 2001). However, consuming less breast milk it is not due to infants rejecting the flavor of ethanol, since research has shown that outside of the breastfeeding situation, infants consume more alcohol-flavored milk (Mennella, 1997). On the other hand, research with

human infants suggests that consuming breast milk contaminated with alcohol can affect sleep patterns in the infant (Mennella & Gerrish, 1998). In comparison, prenatal exposure to alcohol in rats can reduce the reinforcing properties usually associated with milk (Domínguez et al., 1993) and can lead to aversive developmental outcomes. Dams that consumed higher concentrations of ethanol during lactation produced pups that gained body weight at a reduced rate (Murillo-Fuentes, Artillo, Carreras, & Murillo, 2001; Oyama, Couto, Couto, Dâmaso, & Oller do Nascimento, 2000). Research suggests that alcohol administration to the lactating rat affects and inhibits oxytocin and prolactin release, and as a consequence, reduces milk secretion that eventually affects pup growth (Subramanian, 1999; Subramanian & Abel, 1988). Exposure to ethanol in the lactating rat not only results in reduced daily production of milk, but ethanol also affects milk composition (Vilaró, Viñas, Remesar, & Herrera, 1987), which may be another reason for infants consuming less milk after maternal alcohol consumption (Mennella, 1997). Milk therefore does not only serve as a vehicle of exposure of food odorants, but also can be the route of transmission of toxic substances such as ethanol. This finding suggests that, similar to AF, exposure to stimuli through milk can also lead to significant consequences for the expression and development of behavior.

Milk effects on antinociception

Milk has been characterized as having properties that induce antinociception and comfort in newborn and infants rats. For example, 10-day-old rats infused with milk left their paws placed on a hotplate surface for longer duration in comparison to rats that were untreated (Blass & Fitzgerald, 1988). The same effect has been reported in cesarean-

delivered newborn rats (Blass, Jackson, & Smotherman, 1991). The calming effect of milk in rats is comparable to the effects of morphine in infant rats. Morphine administration in infant rats increases latencies of removal of paws from a heated surface (Kehoe & Blass, 1986). Similarly, human newborns exposed to milk and some of its components, including sucrose, fat solutions, and protein (but not lactose) showed reduced crying during and after blood collection (Blass, 1997). In another study, 3-day-old human newborns exposed to the odor of their mother's milk displayed less crying and less grimacing after blood collection in comparison to babies exposed to an unfamiliar odor or a control (Rattaz & Goubet, 2005). This effect seems to be specific to hindmilk, which is the milk secreted later in a suckling episode (Barr, Young, Alkawaf, & Wertheim, 1996). The described effects of milk on antinociception suggest that milk exposure in the newborn does not only has nutritional value but also affects how newborns perceive and interact with other stimuli.

Milk effects on facial wiping

AF is not the only stimulus that can affect the facial wiping response; milk also has been shown to affect this behavioral response. Infusing a small amount of milk into the mouth of the rat fetus before a facial wiping challenge results in a reduced response or elimination of wiping strokes (Korthank & Robinson, 1998; Robinson & Smotherman, 1994). The same effect has been reported in the newborn rat after milk exposure and a facial wiping test (Méndez-Gallardo & Robinson, 2010).

Milk effects on learning

Studies with rats have described that milk can be a strong reinforcing stimulus in support of instrumental conditioning, and that it also can serve as an unconditioned stimulus in support of classical conditioning. Johanson and Hall (1979) described the reinforcing properties of milk in an experiment with 1-day-old rats. In their experiment, newborn rats learned to probe into a paddle that led them to receive an infusion of milk, showing that milk can serve as a reinforcer for the newborn rat. To support this finding, these same results have been reported in rats tested up to 15 and 16 days after birth in an operant conditioning task consisting of pressing a paddle and receiving a reinforcing infusion of milk (Domínguez et al., 1993). Almost identically, another experiment with rat pups tested 5 days after birth showed that they learned to touch a sensor that led to delivery of an intraoral milk infusion (Arias et al., 2007). However, the results of the experiment by Domínguez and collaborators (1993) are especially interesting because they revealed that milk seems to have higher reinforcing properties than milk contaminated with ethanol. Taken together, these results suggest that milk is much more than a stimulus with nutritional value, as it constitutes a powerful behavioral reinforcer in the newborn rat.

Other studies supporting the idea that milk is important during early learning in the newborn and fetal rat have shown that pairing a novel odor with milk exposure can lead to an association and to a conditioned preference response towards the novel odor (Johanson & Teicher, 1980). This conditioned response also can be seen even when the novel odor paired with milk is normally aversive (Brake, 1981; Johanson & Hall, 1982).

These and other studies have shown that milk can mediate learning through classical conditioning in the perinate.

Classical conditioning as a learning mechanism

Classical conditioning is a well-known paradigm that has been demonstrated to be useful to describe learning mechanisms in the fetus, newborn, infant, and adult rats. As an example, rat fetuses have been tested on day 20 of gestation in a conditioning paradigm using lemon as the unconditioned stimulus (US) and sucrose as the conditioned stimulus (CS). Because oral infusion of lemon typically elicits a burst of motor activity and since sucrose does not evoke a similar behavioral response in fetuses, this experiment tested if pairing of these two stimuli would result in higher behavioral activation to sucrose (Smotherman & Robinson, 1991). Results showed that fetuses in the Paired group, where infusion of sucrose was followed immediately by infusion of lemon, showed higher levels of activity after subsequent reexposure to sucrose alone. Similar behavioral activation did not occur, however, if fetuses received unpaired presentations — separated by a 30 s delay — of sucrose and lemon, or were exposed to sucrose (the CS) or lemon (the US) alone.

While this example represents a study in which conditioning trials take place during a short experimental session at one prenatal age, there are other studies that have demonstrated associative learning after pairing two solutions during gestation and testing effects after birth. For example, pairing of an intragastric administration of cineole followed immediately by administration of alcohol in pregnant rats, results in increased grasping of an artificial nipple by their offspring when the nipple is scented with the odor

of cineole (Abate, Varlinskaya, Cheslock, Spear, & Molina, 2002). This effect was not observed in pups whose mothers received administration of cineole followed by administration of alcohol after a 6-hr delay. The authors interpret this finding as consistent with a process of classical conditioning, with cineole serving as the CS and alcohol as the US.

Another set of studies about classical conditioning in the fetal rat has combined presentations of two stimuli that are relevant for their development and that are encountered immediately at the beginning of their postnatal life and during suckling. These stimuli are presentation of an artificial nipple and exposure to milk. Both of these stimuli have been demonstrated to be effective in exerting behavioral effects in the fetal (Robinson, Hoeltzel, Cooke, Umphress, & Smotherman, 1992; Robinson & Smotherman, 1994; Smotherman, Arnold, & Robinson, 1993) and neonatal rat (Petrov, Nizhnikov, Varlinskaya, & Spear, 2006; Varlinskaya, Petrov, & Smotherman, 1996). Presentation of an artificial nipple to the area around the mouth of a rat fetus elicits an oral grasping response and other elements characteristic of suckling behavior (Browne, Robinson, & Smotherman, 1994; Robinson et al., 1992). Milk, on the other hand, has been used as a US since presentation of milk promotes changes in fetal responsiveness to stimulation. Facial wiping has been used as a behavioral measure to demonstrate that an infusion of a small amount of milk in fetuses results in reduced facial wiping after applying a tactile stimulus to the perioral area of the subject (Smotherman & Robinson, 1992a). In a classical conditioning paradigm, the artificial nipple (the CS) was paired with an infusion of milk (the US). Fetuses were later reexposed to the nipple CS and their responsiveness

assessed in the test of facial wiping to lemon. This protocol has been explored most thoroughly in experiments that demonstrate classical conditioning in the fetal rat.

In the initial series of experiments using the artificial nipple as the CS and milk as the US, Robinson and collaborators (1993) presented rat fetuses with an artificial nipple to the perioral area and an intraoral infusion of milk in paired and unpaired conditions. Conditioning consisted of three trials separated by 5-min intervals. Results showed that those subjects that received contingent presentations of the artificial nipple followed by milk showed a diminished facial wiping response when tested for their wiping response to perioral stimulation after reexposure to the nipple. Similarly, when sucrose (CS) was used in contingent presentations with milk (US) in rat fetuses, and then presented alone during reexposure, it resulted in reduced responsiveness to perioral stimulation (Arnold et al., 1993).

Subsequently, it was demonstrated that only a single pairing of the artificial nipple and milk is sufficient to promote classical conditioning in the rat fetus (Smotherman & Robinson, 1994). Learning occurs even when pairing of the CS and US is separated by a delay of 30 s (Varlinskaya, Petrov, Simonik, & Smotherman, 1997). Also, it has been demonstrated that milk is essential for the conditioning to occur. If another neutral stimulus is used as the US — such as isotonic saline — and paired with the artificial nipple, associative learning does not occur (Varlinskaya et al., 1997). This finding supports the conclusion that milk is a salient and important stimulus that can promote associative learning in the perinatal rat.

But the evidence of conditioning in perinatal rats is not limited to the prenatal period, because rat neonates also can learn to prefer and respond positively to a novel stimulus after classical conditioning. This preferential behavior can be learned when rat newborns are exposed to pairings using different novel odors before milk presentation, resulting in a learned response specific to the odor experienced during pairings (Johanson & Hall, 1982). In a similar way as fetuses, rat newborns can develop a preference not only for a novel stimulus but also to a normally aversive odor when the odor is paired and presented just before an infusion of milk. For example, 3-day-old rats that were exposed to the odor of cedar paired with intraoral infusions of milk learned to prefer the novel odor of cedar (Johanson & Teicher, 1980) and showed similar responses to those normally expressed towards milk (Johanson, Hall, & Polefrone, 1984). In a similar way, 11- to 14-day-old rats exposed to pairing of the odor of orange — considered to be mildly aversive for rats — and infusions of milk, learned to prefer the orange odor (Brake, 1981). Johanson and Hall (1982) showed that 3- and 6-day-old rats showed positive behavior (i.e., oriented toward and maintained contact) towards the aversive odor of cedar after pairing it with milk infusions. In another way to look at associative learning mediated by milk, newborn rats conditioned with lemon odor as the CS and milk as the US grasped an artificial nipple scented with lemon after acquiring a conditioned response towards lemon (Cheslock, Varlinskaya, Petrov, & Spear, 2000).

However, milk is not the only biologically relevant stimulus that promotes classical conditioning in the rat. Analogous to the studies using milk as a US, research has reported the use of AF as a US to study the effects of ethanol exposure in the rat. In a

study by Arias and Chotro (2007), rat pups were tested 12 and 13 days after birth in conditioning trials by exposing pups to the odor of ethanol (CS) paired or unpaired with an intraoral infusion of AF (US). Results showed that those pups in the Paired group showed enhanced palatability of ethanol and an increased intake of alcohol after reexposure to the ethanol CS, demonstrating that AF was effective as a US during classical conditioning.

Although more findings about the capability of AF to serve as a US are yet to be discovered, these results potentially suggest that AF can serve as a US in the perinatal rat. In addition, in the same way that newborns are exposed to pairings of milk with a multitude of stimuli during suckling, some studies suggest that regular exposure to AF during gestation may result in repeated association of AF with other flavor components that can lead to postnatal preferences of these novel flavors (Chotro & Molina, 1990; Mennella, Johnson, & Beauchamp, 1995; Schaal et al., 2000; Smotherman, 1982). Potentially, AF could already be a US promoting classical conditioning during normal prenatal development.

Taken together, all of these findings show that AF and milk promote associative learning in the fetus and the neonate. To support the role of both fluids in classical conditioning, further research suggests that the mechanism by which AF and milk exert their effects on behavior in the perinate is through activation of the endogenous opioid system.

CHAPTER 2: THE MEDIATING ROLE OF THE ENDOGENOUS OPIOID SYSTEM

The role of the endogenous opioid system has been widely discussed in the literature about perinatal development in rats. Research has identified kappa, mu, and delta receptors within the CNS of fetal and neonatal rats as part of their opioid system (Leslie & Loughlin, 1993). Mu and kappa receptors are functional and can affect behavior in the rat fetus at the end of gestation (Attali, Saya, & Vogel, 1990; DeVries, Hogeboom, Mulder, & Schoffelmeer, 1990; McDowell & Kitchen, 1987). However, delta receptors appear to develop and become functional later, after the first postnatal week (Leslie & Loughlin, 1993).

In addition to the functionality of different classes of receptors of the opioid system at the end of gestation in rats, there also is research that has described the specific effects of both mu and kappa opioid activity on behavior in the perinatal rat. Moreover, activity at mu and kappa receptors has been documented as mediating some of the effects of AF and milk. These effects include the role of kappa opioid activity during facial wiping responses after AF or milk exposure in rat fetuses and neonates (Korthank & Robinson, 1998; Méndez-Gallardo & Robinson, 2010), the role of mu and kappa activity during the stretch response in rat fetuses (Smotherman & Robinson, 1992b), and the role of mu and kappa opioid activity during classical conditioning with milk (Arnold et al., 1993; Smotherman, 2002a).

Opioid mediation of the stretch response

An example of how the endogenous opioid system affects perinatal behavior is its mediation of the stretch response. In the stretch response, receptors from both kappa and mu are involved. While blockade of mu or kappa receptors with selective antagonist drugs eliminates the expression of the stretch response, only kappa, and not mu, can also block the expression of hindlimb activity that occurs after an infusion of milk (Smotherman & Robinson, 1992b). Conversely, activation of the kappa opioid system with selective agonist drugs promotes hindlimb activity and can facilitate expression of the stretch response to non-milk fluids, such as saline (Andersen, Robinson & Smotherman, 1993). These findings suggest that the kappa opioid system functions as a facilitator of the behaviors that begin after an intraoral infusion of milk and conclude with the stretch response.

Opioid mediation of antinociception

As another example, in adult rats AF enhancement of analgesia produced by morphine injection is mediated by the opioid system. Blockade of the opioid system with the non-selective antagonist naltrexone before morphine injection and followed by ingestion of AF, reduced the opioid-mediated analgesia effect of morphine that normally is enhanced by AF (Kristal et al., 1986). Although this example is described in adult rats, rather than fetuses and newborns, it serves as another example suggesting the presence of opioid properties in AF.

The opioid system also plays a role in milk-induced antinociception. Blass and Fitzgerald (1988) reported that in 10-day-old rats pretreatment with the non-selective

antagonist naltrexone reverses the effect of milk on paw-lift latencies. Rats pretreated with naltrexone and infused with milk withdraw their paws faster from a hotplate surface. Milk-induced analgesia in cesarean delivered pups can also be reversed by the non-selective opioid antagonist naloxone, suggesting mediation of the opioid system in this effect of milk (Blass et al., 1991).

Opioid mediation of facial wiping

Another example of an opioid-mediated behavioral effect is facial wiping. Studies have shown that blocking the opioid system with a non-selective opioid antagonist such as naloxone or naltrexone immediately before infusing the subject with AF, and then testing them for facial wiping to lemon, reinstates higher levels of wiping strokes (Korthank & Robinson, 1998; Méndez-Gallardo & Robinson, 2010). This finding suggests that AF engages the opioid system during its effect on facial wiping. This effect also can be blocked when rats are pretreated with the selective kappa receptor antagonist nor-binaltorphimine di-HC1 (BNI), but not with CTOP (the somatostatin analog, [Cys^2, Tyr^3, Orn^5, Pen^7]-Amide), a selective mu antagonist (Korthank & Robinson, 1998; Méndez-Gallardo & Robinson, 2010). Since the AF effect on the facial wiping response can be reversed with BNI, the effect of AF appears to be specifically mediated by the kappa opioid system.

An important aspect of the opioid effect of AF on facial wiping is that although AF continuously surrounds fetuses, research has shown that AF induces opioid activity only after oral exposure, and that the time course of this effect seems to be relatively brief. In a study described by Robinson and Méndez-Gallardo (2010), rat fetuses tested

on day 20 of gestation (E20; E0 = conception) were infused first with AF and then received an infusion of lemon. Infusions of lemon were presented at different delays after receiving AF. Results showed that when lemon was infused before AF (0 s delay), fetuses showed higher levels of facial wiping. Lemon infused after a 15 s delay resulted in a significant reduction of facial wiping. By 60 s and 120 s after exposure to AF, lemon significantly suppressed facial wiping. Starting at a 180 s delay, fetuses started to show some recovery, in which infusion of lemon resulted in some facial wiping. And by infusing lemon 240 s after exposure to AF, facial wiping was not suppressed in fetuses. This study suggests that AF can exert an effect on facial wiping responses to lemon that lasts only a few minutes and with maximal effects around 1 to 2 minutes after exposure to AF.

However, this first study represents an experimental situation in which exposure to AF can be controlled and monitored. Under normal environmental circumstances, fetuses sample their own AF, since they are continuously surrounded by it. It therefore was important to explore if these findings would also occur while the fetus is immersed in its own environment. To explore this possibility, rat fetuses were tested within their own amniotic sac and while surrounded by their own AF. They were infused with lemon 60 and 300 seconds (s) after their last mouthing movement. Results showed that at 60 s fetuses showed reduced facial wiping, but showed significantly higher levels of wiping 300 s after the most recent mouthing movement (Robinson & Méndez-Gallardo, 2010). This finding suggests not only that AF opioid-activation is short-lasting and not continuous, but also that the way AF activates the opioid system is through the normal

process of oral activity expressed by the fetus. In other words, through the fetus's own mouthing activity and intermittent sampling, AF gains access to the opioid system.

In addition, although the fetus also swallows AF as part of this regulatory process (Brace, 1997), research has shown that AF does not need to be swallowed to exert an effect on behavior. Rat fetuses tested on E20 prepared with a ligature occluding the esophagus and trachea to prevent swallowing and breathing were tested in their facial wiping response to lemon after oral exposure to AF (Robinson & Méndez-Gallardo, 2010). AF exposure continued to reduce the number of wiping strokes to lemon even though the fetus could not swallow AF. These findings suggest that AF does not need to be ingested to activate the opioid system, and that the opioid effects resulting from oral exposure are short-lasting, on the order of 4-5 minutes. However, this finding contrasts with the specific opioid-mediated mechanism involved in the role of AF in analgesia in adult rats. While AF engages kappa receptors during the behavioral effects on facial wiping (Korthank & Robinson, 1998; Méndez-Gallardo & Robinson, 2010), AF effects on analgesia in adult rats engages kappa receptors and also delta receptors (DiPirro & Kristal, 2004). In addition, swallowing and ingestion of AF is not necessary to produce effects on facial wiping in fetuses, as oral exposure is sufficient (Robinson & Méndez-Gallardo, 2010). However, AF must be ingested by the dam in order to produce the opioid-mediated effects on adult analgesia (Kristal et al., 1988). Finally, the duration of behavioral effects following oral exposure to AF in the rat fetus is brief, on the order of 15-240 s. But in the adult rat, AF enhancement of morphine effects on analgesia are evident more than 30 min after exposure to AF. In other words, while AF exerts opioid-

mediated effects on facial wiping in the perinatal rat and also enhances analgesia in the adult rat, both behaviors seem to partly engage similar opioid systems (i.e., kappa) through different routes with very different time courses. It therefore is unlikely that AF effects are governed by the same mechanism in the fetus and adult.

On the other hand, the endogenous opioid system appears to mediate the effects of milk on facial wiping in a manner entirely consistent with the results of AF. In this case, blockade of the opioid system by a non-selective opioid antagonist, or by the selective kappa antagonist BNI and not the selective mu antagonist CTOP, reinstates the response after exposure to milk in the same way as with AF (Korthank & Robinson, 1998; Méndez-Gallardo & Robinson, 2010; Smotherman & Robinson, 1992a). The effects of milk on facial wiping responses also follow a time course remarkably similar to that of AF, with significant effects at 15 s, peak effects at 60 s, and complete recovery by 5 min (Robinson & Smotherman, 1994). These findings suggest that oral exposure to milk also triggers activity at kappa opioid receptors, which mediates the effects of milk exposure on facial wiping to lemon.

Opioid mediation during classical conditioning after reexposure to the CS

Although studies about the effects of AF and milk on facial wiping suggest the involvement of kappa receptors in modulating behavior in the rat fetus (Korthank & Robinson, 1998) and the newborn (Méndez-Gallardo & Robinson, 2010), studies about classical conditioning suggest a more complicated function of the opioid system during associative learning. As discussed above, classical conditioning in the perinatal rat can be

supported when a novel CS is paired with milk as the US. The results of experiments with conditioning in fetal rats further suggest that reexposure to the CS results in activation of the opioid system. Specifically, it was found that the facial wiping response could be reinstated by treating fetuses with the nonselective opioid antagonist naloxone after training and before reexposure to the CS, thereby blocking a conditioned opioid response. Further, the conditioned opioid response could be blocked, reinstating high levels of responsiveness, after treatment with selective opioid antagonists for mu receptors, CTOP and β – funaltrexamine HC1 (FNA), but not after blockade of kappa receptors with BNI (Arnold et al., 1993; Robinson et al., 1993). Taken together, these results suggest that after learning has been acquired during conditioning trials in which both the CS and US are presented in a contingent manner, reexposure to the CS evokes opioid activity specifically at the mu class of opioid receptors, in contrast to the kappa opioid response evoked by the US, which then reduces the behavioral response to perioral cutaneous stimulation.

Opioid mediation during conditioning learning with ethanol

The involvement of the opioid system also has been described in other classical conditioning studies during prenatal development. Studies about ethanol exposure have suggested that the acquisition of a conditioned preference during prenatal exposure to alcohol is mediated by activation of the opioid system (Chotro et al., 2007). Pups tested on postnatal day 14, after ethanol was administered to the mother during pregnancy together with the non-selective opioid antagonist naloxone, did not show an increased intake of ethanol after birth (Chotro & Arias, 2003). In addition, in a similar study where

pregnant rats were administered naloxone with ethanol, pups tested on day 14 did not show an increased intake of ethanol or higher amount of ingestive or appetitive responses to the taste of the drug (Arias & Chotro, 2005a). These results suggest that the prenatal acquisition of a preference for ethanol seems to be mediated by activity in the endogenous opioid system, although the specific class of opioid receptors that are involved in the alcohol preference has not been identified.

In summary, not only do studies such as these provide evidence of a functional opioid system in both mu and kappa receptors, but they also demonstrate that the rat fetus and newborn can learn through simple classical conditioning. Although mu and kappa receptors seem to be involved in the learning process in different ways, they both seem to be important, either for the rat perinate to learn associations between the CS and the US, or for the expression of a conditioned response. Also, although there are not many studies that have shown classical conditioning using AF as a US, the reported similarities between AF and milk suggest that the rat fetus and newborn may learn associations between paired presentations of AF (serving as a US) and stimuli such as presentation of an artificial nipple (as the CS).

Summary: Milk versus amniotic fluid effects on behavior

Given the foregoing review of the literature, it is clear that AF and milk are important elements of the perinatal environment that influence behavioral development before and after birth. Not only do AF and milk contribute to the development of specific behaviors in their respective developmental periods, but they also challenge the conception — at least behaviorally — of each of these stimuli being specific to a function

during growth and maturation of the fetus and the neonate. In other words, while one is a feature of the prenatal environment and the other a feature of the postnatal environment, they exert similar effects on neural and behavioral systems. Studies about the behavioral effects of AF and milk have demonstrated that these two stimuli share similar properties. Not only do similarities in the effects of AF and milk suggest a continuum between prenatal and postnatal life at a behavioral level, but they also suggest that both share similar neural mechanisms.

To demonstrate their similarities, some studies suggest that although milk is not present before birth, AF may be what prepares fetuses for their later postnatal experience with milk (Schaal, 2005). This possibility has been explored in a study with newborn human babies. In this study, conducted with breast-fed infants tested in a two-choice head turning paradigm, 2-day-old infants did not show a preference between AF and their mother's colostrum. In contrast, 4-day-olds showed a distinct preference by orienting more toward colostrum (Marlier, Schaal & Soussignan, 1998b). However, in a different study where bottle-fed infants (i.e., infants fed formula milk) were tested in a similar paradigm, 2-day-old and 4-day-old infants both preferred AF over formula milk (Marlier, Schaal & Soussignan, 1998a). These findings imply that infants shift from a preference for AF to a preference for maternal milk after having more suckling experience, and that experience with infant formula is inadequate to promote the shift away from AF. Another inference that may be drawn from these results is that both AF and colostrum share chemical characteristics that may result in olfactory similarity (they are not discriminated by 2-day-olds), but AF and infant formula do not (Schaal, 2005).

As discussed above, another example in which a behavioral similarity between AF and milk has been systematically explored is in the studies of the facial wiping response after treatment with milk or AF (Korthank & Robinson, 1998; Méndez-Gallardo & Robinson, 2010). Exposure to a small amount of AF or milk results in reduced number of wiping strokes after a lemon infusion, both of which involve activity at kappa receptors of the opioid system.

However, although other studies have not explicitly explored how AF and milk exert similar behavioral responses, independent studies have identified behavioral effects of milk that are also reported in different studies with AF. Table 1 provides a summary of the studies that have describe similar behavioral effects of AF and milk.

Moreover, milk and AF seem to share qualitative properties that have effects on the same neural substrates affecting facial wiping, suggesting a developmental continuum between both fluids, at least on this behavioral response. The effects of AF and milk on facial wiping are mediated by kappa receptors within the endogenous opioid system (Korthank & Robinson, 1998; Méndez-Gallardo & Robinson, 2010).

Finally, milk can promote classical conditioning of opioid responses in the fetal rat (Robinson et al., 1993; Robinson & Smotherman, 1997; Smotherman & Robinson, 1994), suggesting that associative learning may take place *in utero* through AF exposure in conjunction with other substances, and *ex utero* through implicit pairing of milk with other stimuli present during suckling. This evidence not only suggests that fetuses and neonates have the capacity of learning through classical conditioning, but also that AF and milk can play important roles in that process.

Table 1

Comparison of behavioral effects of AF and milk described in the literature

Behavioral effect	Described with AF or milk?	Species	Age assessed	Mediation of opioid system investigated?	Citation
Nipple attachment	AF	Rats	Hours after birth	No	Teicher & Blass, 1977
	AF	Humans	Hours after birth	No	Varendi et al., 1996
Stretch response	Milk	Rats	Day 20 of gestation	Yes	Andersen et al., 1993; Smotherman & Robinson, 1992b
Attraction to the odor	AF & milk	Pigs	Hours after birth	No	Parfet & Gonyou, 1991
	AF	Lambs	Hours after birth	No	Schaal, Orgeur et al., 1995
	AF	Humans	2 days after birth	No	Schaal, Marlier et al., 1995
	Milk	Humans	3 to 4 days after birth	No	Marlier & Schaal, 2005
	Milk	Humans	3 to 4 days after birth	No	Allam et al., 2006
Antinociception	AF	Humans	Minutes after birth	No	Varendi, et al., 1998
	AF	Rats	Adulthood	Yes	Kristal et al., 1986
	Milk	Humans	3 days after birth	No	Rattaz & Goubet, 2005
	Milk	Rats	10 days after birth	Yes	Blass & Fitzgerald, 1988

Table 1 continued

Behavioral effect	Described with AF or milk?	Species	Age assessed	Mediation of opioid system investigated?	Citation
Development of flavor preferences	AF	Rats	Exposure at day 20 of gestation/ testing at day 60 after birth	No	Smotherman, 1982
	Milk	Humans	Exposure at end of gestation/ testing 5 months after birth	No	Mennella et al., 2001
Facial wiping	AF & Milk	Rats	Day 20 of gestation	Yes	Korthank & Robinson, 1998
	AF & Milk	Rats	1 day after birth	Yes	Méndez-Gallardo & Robinson, 2010
Supports associative learning	AF	Rats	12 -13 days after birth	No	Arias & Chotro, 2007
	Milk	Rats	Day 20 of gestation	Yes	Arnold et al., 1993; Robinson et al., 1993

However, apart from the experimental findings described above, there is not much information about how AF and milk can be compared with specific behavioral assays and how the endogenous opioid system may be involved in each of these behaviors. In many of the cited studies, experiments focussed on AF or milk, but rarely both. Opioid involvement has been investigated much more completely in milk effects on perinatal behavior than AF, and the potential role of AF-induced opioid activity in prenatal learning has not been directly evaluated. Transnatal continuity in the effects of AF and milk is the central hypothesis of this study, and filling in many of the missing elements necessary to empirically document such continuity is the goal of this study.

While other studies have described components that are present in AF and also in milk (Underwood et al., 2005; Underwood & Sherman, 2006; Wagner, 2002), the current study does not intend to describe the chemical properties or the biological elements that AF and milk share. Rather, this study serves as a behavioral portrayal of how AF and milk can affect behavior in the newborn rat in three main areas of investigation: (a) behavioral activation after chemosensory stimulation with AF and milk, (b) the attractive properties of AF and milk as measured in two tasks of ecological relevance, and (c) how AF and milk can contribute to associative learning in the newborn rat. Finally, this study explores if the effects of AF and milk in each of these domains are mediated by the endogenous opioid system.

Purpose and description of experiments

The main purpose of this study is to compare the behavioral effects of AF and milk in the neonatal rat and to determine the role of the endogenous opioid system on

these behaviors. To accomplish this purpose, twelve experiments were conducted within three parts of the investigation.

The first part of the study (comprising Experiments 1 to 4) compares the behavioral responses of the neonatal rat to exposure to AF, milk, a novel stimulus (i.e., anise), and a control stimulus (i.e., distilled water). To evaluate behavioral activation, P1 pups (pups tested 24 hours after birth) were exposed to an intraoral infusion of one of these fluids (Experiment 1), or exposed to air suffused with their odor (Experiment 3). In Experiments 2 and 4, behavioral responses were evaluated after opioid blockade through an injection of the non-selective opioid antagonist naloxone.

The second part of the study (comprising Experiments 5 to 8) investigates the attractive properties of AF and milk using two different dependent variables: crawling (Experiment 5) and responses to an artificial nipple (Experiment 7). Two consecutive experiments evaluate if the endogenous opioid system plays a role in AF- or milk-evoked crawling (Experiment 6) and in responses to an artificial nipple (Experiment 8) during exposure to AF or milk odor.

Finally, the third and last part of the study (comprising Experiments 9 to 12) investigates if AF (Experiment 9) or milk (Experiment 11) can be used as a US to support classical conditioning in the neonatal rat. As in parts 1 and 2, the role of the endogenous opioid system is evaluated by pharmacological blockade of opioid receptors after training but before reexposure to the CS, using AF or milk as the US (Experiments 10 and 12).

Hypotheses

The available literature, and previous experiments in our laboratory, suggest two general hypotheses concerning the relationship between AF and milk. The first hypothesis is that both AF and milk exert effects on multiple aspects of behavior in the neonatal rat, including measures of general behavioral activity, specific responsiveness in ecologically relevant contexts (responses to the nipple and crawling locomotion), and associative learning. The second hypothesis is that the behavioral effects resulting from exposure to AF and milk are dependent on activity in the endogenous opioid system. In each of the proposed experiments, specific predictions about experimental results are derived directly from these two hypotheses.

However, although the findings described in the literature support the hypothesis that AF and milk indeed engage similar neural mechanisms and exert similar, or even the same behavioral responses, there is the possibility that, in fact, AF and milk do not affect behavior in the same way. Despite all of the evidence described above, these studies have been conducted with different species of mammals, at different moments in development, and by assessing behavior in different ways. For example, it is possible that while oral exposure of AF and milk engage the opioid system in behavioral responses such as facial wiping (Korthank & Robinson, 1998; Méndez-Gallardo & Robinson, 2010), this may not be the case when AF and milk are presented in odor form. The literature suggests that AF prepares the fetus for the postnatal experience with milk (Schaal, 2005) and not that they constitute the same stimulus or that they are exchangeable. While these two stimuli may share similar chemical components, they also contain many different constituents, and the

way the fetus or the newborn responds to AF or milk also depends on the development of the subject, the surrounding environment, and the experience of the animal with these or other stimuli. In this regard, and with particular relevance to the current study, the response of newborn rats to AF or milk could be different than to the reported findings on rat fetuses. This alternative possibility suggests that some modes of exposure to AF or milk (e.g., oral) may activate the opioid system, whereas other modes (e.g., odor) may not, that fetuses may show robust opioid response to AF and milk, but newborns may not, and that some behavioral responses may be affected by AF- or milk-induced opioid activity, but others may not. While the main hypothesis of this study, as mentioned above, is that AF and milk affect behavior in the fetus and newborn rat in similar ways, this study provides the opportunity to explore whether alternative patterns of effects can be found.

Regardless of the specific results of the experiments of this study, the findings are informative and contribute to a better understanding about the relationship of AF and milk in perinatal development. Although the experiments were conducted postnatally, the comparison of the behavioral effects of AF and milk as well as the exploration of the opioid system as a neural mechanism responsible for these effects contributes to better understanding of the transition of life before and after birth. In addition, this study adds information to the ongoing debate about how AF and milk share properties that not only help fetuses to prepare for life outside the womb, but also how newborns adapt better to postnatal challenges through the influence of AF and milk.

CHAPTER 3: GENERAL METHODS

Subjects

Neonatal subjects were the offspring of Sprague-Dawley laboratory rats (*Rattus norvegicus*; Simonsen Labs, Santa Clara, CA). Approximately 416 neonates from 129 pregnancies were used in this study and bred in our laboratory. Additional rats were used to provide AF samples that were used during behavioral testing. Adult rats were housed in standard breeding cages (38 x 48 x 20 cm) with female rats (three per cage) and an adult male. Cages were stored in the colony room with room temperature maintained at about 23°C with a 12:12 hours light/dark cycle. Food and water were available ad libitum. Anticipated dates of birth and gestational ages for pregnancies that yielded AF samples were determined through daily collection of vaginal smears during the 4-day breeding period. The day that sperm were detected was designated as the day of conception (E0 of gestation). Maintenance of rats was in accordance with the National Institutes of Health (NIH) guidelines for animal care and use (Institute for Laboratory Animal Resources, 1996) and of the Institutional Animal Care and Use Committee (IACUC) and the Association for Assessment and Accreditation of Laboratory Animal Care (AALAC) at Idaho State University, where the proposed experiments were conducted.

Stimulus fluids

AF was used in most of the experiments (i.e., all of experiments except Experiments 11 and 12) and was presented postnatally at the time of testing. Also, AF was used as the Unconditioned Stimulus (US) in the last section of the study where

associative learning was assessed experimentally. Previous studies have documented the collection and experimental presentation of AF to rat fetuses (Korthank & Robinson, 1998) and neonates (Méndez-Gallardo & Robinson, 2010). In addition, other studies have used AF as a US to study the effects of ethanol exposure in rats (Arias & Chotro, 2007).

Milk also was used in most of the experiments (i.e., all of the experiments except Experiments 9 and 10). The milk used was a bovine light cream (commercial Half & Half). This milk is similar in composition to rat milk (i.e., they have similar fat and water content) and previously has been used as the milk stimulus in both rat fetuses (Korthank & Robinson, 1998; Robinson & Smotherman, 1994) and rat neonates (Hall & Rosenblatt, 1977; Méndez-Gallardo & Robinson, 2010). Care was taken to use milk that was fresh and before the expiration date. Milk stayed refrigerated until the time of testing, when it was taken out to warm to room temperature. Milk samples were presented to rat pups at incubator temperature (35°C ± 0.3°).

To compare the effects of AF and milk with a novel stimulus, anise (anethole) was used as another olfactory stimulus to explore responsiveness during testing with pups. Anise odor in another form (aniseed spice) has been used to test prenatal olfactory learning in dogs (Hepper & Wells, 2006; Wells & Hepper, 2006). Similarly, food with anise flavor (i.e., candy, cookies, and syrup) has been used to study prenatal exposure of anise in the human fetus and postnatal responsiveness in the human newborn after reexposure to pure anise flavor (Schaal et al., 2000). In pigs, anise was included in the diet of pregnant sows to study prenatal flavor exposure in piglets (Oostindjer, Bolhuis, van den Brand, & Kemp, 2009; Oostindjer, Bolhuis, van den Brand, Roura, & Kemp,

2010). Although studies were not found that have used anise to study postnatal preferences in rats, the studies discussed above suggest that this is a salient stimulus that could be used in the current study and that can be detected by both fetuses and neonates in rats. In addition, the laboratory of M. Gabriela Chotro reported the use of anise in rats and provided information about how to prepare the anise emulsion (personal communication, April 9, 2010). To prepare a 10 % anethole emulsion, 10 ml of trans-anethole oil were mixed with 0.15g of gum tragacanth, 1.5g of gum arabic from acacia tree, and 1.0 ml of ethanol (ethyl alcohol denatured 95%). A similar process was used to prepare a cineole emulsion (Abate, Pepino, Domínguez, Spear, & Molina, 2000). Once the anethole emulsion was prepared, 10 ml of this emulsion was mixed with 90 ml of distilled water (0.8 % anethole), which was administered as a test stimulus to the neonatal rat subjects.

In addition, a 1:1 dilution of commercial pure lemon odor extract (McCormick brand) in isotonic saline was used as a test stimulus to elicit facial wiping in Experiments 9 to 12. In these experiments, pups received 20 µl volume of lemon solution infused in a 2-s pulse directly into the mouth. Previous research has reported that rat fetuses that are exposed to an intraoral infusion of a lemon-odor infusion show an increase in general activity, including the expression of facial wiping responses (Smotherman & Robinson, 1987, 1988). The effectiveness of a 1:1 dilution of lemon extract in eliciting a wiping response has been previously described in E20 rat fetuses (Brumley & Robinson, 2004). Pilot data collection confirmed the effectiveness of the 1:1 dilution in P1 pups (mean of wiping responses with 1:1 = 12.5 strokes).

In four of the experiments (Experiments 1, 3, 5, and 7), distilled water was used as a control stimulus.

Amniotic fluid collection

AF samples were collected from donor fetuses at gestational age E20. Previous work has reported the effectiveness of AF collected at this gestational age to produce behavioral effects in rat fetuses (Korthank & Robinson, 1998; Robinson & Méndez-Gallardo, 2010) and neonates (Méndez-Gallardo & Robinson, 2010). Donor fetuses were obtained from different pregnancies not used to provide subjects for behavioral testing. The procedure of AF collection involved (a) rapid euthanasia of the pregnant rat by cervical dislocation, (b) externalization of the pregnant female's uterus, (c) careful removal of fetuses and placenta, avoiding rupture of the amnion and chorion, and (d) extraction of AF into a test tube. After collection, AF was immediately stored at -20º C until the day of testing. AF samples were thawed and presented to rat pups at incubator temperature (35°C ± 0.3°).

Administration of opioid antagonist

In six of the experiments (Exp. 2, 4, 6, 8, 10, 12), an opioid receptor antagonist or a vehicle control was administered to neonates to manipulate their opioid system. The non-selective antagonist naloxone hydrochloride (Sigma-Aldrich, St. Louis, MO) was administered directly to neonatal subjects. Before administration, the drug was prepared in an isotonic saline vehicle, kept in refrigeration until testing, and administered to subjects at body temperature (37.5°C). Naloxone or the vehicle control was administered in a fixed volume of 50 µl to neonatal subjects by intraperitoneal (IP) injection with a 30-

ga hypodermic needle and a 1 ml syringe. Naloxone was administered in a single dosage (1.0 mg/kg) based on the mean body weight of P1 pups (6.5 g). This dosage previously has been reported to be effective in blocking the opioid-mediated behavioral effects of milk or AF presented to rat fetuses (Korthank & Robinson, 1998; Smotherman & Robinson, 1992a; Smotherman, Simonik, Andersen, & Robinson, 1993) and neonates (Méndez-Gallardo & Robinson, 2010). A vehicle control of isotonic saline also was administered to neonates in these experiments. To ensure that the observer was blind to the treatments received by subjects, the syringes were color-coded before administration of drugs.

General testing protocol

Rat pups were tested after vaginal delivery. Vaginal births were not interrupted to obtain subject pups. To confirm the correct date of birth, pregnant rats were monitored at term on day 22 of gestation. All experiments were conducted approximately 24-hours after birth, with P1 pups. On the day of testing, four pups were removed to provide subjects for this study, with one or two extras to be used as acclimators. All pups were identified and weighed. Before testing, pups' bladders were voided by gently stroking the anogenital area with a soft paint brush. Pups were placed together in a plastic dish inside a warm humid incubator (35°C ± 0.3°) where experimental testing was conducted. In all of the experiments, pups were acclimated for at least 30-min inside the incubator.

In experiments in which pups received an infusion of a test solution through an intraoral cannula, and immediately before commencement of testing, pups were fitted with a single (Experiments 1 and 2) or dual-channel (Experiments 9 to 12) intraoral

cannula (procedure described below). To test subjects, pups were placed on a horizontal support in a supine posture, where they were held in place with a small piece of hair tape lightly extended over the torso. This posture facilitates testing of the two behavioral assays that were used in parts of this study: facial wiping and the oral grasp response to presentation of an artificial nipple. In Experiments 3 and 4, in which pups were exposed to the odor of different solutions, they were placed in a prone position at the base of the testing chamber and stayed unrestrained for the duration of the testing session. As well, in Experiments 5 and 6, where pups were tested in a runway, they were placed in a prone position and remained unrestrained to facilitate locomotion.

In all experiments, surfaces were cleaned with 70% propanol to eliminate any residue of odor or test solutions. Cleaning and drying of surfaces occurred between subjects to avoid the next subject being exposed to experimental odorants before testing.

Facial wiping protocol

Facial wiping is a behavior that has been studied and characterized in the fetal rat (Korthank & Robinson, 1998; Smotherman & Robinson, 1987) and the newborn rat (Méndez-Gallardo & Robinson, 2010). This behavior involves moving one or both forelimbs along the side of the face while making contact with the ears and the nose (Robinson & Smotherman, 1991b). Facial wiping is a species-typical behavior that occurs in response to chemosensory stimulation such as a lemon infusion (Smotherman & Robinson, 1987) and in response to stimulation with a tactile probe applied to the perioral area (Smotherman & Robinson, 1990; 1992a). This behavioral response has been described in many rodent species (Robinson & Smotherman, 1992b) and appears to be

related and developmentally continuous with face-washing, grooming behavior, and aversion responses in the adult rat (Berridge, 2000; Berridge & Fentress, 1986, Ganchrow, Steiner, & Canetto, 1986; Golani & Fentress, 1985; Johanson & Shapiro, 1986).

In the experiments in which facial wiping was used as the behavioral measure (i.e., Experiments 9 to 12), administration of the test solutions was presented through intraoral cannulae. Single or dual-channel intraoral cannulae were prepared with polyethylene tubing (PE-10, diameter = 0.61 mm; Clay-Adams, length of cannula = 10 cm) with a heat-formed flange at one end. Cannulae were implanted in fetuses and pups following a method originally described by Hall and Rosenblatt (1977). To install the cannula, a fine stainless steel wire was drawn through the lower jaw and tongue of the pup, the cannula friction-fitted to the end of the wire, and the cannula pulled back through the jaw, with the flange resting flat against the dorsal surface of the tongue. In Experiments 1 and 2, a single cannula was installed, because subjects were infused with only one test solution. In the experiments in which two solutions were infused into the mouth of the subject (i.e., Experiments 9 to 12) two parallel tubes were installed. The cannulae were placed with the flanged tips resting on the tongue of the fetal subjects in an anterior position (Kehoe & Blass, 1985). Previous studies have reported the use of an alternative method of cannula implantation in which the cannula is placed in the cheek of the pup rather than in an anterior position on the tongue (Spear, Specht, Kirstein, & Kuhn, 1989). However, placement of the cannula in an anterior tongue position is preferred over other placements to prevent interference of the cannula with the expression

of facial wiping behavior. When the cannula is implanted in the cheek, pups cannot perform facial wiping because the cannula restricts movement of the paw along the side of the face. In addition, the anterior tongue cannula is a standard method that has been used extensively in studies of perinatal rats in which facial wiping is used as a behavioral measure during testing (Brumley & Robinson, 2004; Korthank & Robinson, 1998; Méndez-Gallardo & Robinson, 2010; Robinson & Smotherman, 1991b). Implantation of cannulae was performed without the use of anesthesia and took approximately 30 s to pass each cannula through the jaw. Cannulae were installed immediately before each subject was tested.

To deliver chemosensory stimuli, the external end of the intraoral cannula was attached to polyethylene tubing (PE-50) connected to a 3-ml syringe in an infusion pump that contained the exposure fluid. This procedure permits precise infusion (± 1 µl) of the stimulus solution in a 2-s pulse. Infusions were delivered directly into the mouth of the neonatal subject in a volume of 20 µl, based on previous studies that have used infusions of both milk and AF in fetuses and neonates (Korthank & Robinson, 1998; Méndez-Gallardo & Robinson, 2010).

Protocol for odor exposure

In Experiments 3 and 4, subjects were exposed to the odor of different test solutions through an odor apparatus that delivers the odor of the solution to the chamber where pups were tested. The odor exposure apparatus framework was similar to that described by Alberts and May (1980). The apparatus was constructed using an aquarium air pump and airline tubing (5 mm diameter). As illustrated in Figure 1, the air pump

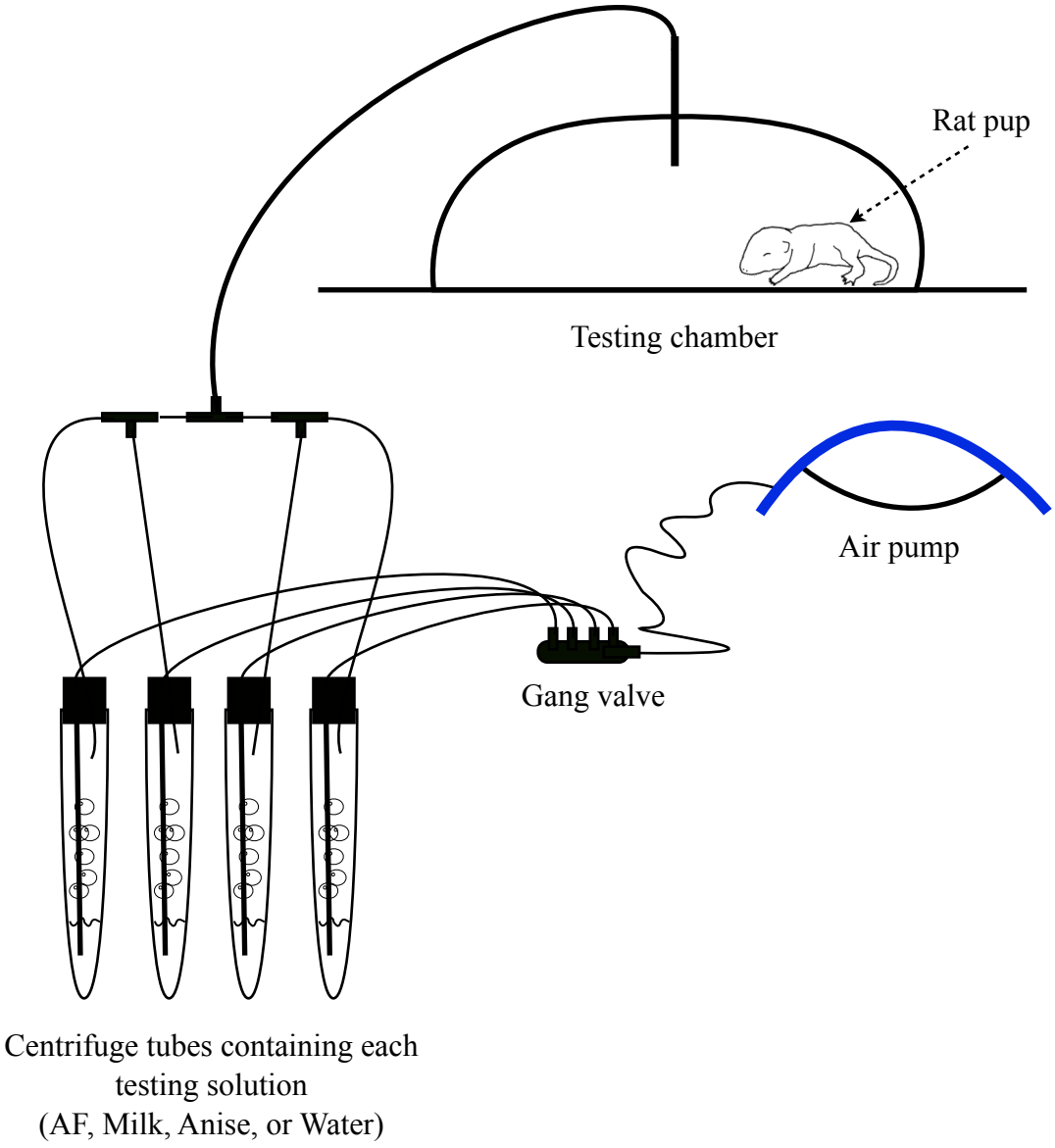

Figure 1. Diagram of odor apparatus. To produce the odor stimulus, the air pump moves air to a gang valve that separates the source air stream into four individual channels that carries the air stream to four separate polypropylene conical centrifuge tubes (15.0 ml) containing each testing solution (AF, milk, anise, or water). The volume of each solution is approximately 1.0 ml. The incoming air stream generate air bubbles from the solution, producing an odor-laden air stream that is collected at the top of the centrifuge tube and carried into the testing chamber. The chamber consists of a closed circular plastic container (12.5 cm diameter x 5 cm height). Odor-laden air enters the chamber through tubing in the center of the ceiling of the chamber, and leaves through one of four small holes at the base of the chamber. During testing, the pup is placed on the floor of the chamber with its nose midway between the chamber wall and the point of entry of the air stream (approximately 3-4 cm from the wall) facing towards the center.

moves air to a gang valve that separates the source air stream into four individual channels that carries the air stream to four separate polypropylene conical centrifuge tubes (15.0 ml) containing each testing solution (i.e., AF, milk, anise, or water). The volume of solution in each centrifuge tube is approximately 1.0 ml. The incoming air stream generates air bubbles in the solution, producing an odor-laden air stream that is collected at the top of the centrifuge tube and carried into the testing chamber. The chamber consists of a closed circular plastic container (12.5 cm diameter x 5 cm height). Odor-laden air enters the chamber through tubing in the center of the ceiling of the chamber, and leaves through one of four small holes at the base of the chamber. During testing, the pup was placed on the floor of the chamber with its nose midway between the chamber wall and the point of entry of the air stream (approximately 3-4 cm from the wall), facing towards the center.

Before testing, the air pump was turned on but the valves remained closed until commencement of testing. This method ensured that there was an air stream running directed into the olfactory solution in the centrifuge tube. The testing procedure involved acclimating subjects to a warm incubator (35ºC) for 30-min. After acclimation to this temperature, subjects were moved to the testing area next to the odor apparatus and remained unmanipulated for 10-min at room temperature (~25ºC). After this period, testing commenced with a 1-min baseline. After baseline, the valve was opened so that odor-laden air was delivered into the testing chamber. Pups were exposed to the odor during 5-min after opening the valve and exposing them to the odor-laden air stream.

Crawling testing protocol

An early form of locomotion in the newborn rat can be observed from the day of birth until about postnatal day 3, when they start showing better organized locomotion. In the normal environment of the nest, the motility of newborn pups is restricted to movement towards the mother, and specifically oriented towards the nipple, which is accomplish by simple motor patterns such as righting and different forms of crawling towards the nipple (Eilam & Smotherman, 1998). Crawling at these early ages has been characterized as moving without postural control, with the body contacting the ground at all times (Gramsbergen, 1998), and as pushing the body mainly with the forelimbs while dragging the hindlimbs (Westerga & Gramsbergen, 1993), although some studies have suggested that all limbs move during crawling even in newborn rats (Eilam & Smotherman, 1998). In addition, during the first week after birth newborn rats commonly show movements in a circular motion known as *pivoting* in which they alternate between movements of the head and the forelimbs (referred to as punting) (Altman & Sudarshan, 1975). Crawling behavior in newborn rats seems to be adapted to the nest environment and appears to serve the function of helping the newborn to access the nipple (Eilam & Smotherman, 1998).

Previous research has reported that olfactory stimulation can elicit crawling and organized limb activity in newborn rats (Fady, Jamon, & Clarac, 1998; Jamon & Clarac, 1998; Jamon, Maloum, Riviere, & Bruguerolle, 2002; Sczerzenie & Hsiao, 1977). Rats tested on postnatal day 3 suspended from the ground and exposed to the odor of nest material, show forward locomotion (crawling) by pushing their bodies with their legs and

slightly raising their bodies (Jamon & Clarac, 1998). Similarly, movements of the forelimbs and hindlimbs can be elicited in pups tested as early as the day of birth (P0) when presented with the odor of bedding material (Fady et al., 1998). In addition, research has shown that air-stepping in 3-day-old rats can be elicited by exposure to the odor of nest materials (Jamon et al., 2002). Comparable to rats, a study by Varendi and Porter (2001) showed that human newborns tested between 36 and 80 hours after birth reached and crawled toward a pad scented with odors of the breast of the mother in comparison to a clean pad without any scents. These studies suggest that newborns crawl and move toward an odor source that is related to their immediate environment within the nest or to features related to nursing or suckling. Moreover, they suggest that other stimuli of ecological relevance, such as AF or milk, also could evoke crawling activity in the newborn.

In Experiments 5 and 6, pups were exposed to the odor of AF, milk, anise, or water to elicit crawling. Testing was conducted on a runway inside a testing chamber that was prepared with an aquarium made of glass (76 cm length X 32 cm width X 32 cm height). The runway consisted of a long strip of soft vinyl material (vinyl dissecting pad, 61 cm long X 8 cm wide). The vinyl strip was placed on a block of wood of the same dimensions to provide a solid base for support and stability. The vinyl pad was prepared with lines marked in 1-cm increments to calibrate the distance that pups moved during the test session. A camera was placed above the runway providing an overhead view of the subject. To expose pups to the odor of the different testing solutions, 0.3 mL of each solution was placed in a 1.5 mL graduated microcentrifuge tube.

During the test session, pups were removed from the home cage and moved to a warm incubator (35°C ± 0.3) to acclimate for 30 min. Before acclimation, pups were weighed, marked, and voided. After acclimation, each subject was moved to the testing chamber (27°C ± 0.5) for 20 min. Testing commenced immediately after this second 20-min period. Testing involved placing the subject at the beginning of the runway, with the nose aligned with a starting line 5 cm from the border of the runway and moving the tube containing the test solution over the pup's snout to expose it directly to the odor. Odor exposure continued for 3 min. If the subject traversed the complete runway (distance = 50 cm) before 3-min, the testing session for that subject was completed. Otherwise, the session was completed after 3-min of exposure to the odor.

As described in a study by Jamon and Clarac (1998), exposure to the odor has to follow specific parameters to guarantee that the odor is presented in a controlled and optimal way. As in the study by Jamon and Clarac (1998), preliminary observations of the testing protocol of the current study revealed that while pups responded to the odor and followed it by crawling towards the odor, if the tube containing the solution rested directly on the surface they lost contact with it by turning their head to the side. When they lost contact with the odor, they stopped moving and rapidly fell asleep. To ensure continuous contact with the tube during testing, the tube was placed over the snout of the subject and was held by the investigator. As the pup responded to the odor by crawling and pushing the tube, the investigator gently moved the tube forward to maintain crawling behavior. In this way, crawling behavior in response to the odor exposure was maintained with minimal interruptions.

Protocol for presentation of an artificial nipple

Research has reported that rat fetuses as young as E18 and E19 presented with an artificial nipple respond by opening the mouth or expressing an oral grasp response (Browne et al., 1994; Robinson et al., 1992). Studies have shown that exposure to an artificial nipple elicits a range of behaviors in the newborn rat that includes suckling, mouthing, licking, and oral grasping (Smotherman, Goffman, Petrov, & Varlinskaya, 1997). In addition, use of the artificial nipple as a CS in paired presentation with milk as a US has demonstrated the capacity of rat fetuses to learn through classical conditioning (Robinson et al., 1993).

To assess responses to the nipple in this study, an artificial nipple was used following some of the parameters used in previous research (Robinson et al., 1992; Smotherman, Goffman et al., 1997). The artificial nipple was constructed using silicone compound and shaped to the nipple form (25mm in length, 5mm wide at the base, and 1 mm wide at the rounded tip). In addition, a round baseplate was fashioned from soft vinyl material (1.5 mm thick, 5 mm diameter) and placed 5mm from the tip of the nipple. This baseplate was used to provide a contact point for the snout of the pup when it grasps the nipple and to control the depth of nipple's entry into the mouth of the pup (Petrov, Varlinskaya, & Smotherman, 1997b; Petrov, Varlinskaya, & Spear, 2003). The artificial nipple was attached to a dissecting needle used as a handle to facilitate manipulation and presentation by the experimenter.

Presentation of the artificial nipple involves holding the tip of the nipple in contact with the oral aperture of the rat pup. This process has to be conducted in a gentle

manner without forcing the tip of the nipple into the mouth of the pup. In Experiments 7 and 8, in which pups were exposed to an artificial nipple containing an odor, a cotton pad (1.0 cm of diameter) was moistened with 0.3 mL of the solution used as testing stimulus (i.e., AF, milk, anise or distilled water) and attached behind the nipple at a distance of 0.8 to 1.0 cm from the tip of the nipple. The nipple was presented for 3 min to each pup. In Experiments 9 to 12, in which the nipple was used as a CS in a conditioning paradigm, the nipple was presented for only 15 s during each period of CS exposure.

Conditioning Protocol

Experiments 9 to 12 used a classical conditioning protocol adapted from previous experimental studies in the fetal rat (Robinson et al., 1993; Smotherman & Robinson, 1991). In these experiments, conditioning and testing occurred in a single observation session lasting 20 min (Experiments 9 and 11) or 22 min (Experiments 10 and 12). Each experiment involved presenting an artificial nipple as the conditioned stimulus (CS) and intraoral infusion of AF (Experiments 9 and 10) or milk (Experiments 11 and 12) as the unconditioned stimulus (US). The CS and the US were presented alone or in combination during conditioning trials and the pup was reexposed to the artificial nipple CS alone during a final test after conditioning.

Conditioning trials involved presenting AF or milk alone (US alone), the artificial nipple alone (CS alone), noncontingent presentation of US followed 2.5 min later by CS (unpaired group), or contingent presentation of CS followed immediately by US (paired group). The three conditioning trials were separated by 5-min intervals. At min 18, all subjects were reexposed to the CS (artificial nipple) for 15 s, and 1 min later, presented

with a 20 µl infusion of a 1:1 dilution of lemon odor extract to evoke a facial wiping response. Changes in the pup's oral responses to the artificial nipple during reexposure to the CS, and in facial wiping responses evoked by lemon, were used as the principal dependent variables to assess conditioned responses. In Experiments 10 and 12, subjects received an IP injection of naloxone (or a saline control) at min 15 to manipulate the endogenous opioid system. In these experiments involving naloxone administration, the session was extended by 2 min, with the final CS reexposure occurring at min 20 and infusion of lemon at min 21 to evoke facial wiping. This extension provided a 5-min delay between the injection and test after conditioning trials. Figures 2 and 3 illustrate the timeline of the conditioning protocols.

Behavioral measures and observations

Behavioral observations consisted of 1 to 22 min sessions. Experiments 1 to 4 consisted of 6-min sessions in which behavioral activation of the subjects was observed. Experiments 5 and 6 consisted of 3-min sessions to evaluate crawling behavior, while Experiments 7 and 8 involved 4-min sessions to evaluate oral grasp responses directed to an artificial nipple. Experiments 9 to 12 consisted of 20-min (Experiments 9 and 11) or 22-min (Experiments 10 and 12) sessions to conduct conditioning trials and evaluate conditioned responses after training. Behavioral scoring in different experiments thus focused on measuring behavioral activation to an intraoral infusion and to odor exposure, oral responses to an artificial nipple, crawling activity, and facial wiping.

Behavioral activation to an infusion (Experiments 1 and 2) was quantified as the total number of movements of all limbs, head, and mouth. Behavioral responses that were

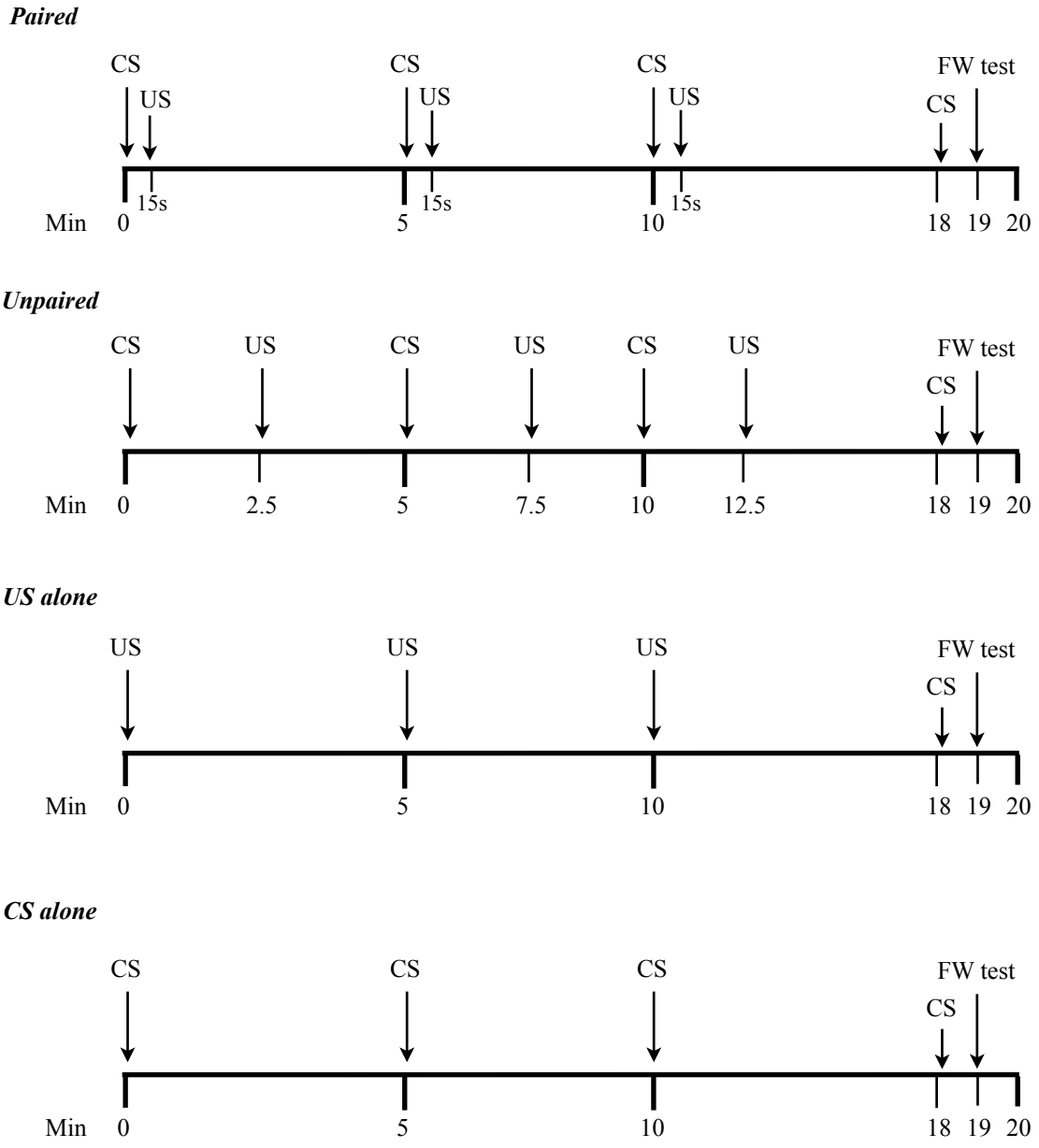

Figure 2. Timeline of events of the four conditioning protocols in Experiments 9 and 11. Stimuli used in these protocols consisted of presentation of an artificial nipple (CS) and an intraoral infusion of AF (US) in Experiment 9 or an intraoral infusion of milk (US) in experiment 11. Subjects in the Paired group received three presentations of the CS followed 15 s later by the US; subjects in the Unpaired group received three presentations of the US followed 2.5 min later by the CS; subjects in the US alone group received three presentations of the US every 5 min; and subjects in the CS alone group received three presentations of the CS every 5 min. At min 18 all subjects were reexposed to the CS and 1 min later received an infusion of lemon to test for facial wiping.

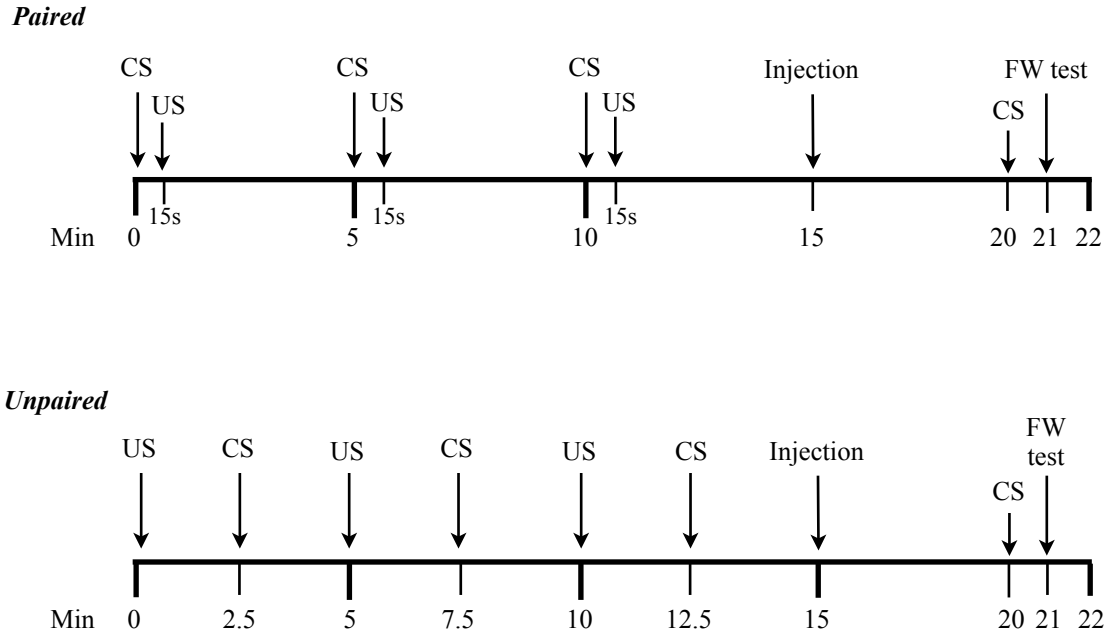

Figure 3. Timeline of events of the two conditioning protocols in Experiments 10 and 12. Stimuli used in these protocols consisted of presentation of an artificial nipple (CS) in both experiments, and an intraoral infusion of AF (US) in Experiment 10 or an intraoral infusion of milk (US) in experiment 12. Drug treatment consisted of IP injection of 50 µl of naloxone (1.0 mg/kg) or isotonic saline. Subjects in the Paired group received three 15-s presentations of the CS followed immediately by the US. Subjects in the Unpaired group received three presentations of the US followed 2.5 min later by the CS. At min 15, all subjects were treated with naloxone or saline. Five min later, at min 20, all subjects were reexposed to the CS and 1 min later received an infusion of lemon to test for facial wiping.

scored include: (a) Forelimb movements – overall movements of right and left forelimbs including movements that could be qualify as facial wiping, alternated or coordinated movements; (b) Hindlimb movements – overall movements of right and left hindlimbs including alternated and coordinated movements; (c) Head turning – turning the head from midline and lifting the head; and (d) Mouthing – opening and closing of the mouth. In Experiments 3 and 4, in which subjects were exposed to the odor of different olfactory fluids, behavioral activation as described above was scored.

Previous reports have described how experimental infusion of milk to the fetal rat results in a systematic reorganization of motor activity expressed in different parts of the body (Robinson & Smotherman, 1992d). Initial responses to infusion include mouthing, but after several minutes, activity shifts from the head and forelimbs to the hindlimbs. This shift in activity is clearly represented by expressing hindlimb movements as a percentage of overall activity (Robinson & Smotherman, 1992c, 1994). Moreover, the change in relative hindlimb activity is dependent on the endogenous opioid system (Andersen et al., 1993). For these reasons, relative hindlimb activity, calculated as the frequency of hindlimb movements expressed as a percentage of all movements, was included as a dependent measure in Experiments 1-4.

In Experiments 5 and 6, crawling was elicited by exposing the P1 rat pup to the odors of AF, milk, anise, or water. Behavioral observations of crawling included scoring the duration (time) required for the pup to move through the runway, as well as the distance completed by each subject within the 3-min of direct odor exposure.

In experiments in which responses toward the artificial nipple was the dependent variable (Experiments 7 and 8), behavioral observations included scoring an array of behavioral categories. Oral grasping of the nipple was counted, and additional behavioral categories were quantified because previous studies have reported that rat fetuses tested on days E19, E20, or E21 express multiple behavioral responses to an artificial nipple, including mouthing, licking, and head-turning (Robinson et al., 1993). Behavioral categories measured in this study included: (a) Oral grasping of the nipple – firm closure of the jaw while the nipple is in the subject's mouth, (b) Head aversion – lateral movement of the head at least 45 degrees away from the tip of the nipple, (c) Head ventriflexion – flexion of neck and body curling in ventral direction, (d) Mouthing – includes behaviors such as opening of the mouth, tongue movements, jaw movements with mouth closed, all while in contact with nipple or away from it, (e) Paw dorsiflexion – dorsiflexion of the forepaw at the wrist rising at least 30 degrees above horizontal, and (f) Paw plantarflexion – forepaw curving inward in a plantarflexion manner with a closing or gripping motion (often occurred when the paws were oriented toward midline).

Behavioral scoring of facial wiping (Experiments 9 to 12) involved identifying individual facial wiping strokes. Facial wiping cycles are defined as movements of the forepaws in a rostral direction (from ear to nose) that maintain contact with the side of the face (Robinson & Smotherman, 1991b). Unilateral paw contact was distinguished from bilateral paw contact during the wiping cycle. Unilateral wipes were scored only when one paw made contact with the face without simultaneous contact by the opposite paw. Accordingly, bilateral wipes were scored only when both paws contacted the face at the

same time during some portion of the wiping cycle. In these experiments, mouthing activity in response to the artificial nipple also was scored as described in the previous paragraph.

Experimental Design and Data Analysis

In each experiment, multiple pups were tested from each pregnancy. In order to avoid confounding treatment effects with litter effects, neonatal subjects from the same pregnancy were assigned to different treatment groups and each treatment group was represented only once in each pregnancy (Abbey & Howard, 1973; Holson and Pearce, 1992). Preliminary analyses revealed no significant differences in the expression of behavior between females and males. Since no gender differences were evident, data from male and female pups were collapsed together in all experiments.

All experimental sessions were recorded from a single camera view directly to computer as digital video files (mp4 files recorded with H.264 video codec, 30 fps). Recording took place during the entire test session in each experiment. These digital video files were used for behavioral scoring. Test sessions were played back on a computer within custom event-recording software (EventCoder, provided by Dr. Michael Goldstein, Cornell University). For some measures (e.g., facial wiping, nipple grasping) video was played back at reduced speed (0.5x or 0.25x of real time). In the experiments in which several behavioral categories were scored, scoring was done in multiple passes to guarantee careful and accurate quantification of each behavior. Behavioral scoring using this software recorded the category and time of occurrence (± 0.1 s) of each

behavioral event, which permitted summaries of the frequency and duration of each behavioral category during the experimental session.

Specific approaches for data analysis are discussed in each experiment (see below). In general, frequency counts of each behavioral category were summed in time bins (e.g., 1-min intervals) over the test session and compared across the different treatment conditions in each experiment. Behavioral data were analyzed in a series of ANOVAs, with time treated as a repeated measure where appropriate. Where main or interaction effects were found, additional tests for simple main effects were performed. Post-hoc comparisons of individual group means used the Fisher PLSD test. Alpha levels were set at .05.

CHAPTER 4: BEHAVIORAL ACTIVATION TO AMNIOTIC FLUID AND MILK

In Chapter 4, Experiments 1, 2, 3, and 4 investigated the behavioral effects of AF and milk and the role of the endogenous opioid system in producing behavioral activation in the neonatal rat. Two of the experiments (1 and 2) presented AF or milk to P1 rat pups in an intraoral infusion. The other two experiments (3 and 4) exposed pups only to the odor of AF or milk.

Experiment 1: Behavioral activation to an intraoral infusion of amniotic fluid or milk

Experiment 1 addressed the question of whether exposure to an intraoral infusion of AF to the neonatal rat results in overall behavioral activation that is similar to that produced by milk. Research has shown that exposure to AF results in overall behavioral activation in the fetal rat. Robinson and Méndez-Gallardo (2011) reported that rat fetuses that receive continuous infusion of AF through an intraoral cannula display greater overall behavioral activation than fetal subjects infused with saline or untreated subjects. This behavioral effect is more evident in hindlimb activity, which exhibits longer periods of activation. On the other hand, rat fetuses infused with a small amount of milk show a series of behavioral effects that starts with mouthing only seconds after the infusion, continues with increased hindlimb activity, and culminates with the stretch response (Robinson & Smotherman, 1994; Robinson & Smotherman, 1992c). Milk infusion also is known to produce general behavioral activation in the infant rat (Hall, 1979a, 1979b). However, the overall behavioral effects of AF have not previously been described in the

neonatal rat, and activational effects of AF and milk after intraoral infusion have not been compared under similar experimental conditions. The purpose of Experiment 1, therefore, was to evaluate the behavioral effects of AF and milk after exposure through an intraoral infusion in the neonatal rat. The hypothesis in this experiment was that exposure to an infusion of AF or milk should result in similar behavioral activation in the neonatal rat.

Methods

Subjects

Neonatal subjects were the offspring of Sprague-Dawley laboratory rats. Thirty-two pups from 11 litters were used in Experiment 1. Pups were tested 24 hours after birth (P1). Subjects' breeding and care followed the same protocol described in the General Methods section.

Conditions

Rat pups were assigned to one of four conditions according to the testing solution they were exposed to (n = 8 pups per group): AF, Milk, Water, or Anise. Females and males rat pups were tested in each of these conditions.

Procedure

Rat pups were tested 24 hours after birth (P1) in a supine posture and were exposed to one of four testing solutions: AF, milk, anise, or water. Before testing, subjects were fitted with an intraoral cannula to allow delivery of the different solutions. Testing involved a 6-min session starting with a 1-min baseline. After the baseline period, and immediately at the beginning of min 2, pups received a 20 µl infusion of either AF, milk,

anise or distilled water. Following exposure, behavioral responses were observed for 5 minutes.

Behavioral observations and data analysis

Behavioral observations included scoring of facial wiping and overall activity, including movements of the forelimbs, hindlimbs, head, and mouth. These observations were made during the entire 6-min test session. Each test session was recorded directly to a digital video file. Video files were scored using event-recording software, summarized, and statistically analyzed as described in the General Methods section. An equal number of female and male subjects were tested in Experiment 1.

Results

Analyses of each behavioral category (i.e., facial wiping, forelimb, hindlimb, head, and mouthing) were performed in 2-factor analysis of variance (4 Exposure groups X 6 1-min intervals), with the Time factor treated as a repeated measure. Results showed that there was no significant expression of facial wiping across groups during the entire testing session. In the case of mouthing responses, there were no significant differences between infusion groups ($p > .05$) and there was only a significant main effect of Time across groups, $F\ (5, 160) = 2.465$, $p < .05$ (Figure 4), with a significant decrease in activity after the infusion during minutes 2 and 3 and an increase of activity at the end of the session during minutes 4, 5, and 6. Analysis of head movements revealed a significant difference between infusion groups, $F\ (3, 31) = 7.77$, $p < .01$ and a significant effect of Time, $F\ (5, 160) = 3.20$, $p < .01$ (Figure 5), with an increase in activity in min 2 after the infusion. Results of forelimb activity showed a significant difference between infusion

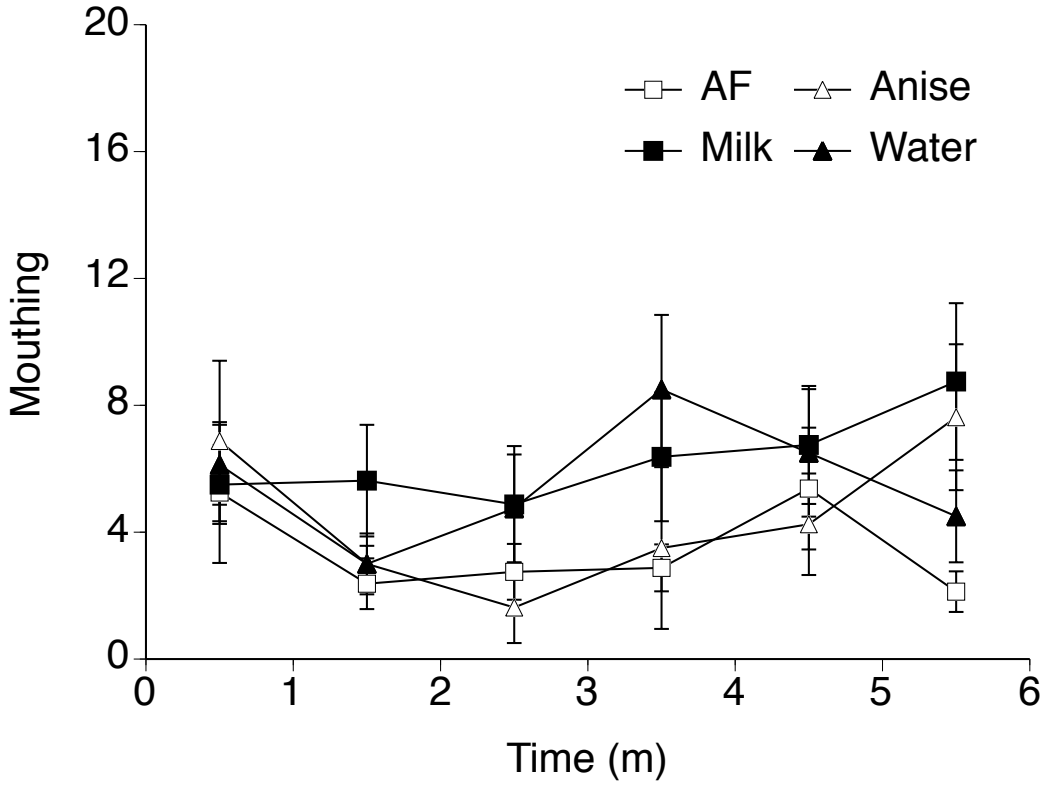

Figure 4. Changes in mouthing activity throughout the 6-min test session of Experiment 1. Pups received a 20 µl intraoral infusion of AF, Milk, Anise, or Water at the end of min 1. Squares and triangles depict mean numbers of mouthing responses per minute. Error bars depict S.E.M.

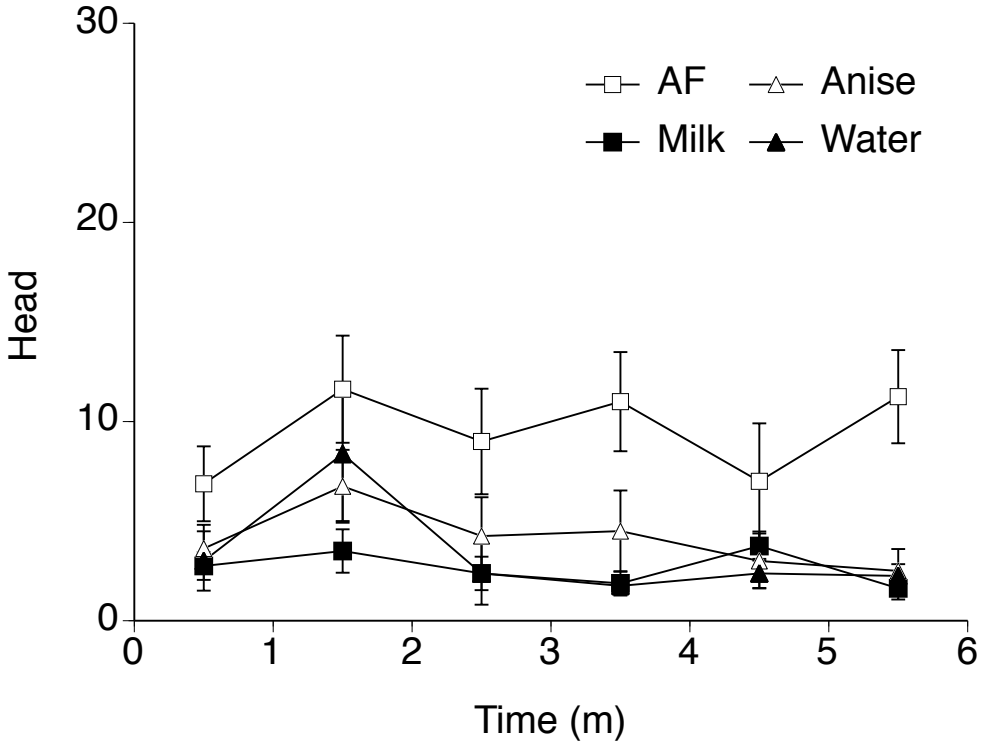

Figure 5. Changes in head activity throughout the 6-min test session of Experiment 1. Pups received a 20 µl intraoral infusion of AF, Milk, Anise, or Water. Squares and triangles depict mean numbers of head movements per minute. Error bars depict S.E.M.

groups, F (3, 31) = 5.51, $p < .01$, and no significant effect of Time (Figure 6). Finally, results of hindlimb activity showed that there were significant differences between groups, F (3, 31) = 5.54, $p < .01$ (Figure 7). One-way ANOVAs were performed to compare group effects in those categories in which a significant main effect of group was found. Activity was collapsed across all minutes after the infusion (min 2 to 6), which were compared between groups in one-way analysis of variance and subsequent post hoc comparisons. Results showed that head movements were significantly elevated in subjects infused AF compared to subjects in the other three groups (ps < .001). Infusion of AF also resulted in higher levels of forelimb activity in comparison to those subjects in the other three groups (ps < .01). Similarly, hindlimb activity was elevated in those subjects infused with AF in comparison to subjects in the milk, anise, and water groups (ps < .01). An additional 3-way ANOVA compared hindlimb activity between the groups by expressing activity as a percentage of overall activity. This analysis did not reveal any significant differences among groups ($p > .05$).

Discussion

The results of Experiment 1 suggest that an intraoral infusion of AF or milk exert different effects on behavioral activity in the newborn rat. Specifically, oral exposure to AF results in higher levels of behavioral activity, as was evident in head, forelimb, and hindlimb movements. However, AF and milk both exerted similar effects on mouthing responses, with higher levels of activation during the minute after the infusion. Increased mouthing immediately after infusion could correspond to oral sampling in response to the oral exposure of the fluids. In addition, facial wiping was not a behavior that was

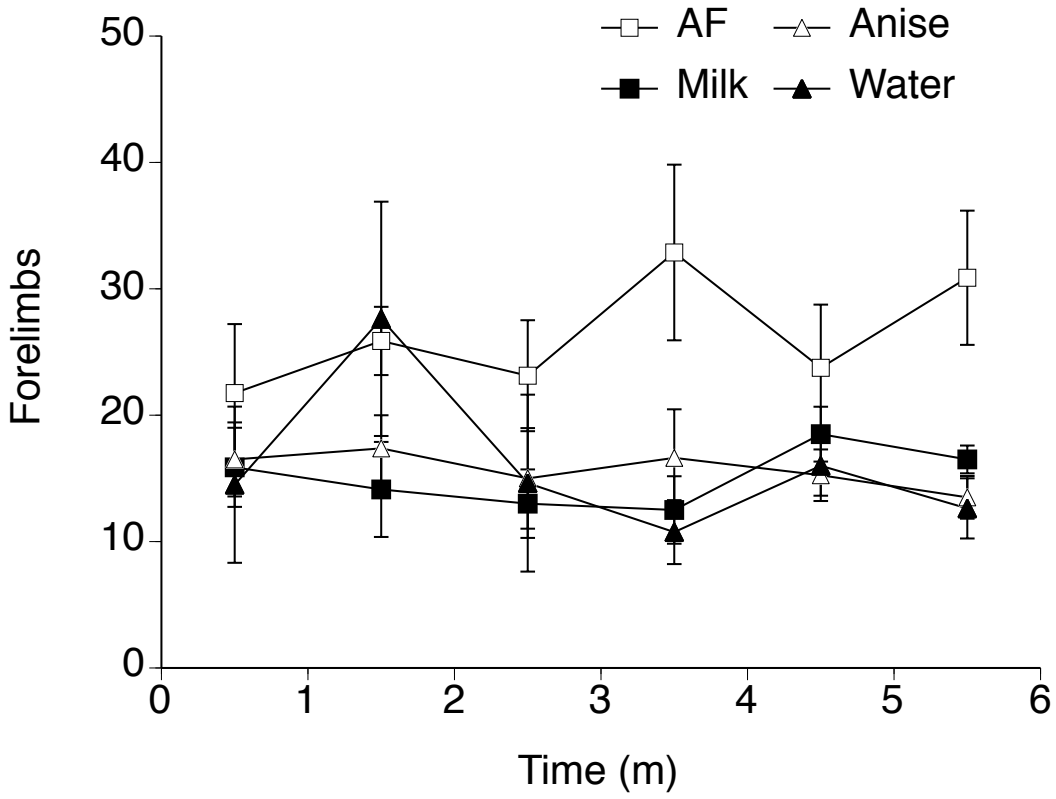

Figure 6. Changes in forelimb activity throughout the 6-min test session of Experiment 1. Pups received a 20 µl intraoral infusion of AF, Milk, Anise, or Water. Squares and triangles depict mean numbers of forelimb movements per minute. Error bars depict S.E.M.

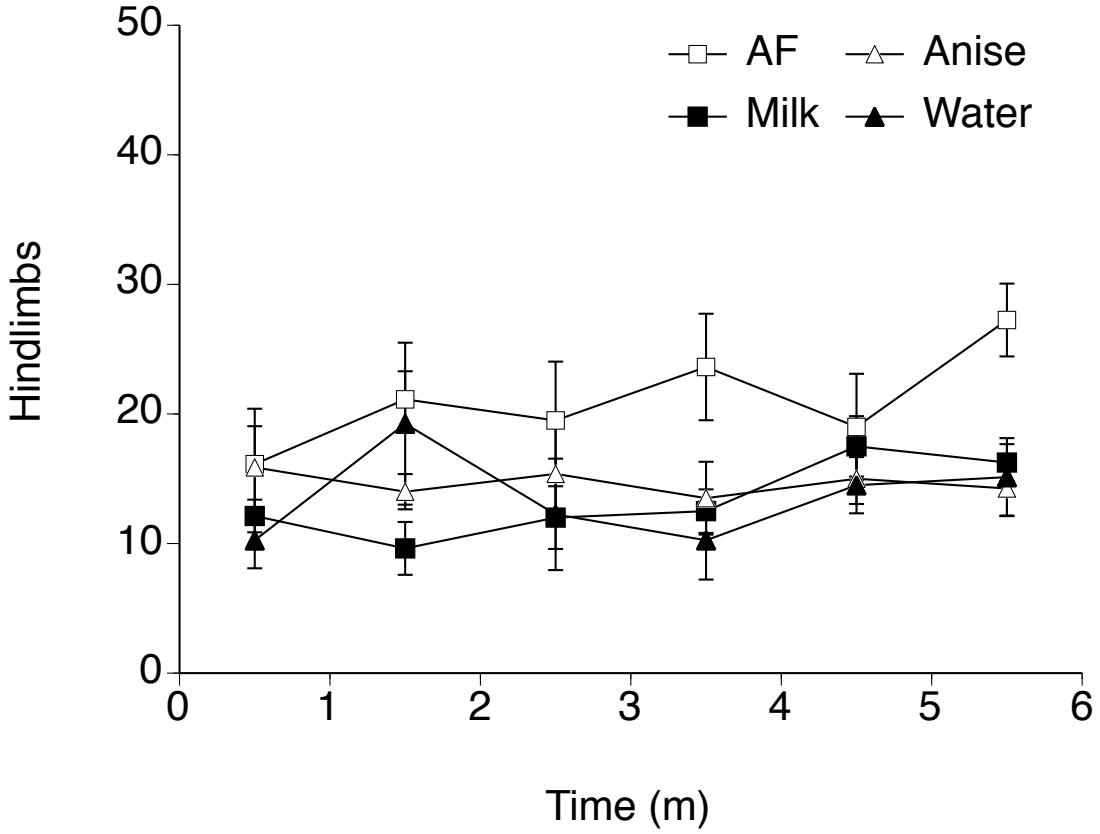

Figure 7. Changes in hindlimb activity throughout the 6-min test session of Experiment 1. Pups received a 20 µl intraoral infusion of AF, Milk, Anise, or Water. Squares and triangles depict mean numbers of hindlimb movements per minute. Error bars depict S.E.M.

significantly expressed after exposure to any of the fluids, including the novel stimulus of anise and the water control.

While overall behavioral activation after an infusion of AF or after oral exposure to milk has been previously described in the fetal rat (Robinson & Méndez-Gallardo, 2011; Robinson & Smotherman, 1994; Robinson & Smotherman, 1992c), the results of this experiment suggest that oral exposure to AF represents a more salient stimulus compared to milk that promotes higher levels of activity in the newborn rat. This experiment not only demonstrates that newborn rats are still responsive to exposure to AF 24 hours after birth, but also that they respond in a different way than to oral exposure to milk. In addition, AF was the only stimulus that exerted a significantly different behavioral response relative to anise, a novel chemosensory stimulus, and water, a control stimulus. While the results did not reveal any significant difference between anise and water, they also did not show that these stimuli resulted in significant activation as was the case for AF. These findings suggest that AF, as a stimulus of ecological relevance, may exert greater effects on behavior than anise as a novel stimulus.

Although the main hypothesis of this study — namely, that AF and milk would produce similar effects on behavioral activity — was not supported with the findings of Experiment 1, it is still relevant to explore whether the opioid system can be engaged during the expression of behavior in response to an intraoral infusion of AF or milk. Based on the findings of Experiment 1, it was expected that if the opioid system is involved during behavioral responses to an intraoral infusion, at least the behavioral

activation to AF should be reversed by blockade of opioid receptors, resulting in reduced activity in the newborn rat. This prediction was evaluated in Experiment 2.

Experiment 2: Opioid mediation of amniotic fluid and milk effects after an intraoral infusion

Experiment 2 addressed the question of whether the behavioral effects resulting from infusion of AF or milk are dependent on activation of the endogenous opioid system in the newborn rat. As already described, exposure to AF through intraoral infusion in the fetal rat results in behavioral activation (Robinson & Méndez-Gallardo, 2010). It also has been reported that this behavioral effect seems to be mediated by the endogenous opioid system, specifically by kappa receptors. Pretreatment with the non-selective antagonist naloxone or the selective kappa antagonist BNI results in reduced behavioral activation after infusion of AF to the rat fetus. In a similar way, research has described that the effects of intraoral infusion of milk in the fetal rat also are opioid mediated. For instance, Smotherman and Robinson (1992b) reported that the stretch response elicited by an infusion of milk can be blocked by pretreatment with naloxone, the kappa specific antagonist BNI, or the mu specific antagonist FNA. However, mouthing behavior as a response to milk exposure cannot be blocked by opioid antagonists, suggesting that the opioid system may not be involved in this behavior. Finally, a specific increase in hindlimb activity in response to an infusion of milk can be blocked by BNI, but not FNA, suggesting involvement of receptors of the kappa opioid system.

While the effects of an intraoral infusion of AF or milk in the newborn rat were described in Experiment 1, the role of the opioid system in general behavioral activation has not been evaluated. The purpose of Experiment 2 was to evaluate if the endogenous opioid system mediates the behavioral effects of AF and milk when they are infused into the mouth of P1 pups. The hypothesis of this experiment was that exposure to an intraoral infusion of AF or milk results in opioid-mediated behavioral activation, which could be blocked by pretreatment with the opioid antagonist naloxone.

Methods

Subjects

Neonatal subjects were the offspring of Sprague-Dawley laboratory rats. Thirty-two pups from 12 litters were used in Experiment 2. Pups were tested 24 hours after birth (P1). Subjects' breeding and care followed the same protocol described in the General Methods section.

Conditions

Rat pups, including one male and one female pup from each litter, were assigned as subjects to one of four conditions resulting from the combination of Pretreatment and Exposure solution. In pretreatment, subjects were assigned to one of two conditions: (a) an intraperitoneal (IP) injection of 50 µl of naloxone (1.0 mg/kg), a nonselective opioid antagonist, or (b) an IP injection of the vehicle control of isotonic saline (50 µl). In exposure, pups were assigned to one of two conditions: AF or milk. Thus, four treatment groups (n = 8 pups per group) resulted from the combination of the pretreatment

conditions (Naloxone or Saline) and the exposure solution (AF or milk): AF-Naloxone, AF-Saline, Milk-Naloxone, and Milk-Saline.

Procedure

The experimental procedure was exactly as described in Experiment 1 with the exception that before testing, pups were pretreated with naloxone or saline. The 6-min test session commenced 5 min after pretreatment.

Behavioral observations and data analysis

Behavioral observations included scoring of facial wiping and overall activity, including movements of forelimbs, hindlimbs, head, and mouth. These observations were made during the entire 6-min testing session, beginning 5 min after pretreatment. Each test session was recorded to a digital video file. Video files were scored using event-recording software, summarized, and analyzed as described in the General Methods section.

Results

Analysis of each behavioral category (i.e., facial wiping, forelimb, hindlimb, head, and mouthing) was performed in 3-factor analysis of variance (2 Pretreatment groups X 2 Exposure groups X 6 1-min intervals), with the Time factor treated as a repeated measure.

Results showed that there was no significant expression of facial wiping across groups during the entire testing session. Analysis of mouthing activity showed that while there were no significant differences between infusion groups ($p > .05$), there was a significant main effect of Time, F (5, 140) = 3.44, $p < .01$ (Figure 8). Within subjects

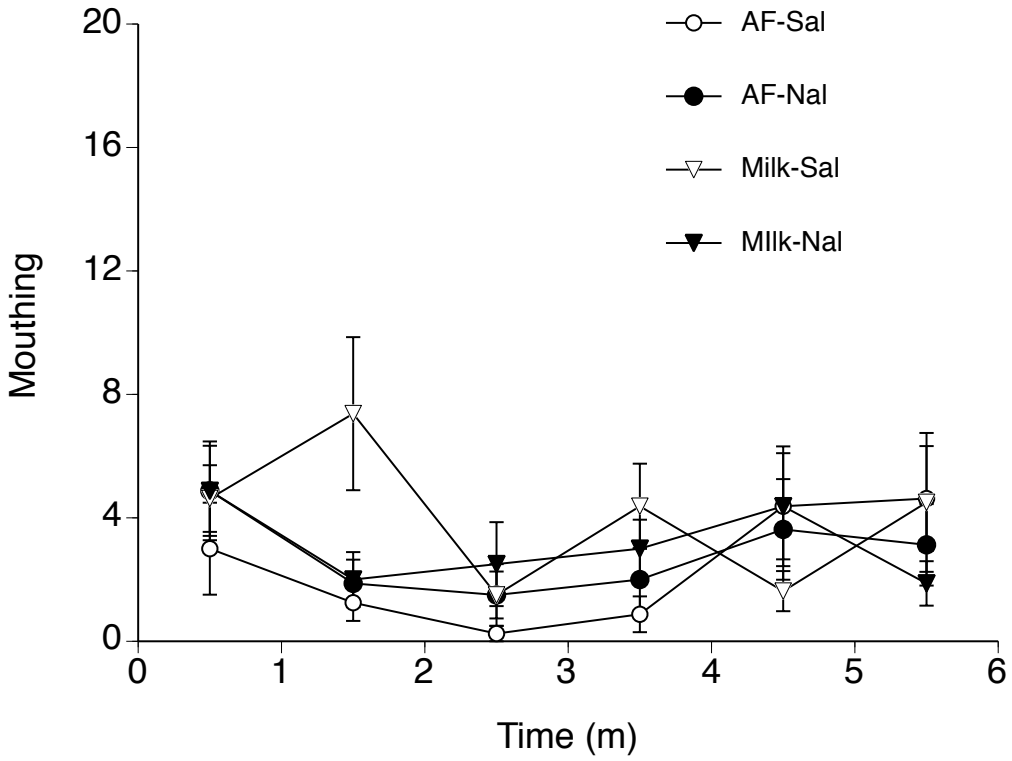

Figure 8. Changes in mouthing activity throughout the 6-min test session of Experiment 2. Pups were pretreated by IP injection of isotonic saline (Sal) or non-selective opioid antagonist naloxone (Nal; 1.0 mg/kg) 5 min before infusion and received 20 µl of an intraoral infusion of AF or milk. Circles and triangles depict mean numbers of mouthing responses per minute. Error bars depict S.E.M.

analysis to evaluate the effect of Time revealed that mouthing activity was higher during the first 2 minutes and after min 2 there was a decreased in activity. Additional two-way analyses of variance performed in each minute of the testing session showed that during min 2 there was a significant main effect of infusion group, F (1, 28) = 4.97, $p < .05$, and an interaction between infusion and drug, F (1, 28) = 4.58, $p < .05$. Additional two-way analyses of variance performed in each minute of the testing session showed that during min 2 there was a significant main effect of infusion group, F (1, 28) = 4.97, $p < .05$, and an interaction between infusion and drug, F (1, 28) = 4.58, $p < .05$. Post hoc comparisons of means by the method of Fisher PLSD indicated significantly more mouthing activity in the saline group and in the milk group during min 2 (ps < .05). Two-way analysis of variance showed that during min 4 there was a significant main effect of infusion group, F (1, 28) = 4.87, $p < .05$. Post hoc comparisons of means by the method of Fisher PLSD indicated significantly more mouthing activity in the milk group during min 4 (ps < .05).

Analysis of head activity revealed that there were significant differences between infusion groups, F (1, 28) = 5.35, $p < .05$, a significant effect of Time, F (5, 140) = 2.84, $p < .05$, a significant interaction between infusion groups and Time, F (5, 140) = 3.69, $p < .01$, and a significant interaction between drug groups and Time, F (5, 140) = 2.66, $p < .05$ (Figure 9). One-way analyses revealed that higher levels of head activity were evident in the AF group than the milk group, F (1, 30) = 8.70, $p < 0.01$. Within subjects ANOVAs performed individually in each infusion group showed that the significant effect of Time in the AF group was evident in higher levels of head activity after the

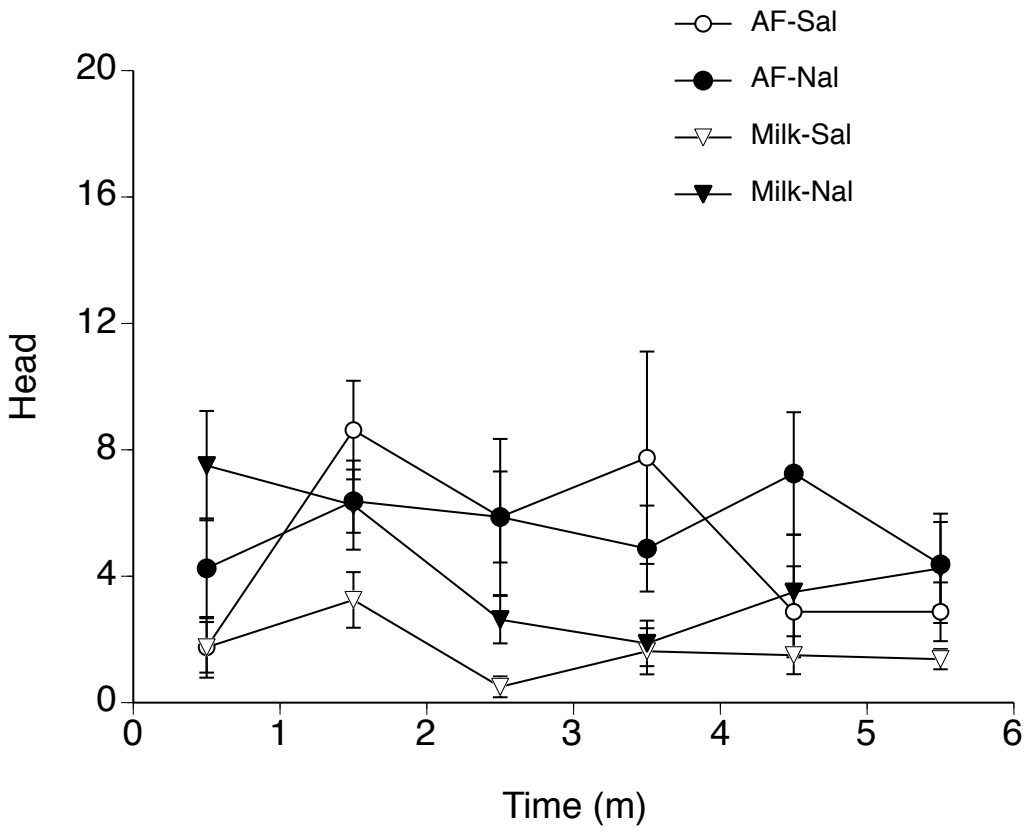

Figure 9. Changes in head activity throughout the 6-min test session of Experiment 2. Pups were pretreated by IP injection of isotonic saline (Sal) or non-selective opioid antagonist naloxone (Nal; 1.0 mg/kg) 5 min before infusion and received 20 µl of an intraoral infusion of AF or milk. Circles and triangles depict mean numbers of head movements per minute. Error bars depict S.E.M

infusion in min 2 and throughout the following minutes with a decrease at the last minute. In the milk group, a significant decrease in head activity was evident after min 2. One-way repeated-measures ANOVAs within each drug group showed an effect of Time only in those subjects pretreated with saline, with a significant increase in head activity immediately after the infusion in min 2. Additional two-way ANOVAs performed in each minute of the testing session showed a significant difference between infusion groups during min 2, F (1, 28) = 4.89, $p < .05$, min 3, F (1, 28) = 8.37, $p < .05$, and min 4, F (1, 28) = 5.86, $p < .05$, with higher levels of head activity in the AF group. A significant difference between drug groups was evident during min 1, F (1, 28) = 9.66, $p < .05$, and min 5, F (1, 28) = 4.26, $p < .05$, where subjects in the naloxone group showed higher levels of head activity. During min 2 there was a significant interaction between infusion and drug, F (1, 28) = 4.45, $p < .05$ where subjects in the AF group in both drug groups showed higher levels of head activity.

Analysis of forelimb activity revealed that there were no significant differences between infusion groups ($p > .05$), or any effect of Time ($p > .05$), or drug ($p > .05$), as well as no significant interaction between infusion group, drug group, or Time (ps > .05) (Figure 10). Similar results were found in the hindlimb activity category (Figure 11). However, an additional 3-way ANOVA compared hindlimb activity between the groups by expressing activity as a percentage of overall activity. This analysis found that hindlimb activity was significantly higher in subjects treated with saline, F (1, 28) = 4.76, $p < .05$, as well as a significant effect of Time, F (5, 140) = 4.10, $p < .01$, in which

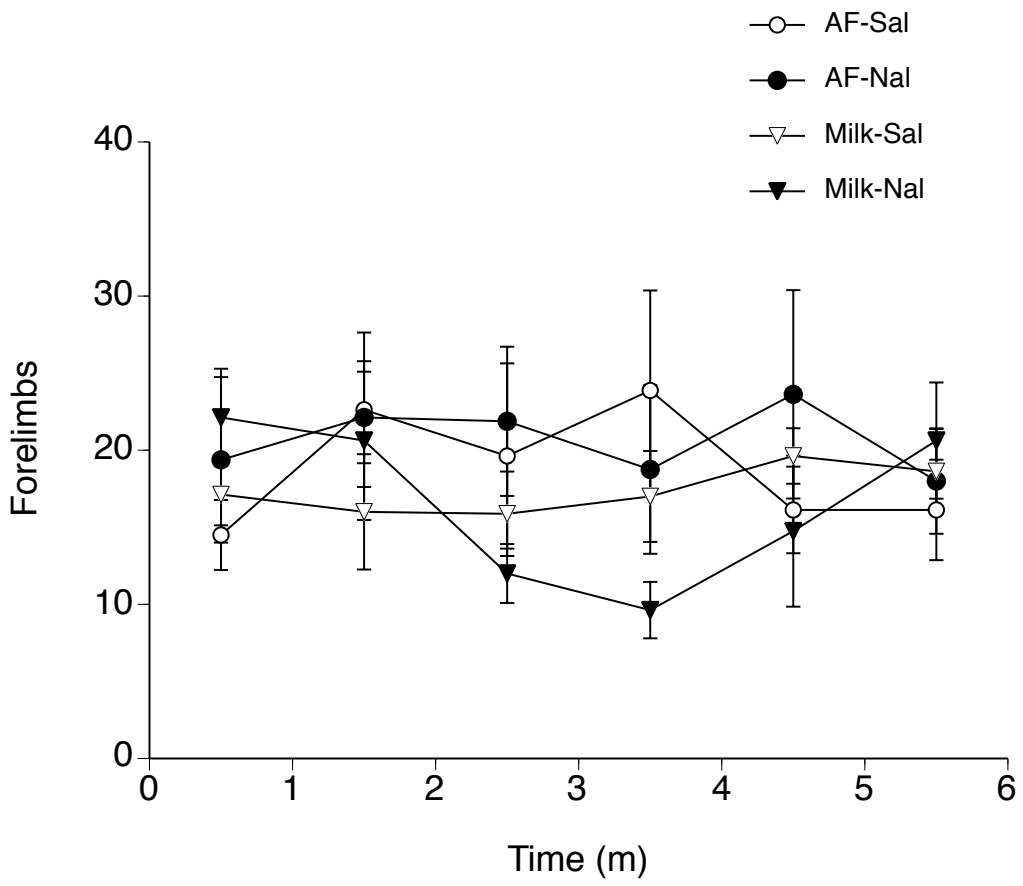

Figure 10. Changes in forelimb activity throughout the 6-min test session of Experiment 2. Pups were pretreated by IP injection of isotonic saline (Sal) or non-selective opioid antagonist naloxone (Nal; 1.0 mg/kg) 5 min before infusion and received 20 µl of an intraoral infusion of AF or milk. Circles and triangles depict mean numbers of forelimb movements per minute. Error bars depict S.E.M.

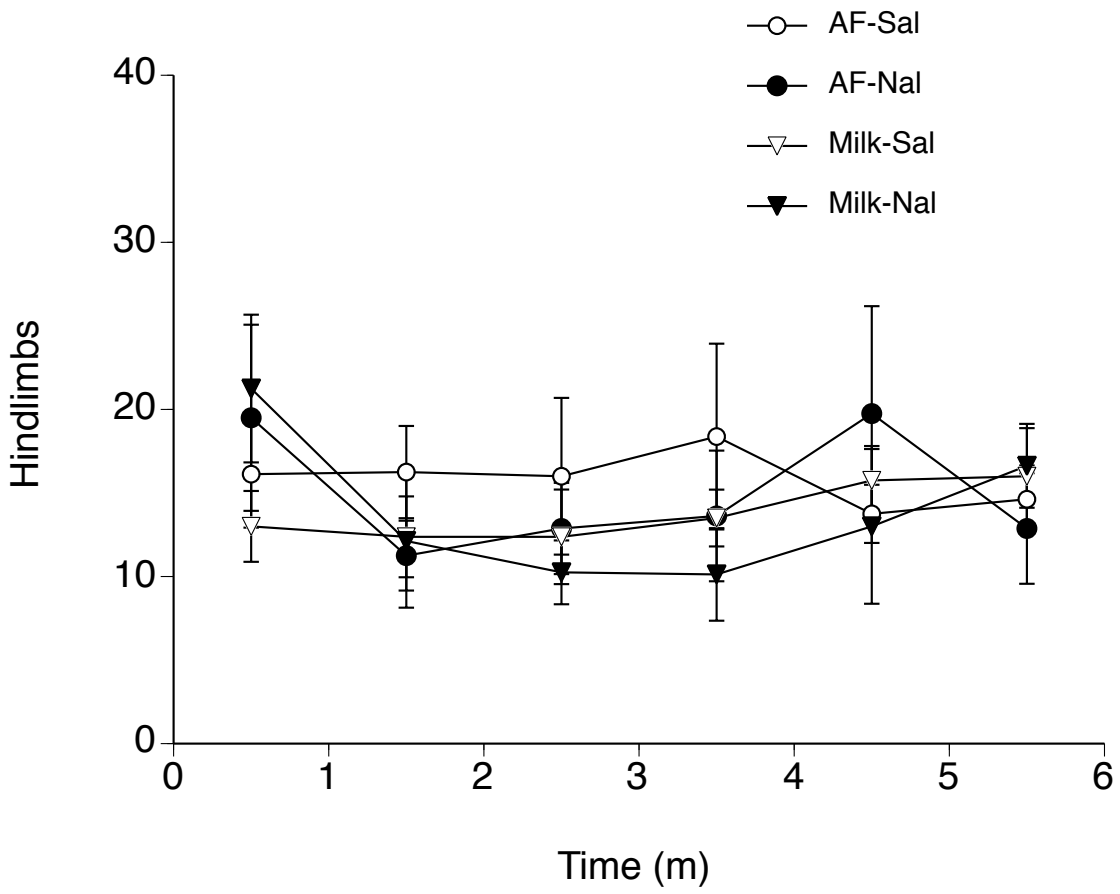

Figure 11. Changes in hindlimb activity throughout the 6-min test session of Experiment 2. Pups were pretreated by IP injection of isotonic saline (Sal) or non-selective opioid antagonist naloxone (Nal; 1.0 mg/kg) 5 min before infusion and received 20 µl of an intraoral infusion of AF or milk. Circles and triangles depict mean numbers of hindlimb movements per minute. Error bars depict S.E.M.

hindlimb activity was reduced significantly after min 1 and then increased on min 3 and remained high for the rest of the session (Figure 12).

Discussion

The results of Experiment 2 revealed that an intraoral infusion of AF or milk resulted in different effects on behavioral activity in the newborn rat. Although some differences were found between saline-injected and naloxone-injected pups, suggesting activation of the endogenous opioid system, the effects of AF- or milk-induced opioid activity may be limited to only some behavioral categories. Findings in this experiment showed significant results only in the categories of mouthing and head movements when analyzing the frequency of the behavioral events. In mouthing, subjects that received an intraoral infusion of AF showed higher levels of activation at the beginning and the end of the testing session. However, during min 2 subjects infused with milk and treated with saline showed higher levels of mouthing activity. These results suggest that an intraoral infusion of milk and pretreatment with saline results in higher levels of mouthing activity in comparison to oral exposure to AF. Since only subjects in the saline group showed higher levels of mouthing activity, it suggests that pretreatment with naloxone reduces activity in those subjects that received an intraoral infusion of milk.

In contrast, the behavioral category of head movements revealed a more complicated pattern of results. Results showed that an intraoral infusion of AF evoked higher levels of head activity in comparison to subjects infused with milk. While subjects in the AF groups showed a significant increase in activity after the infusion in min 2, subjects in the milk groups showed a decrease in head activity during min 2. Results also

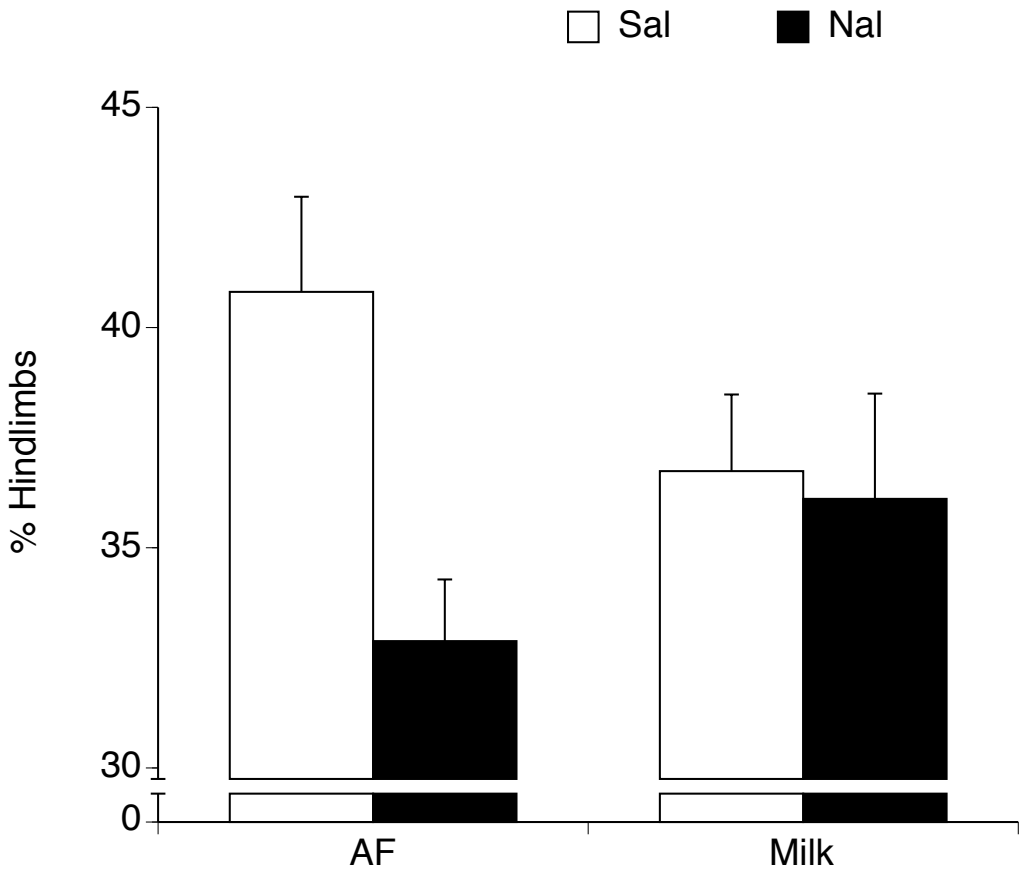

Figure 12. Changes in the frequency of hindlimb activity expressed as a percentage of overall activity in Experiment 2. Pups were pretreated by IP injection of isotonic saline (Sal) or non-selective opioid antagonist naloxone (Nal; 1.0 mg/kg) 5 min before infusion and received 20 µl of an intraoral infusion of AF or milk. Bars show mean percent hindlimb activity over the entire test session. Error bars depict S.E.M.

showed that higher levels of activity after min 2 were only evident in those subjects pretreated with saline. As in the case of milk during mouthing activity, pretreatment with naloxone appeared to reduce head activity in those subjects that received oral exposure to AF. However, pretreatment with naloxone also resulted in higher levels of head activity during min 1, before the infusion, and almost at the end of the session in min 5. The difference during baseline suggests that head activity might be influenced by endogenous opioid activity prior to exposure to AF or milk.

Subjects in all groups did not express significant facial wiping in response to oral exposure to AF or milk. Forelimb and hindlimb activity also did not differ in any of the groups, suggesting not only that AF and milk do not exert different effects on behavioral activity as measured in these categories, but also that opioid responses triggered by AF or milk do not influence these responses.

However, when hindlimb activity was expressed as a percentage of overall activity and compared between groups, results revealed that activity in hindlimbs was significantly higher in the saline group. These results suggest that oral infusion of AF or milk results in opioid activity that alters the distribution of motor activity, with hindlimbs becoming more active.

Taken together, the results of Experiment 2 provide evidence that oral exposure to AF or milk does promote endogenous opioid activity in the newborn rat, which was particularly evident in the analysis of hindlimb activity. These results are consistent with those reported in the fetal rat, in which behavioral activation to exposure to milk can be altered after blockade of the opioid system (Smotherman & Robinson, 1992b). However,

together with results of Experiment 1, Experiment 2 suggests that activity levels are higher after oral exposure to AF and not to milk, although milk was high in some behavioral variables. This last result also demonstrates that oral exposure to AF and milk does not produce similar responses in the newborn rat, contradicting previous reports in which AF and milk play a nearly identical role during the behavioral responses to chemosensory stimulation in the newborn rat (Méndez-Gallardo & Robinson, 2010).

While Experiments 1 and 2 explored whether an intraoral infusion of AF and milk result in similar behavioral responses in the newborn rat, there is the possibility that experiencing AF or milk in odor form differ in its behavioral effects. This possibility was explored in Experiments 3 and 4.

Experiment 3: Behavioral activation to the odor of amniotic fluid or milk

Experiment 3 addressed the question of whether the odor of AF and milk exert similar behavioral effects in the newborn rat. Previous research has described how AF is ecologically relevant after birth because it seems to facilitate the first suckling episode in the newborn rat (Teicher & Blass, 1977), and possibly in the human newborn as well (Varendi et al., 1996). Rat neonates, lambs, and human newborns also appear to recognize the odor of their own AF when compared to the odor of an unfamiliar pregnancy (Hepper, 1987; Schaal et al., 1998; Schaal, Orgeur et al., 1995). These findings suggest that AF has strong olfactory signatures that exert effects on the behavior of the newborn. As Kodama and Smotherman (1997) reported, AF exerts behavioral effects in caesarean delivered rat

pups that lack suckling experience, which show head movements for a longer duration after exposure to the odor of AF. Although studies with newborn rats suggest that they detect the odor of AF and respond to it (Koffman, Petrov, Varlinskaya, & Smotherman, 1998; Teicher & Blass, 1977), there are no studies that thoroughly describe how exposure to the odor of AF affects behavior in the newborn rat.

In a similar way, newborn pups respond to the odor of milk by attaching more quickly to an artificial nipple and engaging in oral grasping for longer duration (Koffman et al., 1998). Similarly, human newborns detect and respond to the odor of breast milk and formula milk (Marlier, Schaal, & Soussignan, 1997, 1998a, 1998b). In addition, Terry and Johanson (1987) described that 3 to 9-day-old rat pups become behaviorally active in response to the odor of milk. Their study did not report these effects in 1-day-old rat pups, but other studies suggest that newborns can detect the odor of milk and respond to it (Koffman et al., 1998). However, there are no studies that have described the effects of exposure to the odor of milk in the newborn rat. The purpose of Experiment 3 was to describe the behavioral response of newborn rats to the odor of AF and milk. The hypothesis in this experiment was that exposure to an infusion of AF or milk should result in similar behavioral activation in the neonatal rat.

Methods

Subjects

Neonatal subjects were the offspring of Sprague-Dawley laboratory rats. Thirty-two pups from 9 litters were used in Experiment 3. Pups were tested 24 hours after birth

(P1). Subjects' breeding and care followed the same protocol described in the General Methods section.

Conditions

Rat pups were assigned to one of four odor conditions (n = 8 pups per group): AF, Milk, Water, or Anise. An equal number of female and male rat pups were tested in each of these conditions.

Procedure

Rat pups were tested 24 hours after birth (P1) and involved exposure to one of the four testing odors: AF, milk, anise, or water. To test the pups, subjects were placed in the base of the testing chamber (as described in the General Methods section) where they were exposed to the odors through an incoming tube directing the odors from the apparatus and placed vertically in the roof of the chamber over the center of the testing area. Pups were tested in an unrestrained prone position. All pups were placed at the same starting point, with their head positioned approximately 3 cm from the wall and the tube. Testing involved a 6-min session starting with a 1-min baseline. After the baseline period and immediately at the beginning of min 2, pups were exposed to the odors of either AF, milk, anise, or water. Behavioral responses were observed during baseline and throughout the 5-min period of odor exposure. After each testing session and removal of each testing subject, the testing chamber was cleared by opening a clean air stream to ventilate any remaining test odors.

Behavioral observations and data analysis

Behavioral observations included scoring of overall activity, including movements of forelimbs, hindlimbs, and head. These observations were made during the entire 6-min testing session. Each test session was recorded to a digital video file. Video files were scored using event-recording software, summarized, and statistically analyzed as described in the General Methods section.

Results

Analysis of each behavioral category (i.e., forelimb, hindlimb, and head) was performed in 2-factor analysis of variance (4 Exposure groups X 6 1-min intervals), with the Time factor treated as a repeated measure. Analysis of head activity showed that while there were no significant differences between odor groups, there was a significant main effect of Time, F (5, 160) = 19.46, $p < .01$ (Figure 13). Within subjects analysis of variance revealed higher levels of head activity during min 1, which significantly decreased after exposure to the odor during min 2 and throughout the rest of the session. Analysis of forelimb activity showed that while there were no significant differences between odor groups, there was a significant main effect of Time, F (5, 160) = 7.61, $p < .01$ (Figure 14). Higher levels of forelimb activity were evident during min 1, which significantly decreased after exposure to the odor during min 2 and throughout the rest of the session. Analysis of hindlimb activity revealed no significant differences between groups or Time, $p > .05$ (Figure 15). An additional 3-way ANOVA compared hindlimb activity between the groups by expressing activity as a percentage of overall activity. This analysis did not reveal any significant differences among groups ($p > .05$).

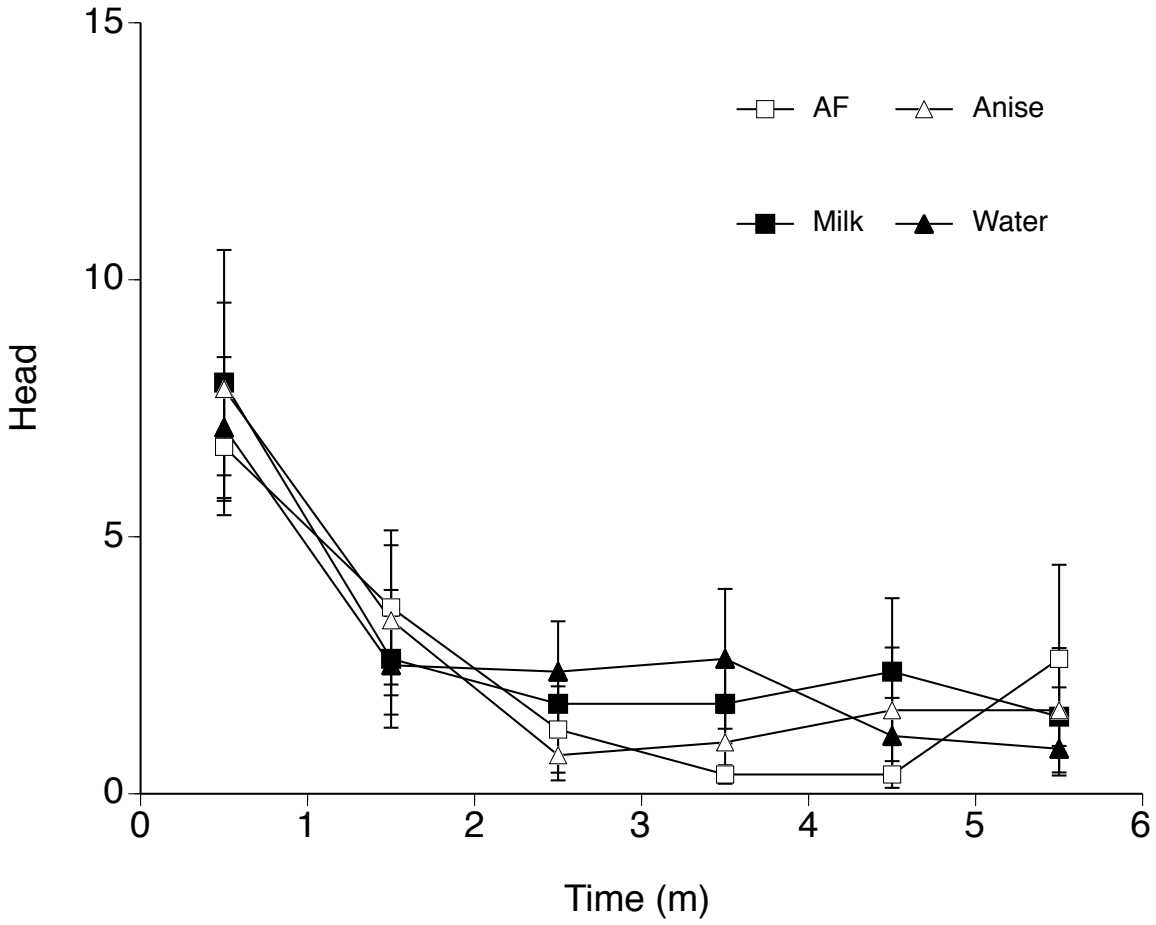

Figure 13. Changes in head activity throughout the 6-min test session of Experiment 3. Pups were exposed to the odor of AF, Milk, Anise, or Water while placed unrestrained inside the testing chamber. Squares and triangles depict mean number of head movements per minute. Error bars depict S.E.M.

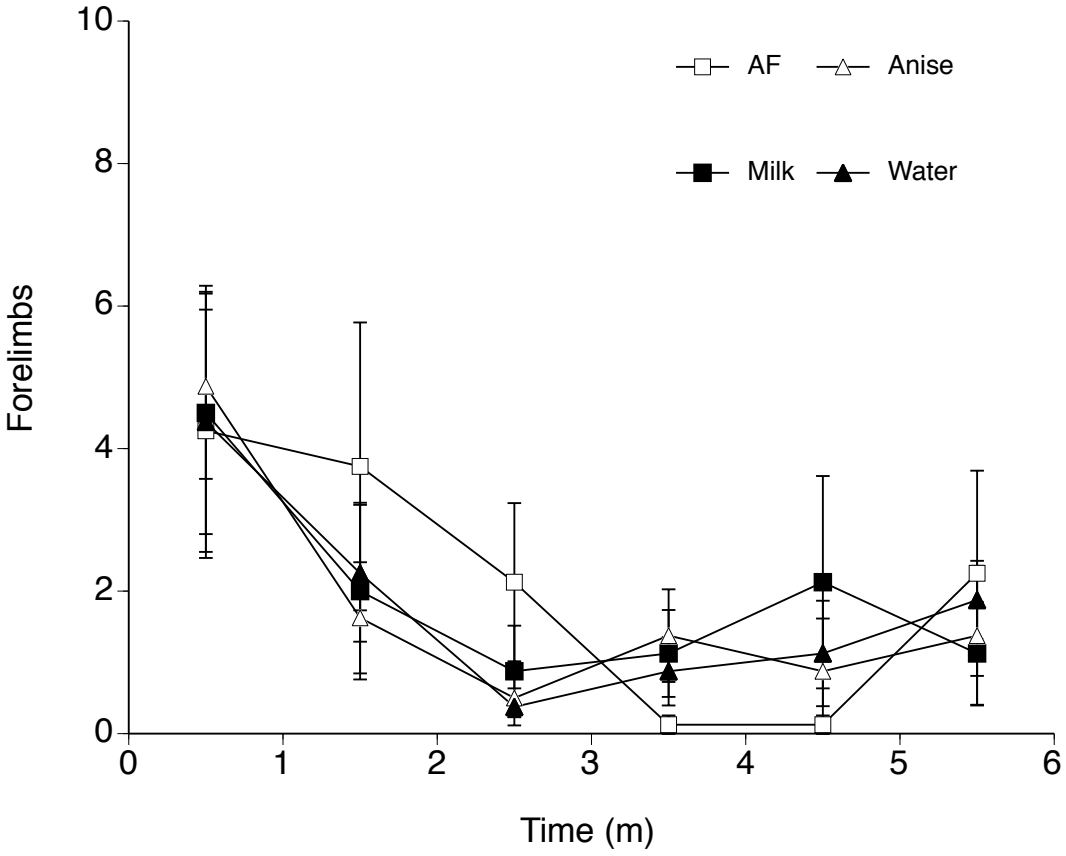

Figure 14. Changes in forelimb activity throughout the 6-min test session of Experiment 3. Pups were exposed to the odor of AF, Milk, Anise, or Water while placed unrestrained inside the testing chamber. Squares and triangles depict mean number of forelimb movements per minute. Error bars depict S.E.M.

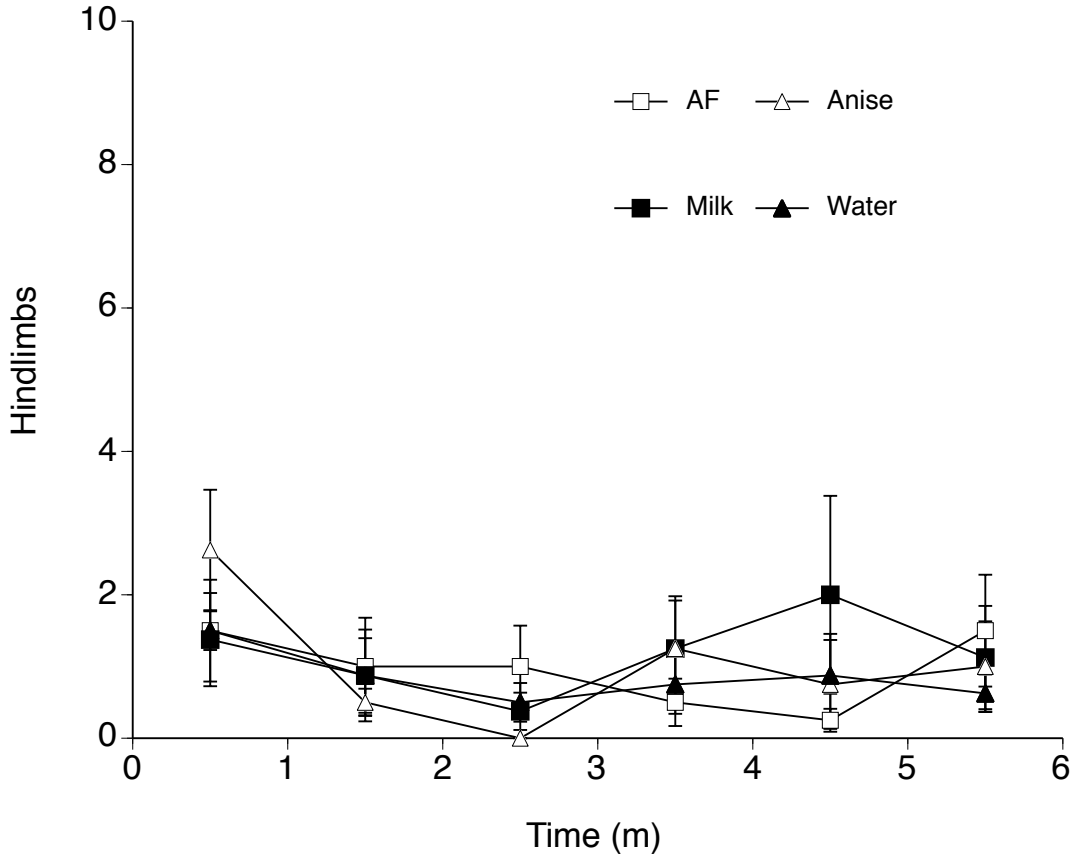

Figure 15. Changes in hindlimb activity throughout the 6-min test session of Experiment 3. Pups were exposed to the odor of AF, Milk, Anise, or Water while placed unrestrained inside the testing chamber. Squares and triangles depict mean number of hindlimb movements per minute. Error bars depict S.E.M.

Discussion

Results of Experiment 3 revealed no evidence of detection of any of the four odors. Results showed that there were no significant differences between any of the four groups in any of the three behavioral categories; head, forelimbs, and hindlimbs. However, an effect of Time was found within the categories of head and forelimb activity where subjects showed a significant decrease in activity after they were exposed to the odor in min 2. Findings of Experiment 3 suggests that newborn rat pups exposed to the odor of AF or milk do not express significant behavioral activation and do not respond differently to any of the odors. On the contrary, odor exposure seems to decrease activity levels.

Experiment 3 provides no evidence that pups respond differentially to the odor of AF or milk, or that odor exposure alone can evoke significant behavioral activation. However, the results of Experiment 3 are not sufficient to conclude that newborns rats cannot detect AF or milk odor such as in a different setting or behavioral context (Hepper, 1987; Teicher & Blass, 1977; Terry & Johanson, 1987). While the lack of significant findings in Experiment 3 may be the result of methodological issues, they may be the consequence of the experience of the newborn pup with these fluids or the context in which these fluids are being presented during this task. Newborn pups may be responsive to the odor of AF or milk only when presented with other stimuli of ecological relevance, such as the nipple during suckling activity. This possibility is explored in Experiments 5 to 8 where the odors of AF and milk are presented in other tasks to evaluate attraction towards the odor.

To follow up Experiment 3, Experiment 4 evaluated whether the endogenous opioid system plays any role during the behavioral response to the odor of AF or milk.

Experiment 4: Opioid mediation of amniotic fluid and milk effects after odor exposure

Experiment 4 addressed the question of whether the behavioral response resulting from exposure to the odor of AF and milk is opioid mediated. Although no effects were found in Experiment 3, Experiment 4 was planned from the outset to evaluate opioid involvement in any responses to odor exposure, and was conducted contemporaneously with Experiment 3. Previous research has described that the odors of AF and milk can be detected by different species of mammals and can affect their behavior. Among these, human newborns (Marlier et al., 1997; 1998a; 1998b; Schaal et al., 1998; Varendi et al., 1996), lambs (Schaal, Orgeur et al., 1995), and rats (Hepper, 1987; Kodama & Smotherman,1997; Koffman et al., 1998; Teicher & Blass, 1977) respond, preferred, and are attracted to the odors of AF and milk. However, although there are studies that suggest that AF and milk exert effects on sensory responsiveness that are mediated by the endogenous opioid system (Korthank & Robinson, 1998; Méndez-Gallardo & Robinson, 2010), there are no reports that describe whether other behavioral effects of the odors of these two important features of pre- and postnatal development may depend upon activity in the endogenous opioid system. The purposes of Experiment 4 was to evaluate whether blockage of opioid activity would influence the behavioral responses of newborn rats to the odor of AF or milk.

Methods

Subjects

Neonatal subjects were the offspring of Sprague-Dawley laboratory rats. Thirty-two pups from 10 litters were used in Experiment 4. Pups were tested 24 hours after birth (P1). Subjects' breeding and care followed the same protocol described in the General Methods section.

Conditions

Rat pups, including one male and one female pup from each litter, were assigned as subjects to one of four conditions resulting from the combination of Pretreatment and Exposure solution. In pretreatment, subjects were assigned to one of two conditions: (a) an intraperitoneal (IP) injection of 50 µl of naloxone (1.0 mg/kg), a nonselective opioid antagonist, or (b) an IP injection of the vehicle control of isotonic saline (50 µl). In exposure, pups were assigned to one of two odor conditions: AF or milk. Thus, four treatment groups (n = 8 pups per group) resulted from the combination of the pretreatment conditions (Naloxone or Saline) and the exposure solution (AF or milk): AF-Naloxone, AF-Saline, Milk-Naloxone, and Milk-Saline.

Procedure

The experimental procedure was exactly as described in Experiment 2 with the exception that before testing pups were pretreated with naloxone or saline. Testing commenced 5 min after pretreatment.

Behavioral observations and data analysis

Behavioral observations included scoring of overall activity, including movements of forelimbs, hindlimbs, and head. These observations were made during the entire 6-min testing session, beginning 5-min after pretreatment. Each test session was recorded to a digital video file. Video files were scored using event-recording software, summarized, and analyzed as described in the General Methods section.

Results

Analysis of each behavioral category (i.e., forelimb, hindlimb, and head) was performed in 3-factor analysis of variance (2 Pretreatment groups X 2 Exposure groups X 6 1-min intervals), with the Time factor treated as a repeated measure. Additional within subjects ANOVAs were performed to identify specific differences in the different variables.

Analysis of head activity showed no significant differences between odor or drug groups, and no significant interactions (ps > .05). There was, however, a significant main effect of Time across all groups, F (5, 140) = 25.96, p < .01 (Figure 16). Within subjects analysis of variance revealed a significant decrease in head activity after exposure to the odor (min 2). Analysis of forelimb activity showed that there were significant differences in activity across all odor groups, F (1, 28) = 10.83, p < .01, as well as significant differences between drug groups, F (1, 28) = 4.97, p < .05, and a significant main effect of Time, F (5, 140) = 2.47, p < .05 (Figure 17).

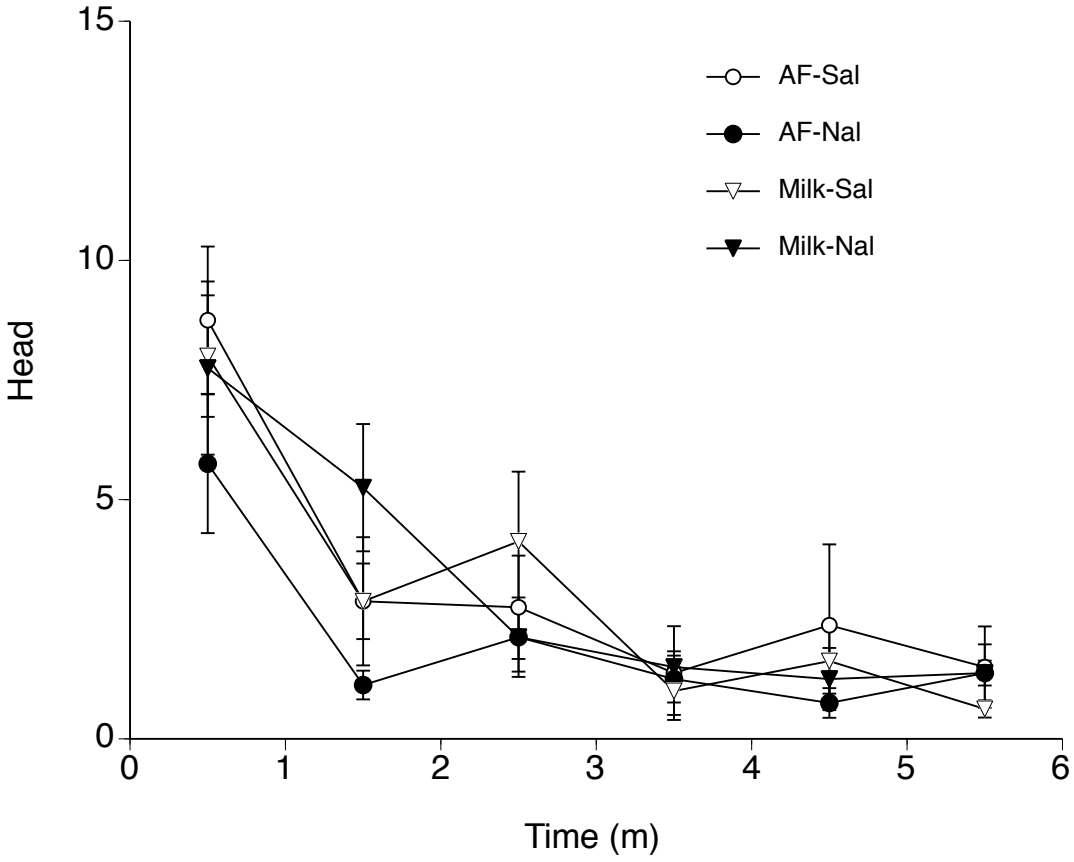

Figure 16. Changes in head activity throughout the 6-min test session of Experiment 4. Pups were pretreated by IP injection of isotonic saline (Sal) or the non-selective opioid antagonist naloxone (Nal; 1.0 mg/kg) before testing. During testing, pups were exposed to the odor of AF or milk while placed unrestrained inside the testing chamber. Circles and triangles depict mean number of head movements per minute. Error bars depict S.E.M.

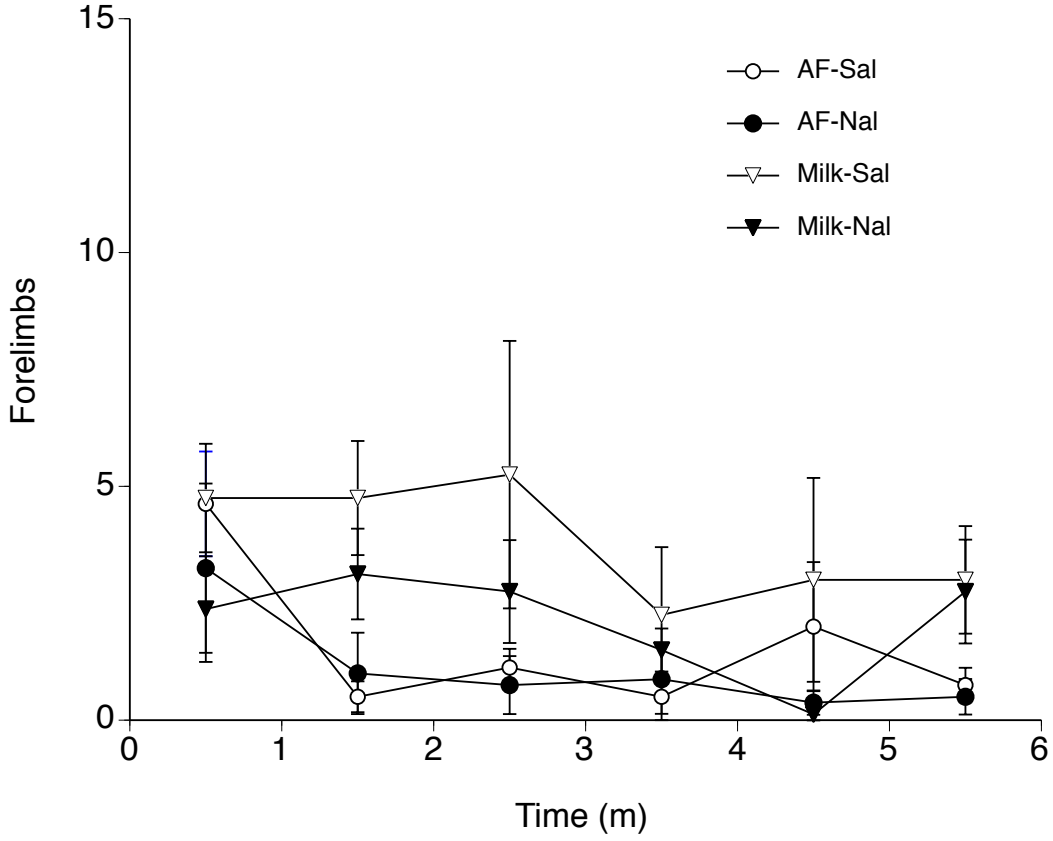

Figure 17. Changes in forelimb activity throughout the 6-min test session of Experiment 4. Pups were pretreated by IP injection of isotonic saline (Sal) or the non-selective opioid antagonist naloxone (Nal; 1.0 mg/kg) before testing. During testing, pups were exposed to the odor of AF or milk while placed unrestrained inside the testing chamber. Circles and triangles depict mean number of forelimb movements per minute. Error bars depict S.E.M.

Analysis did not reveal any significant interactions in the Forelimb category. Higher levels of forelimb activity were evident after exposure to the odor of milk than AF, and higher levels of forelimb activity occurred after pretreatment with saline than naloxone. Within subjects analysis of variance showed a significant difference across Time, F (5, 140) = 2.47, p < . 05, where forelimb activity was higher during min 1 before the infusion and started to decrease at min 2 and throughout the rest of the session.

Analysis of hindlimb activity showed that there were significant differences between odor groups, F (1, 28) = 15.54, p < .001 (Figure 18), with higher levels of hindlimb activity in the milk odor group. An additional 3-way ANOVA compared hindlimb activity between the groups by expressing activity as a percentage of overall activity. This analysis found that relative hindlimb activity was significantly higher in subjects treated with milk odor, F (1, 28) = 7.90, p < .01, (Figure 19). However, no significant main or interaction effects involving pretreatment with saline or naloxone were found.

Discussion

The results of Experiment 4 provided little evidence of opioid involvement during exposure to the odor of AF or milk. Comparable to Experiment 3, in Experiment 4 forelimb and head activity decreased significantly after odor exposure during min 2. However, in this experiment, forelimbs and hindlimbs showed higher levels of activity in those subjects that were exposed to the odor of milk. Forelimb activity also was expressed at higher levels in subjects that were pretreated with saline. This last finding suggests that blockade of the opioid system may reduce general motor activity, at least in

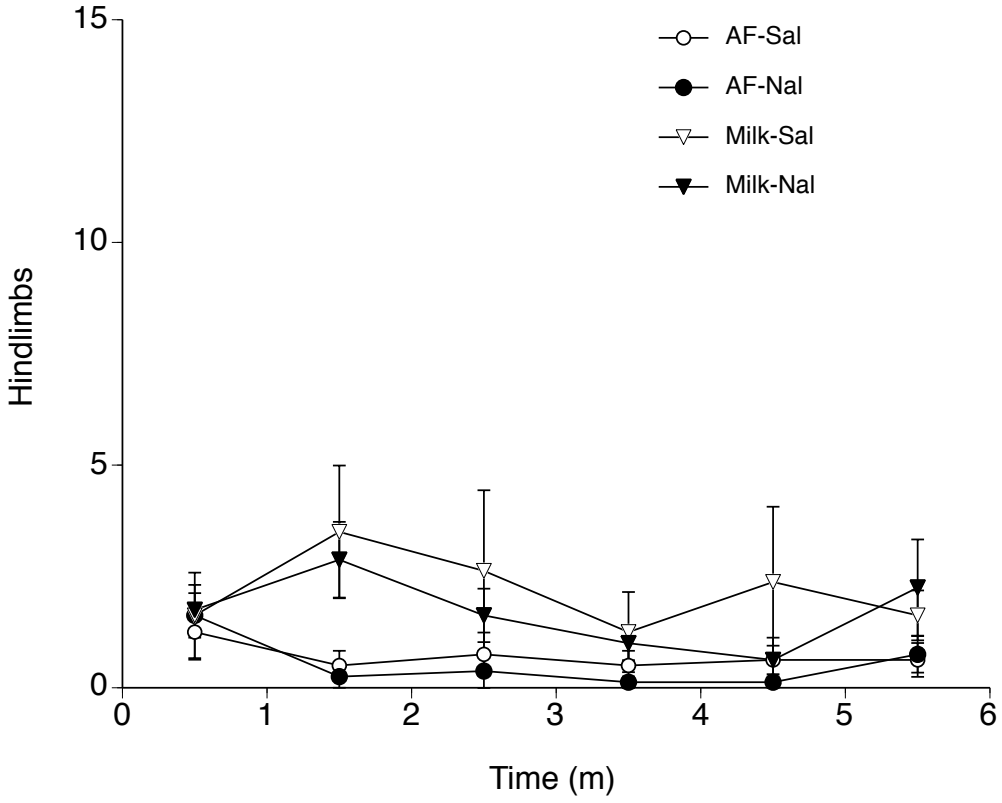

Figure 18. Changes in hindlimb activity throughout the 6-min test session of Experiment 4. Pups were pretreated by IP injection of isotonic saline (Sal) or the non-selective opioid antagonist naloxone (Nal; 1.0 mg/kg) before testing. During testing, pups were exposed to the odor of AF or milk while placed unrestrained inside the testing chamber. Circles and triangles depict mean number of hindlimb movements per minute. Error bars depict S.E.M.

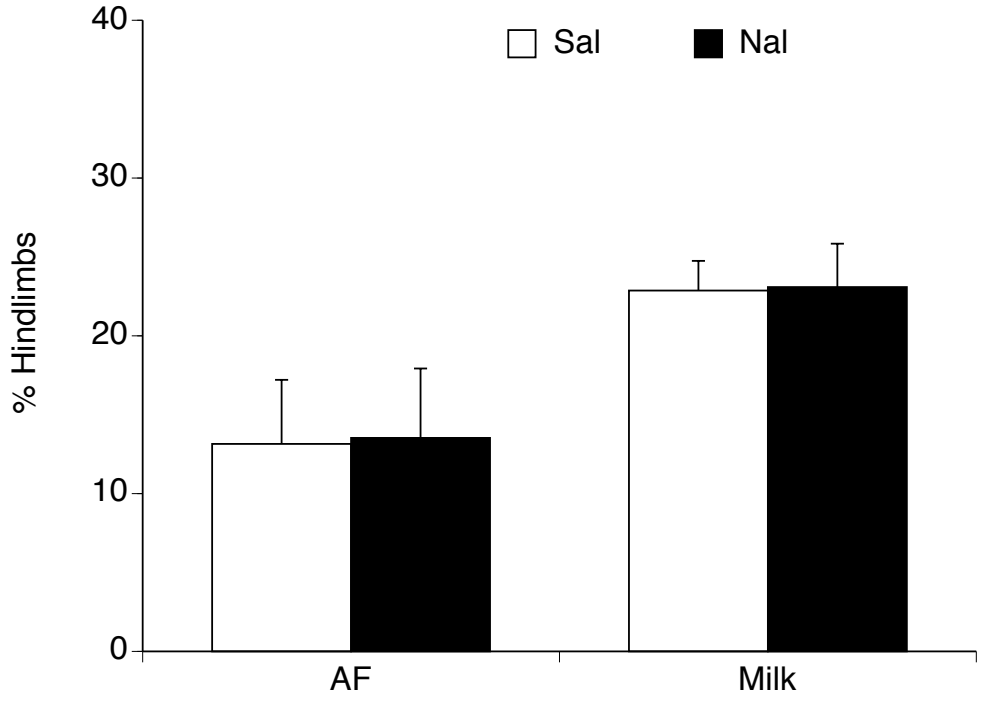

Figure 19. Changes in the frequency of hindlimb activity expressed as a percentage of overall activity in Experiment 4. Pups were pretreated by IP injection of isotonic saline (Sal) or the non-selective opioid antagonist naloxone (Nal; 1.0 mg/kg) before testing. During testing, pups were exposed to the odor of AF or milk. Bars show mean percent hindlimb activity over the entire test session. Error bars depict S.E.M.

the forelimb category. Results of Experiment 4 also suggest that, as described in Experiments 1 and 2, while oral exposure of AF results in higher activation than oral exposure to milk, odor exposure to milk results in higher activation than odor exposure to AF. These results suggest that experiencing AF and milk in odor form results in different effects on behavioral activity in the newborn rat than when AF and milk are presented through oral exposure.

While there are no reports that describe the role of the opioid system during exposure to the odor of AF and milk, results of Experiment 4 do not accord with the results of Experiment 2 or with other reports of opioid involvement during the behavioral response to oral exposure to AF and milk after chemosensory stimulation in the fetal and newborn rat (Korthank & Robinson, 1998; Méndez-Gallardo & Robinson, 2010). Together with Experiment 3, the results from odor exposure to AF or milk do not provide sufficient evidence to suggest that pretreatment with naloxone significantly alters the behavioral response of newborn rat pups to the odor of AF or milk. As a consequence, they provide little support for the hypothesis that the opioid system is activated by exposure to the odor of AF or milk.

Chapter 5 of this study comprises four experiments that continue to evaluate whether the odor of AF and milk are attractive to the newborn rat in other behavioral contexts, including when presented during exposure to an artificial nipple and during a test for quadrupedal locomotion. Together with the findings of Experiment 3 and 4, Experiments 5-8 contribute to a more complete understanding of the behavioral response of the newborn rat to the odor of AF and milk.

CHAPTER 5: ATTRACTIVE PROPERTIES OF AMNIOTIC FLUID AND MILK

In Chapter 5, Experiments 5, 6, 7, and 8 investigated how AF and milk may affect behavioral responses that have biological value to the neonate, and whether these behavioral effects depend on opioid activity. Two of the experiments (5 and 6) determined whether AF, milk or a novel odor facilitates locomotor behavior — quadrupedal crawling — oriented toward the source of the odor. The other two experiments (7 and 8) determined whether AF, milk or a novel odor influence pup responses to an artificial nipple.

Experiment 5: Crawling activity during exposure to the odor of amniotic fluid or milk

Experiment 5 addressed the question of whether AF or milk can elicit crawling activity in the newborn rat. Previous research has described the capacity of newborn rats to express crawling (Altman & Sudarshan, 1975; Eilam & Smotherman, 1998; Gramsbergen, 1998; Westerga & Gramsbergen, 1993). In addition, it has been reported that rats exposed to the odor of soiled nest material on postnatal days 2 and 3 show crawling toward the source of the odor (Fady et al., 1998; Jamon & Clarac, 1998; Sczerzenie & Hsiao, 1977). However, the capacity of newborn rats to show crawling behavior during exposure to the odor of AF or milk has not been documented. Experiment 5 describes the effects of AF and milk exposure in the newborn rat and whether these biologically relevant fluids can elicit locomotion at this early age. The

hypothesis of this experiment was that presentation of the odor of AF or milk would result in a crawling behavioral response in the neonatal rat.

Methods

Subjects

Neonatal subjects were the offspring of Sprague-Dawley laboratory rats. Forty-eight pups from 14 litters were used in Experiment 5. Pups were tested 24 hours after birth (P1). Subjects' breeding and care followed the same protocol described in the General Methods section.

Conditions

Pups were assigned to one of four odor conditions (n = 12 pups per group): AF, Milk, Water, or Anise. Female and male rat pups were tested in each of these conditions.

Procedure

Rat pups were tested 24 hours after birth (P1). Testing involved exposure to the odor of AF, milk, anise, or water to evoke crawling behavior. Pups were tested unrestrained in a prone posture on a 50-cm test runway inside a testing chamber (27°C ± 0.5) as described in the General Methods section. Pups were first acclimated for 30-min in a warm incubator (35°C ± 0.3) and then moved to the testing chamber for another 20-min period at a lower temperature (27°C ± 0.5). Testing involved placing the subject at the beginning of the runway, with the nose aligned with a starting line 5 cm from the border of the runway and moving the tube containing the test solution over the pup's snout to expose them directly to the odor. Pups were exposed to the odor for 3 min. If the subject traversed the complete runway (distance = 50 cm) before 3 min, then the testing

session for that subject was completed. Otherwise, the session was completed 3-min after exposure to the odor.

Behavioral observations and data analysis

Behavioral observations included scoring the time required for the pup to move along the runway, as well as the distance that was completed. These observations were made during the entire 3-min testing session. Each test session was recorded to a digital video file. Video files were scored using event-recording software, summarized, and analyzed as described in the General Methods section.

Results

Analysis of the main dependent variable (i.e., distance traveled in 3 min) was performed in 1-factor analysis of variance (4 Exposure groups). Results revealed that there were significant differences in distance completed between subjects across groups, F (3, 44) = 6.12, $p < .01$ (Figure 20). Post hoc comparison of means by the method of Fisher PLSD indicated significantly more distance completed among subjects that were exposed to the odor of AF and milk compared to subjects in the anise and water groups. The analysis did not show significant differences in distance completed between subjects in the AF and milk groups, but only that these two groups crawled significantly farther than the anise and water groups. Data also showed that some subjects that traversed the complete 50 cm of the runway in response to the odor of AF or milk did it as fast as 1 min 30 sec.

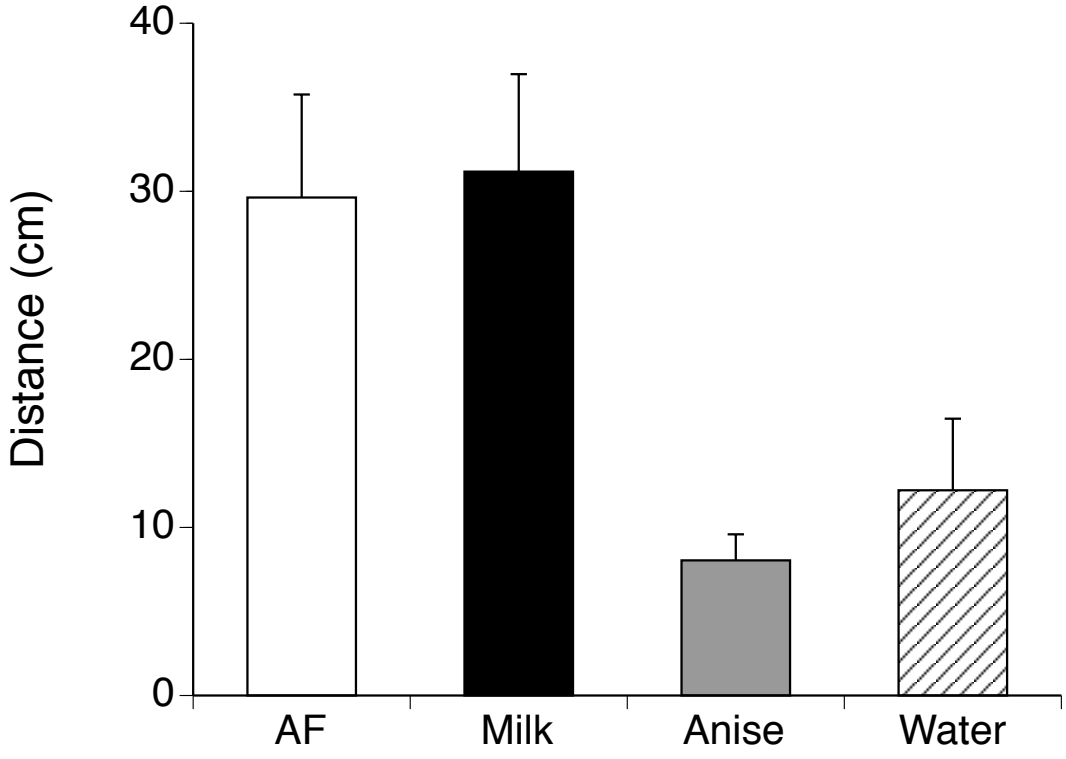

Figure 20. Mean distance traveled (cm) by P1 pups in response to the odors of AF, milk, anise, or water in Experiment 5. Error bars depict S. E. M.

Discussion

Results of Experiment 5 revealed that exposure to the odor of AF or milk induces crawling locomotion in the newborn rat. The findings are in accordance with previous reports that newborn rats tested 2-3 days after birth show crawling in response to the odor of nest material (Fady et al., 1998; Jamon & Clarac, 1998; Sczerzenie & Hsiao, 1977). However, the findings of Experiment 5 are, to our knowledge, the first to document crawling towards the odor of AF and milk in the rat 1 day after birth.

Contrary to Experiment 5, subjects in Experiment 3 did not show significant behavioral activation to the odor of AF and milk when presented in the air inside a testing chamber. This difference between Experiments 3 and 5 may be due to the way in which odors were presented in the task. The mode of presentation in Experiment 5 allowed a direct exposure of the odor to the snout of the pup rather than exposure to the odor in the open air as in Experiment 3. In the current experiment, the effect of odor exposure also may have been augmented by the tactile stimulus of the tubing in which the odors were presented, which was placed directly over the pup's snout. Although perioral tactile stimulation may evoke some level of crawling, significant differences between groups suggest that pups not only detect the odor of AF and milk, but also respond to it in a strong and consistent manner by expressing locomotion towards the odor source.

The next step is to evaluate whether the endogenous opioid system plays a mediating role in the crawling response to the odor of AF and milk in the newborn rat. Experiment 6 investigates this aspect.

Experiment 6: Opioid mediation of crawling activity during exposure to the odor of amniotic fluid or milk

Experiment 6 addresses the question of whether crawling activity evoked by the odor of AF or milk is mediated by the endogenous opioid system. Experiment 6, together with Experiment 5, provides information about the effectiveness of AF and milk odor to evoke and orient crawling activity, and whether AF- or milk-induced crawling is dependent upon activity in the opioid system. The hypothesis of this experiment was that presentation to the odor of AF or milk would result in a crawling behavioral response in the neonatal rat that can be altered by blockade of the endogenous opioid system.

Methods

Subjects

Neonatal subjects were the offspring of Sprague-Dawley laboratory rats. Forty-eight pups from 14 litters were used in Experiment 6. Pups were tested 24 hours after birth (P1). Subjects' breeding and care followed the same protocol described in the General Methods section.

Conditions

Rat pups, including one male and one female pup from each litter, were assigned as subjects to one of four conditions resulting from the combination of Pretreatment and Odor Exposure. In pretreatment, subjects were assigned to one of two conditions: (a) an intraperitoneal (IP) injection of 50 µl of naloxone (1.0 mg/kg), a nonselective opioid antagonist, or (b) an IP injection of the vehicle control of isotonic saline (50 µl). In odor exposure, pups were assigned to one of two conditions: AF or milk. Thus, four treatment

groups (n = 12 pups per group) resulted from the combination of the pretreatment conditions (Naloxone or Saline) and the exposure solution (AF or milk): AF-Naloxone, AF-Saline, Milk-Naloxone, and Milk-Saline.

Procedure

The experimental procedure was the same as described in Experiment 5. However, in Experiment 6 pups first were pretreated with naloxone or saline following the 30-min acclimation period. Pups were pretreated before they were placed in the testing chamber so that they remained undisturbed during the 20-min period before the crawling test. Testing commenced 20 min after pups were placed in the testing chamber, as described in Experiment 5.

Behavioral observations and data analysis

Behavioral observations included scoring the time required for the pup to move along the length of the runway, as well as the distance that was completed. These observations were made during the entire 3-min testing session. Each test session was recorded to a digital video file. Video files were scored using event-recording software, summarized, and analyzed as described in the General Methods section.

Results

Analysis of the main dependent variable (distance traveled in 3 min) was performed in a 2-factor analysis of variance (2 Pretreatment groups X 2 Odor Exposure groups). Results showed that there were no significant differences between the odor or the drugs groups, as well as no interactions, *p*s > .05 (Figure 21). Data also showed that

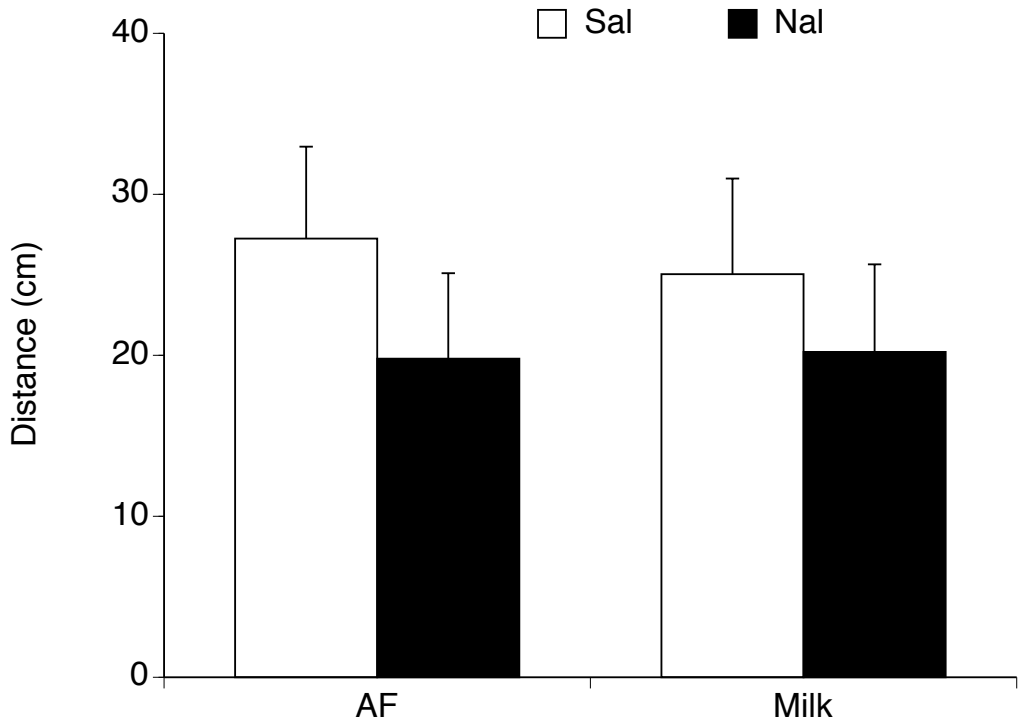

Figure 21. Mean distance traveled (cm) by P1 pups in Experiment 6. Pups were pretreated by IP injection of isotonic saline (Sal) or the non-selective opioid antagonist naloxone (Nal; 1.0 mg/kg) before odor exposure and were exposed to the odor of AF or milk. Error bars depict S.E.M.

some subjects that traversed the complete 50 cm of the runway in response to the odor of AF or milk did it as fast as 1 min 29 sec.

Discussion

Results of Experiment 6 did not provide evidence of mediation of the endogenous opioid system during the crawling response to the odors of AF and milk. In this experiment, the odor of AF and milk continued to evoke a crawling response in the P1 rat, but this response could not be reduced by blockade of receptors in the opioid system. While these results are not consistent with the opioid-induced behavioral effects of oral exposure to AF and milk described in the newborn rat (Korthank & Robinson, 1998; Méndez-Gallardo & Robinson, 2010), they are consistent with the results of Experiment 4, where no opioid effect on general activity was found during odor exposure to AF and milk. Together with Experiment 4, Experiment 6 suggests that if the opioid system is involved in the behavioral response of newborn rats to the odors of AF or milk, the component that activates the opioid system during oral exposure to AF or milk may not be available to the pup in odor form.

Experiments 7 and 8 continue to evaluate the behavioral response of the newborn rat to AF and milk odors in another task of ecological relevance: behavioral responses to an artificial nipple.

Experiment 7: Responses to an artificial nipple during exposure to the odor of amniotic fluid or milk

Experiment 7 addressed the question of whether the odors of AF and milk have an effect on the behavioral responses toward an artificial nipple in the newborn rat. Previous research has described the capacity of rat fetuses (Browne et al., 1994; Robinson et al., 1992) and rat neonates (Smotherman, Goffman et al., 1997) to respond to an artificial nipple by expressing an oral grasp response. Koffman, Petrov, Varlinskaya, and Smotherman (1998) conducted a study in which caesarean-delivered E21 pups were exposed to AF or milk in an oral grasping task. They reported that the presence of AF or milk odor during exposure to an artificial nipple decreased the latency to grasp the nipple, increased the number of grasps, and lengthened the duration of grasping the nipple. Experiment 7 attempted to replicate and extend the study by Koffman and collaborators (1998) by exposing AF or milk to vaginally-delivered pups tested 24 hrs after birth. The findings from this experiment provide information about differential responses to the odors of AF, milk, and anise in an experimental situation that simulates the ecological context of suckling. The hypothesis of this experiment was that exposure to the odor of AF or milk in an artificial nipple would alter behavioral responses towards the nipple.

Methods

Subjects

Neonatal subjects were the offspring of Sprague-Dawley laboratory rats. Thirty-two pups from 10 litters were used in Experiment 6. Pups were tested 24 hours after birth

(P1). Subjects' breeding and care followed the same protocol described in the General Methods section.

Conditions

Rat pups were assigned to one of four odor conditions (n = 8 pups per group): AF, Milk, Water, or Anise. Equal numbers of female and male rat pups were tested in each of these conditions.

Procedure

Rat pups were tested 24-hours after birth (P1). Testing involved experimental presentation of an artificial nipple attached to an absorbant pad containing an odorant to evoke oral behavioral responses toward the nipple. Pups were tested in a supine position inside a warm incubator (35°C ± 0.3). After preparing the pups for testing and acclimation (as described in the General Methods section), they were tested in a 4-min test. The test period included a 1-min baseline, presentation of the artificial nipple at the end of min 1, and exposure to the nipple for 3-min. The nipple had a cotton pad attached containing the odor of AF, milk, anise, or water as a control stimulus.

Behavioral observations and data analysis

Behavioral observations included scoring of behaviors such as oral grasping of the nipple, paw dorsiflexion, paw plantarflexion, head aversion, head ventriflexion, and mouthing. These observations were made during the entire 4-min testing session. Each test session was recorded to a digital video file. Video files were scored at reduced speed using event-recording software, summarized, and analyzed as described in the General Methods section.

Results

Behavioral scoring revealed no occurrence of many of the variables during baseline (min 1), before presentation of the artificial nipple. In addition, two of the variables — oral grasping and head aversion — could not be expressed until presentation of the artificial nipple began. As a consequence, analyses were conducted only during min 2-4, during nipple presentation. Analysis of each behavioral category (i.e., oral grasping, paw dorsiflexion, paw plantarflexion, head aversion, head ventriflexion, and mouthing) was performed in 2-factor analysis of variance (4 Exposure groups X Sum of frequencies in Min 2-4).

Data analysis revealed a very low frequency of oral grasping across all groups. There were no significant differences among groups ($p > .05$).

There was a significant group effect on overall mouthing behavior, $F\,(3, 28) = 8.42$, $p < .001$ (Figure 22). Post hoc comparison of means by the method of Fisher PLSD indicated significantly more mouthing activity in the AF and milk groups than in the anise and water groups (ps $< .05$). Mouthing responses did not differ between subjects in the AF and milk groups, nor between the anise and water groups.

The results showed no significant differences in head ventriflexion among groups. However, the overall effect approached significance ($p = .078$), with the pattern consistent with more ventriflexion in the AF and milk groups (Figure 23). In contrast, head aversion varied significantly among groups, $F\,(3, 28) = 3.58$, $p < .05$ (Figure 24). Post hoc comparison of means by the method of Fisher PLSD indicated that head aversion activity was higher in the anise group than in the AF group ($p < .05$). The

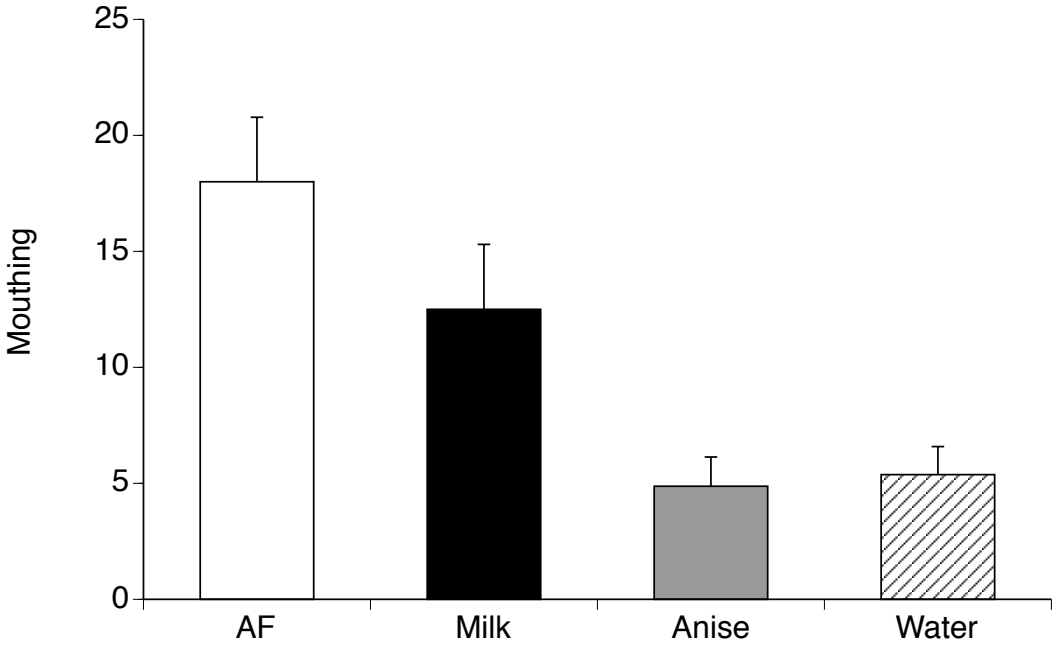

Figure 22. Mouthing responses during exposure to the artificial nipple in Experiment 7. Pups were presented with the nipple along with the odor of AF, milk, anise, or water. Bars depict mean numbers of mouthing movements during the 3-min period of exposure to the artificial nipple. Error bars depict S.E.M.

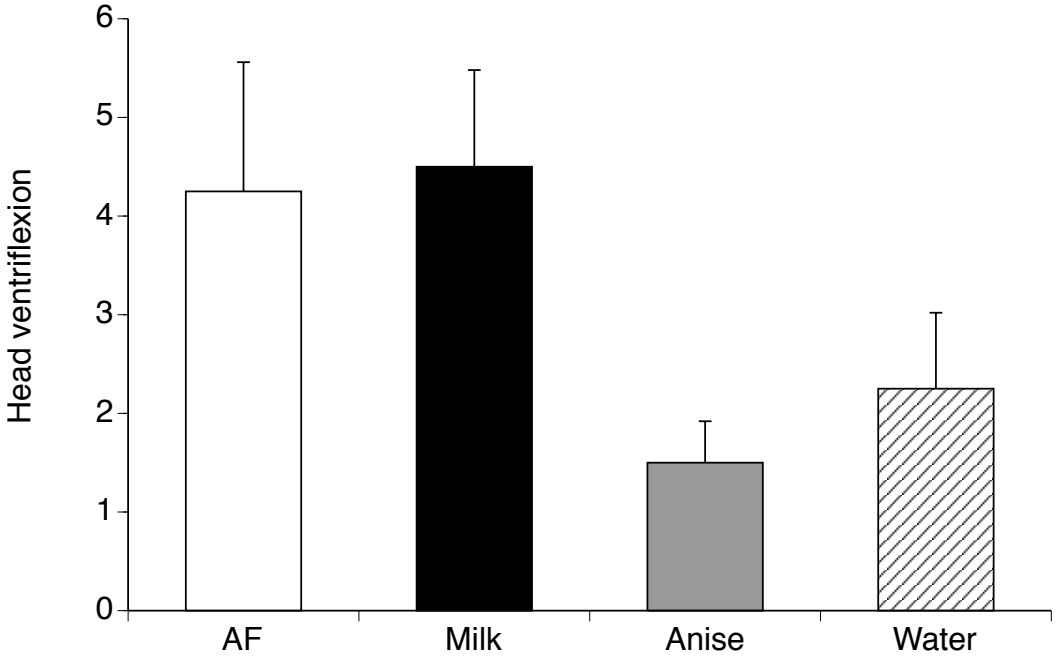

Figure 23. Head ventriflexion responses during exposure to the artificial nipple in Experiment 7. Pups were presented with the nipple along with the odor of AF, milk, anise, or water. Bars depict mean number of head ventriflexion movements during the 3-min period of exposure to the artificial nipple. Error bars depict S.E.M.

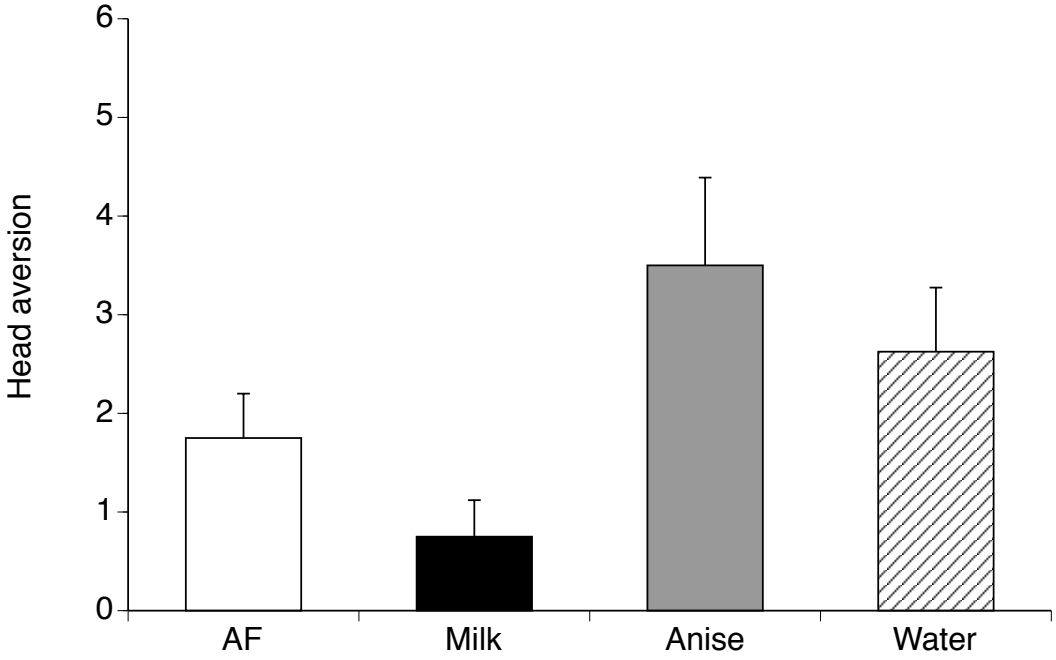

Figure 24. Head aversion responses during exposure to the artificial nipple in Experiment 7. Pups were presented with the nipple along with the odor of AF, milk, anise, or water. Bars depict mean number of head aversion movements during the 3-min period of exposure to the artificial nipple. Error bars depict S.E.M.

analysis of paw plantarflexion revealed significant differences among groups, F (3, 28) = 7.16, $p < .01$ (Figure 25). Post hoc comparison of means by the method of Fisher PLSD indicated significantly more paw plantarflexion activity in subjects in the AF group than in subjects in the milk, anise, or water groups ($p < .05$). Paw dorsiflexion also differed significantly among groups, F (3, 28) = 3.29, $p < .05$ (Figure 26). Post hoc comparison of means by the method of Fisher PLSD indicated significantly more paw dorsiflexion activity in the milk group in comparison to the AF, anise, and water groups.

Discussion

Results of Experiment 7 did not provide evidence of significant oral grasping to the artificial nipple in response to the odor of AF, milk, anise, or water. However, other behaviors in response to the nipple varied in response to the odor the subject was exposed to. For instance, mouthing was expressed at a higher frequency in subjects that were exposed to the odor of AF or milk in comparison the novel odor of anise or the water control. Since mouthing activity did not differ between AF and milk odors, it suggests that mouthing movements may be a response to an odor that is familiar to the neonate.

While there were no differences in head ventriflexion activity between any of the experimental groups, head aversion occurred more often in subjects in the anise group. These results suggest that in comparison to AF or milk, anise is an unfamiliar stimulus. Rat neonates may be less attracted to anise, or even show mild aversion, than to other stimuli with more ecological relevance, such as AF or milk. For this reason, pups may have responded to the odor of anise by moving their head away from the nipple.

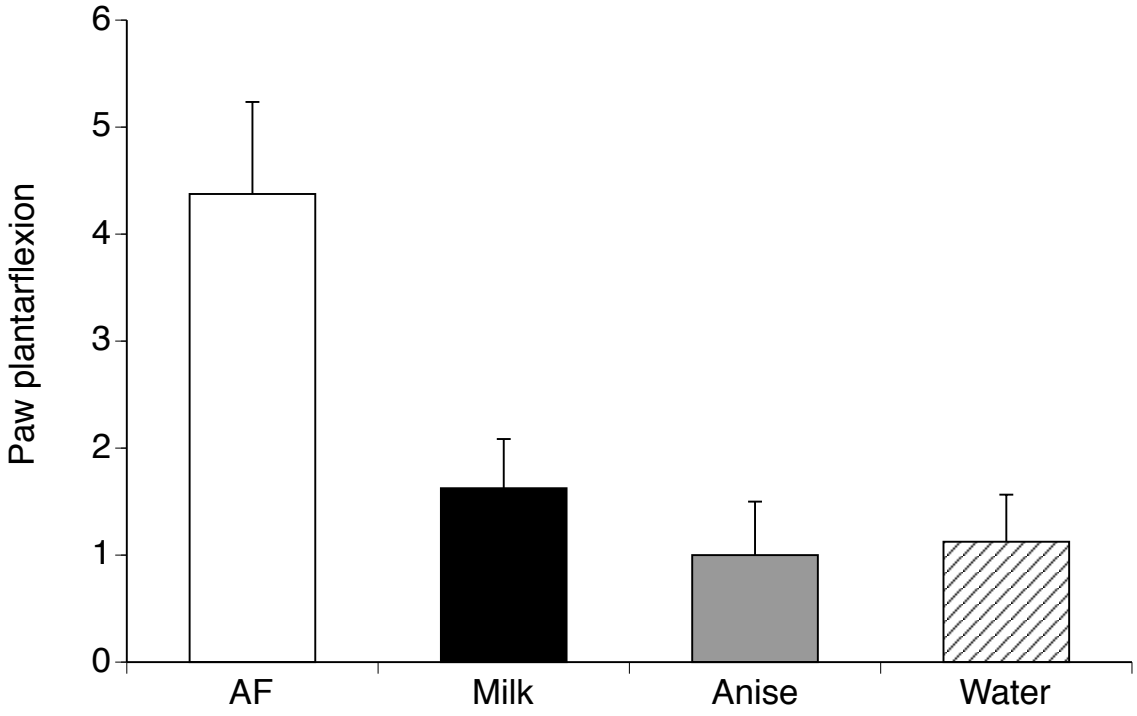

Figure 25. Paw plantarflexion responses during exposure to the artificial nipple in Experiment 7. Pups were presented with the nipple along with the odor of AF, milk, anise, or water. Bars depict mean number of paw plantarflexion movements during the 3-min period of exposure to the artificial nipple. Error bars depict S.E.M.

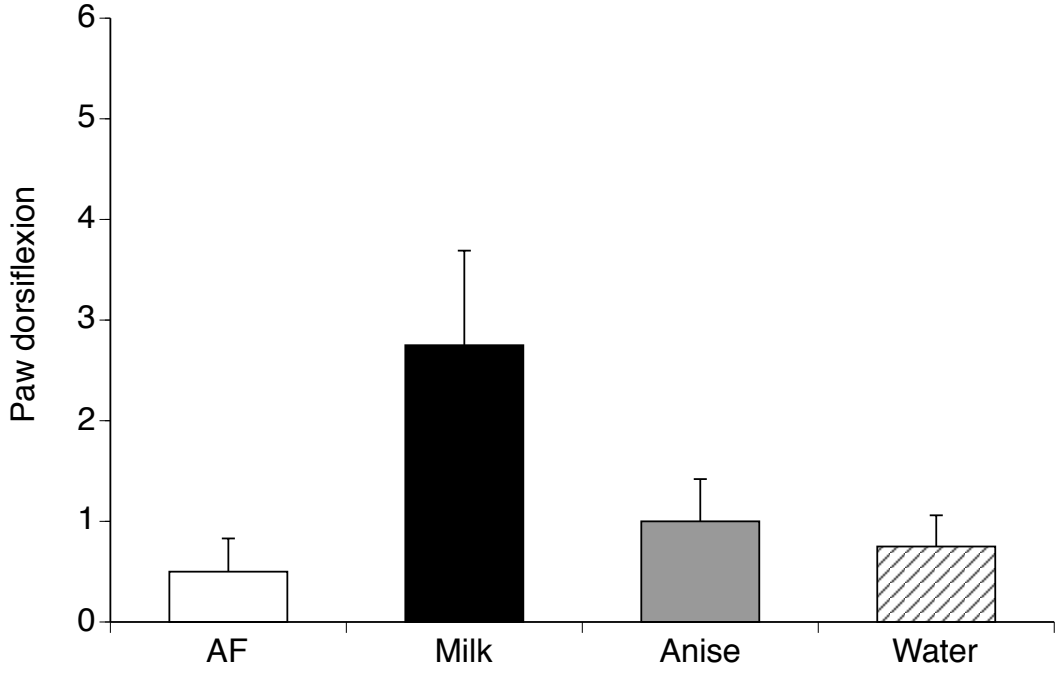

Figure 26. Paw dorsiflexion responses during exposure to the artificial nipple in Experiment 7. Pups were presented with the nipple along with the odor of AF, milk, anise, or water. Bars depict mean number of paw dorsiflexion movements during the 3-min period of exposure to the artificial nipple. Error bars depict S.E.M.

In comparison to head movements, forelimb behavior as quantified by two variables in this experiment — paw plantarflexion and paw dorsiflexion — showed significantly more activity in the AF or milk groups. Specifically, paw plantarflexion occurred more often during nipple exposure in subjects exposed to the odor of AF, while paw dorsiflexion activity was higher in subjects exposed to the odor of milk. These paw behaviors are not well described in the literature, but descriptions of normal behavioral sequences associated with suckling suggest that the occurrence of paw plantarflexion and paw dorsiflexion in response to the artificial nipple and the odors of AF and milk may be behaviors related to suckling and to the experience of the pup with AF and milk. I will return to this possibility in the general discussion in Chapter 7.

Of all of the variables scored in Experiment 7, oral responses to the nipple probably is the most interesting, since newborns must grasp the nipple to suckle. Because exposure to the odor of AF or milk did not consistently evoke oral grasping, these results are not consistent with previous reports that describe that the odors of AF and milk increased oral grasping in caesarean-delivered E21 pups (Koffman et al., 1998). Absence of oral grasping to the artificial nipple in Experiment 7 may be due to methodological differences between this study and previous reports. For instance, the artificial nipple, while similar, was not constructed exactly the same way as in previous studies, and the mode of presentation may have differed as well.

However, other distinctive oral responses during the presentation of the nipple that were scored in Experiment 7 suggest that the behavioral response of the newborn rat to the nipple is dependent on the odor presented in association with the nipple. This does

accord well with reports that the interaction of the subject with the nipple includes other behavioral responses besides oral grasping (Robinson, Arnold et al., 1993). Although in this experiment oral grasping was not reported, many of the other mouthing movements were performed while the artificial nipple rested in contact with the mouth (without being grasped), which suggests that pups were indeed responding to presentation of the nipple. Given that pups showed more mouthing activity to the nipple when presented with the odors of AF and milk, some of these responses may be characteristic of appetitive response or simply responses that reflect the familiarity of the odor. Newborn pups used in this experiment gained hours of experience with milk during suckling between the times of birth and testing (approximately 24-hours), which may have influenced the way they interacted with these stimuli. On the other hand, although AF was not present in the postnatal environment after the process of birth, pups had been exposed to AF during gestation until approximately 24-hours before testing in Experiment 7. As a consequence, pups' oral responses to the odor of AF and milk may be influenced by their recent experience with these stimuli during perinatal life.

Experiment 8: Opioid mediation of responses to an artificial nipple during exposure to the odor of amniotic fluid or milk

Experiment 8 addresses the question of whether the opioid system plays a role during the expression of behavioral responses to an artificial nipple with the odor of AF or milk in the newborn rat. Koffman and collaborators (1998) described the response of caesarean-delivered pups to an artificial nipple presented with the odor of AF or milk.

However, the role of the opioid system during this behavioral response has yet to be described. As in Experiment 6, this experiment explored the effects of exposure to the odor of AF or milk during presentation of an artificial nipple. In addition, Experiment 8 investigated whether the influence of AF or milk odor on the behavioral response of newborn pups to an artificial nipple can be altered by blockade of the endogenous opioid system. The results of this experiment contribute to a more complete understanding of the role of the opioid system during behavioral responsiveness in the context of suckling (i.e., during exposure to an artificial nipple). The hypothesis of this experiment was that blockade of the endogenous opioid system alters the response of the neonatal rat to presentation of an artificial nipple.

Methods

Subjects

Neonatal subjects were the offspring of Sprague-Dawley laboratory rats. Thirty-two pups from 8 litters were used in Experiment 8. Pups were tested 24 hours after birth (P1). Subjects' breeding and care followed the same protocol described in the General Methods section.

Conditions

Rat pups, including one male and one female pup from each litter, were assigned as subjects to one of four conditions resulting from the combination of Pretreatment and Odor Exposure. In pretreatment, subjects were assigned to one of two conditions: (a) an intraperitoneal (IP) injection of 50 µl of naloxone (1.0 mg/kg), a nonselective opioid antagonist, or (b) an IP injection of the vehicle control of isotonic saline (50 µl). During

odor exposure, pups were assigned to one of two conditions: AF or milk. Thus, four treatment groups (n = 8 pups per group) resulted from the combination of the pretreatment conditions (Naloxone or Saline) and the exposure solution (AF or milk): AF-Naloxone, AF-Saline, Milk-Naloxone, and Milk-Saline.

Procedure

The experimental procedure was exactly as described in Experiment 6 with the exception that before testing, pups were pretreated with naloxone or saline. Testing commenced 5 min after pretreatment.

Behavioral observations and data analysis

Behavioral observations included scoring of behaviors such as oral grasping of the nipple, paw dorsiflexion, paw plantarflexion, head aversion, head ventriflexion, and mouthing. These observations were made during the entire 4-min testing session. Each test session were recorded to a digital video file. Video files were scored at reduced speed using event-recording software, summarized, and analyzed as described in the General Methods section.

Results

As in Experiment 7, behavioral scoring revealed no occurrence of many of the variables during baseline (min 1). In addition, two of the variables — oral grasping and head aversion — could not be expressed until presentation of the artificial nipple began. As a consequence, analyses were conducted only during min 2-4, during nipple presentation. Analysis of each behavioral category (i.e., oral grasping, paw dorsiflexion, paw plantarflexion, head aversion, head ventriflexion, and mouthing) was performed in

3-factor analysis of variance (2 Pretreatment groups X 2 Odor Exposure groups X Sum of frequencies in Min 2-4).

Analysis of data revealed a very low frequency of oral grasping across all groups. Results revealed no significant differences between groups ($p > .05$).

Results of mouthing activity, head ventriflexion, or head aversion revealed no significant differences, either as a function of pretreatment, odor exposure, or their interaction ($p > .05$) (Figures 27, 28, and 29). In comparison, results of paw plantarflexion activity showed significant differences between odor groups, F (1, 28) = 23.36, $p < .01$ (Figure 30). Post hoc comparison of means by the method of Fisher PLSD indicated more paw plantarflexion activity in subjects in the AF group in comparison to subjects exposed to the milk odor at the nipple ($p < .05$). Results of paw dorsiflexion activity also showed significant differences between odor groups, F (1, 28) = 8.75, $p < .01$, as well as a significant interaction between odor and drug pretreatment, F (1, 28) = 4.59, $p < .05$ (Figure 31). Post hoc comparisons of simple main effects indicated no difference in paw dorsiflexion activity between saline and naloxone pretreatments in pups exposed to AF odor. However, saline-injected pups exposed to milk odor showed more paw dorsiflexion than naloxone-injected pups ($p < .05$).

Discussion

The results of Experiment 8 provided little evidence that endogenous opioid activity influences behavioral responses to the artificial nipple in the neonatal rat. The behavioral categories of mouthing, head aversion, and head ventriflexion showed no drug

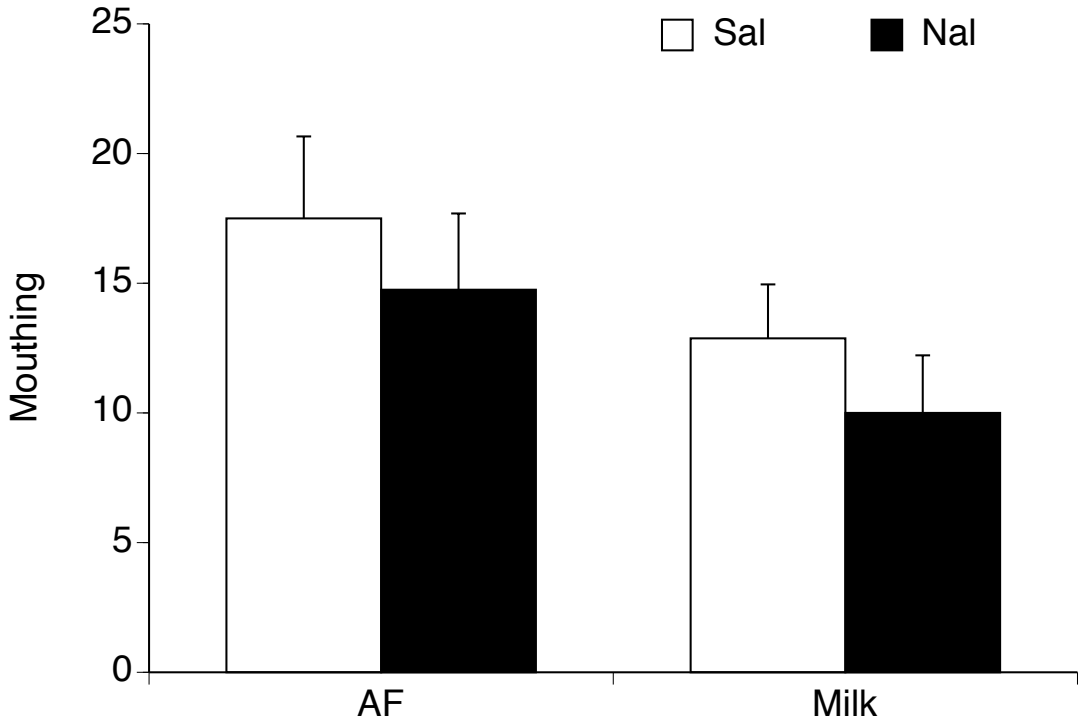

Figure 27. Mouthing responses during exposure to the artificial nipple in Experiment 8. Pups were pretreated by IP injection of isotonic saline (Sal) or the non-selective opioid antagonist naloxone (Nal; 1.0 mg/kg) 5 min before testing and were presented with the nipple along with the odor of AF or milk. Bars depict mean number of mouthing movements during the 3-min period of exposure to the artificial nipple. Error bars depict S.E.M.

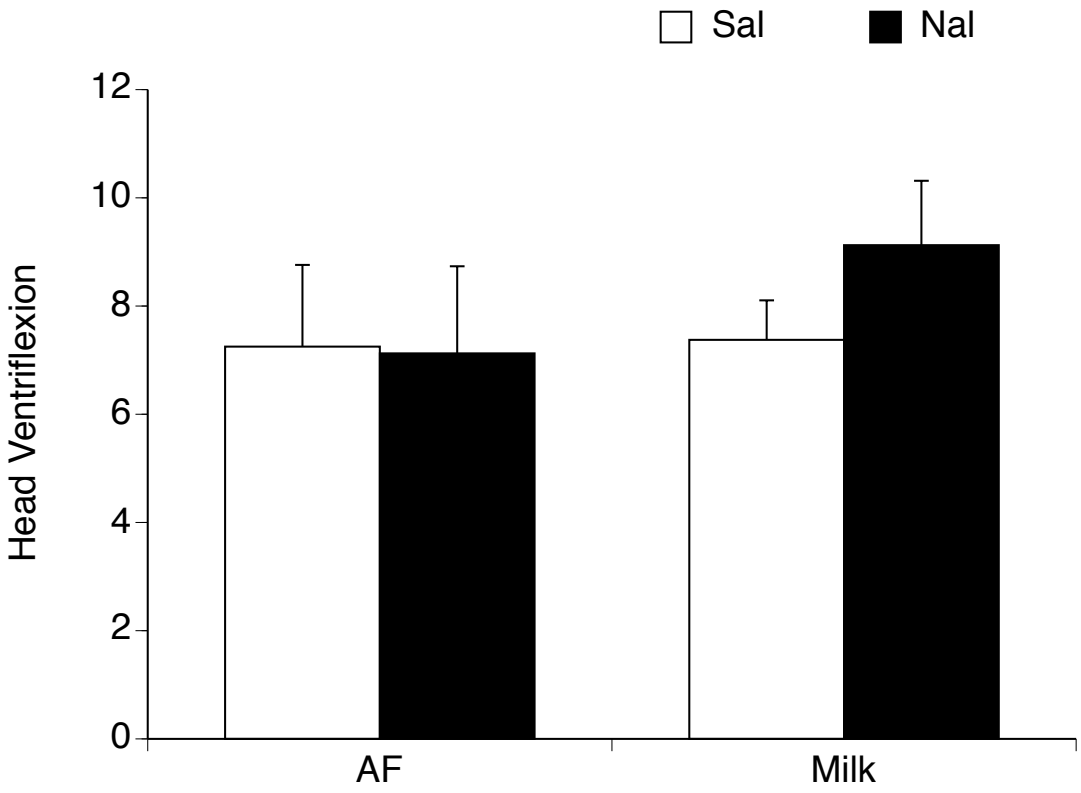

Figure 28. Head ventriflexion responses during exposure to the artificial nipple in Experiment 8. Pups were pretreated by IP injection of isotonic saline (Sal) or the non-selective opioid antagonist naloxone (Nal; 1.0 mg/kg) 5 min before testing and were presented with the nipple along with the odor of AF or milk. Bars depict mean number of head ventriflexion movements during the 3-min period of exposure to the artificial nipple. Error bars depict S.E.M.

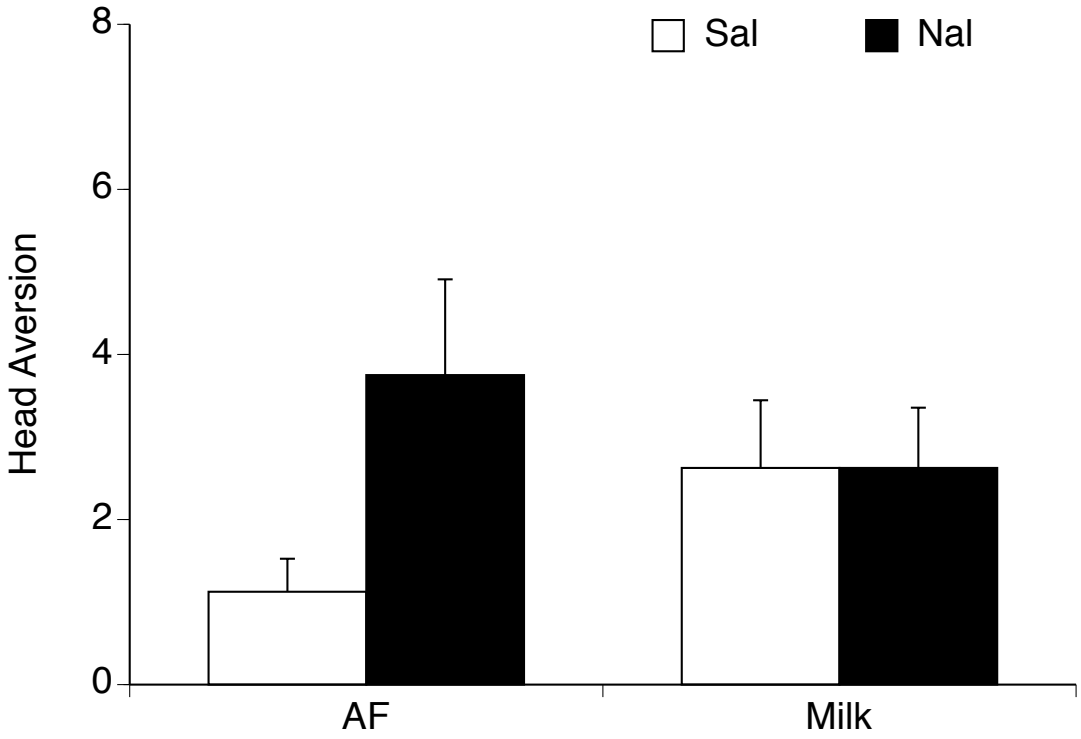

Figure 29. Head aversion responses during exposure to the artificial nipple in Experiment 8. Pups were pretreated by IP injection of isotonic saline (Sal) or the non-selective opioid antagonist naloxone (Nal; 1.0 mg/kg) 5 min before testing and were presented with the nipple along with the odor of AF or milk. Bars depict mean number of head aversion movements during the 3-min period of exposure to the artificial nipple. Error bars depict S.E.M.

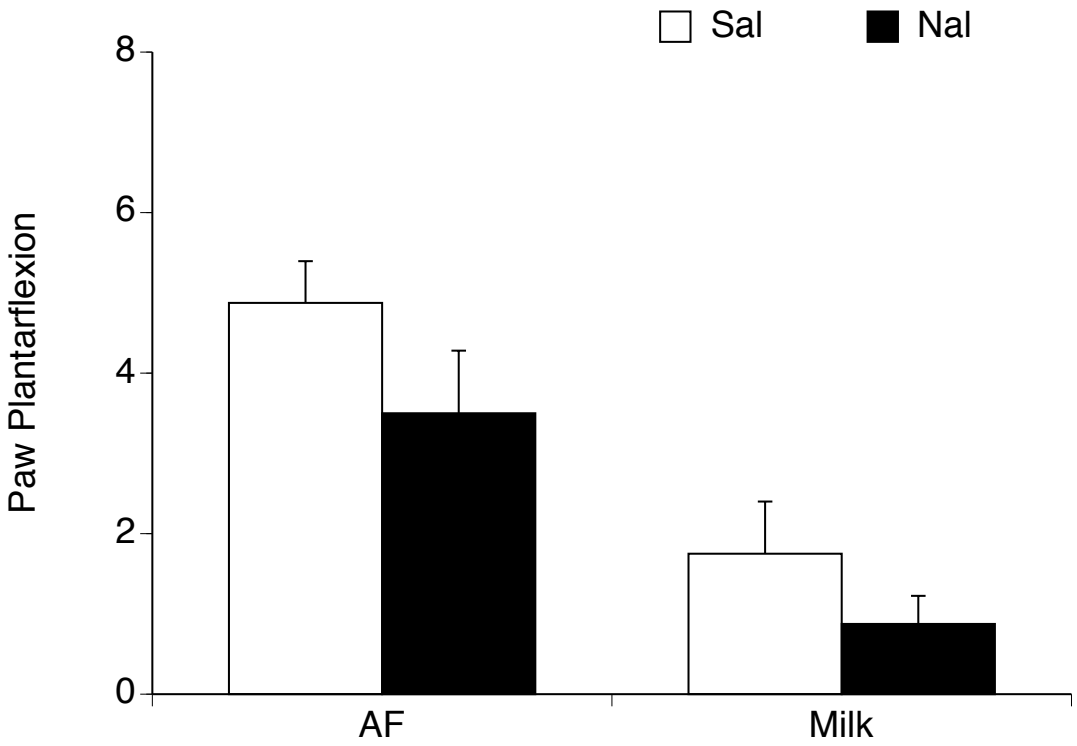

Figure 30. Paw plantarflexion responses during exposure to the artificial nipple in Experiment 8. Pups were pretreated by IP injection of isotonic saline (Sal) or the non-selective opioid antagonist naloxone (Nal; 1.0 mg/kg) 5 min before testing and were presented with the nipple along with the odor of AF or milk. Bars depict mean number of paw plantarflexion movements during the 3-min period of exposure to the artificial nipple. Error bars depict S.E.M.

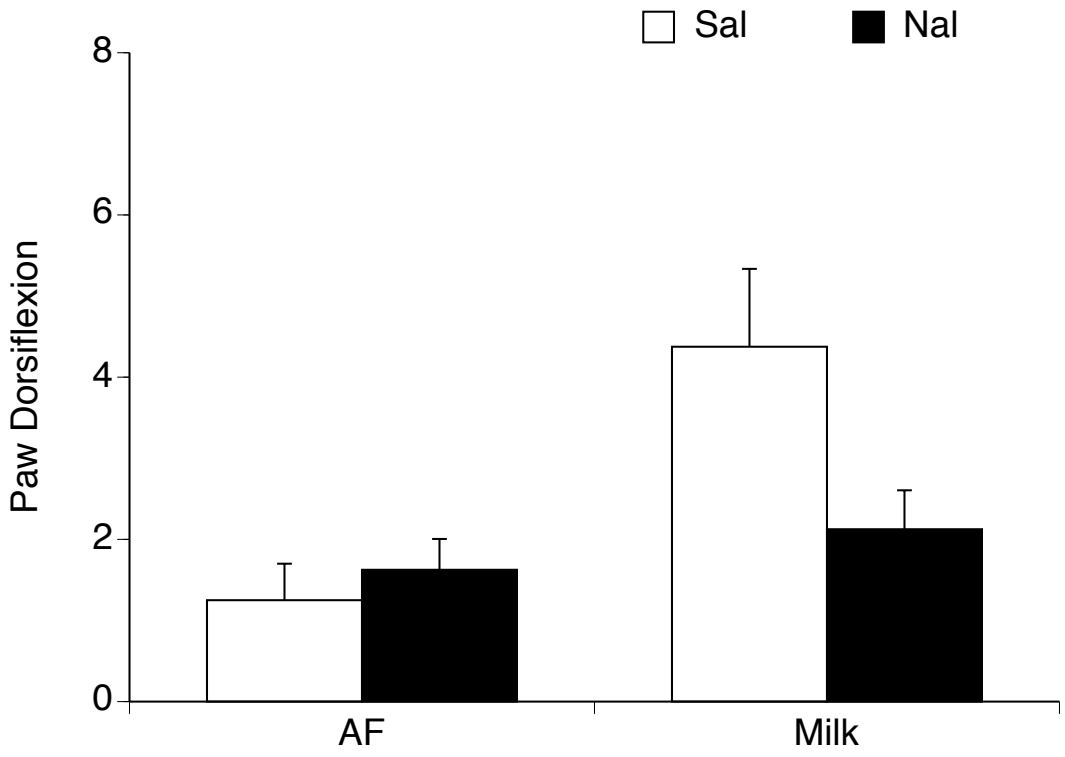

Figure 31. Paw dorsiflexion responses during exposure to the artificial nipple in Experiment 8. Pups were pretreated by IP injection of isotonic saline (Sal) or the non-selective opioid antagonist naloxone (Nal; 1.0 mg/kg) 5 min before testing and were presented with the nipple along with the odor of AF or milk. Bars depict mean numbers of paw dorsiflexion activity during the 3-min period of exposure to the artificial nipple. Error bars depict S.E.M.

effect and did not differ between odor groups. In Experiment 8, only paw plantarflexion and paw dorsiflexion activity revealed significant results. As in Experiment 7, subjects exposed to the odor of AF during presentation of the artificial nipple expressed higher levels of paw plantarflexion activity while subjects in the milk group expressed higher levels of paw dorsiflexion activity. In this last behavioral category, there also was an effect of drug pretreatment. Subjects that were exposed to the odor of milk and pretreated with saline showed higher levels of paw dorsiflexion activity. Together with Experiment 7, the findings of Experiment 8 suggest that paw plantarflexion and paw dorsiflexion are behavioral variables that are affected by presentation of the artificial nipple in association with the odors of AF or milk.

The results of Experiment 8, which failed to show opioid involvement during the exposure of AF and milk odor, contrast sharply with previous reports of opioid involvement during AF and milk-evoked behavioral responses in the neonatal rat (Méndez-Gallardo & Robinson, 2010). However, they do agree with the results of Experiments 4 and 6 of the present study, in which exposure to the odor of AF or milk did not result in activation of the endogenous opioid system in the neonatal rat. Taken together, the results of Experiments 1-8 of the present study and previous reports (Korthank & Robinson, 1998; Méndez-Gallardo & Robinson, 2010) suggest that oral exposure to AF or milk constitutes a different sensorial experience in the neonatal rat than exposure to the odor of AF or milk alone, and that oral vs. odor exposure may evoke distinctive behavioral responses. While experiencing AF or milk through the mouth

activates the opioid system of the neonatal rat, experiencing AF and milk as an airborne odorant does not appear to engage receptors in the opioid system.

CHAPTER 6: CLASSICAL CONDITIONING EFFECTS OF AMNIOTIC FLUID AND MILK

In Chapter 6, Experiments 9, 10, 11, and 12 investigated if AF and milk can serve as an unconditioned stimulus (US) in a classical conditioning paradigm in the neonatal rat, and whether the conditioned response (reduced responsiveness to a facial wiping challenge after reexposure to the CS) is dependent on opioid activity. Two of the experiments (9 and 10) investigate the effectiveness of AF as the US, while the other two experiments (11 and 12) focus on milk.

Experiment 9: Classical conditioning using AF as the unconditioned stimulus

Experiment 9 addressed the question of whether AF can serve as a US in the acquisition of a conditioned response to an artificial nipple. Previous studies have reported effective Pavlovian conditioning when neutral stimuli (sucrose, artificial nipple, novel odor cues), serving as the CS, are paired with milk as the US (Arnold et al, 1993; Johanson & Terry, 1988; Smotherman & Robinson, 1994). In prenatal studies, when fetal subjects were reexposed to the CS after three presentations of the CS paired with milk, they exhibited conditioned changes in responsiveness to a perioral stimulus that typically evokes facial wiping. Learning experiments in neonatal rats, using a somewhat different training protocol, have similarly reported the establishment of odor preferences for a novel odor cue (the CS) after exposure to the cue paired with milk (Brake, 1981; Johanson & Hall, 1982; Johanson & Teicher, 1980). Several laboratories have

documented similarities in the composition and behavioral effects of milk and AF in different species, including rats, sheep and humans, suggesting an important continuity in these biologically relevant fluids (Korthank & Robinson, 1998; Méndez-Gallardo & Robinson, 2010; Schaal, 2005). In addition, research has documented the capacity of AF to act as a US when administered simultaneously with ethanol (Arias & Chotro, 2007), as well as the use of an artificial nipple as a CS in fetuses (Petrov, Varlinskaya, & Smotherman, 2000; Robinson, Arnold et al., 1993; Smotherman, 2002a, 2002b; Smotherman & Robinson, 1994) and in newborn rats (Petrov et al., 2006). This study adapted a training and testing protocol originally developed to study classical conditioning in the rat fetus to evaluate whether AF can act as a US during classical conditioning, and whether pairing AF with an artificial nipple CS can alter the facial wiping response to lemon in the newborn rat after reexposure to the CS. The hypothesis of this experiment was that pairing of the artificial nipple (the CS) with AF (the US) will result in altered oral behavior and reduced facial wiping responsiveness to lemon after reexposure to the CS.

Methods

Subjects

Subjects were neonatal rats tested 24 hours after birth (P1). Thirty-two pups from 10 litters were used in Experiment 9. Subjects' breeding and care followed the same protocol described in the General Methods section.

Conditions

Rat pups were assigned to one of four training conditions (n = 8 pups per group): Paired, Unpaired, CS alone, and US alone. Equal numbers of female and male rat pups were tested in each of these conditions.

Procedure

Testing was conducted on the first postnatal day (P1). The procedure started by fitting pups with a dual intraoral cannula to allow delivery of different stimulus fluids used during conditioning trials and testing (i.e., AF and lemon). Pups were tested in a supine posture inside a warm incubator (35°C ± 0.3). A classical conditioning paradigm closely similar to that reported by Robinson and collaborators (1993) was used to experimentally determine whether AF can serve as an effective US. The procedure involves pairing AF as the US with an artificial nipple as the CS. Experimental testing took place in a single 20-min session that comprises both conditioning and testing. CS presentation consisted of exposure to the artificial nipple for 15 s, following the same methods described in Experiment 7 and in the general methods section. US presentation consisted of a single 20 µl intraoral infusion of AF. The four training conditions differed in the schedule of presentation of stimuli during three conditioning trials of the experiment. The four training conditions included: (a) a paired group exposed for 15 s to the artificial nipple (CS) followed immediately by infusion of AF (US), (b) an unpaired group exposed to AF (US) followed 2.5 min later by presentation of the artificial nipple (CS), (c) a US alone group exposed only to AF, and (d) a CS alone group exposed only to the artificial nipple. In each training condition, the CS and/or US were presented during

three conditioning trials, which were scheduled at 5-min intervals starting at min 0 (Figure 2). At min 18, all subjects were reexposed to the CS (artificial nipple), and 1 min later (45 s after cessation of CS exposure) received a 20 µl infusion of a 1:1 dilution of lemon odor extract to evoke a facial wiping response. After the lemon infusion, facial wiping behavior was observed for 60 s. The dependent variables in this experiment were mouthing activity evoked by presentation of the artificial nipple during reexposure and facial wiping responses evoked by the test infusion of lemon.

Behavioral observations and data analysis

Behavioral observations included scoring of mouthing and facial wiping. Mouthing was scored for 1 min, comprising the 15-s during reexposure to the CS and the following 45-s after reexposure. Facial wiping was observed for 1 min after the lemon infusion. Changes in mouthing provided information about the responses to the CS during reexposure to the nipple and facial wiping provided specific information about the effects of conditioning on chemosensory responsiveness. The rationale for looking at these two dependent variables was the enhanced mouthing behavior (unconditioned response) evoked by AF or milk during exposure to the artificial nipple in Experiments 7 and 8, and reduced facial wiping to lemon infusion after exposure to AF or milk (Méndez-Gallardo & Robinson, 2010). Each test session was recorded to a digital video file. Video files were scored using event-recording software, summarized, and analyzed as described in the General Methods section.

Results

Analysis of facial wiping was performed in 1-factor analysis of variance (4 training conditions). The results of this analysis revealed that there were no significant differences in facial wiping responses among training groups ($p > .05$) (Figure 32).

Mouthing activity was analyzed in a 2-factor analysis of variance (4 training conditions X 4 15-s intervals), with the Time factor treated as a repeated measure. In contrast to the results for facial wiping, mouthing activity showed significant differences among training groups, $F(3, 31) = 6.29$, $p < .05$, as well as a significant difference across Time, $F(3, 96) = 7.84$, $p < .001$ (Figure 33), but no interaction between these factors ($p > .05$). Comparison of group means following the main effect of training condition indicated that subjects in the paired group expressed higher levels of mouthing activity in comparison to the unpaired and CS alone groups. However, mouthing activity in the paired condition did not significantly differ from the US alone group. Comparison of 15-s intervals following the main effect of Time revealed higher levels of mouthing activity during the 15 s during reexposure to the CS and the following 15 s than during the last two intervals.

One additional one-way analysis of variance was performed to compare training conditions during the last 15-s interval, 30 s after reexposure to the CS. The rationale for this comparison derives from previous reports that conditioned opioid responses only become evident 30 s after reexposure to the CS (Smotherman, 2002a). This analysis confirmed a significant difference between training groups in which the paired group differed significantly from the other three training groups.

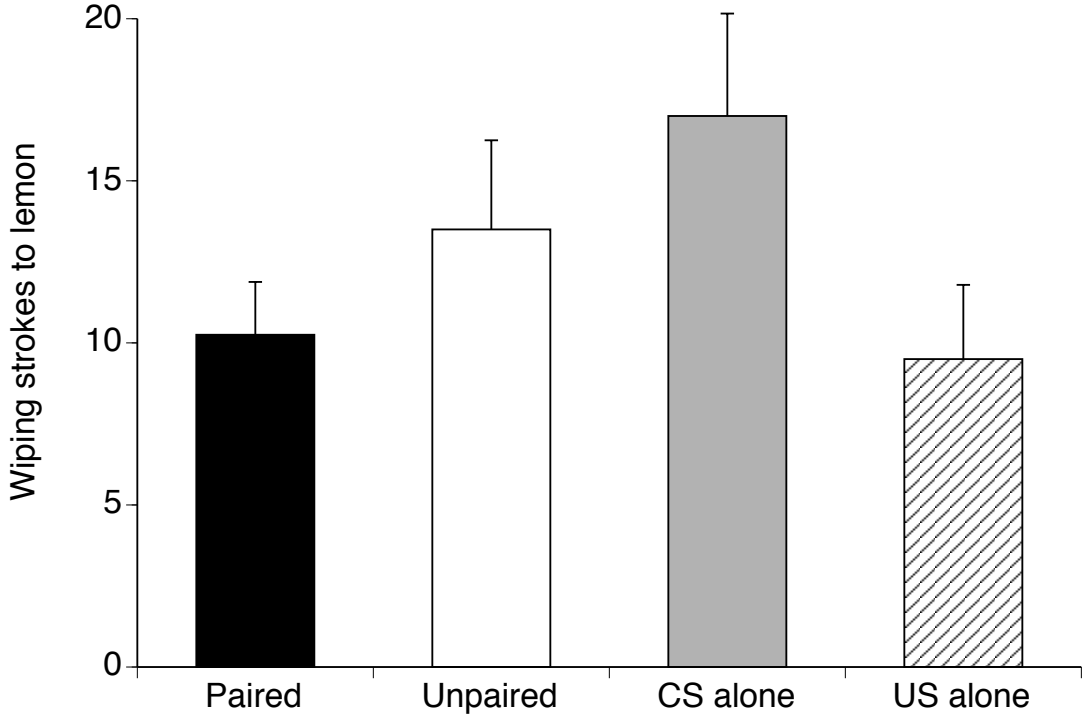

Figure 32. Facial wiping responses to lemon after reexposure to the CS in Experiment 9. Pups were tested in one of four training conditions (Paired, Unpaired, CS alone, and US alone) comprising a 15-s presentation of the artificial nipple (the CS) and/or an intraoral infusion of AF (the US). Bars depict mean number of wiping strokes during the minute after lemon infusion. Error bars depict S.E.M.

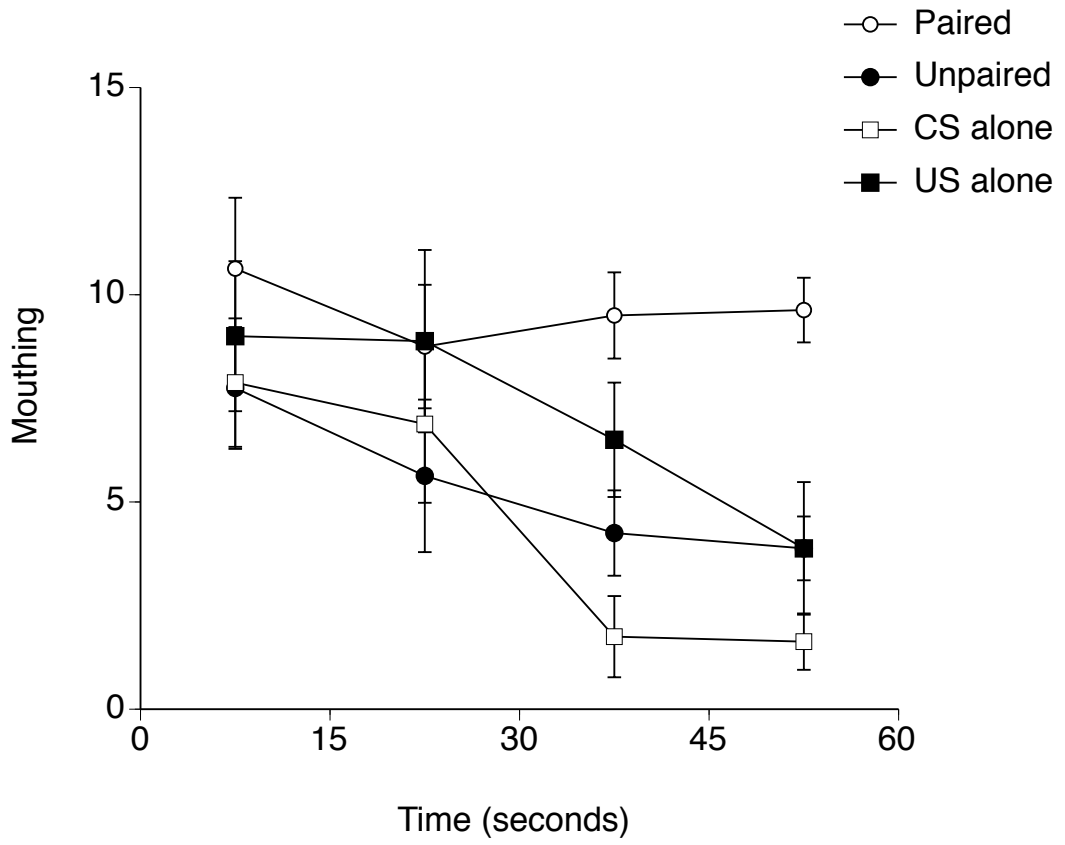

Figure 33. Mouthing movements in response to reexposure to the CS in Experiment 9. Pups were tested in one of four training conditions (Paired, Unpaired, CS alone, and US alone) comprising a 15-s presentation of the artificial nipple (the CS) and/or an intraoral infusion of AF (the US). Lines depict mean number of mouthing movements during the 15 sec of reexposure to the CS and the 45 s following reexposure. Error bars depict S.E.M.

Discussion

The results of Experiment 9 revealed that facial wiping responses to lemon after the reexposure to the CS were not significantly different among all of the four training conditions. It was predicted that contingent presentations of intraoral infusions of AF (as the US) with the artificial nipple (as the CS) should result in the CS taking upon the properties of AF. As AF has been reported to successfully reduce the facial wiping response in the newborn rat (Méndez-Gallardo & Robinson, 2010), it was expected that reexposure to the artificial nipple CS would also result in a reduced facial wiping response to lemon. However, this prediction was not confirmed in this experiment. As reported above, facial wiping was not significantly reduced in subjects in the paired group.

However, the results of mouthing activity after reexposure to the CS confirmed that paired presentations of the CS and the US had a significant effect on this behavior in comparison to unpaired presentations or individual presentations of the CS and the US. Mouthing activity was expressed at higher levels in the paired group. Although mouthing activity in the paired group did not significantly differ from the US alone group overall, the paired group expressed more mouthing activity than any of the other three conditions during the last 15-s interval. These results suggest that although facial wiping as a dependent variable did not successfully indicate associative learning in the newborn rat, mouthing responses to the nipple were modified as a result of conditioning. Mouthing activity was expressed at high levels immediately at the moment of the nipple presentation and remained high at least for 15 s after the nipple was removed. Although at

lower levels, mouthing continued to be expressed for 45 s after presentation of the artificial nipple ended and by the end of this period, the mouthing responses of the paired group remained significantly elevated.

Although previous studies have reported that facial wiping is reduced after reexposure to the CS following a similar conditioning paradigm (Robinson, Arnold et al., 1993), these studies were conducted in rat fetuses with milk — instead of AF — used as the US. The differences between these previous studies and Experiment 9 suggest that facial wiping may not be a good dependent variable to study associative learning in the newborn rat or that the conditioning paradigm may need to be modified.

To follow-up on the findings on Experiment 9, Experiment 10 continued to evaluate the use of AF as a US and also determined whether the endogenous opioid system mediates conditioned responses to the CS in the newborn rat.

Experiment 10: Conditioned opioid activity after association with amniotic fluid as the US

Experiment 10 addressed the question of whether the conditioned response to the artificial nipple after associative learning with AF is opioid mediated. As summarized above, previous reports have documented that contingent pairing of an artificial nipple CS and milk US evokes conditioned opioid activity in the rat fetus. Reduced fetal responsiveness in a facial wiping test is evident only in subjects receiving paired presentations of CS and US, and high levels of responsiveness can be reinstated if subjects are treated with the opioid antagonist naloxone after all conditioning trials but

before reexposure to the CS (Arnold et al. 1993; Robinson, Arnold et al., 1993; Smotherman & Robinson, 1994). While Experiment 9 addressed the possibility of altered responsiveness of the newborn rat after conditioning trials, with AF serving as the US, Experiment 10 determined whether the conditioned response to the artificial nipple is opioid mediated by blocking opioid receptors after three conditioning trials. The hypothesis of this experiment was that classical conditioning with AF as the US would result in activation of the opioid system, with consequent changes in responsiveness in the newborn rat. The prediction, therefore, was that subjects in the paired group treated with naloxone would show higher levels of facial wiping and reduced mouthing to the nipple CS compared to subjects in the paired group treated with saline.

Methods

Subjects

Neonatal subjects were the offspring of Sprague-Dawley laboratory rats. Thirty-two pups from 10 litters were used in Experiment 10. Pups were tested 24 hours after birth (P1). Subjects' breeding and care followed the same protocol described in the General Methods section.

Conditions

Rat pups, including one male and one female pup from each litter, were assigned to one of four experimental conditions that resulted from a combination of training condition and drug treatment. Training conditions included paired or unpaired presentation of the artificial nipple (the CS) and AF (the US). Drug treatments involved IP injection of 50 µl of naloxone (1.0 mg/kg) or 50 µl of isotonic saline. The four

experimental groups (n = 8 pups per group) were as follows: Paired-Saline, Paired-Naloxone, Unpaired-Saline, and Unpaired-Naloxone.

Procedure

The procedure started by fitting pups with a dual intraoral cannula to allow delivery of the different solutions used during conditioning trials and testing (i.e., AF and lemon). Pups were tested in a supine posture inside a warm incubator (35°C ± 0.3). In this experiment, presentation of CS and US in paired and unpaired conditions was similar to that described in Experiment 9. Subjects were exposed to an artificial nipple (the CS) and AF collected on day 20 of gestation (the US). Subjects were assigned to either a paired group that was exposed for 15 s to the artificial nipple (CS) followed immediately by infusion of AF (US), or an unpaired group that was exposed to AF (US) followed 2.5 min later by presentation of the artificial nipple (CS). Both training conditions received presentations of CS and US during three conditioning trials, which were scheduled at 5-min intervals starting at min 0 (Figure 3). After the three conditioning trials and 5-min before reexposure to the CS, subjects were treated with an IP injection of either 50 µl of the nonselective opioid antagonist naloxone or 50 µl of isotonic saline as a vehicle control. To permit this treatment, the experimental session was extended to 22 min, with naloxone/saline injection occurring 5 min after the last exposure to the US (AF) in both the Unpaired and Paired training conditions. As in Experiment 9, the dependent variables in this experiment were mouthing activity evoked by presentation of the artificial nipple during reexposure and facial wiping responses evoked by the test infusion of lemon. The lemon infusion was delivered 60-s after beginning of reexposure to the artificial nipple

CS (45 s after removal of the nipple). Facial wiping responses were observed during 60-s following the infusion of lemon.

Behavioral observations and data analysis

Behavioral observations included scoring of mouthing and facial wiping. Mouthing was scored for 1 min comprising the 15-s during reexposure to the CS and the following 45-s after reexposure. Facial wiping was observed for 1 min after the lemon infusion. Changes in mouthing provided information about the responses to the CS during reexposure to the nipple and facial wiping provided specific information about the effects of conditioning on chemosensory responsiveness. Each test session was recorded to a digital video file. Video files were scored using event-recording software, summarized, and analyzed as described in the General Methods section.

Results

Analysis of facial wiping was performed in 2-factor analysis of variance (2 Drug treatments X 2 Training conditions). The results for facial wiping revealed that there were no significant main effects of training conditions or drug treatments, and no interaction of these factors ($p > .05$) (Figure 34).

Mouthing activity was analyzed in 3-factor analysis of variance (2 Drug treatments X 2 Training conditions X 4 15-s intervals), with the Time factor treated as a repeated measure. In contrast to facial wiping, the results for mouthing activity revealed the significant main effect of training conditions, $F(1, 28) = 15.44$, $p < .001$, the significant effect of drug groups, $F(1, 28) = 6.13$, $p < .05$, a significant interaction between training conditions and drug groups, $F(1, 28) = 8.06$, $p < .05$, and a significant

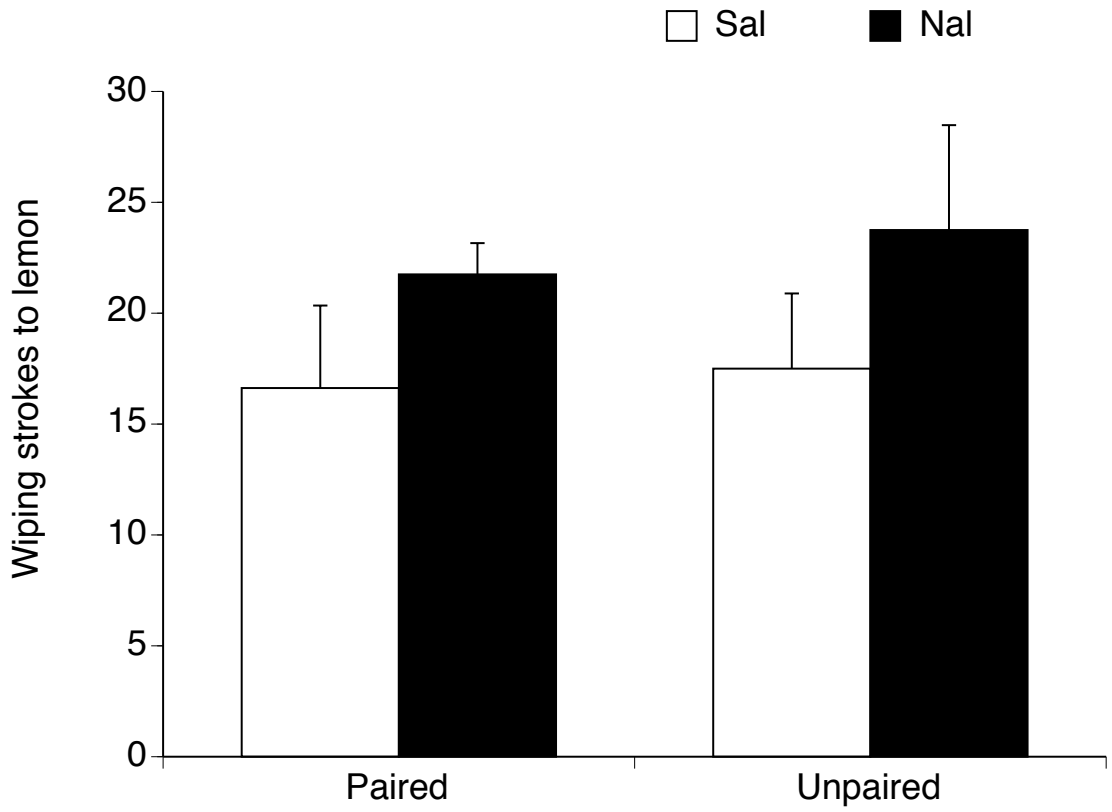

Figure 34. Facial wiping responses to lemon after reexposure to the CS in Experiment 10. Pups were tested in one of two training conditions (Paired or Unpaired) comprising a 15-s presentation of the artificial nipple (the CS) and an intraoral infusion of AF (the US). Subjects received an IP injection of isotonic saline (Sal) or the non-selective opioid antagonist naloxone (Nal; 1.0 mg/kg) 5 min before reexposure to the CS. Bars depict mean number of wiping strokes during the minute after lemon infusion. Error bars depict S.E.M.

effect of Time, F (3, 84) = 4.55, $p < .05$ (Figure 35). Following this pattern of main and interaction effects, additional two-way analyses of variance were performed to analyze group differences in mouthing activity by comparing mouthing activity collapsed across the 4 15-s intervals among the 2 drug treatments and the 2 training conditions. This analysis indicated that subjects in the paired group that were treated with saline expressed higher levels of mouthing activity than paired subjects treated with naloxone or either unpaired training group. In addition, mouthing activity was higher across all groups during the 15 s during reexposure to the CS and the following 15 s after reexposure.

As in Experiment 9, an additional two-way analysis of variance was performed to compare groups during the last 15-s interval (30 s after reexposure to the CS). This analysis revealed a significant difference between training conditions, F (1, 28) = 6.38, $p < .05$, and a significant effect of drug, F (1, 28) = 5.87, $p < .05$, but no interaction ($p > .05$). Specifically, subjects in the paired group showed more mouthing than unpaired subjects, and subjects treated with saline showed more mouthing than subjects treated with naloxone.

Discussion

The findings of Experiment 10 confirmed that the opioid system is activated during expression of a learned response to the CS after association with AF as the US. As in Experiment 9, facial wiping responses were not significantly different in any of the groups. However, mouthing activity after reexposure to the CS was expressed differently in response to training and drug treatment. Specifically, subjects that received contingent

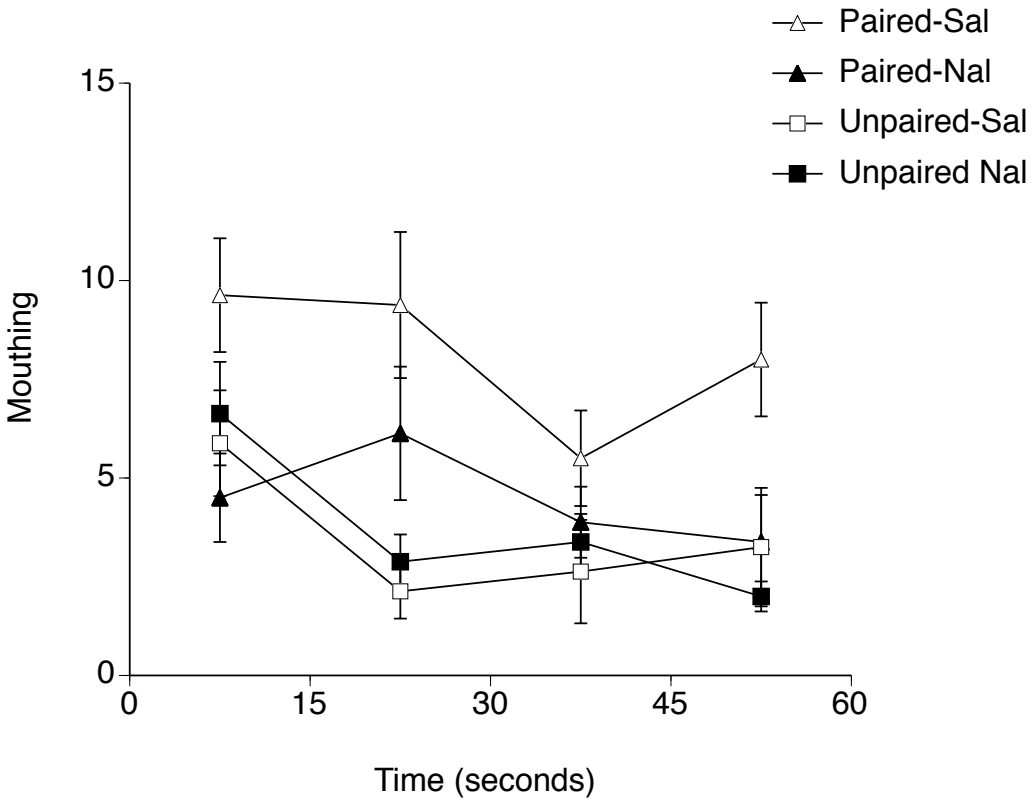

Figure 35. Mouthing movements in response to reexposure to the CS in Experiment 10. Pups were tested in one of two training conditions (Paired or Unpaired) comprising a 15-s presentation of the artificial nipple (the CS) and an intraoral infusion of AF (the US). Subjects received an IP injection of isotonic saline (Sal) or the non-selective opioid antagonist naloxone (Nal; 1.0 mg/kg) 5 min before reexposure to the CS. Lines depict mean number of mouthing movements during the 15 s of reexposure to the CS and the 45 s following reexposure. Error bars depict S.E.M.

presentations of the artificial nipple CS and intraoral infusions of AF, the US, and which were treated with saline, expressed mouthing at higher levels compared to subjects in the other groups. Because mouthing also was significantly elevated in the paired group in Experiment 9, the finding of Experiment 10 that subjects in the saline group showed higher levels of mouthing activity than those treated with naloxone suggests opioid involvement in the conditioned response. In other words, treatment with naloxone seems to reduce mouthing activity in the paired group by blocking receptors in the opioid system. This effect was noticeable throughout the minute of observation, and was confirmed during the last 15-s interval, when a conditioned opioid response should be most pronounced. Even after the nipple was removed, the paired group treated with saline continued to express mouthing at elevated rates.

Together with Experiment 9, Experiment 10 confirms the ability of AF to promote associative learning in the newborn rat. Although there are reports about AF serving as a US during associative learning in older rats (Arias & Chotro, 2007), these two experiments validate the use of AF as a US in this associative learning paradigm. In addition, results of this experiment agree with other studies that have described other opioid-induced behavioral effects of AF in the newborn rat (Méndez-Gallardo & Robinson, 2010). Although this study did not explore the involvement of specific opioid receptors in the AF response, data from this experiment and previously reported studies (Korthank & Robinson, 1998; Méndez-Gallardo & Robinson, 2010) suggest that AF activates the opioid system of the perinatal rat and induces effects in the expression of behavior as well as it plays an important role during associative learning.

Experiment 11: Classical conditioning using milk as the unconditioned stimulus

Experiment 11 addressed the question of whether milk can serve as a US in the acquisition of a conditioned response to an artificial nipple. Although milk has been shown to be an effective US in the rat fetus (Arnold et al, 1993; Smotherman & Robinson, 1994), and can support classical conditioning of odor preferences in the neonatal rat (Johanson & Hall, 1982; Johanson & Terry, 1988), it has not been used in the facial wiping paradigm to study classical conditioning in the newborn rat. Together with results of Experiment 9, this experiment contributes to a better understanding of the effects of milk and AF on associative learning in the neonatal rat. The hypothesis of this experiment was that pairing of the artificial nipple (the CS) with milk (the US) will result in altered oral behavior and reduced facial wiping responsiveness to lemon after reexposure to the CS.

Methods

Subjects

Neonatal subjects were the offspring of Sprague-Dawley laboratory rats. Thirty-two pups from 8 litters were used in Experiment 11. Pups were tested 24 hours after birth (P1). Subjects' breeding and care followed the same protocol described in the General Methods section.

Conditions

Rat pups were assigned to 1 of 4 training conditions (n = 8 pups per group): Paired, Unpaired, CS alone, and US alone. Equal numbers of female and male rat pups were tested in each of these conditions.

Procedure

Testing was conducted exactly in the same way as described in Experiment 9 with the exception that presentation of the US consisted of a 20 µl intraoral infusion of milk.

Behavioral observations and data analysis

Behavioral observations included scoring of mouthing and facial wiping. Mouthing was scored for 1 min comprising the 15-s during reexposure to the CS and the following 45-s after reexposure. Facial wiping was observed for 1-min after the lemon infusion. Changes in mouthing provided information about the responses to the CS during reexposure to the nipple CS and facial wiping provided specific information about the effects of conditioning on chemosensory responsiveness. Each test session was recorded to a digital video file. Video files were scored using event-recording software, summarized, and analyzed as described in the General Methods section.

Results

Analysis of facial wiping was performed in a 1-factor analysis of variance (4 training conditions). Results showed that there were no significant differences in facial wiping to the lemon infusion among training conditions ($p > .05$) (Figure 36).

Mouthing activity was analyzed in a 2-factor analysis of variance (4 training conditions X 4 15-s intervals), with the Time factor treated as a repeated measure. This

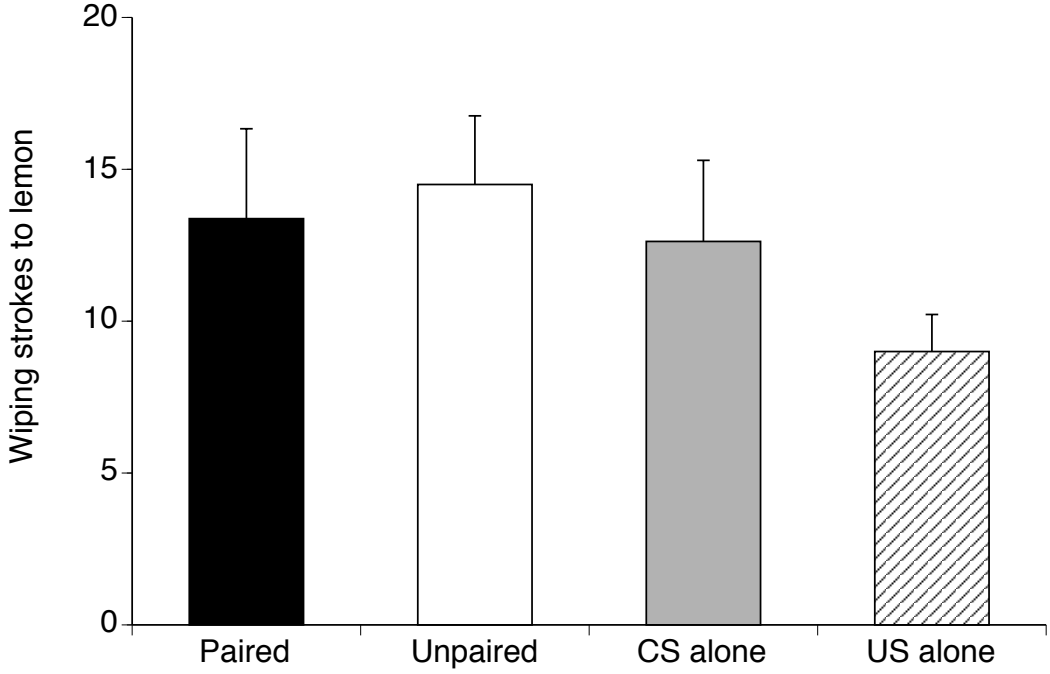

Figure 36. Facial wiping responses to lemon after reexposure to the CS in Experiment 11. Pups were tested in one of four training conditions (Paired, Unpaired, CS alone, and US alone) comprising a 15-s presentation of the artificial nipple (the CS) and/or an intraoral infusion of milk (the US). Bars depict mean number of wiping strokes during the minute after lemon infusion. Error bars depict S.E.M.

analysis indicated significant differences in mouthing activity among training conditions, F (3, 31) = 4.40, $p < .05$, and a significant effect of Time, F (3, 96) = 10.21, $p < .001$ (Figure 37). A one-way analysis for the simple main effect of training condition revealed that mouthing activity was significantly higher in the paired group compared to the unpaired and US alone group. However, mouthing activity in the paired group did not differ from the CS alone group. A within-subjects analysis of variance revealed that mouthing was significantly higher during the first two 15-s intervals than the last two intervals.

As in Experiment 9, an additional one-way analysis of variance was performed to compare training conditions during the last 15-s interval (30 s after reexposure to the CS). This analysis revealed a significant difference between the paired and the US alone groups. However, there were no significant differences between the paired group and the unpaired or CS alone groups.

Discussion

The findings of Experiment 11, which employed milk as the US, were similar to those reported in Experiment 9, in which AF was used as the US. In this experiment, facial wiping responses were not significantly different across the training conditions. However, mouthing activity in response to reexposure to the artificial nipple CS was significantly different among conditioning groups. Specifically, subjects in the paired group showed higher levels of mouthing activity compared to subjects in the unpaired and US alone groups, but was not significantly different than subjects in the CS alone group.

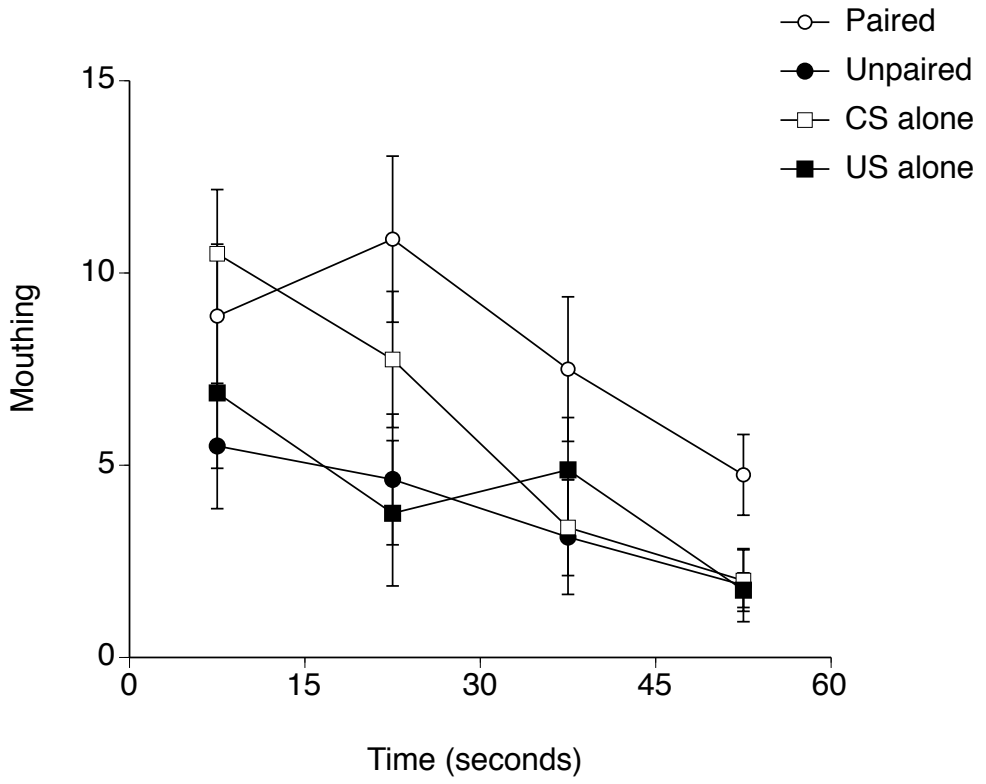

Figure 37. Mouthing movements in response to reexposure to the CS in Experiment 11. Pups were tested in one of four training conditions (Paired, Unpaired, CS alone, and US alone) comprising a 15-s presentation of the artificial nipple (the CS) and/or an intraoral infusion of milk (the US). Lines depict mean number of mouthing movements during the 15 s of reexposure to the CS and the 45 s following reexposure. Error bars depict S.E.M.

In addition, mouthing became elevated during the first 30 s of the reexposure period, which comprised the 15-s presentation of the artificial nipple and the following 15-s interval. Mouthing continued to be expressed during the 45 s after removal of the nipple, and at the end of that period, mouthing activity in the paired group continued to be significantly elevated.

The results of Experiment 11 are not consistent with previous reports in which milk, as the US, paired with presentations of the artificial nipple as the CS, resulted in diminished facial wiping to lemon after reexposure to the CS (Robinson, Arnold et al., 1993). Although these previous studies were conducted in the fetal rat, they used a similar paradigm as the one used in Experiment 11. However, facial wiping to lemon was not reduced in this experiment.

In contrast, results suggest that mouthing activity was modified in the paired group as a result of conditioning. Together with Experiment 9, the results of Experiment 11 confirmed that mouthing activity changes in response to associative learning using AF as the US (in Experiment 9) or milk as the US (in Experiment 11). These findings are especially interesting given the results reported in Experiment 1 of this study. In that experiment, intraoral infusion of AF or milk did not result in significant changes in mouthing activity between groups and higher levels of activity were only reported minutes after the infusion. However, in Experiments 9 and 11, reexposure to the CS after contingent presentations of the US (AF or milk) with the artificial nipple CS was sufficient to exert a significant effect on mouthing behavior in the newborn rat. Not only

was mouthing in the pup elevated immediately during presentation of the artificial nipple, but mouthing responses were altered by training conditions.

As a continuation to Experiment 11, Experiment 12 continued to explore the role of milk during associative learning in the newborn rat and also investigated whether the opioid system mediates the conditioned response after reexposure to the artificial nipple CS.

Experiment 12: Conditioned opioid activity after association with milk as the US

Experiment 12 addressed the question of whether the conditioned response to the artificial nipple after associative learning with milk is opioid mediated. As summarized earlier, previous reports have documented that milk can serve as an effective US to support classical conditioning in both the rat fetus and neonatal rat. Using a paradigm similar to that used here, experiments with fetal rats have confirmed that conditioned responses elicited by reexposure to the CS are opioid dependent (Arnold et al., 1993; Robinson, Arnold et al., 1993; Smotherman & Robinson, 1994). Experiment 12 examines whether conditioned opioid responses also are expressed by neonatal rats after conditioning with milk as the US. Together with Experiment 10, this experiment contributes to a better understanding of the role of the endogenous opioid system during classical conditioning involving AF and milk. The hypothesis of this experiment was that classical conditioning with milk as the US would result in activation of the opioid system, with consequent change in responsiveness in the newborn rat. The prediction, therefore,

was that subjects in the paired group treated with naloxone would show higher levels of facial wiping and reduced mouthing to the nipple CS compared to subjects in the paired group treated with saline.

Method

Subjects

Neonatal subjects were the offspring of Sprague-Dawley laboratory rats. Thirty-two pups from 12 litters were used in Experiment 12. Pups were tested 24 hours after birth (P1). Subjects' breeding and care followed the same protocol described in the General Methods section.

Conditions

Rat pups, including one male and one female pup from each litter, were assigned to one of four experimental conditions that resulted from a combination of training condition and drug treatment. Training conditions included paired or unpaired presentation of the artificial nipple (the CS) and milk (the US). Drug treatments involved IP injection of naloxone or saline. The four experimental groups (n = 8 pups per treatment group) were as follows: Paired-Saline, Paired-Naloxone, Unpaired-Saline, and Unpaired-Naloxone.

Procedure

Testing was conducted in exactly the same way as described in Experiment 10 with the exception that presentation of the US consisted of a 20 µl intraoral infusion of milk.

Behavioral observations and data analysis

Behavioral observations included scoring of mouthing and facial wiping. Mouthing was scored for 1 min comprising the 15-s during reexposure to the CS and the following 45-s after reexposure. Facial wiping was observed for 1-min after the lemon infusion. Changes in mouthing provided information about the responses to the CS during reexposure to the nipple CS and facial wiping provided specific information about the effects of conditioning on chemosensory responsiveness. Each test session was recorded to a digital video file. Video files were scored using event-recording software, summarized, and analyzed as described in the General Methods section.

Results

Analysis of facial wiping was performed in a 2-factor analysis of variance (2 Drug treatments X 2 Training conditions). This analysis revealed no significant differences in facial wiping between training conditions ($p > .05$). However, there was a significant difference in wiping responses between drug groups, $F\ (1, 28) = 6.96$, $p < .05$ (Figure 38). Subjects treated with saline expressed less facial wiping compared to subjects treated with naloxone.

Mouthing activity was analyzed in a 3-factor analysis of variance (2 Drug treatments X 2 Training conditions X 4 15-s intervals), with the Time factor treated as a repeated measure. The results for mouthing activity revealed that there was a significant main effect of training condition, $F\ (1, 28) = 5.48$, $p < .05$, a significant interaction between training conditions and drug groups, $F\ (1, 28) = 4.24$, $p < .05$, a significant effect of Time, $F\ (3, 84) = 12.13$, $p < .001$, and a significant interaction between drug groups

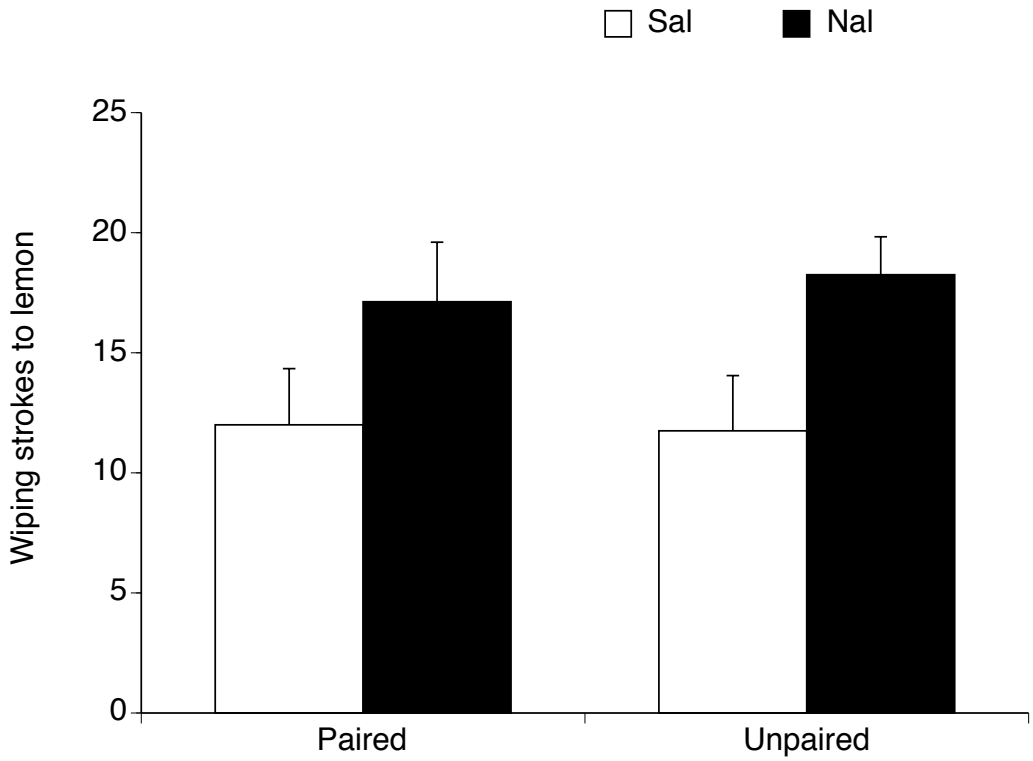

Figure 38. Facial wiping responses to lemon after reexposure to the CS in Experiment 12. Pups were tested in one of two training conditions (Paired or Unpaired) comprising a 15-s presentation of the artificial nipple (the CS) and an intraoral infusion of milk (the US). Subjects received an IP injection of isotonic saline (Sal) or the non-selective opioid antagonist naloxone (Nal; 1.0 mg/kg) 5 min before reexposure to the CS. Bars depict mean number of wiping strokes during the min after lemon infusion. Error bars depict S.E.M.

and Time, F (3, 84) = 3.66, $p < .05$ (Figure 39). The main effect of drug treatment was not significant ($p > .05$). To simplify this pattern of effects, a two-way ANOVA examined the interaction of training condition and drug groups collapsed across time intervals. This comparison indicated a significant main effect of training condition, F (1, 28) = 5.48, $p < .05$), and a significant interaction between training conditions and drug groups, F (1, 28) = 4.24, $p < .05$. The pattern of results indicated that naloxone decreases mouthing in the paired group and increases mouthing in the unpaired group. A second two-way ANOVA examined the simple interaction of drug treatment and time, with the Time factor treated as a repeated measure. This analysis indicated significantly higher levels of mouthing activity in subjects in the naloxone group during the 15 s during reexposure to the CS and the following 15 s after reexposure.

As in the preceding conditioning experiments, an additional two-way analysis of variance was performed to compare groups during the last 15-s interval (30 s after reexposure to the CS). These results revealed that there were significant differences between training conditions, F (1, 28) = 9.86, $p < .05$. Specifically, subjects in the paired group expressed higher levels of mouthing activity compared to subjects in the unpaired group.

Discussion

The results of Experiment 12 confirmed that the opioid system is activated during expression of a learned response to the CS after association with milk as the US. While facial wiping was not significantly different between training conditions, there were differences between drug groups where those treated with saline expressed less facial

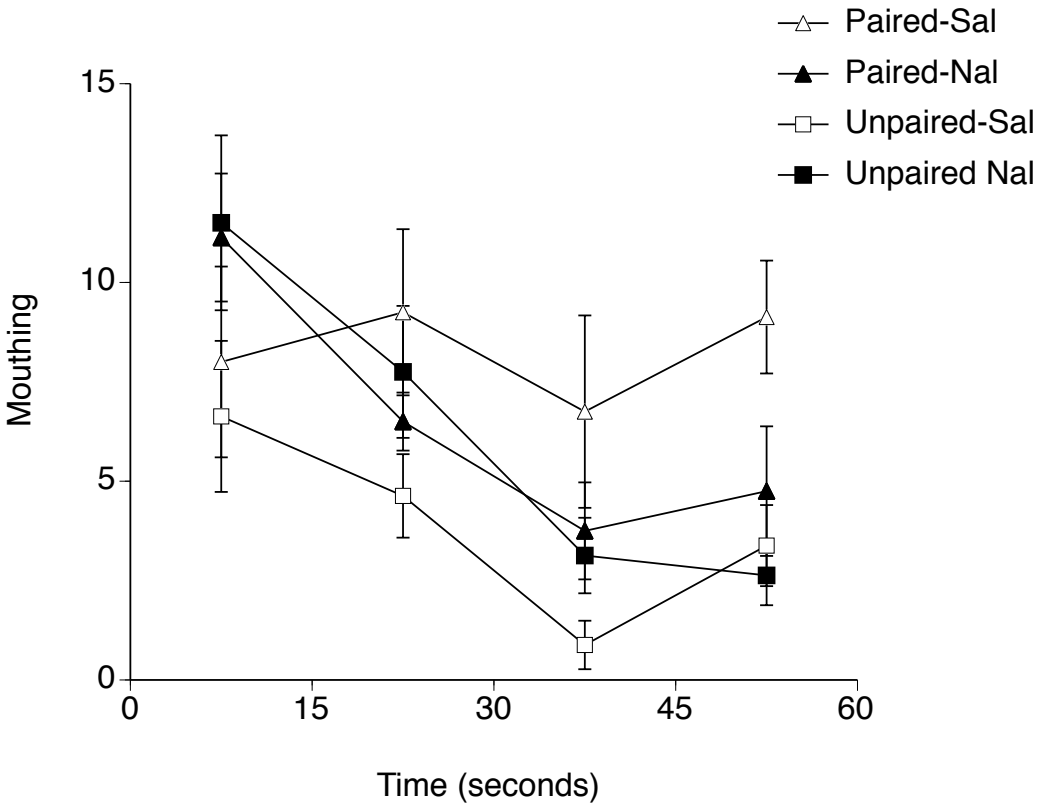

Figure 39. Mouthing movements in response to reexposure to the CS in Experiment 12. Pups were tested in one of two training conditions (Paired or Unpaired) comprising a 15-s presentation of the artificial nipple (the CS) and an intraoral infusion of milk (the US). Subjects received an IP injection of isotonic saline (Sal) or the non-selective opioid antagonist naloxone (Nal; 1.0 mg/kg) 5 min before reexposure to the CS. Lines depict mean number of mouthing movements during the 15 s of reexposure to the CS and the 45 s following reexposure. Error bars depict S.E.M.

wiping in comparison to subjects treated with naloxone. This finding does not suggests that the opioid system was activated during reexposure to the nipple CS.

In contrast, the results for mouthing activity revealed not only a significant difference between drug groups but also a difference between training conditions. Specifically, mouthing activity levels were higher in the paired group treated with saline compared to other groups. This effect was evident during the reexposure to the CS during the last 15-s interval after removal of the nipple. This finding suggests that mouthing activity in response to reexposure to the nipple CS is expressed in different ways as a consequence of training conditions. In addition, it confirms involvement of the opioid system after associative learning with milk as the US in the newborn rat. Treatment with naloxone resulted in reduced mouthing activity in response to reexposure to the CS after contingent presentations of the milk US and the artificial nipple CS. As was reported in Experiment 9, the findings of this experiment are consistent with other reports of opioid-induced behavioral effects of milk in the perinatal rat (Korthank & Robinson, 1998; Méndez-Gallardo & Robinson, 2010).

Finally, Experiments 11 and 12 support the ability of milk to serve as a US in the newborn rat during associative learning. Together with Experiments 9 and 10, these last two experiments suggest that AF and milk not only can successfully be used as a US, but AF and milk appear to produce similar opioid-induced behavioral responses in the newborn rat. Although the results of facial wiping measures were consistent within the present study, they stand in contrast with previous conditioning studies with fetal rats. Some questions therefore remain about the use of facial wiping as a dependent variable to

measure associative learning in the newborn rat. However, mouthing activity in response to reexposure of the artificial nipple CS was sufficient to confirm the expression of opioid responses after associative learning in the newborn rat.

CHAPTER 7: GENERAL DISCUSSION

The study of perinatal development provides an opportunity to explore the origin of behavior. Certainly, the development of behavior does not start at birth, but begins long before *in utero,* when the organism is already gaining experience and interacting with its surroundings. Among all the possibilities of research, understanding how amniotic fluid (AF) and milk affect behavior represents an excellent opportunity to not only explore those roots of behavior and learning, but also to identify points of continuity between life before and after birth.

As described extensively in this study, AF and milk are two features that represent two different developmental periods: the intrauterine environment before birth and the environment of the mother, siblings and nest after birth. However, while AF continues to affect behavior after birth and outside the uterine environment, the fetus also can distinguish and express organized responses to milk, even though it is a feature normally present only after birth. In this way, AF and milk are two aspects that affect behavior in mammals in ways that challenge the conception of perinatal development as represented by two separate periods before and after birth. Rather, research about AF and milk has shown that experiences before birth continue to influence, direct, and mediate behavioral development after birth. AF and milk are essential for the argument of behavioral continuity because research continues to show that fetuses and newborns recognize, respond, and learn in response to exposure to these two stimuli while interacting actively with their environment. And although AF and milk represent two aspects of the complicated interactive process that is development, these are two stimuli that also

represent the source of other behavioral phenomena, such as the development of kin recognition, the establishment of flavor, food, and odor preferences, the development of attachment, as well as learning through basic associations between stimuli of ecological relevance and novel stimuli.

Although the present study did not investigate any of the aspects by which AF and milk can affect later development after the first day of life, this study constitutes a description of several important ways by which AF and milk can affect newborn behavior. Beyond simply describing similarities in behavior, this study also focuses attention on an important neural mechanism — activity in the endogenous opioid system — that may underlie some of these behavioral continuities. In this way, this study invites further research to continue to explore questions not only about perinatal behavior but also the mechanisms by which development happens.

The present study provides important information about the behavioral effects of amniotic fluid (AF) and milk in the newborn rat. Specifically, overall behavioral activation, crawling locomotion, oral responses to an artificial nipple, and associative learning were investigated. To summarize the main findings: (a) oral exposure to AF results in higher levels of behavioral activation than oral exposure to milk; (b) exposure to the odor AF or milk does not produce significant behavioral activation, although the odor of milk seems to evoke higher levels of behavioral activity than exposure to the odor of AF; (c) both AF and milk odor elicited crawling locomotion; (d) odor of AF or milk did not promote oral grasping to an artificial nipple, but produced other behavioral effects, including mouthing responses and movements of the forepaws; (e) contingent

presentations of AF or milk as the unconditioned stimulus (US), with an artificial nipple as the conditioned stimulus (CS), promoted mouthing responses to the reexposure of the CS, but facial wiping was not modified as a result of conditioning; and (f) mediation of the opioid system was evident only during hindlimb activity after oral exposure to AF or milk and during mouthing responses to the CS after associative learning. While these findings provide information that contribute to a more complete understanding of the relationship of AF and milk, they also raise a few questions about how these results are relevant to known information about the behavioral effects of AF and milk in the perinatal rat, as well as to development in general.

Behavioral activation to exposure of AF and milk

The findings of the experiments of the first part of this study (Chapter 4) did not provide compelling evidence about similar behavioral effects of AF and milk during oral or odor exposure. Oral exposure to AF seems to evoke higher levels of behavioral activity (although small in magnitude) than milk, while milk, unlike AF, seems to promote some behavioral activity when exposed in odor form. This study thus suggests that the effects of AF or milk on general behavioral activity are slight. However, there are published reports that document the behavioral effects of AF in the newborn rat during chemosensory stimulation (Méndez-Gallardo & Robinson, 2010), and it is known than milk is a salient stimulus of ecological relevance that can affect behavior in the neonatal rat.

When infused intraorally, milk can produce strong effects in the perinatal rat. Experimental manipulations of the fetal rat have presented milk and investigated the

behavioral response to this stimulus before birth, although milk is normally present only after birth. Rat fetuses exposed to an intraoral infusion of milk show behavioral activation that culminates with a stretch response (Robinson & Smotherman, 1992c) similar to the stretch response reported in newborn pups during suckling at the nipple (Hall & Rosenblatt, 1977). As soon as fetuses receive an intraoral infusion of milk, they express high levels of mouthing activity and lower levels of forelimb and head movements; 2-3 min after infusion, hindlimb activity increases, eventually leading to the stretch response (Robinson & Smotherman, 1992c; Smotherman, Arnold et al., 1993).

In comparison, in young rat pups (3-, 4-, 6-, 9, and 12 day-old), intraoral infusions of milk elicit higher levels of behavioral activity, including mouthing, probing, twisting, locomotion, rolling, curling, reaching, posturing, and performing the stretch response (Camp & Rudy, 1987; Hall, 1979a, 1979b; Specht, Burright, & Spear, 1996). Some of these behaviors are considered characteristic of nipple search or other behavior related to the suckling context (Blass & Teicher, 1980; Hall, 1979b). However, some of these behaviors, including mouthing, are dependent upon deprivation in the infant rat. Increased deprivation results in more mouthing activity in 3-day-old pups when infused with milk (Hall, 1979b).

In Experiments 1 and 2 of the present study, although milk infusion may have produced higher levels of behavioral activity, this activity was not as great as occurred after intraoral infusion of AF. It is possible that after 24 hours of experience with milk, AF may have become less familiar, and perhaps more salient as a stimulus, than milk, a stimulus with which they were much more familiar at the time of testing. In humans

newborns, it has been reported that just hours after birth, they appear to undergo a transition in which their preference for AF switches to a preference for colostrum, and eventually to a preference for breast milk (Marlier et al., 1998a, 1998b; Schaal, 2005). In newborn rat pups, it has been demonstrated that only when pups are deprived of maternal contact, suckling, and milk for hours before testing, do they show high levels of activation to a milk infusion (Bornstein, Terry, Browde, Assimon, & Hall, 1987). In contrast to newborns, there are reports in the fetal rat that AF can promote increased levels of activity. Rat fetuses tested on Day 20 of gestation that received an intraoral infusion of AF showed higher levels of motor activity, which was especially evident in hindlimbs and mouthing activity (Robinson & Méndez-Gallardo, 2011). Although these results are from rat fetuses, they demonstrate that oral exposure to AF can indeed produce behavioral activation.

Experiments 3 and 4 found that the odor of milk or AF did not exert much influence over behavioral activity in the newborn rat. Only in Experiment 4 was some increase in forelimb and hindlimb activity evident in response to the odor of milk. These findings are consistent with reports in which presentation of the odor of milk in 3- and 6-day-old pups elicited very low behavioral activation that occurred at the moment of presentation and lasted only a little after presentation of the odor (Hall, 1979b). Additional research suggests that 1-day-old rat pups respond much less (if any) to the odor of milk (Terry & Johanson, 1987). However, in the current study it was expected that the odor of AF would elicit higher behavioral activation, based on the literature that suggests that AF is a salient stimulus that helps the newborn rat adapt to the postnatal

environment (Hepper, 1987; Teicher & Blass, 1977). In humans, AF odor can be detected and human babies respond to it (Schaal et al., 1998; Schaal, Marlier et al., 1995). In Experiments 3 and 4, AF odor did not significantly affect activity in the newborn rat.

It is important to mention that while research has documented that infant rats rely heavily on chemosensory cues and olfaction to interact with their immediate environment (Alberts, 1978; Hofer, Shair, & Singh, 1976; Pedersen & Blass, 1982; Polan & Hofer, 1998; Rosenblatt, 1983; Teicher & Blass, 1977), their olfactory system also is relatively immature (Mair, Gellman, & Gesteland, 1982; Shafa, Meisami, & Mousavi, 1980), and experience is vital for its continued development (Brunjes, 1994; Todrank, Heth, & Restrepo, 2011). Olfaction in the neonatal rat becomes more sensitive as it experiences its environment, especially within the suckling context (Alberts & May, 1980; Miller & Spear, 2008; Terry & Johanson, 1987). And it also is known that newborn rats learn about their surroundings through the development of associations between stimuli that are relevant to them, as well as other cues that gain access to their environment (Brake, 1981; Cheslock et al., 2000; Johanson, Hall, & Polefrone, 1984; Johanson & Teicher, 1980). In this sense, it is possible that subjects in Experiments 3 and 4 did not respond actively to the odor of AF and milk because it was not presented in a way that was relevant at that age or that represented their normal neonatal environment.

In summary, the first part of this study provided some information about the behavioral activation of the newborn rat to exposure of AF and milk presented orally or through odor alone. Although continuity between AF and milk effects on behavioral activity was not clearly established, future research should continue to explore the effects

of AF and milk on behavior at different moments in development and through different modes of exposure. For instance, while previous research has explored the effect of an intraoral infusion of AF and milk in the fetal rat (Robinson & Méndez-Gallardo, 2011; Robinson & Smotherman, 1992c; Smotherman, Arnold et al., 1993), it will interesting to explore the effect of AF and milk oral exposure in caesarean-delivered pups before they have any suckling experience. This approach, together with findings from this study, could help us understand whether the behavioral response of the perinatal rat to AF or milk changes after leaving the uterine environment and gaining experience with milk after birth. In addition, given the findings that newborn rats (Hepper, 1987; Teicher & Blass, 1977) and human babies (Schaal et al., 1998; Schaal, Marlier et al., 1995) can respond to the odor of AF, it is important to continue exploring the effects of different modes of presentation of AF and milk odors. Dramatic behavioral responses can be elicited by AF or milk odor, as was demonstrated in the second part of this study (Chapter 5), and it will be interesting to explore odor presentations in other contexts.

Effect of AF and milk odor on crawling locomotion

The findings of Experiments 5 and 6 showed that exposure to the odor of AF or milk evokes crawling in the newborn rat. These findings are novel and unusual, given that the earliest age that organized walking has been characterized in the literature is on day 3 after birth (Jamon & Clarac, 1998). Although the ability of milk to produce behavioral activation in the newborn rat has been described (Hall, 1979a, 1979b; Robinson & Smotherman, 1992c; Terry & Johanson, 1987), there is little information about how milk or AF odors can evoke locomotion in the newborn rat. However, there

are some accounts of the capacity of an intraoral infusion to evoke some form of walking or crawling in the 3-day-old rat pup (Hall, 1979a, 1979b). In addition, although crawling in the 3-day-old rat was described by Jamon and Clarac (1998), their study lacked a comparison of the odor that induced crawling (home bedding) with a control stimulus. In Experiment 5 of the present study, the presence of the water control group and anise as a novel odor, which lack any ecological relevance to the newborn pup, provided confirmation that the behavioral crawling response is indeed a response differentially elicited by AF and milk odors.

In an early report, Bolles and Woods (1964) described that newborn rats immediately after birth can express an immature form of locomotion by using only their forelimbs and dragging their hindlimbs, and that only by day 3 after birth can rats crawl with their heads held up. This description broadly agrees with the classic study of locomotor development in the rat by Altman and Sudarshan (1975). In contrast, Experiments 5 and 6 of the present study provided qualitative observations that the crawling response of P1 pups to AF and milk commenced with a characteristic raising of the head and involved coordinated, alternating movement of the hindlimbs as well as the forelimbs (see Figure 40). These observations suggest that basic locomotor behavior can be expressed soon after birth (and perhaps before birth) and that AF and milk may play a role in the expression of locomotion and, more importantly, in its development.

An important observation made during the test of the crawling response is that crawling was maintained only when the tube remained in contact with the snout of the pup. As the data revealed, some of the pups exposed to the novel odor of anise or the

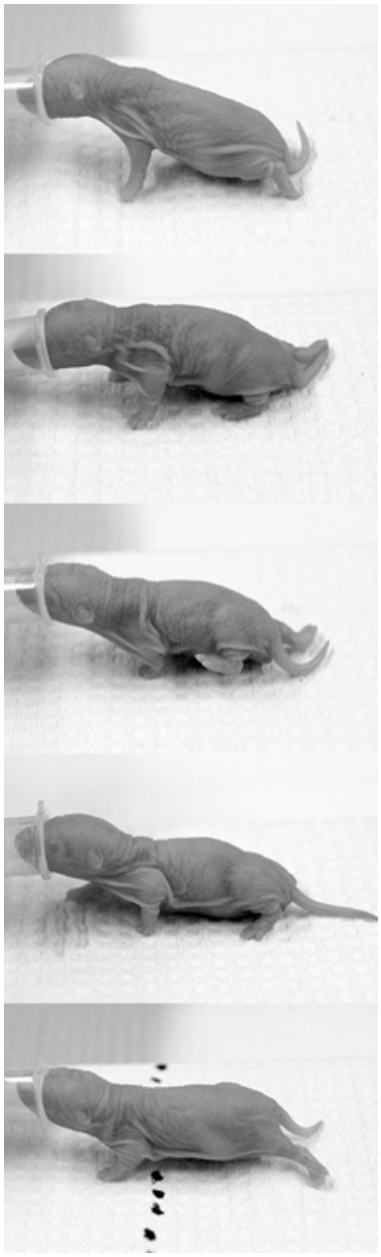

Figure 40. Crawling response of newborn rat pup to the odors of AF or milk in Experiments 5 and 6. Video frames were taken from a single crawling sequence by a P1 rat pup exposed to milk odor.

water control also showed some crawling responses. However, the crawling response to AF and milk was robust and reliable across subjects exposed to these odors, whereas the crawling response to anise and water was sporadic and shown by only some subjects, which suggests that pups definitely discriminated and responded differentially to the odors of AF and milk. But since some subjects crawled to the odor of anise and water, the method of odor exposure also may play a role in triggering a crawling response. It is possible that the microcentrifuge tube placed over the snout of the pup provides tactile stimulation that arouses and activates the pup. Once pups become aroused through perioral tactile stimulation, then they may detect the odor stimulus and continue to respond — through crawling — toward only those stimuli that are relevant to them.

Evidently, the literature in developmental psychobiology is full of examples in which infants of different species of mammals, including rats, respond to tactile stimulation (Rosenblatt, 1983). For example, pups respond faster and with more oral grasp responses to an artificial nipple when they receive tactile stimulation from a conspecific littermate (Koffman et al., 1998). Pups tested 3 days after birth attach to the nipples of an anesthetized dam that have been washed with a novel odor only when they receive tactile stimulation that resembles maternal stimulation that occurred during the original presentation of the odor (Pedersen, Williams, & Blass, 1982). Pairing of alcohol odor and tactile stimulation in neonatal pups affects learning about alcohol and responses to its odor (Domínguez, López, & Molina, 1999). Rat fetuses tested on day 20 of gestation respond to perioral tactile stimulation by expressing a facial wiping response (Smotherman & Robinson, 1990) similar to the one evoked after an intraoral infusion of

lemon (Smotherman & Robinson, 1987). In addition, it has been reported that whiskers in the neonatal rat not only are functional, but also important to process tactile stimulation that is relevant to their immediate environment within the nest and related to suckling (Sullivan et al., 2003). Similarly, application or injection of lidocaine around the perioral area (into the vibrissal pads) of rat pups disrupts nipple attachment, suggesting the importance of tactile cues during suckling (Kenyon, Cronin, & Malinek, 1981; Kenyon, Keeble, & Cronin, 1982). Given the abundance of evidence about the important role of tactile stimulation in general, and perioral stimulation in particular, in the newborn and infant rat, it is important to consider the possibility that the olfactory response to AF and milk during the crawling task used in Experiments 5 and 6 actually represents a multimodal stimulus that includes perioral tactile sensory input. In addition, the presence of the tube around the snout of the pup may provide head support or limit head movement, and therefore may facilitate the crawling response.

Although the crawling response assessed in Experiments 5 and 6 was maintained only during contact with AF and milk odors, qualitative observations made by the investigator after each crawling test suggest that experience with this crawling task may continue to promote crawling even after the tube containing the odor of AF and milk is removed and the task has ended. This observation raises the possible utility of using odor-induced crawling as a method for investigating developmental changes in locomotor abilities and how ecologically relevant odors may contribute to motor development in the newborn rat.

Effect of AF and milk odor on oral responses to the artificial nipple

The findings of Experiments 7 and 8 revealed that the newborn rat did not often grasp the artificial nipple, and that the odors of AF or milk did not facilitate grasping. Although there are reports that indicate that presentation of an artificial nipple to newborn rats tested before any suckling experience results in little or no oral grasping responses (Robinson et al., 1992), other reports suggest that the newborn rat can indeed grasp an artificial nipple (Koffman et al., 1998; Kozlov, Petrov, Kashinsky, Nizhnikov, & Spear, 2003; Petrov et al., 1999; Petrov, Nizhnikov et al., 2000; Smotherman, 2002c; Smotherman, Goffman et al., 1997). However, there are several reasons why the results from Experiments 7 and 8 are not directly comparable to the report by Koffman et al. (1998), in which presentation of the odor of AF or milk promoted oral grasping of an artificial nipple in caesarean-delivered rat pups. To start, while rat pups in this study were tested 24 hours after birth, Koffman et al. (1998) tested pups after caesarean delivery on day 21 of gestation (24 hours before term, and 48 hours earlier than pups in this study), before they had any suckling experience. Although our subjects were tested within a day of birth, it is well known that newborn rats are quite capable of learning about stimuli they encounter and adapting their behavior to new events (Cheslock et al., 2000; Johanson et al., 1984; Miller & Spear, 2008, 2009; Nizhnikov, Petrov, & Spear, 2002). In fact, 24 hours is likely to provide abundant experience, particularly in the context of suckling. In our study, those 24 hours of experience may have been sufficient for them to learn much about milk, lactating nipples, and other sensory features of the suckling environment.

Responsiveness to an artificial nipple is likely to be dependent on experience with milk and other features of the suckling context. While the fetal rat, which lacks suckling experience, does not appear to increase the probability of oral grasping of an artificial nipple after experimental exposure to milk (Smotherman, Arnold et al., 1993), newborn pups express oral grasping faster and for longer duration when presented with an artificial nipple that contains milk (Petrov et al., 1999). Although pups can grasp and become attached to a nipple despite deprivation or satiation (Hall, 1990), frequent milk infusions through an artificial nipple can prevent the pup from attaching to it (Petrov, Nizhnikov et al., 2000). In this study, although the nipple was presented only with the odor of milk or AF and no infusions were made, pups' prior experience with milk in the context of natural suckling may have contributed to their behavioral responses to the artificial nipple.

More importantly, the period of separation of the pup from the mother seems to play a very important role in the oral grasping response to an artificial nipple. As reported by Smotherman, Goffman and collaborators (1997), pups tested 1 day after birth, after separation from their mother at different separation intervals, showed more oral grasp responses to the artificial nipple when tested immediately after separation. However, oral grasping was significantly reduced when presentation of the nipple occurred 1 hour after pups were separated from their mother and only increased when nipple presentation occurred after a 5-hour separation period. Pups in our study were tested after they had been removed from their home cage, separated from their mother, and acclimated in the incubator for 30-min. As a consequence, most pups were tested about 45-min after

removal from their home cage. Considering the findings of Smotherman, Goffman et al. (1997), the results of Experiment 7 and 8 may have been affected by the length of separation of the newborn pup from the home cage and the mother.

An important difference between the present study and Koffman et al. (1998) is that they presented the artificial nipple to pups for 10 min, compared to the 3-min presentation used in Experiments 7 and 8. In their report, some of the oral grasping responses occurred after 3-min of presentation, which raises the question of whether our subjects may have shown more grasp responses if the nipple were presented for longer durations.

Although this study did not find that oral grasping of the artificial nipple was enhanced by exposing rat neonates to the odor of AF or milk, mouthing responses were commonly expressed and were significantly affected by odor exposure in Experiment 7. Mouthing activity is not uncommon during presentation of an artificial nipple. Smotherman, Goffman and collaborators (1997) reported that mouthing activity occurs significantly during presentation of an artificial nipple in the newborn rat. Mouthing activity during presentation of the artificial nipple in the fetal rat also is common (Robinson et al., 1992; Smotherman, Arnold et., 1993). The findings of Experiment 7 suggest that although pups did not grasp the nipple, they distinguished and responded to the odor of AF and milk, as demonstrated by significant changes in their mouthing activity at the time of presentation of the nipple.

In addition to mouthing, two of the behaviors that were expressed in response to the artificial nipple were paw plantarflexion and paw dorsiflexion. Although these

behaviors were reliably shown by the subjects of Experiments 7 and 8, there is no account of these or similar behaviors in the literature in response to presentation of the artificial nipple. Paw treading is the closest behavior that has been described during presentation of the nipple (Robinson et al., 1992) and during exposure to chemosensory solutions including milk (Hall, 1979a, 1979b). Paw treading (or paw pushing) is described as an aversive response to stimulation in newborns (Hall, 1979b; Hall & Bryan, 1981), infants (Johanson & Shapiro, 1986; Kehoe & Blass, 1985), and adult rats (Berridge, 2000; Grill & Norgren, 1978). But in the suckling context, it typically is not considered aversive and is often observed during and after active suckling behavior (Lau & Henning, 1985; Robinson & Smotherman, 1992a). However, paw plantarflexion and paw dorsiflexion do not closely resemble paw treading. Paw plantarflexion was expressed by the pup curving its forepaw in an inward motion with closing or gripping motion, while paw dorsiflexion involved raising the entire forepaw by bending at the wrist in a dorsal direction (Figures 41 and 42). These were distinctive paw behaviors noted in this study, but there appears to be no description of these behaviors in the literature. It therefore is difficult to accurately ascribe the meaning of these behaviors within the context of these experiments. However, it is clear from the findings that pups differentially express paw plantarflexion and paw dorsiflexion in response to the odor of AF or milk, and do not show these responses to the novel odor of anise or the control odor of water. As a consequence, these paw responses may reflect behaviors that are specific to the suckling context and are expressed under natural conditions by pups while sucking on the nipple, ingesting milk, or interacting with AF *in utero*. It will be important

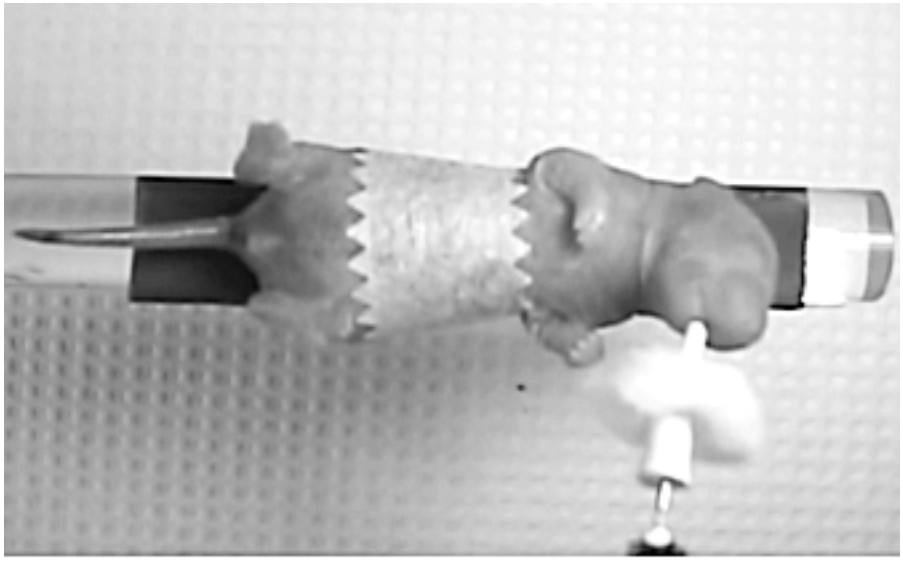

Figure 41. Paw plantarflexion response of newborn rat pup to the odor of AF or milk during presentation of the artificial nipple in Experiments 7 and 8. Note the closure of the forepaws relative to the foreleg.

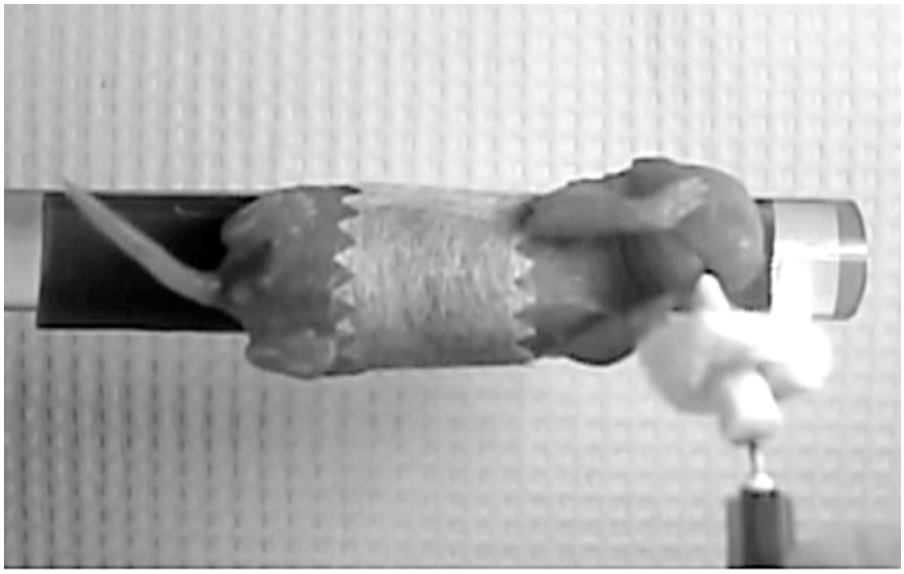

Figure 42. Paw dorsiflexion response of newborn rat pup to the odor of AF or milk during presentation of the artificial nipple in Experiments 7 and 8. Note the sharp upward angle of the right paw at the wrist relative to the foreleg.

to continue to explore the expression of paw plantarflexion and paw dorsiflexion in response to AF and milk exposure and to identify how these paw responses relate to functional behavior in a natural context.

The role of AF and milk during associative learning

Experiments 9 and 10 show not only that AF and milk can act effectively as a US in support of associative learning in the newborn rat, but also that paired presentations of the artificial nipple CS with AF or milk as the US results in conditioned oral responses to the nipple. The use of AF and milk as a US during classical conditioning has been reported in the literature in fetal (Robinson, Arnold et al., 1993; Robinson & Smotherman, 1997; Smotherman 2002a, 2002b; Smotherman & Robinson, 1994; Varlinskaya et al., 1997), newborn (Cheslock et al., 2000), and infant rats (Arias & Chotro, 2007; Johanson et al., 1984; Johanson & Hall, 1982; Johanson & Teicher, 1980; Sullivan & Hall, 1988). In the present study, it originally was expected that facial wiping responses to an intraoral infusion of lemon would be affected in response to conditioning. However, the results of these experiments did not provide evidence of reduced facial wiping after reexposure to the CS after conditioning. Previous research has shown that an intraoral infusion of AF or milk in the newborn rat results in a diminished facial wiping response to a lemon infusion (Méndez-Gallardo & Robinson, 2010). It was expected that after pairings of the CS and AF or milk as the US, reexposure to the CS would result in a diminished facial wiping response to the test infusion of lemon. Since this was not the case, it is important to evaluate why conditioning did not affect the facial wiping response.

One possibility is that pups in this study reacted at a level much higher than usual to the lemon infusion. It has been reported that the concentration of the lemon odor solution affects the facial wiping response (Brumley & Robinson, 2004). Although the concentration used in Experiments 9 to 12 (a 1:1 dilution of pure lemon extract) previously has been used effectively in rat fetuses to elicit facial wiping (Brumley & Robinson, 2004), and to demonstrate reduced responding of fetal rats after exposure to milk or AF (Korthank & Robinson, 1998), it is possible that the 1:1 lemon odor solution elicited higher levels of activation in pups in this study. In fact, the concentration of the lemon stimulus used in this study (1:1 dilution of pure extract) was less than the pure extract used in the previous study of facial wiping in newborn rats (Méndez-Gallardo & Robinson, 2010). Untreated P1 rat pups in the earlier report typically showed 8-10 wiping strokes after lemon infusion, but pups in this study often expressed nearly twice as many wiping strokes (see Figures 32 and 36). Although it is unknown why pups in this study may have been more reactive, greater reactivity may have prevented AF and milk — through reexposure of the CS after pairings — to exert a significant influence on the wiping response.

Another possibility to consider is the effectiveness of the conditioning protocol used in Experiments 9 to 12. Although this conditioning protocol has been used reliably in rat fetuses to evaluate the effect of contingent presentations of the artificial nipple CS and milk US on facial wiping to lemon (Arnold et al., 1993; Robinson, Arnold et al., 1993; Smotherman & Robinson, 1994), it had not been used in newborn rats before this study. It is possible that some parameters of this conditioning protocol are not optimal for

use with newborn rats. The protocol originally was developed taking into account the timeline of the opioid-induced behavioral effects of milk on facial wiping in the fetal rat. As reported by Robinson and Smotherman (1994), the effect of milk on facial wiping in rat fetuses has a timeline of around 5 min, in which milk exerts its maximal effect by 60 s after oral infusion and returns to baseline levels by 300 s after infusion. Although AF had not been used as a US in a similar conditioning protocol in either the fetal rat or the newborn rat before the present study, there are reports that the opioid-induced behavioral effects of AF on facial wiping in rat fetuses have a similar time course as milk, reaching maximal effects within 30 s and returning to baseline within 5-min (Robinson & Méndez-Gallardo, 2010). However, although the effects of AF and milk in the newborn rat have been described (Méndez-Gallardo & Robinson, 2010), no study to date has described the time course of opioid-induced behavioral effects of milk or AF in the newborn rat. Thus, it is possible that the opioid system remains activated for a longer duration after oral infusion of AF or milk in the newborn rat. If so, then scheduling paired presentation of the CS and US every 5 minutes may be less effective in promoting classical conditioning because the CS would not be predictive of the onset of opioid activity during the 2nd and 3rd conditioning trials. This possibility should be explored in the future to investigate whether facial wiping or other dependent variables are differentially affected by the parameters of classical conditioning with AF and milk in the newborn rat.

Nevertheless, the conditioning protocol used in Experiments 9 to 12 was effective in modifying the oral response to the artificial nipple during reexposure of the CS. Mouthing responses during reexposure to the artificial nipple CS were significantly

higher in subjects in the paired group and remained high for at least 15 s after the nipple was removed. As discussed above, mouthing during presentation of an artificial nipple is common and has been reported previously in both the newborn (Smotherman, Goffman et al., 1997) and fetal rat (Robinson et al., 1992; Smotherman, Arnold et., 1993). Similarly, intraoral infusions of milk commonly elicit a mouthing response in the newborn rat (Hall, 1979a, 1979b). Given these previous reports, it is important to consider the possibility that mouthing responses during reexposure to the nipple may not represent classical conditioning, but rather may be a form of sensitization in response to the pairings.

Since mouthing can be evoked by the artificial nipple prior to conditioning, the expression of mouthing activity to the nipple after conditioning does not meet the conventional definition of a classically conditioned response. Holland (1977) described a similar phenomenon using light or tone as CS and food as US. He suggested that the CS can be associated with different stimulus or response events even though the US used is always the same. In this sense, although it may seem that the responses to the CS are the result of classical conditioning, Holland suggests that some of these responses could also be the result of instrumental conditioning. In the current study, a similar situation may be playing a role with the mouthing responses to the nipple after conditioning. These responses may occur due to the continuous reinforcement of the CS during pairing. Mouthing responses to the nipple during reexposure appear to be a response that has been rendered resistant to habituation during pairings. Mouthing can be normally evoked by presentation of the nipple alone and although it would be expected that this behavior

could be altered after the three pairings used in the current paradigm, as already reported, there is a persistence of this behavior in the paired group. However, it is important to also highlight that mouthing activity was observed only in the paired group, which suggests that this is indeed a modification resulting from associative learning. Whether response to the nipple during reexposure to the CS is the result of classical conditioning or of reinforcement after pairing remains to be explored.

Opioid involvement in behavioral responses to AF and milk

The findings of this study provide some evidence that the endogenous opioid system may mediate some of the behavioral effects of AF and milk in the newborn rat. However, evidence of opioid involvement was not found in all experiments, despite reasonable expectations to the contrary drawn from the available literature. Some of the reasons for these mixed results are discussed below.

The findings of Experiments 2 and 4, in which behavioral activation to AF or milk was explored, provide some evidence for the involvement of the opioid system after intraoral infusion, but not after exposure to the odor of AF or milk alone. In Experiment 2, pretreatment with the opioid antagonist naloxone did not appear to affect the frequencies of movements of mouth, head, forelimbs, or hindlimbs relative to saline-treated controls. However, when hindlimb activity was expressed as a percentage of overall activity, naloxone-treated subjects showed reduced activity, implying that oral exposure to milk or AF results in an opioid-dependent shift in the relative occurrence of movement categories. These results agree broadly with previous research that has described the opioid-induced behavioral effects of AF and milk after oral exposure. For

example, blockade of the opioid system with a non-selective opioid antagonist such as naloxone or naltrexone before an intraoral infusion of AF or milk reduce facial wiping to a lemon odor solution in the fetal and newborn rat (Korthank & Robinson, 1998; Méndez-Gallardo & Robinson, 2010). Specifically, the kappa class of opioid receptors, and not mu, appears to mediate the behavioral effects of AF and milk on facial wiping (Korthank & Robinson, 1998; Méndez-Gallardo & Robinson, 2010).

In a similar way, the stretch response elicited by an intraoral infusion of milk in the fetal rat can be blocked by treatment with naloxone or with selective antagonist drugs for either the kappa or mu systems (Smotherman & Robinson, 1992b). However, blockade of kappa receptors, but not mu, prevents the increase in hindlimb activity that typically occurs after an infusion of milk, suggesting that the kappa class of opioid receptors is essential in the expression of behaviors elicited by an intraoral infusion of milk (Andersen et al., 1993). These findings are especially relevant to the results of Experiment 2, since an increase in hindlimb activity relative to other movements was blocked by treatment with naloxone. This finding suggests that the effects of AF and milk on hindlimb activity in the newborn rat are governed by the opioid system, possibly through activation of kappa opioid receptors, in the same way as the stretch response in the rat fetus (Andersen et al., 1993; Smotherman & Robinson, 1992b).

Although the examples described above, as well as the findings of Experiment 2, provide evidence that the behavioral effects of AF and milk infusion are mediated by the endogenous opioid system in the perinatal rat, Experiment 4 failed to provide evidence for opioid modulation of behavioral activity during pup exposure to the odor of AF or

milk. It is possible that oral contact with AF or milk is necessary, and odor exposure alone is not sufficient, to evoke opioid activity in the newborn rat.

The interpretation that AF or milk odor is insufficient to evoke opioid responses was further supported by the results of Experiments 6 and 8. In Experiment 6, blockade of opioid receptors had no evident effect on the crawling response evoked by either AF or milk. This absence of opioid effects on crawling is interesting in light of previous research that has implicated the opioid system, possibly acting in conjunction with other systems such as serotonin or dopamine, in modulating locomotor behavior in infant rats. Administration of the kappa opioid agonist U50,488 in 5- to 10-day-old rat pups results in increased locomotion, suggesting that kappa receptors are involved in this behavior (Jackson & Kitchen, 1989). Activity in the dopamine system also has been implicated in promoting behavioral activity and general locomotion in perinatal rats. Dopamine agonists that act preferentially at D_1 (SKF-38393) and D_2 (quinpirole) receptors induce locomotion in the neonatal (Moody & Spear, 1992) and the infant rat (McDougall, Arnold, & Nonneman, 1990; Van Hartesveldt, Meyer, & Potter, 1994). Moreover, the dopamine system also has been related to behavioral responses to milk in the neonatal (Caza & Spear, 1982) and fetal rat (Moody, Robinson, Spear, & Smotherman, 1993; Robinson, Moody, Spear, & Smotherman, 1993; Smotherman & Robinson, 1995). Furthermore, research in the fetal rat suggests that dopamine and kappa systems interact to promote effects on motor activity (Smotherman, Moody, Spear, & Robinson, 1993). However, the relationship between opioid and dopamine systems in mediating the effects

of AF and milk in the newborn rat remains unexplored, and should be a productive area for future research.

Experiment 8 also found no evidence that the opioid system mediates behavioral responses of the newborn rat to presentation of the artificial nipple in association with the odors of AF and milk. These results are not consistent with previous research that has reported opioid influences on oral grasping of an artificial nipple (Petrov, Varlinskaya, Becker, & Smotherman, 1998; Petrov, Varlinskaya, & Smotherman, 1997a). Specifically, research with fetal and caesarean-delivered newborn rats has suggested that mu receptors of the opioid system play a role during oral grasping, and that this class of receptors has different effects in different parts of the central nervous system, with receptors in rostral parts of the brain promoting oral grasping (Petrov, Varlinskaya, Becker et al., 1998; Petrov et al., 1997a), and receptors in more caudal areas of the brainstem inhibiting grasping (Petrov, Varlinskaya, Becker et al., 1998; Petrov et al., 1997a). However, there are no reports concerned with opioid effects on responses to the nipple during presentation of AF or milk odors. The only report in the literature about presentation of the nipple with the odors of AF and milk in the caesarean-delivered rat pup (Koffman et al., 1998) does not evaluate if the opioid system is involved in the response. Thus, taken together with the results of Experiments 4 and 6, the findings of Experiment 8 suggest that the opioid system may not be involved during the behavioral effects of AF and milk when they are presented in odor form.

Opioid activity after associative learning

Experiments 10 and 12, which evaluated associative learning in the newborn rat using AF and milk as US and the artificial nipple as the CS, confirmed that the endogenous opioid system is involved during the expression of conditioned oral responses to the artificial nipple after associative learning. The next step to be considered in the future in relation to these studies would be to explore which class of opioid receptors is involved during associative learning in the newborn rat. However, review of the literature about opioid effects during associative learning in the fetal rat has revealed a complicated function of the opioid system where not only kappa receptors are active, but also receptors of the mu opioid system. In these studies, milk was used as the US, the artificial nipple as the CS, and the dependent variable was facial wiping responses to a perioral probe after reexposure to the CS. For example, Robinson, Arnold, and collaborators (1993) reported that after pairing of an artificial nipple (CS) and milk (US), blockade of the endogenous opioid system with naloxone — a non-selective opioid antagonist — before reexposure to the CS resulted in higher levels of facial wiping to a perioral probe. Reduced responding in saline-injected subjects was evident after paired presentation of CS and US, but not in unpaired, CS-alone or US-alone groups. These results suggest that reexposure to the CS results in activation of the opioid system, which in turn modulates responsiveness in the facial wiping test.

In subsequent experiments, it was found that the facial wiping response could be reinstated by treating subjects after training and before reexposure to the CS with the selective opioid antagonists for mu receptors, CTOP or FNA (β-funaltrexamine HCl), but

not with the selective kappa receptor antagonist BNI. The conclusion that mu opioid activity, and not kappa, mediates the conditioned opioid response after training has been replicated several times (Arnold et al., 1993; Smotherman & Robinson, 1994; Smotherman, 2002a). Taken together, these studies of associative learning in fetal rats suggest that association of a CS with a stimulus that evokes an unconditioned kappa opioid response (milk) results in a conditioned opioid response to reexposure to the CS that involves the mu opioid system.

Further research has revealed a much more complicated involvement of both mu and kappa opioid systems during associative learning and during expression of conditioned responses (Petrov, Varlinskaya & Smotherman, 2000; Robinson & Smotherman, 1997; Smotherman & Robinson, 1994; Smotherman, 2002a, 2002b), the details of which go beyond the scope of the present study. Evidently, involvement of the endogenous opioid system during associative learning in the perinatal rat is a story that will require much more research to gain a better understanding of the role of opioids during perinatal learning. The present study contributes to this effort in two ways, by documenting that: (a) the newborn rat can acquire and express associative learning when a CS (the artificial nipple) is paired with oral infusion of AF or milk, and (b) reexposure to the nipple CS after training appears to evoke a conditioned opioid response that modifies neonatal behavioral responses to the artificial nipple. It remains for future research to determine whether learned changes in response to the artificial nipple represent classical conditioning or some other form of associative learning, and whether

patterns of involvement of specific opioid receptors also are similar in fetal and neonatal learning.

Conclusion: Do AF and milk exert similar behavioral effects?

The principal question of this study was whether sensory exposure to AF and milk results in similar behavioral effects in the newborn rat. As the results of the 12 experiments comprised by this study demonstrate, the answer to this question is not simple and straightforward. Although some behavioral responses to AF and milk may be similar, others are not the same. And while behavioral responses elicited by AF and milk through oral infusion or odor exposure, in association with presentation of an artificial nipple, or through learned associations, need further research to be fully understood, the results of the present study identify some points of continuity between AF and milk. It seems clear that the behavioral responses elicited by these stimuli are strongly influenced by the context of the experimental task, by the developmental state of the pup, by physiological differences such as temperature, and, most importantly, by experience. The methods and findings of this study amply demonstrate that all of these aspects should be taken into consideration when assessing the behavioral effects of AF and milk.

The present study also provided mixed results concerning the role of the endogenous opioid system in mediating the behavioral effects of AF and milk. The results revealed engagement of the opioid system only during the expression of hindlimb activity after oral infusion of AF or milk in Experiment 2 and during mouthing responses to the nipple CS after associative learning in Experiments 9 to 12. The general hypothesis that the endogenous opioid system underlies all of the behavioral effects of AF and milk in

the perinatal rat cannot be supported. However, opioid activity does appear to play a role in some behavioral variables and with some modes of presentation. Clearly, taking all of the experiments of this study into consideration, the question of how the opioid system is involved in behavioral responses to AF and milk, before and after birth, should continue to be explored.

Something that is very important to emphasize is that although this study did not find evidence of opioid involvement in some of the experiments conducted, that does not indicate that the opioid system does not play important roles in mediating the behavioral effects of AF and milk. As discussed extensively in this study, there are many examples in which exposure to AF or milk results in activation of the opioid system in the fetal, newborn, infant, and adult rat. But how the opioid system contributes to the regulation and development of behavior is also complicated and requires extensive research. The simple hypothesis posed at the outset that opioid activity mediates all of the behavioral effects of AF and milk appears to be falsified by the present study. The simplest modification consistent with these results is that opioids are involved in behavioral effects after direct oral contact with AF or milk, but not after mere exposure to the odor of AF or milk alone. It remains for future research to resolve this possibility.

As a final thought, it may seem redundant to continue to mention the importance of studies, such as the present one, in which a continuum between behavioral life before and after birth continues to be emphasized. This has been a common theme in the field of developmental psychobiology for decades. But continuity in behavior and its underlying mechanisms before and after birth continue to be highly misunderstood and

underappreciated in the larger scientific community. Too often, developmental scientists simply assume that behavior expressed soon after birth comes from nowhere and that behavioral organization in the newborn appears suddenly in an inexplicable and almost magical way. This continuing perspective is why it is important to investigate aspects of behavioral continuity, as in this study. Moreover, the case for developmental continuity is strengthened by not just describing similarities in the expression of behavior, but also by identifying common underlying neural mechanisms, such as involvement of the endogenous opioid system, in the fetus and newborn. It is the aim of this study to contribute to a growing literature that emphasizes understanding the developmental roots of behavior before and after birth. This study demonstrates, once more, that newborns can respond and interact with stimuli in their environment, that they can recognize stimuli of ecological relevance such as AF, milk, and an artificial nipple, that they can learn about these stimuli and associations between them, and that these experiences contribute and are essential to their development. Finally, the capacity of the newborn rat to express behavior in different and adaptive ways in response to both AF and milk — features that represent two different developmental periods — demonstrates once more that continuity in behavior and underlying neural mechanisms bridge the artificial separation between prenatal and postnatal life.

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