Dopamine Action in the Nucleus Accumbens and Medial Preoptic Area and the Regulation of the Hormonal Onset of Maternal Behavior in Rats

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DOPAMINE ACTION IN THE NUCLEUS ACCUMBENS AND MEDIAL PREOPTIC AREA AND THE REGULATION OF THE HORMONAL ONSET OF MATERNAL BEHAVIOR IN RATS

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Abstract:

Postpartum female rats immediately respond to biological or foster offspring with the display of maternal behavior. In contrast, females that are hysterectomized and ovariectomized on day 15 of pregnancy (15HO) and presented with pups 48 hours later show maternal behavior after 2-3 days of pup exposure. The natural onset of maternal behavior in postpartum females is mediated, in part, by the rise in estradiol just prior to birth. When 15HO rats are administered estradiol benzoate (EB) at the time of HO surgery, 48 hours prior to pup presentation, they show an almost immediate onset of maternal behavior. Presumably, EB administration functions to prime neural circuits which regulate maternal behavior such that these circuits respond to pup presentation with increased maternal responsiveness. Two important neural regions which have been shown to interact in order to promote responsiveness toward infant stimuli are the medial preoptic area (MPOA) and the nucleus accumbens (NA). The following series of studies were undertaken to examine how dopamine (DA) activity within these two important neural sites substitutes for the facilitatory effects of chronic (48 hours) EB stimulation of maternal behavior in 15HO rats. Study 1 investigates whether, in the absence of EB treatment, microinjection of

dopamine receptor agonists into either NA or MPOA at the time of pup presentation stimulate maternal behavior in 15HO rats. Study 2 examines the underlying mechanism by which DA receptor stimulation of NA promotes the onset of maternal behavior in 15HO rats. Finally, Study 3 examines the relationship between DA receptor stimulation and estradiol stimulation in the facilitation of maternal responsiveness in 15HO rats.

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I. Introduction:

The goal of my research has been to uncover aspects of the neural circuitry which regulates maternal motivation in rats, or the change in responsiveness toward pup stimuli which occurs naturally at birth. In particular, maternal behavior in rats is an excellent model with which to examine neural regulation of motivation, because female rats display a dramatic change in responsiveness toward infant stimuli, such that a female rat who is unresponsive to infant stimuli prior to birth rapidly approaches and responds to infant stimuli at the time of birth.

Importantly, aside from being an excellent model with which to understand motivation in general, research on maternal behavior in rats provides relevant implications for our understanding of human maternal behavior as well. There are, of course, differences between species, but because maternal motivation is a defining characteristic of all mammals, it is likely that a core neural circuit which regulates maternal behavior has been conserved across species (Numan and Insel, 2003). In this context, individual variation in maternal motivation across species might be explained by the extent to which hormonal, genetic, and experiential factors impact this core neural circuit. Finally, note that this core neural circuit does not include the regulation of species-specific behavioral responses toward infants. For example, humans do not carry infants in their mouths the way that rodents do, and therefore the neural mechanisms underlying the specific behaviors which vary between species are not addressed here, instead the scope of my research has been to understand the core neural circuit

which regulates the change in responsiveness or maternal motivation which is expressed across species.

Maternal behaviors, like all reproductive behaviors, can be dichotomized into appetitive and consummatory components (Ball and Balthazart, 2008). The initial phase of attraction and pursuit, termed the appetitive phase, is comprised of behaviors which bring the organism into contact with the goal stimulus, and is followed by the final phase of consummation, or the consummatory phase, in which the organism interacts with the acquired stimulus. Because the appetitive components of reproductive behavior are variable behavioral responses which depend upon the different environmental constraints or obstacles the organism must overcome to gain access to the goal stimulus, higher cognitive processes are probably recruited to mediate appetitive reproductive behaviors. In contrast, since the consummatory components of reproductive behavior are predictable, invariant, and usually reflexive in nature, they are likely mediated in large part by brainstem and spinal cord mechanisms.

Rat pups are born altricial, which means they have limited sensory and motor capabilities and are not able to regulate their body temperature or urinate and defecate on their own; therefore, infants are entirely dependent on maternal behaviors for their survival. After giving birth, female rats naturally respond to their infants by building a nest and crouching over the pups to keep them warm. Females lick and groom pups, which aids in urination and defecation, and feed them by adopting nursing postures over pups allowing them to suckle and

receive milk. Should pups become displaced, or should the female need to move her nest, she picks the pups up in her mouth and carries them to the nest. This transport behavior is referred to as retrieval behavior.

Typically, approach behaviors which help the female gain access to her pups can be conceptualized as the appetitive components of maternal behavior, and this would include retrieval behavior, during which the female initiates contact with her pups (Numan and Insel, 2003). Note that some question exists as to whether retrieval behavior, which also requires a stereotypical mouthing and picking up of the pups, can also be considered a consummatory component of maternal behavior. However, there are two important aspects of retrieval behavior which classify it as an appetitive behavior. First, retrieval behavior is variable and dependent upon complex cognitive processes. In order to retrieve pups to the nest, the female must find the pups, recall the location of her nest, remember where she has searched for pups and where she has yet to search, determine which route to take to get to her nest, and determine if she has collected all of her pups at the nest or if she must continue to search for her pups. Second, if one views nursing behavior as the goal of maternal interaction, then retrieval behavior, which brings the pups to the nest so they can be nursed, should also be considered an appetitive component of maternal behavior. Furthermore, because the goal of our laboratory has been to examine the brain circuits which regulate mother-initiated maternal responses, retrieval behavior is an important dependent variable in many of our studies. Finally, by studying the

neural mechanisms regulating the transition from pup avoidance to maternal care (onset of maternal behavior) we should be tapping into basic appetitive motivational mechanisms.

While postpartum female rats display maternal behaviors in the presence of offspring (including offspring that are not biologically related), virgin female rats do not display these behaviors when presented with pups, rather they avoid them (Numan and Insel, 2003). However, if nulliparous female rats are continuously cohabitated with freshly nourished pups from a donor mother each day, after about 8 days they will come to display full maternal behavior almost indistinguishable from that of a lactating female (Rosenblatt, 1967). This process in which repeated exposure to pups induces maternal behavior in virgin female rats is called sensitization, and the number of days required before the female exhibits responsiveness is referred to as her sensitization latency. The finding that virgin female rats can be induced to show maternal behavior after a period of pup sensitization indicates that the hormonal events of pregnancy and parturition are not necessary for maternal responsiveness to occur; instead they reduce the amount of pup exposure required for maternal behavior to be initiated (Numan, 2006).

Exposure to the hormonal events of pregnancy, which lasts 22 or 23 days, facilitates the rapid onset of maternal behavior (Numan and Insel, 2003). First, cervical stimulation during mating initiates a twice-daily pattern of prolactin release from the anterior pituitary (for approximately 9-10 days after mating)

which functions to rescue the corpora lutea from degradation, and as a result, steadily increase progesterone (P) levels during the first part of pregnancy (Erskine, 1995). After day 10, a shift takes place such that placental lactogens support the luteal secretion of P that is necessary for the continuation of pregnancy. During early pregnancy, P prepares the uterine endometrium for implantation of the blastocysts and once implanted, it maintains a uterine environment that promotes growth of the embryos and prevents an early expulsion of the fetuses (Turner, 1966; Zakar and Hertelendy, 2007). While rising levels of P promote the maintenance of pregnancy, the decline of P beginning in mid-pregnancy initiates a shift in hormonal events that eventually regulates the timing of labor. Increasing levels of estradiol (E) secreted from the ovary prepare the uterine endometrium for labor by promoting rhythmic contractility of the uterus, and the sharp decline in P just before birth removes the inhibitory tone on the uterine muscles and allows them to respond to the surge in oxytocin (OT) from the posterior pituitary that induces uterine contraction and labor. Finally, as parturition approaches, the hormonal shift from P dominance to E dominance, in combination with peripherally released OT and prolactin functions to prepare the uterus for birth of the fetuses, while E in combination with centrally released OT and prolactin primes neural circuits to respond to infant stimuli at birth (Bridges et al., 1990; Numan and Insel, 2003; Pedersen et al., 1994).

Although the pregnant rat is exposed to circulating levels of pregnancy hormones, responsiveness toward pup stimuli first emerges in the final hours

prior to parturition (Mayer and Rosenblatt, 1984). Therefore the hormonal events which occur at this time probably play a role in stimulating the onset of maternal behavior. The early work of Terkel and Rosenblatt (1968, 1972) empirically explored this idea and reported that factors circulating in the blood at the time of birth and just after birth were responsible for initiating maternal responsiveness. They found that blood transfused from a maternal female rat (taken within 24-48 hours of giving birth) to a virgin female rat produced significantly shortened sensitization latencies in the virgin rat. Research conducted during the next several decades has supported the idea that the precipitous decline in P in combination with the rise in E and lactogens, which occurs during the final 48 hours of pregnancy, is responsible for the immediate maternal responsiveness of parturient females at birth (Numan, 1988).

First, artificial termination of pregnancy by hysterectomy (H; removal of uterus and fetuses) during the latter half of pregnancy (after a period of P secretion) produces a decline in P levels due to removal of the influence of placental lactogens; the drop in P levels combined with rising E secretion (the natural rise in E during late pregnancy is potentiated by removal of P) is strikingly similar to the hormonal profile which occurs just prior to parturition, and this preparation produces an immediate onset of maternal behavior when pups are presented 48 hours after surgery (Rosenblatt and Siegel, 1975). Note that if pups are presented before P has declined (Bridges et al., 1978; Siegel and Rosenblatt,

1978) or if P is maintained (Doerr et al., 1981; Numan, 1978; Siegel and Rosenblatt, 1975b, 1978) the onset of maternal behavior is delayed.

Second, the presence of E is equally as important as the decline in P for the stimulation of maternal behavior because ovariectomy (O) at the time of H (which would produce a decline in E as well as P) prevents the full facilitatory effects of H on the onset of maternal behavior (Siegel and Rosenblatt, 1975a). Female rats that are hysterectomized and ovariectomized (HO) on day 15 of pregnancy (15HO) require about 3 days of exposure to pups prior to showing maternal behavior (significantly more than the immediate responsiveness of the 15H animal). Importantly, the injection of estradiol benzoate (EB) at the time of HO can reinstate immediate onset in 15HO rats. Note that while the 3 day latency of 15HO females is significantly longer than that of 15HO +EB treated females, it is also significantly shorter than the sensitization latency of a virgin female (about 7-8 days). Therefore, 15HO females might be regarded as receiving a sub-optimal or partial hormone priming due to the partial facilitation of this preparation on the onset of maternal behavior.

Third, nulliparous virgin female rats administered a hormone schedule which mirrors the latter half of pregnancy, show a facilitated onset of maternal behavior. Moltz, Lubin, Leon, and Numan (1970) were the first to examine sensitization latencies of ovariectomized virgin female rats given the following hormone injection schedule: EB for days 1-11, P for days 6-9, and prolactin on days 9 and 10. On the morning of day 10 females were presented with pups and

tested for maternal behavior. Females receiving this schedule of injections showed about a 2 day sensitization latency. Note that while this latency is not as short as the 0-1 day latency in 15H or 15HO + EB females, it is still significantly shorter than the latency of a virgin female rat (about 7-8 days). Also note P injection on day 10 prevented a facilitated onset of maternal behavior. Bridges (1984) went on to examine whether virgin females administered physiological doses of E and P needed exogenous prolactin in order to show a facilitated onset of maternal behavior. Results indicated that E and P injection alone are sufficient to induce maternal behavior. However prolactin is still involved since E stimulates the release of prolactin from the pituitary gland and because E and P injections do not stimulate maternal behavior in hypophysectomized rats (Bridges and Dunckel, 1987).

The primary conclusion of the pregnancy termination and virgin sensitization studies presented above, is that high E and lactogens superimposed on a backdrop of P withdrawal facilitate maternal behavior. The role of P decline in the facilitation of maternal behavior is related to its ability to lower the threshold of E stimulation necessary to produce a behavioral effect (Doerr et al., 1981; Siegel and Rosenblatt, 1975a, 1975b 1978); other studies have shown that E alone can stimulate maternal behavior, but only with much higher, supraphysiological doses (Bridges, 1984).

One neural site where hormones act to promote the onset of maternal behavior is the medial preoptic area of the hypothalamus (MPOA). The MPOA

contains estrogen receptors (Shughure et al., 1997), prolactin receptors (Bakowksa and Morrell, 1997), progesterone receptors (Numan et al., 1999), and oxytocin receptors (Champagne et al., 2001). Using the pregnancy termination model, Numan, Rosenblatt, and Komisaruk (1977) showed that 15HO female rats that normally required 2-3 days of pup exposure to induce maternal behavior would show maternal behavior on the first day of pup exposure when implanted with EB directly into MPOA, suggesting that the MPOA is at least one neural site where the rise in E at the end of pregnancy acts to promote the onset of maternal behavior.

Not surprisingly, a large body of evidence indicates that damage to the MPOA impairs maternal behavior (Numan and Insel, 2003). Interference with MPOA activity either with lesions (both electrical and excitotoxic) or pharmacological depression severely disrupts maternal behavior in sensitized virgin, parturient, and postpartum females (Arrati et al., 2006; Fleming et al., 1983; Gray and Brooks, 1984; Jacobson et al., 1980; Kalinchev et al., 2000; Lee et al., 2000; Numan, 1974; Numan and Callahan, 1980; Numan et al., 1988; Numan et al., 1977). Additionally, knife cuts which sever the lateral connections of MPOA produce similar deficits in maternal responding, suggesting the importance of lateral MPOA efferents in the regulation of maternal behavior (Numan, 1974;Numan and Callahan, 1980; Terkel et al., 1979). While MPOA lesions have been shown to affect all aspects of maternal behavior, note that the active components such as retrieval and nest building show the most severe

impairments, as first pointed out in Terkel et al. (1979). Based on the extensive evidence that MPOA damage disrupts maternal behavior and hormonal stimulation of MPOA potentiates maternal behavior, the MPOA is considered a central, critical site for the regulation of maternal behavior (Numan, 2006).

How does the MPOA regulate maternal behavior? While the hormonal events of parturition can induce maternal behavior, this hormonal profile is relatively short lived. Evidence indicates, however, that once maternal behavior is initiated at parturition, its maintenance during the postpartum period is independent of hormonal control (Numan and Insel, 2003). The interaction between hormones and afferent input from pups within MPOA during the onset of maternal behavior might modify MPOA neurons such that they are responsive to pup related stimuli even in the absence of continued hormonal stimulation. From a molecular perspective, one way steroid hormones, like E and P, exert their effects is by altering gene expression. Long term changes in gene expression can change the phenotype of neurons, altering the type of stimuli a particular neuron responds to as well as the type of response elicited (Sheng and Greenberg, 1990). Therefore, hormones might be involved in modifying MPOA neurons such that they are responsive to pup inputs even after the hormonal events of birth wane.

The most common description of the mechanism by which steroid hormones [such as estradiol (E), progesterone (P), and testosterone (T)] alter cellular function is via their slow acting, but long lasting effects on gene

transcription (Beato and Klug, 2000). Their lipid-like structure enables them to permeate the plasma membrane of cells and diffuse into the nucleus where, once bound by their protein receptor, they form a complex that interacts with steroid responsive DNA sequences to activate or repress the expression of particular genes. Steroid receptors can also be found in the cytoplasm, in which case the bound steroid receptor complex translocates to the nucleus to exert its effects. A classic example of this mechanism is the induction of progesterone receptors (PR) by the steroid E (Kraus et al., 1994). In this case, the ligand bound estrogen receptor (ER) binds to an estrogen response element (ERE) in the promoter region of the PR gene where it initiates transcription and, after several hours, results in a high density of PR in the cell.

Steroid hormones are also capable of initiating the transcription of genes which do not contain steroid responsive elements. This newly recognized mechanism involves both recently identified steroid membrane receptors as well as intracellular receptors (Beato and Klug, 2000; Bishop and Stormshak, 2008; Bjornstrom and Sjoberg, 2005; Foradori et al., 2008; Kelly and Levin, 2001; Mhyre and Dorsa, 2006; Toran-Allerand, 2004; Vasudevan and Pfaff, 2007, 2008). For example, while it has been documented that ovariectomized female rats treated with E show increased expression of the neurotransmitter neurotensin in the MPOA which is indicative of increased transcription of the neurotensin gene, the neurotensin gene clearly lacks an ERE (Mhyre and Dorsa, 2006). A series of experiments revealed that E administration increases the

expression of neurotensin by affecting a protein which is capable of initiating transcription of the neurotensin gene. Specifically, E stimulates an intracellular signaling cascade which ultimately phosphorylates or activates the cyclic AMP response element binding protein (CREB), a protein which binds to a calcium or cyclic AMP response element (CRE) in the promoter region of the neurotensin gene (Mhyre and Dorsa, 2006). Importantly, because many genes have CRE in their promoter regions, and because the phosphorylation of CREB is typically mediated by intracellular signaling cascades initiated by neurotransmitter binding at the cell membrane, this finding revealed a myriad of possible genes which steroid hormones might influence, and suggested a common mechanism by which steroids and neurotransmitters might exert their effects.

In addition to altering gene transcription at CRE sites, note that rapid phosphorylation of CREB indicates that steroid hormones activate intracellular signaling cascades that can also produce rapid cellular changes. This last point calls attention to a second important alteration in our conceptualization of steroid hormone action. Whereas it has generally been thought that hormones produce their effects in hours or even days, it is now clear that hormone binding can induce cellular changes measurable in just minutes. For example, E is capable of increasing CREB phosphorylation in minutes (Abraham et al., 2004). This example suggests that cytoplasmic proteins (rather than nuclear proteins) may also be phosphorylated within minutes of E activation of signaling cascades, and therefore promptly modify cellular function.

In summary, two important nonclassical aspects of steroid function should be noted: (1) steroids can influence the transcription of genes which do not have steroid responsive elements via a number of mechanisms, but importantly, (2) one of these mechanisms involves signaling cascades initiated at the cell membrane which can rapidly alter cell function in response to extracellular signals. Finally, while steroids produce their effects via both genomic and nongenomic mechanisms, convergence between these pathways is a key component of steroid action and enables hormones to influence the same behavioral and cellular endpoints through more than one route.

If the hormonal events of pregnancy and parturition, particularly the rise in E, alter MPOA neurons to increase maternal responsiveness, what changes in gene transcription mediate this effect? One family of genes that can be activated by the hormonal events of pregnancy termination is referred to as the immediate early genes (IEGs; Sheehan and Numan, 2002). When a neuron has been stimulated (for example, by a hormone, neurotransmitter, or neuromodulator) IEGs are transcribed within minutes (Sheng and Greenberg, 1990). Generally, IEGs play a regulatory role in the neuron, influencing how extracellular events are translated into genomic activation. IEGs accomplish this by encoding protein transcription factors which bind to DNA and activate or repress the transcription of later responding genes (these later responding genes would serve more specific roles, ie. affect neurotransmitter content or receptor expression).

Because IEGs are transcribed very quickly in response to numerous extracellular events, their protein products can be easily visualized using immunocytochemical techniques. The most commonly visualized member of the IEG family is the protein Fos. The Fos protein can be localized by treating tissue from a sacrificed animal with a labeled antibody raised against the Fos protein. The presence of Fos immunoreactivity (Fos-IR) has proven a reliable marker for neuronal responses during maternal behavior. Mice with a mutation of the fos B gene show a complete abolishment of maternal behavior, while these females show surprisingly normal behavior in other hypothalamically controlled systems (body temperature, food intake, sexual behavior; Brown et al., 1996; also see Kuroda et al., 2008).

In support of the idea that hormones are modifying the phenotype of MPOA neurons with Fos expression being one of the markers for those changes, the hormonal events of pregnancy termination induce Fos expression in MPOA neurons (Sheehan and Numan, 2002). The MPOA also shows increased Fos–IR while a female rat is interacting with her pups during the postpartum period (Fleming et al., 1994b; Lonstein and DeVries, 2000; Numan and Numan, 1994, 1995; Stack and Numan, 2000) and while she is searching an environment that previously contained pups (Mattson and Morrell, 2005). This pup induced Fos expression can be separated from pregnancy hormone- induced Fos expression, as it occurs in sensitized virgin female rats but not in virgins who have been exposed to pups but who have not yet sensitized (Numan and Numan, 1994).

The fact that initially hormone stimulation and subsequently pup stimulation can induce Fos expression in the MPOA suggests that Fos expression in MPOA may alter the responsiveness of MPOA neurons to pup inputs. Although Fos may be involved in modifying the phenotype of MPOA neurons so that they become responsive to inputs derived from pup stimuli, just which late-responding genes are activated by Fos to produce such effects is not known. In addition, hormonal events and pup stimuli are likely to influence many other genetic and cellular processes in order to promote maternal behavior.

What types of neurons in the MPOA express Fos during mother-pup interactions and where do these neurons project in order to influence maternal behavior? Double labeling immunohistochemical procedures can be used to determine the phenotype of neurons expressing Fos as well as where these neurons terminate. Lonstein, Greco, De Vries, Stern, and Blaustein (2000) have shown that MPOA neurons which express estrogen receptor α (ER α) show Fos expression during maternal behavior. MPOA has a strong projection to the ventral tegmental area (VTA: Numan and Numan, 1996), and neurons that bind E in the MPOA terminate in the VTA (Farbach and Pfaff, 1986). MPOA neurons which are activated by mother-pup interaction (as indicated by Fos expression) also have a projection to the VTA (Numan and Numan, 1997). While a triple labeling study has not been completed, the results strongly suggest that MPOA neurons which bind estradiol and show Fos expression during maternal behavior terminate in the VTA. The VTA gives rise to ascending dopaminergic fibers which

include those which terminate in the nucleus accumbens (NA), known as the mesolimbic dopamine (DA) system. Although the MPOA projects to more than one region in order to influence maternal responsiveness, a focus of my research has been to understand the interaction between the MPOA and the mesolimbic DA system in the regulation of proactive responsiveness toward pups.

Importantly, interference with the release of DA into the NA produces similar deficits in maternal behavior as MPOA lesions, suggesting that MPOA efferents might regulate the appetitive components of maternal behavior via activation of the mesolimbic DA system. Electrolytic lesions of the VTA (Numan and Smith, 1984) or pharmacological depression of the VTA (Numan et al., in press) disrupt maternal behavior, and microinjection of 6-hydroxydopamine, a toxin which is taken up by DA neurons and selectively destroys them, produces the same effect when injected into VTA (Hansen et al., 1991a,b). Reversible, pharmacological antagonism of DA in NA, which allows more specificity and causes less damage, also impairs maternal behavior (Keer and Stern, 1999; Silva et al., 2003; Numan et al., 2005a).

Generally speaking, the mesolimbic DA system is widely believed to regulate behavioral responsiveness toward many biologically significant stimuli through its projection to the NA (Numan, 2006). The NA receives most of its other inputs from the limbic system (hippocampus, prefrontal cortex, and amygdala; Pennartz et al., 1994) and it is comprised almost entirely of medium spiny GABAergic output neurons (MSN) which have a major

projection to a basal ganglia motor nucleus called the ventral pallidum (VP; Pennartz et al., 1994; Zahm and Heimer, 1993; Zhou et al., 2003). The NA-VP circuit has been called a limbic-motor interface (Mogenson, 1987), as it is the pathway by which sensory inputs which have first been processed by the limbic system can influence the motor system and direct behavior. DAergic inputs to NA function to increase the responsiveness of the NA-VP circuit to limbic inputs (Heidenreich et al., 1995). The mesolimbic DA system has been referred to as a nonspecific motivational system because DA action at the level of NA increases an organisms responsiveness to a wide variety of biologically significant stimuli (Numan, 2006).

Gordon Mogenson (1987) proposed that DA release into NA functions to depress the responsiveness of MSN output neurons which project to the VP. Therefore DA produces its behavioral effects on motor activity by releasing the VP from NA inhibition. Mogenson proposed this model on the basis of his straightforward findings: increased DA release into NA (either by stimulation of VTA or by exogenous administration) increased locomotor activity in an open field, and this effect could be blocked by pharmacological depression of the VP. Mogenson concluded that while NA to VP connections are important for locomotion, because the NA to VP projection is GABAergic and increasing GABA in VP resulted in depression of motor activity, DA must act to increase motor activity by reducing NA GABA output to VP.

This model has been extended by Numan (1988, 2006) with respect to maternal behavior. Infant stimuli can presumably gain access to the NA-VP circuit through limbic afferents originating in the prefrontal cortex and the amygdala, which are glutamatergic and terminate in both the NA and VP (Charara and Grace, 2003; Maeda et al., 2004; Maslowski-Cobuzzi et al., 1994; Petrovich et al., 1996; Sesack et al., 1989; see Figure I). While infant stimuli are potentially capable of exciting this circuit, they are not likely to elicit motor responsiveness without DAergic intervention because excitation of NA GABAergic output neurons results in inhibition of VP, preventing the VP from processing excitatory pup inputs and stimulating motor output. Therefore, DA action on NA, by depressing NA responsiveness to limbic inputs, would allow VP to respond to such inputs.

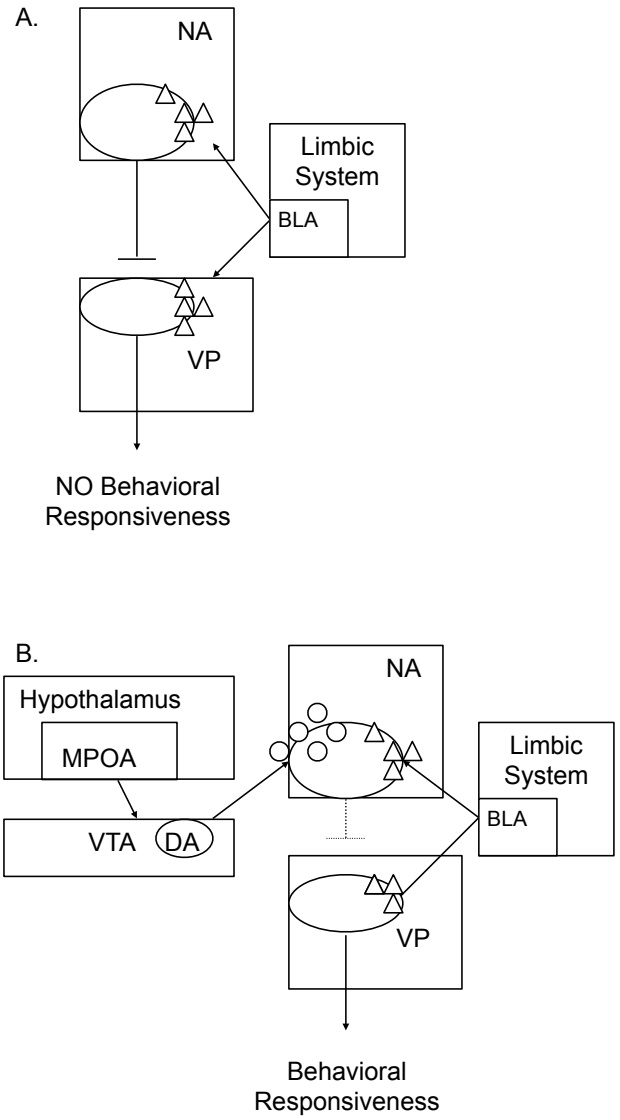

Figure I. Schematic of the mesolimbic dopamine (DA) system: A. The nucleus accumbens (NA) and ventral pallidum (VP) receive excitatory inputs from the basolateral amygdala (BLA; triangles refer to glutamate release). In the absence of DA release, BLA projections to the NA-VP circuit do not result in behavioral reactivity because the projection from NA to VP is inhibitory. Therefore excitation of NA cancels out excitation of VP. B. MPOA activates the VTA and induces DA release into NA (circles refer to DA). In the presence of DA input from the VTA, the impact of excitatory input to NA is dampened and inhibitory output to VP is silenced, allowing excitatory BLA input to the VP to result in behavioral activation. (Adapted from Numan, 2006).

In contrast, other labs contend that DA action on NA output stimulates behavioral reactivity by increasing NA responsiveness to cortical and limbic afferents (Nicola et al., 2000). NA output, therefore, is critical. More specifically, this view proposes that DA release into the NA attenuates weak excitatory inputs from PFC, amygdala, hippocampus, while potentiating strong excitatory inputs. In this way, NA output is driven by strong inputs. DA activity in the NA can therefore be viewed as a modulator that increases the likelihood that strong inputs will evoke a response and reduces the likelihood that weak inputs will evoke a response, which has often been identified as increasing the signal to noise ratio (Horvitz, 2002; Nicola et al, 2000).

If the latter hypothesis is applied to the maternal behavior, two assumptions can be made:(1) NA activity and therefore output would be critical for proactive aspects of maternal behavior; therefore lesions of the NA or pharmacological depressions of NA activity would have a disruptive effect on maternal responsiveness, and (2) VP activity is not critical, rather inhibition of VP activity would be expected because NA output is inhibitory. In contrast, the Mogenson (1987) hypothesis would suggest the opposite, that VP depression rather than NA depression would disrupt maternal behavior.

Recent data from our lab are consistent with Mogenson's view (Numan et al, 2005b). For example, NA lesions do not disrupt the onset (Lee et al., 1999; Li and Fleming, 2003; Numan et al., 2005b) or the maintenance of maternal

behavior (Numan et al., 2005b), suggesting that while DA receptor action in NA is critical for maternal behavior to occur (Keer and Stern, 1999; Numan et al., 2005a), NA activity is not. On the other hand, interference with VP activity, either with excitotoxic lesions or temporary inactivation, disrupts the display of maternal behavior (Numan et al., 2005b).

Given that the mesolimbic DA projection from the VTA to NA is important for maternal behavior, what activates this system so that is can be utilized to control maternal responsiveness? In addition to the anatomical evidence already described, neurobehavioral evidence suggests the importance of MPOA activation: using an asymmetrical design, Numan and Smith (1984) demonstrated that unilateral MPOA lesions severely disrupt maternal responsiveness (particularly retrieving) when paired with a VTA lesion on the opposite side of the brain. When a unilateral MPOA lesion is paired with a VTA lesion on the same side of the brain maternal behavior remains intact. Therefore, not only is VTA activity critical for maternal behavior, but MPOA inputs to VTA appear necessary for mother-initiated responsiveness toward pups.

In further support of the importance of MPOA to NA-VP connectivity in the regulation of maternal behavior, Numan et al. (2005b) found that unilateral lesions of MPOA paired with contralateral depression of VP resulted in severe deficits in maternal responding, while disruption of this system unilaterally did not. In light of the fact that unilateral MPOA lesions alone do not disrupt maternal behavior, this finding suggests that MPOA and VP are functionally linked. From

this data, along with anatomical findings that MPOA neurons which express Fos during maternal behavior project to VTA and MPOA neurons which bind estradiol project to VTA, the following model is proposed: when the MPOA has been properly primed with hormones, pup stimuli activate MPOA neurons which project to VTA DA neurons. DA release into NA disinhibits the VP, allowing VP to respond to pup inputs so that maternal behavior can occur (Numan, 2006; Numan et al., 2005a,b).

The above findings provide support for the idea that MPOA output is necessary for VP activation of maternal behavior. There is other evidence that also shows that MPOA activity is necessary for NA function during maternal behavior. Both MPOA and NA show increased Fos expression during maternal behavior (Stack et al., 2002). Unilateral MPOA lesions (which do not disrupt maternal behavior) reduce maternal behavior induced Fos expression in the NA on the same side of the brain as the MPOA lesion. Fos expression induced by maternal behavior in the contralateral NA, which would presumably still be receiving the presumed MPOA inputs, remains intact.

Note that while the presence of Fos is usually interpreted to mean that a cell has been active, activation does not necessarily equate with Fos expression (Hoffman and Lyo, 2002). In our model, for example, Fos expression in NA might be induced as a result of an intracellular signaling cascade initiated by DA receptor activation. For example, DA exerts its effects by binding to one of the five characterized DA receptor subtypes (Missale et al., 1998). These five

receptors have been classified into two receptor subtype families: D1-like (D1 and D5) and D-2 like (D2, D3, and D4). All DA receptors are G-protein coupled, which means that when DA binds to its receptor, rather than directly inducing changes in membrane electrical conductance, it activates a G protein coupled to the intracellular component of the receptor. This G-protein initiates a second messenger cascade which results in intracellular signaling, activation of protein kinases which phosphorylate proteins, and genomic activation, all which can modulate the way the neuron responds to straightforward excitatory and inhibitory inputs. In other words, one function of this signaling cascade is to modulate a neuron's responsiveness to other neurochemical inputs which directly affect conductance. Therefore, DA receptor activation could result in both a depression in neural responsiveness as well as induction of Fos. Specifically, DA action on D1 receptors has been found to depress NA responses to limbic inputs (Charara and Grace, 2003; Harvey and Lacey, 1997; O'Donnell et al., 1999; Pennartz et al., 1994), and induce Fos expression in NA (Blandini et al,, 2003; Robertson and Jian, 1995). There is also evidence DA action on D1 receptors can induce Fos in striatal neurons which are being depressed (Berretta et al., 1992). The downstream intracellular signaling cascades initiated by DA D1 stimulation can also result in the neuron being less responsive to glutamatergic inputs in the future (Hara and Pickel, 2005; Pei et al., 2004). With respect to these two mechanisms: DA-D1 induced decreases in NA responsiveness and DA-D1 induced activation of Fos, at present it is not clear how they might be

related. It is tempting to speculate that Fos induction might activate genes which result in the synthesis of proteins which cause a long term decrease in the responsiveness of NA neurons to limbic inputs.

Although no one has shown that DA inhibits NA neurons *during maternal behavior*, several studies have shown that NA neurons are inhibited during increased behavioral reactivity toward other biologically important stimuli, like food (Carelli et al., 2000; Taha and Fields, 2006). In support of the idea that neural inhibition of NA neurons during goal-directed behavior is related to DA action at the level of the NA, Cheer et al. (2007) have shown that phasic changes in DA release, on the scale of milliseconds, during operant responding for intracranial self stimulation, specifically correspond to the initiation of goal-directed behavior and these surges in extracellular DA are temporally associated with the inhibition of a majority of NA neurons, and importantly neural inhibition associated with DA release is mediated by DA D1 receptors.

Several researchers have shown that mother-pup interactions result in increased DA release into NA (Hansen et al., 1993), and that the amount of DA release into NA directly corresponds to the amount of time the mother spends interacting with her litter (Champagne et al., 2004). While females spend quite a bit of time tending to their newborn infants, mother-pup interaction gently wanes as the postpartum period progresses.

As indicated earlier, several empirical studies have demonstrated that interference with the mesolimbic DA system impairs maternal responding. Of the

two DA receptor subtypes (D1 and D2) our laboratory recently determined that D1 receptors appear to be mediating the critical effects of DA in NA, and this fits with the data already reviewed about DA-D1 effects on NA responsiveness and Fos induction. Microinjection of the DA D1 receptor antagonist, SCH 23390, into the NA severely disrupted maternal behavior and specifically retrieval behavior, with females taking up to 2 hours to collect their pups back at the nest (Numan et al., 2005a). Note that these females did not show motor impairments and all females promptly approached and sniffed pups. The main deficit was in an inability to complete retrieval. Importantly, eticlopride, a D2 antagonist, did not disrupt maternal behavior after injection into NA. In addition, SCH 23390 injection into a neighboring neural region was not effective.

Despite that plethora of research on the mesolimbic DA system and the regulation of maternal behavior in rats, no one had provided experimental evidence that increased DA activity in the NA mediates the transition from pup avoidance to pup approach which underlies the onset of maternal behavior at parturition. Therefore, the series of experiments included in the first study examine the hypothesis that increased DA release and its subsequent action at DA D1 receptors in NA mediates increased maternal responsiveness, as well as the chemical and anatomical specificity of this idea. The second study examines the neural mechanism by which DA action at D1 receptors in the NA promotes the onset of maternal behavior. The third study examines the role of estradiol stimulation of maternal behavior in an attempt to understand the relationship

between DA and E stimulation of maternal behavior. Before I begin describing the three studies presented here, I would like to briefly describe one of the methods I used in all three studies.

Probably the most compelling way to provide empirical support for the idea that DA release into the NA functions to increase responsiveness would be to show that interference with DA activity in the NA blocks behavioral reactivity, as well as show that increasing DA activity in the NA promotes responsiveness in an unresponsive animal. One challenge in empirically examining the latter is that an optimal amount of DA stimulation is probably required to regulate appropriate responsiveness toward stimuli, and therefore it is not appropriate to predict that increasing DA activity in an animal that is already responding to a particular stimulus will improve responding. Instead, supraphysiological amounts of DA activity can be equally as detrimental as DAergic depletions (Numan et al., in press, Stern and Protomastro, 2000). For example, artificial increases in DA activity in NA via large doses of cocaine (50 µg/µl) disrupt maternal behavior (Vernotica et al.,1999), and upregulation of DA D1 receptor binding in NA is associated with naturally occurring maternal neglect in some strains of mice (Gammie et al., 2008). A better model with which to examine DA regulated increases in behavioral responsiveness would be to examine an animal that is unresponsive toward a particular stimulus, and examine whether increasing DA activity in NA can increase responsiveness toward this particular stimulus.

Maternal behavior in rats is an excellent model to address this issue; recall that female rats display a dramatic change in maternal responsiveness in response to hormonal exposure. To this end, note that females who are exposed to the full hormonal events of birth (fully-primed females) not only show an immediate onset of maternal behavior, but also show elevated DA release in NA in response to pups (Afonso et al., 2009), whereas female rats that are not exposed to the full hormonal events of birth do not show appropriate accumbal DA responses to pups (Afonso et al., 2008). Recall that partial exposure to the hormonal events of pregnancy and parturition via pregnancy termination on day 15 of pregnancy (15HO) results in partial increased maternal responsiveness, such that these females require 2-3 days of pup exposure to show maternal behavior. Therefore, in order to examine whether increased DA activity in NA can elicit increased maternal responsiveness, we chose to utilize a sub-optimally hormone primed female rat, that would not show immediate maternal responsiveness upon exposure to pups. If the increased DA activity in NA of fully-hormone primed rats regulates the immediate onset of maternal behavior, then it can be hypothesized that sub-optimally hormone-primed rats can be induced to show immediate maternal responsiveness after microinjection of DA agonists into the NA.

Importantly, the partially primed 15HO female model was utilized to test our hypothesis because it is not likely that DA action on D1 receptors in NA, acting alone, would stimulate maternal behavior in naïve virgin females who

normally show long sensitization latencies. As reviewed by Numan (2006), multiple neural systems are affected by pregnancy hormones to stimulate the immediate onset of maternal behavior in parturient females, which includes an upregulation of neural systems which promote maternal responsiveness and a downregulation of neural systems which inhibit maternal behavior. The mesolimbic DA system is conceived of as a component of a facilitatory neural system while medial amygdala projections to the caudal anterior hypothalamic region are viewed as components of the inhibitory system (see Numan, 2006). By partially priming females, it was hoped that the inhibitory system would be downregulated sufficiently to allow an experimentally induced upregulation of DA activity at D1 receptors in NA to promote full maternal behavior.

II. **General Methods**

Experimental subjects were nulliparous female rats of the Charles River CD strain (Charles River, Wilmington, MA). Each female, 70-100 days of age, was mated with a male of the same strain. The day of mating was considered day 0 of pregnancy. On the following morning, day 1, females were transferred to clear polycarbonate cages (20 X 45 X 20 cm) that contained wood shavings as bedding material. On day 14 of pregnancy, females were transferred to clear polycarbonate observation cages (50 X 40 X 20 cm), which also contained wood shavings for bedding material. The floors of these observation cages were divided into four equal compartments by 5-cm high Plexiglas dividers. These barriers served to prevent pups from crawling from one quadrant to another. All

subjects were maintained under a 12-hr reversed light-dark cycle (lights off at 0600), and food and water were freely available. Behavioral observations occurred during the dark phase and test rooms were illuminated with dim red light.

An additional group of female rats served as donor mothers that provided test pups for the experimental females. These Charles River CD females were mated and allowed to give birth naturally in polycarbonate cages (20X 45X 20 cm) containing wood shavings. These females and their litters were housed in a different room than the experimental females.

Cannula implantation was performed on day 1 of pregnancy. This stereotaxic surgery was carried out while the rats were under Nembutal anesthesia (50 mg/kg, ip) and atropine sulfate pretreatment (0.2 mg/kg, ip). Following surgery, females were injected with penicillin (50,000 units/kg, sc) and were placed under a warming lamp to recover. Bilateral 22-gauge stainless steel guide cannulas (Plastics One, Roanoke, VA) were implanted into the NA. The DeGroot (1959) stereotaxic atlas was used and interaural zero served as the reference point. Coordinates for NA guide cannula implants were, A 9.5, L ± 1.0, V 7.0. The implanted cannulas were cemented to the skull with dental acrylic and were occluded with stainless steel stylets that extended 2 mm beyond the end of the guide. On day 15 of pregnancy each female was hysterectomized and ovariectomized (HO); the operations were preformed by dorsolateral and ventral incisions with the animal under nembutal anesthesia. Please note, therefore, that

each animal underwent a double survival surgery (cannula implant on day 1 and HO on day 15). These procedures, as well as all the other procedures described in this thesis, were approved by Boston College's Institutional Animal Care and Use Committee.

To acclimate females to the intracranial drug administration procedure that would begin 48 hrs post-HO, the following procedure was performed at 24 hrs post-HO: females were taken to a room outside the maternal behavior testing room, and while hand-held, the experimenter removed and replaced the inner stylet that kept the guide cannula occluded.

To perform intracranial injections, the experimenter removed the inner stylet from the guide cannula of an awake, hand-held rat and replaced it with a 28-gauge injector cannula that extended 2 mm beyond the end of the guide. The injector cannula was attached to a Hamilton microliter syringe with polyethylene tubing, and various doses of a DA agonist were injected into the NA over a 60-s interval with the aid of a Sage syringe pump. The injector remained in place for an additional 45-s, at which time it was removed and the procedure was repeated on the other side of the brain. The injections were performed in a room outside the maternal behavior testing room. These injections occurred on the first 3 days of behavioral testing (see below)

Pup presentation and behavioral observations commenced between 1000 and 1100 on each test day and began 48 hours after HO, deemed day 0 of testing. On the days that females received intracranial injections (days 0,1, and 2

of testing), pup presentation occurred 20 min post-injection. Three stimulus pups aged 2-7 days, provided by donor mothers, were placed throughout the cage, one in each quadrant which the subject had not used as a sleeping quadrant. Care was taken so that younger pups were presented on the earlier days of the testing period, whereas older pups were presented later. During the first hr of pup exposure, subjects were continuously observed for the first 15 min, spot-checked at 30 min, and observed continuously again for the last 15 min of the hour observation. Latency to sniff and approach pups was recorded. Females that did not sniff the pups by the end of the first 15-min observation were manually moved by the experimenter so that they would make snout contact with a pup (forced sniff), and such females were assigned a sniff latency of 15 min. During the one-hour observation, the location of the female and the pups was noted and the occurrence of retrieval responses, nursing behavior (hovering), pup grooming and licking, and infanticide were recorded.

On the first day of behavioral observations, the three stimulus pups were placed one at a time in the female's cage. At the start of the observation period, one pup was placed in the quadrant farthest away from the female's sleeping quadrant, at 15 min, a second pup was placed in a separate quadrant, also not the female's sleeping quadrant, and the third pup was placed at the 30 min spot check. If the female attacked any of the pups on the first test day, all pups were removed and this process was repeated on day 1 of testing. Subjects that attacked pups on two consecutive days were excluded from the experiment.

[Across the various experiments, approximately 10-15% of females were removed from the study because of infanticide. The various groups did not differ in the frequency of this behavior]. Females were subsequently given all three freshly nourished pups at the start of behavioral observation on the remainder of the test days. Approximately 5 hrs post-pup presentation, subjects were spot-checked, and the locations of the female and the pups were recorded. In order to be considered fully maternal in a given test day, subjects had to retrieve all pups to a single location, lick and groom pups, and adopt a nursing posture over the grouped litter by the end of the 5 hr observation. At the 5-hr observation, if a female retrieved and grouped pups, she was observed continuously for 20 min to determine whether nursing behavior and pup grooming occurred. Behaviors observed during this 20 min were considered to have occurred on that test day.

The following morning, the location of the subject and the pups was noted, the pups were removed, and were replaced with 3 freshly nourished pups (20 min post injection). This procedure was repeated until subjects showed complete maternal behavior during the one hr AM observation for two consecutive mornings or until 5 days had elapsed. If complete maternal behavior occurred for two consecutive days during drug injections, an additional test was given without drug injection in order to show that the maintenance of the behavior was not dependent upon drug administration (as it turned out, all females that initiated maternal behavior while receiving intracranial injections maintained such behavior afterwards).

The latency to show maternal behavior was taken as the number of days of pup contact preceding the first day on which full maternal behavior was displayed. Subjects that did not show maternal behavior during test days 0-5 were assigned latencies of day 6 for statistical purposes. If a subject showed maternal behavior for the first time on day 5 of testing, the subject was tested again on day 6 to ensure two consecutive days of complete maternal behavior. Based on our observations we were able to compute two main latency measures in each female: latency to show full maternal behavior during the first hour of pup exposure (hour latency); latency to show full maternal behavior by the end of the test day (day latency).

Females were perfused with saline followed by formalin while under deep anesthesia. Forty-μm thick brain sections, cut on a freezing microtome, were stained with cresyl violet. Microscopic examination mapped the location of the cannula injection sites. Females with excessive brain damage or misplaced cannula were removed from the study.

For all analyses, a minimum significance level of $p = .05$ was used. All tests were 2-tailed, unless noted otherwise. Ordinal scale data were analyzed with nonparametric statistics. The Mann-Whitney *U* test was used to analyze whether median sensitization latencies differed among the independent treatment groups. When more than two independent groups were analyzed, the Kruskal-Wallis test was used first, and if an overall significant difference was detected, then Mann-Whitney U tests were performed for multiple comparisons.

For the maternal behavior latency measure, if an overall difference between groups was not significant by the Kruskal-Wallis test, we performed the following planned comparisons with the Mann-Whitney *U* test: each drug treatment group was compared with the vehicle- injected control. The Fisher exact probability test was used for frequency data and the same planned comparisons described above were analyzed. Interval scale data were analyzed with t tests, or with analysis of variance (ANOVA) followed by the Newman-Keuls test. Interval scale data were analyzed with nonparametric statistics rather than ANOVA in cases in which there was a lack of homogeneity of variance across multiple groups.

III. Study 1: Dopamine D1 Receptor Stimulation of the Nucleus Accumbens or the Medial Preoptic Area Promotes the Onset of Maternal Behavior in Pregnancy Terminated Rats

Experiment 1.1.

Based on the finding that SCH 23390 injection into NA abolished retrieval behavior in postpartum females (Numan et al., 2005a), the central question driving the first experiment was whether microinjection of a DA D1 agonist, SKF 38393, which is considered the compliment to SCH 23390, could produce an immediate onset of maternal behavior in 15HO female rats.

Method

The purpose of Experiment 1.1 was to determine the effects of bilateral injections of various doses of SKF 38393 (a D1 DA receptor agonist obtained from Sigma Chemical, St. Louis, MO) into the medial NA on the sensitization

latencies of 15HO female rats. This experiment was composed of three independent groups receiving either 0μg (vehicle; n = 12), 0.2μg (D1-low; n = 13), or 0.5μg (D1-high; n = 10) of SKF 38393 into the NA. Each dose of SKF 38393 was dissolved in 0.5μl of sterile H_20 and injected bilaterally on days 0, 1, and 2 of behavioral testing. The doses of SKF 38393 were in the range used by other investigators (Ikemoto et al., 1997).

Results

Experiment 1.1 examined the effects of microinjection of SKF 38393 (a D1 DA receptor agonist) into the NA on sensitization latencies to show complete maternal behavior in 15HO rats (see Table 1.1.1). Although statistical analyses did not reveal an overall significant difference among the three groups at either the end of the test day or the end of the first hr of observation (Kruskal-Wallis One Way ANOVA, H(2) = 4.69, p = .09, H(2) 4.92, p = .08, respectively), planned comparisons were made between each dose of SKF 38393 and the vehicle-injected group. The median latency for females receiving the high dose (0.5 μg) of SKF 38393 (n = 10) to show complete maternal behavior by the end of the test day was 0.5 days, significantly shorter than the 2-day latency of the vehicle-treated group (n = 12; Mann-Whitney *U* test, U = 26.5, p <.023). When the median latency to show maternal behavior during the first hr of maternal behavior testing was analyzed (rather than by the end of the test day), the 0.5μg group still showed maternal behavior significantly sooner (median = 1 day) than the 0 μg group (median = 2 days; Mann-Whitney *U* test, U = 25.5, p = 0.019). The median

latency for females receiving the low dose (0.2μg) of SKF 38393 (n = 13) was 1 day, and was not significantly shorter than the 2-day latency shown by the vehicle-treated group (n = 12) during the first hr of observation (Mann-Whitney *U* test, U = 55, p = .19) or by the end of the day (Mann-Whitney *U* test, U = 53.5, p = .16).

In addition to analyzing median latencies to onset of complete maternal behavior for either the day or the hour, we did the same analysis for each group on the latencies to retrieve all pups to the nest. In each group, these latencies were identical to those for complete maternal behavior. In other words, given our observational methods, fragmented maternal behavior did not occur. Once a female retrieved all of her pups to her nest, she also nursed and licked them.

The cumulative percentages of females in each group showing full maternal behavior during the day on each test day are shown in Figure 1.1.1. By day 1 of testing, 90% of 15HO females microinjected with the high dose (0.5μg) of SKF 38393 into NA showed full maternal behavior compared with only 33% of vehicle injected animals. The Fisher exact probability test indicated that this difference was significant ($p \leq .05$). As shown in Table 1.1.1, the mean latency to approach and sniff pups (averaged over days 0-2 of testing) did not differ between any of the groups (One Way ANOVA, $F(2,32) = 0.147$, $p = .86$).

Figure 1.1.2 shows reconstructions of the location of the NA injection sites, drawn on to the appropriate plates taken from the atlas of Paxinos and Watson (1997; on the microtome, the brain sections for both groups were cut in the plane of this atlas). Importantly, microscopic analysis of brain sections indicated that for each group, all cannula placements were located in the NA, either in the shell region or the shell-core border.

Table 1.1.1. Outcome Measures in 15HO Rats that Received 0, 0.2, or 0.5µg doses of SKF 38393 into the Nucleus Accumbens (NA)

SKF 38393 Groups	N	Mean ± SE latency to approach- sniff pups (s)	Median onset to full maternal behavior (days)	
			day	hour
Vehicle	12	232.95 ± 82.82	2 (1-4)	2 (1-4)
D1-low	13	271.06 ± 113.30	1 (0-2.5)	1 (0.5-3.5)
D1-high	10	256.40 ± 103.50	0.5*(0-1)	1* (0-1)

Note: For each female, latency to sniff and approach pups was averaged over days 0-2 of testing. Interquartile ranges are shown in parentheses. Abbreviations: HO = hysterectomized and ovariectomized. D1 refers to SKF 38393, a DA D1 agonist.
* Significantly different from the corresponding vehicle group, Mann-Whitney *U* test.

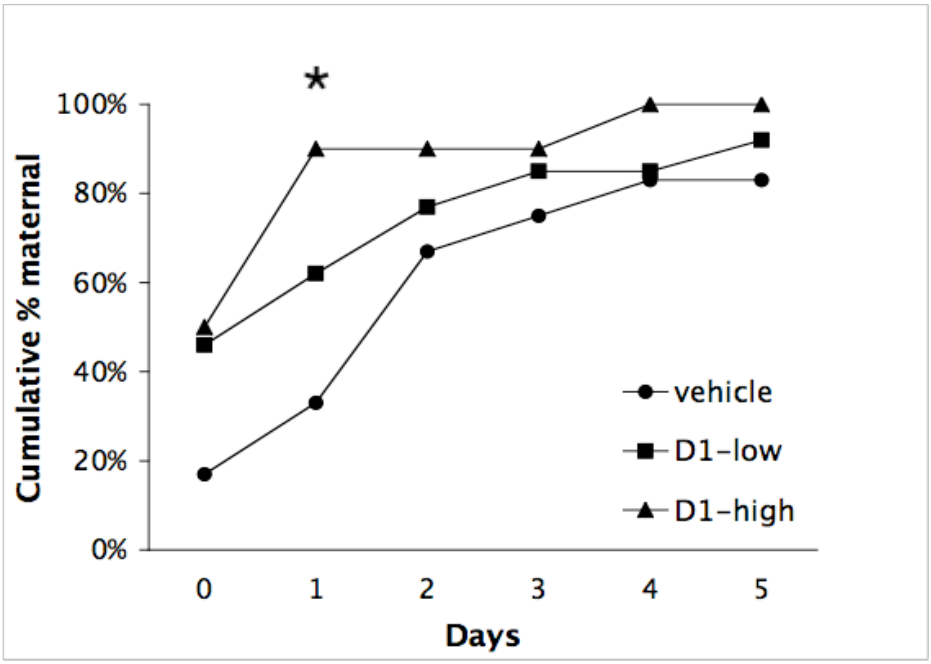

Figure 1.1.1. Cumulative percentage of female rats showing full maternal behavior on each test day. Females received bilateral microinjections of either 0 (vehicle), 0.2 (D1-low), or 0.5 (D1-high) µg of SKF 38393 (n_s= 12, 13, 10, respectively) into the nucleus accumbens (NA) on days 0, 1, and 2 of testing. * Significantly different from vehicle group, Fisher exact probability test.

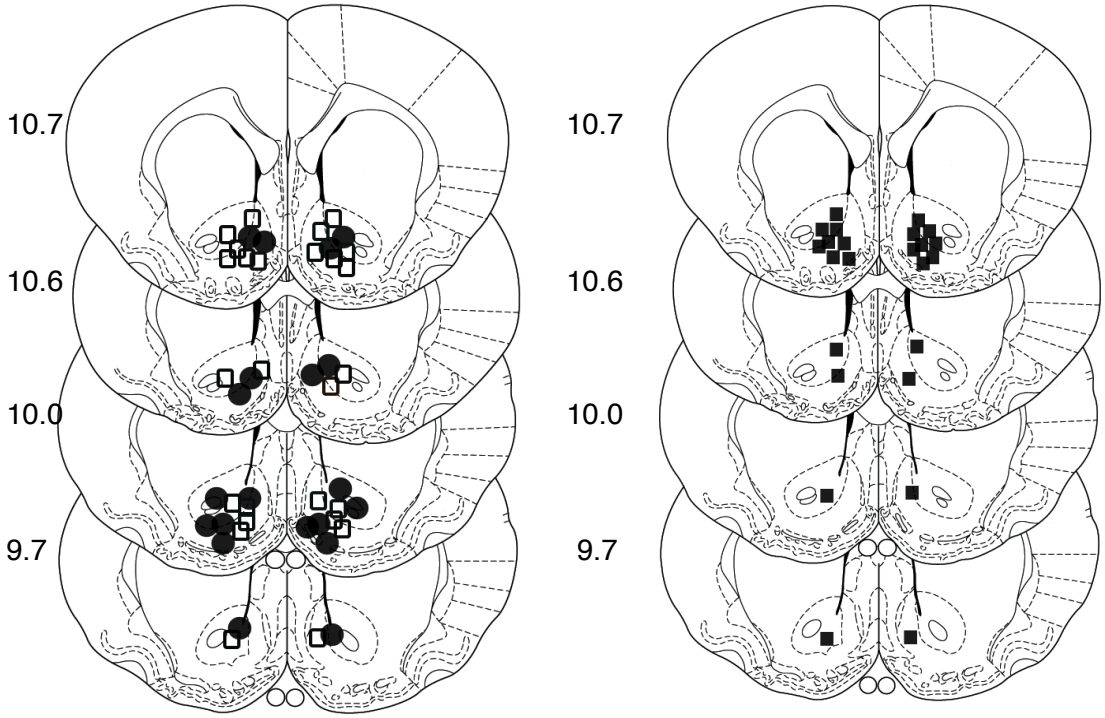

Figure 1.1.2. Reconstructions based on the microscopic analysis of cresyl violet stained sections of the location of SKF 38393 injection sites (solid squares= 0µg dose, open squares= 0.2µg dose, solid circles= 0.5µg dose) into the nucleus accumbens (NA), which were drawn onto plates from the Paxinos and Watson (1997) atlas. The numbers next to the plates indicate the distance in millimeters anterior to the interaural plane. All implant sites were located in either the shell region of the nucleus accumbens or on the shell-core border. However, in order to facilitate viewing, the depicted locations were spread out slightly to eliminate the overlap that actually occurred.

Discussion

Experiment 1.1 indicated that microinjection of a DA D1 agonist, SKF 38393, into the NA promoted maternal responsiveness: the 0.5μg dose resulted in a sensitization latency of about 0.5 days, significantly shorter than the 2-day latency of the control group. Importantly, 90% of females receiving 0.5 μg of SKF 38393 were maternal by day 1 of testing compared with only 33% of females in the control group.

In the present experiment, 9 of the 10 females in the 0.5 μg SKF 38393 treatment group were showing maternal behavior by day 1 of testing. Of these nine females, five initiated maternal behavior on day 0 and four showed maternal behavior on day 1. Given that SKF 38393 was injected into the NA on days 0, 1, and 2 of testing, what accounts for the observed variability in maternal behavior initiation? The best explanation is that there is an interaction between each female's baseline maternal responsiveness and the D1 agonist: a certain level of maternal responsiveness may be necessary to allow DA-D1 activity in NA to push the female over the threshold for full maternal behavior. Some females may have had sufficient maternal responsiveness on day 0, while others may have required a period of pup stimulation before the DA-D1 agonist could be effective. In this context, note that female rats typically give birth on day 22 or 23 of pregnancy. Since all female rats were hysterectomized and ovariectomized on day 15, it is possible that those females who would have given birth on day 22

received a higher level of partial hormonal priming than did the females who would have given birth on day 23.

An interesting question is whether DA stimulation of NA caused a higher level of maternal behavior than would have been induced by sensitization processes alone. To get some information on this point, we examined the time it took a female to show complete maternal behavior on the first day during which maternal behavior occurred within the one hour morning observation period. Most females in the D1-high group were receiving SKF 38393 microinjections into NA at this time while the vehicle group did not receive such stimulation. When measured from the time of pup presentation on the relevant day, females in each of these groups did not differ in the time it took to show complete maternal behavior, most doing so in the first 15-30 min of the observation period. Therefore, although the vehicle-treated females received more days of pup stimulation before showing complete maternal behavior, once the behavior was initiated, it occurred as quickly as that displayed by the 0.5 μg group.

Experiment 1.2.

The purpose of Experiment 1.2 was to examine the chemical specificity of the finding that DA D1 receptor stimulation of NA promotes the onset of maternal behavior in 15HO rats. We hypothesized that agonists at the D2 DA receptor would not facilitate the onset of maternal behavior in 15HO females because our previous work in postpartum animals showed that D1, but not D2, DA receptor

antagonists disrupted maternal behavior when injected into NA (Numan et al., 2005a).

Method

The effects of bilateral injections of quinpirole (a D2 DA receptor agonist obtained from Sigma Chemical, St. Louis, MO) on the sensitization latencies of 15HO female rats were examined. The methods for Experiment 1.2 were identical to those of Experiment 1.1 except that subjects received either 0µg (vehicle; n = 15), 0.2µg (D2-low; n = 10), or 0.5µg (D2-high; n = 12)/0.5µl per side of quinpirole dissolved in sterile H_20. Because quinpirole has a molecular weight close to SKF 38393 (256 and 292, respectively) the molar doses of these two drugs were approximately equivalent across the two experiments.

Results

The effects of microinjection of quinpirole (a D2 DA receptor agonist) into the NA on sensitization latencies to show complete maternal behavior in 15HO are displayed in Table 1.2.1. The Kruskal-Wallis One Way ANOVA indicated there was not an overall significant difference between treatment groups by the end of the test day or the end of the first hr of observation, ($H(2) = 2.65$, $p = .26$, $H(2) = 2.64$, $p = .27$, respectively). Planned comparisons examining each dose of quinpirole and the vehicle-treated group also indicated that group differences did not exist. The median latency for females receiving either the high dose (0.5 µg) of quinpirole (1.5 days; n = 12) or the low dose (0.2 µg) (3 days; n= 10) was not significantly different from females which received vehicle injections (median = 2

days; n = 15; Mann-Whitney *U* test, U = 87, p = .84; U = 48.5, p = .13, respectively). When the median latency to show maternal behavior during the first hr of maternal behavior testing was analyzed (rather than by the end of the test day), the differences were also not significant (Mann-Whitney *U* test, U = 89.5, p = .94; U = 47.5, p = .12, respectively). There were also no significant differences between the groups in mean latency to approach and sniff pups (One Way ANOVA, F(2,34) = 0.55, p = 0.58).

The cumulative percentages of females in each group showing full maternal behavior during the day on each test day are shown in Figure 1.2.1. The Fisher exact probability test did not detect any significant differences. Figure 1.2.2 shows reconstructions of the location of the NA injection sites, which were all located near the shell-core border and within a range similar to NA injection sites for Experiment 1.1.

Table 1.2.1. Outcome Measures in 15HO Rats that Received 0, 0.2, or 0.5µg doses of Quinpirole into the Nucleus Accumbens (NA)

Quinpirole Groups	N	Mean ± SE latency to approach-sniff pups (s)	Median onset to full maternal behavior (days)	
			day	hour
Vehicle	15	278.00 ± 66.41	2 (0-4)	2 (1-4)
D2-low	10	182.00 ± 67.30	3 (2-5)	3 (2-6)
D2-high	12	212.42 ± 64.93	1.5 (0-5)	1.5 (0-6)

Note: For each female, latency to sniff and approach pups was averaged over days 0-2 of testing. Interquartile ranges are shown in parentheses. Abbreviations: HO = hysterectomized and ovariectomized. D2 refers to quinpirole, a DA D2 agonist.

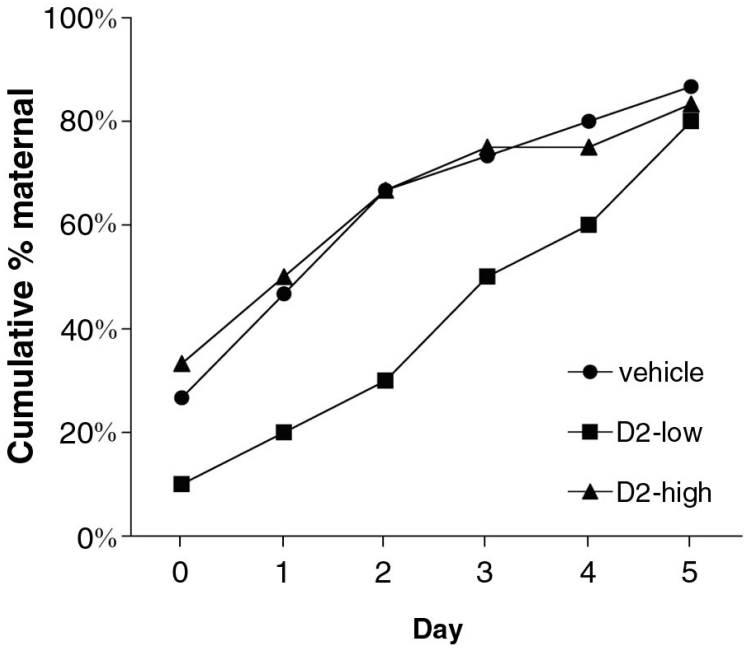

Figure 1.2.1. Cumulative percentage of female rats showing full maternal behavior on each test day. Females received bilateral microinjections of either 0 (vehicle), 0.2 (D2-low), or 0.5 (D2-high) μg of quinpirole (n_s= 15, 10, 12, respectively) into the nucleus accumbens (NA) on days 0, 1, and 2 of testing. Groups did not significantly differ.

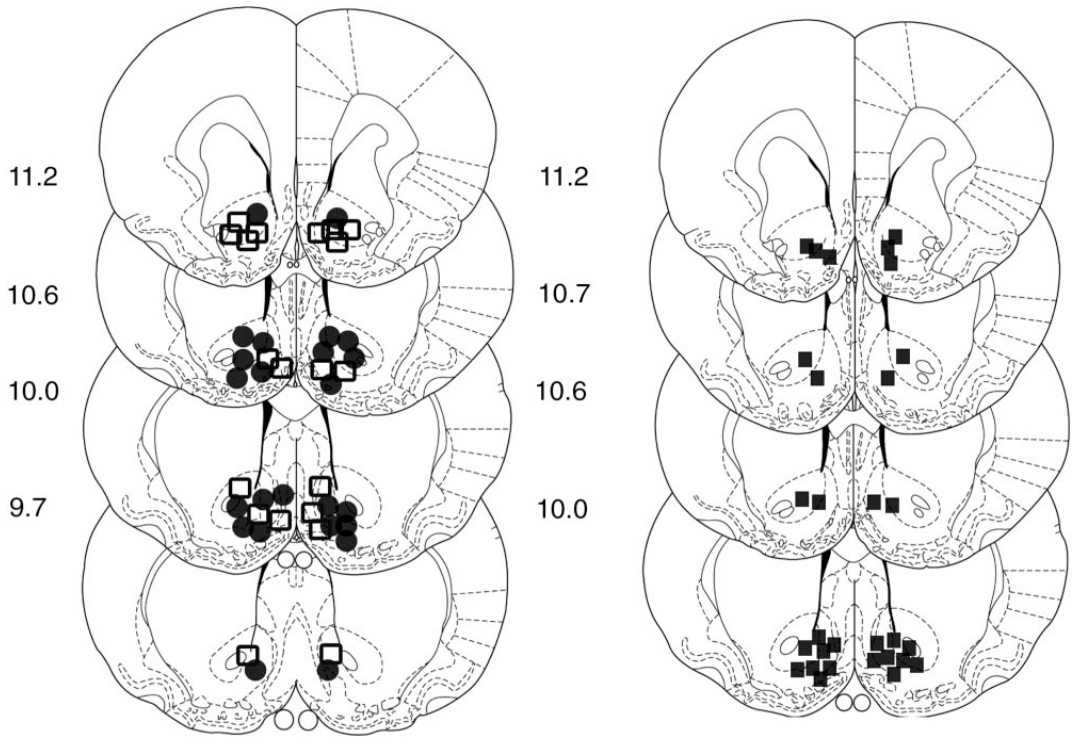

Figure 1.2.2. Reconstructions based on the microscopic analysis of cresyl violet stained sections, of the location of quinpirole injection sites (solid squares= 0µg dose, open squares= 0.2µg dose, solid circles= 0.5µg dose) into the nucleus accumbens (NA), which were drawn onto plates from the Paxinos and Watson (1997) atlas. The numbers to the left of the plates indicate the distance in millimeters anterior to the interaural plane. All implant sites were located in either the shell region of the nucleus accumbens or on the shell-core border. However, in order to facilitate viewing, the depicted locations were spread out slightly to eliminate the overlap that actually occurred.

Discussion

Experiment 1.2 indicated that within the NA, DAergic facilitation of maternal behavior is mediated primarily by D1 receptors, as the D2 receptor agonist, quinpirole, did not produce facilitatory effects at the doses tested, which were approximate molar equivalents of the SKF 38393 doses. Our results, of course, do not rule out the possibility that other doses of quinpirole might have been effective. The fact that quinpirole did not produce any facilitatory effects when injected into the NA is not unexpected based on our previous research which shows that blockade of DA D2 receptors via microinjection of the DA D2 receptor antagonist, eticlopride, in the NA did not produce any disruptive effects on the maternal behavior in postpartum females.

In examining the results of this experiment, although significant differences were not detected, note that the 0.2μg quinpirole-injected females tended to show the poorest maternal behavior, with a difference from the 0μg group that approached significance. In this regard, note that DA D2 receptors are both presynaptic and postsynaptic (Missale et al., 1998). Presynaptic DA receptors are located on DA axon terminals in NA and inhibit DA release, which for the 0.2μg dose, may have caused a slight depression of maternal behavior. To leave open the possibility that postsynaptic D2 receptors in NA may contribute to the onset of maternal behavior, perhaps the 0.5μg dose of quinpirole acted pre and postsynaptically and a positive postsynaptic effect may have subtracted from the

negative presynaptic effect. Therefore, perhaps doses of quinpirole higher than 0.5µg might have been effective in stimulating the onset of maternal behavior.

Experiment 1.3.

The purpose of Experiment 1.3 was to determine the anatomical specificity of the SKF 38393 effect found in the NA by examining the effects of SKF 38393 administration to the dorsal striatum (DS) on maternal behavior. Over days 0-2 of behavioral testing, 15HO female rats received bilateral microinjection of either SKF 38393 (0.5µg/0.5µl sterile H_20/side) or sterile H_20 (0.5µl/side) into DS [D1-DS (n = 8) and vehicle-DS (n = 8) groups, respectively].

Method

The methods for Experiment 1.3 are identical to those for Experiments 1.1-1.2, except that bilateral 22-gauge stainless steel guide cannulas were implanted into the dorsal striatum (DS). The DeGroot (1959) stereotaxic atlas was used and interaural zero served as the reference point. Coordinates for DS guide cannula implants were A 8.6, L ± 2.5, V 8.0. The implanted cannulas were cemented to the skull with dental acrylic and were occluded with stainless steel stylets that extended 2 mm beyond the end of the guide.

Results

Experiment 1.3 examined the effects of microinjection of SKF 38393 into the DS on sensitization latencies to show complete maternal behavior in 15HO rats (Table 1.3.1). The Mann-Whitney *U* test indicated there was not a significant

difference between the vehicle-treated animals and the those treated with a 0.5μg dose of SKF 38393 by the end of the test day (median = 3 days; n = 8; median = 6 days; n = 8; Mann-Whitney *U* test, U = 16.5, p = .08, respectively), or the end of the first hr of observation, (U = 16, p = .07). Note, however, that these differences approached significance, implying that D1 DA receptor stimulation of DS may actually depress maternal behavior. Importantly, there was not a significant difference between the groups in mean latency to approach and sniff pups [t(14) = 0.25, p = 0.80 (Table 1.3.1)].

The cumulative percentages of females in each group showing full maternal behavior during the day on each test day are shown in Figure 1.3.1. Significant differences did not occur. Figure 1.3.2 shows reconstructions of the location of the DS injection sites, drawn on to the appropriate plates taken from the atlas of Paxinos and Watson (1997). Note that the implant sites were located in the ventromedial part of the DS, close to the lateral part of the NA.

Table 1.3.1. Outcome Measures in 15HO Rats that Received 0 or 0.5µg doses of SKF 38393 into the Dorsal Striatum (DS)

SKF 38393 Groups	N	Mean ± SE latency to approach- sniff pups (s)	Median onset to full maternal behavior (days)	
			day	hour
Vehicle-DS	8	249.21 ± 74.44	2.5 (0-5)	3 (0-5)
D1-DS	8	222.17 ± 77.70	6 (1-6)	6 (3-6)

Note: For each female, latency to sniff and approach pups was averaged over days 0-2 of testing. Interquartile ranges are shown in parentheses. Abbreviations: HO = hysterectomized and ovariectomized. D1 refers to SKF 38393, a DA D1 agonist. Groups did not differ significantly.

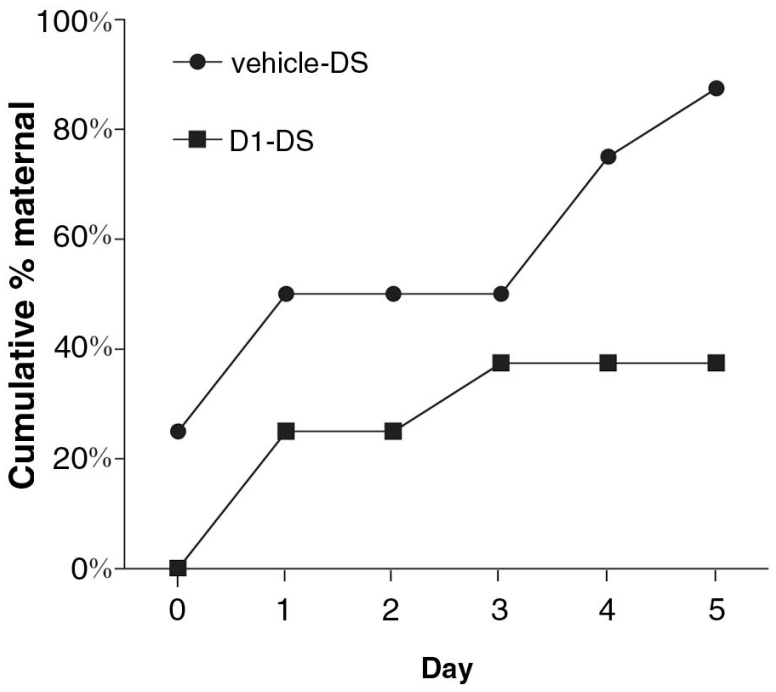

Figure 1.3.1. Cumulative percentage of female rats showing full maternal behavior on each test day. Females received bilateral microinjections of either 0 (vehicle-DS) or 0.5 (D1-DS) µg of SKF 38393 (n_s= 8, 8, respectively) into the dorsal striatum (DS) on days 0, 1, and 2 of testing. Groups did not differ significantly.

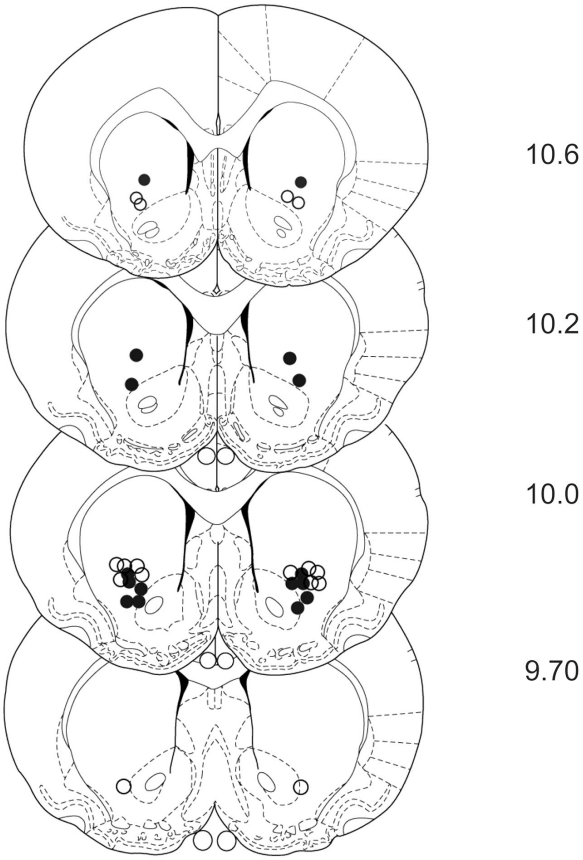

Figure 1.3.2. Reconstructions, based on the microscopic analysis of cresyl violet stained sections, of the location of SKF 38393 injection sites (open circles= 0µg dose, solid circles= 0.5µg dose) into the dorsal striatum (DS), which were drawn onto plates from the Paxinos and Watson (1997) atlas. The numbers next to the plates indicate the distance in millimeters anterior to the interaural plane.

Discussion

The results indicate that the the facilitatory effect of D1 activation in the NA on maternal behavior was also shown to be relatively site specific, as the dose of SKF 38393 which was effective in stimulating maternal behavior when injected into the NA failed to facilitate maternal behavior when injected into the DS. In fact, data obtained from Experiment 3 show that SKF 38393 injections into the DS tended to inhibit maternal responding compared with controls, although this trend was not significant.

Experiment 1.4

Although our hypothesis is that stimulation of DA D1 receptors in NA would promote the onset of maternal behavior in 15HO females, we also examined whether increased DA D1 receptor stimulation in MPOA would promote maternal behavior. As reviewed in the Introduction, recall that the MPOA is critical for the onset and maintenance of maternal behavior. Several pieces of evidence indicate that DA action at the level of the MPOA might also participate in the regulation of the onset and maintenance of maternal behavior in rats; (a) MPOA receives DA input from diencephalic sources (Simerly et al., 1986), (b) expresses both D1 and D2 receptors (Bakowska and Morrell, 1995); (c) DA activity in the MPOA fluctuates throughout pregnancy and lactation (Lonstein et al., 2003; Olazabel et al., 2004); (d) high doses (5 µg) of D1 antagonists injected directly into the MPOA have been found to disrupt maternal

behavior in postpartum rats, and the nature of the disruption is similar to that observed after D1 antagonist injection into NA (Miller and Lonstein, 2005).

Using the 15HO model, we examined whether the 0.5 µg dose of SKF 38393, which effectively stimulated maternal behavior when injected into the medial NA, could produce similar effects when injected into MPOA. We chose to examine four independent groups of females who received either 0 or 0.5µg of SKF 38393 into either NA or MPOA, which allowed us to directly compare the effects of SKF 38393 microinjection into these two important regions.

Method

The methods used were similar to those described for Experiment 1.1, except for the following details. Four groups were formed based on the injections received on days 0-2 of testing. D1-MPOA (n = 10): 0.5µg SKF 38393/0.5µl sterile H_20/side; vehicle-MPOA (n = 10): 0.5 µl sterile H_20/side; D1-NA (n = 8): 0.5µg SKF 38393/ 0.5µl sterile H_20/side; vehicle-NA (n = 8): 0.5µl sterile H_20/ side. Bilateral 22-gauge stainless steel guide cannulas implanted into either the NA or the MPOA. Coordinates (DeGroot, 1959) for MPOA guide cannula implants were, A 7.5, L ± 0.75, V 5.8. The implanted cannulas were cemented to the skull with dental acrylic and were occluded with stainless steel stylets that extended 2 mm beyond the end of the guide.

In this experiment we also determined whether SKF 38393 administration had an effect on general locomotor activity. On days 0-2 of testing, over the 3 minutes prior to pup presentation (17-20 min post-injection), the absolute number

of line crosses (movement of all four feet from one quadrant to another as defined by the Plexiglas dividers) performed by each female was recorded.

Results

The results of Experiment 1.4 indicate two important points: first, the main effect of Experiment 1.1 was replicated in that the high dose of SKF 38393 (0.5μg) was once again effective in reducing sensitization latencies when injected bilaterally into the medial NA, and secondly, this high dose of SKF 38393 was also able to stimulate maternal behavior when injected into MPOA (Table 1.4.1). The Kruskal-Wallis One Way ANOVA indicated that there was an overall significant difference among the four groups during the observation hr, [$H(3) = 9.73$, $p = .02$], and by the end of the observation day [$H(3) = 8.30$, $p < 0.05$]. The median latency for females receiving 0.5μg of SKF 38393 into the NA ($n = 8$) was 0 days for both the hr and the day latency measure, and this was significantly shorter than the 2-day latency of the corresponding vehicle-NA group ($n = 8$) during the first hr of observation (Mann-Whitney *U* test, $U = 11$, $p < 0.05$) or the 1.5 day latency for the vehicle group when measured at the end of the test day (Mann-Whitney *U* test, $U = 12.5$, $p < 0.05$). The median latency for females receiving 0.5μg of SKF 38393 into MPOA ($n = 10$) was also significantly shorter than the vehicle-treated group during the first hr of observation (Mann-Whitney *U* test, $U = 21.5$, $p < 0.05$) or by the end of the observation day (Mann-Whitney *U* test, $U = 24$, $p < 0.05$). There were no significant differences between the median latencies for females receiving SKF 38393 injections into NA versus MPOA, nor

were there significant differences between median latencies of vehicle treated animals who received injections into NA or MPOA. A one way ANOVA indicated there were not significant differences between treatment groups (see Table 1.4.1) in the latency to approach and sniff pups, $F(3,32) = 1.80$, $p = .17$, or in home cage activity in the 3 min prior to pup presentation [(Kruskal-Wallis One Way ANOVA) $H(3) = 3.37$, $p = .29$]. Sniff latencies and home cage line crosses were averaged across days 0-2 of testing.

In addition to analyzing median latencies to onset of complete maternal behavior for either the day or the hour, we did the same analysis for each group on the latencies to retrieve all pups to the nest. In each group, these latencies were identical to those for complete maternal behavior. Therefore, similar to the results obtained for Experiment 1.1, fragmented maternal behavior did not occur in Experiment 1.4. Once a female retrieved all of her pups to her nest, she also nursed and licked them.

The cumulative percentages of females showing complete maternal behavior on each test day are shown in Figure 1.4.1. By the end of day 0 of testing, 80% of the females receiving SKF 38393 into MPOA showed complete maternal behavior compared with only 20% of vehicle-treated animals. Fisher exact probability test indicated that this difference was significant ($p < 0.05$). Although 62.5% of females injected with SKF 38393 into NA showed complete maternal behavior by the end of day 0 of testing, compared with only 14% of vehicle-injected animals, this difference only approached significance ($p = .07$); if

a larger number of subjects had been run it is likely that a significant effect would have been observed. Figure 1.4.2 shows reconstructions of the location of MPOA injection sites and Figure 1.4.3 shows reconstructions of the location of NA injection sites.

In order to examine whether DA stimulation of NA or MPOA caused a higher level of maternal behavior than would have been induced by sensitization processes alone, we examined the time it took a female to show complete maternal behavior on the first day during which maternal behavior occurred within the one-hour morning observation period. Most females in the D1-NA and D1-MPOA groups were receiving SKF 38393 microinjections at this time while the vehicle groups were not receiving such stimulation. Females in each of these four groups did not differ in the time it took them to show complete maternal behavior, most doing so in the first 15-30 min of the observation period. These results conform with those of Experiment 1.1.

Table 1.4.1. Outcome Measures in 15HO Rats that Received 0 or 0.5µg doses of SKF 38393 into the Nucleus Accumbens (NA) or Medial Preoptic Area (MPOA)

Groups	N	Mean ± SE latency to approach- sniff pups (s)	Median onset to full maternal behavior (days)	
			day	hour
Vehicle-NA	8	265.96 ± 82.61	1.5 (1-6)	2 (1-6)
D1-NA	8	153.21 ± 51.64	0* (0-1)	0* (0-1)
Vehicle-MPOA	10	87.37 ± 38.38	1 (1-4)	1.5 (1-4)
D1-MPOA	10	179.29 ± 45.27	0* (0-0)	0* (0-1)

Note: For each female, latency to sniff and approach pups and number of line crosses were averaged over days 0-2 of testing. Interquartile ranges are shown in parentheses. Abbreviations: HO = hysterectomized and ovariectomized. D1 refers to SKF 38393, a DA D1 agonist.
*Significantly different from the corresponding vehicle group, Mann-Whitney *U* test.

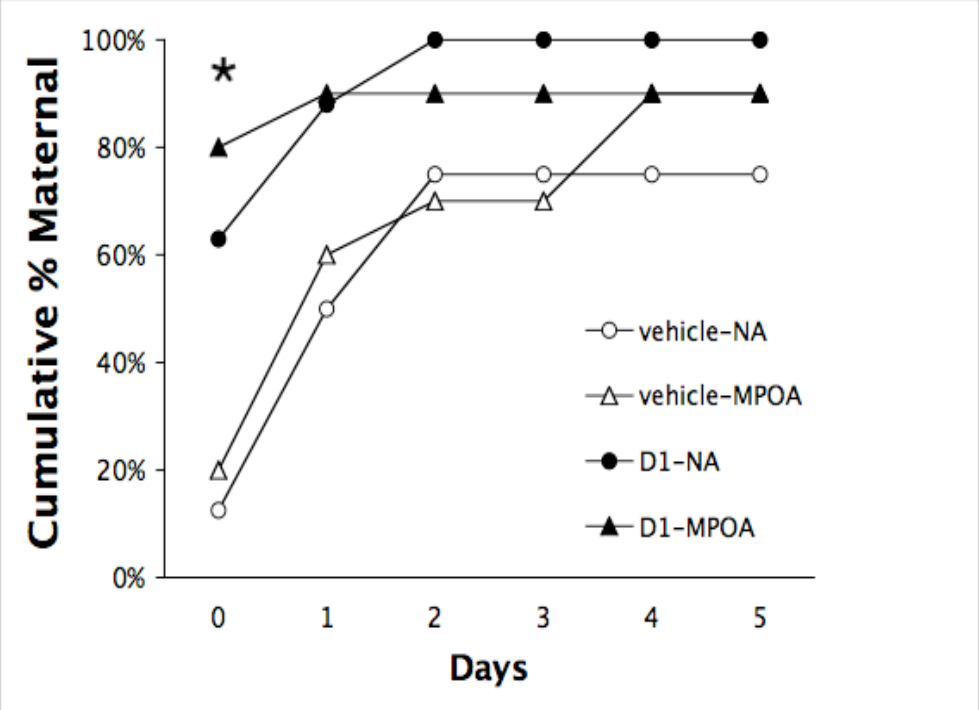

Figure 1.4.1. Cumulative percentage of female rats showing full maternal behavior on each test day. Females received bilateral microinjections of either 0 (vehicle-NA) or 0.5 (D1-NA) µg of SKF 38393 (n_s= 8, 8 respectively) into the nucleus accumbens (NA) on days 0, 1, and 2 of testing, or 0 (vehicle-MPOA) or 0.5 (D1-MPOA) µg of SKF 38393 (n_s= 10, 10 respectively) into the medial preoptic area (MPOA). * Significantly different from vehicle- MPOA group, Fisher exact probability test.

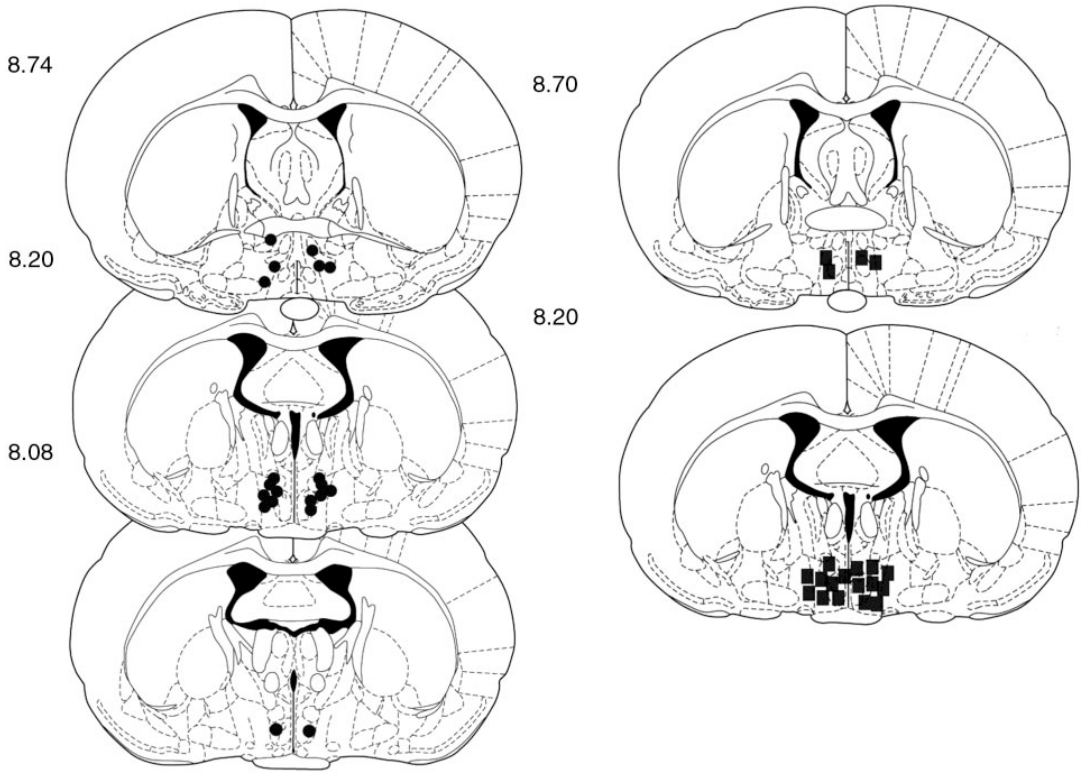

Figure 1.4.2. Reconstructions, based on the microscopic analysis of cresyl violet stained sections, of the location of SKF 38393 injection sites (solid squares= 0µg dose, solid circles= 0.5µg dose) into the medial preoptic area (MPOA), which were drawn onto plates from the Paxinos and Watson (1997) atlas. The numbers to the left of the plates indicate the distance in millimeters anterior to the interaural plane. All implants were located within the medial preoptic area or the ventral bed nucleus of the stria terminalis. However, in order to facilitate viewing, the depicted locations were spread out slightly to eliminate the overlap that actually occurred.

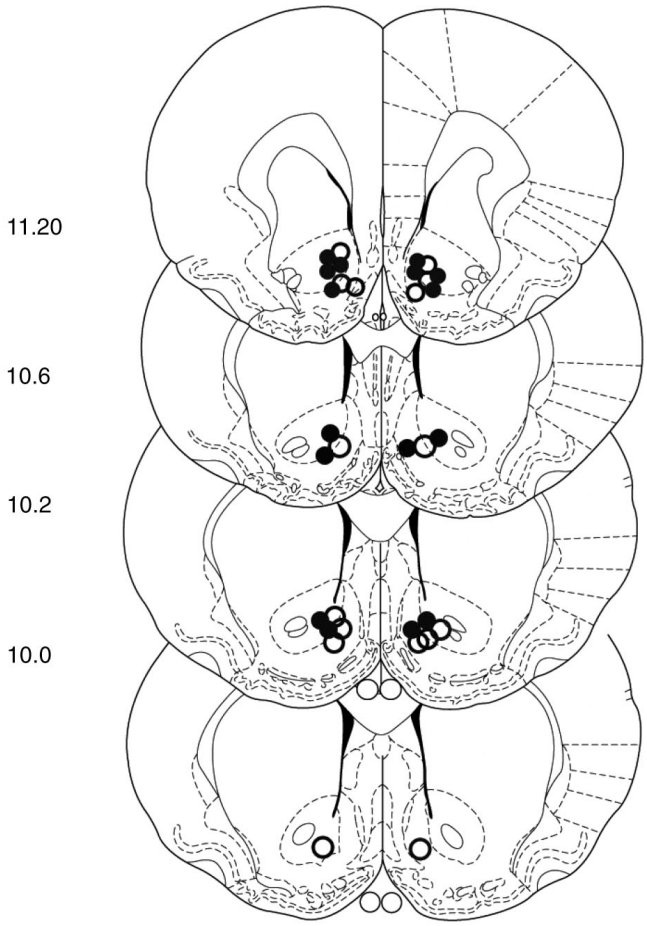

Figure 1.4.3. Reconstructions, based on the microscopic analysis of cresyl violet stained sections, of the location of SKF 38393 injection sites (open circles= 0µg dose, solid circles= 0.5µg dose) into the nucleus accumbens (NA), which were drawn onto plates from the Paxinos and Watson (1997) atlas. The numbers next to the plates indicate the distance in millimeters anterior to the interaural plane.

Discussion

Experiment 1.4 demonstrated that increased D1 receptor activation in either the NA or the MPOA was capable of facilitating maternal behavior. Not only do these findings validate the results of Experiment 1.1, but they also suggest a role for DAergic mechanisms within the MPOA in the regulation of the onset of maternal behavior. Importantly, there were no differences between the effects of SKF 38393 microinjection into the NA and MPOA, indicating that increased DA-D1 activation in each region produced equivalent effects on maternal responding.

One possible interpretation of the finding that SKF 38393 significantly reduced sensitization latencies is that administration of the DA agonist altered motor activity and therefore females treated with SKF 38393 had an increased opportunity to come into contact with pups. This interpretation is highly unlikely, however, because the latency to approach and sniff pups was not significantly different between treatment groups. Additionally, Experiment 1.4 showed that in the three min prior to pup presentation, groups did not differ in home cage activity. Finally, casual observation indicated that differences in general motor activity did not occur.

Concerning the location of the implant sites, histological analysis indicated that MPOA implant sites were similar between vehicle and SKF 38393 injected animals. NA implant sites were all located within the shell region of the NA or the shell-core border in Experiments 1.1, 1.2, and 1.4. This finding fits with previous work that suggests the importance of the shell region for maternal behavior (Keer

and Stern, 1999; Li and Fleming, 2003; Numan et al, 2005a; Stack et al., 2002). Analysis of the rostro-caudal location of the NA implant sites in the SKF 38393 treated females in Experiments 1.1 and 1.4 indicated that there was no relationship between anterior-posterior injection location and onset latency.

Because the present experiment found that SKF 38393 microinjection into either the NA or MPOA was capable of facilitating maternal behavior, probably the most central question that future research will have to resolve is whether SKF 38393 is actually capable of facilitating maternal behavior by acting at either of these sites. In other words, the possibility remains open that the D1 agonist had its primary effect at only one of these two sites and the facilitatory effect observed at the other site was due to spread of the drug to the primary site (the NA and the MPOA implant sites were only separated by about 2 millimeters).

With respect to the possibility that the primary site of action of SKF 38393 is at the level of the NA, the following should be noted. For postpartum females, Numan et al. (2005a) were able to depress maternal behavior with microinjection of SCH 23390 (a D1 antagonist) into the NA at a dosage level (1-2 micrograms) which was ineffective when injected into the MPOA. The depressive effect of SCH 23390 into NA therefore, cannot be explained by spread of the antagonist to the MPOA. Supporting the results of Numan et al. (2005a), Miller and Lonstein (2005) also found that bilateral injections of a 2 µg dose of SCH 23390 into the MPOA did not affect maternal behavior. However, these same authors found that bilateral MPOA injections of 5 µg of the D1 antagonist did disrupt maternal

behavior. These results suggest the possibility that the disruptive effects reported by Miller and Lonstein (2005) were due to spread of the 5 µg dose of SCH 23390 from the MPOA to the NA. We cannot rule out the possibility however, that in postpartum females small doses of SCH 23390 in NA effectively depress maternal behavior, while direct action of this drug in the MPOA requires higher doses.

In spite of the difficulty interpreting the effects on maternal behavior of D1 antagonism at the level of the MPOA, other research supports the possibility that in addition to an action on the NA, DA action on the MPOA is also important for maternal behavior. This research draws on parallels between the neural regulation of maternal behavior and male sexual behavior in rats. In particular, MPOA lesions disrupt both behaviors, and E action on the MPOA not only facilitates maternal behavior, but also promotes male sexual behavior (Numan, 1974,1985). Significantly, DA activity in the MPOA is integral to the regulation of male sexual behavior (Hull and Dominguez, 2006). Blockade of DA receptors in the MPOA has been found to impair copulation, microinjection of DA agonists into the MPOA facilitates copulation, and DA is released into the MPOA both in the presence of a receptive female and during mating behavior (Dominguez and Hull, 2005). Furthermore, DA activity in the MPOA is regulated by male sex hormones and this hormonal regulation of DA release has been linked to a mechanism involving the gaseous neuromodulator nitric oxide (NO) (Hull and Dominguez, 2006). Disruption of NO synthesis by microinjection of L-NAME (NO synthase

inhibitor) not only impairs copulation (Lagoda et al., 2004) but also disrupts DA release in response to a receptive female (Dominguez et al., 2005).

Importantly, there is also evidence to indicate that NO is important for the expression of maternal behavior in rats: Popeski and Woodside (2004) showed that intracerebroventricular injection of L-NAME disrupts maternal behavior. The recent finding that microinjection of L-NAME directly into the MPOA also disrupts maternal behavior (Service and Woodside, 2007), and that this effect can be reversed with simultaneous injections of SKF 38393 (Service and Woodside, 2006), suggests that NO may be similarly involved in the regulation of DA release in the MPOA during maternal behavior, and this may provide a mechanism through which the MPOA regulates the onset of maternal responsiveness.

Interestingly, there is also evidence to suggest that E action on NA can facilitate DA transmission in NA (Becker, 1999) and because the NA also contains nitric oxide synthase (NOS) neurons and NO is involved in regulating NA function (West, Galloway, & Grace, 2002), an important question concerns the similarity in underlying mechanisms between DA action on these two structures.

With respect to this question, it would be to interesting to explore the effects of doses of SKF 38393 into MPOA that are lower than 0.5µg on the onset of maternal behavior in 15HO rats. If 0.2µg were to be effective it could be argued strongly for an MPOA site of action, since this dose was not effective in NA.

Finally, note that the Numan et al (2005a) and Miller and Lonstein (2005) studies deal with the maintenance of maternal behavior during the postpartum period, after its initiation. It is entirely possible that the brain mechanisms regulating the onset of maternal behavior differ from those regulating the maintenance of maternal behavior, in the same manner as hormones being necessary for the onset of maternal behavior, are not required for its maintenance. Therefore, perhaps DA-D1 input to NA is necessary for both the onset and the maintenance of maternal behavior, while DA D1 input to the MPOA has its major influence only on the onset of maternal behavior.

General Discussion

Previous research has shown that depression of DA action on the NA disrupts maternal behavior in postpartum female rats (Keer and Stern, 1999), and has emphasized the involvement of D1 receptors (Numan et al., 2005a). As an important compliment to these findings, study 1 provides substantial evidence that increased DA activity at D1 receptors in the NA promotes maternal behavior. In addition, our findings indicate that the MPOA, but not the DS, is another site where D1 receptor activation promotes maternal behavior. These results suggest that increased DA activity at D1 receptors in the NA or MPOA can substitute for estradiol in the stimulation of maternal behavior in 15HO females.

In the present study we were primarily concerned with the mechanism regulating the onset of complete maternal behavior (retrieving, nursing, and licking pups), and we did not take detailed observations on the intensity of

maternal behavior after it was initiated. An interesting question for future investigation might be to examine whether the intensity of maternal behavior (amount of nursing and licking pups) is superior in females that received SKF 38393 stimulation of either NA or MPOA when compared to the maternal behavior of control females. Based on the fact that once maternal behavior is initiated it is controlled by endogenous DA activity, our inclination, backed up by some observations during the current study, is that there would not be a difference in the nature of maternal behavior between SKF 38393 and vehicle injected females. In particular, on the first day that females showed complete maternal behavior within the one-hour morning observation, females typically showed the behavior within 15-30 min of pup presentation irrespective of whether they were receiving SKF 38393 microinjections into the brain. That is, SKF 38393- and vehicle-treated females did not differ in the speed to show the complete maternal behavior pattern once the behavior had been initiated. In further support, it has been found that in home cage tests, the maternal behaviors shown by sensitized virgins are virtually indistinguishable from those shown by postpartum lactating females (Fleming and Rosenblatt, 1974; Reisbick et al., 1975). Additionally, note that endogenous DA levels are higher in the MPOA of sensitized virgins compared with controls (Olazabel et al., 2004). However, we cannot rule out the possibility that SKF 38393 injection caused supernormal levels of D1 stimulation which might have caused higher levels of

various maternal behaviors in terms of the durations of the behaviors (see Champagne et al., 2004).

While it is clear that DA activity in NA is critical for both the onset and maintenance of maternal behavior, the role of DA in MPOA is less understood. Despite the discrepancy in data with respect to the disruptive effect of SCH 23390 injections into MPOA, our working hypothesis, based on the results of the present study, is that it is likely that D1-DA receptor activation in both MPOA and NA is important for the onset of maternal behavior. In this context, recall that the MPOA and mesolimbic DA system are linked in the control of maternal behavior. Our lab has developed a model in which the MPOA plays a role in the regulation of maternal responsiveness through its activation of the mesolimbic DA system via projections to the VTA (see Numan, 2006; Numan et al., 2005b): as a result of hormonal stimulation, the MPOA is primed to respond to certain pup stimuli. The MPOA, in turn activates the VTA-DA projections to NA which ultimately, through a process of disinhibition, allow the ventral pallidum to process pup stimuli-induced afferent input so that voluntary maternal responses can occur.

Given the linkage between the MPOA and the mesolimbic DA system in the control of maternal behavior, it is certainly possible that D1-DA receptor activation in MPOA is involved in the activation of efferent projections to VTA, which in turn increases D1-DA receptor activation in NA. In the 15HO female that is not treated with estradiol, DA activity in both the MPOA and NA may be relatively low. By upregulating D1-DA activity in either the MPOA or the NA we

circumvent the need for estradiol action on the MPOA and facilitate a rapid onset of maternal behavior.

IV. Study 2: Dopamine D1 receptor activation of adenylyl cyclase, but not phospholipase C, in the nucleus accumbens promotes the onset of maternal behavior in pregnancy-terminated rats

The results of study 1 indicate that increased DA action at D1 receptors is effective in stimulating the onset of maternal behavior in 15HO rats at the level of the NA as well as the MPOA. However, in the context of our neural model, these results suggest that DA action at D1 receptors in MPOA results in *increased* activation of MPOA projections to the VTA while DA D1 stimulation of NA results in *decreased* activity of NA output to the VP. An implication of this proposal is that distinct inter and/or intracellular mechanisms might mediate DA-D1 effects in MPOA and NA.

Interestingly, a relatively new body of literature has challenged the classic view that D1 receptors are only coupled to cAMP signal cascades. Rather, another class of DA D1 receptors exerts its effects by stimulating a different signaling cascade, the phospholipase C (PLC) phosphatidylinositol cascade which is linked to protein kinase C (PKC; see Figure 2.1.1). This sub-class of DA D1 receptors is coupled to a different G protein which stimulates the enzyme PLC rather than AC (Clifford et al., 1999; Jin et al., 2001; Undie et al., 2000; Undie and Friedman, 1994). The result of PLC stimulation is the hydrolysis of phosphatidylinositol biphosphate (PIP2) into diacylglycerol (DAG) and inositol triphosphate (IP3). The function of this intracellular signaling cascade is increased PKC and intracellular Ca^{2+}. It is unclear at this time what proportion of DA D1 receptors are linked to phosphoinositol (PI) signaling, however it appears

that PLC linked DA D1 receptors are located on neurons in the hippocampus, amygdala, and striatum (Jin et al., 2003; Jin et al., 2001; Ming et al., 2006; Wirtshafter and Osborn, 2005).

With respect to the findings that microinjection of SKF 38393, a DA D1 receptor agonist, directly into NA promotes maternal behavior in 15HO rats (Stolzenberg et al., 2007) and microinjection of SCH23390, a DA D1 receptor antagonist, into NA disrupts ongoing maternal behavior in lactating rats (Numan et al., 2005a), note that SKF 38393 is capable of stimulating both signaling cascades and SCH 23390 is capable of blocking both pathways (Jin et al., 2003, Undie et al., 2000). Therefore, study 2 used two selective pharmacological agents: SKF 83959 which is linked to DA D1- PLC (Ming et al., 2006; Jin et al., 2001, 2003; Undie et al., 2000, Zhen et al., 2005), and SKF 83822 which selectively stimulates AC and is not capable of stimulating PLC pathways (Kuroiwa et al., 2008; Undie and Friedman, 1994).

Experiment 2.1.

Recall that several studies have reported that DA release into the NA dampens the effects of excitatory glutamatergic inputs from the limbic system and cortex on medium spiny GABA neurons. Electrophysiological data, both in vivo and in vitro, also suggest that the inhibitory effect of DA stimulation on NA medium spiny neurons is mediated by DA D1 receptors (Calabresi et al., 1987; Charara and Grace, 2003; Chergui and Lacey, 1999; Harvey and Lacey, 1996, 1997; Maeda et al., 2004; Nicola and Malenka, 1997). Whereas DA D1 receptors

are frequently associated with the activation of AC, the present experiment was aimed at determining whether the facilitatory effect of DA D1 receptor stimulation of maternal behavior could be replicated by stimulation of DA D1- PLC receptors because of the evidence that these receptors, in particular, are involved in the depressive effects of DA D1 stimulation on excitatory postsynaptic potentials in NA (Chergui and Lacey, 1999; Harvey and Lacey; Kombian et al., 2003; Noriyama et al., 2006).

Methods

The methods of study 2 are the same as those described for the general methods section, with a few exceptions. available. Bilateral 22-gauge stainless steel guide cannulas were implanted into NA using the DeGroot (1959) stereotaxic atlas coordinates: A 9.5, L ± 1.0, V 7.0. The implanted cannulas were cemented to the skull with dental acrylic and were occluded with stainless steel stylets that extended 2 mm beyond the end of the guide.

Experiment 2.1 examined the effects of bilateral injections of SKF 83959 (a DA D1-PLC linked receptor agonist obtained from Sigma, St.Louis, MO) on the sensitization latencies of 15HO female rats. Two independent groups of female rats received bilateral injections of either 0 μg (Vehicle; n = 7) or 0.5 μg/0.5 μl/ side of SKF 83959 dissolved in sterile water (SKF 83959; n = 8) directly into the NA on days 0-2 of behavioral testing. The doses of SKF 83959 were in the range

used by other investigators (Adachi et al., 1999; Cools et al., 2002; Hasegawa et al., 2001).

Behavioral observations matched those described in the general methods section, except that the first time a female was observed to show full maternal behavior (retrieve all 3 pups to a single nest site, lick/groom pups, and hover/ crouch over pups), a detailed 15 minute nursing observation took place. If full maternal behavior was observed during the morning one hour test, the 15 minute nursing observation occurred from 60-75 min post pup presentation. If maternal behavior first occurred during the PM observation, the 15 minute nursing observation occurred at that time. Note that regardless of whether the nursing observation took place in the morning or in the afternoon, the nursing observation always occurred at the first time on the first day the female showed full maternal behavior. During the 15 minute nursing observation, a female was observed for 10-15 seconds every 30 seconds of the 15 minute (for a total of 30 observations), and it was noted whether she was hovering, sniffing and licking, or crouching over the pups. If a female transitioned from one nursing posture to another, the behavior having the longest duration during the interval was recorded. However, in most cases the female engaged in only one behavior during each 10-15 second interval. Females were recorded as hovering when they were upright over pups (so that pups had access to the female's ventral surface) but also actively sniffing/licking the pups or engaging in self-grooming. In contrast, females were recorded as crouching when in a quiescent, immobile posture, with

all four limbs supporting a slightly arched or highly arched posture over the pups (Stern and Johnson, 1990). In some cases females also adopted a supine or prone position, resting on their side while the pups had access to the female's ventral surface; this posture was recorded as a hover. Incidences of sniffing/ licking were individually noted, but this behavior always occurred while the female was in a hover position. Note that this additional nursing observation was used to address one question raised by study 1: whether DA D1 agonist treated females show a higher quality of maternal behavior than vehicle treated females who naturally sensitize.

Results

Experiment 2.1 examined whether a DA D1-PLC linked receptor agonist would stimulate maternal behavior in 15HO rats when injected into the NA. Table 2.1.1 shows that females receiving SKF 83959 approached and sniffed pups with significantly shorter latencies, when averaged on days 0-2; than did the vehicle-treated females [$t(13)= 8.14$, $p < .01$]. However, other aspects of maternal behavior did not distinguish the two groups. The median sensitization latency for female rats injected with SKF 83959 into the NA (SKF 83959, $n = 8$) was 6 days which was not significantly different from the 5 day median sensitization latency shown by vehicle treated females (Vehicle, $n = 7$; Mann-Whitney *U* test, $U = 24.5$, $p= .6$, see Table 2.1.1). The cumulative percentage of females showing full

maternal behavior on each test day was similar between the SKF 83959 and Vehicle groups (Figure 2.1.2).

Figure 2.1.3. shows reconstructions of the location of the NA injection sites, drawn on to the appropriate plates taken from the atlas of Paxinos and Watson (1997). Importantly, microscopic analysis of brain sections indicated that for each group, all cannula placements were located in the NA, either in the shell region or the shell-core border.

Table 2.1.1. Outcome Measures for 15HO Female Rats that Received injections of SKF 83959 into the Nucleus Accumbens

Groups	N	Mean ± SE latency to approach-sniff pups (s)	Median onset to full maternal behavior (days)	
			hour	day
SKF 83959	8	32.85 (± 13.94)*	6 (2-6)	6 (2-6)
Vehicle	7	157.42 (± 40.84)	5 (2-6)	5 (2-6)

For each female, latency to sniff-approach pups was averaged across days of drug injection (days 0-2). Interquartile ranges are shown in parentheses. Abbreviations: 15HO = hysterectomized and ovariectomized on day 15 of pregnancy. * Significantly different from the Vehicle group, t test.

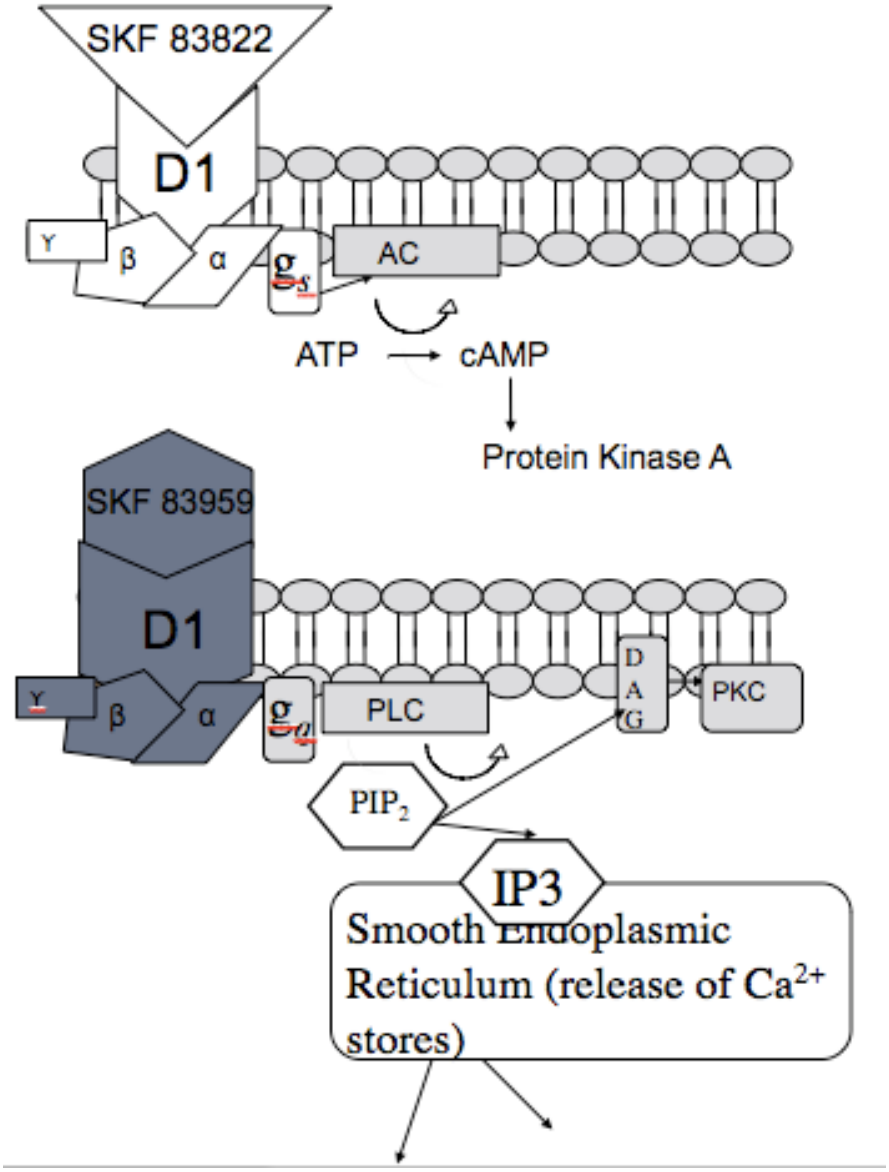

Figure 2.1.1. SKF 83822, a DA D1 receptor agonist that binds exclusively to DA D1 receptors that are liked to the stimulatory G protein (G_s) which activates the enzyme adenylate cyclase (AC). Once activated, AC catalyzes the conversion of ATP to cyclic AMP (cAMP). cAMP binds to the regulatory unit on protein kinase A (PKA) inducing a conformational change which exposes the catalytic unit, allowing PKA to phosphorylate target proteins. SKF 83959, a DA D1 agonist that binds exclusively to DA D1 receptors that are linked to the stimulatory G protein G_q which activates the enzyme phospholipase C (PLC). PLC hydrolyzes phosphatidylinositol 4,5-biphosphate into diacylglycerol (DAG) and inositol 1,4,5, triphosphate (IP3). DAG activates protein kinase C, and IP3 releases intracellular calcium stores.

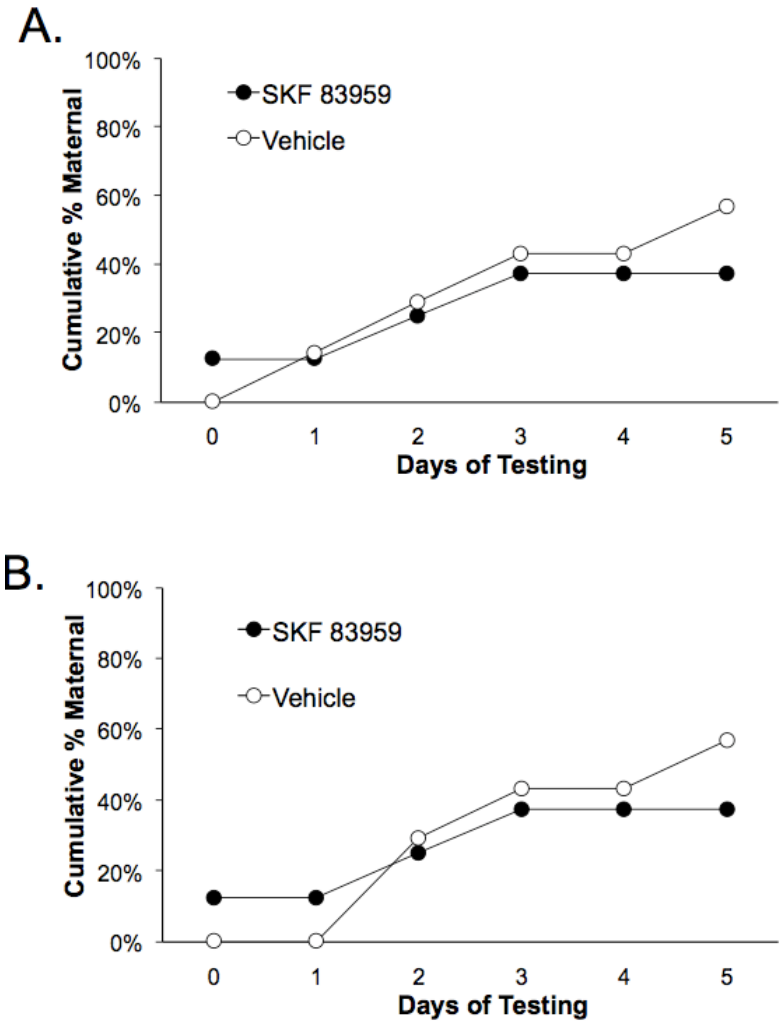

Figure 2.1.2. Cumulative percentage of female rats showing full maternal behavior on each test day (A) when measured at the end of the test hour or (B) when measured at the end of the test day. Females received bilateral microinjections of either 0 (Vehicle) or 0.5 (SKF 83959) μg of SKF 83959 (n_s= 7, 8, respectively) into the nucleus accumbens (NA) on days 0, 1, and 2 of testing. Groups did not differ significantly.

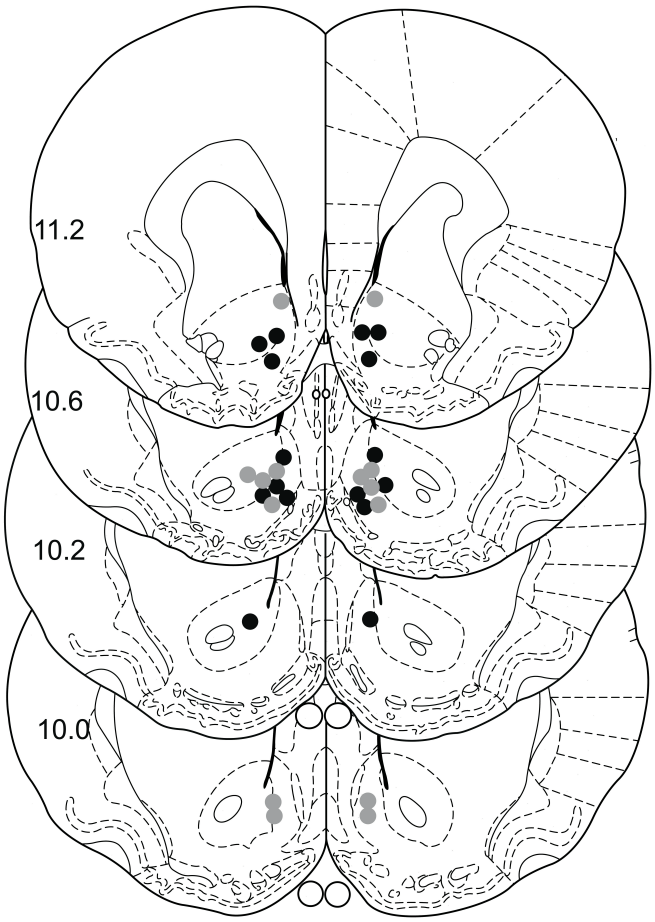

Figure 2.1.3. Reconstructions, based on the microscopic analysis of cresyl violet stained sections, of the location of SKF 83959 injection sites (black circles = 0.5μg dose, grey circles= 0 μg dose) into the nucleus accumbens (NA), which were drawn onto plates from the Paxinos and Watson (1997) atlas. The numbers to the left indicate the distance in millimeters anterior to the interaural plane. All implant sites were located on the shell-core border. However, in order to facilitate viewing, the depicted locations were spread out slightly to eliminate the overlap that actually occurred.

Discussion

The results of the present experiment indicate that DA action at DA D1-PLC receptors does not promote the onset of maternal behavior in 15HO rats. It is interesting to note that SKF 83959 has been shown to inhibit DA-induced AC formation as well as increasing PLC intracellular signaling (Andringa et al., 1999). Therefore, the inhibition of DA activation of AC/cAMP may have contributed to the lack of effectiveness of SKF 83959, suggesting that activation of AC in NA might play an essential in the onset of maternal behavior.

Experiment 2.2.

The purpose of the present experiment was to examine whether the facilitatory effect of SKF 38393, a DA D1 agonist capable of binding to both DA D1-PLC and DA D1-AC linked receptors, was mediated by the latter receptor subtype. Although several studies have shown that DA D1-PLC receptors are associated with neural inhibition in the NA, it is possible that the inhibitory effects of DA D1 stimulation of the excitability of medium spiny neurons are mediated by DA D1 activation of cAMP and PKA, since some of the motivational effects of D1 agonist administration to NA appear to be mediated by the AC-cAMP-PKA cascade (Lynch and Taylor, 2005).

Method

Experiment 2.2 examined the effects of bilateral injections of SKF 83822 (a D1 DA receptor agonist linked to AC obtained from Tocris Bioscience, Ellisville, MO) into the NA on the sensitization latencies of 15HO female rats. Two

independent groups received either 0 μg (Vehicle; n = 10) or 0.5 μg/0.5 μl/side of SKF 83822 (SKF 83822; n = 8) into the medial NA. Each dose of SKF 83822 was dissolved in 5% DMSO and sterile H_20. The method for this experiment is identical to that described for Experiment 2.1 except that female rats were injected bilaterally into NA on days 0 and 1 of behavioral testing (rather than on days 0-2). It was decided that only injections on days 0 and 1 were needed because, based on the data from the previous experiments, these are the days on which SKF 38393 exerted its major facilitatory effects on the onset of maternal behavior. Because SKF 83959 and SKF 83822 have similar molecular weights, the 0.5 μg dose of both drugs is comparable.

Results

The results of Experiment 2.2 indicate that the facilitatory effect of DA D1 receptor activation in the NA on the onset of maternal behavior in 15HO rats is mediated DA D1-AC linked receptors which initiate cAMP intracellular signaling. Microinjection of SKF 83822, a drug which binds selectively to DA D1 receptors linked to AC, directly into the NA of 15HO female rats produced a rapid onset of maternal behavior. Females treated with 0.5 μg of SKF 83822 (SKF 83822, n = 8) on days 0 and 1 of testing showed a median sensitization latency of 1 day, by the end of the hour observation, which was significantly shorter than the 2.5 day latency of the vehicle treated group (Vehicle, n = 10; Mann-Whitney *U* test, U = 17, $p < .05$; Table 2.2.1). The median sensitization latency for SKF 83822 females was also significantly shorter than the Vehicle group when measured at

the end of the test day: 0 days versus 2 days, respectively (Mann-Whitney *U* test, U = 16.5, $p < .05$; Table 2.2.1). Importantly, the mean latency to approach and sniff pups was not significantly different between SKF 83822 and Vehicle rats, indicating that the facilitatory effect of SKF 83822 treatment cannot be the result of SKF 83822 treated females approaching pups more readily than vehicle treated rats.

The cumulative percentage of female rats displaying full maternal behavior on each test day is shown in Figure 2.2.1. Microinjection of SKF 83822 directly into the NA significantly facilitated the onset of maternal behavior in 15HO rats compared vehicle injection. The cumulative percentage of female rats showing full maternal behavior was significantly higher in the SKF 83822 group compared with Vehicle: 88% versus 30%, respectively, on day 0 of testing when measured by the end of the test day (Fig 2.2.1b) and on day 1 of testing when measure by the end of the observation hour (Fig 2.2.1a; Fisher exact probability test, $p < .05$).

Statistical comparisons (t-tests) of nursing behavior between SKF 83822 and Vehicle treated females indicated that no significant differences existed with respect to nursing duration, sniffing/licking duration, hover duration, or crouch duration over the 15 minute nursing observation (Table 2.2.2). This observation occurred for all females on the first observation where full maternal behavior occurred.

Figure 2.2.2. shows reconstructions of the location of the NA injection sites, drawn on to the appropriate plates taken from the atlas of Paxinos and Watson (1997). Importantly, microscopic analysis of brain sections indicated that for each group, all cannula placements were located in the NA, either in the shell region or the shell-core border.

Table 2.2.1. Outcome Measures for 15HO rats that Received SKF 83822 Microinjection into the Nucleus Accumbens

Groups	N	Mean ± SE latency to approach-sniff pups (s)	Median onset to full maternal behavior (days)	
			hour	day
SKF 83822	8	225.81 ± 80.03	1 (0-1)*	0 (0-0)*
Vehicle	10	139 ± 59.73	2.5 (1-6)	2 (0-6)

For each female, latency to sniff and approach pups was averaged across days of drug injection (0-1). Interquartile ranges are shown in parentheses. Abbreviations: 15HO = hysterectomized and ovariectomized on day 15 of pregnancy. * Significantly different than Vehicle group, Mann Whitney *U* test

Table 2.2.2. Outcome measures (with standard errors) for Females that Received SKF 83822 or Vehicle Microinjections into the Nucleus Accumbens

Group	*N*	Mean nursing duration	Mean no. sniffs/licks	Mean no. Hovers	Mean no. Crouches
SKF 83822	8	25.13 ± 1.72	8.5 ± 2.13	13.88 ± 3.0	9.75 ± 3.13
Vehicle	7	26.57 ± 2.19	7.0 ± 2.82	8.14 ± 3.44	15.29 ± 3.14

Groups did not differ significantly. Note that the number of subjects in each group is based on those females that showed maternal behavior during the 5 day test period. Abbreviations: 15HO = hysterectomized and ovariectomized on day 15 of pregnancy.

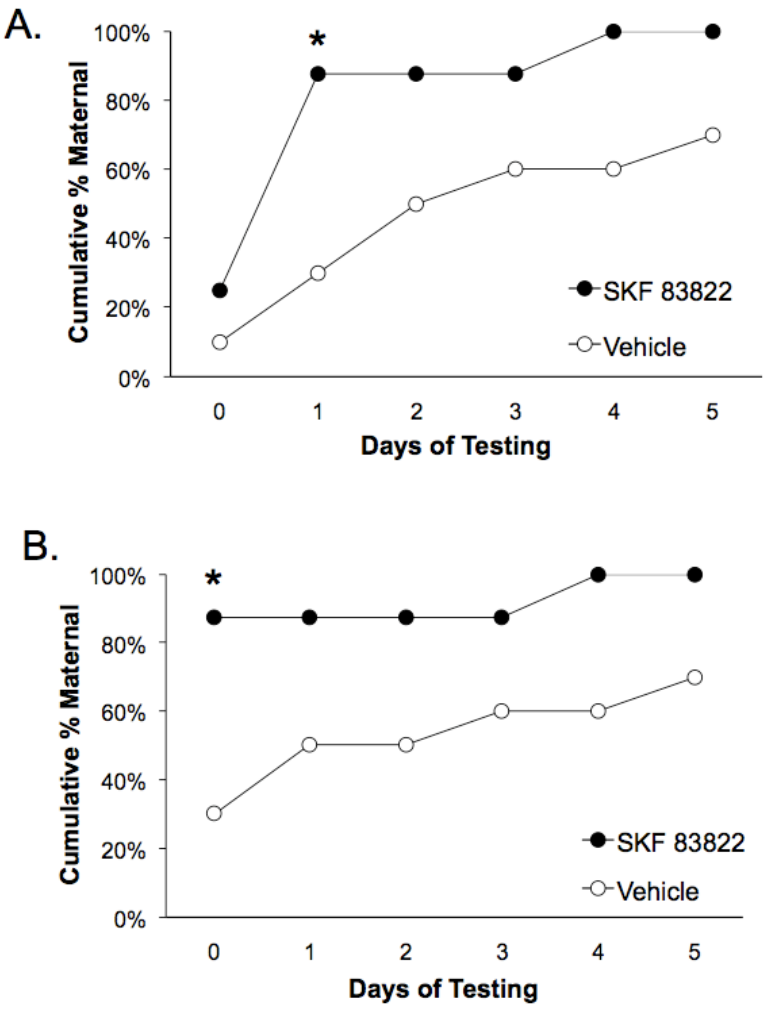

Figure 2.2.1. Cumulative percentage of female rats showing full maternal behavior on each test day (A.) as measured at the end of the test hour or (B.) as measured at the end of the test day. Females received bilateral microinjections of either 0 (Vehicle) or 0.5 μg of SKF 83822 (n_s= 10, 8, respectively) into the nucleus accumbens (NA) on days 0 and 1 of testing. *Significantly different from Vehicle, Fisher Exact Probability.

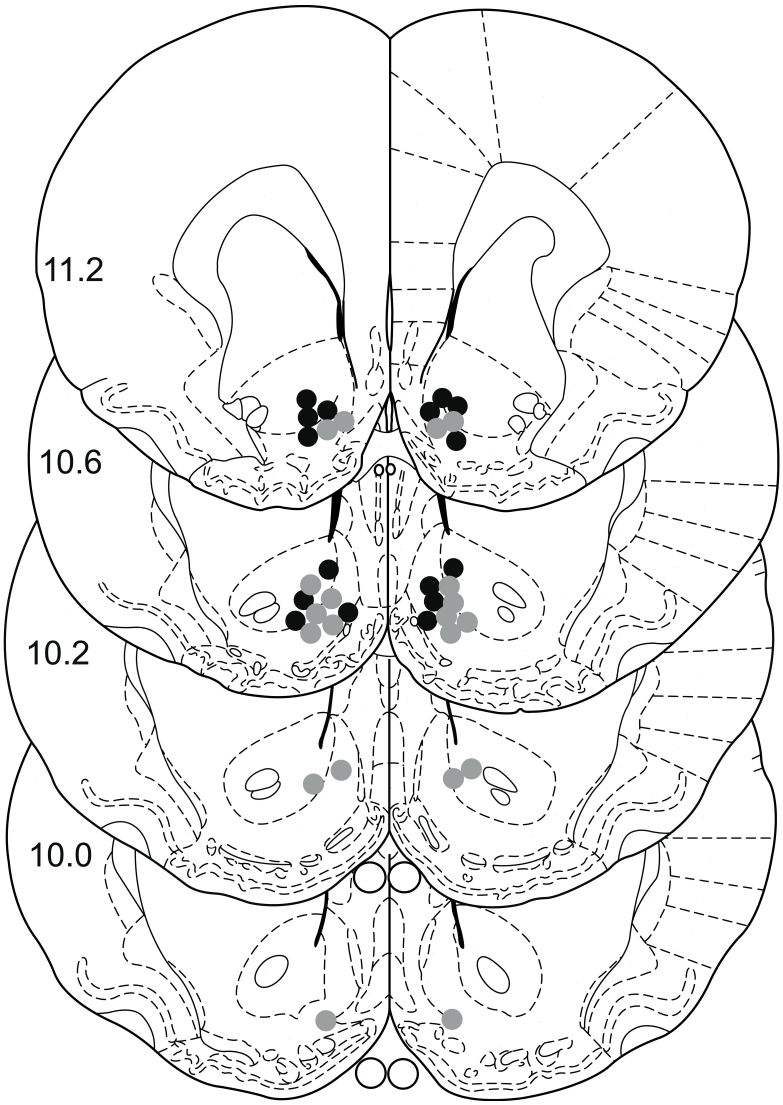

Figure 2.2.2. Reconstructions based on the microscopic analysis of cresyl violet stained sections, of the location of SKF 83822 injection sites (black circles = 0.5μg dose, grey circles= 0 μg dose) into the nucleus accumbens (NA), which were drawn onto plates from the Paxinos and Watson (1997) atlas. The numbers to the left indicate the distance in millimeters anterior to the interaural plane. All implant sites were located on the shell-core border. However, in order to facilitate viewing, the depicted locations were spread out slightly to eliminate the overlap that actually occurred.

Discussion

The results of the present experiment represent the major finding of study 2. The facilitatory effect DA activity in NA on the onset of maternal behavior in 15HO rats is most likely mediated by DA D1-AC receptors and involves increased cAMP signaling. Because the facilitatory effect of SKF 83822 microinjection into NA consistently occurred approximately 5 hours post injection(see figure 2.2.1), one possibility is that cAMP intracellular signaling cascades promote the onset of maternal behavior via activation of a downstream transcription factors which alter gene expression. This hypothesis would be consistent with the time course of DA D1-AC receptor stimulation. Importantly, activation of the transcription factor cAMP response element binding protein (CREB), a downstream target of PKA, in the NA has been associated with the initiation of behavioral reactivity toward biological stimuli in naïve animals (Barrot et al., 2005), suggesting that activation of cAMP signaling cascades in NA might function to increase an organisms responsiveness to pup stimuli during the onset of behavior.

Although the evidence is clear that SKF 83822 activates AC (Kuroiwa et al., 2008; Undie and Friedman, 1994), it is possible that this drug also has other effects which have yet to be detected, and these effects might have also influenced the onset of maternal behavior. Furthermore, since we did not actually measure cAMP production, we are going on conclusions from biochemical findings of others (Kuroiwa eta l., 2008; Undie and Friedman, 1994).

Interestingly, there is some evidence that CREB activity in NA might be associated with the onset of male sexual behavior (Barrot et al., 2005). Whereas its clear that DA D1 receptor activation is necessary for both the onset (Stolzenberg et al., 2007) and maintenance of maternal behavior (Numan et al., 2005a), activation of CREB might be one downstream signal of DA D1 activation which mediates the initial increase in responsiveness necessary to push females over the threshold for full maternal behavior. A good question for future research would be whether or not the maintenance of maternal behavior is also dependent upon activation of DA D1-AC linked receptors and subsequent stimulation of PKA, or whether this intracellular signaling mechanism solely regulates the onset of maternal behavior in rats. With respect to the importance of CREB for maternal behavior, note that CREB knockout mice show severe deficits in maternal behavior (Jin et al., 2005).

The results of Experiment 2.2 also address a question posed by study 1: are DA D1 treated females “better” mothers than their sensitized, Vehicle-treated counterparts? Therefore, the methods of study 2 included detailed nursing observations which were taken for a 15 min period once a female rat had initiated maternal behavior. Therefore the exact day and time in which observations were taken varied for each rat depending on when they first displayed maternal behavior. Comparisons between the duration of nursing behavior, sniffing and licking, hovering, and crouching during the 15 min observation revealed no significant differences between sensitized Vehicle-treated rats and SKF 83822

treated females. These data suggest that SKF 83822 probably functions to increase DA action at D1-AC receptors above a particular threshold required for the initiation of maternal responsiveness, at which point endogenous DA activity probably maintains maternal responsiveness. In this case, it is conceivable that sensitized Vehicle-treated rats would display similar amounts of mother-pup interaction as SKF 83822-treated females, since endogenous DA is most likely involved in the onset of maternal behavior in the vehicle-injected females.

Experiment 2.3

While the finding that DA stimulation of DA D1-AC receptors in NA is necessary for the DAergic facilitation of maternal behavior in 15HO rats is important, this finding is ultimately incomplete without providing evidence that this facilitatory effect of SKF 83822 on maternal behavior is not due to spread of the injected drug to a nearby region, particularly the MPOA. Because DA action at the level of the MPOA also produces facilitatory effects on maternal behavior in 15HO rats, the present experiment makes use of another region which is located in close proximity to both the NA and the MPOA. In this experiment we examined the effects of bilateral application of SKF 83822 to VP. Note that the VP is spatially as close to the MPOA as it is to the NA. Our reasoning was that if VP injection of SKF 83822 did not facilitate maternal behavior, it would be difficult to explain the facilitation of maternal behavior after SKF 83822 application to the NA as due to spread to the MPOA.

Method

The methods for this experiment are identical to those described for Experiment 2.1 except that guide cannula were implanted into the ventral pallidum (VP) using the DeGroot stereotaxic coordinates: A7.5, L2.5,V5.8. Similar to Experiment 2.2, female rats in this experiment received microinjections for 2 days. Over days 0-1 of behavioral testing, independent groups of 15HO female rats received bilateral microinjection of either 0μg (Vehicle VP, n = 11) of SKF 83822 or 0.5 μg/0.5 μl/ side (SKF 83822 VP, n = 11) dissolved in 5% DMSO in sterile water.

Results

Female rats injected with SKF 838322 into the VP (SKF 83822 VP; n = 11) showed a median sensitization latency of 5 days which was not significantly shorter than the 3 day latency shown by vehicle treated rats by the end of the hour or the test day (Vehicle; n = 11; Mann-Whitney U test U = 51.5, p = .5114, Table 2.3.1). The latency to approach and sniff pups was also not significantly different between these two groups of animals (t-test, t(22) = 0.18; Table 2.3.1). Figure 2.3.1 indicates that the cumulative percentage of female rats showing full maternal behavior on each test day was comparable between SKF 83822 VP and Vehicle VP groups. Figure 2.3.2. shows reconstructions of the location of the VP injection sites, drawn on to the appropriate plates taken from the atlas of Paxinos and Watson (1997).

Table 2.3.1. Outcome Measures for 15HO rats that Received SKF 83822 Microinjection Into the Ventral Pallidum

Groups	N	Mean ± SE latency to approach-sniff pups (s)	Median onset to full maternal behavior (days)	
			hour	day
SKF 83822 VP	11	186.22 ± 71.73	5 (1-6)	5 (1-6)
Vehicle VP	11	169.23 ± 64.72	3 (2-6)	3 (1-6)

For each female, latency to sniff and approach pups was averaged across drug injection days (0-1). Interquartile ranges are shown in parentheses. Abbreviations: 15HO = hysterectomized and ovariectomized on day 15 of pregnancy. Significant differences were not detected.

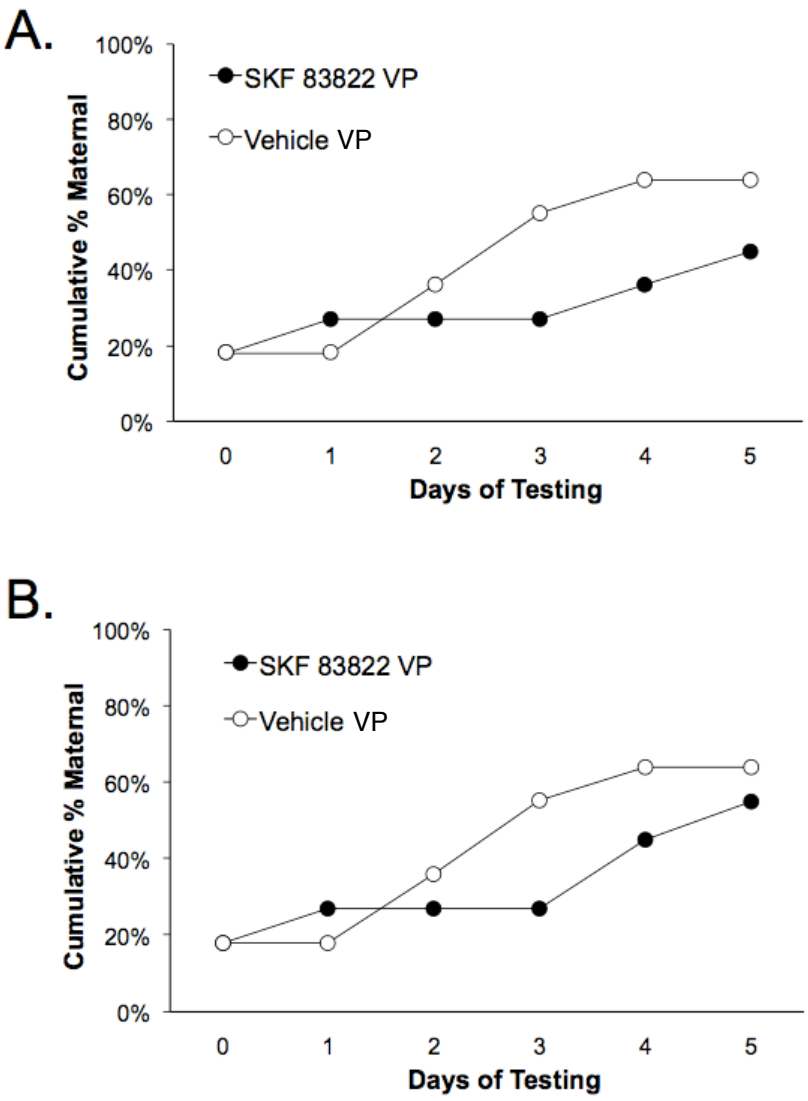

Figure 2.3.1. Cumulative percentage of female rats showing full maternal behavior on each test day, (A) as measured at the end of the test hour or (B) as measured at the end of the test day. Females received bilateral microinjections of either 0 (Vehicle VP) or 0.5 (SKF 83822 VP) μg of SKF 83959 (n_s= 10, 8, respectively) into the ventral pallidum (VP) on days 0 and 1 of testing. Groups did not differ significantly.

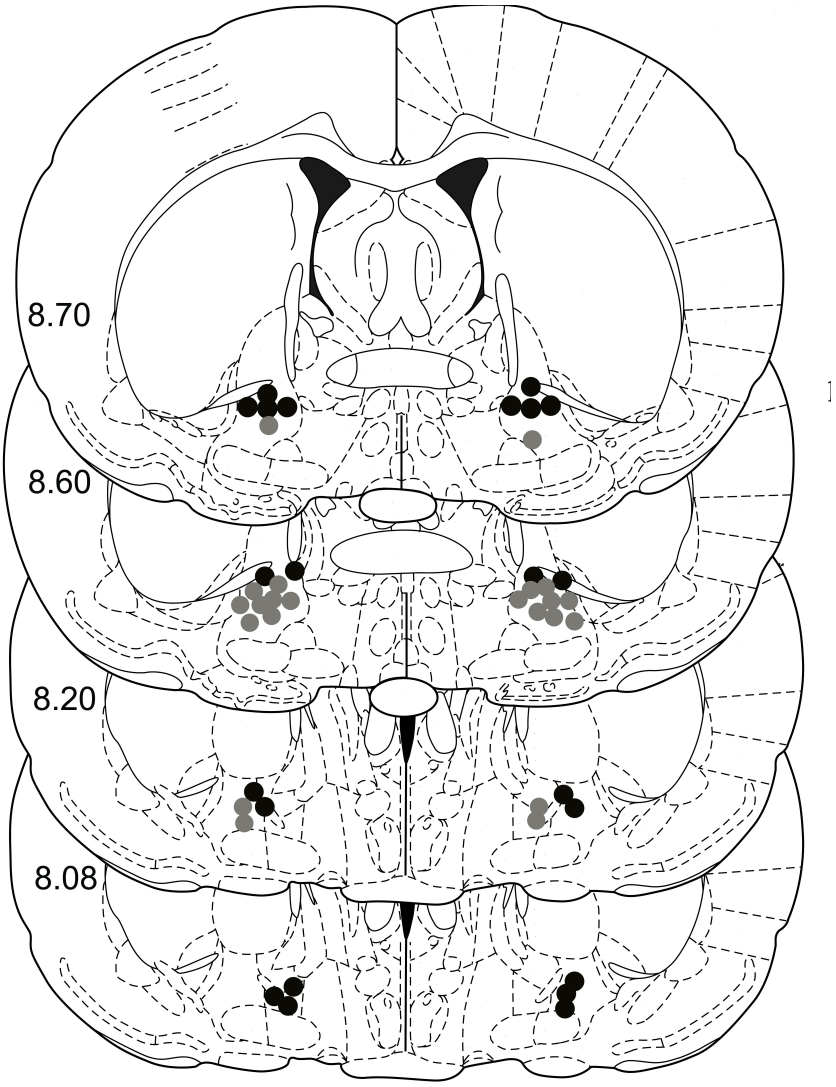

Figure 2.3.2. Reconstructions based on the microscopic analysis of cresyl violet stained sections, of the location of SKF 83822 injection sites (black circles = 0.5μg dose, grey circles= 0 μg dose) into the ventral pallidum (VP), which were drawn onto plates from the Paxinos and Watson (1997) atlas. The numbers to the left indicate the distance in millimeters anterior to the interaural plane.

Discussion

The results of Experiment 2.3 are an important contribution to this study, and they strongly indicate that the facilitatory effect of SKF 83822 in NA on the onset of maternal behavior is localized to the NA, and not due to spread of the injected substance to a nearby neural site. It is important to note that the NA is not the only neural site where DA D1 receptor stimulation has been found to promote the onset of maternal behavior in 15HO rats. For example, DA activity in the MPOA also promotes the onset (Experiment 1.1; Stolzenberg et al., 2007). The MPOA is a critical hypothalamic nuclei which responds to hormonal and pup inputs to regulate maternal responsiveness (Numan and Insel, 2003). One issue with examining the effects of DA activity in NA and MPOA is that the MPOA and NA are only separated by about 2 mm, therefore it is difficult to know whether microinjected substances produced facilitatory effects via spread from one region to the other. In the current study, the ventral pallidum (VP) functions as a key anatomical control site because (1) it is in close proximity to the MPOA (it is located on the same rostro-caudal plane as the MPOA, but roughly 1.5 mm lateral to it), (2) it is critical for maternal behavior (Numan et al., 2005b) and (3) it also receives DA inputs from the VTA (Klitenick et al., 1992).

General Discussion

The present series of experiments made use of two DA D1 receptor agonists which have been reported to be selective for the two distinct forms of DA D1 receptors. The main finding of the present study is that microinjection of

SKF 83822, a DA D1 agonist which selectively binds to DA D1-AC linked receptors, directly into NA promotes a rapid onset of maternal behavior in 15HO rats, whereas microinjection of SKF 83959, a DA D1 agonist which selectively binds to the alternate DA D1-PLC linked receptors, directly into NA is not capable of promoting the onset of maternal behavior in 15HO rats. The results of the present study replicate our previous work which showed that intra-NA microinjection of SKF 38393, a DA D1 receptor agonist which binds both forms of the D1 receptor, promotes the onset of maternal behavior in 15HO rats, and extend this work by pinpointing the particular intracellular signaling cascade by which DA D1 receptor stimulation of NA produces its facilitatory effect on maternal behavior. Importantly, the present study also demonstrates that the facilitatory effect of SKF 83822 on the stimulation of maternal behavior in 15HO rats is not likely to be due to spread of the drug to the MPOA, because microinjection of SKF 83822 into the VP did not facilitate the onset of maternal behavior in 15HO rats.

The finding that DA D1-mediated activation of cAMP intracellular signaling cascades in NA promotes the onset of maternal behavior in 15HO rats has major implications for the intracellular mechanism by which DA produces its facilitatory effect. First, whereas a role for the mesolimbic DA system has been well-established in the regulation of maternal responsiveness, as well as more generally in the regulation of behavioral reactivity toward a wide range of biologically important stimuli (Numan, 2006), the mechanism by which DA action,

particularly at D1 receptors, in NA functions to increase behavioral responsiveness is frequently debated (Nicola et al., 2000, Pennartz et al., 1994). Therefore, the current data, which indicate that the induction of cAMP signaling mediates the facilitatory effects of DA D1 receptor stimulation in NA, provide a starting point for investigating how the release of DA into NA functions to increase behavioral reactivity. If the Mogenson (1987) model, which seems to conform to the data from our laboratory (Numan, 2008), is correct, one can now explore the mechanisms through which the D1-AC-cAMP cascade might function to reduce the responsiveness of NA to glutamatergic inputs from the limbic system.

Secondly, the current work raises the important question of the mechanism through which SKF 38393 application to MPOA promotes the onset of maternal behavior in 15HO females (Experiment 1.4; Stolzenberg et al., 2007). Future work will need to explore the effects of SKF 83822 and SKF 83959 application to MPOA on the onset of maternal behavior. In other words, does DA D1 action at the level of the MPOA stimulate maternal behavior via a PLC or AC signaling cascade? Since MPOA output is essential for maternal behavior, while it is proposed that NA output might be depressed for maternal behavior to occur, it is possible that different signaling cascades are utilized by DA in each region. However, if DA action on D1 receptors in MPOA acts on inhibitory interneurons, which depress MPOA output, then one can conceive of the possibility that SKF 83822 might be effective in MPOA , as it is effective in NA (in NA, the proposal is

that DA D1 linked to AC is acting on GABAergic output neurons to depress their responsiveness.

Thirdly, the finding that stimulation of NA DA D1 receptor-mediated cAMP intracellular signaling promotes the onset of maternal behavior in 15HO rats may provide useful information about the mechanism(s) through which estradiol regulates maternal responsiveness. Recall that when 15HO females are presented with foster pups 48 hours after surgery, they typically show maternal behavior after about 2-3 days of exposure. However a rapid onset of maternal behavior can be induced in 15HO rats when they are treated with estradiol benzoate (EB) at the time of HO surgery (Siegel and Rosenblatt, 1975a). The fact that in the absence of EB treatment, 15HO females treated with SKF 38393 or SKF 83822 directly into the NA could produce a rapid onset of maternal behavior, suggests that activation of DA D1 receptors linked to cAMP in NA substitutes for the facilitatory effect of estradiol and promotes the onset of maternal behavior in 15HO rats (Stolzenberg et al., 2007). Whereas estradiol benzoate has been typically administered at the time of HO surgery, 48 hours prior to pup presentation, with the assumption that genomic effects of E require 48 hours to develop, it is certainly possible that EB was acting closer to the time of pup presentation, since EB injected in oil may remain in the systemic circulation for several days (Numan et al., 1977). Since estradiol action at membrane-associated ERs can stimulate the AC/cAMP/PKA cascade (Aronica et al., 1994; Razandi et al., 2004; Razandi et al., 1999; Watters and Dorsa, 1998), it

is possible that activation of this cascade is one of the common mechanisms through which both E and DA stimulate maternal behavior. Study 3 begins an examination of this important issue.

V. Study 3: A single injection of 17β-estradiol at the time of pup presentation promotes the onset of maternal behavior in pregnancy-terminated rats

According to our model, the onset of maternal behavior is regulated by the hormonal events of pregnancy and parturition. These hormonal events, particularly, the rise in estradiol (E) just before birth, probably function to induce the onset of maternal behavior through actions at multiple neural sites involved in the regulation of maternal behavior. We have focused on the regulation of increased maternal responsiveness which is mediated by MPOA interaction with the mesolimbic DA system, and therefore I am interested in how E acts on this aspect of the neural circuit, in particular. The finding that increased DA action at D1 receptors, at the level of the MPOA or the NA, in the absence of appropriate E stimulation, promotes the onset of maternal behavior indicates that increased DA action at D1-AC receptors might be a downstream effect of E stimulation. In support of this idea, recall that subcutaneous injection of estradiol benzoate (EB) dissolved in oil at the time of HO, or intra-MPOA implant of EB at the time of HO, can reinstate an immediate onset in 15HO rats (Siegel and Rosenblatt, 1975a; Numan et al., 1977, respectively).

If the DA-D1 agonist-treated 15HO rats in studies 1-2 showed a rapid onset of maternal behavior because DA D1 receptor stimulation substituted for the facilitatory effect of E stimulation, a relevant issue concerns the relationship between these two stimulatory effects. Because the long latency and long lasting classical effects of hormones are the best documented within the literature, it has

often been stated that the immediate activation of responsiveness toward biologically significant stimuli by neurotransmitter systems, for example DA, are likely influenced by the prior classical genomic effects of hormonal action (Hull et al., 1997). In support of this idea, note that EB (both systemic and intra-MPOA) has typically been administered at the time of HO surgery, 48 hours prior to pup presentation. Treatment with EB at that time was based on the idea that steroid hormones exert their effects via the transcription of genes at estrogen responsive DNA sites and the subsequent synthesis of new proteins which might take several hours to days. However, recall that steroid hormone action also occurs at membrane-bound receptors or intracellular receptors and is capable of inducing non-classical cellular effects via the activation of intracellular signaling cascades and the phosphorylation of proteins (for reviews see Beato and Klug, 2000; Bishop and Stormshak, 2008; Bjornstrom and Sjoberg, 2005; Foradori et al., 2008; Kelly and Levin, 2001; Mhyre and Dorsa, 2006; Toran-Allerand, 2004; Vasudevan and Pfaff, 2007, 2008).

Importantly, the mechanism by which EB produces a facilitatory effect on the onset of maternal behavior in 15HO rats is not clear. For example, EB was implanted in MPOA at the time of surgery and removed at the time of pup presentation (Numan et al., 1977); therefore, the EB implant was still present in the MPOA at the time of pup presentation. Further, in 15HO females systemically treated with EB at the time of HO, it is also likely that EB was present at the time of pup presentation because EB dissolved in oil results in the slow, long-term

release of EB from the subcutaneous depot; in confirmation of this idea, vaginal cytology of 15HO female rats injected with EB subcutaneously indicates that E is exerting cellular effects up to 7 days post injection (Numan, 1978; Numan et al., 1977). Therefore, the possibility that EB can exert facilitatory effects on maternal behavior by acting at the time of pup presentation cannot be ruled out. Since DA-D1 receptor activation of MPOA or NA at the time of pup presentation stimulates maternal behavior, perhaps E and DA operate via similar mechanisms to influence the onset of maternal behavior.

As a first step to resolving this question, we directly examined whether systemic administration of a water-soluble form of 17β-estradiol (E_2) can facilitate the onset of maternal behavior when injected at the time of pup presentation. Note that water-soluble E_2 is rapidly absorbed and degraded so that it is eliminated within 24 hours of injection (Cross and Roselli, 1999; Taylor, et al., 1989; Trainor et al., 2008).

Experiment 3.1

Method

The methods used in study 3 were similar to those of study 2, except for the following details. In this experiment, females did not undergo stereotaxic surgery on day 1 of pregnancy. However, all females were HO on day 15 of pregnancy as previously described.

Female rats in all the experiments of study 3 were administered either cyclodextrin encapsulated 17β-estradiol (E_2; Sigma cat. # E4389) dissolved in

physiological saline or the cyclodextrin/saline vehicle solution. Both solutions, once prepared, were stored in the refrigerator and used for up to 5 days. Female rats included in Experiment 3.1 were divided into two independent groups and were administered sc 100 μg/kg of E_2 (E_2 100; n = 9) or vehicle (vehicle; n = 9) on days 0 and 1 of behavioral testing, one hour prior to pup presentation.

Results

15HO female rats treated with 100 μg/kg of E_2 (E_2 100, n = 9) on days 0 and 1 of testing showed a median sensitization latency of 1 day, by the end of the hour observation, significantly shorter than the 4 day latency of the vehicle treated group (vehicle, n = 9; Mann-Whitney *U* test, U = 20, $p<.05$, one-tailed, Table 3.1.1). The median sensitization latency for E_2 100 females was also significantly shorter than vehicle treated animals when measured at the end of the test day: 1 day compared with 3 days, respectively (Mann-Whitney *U* test, U = 23, $p<.05$, one-tailed).

The cumulative percentage of female rats displaying full maternal behavior by the end of the test hour and by the end of the test day for females in the $E_2$100 and vehicle treated conditions is shown in Figure 3.1.1. The cumulative percentage of females showing full maternal behavior within the 1 hour morning observation period was significantly higher in the E_2- treated females on day 3 of testing (Fisher exact probability test, $p<.05$). When the criterion used was the end of the test day to show full maternal behavior, the cumulative percentage of

females showing full maternal behavior was significantly higher in the E_2- treated females on days 2 and 3 of testing (Fisher exact probability test, $p_s<.05$).

Statistical comparisons (t-tests) of nursing behavior between E_2 100 and vehicle treated females indicated that no significant differences existed with respect to nursing duration, sniffing/licking duration, hover duration, or crouch duration over the 15 minute nursing observation (Table 3.1.2). This observation occurred for all females on the first observation where full maternal behavior occurred.

Table 3.1.1. Outcome Measures for 15HO rats that Received Subcutaneous Injections of 17β-estradiol at the time of pup presentation

		Median onset to full maternal behavior (days)	
Group	*N*	Hour	Day
E_2 100	9	1 (1-1.5)*	1 (0-1)*
Vehicle	9	4 (1-4.5)	3 (0-4)

Interquartile ranges are shown in parentheses. * Significantly different from the corresponding vehicle group, Mann-Whitney *U* test. Female rats were injected with either 100 μg/kg of 17β-estradiol (E_2 20) or vehicle on days 0-1 of behavioral testing. Abbreviations: 15HO = hysterectomized and ovariectomized on day 15 of pregnancy.

Table 3.2. Outcome Measures (with standard errors) for 15HO rats that Received Subcutaneous Injections of 17β-estradiol at the time of pup presentation

Group	*N*	Mean no. sniffs/licks	Mean no. Hovers	Mean no. Crouches
E_2 100	9	10.3 ± 1.6	12.6 ± 1.8	14.2 ± 2.3
Vehicle	8	7.2 ± 2.1	8.7 ± 2.4	20 ± 2.4

E_2 100 refers to 100 μg/kg of 17β-estradiol, these female rats were treated with estradiol on day 0 of testing one hour prior to pup presentation. * Significantly different than vehicle, t-test. Note the number of subjects in each group is based on those females that showed maternal behavior during the 5 day test period. Abbreviations: 15HO = hysterectomized and ovariectomized on day 15 of pregnancy.

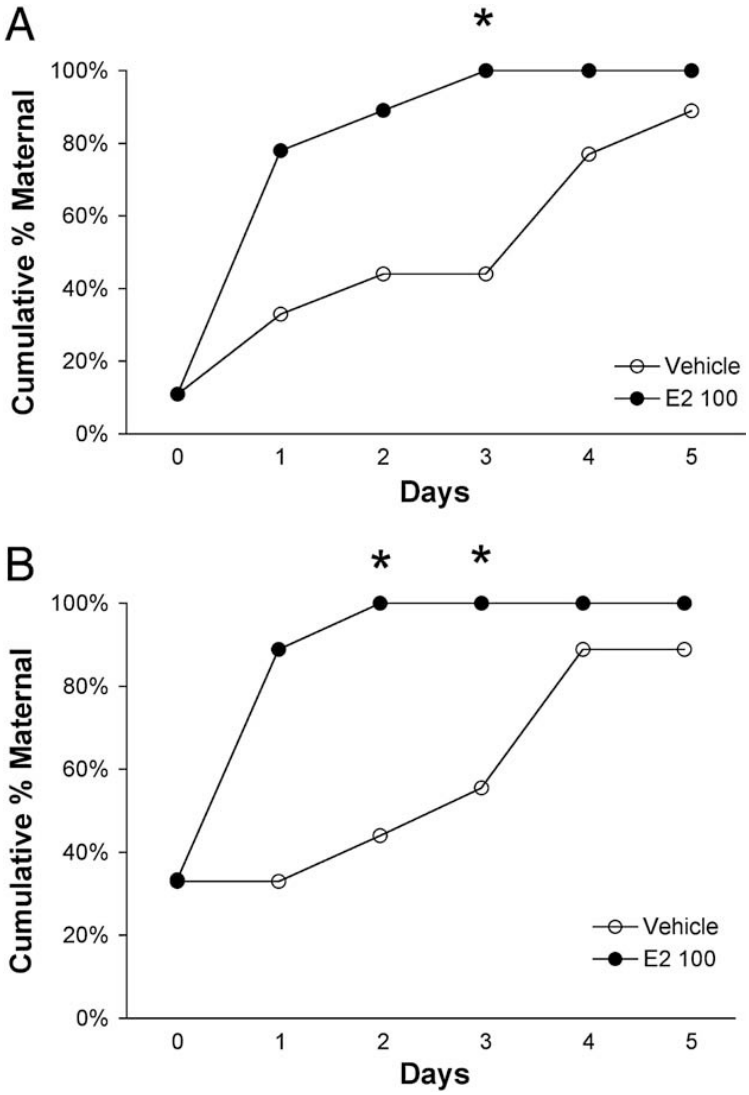

Figure 3.1.1 A. Cumulative percentage of female rats showing full maternal behavior by the end of the hour observation on each test day. B. Cumulative percentage of female rats showing full maternal behavior by the end of the day on each test day. Females received subcutaneous injections of either 100 μg/kg of 17β-estradiol (E_2 100) or vehicle on days 0-1 of behavioral testing.

Discussion

The purpose of the current experiment was to examine whether subcutaneous administration of E_2 could replicate that facilitatory effect of SKF 83822 or SKF 38393 on the onset of maternal behavior in 15HO rats when administered at the time of pup presentation. It is already clear that subcutaneous administration of EB can replicate the facilitatory effect of SKF 83822 or SKF 83959 when administered 48 hours prior to pup presentation. The injection schedule of E_2 in the present experiment was meant mimic the injection schedule of SKF 38393 and SKF 83822 in study 1 and study 2, respectively.

Whereas subcutaneous administration of E_2 promoted the onset of maternal behavior in rats, the effect consistently occurred 6-24 hours after administration Therefore it appears that while E_2 is capable of rapidly promoting the onset of maternal behavior, the mechanism by which E_2 operates likely involves alterations in gene transcription which would be more consistent with a 6-24 hour onset of maternal behavior.

Experiment 3.2.

Given that female rats included in Experiment 3.1 tended to show maternal behavior on day 1 of testing, the day of the second E_2 injection, an interesting question is whether an acute injection of E_2 on only one day of testing would be able to stimulate the onset of maternal behavior 24 hours later. The present experiment was conducted to examine this question. It is identical to Experiment 3.1 except that females received a single injection of E_2 at the time

of pup presentation.

Method

Experiment 3.2 consisted of two independent groups of females administered sc either 100 μg/kg E_2 (E_2 100-single; n = 15) or vehicle (vehicle; n = 14) in a single injection given on day 0 of behavioral testing, one hour prior to pup presentation.

Results

The results of Experiment 3.2 replicated those of Experiment 3.1, indicating that a single dose of E_2 is sufficient to induce the onset of maternal behavior. Female rats in the E_2 100-single group (n = 15) showed a significantly shorter sensitization latency than vehicle treated females (vehicle, n = 14) by the end of the observation hour as well as at the end of the test day (see Table 3.2.1). The median sensitization latency for E_2 100-single rats to show full maternal behavior by the end of the observation hour was 1 day, significantly shorter than 3 days displayed by vehicle treated rats, Mann-Whitney *U* test, U = 65, $p<.05$, one-tailed (Table 3.2.1). When sensitization latencies were based on the occurrence of maternal behavior by the end of the test day, the median sensitization latency for the E_2 100-single treated females (1 day) was significantly shorter than the 2.5 day latency observed in the vehicle-treated females (Mann-Whitney *U* test, U = 60.5, $p<.05$).

During the first hour on the morning of day 1 of testing, 86.7% of the E_2 100-single females showed full maternal behavior, which was significantly higher

than the 28.6% of the vehicle-treated females showing maternal behavior, and this significant difference between E_2 100-single and vehicle-treated rats continued on day 2 (Fisher exact probability, p_s<.05; see Figure 3.2.1A). The cumulative percentage of E_2 100 single females showing full maternal behavior by the end of the test day was also significantly greater than vehicle treated females on days 1 and 2 of testing (Fisher exact probability test, p<.01, p = .05, respectively; see Figure 3.2.1B).

While nursing behavior was relatively similar between E_2 100 single and vehicle treated females, a significant difference was detected with respect to crouch duration. Females treated with E_2 100-single tended to crouch more frequently over the 15 minute observation than those treated with vehicle [t (23)= 1.81, p<.05, one-tailed; see Table 3.2.2].

Table 3.2.1. Outcome Measures for 15HO rats that Received Subcutaneous Injection of 17β-estradiol at the time of pup presentation

		Median onset to full maternal behavior (days)	
Group	*N*	Hour	Day
E_2 100-single	15	1 (1-1)*	1 (0-1)*
Vehicle	14	3 (1-3)	2.5 (0-3)

Interquartile ranges are shown in parentheses. * Significantly different from the corresponding vehicle group, Mann-Whitney *U* test. Females were injected with either 100 µg/kg of 17β-estradiol (E_2 100-single) or vehicle on day 0 of behavioral testing. Abbreviations: 15HO = hysterectomized and ovariectomized on day 15 of pregnancy.

Table 3.2.2. Outcome Measures (with standard errors) for 15HO rats that

Received Subcutaneous Injection of 17β-estradiol at the time of pup presentation

Group	*N*	Mean no. sniffs/licks	Mean no. Hovers	Mean no. Crouches
E_2 100-single	14	8.4 ± 1.3	11.1 ± 1.5	16.7 ± 2.1*
Vehicle	12	12.4 ± 2.3	14.9 ± 2.4	10.7 ± 2.4

E_2 100-single refers to 100 µg/kg of 17β-estradiol and Vehicle refers to vehicle injected females. Females were treated with estradiol on day 0 of testing only one hour prior to pup presentation. * Significantly different than vehicle, t-test. Note the number of subjects in each group is based on those females that showed maternal behavior during the 5 day test period. Abbreviations: 15HO = hysterectomized and ovariectomized on day 15 of pregnancy.

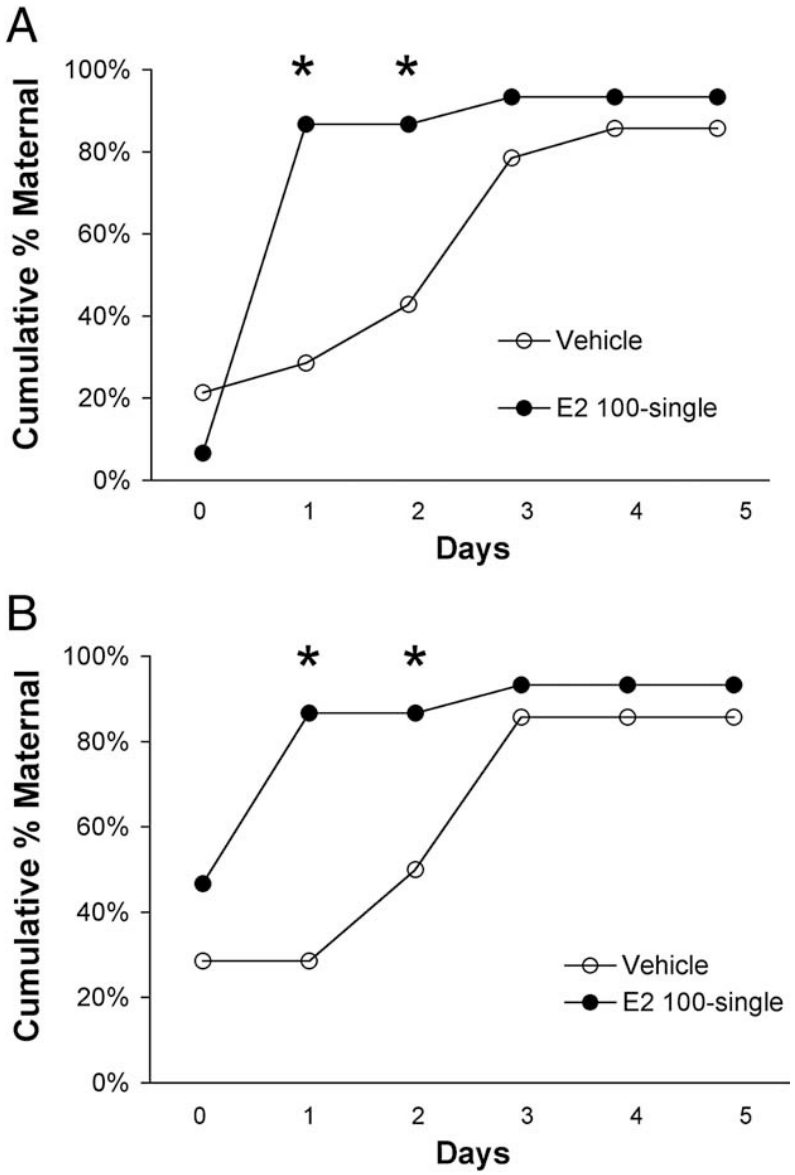

Figure 3.2.1. A. Cumulative percentage of female rats showing full maternal behavior by the end of the hour observation on each test day. B. Cumulative percentage of female rats showing full maternal behavior by the end of the day on each test day. Females received subcutaneous injections of either 100 μg/kg of 17β-estradiol (E_2 100-single) or vehicle on day 0 of behavioral testing. *Significantly different from vehicle group, Fisher exact probability test.

Discussion

In summary, the above results replicated those of Experiment 3.1 and indicated that a second injection of E_2 is not necessary to produce facilitatory effects on the onset of maternal behavior in 15HO rats. Note that the results from Experiments 3.1 and 3.2 indicate that the major facilitatory effect of E_2 treatment occurred by the morning of day 1, which means that prior to the start of maternal behavior testing on day 1, when the experimenter removed the pups from the previous day, almost all females treated with E_2 were already showing maternal behavior. However because maternal behavior observations are not taken between 5 PM and 9 AM, the exact time of maternal behavior onset is not clear. That is, the facilitatory effect of E_2 treatment occurred sometime between between 6-24 hours after injection, but whether it occurred at 6 hours, 12 hours, or 24 hours is not clear.

Experiment 3.3

Method

Experiment 3.3 consisted of two independent groups of females administered sc either 100 µg/kg E_2 (E_2 100 day 15; n = 12) or vehicle (vehicle; n = 13) at the time of HO surgery, 48 hours before day 0 of behavioral testing.

Results

Experiment 3.3 examined whether water-soluble E_2 is capable of facilitating maternal behavior when injected at the time of HO, on day 15 of pregnancy. The results indicated that 100 µg/kg of E_2, a dose that initiated

maternal responsiveness in 15HO females when administered at the time of pup presentation, was not capable of facilitating maternal behavior when injected at the time of HO. The median sensitization latency measured by the test hour for E_2-treated females (E_2 100 day 15, n = 12) was 3 days, and this latency was 2.5 days when measured at the end of the test day, both of which were similar to the 2 day median sensitization latencies shown by vehicle-treated females (vehicle, n = 13) in the hour and in the day (Table 3.3.1). Figure 3.3.1 shows that the cumulative percentage of females showing full maternal behavior on each test day was similar for E_2- and vehicle-treated females.

Table 3.3.1. Outcome Measures for 15HO rats that Received Subcutaneous Injection of 17β-estradiol at the time of pup presentation

		Median onset to full maternal behavior (days)	
Group	*N*	Hour	Day
E_2 100 day 15	12	3 (1-6)	2.5 (0-6)
Vehicle	13	2 (0.5-5.5)	2 (0.5-5)

Interquartile ranges are shown in parentheses. Females were injected with either 100 µg/kg of 17β-estradiol (E_2 100) or vehicle on day 15 of pregnancy at the time of surgery. Abbreviations: 15HO = hysterectomized and ovariectomized on day 15 of pregnancy.

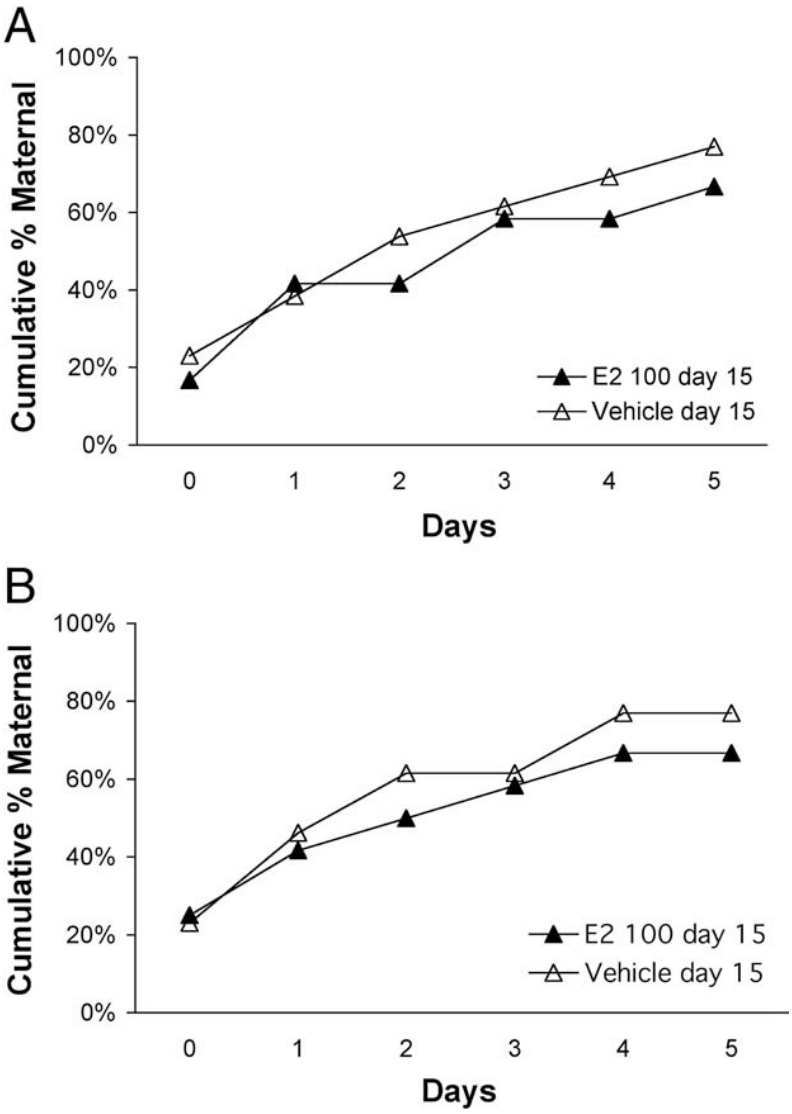

Figure 3.3.1. A. Cumulative percentage of female rats showing full maternal behavior by the end of the hour observation on each test day. B. Cumulative percentage of female rats showing full maternal behavior by the end of the day on each test day. Females received subcutaneous injections of either 100 μg/kg of 17β-estradiol (E_2 100 day 15) or vehicle on day 15 of pregnancy just after hysterectomy-ovariectomy surgery, 48 hours before pup presentation.

Discussion

The fact that 100 μg/kg of water-soluble E_2 was not able to stimulate maternal behavior when injected on day 15 of pregnancy, at the time of HO, indicates that E_2 needs to act closer to the time of pup presentation in 15HO females to produce a facilitatory effect on maternal behavior. The major implication of this finding is that the facilitatory effects observed after EB in oil administration systemically on day 15, or directly into the MPOA, are probably not due to a genomic effect which occurred over the 48 hour period of time between EB administration and pup presentation. Instead, the current data indicate that some of the stimulatory effects of estradiol on maternal behavior, whether genomic or nongenomic, occur closer to the time of pup presentation than previously determined. Because the water-soluble E_2 preparation is quickly metabolized, any effects initiated by the injection of E_2 in saline/cyclodextrin on day 15, post HO, are no longer capable of stimulating maternal behavior 48 hours later, at the time of pup presentation.

Experiment 3.4.

Method

Experiment 3.4 consisted of two independent groups of HO virgin female rats administered sc 100 μg/kg E_2 (E_2 100 virgin; n = 10) or vehicle (vehicle; n = 10) on day 0 of behavioral testing, one hour prior to pup presentation. Note that the the methods for Experiment 3.4 are identical to that of 3.1-3.2, except that virgin females were not mated, and these females were transferred to test cages

24 hours prior to HO. Pups were presented to these females, as previously described, 48 hours post-HO.

Results

Experiment 3.4 examined whether a single subcutaneous injection of E_2 (100 μg/kg) administered one hour prior to pup presentation on day 0 of behavioral testing would facilitate the onset of maternal behavior in HO virgin female rats. HO virgin female rats treated with E_2 (E_2 100-virgin, n = 10) showed similar sensitization latencies as did vehicle-treated virgin females (vehicle, n = 10); the median sensitization latency for E_2 100-virgin females was 6 days, which was not significantly different from the 6 day median sensitization latency displayed by vehicle injected rats (Table 3.4.1). Figure 3.4.1 shows that virgin female rats did not show any facilitation of maternal behavior onset in response to E_2 treatment.

Table 3.4.1. Outcome Measures for 15HO rats that Received Subcutaneous Injection of 17β-estradiol at the time of pup presentation

		Median onset to full maternal behavior (days)	
Group	*N*	Hour	Day
E_2 100-virgin	10	6 (4-6)	6 (4-6)
Vehicle	10	6 (6-6)	6 (6-6)

Interquartile ranges are shown in parentheses. Virgin females were hysterectomized and ovariectomized and injected with either 100 µg/kg of 17β-estradiol (E_2 100) or vehicle on day 0 of behavioral testing. Abbreviations: 15HO = hysterectomized and ovariectomized on day 15 of pregnancy.

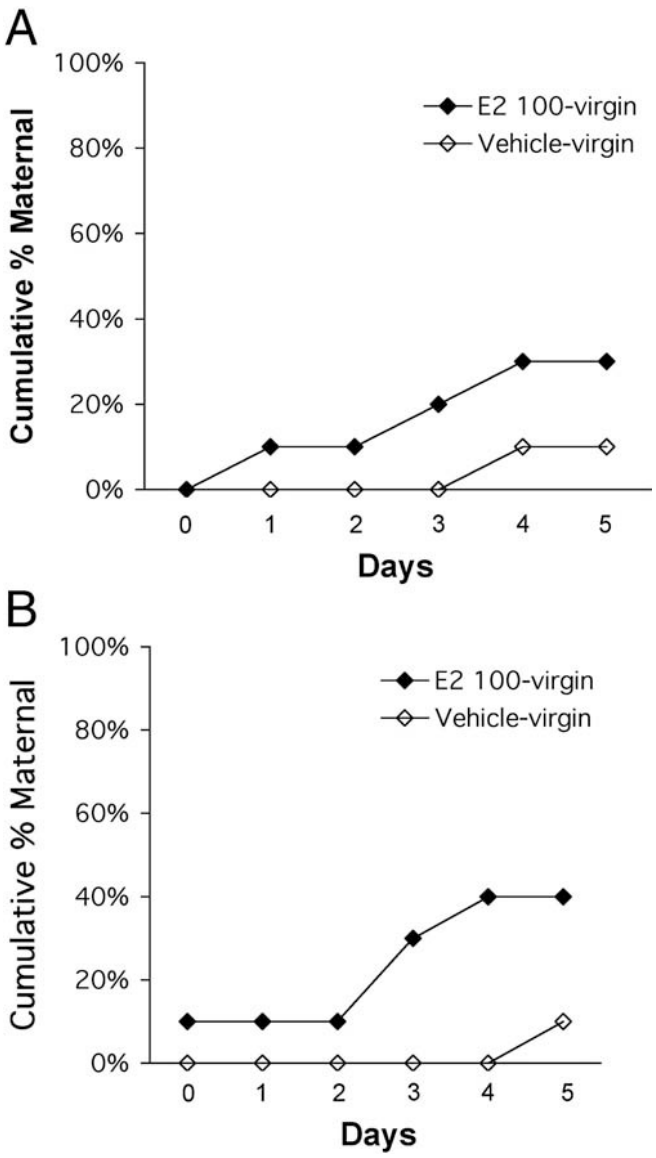

Figure 3.4.1. A. Cumulative percentage of virgin female rats showing full maternal behavior by the end of the hour observation on each test day. B. Cumulative percentage of female rats showing full maternal behavior by the end of the day on each test day. Females received subcutaneous injections of either 100 μg/kg of 17β-estradiol (E_2 100-virgin) or vehicle on day 0 of behavioral testing.

Discussion

Experiment 3.4 indicates that a 100 μg/kg E_2 injection does not produce a facilitatory effect in HO virgin female rats, supporting the view that the facilitation of maternal behavior by an acute injection of E_2 is dependent upon the priming effects of prior exposure to the endocrine events of 15 days of pregnancy plus the endocrine events produced by pregnancy termination. These priming factors are likely to include lactogenic hormones, P withdrawal, and minimal E stimulation (Numan and Insel, 2003). The data of this experiment clearly indicate that the females in both groups behaved like typical naive virgin female rats, with sensitization latencies greater than 5 days. Indeed, most females were not maternal over the 5-day test period.

General Discussion

The major finding of this study is that E_2 injection at the time of pup presentation induces a rapid onset of maternal behavior. Note that while females rapidly sensitize, almost all E_2-treated rats showed a sensitization latency of day 1, indicating that the facilitatory effect of E_2 occurs between 6-24 hours after injection. This finding should be contrasted with the fact that when EB in oil is injected sc on day 15 post HO, most females show maternal behavior on day 0 of testing, 48 hours later (Siegel and Rosenblatt, 1975a, 1975b; Rosenblatt et al., 1998). Therefore, the following facts need to be integrated: (1) EB in oil injected on day 15 stimulates maternal behavior when pups are presented 48 hours later,

with females showing a median sensitization latency of 0 days, (2) E_2 in saline/cyclodextrin injected on day 15 does not stimulate maternal behavior when pups are presented 48 hours later, and (3) E_2 in saline/cyclodextrin injected at the time of pup presentation results in a median sensitization latency of about 1 day. One interpretation, based on the putative degradation pattern of EB in oil compared with E_2 in saline/cyclodextrin, is that E_2 must act several hours (6-24 hours) before pup stimuli can elicit maternal responsiveness, and such an interpretation would be consistent with the idea that E_2 stimulates maternal behavior by exerting classical or non-classical genomic effects. Importantly, the current data indicate that the stimulatory effects of estradiol on maternal behavior occur closer to the time of pup presentation than previously determined, because any genomic effects initiated by the injection of E_2 in saline/cyclodextrin on day 15, post HO, are no longer capable of stimulating maternal behavior 48 hours later, at the time of pup presentation. Therefore, EB in oil probably produced its facilitatory effect on maternal behavior by acting in the last 6-24 hours (rather than 48 hours) prior to pup presentation, and also possibly at the time of pup presentation. Therefore, an alternate interpretation of the finding that both systemic and intra-MPOA EB stimulation produced an immediate onset of maternal behavior after 48 hours of EB stimulation (Numan et al., 1977; Siegel and Rosenblatt 1975a, 1975b) is that EB exerted its effects 6-24 hours prior to pup presentation, and that both classical and non-classical mechanisms of E_2 action might contribute to the onset of maternal behavior. Finally, it should be

emphasized that since the injection of E_2 facilitated the onset of maternal behavior when administered at the time of pup presentation were systemic injections, the actual neural site at which E_2 acted in this type of preparation needs to be explored in future work.

VI. Concluding Discussion

The focus of this paper has been on understanding the neural mechanisms which govern the natural change in maternal responsiveness female rats show at birth, which we have defined as maternal motivation. The hormonal events of pregnancy and parturition act on key neural sites which regulate maternal responsiveness, altering cellular activity to allow infant stimuli access to the neural circuitry which regulates maternal behavior. Importantly, while I have emphasized the role of neurotransmitter/neuromodulators in maternal motivation, particularly the role of mesolimbic DA system, the rise in E at parturition also plays an integral role in maternal motivation. Therefore, I have also investigated the hormonal mediation of the onset of maternal behavior. One objective of this thesis has been to understand the interaction between hormone action and the mesolimbic DA system with respect to maternal motivation. Importantly, knowledge about how hormones interact with neurotransmitter systems in the brain so that behavioral responsiveness remains intact long after the hormonal events have waned may have broader implications for understanding how, in certain species, the brain has evolved to become less

dependent on hormonal stimulation even when initially processing and responding to infant stimuli.

This dissertation was comprised of 3 major aims. First, the hypothesis that increased DA activity in the NA can facilitate the onset maternal behavior in naive rats was evaluated. Importantly, while several researchers have made this claim, prior to study 1 there was no direct empirical support for it. Because the hypothalamic DA system may also be involved in the regulation of established maternal behavior (Miller and Lonstein, 2005; Numan et al., 2005a), the role of DA input to the MPOA on the onset of maternal behavior was also evaluated. The results of the study 1 provided the first evidence that increased DA action at D1 receptors in either the MPOA or the NA promotes the onset of maternal behavior in sub-optimally hormone-primed rats. Second, because the cellular mechanism by which DA release into NA and its subsequent action at D1 receptors functions to increase behavioral reactivity is often debated, the intracellular signaling cascade which mediates the facilitatory effect of DA D1 receptor activation on increased maternal responsiveness was evaluated. The results of study 2 provide important implications with respect to the mechanism by which DA D1 receptor activation might modulate increased behavioral reactivity. Third, the combined results of study 1 and study 2 have indicated that in the absence of E, DA stimulation of the NA or the MPOA is capable of inducing the onset of maternal behavior in 15HO rats. These findings have been interpreted to mean that DA D1 receptor activation is capable of substituting for

E. Therefore, study 3 was undertaken to gain more information about the nature of the relationship between E and DA. For example, several possibilities exist: (1) Does DA substitute for E because increased DA D1 activation is a downstream effect of E stimulation? (2) Does DA D1 activation substitute for E because they are both capable of initiating maternal behavior via the same intracellular signaling cascades, and are therefore interchangeable? (3) Does DA D1 activation substitute for E because activation of intracellular kinases culminates in the phosphorylation of ER, and therefore, DA can activate ER similar to E itself (Gangolli et al.,1997; Olesen et al., 2005; Olesen et al., 2007; Olesen et al., 2008). The results of study 3, therefore, are an important complement to studies 1-2, and have important implications with respect to which of these possibilities is most conceivable.

Taken together, the major findings of these 3 studies indicate that DA D1 stimulation of NA or MPOA produces similar facilitatory effects as systemic E_2 administration on the onset of maternal behavior in 15HO rats. Because all three manipulations were capable of promoting the onset of maternal behavior within 24 hours of injection, the hypothesis that DA D1 receptor stimulation substitutes for E because each compound uses a similar mechanism of action (possibility #2) is plausible. Additionally, because DA D1 agonist application to MPOA or NA produce a more rapid (albeit slight) effect on the onset of maternal behavior in 15HO rats when compared to E_2 injection at the time of pup presentation, the possibility that DA D1 receptor activation is upstream from ER stimulation

(possibility #3) is unlikely; however, the possibility that DA D1 receptor activation is downstream from ER activation is still feasible (possibility #1).

Before I address the important implications of the studies contained herein, a brief review of our hypothetical model of the neural circuitry regulating maternal responsiveness will add the necessary context to this discussion (note that evidence for this model is referenced in the Introduction and a schematic is depicted in Figure I). We have proposed that the MPOA regulates increased maternal responsiveness through its efferent projections to the VTA. These projections allow MPOA output to activate the release of DA into the NA. The NA-VP circuit regulates increased behavioral responsiveness toward a wide range of biologically salient stimuli, and which stimuli are responded to in particular depends on which stimuli elicit DA release into this system at a given time. For example, in the absence of DA release, pup inputs to the NA-VP circuit are unlikely to elicit maternal responsiveness because NA projections to the VP are inhibitory. When the MPOA has been appropriately primed by hormones, pup inputs act on the MPOA, increasing output of MPOA neurons to the VTA and activating DA release into NA. The release of DA into NA is proposed to depress the activity of NA output neurons, thereby releasing the VP from inhibition and allowing it to respond to pup inputs with increased maternal responsiveness.

In the context of this model, we have argued that in naturally parturient female rats, the hormonal events of pregnancy and parturition act on the MPOA to increase output of MPOA neurons which project to the VTA in the presence of

pup inputs (see Figure VI**)**. The resultant increased release of DA into NA mediates the immediate onset of maternal behavior that parturient females naturally show at birth. In the 15HO sub-optimally hormone-primed rat, MPOA neurons do not strongly activate the VTA in response to pup inputs, and therefore the resultant DA release into NA is not enough to activate maternal responsiveness. The results of study 1 and study 2 suggest that SKF 38393 and SKF 83822 microinjection into NA circumvents the need for hormonal mediation by artificially increasing DA action at DA D1-AC receptors. The results of study 1 suggest that SKF 38393 microinjection (which activates both DA D1-AC and DA D1-PLC receptors) into MPOA circumvents the need for hormonal mediation by increasing the output of MPOA neurons which project to the VTA and therefore the resultant DA release into NA is sufficient to induce maternal responsiveness. The results of study 3 indicate that the mechanism by which E_2 acts on this neural system to facilitate maternal behavior might include a nonclassical component, and therefore it is possible that in addition to indirectly facilitating DA release in NA (through an action on MPOA), E_2 might also act at the level of the NA to potentiate DA action directly. Furthermore, at the level of the MPOA, E_2 and DA D1 action may stimulate maternal behavior onset by affecting similar intracellular signaling cascades.

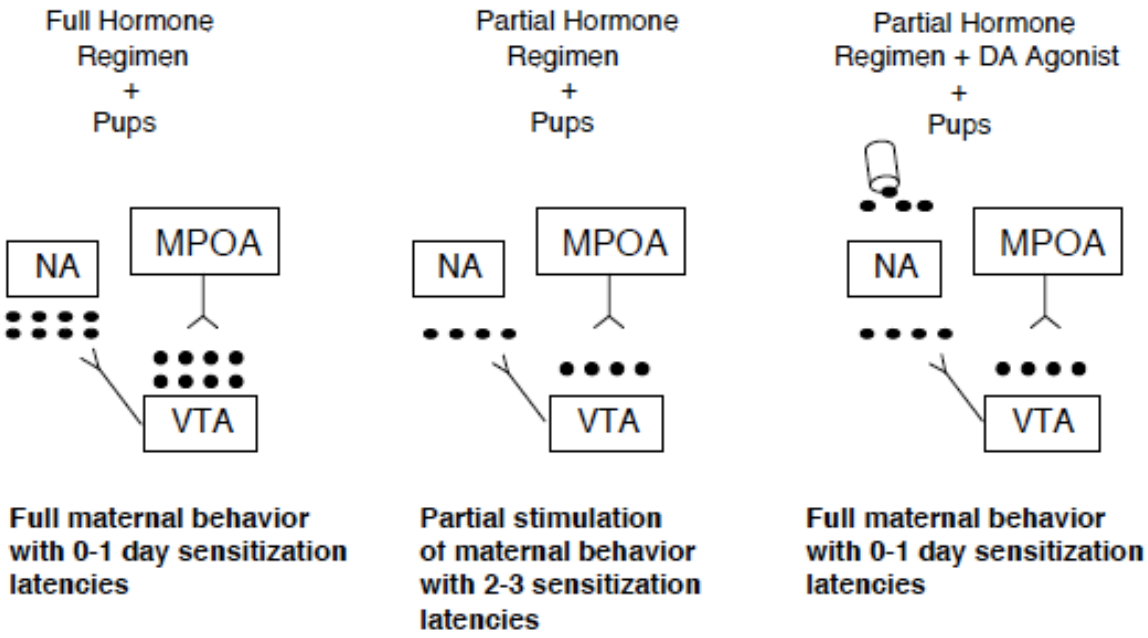

Figure VI. Hypothetical model adapted from Numan and Stolzenberg, (2009). Naturally parturient females immediately respond to infant stimuli because the hormone-primed medial preoptic area (MPOA) responds to pups with increased activation of the ventral tegmental area (VTA) and the subsequent release of dopamine (DA) into the nucleus accumbens (NA) promotes the onset of maternal behavior. When a naive female rat is partly hormone primed (such as the 15HO female) the MPOA weakly activates the VTA and therefore a sub-threshold amount of DA is released into NA, and maternal behavior onset is delayed 2-3 days. However, the effects of hormone action on the MPOA can be circumvented via artificial increase of DA activity at D1 receptors (either by injection of SKF 38393 or SKF 83822) in NA of partly-primed females and this manipulation increases DA activity to threshold amounts and initiates a rapid onset of maternal behavior. Note that microinjection of SKF 38393 directly into the MPOA also circumvents the need for hormone stimulation, presumably by increasing MPOA activation of the VTA and thereby indirectly increasing DA release into the NA (this is not shown here).

The results of this thesis suggest that DA D1 stimulation of either the medial NA or the MPOA is capable of substituting for E in the stimulation of maternal behavior onset in 15HO females either because DA D1 activity is downstream from E_2 effects or because DA D1 activity and E_2 stimulation operate via similar underlying mechanisms. At the present time, we do not know which of these potential mechanisms, which are not mutually exclusive, are actually operative. In the discussion which follows, I will present examples which lend support to each type of mechanism of action at both the level of the NA and the MPOA.

Because DA D1 receptor stimulation of the MPOA or direct EB stimulation of the MPOA have both been found to facilitate a rapid onset of maternal behavior, is there any evidence that a downstream effect of E stimulation in MPOA is related to a potentiation of DA activity? Elaine Hull and colleagues have uncovered a mechanism by which the classical genomic actions of E in MPOA potentiate the release of DA in the MPOA which is critical for the display of male sexual behavior in rats (Hull and Dominguez, 2006). First, recall that the neural circuitry regulating male sexual behavior in rats is very similar to the neural circuitry regulating maternal behavior (Numan, 1974, 1985). Importantly, E and DA have both been found to act in the MPOA to stimulate male sexual behavior (reviewed in Hull and Domiguez, 2006), and I have reviewed the evidence that similar mechanisms operate for maternal behavior. While some sexual dimorphism exists within the MPOA, it is possible that some of the mechanisms

which regulate maternal behavior and male sexual behavior are similar. Therefore, the Hull and Dominguez (2006) work on male sexual behavior may apply to maternal behavior. They report that NO is one link between E and DA action in the MPOA which regulates male sexual behavior. The classic genomic mechanism by which the E-ER complex influences DA activity is through increased transcription of nitric oxide synthase (NOS), which is necessary for the production of NO (Putnam et al., 2005). Once produced, NO potentiates DA activity (Dominguez et al., 2004; West et al., 2002). Therefore, the effects of DA on male sexual behavior in the MPOA are downstream from the effects of E_2. While this idea has been best developed in the male sexual behavior literature (Hull and Dominguez, 2006), note that disruption of NO production in the MPOA has been found to disrupt maternal behavior (Service and Woodside, 2007), and interestingly, SKF 38393 injection into MPOA can reverse these deleterious effects on maternal behavior (Service and Woodside, 2006).

Whereas a role of E potentiation of DA effects has been worked out at the level of the MPOA, there is less evidence for this idea with respect to the NA. Estrogen has well known effects on striatal DA systems, however, most research has examined these effects after systemic administration of E, and therefore it is not clear whether E exerted its effects on NA directly (Becker, 1999; Lammers et al., 1999; Shieh and Yang, 2008). Note that a few studies have show that direct application of E to NA can potentiate DA activity (Lee and Mouradian, 1999; Thompson, 1999; Thompson and Certain, 2005; Thompson and Moss, 1994),

and that direct application of E to NA can produce behavioral effects similar to those reported after DA agonist administration to NA (Roy et al., 1990; Schultz et al., 2009; Walf et al., 2007; Xiao and Becker, 1997). Additionally, while the presence of ERs in the NA are not well-defined (Shughrue et al., 1997), several studies have reported effects of E at ER receptors in NA (Phillips-Farfan et al., 2007; Walf et al., 2007). Note that while multiple mechanisms by which E can potentiate DA in NA have been worked out, no one has investigated whether E exerts its effects at the level of the NA via the activation of increased AC-cAMP intracellular signaling cascades.

With respect to the proposal that E and DA operate through similar underlying mechanisms, the fact that E can produce effects on both gene transcription as well as modify existing protein activity via the induction of intracellular cell signaling cascades, suggests that it can produce cellular effects similar to DA D1 stimulation. In support of this idea, membrane-initiated effects of E can occur at ERα, ERβ, (Abraham et al., 2004; Razandi et al., 1999; Razandi et al., 2004) and at a novel G protein coupled receptor 30 (GPR30) which binds E (Filardo et al., 2007). The MPOA contains both isoforms of the ER (α and β; Shughrue et al., 1997), and GPR30 has also been localized to the MPOA (Canonaco et al., 2008). At the present time it is not clear whether NA expresses GPR30, however it has been recently been reported that GPR30 is expressed in the dorsal striatum (Hammond et al., 2009).

The results of study 2 indicate that the facilitatory effects of DA release in NA on the onset of maternal behavior in 15HO rats are mediated by DA D1 receptors which are linked to the stimulatory G protein, Gs, which activates the enzyme AC and initiates cAMP intracellular signaling cascades. This finding is important for 2 reasons. (1) If systemic injection of E_2 promotes the onset of maternal behavior in 15HO rats by acting, in part, at the level of the NA, it might produce facilitatory effects via membrane-initiated cAMP intracellular signaling cascades. For example, there is evidence that E_2 stimulation, like SKF 83822, is capable of increasing cAMP intracellular signaling at both ERα (Abraham et al., 2004; Aronica et al., 1994; Lagrange et al., 1997; Watters and Dorsa, 1998) as well as GPR30 (Filardo et al., 2007). (2) These findings also conform with our hypothesis that DA functions to inhibit NA projection neurons. Electrophysiological data, both in vivo and in vitro, suggest that the inhibitory effect of DA stimulation on NA medium spiny neurons is mediated by DA D1 receptors (Calabresi et al., 1987; Charara and Grace, 2003; Chergui and Lacey, 1999; Harvey and Lacey, 1996, 1997; Maeda et al., 2004; Nicola and Malenka, 1997). Although it is not clear, at present, whether this D1 inhibition effect on medium spiny neurons is liked to the cAMP cascade, note that downstream targets of cAMP signaling, such as cyclic AMP response element binding protein (CREB), have been shown to critically regulate the onset of sexual behavior in naive or socially isolated male rats (Barrot et al., 2005). This finding suggests a role for CREB activity in NA in the onset of reproductive behavior.

Importantly, E can initiate PLC intracellular signaling cascades as well as cAMP intracellular signaling cascades (Kelly and Levin, 2001; Kow and Pfaff, 2004; Razandi et al., 1999; Qui et al., 2003). While the specific DA D1 receptor subtype which mediates the facilitatory effect of DA D1 stimulation in MPOA on maternal behavior is not known, it might be interesting to explore the possibility that distinct intracellular signaling cascades mediate the facilitatory effects of DA D1 receptor stimulation at the level of the MPOA and the NA. Importantly, because our model predicts the cellular effects of DA D1 action at the level of MPOA function to potentiate MPOA projections to the VTA, but that DA D1 action at the level of the NA functions to depress NA projection neurons to the VP, it is possible that distinct intracellular mechanisms occur in these two regions (however, note that this proposal is also based on the assumption that DA D1 receptors which effect maternal behavior are located on output neurons in both regions). In support of this idea, recall that E participates in NO formation. One mechanism by which E has been found to *rapidly* effect NO formation is via ER-initiated increases in intracellular Ca2+ released from intracellular Ca2+ stores (Stefano et al., 1999), an effect which can be mediated by PLC intracellular signaling cascades. E has also been found to activate membrane ER which interact with the Gq protein- coupled metabotropic glutamate receptor 1 (mGLUR1) in MPOA (Mermelstein, 2009), and the subsequent activation of PKC has been found to act on MPOA neurons to facilitate female sexual receptivity (Micevych, et al., 2009). Therefore a critical question which should be addressed

by future studies is whether or not the facilitatory effects of DA D1 receptor stimulation of MPOA on the onset of maternal behavior in 15HO rats are mediated by DA D1-PLC linked receptors.

In conclusion, the data presented here indicate that hypothalamic DA systems as well as mesolimbic DA systems are involved in the regulation of the onset of maternal motivation, and suggest the possibility that E regulates the increased maternal responsiveness of dams which occurs naturally at birth through a direct potentiation of one or both of these systems, or through action on similar intracellular signaling cascades at one or both of these regions.

VII. References

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