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Brain-derived neurotrophic factor and maternal behavior : neuronal alterations in the medial preoptic area and suppression of pup attacks

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Abstract

Brain-Derived Neurotrophic Factor and Maternal Behavior:

Neuronal Alterations in the Medial Preoptic Area and Suppression of Pup Attacks

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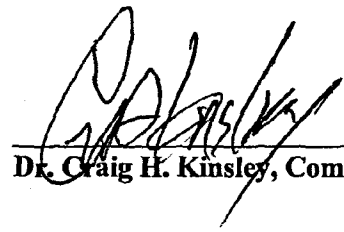
Brain-derived neurotrophic factor (BDNF), by virtue of its relationship to various neurotransmitter systems, hormones, and to estrogen in particular, may play a role in maternal behavior. To explore this possible role, female virgin Sprague Dawley rats received continuous intracerebroventricular infusions of BDNF sense oligonucleotide and were exposed to pups for maternal behavior testing. Behaviorally, BDNF sense had no effect on maternal behavior but did significantly suppress pup attacks during the first 24 hours of exposure. BDNF had a significant effect on neuronal morphology in the medial preoptic area (mPOA) as well, such that neurons in this region exposed to BDNF had more dendritic protrusions per 10 μm of dendritic branch than did control neurons. However, the length of the two longest dendritic spines on each branch measured was not affected. These findings shed light on the differential effects of BDNF in both priming the mPOA for behavior to occur, and in suppressing a specific aspect of maternal behavior.

I certify that I have read this Thesis and find that, in scope and quality, it satisfies the requirements for the degree of Master of Arts.



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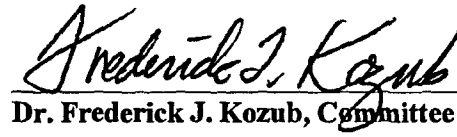

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BRAIN-DERIVED NEUROTROPHIC FACTOR AND MATERNAL BEHAVIOR: NEURONAL ALTERATIONS IN THE MEDIAL PREOPTIC AREA AND SUPRESSION OF PUP ATTACKS

By

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Brain-Derived Neurotrophic Factor and Maternal Behavior: Neuronal Alterations in the Medial Preoptic Area and Suppression of Pup Attacks

A female experiences a myriad of changes, both physical and behavioral, as she progresses through pregnancy, parturition, lactation and caring for her offspring. The exact extent of this transformation's effects, and the mechanisms through which it occurs, continue to intrigue researchers today. The regulation of maternal behavior is one such aspect that is still being explored. In particular, how is maternal behavior correlated to the physical changes in the brain? Can brain chemicals, other than hormones, affect this behavior?

Researchers, in pursuit of these and many other questions, are beginning to shed light on just how reproductive experience affects a female. In addition to hormonal changes, research has indicated that rats with reproductive experience (RE) also experience behavioral changes related to learning and memory (Kinsley, Madonia, Gifford, Yureski, Griffin, Lowry, et al., 1999). These data show that rats with RE are capable of learning to run a radial-arm maze and dry land version of the Morris water maze more quickly and with fewer errors than their nulliparous (virgin) counterparts. These results have been extended to the right angle maze; another test of spatial ability (Kinsley et al., 1999).

The behavioral changes may be related to simultaneous neuronal changes in the rats. In late pregnant females there is increased dendritic spine proliferation in the CA1 area of the hippocampus (an area of the brain associated with learning and memory), which may allow for more connections to adjacent neurons (Trainer, Quadros, Stafisso-

Sandoz, & Kinsley, 1997; Kinsley, Trainer, Quadros, & Stafisso-Sandoz, 1998). Also, glial cells, those cells that support neurons and provide nourishment, show increased number and complexity in the hippocampus and in the medial preoptic area, an area of the brain associated with regulating maternal behavior (Gifford, Miller, Quadros, Lambert, & Kinsley, 1998). These glial changes may indicate a similar increased complexity in the neurons in these brain regions. There have also been observed enhancements of neurogenesis (new neuron growth) in the post partum period, following a significant reduction of neurogenesis during pregnancy as well as the dip of hormones inherent in parturition (Amory & Madonia, unpublished observations).

Evidence suggests that the aforementioned neuronal changes come about via the effects of estrogen. Estrogen is known to promote dendritic spine growth and synaptic plasticity. As McEwen and Woolley (1994) explain, an increase in number of spines is accompanied by an increase in the number of synapses, thus more connections to adjacent neurons. More specifically, dendritic arborization (the tree-like projection of dendrites) and synapse formation increase and decrease rapidly during the rat's 5-day estrous cycle (Woolley, Gould, Frankfurt, & McEwen, 1990; Woolley & McEwen, 1992; Woolley & McEwen, 1993; McEwen, Alves, Bulloch, & Weiland, 1997). This waxing and waning can be reproduced by giving estradiol to ovariectomized rats for several days (Woolley & McEwen, 1993; McEwen & Woolley, 1994). Estrogen replacement therapy has also been shown to increase the number of neurites (also referred to as dendrites), neurite length, total number of branches, and branch length (Brinton, Chen, Montoya, Hsieh, & Minaya, 2000).

Interestingly, estrogen deprivation has been reported as a significant factor affecting nonspatial learning in animals (Singh, Meyer, Millard, & Simpkins, 1994) and humans (Asthana, Baker, Craft, Stanczyk, Veith, Raskind, et al., 2001). Singh and colleagues (1994) reported an impairment of active avoidance learning after as little as 5 weeks of estrogen deprivation in female rats. Further, Asthana and colleagues (2001) worked with postmenopausal women with Alzheimer's disease and reported that short-term administration of estrogen improved verbal memory, visual memory and attention.

The previously described findings regarding how maternal experiences affect the brain and behavior, however, also bear remarkable similarity to the effects of brain-derived neurotrophic factor (BDNF)- a protein that is primarily responsible for promoting neuron survival, enhancing dendritic sprouting, providing neuronal protection against insult, and enhancing recovery of damaged neurons (Kesslak, So, Choi, Cotman, & Gomez-Pinilla, 1998). As previously mentioned, estrogen, too, increases dendritic sprouting. These neuronal changes lead to stronger, healthier neurons that are better able to connect with more neurons and thus increase intercellular communication. Better neuronal communication also enhances learning.

BDNF is thus closely linked to estrogen by its similar neuroprotective effects, and has been implicated in learning and memory (Kesslak, So, Choi, Cotman, & Gomez-Pinilla, 1998). It is therefore possible that the behavioral effects reported in rats with reproductive experience are in some way related to the levels of BDNF in the brain. Further, multiparous rats have been shown to exhibit less BDNF immunoreactivity in the

hippocampus- particularly in the CA3 area (Lambert, Love, Stevens, McNamara, Morgan, Hoffman, et al., 2003).

The knowledge that BDNF is implicated in neuronal and behavioral learning changes (which our laboratory has reported in rats with reproductive experience) points strongly toward the involvement of BDNF in the enhancement of learning and memory in rats with reproductive experience. This study will attempt to further explore the potential role of BDNF in stimulating maternal behavior, a behavior "learned" by virgin rats through exposure to pups. A brief review of background and related literature is provided.

The Transformation From Virgin to Motherhood and Beyond

A nulliparous female experiences constant hormonal fluctuations during the estrous cycle. Estrogen levels are low during estrus, begin to rise from metestrus through the morning of diestrus, reach peak concentrations by the afternoon of proestrus and fall rapidly reaching basal levels by early estrus (Freeman, 1994). Progesterone peaks twice during the cycle- the first peak occurs during the afternoon and evening of proestrus (when estrogen levels peak). A second larger peak begins midway through metestrus and lasts through early morning of diestrus and falls to basal levels shortly thereafter (Freeman, 1994). Please see Figure 1.

During pregnancy, the female experiences a sustained change in hormonal levels Numan (1994). Estradiol levels are relatively low until day 16 of pregnancy, after which they begin to rise and reach peak levels on day 22. Progesterone levels are relatively high through out pregnancy as well, reaching peak levels on days 14 and 15. They begin to

decline sharply beginning on day 19. During the first half of pregnancy, prolactin, a lactogenic hormone that stimulates mammary gland development, is secreted from the anterior pituitary in two daily surges, but in the second half of pregnancy prolactin levels are low. A final peak occurs in the last two days of pregnancy. Oxytocin, a hormone involved in causing uterine contractions, keeps relatively low levels throughout the pregnancy, but increases sharply as parturition nears. Please see Figure 2.

Russell, Douglas and Ingram (2001) summarize how the hormones of pregnancy affect the female physiologically in a number of ways. She experiences a suppression of fertility and metabolic changes that include a 40% increase in blood volume, and an increase in body weight, fluids and adipose tissue. The female's hypothalamo-pituitary-adrenal (HPA) axis is modified as well, which in turn affects metabolism, gene transcription, and lowers responses to stress and fear. Further, there is reduced excitation in the limbic brain regions that process stressors during pregnancy and lactation.

Relatedly, Wartella, Amory, Macbeth, Stevens, Lambert and Kinsley (2003, article in press) found that parous and gravid females not only displayed reduced stress reactivity in an open field maze, but also reduced c-fos activation in the CA3 of the hippocampus and in the basolateral amygdala. Further work by McNamara, Love, Lambert and Kinsley (2003) is extending this line of research by examining stress reactivity throughout the lifespan. Nulliparous, primiparous and multiparous females have so far been tested on an elevated plus maze at 6, 9 and 12 months of age. Results show that at six months there was no difference in anxiety, with all groups showing equal time on the open arm. However, by 9 months the primiparous were spending significantly

more time on the open arm (displaying less anxiety). The multiparous females spent less time on the open arm than the primiparous, and the nulliparous females spent the least amount of time on the open arm. Similar results have been seen at 12 months, with primiparous females displaying the least anxiety. However, the multiparous and nulliparous females were not significantly different from each other, but the trend was similar to that seen at 9 months. Future tests will be conducted as the rats get older. Both these studies reiterate the lower stress reactivity of females with reproductive experience.

The hormonal patterns of pregnancy not only affect the female physiologically, but behaviorally as well. Perhaps the most characteristic change in behavior that the hormones of pregnancy (prolactin and oxytocin in particular) exert on the postpartum female rat is that of the immediate onset of maternal behavior (Keyser-Marcus, Stafisso-Sandoz, Gerecke, Jasnow, Nightingale, Lambert, et al., 2001). Mothers engage in pup-directed behaviors such as constructing elaborate nests, retrieving, grouping, crouching and licking their pups. They also sleep and eat in different patterns than before parturition and forage more efficiently (Keyser-Marcus et al., 2001).

These behaviors during lactation are maintained by a combination of hormones and pup stimulation. Suckling stimulation causes the release of the hormones necessary for lactation and milk ejection; these include prolactin, growth hormone, and adrenocorticotropic hormone from the anterior pituitary and oxytocin from the neurohypophysis (Numan, 1994). Sucking stimulation also inhibits the release of gonadotropic hormones, which results in the suspension of ovulation. Further, as the pups

grow older, they elicit less maternal responsiveness. If younger pups continuously replace older pups, maternal responsiveness is prolonged (Numan, 1994).

However, the hormones of pregnancy also have long lasting effects that extend beyond maternal behavior. As was already mentioned, rats with RE have increased spatial abilities (Kinsley et al., 1999). Gatewood (2002) extended these findings by testing nulliparous, primiparous and multiparous females in a dry land maze longitudinally at 6, 12, 18 and 24 months of age. All the parous animals had had their reproductive experience before testing began. Gatewood found that at all ages, the multiparous animals performed the best, followed by the primiparous. Nulliparous females performed the worst. Her findings show a spatial learning improvement for parous animals that lasts through senescence.

Brain-Derived Neurotrophic Factor

Neurotrophins are proteins that regulate the survival (protection from metabolic insult, brain injury, apoptosis, etc.), differentiation, and plasticity (ability to change in both shape and physiology) of neurons (Berninger & Poo, 1999) by causing rapid changes in neuron morphology and physiology (McAllister, Katz, & Lo, 1999). They can be thought of as intercellular messengers (mainly neuron to neuron communication), which are used by neurons to regulate the gene expression of their innervating neurons (Smith, 1996). They also modulate and mediate chemical transmission at certain synapses by causing membrane depolarization and can modulate the secretion of neurotransmitter-filled vesicles (Berninger & Poo, 1999).

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family, and is one of the most widely expressed in the brain (Fumagalli, Santero, Gennarelli, Racagni, & Riva, 2001). As gathered from studies in rodents, BDNF is expressed in many brain areas, including the hippocampus, dentate gyrus, amygdala, projection areas of the olfactory system, inner and outer pyramidal layers of the neocortex (Phillips, Hains, Laramee, Rosenthal, & Winslow, 1990), claustrum, cerebellum, and the superior colliculus (Hofer, Paglusi, Hohn, Leibrock, & Barde, 1990; Wetmore, Ernfors, Persson, Olson, 1990).

Within the brain, as described by Murer, Boissiere, Yan, Hunot, Villares, Raucheux, et al. (1999), BDNF immunoreactivity was observed in the cytoplasm of neuronal cell bodies and proximal dendrites, axons and nerve terminals, in glial cells and processes, as well as in senile plaques of patients with Alzheimer's disease.

BDNF, as previously mentioned, is primarily responsible for promoting neuron survival, enhancing dendritic sprouting, providing neuronal protection against insult, and enhancing recovery of damaged neurons (Kesslak, So, Choi, Cotman, & Gomez-Pinilla, 1998). It is also involved in a myriad of other neuronal events. For example, BDNF plays a role in the formation and maintenance of long-term potentiation in the hippocampus (Korte, Carroll, Wolf, Brem, Thoenen, & Bonhoeffer, 1995; Johnson, 1999), promotes activity-dependent dendritic growth (McAllister, Katz, & Lo, 1996), provides protection from apoptotic cell death (Courtney, Åkerman, & Coffey, 1997), promotes survival of motor neurons (Jessell & Sanes, 2000), is involved in synaptic plasticity in the adult central nervous system (Hall, Thomas, & Everitt, 2000), mediates glial development

(Pruginin-Bluger, Shelton, & Kalcheim, 1997), and modulates myelination in the peripheral nervous system (Chan, Cosgaya, Wu, & Shooter, 2001).

Additionally, BDNF can enhance the release of various neurotransmitters (Thoenen, 1995), and in fact, BDNF can modulate the opening of a sodium channel on the same timescale as a change in voltage can (Barde, 2002). Further, BDNF is involved in various forms in many different neuronal systems. For example, BDNF has been linked to the GABAergic system. GABA is the most important inhibitory neurotransmitter in the brain. BDNF has been implicated in contributing to the differentiation of GABAergic neurons (Mizuno, Carnahan, & Nawa, 1994), and has been shown to enhance the survival, amount of GABA uptake, and total protein content of forebrain cholinergic neurons (Knüsel, Winslow, Rosenthal, Burton, Seid, Nikolics, et al. 1991). BDNF also regulates $GABA_A$ receptors (Tanaka, Saito, & Matsuki, 1997) by decreasing their number in the postsynaptic membrane (Brünig, Penschuck, Berninger, Benson, & Fritschy, 2001).

GABA is known to decrease BDNF mRNA levels, whereas reciprocally, glutamate has been shown to increase BDNF synthesis (Zafra, Castrén, Thoenen, & Lindholm, 1991; Lindholm, Castrén, Berzaghi, Blöchl, & Thoenen, 1994). BDNF has also been reported to elicit the same excitatory effects (depolarization) as glutamate, but at much lower concentrations (Kafitz, Rose, Thoenen, & Konnerth, 1999). Further, BDNF enhances glutamate release through the use of intracellular calcium stores (Matsumoto, Numakawa, Adachi, Yokomaku, Yagamishi, Takei, et al., 2001).

BDNF stimulates the differentiation of cholinergic neurons (Knüsel et al., 1991; Nonomura & Hatanaka, 1992), and protects serotonergic neurons from injury and promotes their axonal sprouting (Mamounas, Blue, Siuciak, & Altar, 1995). BDNF is also known to increase the survival of dopaminergic neurons (Hyman, Hofer, Barde, Juhasz, Yancopoulos, Squinto, et al., 1991), and protect dopamine-producing neurons from dopamine neurotoxins (Spina, Squinto, Miller, Lindsay, & Hyman, 1992). In its target neurons, BDNF elicits long-term neuronal adaptations by inducing the expression of D_3 dopamine receptors (Guillin, Diaz, Carroll, Griffon, Schwartz, & Sokoloff, 2001). It has also been suggested that stressful and challenging situations involving activation of the dopaminergic system, and higher than normal release of dopamine, results in a reduction of BDNF expression which leads to an increased cellular vulnerability to stress- as the neuroprotection provided by BDNF is lacking (Fumagalli, Santero, Gennarelli, Racagni, & Riva, 2001).

In addition to exerting an influence, BDNF itself is also influenced by a number of events. For example, events that are capable of inducing long-term potentiation (a long-term increase in the excitability of a neuron) in the hippocampus, such as physiological activity and high frequency stimulation, also increase the levels of BDNF messenger ribonucleic acid (mRNA: the half of the genetic code that contains the signal to produce BDNF; Patterson, Grover, Schwartzkroin, & Bothwell, 1992; Poo, 2001). High frequency neuronal activity and synaptic transmission can elevate the number of trkB receptors (the tyrosine kinase receptor that BDNF binds to with the highest affinity) in cultured hippocampal neurons (Du, Feng, Yang, & Lu, 2000), and may therefore

facilitate the synaptic action of BDNF (Poo, 2001). Contextual fear training (a process in which a negative event such as foot shock is paired to a novel environment) has increased hippocampal BDNF expression as well (Hall, Thomas, & Everitt, 2000), yet mild stressors such as immobilization stress reduce BDNF levels in the dentate gyrus in as little as 45 minutes (Smith, 1996).

Exercise (voluntary running in rats) can alone modify BDNF mRNA expression in the hippocampus and other brain areas (Neeper, Gómez-Pinilla, Choi, & Cotman, 1995) such that there is enhanced ability of sensory neurons to compensate for insults such as sciatic nerve crush injury (Twiss, Molteni, Zheng, Ying, Schanen, & Gómez-Pinilla, 2002). Molteni, Ying, and Gómez-Pinilla (2002) also reported that in the rat hippocampus, BDNF, along with a number of genes involved in synaptic trafficking, signal transduction pathways, and transcription regulation, was consistently up-regulated during both acute and chronic exercise. These researchers suggest that BDNF plays a central role in the effects of exercise on brain plasticity.

Whereas the effects BDNF has on the brain are well documented, the mechanisms by which BDNF works are much less understood. Studies have observed that it is transported anterogradely from neuron cell bodies to their terminals (Altar, Cai, Bliven, Juhasz, Conner, Acheson, et al., 1997), is released or secreted through presynaptic mechanisms in an activity dependent manner (Kohara, Kitamura, Morishima, & Tsumoto, 2001), is released on neuron depolarization (Thoenen, 1995), and triggers rapid intracellular signals (Altar & DiStefano, 1998) and action potentials in central neurons (Kafitz, Rose, Thoenen, & Konnerth, 1999).

BDNF has been reported to modulate synaptic vesicle distribution within presynaptic terminals in CA1 excitatory spine synapses of hippocampal slices by increasing the number of docked vesicles at the active zone (Tyler & Pozzo-Miller, 2001). Other studies report that BDNF is found in the releasing vesicles in nerve terminals (Luo, Rush, & Zhou, 1998, as cited in Mu, Li, Yao, & Zhou, 1999).

On a related venue, BDNF activates sodium channels via the TrkB receptor; this receptor might interact directly or indirectly with the channel protein to cause it to open (Berninger & Poo, 1999). Relatedly, calcium channel blockade prevents the upregulation of BDNF (Johnson, 1999), and BDNF mRNA is transiently induced within hours of a voltage sensitive calcium influx (Johnson, 1999).

The Relationship Between BDNF and Estrogen

BDNF and estrogen are quite intertwined regarding their effects and functions. Both estrogens and neurotrophins "have been shown to promote survival and differentiation of their neuronal targets" (Sohrabji, Miranda, & Toran-Allerand, 1995, p. 11110). The BDNF gene contains an estrogen response element (Sohrabji, Miranda, & Toran-Allerand, 1995), and BDNF levels are increased in vivo by estrogen (Singh, Meyer, & Simpkins, 1995). BDNF can modulate synaptic transmission, and enhance synaptogenesis (McAllister, Katz, & Lo, 1999), just as estrogen can (Woolley & McEwen, 1992). Further, BDNF is sensitive to regulation by physical activities and learning (Neeper, Gómez-Pinilla, Choi, & Cotman, 1995; Kesslak, So, Choi, Cotman, & Gomez-Pinilla, 1998), which are also influenced by estrogen levels (McEwen & Alves, 1999). Berchtold, Kesslak, Pike, Adlard, & Cotman (2001) suggest that some of the

beneficial effects of estrogen may be in fact due to increased availability of BDNF; BDNF may provide a pivotal process by which estrogen can exert its positive influence.

Relatedly, BDNF is significantly affected by physiological changes in the levels of gonadal hormones circulating in the body (Gibbs, 1998). Gibbs found that in the CA1, CA3 and CA4 regions of the hippocampus, peak levels of BDNF mRNA were detected on the morning of diestrus 2 when progesterone levels are relatively low. At this point estrogen levels are undetectable (Yoshinga, Hawkins, & Stocker, 1969). On the other hand, the lowest levels of BDNF mRNA were detected on the afternoon of proestrus when progesterone levels were highest (Gibbs, 1998), and levels of estrogen have peaked and begin to decrease (Yoshinga, Hawkins, & Stocker, 1969).

Also, in a study conducted by Sohrabji et al. (1995), it was found that estrogen rapidly up-regulates mRNA expression of BDNF in the cerebral cortex and olfactory bulb of rats. Based on this finding, these researchers suggest that estrogen may be in a position to increase the availability of BDNF by regulating its gene transcription in these areas.

Studies have reported that deprivation of endogenous estrogen results in reduced levels of BDNF mRNA expression in the hippocampus (Singh et al., 1995; Berchtold, Kesslak, Pike, Adlard, & Cotman, 2001). Similarly, estrogen replacement therapy increased BDNF mRNA levels in the hippocampus, cerebral cortex, olfactory bulbs, medial septum, and the nucleus basalis of Meynert after this estrogen deprivation (Singh, Meyer, & Simpkins, 1995; Sohrabji et al., 1995; Gibbs, 1998). BDNF also remains responsive to regulation by exogenous estrogen after 3 weeks of estrogen deprivation (Berchtold, Kesslak, Pike, Adlard, & Cotman, 2001).

In cultured hippocampal neurons, estradiol down-regulates BDNF, which decreases inhibition and increases excitatory tone in pyramidal neurons and increases spine density (Murphy, Cole, & Segal, 1998). These same researchers have found that exogenous BDNF blocks the effects of estradiol on spine formation and BDNF selective antisense oligonucleotide imitates the effect of estradiol.

Berchtold, Kesslak, Pike, Adlard, & Cotman (2001) report that combined exercise and estrogen replacement enhance BDNF gene regulation in the hippocampus above replacement alone.

BDNF's Involvement in Learning and Memory

BDNF is predominantly expressed in the hippocampus, where it is involved in learning and memory (Gibbs, 1998). It has been determined that BDNF expression is rapidly and selectively upregulated in the CA1 region of the hippocampus during contextual learning (Hall, Thomas, & Everitt, 2000). These authors further state that the regulation of BDNF activity is a correlate of hippocampal learning in vivo.

Mizuno, Yamada, Olariu, Nawa, & Nabeshima (2000) report that spatial memory formation, as tested using the radial arm maze, is associated with an increase in BDNF mRNA levels in the hippocampus. These researchers found that antisense BDNF oligonucleotide treatment (administering a short chain of nucleotides produced to bind to BDNF's particular RNA or DNA sequence and thus block its expression) significantly inhibited spatial reference and working memory formation and disrupted previously formed spatial memory.

Further, Ma, Wang, Wu, Wei, and Lee (1998) found an increase in hippocampal BDNF mRNA during the early phase of the memory consolidation process of inhibitory avoidance learning. BDNF antisense oligonucleotide injection to the dentate gyrus impaired retention performance as well, reduced the amplitude and slope of LTP, and also decreased BDNF mRNA in the dentate gyrus. Based on these findings, Ma et al. suggest that BDNF gene expression in the hippocampus is a necessary component for memory processing and LTP induction in rats.

Similarly, Mu, Li, Yao, & Zhou (1999) report that BDNF antibody treated rats displayed significantly impaired learning and spatial memory abilities, as tested using the Morris water maze (a learning task in which a rat is forced to swim to find a submerged platform). Upon this finding they suggest that BDNF may be preferentially involved in the intermediate steps of the learning process by virtue of its neurotrophic effects on cholinergic neurons.

In 1998 Kesslak, So, Choi, Cotman, and Gómez-Pinilla performed a study to investigate the roles that exercise and learning may play in BDNF induction. Using the Morris water maze they found that spatial learning enhances expression of BDNF mRNA in addition to the enhancement already caused by the physical activity associated with swimming in the maze. Additionally, these elevated levels are maintained with repeated exposure. Interestingly, however, intrahippocampal administration of BDNF does not alleviate spatial learning impairments in aged rats (Pelleymounter, Cullen, Baker, Gollub, & Wellman, 1996). Pelleymounter et al. did find though, that BDNF induced a partial,

long-term normalization of the elevated hypothalamic serotonin levels of these aged animals.

Recent research has established the effects of BDNF on human memory as well. Egan, Kojima, Callicott, Goldberg, Kolachana, Bertolino, et al. (2003) studied the effects of a valine to methionine substitution in the human BDNF protein. They report that the methionine allele was associated with poorer episodic memory and abnormal hippocampal activation, as assessed by neuropsychological testing.

An interesting study by Liu et al. (2000) provides a link between both maternal and learning behaviors and the levels of BDNF present in pups. Mother rats were divided into groups of high versus low maternal behavior based on their naturally occurring variations. The culled litters from mothers displaying high maternal behavior were then given to the mothers with low maternal behavior and vice versa, a process called cross-fostering. The cross-fostered pups from these two groups were studied in terms of various neuronal differences, including BDNF levels. Liu and colleagues found increased expression of BDNF mRNA in the dorsal hippocampus of the day 8 pups of high maternal behavior rats compared to the pups of low maternal behavior rats.

Liu and colleagues suggest that variations in maternal behavior cause varying sensory experiences for the pups. The amount of maternal licking and grooming each pup receives may be different, and the differences in sensory experience may in turn alter the pup's levels of hippocampal synaptic development. These researchers believe that maternal care increases N-methyl-D-aspartate receptor (NMDA; receptors to which the excitatory neurotransmitter glutamate binds to) levels, which in turn elevates BDNF

expression and increases hippocampal synaptogenesis, which leads to further enhanced spatial learning in adulthood.

However, Roceri, Hendriks, Racagni, Ellenbroek, and Riva (2002) found significantly reduced expression of BDNF in the hippocampus of adult rats resulting from a 24-hour maternal deprivation period on post-natal day 9. These researchers suggest that the 24 hour maternal deprivation "may interfere with hippocampal maturation and this may lead to a reduction of BDNF levels in adulthood as a consequence of changes in neurotransmitters or hormones involved in the control of the neurotrophin production" (p. 613).

As previously mentioned, BDNF is involved in the formation and maintenance of long-term potentiation in the hippocampus (Korte et al. 1995; Johnson, 1999). Studies have shown that suppression of BDNF expression results in deficiencies in hippocampal long-term potentiation (Korte et al. 1995; Mu, Li, Yao, & Zhou, 1999). Further, these deficiencies can be reversed by acute provision of exogenous BDNF (Patterson, Abel, Deuel, Martin, Rose, & Kandel, 1996; Muller, Djebbara-Hannas, Jourdain, Vutskits, Durbec, Rougon et al., 2000).

Manabe (2002) reports that binding of BDNF to TrkB receptors is vital for induction of LTP in the hippocampus. It has also been suggested that BDNF regulates long-term potentiation by enhancing synaptic responses to high frequency stimulation (Figurov, Pozzo-Miller, Olafsson, Wang, & Lu, 1996; Pozzo-Miller, Gottschalk, Zhang, McDermott, Du, Gopalakrishnan, et al., 1999). More specifically, it is believed that BDNF promotes long-term potentiation induction via presynaptic mechanisms (Figurov,

Pozzo-Miller, Olafsson, Wang, & Lu, 1996; Jovanovic, Czernik, Feinberg, & Greengard, 2000) by preventing the synaptic fatigue that is induced by the same high frequency stimuli that can induce long-term potentiation (Tyler & Pozzo-Miller, 2001).

Other studies, however, have shown that BDNF also has postsynaptic effects. Kovalchuk, Hanse, Kafitz and Konnerth (2002) found an exclusive postsynaptic site of Ca^{2+} signaling in response to BDNF pulses that plays a direct and instructive role in LTP induction.

The Purpose of This Study

BDNF is a powerful neurotrophic factor that has been implicated in numerous neurotransmitter systems, clinical disorders, and spatial abilities. By virtue of its relationship to estrogen in particular, it is possible that BDNF may play a role in maternal behavior as well.

In review, rats with reproductive experience learn faster and show an increase in neuronal morphological complexity. Both these learning and neuronal changes can come about through the effects of estrogen. However, BDNF has effects similar to estrogen on neuronal morphology and learning, and previously mentioned studies report that deprivation of endogenous BDNF can decrease learning.

The non-hormonal onset of maternal behavior is arguably a learned behavior in virgin rats- it takes about five days of sensitization to pups for these to be initiated (Bridges, Zarrow, Gandelman, & Denenberg, 1972). Fleming and Rosenblatt, in 1974, reported that after this sensitization period, virgin females will retrieve foster pups, engage in licking behaviors, adopt nursing positions, and even build maternal nests. The

researchers explain that the quality of these behaviors is different from postpartum rats in that inexperienced females do not hold the arched back nursing positions as long and lick the foster pups more than the postpartum females. This difference has been explained by the different levels of stimulation the rats receive; virgins do not receive the tactile stimulation of the pups nursing and therefore do not hold their nursing positions as long, and virgins are exposed to pups with distinct odors not her own which would increase licking.

Based on BDNF's involvement with spatial learning, it is possible that exogenous BDNF may speed the onset of learned maternal behavior. To examine if BDNF plays a role in regulating the onset of non-hormonal maternal behavior, this study will add exogenous BDNF in virgin females exposed to pups. Neuronal effects will also be examined in the medial preoptic area (mPOA) of the hypothalamus, which is known to regulate maternal behavior.

Method

Animals

Sixteen age-matched (4-5 months old) virgin female Sprague-Dawley rats purchased from Harlan were maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the University of Richmond. The animals were double housed until surgery, after which they were isolated in 20 x 45 x 25 cm polypropylene cages with 1/8th inch cob bedding covering the floors. Food and water were available *ad libitum* and all animals were housed in light- (on from 0500-1900 h) and temperature- (21-24°C) controlled testing rooms for the duration of the study. A

separate group of age-matched lactating donor rats were maintained to provide pups for testing.

Experimental Groups and Outline of Procedure

To explore the role of BDNF in regulating the onset of maternal behavior, we used two groups of virgin female rats. The experimental group consisted of eight females that received intracerebroventricular infusions of BDNF sense oligonucleotide to supplement the endogenous, or naturally occurring, BDNF. The control group consisted of eight females that received infusions of sterile, pyrogen-free physiological saline.

Studies have shown that exogenous BDNF, when administered into the lateral ventricles, has effects that reach circumventricular brain regions. Pencea and colleagues (2001) showed neurogenesis in the striatum, septum, thalamus and hypothalamus, following 16 days of BDNF infusion into the lateral ventricles of adult rats. Yan, Matheson, Sun, Radeke, Feinstein and Miller (1994) also suggest that TrkB receptors line the ependymal layer of the ventricles, thus controlling the absorption of BDNF administered intracerebroventricularly.

Further, previously mentioned studies have shown that the effects of infused BDNF sense and antisense on learning can be detected as early as 4 days (Mizuno, Yamada, Olariu, Nawa, & Nabeshima, 2000) and 7 days (Pelleymounter, Cullen, Baker, Gollub, & Wellman, 1996; Mu, Li, Yao, & Zhou, 1999) after surgical implantation of pumps. Based on the findings by Mizuno et al., in this study the testing of maternal behavior began on the fourth day after surgery.

Foster pups were taken from donor mothers and placed in the home cages of virgins to test maternal behavior. After a maximum of 10 days of testing, each virgin exposed to pups was sacrificed and her brain removed for analysis of neuronal morphology. Please see Appendix A for a flowchart outlining the general sequence of events.

Surgical Procedures and Intracerebroventricular Infusions

The delivery of experimental and control agents were via osmotic pumps. Osmotic pumps are miniature pumps that continuously deliver test agents at controlled rates into laboratory animals. The pumps are made of three concentric layers- an outer semi-permeable membrane, an osmotic salt sleeve, and a reservoir with an impermeable wall. When water crosses the outer semi-permeable membrane, it adds bulk to the salt sleeve and compresses the inner reservoir forcing out the test agent.

The evening before surgeries, pumps were assembled, filled and primed. The cannula was attached to an osmotic pump (Alzet model 2002, infusion rate of 0.5 ml/h for 14 days, Alza, Palo Alto, CA, USA), following Alzet's instructions, via 3 cm of flexible tubing. Although Alzet recommends 12 cm of tubing, we found that the extra slack exerted pressure on the cannula that lifted it from the skull. To eliminate this lifting and allow the cannula to lie flat on the skull, we shortened the length of the tubing. Pilot animals showed no restrictions in movement and appeared comfortable.

Animals that were infused with BDNF sense received a phosphothioate oligonucleotide synthesized by Oligos Etc. Inc. (Wilsonville, OR), and purified with high performance liquid chromatography. The BDNF sense oligonucleotide sequence was 5'-

ACCATTAAAAGGGGAAGA-3'. This sequence was used by Mizuno et al. (2000) and was shown by Acheson and colleagues (1995) to have neuron survival effects similar to that of exogenous BDNF. Further, a BLAST search was conducted on this sequence upon the manufacturer's suggestion to determine if it matched any other sequences. This search revealed that the only matches this sequence had were to BDNF genes of various species, with the exception of two human chromosome 11 clones. However, because this particular oligonucleotide was administered to rats, we believed that it would still have the effects Acheson et al. reported.

Upon arrival, the oligonucleotide was re-suspended in 3.2 ml sterile, pyrogen-free physiological saline to a concentration of 3.6nm/d. Experimental pumps were filled with the BDNF sense, and control pumps were filled with physiological saline. Filled pumps were primed in physiological saline at 37°C for up to 24 hours before surgical implantation.

Surgeries began at 11:00am the following morning. Standard stereotaxic procedures were followed and all surgical pump implantations were performed as aseptically as possible. Each rat was first anesthetized with a mixture of Ketamine and Xylazine (at a 50 mg/kg Ketamine and 4 mg/kg Xylazine dosage administered intraperitoneally) and placed in a stereotaxic head frame for the implantation of the osmotic pump and cannula. After shaving and washing the scalp, a midline sagittal incision about 2.5 cm long was made to expose the skull. The skull was scraped to create a dry and rough surface for the dental cement to take hold. A hole was drilled in the skull

over the right lateral ventricle, 1.5 mm lateral to the midline and 0.8 mm posterior to bregma.

At this point the previously assembled cannula and pump complex was inserted into the animal. The pump was implanted subcutaneoulsy in a previously prepared pocket in the subscapular area of the neck. By opening and closing the jaws of a hemostat, the subcutaneous tissue was spread to make a 4.0 cm long pocket for the pump. The cannula was inserted through the hole into the right lateral ventricle at a depth of 3.5 mm below the skull surface. A drop of superglue was used to secure the cannula to the skull while the dental cement was mixed, poured over the cannula and skull, and allowed to dry.

Each animal was sutured (or in the case of three animals, stapled) and the incision cleaned with Betadine and treated with the topical antibiotic Neosporin. Immediately following surgery, each animal was placed in a clean cage with a lamp over one corner and observed until she awoke from the anesthesia. The females were allowed four days to recover under post-operative observation, and remained singly housed for the duration of the study.

Assessment of Maternal Behavior

The onset of maternal behavior was recorded in all groups beginning at 12 noon on the fourth day following surgery, using methodological adaptations from Bridges, Feder and Rosenblatt (1977). The first step involved priming each rat to the smell of pups. To do this, a 50ml beaker-full of bedding from the donor mother's cage was poured in the virgin's home cage. After an hour, maternal assessment began, and this priming step was not repeated.

To begin observing maternal behaviors, the quality of the nest in the virgin's home cage was first rated from 1 to 4 (1 = poor, 2 = fair, 3 = good, 4 = excellent). After this rating, each virgin had 1 recently fed (as determined by the presence of milk bands) rat pup placed in her cage. Due to variability, some donors delivered their litters on the day testing was supposed to initiate, and so the ages of the pups varied from one to 24 hours. The responses from each rat were continuously recorded for 15 minutes, and then recorded at 30, 45, and 60 minutes after the start of testing. Pup retrieval, grouping and crouching were noted, and for the purposes of this study retrieving was assessed as any behavior during which the female collected one or more pups away from the nest. Grouping was any behavior during which the female placed one or more pups together in one spot. Crouching was any behavior during which the mother huddled over one or all of the pups.

After 60 minutes of observation, the rat pup remained in the virgin's home cage over night. The following day testing resumed and nest quality was rated and the position of the pups and virgin noted. If the virgin female had not cannibalized the one pup, this pup was returned to its mother. Each virgin female then had a 15-minute time period after the pup(s) were removed during which she had no pups in her cage. After this 15-minute period, new recently fed pups were placed in her cage, and the next series of observations continued as outlined above. Each virgin received three new pups after the first 24 hour period when the virgin did not cannibalize the one pup.

Criteria for reaching maternal behavior was met once each virgin showed all three behaviors in the first 60 minutes of testing for two consecutive days, after which she was

sacrificed. If a female failed to reach this criteria within 10 days of testing, the observations were terminated and the rat sacrificed.

Timeline of Surgeries and Testing

Due to the time consuming nature of serial surgeries and maternal observations, this experiment was divided into two rounds. For each round, 4 surgeries a day were performed for 2 consecutive days. On day one, the four females were randomly and blindly assigned to receive pumps such that one female got BDNF sense and three females got saline. On day two, the four females were randomly assigned such that three females got BDNF sense and one got saline. This way, there was an equal number of experimental and control animals per round of testing.

Since the behavior testing involved an initial 15 minutes of continuous observations, animals were tested serially. This required a complex schedule for priming the virgins to pup odors on the first day of testing, and then removing pups, replacing pups, observing continuously for the first 15 minutes, and spot-checking for 45 minutes afterwards. Please see Appendix B for this testing schedule.

Brain Tissue Preparation

After observing maternal behavior, all animals were sacrificed and their brains removed for analysis of neuronal morphology using the Golgi-Cox staining procedure. The protocol used in this study was an adaptation of the Golgi-Cox technique (Ramón-Moliner, 1970) modified for short-term staining (Keyser-Marcus et al., 2001).

Briefly, the brains were blocked in the coronal plane in three sections, beginning 1.0 mm anterior to the optic chiasm, using a brain matrix, immediately upon removal.

The sections were placed in standard Golgi-Cox stain (potassium dichromate and mercuric chloride, plus potassium chromate and sodium tungstate in distilled water) for approximately 30 days. Subsequently, they were super-glued to a metal chuck, and sections were cut using a Vibratome (Series 3000, Technical Products International, Inc., St. Louis, MO) into a .9% physiological saline bath. Four sequential 100μ sections were taken of the medial preoptic area of the hypothalamus as soon as the anterior commissure came together. All sections were placed on "subbed" slides and allowed to dry for approximately 20 minutes, after which they were exposed for roughly 5 minutes to the alkalizing solution of lithium hydroxide, which reacts with the Golgi-Cox heavy metal deposits in the neuron to produce the black product characteristic of the stain. The slides were then run through a dehydrating series of alcohols and xylenes, coverslipped, and allowed to dry.

Verification of cannula placement was assessed via histological track marks observed during slicing of the brain. All track marks were observed to cut through at least part of the corpus callosum above the ventricle.

Analysis of Neuronal Morphology

A Zeiss Axioplan microscope fitted to an Optronics color camera and a software package expressly designed to trace and record neuronal morphometry ("Bioquant Nova," version 5.00.8, R&M Biometrics, Inc., Nashville, TN), was used to measure features of neuronal morphology under a total magnification of 1,260x. Within the mPOA (up to 774 μm from the left or right of the midline, and up to 1,009 μm below the anterior commissure), only neurons with completely stained cell bodies that were easily

discernible from other cells were used. The number of dendritic protrusions was counted on the most clearly defined dendrite between the cell body and first branch. Protrusions from the dendrite of two kinds were included- either the traditional spines with or without bulbous heads as described by Woolley, Gould, Frankfurt and McEwen (1990), or any thin, long, filopodia-like extensions. Maletic-Savatic, Malinow and Svoboda (1999) suggested that filopodia might mature to become spines and often make synaptic contacts in vivo. For this reason, they were included in these analyses. The length of the two longest spines per branch was also measured.

Studies have indicated that the effects of BDNF on neuronal morphology are fast acting- in particular, increases in the number of dendrites, as well as increases in their length and complexity, have been seen as early as 24 hours (Horch, Krüttgen, Portbury, & Katz, 1999) and 36 hours (McAllister et al. 1995, as cited in McAllister, 1999). Since our animals received infusions of BDNF sense for up to 14 days, we anticipated no trouble in detecting any effects of BDNF manipulation.

Results

To first insure that the pumps were working properly, the residual amount of test agents left in the pumps were measured immediately after each animal was sacrificed and compared to the amount that should have remained (according to a formula provided by Alzet technical support that factored in each pump's flow rate and number of days left in the animal). A *t* test revealed no significant difference between the groups, $t(14) = .11$,

$p = .91$. Further, Pearson product-moment and point-biserial correlations indicated no significant relationships between the residual amounts of test agents and any of the measures described below.

Behavioral Data: Maternal Behavior

Due to the increased variability when looking at days to reach criterion, and in keeping with previous research examining latencies to display maternal behavior, a Mann-Whitney U test was performed using SPSS to explore the differences in maternal behavior between those rats infused with BDNF sense and saline. One animal was excluded from this analysis of maternal behavior because she attacked on six consecutive days. Separate analyses were run including this animal, but her inclusion in no way changed any of the results reported. For the remaining 15 females, the test revealed no significant behavioral difference between the groups, Mann-Whitney U = 26.5, $p = .429$. Please see Tables 1 and 2 for descriptive statistics.

Separate analyses were also run for each individual component of maternal behavior. However, there were no significant differences between groups for retrieving one pup, Mann-Whitney U = 26.5, $p = .43$, for retrieving two pups, Mann-Whitney U = 27.5, $p = .48$, for retrieving three pups, Mann-Whitney U = 27.5, $p = .48$, for grouping all three pups together, Mann-Whitney U = 26.5, $p = .43$, nor for crouching over all three pups, Mann-Whitney U = 28.0, $p = .50$.

Pup attacks during the first 24 hours of pup exposure were also examined for all 16 animals, including the female that attacked on six consecutive days. A chi square test was performed using SPSS and it revealed a significant effect of BDNF on decreasing

attacks, $X^2(1) = 4.00, p < .05$. As predicted, more control females attacked during the first 24 hours of pup exposure (6 out of 8; 75%) than did BDNF females (2 out of 8; 25%). Please see Figure 3.

Neuronal Data: Medial Preoptic Area

Within the mPOA, neurons were studied for their number of dendritic protrusions as well as the length of the two longest protrusions on each branch measured. Protrusions were counted on the principal dendrite between the cell body and first branch, and the length of that dendritic branch was measured using Bioquant. It has been reported that in mPOA neurons in the majority of adult (90 days) rats the first 10-30 μm long portion of dendrites are devoid of protrusions (Gerócs, Réthelyi, & Halász, 1986). The dendritic branches we measured were on average 84.51 μm in length, but they ranged from 19.87 μm to 281.64 μm.

Following Woolley and McEwen's (1993) data analysis methodology, the length of the branch was divided by 10, and the number of spines was divided into that number, yielding a numerical value for the number of protrusions per 10 μm of dendritic branch. These values were then averaged for each animal. The lengths of the two longest protrusions per branch for all the branches measured in each animal were also averaged together.

One branch was examined per neuron, two neurons were examined per tissue section, and four sections were taken per animal, resulting in a maximum total of 8 neurons per animal. However, due to staining difficulties, it was possible to get data on only 4 animals per experimental group. Further, the lack of full Golgi impregnation left

one animal in each group with less than 8 data values- one animal in the control group only had 3 data values averaged together, and one animal in the BDNF group only had 6 data values averaged together.

A one-way independent samples *t* test was performed using SPSS to explore the differences in dendritic protrusion number between those rats infused with BDNF sense and saline. Again, one animal was excluded, as she attacked on six consecutive days. This test revealed that BDNF had a significant effect on dendritic protrusion number, $t(6) = -2.09, p < .05$. As predicted, neurons of rats infused with BDNF had more protrusions per 10 μm of dendritic branch ($M = 2.96$, $SD = .56$) than did those neurons of control rats ($M = 1.84$, $SD = .91$). Please see Figures 4 and 5.

A similar one-way independent samples *t* test was also performed to explore the differences in dendritic protrusion length between both groups. This test revealed that BDNF had no effect on the average length of protrusions per branch, $t(6) = -.24, p = .41$. Please see Table 3.

Behavioral Data for the Females that had Neuronal Data

Once the differences in protrusions density mentioned above were found, the behaviors of the animals from which neuronal data were derived were re-analyzed. Within this smaller sub-sample of four rats per group, a Mann-Whitney U test again revealed no significant effect of BDNF on maternal behavior, Mann-Whitney U = 3.0, $p = .06$. Please see Table 4. Further, the previously established difference in attack behaviors was not preserved, $X^2(1) = .00, p = 1.0$, as an equal number of rats attacked and did not attack in both groups. Please see Table 5.

Correlations were also run to examine if there was a relationship between dendritic protrusion density and maternal behavior or the number of attacks (please see Table 6). Pearson product-moment correlations revealed no significant relationships between protrusion density and maternal behavior in control animals, $r = -.604$, $p = .396$; or in BDNF-infused animals, $r = -.836$, $p = .164$. However, point-biserial correlations revealed that within the 4 control animals there was as significant relationship between protrusion density and attacks, such that animals with higher numbers of protrusions per 10 μm branch attacked less, $r_{pb} = -.984$, $p < .05$. This relationship was not significant for the 4 BDNF-infused animals though, $r_{pb} = .540$, $p = .460$.

Discussion

Summary of Findings

Behaviorally, BDNF sense had no effect on facilitating maternal behavior but did significantly suppress pup attacks during the first 24 hours of exposure. BDNF had a significant effect on neuronal morphology in the mPOA as well, such that mPOA neurons exposed to BDNF had more dendritic protrusions per 10 μm of dendritic branch than did control neurons. However, the length of the two longest dendritic protrusions on each branch measured was not affected by BDNF sense. Within the animals for which neuronal data was obtained, there was no significant difference between groups on maternal behavior or attacks. However, for the 4 control animals, there was a significant relationship between protrusion density and attacks, such that animals with higher

numbers of protrusions per 10 μm branch attacked less. This relationship was not present in the 4 BDNF-infused animals.

Discussion of Findings

Finding differences in pup attacks despite no differences in maternal behavior is interesting as it suggests that pup attacks and maternal behavior may rely on separate mechanisms or brain regions. Kimble, Rogers, and Hendrickson (1967) examined the effects of hippocampal lesions on maternal behavior and reported some interesting findings that shed light on the pup attack differences observed here. They reported that following bilateral aspiration lesions of the hippocampus, female rats that had given birth showed significantly increased rates of cannibalism, time spent exploring (as opposed to nursing), and inferior nest-building skills, as compared to control mothers. Hippocampal-lesioned mothers also generally failed to hover over their pups, or if they did, they hovered several inches away from any of the pups. Keeping in mind that our rats did not experience pregnancy, parturition or lactation, and did not have hippocampal damage, the fact that hippocampal lesioning had differential effects on different aspects of maternal behavior is of particular interest because it suggests a division among maternal behaviors; different aspects of maternal behavior may be regulated by different brain regions.

Indeed, a number of studies show that different brain regions regulate different aspects of maternal behavior (please see Table 7). Kimble, Rogers and Hendrickson, specifically, implicated the hippocampus in increased rates of cannibalism. This fits with the data reported in this study, as it suggests that the actions of BDNF on neuronal morphology in the mPOA are independent of BDNF's suppressive nature on pup attacks.

Further, research is starting to identify a neural circuit involved in the inhibition of maternal behavior in virgin rats. Sheehan, Cirrito, Numan and Numan (2000) have identified various regions they believe to inhibit maternal behavior: the anterior hypothalamic nucleus, the principal bed nucleus of the stria terminalis, the ventral subdivision of the lateral septum, the posterodorsal medial amygdala, the ventrolateral medial preoptic area, the dorsal premammillary nucleus, the parvocellular paraventricular nucleus of the hypothalamus, and the dorsomedial and ventral regions of the ventromedial nucleus of the hypothalamus. Sheehan and colleagues believe that the hormonal environment of pregnancy actually decreases pup-induced neural activity in these brain regions, thus inducing maternal behavior.

Finding protrusion density differences despite the lack of behavioral differences is intriguing and strengthens the argument that maternal behavior is steroid dependent. It has been shown that BDNF sense alone cannot exert changes in spatial learning behavior (Mizuno et al., 2000), despite its documented ability to enhance sprouting (Kesslak, So, Choi, Cotman, and Gomez-Pinilla, 1998). The same dichotomous actions of BDNF may hold true for maternal behavior, especially in light of work done by Bridges, Numan, Ronsheim, Mann, and Lupini (1990) showing that prolactin's stimulation of maternal behavior is steroid dependent. These researchers showed that infusions of prolactin into the lateral ventricle failed to stimulate maternal behavior in nonsteroid-treated rats, whereas the same dose of prolactin given with estrogen and progesterone did stimulate the behavior. It is possible that even though BDNF alone may stimulate dendritic protrusion formation in the mPOA, BDNF sense may have to be administered with

estrogen and progesterone levels similar to those of pregnancy to stimulate maternal behavior.

Furthermore, finding neuronal differences in the mPOA that did not translate into maternal behavior also fits with the theory described by Numan (1994) that the mPOA is a site where hormones act to influence maternal behavior. Numan summarizes previous literature and describes how the mPOA is packed with estrogen- and progesterone-binding receptors, and how the rising levels of estradiol during late pregnancy are thought to bind to these receptors and stimulate protein synthesis. This protein synthesis is then thought to affect neurotransmitter levels and possibly change mPOA neural activity in a way that promotes maternal behavior. Our findings are in keeping with this theory- BDNF has been shown to alter neurotransmitter synthesis and release, and in this study enhanced the dendritic protrusion density of mPOA neurons. Further, our failure to see a change in maternal behavior may be due to a lack of pregnancy-like levels of estrogen and progesterone. The mPOA may have been primed for the behavior but needed the hormones to actually make the behavior occur, as in the work done by Bridges and colleagues in 1990.

Perhaps the most intriguing finding was that within the control animals that had neuronal data, there was a relationship between protrusion density and attacks. This suggests that having more protrusions suppresses pup attacks. Our BDNF-infused animals had increased levels of spines and thus no effect was seen on pup attacks- though it is important to note that in the BDNF group of four animals, 2 attacked and 2 did not. It is possible that for this reason the correlation was not significant. However, these

findings may still suggest that cannibalism, one aspect of maternal behavior, is not steroid dependent.

Connections to the Hormones of Pregnancy

As previously mentioned, BDNF is influenced by fluctuating hormones (Gibbs, 1998), as well as estrogen replacement therapy (Singh, Meyer, & Simpkins, 1995; Sohrabji et al., 1995; Gibbs, 1998). Progesterone too has been shown to affect levels of BDNF mRNA and protein. Gibbs (1999) reported that acute treatment of ovariectomized females with estrogen and progesterone produces a significant increase in BDNF mRNA and protein in the pyriform cortex. In the hippocampus however, results were different. Females killed 72 hours after receiving estrogen and 24 hours after receiving progesterone showed a significant increase in BDNF mRNA. A smaller increase was detected in animals killed after 53 hours of receiving estrogen alone, but there was no significant effect noted in animals killed after 53 hours of receiving estrogen and 5 hours after receiving progesterone. These findings indicate that estrogen and progesterone interact with each other in altering BDNF levels.

Oxytocin is another hormone that has been recently implicated in learning and memory processes in the hippocampus. Intracerebroventricular injections of oxytocin have been shown to enhance long-term synaptic plasticity as well as long-term memory through the activation of the MAP kinase cascade and the CREB phosphorylation that follows in virgin mice (Tomizawa, Iga, Lu, Moriwaki, Matsushita, Li, et al., 2003). Linking their findings to the previously mentioned study reporting that rats with reproductive experience learn faster (Kinsley et al., 1999), Tomizawa and colleagues

indicate that reproductive experience strikingly changes a female's biochemical environment- oxytocin is released through pup stimulation and oxytocin's improvement in spatial memory is important in ensuring pups' survival and development.

Further, Tomizawa et al. (2003) report that oxytocin receptors were strongly expressed in the hippocampus, but that their expression level did not change through development and pregnancy. However since estrogen and progesterone, as these researchers report, have been shown to change oxytocin receptor expression levels in other brain regions, this apparent differential regulation of receptor expression in response to sex hormones remains to be determined. As BDNF is influenced by estrogen and progesterone, it is also possible that BDNF and oxytocin have a dual relationship. This, however, has not yet been investigated.

BDNF is a protein so entwined in different systems and hormones that its role in maternal behavior and reproductive experience should not go un-investigated.

Limitations

This experiment is not without its limitations. The brains of the second round of animals tested were not fully impregnated with Golgi, despite having remained in solution for approximately 30 days just as the first round of brains had. These staining difficulties halved our sample size for the analysis of neuronal morphology, and the small number of animals per group may have prevented us from observing the full effects of BDNF sense.

Our definition and quantification of protrusions may have been too general as well. We included filopodia in our analyses, and future studies may want to be more

restrictive in their identification of spines. It is possible that the effects of BDNF sense are different if only spines are measured. It would also be interesting to determine whether or not the filopodia we observed would develop into spines with more exposure to BDNF, as it is probable that our brains were removed midway through a process of change for the mPOA neurons. Further, the region we considered to be mPOA may have been too broad; the effects reported here may not be replicated in a smaller, more strictly defined region.

The fact that there were no differences between groups in maternal behavior may be a result of various factors. First, there was no way of verifying whether or not the BDNF sense was functioning in vivo. Oligos Etc. Inc. did provide a synthesis and analysis report, and although all precautions were taken to avoid contamination, we are not sure if the oligonucleotide was contaminated during transportation, re-suspension, or filling of the pumps. However, because neuronal differences were found in the present work, it can be argued that the BDNF was indeed functioning properly and to its fullest potency.

Second, some of the pups were only hours old and may have had stronger, even more aversive, odors due to the presence of blood and amniotic fluids. Grota (1973) conducted an elaborate experiment in which he manipulated the delivery of pups (normal or surgical), whether or not the pups were washed or had amniotic fluids, and whether or not the foster mother was injected with placental homogenate. He reported that both normally and surgically delivered pups that had been washed with .9% physiological saline had a 78% survival rate. Surgically delivered and washed pups had a 100%

survival rate when the foster mothers had been injected with placental homogenates. Both normally and surgically delivered pups that had been immersed in amniotic fluids had a 66% survival rate, however, when the foster mother had been injected with placental homogenates none of the normally delivered pups survived, and only 39% of the surgically delivered pups survived. Grota suggested that pup survival depends on the interaction of both the fluids surrounding pups at fostering, as well as the substances present in the placenta and liver. Grota used lactating females as foster mothers, and the hormones of pregnancy could play a significant role in the findings he reported. Although we used virgin females, the presence of amniotic fluid on some of the pups may have interacted with the onset of their maternal behaviors.

Third, the animals used in this study were cycling females. It has been well documented that densities of hippocampal dendritic spines fluctuate naturally during the rat's 5-day estrous cycle (Woolley, Gould, Frankfurt, & McEwen, 1990; Woolley & McEwen, 1992). It is thus possible that the waxing and waning in spine densities that the fluctuating hormone levels may have caused could have occluded any of the effects of BDNF itself. However, Murphy, Cole and Segal (1998) showed that exogenous BDNF suppressed estradiol's effect on spine density and even had a small effect of its own to reduce dendritic spine density in hippocampal cultures. It can therefore be argued that in this study, BDNF was able to override the dendritic spine density fluctuations associated with the estrous cycle, and that the increased number of protrusions that we observed was in fact due to the manipulation of BDNF and not to the rat's estrous cycle. However, it is still possible that the hormonal fluctuations occluded BDNF's effect (if any) on maternal

behavior. Further, we did not smear our females at any point, so there was no knowledge of what stage of the estrous cycle the rats were in at various points throughout the study. Future studies should nonetheless control for these hormonal fluctuations by using ovariectomized females, or ovariectomized females receiving estrogen and progesterone.

Fourth, this study only examined retrieving, grouping and crouching. Maternal behavior is composed of a host of other behavioral aspects, such as avoidance, licking, nest building, time spent in the nest, etc., that may have been differentially affected by BDNF.

Further work should be done to replicate these findings and improve on some of our methods by, for example, increasing the sample size, using ovariectomized females (or ovariectomized females with estrogen and progesterone), tighten the definition of dendritic spines and examine other aspects of maternal behavior.

Future Research

Should BDNF prove to be an important factor in speeding the onset of maternal behavior in virgin rats, it would be important to further explore the current findings. One aspect of inquiry would be whether BDNF has its effects on the maternal behavior of post partum rats. To find this out, a similar study can be conducted in which animals receive infusions of BDNF sense, antisense and saline during the last week of pregnancy and first week of lactation. In addition to this, one could further examine whether BDNF has an effect on maternal behavior during pregnancy, lactation or both. It is possible that these different stages affect different aspects of maternal behavior. To explore this idea, some

rats can be infused with BDNF sense or antisense only during pregnancy and not during lactation, and other rats can be infused only during lactation.

Implications for BDNF and Post-Partum Depression / Infanticide

Should BDNF prove to be an important factor in speeding the onset of maternal behavior in virgin rats the implications would be far-reaching. The results could open the doors to further work with BDNF in the realm of the behavioral symptomatology of post-partum depression. Research has shown that intracerebral administration of BDNF to animals may have antidepressant properties. Using the learned helplessness and forced swim animal models of depression, Siuciak, Lewis, Wiegand and Lindsay (1997) reported that BDNF infusion in rats produced anti-depressant like effects. Vehicle-infused rats exposed to the inescapable foot shock of the learned helplessness paradigm showed escape deficits. These deficits were reversed by chronic BDNF infusion. Further, BDNF infusion also decreased the immobility time by 70% in the forced swim task, as compared to vehicle infusion.

BDNF expression also plays an important role in the clinical response to antidepressant treatment (Russo-Neustadt, Ha, Ramirez, and Kesslak, 2001). Altar (1999) suggests that antidepressant medications and even electroconvulsive shock therapy may work by boosting the brain's production of BDNF.

A strong presence of BDNF during pregnancy may prove protective to a female, such that she is in less risk of developing depression. BDNF may also be adversely affected during the rapid and sudden decrease in hormones that occurs at parturition and negatively affect a female. BDNF may thus play a role in post partum depression.

The fact that we found differences in pup attacks in our total sample of rats may also indicate a role for BDNF in decreasing post-partum infanticide. While BDNF has not previously been studied in relation to maternal behavior, our findings do show that females receiving BDNF sense attacked pups significantly less during the first 24 hours of pup exposure. Although it may be possible that BDNF had a direct effect on decreasing cannibalism, it more likely affected areas of the brain that we did not look at, which do influence cannibalism. Kimble, Rogers and Hendrickson's 1967 study, implicated the hippocampus in increased rates of cannibalism- it is possible that BDNF affected hippocampal neurons and thus altered this aspect of maternal behavior via this route.

Implications for BDNF and Alzheimer's Disease

As previously discussed, BDNF and estrogen are closely linked in terms of their neuroprotective effects. Alzheimer's disease is an example of an area of interest in which estrogen and BDNF's roles have been explored in a number of ways, but with mixed results. For example, decreased levels of BDNF have been reported in the hippocampus and parietal cortex of patients with Alzheimer's disease (Hock, Heese, Hulette, Rosenberg & Otten, 2000; Holsinger, Schnarr, Henry, Castelo & Fahnestock, 2000). However, other studies have shown an opposite increase of BDNF levels in patients with Alzheimer's disease (Durany, Michel, Kurt, Cruz-Sánchez, Cervós-Navarro, & Riederer, 2000).

Similarly, administration of estrogen to patients with Alzheimer's has been shown to improve some aspects of cognition related to this disease (Brinton, Chen, Montoya,

Hsieh, Minaya, Kim, et al., 2000; Asthana et al., 2001), but also has reportedly failed to improve these aspects of cognition (Mulnard, Cotman, Kawas, van Dyck, Sano, Doody et al., 2000). Recent findings even indicate that estrogen plus progestin therapy increases the risk of developing dementia and does not prevent mild cognitive impairment in postmenopausal women (Shumaker, Legault, Rapp, Thal, Wallace, Ockene et al., 2003). However, these equivocal findings could be due to methodological confounds.

Research in this lab also promotes further exploration of the link between BDNF and Alzheimer's disease. Gatewood (2002) examined the levels of amyloid precursor protein (APP; a protein that plays an important role in the early stages of neurodegeneration associated with Alzheimer's disease) in the hippocampus and dentate gyrus of aged (24 months) rats with differing levels of reproductive experience. Gatewood found that in the CA1 area of the hippocampus multiparous rats had significantly fewer APP immunoreactive cells than did either nulliparous or primiparous rats. This significant difference, however, was revealed upon post hoc analysis of a non-significant *F* value. Gatewood also found that in the dentate gyrus, multiparous animals had significantly fewer APP immunoreactive cells than did nulliparous rats, but no other significant differences were noted.

Gatewood's study provides strong support for the notion that reproductive experience provides long-lasting neuroprotective properties. BDNF, with its role in neuroplasticity and its link to estrogen, may be an important neurochemical link responsible for these effects. Berchtold, Kesslak, Pike, Adlard, & Cotman (1995) have suggested that some of the beneficial effects of estrogen may in fact be due to increased

availability of BDNF. Thus BDNF may function as an important step involved in treatment programs such as estrogen replacement.

Implications for BDNF and Huntington's Disease

BDNF has even been implicated in playing a therapeutic role in the treatment of Huntington's disease (Zuccato, Ciammola, Rigamonti, Leavitt, Goffredo, Conti, et al., 2001). Interestingly, preliminary DNA microarray data collected by this lab have shown that lactating females express the Huntington's disease mRNA gene 2.8 times more than do nulliparous females. The actions of this gene have yet to be examined, but the increased expression in lactating versus nulliparous females may point to a long-lasting, potentially beneficial and protective, effect of reproductive experience on the females' brains. BDNF and estrogen may work together in mediating this neuroprotective effect.

BDNF as a Promising Therapeutic Agent

BDNF has recently been modified to cross the blood-brain barrier, which has opened the doors to its potentially more accessible use as a therapeutic agent. Stroke research (Wu & Pardridge, 1999; Zhang & Pardridge, 2001) has shown that intravenous administration of BDNF conjugated to the OX26 murine monoclonal antibody (BDNF/OX26) normalized hippocampal CA1 neuronal density, and reduced cortical stroke volume at both 24 hours and 7 days by 68% and 70% respectively. The neuroprotection was long lasting, as it persisted for at least 7 days after a 1-hour middle cerebral artery occlusion.

BDNF's conjugation to a blood-brain barrier drug targeting system could lead to the systemic administration of BDNF for many purposes. The neuroprotective and memory-related effects of BDNF may thus become more widely and painlessly available.

Concluding Remarks

Despite its limitations, this study provides evidence that BDNF sense, when administered centrally into virgin females, increases dendritic protrusion density and suppresses cannibalism during the first 24 hours of pup exposure. These findings add another piece to the puzzle of maternal behavior, opening the doors to a new line of research in the field. BDNF has not yet been studied in relation to maternal behavior, and this pioneering study points to its potential role in both suppressing one aspect of the behavior (cannibalism), and in priming the mPOA for maternal behaviors to occur. Future research should explore the significance of these findings, as well as the mechanisms by which BDNF exerts the effects we report.

References

Acheson, A., Conover, J. C., Fandl, J. P., DeChiara, T. M., Russell, M., Thadani, A., et al. (1995). A BDNF autocrine loop in adult sensory neurons prevents cell death. *Nature, 374*, 450-453.

Altar, C. A. (1999). Neurotrophins and depression. *Trends in Pharmacological Science, 20*, 59-61.

Altar, C. A., Cai, N., Bliven, T., Juhasz, M., Conner, J. M., Acheson, A. L., Lindsay, R. M., & Wiegand, S. J. (1997). Anterograde transport of brain-derived neurotrophic factor and its role in the brain. *Nature, 389*, 856-860.

Altar, C. A., & DiStefano, P. S. (1998). Neurotrophin trafficking by anterograde transport. *Trends in Neuroscience, 21*, 433-437.

Asthana, S., Baker, L. D., Craft, S., Stanczyk, F. Z., Veith, R. C., Raskind, M. A., et al. (2001). High-dose estradiol improves cognition for women with AD. *Neurology, 57*, 605-612.

Barde, Y-A. (2002). Neurotrophin channels excitement. *Nature, 419*, 683-684.

Berchtold, N. C., Kesslak, J. P., Pike, C. J., Adlard, P. A., & Cotman, C. W. (2001). Estrogen and exercise interact to regulate brain-derived neurotrophic factor m RNA and protein expression in the hippocampus. *European Journal of Neuroscience, 14*, 1992-2002.

Berninger, B., & Poo, M. (1999). Exciting neurotrophins. *Nature, 401*, 862-863.

Bridges, R. S., Feder, H. H., & Rosenblatt, J. S. (1977). Induction of maternal behaviors

in primigravid rats by ovariectomy, hysterectomy, or ovariectomy plus hysterectomy: Effect of length of gestation. *Hormones and Behavior, 9*, 156-169.

Bridges, R. S., Numan, M., Ronsheim, P. M., Mann, P. E., & Lupini, C. E. (1990). Central prolactin infusions stimulate maternal behavior in steroid-treated, nulliparous female rats. *Proceedings of the National Academy of Sciences, 87*, 8003-8007.

Bridges, R. S., Zarrow, M. X., Gandelman, R., & Denenberg, V. H. (1972). Differences in maternal responsiveness between lactating and sensitized rats. *Developmental Psychobiology, 5*, 123-127.

Brinton, R. D., Chen, S., Montoya, M., Hsieh, D., & Minaya, J. (2000). The estrogen replacement therapy of the Women's Health Initiative promotes cellular mechanisms of memory and neuronal survival in neurons vulnerable to Alzheimer's disease. *Maturitas, 34*, S35-S52.

Brinton, R. D., Chen, S., Montoya, M., Hsieh, D., Minaya, J., Kim, J., et al. (2000). The Women's Health Initiative estrogen replacement therapy is neurotrophic and neuroprotective. *Neurobiology of Aging, 21*, 475-496.

Brunig, I., Penshuck, S., Berninger, B., Benson, J., & Fritschy, J. M. (2001). BDNF reduces miniature inhibitory postsynaptic currents by rapid downregulation of $GABA_A$ receptor surface expression. *European Journal of Neuroscience, 13*, 1320-1328.

Chan, J. R., Cosgaya, J. M., Wu, Y. J., & Shooter, E. M. (2001). Neurotrophins are key

mediators of the myelination program in the peripheral nervous system. *Proceedings of the National Academy of Sciences, 98*, 14661-14668.

Courtney, M. J., Åkerman, K. E. O., & Coffey, E. T. (1997). Neurotrophins protect cultured cerebellar granule neurons against the early phase of cell death by a two-component mechanism. *The Journal of Neuroscience, 17*, 4201-4211.

Du, J., Feng, L., Yang, F., & Lu, B. (2000). Activity- and Ca^{2+} -dependent modulation of surface expression of brain-derived neurotrophic factor receptors in hippocampal neurons. *Journal of Cell Biology, 150*, 1423-1433.

Durany, N., Michel, T., Kurt, J., Cruz-Sánchez, F. F., Cervós-Navarro, J. & Riederer, P. (2000). Brain-derived neurotrophic factor and neurotrophin-3 levels in alzheimer's disease brain. *International Journal of Developmental Neuroscience, 18*, 807-813.

Egan, M. F., Kojima, M., Callicott, J. H., Goldberg, T. E., Kolachana, B. S., Bertolino, A. et al. (2003). The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. *Cell, 112*, 257-269.

Figurov, A., Pozzo-Miller, L. D., Olafsson, P., Wang, T., & Lu, B. (1996). Regulation of synaptic responses to high-frequency stimulation and LTP by neurotrophins in the hippocampus. *Nature, 381*, 706-709.

Fleming, A. S., & Rosenblatt, J. S. (1974). Maternal behavior in the virgin and lactating rat. *Journal of Comparative and Physiological Psychology, 86*, 957-972.

Freeman, M. E. (1994). The neuroendocrine control of the ovarian cycle of the rat. In E.

Knobil, J. D. Neill (Eds.), The physiology of reproduction (2nd ed., pp. 613-658). New York: Raven Press, Ltd.

Fumagalli, F., Santero, R., Gennarelli, M., Racagni, G., & Riva, M. A. (2001). Decreased hippocampal BDNF expression after acute systemic injection of quinpirole. *Neuropharmacology, 40*, 954-957.

Gatewood, J. D. (2002). *Reproductive experience and aging: Possible neuroprotective effects of motherhood.* Un-published master's thesis, University of Richmond, Virginia.

Geröcs, K., Réthelyi, M., & Halász, B. (1986). Quantitative analysis of dendritic protrusions in the medial preoptic area during postnatal development. *Developmental Brain Research, 26*, 49-57.

Gibbs, R. B. (1998). Levels of trkA and BDNF mRNA, but not NGF mRNA, fluctuate across the estrous cycle and increase in response to acute hormone replacement. *Brain Research, 787*, 259-268.

Gibbs, R. B. (1999). Treatment with estrogen and progesterone affects relative levels of brain-derived neurotrophic factor mRNA and protein in different regions of the adult rat brain. *Brain Research, 844*, 20-27.

Gifford, G., Miller, S., Quadros, P., Lambert, K. G., & Kinsley, C. H. Alterations in glial cells (astrocytes) may accompany changes in reproductive condition in the female rat. Paper presented at the annual meeting of the Virginia Academy of Science, May, 1998.

Grota, L. J. (1973). The effects of the placenta and fetal fluids on the acceptance of foster

young. *Developmental Psychobiology, 6*, 495-502.

Guillin, O., Diaz, J., Carroll, P., Griffon, N., Schwartz, J. C., & Sokoloff, P. (2001). BDNF controls dopamine D_3 receptor expression and triggers behavioural sensitization. *Nature, 411*, 86-89.

Hall, J., Thomas, K. L., & Everitt, B. J. (2000). Rapid and selective induction of BDNF expression in the hippocampus during contextual learning. *Nature Neuroscience, 3*, 533-535.

Hock, C., Heese, K., Hulette, C., Rosenberg, C., & Otten, U. (2000). Region-specific neurotrophin imbalances in Alzheimer disease. *Archives of Neurology, 57*, 846-851.

Hofer, M., Pagliusi, S. R., Hohn, A., Leibrock, J., & Barde, Y-A. (1990). Regional distribution of brain-derived neurotrophic factor mRNA in the adult mouse brain. *The EMBO Journal, 9*, 2459-2464.

Holsinger, R. M. D., Scharr, J., Henry, P., Castelo, V. T., & Fahnestock, M. (2000). Quantitation of BDNF mRNA in human parietal cortex by competitive reverse transcription-polymerase chain reaction: decreased levels in Alzheimer's disease. *Molecular Brain Research, 76*, 347-354.

Horch, H. W., Krüttgen, A., Portbury, S. D., & Katz, L. C. (1999). Destabilization of cortical dendrites and spines by BDNF. *Neuron, 23*, 353-364.

Hyman, C., Hofer, M., Barde, Y-A., Juhasz, M., Yancopoulos, G. D., Squinto, S. P., & Lindsay, R. M. (1991). BDNF is a neurotrophic factor for dopaminergic neurons of the substantia nigra. *Nature, 350*, 230-232.

Jessell, T. M., & Sanes, J. R. (2000). The generation and survival of nerve cells. In Kandel, R. R., Shwartz, J. H., & Jessell, T. M. (Eds.), *Principles of Neuroscience* (pp.1041-1062), New York, NY: McGraw-Hill.

Johnson, J. E. (1999). Neurotrophic factors. In Zigmond, M. J., Bloom, F. Z., Landis, S. C., Roberts, J. L., & Squire, L. R. (Eds.), *Fundamental Neuroscience* (pp. 611-635). San Diego, CA: Academic Press.

Jovanovic, J. N., Czernik, A. J., Feinberg, A. A., & Greengard, P. (2000). Synapsins as mediators of BDNF-enhanced neurotransmitter release. *Nature Neuroscience, 3*, 323-329.

Kafitz, K. W., Rose, C. R., Thoenen, H., & Konnerth, A. (1999). Neurotrophin-evoked rapid excitation through TrkB receptors. *Nature, 401*, 918-921.

Kesslak, J. P., So, V., Choi, J., Cotman, C. W., & Gomez-Pinilla, F. (1998). Learning upregulates brain-derived neurotrophic factor messenger ribonucleic acid: A mechanism to facilitate encoding and circuit maintenance? *Behavioral Neuroscience, 112*, 1012-1019.

Keyser-Marcus, L., Stafisso-Sandoz, G., Gerecke, K., Jasnow, A., Nightingale, L., Lambert, K. G., et al. (2001). Alterations of medial preoptic area neurons following pregnancy and pregnancy-like steroidal treatment in the rat. *Brain Research Bulletin, 55*, 737-745.

Kimble, D. P., Rogers, L., & Hendrickson, C. W. (1967). Hippocampal lesions disrupt maternal, not sexual, behavior in the albino rat. *Journal of Comparative and Physiological Psychology, 63*, 401-407.

Kinsley, C. H., Madonia, L., Gifford, G. W., Yureski, K., Griffin, G. R., Lowry, C., et al. (1999). Motherhood improves learning and memory: Neural activity in rats is enhanced by pregnancy and the demands of rearing offspring. *Nature, 402*, 137-138.

Kinsley, C. H., Trainer, R., Quadros, P. S., & Stafisso-Sandoz, G. Reproductive condition modifies the brain: Differences in concentrations of dendritic spines on hippocampal neurons among virgin, pregnant, and lactating rats. Paper presented at the annual meeting of the International Behavioral Neuroscience Society meeting, Richmond, Virginia, June, 1998.

Knüsel, B., Winslow, J. W., Rosenthal, A., Burton, L. E., Seid, D. P., Nikolics, K., & Hefti, F. (1991). Promotion of central cholinergic and dopaminergic neuron differentiation by brain-derived neurotrophic factor but not neurotrophin 3. *Proceedings of the National Academy of Sciences, 88*, 961-965.

Kohara, K., Kitamura, A., Morishima, M., & Tsumoto, T. (2001). Activity-dependent transfer of brain-derived neurotrophic factor to postsynaptic neurons. *Science, 291*, 2419-2423.

Korte, M., Carroll, P., Wolf, Brem, G., Thoenen, H., & Bonhoeffer, T. (1995). Hippocampal long-term potentiation is impaired in mice lacking brain-derived neurotrophic factor. *Proceedings of the National Academy of Sciences, 92*, 8856-8860.

Kovalchuck, Y., Hanse, E., Kafitz, K. W., & Konnerth, A. (2002). Postsynaptic induction of BDNF-mediated long-term potentiation. *Science, 295*, 1729-1734.

Lambert, K. G., Love, G., Stevens, L., McNamara, I., Morgan, M., Hoffman, A., Wightman, N., Jeannotte, A., Karp, N., Kinsley, C. H. (2003, November). *Long-lasting maternal-induced cognitive enhancement, decreased emotionality, and accompanying hippocampal GFAP- and BDNF- immunoreactivity in long-evans rats.* Poster session to be presented at the annual meeting of the Society for Neuroscience, New Orleans, LA.

Lindholm, D., Castrén, E., Berzaghi, M., Blöchl, A., & Thoenen, H. (1994). Activity-dependent and hormonal regulation of neurotrophin mRNA levels in the brain-Implications for neuronal plasticity. *Journal of Neurobiology, 25*, 1362-1372.

Liu, D., Diorio, J., Day, J. C., Francis, D. D., & Meaney, M. J. (2000). Maternal care, hippocampal synaptogenesis and cognitive development in rats. *Nature Neuroscience, 3*, 799-806.

Ma, Y. L., Wang, H. L., Wu, H. C., Wei, C. L., & Lee, E. H. Y. (1998). Brain-derived neurotrophic factor antisense oligonucleotide impairs memory retention and inhibits long-term potentiation in rats. *Neuroscience, 82*, 957-967.

Maletic-Savatic, M., Malinow, R., & Svoboda, K. (1999). Rapid dendritic morphogenesis in CA1 hippocampal dendrites induced by synaptic activity. *Science, 283*, 1923-1927.

Mamounas, L. A., Blue, M. E., Siuciak, J. A., & Altar, C. A. (1995). Brain-derived neurotrophic factor promotes the survival and sprouting of serotonergic axons in rat brain. *The Journal of Neuroscience, 15*, 7929-7939.

Manabe, T. (2002). Does BDNF have pre- or postsynaptic targets? *Science, 295*, 1651-

1653.

Matsumoto, T., Numakawa, T., Adachi, N., Yokomaku, D., Yagamishi, S., Takei, N., & Hatanaka, H. (2001). Brain-derived neurotrophic factor enhances depolarization-evoked glutamate release in cultured cortical neurons. *Journal of Neurochemistry, 79*, 522-530.

McAllister, A. K., Katz, L. C., & Lo, D. C. (1996). Neurotrophin regulation of cortical dendritic growth requires activity. *Neuron, 17*, 1057-1064.

McAllister, A. K., Katz, L. C., & Lo, D. C. (1999). Neurotrophins and synaptic plasticity. *Annual Review of Neuroscience, 22*, 295-318.

McEwen, B. S., & Alves, S. E. (1999). Estrogen actions in the central nervous system. *Endocrinology Review, 20*, 279-307.

McEwen, B. S., Alves, S. E., Bulloch, K., & Weiland, N. C. (1997). Ovarian steroids and the brain: Implications for cognition and aging. *Neurology, 48*, S8-S15.

McEwen, B. S., & Woolley, C. S. (1994). Estradiol and progesterone regulate neuronal structure and synaptic connectivity in adult as well as developing brain. *Experimental Gerontology, 29*, 431-436.

McNamara, I., Love, G., Lambert, K. G., & Kinsley, C. H. Long Lasting Diminution of Anxiety Responses as a Consequence of Reproductive Experience (RE). Poster Presented at the Mother & Infant Conference, Montreal, Canada, June 2003.

Mizuno, K., Carnahan, J., & Nawa, H. (1994). Brain-derived neurotrophic factor promotes differentiation of striatal GABAergic neurons. *Developmental Biology, 165*, 243-256.

Mizuno, M., Yamada, K., Olariu, A., Nawa, H., & Nabeshima, T. (2000). Involvement of brain-derived neurotrophic factor in spatial memory formation and maintenance in a radial arm maze test in rats. *The Journal of Neuroscience, 20*, 7116-7121.

Mu, J. S., Li, W. P., Yao, Z. B., & Zhou, X. F. (1999). Deprivation of endogenous brain-derived neurotrophic factor results in impairment of spatial learning and memory in adult rats. *Brain Research, 835*, 259-265.

Muller, D., Djebbara-Hannas, Z., Jourdain, P., Vutskits, L., Durbec, P., Rougon, G., & Kiss, J. Z. (2000). Brain-derived neurotrophic factor restores long-term potentiation in polysialic acid-neural cell adhesion molecule-deficient hippocampus. *Proceedings of the National Academy of Sciences, 97*, 4315-4320.

Mulnard, R. A., Cotman, C. W., Kawas, C., van Dyck, C. H., Sano, M., Doody, R., et al. (2000). Estrogen replacement therapy for treatment of mild to moderate Alzheimer's disease. *Journal of the American Medical Association, 283*, 1007-1015.

Murer, M. G., Yan, Q., & Raisman-Vozari, R. (2001). Brain-derived neurotrophic factor in the control human brain, and in Alzheimer's disease and Parkinson's disease. *Progress in Neurobiology, 63*, 71-124.

Murphy, D. D., Cole, N. B., & Segal, M. (1998). Brain-derived neurotrophin mediates estradiol-induced dendritic spine formation in hippocampal neurons. *Proceedings of the National Academy of Sciences, 95*, 11412-11417.

Neeper, S. A., Gómez-Pinilla, F., Choi, J., & Cotman, C. (1995). Exercise and brain neurotrophins. *Nature, 373*, 109.

Nonomura, T., & Hatanaka, H. (1992). Neurotrophic effect of brain-derived neurotrophic factor on basal forebrain cholinergic neurons in culture from postnatal rats. *Neuroscience Research, 14*, 226-233.

Numan, M. (1994). Maternal Behavior. In E. Knobil, J. D. Neill (Eds.), The physiology of reproduction (2nd ed., pp. 221-302). New York: Raven Press, Ltd.

Patterson, S. L., Abel, T., Deuel, T. A. S., Martin, K. C., Rose, J. C., & Kandel, E. R. (1996). Recombinant BDNF rescues deficits in basal synaptic transmission and hippocampal LTP in BDNF knockout mice. *Neuron, 16*, 1137-1145.

Patterson, S. L., Grover, L. M., Schwartzkroin, P. A., & Bothwell, M. (1992). Neurotrophin expression in rat hippocampal slices: A stimulus paradigm inducing LTP in CA1 evokes increases in BDNF and NT-3 mRNAs. *Neuron, 9*, 1081-1088.

Pelleymounter, M. A., Cullen, M. J., Baker, M. B., Gollub, M., & Wellman, C. (1996). The effects of intrahippocampal BDNF and NGF on spatial learning in aged Long Evans rats. *Molecular and Chemical Neuropathology, 29*, 211-226.

Pencea, V., Bingaman, K. D., Wiegand, S. J., & Luskin, M. B. (2001). Infusion of brain-derived neurotrophic factor into the lateral ventricle of the adult rat leads to new neurons in the parenchyma of the striatum, septum, thalamus, and hypothalamus. *Journal of Neuroscience, 21*, 6706-6717.

Phillips, H. S., Hains, J. M., Laramee, G. R., Rosenthal, A., & Winslow, J. W. (1990). Widespread expression of BDNF but not NT3 by target areas of basal forebrain cholinergic neurons. *Science, 250*, 290-294.

Poo, M. (2001). Neurotrophins as synaptic modulators. *Nature Reviews; Neuroscience, 2,* 24-31.

Pozzo-Miller, L. D., Gottschalk, W., Zhang, L., McDermott, K., Du, J., Gopalakrishnan, R., Oho, C., Sheng, Z-H., & Lu, B. (1999). Impairments in high-frequency transmission, synaptic vesicle docking, and synaptic protein distribution in the hippocampus of BDNF knockout mice. *The Journal of Neuroscience, 19,* 4972-4983.

Pruginin-Bluger, M., Shelton, D. L., & Kalcheim, C. (1997). A paracrine effect for neuron-derived BDNF in development of dorsal root ganglia: Stimulation of Schwann cell myelin protein expression by glial cells. *Mechanisms of Development, 61,* 99-111.

Ramón-Moliner, E. (1970). The Golgi-Cox technique. In Nauta, W. J. H., & Ebbesson, S. O. E. (Eds.), Contemporary Research Methods in Neuroanatomy (pp.33-55). New York: Springer-Verlag.

Roceri, M., Hendriks, W., Racagni, G., Ellenbroek, B. A., Riva, M. A. (2002). Early maternal deprivation reduces the expression of BDNF and NMDA receptor subunits in rat hippocampus. *Molecular Psychiatry, 7,* 609-606.

Russell, J. A., Douglas, A. J., & Ingram, C. D. (2001). Brain preparations for maternity-adaptive changes in behavioral and neuroendocrine systems during pregnancy and lactation. An overview. In J. A. Russell, A. J. Douglas, R. J., Windle, & C. D. Ingram (Vol. Eds.), Progress in brain research: Vol. 133. The maternal brain:

Neurobiological and neuroendocrine adaptation and disorders in pregnancy and post partum (1st ed., pp. 1-38). Amsterdam, The Netherlands: Elsevier.

Russo-Neustadt, A., Ha, T., Ramirez, R., & Kesslak, J. P. (2001). Physical activity-antidepressant treatment combination- Impact on brain-derived neurotrophic factor and behavior in an animal model. *Behavioural Brain Research, 120*, 87-95.

Sheehan, T. P., Cirrito, J., Numan, M. J., & Numan, M. (2000). Using c-fos immunocytochemistry to identify forebrain regions that may inhibit maternal behavior in rats. *Behavioral Neuroscience, 114*, 337-352.

Shumaker, S. A., Legault, C., Rapp, S. R., Thal, L., Wallace, R. B., Ockene, J. K. et al. (2003). Estrogen plus progestin and the incidence of dementia and mild cognitive impairment in postmenopausal women. *Journal of the American Medical Association, 289*, 2651-2662.

Singh, M., Meyer, E. M., Millard, W. J., & Simpkins, J. W. (1994). Ovarian steroid deprivation results in a reversible learning impairment and compromised cholinergic function in female Sprague-Dawley rats. *Brain Research, 644*, 305-312.

Singh, M., Meyer, E. M., & Simpkins, J. W. (1995). The effect of ovariectomy and estradiol replacement on brain-derived neurotrophic factor messenger ribonucleic acid expression in cortical and hippocampal brain regions of female Sprague-Dawley rats. *Endocrinology, 136*, 2320-2324.

Siuciak, J. A., Lewis, D. R., Wiegand, S. J., & Lindsay, R. M. (1997). Antidepressant-

like effect of brain-derived neurotrophic factor (BDNF). *Pharmacology Biochemistry and Behavior, 56*, 131-137.

Smith, A. (1996). Hippocampal vulnerability to stress and aging: Possible role of neurotrophic factor. *Behavioural Brain Research, 78*, 25-36.

Sohrabji, F., Miranda, R. C., & Toran-Allerand, C. D. (1995). Identification of a putative estrogen response element in the gene encoding brain-derived neurotrophic factor. *Proceedings of the National Academy of Sciences, 92*, 11110-11114.

Spina, M. B., Squinto, S. P., Miller, J., Lindsay, R. M., & Hyman, C. (1992). Brain-derived neurotrophic factor protects dopamine neurons against 6-Hydroxydopamine and *N*-Methy-4-Phenylpyridinium ion toxicity: Involvement of the glutathione system. *Journal of Neurochemistry, 59*, 99-106.

Stern, J.M., & Lonstein, J. S. (2001). Neural mediation of nursing and related behaviors. In J. A. Russell, A. J. Douglas, R. J., Windle, & C. D. Ingram (Vol. Eds.), Progress in brain research: Vol. 133. The maternal brain: Neurobiological and neuroendocrine adaptation and disorders in pregnancy and post partum (1st ed., pp. 1-38). Amsterdam, The Netherlands: Elsevier.

Tanaka, T., Saito, H., & Matsuki, N. (1997). Inhibition of $GABA_A$ synaptic responses by brain-derived neurotrophic factor (BDNF) in rat hippocampus. *The Journal of Neuroscience, 17*, 2959-2966.

Terlecki, L. J., & Sainsbury, R. S. (1978). Effects of fimbria lesions on maternal behavior in the rat. *Physiology & Behavior, 21*, 89-97.

Thoenen, H. (1995). Neurotrophins and neuronal plasticity. *Science, 270*, 593-598.

Tomizawa, K., Iga, N., Lu, Y-F., Moriwaki, A., Matsushita, M., Li, S-T., Miyamoto, O., Itano, T., & Matsui, H. (2003). Oxytocin improves long-lasting spatial memory during motherhood through MAP kinase cascade. *Nature Neuroscience, 6*, 384-390.

Trainer, R., Quadros, P., Stafisso-Sandoz, G., & Kinsley, C. H. Differences in concentrations of dendritic spines on hippocampal neurons among virgin, pregnant and lactating rats. Paper presented at the International Society for Developmental Psychobiology annual meeting, New Orleans, Louisiana, October, 1997.

Tyler, W. J., & Pozzo-Miller, L. D. (2001). BDNF enhances quantal neurotransmitter release and increases the number of docked vesicles at the active zones of hippocampal excitatory synapses. *The Journal of Neuroscience, 21*, 4249-4258.

Wartella, J., Amory, E., Macbeth, A., McNamara, I., Stevens, L, Lambert, K. G., et al., (2003). Single or multiple reproductive experiences attenuate neurobehavioral stress and fear responses in the female rat. *Physiology and Behavior*, Manuscript in Press.

Wetmore, C., Ernfors, P., Persson, H., Olson, L. (1990). Localization of brain-derived neurotrophic factor mRNA to neurons in the brain by in situ hybridization. *Experimental Neurology, 109*, 141-152.

Woolley, C. S., Gould, E., Frankfurt, M., & McEwen, B. S. (1990). Naturally occurring fluctuation in dendritic spine density on adult hippocampal pyramidal neurons. *The Journal of Neuroscience, 10*, 4035-4039.

Woolley, C. S., & McEwen, B. S. (1992). Estradiol mediates fluctuation in hippocampal synapse density during the estrous cycle in the adult rat. *The Journal of Neuroscience, 12*, 2549-2554.

Woolley, C. S., & McEwen, B. S. (1993). Roles of estradiol and progesterone in regulation of hippocampal dendritic spine density during the estrous cycle in the rat. *The Journal of Comparative Neurology, 336*, 293-306.

Wu, D. & Pardridge, W. M. (1999). Neuroprotection with noninvasive neurotrophin delivery to the brain. *Proceedings of the National Academy of Sciences, 96*, 254-259.

Yan, Q., Matheson, C., Sun, J., Radeke, M. J., Feinstein, S. C., & Miller, J. A. (1994). Distribution of intracerebral ventricularly administered neurotrophins in rat brain and its correlation with trk receptor expression. *Experimental Neurology, 127*, 23-36.

Yoshinga, K., Hawkins, R. A., & Stocker, J. F. (1969). Estrogen secretion by the rat ovary in vivo during the estrous cycle and pregnancy. *Endocrinology, 85*, 103-112.

Zafra, F., Castrén, E., Theonen, H., & Lindholm, D. (1991). Interplay between glutamate and g-aminobutyric acid transmitter systems in the physiological regulation of brain-derived neurotrophic factor and nerve growth factor synthesis in hippocampal neurons. *Proceedings of the National Academy of Sciences, 88*, 10037-10041.

Zhang, Y., & Pardridge, W. M. (2001). Neuroprotection in transient focal brain ischemia

after delayed intravenous administration of brain-derived neurotrophic factor conjugated to a blood-brain barrier drug targeting system. *Stroke,* 32, 1378-1383.

Zuccato, C., Ciammola, A., Rigamonti, D., Leavitt, B. R., Goffredo, D., Conti, L., et al. (2001). Loss of huntingtin-mediated BDNF gene transcription in Huntington's disease. *Science, 293*, 493-498.

Table 1

Maternal Behavior Data: Days to Reach Criteria

Group	N	Range	Mean (SD)	Median
BDNF	8	4-10	7.63 (1.85)	7.5
Control	7	6-10	7.71 (1.89)	7

Table 2

Range Distribution for the Number of Animals in Each Group That Reached Maternal Behavior Criteria Based on Day of Testing

	Days of Testing				
Group	1-2	3-4	5-6	7-8	9-10
BDNF	0	1	0	4	3
Control	0	0	3	1	3

Table 3

Average Length in Microns of the Two Longest Spines in the Medial Preoptic Area of the Hypothalamus

Group	N	Mean (SD)
BDNF	4	1.32 (.35)
Control	4	1.26 (.45)

Table 4

Maternal Behavior Data for the 8 Animals that had Neuronal Data: Days to Reach Criteria

Group	N	Range	Mean (SD)	Median
BDNF	4	7-9	8.25 (0.96)	8.5
Control	4	6-9	6.75 (1.50)	6

Table 5

Pup Attacks for the 8 Animals that had Neuronal Data During the First 24 Hours of Pup Exposure

	Attacks	
Group	Yes	No
BDNF	2	2
Control	2	2

Table 6

Pearson Product Moment and Point-Biserial Correlations Between Neuronal and Behavioral Effects for the Animals with mPOA Data

Group	Days to Reach Maternal Behavior	Pup Attacks During the First 24 Hours of Exposure
Controls (n = 4)		
# of Protrusions	-.604	-.984*
BDNF (n = 4)		
# of Protrusions	-.836	.540

* $p < .05$

Table 7

A Summary of Studies Showing How Damage to Different Brain Regions Affects Different Aspects of Maternal Behavior

Brain Region	Aspect of Behavior Disrupted by Lesions (Reference)
Hippocampus*	Cannibalism, nest building, nursing, & retrieving (Kimble, Rogers & Hendrickson, 1967)
Fimbria	Nest building & retrieving (Terlecki & Sainsbury, 1978)
Periaqueductal Gray	Nursing (Stern & Lonstein, 2001)
Neocortex*	Nest building, nursing, & retrieving (Beach, 1937; In Numan, 1994)
Medial Cortex	Retrieving (Stamm, 1955; Slotnick, 1967; Wilsoncroft, 1963; All in Numan, 1994)
Septum	Retrieving (Fleischer & Slotnick, 1978; In Numan, 1994)
Medial Preoptic Area	Nest building, nursing, & retrieving, (Numerous studies cited in Numan, 1994)

* BDNF has been observed in this brain region

Figure Captions

Figure 1. Estrous Cycle of the Rat

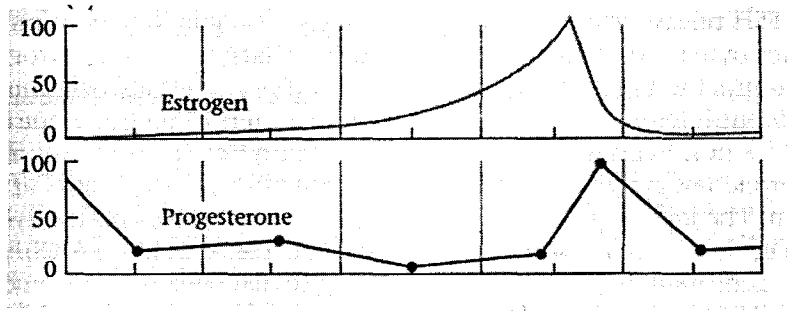
Figure 2. Hormonal Pattern of Pregnancy in the Rat

Figure 3. Percentage of Females that Attacked During the First 24 Hours of Pup Exposure

Figure 4. Mean Number of Dendritic Protrusions per 10 μm of Dendritic Branch

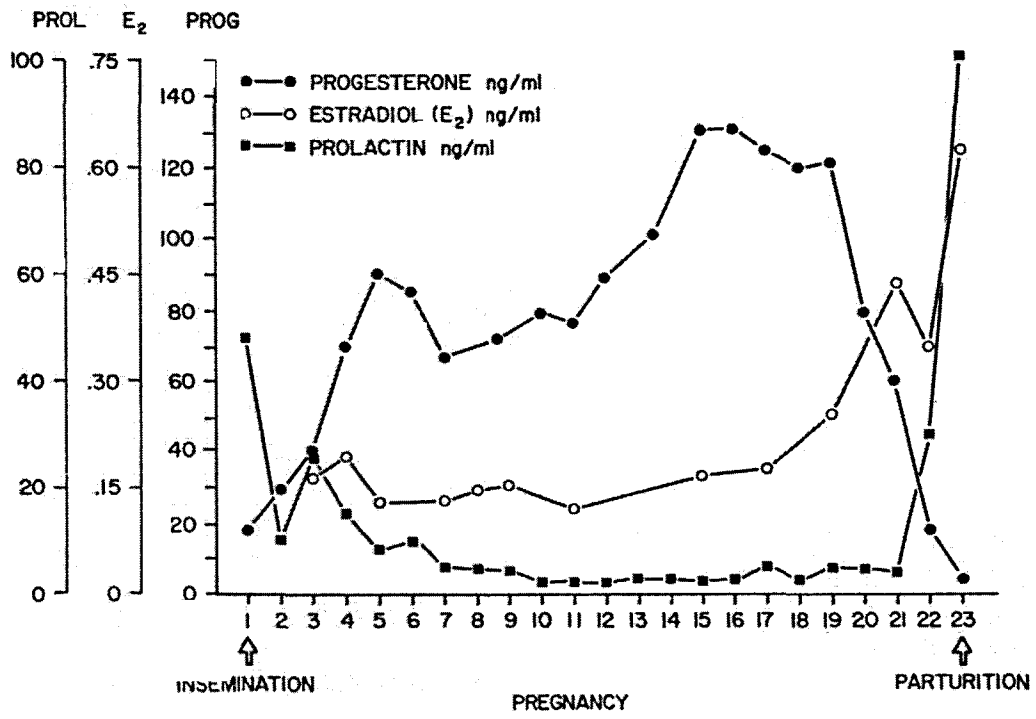
Figure 5. Photomicrographs of Representative Neurons that Received BDNF Sense and Control Neurons at a Total Magnification of 1,260x

Estrous Cycle of the Rat*



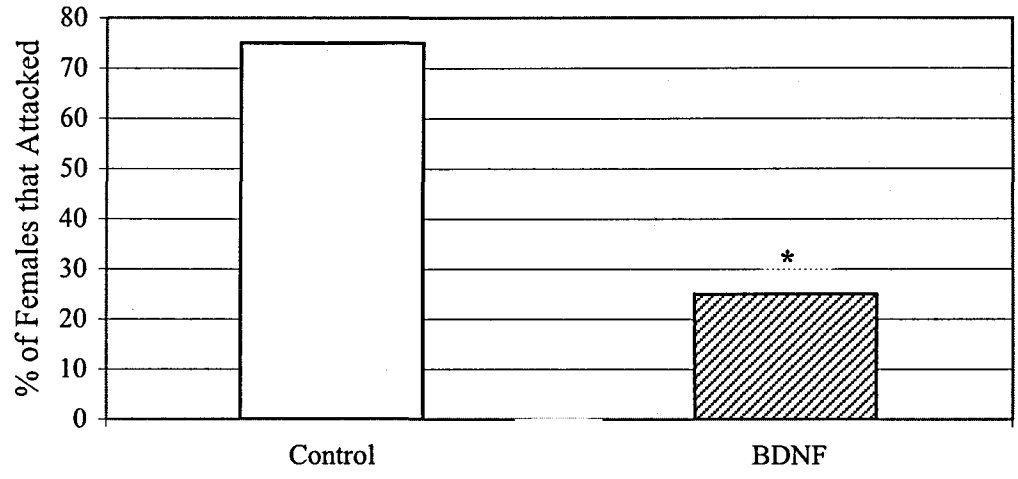
* Adapted from Nelson, R. J. (1995). Female Reproductive Behavior (p. 273). In *An Introduction to Behavioral Endocrinology*. Sunderland, MA: Sinauer Associates, Inc.

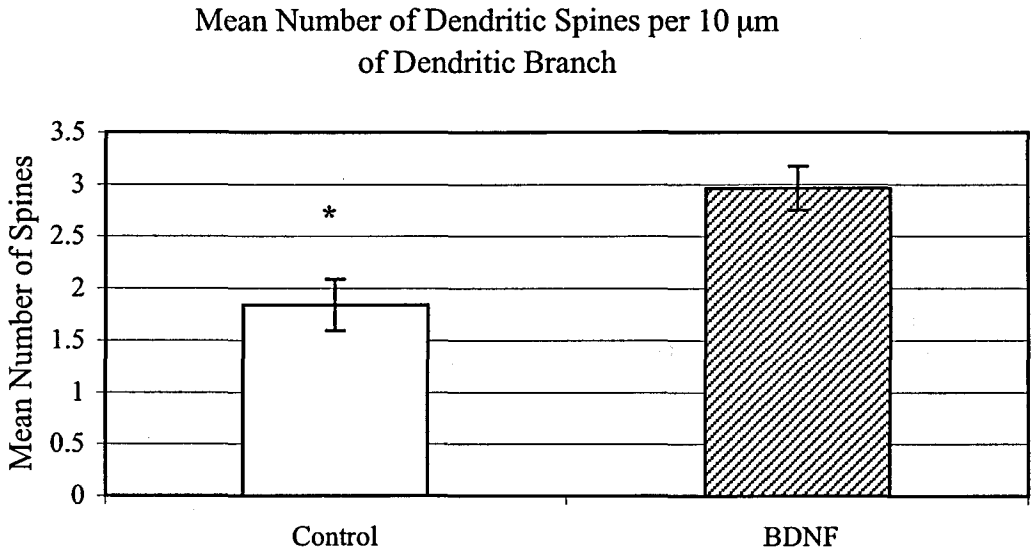
Hormonal Pattern of Pregnancy in the Rat*



* From Rosenblatt, J. S., & Siegel, H. I. (1981). Factors governing the onset and maintenance of maternal behavior among nonprimate mammals: The role of hormonal and nonhormonal factors. In Gubernick, D. J., & Klopfer, P. H. (Eds.), *Parental Care in Mammals* (p. 16). New York: Plenum Press.

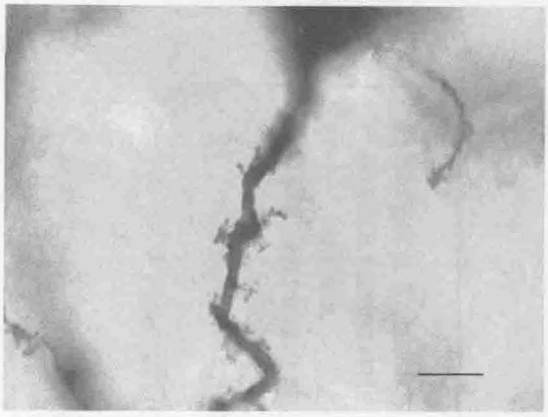
Percentage of Females that Attacked During the First 24 Hours of Pup Exposure



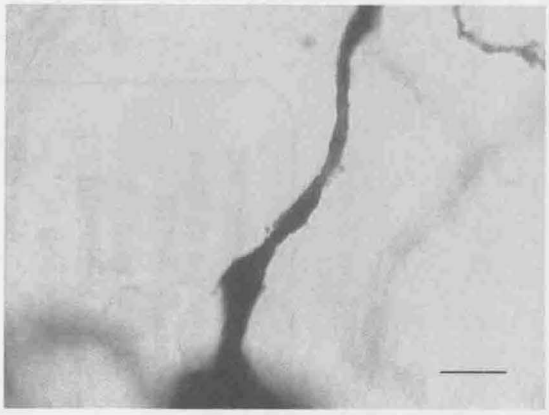

Mean Number of Dendritic Spines per 10 μm of Dendritic Branch
Mean Number of Spines
3.5
3
2.5
2
1.5
1
0.5
0
*
Control
BDNF

Photomicrographs of Representative BDNF Sense Infused Neurons and Control Neurons at a Total Magnification of 1,260x

BDNF Neuron



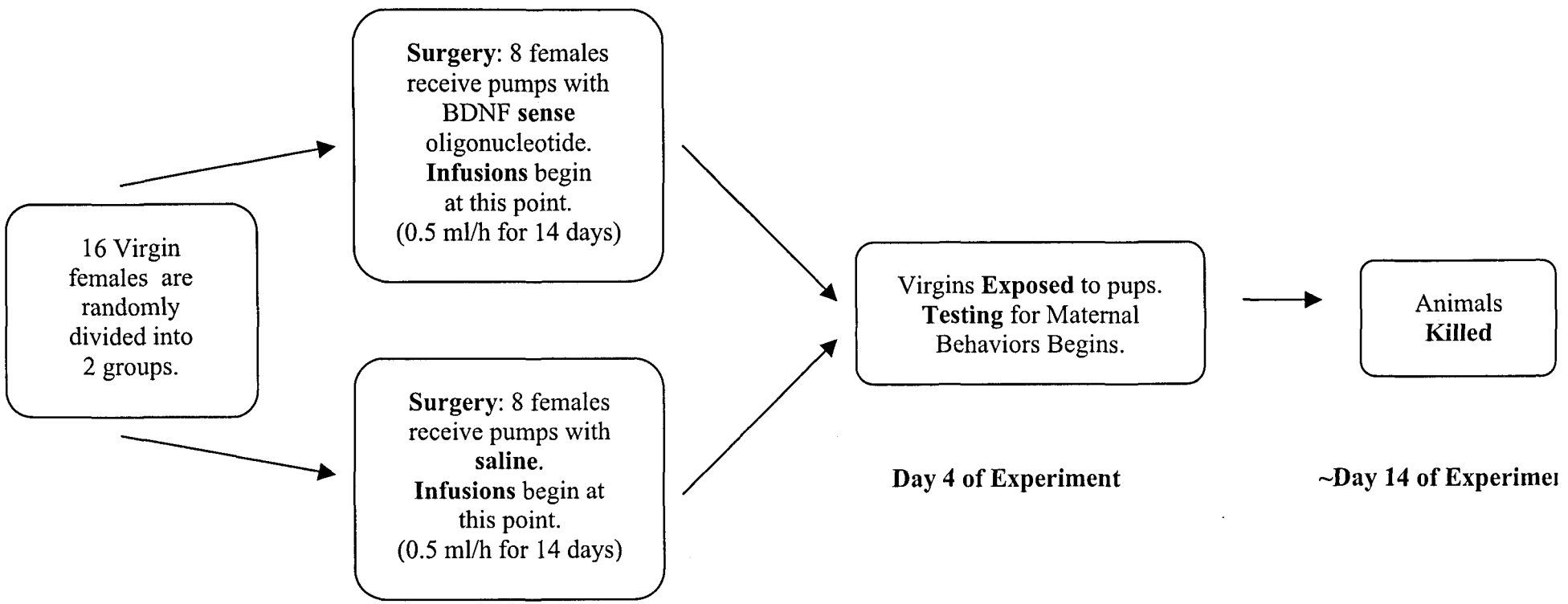
Control Neuron



Scale Bars = 6 μm

Appendix A

General Sequence of Events:



Appendix B

Schedule of Events for Maternal Behavior Testing:

	Day 1			Day 2				Days 3 - 10		
Time	Prime	Test	Spot Check	Prime	Remove	Test	Spot Check	Remove	Test	Spot Check
11:15am	1 and 2									
11:30am	3 and 4									
11:45am				5 and 6						
12:00pm				7 and 8	1 and 2			1 and 2		
12:15pm		1 and 2			3 and 4	1 and 2		3 and 4	1 and 2	
12:30pm		3 and 4				3 and 4		5 and 6	3 and 4	
12:45pm			1 and 2			5 and 6	1 and 2	7 and 8	5 and 6	1 and 2
1:00pm			1, 2, 3 & 4			7 and 8	1, 2, 3 & 4		7 and 8	1, 2, 3 & 4
1:15pm			1, 2, 3 & 4				1, 2, 3, 4, 5 & 6			1, 2, 3, 4, 5 & 6
1:30pm			3 and 4				3, 4, 5, 6, 7 & 8			3, 4, 5, 6, 7 & 8
1:45pm							5, 6, 7, & 8			5, 6, 7 & 8
2:00pm							7 and 8			7 and 8