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The Afferent Circuitry of the Ventromedial Hypothalamus and Its Activation in Paternal Behavior of the Socially Monogamous Prairie Vole

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THE AFFERENT CIRCUITRY OF THE VENTROMEDIAL HYPOTHALAMUS AND ITS ACTIVATION IN PATERNAL BEHAVIOR OF THE SOCIALLY MONOGAMOUS PRAIRIE VOLE

BY

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Abstract:

Paternal behavior is an interesting and important research topic due to its integral contribution to the fitness and well-being of multiple species, including humans. Although paternal behavior is well described in literature, attempts at neurobiological characterization have yielded conflicting results that fail to address brain region interconnectivity. This study was designed to evaluate the relationship between afferent VMH circuitry and the onset of paternal behavior, using the prairie vole (*Microtus ochrogaster*) model. Sexually naïve male prairie voles received injections of the retrograde neurotracer Fluoro-Gold (FG), into the VMH. Two weeks later, subjects were exposed to either conspecific pups, contained within a tea-ball, or an empty tea-ball (control) for 1 hr. Immunohistochemical labeling was conducted for both FG and the neuronal activity marker Egr-1, in order to evaluate neuronal and afferent pathway activation between the ventromedial hypothalamus (VMH) and the amygdala (AMYG), bed nucleus of the stria terminalis (BNST), lateral septum (LS) and ventral tegmental area (VTA). Similar to the pathway implicated in the onset of maternal behavior, the results of this study showed pup exposure-induced neuronal activation in the AMYG and BNST, particularly in the efferent pathways from these two brain areas to the VMH. This effect was not found in the LS and VTA projection neurons to the VMH. Together, the data suggests a brain region-specific neuronal activation by pup exposure in particular brain circuitry, implicating its possible involvement in paternal behavior.

Introduction:

Phylogenetic transitions from uniparental, maternal care, to biparental care often evolve through necessary tradeoffs between offspring survival and paternal investment (Reynolds et al., 2002; Webb et al., 1999). Paternal behavior becomes more important to species fitness as environmental pressures such as high male mortality rate, and limited resource access demand increased paternal investment in offspring care (Charpentier et al., 2008; Huchard et al., 2013; Klug et al., 2013; Wynne-Edwards & Lisk, 1989). The importance of paternal care cannot be overstated in humans; although it is often associated with physical and emotional development in children, paternal care also plays a pivotal role in the overall well-being of young adults. Data collected from young women and men in the United States has implicated early female reproductive development, and lower physical fitness, with paternal absence (Isgor et al., 2013; Quinlan, 2003). Furthermore, significant increases in aggressive behaviors, attention problems and youth incarceration have been documented in children whose fathers are absent (Geller et al., 2012; Harper & McLanahan, 2004). Although the impact of paternal presence and the display of paternal behavior on health and development of adult and children are well documented in humans, the underlying neuronal interactions involved in paternal behaviors are more enigmatic (Wynne-Edwards & Timonin, 2007).

Animal research has also shed light on the importance of paternal presence on offspring well-being and species fitness; even in invertebrates, such as arthropods and insects, paternal investment has been found to increase offspring survival and reproductive success (Tallamy, 2001; Thornhill, 1976). The effects of paternal investment are also documented in vertebrate animals. In some bird species such as the house sparrow (*Passer domesticus*) and the dark-eyed junco (*Junco hyemalis*), the father contributes predominantly through both resource gathering and nest defense, with paternal investment directly correlated with offspring growth, and inversely correlated with nest predation

(Schwagmeyer & Mock, 2008; Wolf et al., 1988). Multi-male primate societies with intricate social hierarchies display paternal behavior in both cases where paternity is certain, and when it is not. In primates, purely paternal behavior reduces risk of infanticide from rival males, increases their offspring's access to vital resources and is correlated with offspring maturation rate; non-paternal male parental behavior is correlated with the overall fitness and reproductive success of the group (Baker et al., 1993; Charpentier et al., 2008; Huchard et al., 2013; Paul et al., 1996; van Schaik & Paul, 1996). Paternal behavior is also present in a variety of rodent species. In bi-parental rodents, such as the California mouse (*Peromyscus californicus*) and the Djungarian dwarf-hamster (*Phodopus sungorus*), fathers contribute to resource gathering and provide necessary body warmth in cold climates (Gubernick et al., 1993; Wright & Brown, 2002; Wynne-Edwards, 1995; Wynne-Edwards & Lisk, 1989). Rodents exhibiting bi-parental care are of particular importance to research due to their ease of cultivation in a laboratory setting, and have contributed greatly to our understanding of the effects of paternal behavior. For example, studies using the Mandarin vole (*Lasiopodomys mandarinus*) have linked parental deprivation with increased anxiety behaviors, as well as detriments in social behavior, in offspring (Jia et al., 2009; L. Wang et al., 2014).

Attempts to characterize the neurobiology of paternal behavior have yielded conflicting results that fail to consider the importance of neuronal connectivity between regions, although it has been demonstrated in previous research that the lateral septum (LS) is of integral importance in the development of paternal behavior (Harris et al., 2011; Kirkpatrick et al., 1994; B. Wang et al., 2015; Z. Wang et al., 1994; Wynne-Edwards & Timonin, 2007). Indeed, understanding of the neurobiology of paternal behavior is limited, but much may be gleaned from the extensive research conducted on maternal behavior. Studies using sexually naïve female rodent models have outlined the importance of several regions within the limbic system in the onset of maternal behavior, including the amygdala (AMYG), and the ventromedial hypothalamus (VMH). During pup exposure and interaction, the

AMYG receives input from both the olfactory system and the VMH before projecting to the medial preoptic area (MPOA) via the bed nucleus of the stria terminalis (BNST) output pathway (Gardner & Phillips, 1977). Activity in the MPOA has been correlated with increased maternal behavior in sexually naïve female rats, while the medial amygdala (MeA) and VMH have been shown to inhibit maternal behavior by decreasing neuronal activity in the MPOA (Bridges et al., 1999a; Mann & Babb, 2004; Morgan et al., 1999; Numan et al., 1998; Olazabal & Ferreira, 1997). These findings are augmented by additional rodent research confirming that the MPOA asserts its effects on maternal and sexual behaviors via interactions with the mesolimbic dopamine pathway, at least in part via specific connections with the ventral tegmental area (VTA). (Geisler & Zahm, 2005; Numan & Numan, 1997; Numan et al., 2009). This discovery is particularly provocative due to its implication that the limbic system works in tandem with the mesolimbic dopamine pathway to regulate the onset maternal behavior, a notion not yet tested in paternal behavior. Combining this knowledge with electrophysiological research showing robust connections between the LS and the VMH and increased neuronal activation of the LS during maternal behavior, the interactions between these regions appear to be an excellent candidate for involvement in the onset of paternal behavior (Blume et al., 1982; Numan & Insel, 2003).

The socially monogamous prairie vole (*M. ochrogaster*) is a superior model for paternal behavior in comparison to traditional rodent models, as it has been shown to exhibit bi-parental care both in the wild and in laboratory settings (Oliveras & Novak, 1986; Thomas & Birney, 1979). After the birth of a litter, male prairie voles exhibit all patterns of the parental behaviors displayed in females, except nursing; these behaviors continue after the birth of subsequent litters (Wang & Novak, 1992). In the present study, we investigated the circuitry of the VMH projecting neurons and their activation in response to paternal behavior. The VMH was chosen as the focus of this study due to its position at the neurobiological forefront of the maternal behavior pathway in sexually naïve female rats, as well

as its afferent connections with the LS and the VTA (Blume et al., 1982; Geisler & Zahm, 2005; Mann & Babb, 2004; Sheehan et al., 2001; Stolzenberg & Numan, 2011).

This investigation tested the hypothesis that ***pup exposure activates the afferent circuitry of the VMH in connection with the AMYG, BNST, MPOA, LS and VTA.*** The retrograde neuronal tracer Fluoro-Gold (FG) was injected into the VMH to map its afferent connections with the AMYG, BNST, LS, MPOA, and the VTA. The immediate gene marker, early growth response protein 1 (Egr-1), was also used to map the brain region-specific neuronal activation due to pup exposure. Together, the data indicates the selective afferent VMH pathways activated by exposure to pup associated cues, implicating its potential role in male parental behavior.

Methods:

Subjects:

The subjects consisted of adult (90 – 150 days of age), sexually naïve male prairie voles (*Microtus ochrogaster),* from the laboratory breeding colony, weaned at 21 days of age and housed in same-sex sibling pairs inside plastic cages (12 x 28 x 16 cm) that contained cedar chip bedding. Water and food were provided *ad libitum*. The cages were maintained at 20 ± 1 °C with a 14:10 h light-dark cycle (lights on at 07:00 h). All subject voles were randomly assigned into experimental groups between the ages of 70 –120 days. All experimental procedures were approved by the Animal Care and Use Committee of Florida State University and conformed to the guidelines set forth by the NIH.

Retrograde Tracer Fluoro-Gold (FG) Injection

FG is a reliable neuronal tracer due to its potent axonal uptake at the site of injection, and its ability to be visualized via immunohistochemistry, cytoplasmically (Persson & Havton, 2009). The subject was anesthetized via sodium pentobarbital (0.1mg/10g body weight) and then received FG

injections into VMH bilaterally, using a stereotaxic coordinate system. The coordinates were as follows: -1.4 mm anterior-posterior, ± 0.6 mm medial-lateral, and 6.9 mm dorsal-ventral. The injections were achieved using glass capillary micropipettes (A-M Systems, Inc., Carlsborg, WA) filled with 2% Fluoro-Gold (FG; Fluorochrome, Englewood, CA) in 0.01 M phosphate buffer solution (PBS; pH 7.4). The micropipette was pre-stretched using a vertical micropipette puller (Sutter Instruments, Novato, CA) to achieve an inner tip diameter between 15-35 μm. The injection volume was 50 μL; FG was allowed to diffuse for 15 min before removal of the micropipette. The FG injected subjects were sent back to the home-cage for 2 weeks allowing tracer to be transferred back to projection cell bodies. Injection placement was evaluated via FG immunohistotology. Only subjects with correct bilateral placement were included in data analysis.

Pup Exposure:

Two weeks post intra-VMH tracer injection, the subject was allowed to habituate to the transparent plastic testing cage (13 x 29 x 16 cm) for ten minutes. Afterwards, empty mesh tea ball (D = 4.5 (cm), control), or tea ball containing two of day-3 pups was introduced to the opposite side of the cage. The test lasted for 1 hr with food and water provided. All tests were video recorded for subsequent behavioral analysis.

Tissue Processing and Immunocytochemistry:

Immediately following the pup exposure test, subjects were deeply anesthetized with 0.1 mL ketamine and transcardially perfused through the ascending aorta, with chilled 0.9% saline followed by chilled 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were harvested, post-fixed in the same fixative at 4 °C for 2 hr, and then transferred to 30% sucrose in 0.1 M phosphate buffer until brains sank. Brains were then sectioned coronally at 40 μm via microtome.

Brain sections were stored in 0.1 M PBS with 1% sodium azide, at 4 °C until processing via immunohistochemistry, to prevent bacterial growth. In order to examine FG labeled neuronal activation during pup-exposure, FG and early growth response protein (Egr-1) double labeling was processed. The expression of Egr-1 is correlated with neuronal activation specifically linked with neuronal plasticity (Knapska & Kaczmarek, 2004).

Brain sections, sliced at 120 um intervals, were then processed. Sections were treated with 3% H_2O_2 in 0.1 M PBS to block endogenous peroxidase activity and then incubated in a blocking solution consisting of 10% normal goat serum (NGS) in 0.3% Triton X-100 in 0.1 M PBS (TPBS) for 1 hr. Afterwards, sections were incubated in 1:5K rabbit anti-Egr-1 (Cell Signaling; Danvers, MA.) in 2% NGS in 0.3% TPBS at 4°C overnight. Thereafter, sections were incubated in 1:300 goat anti-rabbit secondary antibody in 2% NGS in 0.3% TPBS at room temperature for 2 hrs, and then incubated in Vector Elit ABC complex for 1.5 hrs. Egr-1 nuclear labels were visualized by DAB (Sigma-Aldrich; St. Louis, MO.) staining. Next, Egr-1 labeled sections were incubated in 5% NGS in 0.3% TPBS at room temperature for 30 min, then incubated in 1:10K rabbit anti-FG antibody (Fluorochrome LLC; Denver, CO.) at 4°C overnight. Subsequently, sections were incubated in goat anti-rabbit antibody and then Elit ABC complex. Sections were stained with Vector ® SG Substrate (Vectorlabs; Burlingame, CA) Brain sections were mounted on glass microscopy slides and cover-slipped.

Data Analysis:

The video recorded pup exposure test was scored using the JWatcher™ behavioral analysis program. The behaviors scored, for both frequency and duration, were as follows: investigative behavior, defined as approaching the tea-ball; contact behavior, defined as physical contact with the tea-ball and locomotive behavior, defined as any non-stationary activity. These behaviors were

analyzed using a paired Student's *t*-test, with pup vs. empty tea-ball exposure as the independent variable. The criteria of significance was set at $p < 0.05$.

All microscope slides were coded to disguise the treatment group identity until data quantification was complete. The slides were examined using a Zeiss Axioskop II microscope. Egr-1 and FG single-labeled, as well as Egr-1/FG colabeled neurons, were quantified respectively in the following brain regions: the central (CeA), cortical (CoA), and medial (MeA) amygdala; the dorsal and ventral bed nucleus of the stria terminalis (BNST); the dorsal and ventral lateral septum (LS); and the ventral tegmental area (VTA). The quantification was conducted bilaterally on three matched coronal sections confirmed via a stereotaxic rodent-brain atlas, with 95% reliability. The data was analyzed using a paired Student's *t*-test, with pup vs. empty tea-ball exposure as the independent variable. The criteria of significance was set at $p < 0.05$.

Results:

During the pup exposure test, sexually naïve male prairie voles were exposed to either a mesh tea-ball containing two conspecific pups, or an empty tea-ball. The results of the pup exposure test showed male voles that were exposed to a tea-ball containing two conspecific pups showed a significant increase in frequency of investigation ($p = 0.001$; $T_{10} = 4.52$) and contact behavior ($p = 0.0002$; $T_{10} = 5.35$) in comparison to the control males exposed to the empty tea-ball (Fig 2A). The pup exposure group also showed a significant increase in the duration of investigation ($p = 0.0002$; $T_{10} = 5.66$) and contact behavior ($p = 0.0004$; $T_{10} = 5.24$) (Fig 2B). There was no significant difference in total locomotive behavior between the pup exposure and control group (Fig 2 A and B).

After pup exposure, immunohistochemical labeling was conducted for both Egr-1 and FG. The retrograde neuronal tracer FG appears as a cytoplasmic stain and denotes afferent connectivity between the region it was injected into and the region in which labeling is observed (Persson &

Havton, 2009). The marker Egr-1 appears as a nuclear stain, and its presence is correlated with neuronal activity associated with pup exposure (Knapska & Kaczmarek, 2004). Because Egr-1 labeling appears as nickel-intensified dark-brown nuclear staining, whereas FG labeling appears as blue DAB cytoplasmic staining, both of these markers may be observed simultaneously within a colabeled neuron (Fig 1). The presence of colabeled neurons denotes activation of the afferent pathway between the VMH and the region in which the colabeled neurons are present.

Egr-1 staining indicated neuronal activation in many brain areas. In the AMYG, male voles that were exposed to a tea-ball containing two conspecific pups showed a significant increase in the number of Egr-1 labeled cells, compared to control males that were exposed to an empty tea-ball ($p = 0.024$; $T_9 = 2.71$) (Fig 3A). No group differences were found in the number of neurons labeled for FG (Fig 3A). However, the pup exposure group had more neurons that were colabeled for FG and Egr-1, compared to the control males ($p = 0.034$; $T_9 = 2.49$) (Fig 3A), indicating increased neuronal activation of the afferent projection neurons between the AMYG and the VMH due to pup exposure. A similar trend was also found in the percentage of total FG labeled neurons that contained Egr-1 staining but this difference did not reach to statistical significance ($p = 0.129$; $T_9 = 1.67$) (Fig 3B). Finally, no group differences were found in the number of Egr-1 single, FG single, and FG/Egr-1 colabeled cells in any of the subnuclei of the AMYG (Table 1). In the cortical nucleus of the AMYG, pup exposure group had a higher percentage of FG cells that were co-stained for Egr-1, compared to the control (Table 1).

In the BNST, male voles that were exposed to a tea-ball containing a conspecific pup showed a significant increase in the number of Egr-1 labeled cells, compared to control males that were exposed to an empty tea-ball ($p = 0.009$; $T_{10} = 3.17$) (Fig 4A). No group differences were found in the number of neurons labeled for FG (Fig 4A). The pup exposure group had more neurons that were

colabeled for FG and Egr-1, compared to control males ($p = 0.010$; $T_{10} = 3.11$) (Fig 4A), indicating increased neuronal activation of afferent projections between the BNST and the VMH due to pup exposure. Furthermore, there was a significantly higher percentage of total FG labeled neurons that contained Egr-1 staining in the pup exposure group compared to the control group ($p = 0.035$; $T_{11} = 2.41$) (Fig 4B). Lastly, the increased Egr-1 single labeling, colabeling with FG, and percent of total FG neurons colabeled with Egr-1, were most prominent in the ventral portion of the BNST (vBNST) (Table 2).

In both the LS and the VTA, there was no significant difference in Egr-1 single labeled, Egr-1/FG colabeled or percent of total FG labeled neurons colabeled with Egr-1, indicating the afferent pathways between the VMH and these regions were not activated during pup exposure (Fig 5 A, B, C and D). Once more there was no difference in the total number of FG labeled neurons between the control and pup exposure groups for either of these regions. Finally, due to low stain contrast in the MPOA, no data was gathered from this region.

Discussion:

Paternal behavior is an integral contributor to the fitness and well-being of multiple species, including humans, yet remains underrepresented in neurobiological research. Scant attempts at neurobiological characterization have yielded conflicting results that fail to address the importance of brain region interconnectivity. This study was designed to evaluate the relationship between afferent VMH circuitry in the onset of paternal behavior, using the prairie vole model. Our data shows that pup exposure increased neuronal activation both in the AMYG and BNST, particularly in efferent projections with the VMH; such effects were not found in the LS and VTA efferent projections with the VMH. This data indicates region-specific neurocircuitry that is activated during pup exposure and possibly involved in the parental behavior of the male prairie vole.

The behavioral data collected during the pup exposure test showed sexually naïve male prairie voles, exposed to a mesh tea-ball containing two conspecific pups showed both a higher frequency and duration of investigative and contact behaviors in comparison to the control group exposed to the empty tea-ball. This data indicates that although the subject males were not permitted direct contact with the pups, interaction still occurred.

The data showed that exposure to pup associated cues increased neuronal activation in the AMYG and the BNST, as indicated by increased expression of Egr-1. The transcription regulatory protein Egr-1 was chosen as the neural marker for several reasons, most important of which is its expression in concurrence with synaptic activity. In this situation Egr-1 held a distinct advantage over other endogenous markers of neuronal activity, such as the transcription factor c-fos, due to its consideration not only in context with neuronal activity, but its tight linkage with neuronal plasticity (Knapska & Kaczmarek, 2004). Interestingly, a significant increase in Egr-1 expression was found in the AMYG and the BNST, yet this increase was localized to the ventral subnuclei of the BNST (vBNST), but no localized increase was found in the any AMYG subnuclei. The increased neuronal activity seen in the vBNST was consistent with similar findings using neuronal activation markers in female rodents during the onset of maternal behavior, indicating this pathway may function similarly in both male and female parental behavior (Lonstein & De Vries, 2000; Numan & Numan, 1995; Numan et al., 1998). Again, the results of this study showed that the overall AMYG, rather than select subnuclei, was activated during pup exposure. This may indicate that rather than a specific subnuclei, the AMYG as a whole may be activated during pup exposure and paternal behavior. This lack of regiospecific neuronal activity in the AMYG was surprising given previous studies concerning maternal behavior, in which the MeA and CeA specifically were implicated in the regulation of maternal behavior (Lonstein et al., 1997; Numan & Numan, 1995; Sheehan et al., 2001). The differences in regiospecific activation between male and females within the AMYG during pup

exposure may be due the sexually dimorphic nature of AMYG circuitry, although this has yet to be tested (Cooke & Woolley, 2005; Nishizuka & Arai, 1981). There was no altered neuronal activation seen in either the LS or the VTA of male prairie voles in response to pup exposure during the present study. While it has been previously shown that the LS is of integral importance in the development of paternal behavior, its activation in response to pup exposure had yet to be shown (Z. Wang et al., 1994). Additionally, mother-pup interaction has been shown to elicit neuronal activation in the VTA, yet pup exposure did not elicit a similar effect in males (Numan & Insel, 2003). While sex differences may account for the lack of increased neuronal activation in the VTA and LS in the male voles, it is also possible that chemosensory stimuli alone was not sufficient, and direct pup interaction would be necessary to elicit a neuronal response.

The afferent neuronal pathways between the VMH and the AMYG, BNST, VTA, LS and MPOA, were mapped using the retrograde tracer FG, which was injected into the VMH. Immunohistochemical analysis yielded clear FG labeling in the AMYG, BNST, LS and the VTA, confirming afferent neuronal connections between the VMH and these regions, as shown in previous electrophysiological and tracer studies (Blume et al., 1982; Numan & Numan, 1997; Sheehan et al., 2001). More importantly, no significant difference was found between the total number of FG labeled neurons in any of the regions, or subnuclei analyzed. This implied that any significant increase in the number of Egr-1/FG colabeled neurons between the control and pup exposure group was due to the pup exposure condition and not due to increased density of FG labeled cells in the region.

As mentioned previously, this study confirmed the activation of two regions within the limbic system in response to pup exposure, the AMYG and the BNST, but the most critical aspect of this study was the inclusion of markers for neuronal activation (Egr-1) combined with a tracer analysis (FG). Colabeling of both Egr-1 and FG within the same neuron indicates that not only was that

neuron activated in response to the stimulus, but also was part of the afferent pathway between the VMH and the region containing the neuron. Similar to the Egr-1 single-labeled data, activation of the afferent pathway between the VMH and both the AMYG and the BNST was documented. These results seem sensible when compared with previous neuronal tracer studies confirming connectivity between the vBNST and the CeA, and the role of efferent projections between the AMYG and the VMH, via the vBNST, on the regulation of maternal behavior (Numan et al., 1998; Sheehan et al., 2001; Wood & Swann, 2005). Furthermore, although the results of this study show activation of the afferent pathway between the VMH and the AMYG was not localized to any subnuclei, similar findings in female rodents strongly attribute connections between the MeA and the VMH in the regulation of maternal behavior (Sheehan et al., 2001). The sexually dimorphic nature of AMYG synaptic organization has been previously documented, particularly in the MeA. Thus, the equally distributed neuronal and afferent VMH pathway activation, seen within the AMYG, may imply neurobiological differences between pathways responding to paternal versus maternal experience (Cooke & Woolley, 2005; Nishizuka & Arai, 1981). Possible future studies comparing pup exposure effects between males and females, using the prairie vole model, may support this conjecture.

The lack of both neuronal and pathway activation in the LS was quite surprising, but not unanticipated, as increased neuronal activity, as well as changes in vasopressin (AVP) innervation in the LS occurs concurrently with increased paternal responsiveness in the male prairie vole. The rapid increase in AVP immunoreactive fibers happens naturally after three days of pair-bonding with a conspecific female. Additionally, behavioral studies have shown an increase in vole paternal behavior in response to tactile cues from their conspecific female partner (Simoncelli et al., 2010; Z. Wang et al., 1994). Thus, it is not unreasonable to speculate that LS activation in response to pup exposure may have a longer time-course than can be shown using the Egr-1 neural activity marker; or perhaps pair bonding with a female is necessary to expedite this process. Conversely the lack of neuronal

activity and pathway activation in the VTA was much more puzzling. Interactions between the VMH and the mesolimbic dopamine system, specifically the VTA, is heavily implicated in the onset of maternal behavior in sexually naïve female rodents (Numan & Smith, 1984; Numan & Stolzenberg, 2009; Stolzenberg & Numan, 2011). Although these findings were not successfully replicated in males, this does not exonerate the mesolimbic dopamine system from the regulation of male paternal behavior. Similarly to the LS, it is possible that the activation of the VTA also has a longer time-course than can be shown using Egr-1. Potential future studies should colabel neuronal tracer and neural activity markers in pair bonded male prairie voles at various stages of paternal behavior in order to elucidate possible time-dependent pathway activation.

Several caveats should be discussed. First, the MPOA was not examined in the present study, although it has been well implicated in maternal behavior. This was due to very dark background staining, causing low contrast, preventing clear visualization of the MPOA, and thus data collection was not possible in this region. This was most likely due to a combination of factors, including close proximity between and the MPOA and the VMH injection site, causing heavy FG concentration, and close proximity between the MPOA and ventral edge of the brain, causing heavy staining. Second, pup exposure was conducted using a tea ball to protect the pups from infanticide behavior, which occurs in roughly 10-20% of sexually naïve male prairie voles (Carter et al., 1995). Although our data showed neuronal activation associated with "exposure to pup associated cues," we cannot distinguish the effects of the pup sensory stimulation from the display of paternal behavior.

The activation of the afferent pathway between the VMH and the AMYG, as well as between the VMH and the vBNST, in males during exposure to conspecific pups suggests a need for additional studies characterizing this pathway. First, the roles each of these individual regions plays in the onset of paternal behavior must be considered; this has been successfully done with maternal behavior, using

ablation. In sexually naïve female rodents, ablations to the vBNST and MPOA inhibited the onset of maternal behavior. Additionally, ablations to the MeA and VMH induced the onset of maternal behavior, while also decreasing neural activation in the vBNST and MPOA (Bridges et al., 1999b; Kalinichev et al., 2000; Mann & Babb, 2004; Numan & Numan, 1996; Sheehan et al., 2001). A similar procedure using the male prairie vole would reveal whether these pathways exert an inhibitory or excitatory effect on paternal behavior. Next, neurochemical characterization of the afferent pathway between the VMH and the AMYG in response to pup exposure must be conducted. First, it may be prudent to consider the role of the neuropeptides oxytocin (OT) and vasopressin (AVP), as increased expression of both OT and AVP occurs postpartum in both males and females; additionally, both of these hormones are involved in the regulation of maternal behavior (Bosch & Neumann, 2012). OT antagonists have been shown to disrupt the response of the MeA to chemosensory cues in male mice, but whether or not OT expression in the AMYG modulates male chemosensory sensitivity to pups has yet to be tested (Samuelsen & Meredith, 2011). AVP gene expression in the VMH increases in both females and males postpartum, furthermore: maternal behavior has been associated with AVP release in BNST, which is the primary afferent connection between the VMH and the AMYG (Sheehan et al., 2001). A logical future study would include the use of a tracer to observe OT and AVP binding and *in-situ* hybridization for AVP and OT mRNA expression in the AMYG, VMH and BNST during pup exposure.

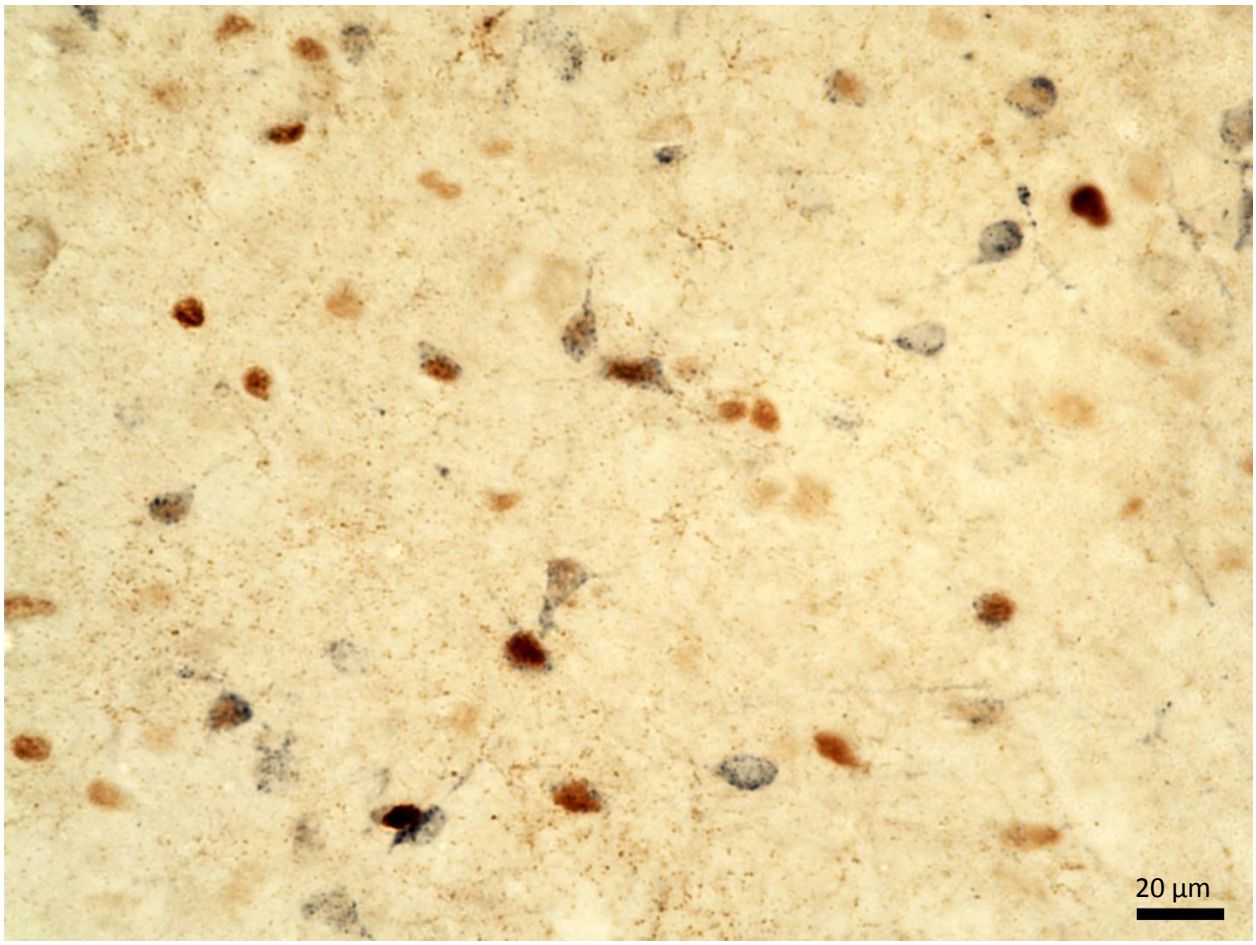

Figure 1: Microscopic slide, 20x magnification, Egr-1 and FG immunohistochemistry. Egr-1 labeled neurons present with a dark brown, nuclear stain. FG labeled neurons present with a blue cytoplasmic stain. Egr-1/FG colabeled neurons present with both brown nuclear and blue cytoplasmic stain.

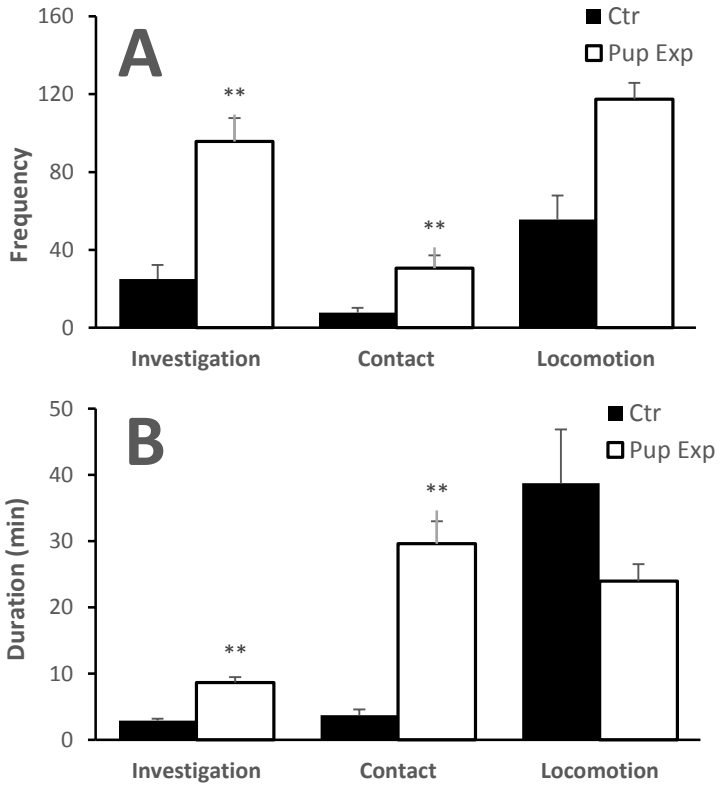

Figure 2: Sexually naïve prairie voles showed increased interaction with tea-ball filled with conspecific pup vs. empty tea-ball. Males that were exposed to a conspecific pup within tea-ball exhibited significantly higher frequency of investigation and contact behavior, with the tea-ball, compared to the control males exposed to the empty tea-ball; no group differences were found in frequency of locomotion (A). Additionally, pup exposure elicited a higher duration of investigation and contact behavior, compared to control; no group differences were found in the duration of locomotion (B) *: $p < 0.001$. Error bars represent SEM.

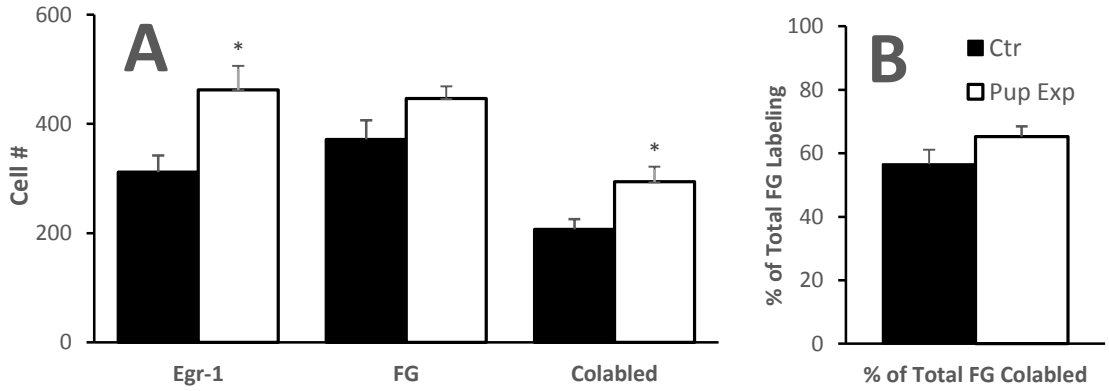

Figure 3: Pup exposure altered neuronal activation of the VMH-projecting neurons in the amygdala (AMYG) of sexually naïve prairie voles. Males that were exposed to a conspecific pup had more cells labeled for Egr-1 and colabeled for FG/Egr-1 in the AMYG, compared to control males (A). No group differences were found in the total number of FG labeled cells (A) or % of FG-labeled cells that colabeled for Egr-1 (B) *: $p < 0.05$. Error bars represent SEM.

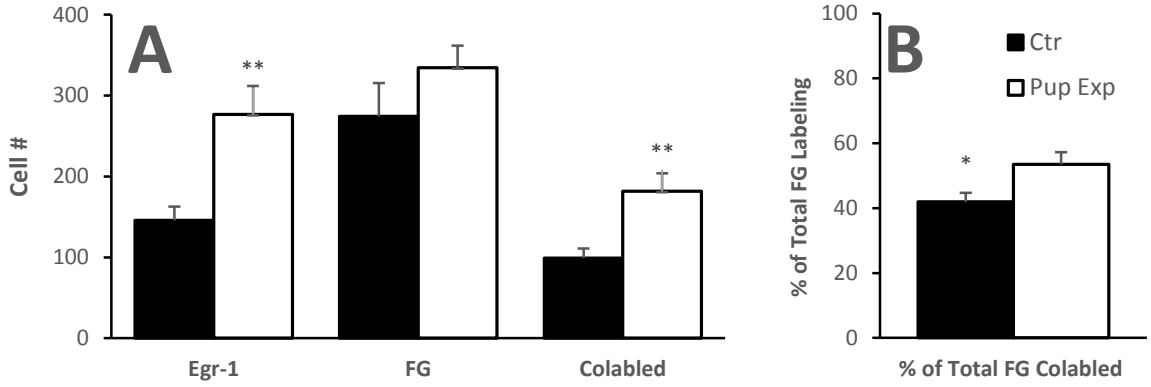

Figure 4: Pup exposure altered neuronal activation of the VMH-projecting neurons in the bed nucleus of the stria terminalis (BNST) of sexually naïve prairie voles. Males that were exposed to a conspecific pup had more cells labeled for Egr-1 and colabeled for FG/Egr-1 in the BNST, compared to control males (A). No group differences were found in the total number of FG labeled cells (A). Males that were exposed to a conspecific pup also had a larger % of total FG-labeled cells colabeled for Egr-1, compared to control males (B) *: $p < 0.05$. Error bars represent SEM.

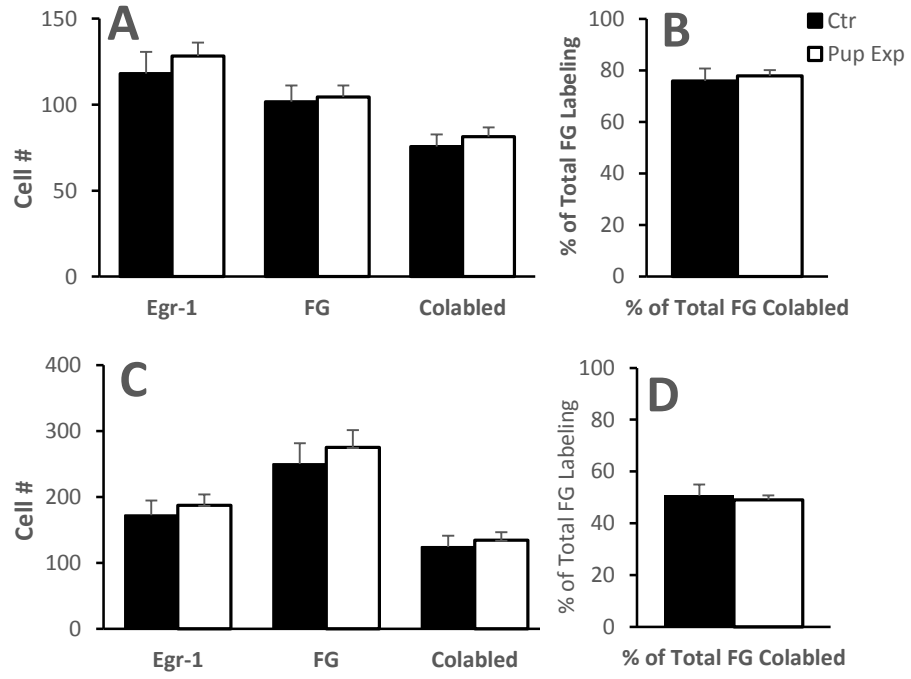

Figure 5: Pup exposure did not alter neuronal activation of the VMH-projecting neurons in the (A) lateral septum (LS) or (B) ventral tegmental area (VTA) of sexually naïve prairie voles. Males that were exposed to a conspecific pup showed no difference in cells labeled for Egr-1 or colabeled for FG/Egr-1 in the either the LS or VTA, compared to control males (A & C). No group differences were found in the total number of FG labeled cells (A & C). Males that were exposed to a conspecific pup also showed no difference in the % of total FG-labeled cells colabeled for Egr-1, compared to control males (B) *: $p < 0.05$. Error bars represent SEM.

Table 1: Total number of Egr-1 single labeled, FG single labeled, and colabeled neurons as wells as the percent of total FG labeled neurons colabled with Egr-1, in the amygdala subnuclei.

Amygdala Subregions	Egr-1			FG			Colabled			% Total FG Colabled		
	Control	Pup Exp.	P Value	Control	Pup Exp.	P Value	Control	Pup Exp.	P Value	Control	Pup Exp.	P Value
CeA	69.4±16	111±19	NS	75.3±12	108±10	NS	45.9±10	74.1±12	NS	60.6±6.6	66.6±5.5	NS
CoA	133±18	183±22	NS	159±9.1	176±13	NS	90.3±12	116±14	NS	56.1±5.0	64.7±3.4	< 0.05
MeA	116±16	145±16	NS	141±21	147±13	NS	75.1±10	90.8±9.2	NS	56.5±7.4	61.7±3.3	NS

Table 2: Total number of Egr-1 single labeled, FG single labeled, and colabled neurons as wells as the percent of total FG labeled neurons colabled with Egr-1, in the BNST subnuclei.

BNST Subregions	Egr-1			FG			Colabeled			% Total FG Colabeled		
	Control	Pup Exp.	P Value	Control	Pup Exp.	P Value	Control	Pup Exp.	P Value	Control	Pup Exp.	P Value
Dorsal	61.0±8.0	89.0±10	NS	121±18	126±12	NS	51±9.3	59.1±6.1	NS	42.1±3.1	46.6±2.4	NS
Ventral	84.8±14	188±27	< 0.01	152±23	208±19	NS	56.3±8.1	123±18	< 0.01	46.1±4.5	57.7±5.1	< 0.05

Note: Both means and standard errors are provided for control and pup exposed subjects. Values for Egr-1, FG and colabled reflect the total number of stained neurons in the amygdala and BNST subnuclei. Groups include the control group exposed to the empty tea-ball and the pup exposure group.

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