

ABSTRACT

Title of Thesis: NEURONAL ACTIVATION FOLLOWING COHABITATION IN THE PRAIRIE VOLE (*MICROTUS OCHROGASTER*)

Degree candidate: Julie L Hazelton

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Thesis directed by: Professor Mary Ann Ottinger
Department of Animal and Avian Sciences

Sexually naïve female prairie voles require exposure to a novel male to activate the neural circuits involved in the formation of stable pair bonds and to stimulate sexual receptivity. The objective of our study was to investigate the neural pathways involved in the formation of pair bonds. Cushing et al. (2003) studied neuronal activation in selected brain regions, as expressed by c-Fos immunoreactivity (ir), during the first hour of cohabitation in prairie voles. In the present study, we extend the findings of this study to examine neuronal activation associated with cohabitation to include 2, 6 and 12 hr. In addition, we examined the potential colocalization of luteinizing hormone releasing hormone (LHRH) and c-Fos to determine if this is a system activated during the initial stages of pair bond formation. The selected time periods include the initiation of sexual activation of the female and pair bond formation. Expression of c-Fos as analyzed in three regions that play a role in early social encounters. These areas included: (1) the sociosexual behavior circuit, (2) the reward pathway, and (3) nuclei whose peptides regulate the actions of those networks.

Based on the previous data, increased c-Fos expression was predicted in the social behavior circuit, including the medial amygdala, bed nucleus of the stria terminalis, medial preoptic nucleus, and ventromedial nucleus of the hypothalamus. The lateral septum was examined due to its role in the social behavior circuit and in the process of pair bond formation.

Next, we predicted that increased c-Fos activity would be observed over time in regions that did not show increases during initial contact, but are involved in pair bond formation. These regions include two components of the reward pathway: the nucleus accumbens and the ventral pallidum. Finally, nuclei known to regulate both the social behavior and reward circuits were examined and included the supraoptic nucleus and the paraventricular nucleus, which produce oxytocin and vasopressin. These neuropeptides are critical in social behavior and the formation of pair bonds.

Immediately following the period of cohabitation (0, 1, 2, 6 or 12 hour in length) animals were separated and brains fixed. Fixed brains were sectioned at 30 μ m and stained with c-Fos and LHRH antibodies using double label immunocytochemistry (ICC) (Berghorn et al., 1994). Significant colocalization of LHRH and c-Fos was not observed in the first twelve hours in either sex of cohabitated prairie voles. Additionally there was no difference in number of LHRH ir neurons between sex or treatments. LHRH ir neurons in male and female prairie voles were predominantly located in the diagonal band of broca, preoptic area, lateral hypothalamus and supraoptic decussation. Individual LHRH neurons that did express c-Fos were predominantly located in the POA and LH.

We observed a sexually dimorphic temporal pattern in c-Fos ir in the circuitry involved in pair bond formation in prairie voles. This pattern suggests that incoming

information is first sorted through the social-sexual circuit and continues to be processed as information is received by nuclei of the reward pathway a short time later in both sexes. An increase in immediate early gene (IEG) immunoreactivity in the social behavior network is reported concurrently with peak activation of the reward circuit. During the same time period, an increase in c-Fos ir is reported in the nuclei involved in the endocrine control of partner preference and pair bond formation. Together these data suggest that in prairie voles, both the social and reward circuits interact early in cohabitation prior to reproductive activation to establish a heterosexual bond and that both circuits may be regulated by neuropeptides produced by the PVN and SON. This research was conducted by funding from the following: NSF IBN-9817024 (MAO), NIH HD 38490 (CSC, GEH, BSC, MAO) and MH 01992 (BSC).

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NEURONAL ACTIVATION FOLLOWING COHABITATION IN THE
PRAIRIE VOLE (*MICROTUS OCHROGASTER*)

by

Julie L Hazelton

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Advisory Committee:

Professor MaryAnn Ottinger, Chair/Advisor
Professor Gloria Hoffman
Associate Professor Carol Keefer

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LIST OF ABBREVIATIONS

ACo	anterior cortical amygdaloid nucleus
ACTH	adrenocorticotropic hormone
ARC	arcuate hypothalamic nucleus
AOB	anterior olfactory bulb
AVP	arginine vasopressin
BnST	bed nucleus of stria terminalis
CORT	corticosterone
CRH	corticotropin releasing hormone
DA	dopamine
DBB	diagonal band of broca
HPG	hypothalamic-pituitary- gonad
ICC	immunocytochemistry
icv	intracerebroventricular
ir	immunoreactivity
LD	laterodorsal thalamic nucleus
LH	luteinizing hormone
LHA	lateral hypothalamic area
LHRH	luteinizing hormone releasing hormone
LS	lateral septum
MeA	medial amygdaloid nucleus
MeAD	medial amygdaloid nucleus, anterodorsal
MePD	medial amygdaloid nucleus, posterodorsal
MePV	medial amygdaloid nucleus, posteroventral
MPOA	medial preoptic area
MPN	medial preoptic nucleus
NAcc	nucleus accumbens
OT	oxytocin
OTR	oxytocin receptor
PLC	prelimbic cortex
PLCo	posterolateral, cortical amygdaloid nucleus
PMv	preammillary nuclei, ventral
POA	preoptic area
poPVN	paraventricular nucleus, posterior
PVN	paraventricular nucleus
SCN	suprachiasmatic nucleus
SON	supraoptic nucleus
sox	supraoptic decussation
SuM	supramammillary nucleus
VMNvl	ventromedial hypothalamic nucleus, venterolateral
VNO	vomer nasal organ
VP	ventral pallidum
VTA	ventral tegmental area

INTRODUCTION

The monogamous prairie vole provides a dynamic social system in which signal integration is essential for pair bond formation and to stimulate sexual receptivity. Female prairie voles do not undergo a spontaneous estrous cycle. Instead they require chemosensory cues from urine and prolonged exposure to a novel male (Carter et al., 1995). During this period males and females form a long-term pair bond, which can occur in the absence of mating (Williams et al., 1992). The initial pattern of neuronal activation, after one hour of cohabitation has been described in prairie voles to include the medial amygdala (MeA), bed nucleus of the stria terminalis (BnST), medial preoptic nucleus (MPN) and the ventromedial hypothalamic nucleus, venterolateral VMNvl (Cushing et al 2003). Pair bond formation does not occur during this period and therefore the goal of this study was to further elucidate the activation of neuroanatomical regions over time, and to determine if there is a sexually dimorphic temporal or regional pattern of cFos expression. Here we extended examination of neural activation over a 12-hour period of cohabitation. This critical timeframe covers a period in which pair bond formation and activation of the hypothalamic-pituitary-gonad (HPG) axis is beginning, transforming the vole from a state of reproductive quiescence into that of sexual competence.

This study provides a unique look into the natural process of pair bond formation, as the animals have not been steroidally manipulated or permitted to mate. This study also differs from other studies in that animals remained in cohabitation for the entire interval. Control animals were taken directly from sibling pairs and sampled. Moreover,

control animals were never isolated, in order to avoid another stressful source of variation to this highly social animal. In this experiment we conduct a temporal investigation into the activation of nuclei involved in three main circuits known to be involved in pair bonding, using c-Fos IR as an indicator of neuronal activation. The first circuit was the social-sexual behavior network including MeA, BnST, MPN, VMNVL and the lateral septum (LS). The LS is known to be involved the social behavior network (Newman, 2002) and also critical to pair bonding in male prairie voles (Liu et al., 2001). The second circuit included components of the reward pathway including the nucleus accumbens (Nacc) and ventral pallidum (VP). Last, we examined c-Fos expression in areas heavily under endocrine control, including the paraventricular nucleus (PVN) and supraoptic nucleus (SON), which produce the peptides vasopressin (AVP) and oxytocin (OT). This provides a clear view of the processes occurring during the time when initiation of pair bonding begins, without confounding the data with neuronal processes activated as a result of isolation or mating. From the information discovered in this experiment, we argue that the initiation of pair bond formation, as evidenced by c-Fos activation in areas known to be involved in this process, actually begins within the first few hours a novel male and female prairie vole are introduced. This experiment is the first to show an approximate timeline for the activation of neuronal areas implicated in (1) social and sexual behaviors, (2) pair bond formation, and (3) production of neuropeptides that regulate those behaviors in the prairie vole.

BACKGROUND

Pair Bonding in the Prairie Vole

Monogamy in natural populations is exceedingly rare with only 3% of mammalian species utilizing this system. There is both a social and a sexual definition of monogamy. Mating systems characterized by sexual exclusivity have been designated as sexually monogamous whereas social monogamy emphasizes long term or pair bond associations and biparental care (Carter et al., 1995). Prairie voles display many of the traits traditionally used to characterize monogamy including (1) biparental care and alloparenting, (2) induction of estrus and subsequent ovulation under regulation of social factors, (3) incest avoidance and reproductive suppression of adult "helpers", and (4) formation of long term stable associations known as pair bonds.

Pair bonding is measured in the laboratory through measurements of partner preference formation, selective aggression, nest site selection and adrenal responses to separation (Carter et al., 1995). In order to form a partner preference leading to the formation of a pair bond, the animal must be able to first recognize and then form a social memory for that individual. Physical or side-by-side contact increases in familiar pairs that have cohabitated or mated (Carter et al., 1988). A preference for a familiar partner can then be measured by time spent in physical contact with that individual in dyadic encounters and/or a choice preference test where they can choose between a strange or familiar partner (Williams et al., 1992).

Selective aggression towards unfamiliar conspecifics can also be used to indicate pair bonding (Carter et al., 1995). This may represent mate guarding in monogamous

systems despite the fact that sexual exclusivity is not an essential characteristic of prairie vole monogamy (Dewsbery, 1982; Carter et al., 1990). Sexually naïve prairie voles are rarely aggressive whereas sexual experience increases aggression in both sexes.

Aggression develops much more quickly in sexually experienced males, within approximately 24 hours after the onset of mating (Getz and Carter, 1980; Getz et al., 1993; Winslow et al., 1993). Females show slightly increased aggression 24-72 hours after mating begins and become highly aggressive around the time of parturition (Gavish et al., 1981; Firestone et al., 1991).

Another indication of pair bonding in prairie voles is nest site selection. Prairie voles use individual odors to recognize their mate and prefer to build nests in areas containing the scent of a familiar partner vs. a stranger (Newman and Halpin, 1988). Female prairie voles have been observed in the laboratory carrying nesting materials to her male partner who in nature would be responsible for nest construction (Carter et al., 1995). Therefore, nest construction may indicate that a pair bond has formed in prairie voles.

Adrenal responses can be monitored as an index of pair bonding in prairie voles. Elevation of corticosterone (CORT) levels is typically viewed as an indication of stress (Levine et al., 1989). Sexually naïve male and female prairie voles show a decrease in CORT levels upon introduction to a novel conspecific (Williams et al., unpublished data). In bonded pairs, separation is followed by an increase in CORT in both sexes whereas reunion is followed by a reduction in CORT to that below baseline. Pair bonded females also responded to an unfamiliar male with an elevation in CORT indicating that

separation from her mate and exposure to a stranger are both stressful situations whereas reunion is a stress-reducing event (Carter et al., 1995).

Social Behavior and Structure of the Family Unit

Much of the evidence of monogamy in prairie voles comes from field studies (Getz et al., 1981). Monogamy is observed in *Microtus ochrogaster* at high and low population densities and across all seasons (Carter et al., 1986). In nature, prairie vole home ranges broadly overlap between one male and one female, but not between one breeding pair and another. The breeding pair, consisting of one male and one female, nest together during breeding and non-breeding seasons and are commonly trapped together. The family group most commonly consists of the breeding pair and one or two of their litters of offspring, who remain in the natal nest, reproductively quiescent, as "helpers". Approximately 70% of juveniles do not disperse from the nest and form philopatric communal groups, usually in late autumn and winter (Getz et al., 1993; McGuire et al., 1993). In spring, many survivors pair off with an unrelated individual with whom they will remain until one partner dies. Prairie voles rarely acquire a new mate (Pizzuto and Getz, 1998) and a substantial pool of wandering males exist (~45% of adult males) who have sexual experience but lack a mate.

Reproduction in Prairie Voles

Social stimuli, including chemosensory cues, play a critical role in reproduction and behavior in many rodent species. Specifically, the accessory olfactory bulb (AOB) is used to detect nonvolatile chemosensory cues in the urine which triggers an endocrine cascade resulting in increased LHRH which stimulates the release of luteinizing and

follicle stimulating hormones from the anterior pituitary into circulation (Keverne, 1983; Singer, 1988; Clancy et al., 1988; Wysocki et al., 1980). Unlike hamsters, mice and rats which exhibit spontaneous puberty and estrous cycles (Richmond and Stehn, 1976), the prairie vole requires prolonged exposure to a novel adult male before she can be induced into estrus (Carter et al., 1995). Prior to that event, the female prairie vole remains in the communal nest and is reproductively quiescent. The cause of this suppression is not fully understood but was once believed to be due to the presence of pheromonal cues of reproductively active females (Getz et al., 1983; Brant et al., 1998). This has since been proved not to be the case in prairie voles where the presence of the mothers had no effect on the breeding success of their juvenile daughters (Wolff et al., 2001). An alternative hypothesis suggests that this suppression may actually be a behavioral mechanism in place for incest avoidance. Prairie voles rarely engage in anogenital investigation with familiar conspecifics and therefore do not come into direct contact with chemical cues capable of activating reproductive processes. It is however, common for prairie voles to investigate the anogenital region of unfamiliar animals, thereby coming into direct contact and ingesting pheromones of a potential mate. This in part provides an explanation as to why a sexually naïve female prairie vole is activated by an unfamiliar male but not by her father or siblings. Further research has shown that if a female and her male sibling are separated for 8-10 days, she can be induced into estrus when a drop of his urine is applied to her nares (Moffatt, 1994). This provides support to the behavioral block theory of reproductive activation and additionally suggests that the length of a social memory in prairie voles is approximately 8-10 days.

Pheromonal cues and tactile stimulation in the form of anogenital grooming are therefore both required for the female prairie vole to become reproductively active (Carter et al., 1987). Both natural cues contribute to a rise in estrogen that is necessary to induce behavioral estrus in the female prairie vole. In the laboratory, priming females with estrogen to those levels found during estrus can bypass the need for physical contact with a male. This hormonal manipulation can induce sexual receptivity in the absence of a natural cue (Hnatzuk and Morrell, 1995). Onset of behavioral estrus usually occurs 24 – 48 hours following initial contact of the pair (Dluzen et al., 1981). The major determinants of the duration of behavioral estrus are the physiological and behavioral condition of the female during cohabitation (Carter et al., 1988). Familiarity of the male and female does not significantly influence frequency or duration of mating but does influence side-by-side contact -suggesting that social and sexual behaviors may be independently regulated. Prairie voles typically begin mating within 20 – 60 hours after initial contact and continue in multiple bouts over a 24-hour period (Carter et al., 1980). With mating, dopamine is released in this species as in most other laboratory rodents (Gingrich et al., 2000). Ovulation occurs within 10 – 12 hours following initiation of mating (Richmond and Conaway, 1969) and prolonged contact with their mate after fertilization significantly increases the probability of a successful pregnancy.

Endocrine Events Following Introduction to a Novel Mate

The female prairie vole requires direct physical contact and chemosensory cues from the urine of a novel male in order to induce and maintain behavioral estrus (Carter et al., 1987). An elevation in serum luteinizing hormone (LH) is measured in female prairie

voles at 1 and 30 minutes after exposure to a drop of male urine (Dluzen et al., 1981). It has also been reported that a female prairie vole can develop a partner preference for the male she is housed with during this initial 30-minute interval (Carter et al., 1992). By 60 minutes, serum LH returns to basal levels while the concentration of luteinizing hormone releasing hormone (LHRH) increases 185% and the concentration of norepinephrine decreases 54% in the posterior olfactory bulb. A 1-hour cohabitation with a novel male leads to an increase in uterine weight 48 hours later that is sustained for 10 days (Carter et al., 1980). In addition to this increase in uterine weight, exposure to male pheromones leads to an increase in uterine protein content and ovarian estrogen content (Carter et al., 1989). Ovarian estrogen production is enhanced and maintained by non copulatory stimulation from the male supporting the hypothesis that male presence and contact are required for the maintenance of ovarian activity in the female prairie vole (Carter et al., 1989). Serum estrogen levels more than double during initial contact and the behaviors that follow. Peak estrogen levels are observed at the time of lordosis (Cohen-Parsons and Carter, 1987) while a dramatic decrease is observed after mating occurs. Dopamine is released with mating (Gingrich et al., 2000) while progesterone levels do not rise until 72 hours after mating, indicating that this hormone is not required for sexual receptivity in the prairie vole (Carter et al., 1989). In females, both estrogen (Cohen-Parsons and Carter, 1987) and progesterone (Cohen-Parsons and Carter, 1988) receptor binding in hypothalamic regions increases with increased exposure to males and male soiled bedding.

Regulation of Pair Bond Formation

Social contact and mating facilitates pair bond formation in the prairie vole - making the hormones released with these actions including OT, AVP, CORT, and the neurotransmitter dopamine (DA), likely candidates in the regulation of the pair bond process. Neuropeptide regulation of behaviors associated with monogamy has been reviewed extensively (Insel et al., 1995; Getz and Carter, 1996; Carter et al., 1997; Insel et al., 1998; Curtis and Wang, 2003; Aragona and Wang, 2004).

Oxytocin

Oxytocin is released with social contact, mating and vaginal cervical stimulation (Carter et al., 1995). For this reason, OT has been extensively studied in the context of social interactions and the regulation of social behaviors. Oxytocin treatments to female prairie voles increase social contact (Cho et al., 1999). Central administration of OT facilitates the formation of a pair bond whereas administration of an OT antagonist interferes with this preference formation even when followed by prolonged cohabitation or OT treatments (Cho et al, 1999; Insel and Hulihan, 1995; Williams et al., 1994). The facilitative effects of OT on the pair bond process have been reported following both central and peripheral injections. Chronic icv infusions of oxytocin to female prairie voles 24 hours prior to cohabitation facilitated a preference for the cohabitated male (Williams et al., 1994). Similar results are reported when acute icv injections are given to male and female prairie voles immediately prior to a one hour cohabitation (Cho et al., 1999). In this same experiment, administration of an oxytocin receptor (OTR) antagonist

alone or in conjunction with OT, prevented partner preference formation and lowered social contact in both male and female prairie voles.

Peripheral injections of OT given in pulses to female prairie voles are successful in facilitating a preference for the cohabitated partner but not when administered as a single injection (Cushing and Carter, 2000).

Vasopressin

Vasopressin has been implicated in the induction of many monogamous traits in the male prairie vole including mate guarding, territory defense and the induction of partner preferences (Cho et al., 1999; Carter et al., 1995; Winslow et al., 1993). AVP production is androgen dependent with studies demonstrating that both testosterone treatments and mating induced testosterone, influence AVP levels in male prairie voles (DeVries and Villalba, 1997; Harrison et al., 2000). Vasopressin is released with mating and both sexes display higher levels of aggression following sexual encounters (Getz et al., 1981; Winslow et al., 1993).

Vasopressin has been shown to facilitate the development of partner preference in male prairie voles after chronic (Winslow et al., 1993) and acute (Cho et al., 1999) treatments. Similar results were noted with acute injections of AVP to females whereby a preference for the cohabitated male developed (Cho et al., 1999). In this same experiment, administration of a vasopressin receptor (V1a) antagonist alone or in conjunction with AVP, prevented partner preference formation and lowered social contact in both male and female prairie voles.

Corticosterone

Prairie voles have basal plasma levels of total CORT 10 times higher than those reported in other vole species and rats (Taymans et al., 1997). They also have significantly higher adrenal to body weight ratios and 2-fold higher levels of baseline adrenocorticotropin hormone (ACTH) and corticosterone binding globulin (CBG), coupled with decreased abundance and affinity to glucocorticoid receptors. Together these data suggest that the prairie vole is glucocorticoid resistant. Although prairie voles hypersecrete glucocorticoids, they express fewer adrenal steroid receptors, with a lower binding affinity in the hippocampus than that seen in laboratory species with conventional CORT levels (Hastings et al, 1999). Despite its hyperactive hypothalamic-pituitary-adrenal (HPA) axis, the prairie vole is responsive to circadian rhythms and external stressors, and therefore CORT levels can be used as an index of stress in this species.

Elevated CORT levels have been reported in prairie voles in response to social isolation, separation from a familiar partner, swim testing, and physiological manipulations. Both prairie vole pups (Shapiro and Insel, 1990) and sexually naïve females (Kim and Kirkpatrick, 1996) show an increase in CORT levels following social isolation. Introduction to a novel male elevates CORT levels in a pair bonded female but reduces CORT levels in sexually naïve females (DeVries et al, 1995). Exposure to a stressor (swim test) increases CRH mRNA (Liu et al., 2001a) and nearly doubles serum CORT levels in both male and female prairie voles (DeVries et al, 1995).

Regulation of pair bonding by corticosterone is sexually dimorphic in prairie voles in that an increase of CORT or central CRH (DeVries et al., 2002) in males and a

decrease of CORT in females facilitates partner preference formation (Williams et al, 1992). Male prairie voles form partner preferences following exposure to a stressor (swim test) and corticosterone injections (DeVries et al., 1996). Both experiences interfere with preference formation in the female prairie vole. Adrenalectomized females, however, develop partner preferences for the male following one hour in cohabitation as compared to three hours required for control females (DeVries et al., 1995). Corticosterone injections to intact and adrenalectomized females prevent this preference. Adrenalectomized males fail to develop partner preferences and replacement of corticosterone reverses this behavioral effect (DeVries et al., 1996). Taken together, these results provide evidence that a reduction in corticosterone is associated with partner preference formation in females while an increase in corticosterone is associated with preference formation in male prairie voles.

Dopamine

Dopamine (DA) is released with mating in multiple laboratory species including the prairie vole (Gingrich et al., 2000) and its involvement in bond formation has subsequently been studied. Female prairie voles injected with vehicle, a dopamine agonist (apomorphine), or a dopamine antagonist (haloperidol), were allowed to cohabitate with a male for six hours without mating (Wang et al.1999). Those females injected with apomorphine developed a partner preference for the male whereas those injected with vehicle or haloperidol did not. Similar results were obtained in male prairie voles although lower doses of apomorphine were capable of inducing a partner preference (Aragona et al 2003a).

Activation of D2-type receptors facilitates partner preference formation in female prairie voles (Wang et al., 1999). A D2-type receptor antagonist (eticlopride) blocked partner preferences in mated females whereas a D1-type receptor antagonist (SCH23390) did not, when injected immediately prior to mating. Likewise a D2-type receptor agonist (quinpirole) induced partner preferences in females whereas a D1-type receptor agonist (SKF38393) did not. With this data, Wang's laboratory suggests not only that social experience activates dopaminergic pathways, but that activation of D2 receptors is necessary for partner preference formation.

Distribution of Oxytocin and Vasopressin Cells and Receptors

OT-ir cells have been located in greatest concentration in the SON and PVN (Wang et al., 1996). Additionally, OT-ir cells have been found in the medial preoptic area (MPOA), paraventricular nucleus posterior (poPVN), and BnST. Regardless of the region of staining, no gender differences have been found in concentration of OT-ir cells in the prairie vole. OT-ir fibers have been located within the MPOA, BnST, and lateral hypothalamic area (LHA). Additionally, OR-ir fibers are localized in components of the reward pathway including the NAcc (but not the VP) where no sex differences are found (Lim et al., 2004).

OTR's have been found in the BnST, PLC, NAcc, midline nuclei of the thalamus, MPOA, VMNVL, VTA, SuM and aspects of the lateral and medial amygdala of prairie voles (Witt et al., 1991; Insel and Shapiro, 1992). Recently, OTR mRNA has been found in the NAcc and OTR's are most likely located on cell bodies within the NAcc rather than on DA nerve terminals (Young et al., 2001; Lim et al., 2004). Estrogen upregulates

OTR's in multiple neuroanatomical regions in many rodent species. The prairie vole shows a different pattern in that only the anterior olfactory nucleus shows an increase in OTR's following estrogen treatment (Witt, Carter and Insel 1991). OTR activation has been shown to upregulate the ppEnk mRNA gene in rats (Bale and Dorsa, 1997). Similarly, icv OT infusions increase preproendkephalin (ppEnk) mRNA in the NAcc of prairie voles with a significant correlation between OTR density and ppEnk mRNA levels (Young et al., 2001). This may provide a mechanism by which neuropeptides can influence the reward pathway in this species.

AVP-ir cells are predominantly found in the SON, PVN, and suprachiasmatic nucleus (SCN) and in small bundles in the preoptic area (POA) of the anterior hypothalamus in prairie voles (Wang et al., 1996). There are few species differences in AVP-ir fiber patterns but there is a large gender difference. Males have a plexus of AVP-ir fibers in the LS that continues into the DBB, BnST, MPOA. Fibers are also located heavily in the lateral habenular nucleus, PVN of the thalamus and medial amygdala.

Prairie voles have a high density of AVP-V1a receptors in the VP (Young et al., 2001; Young et al., 1997; Lim, Murphy and Young, 2004). Distribution of V1a receptors reflects the pattern of V1a receptor mRNA (Young et al., 1997). V1a receptors have been localized in the DBB, laterodorsal thalamus, LS, LD and the VP (Insel, Wang and Ferris, 1994; Young et al., 1997; Lim et al., 2004). V1a receptor mRNA is not sexually dimorphic in the VP.

Hormonal Control of Nuclei Important for Social Behavior in Prairie Voles

Nucleus Accumbens

The NAcc is a critical component of the mesolimbic DA pathway and receives input from the VTA (Young et al., 2001). It plays an important role in conditioned learning as well as in the reinforcing and rewarding effects of both addictive drugs and natural stimuli (McBride, Murphy and Ikemoto, 1999). The NAcc has been identified as important in the formation of pair bonds in female prairie voles. Young et al (1999) reported the probable presence of OTR on the cell bodies of neurons contained within the NAcc. Furthermore, injection of an OT antagonist to the NAcc and prelimbic cortex (PLC) blocked mating induced partner preferences (Young et al., 2001).

Dopamine distribution and release in prairie voles is similar to that of many laboratory species in that (1) prairie voles have a high concentration of DA terminals and receptors in the NAcc (Aragona et al, 2003) and (2) DA is released following mating in the NAcc (Gingrich et al., 2000; Mermelstein and Becker, 1995; Pfaus et al., 1993; Pfaus et al., 1995). Site-specific NAcc injections of a DA agonist apomorphine induced partner preference formation in male prairie voles, whereas the DA antagonist haloperidol blocked them. Two types of DA receptors have been identified, D1 and D2, with apomorphine binding preferentially to the D2 type receptor (Missale et al, 1998). Given that apomorphine induces partner preference formation (Wang et al, 1999) and that it predominantly binds to the D2 type receptor, it has been hypothesized that DA works through the D2 receptor to regulate this behavior. Injection of a D2 but not D1 type receptor agonist into the NAcc of female prairie voles induced a partner preference for a male in

the absence of mating (Gingrich et al, 2000). Further, this preference was blocked by the administration of a D2 but not a D1 type receptor antagonist, even after mating (Young et al, 2001) - providing evidence that DA exerts its effects thru the D2 receptor to regulate the formation of a partner preference. Similar results have since been confirmed in male prairie voles (Aragona et al, 2003a). In this experiment, Aragona showed that activation of D2 type receptors, specifically in the shell of the NAcc, are associated with the induction of partner preference formation. Interestingly, injection of a D1 type receptor agonist (SKF38393) following an injection of a D2 type agonist, blocks this preference. These results suggest that D2 type receptors may be involved in the induction of partner preferences whereas activation of D1 type receptors prevents a new preference from forming, perhaps to protect the existing pair bond (Aragona et al, 2003b).

A comparison of OTR distribution between the polygamous montane vole and the monogamous prairie vole provides insight into a possible mechanism of pair bond formation. The monogamous vole species has a high density of OTR's within the NAcc whereas the polygamous vole species is almost completely devoid in this region. The presence of both OTR and D2-type receptors within the NAcc led to studies exploring their possible interaction. It was discovered that the concurrent activation of OTR and D2-type receptors in the NAcc are required for partner preference formation in female prairie voles (Liu and Wang, 2003). Blocking OTR's in the NAcc blocked D2-agonist induced partner preferences in females cohabitating for 6 hours with a novel male. Likewise, blocking D2-type receptors in the same region blocks OT agonist induced partner preferences in cohabitated females that have not been permitted to mate. Interaction of neuropeptides and dopaminergic pathways potentially stimulate a partner

preference by linking a social stimulus with the reward pathway in prairie but not in montane voles. Neuropeptide receptor distribution may explain why pair bonds form in many monogamous but not polygamous species even though dopamine is released with mating in both.

Ventral Pallidum

The ventral pallidum, which receives major outputs from the NAcc and DA input from the VTA, is important in the rewarding and reinforcing effects of natural stimuli and psychostimulants (Young et al., 2001, McBride et al., 1999). Research has shown that cocaine administration leads to a major increase of DA in the VP (Young et al., 2001). An animal will develop a conditioned place preference for the environment the injection was administered when various psychostimulants are injected into the VP (Gong et al., 1996) Additional evidence linking reward pathway DA to the regulation of social behaviors is shown by the prevention of cocaine-induced place preferences when VP DA is depleted (Gong et al., 1997). In prairie voles, the VP has been implicated in the formation of pair bonds, at least in males (Lim et al., 2001; Pitkow et al., 2001; Young et al., 2001). Vasopressin transmission occurs in the VP during mating (Lim et al., 2004) The monogamous prairie vole has much higher density of V1a receptors in this area as compared to non-monogamous voles perhaps providing a species specific mechanism for the regulation of this process (Young et al., 2001). Access to the V1a receptor within the VP is critical in pair bond formation in male prairie voles (Lim and Young, 2002; Lim and Young, 2003). Infusion of a selective V1aR antagonist into the VP prevents partner preference formation

Lateral Septum

A third area involved in the regulation of pair bond formation in male prairie voles is the lateral septum. This nuclei has far more vasopressin fibers in males than in female prairie voles (Wang et al., 1996). AVP injected into the lateral septum induces pair bond formation in the absence of mating (Liu et al., 2001). Administration of an OTR or an AVP V1a receptor antagonist blocks this attachment even when mating occurs. This evidence suggests that both OT and AVP receptors are required to regulate this process in the lateral septum of male prairie voles.

Neural Pathways Involved in the Response to Social Stimuli

The network implicated in many social behaviors is composed of six main interconnected limbic areas including the following: MeA/ BnST, LS, midbrain, VMN, anterior hypothalamus, and the MPOA (Newman, 1999; Newman, 2002). This pathway links socially relevant stimuli, including chemosensory cues, into a central neural circuit to influence an individual's behavioral response to those stimuli. Chemosensory cues from the urine of a novel mate are transmitted to the vomeronasal organ (VNO), which in turn projects to the AOB (see Appendix A). The AOB sends projections to at least two subdivisions of the medial amygdala, the anterodorsal (MeAD) and posterodorsal (MePD). The MeAD has been implicated in sorting chemosensory cues, while the MePD has been implicated in processing hormonal cues. The MeAD also receives input from the main olfactory bulb and the primary olfactory cortex. Communication between the MeAD and MePD is bi-directional and both innervate the central amygdala and the ventral lateral septum. Two parallel networks leave the medial amygdala. One pathway

projects from the MeAD, to the BnSTpi, through the MPOAi, to the anterior hypothalamus and on to the VMHm. The VMHm in turn, sends projections to the dorsal medial hypothalamus and then on to both the premammillary nuclei ventral (PMv) and the arcuate nucleus (ARC). The other pathway projects from the MePD, to the BnSTpm, through the MPOAm, and on to the VMHl. The VMHl sends projections to both the PMV and the ARC. Both circuits process information critical to normal mating behaviors but regulate different aspects of this behavior (Gomez and Newman, 1992). The MeAD-BnSTpi pathway utilizes olfactory, vomeronasal and somatosensory stimuli to activate a non specific behavioral response and the readiness to differentially respond to specific signals appropriately (Newman et al., 1997).

Recent evidence has shown that neural pathways of incentive behaviors necessary for reproduction, may be utilizing the same reward pathway utilized in substance abuse (Insel, 2003). A putative reward circuit has been shown to include the ventral tegmental area (VTA) that projects through the amygdala, BnST to the NAcc. The circuit continues from the NAcc, to the VP, and on to the thalamus that sends projections to the prefrontal and cingulate cortex to activate cells that feedback to the VTA.

c-Fos

Immediate early genes (IEG's) are one of the first cellular responses to incoming stimuli. Expression of ieg's is by way of second messenger pathways and therefore does not require prior protein synthesis. Of these genes induced by activation, *cfos* is perhaps the best understood and most commonly used. Under basal conditions, the cell produces little if any *cfos* and its presence indicates an activational change in neuronal status that is

usually associated with a strong or novel stimulus and one that induces a long term change in that neuron's function (Sagar and Sharp, 1993). For this reason its protein product, c-Fos, has been used in a multitude of studies as a reliable marker of neuronal activation. It is of particular importance in hypothalamic systems under neuroendocrine control (reviewed in Hoffman et al., 1993; Hoffman and Murphy, 2000; Hoffman and Lyo, 2002)

The protein, Fos is one product of the immediate early gene *cfos*. The *cfos* gene is rapidly induced in neurons following stimulation including depolarization of the cell membrane, activation of Ca channels, and activation of pathways regulating SRF and CREB phosphorylation (Sagar et al., 1988; Sharp et al., 1993; Cahill et al., 1996; Herrera, and Robertson, 1996; Chaudhuri, 1997). Following transcription, *cfos* mRNA is transported to the cytosol where translation into a 56 kDa Fos protein product occurs. The Fos protein contains a leucine zipper motif that promotes dimerization with other gene products of the Jun early gene family (Turner and Tjian, 1989). The Fos-Jun product, known as AP-1, quickly migrates back into the nucleus where it binds to a specific sequence in the promoter region- the AP-1 binding site- to either inhibit or promote transcription of that late response gene. Antibodies for Fos protein therefore yields a stain localized to the nucleus. The Fos protein and similar immediate early gene products may potentially regulate any gene containing an AP-1 binding site. Among these are many target genes that encode neuropeptides including enkephalin, dynorphin, LHRH, and galanin.

FRA's

Chemically similar proteins exist, Fos Related Antigens (FRA's), and have been characterized into three groups: Fos-B, FRA-1, and FRA-2. Although they are produced by other immediate early genes, they have similar leucine zipper motifs, and are capable of dimerizing with Jun products and attaching to the AP-1 binding site. The antibody we used (Oncogene,) specifically recognized amino acid residues of the amino terminus in order to minimize cross-reactivity with other FRA's. Fos antibodies directed towards the leucine zipper may therefore detect FRA's, but those directed more specifically towards the N-terminal of c-Fos are much more specific.

Limitations of c-Fos

Fos is best used as a marker of activation in neurons where a strong or novel stimulus is received that potentiates long term changes in that neuron. Expression of c-Fos indicates neuronal activation but does not indicate when a stimulus ends (Hoffman et al., 1993; Pennypacker et al., 1995). Fos is not a good marker of activity in cells under baseline or under condition if there is persistent activation partly due to the inhibitory feedback of Fos on c-fos mRNA induction (Sassone-Corsi et al., 1988).

While the presence of c-Fos indicates stimulation of individual neurons has taken place, interpreting its absence is more complex. Many neurons have a level of baseline activity where c-Fos is not expressed, perhaps due to differing thresholds. Additionally, a neuron may be using a different second messenger pathway. In both cases, c-Fos expression may be undetectable when the neuron is in fact active.

c-Fos Studies

The protein c-Fos has been used to identify nuclei activated by stimuli associated with chemosensory cues (Fiber et al., 1993; Moffatt et al., 1993; Bressler and Baum, 1996; Tubbiola and Wysocki, 1997; Halem et al., 1999; Inamura et al., 1999; Matsuoku et al., 1999; Yokosuka et al., 1999, Westberry, 2003), stress (Briski and Gillen, 2001; Swann et al., 2001), and mating (Fernandez-Fewell and Merideth, 1994; Heeb and Yahr, 1996; Ramos and DeBold, 2000; Curtis et al., 2003) as well as parental, social (Briski and Gillen, 2001; Ferguson et al., 2001; Cushing et al., 2003), and sexual behaviors (Heeb and Yahr, 1996; Veening and Coolen, 1998).

Elements of the social sexual behavior circuit reliably express c-Fos IR following introduction to a novel or familiar conspecific or their secretions in many species including rats, gerbils, hamsters, mice, and prairie voles (Fiber et al., 1993; Bressler and Baum, 1996; Tubbiola and Wysocki, 1997; Halem et al., 1999; Inamura et al., 1999; Matsuoku et al., 1999; Yokosuka et al., 1999, Westberry, 2003). Sexual interactions and mating also induces c-Fos expression in nuclei of the social behavior network (Fernandez-Fewell and Merideth, 1994; Heeb and Yahr, 1996; Ramos and DeBold, 2000; Curtis et al., 2003). Differences do exist between individual nuclei according to stimuli presented and sex. While activation within the sociosexual behavior network is modulated by social experience, the MeA is involved in integrating and processing socially relevant cues, while the BnST and LS respond to male-related cues (Wang et al., 1997). The MPN is regulated by different magnitudes of sociosexual contact and experience and like the BnST, is involved with mating or the preparation to mate in many

species. c-Fos expression in the VMN may be associated with social contact and the preparation to mate, especially in the female of the species (Cushing et al., 2003).

Studies utilizing c-Fos expression in prairie voles have revealed that chemosensory and cohabitation cues induce activation within the social behavior circuit within the first few hours of the stimulus (Moffatt et al., 1995; Cushing et al., 2003; Hairston et al., 2003). Moffatt first reported increased c-Fos ir within an hour in the MeA of female prairie voles following exposure to male urine. Hairston characterized additional components and provided an approximate time line for chemosensory activation in this species. An increase in c-Fos ir was reported in the MeA, BnST, MPOA, and the VMNVL of female prairie voles within two hours of receiving a single drop of male urine. These same regions show activation in both male and female prairie voles following exposure to social stimuli associated with a one-hour cohabitation (Cushing et al., 2003). The initial stages of social bond formation occur during this time where neuronal activation, associated with heterosexual cohabitation, is similar in males and females but is sexually dimorphic in same-sex unions.

Mating induced activation within the social behavior circuit of male and female prairie voles shows an increase in c-Fos IR in the MeA, BnST, and the MPOA (Curtis and Wang, 2003; Lim and Young, 2003). Neuronal activation was measured in females following 6 hours of mating (Curtis and Wang, 2003) and in males following 2 hrs of mating (Lim and Young, 2003). Analysis of c-Fos expression in the VMN was not provided. c-Fos expression in the LS increases following mating in male (Wang et al., 1997) but not female prairie voles (Curtis and Wang, 2003a). Increased activation in the LS and BnST was also measured in males that did not mate following a heterosexual

cohabitation that were introduced to a male, but not a female intruder (Wang et al., 1997). It is therefore possible that the observed increase in activity is due to male-related cues and not solely derived from mating.

Neuronal activation associated with mating has also been reported in the reward pathway of prairie voles (Lim and Young, 2003). There is an increase in c-Fos expression in the NAcc and VP following two hours of mating in male (Lim and Young, 2003) but not after 6 hours of mating in female prairie voles (Curtis and Wang, 2003). Neuronal activation as measured by increased c-Fos IR in the NAcc of female prairie voles has yet to be recorded.

Increased c-Fos IR has been measured in the SON and PVN due to a multitude of behavioral and physiological manipulations in most laboratory species including prairie voles (Costa et al., 1999; Flanigan et al., 1993; Caba et al., 2000; Caba et al., 2003). c-Fos expression has shown to be a reliable marker of neuronal activation in these neuropeptide producing nuclei of the hypothalamus as a result of diverse stimuli including dehydration (McKinley et al., 1994; Patronas et al., 1998; Morien et al., 1999; Pastuskovas et al., 2003), stress (Briski and Gillen, 2001), maternal aggression (Gammie SC and Nelson RJ, 2001) and cohabitation (Cushing et al., 2003). Additionally, increased c-Fos expression is reported within the SON following application of male urine to the nares of a female prairie vole, suggesting that urinary cues induce activation in this nucleus. Taken together, these data indicate that the PVN and SON are differentially activated by specific stimuli associated with social encounters.

MATERIALS AND METHODS

Animals

Prairie voles used in this experiment were laboratory-reared animals from stock originally caught near Champaign, Illinois. All animals were maintained in long day photoperiod (14 h light, 10 h dark: lights on at 0600 h) and given Lab Rabbit Diet HF 5326 and water *ad libitum*. Animals were kept in polycarbonate cages (20 x 25 x 45 cm) on beta chip bedding. Voles were reared with both parents and weaned into same sex pair cages at day 21. Pairs remained together until 60 - 90 days of age when they were randomly assigned to a treatment group. All animals were sexually naïve and gonadally intact in this experiment.

Experimental Design

Voles were randomly assigned to one of five treatment groups : 0, 1, 2, 6, and 12 hr cohabitations with a novel mate of the opposite sex ($n = 5- 8$ per treatment). Animals remained with their new partner the full length of cohabitation. Cohabitations were performed by moving one female to the male sibling cage, and one male into the female sibling cage. Control (0 h) animals were separated directly from their same sex sibling cages and sampled. Although voles were behaviorally unlikely to mate, twelve-hour cohabitations were recorded with time-lapse video and females were lavaged no later than an hour post mortum (to determine presence of sperm), to verify absence of mating. No animals in this experiment were permitted to mate.

Tissue collection and Immunocytochemistry

Immediately following the end of the designated cohabitation period, the animals were removed from the experimental cage and deeply anaesthetized using a combination of Ketamine and Xylazine. Animals were flushed transcardially with saline and then perfused with a 4% paraformaldehyde and 2.5% acrolein solution. Brains were removed and stored in 25% sucrose at 4^o C until sectioning. Brains were then sectioned on a freezing sliding microtome at 30 µm. Sections were placed in wells containing antifreeze cryoprotectant (Watson et al., 1986) and stored at -20^o C until processing using ABC immunocytochemistry (ICC) staining for the cFos protein (Adams, 1992; Berghorn et al., 1994).

To reduce variability due to different patterns in background staining, sections from all the treatment groups were processed in tandem. Floating sections, taken at 180 µm intervals, were processed for cFos immunoreactivity (ir). Tissue sections were rinsed 6 times (10 min each) in 0.05M KPBS pH 7.4. Sections were incubated at room temperature (20 min) in 1% sodium borohydride in 0.05M KPBS and then rinsed repeatedly in KPBS until bubbling stopped. To remove residual blood, sections were incubated for 15 min in 0.014 % phenylhydrazine and then rinsed in KPBS. Next, sections were incubated with rabbit anti-cFos polyclonal antibody (Oncogene Sci anti-cFos A1-2 #4191 used at 500K) in 0.05M KPBS-0.4% TritonX (1 hr at room temperature and then for 48 hr at 4^o C). Sections were rinsed 6 times (10 min each) in KPBS before being incubated for 1 hr at room temperature in biotinylated goat, anti-rabbit IgG (H + L, BA-1000, 1:600 dilution in KPBS-0.4% TritonX). Sections were rinsed 5 times (10 min each rinse) in KPBS and incubated in an avidin-biotin peroxidase

complex (Vectastain ABC kit-elite pk-6100 standard 4.5 μ l A and 4.5 μ l B per 1 ml solution) diluted in KPBS-0.4% TritonX (1 hr room temperature). Sections were rinsed 3 times in KPBS followed by three rinses in 0.175M sodium acetate (5 min each). Finally, cFos IR was visualized by incubating in a nickel sulfate-diaminobenzine chromogen solution for 12 min, and then rinsed 3 times in sodium acetate followed by 3 KPBS rinses. The process was repeated for the second primary (rabbit anti-LHRH ,LR-1 Benort, 100K), except LHRH ir was visualized by incubating in diaminobenzine chromagen solution for 6 minutes. Following 3 rinses in Tris buffer and 3 rinses of KPBS, sections were placed into a .9% saline solution, then mounted onto subbed glass slides and air dried overnight. Mounted tissue was then dehydrated through ascending ethanol solutions, cleared in HistoClear, and slides coverslipped with Histomount.

STATISTICS

Slides were coded by an experimentally blind observer and LHRH ir cells were recorded as positive or negative for colocalization with c-Fos ir for each section in a 1-12 series. Expression of c-Fos ir was analyzed separately. Images were captured and analyzed using IPLab (Scanalytics, Inc, Fairfax, VA) by an experimentally blind scorer. The number of cells expressing cFos ir from bilateral locations per nuclei or region were averaged and analyzed by treatment for each sex using a one-way ANOVA. If significant difference were found in the ANOVA, post-hoc pair-wise comparisons were made using a Fisher's PLSD. Post-hoc pair-wise differences were considered significant at $P < 0.05$.

RESULTS

c-Fos IR in Elements of the Sociosexual Circuit

There was a significant treatment effect in both female and male prairie voles in each nuclei measured within the social-sexual circuit which includes: MeA ($F_{4,26} = 8.04$, $P < 0.001$; $F_{4,20} = 29.19$, $P < 0.001$), ACo ($F_{4,26} = 7.16$, $P < 0.001$; $F_{4,20} = 8.15$, $P < 0.001$), PLCo ($F_{4,26} = 4.85$, $P < 0.01$; $F_{4,20} = 5.82$, $P < 0.01$), BST ($F_{4,22} = 3.13$, $P < 0.05$; $F_{4,23} = 4.99$, $P < 0.01$), MPN ($F_{4,24} = 2.84$, $P < 0.05$; $F_{4,24} = 7.61$, $P < 0.001$), VMNVL ($F_{4,27} = 9.80$, $P < 0.001$; $F_{4,21} = 7.97$, $P < 0.001$) and LS ($F_{4,27} = 4.25$, $P < 0.01$; $F_{4,25} = 3.40$, $P < 0.05$). Post-hoc comparison with results considered significant at $P < 0.05$ are presented below and are summarized in Table 1.

c-Fos IR in the MeA

Post-hoc comparison indicated that females that cohabitated for 1, 2, and 6 hours expressed significantly higher levels of c-Fos IR than control females, while there was no difference between controls and females cohabitated for 12 hr in the MeA (Fig 1).

Males that cohabitated for 1 and 2 hours expressed significantly higher levels of c-Fos IR than control males, while there was no difference between controls and males that cohabitated for 6 and 12 hours (Fig 1).

While males and females showed activation in all three amygdala areas following initial contact, males returned to baseline levels of c-Fos expression earlier (following a 6 hour cohabitation) than females (following a 12 hour cohabitation).

Table 1 a,b. Summarizes patterns of activation seen during specific intervals within the first twelve hours of cohabitation between a sexually naïve male and female prairie vole. Nuclei of the social behavior network (MeA, ACo, PLCo, BST, MPN, VMNVL, and LS), reward pathway (NAcc and VP), and neuroendocrine regulating nuclei (PVN and SON) are reported for females (a) and males (b) following a cohabitation of 0 hr (baseline), 1 hr, 2 hr, 6 hr and 12 hr durations. Shaded areas display patterns observed in c-Fos expression. += c-Fos IR at 0 hr (baseline) ++ = significant increase from baseline c-Fos IR, $p < 0.05$ +++ = peak level of c-Fos IR, $p < 0.05$

Table 1:

(a) Females

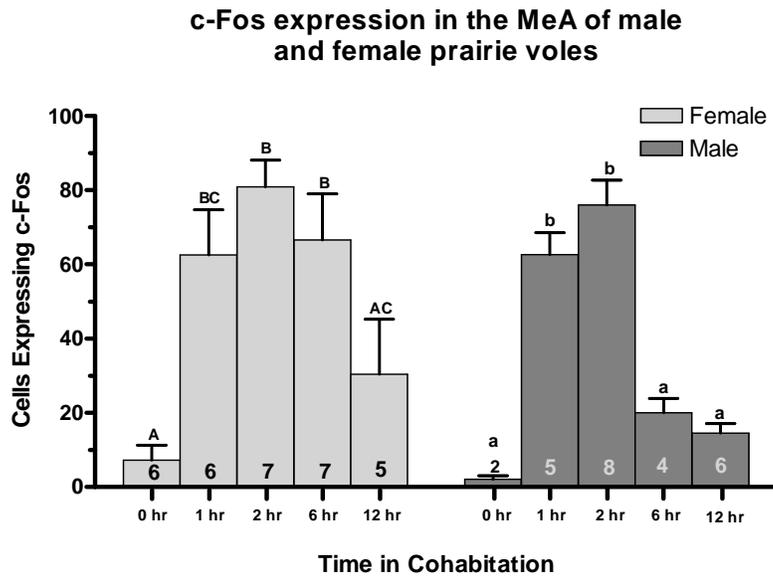
	0 hr	1 hr	2 hr	6 hr	12 hr	ANOVA
MeA	+	++	+++	++	+	$F_{4,26} = 8.04$, $P < 0.001$
ACo	+	++	+++	++	+	$F_{4,26} = 7.16$, $P < 0.001$
PLCo	+	++	+++	++	+	$F_{4,26} = 4.85$, $P < 0.01$
BnST	+	+++	++	++	+	$F_{4,22} = 3.13$, $P < 0.05$
MPN	+	+++	++	+	++	$F_{4,24} = 2.84$, $P < 0.05$
VMNVL	+	+++	++	++	++	$F_{4,27} = 9.80$, $P < 0.001$
LS	+	+	+++	+	+	$F_{4,27} = 4.25$, $P < 0.01$
NAcc	+	+	+++	+	+	$F_{4,23} = 3.75$, $P < 0.05$
VP	+	+	+++	+	+	$F_{4,28} = 4.30$, $P < 0.01$
PVN	+	++	+++	+	+	$F_{4,22} = 3.13$, $P < 0.05$
SON	+	+	+	+	+++	$F_{4,28} = 2.79$, $P < 0.05$

(b) Males

	0 hr	1 hr	2 hr	6 hr	12 hr	ANOVA
MeA	+	++	+++	+	+	$F_{4,20} = 29.19$, $P < 0.001$
ACo	+	++	+++	+	+	$F_{4,20} = 8.15$, $P < 0.001$
PLCo	+	+++	++	+	+	$F_{4,20} = 5.82$, $P < 0.01$
BnST	+	++	+++	++	++	$F_{4,23} = 4.99$, $P < 0.01$
MPN	+	+++	++	++	++	$F_{4,24} = 7.61$, $P < 0.001$
VMNVL	+	++	+++	+	+	$F_{4,21} = 7.97$, $P < 0.001$
LS	+	+	+++	++	+	$F_{4,25} = 3.40$, $P < 0.05$
NAcc	+	++	+++	++	+	$F_{4,23} = 5.38$, $P < 0.01$
VP	+	+	+++	+	+	$F_{4,24} = 3.61$, $P < 0.05$
PVN	+	+	++	++	+++	$F_{4,23} = 4.99$, $P < 0.01$

Figure 1. Mean number of cells expressing c-Fos IR in the MeA of male and female prairie voles by sex and cohabitation length. Females cohabitated for 1, 2, and 6 hours and males cohabitated for 1 and 2 hours expressed significantly higher c-Fos than that of their control counterparts.

Figure 1:



c-Fos IR in the ACo

Post-hoc comparisons identified that females cohabitated for 1, 2, and 6 hours expressed significantly higher levels of c-Fos IR than control females in the ACo. There were no differences between controls and females that cohabitated for 12 hours (Fig 2).

Males cohabitated for 1 and 2 hours expressed significantly higher levels of c-Fos IR than controls in the ACo, while no differences were found between control males and those cohabitated for 6 and 12 hours (Fig 2).

c-Fos IR in the PLCo

Post-hoc comparison revealed that expression of c-Fos IR was significantly higher in females cohabitated for 1, 2, and 6 hours as compared to controls, but not in those cohabitated for 12 hours (Fig 3).

Expression of c-Fos IR was significantly higher in the PLCo of males cohabitated for 1, and 2 hours as compared to controls, while no differences were found between controls and males cohabitated for 6 and 12 hours (Fig 3).

Figure 2. Mean number of cells expressing c-Fos IR in the ACo of male and female prairie voles by sex and cohabitation length. Females cohabitated for 1, 2, and 6 hours and males cohabitated for 1 and 2 hours expressed significantly higher c-Fos than that of their control counterparts.

Figure 2.

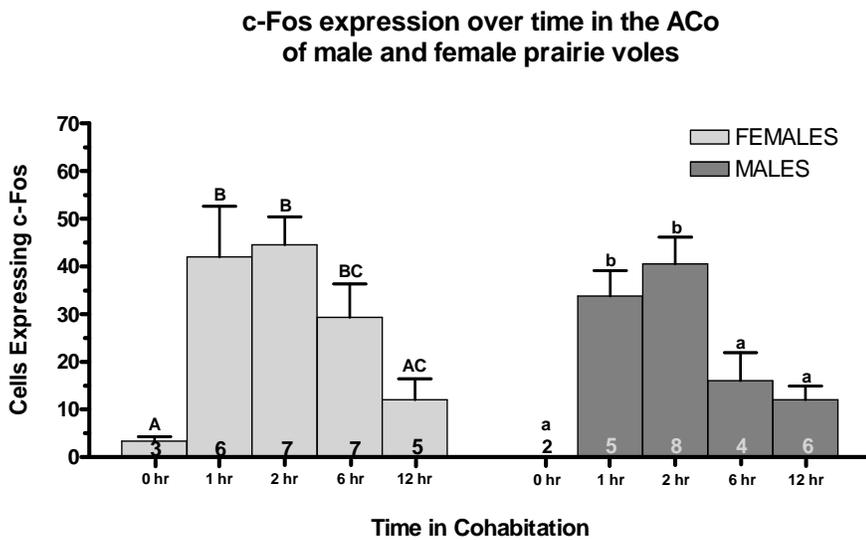
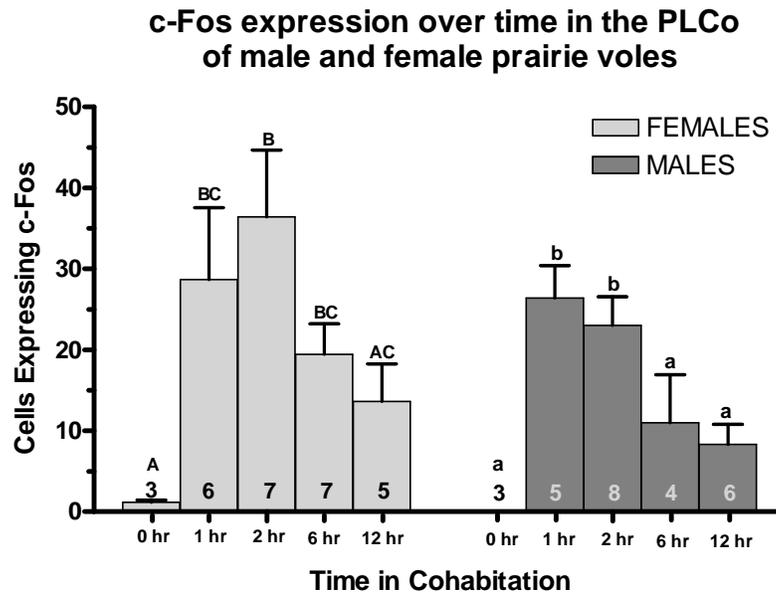


Figure 3. Mean number of cells expressing c-Fos IR in the PLCo of male and female prairie voles by sex and cohabitation length. Females cohabitated for 1, 2, and 6 hours and males cohabitated for 1 and 2 hours expressed significantly higher c-Fos than that of their control counterparts.

Figure 3.



c-Fos IR in the BST

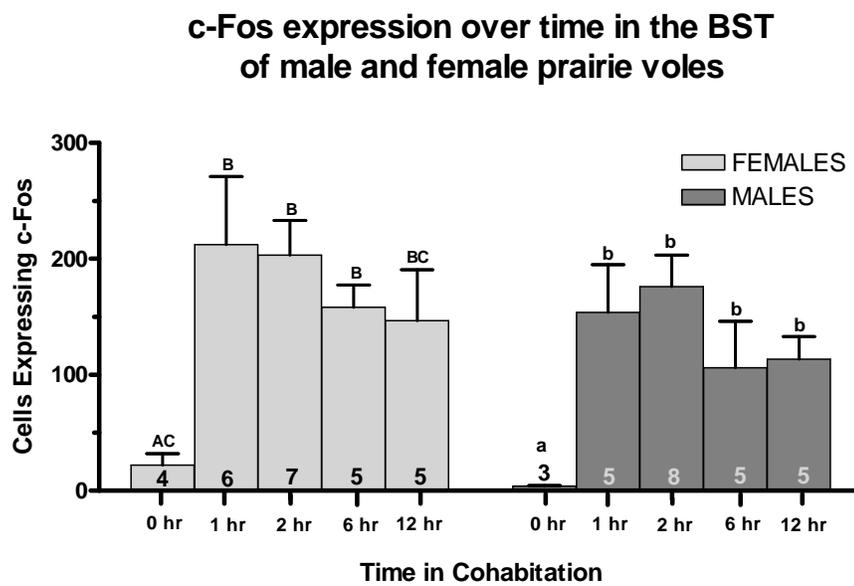
Post-hoc comparison identified that expression of c-Fos IR was significantly higher in females cohabitated for 1, 2, and 6 hours as compared to controls. No differences were seen between controls females and those that cohabitated for 12 hours (Fig 4).

Males cohabitated for 1, 2, 6, and 12 hours expressed significantly higher c-Fos IR in the BST of males as compared to controls (Fig 4).

The expression of c-Fos over time spent in cohabitation reveals similar patterns of activation in the BST and MPN of males and females, where activity significantly increases during initial contact and remains elevated as time for mating approaches.

Figure 4. Mean number of cells expressing c-Fos IR in the BST of male and female prairie voles by sex and cohabitation length. Females cohabitated for 1, 2, and 6 hours and males cohabitated for 1, 2, 6, and 12 hours expressed significantly higher c-Fos than that of their control counterparts.

Figure 4.



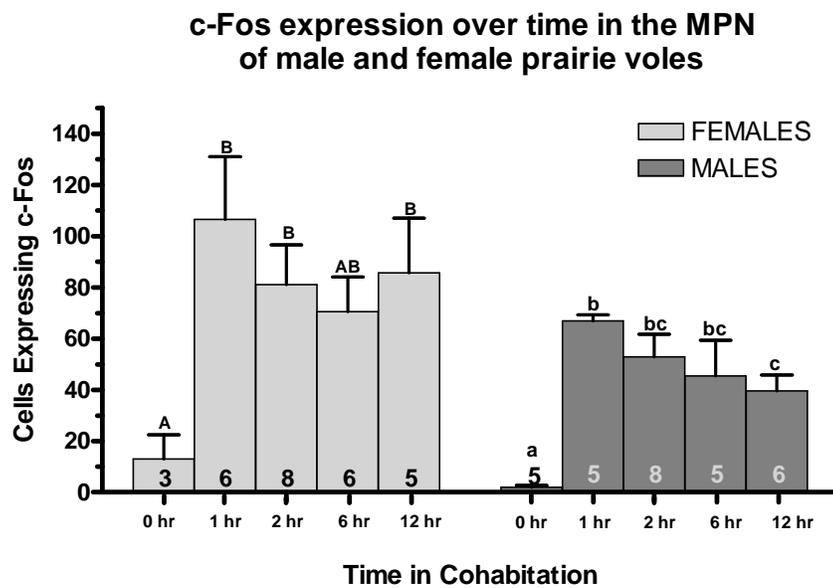
c-Fos IR in the MPN

Females cohabitated for 1, 2, and 12 hours expressed significantly higher levels of c-Fos IR than controls in the MPN, while no differences were found between control females and those cohabitated for 6 hours (Fig 5).

Post-hoc comparisons revealed that males cohabitated for 1, 2, 6 and 12 hours expressed significantly higher levels of c-Fos IR than controls (Figure 5).

Figure 5. Mean number of cells expressing c-Fos IR in the MPN of male and female prairie voles by sex and cohabitation length. Females cohabitated for 1, 2, and 12 hours and males cohabitated for 1, 2, 6, and 12 hours expressed significantly higher c-Fos than that of their control counterparts.

Figure 5.



c-Fos IR in the VMNVL

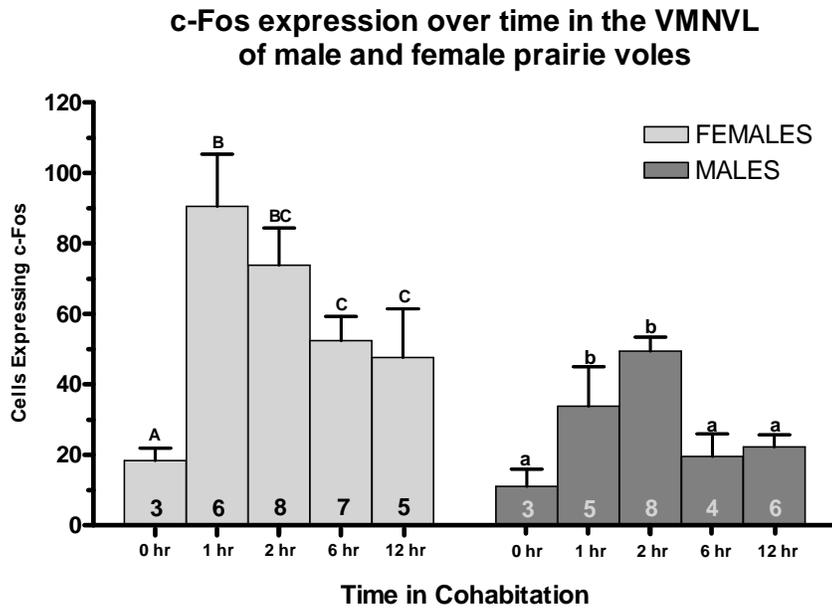
Post-hoc comparison indicated that females across all treatment intervals - cohabitating for 1, 2, 6, and 12 hour intervals - expressed significantly higher levels of c-Fos IR than control females (Fig 6).

Males that cohabitated for 1 and 2 hours expressed significantly higher levels of c-Fos IR than control males, while there was no difference between controls and males that cohabitated for 6 and 12 hours (Fig 6).

Both males and female prairie voles have an increase in VMNVL activity with initial contact but the female has a much more substantial response. Additionally, c-Fos expression remains elevated in the female across all cohabitation intervals, but returns to basal levels in the male, by the conclusion of a 6 hour cohabitation.

Figure 6. Mean number of cells expressing c-Fos IR in the VMNVL of male and female prairie voles by sex and cohabitation length. Females cohabitated for 1, 2, 6 and 12 hours and males cohabitated for 1, and 2 hours expressed significantly higher c-Fos than that of their control counterparts.

Figure 6.



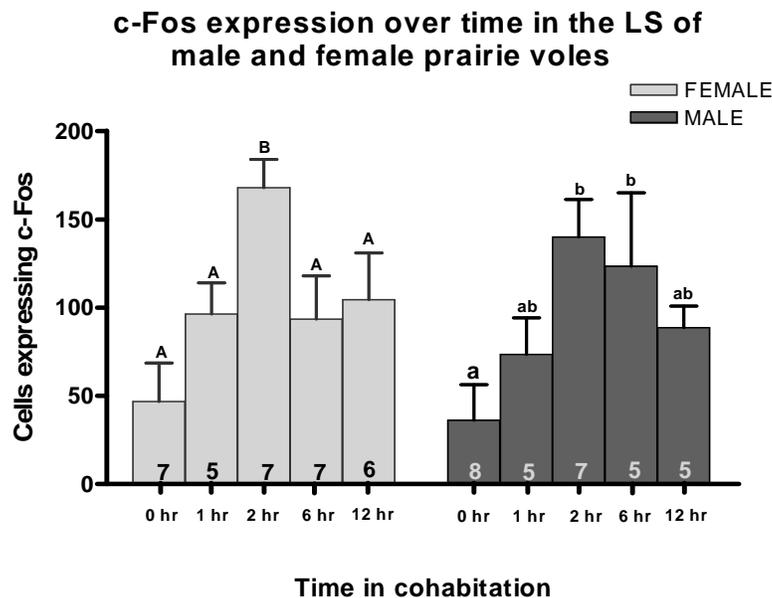
c-Fos IR in the LS

Post-hoc comparison indicated that females that cohabitated for 2 hours expressed significantly higher levels of *c-Fos* IR than control females, while there was no difference between controls and females cohabitated for 1, 6 or 12 hrs in the LS (Fig 7).

Males that cohabitated for 2 and 6 hours expressed significantly higher levels of *c-Fos* IR than control males, while there was no difference between controls and males that cohabitated for 1 and 12 hours (Fig 7). Expression of *c-Fos* IR in the LS peaks more sharply in females but remains active longer in males.

Figure 7. Mean number of cells expressing *c-Fos* IR in the LS of male and female prairie voles by sex and cohabitation length. Females cohabitated for 2 hours and males cohabitated for 2 and 6 hours expressed significantly higher *c-Fos* than that of their control counterparts.

Figure 7.



c-Fos Expression in Elements of the Reward Circuit

There was a significant treatment effect in both female and male prairie voles in both nuclei measured within the reward circuit which includes the NAcc ($F_{4,23}=3.75$, $P<0.05$; $F_{4,23}=5.38$, $P<0.01$) and the VP ($F_{4,28}=4.30$, $P<0.01$; $F_{4,24}=3.61$, $P<0.05$). Post-hoc comparisons are presented below and are summarized in Table 1.

Expression of c-Fos IR in the NAcc doubled within initial contact in both males and females and peaked following a 2 hour cohabitation, but females peaked much sharper. In the VP, males and females showed a sharp peak in c-Fos IR following a 2 hour cohabitation.

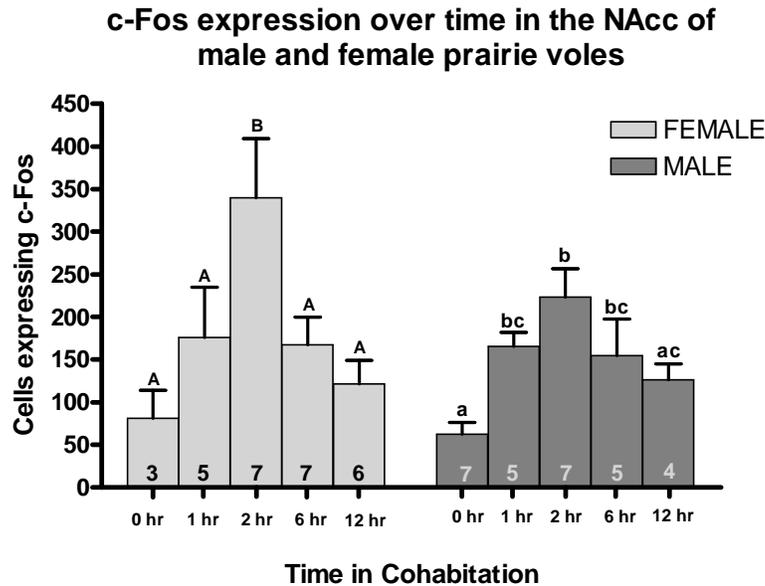
c-Fos IR in the NAcc

Post-hoc comparison revealed that expression of c-Fos IR in the NAcc was significantly higher in females cohabitated for 2 hours as compared to controls, but not in those cohabitated for 1, 6, and 12 hours (Fig 8).

Expression of c-Fos IR was significantly higher in males cohabitated for 1, 2, and 6 hours as compared to controls, while no differences were found between controls and males cohabitated for 12 hours (Fig 8).

Figure 8. Mean number of cells expressing c-Fos IR in the NAcc of male and female prairie voles by sex and cohabitation length. Females cohabitated for 2 and males cohabitated for 1, 2, and 6 hours expressed significantly higher c-Fos than that of their control counterparts.

Figure 8.



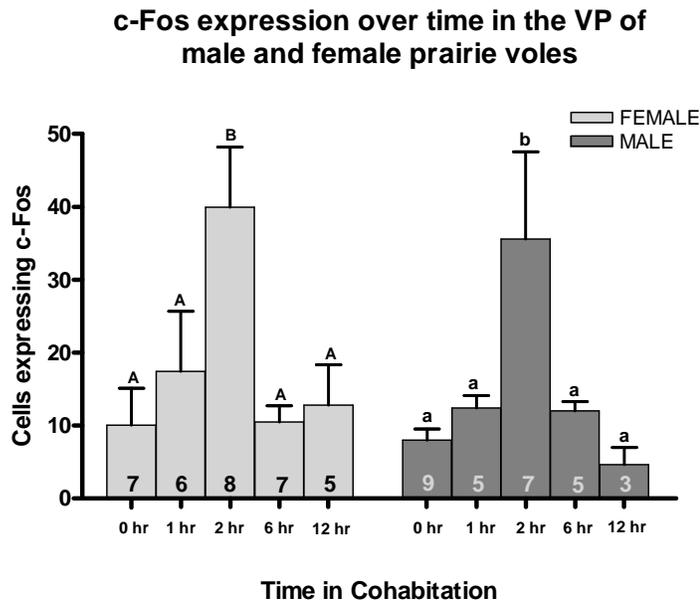
c-Fos IR in the VP

Post-hoc comparison revealed that expression of c-Fos IR in the VP was significantly higher in females cohabitated for 2 hours as compared to controls, while no differences were found between controls and females cohabitated for 1, 6, and 12 hours (Fig 9).

Expression of c-Fos IR was significantly higher in the VP of males cohabitated for 2 hours as compared to controls, while no differences were found between controls and males cohabitated for 1, 6, and 12 hours (Fig 9).

Figure 9. Mean number of cells expressing c-Fos IR in the VP of male and female prairie voles by sex and cohabitation length. Males and females cohabitated for 2 hours expressed significantly higher c-Fos than that of their control counterparts.

Figure 9.



c-Fos Expression in Nuclei Providing Neuroendocrine Regulation

There was a significant treatment effect in both female and male prairie voles in both nuclei that produce peptides involved in the regulation of pair bonding. The nuclei measured here include the PVN ($F_{4,22} = 3.13$, $P < 0.05$; $F_{4,23} = 4.99$, $P < 0.01$) and the SON ($F_{4,28} = 2.79$, $P < 0.05$; $F_{4,22} = 3.57$, $P < 0.05$). Post-hoc comparisons are presented and are summarized in Table 1.

Females cohabitated for 1 hour expressed higher levels of c-Fos IR in the PVN that remained elevated across all time points, whereas males were slower to show an increase in c-Fos IR but also remained elevated at later time points in this study. Activation in the SON followed a similar pattern in males and females in that activation increased with increased time spent in cohabitation and peaked following a 12 hour cohabitation.

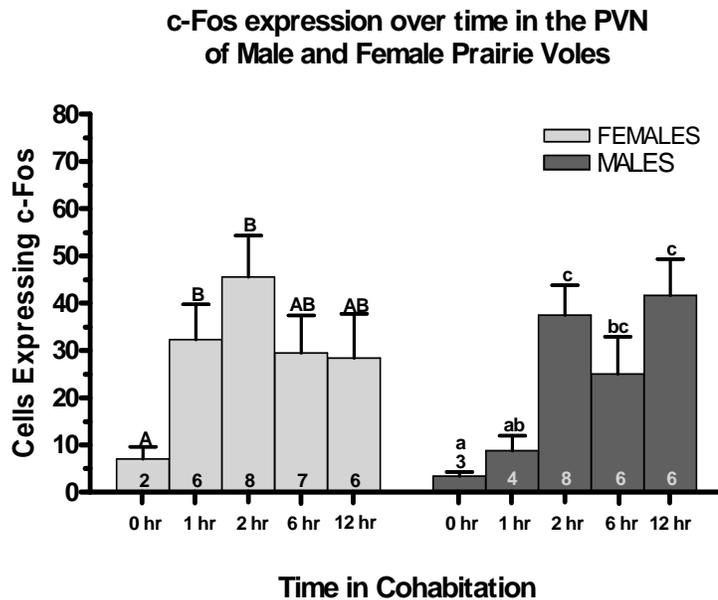
c-Fos IR in the PVN

Post-hoc comparison indicated that females that cohabitated for 1, and 2 hours expressed significantly higher levels of c-Fos IR than control females, while there was no difference between controls and females cohabitated for 6 or 12 hrs in the PVN (Fig 10).

Males that cohabitated for 2, 6 and 12 hours expressed significantly higher levels of c-Fos IR than control males, while there was no difference between controls and males that cohabitated for 1 hour (Fig 10).

Figure 10. Mean number of cells expressing c-Fos IR in the PVN of male and female prairie voles by sex and cohabitation length. Females cohabitated for 1, and 2 hours and males cohabitated for 2, 6, and 12 hours expressed significantly higher c-Fos than that of their control counterparts.

Figure 10.



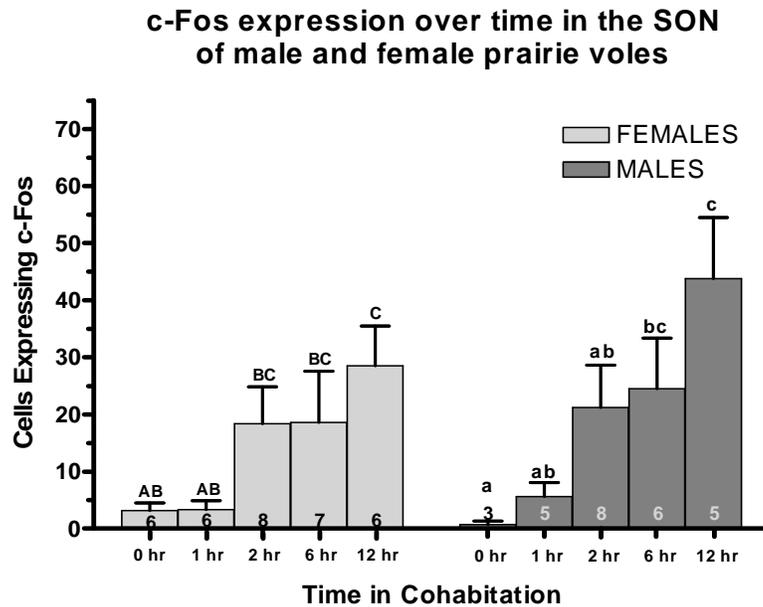
c-Fos IR in the SON

Post-hoc comparison indicated that females that cohabitated 12 hours expressed significantly higher levels of c-Fos IR than control females, while there was no difference between controls and females cohabitated for 1, 2, and 6 hour (Fig 11).

Males that cohabitated for 6 and 12 hours expressed significantly higher levels of c-Fos IR in the SON than control males, while there was no difference between controls and males that cohabitated for 1 and 2 hours (Fig 11).

Figure 11. Mean number of cells expressing c-Fos IR in the SON of male and female prairie voles by sex and cohabitation length. Females cohabitated for 12 hours and males cohabitated for 6 and 12 hours expressed significantly higher c-Fos than that of their control counterparts.

Figure 11.



Colocalization of LHRH and c-Fos Antibodies

In male and female prairie voles, the majority of LHRH neurons were found in the diagonal band of broca (DBB), preoptic area (POA) and the lateral hypothalamus (LH). Few ir neurons were recorded in the sox. Table 2 summarizes the average percentage distribution of total neurons found by neuroanatomical area per sex and treatment. Significant levels of c-Fos expression were not seen across treatments for both sexes. No males in cohabitation for 0 or 1 hour showed LHRH neurons double labeled with c-Fos. However, 87% of males in cohabitation for 2 hours, 67% of 6 hour males, and 33% of 12 hour males expressed at least some double labeled neurons (Table 3). No females in cohabitation for 0, 1 or 12 hours showed LHRH neurons double labeled with c-Fos. However, 38% of 2 hour cohabitated females and 50% of 6 hour cohabitated females expressed at least some double labeled neurons. The majority of neurons showing colocalization for c-Fos and LHRH in both sexes were located in preoptic areas of the hypothalamus.

Table 2. Summarizes location of LHRH ir neurons recorded during specific intervals within the first twelve hours of cohabitation between a sexually naïve male and female prairie vole. Numbers represent average percentage of LHRH ir neurons per area. No sex differences exist between LHRH ir neuron number by sex, treatment, or area examine. Treatments were cohabitation intervals of 0 hr (baseline), 1 hr, 2 hr, 6 hr and 12 hr durations.

Table 2. Location of LHRH ir neurons in male and female prairie voles following designated cohabitation intervals (Trts: 0 hr controls, 1, 2, 6 or 12 hours in length)

Sex	Trt	n	% in DBB	% in POA	% in LH	% in sox	% in OTHER
M	0	3	54.0	16.9	18.6	8.0	2.5
M	1	5	37.8	14.7	23.3	18.3	6.0
M	2	8	60.0	18.5	11.7	6.4	3.4
M	6	6	52.1	22.5	13.8	6.4	5.2
M	12	6	54.7	12.2	17.2	12.7	3.1
F	0	3	56.5	11.8	18.8	6.7	6.2
F	1	6	47.1	18.6	14.9	11.3	8.0
F	2	8	46.0	17.6	19.0	12.2	5.2
F	6	7	52.8	18.0	17.7	7.9	3.5
F	12	6	62.7	14.7	15.4	5.6	1.6

Table 3. Summarizes the number of male and female prairie voles expressing colocalization of c-Fos and LHRH immunoreactivity over the course of a timed cohabitation interval. Neither sex expressed colocalization in the control groups or following a one hour cohabitation with a member of the opposite sex. Some males and females expressed colocalization of c-Fos and LHRH following cohabitations of two hours or longer in length.

Table 3. Number of male and female prairie voles expressing colocalization of c-Fos and LHRH immunoreactivity following designated cohabitation intervals.

	0 hr	1 hr	2 hr	6 hr	12 hr
Females	0 / 3	0 / 6	3 / 8	3 / 6	0 / 6
Males	0 / 3	0 / 5	7 / 8	4 / 6	2 / 6

DISCUSSION

Social Behavior Network

The results from this study indicate that male-female cohabitation for durations ranging from 1 to 12 hours stimulates a pattern of immediate early gene activation that is initially similar in male and female prairie voles. These changes were seen in brain areas that have been implicated in both social behavior and reward. In this paper I have considered the MeA, BnST, MPN, LS, and VMN as components of a “social behavior network” (Newman, 2002). In addition, the NAcc, and VP, which have been implicated in reward, are combined into a “reward” pathway (Lim et al., 2004). This is done to facilitate discussion of the functional significance of my findings, recognizing that all of these regions have many additional functions.

Immediate early gene activation, measured by c-Fos immunocytochemistry, in the MeA, BST, MPN, and VMN was significantly elevated during initial contact in both males and females. The reported results are consistent with previous research showing that chemosensory, social and sexual cues induce activity in these regions of many rodents including mice, rats, gerbils and hamsters (Halem et al., 1999; Ramos and DeBold, 2000; Kondo et al., 2003; Bresslor and Baum 1996, Heeb and Yahr 1996, Pfaus and Heeb 1997). Chemosensory cues alone have been shown to induce immediate early genes in the MeA, BST and MPN but were not sufficient to induce early gene activation in the VMN of sexually naive prairie voles (Moffatt et al., 1995). However, urinary cues coupled with social contact associated with cohabitation, are sufficient to stimulate immediate early gene activation in the VMN; these observations support the findings of

Cushing et al 2003. The neural circuit activated by male-female cohabitation also is crucial for general attention, arousal and novelty (Newman, 2002).

Activation of c-Fos, as observed in the present study suggests that initial processing of social stimuli and other forms of neural information occurs in the first few hours of cohabitation and follows a similar pattern in both sexes, although amygdala activity declined faster in males than females. Such changes may reflect neural events associated with determining whether a preference will develop for the partner. Previous research, using an identical paradigm and various kinds of stimulus animals (same or opposite sex, familiar or unfamiliar) revealed that c-Fos changed as a function of the stimulus properties of the partner (Cushing, et al., 2003). Thus, this circuit may be utilized by both sexes in the early establishment of heterosexual bonds. However, sexually dimorphic patterns in c-Fos expression emerge over time spent in cohabitation.

Sex Difference in Temporal Patterns of c-Fos

Although both sexes showed activation of this circuit following initial contact, immediate early gene activation in the MeA, ACo, and PLCo extends over a longer period of time in females than in male prairie voles. A similar pattern is seen in mice where a lower baseline of investigatory behavior is reported in females than in males. However, females retain recognition responses significantly longer than males (Bluthe and Dantzer, 1990)

Both sexes showed activation in nuclei critical to sex behavior, including the BST, MPN and VMNVL, following the first hour of cohabitation. The VMNVL remained active longer in females than in male prairie voles where c-Fos IR following a 6

hour cohabitation returned to that of baseline. Sustained immediate early gene activation is reported in the BnST and MPN for both sexes. These nuclei play an initial role in processing incoming social information in both sexes and play an additional role in sex behavior as the opportunity to mate approaches. This may explain the pattern of sustained c-Fos expression to be initial social activation followed by endocrine activation.

Continuous activation reported in these nuclei may reflect overlapping endocrine control during the time period surrounding behavioral activation in some regions. This would in part explain the pattern of neuronal activity observed in the male BST and the female VMNVL. These data support evidence indicating the BnST and MPN may play a role in both sexes, while the VMNVL in females may play more prominent roles in the expression of sexual behavior (Hansen et al., 1981; Emery and Moss, 1984; Everitt, 1990; Dominquez and Hull, 2001).

The LS is important both in the social behavior network (Newman, 2002) and in pair bond formation, at least in male prairie voles (Liu, Thomas and Wang, 2001). Initial activation is reported in both sexes shortly after activation of components in the social behavior network and concurrently with initial activation of nuclei involved in the reward pathway. It is possible that the initial activation measured following a 2 hr cohabitation reflects the participation of this nuclei in the social behavior network of both sexes.

Immediate early gene expression peaks more sharply in females but remains elevated from baseline for a longer period of time in males and may reflect the activity of a large population of AVP-IR fibers in this area that is almost absent in females (Young et al., 1997). Additional activation in this area in males may indicate an initial social response

followed by a more slowly appearing endocrine stimulated response. Vasopressin-dependent activity in the LS in males could contribute to this sex difference.

Nuclei Associated with Pair Bonding

Partner preferences can form in both sexes even in the absence of mating (De Vries et al., 1997). However, the establishment of pair bonds in prairie voles, and especially the onset of male-male mate guarding, may be facilitated following mating (Williams, et al., 1992; Winslow, et al., 1993; Insel, et al., 1995). Little research has been done to examine the initial activation of immediate early genes within the reward pathway, prior to mating. Brain regions implicated in pair bond formation in prairie voles, including the LS (Liu et al, 2001), NAcc (Gingrich et al 2000, Young et al., 2001) and VP (Young et al., 2001; Pitkow et al., 2001), that did not show activation following one hour in cohabitation (Cushing et al 2003), show peak levels of immediate early gene expression following a two hour cohabitation in both sexes in the present study. This dopaminergic utilizing circuit is involved in the rewarding effects of natural stimuli (McBride et al., 1999) and potentially mediates the limbic and motivational aspects of pair bond formation (Lim et al., 2004). Activation in these areas suggests that this process begins in the first few hours of cohabitation in both sexes. Social bonds tend to form when a stressful experience is followed by a potentially positive experience (Carter, 1998). It is therefore likely that the initial stages of partner preference formation will commence after these social voles are stressed by sibling separation followed by a period of reduced anxiety as the animal recognizes the new partner as a potential mate. It is possible that neuronal activity stimulated by social contact in the NAcc and VP and is

regulated by neuropeptides acting in conjunction with dopamine (Liu and Wang, 2003; Lim et al., 2004). Recent studies report concurrent activation of OT and DA D2-type receptors in the NAcc of female prairie voles are critical in the formation of a pair bond (Liu and Wang 2003; Aragona et al., 2003a). DA release with social contact and mating is not limited to monogamous mammals. DA is released in polygynous species that do not form pair bonds (Mermelstein and Becker 1995, Pfaus et al., 1995) and is released in equal magnitudes in monogamous and polygynous voles (Curtis et al., 2003). Liu and Wang have therefore suggested that it is the OT systems or the interaction of OT and DA present in prairie voles that is responsible for the establishment of pair bonds. This hypothesis is supported by the findings that OT receptors are found in the highest densities in the NAcc of monogamous voles whereas they are almost absent in the same region in polygynous voles (Insel and Shapiro, 1992; Young et al., 2001; Lim et al., 2004). Additionally, V1a receptors are concentrated in the VP of monogamous prairie voles and are almost absent in polygynous species (Insel et al., 1994; Young et al 2001; Lim et al., 2004). It is therefore possible that in females, OT and DA interactions within the NAcc are key whereas in males, V1a and DA interactions within the VP may play a more prominent role in establishing a pair bond (Winslow et al., 1993; Insel and Hulihan, 1995; Cushing and Carter, 2000; Lim et al., 2004). The present study reveals that the brain regions necessary for this to be the case appear to be simultaneously activated. Nuclei implicated in pair bonding including LS, VP, and NAcc showed simultaneous peak ieg expression and were concurrent with activation in the PVN in both sexes.

Dopamine systems may also interact with neural circuits involved in reproductive activation in the prairie vole (Demas et al., 1997). Activation of tuberoinfundibular

neurons following chemosensory stimulation induces a subsequent release of DA into the hypophysial portal system (Keverne, 1983). This increase in hypothalamic DA, leading to a decrease in prolactin may stimulate LHRH neurons and increase the secretion of LHRH. These results may in part explain the activity observed in the present study. There were no sex differences in number of LHRH ir neurons per area or treatment, and very few neurons were colocalized with c-Fos. However, the majority of males, and some females, in cohabitation for 2 hours that did express LHRH/c-Fos colocalized neurons were primarily located in preoptic and lateral hypothalamic areas. It is interesting to note that while neither sex expressed colocalized neurons during initial contact, that both expressed at least some activation concurrent with activity recorded in nuclei that are dopamine targets including the NAcc and VP. At least some males at each further time point expressed colocalized neurons whereas female prairie voles did not. Preliminary results indicate that prairie voles may require longer than a twelve-hour cohabitation to fully activate the HPG axis.

Endocrine stimulated activity, for example OT and/or AVP, may be the initial event that primes the reward circuit. Concurrent or subsequent release of DA may solidify the evolving pair bond. This hypothesis is supported in part by data from other studies showing an increase in immediate early gene activation in these same areas following mating. An increase c-Fos ir was measured two hours after mating in the NAcc and VP of male prairie voles (Lim and Young 2003) but was not observed following 6 hours of mating in females of this species (Curtis and Wang 2003). This may indicate that neurons in the reward circuit, activated by mating, remain active for of some time following sexual activity. It also may mean that additional neurons need not be recruited

for the pair bond to progress, and thus a change in neuronal status is not measured. Alternatively, these areas may be more important for the formation rather than the maintenance of a pair bond. These data indicate that the reward pathway is utilized by both sexes during the time when partner preferences are forming, and a sexually dimorphic temporal pattern emerges over time when animals remain in contact, as was seen during the cohabitation used in the present study.

Neuronal activation in the NAcc, as indexed by c-Fos IR, doubled with initial contact and peaked following a 2 hour cohabitation in both sexes, but females peaked much sharper. The NAcc is involved in pair bond formation in male and female prairie voles (Young et al., 2001; Aragona et al., 2003a; Liu and Wang, 2003) and sends major projections to the VP. The present experiment is the first to show c-Fos expression in the NAcc of female prairie voles. Its timing coincides with activity in other components of the reward pathway and additionally with activation in the PVN.

The VP showed activation in both sexes shortly after activation of the social behavior network. Immediate early gene expression was measured within the first hours of cohabitation and only after activity was recorded in the social behavior network. No temporal or sex differences in activation were reported in the VP.

Male and female prairie voles displayed increased neuronal activation in nuclei containing dense OTR and V1a receptor binding, supporting data implicating the importance of both OT and VP systems in pair bond formation (Cho et al., 1999). Further staining will reveal (1) if c-Fos expression in these nuclei represents activation of dopaminergic pathways and (2) if neuropeptide receptor binding changes as cohabitation commences. Additionally, staining will reveal if sex differences exist and provide a

timeline for OT/DA interactions in NAcc and potentially, AVP/DA interactions in the VP. Concurrent activation of the reward pathway and neuropeptide producing nuclei early in cohabitation may be the initial step in pair bond formation in prairie voles.

Patterns seen in neuropeptide producing nuclei

Significant increases from basal c-Fos ir are measured in the PVN and SON with time spent in cohabitation in male and female prairie voles. Although both sexes utilize these nuclei during the time when partner preferences are forming, sexually dimorphic patterns exist in c-Fos expression. This difference is more striking in the PVN where we observed earlier activation in the female prairie vole and sustained activation in the both sexes. Generally speaking, activation in the PVN was recorded in both sexes concurrently with activation in the reward pathway but before activation of the SON. Both sexes showed a similar pattern of c-Fos expression in the SON where activation is reported later in cohabitation as the time for mating would normally approach. Activity in these nuclei suggests neuropeptide involvement in early stages of pair bond formation. The sexually dimorphic pattern of c-Fos expression in the PVN and SON may reflect the sexually dimorphic nature of pair bond formation in prairie voles.

Neuronal activity within the PVN and SON may be regulating activity measured at later time points in both the sociosexual and reward circuits. An increase in activity is measured in neuropeptide producing nuclei as AVP and OT dependent behaviors emerge. This later activation in males could reflect the androgen dependent action of AVP neurons capable of influencing activity in the LS and related systems. This study indicates that the nuclei necessary for this to be the case may undergo concurrent activation. The

pattern shared by the PVN and LS in males is absent in females; possibly because females lack AVP-ir fibers in this area. An increase in AVP facilitates behaviors typical to social monogamy, including selective aggression and paternal care, in males. Selective aggression develops earlier in males than in female prairie voles where aggression usually occurs around the time of parturition. Increased c-Fos expression in the SON occurs as cohabitation commences. This may reflect an increase in neuropeptide synthesis or release required for the appropriate display of sex behaviors and behaviors leading to the formation of pair bonds. Alternatively, this increase in activity may be due to relative dehydration. Although the animals had access to water for the duration of cohabitation, interest in the novel partner may have overcome the desire to drink.

Further staining may reveal which neuropeptide populations are responsible for the increase in c-Fos activity and may provide approximate time lines for the onset of neuropeptide regulated behaviors associated with monogamy including parental care, selective aggression, and partner preference formation.

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APPENDIX A:

