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CHARACTERIZATION OF NOVEL GROUPS OF
CATECHOLAMINERGIC CELLS IN THE MALE PRAIRIE VOLE
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CHARACTERIZATION OF NOVEL GROUPS OF CATECHOLAMINERGIC CELLS IN THE MALE PRAIRIE VOLE EXTENDED AMYGDALA

By

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ABSTRACT

CHARACTERIZATION OF NOVEL GROUPS OF CATECHOLAMINERGIC CELLS IN THE MALE PRAIRIE VOLE EXTENDED AMYGDALA

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The neurobiology of copulatory and maternal behaviors has been well studied in mammals, but much less is known about gregarious and monogamous social behaviors. Prairie voles (*Microtus ochrogaster*) are one of few mammalian species that are highly gregarious, monogamous, and biparental, and have been a useful rodent model for studying the neural networks influencing these behaviors. Comparisons between the prairie vole brain and closely related nonmonogamous rodents have revealed species differences in neurotransmitter and receptor expression that contribute to differences in social behavior. Recently, large populations of cells expressing tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis, were discovered in the prairie vole principal nucleus of the bed nucleus of the stria terminalis (pBST) and posterodorsal medial amygdala (MeApd). Large numbers of TH-immunoreactive (TH-ir) cells have never been described in these sites in other species. The pBST and MeApd are important for the expression of sociosexual behaviors, and these cells may affect the social behaviors typical of prairie voles. The experiments in this dissertation characterize the distribution, hormone regulation, immediate-early gene (IEG) responses, and neuroanatomical projections of these unique groups of cells in the male prairie vole brain.

The results of the first experiment (Chapter 2) indicated that several nonmonogamous species, laboratory rats, Syrian hamsters, and meadow voles, did not have these large populations of TH-ir cells, further supporting the idea that these cells

may influence species-specific social behaviors. The results also indicated that male prairie voles had 3-5 times more TH-ir cells in the pBST and the MeApd than did females, and this sex difference was almost entirely due to differences in circulating gonadal hormones during adulthood. The experiments in Chapter 3 examined immediate-early gene expression in these TH-ir cells after males engaged in sociosexual behaviors, and these experiments revealed that these groups of TH-ir neurons likely influence gregariousness in male prairie voles, but may also be involved more specifically in mating and its facilitation of pair bonding. Finally, neuroanatomical tracing (Chapter 4) revealed that many TH-ir cells in the pBST and MeApd project to the medial preoptic area (MPO), an area important for the expression of many sociosexual behaviors.

These experiments demonstrate that TH expression in the pBST and MeApd remains plastic in adulthood and is sensitive to circulating gonadal hormones, much like other morphological and neurochemical features of the pBST and MeApd in rats, mice, and hamsters. Furthermore, these cell groups are unique in that they may promote the expression of all social behaviors, but also specific behaviors related to mating and pair bonding. They also provide a species-specific catecholaminergic input to the MPO, which has connections with other brain areas known to be important for pair bonding. These experiments have provided information necessary for many future experiments that can explore the precise function of these cells and their interactions with other neurochemicals. This catecholaminergic network is likely essential for the affiliative and monogamous behaviors typical of male prairie voles.

I dedicate this dissertation to Rascal. You were my best friend and partner in crime, and thank you for always reminding me to embrace every moment.

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LIST OF ABBREVIATIONS

ACB	nucleus accumbens
aco	anterior commissure, olfactory limb
adBST	bed nuclei of the stria terminalis, anterior division, anterodorsal area
ADP	anterodorsal preoptic nucleus
AHA	anterior hypothalamic area
AHN	anterior hypothalamic nucleus
alBST	bed nuclei of the stria terminalis, anterior division, anterolateral area
ARH	arcuate nucleus hypothalamus
AV	anteroventral nucleus thalamus
avBST	bed nuclei of the stria terminalis, anterior division, anteroventral area
AVP	anteroventral preoptic nucleus
AVPV	anteroventral periventricular nucleus
BDA	biotinylated dextran amine
BMA	basomedial nucleus amygdala
BST	bed nuclei of the stria terminalis
ccBST	bed nuclei of the stria terminalis, anterior division, anterodorsal area, central core
CEA	central nucleus amygdala
COA	cortical nucleus amygdala
DA	dopamine
DAB	3,3'-diaminobenzidine
dBST	bed nuclei of the stria terminalis, posterior division, dorsal nucleus
dlBST	bed nuclei of the stria terminalis, anterior division, dorsolateral nucleus

dmBST	bed nuclei of the stria terminalis, anterior division, dorsomedial nucleus
DMH	dorsomedial nucleus hypothalamus
ENT	entorhinal area
FG	FluoroGold
fi	fimbria
fuBST	bed nuclei of the stria terminalis, anterior division, fusiform nucleus
fx	columns of the fornix
IEG	immediate-early gene
ifBST	bed nuclei of the stria terminalis, posterior division, interfascicular nucleus
-ir	immunoreactive
juBST	bed nuclei of the stria terminalis, anterior division, juxtacapsular nucleus
LH	lateral habenula
LHA	lateral hypothalamic area
LM	lateral mammillary nucleus
LPO	lateral preoptic area
LS	lateral septal nucleus
LSc	lateral septal nucleus, caudal part
LSc.d	lateral septal nucleus, caudal part, dorsal zone
LSc.v	lateral septal nucleus, caudal part, ventral zone
LSr	lateral septal nucleus, rostral part
LSr.d	lateral septal nucleus, rostral part, dorsal zone
LSr.v	lateral septal nucleus, rostral part, ventral zone
LSv	lateral septal nucleus, ventral part

MA	magnocellular preoptic nucleus
MD	mediodorsal nucleus thalamus
MeA	medial nucleus amygdala
MeAad	medial nucleus amygdala, anterodorsal part
MeAav	medial nucleus amygdala, anteroventral part
MeApd	medial nucleus amygdala, posterodorsal part
MeApv	medial nucleus amygdala, posteroventral part
MEPO	median preoptic nucleus
mgBST	bed nuclei of the stria terminalis, anterior division, magnocellular nucleus
MH	medial habenula
MM	medial mammillary nucleus
MPN	medial preoptic nucleus
MPO	medial preoptic area
NaPB	sodium phosphate buffer
NDB	nucleus of the diagonal band
NGS	normal goat serum
opt	optic tract
ovBST	bed nuclei of the stria terminalis, anterior division, oval nucleus
PA	posterior nucleus amygdala
PAG	periaqueductal gray
pBST	bed nuclei of the stria terminalis, posterior division, principal nucleus
PH	posterior hypothalamic nucleus
PM	premammillary nucleus

PS	parastrial nucleus
PSCH	suprachiasmatic preoptic nucleus
PV	periventricular nucleus hypothalamus
PVH	paraventricular nucleus hypothalamus
PVT	paraventricular nucleus thalamus
RCH	retrochiasmatic area
rhBST	bed nuclei of the stria terminalis, anterior division, rhomboid nucleus
RT	reticular nucleus thalamus
SBPV	subparaventricular zone hypothalamus
scBST	bed nuclei of the stria terminalis, anterior division, subcommissural zone
seBST	bed nuclei of the stria terminalis, posterior division, stria extension
SF	septofimbrial nucleus
SI	substantia innominata
sm	stria medullaris
SN	substantia nigra
st	stria terminalis
SUM	supramammillary nucleus
TBS	tris-buffered saline
TEP	temporal pole
TH	tyrosine hydroxylase
TMv	tuberomammillary nucleus, ventral part
trBST	bed nuclei of the stria terminalis, posterior division, transverse nucleus
TRS	triangular nucleus septum

TU	tuberal nucleus
V3	third ventricle
vBST	bed nuclei of the stria terminalis, anterior division, ventral nucleus
VL	lateral ventricle
VMH	ventromedial nucleus hypothalamus
VP	ventral pallidum
VTA	ventral tegmental area
zl	zona limita

CHAPTER 1:

Introduction

Monogamy is defined as "a prolonged association and essentially exclusive mating relationship between one male and one female," and often occurs when breeding opportunities are limited and offspring benefit from biparental care (Wittenberger and Tilson, 1980). Mating exclusivity is difficult to determine, and individuals of "socially monogamous" species do occasionally mate with conspecifics other than their partner (Wittenberger and Tilson, 1980). In fact, in some monogamous species one litter can contain pups from different fathers (Borkowska et al., 2009; Glenn et al., 2009; Goossens et al., 1998; Solomon et al., 2004). Therefore, Kleiman (1977) has described four characteristics that are often used to recognize monogamous species: 1) members of the pair remain in close proximity during breeding and nonbreeding periods, 2) members of the pair prefer to mate with their partner as opposed to an unfamiliar opposite-sex conspecific, 3) unrelated conspecifics are rarely or never found in the pair's territory, and 4) only one pair in a family group breeds. Most bird species are monogamous (Lack, 1968), but monogamy is rare in mammals, fish, reptiles, and amphibians (Bull, 2000; Kleiman, 1977; Whiteman and Cote, 2004; Wittenberger and Tilson, 1980).

Monogamy can be further divided into two types, facultative (Type I) and obligate (Type II) (Kleiman, 1977). Facultative monogamy occurs only at times when the population density is so low that only one opposite-sex conspecific is available, and in this type of monogamy males typically do not directly provide care to their offspring. In contrast, obligately monogamous species require male participation in rearing young.

Obligate monogamy often co-occurs with cooperative breeding, a social structure in which animals live in groups and some members take care of young that are not their own (Bergmuller et al., 2007; Jennions and MacDonald, 1994). In obligately monogamous family groups, adult and juvenile offspring of the breeding pair will typically help raise younger litters, and often forego their own breeding opportunities (Carter and Roberts, 1997). Cooperatively breeding species usually live in environments with ecological constraints that allow some members of the group to have greater reproductive success by caring for other members of the group than breeding independently, such as low availability of mates, high predator density that make dispersal risky, or poor quality of breeding opportunities (Komdeur et al., 2008; Solomon and Getz, 1997).

The brains of obligately monogamous and cooperatively breeding species are surely different than those of nonmonogamous species because their social behaviors drastically differ. Neural networks must exist that allow breeding individuals to form bonds with their partner, sires to care for their pups, nonbreeding individuals to display alloparental behavior towards offspring that are not their own, and all individuals to show gregarious behaviors necessary to live in a large social group. Prairie voles (*Microtus ochrogaster)* have become a popular rodent model for investigating the neurobiology of complex sociosexual behaviors more typical of humans and other primates because prairie voles are obligately monogamous cooparative breeders that form lifelong pair bonds after mating and engage in biparental care (Carter et al., 1995; Getz and Hoffman, 1986). Investigations of the prairie vole brain have revealed that it contains some neural networks not present in non-monogamous rodents, and many of these networks influence social motivation.

The prairie vole model

Prairie voles are highly gregarious, both in the wild and in captivity, making them a valuable model for laboratory and field research (Carter et al., 1995; Getz and Hoffman, 1986). As cooperative breeders, they are often found in large social groups composed of a reproductively active male and female, and several generations of their offspring (Getz and Carter, 1980; Getz and Hofmann, 1986; Getz et al., 1993). In fact, as many as 70% of males and females remain in the natal nest for life and forego opportunities for reproduction (McGuire et al., 1993). Although many voles are in close contact with their opposite-sex relatives throughout their lives, incest is prevented because females avoid sniffing the anogenital region of their male relatives and the absence of exposure to these male olfactory cues prevents the induction of estrus (Carter et al., 1980, 1987b, 1989; Gavish et al., 1984). The prairie voles that do disperse from the nest become reproductively active, and many of these animals form life-long pair bonds after mating, as indicated by field studies in which the same two voles were repeatedly captured in the nest together until one or both members of the pair died (Getz and Hofmann, 1986; Getz et al., 1993). Even after the death of a partner, only 10% of survivors form a new pair bond (Getz et al., 1981). In the laboratory, pair bonding is inferred when a vole forms a "partner preference" (*e.g.* Williams et al., 1992b). After a period of cohabitation with an opposite-sex "partner," the subject (either male or female) is placed in a three-chambered apparatus with the partner tethered in one chamber and an unfamiliar opposite-sex "stranger" tethered to another chamber. Subjects have access to all three chambers, and prairie voles that have formed a partner preference spend significantly more time in contact with their partner than with the stranger. Mating with the partner prior to the test

facilitates the formation of partner preferences, particularly in males, but extended cohabitation without mating is sometimes sufficient for partner preference formation (Insel et al., 1995; Williams et al., 1992a). Once the pair bond has formed, both males and females are aggressive toward intruders, which may limit contact with unfamiliar adults and help maintain the pair bond (Getz et al., 1981; Gobrogge et al., 2007, 2009).

Even in the laboratory, juvenile prairie voles are highly parental and often provide care towards younger siblings (Lonstein and De Vries, 2001; Olazabal and Young, 2005; Roberts et al., 1998; Wang and Novak, 1994). Interestingly, when males and females are weaned from their natal cage before they are exposed to subsequent litters (and presumably when they emigrate from the nest in the wild), adult virgin males are highly paternal but most virgin females ignore or attack pups (Hayes and De Vries, 2007; Lonstein and De Vries, 1999b, 2001; Olazabal and Young, 2005; Williams et al., 1992c). However, after parturition, both sexes provide parental care, and males show the same complement of parental behaviors as females, with the obvious exception of nursing (Lonstein and De Vries, 1999a; McGuire and Novak, 1984; Oliveras and Novak, 1986). Paternal care does influence survival of the offspring; female prairie voles are more likely to maintain a pregnancy when their partners are present (Dewsbury, 1994; McGuire et al., 1992), and litters develop more rapidly in the presence of the father (Wang and Novak, 1992). Thus, male prairie voles gain more reproductive success if they remain with their mate, and this is likely one of the reasons that monogamy evolved in this species.

The neurobiology of prairie vole sociosexual behaviors

Studies examining the neurobiology of pair bonding and parental behavior in prairie voles have highlighted the importance of several neurotransmitters, most notably dopamine (DA), vasopressin (primarily in males), and oxytocin (primarily in females), in the regulation of social behaviors (Wang and Aragona, 2004; Young et al., 2001, 2005, 2008). These neurotransmitters interact with one another to regulate pair bonding (Cho et al., 1999; Lim et al., 2004c; Liu et al., 2001, 2003), and Young and colleagues (1998, 2001) have proposed that DA release during mating interacts with vasopressin or oxytocin release from olfactory regions to allow animals to associate the rewarding aspects of mating with the characteristics of that particular mate. In males, research has focused on the release of DA in the nucleus accumbens (ACB) and vasopressin in the ventral pallidum (VP) and lateral septum (LS) (Young et al., 2008), but little is known about the neurons providing this input, or other areas of the brain that may also influence social behaviors (see Figure 1.1). DA, vasopressin, and oxytocin are also important for the expression of parental behaviors (Lim et al., 2004c; Lonstein, 2002; Olazabal and Young, 2006; Wang et al., 1994a), but the neural networks involved are unclear.

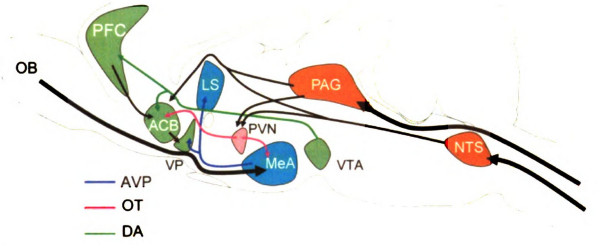

Figure 1.1. Proposed neural network involved in pair bonding. Somatosensory input from the penis and vagina likely projects to the periaqueductal gray (PAG) and nucleus of the solitary tract (NTS), which in turn communicate with other areas of the forebrain. In addition, vasopressin (AVP) release in the VP and LS, oxytocin (OT) release in the ACB and medial amygdala (MeA), and dopamine (DA) in the ACB and prefrontal cortex (PFC) are important for pair bonding. The location of cell bodies of these neurotransmitters in prairie voles, as well as the connections with other brain sites, is largely unknown. Figure adapted from Young et al., 2005.

The *Microtus* genus consists of a number of highly social species, such as the prairie vole, but also many species that are neither monogamous nor biparental. This diversity in social strategy has allowed a number of comparisons of neurotransmitter and receptor expression among monogamous and nonmonogamous species to better understand the neurobiology of social behaviors. These studies have revealed complex species differences in the expression of vasopressin (Wang, 1995), vasopressin receptors (Insel et al., 1994; Young et al., 1997), DA receptors (Aragona et al., 2006; Smeltzer et al., 2006), oxytocin receptors (Insel and Shapiro, 1992; Olazabal and Young, 2006; Smeltzer et al., 2006), corticotropin-releasing factor receptors (Lim et al., 2005), and cocaine- and amphetamine-regulated transcript (Hunter et al., 2005) that may be related to social structure. All of these species differences probably contribute to differences in

behavior, but interspecies variations in vasopressin receptor density have been the focus of most research to date. Indeed, blocking vasopressin receptors in the ventral pallidum (VP), a brain area with many more vasopressin receptors in social voles compared to non-social voles, inhibits partner preference formation in male prairie voles (Lim and Young, 2004). Interestingly, increasing the expression of vasopressin receptors in the VP promotes social interactions in relatively non-social meadow voles, rats, and mice, and can even facilitate partner preference formation in some animals (Landgraf et al., 2003; Lim et al., 2004c, Young et al., 1999). These drastic changes in social interactions are remarkable, but the complete repertoire of gregarious and monogamous behaviors typical of prairie voles are not induced after these manipulations. In addition, although differences in microsatellite length in the promoter region of the vasopressin V1a receptor gene have been found to underlie species differences in receptor expression (Hammock et al., 2004), Fink et al. (2006) reported that microsatellite length and V1a receptor expression cannot completely explain differences in social behavior across the *Microtus* genus. Furthermore, microsatellite length does not reflect the mating strategy used by individual prairie voles (Ophir et al., 2008a, b). There are likely a number of neural systems contributing to the expression of social behavior, and some of these have yet to be discovered.

Species differences in DAergic systems have also been reported, and may contribute to differences in social behavior. For example, male meadow voles have a greater density of D1 DA receptors in the ACB and prefrontal cortex (PFC) than do male prairie voles, while prairie voles have more D2 receptors in the PFC than do meadow voles (Aragona et al., 2006; Smeltzer et al., 2006). Furthermore, while baseline

extracellular DA levels are equivalent, male prairie voles have a greater increase in DA release in the ACB after peripheral amphetamine adminstration than do meadow voles (Curtis and Wang, 2007). This species difference in response to amphetamine could be due to differences in numbers of DA transporters or DA autoreceptors on presynaptic terminals in the ACB. The behavioral effects of these species differences are unknown, but may contribute to variations in social structure given the role of DA in pair bonding and parental behavior (see below).

Dopamine and sociosexual behaviors

DA, synthesized from tyrosine via the rate-limiting enzyme tyrosine hydroxylase (TH) and L-amino acid decarboxylase, was one of the first neurotransmitters proposed to influence prairie vole social behaviors, and its release is necessary for the formation and maintenance of pair bonds in both sexes of prairie voles. DA release in the ACB increases during mating, and peripheral administration of the DA antagonist haloperidol eliminates partner preference formation (Aragona et al., 2003). Furthermore, partner preference formation can be elicited in the absence of mating if a D2 DA receptor agonist is injected into the ACB (Aragona et al., 2006; Gingrich et al., 2000). However, activation of D1 receptors in the ACB prevents partner preference formation, and an upregulation of D1 receptors after pair bond formation is thought to ensure the stability of the initial pair bond (see Figure 1.2, Aragona et al., 2006). Although DAergic projections to the ACB necessary for DA's facilitation of pair bonding are assumed to come from the ventral tegmental area (VTA) (Curtis and Wang, 2005), it is likely that DA originating

from multiple brain sites might be important (Dominguez and Hull, 2005; Melis and Argiolas, 1995; Miller and Lonstein, 2005).

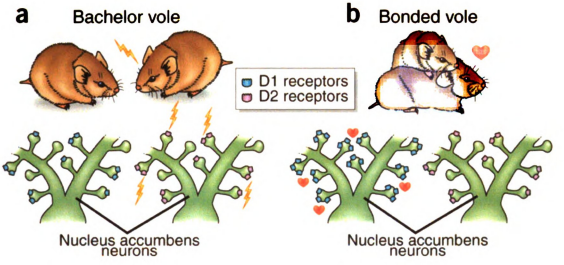

Figure 1.2. Depiction of DA receptor distribution in the nucleus accumbens in naïve and pair bonded male prairie voles. The upregulation of D1 receptors during pair bond formation is thought to prevent the formation of additional pair bonds. Figure from Edwards and Self, 2006.

DA neurotransmission is also necessary for the typical expression of parental behavior in prairie voles. Systemically administered haloperidol increases the latency for males to contact pups, decreases the amount of time that male and female prairie voles spend licking their pups, and decreases the amount of time spent actively hovering over pups without affecting motor systems (Lonstein, 2002). However, investigations of the specific DAergic systems responsible for parental behaviors in prairie voles have not been carried out. In rats, DA release (measured by microdialysis) increases in the ACB after lactating females interact with pups (Champagne et al., 2004; Hansen et al., 1993), and is also higher in the medial preoptic area (MPO) during lactation compared to the end

of pregnancy (Lonstein et al., 2003). D1 agonists injected into either the ACB or the MPO decrease the time needed for females to show maternal behavior (Stolzenberg et al., 2007). Conversely, selective lesions of DAergic terminals in the ACB or local injections of DA antagonists into the ACB or MPO disrupt maternal behaviors (Hansen et al., 1991a, b, 1994; Keer and Stern, 1999; Miller and Lonstein, 2005; Numan et al., 2005). Similar to the divergent role of D1 and D2 receptors in pair bonding, these receptors have different functions in maternal behavior, at least in the MPO, as D1 receptor activation stimulates retrieving and licking while D2 receptor activation inhibits quiescent nursing behaviors (Miller and Lonstein, 2005). This relevant DA release likely arises from cell bodies in the VTA and hypothalamus (Champagne et al., 2004; Keer and Stern, 1999; Miller and Lonstein, 2005, 2009; Silva et al., 2003; Stolzenberg et al., 2007). Similar mechanisms may be responsible for the expression of maternal and paternal behaviors in prairie voles.

DA neurotransmission is also critical for male sexual behavior in at least some rodents. In male rats, DA release in the MPO increases when males are exposed to estrous females and during copulation (Dominguez et al., 2001; Hull et al., 1995), and microinjections of DA agonists increase intromissions and ejaculations (Hull et al., 1986), while microinjections of DA antagonists decrease ejaculations and penile reflexes (Pehek et al., 1988; Warner et al., 1991). Interestingly, activity in the medial amygdala (MeA) resulting from olfactory and other sensory inputs from females enhances DA release in the MPO to facilitate masculine copulatory behaviors (Dominguez and Hull, 2001). DAergic cells are not found in the rat MeA, but glutamatergic neurons in the MeA synapse on terminals of DAergic cells coming from other regions, such as the

periventricular hypothalamus or posterior hypothalamus (Miller and Lonstein, 2009; Moore and Lookingland, 1995), to increase DA release (Dominguez and Hull, 2005). DA release is also required in additional areas, including the dorsal striatum and ACB, for the normal expression of copulatory behaviors in male rats (Melis and Argiolas, 1995). Interestingly, DA may be one of the mediators of hormonal effects on male sexual behavior, as castration eliminates increases in DA release in the MPO (Hull et al., 1995), and DA agonists can restore sexual behaviors in castrated rats or mice lacking the estrogen receptor α gene (Malmnas, 1977; Scaletta and Hull, 1990; Wersinger and Rissman, 2000).

DA's role in sexual behavior in male prairie voles is less clear, as initial investigations surprisingly found that DA may not be necessary. DA release is enhanced in some brain areas, including the ACB, during mating in both male and female prairie voles (Aragona et al., 2003; Curtis et al., 2003). However, Aragona and colleagues (2003) reported no reduction in the number of mating bouts in males given systemic haloperidol. The details of mating interactions resulting from this manipulation were not reported, however, and it is possible that DA may be required for some aspects of their mating, as it is for male and female rats.

Recently, during an investigation of DAergic systems that may be important for the expression of parental behavior in prairie voles (Lansing and Lonstein, 2006), the Lonstein laboratory discovered groups of neurons in the principal nucleus of the bed nucleus of the stria terminalis (pBST) and posterodorsal medial amygdala (MeApd) that contained TH immunoreactivity (Figure 1.3; Northcutt et al., 2007). TH-immunoreactive (TH-ir) cells were particularly numerous in males. Interestingly, large numbers of TH-ir

cells had never before been described in the pBST or MeApd of other vertebrates that have been studied (Albanese et al., 1986; Hokfelt et al., 1984; Kalsbeek et al., 1992; Smeets and Gonzalez, 2000), and may be part of neural networks influencing social behaviors. Indeed, the BST and MeA process chemosensory and hormonal information, have widespread projections throughout the forebrain (including extensive projections to one another), and regulate sociosexual behaviors in mammals (Coolen and Wood, 1998; Kling, 1972; Newman, 1999; Numan and Insel, 2003; Wood and Swann, 2005).

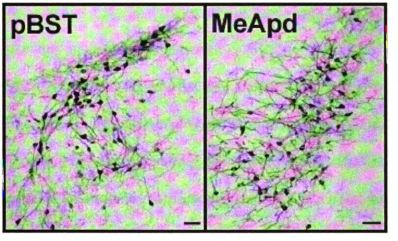

Figure 1.3. TH-ir cells in the pBST (left) and MeApd (right) of a male prairie vole. Scale bar = 50 μm.

TH-expressing neurons have never been described in the rat pBST or MeApd, but similar groups may exist in male hamsters. Wommack et al. (2002) found TH-ir cells in the pBST and MeApd of male hamsters, especially under conditions of social stress, but in very low numbers (~10-11 cells per region). Many more TH-expressing cells are seen in the pBST and MeApd in hamsters after axonal transport is inhibited by colchicine (Asmus et al., 1992; Asmus and Newman, 1993), but the need for colchicine indicates either that prairie voles have higher TH levels than hamsters under normal conditions or

that axonal transport of TH is different between the two species. Similar to previously-discovered differences in neurotransmitter or receptor distribution, this species difference in TH-immunoreactivity may also reflect differences in behavior, and the lack of large numbers of TH-ir cells in rats and hamsters may partially explain why they do not engage in high levels of affiliative behaviors, pair bonding, and paternal behavior. Furthermore, these cell groups may contribute to DA release necessary for pair bonding and paternal behavior, and may interact with other neurotransmitters known to be involved in facilitating social interactions.

The olfactory system and sociosexual behaviors

The BST and MeA, as well as olfactory areas projecting to these regions, are already implicated in regulating sociosexual behaviors in prairie voles and other rodents. Lesions of the olfactory bulbs prevent partner preference formation in females and reduce general affiliative behavior in male prairie voles (Curtis et al., 2001; Kirkpatrick et al., 1994c). Furthermore, the expression of Fos, an immediate-early gene (IEG) product, increases in the BST and MeA after male prairie voles cohabitate with a female, which indicates that these areas may play a role in consolidating partner preferences (Cushing et al., 2003; Lim and Young, 2004). The MeA may also be involved in pair bond formation in primates, as the MeA of pair bonded titi monkey males shows greater glucose uptake in PET scans than that of lone males (Bales et al., 2007). However, the specific role of the BST and MeA in pair bond formation or maintenance in prairie voles or other monogamous species is not known.

The BST and MeA are also involved in the expression of paternal behavior in prairie voles. Lesions of the olfactory bulbs or the MeA decrease paternal behavior (Kirkpatrick et al., 1994a, c), and Fos expression increases in the BST and MeA of male prairie voles after exposure to pups (Kirkpatrick et al., 1994b). However, olfactory bulb lesions have no effect on maternal behavior in females (Williams et al., 1992c), suggesting that olfactory information is more important for promoting paternal behavior than maternal behavior in prairie voles.

Although there is limited information about the function of the BST and MeA in pair bonding and parental behavior, these regions clearly influence sexual behavior in prairie voles and other rodents. Lesions of the main and accessory olfactory bulbs prevent estrus induction in female prairie voles and eliminate mating in males (Curtis et al., 2001; Kirkpatrick et al., 1994c; Lepri and Wysocki, 1987). Additionally, Fos expression increases in the BST and MeA after male prairie voles cohabitate or mate with a female (Cushing et al., 2003; Lim and Young, 2004). The roles of subregions of the prairie vole BST and MeA are unknown, but the pBST and MeApd are especially important for chemosensory investigation of females and ejaculation in male rats (Bressler and Baum, 1996; Coolen et al., 1997, 1998).

To date, research on BST and MeA neurochemistry important for prairie vole sociosexual behaviors has focused on vasopressin projections originating in these regions. Males have more vasopressinergic cells in the BST and MeA than do female prairie voles, and more vasopressin-ir fibers, presumably from BST and MeA cells, in the lateral septum (LS) and lateral habenula (Bamshad et al., 1993; Lonstein and De Vries, 1999b; Wang, 1995). The first idea that the extra-hypothalamic vasopressin system plays

a role in sociosexual behaviors in prairie voles came from evidence that microinjections of vasopressin into the LS facilitate paternal behavior (Wang et al., 1994a). Furthermore, injection of a V1a vasopressin receptor antagonist into the LS or VP blocks the formation of partner preferences (Lim and Young, 2004; Liu et al., 2001), while injection into the LS or MeA interferes with paternal behavior (Lim et al., 2004c; Wang et al., 1994a). Finally, over-expression of V1a receptors in the VP facilitates partner preference formation in male prairie vole and in the typically non-monogamous male meadow vole (Lim et al., 2004c; Pitkow et al., 2001). Thus, the BST and MeA probably influence pair bonding and paternal behaviors by regulating vasopressin production and release, but there are likely other neurochemicals, such as DA, in these regions that regulate species-specific social behaviors in prairie voles.

Hormone regulation of the pBST and MeApd

The apparent sex difference in TH expression in the prairie vole pBST and MeApd is not particularly surprising because pBST and MeApd morphology and neurochemistry are sexually dimorphic in many rodents (Cooke et al., 1999; De Vries and Miller, 1998; Hines et al., 1992; Malsbury and McKay, 1987, 1989; Micevych et al., 1988). In rats and mice, males have a greater overall pBST and MeApd volume in both regions, and larger neuronal soma sizes in the MeApd (Cooke et al., 1999; Durazzo et al., 2007; Hines et al., 1992; Morris et al., 2005, 2008). Astrocyte number and complexity are also sexually dimorphic in the MeApd (Johnson et al., 2008), as is the expression of numerous neurotransmitters in both sites (De Vries et al., 1984, 1985; Malsbury and McKay, 1994; Simerly and Swanson, 1987; Swann and Newman, 1992). Most sex

differences in anatomy, physiology, and behavior arise due to sex differences in gonadal hormone levels in adulthood or during a critical period in development (usually the perinatal period or puberty) (*e.g.* De Vries and Simerly, 2002), and the pBST and MeApd seem exquisitely sensitive to circulating hormone levels in adulthood (Cooke et al., 1999, 2003; De Vries et al., 1984, 1985; Malsbury and McKay, 1994; Morris et al., 2008; Simerly and Swanson, 1987; Swann and Newman, 1992).

Sex differences in pBST and MeApd morphology and neurochemistry are thought to regulate many aspects of sociosexual behaviors in rodents, including mating and odor preferences. Positive correlations between neuronal soma size in the MeApd and measures of sexual arousal in castrated and hormone-treated male rats suggest that hormone-mediated changes in the morphology of MeApd cells may result in changes in sexual behaviors (Cooke et al., 2003). Additionally, testosterone or estradiol implants in the pBST or MeApd are able to reverse castration-induced declines in male sex behavior (Huddleston et al., 2003; Rasia-Filho et al., 1991; Wood and Newman, 1995b, c), and estradiol implants in the MeA induce receptive sexual behaviors in some female rats and hamsters (Lisk and Barfield, 1975; Sterner et al., 1992).

Adult gonadal steroids also alter the neurochemistry of the prairie vole BST and MeA, which may change the expression of sociosexual behaviors. The number of vasopressinergic cells in the BST and MeA and the number of vasopressinergic fibers in the areas they innervate dramatically declines after castration, but not if testosterone replacement is given (Lonstein and De Vries, 1999b; Wang and De Vries, 1993). Furthermore, vasopressinergic fiber density increases in females given estradiol (Lonstein

and De Vries, 1999b). Thus, similar to other rodents, the prairie vole BST and MeA are sensitive to hormones in adulthood.

Hormonal influences on sociosexual behaviors in prairie voles

Hormone levels also influence the display of sociosexual behaviors in prairie voles. Similar to other rodents, the ability to copulate is under gonadal hormone control in male and female prairie voles (Carter et al., 1987a, b, 1988; Cushing et al., 2004; Dluzen and Carter, 1979). The role of gonadal hormones in the post-copulatory formation of pair bonds has not yet been investigated, but other steroid hormones, such as glucocorticoids, modulate partner preference formation (DeVries et al., 1995, 1996a, b). Finally, gonadal hormones also influence parental behaviors in prairie voles; the effects of castration on paternal behavior are controversial (Lonstein and De Vries, 1999b; Wang and De Vries, 1993), but virgin females given testosterone or estradiol in adulthood show much higher levels of parental behavior than untreated females (Lonstein and De Vries, 1999b; Lonstein et al., 2002). These hormone-mediated changes in sociosexual behaviors may result from changes in morphology or neurochemistry of cells in the pBST or MeApd, as these regions also contain many androgen and estrogen receptors in prairie voles (Cavanaugh and Lonstein, 2010a; Cushing et al., 2004; Hnatczuk et al., 1994; Northcutt and Lonstein, 2008). Indeed, high levels of vasopressin immunoreactivity in the BST and MeA are only seen when testosterone levels are high (Bamshad et al., 1993, 1994; Wang and De Vries, 1993; Wang et al., 1994b), which may affect paternal behavior (Wang and De Vries, 1993; but see Lonstein and De Vries, 1999b). Other neurochemicals regulated by hormones in adult prairie voles, such as DA (Lansing and

Lonstein, 2006), may also contribute to alterations in the expression of sociosexual behaviors.

Although most research to date has examined neurochemistry or behavior after drastic hormone manipulations, gonadal hormones do, indeed, fluctuate in adult male and female prairie voles. Female prairie voles are induced ovulators and, in the absence of an unfamiliar male, their ovaries are quiescent and release very low amounts of estradiol and progesterone (Carter et al., 1989). Within 48 hours of male exposure, serum estrogen levels increase to about five times that of unexposed females (Cohen-Parsons and Carter, 1987), and progesterone levels rise dramatically after about 72 hours of mating (Carter et al., 1989). In contrast, the testes secrete significant amounts of testosterone even in the absence of unfamiliar females (Wang et al., 1994b), but testosterone levels may slightly increase after initial exposure to a female and after a male's partner gives birth and undergoes a postpartum estrus (Bamshad et al., 1994; Gaines et al., 1985; Wang et al., 1994b). These natural changes in hormone levels likely affect neurochemistry and sociosexual behaviors across different reproductive states.

Overview of dissertation chapters

Groups of TH-ir cells in the pBST and MeApd have never before been described in other vertebrates, and are likely to influence the monogamous behaviors of prairie voles, given the role of DA in pair bonding and parental behavior and the importance of the pBST and MeApd in sociosexual behaviors in all mammals. The projects in this dissertation further explore whether these cells could be specific to prairie voles and characterize the hormone regulation, IEG responses, and neuroanatomical projections of

TH-ir cells in the prairie vole pBST and MeApd. Chapter 2 describes species differences in TH-ir expression in these regions among prairie voles and three non-monogamous rodents, as well as sex differences and adult gonadal hormone regulation of these cell groups in prairie voles. Chapter 3 investigates possible functions of these TH-ir cell groups in male prairie voles by examining IEG expression after one of several social interactions. Finally, Chapter 4 details the anatomical projections of TH-ir cells in the pBST and MeApd, and proposes ways that these neurons may interact with cells of other phenotypes to facilitate prairie vole social behaviors. These experiments provide insight into the possible role of these species-specific TH-ir cells and expand the understanding of how they may be integrated within other social behavior networks in the brain.

CHAPTER 2

Northcutt, K. V., Wang, Z., and Lonstein, J. S. (2007). Sex and species differences in tyrosine hydroxylase-synthesizing cells of the rodent olfactory extended amygdala. J Comp Neurol 500(1), 103-115.

CHAPTER 2:

Sex and species differences in tyrosine hydroxylase-synthesizing cells of the rodent extended amygdala

Abstract

The bed nucleus of the stria terminalis (BST) and the medial amygdala (MeA) are anatomically connected sites necessary for chemosensory regulation of social behaviors in rodents. Prairie voles (*Microtus ochrogaster*) are a valuable model for studying the neural regulation of social behaviors because, unlike many other rodents, they are gregarious, pair bond after copulating, and are biparental. In this chapter, sex and species differences in immunoreactivity for tyrosine hydroxylase (TH), the rate-limiting enzyme for catecholamine synthesis, in the BST and MeA are described. Virgin male prairie voles had a large number of TH-immunoreactive (TH-ir) cells in areas analogous to the rat principal nucleus of the BST (pBST) and the posterodorsal medial amygdala (MeApd). Virgin female prairie voles had far fewer TH-ir cells in these sites (~17% of the number of cells as males in the pBST, ~35% of the number of cells in the MeApd). A few TH-ir cells were found in the BST of male and female hamsters and meadow voles, but not in rats. The MeApd also contained a few TH-ir cells in male and female hamsters and male meadow voles, but not rats. Castration greatly reduced the number of TH-ir cells in the male prairie vole pBST and MeApd, an effect that could be reversed with testosterone. Furthermore, treating ovariectomized females with testosterone substantially increased TH-ir cells in both sites. Therefore, a species-specific sex difference in TH expression is found in a chemosensory pathway in prairie voles. Expression of TH in

these sites is influenced by circulating gonadal hormones in adults, which may be related to changes in their display of social behaviors across the reproductive cycle.

Introduction

Neurons expressing tyrosine hydroxylase (TH), the rate-limiting enzyme for catecholamine synthesis, are widespread throughout the vertebrate brain. In most species, cells producing epinephrine or norepinephrine are invariably found in the brainstem, whereas cells producing dopamine (DA) are located in both the brainstem and the forebrain (Smeets and Gonzalez, 2000). The specific distribution of these catecholaminergic cells, however, differs between vertebrates (Smeets and Gonzalez, 2000; Tillet and Kitahama, 1998). In fact, differences are even found within the order Rodentia. For example, the anterior and posterior medial amygdala (MeA) and posteromedial bed nucleus of the stria terminalis (BSTpm) of Syrian hamsters (*Mesocricetus auratus*) contain catecholaminergic cells not found in rats, mice, or Siberian hamsters (Asmus et al., 1992; Asmus and Newman, 1993; Shi and Bartness, 2000; Wommack and Delville, 2002). Furthermore, species-specific TH-immunoreactive (TH-ir) cells are found in the Syrian hamster diagonal band of Broca, lateral preoptic area, and cortex (Vincent, 1988). There also may be differences between laboratory mice and rats in the distribution and density of TH-expressing cells in the preoptic area of the hypothalamus (Ruggiero et al., 1984).

Not only are there differences between rodent species, but sex differences in forebrain TH expression can be found within a species. TH-expressing cells of the anteroventral preoptic area (AVPV), necessary for gonadotropin release and ovulation (Weigand and Terasawa, 1982), are two to four times more numerous in gonadally intact female rats and mice than in intact males (Simerly et al., 1985a, b, 1997; Simerly, 1989; Zup et al., 2003). A sex difference in TH immunoreactivity in the AVPV also exists in

prairie voles (*Microtus ochrogaster*), although subjects must be gonadectomized for this sex difference to be revealed (Lansing and Lonstein, 2006).

Other areas of the rat, mouse, and prairie vole forebrain have not been reported to be sexually dimorphic in the number of TH-ir cells (Simerly et al., 1985a, b; Simerly, 1989; Lansing and Lonstein, 2006). During a previous examination of sex differences in the number of TH-ir cells in the hypothalamus of prairie voles (Lansing and Lonstein, 2006), however, two unexpected populations of TH-ir cells that appeared sexually dimorphic were noticed (but not reported). One population was found in an area analogous to the principal bed nucleus of the stria terminalis (pBST) of the rat or the posteromedial bed nucleus of the stria terminalis (BSTpm) of the hamster (Swanson, 1998; Wood and Swann, 2005). The other population of TH-ir cells was found in the area analogous to the posterodorsal medial amygdala (MeApd) of both rats and hamsters. In both cases, a large number of TH-ir cells were found in males, but relatively few in females.

The pBST/BSTpm and MeApd have dense reciprocal connections in male rats and hamsters (Canteras et al., 1995; Coolen and Wood, 1998; Wood and Swann, 2005) and are involved in chemosensory processing necessary for their sociosexual behaviors (for reviews see Hull et al., 2002; Newman, 1999; Wood, 1998). The presence of TH-ir cells in the pBST/BSTpm and MeApd of male, but not female, prairie voles is intriguing given the complex and unique social structure of this species (for review see Carter et al., 1995). Unlike many male rodents, male prairie voles form life-long pair bonds with their mates and later display high levels of parental care toward their offspring. Pair bonding and paternal behavior in male prairie voles require processing of olfactory inputs, insofar

as either olfactory bulbectomy or lesions encompassing the MeApd disrupt these behaviors (Kirkpatrick et al., 1994a, c). Furthermore, impeding DAergic neurotransmission also impairs these social behaviors in male prairie voles (Lonstein, 2002; Wang and Aragona, 2004). Here, populations of TH-ir cells in the pBST/BSTpm and MeApd of male prairie voles are described, and the effects of gonadal hormones on the TH-ir cells are examined in these sites. It may be that these relatively unique populations of catecholaminergic cells in the male prairie vole brain are involved in the chemosensory control of their pair bonding, parental, or other social behaviors.

Methods

Subjects

Male and female prairie voles (*Microtus ochrogaster*) were born and raised in the colony, which was established in 2002 at Michigan State University, from breeding stock that originated from offspring of voles captured in 1994 in Urbana, Illinois, provided by Dr. Betty McGuire (Smith College, Northampton, MA) and Dr. Zuoxin Wang (Florida State University, Tallahassee, FL). These voles were outbred in 2000 at the University of Massachusetts with voles of Illinois origin provided by Dr. C. Sue Carter (University of Illinois at Chicago) and brought to Michigan State University in 2002. Animals were mated by socially isolating adult virgin female and male prairie voles for 4 days, after which females were placed in the cage of an unfamiliar male. Animals were maintained on a 14:10-hour light:dark cycle with an ambient temperature maintained at ~21°C. At all ages, animals were housed in clear plastic cages (48 × 28 × 16 cm) containing wood chips, wood shavings, and a substantial hay covering. Water and a food mixture

containing cracked corn, whole oats, sunflower seeds, and rabbit chow (Teklad rodent diet No. 2031) in a ratio of 1:1:2:2 were freely available. Pups were weaned from their parents at 20 days of age, placed in same-sex sibling groups of two or three animals/cage between 50 and 60 days of age, and housed in these groups until they were killed at least 6 weeks later. The comparative study used eight voles of each sex, whereas the hormone manipulation study (see below) used 24 voles of each sex.

The eight male and five female meadow voles were born in the breeding colony of the Wang laboratory. Voles were weaned at 21 days of age and placed in same-sex sibling pairs in clear plastic cages (29 × 18 × 13 cm) containing cedar chip bedding until the time of death during adulthood. Food (Purina rabbit chow No. 5326), sunflower seeds, and water were provided *ad libitum*. All cages were maintained in a 14:10-hour light:dark cycle while the ambient temperature was maintained at about 21°C.

Six male and six female Long-Evans rats (*Rattus norvegicus*) were purchased from Harlan Laboratories (Indianapolis, IN) and sent to the laboratory when approximately 75 days of age. Rats were housed in same-sex groups of two or three animals per cage in clear polypropylene cages (48 × 28 × 16 cm) with wood shavings for bedding. Food (Teklad rodent diet No. 8640) and water were continuously available, lights were on a 12:12-hour light:dark cycle with onset at 0800 hours daily, and the ambient temperature was maintained at ~21°C. Rats were maintained in the laboratory for at least 3 weeks before death.

Seven male and seven female Syrian hamsters (*Mesocricetus auratus*) were also purchased from Harlan Laboratories and sent to the laboratory when approximately 75 days of age. Hamsters were singly housed in clear polycarbonate cages (12 × 4 × 8 in.)

with food (Teklad rodent diet No. 8640) and water freely available. Animals were exposed to a 14:10-hour light:dark cycle, and the ambient temperature was maintained at ~21°C. Hamsters were maintained in the laboratory for at least 2 weeks prior to death.

Gonadectomy and hormone replacement

To determine the effects of circulating gonadal hormones on TH expression in prairie voles, voles of both sexes were weighed and anesthetized with an IP injection of an anesthetic cocktail containing ketamine (62.5 mg/kg), xylazine (7.5 mg/kg), and acepromazine (0.8 mg/kg). Males were either castrated or received a sham surgery in which an incision was made in the scrotum, but the testes were not removed. Similarly, female prairie voles were anesthetized and either were ovariectomized through a single midline ventral incision or received a sham surgery in which the ovaries were visualized but not removed. Half of the castrated males and ovariectomized females were subcutaneously implanted with a 2.5-cm-long Silastic capsule containing crystalline testosterone (Sigma, St. Louis, MO). This capsule provides supraphysiological levels of testosterone that maintain masculine behavior and neurochemistry in male prairie voles (e.g., Wang and DeVries, 1993; Lonstein et al., 2005). The other half of the castrated males and ovariectomized females, and the sham surgery controls of both sexes, received an empty Silastic capsule. Four weeks after surgery, subjects were killed and their brains collected as described below. Each of the six groups of prairie voles contained eight animals. All procedures were in accordance with the Institutional Animal Care and Use Committees at Michigan State University and Florida State University.

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Perfusion, tissue collection, and immunocytochemistry

All voles, rats, and hamsters were weighed, overdosed with sodium pentobarbital, and perfused through the heart with 0.9% saline (100 ml for voles, 150 ml for hamsters and rats), followed by 4% paraformaldehyde in sodium phosphate buffer (NaPB; pH 7.6, 100 ml for voles, 150 ml for hamsters and rats). Brains were removed, postfixed for 1-4 hours (species comparison) or overnight (sex and hormonal condition comparison) in 4% paraformaldehyde in NaPB, and then submerged in a 20% sucrose/NaPB solution for at least 3 days before sectioning into 40-μm coronal sections with a freezing microtome. Immunocytochemistry for TH was performed on every other section through the brain for the prairie voles, meadow voles, and hamsters and on every third section through the rat brains. Immunocytochemical procedures were identical to a procedure described previously (Lansing and Lonstein, 2006). Briefly, sections were rinsed three times for 5 minutes each in Trisma-buffered saline (TBS; pH 7.6), incubated in 0.1% sodium borohydride for 15 min, rinsed three times in TBS, incubated in 0.3% Triton X-100 and 1% hydrogen peroxide in TBS for 10 min, rinsed three times in TBS, blocked with 20% normal goat serum in 0.3% Triton X in TBS for 15 min, and then incubated with a rabbit anti-TH polyclonal primary antiserum (AB152; 1:2,000; Chemicon, Temecula, CA) in 0.3% Triton X and 2% NGS in TBS at room temperature for approximately 18 hours. According to the manufacturer, the immunogen for this primary antibody is denatured TH from rat pheochromocytoma and has been shown by Western blot analysis not to recognize other monoaminergic synthetic enzymes. Indeed, labeling was found for all four species in the sites previously described to express TH in rodents, including the

periventricular hypothalamus, zona incerta, substantia nigra, and ventral tegmental area. Furthermore, in rats and hamsters, no labeling was seen in any site not previously described to express TH in these species. Sections were then rinsed three times in TBS, incubated in a biotinylated goat anti-rabbit secondary antibody (1:500; Vector Laboratories, Burlingame, CA) in 0.3% Triton-X and 2% NGS, rinsed three times in TBS for 5 minutes each, and incubated with avidin-biotin complex (Vectastain Elite, Vector Laboratories) for 60 minutes. After rinsing three times with TBS, visualization of TH immunoreactivity occurred with the use of Vector SG chromagen (Vector Laboratories) according to the manufacturer's instructions, which provided a light blue cytoplasmic label. Sections were mounted on microscope slides, dehydrated, and coverslipped. Immunocyotchemical control procedures included omission of the primary or secondary antiserum, which abolished any specific labeling. A single immunocytochemical run including the tissue from all four species was used for the species comparison, and a separate immunocytochemical run was used for the gonadectomy and hormone-replacement study in prairie voles.

To determine whether TH-ir cells in the pBST and MeApd of prairie voles might be DAergic or noradrenergic, the expression of DA-β-hydroxylase (DBH), the enzyme that converts DA into norepinephrine, was examined in these sites. An additional series of brain sections that included the forebrain and brainstem of two gonadally intact male and two intact female prairie voles was processed immunocytochemically as described above but incubated for 3 days at 4°C in a rabbit polyclonal anti-DBH primary antiserum (AB1585; 1:500; Chemicon). According to the manufacturer, the immunogen for this primary antibody is DBH obtained from bovine adrenal glands and has been shown by

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Western blot analysis to be specific for DBH. Indeed, many DBH-ir cells were found in the locus coeruleus of both the male and the female prairie vole brainstem, but none were found in pBST or MeApd, or in the hypothalamus and zona incerta on these same sections. Omission of the primary or secondary antiserum abolished all specific labeling.

Analyses

To compare the presence of TH immunoreactivity in the BST and MeA of prairie voles, meadow voles, hamsters, and rats of both sexes, the entire rostrocaudal extents of the BST and MeA were examined for each subject. The presence or absence of TH-ir cells at any rostrocaudal level within these areas was noted.

Slides for the gonadectomy and hormone-replacement study in prairie voles were masked and coded for analysis, which was performed by only one observer (K.V.N.). The three areas examined were analyzed bilaterally from consecutive sections in a one-in-two series, with the number of TH-ir cells totaled from these sections. The number of cells containing detectable TH immunoreactivity was counted by eye with the use of a Nikon E400 microscope at ×200 magnification with the aid of a reticle placed in one of the ocular lenses. The area of the BST analyzed included the area of the prairie vole brain that had the densest cluster of TH-ir cells and corresponded to the dorsal principal BST of the rat or the dorsal posteromedial BST of the hamster. The total number of TH-ir cells was quantified from four consecutive sections in a one-in-two series through the middle and caudal pBST, roughly corresponding to plates 20-22 of Swanson's (1998) atlas of the rat brain. Males rarely had TH-ir cells in the dorsal pBST rostral or caudal to these four sections. The area of the MeApd analyzed began with the section roughly corresponding

to plate 28 of Swanson's rat atlas, where TH-ir cells first appeared for most subjects, and continued for six consecutive sections in a one-in-two series through the MeApd, ending approximately at the level corresponding to Swanson's atlas plate 30. Subjects reliably had six MeApd sections represented, but not necessarily more, because the amygdala was often lost in the most caudal sections. In addition, a small cluster of TH-ir cells in the dorsal reticular nucleus of the thalamus was also found, at the level roughly corresponding to Swanson's atlas plate 27. These cells are also found in the hamster brain (Asmus et al., 1992). These thalamic cells were found only in two sections of the one-in-two series for each subject and did not appear to differ between the sexes, so this was used as a control site not likely to be affected by the hormone manipulations. Average soma size of TH-ir cells in the pBST and MeApd was evaluated for each subject by nonsystematically choosing for each subject one hemisection through dorsal pBST and MeApd and then tracing the perimeter of every TH-ir soma under ×200 magnification with the use of an image analysis system (Image Pro Plus; Media Cybernetics). Stereology was not used to analyze these TH-ir cells for numerous reasons. First, these TH-ir populations were found on only a small number of sections through each brain region. Second, it was possible to analyze every other section through these sites. Third, the TH-ir cells were not very densely packed, and, fourth, the total number of TH-ir cells per section was relatively small and easily quantified. Cell counts were subjected to an Abercrombie correction factor of 0.83 based on the finding of a z-plane average soma diameter of ~8 μm and are presented as corrected counts. Photomicrographs were arranged with Adobe Photoshop 6.0, with the Dodge tool used to reduce any

inconsistencies in illumination within a given panel, and the brightness and contrast were adjusted to maintain consistency across panels.

Data from the sex and hormone treatment comparison in prairie voles were analyzed for each site with a 2 (sex) × 3 (treatment) ANOVA, followed by Fisher LSD post-hoc tests on main effects. Separate Fisher LSD post-hoc tests were then used as planned comparisons to compare treatment groups within each sex. Because a single hemisection was analyzed for somal area, the number of subjects included in this analysis was greater for some groups than the analysis of the total number of TH-ir cells, which required many intact sections for us to include a subject in the analysis. Statistical significance was indicated at $P = 0.05$.

Results

Species comparison

All male prairie voles had a distinct cluster of intensely TH-ir cells in the dorsal pBST, with the majority of cells appearing at the rostrocaudal level where the third ventricle appears both dorsal and ventral to the anterior commissure. Many fewer cells were found in sections of the male dorsal pBST anterior or posterior to this dense cluster, although some TH-ir cells were found adjacent to the stria medullaris and fornix in more caudal sections, as the pBST extended ventromedially. In contrast to males, female prairie voles had many fewer TH-ir cells in the dorsal pBST (Figure 2.1), and the intensity of immunoreactive labeling in most of these cells was relatively weak. Some females also had a few weakly immunoreactive cells in the ventromedial pBST more caudally. Small numbers (one to eight cells) of very weakly TH-ir cells were also found

in each section of the dorsal pBST of most male and female meadow voles, and one to four moderately immunoreactive cells per section were found in the dorsal pBST of male and female hamsters (Figure 2.2). No TH-ir cells were found in the dorsal pBST of any male or female rat (Figure 2.2). Although relatively few or no TH-ir cells were found in the dorsal pBST of female prairie voles or meadow voles, hamsters, and rats of both sexes, the periventricular hypothalamus on these same sections contained a large number of intensely TH-ir cells, as previously described (e.g., Chan-Paley et al., 1984; Vincent, 1988; Lansing and Lonstein, 2006).

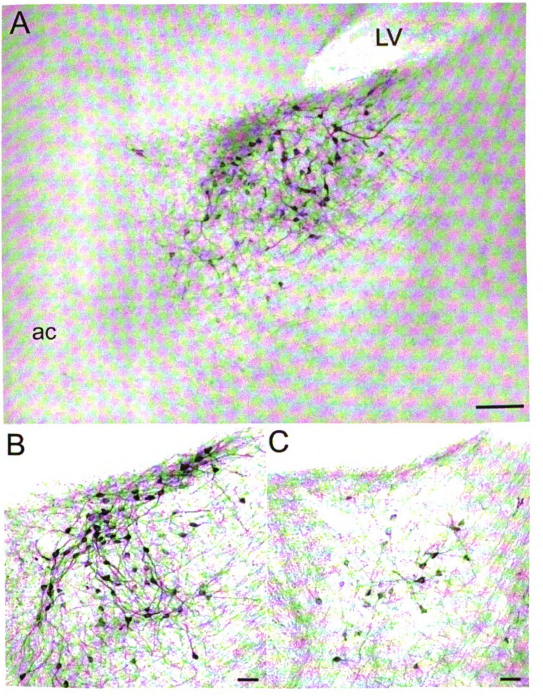

Figure 2.1. Photomicrographs of TH-ir cells in the pBST of representative prairie voles. A: Male prairie vole at ×100 magnification. B: Male at ×200 magnification. C: Female at ×200 magnification. LV, lateral ventricle; ac, anterior commissure. Scale bars = 100 μm in A; 50 μm in B and C.

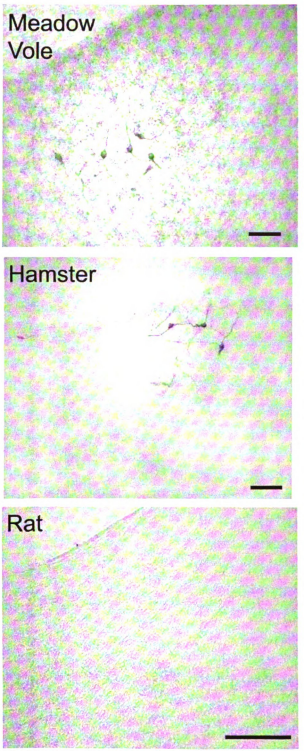

Figure 2.2. Photomicrographs (×200 magnification) of TH-ir cells in the dorsal pBST of representative male meadow voles, hamsters, and rats. Males of these species were similar to females. Scale bars = 50 μm.

All male prairie voles showed a large number of highly TH-ir cells throughout the entire rostrocaudal extent of the MeApd, and some males also had a few TH-ir cells in the posteroventral MeA (MeApv; Figure 2.3). The MeApd of all female prairie voles also contained TH-ir cells, but there were far fewer compared with males, and many of these cells were only weakly immunoreactive (Figure 2.3). Some (one to 12 cells/section) very weakly TH-ir cells were also found in the MeApd of most male and female meadow voles. The MeApd of all hamsters contained a smaller number (one to four cells/section) of moderately TH-ir cells, with no obvious difference between the sexes in the number of cells. No TH-ir cells were found in the MeApd of male or female rats (Figure 2.4).

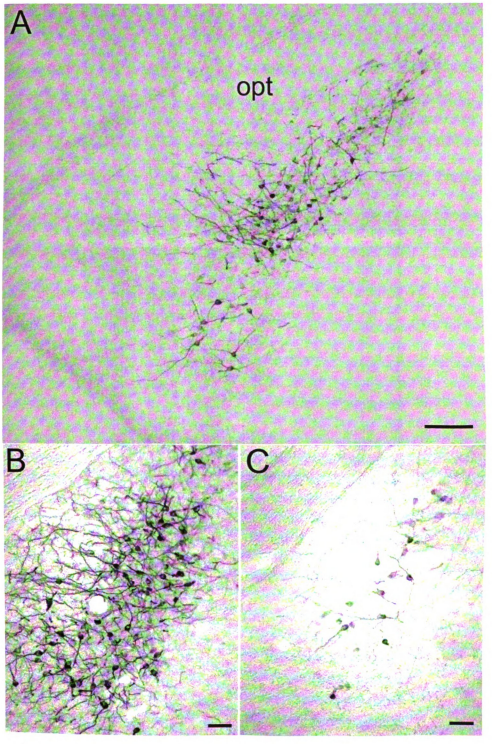

Figure 2.3. Photomicrographs of TH-ir cells in the MeApd of representative prairie voles. A: Male prairie vole at ×100 magnification. B: Male at ×200 magnification. C: Female at ×200 magnification. opt, Optic tract. Scale bars = 100 μm in A; 50 μm in B,C.

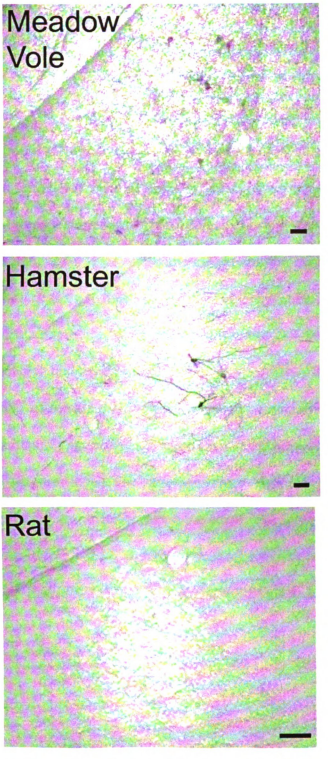

Figure 2.4. Photomicrographs (×200 magnification) of TH-ir cells in the MeApd of representative male meadow voles, hamsters, and rats. Males of these species were similar to females. Scale bars = 50 μm.

Sex difference and effects of gonadal hormones on TH immunoreactivity in prairie voles

In the dorsal pBST, there were significant main effects of sex ($F(1,33) = 11.63$, $P < 0.002$) and treatment ($F(2,33) = 18.79$, $P < 0.0001$) on the number of TH-ir cells. The interaction between sex and treatment was not significant ($F(2,33) = 1.69$, $P > 0.2$). Post-hoc analysis on the main effects revealed that males had significantly more TH-ir cells than females and that testosterone-treated animals had significantly more TH-ir cells than gonadally intact and gonadectomized animals, which did not significantly differ from each other (Figures 2.5, 2.6). Planned comparisons within each sex revealed that castration of males significantly reduced the number of TH-ir cells, whereas testosterone treatment maintained the number of cells in castrated males at a level similar to that of sham males. Ovariectomy had no significant effect on the already low number of TH-ir cells in the dorsal pBST of female prairie voles, whereas treating ovariectomized females with testosterone significantly increased the number of cells compared with the other two groups of females (Figure 2.5). The average cross-sectional area of these TH-immunoreactive somata did not differ between the sexes ($F(1,39) = 0.35$, $P > 0.8$) or by treatment ($F(2,39) = 2.05$, $P > 0.1$), and there was no significant interaction between these factors ($F(2,39) = 2.40$, $P > 0.1$; group averages = 109 ± 4 to 135 ± 5 μm^2).

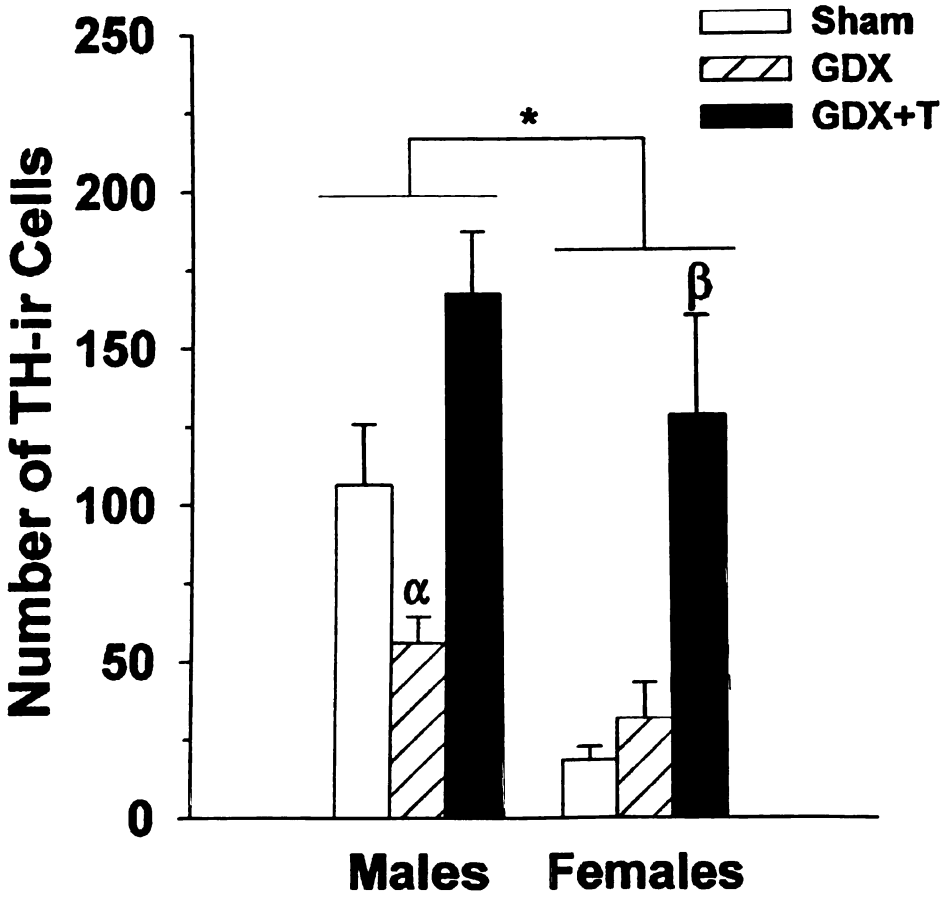

Figure 2.5. Number (Mean ± SEM) of TH-ir cells in every other section of the dorsal pBST of male and female prairie voles that were gonadally intact (Sham), gonadectomized (GDX), or gonadectomized and implanted with a capsule filled with testosterone (GDX + T). *Significant difference between the sexes, collapsed across treatment. α, GDX males significantly different from other groups of males; β, GDX + T females significantly different from other groups of females.

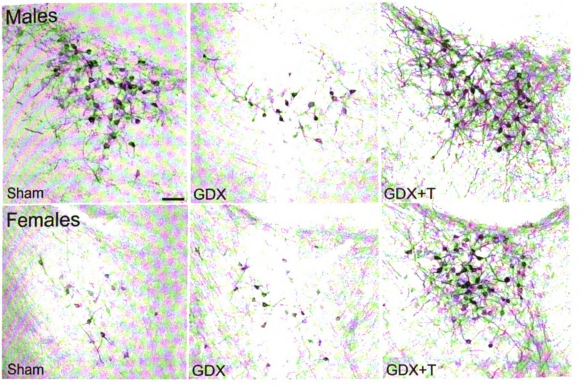

Figure 2.6. Photomicrographs (×200 magnification) of TH-ir cells in the dorsal pBST of male (top) and female (bottom) prairie voles that were gonadally intact (Sham), gonadectomized (GDX), or gonadectomized and implanted with a capsule filled with testosterone (GDX + T). Scale bar = 50 μm.

In the MeApd, there were significant main effects of sex ($F(1,35) = 25.35$, $P < 0.0001$) and treatment ($F(2,35) = 22.47$, $P < 0.0001$), but no significant interaction between them ($F(2,35) = 2.17$, $P > 0.12$). Post-hoc analysis on the main effects revealed that the number of TH-ir cells was greater in males than females and that all three hormone treatments significantly differed from each other, with the number of TH-ir cells lowest in gonadectomized animals, intermediate in intact animals, and highest in testosterone-treated animals (Figures 2.7, 2.8). Similar to the case in the dorsal pBST, planned comparisons of the treatment groups within each sex revealed that castration of males significantly reduced TH-ir cells in the MeApd, whereas testosterone treatment maintained a high number of these cells. Ovariectomy had no significant effect on the number of TH-ir cells in the MeApd of females, whereas treating ovariectomized females with testosterone significantly increased it (Figure 2.7). The average cross-sectional area of the TH-ir somata did not differ between the sexes ($F(1,37) = 0.22$, $P > 0.64$) or by treatment ($F(2,37) = 0.42$, $P > 0.65$), and there was no significant interaction between these factors ($F(2,37) = 0.01$, $P > 0.99$; group averages = 132 ± 7 to 143 ± 9 μm^2).

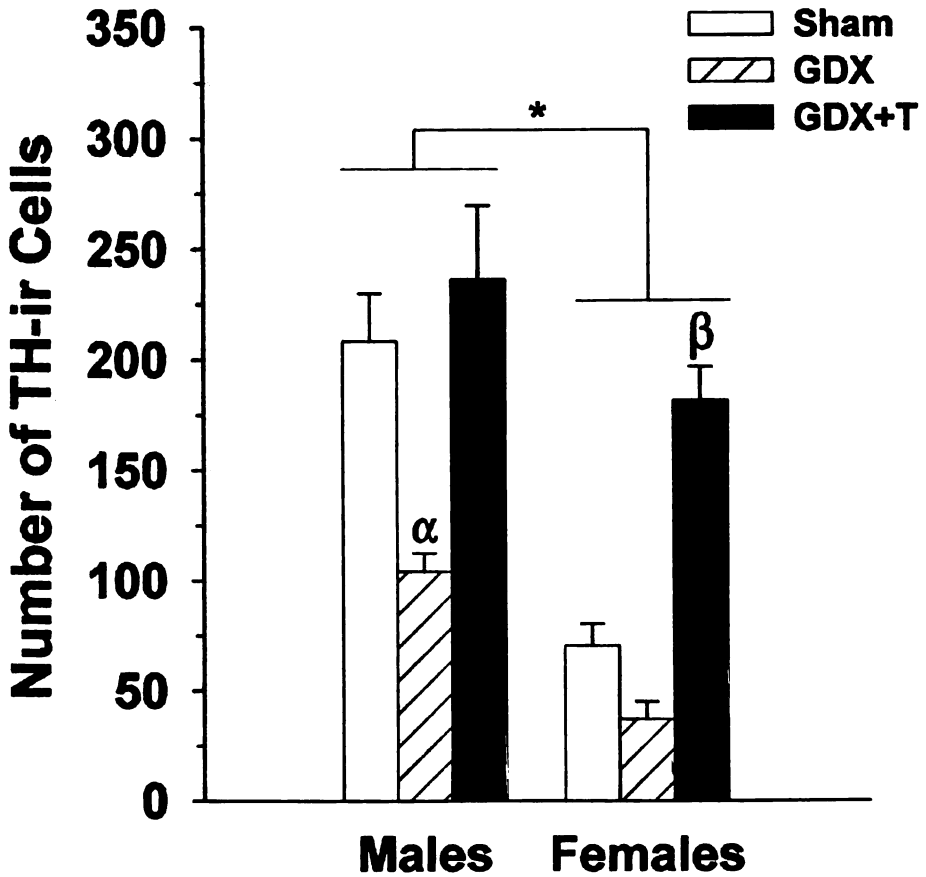

Figure 2.7. Number (Mean ± SEM) of TH-ir cells in every other section of the MeApd of male and female prairie voles that were gonadally intact (Sham), gonadectomized (GDX), or gonadectomized and implanted with a capsule filled with testosterone (GDX + T). *Significant difference between the sexes, collapsed across treatment. α, GDX males significantly different from other groups of males; β, GDX + T females significantly different from other groups of females.

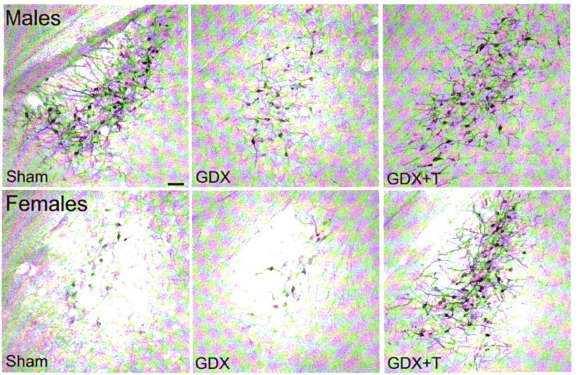

Figure 2.8. Photomicrographs (×200 magnification) of TH-ir cells in the MeApd of male (top) and female (bottom) prairie voles that were gonadally intact (Sham), gonadectomized (GDX), or gonadectomized and implanted with a capsule filled with testosterone (GDX + T). Scale bar = 50 μm.

In the small cluster of TH-ir cells in the dorsal reticular nucleus of the thalamus, there were no significant main effects of sex ($F(1,34) = 1.31$, $P > 0.26$) or treatment ($F(2,34) = 0.33$, $P > 0.71$), and no significant interaction between them ($F(2,34) = 0.38$, $P > 0.69$; Figure 2.9).

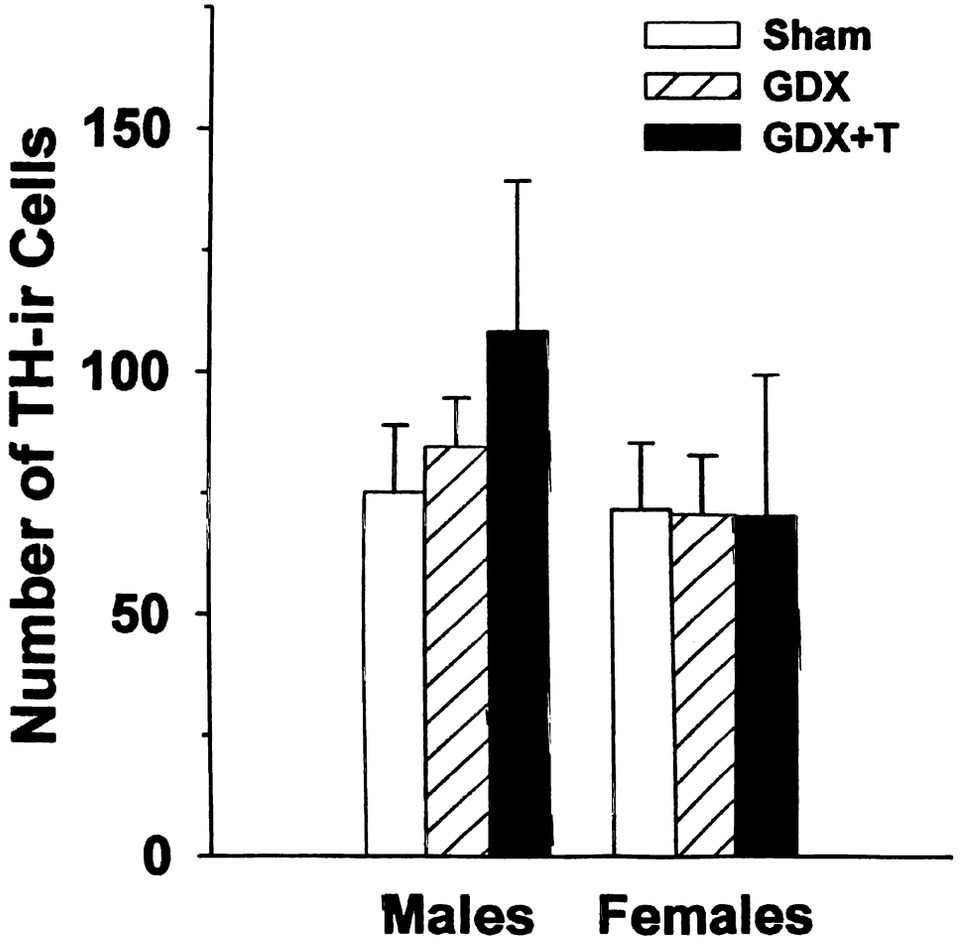

Figure 2.9. Number (mean ± SEM) of TH-ir cells in every other section of the dorsolateral thalamus of male and female prairie voles that were gonadally intact (Sham), gonadectomized (GDX), or gonadectomized and implanted with a capsule filled with testosterone (GDX + T).

Discussion

These experiments demonstrate that large numbers of TH-ir cells were found in the dorsal pBST and MeApd of male prairie voles. Female prairie voles and meadow voles, hamsters, and rats of both sexes had relatively few or no TH-ir cells in these sites. Furthermore, these populations of cells were sensitive to manipulations in gonadal hormones in adult prairie voles, in that castration reduced the number of TH-ir cells in males, an effect that could be prevented by chronic treatment with testosterone. Females treated with testosterone during adulthood also showed a sharp increase in the number of TH-ir cells. Differences between the sexes in TH-ir cells within a known chemosensory pathway, and the sensitivity of these cells to circulating hormones, suggest that catecholamine release from these sites may be involved in regulating sex differences in social behaviors in prairie voles as well as changes in these social behaviors across the reproductive cycle at times when hormones dramatically fluctuate.

Species differences in TH immunoreactivity

There seems to be notable species differences in TH expression in the pBST and MeApd. However, the absence of large numbers of TH-ir cells in the BST and MeA of meadow voles, hamsters, and rats of both sexes does not necessarily indicate that TH-producing cells do not exist in the BST and MeA of these species. In fact, Syrian hamsters of both sexes can express a large number of TH-ir cells in these sites, apparently almost as many as were found for male prairie voles, if treated with colchicine 48 hours prior to sacrifice (Asmus et al., 1992; Asmus and Newman, 1993). The "appearance" of these immunocytochemically identifiable cells in hamsters likely is due to increased

accumulation of TH in the somata after colchicine treatment, rather than a *de novo* increase in TH synthesis that can occur as a result of colchicine (Cortes et al., 1990). This is suggested by the finding that these cells are present in high numbers when TH mRNA is visualized with in situ hybridization in hamsters not treated with colchicine (Asmus and Newman, 1993). Nonetheless, under normal conditions, only male prairie voles express high enough levels of TH in the dorsal pBST and MeApd to be visualized with immunocytochemistry, although other rodents may express relatively low levels of TH in these sites. The comparison between species in TH immunoreactivity in the pBST and MeApd also revealed that male prairie voles, meadow voles, and hamsters have at least some TH-ir cells in both sites, whereas rats do not. All of the rodents examined herein are within the superfamily Muroidea. However, based on recent genetic examination, voles and hamsters are both members of the family Cricetidae, whereas Old World (laboratory) rats are within the family Muridae (Steppan et al., 2004). Hamsters and voles are more phylogenetically related to each other than either species is to laboratory rats, and cells expressing TH in these regions of the brain may be relatively specific to Cricetidae. Nonetheless, there are some notable differences between voles and hamsters. TH-ir cells were rare in the anterior MeA of male voles, but are quite dense in both the anterior and the posterior MeA of colchicine-treated male hamsters (Asmus et al., 1992), indicating that the distribution of TH-ir cells in these sites is similar, but not identical, among relatively closely related species.

Possibly more surprising, based on degree of relatedness, is the difference in the level of TH expression between male prairie and male meadow voles. The brains of these two very closely related species differ in numerous ways, including expression of

receptors for oxytocin (Insel and Shapiro, 1992), vasopressin (Insel et al., 1994), corticotropin-releasing factor (Lim et al., 2005), estrogen (Fowler et al., 2005), and DA (Curtis, Fowler, Lonstein, and Wang, in preparation). Male prairie and meadow voles also differ in how social interactions alter neurochemistry (Bamshad et al., 1993; Wang et al., 1994; Curtis et al., 2003). This divergent neurochemistry has often been suggested to be related to differences between these species in their social organization, with prairie voles highly gregarious and socially monogamous and meadow voles nongregarious and polygamous (Young et al., 2001). An examination of TH expression in the pBST and MeApd of males from other monogamous species, such as California mice (*Peromyscus californicus*), Djungarian hamsters (*Phodopus campbelli*), or Mongolian gerbils (*Meriones unguiculatus*), would be useful to determine whether very high TH expression in these sites is typical of monogamous male rodents.

Sex differences and hormone sensitivity of TH-immunoreactive cells

There was a large sex difference in the number of TH-ir cells in the prairie vole dorsal pBST and MeApd. As noted above with regard to differences between species, the absence of TH-ir cells in untreated female prairie voles does not necessarily mean that TH-synthesizing cells do not exist, but rather that levels of TH are below the threshold of immunocytochemical detection. It is valuable to note that males do not have greater levels of TH-ir cells everywhere in the brain; sex differences in detectable cells are not found in the dorsal reticular thalamus (present results) or hypothalamus and zona incerta (Lansing and Lonstein, 2006). Furthermore, the sex difference in TH expression in the

AVPV is the reverse of that found in the pBST or MeApd (Lansing and Lonstein, 2006). The sex differences in the dorsal pBST and MeApd are, therefore, quite unique.

These data also demonstrate that these TH-ir populations are sensitive to gonadal hormones. When treatment groups were compared within each sex, castration of male prairie voles demasculinized (i.e., reduced) the number of TH-ir cells in both sites, whereas exogenous testosterone maintained the numbers of these cells at a level similar to that of gonadally intact males. Conversely, the number of TH-ir cells was masculinized (i.e., increased) in females given testosterone. The lack of an effect of ovariectomy on these cells in females was expected, insofar as prairie voles are induced ovulators and, in the absence of sensory cues from males, have little circulating estradiol (Cohen-Parsons and Carter, 1987). This does not necessarily mean that estradiol is not responsible for modulating TH expression in these sites. Many effects of testosterone on sex differences in the rodent brain are mediated through its aromatization to estradiol and subsequent activity of the estradiol receptor (DeVries and Simerly, 2002; Wallen and Baum, 2002). The pBST and MeApd are extremely steroid sensitive and express high levels of estradiol receptors in prairie voles of both sexes (Cushing and Wynne-Edwards, 2005; Fowler et al., 2005; Hnatczuk et al., 1994). Aromatization of testosterone into estradiol may be largely responsible for testosterone's effects on TH-ir cells in these sites, a detail that should be examined in future experiments.

The effects of hormone manipulations in male prairie voles were somewhat dissimilar to those found in colchicine-treated male hamsters. Castration of hamsters reduces TH immunoreactivity only in the anterior MeA, not the posterior MeA or BSTpm (Asmus and Newman, 1993). Furthermore, although exogenous testosterone fully

maintained of the number of TH-containing cells in the MeApd of male prairie voles, it does not in the hamster anterior MeA. Asmus and Newman (1993) also found that the decrease in TH immunoreactivity in the hamster anterior MeA after castration was transient, insofar as high numbers of TH-ir cells were again found in males examined 12 weeks after castration. Similar long-term castration effects may occur in the MeApd of male prairie voles. It appears that differences in TH expression between members of Cricetidae are apparently not only in distribution but also in sensitivity to gonadal hormones.

Even though castration greatly reduced TH expression in males and testosterone greatly increased it in females, the sex difference was not completely eliminated by equating adult circulating hormones. Indeed, adult female prairie voles treated with testosterone still had ~25% fewer TH-expressing cells in the dorsal pBST than testosterone-treated males. Conversely, castrated males still had almost twice as many cells as ovariectomized females. However, the *magnitude* of the sex difference is greatly reduced when gonadal hormones are equated in adult animals. In the dorsal pBST, the magnitude of the sex difference changed from an almost 600% difference in intact animals to a 25% difference when animals were similarly treated with testosterone. The sex difference in the MeApd went from almost 300% to 25%. A similar reduction in the magnitude of the sex difference in MeA volume occurs when adult male and female rats are given the same testosterone treatment (Cooke et al., 1999), so prairie voles are not the only species in which adult gonadal hormones can greatly alter the magnitude of a neural sex difference.

Given that the sex differences in TH expression were not completely eliminated, gonadal hormones acting on these sites during perinatal development also probably influence this sex difference to some extent. Perinatal exposure to testicular hormones may render adult males more sensitive to a given amount of testosterone, leading to the persistence of a sex difference in TH immunoreactivity even when adult males and females receive the same hormone treatment. Adult female prairie voles have greater estrogen receptor expression in these sites than males (Hnatczuk et al., 1994), so reduced effectiveness of testosterone in adult females may instead be due to reduced intraneuronal aromatization of testosterone to estradiol (Roselli et al., 1985). It is also possible that testosterone actions on the androgen receptor, which is sexually dimorphic in these sites (Wood and Newman, 1999), also contributes to the sex differences (Morris et al., 2005).

Functional considerations

Differences in TH immunoreactivity between individual cells within a given structure reflect functional and anatomical heterogeneity (Bayer and Pickel, 1990; Benno et al., 1982; Weiss-Wunder and Chesselet, 1990, 1991; Zigmond and Ben-Ari, 1977). It is reasonable to believe that differences between animals in TH immunoreactivity in a given site also reflects such heterogeneity. The presence of a large number of TH-ir cells in the pBST and MeApd of male prairie voles is presumably associated with unique anatomy and neural processing compared with female prairie voles, as well as meadow voles, hamsters, and rats of both sexes. Indeed, these populations in male prairie voles do not contain an insignificant number of cells. The quantification of all TH-ir cells in every other section suggests that the male prairie vole dorsal pBST contains over 200 TH-ir

cells, and the MeApd contains at least twice that many. The absence of DBH immunoreactivity the pBST and MeApd indicates that these cells may be DAergic, rather than noradrenergic, which is consistent with what is found in colchicine-treated hamsters (Asmus et al., 1992; Asmus and Newman, 1993; Vincent, 1988).

In hamsters, lesions encompassing the BSTpm reduce olfactory investigation of estrous females, although many lesioned males continue to copulate, albeit at a reduced level (Powers et al., 1987). Lesions of the MeA that include the MeApd, particularly its rostral component, also severely reduce males' olfactory investigation of females and virtually eliminate copulation (Lehman et al., 1980; Powers et al., 1987). Various impairments in copulation are also found in male laboratory rats after pBST or MeApd lesions (Claro et al., 1995; Emery and Sachs, 1976; Kondo, 1992; McGregor and Herbert, 1992; Valcourt and Sachs, 1979). Not much is known about the prairie vole pBST and MeApd, but the anatomy and function of these areas in other rodents suggests that these TH-ir populations in male prairie voles could be related to the chemosensory control of copulation, as well as to the more unique social behaviors they display, such as pair bonding and paternal care.

Both male and female prairie voles pair bond and show parental care, though, which complicates the suggestion that pBST/MeApd TH expression in males is involved in their ability to display these behaviors, as one might expect that the sexes employ similar neural mechanisms to achieve these similar behavioral endpoints. De Vries (2004) has suggested that the opposite may be possible, such that sex differences in the brain, rather than similarities, produce similar behavioral or physiological endpoints by compensating for sex differences in circulating gonadal hormones. A sex difference in

TH expression in the pBST and MeApd may allow males to show monogamous behaviors even in light of their higher circulating androgens, which are sometimes regarded as inconsistent with monogamous behaviors (Wingfield et al., 2001).

Nonetheless, any function for the dorsal pBST and its catecholaminergic cells in the formation and expression of pair bonds in male prairie voles remains to be examined. The pBST and surrounding area show increased activity of the immediate early gene *c-fos* when male prairie voles copulate with females (Lim and Young, 2004), which normally is necessary for their pair bonding (see Carter et al., 1995). Furthermore, peripheral injection of the DA antagonist haloperidol eliminates males' social preferences for their mates (Aragona et al., 2003). Conversely, injection of the DA agonist apomorphine promotes males' social preferences for familiar females in the absence of mating (Aragona et al., 2003). Areas of the brain where changes in DAergic neurotransmission produce these effects include the mesolimbic system (Aragona et al., 2003, 2006; Curtis and Wang, 2005), but, as with other social behaviors, multiple catecholaminergic systems are likely to be involved (Dominguez and Hull, 2005; Miller and Lonstein, 2005). A function for the dorsal pBST in parental behavior in prairie voles is also unknown. It may be sex-specific, in that paternal behavior in prairie voles requires intact olfaction, whereas maternal behavior does not (Kirkpatrick et al., 1994c; Williams et al., 1992c), suggesting that olfactory inputs to the pBST may be more important for paternal care than maternal care. In partial support, lesions of the entire dorsal BST (including the dorsal pBST) of lactating female rats do not significantly affect their maternal behavior (Numan and Numan, 1996).

In contrast to the pBST, the MeApd has been investigated for roles in both pair bonding and paternal behavior in male prairie voles. Although large lesions of the corticomedial amygdala (which include the MeApd) reduce contact with a familiar female, lesions specifically of the medial amygdala do not (Kirkpatrick et al., 1994a). Such lesions do reduce their paternal behavior, though (Kirkpatrick et al., 1994a). Inhibiting DAergic neurotransmission with peripheral injection of haloperidol also impairs their paternal behavior (Lonstein, 2002), and the MeApd may be one source of critical DAergic neurotransmission.

In summary, species-specific catecholaminergic activity originating from cells in the pBST and MeApd of male prairie voles may be involved in the display of their unique social behaviors. Direct examination of how catecholamines originating from these two sites influence any behavior in male prairie voles remains to be determined.

Acknowledgements

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CHAPTER 3

Northcutt, K. V. and Lonstein, J. S. (2009). Social contact elicits immediate-early gene expression in dopaminergic cells of the male prairie vole extended olfactory amygdala. Neuroscience 163(1), 9-22.

CHAPTER 3:

Social contact elicits immediate-early gene expression in catecholaminergic cells of the male prairie vole extended amygdala

Abstract

Male prairie voles (*Microtus ochrogaster*) are a valuable model in which to study the neurobiology of sociality because, unlike most mammals, they pair bond after mating and display paternal behaviors. Research on the regulation of these social behaviors has highlighted dopamine (DA) neurotransmission in both pair bonding and parenting. Recently, large numbers of tyrosine hydroxylase-immunoreactive (TH-ir) cells were described in the male prairie vole principal nucleus of the bed nucleus of the stria terminalis (pBST) and posterodorsal medial amygdala (MeApd), but such cells were very few in number or absent in the non-monogamous species that were examined, including meadow voles. This suggests that TH-ir cells in these sites may be important for sociosexual behaviors in male prairie voles. To gain some insight into the function of these TH-ir neurons in male prairie voles, expression of the immediate-early genes (IEGs) Fos and Egr-1 were examined in TH-ir cells of the pBST and MeApd after males interacted (or not) with one of several social stimuli. IEGs were constitutively expressed in some TH-ir neurons under any social condition, but that the number of cells expressing IEGs decreased after a 3.5-h social isolation. Thirty-minute mating bouts (but not 6- or 24-h bouts) that included ejaculation elicited more TH-ir cells expressing IEGs than did non-ejaculatory mating, interactions with a familiar female sibling, or interactions with pups. Furthermore, Fos expression in TH-ir cells was positively correlated with the

display of copulatory, but not parental, behaviors. These effects of mating were not found in other DA-rich sites of the forebrain (including the anteroventral periventricular preoptic area, periventricular anterior hypothalamus, zona incerta, and arcuate nucleus). Thus, activity in TH-ir cells of the male prairie vole pBST and MeApd is influenced by their social environment, and may be particularly involved in mating and its consequences, including pair bonding.

Introduction

Prairie voles (*Microtus ochrogaster*) are an invaluable rodent model to study the neurobiology of sociality because they form lifelong pair bonds after mating and show biparental behavior after the birth of pups (Carter et al., 1995). Release of numerous neurochemicals, including dopamine (DA), is required for the display of these social behaviors (Young et al., 2008). D2 receptor antagonism prevents the formation of partner preferences after mating in both sexes of prairie voles, while D2 stimulation induces partner preference formation in the absence of mating (Aragona et al., 2006; Wang et al., 1999). DA receptor antagonism also impairs parental responding in both sexes of prairie voles, with the mixed D1/D2 receptor antagonist haloperidol reducing voles' motivation to make contact with pups and lick them (Lonstein, 2002). The nucleus accumbens (ACB) is one site where dopaminergic (DAergic) activity is critical for pair bond formation in prairie voles (Aragona et al., 2003; Gingrich et al., 2000), and this is probably also true for their biparental behaviors (see Numan and Stolzenberg, 2009).

The ventral tegmental area (VTA) is likely a major source of DA to the ACB and other forebrain sites necessary for social behaviors in prairie voles (Curtis and Wang, 2005), but the involvement of other regions that may provide DAergic signaling necessary for sociality has not been investigated. Novel populations of neurons expressing high levels of tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis, were recently described in the prairie vole principal nucleus of the bed nucleus of the stria terminalis (pBST) and posterodorsal medial amygdala (MeApd) (Northcutt et al., 2007). These cells do not contain DA-beta-hydroxylase and, thus, may be DAergic (Northcutt et al., 2007). Few or no TH-immunoreactive (TH-ir)

cells were found in these sites in three non-monogamous species that were examined, including the closely related meadow vole, suggesting that these cells may be involved in how DA influences social behaviors relatively unique to prairie voles. In addition, male prairie voles have three- to five-fold more TH-ir cells in these sites than do female prairie voles, indicating particular importance of these cells for social behaviors in males.

The pBST and MeApd have dense connections with the main and accessory olfactory systems, as well as with each other (Aldheid et al., 1995; Coolen and Wood 1998; Davis et al., 1978; Scalia and Winans 1975). They process and transmit olfactory information essential for conspecific identification and appropriate social responding in laboratory rats, hamsters, and mice (Newman, 1999). The same is true for male prairie voles, as indicated by the increased expression of the immediate-early gene (IEG) Fos in both the pBST and MeApd after male prairie voles cohabitate with a female, mate with her, or interact with pups (Cushing et al., 2003; Kirkpatrick et al., 1994b; Lim and Young 2004). Furthermore, lesions of the entire MeA decrease males' paternal behaviors (Kirkpatrick et al., 1994a). How the TH-ir cells of the pBST and MeApd are specifically involved in these effects is unknown.

To begin investigating potential roles for the TH-ir cells of the male prairie vole pBST and MeApd, Fos expression in TH-ir neurons was examined in these brain sites after males interacted for 30 min with one of several social stimuli, including a sexually receptive female prairie vole or conspecific pups. In addition, Fos in other forebrain TH-ir cell groups was examined to determine the selectivity of Fos expression in TH-ir cells in the pBST and MeApd after sociosexual interactions. Because many, but not most, TH-ir cells in these two sites contained Fos immunoreactivity after social interactions, a

subsequent experiment was designed to determine if even more of these TH-ir cells would contain another IEG, Egr-1, after particular sociosexual interactions. Egr-1 was chosen because it is expressed in many brain sites after the display of sexual or maternal behaviors, including in cells that do not express Fos (Numan et al., 1998; Polston and Erskine 1995; Wersinger and Baum 1996). Because the formation of partner preferences in male prairie voles requires rather long sexual interactions with a female (up to 24 h; Insel et al., 1995; Winslow et al., 1993), and surprisingly prolonged Fos expression can be seen after acute or chronic social or other stimulation (Bullitt et al., 1992; Matsuda et al., 1996; Quattrochi et al., 2005; Ricci et al., 2007; Stack and Numan 2000; Wrynn et al., 2000; Xu et al., 2006), Fos expression was also examined in TH-ir cells of the pBST and MeApd after males cohabitated with a sexually receptive female for 6 or 24 h.

Methods

Subjects

Subjects were adult prairie voles (*Microtus ochrogaster*) that were born and raised in the colony at Michigan State University. The colony originated with voles originally captured in Urbana, IL, USA, in 1994, and since maintained in laboratory environments and interbred with voles from the colonies of Drs. C. Sue Carter, Zuoxin Wang, Geert De Vries, and Betty McGuire. This stock was brought to Michigan State University in 2002. Animals were housed in clear plastic cages (48×28×16 cm), containing wood chips, wood shavings, and a layer of hay. Food and water were provided *ad libitum*; food consisted of a mixture of cracked corn, whole oats, sunflower seeds, and rabbit chow (Teklad rodent diet # 2031, Harlan, Madison, WI, USA) mixed in a 1:1:2:2

ratio. The colony room was maintained with an ambient temperature of approximately 21°C and a 14:10-h light/dark cycle. Litters were weaned from breeding pairs at 20 days of age and housed with their siblings in mixed-sex groups consisting of the entire litter until adulthood (60–120 days old) when they were used in one of the experiments. While exposure to male siblings affects sensitivity to estradiol in female prairie voles (Cushing and Carter, 1999), the effects of the postweaning social environment on males' reproductive potential are less pronounced (Mateo et al., 1994). In fact, the sex ratio was retrospectively determined for 75% of the subjects' natal litters, and found that neither the total number of siblings nor the litter sex ratio was significantly correlated with any sociosexual behavior or the IEG expression found in any site examined in the present experiments (data not shown). The experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80–23), as well as the Institutional Animal Care and Use Committee at Michigan State University. The minimal number of subjects per group that would provide sufficient statistical power was used, and every effort was made to ensure that subjects did not experience any pain during any of the behavioral tests or procedures.

Experiment Ia: effects of 30-min social interactions on Fos expression

Sexually naïve male prairie voles were placed individually into a clean cage (48×28×16 cm) with bedding, food, and water, and transported to a nearby room illuminated with dim ambient light (20 lx) for behavioral testing. After a 3-hour habituation to the testing cage and room, each subject was presented with a social stimulus (or nothing) for 30 min. After 30 min, the stimulus animal was removed and the

male subject remained in the cage and testing room for 1 h before being sacrificed. An additional control group was not placed in a novel cage or brought to the testing room, but instead sacrificed directly after removal from their home cage in the colony room. The social stimuli presented were:

Groups 1 and 2: sexually receptive female

Males (n = 40) were allowed to interact with an adult female that was ovariectomized, allowed to recover for at least 2 weeks, and injected s.c. with estradiol benzoate (10 μg in 50 μl sesame oil; Sigma, St. Louis, MO, USA) 48 and 24 h before testing to induce sexual receptivity (Carter et al., 1987a; Northcutt and Lonstein 2008). Stimulus females were tested for receptivity with a sexually experienced male from the colony immediately before testing, and only females displaying lordosis when mounted were used. Because Fos expression in the pBST and MeApd of male rats is greatly increased after ejaculation, but this increase does not necessarily occur after mating bouts that do not include ejaculation (Coolen et al., 1997, 1998; Veening and Coolen 1998), male prairie voles interacting with receptive females were screened until two groups were filled: males that mated to ejaculation within the 30-min test (EJAC; n = 8) and males that mated but did not ejaculate (NON EJAC; n = 8). Previous work demonstrated that up to one third of the sexually inexperienced males in the colony ejaculate during a 30-min sexual behavior test (Northcutt and Lonstein, 2008). After eight NON EJAC males were obtained, the subsequent males that were screened but did not ejaculate within 30 min were unnecessary, so were not sacrificed or included in the experiment.

Group 3: pups

To examine the effects of other unfamiliar social stimuli on Fos expression, a group of parentally naïve adult virgin males was allowed to interact with two 2–3 day old conspecific pups (PUPS; $n = 9$). Pups were taken from breeding pairs in the colony just prior to testing, and were wiped clean with distilled water before being placed in the testing cage with the subject for 30 min. One subject attacked the pups, so testing was terminated and this male was not included in the study (final $n = 8$).

Group 4: familiar female sibling

To control for the effects of general social interaction on Fos expression, a fourth group of males interacted for 30 min with a familiar female sibling that he had been housed with until 3 hours before testing (SIBLING; $n = 9$). Female prairie voles are induced ovulators and do not come into behavioral estrus unless exposed to an unfamiliar male (Carter et al., 1980, 1987b). Incest is avoided in this species because females do not sniff the anogenital region of their familiar male siblings (Carter et al., 1980), which prevents estrus induction, so expectedly none of these males attempted to mate with his sibling.

Group 5: alone control

To determine the effects of handling and habituation to a clean cage and the testing room, these males were removed from the colony but remained alone in the testing cage and room (ALONE; $n = 8$). The cage lid was removed and a hand placed into the cage after the 3 h habituation, and again 30 min later, to mimic inserting and removing a social stimulus.

Group 6: colony control

To determine baseline Fos expression in socially housed males, these subjects were removed from their home cages in the colony room, immediately overdosed with anesthetic, and perfused (COLONY; $n = 8$).

All behavioral interactions for the socially stimulated groups were continuously recorded using a Panasonic low-light-sensitive camera connected to a Panasonic VCR (Panasonic, Secaucus, NJ, USA). Sexual behaviors, paternal behaviors, social contact, and non-social behaviors (including feeding, drinking, and exploring) were scored similarly to what the laboratory has previously described (Lonstein 2002; Northcutt and Lonstein 2008).

One hour after the stimulus was removed, or 4.5 h after ALONE males were placed in the novel cage, subjects were overdosed with an anesthetic cocktail containing ketamine (62.5 mg/kg; Butler, Dublin, OH, USA), xylazine (7.5 mg/kg; Butler), and acepromazine (0.8 mg/kg; Butler). They were perfused transcardially with 100 ml of 0.9% saline and 100 ml of 4% paraformaldehyde in 0.1 M sodium phosphate buffer. After perfusion, brains were postfixed overnight in 4% paraformaldehyde, and then cryoprotected in a 20% sucrose solution for at least 2 days. Brains were sectioned into 35-μm coronal sections on a freezing microtome and stored in cryoprotectant at −20 °C until further processing. One series containing every other section throughout each brain was processed immunocytochemically for both TH and Fos.

Fos and TH dual immunocytochemistry

Sections were rinsed three times in Tris-buffered saline (TBS; pH 7.6) between each step. First, sections were incubated in 0.1% sodium borohydride for 15 min, and then incubated in 1% hydrogen peroxide and 0.3% Triton X-100 in TBS for 10 min. Sections were blocked in 20% normal goat serum (NGS) and 0.3% Triton X-100 in TBS for 30 min before being placed in rabbit anti-Fos polyclonal primary antiserum (1:10,000; cat. # sc52; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 2% NGS and 0.3% Triton X-100 in TBS at 4 °C for 48 h. Next, sections were incubated in a biotinylated goat anti-rabbit secondary antiserum (1:500; Vector Laboratories, Burlingame, CA, USA) in 2% NGS and 0.3% Triton X-100 in TBS for 1 h, followed by a 1-h incubation in avidin–biotin complex (Vectastain Elite, Vector Laboratories). Fos was visualized by incubating sections in 3,3′-diaminobenzidine (Sigma) in TBS, which produced a brown nuclear reaction product. Brains were then blocked again in 20% NGS for 30 min, and were incubated in a mouse anti-TH monoclonal primary antiserum (1:2000; cat. # MAB318; Chemicon, Temecula, CA, USA) in 2% NGS and 0.3% Triton X-100 in TBS at room temperature overnight for ~18 h. Sections were then incubated in goat anti-mouse secondary antiserum (1:500; Vector Laboratories) in 2% NGS and 0.3% Triton X-100 in TBS for 1 h, followed by a 1-h incubation in avidin–biotin complex. TH was visualized with Vector SG (Vector Laboratories) diluted in TBS, which resulted in a blue cytoplasmic reaction product. Cross-reactivity was not expected, given that the primary antisera were obtained from different species, and visual inspection of the tissue revealed that numerous single-labeled cells of both types were clearly visible in many brain areas. Immunocytochemical controls that included omitting one or both of the primary and

secondary antisera eliminated the corresponding specific labeling. After processing, brain sections were mounted onto slides, dehydrated, and coverslipped. Two immunocytochemical runs were performed, with a similar number of subjects from each group represented in each run.

Analysis of Fos and TH-ir cells

Slides were randomized and coded for analysis. Sections were analyzed by a single observer blind to subject condition (K.V.N.). The number of Fos-ir, TH-ir, and dual-labeled cells was counted in each site examined (see below). All cells expressing TH were counted at 200× magnification using a Nikon E400 light microscope (Nikon Inc., Melville, NY, USA) with the aid of a reticle in one ocular lens. A cell was counted if it contained any blue cytoplasmic labeling. Fos-expressing cells were also examined at 200× magnification, and included in the analysis if they contained brown nuclear labeling. To determine the number of dual-labeled cells, TH-ir cells were re-examined at 400× magnification and were considered to also contain Fos immunoreactivity if they clearly contained a brown nuclear label surrounded by a blue cytoplasmic label.

Cells were examined bilaterally from four consecutive sections containing the pBST (roughly corresponding to Swanson's plates 21–22; Swanson, 1998) and from seven sections containing the MeApd (Swanson's atlas plates 28–30). To determine if the effect of social stimulation on IEG expression within TH-ir cells of the pBST and MeApd was unique, numerous other forebrain regions were examined where TH-ir cells are found in prairie voles and other rodents (Bjorklund and Dunnett 2007; Lansing and Lonstein 2006; Smeets and Gonzalez 2000). This involved bilaterally examining two

sections containing the anteroventral periventricular nucleus of the preoptic area (AVPV) and ventrolateral region of the anteroventral preoptic area (AVP) (Swanson's atlas plate 19), periventricular anterior hypothalamus (PVa; atlas plate 24), zona incerta (ZI; atlas plate 28), and arcuate nucleus of the hypothalamus (ARH; atlas plate 30). For all regions, cells were counted from the entire area containing TH-ir cells, with the size of the area examined remaining constant across all subjects.

Statistical analyses

Behavioral differences between EJAC and NON EJAC males were compared with unpaired *t*-tests. The duration of time that socially stimulated groups spent with their stimulus was compared with a one-way ANOVA, followed by Fisher's LSD post-hoc tests to compare differences between individual groups.

To analyze TH and IEG expression in each brain site, the Shapiro–Wilk test was used to determine whether the data were normally distributed. The TH immunoreactivity data from every site were normally distributed, so one-way ANOVAs were used to compare groups in the number of TH-ir cells. The IEG measures were not normally distributed, so data were subjected to a square root transformation, and normality was assessed again. If the transformation resulted in a normal distribution, one-way ANOVAs were used to analyze the transformed data. This was the case for Fos immunoreactivity in the AVP, ZI, and ARH, the number of cells containing both TH immunoreactivity and Fos immunoreactivity in the pBST, MeApd, AVP, PVa, and ZI, and the percentage of TH-ir cells containing Fos immunoreactivity in the pBST, MeApd, AVP, PVa, and ZI. If these omnibus ANOVAs were significant, Fisher's LSD post-hoc tests were then used to

compare differences between individual groups. If square root transformation did not result in a normal distribution, Kruskal–Wallis tests were used to compare treatment groups, followed by Mann–Whitney tests to determine which individual groups differed from each another. This was the case for Fos immunoreactivity in the pBST, MeApd, AVPV, and PVa, the number of dual-labeled cells in the AVPV and ARH, and the percentage of TH-ir cells expressing Fos in the AVPV and ARH. Pearson's *r* was used to correlate the percentage of TH-ir cells expressing Fos in the pBST and MeApd with the latencies, frequencies, and durations of sexual behaviors displayed by EJAC and NON EJAC males, paternal behaviors in PUPS males, and total duration of all social behaviors in males tested with a stimulus animal. Based on the previous studies (Coolen et al., 1997, 1998; Veening and Coolen 1998), EJAC males were expected to have greater IEG expression than NON EJAC males, so one-tailed post-hoc tests were used to compare IEG expression between these two particular groups (significance indicated by $P \leq 0.10$). In all other cases, statistical significance was indicated by $P \leq 0.05$. Tissue from the AVPV and AVP of one NON EJAC male was damaged and could not be analyzed, resulting in a sample size of seven subjects for this group for these sites.

Experiment Ib: effects of 30-min social interactions on Egr-1 expression in TH-ir cells

Egr-1 and TH immunocytochemistry and analysis

Because mating to ejaculation was the most effective stimulus for eliciting Fos in many, but still not most, TH-ir cells in the pBST and MeApd (see Results), an additional experiment determined if mating-induced modulation of even more TH-ir cells could be reflected by expression of another IEG, Egr-1. As noted above, Egr-1 is expressed in

many brain sites after the display of social behaviors (Numan et al., 1998; Polston and Erskine 1995; Wersinger and Baum 1996), including in cells that do not express Fos. To do this, the alternate series of brain sections for ALONE, SIBLING, NON EJAC, and EJAC males from Experiment Ia were examined. Egr-1 and TH immunocytochemistry was performed identically to that described above for Fos and TH, except that the Fos primary antiserum was replaced with a rabbit polyclonal primary antiserum against Egr-1 (1:1000; cat. # sc189; Santa Cruz Biotechnology). Immunocytochemical controls that omitted the primary or secondary antisera abolished specific labeling. The number of cells containing TH immunoreactivity, the number containing Egr-1 immunoreactivity, and the number containing both TH immunoreactivity and Egr-1 immunoreactivity were counted bilaterally from the two sections through the pBST and three sections through the MeApd that contained the most TH-ir cells in these sites.

Statistical analyses

Statistical analyses of the number of TH-ir cells, the number of Egr-1-ir cells, and the percentage of TH-ir cells also expressing Egr-1 were performed as described for Experiment Ia. The number of TH-ir cells among groups was normally distributed, so one-way ANOVAs were used to analyze these data. The number of Egr-1-ir cells was normally distributed after square root transformation of the data, so one-way ANOVAs were performed on these transformations. All other data sets were not normally distributed even after square root transformation, so Kruskal–Wallis analyses were used to compare groups. Some tissue processed for TH and Egr-1 was damaged during immunocytochemical processing, resulting in final sample sizes for the pBST of ALONE

and SIBLING $n = 8$, NON EJAC $n = 6$, EJAC $n = 5$ and for the MeApd of ALONE $n = 8$, SIBLING $n = 6$, NON EJAC $n = 5$, and EJAC $n = 6$.

Experiment II: effects of 6 or 24-h social interactions on Fos expression

Prolonged bouts of mating up to 24 h in duration are typically necessary to induce partner preferences in male prairie voles (Insel et al., 1995; Winslow et al., 1993). To determine if prolonged sexual interactions could induce persistently high numbers of TH-ir cells expressing Fos in the pBST and MeApd that might reflect establishment of a pair bond, sexually naïve male prairie voles were exposed to sexually receptive females or a control stimulus for 6 or 24 h. Males were placed in a novel cage and moved to a testing room as described in Experiment Ia. After a 3-h habituation, one group of males interacted with a sexually receptive female (MATED; 6 h: $n = 12$, 24 h: $n = 9$). Receptivity in females was induced and verified before testing as described in Experiment Ia. Another group of males interacted with a familiar female sibling (SIBLING; 6 h: $n = 8$; 24 h: $n = 7$). Behavioral interactions were recorded at 5:1 time-lapse using a Panasonic video recorder, and were viewed to ensure that males mated with sexually receptive females within 2 h after the beginning of testing, but did not mate with their siblings. It was observed that five males in the MATED groups (four 6-h males, and one 24-h male) failed to mate in the first 2 h, and were excluded from the study, but the remaining males began mounting the stimulus female within 40 min of the beginning of the test (final $n = 8$ for both time points). None of the SIBLING males attempted to mate with their sisters. A third group of males remained alone in the observation cage (ALONE; 6 h: $n = 7$, 24 h: $n = 8$), but had the cage lid removed and a hand placed inside

the cage after habituation to imitate inserting a stimulus animal. In the 6-h groups, 6 hours after the stimulus was introduced to the cage, it was removed (or a hand was placed into the cage for the ALONE group) and the male remained alone in the cage for 1 h before perfusion. In the 24-h groups, the male subject was perfused 24 h after the stimulus was placed in the cage. Tissue collection, processing, and analysis, as well as statistical analyses, were identical to that described in Experiment Ia. TH immunoreactivity data were normally distributed, so one-way ANOVAs were used to analyze these data. All other data were not normally distributed, even after square root transformation, so Kruskal–Wallis tests were used instead.

Results

Experiment Ia: Fos and TH expression after 30-min social interactions

Behavior

All males in the NON EJAC and EJAC groups mounted the stimulus female with a latency and frequency that did not significantly differ between the two groups (latency to mount: NON EJAC = 346 ± 78 s, EJAC = 182 ± 35 s, $t(14) = -1.93$, $P = 0.075$; frequency of mounts: NON EJAC = 19 ± 6, EJAC = 34 ± 10, $t(14) = 1.36$, $P = 0.194$). However, EJAC males displayed more thrusting bouts than did NON EJAC males (110 ± 19 vs. 30 ± 13, $t(14) = 3.50$, $P = 0.004$). Males in the EJAC group ejaculated a mean of 1.9 ± 0.3 times, with the first ejaculation occurring 389 ± 182 s after the first mount and 383 ± 182 s after the first thrust.

All males in the PUPS group licked and hovered over pups. Males spent 777 ± 72 s licking pups, and began licking 64 ± 27 s after the beginning of testing. They spent

1061 ± 156 s huddled over pups, with an average latency of 99 ± 20 s after they began licking. Males retrieved pups an average of 2 ± 1 times, but 37% of males did not retrieve at all, which was not surprising given that prairie voles rarely retrieve pups (see Lonstein and De Vries, 1999a).

The duration of time that males spent in physical contact with their stimulus (including the durations of sniffing, grooming, and huddling) significantly differed among groups ($F(3, 29) = 4.47$, $P = 0.011$), and was greatest in the PUPS group (1207 ± 133 s). This was significantly more time spent with the stimulus than the duration of time that SIBLING and EJAC males spent in physical contact with their stimulus females (SIBLING: 682 ± 137 s, EJAC: 725 ± 53 s), but it did not differ from NON EJAC males (951 ± 103 s). SIBLING, NON EJAC, and EJAC males spent a similar amount of time in contact with their respective stimuli.

TH expression

No significant differences were found between groups in the number of TH-ir cells in the pBST, MeApd, AVPV, AVP, PVa, or ZI. Unexpectedly, EJAC and SIBLING males had significantly more TH-ir cells in the ARH (by approximately 30%) than the number of TH-ir cells found in the ARH of ALONE and NON EJAC males (Table 3.2).

Table 3.1. The number of cells expressing TH and the number of dual-labeled cells (Mean ± SEM) in the pBST and MeApd after males interacted with one of several social stimuli for 30 min. Groups with any of the same superscript letters do not significantly differ from each other.

		# of TH-ir cells	# of Dual-labeled cells
pBST	COLONY	154 ± 20	4 ± 1^{b}
	ALONE	184 ± 31	1 ± 0^{a}
	SIBLING	175 ± 21	4 ± 1^{b}
	PUPS	143 ± 26	2 ± 1^{b}
	NON EJAC	167 ± 22	5 ± 1^{b}
	EJAC	169 ± 12	9 ± 2^{c}
	$F(5, 43)$	0.425	5.72
	p	0.829	<0.001
MeApd	COLONY	336 ± 33	9 ± 3^{bc}
	ALONE	330 ± 33	1 ± 1^{a}
	SIBLING	334 ± 27	13 ± 3^{cd}
	PUPS	343 ± 52	4 ± 1^{ab}
	NON EJAC	289 ± 34	6 ± 2^{bc}
	EJAC	333 ± 42	23 ± 6^{d}
	$F(5, 43)$	0.278	7.26
	p	0.926	<0.001

Table 3.2. The number of cells expressing TH, Fos, both Fos and TH, and the percentage of TH-ir cells also expressing Fos (Mean ± SEM) after subjects interacted with one of several social stimuli for 30 min. Groups with any of the same superscript letters do not significantly differ from each other.

		# of TH-ir cells	# of Fos-ir cells	# of Dual-labeled cells	% of TH-ir cells expressing Fos
AVPV	COLONY	342 ± 15	25 ± 9^{a}	3 ± 1^{b}	1 ± 0^{b}
	ALONE	276 ± 18	18 ± 6^{a}	1 ± 0^{a}	0 ± 0^{a}
	SIBLING	293 ± 29	19 ± 2^{a}	1 ± 0^{ab}	0 ± 0^{ab}
	PUPS	297 ± 35	125 ± 23^{b}	3 ± 1^{b}	1 ± 0^{bc}
	NON EJAC	293 ± 27	181 ± 40^{b}	7 ± 3^{b}	2 ± 1^{bc}
	EJAC	277 ± 19	373 ± 57^{c}	7 ± 2^{b}	3 ± 1^{c}
		$F(5, 42) = 0.916$	$H(5) = 35.72$	$H(5) = 12.33$	$H(5) = 13.40$
	p	0.480	<0.001	0.031	0.020
AVP	COLONY	185 ± 15	95 ± 22^{a}	6 ± 3	3 ± 1
	ALONE	166 ± 18	114 ± 16^{a}	2 ± 1	1 ± 0
	SIBLING	176 ± 22	114 ± 15^{a}	3 ± 1	2 ± 1
	PUPS	188 ± 15	384 ± 45^{c}	7 ± 1	4 ± 1
	NON EJAC	163 ± 25	242 ± 46^{b}	3 ± 1	2 ± 1
	EJAC	202 ± 18	314 ± 48^{bc}	6 ± 2	2 ± 1
	$F(5, 42)$	0.551	11.85	1.54	1.58
	p	0.736	<0.001	0.199	0.186
PVa	COLONY	508 ± 19	117 ± 49^{ab}	5 ± 2^{a}	1 ± 0^{a}
	ALONE	449 ± 30	92 ± 18^{a}	5 ± 1^{ab}	1 ± 0^{a}
	SIBLING	498 ± 20	162 ± 26^{b}	8 ± 1^{bcd}	2 ± 0^{abc}
	PUPS	477 ± 37	472 ± 58^{cd}	7 ± 1^{abc}	1 ± 0^{ab}
	NON EJAC	430 ± 31	410 ± 44^{c}	10 ± 2^{cd}	2 ± 0^{bc}
	EJAC	480 ± 27	576 ± 91^{d}	14 ± 3^{d}	3 ± 1^{c}
		$F(5, 43) = 1.138$	$H(5) = 30.84$	$F(5, 43) = 4.03$	$F(5, 43) = 3.95$
	p	0.355	<0.001	0.004	0.005

Table 3.2 (cont'd)

		# of TH-ir cells	# of Fos-ir cells	# of Dual-labeled cells	% of TH-ir cells expressing Fos
ZI	COLONY	732 ± 53	172 ± 38^{a}	20 ± 5^{a}	3 ± 1^{a}
	ALONE	586 ± 58	208 ± 27^{a}	21 ± 4^{a}	4 ± 1^{a}
	SIBLING	585 ± 46	242 ± 17^{ab}	24 ± 6^{a}	4 ± 1^{a}
	PUPS	647 ± 29	347 ± 41^{b}	44 ± 3^{b}	7 ± 1^{b}
	NON EJAC	575 ± 41	362 ± 62^{b}	37 ± 4^{b}	7 ± 1^{b}
	EJAC	610 ± 33	266 ± 44^{ab}	24 ± 5^{a}	4 ± 1^{a}
	$F(5, 43)$	1.800	3.08	4.50	5.60
	p	0.133	0.004	0.002	0.001
ARH	COLONY	194 ± 19^{abc}	31 ± 7^{a}	1 ± 1	1 ± 0
	ALONE	152 ± 15^{a}	66 ± 12^{ab}	3 ± 1	2 ± 1
	SIBLING	230 ± 12^{c}	62 ± 13^{ab}	2 ± 1	1 ± 1
	PUPS	208 ± 12^{bc}	76 ± 10^{b}	3 ± 1	1 ± 0
	NON EJAC	177 ± 25^{ab}	105 ± 25^{b}	6 ± 3	3 ± 2
	EJAC	240 ± 20^{c}	103 ± 26^{b}	7 ± 3	3 ± 1
		$F(5, 43) = 3.51$	$F(5, 43) = 2.83$	$H(5) = 3.98$	$H(5) = 3.94$
	p	0.010	0.027	0.552	0.558

Fos expression

In the pBST and MeApd, as well as the AVPV and PVa, the number of Fos-ir cells significantly differed among groups of males (Figure 3.1, Table 3.2; pBST, $H(5) = 39.96$, $P < 0.001$; MeApd, $H(5) = 38.46$, $P < 0.001$; AVPV, $H(5) = 35.72$, $P < 0.001$; PVa $H(5) = 30.84$, $P < 0.001$). In the pBST, MeApd, and AVPV, EJAC males had 15–18 times more Fos-ir cells than ALONE, SIBLING, and COLONY males, while NON EJAC and PUPS males had approximately eight times more Fos-ir cells than these three control groups. In the PVa, EJAC and PUPS males had approximately five times more Fos-ir cells, and NON EJAC males had approximately four times more Fos-ir cells, than that found in the three control groups.

For the other forebrain sites examined, males exposed to any novel social stimulus (EJAC, NON EJAC, and PUPS groups) generally had more Fos-ir cells compared to SIBLING, ALONE, and COLONY control males, with the pattern of group differences in Fos expression different in each brain site. Notably, the pattern of Fos expression seen in the pBST, MeApd, AVPV (i.e. EJAC males having more Fos-ir cells than all other groups) was not found in any of the other brain sites that were examined (see Table 3.2).

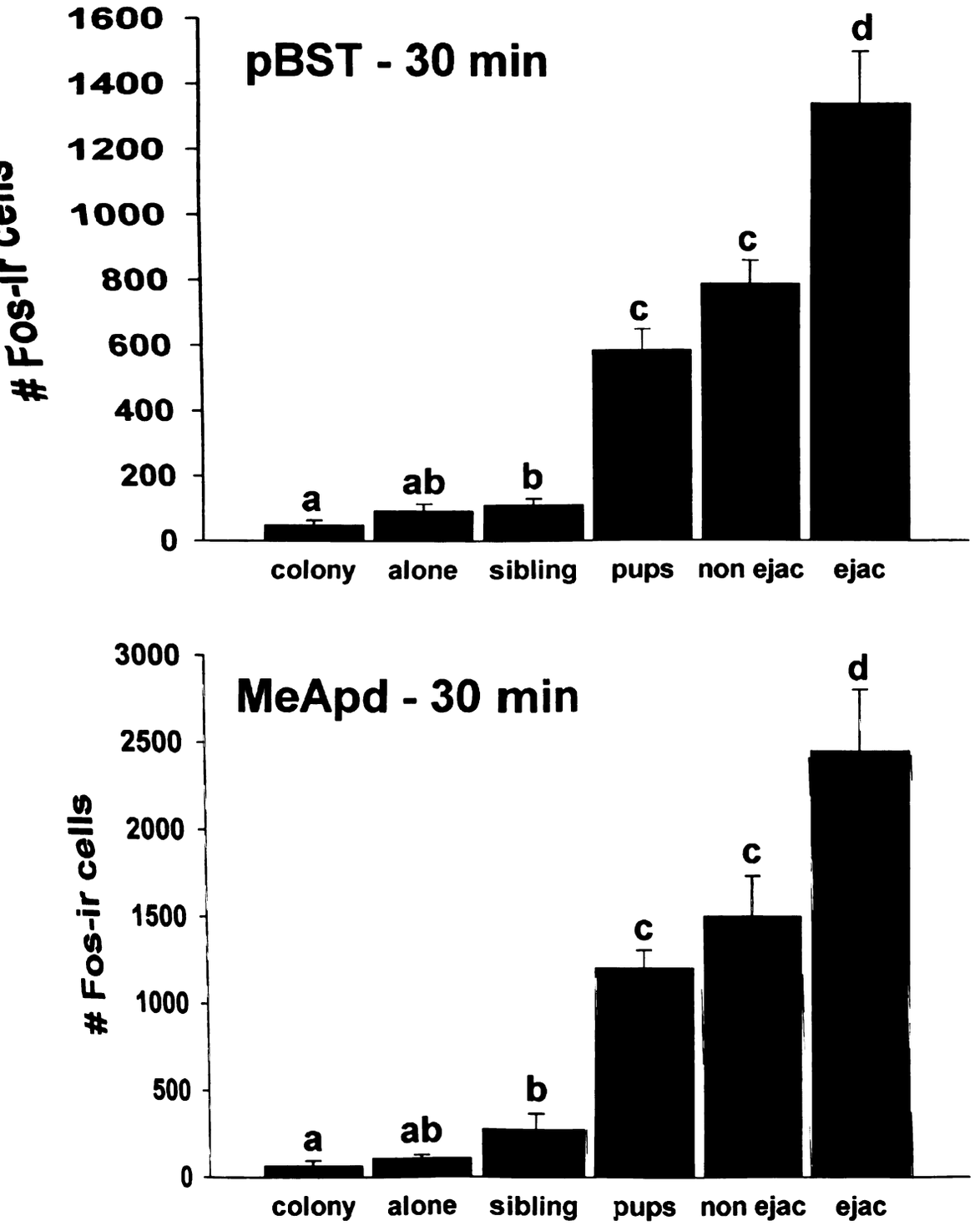

Figure 3.1. Number of Fos-ir cells (Mean ± SEM) in four sections through the pBST and seven sections through the MeApd after male subjects interacted with one of several stimuli for 30 min. Bars with any of the same letters above them do not significantly differ from each other.

Fos expression within TH-ir cells

The percentage of TH-ir cells in the pBST and MeApd that also expressed Fos significantly differed among groups (Figure 3.2 and Figure 3.3; pBST, $F(5, 43) = 5.44$, $P = 0.001$; MeApd, $F(5, 43) = 7.29$, $P < 0.001$). In both sites, EJAC males had a greater percentage of TH-ir cells expressing Fos (~6% of all TH-ir cells) than did ALONE, PUPS, and NON EJAC males. This pattern was unique, because EJAC males did not have a greater percentage of TH-ir cells expressing Fos than NON EJAC males in any of the other forebrain regions analyzed, including the AVPV and PVa (see Table 3.2). In addition, the percentage of TH-ir cells in the pBST and MeApd also expressing Fos was higher in all groups exposed to an adult social stimulus (COLONY, SIBLING, NON EJAC, and EJAC males) when compared to ALONE males.

The number of dual-labeled cells in the pBST and MeApd (Table 3.1) showed a very similar pattern as the percentage of TH-ir cells expressing Fos, with EJAC males having significantly more dual-labeled cells than all other groups in the pBST and more than the COLONY, ALONE, PUPS, and NON EJAC males in the MeApd.

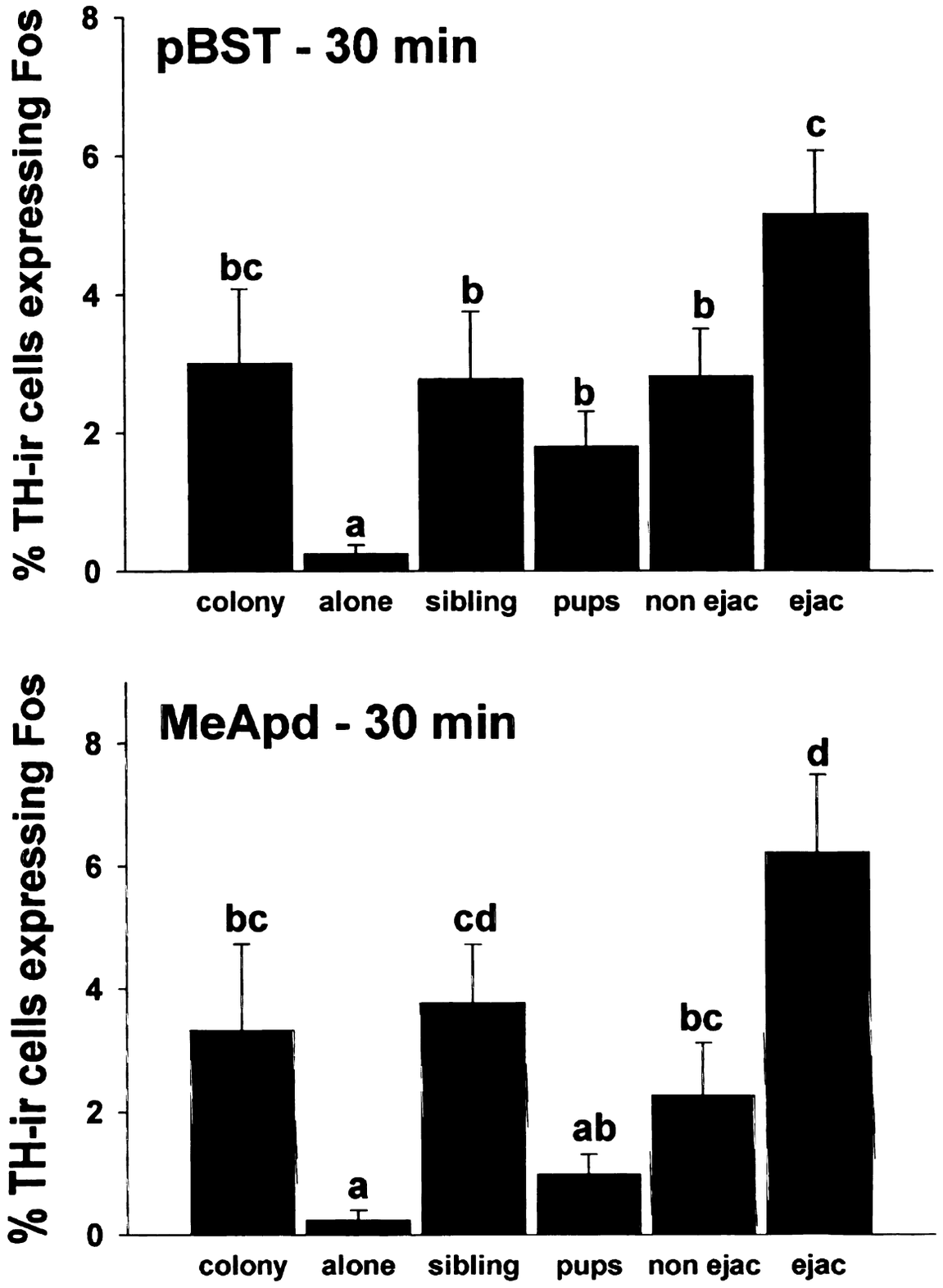

Figure 3.2. Percentage of TH-ir cells expressing Fos (Mean ± SEM) in the pBST and MeApd after male subjects interacted with one of several stimuli for 30 min. Bars with any of the same letters above them do not significantly differ from each other.

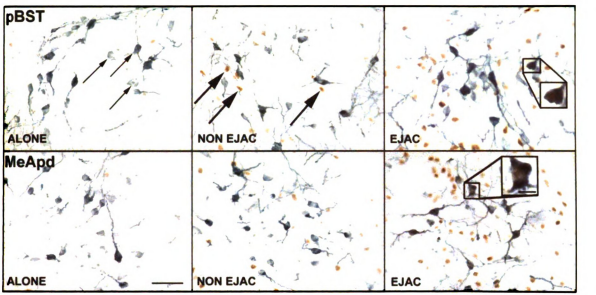

Figure 3.3. Photomicrographs of the pBST (top row) and MeApd (bottom row) from representative males in the ALONE, NON EJAC, and EJAC groups in Experiment Ia. Small arrows in top left panel indicate cells that are single-labeled with TH-immunoreactivity (blue cytoplasmic label). Large arrows in top middle panel indicate cells that are single-labeled with Fos-immunoreactivity (brown nuclear label). Dual-labeled cells are highlighted at higher magnification in the two panels showing EJAC males. Scale bar in bottom left panel = 50 μm. Scale bar in bottom right panel inset = 10 μm.

Correlations between the percentage of TH-ir cells expressing Fos and behavior

The percentage of TH-ir cells expressing Fos in the pBST and MeApd was significantly correlated with the expression of numerous sexual behaviors (see Figure 3.4, Table 3.3). In the pBST, the percentage of TH-ir cells expressing Fos was positively correlated with the number of mounts, number of thrusting bouts, number of ejaculations, duration of time spent mounting, and total time engaged in all sexual behaviors. In the MeApd, the percentage of TH-ir cells expressing Fos was positively correlated with the number of thrusting bouts, duration of time spent mounting, duration of time spent thrusting, and total amount of time engaged in sexual behaviors. The percentage of TH-ir cells in the pBST that expressed Fos was also positively correlated with the latency to lick pups in PUPS males, but there were no significant correlations between the percentage of TH-ir cells expressing Fos in the MeApd and any paternal behaviors.

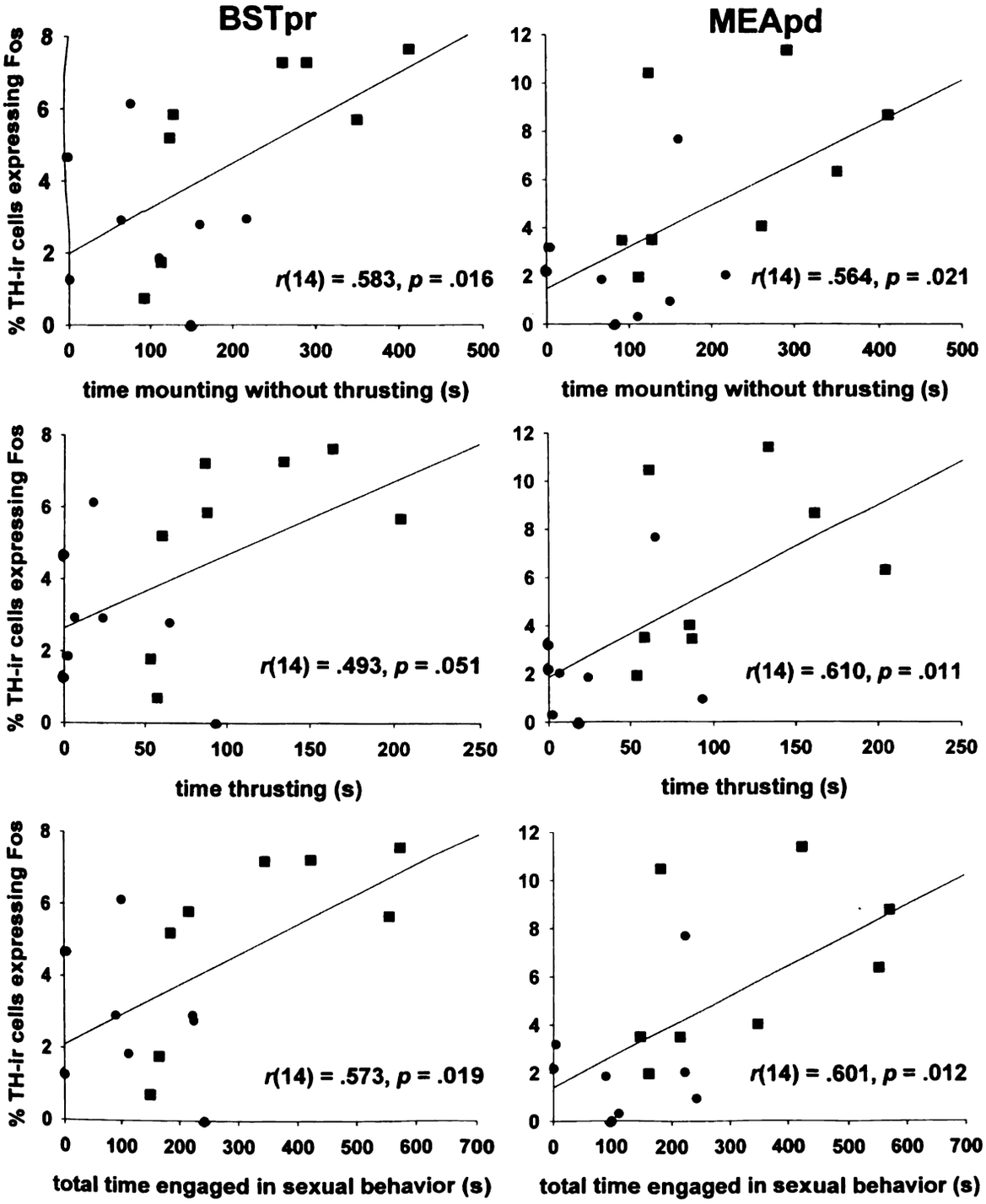

Figure 3.4. Correlations between the percentage of TH-ir cells in the pBST (left) or MeApd (right) that also expressed Fos and the duration of time spent mounting and thrusting by EJAC males (squares) and NON EJAC males (circles) during a 30-min test with a sexually receptive female. Time engaged in male sexual behavior (bottom row) is the summed duration of mounting, thrusting, and ejaculating. See Table 3.3 for correlations between other behaviors and the percentage of TH-ir cells that also expressed Fos.

Table 3.3. Correlations between the percentage of TH-ir cells expressing Fos and the expression of sexual behavior, paternal behavior, and all social behaviors during 30-min interactions with a sexually receptive female, pups, or a familiar female sibling. See Figure 3.4 for additional correlations associated with the duration of sexual behaviors.

	% TH-ir cells in pBST expressing Fos	% TH-ir cells in MeApd expressing Fos
Sexual behaviors		
Latency (s)		
Mount	-.481	-.184
Thrust from first mount	-.063	.112
Ejaculation from 1st thrust	.469	.434
Frequency		
Mounts	.515*	.473
Thrusting bouts	.517*	.646**
Ejaculations	.516*	.410
Paternal behaviors		
Latency (s)		
Lick pups	.753*	-.328
Huddle from first lick	-.112	.390
Frequency		
Retrieve pups	-.160	.210
Duration (s)		
Lick pups	-.390	.537
Huddle over pups	-.309	.016
Time in contact with stimulus (s)	.141	.116

* $p \leq 0.05$, ** $p \leq 0.01$

Experiment Ib: Egr-1 expression after 30-min social interactions

TH expression

The number of TH-ir cells in the pBST or MeApd did not differ among groups (pBST, $F(3, 23) = 0.119$, $P = 0.948$; MeApd, $F(3, 21) = 0.915$, $P = 0.451$) (Table 3.4).

Table 3.4. The number of cells expressing TH, and the number of cells containing both TH and Egr-1 (Mean ± SEM) in two sections through the pBST and three sections through the MeApd after males interacted with one of several stimuli for 30 min. Groups with any of the same superscript letters do not significantly differ from each other.

		# of TH-ir cells	# of Dual-labeled cells
pBST	ALONE	88 ± 6	1 ± 0^{a}
	SIBLING	82 ± 10	4 ± 1^{b}
	NON EJAC	92 ± 18	3 ± 1^{b}
	EJAC	87 ± 16	9 ± 4^{b}
		$F(3, 23) = 0.119$	$H(3) = 10.31$
	p	0.948	0.016
MeApd	ALONE	113 ± 12	1 ± 0^{a}
	SIBLING	80 ± 12	8 ± 3^{b}
	NON EJAC	112 ± 19	7 ± 1^{b}
	EJAC	97 ± 22	13 ± 4^{b}
		$F(3, 21) = 0.915$	$H(3) = 15.04$
	p	0.451	0.002

Egr-1 expression

All groups of males had more Egr-1-expressing cells in the pBST and MeApd than the number of Fos-expressing cells they had in Experiment Ia. In both sites, Egr-1 expression differed among groups (Figure 3.5; pBST, $F(3, 23) = 53.52$, $P < 0.001$; MeApd, $F(3, 21) = 26.11$, $P < 0.001$), with all groups being significantly different from one another. SIBLING males had approximately 1.5 times the number of Egr-1-ir cells than did ALONE males, and NON EJAC males had almost twice the number found in ALONE males. EJAC males had significantly more Egr-1 expressing cells than all other groups, including 1.5 to two times more than that seen in NON EJAC males.

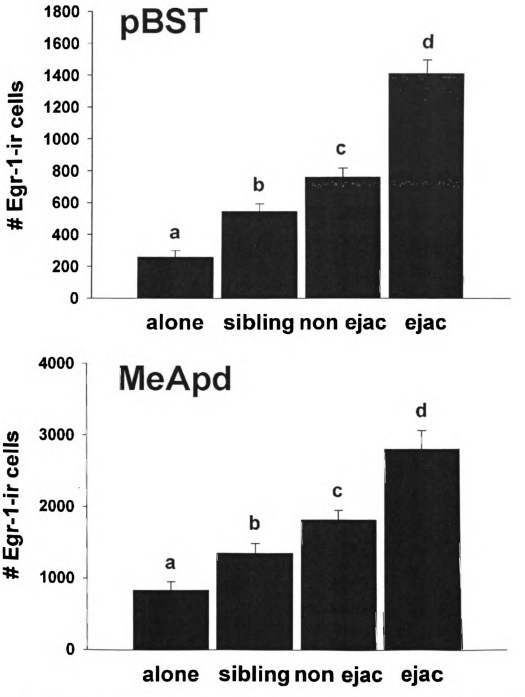

Figure 3.5. Number of Egr-1-ir cells (Mean ± SEM) in two sections of the pBST and three sections through the MeApd after male subjects interacted with one of several stimuli for 30 min. Bars with any of the same letters above them do not significantly differ from each other.

Egr-1 expression within TH-ir cells

The percentage of TH-ir cells in the pBST and MeApd that also contained Egr-1 immunoreactivity significantly differed among groups (Figure 3.6; pBST, $H(3) = 9.58$, $P = 0.023$; MeApd, $H(3) = 18.96$, $P < 0.001$). In these regions, EJAC males had nine and 23 times the percentage of TH-ir cells expressing Egr-1 than did ALONE males and twice that of NON EJAC males. Notably, the total percentage of TH-ir cells in these sites that also contained Egr-1 was higher than the percentage that contained Fos in Experiment 1a, but it was still quite moderate (up to ~10%–15% of all TH-ir cells). The raw number of dual-labeled cells also differed among groups in both sites (Table 3.4), with a generally similar pattern as the percentage of TH-ir cells that were dual-labeled.

Correlations between the percentage of TH-ir cells expressing Egr-1 and behavior

The percentage of TH-ir cells in the pBST that also contained Egr-1 immunoreactivity was not significantly correlated with any measure of males' sexual or other social behaviors. In the MeApd, the percentage of TH-ir cells expressing Egr-1 was positively correlated with the number of ejaculations ($r(9) = 0.853$, $P < 0.001$) and negatively correlated with the latency to ejaculate after the first thrust ($r(4) = -0.814$, $P = 0.049$).

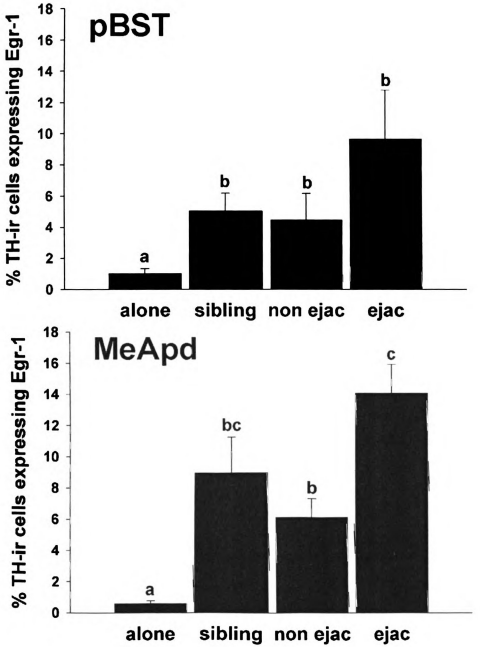

Figure 3.6. Percentage of TH-ir cells (Mean ± SEM) in the pBST and MeApd that also expressed Egr-1 after male subjects interacted with one of several stimuli for 30 min. Bars with any of the same letters above them do not significantly differ from each other.

Experiment II: Fos expression after 6- or 24-h social interactions

TH expression

The number of TH-ir cells in the pBST and MeApd did not significantly differ among groups at either the 6- or 24-h time point (Table 3.5).

Fos expression

After 6-h interactions, the number of Fos-ir cells in the pBST and MeApd significantly differed among groups (Figure 3.7; pBST, $H(2) = 15.05$, $P = 0.001$; MeApd, $H(2) = 14.71$, $P = 0.001$), with MATED males having five-fold more Fos-ir cells in both brain sites than did ALONE and SIBLING males.

Fos expression in the pBST and MeApd also differed among groups after 24-h interactions (Figure 3.7; pBST, $H(2) = 15.85$, $P < 0.001$; MeApd, $H(2) = 16.73$, $P < 0.001$). In both brain sites, MATED males had twice the number of Fos-ir cells than did SIBLING males, and SIBLING males had two to three times as many Fos-expressing cells as did ALONE males.

Table 3.5. The number of cells expressing TH and the number of cells containing both TH and Fos (Mean ± SEM) in four sections through the pBST and seven sections through the MeApd after males interacted with one of several stimuli for 6 or 24 h.

		# of TH-ir cells	# of Dual-labeled cells
pBST 6 h	ALONE	236 ± 29	0 ± 0^{a}
	SIBLING	226 ± 22	1 ± 0^{a}
	MATED	266 ± 18	6 ± 3^{b}
		$F(2, 20) = 0.851$	$H(2) = 13.27$
	p	0.442	0.001
pBST 24 h	ALONE	221 ± 19	0 ± 0^{a}
	SIBLING	221 ± 17	4 ± 2^{b}
	MATED	285 ± 24	3 ± 1^{b}
		$F(2, 20) = 3.23$	$H(2) = 6.21$
	p	0.567	0.045
MeApd 6 h	ALONE	426 ± 12	0 ± 0^{a}
	SIBLING	378 ± 25	6 ± 2^{b}
	MATED	363 ± 50	4 ± 2^{b}
		$F(2, 20) = 0.888$	$H(2) = 9.94$
	p	0.427	0.007
MeApd 24 h	ALONE	387 ± 18	0 ± 0^{a}
	SIBLING	351 ± 32	5 ± 3^{b}
	MATED	413 ± 32	2 ± 1^{b}
		$F(2, 20) = 1.31$	$H(2) = 6.98$
	p	0.292	0.031

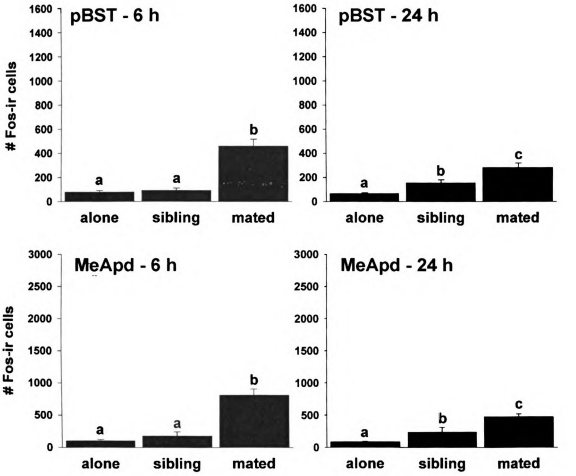

Figure 3.7. Number of Fos-ir cells (Mean ± SEM) in four sections through the pBST and seven sections through the MeApd after male subjects mated, interacted with a familiar female sibling, or remained alone for 6 or 24 h. Note that the Y-axis scales for both sites are the same as that in Figure 3.1. Bars with any of the same letters above them do not significantly differ from each other.

Fos expression within TH-ir cells

The percentage of TH-ir cells in the pBST and MeApd that also contained Fos immunoreactivity significantly differed among groups after 6-h interactions (Figure 3.8; pBST, $H(2) = 12.77$, $P = 0.002$; MeApd, $H(2) = 10.35$, $P = 0.006$), with MATED males having a significantly greater percentage than that found in ALONE males, although this percentage was very low (~2% of all TH-ir cells).

A similar pattern of Fos expression within TH-ir cells was seen after 24 h interactions with the stimuli. The percentage of TH-ir cells expressing Fos differed among all groups in the pBST and MeApd (Figure 3.8; pBST, $H(2) = 6.40$, $P = 0.041$; MeApd, $H(2) = 7.17$, $P = 0.028$), but was very low (less than 2% in all groups).

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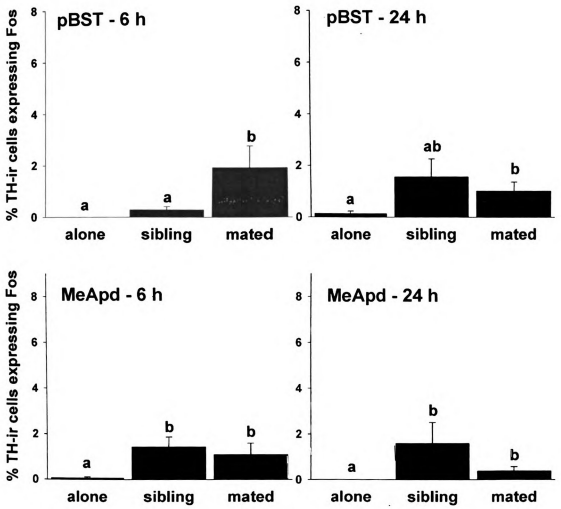

Figure 3.8. Percentage of TH-ir cells also expressing Fos (Mean ± SEM) in the pBST and MeApd after male subjects mated, interacted with a familiar female sibling, or remained alone for 6 or 24 h. Note that the Y-axis scales for both sites are the same as that in Figure 3.2. Bars with any of the same letters above them do not significantly differ from each other.

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Discussion

IEG expression after sociosexual interactions

The pBST and MeApd are necessary for sexual and parental behaviors in many rodents (reviewed in Newman 1999; Numan and Insel 2003). Consistent with this, and similar to other studies of male prairie voles (Cushing et al., 2003; Kirkpatrick et al., 1994b; Lim and Young 2004), there were more cells expressing IEGs in these brain sites after male prairie voles interacted with either a sexually receptive female or pups. These data are the first evidence in prairie voles, though, that mating to ejaculation induces even greater Fos and Egr-1 expression in these sites than do mating bouts that do not result in ejaculation. Similar results have been found in male rats, with Fos expression higher after ejaculation compared to that observed after only intromissions, even when the amount of mating stimulation required for ejaculation is drastically reduced (Baum and Everitt 1992; Coolen et al., 1997; Veening and Coolen 1998). This suggests that some cells in the pBST and MeApd may express IEGs only after males ejaculate and possibly approach sexual satiety.

The effects of social interactions on Fos expression in the two rostral periventricular regions that were examined (AVPV and PVa) were very similar to what was found in the pBST and MeApd. This is similar to findings that Fos expression increases in the AVPV of other rodents after mating, and particularly after ejaculation (Simmons and Yahr, 2002). Because the AVPV receives projections from cells in the MeApd that also express Fos after ejaculation (Simmons and Yahr, 2002), the AVPV and possibly the PVa may work with other sites to regulate physiological or behavioral events occurring after ejaculation, such as hormone secretion or the sexual refractory period. Of

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the forebrain sites examined, only in the pBST, MeApd, AVPV, and PVa did mating to ejaculation increase the number of cells expressing Fos above and beyond mating without ejaculation, so these sites may be part of a neural network regulating ejaculation-related events in male prairie voles, as is true in other rodents. Because mating greatly facilitates partner preference formation in male prairie voles (Insel et al., 1995; Winslow et al., 1993), these four neural sites may be particularly influential in the formation of social bonds with mates.

IEG expression within TH-expressing cells after sociosexual interactions

The behavioral or physiological functions of the hundreds of TH-ir cells in the male prairie vole pBST and MeApd are unknown. Because cells containing immunocytochemically detectable levels of TH are few or non-existent in the pBST and MeApd of the non-monogamous species that have been examined (including meadow voles), these cells were hypothesized to contribute to the characteristic social behaviors of prairie voles (Northcutt et al., 2007). These data support this hypothesis, although they further suggest that these cells may have a somewhat non-specific role in their sociality. Interaction with any social partner maintained at least baseline levels of Fos or Egr-1 within the TH-ir cells of the pBST and MeApd. In fact, social isolation (ALONE group) caused a significant decrease in the number of TH-ir cells of these sites expressing IEGs compared to that found in COLONY and SIBLING controls. These results highlight the importance of including a group of completely unmanipulated COLONY controls in studies such as these, because this group allowed us to discover the constitutive Fos expression in TH-ir cells of the pBST and MeApd of socially-housed prairie voles.

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Unexpectedly high constitutive expression of IEGs within TH-ir cells has also been found in the male zebra finch brain, although in zebra finches the number of dual-labeled cells decreases after social interactions rather than being maintained or increasing (Bharati and Goodson, 2006). A similar example of high constitutive expression of IEGs is provided by the visual cortex, where expression of these transcription factors decreases after adaptation to the dark (Worley et al., 1991). The pBST and MeApd were the only brain sites examined where both COLONY and SIBLING males differed from ALONE males in Fos expression within TH-ir cells, which highlights these regions as possibly unique among brain sites containing TH-ir cells. These cells may be part of a neural network monitoring the ongoing status of a vole's social environment; their decreased activity resulting from even a few hours of social isolation may increase the motivation to seek out a social partner, or if unsuccessful, contribute to the stressful effects of social isolation in this highly gregarious species (Grippo et al., 2007; Kim and Kirkpatrick 1996; Klein et al., 1997).

While any social contact maintained at least baseline numbers of TH-ir cells of the pBST and MeApd expressing IEGs, mating to ejaculation was particularly effective in inducing further IEG expression in additional cells. The pBST and MeApd were the only forebrain sites examined where EJAC males had more TH-ir cells expressing Fos than NON EJAC males. As noted above, EJAC males had more Fos-ir cells in the AVPV and PVa than did NON EJAC males, but not specifically within TH-ir cells. Thus, of the forebrain DA-rich sites examined, IEG expression preceding or resulting from ejaculation was specific to the TH-ir cells of the pBST and MeApd. Furthermore, the percentage of TH-ir cells expressing Fos in both regions was positively correlated with males' sexual

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behaviors, but not males' paternal behaviors or total duration of social contact. In other rodents, the pBST and MeApd receive olfactory information from both the main and accessory olfactory systems, and process this information in a manner that promotes the appropriate expression of sociosexual behaviors (*e.g.* Newman 1999; Numan and Insel 2003). TH-ir cells in these sites might help males establish olfactory-based memory of mates, which is necessary for the formation and maintenance of partner preferences (Curtis et al., 2001; Kirkpatrick et al., 1994c).

Mating bouts extending up to 24 h are typically necessary for establishing social bonds in prairie voles (Insel et al., 1995; Winslow et al., 1993). Because of this, prolonged sexual interactions were hypothesized to be even more effective than 30-min mating bouts in eliciting Fos expression in TH-ir cells of the pBST and MeApd. This was not the case, as males mating for 6 or 24 h had even less Fos expression in TH-ir cells than did males sacrificed after a 30-min bout (although MATED males did have a greater percentage of TH-ir cells expressing Fos than did ALONE males at both extended time points). Thus, mating elicits Fos expression in these TH-ir cells soon after initial mating and ejaculation, but does not appear to produce chronic or repeated waves of Fos expression, although it remains possible this might occur at times points that were not investigated. TH-ir cells in the pBST and MeApd may be involved in males' display of copulatory behaviors and initiating the neural cascades that lead to the development of social bonds after males ejaculate. Additional mating interactions over the following 24 h may instead have a greater impact on downstream targets in this pathway, such as the ACB and ventral pallidum, which may be involved in the solidification, rewarding qualities, and later expression of the social bond (Lim and Young, 2004).

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Ejaculating males had the greatest percentage of TH-ir cells in the pBST and MeApd expressing IEGs, but it is clear that only a relatively small percentage of all TH-ir cells in either site was dual-labeled. This is completely consistent with studies in birds and rats investigating neural Fos expression after mating, which report that fewer than 5%–10% of TH-ir cells in any site also express Fos (Balfour et al., 2004; Bharati and Goodson 2006; Charlier et al., 2005). In addition, it is clear that the majority of IEG-expressing cells in the pBST and MeApd of ejaculating males do not synthesize TH and are not DAergic. Therefore, many neurons in the pBST and MeApd of yet unknown phenotype are also modulated in response to sociosexual interactions in male prairie voles. If prairie voles are similar to gerbils, these other cells are probably glutamatergic or GABAergic (Simmons and Yahr, 2003).

DA and prairie vole sociosexual behaviors

These data demonstrate that mating to ejaculation is more potent than some other social stimuli in eliciting IEG expression in TH-ir cells of the prairie vole olfactory extended amygdala. In addition to being convenient markers for cellular modulation including depolarization (Hoffman and Lyo 2002; Morgan and Curran 1991), both Fos and Egr-1 can induce TH transcription, which may restore DA stores after cells have been active (Ghee et al., 1998; Guo et al., 1998; Nakashima et al., 2003; Stefano et al., 2006). It is possible these cells contribute to DAergic regulation of pair bonding, as D2 receptor activation is necessary for inducing partner preferences (Aragona et al., 2006). In addition, changes in DA release from these cells may help upregulate D1 receptors in the ACB and elsewhere after pair bond formation to prevent the formation of extra-pair

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bonds (Aragona et al., 2006). DAergic neurons in the VTA may be the predominant source of DA release in the ACB during mating (Curtis and Wang, 2005), but very little Fos expression was found in either TH-ir or non-TH-ir cells of the VTA (or the substantia nigra; data not reported) in any group of males. This is similar to what is found in the VTA after lactating female rats display maternal behaviors (Lonstein and Stern, 1997), and indicates that not all DAergic cells involved in behavioral displays show increased IEG expression. Ascending projections from the VTA to the ACB are surely involved in DAergic control of social behaviors in prairie voles, but it is intriguing to consider that that TH-ir cells in the pBST and MeApd may also project to the ACB and other sites (e.g. lateral septum, ventral pallidum, MPO, each other) to modulate sociosexual behaviors in prairie voles.

These studies provide initial insight into how TH-ir cells of the male prairie vole pBST and MeApd are influenced by, and might influence, social interactions. DA affects olfactory processing and increases olfactory discrimination in rats, mice, and primates (Miwa et al., 2004; Pavlis et al., 2006; Tillerson et al., 2006; Yue et al., 2004). Furthermore, DA is involved in learning about olfactory stimuli (Kruzich and Grandy 2004; Rosenkranz and Grace 2002; Weldon et al., 1982), including learning about socially-relevant odors (Cornwell-Jones and Bollers, 1983). Thus, a system of DAergic cells existing within the male prairie vole pBST and MeApd may be a unique neural mechanism through which these animals process socially relevant olfactory information, and then transmit this information to DA-sensitive areas of the brain necessary for social bonding. These could include sites essential for memory, reward, and emotional regulation. Current studies in the laboratory are examining the anatomical projections of

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TH-ir cells of the male prairie vole pBST and MeApd, which will determine if there is an anatomical basis to support this hypothesis.

Acknowledgments

We would like to thank Drs. Lyn Clemens and Tony Nunez for the use of their electronic video recording equipment, and Dr. Juli Wade for providing the Egr-1 primary antiserum. This research was supported by NSF grant # 0515070 to J.S.L., and an NSF graduate research fellowship to K.V.N.

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CHAPTER 4:

Unique catecholaminergic projections from the extended olfactory amygdala to the medial preoptic area in male prairie voles

ABSTRACT

The principal nucleus of the bed nucleus of the stria terminalis (pBST) and posterodorsal medial amygdala (MeApd) are densely interconnected sites that transmit olfactory information via parallel projections to many brain areas mediating sociosexual behaviors in mammals. In male prairie voles (*Microtus ochrogaster*), the pBST and MeApd each contain hundreds of cells intensely immunoreactive for tyrosine hydroxylase (TH). These large populations of TH-immunoreactive (TH-ir) cells do not exist in other rodents examined, and studies from the laboratory suggest these cells could be part of a unique catecholaminergic network necessary for mating and pair bonding in monogamous prairie voles. To gain information about how TH-ir cells in the prairie vole pBST and MeApd communicate with other brain areas involved in social behaviors, and with each other, anterograde tracing from the pBST was performed using biotinylated dextran amine (BDA) and analyzed where these pBST projections overlapped with TH-ir fibers. Only in the medial preoptic area (MPO) and MeApd did dense plexuses of BDA-labeled fibers co-exist with TH-ir fibers. To examine whether these sites receive input from TH-ir cells of the pBST, the retrograde tracer FluoroGold was infused into the MPO or MeApd, and the location of cells throughout the brain containing both FG-ir and TH-ir determined. Approximately 77% of all TH-ir projections to the MPO originated from the pBST or MeApd, and these projections involved over 40% of all TH-ir cells in the latter

two sites. The remaining TH-ir projections to the MPO originated in the supramammillary nucleus, arcuate nucleus, anteroventral periventricular nucleus, and posterior hypothalamus. In contrast, the MeApd received almost no input from TH-ir cells in the pBST, and instead received most of its TH-ir input from the ventral tegmental area (VTA). Retrograde tracing from the pBST itself revealed substantial input from TH-ir cells of the MeApd, and some additional input from TH-ir cells in the VTA and caudal periventricular hypothalamus. Thus, the male prairie vole brain contains a unique catecholaminergic network involving the pBST, MeApd, and MPO. This species-specific catecholaminergic network connecting brain sites involved in olfaction, social behaviors, and motivation (through input from the VTA) may be essential for the monogamous behaviors of prairie voles.

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Introduction

The principal nucleus of the bed nucleus of the stria terminalis (pBST) and posterodorsal medial amygdala (MeApd) are highly interconnected brain sites processing chemosensory and hormonal information necessary for sociosexual behaviors in a number of mammalian species (Coolen and Wood, 1998; Kling, 1972; Newman, 1999; Numan and Insel, 2003; Wood and Swann, 2005). The chemical phenotype of neurons in the pBST and MeApd is heterogeneous, and includes cells expressing GABA, glutamate, vasopressin, cholecystokinin, galanin, opioids, and substance P (De Vries et al., 1984; DeVries et al., 1985; Malsbury and McKay, 1989; Miller et al., 1993; Muganini and Oertel, 1985; Poulin et al., 2009; Simerly and Swanson, 1987; Simmons and Yahr, 2003; Swann and Newman, 1992). In male prairie voles (*Microtus ochrogaster*), the pBST and MeApd also contain hundreds of neurons intensely immunoreactive for tyrosine hydroxylase (TH), and because these TH-immunoreactive (TH-ir) cells do not contain dopamine-β-hydroxylase (Northcutt et al., 2007), they could be dopaminergic (DAergic). These large populations of densely immunoreactive TH-ir cells do not exist in the pBST and MeApd of any non-monogamous rodents examined to date (Albanese et al., 1986; Kalsbeek et al., 1992; Northcutt et al., 2007), and this species difference has led us to suggest that these cells may influence species-specific social behaviors in prairie voles, including high gregariousness, pair bonding, and biparental care (Northcutt and Lonstein, 2009; Northcutt et al., 2007).

Recently, social interaction with familiar conspecifics was found to maintain immediate-early gene (IEG) expression in TH-ir cells of the male prairie vole pBST and MeApd, and that the number of cells expressing IEGs is even further increased by mating

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and ejaculation (Northcutt and Lonstein, 2009). These results suggest that TH-ir cells in the pBST and MeApd may be part of the neural networks required for high levels of affiliation in prairie voles, as well as for mating and its consolidation of pair bonds. Although IEG experiments provide insight into the possible function of these cells, the absence of anatomical information about this system's placement within the brain's social behavior network prevents a more comprehensive understanding of their potential roles (Young et al., 2008; Young et al., 2005).

The anatomical projections of the pBST and MeApd have been well studied in laboratory rats, hamsters, and mice. They have tremendous reciprocal projections to each other and a very similar termination pattern throughout the rest of the brain (Coolen and Wood, 1998; Dong and Swanson, 2004; Gomez and Newman, 1992; Gu et al., 2003; Usunoff et al., 2009; Wood and Swann, 2005). In the forebrain, both sites have prominent projections to the medial preoptic area (MPO), medial hypothalamus, lateral septum (LS), and locally within the BST and amygdala. The MPO and LS are implicated in social behaviors in many rodents (Clarke and File, 1982; Heimovics et al., 2009; Korzan et al., 2006; Sheehan et al., 2004), including prairie voles; IEG expression in the MPO increases dramatically after male prairie voles mate or interact with pups (Lim and Young, 2004) and the LS is involved in their pair bonding and parenting (Liu et al., 2001). The TH-ir cells in the prairie vole pBST and MeApd may provide a unique catecholaminergic input to the MPO or LS, and may also contribute novel projections to areas of the social behavior network that receive no or only sparse innervation from the pBST and MeApd in other rodents. One intriguing possibility is that these cells terminate

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in the nucleus accumbens (ACB), where DA release and D2 receptor activity is required for pair bonding in male prairie voles (Aragona et al., 2003; Aragona et al., 2006).

In the experiments presented here, biotinylated dextran amine (BDA) was used to first determine the efferent projections of the male prairie vole pBST, and visually compare those projections with the distribution of TH-ir fibers in their brain. The pBST was chosen for anterograde tracing because in other rodents it projects to all of the areas innervated by the MeApd, but has some additional projections, including to the ACB and VTA (Wood and Swann, 2005). Sites receiving input from TH-ir cells were expected to have a moderate to extremely dense plexus of BDA-labeled fibers because of the hundreds of TH-ir cells located in the pBST (Northcutt et al., 2007). Two sites (the MPO and MeApd) were found to have greatly overlapping BDA-labeled fibers and TH-ir fibers, so a second experiment investigated whether TH-ir neurons of the pBST specifically projected to these sites. To accomplish this, the retrograde tracer FluoroGold (FG) was injected into the MPO or MeApd in separate groups of males and neurons in the pBST and elsewhere in the brain that contained both TH-ir and FG-ir were identified. Lastly, because the rat, hamster, and mouse pBST and MeApd are so densely interconnected (Coolen and Wood, 1998; Dong and Swanson, 2004; Gomez and Newman, 1992; Gu et al., 2003; Usunoff et al., 2009; Wood and Swann, 2005), retrograde tracing was used to determine if the MeApd sends TH-ir input to the pBST. These results demonstrate a unique catecholaminergic network among the pBST, MeApd, MPO, and VTA that is likely essential for the monogamous social behaviors of this species.

Experiment 1: Anterograde projections of the prairie vole pBST

Methods

Subjects

Subjects were adult (90-140 day old) male prairie voles (*Microtus ochrogaster*) born and raised in the colony at Michigan State University. The colony originated with voles captured in Urbana, IL and animals were last outbred in 2000 with voles from Illinois. The colony was maintained on a 14:10 light:dark cycle with an ambient temperature of 21°C. After weaning at 20 days of age, subjects were housed with male and female littermates in clear plastic cages (48 x 28 x 16 cm) containing wood chips, wood shavings, and a covering of hay. Water and a food mixture containing cracked corn, whole oats, sunflower seeds, and rabbit chow (Teklad rodent diet No. 2031; Harlan, Madison, WI) in a 1:1:2:2 ratio were provided *ad libitum*. All procedures were in accordance with the Institutional Animal Care and Use Committees at Michigan State University.

Stereotaxic surgery and tissue collection

Subjects (n = 19) were anesthetized with an i.p. injection of a mixture of ketamine (44 mg/kg; Butler Co., Dublin, OH), xylazine (5 mg/kg; Butler), and acepromazine (0.6 mg/kg; Butler). Biotinylated dextran amine (BDA; 40 nl of 10% solution in dH2O; Invitrogen, Carlsbad, CA) was slowly injected over the course of 10 min into the right pBST (A/P +0.3 mm, M/L -0.9 mm, D/V -5.0 mm) via a 0.5 µl Hamilton syringe.

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Tissue collection and immunohistochemistry

Seven days after surgery, males were overdosed with the anesthetic mixture (see above), and perfused through the heart with 100 ml 0.9% saline, followed by 100 ml 4% paraformaldehyde in 0.1 M sodium phosphate buffer (NaPB; pH = 7.6). Brains were removed and postfixed overnight in 4% paraformaldehyde, and then submerged in 20% sucrose in NaPB for at least two days before being sectioned into 35 μm sections on a freezing microtome. During sectioning, a small hole was made in the cortex on the side contralateral to the injection to help maintain section orientation during tissue histology and microscope analysis.

BDA histochemistry and tyrosine hydroxylase (TH) immunohistochemistry were performed on every third section throughout the brain. On the first day of processing, sections were rinsed three times with 0.1 M NaPB (pH = 7.4) between incubations. First, sections were incubated in 1% hydrogen peroxide in NaPB for 10 min, followed by 1 h in 0.5% Triton X-100 in NaPB. Then, sections were incubated in avidin-biotin complex (1:250; Vectastain Elite; Vector Laboratories, Burlingame, CA) and 0.5% Triton X-100 in NaPB for 1 h. BDA-labeled cells and fibers were visualized by incubating sections in Ni-enhanced 3,3'-diaminobenzidine (Sigma; 0.05% DAB and 0.4% nickel ammonium sulfate) and 0.01% hydrogen peroxide in Tris-buffered saline (TBS; pH = 7.6), which produced a dark blue reaction product in cell bodies at the injection site and in processes of these cells. From this point on, sections were rinsed in TBS between incubations. Sections were blocked with 20% normal goat serum (NGS) and 0.3% Triton X-100 in TBS for 30 min, and were incubated for 18 h in mouse anti-TH primary monoclonal

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antiserum (1:2000; cat.# MAB318; Chemicon, Temecula, CA) in 2% NGS and 0.3% Triton X-100 in TBS at room temperature. Next, sections were incubated in a goat anti-mouse secondary antiserum (1:500; Vector Laboratories) in 2% NGS and 0.3% Triton X-100 in TBS for 1 h, followed by 1 h in avidin-biotin complex in TBS. TH was visualized with a solution of hydrogen peroxide (0.09%) and DAB (0.02%) in TBS, which produced a light brown cytoplasmic label. Omission of the primary or secondary antisera abolished specific labeling. After tissue processing, sections were mounted onto slides, dehydrated, and coverslipped.

BDA Microscope Analysis

Seven subjects were chosen for analysis because their BDA injection included most or all of the region where TH-ir cells are found in the prairie vole pBST, but did not extend far beyond this region. The entire forebrain and midbrain of these subjects were examined for the presence of BDA-labeled fibers, which were mapped onto images from an atlas of the laboratory rat brain (Swanson, 1998); an atlas of the vole brain does not exist. Dense collections of parallel fibers were assumed to be fibers of passage, and are not reported in the results. The location of TH-ir fibers on the contralateral side of the brain was compared to the location of BDA-labeled fibers on the injected side to determine the areas most likely to receive TH-ir input from the pBST.

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Based on the pattern of somata labeled with BDA, seven subjects had injections that covered almost all of the area of the pBST where TH-ir cells are found (Figure 4.1). In two subjects (1, 2), no cells containing only TH-immunoreactivity were visible, indicating that the BDA injection covered every TH-ir cell in that region. In the remaining five subjects (3-7), a small number (4-20 cells) of TH-ir cells in the pBST were still visible that did not contain BDA labeling. The pBST was the center of the injection site in all cases, but there was minimal spread to the anterodorsal BST (adBST) in all subjects. Additionally, some BDA was found in the transverse nucleus of the BST (trBST) of five subjects (1, 2, 4, 6, 7), the interfascicular nucleus of the BST (ifBST) of four subjects (1, 2, 4, 7), the reticular thalamus (RT) of one subject (2), and in very small portions of the anterolateral (avBST), anteroventral (alBST), and rhomboid (rhBST) nuclei of the BST of one subject (4). Projections are detailed below in rostrocaudal order.

Projections to the lateral septum

The projections of the pBST were almost exclusively ipsilateral to the injection site, with the few exceptions noted below. pBST projections to the LS were primarily ventral (LSv; Figure 4.2). However, some labeled fibers were also seen in the ventral part of the rostral LS (LSr.v), and a few fibers were found bilaterally in the dorsal LSr (LSr.d) and the ventrocaudal LS (LSc.v) in every subject. In all subjects, the dorsal LSc contained few or no BDA-labeled fibers.

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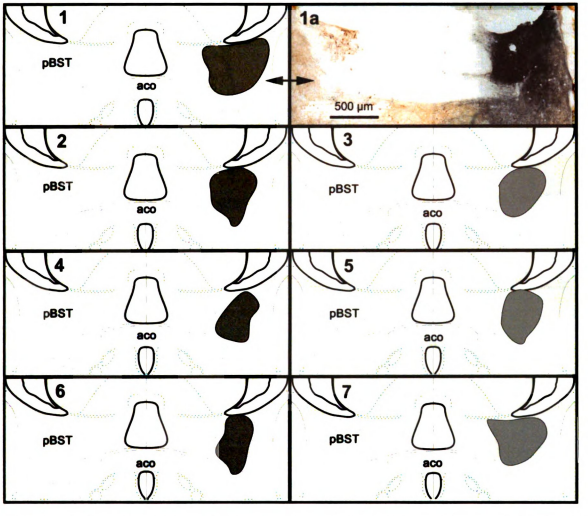


Figure 4.1. Representation of BDA injection sites (gray shading) in the pBST for the seven subjects (1-7) mapped onto atlas plate 21 from Swanson's rat brain atlas (Swanson, 1998), and a photomicrograph of the BDA injection site (dark blue staining on right) compared to TH-ir cell distribution (brown staining on left) of subject 1 (1a).

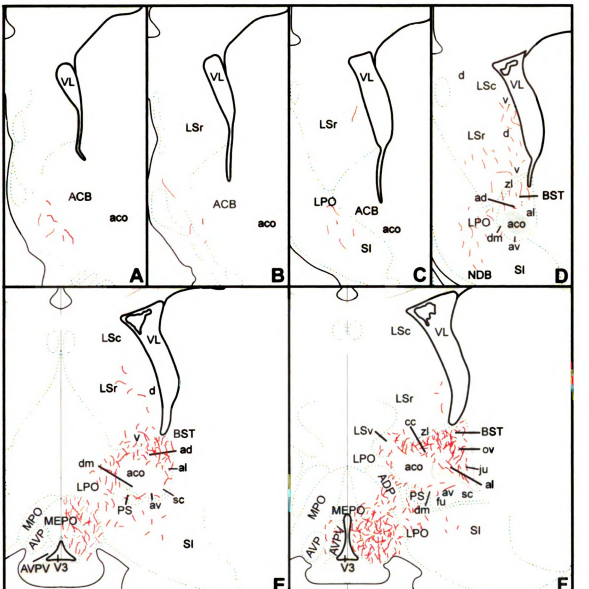
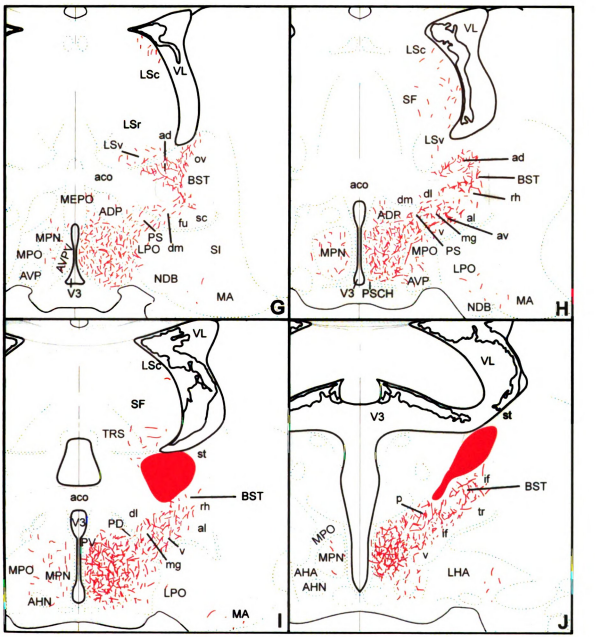


Figure 4.2. Relative distribution of BDA-labeled fibers throughout the forebrain in one representative subject receiving a BDA injection into the pBST, mapped onto plates 13-37 (A-Y) of Swanson's rat brain atlas (Swanson, 1998).

Figure 4.2 (cont'd)



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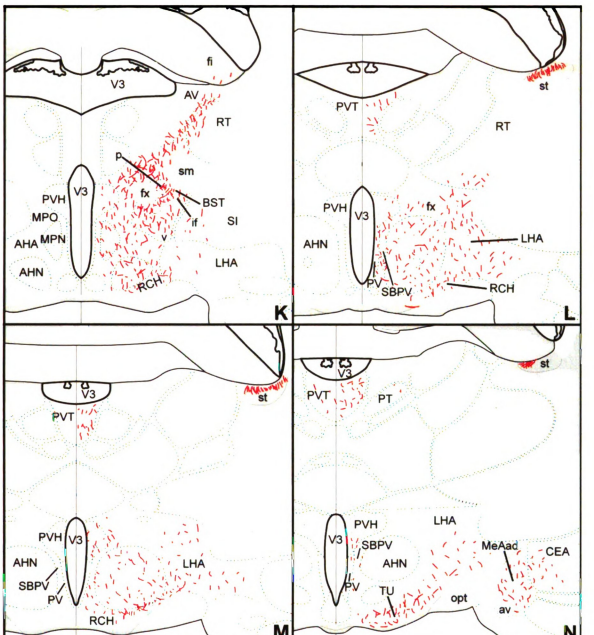

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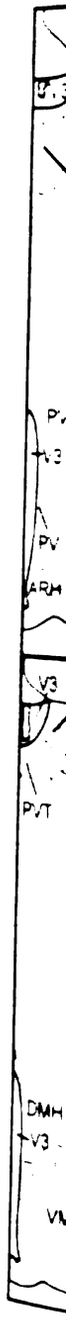

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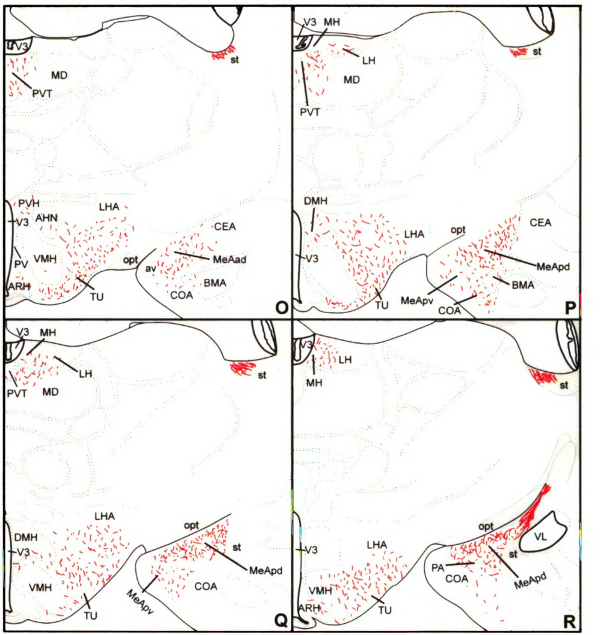

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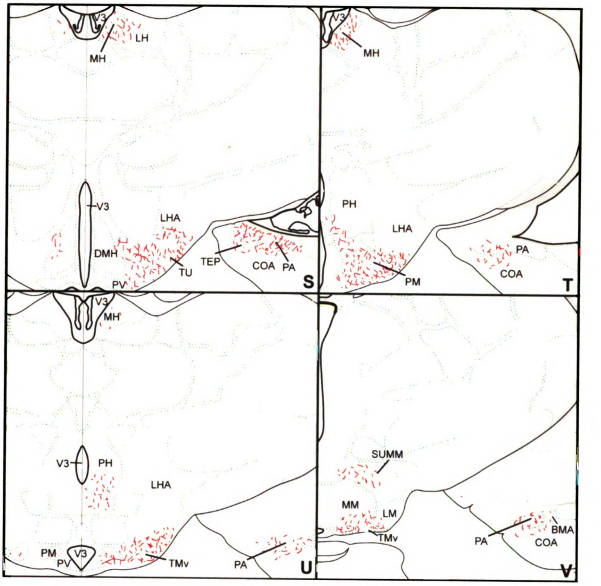

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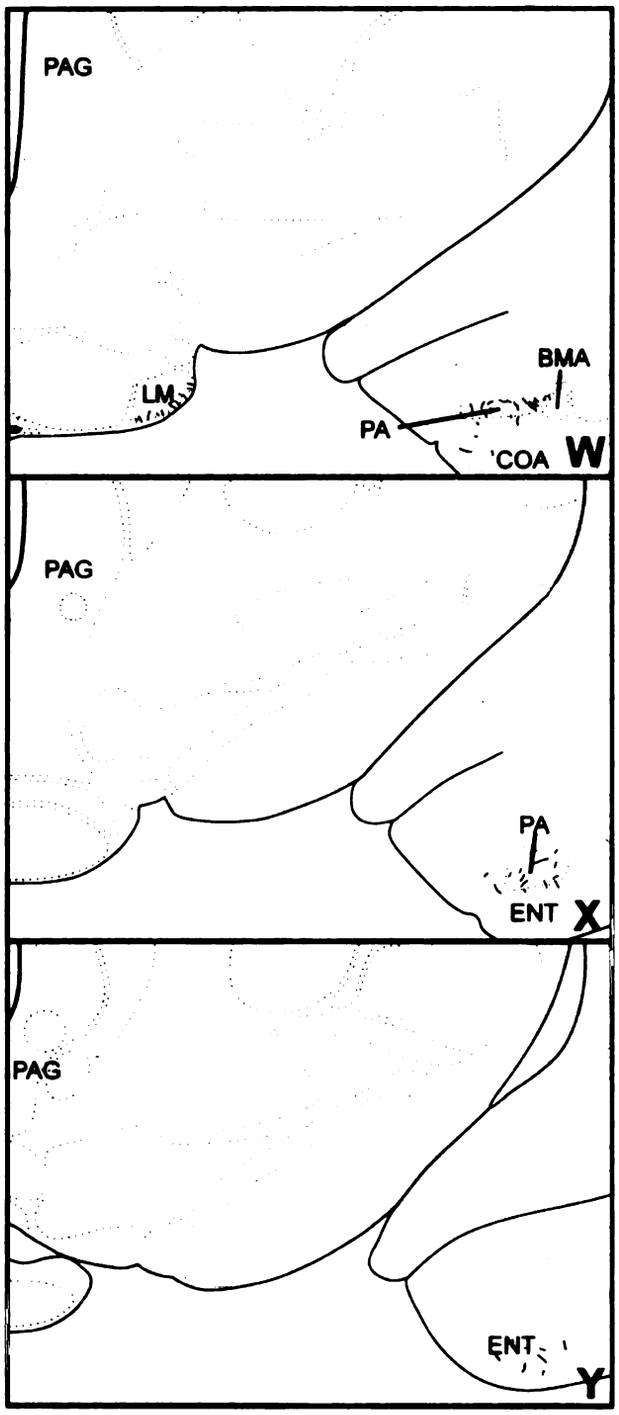

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Projections to the preoptic area and hypothalamus

The pBST provided very dense projections to the ipsilateral MPO and anteroventral periventricular nucleus (AVPV) in every subject, with some minor projections contralaterally to both sites (Figure 4.3). Hypothalamic regions receiving dense innervation from the pBST included the lateral hypothalamus (LHA), paraventricular hypothalamus (PVH), anterior hypothalamus (AHN), and tuberal nucleus (TU). The lateral preoptic area (LPO), arcuate nucleus (ARH), dorsomedial nucleus (DMH), ventromedial nucleus (VMH), and posterior hypothalamus (PH) contained some sparse labeling in all subjects. No labeled fibers were found in the supraoptic or suprachiasmatic nuclei of the hypothalamus.

Projections to other regions of the BST

The pBST also densely projected to subregions within the anterior BST, including the anterodorsal (adBST), anterolateral (alBST), anteroventral (avBST), rhomboid (rhBST), dorsolateral (dlBST), magnocellular (mgBST), and ventral (vBST) divisions. A few labeled fibers were found in all subjects in the dorsomedial (dmBST), fusiform (fuBST), and oval (ovBST) nuclei, as well as in the subcommissural zone (scBST), but none were found in the juxtacapsular nucleus (juBST) of any subject. Extensive labeling was observed throughout the entire posterior BST of all subjects, including the ventral portion of the pBST that was outside the injection site.

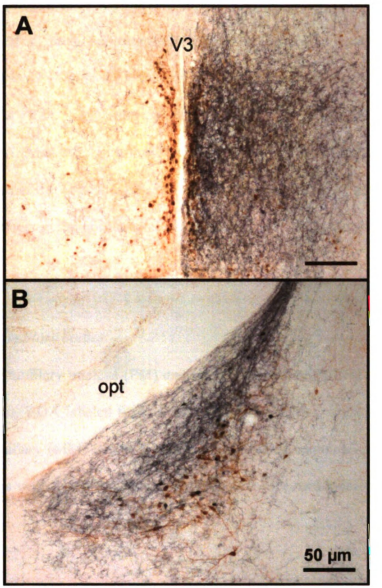

Figure 4.3. Photomicrograph showing BDA-labeled fibers (dark blue staining) and TH-ir (brown staining) in the MPO (A) and MeApd (B). Both regions received dense innervation from the BST that overlapped with the distribution of TH-ir fibers.

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Projections to the amygdala

The pBST of all seven subjects projected densely to the amygdala (Figure 4.2), particularly the MeApd (Figure 4.3), but also to the anterodorsal MeA (MeAad) and posterior amygdala (PA). All subjects also had some BDA-labeled fibers in the posteroventral MeA (MeApv). In a few subjects (1-4), fibers extended into the central and medial portions of the central nucleus of the amygdala (CEA), and most subjects (1-4, 7) had labeled fibers in the cortical and basomedial nuclei of the amygdala (COA and BMA).

Projections to mammillary bodies

The premammillary nucleus (PM) and ventral tuberomammillary nucleus (TMv) each contained many BDA-labeled fibers in every subject. The supramammillary (SUM) and medial mammillary (MM) nuclei of a few subjects (2-5) contained labeling, but no BDA-labeled fibers were found in any other subdivision of the mammillary bodies.

Other projections

A moderate number of BDA-labeled fibers were found in the nucleus accumbens (ACB) of only two subjects (1, 3), primarily in the medial portion of the shell. Two other subjects (2, 7) had only sparse BDA-containing fibers in the ACB, and BDA-labeled fibers were non-existent in the ACB of the remaining three voles. There was a sparse to moderate projection to the substantia innominata (SI) in all subjects. In the ventral pallidum (VP) region of the SI, two subjects (1, 7) had a moderate number of BDA-labeled fibers, four had a few labeled fibers (2-5), and one subject had none (6). Finally,

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the paraventricular thalamus (PVT) and medial and lateral habenula (MH and LH) had many BDA-labeled fibers in all subjects, and these fibers occasionally extended into the mediodorsal thalamus (MD); a few labeled fibers were seen in the contralateral PVT.

Overlap between BDA-labeled fibers and TH-ir fibers

Two areas, the MeApd and MPO/AVPV region, stood out as receiving very dense projections from the pBST and also containing many TH-ir fibers. Other areas of the amygdala, BST, and hypothalamus/preoptic region receiving input from the pBST had very few or no TH-ir fibers and, thus, may be unlikely to receive meaningful input from TH-ir cells in the pBST. Although the LS contained a dense plexus of TH-ir fibers, these were found in the dorsal portion of the caudal LS (LScd), while BDA-labeled fibers coming from the pBST existed ventrally (Figure 4.4). The ACB contained a tremendous number of TH-ir fibers, but the number of BDA-labeled fibers was very sparse or non-existent in most subjects. Finally, some subjects had a sparse to moderate number of BDA-labeled fibers in the VP, but there was very little overlap with the few TH-ir fibers in this region.

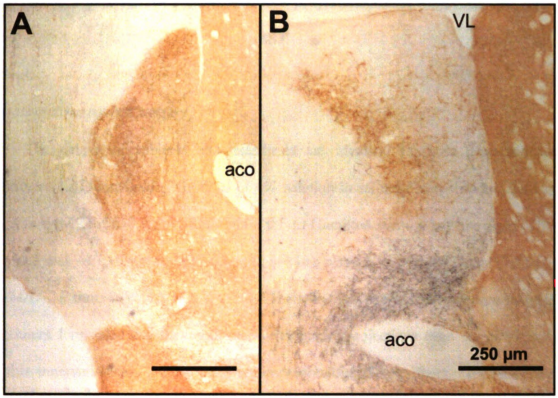

Figure 4.4. Photomicrograph showing BDA-labeled fibers (dark blue staining) and TH-ir (brown staining) in the ACB (A) and LS (B). The ACB contained a dense plexus of TH-ir fibers, but few BDA-labeled fibers. In the LS, BDA-labeled fibers were primarily found in the LSv and LSr subregions, and did not overlap with TH-ir fibers in the LSc (B).

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Experiment 2: Retrograde analysis of TH-ir projections to the MPO, MeApd, and pBST

Methods

Subjects and stereotaxic surgery

The details of subjects and anesthesia are identical to those described for Experiment 1. FluoroGold (FG; 40 nl of 4% solution in saline; Fluorochrome, Denver, CO) was slowly infused over 10 min via a 0.5 µl Hamilton syringe into the right MPO (A/P +0.7 mm, M/L -0.4 mm, D/V -6.1 mm; n = 10), pBST (n = 14), or MeApd (A/P -0.9 mm, M/L -2.7 mm, D/V -6.1 mm; n = 10). The MPO and MeApd were chosen because Experiment 1 revealed that they both receive extensive projections from the pBST, and that this innervation was in the same regions containing TH-ir fibers. Based on the results of tracing studies in other rodents demonstrating that the pBST receives the majority of output from the MeApd (Coolen and Wood, 1998; Gomez and Newman, 1992), possibility that the pBST receives input from TH-ir cells in the MeApd was also investigated.

Tissue collection and immunohistochemistry

Brains were collected, postfixed, and sectioned similar to that described in Experiment 1. FG and TH immunohistochemistry was performed on every other section throughout the brain similar to previous work from the laboratory (Miller and Lonstein, 2009). Sections were rinsed with TBS between each incubation. First, sections were incubated with 0.1% sodium borohydride in TBS for 15 min, 1% hydrogen peroxide and 0.3% Triton X-100 in TBS for 10 min, and 20% NGS and 0.3% Triton X-100 in TBS for

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30 min. Sections were then incubated for 18 h in a rabbit anti-FG polyclonal antiserum (1:3000; cat.# AB153; Chemicon, Temecula, CA) in 2% NGS and 0.3% Triton X-100 in TBS at room temperature. Next, sections were incubated with a goat anti-rabbit secondary antiserum (1:500; Vector Laboratories) in 2% NGS and 0.3% Triton X-100 in TBS for 1 h, and avidin-biotin complex in TBS for 1 h. FG was visualized with a solution of hydrogen peroxide (0.09%) and DAB (0.02%) in TBS, which produced brown puncta in the cytoplasm. Sections were then blocked and incubated in anti-TH primary antiserum, goat anti-mouse secondary antiserum, and avidin-biotin complex as described in Experiment 1. TH was visualized with Vector SG (Vector Laboratories), which produced a dark blue cytoplasmic reaction product. Omission of any of the primary or secondary antisera abolished specific labeling for the antigen. After tissue processing, sections were mounted onto slides, dehydrated, and coverslipped.

FG Microscope Analysis

Five subjects with accurately placed FG injections in the MPO, six with accurately placed injections in the pBST, and four with accurately placed injections in the MeApd were chosen for detailed analysis. In all cases, the injection covered the entire area of interest and did not extend much beyond the intended site. The number of TH-ir cells was counted bilaterally in four sections of the pBST and bilaterally in seven sections of the MeApd, excluding the site of injection, at 200x on a Nikon E400 light microscope. These areas were then examined at 400x, and the number of cells containing both TH- and FG-labeling were counted. Cells were considered double-labeled if they clearly contained both dark-blue (TH) and brown (FG) reaction products. The number and

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percentage of TH-ir cells containing FG-ir was then calculated for each side of the brain in each brain area. Finally, TH-ir cells in other brain sites, including the anteroventral periventricular nucleus (AVPV), periventricular nucleus (PV), zona incerta, arcuate nucleus (ARH), posterior hypothalamus (PH), other hypothalamic regions containing scattered TH-ir cells, supramammillary nucleus (SUM), ventral tegmental area (VTA), substantia nigra, and periaqueductal gray (PAG), were scanned at 400x, and double-labeled cells were counted in each site. To estimate the total number of double-labeled cells in each site, the number of double-labeled cells counted was multiplied by two for each subject, and an Abercrombie correction factor of 0.78 was applied (Abercrombie, 1946). This correction was calculated as has previously been done (Northcutt et al., 2007) using an average somal diameter of 10 μm, which is a conservative estimate given that TH-ir cells across these regions had average diameters of 8-10 μm.

Results

Injection sites

Most injections targeting the pBST or MeApd extended to other nearby subregions, but never included other areas containing TH-ir fibers, such as the lateral portions of the BST (including juBST, alBST, and rhBST) or basolateral amygdala (Asan, 1998; Brummelte and Teuchert-Noodt, 2006; Kozicz, 2001; Phelix et al., 1992; Pinard et al., 2008). Injections extended into adjacent regions of the central amygdala, but no TH-ir fibers were observed on the contralateral side of the brain in the corresponding location as the injection site. Furthermore, there was no spread of FG to the pBST after injection into the MPO or vice versa.

FG injection into MPO

In the five animals receiving accurately placed FG injections in the MPO (Figure 4.5), an average of 204 ± 34 TH-ir cells projected to the MPO, and 77% of these originated in the pBST or MeApd. Many double-labeled cells were found in the ipsilateral pBST (50 ± 10 cells, 38 ± 5% of TH-ir cells in the ipsilateral pBST; Table 4.1; Figures 4.6 and 4.7) and MeApd (107 ± 19 cells, 42 ± 3% of TH-ir cells in the ipsilateral MeApd), although a very small number were also found in the contralateral pBST (3 ± 1 cells, 2 ± 0.5% of TH-ir cells in the contralateral pBST) and MeApd (8 ± 2 cells, 3 ± 0.4% of TH-ir cells in the contralateral MeApd). In all subjects, the remaining TH-ir/FG-ir cells were found bilaterally in the supramammillary nucleus (SUM; 20 ± 7 cells) and arcuate nucleus (ARH; 5 ± 2 cells), and contralaterally in the anteroventral periventricular nucleus (AVPV; 7 ± 1 cells). A small number of double-labeled cells (3 ± 1) were also found in the posterior hypothalamus (PH) of three subjects (2, 4, 5), in the medial mammillary nucleus of one subject (1; nine TH-ir cells), and in the lateral hypothalamus of one subject (3; three TH-ir cells).

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Table 4.1. Estimated number of TH-ir neurons (± SEM) in regions throughout the forebrain that project to the MPO, pBST, or MeApd in all subjects examined (also see Figure 4.7).

	Projecting to:		
TH-ir neuron in:	**MPO**	**pBST**	**MeApd**
pBST	50 ± 10	-	8 ± 4
MeApd	107 ± 19	112 ± 18	-
AVPV (contralateral)	7 ± 1	6 ± 3*	3 ± 2*
ARH	5 ± 2	0.3 ± 0.3*	0
SUM	20 ± 7	0	1 ± 1*
PV (bilateral)	0	11 ± 4	0
VTA	0	40 ± 9	19 ± 5

* indicates that double-labeled cells were not found in that site in every subject, but the number of double-labeled cells was averaged across all subjects (see text for details).

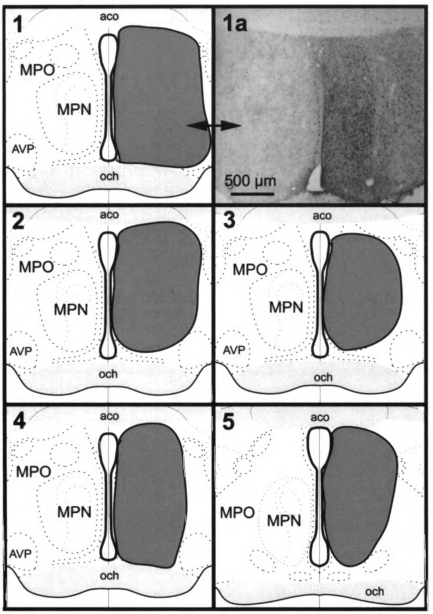

Figure 4.5. Representation of the center of FG injection sites (gray shading) in the MPO for the five subjects mapped onto plates 20 (1-4) and 21 (5) of Swanson's rat brain atlas (Swanson, 1998), and a photomicrograph of the FG injection site (brown staining on right) in subject 1 (1a). TH-ir cells and fibers (blue staining) can also be seen on the left.

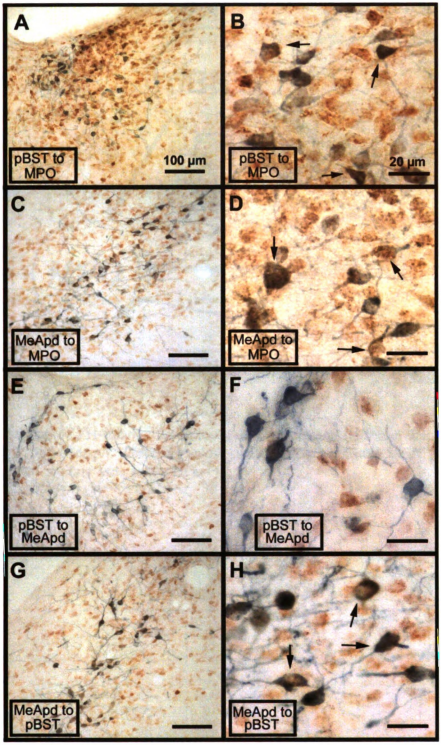

Figure 4.6. Photomicrographs of TH-ir (blue labeling) and FG-ir (brown labeling) cells and fibers in the pBST (panels A, B) and MeApd (panels C, D) in voles with FG injected into the MPO (scale bars = 20 or 100 µm). Some double-labeled cells are indicated by arrows. Photomicrographs of TH-ir cells in the pBST after FG injection in the MeApd (panels E, F), and TH-ir cells in the MeApd after FG injection in pBST (panels G, H) (scale bars = 20 or 100 µm). Note the lack of double-labeled cells in panels E and F.

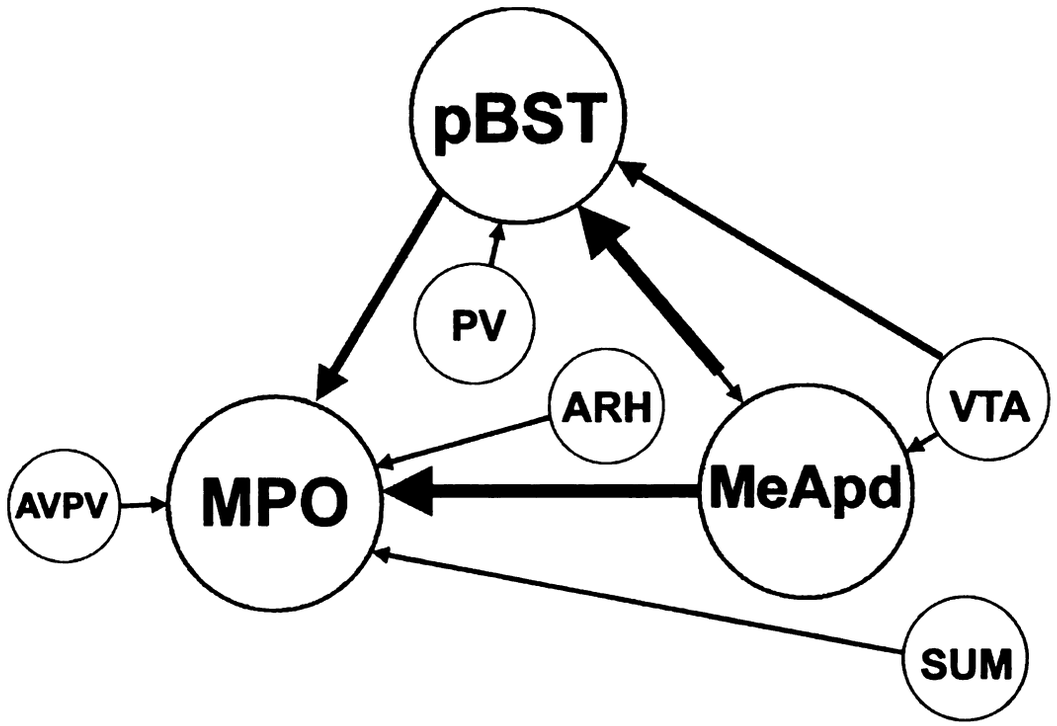

Figure 4.7. Diagram of TH-ir cells throughout the forebrain and midbrain that project to the MPO, pBST, and MeApd. Different arrow sizes represent the number of TH-ir cells connecting the two regions (see Table 4.1).

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In animals receiving accurately placed FG injections in the pBST (Figures 4.8 and 4.9), there were an average of 172 ± 28 TH-ir cells projecting to the pBST, with most found in the ipsilateral MeApd (112 ± 18 double-labeled cells; 49 ± 4% of all TH-ir cells in the ipsilateral MeApd). Similar to what was observed in MPO-injected subjects, a very small number of double-labeled cells were found in the contralateral pBST (1 ± 1 double-labeled cells, 0.6 ± 0.3% of TH-ir cells in the contralateral pBST) or MeApd (12 ± 7 double-labeled cells, 3 ± 2% of TH-ir cells in the contralateral MeApd). Other TH-ir cells containing FG-ir were found for all subjects in the ipsilateral ventral tegmental area (VTA; 40 ± 9 cells) and bilaterally in the caudal periventricular region (PV; 11 ± 4 cells). Four subjects also had some cells containing TH-ir and FG-ir in the periaqueductal gray (PAG; 12 ± 6 cells; subjects 2-5) and in the substantia nigra (SN; 7 ± 2 cells; subjects 1-3, 5). Lastly, three subjects (2, 4, 6) had an average of 6 ± 3 double-labeled cells in the paraventricular nucleus of the hypothalamus (PVH), three other subjects (3, 4, 6) had an average of 11 ± 1 double-labeled cells in the AVPV, and one subject (4) had two double-labeled cells in the ARH.

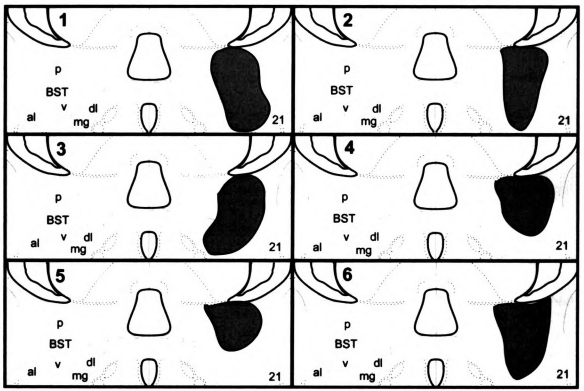

Figure 4.8. Representation of FG injection sites in the pBST (gray shading) for the six subjects (1-6) mapped onto plate 21 of Swanson's rat brain atlas (Swanson, 1998).

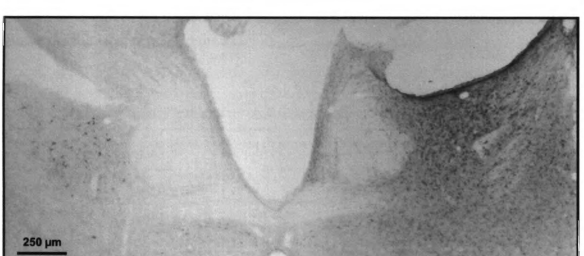

Figure 4.9. Photomicrograph of the FG injection site in the pBST of subject 4 (Figure 4.8) showing FG-labeled cells (brown staining) and TH-ir cell and fiber distribution (blue staining).

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Four subjects received accurately placed FG injections in the MeApd (Figure 4.10). An average of 52 ± 16 TH-ir cells projected to the MeApd, but relatively few double-labeled cells were found in the ipsilateral pBST (8 ± 4 cells, 4.5 ± 1.2% of all TH-ir cells in the ipsilateral pBST) or contralateral MeApd (2 ± 1 cells, 0.5 ± 0.3% of all TH-ir cells in the contralateral MeApd); no double-labeled cells existed in the contralateral pBST. However, there was extensive FG labeling in other pBST cells of unknown phenotype (see Figure 4.6E), indicating that terminals in the MeApd did take up the FG and retrogradely transported it to the soma. Instead of the pBST, all four subjects had more cells containing TH-ir and FG-ir in their ipsilateral VTA (19 ± 5 cells). Furthermore, dual-labeled cells were found bilaterally in the PAG in three of the subjects (21 ± 7 cells; subjects 1-3), bilaterally in the PVH of three subjects (3 ± 1; subjects 1, 3, 4), and in the contralateral AVPV of two subjects (5 ± 2 cells; subjects 1, 3). One subject (4) had five double-labeled cells in the SUM and two in the SN, and another subject (2) had two double-labeled cells in the PH.

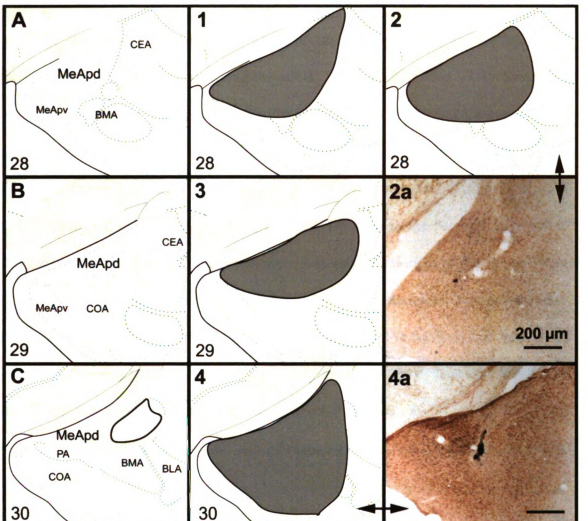

Figure 4.10. Representation of MeA and surrounding regions at plates 28 (A), 29 (B), and 30 (C) of Swanson's brain atlas (Swanson, 1998), and FG injection sites in the MeApd that were centered on corresponding plate (1, 2, 3, 4). 2a and 4a are photomicrographs of the FG injection site (brown staining on right) in subjects 2 and 4. TH-ir cells and fibers (blue staining) can also be seen on the left.

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Discussion

The results of these experiments demonstrate a species-specific network of catecholaminergic projections involving the pBST, MeApd, MPO, and VTA. Similar to projections of the entire pBST and MeApd in other rodents (Dong and Swanson, 2004; Gu et al., 2003; Wood and Swann, 2005), many TH-ir cells in both sites terminated in the MPO. In fact, over 75% of the TH-ir input to the male prairie vole MPO originated in the pBST and MeApd, and almost all (91%) of the TH-ir cells in the MeApd projected to either the pBST or the MPO. In addition, there was substantial input to this system from TH-ir cells of the VTA as well as other areas (posterior hypothalamus, supramammillary nucleus, and ventral PAG) containing TH-ir cells considered to be from the "extended" A10 and A11 groups (Albanese et al., 1986; Hokfelt et al., 1984). This unique network (see Figure 4.7) may process information about olfaction, social recognition, and motivation to regulate the gregarious and monogamous behaviors found in male prairie voles but not most other mammals.

Methodological considerations

In the anterograde tracing experiment, minimal tracer spread to other subregions of the BST adjacent to the pBST was seen, but this was not likely to dramatically affect these results, as only areas containing FG-ir fibers across all subjects were considered as potential targets of TH-ir cells. Furthermore, these results are very similar to what others have reported using other anterograde tracers and injections in various parts of the pBST, with a few exceptions discussed in detail below (Dong and Swanson, 2004; Gu et al., 2003; Wood and Swann, 2005). Previous investigations in rats and hamsters have used

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the anterograde tracer PHA-L, but this was not likely to contribute to subtle differences in projection patterns. PHA-L and BDA typically produce similar patterns of labeled fibers (Raju and Smith, 2006; Reiner et al., 2000; Wouterlood et al., 2002), but BDA does have a slower rate of transport (Raju and Smith, 2006). This is unlikely to have impacted these results, however, because BDA can travel 15-20 mm a week (Renier et al., 2000), which is ample time for it to be transported throughout the prairie vole forebrain and midbrain. Some BDA-labeled cells were found in the LS in most subjects, and it is possible that tracer was deposited in the LS as the syringe was lowered into the pBST. However, brain sites receiving projections from the LS and not the pBST in other rodents, such as CA1 and CA3 subfields of the hippocampus, VTA, anterior cingulate cortex, olfactory tubercle, and PAG (Meibach and Siegel, 1977; Staiger and Nurnberger, 1991; Tsukahara and Yamanouchi, 2001), had minimal or no BDA-labeled fibers in these subjects. Furthermore, BDA-labeled LS cells were widely distributed, suggesting that they were likely labeled through retrograde transport, which does occur to some degree with BDA (Reiner et al., 2000). Spread to the nearby fornix is also not likely, given that there were few or no labeled fibers in the hippocampus. Finally, BDA probably did not spread to the MPO, because no labeled fibers were seen in the lateral parabrachial nucleus, zona incerta, VTA, pedunculopontine nucleus, PAG, or laterodorsal tegmental area, which receive input from the MPO (Finn et al., 1993; Simerly and Swanson, 1988; Swanson et al., 1987). In the retrograde tracing experiment, almost all of the MPO, pBST, or MeApd were included in the injections, but there are very few TH-ir fibers in areas adjacent to the FG injection sites, therefore even if the tracer spread to outlying areas, it is unlikely that it would have been taken up by TH-ir fibers not of interest in the current study.

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These retrograde tracing studies have accounted for much of the output from TH-ir cells of the male prairie vole pBST and MeApd. This is particularly true for the MeApd, with 91% of the TH-ir cells in this site targeting either the pBST or MPO (assuming no collateralization). The termination site(s) of the remaining TH-ir cells in the MeApd is unknown, but would be very minor compared to the dense innervation these cells provide to the pBST or MPO. On the other hand, just under half of the TH-ir cells in the pBST project to either the MPO or MeApd, and the projections of the remaining cells are unknown. No other brain sites contained notable comingling of innervation from the pBST and TH-ir fibers, so it is probably unlikely that one site receives the other half of TH-ir input from the pBST. Instead, an array of brain sites that have not yet identified probably each receive a small amount of input from this population of cells.

Comparison of pBST projections in prairie voles and other rodents

Overall, the pattern of projections from the prairie vole pBST was extremely similar to what has been reported from the pBST of laboratory rats (Dong and Swanson, 2004; Gu et al., 2003; Wood and Swann, 2005) and the homologous BSTpm of hamsters (Wood and Swann, 2005). This included that the densest projections from the pBST terminated in the MPO and MeApd. In fact, there were no projections from the pBST that were unique to prairie voles, and some minor projections reported in other species were not found in prairie voles. For example, the PAG is consistently reported to receive some sparse input from the pBST or BSTpm in rats and hamsters but there were no BDA-labeled fibers in prairie voles (Dong and Swanson, 2004; Gu et al., 2003; Wood and

Swann, 2005). There were also fewer BDA-labeled fibers in the ARH and PV than what has been found in rats and hamsters. These minor differences are likely due to variation among studies in tracer injection site; two previous studies examined projections of the ventral portion of the pBST or BSTpm (Dong and Swanson, 2003; Wood and Swann, 2005), Gu et al. (2003) examined projections from the center of the pBST, and the present study involved primarily the rostral dorsal pBST (where most TH-ir cells are found) (Northcutt et al., 2007). It is possible that the ventral part of the prairie vole pBST does project to the PAG, and more heavily than the rostrodorsal pBST to the ARH and PV.

Wood and Swann (2005) describe differences between the hamster BSTpm and the rat pBST, including more elaborate projections to the ACB and SI, as well as projections to the entire LS, medial septum (MS), nucleus of the diagonal band (NDB), supraoptic nucleus (SON), and VTA that are not found in the rat brain or in the present results. These differences are particularly notable given the small tracer injection site in their experiment. Again, variation in tracer injection site may account for these differences; their injections were caudal and ventral to these and to those of Gu et al. (2003), but were similar to Dong and Swanson's (2004). Wood and Swann (2005) also propose that the hamster BST has only three divisions, as opposed to the four described in rats (Aldheid et al., 1995), because a different division of the posterior BST, the BSTtr, innervates many of these sites in rats. Interestingly, these results suggest that the prairie vole BST is more similar to that of rats than hamsters, as there was no visible innervation of any of these sites, at least from the dorsal portion of the pBST.

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Implications of species-specific catecholaminergic circuit

The finding that many TH-ir cells in the prairie vole pBST and MeApd project to the MPO is particularly interesting because DAergic neurotransmission in the MPOA influences the expression of sociosexual behaviors in other species (Dominguez and Hull, 2005; Miller and Lonstein, 2005). In male rats, DA neurotransmission within the MPO is necessary for consummatory and motivational aspects of male sexual behavior (Dominguez and Hull, 2005; Hull and Dominguez, 2007). Furthermore, DA receptor activity in the MPO is required for maternal behavior in female rats, with D1 receptor antagonism in the MPO impairing retrieval and licking of pups (Miller and Lonstein, 2005), whereas D1 agonists facilitate the onset of these behaviors (Stolzenberg et al., 2007). Previous work in the Lonstein laboratory has described the DAergic projections to the MPO of female rats (Miller and Lonstein, 2009), and found that most of it arises from the posterior hypothalamus (PH), supramammillary bodies (SUM), and VTA (Miller and Lonstein, 2009). Surprisingly, two of these sites, the PH and VTA, did not consistently provide DAergic innervation in male prairie voles. This could be attributed to a species difference, although sex or reproductive state differences could also be responsible. Some TH-ir cells in the SUM did project to the MPO, and the pBST and MeApd both received TH-ir input from the VTA, which may provide the same information that is relayed directly to the MPO in postpartum rats. Of course, because laboratory rats do not have TH-ir cells in their pBST and MeApd, input from these areas to the MPO may be unique to prairie voles. Even so, the rat MeA interacts with other sites to enhance DAergic activity in the MPO necessary for sociosexual behaviors; MeA stimulation increases DA release in the MPO, and DA receptor activation in the MPO

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restores sexual behavior in male rats after MeA lesions (Dominguez et al., 2001; Dominguez and Hull, 2001). This may also occur in male prairie voles, but at least some of the catecholaminergic tone in the MPO appears to arise directly from the MeA itself and may allow faster communication between the two sites or may increase DA release above and beyond what is found in rats to facilitate behaviors that are specific to prairie voles.

In prairie voles, direct catecholaminergic input to the MPO from the pBST and MeApd may facilitate high gregariousness and initiate pair bonding after mating. Given the role of DA release in the MPO in two very different sociosexual behaviors in rats, it is interesting to consider that DAergic input may be necessary for social motivation, whereas other networks regulate the specific behaviors displayed. If this is the case, TH-ir cells in the pBST and MeApd may relay olfactory information to the MPO through DA release during all social interactions in male prairie voles, in addition to the DA release that occurs only in specific situations (mating or maternal care) in a number of rodent species. Thus, TH-ir cells in the prairie vole pBST and MeApd may enhance catecholaminergic neurotransmission to the point that prairie voles will initiate social interactions with many individuals, including pups and partners, but also siblings and in some cases unfamiliar conspecifics (Bowler et al., 2002; Firestone et al., 1991; Olazabal and Young, 2005; Roberts et al., 1996). Furthermore, DA release in the MPO may contribute to specific prairie vole social behaviors. Many neurons in the rat pBST and MeApd that express IEGs after mating project to the MPO (Coolen et al., 1998), and TH-ir cells expressing IEG expression after ejaculation in prairie voles may also project to the MPO. If so, additional DA input to the MPO may motivate males to initiate the next

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mating bout, as prairie voles have protracted mating encounters of 1-2 days (Carter and Getz, 1985), or may directly initiate neural cascades involved in the formation of pair bonds.

The MPO has widespread connections throughout the forebrain (Anderson and Shen, 1980; Chiba and Murata, 1985; Coolen et al., 1998; Simerly and Swanson, 1988), projects to many brain sites involved in social behavior, and may link input from TH-ir cells in the pBST and MeApd with networks already known to be involved in regulating male prairie vole sociosexual behaviors. The extra-hypothalamic vasopressin (AVP) system and the mesolimbic DA system are both critical for the formation of pair bonds (see Young et al., 2008), and the MPO may integrate information from these networks with catecholaminergic input from the pBST and MeApd. Indeed, the MPO has connections with the BST and MeA that contain AVP cell bodies (Chiba and Murata, 1985; Coolen and Wood, 1998; Wood and Swann, 2005), and their targets in the VP and LS (Anderson and Shen, 1980; Chiba and Murata, 1985; Finn et al., 1993; Grove, 1988a, b; Staiger and Nurnberger, 1991; Varoqueaux and Poulain, 1999). In addition, projections between the MPO and ACB or VTA may allow multiple catecholaminergic networks to communicate with one another (Chiba and Murata, 1985; Fahrbach et al., 1986; Groenewegen and Russchen, 1984).

A similar percentage of TH-ir cells in the pBST and MeApd project to the MPO, but there was a remarkably uneven projection between these two sites; about half of TH-ir cells in the MeApd project to the pBST, but very few TH-ir cells in the pBST terminate in the MeApd. Generally, overall projections from these two brain sites seem to follow this trend (Coolen et al., 1998; Dong and Swanson, 2004; Gu et al., 2003; Wood and

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Coolen, 2005), but there were numerous non-TH-ir pBST cells projecting to the MeApd. Some TH-ir cells in the MeApd communicate directly with the MPO, but others may indirectly enhance catecholamine release via connections with TH-ir cells in the pBST, or may coordinate sociosexual behaviors by influencing non-TH-ir cells. On the other hand, the pBST may communicate with the MeApd through other neurotransmitters, likely GABA given that the majority of pBST neurons in other rodents are GABAergic (Simmons and Yahr, 2003; Stefanova, 1998), but possibly any of the numerous other neurotransmitters found in the pBST (De Vries et al., 1984; DeVries et al., 1985; Malsbury and McKay, 1989; Miller et al., 1993; Muganini and Oertel, 1985; Poulin et al., 2009; Simerly and Swanson, 1987; Simmons and Yahr, 2003; Swann and Newman, 1992). In the present experiment, the termination site of approximately 45% of TH-ir cells in the pBST was accounted for, and it is likely that the remainder have an array of small projections throughout the forebrain, and possibly also connections with nearby BST cells that were within the injection site in the retrograde tracing experiment (Dong and Swanson, 2004; Gu et al., 2003; Wood and Coolen, 2005).

The VTA may also provide motivational information to the pBST and MeApd, and could influence catecholamine release from these sites. DA release in the ACB, probably from the VTA (Chronister et al., 1980; Curtis and Wang, 2005), increases during mating in male prairie voles, and activation of D2 receptors is critical for the consolidation of pair bonds (Aragona et al., 2003; Aragona et al., 2006). The VTA may simultaneously release DA in the pBST and MeApd to alter catecholamine release in the MPO or to influence pBST and MeApd cells of other phenotypes. Interestingly, TH-ir projections from the VTA to the pBST and MeApd are also unique to prairie voles, as the

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pBST and MeApd of other rodents receive very little input from the VTA (Coolen and Wood, 1998; Herbert et al., 1997; Wood and Swann, 2005). Other regions of the BST and amygdala, including the lateral BST and basolateral and central amygdala, may also receive some DAergic innervation from the VTA (Asan, 1998; Brummelte and Teuchert-Noodt, 2006; Kozicz, 2001; Phelix et al., 1992; Pinard et al., 2008). DAergic projections from the VTA to the ACB are part of complex networks mediating motivation and reward (Carlezon and Thomas, 2009; Ikemoto, 2010; Kiyatkin, 2002), and projections to the prairie vole pBST and MeApd may link motivational information with olfactory input to promote social interactions in this highly social species.

Conclusions

A unique catecholaminergic network consisting of the pBST, MeApd, MPO, and VTA has been discovered that probably affects the unique social behaviors of prairie voles. DA input from the VTA may be integrated with olfactory information in the pBST to MeApd to influence social motivation. Projections between TH-ir cells in the pBST/MeApd and MPO may then influence the desire for all types of social interaction, but also impact mating and the initiation of pair bonding. This unique catecholaminergic network may also interact with AVP and other DA systems to facilitate high levels of gregariousness and monogamous behaviors in this species.

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CHAPTER 5:

Conclusions

This dissertation describes a species-specific, steroid hormone sensitive, catecholaminergic system that likely influences monogamous social behaviors characteristic of male prairie voles. This network probably integrates olfactory, endocrine, and motivational input necessary for males' social behavior, and interacts with other brain areas already implicated in pair bonding in this species.

Significance of sex difference in TH-ir cells

Given the relative sexual monomorphism of prairie vole behaviors, it is at first surprising that males have many more TH-ir cells than do females. However, De Vries (2004) proposed that neural sex differences sometimes function to compensate for sex differences in physiology, such as circulating gonadal hormones, to eliminate sex differences in behavior. Indeed, gonadal hormone activity has been associated with the expression of vole social behaviors; males of non-social vole species or less social prairie vole populations have more estrogen receptors in the BST and MeA and are more sensitive to testosterone than males from highly social species and populations (Cushing et al., 2004; Cushing and Wynne-Edwards, 2006; Kramer et al., 2006). High numbers of TH-ir cells may also compensate for high testosterone levels in male prairie voles to allow social interactions.

These studies have focused on characterizing TH-ir cells in male prairie voles, but it is important to note that female prairie voles have more TH-ir cells than rats, hamsters,

or meadow voles of either sex (Northcutt et al., 2007), and these cells may be important for the expression of some female social behaviors. Given the ability of exogenous testosterone and estrogen to increase the number of TH-ir cells in the pBST and MeApd of females (Cavanaugh and Lonstein, 2010a; Northcutt et al., 2007), dramatic changes in hormone levels throughout different reproductive states were hypothesized to change TH expression in females (Northcutt et al., 2007). Indeed, the number of TH-ir cells does slightly increase at times when estrogen levels are normally high (such as after pairing with a male), but ovarian hormones are surprisingly not required, as the number of TH-ir cells is greater after pairing even in ovariectomized females (Cavanaugh and Lonstein, 2010b). Despite this increase, the number of TH-ir cells never approaches the number found in males (Cavanaugh and Lonstein, 2010b), and they are likely more critical for the expression of monogamous behaviors in males.

Species comparison between TH-ir cells and others in pBST and MeApd

Although non-monogamous rodents studied to date do not have large numbers of TH-ir cells in the pBST and MeApd (Albanese et al., 1986; Hokfelt et al., 1984; Kalsbeek et al., 1992; Northcutt et al., 2007), the regulation and projections of TH-ir cells in these regions in prairie voles is remarkably similar to that of other cell groups found in the BST and MeA of other rodents. In particular, the male-biased sexual dimorphism in TH expression and its maintenance by testosterone in adulthood parallels morphological (overall volume, soma size, astrocyte number and complexity) and neurochemical (expression of vasopressin, CCK, and substance P) features of these two brain sites in rats, hamsters, and mice (Cooke et al., 1999, 2003; De Vries et al., 1984, 1985; Malsbury

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and McKay, 1994; Morris et al., 2008; Simerly and Swanson, 1987; Swann and Newman, 1992). Recent data demonstrate that testosterone must be aromatized to estradiol to influence TH expression in prairie voles; estradiol maintains TH expression in castrated males and increases TH expression in females, whereas testosterone's androgenic metabolite DHT does not have these effects (Cavanaugh and Lonstein, 2010a). Similarly, estrogenic metabolites of testosterone are largely responsible for the regulation of many neurochemicals in the rat and mouse BST and MeA (De Vries et al., 1986, 1994; Pak et al., 2009; Wang and De Vries, 1995), though often the activation of both estrogen and androgen receptors is required to fully maintain a masculine phenotype (Cooke et al., 2003; De Vries et al., 1986; Durazzo et al., 2007; Johnson et al., 2008; Morris et al., 2005, 2008; Plumari et al., 2002; Scordalakes and Rissman, 2004). The projections of the prairie vole pBST are also very similar to what has been reported in rats and hamsters (Dong and Swanson, 2004; Gu et al., 2003; Wood and Swann, 2005), and TH-ir cells in the pBST and MeApd terminate in areas receiving the densest innervation from these areas in rats, mice, and hamsters (Coolen and Wood, 1998; Dong and Swanson, 2004; Gomez and Newman, 1992; Gu et al., 2003; Usunoff et al., 2009; Wood and Swann, 2005). These remarkable similarities suggest that prairie voles may not have more cells in the pBST and MeApd than non-social rodents (which could be determined through a stereological analysis), but that some of the cells common to all rodents began to express TH at some point in the evolutionary history of the prairie vole. In environments with low population density and many predators, voles with random mutations causing TH to be expressed in the pBST and MeApd may have had more reproductive success due to their ability to be highly social and adopt a cooperative breeding strategy. Because

catecholaminergic input to the MPO is necessary for a wide variety of sociosexual behaviors (Dominguez and Hull, 2005; Miller and Lonstein, 2005; Stolzenberg et al., 2007), additional catecholaminergic projections that originate in areas receiving olfactory input and involved in male sexual behavior could promote the formation of social bonds after mating.

One of the few differences between TH-ir cells in the male prairie vole pBST and MeApd and cells of all phenotypes in these sites in rats and mice is their IEG production in response to social stimuli. In male rats, IEG expression in the pBST and MeApd remains at baseline levels when they are exposed to anestrous females (Coolen et al., 1997), and in prairie voles, increases in IEG expression after interactions with siblings were specific to TH-ir cells in the pBST and MeApd (Lim and Young, 2004; Northcutt et al., 2009). TH-ir cells in the pBST and MeApd may receive very specific input from olfactory regions that do not innervate other cell types in the pBST and MeApd to communicate information about non-reproductive social contexts. Alternatively, enhanced signaling from areas involved in processing of goal-oriented behaviors and motivation may facilitate responses in these cells. The latter possibility is especially intriguing given the species-specific innervation of the pBST and MeApd by TH-ir cells in the VTA; however, it is unknown whether TH-ir cells receive this input.

Although male Syrian hamsters do contain a few TH-ir cells in the BSTpm and MeApd (Asmus et al., 1992; Asmus and Newman, 1993; Davis and Macrides, 1983; Northcutt et al., 2007; Vincent, 1988; Wommack and Delville, 2002), the distribution and number of cells, as well as their hormone sensitivity and IEG expression, is very different from what has been seen in the present experiments in prairie voles. First, hamsters have

TH-ir cells throughout the MeA and some scattered in lateral regions of the BST (Asmus and Newman, 1993), whereas TH-ir cells are largely confined to the pBST and MeApd in prairie voles. In addition, axonal transport must be blocked to see more than ~10 TH-ir cells in hamsters (Northcutt et al., 2007; Vincent, 1988; Wommack and Delville, 2002). The hormonal regulation of these cell groups is also dramatically different, further supporting the idea that these are functionally different groups of cells. Approximately 70% of TH-ir cells in the male hamster BSTpm and MeApd contain androgen receptors (Asmus and Newman, 1993), similar to what has been shown in prairie voles (Cavanaugh and Lonstein, 2010a), but the number of TH-ir cells in the BSTpm and MeApd does not change after castration, and only transiently decreases in the anterior MeA (Asmus and Newman, 1993). Experiments examining IEG expression also suggest that the cell groups have very different functions in the two species. Approximately 60% of the TH-ir cells in the male hamster MeApd express Fos in males that have been socially housed or left alone until sacrifice, and mating does not change Fos expression (Asmus and Newman, 1994). In contrast, less than 1% of TH-ir cells expressed IEGs when male prairie voles were tested alone, and dramatic increases in IEG expression were seen after mating (Northcutt and Lonstein, 2009). These drastic differences between the two species provides further evidence that TH-ir cells in the male prairie vole pBST and MeApd are involved in their unique social behaviors.

The function of TH-ir cells in the pBST and MeApd in hamsters is unknown, but chronic social stress increases TH expression and these neurons may affect aggression (Wommack and Delville, 2002). In addition to previous results implicating their role in affiliative, mating, and/or bonding behaviors, other investigations in prairie voles

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demonstrate that TH-ir cells are unlikely to be involved in at least some forms of aggression because IEG expression in TH-ir cells in the pBST and MeApd is similar among pair bonded males that fight with an intruder male or female and bonded males that remain with their partner (Gobrogge et al., 2007). It is interesting to consider that two neurochemically similar, but functionally different groups of cells exist in similar sites in these two species. A similar number of TH-ir cells was found in meadow voles and hamsters, and meadow voles may provide an interesting model in which to study the diversity in function in TH-ir cells in the pBST and MeApd. Meadow voles are relatively non-social during the summer, but become more gregarious in short day environments (Beery et al., 2008, 2008; Parker and Lee, 2001, 2002), and seasonal plasticity in TH expression and function would be fascinating.

Phenotype of TH-ir cells of the male prairie vole pBST and MeApd

The absence of dopamine-β-hydroxylase in the pBST and MeApd (Chapter 1) led us to tentatively conclude that TH-ir cells in these sites could be dopaminergic (DAergic). TH is the rate-limiting enzyme in DA synthesis and is very often used as a marker for DAergic cells in the vertebrate brain (Smeets and Gonzalez, 2000). However, not all TH-ir cells in the rat brain contain L-amino acid decarboxylase (AADC), which is the enzyme required to convert L-DOPA to DA (Jaeger et al., 1984; Ugrumov, 2009). In fact, cells expressing TH but not AADC are found in many sites throughout the brain in rats, hamsters, mice, guinea pigs, monkeys, and humans, and have a number of functions that have only recently been explored (reviewed in Ugrumov, 2009).

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Although often considered to only be a DA precursor, L-DOPA itself can act as a neurotransmitter and neuromodulator itself when released from cells that do not contain the AADC required to convert it into DA (Misu et al., 2002, 2003; Opacka-Juffry and Brooks, 1995). L-DOPA is released in the ventrolateral medulla, striatum, and hippocampus in a calcium-dependent manner and acts at postsynaptic targets or nearby presynaptic terminals (reviewed in Misu et al., 2003; Opacka-Juffry and Brooks, 1995). A specific receptor for L-DOPA has yet to be identified, but L-DOPA can induce depolarization and glutamate release from striatal neurons (Goshima et al., 1993). L-DOPA also acts on presynaptic terminals to influence DA release in the striatum (low L-DOPA concentrations enhance release via actions at β-adrenergic receptors while high L-DOPA concentrations inhibit it via D2 DA receptors), and also potentiates postsynaptic D2 receptors (Hume et al., 1993; Misu et al., 1986). In other neural networks, L-DOPA can influence the release of norepinephrine, acetylcholine, and glutamate, as well as the postsynaptic response to GABA (Goshima et al., 1986, 1991, 1993; Honjo et al., 1999; Misu et al., 1985; Ueda et al., 1995).

In some brain sites, TH-ir cells only express AADC and produce DA in specific developmental periods or in response to environmental cues. In the arcuate nucleus, very few cells express both TH and AADC in embryonic and early postnatal rats, but the number of bi-enzymatic neurons increases dramatically throughout postnatal development (Ershov et al., 2002a, b). Additionally, environmental cues can affect AADC expression; hamsters with short photoperiods have approximately half of the AADC-ir cells of their long photoperiod counterparts in the arcuate nucleus and median eminence (Krajnak and Nunez, 1996). Finally, in some brain sites L-DOPA can be taken

up by neighboring neurons expressing AADC, and sometimes dopamine-β-hydroxylase, and converted to DA or norepinephrine (Ugrumov, 2009). Preliminary experiments show that many AADC-ir cells exist in the BST, and these may produce DA from L-DOPA that is released in this site from the MeApd, VTA, and possibly pBST.

This is particularly relevant to this dissertation, because there is now preliminary evidence that most of the TH-ir cells in the pBST and MeApd of virgin male prairie voles do not express immunocytochemically-detectable levels of AADC. AADC may be found in the terminals of these TH-ir cells or at low levels that are unable to be detected using immunocytochemical techniques, but this recent finding opens up fascinating possibilities about the phenotype of these cells. TH-ir cells in the pBST and MeApd may influence MPO neurons through direct actions at postsynaptic receptors and/or modulations of the release of other neurotransmitters, including DA from other sites. Another possibility is that TH-ir in the pBST and MeApd neurons only produce AADC in response to specific developmental, environmental, or acute physiological cues, and that the enzyme is nonexistent or below detectable levels in adult virgin males. It is particularly intriguing to consider that AADC might be produced only when a male first encounters a female and that subsequent DA release in the MPO during mating would then facilitate the consolidation of a pair bond. Although this is not part of this dissertation, current experiments are exploring this possibility.

Possible role of pBST and MeApd TH-ir cells in social motivation or ability

The results of the IEG experiments suggest that some TH-ir cells in the pBST and MeApd are tonically active in any social situation. This is not surprising given the role of

the pBST and MeApd in a wide range of social behaviors, including mating, parental care, and social recognition, in other rodents (Newman, 1999; Numan and Insel, 2003; Petrulis, 2009). Many investigations of prairie vole social behavior have focused on the role of neurotransmitter systems in single social behaviors, typically pair bonding or parental behavior (see Young et al., 2008). In addition to these specific behaviors that have received much attention, prairie voles are highly gregarious (Bowler et al., 2002; Firestone et al., 1991; Olazabal and Young, 2005; Roberts et al., 1996). When left undisturbed in the laboratory, they spend the majority of time in close contact with siblings, either grooming one another or huddling together (Chapter 2; Firestone et al., 1991), and virgin males and females will often show high levels of affiliative behavior towards unfamiliar same-sex voles (Bowler et al., 2002; Firestone et al., 1991; Olazabal and Young, 2005). The high levels of social behavior in many settings indicate that there may be neural mechanisms promoting all social interactions in addition to networks specific for some behaviors. These initial experiments suggest that the TH-ir cells in the pBST and MeApd may be playing this role, and it will be very interesting to know if disrupting L-DOPA or DA release from these cell groups disrupts the motivation or ability to engage in all social interactions.

Similarly, Newman (1999) proposed that the BST and MeA are part of an "integrated social behavior circuit" along with the LS, MPO, anterior hypothalamus, ventromedial hypothalamus, and periaqueductal gray. She suggests that all of these areas are important for the expression of all mammalian sociosexual behaviors, but the relative activity among the regions differs depending on the behavior (see Figure 5.1). When applying this model to prairie voles, it seems necessary to expand the network to include

other areas critical for social behavior expression, particularly the ACB, VP, and VTA, and the integration across regions is likely to be more complex given the greater array of social interactions. Furthermore, TH-ir cells in the pBST and MeApd may act as a separate node, as their IEG responses are very different than those of the entire pBST and MeApd to some social stimuli, particularly siblings and pups (Chapter 3). Responses in TH-ir cells would be integrated with input from the other nodes to determine the appropriate behavior.

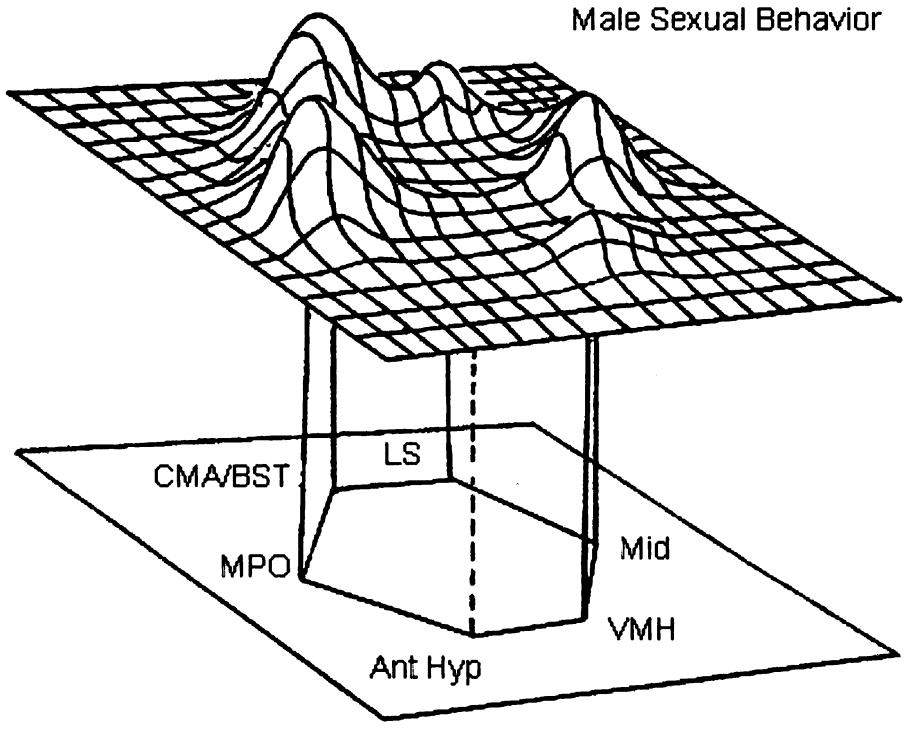

Figure 5.1. Hypothetical representation of neural activity in Newman's "integrated social behavior circuit" during male sexual behavior. Higher peaks indicate greater neuronal activity in that brain site. Figure from Newman, 1999.

An alternative explanation is that two functionally different, but anatomically overlapping groups of TH-ir cells exist in the male prairie vole brain. One group of TH-ir cells may be active after any social situation, while a second group is active only after ejaculation. This would be interesting given that subregions of the pBST and MeApd are differentially activated after male rats mount, intromit, or ejaculate (Coolen et al., 1996, 1997). IEG expression appeared to be scattered throughout TH-ir cells in the entire pBST and MeApd, but this does not rule out the possibility of two overlapping, but functionally separate groups. This may also explain why males have many more TH-ir cells than do females; females may have a moderate number of TH-ir cells because they only need one of these groups. Females form partner preferences more readily than males and their preference formation is less dependent on mating (DeVries et al., 1996b; DeVries and Carter, 1999; Insel et al., 1995; Winslow et al., 1993), and this may be independent of pBST and MeApd TH-ir cells in females.

Both of these possibilities are interesting, and future work will elucidate more about the role of these cells in monogamous behaviors. Initial attempts to lesion these cells by infusing a DA-specific toxin, 6-hydroxydopamine, into the pBST were unsuccessful, likely due to a lack of DA transporter in the cell bodies of TH-ir cells in the pBST and axon terminals from TH-ir cells in the MeApd, or because they are resistant to oxidative stress as are some other catecholaminergic cell groups in the forebrain (Behrouz et al., 2007). As an alternative, others in the laboratory are currently exploring the effects of intra-MPO infusions of a DA transporter (DAT) antibody conjugated to saporin toxin, which will be retrogradely transported to the soma and destroy DAT-synthesizing cells projecting to the MPO (Wiley et al., 2003). This work suggests that

this could result in the death of approximately 40% of TH-ir cells in the pBST and MeApd. Results of the current and future experiments on these TH-ir populations will provide insight into the neural networks that evolved to allow monogamous behaviors and cooperative breeding

Conclusions

The experiments in this dissertation describe a unique network in the prairie vole brain that is likely required for the high gregariousness and pair bonding characterisitic of this species. These species-specific groups of TH-ir cells probably work in conjunction with other "social network nodes" to motivate all social interactions and regulate mating and pair bonding. Now that functional possibilities and the anatomical projections of these cells are known, future experiments can determine the precise role of TH-ir cells in prairie vole sociosexual behaviors, and determine how they interact with other neurotransmitter systems that influence both general and specific social behaviors. Future studies that can now be performed because the projections of pBST and MeApd TH-ir cells are known, and will reveal the role of these cell populations in the complex sociosexual behaviors typical of male prairie voles.

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