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L-Amino acid decarboxylase- and tyrosine hydroxylase-immunoreactive cells in the extended olfactory amygdala and elsewhere in the adult prairie vole brain

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ABSTRACT

Neurons synthesizing dopamine (DA) are widely distributed in the brain and implicated in a tremendous number of physiological and behavioral functions, including socioreproductive behaviors in rodents. We have recently been investigating the possible involvement of sex- and species-specific TH-immunoreactive (TH-ir) cells in the male prairie vole (*Microtus ochrogaster*) principal bed nucleus of the stria terminalis (pBST) and posterodorsal medial amygdala (MeApd) in the chemosensory control of their monogamous pairbonding and parenting behaviors. These TH-ir cells are not immunoreactive for dopamine-beta-hydroxylase (DBH), suggesting they are not noradrenergic but possibly DAergic. A DAergic phenotype would require them to contain aromatic L-amino acid decarboxylase (AADC) and here we examined the existence of cells immunoreactive for both TH and AADC in the pBST and MeApd of adult virgin male and female prairie voles. We also investigated the presence of TH/AADC cells in the anteroventral periventricular nucleus (AVPV), medial preoptic area (MPO), arcuate nucleus (ARH), zona incerta (ZI), substantia nigra (SN) and ventral tegmental area (VTA). Among our findings were: (1) the pBST and MeApd each contained completely non-overlapping distributions of TH-ir and AADC-ir cells, (2) the AVPV contained surprisingly few AADC-ir cells and almost no TH-ir cells contained AADC-ir, (3) approximately 60% of the TH-ir cells in the MPO, ARH, and ZI also contained AADC-ir, (4) unexpectedly, only about half of TH-ir cells in the SN and VTA contained AADC-ir, and (5) notable populations of AADC-ir cells were found outside traditional monoamine-synthesizing regions, including some sites that do not contain AADC-ir cells in adult laboratory rats or cats (medial septum and cerebral cortex). In the absence of the chemical requirements to produce DA, monoenzymatic TH-ir cells in the virgin adult prairie vole pBST, MeApd, and elsewhere in their brain may instead produce L-DOPA as an end product and use it as a neurotransmitter or neuromodulator, similar to what has been observed for monoenzymatic TH-synthesizing cells in the laboratory rat brain.

1. Introduction

Prairie voles (*Microtus ochrogaster*) are unusual mammals because their social organization includes hallmarks of monogamy including pairbonding after mating, biparental care of offspring, and alloparenting of younger siblings by non-reproducing adults (Carter et al., 1995; Young et al., 2011). Neurochemicals necessary for the display of these social behaviors in prairie voles include the neuropeptides vasopressin and oxytocin, as well as the neurotransmitter dopamine (DA) (Carter et al., 2008; Phelps et al., 2010; Ross and Young, 2009; Young et al., 2011). The essential role for DA in prairie vole social bonding is evident by the ability of peripheral or intra-accumbens administration of a D2 receptor antagonist to block partner preferences in mated prairie voles, while D2 receptor agonism forges social preferences even without the need for mating (Aragona et al., 2003; Gingrich et al., 2000; Wang et al., 1999). Furthermore, stimulating the second messenger systems downstream of the D1 receptor inhibits partner preferences while downregulating them (such as that seen after D2 agonism) promotes social bonding (Aragona et al., 2006; Aragona and Wang, 2007). With regards to parenting, DA receptor blockade with the mixed D1/D2 receptor antagonist haloperidol suppresses pup licking and general contact with the litter in both sexes of prairie voles, although the neural sites of action for this effect remain unknown (Lonstein, 2002).

Almost all studies of DA's effects on social behaviors in prairie voles have focused on the mesocorticolimbic system (Young et al., 2011). Known and putative DA-synthesizing cells are found throughout the vertebrate forebrain (Smeets and González, 2000), however, and project to brain areas essential for socio-reproductive functions (*e.g.*, Cheung et al., 1998; Li et al., 1993; Lindvall and Stenevi, 1978; Miller and Lonstein, 2009; Northcutt

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and Lonstein, 2011; Palkovits et al., 1977). In this context, it is particularly interesting that in male prairie voles the densely interconnected principal nucleus of the bed nucleus of the stria terminalis (pBST) and posterodorsal medial amygdala (MeApd) each contain hundreds of neurons intensely immunoreactive for tyrosine hydroxylase (TH), the enzyme catalyzing the conversion of tyrosine to the dihydroxyphenylalanine (DOPA) necessary for catecholamine synthesis (Northcutt et al., 2007; Northcutt and Lonstein, 2009, 2011). These TH-immunoreactive (TH-ir) cells are not immunoreactive for dopamine beta-hydroxylase (DBH) (Northcutt et al., 2007) so we have proposed they may instead be dopaminergic (DAergic). In addition, because the numerous other adult rodents that have been examined have few or no TH-ir cells in these sites, we have proposed these cells contribute to male prairie voles' monogamous behaviors (Northcutt et al., 2007; Northcutt and Lonstein, 2009, 2011). This may occur through pBST and MeApd integration of olfactory and hormonal information, which is known to be necessary for many socioreproductive behaviors in mammals (Coolen and Wood, 1998; Newman, 1999; Numan and Insel, 2003; Wood and Swann, 2005). In support, we previously found that general social contact maintains basal immediate-early gene (IEG) expression in TH-ir cells of the male prairie vole pBST and MeApd and that mating to ejaculation is particularly potent (compared to other social stimuli including pups) in further increasing this IEG expression (Northcutt and Lonstein, 2009).

Many of the TH-ir cells in the male prairie vole pBST and MeApd project to the medial preoptic area (Northcutt and Lonstein, 2011), creating a unique TH-containing neural network among sites essential for social discrimination, copulation, parenting, and aggression (see Newman, 1999). Although it is intriguing to suggest that TH-ir cells projecting from the male prairie vole pBST and MeApd to the MPO comprise a newly discovered DAergic system necessary for monogamous behaviors in this species, it is unknown if these TH-ir cells contain the full complement of enzymes necessary to produce DA. Indeed, many TH-ir cells in the brains of other mammals do *not* synthesize DA or other catecholamines (Björklund and Lindvall, 1984) because they do not contain the enzyme aromatic L-amino acid decarboxylase (AADC) necessary to convert L-DOPA to DA. The distribution of these TH-positive/AADC-negative cells is generally similar across species (Jaeger et al., 1984a,b; Karasawa et al., 2007; Kitahama et al., 1988, 1990a,b; Skagerberg et al., 1988) and, instead of DA, these cells are thought to produce L-DOPA as an end product and utilize it as a neurotransmitter and neuromodulator (see Misu and Goshima, 2006; Ugrumov, 2009).

To better understand the phenotype and possible function of the tremendous number of TH-ir cells in the male prairie vole pBST and MeApd, we here used dual-label immunohistochemistry to examine the overlap between the distribution of TH-ir cells and AADC-ir cells in these brain sites. We also examined other brain sites that more traditionally contain TH-ir cells, including the anteroventral preoptic area (AVPV), medial preoptic area (MPO), arcuate nucleus (ARH), zona incerta (ZI), substantia nigra (SN) and ventral tegmental area (VTA). Lastly, we describe numerous populations of AADC-containing cells distributed widely outside these TH-synthesizing regions of the brain.

2. Methods

2.1. Subjects

Subjects were adult male and female prairie voles (*M. ochrogaster*) born and raised in our colony at Michigan State University, which originated with voles captured in Urbana, IL and last outbred in 2000 with voles from Illinois. Voles were housed in a 14:10 light:dark cycle with an ambient temperature of 21 °C. After weaning at 20 days old, subjects were housed in mixed-sex groups of littermates in clear plastic cages (48 × 28 × 16 cm) containing wood chips, wood shavings, and a covering of hay. Voles were later rehoused at least 10 days before sacrifice into cages containing only their same-sex siblings. Water and a food mixture containing cracked corn, whole oats, sunflower seeds, and rabbit chow (Tekland rodent diet No. 2031; Harlan, Madison, WI) in a 1:1:2:2 ratio were provided *ad libitum*. All procedures were performed in accordance with the Institutional Animal Care and Use Committee at Michigan State University and the EU Directive 2010/63/EU for animal experiments.

2.2. Tissue collection and immunohistochemistry

Voles were overdosed with sodium pentobarbital 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 the forebrain and midbrain of each were cut into 35 μm sections on a freezing microtome.

TH and AADC dual-immunohistochemistry was performed on every other section throughout the brain using methods standard in our laboratory (Cavanaugh and Lonstein, 2010; Northcutt et al., 2007; Northcutt and Lonstein, 2011). The free-floating sections were rinsed three times with 0.1 M Trisma-buffered saline (TBS; pH = 7.4) between each incubation. Sections were incubated in 0.1% sodium borohydride in TBS for 15 min, incubated in 1% hydrogen peroxide in 0.3% Triton X-100 in TBS for 10 min, blocked with 20% normal goat serum (NGS) in 0.3% Triton X-100 in TBS for 30 min, and incubated overnight at room temperature with rabbit anti-AADC polyclonal antiserum (1:1000, AB1569; Millipore, Temecula, CA). The next day, sections were rinsed in TBS then incubated in goat anti-rabbit biotinylated secondary antiserum in 2% NGS and 0.3% Triton X-100 in TBS for 1 h at room temp (1:500; Vector Laboratories) and incubated in avidin–biotin complex (Vectastain Elite; Vector Laboratories, Burlingame, CA) in TBS for 1 h. Immunoreactivity was visualized with 0.05% 3,3′-diaminobenzidine (Sigma) and 0.01% hydrogen peroxide in TBS, which produced a light brown reaction product. After rinsing, sections were re-blocked with 20% NGS and 0.3% Triton X-100 in TBS, and incubated overnight in a mouse anti-TH primary monoclonal antiserum (1:2000, MAB318; Chemicon, Temecula, CA) in 2% NGS and 0.3% Triton X-100 in TBS at room temperature. Sections were then 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 using Vector SG (Vector Laboratories), which provided a light blue label. Omission of the primary or secondary antisera abolished specific immunohistochemical labeling. After tissue processing, sections were mounted onto slides, dehydrated, and coverslipped.

We previously demonstrated using Western blot analysis that the TH antiserum used here detects a single band at the expected size in the prairie vole forebrain and midbrain, and this band is at the same location as that found in the laboratory rat forebrain and midbrain (Northcutt and Lonstein, 2011). A recent Western blot analysis performed in our laboratory using the AADC antiserum from the present study verified a band at the expected size (~55 kD) in the forebrain and midbrain of both prairie voles and laboratory rats, as well as a very heavy band in both species at ~110 kD that is likely the dimerized AADC protein found in central nervous system tissue (Kubrusly et al., 2008; Siow and Dakshinamurti, 1990; Zhu and Juorio, 1995). Control blots that omitted either the primary or secondary antisera did not contain any immunoreactive bands.

2.3. Microscope analysis

Slides were coded so the single observer (EIA) was blind to subject sex. Brain sections containing eight TH-rich regions of interest (pBST, MeApd, AVPV, MPO, ARH, ZI, SN and VTA) were examined bilaterally with a Nikon E400 light microscope at up to 200× magnification with the aid of a reticle in one ocular lens. The areas of analysis were standardized for each brain site, remained consistent for each subject, and covered the area where TH-ir cells are found within each site of interest. The area of analysis for each forebrain site was similar to what we have done in our previous work studying TH-ir cells in the prairie vole brain (Cavanaugh and Lonstein, 2010; Lansing and Lonstein, 2006; Northcutt et al., 2007; Northcutt and Lonstein, 2011) and involved three sections in the series through the pBST, four sections through the MeApd, three sections through the AVPV, three sections through the MPO, and four sections each through the ARH and ZI (Fig. 1). The VTA was analyzed from four sections and SN from three sections corresponding to approximately Plate 38 from Swanson's (1998) atlas of the rat brain (Fig. 1). The location and number of cells in each section that contained TH immunoreactivity (contained blue reaction product), AADC immunoreactivity (contained brown reaction product), and both immunoreactive products were recorded and totaled across sections. Cells were considered double-labeled only if their cytoplasm contained unmistakably overlapping blue and brown immunoreactivity.

Three to six voles per sex were examined for each brain site (see Table 1), although females are mentioned below only for the two sites where sex differences were detected (pBST, MeApd). Some additional brain regions were examined because they contain cells immunoreactive for AADC (but not TH) in other small mammals (Jaeger et al., 1984a,b; Kitahama et al., 1988, 1990a,b), or we noticed large populations of AADC-ir cells that had not been previously described in any animal. These sites were outside our primary interest in TH-rich brain areas, but because there has never been any examination of AADC expression in the prairie vole brain,