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Comparative distribution of central neuropeptide Y (NPY) in the prairie (*Microtus ochrogaster*) and meadow (*M. pennsylvanicus*) vole

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Abstract

Neuropeptide Y (NPY) has been implicated as a modulator of social behavior, often in a species-specific manner. Comparative studies of closely related vole species are particularly useful for identifying neural systems involved in social behaviors in both voles and humans. In the present study, immunohistochemistry was performed to compare NPY-like immunoreactivity (-ir) in brain tissue of the socially monogamous prairie vole and non-monogamous meadow vole. Species differences in NPY-ir were observed in a number of regions including the cortex, extended amygdala, septal area, suprachiasmatic nucleus, and intergeniculate leaf. Meadow voles had higher NPY-ir in all these regions as compared to prairie voles. No differences were observed in the striatum or hippocampus. The extended amygdala and lateral septum are regions that play a key role in regulation of monogamous behaviors such as pair bonding and paternal care. The present study suggests NPY in these regions may be an additional modulator of these species-specific social behaviors. Meadow voles had moderately higher NPY-ir in a number of hypothalamic regions, especially in the suprachiasmatic nucleus. Meadow voles also had much higher levels of NPY-ir in the intergeniculate leaflet, another key region in the regulation of circadian rhythms. Overall, species differences in NPY-ir were observed in a number of brain regions implicated in emotion, stress, circadian, and social behaviors. These findings provide additional support for a role for the NPY system in species-typical social behavior.

Keywords

neuropeptide Y; prairie vole; meadow vole; social behavior; circadian patterns

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1. Introduction

Social behaviors such as selective emotional attachment (“pair bonding”) and biparental care play a critical role in human behavior and society, but are rare among mammals. Only 3% of mammalian species display these and other behaviors associated with monogamy [35]. Monogamous animal models are a useful tool for understanding the neurobiology of social behaviors not present in more common laboratory animals such as rats and mice [42]. The prairie vole (*Microtus ochrogaster*) is an arvicoline rodent that displays a suite of behaviors associated with social monogamy, including pair bonding, biparental care, and mate guarding [9,48]. In contrast, the closely related meadow vole species (*Microtus pennsylvanicus*), displays typical mammalian behavior associated with a promiscuous breeding system [48,58]. Despite these striking differences in social organization, these species are similar in morphology and in non-social behaviors [54,58].

Comparative studies between these species are particularly useful for identifying neural systems involved in social behaviors associated with monogamy. For example, *Microtus* species show species differences that reflect social organization in a number of peptide systems including arginine vasopressin (V1a receptor: [33,37]), oxytocin (OT receptor: [32,37], corticotrophin releasing factor (CRF, urocortin 1, and receptor subtypes 1 and 2: [38,39] and CART peptide [28]. This approach has been largely successful in identifying neural systems involved in modulating social behavior in prairie voles [26,31,50,53,65] with consistent findings in humans [17,25,62], and has demonstrated high translational and clinical potential [30,44]

Neuropeptide Y (NPY) is involved in a wide array of behavioral processes including memory [60], stress and anxiety [60,67], circadian rhythms [2], hypertension [5], feeding [13,43], and alcohol drinking [12,59].

There are also a number of studies in a diversity of species implicating a role of this peptide in social behavior. Among invertebrates, the NPY system is implicated in social feeding behaviors. Wild strains of *C. elegans* display either social or solitary feeding behavior. This naturally occurring variation in feeding strategy is directly associated with two isoforms of the NPY receptor homolog gene *npr-1* [15]. Experimental manipulation of this gene can entirely shift the feeding behavior of the organism to an opposite strategy. Thus, a single gene in the NPY system can induce or prevent expression of social feeding behavior in this species. Social feeding strategies are also regulated developmentally by the NPY homolog neuropeptide F (NPF) in *Drosophila* [66]. The NPF system of the fruit fly is also highly responsive to the social environment, as mating increases central levels of each NPF and NPF mRNA, whereas sexual deprivation or rejection decreases these measures [55]. Research on social behavior and NPY in mammals has been primarily limited to the social interaction test in rats and mice. In this test, social investigation of a novel conspecific is measured. The traditional interpretation is that increased exploration is indicative of decreased anxiety. There is robust evidence for a role of NPY and its receptors in the social interaction test, with NPY promoting social interaction or preventing stress-induced decreases in interaction [20,45,52]. NPY has also been shown to decrease sexual behavior in male rats [13]. These studies across a variety of taxa and behaviors support a modulatory role for NPY in social behavior, often in a species-specific manner.

Given the likely role of NPY in social behavior, we aimed to compare the distribution of NPY in two closely related species that differ in social systems: the meadow vole (*Microtus pennsylvanicus*) and prairie vole (*M. ochrogaster*). In the present study, we analyzed brain tissue of meadow and prairie voles to perform a semi-quantitative comparison of NPY-like immunoreactivity (NPY-ir) between these species.

2. Experimental Procedure

2.1 Animals

Voles used in these experiments were bred in the colony at Emory University, and housed in same-sex duos or trios until adulthood. At 100–120 days of age, 3 experimentally naïve voles of each sex and species (12 total) were euthanized by CO₂ inhalation followed by transcardial perfusion with 4% paraformaldehyde in phosphate-buffered saline (PBS). Brains were removed and post-fixed in the same solution, and then preserved in 30% sucrose in PBS. The brains were shipped on ice to Oregon Health & Science University where the immunohistochemistry and analyses were performed. The protocol was approved by the Emory Institutional Animal Care and Use Committee (protocol number 205–2007Y).

2.2 Immunohistochemistry

Tissue was sliced into 40-micron sections and stored in 0.1% sodium azide (NaN₃) in PBS until time of assay. Floating sections were rinsed in PBS and then incubated in 0.3% hydrogen peroxide for fifteen minutes. Sections were then rinsed again in PBS and incubated in a block solution of 2% bovine serum albumin (BSA) and 5mg/mL heparin in a 0.003% Triton X100 and PBS solution for 5 hours. After blocking, sections were incubated overnight with a 1:50,000 dilution of rabbit polyclonal primary antibody (Anti-Neuropeptide Y, N9528, Sigma Aldrich, St. Louis, Missouri) in PBS with Triton and BSA. A parallel reaction to investigate non-specific binding was performed without the primary antibody, and no immunoreactivity was observed in the regions of interest described herein. Following overnight incubation, tissue was washed in PBS and incubated with a biotinylated-goat anti-rabbit secondary antibody in PBS and Triton for one hour. Tissue was then washed in PBS and incubated for one hour in A/B solution, then washed again in PBS. Tissue was stained using a DAB reaction and mounted the day of assay. All immunohistochemistry was performed at room temperature, and all tissue was assayed at the same time. All slides were coded by a party not performing analysis, and the code was not broken until data collection was complete.

For each region, we aimed to analyze photographs from both hemispheres of two serial sections, for an average of four sections per subject. In only a few cases, less than four sections were analyzed due to tissue quality or availability. Regions were then analyzed using NIH ImageJ software. Each region of interest was manually selected according to a mouse brain atlas [18]. To allow a more accurate assessment of staining, photographs were analyzed using a standardized background subtraction and the threshold function in ImageJ. For regions in which cell bodies were present in the absence of fibers (i.e. cortical regions), semi-quantitation represents the relative density of soma in the region of interest. For all other regions, the semi-quantitation of fiber density is presented. As counting of cell bodies in densely stained regions may be unreliable, cell bodies in these areas are described in the Results section. Fiber density was ranked on a scale of highest/maximum (++++) to low (+) density, as well as few to no (+/–) or no fibers present at all (0), as reported in Table 1.

3. Results

Semi-quantitative comparisons of NPY-ir in each meadow voles and prairie voles are presented in Table 1, with representative photomicrographs in Figure 1. The cell types and distribution of both cells and fibers in these vole species are consistent with previous reports in other rodent species. Numerous large (12–16µm in diameter) cell bodies were apparent in cortical areas, particularly in motor and somatosensory cortices (Figures 2,3a). Perikarya were observed in layers II through VI, with layer V having a slightly higher density than all other layers. Cells were almost exclusively multipolar or bipolar.

Cell bodies were also observed in a number of subcortical regions. In the intermediate region of the lateral septum (LS), cells of moderate size (10 μ m diameter) were rare but observed in some tissue. However, the presence of perikarya in the LS was an exception rather than typical for this region. Perikarya were also observed throughout the striatum, although cell types differed between the dorsal striatum and nucleus accumbens (NAc). In the dorsal striatum, there was a uniform distribution of low to moderately stained cells. These cells ranged from small to medium size (7–12 μ m diameter), lacked uniform orientation, and processes were not clearly observed. The NAc was characterized by darkly stained cells of moderate size (8 μ m diameter; Figure 3b with multipolar processes, and were observed throughout the structure although more densely in the shell region.

Small, darkly stained cell bodies (2.5–5 μ m diameter) were observed in a number of regions including the paraventricular nucleus of the hypothalamus (PVN; Figure 3e), arcuate nucleus, paraventricular thalamic nucleus (PV thal), and intergeniculate leaflet (IGL). Each of these regions were also characterized by very high fiber density, which likewise makes an accurate count of cell density unreliable (see Figure 3e).

Although NPY-ir was observed in the hippocampus, the staining for each cell bodies and fibers was low, and no species differences were observed (Figure 4). Moderately sized cell bodies (6 μ m) were present in low quantities in all CA regions, with somewhat higher concentrations in CA1 relative to CA3 and CA4. The staining of these cells was lighter compared to cells in cortical regions, as can be seen in Figure 4. The processes of these cells were likewise faintly stained and generally appeared very short, without uniform orientation. Smaller cells lacking distinct processes (2.5–5 μ m diameter) were observed in the dentate gyrus (DG), but these cells were not present in all sections examined.

The areas with the highest density of fibers included the nucleus accumbens, bed nucleus of the stria terminalis (BNST), hypothalamic areas (including the arcuate nucleus), and the IGL. Fibers in the BNST and amygdala were characterized by beaded varicosities, as in Figures 3c & d.

Marked species differences were observed in the amygdala, BNST, medial septum (MS) and parts of the LS, with meadow voles having higher density staining in all areas (Figure 5). In the hypothalamus, meadow voles had noticeably stronger NPY-ir in the suprachiasmatic nucleus (SCN), PVN, supraoptic nucleus (SON), ventromedial hypothalamus (VMH) and dorsomedial hypothalamus (DMH). Meadow voles also had more NPY-ir in the IGL as compared to prairie voles, but there was no difference in the lateral geniculate nucleus (LGN).

NPY-ir was similar across species in the NAc and dorsal striatum, hippocampus, thalamus, and many hypothalamic regions. We found no difference in the arcuate nucleus.

4. Discussion

We investigated species differences in NPY-ir between the socially monogamous prairie vole and promiscuous meadow vole. NPY has been characterized in detail in a broad array of species, and among mammals the best descriptions of NPY have been performed in rats [11], hamster [51], cat [27,41], and primates (lemur: [8]; squirrel monkey: [56]; rhesus macaque: [36]; humans: [1]). Overall, the cell types and distribution seen here in voles is consistent with that seen in non-primate mammals. Species differences in NPY-ir were observed in a number of regions including the extended amygdala, septal area, SCN, and IGL. Meadow voles had higher NPY-ir in all these regions as compared to prairie voles. Many of these regions are implicated in emotion, stress, and social behavior, including species-typical behaviors in voles.

Among its many functions, NPY is anxiolytic and modulates anxiety-like behaviors in part through the extended amygdala. The vole species examined here differ in behavior in the elevated plus maze, with meadow voles showing more anxiety-like behavior [57]. Paradoxically, prairie voles, but not meadow voles, show an increase in plasma corticosterone after this test. These species clearly differ in both their behavioral and physiological responses to stressors, and this may be reflected in some of the NPY-ir differences observed in this study.

NPY-ir in the amygdala of prairie voles was low, both in fiber density and cell numbers. In contrast, meadow voles showed moderate to strong fiber density, although cell numbers were still low. Within each species, these differences were consistent in all amygdala regions examined. Across mammals, there is substantial variation in patterns of NPY in the amygdala. For example, Chronwall et al. [11] noted few perikarya and low to moderate fiber density in amygdala regions of the rat, with the exception of a high density of fibers in the medial amygdala (MeA). In contrast, the cat shows strong cell and fiber staining throughout the amygdala, but low immunoreactivity in the MeA [41]. It appears that NPY in the amygdala is highly variable between species, and our findings are in agreement. Even within species, NPY in the amygdala is highly variable, as notable differences are seen within lines of rats selected for specific behaviors. NPY-ir is reduced in the amygdala of rat lines that have been selected for high alcohol preference and drinking compared to lines selected for low preference and drinking [29]. In intriguing agreement with these findings, prairie voles consume larger doses of alcohol than meadow voles [4]. Our findings are consistent with broad variation in amygdalar NPY both across and within species.

Similar differences in NPY-ir were also noted in regions of the extended amygdala: the interstitial nucleus of the posterior limb of the anterior commissure (IPAC) and BNST. The IPAC is considered a dopaminergic component of the extended central amygdala (CeA) [3]. Research in this region has been restricted to anatomical reports, and behavioral studies regarding IPAC are lacking. The anatomical evidence of this region as a component of the extended amygdala suggests that the IPAC should be involved with emotionally regulated behaviors. Although the IPAC has not been studied in voles, a number of studies in prairie voles have identified the BNST as a key region for social behavior. Exposure to an opposite sex partner (regardless of mating) increased c-fos expression in this region [14,24]. Species differences in a number of other neuromodulator systems have been noted, including the oxytocin receptor [32], CART peptide [28], and tyrosine hydroxylase (TH; [46]). Similar to c-fos studies, levels of both CART peptide and TH in this region are responsive to social novelty in prairie voles (CART: [26]; TH: [10]). The present study suggests BNST NPY may be an additional modulator of species-specific social behaviors. Overall, NPY is greatly increased in the extended amygdala of meadow voles as compared to prairie voles, and this is consistent across many regions.

We also found species differences in NPY-ir in the lateral and medial septum. The LS has been implicated in a number of species-specific vole behaviors including partner preference formation [40] and paternal behavior [63]. Species differences in both oxytocin and δ -opioid receptor binding have also been observed between prairie voles and the promiscuous montane vole [32]. Among prairie voles, individual differences in oxytocin and vasopressin V1a receptors correspond to willingness to engage in social investigation [23,49]. Clearly, peptides in this region are variable both within and between vole species, with consequences for social behavior. Our findings reported here support a potential role of NPY in the LS in social behavior of voles.

The MS is primarily implicated in arousal [7] and memory [21], including social transmission of food preferences [6]. This region receives afferents from the arcuate nucleus

[34] and projects to the hippocampus [19]. It is therefore interesting to note that there are no differences in NPY-ir in either the arcuate nucleus or hippocampus between species. The MS has not been previously implicated in vole behavior, although this may be primarily due to a lack of examination of this region in other comparative studies in voles. We therefore identify the MS as a novel region of interest for exploring species-typical behavior in voles, with emphasis on NPY.

Meadow voles had moderately higher NPY-ir in a number of hypothalamic regions, especially in the SCN. Our observation of high fiber density in the SCN region is consistent with that seen in the rat, hamster, and chipmunk, and may reflect a common trait among rodents [61] [11]. The SCN projects to a number of regions in which meadow voles had higher NPY-ir, including the BNST, LS, MPOA, MPN, PVN, DMH and VMH [64]. Meadow voles also had much higher levels of NPY-ir in the IGL, a key region in the regulation of circadian rhythms. There are reciprocal connections between the SCN and the IGL [64]. The increased NPY-ir in regions receiving projections from the SCN suggests the possibility that this region may be a critical source of species differences in NPY-ir. It is unclear what the source of this species difference is, as the voles examined were housed under similar day length conditions and are derived from wild caught populations of similar latitudes. However, under controlled conditions, meadow voles display a stronger nocturnal activity pattern than prairie voles [16], suggesting circadian rhythms differ between these species. Additionally, circadian rhythms are influenced in vole species in a more complex manner than in many other rodents. For example, voles display polyphasic rhythms, with bouts of activity and rest occurring throughout the day. The relative distribution of the activity bouts to either the dark or light phase is dependent on season [22]. The species differences noted here in the SCN and other regions involved in circadian rhythms support further research into species differences of activity patterns and their relationship to other NPY-mediated behaviors.

Often species differences in peptide or receptor expression differ in direction based on the region of interest. We did not observe any brain regions where NPY-ir was higher in prairie voles compared to meadow voles. Differences in immunoreactivity were not global but were restricted to specific brain regions, while in other brain regions the levels of NPY-ir were similar between species. Therefore, these findings could not reflect general differences in recognition of the ligand by the antibody between the species. Additionally, our present findings of differences in a single direction are not uncommon in studies of NPY-ir. For example, comparisons between selected lines of rats have shown similar patterns of region-specific differences in NPY-ir that occur in a single direction [29]. An additional possible explanation for differences in binding is that these vole species may differ in the amino acid sequence of NPY. However, the sequence of NPY is highly conserved across animals, with exact homology among other mammalian species closely related to voles [47].

We found that differences in organization of social behaviors in voles is reflected in species differences of NPY-ir in the amygdala, extended amygdala, septal areas, and regions involved in circadian regulation. These findings further support a role for the NPY system in regulating social behavior across a diversity of taxa, often in a species-specific manner [15,52,66]. However, it is also possible that these differences in NPY-ir are independent of social organization and reflect other species differences in behavior or physiology. A fuller understanding of the neuroanatomy of the NPY system in *Microtus* species requires similar comparative studies on NPY mRNA and NPY receptor subtypes. Further experimental studies are an exciting direction for future research.

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Abbreviations

AHA	anterior hypothalamic area
BLA	basolateral amygdala
BNST	bed nucleus of the stria terminalis
CeA	central amygdala
DG	dentate gyrus
DMH	dorsomedial hypothalamus
GP	globus pallidus
IGL	intergeniculate leaflet
IPAC	interstitial nucleus of the posterior limb of the anterior commissure
LGN	lateral geniculate nucleus
LPOA	lateral preoptic area
LS	lateral septum (d, dorsal
i	intermediate
v	ventral)
MeA	medial amygdala
MPN	medial preoptic nucleus
MPOA	medial preoptic area
MS	medial septum
NAc	nucleus accumbens
NPF	neuropeptide F
NPY	neuropeptide Y
NPY-ir	NPY-like immunoreactivity
PeV	periventricular nucleus of the hypothalamus
PVN	paraventricular nucleus of the hypothalamus
PV thal	paraventricular thalamic nucleus
SCN	suprachiasmatic nucleus
SON	supraoptic nucleus
VMH	ventromedial hypothalamus
VP	ventral pallidum

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Highlights

- Comparison of brain NPY-ir of promiscuous meadow vole and monogamous prairie vole
- Meadow voles have higher NPY-ir in the amygdala, extended amygdala and septal areas
- Meadow voles also have higher NPY-ir in regions involved in circadian regulation
- There were no species differences in the striatum or hippocampus
- Findings support a role for NPY in regulating species-typical social behavior

Figure 1.
Representative photomicrographs of NPY-ir in coronal sections of the meadow and prairie vole brain.

Figure 2.
The prefrontal (a), motor (b), and somatosensory (c) cortex of the meadow vole. Cortical layers are labeled (b) and scale bar (c) represents 250 μ m. Note the large cell bodies throughout layers II-IV, which are concentrated in layer V.

Figure 3. High-resolution photomicrographs of cell bodies and processes in the motor cortex (a), NAc (b), BNST (c), medial amygdala (d), and PVN (e). Adjacent structures are identified for orientation: corpus callosum (cc), anterior commissure (ac), and optic tract (ot). Scale bar represents 100 μ m.

Figure 4.
The hippocampal formation of a prairie vole. No species differences in NPY-ir were observed in this region. Scale bar represents 500 μ m.

Figure 5. Representative photomicrographs of the septal area (a,b), BNST (c,d), and amygdala regions (e,f) of the meadow vole (a,c,e) and prairie vole (b,d,f). Scale bar (f) represents 1mm. Meadow voles had higher NPY-ir in all three regions.

Table 1

Semi-quantitative comparisons of NPY-ir in the meadow and prairie vole. The presence of cells (c) and fibers (f) is also noted. Cell (cortical areas) and fiber density (all other regions) are ranked on a scale of highest/maximum (+++++) to low (+) density, as well as few to no (+/-) or no fibers present at all (0).

		meadow vole	prairie vole
<i>Cortex</i>			
Prefrontal	c	++	+
Motor	c	+++	++
Somatosensory	c	+++	++
Piriform	c	+	+
<i>Septum</i>			
LSd	f	+/-	+/-
LSi	c,f	+/-	+/-
LSv	f	+++	+
MS	f	+++	+
<i>Striatum</i>			
NAc shell	c,f	+++++	+++++
NAc core	c,f	+++	+++
Dorsal striatum	c	+	+
BNST	f	+++++	++
VP	c	+	+
IPAC	f	+++	++
GP		0	0
<i>Amygdala</i>			
BLA	f	++	+/-
CeA	f	++	+/-
MeA	f	++	+/-
<i>Hypothalamus</i>			
MPN	f	+++	+++
MPOA	f	+++	+++
LPOA	f	++	+
PeV	f	+++++	+++++
SCN	f	+++++	++
PVN	c,f	+++	++
AHA	f	++	+
SON	f	+++	++
Arcuate	c,f	+++++	+++++
VMH	f	+++	++
DMH	f	+++	++
Mammillary		0	0
<i>Hippocampus</i>			
DG	c,f	++	++
CA1	c	+	+

		meadow vole	prairie vole
CA3	c	+	+
CA4	c,f	++	++
<i>Thalamus</i>			
PV thal	c,f	++	++
LGN	f	+	+
IGL	c,f	++++	++