

**Monoaminergic integration of diet and social signals in the brains of
juvenile spadefoot toads**

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Summary statement

Early effects of diet modify monoamine levels and the monoamine response to social signals in the brain of Plains spadefoot toads, a species in which diet and body condition influence social preferences.

Abstract

Social behavior often includes the production of species-specific signals (e.g., mating calls or visual displays) that evoke context-dependent behavioral responses from conspecifics. Monoamines are important neuromodulators that have been implicated in context-dependent social behavior yet we know little about development of the monoaminergic systems and whether they mediate the effects of early life experiences on adult behavior. We examined the effects of diet and social signals on monoamines early in development in the Plains spadefoot toad (*Spea bombifrons*), a species in which diet affects the developmental emergence of species recognition and body condition affects the expression of adult mating preferences. To do so, we manipulated the diet of juveniles for six weeks following metamorphosis and collected their brains 40 minutes following the presentation of either a conspecific or heterospecific call. We measured levels of monoamines and their metabolites using high pressure liquid chromatography from tissue punches of the auditory midbrain (i.e., torus semicircularis), hypothalamus, and preoptic area. We found that call type affected dopamine and norepinephrine signaling in the auditory midbrain and that diet affected dopamine and serotonin in the hypothalamus. In the preoptic area, we detected an interaction between diet and call type, indicating that diet modulates how the preoptic area integrates social information. Our results suggest that the responsiveness of monoamine systems varies across the brain and highlight preoptic dopamine and norepinephrine as candidates for mediating effects of early diet experience on later expression of social preferences.

Introduction

Social behavior can be defined broadly as any interaction between conspecifics in which one individual benefits, and it often includes the production of species-specific signals that evoke context-dependent responses from conspecifics (Insel and Fernald, 2004). The context-dependent expression of social behavior is particularly important for the adaptive expression of mating preferences (Cummings, 2015). For example, mate preferences may vary depending on predation risk (Won Kim et al., 2009), social experience (Kodric-Brown and Nicoletto, 2001), or physiological state (Cotton et al., 2006; Lynch et al., 2006).

In the Plains spadefoot toad (*Spea bombifrons*), body condition (mass relative to length) modifies the expression of mating preferences: females in poor body condition are more likely to prefer the mating calls of the heterospecific *S. multiplicata* than are good condition-females (Pfennig, 2007). While hybrid offspring have reduced fertility and fecundity, they develop faster, thus producing a net benefit for poor condition-females living in fast drying ponds (Pfennig, 2007; Pfennig and Simovich, 2002). In contrast, good condition-females are more likely to produce faster developing offspring and, therefore, are less likely to benefit from hybridizing. Thus, females in good body condition more consistently prefer conspecifics. While it is still unknown how plasticity in these mating preferences develops, early experiences with diet may play an important role (Pfennig et al., 2013). This species is therefore ideal for evaluating the neural mechanisms linking diet and condition with development of social preferences.

Because neuromodulators act to alter the efficacy of synaptic transmission, they are poised to play an important role in context-dependent behavior. The monoamines – norepinephrine (NE), dopamine (DA), and serotonin (5HT) – share a number of functions, including sensory processing, attention, arousal, and synaptic plasticity (Hurley et al., 2004; Maney, 2013; Sara, 2009) that affect social behavior broadly, and its context-dependent expression, specifically. For example, NE plays an important role in auditory discrimination of conspecific signals in songbirds (Appeltants et al., 2002; Castelino and Schmidt, 2010;

Lynch and Ball, 2008; Maney, 2013) and 5-HT modifies sensory processing of conspecific social signals in mammals (Hurley and Hall, 2011; Hurley and Pollak, 2005) and fish (Deemyad et al., 2013). In the basal forebrain, monoamines are sensitive to physiological conditions, such as gonadal development, that influence social interactions (Chu and Wilczynski, 2002; Etgen et al., 1999; Hull et al., 1995). In addition, it has been found that early dietary experiences can modify the development of monoamine signaling (Levin, 2006). Thus, monoamines may provide a link between diet and the later development of social preferences.

Our long-term goal is to understand how early life experiences affect the development of context-dependent mating preferences in Plains spadefoot toads. In the present study we examined monoamine levels in response to diet and social signals in the brains of juveniles. We measured monoamines in the auditory midbrain, which is responsible for processing social signals in anurans (Wilczynski and Endepols, 2006), the hypothalamus, which is central to the control of food intake and satiety in vertebrates (Fischer and O'Connell, 2017), and the preoptic area, a central part of the social decision making network (O'Connell and Hofmann, 2012) that has been implicated in mating preferences (Martinez and Petrulis, 2013).

Materials and Methods

Subjects

To generate our subjects, we bred four pairs of *S. bombifrons* males and females. All adults were wild-caught from sympatric populations near Portal, Arizona, USA. The resulting tadpoles were fed tadpole chow *ad libitum*. At metamorphosis, juvenile toads were randomly assigned to either a high food ($n = 15$) or low food ($n = 15$) diet. We interspersed siblings among treatment groups such that any treatment effect would not be confounded by a family effect. However, due to small sample sizes, we were unable to account for family effects in our models. Because our subjects were only one generation removed from wild populations, they still maintain significant levels of genetic variation. Nonetheless, the use of four families may reduce the generalizability of our results beyond the families included here. Because live juveniles cannot be sexed, both males and females were randomly included in our sample (sex ratios of sibships are generally 50:50; K. Pfennig, unpubl. data). From metamorphosis until 6 weeks of age, juveniles on the high diet were fed five 1/8-inch crickets daily while those on the low diet were fed five 1/8-inch crickets three times per week. At 6-weeks of age, we measured body mass and snout-vent length (SVL). We calculated body condition for individuals relative to others of their species by regressing mass on SVL and using the resulting residuals as our measure of body condition (Pfennig, 2007).

At 6-weeks post-metamorphosis, we presented individual toads with either 40 minutes of *S. bombifrons* mating calls or 40 minutes of *S. multiplicata* mating calls in a dark acoustic chamber as previously described (Rodriguez Moncalvo et al., 2013). We tested animals in a dry environment (on moist towel, but no standing water). The call stimuli were identical to those used previously (Pfennig, 2007), and consisted of calls that were average for the call characters of each species (Pfennig, 2000; Pfennig, 2007). For these sympatric populations, the pulse and call rates of the two species show no overlap (Pfennig, 2000) and are, thus, readily distinguishable (see Pfennig 2007 for audio files). The stimuli were synthesized with Audacity 1.2.4 sound editor software by generating a triangular pulse by

applying the “Fade out” effect to a pure sine tone. These pulses were repeated to create a single call and the calls were repeated to produce a 29 second call bout for each species (Pfennig 2007).

Quantification of monoamines

Immediately following the 40-minute sound exposure, we decapitated each animal, quickly dissected its cranium, embedded it in OCT embedding medium (Sakura, Finetek, Torrance CA), and froze it in liquid nitrogen (see Rodriguez Moncalvo et al., 2013 for details). We stored craniums at -80° C until sectioning. We sectioned craniums in the coronal plane in a caudal-to-rostral direction at -15° C, obtaining 300- μ m brain sections for microdissection. We collected 350- μ m diameter punches from the auditory midbrain (including posterior, laminar, and magnocellular nuclei of the torus semicircularis), hypothalamus (including ventral hypothalamus, dorsal hypothalamus and posterior tuberculum), and preoptic area following Rodriguez Moncalvo et al. (2013) (Fig. 1). For the auditory midbrain, we combined punches from the two hemispheres to create one representative sample, while we took single punches from the midline for the hypothalamus and preoptic area. During punching, we maintained the section at -20° C. We expelled the tissue punches into microcentrifuge tubes containing 100 μ l of mobile phase and 0.1 pg/ μ l of an internal standard (see Rodriguez Moncalvo et al., 2013 for details) and rapidly froze them on dry ice. We stored all samples at -80°C until HPLC analysis.

While the neuroanatomy of monoaminergic systems have not been well studied in juvenile anurans, the limited amount of data suggest that these systems are well developed early in postmetamorphosis and are consistent with adult distributions (Rodriguez Moncalvo et al., 2013). Therefore, based on the adult anatomical studies (reviewed in Rodriguez Moncalvo et al., 2013), we infer that our auditory midbrain samples contained terminals for NE, DA, and 5-HT fibers. Our hypothalamus tissue sample likely included terminals for NE, DA, and 5-HT fibers, as well as DA cell bodies and, potentially, 5-HT cell bodies. Finally, the

preoptic area sample likely included terminals for NE, DA, and 5-HT fibers, as well as DA cell bodies.

We used high pressure liquid chromatography (HPLC) with electrochemical detection to determine tissue concentrations of norepinephrine (NE) and its metabolite MHPG (3-Methoxy-4-hydroxyphenylglycol), dopamine (DA) and its metabolite DOPAC (3,4-Dihydroxyphenylacetic acid), and serotonin (5-HT; 5-Hydroxytryptamine) and its metabolite 5HIAA (5-Hydroxyindoleacetic acid) (see Rodriguez Moncalvo et al., 2013 for details). Tissue levels of monoamines reflect amounts that are both stored (in cells and fibers) and released, whereas levels of metabolites are thought to primarily reflect the amount of monoamine that is released from nerve terminals (Meiser et al., 2013; Moore, 1986). We were not able to normalize concentrations per μg of protein because the protein content of each sample was too low to be detected using standard protein assays. If anything, our inability to normalize for protein concentration would decrease our ability to detect differences among groups because protein content of punches should account for error due to variation in section thickness or placement of the punch. Thus, our results likely represent conservative estimates of differences among groups.

Statistical Analysis

We used t-tests to examine the effects of diet on final length (SVL) and body condition. We used two-way ANOVAs to examine the effects of diet, call type, and their interaction on concentrations of monoamines for each brain region separately. For the hypothalamus (MHPG, DOPA) and preoptic area (MHPG), some metabolite concentrations fell below detectable limits for our HPLC system and so are not reported here.

Results

Six-week old toads on the high diet were longer ($t_{28} = 4.16$, $p = 0.0003$) and had better body condition ($t_{28} = 3.8$, $p = 0.0007$) than toads on the low diet (Fig. 2).

In the auditory midbrain, we found a strong relationship between the call type presented and levels of DA and NE: call type affected levels of NE ($p = 0.007$) and its metabolite MHPG ($p = 0.01$) as well as DA's metabolite DOPAC ($p = 0.04$) (Fig. 3, Table 1). Diet had no detectable effect on monoamine levels in the auditory midbrain (Fig. 3, Table 1). Taken together, we conclude that DA and NE release in the auditory midbrain responded to intrinsic features of the call (e.g., call energy) in a way that could contribute to encoding information about call type.

In the hypothalamus, animals receiving the high food diet had lower levels of DA ($p = 0.02$), 5-HT ($p = 0.005$), and 5-HT's metabolite 5HIAA ($p = 0.01$) than animals in the low food diet (Fig. 4, Table 1). Even though the hypothalamus is known to be acoustically sensitive (Allison, 1992), monoamine levels here were not influenced by the type of call heard.

Finally, in the POA, we detected a significant interaction between diet and call type for both NE ($p = 0.03$) and DA ($p = 0.04$), indicating that diet affected how the POA responded to call type (Fig. 5, Table 1). Intriguingly, diet reversed the relationship between call type and NE/DA levels in a manner that is reminiscent of the relationship between body condition and female mate choice behavior in adults (Pfennig, 2007). That is, under conditions that would promote a conspecific preference in adults (high diet), NE and DA levels were higher in response to the conspecific call, whereas under conditions that would promote a heterospecific preference diet in adults (low diet), NE and DA levels were high in response to the heterospecific call.

Discussion

Overall, the effects of diet and social signals on monoamine levels showed neuroanatomical heterogeneity in the brains of juvenile spadefoot toads. We found that monoamines levels were sensitive to call type within the auditory midbrain, to diet in the hypothalamus, and to both calls and diet in the preoptic area. In the preoptic area, the interaction between diet and call type reflected the relationship between body condition and female mating preferences in adults.

In the auditory midbrain, we found changes in the concentrations of both metabolites (MHPG, DOPAC) and neurotransmitter (NE, but not DA), suggesting changes in synaptic transmission (Meiser et al., 2013; Moore, 1986) with greater neurotransmitter release in response to the conspecific call compared to the heterospecific call. In the preoptic area, we only had evidence for changes in neurotransmitter concentration (NE, DA), but not metabolites (DOPAC; MHPG was not measured). Increases in neurotransmitter concentrations likely reflect changes in monoaminergic tone. That is, the increased production and release of the neurotransmitter over a longer period of time. Changes in monoaminergic tone often occur when longer-term modulation of neurotransmission is required (e.g., Krenz et al., 2014). These two patterns are consistent with the factors that influenced the monoamines in the two brain areas. That is, auditory processing of call information in the midbrain may not depend on changes in neurotransmitter tone, whereas integration of physiological cues such as food availability with social information is more likely to involve longer-term modulatory mechanisms.

Amphibians, unlike most vertebrates, have a larval stage followed by metamorphosis that leads to a juvenile in the form of the adult. The cytoarchitecture of tadpole and adult brains is qualitatively similar (Lannoo, 2000), although the metamorphic transition from aquatic to terrestrial hearing is characterized by plasticity in the auditory system (Boatright-Horowitz and Simmons, 1997; Simmons, 2015). Once metamorphosis is complete, the juvenile anuran brain appears to be similar anatomically and functionally to that of adults.

This is supported by the fact that auditory discrimination and phonotaxis appear early in life (Baugh and Ryan, 2010; Pfennig et al., 2013), gradually increase in frequency through postmetamorphic growth, and are expressed in their full form at sexual maturity (Baugh and Ryan, 2010). In Plains spadefoot toads, the emergence of call preferences (conspecific vs. heterospecific) is modified by diet (Pfennig et al., 2013), indicating plasticity in the ontogeny of social behavior in this species. Our findings that diet modified the call-evoked levels of monoamines in the preoptic area suggest that the preoptic area could be an important site for mediating the developmental emergence of mating preferences and/or context-dependent behavior in adults.

While the preoptic area is a highly conserved node of the social behavior network of vertebrates (Newman, 1999; O'Connell and Hofmann, 2012), relatively little is known about its role in social behavior in anurans. Because the preoptic area is acoustically sensitive (Allison, 1992) and its neuroendocrine cells respond to social signals (Burmeister and Wilczynski, 2005) it has long been thought critical in mediating the effects of social interactions on gonadal development (Brzoska and Obert, 1980; Burmeister and Wilczynski, 2000), as part of the so-called audioendocrine circuit (Wilczynski and Endepols, 2006). However, the preoptic area might also have direct influence on motor circuits through its descending projections to the midbrain and spinal cord (Sánchez-Camacho et al., 2001). Indeed, in males, the preoptic area is critical for producing vocal responses to mating calls (Schmidt, 1984), and it is required for selective phonotaxis in females (Walkowiak et al., 1999). Furthermore, neural responses of the preoptic area – but not other brain regions examined – correlate with the expression of mating preferences in both túngara frogs (Chakraborty and Burmeister, 2015) and spadefoot toads (present study). A role for the preoptic area in mating preferences of anurans is consistent with data from mammals (Henley et al., 2011; Martinez and Petrulevicius, 2013). However, whether the changes in norepinephrine and dopamine signaling we observed in the preoptic area are causally related to selective social responses awaits further study.

In the hypothalamus, we found that diet restriction was associated with increased levels of dopamine, serotonin and its metabolite 5HIAA, with smaller animals having higher levels of these monoamines. In mammals, serotonin produced by the raphe nuclei acts as a satiety signal when released in the hypothalamus (Lam et al., 2010; Voigt and Fink, 2015) and diet can affect development of serotonin signaling (Hassanain and Levin, 2002). While little is known about the control of appetite in anurans, the neuroanatomy of both the hypothalamus (Domínguez et al., 2014; Domínguez et al., 2013) and serotonin systems (Zhao and Debski, 2005) are highly conserved in vertebrates and are likely to be functionally similar. Indeed, our results are consistent with the causal relationship between serotonin and satiety in mammals (Voigt and Fink, 2015): we found that toads in the high food group and, we infer, with greater levels of satiety, had lower concentrations of hypothalamic serotonin.

Monoamines have been shown to be important in auditory processing in mammals (Hurley et al., 2004) and birds (Castelino and Schmidt, 2010; Maney, 2013), where they increase the signal to noise ratio in such a way to enhance processing of social signals. While the auditory midbrain of anurans (torus semicircularis) has been studied extensively in the context of social communication (Wilczynski and Endepols, 2006), our study is the first to identify a potentially important role for monoamines. The auditory midbrain is believed to be important for audio-motor integration (Wilczynski and Endepols, 2006). Descending projections from the forebrain, including the septum, striatum, preoptic area and hypothalamus are thought to modulate processes within the torus semicircularis that are, in turn, capable of generating behavioral responses to mating calls (reviewed in Wilczynski and Endepols, 2006). However, in the present study, monoaminergic responses to mating calls in the auditory midbrain lacked plasticity of the kind that reflects the behavioral plasticity we observe in response to diet. At least with regards to monoaminergic signaling, then, the auditory midbrain does not appear to be a candidate for the development of social preferences as mediated by physiological cues such as diet.

In summary, we examined the effects of diet and social signals on monoamines in a species where diet has the potential to influence the development of social preferences. Our

results identified the preoptic area as a candidate for mediating plasticity of social behavior, while the hypothalamus and auditory midbrain showed no plasticity in their response to diet and mating calls, respectively. By using a broad approach that examined multiple neuromodulators across multiple brain regions, we have created a strong foundation for future investigations of the early development of social preferences.

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Table 1. Results from two-way Anovas examining the effects of Diet (high vs. low), mating call type (conspecific vs. heterospecific), and their interaction on levels of monoamines and their metabolites.

	Diet			Call			Diet x Call		
	F	df	p	F	df	p	F	df	p
Auditory Midbrain									
Norepinephrine	0.15	1,28	0.70	8.5	1,28	0.007	0.75	1,28	0.40
MHPG	0.01	1,28	0.90	7.2	1,28	0.01	0.03	1,28	0.86
Dopamine	2.5	1,28	0.13	0.00	1,28	0.97	0.15	1,28	0.70
DOPAC	0.07	1,28	0.79	4.8	1,28	0.04	2.1	1,28	0.15
Serotonin	1.98	1,28	0.17	0.13	1,28	0.72	0.37	1,28	0.55
5HIAA	.36	1,28	0.55	0.30	1,28	0.59	0.49	1,28	0.49
Hypothalamus									
Norepinephrine	0.05	1,28	0.82	1.68	1,28	0.21	0.65	1,28	0.43
Dopamine	6.4	1,28	0.02	0.67	1,28	0.42	1.15	1,28	0.29
Serotonin	9.24	1,28	0.005	1.8	1,28	0.19	<0.01	1,28	0.98
5HIAA	7.1	1,28	0.01	1.86	1,28	0.18	0.01	1,28	0.94
Preoptic area									
Norepinephrine	0.17	1,26	0.69	0.15	1,26	0.70	5.0	1,26	0.03
Dopamine	0.01	1,26	0.91	0.10	1,26	0.76	4.9	1,26	0.04
DOPAC	0.04	1,26	0.85	2.1	1,26	0.16	0.15	1,26	0.70
Serotonin	<0.01	1,26	0.97	2.2	1,26	0.15	2.2	1,26	0.15
5HIAA	3.8	1,26	0.06	1.82	1,26	0.19	0.52	1,26	0.48

Figures

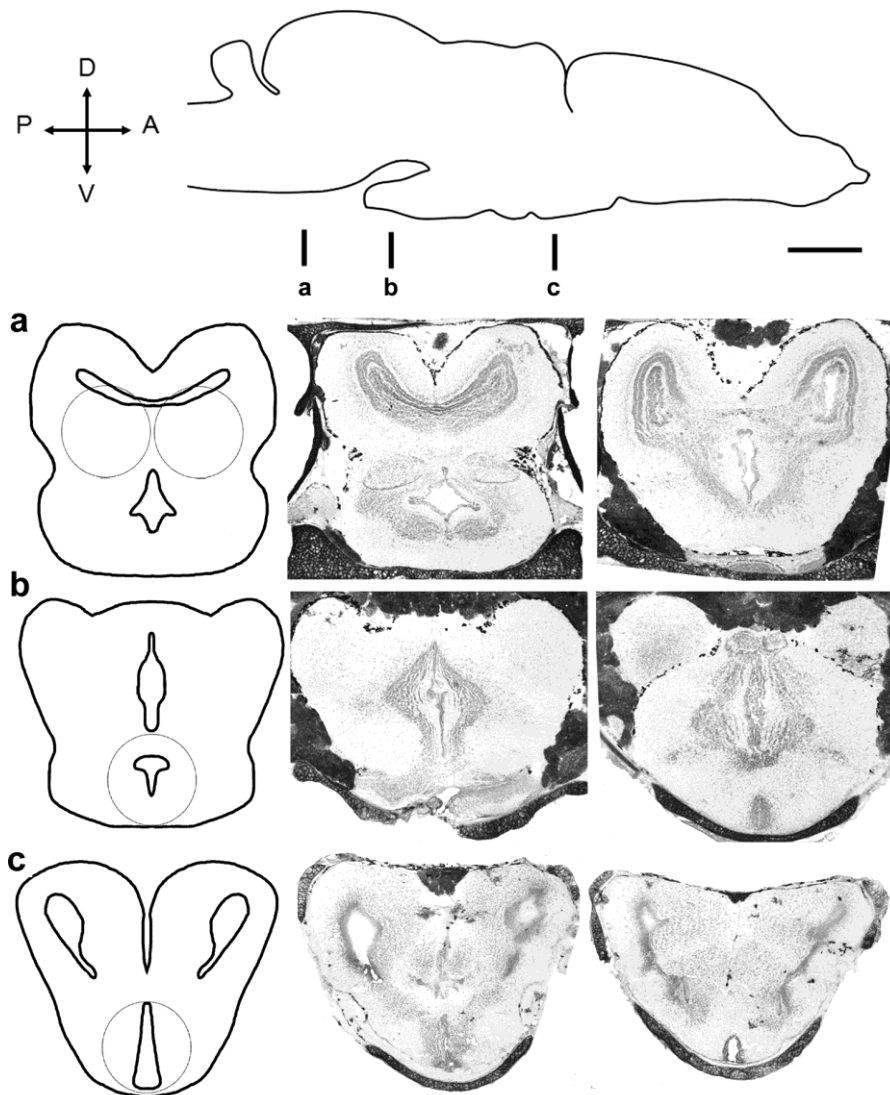


Figure 1: Schema of the juvenile spadefoot brain and diagrams of transverse sections illustrating the brain regions studied. Vertical bars indicate the starting point of each 300 micron-transverse section from which we micropunched the brain regions of interest, shown in corresponding rows (a. auditory midbrain; b. hypothalamus; c. preoptic area). The left side of each row shows schematic diagrams of the caudal position of each micropunch (indicated by circles), and the photomicrographs of the Thionin-stained sections represent the 'before' (center) and 'after' (right) sections of each punch. Scale bar = 300 microns.

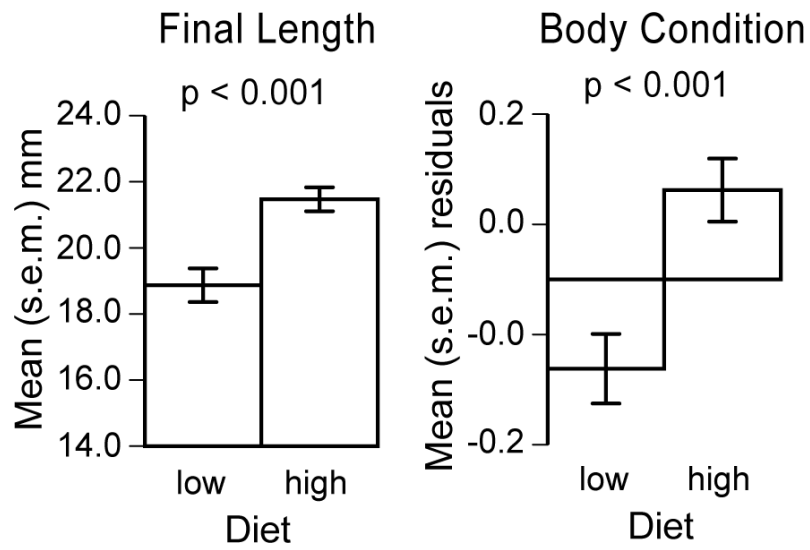


Figure 2: Diet manipulation affected growth (final length) and body condition (residuals of mass regressed on length). There were 15 animals in each diet treatment; p values correspond to two-tailed t tests.

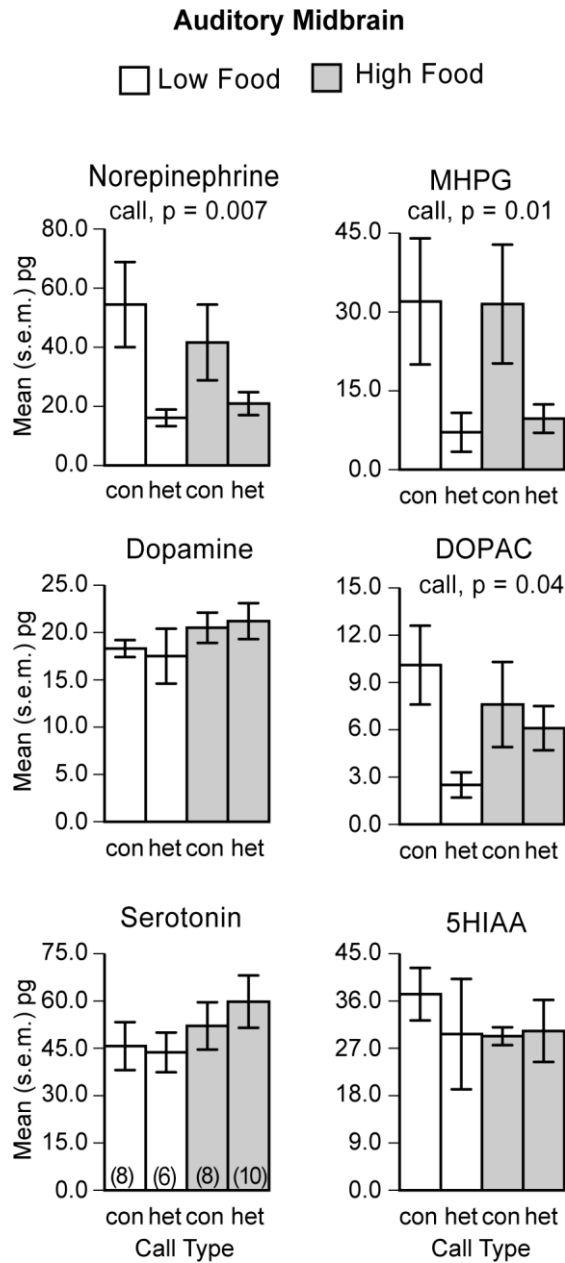


Figure 3: The type of mating call heard (con, conspecific; het, heterospecific) affected noradrenergic and dopaminergic signaling in the auditory midbrain, while diet manipulation (low food, high food) had no detectable effect. The number of animals in each group is indicated parenthetically; p values correspond to two-way ANOVA.

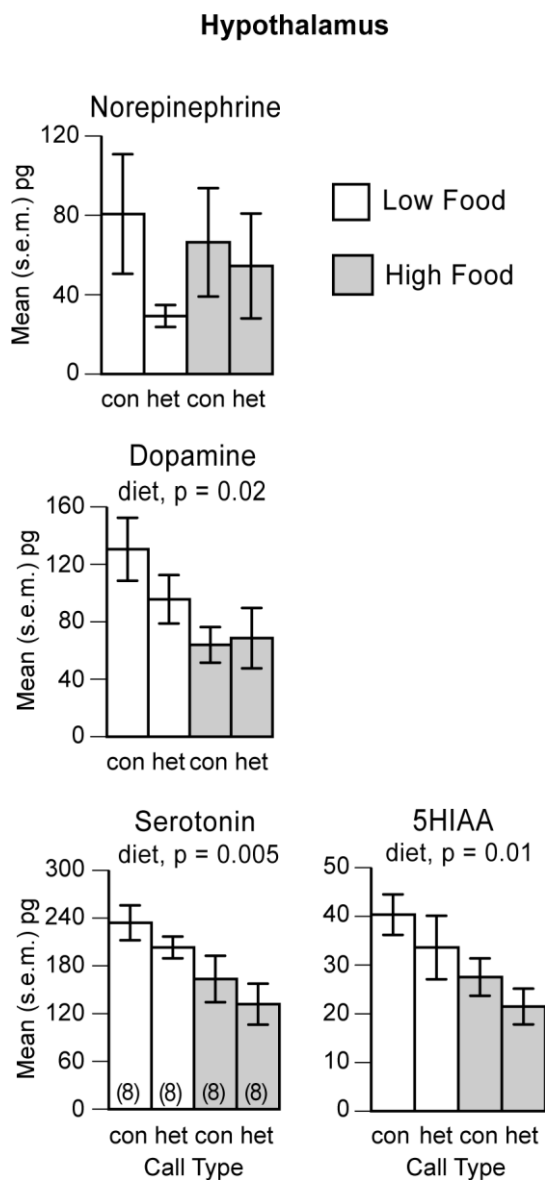


Figure 4: Diet manipulation (low food, high food) affected concentrations of dopamine, serotonin, and serotonin's metabolite (5HIAA) in the hypothalamus, while mating call type (con, conspecific; het, heterospecific) had no effect. The number of animals in each group is indicated parenthetically; p values correspond to two-way ANOVA.

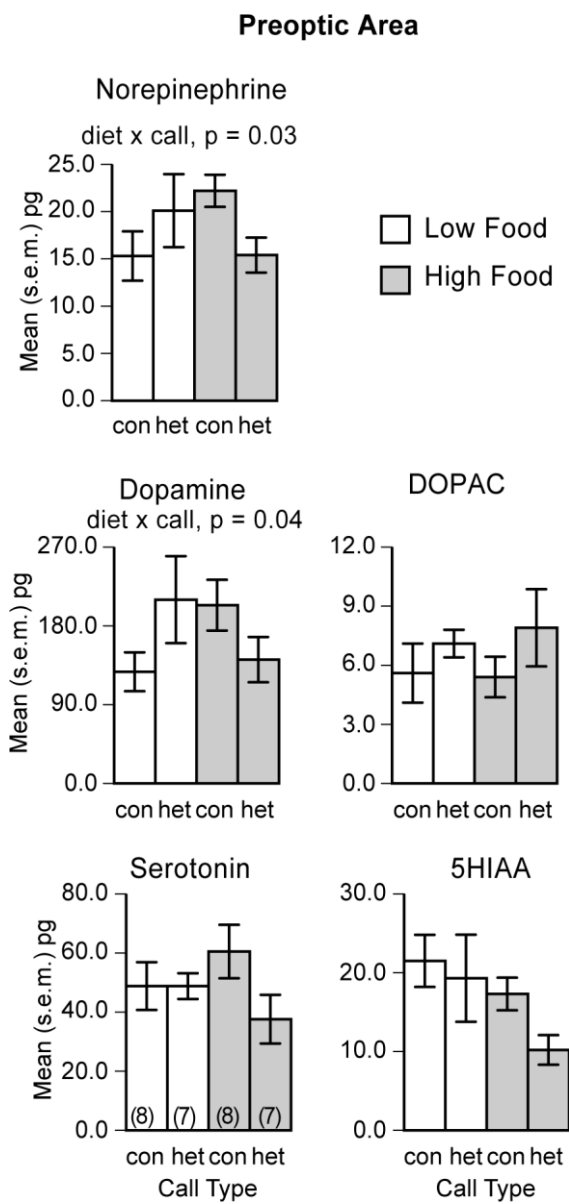


Figure 5: In the preoptic area, diet manipulation (low food, high food) reversed the relative response of norepinephrine and dopamine to mating call type (con, conspecific; het, heterospecific). The number of animals in each group is indicated parenthetically; p values correspond to two-way ANOVA.