Published in final edited form as:

Horm Behav. 2008 February ; 53(2): 342–350.

Neuropeptide Y influences acute food intake and energy status affects NPY immunoreactivity in the female musk shrew (*Suncus murinus*)¹

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Abstract

Neuropeptide Y (NPY) stimulates feeding, depresses sexual behavior, and its expression in the brain is modulated by energetic status. We examined the role of NPY in female musk shrews, a species with high energetic and reproductive demands; they store little fat, and small changes in energy can rapidly diminish or enhance sexual receptivity. Intracerebroventricular infusion of NPY enhanced acute food intake in shrews, however, NPY had little effect on sexual receptivity. The distribution of NPY immunoreactivity in the female musk shrew brain was unremarkable, but energy status differentially affected NPY immunoreactivity in several regions. Similar to what has been noted in other species, NPY immunoreactivity was less dense in brains of *ad libitum* shrews and greater in shrews subjected to food restriction. In two midbrain regions, both of which contain high levels of gonadotropin releasing hormone II (GnRH II) which has anorexigenic actions in shrews, NPY immunoreactivity was more sensitive to changes in food intake. In these regions, acute refeeding (90–180 minutes) after food restriction reduced NPY immunoreactivity to levels noted in *ad libitum* shrews. We hypothesize that interactions between NPY and GnRH II maintain energy homeostasis and reproduction in the musk shrew.

Keywords

obesity; anorexia; orexins; energy; feeding disorder; mating behavior; reproduction; gonadotropin releasing hormone; GnRH II

Introduction

The two behaviors that are the most highly conserved in all animals are feeding and mating (Bronson, 1985; Schneider, 2004). One neuropeptide that is abundant in brains and influences both behaviors is Neuropeptide Y (NPY), a 36 amino acid peptide member of the pancreatic hormone family (Allen, Adrian, Allen, Tatamoto, Crow, Bloom, and Polak, 1983; de Quidt

¹This work is supported by NIH grant R01 MH068729.

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and Emson, 1986; Tatemoto, 1982). NPY is a potent stimulant of feeding behaviour and lipogenesis (Clark, Kalra, Crowley, and Kalra, 1984; Stanley and Leibowitz, 1985; Zarjevski, Cusin, Vettor, Rohner-Jeanrenaud, and Jeanrenaud, 1993). It also plays an important role in maintaining energy intake and body weight under conditions of food restriction and decreased energy expenditure (Herzog, 2003; Kalra and Kalra, 2003; Thorsell and Heilig, 2002). In contrast in rats and hamsters NPY infusions inhibit sexual behaviors (Clark, Kalra, and Kalra, 1985; Corp, Greco, Powers, Marin Bivens, and Wade, 2001; Jones, Pick, Dettloff, and Wade, 2004; Kalra, Clark, Sahu, Dube, and Kalra, 1988). These data taken together suggest that NPY is one of the neuropeptides that acts as an interface between reproduction and energy intake.

NPY is widely distributed within vertebrate species as its presence in the brain and role in stimulating food intake has been confirmed in various vertebrates from fish to humans (Allen et al., 1983; Crespi, Vaudry, and Denver, 2004; Goldstone, 2006; Grove, Chen, Koegler, Schiffmaker, Susan Smith, and Cameron, 2003; Inui, Okita, Nakajima, Inoue, Sakatani, Oya, Morioka, Okimura, Chihara, and Baba, 1991; Kuenzel, Douglass, and Davison, 1987; Morley, Hernandez, and Flood, 1987; Volkoff, Canosa, Unniappan, Cerda-Reverter, Bernier, Kelly, and Peter, 2005). While the function of NPY has been extensively studied in a few temperate zone mammals, to our knowledge no data have been collected in tropical or semi-tropical mammals which have less predictable food supplies and as such may have differences in neuropeptide regulation of appetite and feeding. Here we addressed the role of this well characterized peptide in the musk shrew (*Suncus murinus*). Shrews are primitive mammals belonging to order Insectivora. Musk shrews reside in the tropics and semitropics where they breed year round. Their reproduction is exquisitely tied to food availability, having low body fat stores and high metabolic rates (Temple, 2004). The hypothalamic-pituitary-gonadal (HPG) axis can be shut down by two days of mild food restriction (60% of *ad libitum*) and re-activation begins after 90-minutes of *ad libitum* feeding (Temple and Rissman, 2000a). To date the only neuropeptide that has been examined for its role in feeding in this species is gonadotropin releasing hormone II (GnRH II), which depresses food intake and enhances female sexual behavior (Kauffman and Rissman, 2004; Temple, Millar, and Rissman, 2003; Temple and Rissman, 2000a). NPY stimulates feeding and inhibits sexual behavior (Clark et al., 1985), and thus appears to act in an opposite direction to GnRH II.

In the first two experiments we evaluated several doses of NPY, infused into the lateral ventricles, on acute food intake and sexual receptivity. Next the distribution of NPY immunoreactivity in the musk shrew brain, and the effect of nutritional status on staining densities was quantified.

Materials and Methods

Animals

All experiments were conducted with adult (2–3 months of age), sexually naïve, female musk shrews (*Suncus murinus*) weighing between 20 and 28 g. All animals were born in the breeding colony at the University of Virginia (Charlottesville, VA). After weaning at 21 days of age, the animals were housed individually in cages (28 × 17 × 12 cm) with food (Purina Cat Chow, Nestle Purina PetCare, St. Louis, MO) and water available *ad libitum* (except in Experiment 3 in which food restriction was required). The room, containing only females, was maintained on a 14:10 light:dark photoperiod at temperature of 23 ± 2 °C. All experiments were performed in compliance with regulations of the Animal Care and Use Committee of the University of Virginia.

Experiment 1: Food Intake and NPY

In Experiment 1 the impact of NPY on acute feeding was examined. Adult females were subjected to stereotaxic surgery and a cannula was placed in the lateral ventricle. After 7–10 days of recovery from the surgery, basal food intake was measured for 4 days. Animals were briefly anesthetized and received an intracerebroventricular (*icv*) infusion with one of four doses of NPY (20, 2, 0.2 and 0.02 nmol; n=8–10 per dose group). Control animals received an infusion of vehicle, artificial cerebral spinal fluid (aCSF; n=8). Next, food intake was measured one, three, and 24 hours after the infusion. All shrews were infused at the same time of the day about 30 minutes before lights in the animal room go off. This time was selected because the majority of feeding occurs in the dark portion of the day (Kauffman and Rissman, 2004). All feeding groups were initially matched for average body weight and baseline food intake.

Experiment 2: Sexual behavior and NPY

In Experiment 2 the effect of NPY on female receptivity was examined. Adult females were subjected to stereotaxic surgery and a cannula was placed in the lateral ventricle. After 7–10 days of recovery from the surgery, animals were briefly anesthetized and received an *icv* infusion with one of two doses of NPY (2 nmol; n=12 and 0.2 nmol; n=10). Control animals received an infusion of vehicle, aCSF (n=12). Fifteen minutes after infusion females were paired with a stud male that was habituated to a neutral test box. All shrews were infused and tested at the same time of the day, during the last 4 hours prior to when the room lights went off; musk shrews can display mating behavior at any time in the day (Rissman, unpublished observation). All groups were initially matched for average body weight.

Experiment 3: Immunocytochemistry for NPY

Adult female musk shrews were assigned to one of four feeding groups (n=6 per group); AL, receiving unlimited access to food at all times, FR, food restricted to 60% of baseline intake for 48 hours prior to sacrifice, RF-90, or RF-180, receiving *ad libitum* access to food for 90 or 180 min, respectively, after 48 hours of food restriction. These time points were selected based on our past work in which we found that this schedule of food restriction suppressed mating behavior which was reversed in the majority of females by 90 minutes of *ad libitum* access to food (Temple and Rissman, 2000b). Upon sacrifice brains were removed, fixed, and the tissue was processed for immunocytochemistry with a primary antibody against NPY peptide. Pattern of NPY immunoreactivity and differences in the staining between groups was determined. All musk shrews were sacrificed at the same time of the day.

Experiment 4: Immunocytochemistry for NPY and GnRH II

To assess the colocalization of NPY and GnRH II we conducted a study with brains from 4 females that were food restricted for 12 hours prior to perfusion. The tissue was processed for dual-labeled immunocytochemistry in sequence with first a primary monoclonal antibody against NPY peptide and then a primary rabbit polyclonal antibody made against GnRH II. Presence of NPY- immunoreactive (ir) fibers near GnRH II-ir perikarya was assessed qualitatively. All musk shrews were sacrificed at the same time of the day.

Stereotaxic Surgery

In Experiments 1 and 2, the animals underwent stereotaxic implantation of cannula into the lateral ventricle as previously described (Temple et al., 2003). Briefly, animals were anesthetized with sodium pentobarbital (4.5mg/ml/kg body weight) and/or isoflurane inhalant. Shrews received a midline incision along the top of the head and bupivacaine (0.25% in 0.1 ml) was injected into the muscles above the skull. Shrews were fitted into a modified mouse stereotaxic apparatus and a guide cannula (26-gauge from Plastics One) containing an internal dummy cannula was centered on bregma and positioned –4.5mm rostral-caudal, –1.0mm

medial-lateral. A hole was drilled in the skull and the cannula was lowered to a depth of 2.2mm aimed at the lateral ventricle. The cannula was fixed to the skull with glue and dental acrylic and the tip of cannula was secured with dummy cannula (33-gauge). After the surgery the animal received an injection of saline (sc) and analgesic (Ketoprofen, 2 mg/kg body weight).

Peptide Infusions

In Experiments 1 and 2, on the day of infusion females were briefly anesthetized with isoflurane inhalant (Burns Veterinary Supplies, Inc. Paul, MN, USA) and infused with 2 µl of either artificial cerebral spinal fluid (aCSF, control group) or different doses of NPY. Using an internal cannula (33 gauge) with a 0.5 mm projection attached to a syringe and delivered slowly with an infusion pump over the course of 1 minute in Experiment 1 and 2 minutes in Experiment 2. The internal cannula was left in place for an additional 30–45 secs after the infusion in order to prevent backflow. Then the dummy cannula was replaced and the shrew returned to its cage. At the end of the study to confirm the placement of cannula shrews were anesthetized with an overdose of sodium pentobarbital and 10 µl of India ink was injected into the cannulas. The brains were removed and sectioned on a cryostat. Cannulas were considered placed correctly if ink was present in the ventricular linings.

Food intake measurement and food restriction

In Experiments 1 and 3 animals received pre-weighed food in excess of their normal 24-h intake. The uneaten food was weighed at 24 hours intervals for 4 days and the difference used to calculate the daily average baseline food intake for each individual. In Experiment 1, after the *icv* infusion, individual food intake was recorded one, three and 24 hours later. Animals received pre-weighed food and at the specific time points the remaining food was weighed and the difference used to calculate the food intake. In Experiment 3, once the baseline food intake was determined, animals in groups FR, RF-90 and RF-180 were food restricted for 48 hours (they received 60% of their baseline food intake). After the food restriction period, animals in groups RF-90 and RF-180 received food available *ad libitum* for 90 and 180 min, respectively.

Sexual behavior testing

Female musk shrews do not have estrous cycles and their follicular development and ovulation are both induced by mating (Clendenon and Rissman, 1990; Rissman, 1992; Rissman, Silveira, and Bronson, 1988). Receptivity is initiated by contact with a male. During a typical mating event, sexually naïve females are initially aggressive toward males. The onset of sexual receptivity is marked by a significant reduction in aggression accompanied by tail wagging at which time the male begins to mount and intromit. Usually after a series of mounts that include a single missed, placed or deep intromissions the male ejaculates.

Each female in Experiment 2 was tested once for sexual receptivity 7–10 days after surgery. Tests were conducted between 0900–1300 EST (in the second half of the light portion of the day). Directly after *icv* infusions females were placed in their home cage for 14 minutes and then introduced into a Plexiglas (dimensions: 39 cm × 18 cm × 10 cm high) testing cage containing a sexually experienced male which had habituated to the test box for at least ten minutes. Each test lasted for 60 minutes or until the male attained an ejaculation. If at the end of 60 minutes the animals were engaged in mounting the test was extended until 2 minutes elapsed without any contact between the two shrews. Latencies for the female to begin tail wagging, receive the first mount, first missed and placed intromissions and the latency to the male's ejaculation were recorded. Frequencies of male behaviors including the numbers of missed, placed, and deep intromissions were recorded. Numbers of female vocalizations ("yelling") after the onset of receptive tail-wagging were also recorded.

Brain tissue collection and processing

In Experiment 3 after the appropriate period of food restriction or re-feeding shrews were deeply anesthetized with isoflurane inhalant (Burns Veterinary Supplies, Inc. Paul, MN, USA) and sacrificed by cervical dislocation. The brains were quickly removed, placed into 5% acrolein and incubated on a shaker (160 rpm). After two hours, acrolein was replaced by fresh solution and the brains were fixed overnight at 4°C on the shaker. The next day, brains were placed into 30% sucrose for cryoprotection at 4°C. In Experiment 4 shrews were deeply anesthetized with an overdose of sodium pentobarbital and rapidly perfused first with heparinized saline followed by Zamboni's fixative (15% picric acid in 4% paraformaldehyde). Brains were removed and placed into 30% sucrose.

The fixed brains were cut into 30 µm thin coronal sections in a cryostat into 2 or 3 series. All rinses and solutions were made in 0.02 M Tris-buffered saline (TBS, pH 7.8). The sections were pretreated in 0.3 % hydrogen peroxide in TBS buffer and then incubated in 1 % sodium borohydride to remove residual aldehydes. The sections were then transferred into avidin and subsequently into biotin blocking solution (Avidin Biotin Blocking Kit, Vector Laboratories, Burlingame, CA) to block endogenous biotin. Next, the tissue was incubated in the primary NPY antiserum (1:10,000; IHC 7180, Peninsula Lab., Belmont, CA) overnight at room temperature. After rinses, tissue was incubated in secondary biotinylated anti-rabbit IgG antiserum made in goat (1:500; Vector Laboratories) and then treated with avidin-biotin complex (1:1,000; Vectastain ABC Kit, Vector Laboratories). Immunoreactivity was visualized with nickel intensified diaminobenzidine solution (0.25 % nickel ammonium sulfate and 0.05 % diaminobenzidine) and activated by 0.001% hydrogen peroxide. In order to minimize the differences in staining all the sections were processed in a single run.

In Experiment 4, to visualize both NPY and GnRH-II in the musk shrew brain sections were cut as described and sequential dual labeling was conducted. We used a monoclonal NPY antibody (1:500; Abnova Corp. Taipei, Taiwan) that was validated by western blot (data not shown) and biotinylated horse anti-mouse (1:200; Vector Laboratories) followed by ABC (1:500; Vectastain ABC Kit, Vector Laboratories) and developed with nickel DAB to yield a black appearance. After rinses in TBS, the tissues were incubated overnight in a GnRH II specific polyclonal antibody (#741 generously provided by Dr. Robert Millar; 1:500) which we have validated for use in musk shrew brain previously (Rissman and Li, 1998). Next tissues were incubated in biotinylated goat anti-rabbit followed by ABC at the same concentrations listed above, and developed in DAB to give a brown appearance.

For the NPY polyclonal antibody negative controls from three series of brain sections were processed: the first without the primary antibody, the second control was run without the secondary antibody and the third one was run following the regular procedure except the primary antibody was pre-incubated with 50 µg of NPY peptide prior to staining the brain tissue. After the staining, the sections were mounted on gel-coated glass slides (Superfrost Microscope Slides, Fisher), dehydrated, coverslipped with mounting medium (Cytoseal XYL, Apogent, Kalamazoo, MI) and dried. The staining was analyzed with Olympus BX60 light microscope and the fibers density was measured with MetaMorph Series Software (Molecular Devices Corp. Downingtown, PA).

Image analysis

The sections with brain regions of interest were captured and the images were analyzed with MetaMorph Series Software (Molecular Devices, West Chester, PA). Preoptic area (POA), periaqueductal central grey (PAG) and the region in the musk shrew brain containing the GnRH II neuronal cell bodies were examined with a 10x magnification, while bed nucleus of stria terminalis (BNST), medial habenula (mHB), paraventricular hypothalamic nucleus (PVN),

arcuate nucleus (ARC), median eminence (ME), and paraventricular thalamic nucleus (PVT) were captured with a 20x magnification. The landmarks used to define brain regions were based on the mouse brain atlas (Franklin and Paxinos, 1997) as follows: BNST and POA (bregma 0.38 mm to -0.10 mm), mHB and PVT (bregma -0.82 mm to -1.82 mm), PVN (bregma -0.58 mm to -1.22 mm), ARC (bregma -1.34 mm to -2.46 mm), and PAG (bregma -2.70 mm to -4.16 mm). The region that contains the GnRH II cell bodies is defined by the following landmarks; it is dorsal to the PAG, runs medial to the fasciculus retroflexus in the anterior portion of its extent into a region similar in location to the rostral linear nucleus of the raphe in the mouse (bregma -2.92 to -3.88 mm; Dellovade, King, Millar, and Rissman, 1993).

For each brain region fiber densities of immunoreactivity were analyzed by computerized gray-level thresholding using MetaMorph image analysis. The light intensity and camera settings were kept constant across all sections and areas to standardize the measurements. Immunoreactivity was expressed as the amount of NPY-ir staining (μm^2). A series of sections was used and the data are based on average area covered with NPY-ir per section per animal. The size of the area varied by region but not between subjects.

Statistical analysis

In Experiments 1 and 3 differences between groups were assessed using analysis of variance (ANOVA) and planned comparisons were conducted with Fisher's LSD multiple-comparison test. For latency measurements in Experiment 2, only animals that engaged in the behaviors were included in the analysis. Kruskal-Wallis One way ANOVA on ranks were used followed by Z-value tests.

Results

Food intake is modified by NPY dose

Food intake was acutely affected by NPY infusion in an inverted U dose-response manner. Shrews receiving the middle two NPY doses (0.2 and 2 nmol) ate more in the first hour after infusion than the females receiving the other doses as well as the controls ($F_{(4, 46)} = 3.60$, $p < 0.013$, Figure 1A). At the three hour post-infusion time point no differences in food intake were noted ($F_{(4, 46)} = 1.46$, Figure 1B). By 24 hours after the infusions the females in the highest dose group (20 nmol) had consumed less food than the controls, the 0.2 and the 2 nmol infused females ($F_{(4, 46)} = 2.60$, $p < 0.05$, Figure 1C). When pre-infusion 24 hour baseline intake was compared with the 24 hours post-infusion again no differences were detected ($F_{(4, 46)} = 0.44$) suggesting that the acute increase in feeding was transient.

Sexual behavior not modified by NPY

Only one measure of female receptivity was affected by NPY, male latency to mount the female was significantly decreased ($H = 6.53$, $p < 0.04$), in males paired with females that received the highest dose (2 nmol) of NPY ($p < 0.05$, Table 1). None of the other behaviors we quantified were different between the groups.

NPY immunoreactivity is wide-spread in the musk shrew brain

Immunoreactive NPY was present in many regions in the musk shrew brain. Dense NPY positive fibers were detected in the PVN, PeN, PVT, mHB, suprachiasmatic nucleus (SCN), POA, BNST, ARC, nucleus accumbens (Acb), PAG, and midbrain, where GnRH II cells are located (Table 2). Scattered fibers were observed in the cortex and hippocampus. Immunopositive cells were found in the BNST, PVT, ARC and ME in most animals and cell bodies were found in the cortex and hippocampus of all animals regardless of their feeding condition. No positive staining was detected in any tissues from the negative control groups,

including the preabsorption control. This indicates that the observed immunoreactivity was specific for NPY.

NPY immunoreactivity is correlated with food intake

NPY-ir fiber density was quantified in nine brain regions; the POA, BNST, PVN, ARC, ME, PVT, PAG, the GnRH II cell region, and mHB. In the majority of these areas feeding condition affected NPY-ir. To determine if the amount of time the animals were allowed to eat (for 90 versus 180 minutes) after the 48 hour food restriction had an effect we first compared data from the RF-90 and RF-180 groups using two sample t-tests. No differences were noted between these groups in any region [the range of T values (df=10) were from 0.02 (POA) to 1.66 (mHB)] thus these data were combined and the one way ANOVA was conducted on three groups: AL, FR and RF. NPY-ir area varied with feeding condition in the BNST, PVN, and PVT ($F_{(2,23)} = 13.15, 12.21, 4.82$ respectively; $p < 0.02$ at least, Figures 2 and 3A). The NPY-ir area in all three brain regions was the greatest in the brains of food restricted (FR) and refed (RF) shrews, and least dense in the AL brains ($p < 0.05$).

Another pattern of NPY-ir was noted in the PAG and the vicinity of the GnRH II cell bodies (Dellovade et al., 1993; Rissman, Alones, Craig-Veit, and Millam, 1995). In both locations an overall effect of nutritional status was noted ($F_{(2,23)} = 5.55, 4.49$ respectively; $p < 0.025$ at least, Figures 2 and 3B). However in these two midbrain locations the short refeeding period had an impact on NPY-ir. Planned comparisons revealed that the AL and RF groups had similar low levels of NPY-ir and both were significantly different from FR brains ($p < 0.05$). A final pattern was seen in the ARC and ME. In these areas an overall effect was noted ($F_{(2,23)} = 4.62, 3.60$; $p < 0.05$ at least; Figures 2 and 3C) but the only group differences were between AL and RF brains in the ARC ($p < 0.05$). No effect of nutrition on NPY-ir was noted in the POA or the mHB ($F_{(2,23)} = 2.70, 2.22$ respectively).

Dual labeled brain tissues confirmed that NPY-ir fibers were intermingled within and around the GnRH II-ir cells in the midbrain (Figure 4).

Discussion

The results of this study highlight some similarities and differences in the NPY system between musk shrews and other animals. As described in rats and sheep (Clark et al., 1985; Meister, 2007; Miner, Della-Fera, Paterson, and Baile, 1989; van Dijk and Strubbe, 2003) we found that *icv* infusion of NPY in female musk shrews results in an acute increase in food intake. The most effective doses of NPY are similar to the doses typically given *icv* in hamsters, rats and sheep (Clark et al., 1984; Clark et al., 1985; Corp et al., 2001; Kulkosky, Glazner, Moore, Low, and Woods, 1988; Miner et al., 1989). Treatment with NPY had only a mild influence on female musk shrew receptive behavior. Females treated with 2 nmol NPY (the higher of two doses used) were mounted by males more quickly than females in the vehicle and lower dose group. Female musk shrews do not display lordosis during mating (Rissman et al., 1988), but they do engage in tail wagging which typically precedes the initiation of mating and mounting attempts by the male. In rats and hamsters the aspect of receptivity affected by NPY is specifically the lordotic posture which is inhibited at doses lower than we used (Clark et al., 1985; Corp et al., 2001; Jones et al., 2004; Kalra et al., 1988; Keene, Jones, Wade, and Corp, 2003). NPY-treated female shrews, if anything, tend to display enhanced sexual receptivity, which is opposite to the effect noted in rodents.

Distribution of NPY-ir in the shrew brain was similar to that previously described in the rat brain (Allen et al., 1983; Chronwall, DiMaggio, Massari, Pickel, Ruggiero, and O'Donohue, 1985; de Quidt and Emson, 1986) with positive staining in many regions, including basal forebrain, thalamus, hypothalamus and the midbrain. In addition our data confirm that as in

rats, NPY-ir in several parts of the musk shrew brain is modified by energetic status (Beck, Jhanwar-Uniyal, Burlet, Chapleur-Chateau, Leibowitz, and Burlet, 1990; Sahu, Kalra, and Kalra, 1988). The area of NPY-ir fibers and cells was elevated in food restricted female shrews within several nuclei, notably, the BNST, PVN, PTV, PAG, ARC, and the GnRH II cell region. A trend was present in the ME and no effect of nutrition on NPY-ir was noted in the POA or the mHB. In rat and rhesus monkey brains fasting for 2–3 days elevated NPY immunoreactivity or mRNA consistently in the ARC and PVN (Grove et al., 2003; Sahu et al., 1988). Interestingly, in the male rat PVN, NPY-ir decreased to *ad libitum* levels one day, but not 6 hours, after the fast ended (Beck et al., 1990; Sahu et al., 1988). In our study NPY-ir in the shrew PVN was not affected by 90–180 minutes of refeeding. In rats, NPY mRNA and immunoreactivity have been reported to change quickly after fasting and refeeding in the ARC (Beck et al., 1990; Brady, Smith, Gold, and Herkenham, 1990; Sahu et al., 1988). Our data reveal that the NPY-ir in the musk shrew ARC is less sensitive to energetic status. It is likely that in rats, and other animals with spontaneous estrous cycles, changes in estradiol (E2) caused by food restriction produce increased levels of NPY in the ARC (Bonavera, Dube, Kalra, and Kalra, 1994; Pelletier, Li, Luu-The, and Labrie, 2007). Musk shrews display mating-induced ovulation and very low plasma levels of E2 up until 24 hours after mating (Fortune, Eppig, and Rissman, 1992), thus sexually naïve females in this study had very low levels of plasma E2 and estrogen receptor in brain that did not change after food restriction (Temple and Rissman, 2000b). It would be interesting to examine interactions between E2, NPY and food intake in other mating-induced ovulating species.

One of the novel findings in our study is that NPY-ir in two areas associated with the GnRH II cells was highly responsive to energy status. NPY has been previously reported in the rat PAG and the region where the GnRH II neurons are observed in shrews but not rats (de Quidt and Emson, 1986). In the musk shrew not only did these two areas have elevated NPY-ir after food restriction the NPY-ir decreased rapidly after refeeding. The third GnRH II-related area we examined, the mHB, was non-responsive to food condition and this region also has little or no NPY-ir in the rat (de Quidt and Emson, 1986). GnRH II mRNA and peptide content are likewise affected by energy status, both decrease after 2 days of food restriction (the same feeding paradigm used here) and GnRH II mRNA was reversed by 90 minutes of *ad libitum* access to food (Kauffman, Bojkowska, Wills, and Rissman, 2006). GnRH II content in the PAG showed the inverse response to restriction and refeeding as compared with NPY-ir; GnRH II peptide levels dropped in restricted females and increased after 180 minutes (but not sooner) of food intake. In the mHB, the major terminal field for GnRH II neurons (Rissman et al., 1995) GnRH II peptide displayed the same highly plastic response to energy status which is at odds with the lack of change in NPY-ir in this area.

Previous studies in musk shrews have shown when the energy resources are scarce, females are unlikely to mate and one factor responsible for this effect is the low level of GnRH II (Kauffman and Rissman, 2004). In contrast, when females are on *ad libitum* diets or they are food restricted, acute infusions of GnRH II depressed food intake (Kauffman, Wills, Millar, and Rissman, 2005). Since GnRH II is an evolutionarily conserved neuropeptide found in various vertebrates and non-vertebrate species, it is possible that it interacts with some of the other highly conserved hypothalamic peptides that modulate feeding behavior; such as NPY. Since the PAG and the midbrain GnRH II cells area are mirror images in terms of alterations in NPY and GnRH II content after food restriction and refeeding, we suggest that these may act as integration sites that coordinate the signals from GnRH II and NPY. Distribution of GnRH type II receptors are not well characterized but like NPY, are fairly wide spread in musk shrew brains (Temple et al., 2003). In rats the NPY Y1 and Y2 receptors are present in the PAG and the analogous area to the GnRH II cells in shrews (Stanic, Brumovsky, Fetissov, Shuster, Herzog, and Hokfelt, 2006; Wolak, DeJoseph, Cator, Mokashi, Brownfield, and Urban, 2003). It is possible that the GnRH II neurons, and/or the PAG serve to coordinate

multiple neurotransmitter inputs which respond to energy balance to affect reproductive behavior.

Many studies have addressed the interactions between NPY and the other feeding regulators. Other hormones or peptides, such as AgRP are known to stimulate the ingestive behavior in a similar manner as NPY (Swart, Jahng, Overton, and Hout, 2002). In contrast, α -MSH, norepinephrine and serotonin decrease food intake and are believed to antagonize the actions of NPY (Ramos, Meguid, Campos, and Coelho, 2005). In musk shrews, both GnRH I and GnRH II fibers are present in the hypothalamus. Protein and mRNA levels of both peptides are affected by food intake (Kauffman et al., 2006; Temple and Rissman, 2000a). The amount of GnRH I peptide in the median eminence is reduced by food restriction and this affects ovulation (Temple and Rissman, 2000a), and GnRH II reduces food intake and is permissive for reproduction (Temple et al., 2003). Since NPY is modulated by food and the NPY neurons are in the same brain areas as the GnRH I cell bodies, these peptides might interact and our future studies will concentrate on the determining the hierarchy and interactions between these neuropeptides in the musk shrew.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Ms. Aileen Wills and Dr. Alexander Kauffman for technical assistance.

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Figure 1.

Mean ($\pm$ S.E.M) food intake (in grams) of animals that received NPY or vehicle infusions. Presented are data from animals 1 hour (A), 3 hours (B), and 24 hours (C) after infusion. Five doses were given, each dose group contained 10 female shrews, except the 0.02 nmol dose had only 9 animals, and the control group had 8 females. The doses of NPY were 0.02, 0.2, 2, or 20 nmol, controls received vehicle (0 nmol NPY in aCSF). * Significantly greater food intake at 1-hour post infusion as compared with the other dose groups ($p < 0.05$). ** Significantly more food consumed in 24 hours than females in the 20 nmol group ($p < 0.05$).

Figure 2.

Images of NPY immunoreactivity in the musk shrew brain. Representative images are taken from four locations: the bed nucleus of stria terminalis (BNST), paraventricular nucleus of thalamus (PVT), paraventricular hypothalamic nucleus (PVN), and the periaqueductal grey (PAG). Representative sections from animals in each feeding condition are presented. Shrews were maintained on *ad libitum* (AL) access to food or were food restricted (FR). Another group of the food restricted animals were subsequently re-fed (RF) for 90 or 180 minutes prior to sacrifice. ac=anterior commissure, d3V=dorsal third ventricle, 3V=third ventricle, Aq=aqueduct. Scale bar=100 μ m.

Figure 3.

Mean ($\pm$ S.E.M) area of NPY fibers (μm^2) in the female musk shrew brain. Animals were in 3 feeding groups: *ad libitum* (white bars; $n=6$), food restricted for 48 hours to 60% of baseline intake (cross-hatched bars, $n=6$), food restricted and re-fed for either 90 or 180 minutes (RF, black bars, $n=12$). A) data from the bed nucleus of stria terminalis (BNST), the paraventricular nucleus of hypothalamus (PVN), and paraventricular nucleus of thalamus (PVT), B) data from the periaqueductal gray (PAG), and region surrounding the GnRH II cells containing neurons (GnRH II cells), C) NPY-ir data from the arcuate nucleus (ARC), and the median eminence (ME). * Significantly different from the Food Restricted and ReFeed groups in the same area

($p < 0.05$). ** Significant difference between the Ad Lib and ReFeed groups in the same area ($p < 0.05$). + Significant differences between the Ad Lib and ReFeed groups ($p < 0.05$).

Figure 4. Low (A) and high (B) magnification photomicrographs of dual labeled GnRH II neurons and NPY fibers in the female musk shrew brain. The brown cells are labeled for GnRH II-ir and the black fibers are labeled for NPY-ir. The scale bar in A=100 microns and that in B=10 microns.

Table 1

Sexual behavior in paired musk shrews. Females were treated *icv* with vehicle or NPY prior to testing. Data are mean $\pm$ SEM frequencies and latencies (in seconds). Numbers per group are listed in *italics*.

Musk Shrew Mating Behaviors:	aCSF	0.2 nmol NPY	2.0 nmol NPY
Latency for female to tail wag (sec)	957 $\pm$ 139 <i>12</i>	945 $\pm$ 173 <i>9</i>	621 $\pm$ 110 <i>11</i>
Latency for male to mount (sec)	1146 $\pm$ 134 <i>12</i>	1084 $\pm$ 267 <i>7</i>	651 $\pm$ 160* <i>11</i>
Latency for first intromission (sec)	1265 $\pm$ 129 <i>12</i>	1225 $\pm$ 248 <i>7</i>	937 $\pm$ 186 <i>11</i>
Latency to ejaculation (sec)	2746 $\pm$ 364 <i>6</i>	2569 $\pm$ 332 <i>5</i>	2282 $\pm$ 419 <i>7</i>
Latency between female tail wag and male mounting (sec)	189 $\pm$ 84 <i>12</i>	92 $\pm$ 144 <i>7</i>	30 $\pm$ 85 <i>11</i>
Number of yells emitted by female	28 $\pm$ 5.5 <i>12</i>	34.9 $\pm$ 11.1 <i>10</i>	27.6 $\pm$ 10.9 <i>12</i>
Total number of intromissions	43.3 $\pm$ 6.6 <i>12</i>	43.8 $\pm$ 12.8 <i>10</i>	48.8 $\pm$ 8.6 <i>12</i>

*
p<0.05 different from control group

Table 2

Selected distribution of NPY-ir fibers and cells in the musk shrew forebrain and midbrain. Cell density and fiber staining intensity were evaluated in brains from food restricted animals (high, +++; moderate, ++; low, +; and not detected, nd)

Region	Fibers	Cells
Forebrain		
<i>Nucleus accumbens (Acb)</i>	+	+
<i>Caudate putamen (CPu)</i>	+	+
<i>Medial preoptic area (mPOA)</i>	++	nd
<i>Clastrum (Cl)</i>	+	+
<i>Bed nucleus of the stria terminalis (BNST)</i>	++	+
Medial Basal Region		
<i>Periventricular nucleus (PeN)</i>	++	nd
<i>Paraventricular nucleus (PVN)</i>	+++	+
<i>Arcuate nucleus (ARC)</i>	++	++
<i>Dorsomedial ventricular nucleus (DM)</i>	+++	nd
<i>Medial habenula (mHB)</i>	+	nd
<i>Ventromedial Nucleus (VMN)</i>	+	nd
<i>Medial amygdala (MePD)</i>	+	+
<i>Lateral hypothalamus (LH)</i>	+	nd
<i>Paraventricular thalamic nucleus (PVT)</i>	+	nd
<i>Hippocampus CA1,2 3</i>	+	++
<i>Median eminence (ME)</i>	++	++
Midbrain		
<i>Periaqueductal grey (PAG)</i>	++	nd
<i>Anterior and posterior pretectal nucleus (PPT)</i>	+	++
<i>GnRH II cell body region (Similar to the rostral linear raphe nucleus)</i>	+	nd