Terminal Nerve-Derived Neuropeptide Y Modulates Physiological Responses in the Olfactory Epithelium of Hungry Axolotls (*Ambystoma mexicanum*)

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The vertebrate brain actively regulates incoming sensory information, effectively filtering input and focusing attention toward environmental stimuli that are most relevant to the animal’s behavioral context or physiological state. Such centrifugal modulation has been shown to play an important role in processing in the retina and cochlea, but has received relatively little attention in olfaction. The terminal nerve, a cranial nerve that extends underneath the lamina propria surrounding the olfactory epithelium, displays anatomical and neurochemical characteristics that suggest that it modulates activity in the olfactory epithelium. Using immunocytochemical techniques, we demonstrate that neuropeptide Y (NPY) is abundantly present in the terminal nerve in the axolotl (*Ambystoma mexicanum*), an aquatic salamander. Because NPY plays an important role in regulating appetite and hunger in many vertebrates, we investigated the possibility that NPY modulates activity in the olfactory epithelium in relation to the animal’s hunger level. We therefore characterized the full-length NPY gene from axolotls to enable synthesis of authentic axolotl NPY for use in electrophysiological experiments. We find that axolotl NPY modulates olfactory epithelial responses evoked by L-glutamic acid, a food-related odorant, but only in hungry animals. These results suggest that expression or activity of NPY receptors in the olfactory epithelium may change with hunger level, and that terminal nerve-derived peptides modulate activity in the olfactory epithelium in response to an animal’s changing behavioral and physiological circumstances.

Key words: *Ambystoma*; electro-olfactogram; modulation; NPY; olfaction; patch clamp

Introduction

In recent years, great strides have been made in elucidating the molecular and physiological basis of odorant transduction and processing (Firestein, 2001; Kauer, 2002; Laurent, 2002). Most such research focuses on mechanisms involving single receptor neurons, and the role of multicellular interactions, including those involved in neuromodulation, receives little attention. Nevertheless, a complete understanding of peripheral odorant processing must encompass the role of neuromodulators.

The terminal nerve is the most anterior vertebrate cranial nerve, extending between the nasal cavity and basal forebrain (Fig. 1). Although biologists have long known of its existence (Fritsch, 1878), its function has remained mysterious. Because of its anatomical projections, the nerve has been suggested to function as a chemosensory system (Rossi et al., 1972; Demski and Northcutt, 1983), although behavioral and electrophysiological studies have failed to demonstrate a sensory role (Bullock and Northcutt, 1984; Fujita et al., 1991; Wirsig-Wiechmann, 1993; White and Meredith, 1995). Instead, the terminal nerve probably serves a neuromodulatory function: its ganglion cells are rhythmically active (Oka, 1992, 2002), and the undifferentiated fibers contain acetylcholine and potentially neuromodulatory peptides (Wirsig-Wiechmann et al., 2002).

One such peptide that has been positively identified is gonadotropin-releasing hormone (GnRH) (Sherwood et al., 1986; King and Millar, 1992). In previous work, we showed that GnRH modulates both odorant responses and voltage-activated currents in the olfactory epithelium in salamanders (Eisthen et al., 2000; Park and Eisthen, 2003). In teleost fishes, an additional branch of the nerve projects to the retina, and both GnRH and olfactory input modulate retinal activity (Walker and Stell, 1986; Maaswinkel and Li, 2003; Behrens and Wagner, 2004; Huang et al., 2005).

A second class of terminal nerve fibers contains a compound that has not been identified, but which displays immunoreactivity to neuropeptide Y (NPY) and to a smaller peptide, Phe-Met-Arg-Phe-NH2 (FMRFamide), that resembles the C terminus of the NPY molecule (Chiba et al., 1994, 1996; Chiba, 2000, 2005).
NPY-containing fibers are widely distributed in vertebrate brains, and the peptide plays an important role in the control of appetite and feeding (Kalra and Kalra, 2004; Levens et al., 2004).

We used immunocytochemical methods to demonstrate that the terminal nerve in an aquatic salamander, the axolotl (*Ambystoma mexicanum*), displays NPY-like immunoreactivity; control experiments indicate that the labeling obtained is not attributable to the presence of a FMRFamide-like compound. We then examined the possibility that NPY modulates activity in the olfactory epithelium. Because exogenous forms of NPY can produce non-physiological responses when interacting with receptors, we sequenced the NPY gene in axolotls to allow synthesis of endogenous axolotl NPY for use in electrophysiological experiments. Using electro-olfactogram (EOG) recording techniques, we found that synthetic axolotl NPY enhances responses evoked by a feeding cue, t-glutamic acid, but only in hungry axolotls. In previous work, we found that GnRH modulates a tetrodotoxin (TTX)-sensitive inward current in salamander olfactory receptor neurons (Eisthen et al., 2000). Here, we report that synthetic axolotl NPY also modulates the magnitude of this current, but only in hungry animals.

**Materials and Methods**

**Subjects.** In all experiments, equal numbers of adult male and female axolotls were used as subjects. Axolotls were obtained from the Indiana University Axolotl Colony and maintained at 20°C in Holtfreter's solution, which contained the following (in mM): 60 NaCl, 2.4 NaHCO3, 0.67 KCl, 0.81 MgSO4, and 0.68 CaCl2, pH 7.5–7.6.

Except where noted, animals were fed commercial salmon pellets (Rangen, Buhl, ID) twice each week. All procedures were conducted under the supervision of the institutional animal care and use committee of Michigan State University (East Lansing, MI) and Queen's University Belfast (Belfast, UK), in accordance with guidelines established by the United States Public Health Service and the Animals (Scientific Procedures) Act, United Kingdom.

**Immunocytochemistry.** Because the terminal nerve consists of bipolar neurons with long, tapering fibers, we chose to use immunocytochemical methods to visualize the full extent of NPY-containing cells and fibers in the brain and olfactory epithelium. In addition, because the deduced sequence of NPY in axolotls is largely identical to those found in other vertebrates, we were able to use a commercially available antiserum generated against porcine NPY to examine the distribution of the peptide in the CNS.

Four male and four female axolotls were transcardially perfused with PBS followed by 4% paraformaldehyde in the same buffer. Brains with olfactory nerves and nasal cavities attached were dissected out and postfixed for 96 h at 4°C. After cryoprotection with 30% sucrose for 12 h followed by 30% sucrose for 24 h, horizontal sections (60 μm) were cut, mounted on slides, incubated in primary antisera at a 1:2000 dilution [anti-porcine NPY, shown to cross-react with the C terminus (13–36 and 18–36) of NPY in dot blots; N-9528; Sigma, Poole, UK] for 24 h, washed, and then incubated in secondary antibody (FITC-labeled swine anti-rabbit; 1:100; Sigma) for 24 h. For clarity, some sections were counterstained with Hoechst 33258, a fluorescent Nissl stain (Sigma). Sections were viewed and photographed using a Leica (Nussloch, Germany) acousto-optical beam splitter confocal scanning laser microscope. Controls, all of which were performed on at least two of the eight specimens, included (1) omission of primary antibody, (2) preabsorption (12 h) of anti-NPY (1:2000) with synthetic axolotl NPY (250 ng/ml), and (3) omission of secondary antibody, all of which were performed on at least two of the eight specimens, included (1) omission of primary antibody, (2) preabsorption (12 h) of anti-NPY (1:2000) with synthetic axolotl NPY (250 ng/ml = 0.05 nm; American Peptide, Sunnyvale, CA), (3) preabsorption (12 h) of anti-NPY (1:2000) with synthetic Gly-Tyr-Ile-Arg-Phe-NH2 (GyrFamide; 250 ng/ml = 0.4 nm; American Peptide) and, (4) in separate experiments, preabsorption (12 h) of anti-NPY (1:1000) with synthetic FMRFamide (4.95 μg/ml = 10 μm; Sigma).

The primary antisera was generated by conjugating porcine NPY to keyhole limpet hemocyanin (KLH), which can lead to artifactual staining in olfactory areas (Riddle and Oakley, 1992). We therefore compared labeling obtained with anti-NPY with that obtained using an anti-KLH antisera (A150–104A; Bethyl Laboratories, Montgomery, TX) to ensure specificity of labeling. Anti-KLH produced labeling in peripheral structures, such as the Bowman’s glands surrounding the olfactory epithelium, but not in neural tissue.

**RNA extraction and standard PCR analysis.** Poly A+ RNA was extracted from axolotl brain (62.5 mg) using Dynabeads mRNA Direct kit (Dynal Biotech, Wirral, UK) and converted to 5’ and 3’ rapid amplification of cDNA ends (RACE)-ready cDNA (SMART RACE kit protocol; BD Biosciences, Oxford, UK). Gene specific primers (GSPs) and nested GSPs were designed to the peptide-encoding region of the *Typhlonectes natans* NPY gene (Ebersole et al., 2001) (Table 1) and manufactured by Sigma-Genosys (Haverhill, UK). First-strand cDNA was used in an initial PCR with a sense primer corresponding to the amino acid sequence PSKDPDN (TN-NPY-S1) and an antisense primer corresponding to the amino acid sequence LITRORY (TN-NPY-A1) of *T. natans* NPY. Products were reamplified with nested sense and antisense primers designed to the internal sequences NPGE/DA (TN-NPY-S2) and RHY/INLI (TN-NPY-A2), respectively. Components of each 50 μl primary PCR were as follows: 5 μl 10× PCR buffer (Applied Biosystems, Warrington, UK), 6 μl MgCl2 (25 mM; Applied Biosystems), 1 μl deoxyribonucleotide triphosphate (dNTP) mix (10 mM; Promega, Southampton, UK), 2.5 μl cDNA, 1 μl sense GSP (20 μM), 1 μl antisense GSP (20 μM), 0.25 μM AmpliTaq Gold DNA polymerase (5 U/μl; Applied Biosystems), diH2O to 50 μl. Secondary nested reactions were identical except that the template consisted of 2 μl of the primary reaction ( neat) and sense and antisense nGSPs were used. Thermal cycling conditions for standard PCRs were as follows: initial denaturation and “hot start” at 94°C for 7 min, 40 cycles of 94°C for 1 min, 45°C for 1 min, and 72°C for 1 min followed by a final extension step of 72°C for 7 min.

**Table 1. PCR primers**

<table>
<thead>
<tr>
<th>Oligonucleotide code</th>
<th>Nucleotide Sequence (5′→3′)</th>
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<tbody>
<tr>
<td>1. TN-NPY-S1</td>
<td>CATTCAAAACCGGAGCATCCT</td>
</tr>
<tr>
<td>2. TN-NPY-S2</td>
<td>AACTCTTGGAGAGGGAGG</td>
</tr>
<tr>
<td>3. TN-NPY-A1</td>
<td>ATATCTTCATCTGTATGAGAG</td>
</tr>
<tr>
<td>4. TN-NPY-A2</td>
<td>GATGGATTGATGTAAATGC</td>
</tr>
<tr>
<td>5. AM-NPY-RS1</td>
<td>GGGCAGAATACCTGGCGACTGAG</td>
</tr>
<tr>
<td>6. AM-NPY-RS2</td>
<td>ACTGGACATGGATCATTACCT</td>
</tr>
<tr>
<td>7. AM-NPY-RS3</td>
<td>TCAATGGCGAGATTTGGGCC</td>
</tr>
<tr>
<td>8. AM-NPY-RA1</td>
<td>ATGATTTCGCGATCTTCCCGAG</td>
</tr>
<tr>
<td>9. AM-NPY-RA2</td>
<td>TCCCTCGGGGCGGCTGCTCTC</td>
</tr>
</tbody>
</table>

Oligonucleotide sequences designed against *Typhlonectes natans* NPY-encoding gene (1–40) were used in standard PCR, and those against *Ambystoma mexicanum* NPY-encoding gene fragments (5–10) were used in 5′ and 3′ RACE.
**Table 2. Responses of all cells examined**

<table>
<thead>
<tr>
<th>Hunger level</th>
<th>Treatment</th>
<th>Responders</th>
<th>Nonresponders</th>
<th>Uncategorizable</th>
<th>Total cells</th>
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</thead>
<tbody>
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<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>5 μM NPY</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>1 μM NPY</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>100 nM NPY</td>
<td>0</td>
<td>6</td>
<td>2</td>
<td>8</td>
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<tr>
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<td>2</td>
</tr>
<tr>
<td></td>
<td>5 nM NPY</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1 nM NPY</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>2</td>
<td>9</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>Hungry</td>
<td>1 μM NPY</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>100 nM NPY</td>
<td>7</td>
<td>11</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>4</td>
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<td>Total cells</td>
<td>12</td>
<td>41</td>
<td>3</td>
<td>56</td>
<td>76</td>
</tr>
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</table>

Response of the inward current in olfactory receptor neurons from well fed and hungry animals to different concentrations of synthetic axolotl NPY, as well as a control solution (last green). Significantly more neurons from hungry animals responded to application of NPY with an increase in the magnitude of the inward current, although neurons from well fed animals were exposed to higher concentrations of NPY. For categorization criteria, see Materials and Methods.

**RACE.** First-round 3′ and 5′ RACE reactions were performed using the manufacturer's Universal Primer Mix (UPM; BD Biosciences) and sense (AM-NPY-RS1) or antisense (AM-NPY-AS1) GSPs. Reamplification was performed using nGSPs [sense (AM-NPY-RS2 or AM-NPY-RS3)/antisense (AM-NPY-RA2 or AM-NPY-RA3)] (Table 1) and nested UPM. Components of each 50 μl primary RACE reaction were: 5 μl 10X PCR buffer, 6 μl MgCl2 (25 mM), 1 μl dNTP mix (10 mM), 2.5 μl RACE-ready 5′ or 3′ cDNA, 5 μl 10X UPM, anchor primer, 1 μl GSP (20 μM), 0.25 μl AmpliTaq Gold DNA polymerase (5 U/μl), ddH2O to 50 μl. Secondary nested reactions had identical components to primary reactions, except that the template consisted of 5 μl of the primary reaction (1:50 dilution in Tricine EDTA buffer) and 10X UPM was replaced by 1 μl nested UPM (nUPM). The cycle profile for all RACE reactions was as follows: 1 cycle of 94°C for 7 min, 40 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min followed by 1 cycle of 72°C for 7 min.

**Cloning and sequence analysis.** Discrete bands from standard PCR and RACE reactions were gel purified using a Qiagen gel extraction kit (Qiagen, Crawley, UK) and cloned using a TOPO TA cloning kit (Invitrogen, Paisley, UK). Plasmid DNA was extracted using a miniprep kit (Sigma) and sequences were confirmed using M13 forward and reverse primers using Applied Biosystems Big-Dye version 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer. At least three independently derived clones were sequenced for each PCR.

**Electro-olfactogram recordings.** We examined the effects of synthetic axolotl NPY on odorant responses in the olfactory epithelium. NPY is involved in many activities in the CNS, but most attention has been focused on its role in regulating appetite and hunger (Michel, 2004). We therefore investigated the possibility that modulatory effects of NPY on odorant responses in the olfactory epithelium. NPY is found in a previous experiment that bath application of GnRH decreases the magnitude of odorant responses evoked by amino acid odorants, as described in Eisthen et al. (2000). Briefly, animals were decapitated, the nasal sac dissected out of its capsule, slit longitudinally, opened flat, and then attached to a support with cyanoacrylate glue. Slices 150–250 μm thick were cut with a Vibratome (Leica model VT1000) and stored at 4°C up to 48 h, until use.

Slices were stored in amphibian physiological saline (APS) containing the following (in mM): 120 NaCl, 10 HEPES, 10–20 CaCl2, 5 glucose, 5 pyruvic acid, 2.5 KCl, and 1 MgCl2, pH 7.4. For some recordings, we did not use ionic substitutions in the intracellular solution; in these experiments, the recording pipette was filled with a solution containing the following (in mM): 105 K gluconate, 30 KCl, 10 HEPES, 5 ATP, 3 MgSO4, 1 EGTA, 0.5 GTP, and 0.5 CaCl2. Our preliminary experiments suggested that axolotl NPY strongly affected an inward current, so we chose to concentrate our efforts on characterizing this effect. In most experiments, then, CsCl was substituted for KCl to block large K+ currents. In addition, Ca2+-dependent K+ currents were blocked by 10−4 M apamin. The inward current carried by Ba2+ is slowly inactivating and readily distinguishable from the Na+ current. In such experiments, the intracellular solution contained the following (in mM): 135 CsCl, 10 HEPES, 5 ATP, 3 MgSO4, 1 EGTA, 0.5 GTP, and 0.085 CaCl2. Synthetic axolotl NPY dissolved in APS was used at concentrations ranging from 1 nM to 10 μM (Table 2).

Epithelial slices were mounted in a recording chamber and viewed with a 40× water immersion objective on a Zeiss (Thornwood, NY)
Axioskop 2 FS microscope. Patch electrodes of borosilicate glass (LE-16; Dagan, Minneapolis, MN) were pulled on a Flaming-Brown micropipette puller (Sutter Instruments, Novato, CA) and coated with wax to reduce electrode capacitance; initial pipette resistance was generally 3–6 MΩ. All recordings were conducted at room temperature using an Axopatch 200B amplifier (Molecular Devices) with a low-pass Bessel filter set at 5–10 kHz. Raw data were collected and leak current was subtracted before analysis using AxoGraph software (version 4.6; Molecular Devices).

For each recording, we followed a standard protocol to ensure that data collected from all cells could be subjected to statistical analysis. Specifically, once a seal of 1–4 GΩ was attained, the membrane under the electrode was ruptured and a holding potential of −60 mV was applied. We recorded responses to a series of 17.5 ms voltage pulses ranging from −100 to 100 mV in 10 mV steps, then initiated the flow of bath solution (APS) over the slice and recorded responses to the same set of pulses 1 min later. Recordings were made every 2.5 min in flowing bath until the recording stabilized; for most cells, only two sets of recordings were made in flowing bath before NPY was applied. After the flow of NPY was initiated, recordings were made at 2.5 min intervals for 15–20 min before the NPY was washed off with plain APS. Recordings were made at 2.5 min intervals for 20–30 min during the wash period. In control recordings, APS containing fast green was substituted for NPY.

The axolots used in this experiment were subjected to the same feeding regime described above; overall, data were collected from 24 cells from six hungry animals (three male, three female) and 31 cells from 11 well-fed animals (seven male, four female). We included in our analyses only recordings for which we obtained at least 15 min of data in NPY or the control solution and 10 min of data in the wash, the measured series resistance did not vary by >10 MΩ, and the seal was at least 1 GΩ throughout the recording.

In a separate experiment, the identity of the inward current was examined through the application of 100 nM TTX (Sigma) in the bath solution. For these recordings, we included 100 nM NPY in the TTX solution to ensure that the response obtained was caused by the TTX and not simply to washing off the NPY solution. Only animals in the hungry condition were used for these recordings.

For most concentrations of NPY, the number of cells examined is too small for statistical analysis. However, many recordings were obtained from cells exposed to 100 nM NPY and these data were subjected to statistical analysis, performed using the JMP software package (v. 5.0; SAS Institute). Before conducting analyses of variance, Levene’s test was used to assess homogeneity of variances among groups being compared; because the results were not significant for the magnitude of the inward current, for the voltage sensitivity of the inward current, and for the analysis of rise time among cells classified as responders (F values ≤ 1.5; p values ≥ 0.25), untransformed data were used in these analyses. Because Levene’s test indicated that the variances were not homogeneous for rise time data (F = 13.6; p = 0.0003), log-transformed data were used in this analysis (F = 1.16; p = 0.29). All statistical tests were two-tailed.

**Results**

**The terminal nerve shows abundant NPY-like immunoreactivity**

In salamanders, unlike most other jawed vertebrates, the cells of the terminal nerve do not form a discrete ganglion; rather, the ganglion cells are scattered in small clusters between the nasal cavity and anterior olfactory bulb (Wirsig and Getchell, 1986). We found that NPY-immunoreactive cells and fibers are present throughout the terminal nerve pathway in axolots (Fig. 2): labeled cells were observed in fascicles of olfactory nerve fibers underneath the olfactory epithelium (Fig. 2A), in the olfactory nerve proper (Figs. 2B, C1), in the olfactory nerve layer on the surface of the olfactory bulb (Fig. 2J), in the ventral olfactory bulb (Fig. 2D), and in the anterior preoptic area (Figs. 2E, L). Additional labeled cells were observed beyond the pathway of the terminal nerve (Northcutt and Muske, 1994; Fiorentino et al., 2001) in the posterior preoptic area, hypothalamus, and infundibulum (Figs. 2E, L–N). No labeled cell bodies were observed in the midbrain and hindbrain.

Peripherally, immunoreactive terminal nerve fibers could be traced within the olfactory nerve to the lamina propria of the olfactory epithelium, where they appeared to terminate. No labeled cells or fibers were observed in the olfactory epithelium (Fig. 2F). In the brain, fibers were densely labeled in the olfactory bulb and tectum (Figs. 2J, L). Labeled fibers were observed in the lateral portion of the dorsal telencephalon (Fig. 2J), and in the medial portion only more ventrally (Figs. 2K, L). Sparsely labeled fibers were also observed in the medulla and tegmentum (Figs. 2J, K, respectively), and in the hippocampal, posterior, and cerebellar commissures. Some labeled fibers were present in the medial forebrain bundle, but were conspicuously absent from the lateral forebrain bundle. Fiber labeling was particularly dense in the neurohypophysis (data not shown).

Immunostaining was not observed in control sections for which the primary antiserum was omitted. Importantly, labeling was not abolished after preadsorption with synthetic GTTRFamide (0.4 nM) (Fig. 2G) or FMRFamide (10 μM) (Fig. 2H) but was eliminated after preadsorption of the primary antiserum with synthetic axolotl NPY (0.05 nM). These data indicate that the labeling observed with the anti-NPY antiserum is unlikely to be attributable to cross-reaction with a FMRFamide-like peptide (Chiba, 2000).

**The sequence of the NPY gene in axolots is similar to that of other amphibians**

Using GSPs designed against the NPY-encoding region from *T. natans* (a caecilian), we amplified a 90 bp fragment from cdNA synthesized from axolotl mRNA. Subsequent 3’ and 5’ RACE resulted in the identification of 341 bp and 257 bp sequences, respectively, facilitating full-length characterization of the axolotl NPY-encoding gene (*amnpy*; GenBank accession number: AY660754). The axolotl NPY gene contains an open reading frame of 297 nucleotides encoding a prepropeptide of 98 amino acids. Also present are a 5’UTR of 133 bp and a 3’UTR of 181 bp. The 3’ UTR contains a poly-A tail and a polyadenylation signal. The putative open reading frame is marked by consensus start and stop sequences, as indicated in Figure 3A.

The organization of *amnpy* conforms to all other vertebrate NPY genes in that it comprises a characteristic signal peptide, a distinct N-terminal processing site, a C-terminal glycolyl residue, a dibasic processing site, and a C-terminal flanking peptide (CPON). The deduced 36-amino acid NPY primary sequence, YSPKPNAPGEDAPAEWMKYSALRHYINLRQRQY-NH2, is similar to all known amphibian NPYs (94.4% identity). Substitutions occur in positions 6 and 7, where the Asp-Asn common to similar to all known amphibian NPYs (94.4% identity). Substitutions occur in positions 6 and 7, where the Asp-Asn common to all other vertebrate NPY genes is replaced by Asp-Asp in *amnpy*. The deduced amino acid sequence of the NPY gene in axolots is similar to that of other amphibians.
whether the application of NPY versus the control solution had a significant effect independent of feeding condition, and to determine whether the manipulation of hunger level affected responses independent of the application of NPY. Neither effect was significant: the response magnitude did not differ significantly between NPY and control treatments \((F = 0.21; p = 0.72)\), nor between hungry and well fed animals \((F = 0.77; p = 0.54)\). Importantly, however, the interaction between feeding condition and NPY/control treatment was statistically significant \((F = 709.5; p = 0.024)\), indicating that the effect of NPY on the magnitude of the EOG response depends on the animal’s hunger condition. Post hoc tests indicate that the difference in the magnitude of the EOG response in the hungry-NPY group differs significantly from that in other groups during the treatment period \((Student’s t > 2.09; p < 0.05)\), but not during the wash period \((Student’s t < 1.72; p > 0.10)\).

Among control animals, EOG responses did not differ between hungry and well fed animals \((two-way repeated-measures ANOVA, F = 0.22, p = 0.72)\), and did not change over the course of the testing period \((F = 0.21; p = 0.72)\); the interaction between these variables was also not significant \((F = 1.37; p = 0.26)\). These results from the two control groups demonstrate that food deprivation alone does not alter EOG responses under our testing conditions, and that the change of solutions between testing periods does not significantly alter EOG responses.

In previous experiments, we found that axolotls that are deprived of food for 10 d spend more time searching for food, particularly in the presence of food odorants, than do well fed animals \(\text{(Eisheiten and Park, 2005)}\). Although in the present experiment the animals in the hungry condition appeared to be hungry, snapping at experimenters’ fingers and disturbances in the water, these animals did not lose significant body mass relative to animals in the well fed condition \((\text{Student’s} t = 1.39; p = 0.18)\). Nevertheless, we also analyzed the data by dividing the subjects into two groups: those that lost >5% of their initial mass during the course of the feeding regime \((\text{mean} \pm \text{SEM} = 9.9 \pm 1.2\% \text{loss in body mass}; n = 12)\), and those that maintained a fairly steady body mass \((1.6 \pm 0.8\% \text{loss}; n = 12)\). Using these groupings, we found that the effect of applying NPY magnitude \((\text{SEM} = 14.4\%; n = 6)\) relative to baseline, and the magnitude of the EOG response returned to baseline within 8 min of the beginning of the wash period. We used a repeated-measures multivariate ANOVA \(\text{(MANOVA)}\) to determine

Figure 2. Distribution of NPY-like immunoreactivity in the brain and snout of adult axolotls. A–H, Confocal images of NPY-immunoreactive terminal nerve cell bodies and fibers (green); for clarity, some sections have been counterstained with Hoechst 33258, a fluorescent Nissl stain (blue). Anterior is toward the left in A–D and G, and toward the top in E and H–N; the lumen is toward the top in F. Scale bars, 100 μm. A, Olfactory nerve fascicles underneath the olfactory epithelium containing labeled terminal nerve cells. B, Labeled terminal nerve neurons inside the olfactory nerve. C, A cluster of labeled neurons in the ventral olfactory bulb. D, Labeled cells in the proximal portion of the olfactory nerve, adjacent to the olfactory bulb. E, Montage of three images illustrating labeled cells in the anterior and posterior preoptic area. F, No NPY-like immunoreactivity was observed in the olfactory epithelium. G–H, Images showing the distribution of NPY immunoreactivity in control experiments. G, NPY-immunoreactive terminal nerve cells in olfactory nerve fascicles after preincubation of anti-NPY antiserum with GYIRFamide. H, Labeled cells in the posterior preoptic area after preincubation of anti-NPY antiserum with FMRFamide. I–N, Illustrations showing the distribution of labeled cells and fibers in horizontal sections \(\text{(from dorsal (I) to ventral (N))} \text{through the brain of an adult axolotl. Shaded areas represent locations of large groups of neurons. Filled circles indicate regions in which labeled neurons were observed. apoa, Anterior preoptic area; dh, dorsal hypothalamus; ob, olfactory bulb; on, olfactory nerve; ppoa, posterior preoptic area; vh, ventral hypothalamus.}

The absolute magnitude of the EOG response did not differ significantly between animals that lost mass and those that maintained their mass \((\text{repeated-measures MANOVA,} p > 0.05 \text{for all main effects and interactions})\). In addition, the absolute magnitude of the EOG
response did not differ between control animals in the hungry and well fed groups (two-way repeated-measures ANOVA, \( F = 0.02, p = 0.89 \)). Similarly, the absolute magnitude of the EOG response did not differ among testing periods (\( F = 0.44; p = 0.69 \)), and no interaction effect was observed (\( F = 0.36; p = 0.70 \)). Thus, differences in responses to NPY between the hungry and well fed groups are not attributable to changes in body mass.

**NPY modulates a TTX-sensitive inward current in hungry animals**

At the single-cell level, the most dramatic result of NPY application was an increase in the magnitude of a large, rapidly inactivating voltage-activated inward current in a subset of neurons examined; the current partially recovered during the wash, as illustrated in Figure 5. Because the effect on the inward current was the first we observed in whole-cell recordings, subsequent recordings were made with CsCl intracellular solution in the recording pipette and BaCl\(_2\) in the bath solution to facilitate examination of this current.

We did not observe any cases in which the magnitude of the inward current decreased in NPY and then increased during the wash period. Nevertheless, our observations indicated that not all cells responded to NPY with an increase in the inward current. We categorized cells as “responders” or “nonresponders” based on the dynamics of inward current during the treatment period, in which the cells were exposed to NPY or the control (fast green) solution. Cells were classified as responders if the inward current elicited by a \(-10\) mV voltage pulse was elevated above baseline, defined as the magnitude of the current recorded immediately before application of NPY or the control solution, by at least 5% for at least five consecutive readings (10 min) during the treatment period. Cells were classified as nonresponders if the inward current was equal to or below baseline for at least 10 min during the treatment period.

Using these criteria, we were able to categorize 53 of 56 cells that were exposed to different concentrations of NPY or the control solution; in the remaining three cells, the inward current was variable. Overall, 10 of the 41 cells exposed to NPY (24%) responded with a sustained increase in the magnitude of the inward current. Interestingly, 8 of the 10 responders were from hungry animals, and the difference in probability of responding to NPY between hungry and well fed animals was statistically significant (\( \chi^2 = 7.15; p = 0.007 \)). Within hungry animals, the probability that the inward current increased during treatment was greater in NPY than the control treatment (\( \chi^2 = 3.63; p = 0.057 \)). Within well fed animals, this difference was not significant, although we tested cells from well fed animals with higher concentrations of NPY than were used with cells from hungry animals (\( \chi^2 = 0.50; p = 0.48 \)). In addition, two of the nine control cells recorded from well fed animals also met our criteria for classification as responders, suggesting that the magnitude of the inward current is subject to inherent variability. Details concerning the hunger level of the animal, concentration of NPY applied, and responses of all cells examined are provided in Table 2.

In recordings from hungry animals, we collected data from a large number of cells exposed to 100 nM NPY (\( n = 18 \)). We used these data for quantitative analysis of the effects of NPY on olfactory receptor neurons. The recorded magnitude of the inward current varied considerably among cells; therefore, for statistical analysis and data display, we normalized all data to the magnitude of the inward current measured immediately before applying NPY to the slice. Figure 6 illustrates the time course and magnitude of the response to NPY among cells categorized as responders and nonresponders, as well as controls.

The magnitude of the inward current differed significantly among the three groups of cells (two-way repeated-measures ANOVA, \( F = 113.4, p = 0.004 \)) and between the treatment and wash periods (\( F = 112.1; p = 0.009 \)); no significant interaction was observed (\( F = 0.669; p = 0.51 \)). In general, the inward current began to increase in magnitude almost immediately after NPY was first washed onto the slice. The difference between responders and other cells was statistically significant after 2.5 min in NPY (Student’s \( t \) values \( > 4.31; p < 0.001 \)), but the magnitude of the current in nonresponders and controls did not differ (Student’s \( t \) values \( > 0.99; p = 0.34 \)). The inward current remained elevated throughout the period of NPY exposure, and did not recover completely in the wash. The difference in magnitude between the responders and other groups was still significant toward the end of the wash period (at 7.5 min in wash, responders vs nonresponders and controls, \( t \) values \( > 2.19 \),
p \leq 0.05; at 10 min in wash, responders vs nonresponders, Student's t = 1.73, p = 0.10, and responders vs controls, Student's t = 3.77, p = 0.004). As reflected in Figure 6, for most cells classified as responders, the inward current reached its maximum after \sim 10\,\text{min} of NPY exposure, and began to decline somewhat before the wash period began. Among responsive cells, the inward current was \sim 10\% larger than baseline after 10\,\text{min} of exposure to NPY (mean = 9.96\%; SEM = 3.04); at this same time point, the inward current had declined an average of 15.1\% (SEM = 3.46) in cells exposed to the control solution.

The 18 neurons from hungry animals that were exposed to 100\,\text{nm} were derived from six animals, detailed in Table 3. Seven of these cells (38.9\%) responded to NPY application with an increase in the inward current. The difference between cells that responded to NPY and those that did not is not attributable to the sex of the animal from which the neurons were derived (Pearson's \chi^2 = 0.23; p = 0.63). In addition, the probability that a cell responded to NPY did not differ significantly among individuals (\chi^2 = 2.92; p = 0.71). Thus, we found no evidence that the difference in probability of a neuron responding to NPY varies among individuals; rather, some neurons within an individual respond to NPY, and some do not.

Although NPY clearly altered the magnitude of the inward current, it did not alter other aspects of the current. A within-subjects ANOVA indicates that although the voltage sensitivity of the peak of the inward current differed significantly among cells (F = 25.4; p < 0.0001), it did not change among the baseline, treatment, and wash recordings (F = 2.32; p = 0.14); the interaction between these variables was also not significant (F = 1.21; p = 0.29). We also examined the possible effect of NPY application on the rise time for the inward current, defined as the time required for the inward current to increase from 10 to 90\% of its maximal level. Although we measured the rise time for both the peak of the inward current for each cell and the inward current elicited by a \sim 10\,\text{mV} voltage pulse, we were unable to find any statistically significant effect of NPY application. For example, a repeated-measures two-way ANOVA indicated that the rise time of the current elicited by a \sim 10\,\text{mV} voltage pulse did not differ between NPY-treated and control cells (F = 7.53; p = 0.094); the rise time also did not vary across the baseline, treatment, and wash recordings (F = 0.14; p = 0.88), and the interaction between these two variables was not significant (F = 1.49; p = 0.23). Even within the cells classified as responders, a repeated-measures one-way ANOVA indicated no difference across the baseline, treatment, and wash recordings (F = 1.45; p = 0.24).

The voltage-activated inward current that increased in the presence of NPY was large and rapidly inactivating, resembling a sodium current. To further characterize this current, we used...
TTX in some recordings, as illustrated in Figure 7. For this experiment, we followed the standard recording protocol described above, with CsCl-containing intracellular solution in the recording pipette and BaCl\textsubscript{2} rather than CaCl\textsubscript{2} in the bath solution. Three hungry individuals were used, and of the nine cells to which NPY was applied, three cells met our criteria for being classified as responders. In all three cells, the inward current decreased significantly or was abolished within 2–5 min of application of the TTX-NPY. The inward current recovered to its baseline level within 15 min during the wash period. These data suggest that the inward current that is affected by application of NPY is the TTX-sensitive sodium current, which in olfactory receptor neurons is also modulated by the application of GnRH (Eisthen et al., 2000).

Discussion

NPY-immunoreactive fibers are extensively distributed throughout the brain in axolotls (Fig. 2). However, labeled cell bodies were found only in the terminal nerve pathway, hypothalamus, and infundibulum, a more restricted distribution than has been described in other amphibians, including another salamander species, *Triturus cristatus* (Perroteau et al., 1988; Danger et al., 1990; Tuinhof et al., 1994; Ebersole et al., 2001). In the preoptic area and hypothalamus, many immunoreactive cells had processes that extend to the ventricular surface (Fig. 2E), suggesting that in axolotls NPY may be released into the ventricles, as is GnRH. The presence of NPY in the olfactory epithelium proper varies across species, as NPY and NPY-like immunoreactivity have been observed in the olfactory epithelium of zebrafish and mice (Hansel et al., 2001; Mathieu et al., 2002), but not in gars (Chiba, 2005) nor, in this study, in axolotls.

Clearly, the amino acid sequence of NPY has been highly conserved during the evolution of vertebrates. The deduced axolotl NPY sequence conforms with this extraordinary sequence conservation in that it displays profound similarity (94.4%) to isolated and/or deduced sequences from other amphibians including the frogs *Rana ridibunda* (Chartrel et al., 1991), *Rana temporaria* (McKay et al., 1992), and *X. laevis* (Griffin et al., 1994), and the caecilian *T. natans* (Ebersole et al., 2001). In addition, putative axolotl NPY possesses conserved prolines at positions 5, 8, and 27 and tyrosines at positions 4 and 11. After 2.5 min in NPY, the magnitude of the inward current was significantly larger in cells responding to NPY than in control cells and nonresponders; the current decreased but did not recover during the 10 min wash period. All recordings were made with CsCl in the intracellular solution and BaCl\textsubscript{2} in the extracellular solution.

Table 3. Responses in cells from hungry animals

<table>
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Responses to 100 nM NPY in olfactory receptor neurons from hungry animals only. The probability of a cell responding does not appear to be related to the sex of the animal; in addition, the probability of responding does not vary among animals. For categorization criteria, see Materials and Methods.

We found that NPY modulates responses evoked by \textgamma-glutamatic acid as well as a voltage-sensitive current in olfactory receptor neurons. Several lines of evidence indicate that these effects are not simply caused by activation of odorant receptors by NPY. First, application of NPY alone does not produce a signal that can be recorded with our EOG electrodes, although EOG responses are caused in part by odorant-stimulated receptor potentials (Scott and Scott-Johnson, 2002). In addition, no odorant or odorant transduction pathway has been shown or suggested to activate or modulate the TTX-sensitive, voltage-activated inward current in olfactory receptor neurons. Finally, olfactory receptor neurons generally begin to adapt or desensitize to the presence of individual odors within seconds, but we recorded consistent changes in both responses evoked by \textgamma-glutamatic acid and in voltage-activated currents during bath application of NPY that lasted a minimum of 12–15 min.

Increased levels of NPY in the CNS stimulate feeding in a variety of vertebrates, including *Xenopus*, teleosts, birds, rodents, and other amphibians.
as responders, making it impossible to draw conclusions con-

cerning the effect of concentration on such a small number of cells.

In the olfactory epithelium, the effects of NPY and GnRH differ. Most conspicuously, in our EOG experiment, NPY increased the magnitude of responses elicited by L-glutamic acid, but only in hungry animals. In contrast, we have previously found that GnRH suppresses EOG responses evoked by L-glutamic acid and other amino acids, regardless of the animal’s hunger level (Park and Eisthen, 2003). Curiously, in that experiment, we also found that the magnitude of the EOG response elicited by L-glutamic acid did not simply return to baseline during the wash period but was significantly larger than before GnRH application (Park and Eisthen, 2003).

In our patch-clamp experiments, we did not find a significant number of cells from well fed animals responding to NPY, thereby altering odorant responses. However, because we applied a constant dose of NPY to all subjects and cells in physiological experiments, the observed hunger-based differences in responding are likely caused by differences in the expression or activity of NPY receptors or other elements of the NPY signal transduction pathway. Although the relative roles of NPY receptor subtypes in mediating food intake have received much attention (Chamorro et al., 2002), to our knowledge, hunger-related changes in NPY receptor expression or transduction have not been documented in previous studies.

In our patch-clamp experiments, we did not find a significant number of cells from well fed animals responding to NPY, although we tested a wider range of concentrations with well fed animals than with hungry animals. We note with some interest that of the four cells from well fed animals tested at high concentrations (5 and 10 μM), two cells were categorized as responders and one as a nonresponder (Table 2). The data from the fourth cell were ambiguous, because the inward current increased for the first three readings in NPY and then remained near baseline before declining in the wash. Thus, it seems possible that cells from hungry animals are more responsive to NPY, perhaps because of increased receptor expression or other changes in the transduction pathway, but that cells in well fed animals will also respond to NPY if presented with sufficiently high doses. Nevertheless, 2 control cells also met our criteria for being categorized as responders, making it impossible to draw conclusions con-

Figure 7. Whole-cell recordings from an olfactory receptor cell, illustrating the effect of TTX on the inward current in a cell that responds to NPY. A, Currents elicited by stepping from a holding potential of −60 mV to a series of voltage pulses ranging from −100 to +100 mV, in 10 mV increments. These currents were recorded in barium-APS, 3 min before NPY was applied to the cell. B, Currents elicited after 5 min exposure to a bath solution containing 100 nM NPY. The peak magnitude of the inward current is −12% larger than in A. C, Currents elicited after 5 min exposure to 100 nM NPY plus 100 nM TTX. Note that the large, rapidly inactivating, voltage-activated inward current that is probably carried by sodium has been blocked; the inward current is −25% of its initial magnitude. D, Currents elicited after 12.5 min of washing in barium-APS. The inward current has returned to 97% of its initial magnitude.


