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# Gonadotropin Releasing Hormone (GnRH) Modulates Odorant Responses in the Peripheral Olfactory System of Axolotls

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**Park, Daesik and Heather L. Eisthen.** Gonadotropin releasing hormone (GnRH) modulates odorant responses in the peripheral olfactory system of axolotls. *J Neurophysiol* 90: 731–738, 2003. First published April 2, 2003; 10.1152/jn.01162.2002. Peripheral signal modulation plays an important role in sensory processing. Activity in the vertebrate olfactory epithelium may be modulated by peptides released from the terminal nerve, such as gonadotropin releasing hormone (GnRH). Here, we demonstrate that GnRH modulates odorant responses in aquatic salamanders (axolotls, *Ambystoma mexicanum*). We recorded electrical field potentials (electro-olfactograms, or EOGs) in response to stimulation with four different amino acid odorants, L-lysine, L-methionine, L-cysteine, and L-glutamic acid. EOG responses were recorded from the main olfactory epithelium before, during, and after application of 10 $\mu$M GnRH. This protocol was repeated for a total of three trials with 60–80 min between trials. The effect of GnRH on EOG responses was broadly similar across odorants and across trials. In general, EOG responses were reduced to 79% of the initial magnitude during application of GnRH; in some trials in which glutamic acid served as the odorant, EOG responses were enhanced during the wash period. Although the 4-min interstimulus interval did not lead to adaptation of EOG responses during the first trial, we frequently observed evidence of adaptation during the second and third trials. In addition, we found that lower concentrations of GnRH produced a smaller effect. These results demonstrate that GnRH can modulate odorant responses in the peripheral olfactory system.

## INTRODUCTION

Peripheral signal modulation plays an important role in sensory processing. In the visual and auditory systems of vertebrates, transduction of sensory stimuli into neural signals is modulated both locally and by centrifugal inputs in the retina and cochlea (Akopian 2000; Ashmore et al. 2000). Although understanding peripheral events in odorant processing is essential to understanding olfactory system function, little is known about signal modulation in peripheral olfactory systems. Recent studies have found that peptides and other molecules modulate the activity of olfactory receptor neurons (Bouvet et al. 1988; Frings 1993; Grosmaitre et al. 2001; Kawai et al. 1999; Vargas and Lucero 1999). However, because the physiological source and means of access to the olfactory epithelium have not been established for most of these chemicals, it is not yet clear whether or not odorant responses are modulated by endogenous chemicals in the peripheral olfactory system.

The terminal nerve is an anterior cranial nerve that extends between the nasal cavity and preoptic area in the ventral forebrain of most jawed vertebrates (Wirsig-Wiechmann et al. 2002). The terminal nerve seems to play a role in reproductive behavior, although the nature of this role has not been clearly established. For example, in dwarf gouramis (*Colisa lalia*), lesions of terminal nerve cells inhibit initial nest-building behaviors (Yamamoto et al. 1997). In rough-skinned newts (*Taricha granulosa*), gonadotropin releasing hormone (GnRH) concentrations in the terminal nerve are higher in previously courted females than in uncourted females (Propper and Moore 1991). In male hamsters (*Mesocricetus auratus*), lesions of the terminal nerve decrease mating frequency, and reduce responses to female vaginal odors (Wirsig and Leonard 1987). Recent work suggests that the terminal nerve may play a modulatory role in peripheral olfactory systems (Oka and Matsushima 1993) and that this modulation may underlie its behavioral effects. The terminal nerve contains several potentially modulatory compounds, including acetylcholine (Wirsig and Leonard 1986; Wirsig-Wiechmann 1990) and unidentified compounds that display immunoreactivity to FMRFamide and neuropeptide Y (NPY) (Chiba 2000; Eisthen and Northcutt 1996) and tyrosine hydroxylase (White and Meredith 1995).

One peptide that has been unambiguously identified in the terminal nerve of many jawed vertebrates is GnRH (King and Millar 1992; Schwanzel-Fukuda and Silverman 1980; Sherwood et al. 1986). GnRH-containing neurons in the terminal nerve may release GnRH into the main olfactory and vomeronasal epithelia (Wirsig-Wiechmann and Jennes 1993; Wirsig-Wiechmann and Wiechmann 2001). Although the frequency of GnRH release and the amount of peptide that reaches the olfactory epithelium are not known, these studies suggest that GnRH released from the terminal nerve may gain access to receptor neurons, where it may modulate odorant processing. One study suggests that GnRH can increase the excitability of olfactory receptor neurons: GnRH applied to olfactory neurons of mudpuppies (*Necturus maculosus*) increases the magnitude of a tetrodotoxin-sensitive sodium current and alters outward currents (Eisthen et al. 2000). In other neural systems, GnRH has been demonstrated to modulate the excitability of neurons; for example, GnRH excites goldfish (*Carassius auratus*) retinal ganglion cells (Walker and Stell 1986) and modulates N-type calcium channels in bullfrog (*Rana catesbeiana*) sympathetic neurons (Boland and Bean 1993). To date, the direct

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evidence demonstrating that GnRH modulates odorant responses in peripheral olfactory systems has not been obtained, although one study in abstract form has reported that application of GnRH to rodent olfactory receptor neurons can reduce and/or enhance odorant responses, depending on the type of odorant used (Wirsig-Wiechmann et al. 2000).

In the present study, we recorded electrical field potentials, called electro-olfactograms (EOGs) (Ottoson 1956), from the main olfactory epithelium of axolotls, *Ambystoma mexicanum*. Axolotls are essentially a subspecies of tiger salamander (Shaffer 1993). As nonmetamorphosing, aquatic amphibians, axolotls are excellent research animals because they are easily maintained in the laboratory and have large, accessible olfactory receptor neurons. To investigate whether GnRH affects odorant responses, EOG responses were elicited by application of one of four different amino acid odorants before, during, and after GnRH exposure. Our data demonstrate that application of GnRH alters odorant responses in the olfactory epithelium.

## METHODS

### *Animal maintenance, anesthesia, and immobilization*

Fifty-nine adult axolotls (*A. mexicanum*) obtained from the Indiana University Axolotl Colony (34 female, 15 male, 10 of undetermined sex) were kept in aquaria (80 × 40 × 50 cm) containing 100% Holtfreter's solution, the most commonly used medium for maintaining axolotls (Armstrong et al. 1989; Mattison 1982). Holtfreter's solution contains (in mM) 60 NaCl, 2.4 $NaHCO_3$, 0.67 KCl, 0.81 $MgSO_4$, and 0.68 $CaCl_2$ in deionized water (pH 7.5). Aquaria were equipped with a recirculating filter system in which Holtfreter's solution from groups of tanks passed through mechanical and biological filters and an ultraviolet sterilizer before being returned to tanks.

To minimize stress, no more than six same-sex individuals were housed in each tank. Axolotls were fed commercial salmon chow (Rangen, Buhl, ID) twice each week. The temperature of the tanks ranged between 18 and 22°C, and the photoperiod was altered monthly to match that of the animals' native habitat in Mexico City.

Before surgery, axolotls were anesthetized with pH-corrected 0.1% MS 222 (tricaine methanesulfonate, Sigma Chemical, St. Louis, MO, pH 7.5) in Holtfreter's solution, and immobilized with an intramuscular injection of gallamine triethiodide dissolved in amphibian Ringer solution (Flaxedil, Sigma Chemical; 0.1–0.3 mg/100 g body weight, pH 7.6). Supplemental doses of MS 222 were delivered to the gills, and additional Flaxedil injected intramuscularly as necessary throughout the experiment.

The original experiments described in this paper were conducted in accordance with guidelines established by the Society for Neuroscience, and were approved by the Michigan State University animal care and use committee.

### *EOG recording*

The main olfactory epithelium was exposed by removing the tissue dorsal to the nasal capsule. To record electrical field potentials, a glass capillary electrode (100- to 200-$\mu$m tip diameter) was filled with 1% agar in Ringer solution bridged to a chloride-coated silver wire. An Ag-AgCl reference electrode was placed under the skin on the head and isolated from both the Holtfreter's and odorant solutions with petroleum jelly (Park et al. 2001). Electrodes were coupled to a differential amplifier (DP-301, Warner Instruments, Hamden, CT). Signals were digitized via an ITC-18 interface (Instrutech, Great Neck, NY), and recorded and analyzed on a Macintosh computer using AxoGraph software (v. 4.4, Axon Instruments, Foster City, CA).

The magnitude of the EOG response was measured as the maximal height of phasic displacement from the baseline level. Absolute response values in millivolts were obtained by comparison with the deflection elicited by a known calibration voltage.

### *Stimulus compounds and delivery*

To determine whether the effects of GnRH are odorant-specific, we selected stimuli to represent four broad categories of amino acids, with minimum cross reactivity among odorants, as described by Caprio and Byrd (1984). Odorant stimuli consisted of 1 mM solutions containing one of four amino acids (Sigma Chemical): L-lysine (Lys, a basic amino acid), L-methionine (Met, a neutral amino acid with a long side chain), L-cysteine (CysH, a neutral amino acid with a short side chain), or L-glutamic acid (Glu, an acidic amino acid). Stock solutions containing 10 mM odorant dissolved in Holtfreter's solution were prepared weekly, stored at 4°C, and diluted in Holtfreter's solution before each experiment. The pH of each solution was adjusted to 7.5–7.6 using 1 N HCl or 1 M Tris base to match the Holtfreter's solution in which axolotls were maintained and that bathed the olfactory mucosa during experiments.

During each trial, a continuous flow (3.5–4 ml/min) of Holtfreter's solution bathed the olfactory mucosa. For each EOG recording, ~70 $\mu$l of a 1 mM stimulus solution at room temperature (23–25°C) was injected into the flow of the Holtfreter's solution from a 1 ml syringe connected to a pressure injector (Picospritzer II, General Valve, Fairfield, NJ). The time of arrival of the stimulus at the olfactory mucosa was measured by adding a dye solution (fast green) to the odorant solution on some trials. Using this method, we found that the odorant arrived at the epithelium ~10 s after injection into the carrier stream and remained on the epithelium ~2–3 s.

### *Experimental protocol*

The mammalian form of GnRH (also called mGnRH or LHRH; Peninsula Labs, Belmont, CA), which is the form present in the terminal nerve of amphibians (Sherwood et al. 1986), was dissolved in $dH_2O$ and stored in 100 $\mu$l aliquots at −20°C. At the beginning of each day of recording, one aliquot of GnRH was dissolved in Holtfreter's solution at a final concentration of 10 $\mu$M. This concentration of GnRH was selected to match that used in a previous study in which we found that 10 $\mu$M GnRH increased voltage-dependent inward currents in salamander olfactory cells (Eisthen et al. 2000). The pH was then adjusted to 7.5–7.6 using small amounts of 1 N HCl or 1 M Tris base that were not sufficient to substantially alter the concentration of the solution.

To determine whether GnRH affects EOG responses, we recorded EOG responses before, during, and after GnRH application; the experimental protocol is illustrated schematically in Fig. 1. The interval between consecutive odorant presentations was 4 min and did not produce any indication of odorant adaptation during baseline recordings of EOG responses during initial trials. To determine the baseline response level, we recorded at least two to four EOG responses to the stimulus odorant before GnRH application. Once the EOG responses were relatively consistent (<10% difference in EOG magnitude for ≥2 consecutive recordings), 10 $\mu$M GnRH prepared in Holtfreter's solution was delivered to the olfactory epithelium continuously for 12 min. This time frame was selected because previous work indicated that the effects of GnRH on single olfactory receptor neurons are significant beginning ~10–15 min after initial GnRH exposure (Eisthen et al. 2000). Three EOG responses to the stimulus odorant were recorded during GnRH application. During the period after GnRH was applied ("wash"), we recorded another six EOG responses to the stimulus odorant while bathing the olfactory epithelium in running Holtfreter's solution. To investigate the effects of consecutive exposures to GnRH, we repeated this procedure for another two trials for each animal with a 60- to 80-min interval between trials. To optimize

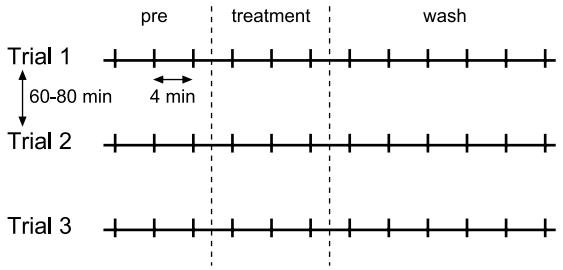


FIG. 1. Schematic illustration of the experimental protocol used with each animal. Pulses of odorant were delivered at 4-min intervals, and the electro-olfactogram (EOG) response recorded. During each trial, ≥2–4 EOG responses were recorded before application of gonadotropin releasing hormone (GnRH) (pre), 3 responses were recorded during application of GnRH or Holtfreter's solution (treatment), and 6 responses were recorded during the period after application of GnRH (wash). After an interval of 60–80 min, a 2nd trial was conducted, and after another interval of 60–80 min, a 3rd trial was conducted. In statistical analyses and subsequent figures, EOG responses are sometimes averaged within testing periods (pre, treatment, or wash) or across the 3 trials.

the signal, the recording electrode was sometimes relocated at the beginning of a trial. Thirty-three animals were used in these experiments: 9 for Lys, 10 for Met, 7 for CysH, and 7 for Glu. Seven animals were used in a control experiment in which plain Holtfreter's solution was substituted for the GnRH solution, and 19 additional animals were used in experiments to examine the effects of 1 and 5 $\mu$M GnRH.

### *Data analysis*

For purposes of statistical analysis and data display, we designated the mean magnitude of the two to four EOG responses recorded before the GnRH application as 100% and normalized all other data collected in each trial relative to this mean level. EOG responses from each trial were grouped into blocks of three categories, as follows: *baseline*, the average magnitude of two to four EOG responses recorded before GnRH exposure; *treatment*, the average magnitude of three EOG responses recorded during GnRH exposure; and *wash*, the average magnitude of six consecutive EOG responses recorded during the wash period.

To determine whether EOG responses differed before, during, and after application of GnRH, we used one-way ANOVA tests to compare data within trials. To determine whether the effect of GnRH on EOG responses differed among the three trials, we used a two-way ANOVA (effect of GnRH application × trial number). Two-way ANOVAs were also used to determine whether EOG responses elicited by the four odorant stimuli differed (effect of GnRH application × odorant) and whether the three different concentrations of GnRH produced different effects (effect of GnRH application × GnRH concentration). In cases in which an ANOVA indicated a significant difference, we used Tukey's post hoc test to perform two-point comparisons. To determine whether the percent of trials resulting in reduction of the EOG response during GnRH application differs among odorants or among the three trials for each individual, $\chi^2$ tests were used. For some two-point comparisons, Student's *t*-test was used.

Because of the relatively long time required to complete a trial, ~1 h, we were concerned about obtaining spurious results due to changes in the animal's state. In addition, we did not want to include data from trials in which the GnRH might have been ineffectively removed during the wash period. We therefore excluded from analysis data from any trial in which the magnitude of the EOG response during the wash period did not recover to ≥90% of baseline. Using this criterion, 125 of 147 trials (85.0%) conducted with 59 animals produced analyzable data. The percent of trials producing analyzable data did not vary among odorants ($\chi^2$, $P = 0.52$, $n = 98$ trials), among trials ($\chi^2$, $P = 0.43$, $n = 98$ trials) or among concentrations of GnRH ($\chi^2$, $P = 0.38$, $n = 70$ trials) nor between the sexes ($\chi^2$, $P = 0.56$, $n = 147$ trials). Our observations indicate that the few trials excluded from analysis involved technical problems such as difficulties with solution delivery or changes in the level of anesthesia. Thus we did not discard all data from an animal for which one trial was problematic.

## RESULTS

As illustrated in Fig. 2*A*, EOG responses varied within ~10% of the baseline magnitude in control experiments. During the period in which Holtfreter's solution was applied instead of GnRH, small increases or decreases in the magnitude of the EOG response were observed. In the majority of trials (13 of 19), EOG responses were slightly reduced during the wash period, although in the other six trials, a small enhancement in EOG responses was observed. Because EOG responses did not differ significantly across three trials [$F(4,57) = 0.49$, $P = 0.74$], we pooled data and then determined whether EOG responses were different before, during, and after Holtfreter's solution application. As indicated in Fig. 3, no significant changes were observed [$F(2,18) = 1.09$, $P = 0.36$].

### *Effects of GnRH are similar across odorants*

EOG responses to each odorant are illustrated in Fig. 2, *B–E*. In experiments in which 10 $\mu$M GnRH was applied to the olfactory epithelium, odorant responses were generally reduced during GnRH application. During the wash period, the

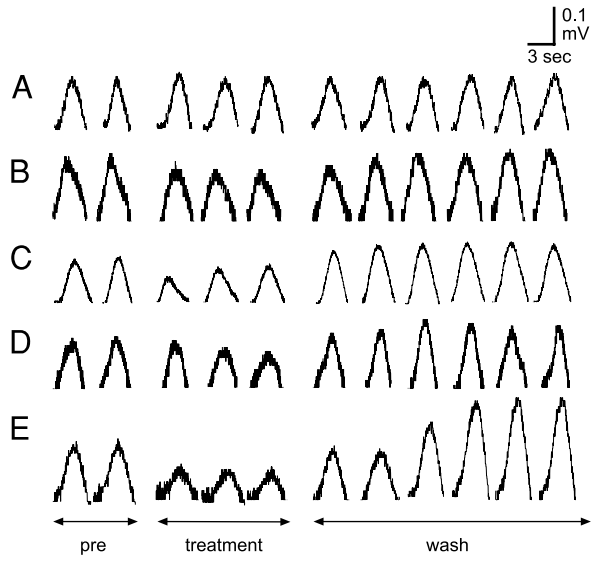


FIG. 2. Representative examples of EOG responses before (pre), during (treatment), and after (wash) application of Holtfreter's solution (*A*) or GnRH (*B–E*). EOG responses were evoked by delivery of ~70 $\mu$l of 1 mM L-methionine (Met, *A* and *C*), L-lysine (Lys, *B*), L-cysteine (CysH, *D*), and L-glutamic acid (Glu, *E*). *A*: in control experiments Holtfreter's solution was washed into the nasal cavity instead of GnRH. EOG response magnitudes varied within ~10% of the baseline. *B–E*: in experiments in which GnRH was applied, EOG response magnitudes were decreased during the period of GnRH application: 19% reduction in mean EOG magnitude (*B*); 29.4% reduction (*C*); 25.4% reduction (*D*); and 40.3% reduction (*E*) of EOG magnitude relative to the baseline. During the wash period, EOG responses recovered to the baseline and were sometimes enhanced. The enhanced magnitude of EOG responses varied, and was 2.8% (*B*), 19.6% (*C*), 19.2% (*D*), and 106% (*E*) above baseline during the wash period.

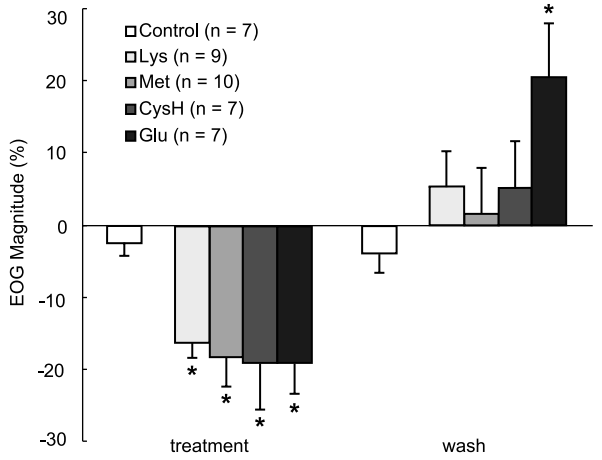


FIG. 3. GnRH affects EOG responses to L-amino acids. EOG responses obtained were grouped into the GnRH or control treatment and wash EOG responses and were normalized to the mean baseline EOG and pooled across trials. Bar height indicates mean percent deviation from baseline for each amino acid, and vertical lines indicate ±SE. In control experiments, EOG responses did not differ significantly from baseline ($P = 0.40$). During GnRH application, EOG responses were significantly reduced relative to baseline for all amino acids tested (all $P$s $< 0.05$). Only responses to Glu were significantly enhanced during the wash period ($P = 0.03$). Overall, the effect of GnRH did not differ among odorants ($P = 0.40$). *, a significant difference ($P < 0.05$) compared with the baseline. $n$ = the number of animals.

response recovered to approximately the initial magnitude, and was enhanced relative to baseline in some trials.

The pattern of EOG responses during a trial did not differ significantly across trials [$F(4,63) = 0.55$, $P = 0.70$ for Lys, $F(4,72) = 0.28$, $P = 0.89$ for Met, $F(4,46) = 0.06$, $P = 0.99$ for CysH, and $F(4,54) = 0.10$, $P = 0.98$ for Glu], so we pooled the data across trials for each animal and then determined whether the effect of GnRH differed for the four odorant stimuli. Within the experiments conducted with each odorant, EOG responses differed significantly throughout the course of the trial for all odorants [$F(2,24) = 12.9$, $P < 0.001$ for Lys, $F(2,27) = 122.9$, $P < 0.001$ for Met, $F(2,18) = 6.66$, $P = 0.007$ for CysH, and $F(2,18) = 15.4$, $P < 0.001$ for Glu]. Specifically, during GnRH application, EOG responses were reduced relative to baseline for all odorant stimuli (Fig. 3, all $P$s $<0.05$). During the subsequent wash period, EOG responses evoked by Glu were significantly enhanced relative to baseline (Fig. 3, $P = 0.03$), but responses to Lys, Met, and CysH were not significantly different from baseline (Fig. 3, $P > 0.05$). The overall effect of GnRH on EOG responses did not differ significantly among the different types of amino acids tested [Fig. 3, $F(6,99) = 1.04$, $P = 0.41$].

Although the overall effect of GnRH application was a reduction in EOG responses, in some cases, EOG responses appeared to be enhanced in the presence of GnRH. During GnRH application, the magnitude of the mean EOG response was reduced in 62 of 78 trials (79.5%). In these trials, the magnitude of the EOG responses averaged 79.4 ± 1.6% of baseline EOG magnitude (range: 47.0–99.4%). In contrast, EOG responses during GnRH application were enhanced in the remaining 16 trials with an average magnitude of 112.3 ± 3.4% of the baseline (range: 100.0–140.7%). The difference in effect of GnRH could not be attributed to individual differences, differences across trials, or differences across odorant stimuli. Fourteen of 33 animals (42.4%) showed reduced responses during GnRH application in some trials and enhanced responses during other trials. The percentage of trials in which reduction of EOG responses was observed did not differ: reduction occurred in 81.5% of cases in the first GnRH trial (22 of 27 trials), 87.0% in the second (20 of 23 trials), and 71.4% in the third (20 of 28 trials; $\chi^2$, $P = 0.89$). The effect of GnRH did not depend on the stimulus used: reduction was observed in 85.0% of trials with Lys (18 of 21 trials), 75.0% with Met (18 of 24 trials), 66.4% with CysH (10 of 15 trials), and 89.9% with Glu (16 of 18 trials; $\chi^2$, $P = 0.94$).

### *GnRH may affect temporal properties of odorant adaptation*

Although we did not observe evidence of odorant adaptation during EOG recordings on the first trial for any individual, we frequently observed a successive decrement in EOG response magnitude over the first few recordings during the second and third trials. We interpret this progressive decrement in magnitude, illustrated in Fig. 4, as indicating that odorant adaptation is occurring. Interpretable data consisting of at least three baseline EOG responses were collected for 17 animals; of these, odorant

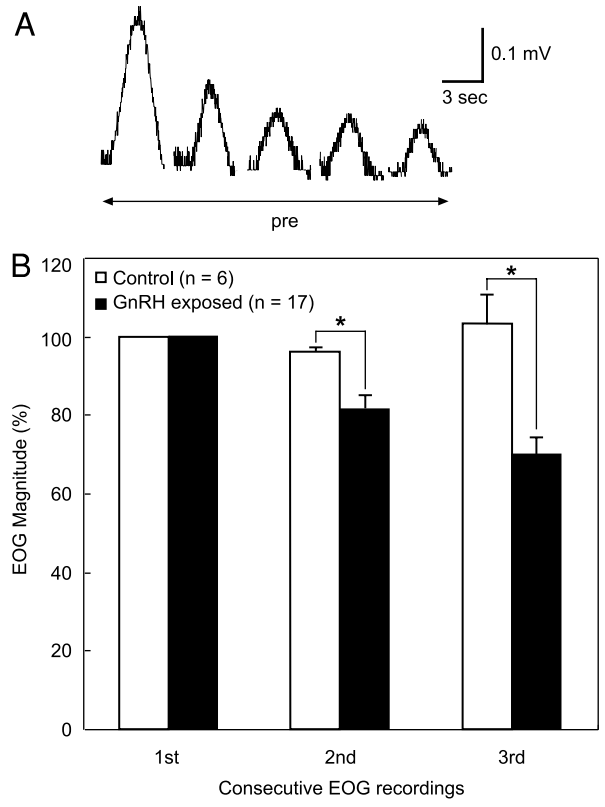


FIG. 4. GnRH may affect temporal properties of odorant adaptation. Before GnRH application and in control experiments, no significant odorant adaptation was observed with a 4-min inter-stimulus interval, but in experiments where GnRH was applied, the magnitude of EOG responses frequently decreased over the first few recordings on the 2nd or 3rd trials. *A*: successive decrement in the magnitude of EOG responses evoked by Glu at the beginning of the 2nd trial for 1 animal. *B*: the magnitude of EOG responses in GnRH-exposed animals decreased significantly over the 1st 3 consecutive recordings ($P < 0.001$). The magnitude of successive EOG responses differed significantly between control and GnRH-exposed animals: the magnitude of the 2nd and 3rd EOG responses in GnRH-exposed animals were significantly smaller than in control animals ($P$s $< 0.001$). *, a significant difference ($P < 0.05$) between control and GnRH-exposed animals. $n$ = the number of animals for which data were available.

adaptation was observed on the second or third trial, or both, for 13 animals (76.5%). The frequency of occurrence of adaptation was similar among odorants: adaptation occurred in five of five animals (100%) for which Lys served as the stimulus, three of five animals (60%) tested with Met, two of three animals (66.7%) tested with CysH, and three of four animals (75%) tested with Glu. (Because we noticed this unexpected phenomenon toward the middle of our experiments, the sample sizes obtained using different odorants varies.) Differences among odorants were not significant ($\chi^2$, $P = 0.96$).

For further analysis of this phenomenon, we examined all 17 sets of EOG recordings for which data were available and analyzed the first three EOG responses at the beginning of the second trial before the second application of GnRH. For comparison, we also analyzed the first three EOG responses of the second trial for all control subjects for which such data were available ($n = 6$). EOG responses were normalized such that the magnitude of the very first EOG response was designated 100% and the magnitudes of the second and third EOG responses were expressed as a percent of this value.

Because the pattern of reduction in EOG response magnitude did not differ among the four stimuli used [$F(6,51) = 0.21$, $P = 0.97$], we pooled the data obtained using different odorants. As illustrated in Fig. 4, the overall pattern of EOG response magnitude during the first three recordings differed significantly between the control and experimental groups [$F(2,69) = 8.42$, $P < 0.001$]. In addition, we found that the magnitude of the second and third EOG responses in GnRH-exposed animals were significantly smaller than those obtained from animals in the control group (Fig. 4; $t = 2.68$, df = 21, $P = 0.01$ for the 2nd EOG response, $t = 4.03$, df = 21, $P < 0.001$ for the 3rd EOG response). Within-group analyses indicate that the magnitude of the first three EOG responses differed significantly in GnRH-exposed animals [Fig. 4; $F(2,48) = 24.6$, $P < 0.001$], but not within the control group [Fig. 4; $F(2,15) = 0.73$, $P = 0.50$]. Post hoc tests demonstrate that the magnitude of the second and third EOG responses in the experimental group were significantly smaller than that of the first, and that the magnitude of the third EOG response was smaller than that of the second (Fig. 4; both $P < 0.04$).

### *Effects of GnRH on EOG responses may be concentration-dependent*

As in the preceding text, all data obtained using varying concentrations of GnRH were normalized relative to the magnitude of the baseline EOG response for each trial. Because the pattern of EOG responses during a trial did not differ significantly across trials, we pooled the data across trials for each animal [$F(4,63) = 0.79$, $P = 0.54$ for 1 $\mu$M GnRH, $F(4,72) = 0.56$, $P = 0.69$ for 5 $\mu$M GnRH]. As illustrated in Fig. 5*A*, 5 and 10 $\mu$M GnRH produced similar effects on EOG responses, but the effect of 1 $\mu$M GnRH was not as pronounced. Overall, EOG responses before, during, and after GnRH application differed significantly with GnRH concentration [$F(4,78) = 4.09$, $P = 0.005$]. We further determined whether EOG responses during and after GnRH application differ across GnRH concentrations. EOG responses during GnRH application did not differ across groups [$F(2,23) = 0.32$, $P = 0.73$], but responses during the wash period differed significantly [$F(2,23) = 7.62$, $P = 0.003$]. The maximum EOG magnitude during the wash period was 96.5 ± 2.6% ($n = 9$) in 1 $\mu$M GnRH, 120.9 ± 6.9% ($n = 10$) in 5 $\mu$M GnRH, and 122.0 ± 4.3% ($n = 7$) in 10 $\mu$M GnRH. Post hoc tests revealed that EOG responses in experiments using 5 and 10 $\mu$M GnRH were significantly different from those in which 1 $\mu$M GnRH was used, and were significantly larger during the wash period than in trials in which 1 $\mu$M GnRH was used (Fig. 5, all $P$s $< 0.02$).

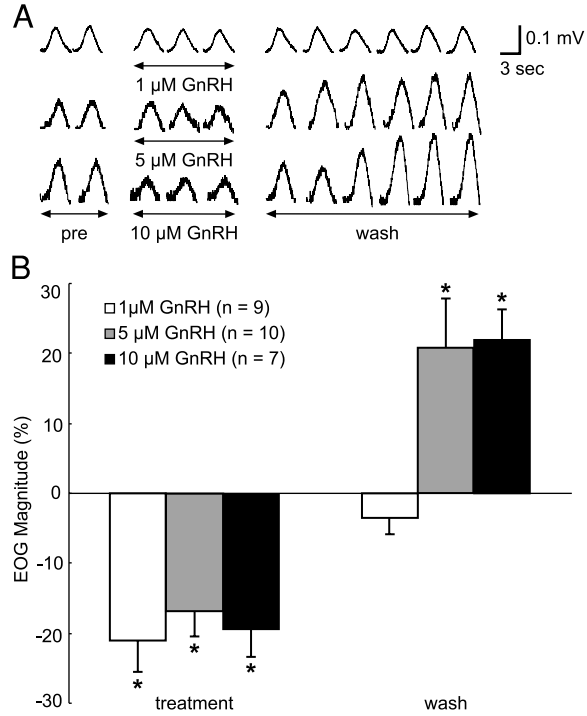


FIG. 5. The effect of GnRH on EOG responses may be concentration-dependent. *A*: representative EOG responses to Glu before, during, and after the application of 1 $\mu$M GnRH (*top*), 5 $\mu$M GnRH (*middle*), and 10 $\mu$M GnRH (*bottom*). *B*: summary of the differences observed. For each trial, mean EOG responses obtained during GnRH treatment and wash were normalized to the mean baseline EOG and then data were pooled across trials. Bar height indicates mean percent deviation from baseline, and vertical lines indicate the SE. The effect of GnRH on EOG responses differed significantly with concentration ($P = 0.005$). During GnRH application, EOG responses were similarly reduced at all concentrations ($P = 0.73$); however, during the wash period, EOG responses differed significantly across GnRH concentrations ($P = 0.003$). Enhanced EOG responses were observed during the wash period after exposure to 5 and 10 $\mu$M GnRH ($P < 0.05$), but not after exposure to 1 $\mu$M GnRH. *, a significant difference ($P < 0.05$) compared with the baseline. $n$ = the number of animals.

The results of within-group analyses indicate that EOG responses before, during, and after GnRH application differed significantly for all three concentrations of GnRH [$F(2,24) = 14.77$, $P < 0.001$ for 1 $\mu$M GnRH, $F(2,27) = 16.55$, $P < 0.001$ for 5 $\mu$M GnRH, and $F(2,18) = 36.45$, $P < 0.001$ for 10 $\mu$M GnRH]. Post hoc tests indicate that EOG responses during GnRH application were significantly reduced relative to baseline at all three concentrations (Fig. 5; all $P$s $< 0.05$). EOG responses during the wash period were significantly enhanced in trials using 5 and 10 $\mu$M GnRH (Fig. 5; both $P$s $< 0.01$) but not using in 1 $\mu$M GnRH (Fig. 5, $P = 0.69$).

## DISCUSSION

To determine whether GnRH affects odorant responses in peripheral olfactory systems, we recorded electrical field potentials (EOGs) in response to different amino acid odorants

before, during, and after application of GnRH. In general, we found that 10 $\mu$M GnRH reduces odorant responses during GnRH application and that the effect appears to be independent of the odorant stimulus used. We did not observe any obvious sex differences in the effects of GnRH, although we did not perform statistical analyses to compare males and females because of the small numbers of animals of each sex used in each experimental condition. Previous work has also demonstrated that the effects of GnRH on olfactory receptor cells are not sex-specific in salamanders (Eisthen et al. 2000).

The magnitude of EOG responses to 1 mM L-amino acids is relatively small in axolotls, ranging from 100 $\mu$V to ~1–2 mV, and is comparable to that of EOG responses recorded from a marine elasmobranch (*Dasyatis sabina*) and marine ariidae catfish (*Arius felis*) (Caprio 1980; Silver 1979). In the marine catfish, the magnitude of EOG responses to some amino acids, like L-glutamic acid, is smaller than to others, such as L-cysteine, L-methionine, and L-alanine (Caprio 1980). The relatively small EOG magnitude that we recorded in this study may be due to the use of relatively small amounts of odorants or may be due to the use of Holtfreter's solution, which contains NaCl and may cause electrical shunting of EOG signals (Caprio 1980; Silver 1979). Although the magnitude of EOG responses we obtained was relatively small, the signal-to-noise ratio was good, producing reliable, analyzable data throughout the study.

In our preliminary studies, we delivered 10 $\mu$M GnRH to the main olfactory epithelium of axolotls using the same method we later used to apply odorants and found that GnRH alone did not induce any EOG response. This result is consistent with another report that GnRH application alone does not elicit odorant responses in single olfactory receptor neurons from rodents (Wirsig-Wiechmann et al. 2000). Although application of GnRH to the olfactory epithelium of rainbow trout (*Oncorhynchus mykiss*) has previously been reported to elicit EOG responses (Andersen and Døving 1991), more recent evidence suggests that Andersen and Døving's result may have been caused by contamination (K. B. Døving, personal communication). Thus we conclude that GnRH does not serve as an odorant stimulus, and that any effects of GnRH application that we observed were due to modulatory rather than sensory processes.

GnRH alters EOG responses to L-amino acid odorants. During GnRH application, reduction of EOG responses was the most common effect, although we observed both reduction and enhancement of EOG responses. The effect of GnRH did not differ substantially among odorants nor among trials for each individual. Similar inhibitory effects of GnRH application have been observed in isolated rat uterine muscles, although the mechanism underlying this inhibition is not known (Medeiros et al. 1988). In uterine muscles, GnRH does not directly affect contractility of the muscles but decreases contractile responses to acetylcholine and oxytocin (Medeiros et al. 1988). This modulatory effect of GnRH is detectable 10 min after initial GnRH exposure and reaches a plateau within 30–60 min, reducing the response to 80–85% of the control response (Medeiros et al. 1988).

In single neurons, inhibitory effects of GnRH on ion channels have been reported in bullfrog sympathetic neurons (Bley and Tsien 1990; Boland and Bean 1993; Elmslie et al. 1990; Lewis and Ikeda 1997). Neurons exposed to 300 nM GnRH show an ~80% reduction in the magnitude of N-type $Ca^{2+}$ inward currents (Boland and Bean 1993). The inhibition begins almost immediately after GnRH application, develops over several seconds, and reverses completely after peptide removal. In addition, a decrease in the magnitude of $Ca^{2+}$-dependent $K^+$ currents has been observed in sympathetic neurons, most likely as a consequence of inhibition of $Ca^{2+}$ influx by GnRH exposure (Boland and Bean 1993). In this system, the decrease in the magnitude of the $Ca^{2+}$-dependent $K^+$ current results in reduced neurotransmitter release evoked by $K^+$ depolarization and in a late slow excitatory postsynaptic potential (Lewis and Ikeda 1997).

Additional inhibitory actions of GnRH have been reported in other types of neurons. For example, in GT1 neurons, GnRH transiently hyperpolarizes membrane potentials by increasing the internal concentration of $Ca^{2+}$; during GnRH application, spike amplitude is significantly decreased, although the frequency and duration of spikes are increased (Van Goor et al. 1999). In a previous study using whole cell recordings from mudpuppy olfactory neurons, we observed a few cases suggesting that 10 $\mu$M GnRH alters an outward current 5–10 min after GnRH application (Eisthen et al. 2000). These results imply that GnRH may reduce odorant responses via inhibitory effects.

We found that EOG responses are enhanced during the wash period in trials in which Glu was used as the stimulus but not in trials with other odorants. This enhancement was observed after application of both 5 and 10 $\mu$M GnRH but not after application of 1 $\mu$M GnRH. These results suggest that GnRH could increase the sensitivity of peripheral olfactory systems to selected odorants. In rainbow trout (*Salmo gairdneri*) and catfish (*Ictalurus punctatus*), the four L-amino acids used in our study show relatively low cross-adaptation at the olfactory epithelium (Caprio and Byrd 1984; Rhein and Cagan 1983); thus these amino acids probably bind to different receptors and may activate different signal pathways. GnRH may therefore interact with specific receptors or signaling pathways, altering responses to specific odorants.

Two possible mechanisms may underlie the enhanced response that we observed during the wash period. First, enhanced EOG responses may simply represent over-recovery of the responses. Previous studies of a variety of cell types demonstrate over-recovery of the response during the wash period after GnRH application in about half the individual cells (Bley and Tsien 1990; Boland and Bean 1993; Bosma and Hille 1989). Over-recovery is also observed after GnRH inhibition of both $Ca^{2+}$ (Bley and Tsien 1990) and $K^+$ currents (Boland and Bean 1993; Bosma and Hille 1989), but the mechanism underlying the over-recovery is not known. This explanation does not completely explain our results, as enhanced EOG responses during the wash period were only observed in experiments in which Glu served as the stimulus.

Alternatively, GnRH may sensitize odorant responses to specific stimuli. Enhancement of specific odorant responses was reported in a study of frog (*Rama esculenta*/*ridibunda*) olfactory receptor neurons: enhanced responses to odorants that generate adenosine 3:5-cyclic monophosphate (cAMP) are observed 10 min after the application of compounds such as carbachol and serotonin (Frings 1993). Protein kinase C (PKC) activated by intracellular $Ca^{2+}$ serves as a key factor in determining the responsiveness of adenylyl cyclase (AC) in response to odorant stimulation (Anholt and Rivers 1990; Frings 1993). In GnRH-releasing neurons in the terminal nerve,

GnRH induces the release of $Ca^{2+}$ from intracellular stores (Abe and Oka 2000). In salamander olfactory receptor neurons, GnRH enhances voltage-activated $Na^+$ currents and also alters outward currents (Eisthen et al. 2000). If GnRH interacts with one of the second-messenger-mediated processes that sensitize odorant responses in olfactory neurons, the result may be enhanced EOG responses as observed in this study.

In our experiments, we did not detect any signs of odorant adaptation before GnRH application. After the first trial, however, odorant adaptation to consecutive odorant presentations was often observed, even though the inter-stimulus interval and the volume and concentration of stimulus were kept constant among trials. To date, there are no similar reports. Two different mechanisms may account for the induction of odorant adaptation after GnRH application. First, odorant adaptation may be a long-lasting effect of enhanced EOG responses from GnRH exposure on the previous trial. EOG responses may adapt during consecutive odorant presentations if some components that were activated by GnRH and led to enhanced EOG responses are gradually inactivated during the next trial. If this hypothesis is correct, we should not find odorant adaptation occurring during trials in which the previous exposure to GnRH did not enhance EOG responses. Nevertheless, we have detected odorant adaptation after trials in which EOG responses were not enhanced, suggesting that this hypothesis is incorrect. Alternatively, GnRH may directly affect pathways involved in odorant adaptation. For example, in GnRH-releasing neurons, GnRH releases $Ca^{2+}$ from intracellular stores (Abe and Oka 2002). $Ca^{2+}$ activates $Ca^{2+}$-dependent calmodulin kinase II (CaMKII) in olfactory receptor neurons (Zufall and Leinders-Zufall 2000). In salamander olfactory neurons, CaMKII determines the temporal properties of odorant adaptation by altering the sensitivity of AC (Leinders-Zufall et al. 1999). Thus it is possible that GnRH may affect one of the mechanisms of odorant adaptation, for example by causing release of intracellular $Ca^{2+}$ which could stimulate CaMKII, altering the sensitivity of AC.

The effect of GnRH on EOG responses appears to be concentration-dependent. In our study, the pattern of EOG responses following the application of 5 and 10 $\mu$M GnRH was similar, whereas application of 1 $\mu$M GnRH produced only a subset of these effects. The reduction of EOG responses during GnRH application did not differ with GnRH concentration, but during the wash period EOG responses were enhanced more by application of 5 and 10 $\mu$M GnRH than by 1 $\mu$M GnRH. A similar concentration effect has been observed in response to GnRH application in other neurons. For example, in terminal nerve GnRH neurons, the increased rate of firing is correlated with the concentration of GnRH applied (Abe and Oka 2000). In pituitary gonadotrophs, low (picomolar) concentrations of GnRH induce irregular and low-amplitude changes of intracellular $Ca^{2+}$ concentrations, whereas at higher concentrations, GnRH induces high-amplitude changes (Krsmanovic et al. 2000). In intact animals, inputs from hormones and from other neurons may increase or decrease the amount of GnRH released (Wirsig-Wiechmann 1993; Yamamoto and Ito 2000).

Given that we have only recorded from semi-intact preparations of olfactory epithelium, it is possible that nonneural cells within the olfactory epithelium may change the physiological environment of olfactory receptor cells in response to stimulation by GnRH, altering the EOG response indirectly. For example, supporting cells in the olfactory epithelium of frogs and mice possess voltage-dependent $K^+$ and $Na^+$ conductances and are highly permeable to $K^+$ (Ghiaroni et al. 2003; Trotier 1998). In addition, supporting cells in the main olfactory epithelium can be depolarized by odorant stimuli or an increase in extracellular $K^+$ concentrations (Trotier 1998). These studies suggest that supporting cells in the olfactory epithelium may play an active role in signal transduction in the olfactory epithelium. If peptides that are present in the nasal epithelium activate nonneural cells, such as sustentacular cells or Bowman's cells (Getchell et al. 1989), the physiological milieu of the olfactory receptor neurons could be altered, possibly resulting in altered EOG responses.

Odorant responses in the olfactory epithelium may be modulated by endogenous chemicals released from the terminal nerve or from the other, as yet unidentified, sources. The effects of GnRH on EOG responses that we observed are comparable to those resulting from the application of adrenaline. In our study, EOG responses during GnRH application were reduced to ~80% of the baseline, and some enhancement was observed during the wash period. In experiments in which adrenaline has been used, EOG responses are enhanced 20–50% above baseline magnitude within 5−10 min after adrenaline application, but no reduction is observed (Arechiga and Alcocer-Cuaron 1969). In studies of single salamander olfactory neurons, GnRH increases the voltage-activated $Na^+$ current, and may reduce outward currents (Eisthen et al. 2000). Adrenaline exposure increases the inward $Na^+$ current to ~18% above the baseline level in newt olfactory neurons, but no information about outward currents is available (Kawai et al. 1999). These results demonstrate that both GnRH and adrenaline can modulate activity of olfactory receptor neurons, but the mechanisms of modulation may differ.

Signal modulation in peripheral olfactory systems may be important for odorant information encoding in the CNS, but few studies have examined the modulation of odorant responses in the olfactory epithelium. Our results indicate that GnRH reduces odorant responses in the olfactory epithelium in the early phase of its application but in some trials enhances responses during the wash period, possibly in an odorant-specific manner. Given that the terminal nerve may also release other chemicals into the nasal cavity (White and Meredith 1995; Wirsig-Wiechmann 1990; Wirsig-Wiechmann et al. 2002), understanding peripheral signal modulation by multiple endogenous chemicals in olfactory systems could greatly increase our understanding of signal processing in the olfactory epithelium.

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## DISCLOSURES

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