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Biogenic Amines in the Vomeronasal Organ

Carlo Zancanaro, Carla Mucignat Caretta[1], Andrea Bolner[2], Andrea Sbarbati, Gian Pietro Nordera[2] and Francesco Osculati

Institute of Anatomy and Histology, University of Verona, [1]Institute of Human Physiology, University of Parma and [2]A. Benedetti Institute Villa Margherita, Arcugnano, Italy

Correspondence to be sent to: C. Zancanaro, Istituto di Anatomia ed Istologia, Strada Le Grazie 8, I-37134 Verona, Italy

Abstract

The vomeronasal organ of frog and mouse was investigated for the presence and content of serotonin and catecholamines by means of high-performance liquid chromatography. Measurable amounts of serotonin, adrenaline and noradrenaline were found in the vomeronasal organ of adult individuals of both species. The amine content varied with sex of adult frogs and mice and sexual maturity of mice. In preliminary experiments, acute exposure to male urine containing pheromone affected the amine content in the vomeronasal organ of adult female mice. These data suggest that functional sex dimorphism is present in the vomeronasal organ, and biochemical changes therein take place according to stage of sexual maturity. The role of biogenic amines in the vomeronasal organ deserves further study. **Chem. Senses 22: 439–445, 1997.**

Introduction

A chemoreceptor structure called the Jacobson's organ or vomeronasal organ (VNO) is located on both sides of the nasal septum in most terrestrial vertebrates, including humans (reviewed in Halpern, 1987; Wysocki and Meredith, 1987; Monti-Bloch *et al.*, 1994). According to the large body of evidence which has emerged since the seminal work of Powers and Winans (1975), the VNO is involved in reproductive physiology and behaviour (reviewed in e.g. Wysocki, 1979). In the VNO, chemical signals (pheromones) secreted in the environment by a conspecific are transduced in an as yet unknown manner and transmitted to the basal forebrain and anterior hypothalamus via the vomeronasal nerves. The interconnected vomeronasal structures (VNO, VN nerves and central projections) have been considered as the vomeronasal system (VNS). Centrifugal noradrenergic nerves (from e.g. the locus coeruleus; Shipley *et al.*, 1985) play a key role in VNS-mediated activities (Keverne and de la Riva, 1982; Cornwell-Jones, 1988; Guan *et al.*, 1993).

Sexual dimorphism has been shown in the VNS network, and the VNS has been proposed as 'a model that successfully facilitates the establishment of a connection between sexual dimorphism in the brain and the dimorphism observed in reproductive physiology and behaviour' (Segovia and Guillamon, 1993). Morphological sexual dimorphism has been demonstrated in structures receiving vomeronasal input and in the VNO itself (Segovia and Guillamon, 1993). Such dimorphism is influenced by gonadal hormones present shortly after birth (Segovia and Guillamon, 1982; Segovia *et al.*, 1984). Investigation of possible sex- and age-related biochemical and functional changes in the VNO is lacking. As a first step in such

an investigation, the amount of biogenic amines [adrenaline, noradrenaline and 5-hydroxytryptamine (5-HT)] was determined by means of high-performance liquid chromatography (HPLC) in VNOs of adult frogs and mice of both sexes, and also in prepubertal and pubertal mice. Preliminary investigation of the effect of exposure to male urine on the amines content in the VNO was also carried out in adult female mice.

Materials and methods

Animals and organ procurement

A total of 20 frogs and 70 mice of both sexes were used in this work. Frogs (*Rana esculenta*) were obtained from a local supplier during summer and used for experiments within 2 weeks. The frogs were adult individuals, as indicated by body size and inspection of gonads. Swiss mice (Morini, San Polo d'Enza, Italy) were maintained in an animal house under controlled light (12:12, light on at 06:00 a.m.) and temperature (24 ± 1°C) conditions, in 27 × 42 × 15 cm plastic cages. Food (mouse diet chow; Mucedola, Milan, Italy) and water were allowed *ad libitum*. The litter of wood shavings was changed every 5 days. Mice were weaned at 21 days of age and then kept in groups of a maximum of six animals of the same age and sex. Prepubertal male and female mice were killed at 35–40 and 30 days of age respectively. Pubertal mice were killed at 50 (males) and 35–40 (females) days of life. Adult male and female mice were killed at 5 months of age; female individuals were in dioestrus. The VNOs were carefully dissected out from frogs and mice under light anaesthesia induced by immersion in MS222 [3-aminobenzoic acid ethyl ether (Sandoz, Milan, Italy), 1:1000 in tap water] or after killing by cervical dislocation respectively. The VNOs were rapidly washed in isotonic saline and immediately used for HPLC analysis (*vide infra*).

In a further experiment, 14 adult female mice were acutely exposed (10 times intranasally at 10 min intervals) to urine which had been collected over a tray immediately prior to use from a pool of five males aged 4–6 months. The VNO was taken immediately after the last exposure and used for HPLC analyses. Control female mice ($n = 5$) were exposed to saline according to the same protocol.

General procedures

The VNOs were placed in 200 µl of an acidic solution (perchloric acid 0.4 M, ascorbic acid 15.0 mM), sonicated for 2 min, centrifuged (10 min at 10 000 *g*), and the supernatant was deep frozen at –80°C until analysis. Protein determination (Lowry *et al.*, 1951; Peterson, 1979) was performed by Coomassie Brilliant Blue G-250 (Protein Assay-Microassay Procedure; Bio-Rad, Milan, Italy) in 25 µl of thawed samples previously treated with 5% (w/v) Triton X-100 (10 volumes of sample mixed with 1 volume of surfactant). All results were corrected for analytical losses and expressed as pg of the given amine/µg protein. Data are presented as means ± SEM. One-way analysis of variance was used for statistical analysis, except where indicated. Where signif- icant differences were found among groups, the Bonferroni's test was used for comparisons between groups.

Biogenic amine assay

Excised VNOs were assayed for adrenaline, noradrenaline and 5-HT. Catecholamine determination was performed according to He *et al.* (1992) with slight modifications. A volume of 50 µl of thawed supernatant was diluted with 950 µl distilled water and 400 µl of 2 M Tris buffer, pH 8.6. Ten microliters of 1 M sodium metabisulphite and 20 ng/ml of dihydroxybenzyl-amine (Sigma, St Louis, MO) were added to the sample as antioxidant and the internal standard, respectively. The sample was than mixed for 10 min on a rotating stirrer in the presence of alumina (Sigma, 10 mg). After mixing, the supernatant was removed and the alumina-absorbed material was washed twice by addition of distilled water (1 ml), short vortex mixing and centrifugation. The alumina-absorbed amines were then eluted by mixing the sample with 0.2 M acetic acid (200 µl) for 10 min on a rotating stirrer. After short centrifugation, 100 µl of the supernatant was injected via a model 234 autosampler (Gilson, Villiers-le-Bel, France) in a HPLC apparatus (Gilson, model 307 pump). Chromatographic separation was performed on a reversed phase column (ODS2 Nucleosil 5 m, 250 × 4.6 mm; Macherey Nagel, Darmstadt, Germany). The mobile phase consisted of an aqueous solution of 0.1 M formic acid containing 1 mM citric acid, 0.5 mM EDTA, 50 mM diethylamine, 5.8 mM sodium azide, 14% (v/v) acetonitrile and 0.5 mM sodium dodecylsulphate, pH 3.2. The flow rate was 1 ml/min.

Detection of catecholamine was achieved using a Coulochem 5100A electrochemical detector (ESA, Bedford, MA, USA) equipped with an analytical cell model 5011 (parameters of choice: first electrode +0 mV, second

electrode +550 mV, gain 100 × 20 corresponding to a full scale current of 50 nA). For 5-HT determination, the procedure was as previously described (Zancanaro *et al.*, 1995) with slight modifications. A 75 µl aliquot of the thawed supernatant was added with 75 µl of 100 ng/ml 5-hydroxy-*N*-methyl-tryptamine [internal standard (Sigma, Milan, Italy)] and 40 µl sodium acetate (5 M). After mixing and short centrifuga tion, 20 µl of the sample was injected via autosampler into the chromatograph equipped with a reversed phase column (ODS2 Nucleosil 5 µm, 150 × 4.6 mm) and a Coulochem 5100A electrochemical detector (ESA) coupled with an analytical cell model 5011 (first electrode +250 mV, second electrode +520 mV, gain 10 × 20 corresponding to a full scale current of 500 nA). The mobile phase was an acidic solution (formic acid 0.1 M, citric acid 1 mM, EDTA 0.5 mM, diethylamine 50 mM, sodium azide 5.8 mM, acetonitrile 95 mM) delivered at a flow rate of 1 ml/min. pH was adjusted to 3.2. Under the described conditions, the minimum detectable amount of catecholamine and 5-HT was 5 pg, with a S/N ratio of 2.

Results

Figure 1 shows the amine concentration in the VNO of adult frogs. The mean catecholamine concentration was higher in female than in male individuals (difference statistically significant for adrenaline, $P < 0.025$); 5-HT concentrations were similar in the two sexes.

Figure 2A shows the amine content of VNO in prepubertal, pubertal and adult male mice. Analyses of these data showed that the concentrations of the three amines significantly differed among groups (F = 14.8, 22.2 and 23.9 for adrenaline, noradrenaline and 5-HT respectively; $P < 0.001$). Analysis of differences between groups showed that the concentration of the three amines was lower in pubertal than in prepubertal and adult mice ($P < 0.05$–$P < 0.005$). Moreover, the amine concentrations were lower in pubertal than in adult individuals (noradrenaline, $P < 0.025$, 5-HT, $P < 0.05$; adrenaline, not significant).

Figure 2B shows the amine content of VNOs in prepubertal, pubertal and adult female mice. The concentration of adrenaline and 5-HT (but not noradrenaline) significantly differed among groups ($F = 4.4$, $P < 0.025$; $F = 6.7$, $P < 0.005$ respectively). The concentration of adrenaline was significantly higher in pubertal than in prepubertal

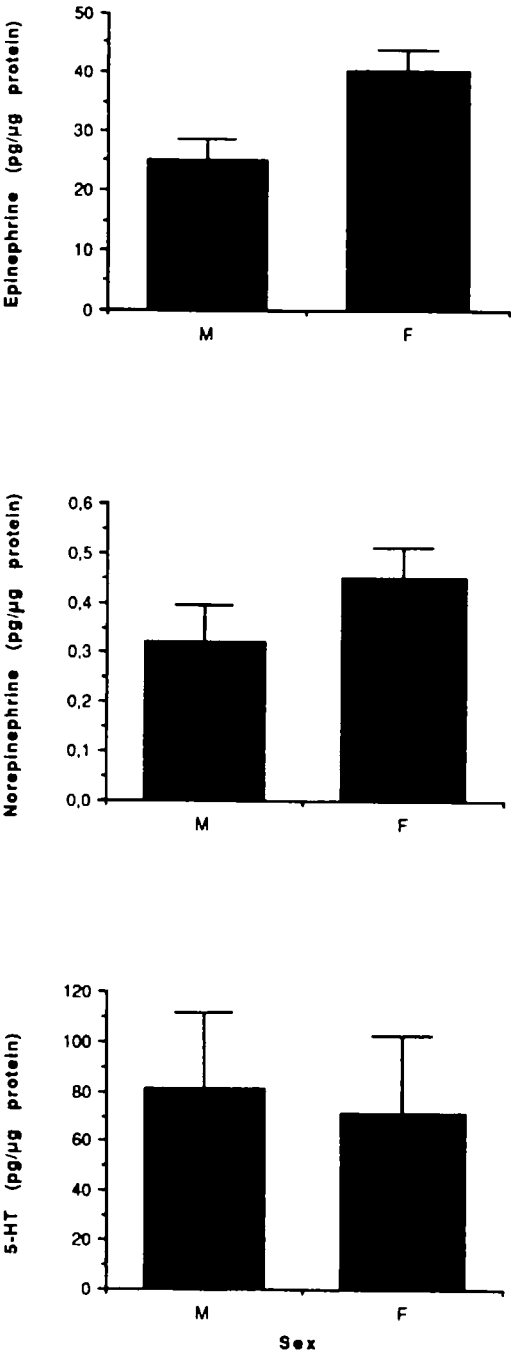

Figure 1 Amine concentration in the VNO of adult frogs (n = 4). The mean concentrations of catecholamines (adrenaline and noradrenaline) were higher in females ($P < 0.025$ for adrenaline, Wilcoxon rank test). M, male, F, female

($P < 0.001$) and adult ($P < 0.002$) individuals. The VNO content of 5-HT was significantly higher in adult females in comparison with both prepubertal and pubertal individuals ($P < 0.01$).

Data shown in Figure 2A and B were also analysed (Table 1) to compare the concentration of adrenaline,

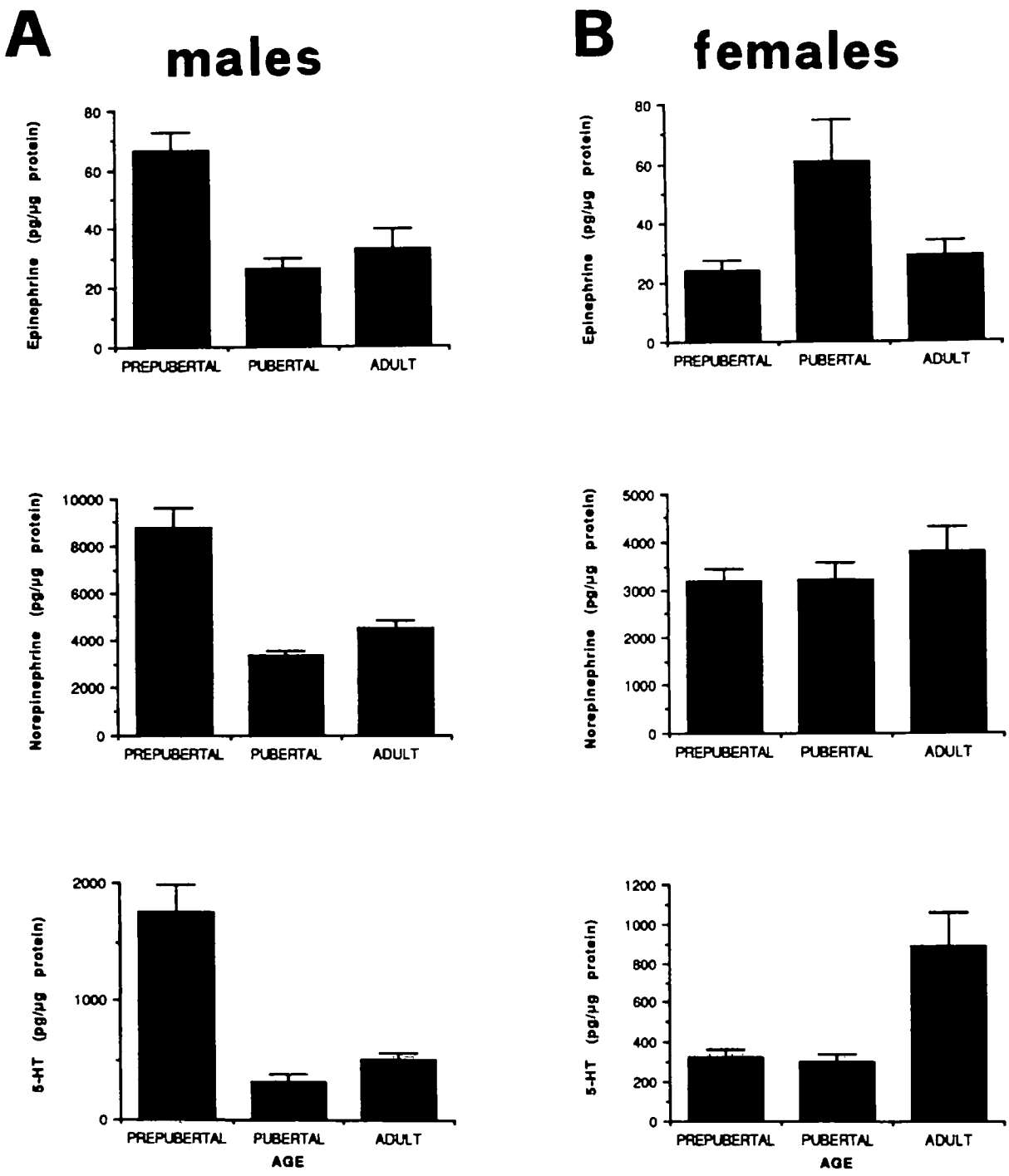

Figure 2 (A) Amine concentrations in the VNO of prepubertal (n = 13), pubertal (n = 9) and adult (n = 10) male mice Analysis of variance showed significant differences among the three groups for all the measured amines (P < 0 001) Pubertal mice showed lower concentration of the three amines in comparison with both prepubertal (P < 0.005) and adult individuals (adrenaline, P < 0 05, noradrenaline and 5-HT, P < 0 005, Bonferroni's test) (B) Amine concentrations in the VNO of prepubertal (n = 10), pubertal (n = 14) and adult (n = 13) female mice Analysis of variance showed significant differences among the three groups for adrenaline (P < 0 025) and 5-HT (P < 0.005) Pubertal mice showed a higher concentration of adrenaline in comparison with both prepubertal (P < 0 0001) and adult (P < 0 002) individuals (Bonferroni's test) The concentration of 5-HT was higher in adult mice than in prepubertal and pubertal individuals (P < 0.01, Bonferroni's test)

noradrenaline and 5-HT in male and female mice at each stage of sexual development (prepubertal, pubertal, adult). In prepubertal mice, the concentration of any of the three amines were obviously lower in female than in male individuals and the difference was highly significant (P < 0.005–P < 0.0001). In pubertal mice, female individuals

Table 1 Concentration of biogenic amines in the VNO of male and female mice at different stage of sexual maturity (pg/μg protein; means ± SEM)

Amine	Age								
	Prepubertal			Pubertal			Adult		
	Male	Female	*P*	Male	Female	*P*	Male	Female	*P*
Adrenaline	66 8 ± 5 86	24 3 ± 3.57	<0.0001	26.4 ± 3.36	61 1 ± 13.60	<0.00001	32.9 ± 6.67	29.3 ± 5 46	NS
Norepinephrine	8752 ± 830	3207 ± 257	<0.0005	3332 ± 205	3237 ± 327	NS	4512 ± 325	3808 ± 473	NS
Serotonin	1761 ± 226	323 ± 40.2	<0.00001	329 ± 52 2	305 ± 34 7	NS	522 ± 48	890 ± 160	<0.0004

showed a statistically significant difference in adrenaline concentration ($P < 0.00001$). In adult mice, 5-HT concentration was higher in female individuals ($P < 0.0004$); adrenaline and noradrenaline concentrations did not significantly differ.

In the VNO of adult females ($n = 14$), acute exposure to male urine reduced the amine concentration, expressed as percent of the relevant control, to 87.4 ± 9.86, 88.6 ± 13.66 and 55.2 ± 9.86% for adrenaline, noradrenaline and 5-HT respectively. In the control group ($n = 18$), data from saline-exposed ($n = 5$) and not-exposed ($n = 13$) individuals were pooled, since their amine concentrations were similar.

Discussion

It is well known that biogenic amines play important roles in both the central and peripheral nervous system. Discrete amounts of biogenic amines have been found in a chemoreceptor organ, the taste organ (Zancanaro *et al.*, 1995), where they are possibly involved in stimulus transduction/modulation. To the best of our knowledge, this is the first quantitative determination of biogenic amines in the vomeronasal organ.

Results presented in this paper demonstrate that: (i) the VNO of frogs (Amphibia) and mice (Mammalia) contains discrete amounts of catecholamine and 5-HT in both sexes, and (ii) the amine content of the VNO shows changes related to age and sexual maturity of mice and a clear sexual dimorphism is present in at least prepubertal mice.

In the frog, the adrenaline/noradrenaline ratio was found to be much higher than in the mouse. This is consistent with the ubiquitous larger presence of adrenaline in frog tissues and fluids (see e.g. Zancanaro *et al.*, 1995).

It should be noted that the surgically removed VNO is composed of the neurosensory and non-neurosensory epithelium, VN glands, blood vessels, etc. Therefore, in this work, any individual HPLC measurements of biogenic amines in the VNO were obtained in a pool from varied structures. Nevertheless, the VNO is enclosed in a bony capsule, and therefore it is easily dissected free of external, contaminating structures, thereby ensuring inter-individual reproducibility. The chief possible sources of biogenic amines in the surgically removed VNO are blood trapped in vessels, nerves and amine-containing local cells. The distribution of biogenic amines in the VNO was not investigated in this study. However, the morphological evidence available indicates the lack of amine stores in perikaria of VNO neurons both in the mouse (Mendoza, 1993) and the frog (Franceschini *et al.*, 1991). Instead, dense-cored vesicles (possibly containing amines) have been shown in terminal axons lying between the receptor cell perikaria, in the absence of synaptic specialization of the receptor membrane (Mendoza, 1993). A possibly larger set of efferent nerve fibres containing dense-cored granules is in the nerve supply of VN glands (Mendoza and Kunel, 1987). These are of serous (Mendoza, 1986; Salazar *et al.*, 1996) or mucous (Vaccarezza *et al.*, 1981) acinus type and are possibly related to the activity of the VNO pump (Meredith and O'Connel, 1979). In many vertebrates (inclusive of the mouse but exclusive of the frog) the VNO receives sympathetic and parasympathetic fibres that regulate the so-called 'vascular pump' of the organ (Meredith and O'Connel, 1979), which is supposed to take part in the stimulus access to the VNO. Therefore, according to the available evidence, the catecholamine content of the VNO is better accounted for by efferent nerve projections to the VNO. Serotonin is released by neurons (acting as a transmitter) and by other cell types in a variety of organs (exerting 'paracrine' actions). Serotonin-containing cells have been demonstrated histochemically in a chemoreceptor organ, the taste organ (see e.g. Sbarbati *et al.*, 1989), but not in the VNO. Results presented here suggest that further

morphological investigation is needed to localize the 5-HT-containing cell(s) and/or structures in the VNO.

According to results obtained in adult individuals (Figure 1; Table 1), the concentration of biogenic amines in the VNO showed a tendency to sex-related changes in both frogs and mice. The catecholamine (adrenaline and noradrenaline) content of VNO was higher in female than in male frogs (difference significant for noradrenaline), whereas 5-HT showed a tendency to be higher in males. A somewhat inverse pattern was found in mice, where the catecholamine content was slightly higher in males and 5-HT was higher in females ($P < 0.0004$, Table 1). In the central nervous system, sex differences have been found in neurotransmitter content and enzyme activities of the noradrenergic, dopaminergic and serotoninergic systems (Gordon and Shellenberger, 1974; Gilmore and Wilson, 1983; Siddiqui and Gilmore, 1988), but these have not been investigated in the VNO. Data presented herein suggest that the VNO of adult frogs and mice is sexually dimorphic as far as the biogenic amines content is concerned. The functional significance of such differences remains to be elucidated.

In young mammals the development of VNO sexual dimorphism is influenced by androgen hormones and the VNO plays important roles in the pheromonal modulation of puberty onset (Kaneko *et al.*, 1980). Therefore, we also investigated the VNO amines content in prepubertal and pubertal mice of both sexes. In prepubertal mice the concentration of adrenaline, noradrenaline and 5-HT in the VNO was obviously higher in males than in females (Table 1). Therefore, a marked sexual dimorphism is apparently present in prepubertal mice, in this regard. Pubertal male mice show depletion of biogenic amines in comparison with prepubertal (Figure 1). In adult males, the amine concentrations increased again (Figure 1), but did not reach the prepubertal levels. In females, puberty is only associated with a striking rise of the VNO content of adrenaline (Figure 1). Therefore, it seems that the surge of androgen hormones during puberty associates with amines depletion in the VNO of male mice. Definition of the androgen dependency of such a phenomenon requires further study.

Preliminary data presented here show that acute exposure to adult male urine (containing pheromones) was associated with lower amine content in the VNO of adult female mice in comparison with pooled saline- and not-exposed individuals. In mice, several pheromonal effects are dependent on a functional VNS, for example suppression of oestrus induced by female group housing (Reynolds and Keverne, 1979) or the failure of implantation of fertilized ova in the uterus following exposure to a novel male (Keverne, 1983). Male pheromones are known to induce effects in the central nervous system (via the VNS from the accessory olfactory bulb to the corticomedial amygdala and to the neuroendocrine hypothalamus), resulting in reduced prolactin secretion and increased luteinizing hormone (LH) levels (Keverne and de la Riva, 1982). Data presented herein suggest that exposure to adult male urine, which is a major source of pheromones, can induce biochemical changes in the peripheral receptor organ of the VNS, the VNO.

In conclusion, results presented in this paper demonstrate, in frogs and mice VNO, the presence of biogenic amines. The amine concentration changed in association with sex and sexual maturity, thereby suggesting a physiological role of adrenaline, noradrenaline and 5-HT in VNO. Further work is required to elucidate the tissue distribution and the precise role of biogenic amines in the VNO.

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