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Olfactory Sensitivity to Catecholamines and their Metabolites in the Goldfish

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Abstract

The current study assessed the olfactory sensitivity of the goldfish (*Carassius auratus* L.) to the catecholamines, their immediate precursors and metabolites by use of the electro-olfactogram (EOG). The olfactory system of the goldfish was found to be sensitive to both adrenaline and dopamine with thresholds of detection of $10^{-7.8}$ and $10^{-7.9}$ M respectively, but less so to noradrenaline (threshold of detection $10^{-6.3}$ M). The 3-*O*-methoxy metabolites (metadrenaline, normetadrenaline and 3-*O*-methoxytyramine) evoked larger amplitude EOGs than the non-metabolized form with lower thresholds of detection. However, the olfactory system was less sensitive to the amino acid precursors L-tyrosine and L-DOPA, and markedly less so to the α-deaminated metabolites (3,4-dihydroxyphenyl glycol, 3,4-dihydroxy mandelic acid and dihydroxyphenyacetic acid). Sensitivity to metabolites, both α-deaminated and 3-*O*-methoxylated, was similar to the α-deaminated forms. Cross-adaptation studies suggested that, while there is some degree of commonality of the receptor mechanisms with L-tyrosine and L-serine, a proportion of the response to the catecholamines is due to distinct receptor subtypes. Similarly, the 3-*O*-methoxy metabolites also had (a) separate receptor mechanism(s), although, again, there was overlap with the adrenaline/dopamine receptor site(s). Presence of the α-adrenoreceptor antagonist prazosin or the peripheral DA_2 dopamine receptor antagonist domperidone caused partial attenuation of the EOG responses to adrenaline and dopamine, but had much less effect on the responses to their 3-*O*-methoxy metabolites. The β-adrenoreceptor antagonist sotalol had no such effect. This suggests that the olfactory catecholamine receptors are structurally and functionally distinct from systemic adreno- and dopamine receptors. The current study raises the possibility that release of catecholamines or their 3-*O*-methoxy metabolites to the water may play a role in chemical communication.

Key words: adrenaline, dopamine, electro-olfactogram (EOG), metabolites, teleost.

Introduction

The olfactory system of many species of fish has been shown to be sensitive to amino acids (Hara, 1994; Sorensen and Caprio, 1998). These are detected by at least four main receptor types (Caprio and Byrd, 1984; Friedrich and Korsching, 1997), recognizing amino acids with neutral (subdivided into short chain and long chain), basic or acidic side chains. Individual amino acids may be recognized centrally by the pattern of activity evoked in the olfactory bulb in a combinatorial manner (Friedrich and Korsching, 1997). However, it is the presence of the α-amino group which is of prime importance in conferring olfactory potency (Lipschitz and Michel, 1999). Another group of biologically important compounds with an α-amino group is the catecholamines: the olfactory potency of this group in fish is unknown.

Adrenaline, dopamine and noradrenaline have well characterized roles as neurotransmitters, neuromodulators, paracrine agents and circulating hormones. Given that the plasma concentrations of catecholamines (principally adrenaline, but to a lesser extent noradrenaline) undergo massive and rapid increases in response to various forms of stress in fish (Wendelaar Bonga, 1997; Reid *et al.*, 1998), and these in turn have very marked effects on many aspects of physiology, especially cardiovascular effects (Fabbri *et al.*, 1998), it is possible that a proportion of these, or their metabolites, may be released into the water where they may have some communicative function. To test this hypothesis, we investigated the olfactory sensitivity of goldfish (*Carassius auratus*, L., a freshwater cyprinid inhabiting still-waters and slow-moving reaches of rivers) to the catecholamines, their precursors (L-tyrosine and L-DOPA) and their metabolites (both 3-*O*-methoxylated and α-deaminated forms; Figure 1) by use of the electro- olfactogram (EOG).

The half-life of circulating catecholamines is very short: they are rapidly metabolized to inactive forms. In mammals, catecholamines are firstly metabolized by the enzymes monoamine oxidase (MAO) and catechol-*O*-methyltransferase (COMT) which convert them to the corresponding

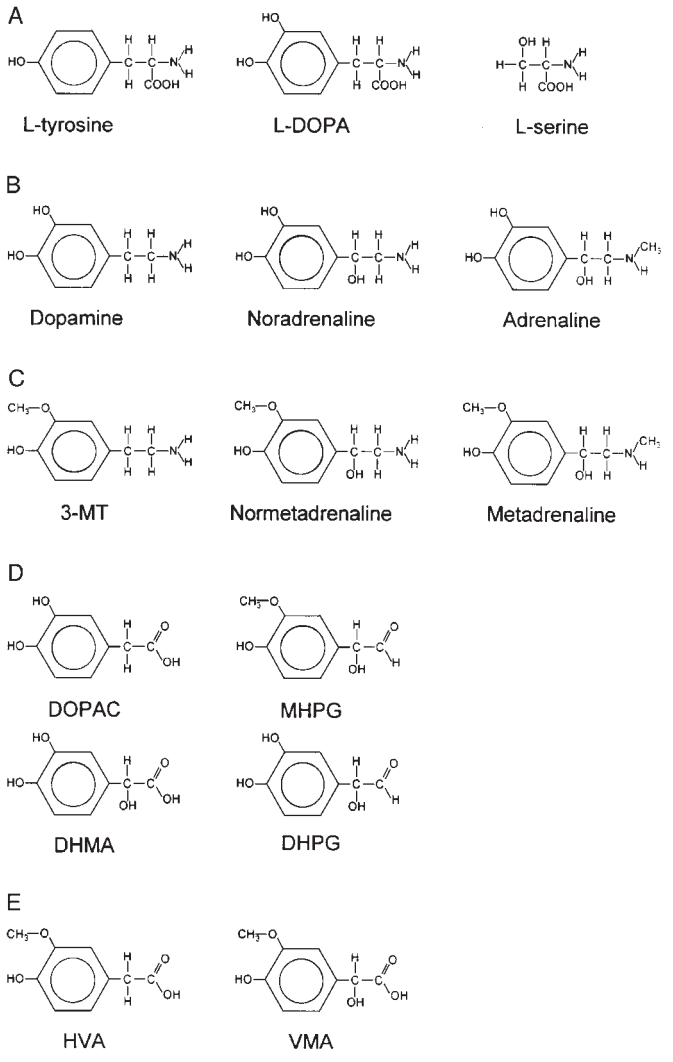

Figure 1 Chemical structures of the odorants used in this study. **(A)** Amino acids, including the immediate precursors of the catecholamines (L-tyrosine and L-DOPA; L-3,4-dihydroxyphenylalanine). **(B)** The catecholamines. **(C)** 3-*O*-methoxy metabolites of the catecholamines (3-MT; 3-methoxytyramine). **(D)** Some α-deaminated metabolites of the catecholamines (DOPAC; 3,4-dihydroxyphenylacetic acid, MHPG; 3-methoxy-4-hydroxyphenylglycol, DHMA; 3,4-dihydroxymandelic acid, DHPG; 3,4-dihydroxyphenylglcol). **(E)** 'End products' of catecholamine metabolism (α-deaminated, oxidized and 3-*O*-methylated, HVA; homovanillic acid or 4-hydroxy–3-methoxyphenylacetic acid, VMA; vanilmandelic acid or 3-methoxy–4-hydroxymandelic acid).

aldehydes (e.g. DHPG, Figure 1) and 3-*O*-methyl metabolites (e.g. metadrenaline, Figure 1) respectively (Cooper *et al.*, 1996). The aldehydes are rapidly oxidized to the corresponding acid (e.g. DHMA, Figure 1). As both MAO and COMT are relatively non-specific, both enzymes can act on the products of the other to produce 3-*O*-methylated and α-deaminated metabolites (e.g. HVA and VMA, Figure 1). These are the chief metabolites excreted via the urine. Whether the same pathways predominate in fish is much less well studied, although the limited data available suggest that they do (Mazeaud and Mazeaud, 1973b; Sloley *et al.*, 1992). For example, a trout MAO has been cloned (Chen *et al.*, 1994). However, the rates and routes of release of these metabolites are not yet known (Mazeaud and Mazeaud, 1973a).

Materials and methods

Experimental animals

Goldfish of both sexes (nose to fork length: 76–204 mm; weight: 8–207 g) were kept outside in 1000 l tanks under semi-natural conditions (i.e. under natural photoperiod and temperature variation) and fed once or twice a day (depending on temperature) on commercial pond-fish food (TetraPond Pond Sticks®, TetraWerke, Melle, Germany).

Recording of the EOG

The method used for recording EOGs from goldfish has been described in detail previously (Hubbard *et al.*, 2002). Briefly, the goldfish were anaesthetized by immersion in water containing 3-aminobenzoic acid ethyl ester (MS222; 80 mg/l) and immobilized with intramuscular injection of neuromuscular blocker (gallamine triethiodide; 1 mg/kg in 0.9% NaCl), and maintained with water flowing over the gills (containing 40 mg/l MS222) in a padded V-clamp. The flap of skin overlying the nostril was removed and the recording electrode placed near the raphe, between two adjacent olfactory lamellae—a site previously shown to give the best response to known odorants (e.g. amino acids). The reference electrode was placed lightly on the skin of the head and connected to earth via the headstage of the amplifier. The DC voltage signal was amplified (gain × 1000), filtered above 50 Hz, digitized and recorded by computer running the appropriate software. Electrodes were made from borosilicate glass micropipettes containing 4% agar in 0.9% NaCl, connected to solid-state electronics via an Ag/AgCl pellet.

Stimuli preparation and delivery

All odorants were bought from SigmaAldrich Chemical Co. (Madrid, Spain). All stimuli were dissolved in distilled water (10^{-3} M), although some catecholamines were previously dissolved in a small volume of 0.1 M HCl prior to this, and stored aliquoted at –20ºC. All test solutions were made up immediately prior to use (to avoid possible oxidation of the catecholamines) in dechlorinated, charcoal-filtered tap-water; the same water used to irrigate the nostril (see below) in acid-washed glassware. The nostril of the fish was continuously irrigated via a glass tube, the opening of which was held immediately above the olfactory rosette, at a flow-rate of 6 ml/min under gravity with dechlorinated, charcoal-filtered tap-water. Odorant containing solutions were introduced into this flow via a computer-controlled three-way solenoid valve for a period of 10 s.

Experimental design

The olfactory responses of the goldfish to the catecholamines (dopamine, adrenaline and noradrenaline) were first assessed by concentration/response experiments. These were carried out by stimulating the olfactory epithelium with increasing concentrations (10^{-9}–10^{-4} M), allowing at least 1 min to elapse between stimuli to ensure washout of the stimulus from the nasal cavity, and to counteract any possible adaptation. Once it was clear that goldfish had an acute olfactory sensitivity to dopamine and adrenaline, the sensitivity to a range of precursors and metabolites (Figure 1) was assessed using the same experimental approach. Having established the most potent odorants (dopamine, adrenaline and their 3-*O*-methoxy metabolites; 3-MT and metadrenaline, respectively) within this group of compounds, the olfactory selectivity was investigated by means of cross-adaptation experiments (e.g. Lipschitz and Michel, 1999). The adapting solutions (all at 10^{-5} M) were continuously superfused over the olfactory epithelium for at least 1 min before the odorant in question (in the presence 10^{-5} M adapting odorant) was applied as stimulus. Ideally, the concentration of each odorant should be chosen to evoke EOGs of similar magnitude. However, due to variability in the relative magnitude of the responses to the amino acids (L-tyrosine and L-DOPA) and catecholamines among different fish, this was not possible. The 'self-adapted control' (SAC) consisted of the odorant in question (at 2×10^{-5} M; the same total concentration of odorant as in the test solutions) against a background of 10^{-5} M. The amplitudes of these responses were compared to the means of controls (10^{-5} M of each odorant alone) run before and after the cross-adaptation experiments. Finally, the possibility that the olfactory responses are mediated by 'conventional' dopamine and/or adreno- receptors was investigated by continually superfusing the olfactory epithelium with a DA_2 dopamine receptor antagonist (domperidone), an α-adrenoreceptor antagonist (prazosin) or a β-adrenoreceptor antagonist (sotalol), and comparing the responses to the catecholamines, their 3-*O*-methoxy metabolites and L-tyrosine (plus L-serine at 10^{-5} M, and the structurally unrelated steroid goldfish pheromone, 4-pregnene–17α,20β-diol-3-one (17,20β-P at 10^{-9} M), as controls) in the presence of these antagonists (10^{-7} and 10^{-6} M) to those under control conditions (absence of antagonist). Only one antagonist was used in each experiment, and the order of treatments was randomized.

Data treatment and statistical analysis

The amplitude of the initial sharp peak of the EOG was measured in millivolts. This was then blank-subtracted (the amplitude of EOG response to water treated in the same way as the odorant solutions but containing no odorant: this was generally <10% of the amplitude of the response to 10^{-5} M L-serine). To reduce the variation of responses of different fish, this was then normalized to a previously run 'standard' of 10^{-5} M L-serine, blank subtracted in the same way. These standard responses were run at regular intervals throughout the experiment (every 10–20 min). Thresholds of detection were calculated by linear regression on log-transformed data using the formula $\log(N + 1.5) = a \log C + b$, where N is the normalized response, C is the concentration, and a and b are constants. The threshold of detection is then the value for x where $y = 0.1761$ (i.e. log 1.5; $N = 0$). For each odorant, only the concentrations that elicited EOG responses significantly greater than zero were used. This revealed that the odorants used could be divided into groups according to chemical structure, discarding the outliers noradrenaline and normetadrenaline (see below). Thus the data for adrenaline and dopamine were pooled (catecholamines), likewise 3-MT and metadrenaline (3-*O*-methoxy metabolites), DOPAC and DHMA (α-deaminated metabolites), HVA and VMA (α-deaminated and 3-*O*-methoxy metabolites) and L-DOPA and L-tyrosine (amino acids). The aldehydes, DHPG and MHPG, were not included as they are short-lived intermediates and rapidly metabolized to their respective acid forms. Linear regressions (slopes and line elevations) were compared within and between groups of compounds by Student's *t*-test (two lines) or by ANOVA followed by Tukey's test (Zar, 1996). Pooled, normalized data from the cross-adaptation experiments were subjected to repeated-measures ANOVA followed by Dunnett's test (SigmaStat 2.0, Jandel Scientific), comparing to both controls and self-adapted controls. Pooled, normalized data from the antagonist experiments were compared by repeated-measures ANOVA followed by Dunnett's test for each concentration of odorant. In all cases, a P value of <0.05 was considered significant.

Results

Olfactory sensitivity to catecholamines

The olfactory system of the goldfish proved to be highly sensitive to adrenaline and dopamine (Figure 2A–C), but less so to noradrenaline (Figure 2D), evoking typical, biphasic fish EOGs with a rapid negative deflection at stimulus onset, followed by a period of adaptation during which the EOG fell to approximately half of the peak amplitude (Figure 2A). After the end of the stimulus period the potential returned to baseline levels within a few seconds. The amplitude of the EOG responses was strongly concentration dependent, increasing by a factor of approximately two for each tenfold increase in odorant concentration. Linear regression of log-transformed data revealed highly significant relationships [adrenaline: $F(1,162) = 371.97$, $P < 0.001$, $R^2 = 0.697 \pm 0.080$, $a = 0.086 \pm 0.005$, $b = 0.845 \pm 0.027$; dopamine: $F(1,158) = 312.55$, $P < 0.001$, $R^2 = 0.664 \pm 0.082$, $a = 0.082 \pm 0.005$, $b = 0.822 \pm 0.028$; noradrenaline: $F(1,39) = 105.39$, $P < 0.001$, $R^2 = 0.730 \pm 0.074$, $a = 0.146 \pm 0.014$, $b = 1.094 \pm 0.072$; values ±

SEM). Thresholds of detection were estimated as $10^{-7.8}$ M for adrenaline, $10^{-7.9}$ for dopamine and $10^{-6.3}$ M for noradrenaline. No significant differences were found between the slope and elevation of the linear regression lines calculated for adrenaline and dopamine [Tukey's test, $q(\infty,3) = 0.854$, $P > 0.05$ for comparison among slopes; $q(\infty,3) = 0.105$, $P > 0.05$ for comparison among elevations], whereas those parameters in the linear regression for noradrenaline were significantly different from the other two catecholamines [comparison among slopes: dopamine, $q(\infty,3) = 5.616$, $P < 0.001$; adrenaline, $q(\infty,3) = 5.283$, $P < 0.001$; comparison among elevations: dopamine, $q(\infty,3) = 4.754$, $P < 0.01$; adrenaline, $q(\infty,3) = 4.833$, $P < 0.01$]. Thus the olfactory system of the goldfish is equally sensitive to adrenaline and dopamine but significantly less so to noradrenaline.

Olfactory sensitivity to catecholamine metabolites

The 3-*O*-methoxy metabolites, metadrenaline and 3-MT, also proved to be potent odorants to goldfish, again evoking typical fish EOGs in a concentration-dependent manner (Figure 2A). Significant linear relationships were obtained for metadrenaline [$F(1,111) = 248.09$, $P < 0.001$, $R^2 = 0.691 \pm 0.089$, $a = 0.099 \pm 0.006$, $b = 0.936 \pm 0.039$], 3-MT [$F(1,114) = 385.79$, $P < 0.001$, $R^2 = 0.772 \pm 0.073$, $a = 0.095 \pm 0.005$, $b = 0.933 \pm 0.030$) and normetadrenaline [$F(1,41) = 141.70$, $P < 0.001$, $R^2 = 0.776 \pm 0.059$, $a = 0.098 \pm 0.008$, $b = 0.885 \pm 0.045$]. The thresholds of detection were estimated as $10^{-7.7}$ M (metadrenaline) and $10^{-7.9}$ M (3-MT). No significant differences were found among the slopes of the regression lines obtained for the three metabolites [$F(3,266) = 0.098$, $P > 0.05$]. Also, no significant differences were found between the elevations of the linear regression lines calculated for metadrenaline and 3-MT [Tukey's test, $q(\infty,3) = 2.383$, $P > 0.05$ for comparison among elevations], but the elevation in the linear regression for normetadrenaline was significantly different from the other two metabolites [metadrenaline, $q(\infty,3) = 3.861$, $P < 0.05$; 3-MT, $q(\infty,3) = 5.670$, $P < 0.001$]: the olfactory system is equally sensitive to 3-MT and metadrenaline, but less so to normetadrenaline. The elevation of the linear regression line obtained for the pooled 3-*O*-methoxy metabolite data was significantly different from the one obtained with the catecholamines [Tukey's test, $q(\infty,5) = 8.086$, $P < 0.01$] but no significant difference was found between the slopes [$F(5,797) = 0.000004$, $P > 0.05$]. Also, both the slopes and elevations of the linear regression line obtained for noradrenaline and normetadrenaline differed significantly ($t = 3.159$, d.f. = 80, $P < 0.01$, for comparison between slopes; $t = 3.051$, d.f. = 81, $P < 0.01$, for comparison between elevations). Thus, in all cases, the sensitivity is greater for the 3-*O*-methoxy-metabolites than the unmetabolized forms.

A significant linear regression was found for the acid metabolites of adrenaline and dopamine [DHMA: $F(1,19) = 9.414$, $P < 0.01$, $R^2 = 0.331 \pm 0.089$, $a = 0.073 \pm 0.024$, $b = 0.649 \pm 0.120$; DOPAC: $F(1,21) = 17.741$, $P < 0.001$, $R^2 = 0.446 \pm 0.064$, $a = 0.067 \pm 0.016$, $b = 0.591 \pm 0.081$], with detection thresholds estimated as $10^{-6.5}$ M for DHMA and $10^{-6.2}$ M for DOPAC. No significant differences were found among the slopes and the elevations of the regression lines obtained for these two compounds ($t = 0.212$, d.f. = 41, $P > 0.05$, for comparison between slopes; $t = 1.228$, d.f. = 42, $P > 0.05$, for comparison between elevations). Significant differences were found between the elevations of the regression lines calculated for catecholamines and for the α-deaminated metabolites [Tukey's test, $q(\infty,5) = 28.725$, $P < 0.0001$], but no significant difference was found between the slopes [$F(5,797) = 0.000004$, $P > 0.05$]. In addition, the linear regression obtained for the α-deaminated metabolite of noradrenaline [DHPG; $F(1,19) = 13.331$, $P < 0.01$, $R^2 = 0.412 \pm 0.055$, $a = 0.054 \pm 0.015$, $b = 0.512 \pm 0.075$] was significantly different from the one obtained for noradrenaline ($t = 4.081$, d.f. = 58, $P < 0.01$, for comparison between slopes; $t = 5.905$, d.f. = 59, $P < 0.001$, for comparison between elevations), and indicated a lower estimated threshold of detection ($10^{-6.2}$ M). Thus, α-deamination markedly reduces olfactory potency compared to the parent compound. The linear regressions obtained for those metabolites both 3-*O*-methylated and α-deaminated [MHPG, $F(1,19) = 76.741$, $P < 0.001$, $R^2 = 0.802 \pm 0.068$, $a = 0.159 \pm 0.018$, $b = 1.122 \pm 0.092$; HVA; $F(1,26) = 47.285$, $P < 0.001$, $R^2 = 0.645 \pm 0.063$, $a = 0.073 \pm 0.011$, $b = 0.674 \pm 0.060$; VMA, $F(1,30) = 32.908$, $P < 0.001$, $R^2 = 0.523 \pm 0.075$, $a = 0.068 \pm 0.012$, $b = 0.635 \pm 0.067$] showed similar thresholds of detection [$F(3,77) = 0.650$, $P > 0.05$] for comparison among elevations (MHPG $10^{-6.0}$ M, HVA $10^{-6.8}$ M and VMA $10^{-6.8}$ M), but different slopes between MHPG and the other two compounds [HVA, Tukey's test, $q(75,3) = 5.517$, $P < 0.001$; VMA, $q(75,3) = 5.978$, $P < 0.001$]. The sensitivity is similar, but the amplitude of response is higher for HVA. Addition of the 3-*O*-methoxy group does not, therefore, restore olfactory potency to the α-deaminated metabolite.

Olfactory sensitivity to precursors

It is well established that the olfactory system of fish is highly sensitive to amino acids (e.g. Hara, 1994). The catecholamines are derived from the amino acid L-tyrosine (Figure 1) via L-3,4-dihydroxyphenylalanine (L-DOPA). As expected, therefore, goldfish had olfactory sensitivity to both L-tyrosine and L-DOPA (Figure 2E). Significant linear relationships were obtained for L-tyrosine [$F(1,118) = 202.50$, $P < 0.001$, $R^2 = 0.632 \pm 0.077$, $a = 0.072 \pm 0.005$, $b = 0.834 \pm 0.031$] and L-DOPA [$F(1,42) = 120.59$, $P < 0.001$, $R^2 = 0.742 \pm 0.050$, $a = 0.074 \pm 0.007$, $b = 0.714 \pm 0.038$], with thresholds of detection estimated as $10^{-7.8}$ and $10^{-7.3}$ M for L-tyrosine and L-DOPA respectively. These two linear relationships were not significantly different ($t = 0.117$, d.f. = 70, $P > 0.05$, for comparison between slopes; $t = 1.155$, d.f. = 71, $P > 0.05$, for comparison between elevations). The elevation of the linear regression line obtained for the

catecholamines (pooled data of adrenaline and dopamine) was significantly different from that of the amino acids [Tukey's test, $q(\infty,5) = 20.671$, $P < 0.0001$] but no significant difference was found between the two slopes [$F(5,797) = 0.000004$, $P > 0.05$): the sensitivity is greater for the catecholamines than their amino acid precursors.

Cross-adaptation

In the presence of dopamine, metadrenaline, 3-MT and the amino acids L-tyrosine and L-serine (at 10^{-5} M), the amplitude of EOG responses to adrenaline (10^{-5} M) was significantly lower than that of controls (Figure 3A). This suggests that part of the response olfactory response to adrenaline is mediated by (a) common receptor mechanism(s) to the other odorants. However, the responses to adrenaline in the presence of L-tyrosine or L-serine were significantly larger than adrenaline self-adapted controls (SACs): the EOG amplitudes of responses to dopamine, metadrenaline and 3-MT were statistically indistinguishable from the adrenaline SACs. This suggests that a large part of the olfactory response to adrenaline is independent of L-serine and L-tyrosine receptors, but most, if not all of the olfactory receptor sites for adrenaline can also be activated by dopamine, metadrenaline and 3-MT. Similarly, the amplitude of EOG responses to dopamine were also significantly attenuated by the presence of the other odorants when compared to controls (Figure 3B), suggesting that there is (a) common receptor mechanism(s) for all these odorants. Nevertheless, responses to dopamine in the presence of L-tyrosine, L-serine and adrenaline were significantly greater than the dopamine SACs. This is indicative of dopamine being detected by a sub-set of different receptor types to L-tyrosine and L-serine. Conversely, dopamine seems to be detected by the same receptor type(s) as 3-MT and metadrenaline. The responses to metadrenaline, however, were greater than metadrenaline SACs in the presence of all odorants other than 3-MT (Figure 3C). Again, all cross-adapted responses were lower than controls. Taken together, this suggests that whilst there is a degree of commonality among the receptor sites for metadrenaline, only 3-MT is capable of competing with metadrenaline for all of these sites. This is supported by the pattern of

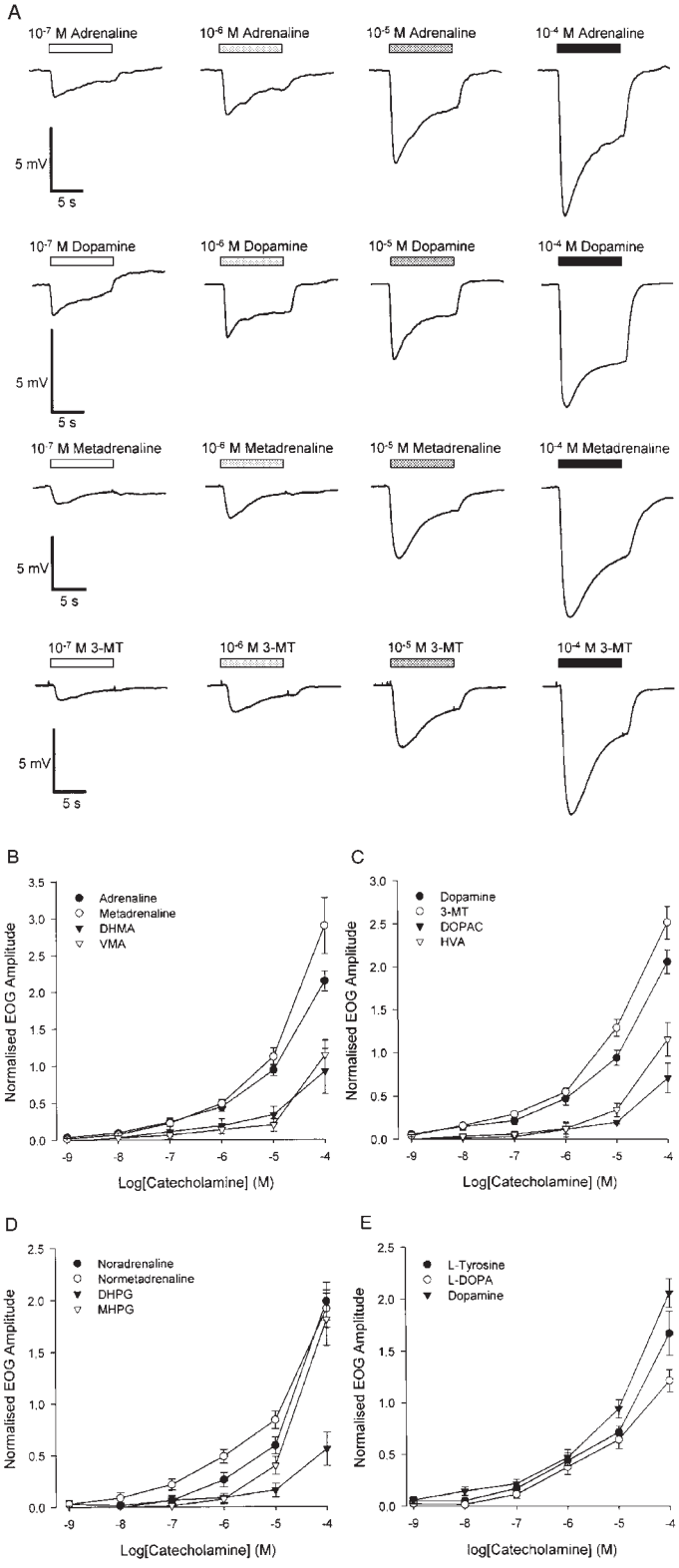

Figure 2 Olfaction of catecholamines, their precursors and metabolites by goldfish. **(A)** Typical EOG responses to adrenaline, dopamine and their 3-*O*-methoxy metabolites, metadrenaline and 3-MT respectively. Responses are from four independent experiments. A downward deflection of the EOG trace is negative. **(B)** Semi-logarithmic plot of normalized EOG amplitude against concentration of adrenaline (filled circles; $n = 34$) and its metabolites, metadrenaline (open circles; $n = 25$), DHMA (filled triangles; $n = 7$) and VMA (open triangles; $n = 8$). Data are shown as mean ± SEM. **(C)** Semi-logarithmic plot of normalized EOG amplitude against concentration of dopamine (filled circles; $n = 33$) and its metabolites, 3-MT (open circles; $n = 24$), DOPAC (filled triangles; $n = 8$) and HVA (open triangles; $n = 7$). Data are shown as mean ± SEM. **(D)** Semi-logarithmic plot of normalized EOG amplitude against concentration of noradrenaline (filled circles; $n = 14$) and its metabolites, normetadrenaline (open circles; $n = 11$), DHPG (filled triangles; $n = 7$) and MHPG (open triangles; $n = 7$). Data are shown as mean ± SEM. **(E)** Semi-logarithmic plot of normalized EOG amplitude against concentration of catecholamine precursors L-tyrosine (filled circles; $n = 25$) and L-DOPA (open circles; $n = 11$). The data for dopamine are included for comparison (filled triangles). In all figures, data are shown as mean ± SEM.

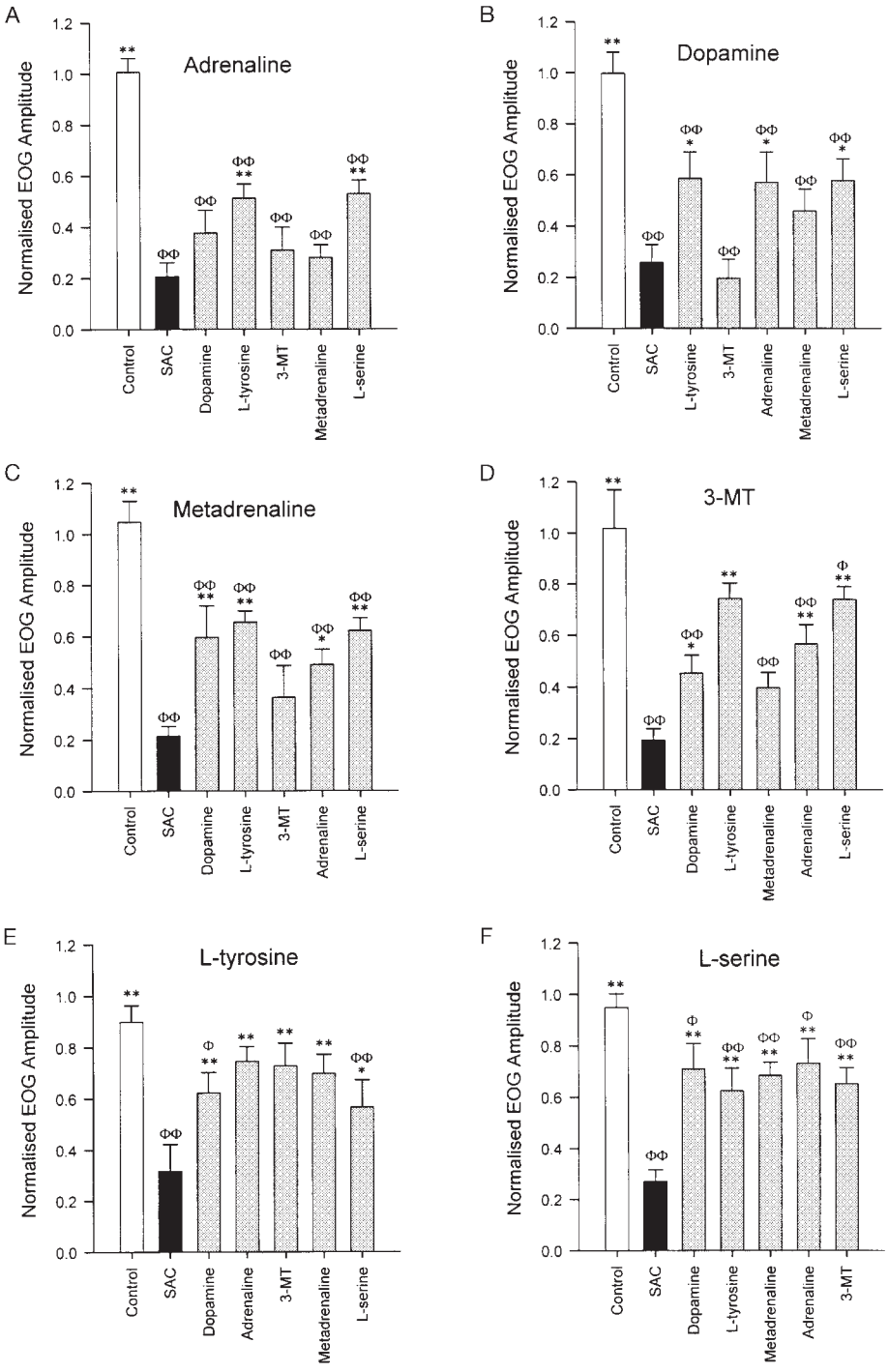

Figure 3 Olfactory selectivity of the catecholamines by the goldfish as assessed by cross-adaptation studies. **(A)** Amplitude of EOG responses to 10^{-5} M adrenaline in the absence (control) and presence of adapting solutions of adrenaline (SAC; self-adapted control), dopamine, L-tyrosine, 3-MT, metadrenaline and L-serine (all at 10^{-5} M). In this, and subsequent histograms, data (mean ± SEM, $n = 8$) are normalized to the mean of two control EOG responses run before and after experimental EOGs. **(B)** Amplitude of EOG responses to 10^{-5} M dopamine in the absence (control) and presence of adapting solutions of dopamine (SAC; self-adapted control), L-tyrosine, 3-MT, adrenaline, metadrenaline and L-serine (all at 10^{-5} M). **(C)** Amplitude of EOG responses to 10^{-5} M metadrenaline in the absence (control) and presence of adapting solutions of metadrenaline (SAC; self-adapted control), dopamine, L-tyrosine, 3-MT, adrenaline and L-serine (all at 10^{-5} M). **(D)** Amplitude of EOG responses to 10^{-5} M 3-MT in the absence (control) and presence of adapting solutions of 3-MT (SAC; self-adapted control), dopamine, L-tyrosine, metadrenaline, adrenaline and L-serine (all at 10^{-5} M). **(E)** Amplitude of EOG responses to 10^{-5} M L-tyrosine in the absence (control) and presence of adapting solutions of L-tyrosine (SAC; self-adapted control), dopamine, adrenaline, 3-MT, metadrenaline and L-serine (all at 10^{-5} M). **(F)** Amplitude of EOG responses to 10^{-5} M L-serine in the absence (control) and presence of adapting solutions of L-serine (SAC; self-adapted control), dopamine, L-tyrosine, metadrenaline, adrenaline and 3-MT (all at 10^{-5} M). Φ$P < 0.05$, ΦΦ$P < 0.01$ compared to control, *$P < 0.05$, **$P < 0.01$ compared to SAC (one-way repeated-measures ANOVA followed by Dunnett's test).

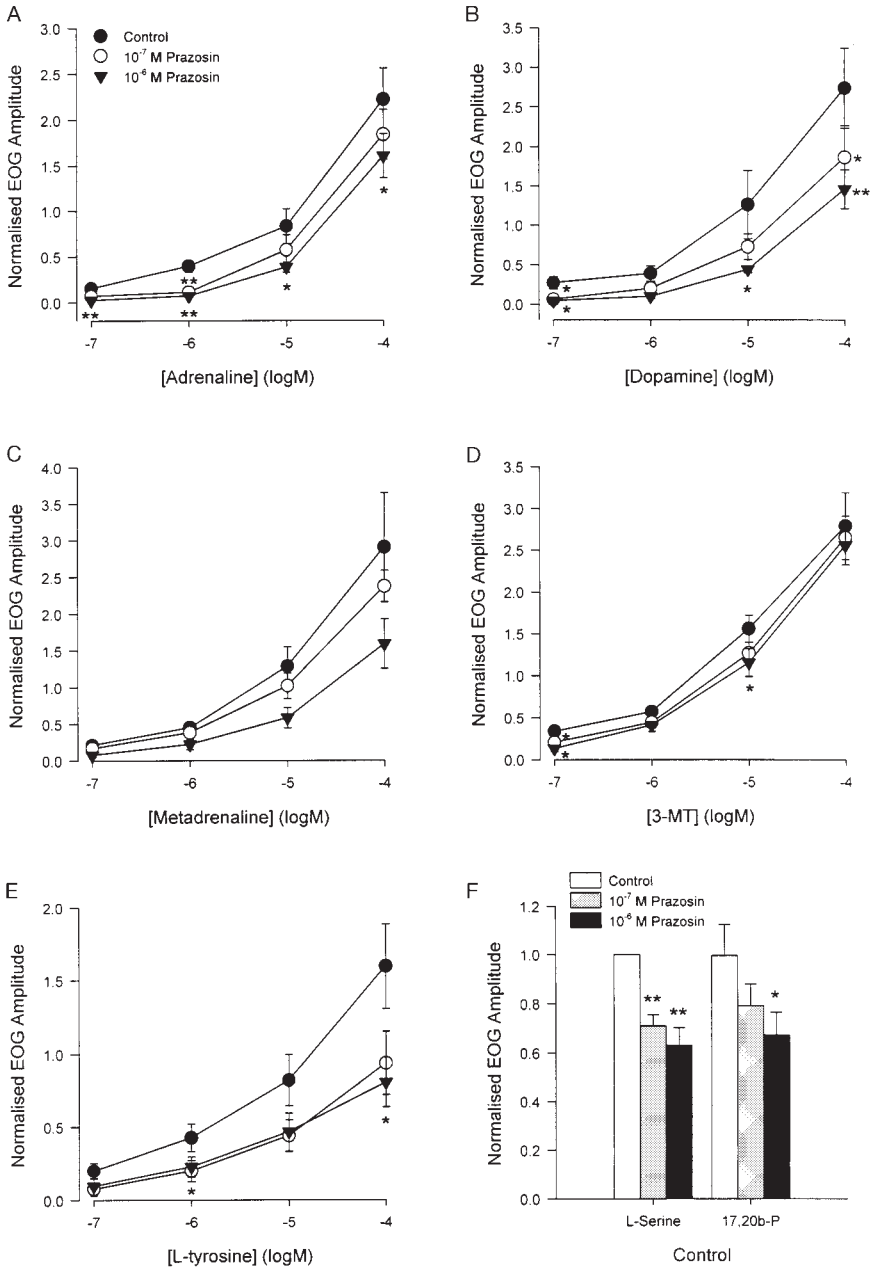

Figure 4 Effect of the α-adrenoreceptor antagonist prazosin on the olfactory responses to the catecholamines in the goldfish. Semi-logarithmic plots of normalized EOG amplitude in response to **(A)** adrenaline (10^{-7}–10^{-4} M), **(B)** dopamine, **(C)** metadrenaline, **(D)** 3-MT and **(E)** L-tyrosine in the absence (control; filled circles) and presence of 10^{-7} M (open circles) and 10^{-6} M prazosin (filled triangles). **(F)** Normalized EOG amplitudes in response to L-serine (10^{-5} M) and 17,20β-P (10^{-9} M) in the absence (open bars) and presence of 10^{-7} M (grey bars) and 10^{-6} M (black bars) prazosin. In each graph, data are shown as mean ± SEM ($n = 5$) and $^{*}P < 0.05$, $^{**}P < 0.01$ compared to control (one-way repeated-measures ANOVA followed by Dunnett's test).

responses to 3-MT in the presence of the other odorants (Figure 3D); although amplitudes of responses to 3-MT were reduced compared to controls, only the responses in the presence of metadrenaline were similar to 3-MT SACs. Thus it appears that there is/are (a) specific olfactory binding site(s) for 3-*O*-methylated catecholamines. In contrast, the olfactory responses to L-tyrosine and L-serine were somewhat less attenuated by the presence of the catecholamines. The responses to L-tyrosine (Figure 3E) were only significantly attenuated by the presence of dopamine and L-serine, suggesting a degree of overlap in the receptor mechanisms for these odorants. However, the cross-adapted responses were all larger than L-tyrosine SACs, indicative of (a) L-tyrosine specific binding site(s) at which the other odorants are without effect. In a similar manner, the responses to L-serine (Figure 3F) were only slightly (but significantly) reduced by the presence of the other odorants. The amplitudes of cross-adapted responses

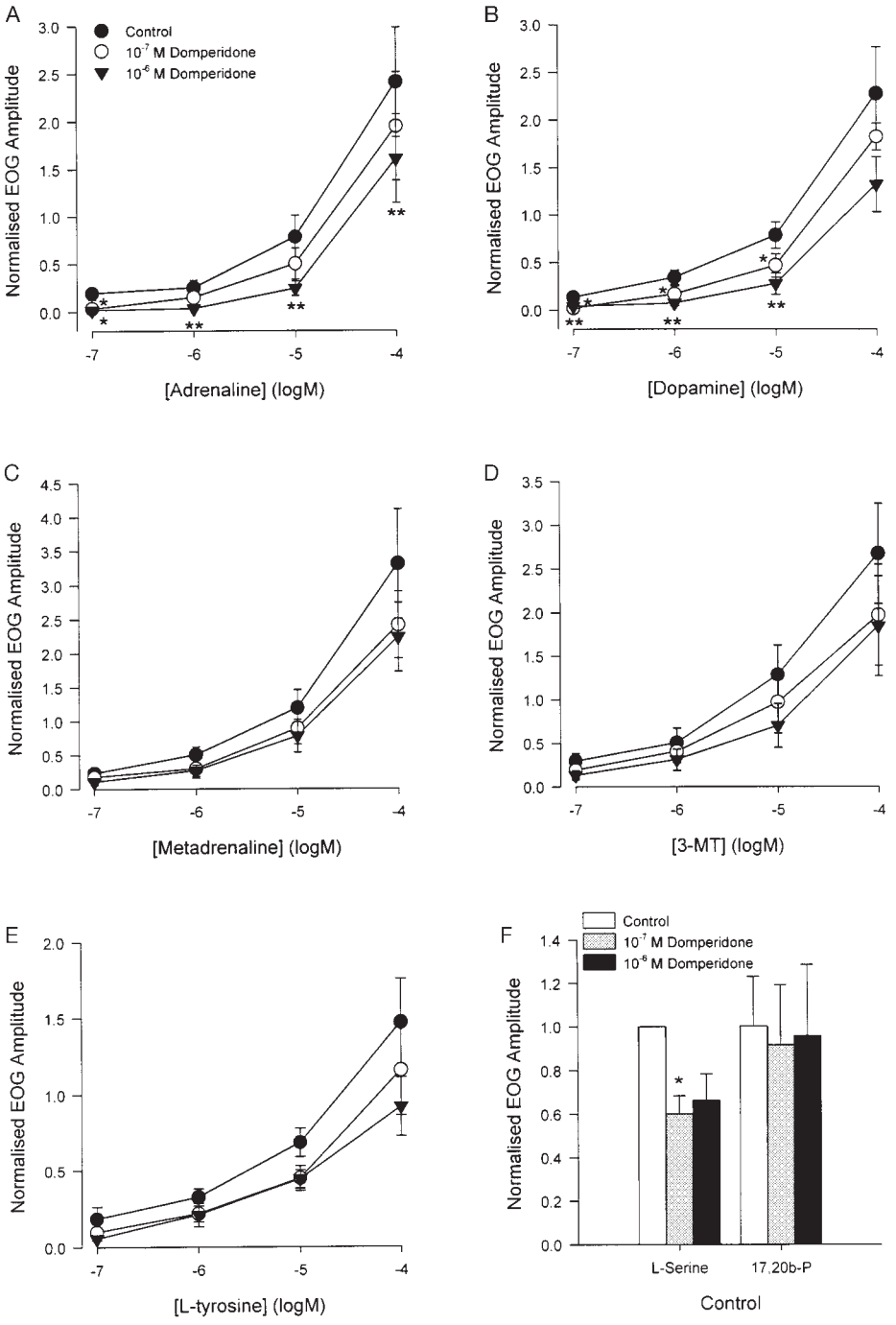

Figure 5 Effect of the dopamine receptor antagonist domperidone on the olfactory responses to the catecholamines in the goldfish. Semi-logarithmic plots of normalized EOG amplitude in response to **(A)** adrenaline (10^{-7}–10^{-4} M), **(B)** dopamine, **(C)** metadrenaline, **(D)** 3-MT and **(E)** L-tyrosine in the absence (control; filled circles) and presence of 10^{-7} M (open circles) and 10^{-6} M domperidone (filled triangles). **(F)** Normalized EOG amplitudes in response to L-serine (10^{-5} M) and 17,20β-P (10^{-9} M) in the absence (open bars) and presence of 10^{-7} M (grey bars) and 10^{-6} M (black bars) dopmperidone. In each graph, data are shown as mean ± SEM (n = 5) and $*P < 0.05$, $**P < 0.01$ compared to control (one-way repeated-measures ANOVA followed by Dunnett's test).

were, again, all significantly larger than the L-serine SACs. In a similar way to L-tyrosine, this is indicative of (a) specific olfactory receptor mechanism(s) for L-serine.

Taken together, these data suggest a degree of commonality in the receptor sites for these structurally diverse odorants, but indicate that there are specific olfactory catecholamine receptors, of which a sub-population is relatively specific for the 3-*O*-methoxy metabolites.

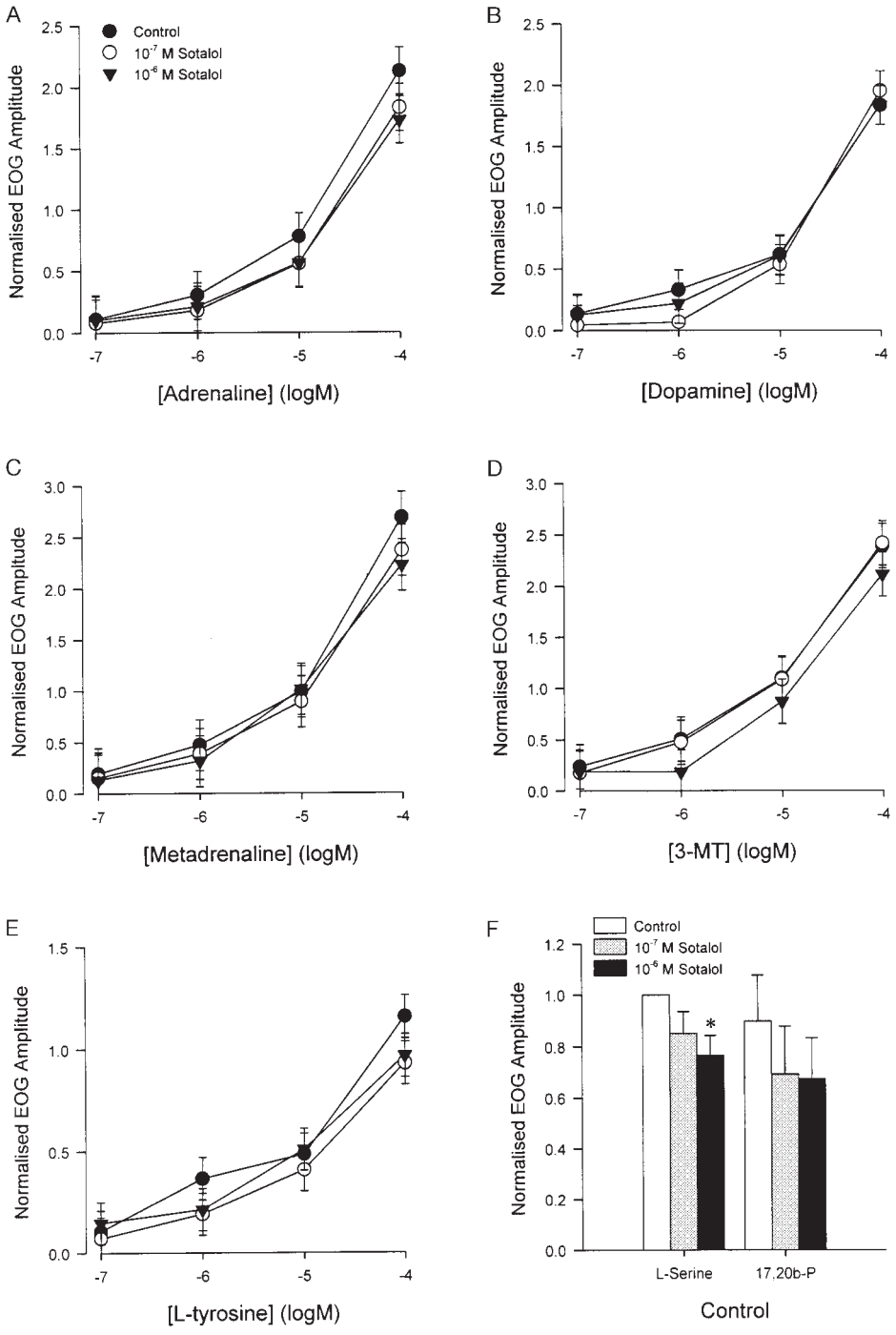

Figure 6 Effect of the β-adrenoreceptor antagonist sotalol on the olfactory responses to the catecholamines in the goldfish. Semi-logrithmic plots of normalized EOG amplitude in response to **(A)** adrenaline (10^{-7}–10^{-4} M), **(B)** dopamine, **(C)** metadrenaline, **(D)** 3-MT and **(E)** L-tyrosine in the absence (control; filled circles) and presence of 10^{-7} M (open circles) and 10^{-6} M sotalol (filled triangles). **(F)** Normalized EOG amplitudes in response to L-serine (10^{-5} M) and 17,20β-P (10^{-9} M) in the absence (open bars) and presence of 10^{-7} M (grey bars) and 10^{-6} M (black bars) sotalol. In each graph, data are shown as mean ± S.E.M. (n = 5) and $^*P < 0.05$, compared to control (one-way repeated-measures ANOVA followed by Dunnett's test).

Effect of adrenergic and dopaminergic antagonists

Having established that there is a specific olfactory response to the catecholamines in the goldfish, an attempt was made to pharmacologically characterize the receptor type(s) responsible by use of the α-adrenoreceptor antagonist prazosin, the β-adrenoreceptor antagonist sotalol and the dopamine receptor antagonist domperidone. At concentrations of 10^{-7} and 10^{-6} M, prazosin caused a clear,

concentration-dependent reduction in the amplitude of EOGs evoked by both adrenaline (Figure 4A) and dopamine (Figure 4B). This was more manifest at lower concentrations of agonist (i.e. at equimolar concentrations to prazosin) where the response was often completely obliterated. Although a similar trend was seen in the responses to metadrenaline (Figure 4C), this failed to reach statistical significance. Similarly, little effect was seen in the responses to 3-MT (Figure 4D) in the presence of prazosin. Somewhat surprisingly, prazosin also caused a slight concentration-dependent attenuation of EOG amplitude evoked by L-tyrosine (Figure 4E) and the structurally unrelated controls, L-serine and the steroid pheromone 17,20β-P (Figure 4F). These data suggest that prazosin is an effective antagonist at some of the receptor sites to the catecholamines and amino acids, but provides further evidence that there is a specific olfactory receptor mechanism for the 3-*O*-methoxy metabolites of the catecholamines, at which it is relatively ineffective.

Perhaps surprisingly, the DA_2-receptor antagonist domperidone had a similar effect to prazosin. While domperidone caused a clear, concentration dependent reduction in the EOG amplitude in response to adrenaline (Figure 5A) and dopamine (Figure 5B), such an effect was much less marked in responses to metadrenaline (Figure 5C) and 3-MT (Figure 5D) and only significant at the higher concentration of antagonist (10^{-6} M). A similar slight effect was seen on the amplitude of responses to L-tyrosine (Figure 5E). Again, an unexpected reduction in amplitude of response to L-serine was found (Figure 5F), but the responses to the steroid odorant 17,20β-P were unaffected.

In contrast, the β-adrenergic antagonist, sotalol, had little or no effect on the responses to adrenaline (Figure 6A), dopamine (Figure 6B), metadrenaline (Figure 6C), 3-MT (Figure 6D) or L-tyrosine (Figure 6E). Only a slight reduction in the amplitude of responses to 10^{-5} M L-serine was seen (Figure 6F), with a similar trend in the responses to 10^{-9} M 17,20β-P which failed to reach statistical significance.

Discussion

Olfactory sensitivity to the catecholamines

To the authors' knowledge, this is the first study to address the olfactory sensitivity of a fish to the catecholamines or their metabolites. The olfactory system of the goldfish is clearly sensitive to both adrenaline and dopamine with thresholds of detection around 10 nM, similar to the most potent amino acid (L-cysteine) and bile acid (taurocholic acid) in the zebrafish (Michel and Lubomudrov, 1995), and well within the range of sensitivity of fish in general to amino acids (10^{-7}–10^{-9} M) (Hara 1994; Sorensen and Caprio, 1998). However, removal of the α-carboxylic acid group (e.g. L-DOPA to dopamine) actually increases the olfactory potency: the catecholamines are more potent odorants (in the goldfish, at least) than their amino acid precursors. Surprisingly, the sensitivity to noradrenaline was much less acute, both in terms of the amplitude and threshold of the response (nearly two orders of magnitude higher). This was the first indication that the olfactory receptor mechanism(s) for adrenaline may not be via conventional adrenoreceptor(s), as these tend to have similar affinities for both adrenaline and noradrenaline (Fabbri *et al.*, 1998), and much lower affinity for dopamine. This suggests that the olfactory system seems to be able to discriminate—at the level of the receptor—between catecholamines differing only in the β-hydroxyl group and the methylated α-amino group. In contrast, amino acids are believed to be discriminated in a combinatorial manner by the pattern of activity evoked in the olfactory bulb (Friedich and Korching, 1997), presumably integrating the input from a range of at least four types of olfactory amino acid receptors (Caprio and Byrd, 1984). The sensitivity to either adrenaline or dopamine cannot be entirely explained by activation of olfactory neutral amino acid receptor receptor(s), as cross-adaptation with L-tyrosine (neutral amino acid with cyclic group) or L-serine (neutral amino acid with short aliphatic residue) does not completely block the response to the catecholamines (Figure 3). Furthermore, the responses to adrenaline and dopamine can be partially blocked by the α-adrenoreceotor antagonist prazosin (Figure 4) and DA_2 antagonist domperidone (Figure 5), whereas these had little effect on the responses to L-tyrosine.

Olfactory sensitivity to catecholamine metabolites

Goldfish also had a high olfactory sensitivity to the 3-*O*-methoxy metabolites of the catecholamines; metadrenaline and 3-MT consistently evoked larger responses than adrenaline and dopamine, with slightly greater sensitivity. As the EOG is believed to be a summation of receptor neurone generator potentials (Scott and Scott-Johnson, 2002), this is suggestive that more olfactory receptors are responsive to the 3-*O*-methoxy metabolites than their catecholamine precursors. This could be indicative that the 3-*O*-methoxy metabolites are more important in nature. The α-deaminated metabolites, however, were much less effective odorants, whether in the aldehyde (e.g. DHPG) or acid (e.g. DHMA) form. Furthermore, 3-*O*-methylation of any α-deaminated metabolites (e.g. HVA, VMA and MHPG) did not restore their olfactory potency to anywhere near that of the unmetabolized forms. This strongly suggests that the α-amino group is important in ligand binding/recognition (see below).

Olfactory selectivity

From the cross-adaptation experiments (Figure 3), it is clear that both amino acids (L-tyrosine and L-serine) and the catecholamines share some common olfactory binding sites. The one feature that all these molecules have in common is the α-amino group (Figure 1). As previously discussed, the α-deaminated catecholamine metabolites have much lower

olfactory potency than their α-aminated equivalents. This is in agreement with Lipschitz and Michel (1999) who found that removal of the α-amino group in structural analogues of L-arginine dramatically reduced their effectiveness as odorants in the zebrafish. This may indicate that there are relatively non-specific olfactory receptors for compounds with an α-amino group that are relatively indifferent to the structure of the rest of the molecule. However, the cross-adaptation experiments also suggest that there are olfactory receptors that do not respond to either L-serine or, more importantly, L-tyrosine which do respond to the catecholamines or their 3-*O*-methoxy metabolites: the presence of either amino acid cannot completely block the response to adrenaline or dopamine, even less metadrenaline or 3-MT. Thus goldfish must have olfactory receptors relatively specific to the catecholamines. Surprisingly, a proportion of these receptors seems unable to discriminate between dopamine and adrenaline (Figure 3A,B). The olfactory catecholamine receptors must therefore be structurally and functionally different from 'conventional' adrenoreceptors and dopamine receptors, although they might share a common evolutionary origin (Berghard and Dryer, 1998): vertebrate olfactory receptors (Buck, 1996; McClintock, 2000), adrenoreceptors and dopamine receptors all belong to the G-protein linked, seven transmembrane region super-family of cell-surface receptors (e.g. Sibley and Monsma, 1992; Aantaa *et al.*, 1995; Johnson, 1998; Zhong and Minneman, 1999). Furthermore, a proportion of these receptors seems to be relatively specific for the 3-*O*-methoxy metabolites (Figure 3C,D). This contention is supported by the ability of the α-adrenoreceptor antagonist prazosin to competitively block the olfactory response to both adrenaline and dopamine (Figure 4A,B), whilst having little or no effect on the responses to metadrenaline and 3-MT (Figure 4C,D). Similarly, the dopamine receptor antagonist, domperidone, was also able to attenuate the olfactory responses to adrenaline and dopamine (Figure 6A,B) whilst having a much lesser effect on the responses to metadrenaline and 3-MT (Figure 6C,D). Somewhat surprisingly, both these antagonists were able to slightly reduce the response to L-serine (Figures 4F and 6F), while prazosin was also able to antagonize the response to L-tyrosine and the steroid 17,20β-P. The β-andrenoreceptor antagonist sotalol, however, had very little effect on the olfactory responses to either the catecholamines or the 3-*O*-methoxy metabolites (Figure 5), while slightly reducing the response to L-serine. The most conservative explanation for these observations is that there are at least four different receptor types for the odorants used in the current study; one that recognizes principally L-serine, one that recognizes principally L-tyrosine, one that recognizes dopamine and adrenaline plus (to a lesser extent) the 3-*O*-methoxy metabolites and, finally, one that is relatively specific for the 3-*O*-methoxy metabolites. The first could be the receptor for neutral amino acids with aliphatic residues, the second for neutral amino acids with cyclic residues receptor as proposed by Friedich and Korsching (1997). This is represented schematically in Figure 7. Prazosin and domperidone appear to principally antagonize the catecholamine receptor(s). We strongly suspect that there are sub-populations of receptors within these groups.

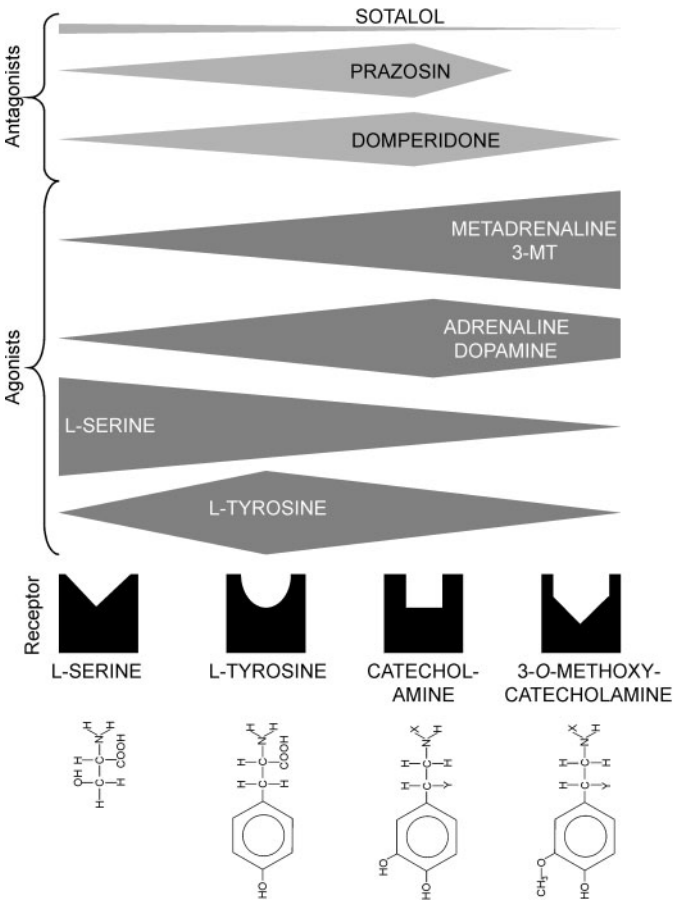

Figure 7 Olfactory catecholamine receptors in the goldfish. Schematic representation of receptor types and their relative sensitivity to some of the odorants (agonists) and antagonists used in this study. The selectivity is represented by the width of band, and sensitivity by depth e.g. L-serine is a more effective agonist at the L-serine receptor than at the L-tyrosine, or catecholamine receptor, but is able to activate all receptors to some extent. The chemical structures of the relevant ligand are depicted below the receptors (X = -H or -CH_3, Y = -H or -OH). It is possible that there are sub-populations within the four groups of receptors.

Possible functional significance of olfaction of catecholamines

The possible functional significance of the ability of the goldfish to smell catecholamines was not addressed. Nevertheless, given the roles of circulating adrenaline in fish, it might be involved in the communication of the alarm response. Evidence exists for a 'disturbance' pheromone in fish which alerts conspecifics to the presence of danger such as predators (Wisenden *et al.*, 1995; Chivers and Smith,

1998; Kats and Dill, 1998; Jordão and Volpato, 2000; Mirza and Chivers, 2001), without the prerequisite of damage to the sender. This response can also occur between different species. The current study raises the possibility that goldfish might be using catecholamines or their 3-*O*-methoxy metabolites as chemical messengers of some sort. Whether or not goldfish release catecholamines or their metabolites in quantities large enough for conspecifics to detect remains to be investigated.

Summary

The present study demonstrates that goldfish have acute olfactory sensitivity to catecholamines (principally adrenaline and dopamine) and their 3-*O*-methoxy metabolites, metadrenaline and 3-MT. This olfactory sensitivity is likely to be mediated by at least two specific receptor types functionally distinct from both olfactory amino acid receptors and systemic/neuronal adreno- and dopamine receptors.

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