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Pharmacology Biochemistry and Behavior, Vol. 54, No. 1, pp. 85–91, 1996

0091-3057/96 $15.00 + .00

SSDI 0091-3057(95)02170-1

Behavioural and Neurochemical Responses of Male and Female Chicks to Cat Odour

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FLUCK, E., S. HOGG, P. S. MABBUTT AND S. E. FILE. *Behavioural and neurochemical responses of male and female chicks to cat odour.* PHARMACOL BIOCHEM BEHAV **54**(1) 85–91, 1996.—In the first experiment male chicks were exposed to neutral and cat odours at days 4, 7, or 10 after hatching. Of the chicks tested at day 4, few made contact with either odour cloth, but those tested at day 7 made fewer contacts with the cat odour cloth, compared with the neutral odour, spent less time in contact with it, and spent more time in the zone furthest from the cloth. These clear differences were not seen in the group tested at day 10. In a second experiment, the behaviour of day 7 male chicks was compared in the presence of neutral, disinfectant, chick blood or cat odours, and the most extreme differences were between neutral and cat odours. In a third experiment, both male and female chicks were exposed to cat odour at day 7 and both showed similar avoidance. After exposure to cat odour both sexes showed significantly reduced GABA enhancement of benzodiazepine binding; which is a change associated with increased fear. However, after exposure to cat odour, they also showed significant decreases in 5-HT availability evidenced by lower basal and K^+-evoked [^{3}H]-5-HT release and, in the male chicks only, by an increased [^{3}H]-5-HT uptake from archistriatal slices. These changes in 5-HT function are in the direction associated with reduced fear and would, therefore, seem to be adaptive and compensatory in function. Neither male nor female chicks showed any differences in [^{14}C]-GABA release or uptake as a result of exposure to cat odour. Although the pattern of response to cat odour was the same in both male and female chicks at day 7, there were significant sex differences in 5-HT and GABA tone and benzodiazepine binding; these sex differences were also found in day 10 chicks. The importance of these for sex differences in trait anxiety is discussed.

Anxiety Fear Poultry GABA 5-HT Benzodiazepine

THE main purpose of this study was to examine neurochemical correlates of exposure of chicks to the odour of a predator. From clinical studies and mammalian work the 5-HT and GABA-benzodiazepine systems have been widely implicated in the control of fear and anxiety (7,16,17), and changes in these systems have been found in rats exposed to the odour of a predator (5). There is evidence that both the 5-HT and GABA-benzodiazepine systems exert similar behavioural control in avian tests of anxiety that are analagous to those used in rats (3), but there has been no work on the neurochemical correlates in birds of exposure to predators. It has been shown that chicks respond to the visual cues of a predator with alarm calls (14), but their response to predator odours has not yet been reported. However, there is evidence that chicks respond to olfactory cues as early as 3 days of age (18), and conspecific blood elicits avoidance and fear in 7-day-old chicks (11) and in 2-week-old pheasants (12). The avoidance of conspecific blood is replaced by attack and cannibalism in older birds (4).

Because of the lack of information regarding the response of birds to odours, the the purpose of Experiment 1 was to determine the age at which there was a clear differential behavioural response to a predator (cat) compared with a neutral odour. On the basis of the results, day 7 was chosen for a further exploration of responses to novel odours. In Experiment 2, the response to cat odour was compared with that to conspecific blood and to disinfectant, which was included as a strong novel nonbiological odour. The results showed that the most extreme behavioural response was to the cat odour.

In Experiment 3 we, therefore, measured benzodiazepine binding, GABA enhancement of benzodiazepine binding, and GABA and 5-HT release in chicks exposed to neutral or cat odour. Since this experiment revealed marked sex differences in GABA and 5-HT tone (even in the control condition of exposure to neutral odour), Experiment 4, therefore, investigated whether similar sex differences could also be seen in day 10 chicks. The chicks in Experiment 4 were handled as in Experiment 3, but not exposed to test odours.

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TABLE 1

MEAN (±SEM) NUMBER OF CONTACTS AND TIME (s) IN CONTACT WITH THE ODOUR CLOTH AND TIME (s) IN ZONE 3 (FURTHEST FROM THE ODOUR) FOR 7- AND 10-DAY-OLD CHICKS EXPOSED FOR 5 min TO NEUTRAL OR CAT ODOUR

	7 Days		10 Days	
	Neutral (n = 8)	Cat (n = 8)	Neutral (n = 10)	Cat (n = 10)
No. contacts	6.0 ± 1.7	1.0 ± 0.9*	4.1 ± 1.4	6.3 ± 1.2
Time in contact	61.5 ± 16.4	2.6 ± 2.4**	19.4 ± 11.6	21.6 ± 7.4
Time in zone 3	53.0 ± 32.0	176.6 ± 37.4**	75.1 ± 30.8	25.4 ± 11.7

*$p < 0.05$; **$p < 0.01$ compared with neutral odour (Duncan's tests after analysis of variance).

METHOD

Animals and Housing

Newly hatched Isa Brown chicks (a commercial medium-hybrid line originally derived from Rhode Island Red × Rhode Island White cross) were obtained from ISA, Peterborough, UK. They were housed in groups of four in wooden boxes measuring 70 × 40 × 30 cm. The floors of the boxes were covered with wood litter beneath a 2 cm high wire mesh grid (1 cm mesh), which allowed the passage of excreta but denied the chicks access to the litter. Food (chick starter mash) and water were supplied ad lib in semicircular plastic hoppers attached to grids suspended to the tops of the walls. The photoperiod was 0700 to 1900 h, warmth was provided by dull-emitter heaters fitted with 250 W bulbs, and wire-mesh lids prevented the chicks from jumping out.

All chicks received gentle handling twice a day from day 1 of age to the time of experiment. Regular handling has been widely reported to reduce chick's fear of human beings (9,10), and as our experiments were designed to measure a fear response to cat and other odours, the chicks were handled so as to eliminate a response due to handling. A cloth (similar to the one that was later used for the odour exposure) was left in their cages from day 1 until all chicks were culled to allow them to habituate to it.

Experimental Procedure

Odour exposure. Cat odour was obtained by rubbing damp pieces of towelling cloth against the fur of a laboratory cat for 5 min; the disinfectant odour was obtained by soaking pieces of cloth in Dettol diluted 1 : 1 with water (Reckitt & Colman Ltd.); the blood odour was obtained by diluting blood from a freshly killed chick in 1 ml of water and soaking the cloth in this. Neutral odour was provided by damp pieces of the same original cloth.

Odour exposure was performed in a cage, identical to the home cages, and chicks were exposed in pairs, both partners being home-cage mates. Pilot experiments had shown that all chicks showed high levels of immobility if they were tested singly in a novel environment. A similar method has been used for testing the responses of young pheasants (13). The cloth was wedged between the food and water hoppers at one end of the cage and was changed between every fourth pair of animals. For scoring purposes the grid floor was marked into three equal zones of 23.3 cm from the food. Each odour exposure lasted 5 min, and the time in contact and number of contacts with the odour cloth and the time spent in the zone furthest from the cloth were scored for one of each pair of chicks, which was randomly determined prior to the start of testing.

Chemicals

[^{14}C]-GABA (228 mCi/mmol), [^{3}H]-5-HT creatinine sulphate (24.4 Ci/mmol), and [^{3}H]-flunitrazepam (84.3 Ci/mmol) were purchased from DuPont, NEN (Stevenage, UK). The Krebs bicarbonate buffer was of the following composition (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.4, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 9.5 (Sigma Chemical Co., Poole, Dorset, UK) and (μM) aminooxyacetic acid 50 (Sigma), pargyline 50 (Sigma), ascorbic acid 100 (Sigma), and EDTA 30 (BDH Ltd., Dagenham, Essex). The medium was gassed continuously with 5% CO_2 in O_2.

Neurochemical Studies

In Experiment 3, one member of each test pair of birds was killed by decapitation immediately after the test, between 0900

TABLE 2

MEAN (±SEM) NUMBER OF CONTACTS AND TIME (s) IN CONTACT WITH THE ODOUR CLOTH AND TIME (s) IN ZONE 3 FOR 7-DAY-OLD CHICKS EXPOSED FOR 5 min TO NEUTRAL, DISINFECTANT, BLOOD, OR CAT ODOUR

	Neutral (n = 20)	Disinfectant (n = 16)	Blood (n = 8)	Cat (n = 16)
No. contacts	9.2 ± 1.1	3.8 ± 1.5**	2.4 ± 1.1**	1.5 ± 0.4**
Time in contact	73.0 ± 9.1	36.6 ± 12.2**	13.5 ± 7.9**	8.4 ± 3.2‡
Time in zone 3	18.3 ± 7.8	53.1 ± 26.3	110.4 ± 39.2*	104.4 ± 28.6*

**$p < 0.01$; *$p < 0.05$ compared with neutral odour; ‡$p < 0.05$ compared with disinfectant (Duncan's tests after analysis of variance).

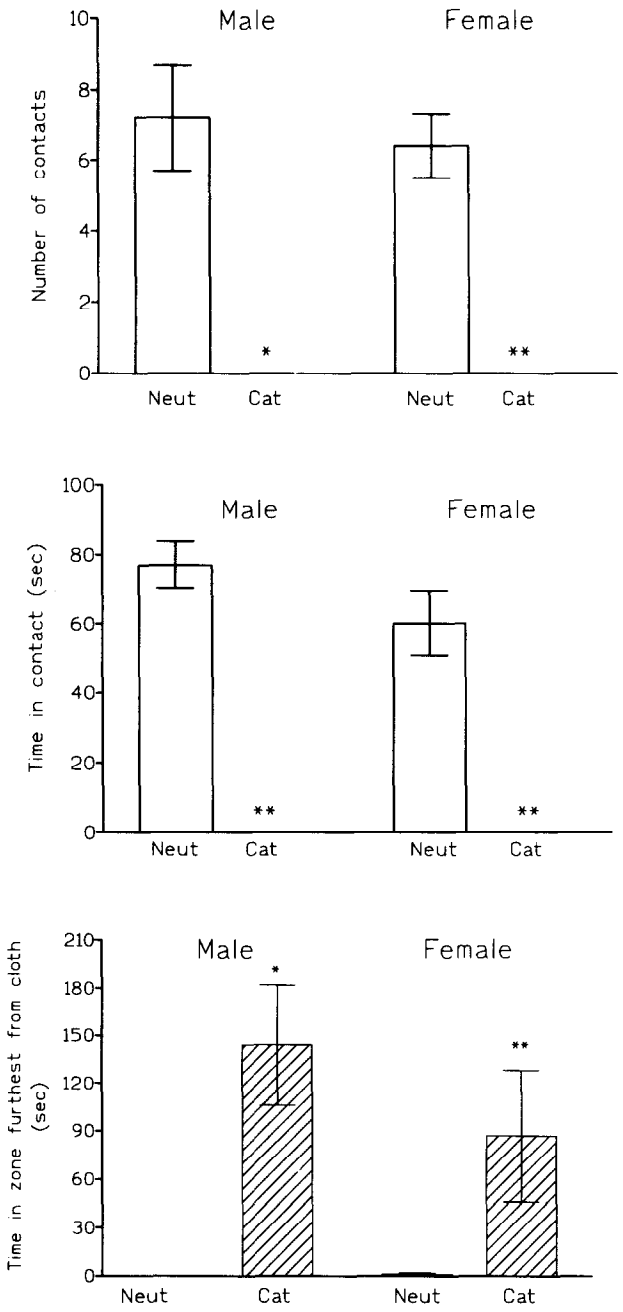

FIG. 1. Mean (±SEM) number of contacts, time in contact, and time in zone furthest from the cloth for day 7 male and female chicks exposed to neutral or cat odour (n = 5/group). **$p < 0.01$; *$p < 0.05$ compared with appropriate neutral odour group, Mann-Whitney U-tests.

and 1400 h. The forebrain was removed and the left archistriatum dissected for neurotransmitter release. The rest of the forebrain was frozen (−20°C) for benzodiazepine binding. In Experiment 4 all the day 10 chicks had experienced the same twice daily handling as in Experiment 3 and were removed from their home cages and culled at the same time of day as in Experiment 3, but were not exposed to the test situation. The forebrain was removed, the left archistriatum was dissected for neurotransmitter release, and the rest of the forebrain was frozen (−20°C) for benzodiazepine binding.

Benzodiazepine Binding

The tissue was thawed and homogenized in distilled water (tissue concentration 50 mg/ml) using a polytron (setting 3 for 15 s) and centrifuged at 26,000 × g for 20 min. The pellet was resuspended in assay buffer (50 mM Tris HCl pH 7.4 at 4°C) and centrifuged at 48,000 × g for 20 min; this process was repeated three times with the final homogenate frozen at −20°C for at least 24 h. On the day of the assay the homogenates were thawed, centrifuged at 48,000 × g for 20 min, and resuspended in assay buffer.

Saturation assays were carried out incubating 100 μl of homogenate with [^{3}H]-flunitrazepam (0.005–12 nM; 100 μl) in a final volume of 1 ml. Non specific binding was determined with 3 μM diazepam (100 μl).

The effect of GABA on benzodiazepine binding was determined by incubating 100 μl of homogenate with an approximate K_d concentration of [^{3}H]-flunitrazepam (0.75 nM; 100 μl) and GABA (10^{-4}–10^{-7} M; 100 μl) in a final volume of 1 ml. Nonspecific binding was determined with 3 μM diazepam (100 μl).

Incubation on ice for 60 min was terminated by rapid filtration through Whatman GF/B filters (presoaked for 1 h in 0.1% polyethylenimine) and washed three times with 5 ml of ice-cold 50 mM Tris HCl (pH 7.4) buffer. The filters were placed in 3 ml of scintillation fluid (Emulsifier-Safe; Packard) and left overnight before counting in a LKB Rackbeta 1214 liquid scintillation counter. The binding was quantified per milligram of membrane protein (0.15–0.19 mg/ml) using the method of Lowry (15). K_d and B_{max} were calculated for each animal from Scatchard analyses and the values within each odour group expressed as mean ± SEM.

Neurotransmitter Release

Following dissection the archistriatum was sliced (0.2 mm) using a MacIlwain tissue chopper. After a preliminary incubation for 10 min at 37°C in normal Krebs bicarbonate medium [^{14}C]-GABA and [^{3}H]-5-HT were added to the medium to give final concentrations of 0.23 μM and 0.07 μM, respectively.

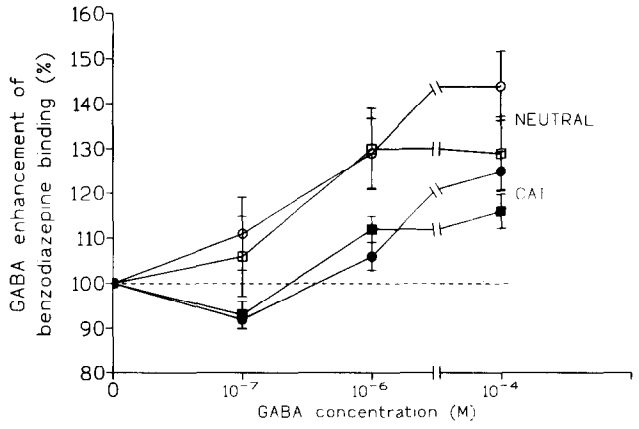

FIG. 2. Mean (±SEM) % GABA enhancement of benzodiazepine binding for day 7 male chicks exposed to neutral (○) or cat (●) odour and for day 7 female chicks exposed to neutral (□) or cat (■) odour (n = 5/group). See text for statistical analyses.

TABLE 3

MEAN (±SEM) K_d (nM) AND B_{max} (fmol/mg PROTEIN) [^{3}H]-FLUNITRAZEPAM BINDING, [^{14}C]-GABA, BASAL (FRC), AND K^+-EVOKED RELEASE (SUM OF FRCs ABOVE BASELINE) AND UPTAKE (dpm × 10^{-3}) FROM ARCHISTRIATAL SLICES FROM DAY 7 MALE AND FEMALE CHICK FOREBRAINS

	Male		Female	
	Neutral (n = 6)	Cat (n = 8)	Neutral (n = 7)	Cat (n = 7)
[^{14}C]-GABA				
Basal release	0.4 ± 0.06	0.4 ± 0.09	0.4 ± 0.1	0.3 ± 0.04
K^+-evoked release	12.8 ± 2.9	14.0 ± 2.7	35.3 ± 4.2	36.0 ± 3.9
Uptake	21.4 ± 2.0	25.8 ± 6.3	13.6 ± 1.4	14.2 ± 1.4
BDZ binding				
K_d	1.3 ± 0.07	1.6 ± 0.07	2.0 ± 0.07	1.9 ± 0.08
B_{max}	1172.6 ± 68.3	1307 ± 105.8	865.6 ± 39.7	879.8 ± 39.1

After a further 30-min incubation at 37°C, five slices (approximately 10 mg tissue wet weight) were placed in perfusion chambers (0.3 ml) between GF/B filters, where they were superfused with Krebs buffer at a rate of 1 ml/min for 15 min before fractions (2 ml each) were collected. A stimulated release was achieved by perfusing the slices for 2 min, during fraction 7 (in a total of 20 fractions) with perfusing buffer containing 30 mM KCl, which was then replaced with the normal perfusing buffer for the remainder of the collection period.

Statistics

The data from Experiments 1 and 2 were analyzed with a one-way analysis of variance (ANOVA) followed by Duncan's post hoc tests between individual groups. The behavioural data for Experiment 3 were analysed by Mann-Whitney U-tests, because of the high incidence of zero scores. The neurochemical data were analysed by two-way ANOVAs (with sex and odour as the two factors) followed by Duncan's post hoc tests.

RESULTS

Experiment 1

Very few of the day 4 chicks made contact with the neutral cloth (2 out of 8) and, therefore, it was impossible to measure any significant decreases to the cat odour. The groups did not differ in the times they spent in the zone furthest from the cloth (145 s for neutral odour, 94 s for cat odour). However, as can be seen in Table 1, at day 7 the chicks made significantly fewer contacts with the cat cloth, spent less time in contact with it, and spent more time in the zone furthest from the cloth, thus avoiding it as much as possible. These clear differences were not seen in the groups tested at day 10 (Table 1).

Experiment 2

The chicks made a decreased number of contacts and spent significantly less time in contact with all the odour cloths, compared with the neutral odour (Table 2); the time in contact with the cat odour was also significantly less compared with the disinfectant odour. The chicks spent significantly longer in the zone furthest from the cloth when exposed to blood or cat odour, compared with the neutral odour (Table 2).

Experiment 3

Both day 7 male and female chicks showed significantly fewer contacts and spent significantly less time in contact with the cat cloth than with the neutral cloth and spent significantly more time in the zone furthest from the cat odour cloth (see Fig. 1).

There were no significant differences in benzodiazepine binding between the chicks exposed to cat and neutral odours, $F(1, 16) = 1.1$ (see Table 3). GABA (10^{-7}–10^{-4}) significantly enhanced benzodiazepine binding, $F(2, 32) = 106$, $p < 0.0001$, and the GABA enhancement was significantly less in those exposed to cat odour, $F(1, 16) = 9.4$, $p < 0.01$ (see Fig. 2).

There was evidence for a sex difference in benzodiazepine receptor function. Thus, the female chicks had a lower affinity ($1/K_d$) and fewer receptors (lower B_{max}) than the males, $F(1, 15) = 42.4$, $p < 0.0001$, $F(1, 15) = 25.9$, $p < 0.0001$, respectively (see Table 3). There was also a significant sex × GABA enhancement of benzodiazepine binding, $F(2, 30) = 8.0$, $p < 0.01$, which arose because the females showed no further enhancement of binding at the highest concentration whereas the males did (see Fig. 2).

There were no significant changes in [^{14}C]-GABA uptake or release as a result of exposure to cat odour (see Table 3). However, there were sex differences in GABA tone. There was a higher K^+-evoked release in female chicks compared with the male chicks, $F(1, 24) = 11.7$, $p < 0.01$, $F(1, 24) = 18.8$, $p < 0.001$, respectively, and a greater uptake in male chicks, $F(1, 24) = 6.3$, $p < 0.05$.

Both male and female chicks showed decreased 5-HT release after exposure to cat odour [basal release, $F(1, 24) = 9.2$, $p < 0.01$, K^+-evoked release, $F(1, 24) = 5.3$, $p < 0.05$]; the sex × odour interactions did not reach significance, $F(1, 24) = 0.05$ and 2.4, $p = 0.13$. However, as is shown in Fig. 3, on post hoc tests the decrease in evoked release was significant only in females. An increase in 5-HT uptake was shown only in the male chicks [sex × odour interaction, $F(1, 24) = 5.2$, $p < 0.05$] (see Fig. 3).

There were also sex differences with respect to 5-HT tone. There was an overall lower basal and K^+-evoked release in the male chicks compared with the female chicks, $F(1, 24) = 6.1$, $p < 0.05$, and 18.8, $p < 0.001$, respectively.

Experiment 4

At day 10, there was a significant sex difference in benzodiazepine binding with females having significantly lower receptor density than males, $F(1, 13) = 6.6$, $p < 0.05$ (see Table

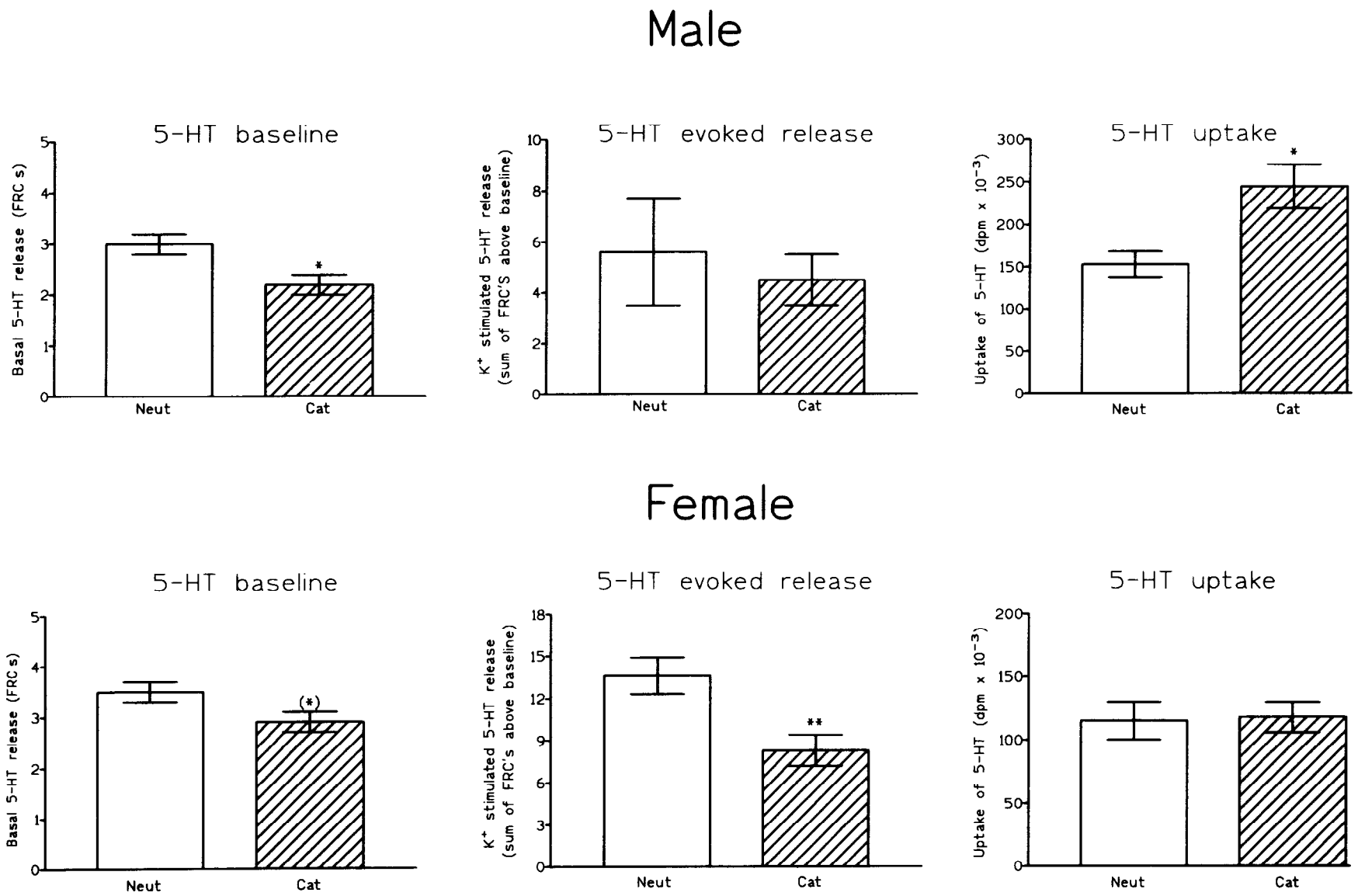

FIG. 3. Mean (±SEM) basal and K^+-evoked 5-HT release and 5-HT uptake from archistriatal slices from day 7 male and female chicks exposed to neutral or cat odour (n = 6-8/group). $^{**}p < 0.01$, $^{*}p < 0.05$, $(^{*})p = 0.06$ compared with appropriate neutral odour group, Duncan's test after analysis of variance.

TABLE 4

MEAN (±SEM) [^{14}C]-GABA AND [^{3}H]-5-HT, BASAL RELEASE (FRC), K^+-EVOKED RELEASE (SUM OF FRCs ABOVE BASELINE), AND UPTAKE (dpm × 10^{-3}) FROM ARCHISTRIATAL SLICES AND BENZODIAZEPINE BINDING IN DAY 10 MALE AND FEMALE CHICK FOREBRAINS

	Male (n = 7)	Female (n = 8)
[^{14}C]-GABA		
Basal release	0.2 ± 0.06	0.6 ± 0.09**
Evoked release	15.5 ± 4.7	25.8 ± 6.7
Uptake	22.2 ± 2.9	14.0 ± 2.0†
[^{3}H]-5-HT		
Basal release	2.2 ± 0.2	3.7 ± 0.2**
Evoked release	3.3 ± 0.4	7.0 ± 1.1**
Uptake	211.9 ± 21.7	41.7 ± 4.7**
Benzodiazepine binding		
K_d (nM)	1.7 ± 0.1	1.9 ± 0.2
B_{max} (fmol/mg)	2184 ± 127	1812 ± 53.2*

**$p < 0.01$; *$p < 0.05$ compared with males.

4). There was also significantly less GABA enhancement of benzodiazepine binding in females [sex × GABA interaction, $F(3, 36) = 3.1$, $p < 0.05$]. The females had significantly higher basal GABA release, $F(1, 13) = 17.3$, $p < 0.01$, and lower GABA uptake, $F(1, 13) = 5.8$, $p < 0.05$, than the males (see Table 4). Finally, the females had significantly higher basal and K^+-evoked 5-HT release, $F(1, 13) = 28.6$, $p < 0.001$, $F(1, 13) = 11.2$, $p < 0.01$, respectively, and lower 5-HT uptake, $F(1, 13) = 8.8$, $p < 0.01$, than the males.

DISCUSSION

The results of the present experiments show that at day 7 both male and female chicks show behavioural avoidance of a cloth impregnated with the odour of a cat. This response could not be demonstrated at day 4 because of the strong avoidance even of the neutral odour cloth. The avoidance of the cat odour cloth was no longer evident at day 10. At day 10 there is an abrupt increase in exploration and approach to novel stimuli (19,20) and, hence, an increased interest in the cat odour as a novel stimulus could account for the higher scores at day 10. It is possible that at this age there is a shift from novel odours primarily inducing fear to primarily inducing exploration. Between days 8–10 there are rapid neurological and behavioural changes that could influence fear responses (1,2). An additional factor is that by day 10 chicks may no longer need to use odour cues to avoid or shelter from predators because of the rapid development in the organization of the visual system; thus, at this age it is probable that visual stimuli predominate over olfactory ones in eliciting fear responses. Our data suggested a marked increase in the number of benzodiazepine receptors between days 7 and 10, but direct age comparisons for the biochemical measures cannot really be made because the day 7 and 10 chicks for Experiments 3 and 4 came from different batches, tested at different times, and, furthermore, the day 10 chicks would have also received 3 day's more handling.

The avoidance of cat odour was shown by both male and female chicks, and as a result of exposure to cat odour there was a significant decrease in synaptic availability of 5-HT and a decreased GABA enhancement of benzodiazepine binding. However, these biochemical changes are not both in the direction that would be associated from increases in fear. A decreased synaptic availability of 5-HT is normally associated with a reduction in fear/anxiety in rats (16) and birds (6), whereas the reduced activity of the GABA-benzodiazepine system is a change in the direction that would be associated with enhanced anxiety (8). Thus, the change in 5-HT function would be considered to be a compensatory reaction limiting the fear response to cat odour, whereas the change in the GABA-benzodiazepine function would seem a correlate of the increased fear response to the odour. In the rat, changes in the GABA and 5-HT systems have been found both after exposure to a novel test situation with a neutral odour and after exposure to cat odour (5). The changes in response to cat odour did not follow those found in the present study, but the changes were complex and depended both on the time since test exposure and the brain area examined (e.g., hippocampus or frontal cortex). More studies are needed before meaningful species comparisons can be made. Further studies are also needed before it is possible to attribute the neurochemical changes to the nature of the stimulus (i.e., predator odour) rather than to its degree of novelty. However, the results of the present study show clear behavioural and neurochemical responses to odours in day 7 chicks and, thus, demonstrate the importance of the olfactory system at this stage of development. Another potentially important finding was the sex difference in tone in both the 5-HT and GABA systems. These were found at both days 7 and 10, and if similar differences persist into adulthood, they could underlie sex differences in fear responding.

ACKNOWLEDGEMENT

We are grateful to Fiona Sanders for expert technical assistance.

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