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Brain Benzodiazepine Receptors in Fathead Minnows and the Behavioral Response to Alarm Pheromone

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REHNBERG, B. G., E. H. BATES, R. J. F. SMITH, B. D. SLOLEY AND J. S. RICHARDSON. *Brain benzodiazepine receptors in fathead minnows and the behavioral response to alarm pheromone.* PHARMACOL BIOCHEM BEHAV 33(2) 435–442, 1989.—Fathead minnows (*Pimephales promelas*) exposed to 1 or 10 mg/l chlordiazepoxide showed normal alarm behavior during the presentation of alarm pheromone. Fish exposed to 20 mg/l drug, however, showed little or no behavioral alarm and did not appear sedated. A food extract stimulus presented after alarm pheromone led to a large foraging response by fish exposed to 20 mg/l chlordiazepoxide. Control fish or fish exposed to 1 to 10 mg/l drug showed less tendency to begin foraging. Exposure to 1, 10, or 20 mg/l chlordiazepoxide for 3 hr did not affect whole-brain concentrations of tryptophan, 5-hydroxytryptamine, 5-hydroxyindoleacetic acid, tyrosine, or dopamine. The binding of [^{3}H]diazepam to specific brain receptor sites ($K_D = 10$ nM, estimated $B_{max} = 3.3$ fmol/mg wet weight) could be displaced by chlordiazepoxide ($IC_{50} = 1.6$ μM). These results suggest that benzodiazepine receptors in the central nervous system of lower vertebrates may function in ways similar to those in mammals, i.e., in the modulation of behavior in anxiety-like states.

Benzodiazepines Receptors Behavior Alarm pheromone Ostariophysan fishes Fathead minnows Neurochemicals

OSTARIOPHYSAN fishes possess an alarm pheromone system that is thought to warn conspecifics about predator activity (7,38). The alarm pheromone, which may be a hypoxanthine derivative (24), resides in large club cells that can be found in the epidermis covering much of the body (37). The attack of a predator has been shown to release enough pheromone to induce alarm behaviors in nearby conspecifics (45). The alarm behavior, classically termed a "Schreckreaktion" or fright reaction by von Frisch, consists of behavior that may protect the responder from nearby active predators. Although it varies from species to species, the fright reaction often includes rapid dashing, C-turns, hiding, immobility, and avoidance of the area. The initial behavioral display of alarm by responding fish becomes a visual alarm stimulus that insures a rapid transmission of alarm to the rest of the shoal (44). Other than the initial involvement of olfactory (23) and visual pathways (44), nothing is known about the neurobiology of these behaviors (27).

Although assessment of psychological states in nonhumans is difficult, the behavioral condition of alarmed fish gives the subjective impression of fright or anxiety. Mammals respond to frightening stimuli with a stereotypic physiological stress response (1) and there is now evidence to suggest that fish respond similarly (26–28). Pearl dace (*Semotilus margarita*) respond to alarm pheromone with a biphasic behavioral alarm response and a simultaneous increase in concentrations of plasma cortisol and glucose (28). Elevations of plasma cortisol and glucose have been established as components of a physiologically complex stress response in teleost fishes (32).

Mammalian animal models have been important in developing pharmacological agents that reduce anxiety in humans (2). The property of reducing anxiety was given a biochemical basis when specific binding sites for diazepam and other antianxiety drugs belonging to the benzodiazepine class were discovered in the brain of rats (20,40). Most importantly, there appeared to be a relationship between binding events and behavioral effects. Sepinwall and Cook (33) used rat conflict tests to assess the anxiety-reduction potency of 15 benzodiazepines. The potency of these compounds as anxiolytic agents correlated (+0.832) with their ability to inhibit [^{3}H]diazepam binding in rat cortex preparations. Although controversial, there is evidence to suggest that the anxiolytic effects of benzodiazepines may involve a reduction in serotonergic neural activity (14,42).

Our working hypothesis has been that the concepts of fear and

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anxiety, though difficult to define, are not unique to mammals and can be applied to lower vertebrates including fishes. Specific binding sites for benzodiazepines have been found in the brains of fishes and higher vertebrates (21) as well as elasmobranchs (9) and insects (30). The benzodiazepine chlordiazepoxide has been shown to reduce a variety of intraspecific attack behaviors in male Siamese fighting fish without inducing noticeable toxic or sedative effects (6). In this paper we evaluate the effects of a benzodiazepine drug on the behavioral alarm reaction of the fathead minnow, *Pimephales promelas* (Ostariophysi, Cyprinidae). Experimental objectives were to determine 1) if alarm behaviors are selectively altered or eliminated when under the influence of chlordiazepoxide, 2) if the drug can accelerate the recovery from the alarmed state, 3) if fathead minnows have benzodiazepine binding sites in the brain, and 4) if brain concentrations of tryptophan, 5-hydroxytryptamine (serotonin), 5-hydroxyindoleacetic acid, tyrosine, or dopamine covary with drug treatment or behavioral changes.

METHOD

Fathead minnows were collected from Eagle Creek near Saskatoon, Saskatchewan, Canada and held in large outdoor pools. Test fish were transferred to indoor glass holding tanks (150 l) at least two weeks before testing. Fish being acclimated to laboratory conditions were fed Tetramin Staplefood flakes to satiation twice a day. A physical and chemical description of the dechlorinated tapwater used in the outdoor pools and indoor holding and testing facilities has been previously published (19). Fatheads were selected as a test species because they are locally abundant, their alarm pheromone system is relatively well-described (17,36), and they are amenable to the testing of single fish in activity tracking meters (19).

Behavioral Experiments

Behavioral experiments were done using the Opto-Varimex-Aqua activity tracking meter (ATM) (Columbus Instruments, Columbus, OH) described by Lemly and Smith (18). The ATM surrounds a Plexiglas test tank ($45 \times 45 \times 20$ cm) and is connected to a microcomputer by an analog/digital integrator. The tank has a recirculation system in which water exits through a manifold along one side of the tank and passes through a power filter. The filter canister contained only glass wool for the removal of particulate matter. Tygon tubing left the power filter and entered an adjacent room (housing the computer and observer) before returning to an intake manifold on the opposite side of the tank. This arrangement allowed the observer to inject drugs or chemical stimuli into the recirculation system from a remote location.

Photocells emitting visible red light are positioned every 2.6 cm along two adjacent sides of the ATM, thereby creating an intersecting grid of light beams. Photoreceptor cells, located on the opposite side of the tank from each photocell, detect any interruption of the beams and provide electrical signals that the computer uses to calculate fish movements. The computer was programmed to scan the grid every 0.125 seconds and provide data on the following 4 behavioral parameters: 1) total distance traveled (cm), 2) number of stereotypic movements (any activity in which the fish remains within a "box" of beams of preset dimensions), 3) time spent in stereotypic activity (sec), and 4) time spent resting (sec). The four parameters are neither fully independent measures nor are they directly correlated with each other. Taken together, they provide a composite assessment of activity with a rigor not possible using conventional observations.

Test fish were transferred in pairs from the indoor holding tank to a Plexiglas acclimation tank containing water 8 cm in depth. Twenty-four hr later, the fish were transferred to a second acclimation tank containing water 5 cm in depth. Twenty-four hr later, one of the two fish was transferred to an ATM tank (7.6 l, 3.8 cm in depth). After residing in a test tank for 24 hr, the fish were tested singly in experiments conducted between 0900 and 1300 hours. This procedure resulted in most fish being acclimated to shallow water and to being isolated from conspecifics. Fish being acclimated were fed after the morning tests. Test fish were not fed on the day of an experiment. The acclimation and test tanks were similarly constructed and all had a centrally moored floating shelter. Mean water temperature in the acclimation and test tanks was 21.5°C (range: 20.0–23.0°C).

The ATM was used to investigate the effects of chlordiazepoxide hydrochloride on the response of single fathead minnows to control stimulus (water), alarm pheromone, and food extract. Each test ran 84 min with all behavioral data being collected and summarized by the computer every 2 min. Fish behavior was also visually monitored using overhead television cameras. All tests began by collecting baseline data on each animal for an initial 8-min pretreatment period (min 0–8). At the end of this period, a drug control (water) or drug solution was injected into the recirculation system in control and experimental tests, respectively. Data collection was resumed at min 52 to monitor the effects of drug exposure before presentation of any test stimuli. At min 60, the fish was presented with a control chemical stimulus (1 ml distilled water) and data were collected for 8 min. At min 68, 1 ml of the alarm pheromone stimulus was presented and data were collected for 8 min. At min 76, the food extract stimulus (1 ml) was presented and data were collected for a final 8 min. At the end of each test, the fish was measured, weighed, sexed, and its brain removed and weighed. Fish used in behavioral experiments had a mean weight of 3.40 g (range: 2.01–5.22 g), a mean fork length of 6.6 cm (range: 5.8–7.6 cm), and consisted of 29 males, 36 females, and 7 sexually immature individuals. Each brain was quickly frozen in liquid nitrogen and stored at −70°C. After a test, the tank was drained, rinsed, and refilled with aged water in preparation for the next test animal. Fish were tested only once in control tests ($n = 27$), or in tests using 1 mg/l ($n = 13$), 10 mg/l ($n = 14$), or 20 mg/l ($n = 11$) chlordiazepoxide.

To help correct for the large range in activity across animals, all behavioral parameters for each fish were normalized to its own baseline activity. To do this, the mean score for min 0–8 was calculated for each animal. All scores for that parameter and animal were then divided by this baseline correction factor to obtain the normalized score. Mann-Whitney U-tests were used to compare the normalized behavioral scores obtained during the 4 min before a stimulus with scores recorded 4 min after the stimulus.

Drugs and Chemical Stimuli

Stock solutions of chlordiazepoxide hydrochloride (Hoffmann-La Roche, Calgary, Alberta, Canada) were made with distilled water so that 5 ml injections would result in nominal test concentrations of 1, 10, or 20 mg/l. Pilot bioassays and the results of Figler (6) suggested that these concentrations could be effective without being sedative. The drug control solution was 5 ml distilled water.

The control chemical stimulus was 1 ml of aged dechlorinated tapwater similar to that used in the acclimation and test tanks.

Alarm pheromone stimulus was obtained by making an extract of skin samples from 12 fish (8 male, 4 female, mean wt. = 3.47 g). Minnows were killed by a blow to the head and a patch of skin (1.0×0.5 cm) was separated from underlying muscle and re-

moved from both sides of the caudal peduncle. Histological examination of tissue subsamples revealed that all but one fish had epidermal alarm pheromone cells. The pooled tissue was homogenized in 100 ml distilled water for 60 sec in a Polytron. The extract was diluted 1:10 and frozen in 7-ml aliquots. Frozen extract was thawed on the day of an experiment and used in 1 ml quantities as the pheromone stimulus.

The food extract was made by stirring finely-chopped Tetramin Staplefood flakes for 10 min in enough water to make a 0.1 g/100 ml slurry. The slurry was filtered through Whatman No. 2 qualitative filter paper under vacuum and frozen in 7-ml aliquots. One-ml quantities were used during experiments as the chemical feeding stimulus.

Neurochemical Analysis

Whole-brain concentrations of tryptophan, 5-hydroxytryptamine (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), tyrosine, and dopamine were analyzed using high performance liquid chromatography with electrochemical detection. A description of methodology has been previously published (28,35).

Benzodiazepine Receptor Assay

Whole brains, including medulla and olfactory bulbs, were removed from fathead minnows that had not been exposed to any drug or test situation. Each brain was quickly frozen in liquid nitrogen and then pooled with other brains for storage at −70°C. The preparative procedures and binding assay were based on the methods of Nielsen *et al.* (21). One gram of frozen tissue was homogenized in 20 ml of iced sucrose buffer (0.32 M) for 30 sec with a Polytron. The homogenate was centrifuged at 2000 × g for 5 min. The resulting supernatant was decanted and centrifuged at 30,000 × g for 15 min. The pellet was resuspended in 20 ml of iced Tris buffer [Tris(hydroxymethyl)methylamine, 0.05 M, pH 7.4] and used in the binding assay. Total binding was found by incubating 0.2 ml of the membrane preparation (= 10 mg original wet weight/tube) with 0.20 ml water, 0.01–0.08 ml [methyl-^{3}H]-diazepam (New England Nuclear, 74.9 Ci/mmol) and enough Tris buffer to reach a 0.5 ml reaction volume. Nonspecific binding, defined as [^{3}H]diazepam binding in the presence of excess nonradioactive diazepam, was found by incubating 0.2 ml of membranes with 0.2 ml nonradioactive diazepam (10^{-5} M final concentration), 0.08 ml water, 0.01–0.08 ml [^{3}H]diazepam and enough Tris buffer to reach a 0.5 ml total volume. After incubating on ice for 40 min, the contents of the incubation tubes were filtered through Whatman GF/C filters using a modified Brandel Cell Harvester. Each filter with trapped tissue membranes was washed 4 times with 4 ml iced Tris buffer and placed in a scintillation vial along with 10 ml DuPont Formula 963 scintillation counting cocktail. The vials were shaken for 2 hr and then counted for radioactivity in a Beckman 3801 liquid scintillation counter with 45 percent efficiency. Specific receptor binding was calculated by subtracting nonspecific binding from total binding. A Scatchard analysis was performed on the specific binding of 6 concentrations of [^{3}H]diazepam ranging from 1 to 24 nM. The ability of chlordiazepoxide to compete with 1.5 nM [^{3}H]diazepam for the benzodiazepine receptor was determined with 6 concentrations of chlordiazepoxide ranging from 4 nM to 62.5 μM.

RESULTS

Activity patterns of fathead minnows before presentation of chemical stimuli were variously affected by exposure to control water and chlordiazepoxide (Table 1). Fish in each experimental group, including controls, were generally more active after the "exposure" period suggesting either the involvement of diel patterns of motor activity or a gradual acclimation to the test conditions. Distance traveled increased after fish were exposed to the drug control (water), whereas the other behaviors were not significantly altered. Exposure to 1 or 20 mg/l chlordiazepoxide did not significantly affect any of the behavioral measures. Exposure to 10 mg/l, however, resulted in a significant increase in stereotypic movements and stereotypic time, a near-significant increase in distance traveled ($p = 0.052$), and a significant decrease in time resting (Table 1).

TABLE 1

EFFECTS OF EXPOSURE TO CHLORDIAZEPOXIDE ON FOUR TYPES OF BEHAVIOR

Exposure	Distance Traveled	Stereotypic Movements	Stereotypic Time	Time Resting
Control Water	B/A = 1.83/5.06	1.96/2.65	1.97/2.49	2.00/1.91
	U = 237.0	321.0	299.0	431.0
	$p = 0.027$*	0.452	0.257	0.250
1 mg/l	2.01/4.83	2.18/2.87	2.14/3.02	2.07/1.72
	63.0	58.0	63.5	111.1
	0.270	0.174	0.282	0.174
10 mg/l	1.67/4.09	1.89/3.91	1.94/3.83	2.10/1.51
	51.0	41.0	41.0	168.0
	0.052	0.009†	0.009†	0.001†
20 mg/l	2.02/2.13	2.01/2.68	1.97/2.75	2.00/2.26
	61.0	61.0	56.0	62.5
	0.974	0.974	0.768	0.896

"B/A" represents means of measurements made during the 4 min before drug treatments (Min 4–8) and during the 4 min before the control stimulus (Min 56–60). Also shown are Mann-Whitney U-statistics and corresponding probability values with * and † indicating $p<0.05$ and 0.01, respectively. Sample sizes were n = 27 for controls, n = 13 for 1 mg/l, n = 14 for 10 mg/l, and n = 11 for 20 mg/l chlordiazepoxide.

Exposure to chlordiazepoxide clearly influenced the manner in which minnows responded to chemical stimuli. Distance traveled by fish in control or drug-treated groups did not change when presented with water stimulus (Fig. 1, Table 2). Presentation of alarm pheromone, however, resulted in significantly lower distance traveled scores in fish exposed to control water, 1 mg/l, or 10 mg/l chlordiazepoxide. Fish exposed to 20 mg/l chlordiazepoxide did not significantly respond to alarm pheromone (Fig. 1). Statistical tests were based on a comparison of the 4 min before and after presentation of chemical stimuli. Unlike the other fish, minnows exposed to 20 mg/l drug increased their distance traveled during the first 2 min after detecting alarm pheromone and then became less active in the minutes that followed. The observed increase in distance traveled was not significant when compared to the 2 min before presentation of alarm pheromone (Mann-Whitney U = 56.5, $p = 0.793$). When presented with food stimulus, distance traveled increased as a function of chlordiazepoxide exposure (Fig. 1). The increased activity in fish exposed to drug control, 1 mg/l, or 10 mg/l drug was not significant (Table 2). In contrast, fish exposed to 20 mg/l showed a tremendous increase in swimming activity that was characterized by persistent exploration of all regions of the tank (Fig. 1). Distance traveled generally peaked during the first 2 min after detecting food stimulus and then rapidly decreased.

Neither control nor drug-treated fish changed their frequency of stereotypic movements or stereotypic time in response to water

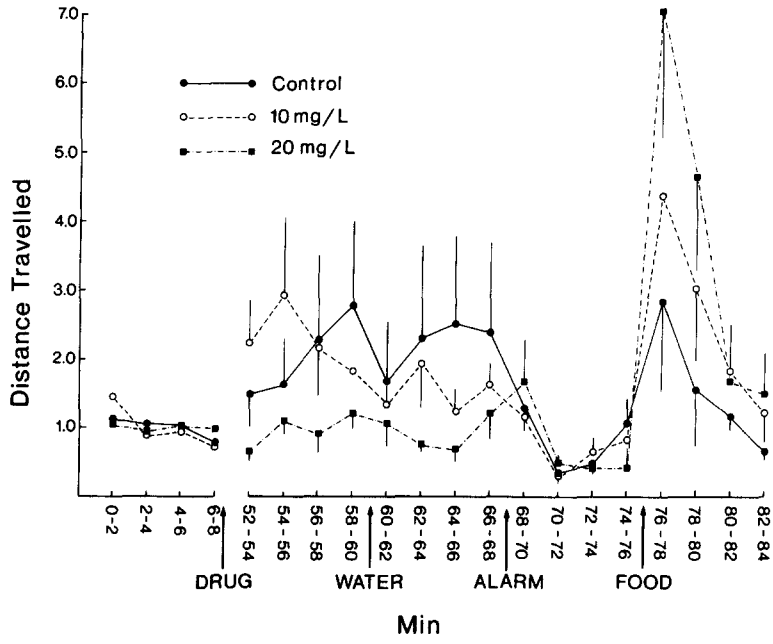

FIG. 1. Distance traveled by fish exposed to drug control (n = 27) or to 10 mg/l (n = 14) or 20 mg/l (n = 11) chlordiazepoxide. Standard error bars that overlap have been omitted for clarity of presentation.

stimulus (Tables 3 and 4). Both behavioral measurements indicate that fish exposed to control water, 1 mg/l, or 10 mg/l chlordiazepoxide showed significantly less activity in response to alarm pheromone but did not change their activity in response to food stimulus. Fish exposed to 20 mg/l behaved oppositely: activity did not change in response to alarm pheromone but stereotypic movements strongly increased following presentation of food stimulus. Stereotypic time also increased after detecting food stimulus ($p = 0.053$) (Tables 3 and 4).

Measurements of time resting fully support the trends established by analyzing the other behaviors (Table 5). Fish exposed to control water, 1 mg/l, or 10 mg/l chlordiazepoxide responded strongly to alarm pheromone (decreased activity) but not to food stimulus. Fish exposed to 20 mg/l responded to food stimulus but not to alarm pheromone. Time resting did not change in any of the groups in response to water stimulus (Table 5).

TABLE 2

EFFECTS OF CHLORDIAZEPOXIDE ON DISTANCE TRAVELED WHEN MEASURED BEFORE AND AFTER PRESENTATION OF WATER, ALARM PHEROMONE, AND CHEMICAL FEEDING STIMULUS

Exposure	Water	Alarm	Food
Control	B/A = 5.06/3.97	4.89/1.63	1.54/4.39
Water	U = 466.0	531.0	288.0
	$p = 0.079$	0.004†	0.186
1 mg/l	4.83/3.74	4.84/2.59	1.41/2.33
	88.5	531.0	68.5
	0.838	0.004†	0.412
10 mg/l	4.09/3.26	2.85/1.45	1.47/7.36
	104.0	142.0	61.0
	0.528	0.043*	0.089
20 mg/l	2.13/1.82	1.87/2.14	0.86/11.70
	69.0	60.0	10.0
	0.577	0.974	0.001†

Before and after values (B/A), Mann-Whitney U-statistics, and associated probabilities are shown for each exposure × stimulus case. * and † indicate $p < 0.05$ and 0.01, respectively. Sample sizes were n = 27 for controls, n = 13 for 1 mg/l, n = 14 for 10 mg/l, and n = 11 for 20 mg/l chlordiazepoxide.

TABLE 3

EFFECTS OF CHLORDIAZEPOXIDE ON STEREOTYPIC MOVEMENTS WHEN MEASURED BEFORE AND AFTER PRESENTATION OF WATER, ALARM PHEROMONE, AND CHEMICAL FEEDING STIMULUS

Exposure	Water	Alarm	Food
Control	B/A = 2.65/2.37	2.62/1.30	1.53/2.25
Water	U = 395.5	538.5	293.0
	$p = 0.592$	0.003†	0.216
1 mg/l	2.87/3.64	3.23/1.95	2.51/3.51
	96.5	132.0	72.5
	0.538	0.015*	0.538
10 mg/l	3.91/3.81	3.74/2.08	2.34/4.20
	95.0	147.0	77.0
	0.890	0.024*	0.335
20 mg/l	2.68/2.56	2.27/1.70	1.68/4.34
	64.0	62.0	25.0
	0.818	0.922	0.020*

Before and after values (B/A), Mann-Whitney U-statistics, and associated probabilities are shown for each exposure × stimulus case. * and † indicate $p < 0.05$ and 0.01, respectively. Sample sizes were n = 27 for controls, n = 13 for 1 mg/l, n = 14 for 10 mg/l, and n = 11 for 20 mg/l chlordiazepoxide.

TABLE 4

EFFECTS OF CHLORDIAZEPOXIDE ON STEREOTYPIC TIME WHEN MEASURED BEFORE AND AFTER PRESENTATION OF WATER, ALARM PHEROMONE, AND CHEMICAL FEEDING STIMULUS

Exposure	Water	Alarm	Food
Control	B/A = 2.49/2.08	2.27/1.30	1.63/2.02
Water	U = 402.0	520.0	317.0
	$p = 0.517$	0.007†	0.411
1 mg/l	3.02/4.73	3.49/2.27	3.42/5.41
	90.5	135.5	62.0
	0.758	0.009†	0.249
10 mg/l	3.83/3.68	4.04/2.19	2.69/3.49
	99.0	142.0	87.0
	0.963	0.043*	0.613
20 mg/l	2.75/2.63	2.38/1.61	1.77/2.90
	71.5	70.0	31.0
	0.470	0.533	0.053

Before and after values (B/A), Mann-Whitney U-statistics, and associated probabilities are shown for each exposure × stimulus case. * and † indicate $p<0.05$ and 0.01, respectively. Sample sizes were n = 27 for controls, n = 13 for 1 mg/l, n = 14 for 10 mg/l, and n = 11 for 20 mg/l chlordiazepoxide.

Exposure to chlordiazepoxide did not induce alterations in whole-brain concentrations of tryptophan, 5-HT, tyrosine, or dopamine (Table 6). One-way analysis of variance across exposures was not significant for tryptophan, $F(3) = 1.30$, $p = 0.301$; 5-HT, $F(3) = 0.10$, $p = 0.998$; tyrosine, $F(3) = 1.63$, $p = 0.212$; or dopamine, $F(3) = 0.66$, $p = 0.520$. Concentrations of 5-HIAA were generally at or below detection limits (20 ng/g wet weight) for all experimental groups.

Specific binding sites for [^{3}H]diazepam were found in membranes prepared from the brains of fathead minnows. The receptor saturation experiment suggests that specific binding is saturable, whereas nonspecific binding is linearly related to ligand concentration (Fig. 2a). A Scatchard analysis of these data resulted in a K_D estimate of 10 nM (Fig. 2b). Although the total number of binding sites could not be precisely determined from these data (15), the Scatchard plot estimates B_{max} to be about 3.3 fmol/mg wet weight. Chlordiazepoxide was able to displace [^{3}H]diazepam from its binding site in a concentration-dependent manner (Fig. 2c). The IC_{50} for the mean displacement curve is about 1.6 μM.

TABLE 5

EFFECTS OF CHLORDIAZEPOXIDE ON TIME RESTING WHEN MEASURED BEFORE AND AFTER PRESENTATION OF WATER, ALARM PHEROMONE AND CHEMICAL FEEDING STIMULUS

Exposure	Water	Alarm	Food
Control	B/A = 1.91/2.08	2.19/3.87	3.20/2.72
Water	U = 360.0	193.0	381.0
	$p = 0.938$	0.003†	0.775
1 mg/l	1.72/1.78	1.82/2.39	2.29/2.12
	78.5	21.0	103.0
	0.758	0.001†	0.343
10 mg/l	1.51/1.36	1.39/3.00	2.72/2.55
	108.0	21.0	103.0
	0.646	0.0004†	0.818
20 mg/l	2.26/2.18	2.32/2.54	2.80/1.73
	51.0	49.0	97.0
	0.533	0.450	0.017*

Before and after values (B/A), Mann-Whitney U-statistics, and associated probabilities are shown for each exposure × stimulus case. * and † indicate $p<0.05$ and 0.01, respectively. Sample sizes were n = 27 for controls, n = 13 for 1 mg/l, n = 14 for 10 mg/l, and n = 11 for 20 mg/l chlordiazepoxide.

TABLE 6

WHOLE-BRAIN CONCENTRATIONS (ng/g WET WEIGHT) OF TRYPTOPHAN (TRYP), 5-HYDROXYTRYPTAMINE (5-HT), TYROSINE (TYR), AND DOPAMINE (DOP) AFTER 3-HR EXPOSURES TO CHLORDIAZEPOXIDE

		Chlordiazepoxide		
Chemical	Control	1 mg/l	10 mg/l	20 mg/l
TRYP	3995.6	4762.8	4993.0	3790.7
	(7, 370.6)	(6, 854.7)	(7, 262.3)	(5, 393.9)
5-HT	228.8	228.5	221.5	231.7
	(7, 5.99)	(6, 9.89)	(7, 16.82)	(5, 21.93)
TYR	5138.4	6795.9	7010.2	7009.9
	(7, 663.9)	(6, 508.4)	(7, 765.1)	(5, 1002.2)
DOP	117.4	107.1	121.7	105.5
	(7, 7.81)	(6, 13.60)	(7, 7.83)	(5, 8.31)

Sample sizes and standard errors are shown in parentheses.

DISCUSSION

Research on benzodiazepines has been primarily directed toward understanding their mechanism of action and toward developing drugs that reduce anxiety in humans without causing undesirable side effects. Because almost all research has used rodents, there is relatively little understanding of the comparative aspects of anxiety-like states in other species. However, there seems to be no obvious reason why lower vertebrates should not experience such states when facing novel environments or anticipating aversive events. All classes of living vertebrates have limbic system structures although precise homologies have not been established (10). The existence of exact homologies may not be relevant, however, since there is reason to believe that emotion is not necessarily "produced" by discrete anatomical structures in the limbic system or elsewhere in the brain (43). Rather, it may be an experiential phenomenon reflecting global limbic activity whereby survival value is attached to environmental situations (12, 13, 29). Emotion viewed in this way has a neurologically complex origin and functions to adjust behavioral responses to biologically relevant stimuli.

Our results support the notion that anxiety-like states exist and have survival significance in lower vertebrates. Fathead minnows exposed to the drug control responded "properly" to the presentation of alarm pheromone by displaying a behavioral alarm reaction. When presented with the chemical feeding stimulus while still in an alarmed state, control fish showed relative restraint in increasing exploratory activity. Fish exposed to low levels of chlordiazepoxide behaved similar to the controls. Exposure to 20 mg/l drug, however, induced nonsedative behavioral changes in the minnows that could be interpreted as a reduction in anxiety. Those fish did not show a fright response when presented with alarm pheromone and they displayed vigorous exploration when presented with the chemical feeding stimulus. In both situations, the high dose of chlordiazepoxide resulted in behaviors having potentially lower survival value. Lawrence and Smith (17)

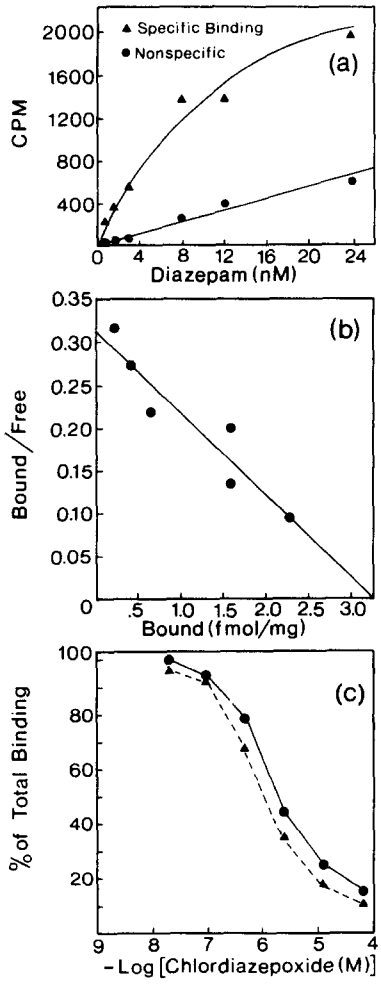

FIG. 2. Binding of [^{3}H]diazepam to a sedimentable fraction from the brains of fathead minnows. Results of saturation experiments are shown in (a). Specific and nonspecific binding data points represent means from two experiments run in quadruplicate. A Scatchard analysis of the saturation data is shown in (b). The ability of chlordiazepoxide to displace [^{3}H]diazepam (1.5 nM) is shown in (c). Each line represents one experiment run in triplicate.

found that solitary fathead minnows showed an increased latency of response and more exploratory behavior as the concentration of alarm pheromone was lowered. An ability of chlordiazepoxide to raise the sensory threshold for alarm pheromone is a possible alternative to the anxiolytic mechanism already presented.

The laboratory rat has been a popular animal model for studying anxiety using conflict tests. Rats are trained to press a lever to obtain food or water. After learning this task, they are then electrically shocked when they approach the lever. Hence, a conflict arises between the drive to eat or drink and the desire to avoid being shocked. Lever pressing for food or water can become totally suppressed by electric shock, but it can also be restored by administering benzodiazepines (3). Shock-induced suppression of food-reinforced lever pressing and its subsequent release by benzodiazepines might be analogous to the low levels of foraging in alarmed fathead minnows and its release by preexposure to chlordiazepoxide. For both the rat and the fathead minnow, the normal adaptive response to an aversive event is reduced by benzodiazepines.

Other widely-used paradigms in anxiety research investigate the effects of drugs on exploratory behaviors of mice (5). In light/dark transition tests, mice are placed in a container that has both brightly-lit and dark compartments. A conflict arises due to the aversion toward the brightly-lit compartment and the natural exploratory drive in mice. Antianxiety drugs increase movements between compartments without inducing general hyperactivity. Benzodiazepines also reverse neophagia in mice. Mice consume abnormally small amounts of food when in new surroundings. The willingness to feed is increased by administration of benzodiazepines. In our study, exploratory behavior in response to food stimuli was clearly enhanced in fish previously exposed to 20 mg/l chlordiazepoxide. It is likely that the increased exploration during the presentation of food stimulus was foraging behavior although we cannot exclude the possibility of it being a nonspecific response to a novel stimulus. In any case, both rodents and fathead minnows showed increased exploration and possibly more foraging behavior in "anxiogenic" environments after treatment with benzodiazepine drugs.

Behavioral effects of benzodiazepines are preceded by the binding of the drug to specific receptor sites in the brain. The affinity of [^{3}H]diazepam for binding sites in membrane preparations is similar for fathead minnows ($K_D = 10$ nM), rats [3.6 nM (20)], eel (5.8 nM), plaice (5.9 nM), and codfish (6.3 nM) (21). Whole brains were used in the fathead minnow experiments, cerebral cortex for the rat, and forebrains for the other fishes. The ability of chlordiazepoxide to inhibit [^{3}H]diazepam binding was similar in codfish [$IC_{50} = 0.6$–1.0 μM (21)] and fathead minnows ($IC_{50} = 1.6$ μM). Chlordiazepoxide has lower affinity for binding sites in rat cortex than diazepam and has a correspondingly lower potency in rat conflict tests (33).

At present, the most likely mechanism of action for the anxiolytic effects of the benzodiazepines is that they enhance the actions of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) (3). Such an action in the serotonergic raphe nuclei in the rostral brain stem of mammals may contribute to their clinical effects (11). There is considerable evidence that the benzodiazepine receptor is part of a macromolecular complex that includes a GABA receptor and chloride channel (3). The presence of chloride anion or GABA has been shown to enhance [^{3}H]diazepam binding to membranes prepared from rat cortex (4,41). Preliminary data suggest a small enhancement of [^{3}H]diazepam binding to fathead minnow receptors in the presence of 10^{-4} M GABA, but no effect in the presence of chloride at concentrations ranging from 1–100 mM.

There exists some evidence that benzodiazepines bind to a site that is a receptor for an endogenous ligand. Diazepam binding inhibitor is a neuropeptide found in the rat and human brain that displaces diazepam from its binding site and may also have anxiogenic properties (8). If there is an endogenous ligand and corresponding receptor in mammals, it is not clear that a homologous system would necessarily be expected in lower vertebrates. Nevertheless, Nielsen *et al.* (21) found diazepam binding sites in central nervous systems of all classes of vertebrates tested except the Agnatha. Invertebrates were found to have no detectable binding sites. More recent investigations have shown benzodiazepine receptors to be present in the CNS of elasmobranchs (9) and insects (30). Autoradiographic data show that [^{3}H]flunitrazepam binding sites in the pond turtle (*Pseudemys scripta*) have a regional distribution similar to that found in mammals (31). Opiate receptor binding also has a broad phylogenetic distribution that includes primitive fishes (22,34). Our findings with the fathead minnow suggest that the benzodiazepine receptors found in lower vertebrates are functional in ways that may be similar to those in mammals.

The specific neural pathways that benzodiazepines affect in

altering anxiety levels are currently unknown. A great deal of research has focused on the functioning of serotonergic neurons, particularly those in the raphe nuclei. Among the many findings are that benzodiazepine administration results in 1) reduced amounts of 5-HT released from nerve endings (39), 2) reduced firing rates by 5-HT neurons (16), 3) reduced turnover of 5-HT (46), and 4) increased brain concentrations of tryptophan (the precursor of 5-HT), 5-HT, and 5-HIAA (the metabolite of 5-HT) (25). Two recent reviews (14,42), however, stress the abundance of conflicting and inconsistent results and caution against an uncritical acceptance of a causal link between benzodiazepines and serotonergic function. We found no significant trends between chlordiazepoxide exposure and brain levels of tryptophan, 5-HT, 5-HIAA, tyrosine, or dopamine. Our use of whole brains and short drug exposures (3 hr) may have reduced the likelihood of observing significant changes in these neurochemicals.

In conclusion, our findings support the notion that benzodiazepine receptors are present in the central nervous system of lower vertebrates. More importantly, it appears that benzodiazepine receptors in fathead minnows may mediate physiological processes that modulate behavior in anxiety-like states. Whether or not the established phylogenetic distribution of receptors implies a correspondingly broad distribution of anxiety-modulating endogenous ligands is an intriguing question.

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