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Scotophobin A Causes Several Responses in Goldfish if the Pineal Gland is Present

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SATAKE, N. AND B. E. MORTON. *Scotophobin A causes several responses in goldfish if the pineal gland is present.* PHARMAC. BIOCHEM. BEHAV. 10(2) 183–188, 1979.—Rat scotophobin A increased dark avoidance in goldfish in dark and light avoidance shuttlebox experiments, controlled for general and light cycling-induced swimming activity. A possible site of action for scotophobin was suggested by the reports that dark avoidance was also increased in goldfish by pinealectomy, a treatment which increased shock sensitivity as well. It was found that scotophobin alone decreased the voltage required to induce tail-flip contractures in goldfish. The pineal gland was further implicated in the mode of action of scotophobin when it was found that this peptide suppressed the norepinephrine-induced aggregation of goldfish chromatophores whose state is in part controlled by pineal melatonin. Pinealectomized goldfish became insensitive to the effects of scotophobin upon both light-dark preference and chromatophore aggregation state. These observations strongly suggest that the pineal gland is required for the action of scotophobin.

Scotophobin Pineal gland Dark avoidance Shock threshold Chromatophores

SCOTOPHOBIN (G. dark+fear) is a peptide isolated from the brains of rats trained with electric shock to avoid the dark [13]. Both native and synthetic scotophobins cause dark avoidance in naive rats [5]. Synthetic rat scotophobin has also been reported to cause dark avoidance in naive goldfish by Guttman *et al.* [8] and to enhance dark-avoidance learning and to inhibit light-avoidance learning in fish being trained with electric shock by Bryant *et al.* [3]. However, although these reports on goldfish showed promise, they were subject to certain methodological criticisms. Here, using a different approach, we sought to avoid these problems and to confirm whether or not synthetic rat scotophobin indeed causes dark avoidance behavior in goldfish.

If specific dark avoidance-dark escape behavior was found to be produced in goldfish by scotophobin, the next question would concern the site of action of this behavioral peptide. Dark avoidance behavior in goldfish is also caused by pinealectomy [7]. This might appear reasonable due to the known influence of environmental lighting on the pineal gland [2]. Pinealectomy also increases the sensitivity of goldfish to shock [7]. This led to the discovery, reported here, that scotophobin lowers the threshold voltage required to induce tail-flip contractures in goldfish; a result consistent with the possibility that scotophobin may in some unknown manner carry information regarding shock.

These results implying possible pineal gland involvement in scotophobin action led us to investigate whether goldfish chromatophore aggregation, a process controlled in part by pineal melatonin production [4,6], might also be sensitive to scotophobin interference. That this was the case is also shown here.

Finally, data are presented demonstrating that the effect of scotophobin upon goldfish light-dark behavior and chromatophore aggregation appears to be dependent upon the presence of the pineal gland.

METHOD

Biochemicals

Synthetic scotophobin A [12] was generously supplied by the late Georges Ungar, who stated it to be 25% as active as native scotophobin. Norepinephrine bitartrate was purchased from Sigma Chemical Company.

Fish

The experimental animals used in these studies were common goldfish *(Carassius auratus)* of both sexes supplied throughout the year by Ozark Fisheries. The goldfish were between 7.5 and 8 cm in length. After about a month in a large holding tank, they were then separated and individually maintained in two gallon tanks whose water was filtered, aerated and kept at about 18°C. The fish were each fed two Purina Koi pellets at 5:00 p.m. daily and illuminated from 8:00 a.m. to 8:00 p.m. All experiments were carried out between 10:00 a.m. and 3:00 p.m.

Scotophobin and norepinephrine, dissolved in 0.9% NaCl, pH 7.4, were administered to the fish by way of 10 μl intracranial injections using a 25 μl Hamilton microsyringe with the guard of the No. 30 gauge needle set at 3 mm to prevent insertion beyond the supracerebral space [1]

Pinealectomy

When it became appropriate to determine the involvement of the pineal gland in the action of scotophobin, goldfish were pinealectomized under the anesthetic ethyl-

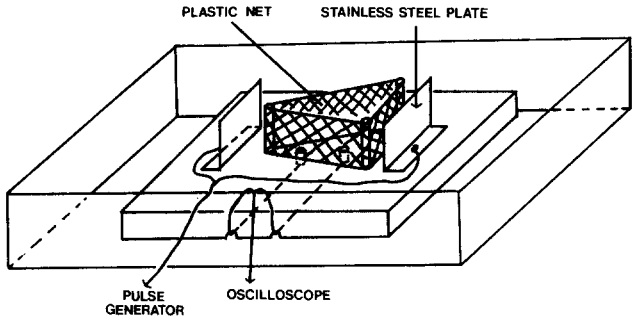

FIG. 1. The apparatus used to investigate the voltage necessary to induce tail-flip contractures in goldfish. Each fish was aligned in the plastic net cage between two stainless steel plates. A pulse of electric current was applied to each subject through these two plates. Day-to-day fluctuation of the current was monitored by an oscilloscope. The details of the construction and utilization of this apparatus are given in the Method section.

m-aminobenzoate (Tricaine; 1/8,000 dilution). The pineal glands were exposed by making a circular cut in the parietal bone (less than 5 mm in dia.) such that the center of the circle bisected an imaginary line connecting the posterior margins of the orbital cavities. Under a dissecting microscope (20 magnifications), the pineal gland was removed with a forcep. After the operation, the fish were kept in their home tanks for a month to permit recovery and the resealing of the skull opening. Post-experimental inspection of these fish verified that no pineal gland regeneration had occurred. In sham operations all steps were carried out except the gland was not excised. There were no mortalities resulting from these operations.

Light and Dark Avoidance Behavior Assay

Equipment. Light preference behavior was produced and measured in two similar sets of shuttleboxes. The first set were shuttleboxes used by Potts and Bitterman [10] that were made of black Plexiglas (25.5×11×10 cm deep) with black Plexiglas covers. Each box was transected through the center by a partial barrier 5 cm high that stopped 2.5 cm from the water's surface. Fish movement (response) across this barrier was monitored by a pair of photocells, 5.7 cm apart, placed upon the sides of the box 0.8 cm above the barrier. A white light (stimulus) could be presented at either end of the box through frosted glass panels containing a 7 W Christmas tree light.

The second set of shuttleboxes were wooden and larger (52×18×15.5 cm deep). These also contained a 5 cm central underwater barrier that was submerged 2.5 cm beneath the water level. Here the photocell pair was 4.5 cm apart on the sides 1.5 above the barrier. The use of the larger shuttleboxes permitted the utilization of bigger fish and also reduced random barrier crossing.

Experimental Procedure

In order to determine the normal baseline light-dark preference of the individual fish and to establish their basal swimming activity levels, each fish was monitored in baseline sessions for 4 days using the shuttleboxes. Each baseline section consisted of 3 trials of 5 min dark or light avoidance measurement alternating with 3 trials of 5 min of lights off general activity measurement. An intertrial darkness interval of 10 sec separated the alternating measurement periods. In the avoidance trial periods, one chamber of the shuttlebox was always darkened. For example in the dark avoidance studies, it was the side of the shuttlebox occupied by the fish while in the light avoidance studies it was the chamber opposite the fish. Crossing the barrier automatically tripped the photocells and switched the lighting to maintain the same light-dark relationship to the fish.

After the basal light-dark preference and swimming activity levels were determined for the set of fish involved in an experiment, the fish were sorted into groups in such a way that the average basal activities of each group were as close to the other as possible. Then the fish of each group were injected with scotophobin or saline and tested on the following 4 days for their dark or light avoidance responses and for general activity in exactly the same way as baseline studies above.

Tail-Flip Contracture Threshold Assay

The apparatus shown in Fig. 1 was set up in a 25×20×10 cm deep tank with a water level of 4.5 cm. Each subject was placed within a triangular net restraining area (2.5 cm "base" and 11.5 cm "altitude") created by three plastic rods. Electric current was applied through two stainless steel plate (3×4 cm high) mounted 0.5 cm underwater on either end of the restraining net. These plates were 15.5 cm apart and perpendicular to the longitudinal axis of the fish within. The electric current was a single-monopolar squarewave of 20 msec duration produced with a Model SD-5 pulse generator manufactured by Grass Instruments Incorporated. Day to day pulse voltage variation was monitored by an oscilloscope attached to electrodes directly under the subject area.

Fish were aligned and protected from physical damage by the emplacement of a fixed amount of cotton along their sides. The tail flip response consisted of a quick asymmetric body contraction. All fish were first tested for their minimum shock threshold by determining for each the voltage causing the tail-flip response. This was done over two days with 20 measurements per fish on each day, using 20 mV incremental increases until the response was elicited. On the third day the fish were sorted into two groups, matched according to the results of the prvious two days. The fish of these groups were injected with scotophobin or saline and tested the following 4 consecutive days for shock threshold, again employing 20 measurements per fish each day.

Chromatophore Aggregation Assay

The state of aggregation of fish scale xanthophores was determined at the indicated times in the experiments as follows: a fish scale from the dorso-lateral area below the beginning of the dorsal fin of each fish was removed from a forcep and immersed in a drop of 0.9% saline on a microscope slide.

The average state of aggregation of the xanthophores, located in the anterior portion of the scale which is normally covered by the overlap of the preceding scale, was rated microscopically (100 magnifications) on a scale of 1 to 5. This arbitrary scale is based upon the 5 stages of a melanophore index [9] where 5 is the most expanded and 1 is the most aggregated. All experiments were performed by a blind pro-

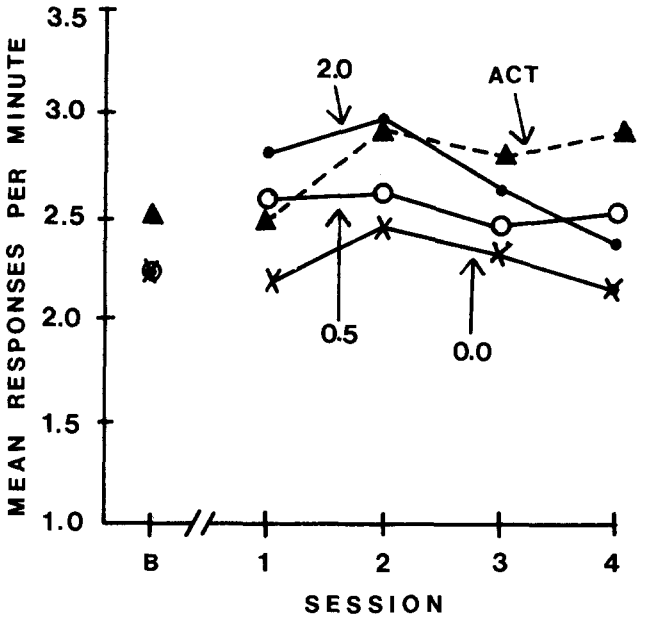

FIG. 2. The effect of rat scotophobin upon the dark avoidance behavior of goldfish. Three groups of 16 fish each were injected immediately before session one as follows: saline (X), 0.5 μg scotophobin (○), 2 μg scotophobin (●) and average basal swimming activity (in the dark) for all the fish (▲). Data points at B represent the mean of the four days baseline sessions for both general activity (in the dark) and dark avoidance responses. The smaller shuttleboxes were used in this experiment. Since there was no significant heterogeneity of variance in this and the following experiments, only the average SEMs are presented. The average SEM for this experiment was about 0.31.

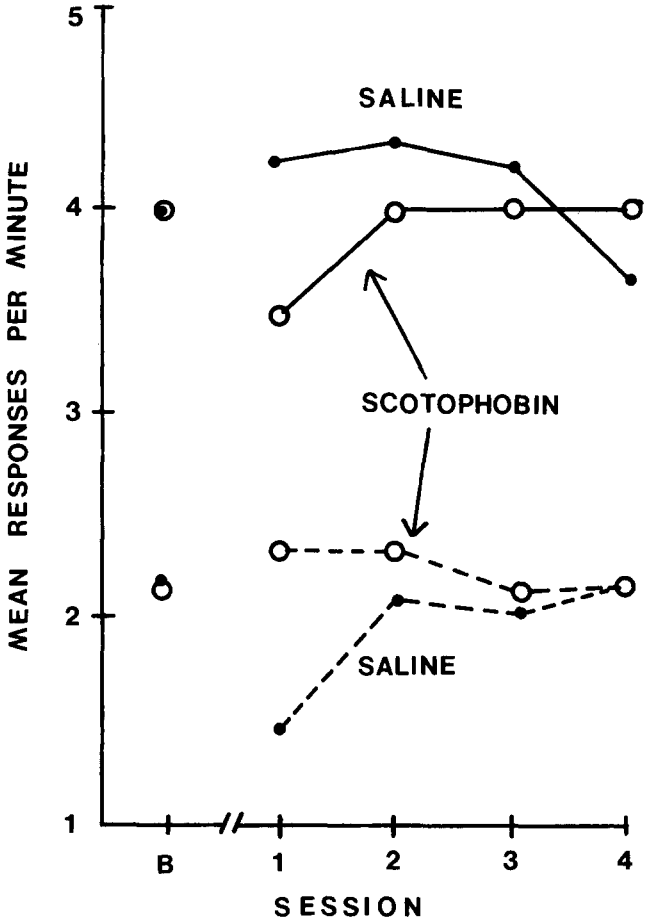

FIG. 3. The effect of scotophobin upon light avoidance behavior in goldfish. The two groups of 11 fish each were injected immediately before session one with saline (●) or 2 μg scotophobin (○). The solid lines represent light avoidance responses while the broken lines depict general swimming activity in darkness. Data points at B represent the mean of four days baseline sessions for both general activity (in the dark) and light avoidance responses. The larger shuttleboxes were used in this experiment. Average SEM was 0.39.

cedure where the experimenter was not informed of the drug treatment of the subjects.

RESULTS

We wished to verify that in our hands rat scotophobin would produce dark avoidance behavior in goldfish. We also wished to eliminate certain questions that could be raised regarding the previous reports that scotophobin produced dark avoidance in goldfish [3,8]. These were that scotophobin may have only elevated the basal swimming activity level of the fish [8] or that scotophobin modified some process of learning rather than affecting fish light-dark preference behavior independently of ongoing learning [3]. To accomplish these goals we used a different approach.

In the procedures used here, there were no actual acquisition sessions of avoidance responses. In order to maximize the performance of the fish, the illumination of light was programmed to follow the empty compartment opposite from the fish so that at any moment during the period of measurement the fish would be in the dark compartment opposite from the light compartment and thus was in the position to make a dark avoidance (escape) response. Besides this measurement, the general activity of the subject in complete darkness was measured. To eliminate the objection that scotophobin only created an increase of swimming activity associated with the switching of the location of the light source, light avoidance experiments were also run where darkness was programmed to follow the empty compartment and the light was shifted to whichever side of the shuttlebox the fish chose to occupy. Again the general activity of the subject was monitored during periods of darkness alternating with the avoidance periods.

The results of these experiments are shown in Figs. 2 and 3. In Fig. 2, the effect of 10 μl intracranial injections of either saline, 0.5 μg or 2 μg scotophobin per animal upon the dark avoidance behavior of three groups of 16 fish is shown. The average number of responses per minute during the dark avoidance period for the three groups and the grouped mean responses of the general activity level of the three groups are plotted. Scotophobin caused an increased dark avoidance response compared to the saline injected group, even though the activity levels were not significantly different among three groups. An overall analysis of variance showed a significant interaction between groups and two response measures (dark avoidance and general activity), $F(2,45)=3.62$, $p<0.05$. Subsequent analysis based on each response measure revealed a significant groups effect, $F(2,45)=3.6$,

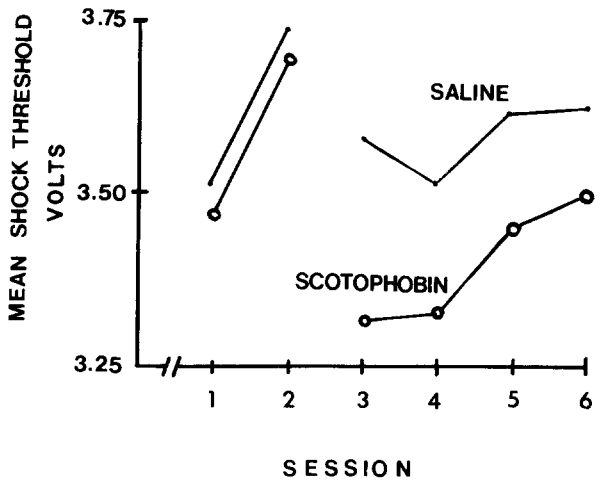

FIG. 4. The mean voltage required to produce tail-flip contraction in goldfish. The two groups of 7 animals each were injected one day prior to session three with saline (●) or 2 μg scotophobin (○). Sessions one and two represent preinjection data as described in Method. Average SEM of the raw scores was 0.15.

$p<0.05$, in dark avoidance responses but not in the general activity levels in darkness ($F<1.0$). These results indicate that scotophobin did not simply increase the general activity levels but activated a specific dark avoidance response.

In the light avoidance experiments of Fig. 3, designed to differentiate whether a simple activation of swimming associated with light switching activity could account for these results, two groups of 11 fish received either 10 μg intracranial injections of saline or 2 μg scotophobin. The mean number of responses per minute during the light avoidance trials and the general activity measurement periods are plotted for the two groups. It shows that the scotophobic-injected group decreased its light avoidance responses even though activity of the fish in darkness was not different from the saline injected control group. An overall analysis of variance showed a significant interaction among groups, two response measures (light avoidance responses and general activity levels) and session, $F(3,60)=4.68$, $p<0.006$. Subsequent analysis for each response measure revealed a significant difference in light avoidance responses, $F(3,60)=3.44$, $p<0.05$, but not in the activity levels ($F<1.0$).

Since scotophobin-induced increases in basal swimming activity or of swimming in response to flashing lights would have been registered as both increased dark and light avoidance, the asymmetric behavioral results of Figs. 2 and 3 support the idea that scotophobin indeed produces a specific dark avoidance (light preference) behavior and in a species far removed from rodents.

It came to our attention that pinealectomy increased the dark avoiding behavior of goldfish and lowered the threshold to convulsion induced by voltage gradients applied across their bodies [7]. Since shock was associated with the genesis of scotophobin in rats, we wondered if these data might not suggest that scotophobin could also alter the susceptibility of goldfish to shock-induced phenomena. In Fig. 4 is shown the effect of longitudinally applied whole body voltage gradients upon the tail-flip response thresholds in two groups of 7 goldfish, one injected with 10 μg saline and the other with 2 μg scotophobin. A preliminary experiment (data not shown) indicated that in goldfish a sudden body contraction (tail-flip convulsion) occurred when a specific threshold voltage was exceeded.

The mean shock thresholds for the two groups are plotted in Fig. 4. Treatment with scotophobin decreased the shock threshold after which time the threshold slowly approached the mean preinjection level. The differences between the mean pre- and the daily post-injection data was used for the statistical analysis which showed a significant groups effect, $F(1,12)=6.37$, $p<0.03$. These results indicated that the injection of scotophobin lowered the shock threshold in goldfish. These data together with the results of the previous two experiments share a resemblance to the behavioral effects caused by pinealectomy. It seemed possible, then that scotophobin might have interrupted some functions of the pineal gland to produce dark avoidance behavior.

To further test this hypothesis we utilized the fact that the pineal gland, via melatonin secretion, is involved in the control of the aggregation state of goldfish scale chromatophores (xanthophores) [4,6]. Preliminary experiments showed that scotophobin injection did not cause any observable change in the chromatophores when it was injected into naive goldfish. Since xanthophores (yellow chromatophores) of naive goldfish were usually in the expanded state, it was considered necessary first to aggregate the xanthophores in order to observe any possible effect of scotophobin.

In the two experiments of Fig. 5, 96 goldfish were utilized, 80 of which were injected intracranially at the start with 0.5 μg scotophobin in 10 μl saline. On the 5 subsequent days separate groups of 8 fish received for the first time 10 μl intracranial injections of 6 μg norepinephrine or saline. The aggregation state of their chromatophores was inspected one hour after this injection. In addition, 8 fish not receiving scotophobin were similarly injected with norepinephrine the day after the experiment began and compared with 8 scotophobin injected control fish for their one hour chromatophore state.

The results of these experiments (Fig. 5) show, first, that scotophobin alone had no effect upon the normal expanded state of goldfish scale chromatophores for the 5 days of the experiment. Second, in the absence of scotophobin, norepinephrine strongly contracted (aggregated) the fish chromatophores on Day 1, $F(1,4)=7.98$, $p<0.0135$, as was reported earlier for epinephrine [4]. Third, scotophobin strongly suppressed the ability of norepinephrine to aggregate the chromatophores, an effect which lasted for three days after the scotophobin was first injected. An overall analysis of variance, with the two treatments (one with scotophobin plus norepinephrine injections and the other with scotophobin plus saline injections) over five days, showed a significant interaction between groups and days, $F(4,70)=10.67$, $p<0.05$, and a significant groups effect, $F(1,70)=307.3$, $p<0.05$. These results indicate that the injection of scotophobin, indeed, suppressed the effect of norepinephrine.

We next wished to know whether the ability of norepinephrine to aggregate goldfish chromatophores required the presence of the pineal gland. To determine this 6 pinealectomized and 6 sham operated fish were injected with 6 μg of norepinephrine and the state of their chromatophores observed over the subsequent hour. The mean aggregation state of both groups fell from an initial rating of 4 to that of 2 during this time period. An analysis showed a significant effect of norepinephrine over the time period, $F(2,6)=10.34$, $p<0.015$, but did not show any effect caused by pinealec-

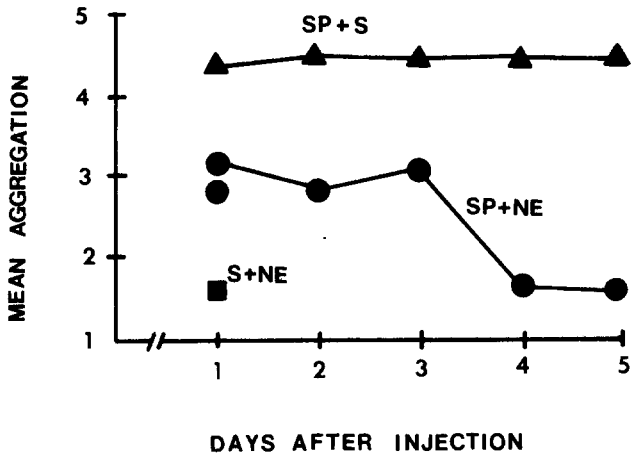

FIG. 5. The effect of scotophobin upon the norepinephrine induced aggregation of goldfish xanthophores. The mean state of xanthophore aggregation is represented on a scale with 1 as the most aggregated and 5 as the most expanded state. Eighty fish were first injected with scotophobin and each day thereafter subgroups of 8 were injected with saline (SP+P) or with norepinephrine (SP+NE), each day with different fish. The other two groups of 8 fish each were injected first with saline (S+NE) or with scotophobin (SP+NE) and a day later they were injected with norepinephrine. In every case the state of xanthophore aggregation was observed one hour after the second injection. Average SEM was 0.14.

tomy. Thus, the pineal gland was not apparently required for the aggregation goldfish chromatophores by norepinephrine.

However the pineal gland was required for scotophobin to inhibit the action of norepinephrine. This was shown in two experiments: in the first, 12 pinealectomized fish were injected with either saline or 2.5 μg scotophobin followed the next day by an injection of 6 μg norepinephrine. One hour after the norepinephrine injection the mean aggregation state of the chromatophores of both groups were 2.05 and 2.10, respectively ($F<1.0$) indicating no inhibitory effect by scotophobin. In the second experiment the injection of 2.5 μg scotophobin the day before 6 μg norepinephrine was injected suppressed chromatophore aggregation in 6 sham operated fish but failed to do so in 7 pinealectomized fish. The analysis showed a significant interaction between groups and measurement times, $F(3,18)=5.31$, $p<0.01$, and a significant times effect, $F(3,18)=5.19$, $p<0.01$.

These results, indicating the requirement of the pineal gland for scotophobin to act in the chromatophore system, encouraged us to ask a final question: Is the pineal gland required for scotophobin to produce light-dark preference behavior in goldfish? The answer to this was obtained in a final experiment using 12 pinealectomized goldfish, 6 injected with 2.5 μg scotophobin and 6 injected with saline. These animals were then tested over six daily blocks of 20, 1 min light avoidance trials separated by the usual 10 sec of intertrial darkness. Scotophobin had no effect upon the light-dark preference behavior of these pinealectomized animals. Scotophobin and saline groups made 1.64 and 1.58 mean responses per minute, respectively ($F<1.0$). These rates were lower than that of normal animals, in agreement with the report [7] that pinealectomy itself increases dark avoidance. These results suggest that the pineal may be required in order for scotophobin to cause dark avoidance in goldfish.

DISCUSSION

In this report we have confirmed that rat scotophobin A increases specific dark avoidance behavior in goldfish. This was manifested by an approximately 25% greater tendency for fish, injected with 2 μg scotophobin the day before, to leave a darkened shuttlebox chamber, and an approximately 20% lesser tendency for them to enter a darkened chamber than saline injected control subjects would have. Regarding the report by Guttman *et al.* [8] the objection that scotophobin could have produced dark avoidance results by the elevation of general activity levels has been eliminated here by the demonstration that scotophobin does not produce hyperactive swimming in goldfish. The objection to the report by Bryant *et al.* [3], that the effect of scotophobin was examined in grouped fish instead of individual fish, was eliminated here by the use of light and dark avoidance experiments which tested the effect of scotophobin in individual fish. These showed that the scotophobin-induced avoidance responses of the fish were specific to darkness only. Thus, it appears that rat scotophobin can elicit behavioral responses in species widely separated from rodents. This supports a generality in the mode of action of this behavioral peptide.

Turning to the subject of the possible site of scotophobin action, the observations that scotophobin creates increased dark avoidance and shock sensitivity in a manner equivalent to pinealectomy [7] suggested a possible role for the pineal gland in the action of scotophobin. Melatonin formation is a well known function of the pineal gland [2]. One of the systemic effects associated with melatonin action involves the control of the aggregation state of chromatophores in goldfish [4]. If scotophobin acts via the pineal gland it would be expected that it would alter the aggregation state of the pigment within goldfish xanthophores. Although scotophobin itself did not reduce the completely expanded xanthophores, we discovered that norepinephrine would cause the aggregation of these chromatophores [4]. Scotophobin was found to suppress the aggregation produced by norepinephrine. Pinealectomy abolished this effect of scotophobin while not altering the ability of norepinephrine to aggregate the chromatophores. While it is not clear how scotophobin blocks the norepinephrine-dependent chromatophore aggregation, these results do appear to demonstrate the requirement of the pineal for scotophobin action. This is further reinforced by the apparent absence of all scotophobin activity in the light-dark preference of pinealectomized goldfish. Although the pineal gland was not implicated in the two studies on the binding distribution of labeled scotophobin within the brains of rodents [11] and fish [8], the difficulty in utilizing the results of such studies to establish the site of action of a compound is well known.

The specific site of scotophobin interaction within the pineal gland has recently been elucidated and will be the subject of subsequent reports in this series (Satake and Morton, to be published).

ACKNOWLEDGEMENTS

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